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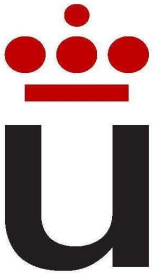
FACULTAD DE CIENCIAS DE LA SALUD



TESIS DOCTORAL

1. EFECTO DEL ENVEJECIMIENTO EN LA ESTRUCTURA Y FUNCIONALIDAD DEL PLEXO MIENTÉRICO DE ÍLEON DE COBAYO.

2. EFECTO DE LOS CANNABINOIDES EN LAS ALTERACIONES CAUSADAS POR EL TRATAMIENTO CRÓNICO CON CISPLATINO EN RATA.

GEMA VERA PASAMONTES

Madrid, 2009

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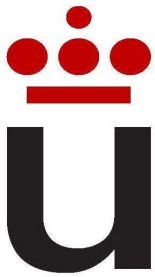

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Facultad de Ciencias de la Salud
Departamento Farmacología y Nutrición

Dña. MARIA ISABEL MARTÍN FONTELLES, CATEDRÁTICA DE FARMACOLOGÍA DE LA UNIVERSIDAD REY JUAN CARLOS, Y Dña RAQUEL ABALO DELGADO, PROFESORA CONTRATADA DOCTORA DE LA UNIVERSIDAD REY JUAN CARLOS

CERTIFICAN:

Que el trabajo de investigación titulado

"1. Efecto del envejecimiento en la estructura y funcionalidad del plexo mientérico de íleon de cobayo. 2. Efecto de los cannabinoides en las alteraciones causadas por el tratamiento crónico con cisplatino en rata" ha sido realizado por Dña. Gema Vera Pasamontes en el Departamento de Farmacología y Nutrición de la Universidad Rey Juan Carlos y en el Departamento de Medicina Interna y Gastroenterología de la Università di Bologna, Alma Mater Studiorum, bajo nuestra dirección como Tesis Doctoral Europea, y como tal autorizamos su lectura.

Y para que así conste donde proceda se firma este certificado en Alcorcón, a 8 de septiembre de 2009.

Fdo. M. I. Martín Fdo. R. Abalo Fdo. G. Vera

Avda. de Atenas s/n E 28922 Alcorcón Madrid España Tel. 34 91 4888855 Fax 34 91 4888831

A mi familia

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Justificación del trabajo realizado.

En esta tesis doctoral se presentan trabajos de dos líneas de investigación. Cuando llegué al laboratorio, el grupo de gastrointestinal estaba investigando el envejecimiento en el sistema nervioso entérico. Más tarde, nuestro trabajo se encaminó hacia el estudio de los antitumorales y el dolor neuropático.

La investigación en el Sistema Nervioso Entérico (SNE) y sus cambios con la edad está justificada por el rápido envejecimiento que está sufriendo la población. Este segmento de población es el que más visita al médico por problemas intestinales crónicos y los trastornos del SNE tienen efectos deletéreos en el bienestar y la autoestima de los mayores. Un mejor conocimiento de los efectos del envejecimiento en el SNE puede contribuir a la consecución de estrategias terapéuticas que mantengan la función gastrointestinal (GI) y así mejorar la calidad de vida de los ancianos.

Por otra parte, el tratamiento del dolor en las personas mayores está dificultado porque, con la edad, son más frecuentes las respuestas alteradas a los medicamentos, las interacciones farmacológicas y las reacciones adversas. Una de las alteraciones más frecuentes que producen los fármacos son las alteraciones gastrointestinales. El conocimiento de las alteraciones que produce la edad en el tracto gastrointestinal es fundamental para realizar un control adecuado del dolor.

La quimioterapia, desafortunadamente, se conoce por sus efectos adversos a nivel gastrointestinal y la neuropatía que produce. El desarrollo de modelos animales que asemejen las condiciones y pautas de administración de los antitumorales usados en humanos es esencial para que el estudio de nuevos fármacos sea más efectivo a la hora de tratar estos efectos adversos.

Los cannabinoides están siendo objeto de gran cantidad de estudios por su potencial terapéutico en el tratamiento de síntomas y signos de diversas patologías. Sería útil evaluar los cannabinoides en el tratamiento de las alteraciones asociadas al uso de antineoplásicos como el cisplatino.

Nuestros objetivos de trabajo han sido, estudiar:

1. El envejecimiento del sistema nervioso entérico. Se han incluido 3 publicaciones en este estudio.

2. Las alteraciones digestivas y neuropatía causadas por antineoplásicos y el efecto de los cannabinoides en dichas alteraciones. Se presentan 4 publicaciones.

Parte I

Introducción: La población está sufriendo un rápido envejecimiento. Los ancianos son los que más visitan al médico por problemas intestinales, además toman mucha medicación y una de las alteraciones más frecuentes que producen los fármacos es a nivel gastrointestinal. El conocimiento de las alteraciones que produce la edad en el tracto gastrointestinal puede contribuir a la consecución de estrategias terapéuticas que mantengan la función gastrointestinal y así mejorar la calidad de vida de los ancianos.

Objetivo: Estudiar el efecto del envejecimiento en la funcionalidad y estructura de la preparación fibra longitudinal-plexo mientérico (FL-PM) de íleon de cobayo.

Métodos: Se utilizaron preparaciones de fibra FL-PM de íleon procedentes de cobayos de distintas edades. Se estudió su funcionalidad en baño de órganos y se analizaron, mediante tinción inmunohistoquímica, distintas poblaciones de neuronas mientéricas y cómo se afectaban durante el envejecimiento.

Resultados: El envejecimiento afecta de distinta manera a las distintas poblaciones de neuronas del plexo mientérico, las neuronas colinérgicas están más afectadas, mientras que las nitrérgicas parecen estar preservadas. En cuanto a la funcionalidad, la respuesta a los agonistas excitadores está aumentada en las preparaciones FL-PM de cobayos viejos. Sin embargo, la respuesta a los agonistas inhibidores no se modifica con la edad.

Conclusiones: Con la edad, se produce una pérdida neuronal en el plexo mientérico de íleon de cobayo que no afecta por igual a las distintas poblaciones neuronales. La pérdida neuronal que se produce con el envejecimiento afecta en mayor grado a las neuronas excitadoras, mientras que las neuronas inhibidoras parecen estar preservadas. Hay una mayor respuesta a los estímulos excitadores en las preparaciones procedentes de animales viejos. Además el efecto de los agonistas inhibidores (tanto opioides como cannabinoides) no se modifica con el envejecimiento. La mayor respuesta a agonistas excitadores podría ser responsable de los episodios de hipermotilidad frecuentes a edades avanzadas. En conclusión, las alteraciones que se producen con la edad en el sistema nervioso entérico podrían hacer que el equilibro de la función GI sea más frágil, lo que haría más susceptibles a las patologías a los individuos viejos.

Parte II

Introducción: Los cannabinoides están siendo objeto de gran cantidad de estudios por su potencial terapéutico en el tratamiento de síntomas y signos en diversas patologías, como por ejemplo, los efectos adversos causados por la quimioterapia. El desarrollo de modelos animales que asemejen las condiciones y pautas de administración de los antitumorales usados en humanos es esencial para que el estudio de nuevos fármacos sea más efectivo a la hora de utilizarlos en estas patologías.

Objetivo: Estudiar las alteraciones gastrointestinales y la neuropatía (tanto entérica como sensorial) causadas por el uso de cisplatino y determinar el efecto de los cannabinoides en dichas alteraciones.

Material y métodos: Se desarrolló un modelo de administración crónica de cisplatino en el que se estudiaron las alteraciones alimentarias (anorexia; pica, que consiste en la ingesta de sustancias no nutritivas y es marcador indirecto de náusea), gastrointestinales y el desarrollo de neuropatía autonómica. Se analizó el efecto de los cannabinoides en las alteraciones alimentarias y en la neuropatía periférica producida por el cisplatino. También se desarrolló un método radiológico para estudiar las alteraciones en la motilidad gastrointestinal que producía la administración aguda de cisplatino y su relación temporal con la pica.

Resultados: La administración de cisplatino produjo anorexia, disminución del peso corporal de los animales y pica. Además produjo neuropatía sensorial y entérica (retraso persistente del tránsito gastrointestinal y alteraciones en el plexo mientérico). La administración de cannabinoide no modificó las alteraciones alimentarias, ni la disminución del peso corporal, pero sí previno el desarrollo de neuropatía periférica. Por otro lado, hemos desarrollado un método radiológico que permite el estudio de las alteraciones gastrointestinales (retraso en el vaciamiento gástrico y tránsito gastrointestinal) que produce la administración de cisplatino. El fármaco alteró tanto el vaciamiento gástrico como el tránsito GI en los animales tratados; de forma paralela indujo la aparición de pica.

Conclusiones: Nuestro modelo de administración de cisplatino, podría ser útil para el estudio de los mecanismos de la náusea y el vómito y el desarrollo de nuevas terapias antieméticas en tratamientos crónicos, más resistentes a los antieméticos

convencionales. El efecto de los cannabinoides en la neuropatía periférica podría deberse al efecto neuroprotector de estos fármacos.

El método radiológico desarrollado permite relacionar las alteraciones de la motilidad con la pica, por lo que ambos métodos podrían ser complementarios para el estudio de nuevos fármacos emetógenos y el desarrollo de terapias antieméticas.

Part I

Introduction: The population is ageing rapidly. Elder people are those who more often go to the doctor for intestinal problems. Moreover, they take many medications, and drug-induced alterations in the functions of the gastrointestinal tract (GIT) are amongst the most common side effects. A deeper knowledge of age-related alterations in the GIT could contribute to the development of therapeutic strategies in order to maintain gastrointestinal function and improve the quality of life in the elderly.

Aim: To determine the effect of ageing on structure and function of the longitudinal muscle-myenteric plexus (LM- MP) preparation from guinea-pig ileum.

Material and Methods: LM-MP preparations from guinea-pigs of three different ages were used. Functionality was studied in organ bath and the different neuronal populations were analyzed by immunohistochemistry.

Results: The different neuronal subpopulations in the myenteric plexus are affected in different ways by ageing: a decrease in the number of the cholinergic neurons was observed, whereas nitrergic neurones seemed to be preserved. The functional study showed an increased response to exogenous agonists in the LM-MP preparations from aged guinea-pigs, whereas age did not modify the response to inhibitory agonists.

Conclusions: Even though there is a general loss of myenteric neurones with age, this does not affect all neuronal populations equally. Excitatory neurons are more affected, whereas the inhibitory population seems to be preserved. An increased response to excitatory stimuli in the aged guinea-pig preparations was observed, whereas the response to inhibitory agonists (opioids and cannabinoids) did not vary during ageing. On the other hand, hyperresponsiveness to excitatory agonists could underlie episodes of hypermotility, frequently seen at old ages.

In conclusion, the alterations induced by age in the enteric nervous system seem to make the equilibrium of gastrointestinal function extremely fragile, leading to an increased sensitivity to pathology in older individuals.

Part II

Introduction: Cannabinoids are being studied due to their potential therapeutic effects in the treatment of some signs and symptoms of many diseases, including chemotherapy side effects. The development of new animal models resembling the conditions and guidelines of administration of antineoplastic drugs used in humans is essential for the development of more effective drugs capable of preventing these symptoms.

Aim: To study the development of anorexia, pica and gastrointestinal alterations and the enteric neuropathy caused by cisplatin treatment in the rat. And to determine the effect of cannabinoids in feeding alterations (anorexia and pica) and sensorial neuropathy caused by chronic cisplatin in rat.

Material and methods: A model of chronic administration of cisplatin was developed, in order to study gastrointestinal and feeding alterations (anorexia; pica, which is an indirect marker of nausea and consists of the ingestion of non-nutritive substances), and the development of neuropathy. The effect of cannabinoids was analyzed in feeding behavior and in cisplatin-induced peripheral neuropathy. A radiological method was also developed to study the alterations in gastrointestinal motility induced by acute administration of cisplatin in the rat, and its temporal relationship with pica.

Results: Cisplatin administration induced anorexia, body weight loss and pica. It also produced sensory and enteric neuropathy (persistent gastrointestinal transit delay and alterations in the myenteric plexus). Cannabinoid administration did not counteract feeding alterations or body weight loss, but prevented the development of peripheral neuropathy. The radiological method allowed the study of cisplatin-induced alterations in gastrointestinal motor function. Cisplatin induced both gastric emptying and gastrointestinal transit delay in a dose-dependent fashion, showing a strict temporal relationship with the induction of pica.

Conclusions: The experimental approach proposed in the present work could be useful in both the study of the mechanisms underlying nausea and vomit and the development of new antiemetic therapies in chronic treatments, more resistant to conventional antiemetic drugs. The effect of cannabinoids in peripheral neuropathy could be due to the neuroprotectant effect of these drugs.

The radiological method allows correlating the alterations in gastrointestinal motor function induced by acute cisplatin with pica. Both methods could be useful and complementary to study the effect of emetogenic drugs and to develop new antiemetics therapies.

Indice:

Abreviaturas

ACh: Acetilcolina

AChT ó ChAT: Acetilcolina transferasa

ADN: Ácido desoxirribonucleico

ARN: Ácido ribonucleico

ATP: Adenosin trifosfato

CB: Calbindina

CCK: Colecistoquinina

CGRP: Péptido relacionado con el gen de la calcitonina, Calcitonin gene related peptide

CR: Calretinina

DMSO: Dimetilsulfóxido

DYN: Dinorfina

DRG: Ganglios del asta dorsal de la médula, Dorsal root ganglia

EEM: Error estándar de la media

ENK: Encefalina

FL-PM: Fibra longitudinal-plexo mientérico

GAL: Galanina

GI: Gastrointestinal

GRP: Péptido liberador de gastrina, Gastrin-releasing peptide

5-HT: Serotonina

ICC: Células intersticiales de Cajal

NeuN: Neuronal Nuclei , proteína específica neuronal nuclear

NFP: Neurofilament protein

NFT: Triplete de filamentos específico de neuronas

NO: Óxido nítrico

NOS: Óxido nítrico sintasa

NPY: Neuropéptido Y

NSE: Enolasa específica de neuronas, Neurone specific enolase

PBS: Solución salina tamponada con fosfato, Phosfate buffered saline

SNC: Sistema Nervioso Central

SNE: Sistema nervioso entérico

SOM: Somatostatina

SP: Sustancia P

THC: Δ^9 tetrahidrocannabinol

VIP: Péptido intestinal vasoactivo, Vasoactive intestinal peptide

PARTE 1

EFECTO DEL ENVEJECIMIENTO EN LA ESTRUCTURA Y FUNCIONALIDAD DEL PLEXO MIENTÉRICO DE ÍLEON DE COBAYO

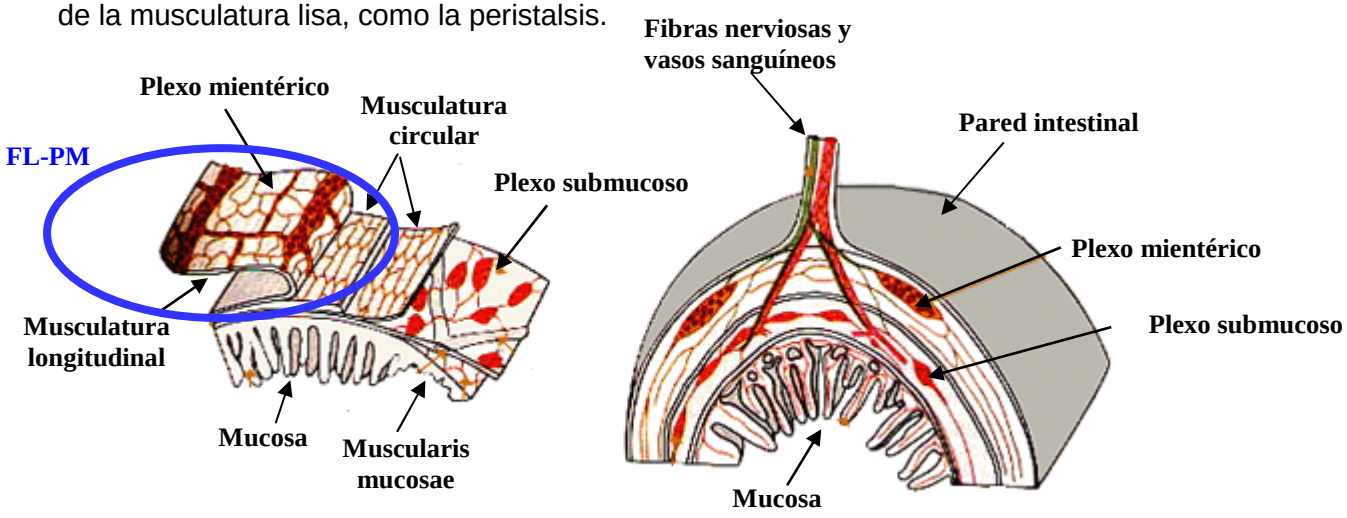
1. Introducción

El envejecimiento está asociado a una serie de trastornos gastrointestinales (Talley *et al.,* 1993; Majumdar *et al.,* 1997). A edades avanzadas la incidencia de trastornos de la motilidad es mayor, como retrasos en el vaciamiento gástrico y tránsito intestinal prolongado y acompañado de estasis fecal (Jost, 1997; O'Mahony *et al.,* 2002; Hays y Roberts, 2006; Norton 2006). Las causas de estas alteraciones no están claras, pero la sintomatología sugiere una pérdida o alteración en los mecanismos que controlan la función gastrointestinal (GI) (para revisión, ver: Phillips y Powley, 2007).

1.1. El Sistema nervioso entérico (SNE)

El tracto GI está inervado por neuronas intrínsecas del Sistema Nervioso Entérico (SNE) y por nervios extrínsecos parasimpáticos, simpáticos y sensoriales. Tanto la inervación intrínseca como la extrínseca están afectadas en mayor o menor grado por la edad.

Las neuronas entéricas son las encargadas del control neuronal de la función GI, se localizan en pequeños ganglios unidos por fibras nerviosas, formando una red, que discurre a lo largo del tracto GI. En muchas regiones del intestino de los vertebrados, la red se divide en dos partes: el plexo mientérico, que discurre entre las capas de musculatura lisa, y el plexo submucoso situado en el tejido conectivo que separa la mucosa y la musculatura lisa (ver Furness y Costa, 1987, ver figura 1). El plexo mientérico es el encargado de la iniciación y el control de los patrones motores de la musculatura lisa, como la peristalsis.



1.- Disposición anatómica de los plexos del SNE (Modificado de http://www.puc.cl/sw_educ/neurociencias/html/029.html) y preparación fibra longitudinal-plexo mientérico (FL-PM).

Los ganglios entéricos son irregulares en tamaño: en el intestino delgado del cobayo, los ganglios del plexo mientérico contienen en general, entre 10 y 100 neuronas, mientras que los submucosos son más pequeños. Como están presentes a lo largo de todo el tracto GI, el número total de neuronas entéricas es elevado; se ha estimado que es similar a las neuronas presentes en la médula espinal (Saffrey, 2004). Además de neuronas, en los ganglios hay células gliales, similares a los astrocitos del sistema nervioso central (SNC). Al contrario que otros ganglios autonómicos, los ganglios entéricos no contienen vasos sanguíneos ni tejido conectivo, pero sí tienen una densa red sináptica. Los ganglios entéricos están rodeados por una delgada membrana basal que está íntimamente asociada con un tipo especial de células llamadas células intersticiales de Cajal, que son células marcapasos, intermedias entre el plexo mientérico y la capa de musculatura lisa (Ward *et al.,* 2004).

1.2. El envejecimiento del SNE

El estudio del envejecimiento del SNE en humanos es complicado debido a la dificultad de obtener muestras y porque en la vejez pueden aparecer enfermedades que, aunque afectan a otros sistemas, producen alteraciones a nivel del tracto GI. Ejemplos de esto son la enfermedad de Parkinson y la neuropatía diabética, en las cuales se afectan las neuronas entéricas y otras neuronas autónomas (Wakabayashi y Takahashi, 1997; Micieli *et al.,* 2003; Vinik *et al.,* 2003). Además, alteraciones de otros sistemas (como el vascular) y las debidas al uso de medicación, pueden confundirse con los cambios producidos por la edad en la fisiología gastrointestinal. Por todo ello, muchos trabajos para estudiar el envejecimiento del SNE se han realizado en animales de laboratorio.

Desde la antigüedad se sabe que el tracto gastrointestinal sufre cambios funcionales con el envejecimiento, Hipócrates afirmaba que “los intestinos tienden a hacerse vagos con la edad”. En el tracto digestivo, se han observado cambios morfológicos, neuronales y bioquímicos que podrían subyacer a dichas alteraciones funcionales.

1.2.1. Cambios morfológicos

Se ha detectado un aumento en la cantidad de colágeno de las vellosidades intestinales, lo cual limita su movilidad (Hollander *et al.,* 1989). También se han encontrado cambios en las estructuras mitocondriales y cambios vasculares, sobre todo de naturaleza arteriosclerótica (Timiras, 1994).

Respecto a la musculatura lisa, las células presentan alteraciones que afectan a las mitocondrias y lisosomas. Las mitocondrias sufren daños en el ADN y las proteínas, mientras que los lisosomas acumulan lipofuscina. Además, las células musculares se hacen más alargadas y su densidad espacial es menor pues aumentan los espacios entre células (Gabella, 2001). La distribución de filamentos en los ancianos es similar a los jóvenes aunque aumentan los filamentos gruesos (Aniansson *et al.,* 1992) y el colágeno en el estroma (Gabella, 2001). Todo esto hace que el aspecto de la musculatura lisa en general, y la intestinal en particular, sea hipertrófico.

1.2.2. Cambios neuronales

En diversas partes del Sistema Nervioso Central, y del Sistema Nervioso Autónomo, se han visto cambios en la estructura neuronal, en el cuerpo celular, fibras, y terminales nerviosos de animales viejos. Algunos estudios han descrito un aumento del tamaño de las neuronas mientéricas (Phillips *et al.,* 2003), mientras que en otros no se ve tal aumento (Gomes *et al.,* 1997). Esta controversia puede deberse a las distintas especies utilizadas en los estudios o a la diversa metodología. Respecto a las fibras nerviosas intrínsecas algunos estudios describen fibras hinchadas (Philips *et al.,* 2003), y por microscopía electrónica se ha visto que el número de fibras inmunorreactivas en el intestino delgado de ratas viejas es menor (Feher y Penzes, 1987). Estos autores también han descrito una disminución en el número y extensión de las fibras nerviosas en animales envejecidos, que podría deberse a la neurodegeneración. El área ganglionar que ocupa el plexo mientérico en la rata F344 disminuye en animales de 26 meses (Phillips *et al.,* 2004a), lo mismo se encontró en el área ganglionar del plexo submucoso (Phillips *et al.,* 2007).

En cuanto al número de neuronas, se ha descrito una reducción en el SNE tanto de humanos como de roedores, durante el envejecimiento. Sin embargo, en otras partes del sistema nervioso sólo hay un descenso neuronal en situaciones patológicas (Turlejski y Djavadian, 2002; Wade y Cowen, 2004). El análisis de los cambios neuronales en el SNE con la edad está dificultado por varias razones: no hay marcadores que realmente marquen todas las neuronas entéricas (ver Philips *et al.,* 2004b), las dimensiones del tracto GI cambian con el envejecimiento, y por último, el tamaño de los ganglios es desigual. Por ello, la preparación de fibra longitudinal-plexo mientérico (FL-PM) o whole-mount, que consiste en la musculatura longitudinal con el plexo mientérico adherido a ella (ver figura 1), es la más adecuada para realizar recuentos neuronales (mejor que las secciones), ya que en una única preparación se pueden observar numerosos ganglios.

Santer y Baker (1988) fueron los primeros en describir la pérdida neuronal del plexo mientérico con la edad en rata, y se ha verificado en diferentes especies, como el cobayo (Gabella, 1989), ratón (El-Salhy *et al.,* 1999) y el humano (de Souza *et al.,* 1993). La muerte celular en el plexo mientérico se ha descrito en el intestino delgado y grueso de distintas cepas de ratas (Fischer 344: Phillips y Powley, 2001, Phillips *et al.,* 2003, 2004a; Sprague-Dawley: Cowen *et al.,* 2000; Thrasivoulou *et al.,* 2006; Wistar: Santer y Baker, 1988). No se conoce bien cuándo comienza esta pérdida neuronal ni el patrón que sigue. Phillips y Powley (2001) realizaron un experimento con ratas Fischer 344 de hasta 27 meses de edad y observaron que la pérdida neuronal en el intestino delgado y grueso comienza en la edad adulta y sigue un patrón lineal a lo largo de la vida de los roedores. En íleon de ratas Sprague-Dawley también se ha visto lo mismo (Thrasivoulou *et al.,* 2006). En ratones de 12 meses (El-Salhy *et al.,* 1999) también se observó pérdida neuronal, pero a los 24 meses, la pérdida no aumentaba. Quizá estas diferencias se deban a la distinta especie y metodología utilizadas para realizar el recuento neuronal. En cuanto al estómago, los resultados son también controvertidos. En ratas Fischer 344 se ha visto pérdida neuronal en el fundus a los 27 meses (Phillips y Powley, 2001). En el antro de ratón, el número de neuronas por ganglio descendió a los 12 meses, pero a los 24 meses la pérdida no era mayor (El-Salhy *et al.,* 1999).

En humanos, se ha descrito pérdida neuronal en el plexo mientérico del esófago (Meciano Filho *et al.,* 1995), el intestino delgado (de Souza *et al.,* 1993) y el colon (Gomes *et al.,* 1997). Estas disminuciones son variables y pueden llegar hasta el 60% en individuos mayores de 70 años (Meciano Filho *et al.,* 1995). En colon, también de humano, Hanani *et al.,* (2004) describieron un aumento en la proporción de ganglios con cavidades, que se relacionan con la pérdida neuronal descrita en otros estudios.

Esta reducción del número de neuronas con la edad, tanto en humanos como en animales, podría relacionarse con alteraciones funcionales frecuentes en la vejez.

1.2.3. Cambios bioquímicos

La preparación de FL-PM de íleon de cobayo es la preparación mejor conocida morfológica y funcionalmente. En los años 80 se vio que ciertos marcadores estaban asociados con poblaciones morfológicas y funcionales específicas y que la combinación de marcadores era mejor que un marcador solo para la identificación de cada población. Es lo que se denominó el código químico (Costa y Brookes, 2008. Ver

tabla 1). Aunque se ha estudiado el efecto del envejecimiento en la población total neuronal, no hay demasiados estudios que analicen los cambios en el código químico con la edad en el íleon de cobayo, aunque sí hay algunos estudios en rata (para revisión ver Phillips y Powley, 2007). Éste podría ser un buen punto de partida para entender la pérdida neuronal que sucede con la edad, ver si afecta a toda la población o es específica de uno o más subtipos celulares.

El análisis de las neuronas nitrérgicas (aquellas que liberan óxido nítrico, sintetizado por la óxido nítrico sintasa, NOS) y las neuronas colinérgicas (que liberan acetilcolina (ACh), sintetizada por la acetilcolina transferasa, AChT ó ChAT) nos puede dar información de prácticamente todas las neuronas entéricas. La mayoría de las neuronas positivas para la NOS en el plexo mientérico son motoneuronas inhibidoras de la musculatura lisa; en el íleon de cobayo, un pequeño porcentaje son interneuronas descendentes (Costa *et al.,* 1996). Sin embargo, las neuronas colinérgicas incluyen distintos grupos funcionales: interneuronas –tanto ascendentes como descendentes-, neuronas aferentes primarias y motoneuronas excitadoras de la musculatura lisa. Así que el análisis de los cambios en las neuronas colinérgicas durante el envejecimiento no identifica qué tipo funcional de neuronas se pierde, a menos que se estudien incluyendo otros marcadores.

FUNCIÓN	TIPO DOGIEL	CÓDIGO QUÍMICO	%
Motoneuronas ascendentes excitadoras de la musculatura circular	I	SP/ChAT/±ENK/NFP	10
Motoneruonas ascendentes excitadoras de la musculatura longitudinal	I	SP/ChAT/CR	25
Motoneuronas descendentes inhibidoras de la musculatura circular	I	VIP/NOS/ATP/DYN/GRP/NFP	12
		VIP/NOS/ATP/±ENK/±NPY	
Interneuronas ascendentes	I	SP/ChAT/ENK/NFP/CR	5
Interneuronas descendentes	I	5-HT/ChAT/NFP	1
Interneuronas descendentes filamen.	III	SOM/ChAT	4
Interneuronas descendentes	I	VIP/NOS/±ChAT/DYN/GRP/NFP	1
Neuronas aferentes primarias	II	NeuN/CB/SP/ChAT	24
	II	NeuN/ SP/ ChAT	14
Neuronas secretomotoras	III	VIP/DYN/GAL	1
	III	NPY/ChAT/SOM/CGRP/CCK	1
	I	VIP/ChAT/ENK/DYN/GRP/CCK	1
Neuronas intestinofugales	I	ChAT/GRP/VIP/ENK/±NOS/CCK	1
TOTAL			100

TABLA 1. *Clases de neuronas mientéricas en el intestino delgado de cobayo (*Modificada de Costa y Brookes, 2008).

De todas formas, los resultados de diversos grupos indican que las distintas poblaciones neuronales reaccionan de diferente forma ante la edad. Las neuronas colinérgicas parecen ser más vulnerables al envejecimiento, mientras que las nitrérgicas están preservadas, o se ven menos afectadas (ver Wade y Cowen, 2004).

En ratas F344, tanto en intestino delgado como en grueso (Phillips *et al.,* 2003), se analizaron la población neuronal total y las neuronas nitrérgicas, y no se encontró reducción en las neuronas nitrérgicas, mientras que la población general sí que estaba disminuida. Resultados similares se han descrito en el íleon (Belai *et al.,* 1995; Cowen *et al.,* 2000), colon proximal (Belai *et al.,* 1995) y yeyuno de ratas (Santer, 1994), y en humanos (Belai y Burnstock, 1999).

Parece, por tanto, que son las neuronas NOS negativas las que se ven más afectadas por el envejecimiento. Cowen *et al., (*2000), utilizando un anticuerpo frente a la AChT, vieron que en ratas de 24 meses había una pérdida del 64% de las neuronas colinérgicas.

Otras proteínas que juegan un papel importante son las proteínas tamponadoras de calcio, como la calretinina (CR) y la calbindina (CB). En rata, las neuronas que expresan CR son más abundantes que las que expresan CB (Thrasivoulou *et al.,* 2006). En íleon de rata se ha visto que el 30% de las neuronas que expresan CR se pierde a los 17 meses. Resultados similares se han visto en intestino delgado y grueso de ratas viejas (Corns *et al.,* 2002), y en el plexo submucoso (Wade *et al.,* 2001). Sin embargo, en humanos, se ha descrito un aumento en la proporción de neuronas mientéricas inmunorreactivas para CR en el intestino delgado (Belai y Burnstock, 1999). Respecto a la CB, se ha descrito una disminución con la edad en duodeno de jerbo (Choi *et al.,* 2008) y en íleon de rata (Thrasivoulou *et al.,* 2006).

De todas formas, no está claro por qué las neuronas colinérgicas y las que expresan proteínas tamponadoras de calcio son más sensibles al envejecimiento. Thrasivoulou *et al.,* (2006) han sugerido una alteración en la homeostasis del calcio, lo cual estaría relacionado con niveles elevados de especies reactivas de oxígeno. De hecho, se ha visto que la restricción calórica, que disminuye su producción, limita o previene la pérdida de neuronas mientéricas que ocurre en el envejecimiento (Thrasivoulou *et al.,* 2006).

1.2.4. Cambios en la inervación extrínseca

Además de la inervación intrínseca del SNE, el tracto GI está inervado por fibras extrínsecas simpáticas, parasimpáticas y sensoriales. En el intestino delgado de ratas de 12-18 meses se ha descrito una reducción de la densidad de fibras simpáticas que inervan el plexo mientérico (Baker y Santer, 1988). Hay evidencias farmacológicas que sugieren que estas alteraciones simpáticas se reflejan en el control de la

relajación del músculo liso del íleon de rata (Kadowaki *et al.,* 2003). Por otro lado, las regiones superiores del tracto GI reciben inervación a través del nervio vago. El vago tiene tanto función sensorial (fibras aferentes) como motora (fibras eferentes). Hacia la mucosa envía terminales arborescentes principalmente a las criptas y la lámina propia (Powley *et al.,* 1994; Berthoud *et al.,* 1995; Phillips y Powley, 2005). Aunque no existen demasiados estudios al respecto, no se han visto alteraciones con la edad en las aferencias vagales (Phillips y Powley, 2001), ni tampoco en el número de neuronas mientéricas que están rodeadas por eferencias vagales (Phillips y Powley, 2005), aunque no se pueden descartar cambios a nivel ultraestructural. Otro problema podría ser la metodología utilizada para realizar estos experimentos. En la mayoría se han utilizado anticuerpos conjugados con la peroxidasa de rábano (Phillips y Powley, 2001), y puede que estos cambios no se observen con este tipo de técnica.

1.2.5. Cambios funcionales con la edad.

Todos estos cambios en el SNE estarían relacionados con las principales alteraciones funcionales que afectan al tracto GI en las personas ancianas. Aunque la función GI se mantiene en el envejecimiento, su efectividad puede estar comprometida por pérdida neuronal y por cambios a nivel de las células musculares. Esto, junto a factores extrínsecos como la dieta, el sedentarismo, y los efectos de la medicación, podrían modificar la función GI. Se han observado cambios en la motilidad GI (Bitar y Patil, 2004): en particular, el tránsito GI está retrasado en la vejez (Smits y Lefebvre, 1996) y el vaciamiento gástrico está prolongado y la amplitud y frecuencia de ondas peristálticas está reducida (Horowitz *et al.,* 1984). Esto podría contribuir al desarrollo de problemas funcionales comunes en la vejez, como el estreñimiento, la disfagia o el síndrome del colon irritable (De Lillo y Rose, 2000; Firth y Prather, 2002).

La mucosa gástrica también se altera con la edad, aumenta su espesor (Hollander *et al.,* 1989) y esto podría afectar a su función secretora y a la absorción de nutrientes y líquidos. Estas alteraciones podrían estar relacionadas con otros problemas frecuentes en la vejez como el síndrome de mala absorción, la malnutrición y la anorexia (Firth y Prather, 2002).

En animales también se han encontrado cambios similares, el tránsito intestinal está retrasado en el colon de rata (Madsen, 1992; Smits y Lefebvre, 1996). En cuanto a la preparación FL-PM, en estudios in vitro, Roberts *et al.* (1994) encontraron una reducción en la contracción muscular en respuesta a campos eléctricos en el colon; en el mismo estudio se vio que la liberación de ACh estaba disminuida (Roberts *et al.,*

1994). La contracción de la fibra muscular se debe a la acción de las motoneuronas excitadoras de la musculatura longitudinal que, en íleon de cobayo, son inmunorreactivas para CR y no expresan el triplete de filamentos específico de neuronas (NFT) (Brookes *et al.,* 1991). En ratas con un marcado descenso en el número de neuronas mientéricas, la contracción de la musculatura lisa en respuesta a estimulación eléctrica no se modificó (Hoyle *et al.,* 2002). Esto sugiere que en esta parte del tracto GI las neuronas sensoriales intrínsecas y las interneuronas podrían estar disminuidas mientras que las motoneuronas no. Podría explicarse por plasticidad del sistema neuromuscular en esta parte del intestino (las neuronas que permanecen inalteradas son capaces de compensar las que se han perdido), o porque cambien las propiedades del músculo liso.

Los compuestos del tipo de la morfina pueden inhibir las contracciones inducidas eléctricamente en las preparaciones de FL-PM de íleon de cobayo, de modo dependiente de la dosis y reversible por naloxona (Paton, 1957; Kosterlitz *et al.,* 1975). El mecanismo subyacente a esta inhibición es una depresión presináptica de la liberación de ACh por las neuronas motoras del plexo mientérico (Leslie, 1987). Los opioides inhiben también la liberación de sustancia P y de otros transmisores, como la serotonina (5-HT), el péptido intestinal vasoactivo (VIP), el óxido nítrico (NO) y los propios opioides (Holzer, 1984). Los opioides actúan sobre receptores localizados a lo largo del tracto GI y tienen numerosos y complejos efectos en él: el control del dolor visceral, la regulación del transporte de fluidos y electrolitos en la mucosa, y efectos sobre la motilidad, como retraso del tránsito y vaciamiento gástrico, así como inhibición de las contracciones gástricas, como consecuencia, se producen síntomas como el estreñimiento (Kromer, 1988). Pero no se ha estudiado si estos efectos se modifican o no durante el envejecimiento.

De forma similar a los opioides, los agonistas cannabinoides inhiben la contracción inducida eléctricamente de manera concentración-dependiente en íleon de cobayo y de humano, actuando como neuromoduladores (Pertwee, 2001). Esta acción se produce presinápticamente por inhibición de la liberación de neurotransmisores excitadores como ACh, noradrenalina, 5-HT, aspartato, glutamato y colecistoquinina (Pertwee y Ross, 2002). El receptor cannabinoide, durante el desarrollo del tracto digestivo, aparece en una época temprana, tanto en el plexo submucoso como en el mientérico (Buckley *et al.,* 1998). Sin embargo, no se sabe qué sucede con la edad en la funcionalidad y en la expresión de dicho receptor en el tracto digestivo.

2. Objetivos

El objetivo de la primera parte de esta tesis es determinar cómo afecta el envejecimiento a la estructura y funcionalidad del plexo mientérico de íleon de cobayo.

Los resultados se encuentran recogidos en tres publicaciones:

1. *Ileal myenteric plexus in aged guinea-pigs: loss of structure and calretinin-immunoreactive neurones.* Neurogastroenterol Motil. 2005; 17(1): 123-132.
2. *Evaluation of the effect of age on cannabinoid receptor functionality and expression in guinea-pig ileum longitudinal muscle-myenteric plexus preparations.* Neurosci Lett. 2005; 383(1-2):176-181.
3. *Age-related changes in the gastrointestinal tract: a functional and immunohistochemical study in guinea-pig ileum.* Life Sci. 2007; 80(26):2436-2445.

3. Material y métodos

Este trabajo se llevó a cabo en cobayos Dunkin–Hartley hembras de 3 edades diferentes: 1-3 meses (JÓVENES, peso 330-450 g), 8–10 meses (ADULTOS, peso 800–950 g) y 22–26 meses (VIEJOS, peso 900–1200 g). Todos los protocolos experimentales se aprobaron por el comité ético de la Universidad Rey Juan Carlos y están de acuerdo con la normativa vigente de la CE para el cuidado y uso de animales de experimentación (EEC N° 86/609). Para minimizar el sufrimiento de los animales, éstos se sacrificaron mediante dislocación cervical y se extrajeron segmentos de íleon distal, al menos a 10 cm de la válvula ileocecal, se abrieron por la línea mesentérica, se lavaron y se fijaron a una placa petri con base de Sylgard y se mantuvieron en una solución de Krebs modificada (mM: NaCl; 118, KCl; 4.75, NaH_2PO4; 1.0, $NaHCO_3$; 25, $MgSO_4$; 1.2, $CaCl_2$; 2.5, glucosa; 11, pH 7.4). Las preparaciones de FL-PM se obtuvieron quitando la mucosa, la submucosa y la musculatura circular. Estas preparaciones se usaron en dos tipos de experimentos.

1. En el estudio funcional, las preparaciones FL-PM se dispusieron en un baño de órganos a una tensión de 1 g, con solución de Krebs a 37ºC burbujeada con carbógeno (95% O_2–5% CO_2). La actividad contráctil de la preparaciones se registró por medio de un transductor isométrico conectado a un sistema PowerLab/4e. Se usó estimulación eléctrica (pulsos de 2 ms de duración, 0.3 Hz y voltaje supramaximal) para producir contracciones sensibles a atropina (colinérgicas). Las preparaciones cuya contracción no llegaba a 0.5 g se descartaron. Los protocolos particulares para cada agonista y antagonista se explican con detalle en las distintas publicaciones. Brevemente, para sustancia P y acetilcolina (agonistas excitadores directos de la fibra muscular) se realizaron curvas no acumulativas en preparaciones no estimuladas eléctricamente. Las curvas de los agonistas cannabinoides y opioides (agonistas inhibidores de la liberación de acetilcolina por las neuronas presinápticas) se realizaron en preparaciones estimuladas eléctricamente a concentraciones acumulativas y se evaluaron como el porcentaje de inhibición de la contracción inicial.

2. La metodología para el marcaje inmunohistoquímico de preparaciones FL-PM está descrito en detalle en las publicaciones 1-3. Brevemente, las preparaciones se estiraron hasta su extensión máxima y fueron fijadas con Zamboni y lavadas con dimetilsulfóxido. Tras varios lavados en suero salino tamponado con fosfato (PBS), los tejidos se incubaron toda la noche a temperatura ambiente con una mezcla de anticuerpos (según publicaciones). Después de lavar con PBS se expusieron durante

90 minutos a temperatura ambiente a los distintos anticuerpos secundarios (según protocolo de las distintas publicaciones). Todos los anticuerpos se diluyeron en PBS hipertónico (1.7% de NaCl) (Costa *et al*, 1986). Las preparaciones se observaron con un microscopio confocal (LSM 510 Zeiss, laser: 488 y 453 nm) o un microscopio de fluorescencia (Nikon Eclipse TE2000-U) y se realizaron mosaicos con microfotografías que fueron analizadas de forma ciega a la edad de los cobayos de los cuales procedía el tejido. Los controles de doble marcaje se hicieron combinando el anticuerpo primario con el secundario erróneo o incubando el anticuerpo secundario sin haber puesto el anticuerpo primario. En ninguno de los casos se observó marcaje específico.

El análisis estadístico se llevó a cabo mediante el paquete estadístico GraphPad Prism® (GraphPad Software, Inc.). Los resultados están expresados como la media ± E.E.M. La letra n indica el número de muestras y si no se dice lo contrario, es igual al número de animales (N). Las comparaciones se realizaron tomando el grupo de animales JÓVENES como referencia, debido a que esta edad es la más utilizada para propósitos experimentales. Las diferencias entre grupos se analizaron mediante una ANOVA de una o dos vías (seguido de un test *post hoc* de Bonferroni) o una t de Student de datos desapareados (con corrección de Welch cuando las varianzas no eran homogéneas). Se ha considerado como diferencia estadísticamente significativa una probabilidad de $p<0.05$.

4. Publicaciones

4.1. Primera publicación:

Abalo R, José Rivera A, Vera G, Martín MI. ***Ileal myenteric plexus in aged guinea-pigs: loss of structure and calretinin-immunoreactive neurones*.** Neurogastroenterol Motil. 2005; 17(1): 123-132.

4.1.1. Objetivo general

Estudiar el efecto del envejecimiento en el plexo mientérico de íleon de cobayo.

4.1.2. Objetivos específicos

Estudiar:

- La densidad ganglionar total en las distintas edades estudiadas
- El área ocupada por los ganglios
- La densidad de ganglios grandes, medianos y pequeños
- La densidad y proporción de neuronas inmunorreactivas para CR en cada edad
- La proporción de neuronas CR^{+}-NFT^{+} y CR^{+}-NFT^{-}

4.1.3. Resultados

La densidad ganglionar era igual en cobayos jóvenes y viejos, pero se encontró una reducción significativa en el área ganglionar total. Específicamente, la densidad de ganglios medianos y grandes se redujo en individuos de mayor edad y parecía haber una tendencia a incrementar el número de ganglios pequeños (aunque no fue estadísticamente significativa) en los cobayos viejos. Tanto en los cobayos jóvenes como en los viejos, la mayoría de las neuronas se encontraban dentro de los ganglios. La densidad de neuronas dentro de los mismos, o densidad de empaquetamiento, se redujo en los animales de mayor edad. En contraste, la proporción de neuronas extra ganglionares (neuronas aisladas, fuera de los ganglios, relativamente poco frecuentes en los individuos jóvenes) se incrementó con la edad.

Además, la densidad y la proporción de neuronas inmunorreactivas para CR se redujo significativamente con la edad, especialmente las que carecían de marcaje para NFT (probablemente motoneuronas excitadoras de la musculatura longitudinal) eran más sensibles a esta disminución asociada a la edad. Sin embargo, las neuronas

inmunorreactivas para CR que también expresaban NFT (probablemente interneuronas ascendentes) aumentaron en los animales viejos.

Por último, en los tejidos de animales envejecidos, aparecían puntos de material autofluorescente, probablemente lipofuscina, y eran más frecuentes en las neuronas inmunorreactivas para CR.

4.1.4. Conclusiones

Con la edad se producen modificaciones en el plexo mientérico de íleon de cobayo, tanto en la estructura general como en la inmunorreactividad para el marcador neuronal CR. Hay una pérdida neuronal dependiente de la edad, aunque los distintos fenotipos neuronales parecen tener una sensibilidad diferente al envejecimiento, lo cual, bajo determinadas circunstancias podría repercutir en la motilidad intestinal en los individuos envejecidos.

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Ileal myenteric plexus in aged guinea-pigs: loss of structure and calretinin-immunoreactive neurones

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Abstract *Myenteric plexus controls gastrointestinal motility by means of well organized circuits which are comprised of sensory neurones, interneurones and motor neurones to the muscular layers. Calretinin (CR) is a calcium-binding protein that, in guinea-pig ileum, has only been found in ascending interneurones, which also express neurofilament triplet proteins (NFT), and excitatory longitudinal muscle motor neurones, which do not. In spite of some evidence that age affects both function and structure of the myenteric plexus, little is known about the possible selectivity of the process regarding specific myenteric neuronal phenotypes. The influence of age on both the structure of the myenteric plexus and the presence of CR-immunoreactive (CR-IR) neurones was studied using conventional immunohistochemical procedures applied to ileal whole-mount preparations from guinea-pigs. Both a reduction in ganglionic size and changes in the distribution of neurones inside and outside the ganglia, together with a general neuronal loss were found in preparations from aged guinea-pigs. More interestingly, a relatively more pronounced age-related loss of CR-IR neurones, especially those lacking of NFT expression, was found. Specific myenteric neuronal phenotypes may show differential sensitivity to ageing, and this could, under certain circumstances, alter the functional balance of gastrointestinal motility in aged individuals.*

Keywords *ageing, calretinin, guinea-pig, immunohistochemistry, myenteric neurone.*

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INTRODUCTION

The myenteric plexus is responsible for the control of motility of the gastrointestinal tract. Myenteric ganglia, which contain most of the neuronal somata, are variable in size, depending on the number of neurones of which they are comprised.[1–3] To further complicate the analysis of the myenteric plexus structure, neurones lying outside the ganglia have also been found.[4,5]

Myenteric neurones have been classified mainly according to their morphology, electrophysiology and chemical coding.[6–8] Calretinin (CR) is a calcium-binding protein that has been found solely in two classes of guinea-pig ileum myenteric neurones: ascending interneurones, which also express neurofilament triplet proteins (NFT), and motor neurones to the longitudinal muscle, which lack of it.[9] Ascending interneurones are involved in the control of the excitatory component of peristalsis [10] and longitudinal muscle motor neurones expressing CR are also excitatory.[11]

Age-related changes in gastrointestinal motility have previously been demonstrated[12] and are mainly supposed to be a consequence of altered neuronal function and morphology.[12] Nevertheless, little is known about the effect of ageing on both the general structure of the plexus and the selectivity of the process with regard to different myenteric neuronal subpopulations.

METHODS

Female guinea-pigs of three different ages were used: 1–3 months (young, weight 330–450 g), 8–10 months (adult, weight 800–950 g) and 22–26 months (old, weight 900–1200 g). Animals were killed by cervical dislocation (approved by the Ethical Committee of the Facultad de Ciencias de la Salud, Universidad Rey Juan Carlos). Segments of distal ileum, at least 10 cm oral to the ileocaecal junction, were obtained, opened along the mesenteric border, rinsed and pinned flat on a Sylgard-coated Petri-dish filled with phosphate buffered

saline (PBS). Care was taken to stretch the tissue to its maximal extension. After conventional fixation and clearing of tissues,[9] mucosa, submucosa and circular muscle layers were removed. These whole-mount preparations were stored at 4 °C in PBS with sodium azide (0.1%), until their immunohistochemical processing.

Conventional indirect immunofluorescence procedures were applied for double-labelling of the whole-mount preparations.[13] Tissues were incubated overnight at room temperature with a mixture of mouse streptavidin-conjugated anti-Hu (1 : 500, Molecular Probe, Inc., Eugene, OR, USA), and goat anti-CR (1 : 1000, Chemicon International, Inc., Temecula, CA, USA). After washing with PBS (3 × 10 min), tissues were exposed for 90 min at room temperature to a mixture of avidin-AlexaFluor 488 (1 : 1000, Molecular Probes) and donkey anti-goat-RRX (1 : 100, Jackson Immuno Research Europe Ltd, Cambridgeshire, UK). In another set of experiments, tissues were double-labelled with a mixture of goat anti-CR and mouse anti-NFT (1 : 500, generously gifted by Dr Vitadello, Italy); the corresponding secondary antibodies were donkey anti-goat-RRX (1 : 100) and donkey anti-mouse-CY2 (1 : 100, Jackson). All mixtures of antibodies were diluted with hypertonic PBS (1.7% NaCl).[7,14]

The preparations were first observed under a fluorescent microscope (Nikon Eclipse TE2000-U, with appropriate filters, Nikon Instech Co. Ltd, Kanagawa, Japan). Quantitative analysis was carried out with a confocal microscope (LSM 510 Zeiss, lasers: 488 and 453 nm, Pacisa and Giralt, Madrid, Spain), by taking 25 micrographs at 20× which were mounted as a mosaic of 0.5 cm^2. Three mosaics were made for each preparation, separated 15 μm in depth, in order to ensure that every neuron was counted.

All counts of myenteric neurones were made by an experimenter blind to the age of the guinea-pig from which the tissue originated. For each preparation labelled for Hu and CR no less than 400 cells immunoreactive to Hu were counted and the proportion of CR-immunoreactive (CR-IR) neurones was obtained. For each preparation labelled for CR and NFT, at least 100 cells immunoreactive to CR were counted and the proportions of CR^+-NFT^+ or CR^+-NFT^- immunoreactive neurones were determined.

The general structure of the plexus was analysed. Ganglia were considered as agglomerates of three or more juxtaposed nerve cell bodies separated by about 50 μm, which is the diameter of a cell body and its surrounding nerve fibre varicosities.[5] Ganglia were classified on the basis of their surface area as large (L, > 45 000 μm^2), medium (M, between 20 000 and 45 000 μm^2) and small (S, <20,000 μm^2) ganglia. The density of each kind of ganglia was evaluated. The ganglionic area was also measured and expressed as percentage of the total area visualized.

To quantitatively analyse the general myenteric neuronal population, different parameters were measured and averaged: the number of cells per ganglion (either as previously defined, or, for comparison with previous data, including isolated neurones or pairs of neurones), the spatial density (number of neurones per serosal surface unit), the density of intra-ganglionic neurones per ganglionic area (intra-ganglionic neuronal density, I-GND), and the spatial density of extra-ganglionic neurones (extra-ganglionic neuronal density, E-GND).

Controls for double-labelling were performed by pairing the wrong primary and secondary antibodies or by avoiding exposure to the primary antibody. No specific labelling was observed in either case.

All results are expressed as mean ± SEM. *n* indicates the number of samples and is equivalent to the number of experimental animals. Comparisons across groups were made using one-way ANOVA followed by the Bonferroni *post hoc* test, or the unpaired Student's *t*-test where necessary. $P < 0.05$ was considered to be statistically significant.

RESULTS

A comparison of the general structure of the myenteric plexus from young and old guinea-pigs is presented in Fig. 1.

Although spatial density of ganglia was not significantly altered in aged guinea-pigs (Fig. 2A), a statistically significant age-dependent reduction in ganglionic area was found (Fig. 2B). The density of large and medium ganglia was significantly reduced in adult and old guinea-pigs, and there was a tendency to an increase in the proportion of small ganglia (Fig. 2C).

There was an age-related reduction in the mean number of cells per ganglion, with slight differences depending on the criteria to define it (Fig. 3A and B). The maximum number of neurones per ganglion found in young animals was 222 neurones (the ganglionic area of this ganglion was 160 487 μm^2), meanwhile in adult animals it was 197 (ganglionic area = 131 416 μm^2) and in old guinea-pigs it was 157 (ganglionic area = 169 342 μm^2).

There was an age-dependent decrease in the spatial neuronal density (Fig. 3C). Most cells were found inside the ganglia and their I-GND was also age-dependently reduced (Figs 4A, C and 5A). In contrast, the E-GND (Fig. 5B) and the proportion of extra-ganglionic neurones (Fig. 5C) significantly increased with age.

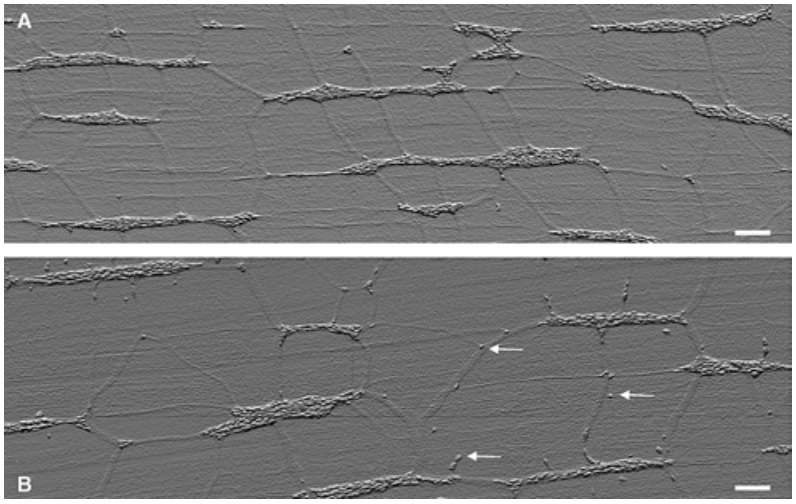

Figure 1 General structure of ileal myenteric plexus in young (A) and old (B) guinea-pigs. Myenteric neurones were labelled with the pan-neuronal marker anti-Hu (black and white micrographs were embossed with ACDSee v5.0). Note the reduction in area occupied by ganglia and the higher presence of extra-ganglionic neurones in B (*arrows*). Scale bar: 200 μm.

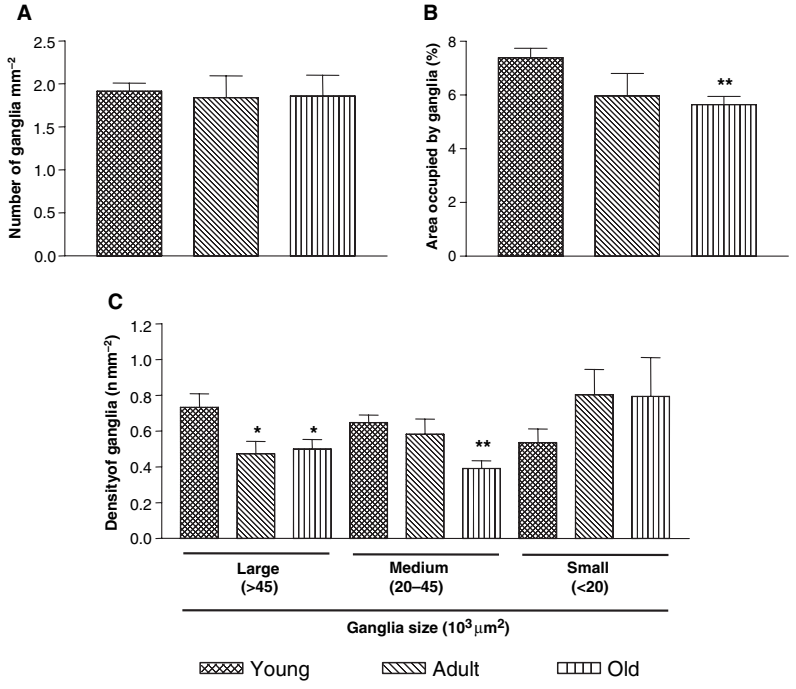

Figure 2 Age-related changes in the structure of guinea-pig ileum myenteric plexus: (A) number of ganglia per surface unit; (B) area occupied by ganglia; (C) density of large, medium and small ganglia. Values are mean ± SEM for young ($n = 7$), adult ($n = 5$) and old ($n = 5$) guinea-pigs. $^*P < 0.05$, $^{**}P < 0.01$ *vs* young.

The density of neurones expressing CR was significantly reduced with age (Figs 4B, D and 6A), as was the proportion of this population in the general neuronal population (Fig. 6B). The proportion of CR-IR neurones that also expressed NFT increased in aged animals (Figs 7 and 8).

Finally, in tissues from aged guinea-pigs, either control or labelled with anti-Hu, dots of an autofluorescent material, optically similar to lipofuscin, were patent in some neurones (Fig. 9). This material appeared more frequently in CR-IR neurones than in the general neuronal population (Table 1).

DISCUSSION

Within the longest segment of the guinea-pig small intestine, the pattern of the myenteric plexus has been described as being uniform.[5,15] Guinea-pig ileum is the gastrointestinal region best characterized to date both morphologically and functionally.[16] Therefore, we concentrated on this region, particularly the distal section of the ileum, far enough from both the duodenum and the ileocaecal junction, where this uniformity might somehow be compromised.

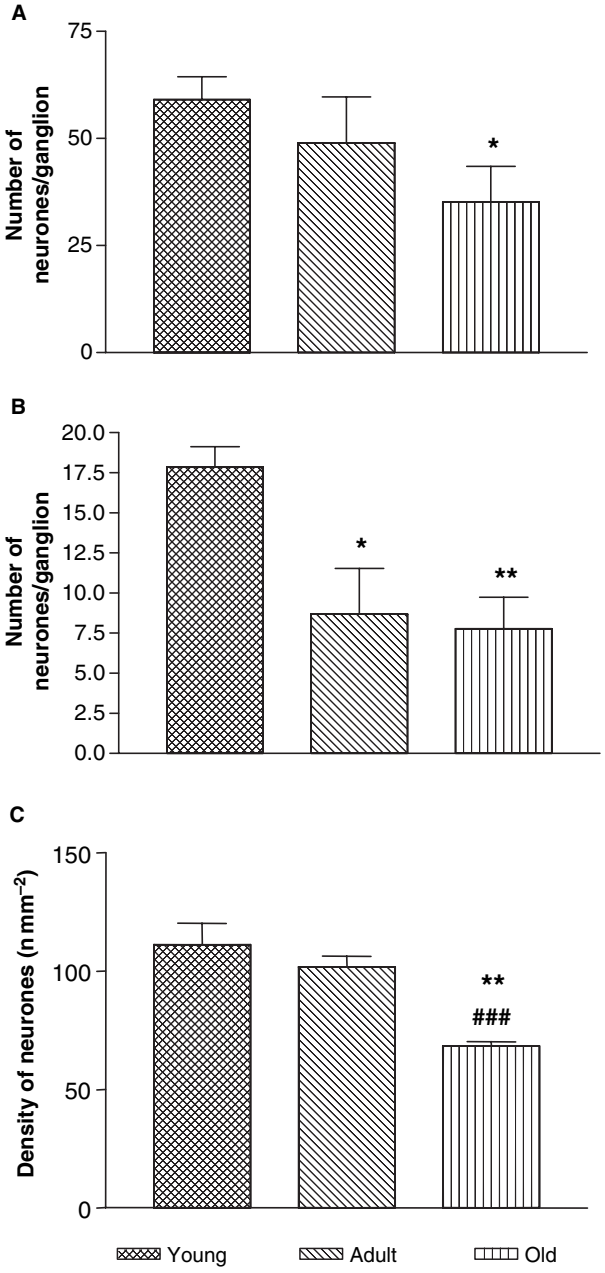

Figure 3 Age-related changes in the general neuronal population (Hu- immunoreactive, Hu-IR) in guinea-pig ileum myenteric plexus: (A) number of Hu-IR neurones per ganglion with three or more cells; (B) number of Hu-IR neurones per ganglion with one or more cells; (C) spatial neuronal density or number of neurones per serosal surface unit. Values are mean ± SEM for young ($n = 7$), adult ($n = 4$) and old ($n = 4$) guinea-pigs. *$P < 0.05$, **$P < 0.01$ *vs* young, ###$P < 0.001$ *vs* adult.

It is relatively easy to establish the spatial density of neurones in whole-mount preparations with a fair degree of accuracy because of the longitudinal and

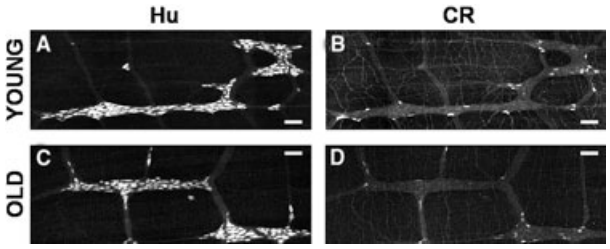

Figure 4 Myenteric ganglia in preparations from young (A, B) and old (C, D) guinea-pigs. Myenteric neurones were double-labelled with anti-Hu (A, C) and anti-CR (B, D). Note the reduced packing density (I-GND) of myenteric neurones and the higher presence of extraganglionic neurones in C. Some of both the intraganglionic and extraganglionic neurones were immunoreactive to CR (B, D). Scale bar: 100 μm.

circumferential uniformity of the pattern of the myenteric plexus in the ileum.[15] Whole-mount preparations were stretched for counting so that the neurones almost formed a cellular monolayer within the ganglia, thus no cells would be missed because of cellular overlap.[17] This was further ensured by taking pictures of myenteric plexus at three levels of depth: the level in the middle provided most of the neurones for the counting, and no more than 0.1% of neurones were found to be present only in the upper or the lower levels.

Several methods have been used to estimate the general neuronal population. A comparison of our results for young animals with those previously reported can be found in Table 2.

The analysis of the number of neurones per myenteric ganglion [2] requires a precise definition of ganglion for its applicability. This method has been questioned, for it could provide only a rough approximation of the innervation density.[17] Thus, many investigations may have favoured large and intermediate ganglia and underestimated most small ganglia.[3,20] In fact, we have found much higher ganglionic density than other authors.[5,21]

Regarding the spatial density, the major difference between our results and those of others, seems to be due to the different marker used to label the total population of neurones. Thus, the NADH diaphorase histochemical technique[4] has already been demonstrated to stain only 80% of the total population of neurones.[3] On the contrary, the spatial neuronal density obtained with Hu[20] and other similar markers such as 'nerve cell body' (NCB),[3] protein gene product 9.5 (PGP 9.5), neurone specific enolase (NSE), c-myc and fos-related antigens (FRA)[2,20] is quite lower than that obtained with cuprolinic blue.[17] The nature of those hypothetical neurones (35%), that cuprolinic

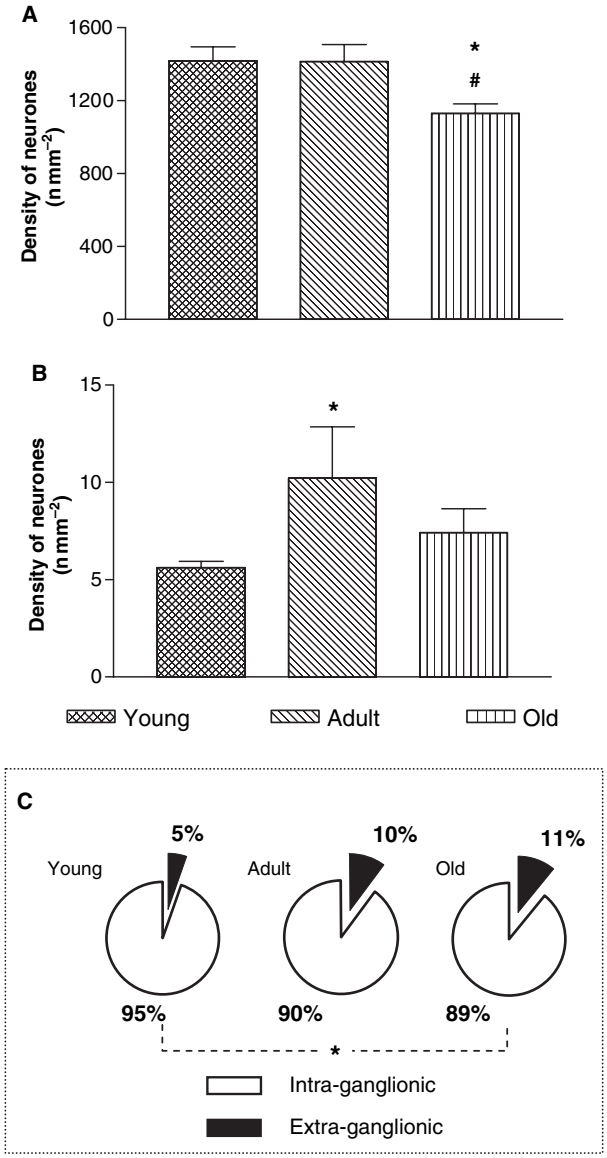

Figure 5 Age-related changes in the distribution of myenteric neurones in guinea-pig ileum: (A) intra-ganglionic neuronal density or packing density (I-GND); (B) extra-ganglionic neuronal density (E-GND); (C) distribution of neurones inside and outside the ganglia. Values are mean ± SEM for young ($n = 7$), adult ($n = 4$) and old ($n = 4$) guinea-pigs. $^{*}P < 0.05$ *vs* young, $\#P < 0.05$ *vs* adult.

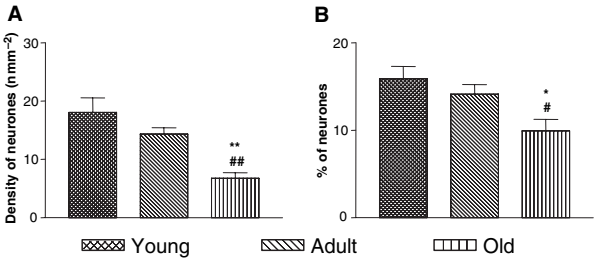

Figure 6 Age-related changes in the population of CR-immunoreactive (CR-IR) neurones in guinea-pig ileum myenteric plexus: (A) number of CR-IR neurones per serosal surface unit; (B) proportion of CR-IR neurones in the general population of myenteric neurones (Hu-IR). Values are mean ± SEM for young ($n = 7$), adult ($n = 4$) and old ($n = 4$). $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* young, $\#P < 0.05$, $\#\#P < 0.01$ *vs* adult.

blue stains but are not immunoreactive to markers traditionally considered as pan-neuronal is unknown.

Finally, the I-GND, also called packing density,[17] has been shown to remain constant throughout the gut, regardless of the intestinal region considered or the degree of stretching of the tissue.[17] We obtained a very similar packing density to the packing density obtained when FRA was used as a pan-neuronal marker.[17]

Several investigations have reported changes in the numbers of myenteric neurones with age, but the structure of the plexus itself has, to date, received much less attention, partly because of the conceptual difficulties in defining a ganglion and its boundaries. In order to accurately study the effect of age on the structure of the plexus, we have considered four groups of 'ganglia': those containing isolated neurones or pairs of neurones (extra-ganglionic neurones), and those containing sets of three or more neurones ('true' ganglia), which we have divided into large, medium and small ganglia.

With regards to these 'true' ganglia, we have found an age-related reduction in the area they occupy, mainly because of a decrease in their size. In aged animals there is an increase in length and diameter of the small intestine, that could produce a 'diluting' effect of ganglia, fibres and neurones in the myenteric plexus.[22] Therefore, one would expect a lower number of ganglia per surface unit, if their size remained constant, or an increase in the size, if the density remains constant. It has been shown that experimental stretching of ileal preparations at the time of fixation does not affect the myenteric ganglia, but changes the area of the non-neuronal elements of the tissue.[17] Age-dependent 'stretching' of the gut could similarly affect only the non-neural components of the tissue, and one would expect no change in the size of the ganglia, implying a reduction in ganglionic density. With the same ganglionic density, a reduction in the ganglionic area could be partly attributed to the breakage of the ganglia into smaller units.

On the contrary, both E-GND and proportion of myenteric neurones lying outside the ganglia significantly increased with age. Gabella mentioned the

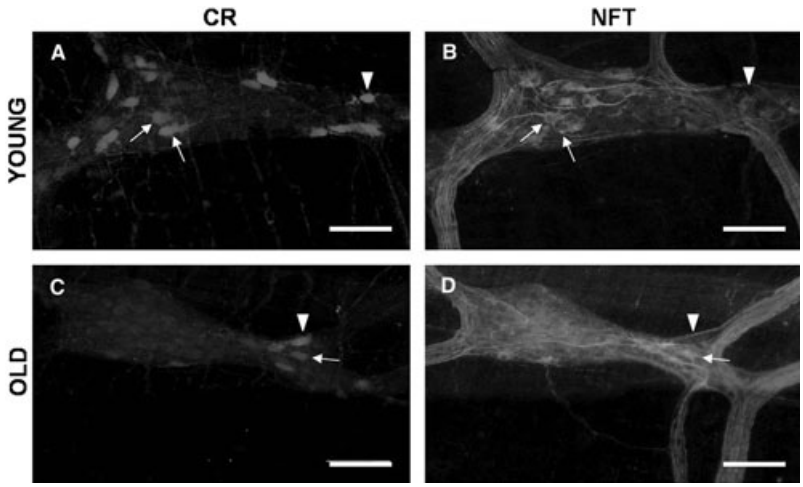

Figure 7 Myenteric ganglia in preparations from young (A, B) and old (C, D) guinea-pigs. Myenteric neurones were double-labelled with anti-CR (A, C) and anti-NFT (B, D). Note the neurones inmunoreactive for both CR and NFT (*arrows*), and neurones inmunoreactive for CR but not for NFT (*arrow heads*). Scale bar: 100 μm.

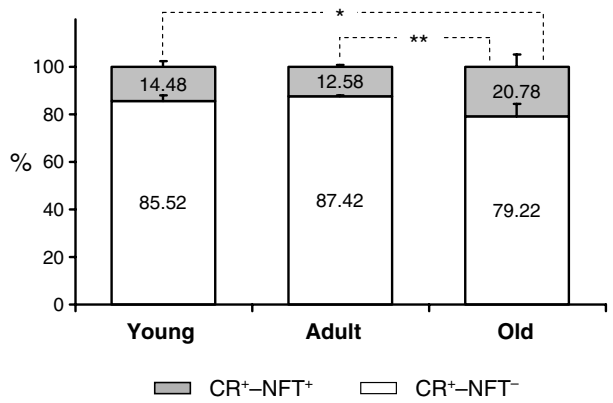

Figure 8 Age-related changes in the subpopulations of CR-immunoreactive (CR-IR) myenteric neurones revealed by neurofilament triplet protein (NFT) immunohistochemistry in guinea-pig ileum. Bars represent the proportions of CR-IR neurones with (CR^+-NFT^+) or without (CR^+-NFT^-) NFT expression. Values are mean ± SEM for young ($n = 5$), adult ($n = 6$) and old ($n = 6$). $^*P < 0.05$, $^{**}P < 0.01$.

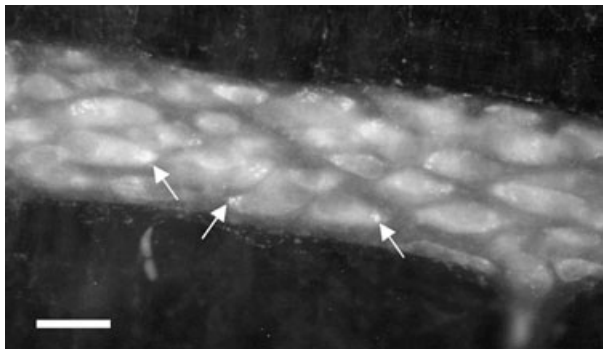

Figure 9 Lipofuscin-like material in old guinea-pigs. Myenteric neurones were labelled with anti-Hu. This microphotograph was taken at 40× amplification with a fluorescence microscope fitted with a filter cube adequate for visualisation of AlexaFluor 488. Note the accumulation of dots of this material in the perikarya of some neurones (*arrows*).

presence of two kinds of isolated, also called extra-ganglionic,[5] neurones in the plexus, those lying along connecting meshes, usually at branching points, and those lying outside the outline of a ganglion, almost as if adhering to its surface, or connected to it by a short peduncle, which seemed to be more numerous in older animals.[4] We have also observed this phenomenon and pooled both kinds of neurones together for our analysis. Once again, the increase in the population of extra-ganglionic neurones of the first kind could easily be attributed to a circumferential breakage of the smallest ganglia into even smaller units, whilst the increase in the population of extra-ganglionic neurones of the second kind seems more related to the 'escape' of ganglionic neurones because of the elongation of the intestine. In both cases, mechanistic forces could exert a disaggregating effect because of ageing. A similar disaggregating effect in the myenteric plexus has also been suggested in developmental studies of the enteric nervous system.[23] Myenteric neuronal progenitors (neural crest cells) and later myenteric neurones seem to tend to aggregate (population pressure) in the intestine,[23] while at the same time, the growing process of the intestine in length and diameter, forces the aggregations to break and form smaller groups and, sometimes, well-defined ganglia soon after birth and postnatally.[24] It is conceivable then, that this process continues until the maximum size of the intestine is reached in adult/old age.

The reduction in ganglionic size and the increase in extra-ganglionic neurones with age could also be due to other reasons, mainly a general loss of neurones. If age induced such a process, then neuronal density inside

Table 1 Age-related changes in the content of lipofuscin-like autofluorescent material in the general neuronal population (Hu-IR) and CR-immunoreactive (CR-IR) neurones in guinea-pig ileum myenteric plexus

	1–2 months		9–10 months		22–26 months	
	Hu-IR ($n = 6$)	CR-IR ($n = 5$)	Hu-IR ($n = 4$)	CR-IR ($n = 4$)	Hu-IR ($n = 5$)	CR-IR ($n = 5$)
0	99.68 ± 0.20	90.60 ± 0.97*	46.75 ± 12.68	48.99 ± 2.21 ns	31.04 ± 10.80	30.30 ± 5.54 ns
1	0.34 ± 0.24	9.40 ± 0.97*	52.58 ± 12.61	46.01 ± 3.68 ns	39.52 ± 8.98	30.54 ± 7.78 ns
2	0.00 ± 0.00	0.00 ± 0.00	0.67 ± 0.32	5.00 ± 3.97 ($P = 0.0726$)	29.44 ± 13.57	39.16 ± 11.88 ns

Each value in the table represents the mean ± SEM proportion of myenteric neurones containing no (0), a little (1) or a great (2) amount of lipofuscin-like material. At least 300 neurones immunoreactive to Hu and 100 neurones expressing CR were counted in each preparation.
*$P < 0.001$, ns = not significant.

