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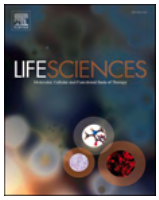

Generation and phenotypic characterization of a sigma-1 receptor knockout rat

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ABSTRACT

The sigma-1 receptor (σ1R) is a chaperone involved in multiple physiological and pathological processes, including pain modulation, neuroprotection, and neurodegenerative diseases. Despite its functional significance, its precise roles remain unclear due to the lack of suitable models for detailed mechanistic studies. In this work, we describe the generation and phenotypic characterization of a novel σ1R knockout (σ1R KO) rat model. Using CRISPR/Cas9 technology, we introduced a specific 218-base-pair deletion into the σ1R gene, resulting in a complete loss of receptor expression, as confirmed by Western blot, immunohistochemistry, and binding assays. Comprehensive phenotypic analyses revealed no major developmental or behavioral abnormalities in σ1R KO rats under baseline conditions, suggesting that σ1R is not essential for development or survival. Additionally, no genotype-related differences were observed in cellular or biochemical blood parameters. Motor function tests (rotarod, grip strength, and wheel running) showed no deficits; however, σ1R KO rats displayed reduced exploratory behavior in actimetry and markedly diminished burrowing behavior. By contrast, no anxiodepressive-like behaviors were observed in the open field, startle, or forced swim tests. Sensory testing of naive rats revealed no significant genotype-related differences in responses to mechanical, heat, or cold stimuli, or in the formalin test (chemical-induced pain). However, σ1R KO rats displayed attenuated neuropathic pain after traumatic nerve injury (spared nerve injury), highlighting the role of σ1R in pain sensitization pathways. This study establishes the σ1R KO rat as a valuable tool for investigating σ1R-mediated mechanisms and for developing therapeutic strategies targeting σ1R for chronic pain, neurodegeneration, and psychiatric disorders.

1. Introduction

The sigma-1 receptor (σ1R) is a ligand-regulated chaperone expressed in a wide range of tissues, with important roles in the nervous system [1–3]. In this regard, the σ1R has been proposed as a therapeutic target for the treatment of several neurological disorders including pain [4], neurodegenerative diseases [3], and anxio-depressive behaviors [2], among others.

Initially misclassified as an opioid receptor [5], it was reclassified as a non-opioid receptor in 1992 [6]. However, its structure was not described in detail until 2016 [7]. Although it has been extensively studied and various ligands (agonists and antagonists) with therapeutic properties have been developed [8], its physiological function in different contexts is still not fully elucidated, highlighting the need for further research.

The creation and phenotypic characterization of a σ1R knockout

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(σ1R KO) mouse, completed in 2003 [9], was fundamental for studying the role of the σ1R in different pathological contexts. For example, σ1R KO mice have shown reduced pain development in different contexts [10], displayed depressive-like behaviors [11], increased susceptibility to seizures [12], and exacerbated neurodegeneration in models of amyotrophic lateral sclerosis [13,14], Parkinson's disease [15,16], and Alzheimer's disease [17,18]. Moreover, the use of this knockout model, together with studies using antagonists (e.g. [19–21]) and agonists (e.g. [22–24]) in wild type (WT) animals, has been key for the preclinical development of the σ1R as a clinical target, enabling several molecules to reach clinical trials (e.g., S1RA for neuropathic pain [25,26], and pridopidine for neurodegenerative diseases [27,28]).

Despite the importance of the knockout mouse model, a σ1R KO rat model had not been developed to date, representing a limitation from a translational perspective. Rats offer several advantages for biomedical research, including larger body size, higher cognitive abilities,and greater genomic similarity to human beings [29]. In fact, neural and immunological differences between rats and mice have been documented, affecting the extrapolation of preclinical results to humans [30,31]. In this regard, rats may serve as a more translational model for certain human pathologies [29,32], and the use of this knockout model could improve our understanding of the role of the σ1R in various pathological contexts, potentially revealing new therapeutic indications for this receptor. Furthermore, obtaining equivalent results in knockout models across two different species strengthens the robustness of findings and increases their potential clinical relevance.

