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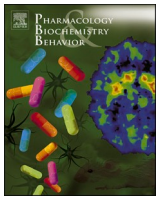

SGK1.1 isoform is involved in nociceptive modulation, offering a protective effect against noxious cold stimulus in a sexually dimorphic manner

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ABSTRACT

The serum and glucocorticoid-regulated kinase 1 (SGK1) is a widely expressed protein in the Central Nervous System (CNS), involved in regulating the activity of a wide variety of ion channels and transporters and physiological functions, such as neuronal excitability. SGK1.1 is a neuronal splice isoform of SGK1, expressed exclusively in the CNS, distributed in brain and cerebellum, that decreases neuronal excitability *via* up-regulation of M-current, linked to Kv7.2/3 potassium channels. Strategies to maintain increased SGK1.1 activity could be helpful in decreasing neuronal hyperexcitability, as occurs in neuropathic pain. Transgenic mice overexpressing SGK1.1 (B6.Tg.sgk1) offer a particularly relevant opportunity to assess the physiological involvement of this protein in nociception.

Behavior and physiological nociception were evaluated in male and female B6.Tg.sgk1 and wild-type mice (B6.WT), characterizing nociceptive thresholds to different nociceptive stimuli (thermal, chemical and mechanical), as well as the electrophysiological properties of cutaneous sensory Aδ-fibres isolated from the saphenous nerve. The acute antinociceptive effect of morphine was also evaluated.

Compared with B6.WT animals, male and female B6.Tg.sgk1 mice showed increased spontaneous locomotor activity. Regarding nociception, there were no differences between transgenic and wild-type mice in heat, chemical and mechanical thresholds, but interestingly, male B6.Tg.sgk1 mice were less sensitive to cold stimulus; B6.Tg.sgk1 animals showed lower sensitivity to morphine. Electrophysiological properties of cutaneous primary afferent fibres were maintained.

This is the first demonstration that the SGK1.1 isoform is involved in nociceptive modulation, offering a protective effect against noxious cold stimulus in a sexually dimorphic manner. B6.Tg.sgk1 mice offer a particularly relevant opportunity to further analyze the involvement of this protein in nociception, and studies in models of chronic, neuropathic pain are warranted.

1. Introduction

The serum and glucocorticoid-regulated kinase 1 (SGK1) is a protein widely expressed in the Central Nervous System (CNS), involved in regulating the activity of a wide variety of ion channels and transporters and underlying physiological functions, such as hormone release, neuronal excitability, cell proliferation and apoptosis (Arteaga et al., 2008). This protein has a recognized role in neuronal plasticity underlying memory and learning, and has been related with nociception-associated plasticity (Lin et al., 2015; Peng et al., 2012, 2013).

SGK1.1 is a neuronal splice isoform of SGK1 kinase protein, exclusively expressed in the CNS, distributed in brain and cerebellum (Arteaga et al., 2008; Martin-Batista et al., 2021; Wesch et al., 2010). SGK1.1 down-regulates the activity of the neuronal ASIC1 channel, by

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decreasing its expression at the cell surface (Arteaga et al., 2008). In addition, SGK1.1 decreases neuronal excitability *via* up-regulation of neuronal M-current, linked to Kv7.2/3 potassium channels (Armas-Capote et al., 2020; Miranda et al., 2013). M-current is a subthreshold voltage-gated K^+ current acting as a "brake" that suppresses abnormal ectopic discharges of neurons and controls neuronal hyperexcitability (Bi et al., 2011; Miranda et al., 2013). Transgenic mice expressing a constitutively active form of SGK1 and its isoforms (B6.Tg.sgk1 mice) show increased SGK1.1 activity in the CNS and enhanced M-current levels in superior cervical ganglion neurons (Miranda et al., 2013) and hippocampal neurons (Armas-Capote et al., 2020). They have been shown to be resistant to kainic acid-induced seizures, which supports a role for SGK1.1 protein in reducing neuronal excitability (Armas-Capote et al., 2020; Arteaga et al., 2008; Miranda et al., 2013).

Strategies to maintain increased SGK1.1 activity could be helpful in decreasing neuronal hyperexcitability, as occurs in neuropathic pain (Sałat et al., 2018; St. John Smith, 2018). Loss of function of the Kv7 potassium channel is thought to be a mechanism responsible for the development and maintenance of neuropathic pain (Abd-Elsayed et al., 2019). This type of pain is a highly prevalent global health problem affecting almost 6.9–10% of the population worldwide; since the currently available pharmacological treatment is only partially effective and is accompanied by undesired side effects, the search for new targets and therapeutic strategies remains a great challenge. Pain research through behavioral models in transgenic mice is a necessity and a very interesting strategy to understand the fundamental mechanisms of nociception, as well as to identify new therapeutic lines (Minett et al., 2014). Considering the role of SGK1.1 protein in the regulation of neuronal excitability, B6.Tg.sgk1 mice offer a particularly relevant opportunity to assess the physiological involvement of this protein in nociception.

In this work we aimed to evaluate the role of SGK1.1 in nociception, by characterizing the behavior of male and female mice with increased SGK1.1 activity. To this end, B6.Tg.sgk1 mice were compared to wild-type animals, B6.WT, of the same genetic background (C57BL/6), and analyzed: 1) general locomotor activity, 2) pain-related responses to acute thermal, chemical and mechanical and 3) the antinociceptive effect of morphine. Additionally, the electrophysiological properties of cutaneous Aδ-primary afferents of B6.Tg.sgk1 and B6.WT were evaluated.

2. Material and methods

2.1. Ethical statement

All experimental animal procedures were carried out according to a protocol approved by the Research Ethics Committee of Rey Juan Carlos University (Ref. 2107201711517). They followed the guidelines for the Care and Use of Laboratory Animals of the European Community (European Directive 2010/63/EU) and those of the International Association for the Study of Pain on ethical standards for investigation in animals (Zimmermann, 1983). All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.2. Animals

Experiments were conducted using both male and female wild-type (B6.WT) and SGK1 transgenic mice (B6.Tg.sgk1), all of which were at least 8–10 weeks old when tested. C57BL/6 J (B6) mice were obtained from Rey Juan Carlos University (URJC)/Charles River (France). B6.Tg.sgk1 mice were created and initially provided by La Laguna University, Tenerife (Armas-Capote et al., 2020; Martin-Batista et al., 2021; Miranda et al., 2013), and the breeding continued at the URJC. Control animals were age-matched, co-housed wild type mice from the inbred C57BL/6 J strain.