Table 2 Comparison of the methods used for estimation of the myenteric neuronal population

Method for neuronal counting	Marker used	Results	Reference
Maximum number of neurones/ganglion	Hu	220	Present results
	Toluidine blue	167	(18)
	*	200	(1)
	Anti-FOS 4	461	(2)
Mean number of neurones/ganglion	Hu	18	Present results (One or more cells/ganglion)
	Hu	59	(Three or more cells/ganglion)
	Toluidine blue	43	(18) (four or more cells/ganglion)
	FRA	53	(2)†
	NCB	105	(3)†
	Propidium iodide	100	(19)†
	Hu	93	(20)†
Spatial density (neurones cm^{-2})	Hu	11 300	Present results
	NADH diaphorase	8600	(4)
	NCB	17 300	(3)‡
	NCB	12 400	(3)§
	Cuprolinic blue	16 000	(17)
	FRA	10 400	(17)
I-GND or packing density (neurones cm^{-2} of ganglionic area)	Hu	140 000	Present results
	Cuprolinic blue	250 000	(17)
	FRA	160 000	(17)

*Analysis of ultrastructure (electronic microscopy).
†Minimum number of neurones/ganglion not determined.
‡Measured in relaxed undistended ileum.
§Estimated for strongly distended ileum.
FRA, fos-related antigen; NCB, nerve cell body; I-GND, intra-ganglionic neuronal density.

the ganglia could be reduced (in fact, I-GND was significantly reduced in a parallel way to the total neuronal density) and eventually some of these ganglia could result in several smaller ganglia. In addition, ganglia which are already small enough in the young animals could easily result in isolated neurones or pairs of neurones. This would explain the relative increase in E-GND shown in Fig. 3C. The loss of neurones outside the ganglia would explain the reduction in density of extra-ganglionic neurones from adult to old animals (Fig. 3C), and a concomitant loss of neurones inside the ganglia would explain the increase in the proportion of extra-ganglionic neurones from young to old guinea-pigs (Fig. 3D).

Neuronal loss with age in the myenteric plexus of the gastrointestinal tract has been observed in a variety of species including humans,[25,26] sheep,[27] guinea-pigs,[12,22,28] rats[29–31] and mice.[32] In small mammals, this neuronal loss is already significant by 12 months of age.[31] The process of neuronal loss is likely to start even earlier, as we have shown significant reductions in guinea-pigs as early as 9–10 months old compared with 2–3 month-old guinea-pigs.

A general loss of myenteric intrinsic innervation could be involved in the physiopathological alterations of gastrointestinal motility associated with the ageing process.[12,33,34] Most of these alterations would be much better explained, though, if some specificity existed in the process of neuronal loss, that is, if the various specific phenotypes of myenteric neurones had different sensitivity to ageing. Up to 16 functional populations of myenteric neurones have been proposed to exist in the guinea-pig ileum.[7,8] This functional classification is based on the morphology, electrophysiology and the combination of markers (chemical coding) expressed by the neurones. The expression of some enteric markers has been shown to change with age, meaning that the functional subpopulations of neurones immunoreactive to them could be quantitatively altered. It has been suggested that serotonin-immunoreactive neurones [28] and nitrergic neurones [35,36] could be somehow spared, while vasoactive intestinal peptide-, substance P- and somatostatin-immunoreactive fibres,[37] cholinergic neurones[36] and calbindin-immunoreactive neurones[28] could be relatively more sensitive to the ageing process.

Calretinin is a calcium-binding protein that is expressed by neurones in a tissue-specific fashion in both central and peripheral nervous systems.[38–40] It has been used as a marker for functional classification of enteric neurones.[7,41] It is present in approximately 25% of the whole myenteric neuronal population.[9] We have found only 16% of neurones expressing CR in young guinea-pigs. This discrepancy may be due to differences in guinea-pig strains, sex, age or weight. Additionally, the previous report could have miscounted some of the extraganglionic neurones or neurones inside relatively small ganglia. On the contrary, as our analysis was photograph-based, we could have miscounted some of the CR-IR neurones if this immunoreactivity was relatively faint.

We have found an age-related reduction in the expression of CR in myenteric neurones from guinea-pig ileum. Furthermore, from our results it could be suggested that CR-IR neurones are more sensitive to the process of ageing than other myenteric neuronal populations, as not only did the density but also the proportion of these neurones decreased with age. CR, like other calcium binding proteins, has been shown to have specific temporal expression patterns in the central nervous system.[40] To our knowledge, though, no study so far has addressed the age-related changes in the expression of CR in the guinea pig enteric nervous system. Gabella[22] found a smaller proportion of large myenteric neurones in aged guinea-pigs. In guinea-pig ileum, the largest myenteric neurones, which are immunoreactive for calbindin, another calcium binding protein, belong mainly to the intrinsic sensory neuronal class. The finding by Gabella suggests that this functional class could be relatively more affected by the age-related neuronal loss. In fact, some evidence of specific age-dependent loss of neurones expressing calbindin has been found in the submucous plexus.[28] On the contrary, immunoreactivity to neurocalcin, which is another calcium binding protein, has also been shown to decrease in aged animals, although the proportion of neurocalcin-immunoreactive cells was not analysed.[42]

From these data it seems likely that cells expressing calcium binding proteins are relatively more sensitive to the process of ageing than others. Calcium binding proteins have been shown to be essential for intracellular calcium homeostasis. Their major role is assumed to be buffering, transport of Ca^{2+} and regulation of various enzyme systems. As cellular degeneration is accompanied by impaired Ca^{2+} homeostasis, a protective role for Ca^{2+}-binding proteins in certain neuronal populations has been postulated.[43,44] More recently, though, CR, calbindin and parvalbumin have been suggested to be involved in calcium sensing and regulation of calcium pools critical for synaptic plasticity,[45] and CR calcium-buffering protective role has been questioned.[46] If such is the case in the myenteric plexus, those neurones expressing CR could not be protected against ageing, but even relatively more sensitive to this process.

In guinea-pig small intestine, CR is expressed only by two functional subpopulations of myenteric neurones: ascending interneurones and excitatory longitudinal muscle motor neurones.[7–9] Ascending interneurones were found to be located in clusters of two to eight cells, on either surface of the ganglia, and comprised approximately 5% of all cells in the myenteric plexus.[9] On the contrary, longitudinal muscle motor neurones, which accounted for 20% of the total myenteric neuronal population, were often found located in small clusters of two to five cells near the roots of the internodal strands, and, occasionally, in isolated 'microganglia' of one to three cells in the tertiary plexus or on internodal strands.[11] More important than their anatomical location within the

plexus, their expression or not of NFT, a set of three intermediate filament proteins of the neuronal cytoskeleton, seems to be the immunohistochemical key for differentiation of both CR-IR subpopulations. Thus, ascending interneurones express NFT and excitatory longitudinal muscle motor neurones do not.[7,11] The proportions of CR^+-NFT^+ and CR^+-NFT^- neurones found in the present work were compatible with previous reports.[7] This distribution, though, changed with age in a significant manner and the proportion of CR^+-NFT^+ neurones increased by 40%, meanwhile a relative decrease of 6% was appreciated for CR^+-NFT^- neurones. It could be interesting to determine to which extent these changes could affect functionality.

Nevertheless, from our results it is not possible to discard that the changes in the proportion of CR-IR neurones might only be due to temporal changes in the expression of this marker. In other words, some neurones that expressed CR in the young guinea-pigs, could stop doing so at a later stage; likewise, some neurones that did not express it in the young animals, could do so in the aged ones. Further studies, such as retrograde labelling of organotypic cultured tissues from aged guinea-pigs combined with immunohistochemistry for CR and other markers, especially NFT, would be necessary to ascertain whether the CR-IR cells found in this work actually keep their functional role as ascending interneurones or longitudinal muscle motor neurones.

Dots of an autofluorescent material were found in some cells when the tissue was observed under fluorescence microscopy. This yellow/gold material, which has been previously named 'the age-pigment' and assumed to be lipofuscin,[42] could be seen in adult and old animals also in non-labelled tissues. Lipofuscin is a fluorescent substance (excitation maximum ≈440 nm, emission maximum ≈600 nm) that accumulates in lysosomes and is thought to be an indicator of oxidative stress and ageing.[47] Our results show that the autofluorescent pigment accumulate in a relatively greater and faster way in CR-IR neurones as compared with the general myenteric neuronal population. Lipofuscin was found to be absent in neurocalcin-immunoreactive myenteric neurones from aged rats.[42] These differences in the accumulation of lipofuscin-like material further suggest that the different functional classes of myenteric neurones could age at different rates. Further studies are needed in order to elucidate whether the presence of this pigment is a marker of selective preservation or predegeneration of specific functional classes of myenteric neurones.

In conclusion, the present work describes age-related modifications in both the general structure of the myenteric plexus of guinea-pig ileum and in the expression of the neuronal marker CR. A general age-dependent neuronal loss underlies these modifications. Nevertheless, phenotypically different myenteric neurones seem to have differential sensitivity to age. Whether or not this fact is a reflection of age-related alterations in gastrointestinal motility remains to be elucidated.

ACKNOWLEDGMENTS

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REFERENCES

1 Gabella G, Trigg P. Size of neurons and glial cells in the enteric ganglia of mice, guinea-pigs, rabbits and sheep. *J Neurocytol* 1984; **13**: 49–71.
2 Parr EJ, Sharkey KA. The use of constitutive nuclear oncoproteins to count neurons in the enteric nervous system of the guinea pig. *Cell Tissue Res* 1994; **277**: 325–31.
3 Young HM, Furness JB, Sewell P, Burcher EF, Kandiah CJ. Total numbers of neurons in myenteric ganglia of the guinea-pig small intestine. *Cell Tissue Res* 1993; **272**: 197–200.
4 Gabella G. The number of neurons in the small intestine of mice, guinea-pigs and sheep. *Neuroscience* 1987; **22**: 737–52.
5 Furness JB, Trussell DC, Pompolo S, Bornstein JC, Smith TK. Calbindin neurons of the guinea-pig small intestine: quantitative analysis of their numbers and projections. *Cell Tissue Res* 1990; **260**: 261–72.
6 Bornstein JC, Furness JB, Kunze WA. Electrophysiological characterization of myenteric neurons: how do classification schemes relate? *J Auton Nerv Syst* 1994; **48**: 1–15.
7 Costa M, Brookes SJ, Steele PA, Gibbins I, Burcher E, Kandiah CJ. Neurochemical classification of myenteric neurons in the guinea-pig ileum. *Neuroscience* 1996; **75**: 949–67.
8 Brookes SJ. Classes of enteric nerve cells in the guinea-pig small intestine. *Anat Rec* 2001; **262**: 58–70.
9 Brookes SJ, Steele PA, Costa M. Calretinin immunoreactivity in cholinergic motor neurones, interneurones and vasomotor neurones in the guinea-pig small intestine. *Cell Tissue Res* 1991; **263**: 471–81.
10 Brookes SJ, Meedeniya AC, Jobling P, Costa M. Orally projecting interneurones in the guinea-pig small intestine. *J Physiol* 1997; **505**: 473–91.
11 Brookes SJ, Song ZM, Steele PA, Costa M. Identification of motor neurons to the longitudinal muscle of the guinea pig ileum. *Gastroenterology* 1992; **103**: 961–73.
12 Wade PR. Aging and neural control of the GI tract. I. Age-related changes in the enteric nervous system. *Am J Physiol Gastrointest Liver Physiol* 2002; **283**: G489–95.

13 Costa M, Furness JB, Gibbins IL. Chemical coding of enteric neurons. *Prog Brain Res* 1986; **68**: 217–39.
14 Grube D. Immunoreactivities of gastrin (G-) cells. II. Non-specific binding of immunoglobulins to G-cells by ionic interactions. *Histochemistry* 1980; **66**: 149–67.
15 Gabella G. On the plasticity of form and structure of enteric ganglia. *J Auton Nerv Syst* 1990; **30** (Suppl): S59–66.
16 Furness JB, Costa M. *The Enteric Nervous System*. Edinburgh, London, Melbourne, New York: Churchill Livingston, 1987.
17 Karaosmanoglu T, Aygun B, Wade PR, Gershon MD. Regional differences in the number of neurons in the myenteric plexus of the guinea pig small intestine and colon: an evaluation of markers used to count neurons. *Anat Rec* 1996; **244**: 470–80.
18 Wilson AJ, Furness JB, Costa M. The fine structure of the submucous plexus of the guinea-pig ileum. I. The ganglia, neurons, Schwann cells and neuropil. *J Neurocytol* 1981; **10**: 759–84.
19 Takaki M, Wood JD, Gershon MD. Heterogeneity of ganglia of the guinea pig myenteric plexus: an in vitro study of the origin of terminals within single ganglia using a covalently bound fluorescent retrograde tracer. *J Comp Neurol* 1985; **235**: 488–502.
20 Lin Z, Gao N, Hu HZ *et al.* Immunoreactivity of Hu proteins facilitates identification of myenteric neurones in guinea-pig small intestine. *Neurogastroenterol Motil* 2002; **14**: 197–204.
21 Tafuri WL. Auerbach's plexus in the guinea-pig. I. A quantitative study of the ganglia and nerve cells in the ileum, caecum and colon. *Acta Anat (Basel)* 1957; **31**: 522–30.
22 Gabella G. Fall in the number of myenteric neurons in aging guinea pigs. *Gastroenterology* 1989; **96**: 1487–93.
23 Young HM, Bergner AJ, Anderson RB, Newgreen DF, Whitington PF. Dynamics of neuronal crest cell migration in the embryonic mouse gut. *Little-Brain Big-Brain, Meeting VIII* 2003; 23.
24 Young HM, Newgreen D. Enteric neural crest-derived cells: origin, identification, migration, and differentiation. *Anat Rec* 2001; **262**: 1–15.
25 de Souza RR, Moratelli HB, Borges N, Liberti EA. Age-induced nerve cell loss in the myenteric plexus of the small intestine in man. *Gerontology* 1993; **39**: 183–8.
26 Gomes OA, de Souza RR, Liberti EA. A preliminary investigation of the effects of aging on the nerve cell number in the myenteric ganglia of the human colon. *Gerontology* 1997; **43**: 210–7.
27 Pfannkuche H, Schellhorn C, Schemann M, Aschenbach JR, Gabel G. Age-associated plasticity in the intrinsic innervation of the ovine rumen. *J Anat* 2003; **203**: 277–82.
28 Wade PR, Scheer G, Regan K, Lieb J. Age-related changes in colonic motility and myenteric neuronal populations. *Soc Neurosci Abstr* 2001; **27**: 840.812.
29 Santer RM, Baker DM. Enteric neuron numbers and sizes in Auerbach's plexus in the small and large intestine of adult and aged rats. *J Auton Nerv Syst* 1988; **25**: 59–67.
30 Cowen T, Johnson RJ, Soubeyre V, Santer RM. Restricted diet rescues rat enteric motor neurones from age related cell death. *Gut* 2000; **47**: 653–60.
31 Phillips RJ, Powley TL. As the gut ages: timetables for aging of innervation vary by organ in the Fischer 344 rat. *J Comp Neurol* 2001; **434**: 358–77.
32 El Salhy M, Sandstrom O, Holmlund F. Age-induced changes in the enteric nervous system in the mouse. *Mech Ageing Dev* 1999; **107**: 93–103.
33 O'Mahony D, O'Leary P, Quigley EM. Aging and intestinal motility: a review of factors that affect intestinal motility in the aged. *Drugs Aging* 2002; **19**: 515–27.
34 Firth M, Prather CM. Gastrointestinal motility problems in the elderly patient. *Gastroenterology* 2002; **122**: 1688–700.
35 Santer RM. Survival of the population of NADPH-diaphorase stained myenteric neurons in the small intestine of aged rats. *J Auton Nerv Syst* 1994; **49**: 115–21.
36 Phillips RJ, Kieffer EJ, Powley TL. Aging of the myenteric plexus: neuronal loss is specific to cholinergic neurons. *Auton Neurosci* 2003; **106**: 69–83.
37 Feher E, Penzes L. Density of substance P, vasoactive intestinal polypeptide and somatostatin-containing nerve fibers in the ageing small intestine of the rats. *Gerontology* 1987; **33**: 341–8.
38 Rogers J, Khan M, Ellis J. Calretinin and other CaBPs in the nervous system. *Adv Exp Med Biol* 1990; **269**: 195–203.
39 DeFelipe J. Types of neurons, synaptic connections and chemical characteristics of cells immunoreactive for calbindin-D28K, parvalbumin and calretinin in the neocortex. *J Chem Neuroanat* 1997; **14**: 1–19.
40 Kwong WH, Chan WY, Lee KK, Fan M, Yew DT. Neurotransmitters, neuropeptides and calcium binding proteins in developing human cerebellum: a review. *Histochem J* 2000; **32**: 521–34.
41 Jenkinson KM, Morgan JM, Furness JB, Southwell BR. Neurons bearing NK(3) tachykinin receptors in the guinea-pig ileum revealed by specific binding of fluorescently labelled agonists. *Histochem Cell Biol* 1999; **112**: 233–46.
42 Corns RA, Hidaka H, Santer RM. Neurocalcin-alpha immunoreactivity in the enteric nervous system of young and aged rats. *Cell Calcium* 2002; **31**: 53–8.
43 Baimbridge KG, Celio MR, Rogers JH. Calcium-binding proteins in the nervous system. *Trends Neurosci* 1992; **15**: 303–8.
44 Heizmann CW. Calcium-binding proteins: basic concepts and clinical implications. *Gen Physiol Biophys* 1992; **11**: 411–25.
45 Schwaller B, Meyer M, Schiffmann S. 'New' functions for 'old' proteins: the role of the calcium-binding proteins calbindin D-28k, calretinin and parvalbumin, in cerebellar physiology. Studies with knockout mice. *Cerebellum* 2002; **1**: 241–58.
46 Billing-Marczak K, Kuznicki J. Calretinin-sensor or buffer-function still unclear. *Pol J Pharmacol* 1999; **51**: 173–8.
47 Harman D. Lipofuscin and ceroid formation: the cellular recycling system. *Adv Exp Med Biol* 1989; **266**: 3–15.

4.2. Segunda publicación:

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4.2.1. Objetivo general

El objetivo general del trabajo fue describir cómo afecta el envejecimiento a la funcionalidad y la inmunorreactividad del receptor cannabinoide CB1 en preparaciones FL-PM de íleon de cobayo.

4.2.2. Objetivos específicos

Estudiar:

- La influencia de la edad en la inhibición, por agonistas cannabinoides, de la contracción inducida eléctricamente.
- La población de neuronas mientéricas inmunorreactivas para el receptor cannabinoide CB1.

4.2.3. Resultados

El estudio de la funcionalidad del receptor cannabinoide CB1 con la edad se realizó en preparaciones FL-PM de íleon de cobayo de diferentes edades. Tanto el agonista cannabinoide endógeno anandamida como el agonista no selectivo CB1-CB2 WIN 55, 212-2 (WIN) inhibieron la contracción inducida eléctricamente de manera concentración-dependiente. Las curvas dosis-respuesta obtenidas fueron similares, independientemente de la edad.

Respecto a la expresión del receptor cannabinoide, se vio que la densidad de las neuronas inmunorreactivas para dicho receptor era muy elevada, al igual que la proporción de dichas neuronas. Con la edad, la densidad de neuronas mientéricas que expresaban el receptor cannabinoide disminuyó. Sin embargo, la proporción de neuronas que expresaban el receptor no se alteró con la edad y además la proporción de neuronas inmunoreactivas para CR que también expresaban el receptor cannabinoide tampoco se modificó con la edad.

4.2.4. Conclusiones

La proporción de neuronas inmunorreactivas para el receptor CB1 no varía en los cobayos viejos, lo cual podría justificar que se mantenga el efecto inhibidor de los cannabinoides en la preparación de íleon de cobayo. Este hecho debería tenerse en cuenta a la hora de utilizar estos fármacos, bien sea como analgésicos, antieméticos u otras aplicaciones, en individuos viejos.

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Evaluation of the effect of age on cannabinoid receptor functionality and expression in guinea-pig ileum longitudinal muscle–myenteric plexus preparations

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Abstract

Cannabinoid drugs exert a wide range of biological effects and are currently under study for their multiple potential therapeutic uses. Cannabinoids reduce gastrointestinal (GI) motility and this is mediated by the CB1 cannabinoid receptor (CB1R) present in the myenteric neurones. GI motility can also be affected by a variety of pathophysiological situations, including ageing. The purpose of this work was to study the influence of age on the functionality and expression of CB1R in the myenteric plexus. Ileal longitudinal muscle–myenteric plexus (LMMP) preparations from young, adult and old guinea-pigs were used in two sets of experiments: in vitro assessment of the inhibitory cannabinoid effect upon electrically stimulated contractions and immunohistochemical quantification of myenteric neurones expressing CB1R. LMMP preparations responded to the synthetic cannabinoid WIN 55,212-2, and the endogenous cannabinoid ligand anandamide in an age-independent manner. The total number of CB1R-immunoreactive (IR) myenteric neurones, which included at least part of the motor neurones to the longitudinal smooth muscle, decreased in proportion to the general neuronal population; however, the proportion of CB1R-IR neurones was preserved in old animals. These data may justify the preservation of the effectiveness of the cannabinoids in the isolated guinea-pig ileum. This age-related independency of CB1R expression and effect on GI motility could be of interest if cannabinoids are to be used therapeutically.

Keywords: Cannabinoid; Myenteric plexus; Ageing; Guinea-pig; Isolated ileum

Marijuana (*Cannabis sativa*) has been used for recreational and medicinal purposes for centuries. The therapeutic effects of marijuana derivatives and synthetic analogues, known as cannabinoid drugs, are surprisingly broad. This variety of effects is related to the widespread distribution of cannabinoid receptors in the brain and throughout the periphery [13,24].

In the gastrointestinal (GI) tract, it has been shown that cannabinoids exert many biological functions, including gastroprotection, reduction of intestinal secretion and reduction of gastric and intestinal motility. Most cannabinoid effects on gastrointestinal motility depend on the activation of CB1 receptors (CB1R; Table 1). CB1R mRNA has been detected in the guinea-pig ileum and rat colon [2,11], and numerous myenteric neurones, responsible for GI motility, show immunoreactivity for the receptor itself [4,20].

Although the CB1R has been found in the rat GI tract as early as in prenatal development [2], the influence of ageing on expression and functionality of CB1R in the enteric nervous system has not been addressed so far. However, it also deserves attention because cannabinoids are being studied and likely to be used in the near future for the treatment of a variety of health challenges frequently encountered in the elderly. Thus, some cannabinoid agonists are being used for the treatment of emesis induced by cancer chemotherapy [25]. Furthermore, the CB1R antagonist, rimonabant, is expected to be imminently approved for the treatment of obesity [7]. It is not known, though, to what extent responses to cannabinoid drugs could be altered in aged patients. Furthermore, in the GI tract, ageing has been associated with functional impairments

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Table 1
Summary of experimental data reporting cannabinoid effects on gastrointestinal motility

Cannabinoid effect	Species
CB1 cannabinoid receptor	
Prejunctional inhibition of electrically-evoked twitches of small intestine longitudinal smooth muscle	Guinea-pig [30], Human [6]
Inhibition of intestinal peristalsis	Guinea-pig [12]
Depression of fast cholinergic neurotransmission in S-myenteric neurones	Guinea-pig [23]
Reduction in gastric emptying	Mouse, rat [36], Rat [21]
Reduction in gastrointestinal transit	Mouse [17], Rat [21]
Reduction in faecal pellet output	Rat [17,18]
Involvement of enteric endocannabinoid system in the induction of experimental paralytic ileus by peritoneal irritation	Mouse [26]
CB2 cannabinoid receptor	
Reduction of intestinal motility induced by endotoxic inflammation	Rat [28]

such as decreased oesophageal and colonic motility, delayed gastric emptying and increased intestinal transit times [9,37]. These alterations have generally been attributed to a loss of myenteric neurones, which could be particularly greater in the case of those mediating excitatory responses [1,10,33]. It is likely that a higher endocannabinoid tone could also be involved in these age-related alterations in GI motility.

Therefore, we aimed to study the influence of age on the expression and functionality of CB1R in the myenteric plexus, which is responsible for GI motility. As a first approach to reach this objective, we used guinea-pig ileum longitudinal muscle myenteric plexus (LMMP) preparations. Myenteric neurones in this preparation have been well characterised functionally in terms of their immunohistochemical contents [3].

Dunkin–Hartley female guinea-pigs of three different ages were used: 1–3 months (YOUNG, weight 330–450 g), 8–10 months (ADULT, weight 800–950 g) and 22–26 months (OLD, weight 900–1200 g). All experimental protocols were approved by the Ethical Committee at the Universidad Rey Juan Carlos and performed in strict accordance with the EC regulation for care and use of experimental animals (EEC N° 86/609). In order to minimize pain and discomfort, animals were killed by cervical dislocation and segments of distal ileum, at least 10 cm oral to the ileocaecal junction, were obtained, opened along the mesenteric border, rinsed and pinned flat on a Sylgard-coated Petri-dish filled with modified Krebs solution (mM: NaCl; 118, KCl; 4.75, NaH_2PO4; 1.0, $NaHCO_3$; 25, $MgSO_4$; 1.2, $CaCl_2$; 2.5, glucose; 11, pH 7.4). Whole-mount LMMP preparations were obtained by removal of mucosa, submucosa and circular muscle layers. LMMP preparations were used in two different sets of experiments.

For the functional studies, LMMP preparations were suspended, under a tension of 1 g, in an organ bath containing Krebs solution (36 °C) and aerated with carbogen (95% O_2–5% CO_2). Contractile activity of the preparations was recorded by means of an isometric transducer connected to a PowerLab/4e system. Electrical field stimulation (pulses of 2 ms duration, 0.3 Hz and supramaximal voltage) was used to induce atropine-sensitive twitches of the preparations. Those preparations whose contractions did not reach 0.5 g were discarded. No atropine-sensitive differences were apparent in the number of usable preparations. The influence of age on the responses to two well-known cannabinoid agonists was tested in electrically stimulated preparations. Either WIN 55,212-2 (WIN, non-selective CB1-CB2 synthetic cannabinoid drug) or anandamide (endogenous ligand of CB1R) were added in increasing cumulative concentrations (10^{-8} to 2.4×10^{-6} M) at intervals of 15 min; the effect of the cannabinoids was evaluated as the percentage of inhibition of the initial twitch amplitude. The CB1R-selective antagonist SR141716A (10^{-6} M) was added at the end of the experiment to revert the effect of the cannabinoids. All drugs were purchased from Tocris and dissolved in ethanol following the method previously described by Pertwee et al. [32]. The effect of the vehicle was tested in preparations from young animals. At the higher concentration used, it produced a slight inhibition (less than 10%) of the electrically stimulated contractions.

Methods for the immunohistochemical study of whole-mount LMMP preparations have been described in detail in a previous report [1]. Briefly, each LMMP preparation was stretched to its maximal extension, fixed in Zamboni's fixative and cleared with dimethylsulfoxide. After several washes with phospate buffer saline (PBS), tissues were incubated overnight at room temperature with a mixture of mouse anti-Hu C/D (1:500, Molecular Probes) and rabbit anti-CB1 (1:100, generous gift from Dr Mackie, University of Washington [4]). After washing with PBS (3×10 min), tissues were exposed for 90 min at room temperature to a mixture of avidin-AlexaFluor 488 (1:1000, Molecular Probes) and donkey anti-rabbit-CY5 (1:100, Jackson). All mixtures of antibodies were diluted with hypertonic PBS (1.7% NaCl) [3]. Some preparations were further labelled for calretinin (CR), which is present in the motor neurones that innervate the longitudinal muscle in guinea-pig ileum [3]. In this case, goat anti-CR (1:1000, Chemicon) and donkey anti-goat (1:100, Jackson) antibodies were added to the mixture of primary and secondary antibodies, respectively. The preparations were first observed under a fluorescent microscope (Nikon Eclipse TE2000-U, with appropriate filters). Using a confocal microscope (LSM 510 Zeiss, lasers: 488 and 453 nm), micrographs were taken and mounted on mosaics for the quantitative analysis [1]. All counts of myenteric neurones were made by an experimenter blind to the age of the guinea-pig from which the tissue originated. For each preparation labelled for Hu and CB1 no less than 400 cells immunoreactive to Hu were counted and the proportion of CB1R-immunoreactive (IR) neurones was obtained. In preparations immunolabelled for CR, the proportion of CR-IR neurones also positive for CB1R was determined. Controls for double-labelling were performed by pairing the wrong primary and

secondary antibodies or by avoiding exposure to the primary antibody. No specific labelling was observed in either case.

All results are expressed as mean ± S.E.M. The letter *n* indicates the number of samples and is equivalent to the number of experimental animals. Comparisons were made taking the group of YOUNG animals as reference, because this age is the most commonly used for experimental purposes. Comparisons across groups were made using one-way ANOVA followed by the Bonferroni post hoc test. $P < 0.05$ was considered to be statistically significant.

Both cannabinoid agonists, WIN (Fig. 1A) and anandamide (Fig. 1B), were able to concentration-dependently inhibit the amplitude of the electrically stimulated contractions of the guinea-pig ileum LMMP preparations. The effect of cannabinoids was reverted by the CB1R-selective antagonist SR141716A (Fig. 1C). No significant influence of age was found on the effect of either of the cannabinoid agonists.

Most myenteric neurones were immunoreactive to CB1R antibody (Fig. 2A) and most of the myenteric neurones immunoreactive for CR, also expressed CB1R (Fig. 2B). An age-related decrease in the density of myenteric neurones labelled with anti-CB1R (Fig. 3A) was found. Nevertheless, the proportion of myenteric neurones that expressed CB1R remained unaltered throughout the different age-groups (Fig. 3B) and the proportion of CR-IR neurones that expressed CB1R did not change significantly with age either (Fig. 3C).

Our results support and extend previous data on the effect of cannabinoid agonists on LMMP preparations of the guinea-pig small intestine. In these preparations, low-frequency electrical field stimulation results in cholinergic contractions of the longitudinal muscle, which are sensitive to cannabinoid agonists [31,32]. Several pharmacological evidences suggest that the inhibitory effect exerted by cannabinoids is due to the activation of prejunctional CB1R followed by a reduction in acetylcholine release. Thus, cannabinoids inhibit the electrically evoked response in a SR-141716A reversible manner, but have no effect on responses to exogenous ACh [5,30,31], or on contractions directly induced in the smooth muscle by indomethacin [12]. Besides, the inhibitory effect of cannabinoids is not affected by the ganglionic blocker hexamethonium, which further implies the participation of CB1R located on the final motor neurones [4].

In agreement with previous reports [4,20], the CB1R was found to be widely expressed in myenteric neurones (see Fig. 2A and B). Amongst those neurones expressing CR, immunoreactivity for CB1R was found in nearly 65% of them (see Fig. 2C), which is similar to the figures given by Coutts et al. [4]. Most (about 80%) CR-IR myenteric neurones in the guinea-pig ileum are excitatory longitudinal muscle motor neurones [3]. Since most CR-IR neurones expressed CB1R, it is likely that a great part (if not all) of the motor neurones to the longitudinal muscle express this receptor, which further supports the functional results. It would be interesting to ascertain whether or not those CR-IR neurones lacking CB1R correspond to the other class of CR-IR myenteric neurones (i.e., ascending interneurones, [3]). In other words, more work is needed to investigate how dependent CB1R expression is on the particular functional class of myenteric neurones, or to the needs of the particular physiological situation. In fact, CB1R expression has been shown to increase in the gut after experimentally-induced inflammation [16,27] or secretory diarrhea [15], and the other main cannabinoid re-

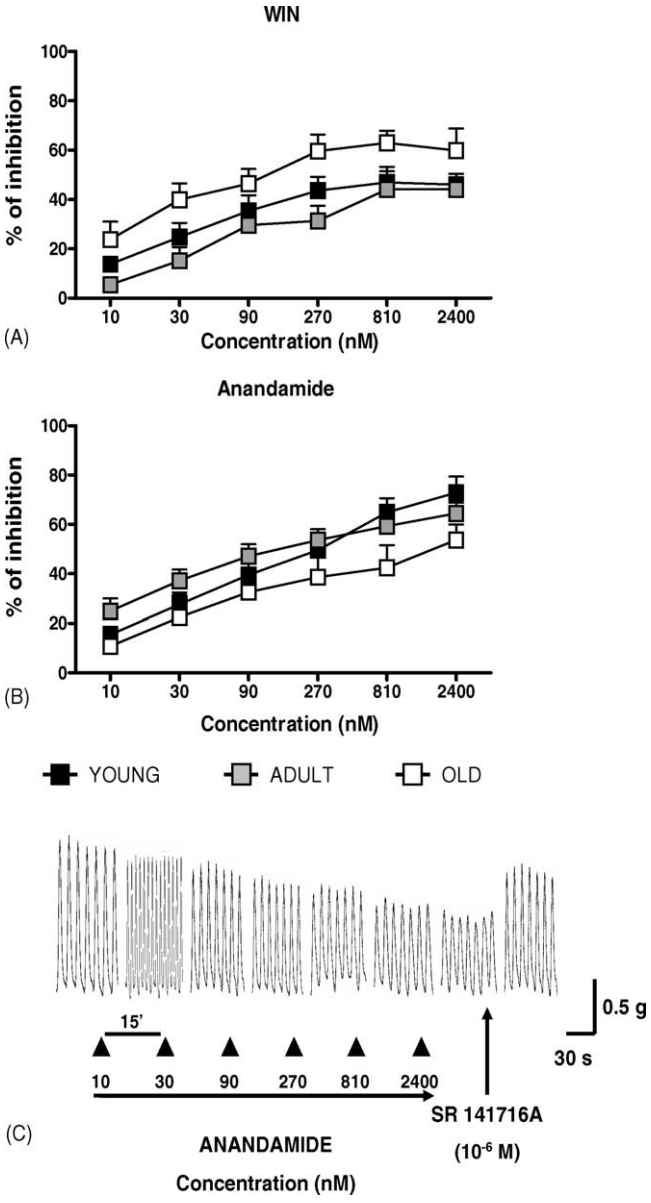

Fig. 1. Influence of age on the in vitro cannabinoid inhibition of electrically stimulated twitches in guinea-pig ileum longitudinal muscle–myenteric plexus preparations. Acute inhibitory effect of (A) the synthetic cannabinoid drug WIN 55-212,2 and (B) the endogenous cannabinoid ligand anandamide. Values represent the mean percent of inhibition ± S.E.M. for YOUNG ($n = 10$, $N = 6$), ADULT ($n = 9$, $N = 6$) and OLD ($n = 9$, $N = 6$) guinea-pigs (two-way ANOVA test). Panel C shows a representative recording of the inhibitory effect of anadamide administered at increasing cumulative concentrations on electrically stimulated twitches, plus its reversion by SR 141716A (arrow).

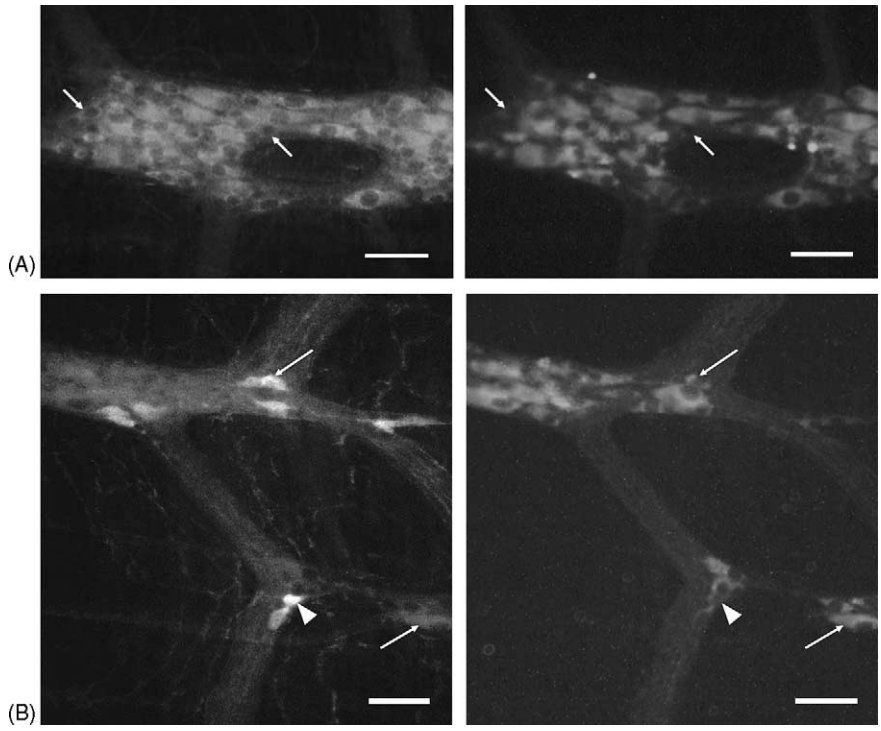

Fig. 2. Microphotographs showing the immunohistochemical appearance of guinea-pig ileum myenteric neurones expressing cannabinoid CB1 receptor (CB1R). (A) longitudinal muscle–myenteric plexus (LMMP) preparation double-labelled for both the pan-neuronal marker HuC/D (left) and CB1R (right); although most neurones expressed CB1R, some did not (arrows). (B) LMMP preparation double-labelled for both the marker calretinin (CR) (left) and CB1R (right); note the cells that expressed both CR and CB1R (arrows) and those that expressed only CR (arrowhead). Scale bar: 100 μm.

ceptor, CB2R, seems to be expressed and functional in the GI tract only under certain conditions, such as inflammation [14,28].

Neurotransmission and ageing in the guinea-pig ileum has only been poorly studied so far [19,38,39]. With regards to the cannabinoid system, the expression of CB1R in the GI tract has been demonstrated to occur as early as in prenatal development in the rat [2], but, to the best of our knowledge, this is the first report on the influence of ageing on its expression and functionality in the enteric nervous system. In the central nervous system, expression of cannabinoid receptors has been described to change extensively with ontogeny [8,40], age [22] and pathological conditions (e.g., Alzheimer's disease, [29]). Thus, an age-induced reduction in cannabinoid receptor binding and mRNA levels has been shown in the striatum and other extrapyramidal brain regions, particularly in the basal ganglia [35], but is less marked in other brain regions including the cerebral cortex or the hippocampus [22]. Not surprisingly, no significant difference was found between young and aged mice with respect to the cannabinoid receptor-mediated modulation of acetylcholine release in the hippocampus [34]. Likewise, the inhibitory effect of the synthetic cannabinoid WIN or the endogenous ligand anandamide on guinea-pig ileum LMMP preparations did not significantly change with age either. This lack of influence of age on cannabinoid responses, could be explained by the results from our immunohistochemical study: even though there was a reduction in the density of neurones expressing CB1R, their proportion versus the total neuronal population (or the population of myenteric neurones expressing CR, most of them longitudinal muscle motor neurones, see above) remained constant. Note that the effect of cannabinoids in this preparation is measured as the percentage of inhibition of the cholinergic twitch elicited by supramaximal electrical stimulation. Neither the proportion of neurones chemically identified as longitudinal muscle motor neurones (unpublished observations) nor that of those expressing CB1R (present results) significantly changed from young to old guinea-pigs. If the proportion of motor neurones releasing ACh does not change, and those neurones express CB1R, then it is reasonable that the inhibitory effect of cannabinoids would not change with age either.

Age-related alterations in GI motility seem to be associated with a generalised neuronal loss in the myenteric plexus [1,10]. Inhibitory neurones, which express nitric oxide synthase [3], seem to be somehow preserved, while at least some cholinergic neurones [1,33], are more affected by age. Since the proportion of CB1R-IR neurones did not change significantly, it could be suggested that some of the functional subpopulations of neurones that do not express CB1R in the young animals, could do so in the old ones.

The sensitivity of the LMMP preparation to cannabinoid drugs could be considered as a reflexion of the physiopharmacological effects these drugs produce in the GI tract and

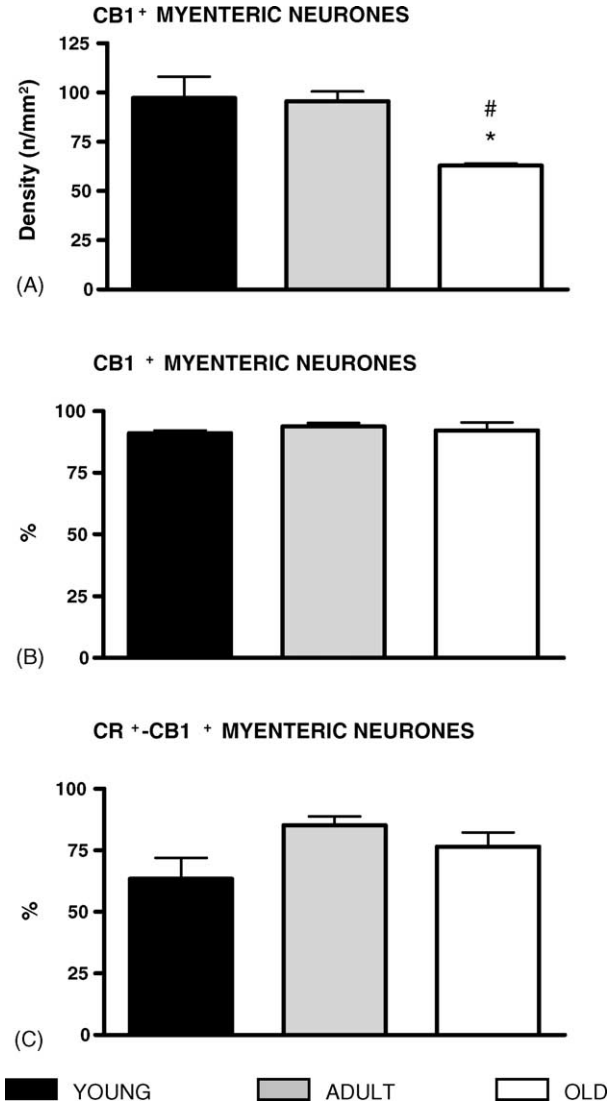

Fig. 3. Influence of age on the population of neurones immunoreactive (IR) for the CB1 cannabinoid receptor (CB1R) in guinea-pig ileum myenteric plexus. (A) number of CB1R-IR neurones/mm of serosal surface (density). (B) Percent of CB1R-IR neurones in the general population of myenteric neurones. (C) Percent of myenteric neurones expressing both calretinin (CR) and CB1R. Values are mean ± S.E.M. for YOUNG ($n = 5$), ADULT ($n = 5$) and OLD ($n = 5$) guinea-pigs. $^{*}p < 0.05$ vs. YOUNG, $^{\#}p < 0.05$ vs. ADULT (one-way ANOVA test).

the fact that it remains unchanged with age opens up interesting possibilities. Nevertheless, more investigative work will be required to confirm this age-independency of the cannabinoid effect on GI motility.

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References

[1] R. Abalo, A.J. Rivera, G. Vera, M.I. Martín, Ileal myenteric plexus in aged guinea-pigs: loss of structure and calretinin-immunoreactive neurones, Neurogastroenterol. Motil. 17 (2005) 123–132.
[2] N.E. Buckley, S. Hansson, G. Harta, E. Mezey, Expression of the CB1 and CB2 receptor messenger RNAs during embryonic development in the rat, Neuroscience 82 (1998) 1131–1149.
[3] M. Costa, S.J. Brookes, P.A. Steele, I. Gibbins, E. Burcher, C.J. Kandiah, Neurochemical classification of myenteric neurons in the guinea-pig ileum, Neuroscience 75 (1996) 949–967.
[4] A.A. Coutts, A.J. Irving, K. Mackie, R.G. Pertwee, S. Anavi-Goffer, Localisation of cannabinoid CB(1) receptor immunoreactivity in the guinea pig and rat myenteric plexus, J. Comp. Neurol. 448 (2002) 410–422.
[5] A.A. Coutts, R.G. Pertwee, Inhibition by cannabinoid receptor agonists of acetylcholine release from the guinea-pig myenteric plexus, Br. J. Pharmacol. 121 (1997) 1557–1566.
[6] T. Croci, L. Manara, G. Aureggi, F. Guagnini, M. Rinaldi-Carmona, J.P. Maffrand, G. Le Fur, S. Mukenge, G. Ferla, In vitro functional evidence of neuronal cannabinoid CB1 receptors in human ileum, Br. J. Pharmacol. 125 (1998) 1393–1395.
[7] J.R. Fernández, D.B. Allison, Rimonabant Sanofi-Synthelabo, Curr. Opin. Investig. Drugs 5 (2004) 430–435.
[8] J. Fernández-Ruiz, F. Berrendero, M.L. Hernández, J.A. Ramos, The endogenous cannabinoid system and brain development, Trends Neurosci. 23 (2000) 14–20.
[9] M. Firth, C.M. Prather, Gastrointestinal motility problems in the elderly patient, Gastroenterology 122 (2002) 1688–1700.
[10] G. Gabella, Fall in the number of myenteric neurons in aging guinea pigs, Gastroenterology 96 (1989) 1487–1493.
[11] G. Griffin, S.R. Fernando, R.A. Ross, N.G. McKay, M.L. Ashford, D. Shire, J.W. Huffman, S. Yu, J.A. Lainton, R.G. Pertwee, Evidence for the presence of CB2-like cannabinoid receptors on peripheral nerve terminals, Eur. J. Pharmacol. 339 (1997) 53–61.
[12] A. Heinemann, A. Shahbazian, P. Holzer, Cannabinoid inhibition of guinea-pig intestinal peristalsis via inhibition of excitatory and activation of inhibitory neural pathways, Neuropharmacology 38 (1999) 1289–1297.
[13] M. Herkenham, A.B. Lynn, M.R. Johnson, L.S. Melvin, B.R. de Costa, K.C. Rice, Characterization and localization of cannabinoid receptors in rat brain: a quantitative in vitro autoradiographic study, J. Neurosci. 11 (1991) 563–583.
[14] A. Izzo, Cannabinoids and intestinal motility: welcome to CB2 receptors, Br. J. Pharmacol. 142 (2004) 1201–1202.
[15] A.A. Izzo, F. Capasso, A. Costagliola, T. Bisogno, G. Marsicano, A. Ligresti, I. Matias, R. Capasso, L. Pinto, F. Borrelli, A. Cecio, B. Lutz, N. Mascolo, V. Di Marzo, An endogenous cannabinoid tone attenuates cholera toxin-induced fluid accumulation in mice, Gastroenterology 125 (2003) 765–774.
[16] A.A. Izzo, F. Fezza, R. Capasso, T. Bisogno, L. Pinto, T. Iuvone, G. Esposito, N. Mascolo, V. Di Marzo, F. Capasso, Cannabinoid CB1-receptor mediated regulation of gastrointestinal motility in mice in a model of intestinal inflammation, Br. J. Pharmacol. 134 (2001) 563–570.
[17] A.A. Izzo, N. Mascolo, F. Borrelli, F. Capasso, Defaecation, intestinal fluid accumulation and motility in rodents: implications of cannabinoid CB1 receptors, Naunyn Schmiedebergs Arch. Pharmacol. 359 (1999) 65–70.
[18] A.A. Izzo, N. Mascolo, L. Pinto, R. Capasso, F. Capasso, The role of cannabinoid receptors in intestinal motility, defaecation and diarrhoea in rats, Eur. J. Pharmacol. 384 (1999) 37–42.

[19] M. Koutsoviti-Papadopoulou, G. Kounenis, V. Elezoglou, N. Kokolis, Effect of cimetidine on the histamine and dimaprit induced responses on the guinea pig ileum during development, Gen. Pharmacol. 24 (1993) 1021–1026.
[20] A. Kulkarni-Narla, D.R. Brown, Opioid, cannabinoid and vanilloid receptor localization on porcine cultured myenteric neurons, Neurosci. Lett. 308 (2001) 153–156.
[21] M. Landi, T. Croci, M. Rinaldi-Carmona, J.P. Maffrand, G. Le Fur, L. Manara, Modulation of gastric emptying and gastrointestinal transit in rats through intestinal cannabinoid CB(1) receptors, Eur. J. Pharmacol. 450 (2002) 77–83.
[22] P. Liu, D.K. Bilkey, C.L. Darlington, P.F. Smith, Cannabinoid CB1 receptor protein expression in the rat hippocampus and entorhinal, perirhinal, postrhinal and temporal cortices: regional variations and age-related changes, Brain Res. 979 (2003) 235–239.
[23] F. López-Redondo, G.M. Lees, R.G. Pertwee, Effects of cannabinoid receptor ligands on electrophysiological properties of myenteric neurones of the guinea-pig ileum, Br. J. Pharmacol. 122 (1997) 330–334.
[24] A.B. Lynn, M. Herkenham, Localization of cannabinoid receptors and nonsaturable high-density cannabinoid binding sites in peripheral tissues of the rat: implications for receptor-mediated immune modulation by cannabinoids, J. Pharmacol. Exp. Ther. 268 (1994) 1612–1623.
[25] B.R. Martin, J.L. Wiley, Mechanism of action of cannabinoids: how it may lead to treatment of cachexia, emesis, and pain, J. Support Oncol. 2 (2004) 305–316.
[26] N. Mascolo, A.A. Izzo, A. Ligresti, A. Costagliola, L. Pinto, M.G. Cascio, P. Maffia, A. Cecio, F. Capasso, V. Di Marzo, The endocannabinoid system and the molecular basis of paralytic ileus in mice, FASEB J. 16 (2002) 1973–1975.
[27] F. Massa, G. Marsicano, H. Hermann, A. Cannich, K. Monory, B.F. Cravatt, G.L. Ferri, A. Sibaev, M. Storr, B. Lutz, The endogenous cannabinoid system protects against colonic inflammation, J. Clin. Invest. 113 (2004) 1202–1209.
[28] R. Mathison, W. Ho, Q.J. Pittman, J.S. Davison, K.A. Sharkey, Effects of cannabinoid receptor-2 activation on accelerated gastrointestinal transit in lipopolysaccharide-treated rats, Br. J. Pharmacol. 142 (2004) 1247–1254.
[29] M.R. Pazos, E. Núñez, C. Benito, R.M. Tolon, J. Romero, Role of the endocannabinoid system in Alzheimer's disease: new perspectives, Life Sci. 75 (2004) 1907–1915.
[30] R.G. Pertwee, Cannabinoids and the gastrointestinal tract, Gut 48 (2001) 859–867.
[31] R.G. Pertwee, S.R. Fernando, J.E. Nash, A.A. Coutts, Further evidence for the presence of cannabinoid CB1 receptors in guinea-pig small intestine, Br. J. Pharmacol. 118 (1996) 2199–2205.
[32] R.G. Pertwee, L.A. Stevenson, D.B. Elrick, R. Mechoulam, A.D. Corbett, Inhibitory effects of certain enantiomeric cannabinoids in the mouse vas deferens and the myenteric plexus preparation of guinea-pig small intestine, Br. J. Pharmacol. 105 (1992) 980–984.
[33] R.J. Phillips, E.J. Kieffer, T.L. Powley, Aging of the myenteric plexus: neuronal loss is specific to cholinergic neurons, Auton. Neurosci. 106 (2003) 69–83.
[34] A. Redmer, M. Kathmann, E. Schlicker, Cannabinoid CB(1) receptor-mediated inhibition of hippocampal acetylcholine release is preserved in aged mice, Br. J. Pharmacol. 138 (2003) 1425–1430.
[35] J. Romero, F. Berrendero, L. García-Gil, P. de la Cruz, J.A. Ramos, J.J. Fernández-Ruiz, Loss of cannabinoid receptor binding and messenger RNA levels and cannabinoid agonist-stimulated [35*S*]guanylyl-5′*O*-(thio)-triphosphate binding in the basal ganglia of aged rats, Neuroscience 84 (1998) 1075–1083.
[36] J.E. Shook, T.F. Burks, Psychoactive cannabinoids reduce gastrointestinal propulsion and motility in rodents, J. Pharmacol. Exp. Ther. 249 (1989) 444–449.
[37] G.J. Smits, R.A. Lefebvre, Influence of aging on gastric emptying of liquids, small intestine transit, and fecal output in rats, Exp. Gerontol. 31 (1996) 589–596.
[38] I. Takayanagi, O. Maeda, K. Koike, Effect of aging on presynaptic alpha 2-adrenoceptor mechanisms in guinea pig ileum, Comp. Biochem. Physiol. C 92 (1989) 419–423.
[39] F.G. van den Brink, Influence of combined cooling and aging on the response of the guinea-pig ileum to histamine. Analysis of the influence of cold storage of the isolated organ on drug parameters, Eur. J. Pharmacol. 2 (1967) 224–228.
[40] X. Wang, D. Dow-Edwards, E. Keller, Y.L. Hurd, Preferential limbic expression of the cannabinoid receptor mRNA in the human fetal brain, Neuroscience 118 (2003) 681–694.

4.3. Tercera publicación:

Abalo R, Vera G, Rivera AJ, Martín MI. ***Age-related changes in the gastrointestinal tract: a functional and immunohistochemical study in guinea-pig ileum.*** Life Sci. 2007; 80(26):2436-2445.

4.3.1. Objetivo general

El objetivo general fue profundizar en el efecto del envejecimiento en la funcionalidad e inmunorreactividad de distintos marcadores neuronales en el plexo mientérico de íleon de cobayo.

4.3.2. Objetivos específicos

Se realizó un estudio funcional para determinar la influencia de la edad en:

- La respuesta a la estimulación excitadora inducida por la liberación de neurotransmisores endógenos (mediante estimulación eléctrica o durante el signo de abstinencia opioide inducido *in vitro* por naloxona) o por administración exógena de acetilcolina (ACh) y Sustancia P (SP).
- La respuesta a morfina en preparaciones estimuladas eléctricamente y en preparaciones previamente incubadas con el opioide para evaluar el desarrollo de tolerancia *in vitro*.

Se realizó un estudio inmunohistoquímico y se determinó la influencia de la edad en:

- La población de neuronas inmunorreactivas para CR pero no para NFT, que en el cobayo son neuronas excitadoras de la musculatura longitudinal
- Las inmunorreactivas para CR y NFT, que se han identificado en el cobayo como interneuronas ascendentes
- Las inmunorreactivas para calbindina (CB), marcador representativo de neuronas intrínsecas aferentes primarias en este tejido.
- Las inmunorreactivas para óxido nítrico sintasa (NOS), presente principalmente en motoneuronas inhibidoras de la musculatura longitudinal.

4.3.3. Resultados

El umbral de estimulación eléctrica para la respuesta contráctil de las preparaciones no cambió de manera significativa con la edad, y cuando se alcanzaba una respuesta contráctil estable, la amplitud era similar a la obtenida con el voltaje supramaximal.

La respuesta a los agonistas excitadores, tanto ACh como SP, aumentó en las preparaciones de animales viejos, al igual que el signo de abstinencia opioide *in vitro*. Por otro lado, la respuesta a agonistas inhibidores (morfina) no varió de manera significativa con la edad. En cambio, la respuesta a la morfina en preparaciones previamente incubadas con el agonista opioide fue mayor en los cobayos viejos, lo que indica que el desarrollo de tolerancia al efecto de morfina fue menor en las preparaciones de estos animales.

El estudio inmunohistoquímico reveló que, independientemente del marcador analizado o la edad de los cobayos de los que procedían las preparaciones, la mayoría de las neuronas estaban en el interior de los ganglios. La densidad de empaquetamiento de las neuronas inmunorreactivas para CB, NOS, CR y NFT descendió con la edad. La población general de neuronas (marcadas con Hu) no cambió significativamente en los cobayos adultos, aunque tendió a decrecer (sin llegar a alcanzar significación estadística) en los cobayos viejos. Aunque comparado con la situación de los cobayos jóvenes, la densidad de todas las poblaciones descendió, aquella inmunorreactiva para CR pero no para NFT (CR+NFT-) (motoneuronas excitadoras de la musculatura longitudinal), lo acusó en mayor medida. La densidad de fibras del componente terciario no varió con la edad.

4.3.4. Conclusiones

El hecho de que la densidad de las motoneuronas excitadoras descienda de manera más significativa que la de las inhibidoras junto con el hecho de que la tolerancia a la morfina se reduzca en los animales envejecidos, puede ser indicativo de un aumento del tono inhibidor en el tracto GI, y estar relacionado con la mayor incidencia de constipación con la edad. Por otro lado, la respuesta aumentada a los agonistas excitadores podría ser responsable de los episodios de hipermotilidad, también frecuentes a edades avanzadas. Estas alteraciones producidas por la edad podrían hacer más inestable el equilibrio de la función GI, haciendo a los ancianos más susceptibles a las patologías de este sistema.

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Age-related changes in the gastrointestinal tract: a functional and immunohistochemical study in guinea-pig ileum

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Abstract

It is known that there is an age-related increase in gastrointestinal diseases. However, there is a lack of studies dealing with the correlation between age-related changes in function and intrinsic innervation in the gastrointestinal tract. The purpose of this work was to study this subject in the guinea pig ileum, whose functional and structural features are well known in the young age. Ileal longitudinal muscle — myenteric plexus (LMMP) preparations were obtained from 3-to 24-month-old guinea pigs. Both functional and immunohistochemical techniques were applied. The force of the contraction elicited by excitatory stimuli (electrical stimulation, acetylcholine, substance P, and opioid withdrawal) increased in parallel with an age-dependent reduction in the density of excitatory motor neurones to the longitudinal muscle, whereas other subpopulations of neurones, including inhibitory motor neurones, decreased much more slowly. Although the increase in responsiveness could be related to the age/weight-related increment in muscle bulk, some compensatory modifications to the lowered density of excitatory neurones could also be involved. On the other hand, the acute inhibitory response to morphine remained unaltered in old animals, whilst in vitro tolerance was lower. These results suggest that although age-dependent neuronal loss does not cause dramatic changes in intestinal motility, it is a factor that could contribute to disturbing normal responsiveness and, perhaps, underlie the higher frequency of gastrointestinal diseases encountered in the elderly.

Keywords: Ageing; Guinea-pig ileum; Immunohistochemistry; Acetylcholine; Substance P; Morphine

Introduction

Gastrointestinal motor function declines with age and this has been attributed to autonomic dysfunction (Pilotto et al., 2003). Although a general loss of myenteric neurones has been reported to occur (Gabella, 1989; Gomes et al., 1997; Abalo et al., 2005a,b), some alterations in gastrointestinal function would be better explained if susceptibility to age were different among the different subpopulations of enteric neurones, leading to a certain degree of misbalance in gut activity.

A widely used and well characterised experimental preparation that could be helpful in order to correlate age-induced structural and functional alterations in the gut is the longitudinal muscle-myenteric plexus (LMMP) preparation obtained from the guinea pig ileum. Functionally, this tissue shows some interesting characteristics. Thus, upon electrical stimulation, a large number of neurones are synchronously activated, leading to the release of excitatory neurotransmitters, which, acting on their corresponding postsynaptic receptors, induce the contraction of the longitudinal muscle. The main excitatory neurotransmitters released are acetylcholine (ACh; Paton and Zar, 1968), and substance P (SP; Taylor and Bywater, 1986; Franco, 1979), and it is possible to mimic their contractile effects by exposing the preparation directly to these agonists or their analogues. In addition, neurotransmitter release in this preparation can be reduced by the stimulation of inhibitory systems, such as the opioid system. Thus, morphine presynaptically inhibits the electrically-stimulated release of ACh and the force of the cholinergic twitch (Paton, 1957; Kosterlitz and Waterfield, 1975; Gintzler and Scalisi, 1982). Lastly, in LMMP preparations that have developed tolerance to morphine, opioid antagonists like naloxone are able to induce the acute release of excitatory neurotransmitters, mainly ACh and SP, from myenteric neurones, leading to a sudden contraction of the preparation (opioid withdrawal sign: Paton, 1970; Aleixandre et al., 1983; Collier et al., 1981; Mehr et al., 2003).

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Interestingly, the neurones primarily involved in the functionality of this tissue, the excitatory motor neurones to the longitudinal muscle, are easily identifiable by immunohistochemical methods (Costa et al., 1996). This particular population of neurones could decrease with age to a higher degree than the general population of myenteric neurones (Abalo et al., 2005a). However, the effects of ageing on the response of the LMMP preparation to excitatory or inhibitory stimuli have not yet been well established.

Therefore, an attempt was made to correlate functional and structural modifications induced by age in the guinea pig ileum LMMP preparation. Female guinea pigs were used because their reproductive behaviour allows a clear separation of differently-aged specimens in three distinct physiological stages (Terril and Clemons, 1998). In preparations originating from these animals, we performed both functional and immunohistochemical studies. Thus, we studied the influence of age on (1) excitatory stimulation induced by either neuronal release of endogenous neurotransmitters (electrical stimulation and naloxone-induced opioid withdrawal) or by exogenous administration of ACh and SP; and (2) the response to morphine, which inhibits the release of excitatory neurotransmitters. Additionally, we analysed the influence of age on the subpopulation of excitatory longitudinal muscle motor neurones that are immunoreactive to calretinin (CR) but not to neurofilament triplet proteins (NFT) (Costa et al., 1996; Brookes et al., 1992). A comparison was made amongst this subpopulation, the general population and three additional subpopulations of myenteric neurones: (1) those showing immunoreactivity to both CR and NFT, which in the guinea pig ileum have been identified as ascending interneurones; (2) those immunoreactive to calbindin (CB), a representative marker of intrinsic primary afferent neurones in this tissue; and (3) those immunoreactive to nitric oxide synthase (NOS), mainly present in inhibitory muscle motor neurones (Costa et al., 1996).

Materials and methods

All experimental protocols were approved by the Ethical Committee at the Universidad Rey Juan Carlos and performed in strict accordance with the EC regulation for care and use of experimental animals (EEC N° 86/609).

Female Dunkin-Hartley guinea pigs of the following ages (related to distinct physiological stages) were used: 2–3 months (YOUNG, weight 330–450 g; at this age, females have reached puberty and are about ready to start breeding), 8–10 months (ADULT, weight 800–950 g; at this age, reproductive maturity is consolidated) and 22–26 months (OLD, weight 900–1200 g; at this age, it is recommended to retire females from breeding). In order to minimize pain and discomfort, animals were killed by cervical dislocation. Segments of distal ileum, at least 10 cm oral to the ileocaecal junction, were obtained, opened along the mesenteric border, rinsed and pinned flat on a Sylgard-coated Petri-dish filled with modified Krebs solution (mM: NaCl; 118, KCl; 4.75, NaH_2PO_4; 1.0, $NaHCO_3$; 25, $MgSO_4$; 1.2, $CaCl_2$; 2.5, glucose; 11, pH 7.4). Whole-mount LMMP preparations were obtained by removal of mucosa, submucosa and circular muscle layers. Tissues were used in two different sets of experiments. Unless otherwise stated, each experiment involved the use of 1–2 preparations from 4–6 different animals per age.

Functional studies

LMMP preparations (length 0.7–1.3 cm) were unpinned and suspended, under a tension equivalent to a 1 g load, in an organ bath containing 5 ml of Krebs solution kept at 36 °C and aerated with carbogen (95% O_2–5% CO_2). The preparation was fixed by a thread to a lower electrode, and passed through a second, ring-shaped one (separation between electrodes: 0.7–1 cm). Basal and electrically-stimulated contractile activities of the preparations were recorded by means of an isometric transducer connected to a PowerLab/4e system. Field electrical stimulation (pulses of 2 ms duration, 0.3 Hz and increasing voltage until supramaximal stimulation was reached) was used to induce cholinergic twitches (Abalo et al., 2000). These twitches were atropine-sensitive, excluding a direct effect of electrical stimulation on the muscle. Those preparations whose contractions did not reach 0.5 g were removed from the study (the possibility existed that they had been damaged by the experimental manipulation).

The influence of age on the contractile activity was tested on:

1. The response of the preparation to electrical stimulation. Two parameters were measured (Fig. 1):
   - threshold, measured as the minimum voltage that produced a stable contractile response of the preparations,
   - force of the contraction elicited by supramaximal electrical stimulation
2. The contractile responses induced by in vitro administration of ACh and SP, the two major excitatory neurotransmitters of the longitudinal muscle. These responses were evaluated in the absence of electrical stimulation. Increasing concentrations (10^{-8}, 5×10^{-8}, 2.5×10^{-7}, 10^{-6} and 5×10^{-6} M) of either ACh or SP were added to the organ bath in a non-cumulative fashion; each concentration was added after

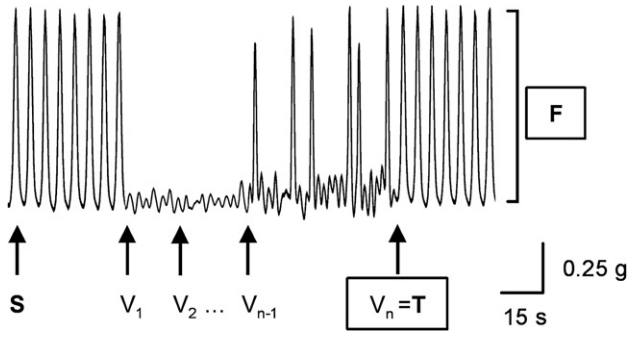

Fig. 1. Evaluation of the response of guinea pig ileum longitudinal muscle — myenteric plexus preparation to electrical stimulation. Rectangular pulses of 2 ms, 0.3 Hz were applied. Voltage of the pulses was initially 4.5 V (*S*, supramaximal). Thereafter, voltage was reduced to a non-stimulating value (V_1) and then increased in steps of 0.1 V (V_2,.., V_{n-1}), until a stable response was elicited. The voltage that produced such response was considered the threshold ($V_n = T$). The force of the stable contraction obtained with either supramaximal or threshold voltage was also measured (*F*).

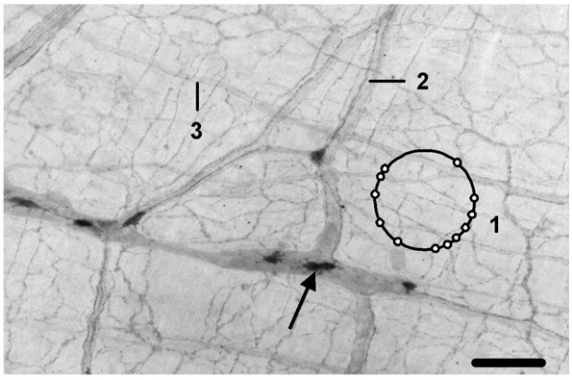

Fig. 2. Estimation of the degree of innervation of the longitudinal muscle in guinea pig ileum longitudinal muscle — myenteric plexus preparations. Density of tertiary plexus (1) was estimated in microphotographs of longitudinal muscle — myenteric plexus preparations immunostained for calretinin (CR; arrow, example of a CR-immunoreactive neurone), by counting the number of tertiary fibres crossing a 500 μm circumference drawn on top of the microphotograph (dots). Internodal strands (2) and secondary branches (3) of the plexus are also shown. Scale bar: 100 μm.

washing out with fresh Krebs; the recording was allowed to stabilise for 60 s after each administration. The force of the contraction induced by each administration was measured.

3. The effect of the opioid antagonist naloxone on LMMP preparations preincubated with morphine (opioid withdrawal sign: Collier et al., 1981). In the absence of electrical stimulation, tissues were pre-incubated with 5×10^{-7} M morphine. After 90 min, naloxone (10^{-6} M) was added to the organ bath and the mean force (g) of the contractile response induced was recorded.
4. The responses to the opioid agonist morphine. The acute effect of morphine was tested in naïve preparations under electrical stimulation: increasing concentrations (5×10^{-8}, 10^{-7}, 2×10^{-7}, 4×10^{-7}, 8×10^{-7} and 1.6×10^{-7} M) of morphine were added to the organ bath in a cumulative fashion at intervals of 5 min; the effect of the opioid was evaluated as the percentage of inhibition of the initial twitch amplitude (Abalo et al., 2000). The effect of morphine was also assayed in a similar way in preparations pre-incubated with morphine as a means of evaluating the degree of tolerance developed by the tissue. As previously described for evaluation of naloxone-induced opioid withdrawal, preparations were incubated with morphine 5×10^{-7} M and the effect of this opioid (5×10^{-8} M to 1.6×10^{-7} M) was assayed 90 min after (Colado et al., 1991).

All drugs were provided by Sigma-Aldrich and dissolved in distilled water.

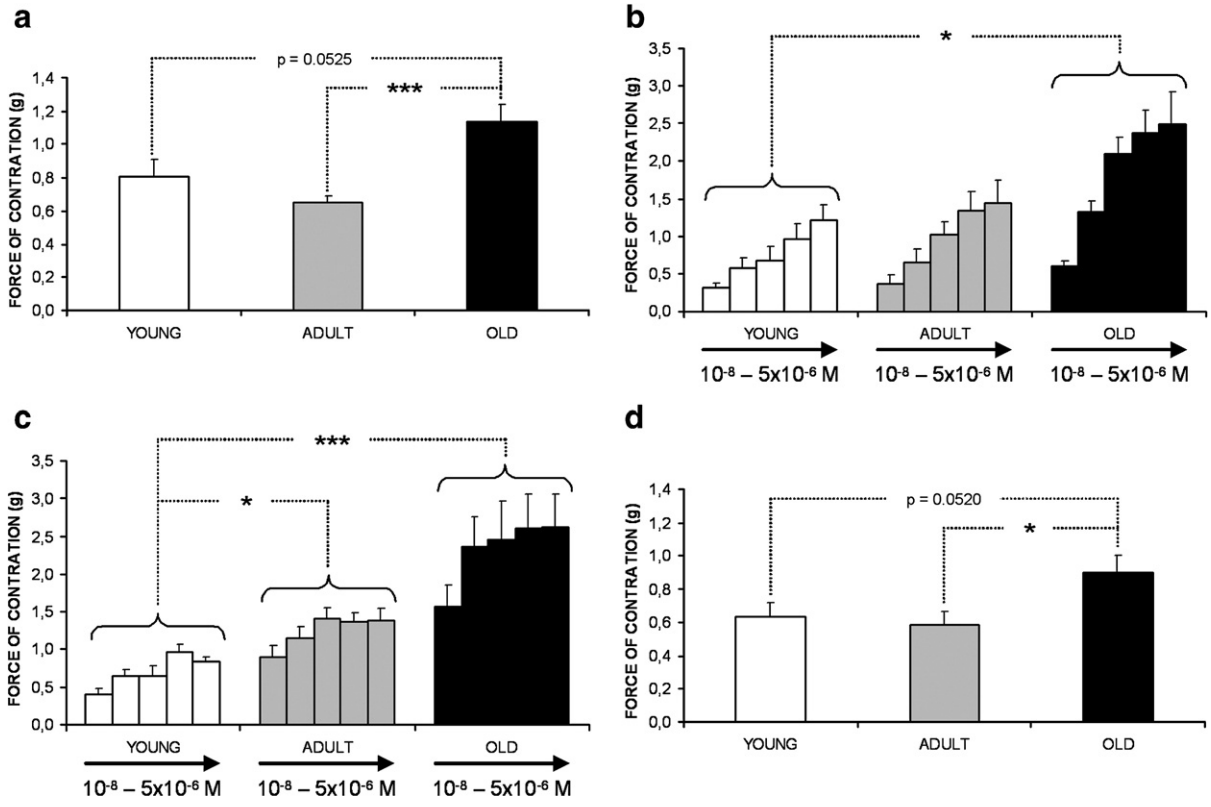

Fig. 3. Influence of age on the response of guinea pig ileum longitudinal muscle — myenteric plexus preparations to excitatory stimulation. a: force of electrically-stimulated contraction; values are mean±SEM for YOUNG (n=12, N=6), ADULT (n=14, N=6) and OLD (n=20, N=6) guinea pigs. b–c: response to excitatory agonists: acetylcholine (10^{-8} to 5×10^{-6} M, B) or substance P (10^{-8} to 5×10^{-6} M, C); force of the contraction elicited by each concentration is shown as mean±SEM for YOUNG (n=6), ADULT (n=9) and OLD (n=7) guinea-pigs. d: sign of withdrawal in response to the opioid antagonist naloxone (10^{-6} M) in preparations preincubated with morphine (5×10^{-7} M, 90 min). Values are mean±SEM for YOUNG (n=12, N=6), ADULT (n=12, N=5) and OLD (n=12, N=6). n=samples; N=animals. *$p<0.05$, ***$p<0.001$ (a and d: Student's t-test; b and c: two-way ANOVA test).