The aim of this study is the generation and phenotypic characterization of a σ1R KO rat. For this purpose, after confirming the effective σ1R depletion, we conducted a wide variety of behavioral tests to evaluate the implications of σ1R absence on viability and survival, general well-being, locomotion, anxiety, depression, and nociception/pain.

2. Materials and methods

2.1. Generation of the σ1R KO rat

The σ1R KO rat was generated using CRISPR/Cas9 technology by Horizon Discovery (2033 Westport Center Drive, USA) on a Wistar Han genetic background. The σ1R KO rats were produced using the SAGE-speed® platform, which involves pronuclear microinjection of CRISPR/Cas9 reagents, followed by embryo transfer into pseudopregnant females. A single-guide RNA (sgRNA) targeting exon 2 of the σ1R gene (see Supplementary Fig. 1), together with Cas9 protein, was co-injected to induce double-strand breaks in the DNA. This resulted in a specific 218 base-pair (bp) deletion (highlighted in blue in Supplementary Fig. 1), encompassing 46 bp of exon 1 (green in Supplementary Fig. 1) and 34 bp of exon 2 (yellow in Supplementary Fig. 1). This deletion disrupted the reading frame, effectively abolishing the production of a functional σ1R protein. Several founder rats carrying distinct mutations (including deletions of 218 bp, 7 bp, and 8 bp) were identified. Multiple F1 breeding pairs were then established from the founder carrying the 218 bp deletion. Heterozygous intercrossing was subsequently carried out and expanded by Envigo/Inotiv (The Netherlands), yielding WT, heterozygous, and homozygous KO offspring.

2.2. Experimental animals

Experiments were conducted using σ1R KO and WT rats of both sexes from heterozygous matings supplied by Envigo/Inotiv. Immunohistochemistry was also performed on WT (Charles River, Spain) and σ1R KO CD-1 mice (Esteve Pharmaceuticals, Spain) weighing 26–32 g (8 to 11 weeks old) [33]. Most experiments were carried out on young rats (9–10 weeks old), while certain experiments were performed on older rats (1 year old). Average body weights are specified in Supplementary Fig. 2. Rats were housed in colony cages (6 per cage) in a temperature-controlled room (22 ± 2 °C) with an automatic 12-h light/dark cycle (08:00–20:00). A plastic tunnel was placed in each cage for environmental enrichment. The rats were fed a standard laboratory diet and had access to tap water until the experiments began. Behavioral testing was performed during the light phase (09:00–15:00). The rats were randomized to treatment groups, with a balanced number of rats from each group tested daily. Random testing was also conducted throughout the estrous cycle. The rats were handled in accordance with international guidelines (European Communities Council directive 2010/63), and the experimental protocols were approved by regional (Junta de Andalucía) and institutional (Research Ethics Committee of the University of Granada) authorities. To minimize the number of rats used, the same ones were used for behavioral, biochemical, and immunostaining evaluation whenever possible.

2.3. Polymerase chain reaction (PCR) genotyping

To ensure the proper selection of the genetic background (WT or σ1R KO), genotyping was periodically performed at the end of the behavioral testing. Genomic deoxyribonucleic acid (DNA) was obtained from tail tips using the DNeasy Blood & Tissue kit (QIAGEN, Germany) according to the manufacturer's instructions. PCR amplifications were conducted using the HotStarTaq Plus Master Mix Kit (QIAGEN, Germany) and with 0.5 μM of each primer (Invitrogen Ltd., UK). The PCR was performed with a thermal controller using an initial template denaturation at 94 °C, followed by 35 cycles: 30 s at 94 °C, 30 s at 57 °C and 20 s at 70 °C; and, as a final extension step, 10 min at 72 °C. To differentiate WT and σ1R KO alleles, the primer pair composed of 1RS (5′-ACG TTG GTG GTA CCA GGC TGC-3′) and 2RA (5′-GCA CGT ACT CAG ACA GCG AGG-3′) was used as the primary genotyping strategy, producing DNA fragments of 476 bp for the WT allele and 258 bp for the σ1R KO allele. A second primer pair combining 1RS and 3RA (5′-AGC CTC CGC AGC TCC ACG-3′) was used as a validation method, generating fragments of 368 bp (WT) and 150 bp (σ1R KO). Amplified products were analyzed by electrophoresis on 4 % agarose gel containing ethidium bromide. The gels were then photographed with a UV transilluminator to visualize the ethidium bromide-stained bands. At the beginning of colony expansion, the σ1R KO sequence was further validated by sequencing using the primer 4RA (5′-ACCCACTGCAGCTCCTCATCC-3′) to confirm the accuracy of the edited allele (data not shown). All primers were synthesized by Sigma-Aldrich (USA).