Transgenic mice were developed by pronuclear injection of embryos derived from breeding (C57BL/6 J X SJL/J)F2. Founder mice, which were already 50% C57BL/6, were backcrossed from 10 generations with C57BL/6 mice, making the resulting strain >99.95% C57BL/6. Subsequently, we have periodically crossed the homozygous transgenic strain with C57BL/6 and use the F1 to generate homozygous again, thus avoiding possible genetic drift in the strain.

Animals were ordered at least two weeks prior to study to allow for acclimatization; mice of each genotype were housed in clear plastic cages (2–4 mice/cage) in an enriched environment that included carton tubes and maintained at 23 ± 1 °C, 50–55% humidity, with a regular 12 h light/dark cycle (lights on at 7:00 am); animals were fed a standard laboratory diet and water *ad libitum*.

2.3. Experimental procedure

The first set of experiments was carried out to characterize the general behavior of B6.Tg.sgk1 *vs* B6.WT mice evaluating their spontaneous locomotor activity and measuring their body weight. The second set of experiments were conducted to characterize nociceptive responses; for this, the nociceptive threshold to different thermal, chemical and mechanical sensory stimuli were studied. Each sensorial stimulus was tested in a different group of animals. The third set of experiments were conducted to compare morphine antinociceptive effect on the hot-plate test both on B6.Tg.sgk1 and B6.WT mice. An additional group of animals was used to perform electrophysiological assays to study cutaneous Aδ-primary afferents activity. All assays were conducted in male and female mice although the oestrus cycle of the female mice was not considered when analysing results. All experiments were performed between 9:00 and 15:00 h. The researchers were blinded to genotype of mice.

2.4. Drugs

Morphine sulphate was purchased from Sigma-Aldrich, dissolved in 0.9% saline solution. It was intraperitoneally (i.p.) administered in a volume of 10 ml/kg at 5, 10 and 20 mg/kg. Doses were chosen based on previous studies of our group (Girón et al., 2002; Goicoechea et al., 2008) and on the data of our own pilot experiments in mice (not shown). Each mouse was tested in only one experiment and received a single dose of morphine.

2.5. Behavioral assays

2.5.1. Locomotor activity monitoring

Monitoring of the spontaneous locomotor activity was carried out using the infrared beam-based activity meter or actimeter (Cibertec S.A., Spain). The activity of four mice were simultaneously evaluated in four individual chambers; mice were allowed to run freely for 30 min. The system has infrared sensors located along the perimeter and analyzes the number of crosses (interruptions of photocell beams) performed by each animal (Bagüés et al., 2014; Kayser et al., 2015).

2.5.2. Hot-plate test and evaluation of antinociceptive effect of morphine

Mice were placed on the hot-plate (hot/cold plate, Ugo Basile, Italy), in a Plexiglas cylinder, with the temperature adjusted to 50 °C, 55 °C or 58 °C ± 0.1. The latency (s) to first observable response (fore/hind paws licking or jump) was taken as an index of nociceptive threshold. The cut-off time was set at 60, 30 and 20 s, respectively, to avoid damage in the paw. All mice were tested for the three temperatures but on different days, to avoid a possible accustoming to the plate.

This test was also used, in a different group of animals, to compare the antinociceptive effect of morphine on B6.Tg.sgk1 and B6.WT mice. Different doses of morphine (5, 10 and 20 mg/kg) were administered i.p. and the effect was tested 30 min after injection on the hot-plate test maintained at 55 °C (Goicoechea et al., 2008). Each dose of morphine was tested on a different group of animals. The antinociceptive effect

was expressed as percentage of the maximal possible effect (% MPE) using the following formula: % MPE = [post-drug latency] − [baseline latency] / [cut-off time − baseline latency] × 100.

2.5.3. Cold-plate test

The cold-plate (hot/cold plate, Ugo Basile, Italy) was used as previously described (Di Cesare Mannelli et al., 2017). Mice were placed on plate, in a Plexiglas cylinder, with the temperature adjusted to 4 ± 0.1 °C. The latency to the first response (hind paw lifting/licking or jump) was taken as an index of nociceptive threshold (Kanda and Gu, 2017). The cut-off time was set at 60 s.

Subsequently, the total number of nociceptive responses (brisk lifts of either hind paw + jumps) was counted for 5 min (Gopalsamy et al., 2017).

2.5.4. Writhing test

To assess the sensitivity to chemical noxious stimulus, a model of visceral pain induced by acetic acid 0.6% was used (Bi et al., 2011). Animals were injected i.p. with 0.3 ml of this solution and placed into a ventilated chamber with wooden bedding; 5 min after injection, the number of abdominal writhes was counted for 10 min. Each animal was tested only once.

2.5.5. von Frey test

Mice were placed inside ventilated Plexiglas chambers on an aluminium wire mesh floor for assessment of mechanical allodynia and were pre-habituated to these chambers 3 days before testing. von Frey filaments (Ugo Basile, Italy) ranging from 4 to 10 g were used; they were applied to the plantar skin of each hind paw alternately 5 times, allowing at least 30 s between each measurement; when the animal lifted paw 3 out of 5 times, that applied force was considered as nociceptive threshold (Garcia et al., 2019). Measurements were performed on both paws and the final threshold was adjusted to the average of the two paws.

2.6. Electrophysiological assays

The *in vitro* skin-saphenous nerve preparation (Reeh, 1986) contains sensory axons that innervate the skin of the hindpaws of the mice. It was used as a first approach to characterize the electrophysiological properties of cutaneous Aδ-primary afferents of B6.Tg.sgk1 mice and to check a possible correlation between somatic sensitivity of the neurons of these mice and nociceptive behavior.