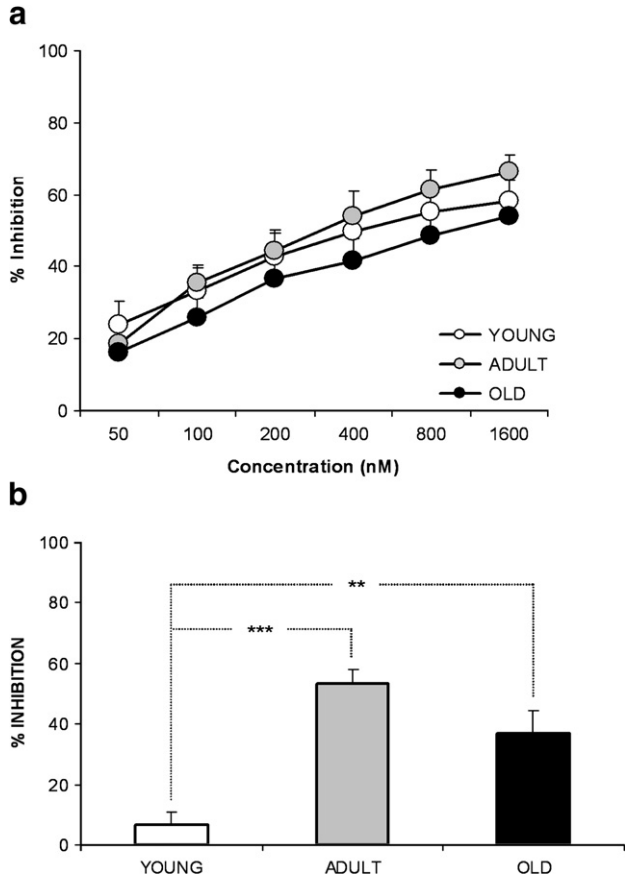

Fig. 4. Influence of age on the in vitro opioid inhibition of electrically-stimulated twitches on guinea pig ileum longitudinal muscle — myenteric plexus preparations. a: acute inhibitory effect of morphine (5×10^{-8} M to 1.6×10^{-7} M) in naïve preparations; values represent the mean % of inhibition ± SEM for YOUNG ($n=11$, $N=8$), ADULT ($n=10$, $N=6$) and OLD ($n=10$, $N=5$); two-way ANOVA test. b: inhibitory effect of morphine (1.6×10^{-7} M) in preparations preincubated with morphine (5×10^{-7} M, 90 min) versus that obtained in naïve preparations (100%); values are mean ± SEM for YOUNG ($n=8$, $N=5$), ADULT ($n=9$, $N=5$) and OLD ($n=7$, $N=5$). n = samples; N = animals. $^{**}p<0.01$, $^{***}p<0.001$ (Student's t-test).

Immunohistochemical study

Each LMMP was stretched to its maximal extension. After conventional fixation and clearing (Brookes et al., 1991), these whole-mount preparations were stored at 4 °C in phosphate buffered saline (PBS) with sodium azide (0.1%), until their immunohistochemical processing.

Conventional indirect immunofluorescence procedures were applied for double-labelling of the whole-mount preparations (Costa et al., 1986). At least two adjacent preparations per animal were used. One of the preparations was incubated with a mixture of mouse anti-CB (1:500, Sigma) and sheep anti-NOS (1:500, Chemicon), and the other one, with mouse anti-NFT (1:500, a generous gift from Dr Vitadello, Italy) and goat anti-CR (1:1000, Chemicon). Incubation with these primary antibodies was performed overnight at room temperature. After washing with PBS (3 × 10 min), tissues were exposed for 90 min at room temperature to a mixture of the following, corresponding secondary antibodies: donkey anti-mouse-CY2 (1:500, Jackson), donkey anti-sheep-CY3 (1:500, Jackson) and donkey anti-goat-RRX (1:500, Jackson). Some additional tissues were labelled with the pan-neuronal marker HuC/D, by using mouse streptavidin-conjugated anti-HuC/D (1:1000, Molecular Probes), as primary antibody, and the avidin-AlexaFluor 488 complex (1:1000, Molecular Probes) to develop the reaction. All mixtures of antibodies were diluted with hypertonic PBS (1.7% NaCl) (Costa et al., 1996; Grube, 1980).

The preparations were observed under a fluorescence microscope (Nikon Eclipse TE2000-U, with appropriate filters). An experimenter blind to the age of the guinea pig from which the tissue originated performed the immunohistochemical analysis, both by eye and by using micrographs taken at 10× or 20× with a DXM1200 camera (Nikon, Spain).

Packing density (number of neurones per ganglionic surface unit: Karaosmanoglu et al., 1996; Abalo et al., 2005a) of neurones showing immunoreactivity to CB, NOS, CR and NFT was measured on mosaics of 0.5 cm^2 consisting of 25 micrographs obtained at 20×, using the program LSM (Zeiss) for the quantitative analysis as described elsewhere (Abalo et al., 2005a). Packing density in YOUNG animals was considered as 100%, so that the age-related degrees of change of the following four populations of immunohistochemically-identified myenteric neurones (Costa et al., 1996) could be compared: sensory neurones (CB-immunoreactive), inhibitory motor neurones (NOS-immunoreactive), ascending interneurones (CR^+- NFT^+-neurones) and excitatory longitudinal muscle motor neurones (CR^+- NFT^--neurones).

The influence of age on innervation of the longitudinal muscle was studied in preparations showing immunoreactivity to CR, by analysing the density of the tertiary plexus, which, in the guinea pig ileum, is a fine network of CR-immunoreactive fibres containing the axonic processes originating from the excitatory motor neurones to the longitudinal muscle (Furness and Costa, 1987). For each preparation, 32 non-overlapping photographs were taken at 10× and 10 of them were randomly chosen for the analysis. As shown in Fig. 2, a circle with a circumference of 500 μm was drawn on the top of each micrograph and the number of CR-immunoreactive tertiary axon bundles crossing the line were counted; care was taken to draw the circumference at least 50 μm apart from the first component of the plexus (ganglia and internodal strands: Furness and Costa, 1987); secondary branches crossing the circumference were not counted.

Controls for the immunohistochemical analysis were performed by pairing the wrong primary and secondary antibodies or by avoiding exposure to the primary antibody. No specific labelling was observed in either case.

Statistical analysis

Statistical analysis was carried out using GraphPad Prism® (GraphPad Software, Inc.). All results are expressed as mean ± SEM; n refers to number of preparations, and, unless otherwise stated, is equivalent to the number of experimental animals (N). Differences between groups were analysed using either an unpaired Student's t-test (with Welch's correction when variances were not homogeneous), or a two-way ANOVA followed by post hoc Bonferroni multiple comparison test. Values of at least $p<0.05$ were regarded as being significantly different.

Results

Functional study

Responses to electrical stimulation

The threshold of electrical stimulation for the contractile response of the preparations did not change in a significant manner with age (YOUNG, 1.97±0.02 V, n=12; ADULT, 1.97±0.01 V, n=14; OLD, 1.96±0.02 V, n=20; N=5–6 animals per age). In all cases, once the contractile response stabilised, its amplitude was similar to that obtained with supramaximal voltage. However, the force of contraction (induced by either threshold or supramaximal voltage) increased with age (Fig. 3a).

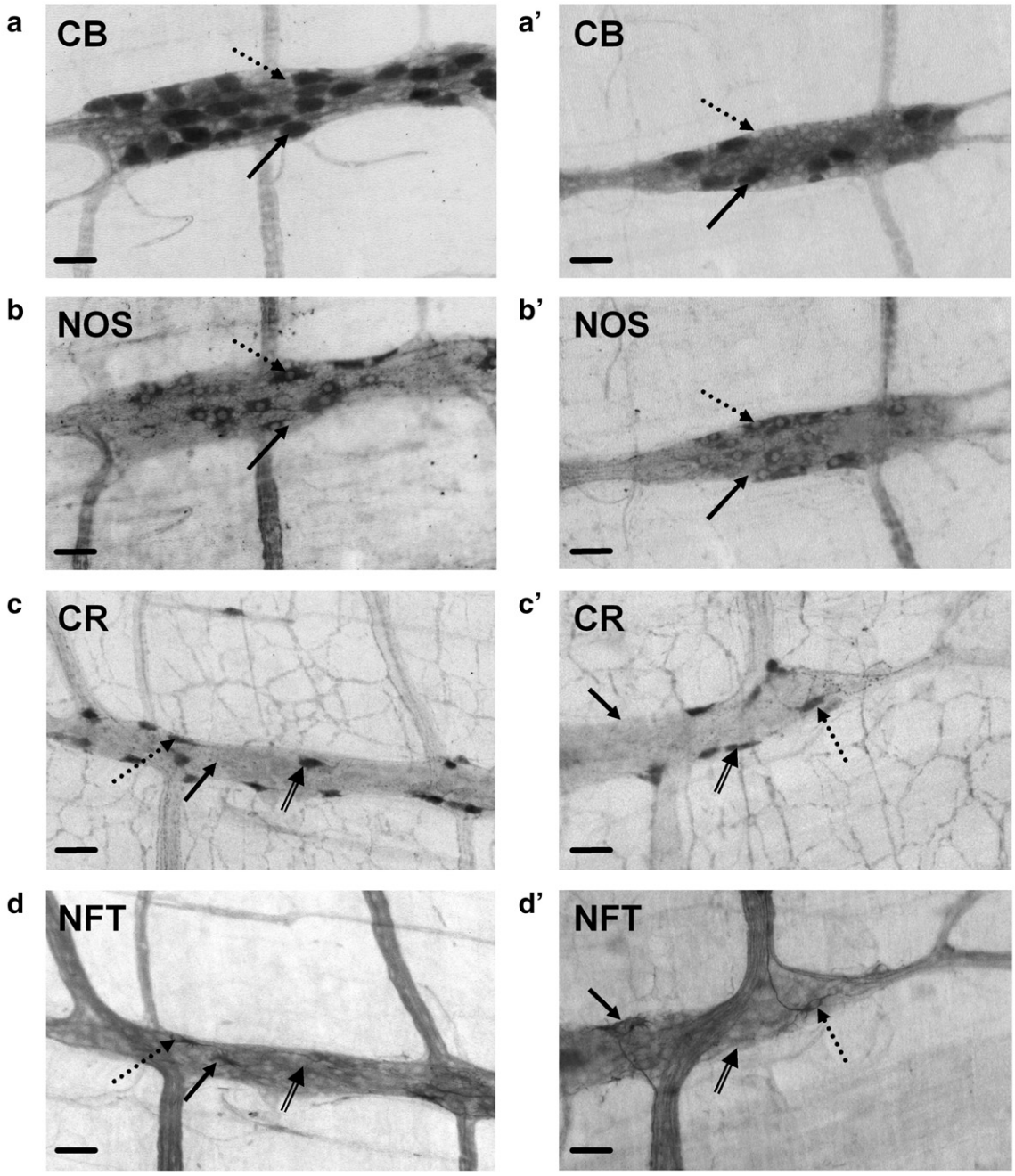

Fig. 5. Representative micrographs of myenteric ganglia from YOUNG and OLD guinea pigs showing neurones immunoreactive for different markers: calbindin (CB); nitric oxide synthase (NOS), calretinin (CR), and neurofilament triplet protein (NFT). For each age, two pairs of photographs are shown, corresponding to two preparations. One pair of photographs corresponds to a preparation labelled for both CB and NOS (a-b=YOUNG; a'-b'=OLD). Note that immunoreactivity to CB (intrinsic primary afferents, solid arrow) and NOS (inhibitory motor neurones, dotted arrow) do not coexist in the same neurones in either age. The other pair of photographs corresponds to a preparation labelled for both CR and NFT (c-d=YOUNG; c'-d'=OLD). In these preparations, three kinds of neurones were found: those showing immunoreactivity to both CR and NFT (ascending interneurones, dotted arrow), those showing immunoreactivity to CR but not to NFT (excitatory motor neurones to the longitudinal muscle, double arrow) and those showing immunoreactivity to NFT but not to CR (different kinds of neurones, solid arrow). Calibration bar: 50 μm.

Responses to exogenously administered excitatory agonists

Both the cholinergic (ACh) and tachykinergic (SP) excitatory agonists induced a concentration-dependent contraction of the non-stimulated preparations. In both cases the contraction was significantly stronger in preparations obtained from aged animals (Fig. 3b and c).

In vitro opioid withdrawal sign

After preparations were exposed to morphine for 90 min, naloxone induced a contraction (in vitro opioid withdrawal sign) whose force was significantly higher in OLD than in YOUNG or ADULT animals (Fig. 3d).

Responses to inhibitory stimulation

Morphine induced a concentration-dependent inhibition of electrically stimulated contractions of the naïve LMMP preparations and this effect did not change in a significant manner with age (Fig. 4a). However, in morphine-incubated LMMP preparations, the effect of morphine was higher in aged guinea pigs, meaning that the degree of tolerance was lower in preparations from these animals (Fig. 4b).

Immunohistochemical study

A qualitative analysis of the preparations confirmed that, irrespective of the age of the animal from which the tissue originated: (1) no neurone showed immunoreactivity to NOS and CB simultaneously; (2) although in each preparation labelled for CR and NFT there were always a few neurones immunoreactive to both markers, most CR-labelled neurones lacked immunoreactivity to NFT; (3) immunoreactivity to CR, but not to NFT, was also found in the fibres of the tertiary component of the plexus; (4) CB-immunoreactive neurones showed large smooth cell bodies; (5) NOS-, CR-and NFT-neurones had relatively smaller somas and the corresponding labelling was also evident in their processes (see Fig. 5). Finally, the presence of dots of lipofuscin-like material (Abalo et al., 2005a) was apparent in some myenteric neurones from ADULT and OLD guinea pigs (Fig. 6).

Whichever the marker analysed or the age of the guinea pig, most myenteric neurones were found inside the ganglia. More specifically, the average proportion of intraganglionic neurones in YOUNG guinea pigs was, 95.71±1.18 (n=6), 97.37±0.46 (n=5), 96.11±2.05 (n=4) and 94.11±0.63 (n=3) for CB-, NOS-, NFT-and Hu-immunoreactive neurones, respectively. CR neurones were also mainly found inside the ganglia, although in a slightly lower proportion, namely 85.08±3.28 (n=4). Nevertheless, these proportions remained relatively constant and always above 85% (80% for CR-immunoreactive neurones), regardless of the age of the guinea pig. Therefore, we concentrated on the analysis of intraganglionic neuronal density or packing density, which has been shown to be independent of the degree of stretching (Karaosmanoglu et al., 1996).

Packing density decreased with age for CB-, NOS-, CR-and NFT-immunoreactive neurones, although different degrees of change for each marker seemed to exist (Fig. 7a–d). A closer comparison of the degrees of change in packing density was made for the four immunohistochemically identified subpopulations of neurones versus that of the general neuronal population (Fig. 8a). The packing density of HuC/D-labelled neurones (general population of neurones), studied in 3 preparations per age, did not significantly change in ADULT animals but tended to decrease in OLD guinea pigs (p=0.0874, vs YOUNG). Changes in NOS-immunoreactive neurones (inhibitory motor neurones) followed the pattern of the general population of neurones, but in this case statistical significance was actually reached in OLD vs YOUNG animals. Neurones immunoreactive to both CR and NFT (ascending interneurones) and those immunoreactive to CB (sensory neurones) decreased from YOUNG to ADULT and did not change any further from ADULT to OLD animals. In the case of CR^{+}- NFT^{-}-neurones (excitatory longitudinal muscle motor neurones), packing density decreased almost at a constant degree

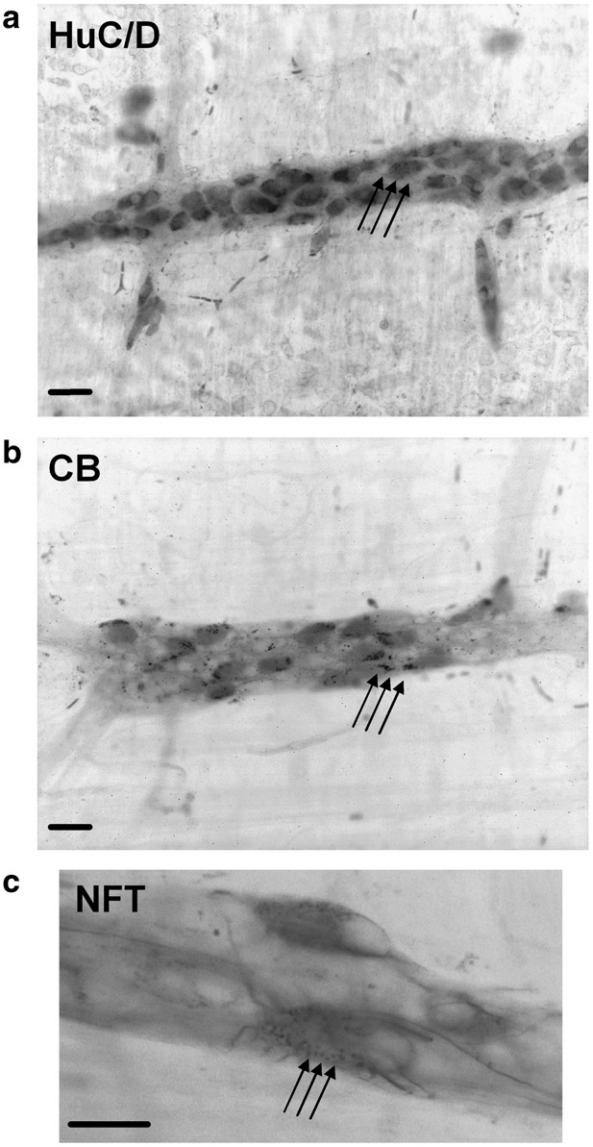

Fig. 6. Presence of lipofuscin-like dots in myenteric neurones from OLD guinea pigs. The preparations were observed with a B-2A filter, which allowed for lipofuscin-like material to be observed (arrows) in: Panel a, the general population of neurones (immunoreactive for HuC/D); Panel b, neurones immunoreactive for calbindin (CB); and Panel c, neurones immunoreactive for neurofilament triplet protein (NFT). Calibration bar: 50 μm.

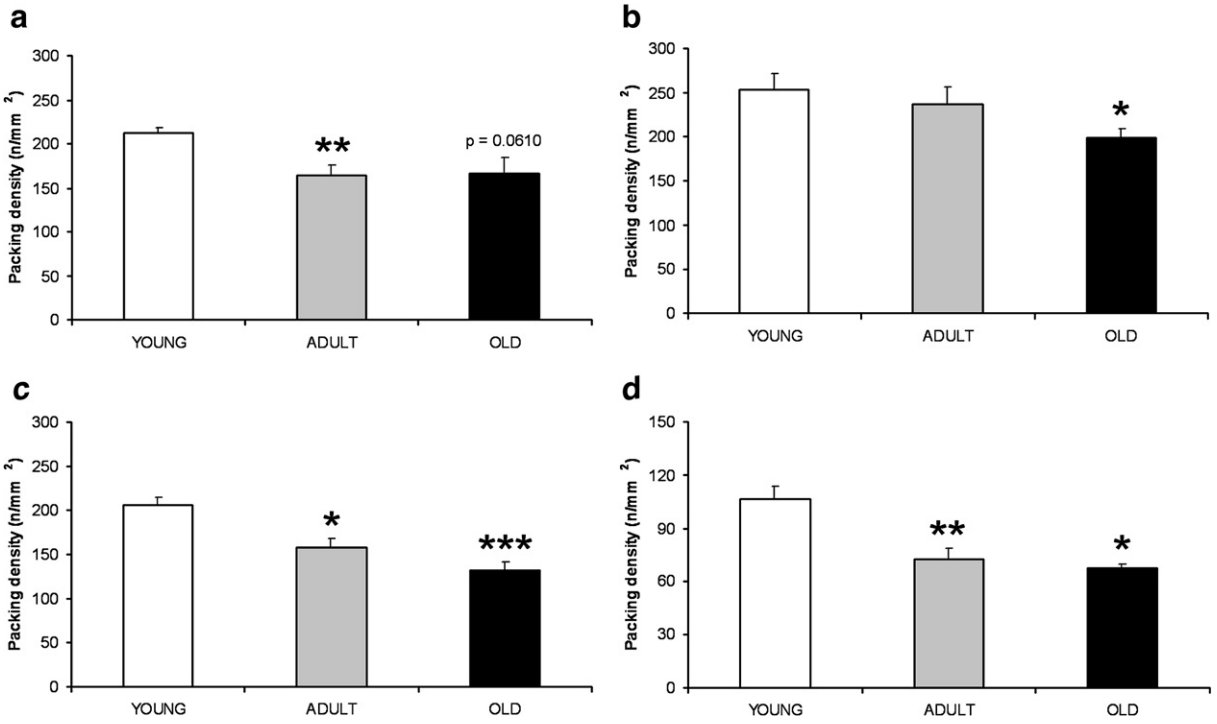

Fig. 7. Influence of age on packing density of myenteric neurones immunoreactive for different markers: Panel a, calbindin (CB, marker of intrinsic primary afferent neurones in this tissue); Panel b, nitric oxide synthase (NOS, marker of inhibitory motor neurones), Panel c, calretinin (CR, marker found in ascending interneurones and excitatory motor neurones to the longitudinal muscle in this tissue), and Panel D, neurofilament triplet protein (NFT, marker present in ascending interneurones, but not in excitatory motor neurones to the longitudinal muscle, in this tissue). Values are mean±SEM for YOUNG, ADULT and OLD guinea pigs (*N*, n=4–6). *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs YOUNG (Student's *t*-test).

from one age to the following one, so that in OLD animals packing density dropped to approximately half of that found in YOUNG guinea pigs. However, the density of axon bundles in the tertiary component of the plexus, which are known to arise from these neurones (Brookes et al., 1992), did not significantly change with age (Fig. 8b).

Discussion and conclusions

In the present work, age-induced changes in function and in myenteric immunohistochemistry of guinea pig ileum LMMP preparations were found to be inversely correlated. Thus, responses to several different excitatory stimuli increased in parallel to an intense decrease in density of excitatory longitudinal muscle motor neurones. In addition, the inhibitory effect of morphine acutely administered and density of the tertiary plexus remained unaltered throughout age, whereas in vitro tolerance to morphine decreased.

In guinea pig ileum, the contraction produced by electrical stimulation of low frequency is known to be due to the release of ACh from the myenteric motor neurones and its action on muscarinic receptors present in the muscle (Daniel, 1982). We found no age-related changes in the electrical threshold for contraction, which could imply that, with each electrical pulse, sufficient ACh from enough motor neurones was being released to activate enough receptors in the muscle, at each of the three ages studied. In fact, although the release of endogenous ACh could decrease with age (Roberts et al., 1994; Tezuka et al., 2004), the occupation of only a small number of receptors might be sufficient to provoke the contraction of the muscle (Ek and Nahorski, 1988).

However, the force of the responses to electrically-released ACh, and other excitatory stimuli, including exogenous administration of ACh, increased with age. The influence of age on cholinergic responses of gastrointestinal muscle seems to be highly dependent on the gastrointestinal region and the experimental animal used. Thus, controversial results have been obtained in the rat (Roberts et al., 1994; Tezuka et al., 2004; Kobashi et al., 1985; Michalek et al., 1993). Nevertheless, in agreement with our results, a report by Tsai and Ochillo (1987) in longitudinal muscle from guinea pig ileum showed an age-related decrease in the EC_{50} for ACh-mediated contractions.

The response to exogenous SP and naloxone also increased in OLD animals. SP is the other main excitatory neurotransmitter of the myenteric plexus and, in the guinea pig ileum, it colocalises with choline acetyl transferase (ChAT) in sensory neurones, ascending interneurones and excitatory motor neurones to both circular and longitudinal muscle (Furness and Costa, 1987; Costa et al., 1996), suggesting that the age-related increase in responses to ACh and SP found in this preparation could be due, at least partially, to changes in cholinergic-peptidergic (SP) innervation. On the other hand, naloxone induced-contraction of the LMMP preparation is due to the sudden release of excitatory neurotransmitters, mainly ACh and SP (Mundey et al., 1998). Therefore, the age-related increase in the effect of naloxone could be due to the increased response to

ACh and SP (and maybe other excitatory neurotransmitters also released after addition of naloxone).

Taking into consideration that the force of the contraction of the LMMP strips obtained from aged guinea pigs increased in a similar way in response to the release of endogenous neurotransmitters (electrically-or naloxone-induced) or to the exogenous administration of ACh or SP, these changes could be a consequence of alterations in the structure and/or function of the longitudinal muscle, or, alternatively, in the numbers, structure and/or function of the interstitial cells of Cajal. Alterations in the muscle could very easily be involved, because, in fact, some hypertrophic structural changes of the muscular fibres have been found in ileum and taenia coli from old guinea pigs (Gabella, 1990, 2001). With regard to the interstitial cells of Cajal, these are functionally interposed between the terminals of motor neurones and the muscle (Ward and Sanders, 2001) and, in addition to their role as gastrointestinal pacemakers, these cells are principal targets of excitatory and inhibitory motor neurones that control the motility of the gastrointestinal tract (Furness, 2006). Impaired neurotransmission to the smooth gastrointestinal muscle has been shown to occur when interstitial cells of Cajal are blocked or lacking (such as in certain pathologies: see Streutker et al., 2007, for review). Therefore, the increase in the response to excitatory stimulation shown in this work, could indicate that interstitial cells of Cajal are rather preserved than lost in aged animals. The use of immunohistochemical methods specific for detecting these cells (c-kit) would be of great help to clarify this.

Age-related changes in the sensitivity and response of the muscle to excitatory stimuli may be due, at least in part, to primary alterations in the myenteric neurones that innervate the guinea pig ileum. In fact, in a previous work, we found several signs of structural alterations in the myenteric plexus (Abalo et al., 2005a). Thus, myenteric ganglia not only were less dense, but contained fewer neurones and were smaller in old than in young guinea pigs.

In the guinea pig ileum, myenteric neurones have been classified in different types according to their chemical code (Costa et al., 1996). The analysis of changes in neurones labelled for each marker could be helpful in understanding the influence of age in gastrointestinal function. Thus, the effect of aging in the density of both the general neuronal population and of four particular immunohistochemically identified subpopulations of neurones was analysed. Packing density was the parameter chosen for this analysis because it has been shown to be relatively independent of the degree of stretching of the tissue (Karaosmanoglu et al., 1996), thus eliminating the effect of age in physical resistance to stretch, and also because most neurones are found inside the ganglia, which also occurs in aged animals (present work; Abalo et al., 2005a).

It has been reported that age induces a reduction in the general population of myenteric neurones (Gabella, 1989; Gomes et al., 1997; Abalo et al., 2005a). In addition, in the present work, in preparations from old animals, lipofuscin, the ageing pigment, was found in part of the neurones immunoreactive to HuC/D, CB or NFT (coupled to green fluorophores), and it is likely that it was also present in part of the neurones labelled with CR or NOS (since these markers were coupled to red fluorophores, the presence of lipofuscin could not be assessed). Indeed, the presence of lipofuscin has been previously reported at least in myenteric neurones immunoreactive to CR in the guinea pig ileum (Abalo et al., 2005a) and to NOS in the human small intestine (Brehmer et al., 2004).

In any case, alterations in particular subpopulations of myenteric neurones could explain functional changes better. Some selective age-related modifications have actually been found. Thus, inhibitory neurones, which express NOS (Costa et al., 1996), seem to be somehow preserved, whilst at least some cholinergic neurones (Phillips et al., 2003; Abalo et al., 2005a), are more affected by age. Our results further support these findings. Although all subpopulations of neurones studied decreased with age, the different subpopulations of neurones analysed showed different sensitivity to age (different degrees of change).

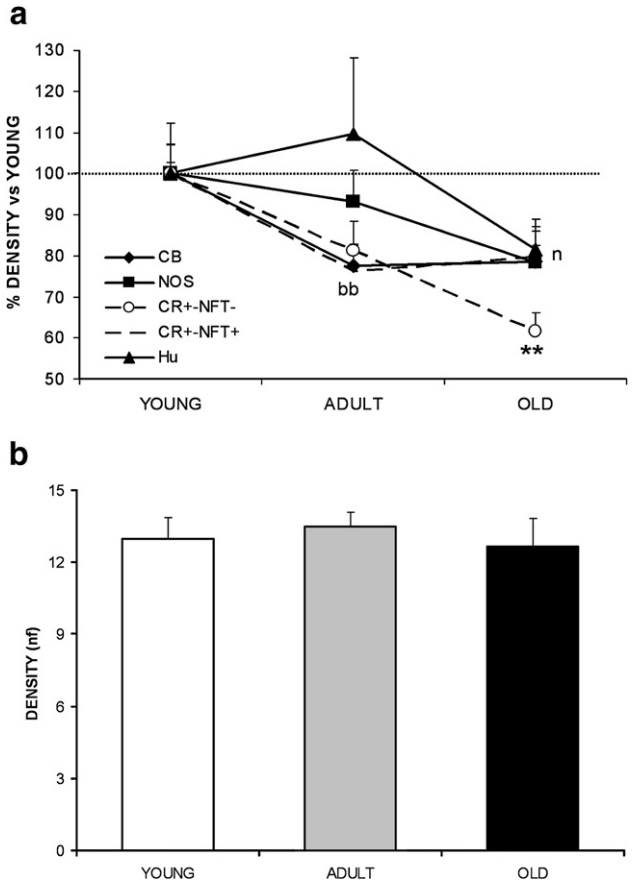

Fig. 8. Panel a: influence of age on the degree of change in packing density of the different populations of immunohistochemically identified neurones in guinea-pig ileum myenteric plexus: neurones immunoreactive to Hu (general population of neurones), CB (sensory neurones), NOS (inhibitory), CR and NFT (ascending interneurones) and CR but not NFT (CR^+ -NFT^--neurones: excitatory longitudinal muscle motor neurones). Values are mean±SEM for YOUNG, ADULT and OLD guinea pigs (N, n=4–6). bb=$p<0.01$ for CB-immunoreactive neurones; n=$p<0.05$ for NOS-immunoreactive neurones; **$p<0.01$ for CR^+ -NFT^--neurones vs YOUNG (Student's t-test). Panel b: influence of age in density of the bundles in the tertiary plexus (fibres arising from CR^+ -NFT^--neurones). Values are mean±SEM for YOUNG (n=6), ADULT (n=6) and OLD (n=6) guinea pigs (Student's t-test). n= samples; N=animals.

Thus, in ADULT animals no change in density of NOS-immunoreactive neurones was found. In the guinea pig, these neurones include some descending interneurones (less than 5% of the total population of myenteric neurones) but the main population of NOS-immunoreactive neurones (16%) is that of inhibitory motor neurones (Costa et al., 1996). In the immunological study, NOS-immunoreactive neurones were analysed as a whole because, due to the fact that these particular interneurones seem to constitute the final link to the inhibitory motor neurones in local descending inhibitory reflexes (Furness, 2006), they could be considered as a functional unit capable of regulating gastrointestinal motility in a highly coordinated, inhibitory fashion.

In contrast to NOS-immunoreactive neurones, density of the other populations of neurones studied in ADULT guinea pigs, namely CB^+ (sensory neurones), CR^+- NFT^+ (ascending interneurones), and CR^+- NFT^--neurones (excitatory motor neurones to the longitudinal muscle), all likely to be cholinergic (Costa et al., 1996), had already decreased. Interestingly, in OLD animals, in which hyperresponsiveness to excitatory stimulation of the LMMP preparations was more evident, the neurones immunohistochemically identified as excitatory longitudinal muscle motor neurones had decreased to almost half the density found in YOUNG guinea pigs.

In spite of the age-related reduction in the density of CR^+-NFT^--neurones, that of the axon bundles in the tertiary plexus, whose fibres are also immunoreactive to CR and known to arise from these neurones (Brookes et al., 1992), did not significantly change with age. In any case, the possibility that these fibres were not as efficacious at stimulating the muscle as in YOUNG guinea pigs should be kept in mind. In fact, an age-dependent reduction in the release of endogenous ACh has been reported both in rat colon (Roberts et al., 1994) and ileum (Tezuka et al., 2004). Even though the number of bundles remained unchanged, the number of axons per bundle could have decreased and contributed to this reduced ability to stimulate the muscle. Electron microscopy studies will help to more accurately determine the effect of age in innervation of the muscle. Whatever the case may be, although hypertrophy of the ileal muscle may justify the increase of the response, the chronic reduction in the release of ACh may induce some degree of hypersensitivity and contribute to the muscle hyperresponsiveness.

Finally, the inhibitory effect of morphine was not altered in aged guinea pigs. Although the reason for this is unknown, it is worth mentioning that a similar result was found with other inhibitory drugs, such as cannabinoids, which share the mechanisms of action of opioids in the myenteric plexus (Abalo et al., 2005b). In addition, in vitro tolerance to morphine in preparations from aged guinea pigs was much lower than in those from young animals, which could be due to an age-related deterioration of the adaptive mechanisms to this opioid. In any case, the in vitro effects of morphine in guinea pig ileum LMMP correlate well with the antinociceptive/analgesic effect of morphine-like compounds (Kosterlitz and Waterfield, 1975), and, very interestingly, the antinociceptive effects of morphine in the rat (Van Crugten et al., 1997; Smith and Gray, 2001; Jourdan et al., 2000) and density and efficacy of opioid receptors in different cerebral and spinal structures (Rabin, 1986; Ueno et al., 1988; Hoskins et al., 1998) did not change with age either. Furthermore, the reduction in tolerance and the increase in the naloxone-induced sign of withdrawal found in vitro in the present work also correlated with the effects of ageing in vivo: both an age-related reduction of tolerance to the antinociceptive (Nozaki et al., 1975) and hypothermic (McDougal et al., 1981) effects of morphine and an increase in the signs of abstinence (Simpkins, 1994) have been found in the rat.

More research is needed to evaluate the gastrointestinal consequences of age-related changes in excitatory and inhibitory neurotransmission in the myenteric plexus — longitudinal muscle system and to try to correlate them with the physiopathological changes commonly seen in aged individuals. However, the fact that excitatory neurones decrease at a higher degree than inhibitory neurones and that tolerance to morphine is reduced with age could imply that the inhibitory tone suggested to exist in the gastrointestinal tract in basal conditions (Abalo et al., 2004) could increase with age, maybe leading to or at least increasing the risk of constipation. On the other hand, hyperresponsiveness to excitatory agonists could underlie the episodes of hypermotility also frequently seen at old ages.

In conclusion, the relatively subtle alterations induced by age in the enteric nervous system could make the equilibrium of gastrointestinal function extremely fragile, leading to an increased sensitivity to pathology in older individuals.

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References

Abalo, R., Pascual, D., Goicoechea, C., Martin, M.I., 2000. Blockade by pertussis toxin of the opioid effect on guinea pig ileum. Contractility and electrophysiological neuronal recording. Neuroscience Letters 291 (3), 131–134.

Abalo, R., Goicoechea, C., Martin, M.I., 2004. Blockade of Gi/o proteins modifies electrical activity of S-myenteric neurons from guinea-pig ileum. Neuroscience Letters 356 (3), 175–178.

Abalo, R., Rivera, A.J., Vera, G., Martín, M.I., 2005a. Ileal myenteric plexus in aged guinea-pigs: loss of structure and calretinin-immunoreactive neurones. Neurogastroenterology and Motility 17 (1), 123–132.

Abalo, R., Rivera, A.J., Vera, G., Suardiaz, M., Martin, M.I., 2005b. Evaluation of the effect of age on cannabinoid receptor functionality and expression in guinea-pig ileum longitudinal muscle-myenteric plexus preparations. Neuroscience Letters 383 (1-2), 176–181.

Aleixandre, M.A., Colado, M.I., Garcia de Jalon, P.D., Martin, M.I., 1983. Influences on morphine action in the myenteric plexus from guinea pig of alpha 2 receptor stimulation. Archivos de Farmacología y Toxicología 9 (3), 217–224.

Brehmer, A., Blaser, B., Seitz, G., Schrodl, F., Neuhuber, W., 2004. Pattern of lipofuscin pigmentation in nitrergic and non-nitrergic, neurofilament immunoreactive myenteric neuron types of human small intestine. Histochemistry and Cell Biology 121 (1), 13–20.

Brookes, S.J., Steele, P.A., Costa, M., 1991. Calretinin immunoreactivity in cholinergic motor neurones, interneurones and vasomotor neurones in the guinea-pig small intestine. Cell and Tissue Research 263 (3), 471–481.

Brookes, S.J., Song, Z.M., Steele, P.A., Costa, M., 1992. Identification of motor neurons to the longitudinal muscle of the guinea pig ileum. Gastroenterology 103 (3), 961–973.

Colado, M.I., Alfaro, M.J., del Val, V.L., Martin, M.I., 1991. Effect of cinitapride in isolated ileum obtained from guinea-pigs treated with morphine. General Pharmacology 22 (5), 863–866.

Collier, H.O., Cuthbert, N.J., Francis, D.L., 1981. Model of opiate dependence in the guinea-pig isolated ileum. British Journal of Pharmacology 73 (4), 921–932.

Costa, M., Furness, J.B., Gibbins, I.L., 1986. Chemical coding of enteric neurons. Progress in Brain Research 68, 217–239.

Costa, M., Brookes, S.J., Steele, P.A., Gibbins, I., Burcher, E., Kandiah, C.J., 1996. Neurochemical classification of myenteric neurons in the guinea-pig ileum. Neuroscience 75 (3), 949–967.

Daniel, E.E., 1982. Pharmacology of adrenergic, cholinergic, and drugs acting on other receptors in gastrointestinal muscle. In: Bertaccini, G. (Ed.), Mediators and drugs in gastrointestinal motility I. (Handbook of Experimental Pharmacology). Springer-Verlag, Berlin, pp. 249–322.

Ek, B., Nahorski, S., 1988. Muscarinic receptor coupling to inositol phospholipid metabolism in guinea-pig cerebral cortex, parotid gland and ileal smooth muscle. Biochemical Pharmacology 37 (23), 4461–4467.

Franco, R., Costa, M., Furness, J.B., 1979. Evidence for the release of endogenous substance P from intestinal nerves. Naunyn-Schmiedeberg's Archives of Pharmacology 306 (3), 195–201.

Furness, J.B., Costa, M., 1987. The enteric nervous system. Churchill Livingstone, Edinburgh.

Furness, J.B. 2006. The enteric nervous system. Blackwell Publishing, Narayana Press, Odder, Denmark.

Gabella, G., 1989. Fall in the number of myenteric neurons in aging guinea pigs. Gastroenterology 96 (6), 1487–1493.

Gabella, G., 1990. Hypertrophy of visceral smooth muscle. Anatomy and Embryology 182 (5), 409–424.

Gabella, G., 2001. Development and ageing of intestinal musculature and nerves: the guinea-pig taenia coli. Journal of Neurocytology 30 (9-10), 733–766.

Gintzler, A.R., Scalisi, J.A., 1982. Effects of opioids on noncholinergic excitatory responses of the guinea-pig isolated ileum: inhibition of release of enteric substance P. British Journal of Pharmacology 75 (1), 199–205.

Gomes, O.A., de Souza, R.R., Liberti, E.A., 1997. A preliminary investigation of the effects of aging on the nerve cell number in the myenteric ganglia of the human colon. Gerontology 43 (4), 210–217.

Grube, D., 1980. Immunoreactivities of gastrin (G-) cells. II. Non-specific binding of immunoglobulins to G-cells by ionic interactions. Histochemistry 66 (2), 149–167.

Hoskins, D.L., Gordon, T.L., Crisp, T., 1998. The effects of aging on mu and delta opioid receptors in the spinal cord of Fischer-344 rats. Brain Research 791 (1-2), 299–302.

Jourdan, D., Boghossian, S., Alloui, A., Veyrat-Durebex, C., Coudore, M.A., Eschalier, A., Alliot, J., 2000. Age-related changes in nociception and effect of morphine in the Lou rat. European Journal of Pain (London, England) 4 (3), 291–300.

Karaosmanoglu, T., Aygun, B., Wade, P.R., Gershon, M.D., 1996. Regional differences in the number of neurons in the myenteric plexus of the guinea pig small intestine and colon: an evaluation of markers used to count neurons. The Anatomical Record 244 (4), 470–480.

Kobashi, Y.L., Breuing, E.P., Markus, R.P., 1985. Age-related changes in the reactivity of the rat jejunum to cholinoceptor agonists. European Journal of Pharmacology 115 (2-3), 133–138.

Kosterlitz, H.W., Waterfield, A.A., 1975. In vitro models in the study of structure-activity relationships of narcotic analgesics. Annual Review of Pharmacology 15, 29–47.

McDougal, J.N., Marques, P.R., Burks, T.F., 1981. Reduced tolerance to morphine thermoregulatory effects in senescent rats. Life Sciences 28 (2), 137–145.

Mehr, S.E., Samini, M., Namiranian, K., Homayoun, H., Gaskari, S.A., Dehpour, A.R., 2003. Inhibition by immunophilin ligands of morphine-induced tolerance and dependence in guinea pig ileum. European Journal of Pharmacology 467 (1-3), 205–210.

Michalek, H., Fortuna, S., Pintor, A., 1993. Age-related changes in muscarinic receptor and post-receptor mechanisms in brain and ileum strip of rats. Acta Neurobiologiae Experimentalis 53 (1), 93–101.

Mundey, M.K., Mason, R., Wilson, V.G., 1998. Selective potentiation by ouabain of naloxone-induced withdrawal contractions of isolated guinea-pig ileum following acute exposure to morphine. British Journal of Pharmacology 124 (5), 911–916.

Nozaki, M., Akera, T., Lee, C.Y., Brody, T.M., 1975. The effects of age on the development of tolerance to and physical dependence on morphine in rats. The Journal of Pharmacology and Experimental Therapeutics 192 (3), 506–512.

Paton, W.D., 1957. The action of morphine and related substances on contraction and on acetylcholine output of coaxially stimulated guinea-pig ileum. British Journal of Pharmacology and Chemotherapy 12 (1), 119–127.

Paton, W.D., 1970. Drug dependence: pharmacological and physiological aspects. Journal of the Royal College of Physicians of London 4 (3), 247–254.

Paton, W.D., Zar, M.A., 1968. The origin of acetylcholine released from guinea-pig intestine and longitudinal muscle strips. The Journal of Physiology 194 (1), 13–33.

Phillips, R.J., Kieffer, E.J., Powley, T.L., 2003. Aging of the myenteric plexus: neuronal loss is specific to cholinergic neurons. Autonomic Neuroscience: Basic and Clinical 106 (2), 69–83.

Pilotto, A., Malfertheiner, P., Holt, P.R., 2003. Aging and the gastrointestinal tract. In: Holt, P.R. (Ed.), Interdisciplinary Topics in Gerontology. Karger, Basel.

Rabin, R.A., 1986. Senescence does not alter activation or inhibition of striatal adenylate cyclase. Neurobiology of Aging 7 (1), 49–51.

Roberts, D., Gelperin, D., Wiley, J.W., 1994. Evidence for age-associated reduction in acetylcholine release and smooth muscle response in the rat colon. The American Journal of Physiology 267 (4 Pt 1), G515–G522.

Simpkins, J.W., 1994. Effects of aging on morphine withdrawal in the rat. Experimental Gerontology 29 (1), 67–76.

Smith, M.A., Gray, J.D., 2001. Age-related differences in sensitivity to the antinociceptive effects of opioids in male rats. Influence of nociceptive intensity and intrinsic efficacy at the mu receptor. Psychopharmacology 156 (4), 445–453.

Streutker, C.J., Huizinga, J.D., Driman, D.K., Riddell, R.H., 2007. Interstitial cells of Cajal in health and disease. Part I: Normal ICC structure and function with associated motility disorders. Histopathology 50 (2), 176–189.

Taylor, G.S., Bywater, R.A., 1986. Antagonism of non-cholinergic excitatory junction potentials in the guinea-pig ileum by a substance P analogue antagonist. Neuroscience Letters 63 (1), 23–26.

Terril, L.A., Clemons, D.J., 1998. The laboratory guinea pig. CRC Press, Boca Raton. (Chapter 1).

Tezuka, A., Ishihata, A., Aita, T., Katano, Y., 2004. Aging-related alterations in the contractile responses to acetylcholine, muscarinic cholinoceptors and cholinesterase activities in jejunum and colon of the male Fischer 344 rats. Experimental Gerontology 39 (1), 91–100.

Tsai, C.S., Ochillo, R.F., 1987. The effects of aging on the responsiveness of the cholinergic receptor of the longitudinal muscle of guinea-pig isolated ileum. Research Communications in Chemical Pathology and Pharmacology 55 (2), 275–278.

Ueno, E., Liu, D.D., Ho, I.K., Hoskins, B., 1988. Opiate receptor characteristics in brains from young, mature and aged mice. Neurobiology of Aging 9 (3), 279–283.

Van Crugten, J.T., Somogyi, A.A., Nation, R.L., Reynolds, G., 1997. The effect of old age on the disposition and antinociceptive response of morphine and morphine-6 beta-glucuronide in the rat. Pain 71 (2), 199–205.

Ward, S.M., Sanders, K.M., 2001. Physiology and pathophysiology of the interstitial cell of Cajal: from bench to bedside. I. Functional development and plasticity of interstitial cells of Cajal networks. American Journal of Physiology: Gastrointestinal and Liver Physiology 281 (3), G602–G611.

5. Discusión

En la preparación whole-mount es relativamente fácil establecer la densidad espacial de neuronas por la uniformidad longitudinal del plexo mientérico. Además, es una preparación de fácil manejo; quizá por ello, el íleon de cobayo es la región GI mejor caracterizada morfológica y funcionalmente (Furness y Costa, 1987; Costa y Brookes, 2008). Pero hay pocos estudios que relacionen los cambios funcionales y los morfológicos que suceden con el envejecimiento en el tracto gastrointestinal.

El parámetro que hemos utilizado para establecer diferencias con la edad en la densidad neuronal ha sido la densidad de empaquetamiento neuronal. Permanece constante a lo largo de la longitud del intestino, independientemente de la región analizada y del grado de estiramiento (Karaosmanoglu *et al.,* 1996) y además la mayoría de las neuronas se encuentran dentro de los ganglios, hecho que también ocurre en los animales viejos.

Nuestro resultado acerca de la estructura del plexo mientérico y una disminución en la población general neuronal está de acuerdo con los datos previos de la literatura. La pérdida neuronal con la edad en el plexo mientérico del tracto digestivo se ha observado en variedad de especies, incluyendo humanos (de Souza *et al.,* 1993; Gomes *et al.,* 1997), oveja (Pfannkuche *et al.,* 2003), cobayos (Gabella 1989; Wade *et al.* 2001; Wade 2002), ratas (Santer *et al.,* 1988; Cowen *et al.,* 2000; Philips y Powley, 2001) y ratón (El-Salhy *et al.,* 1999). Esta pérdida neuronal es significativa a partir de los 9-10 meses de edad; en mamíferos más pequeños se había visto a partir de los 12 meses (Philips y Powley, 2001). Una pérdida de la inervación intrínseca mientérica podría estar relacionada con alteraciones fisiopatológicas de la motilidad intestinal asociadas con el envejecimiento (Firth y Prather, 2002; O'Mahony *et al.,* 2002; Wade 2002, ver más adelante).

Las neuronas del plexo mientérico se clasifican en función de la morfología, electrofisiología, y combinación de marcadores neuronales que expresan (código químico). Se ha visto que los marcadores neuronales cambian con la edad, lo que significa que las distintas poblaciones funcionales pueden verse alteradas. Las neuronas inmunorreactivas para la NOS, en el cobayo, incluyen algunas interneuronas descendentes y las motoneuronas inhibidoras (Costa *et al.,* 1996). Debido a que su función es predominantemente inhibidora, las hemos estudiado en conjunto. Hemos encontrado una relativa preservación de estas neuronas inhibidoras. Resultados similares se han descrito en intestino delgado (Santer, 1994; Belai *et al.,* 1995; Cowen

et al., 2000; Phillips *et al.,* 2003), y grueso (Belai *et al.,* 1995) de rata; y en humanos (Belai y Burnstock, 1999).

En contraste con la preservación de neuronas inhibidoras en cobayos viejos, otras poblaciones neuronales, como las neuronas aferentes primarias intrínsecas (calbindina, CB+), interneuronas ascendentes (CR+/NFT+) y motoneuronas excitadoras de la musculatura longitudinal (CR+/NFT-), todas colinérgicas (Costa *et al.,* 1996), parecen ser más sensibles al envejecimiento, lo que está de acuerdo con estudios previos (Phillips *et al.,* 2003). Aunque recientemente se ha visto que la calbindina no marca la totalidad de las neuronas sensoriales (hay un 14 % de neuronas mientéricas que no expresan este marcador y también son aferentes primarias, Costa y Brookes, 2008), las que sí expresan este marcador disminuyen con la edad, como también sucede en el duodeno de jerbo (Choi *et al.,* 2008) y en íleon de rata (Thrasivoulou *et al.,* 2006).

Las proteínas tamponadoras de calcio, como la CR y CB, son esenciales para la homeostasis de este ión. Una disminución en las neuronas inmunorreativas para CR puede ser signo de degeneración celular (Baimbridge, 1992; Heizmann, 1992). Es interesante la disminución de las motoneuronas excitadoras de la musculatura longitudinal en los animales viejos, ya que en el estudio funcional se vio una hipersensibilidad a la estimulación excitadora (ver más adelante). A pesar de esta reducción de la densidad de las motoneuronas excitadoras de la musculatura longitudinal (CR+/NFT-), las fibras inmunorreactivas para CR que constituyen el plexo terciario y se sabe que surgen de estas neuronas (Brookes *et al.,* 1992), no cambian significativamente con la edad. En cualquier caso, puede que, aunque no descienda el número de fibras (para determinarlo habría que realizar estudios de microscopía electrónica), éstas no sean capaces de estimular el músculo como en los cobayos jóvenes.

En las preparaciones procedentes de animales viejos hemos observado un material autofluorescente, probablemente lipofuscina, que se acumula en los lisosomas y puede ser indicador de estrés oxidativo y envejecimiento (Harman, 1989). Aunque aparece en la población neuronal general, se acumula en mayor medida y más rápido en las neuronas CR+. Podría ser un indicador de neurodegeneración incipiente.

En cuanto a la funcionalidad de la preparación, la respuesta a estímulos excitadores aumenta, mientras que el efecto de los agonistas inhibidores, tanto la

morfina como los cannabinoides, no se altera con la edad. Además, la tolerancia *in vitro* a la morfina disminuye.

En el íleon del cobayo, la contracción producida por la estimulación eléctrica de baja frecuencia es debida a la liberación de ACh desde las motoneuronas mientéricas y su acción en los receptores muscarínicos presentes en el músculo (Daniel, 1982). La fuerza de la respuesta a la liberación de ACh por estimulación eléctrica (o por acción de naloxona en preparaciones incubadas con morfina) y otros estímulos excitadores (incluyendo la administración exógena de ACh y SP), aumenta con la edad. Este aumento podría deberse a cambios en la inervación colinérgica o cambios en la respuesta a estos neurotransmisores. El plexo terciario no se modifica en los animales viejos, pero estos axones pueden no liberar adecuadamente ACh, lo que podría inducir hipersensibilidad y ser responsable de la respuesta aumentada que hemos visto en el músculo. Otra explicación podrían ser cambios en la estructura y/o función del músculo longitudinal, o de las células intersticiales de Cajal (ICC). En íleon y tenia coli de cobayos viejos se han visto alteraciones hipertróficas en el músculo (Gabella, 1990; 2001). Por otro lado, las células intersticiales de Cajal son la principal diana de las motoneuronas excitadoras e inhibidoras que controlan la motilidad del tracto gastrointestinal (Furness, 2006). Se ha detectado pérdida de ICC en desórdenes de la motilidad, como la gastroparesis, pseudobstrucción intestinal y constipación (He *et al.,* 2000; Feldstein *et al.,* 2003; Forster *et al.,* 2005), pero no se sabe qué sucede en el envejecimiento con las ICC, aunque nuestros resultados podrían indicar que están bastante conservadas.

Estos cambios con el envejecimiento en la sensibilidad y la respuesta del músculo a estímulos excitadores pueden relacionarse con las alteraciones primarias en las neuronas mientéricas que hemos mencionado anteriormente. Puede influir el hecho de que los ganglios mientéricos sean menos densos, contengan menos neuronas y sean más pequeños en los cobayos viejos que en los jóvenes. En diversos estudios se ha visto que la muerte celular durante el envejecimiento parece afectar más a las neuronas colinérgicas que a las nitrérgicas (Johnson *et al.,* 1998; Cowen *et al.,* 2000). Y también disminuye la liberación de ACh endógena en colon (Roberts *et al.,* 1994) e íleon de rata (Tezuka *et al.,* 2004). Se ha postulado que la denervación puede originar una mayor sensibilidad en las células en respuesta a cambios crónicos en los estímulos que reciben (Goto *et al.,* 1978). Esto podría ser responsable de la mayor respuesta a los estímulos excitadores que hemos observado.

Con respecto a los agonistas inhibidores, el efecto de la morfina no se alteró en los cobayos viejos. En cambio, la tolerancia *in vitro* en preparaciones de animales viejos, fue menor que en los animales jóvenes, lo que puede deberse a un deterioro con la edad en los mecanismos adaptativos (quizá alteraciones en las proteínas kinasas, Gabra *et al.,* 2008) a estos fármacos. En cualquier caso, estos efectos *in vitro* de la morfina son paralelos a los efectos antinociceptivos/analgésicos de los compuestos opioides (Kosterlitz y Waterfield, 1975) y de manera interesante, los efectos antinociceptivos de la morfina en rata (Van Crugten *et al.,* 1997; Jourdan *et al.,* 2000; Smith y Gray, 2001) tampoco cambian con la edad. Además, *in vivo* se produce una reducción de la tolerancia a los efectos antinociceptivos de la morfina con el envejecimiento (Nozaki *et al.,* 1975) y un aumento del signo de abstinencia en rata (Simpkins, 1994).

Por último, la respuesta de la preparación de FL-PM a los cannabinoides no se modificó con la edad, lo cual concuerda con los resultados del estudio inmunohistoquímico: las neuronas mientéricas inmunorreactivas para el receptor CB1, entre las que se incluyen al menos parte de las motoneuronas excitadoras de la musculatura lisa longitudinal, disminuyen de manera similar a la población neuronal total; sin embargo, la proporción de neuronas que lo expresan no cambia con la edad. La expresión del receptor CB1 es amplia en las neuronas mientéricas (Kulkarni-Narla y Brown, 2001; Coutts *et al.,* 2002), como se produce una disminución de las motoneuronas (cuya gran mayoría expresan el receptor CB1, Costa *et al.,* 1996), se podría sugerir que algunas de las subpoblaciones funcionales de neuronas que no expresan el receptor CB1 en los animales jóvenes, podrían hacerlo en los viejos. Esto podría justificar que el efecto de los cannabinoides en el íleon aislado se mantenga. Sin embargo, en otras partes del Sistema nervioso no sucede lo mismo, en el SNC (Romero *et al.,* 1998), por ejemplo, se ha visto una disminución con la edad del ARN mensajero para el receptor CB1.

Habría que tener en cuenta la independencia de la edad en la expresión del receptor CB1 y en su efecto en la motilidad gastrointestinal si los cannabinoides se usasen terapéuticamente

6. Conclusiones

1. Con la edad, en el plexo mientérico de íleon de cobayo, se produce una pérdida neuronal que no afecta por igual a las distintas poblaciones neuronales. Afecta en mayor grado a las neuronas excitadoras, mientras que las neuronas inhibidoras parecen estar preservadas.

2. Hay una mayor respuesta a los estímulos excitadores en las preparaciones procedentes de animales viejos. Además el efecto de los agonistas inhibidores (tanto opioides como cannabinoides) no se modifica con el envejecimiento. Todo esto podría ser responsable de los episodios de hipermotilidad y constipación frecuentes a edades avanzadas.

En conclusión, las alteraciones que se producen con la edad en el SNE podrían hacer que el equilibro de la función GI sea más frágil, lo que haría más susceptibles a las patologías a los individuos viejos.

PARTE 2

EFECTO DE LOS CANNABINOIDES EN LAS ALTERACIONES CAUSADAS POR EL TRATAMIENTO CRÓNICO CON CISPLATINO EN RATA.

1. Introducción

Entre los efectos adversos que causa el uso de antineoplásicos en la práctica clínica se incluyen la náusea y/o emesis, anorexia, y neuropatía periférica sensorial (Van Cutsem y Arends, 2005; Stillman y Cata, 2006; Navari, 2007; Schwartzberg, 2007). Pero sin duda, el efecto adverso más desagradable y uno de los más temidos por los pacientes tratados con quimioterapia (incluso antes de comenzar el tratamiento), es la náusea y el vómito. Se estima que un 75% de los pacientes sin tratamiento antiemético presentan síntomas (Malik *et al.,* 1995). Estos efectos pueden desembocar en trastornos metabólicos serios, carencias nutricionales y anorexia, problemas esofágicos y deterioro del estatus físico y mental de los pacientes, lo cual puede provocar la interrupción del tratamiento (Craig y Powell, 1987; Passik *et al.,* 2001). Las guías del tratamiento antiemético publicadas por la Sociedad Americana de Oncología (ASCO) recomiendan inhibidores de la serotonina en combinación con corticoides en las náuseas y vómitos agudos en pacientes que reciben cisplatino (Jantunen *et al.,* 1997). En las náuseas y vómitos tardíos se recomiendan metoclopramida y corticoides (Mantovani *et al.,* 1998) y en las náuseas y vómitos anticipatorios, terapia conductual y benzodiacepinas (Malik *et al.,* 1995). Aunque los antagonistas de la serotonina (ondansetrón, por ejemplo) han supuesto una gran herramienta terapéutica, su eficacia en la prevención de las náuseas es limitada ya que sólo alrededor del 50% de los pacientes incluidos en los ensayos clínicos experimentan una mejoría y su eficacia en tratamiento crónico se reduce (Warr, 2008). Desafortunadamente, el uso de los fármacos antieméticos (ondansetrón y otros) a menudo provoca la aparición de resistencia a dichos fármacos (De Wit *et al.,* 2004) y el hecho de administrar la terapia anticancerosa en ciclos hace que la eficacia de estos fármacos se vea muy mermada. Por ello, se deben hacer esfuerzos para desarrollar nuevos fármacos capaces de mantener su eficacia durante toda la terapia o que sirvan de alternativa cuando otras terapias fallan.

Otra de las alteraciones asociadas al cáncer y a las terapias anticancerosas es la anorexia y caquexia. Los pacientes tienen muchas veces reducida la ingesta de alimentos (debido a la propia enfermedad, a efectos psicológicos o a los efectos secundarios del tratamiento), así como alteraciones en el metabolismo de los nutrientes que pueden modificar el estado nutricional. Agentes producidos por el tumor o liberados sistémicamente en respuesta al tumor, como citoquinas y hormonas, se han implicado en la malnutrición y caquexia. Las consecuencias son alteraciones de la función inmune, de la función muscular y de la calidad de vida. Además puede

disminuir la respuesta a la quimioterapia. En consecuencia, hay que dar al paciente soporte nutricional, y asesorarle sobre las necesidades alimentarias específicas (Van Cutsem y Arends, 2005).

Otro efecto adverso derivado del uso de los antineoplásicos es la neurotoxicidad (Warner, 1995), generalmente no es limitante, pero puede afectar a las actividades diarias de los pacientes y a su calidad de vida. Se caracteriza por una neuropatía periférica axonal con pérdida sensorial e hiperexcitabilidad nerviosa que incluye parestesia, disestesia y dolor (Park *et al.,* 2008). Los mecanismos subyacentes a la neurotoxicidad causada por antineoplásicos son diversos e incluyen daño de los cuerpos celulares de las neuronas de los ganglios del asta dorsal de la médula (DRG) y toxicidad por alteraciones en el transporte axonal y fallos en la obtención de energía. Se han propuesto gran cantidad de fármacos para paliar o prevenir estas alteraciones producidas por la quimioterapia, pero hasta el momento no se ha encontrado un fármaco suficientemente eficaz (Park *et al.,* 2008).

1.1. Cisplatino

Los complejos coordinados de platino se han usado en la terapia contra el cáncer desde hace más de 30 años. Sus propiedades antiproliferativas se observaron por primera vez en 1965 por Barnett Rosenberg en la Universidad de Michigan (Rosenberg *et al.,* 1965). Tras las observaciones de Rosenberg, se probó la utilidad del cisplatino en diversos tipos de sarcoma, y posteriormente en otros tipos de tumor. Actualmente, el cisplatino y sus análogos se usan en la clínica para el tratamiento de diversos tumores (ovario, testículo, pulmón, colon y otros). El problema de los derivados del cisplatino es su elevada toxicidad.

1.2. Alteraciones gastrointestinales inducidas por cisplatino.

Entre los síntomas que produce el cisplatino, están la náusea y la emesis tanto aguda como retrasada. De hecho, es el fármaco antitumoral más emetógeno (Jordan et al*.,* 2005), y se utiliza como referencia para el estudio de fármacos antieméticos en animales de experimentación (Andrews y Horn, 2006). También produce anorexia, pérdida de peso, sensación de saciedad (Donnelly y Walsh, 1995) y alteraciones de la función GI (Kris *et al.,* 1988). El retraso en el vaciamiento gástrico que producen los fármacos antineoplásicos, podría ser el responsable, al menos en parte, de estos síntomas (Nelson *et al.,* 1993; Nelson y Walsh, 1993), lo cual podría ser una complicación de la enfermedad en sí misma y de su tratamiento (Liu *et al.,* 2006).

Detrás de estas alteraciones podría haber disfunciones del sistema nervioso autónomo y estasis gástrica (Nelson *et al.,* 2002).

Las anormalidades autonómicas también se han observado en pacientes tratados con cisplatino (Boogerd *et al.,* 1990), pero la controversia surge del limitado número de estudios experimentales, que no han sido capaces de establecer suficientes evidencias del cisplatino como inductor de neuropatía autonómica (Vandertop *et al.,* 1996). De todas formas, estos estudios se enfocan en los efectos cardiovasculares y sudomotores del cisplatino. Hasta ahora no se ha realizado ningún estudio para establecer si el cisplatino puede inducir signos de neuropatía autonómica en el tracto gastrointestinal. No obstante, los síntomas gastrointestinales aparecen en otras neuropatías autonómicas, por ejemplo en la neuropatía diabética (Wegener *et al.,* 1990), en neuropatía producida por antitumorales como la vincristina, que provoca íleo paralítico (Legha, 1986), o por acridina (Belai y Burnstock, 1996). Más aún, en estas neuropatías autonómicas, las alteraciones de la función gastrointestinal ocurren junto con alteraciones de la innervación del tracto digestivo (Belai y Burnstock, 1996; Phillips *et al.,* 2006).

1.2.1. Modelos animales para el estudio de las alteraciones gastrointestinales y de la motilidad inducidas por cisplatino

Los humanos y otros mamíferos tienen mecanismos fisiológicos para eliminar sustancias tóxicas presentes en los alimentos, como evitar la comida por su sabor u olor, y comportamientos evocados como náusea, vómito y diarrea. El vómito es un reflejo protector para la supervivencia que sirve para expulsar toxinas ingeridas accidentalmente. La náusea y vómito inducidos por cisplatino, se han estudiado en numerosas especies animales que poseen el reflejo del vómito (emético): paloma (Tanihata *et al.,* 2000), perro (Fitzpatrick *et al.,* 1990), gato (King, 1990; Rudd *et al.,* 2000), musaraña (Matsuki *et al.,* 1988; Andrews *et al.,* 2000) y hurón (Rudd *et al.,* 1994). Estos experimentos son muy laboriosos y costosos porque utilizan animales no roedores. Las especies usadas habitualmente en el laboratorio, ratas, ratones y cobayos, carecen de la respuesta motora frente a estímulos emetógenos. Estas especies han desarrollado un comportamiento diferente, que consiste en la ingesta de material no nutritivo, como el caolín. Este comportamiento se ha denominado *pica* (Mitchell *et al.,* 1976; Takeda *et al.,* 1993, 1995a,b). Se supone que, cuando la rata ingiere sustancias tóxicas experimenta algo similar a la náusea, y esta experiencia induce la pica (al parecer, el caolín adsorbería las sustancias tóxicas). Además, los

mismos agentes que causan náusea y vómito en humanos producen pica en ratas, como la radiación (Yamamoto *et al.,* 2002b), el movimiento (Uno *et al.,* 2000), el sulfato de cobre (Yamamoto *et al.,* 2004), cloruro de litio (Yamamoto *et al.,* 2004), apomorfina (Takeda *et al.,* 1993), opioides (Aung *et al.,* 2004) y diversos fármacos antitumorales como el cisplatino (Takeda *et al.,* 1993). Más aún, el ondansetrón, la dexametasona y los bloqueantes NK1, que son antieméticos en humanos (de Wit *et al.,* 2004), reducen la pica en ratas (Takeda *et al.,* 1993; Saeki *et al.,* 2001; Rudd *et al.,* 2002).

Hasta la fecha, el modelo de pica se había utilizado sólo en el estudio de emesis asociada a una sola administración de cisplatino (Takeda *et al.,* 1993; Saeki *et al.,* 2001; Rudd *et al.,* 2002). Sin embargo, un número considerable de protocolos terapéuticos utilizan la administración del fármaco en ciclos de más de un día, en los cuales, la eficacia de los antieméticos, se reduce considerablemente. Por todo ello, es interesante estudiar la emesis asociada a administraciones repetidas de cisplatino en modelos experimentales.

En modelos animales también se ha visto que, al igual que en humanos, el tratamiento con antineoplásicos produce retraso en el vaciamiento gástrico y el tránsito intestinal. En rata, una dosis única de cisplatino 10 mg/kg es capaz de disminuir el vaciamiento gástrico a la mitad (Lee *et al.,* 2008). El cisplatino produce una inhibición del vaciamiento gástrico lo cual provoca acumulación de comida en el estómago (Aggarwal *et al.,* 1994; Malik *et al.,* 2007) y un aumento del peso del estómago (Malik *et al.,* 2007). La distensión del estómago que se produce es paralela a la náusea y vómito que ocasiona la administración del antitumoral (Ladabaum *et al.,* 1998).

Las técnicas utilizadas hasta el momento para determinar alteraciones en el vaciamiento gástrico y en el tránsito GI como el azul de Evans, el carbón vegetal o el rojo fenol, requieren el sacrificio de los animales. Otros métodos utilizan marcadores no absorbibles (Scarpignato *et al.,* 1980; Depoortere *et al.,* 2005) o isótopos radiactivos (Miller *et al.,* 1981), pero estos métodos son invasivos y no permiten estudiar el efecto de los fármacos a distintos tiempos en el mismo animal. Los métodos no invasivos, como la detección en aire expirado de C13 del ácido octanoico (Schoonjans *et al.,* 2002), son caros y requieren isótopos radiactivos. La resonancia magnética también puede ser útil para detectar cambios en la morfología y probablemente en la motilidad, pero los equipos necesarios son costosos y el animal debe estar anestesiado y monitorizado (Driehuys *et al.,* 2008; Hildebrandt *et al.,* 2008). Por tanto, el desarrollo de un método alternativo que permita la evaluación del tránsito

intestinal y el vaciamiento en animales vivos sería de gran ayuda y un avance desde el punto de vista ético (reduciría el número de animales necesarios para obtener resultados).

Aunque se utiliza mucho en gastroenterología en humanos, la radiología se ha utilizado poco para estudiar la motilidad en animales de laboratorio (Diani *et al.,* 1979, Perry *et al.,* 1993; Turiiski *et al.,* 2004), y los estudios que se han realizado han utilizado anestesia (Perry *et al.,* 1993; Turiiski *et al.,* 2004), que puede interferir con la motilidad intestinal (Torjman *et al.,* 2005).

1.3. Neuropatía causada por antineoplásicos

Otro efecto adverso derivado del uso de fármacos antineoplásicos, incluidos los derivados del platino, es la neurotoxicidad (Warner, 1995). Aunque no suelen ser limitantes, las neuropatías sensoriales y motoras inducidas por quimioterápicos (incluso si son leves) tienen importantes implicaciones clínicas, pueden ser invalidantes y causar malestar persistente en los pacientes. La neuropatía periférica se define como una disfunción de las neuronas periféricas (motoras, sensoriales y autonómicas, Armstrong *et al.,* 2005) que tiene como resultado signos y síntomas sensoriales (parestesia, disestesia, hipoestesia, hiperestesia, pérdida de propiocepción, y de discriminación del tacto y la temperatura, arreflexia) y motores (dolor y/o debilidad). Aunque generalmente las repercusiones son leves o subclínicas (Freeman, 2005), podrían empeorar la susceptible salud de los pacientes.

Al igual que otros tipos de dolor neuropático, las neuropatías inducidas por antineoplásicos carecen de tratamiento farmacológico eficaz, y por ello suelen ser motivo de abandono del tratamiento anticanceroso. Se recomienda el uso de fármacos habituales para paliar otros tipos de dolor neuropático como la amifostina (Hilpert *et al.,* 2005), vitamina E (Weijil *et al.,* 1998; Argyriou *et al.,* 2005), infusiones de calcio y magnesio (Gamelin *et al.,* 2004), antidepresivos tricíclicos como la nortriptilina (Hammack *et al.,* 2002), anticonvulsivantes como la carbamazepina (Eckel *et al.,* 2002), acetil-L-carnitina (Bianchi *et al.,* 2005; Maestri *et al.,* 2005); glutamina (Stubblefield *et al.,* 2005), glutatión (Ocean y Vahdat, 2004) entre otros. Pero, al igual que sucede con otros tipos de dolor neuropático, estos tratamientos no siempre consiguen resultados satisfactorios.

1.3.1. Modelos animales para el estudio de la neuropatía inducida por tratamiento quimioterápico

Para estudiar los mecanismos responsables del desarrollo de neuropatías asociadas al uso de quimioterápicos se han propuesto diversos modelos animales, tales como la administración de vincristina en ratas de forma intravenosa continua (Authier *et al.,* 1999; Nozaki-Taguchi *et al.,* 2001) o intraperitoneal (i.p) (Rahn *et al.,* 2007); la administración de paclitaxel (Polomano *et al.,* 2001) i.p o la administración de cisplatino, tanto de forma aguda (García *et. al.,* 2008) como crónica (Authier *et al.,* 2000). Estos modelos han sido capaces de reproducir en los animales de laboratorio las neuropatías periféricas dolorosas que inducen diferentes quimioterapias, haciendo posible estudiar las causas que las provocan y buscar nuevas terapias farmacológicas para su tratamiento (Polomano y Bennett, 2001). En los animales se desarrollan alteraciones sensoriales similares a las manifestaciones clínicas que acompañan al dolor neuropático de los pacientes tratados con los antitumorales, como la hiperalgesia térmica y la alodinia mecánica. Ambas se pueden medir en la superficie plantar de las patas traseras del animal, que está inervada por el nervio ciático, y así evaluar la eficacia de fármacos antinociceptivos (Polomano *et al.,* 2001)

La hiperalgesia térmica se puede evaluar mediante el método descrito por Hargreaves *et al.* (1988), llamado plantar test. Evalúa el tiempo que tarda el animal en retirar la pata trasera cuando se la somete a un foco de calor constante. Para evitar lesión tisular, el estímulo nocivo es de duración limitada. Los filamentos de von Frey se pueden utilizar para valorar la alodinia mecánica (Fox *et al.,* 2001). Mediante estos filamentos, que ejercen una presión variable, se determina el umbral de respuesta a la estimulación mecánica de cada animal.

1.4. Cannabinoides

Los cannabinoides constituyen un conjunto de compuestos presentes en la resina de las hojas y brotes florecidos de la planta *Cannabis sativa*. Los preparados obtenidos a partir de la planta, como el hachís y la marihuana, se encuentran entre las drogas de abuso más consumidas en el mundo, lo que ha influido en que el estudio científico de sus propiedades haya sido escaso hasta las últimas décadas.

En el siglo XIX aparecieron los primeros datos contrastados sobre el cannabis y se extendió su uso con fines curativos en Gran Bretaña, gracias a la divulgación de un médico inglés, O´Shaughnessy (Nahas *et al.,* 1973: Fernández-Ruiz *et al.,* 2000). Este

doctor obtuvo resultados positivos en el tratamiento del dolor, pérdida de apetito, rabia, tétanos, etc., que facilitaron la incorporación del cáñamo hindú a la Farmacopea inglesa, y, posteriormente, a la americana.

Durante las primeras décadas del siglo XX disminuyeron las aplicaciones farmacológicas del cannabis, e incluso fue prohibido. Pudo deberse a que era necesario usar preparaciones de la planta cruda o de sus extractos, lo que no estaba bien visto socialmente.

En los últimos años, han aumentado mucho los estudios sobre las aplicaciones terapéuticas del cannabis, impulsados principalmente por el descubrimiento de la existencia de un sistema cannabinoide endógeno, del que también forman parte las dianas moleculares sobre las que actúan tanto los cannabinoides endógenos, como los presentes en la *Cannabis sativa* y los sintetizados en el laboratorio. Gaoni y Mechoulam (1964) aislaron el principal componente psicotrópico, Δ^9 tetrahidrocannabinol (Δ^9-THC). Veinticinco años después, los receptores específicos en cerebro para estos compuestos, los receptores CB1 y CB2, fueron identificados (Matsuda *et al.,* 1990) y clonados (Munro *et al.,* 1993).

1.4.1. Uso de los cannabinoides en el tratamiento de las alteraciones gastrointestinales causadas por quimioterápicos.

Aunque los cannabinoides se han usado tradicionalmente para el tratamiento y/o prevención de los síntomas producidos por la administración de antitumorales, hasta hace poco no se han encontrado evidencias científicas sobre su utilidad (Davis *et al.,* 2007).

Los efectos antieméticos de los cannabinoides, parecen estar mediados por acción del receptor CB1. Estos receptores se han encontrado en el tracto gastrointestinal y en el sistema nervioso entérico (Pertwee, 2001) así como en el cerebro de ratas, hurones y musarañas (Simoneau *et al.,* 2001; Van Sickle *et al.,* 2001; Van Sickle *et al.,* 2003). A nivel gastrointestinal, disminuyen la motilidad intestinal (Pertwee, 2001), pero también actúan en el Sistema Nervioso Central disminuyendo la emesis (Van Sickle *et al.,* 2001; 2003).

El constituyente principal del cannabis, el Δ^9-THC, y varios agonistas sintéticos CB1/CB2 (CP 55,940 y WIN 55, 212-2) han resultado eficaces como antieméticos, e incluso son capaces de prevenir la emesis inducida por cisplatino en diferentes

modelos animales (Darmani, 2001 a,b,c; Van Sickle *et al.,* 2003). De hecho dos análogos estructurales del Δ^9-THC (el dronabinol y la nabilona), y el levonantradol se utilizan como antieméticos para el tratamiento de las náuseas y vómitos inducidos por citostáticos que no responden al tratamiento habitual. En un estudio, Tramer *et al.,* (2001) compararon la eficacia de estos fármacos con placebo y otros antieméticos (fundamentalmente antagonistas 5-HT3) y concluyeron que eran más eficaces. También se han evaluado en la profilaxis de las náuseas y vómitos en pacientes con SIDA (Green *et al.,* 1989; Flynn y Hanif, 1992), pero su uso no está autorizado actualmente con esta indicación.