2.4. Western blot

Dissected spinal cord, dorsal root ganglia (DRG), and liver samples from WT and σ1R KO rats (n = 9–10 per group) were homogenized in radioimmunoprecipitation assay (RIPA) buffer, and the supernatant was collected. Equal amounts of protein (50, 20, and 30 μg for spinal cord, DRG and liver samples, respectively) were separated by 12 % (w/v) SDS-PAGE and transferred onto polyvinylidene difluoride membranes, which were then blocked for 1 h with 5 % non-fat dry milk in Tris-buffered saline containing Tween-20 (TBS-T). They were then incubated overnight at 4 °C in 1 % non-fat dry milk in T-TBS containing a rabbit polyclonal σ1R antibody (IgG; ab223702) from Abcam (UK) [34]. Immunoreactive bands were detected using a peroxidase reaction with an enhanced chemiluminescence method (Clarity Western ECL Substrate, Bio-Rad, USA) and ChemiDoc Imaging System (Bio-Rad, USA). The housekeeping protein GAPDH (hFAB™ Rhodamine Anti-GAPDH Primary Antibody, 12004168, Bio-Rad, USA) was used as a loading control. No densitometric quantifications were needed because we did not observe any detectable bands in σ1R KO rat samples (see Results for details).

2.5. Immunohistochemistry and microscopy

We used a previously described methodology [35,36], with minor

modifications. Rats and mice were anesthetized with 4 % isoflurane in oxygen and perfused with saline followed by paraformaldehyde (Sigma-Aldrich, USA). L4 DRGs were post-fixed, paraffin-embedded, sectioned at 5 μm, mounted on microscope slides (Sigma-Aldrich, USA), deparaffinized in xylol (Panreac Quimica, Spain), and rehydrated before antigen retrieval (steam heating for 22 min with 1 % citrate buffer, pH 8). The sections were incubated for 1 h in blocking solution containing 5 % normal goat serum, 0.3 % Triton X-100, and 0.1 % Tween 20 in Tris buffer. The slides were then incubated with primary antibodies in blocking solution for 1 h at room temperature. The primary antibodies were rabbit anti-NeuN (neuronal nuclei) (1:500, ABN78, Merck Millipore, USA) or mouse anti-NeuN (1:500, MAB377, Merck Millipore, USA), depending on the immunohistochemistry, mouse anti-σ1R (1:200, sc-137075, Santa Cruz Biotechnology, Germany), and rabbit anti-CGRP (1:800, T-4032, BMA Biomedicals, Switzerland). After primary antibody incubation, the sections were washed again three times for 10 min and incubated with the appropriate secondary antibodies: Alexa Fluor-488 goat anti-mouse (A11017), Alexa Fluor-647 goat anti-mouse (A21236), Alexa Fluor-488 goat anti-rabbit (A11008) and Alexa Fluor-594 goat anti-rabbit (A11012) (all 1:500, Thermo Fisher Scientific, USA). Tissue sections were also stained with *Bandeiraea simplicifolia lectin I*, isolectin B4 (IB4) conjugated with Dylight-594 (1:100, DL-1207, Lot ZG0123; Vector Laboratories Ltd., UK) for 1 h at room temperature. The slides were incubated for 5 min with Hoechst 33342 for nucleic acid staining (1:1000, Life Technologies, USA) and washed three times before mounting. Finally, they were coverslipped with ProLong Gold Antifade mounting medium (Thermo Fisher Scientific, USA). Images were acquired with a confocal laser-scanning microscope (Model A1, Nikon Instruments Europe BV, Netherlands).