Recordings from single Aδ-mechanosensitive fibres were performed in preparations isolated from male and female B6.Tg.sgk1 and B6.WT mice (6 male B6.Tg.sgk1: 11 fibres, 6 female B6.Tg.sgk1: 10 fibres, 6 male B6.WT: 10 fibres and 10 female B6.WT: 13 fibres).

The preparations were obtained as it has been described (Paniagua et al., 2020; Reeh, 1986). After the sacrifice of mice, the preparation (the hindpaw branch of the saphenous nerve and a piece of innervated hairy skin) was subcutaneously dissected and placed in an organ bath chamber, where the skin was pinned with the corium-side up and superfused (16 ml/min) with synthetic interstitial fluid (SIF) [(in mM): 108 NaCl, 3.5 KCl, 0.7 $MgSO_4$, 26 $NaHCO_3$, 1.7 NaH_2PO_4, 1.5 $CaCl_2$, 9.6 sodium gluconate, 5.5 glucose, 7.6 sucrose] saturated with carbogen (95% O_2–5% CO_2), (pH: 7.38, temperature: 32 ± 0.5 °C). The saphenous nerve was desheated under binocular control and single unit activity was recorded using a glass suction electrode containing a silver chloride filament and with the tip placed contacting the nerve (Martinez-Gomez and Lopez-Garcia, 2005). Extracellular activity of individual axons was amplified, recorded and analyzed off-line (CED Micro1401 and Spike 2 software, Cambridge Electronic Design Ltd., UK). Mechanosensitive fibres were first identified by manually probing the skin with a blunt rod to search for their receptive field (RF), and their conduction velocity (CV) was assessed by electrical stimulation of the RF with supramaximal square-wave pulses with a Teflon-coated steel microneurography electrode. The CV of a fibre was estimated using the distance between the recording and the stimulating electrode placed on the RF and the conduction delay.

As the CV of the studied fibres was <13.5 m/s, they were classified as Aδ-fibres (Wenk et al., 2006). In each fibre, the measured parameters were the presence of spontaneous activity and the responsiveness to mechanical stimulation. Thus, after a single unit identification, the possible presence of spontaneous discharge (defined as a discharge rate ≥ 1 spike/min) was recorded for a control period of 1 min, then its mechanical threshold was determined using von Frey filaments (0.08–14 mN, Ugo Basile®) and next, a mechanical stimulation protocol was applied using a stimulator with a plastic cylindrical probe (flat tip; diameter: 1 mm, Cibertec®) and feed-back regulation of the force, that was perpendicularly placed with a micromanipulator on the most sensitive spot of the skinny RF of the fibre. Each stimulus began with an adaptation period (3 s) in which the stimulator probe was touching the skin but not delivering any pressure. After the offset of any stimuli, the probe was lifted off the tissue to avoid fibre damage (desensitization). Three types of stimulus were applied: first, a ramp-shaped pressure stimulus (constantly increasing pressure stimulus from 0 to 200 mN; speed: 8 mN/s) and second, a constant supra-threshold non-noxious step-shape pressure stimulus of 5 s and third, a constant noxious step-shape pressure stimulus of 300 mN that was delivered for 10 s. The time interval between two consecutive stimuli was 5 min throughout the experiment.

The response of the fibres to the ramp-pressure stimulation allowed us to determine their electromechanical threshold (defined as the pressure that evoked the first spike that was followed by another spike within the next increment of force) and their supra-threshold response. In fibres that presented ongoing spontaneous activity, the electromechanical threshold was determined as the lowest force at which the instantaneous frequency of spikes continuously exceeded the mean basal activity +1 standard deviation. To analyze the response of the fibres to the ramp-pressure stimulation, the total number of spikes induced by each pressure increment and the total response to the ramp were counted. The response to the step-pressure stimuli was analyzed by quantifying the total number of spikes evoked during the step stimulation and for the ongoing discharge fibres, the basal activity (mean imp/s during the 30 s preceding the stimulus) was multiplied by the stimulus duration to calculate the ongoing discharge level throughout the stimulation period. This ongoing count was subtracted from the total count during the stimulus (Paniagua et al., 2017).

2.7. Statistical analysis

The sample size was calculated with G-power software; taking into account an alpha error of 0.05, a power of 0.8 and an effect size of 0.25, the minimum number of animals in each experimental group was 10; therefore, we considered 10 to be the minimum number of animals necessary to obtain reliable results.

Results were illustrated and analyzed using GraphPad Prism 7.0 (GraphPad Software, San Diego, USA). All data were checked for normality by D'Agostino-Pearson test and expressed as mean ± Standard Error of the Mean (S.E.M.). Two-tailed Unpaired *t*-test (for following normality data) or Mann Whitney test (for non-following normality data) were used to compare results obtained in B6.Tg.sgk1 *vs* B6.WT animals in the evaluation of nociceptive thresholds and locomotor activity. Values of $p < 0.05$ were considered statistically significant.

3. Results

B6.Tg.sgk1 and B6.WT mice showed no significant differences in body weight (male: B6.WT: 25.8 g ± 0.9 *vs* B6.Tg.sgk1: 25.9 g ± 0.8; female: B6.WT: 19.8 g ± 0.3 *vs* B6.Tg.sgk1: 19.3 g ± 0.4).

3.1. Locomotor activity

Both male and female B6.Tg.sgk1 mice showed significantly higher locomotor activity in the actimeter test than the B6.WT animals ($t = 2.9$, $df = 58$, $p < 0.01$ and, $t = 6.6$, $df = 58$, $p < 0.0001$, respectively *vs* WT, Unpaired *t*-test) (Fig. 1).