El uso de quimioterápicos también está asociado a una reducción del peso corporal debida a la anorexia (pérdida de apetito), tanto en humanos (Van Cutsem y Arends, 2005), como en animales de experimentación (Authier *et al.,* 2003 a,b). Los cannabinoides han sido propuestos y usados para reducir la caquexia asociada al SIDA o a cáncer avanzados (Ben Amar, 2006). También se introdujo el antagonista del receptor cannabinoide CB1, rimonabant, en estudios clínicos en pacientes obesos como anorexígeno (Halford, 2006); sin embargo, ha tenido que ser retirado del mercado por las alteraciones psiquiátricas que producía (Stapleton, 2009). Un modelo para estudiar la pica inducida por el cisplatino crónico en rata podría ser útil para determinar los efectos a largo plazo de los cannabinoides en las alteraciones del comportamiento alimentario, tanto en la anorexia como en la náusea.

1.4.2. Uso de los cannabinoides en la neuropatía causada por antineoplásicos

El tratamiento del dolor neuropático es uno de los desafíos en la terapia del dolor. Los opioides, los analgésicos más potentes usados en clínica, son relativamente ineficaces en este tipo de dolor. Por ello, sería interesante estudiar si los derivados cannabinoides son una herramienta válida para su tratamiento.

Hasta ahora, el tratamiento de la neuropatía causada por cisplatino ha consistido en limitar la dosis total del fármaco, reducir dosis individuales o interrumpir el tratamiento para moderar los síntomas (Ocean y Vahdat, 2004). Además, los fármacos utilizados no siempre han sido eficaces para mitigar o prevenir la neuropatía inducida por quimioterápicos (Umapathi y Chaudhry, 2005; Albers *et al.,* 2007).

En animales, se ha demostrado la eficacia de los cannabinoides en la hiperalgesia térmica y la alodinia mecánica en modelos de dolor crónico en ratas

(Herzberg *et al.,* 1997; Fox *et al.,* 2001; Scott *et al.,* 2004; Costa *et al.,* 2006; Costa *et al.,* 2007; Comelli *et al.,* 2008). Estos efectos se obtienen a dosis subanalgésicas y que no producen efectos psicoactivos. Además, también han sido útiles en la reversión de la alodina e hiperalgesia inducidas por la administración de paclitaxel en rata (Pascual *et al.,* 2005). En el tratamiento de la alodinia e hiperalgesia causadas por vincristina, los cannabioides han demostrado ser más potentes que la morfina (Rahn *et al.,* 2007).

Con estos antecedentes y debido a las propiedades neuroprotectoras que han demostrado los cannabinoides, es tentador investigar si pueden también prevenir el desarrollo de la neuropatía periférica inducida por cisplatino.

2. Objetivos

Estudiar:

1. El desarrollo de anorexia, pica, alteraciones de la motilidad gastrointestinal y neuropatía entérica producidas por tratamiento con cisplatino en la rata. Este objetivo se recoge en los siguientes trabajos:

a. *Altered feeding behaviour induced by long-term cisplatin in rats.* Auton Neurosci. 2006; 126-127:81-92.
b. *Radiological study of gastrointestinal motor activity after acute cisplatin in the rat. Temporal relationship with pica.* Auton Neurosci. 2008; 141(1-2): 54-65.
c. *Enteric neuropathy induced by chronic cisplatin in the rat.* Manuscrito en preparación

2. El efecto de los cannabinoides en las alteraciones alimentarias (anorexia y pica) y la neuropatía sensorial producidas por cisplatino en un modelo de administración crónica en rata

a. *WIN 55,212-2 prevents mechanical allodynia but not alterations in feeding behaviour induced by chronic cisplatin in the rat.* Life Sci. 2007; 81(6):468-79.

3. Material y métodos

Para el estudio se han utilizado ratas Wistar macho (230-270 g) obtenidas de Harlan- Ibérica (Barcelona, Spain). Los animales se estabularon aislados (para estudiar la pica y la anorexia producida por administración aguda o crónica de cisplatino) o agrupados (para el estudio de neuropatía periférica y para el estudio radiográfico). Cuando se analizó la pica (la ingesta de sustancias no nutritivas ante un estímulo emetógeno), las ratas tenían libre acceso al caolín.

En los tratamientos crónicos, los animales se acostumbraron la primera semana del experimento a los tests y a la manipulación por parte del investigador y en los animales en los que se estudió la ingesta de sustancias no nutritivas (pica), al caolín y al aislamiento. Tras el periodo de adaptación se administró cisplatino a 1, 2 ó 3 mg/kg, ip. En los estudios en los que se quería ver el efecto del agonista no selectivo WIN 55, 212-2 (WIN), éste se administró 30 minutos antes del antitumoral. Los fármacos se administraron durante 4 ó 5 semanas (T4-T5). Para prevenir la nefrotoxicidad producida por el cisplatino, previamente a la administración del antitumoral se inyectaron 2 ml de suero salino por vía subcutánea.

El peso corporal, la comida y caolín ingeridos (en el caso de medir la pica) y la temperatura rectal se registraron diariamente.

La actividad locomotora espontánea se estudió en los animales el día después de la administración del cisplatino, mediante el test del actímetro.

La alodinia mecánica se midió mediante el test de los filamentos de von Frey (Authier *et al.,* 2003), al menos cuatro días después de cada administración. Se definió la alodinia mecánica como un descenso significativo en el umbral a los estímulos mecánicos en el test de los filamentos de von Frey.

Para comprobar si las dosis de cannabinoide utilizadas tenían efectos psicoactivos se realizó la tétrada cannabinoide, para lo cual se evaluó la temperatura, antinocicepción, catalepsia y locomoción espontánea en los animales tras la administración aguda del fármaco (Compton *et al.,* 1993).

El estudio radiológico se realizó en ratas agrupadas a las que se administró salino o cisplatino (3 ó 6 mg/kg). En estos animales, se administró por vía intragástrica sulfato de bario como medio de contraste inmediatamente, 24 ó 48 horas después de

la administración del fármaco. Se tomaron 7 radiografías de cada animal, a tiempo 0, 30', 1, 2, 4, 6 y 24 horas después de la administración del sulfato de bario. En cada radiografía se evaluaron las alteraciones en la motilidad gastrointestinal de manera semicuantitativa mediante la asignación de un valor numérico a cada región del tracto digestivo (la metodología se explica de forma detallada en el artículo 3). También se registraron las diferencias en la forma y tamaño del estómago. Se trazaron los perfiles de los estómagos sobre transparencias, se fotocopiaron y escanearon y así se estudiaron detalladamente las modificaciones en el tamaño del estómago y en su contenido en bario. Para el estudio de pica aguda que se realizó de forma paralela se utilizaron animales aislados que se acostumbraron a la presencia del caolín durante 3 días y a los que se administró cisplatino a 3 y 6 mg/kg.

En los grupos de animales en los que se iba a evaluar la neuropatía entérica inducida por la administración crónica de cisplatino, una semana después de finalizar el protocolo se dejaron en ayunas durante 18-24 h. Se evaluó el tránsito gastrointestinal (GI) mediante la técnica del carbón vegetal (ver más abajo). El tránsito GI se evaluó como el porcentaje de la distancia recorrida por el carbón vegetal respecto a la longitud total del intestino delgado. En ratas tratadas crónicamente con cisplatino, se analizó también el vaciamiento gástrico por métodos radiográficos a diferentes tiempos durante el tratamiento.

En estos mismos animales, se obtuvieron preparaciones de la siguiente manera. Se extrajo la totalidad del intestino grueso. Los 2 primeros centímetros del colon proximal se destinaron a preparaciones whole-mount para la realización del marcaje inmunohistoquímico; 0,5 cm siguientes se fijaron con formalina para su inclusión en parafina y posterior realización de cortes histológicos en los que se evaluó el daño histológico (Galeazzi *et al.,* 1999) y el número de neuronas por ganglio en el plexo mientérico (Boyer *et al.,* 2005), y 0,5 cm se fijaron con glutaraldehído para realizar un estudio ultraestructural mediante microscopía electrónica. Del mismo modo se procedió con el colon distal (a cuatro centímetros del ciego) y el recto (adyacente al ano), aunque en este caso, se realizó el marcaje inmunohistoquímico en el fragmento más distal.

En las preparaciones whole mount se realizó doble marcaje inmunohistoquímico según la metodología convencional para inmunofluorescencia indirecta (Costa *et al.,* 1986). Para ello, los tejidos se incubaron durante al menos 36 h a temperatura ambiente con una mezcla de anticuerpo anti-HuC/D desarrollado en ratón y conjugado con estreptavidina (1:500, Molecular Probes, Inc., Eugene, OR,

USA), y un anticuerpo primario anti NPY, SP, NOS, CGRP, VIP ó CR. Después de lavar con suero salino tamponado con fosfato (PBS), los tejidos se incubaron 5 h a temperatura ambiente, en agitación y oscuridad con una mezcla de Alexafluor 488 conjugado con biotina (1:500, Molecular probes) y el correspondiente anticuerpo secundario conjugado con RRX (1:100, Jackson Immuno Research Europe Ltd, Cambridgeshire, UK). Estos anticuerpos secundarios llevan incorporados fluoróforos que permiten visualizarlos mediante técnicas de microscopía de fluorescencia. La dilución de los anticuerpos se realizó en PBS hipertónico (1.7% Na Cl) (Costa *et al.,* 1996).

Preparación de caolín, sulfato de bario y fármacos.

El caolín se preparó según el método descrito previamente (Yamamoto *et al.,* 2002a). Se mezcló caolín (silicato de aluminio hidratado, 98,5%), 0.5 % de carmín y 1% de goma arábiga en agua destilada hasta formar una pasta que se moldeaba hasta tener el tamaño y la forma de los pellets de comida normal. El carbón vegetal se preparó con 10% carbón vegetal y 5% de goma arábiga en agua destilada. El caolín, carmín, goma arábiga y el cisplatino fueron suministrados por Sigma-Aldrich (España). El cisplatino se disolvió en salino y se sonicó durante 25 minutos aproximadamente.

El sulfato de bario (Barigraf ® AD, Juste SAQF, España) se suspendió en agua del grifo a una concentración de 2 g/ml y se agitó hasta su administración. A cada animal se le suministraron 2.5 ml de la suspensión.

El WIN 55,212-2 (Tocris, Bristol, U.K.), se preparó siguiendo el método descrito previamente (Pertwee *et al.,* 1992). Se disolvió el fármaco en etanol a una concentración de 1 mg: 1 ml y posteriormente en etanol y Tween 80 (1:2) tras lo cual se evaporó el etanol y se añadió suero salino hasta la concentración final deseada (1 ó 2 mg/ml).

Análisis estadístico.

Los datos representan la media ± EEM. Las diferencias se estudiaron usando el test de la t de Student (aplicando la corrección de Welch en caso necesario), y para comparaciones múltiples se utilizó un análisis de la varianza (ANOVA) de dos vías seguido de un test *post hoc* de Bonferroni. Se tomó como diferencia significativa $p < 0.05$.

4. Publicaciones

4.1. Primera publicación

Vera G, Chiarlone A, Martín MI, Abalo R ***Altered feeding behaviour induced by long-term cisplatin in rats.*** Auton Neurosci. 2006; 126-127:81-92.

4.1.1. Objetivo general

El objetivo general fue el desarrollo de un modelo para el estudio de las alteraciones del comportamiento alimentario que producía la administración crónica de cisplatino en la rata.

4.1.2. Objetivos específicos

- Desarrollar un modelo de pica inducida por cisplatino crónico en ratas, usando una dosis efectiva de cisplatino cuya toxicidad fuera aceptable para los experimentos crónicos.
- Estudiar la influencia del aislamiento crónico y la ingesta de caolín en la temperatura y ganancia de peso.
- Investigar la toxicidad del cisplatino crónico, usando las dosis de 3 mg/kg por semana y 1 mg/kg por semana.

4.1.3. Resultados

El aislamiento y la exposición al caolín no modificaron la temperatura, el peso corporal y la ingesta diaria de comida. La administración de cisplatino a 3 mg/kg fue demasiado tóxica, se produjo hipotermia, pérdida de peso y anorexia tanto en ratas aisladas como agrupadas, y se registró un 50% de mortalidad entre los animales aislados. La toxicidad asociada a la administración de cisplatino a 1 mg/kg/semana fue aceptable, con una pérdida de peso moderada. En estas ratas, la administración del antitumoral produjo anorexia aguda y una respuesta hiperfágica tras cada administración. Además la administración del antitumoral produjo un aumento de la ingesta aguda y basal de caolín, de forma parecida a la náusea que se produce en humanos después de la administración de la quimioterapia. El tratamiento crónico produjo una intensificación de las alteraciones alimentarias.

4.1.4. Conclusiones

Este modelo puede ser útil para estudiar los mecanismos relacionados con la náusea en tratamientos crónicos con fármacos antitumorales y para valorar la eficacia de nuevos fármacos antieméticos de manera similar a la clínica.

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Altered feeding behaviour induced by long-term cisplatin in rats

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Abstract

In animals without the emetic reflex, several emetogenic stimuli induce pica, an altered feeding behaviour consisting of the ingestion of non-nutritive substances. The development of pica in response to an emetogenic stimulus has been proposed to be useful as an indirect marker of nausea in the rat. In fact, like nausea and emesis in humans, it is accompanied by serotonin release from the enterochromaffin cells, increased c-*fos* labelling in the area postrema and the nucleus tractus solitarius, and a delay in gastric emptying. Furthermore, pica, measured as kaolin intake, is reduced by anti-emetic drugs. Pica has been demonstrated after single doses of cisplatin, the most emetogenic chemotherapeutic drug. However, cisplatin, as other antineoplastic drugs, is generally given in cycles, where conventional anti-emetics tend to lose efficiency. The aim of this work was to evaluate the pica induced by long-term treatment with cisplatin. Saline or cisplatin was administered once a week for 5 consecutive weeks, and temperature, body weight, food ingestion and kaolin intake were measured on a daily basis. The influence of isolation (pica is necessarily studied in isolated animals) and exposure to kaolin (basal kaolin intake could modify pica itself and other parameters) on temperature, body weight and daily food ingestion was negligible in saline-treated rats. Cisplatin administered at 3 mg/kg/week was too toxic: it produced hypothermia, weight drop and anorexia in both grouped and isolated rats, and 50% mortality in isolated animals. Toxicity associated with cisplatin administered at 1 mg/kg/week was acceptable, with a slower rate of weight gain being the major effect. In these rats, each cisplatin injection produced both acute anorexia and rebound hyperphagic responses. In addition, each administration induced both acute pica and an increase in basal kaolin intake, resembling the development of nausea in humans. This model could be useful for studying both the mechanisms leading to nausea associated with a long-term antineoplastic treatment and the efficiency of new anti-emetic drugs.

Keywords: Cisplatin; Feeding behaviour; Anorexia; Nausea; Pica; Rat

1. Introduction

Humans and other mammals have physiological mechanisms to avoid and to eliminate toxic substances in foods. These include food avoidance triggered by smell or taste, behaviours induced by nausea, vomiting and diarrhoea. Some species do not have a vomiting (emetic) reflex. These include the commonly used laboratory animals, rats and mice, which are very convenient for initial trials of potential therapeutic compounds. These species have evolved a different mechanism, which is to eat non-digestible material, such as clays. This behaviour has been termed pica (Mitchell et al., 1976; Takeda et al., 1993, 1995a,b). Pica is presumed to provide substances to the digestive tract that dilute or bind toxins. It is supposed that rats experience something akin to nausea when they eat toxins, and that this experience causes the pica. That this is the case is supported by the observation that agents that cause nausea and vomiting in humans cause pica in rats. These include radiation (Yamamoto et al., 2002b), motion (Uno et al., 2000), copper sulphate (Yamamoto et al., 2004), lithium chloride (Yamamoto et al., 2004), apomorphine (Takeda et al., 1993), opioids (Aung et al., 2004) and the antineoplastic drug cisplatin (Takeda et al., 1993). In addition, other events typically related to nausea and vomiting in humans, ferrets or shrews are also induced in the rat by emetogenic stimuli. These include the release of serotonin from enterochromaffin cells (Endo et al., 2002), activation of neurons in the area postrema or the nucleus tractus solitarius (Yamada et al., 2000) and a delay in

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gastric emptying (Ozaki and Sukamoto, 1999). Thus, the study of pica in rats provides a simple approach to studying the mechanisms that may be relevant to the emetic action of different emetogenic stimuli. Furthermore, drugs that inhibit pica in rats may have the potential to be anti-nauseants and anti-emetics in humans. In fact, ondansetron, dexamethasone and NK1 blockers, which are anti-emetics in humans (de Wit et al., 2004), have been shown to reduce pica in rats (Takeda et al., 1993; Rudd et al., 2002; Saeki et al., 2001).

Cisplatin is the single most emetogenic chemotherapeutic agent currently in use (Hesketh et al., 2003), and it is frequently used as the reference drug for the study of emesis and anti-emetics in different animal models (Nakayama et al., 2005). Chemotherapy-induced nausea and vomiting are usually classified into 3 categories (Jordan et al., 2005): acute onset, which occurs within the first 24 h after chemotherapy; delayed onset, which begins 24 h to 5 days after the start of chemotherapy; and anticipatory nausea and vomiting, which can be observed after 1–4 treatment cycles of chemotherapy and involve the elements of classic-conditioning (they are triggered by taste, odour, sight, thoughts, or anxiety secondary to a history of poor response to antiemetic agents). To date, the pica model has only been applied to the study of acute and delayed emetic-like effects associated with a single cisplatin dose (Takeda et al., 1993; Rudd et al., 2002; Saeki et al., 2001). Emesis associated with repeated administrations of cisplatin has not been studied previously in experimental animals. Nevertheless, a considerable number of chemotherapeutic protocols are given over more than 1 day (in cycles), and phenomena such as anticipatory and refractory emesis may develop. In such protocols, conventional anti-emetic therapy often fails to provide adequate prevention and/or control of nauseant and emetic episodes (de Wit et al., 2004).

Therefore, the general objective of the present work was to adapt the pica model to the study of a long-term treatment with cisplatin, in an attempt to better mimic the chemotherapeutic regimes applied to patients. More specifically, we aimed to: (1) study the influence of mild isolation and basal kaolin intake on temperature and weight gain; (2) investigate the long-term toxicity of the lowest dose of cisplatin generally used for the study of acute pica in the rat (3 mg/kg, Rudd et al., 2002), which is also one of the most common doses given chronically for the experimental study of cisplatin-induced peripheral neuropathy (Authier et al., 2003); (3) establish a long-term model of cisplatin-induced pica in rats, using an effective dose of cisplatin whose toxicity was acceptable for chronic experiments.

Part of the present work has been recently published in abstract form (Vera et al., 2005).

2. Materials and methods

The experiments in the present study which were designed to minimize the number of animals used and their suffering, were performed in strict accordance with the EC regulation for care and use of experimental animals (EEC No. 86/609) and approved by the Ethical Committee at the Universidad Rey Juan Carlos.

2.1. Animals

Wistar rats (230–270 g) were obtained from Harlan-Iberica (Barcelona, Spain). Upon arrival to our laboratory, animals were housed, either grouped (4–6/cage) or isolated, in standard transparent cages (40 cm × 28 cm × 25 cm), furnished with wood shaving bedding, which was changed every 1–2 days. Cages were placed adjacent to each other, in environmentally controlled conditions (temperature = 20 °C; humidity = 60%) with a 12-h light/12-h dark cycle (lights on between 08:00 and 20:00 h). Animals were allowed free access to tap water and standard laboratory rat chow (Harlan-Iberica, Barcelona, Spain). In studies in which "pica" was to be analysed, rats also had free access to kaolin pellets (see below). Food and kaolin were placed in adjacent separate compartments in a divided food hopper and were continuously available throughout the experiment. Every day, water bottles were refreshed and the remaining amount kaolin and food was collected, including that spilt outside the containers. Food and kaolin in each cage were topped up to 150 ± 1 and 15 ± 0.1 g of pellets, respectively.

Rats were distributed in eight different experimental groups. Four groups of rats were treated with saline (control): (1) grouped, not exposed to kaolin ($n = 6$); (2) isolated, not exposed to kaolin ($n = 6$); (3) grouped, exposed to kaolin ($n = 6$); (4) isolated, exposed to kaolin ($n = 6$). Two groups of rats not exposed to kaolin were treated with cisplatin 3 mg/kg: (5) grouped ($n = 5$); (6) isolated ($n = 4$). Two groups of isolated rats were treated with cisplatin 1 mg/kg: (7) not exposed to kaolin ($n = 4$); (8) exposed to kaolin ($n = 6$).

2.2. Experimental protocol

During the 2 weeks prior to the experiment, rats were habituated to isolation and/or kaolin, as well as to the testing procedures and to daily handling by the investigator. After this period of adaptation, either cisplatin or vehicle (saline 0.9% w/v, 4–5 ml/kg) was administered intraperitoneally, once per week for 5 weeks. To further study the effects of long-term cisplatin, the anti-neoplastic was also injected (1 mg/kg/week for 5 weeks) in those rats that were isolated, exposed to kaolin and treated with saline once per week for 5 weeks, after completion of the protocol previously described. In order to prevent eventual nephrotoxicity induced by chronically administered cisplatin, 2 ml of saline were injected subcutaneously just before intraperitoneal saline or cisplatin (Authier et al., 2003). Kaolin and chow pellets were provided again immediately after drug administration.

Examination of the rats and measurement of weight, rectal temperature, and food and kaolin intake were recorded on 5 consecutive days per week between 9:00 and 11:00 am. Saline or cisplatin were administered on the first observational day each week, immediately after completion of data recording. Kaolin and food consumption were measured by subtracting the amount remaining from the amount provided the day before for each cage. Care was taken to collect all particles, which were weighed to correct the values of food and kaolin consumption to the nearest 1 and 0.01 g, respectively. In the grouped rats, food consumption represented the combined data from 5 to 6 rats. In these rats, individually recorded weight served as an indirect indicator of individual food consumption (a similar weight gain in all grouped rats was assumed to be indicative of similar food consumption, see O'Connor and Eikelboom, 2000). No such indicator existed for individual kaolin intake in grouped rats; therefore, this parameter was only measured in isolated animals.

2.3. Kaolin

Kaolin pellets were prepared according to the method described by Yamamoto et al. (Yamamoto et al., 2002a,b; Takeda et al., 1993). Briefly, pharmaceutical grade kaolin (hydrated aluminum silicate; 98.5%) was mixed with 0.5% carmine and 1% gum arabic in distilled water to form a thick paste. Pellets of the resulting kaolin mixture were shaped to resemble the dimensions of the rats' normal laboratory diet. The pellets were completely dried at room temperature for up to 48 h. Carmine allowed for visual control of kaolin intake in faeces. The presence of carmine-coloured (pink) faeces in the cage was considered as an indirect marker of significant (physiopathological) kaolin intake. The presence of pink faeces in the cages was semiquantitatively analysed by assigning 0 to those cages in which no pink faeces were apparent and 1 to those in which pink faeces were present. This analysis was carried out in the group of isolated, kaolin-exposed rats that were treated with saline (4–5 ml/kg/week for 5 weeks) first and with cisplatin afterwards (1 mg/kg/week for 5 additional weeks).

2.4. Compounds and drugs

Kaolin, carmine, gum arabic and cisplatin were purchased from Sigma-Aldrich (Spain). Cisplatin was dissolved in saline (sonicated for about 15 min).

2.5. Statistical analysis

Data are presented as the mean values ± S.E.M. Intragroup differences were analysed using a paired Student's *t*-test. Differences between groups were analysed using a two-way ANOVA followed by post hoc Bonferroni's multiple comparison test. p values <0.05 were considered significantly different.

3. Results

3.1. Week-by-week analysis of temperature, weight and food ingestion

3.1.1. Influence of mild chronic isolation and exposure to kaolin

Neither isolation nor exposure to kaolin per se altered, in a significant manner, the rate of weight gain from the first to the fifth week or the average amount of food ingested per day. In saline-treated rats, a slight decrease in the temperature of isolated animals was found at the beginning of the experiment, but this parameter reached similar values to those of grouped animals by the end of the 5 weeks.

3.2. Effects of cisplatin

3.2.1. Cisplatin 3 mg/kg

The effect of repeated administrations of cisplatin 3 mg/kg on temperature, weight and food ingestion in grouped and isolated rats not exposed to kaolin is shown in Fig. 1.

Cisplatin reduced rectal temperature in a significant manner, as early as in the second week in grouped rats and by the fourth week in isolated animals. At the end of the experiment, the difference in temperature between saline-treated and cisplatin-injected rats was approximately two degrees (Fig. 1A, B).

Cisplatin altered the weight gain of grouped and isolated rats in a similar manner (Fig. 1C, D). During the first 3 weeks their weight remained essentially constant but thereafter a significant decrease in weight resulted in even lower weights than those found at the beginning of the experiment.

With regard to food ingestion, saline-treated grouped and isolated rats not exposed to kaolin ingested an average of 24.0 ± 0.5 g and 22.9 ± 1.3 g of food per day, respectively.

Food ingestion was already lower from the first week after cisplatin injection, in both grouped and isolated rats (Fig. 2E, F). From this first week to the third one, grouped and isolated rats ate an average of 16.9 ± 1.3 g and 16.5 ± 1.5 g of food, respectively. From that point onwards, a significant drop in food ingestion was found in both grouped and isolated rats. In isolated rats, daily food ingestion was reduced to less than 5 g/day in the fifth week.

Additional clinical signs of toxicity in these cisplatin-injected rats, especially in those isolated, were evident from the third week: aggressiveness, difficulties in handling, piloerection, vocalisation whilst being handled and diarrhoeas. One isolated rat had alterations in the rhythm of defaecation (severe constipation followed by diarrhoea) from the third cisplatin injection, and died 2 days later. Another isolated rat was sacrificed when its temperature fell below 33 °C (4 days after the fourth injection). Macroscopic analysis of the abdominal cavity of this rat showed signs of peritonitis. Another isolated rat occasionally had black or yellow faeces.

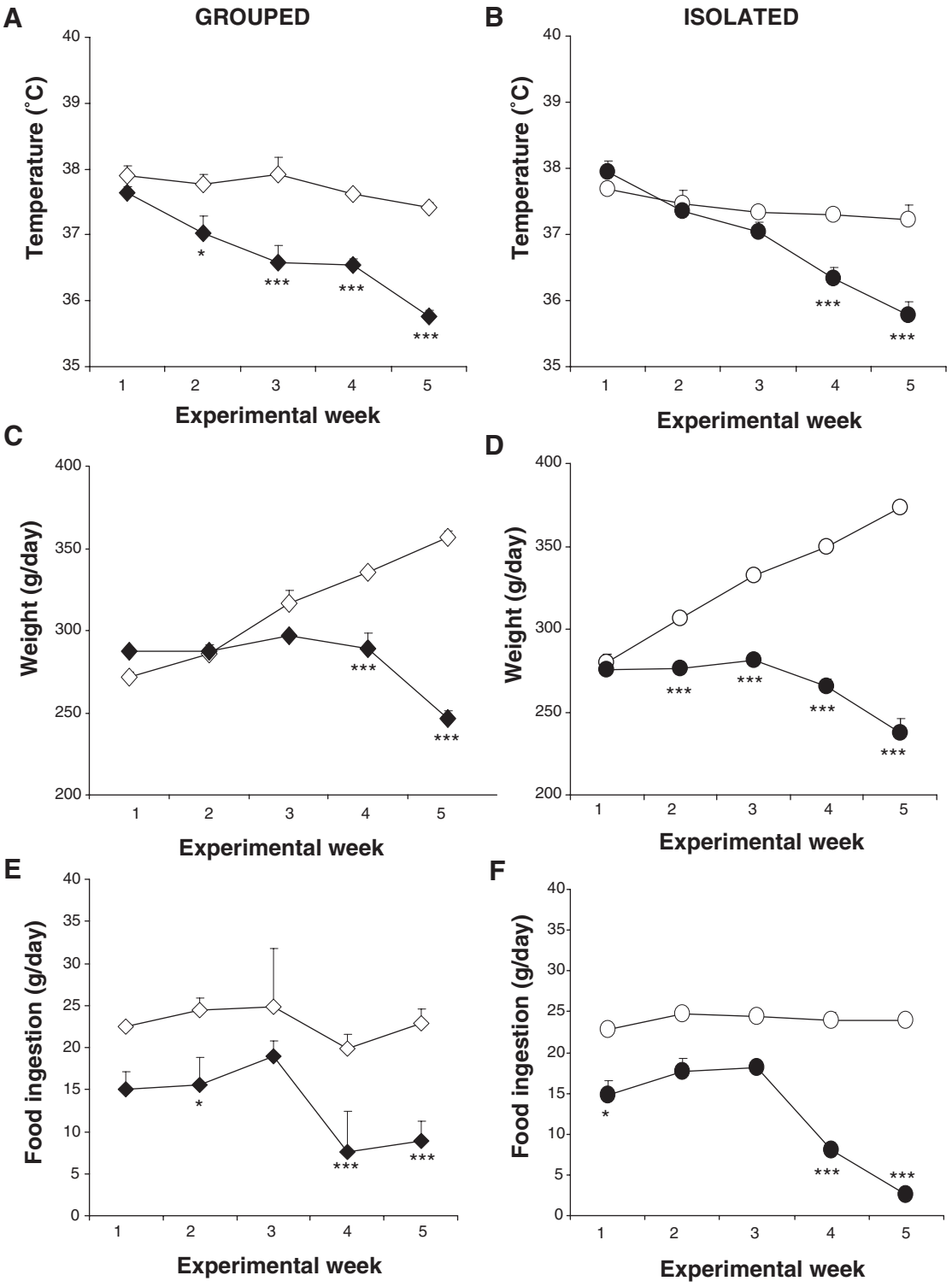

Fig. 1. Effects of cisplatin 3 mg/kg. Rectal temperature (A, B), body weight (C, D) and food ingestion (E, F) were measured in rats not exposed to kaolin injected with cisplatin 3 mg/kg/week (closed symbols) and compared with control rats injected with saline (4–5 ml/kg/week, i.p., open symbols), for 5 weeks. A, C and E show the effect of cisplatin in grouped rats. B, D and F show the effect of cisplatin in isolated rats. Data represent the mean (average of the measurements taken during each week) ± S.E.M. $^{*}p<0.05$, $^{***}p<0.001$ vs. saline-treated (two-way ANOVA followed by Bonferroni's test).

3.2.2. Cisplatin 1 mg/kg

In view of the high toxicity of cisplatin 3 mg/kg, a lower dose of cisplatin, 1 mg/kg, was tested. The effects of cisplatin 1 mg/kg/week (cumulative dose of 5 mg/kg) on temperature, weight and food ingestion in isolated rats, with or without kaolin, are shown in Fig. 2. In these isolated

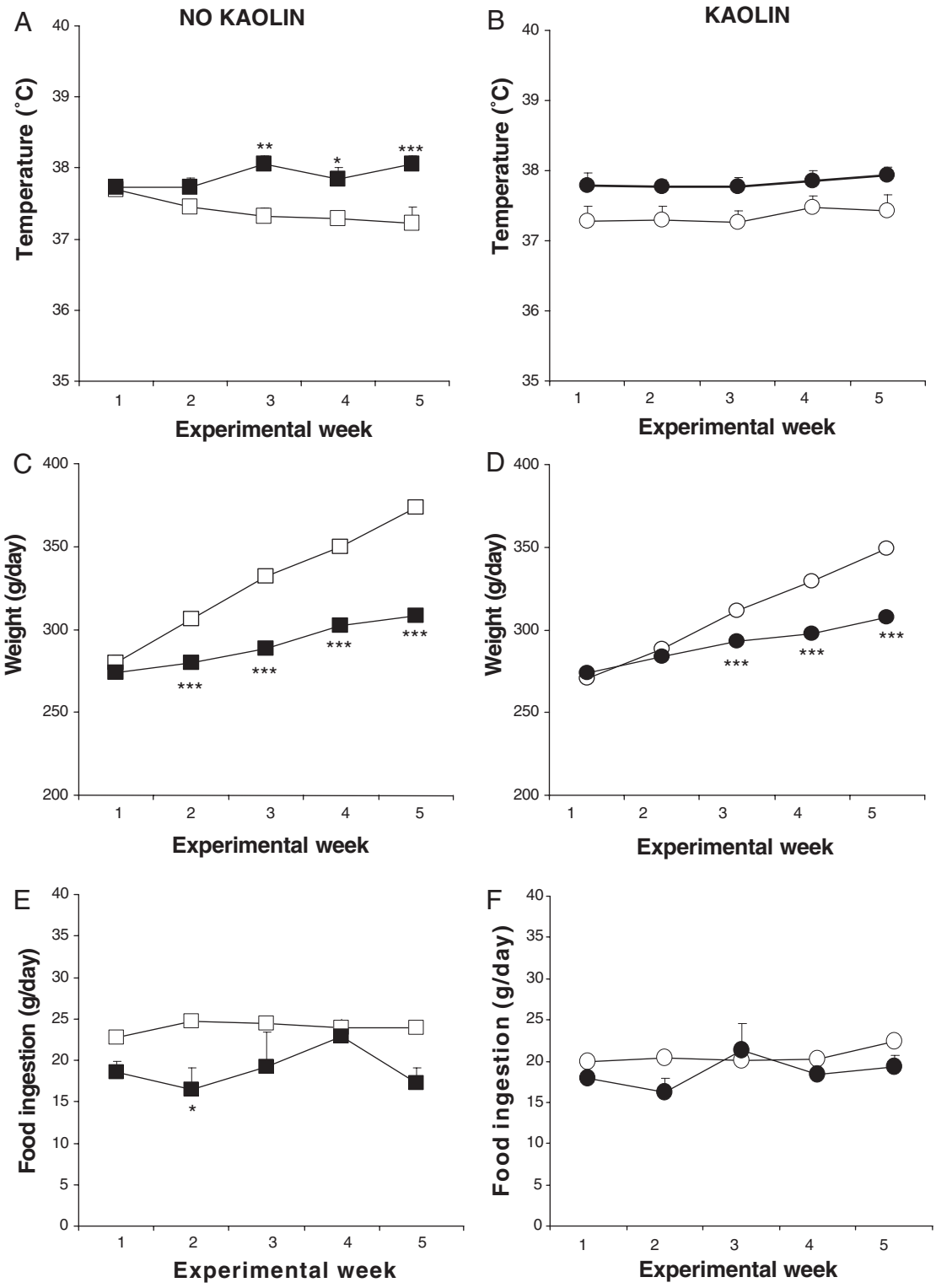

Fig. 2. Effects of cisplatin 1 mg/kg. Rectal temperature (A, B), body weight (C, D) and food ingestion (E, F) were measured in isolated rats injected with cisplatin 1 mg/kg/week (closed symbols) and compared with control rats injected with saline (4–5 ml/kg/week, i.p., open symbols), for 5 weeks. A, C and E show the effect of cisplatin in rats not exposed to kaolin. B, D and F show the effect of cisplatin in rats exposed to kaolin. Data represent the mean (average of the measurements taken during each week) ± S.E.M. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ vs. saline-treated (two-way ANOVA followed by Bonferroni's test).

cisplatin-treated rats, the effect of exposure to kaolin was per se essentially negligible when compared with the effect of cisplatin 1 mg/kg.

Cisplatin 1 mg/kg did not induce a reduction in body temperature. A trend towards higher temperatures was found, but differences were not consistently significant

throughout the experiment in kaolin-exposed animals (Fig. 2A, B).

Weight gain in saline-treated isolated rats, whether exposed or not to kaolin was 23.5±3.6 and 20.6±2.2 g/week, respectively. Weight gain in cisplatin-injected isolated rats was significantly delayed (average rate of weight gain: 8.68±2.22 and 8.38±1.38 g/week for rats not exposed and exposed to kaolin, respectively), although no extreme reduction in weight was found (Fig. 2C, D).

In rats not exposed to kaolin, an overall non-significant reduction in food ingestion was induced by cisplatin, although it did reach statistical significance during the second experimental week (Fig. 2E). In rats exposed to kaolin, the pattern of food ingestion did not differ significantly between cisplatin-injected and saline-treated rats (Fig. 2F).

Occasional diarrhoea and presence of black faeces were observed in 3 of these cisplatin-treated rats. Vocalisation

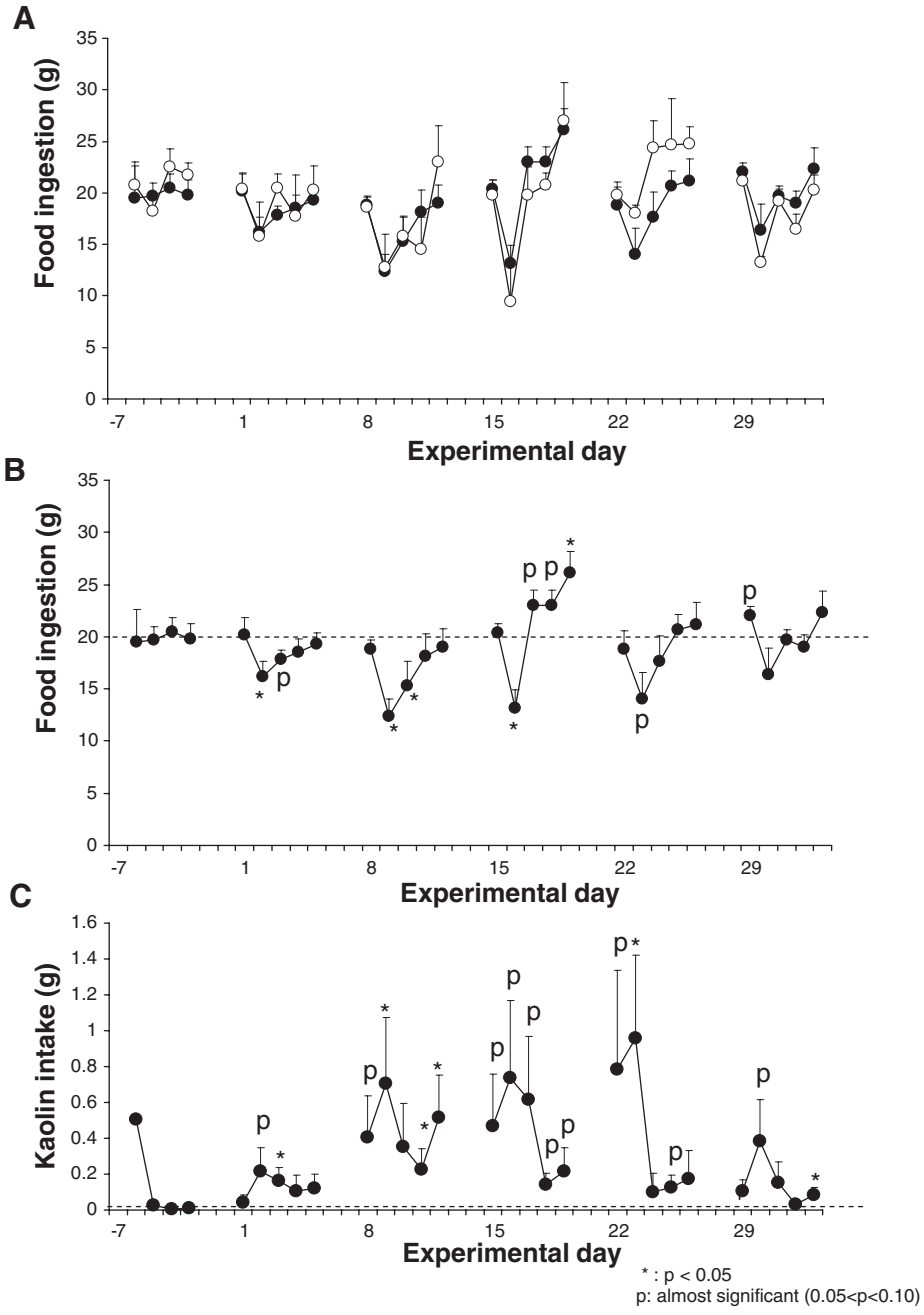

Fig. 3. Day-by-day analysis of food ingestion and kaolin intake in cisplatin-treated rats. Isolated rats were exposed (closed circles) or not (open circles) to kaolin. Cisplatin 1 mg/kg/week was administered on days 1, 8, 15, 22 and 29, immediately after recording of data: (A) daily food ingestion in both groups of rats; (B) daily food ingestion in kaolin-exposed rats; (C) daily kaolin intake (kaolin-exposed rats). Data represent the mean±S.E.M ($n=6$). $^{*}p<0.05$ vs. mean of experimental days −5 to −3. "p" stands for an almost statistically significant difference (0.05–0.1). A: no statistics. B: two-tailed paired Student's t-test. C: one-tailed paired Student's t-test.

with handling and piloerection were also present in practically all of these rats, at least after the third cisplatin injection. No rat treated with cisplatin 1 mg/kg died during the experiment.

3.3. Day-by-day analysis of food ingestion and kaolin intake

Day-by-day modifications in food and kaolin ingestion were studied using the values obtained during the week prior to the first saline or cisplatin injection at 1 mg/kg as a reference.

In saline-treated animals, food ingestion showed certain daily variability, not significantly influenced by the presence of kaolin (in the week prior to the first saline injection, mean values for isolated rats not exposed to kaolin and exposed to kaolin were 22.8 ± 0.5 and 21.1 ± 0.3 g, respectively). In cisplatin-injected animals, each cisplatin injection induced an acute reduction in food ingestion over the

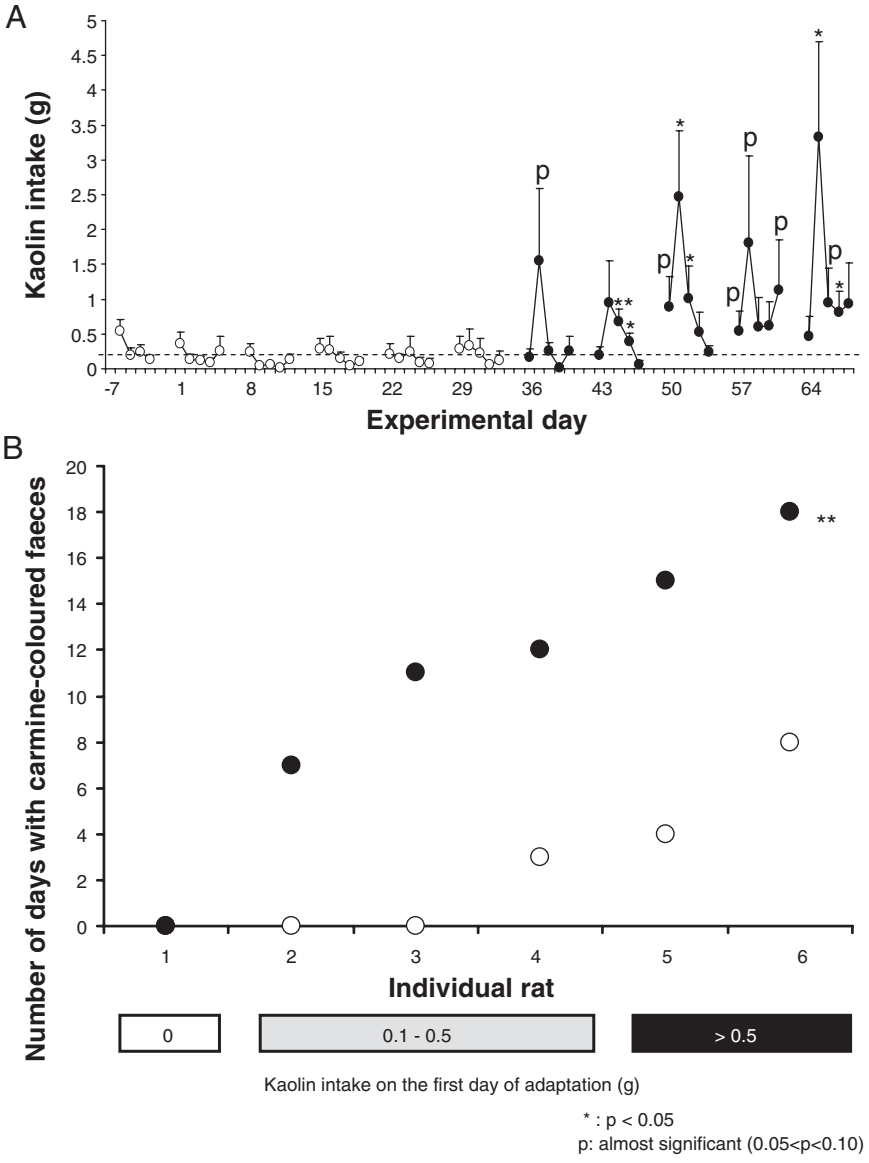

Fig. 4. (A) Day-by-day analysis of kaolin intake in isolated rats exposed to kaolin which were injected with saline (4–5 ml/kg/week, open circles) for 5 weeks and with cisplatin 1 mg/kg (closed circles) for 5 additional weeks. Saline was injected on days 1, 8, 15, 22 and 29, immediately after recording of data. Cisplatin was injected on days 36, 43, 50, 57 and 64, immediately after recording of data. Data represent the mean ± S.E.M. ($n = 6$). $^{*}p < 0.05$, $^{**}p < 0.01$ vs. mean of experimental days −5 to −3. "p" stands for an almost statistically significant difference (0.05–0.1). One-tailed paired Student's t-test. B: Semiquantitative analysis of carmine-coloured faeces (indirect marker of significant kaolin intake) in 6 isolated rats exposed to kaolin. Analysis of faeces was made 4 days each experimental week (40 observational days in total). For each rat, data represent the individual relative frequency of carmine-coloured faeces in the cage when the rats were injected with saline (4–5 ml/kg/week, open circles) for 5 weeks and with cisplatin 1 mg/kg (closed circles) for 5 additional weeks. $^{**}p < 0.01$ vs. saline (one-tailed paired Student's t-test). Individual pica on the first day of adaptation to kaolin is also represented by the white, gray and black bars. Note the correlation between kaolin intake on the first day of adaptation and the presence of faeces in the cage, both during the treatment with saline and during the treatment with cisplatin.

following 24 h, and this day-by-day pattern of food ingestion was the same irrespective of the presence of kaolin (Fig. 3A). In kaolin-exposed rats, this acute reduction in food ingestion was 5.66 ± 0.63 g on average and reached statistical significance from the first to the third injection (Fig. 3B). After each acute response, food ingestion turned to basal values for the rest of the experimental week. Occasionally, significant rebound hyperphagic responses were also detected (Fig. 3B).

During the first day of exposure to kaolin, kaolin intake was 0.503 ± 0.020 g but it decreased immediately after and, by the end of the first week of exposure, it was 0.010 ± 0.003 g. The values obtained in that week (excluding the first day) were used as reference for intragroup comparisons. In this group of animals, each cisplatin injection during the following weeks induced an acute increase in kaolin intake that was almost significant after the first, third and fifth injections and significant after the second and the fourth injections (Fig. 3C). Kaolin intake on the day before the second to fourth cisplatin administrations (considered as basal kaolin intake) tended to be higher than basal values before any injection, although this difference did not reach statistical significance (Fig. 3C).

The effect of long-term cisplatin 1 mg/kg, on kaolin intake was further confirmed when it was injected in the group of isolated kaolin-exposed rats that had been treated with saline (4–5 ml/kg/week) for 5 weeks. As shown in Fig. 4A, spontaneous daily kaolin intake throughout the 6 weeks in which no cisplatin was injected was quite variable, but usually under 0.5 g. No tendency to an increase in the average daily kaolin intake was found during these weeks. However, in the following weeks, kaolin intake increased acutely during the 24 h after each cisplatin injection (Fig. 4A). These acute increases were almost significant after the first and the fourth injections and significant after the third and fifth ones. Once again, basal kaolin intake tended to increase, although this increase did not reach statistical significance (Fig. 4A).

The presence of pink faeces during the 5 weeks in which these rats were treated with saline and the 5 weeks in which they were treated with cisplatin was highly variable (Fig. 4B). However, the difference in the number of days with pink faeces during saline and cisplatin treatment was quite

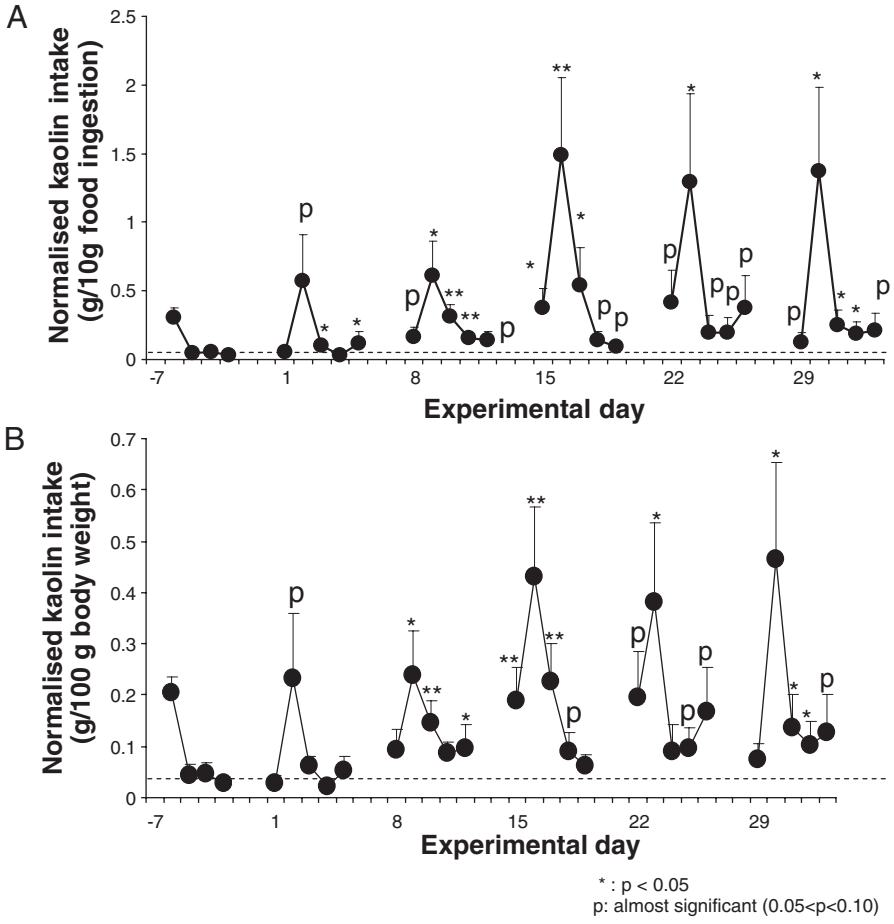

Fig. 5. Combined day-by-day analysis of normalised kaolin intake in isolated rats exposed to kaolin and injected with cisplatin 1 mg/kg for 5 weeks after at least 1 week of adaptation to kaolin. Cisplatin was injected on days 1, 8, 15, 22 and 29, immediately after recording of data: (A) kaolin intake normalised to food ingestion; (B) kaolin intake normalised to body weight. Data represent the mean ± S.E.M. ($n = 12$). $^{*}p < 0.05$, $^{**}p < 0.01$ vs. mean of experimental days −5 to −3. "p" stands for an almost statistically significant difference (0.05–0.1). One-tailed paired Student's t-test.

similar from rat to rat (during cisplatin treatment, pink faeces were found 7 to 11 days more than during saline treatment), as long as it had eaten at least 0.1 g of kaolin during the first day of adaptation. One rat that did not eat any kaolin during this day never showed pica in response to cisplatin and never had pink faeces in its cage (Fig. 4B).

Finally, in an attempt to reduce the effect of individual variability by increasing the number of samples, daily kaolin intake values from both groups of cisplatin-injected rats were pooled together after normalisation for food ingestion (Fig. 5A) or body weight (Fig. 5B). These mathematical operations further confirmed and extended our results. Thus, each cisplatin injection induced an acute increase in pica, which was statistically significant from the second cisplatin injection on. In addition, kaolin intake on the day before each cisplatin injection tended to be higher than basal kaolin intake at the end of the week of adaptation and was significantly different before the third cisplatin injection. Furthermore, delayed pica, occurring from 24 to 72 h after cisplatin treatment, was significant at least after the second, third and fifth cisplatin administrations (Fig. 5).

4. Discussion

The present work shows that chronic cisplatin alters feeding behaviour in the rat, inducing both anorexia and an increase in kaolin intake (indirect marker of nausea).

In rodents, which do not vomit, cisplatin and other emetogenic stimuli induce a characteristic altered feeding behaviour consisting of the ingestion of non-nutritive substances such as kaolin. This behaviour is termed pica and has been demonstrated to be sensitive to anti-emetic drugs (Mitchell et al., 1976; Takeda et al., 1993; Rudd et al., 2002; Saeki et al., 2001). The pica model has only been applied to the study of the effects induced by a single dose of cisplatin. Nevertheless, chemotherapy is usually given in cycles, where control of nausea and vomiting becomes far more complicated (de Wit et al., 2004). In the present work, the long-term changes in feeding behaviour, including the induction of pica, associated with repeated administrations of cisplatin, have been studied in the rat for the first time.

4.1. Experimental conditions

Although some reports do exist on acute pica induced by emetogenic stimuli in mice (Santucci et al., 2000; Yamamoto et al., 2002a, 2005), the rat is generally preferred to study this response, probably because in this species pica is a more robust phenomenon (Liu et al., 2005). Therefore, rats were chosen to develop the present work.

Individual consumption of kaolin in response to emetogenic stimuli, including cisplatin, could obviously be very heterogeneous. The expected variability in the response, together with the fact that there is no alternative marker of such consumption that could reliably help to individualise kaolin intake, has forced investigators to study pica in isolated rats (Takeda et al., 1993; Rudd et al., 2002). Since isolation could produce stressing effects in rodents (Sharp et al., 2002), the first concern of the present work was to analyse the influence of this factor on the different parameters that were to be measured throughout the whole duration of the experiment (5–6 weeks).

Under our conditions of mild chronic isolation, in which a relatively high degree of olfactory, auditory and visual contact with conspecifics is preserved (Brain and Benton, 1979), only minor occasional reductions in body temperature were recorded, in agreement with findings in isolated mice (Spani et al., 2003). Body weight and food intake were not significantly altered by mild chronic isolation. It has been suggested that individual housing does not modify weight gain or food ingestion, as long as it starts well after weaning (O'Connor and Eikelboom, 2000; Lopak and Eikelboom, 2000), and that isolation of male rodents could not be stressful per se but induce an increased reaction to subsequent stressful events, such as changes in housing conditions or even handling (Bartolomucci et al., 2003). Daily experimental procedures and handling in the present work seems not to have induced a significantly meaningful degree of stress in isolated animals. Quite the opposite, daily handling may even have prevented the development of at least some isolation-induced stressing effects (Gentsch et al., 1988).

The other experimental factor that had to be taken into account in our study was the effect of prolonged exposure to kaolin. When pica is to be used as an index of unease after a single administration of cisplatin, rats need to get accustomed to the presence of kaolin in their cages. Thus, after 1 to 3 days of exposure to kaolin, basal kaolin intake fell below 0.5 g/day (Figs. 3 and 4; Rudd et al., 2002). Kaolin is not absorbed in the gastrointestinal tract, but it has antidiarrhoeal properties (Beck et al., 1977). In any case, temperature, body weight and food ingestion were not significantly altered by the basal amounts of kaolin consumed by saline-treated rats during this long-term study.

4.2. Long-term effects of cisplatin

In agreement with previous reports (Authier et al., 2003), cisplatin injected at a dose of 3 mg/kg once a week for 5 weeks in rats not exposed to kaolin-produced hypothermia, a reduction in weight gain, a reduction in food ingestion, and several other clinical signs of toxicity, including allodynia (data not shown). These effects were similar in surviving rats, irrespective of whether they were grouped or isolated. Nevertheless, 2 out of 4 isolated rats could not reach the end of the experiment, while all grouped rats survived up to the end of the study. Mortality associated with long-term cisplatin treatments used for the induction of peripheral neuropathy, in which grouped rats

were used, has been reported in some studies (Authier et al., 2003) but not in others (Authier et al., 2000). Isolation could increase reactivity to a further stressor (Bartolomucci et al., 2003), which, in this study, would have been cisplatin. However, other factors, such as the pattern of administration, experimental conditions or rat strain, sex or weight at the beginning of the experiment can also influence the rate of mortality. In any case, mortality and general toxicity associated with repeated administrations of cisplatin 3 mg/kg were considered unacceptable for our study.

Thus, a lower dose of cisplatin was chosen upon analysis of the results obtained from the long-term treatment with cisplatin 3 mg/kg/week. First, no rat died before the third injection (cumulative dose of 9 mg/kg). Second, it was after the fourth injection when temperature, body weight and food ingestion experienced an extreme decrease. After the second injection (cumulative dose of 6 mg/kg, well under the different LD_{50} reported for single doses of intraperitoneal cisplatin in rats: Khandekar, 1983; Tokuda et al., 1998), both grouped and isolated rats still maintained similar temperature and weight as non-injected animals. Food ingestion, although already lower, had not started to decrease drastically. Therefore, cisplatin 1 mg/kg (once per week, up to a cumulative dose of 5 mg/kg) was chosen as a probable adequate dose.

The effects of cisplatin 1 mg/kg in isolated rats were similar irrespective of the presence of kaolin in the cage. The only significant and consistent alteration induced by cisplatin, a delay in weight gain from the second injection on, could be due to: (1) a reduction in food ingestion (anorexia); (2) a reduced capacity of the body to efficiently use the food ingested.

Anorexia is one important adverse effect associated with chemotherapy, which can affect practically all patients and impair their health, contributing to cancer-induced cachexia and death. In experimental animals, reductions in food ingestion have also been reported after treatment with antineoplastic drugs, including cisplatin (Fig. 2; Rudd et al., 2002). However, the acute reductions in food ingestion induced by each injection of cisplatin 1 mg/kg in the present work were occasionally followed by rebound hyperphagic responses, which probably helped to maintain practically constant the weekly average of daily food ingestion and allowed a partial recovery of the health status of the animals.

As mentioned above, the efficiency of the body to adequately use the food ingested could have also decreased when cisplatin 1 mg/kg was used. Different factors could contribute to this effect: impairment of absorption of nutrients, either direct (Bearcroft et al., 1999) or indirect, by the induction of alterations in gastric emptying (Ozaki and Sukamoto, 1999) or diarrhoeas (this work; Kris et al., 1988); glucose intolerance (Goldstein et al., 1982); toxicity in tissues involved in metabolic use of the nutrients absorbed, such as the liver (Naziroglu et al., 2004).

4.3. Pica induced by long-term cisplatin

If cumulative doses work as single doses do in pica studies, one would expect significant pica behaviour at least from the third administration of cisplatin 1 mg/kg/week (Rudd et al., 2002; Yamamoto et al., 2004). In addition, some signs of delayed emesis could also be expected at least around that point (Rudd et al., 2002).

The induction of pica by cisplatin was similar in both groups of rats studied (Figs. 3–5), which further confirms the robustness of this behaviour in response to emetogenic stimuli. An inspection of faeces confirmed that cisplatin induced physiologically significant kaolin intake, and also that this response to cisplatin was highly variable depending on the particular rat (Fig. 4B). In spite of this apparent individual variability, every single rat increased both pica and frequency of pink faeces in response to cisplatin to quite a similar degree. Surprisingly, pica and frequency of pink faeces correlated well with the amount of kaolin ingested during the first day of adaptation.

Several methods can help reduce variability in kaolin intake induced by an emetogenic stimulus. First, one could develop preliminary experiments in order to select only adequately responsive rats (Yamamoto et al., 2002a,b). Most reports on pica induced by cisplatin and other emetogenic stimuli either do not take variability into consideration or exclude those rats that do eat more than a certain amount of kaolin before the stimulus (Fujisaki et al., 2001), but no previous reports on cisplatin-induced pica have mentioned the possibility that some rats might not eat kaolin at all from the beginning. We propose that those rats should be removed from all pica studies. In any case, a systematic investigation of susceptibility of rats to cisplatin focussing on pica would be very helpful. Second, one can always try to reduce variability by increasing the number of samples. In this study, in order to achieve that, data from the two groups of cisplatin-treated rats were normalised to body weight (Fig. 5B; this operation discards the effect of different body size) or to food ingestion (Fig. 5A; to reduce the effect of anorexia on kaolin intake). These mathematical operations led to similar results in both cases, implying that the factor that contributes more to reducing variability is increasing the number of samples.

4.4. Physiopharmacological implications

The value of pica as an indirect marker of nausea and emesis is supported by the following facts:

(1) Stimuli which are emetogenic in humans, also induce pica in the rat: radiation (Yamamoto et al., 2002a,b), motion (Uno et al., 2000), copper sulphate (Yamamoto et al., 2004), morphine (Aung et al., 2004), cyclosporine A (Fujisaki et al., 2001), apomorpine (Takeda et al., 1993). Our results suggest that pica

could also be valuable as an indirect marker of nausea and emesis induced by chemotherapy given in cycles. As in previous studies (Rudd et al., 2002; Saeki et al., 2001), acute pica occurring during the 24 h after each cisplatin injection could be analogous to chemotherapy-induced nausea and vomiting of acute onset. The fact that basal kaolin intake tended to be higher during chronic treatment with cisplatin could be at least partially due to the development of delayed pica in the rat. Further research is needed to elucidate the mechanisms involved in this facilitation of pica.

(2) Drugs which block or prevent nausea and emesis induced by different emetogenic stimuli in humans (anti-emetics) are also able to block or prevent pica in the rat: ondansetron and other 5-HT_3 antagonists (Rudd et al., 2002), dexamethasone (Rudd et al., 2002), NK1-antagonists (Saeki et al., 2001), and anti-histamines (Takeda et al., 1995a,b). Whether or not acute and facilitated pica induced by chronic cisplatin are sensitive to these anti-emetics or others remains to be investigated.

(3) Events involved in the development of emesis in humans are also triggered in the rat after exposure to emetogenic stimuli: serotonin release (Endo et al., 2002), activation of the vagus nerve (Yamada et al., 2000), activation of neurones in the area postrema and nucleus tractus solitarius (Yamada et al., 2000), reduction in gastric emptying (which, due to the lack of a proper emetic reflex, has often been used as an indirect index of cisplatin-induced malaise in the rat: Ozaki and Sukamoto, 1999). In a preliminary study, we have shown that gastrointestinal transit decreased after 5 weeks of treatment with cisplatin 3 mg/kg/week, but no significant effect was found on this parameter when cisplatin was used at 1 mg/kg (Abalo et al., 2005). Further research is needed to more precisely define the events accompanying the development of acute pica and increase in basal kaolin intake induced by chronic cisplatin.

4.5. Concluding remarks

A long-term (cyclic) treatment with low doses of the antineoplastic drug, cisplatin induced significant alterations in feeding behaviour in the isolated rat. In addition to acute anorexia, each cisplatin challenge induced acute increases in kaolin intake (pica), which parallels acute nausea and emesis in humans. Furthermore, basal kaolin intake seemed to be facilitated in the long run, which could at least be partially due to the development of delayed pica. The experimental approach proposed in the present work could be useful in both the study of the mechanisms underlying the induction of nausea and the development of new anti-emetic therapies.

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References

Abalo, R., Vera, G., Chiarlone, A.P., Rivera, A.J., Martin, M.I., 2005. Alterations of gastrointestinal transit induced by long-term cisplatin in the rat. Auton. Neurosci. Basic Clin. 119, 114.

Aung, H.H., Mehendale, S.R., Xie, J.T., Moss, J., Yuan, C.S., 2004. Methylnaltrexone prevents morphine-induced kaolin intake in the rat. Life Sci. 74, 2685–2691.

Authier, N., Fialip, J., Eschalier, A., Coudore, F., 2000. Assessment of allodynia and hyperalgesia after cisplatin administration to rats. Neurosci. Lett. 291, 73–76.

Authier, N., Gillet, J.P., Fialip, J., Eschalier, A., Coudore, F., 2003. An animal model of nociceptive peripheral neuropathy following repeated cisplatin injections. Exp. Neurol. 182, 12–20.

Bartolomucci, A., Palanza, P., Sacerdote, P., Ceresini, G., Chirieleison, A., Panerai, A.E., Parmigiani, S., 2003. Individual housing induces altered immuno-endocrine responses to psychological stress in male mice. Psychoneuroendocrinology 28, 540–558.

Bearcroft, C.P., Domizio, P., Mourad, F.H., Andre, E.A., Farthing, M.J., 1999. Cisplatin impairs fluid and electrolyte absorption in rat small intestine: a role for 5-hydroxytryptamine. Gut 44, 174–179.

Beck, I.T., Jenkins, N., Thurber, L., Ambrus, J.L., 1977. Methods for the study of antidiarrhoeal agents: study of commonly used protective and adsorbent agents. J. Med. 8, 135–158.

Brain, P., Benton, D., 1979. The interpretation of physiological correlates of differential housing in laboratory rats. Life Sci. 24, 99–115.

de Wit, R., Herrstedt, J., Rapoport, B., Carides, A.D., Guoguang-Ma, J., Elmer, M., Schmidt, C., Evans, J.K., Horgan, K.J., 2004. The oral NK(1) antagonist, aprepitant, given with standard antiemetics provides protection against nausea and vomiting over multiple cycles of cisplatin-based chemotherapy: a combined analysis of two randomised, placebo-controlled phase III clinical trials. Eur. J. Cancer 40, 403–410.

Endo, T., Hamaue, N., Ihira, E., Teramoto, Y., Liu, Y., Hirafuji, M., Minami, M., 2002. Effects of granisetron, a 5-HT_3 receptor antagonist, on 5-hydroxytryptamine (5-HT) release from the isolated ileum in a delayed-emesis rat model. Res. Commun. Mol. Pathol. Pharmacol. 111, 55–68.

Fujisaki, Y., Yamauchi, A., Shuto, H., Niizeki, M., Makino, K., Kataoka, Y., Oishi, R., 2001. Pharmacological characterization of cyclosporine A-induced kaolin intake in rats. Pharmacol. Biochem. Behav. 70, 267–271.

Gentsch, C., Lichtsteiner, M., Frischknecht, H.R., Feer, H., Siegfried, B., 1988. Isolation-induced locomotor hyperactivity and hypoalgesia in rats are prevented by handling and reversed by resocialization. Physiol. Behav. 43, 13–16.

Goldstein, R.S., Mayor, G.H., Rosenbaum, R.W., Hook, J.B., Santiago, J.V., Bond, J.T., 1982. Glucose intolerance following *cis*-platinum treatment in rats. Toxicology 24, 273–280.

Hesketh, P.J., Van Belle, S., Aapro, M., Tattersall, F.D., Naylor, R.J., Hargreaves, R., 2003. Differential involvement of neurotransmitters through the time course of cisplatin-induced emesis as revealed by therapy with specific receptor antagonists. Eur. J. Cancer 8, 1074–1080.

Jordan, K., Kasper, C., Schmoll, H., 2005. Chemotherapy-induced nausea and vomiting: current and new standards in the antiemetic prophylaxis and treatment. Eur J. Cancer 41, 199–205.

Khandekar, J.D., 1983. Improved therapeutic index of *cis*-diammine dichloro platinum by diethyldithiocarbamate in rodents. Res. Commun. Chem. Pathol. Pharmacol. 40, 55–66.

Kris, M., Gralla, R., Clark, R., 1988. Control of chemotherapy induced diarrhoea with a synthetic enkephalin BW942C: a randomised trial with placebo in patients receiving cisplatin. J. Clin. Oncol. 6, 663–668.

Liu, Y.L., Malik, N., Sanger, G.J., Friedman, M.I., Andrews, P.L., 2005. Pica – a model of nausea? Species differences in response to cisplatin. Physiol. Behav. 85, 271–277.

Lopak, V., Eikelboom, R., 2000. Pair housing induced feeding suppression: individual housing not novelty. Physiol. Behav. 71, 329–333.

Mitchell, D., Wells, C., Hoch, N., Lind, K., Woods, S.C., Mitchell, L.K., 1976. Poison induced pica in rats. Physiol. Behav. 17, 691–697.

Nakayama, H., Yamakuni, H., Higaki, M., Ishikawa, H., Imazumi, K., Matsuo, M., Mutoh, S., 2005. Antiemetic activity of FK1052, a 5-HT_3- and 5-HT_4-receptor antagonist, in *Suncus murinus* and ferrets. J. Pharmacol. Sci. 98, 396–403 (Epub 2005 Jul 29).

Naziroglu, M., Karaoglu, A., Aksoy, A.O., 2004. Selenium and high dose vitamin E administration protects cisplatin-induced oxidative damage to renal, liver and lens tissues in rats. Toxicology 195, 221–230.

O'Connor, R., Eikelboom, R., 2000. The effects of changes in housing on feeding and wheel running. Physiol. Behav. 68, 361–371.

Ozaki, A., Sukamoto, T., 1999. Improvement of cisplatin-induced emesis and delayed gastric emptying by KB-R6933, a novel 5-HT_3 receptor antagonist. Gen. Pharmacol. 33, 283–288.

Rudd, J.A., Yamamoto, K., Yamatodani, A., Takeda, N., 2002. Differential action of ondansetron and dexamethasone to modify cisplatin-induced acute and delayed kaolin consumption ("pica") in rats. Eur. J. Pharmacol. 454, 47–52.

Saeki, M., Sakai, M., Saito, R., Kubota, H., Ariumi, H., Takano, Y., 2001. Effects of HSP-117, a novel tachykinin NK1-receptor antagonist, on cisplatin-induced pica as a new evaluation of delayed emesis in rats. Jpn. J. Pharmacol. 86, 359–362.

Santucci, D., Corazzi, G., Francia, N., Antonelli, A., Aloe, L., Alleva, E., 2000. Neurobehavioral effects of hypergravity conditions in adult mouse. NeuroReport 11, 3353–3356.

Sharp, J.L., Zammit, T.G., Azar, T.A., Lawson, D.M., 2002. Stress-like responses to common procedures in male rats housed alone or with other rats. Contem. Top. Lab. Anim. Sci. 41, 8–14.

Spani, D., Arras, M., Konig, B., Rulicke, T., 2003. Higher heart rate of laboratory mice housed individually vs. in pairs. Lab. Anim. 37, 54–62.

Takeda, N., Hasegawa, S., Morita, M., Matsunaga, T., 1993. Pica in rats is analogous to emesis: an animal model in emesis research. Pharmacol. Biochem. Behav. 45, 817–821.

Takeda, N., Hasegawa, S., Morita, M., Horii, A., Uno, A., Yamatodani, A., Matsunaga, T., 1995a. Neuropharmacological mechanisms of emesis: I. Effects of antiemetic drugs on motion- and apomorphine-induced pica in rats. Methods Find. Exp. Clin. Pharmacol. 17, 589–590.

Takeda, N., Hasegawa, S., Morita, M., Horii, A., Uno, A., Yamatodani, A., Matsunaga, T., 1995b. Neuropharmacological mechanisms of emesis: II. Effects of antiemetic drugs on cisplatin-induced pica in rats. Methods Find. Exp. Clin. Pharmacol. 17, 647–652.

Tokuda, K., Natsugoe, S., Shimada, M., Kumanohoso, T., Baba, M., Takao, S., Nakamura, K., Yamada, K., Yoshizawa, H., Hatate, Y., Aikou, T., 1998. Design and testing of a new cisplatin form using a base material by combining poly-D,L-lactic acid and polyethylene glycol acid against peritoneal metastasis. Int. J. Cancer 76, 709–712.

Uno, A., Takeda, N., Horii, A., Sakata, Y., Yamatodani, A., Kubo, T., 2000. Effects of amygdala or hippocampus lesion on hypergravity-induced motion sickness in rats. Acta Oto-laryngol. 120, 860–865.

Vera, G., Chiarlone, A.P., Rivera, A.J., Martín, M.I., Abalo, R., 2005. Development of a chronic model to study antiemetic drugs: long-term cisplatin-induced "emesis" in the rat. Auton. Neurosci. Basic Clin. 119, 125.

Yamada, Y., Tsukamoto, G., Kobashi, M., Sasaki, A., Matsumura, T., 2000. Abdominal vagi mediate c-Fos expression induced by X-ray irradiation in the nucleus tractus solitarii of the rat. Auton. Neurosci. 83, 29–36.

Yamamoto, K., Matsunaga, S., Matsui, M., Takeda, N., Yamatodani, A., 2002a. Pica in mice as a new model for the study of emesis. Methods Find. Exp. Clin. Pharmacol. 24, 135–138.

Yamamoto, K., Takeda, N., Yamatodani, A., 2002b. Establishment of an animal model for radiation-induced vomiting in rats using pica. J. Radiat. Res. (Tokyo) 43, 135–141.

Yamamoto, K., Ngan, M.P., Takeda, N., Yamatodani, A., Rudd, J.A., 2004. Differential activity of drugs to induce emesis and pica behavior in *Suncus murinus* (house musk shrew) and rats. Physiol. Behav. 83, 151–156.

Yamamoto, K., Nohara, K., Furuya, T., Yamatodani, A., 2005. Ondansetron, dexamethasone and an NK(1) antagonist block radiation sickness in mice. Pharmacol. Biochem. Behav. 82, 24–29.

4.2. Segunda publicación:

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4.2.1. Objetivo principal

Determinar el efecto del cannabinoide no selectivo WIN 55,212-2 (WIN) en la alodinia mecánica y las alteraciones del comportamiento alimentario producidas por la administración crónica de cisplatino en rata.

4.2.2. Objetivos específicos

Estudiar si:

- El agonista cannabinoide WIN 55,212-2 administrado de forma semanal era capaz o no de prevenir el desarrollo de anorexia, pica y alodinia mecánica producida por la administración crónica de cisplatino en la rata.
- La dosis de cannabinoide utilizada tenía efectos psicoactivos.

4.2.3. Resultados

El WIN no mejoró la ingesta de comida, ni la reducción del peso corporal, ni disminuyó la ingesta de caolín que producía la administración crónica de cisplatino; incluso, empeoró la pérdida de peso. Sin embargo, fue capaz de prevenir el desarrollo de alodinia mecánica.

Este efecto del cannabinoide en la neuropatía se producía a dosis a las que no tenía efectos psicoactivos.