2.6. Binding assay

Rats were killed by decapitation, and their brains (excluding the cerebellums) were quickly removed and frozen at −80 °C, until use. The frozen brains were thawed and homogenized in approximately 10 volumes of ice-cold Tris-HCl buffer (10 mM, sucrose 0.32 M, pH 7.4) using a Potter homogenizer at 900 rpm for 10 strokes. The homogenate was then centrifuged at 5000*g* for 10 min at 4 °C, and the resulting supernatant was further centrifuged at 60,000*g* for 20 min at 4 °C.The final membrane pellet was collected and frozen at −80 °C until use. Protein concentrations were determined by using the Bradford protein assay (Bradford, 1976) (Kit for the determination of total protein, Sigma-Aldrich, USA). We used the method described by Cagnotto et al. (1994) [37], with minor modifications. Incubations were performed in 96-well flat-bottom plates using Tris-HCI 50 mM, PH 8.0 as binding buffer. Each well contained 25 μl of [^{3}H](+)-pentazocine (spec act. 26.9 Ci/mmol, NEN) at concentrations ranging from 1 to 14 nM, 25 μl of the membrane suspension, yielding a final assay volume of 250 μl (final tissue concentration of approximately 160 μg protein/well). [^{3}H](+)-pentazocine is considered a standard radioligand for σ1R KO binding assays [38]. Non-specific binding was determined by the addition of a final concentration of 10 μM haloperidol. Haloperidol is a high-affinity σ1R ligand [39], which is widely used as a cold drug to determine [^{3}H](+)-pentazocine non-specific binding [38–40]. Plates were incubated at 37 °C for 120 min. 200 μl of the reaction mixture was transferred to a pre-treated 0.5 % PEI, Millipore filter 96-well plate (GFC) (Millipore, USA), filtered, and washed three times with 10 mM Tris-HCI buffer solution, pH 7.4. Filters were allowed to dry at 60 °C for 2 h and 30 μl of scintillation liquid was added to the wells. Following the addition of a scintillation cocktail, the samples were allowed to equilibrate overnight. The amount of bound radioactivity was measured using a Microbeta (PerkinElmer, USA) liquid scintillation counter.

2.7. Hematology and biochemistry

The whole blood and plasma of both genotypes were collected from WT and σ1R KO rats (2–4 months old; n = 8 per group) via the jugular vein under isoflurane anesthesia. Whole blood was collected in K2-EDTA tubes, while plasma was obtained from blood collected in lithium-heparin tubes and then centrifuged at 2060*g* for 10 min at 4 °C. Samples were sent to the PCB Animal Facility laboratories for analysis. Whole blood was analyzed using a Spincell5 (Spinreact™, Spain), a 5-part differential hematology instrument based on the principle of flow cytometry to differentiate white blood cells (WBC) into neutrophils, lymphocytes, monocytes, eosinophils, and basophils according to cell size and complexity (granularity). Plasma cholesterol and triglycerides were measured with the Spinlab 100 (Spinreact™, Spain) auto-analyzer, and plasma biochemistry measurements were performed using a Vetscan V2 analyzer (Abaxis, USA).

2.8. General status

2.8.1. Growth and survival

Rats were monitored from birth to death to establish the growth and survival curves. Young rats (2–4 months old) were weighed weekly, adult rats (1–1.5 years old) were weighed monthly, and old rats (2 years old) were weighed twice a month, using a Mettler-Toledo precision balance.

2.8.2. Food consumption

Food pellets in the rat box feeders were weighed daily from Monday to Friday for one week. The difference in the amount of food from one day to the next was recorded and divided by the number of rats in each cage to calculate 24 h food consumption per rat.