3.2. Nociceptive behavior to heat stimuli: hot-plate test

To investigate whether cutaneous nociceptive response to heat stimuli of B6.Tg.sgk1 mice was different from that of B6.WT animals, the hot-plate test was used. Both male and female mice showed lower withdrawal threshold as the temperature increased. B6.Tg.sgk1 and B6.WT male mice exhibited an equal latency in response to nociceptive stimulation applied to paws, at 50, 55 and 58 °C, with no significant differences between them ($t = 1.45$, $df = 18$, $p = 0.16$; $t = 0.70$, df = 18, $p = 0.38$ and $t = 1.81$, $df = 18$, $p = 0.11$ *vs* B6.WT, respectively, Unpaired *t*-test), showing similar heat pain sensitivity (Fig. 2A). In females, B6.Tg.sgk1 and B6.WT mice showed similar latencies B6.WT at the three temperatures tested (50 °C: $t = 0{,}98$, $df = 22$, $p = 0.30$; 55 °C: $t = 1.64$, $df = 22$, $p = 0.11$ and 58 °C: $t = 1.96$, $df = 22$ $p = 0.19$ *vs* B6.WT, Unpaired *t*-test) (Fig. 2B).

3.3. Nociceptive behavior to cold stimulus: cold-plate test

To investigate whether cutaneous nociceptive response to 4 °C cold stimuli of B6.Tg.sgk1 mice was different from that of B6.WT animals, we used the cold-plate test. B6.Tg.sgk1 male mice showed significantly higher withdrawal thresholds than B6.WT animals ($p < 0.01$ *vs* WT, Mann Whitney test) (Fig. 3A), whereas in female mice there were no significant differences *vs* B6.WT ($t = 0.40$, $df = 22$, $p = 0.69$, Unpaired *t*-test), and the latencies to noxious cold were very similar.

In line with these results, B6.Tg.sgk1 males exhibited less nociceptive responses than B6.WT ones, reaching significant statistically differences ($p < 0.0001$ *vs* WT, Mann Whitney test) (Fig. 3B) whereas the nociceptive responses in B6.Tg.sgk1 and B6.WT females were not statistically different ($p = 0.51$ *vs* B6.WT, Mann Whitney test).

These results suggest that B6.Tg.sgk1 and B6.WT female mice show normal painful responses to cold, whereas B6.Tg.sgk1 males are less sensitive to cold stimuli than their B6.WT mates.

3.4. Nociceptive behavior to chemical stimulus: Writhing test

I.p. injection of acetic acid caused the typical abdominal writhes in all animals, without significant differences between B6.Tg.sgk1 and B6.WT mice (males: $t = 0{,}74$, $df = 18$, $p = 0.61$; females: $t = 0.50$, $df = 18$, $p = 0.44$ *vs* B6.WT, Unpaired *t*-test) (Fig. 4A). Thus, B6.Tg.sgk1 animals were just as sensitive to chemical stimulus-induced visceral pain as B6.WT animals.

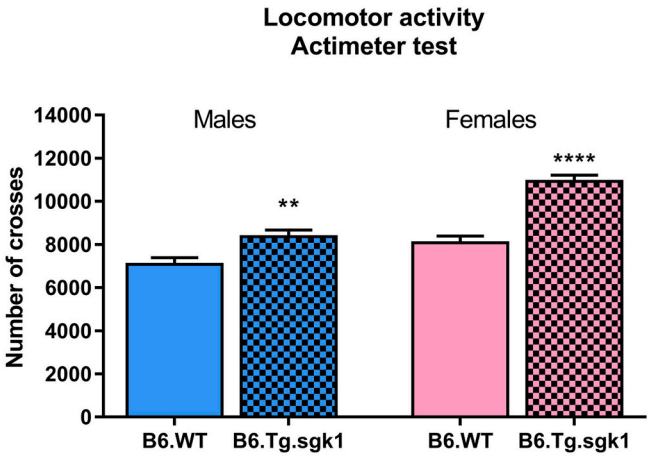

Fig. 1. Locomotor activity in B6 Wild-type (B6.WT) and B6.Tg.sgk1 male and female mice. Each bar shows the mean ± S.E.M of crosses registered by the actimeter test for 30 min. $^{**}p < 0.01$, $^{****}p < 0.0001$ *vs* WT mice, Unpaired *t*-test, $N = 30$.

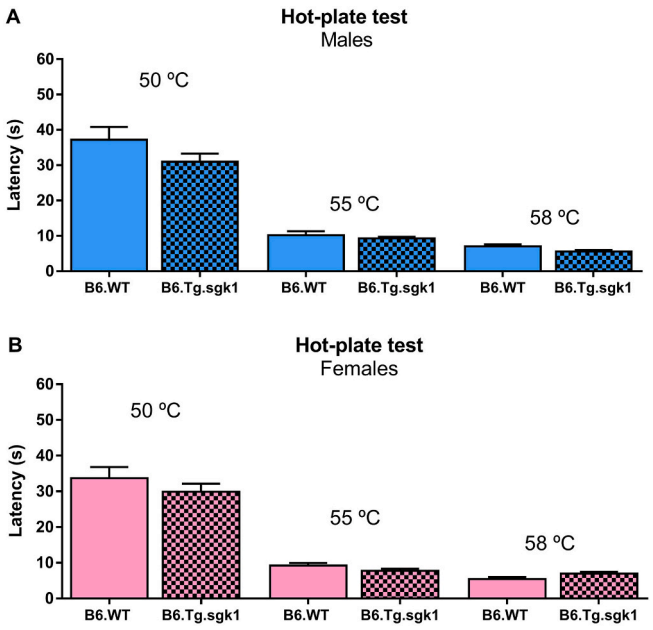

Fig. 2. Nociceptive withdrawal thresholds to heat stimuli in B6 Wild-type (B6.WT) and B6.Tg.sgk1 male and female mice. Figs. A and B show the latency (s) to the first observable response in the hot plate test at 50 °C, 55 °C and 58 °C. Each bar shows the mean ± S.E.M. of the latency, N = 10 for males, N = 12 for females.

3.5. Nociceptive behavior to mechanical stimulus: von Frey test

The mechanical thresholds to von Frey filaments applied on paws were very similar between B6.Tg.sgk1 and B6.WT mice, both in males as in females. There were no significant differences between B6.Tg.sgk1 and B6.WT mice in either sex (males: $t = 0.42$, $df = 20$, $p = 0.68$; females: $t = 0.15$, $df = 20$, $p = 0.88$ *vs* WT, Unpaired *t*-test) (Fig. 4B). Thus, B6.Tg.sgk1 animals were just as sensitive to mechanical stimulus as B6.WT animals.