4.2.4. Conclusiones

Aunque la administración del agonista cannabinoide no tiene efecto orexigénico, ni tampoco disminuye la pica ni contrarresta la pérdida de peso corporal de los animales, sí previno el desarrollo de alodinia mecánica que produce el cisplatino. Este efecto en la alodinia mecánica podría deberse a un efecto neuroprotector, como se ha sugerido en otras patologías. Estos resultados son de

interés para la posible utilización de los cannabinoides en los pacientes que sufren dolor neuropático.

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WIN 55,212-2 prevents mechanical allodynia but not alterations in feeding behaviour induced by chronic cisplatin in the rat

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Abstract

Anorexia, nausea/emesis and peripheral sensorial neuropathy are frequent adverse effects associated with chemotherapy. Cannabinoids have been proposed to alleviate these effects, but their preventive properties in long-term experimental models have not been tested. This study was conducted to determine whether or not a cannabinoid agonist (WIN-55,212-2) can prevent anorexia, pica (an indirect marker of nausea in non-vomiting species, consisting of the ingestion of non-nutritive substances such as kaolin) and mechanical allodynia (a marker of peripheral neuropathy) induced by the antineoplastic drug cisplatin chronically administered. Isolated rats with free access to food and kaolin received either saline, cannabinoid vehicle, WIN-55,212-2 (1–2 mg kg^{-1}), cisplatin (1–2 mg kg^{-1}), or both drugs once per week for five consecutive weeks. Modifications in temperature, body weight gain, food and kaolin intake, and the threshold for mechanical allodynia were recorded. Additionally, the acute psychoactive effects of the cannabinoid (hypomotility, hypothermia, analgesia and catalepsia) were assayed by means of the cannabinoid tetrad. WIN 55,212-2 prevented the development of mechanical allodynia but not anorexia, pica and reduction in weight gain induced by chronic cisplatin. The effect of WIN 55,212-2 was evident even at a dose lacking activity in the cannabinoid tetrad. The preventive effect on cisplatin-induced mechanical allodynia exerted by the cannabinoid could be due to a neuroprotective role, as has been suggested for other conditions. The present results support the interest in the evaluation of cannabinoids for treatment of patients suffering or likely to suffer neuropathic pain.

Keywords: Cannabinoid; Cisplatin; Anorexia; Nausea; Emesis; Pica; Neuropathic pain; Rat

Introduction

Clinical use of antineoplastic drugs is associated with the development of numerous adverse effects that many patients find intolerable, including nausea and/or emesis, anorexia, and peripheral neuropathy (Schwartzberg, 2007; Navari, 2007; Stillman and Cata, 2006; Markman, 2006; Van Cutsem and Arends, 2005). Although cannabinoids have traditionally been used for the treatment and/or prevention of such symptoms, it was not until recently that scientific evidence of their usefulness was obtained (Davis et al., 2007).

Nausea and vomiting remain amongst the most unpleasant side effects of chemotherapy for cancer patients. These effects can result in serious metabolic derangements, nutritional depletion and anorexia, esophageal tears and deterioration of patient's physical and mental status, which prompts the patient to discontinue the potentially useful and curative antineoplastic treatment (Craig and Powell, 1987; Passik et al., 2001). In spite of the progress since the introduction of 5-HT_3 antagonists, a significant number of patients still develop chemotherapy-induced nausea and vomiting, particularly during the 2–5 day period of delayed emesis following chemotherapy (De Wit et al., 2004; Jordan et al., 2005). Furthermore, as chemotherapy consists of multiple (generally 4–6) cycles, the efficacy of antiemetic protection must be robust not only throughout the initial cycle, but over subsequent cycles. Unfortunately, resistance to ondansetron and other antiemetic drugs often develops with use (De Wit et al., 2004) and numerous efforts are still being made to find drugs capable of maintaining their efficacy throughout the whole course of therapy.

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Cisplatin, as well as its analogues (carboplatin, oxaliplatin) are used clinically for a variety of malignancies (ovarian, testicular, lung, colon and others). It is amongst the most emetogenic antitumoral drugs (Jordan et al., 2005) and serves as reference for the study of antiemetics in experimental animals (Andrews and Horn, 2006). In the rat, which does not vomit in response to emetogenic stimuli, cisplatin induces an alteration of feeding behaviour called pica, consisting of the ingestion of non-nutritive substances, i.e., kaolin (Mitchell et al., 1976; Takeda et al., 1993, 1995a,b; Vera et al., 2006). Recently, we have used the pica model to study the effect of low doses of cisplatin chronically administered, in an attempt to better mimic the clinical situation (Vera et al., 2006). In this model, the antineoplastic drug induced an increase in kaolin intake both in the 24 h after each administration (acute pica) and throughout the five weeks of chronic treatment (facilitatory effect). We suggested that this pattern resembles that found in humans, with both acute and delayed/facilitated responses to the antineoplastic drug, which could be useful for a more realistic study of the effect of different antiemetics.

The antiemetic potential of marijuana (*Cannabis sativa*) against chemotherapy-induced vomiting in cancer patients has been known for several decades (Tramer et al., 2001). Thus, its major constituent, Δ^9-tetrahydrocannabinol (THC), and also several synthetic cannabinoid CB_1/CB_2 receptor agonists (CP 55,940 and WIN 55,212-2) have exhibited broad-spectrum antiemetic action, including prevention of cisplatin-induced emesis (Darmani, 2001a,b,c; Darmani et al., 2003; Van Sickle et al., 2003). However, these studies in vomiting species (e.g., ferret and shrew) have focused on acute and delayed emesis induced after a single administration of the antineoplastic drug. To our knowledge, no study has been conducted so far to analyse the effect of cannabinoids on emesis induced by chronic cisplatin in such vomiting species or on cisplatin-induced pica in the rat.

In addition, when chemotherapy is administered, a reduction of weight gain due to anorexia (loss of appetite) is almost unavoidably encountered, both in humans (Van Cutsem and Arends, 2005) and experimental animals (Authier et al., 2003a,b). Cannabinoids have been proposed and used to reduce cachexia associated with AIDS or advanced cancer (Ben Amar, 2006), and the cannabinoid antagonist rimonabant is currently undergoing clinical development as an anorexigenic drug in obese patients (Halford, 2006). However, very little is known about the effect of cannabinoids on food intake and weight gain of experimental animals chronically administered with antineoplastic drugs. Our model to study chronic cisplatin-induced pica in the rat could be useful to determine the long-term effects of cannabinoids on feeding behaviour (both anorexia and pica).

Another frequent adverse effect derived from the use of antineoplastic drugs, including platinum compounds, is neurotoxicity (Warner, 1995). Although infrequently dose-limiting, chemotherapy-induced sensory and motor neuropathies are clinically relevant (even if mild in severity), can be disabling, and cause persistent discomfort to patients and negative impact on quality of life. Moreover, high chemotherapy doses and high intensity of administration are often used, and increased the risk of neurological symptoms (Ocean and Vahdat, 2004).

More specifically, cisplatin induces a duration, dose, and time-dependent axonal sensorimotor polyneuropathy affecting large and small diameter sensory fibres. It usually causes clinical signs and symptoms after a cumulative dose of 300 mg m^{-2}, and neuropathy may persist for years (Chaudhry et al., 2003; Markman, 2003). Moreover, about 20% of patients are unable to complete a full course of cisplatin therapy due to sensory neuropathy. To date, the most effective approach to management of drug-induced neuropathy has been that of limiting the total dose, or reducing individual doses or even discontinuing the neurotoxic drugs at appearance of moderate symptoms (Ocean and Vahdat, 2004). In order to prevent or mitigate chemotherapy-induced neuropathy, drugs such as amifostine (Openshaw et al., 2004), reduced glutathione (Colombo et al., 1995), calcium and magnesium infusions, glutamine (Savarese et al., 2003; Vahdat et al., 2001), acetyl-L-carnitine (Bianchi et al., 2005) or vitamin E (Pace et al., 2003; Argyriou et al., 2005) have been tested, but results have not always been satisfactory (Umapathi and Chaudhry, 2005; Albers et al., 2007).

In view of the neuroprotective properties demonstrated for different cannabinoids in animal models of ischemia, seizures, hypoxia, multiple sclerosis, Huntington's and Parkinson's diseases (Van der Stelt and Di Marzo, 2005), it is tempting to investigate whether cannabinoids can also prevent the development of chemotherapy-induced peripheral neuropathy, if given before the antineoplastic drug. In fact, daily treatment with the synthetic cannabinoid agonist WIN 55,212-2 or the inhibitor of anandamide uptake AM404 prevented nociceptive behaviour in rats subjected to chronic constriction injury of the sciatic nerve (Costa et al., 2004, 2006; La Rana et al., 2006; Palazzo et al., 2006), which is a procedure classically used for the experimental induction of neuropathic pain (Bennett and Xie, 1988).

Therefore, the aim of this work was to determine whether the non-selective cannabinoid agonist WIN 55,212-2, administered on a weekly basis, is able to prevent some adverse effects (anorexia, pica and mechanical allodynia) induced by chronic cisplatin. Additionally, the acute psychoactive effects of the cannabinoid at the doses tested were checked.

Materials and methods

The experiments in the present study, which were designed to minimize the number of animals used and their suffering, were performed in strict accordance with the EC regulations for care and use of experimental animals (EEC no. 86/609) and approved by the Ethical Committee at the Universidad Rey Juan Carlos.

Animals

Male Wistar rats (250–300 g) were obtained from Harlan-Iberica (Barcelona, Spain). Upon arrival to our laboratory, animals were stored in standard transparent cages (40 cm × 28 cm × 25 cm), furnished with wood shaving bedding, which was changed every

Table 1
Distribution of experimental subjects included in the chronic study (see text for details)

Experimental group	1st injection	2nd injection
Saline (n=15)	Saline	Saline
Cisplatin 1 (n=11)	Saline	Cisplatin (1 mg kg^{-1}) week^{-1}
Cisplatin 2 (n=11)	Saline	Cisplatin (2 mg kg^{-1}) week^{-1}
WIN 1–cisplatin 1 (n=10)	WIN 55,212-2 (1 mg kg^{-1}) week^{-1}	Cisplatin (1 mg kg^{-1}) week^{-1}
WIN 2–cisplatin 2 (n=6)	WIN 55,212-2 (2 mg kg^{-1}) week^{-1}	Cisplatin (2 mg kg^{-1}) week^{-1}
WIN 1 (n=7)	WIN 55,212-2 (1 mg kg^{-1}) week^{-1}	Saline
WIN 2 (n=6)	WIN 55,212-2 (2 mg kg^{-1}) week^{-1}	Saline
Vehicle (n=3)	Vehicle	Saline

1–2 days. Cages were placed adjacent to each other, in environmentally controlled conditions (temperature=20 °C; humidity=60%) with a 12 h light/12 h dark cycle (lights on between 08:00 and 20:00 h). Animals were allowed free access to tap water and standard laboratory rat chow (Harlan-Iberica, Barcelona, Spain).

Effect of chronic cisplatin and/or WIN 55,212-2 on feeding behaviour and peripheral neuropathy

In order to analyse individual feeding behaviour, these experiments were conducted in rats habituated to isolation and presence of kaolin in their cages. Isolation of the rats started upon arrival to the laboratory and lasted for the whole duration of the experiment. After the first week of isolation, free access to kaolin pellets was also allowed so that "pica" could be analysed. Food and kaolin were placed in adjacent separate compartments in a divided food hopper and were continuously available throughout the experiment. Every day, water bottles were refreshed and the remaining amount of kaolin and food was collected, including that spilt outside the containers. Food and kaolin in each cage were topped up to 150±1 and 15±0.1 g of pellets, respectively.

Chronic treatment

As mentioned above, during the two weeks prior to the experiment, rats were habituated to isolation and kaolin, as well as to the testing procedures and to daily handling by the investigator. The week of adaptation to kaolin was considered as control and is termed as experimental week 0 (T_0) from now onwards. After this period of adaptation, rats received two intraperitoneal injections once per week for 5 experimental weeks: the first one was WIN 55,212-2 (1 or 2 mg kg^{-1}), its vehicle (see below) or saline (0.9% NaCl weight/volume); the second one was either cisplatin (1 or 2 mg kg^{-1}) or saline. Therefore, rats were distributed in eight different experimental groups, as shown in Table 1. Drug doses were selected according to references found in the literature and our own experience (Authier et al., 2003a; Vera et al., 2006; Pascual et al., 2005). Drug volumes were adjusted to a maximum of 4–5 ml kg^{-1}.

In an attempt to assay the effect of manipulation in feeding behaviour, a number of the rats included in the groups treated with cisplatin at 1 (n=5) and 2 mg kg^{-}1 week^{-1} (n=5) received an additional injection of saline on experimental week 6 (placebo, P); to encourage conditioning, a visual cue was placed in the cage of these animals after each administration (T_0-P) and was removed 24 h later. In order to prevent eventual nephrotoxicity induced by chronically administered cisplatin, 2 ml of saline were also injected subcutaneously just before intraperitoneal saline or cisplatin (Authier et al., 2003a). Kaolin and chow pellets were provided again immediately after drug administration.

Evaluation of overall health parameters (temperature and body weight) and feeding behaviour

Examination of the rats and measurement of weight, rectal temperature, and food and kaolin intake were performed from T_0 to T_5 (or T_6-P) on 5 consecutive days per week between 9.00 and 11.00 am. The drugs were administered on the first observational day each week, immediately after completion of data recording. Core temperatures in the rat were measured using a P6 thermometer and a lubricated rectal probe (Cibertec, Spain) inserted into the rectum to a constant depth of 5 cm. Kaolin and food consumption were measured by subtracting the amount remaining from the amount provided the day before, for each cage. Care was taken to collect all particles, which were weighed to correct the values of food and kaolin consumption to the nearest 1 and 0.01 g, respectively.

Evaluation of mechanical allodynia

Mechanical allodynia was assessed at the end of experimental week 0 (T_0) and on the fourth day after the last injection (experimental week 5, T_5). On those days, rats were placed individually on an elevated iron mesh in a clear plastic cage and were allowed to adapt to the testing environment for at least 10 min. Habituation to this environment was also performed on the two days before assessment. Calibrated von Frey Hairs (0.9, 1.4, 2.1, 2.5, 3, 4, 5.5, 7.5, 8, 10.5, 13, 14, 15, 17, 25, 27, 32 and 40 g) were applied to the plantar aspect of each hindpaw, from below the mesh floor. Each stimulus was applied for

Table 2
Control values

	Range	Mean±s.e. mean
Temperature (°C)	37.4–38.3	37.8±0.1
Body weight (g)	252–289	272±4
Basal food ingestion/day (g)	21.8–24.3	23.1±0.3
Basal kaolin intake/day[a] (g)	0.008–0.331	0.144±0.044
Threshold for paw withdrawal (g)	14.0–24.9	20.8±1.77

[a] Excludes the first day of adaptation.

approximately 1 s with an interstimulus interval of approximately 3 s. Only robust and immediate withdrawal responses from the stimulus were recorded. A positive result was considered when 3 or more withdrawal responses were obtained from 5 consecutive trials with each monofilament. Mechanical allodynia was defined as a significant decrease in von Frey hair threshold evoked by mechanical stimuli. The 40 g hair was selected as the upper cut-off limit for testing.

Psychoactive effects of WIN 55,212-2 (cannabinoid tetrad)

The classical cannabinoid tetrad test evaluates temperature, antinociception, catalepsy and motility in the same animal after cannabinoid administration (Pertwee, 1972). An observer, unaware of the treatments, recorded the test values in grouped rats (6 animals/cage), at least one week after arrival of the animals to the laboratory. On the day of the experiment, rats received one intraperitoneal injection of either saline ($n=6$), WIN 55,212-2 at 1 ($n=6$) or 2 mg kg^{-1} ($n=6$) or cannabinoid vehicle ($n=6$) and the cannabinoid tetrad was subsequently assessed.

Rectal temperature was recorded as described above for the isolated animals. Data were recorded both before drug administration and 30 min after injection.

Heat-antinociception of the hindpaw was tested 20 min after drug administration, using methods described by Bennett and Hargreaves (1990). It was examined by measuring the latency (withdrawal time) of the hindpaws from a focused beam of radiant heat applied to the plantar surface using a plantar test apparatus (Ugo Basile). Briefly, the rat was placed within a plastic compartment on a glass floor; a light source beneath the floor was aimed at the mid plantar surface of the hindpaw. The withdrawal reflex interrupts the light and automatically turns off the light and a timer. The intensity of the light was adjusted at the start of the experiment such that the control average baseline latencies were about 8 s and a cut-off latency of 30 s was imposed. The withdrawal latency of each paw was measured during three trials at 2 min intervals and the mean of the three readings was used for data analysis.

Catalepsy was measured using a modification of the "ring test" (Fox et al., 2001) originally described by Pertwee (1972). Rats were hung by their front paws from a rubber coated metal ring (12 cm diameter) fixed horizontally at a height allowing their hindpaws to just touch the bench, and the time taken for the rat to move off the ring was measured with a cut-off of 30 s. Data are expressed as an immobility index defined as percentage of the total time spent on the ring during which the animal remains motionless. Latencies were measured after temperature evaluation, approximately 35 min after drug or vehicle administration.

Spontaneous locomotor activity was evaluated using individual photocell activity chambers (CIBERTEC, Spain). Rats were placed in the recording chambers (55×40 cm, spacing between beams 3 cm) 40 min after drug administration and the number of interruptions of photocell beams was recorded over a 30-min period. The mean number of crossings of the photocell beams was used for comparison.

Compounds and drugs

Kaolin, carmine, gum arabic and cisplatin were purchased from Sigma-Aldrich (Spain). Cisplatin was dissolved in saline (sonicated for about 15 min). WIN 55,212-2 was obtained from Tocris (Spain) and dissolved in ethanol 1 mg:1 ml and subsequently in ethanol and Tween 80 (1:2) after which the ethanol was evaporated and saline added to reach final concentration (Pertwee et al., 1992).

Kaolin pellets were prepared as previously described (Vera et al., 2006; Yamamoto et al., 2002; Takeda et al., 1993). Briefly, pharmaceutical grade kaolin (hydrated aluminum silicate; 98.5%) was mixed with 0.5% carmine and 1% gum arabic in distilled water to form a thick paste. Pellets of the resulting kaolin mixture were shaped to resemble the dimensions of the rats' normal laboratory diet. The pellets were completely dried at room temperature for at least 48 h.

Statistical analysis

Data are presented as the mean values±SEM. Differences were analysed using Student's *t*-test, with Welch's correction where appropriate, or Two-Way ANOVA followed by post hoc Bonferroni multiple comparison test. Values of $p<0.05$ were regarded as being significantly different.

Mean values obtained in the chronic experiments are representative for each week of the experiment, from week 0 (T_0) to week 5 (T_5) or 6 (placebo, P). In the case of rectal temperature and body weight, daily measures were averaged for each week. For a more accurate comparison, changes in these parameters are shown as percentage of change versus the average obtained for T_0. Finally, two parameters related to food and kaolin ingestion were analysed each week: food/kaolin intake in the 24 h s after drug administration (to measure acute anorexia and acute pica respectively) and basal food/kaolin intake (to measure basal anorexia and facilitated pica respectively; for each substance, this parameter was obtained from the average daily intake for the whole week, excluding the day after drug administration).

Results

Effects of chronic cisplatin and/or WIN 55,212-2 on feeding behaviour and peripheral neuropathy

Control values for temperature, body weight, daily basal food ingestion, daily basal kaolin intake and threshold for paw withdrawal are shown in Table 2. These values were obtained using the averages for the eight experimental groups during T_0 ($n=69$).

Neither basal food nor kaolin ingestion nor the threshold for paw withdrawal was significantly modified after chronic administration of saline (see below) or vehicle of WIN 55,212-2 (not shown). The rate of body weight gain was also similar for saline-treated (see below) and vehicle-treated rats (not shown). A significant reduction in temperature was found when rats received the cannabinoid vehicle (not shown). However, this modification was similar to that induced by the cannabinoid itself at either dose (see below).

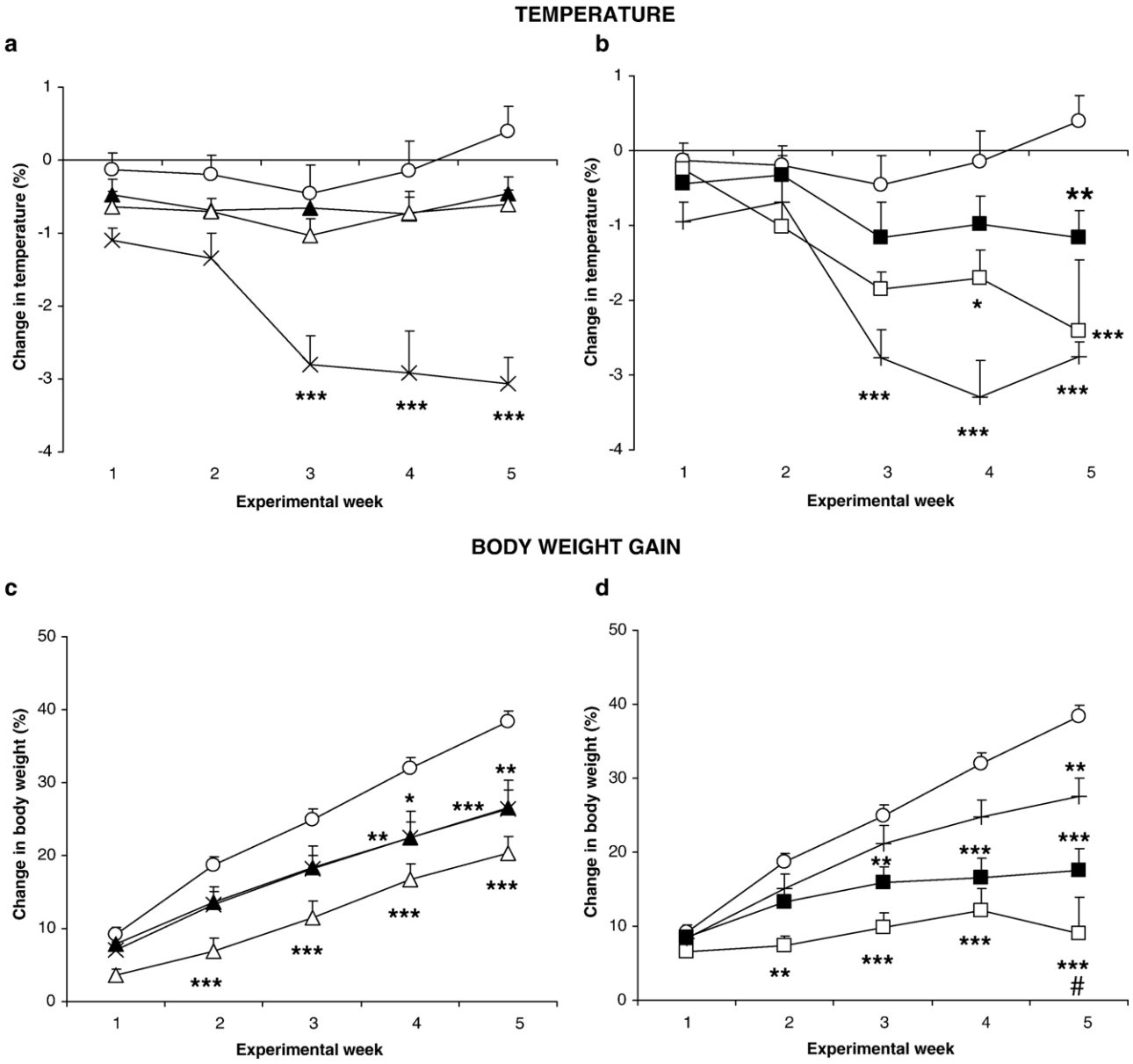

Fig. 1. Effect of chronic cisplatin and/or the cannabinoid agonist WIN 55,212-2 on temperature and body weight in the rat. Change in rectal temperature (A, B) and body weight (C, D) were measured in isolated rats exposed to kaolin and injected for 5 weeks with: saline (4–5 ml kg^{-1} $week^{-1}$, i.p., open circles, $n=15$; in A–D), WIN 55,212-2 at 1 (*X*, $n=7$; in A, C) or 2 mg kg^{-1} $week^{-1}$ (+, $n=6$; in B, D), cisplatin at 1 (closed triangles, $n=11$; in A, C) or 2 mg kg^{-1} $week^{-1}$ (closed squares, $n=11$; in B, D), both WIN 55,212-2 and cisplatin at 1 mg kg^{-1} $week^{-1}$ each (open triangles, $n=10$; in A, C) or both WIN 55,212-2 and cisplatin at 2 mg kg^{-1} $week^{-1}$ each (open squares, $n=6$; in B, D). WIN 55,212-2 was administered 30 min before each cisplatin administration. Data represent the mean (average of the measurements taken during each week)±s.e.mean. *($p<0.05$), **($p<0.005$), ***($p<0.001$) vs saline; # ($p<0.05$) vs cisplatin (two-way ANOVA followed by Bonferroni test).

Temperature and body weight

Rectal temperature decreased after chronic administration of WIN 55,212-2 (1 or 2 mg kg^{-1} $week^{-1}$). In cisplatin-treated rats, temperature was not consistently different from saline-treated animals. When the cannabinoid was administered 30 min before cisplatin, a reduction in temperature could still be seen but only when each drug was used at 2 mg kg^{-1} week. In any case, this effect was lower than in rats treated with the cannabinoid alone (Fig. 1A, B).

Chronic treatment with the antitumoral drug delayed body weight gain in a dose-dependent manner. WIN 55,212-2 at either dose also delayed body weight gain when used alone, and tended to increase the effect of cisplatin, although the difference was not statistically significant in a consistent manner when animals only received the antineoplastic drug (Fig. 1C, D).

Feeding behaviour

Neither acute nor basal food ingestion was modified by chronic administration of WIN 55,212-2. Significant acute anorexia was induced in animals treated with cisplatin at either 1 or 2 mg kg^{-} 1 $week^{-1}$ whereas basal food ingestion decreased

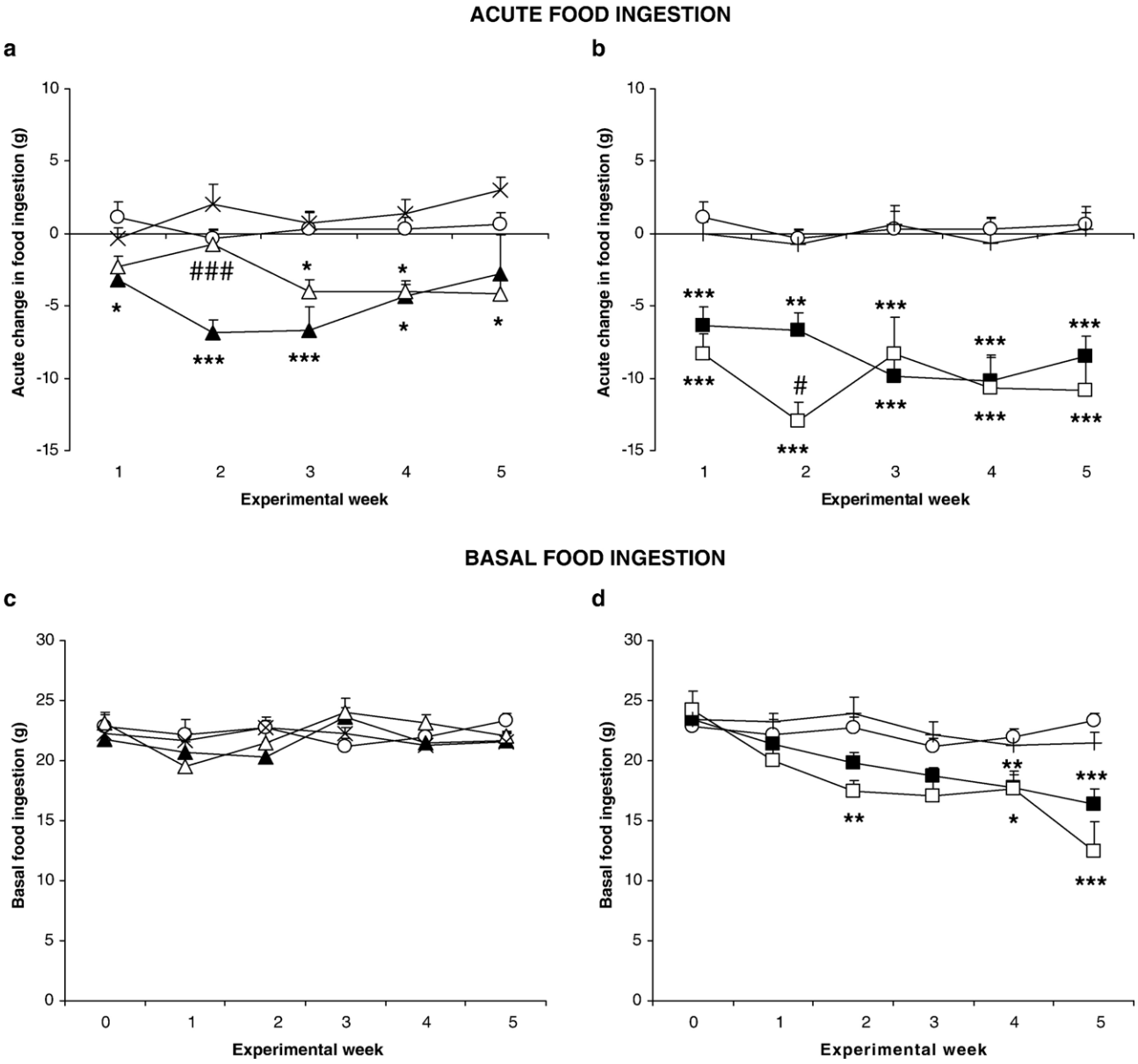

Fig. 2. Effect of chronic cisplatin and/or the cannabinoid agonist WIN 55,212-2 on food ingestion in the rat. Acute (A, B) and basal food ingestion (C, D) were measured in isolated rats exposed to kaolin and injected for 5 weeks with: saline (4–5 ml kg^{-1} $week^{-1}$, i.p., open circles, $n=15$; in A–D), WIN 55,212-2 at 1 (*X*, $n=7$; in A, C) or 2 mg kg^{-1} $week^{-1}$ (+, $n=6$; in B, D), cisplatin at 1 (closed triangles, $n=11$; in A, C) or 2 mg kg^{-1} $week^{-1}$ (closed squares, $n=11$; in B, D), both WIN 55,212-2 and cisplatin at 1 mg kg^{-1} $week^{-1}$ each (open triangles, $n=10$) or both WIN 55,212-2 and cisplatin at 2 mg kg^{-1} $week^{-1}$ each (open squares, $n=6$) . WIN 55,212-2 was administered 30 min before each cisplatin administration. Data represent the mean (average of the measurements taken during each week)±s.e.mean. *($p<0.05$), **($p<0.005$), ***($p<0.001$) vs saline; # ($p<0.05$), ### ($p<0.001$) vs cisplatin (two-way ANOVA followed by Bonferroni test).

only when cisplatin was administered at 2 mg $kg^{-}1$ $week^{-1}$. Administration of WIN 55,212-2 was not able to prevent the effect of cisplatin in either basal food ingestion or acute anorexia (Fig. 2).

Chronic administration of WIN 55,212-2 did not induce significant acute or basal kaolin intake. Compared with saline-treated animals, significant acute and basal pica was induced by administration of cisplatin but only at 2 mg kg^{-1} $week^{-1}$. As in the case of food ingestion, neither acute nor basal pica induced by cisplatin was prevented by previous administration of the cannabinoid (Fig. 3).

Finally, to rule out that handling may condition a potentiation of feeding modifications, a group of cisplatin-treated rats were further injected with saline on the sixth week. This treatment did not induce acute anorexia or pica or significant changes in the values of basal ingestion of food and kaolin when compared to those recorded in the previous week (data not shown).

Mechanical allodynia

Chronic administration of WIN 55, 212-2 (Fig. 4) did not significantly alter the threshold for mechanical

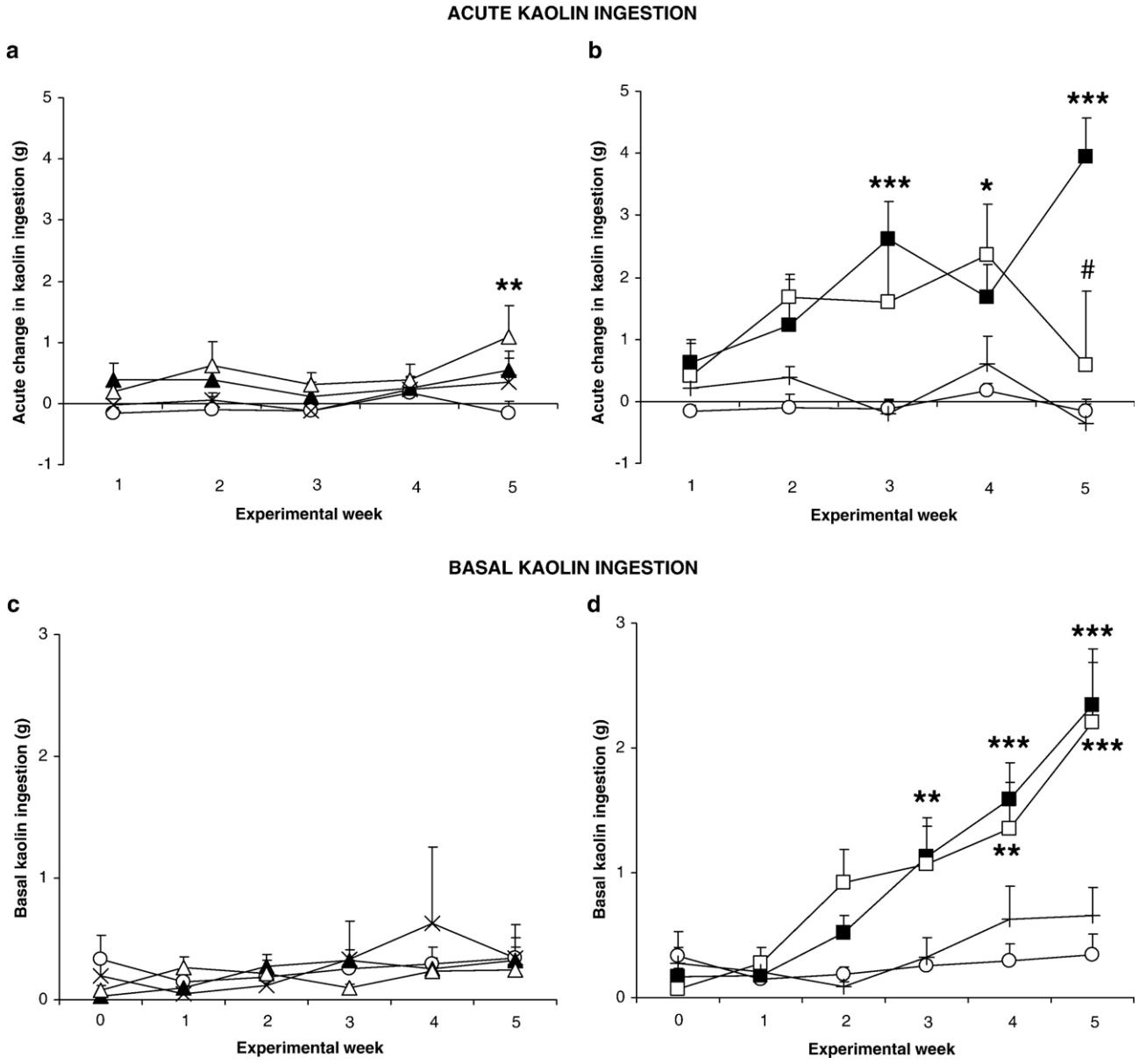

Fig. 3. Effect of chronic cisplatin and/or the cannabinoid agonist WIN 55,212-2 on kaolin ingestion (pica) in the rat. A–D: Acute (A, B) and basal kaolin ingestion (C, D) were measured in isolated rats exposed to kaolin and injected for 5 weeks with: saline (4–5 ml kg^{-1} $week^{-1}$, i.p., open circles, $n=15$; in A–D), WIN 55,212-2 at 1 (*X*, $n=7$; in A, C) or 2 mg kg^{-1} $week^{-1}$ (+, $n=6$; in B, D), cisplatin at 1 (closed triangles, $n=11$; in A, C) or 2 mg kg^{-1} $week^{-1}$ (closed squares, $n=11$; in B, D), both WIN 55,212-2 and cisplatin at 1 mg kg^{-1} $week^{-1}$ each (open triangles, $n=10$; in A, C) or both WIN 55,212-2 and cisplatin at 2 mg kg^{-1} $week^{-1}$ each (open squares, $n=6$; in B, D). WIN 55,212-2 was administered 30 min before each cisplatin administration. Data represent the mean (average of the measurements taken during each week)±s.e.mean. *($p<0.05$), **($p<0.005$), ***($p<0.001$) vs saline; # ($p<0.05$) vs cisplatin (two-way ANOVA followed by Bonferroni test).

allodynia. As expected, cisplatin administered weekly induced a significant reduction in this parameter when tested 4 days after the last injection. Interestingly, administration of WIN 55,212-2, 30 min before each administration of cisplatin, prevented the induction of mechanical allodynia by cisplatin (Fig. 4).

Psychoactive effects of WIN 55,212-2 (cannabinoid tetrad)

The cannabinoid at 1 mg kg^{-1} did not induce any behavioural modification in the cannabinoid tetrad. However, at the highest dose tested, 2 mg kg^{-1}, WIN 55,212-2 induced both catalepsy and heat antinociception (Fig. 5).

Discussion and conclusions

The present results show that the synthetic cannabinoid agonist WIN 55,212-2 prevents the development of mechanical allodynia induced by chronic cisplatin, in a rat model of peripheral neuropathy (Authier et al., 2003a). In addition, prevention of chemotherapy-induced allodynia seems to be a selective effect because other alterations induced by the

antineoplastic given chronically (namely anorexia, pica and reduction in weight gain) were not avoided. Remarkably, the preventive effect of WIN 55,212-2 was evident even at a dose lacking activity in the cannabinoid tetrad, which is an accepted index of cannabinoid psychoactivity (Pertwee, 1972).

Adverse effects of chronic cisplatin

The present work confirms and extends our previous observations on the effects of chronic cisplatin on general health and feeding behaviour (Vera et al., 2006). Cisplatin was tested at 1 mg kg^{-1} $week^{-1}$, but differences with saline-treated rats were not consistently significant. At this dose, cisplatin has been reported to induce pica (Yamamoto et al., 2004). However, in our hands, induction of significant pica requires the use of higher doses, if a single administration of cisplatin is applied (unpublished observations), or chronic administration of this dose (and even then significance is reached only occasionally, see Vera et al., 2006). Inconsistency of results when using this low dose of the antineoplastic drug could be related to differences in the experimental approach (e.g., animals used, preparation of kaolin, precise composition of food or drinking water). The dose of 2 mg kg^{-1} $week^{-1}$ (cumulative dose of 10 mg kg^{-1}) was then tried and found to be more effective and reliable, without excessive toxicity (no rat died and only a slight hypothermia was observed at the end of the experiment). Thus, cisplatin at 2 mg^{-1} kg^{-1} induced a greater delay in body weight gain and more intense acute anorexia. In addition, it induced a reduction in basal food intake and intense increases in basal and acute kaolin intake. Therefore, this dose seems more adequate to study the "anti-pica" (antinauseant?) effects of antiemetic drugs in this model. We declined to use cisplatin at a higher dose because in a previous report, it was too toxic (Vera et al., 2006).

One week of rest after chronic treatment with cisplatin 1 or 2 mg kg^{-1} was not enough to recover basal food and kaolin

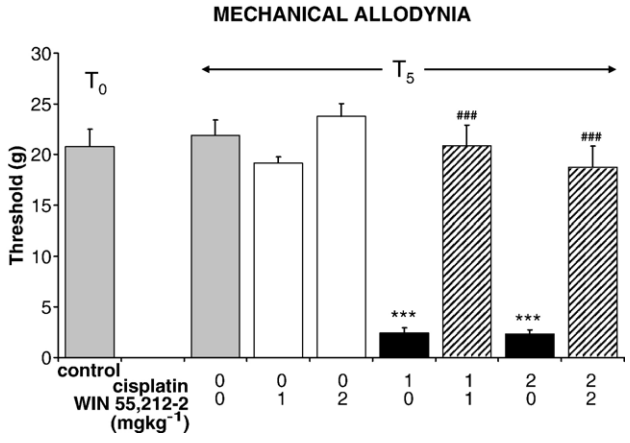

Fig. 4. Prevention of chronic cisplatin-induced mechanical allodynia by the cannabinoid agonist WIN 55,212-2. Bars show the threshold for mechanical allodynia (mean ± s.e.mean), measured in isolated rats before (T_0) and after chronic treatments (four days after the last administration of the drugs, T_5). Grey bars correspond to control animals before treatment and after saline injection (4–5 ml kg^{-1} $week^{-1}$, i.p.), white bars, to rats treated with WIN 55,212-2 at 1 or 2 mg kg^{-1} $week^{-1}$, black bars, to rats treated with cisplatin at 1 or 2 mg kg^{-1} $week^{-1}$ and hatched bars, to rats treated with both cisplatin (1 or 2 mg kg^{-1} $week^{-1}$ and WIN 55,212-2 (1 or 2 mg kg^{-1} $week^{-1}$). WIN 55,212-2 was administered 30 min before each cisplatin administration. ***$p<0.001$ vs saline (grey bar) at experimental week 5 (T_5); ###$p<0.001$ vs cisplatin (unpaired t-test). $n \geq 6$ for all groups.

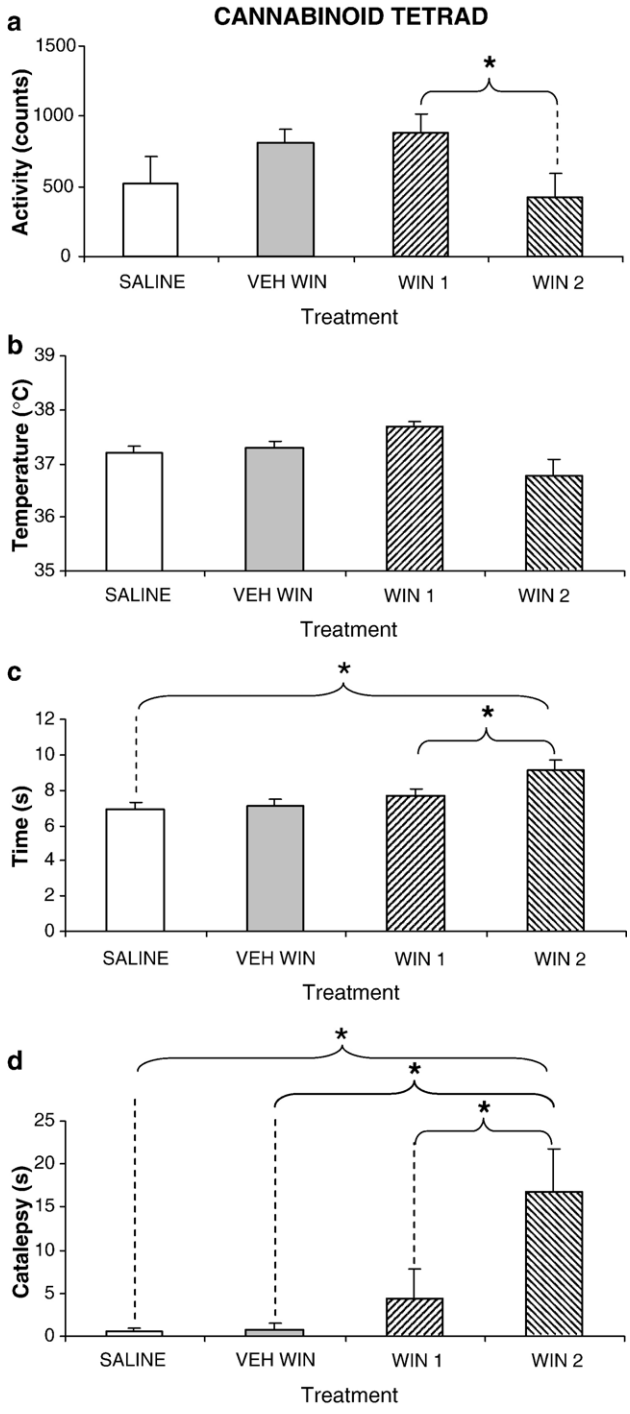

Fig. 5. Acute psychoactive effects of the cannabinoid agonist WIN 55,212-2. The following tests were used to evaluate the appearance of the characteristic cannabinoid psychoactive effects (cannabinoid tetrad): A-spontaneous locomotor activity (for hypolocomotion); B-rectal temperature (for hypothermia); C-plantar test (for heat-analgesia); D-ring test (for catalepsy). The tests were applied to grouped rats after a single dose of WIN 55,212-2 at 1 or 2 mg kg^{-1}, vehicle or saline. Bars show mean values±s.e.mean. *$p<0.05$ (unpaired t-test). $n=6$ for all groups.

intake to control values, suggesting that delayed/facilitated effects of cisplatin are more robust than acute effects. In fact, delayed nausea and emesis are well known for their resistance against traditional pharmacological approaches (Jordan et al., 2005), which encourages the use of long-term models like the present one for the evaluation of drugs with antiemetic properties.

Since basal pica and anorexia increased with each administration of cisplatin, a dose of saline (placebo) on the sixth week was used, to discard the effect of manipulation. This treatment did not induce acute anorexia or pica, not even when a visual cue was used to reinforce pica, indicating that this response is not conditioned by handling.

With regard to the development of chemotherapy-induced peripheral neuropathy, the study conducted here agrees with data from others (Authier et al., 2003a). Different antineoplastic drugs have been used to induce peripheral neuropathy or its generally associated signs in experimental animals (cisplatin, see Authier et al., 2003a; paclitaxel, see Polomano et al., 2001; vincristine, see Authier et al., 2003b). Different functional signs have been shown to be associated with the development of peripheral neuropathy after chronic cisplatin (namely, hyperalgesia and allodynia to mechanical and/or cold stimuli: Authier et al., 2003a). In the present work, the threshold for mechanical allodynia was the parameter chosen to assay the development of peripheral neuropathy, due to the relatively low level of stress associated to its evaluation. Interestingly, cisplatin at 1, 2 (present results) or 3 (unpublished observations) mg kg^{-1} $week^{-1}$ reduced the threshold to similar values, indicating that the dose of 1 mg kg^{-1} is, in our hands, maximal to induce this effect, and adequate to study the palliative and/or preventive effect of different drugs with analgesic and/or neuroprotective properties.

Effects of WIN 55,212-2 alone or in combination with cisplatin

Studies with the cannabinoid or its vehicle alone produced some unexpected results. First, a reduction in the average temperature was detected for WIN 55,212-2 at either dose or its vehicle. The hypothermic effect of WIN 55,212-2 when acutely administered is well-known and justifies the inclusion of temperature recording in the cannabinoid tetrad (Pertwee, 1972). However, we found no acute hypothermic effect of WIN 55,212-2 in grouped animals at the doses tested. This slight although significant hypothermic effect could be due to the vehicle itself. This vehicle was proposed long ago (Pertwee et al., 1992) and it does not alter temperature acutely (present results; Pascual et al., 2005). However, to our knowledge, it has not been tested chronically before (reduction in temperature was significant only from the third experimental week). It could be speculated that some cumulative effect of the vehicle in this particular parameter could be responsible for the effect of the cannabinoid. In any case, the effects of vehicle in the long run were restricted to temperature and no other parameter was significantly different from saline-treated rats.

Second, the cannabinoid induced a reduction in weight gain (without a significant change in food consumption), that was significant at least from the fourth experimental week. One previous report, in which cannabinoids were given at higher doses, higher frequency and for longer duration, has also described a decrease in body weight gain without a change in food consumption (Chan et al., 1996). To explain this effect one could argue that WIN 55,212-2-treated rats could be either physically or metabolically more active. These explanations seem unlikely, because cannabinoids reduce both spontaneous locomotor activity and temperature (present results; Pertwee, 1972). Another possible explanation could be that diarrhoea was induced by WIN 55,212-2, but cannabinoids reduce gastrointestinal motility (Izzo and Coutts, 2005). Lastly, since cannabinoids also decrease gastrointestinal secretion (Izzo and Coutts, 2005), rats treated with WIN 55,212-2 could have nutrient absorption uncoupled. This option deserves further investigation.

Whatever the case may be, it seems that the reductions in body weight gain induced by cisplatin and WIN 55,212-2 could be due to different mechanisms, first because effects were practically additive, and second, and more importantly, because in the case of cisplatin-only treated animals the reduction in weight gain was associated with, at least, acute anorexia, not present in cannabinoid-only treated rats.

WIN 55,212-2 did not increase food ingestion or kaolin intake when used alone. Furthermore, it did not consistently prevent the reduction in food ingestion or the increase in kaolin intake associated with cisplatin administration. This was to some extent surprising, because cannabinoids are empirically used to both increase appetite and reduce chemotherapy-induced nausea and emesis and have been claimed as useful in both cancer and AIDS patients (Ben Amar, 2006).

The lack of effect of WIN 55,212-2 per se and as a means to prevent feeding alterations induced by chronic cisplatin could be due to several factors. Thus, it has been suggested that the cannabinoid-induced increase in appetite could affect preferentially sweet and palatable foods (Cota et al., 2003; Vickers and Kennett, 2005), to which our animals were not exposed. However, there could be other factors more likely involved, such as, the experimental design to measure modifications in feeding and signs of nausea. The orexigenic effect of cannabinoids has been studied using other agonists (THC, HU-210 — Higgs et al., 2005) and other procedures, mostly in previously food-deprived/partially satiated animals and after a high single dose of the drug (Gómez et al., 2002; Higgs et al., 2005). Likewise, this and other cannabinoid agonists have been shown to prevent kaolin intake in the rat (unpublished observations) and nausea and emesis in other animal models (Kwiatkowska et al., 2004; Darmani, 2001a; Van Sickle et al., 2003), but these studies only assayed the effect of a single dose of cisplatin, too high to be used chronically, and too toxic to be interesting from the clinical point of view. An additional factor could be the specific drug, dose and/or frequency of administration. It could be interesting to ascertain whether THC or commercially available extracts such as Sativex®, a higher (although highly psychoactive) dose of WIN 55,212-2 or THC and/or other frequencies of administration (i.e., daily) could be more effective than WIN 55,212-2 at 1-2 mg^{-1} kg^{-1} $week^{-1}$ to prevent cisplatin-induced feeding alterations in our

model. In any case, it is very likely that this kind of approach may induce additional undesired (psychoactive) effects, as has been noticed in cancer patients given high doses of THC or other cannabinoids (Tramer et al., 2001).

Finally, the most interesting result was that WIN 55,212-2 did not alter the responses to mechanical stimulation when used alone, but completely prevented the development of mechanical allodynia induced by chronic cisplatin. This effect has been consistently found in our laboratory and has already been comunicated (Martín et al., 2006). Since the drugs were administered intraperitoneally within only 30 min, one could argue that they could have interacted physically, thus leading to a reduced effect of cisplatin, but this is unlikely, because the antitumoral drug kept exerting all the other adverse effects evaluated here.

The search for therapeutic tools able to prevent the development of neuropathic signs associated with cancer therapy is extremely important. Thus, whilst other causes of neuropathic pain (nerve injury, diabetes) are not predictable, the use of antineoplastic drugs is always something planned, and administration of these preventive tools could be initiated even days before chemotherapy starts, in order to exert a preparatory function in the body.

Cannabinoids, including the synthetic agonist WIN 55,212-2, had already shown acute analgesic properties in different models of established neuropathic pain, such as that induced by the antitumoral drug paclitaxel (Pascual et al., 2005). These effects were mediated by activation of CB1 receptors and obtained also with non-psychoactive doses, like those used in the present work.

The use of cannabinoids as a preventive tool against neuropathic pain has also recently been addressed by different investigators (Costa et al., 2004, 2006; La Rana et al., 2006; Palazzo et al., 2006). It was reported that both the same cannabinoid agonist used here (Costa et al., 2004) and a drug increasing the endogenous cannabinoid tone (Costa et al., 2006; La Rana et al., 2006; Palazzo et al., 2006), were able to prevent pain behaviour in a rat model of peripheral neuropathy induced by chronic sciatic nerve injury. In those reports, cannabinoid treatment was given daily and the reduction in nociceptive signs could have always at least partially been related to the last dose administered before the tests (it could be that some accumulation of the drug induced reversion more than prevention of neuropathic signs). Even though we have used a higher dose than those studies, it should be remembered that allodynia was evaluated four days after the last drug administration and prevention seems more likely involved in this case, especially when WIN 55,212-2 was used at 1 mg kg^{-1}, because this dose did not induce significant acute antinociception (Fig. 5). In any case, the fact that a similar effect can be reached using much lower frequencies of administration reinforces the interest of cannabinoids as a potential therapeutic tool.

Regardless of the specific mechanisms involved in this effect, the fact that cannabinoids were useful to prevent neuropathic signs in two very different animal models, chemotherapy and nerve injury (present results; Costa et al., 2004, 2006), suggests that the cannabinoid effect could be neuroprotective and independent of the origin of the pathology and further supports the ongoing evaluation of the effectiveness of cannabinoids in the treatment of patients suffering or likely to suffer neuropathic pain.

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References

Albers, J., Chaudhry, V., Cavaletti, G., Donehower, R., 2007. Interventions for preventing neuropathy caused by cisplatin and related compounds. Cochrane Database of Systematic Review 24 (1), CD005228.

Andrews, P.L., Horn, C.C., 2006. Signals for nausea and emesis: implications for models of upper gastrointestinal diseases. Autonomic Neuroscience: Basic & Clinical 125 (1–2), 100–115.

Argyriou, A.A., Chroni, E., Koutras, A., Ellul, J., Papapetropoulos, S., Katsoulas, G., Iconomou, G., Kalofonos, H.P., 2005. Vitamin E for prophylaxis against chemotherapy-induced neuropathy: a randomized controlled trial. Neurology 64 (1), 26–31.

Authier, N., Gillet, J.P., Fialip, J., Eschalier, A., Coudore, F., 2003a. An animal model of nociceptive peripheral neuropathy following repeated cisplatin injections. Experimental Neurology 182 (1), 12–20.

Authier, N., Gillet, J.P., Fialip, J., Eschalier, A., Coudore, F., 2003b. A new animal model of vincristine-induced nociceptive peripheral neuropathy. Neurotoxicology 24 (6), 797–805.

Ben Amar, M., 2006. Cannabinoids in medicine: a review of their therapeutic potential. Journal of Ethnopharmacology 105 (1–2), 1–25.

Bennett, G.J., Xie, Y.K., 1988. A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. Pain 33 (1), 87–107.

Bennett, G.J., Hargreaves, K.M., 1990. Reply to Hirata and his colleagues. Pain 42, 255.

Bianchi, G., Vitali, G., Caraceni, A., Ravaglia, S., Capri, G., Cundari, S., Zanna, C., Gianni, L., 2005. Symptomatic and neurophysiological responses of paclitaxel-or cisplatin-induced neuropathy to oral acetyl-L-carnitine. European Journal of Cancer 41 (12), 1746–1750.

Chan, P.C., Sills, R.C., Braun, A.G., Haseman, J.K., Bucher, J.R., 1996. Toxicity and carcinogenicity of delta 9-tetrahydrocannabinol in Fischer rats and B6C3F1 mice. Fundamental and Applied Toxicology 30 (1), 109–117.

Chaudhry, V., Chaudhry, M., Crawford, T.O., Simmons-O'Brien, E., Griffin, J.W., 2003. Toxic neuropathy in patients with pre-existing neuropathy. Neurology 60 (2), 337–340.

Colombo, N., Bini, S., Miceli, D., Bogliun, G., Marzorati, L., Cavaletti, G., Parmigiani, F., Venturino, P., Tedeschi, M., Frattola, L., Buratti, C., Mangioni, C., 1995. Weekly cisplatin±gluthatione in relapsed ovarian carcinoma. International Journal of Gynecological Cancer 5 (2), 81–86.

Costa, B., Colleoni, M., Conti, S., Trovato, A.E., Bianchi, M., Sotgiu, M.L., Giagnoni, G., 2004. Repeated treatment with the synthetic cannabinoid WIN 55,212-2 reduces both hyperalgesia and production of pronociceptive mediators in a rat model of neuropathic pain. British Journal of Pharmacology 141 (1), 4–8.

Costa, B., Siniscalco, D., Trovato, A.E., Comelli, F., Sotgiu, M.L., Colleoni, M., Maione, S., Rossi, F., Giagnoni, G., 2006. AM404, an inhibitor of anandamide uptake, prevents pain behaviour and modulates cytokine and apoptotic pathways in a rat model of neuropathic pain. British Journal of Pharmacology 148 (7), 1022–1032.

Cota, D., Marsicano, G., Lutz, B., Vicennati, V., Stalla, G.K., Pasquali, R., Pagotto, U., 2003. Endogenous cannabinoid system as a modulator of food

intake. International Journal of Obesity and Related Metabolic Disorders 27 (3), 289–330.
Craig, J.B., Powell, B.L., 1987. The management of nausea and vomiting in clinical oncology. The American Journal of the Medical Sciences 293 (1), 34–44.
Darmani, N.A., 2001a. The cannabinoid CB1 receptor antagonist SR 141716A reverses the antiemetic and motor depressant actions of WIN 55, 212-2. European Journal of Pharmacology 430 (1), 49–58.
Darmani, N.A., 2001b. Delta-9-tetrahydrocannabinol differentially suppresses cisplatin-induced emesis and indices of motor function via cannabinoid CB (1) receptors in the least shrew. Pharmacology, Biochemistry and Behavior 69 (1–2), 239–249.
Darmani, N.A., 2001c. Cannabinoids of diverse structure inhibit two DOI-induced 5-HT(2A) receptor-mediated behaviors in mice. Pharmacology, Biochemistry and Behavior 68 (2), 311–317.
Darmani, N.A., Sim-Selley, L.J., Martin, B.R., Janoyan, J.J., Crim, J.L., Parekh, B., Breivogel, C.S., 2003. Antiemetic and motor-depressive actions of CP55,940: cannabinoid CB1 receptor characterization, distribution, and G-protein activation. European Journal of Pharmacology 459 (1), 83–95.
Davis, M., Maida, V., Daeninck, P., Pergolizzi, J., 2007. The emerging role of cannabinoid neuromodulators in symptom management. Supportive Care in Cancer 15 (1), 63–71.
De Wit, R., Herrstedt, J., Rapoport, B., Carides, A.D., Guoguang-Ma, J., Elmer, M., Schmidt, C., Evans, J.K., Horgan, K.J., 2004. The oral NK(1) antagonist, aprepitant, given with standard antiemetics provides protection against nausea and vomiting over multiple cycles of cisplatin-based chemotherapy: a combined analysis of two randomised, placebo-controlled phase III clinical trials. European Journal of Cancer 40 (3), 403–410.
Fox, A., Kesingland, A., Gentry, C., McNair, K., Patel, S., Urban, L., James, I., 2001. The role of central and peripheral Cannabinoid1 receptors in the antihyperalgesic activity of cannabinoids in a model of neuropathic pain. Pain 92 (1–2), 91–100.
Gómez, R., Navarro, M., Ferrer, B., Trigo, J.M., Bilbao, A., Del Arco, I., Cippitelli, A., Nava, F., Piomelli, D., Rodríguez de Fonseca, F., 2002. A peripheral mechanism for CB1 cannabinoid receptor-dependent modulation of feeding. Journal of Neuroscience 22 (21), 9612–9617.
Halford, J.C., 2006. Obesity drugs in clinical development. Current Opinion in Investigational Drugs 7 (4), 312–318.
Higgs, S., Barber, D.J., Cooper, A.J., Terry, P., 2005. Differential effects of two cannabinoid receptor agonists on progressive ratio responding for food and free-feeding in rats. Behavioural Pharmacology 16 (5–6), 389–393.
Izzo, A.A., Coutts, A.A., 2005. Cannabinoids and the digestive tract. Handbook of Experimental Pharmacology 168, 573–598.
Jordan, K., Kasper, C., Schmoll, H.J., 2005. Chemotherapy-induced nausea and vomiting: current and new standards in the antiemetic prophylaxis and treatment. European Journal of Cancer 41 (2), 199–205.
Kwiatkowska, M., Parker, L.A., Burton, P., Mechoulam, R., 2004. A comparative analysis of the potential of cannabinoids and ondansetron to suppress cisplatin-induced emesis in the *Suncus murinus* (house musk shrew). Psychopharmacology 174 (2), 254–259.
La Rana, G., Russo, R., Campolongo, P., Bortolato, M., Mangieri, R.A., Cuomo, V., Iacono, A., Raso, G.M., Meli, R., Piomelli, D., Calignano, A., 2006. Modulation of neuropathic and inflammatory pain by the endocannabinoid transport inhibitor AM404 [*N*-(4-hydroxyphenyl)-eicosa-5,8,11,14-tetraenamide]. Journal of Pharmacology and Experimental Therapeutics 317 (3), 1365–1371.
Markman, M., 2003. Toxicities of the platinum antineoplastic agents. Expert Opinion on Drug Safety 2 (6), 597–607.
Markman, M., 2006. Chemotherapy-induced peripheral neuropathy: underreported and underappreciated. Current Pain and Headache Report 10 (4), 275–278.
Martín, M.I., Vera, G., Chiarlone, A., Cabezos, P.A., Abalo, R., 2006. The cannabinoid agonist WIN 55,212-2 prevents the development of mechanical allodynia induced by chronic cisplatin in the rat. European Journal of Pain 10 (Suppl 1), 213.
Mitchell, D., Wells, C., Hoch, N., Lind, K., Woods, S.C., Mitchell, L.K., 1976. Poison induced pica in rats. Physiology & Behavior 17 (4), 691–697.
Navari, R.M., 2007. Prevention of emesis from multiple-day and high-dose chemotherapy regimens. Journal of the National Comprehensive Cancer Network 5 (1), 51–59.
Ocean, J.A., Vahdat, L.T., 2004. Chemotherapy-induced peripheral neuropathy: pathogenesis and emerging therapies. Supportive Care in Cancer 12 (9), 619–625.
Openshaw, H., Beamon, K., Synold, T.W., Longmate, J., Slatkin, N.E., Doroshow, J.H., Forman, S., Margolin, K., Morgan, R., Shibata, S., Somlo, G., 2004. Neurophysiological study of peripheral neuropathy after high-dose paclitaxel: lack of neuroprotective effect of amifostine. Clinical Cancer Research 10 (2), 461–467.
Pace, A., Savarese, A., Picardo, M., Maresca, V., Pacetti, U., Del Monte, G., Biroccio, A., Leonetti, C., Jandolo, B., Cognetti, F., Bove, L., 2003. Neuroprotective effect of vitamin E supplementation in patients treated with cisplatin chemotherapy. Journal of Clinical Oncology 21 (5), 927–931.
Palazzo, E., de Novellis, V., Petrosino, S., Marabese, I., Vita, D., Giordano, C., Di Marzo, V., Mangoni, G.S., Rossi, F., Maione, S., 2006. Neuropathic pain and the endocannabinoid system in the dorsal raphe: pharmacological treatment and interactions with the serotonergic system. European Journal of Neuroscience 24 (7), 2011–2020.
Pascual, D., Goicoechea, C., Suardiaz, M., Martin, M.I., 2005. A cannabinoid agonist, WIN 55,212-2, reduces neuropathic nociception induced by paclitaxel in rats. Pain 118 (1–2), 23–34.
Passik, S.D., Kirsh, K.L., Rosenfeld, B., McDonald, M.V., Theobald, D.E., 2001. The changeable nature of patients' fears regarding chemotherapy: implications for palliative care. Journal of Pain and Symptom Management 21 (2), 113–120.
Pertwee, R.G., 1972. The ring test: a quantitative method for assessing the 'cataleptic' effect of cannabis in mice. British Journal of Pharmacology 46 (4), 753–763.
Pertwee, R.G., Stevenson, L.A., Elrick, D.B., Mechoulam, R., Corbett, A.D., 1992. Inhibitory effects of certain enantiomeric cannabinoids in the mouse vas deferens and the myenteric plexus preparation of guinea-pig small intestine. British Journal of Pharmacology 105 (4), 980–984.
Polomano, R.C., Mannes, A.J., Clark, U.S., Bennett, G.J., 2001. A painful peripheral neuropathy in the rat produced by the chemotherapeutic drug, paclitaxel. Pain 94 (3), 293–304.
Savarese, D.M., Savy, G., Vahdat, L., Wischmeyer, P.E., Corey, B., 2003. Prevention of chemotherapy and radiation toxicity with glutamine. Cancer Treatment Reviews 29 (6), 501–513.
Schwartzberg, L.S., 2007. Chemotherapy-induced nausea and vomiting: clinician and patient perspectives. The Journal of Supportive Oncology 5 (2 Suppl 1), 5–12.
Stillman, M., Cata, J.P., 2006. Management of chemotherapy-induced peripheral neuropathy. Current Pain and Headache Report 10 (4), 279–287.
Takeda, N., Hasegawa, S., Morita, M., Matsunaga, T., 1993. Pica in rats is analogous to emesis: an animal model in emesis research. Pharmacology, Biochemistry and Behavior 45 (4), 817–821.
Takeda, N., Hasegawa, S., Morita, M., Horii, A., Uno, A., Yamatodani, A., Matsunaga, T., 1995a. Neuropharmacological mechanisms of emesis. II. Effects of antiemetic drugs on cisplatin-induced pica in rats. Methods and Findings in Experimental and Clinical Pharmacology 17 (10), 647–652.
Takeda, N., Hasegawa, S., Morita, M., Horii, A., Uno, A., Yamatodani, A., Matsunaga, T., 1995b. Neuropharmacological mechanisms of emesis. I. Effects of antiemetic drugs on motion-and apomorphine-induced pica in rats. Methods and Findings in Experimental and Clinical Pharmacology 17 (9), 589–590.
Tramer, M.R., Carroll, D., Campbell, F.A., Reynolds, D.J., Moore, R.A., McQuay, H.J., 2001. Cannabinoids for control of chemotherapy induced nausea and vomiting: quantitative systematic review. BMJ 323 (7303), 16–21.
Umapathi, T., Chaudhry, V., 2005. Toxic neuropathy. Current Opinion in Neurology 18 (5), 574–580.
Vahdat, L., Papadopoulos, K., Lange, D., Leuin, S., Kaufman, E., Donovan, D., Frederick, D., Bagiella, E., Tiersten, A., Nichols, G., Garrett, T., Savage, D., Antman, K., Hesdorffer, C.S., Balmaceda, C., 2001. Reduction of paclitaxel-induced peripheral neuropathy with glutamine. Clinical Cancer Research 7 (5), 1192–1197.
Van Cutsem, E., Arends, J., 2005. The causes and consequences of cancer-associated malnutrition. European Journal of Oncology Nursing 9 (Suppl 2), S51–S63.
Van der Stelt, M., Di Marzo, V., 2005. Cannabinoid receptors and their role in neuroprotection. Neuromolecular Medicine 7 (1–2), 37–50.

Van Sickle, M.D., Oland, L.D., Mackie, K., Davison, J.S., Sharkey, K.A., 2003. Delta9-tetrahydrocannabinol selectively acts on CB1 receptors in specific regions of dorsal vagal complex to inhibit emesis in ferrets. American Journal of Physiology: Gastrointestinal and Liver Physiology 285 (5), G566–G576.

Vera, G., Chiarlone, A., Martin, M.I., Abalo, R., 2006. Altered feeding behaviour induced by long-term cisplatin in rats. Autonomic Neuroscience: Basic & Clinical 126–127, 81–92.

Vickers, S.P., Kennett, G.A., 2005. Cannabinoids and the regulation of ingestive behaviour. Current Drug Targets 6 (2), 215–223.

Warner, E., 1995. Neurotoxicity of cisplatin and taxol. International Journal of Gynecological Cancer 5 (3), 161–169.

Yamamoto, K., Matsunaga, S., Matsui, M., Takeda, N., Yamatodani, A., 2002. Pica in mice as a new model for the study of emesis. Methods and Findings in Experimental and Clinical Pharmacology 24 (3), 135–138.

Yamamoto, K., Ngan, M.P., Takeda, N., Yamatodani, A., Rudd, J.A., 2004. Differential activity of drugs to induce emesis and pica behavior in *Suncus murinus* (house musk shrew) and rats. Physiology and Behavior 83 (1), 151–156.

4.3. Tercera publicación:

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4.3.1. Objetivo principal

Determinar el efecto de la administración aguda de cisplatino en la motilidad gastrointestinal (GI) mediante un método radiológico y compararlo con el desarrollo de pica.

4.3.2. Objetivos específicos

Estudiar:

- Las modificaciones en la temperatura, ingesta de comida y ganancia de peso corporal en los animales tratados con el cisplatino.
- Las alteraciones del vaciamiento gástrico y del tránsito GI, producidas por la administración aguda de cisplatino, mediante la obtención de imágenes radiográficas.
- La relación temporal entre la pica producida por la misma dosis de cisplatino y las alteraciones gastrointestinales observadas.

4.3.3. Resultados

El aislamiento afectó ligeramente a la ganancia de peso de los animales. La temperatura corporal de los animales aislados también descendió, pero sólo el primer día tras la administración de salino.

La administración de cisplatino produjo una reducción de la ganancia de peso corporal y la temperatura en las ratas aisladas, así como una reducción dosis dependiente de la ingesta de comida. La ingesta de caolín aumentó tras la administración de cisplatino. La pica aguda fue independiente de la dosis de cisplatino administrada, mientras que la retardada se vio aumentada sobre todo a la dosis más alta.

Con respecto al vaciamiento gástrico y al tránsito gastrointestinal, en el estudio radiológico se vio que el cisplatino redujo el vaciamiento gástrico. Pasadas 24 horas

de la administración aún podían verse restos del medio de contraste en el estómago. Este efecto era independiente de la dosis de cisplatino administrada. Cuando las radiografías se tomaron el segundo o tercer día tras la administración del fármaco en ratas tratadas con cisplatino 3 mg/kg el vaciamiento gástrico volvía a los valores control e incluso podía ser un poco más rápido que en las ratas tratadas con salino. Sin embargo, con cisplatino 6 mg/kg la curva del vaciamiento gástrico estaba más retrasada que en las ratas control, aunque al tercer día la diferencia no fue estadísticamente significativa.

La administración de cisplatino, sobre todo a la dosis de 6 mg/kg produjo un aumento significativo del tamaño del estómago que era evidente durante los 3 días.

El tránsito se modificó por el retraso del vaciamiento gástrico. Así durante el primer día de la administración, el intestino delgado se vació lentamente, el ciego se llenó más tarde que en las ratas control y los bolos fecales no eran frecuentes 6 horas después de la administración del fármaco. Además tanto el ciego como los bolos fecales se podían ver claramente a las 24 horas de la administración del fármaco. Como en el estómago, durante el segundo y tercer día, las ratas tratadas con cisplatino a 3 mg/kg recobraron la situación inicial. Sin embargo, las ratas tratadas con cisplatino 6 mg/kg no se recuperaron.

4.3.4. Conclusiones

El método radiológico desarrollado en nuestro laboratorio es útil para el estudio del vaciamiento gástrico y el tránsito gastrointestinal. Las alteraciones en la motilidad que ocasiona la administración de cisplatino, se relacionan con el aumento en la ingesta de caolín que se produce tras la administración del antineoplásico. La radiología podría ser un método alternativo y/o complementario a los estudios de pica para el estudio de los efectos de fármacos emetógenos y antieméticos, ya que permite la utilización de un número más reducido de animales, no requiere su aislamiento y la obtención de resultados es más rápida.

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Radiological study of gastrointestinal motor activity after acute cisplatin in the rat. Temporal relationship with pica

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Abstract

Nausea and vomiting are amongst the most severe dose-limiting side effects of chemotherapy. Emetogenic activity in rats can only be evaluated by indirect markers, such as pica (kaolin intake), or delay in gastric emptying. The aim of this work was to study, by radiological methods, the alterations in gastrointestinal motility induced by acute cisplatin in the rat, and to compare them with the development of pica. Rats received cisplatin (0–6 mg kg^{-1}) at day 0. In the pica study, individual food ingestion and kaolin intake were measured each day (from day −3 to day 3). In the radiological study, conscious rats received an intragastric dose of medium contrast 0, 24 or 48 h after cisplatin injection, and serial X-rays were taken 0–24 h after contrast. Cisplatin dose-dependently induced both gastric stasis and stomach distension, showing a strict temporal relationship with the induction of both acute and delayed pica. Radiological methods, which are non-invasive and preserve animals' welfare, are useful to study the effect of emetogenic drugs in the different gastrointestinal regions and might speed up the search for new anti-emetics.

Keywords: Anorexia; Chemotherapy; Gastrointestinal motility; Gastric emptying; Pica; Radiology; Rat

1. Introduction

Chemotherapy-induced adverse effects include anorexia, weight loss, nausea and emesis (Van Cutsem and Arends, 2005; Schwartzberg, 2007). To test the effect of emetogenic (and anti-emetic) drugs in rodents, which are the most convenient experimental animals in pre-clinical studies, indirect markers need to be used because the vomiting reflex is not present in these species. Thus, pica (an alteration of feeding behaviour consisting of the intake of non-nutritive substances, such as kaolin) is induced in rats by different emetogenic stimuli (Mitchell et al., 1976; Takeda et al., 1993, 1995a,b; Uno et al., 2000; Aung et al., 2004) and has been extensively used as an index of nausea (Mitchell et al., 1976; Takeda et al., 1993; Saeki et al., 2001; Rudd et al., 2002). As with nausea/emesis induced in humans by chemotherapy (Jordan et al., 2005), two phases can be distinguished: acute pica, which occurs in the 24 h after stimulus; and delayed pica, occurring 24 h at least after the start of chemotherapy. Accordingly, cisplatin, which is one of the most emetogenic drugs and used as reference in this kind of studies, induces both acute and delayed pica when acutely administered (Takeda et al., 1993; Saeki et al., 2001; Rudd et al., 2002).