2.8.3. Irwin test

The methodology used was a modification of the protocol described by Samuel Irwin [41,42], providing a systematic and quantitative methodology for measuring the behavioral changes and/or the physiological modifications associated with the σ1R absence. Prior to testing, all the rats were examined to confirm general health. They were placed in makrolon cages (17 × 17 × 17 cm) with wood shavings on the floor, and remained there for at least 1 h, before the experiment started. The following parameters were evaluated:

- CNS depression: passivity, prostration and sedation; ataxia; palpebral ptosis; reduced reflexes; corneal, pupilar, auricular (ear) and righting reflex; decreased muscle tone; hypnosis; hypothermia; catalepsy.
- CNS stimulation: excitation; stereotypy; exophthalmia; tremors; increased muscle tone; convulsions (clonic or tonic); hyperthermia; Straub tail.
- PNS: lacrimation and salivation; diarrhea or urination; miosis or mydriasis; piloerection.
- Toxicity: lethality

Additional observations not described above such as colored urine, dyspnea, corneal opacity, priapism, hyperemia, ischemia, etc., were also recorded.

All parameters, except for body temperature and pupil diameter, were scored semi-quantitatively using the following scale: 0: Absent; 1: Light or doubtful; 2: Clear; 3: Intense. Rectal temperature was measured using an electronic thermometer (Cibertec, Mod. CITER, Spain) attached to a rectal probe. Mydriasis or miosis, was assessed measuring the pupil diameter using a USB Digital Microscope with LED dimming control, connected to a computer. A transparency with a millimeter scale divided into tenths was placed on the computer screen for measurement.

2.9. Locomotor performance and functionality assessment

2.9.1. Rotarod

Coordination was assessed using a rotarod device (Model 47750, Ugo

Basile, Italy), following previously described protocols with minor modifications [43,44]. In phase I, rats were trained to stay on the rod for 240 s at a constant speed of 10 rpm. The number of falls was recorded. Rats that failed to stay on the rod for 240 s were excluded from the study. In phase II, just after the last rat in a group of four completed phase I, rats were placed back on the rod for recording the time on equilibrium on an accelerating speed test. The rod speed increased continuously from 4 to 40 rpm (1 rpm every 1.5 s), and the latency to fall was recorded. This test was only carried out on female rats, because the size of the male rats impeded them from being properly evaluated.

2.9.2. Actimetry (locomotion)

Locomotor activity was measured using eight Linton AM548 Standard (Dual Layer) X, Y, Z IR Activity Monitors (Linton Instrumentation, UK) equipped with infrared beams, along with transparent polycarbonate Tecniplast rat cages, model 2154F, measuring 482 × 267 × 210 mm. The top metal grid was not used to allow rats to perform rearing in full extension. Each system had two levels of infrared beams, enabling the measurement of forward locomotion (bottom level) and rearing activity (upper level). These two parameters were recorded as mobile time (s), and rearing time (s) for 1 h at 5 min intervals, as previously performed [45].

2.9.3. Voluntary wheel running

Voluntary wheel running was assessed using eight Makrolon® cages equipped with stainless steel wheels (SKU 1800 — 1800-S, 33 cm inter. diam., 96 mm width, 2 mm bars, placed 9 mm apart, from Ugo Basile, Italy). Data were collected by connecting the wheels to a PC, via a Multifunction Interface using ANYmaze software (Ugo Basile, Italy), which recorded rotations every 30 min over a 24 h period. Experiments started at 09:00.

2.9.4. Burrowing test

Burrowing behavior was assessed as previously described [33,46,47]. A burrow tube was constructed using a PVC pipe measuring 30 cm in length, with two steel screws placed at one end to elevate the open end of the tube off the ground. A PVC cap was used to seal the other end. The tube was filled with 2000 g of pea-sized stones (2–5 mm) and placed in an empty cage without floor bedding. To minimize distractions from unfamiliar odors during testing, each rat was provided with its own burrow tube, stones, and cage. Prior to the test, the rats underwent two training sessions to acclimate them to the experimental setup. During each trial, a single rat was placed in its respective cage with the filled burrow tube set up for 30 min. No food or water was provided during the session to avoid distractions. The weight of the burrow tubes was recorded before and after each session to calculate the weight of the stones displaced from the tube.