3.6. Antinociceptive effect of morphine on hot-plate test

Morphine 5, 10 and 20 mg/kg, administered i.p., induced a dose-dependent effect in all experimental groups (Fig. 5). This effect was similar in B6.Tg.sgk1 and B6.WT male mice for doses of 5 and 10 mg/kg ($t = 0.32$, $df = 20$, $p = 0.75$ and $t = 1.00$, $df = 20$, $p = 0.34$ *vs* B6.WT, respectively, Unpaired *t*-test); however, B6.Tg.sgk1 mice showed a significantly lower antinociceptive effect of morphine 20 mg/kg ($t = 3.70$, $df = 20$, $p < 0.01$ *vs* B6.WT, Unpaired t-test) (Fig. 5A). In female mice, the morphine effect was similar in B6.Tg.sgk1 and B6.WT animals for doses of 5 and 10 mg/kg ($p = 0.91$ and $p = 0.57$ *vs* B6.WT, respectively, Mann Whitney test); in contrast, B6.Tg.sgk1 mice showed an antinociceptive effect of morphine 20 mg/kg significantly lower than wild-type mice ($p < 0.05$ *vs* WT, Mann Whitney test) (Fig. 5B). These results indicate that in B6.Tg.sgk1 mice the efficacy of morphine is lower than in B6.WT mice.

3.7. Electrophysiological activity of cutaneous sensory fibres

In order to further determine whether B6.Tg.sgk1 mice show differences in their sensory properties compared to B6.WT mice, we have

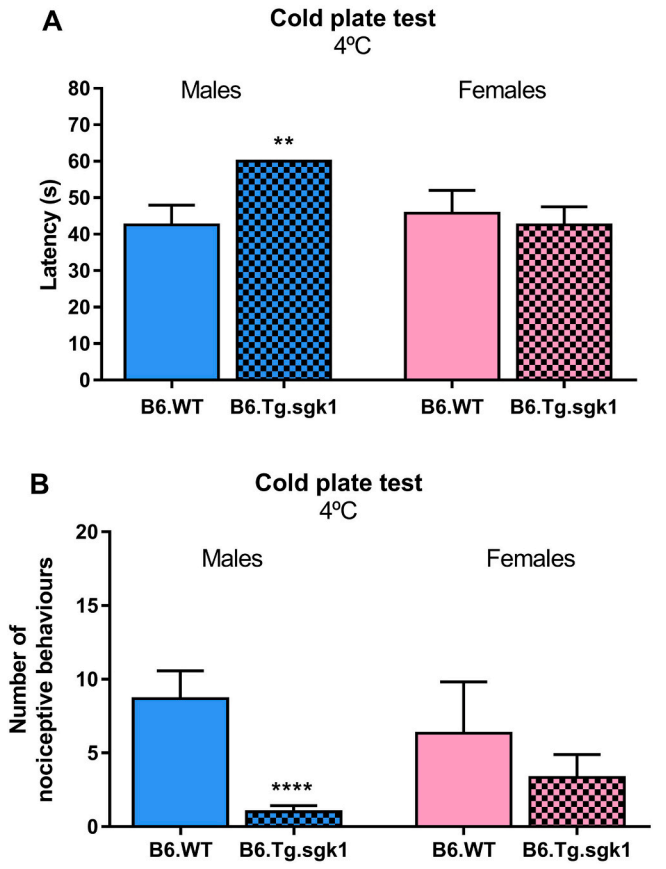

Fig. 3. Nociceptive withdrawal thresholds to cold nociceptive stimulus in the plate at 4 °C in wild-type (B6.WT) and transgenic (B6.Tg.sgk1) male and female mice. Panel A shows the latency (*s*) of paw withdrawal and panel B the number of nociceptive responses (hind paw lifts + jumps) of each animal for 5 min. Each bar shows data expressed as mean ± S.E.M. ** $p < 0.01$, **** $p < 0.0001$ *vs* B6.WT mice, Unpaired *t*-test or Mann Whitney test, $N = 13$ for males, $N = 12$ for females.

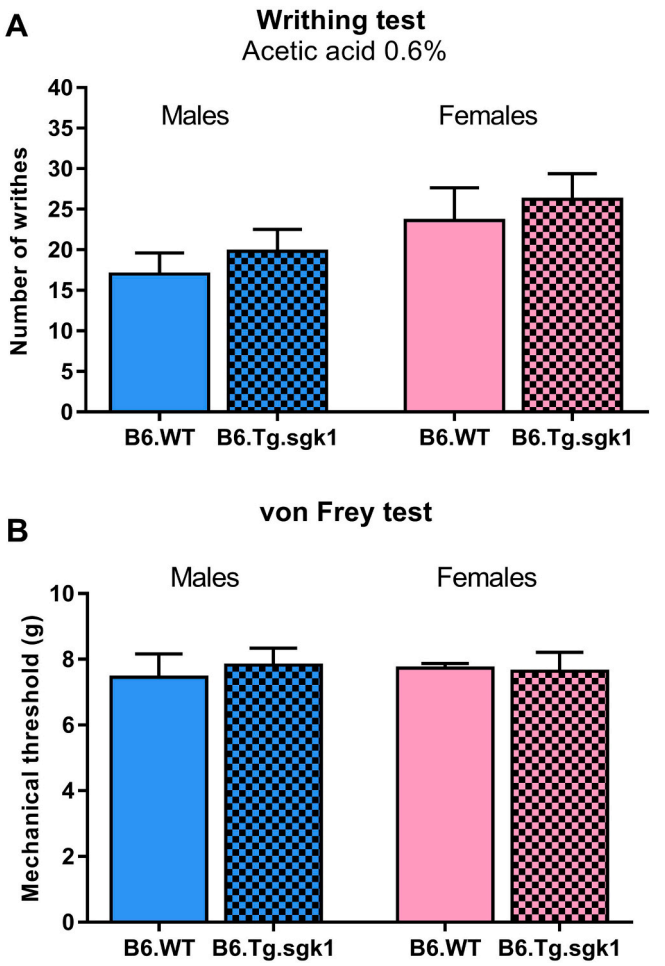

Fig. 4. Nociceptive behavior to chemical and mechanical stimuli in B6 wild-type (B6.WT) and B6.Tg.sgk1 male and female mice. Graph A shows the number of abdominal writhes, induced by acetic acid 0.6% i.p. injection, and graph B illustrates the withdrawal thresholds (g) to mechanical stimulation by von Frey filaments; each bar shows the results as the mean ± S.E.M, N = 10 for writhing test, N = 11 for von Frey test.