Gastric dysrhythmia has been found to be closely correlated with reported nausea in a variety of conditions that produce it, including surgery, pregnancy and chemotherapy (Riezzo et al., 1992; Resnick et al., 1997a,b; DiBaise et al., 2001). Gastric stasis has also been demonstrated after

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administration of cisplatin and other antineoplastic drugs in laboratory animals (Jarve and Aggarwal, 1997) and used as an indirect marker of nausea in rats (Badary et al., 2006). Although essential in human gastroenterology, radiology has rarely been used to assay the effect of drugs on gastrointestinal motility in laboratory animals (Diani et al., 1979; Perry et al., 1993; Turiiski et al., 2004), and, to our knowledge, the few studies reported so far in the rat have been conducted using deep anaesthesia (Perry et al., 1993; Turiiski et al., 2004), which could also interfere with gastrointestinal motor function (Torjman et al., 2005).

Therefore, the present study was designed to determine, using radiological methods, the alterations in gastrointestinal motility induced by a single dose of cisplatin in conscious rats at different times after drug administration. In addition, a parallel study of cisplatin-induced pica was conducted so that both kinds of effects could be compared.

2. Materials and methods

The experiments in the present study, which were designed to minimize the number of animals used and their suffering, were performed in strict accordance with the EC regulation for care and use of experimental animals (EEC N° 86/609) and approved by the Ethical Committee at the Universidad Rey Juan Carlos.

2.1. Animals

Male Wistar rats (240–300 g) were obtained from Harlan-Iberica (Barcelona, Spain). Upon arrival to our laboratory, animals were housed, isolated (for the pica study) or grouped (4–6/cage, for the radiographic study), in standard transparent cages (40 cm × 28 cm × 25 cm), furnished with wood shaving bedding, which was changed every 1–2 days. Cages were placed adjacent to each other, in environmentally controlled conditions (temperature = 20 °C; humidity = 60%) with a 12 h light/12 h dark cycle (lights on between 08:00 and 20:00 h). Animals were allowed free access to standard laboratory rat chow (Harlan-Iberica, Barcelona, Spain) and tap water, which was refreshed every day.

2.2. Evaluation of feeding behaviour (anorexia and pica)

In these studies, rats were first habituated to isolation for at least five days, and then exposed to kaolin pellets (see below) for six additional days. Food and kaolin were placed in adjacent separate compartments in a divided food hopper and were continuously available throughout the experiment. Examination of the rats and measurement of weight, rectal temperature, and food and kaolin intake were undertaken each day between 9.00 and 11.00 am, as described elsewhere (Vera et al., 2006). After three days of adaptation to the presence of kaolin in their cages (day 0), rats received an intraperitoneal (IP) injection of either cisplatin (3 or 6 mg kg^{-1}) or vehicle (saline 0.9% w/v, 4–5 mL kg^{-1}). Kaolin and food were provided again immediately after drug administration. Higher doses of cisplatin were not tested because in a pilot study some rats treated with the drug at 6 mg/kg died on days 4 and 5 after administration (not published).

To avoid the effect of cisplatin-induced anorexia on pica, kaolin intake was normalised to food ingestion (Normalised kaolin intake = [Kaolin intake / Food ingestion] × 100). This allowed determining the proportion of kaolin that was ingested instead of food. In addition, due to the high variability of kaolin intake during the adaptation period (Vera et al., 2006), strict inclusion criteria, similar to those applied by other investigators (Davey and Biederman, 1998; Fujisaki et al., 2001), were applied so that a homogeneous group of animals could be used: 1 — during the three days of adaptation to kaolin, the total amount of kaolin eaten by the rat should be more than 0.2% of the total food ingested in the same days; 2 — on day 0 (for the 24 h before drug administration) pica should not exceed 4% of the food ingested that day. Using these criteria, a total of 24 out of 30 rats (80%) were finally included in the study.

2.3. Gastric emptying and gastrointestinal transit

These parameters were studied by radiological methods in grouped rats. At least one week after arrival to the laboratory, rats received an IP injection of saline or cisplatin (3 or 6 mg kg^{-1}). Immediately, 24 or 48 h after drug administration, 2.5 mL of a suspension of barium sulfate (Barigraf®, 2 g mL^{-1}, $t°$ = 22 °C) were administered per os. Plain facial radiographs of the gastrointestinal tract were performed using a Helident DS X-ray apparatus (Sirona, Spain; 60 kV, 7 mA) with a focus distance manually fixed to 50 ± 1 cm. Exposure time was adjusted to 0.06 s. Immobilisation of the rats in prone position was achieved by placing them inside hand-made transparent plastic tubes, which were adjusted to the size of the rat, so that they could not move, escape or turn around (after the first trial, most rats tended to enter the immobilisation device by themselves; in addition, in a pilot experiment designed to check the effect of adaptation to the immobilisation procedure, gastrointestinal motility was found to be identical in those rats trained to enter the tube and those that received no previous training). X-rays were recorded on Kodak Ektavision G film (15 × 30 cm) housed in a hand-made cassette provided with regular intensifying screen, at different times (immediately and 0.5, 1, 2, 4, 6 and 24 h: T0–T24) after administration of the contrast medium. The film cassette was located directly beneath the restraining tube. While taking the radiographs, the qualified investigator remained at least 2 m away from the X-ray source, where radioactivity while shooting was not different from environmental reading. Films were developed in a Kodak X-omat 2000 automatic processor. Analysis of the radiographs was performed by a trained investigator blind to the drug administered. Alterations in gastrointestinal motility were quantitatively evaluated in each radiograph by assigning a compounded value to each region of the gastrointestinal

Table 1
Evaluation of gastrointestinal motility

	Proportion of the organ labelled	Intensity of labelling	Profile of the organ*	Homogeneity of labelling in the organ*
0	No labeling			
0–1	<25%	Faint (shadow)	Not well-defined	Heterogeneous
2	25–50%	Light	Well-defined	Homogeneous
3	50–75%	Moderate	–	–
4	75–100%	Strong	–	–

Four gastrointestinal regions were evaluated: stomach, small intestine, caecum, colorectal region (faecal pellets). Each region was scored considering four parameters, to give a final compounded value from 0 to 12. Evaluation focused on the more intensely labelled area in each region. As an exemption, stomach and caecum were considered as a whole when profile and homogeneity (*) were evaluated.

tract, according to the score shown in Table 1. Qualitative differences in gastric and intestinal shape and size were also recorded. Small intestinal activity was qualitatively analysed by determining whether contractions indicative of peristalsis and/or segmentation were absent, weaker or stronger after treatment. In addition, the profiles of the stomachs were traced over transparencies, photocopied and scanned so that alterations in their size and barium content could be more accurately studied. Scanned profiles were analysed with the aid of an image analysis system (ImageJ 1.38 for Windows, National Institute of Health, USA, free software: http://rsb.info.nih.gov/ij/).

2.4. Kaolin, compounds and drugs

Kaolin pellets were prepared as previously described (Yamamoto et al., 2002; Vera et al., 2006). Briefly, pharmaceutical grade kaolin (hydrated aluminum silicate; 98.5%) was mixed with 0.5% carmine and 1% gum arabic in distilled water to form a thick paste. Pellets of the resulting kaolin mixture were shaped to resemble the dimensions of the rats' normal laboratory diet. The pellets were completely dried at room temperature for up to 48 h.

Barium sulfate (Barigraf® AD, Juste SAQF, Spain) was suspended in tap water and continuously hand-stirred until administration. Kaolin, carmine, gum arabic and cisplatin were purchased from Sigma-Aldrich (Spain). Cisplatin was dissolved in saline (sonicated for about 15 min). Saline/cisplatin volumes were adjusted to a maximum of 4–5 mL kg^{-1}.

2.5. Statistical analysis

Data are presented as the mean values ± SEM. Differences between groups were analysed using a two-way ANOVA followed by post hoc Bonferroni multiple comparison test. Values of $p<0.05$ were regarded as being significantly different.

3. Results

3.1. Effects of isolation on body weight gain and temperature

Body weight gain was slightly lower in isolated than in grouped rats, but cisplatin did not alter this difference any further (Fig. 1A; only the effect of the higher dose of cisplatin in grouped rats is shown for clarity). Temperature was slightly lower only on the first day after saline administration in isolated *vs* grouped rats (Fig. 1B).

3.2. Feeding behaviour (anorexia and pica)

Acute administration of cisplatin induced a significant reduction in body weight gain and temperature in isolated rats (Fig. 1), as well as a significant dose- and time-dependent reduction in food ingestion (Fig. 2A). Kaolin intake was also significantly increased by cisplatin. However, whilst acute pica was similar irrespective of the dose administered (Fig. 2B), delayed pica was more intense for

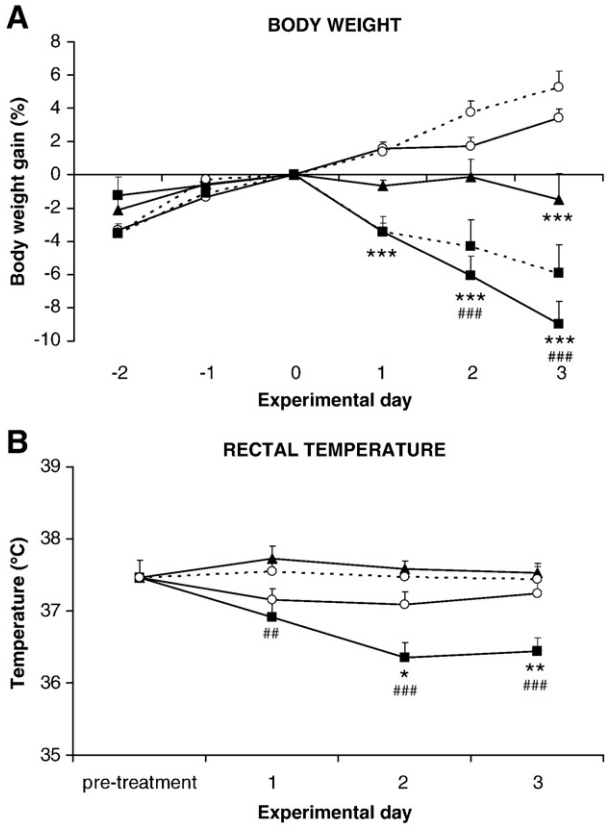

Fig. 1. Effect of cisplatin on body weight gain and temperature in the rat. Body weight gain (A) and rectal temperature (B) were measured in isolated (solid lines) or grouped (dotted lines) rats exposed to kaolin and injected at day 0 with: saline (4–5 mL kg^{-1}, IP, open circles) or cisplatin at 3 (closed triangles) or 6 mg kg^{-1} (closed squares). Data represent the mean ± s.e.mean. * ($p<0.05$), ** ($p<0.005$), *** ($p<0.001$) *vs* saline; ## ($p<0.01$), ### ($p<0.001$) *vs* cisplatin 3 mg kg^{-1} (two-way ANOVA followed by Bonferroni test). $n=4–8$ animals each group.

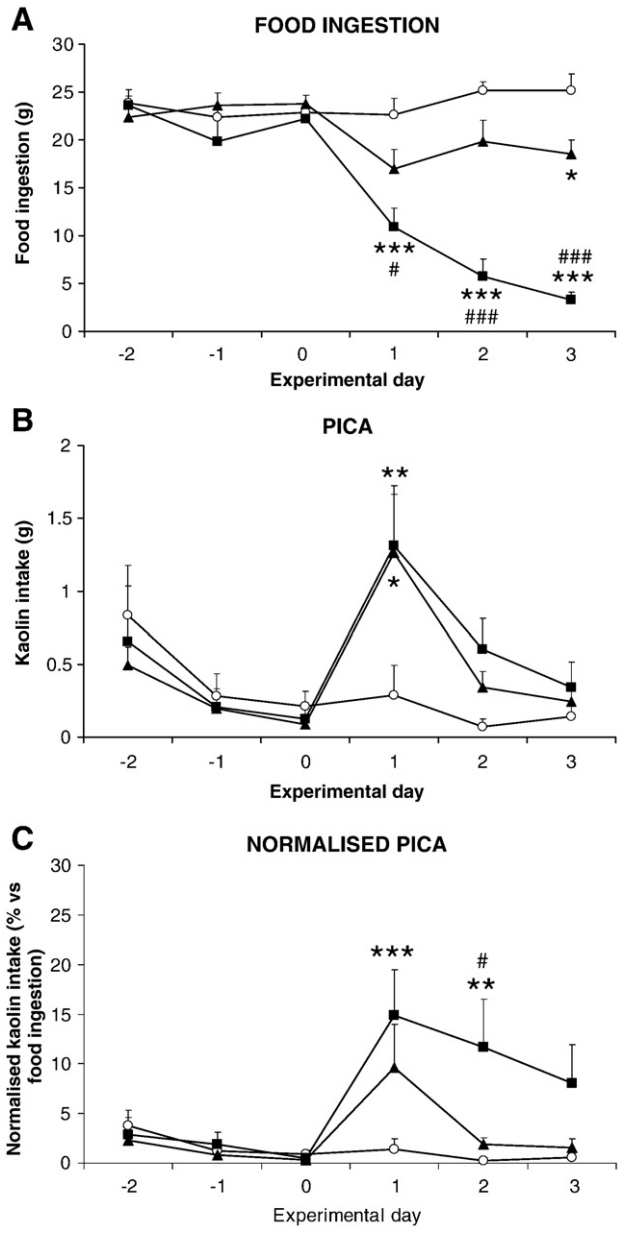

Fig. 2. Effect of cisplatin on feeding behaviour (anorexia and pica) in the rat. Food ingestion (A), kaolin intake (B) and kaolin intake, normalised to food ingestion (C), were measured in isolated rats exposed to kaolin and injected at day 0 with: saline (4–5 mL kg^{-1}, IP, open circles, $n=8$) or cisplatin at 3 (closed triangles, $n=8$) or 6 mg kg^{-1} (closed squares, $n=8$). Data represent the mean±s.e.mean. * ($p<0.05$), ** ($p<0.005$), *** ($p<0.001$) *vs* saline; # ($p<0.05$), ### ($p<0.001$) *vs* cisplatin 3 mg kg^{-1} (two-way ANOVA followed by Bonferroni test).

the higher dose, especially after normalisation to food ingestion (Fig. 2C).

3.3. Gastric emptying and intestinal transit

In a pilot radiological study ($n=8$) the effect of isolation in gastric emptying, with which pica was to be compared, was negligible. Therefore, the remaining radiological study was undertaken in grouped rats.

Since gastric emptying and intestinal transit were similar in saline-treated animals, irrespective of the day of administration, a mean motor function pattern (control) was obtained for each gastrointestinal region (Fig. 3A). These patterns were used as a control for the rest of the experiments. Gastric emptying started soon after administration of contrast (Fig. 3B) and was practically complete already 4 h after oral administration of barium (Fig. 3D). The small intestine started to fill already at T0 (Fig. 3B), but was better observed 30 to 120 min after contrast (Fig. 3C). The contrast medium reached the ceacum some time between 1 and 2 h after barium and completely filled this organ by T4 (Fig. 3D). Faecal pellets could be seen at T4 in some cases (Fig. 3D) but were much more abundant at T6. At T24 stomach, small intestine or ceacum were not apparent any more, whilst in some cases shadows of faecal pellets in colorectum could still be guessed (Fig. 3E).

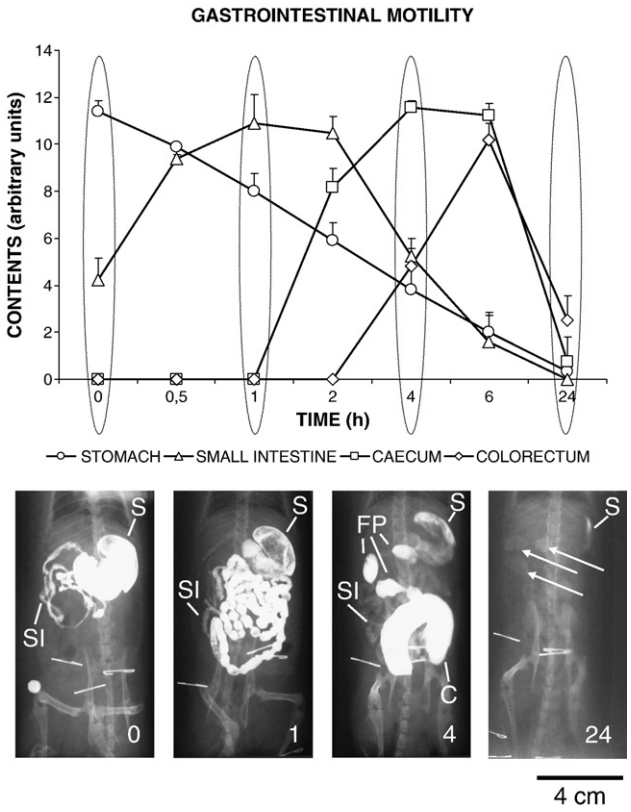

Fig. 3. Radiological analysis of gastrointestinal motor function in the rat. A dose of barium sulfate (2.5 mL, 2 g mL^{-1}) was intragastrically administered at time 0 and X-rays were taken immediately and 0.5, 1, 2, 4, 6 and 24 h after administration. Panel A: a characteristic motor function pattern for the stomach (open circles), small intestine (open triangles), caecum (open squares) and colorectum (open diamonds) was obtained from control rats which received an IP injection of saline immediately ($n=4$), 24 ($n=4$) or 48 ($n=4$) hours before barium administration; data represent the mean±s.e. mean. Panel B: representative X-rays obtained from a saline-treated rat at 0, 1, 4 and 24 h after administration of barium sulfate. At 24 h contrast and brightness were adjusted so that shadows of faecal pellets can be appreciated (arrows). S, stomach; SI, small intestine; C, caecum; FP, faecal pellets in the colorectum.

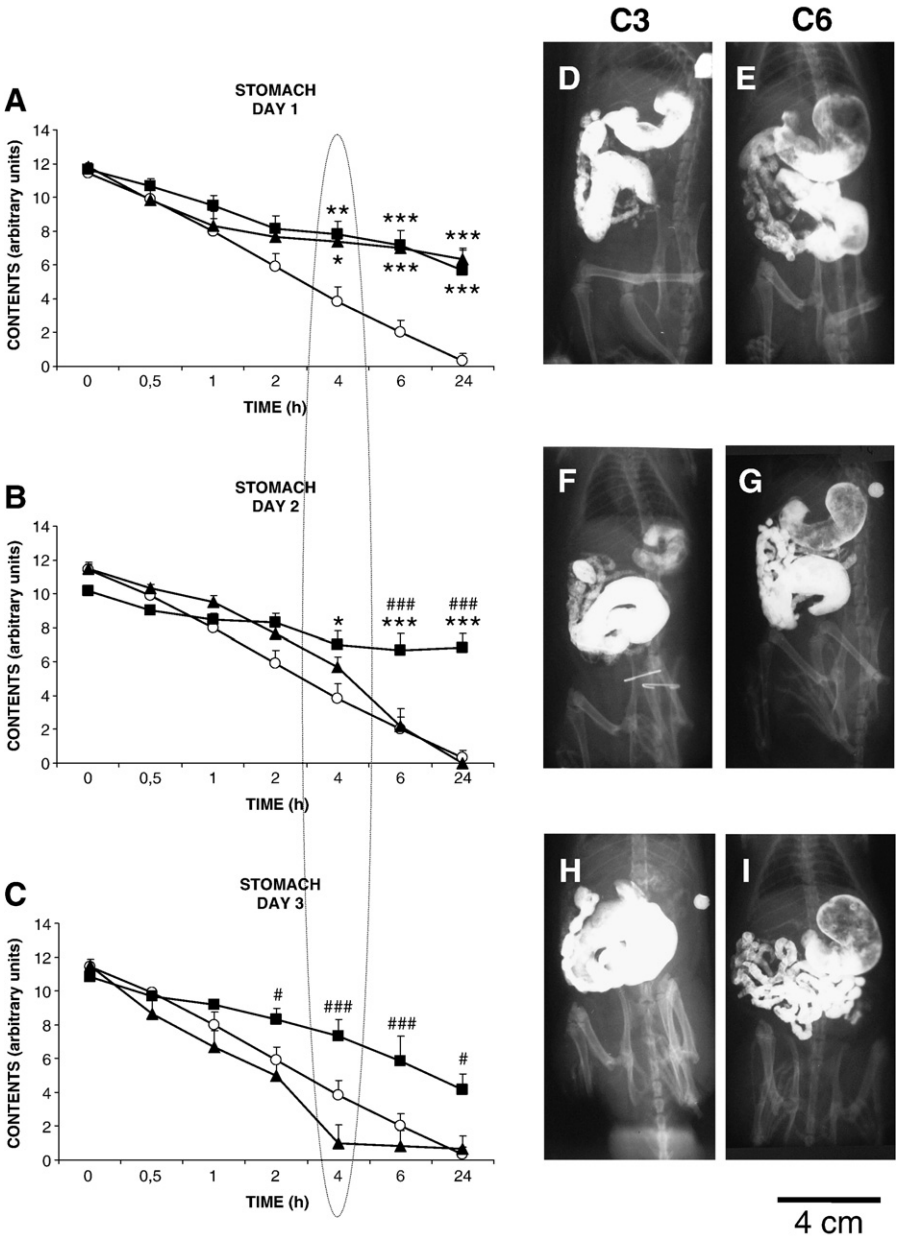

Fig. 4. Effect of cisplatin on gastric emptying in the rat. Barium sulfate (2.5 mL, 2 g mL^{-1}) was intragastrically administered immediately (A), 24 (C) or 48 (E) hours after saline (4–5 mL kg^{-1}, IP, open circles, $n=4$ each experiment) or cisplatin at 3 (closed triangles, $n=6$ each experiment) or 6 mg kg^{-1} (closed squares $n=6$ each experiment), and X-rays were taken 0, 0.5, 1, 2, 4, 6 and 24 h after barium administration. Gastric emptying was measured by radiological methods (see text). Data represent the mean±s.e.mean. * ($p<0.05$), ** ($p<0.005$), *** ($p<0.001$) *vs* saline; # ($p<0.05$), ### ($p<0.001$) *vs* cisplatin 3 mg kg^{-1} (two-way ANOVA followed by Bonferroni test). B, D, F: representative X-rays of rats treated with cisplatin at 3 (C3) or 6 mg kg^{-1} (C6), taken 4 h after barium administration, during the first (B), second (D) and third (F) days after cisplatin injection.

As expected, cisplatin induced a delay in gastric emptying that was significant at least from T4 (first day, Fig. 4A). The contrast medium could still be seen in the stomach at T24 and in some cases gastric profiles remained practically unchanged throughout the whole experiment (T0–T24). This effect was similar irrespective of the dose used during the first day after administration, but when radiographs were taken on the second (Fig. 4B) or the third (Fig. 4C) days after cisplatin, in rats treated with cisplatin at 3 mg kg^{-1} gastric emptying turned to control values and even tended to be a bit faster than in saline-treated rats by day 3 (Fig. 4C). In rats treated with cisplatin at 6 mg kg^{-1}, the curve for gastric emptying was always above that from control rats, although on day 3 the difference did not reach statistical significance (Fig. 4C). The analysis of the proportion of intensely labelled gastric area over the gastric profiles showed similar results (Fig. 5A, B, C).

Administration of cisplatin, especially at 6 mg kg^{-1}, also induced a significant increase in stomach size which was evident during the first, second and third days (Fig. 5A′, B′, C′). Even though barium was frequently scarce in the fore

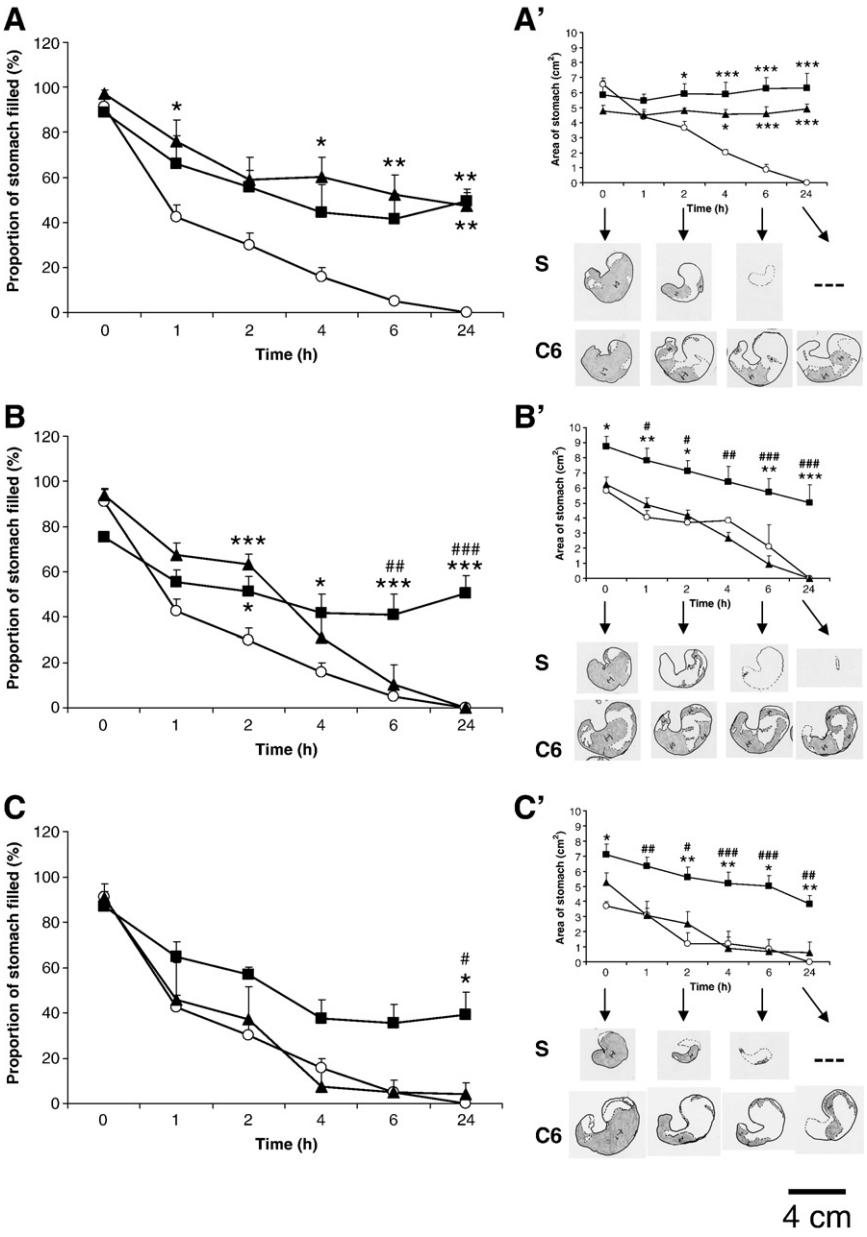

Fig. 5. Effect of cisplatin on gastric profile in the rat. Barium sulfate (2.5 mL, 2 g mL^{-1}) was intragastrically administered immediately (A, A′), 24 (B, B′) or 48 (C, C′) hours after saline (4–5 mL kg^{-1}, IP, open circles, $n=4$ each experiment) or cisplatin at 3 (closed triangles, $n=6$ each experiment) or 6 mg kg^{-1} (closed squares $n=6$ each experiment), and X-rays were taken 0, 1, 2, 4, 6 and 24 h after barium administration. Gastric profiles were traced, scanned and analysed with an image processor (NIH). A, B, and C show the temporal change in the proportion of stomach intensely labelled. A′, B′ and C′ show the temporal change in the size of the stomach and representative images obtained for animals treated with saline (S) or cisplatin at 6 mg kg^{-1} (C6), at 0, 2, 6 and 24 h after contrast (in these images, grey means "intensely labelled"). Data represent the mean±s.e.mean. * ($p<0.05$), ** ($p<0.005$), *** ($p<0.001$) *vs* saline; # ($p<0.05$), ## ($p<0.005$), ### ($p<0.001$) *vs* cisplatin 3 mg kg^{-1} (two-way ANOVA followed by Bonferroni test).

stomach, its profile could be clearly delimited in distended stomachs. In addition, persistent barium stains in the stomach of some of the rats treated with cisplatin, particularly at the highest dose, were also found, especially in radiographs taken during the second or the third days (Fig. 4E, G, I; 6E, G, I; 7E, G, I; 8E, G, I).

Transit of barium contrast in the small and large intestines was influenced by the delay in gastric emptying. Thus, during the first day after cisplatin administration, small intestine emptied more slowly (Fig. 6A), caecum was reached and filled later than in saline-treated rats (Fig. 7A), and faecal pellets were not as abundant at T6 (Fig 8A). Furthermore, both caecum and faecal pellets were clearly seen at T24 after administration of the drug (Fig. 8A, D, E). As in the case of the stomach, during the second and third days, rats treated with cisplatin at 3 mg kg^{-1} showed a recovery to control situation in motor function of small intestine (Fig. 6B, C), caecum (Fig. 7B, C) and colorectum (Fig. 8B, C). In rats

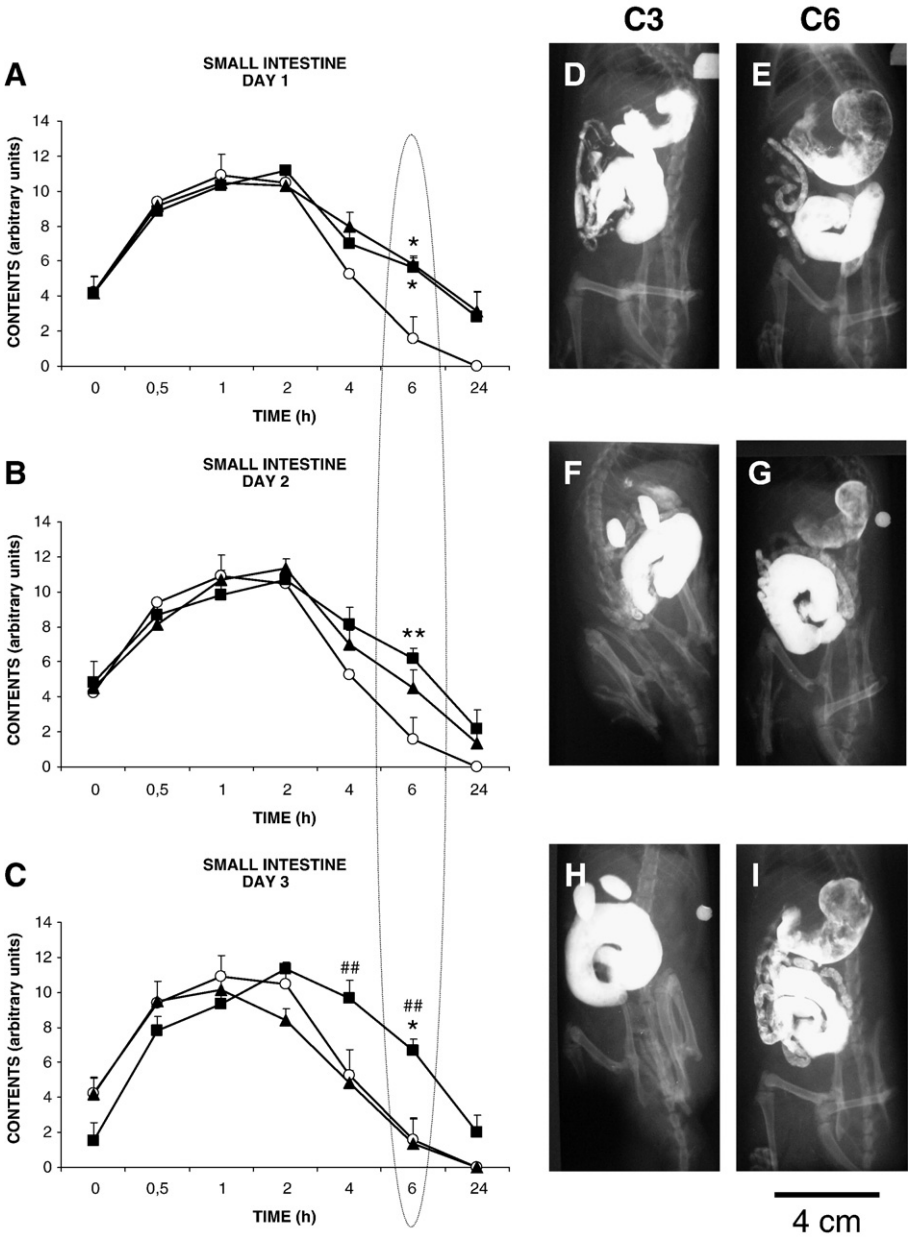

Fig. 6. Effect of cisplatin on motor function of small intestine in the rat. Barium sulfate (2.5 mL, 2 g mL^{-1}) was intragastrically administered immediately (A), 24 (C) or 48 (E) hours after saline (4–5 mL kg^{-1}, IP, open circles, $n=4$ each experiment) or cisplatin at 3 (closed triangles, $n=6$ each experiment) or 6 mg kg^{-1} (closed squares $n=6$ each experiment), and X-rays were taken 0, 0.5, 1, 2, 4, 6 and 24 h after barium administration. Motor function was measured by radiological methods (see text). Data represent the mean±s.e.mean. * ($p<0.05$), ** ($p<0.005$) *vs* saline; ## ($p<0.01$) *vs* cisplatin 3 mg kg^{-1} (two-way ANOVA followed by Bonferroni test). B, D, F: representative X-rays of rats treated with cisplatin at 3 (C3) or 6 mg kg^{-1} (C6), taken 6 h after barium administration, during the first (B), second (D) and third (F) days after cisplatin injection.

treated with cisplatin at 6 mg kg^{-1}, no recovery was found; furthermore, filling of caecum during the third day was even slower than during the first and second days (Fig. 7A–C).

Finally, peristalsis and segmentation were visualized in the mid to lower portion of the small intestine as clustered circular contractions of a "beading" appearance which were similar in all rats, although perhaps not as prominent in some cisplatin-treated animals as compared to saline-treated ones (Fig. 3C; 7D–I). No evident alteration was found in the caecum gross morphology which appeared as a large sac (Fig. 3D; 6D–I), and faecal pellets in colon and rectum were also similar irrespective of the treatment received by the rats (Fig. 3D; 6F, H; 8D–I).

4. Discussion

This work shows that cisplatin-induced alterations in gastric emptying, evaluated by non-invasive radiological methods, are temporally related to the induction of pica, a behavioural parameter traditionally used as an indirect marker of nausea

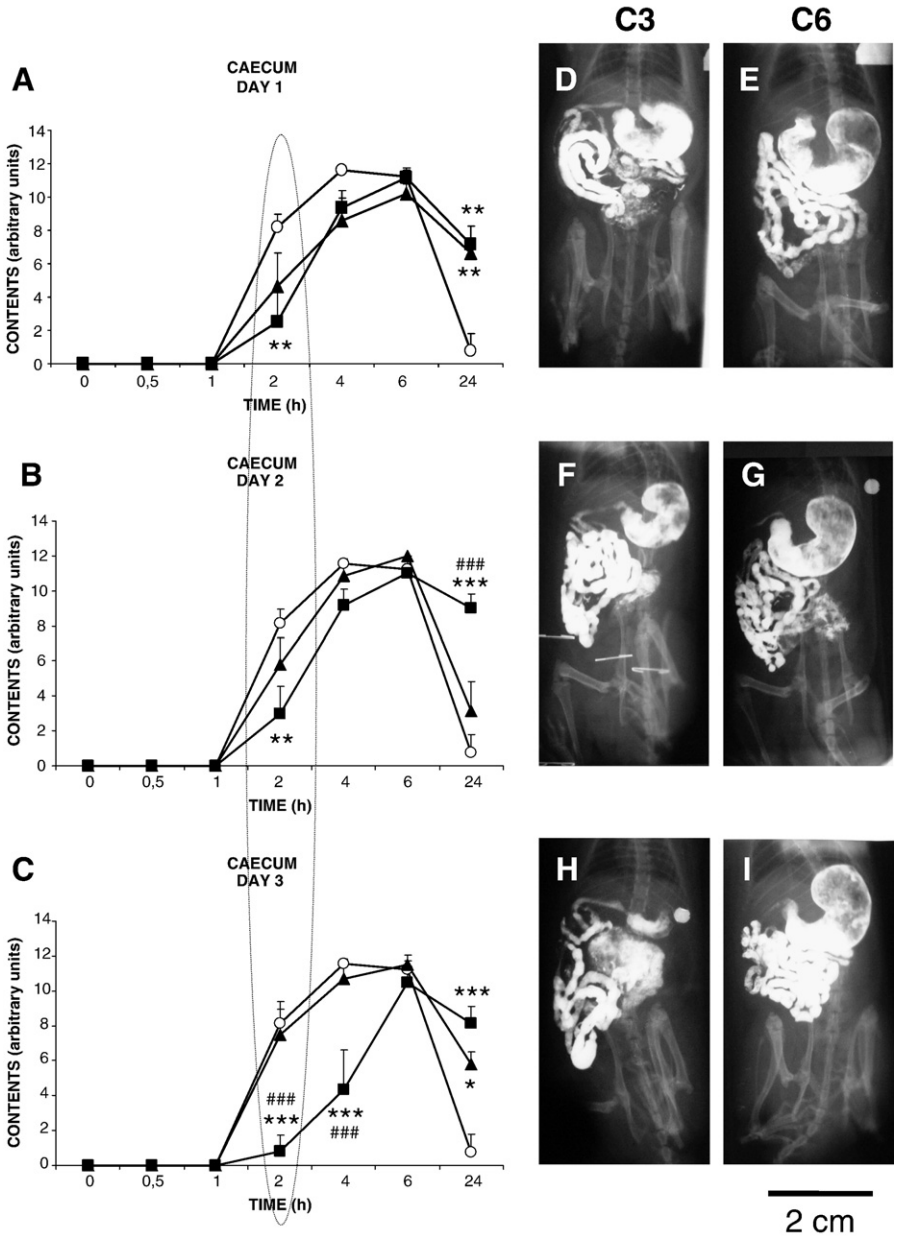

Fig. 7. Effect of cisplatin on motor function of the caecum in the rat. Barium sulfate (2.5 mL, 2 g mL^{-1}) was intragastrically administered immediately (A), 24 (C) or 48 (E) hours after saline (4–5 mL kg^{-1}, IP, open circles, $n=4$ each experiment) or cisplatin at 3 (closed triangles, $n=6$ each experiment) or 6 mg kg^{-1} (closed squares $n=6$ each experiment), and X-rays were taken 0, 0.5, 1, 2, 4, 6 and 24 h after barium administration. Motor function was measured by radiological methods (see text). Data represent the mean±s.e.mean. * ($p<0.05$), ** ($p<0.005$), *** ($p<0.001$) *vs* saline; ### ($p<0.001$) *vs* cisplatin 3 mg kg^{-1} (two-way ANOVA followed by Bonferroni test). B, D, F: representative X-rays of rats treated with cisplatin at 3 (C3) or 6 mg kg^{-1} (C6), taken 2 h after barium administration, during the first (B), second (D) and third day (F) after cisplatin injection.

and/or emesis in rodents, and supports that both methods may be useful and complementary to study the effect of emetogenic stimuli in the rat. Advantages and disadvantages associated to the use of these and other methods for the study of gastrointestinal motor activity are discussed.

4.1. Acute and delayed effects of cisplatin

As expected, cisplatin induced hypothermia, reduction in body weight gain (or even body weight loss) and anorexia in a dose-dependent manner. A number of previous reports have already described and dealt with these well-known effects (Authier et al., 2000; Rudd et al., 2002; Vera et al., 2006; Malik et al., 2006, 2007).

Together with these effects, both acute and delayed pica were induced. Our absolute values for pica were lower than those reported by others at the same doses (Rudd et al., 2002; Malik et al., 2006, 2007). It is likely that a differential sensitivity to cisplatin accounts for this discrepancy, but our results on food ingestion, body weight gain and temperature

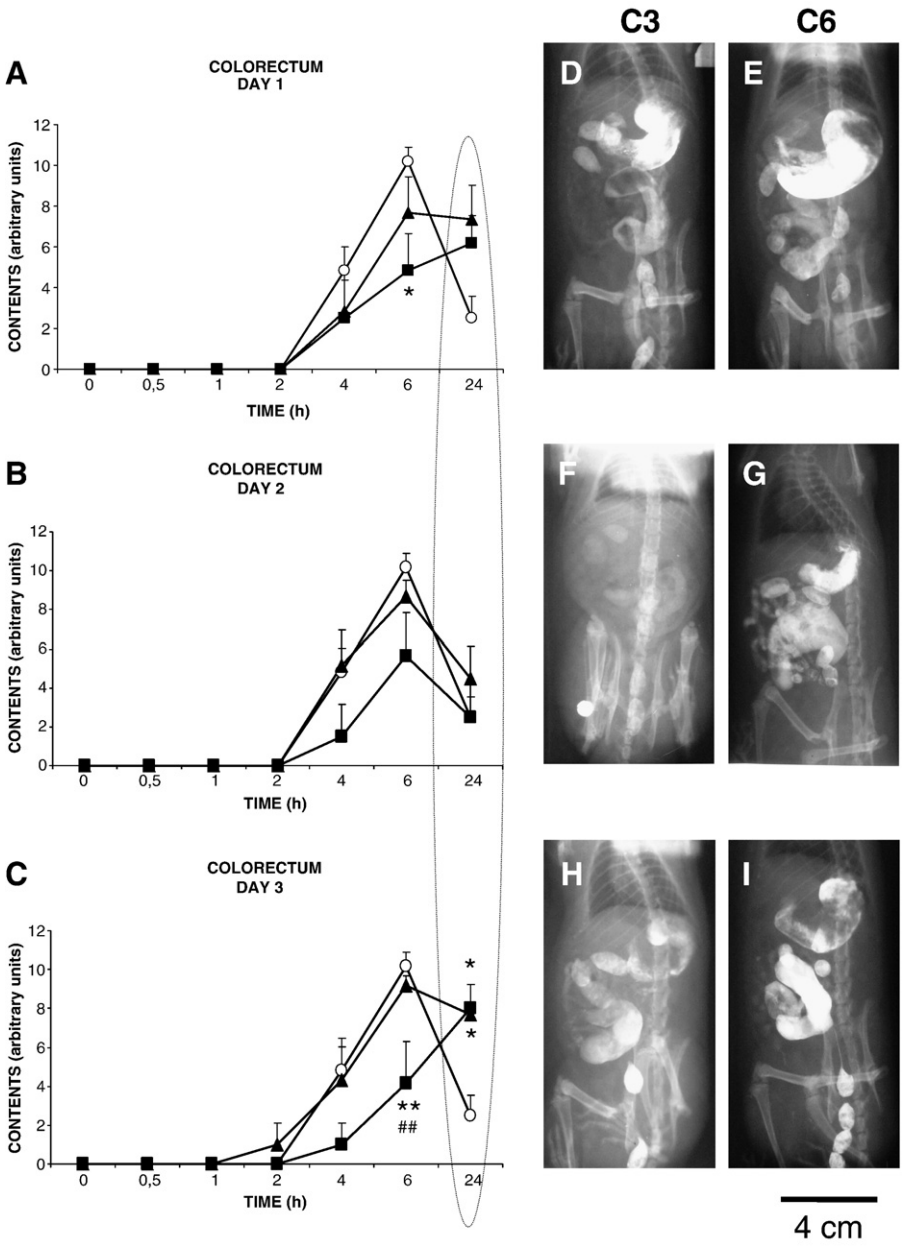

Fig. 8. Effect of cisplatin on motor function of the colorectum in the rat. Barium sulfate (2.5 mL, 2 g mL^{-1}) was intragastrically administered immediately (A), 24 (C) or 48 (E) hours after saline (4–5 mL kg^{-1}, IP, open circles, $n=4$ each experiment) or cisplatin at 3 (closed triangles, $n=6$ each experiment) or 6 mg kg^{-1} (closed squares $n=6$ each experiment), and X-rays were taken 0, 0.5, 1, 2, 4, 6 and 24 h after barium administration. Motor function was measured by radiological methods (see text). Data represent the mean±s.e.mean. * ($p<0.05$), ** ($p<0.005$) *vs* saline; ## ($p<0.01$) *vs* cisplatin 3 mg kg^{-1} (two-way ANOVA followed by Bonferroni test). B, D, F: representative X-rays of rats treated with cisplatin at 3 (C3) or 6 mg kg^{-1} (C6), 24 h after barium administration, during the first (B), second (D) and third day (F) after cisplatin injection.

suggest that our rats are sensitive enough to cisplatin. Other experimental factors, including the availability of wood shavings in the cages as a non-nutritive material, more easily accessible, could have had some influence. In fact, in many pica experiments wood shavings are removed from the cages and substituted by a grid floor; however, grid-floored cages have been shown to induce significant signs of stress (Heidbreder et al., 2000) and we decided not to use them in order to avoid this source of stress.

Dose-dependency in kaolin intake was clearly achieved only after normalisation of data to food ingestion, suggesting that anorexia could interfere with the induction of pica, maybe due to a concomitant decrease in locomotor activity and the development of chemotherapy-induced "fatigue", particularly after 24 h from cisplatin administration (delayed pica; Malik et al, 2006). Since both food and kaolin are given in pelleted form, a similar amount of effort is needed to ingest them, and normalisation allows determining how

much kaolin is eaten instead of food. In fact, in agreement with a previous report (Malik et al, 2007), which showed values for cisplatin-induced anorexia similar to ours, we recorded delayed pica during the second day after cisplatin at 6 mg/kg. When normalisation is applied to those data (Malik et al, 2007), delayed pica is even further increased. When it is applied to the data reported elsewhere (Rudd et al., 2002), delayed pica during the second day after cisplatin is also unmasked. Thus, normalisation to food ingestion (or even to body weight, Vera et al., 2006) in pica studies is an easy and reliable method to dilute experimental differences between laboratories.

In agreement with other studies (Hecht et al., 1997; Badary et al., 2006; Malik et al., 2007), cisplatin induced a delay in gastric emptying. Gastric emptying in the rats treated with the smallest dose recovered to control values throughout the second and third days, whereas that of the animals treated with the highest dose used remained delayed. These results showed a very good temporal relationship with those obtained in the pica experiments. Although pica and gastric emptying are measures of different effects of cisplatin, both have traditionally been used as markers of nausea in non-vomiting species (Mitchell et al., 1976; Takeda et al., 1993; Saeki et al., 2001; Rudd et al., 2002 for pica; Badary et al., 2006; Malik et al., 2007 for delay in gastric emptying) and our results confirm they are related and, probably, mediated by similar mechanisms (Ozaki and Sukamoto, 1999).

It is generally thought that nausea and vomiting are associated and could be at least partly due to gastric distension (Ladabaum et al., 1998). Stomachs from cisplatin-treated rats showed indeed a much more distended profile in the X-rays, especially for the highest dose, even during the second and the third days after administration, and an increased wet weight in stomach content was found in cisplatin-treated rats at least 2 days post-injection (Malik et al., 2007). Thus, gastric distension is associated to food retention (Aggarwal et al, 1994; Malik et al., 2007) and, probably, to gas accumulation, especially in the fore stomach, as has been recently shown (Malik et al., 2007) and is supported by the X-ray analysis (Fig. 5A′, B′, C′).

Our results confirm that a link exists between the different effects of cisplatin. It is likely that gastric dismotility (decrease in gastric emptying and induction of gastric distension) underlies the alterations in feeding behaviour (decrease in food ingestion, increase in kaolin intake), because these involve more complex tasks. In these regards, it is interesting to note that, in non-treated animals, anorectic signals originating from the stomach occur almost exclusively in response to volumetric distension of the organ (Phillips and Powley, 1996; Powley and Phillips, 2004). Remarkably, this "distention-induced anorexia" is mediated by both gastric and hepatic vagal branches (Phillips and Powley, 1998), whereas cisplatin-induced pica and anorexia involve only the common hepatic branch of the vagus (De Jonghe and Horn, 2008).

The effects of cisplatin in gastric emptying influenced transit of barium in both small and large intestine, and few direct effects could be appreciated in these regions of the gastrointestinal tract. However, on the third day after cisplatin administration, the cisplatin-induced delay in reaching the caecum was potentiated, suggesting a possible direct effect of cisplatin on motility of the small intestine. Cisplatin is known to generate free radicals in the intestine (Sangeetha et al., 1990), and this may lead to subsequent release of 5-HT (Matsuki et al., 1993), contributing to the delay in gastric emptying, maybe especially in the 48–72 h after administration (Endo et al., 2002). Interestingly, antioxidant compounds have shown efficacy to reduce cisplatin-induced pica in the rat (Mehendale et al., 2004), possibly involving a reduction in gastric distension and avoiding the delay in gastric emptying. However, free radicals could also be toxic to the circular muscle or the myenteric nerves (Hall and Wiley, 1998) in the small intestine and lead to reduced motility. Histopathological alterations of intestinal mucosa 72 h after cisplatin administration have been reported to occur in the rat (Endo et al., 2002), but the possible effects in the myenteric plexus, directly involved in intestinal motility, are unknown. The effects of cisplatin on intestinal motility deserve more investigation.

4.2. Radiological versus other methods to study emetogenic stimuli and their effects in the gastrointestinal tract

Our results suggest that both the radiological methods (and the analytical score) applied here, and the pica method are useful (and complementary) to indirectly study nausea/emesis in the rat. Some advantages of the radiological methods make them more useful, at least at the initial stages, to study the effects of emetogenic and/or anti-emetic stimuli. First, isolation, which may be to some extent stressful (Sharp et al., 2002), is not necessary. Second, it is possible to determine the effect of the drugs in the same animal at different time-points throughout the following 24 h (or even more) after administration of contrast medium, so that it is possible to identify the specific times at which the drug starts to work, works maximally or does not work any more. Third, it is possible to identify whether the drug acts in one or more gastrointestinal regions. Fourth, reproducibility is excellent and a very low number of animals is required (4–6 animals can be enough), whilst pica is quite variable and a relative big number of animals (8–12) is often needed to reach statistically significant effects. A last consideration would be that, as the pica method (Vera et al., 2006), radiology can also be applied to the same animal at different time-points throughout a long-term experiment (once each week, for example), just making sure that no contrast medium is present any more in the gastrointestinal tract before a new barium administration.

Radiological methods also offer some advantages over other approaches used to study gastric emptying and gastrointestinal transit. The most popular ones are marker dilution studies using different nonabsorbable dyes (Scarpignato et al., 1980; Depoortere et al., 2005) or radionuclides (Miller et al., 1981). The main advantage of radiological over dilution methods is that they are not invasive and allow studying the effects of a drug or any other stimulus at

different time-points in the same animal, thus making them cheaper and ethically more convenient. In addition, non-radiological methods, including the non-invasive ^{13}C-octanoic acid breath test (Schoonjans et al., 2002) involve the use of expensive instrumentation, radionuclides, and/or lengthy extraction/quantification steps. In contrast, both automatic developing and analysis of X-rays using the score proposed are very fast. Even in the small intestine (the gastrointestinal region more difficult to study with radiological methods) an objective parameter can be measured to determine total motility, i.e. "arrival to caecum" (Perry et al., 1993). Furthermore, alterations in morphology and/or size of the different gastrointestinal regions can be detected. Although magnetic resonance imaging could have been more useful to detect subtle changes in morphology and, probably, motility, it involves the use of relatively expensive and not easily accessible equipment, specialized MR physics knowledge and, very important, a relative high amount of time to acquire an image, which makes compulsory the use of anaesthesia, together with body temperature support and physiologic monitoring (Hildebrandt et al., 2008; Driehuys et al., 2008). Finally, rats are generally fasted for technical reasons when invasive methods are used. In this work, rats were not fasted, but still clear effects were obtained which tightly agreed with results on pica and previous reports (see above). Moreover, anatomic and functional features analysed in saline-treated animals closely resembled those previously described in rats fasted for 24 h (Perry et al., 1993), suggesting that fasting is not crucial for the radiographic study of gastrointestinal motility.

Lastly, the specific radiological methods used here offer some advantages over other radiological methods used in experimental animals (Perry et al., 1993; Turiiski et al., 2004). The main advantage is that there was no need for anaesthesia, which could interfere with gastrointestinal motility (Torjman et al., 2005), because the conscious animal was simply immobilised in a tube for a very short time (5 min approximately) whenever an X-ray was to be taken (Diani et al., 1979). Once the rats get adapted to entering the tube (after only 2–3 trials, the rats will enter the tube by themselves), no added stress interferes with the study. In addition, exposure time was reduced to 60 ms, further diminishing the possibility that spontaneous movements altered the image (Diani et al., 1979). Other improvements applied in this work relative to previous radiological studies (Turiiski et al., 2004) are the higher barium concentration, which allowed for a better visualisation of all gastrointestinal regions, and a more detailed score, which proved to be simple and provided a clear pattern of motor function in all gastrointestinal regions. Moreover, this score proved to be as accurate as quantitative analysis of scanned gastric profiles, further supporting its reliability.

In conclusion, radiological methods were applied for the first time to study the alterations in gastrointestinal motor function induced by acute cisplatin in the rat. The development of cisplatin-induced delay in gastric emptying closely coincided with the induction of pica, further supporting that both methods are useful and may be complementary to study the effect of emetogenic drugs in the gastrointestinal tract. The fact that radiographic techniques are not invasive and offer additional morphological information on the alterations in size and shape of the different GI regions may speed up the search for new pharmacological tools in the emesis field.

Acknowledgements

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References

Aggarwal, S.K., San Antonio, J.D., Sokhansanj, A., Miller, C., 1994. Cisplatin-induced peptic ulcers, vagotomy, adrenal and calcium modulation. Anticancer Drugs 5, 177–193.

Aung, H.H., Mehendale, S.R., Xie, J.T., Moss, J., Yuan, C.S., 2004. Methylnaltrexone prevents morphine-induced kaolin intake in the rat. Life Sci. 74, 2685–2691.

Authier, N., Fialip, J., Eschalier, A., Coudore, F., 2000. Assessment of allodynia and hyperalgesia after cisplatin administration to rats. Neurosci. Lett. 291, 73–76.

Badary, O.A., Awad, A.S., Sherief, M.A., Hamada, F.M., 2006. In vitro and in vivo effects of ferulic acid on gastrointestinal motility: inhibition of cisplatin-induced delay in gastric emptying in rats. World J. Gastroenterol. 12, 5363–5367.

Davey, V.A., Biederman, G.B., 1998. Conditioned antisickness: indirect evidence from rats and direct evidence from ferrets that conditioning alleviates drug-induced nausea and emesis. J. Exp. Psychol. Anim., Behav. Process. 24, 483–491.

De Jonghe, B.C., Horn, C.C., 2008. Chemotherapy-induced pica and anorexia are reduced by common hepatic branch vagotomy in the rat. Am. J. Physiol. Regul. Integr. Comp. Physiol. 294, R756–R765.

Depoortere, I., De Winter, B., Thijs, T., De Man, J., Pelckmans, P., Peeters, T., 2005. Comparison of the gastroprokinetic effects of ghrelin, GHRP-6 and motilin in rats in vivo and in vitro. Eur. J. Pharmacol. 515, 160–168.

Diani, A.R., Grogan, D.M., Yates, M.E., Risinger, D.L., Gerritsen, G.C., 1979. Radiologic abnormalities and autonomic neuropathology in the digestive tract of the ketonuric diabetic Chinese hamster. Diabetologia 17, 33–40.

DiBaise, J.K., Brand, R.E., Lyden, E., Tarantolo, S.R., Quigley, E.M., 2001. Gastric myoelectrical activity and its relationship to the development of nausea and vomiting after intensive chemotherapy and autologous stem cell transplantation. Am. J. Gastroenterol. 96, 2873–2881.

Driehuys, B., Nouls, J., Badea, A., Bucholz, E., Ghaghada, K., Petiet, A., Hedlund, L.W., 2008. Small animal imaging with magnetic resonance microscopy. ILAR J. 49, 35–53.

Endo, T., Hamaue, N., Ihira, E., Teramoto, Y., Liu, Y., Hirafuji, M., Minami, M., 2002. Effects of granisetron, a 5-HT3 receptor antagonist, on 5-hydroxytryptamine (5-HT) release from the isolated ileum in a delayed-emesis rat model. Res. Commun. Mol. Pathol. Pharmacol. 111, 55–68.

Fujisaki, Y., Yamauchi, A., Shuto, H., Niizeki, M., Makino, K., Kataoka, Y., Oishi, R., 2001. Pharmacological characterization of cyclosporine A-induced kaolin intake in rats. Pharmacol. Biochem. Behav. 70, 267–271.

Hall, K.E., Wiley, J.W., 1998. Neural injury, repair and adaptation in the GI tract. I. New insights into neuronal injury: a cautionary tale. Am. J. Physiol. 274, G978–G983.

Hecht, J.R., Lembo, T., Chap, L., 1997. Prolonged nausea and vomiting after high dose chemotherapy and autologous peripheral stem cell transplantation in the treatment of high risk breast carcinoma. Cancer 79, 1698–1702.

Heidbreder, C.A., Weiss, I.C., Domeney, A.M., Pryce, C., Homberg, J., Hedou, G., Feldon, J., Moran, M.C., Nelson, P., 2000. Behavioral, neurochemical and endocrinological characterization of the early social isolation syndrome. Neurosci. 100, 749–768.

Hildebrandt, I.J., Su, H., Weber, W.A., 2008. Anesthesia and other considerations for in vivo imaging of small animals. ILAR J. 49, 17–26.

Jarve, R.K., Aggarwal, S.K., 1997. Cisplatin-induced inhibition of the calcium–calmodulin complex, neuronal nitric oxide synthase activation and their role in stomach distension. Cancer Chemother. Pharmacol. 39, 341–348.

Jordan, K., Kasper, C., Schmoll, H., 2005. Chemotherapy-induced nausea and vomiting: current and new standards in the antiemetic prophylaxis and treatment. Eur. J. Cancer 41, 199–205.

Ladabaum, U., Koshy, S.S., Woods, M.L., Hooper, F.G., Owyang, C., Hasler, W. L., 1998. Differential symptomatic and electrogastrographic effects of distal and proximal human gastric distension. Am. J. Physiol. 275, G418–G424.

Malik, N.M., Moore, G.B., Smith, G., Liu, Y.L., Sanger, G.J., Andrews, P.L., 2006. Behavioural and hypothalamic molecular effects of the anti-cancer agent cisplatin in the rat: a model of chemotherapy-related malaise? Pharmacol. Biochem. Behav. 83, 9–20.

Malik, N.M., Liu, Y.L., Cole, N., Sanger, G.J., Andrews, P.L., 2007. Differential effects of dexamethasone, ondansetron and a tachykinin NK1 receptor antagonist (GR205171) on cisplatin-induced changes in behaviour, food intake, pica and gastric function in rats. Eur. J. Pharmacol. 555, 164–173.

Matsuki, N., Torii, Y., Kaji, T., Nishiyama, N., Ishiyama, J., Chida, N., Saito, H., 1993. Emetic responses of *Sorex unguiculatus*. Jikken Dobutsu 42, 225–228.

Mehendale, S.R., Aung, H.H., Yin, J.J., Lin, E., Fishbein, A., Wang, C.Z., Xie, J.T., Yuan, C.S., 2004. Effects of antioxidant herbs on chemotherapy-induced nausea and vomiting in a rat-pica model. Am. J. Chin. Med. 32, 897–905.

Miller, M.S., Galligan, J.J., Burks, T.F., 1981. Accurate measurement of intestinal transit in the rat. J. Pharmacol. Meth. 6, 211–217.

Mitchell, D., Wells, C., Hoch, N., Lind, K., Woods, S.C., Mitchell, L.K., 1976. Poison induced pica in rats. Physiol. Behav. 17, 691–697.

Ozaki, A., Sukamoto, T., 1999. Improvement of cisplatin-induced emesis and delayed gastric emptying by KB-R6933, a novel 5-HT3 receptor antagonist. Gen. Pharmacol. 33, 283–288.

Perry, R.L., Carrig, C.B., Williams, J.F., Johnson, C.A., Kaneene, J.B., 1993. Anatomic features and radiographic observations of gastric emptying and small intestinal motility in the rat. Lab. Anim. Sci. 43, 586–593.

Phillips, R.J., Powley, T.L., 1996. Gastric volume rather than nutrient content inhibits food intake. Am. J. Physiol. Regul. Integr. Comp. Physiol. 271, R766–R769.

Phillips, R.J., Powley, T.L., 1998. Gastric volume detection after selective vagotomies in rats. Am. J. Physiol. Regul. Integr. Comp. Physiol. 274, R1626–R1638.

Powley, T.L., Phillips, R.J., 2004. Gastric satiation is volumetric, intestinal satiation is nutritive. Physiol. Behav. 82, 69–74.

Resnick, J., Greenwald, D.A., Brandt, L.J., 1997a. Delayed gastric emptying and postoperative ileus after nongastric abdominal surgery: part I. Am. J. Gastroenterol. 92, 751–762.

Resnick, J., Greenwald, D.A., Brandt, L.J., 1997b. Delayed gastric emptying and postoperative ileus after nongastric abdominal surgery: part II. Am. J. Gastroenterol. 92, 934–940.

Riezzo, G., Pezzolla, F., Darconza, G., Giorgio, I., 1992. Gastric myoelectrical activity in the first trimester of pregnancy: a cutaneous electrogastrographic study. Am. J. Gastroenterol. 87, 702–707.

Rudd, J.A., Yamamoto, K., Yamatodani, A., Takeda, N., 2002. Differential action of ondansetron and dexamethasone to modify cisplatin-induced acute and delayed kaolin consumption ("pica") in rats. Eur. J. Pharmacol. 454, 47–52.

Saeki, M., Sakai, M., Saito, R., Kubota, H., Ariumi, H., Takano, Y., 2001. Effects of HSP-117, a novel tachykinin NK1-receptor antagonist, on cisplatin-induced pica as a new evaluation of delayed emesis in rats. Jpn. J. Pharmacol. 86, 359–362.

Sangeetha, P., Das, U.N., Koratkar, R., Suryaprabha, P., 1990. Increase in free radical generation and lipid peroxidation following chemotherapy in patients with cancer. Free Radic. Biol. Med. 8, 15–19.

Scarpignato, C., Capovilla, T., Bertaccini, G., 1980. Action of caerulein on gastric emptying of the conscious rat. Arch. Int. Pharmacodyn. Ther. 246, 286–294.

Schoonjans, R., Van Vlem, B., Van Heddeghem, N., Vandamme, W., Vanholder, R., Lameire, N., Lefebvre, R., De Vos, M., 2002. The 13C-octanoic acid breath test: validation of a new noninvasive method of measuring gastric emptying in rats. Neurogastroenterol. Motil. 14, 287–293.

Schwartzberg, L.S., 2007. Chemotherapy-induced nausea and vomiting: clinician and patient perspectives. J. Support. Oncol. 5, 5–12.

Sharp, J.L., Zammit, T.G., Azar, T.A., Lawson, D.M., 2002. Stress-like responses to common procedures in male rats housed alone or with other rats. Contemp. Top. Lab. Anim. Science 41, 8–14.

Takeda, N., Hasegawa, S., Morita, M., Matsunaga, T., 1993. Pica in rats is analogous to emesis: an animal model in emesis research. Pharmacol. Biochem. Behav. 45, 817–821.

Takeda, N., Hasegawa, S., Morita, M., Horii, A., Uno, A., Yamatodani, A., Matsunaga, T., 1995a. Neuropharmacological mechanisms of emesis. I. Effects of antiemetic drugs on motion and apomorphine-induced pica in rats. Methods Find. Exp. Clin. Pharmacol. 17, 589–590.

Takeda, N., Hasegawa, S., Morita, M., Horii, A., Uno, A., Yamatodani, A., Matsunaga, T., 1995b. Neuropharmacological mechanisms of emesis. II. Effects of antiemetic drugs on cisplatin-induced pica in rats. Methods Find. Exp. Clin. Pharmacol. 17, 647–652.

Torjman, M.C., Joseph, J.I., Munsick, C., Morishita, M., Grunwald, Z., 2005. Effects of isoflurane on gastrointestinal motility after brief exposure in rats. Int. J. Pharm. 294, 65–71.

Turiiski, V.I., Krustev, A.D., Sirakov, V.N., Getova, D.P., 2004. In vivo and in vitro study of the influence of the anticholinesterase drug galantamine on motor and evacuative functions of rat gastrointestinal tract. Eur. J. Pharmacol. 498, 233–239.

Uno, A., Takeda, N., Horii, A., Sakata, Y., Yamatodani, A., Kubo, T., 2000. Effects of amygdala or hippocampus lesion on hypergravity-induced motion sickness in rats. Acta Otolaryngol. 120, 860–865.

Van Cutsem, E., Arends, J., 2005. The causes and consequences of cancer-associated malnutrition. Eur. J. Oncol. Nurs. 9, S51–S63.

Vera, G., Chiarlone, A., Martin, M.I., Abalo, R., 2006. Altered feeding behaviour induced by long-term cisplatin in rats. Auton. Neurosci. 126–127 81-92.

Yamamoto, K., Matsunaga, S., Matsui, M., Takeda, N., Yamatodani, A., 2002. Pica in mice as a new model for the study of emesis. Methods Find. Exp. Clin. Pharmacol. 24, 135–138.

4.4. Cuarta publicación:

Gema Vera, Roberto De Giorgio, Anna Chiarlone, Pablo Antonio Cabezos, Mónica Castillo, Alessandra Gori, Gianandrea Pasquinelli, Giovanni Barbara, Ramón Fernández-Pujol, Vincenzo Stanghellini, María Isabel Martín and Raquel Abalo. ***Enteric neuropathy induced by chronic cisplatin in the rat.*** (Manuscrito en preparación)

4.4.1. Objetivo principal

Estudiar el desarrollo de neuropatía autonómica producida por la administración crónica de cisplatino en el tracto gastrointestinal (neuropatía entérica).

4.4.2. Objetivos específicos

Estudiar:

- Las modificaciones en la temperatura, ingesta de comida, ganancia de peso corporal y actividad locomotora en los animales tratados con cisplatino.
- Las alteraciones del tránsito gastrointestinal, producidas por la administración de cisplatino, mediante el método de carbón vegetal.
- La estructura y ultraestructura del tracto gastrointestinal de los animales tratados con cisplatino, así como la expresión de diversos marcadores neuronales (NOS, VIP, NPY, CGRP, SP y CR).

4.4.3. Resultados

El tratamiento crónico con cisplatino produjo una disminución del peso corporal, y la ingesta. También se desarrolló alodinia mecánica y un retraso en el tránsito gastrointestinal, sin que se produjera alteración de vaciamiento gástrico, una semana después de finalizar el tratamiento.

El estudio histológico y ultraestructural realizado reveló alteraciones en el tracto GI de los animales tratados con cisplatino. La proporción de neuronas mientéricas inmunorreactivas para NOS está aumentada. Las neuronas que expresan VIP y NOS, aunque tienen tendencia a estar aumentadas en los animales tratados con cisplatino, no alcanzan diferencias significativas. Las poblaciones neuronales que expresan CR y SP no se modifican tras un tratamiento crónico con cisplatino y la proporción de

neuronas que expresan CGRP disminuye, así como las fibras varicosas inmunorreactivas para este marcador.

4.4.4. Conclusiones

La administración de cisplatino, además de neuropatía periférica, produce alteraciones en la estructura de la pared gastrointestinal y del plexo mientérico, que podrían ser las responsables de las alteraciones persistentes en el tránsito gastrointestinal que hemos observado. Los resultados de este trabajo son de relevancia para el estudio y tratamiento de los efectos adversos que el cisplatino produce a nivel gastrointestinal.

Enteric neuropathy induced by chronic cisplatin in the rat.

Gema Vera, Roberto De Giorgio, Anna Chiarlone, Pablo Antonio Cabezos, Mónica Castillo, Alessandra Gori, Gianandrea Pasquinelli, Giovanni Barbara, Ramón Fernández-Pujol, Vincenzo Stanghellini, María Isabel Martín and Raquel Abalo.

1.- INTRODUCTION.

An autonomic neuropathy is a disorder associated to damage of the small, lightly myelinated and unmyelinated autonomic nerve fibres. Due to the extensive distribution of the autonomic nerves, autonomic neuropathies are characterised by a variable combination of signs and symptoms including impairment of cardiovascular, gastrointestinal, urogenital, thermoregulatory, sudomotor, and pupillomotor autonomic function. Although repercussion is generally mild or subclinical (Freeman, 2005), they could put susceptible patients' health to an increased risk.

Cisplatin is an antineoplastic drug widely used in the treatment of different cancers (testicular, ovarian, bladder, lung). It is a very toxic agent capable of inducing nephrotoxicity, nausea and emesis, anorexia and weight loss, ototoxicity and peripheral sensorial neuropathy. Some autonomic abnormalities have also been observed in patients treated with cisplatin (Boogerd *et al.,* 1990), but controversy arises from the very scarce experimental studies, which have not been able to identify enough evidences of cisplatin as an inductor of autonomic dysfunction (Vandertop *et al.,* 1996). However, those studies focused on the cardiovascular and sudomotor effects of cisplatin. To our

knowledge, no study has been conducted so far to ascertain whether cisplatin could induce signs of autonomic neuropathy in the gastrointestinal tract. Nevertheless, gastrointestinal symptoms are frequently encountered in other autonomic neuropathies (i.e., those induced by diabetes – Wegener *et al.,* 1990; vincristine – Legha, 1986; or acrylamide– Kucuk and Apostol, 1985; Belai and Burnstock, 1996). Furthermore, in these autonomic neuropathies, gastrointestinal functional alterations occur together with alterations in innervation of the gastrointestinal tract (Belai and Burnstock, 1996; De Giorgio *et al.,* 2004; Phillips *et al.,* 2006).

Therefore, the aim of the present work was to study the development of autonomic neuropathy induced by chronic cisplatin administration in the gastrointestinal tract (enteric neuropathy). To reach this aim, we studied the effects of cisplatin in both gastrointestinal transit (in vivo) and neurones and fibres of the myenteric plexus (immunohistochemistry). The study was conducted in a rat model of cisplatin-induced peripheral nociceptive neuropathy (Authier *et al.,* 2003), in which the effects of cisplatin in general health (Vera *et al.,* 2006) and the responses to mechanical non-painful stimulation (positive control) were also evaluated. Since peripheral nociceptive neuropathy is associated to primary damage of sensory neurones in the dorsal root ganglia (Barajon *et al.,* 1996), the immunohistochemical study included those myenteric neurones and structures related to sensory functions in the gastrointestinal tract, mainly those immunoreactive to calretinin (CR), or calcitonin gene-related peptide (CGRP), which are known to be present in the rat intrinsic and extrinsic primary afferents, respectively, Furness *et al.,* 2003). In addition, the neurones immunorreactive to nitric oxide synthase (NOS) were also evaluated, since they

constitute a significant subpopulation of motor (inhibitory) neurones in the gut (Costa *et al.,* 1996). Finally, neurones immunoreactive to substance P (SP), neuropeptide Y (NPY) and vasoactive intestinal peptide (VIP) were also studied, because their numbers are frequently altered in other models of enteric neuropathy (Belai and Burnstock, 1996; Belai *et al.,* 1996).

2.- METHODS.

The experiments were designed and performed in strict accordance with the EC regulations for care and use of experimental animals (EEC Nº 86/609) and were approved by the Ethical Committee at the Universidad Rey Juan Carlos.

2.1.- Animals.

Male Wistar rats (250-275 g) were obtained from Harlan-Iberica (Barcelona, Spain) and housed (4-6/cage) in standard transparent cages (60 x 40 x 20 cm) that were furnished with wood shaving bedding, which was changed every 1-2 days. Cages were placed adjacent to each other under environmentally controlled conditions (temperature = 20ºC; humidity = 60%) with a 12 h light/12 h dark cycle (lights on between 08:00 and 20:00 h). Animals had free access to standard laboratory rat chow (Harlan-Iberica) and tap water. Every day, water bottles were refreshed and food in each cage was topped up to 250 ± 1 g of chow pellets. Experiments started at least one week after arrival of animals to the laboratory.

2.2.- Experimental protocol.

During the first week, rats were habituated to the testing procedures and to daily handling by the investigator. This week of adaptation was considered as control and is termed as experimental week 0 (T_0) from now onwards. After this period

of adaptation, rats received one intraperitoneal injection once per week, on the first day of each experimental week (T_1-T_5), of either cisplatin (at 1 or 3 mg/kg; termed Cispt 1 or Cispt 3 from now onwards) or saline (0.9% w/v, 4-5 mL/kg). In order to prevent eventual nephrotoxicity induced by chronically administered cisplatin, 2 mL of saline were also injected subcutaneously just before intraperitoneal saline or cisplatin (Authier *et al.,* 2003). Water and chow pellets were provided again immediately after drug administration.

2.3.- Evaluation of overall health.

Examination of the rats and measurement of weight, rectal temperature, and food intake were performed from T_0 to T_5, on 5 consecutive days per week between 9.00 and 11.00 am. Cisplatin or saline were administered on the first observational day each week, immediately after completion of data recording. Core temperatures were measured using a P6 thermometer and a lubricated rectal probe (Cibertec, Spain) inserted into the rectum to a constant depth of 5 cm. Food consumption was measured every day by subtracting the amount remaining from the amount provided the day before, for each cage. Care was taken to collect all the remaining food particles (including that spilt outside the containers), which were weighed to correct the values of food consumption to the nearest 1 g.

Spontaneous locomotor activity was evaluated once a week, on the day after drug administration, using individual photocell activity chambers (Cibertec, Spain). Rats were placed in the recording chambers (55 x 40 cm, with a 3 cm spacing between beams), and starting 5 min later, the number of interruptions

of photocell beams was recorded over a 30-min period. The mean number of crossings of the photocell beams was used for comparison.

2.4.- Evaluation of peripheral nociceptive neuropathy.

The development of peripheral nociceptive neuropathy was evaluated by measuring mechanical allodynia, as one of the characteristic nociceptive signs induced by chronic cisplatin (Authier *et al.,* 2003). Mechanical allodynia was assessed on the last observational day of every experimental week (T_0 to T_5). On those days, rats were placed individually on an elevated iron mesh in a clear plastic cage and were allowed to adapt to the testing environment for at least 10 minutes. Habituation to this environment was also performed on the two days before assessment. Calibrated von Frey hairs ranging from 0.9 to 40 g (0.9, 1.4, 2.1, 2.5, 3, 4, 5.5, 7.5, 8, 10.5, 13, 14, 15, 17, 25, 27, 32 and 40 g) were applied to the plantar aspect of each hindpaw, from below the mesh floor. Each stimulus was applied for a maximum duration of approximately 2 s, this was repeated five times with 1-3 s intervals. Only robust and immediate withdrawal responses to the stimulus were recorded. A positive result was considered when at least 3 out of 5 responses were obtained (60%) with each filament, and this value was considered as the tactile threshold. If less than 3 positive responses were noted to any hair trial, the process was repeated with the next higher force hair. Mechanical allodynia was defined as a significant decrease in von Frey hair threshold evoked by mechanical stimuli. The 40 g hair was selected as the upper cut-off limit for testing.

2.5.- Evaluation of enteric neuropathy

2.5.1.- Functional studies: gastrointestinal transit and gastric emptying

Gastrointestinal transit was measured on the sixth experimental week (T_6), that is, one week after last cisplatin administration, using the charcoal meal test (Pascual *et al.,* 2003). Briefly, rats were fasted (but had free access to water) for 24 h, and received 1 mL of a 10% (w/v) charcoal suspension in a 5% (w/v) gum arabic solution via an orogastric cannula. Twenty minutes after receiving the charcoal meal, the rats were euthanized by cervical dislocation and exsanguination and the whole intestine was removed *en bloc*. Small bowel transit was determined for each rat by calculating the ratio between the distance travelled by the charcoal meal and the total length of the small bowel.

Since acute cisplatin administration slows down gastric emptying (Sharma and Gupta, 1998; Ozaki and Sukamoto, 1999; Malik *et al.,* 2007; Cabezos *et al.,* 2008), this parameter might also be altered due to chronic administration of the drug and account, at least partially, for the alterations in gastrointestinal transit. Therefore, gastric emptying was analysed three times throughout the experiment: after the first (T_1: acute effect) and last administrations (T_5: chronic effect) and one week after treatment finalization (T_6: residual effect). Radiological non-invasive methods were applied as recently described (Cabezos *et al.,* 2008). Briefly, 2.5 mL of a suspension of barium sulphate (Barigraf®, 2 g/mL, tº=22ºC) was orally administered and serial X-rays were taken immediately and 1, 2, 4, 6 and 8 hours after contrast. Gastric emptying was semiquantitatively determined from the images by assigning a compounded value to the stomach, from 0 to 12 points, considering the

following parameters: percentage of the stomach filled by contrast (0-4); intensity of contrast (0-4); homogeneity of contrast (0-2); sharpness of the gastric profile (0-2).

2.5.2.- Morphological studies in whole-gut sections.

The morphological study was carried out in the small and large intestines. For each region (ileum, proximal colon, distal colon and rectum), two small whole-gut samples (up to 5 mm each: ileum, 10 cm orally from caecum; proximal colon, immediately adjacent to caecum; distal colon, 4 cm aborally from caecum; rectum, immediately adjacent to anus) were taken for these studies. One was fixed in buffered 10% formalin, paraffin-embedded, cut at 4-µm-thick sections with a microtome and routinely processed either for hematoxylin-eosin staining (H&E) or immunohistochemistry.

Histological damage was evaluated in sections processed for H&E. It was determined using criteria adapted from Galeazzi *et al.* (1999). Briefly, the histological damage score consisted of loss of mucosal architecture (graded 0-3, for absent to severe), the extent of inflammatory cell infiltrate (graded 0-3, for absent to transmural) and the absence or presence of epithelialitis and crypt abscess formation (0-1), goblet cell depletion (0-1), muscular layer thickness (0-1, normal to reduced). Thus, for each section a numerical score of 0-9 was assigned.

The number of cells per ganglia was evaluated in the myenteric plexus using immunohistochemistry. Microtome sections were placed on gelatin-coated slides, and the paraffin was removed by xylene. The sections were then rehydrated through graded ethanols, and finally rinsed in 0.1 M phosphate-

buffered saline (PBS, pH 7.4). Endogenous peroxidase activity was blocked by incubating sections with 1% H_2O_2 for 10 min. To identify neurones, randomly selected sections were incubated with primary monoclonal antibody anti-NSE (Neuronal Specific Enolase, Dako, Glostrup, Denmark) overnight at 4°C. The sections were washed in PBS and incubated with biotinylated secondary antibodies (anti-rabbit, 1:200 Dakopatts Glostrup, Denmark) for 30 minutes at room temperature. The immunologic reaction was revealed through avidin-biotin complex and visualized by diaminobenzidine. The sections were finally dehydrated in graded ethanols, cleared in xylene, and cover slipped with Permount. Counting was performed using a transmission light Olympus AX70 microscope and quantitatively assessed by an independent, double-blinded observer. Only those sections where the outer muscle layer was circumferentially intact were considered. Myenteric plexus ganglia were identified as discrete and encapsulated aggregates of NSE-positive cells, located between the inner circular and outer longitudinal muscle layers.

The other biopsy specimens of each region were processed for electron microscopy. The whole-gut samples were fixed in cacodylate-buffered 2.5% glutaraldehyde for 4 to 6 hours at 4°C and postfixed in 1% osmium tetroxide for 1 hour at 4°C. After dehydration and embedding in araldite, semithin sections were stained with toluidine blue and examined under a light microscope. A pathologist (Dr Pasquinelli) performed a blind analysis of all sections. Representative portions of the myenteric plexus (i.e., absence of artifacts) were selected for ultrathin sectioning. Thin sections were collected on 200 mesh grids, stained with uranyl acetate and lead citrate, and observed with a Philips 410 T transmission electron microscope.

2.5.3.- Immunohistochemistry of myenteric neurones and fibres in whole-mount preparations

Conventional methods for immunohistochemistry of whole-mount intestinal preparations for analysis of the myenteric plexus were used (Costa *et al.,* 1986). These experiments were performed using samples of the distal colon. One sample of 2 cm length was obtained from the distal colon of each rat, 4 cm aborally from ceacum.The segments were introduced in Krebs solution (mM: NaCl; 118, KCl; 4.75, NaH_2PO4; 1.0, $NaHCO_3$; 25, $MgSO_4$; 1.2, $CaCl_2$; 2.5, glucose; 11, pH 7.4) bubbled with carbogen (95%O_2/5%CO_2). The samples were opened along the mesenteric border, rinsed, stretchted maximally and pinned flat on a Sylgard-coated Petri-dish filled with Krebs solution. After conventional fixation and clearing (Brookes *et al.,* 1991; Abalo *et al.,* 2005), mucosa, submucosa and circular muscle were carefully removed with fine forceps. Some preparations were cut into two pieces so that several markers could be tested. These whole-mount preparations were stored at 4ºC in PBS with sodium azide (0.1%), until their immunohistochemical processing.

Each preparation was incubated (36 hours at room temperature, RT) with both the pan-neuronal marker HuC/D (mouse streptavidin-conjugated anti-HuC/D, 1:1000, Molecular Probes), and at least one of the markers shown in table 1. After washing with PBS (3x10 min), tissues were exposed for at least 3 hours (at RT) to both avidin-AlexaFluor 488 (1:1000, Molecular Probes) and the corresponding secondary antibody (table 1). All mixtures of antibodies were diluted with hypertonic PBS (1.7% NaCl) (Costa *et al.,* 1996).

The preparations were observed under a fluorescent microscope (Nikon Eclipse TE2000-U, with appropriate filters). Fluorescently labelled, double-stained whole-mount preparations were analysed by eye, by an experimenter blind to the treatment received by the rat from which the tissue originated. For each staining combination, four to six whole-mount preparations of the myenteric plexus, from different animals, were used. The proportion of neurones labelled with each marker per 100 HuC/D-labelled neurones was evaluated. At least 400 HuC/D-labelled neurones were counted per preparation. The proportions of neurochemically identified populations were then expressed relative to the number of HuC/D -IR neurones, which was considered to represent 100% of the enteric neurones. In other studies, it was considered that the myenteric neurones stained with the general marker HuC/D was effective in labelling all neurones or a large proportion of the neuronal population in the rat (Phillips *et al.,* 2004). In addition, a semiquantitative analysis of density of smooth and varicose CGRP-immunoreactive (IR) fibres was performed, by assigning different values to visually different densities: 0-non-existing; 1-low; 2-normal; 3-high.

Controls for the immunohistochemical analysis were performed by pairing the wrong primary and secondary antibodies or by avoiding exposure to the primary antibody. No specific labelling was observed in either case.

2.6.- Statistical analysis

Data are presented as the mean values ± SEM. Mean values are representative for each week of the experiment, from week 0 (T_0) to week 5 (T_5). For a more accurate comparison, changes in body weight are shown as percentage of

change *versus* the average obtained for T_0. Since rats were grouped, food consumption represented the combined data from 4-6 animals and individually recorded weight served as an indirect indicator of individual food consumption (a similar weight gain in all grouped rats was assumed to be indicative of similar food consumption, see O'Connor *et al.,* 2000). Differences were analysed using student's *t*-test, with Welch's correction where appropriate, or Two-Way ANOVA followed by *post hoc* Bonferroni multiple comparison test. Values of $p<0.05$ were regarded as being significantly different.

3.- Results

3.1.- *Overall health.*

At the beginning of the experiment, the mean weight was 254.59 ± 13.63 g. In the control group, body weight increased approximately by 40% from T_0 to T_5. In contrast, cisplatin dose-depently reduced body weight gain and, at the highest dose, body weight decreased to values even lower than dose recorded at the beginning of the experiment (Fig. 1A). Daily food ingestion during T_0 was 22.08 ± 0.94 g and did not significantly change for saline-treated rats throughout the experiment. As for weight gain, cisplatin reduced food ingestion, but at the end of the treatment, differences were statistically significant only in rats treated with Cispt 3 (Fig. 1B).

As compared to saline, Cispt 3 but not 1 induced a reduction in rectal temperature which was significant already from the second week (Fig. 2A). Spontaneous locomotor activity was also reduced in rats treated with Cispt 3 as compared to animals treated with saline or Cispt 1 (Fig. 2B).

Additional clinical signs of toxicity in cisplatin-injected rats were evident from the third week: difficulties in handling, aggressiveness, piloerection, vocalisation whilst being handled and, occasionally, diarrhoeas.

3.2.- *Peripheral nociceptive neuropathy*

At the beginning of the experiment (T_0), the threshold for mechanical allodynia was 18,07 ± 0,88 g. Along the experiment, the threshold for mechanical allodynia did not change in the saline-treated group. However, cisplatin weekly administered at both doses (1 and 3 mg/kg) induced a significant reduction in the withdrawal threshold compared to saline, already from the first administration (Fig. 3A).

3.3.- Evaluation of enteric neuropathy

3.3.1.- Gastrointestinal transit and gastric emptying

In comparison with saline-treated rats, cisplatin induced a dose-dependent decrease in upper gastrointestinal transit, measured as the relative distance travelled by charcoal in the gut (Fig. 3B).

As shown in figure 4, gastric emptying was time-dependently delayed (Fig. 4 A, B, D-G) whenever cisplatin was administered, particularly at the highest dose. However, one week after treatment finalization, gastric emptying in cisplatin-treated rats (Fig. 4C, H, I) was not different from control, suggesting that the reduction in small intestinal transit described above is not due to any residual delayed gastric emptying. Thus, the remaining histological and immunohistochemical studies were performed in the intestine, where motor function alterations were more persistent.

3.3.2.- Morphological studies in whole-gut preparations.

Figure 5 shows the histological damage in H&E-stained sections of small and large intestines. Histological damage was found in the large intestine but not in the ileum of rats treated with Cispt 1. When cisplatin was given at a higher dose (3 mg/kg), statistical significance was reached in all regions examined. Figures 5E and 5F show representative images of distal colon since in this region cisplatin exerted the clearest damaging effect (both in terms of dose-dependency and intensity).

In the large intestine, paraffin-embedded cross-sections of cisplatin-treated rats immunostained for NSE had a significantly reduced number of myenteric neurones per ganglion relative to saline-treated rats (Fig. 6A-D). There was no significant change in the ileum. Figures 6E and 6F show representative images of distal colon since in this region cisplatin exerted the clearest effect (at 3 mg/kg).

The ultrastructural study of preparations from distal colon and rectum confirmed the histological results. The analysis showed vacuolated ganglia and altered nervous terminal endings in tissues from cisplatin-treated animals. Some alterations in smooth muscle cells were also found: atrophic muscular cells, altered microfilaments… Eosynophil infiltration was also found in one preparation (Fig. 7).

3.3.3.- Immunohistochemistry of myenteric neurones and fibres in whole-mount preparations.

Since the number of myenteric neurones per ganglion in the large intestine was reduced in cisplatin-treated animals, we focused in this region to perform a more accurate analysis. We concentrated on the distal colon, which was the region more easily accessible to obtain whole-mount preparations and in which cisplatin-induced histological changes were more intense.

In preparations from Cispt 3-treated rats, there was an increase in the proportion of NOS-IR (Fig 8A) neurones. The proportion of VIP- AND NPY-IR neurones (Fig 8B, 8C) remained unchanged in rats treated with the drug at either dose.

Chronic cisplatin did not alter the proportion of CR-IR intrinsic primary afferent neurones (that had Dogiel type II morphology) neither SP-IR neurones (Fig 9), but, at the highest dose, reduced that of CGRP-IR neurones in the myenteric plexus of the rat distal colon (Fig 10B).

Density of CGRP-IR smooth fibers remained unaltered, whereas that of CGRP-IR varicose fibers decreased (Fig 10 C, D).

4.- Discussion.

In agreement with previous reports from our laboratory (Vera *et al.,* 2006, 2007; Cabezos *et al.,* 2008) and other's (Rudd *et al.,* 2002; Authier *et al.,* 2003; Malik *et al.,* 2007), cisplatin induced a decrease in body weight gain, food intake, rectal temperature, spontaneous locomotor activity and nociceptive tactile threshold (mechanical allodynia). In addition, our functional (decreased

intestinal transit one week after treatment in the absence of significant changes in gastric emptying), morphological (histological damage, decreased number of myenteric neurones, vacuolated ganglia, atrophic muscular cells...) and immunohistochemical (increased and reduced proportions of NOS- and CGRP-IR neurones, respectively) results in the gastrointestinal tract suggest that cisplatin could induce an enteric neuropathy in the rat.

It is known that cisplatin causes profound gastrointestinal symptomatology (Tally *et al.,* 1973; Hill *et al.,* 1975; Von Hoff *et al.,* 1979) and chemotherapy-induced GI toxicity is often a major cause of morbidity (Magne *et al.,* 2001).The effects induced in the few hours/days following cisplatin administration are well known, but the occurrence of gastrointestinal permanent sequelae after chronic treatment has received much less attention. Thus, acute cisplatin administration slows down gastric emptying and induces distension of the stomach (Sharma and Gupta, 1998; Ozaki and Sukamoto, 1999; Badary *et al.,* 2006; Malik *et al.,* 2007; Cabezos *et al.,* 2008). Chronic treatment also resulted in delayed gastric emptying, but only immediately after administration (Figure 4). This effect was not persistent, and one week after treatment finalization there were no residual alterations in gastric emptying.

In addition to its gastric effects, in the few hours/days after administration, cisplatin may also induce alterations in both function and structure of the intestinal wall. The histological analysis of the intestine showed mucosal alterations (see Figure 6), which is in agreement with previous results obtained in the small intestine of both rat (Bearcroft *et al.,* 1999) and mouse (Allan and Smyth, 1986). In breast cancer patients on combination chemotherapy, crypt cell vacuolation was observed in the small intestine (Cunningham *et al.,* 1985).

Moreover, the structure of the intestinal mucosa of cisplatin-treated rats is similar to that characteristic of the regenerative state of irritable bowel syndrome (IBS): atrophic mucosa with fibrosis, inflammatory cell infiltration and lymph dilatation (Minamiyama *et al.,* 2002). These alterations in the intestinal mucosa could be at least in part responsible for the occasional diarrhoeas observed in this and previous studies (Vera *et al.,* 2006).

However, the main functional result of this work was that, in absence of delayed gastric emptying (evaluated using radiological methods one week after the end of the treatment), a dose-dependent delay in intestinal transit was observed. This delay must be due to persistent changes produced by chronic cisplatin administration. These changes might affect the smooth muscle and/or the neurones of the myenteric plexus (responsible for gastrointestinal motility).

Regarding the muscle, there are no previous studies showing structural alterations in smooth muscle induced by chronic cisplatin administration. However, we observed several qualitative structural alterations, like decreased thickness of the muscular layer in the ileum and the large intestine. In addition, the ultrastructural study revealed changes in the muscular cells of the distal colon, which showed alterations in the microfilaments and atrophic cells (see Figure 7). It has been described that cisplatin inhibits the release of acetylcholine from the axonal endings of the stomach smooth muscle resulting in bloating (Aggarwal *et al.,* 1994). Also, cisplatin exerted a concentration-dependent inhibition of spontaneous contractions of the isolated fallopian tubes (Bugarcić *et al.,* 2008). It can not be rule out that, upon chronic treatment, regular repetition of this kind of inhibitory actions might induce damage to the muscular cells. However, the delay in intestinal transit observed here might also

be due to neurological damage, which might have secondarily, altered muscular function and structure.

In fact, we found a decreased number of myenteric neurones per ganglion in the large intestine but not the ileum of cisplatin-treated animals. There are no previous studies on the effect of chronic cisplatin on the populations of enteric neurones. However, in the dorsal root ganglia (DRGs) of chronically cisplatin-treated rats a decline in medium and large neurones was observed, and this is indicative of neuronal atrophy (Schmidt *et al.,* 1995). Neuronal loss in the gastrointestinal tract has also been described in neuropathies of other ethiologies such as that associated to diabetes (Buttow *et al.,* 1997; Zanoni *et al.* 1997; Fregonesi *et al.,* 2001), and in other pathologies such as IBS (Boyer *et al.,* 2005). Neuronal loss could be due to apoptosis. Cisplatin-induced apoptosis in DRG neurones involves an attempt to re-enter the cell cycle by Bax-cytochrome C pathway, resulting in a loss of mitochondrial function (Gill and Windebank, 1998; McDonald *et al.,* 2005, Ta *et al.,* 2006). Similar mechanisms could account for neuronal loss in the myenteric plexus.

In the DRGs of cisplatin-treated mice, the neuronal loss was selective of large and medium neurones (Schmidt *et al.,* 1995). To study whether the neuronal loss observed in the myenteric plexus was also selective, we performed an immunohistochemical study of the distal colon, the region where the changes were more intense in the studies previously carried out.

We observed an enhanced proportion of myenteric neurones immunoreactive to NOS, which are inhibitory motor neurones to both longitudinal and circular muscle (Costa *et al.,* 1996). However, in the digestive tract of cisplatin-treated

rats, NOS immunoreactivity in the muscle of the pyloric sphincter and stomach was not affected (Järve and Aggarwal, 1997). More recent studies (Jung *et al.,* 2009) have shown that cisplatin administration induces the transcription of mRNA for neuronal NOS (nNOS) in renal mitochondria, suggesting that the up-regulation of nNOS may be directly involved in the pathology induced by cisplatin in the kidney. In diabetic neuropathy, NO levels are also increased in the brain, but this effect seems to involve not the neuronal but the inducible isoform of NOS (Celik and Erdogan, 2008). In agreement with our results for cisplatin-induced alterations, in diabetic neuropathy, NOS-IR neurones are increased in the myenteric plexus of the duodenum and the antrum (Spångéus *et al.,* 2000), and NOS-IR fibers were also increased in the ileum (Shotton et al. 2003). Schicho *et al.,* (2001) have shown that exposure of the rat gastric mucosa to HCl activates a subpopulation of NOS-positive myenteric plexus neurones. This suggests that injuries could activate the expression of NOS. An increase in NOS-IR myenteric neurones might slow down intestinal transit and thus contribute to explain our functional results.

The proportion of NPY-IR neurones did not significantly change in cisplatin-treated rats. In other neuropathies, like that induced by streptozotocine (STZ), NPY expression was not altered in the colon of diabetic mice (Spångéus *et al.,* 2000). On the other hand, in acrylamide-intoxicated rats, NPY significantly increased in the submucous plexus from the ileum (Belai and Burnstock, 1996). Similar results were found in the DRG after sciatic nerve injury (Tarpley, 2004; Son *et al.,* 2007).

In our study, we have not detected any significant change in VIP-IR neurones in the distal colon either. There are no previous studies in the GI tract of cisplatin-

treated animals, but in diabetic neuropathy, similar results have been observed in the colon of mice with type 1diabetes (Spångéus *et al.,* 2000). In contrast, an increase in VIP-IR myenteric neurones was reported in the ileum from diabetic rats (Belai *et al.,* 1996).

The proportion of SP-IR neurones remained unchanged. Although there are no previous data regarding the effect of cisplatin on the enteric SP-IR neurones, in cisplatin-treated mice, the proportion of SP-IR cells increased in the DRG sensory ganglia (Apfel *et al.,* 1992, Schmidt *et al.,* 1995). In other neuropathies, like that induced by STZ in rats, the pattern of distribution of cell bodies containing SP does not change in either myenteric or submucous plexuses in the ileum (Belai *et al.,* 1996) and similar results were obtained in acrylamide-intoxicated rats (Belai and Burnstock, 1996)

Regarding CR, we have not found any significant differences in the proportion of neurones IR for this marker. There are no studies on the expression of CR after treatment with cisplatin or in diabetic or other neuropathies.

We observed a decrease in CGRP-IR neurones. In cisplatin-treated animals, there was a reduction of CGRP in the DRG (neurosecretor peptide, Verdú *et al.,* 1999). Similar results have been found in the ileum from diabetic (Belai *et al.,* 1996) or acrylamide-treated rats (Belai and Burnstock, 1996).

Varicose CGRP-IR fibers were also studied and a dose-dependent decrease was found. There are no previous data in the literature of the effect of cisplatin on these fibers in the digestive tract, but in the DRG of mice chronically treated with cisplatin, a qualitative reduction of varicose fibers was also found (Verdú et

al. 1999). Similar results were found for fibers innervating the ileum (Shotton *et al.,* 2003) and the stomach (Lin *et al.,* 2008) of diabetic animals.

Altogether, it can be concluded that chronic cisplatin treatment induced alterations in the motor function, structure and innervation of the gastrointestinal tract. The persistent delay in gastrointestinal transit could be due to an enteric neuropathy caused in the long run by cisplatin administration. Interestingly, our results are in good agreement to those found in diabetic rats and mice, and other neuropathies (like that induced by acrylamide) or pathological conditions such us irritable bowel syndrome, suggesting that common mechanisms could underlie the development of neuropathic changes induced by different agents and pathological situations in the enteric nerves. The results obtained in this work could have relevance to the study and treatment of gastrointestinal side effects in cisplatin-treated patients.

TABLE 1. Antibodies used for the immunohistochemical study in whole-mount preparations

Neuronal Marker	Primary antibody		Secondary antibody	
	Host (source)	Dilution	Host (source)	Dilution
NOS	Sheep α-NOS (Chemicon)	1:1000	Donkey α-sheep RRX (Jackson)	1:500
VIP	Rabbit α-VIP (antibody code: 7913) *	1:1000	Donkey α-rabbit RRX (Dakopatts Glostrup)	1:500
CGRP	Rabbit α-CGRP (Chemicon)	1:1000	Donkey α-rabbit RRX (Dakopatts Glostrup)	1:500
CR	Goat α-CR (Chemicon)	1:1000	Donkey α-goat-RRX (Jackson)	1:500
SP	Rabbit (antibody code: 8701) *	1:1000	Donkey α-rabbit RRX (Dakopatts Glostrup)	1:500
NPY	Rabbit (antibody code: 8711) *	1:1000	Donkey α-rabbit RRX (Dakopatts Glostrup)	1:500

* Antibodies kindly supplied by Dr. Catia Sternini.
NOS= Nitric oxide synthase; VIP= Vasoactive intestinal peptide; CGRP= Calcitonin gene-related peptide; CR= Calretinin; SP= Substance P; NPY= Neuropeptide Y; RRX= Red Rhodamine.

Fig.1. Effect of chronic cisplatin on body weight gain and food intake in the rat. Change in body weight (A) and food intake (B) were measured in grouped rats injected for 5 weeks with: saline (4–5 mL/kg, i.p., open circles, n=10), or cisplatin (Cispt) at 1 (closed triangles, n=11) or 3 mg/kg (closed squares, n=13). Data represent the mean (average of the measurements taken during each week) ± SEM. * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$) *vs* saline; ## ($p<0.01$), ### ($p<0.001$) *vs* Cispt 1 (two-way ANOVA followed by Bonferroni *post hoc* test).

Fig.2. Effect of chronic cisplatin on rectal temperature and spontaneous locomotor activity in the rat. Rectal temperature (A) and locomotor activity (B) were measured in rats injected for 5 weeks with: saline (4–5 mL/kg, i.p., open circles, n=10), or cisplatin (Cispt) at 1 (closed triangles, n=11) or 3 mg/kg (closed squares, n=13). Data represent the mean (average of the measurements taken during each week) ± SEM. *($p<0.05$), *** ($p<0.001$) *vs* saline; # ($p<0.05$), ## ($p<0.01$), ### ($p<0.001$) *vs* Cispt 1 (two-way ANOVA followed by Bonferroni *post hoc* test).

Fig.3. Effect of chronic cisplatin on mechanical allodynia and gastrointestinal transit. Mechanical allodynia (A) and gastrointestinal transit (B) were measured in rats injected for 5 weeks with: saline (4–5 mL/kg, i.p., open circles, n=6 in A; n=12 in B), or cisplatin (Cispt) at 1 (closed triangles, n=8 in A; n=8 in B) or 3 mg/kg (closed squares, n=10 in A; n=11 in B). Analyses were carried out 4 (mechanical allodyinia) or 7 (gastrointestinal transit) days after last cisplatin administration. Von Frey hairs were used to determine the threshold for mechanical allodynia. Gastrointestinal transit was measured 20 minutes after

charcoal administration (see text); the bars show the relative distance travelled by charcoal in the gut. *($p<0.05$), *** ($p<0.001$) *vs* saline; ## ($p<0.01$) *vs* Cispt 1. (A: two-way ANOVA followed by Bonferroni *post hoc* test; B: unpaired *t* test).

Fig. 4. Effect of cisplatin on gastric emptying in the rat. Rats were treated with saline (4–5 mL/kg, *i.p.*, open circles, n=4 each experiment) or cisplatin (Cispt) at 1 (closed triangles, n=6 each experiment) or 3 mg/kg (closed squares, n=6 each experiment) for 5 weeks. Gastric emptying was measured by radiological methods (see text). Barium sulphate (2.5 mL, 2 g/mL) was intragastrically administrated after the first (A), the last (B) or 1 week after the last drug administration (C) and X-rays were taken 0, 1, 2, 4, 6 and 8 h after barium administration. Data represent the mean ± SEM. * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$) *vs* saline (two-way ANOVA followed by Bonferroni *post hoc* test). D-I: representative X-rays of rats treated with saline (D, F, H) or Cispt 3 (E, G, I), taken 4 h after barium administration, showing the acute (D, E), chronic (F, G) and residual effects (H, I) of cisplatin treatment. Scale bar: 4 cm.

Fig. 5. Histological damage induced by chronic cisplatin in the gut wall. Histological damage was evaluated in paraffin-embedded whole-gut sections from ileum (A), proximal colon (B), distal colon (C) and rectum (D) of rats treated with saline (white bars), or cisplatin (Cispt) at 1 (hatched bars) or 3 mg/kg (black bars). Tissues were stained with hematoxilyn & eosin (H&E), and assigned a histological damage score based on predetermined criteria (0 to 9, arbitrary units, see text). Values are mean score ± SEM; each group consisted of 4-6 rats. * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$) *vs* saline; ## ($p<0.01$) *vs* Cispt

1 (unpaired *t* test with Welch's correction). E and F: representative photographs of distal colon stained with H&E from rats treated with saline (E) and Cispt 3 (F). Scale bar: 100 μm. In rats treated with cisplatin at 3 mg/kg (CISPT 3), loss of mucosal architecture (+) crypt abscess formation (#) and reduced muscular layer thickness (*), could be seen.

Fig. 6. Effect of chronic cisplatin in the population of myenteric neurones. The number of myenteric neurones per ganglion was evaluated in paraffin-embedded tissues from ileum (A), proximal colon (B), distal colon (C) and rectum (D) of rats treated with saline (white bars), or cisplatin (Cispt) at 1 (hatched bars) or 3 mg/kg (black bars). Paraffin-embedded sections were stained with the general neuronal marker NSE and stained cells in the myenteric plexus ganglia were counted and expressed as the number of neurones per ganglion. Each bar represents mean±SEM. Each group consisted of 4-6 animals. ** ($p<0.01$), *** ($p<0.001$) *vs* saline; ## ($p<0.01$), ### ($p<0.001$) *vs* Cispt 1 (unpaired *t* test). E and F: representative photographs of myenteric neurones in the distal colon stained with NSE from rats treated with saline (E) and Cispt 3 (F). Scale bar: 50 μm.

Fig 7. Electron microscopy of colonic and rectal preparations from control and cisplatin-treated rats. Microphotographs of colon (A, B; 8000x) and rectum (C, D; 6000x) of saline (A, C) and cisplatin-treated rats (B, D). In rats treated with cisplatin at 3 mg/kg (CISPT 3), atrophic muscular cells (B) and polimorfonuclear infiltrate (D) could be seen (shown as *).

Fig 8. Effect of chronic cisplatin on the populations of myenteric neurones immunoreactive to NOS, VIP and NPY in the rat distal colon. The proportions of myenteric neurones immunoreactive (IR) to NOS (A), VIP (B) and NPY (C) relative to the general neuronal population were analyzed in whole-mount preparations from the distal colon. Data represent the mean ± SEM. White bars represent saline-treated rats (n= 4-6); hatched bars, rats treated with cisplatin (CISPT) at 1 mg/kg (n= 4-5); black bars, rats treated with CISPT at 3 mg/kg (n= 3-4). **($p<0.01$) *vs* saline; ## ($p<0.01$) *vs* CISPT 1 (unpaired *t* test with Welch correction).

Fig. 9. Analysis of myenteric neurones immunoreactive to CR and SP in distal colon. The proportions of myenteric neurones immunoreactive (IR) to CR (A) and SP (B) were analyzed in whole-mount preparations of distal colon. Data represent the mean ± SEM. White bars represent saline-treated rats (n= 4-6); hatched bars, rats treated with cisplatin (CISPT) at 1 mg/kg (n= 4-5); black bars, rats treated with CISPT at 3 mg/kg (n= 3-4).

Fig.10. Effect of chronic cisplatin on the myenteric structures (neurones and fibers) immunoreactive to CGRP in the rat distal colon. A: representative photograph of a whole-mount preparation from distal colon of a saline-treated rat showing both myenteric neurones (cell) and both varicose and smooth fibers immunoreactive (IR) for CGRP. The proportions of myenteric neurones IR to CGRP (B) were analyzed in whole-mount preparations of distal colon. A semiquantitative analysis of smooth (C) and varicose (D) CGRP-IR fibers was also carried out. White bars represent saline-treated rats (n=5); hatched bars,

rats treated with cisplatin (CISPT) at 1 mg/kg (n=5); black bars, rats treated with CISPT at 3 mg/kg (n=3,). Data represent the mean ± SEM. *($p<0.05$); **($p<0.01$); ***($p<0.001$) *vs* saline; # ($p<0.05$), ## ($p<0.01$) *vs* Cispt 1, (unpaired *t* test).

References

Abalo R, José Rivera A, Vera G, Isabel Martín M. Ileal myenteric plexus in aged guinea-pigs: loss of structure and calretinin-immunoreactive neurones. *Neurogastroenterol Motil*. 2005; 17(1): 123-132.

Aggarwal SK, Antonio JDS, Sokhansanj A, Miller CM. Cisplatin induced peptic ulcer, vagotomy adrenal and calcium modulation. *Anticancer Drugs.* 1994; 5(2): 177-193.

Allan S, Smyth J. Small intestinal mucosal toxicity of cis-platinum: comparison of toxicity with platinum analogues and dexamethasone. *Br J Cancer.* 1986; 53(3): 355–360.

Apfel SC, Arezzo JC, Lipson LA, Kessler JA. Nerve growth factor prevents experimental cisplatin neuropathy. *Ann Neurol.* 1992; 31(7): 76–80.

Authier N, Gillet JP, Fialip J, Eschalier A, Coudore F. An animal model of nociceptive peripheral neuropathy following repeated cisplatin injections. *Exp Neurol.* 2003; 182(1): 12–20.

Badary OA, Awad AS, Sherief MA, Hamada FM. In vitro and in vivo effects of ferulic acid on gastrointestinal motility: inhibition of cisplatin-induced delay in gastric emptying in rats. *World J Gastroenterol.* 2006; 12(33): 5363-5367.

Barajon I, Bersani M, Quartu M, Del Fiacco M, Cavaletti G, Holst JJ, Tredici G. Neuropeptides and morphological changes in cisplatin-induced dorsal root ganglion neuronopathy. *Exp Neurol.* 1996; 138(1): 93-104.

Bearcroft CP, Domizio P, Mourad FH, André EA, Farthing MJ. Cisplatin impairs fluid and electrolyte absorption in rat small intestine: a role for 5-hydroxytryptamine. *Gut.* 1999; 44(2):174-179.

Belai A, Burnstock G. Acrylamide-induced neuropathic changes in rat enteric nerves: similarities with effects of streptozotocin-diabetes. *J Auton Nerv Syst.* 1996; 58(1-2): 56-62.

Belai A, Calcutt NA, Carrington AL, Diemel LT, Tomlinson DR, Burnstock G. Enteric neuropeptides in streptozotocin-diabetic rats; effects of insulin and aldose reductase inhibition. *J Auton Nerv Syst.* 1996; 58(3):163-169.

Belai A, Burnstock G. Distribution and colocalization of nitric oxide synthase and calretinin in myenteric neurones of developing, aging, and Crohn's disease human small intestine. *Dig Dis Sci.* 1999; 44(8): 1579-1587.

Boogerd W, ten Bokkel Huinink WW, Dalesio O, Hoppenbrouwers WJ, van der Sande JJ. Cisplatin induced neuropathy: central, peripheral and autonomic nerve involvement. *J Neurooncol.* 1990; 9(3): 255-263.

Boyer L, Ghoreishi M, Templeman V, Vallance BA, Buchan AM, Jevon G, Jacobson K. Myenteric plexus injury and apoptosis in experimental colitis. *Auton Neurosci.* 2005; 117(1): 41-53.

Brookes SJ, Steele PA, Costa M. Calretinin immunoreactivity in cholinergic motor neurones, interneurones and vasomotor neurones in the guinea-pig small intestine. *Cell Tissue Res.* 1991; 263(3): 471–481.

Bugarcić ZD, Petrović B, Bugarcić ZM, Janković SM, Janković SV, Lukić G, Novaković M, Kostović V, Popović S, Djurdjević P, Baskić D, Arsenijević N. Effects of cisplatin and other Pt(II) complexes on spontaneous motility of isolated human oviduct. *Toxicol In Vitro.* 2008; 22(8): 1878-1882.

Buttow NC, Miranda-Neto MH, Bazotte RB. Morphological and quantitative study of the myenteric plexus of the duodenum of streptozotocin-induced diabetic rats. *Arq Gastroenterol.* 1997; 34: 34-42.

Cabezos PA, Vera G, Castillo M, Fernández-Pujol R, Martín MI, Abalo R. Radiological study of gastrointestinal motor activity after acute cisplatin in the rat. Temporal relationship with pica. *Auton Neurosci.* 2008; 141(1-2): 54-65.

Celik S, Erdogan S. Caffeic acid phenethyl ester (CAPE) protects brain against oxidative stress and inflammation induced by diabetes in rats. *Mol Cell Biochem.* 2008; 312(1-2): 39-46.

Costa M, Furness JB, Gibbins IL. Chemical coding of enteric neurones. *Prog Brain Res.* 1986; 68: 217-239.

Costa M, Brookes SJ, Steele PA, Gibbins I, Burcher E, Kandiah CJ. Neurochemical classification of myenteric neurones in the guinea-pig ileum. *Neuroscience.* 1996; 75(3): 949-967.

Cunningham D, Morgan R, Mills P, et al. Functional and structural changes of the human proximal small intestine after cytotoxic therapy. *J Clin Pathol.* 1985; 38(3): 265–270.

De Giorgio R, Guerrini S, Barbara G, Stanghellini V, De Ponti F, Corinaldesi R, Moses PL, Sharkey KA, Mawe GM. Inflammatory neuropathies of the enteric nervous system. *Gastroenterology.* 2004; 126(7): 1872-1883.

Freeman R. Autonomic peripheral neuropathy. *Lancet.* 2005; 65(9466): 1259-1270.

Fregonesi CE, Miranda-Neto MH, Molinari SL, Zanoni JN. Quantitative study of the myenteric plexus of the stomach of rats with streptozotocin-induced diabetes. *Arq Neuropsiquiatr.* 2001; 59(1): 50-53.

Furness JB, Robbins HL, Selmer IS, Hunne B, Chen MX, Hicks GA, Moore S, Neylon CB. Expression of intermediate conductance potassium channel immunoreactivity in neurones and epithelial cells of the rat gastrointestinal tract. *Cell Tissue Res.* 2003; 314(2): 179-189.

Galeazzi F, Blennerhassett PA, Qiu B, O'Byrne PM, Collins SM. Cigarette smoke aggravates experimental colitis in rats. *Gastroenterology.* 1999; 117(4): 877-883.

Gill JS, Windebank AJ. Cisplatin-induced apoptosis in rat dorsal root ganglion neurones is associated with attempted entry into the cell cycle. *J Clin Invest.* 1998; 101(12): 2842-2850.

Hill JM, Loeb E, MacLellan A, Hill NO, Khan A, King JJ. Clinical studies of Platinum Coordination compounds in the treatment of various malignant diseases. *Cancer Chemother Res.* 1975; 59(3): 647-659.

Jarve RK, Aggarwal SK. Cisplatin-induced inhibition of the calcium-calmodulin complex, neuronal nitric oxide synthase activation and their role in stomach distention. *Cancer Chemother Pharmacol.* 1997; 39(4): 341-348.

Jung M, Hotter G, Viñas JL, Sola A. Cisplatin upregulates mitochondrial nitric oxide synthase and peroxynitrite formation to promote renal injury. *Toxicol Appl Pharmacol.* 2009; 234(2): 236-246.

Kucuk O, Apostol JV. Amsacrine and etoposide induced paralytic ileus in a patient with acute myelomonocytic leukemia. *J Surg Oncol* 1985; 28(3): 172-173.

Legha SS. Vincristine neurotoxicity. Pathophysiology and management. *Med Toxicol.* 1986; 1(6): 421-427.

Lin YY, Tseng TJ, Hsieh YL, Luo KR, Lin WM, Chiang H, Hsieh ST. Depletion of peptidergic innervation in the gastric mucosa of streptozotocin-induced diabetic rats. *Exp Neurol.* 2008; 213(2): 388-396.

McDonald ES, Randon KR, Knight A, Windebank AJ. Cisplatin preferentially binds to DNA in dorsal root ganglion neurones in vitro and in vivo: a potential mechanism for neurotoxicity. *Neurobiol Dis.* 2005; 18(2): 305-313.

Magne N, Marcy PY, Chamorey E, Guardiola E, Pivot X, Schneider M, Demard F, Bensadoun RJ. Concomitant twice-a-day radiotherapy and chemotherapy in unresectable head and neck cancer patients: A long-term quality of life analysis. *Head Neck*. 2001; 23(8): 678-682.

Malik NM, Liu YL, Cole N, Sanger GJ, Andrews PL. Differential effects of dexamethasone, ondansetron and a tachykinin NK1 receptor antagonist (GR205171) on cisplatin-induced changes in behaviour, food intake, pica and gastric function in rats. *Eur J Pharmacol.* 2007; 555(2-3): 164-173.

Minamiyama Y, Takemura S, Toyokuni S, Nishino Y, Yamasaki K, Hai S, Yamamoto S, Okada S. Amelioration of cisplatin toxicity by a fermented grain food product. *Biofactors.* 2002; 16(3-4): 105-115.

O'Connor R, Eikelboom R. The effects of changes in housing on feeding and wheel running, *Physiol Behav.* 2000; 68**:** 361–371.

Ozaki A, Sukamoto T. Improvement of cisplatin-induced emesis and delayed gastric emptying by KB-R6933, a novel 5-HT3 receptor antagonist. *Gen Pharmacol.* 1999; 33(3): 283-288.

Pascual D, Girón R, Alsasua A, Benhamú B, López-Rodríguez ML, Martín MI. New benzimidazole derivatives: selective and orally active 5-HT3 receptor antagonists. *Eur J Pharmacol.* 2003; 462(1-3): 99-107.

Phillips RJ, Hargrave SL, Rhodes BS, Zopf DA, Powley TL. Quantification of neurones in the myenteric plexus: an evaluation of putative pan-neuronal markers. *J Neurosci Methods.* 2004; 133(1-2): 99-107.

Phillips LK, Rayner CK, Jones KL, Horowitz M. An update on autonomic neuropathy affecting the gastrointestinal tract. *Curr Diab Rep.* 2006; 6(6): 417-423.

Rudd JA, Yamamoto K, Yamatodani A, Takeda N. Differential action of ondansetron and dexamethasone to modify cisplatin-induced acute and delayed kaolin consumption (“pica”) in rats. *Eur J Pharmacol.* 2002; 454(1): 47–52.

Schicho R, Schemann M, Holzer P, Lippe IT. Mucosal acid challenge activates nitrergic neurones in myenteric plexus of rat stomach. *Am J Physiol Gastrointest Liver Physiol.* 2001; 281(5): G1316-G1321.

Schmidt Y, Unger JW, Bartke I, Reiter R. Effect of nerve growth factor on peptide neurones in dorsal root ganglia after taxol or cisplatin treatment and in diabetic (db/db) mice. *Exp Neurol*. 1995; 132(1): 16-23.

Sharma SS, Gupta YK. Reversal of cisplatin-induced delay in gastric emptying in rats by ginger (Zingiber officinale). *J Ethnopharmacol*. 1998; 62(1): 49-55.

Shotton HR, Clarke S, Lincoln J. The effectiveness of treatments of diabetic autonomic neuropathy is not the same in autonomic nerves supplying different organs. *Diabetes.* 2003; 52(1): 157-164.

Son SJ, Lee KM, Jeon SM, Park ES, Park KM, Cho HJ. Activation of transcription factor c-jun in dorsal root ganglia induces VIP and NPY upregulation and contributes to the pathogenesis of neuropathic pain. *Exp Neurol.* 2007; 204(1): 467-472.

Spångéus A, Suhr O, El-Salhy M. Diabetic state affects the innervation of gut in an animal model of human type 1 diabetes. *Histol Histopathol.* 2000; 15(3): 739-744.

Ta LE, Espeset L, Podratz J, Windebank AJ.Neurotoxicity of oxaliplatin and cisplatin for dorsal root ganglion neurones correlates with platinum-DNA binding. *Neurotoxicology.* 2006; 27(6): 992-1002.

Talley RW, O'Bryan RM, Gutterman JU, Brownlee RW, McCredie KB.Clinical evaluation of toxic effects of cis-diamminedichloroplatinum (NSC-119875)--phase I clinical study. *Cancer Chemother Rep.* 1973; 57(4): 465-471.

Tarpley JW, Kohler MG, Martin WJ. The behavioral and neuroanatomical effects of IB4-saporin treatment in rat models of nociceptive and neuropathic pain. *Brain Res.* 2004; 1029(1): 65-76.

Vandertop WP, de Vries WB, Notermans NC, Nijsen MJ, de Wildt DJ, Tulleken CA, Gispen WH. Cisplatin-induced autonomic neuropathy: does it really exist? *J Neurooncol*. 1996; 27(1): 53-60.

Vera G, Chiarlone A, Martín MI, Abalo R. Altered feeding behaviour induced by long-term cisplatin in rats. *Auton Neurosci.* 2006; 126-127: 81-92.

Vera G, Chiarlone A, Cabezos PA, Pascual D, Martín MI, Abalo R. WIN 55,212-2 prevents mechanical allodynia but not alterations in feeding behaviour induced by chronic cisplatin in the rat. *Life Sci.* 2007; 81(6): 468-479.

Verdú E, Vilches JJ, Rodríguez FJ, Ceballos D, Valero A, Navarro X. Physiological and immunohistochemical characterization of cisplatin-induced neuropathy in mice. *Muscle Nerve.* 1999; 22(3): 329-340.

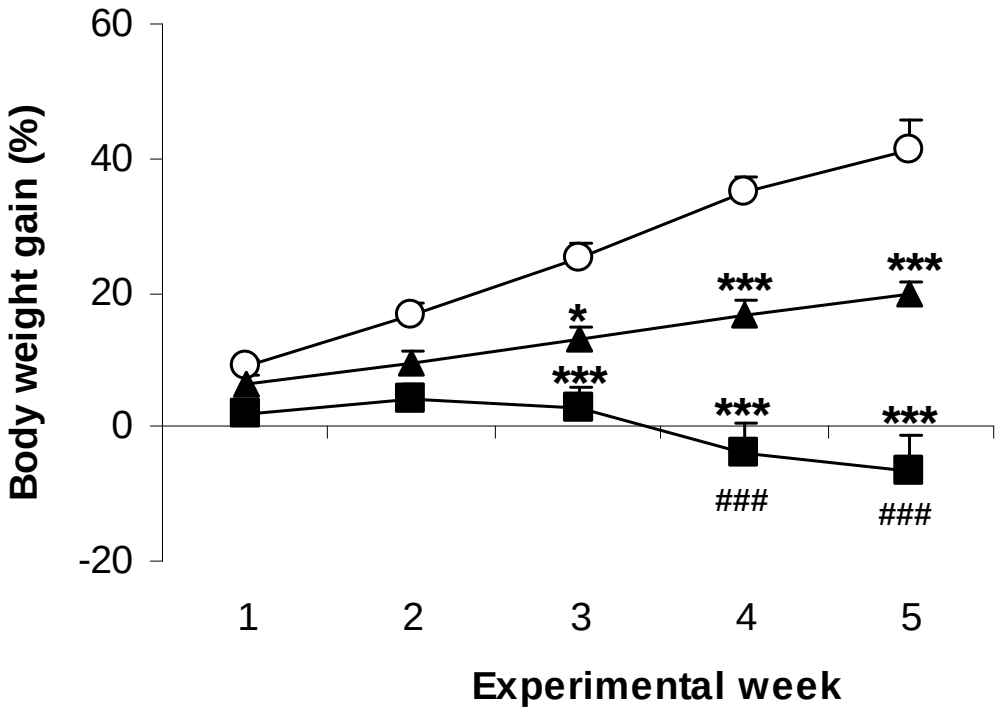
Von Hoff DD, Schilsky R, Reichert CM, Reddick RL, Rozencweig M, Young RC, Muggia FM. Toxic effects of cis-dichlorodiammineplatinum(II) in man. *Cancer Treat Rep.* 1979; 63(9-10): 1527-1531.

Wegener M, Börsch G, Schaffstein J, Luerweg C, Leverkus F. Gastrointestinal transit disorders in patients with insulin-treated diabetes mellitus. *Dig Dis.* 1990; 8(1): 23-36.

Zanoni JN, Miranda-Neto MH, Bazotte RB, Souza RR. Morphological and quantitative analysis of the neurones of the myenteric plexus of the cecum of streptozotocin-induced diabetics rats. *Arq Neropsiquiatr.* 1997; 55: 696-702.

Fig. 1

A


60
40
20
0
-20
Body weight gain (%)
1
2
3
4
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Experimental week
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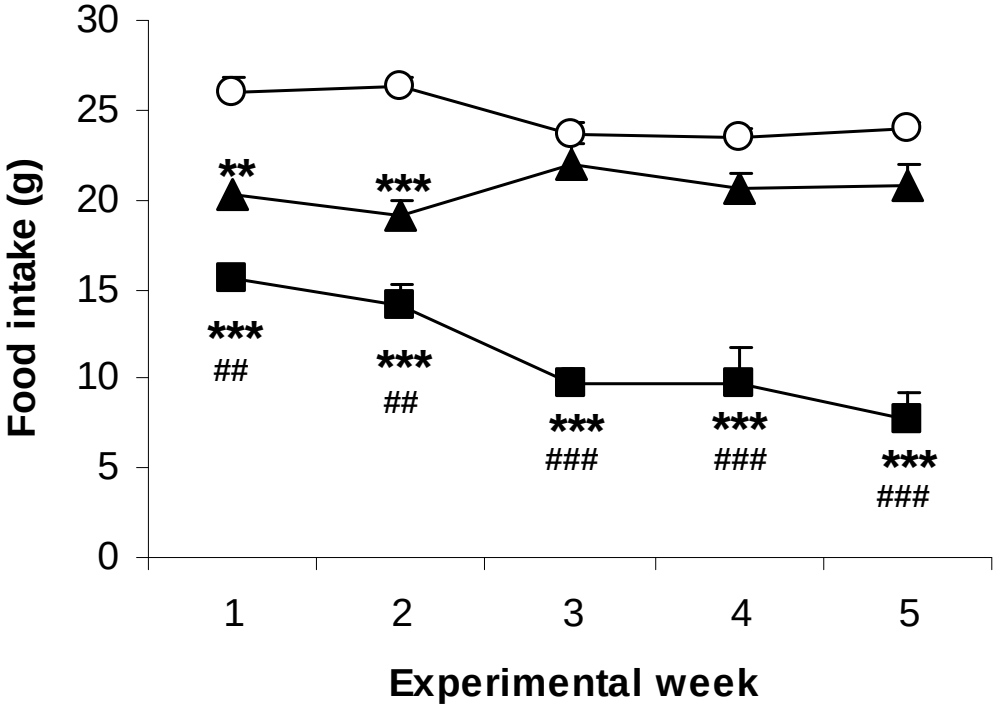
B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25
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Food intake (g)
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Experimental week
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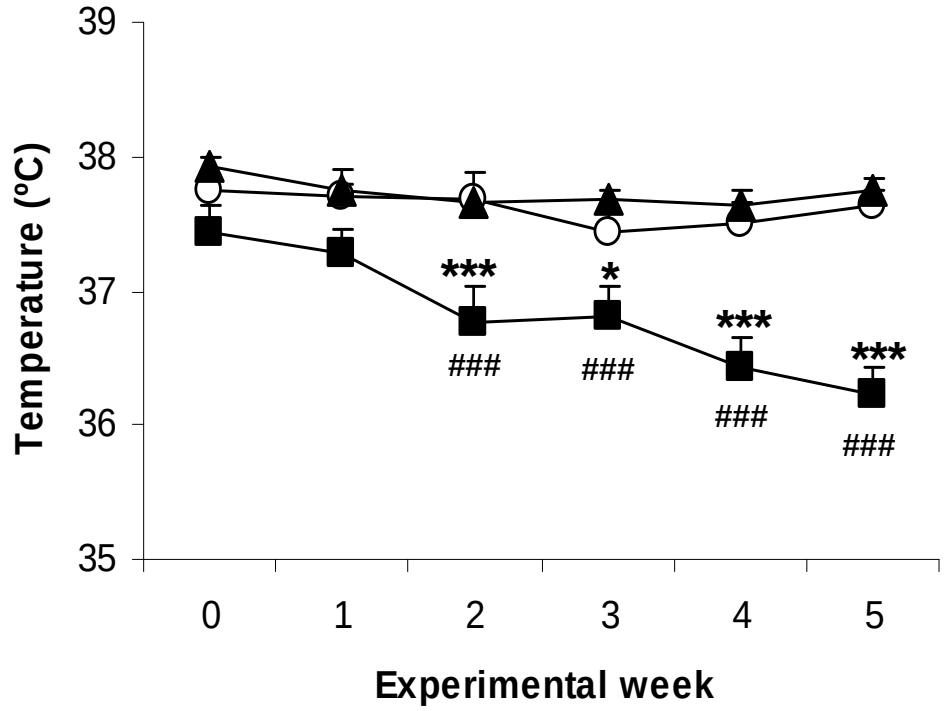
Fig. 2

A


39
38
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35
Temperature (ºC)
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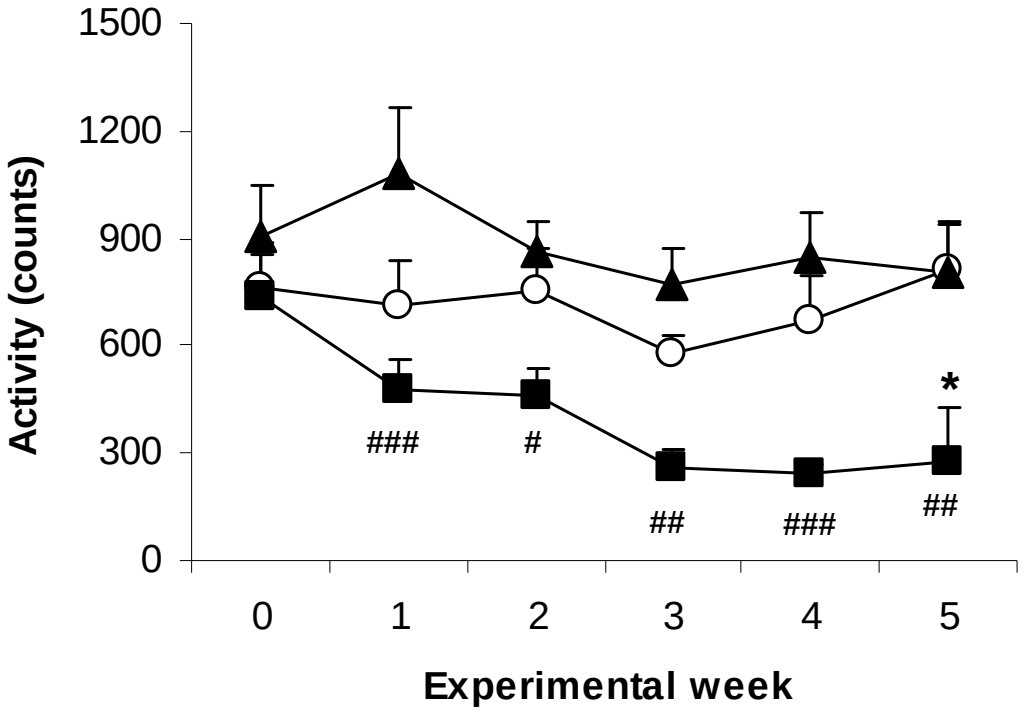
Experimental week

B


1500
1200
900
600
300
0
Activity (counts)
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0
1
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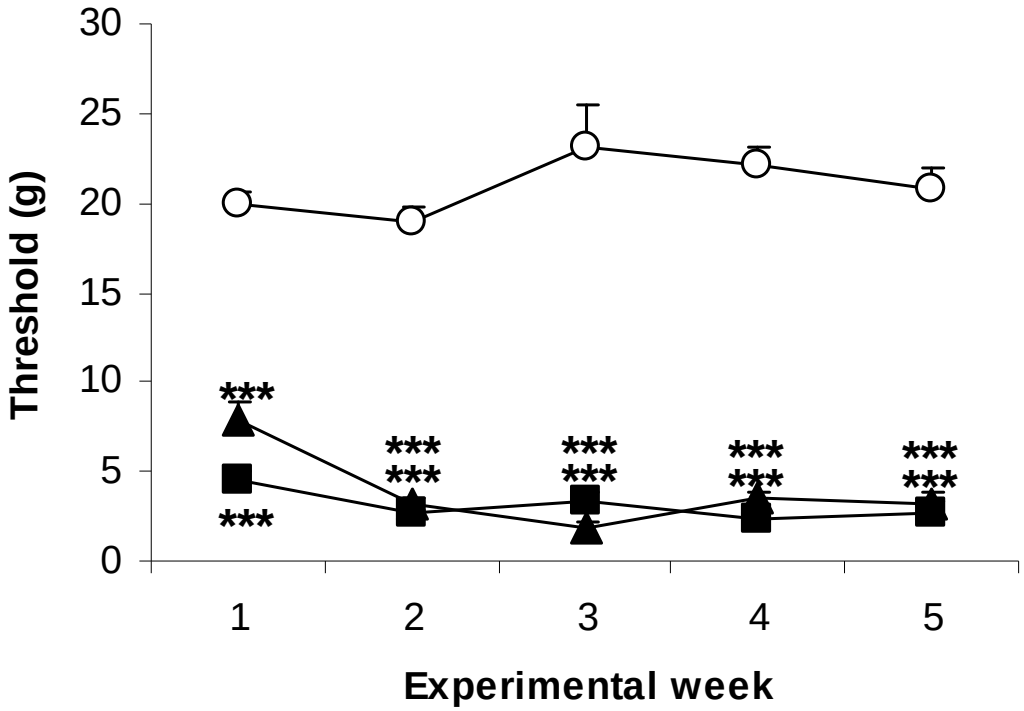
3
4
5
Experimental week

Fig. 3

A


30
25
20
15
10
5
0
Threshold (g)
***
***
***
***
***
***
***
***
***
1
2
3
4
5
Experimental week

B

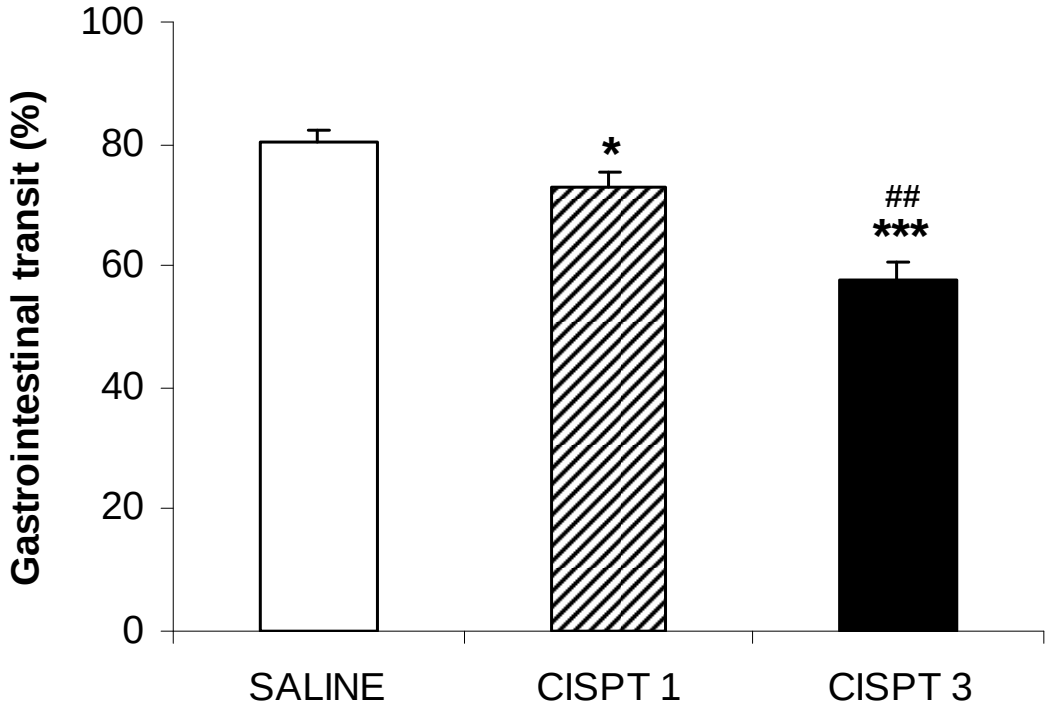

100
80
60
40
20
0
Gastrointestinal transit (%)
*
##
***
SALINE
CISPT 1
CISPT 3

Fig. 4

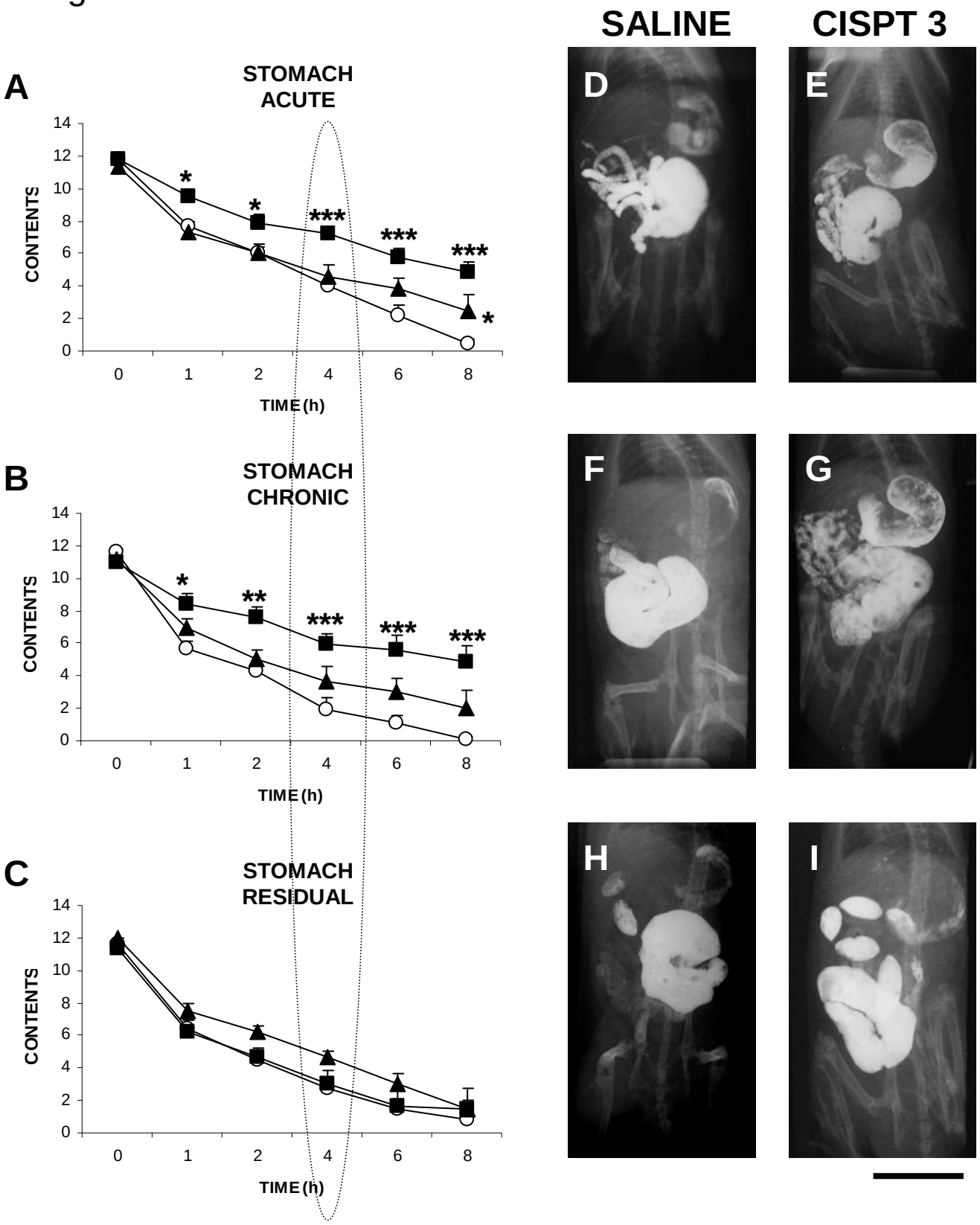

A
STOMACH
ACUTE
CONTENTS
TIME (h)
B
STOMACH
CHRONIC
CONTENTS
TIME (h)
C
STOMACH
RESIDUAL
CONTENTS
TIME (h)
SALINE
CISPT 3
D
E
F
G
H
I

Fig. 5

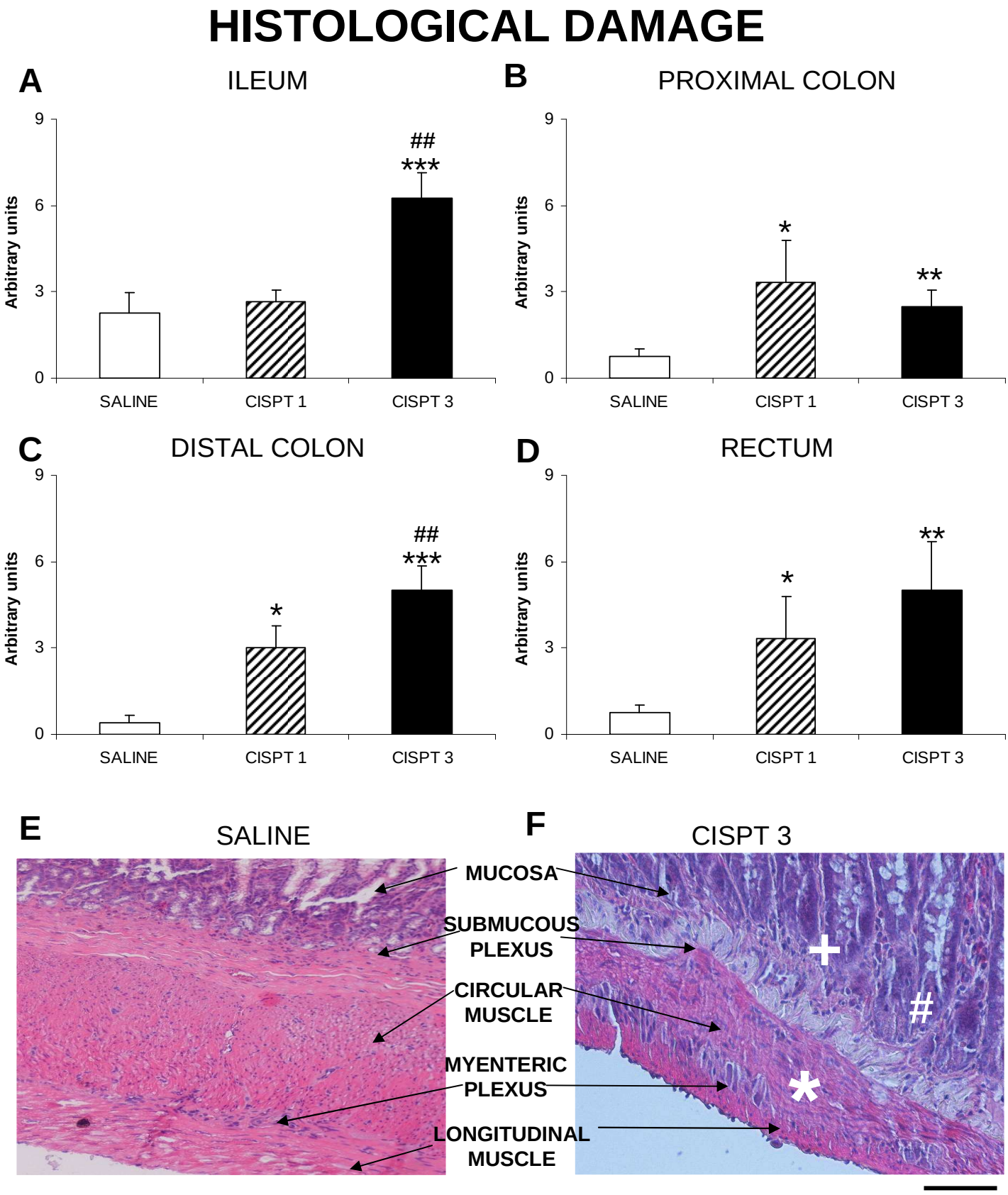
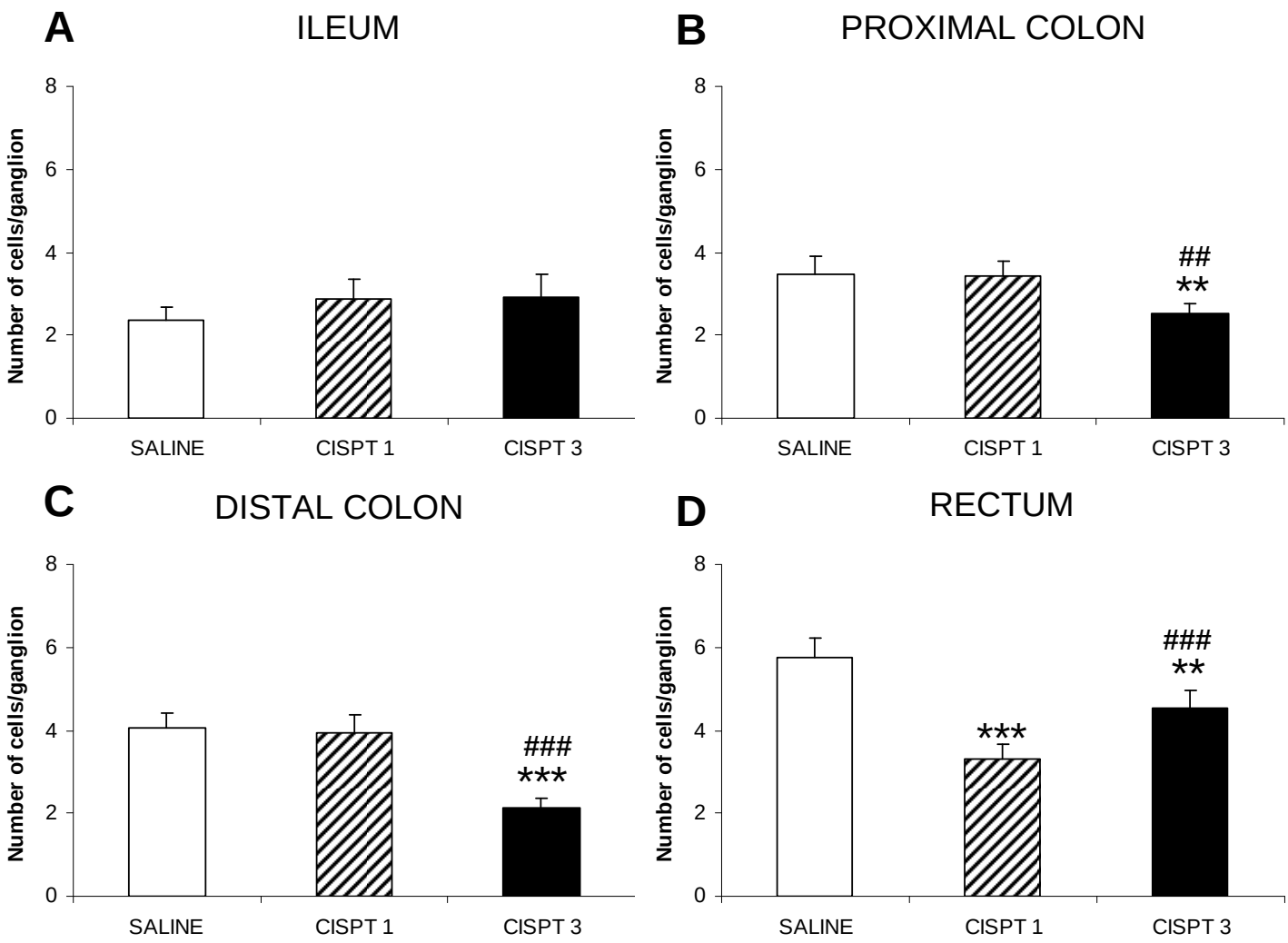

HISTOLOGICAL DAMAGE
A
ILEUM
B
PROXIMAL COLON
C
DISTAL COLON
D
RECTUM
Arbitrary units
0
3
6
9
SALINE
CISPT 1
CISPT 3
##
***
*
**
E
SALINE
F
CISPT 3
MUCOSA
SUBMUCOUS PLEXUS
CIRCULAR MUSCLE
MYENTERIC PLEXUS
LONGITUDINAL MUSCLE
+
#
*

Fig. 6

CELLS PER GANGLION



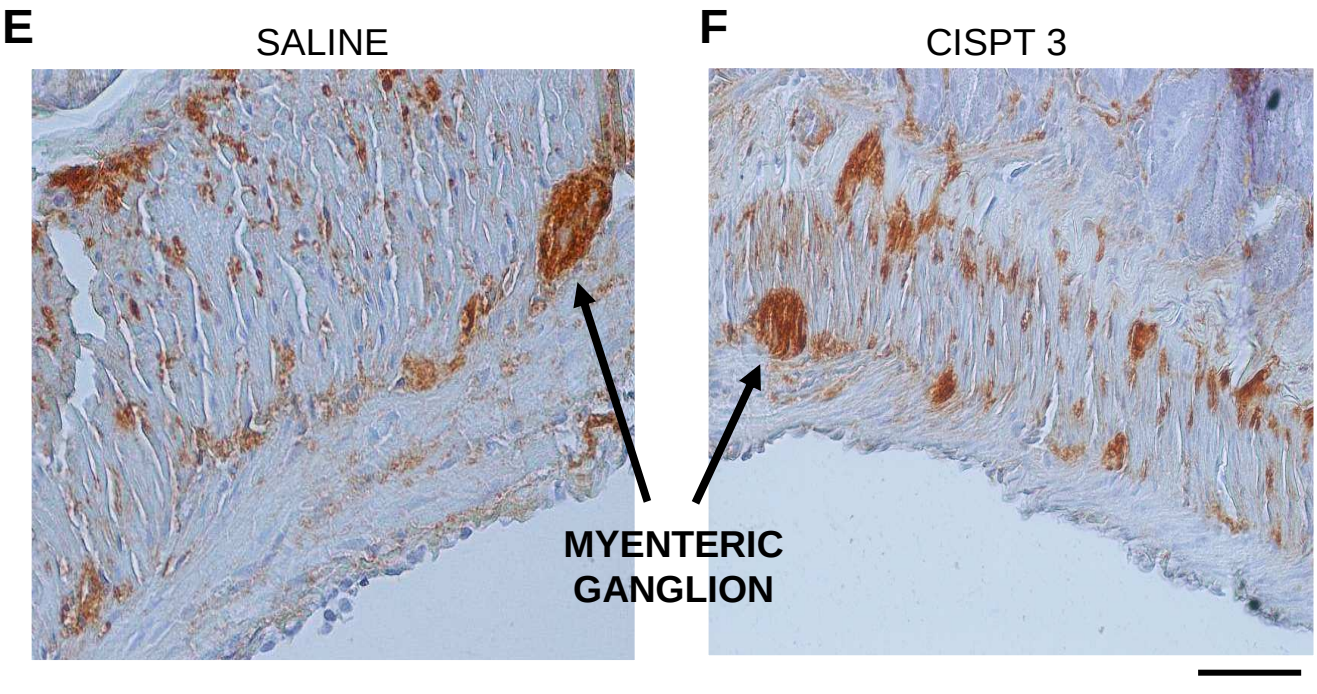
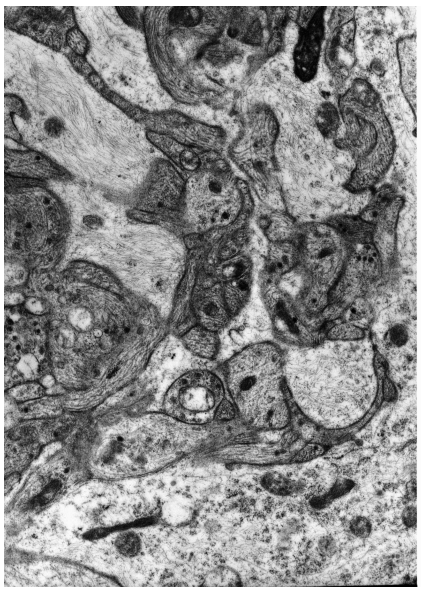