studied the electrophysiological activity of a subgroup of neurons of the saphenous nerve. Thus, the characteristics of the Aδ-fibres studied are shown in Table 1. Their CVs ranged from 3.0–13.1 m/s, they provided stable recordings throughout the entire experiment and all fibres had a single cutaneous RF.

The results show that there were no significant differences in CV between the fibres of B6.Tg.sgk1 and B6.WT mice, neither in males ($t = 1.502$, $df = 19$, $p = 0.1496$, Unpaired *t*-test) nor in females ($t = 0.7502$, $df = 21$, $p = 0.4615$, Unpaired t-test). Furthermore, the mechanical thresholds of the fibres determined with the von Frey filaments, were not statistically different either (males: $t = 0.03790$, $df = 19$, $p = 0.9702$; females: $t = 0.7973$, $df = 21$, $p = 0.4342$, Unpaired t-test). The percentage of fibres that showed spontaneous activity was slightly higher in both WT males and females.

When the responsiveness of the fibres of B6.Tg.sgk1 mice to the mechanical stimulation protocol was analyzed, the results of the ramp stimulation (Fig. 6A, B) did not show significant differences in the fibre electromechanical thresholds of B6.Tg.sgk1 *vs* B6.WT mice (males: $t = 0.1956$, $df = 19$, $p = 0.8470$; females: $t = 0.9149$, $df = 21$, $p = 0.3706$, Unpaired *t*-test) (Fig. 6C). However, the total response to the ramp was slightly higher for the fibres of B6.Tg.sgk1 mice than for those of B6.WT mice, although not significantly different (males: $t = 0.8979$, $df = 19$, $p = 0.3805$; females: $t = 1.184$, $df = 21$, $p = 0.2498$, Unpaired t-test) (Fig. 6D), and it is also shown in the response of the fibres of B6.Tg.sgk1 males to the noxious step stimulus (males: $t = 0.1042$, $df = 18$, $p = 0.9181$; females: $t = 0.2223$, $df = 21$, $p = 0.8263$, Unpaired t-test) (Fig. 6E).

4. Discussion

The general objective of this investigation is to study the role of the SGK1.1 protein in acute pain by characterizing the physiological nociception of mice with increased kinase activity (B6.Tg.sgk1 mice). Compared to B6.WT mice, male and female B6.Tg.sgk1 mice showed greater spontaneous locomotor activity. In relation to nociception, B6.Tg.sgk1 male mice were less sensitive to cold stimulus than B6.WT mice. Interestingly, B6.Tg.sgk1 animals showed lower morphine efficacy than B6.WT mates.

Both male and female B6.Tg.sgk1 mice exhibited greater spontaneous locomotion than WT mates, suggesting a role of SGK1.1 isoform on modulating motor activity. This result is in accordance with previous results, because SGK1.1 decreases the expression of the acid-sensing ion channel-1 (ASIC1) (Arteaga et al., 2008), and the functional activity of ASIC1 receptors is needed for a normal locomotor activity, since it has been described that ASIC1a−/− mice displayed increased locomotor activity (Jiang et al., 2013).

The hot-plate test was used to assess heat sensitivity; heat is applied on the paws and the painful behavioral responses are supraspinally integrated (Bars et al., 2001). B6.Tg.sgk1 mice behaved very similarly to B6.WT mice, at all the temperatures studied. Traditionally, the most

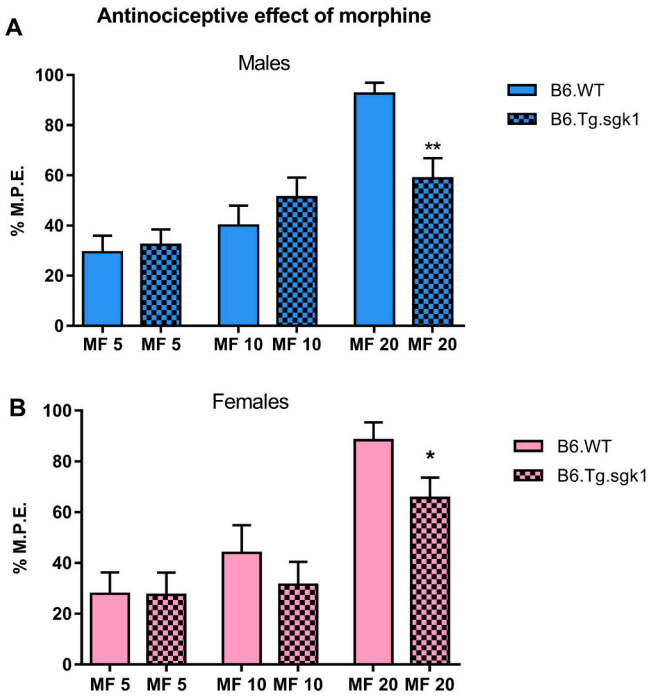

Fig. 5. Antinociceptive effect of morphine at 5, 10 and 20 mg/kg in B6 Wild-type (B6.WT) and B6.Tg.sgk1 male and female mice. Each bar shows the mean ± S.E.M of maximum possible effect induced by morphine on hot-plate test. * $p < 0.05$, ** $p < 0.01$ *vs* B6.WT mice, Unpaired *t*-test or Mann Whitney test, $N = 11$ for males, $N = 12$ for females.

Table 1
Summary of the characteristics of the Aδ-fibres studied from each experimental group of mice.

Group	CV (m/s)	n	% Fibres with SA	von Frey threshold (mN)
B6.WT males	5.6 ± 0.9	10	20	4.0 ± 0.69
B6.Tg.sgk1 males	7.7 ± 1.1	11	9.1	4.0 ± 0.31
B6.WT females	6.8 ± 0.9	13	7.7	5.9 ± 0.83
B6.Tg.sgk1 females	5.8 ± 1.0	10	0	5.0 ± 0.65

n: number of fibres per group, CV: conduction velocity, SA: spontaneous activity. B6.WT: B6 Wild-type mice, B6.Tg.sgk1: transgenic mice. The von Frey thresholds and CVs are expressed as the mean ± S.E.M. of each group of fibres.

valued painful behavior in the hot plate test is the licking of the forelegs (Goicoechea et al., 2008). However, in this study measuring the latency to this response in B6.WT and B6.Tg.sgk1 mice was highly variable so we decided to consider, as a positive result, the first behavior to appear (jump, lick, or lift). Different strains of mice have shown different sensitivity to pain in behavioral assays (Mogil et al., 1999a, Mogil et al., 1999b). Some peculiarities of B6 mice are known, such as they exhibit sensitivity in measures of acute nociception, high rates of voluntary wheel running (Carrigan and Dykstra, 2007), low mechanical allodynia, low hypersensitivity to heat, but suffering from cold allodynia (Smith et al., 2004).

In the cold plate test, the first observable response (jump, lick, or lift) was again considered as a positive response. The aforementioned variability in responses has also been noted by other researchers (Brenner et al., 2015). Greater reproducibility was obtained when counting the number of paw lifts or jumps on a defined period, such as 5 min. The results obtained from female mice showed greater variability (S.E.M.) because some of them jumped many times compared to males; however, they did not show different nociceptive behavior to the cold compared to wild-type mice.

Interestingly, there is a marked sex-dependent response to the cold stimulus; male animals showed lower responsiveness, as they had shorter latency and fewer painful responses to cold, whereas transgenic and WT female mice responded similarly. These results suggest that SGK1.1 protein-mediated reduction in cold sensitivity may be due to the increased activity of the M-current that characterizes these animals (Arteaga et al., 2008; Miranda et al., 2013). Previous results have demonstrated that pharmacological blockade of the M-current increases the excitability of a large fraction of C-fibres in response to cold stimuli, in which TRPM8 channels activation is required (Vetter et al., 2013). Although sex-related differences cannot be fully explained by this model, this decrease in cold-induced pain response supports a relevant role of SGK1.1 protein in the regulation of the response to the noxious cold stimulus. This suggests that it may constitute an interesting pharmacological target for the development of new analgesics.

Our results suggest that B6.Tg.sgk1 and B6.WT animals respond similarly to visceral nociceptive stimuli. The role of ASIC channels in visceral pain has been described previously (Deval et al., 2010); in particular, ASIC3 subtype receptors appear to be especially involved in proinflammatory responses (Davis, 2012). Since the SGK1.1 protein is mainly related to ASIC1 channels (Arteaga et al., 2008), this could explain the lack of differences between wild-type and B6.Tg.sgk1 mice, and may suggest that ASIC1 activation in not directly involved in acetic acid-induced pain behavior.

It should be considered that genetic background has been shown to modify nociception in mice (Wilson and Mogil, 2001). Tg.sgk1 mice were originally obtained by pronuclear injection of (C57BL/6 J X SJL/J) embryos and subsequently backcrossed for 10 generations into the C57BL/6 strain, obtaining a genetic background >99.95% pure. However, we cannot discard that small genomic fragments from the SJL/J background may be retained, particularly at the site of the transgene insertion, exerting some influence on behavioral tests.

The antinociceptive effect induced by morphine in B6.WT animals was very similar to that obtained by other authors (Carrigan and Dykstra, 2007). Male and female B6.Tg.sgk1 mice also showed a similar response to morphine administration, except for the highest dose tested. The spinal SGK1 protein has been described to contribute to the development of morphine tolerance by enhancing NF-kb p65/NMDA receptor signaling (Xiao et al., 2019), and it has been proposed as a potential target in the development of new strategies to reduce morphine tolerance. However, from present results, the SGK1.1 protein seems not to be involved in this modulation, indicating that the physiological effects mediated by SGK1 are not identical to those mediated by SGK1.1. Morphine signals mainly through mu-opioid receptors, which are coupled to the canonical $G\alpha_{i/o}$ pathway, resulting in decreased cAMP levels, and modulation of voltage-gated Ca^{2+} channels and GIRK K^{+} channels *via* βγ dimers (Al-Hasani and Bruchas, 2011; Pasternak and Pan, 2013). Kv7.2/3 channels increased their activity in response to intracellular cAMP, an effect that depends on their phosphorylation state (Schroeder et al., 1998). It is conceivable that the convergence of opposing signaling pathways mediated by SGK1.1 and opioid receptors on Kv7.2/3 channels explains the distinct response of the transgenic animals to high dose of morphine. It is also possible that increased SGK1.1 activity changes the expression or sensitivity of mu-opioid receptors, altering the maximal response achieved by morphine.

The results of the electrophysiological experiments showed that the studied characteristics of the cutaneous axonal Aδ-fibres of B6.Tg.sgk1 mice were very similar to those of their litter mates. Although the percentage of fibres that showed spontaneous activity in both male and female transgenic mice was lower and the response of the fibres to innocuous and noxious pressure stimulation (ramp and noxious step stimuli) was slightly higher than in B6.WT animals, these differences were not statistically significant. These findings are consistent with