Fig. 7

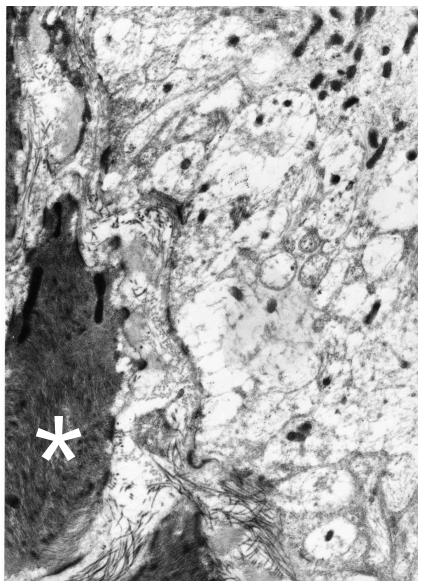
ELECTRON MICROSCOPY

DISTAL COLON

A

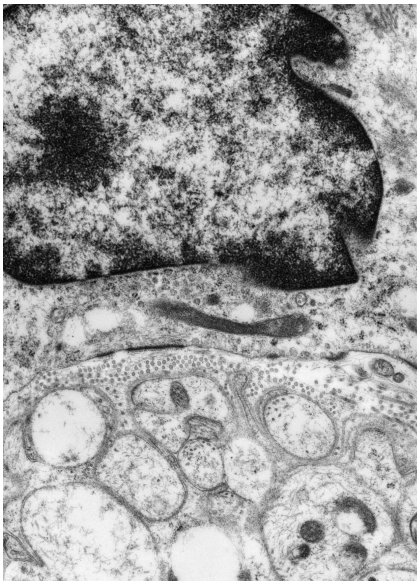


B

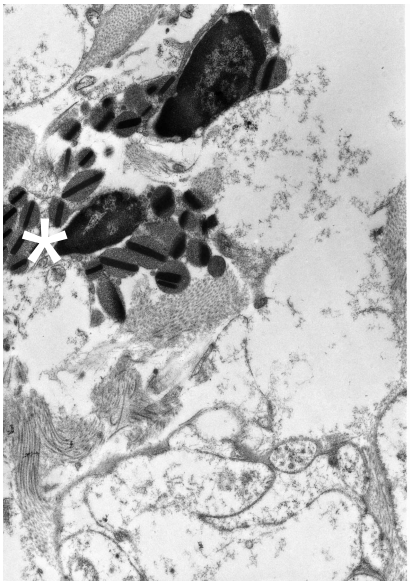


RECTUM

C



D



SALINE

CISPT3

Fig. 8

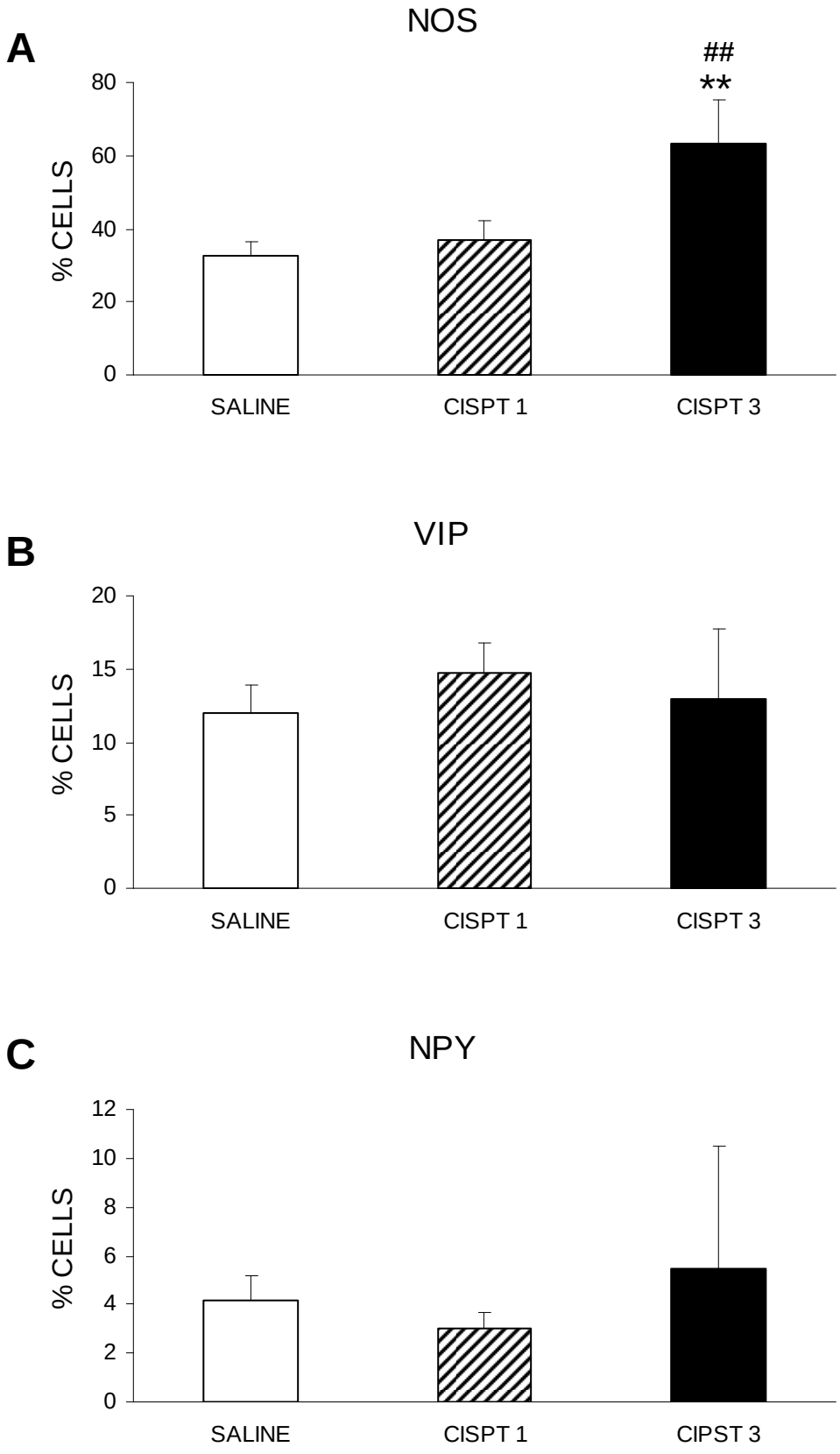

A
NOS
##
**
% CELLS
80
60
40
20
0
SALINE
CISPT 1
CISPT 3
B
VIP
% CELLS
20
15
10
5
0
SALINE
CISPT 1
CISPT 3
C
NPY
% CELLS
12
10
8
6
4
2
0
SALINE
CISPT 1
CIPST 3

Fig. 9

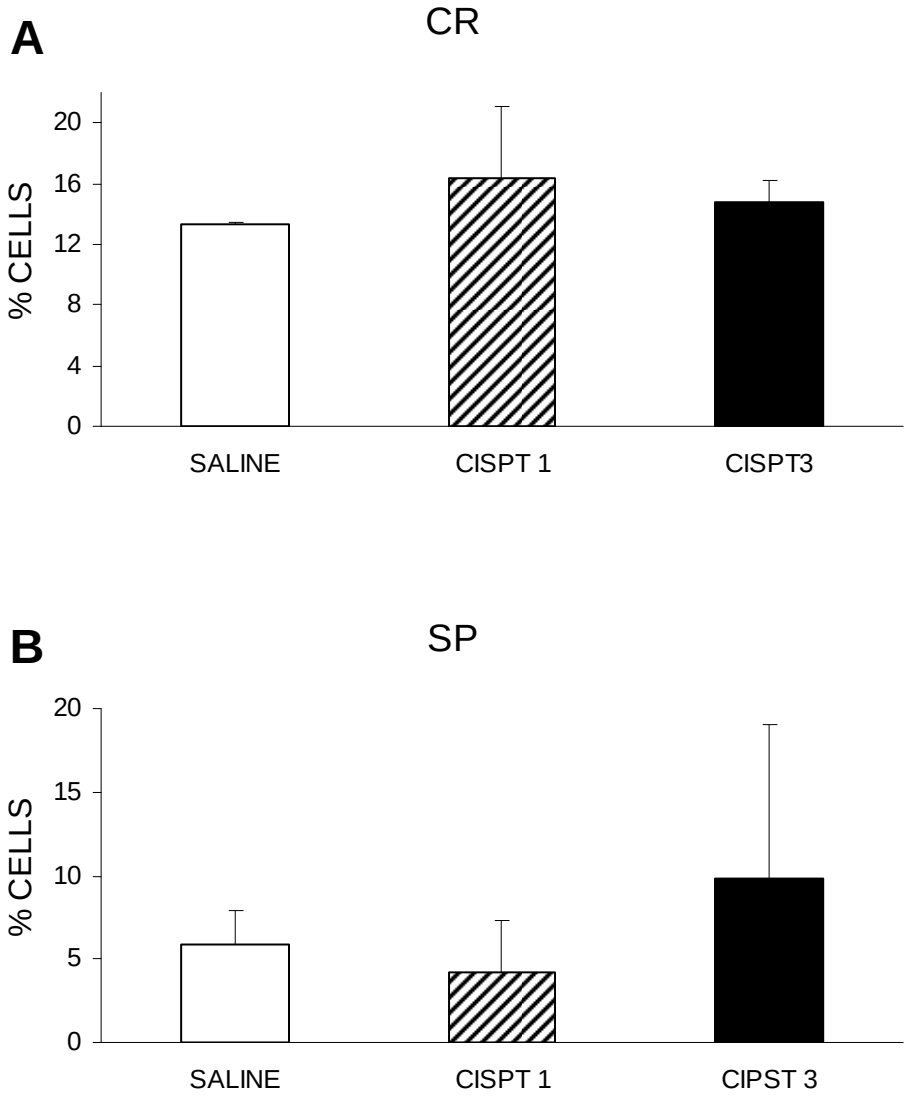
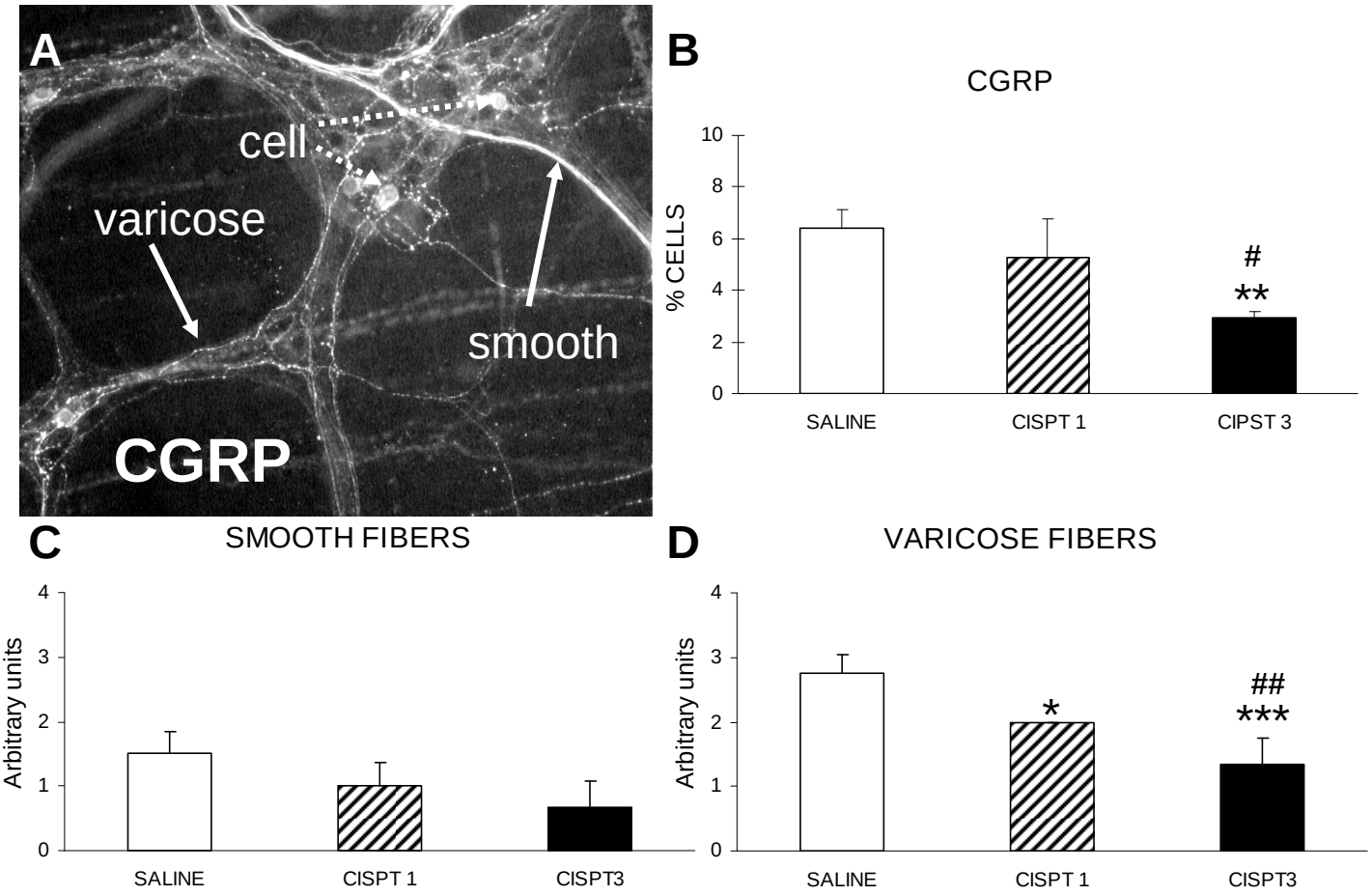

A
CR
% CELLS
20
16
12
8
4
0
SALINE
CISPT 1
CISPT3
B
SP
% CELLS
20
15
10
5
0
SALINE
CISPT 1
CIPST 3

Fig. 10


A
cell
varicose
smooth
CGRP
B
CGRP
% CELLS
10
8
6
4
2
0
#
**
SALINE
CISPT 1
CIPST 3
C
SMOOTH FIBERS
Arbitrary units
4
3
2
1
0
SALINE
CISPT 1
CISPT3
D
VARICOSE FIBERS
Arbitrary units
4
3
2
1
0
*
##
***
SALINE
CISPT 1
CISPT3

5. Discusión

Nuestros trabajos son los primeros en los que se utiliza el modelo de pica para estudiar el efecto del cisplatino en tratamiento crónico. Como era de esperar, el cisplatino alteró el comportamiento alimentario en la rata, produciendo anorexia y un aumento en la ingesta de caolín (como marcador indirecto de náusea).

La utilización crónica de cisplatino a 3 mg/kg produjo una elevada toxicidad. Los animales mostraron hipotermia, reducción de la ganancia de peso corporal y de la ingesta de comida, así como alodinia. Todo esto se produjo tanto en ratas aisladas como agrupadas, pero en los animales aislados se registró una mortalidad elevada (del 50%). El aislamiento puede aumentar la susceptibilidad a un estresante más fuerte (Bartolomucci *et al.,* 2003), como puede ser el cisplatino. Por eso se redujo la dosis a 1 mg/kg. Esta dosis, aunque reducía la ganancia de peso corporal en los animales, probablemente debido a la anorexia que producía la administración del fármaco, no tenía una toxicidad tan elevada como la dosis de 3 mg/kg (ningún animal falleció).

Respecto a la dosis de cisplatino 2 mg/kg, ésta mostró menor toxicidad que la de 3 mg/kg, ninguna rata murió y se observó sólo una ligera hipotermia al final del experimento. Los efectos en la ganancia de peso corporal y en la ingesta de comida fueron mayores que los de 1 mg/kg y el incremento en la ingesta basal y aguda de caolín fue más intenso. Por todo ello, parece que esta dosis es la más adecuada para estudiar el efecto antinauseoso (anti-pica) de fármacos antieméticos en este modelo.

Al analizar el efecto de los cannabinoides en las alteraciones alimentarias causadas por el cisplatino se vio que el WIN produjo una reducción de la ganancia de peso estadísticamente significativa desde la cuarta semana experimental. Esta disminución del peso corporal fue aditiva a la que produjo el cisplatino. Este efecto fue inesperado, pues los cannabinoides se usan empíricamente para reducir la náusea y emesis producidos por quimioterapia y también porque aumentan el apetito; de hecho, se piensa que pueden ser útiles para el tratamiento de pacientes con cáncer o SIDA (Ben Amar, 2006). Estas diferencias podrían deberse a las distintas metodologías utilizadas para estudiar el efecto orexígeno de los cannabinoides; generalmente se utilizan animales sometidos a periodos de ayuno o parcialmente saciados y tras una dosis elevada de fármaco. Así mismo, cuando se utilizan éste y otros agonistas cannabinoides para la prevención de la ingesta de caolín en rata y la náusea y la

emesis en otros modelos animales (Darmani, 2001a; Van Sickle *et al.,* 2003; Kwiatkowska *et al.,* 2004), se usan a dosis que tienen efecto a nivel central (Darmani y Crim, 2005), poco compatibles con un tratamiento crónico. Por último, la falta de efecto orexigénico en nuestro trabajo puede ser típica de este y otros agonistas cannabinoides no selectivos CB1/CB2 (Giuliani *et al.,* 2000; Aceto *et al.,* 2001; Dalton *et al.,* 2009).

Otro de los síntomas más molestos de los tratamientos con antineoplásicos es la neuropatía periférica. El desarrollo de neuropatía periférica se ha asociado con la aparición de diferentes signos funcionales tras el tratamiento crónico con cisplatino, como hiperalgesia y alodinia a estímulos mecánicos y/o estímulos fríos (Authier *et al.,* 2003b). Para probar el desarrollo de neuropatía periférica hemos elegido como parámetro el umbral para la alodinia mecánica, debido a los relativamente bajos niveles de estrés asociados a esta prueba. El cisplatino redujo el umbral de estimulación ya desde la primera administración y esta reducción llega a ser máxima tras la segunda o tercera. La administración de WIN previno la caída del umbral de estimulación. Este efecto es selectivo, ya que otras alteraciones producidas por la administración de cisplatino, como la anorexia, la pica y la reducción de la ganancia de peso corporal, no fueron abolidas. Además, éste efecto preventivo del WIN era evidente a dosis que no tenían efecto en la tétrada cannabinoide. Este hecho es beneficioso, ya que uno de los limitantes del uso terapéutico de los agonistas cannabinoides son sus efectos psicoactivos.

En otros modelos de dolor neuropático, como la administración de paclitaxel (Pascual *et al.,* 2005) o vincristina (Rahn *et al.,* 2007), el agonista sintético WIN ha demostrado tener propiedades analgésicas en administración aguda. En otros estudios de dolor neuropático en los que se han utilizado cannabinoides (Costa *et al.,* 2004, 2006; La Rana *et al.,* 2006; Palazzo *et al.,* 2006), éstos se administraban diariamente y se podría estar produciendo analgesia o reversión de los signos neuropáticos. En nuestro modelo parece más bien una prevención de la alodinia mecánica, ya que se evalúa al menos 4 días después de la administración del fármaco y la vida media de WIN es de 24-36 horas (Aceto *et al.,* 2001; Page *et al.,* 2007;). Además la dosis de 1 mg/kg de WIN, al administrarse de forma aguda, no produjo antinocicepción (ver resultado de la tétrada cannabinoide), pero sí tenía efecto en la prevención de la alodinia. Nuestros resultados refuerzan el interés de estos fármacos como herramienta terapéutica, ya que los cannabinoides podrían utilizarse de manera preventiva (incluso

días antes de la quimioterapia) para así ejercer una función preparatoria en el organismo.

La administración aguda de cisplatino además de alteraciones alimentarias (anorexia y aumento de la ingesta de caolín de manera dosis dependiente), produjo retraso en el vaciamiento gástrico inmediatamente después de la administración, lo que concuerda con otros estudios (Hecht *et al.,* 1997; Badary *et al.,* 2006; Malik *et al*, 2007). Cuando el cisplatino se utilizó a dosis bajas, las ratas se recuperaban a los dos o tres días de la administración, pero no sucedía así a dosis más elevadas. Estas alteraciones son similares a lo que hemos encontrado en el estudio de pica. Hay estudios que relacionan la náusea y el vómito con la distensión gástrica (Ladabaum *et al.,* 1998). Los estómagos de las ratas tratadas con dosis elevadas de cisplatino están distendidos, lo cual se asocia con retención de comida y acumulación de gas (Malik *et al.,* 2008). Por tanto, el descenso en la ingesta de comida y el aumento de la ingesta de caolín, podrían relacionarse con el retraso del vaciamiento gástrico y la distensión gástrica que hemos observado.

De nuestros resultados se desprende que para el estudio de la náusea y emesis en la rata, tanto la radiología como los estudios de pica son adecuados y complementarios. La radiología presenta una serie de ventajas: no es necesario que los animales estén aislados (con lo cual disminuye el estrés) y podemos ver el efecto de un fármaco en distintas partes del tracto gastrointestinal y a distintos tiempos desde la administración. Por otro lado, es un método muy reproducible y requiere pocos animales (6-8), mientras que los estudios de pica requieren un mayor número de animales (8-12) para conseguir diferencias estadísticamente significativas. Como es un método no invasivo, no tiene por qué utilizarse un animal para cada punto del estudio, así es más barato y ético. Nuestra técnica permite detectar alteraciones morfológicas o en el tamaño de las distintas regiones gastrointestinales. Aunque la resonancia magnética puede ser más útil en determinadas circunstancias, los instrumentos necesarios son más caros y menos accesibles. Además, a diferencia de los métodos invasivos, los animales no necesitan estar en ayunas. Otra ventaja es que al inmovilizar al animal en un cepo durante un breve periodo de tiempo, no necesitamos anestesia que pueda interferir con la motilidad GI (Torjman *et al.,* 2005). Nuestra metodología es poco estresante, pues una vez habituadas al habitáculo, las ratas entran por sí solas. Todo esto hace que nuestro método radiológico sea interesante para el estudio de alteraciones de la motilidad GI.

Por último, la administración crónica de cisplatino produjo, además de neuropatía periférica, alteraciones a nivel gastrointestinal compatibles con el desarrollo de neuropatía entérica. Hemos observado un retraso en el tránsito gastrointestinal que podría relacionarse con las alteraciones estructurales y ultraestructurales que hemos detectado en el tracto digestivo. La administración de cisplatino produjo daño histológico y una reducción del número de neuronas por ganglio. Este tipo de alteraciones del tracto GI habían sido descritas anteriormente en otras patologías (como la neuropatía diabética y el intestino irritable Boyer *et al.,* 2005), pero hasta ahora no se habían descrito por la utilización de cisplatino.

En el estudio del plexo mientérico, la proporción de neuronas que expresan NOS está aumentada; las inmunorreactivas para VIP y NPY tienden a estar aumentadas aunque no hay diferencias significativas; las neuronas que expresan SP y CR no se modifican y las que expresan CGRP han disminuido. Estas alteraciones son similares a las encontradas en otro tipo de neuropatías, como la diabética (tanto en rata, como en ratón) o las causadas por acrilamida o las que se presentan en modelos de síndrome de colon irritable (Belai y Burnstock, 1996; Boyer *et al.,* 2005). Esto podría indicar mecanismos similares en la inducción de estas alteraciones.

Estos trabajos abren nuevas perspectivas al tratamiento de los efectos adversos que la terapia con cisplatino causa en el tracto gastrointestinal y proporciona métodos para detectar dichas alteraciones.

6. Conclusiones

1. La administración aguda de cisplatino produce, además de anorexia y pica, reducción del vaciamiento gástrico y distensión del estómago. Estos signos presentan una relación temporal entre sí muy estrecha.

2. Con el tratamiento crónico, el efecto de cisplatino sobre la ingesta de comida y de caolín, el peso y la motilidad gástrica se intensifica. Además, junto con la neuropatía sensorial, se evidencian signos de neuropatía entérica similares a los presentes en otras situaciones patológicas.

3. Los cannabinoides, a dosis no psicoactivas y en tratamiento crónico semanal, previenen el desarrollo de los signos de neuropatía sensorial pero no los efectos en el comportamiento alimentario. Estos efectos sugieren que los cannabinoides podrían constituir una alternativa terapéutica eficaz en neuropatías dolorosas.

Anexo

En este anexo debo hablar de todas las personas con las que he colaborado, coautoras de las distintas separatas y que me han ayudado a la realización de todo el trabajo, aunque debo decir que éste no es el apartado de agradecimientos.

A mi llegada al laboratorio, compartí trabajo con el Dr. Antonio Rivera y la Dra. Margarita Suardíaz. De Antonio aprendí los “secretos” de las preparaciones whole-mount y la realización de los contajes neuronales. A Marga le debo mis conocimientos de baño de órganos y fue la primera que me enseñó los secretos del Excel y del Prisma.

Poco a poco fui tomando independencia y en la parte comportamental y del estudio de los antineoplásicos me ayudó Anna Chiarlone. Con ella empezamos los estudios de pica y la medida de la alodinia mecánica con los “filamentos de *von* Frey”. Debo agradecer al Dr. Pascual por enseñarme a valorar la alodinia mecánica y dónde conseguir los “filamentos de *von* Frey”. Cuando Anna terminó su estancia con nosotros, la sustituyeron Mónica Castillo y Pablo Cabezos. Sin ellos no hubiera sido posible la realización de este trabajo.

De mi etapa italiana tengo que agradecer al Dr. De Giorgio toda la ayuda prestada y toda la buena gente que he conocido gracias a él. Sin las técnicos del laboratorio italiano, no hubiera sido posible la realización de las tinciones inmunohistoquímicas ni toda la histología que he aprendido gracias a ellas, y el trabajo del día a día hubiera sido mucho más aburrido.

Por último, pero no menos importante, este trabajo no hubiera sido posible sin la coordinación de mis directoras de tesis, las Dras. Martín y Abalo.

Bibliografía

Aceto MD, Scates SM, Martin BB. Spontaneous and precipitated withdrawal with a synthetic cannabinoid, WIN 55212-2. *Eur J Pharmacol.* 2001; 416(1-2): 75-81.

Aggarwal SK, Antonio JDS, Sokhansanj A, Miller CM. Cisplatin induced peptic ulcer, vagotomy adrenal and calcium modulation. *Anticancer Drugs.* 1994; 5(2): 177-193.

Albers J, Chaudhry V, Cavaletti G, Donehower R. Interventions for preventing neuropathy caused by cisplatin and related compounds. *Cochrane Database of Syst Rev.* 2007; 24 (1): CD005228.

Andrews PL, Horn CC. Signals for náusea and emesis: Implications for models of upper gastrointestinal diseases. *Auton Neurosci.* 2006; 125(1-2): 100-115.

Andrews PLR, Okada F, Woods AJ, Hagiwara H, Kakaimoto S, Toyoda M, et al. The emetic and anti-emetic effects of the capsaicin analogue resiniferatoxin in *Suncus murinus,* the house musk shrew. *Br J Pharmacol.* 2000; 130: 1247–1254.

Aniansson A, Grimby G, Hedberg M. Compensatory muscle fiber hypertrophy in elderly men. *J Appl Physiol.* 1992; 73(3): 812-816.

Argyriou AA, Chroni E, Koutras A, Ellul J, Papapetropoulos S, Katsoulas G, Iconomou G, Kalofonos HP. Vitamin E for prophylaxis against chemotherapy-induced neuropathy: a randomized controlled trial. *Neurology.* 2005; 64(1): 26-31.

Armstrong T, Almadrones L, Gilbert MR. Chemotherapy-induced peripheral neuropathy. *Oncol Nurs Forum.* 2005; 32(2): 305-311.

Aung HH, Mehendale SR, Xie JT, Moss J, Yuan CS. Methylnaltrexone prevents morphine-induced kaolin intake in the rat. *Life Sci*. 2004; 74(22)**:** 2685–2691.

Authier N, Coudore F, Eschalier A, Fialip J. Pain related behaviour during vincristine-induced neuropathy in rats. *Neuroreport.* 1999; 10(5): 965-968.

Authier N, Fialip J, Eschalier A, Coudoré F. Assessment of allodynia and hyperalgesia after cisplatin administration to rats. *Neurosci Lett*. 2000; 291(2): 73-76.

Authier N, Gillet JP, Fialip J, Eschalier A, Coudore F. A new animal model of vincristine-induced nociceptive peripheral neuropathy. *Neurotoxicology.* 2003a; 24 (6): 797–805.

Authier N, Gillet JP, Fialip J, Eschalier A, Coudore F. An animal model of nociceptive peripheral neuropathy following repeated cisplatin injections. *Exp Neurol.* 2003b; 182 (1): 12–20.

Badary OA, Awad AS, Sherief MA, Hamada FM. In vitro and in vivo effects of ferulic acid on gastrointestinal motility: inhibition of cisplatin-induced delay in gastric emptying in rats. *World J Gastroenterol.* 2006; 12(33): 5363-5367.

Baimbridge KG, Celio MR, Rogers JH. Calcium-binding proteins in the nervous system. *Trends Neurosci.* 1992; 15(8): 303–308.

Baker DM, Santer RM. A quantitative study of the effects of age on the noradrenergic innervation of Auerbach's plexus in the rat. *Mech Ageing Dev.* 1988; 42(2): 147–158.

Bartolomucci A, Palanza P, Sacerdote P, Ceresini G, Chirieleison A, Panerai AE, Parmigiani S. Individual housing induces altered immuno-endocrine responses to psychological stress in male mice. *Psychoneuroendocrinology.* 2003; 28(4): 540-558.

Belai A, Burnstock G. Acrylamide-induced neuropathic changes in rat enteric nerves: similarities with effects of streptozotocin-diabetes. *J Auton Nerv Syst.* 1996; 58(1-2): 56-62.

Belai A, Burnstock G. Distribution and colocalization of nitric oxide synthase and calretinin in myenteric neurons of developing, aging, and Crohn's disease human small intestine. *Dig Dis Sci.* 1999; 44(8): 1579–1587.

Belai A., Cooper S, Burnstock G. Effect of age on NADPH-diaphorase-containing neurons of rat ileum and proximal colon. *Cell Tissue Res.* 1995; 279(2): 379–383.

Ben Amar M. Cannabinoids in medicine: a review of their therapeutic potential. *Journal of Ethnopharmacology*. 2006; 105(1–2): 1–25.

Berthoud HR. Anatomical demonstration of vagal input to nicotinamide acetamide dinucleotide phosphate diaphorase-positive (nitrergic) neurons in rat fundic stomach. *J Comp Neurol.* 1995; 358(3): 428-39.

Bianchi G, Vitali G, Caraceni A, Ravaglia S, Capri G, Cundari S, Zanna C, Gianni L. Symptomatic and neurophysiological responses of paclitaxel- or cisplatin-induced neuropathy to oral acetyl-L-carnitine. *Eur J Cancer.* 2005; 41(12): 1746-1750.

Bitar KN, Patil SB. Aging and gastrointestinal smooth muscle. *Mech Ageing Dev*. 2004; 125(12): 907-910.

Boogerd W, ten Bokkel Huinink WW, Dalesio O, Hoppenbrouwers WJ, van der Sande JJ. Cisplatin induced neuropathy: central, peripheral and autonomic nerve involvement. *J Neurooncol.* 1990; 9(3): 255-263.

Boyer L, Ghoreishi M, Templeman V, Vallance BA, Buchan AM, Jevon G, Jacobson K. Myenteric plexus injury and apoptosis in experimental colitis. *Auton Neurosci.* 2005; 117(1): 41-53.

Brookes SJ, Song ZM, Steele PA, Costa M. Identification of motor neurons to the longitudinal muscle of the guinea pig ileum. *Gastroenterology.* 1992; 103(3): 961–973.

Brookes SJ, Steele PA, Costa M. Calretinin immunoreactivity in cholinergic motor neurones, interneurones and vasomotor neurones in the guinea-pig small intestine. *Cell Tissue Res.* 1991; 263: 471–81.

Buckley NE, Hansson S, Harta G, Mezey E. Expression of the CB1 and CB2 receptor messenger RNAs during embryonic development in the rat. *Neuroscience.* 1998; 82(4): 1131-1149.

Choi JH, Lee CH, Chung DW, Hwang IK, Won MH, Seong JK, Yoon YS, Lee IS. Age-related changes of calbindin D-28k-immunoreactive neurons in the myenteric plexus of gerbil duodenum. *J Vet Med Sci.* 2008; 70(4): 343-348.

Comelli F, Giagnoni G, Bettoni I, Colleoni M, Costa B. The inhibition of monoacylglycerol lipase by URB602 showed an anti-inflammatory and anti-nociceptive effect in a murine model of acute inflammation. *Br J Pharmacol.* 2007;152(5):787-794.

Compton DR, Rice KC, De Costa BR, *et al.* Cannabinoid structure-activity relationships: correlation of receptor binding and in vivo activities. *J Pharmacol Exp Ther.* 1993; 265: 218-226.

Corns RA, Hidaka H, Santer RM. Neurocalcin-alpha immunoreactivity in the enteric nervous system of young and aged rats. *Cell Calcium.* 2002; 31 (2): 53–58.

Costa B, Colleoni M, Conti S, Trovato AE, Bianchi M, Sotgiu ML, Giagnoni G. Repeated treatment with the synthetic cannabinoid WIN 55,212-2 reduces both hyperalgesia and production of pronociceptive mediators in a rat model of neuropathic pain. *Br J Pharmacol.* 2004; 141(1): 4-8.

Costa B, Siniscalco D, Trovato AE, Comelli F, Sotgiu ML, Colleoni M, Maione S, Rossi F, Giagnoni G. AM404, an inhibitor of anandamide uptake, prevents pain behaviour and modulates cytokine and apoptotic pathways in a rat model of neuropathic pain. *Br J Pharmacol.* 2006; 148(7): 1022-1032.

Costa B, Trovato AE, Comelli F, Giagnoni G, Colleoni M. The non-psychoactive cannabis constituent cannabidiol is an orally effective therapeutic agent in rat chronic inflammatory and neuropathic pain. *Eur J Pharmacol*. 2007; 556(1-3): 75-83.

Costa M, Brookes SH. Architecture of enteric neural circuits involved in intestinal motility. *Eur Rev Med Pharmacol Sci.* 2008; 12 (Suppl 1):3-19.

Costa M, Brookes SJ, Steele PA, Gibbins I, Burcher E, Kandiah CJ. Neurochemical classification of myenteric neurons in the guinea-pig ileum. *Neuroscience.* 1996; 75(3): 949-967.

Costa M, Furness JB, Gibbins IL. Chemical coding of enteric neurons. *Prog Brain Res*. 1986; 68: 217-239

Coutts AA, Irving AJ, Mackie K, Pertwee RG, Anavi-Goffer S. Localisation of cannabinoid CB(1) receptor immunoreactivity in the guinea pig and rat myenteric plexus. *J Comp Neurol*. 2002; 448(4): 410–422.

Cowen T, Johnson RJ, Soubeyre V, Santer RM. Restricted diet rescues rat enteric motor neurones from age related cell death. *Gut*. 2000; 47(5): 653-660.

Craig JB, Powell BL. The management of náusea and vomiting in clinical oncology. *Am J Med Sci.* 1987; 293(1): 34-44.

Dalton VS, Wang H, Zavitsanou K. HU210-induced downregulation in cannabinoid CB1 receptor binding strongly correlates with body weight loss in the adult rat. *Neurochem Res* 2009; 34(7):1343-1353.

Daniel EE. Pharmacology of adrenergic, cholinergic, and drugs acting on other receptors in gastrointestinal muscle. En: Mediators and drugs in gastrointestinal motility I. (Handbook of Experimental Pharmacology). Editor: Bertaccini G. Ed: Springer-Verlag (Berlin). 1982. pp. 249–322.

Darmani NA, Crim JL. Delta-9-tetrahydrocannabinol differentially suppresses emesis versus enhanced locomotor activity produced by chemically diverse dopamine D2/D3 receptor agonists in the least shrew (Cryptotis parva). *Pharmacol Biochem Behav. 2*005; 80(1): 35-44.

Darmani NA. The cannabinoid CB1 receptor antagonist SR 141716A reverses the antiemetic and motor depressant actions of WIN 55, 212-2. *Eur J Pharmacol.* 2001a; 430(1): 49-58.

Darmani NA. Delta-9-tetrahydrocannabinol differentially suppresses cisplatin-induced emesis and indices of motor function via cannabinoid CB(1) receptors in the least shrew. *Pharmacol Biochem Behav.* 2001b; 69(1-2): 239-249.

Darmani NA. Delta(9)-tetrahydrocannabinol and synthetic cannabinoids prevent emesis produced by the cannabinoid CB(1) receptor antagonist/inverse agonist SR 141716A. *Neuropsychopharmacology.* 2001c; 24(2): 198-203.

Davis M, Maida V, Daeninck P, Pergolizzi J. The emerging role of cannabinoid neuromodulators in symptom management. *Supportive Care in Cancer*. 2007; 15(1): 63–71.

De Lillo AR, Rose S. Functional bowel disorders in the geriatric patient: constipation, fecal impaction, and fecal incontinence. *Am J Gastroenterol.* 2000; 95(4): 901-905.

de Souza RR, Moratelli HB, Borges N, Liberti EA. Age-induced nerve cell loss in the myenteric plexus of the small intestine in man. *Gerontology.* 1993; 39(4): 183–188.

Depoortere I, De Winter B, Thijs T, De Man J, Pelckmans P, Peeters T.Comparison of the gastroprokinetic effects of ghrelin, GHRP-6 and motilin in rats in vivo and in vitro. *Eur J Pharmacol.* 2005; 515(1-3): 160-168.

De Wit R, Herrstedt J, Rapoport B, Carides AD, Guoguang-Ma J, Elmer M, Schmidt C, Evans JK, Horgan KJ. The oral NK(1) antagonist, aprepitant, given with standard antiemetics provides protection against nausea and vomiting over multiple cycles of cisplatin-based chemotherapy: a combined analysis of two randomised, placebo-controlled phase III clinical trials. *Eur J Cancer.* 2004; 40(3): 403-410.

Diani AR, Grogan DM, Yates ME, Risinger DL, Gerritsen GC.Radiologic abnormalities and autonomic neuropathology in the digestive tract of the ketonuric diabetic Chinese hamster. *Diabetologia.* 1979;17(1):33-40.

Donnelly S, Walsh D. The symptom of advanced cancer. *Semin Oncol.* 1995; 22(2 Suppl 3): 67–72.

Driehuys B, Nouls J, Badea A, Bucholz E, Ghaghada K, Petiet A, Hedlund LW. Small animal imaging with magnetic resonance microscopy. *ILAR J*. 2008; 49(1): 35-53.

Eckel F, Schmelz R, Adelsberger H, Erdmann J, Quasthoff S, Lersch C. Prevention of oxaliplatin-induced neuropathy by carbamazepine. A pilot study. *Dtsch Med Wochenschr.* 2002; 127(3): 78-82.

El-Salhy M, Sandstrom O, Holmlund F. Age-induced changes in the enteric nervous system in the mouse. *Mech Ageing Dev.* 1999; 107(1): 93–103.

Feher E, Penzes L. Density of substance P, vasoactine intestinal polypeptide and somatostatin-containing nerve fibres in the ageing small intestine of the rats. *Gerontology.* 1987; 33(6): 341–348.

Feldstein AE, Miller SM, El-Youssef M, Rodeberg D, Lindor NM, Burgart LJ, Szurszewski JH, Farrugia G. Chronic intestinal pseudoobstruction associated with altered interstitial cells of Cajal networks*. J Pediatr Gastroenterol Nutr.* 2003; 36: 492–497.

Fernández-Ruiz J, Berrendero F, Hernández ML, Ramos JA. The endogenous cannabinoid system and brain development. *Trends Neurosci.* 2000; 23(1):14-20.

Firth M, Prather CM. Gastrointestinal and motility problems in the elderly patient. *Gastroenterology.* 2002; 122 (6): 1688-1700.

Fitzpatrick LR, Lambert RM, Pendley CE, Martin GE, Bostwick JS, Gessner GW, Airey JE, Youssefyeh RD, Pendleton RG, Decktor DL. RG 12915: a potent 5-hydroxytryptamine-3 antagonist that is an orally effective inhibitor of cytotoxic drug-induced emesis in the ferret and dog. *J Pharmacol Exp Ther.* 1990; 254(2):450-455.

Flynn J, Hanif N. Nabilone for the management of intractable nausea and vomiting in terminally staged AIDS*. J Palliat Care*. 1992; 8(2): 46-47.

Forster J, Damjanov I, Lin Z, Sarosiek I, Wetzel P, McCallum RW. Absence of the interstitial cells of Cajal in patients with gastroparesis and correlation with clinical findings. *J Gastrointest Surg.* 2005; 9: 102–108.

Fox A, Kesingland A, Gentry C, McNair K, Patel S, Urban L, James I. The role of central and peripheral Cannabinoid1 receptors in the antihyperalgesic activity of cannabinoids in a model of neuropathic pain. *Pain.* 2001; 92(1–2): 91–100.

Freeman R. Autonomic peripheral neuropathy. *Lancet.* 2005; 365(9466):1259-1270.

Furness JB. The enteric nervous system. Ed: Blackwell Publishing. Narayana Press, Odder (Dinamarca). 2006.

Furness JB, Costa M. The Enteric Nervous System. Ed: Churchill Livingstone (Edimburgo). 1987.

Gabella G. Fall in the number of myenteric neurons in ageing guinea-pigs. *Gastroenterology.* 1989**;** 96(6)**:** 1493–1497.

Gabella G. Hypertrophy of visceral smooth muscle. *Anat Embryol (Berl).* 1990; 182(5): 409-424.

Gabella G. Development and ageing of intestinal musculature and nerves: the guinea-pig taenia coli. *J Neurocytol.* 2001; 30(9-10): 733-766.

Gabra BH, Bailey CP, Kelly E, Smith FL, Henderson G, Dewey WL. Pre-treatment with a PKC or PKA inhibitor prevents the development of morphine tolerance but not physical dependence in mice. *Brain Res.* 2008; 1217: 70-77.

Galeazzi F, Blennerhassett PA, Qiu B, O'Byrne PM, Collins SM. Cigarette smoke aggravates experimental colitis in rats. *Gastroenterology.* 1999; 117(4): 877-83.

Gamelin L, Boisdron-Celle M, Delva R, Guérin-Meyer V, Ifrah N, Morel A, Gamelin E. Prevention of oxaliplatin-related neurotoxicity by calcium and magnesium infusions: a retrospective study of 161 patients receiving oxaliplatin combined with 5-Fluorouracil and leucovorin for advanced colorectal cancer. *Clin Cancer Res.* 2004; 10(12 Pt 1): 4055-4061.

Gaoni Y, Mechoulam R. Isolation, structure and partial synthesis of an active constituent of hashish. *J Am Chem Soc.* 1964, 86: 1646-1647.

Garcia JM, Cata JP, Dougherty PM, Smith RG. Ghrelin prevents cisplatin-induced mechanical hyperalgesia and cachexia. *Endocrinology.* 2008; 149(2): 455-460.

Giuliani D, Ottani A, Ferrari F. Effects of the cannabinoid receptor agonist, HU 210, on ingestive behaviour and body weight of rats. *Eur J Pharmacol.* 2000; 391: 275–279

Gomes OA, de Souza RR, Liberti EA. A preliminary investigation of the effects of ageing on the nerve cell number in the myenteric ganglia of the human colon, *Gerontology*. 1997; 43(4): 210–217.

Goto K, Westfall DP, Fleming WW. Denervation-induced changes in electrophysiologic parameters of the smooth muscle of the guinea-pig and rat vas deferens. *J Pharmacol Exp Ther.* 1978; 204(2):325-333.

Green ST, Nathwani D, Goldberg DJ, Kennedy DH. Nabilone as effective therapy for intractable nausea and vomiting in AIDS. *Br J Clin Pharmacol.* 1989; 28(4): 494-495.

Halford JC. Obesity drugs in clinical development. *Current Opinion in Investigational Drugs.* 2006; 7**(**4): 312–318.

Hammack JE, Michalak JC, Loprinzi CL, Sloan JA, Novotny PJ, Soori GS, Tirona MT, Rowland KM Jr, Stella PJ, Johnson JA. Phase III evaluation of nortriptyline for alleviation of symptoms of cis-platinum-induced peripheral neuropathy. *Pain.* 2002; 98(1-2): 195-203.

Hanani M, Fellig Y, Udassin R, Freund HR. Age-related changes in the morphology of the myenteric plexus of the human colon. *Auton Neurosci.* 2004; 113(1-2): 71-78.

Hargreaves K, Dubner R, Brown F, Flores C, Joris J. A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. *Pain.* 1988; 32(1): 77-88.

Harman D. Lipofuscin and ceroid formation: the cellular recycling system. *Adv Exp Med Biol.* 1989; 266: 3–15.

Hays NP, Roberts SB. The anorexia of aging in humans. *Physiol Behav.* 2006; 88(3): 257–266.

He CL, Burgart L, Wang L et al. Decreased interstitial cell of Cajal volume in patients with slow-transit constipation. *Gastroenterology.* 2000; 118: 14–21

Hecht JR, Lembo T, Chap L. Prolonged nausea and vomiting after high dose chemotherapy and autologous peripheral stem cell transplantation in the treatment of high risk breast carcinoma. *Cancer.* 1997; 79(9): 1698-1702.

Heizmann CW. Calcium-binding proteins: basic concepts and clinical implications. *Gen Physiol Biophys.* 1992; 11(5): 411–425.

Herzberg U, Eliav E, Bennett GJ, Kopin IJ. The analgesic effects of R(+)-WIN 55,212-2 mesylate, a high affinity cannabinoid agonist, in a rat model of neuropathic pain. *Neurosci Lett.* 1997; 221(2-3): 157-160.

Hildebrandt IJ, Su H, Weber WA. Anesthesia and other considerations for in vivo imaging of small animals. *ILAR J.* 2008; 49(1):17-26.

Hilpert F, Stähle A, Tomé O, Burges A, Rossner D, Späthe K, Heilmann V, Richter B, du Bois A; Arbeitsgemeinschaft Gynäkologische Onkologoie (AGO) Ovarian Cancer Study Group. Neuroprotection with amifostine in the first-line treatment of advanced ovarian cancer with carboplatin/paclitaxel-based chemotherapy--a double-blind, placebo-controlled, randomized phase II study from the Arbeitsgemeinschaft Gynäkologische Onkologoie (AGO) Ovarian Cancer Study Group. *Support Care Cancer*. 2005; 13(10): 797-805.

Hollander D, Tarnawski A, Stachura J, Gergely H. Morphologic changes in gastric mucosa of aging rats. *Dig Dis Sci.* 1989; 34 (11): 1692-1700.

Holzer P. Characterization of the stimulus-induced release of immunoreactive substance P from the myenteric plexus of the guinea-pig small intestine. *Brain Res.* 1984; 297(1): 127-136.

Horowitz M, Maddern GJ, Chatterton BE, Collins PJ, Harding PE, Shearman DJC. Changes in gastric emptying rates with age. *Clin Sci.* 1984; 67(2): 213–218.

Hoyle CHV, Cowen T, Saffrey MJ. Effects of age and diet on cholinergic neurotransmission in rat ileum. *S Neurosci.* 2002; 28 (72), p. 11 abstract.

Jantunen IT, Kataja VV, Muhonen TT. An overview of randomised studies comparing 5-HT3 receptor antagonists to conventional anti-emetics in the prophylaxis of acute chemotherapy-induced vomiting. *Eur J Cancer.* 1997; 33(1): 66-74.

Johnson RJ, Schemann M, Santer RM, Cowen T. The effects of age on the overall population and on sub-populations of myenteric neurons in the rat small intestine. *J Anat.* 1998; 192: 479–88.

Jordan K, Kasper C, Schmoll H. Chemotherapy-induced *náusea* and vomiting: current and new standards in the antiemetic prophylaxis and treatment. *Eur J Cancer.* 2005; 41(2): 199–205.

Jost WH. Gastrointestinal motility problems in patients with Parkinson's disease. Effects of antiparkinsonian treatment and guidelines for management. *Drugs Aging.* 1997; 10(4): 249–258.

Jourdan D, Boghossian S, Alloui A, Veyrat-Durebex C, Coudore MA, Eschalier A, Alliot J. Age-related changes in nociception and effect of morphine in the Lou rat. *Eur J Pain.* 2000; 4(3): 291-300.

Kadowaki M, Yoneda S, Takaki M. Involvement of a purinergic pathway in the sympathetic regulation of motility in rat ileum. *Auton Neurosci*. 2003; 104(1):10-16.

Karaosmanoglu T, Aygun B, Wade PR, Gershon MD. Regional differences in the number of neurons in the myenteric plexus of the guinea pig small intestine and colon: an evaluation of markers used to count neurons. *Anat Rec.* 1996; 244(4): 470–480.

King GL. Animal models in the study of vomiting. *Can J Physiol Pharmacol.* 1990; 68, 260–268.

Kosterlitz HW, Waterfield AA. In vitro models in the study of structure- Activity relationships of narcotic analgesics. *Annu Rev Pharmacol.* 1975; 15: 29–47.

Kris M, Grall R, Clark R, Tyson L, Groshen S. Control of chemotherapy-induced diarrhoea with synthetic enkephalin BW942C: a randomised trial with placebo in patients receiving cisplatin. *J Clin Oncol* 1988; 6: 663–668

Kromer W. Endogenous and exogenous opioids in the control of gastrointestinal motility and secretion. *Pharmacol Rev.* 1988; 40(2): 121-162.

Kulkarni-Narla A, Brown DR. Opioid, cannabinoid and vanilloid receptor localization on porcine cultured myenteric neurons. *Neurosci Lett.* 2001; 308(3): 153–156.

Kwiatkowska M, Parker LA, Burton P, Mechoulam R. A comparative analysis of the potential of cannabinoids and ondansetron to suppress cisplatin-induced emesis in the Suncus murinus (house musk shrew). *Psychopharmacology (Berl).* 2004; 174(2): 254-259.

Ladabaum U, Koshy SS, Woods ML, Hooper FG, Owyang C, Hasler WL. Differential symptomatic and electrogastrographic effects of distal and proximal human gastric distension. *Am J Physiol.* 1998; 275(3 Pt 1): G418-G424.

La Rana G, Russo R, Campolongo P, Bortolato M, Mangieri RA, Cuomo V, Iacono A, Raso GM, Meli R, Piomelli D, Calignano A. Modulation of neuropathic and inflammatory pain by the endocannabinoid transport inhibitor AM404 [N-(4-hydroxyphenyl)-eicosa-5,8,11,14-tetraenamide]. *J Pharmacol Exp Ther.* 2006; 317(3): 1365-1371.

Lee TH, Choi JJ, Kim DH, Choi S, Lee KR, Son M, Jin M. Gastroprokinetic effects of DA-9701, a new prokinetic agent formulated with Pharbitis Semen and Corydalis Tuber. *Phytomedicine.* 2008; 15(10): 836-843.

Legha SS. Vincristine neurotoxicity. Pathophysiology and management. *Med Toxicol.* 1986; 1(6):421-7.

Leslie FM. Methods used for the study of opioid receptors. *Pharmacol Rev.* 1987; 39(3): 197-249.

Liu YL, Malik NM, Sanger GJ, Andrews PL. Ghrelin alleviates cancer chemotherapy-associated dyspepsia in rodents. *Cancer Chemother Pharmacol.* 2006; 58(3): 326-333.

Madsen JL. Effects of gender, age and body mass index on gastrointestinal transit times. *Dig Dis Sci.* 1992; 37(10): 1548–1553.

Maestri A, De Pasquale Ceratti A, Cundari S, Zanna C, Cortesi E, Crinò L. A pilot study on the effect of acetyl-L-carnitine in paclitaxel- and cisplatin-induced peripheral neuropathy. *Tumori.* 2005; 91(2): 135-138.

Majumdar AP, Jaszewski, Dubick MA. Effect of aging on the gastrointestinal tract and the pancreas. *Proc Soc Exp Biol Med.* 1997; 215(2)**:** 134–144

Malik IA, Khan WA, Qazilbash M, Ata E, Butt A, Khan MA. Clinical efficacy of lorazepam in prophylaxis of anticipatory, acute, and delayed *náusea* and vomiting induced by high doses of cisplatin. A prospective randomized trial. *Am J Clin Oncol.* 1995; 18(2): 170-175.

Malik NM, Liu YL, Cole N, Sanger GJ, Andrews PL. Differential effects of dexamethasone, ondansetron and a tachykinin NK1 receptor antagonist (GR205171) on cisplatin-induced changes in behaviour, food intake, pica and gastric function in rats. *Eur J Pharmacol.* 2007; 555(2-3): 164-173.

Malik NM, Moore GB, Kaur R, Liu YL, Wood SL, Morrow RW, Sanger GJ, Andrews PL. Adaptive upregulation of gastric and hypothalamic ghrelin receptors and increased plasma ghrelin in a model of cancer chemotherapy-induced dyspepsia. *Regul Pept.* 2008; 148(1-3): 33-38.

Mantovani G, Macciò A, Curreli L, Lampis B, Ghiani M, Bianchi A, Contu P. Comparison of oral 5-HT3-receptor antagonists and low-dose oral metoclopramide plus i.m. dexamethasone for the prevention of delayed emesis in head and neck cancer patients receiving high-dose cisplatin. *Oncol Rep.* 1998; 5(1): 273-280.

Matsuda LA, Lolait SJ, Browstein MJ, Young AC, Bonner TI. Structure of a cannaboid receptor and functional expression of the cloned cDNA. *Nature.* 1990; 346 (6284): 561-564.

Matsuki N, Ueno S, Kaji T, Ishihara A, Wang CH, Saito H. Emesis induced by cancer chemotherapeutic agents in the Suncus murinus: a new experimental model. *Jpn J Pharmacol.* 1988; 48: 303–306.

Meciano Filho J, Carvalho VC, de Souza R.R. Nerve cell loss in the myenteric plexus of the human eosophagus in relation to age: a preliminary investigation. *Gerontology.* 1995; 41(1): 18–21.

Micieli G, Tosi P, Marcheselli S, Cavallini A. Autonomic dysfunction in Parkinson's disease. *Neurol Sci.* 2003; 24 (Suppl 1): S32–S34.

Miller MS, Galligan JJ, Burks TF. Accurate measurement of intestinal transit in the rat. *J Pharmacol Methods.* 1981; 6(3): 211-217.

Mitchell D, Wells C, Hoch N, Lind K, Woods SC, Mitchell LK. Poison induced pica in rats. *Physiol Behav.* 1976; 17 (4): 691–697.

Munro S, Thomas KL, Abu-Shaar M. Molecular characterization of a peripheral receptor for cannabinoids. *Nature.* 1993; 365(6441): 61-65.

Nahas GG, Schwartz IW, Adamec J, Manger WM. Tolerance of delta-9-tetrahydrocannabinol in the spontaneously hypertensive rat. *Proc Soc Exp Biol Med*. 1973; 142(1): 58-60.

Navari RM.Prevention of emesis from multiple-day and high-dose chemotherapy regimens. *J Natl Compr Canc Netw.* 2007; 5(1): 51-59.

Nelson K, Walsh D, Sheehan F. Cancer and chemotherapy-related upper gastrointestinal symptoms: the role of abnormal gastric motor function and its evaluation in cancer patients. *Support Care Cancer.* 2002; 10(6) :455-461.

Nelson KA, Walsh TD. The use of metoclopramide in anorexia due to the cancer associated dyspepsia syndrome (CADS). *J Palliat Care* 1993; 9: 14–18

Nelson KA, Walsh TD, Sheehan FG, O'Donovan PB, Falk GW. Assessment of upper gastrointestinal motility in the cancer-associated dyspepsia syndrome (CADS). *J Palliat Care* 1993; 9: 27–31.

Norton C. Constipation in older patients: effects on quality of life. *Br J Nurs.* 2006; 15(4): 188–192.

Nozaki M, AkeraT, Lee CY, Brody TM. The effects of age on the development of tolerance to and physical dependence on morphine in rats. *J Pharmacol Exp Ther.* 1975; 192(3): 506-512.

Nozaki-Taguchi N, Chaplan SR, Higuera ES, Ajakwe RC, Yaksh TL. Vincristine-induced allodynia in the rat. *Pain.* 2001; 93(1): 69-76.

Ocean JA, Vahdat LT. Chemotherapy-induced peripheral neuropathy: pathogenesis and emerging therapies. *Support Care Cancer.* 2004; 12(9): 619–625.

O'Mahony D, O'Leary P, Quigley EM. Aging and intestinal motility: a review of factors that affect intestinal motility in the aged. *Drugs Aging.* 2002; 19(7): 515–527.

Page ME, Oropeza VC, Sparks SE, Qian Y, Menko AS, Van Bockstaele EJ. Repeated cannabinoid administration increases indices of noradrenergic activity in rats. *Pharmacol Biochem Behav.* 2007; 86(1): 162-8.

Palazzo E, de Novellis V, Petrosino S, Marabese I, Vita D, Giordano C, Di Marzo V, Mangoni GS, Rossi F, Maione S. Neuropathic pain and the endocannabinoid system in the dorsal raphe: pharmacological treatment and interactions with the serotonergic system. *Eur J Neurosci*. 2006; 24(7): 2011-2020.

Park SB, Krishnan AV, Lin CS, Goldstein D, Friedlander M, Kiernan MC. Mechanisms underlying chemotherapy-induced neurotoxicity and the potential for neuroprotective strategies. *Curr Med Chem.* 2008; 15(29): 3081-3094.

Pascual D, Goicoechea C, Suardiaz M, Martin MI. A cannabinoid agonist, WIN 55,212-2, reduces neuropathic nociception induced by paclitaxel in rats. *Pain.* 2005; 118(1–2): 23–34.

Passik SD, Kirsh KL, Rosenfeld B, McDonald MV, Theobald DE. The changeable nature of patients' fears regarding chemotherapy: implications for palliative care. *J Pain Symptom Manage*. 2001; 21(2):113-120.

Paton WD. The action of morphine and related substances on contraction and on acetylcholine output of coaxially stimulated guinea-pig ileum. *Br J Pharmacol Chemother. 1957;* 12(1): 119–127.

Perry RL, Carrig CB, Williams JF, Johnson CA, Kaneene JB.Anatomic features and radiographic observations of gastric emptying and small intestinal motility in the rat. *Lab Anim Sci.* 1993; 43(6): 586-593.

Pertwee RG. Canabinoids and the gastrointestinal tract. *Gut.* 2001; 48(6): 849-867.

Pertwee RG, Ross RA. Cannabinoid receptors and their ligands. *Prostaglandins Leukot Essent Fatty Acids.* 2002; 66(2-3): 101-121.

Pertwee RG, Stevenson LA, Elrick DB, Mechoulam R, Corbett AD. Inhibitory effects of certain enantiomeric cannabinoids in the mouse vas deferens and the myenteric plexus preparation of guinea-pig small intestine. *Br J Pharmacol*. 1992; 105(4): 980-984.

Pfannkuche H, Schellhorn C, Schemann M, Aschenbach JR, Gabel G. Age-associated plasticity in the intrinsic innervation of the ovine rumen. *J Anat*. 2003; 203: 277–282.

Phillips LK, Rayner CK, Jones KL, Horowitz M. An update on autonomic neuropathy affecting the gastrointestinal tract. *Curr Diab Rep.* 2006; 6(6):417-23.

Phillips R, Powley T. Innervation of the gastrointesintinal tract: Patterns of aging. *Auton Neurosci.* 2007; 136(1-2): 1-19.

Phillips RJ, Hargrave SL, Rhodes BS, Zopf DA, Powley TL. Quantification of neurons in the myenteric plexus: an evaluation of putative pan-neuronal markers. *J Neurosci Methods.* 2004b;133(1-2): 99-107.

Phillips RJ, Kieffer EJ, Powley TL. Aging of the myenteric plexus: neuronal loss is specific to cholinergic neurons. *Auton Neurosci.* 2003; 106(2): 69-83.

Phillips RJ, Kieffer EJ, Powley TL. Loss of glia and neurons in the myenteric plexus of the aged Fischer 344 rat. *Anat Embryol (Berl).* 2004a; 209(1): 19-30.

Phillips RJ, Pairitz JC, Powley TL. Age-related neuronal loss in the submucosal plexus of the colon of Fischer 344 rats. *Neurobiol Aging.* 2007; 28: 1124–1137.

Phillips RJ, Powley TL. As the gut ages: timetables for aging of innervation vary by organ in the Fischer 344 rat. *J Comp Neurol.* 2001; 434(3): 358–377.

Phillips RJ, Powley TL. Plasticity of vagal afferents at the site of an incision in the wall of the stomach. *Auton Neurosci.* 2005; 123(1-2):.44-53.

Polomano RC, Bennett GJ. Chemotherapy-evoked painful peripheral neuropathy. *Pain Med.* 2001; 2(1): 8-14.

Polomano RC, Mannes AJ, Clark US, Bennett GJ. A painful peripheral neuropathy in the rat produced by the chemotherapeutic drug, paclitaxel. *Pain.* 2001; 94 (3): 293–304.

Powley TL, Holst MC, Boyd DB, Kelly JB. Three-dimensional reconstructions of autonomic projections to the gastrointestinal tract. *Microsc Res Tech.* 1994; 29(4):297-309.

Rahn EJ, Makriyannis A, Hohmann AG. Activation of cannabinoid CB1 and CB2 receptors suppresses neuropathic nociception evoked by the chemotherapeutic agent vincristine in rats. *Br J Pharmacol.* 2007; 152(5): 765-777.

Roberts D, Gelperin D, Wiley JW. Evidence for age-associated reduction in acetylcholine release and smooth muscle response in the rat colon. *Am J Physiol.* 1994; 267(4 Pt 1): G515-G522.

Romero J, Berrendero F, García-Gil L, de la Cruz P, Ramos JA, Fernández-Ruiz JJ. Loss of cannabinoid receptor binding and messenger RNA levels and cannabinoid agonist-stimulated [35S]guanylyl-5_O-(thio)-triphosphate binding in the basal ganglia of aged rats. *Neuroscience.* 1998; 84: 1075–1083.

Rosenberg B, Van Camp L, Krigas T. Inhibition of cell division in Escherichia coli by electrolysis products from a platinum electrode. *Nature.* 1965; 205 (4972): 698-699.

Rudd JA, Tse JY, Wai MK. Cisplatin-induced emesis in the cat: effect of granisetron and dexamethasone. *Eur J Pharmacol.* 2000; 391(1-2): 145-150.

Rudd JA, Jordan CC, Naylor RJ. Profiles of emetic action of cisplatin in the ferret: a potential model of acute and delayed emesis. *Eur J Pharmacol.* 1994; 262(1-2): R1-2.

Rudd JA, Yamamoto K, Yamatodani A, Takeda N. Differential action of ondansetron and dexamethasone to modify cisplatin-induced acute and delayed kaolin consumption (“pica”) in rats. *Eur J Pharmacol.* 2002; 454(1): 47–52.

Saeki M, Sakai M, Saito R, Kubota H, Ariumi H, Takano Y. Effects of HSP-117, a novel tachykinin NK1-receptor antagonist, on cisplatin-induced pica as a new evaluation of delayed emesis in rats. *Jpn J Pharmacol.* 2001; 86(3)**:** 359–362.

Saffrey MJ. Ageing of the enteric nervous system. *Mech Ageing Dev.* 2004; 125(12): 899-906.

Santer RM. Survival of the population of NADPH-diaphorase stained myenteric neurons in the small intestine of aged rats. *J Auton Nerv Syst.* 1994; 49(2): 115–121.

Santer RM, Baker DM. Enteric neuron numbers and sizes in Auerbach's plexus in the small and large intestine of adult and aged rats. *J Auton Nerv Syst.* 1988; 25(1):59-67.

Scarpignato C, Capovilla T, Bertaccini G. Action of caerulein on gastric emptying of the conscious rat. *Arch Int Pharmacodyn Ther.* 1980; 246(2): 286-294.

Schoonjans R, Van Vlem B, Van Heddeghem N, Vandamme W, Vanholder R, Lameire N, Lefebvre R, De Vos M. The 13C-octanoic acid breath test: validation of a new noninvasive method of measuring gastric emptying in rats. *Neurogastroenterol Motil*. 2002; 14(3): 287-293.

Schwartzberg LS. Chemotherapy-induced *náusea* and vomiting: clinician and patient perspectives. *J Support Oncol.* 2007; 5(2 Suppl 1): 5–12.

Scott DA, Wright CE, Angus JA. Evidence that CB-1 and CB-2 cannabinoid receptors mediate antinociception in neuropathic pain in the rat. *Pain.* 2004; 109(1-2): 124-131.

Simoneau II, Hamza MS, Mata HP, Siegel EM, Vanderah TW, Porreca F, Makriyannis A, Malan TP Jr. The cannabinoid agonist WIN55, 212-2 suppresses opioid-induced emesis in ferrets. *Anesthesiology.* 2001; 94(5): 882-887.

Simpkins, JW. Effects of aging on morphine withdrawal in the rat. *Exp Gerontol.* 1994; 29(1): 67-76.

Smith MA, Gray JD. Age-related differences in sensitivity to the antinociceptive effects of opioids in male rats. Influence of nociceptive intensity and intrinsic efficacy at the mu receptor. *Psychopharmacology (Berl).* 2001; 156 (4): 445–453.

Smits GJM, Lefebvre RA. Influence of aging on gastric emptying of liquids, small intestine transit, and fecal output in rats. *Exp Gerontol.* 1996; 31(5): 589–596.

Stapleton JA. Trial comes too late as psychiatric side effects end hope for rimonabant. *Addiction.* 2009; 104(2): 277-278.

Stillman M, Cata JP. Management of chemotherapy-induced peripheral neuropathy. *Curr Pain Headache Rep*. 2006; 10(4): 279-287.

Stubblefield MD, Vahdat LT, Balmaceda CM, Troxel AB, Hesdorffer CS, Gooch CL. Glutamine as a neuroprotective agent in high-dose paclitaxel-induced peripheral neuropathy: a clinical and electrophysiologic study. *Clin Oncol (R Coll Radiol).* 2005; 17(4): 271-276.

Takeda N, Hasegawa S, Morita M, Horii A, Uno A, Yamatodani A, Matsunaga T. Neuropharmacological mechanisms of emesis. I. Effects of antiemetic drugs on motion- and apomorphine-induced pica in rats. *Methods Find Exp Clin Pharmacol.* 1995a; 17 (9): 589–590.

Takeda N, Hasegawa S, Morita M, Horii A, Uno A, Yamatodani A, Matsunaga T. Neuropharmacological mechanisms of emesis. II. Effects of antiemetic drugs on cisplatin-induced pica in rats. *Methods Find Exp Clin Pharmacol.* 1995b; 17(10): 647–652.

Takeda N, Hasegawa S, Morita M, Matsunaga T. Pica in rats is analogous to emesis: an animal model in emesis research. *Pharmacol Biochem Behav*. 1993; 45(4): 817–821.

Talley NJ, Weaver AL, Zinsmeister AR, Melton LJ 3rd. Functional constipation and outlet delay: a population-based study. *Gastroenterology.* 1993; 105(3): 781-790.

Tanihata S, Igarashi H, Suzuki M, Uchiyama T. Cisplatin-induced early and delayed emesis in the pigeon. *Br J Pharmacol.* 2000; 130(1): 132-138.

Tezuka A, Ishihata A, Aita T, Katano Y. Aging-related alterations in the contractile responses to acetylcholine, muscarinic cholinoceptors and cholinesterase activities in jejunum and colon of the male Fischer 344 rats. *Exp Gerontol. 2004*; 39(1): 91-100.

Thrasivoulou C, Soubeyre V, Ridha H, Giuliani D, Giaroni C, Michael GJ, Saffrey MJ, Cowen T. Reactive oxygen species, dietary restriction and neurotrophic factors in age-related loss of myenteric neurons. *Aging Cell.* 2006; 5(3): 247-257.

Timiras PS. Disuse and aging: same problem, different outcomes. *J Gravit Physiol*. 1994; 1(1): P5-7.

Torjman MC, Joseph JI, Munsick C, Morishita M, Grunwald Z. Effects of isoflurane on gastrointestinal motility after brief exposure in rats. *Int J Pharm.* 2005; 294(1-2): 65-71.

Tramer MR, Carroll D, Campbell FA, Reynolds DJ, Moore RA, McQuay HJ. Cannabinoids for control of chemotherapy induced *náusea* and vomiting: quantitative systematic review. *BMJ.* 2001; 323(7303): 16–21.

Turiiski VI, Krustev AD, Sirakov VN, Getova DP. In vivo and in vitro study of the influence of the anticholinesterase drug galantamine on motor and evacuative functions of rat gastrointestinal tract. *Eur J Pharmacol.* 2004; 498(1-3): 233-239.

Turlejski K, Djavadian R. Life-long stability of neurons: a century of research on neurogenesis, neuronal death and neuron quantification in adult CNS. *Prog Brain Res.* 2002; 136: 39-65.

Umapathi T, Chaudhry V. Toxic neuropathy. *Curr Opin Neurol.* 2005; 18(5): 574–580.

Uno A, Takeda N, Horii A, Sakata Y, Yamatodani A, Kubo T. Effects of amygdala or hippocampus lesion on hypergravity-induced motion sickness in rats. *Acta Otolaryngol.* 2000; 120(7)**:** 860–865.

Van Crugten JT, Somogyi AA, Nation RL, Reynolds G. The effect of old age on the disposition and antinociceptive response of morphine and morphine-6 beta-glucuronide in the rat. *Pain.* 1997; 71(2): 199–205.

Van Cutsem E, Arends J. The causes and consequences of cancer-associated malnutrition. *Eur J Oncol Nurs*.2005; 9 (Suppl 2): S51–S63.

Vandertop WP, de Vries WB, Notermans NC, Nijsen MJ, de Wildt DJ, Tulleken CA, Gispen WH. Cisplatin-induced autonomic neuropathy: does it really exist? *J Neurooncol.* 1996; 27(1): 53-60.

Van Sickle MD, Oland LD, Ho W, Hillard CJ, Mackie K, Davison JS, Sharkey KAA. Cannabinoids inhibit emesis through CB1 receptors in the brainstem of the ferret. *Gastroenterology.* 2001; 121(4): 767-774.

Van Sickle MD, Oland LD, Mackie K, Davison JS, Sharkey KA. Delta9-tetrahydrocannabinol selectively acts on CB1 receptors in specific regions of dorsal vagal complex to inhibit emesis in ferrets. *Am J Physiol Gastrointest Liver Physiol*. 2003; 285(3): G566-G576.

Vinik AI, Maser RE, Mitchell BD, Freeman R. Diabetic autonomic neuropathy. *Diabetes Care.* 2003; 26(5): 1553-1579.

Wade PR. Aging and neural control of the GI tract. I. Age related changes in the enteric nervous system. *Am J Physiol Gastrointest Liver Physiol.* 2002; 283: G489–495.

Wade PR, Cowen T. Neurodegeneration: a key factor in the ageing gut. *Neurogastroenterol Motil.* 2004; 16 (Suppl. 1): 19–23.

Wade PR, Scheer G, Regan K, Lieb J. Age-related changes in colonic motility and myenteric neuronal populations. *Soc Neurosci Abstr.* 2001; 27: 840.812.

Wakabayashi K, Takahashi H. Neuropathology of autonomic nervous system in Parkinson's disease. *Eur Neurol.* 1997; 38 (Suppl. 2): 2–7

Ward SM, Sanders KM, Hirst GD. Role of interstitial cells of Cajal in neural control of gastrointestinal smooth muscles. *Neurogastroenterol Motil.* 2004; 16(Suppl. 1): 112–117.

Warner E. Neurotoxicity of cisplatin and taxol. *Int J Gynecol Cancer.* 1995; 5(3): 161-169.

Warr DG. Chemotherapy-and cancer-relates nausea and vomiting. *Current Oncology.* 2008; 15 (Suppl. 1); S4-S9.

Wegener M, Börsch G, Schaffstein J, Luerweg C, Leverkus F. Gastrointestinal transit disorders in patients with insulin-treated diabetes mellitus. *Dig Dis.* 1990; 8(1): 23-36.

Weijl NI, Hopman GD, Wipkink-Bakker A, Lentjes EG, Berger HM, Cleton FJ, Osanto S. Cisplatin combination chemotherapy induces a fall in plasma antioxidants of cancer patients. *Ann Oncol.* 1998; 9(12): 1331-1337.

Yamamoto K, Matsunaga S, Matsui M, Takeda N, Yamatodani A. Pica in mice as a new model for the study of emesis. *Methods Find Exp Clin Pharmacol.* 2002a; 24(3): 135-138.

Yamamoto K, Ngan MP, Takeda N, Yamatodani A, Rudd JA. Differential activity of drugs to induce emesis and pica behavior in *Suncus murinus* (house musk shrew) and rats. *Physiol Behav.* 2004; 83 (1): 151–156.

Yamamoto K, Takeda N, Yamatodani A. Establishment of an animal model for radiation-induced vomiting in rats using pica. *J Radiat Res (Tokyo).* 2002b; 43(2): 135-141.