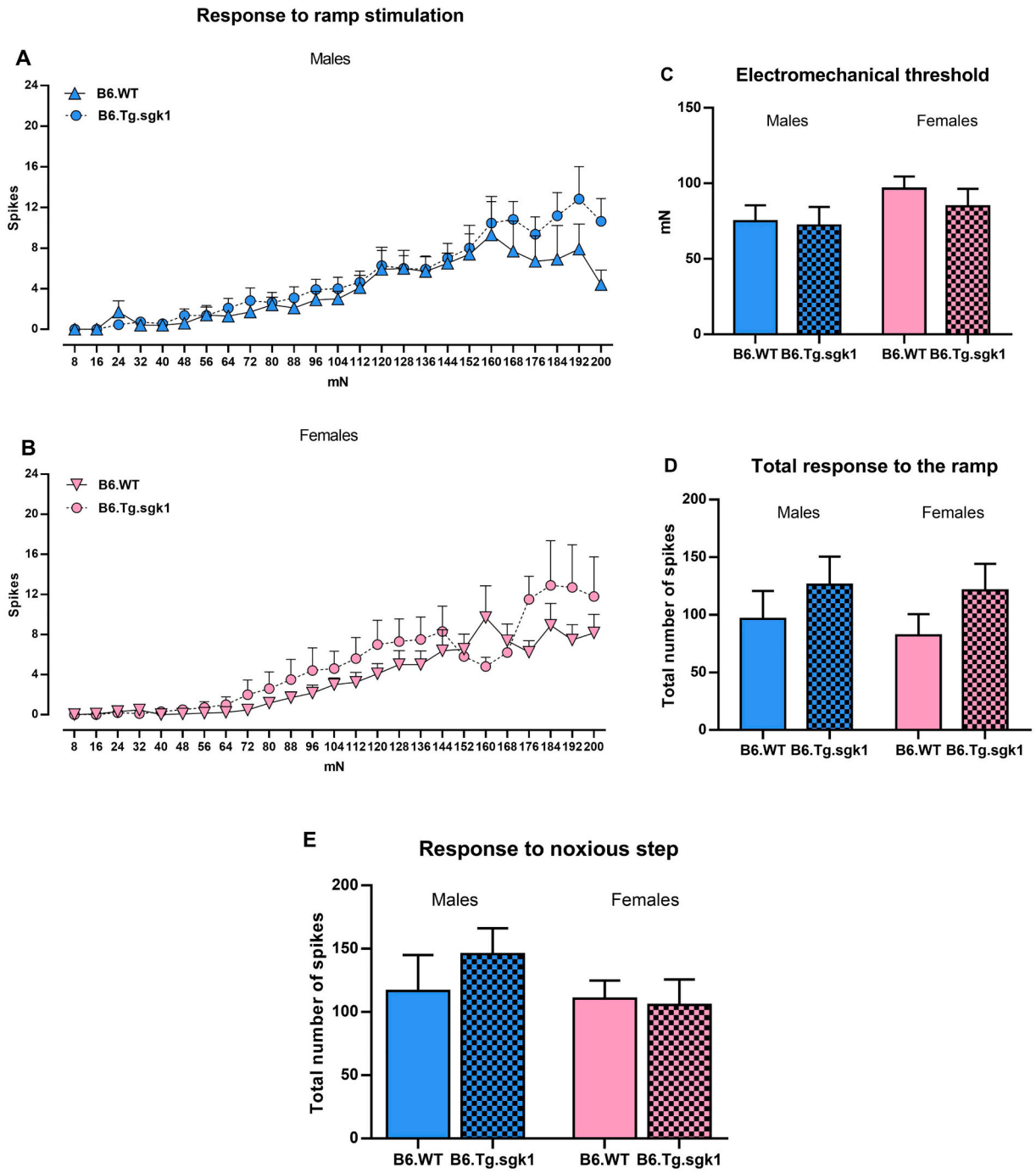

Fig. 6. Response of wild-type (B6.WT) and transgenic (B6.Tg.sgk1) mice Aδ-fibres to the pressure stimulation (ramp- and step-shape pressure stimuli). Graphs A and B show the response of the fibres of males and females, respectively to the ramp stimulus; each point of the lines shows the total number of spikes elicited by each pressure increment of the ramp (0–200 mN). Graph C describes the electromechanical thresholds of the fibres obtained in the ramp. Graph D shows the total response of the fibres to the entire ramp. Graph E shows the response of the fibres to a noxious step stimulus of 300 mN. Results are expressed as the mean response ± S.E.M. of 10–13 fibres from 11 animals/group.

those of previous studies carried out in both peripheral and central sensory neurons (Armas-Capote et al., 2020; Miranda et al., 2013) and correlate with the results obtained in the von Frey test. We have not studied other cutaneous sensory neurons (unmyelinated and thick myelinated), but our results suggest that the overexpression of the SGK1.1 isoform does not modify the physiological transmission or the sensibility of somatic primary afferents to mechanical stimuli.

Since results obtained from the fibres records do not allow us to explain the differences obtained between B6.WT and B6.Tg.sgk1 mice, those differences could be due to supraspinal actions related with pain control. Once nociceptive information reach the brain, regions like nucleus accumbens, periaqueductal grey matter, as well as somatosensorial cortex and prefrontal cortex play a critical role regulating acute pain (Bushnell et al., 2013), both intracerebrally (Vogt, 2005) and in a "top-bottom" manner (Tobaldini et al., 2019). In fact, the presence of SGK1 has been widely demonstrated in pain-related regions, both peripherally and centrally (for review, Liu et al., 2021), including the aforementioned areas; however the presence of SGK1.1 in these brain regions has not yet been reported. Nevertheless we do have recently described that SGK1.1 is expressed in pyramidal neurons of the CA1 and CA3 regions from

hippocampus that constitute a glutamatergic circuit involved in excitatory synaptic transmission (Martin-Batista et al., 2021). Since it has been described that, in an animal model of neuropathic pain, there is a decrease in glutamate and BDNF levels in these same CA1 region of the hippocampus (Saffarpour et al., 2017), a link between the increase in nociceptive threshold observed in our experiments and the presence of SGK1.1 in cortical/lymbic regions cannot be discarded. Recently, in an animal model of acute postoperative pain, c-Fos increased in the hippocampus and mPFC, confirming that acute postoperative pain can directly affect the function of the hippocampus and mPFC (Zhao et al., 2021).

In summary, our results indicate that SGK1.1 isoform is related to nociceptive modulation, offering a protective effect against noxious cold stimulus. Additional work analysing these results in animal models of chronic pain is warranted.

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