2.9.5. Grip strength

Grip strength was measured using a computerized grip strength meter (Model 47200, Ugo-Basile, Italy), as previously described [48]. The device consists of a T-shaped metal bar connected to a force transducer. To measure hindlimb grip strength, the rats were gently held by their back with a cloth, allowing them to grasp the metal bar with their hind paws. Once their hind paws were properly positioned on the metal bar (i.e., perpendicular to the transducer), the experimenter pulled the rat backwards until it lost its grip. This procedure was always performed from the same position, using the same chair to maintain the angle and avoid fluctuations in the measurements. The device automatically records the maximum force of each measurement in grams (g). Each rat was tested twice, and the average of both measurements was used of the final value.

2.10. Anxiodepressive-like behaviors

2.10.1. Open field test (anxiety-related behavior)

We used a modified version of a procedure previously described [49]. The open field arena (70 × 70 × 30 cm) was made from white plexiglass material. Rat activity was recorded using a video tracking system (SMART), which allowed the open field to be virtually divided into several areas (center, wide-center, sidewalls and corners) for a detailed analysis of the rat's trajectory. This trajectory was captured by a digital camera and analyzed by the SMART software (Panlab, Spain) to obtain several parameters such as the time spent in each area and the number of transitions, both overall and for each area. The exploration time was 10 min, and experiments were performed under attenuated light conditions (<200 lx).

2.10.2. Startle response and prepulse inhibition

Four SR-LAB Startle Response Systems (San Diego Instruments, USA) were used. SR-LAB boxes were sound-attenuated and equipped with ventilation, light, and viewing lenses. The system generates white noise and software-controlled sound stimuli to measure startle responses and prepulse inhibition. Animal adjustable enclosures allow free movement, which is monitored using an accelerometer sensor underneath. We used a standard procedure [50]. Briefly, after a 5 min acclimation period in the startle chamber (background noise of 65 dB), rats were exposed to a sequence of combined stimuli: pulse alone (120 dB), no-stimulus (only background noise), and three pre-pulse intensities of 3, 6 and 12 dB followed 100 ms later by the 120 dB pulse. Each of the five types of stimuli were presented 10 times per session in a pseudorandom order. The time between the different stimuli varied, averaging 15 s. The SR-LAB system has software that registers the intensity of the startle response under each stimulus condition.

2.10.3. Forced swim test (depression-related behavior)

The test was performed as previously described [51,52]. During the training session, rats were individually forced to swim for 15 min in a glass beaker (diameter 22 cm, height 36 cm) containing water at 24 ± 1 °C and 22 cm depth. After the pre-test session, rats were removed and dried with a towel. Twenty-four hours later, they were placed in the beaker again for a 5 min test session, during which the total duration of mobility was recorded. In both the training session and the test session, immobility and struggling were recorded by an observer using two stopwatches. Rats were considered immobile when they ceased struggling and remained floating, making only the necessary movements to keep their heads above the water. In the training session, only the last 5 min were used for analysis.

2.11. Sensory testing, nociception and pain-related behaviors

2.11.1. von Frey test (mechanical sensitivity)

Mechanical sensitivity was quantified as previously described [53]. To assess pain sensitivity, rats were placed on a metal grid in a transparent methacrylate cylinder (200 mm diameter, 300 mm high, 3 mm thick) and allowed to acclimate to their new environment for at least 15 min before testing. Tactile sensitivity was determined by measuring the paw withdrawal threshold (PWT) in response to von Frey filaments stimulation, ranging from 1 to 26 g, on the plantar surface of one hind paw (Touch-Test von Frey monofilaments, North Coast Medical, Inc., USA). Each filament was applied for 3 s until a withdrawal response occurred. A single response indicated a positive response. Templates were analyzed using the Up-Down Reader software [54].

2.11.2. Paw pressure test (mechanical sensitivity)

The test was performed as previously described by Randall and Selitto [55]. Rats were gently restrained with a cloth and a gradually increasing mechanical nociceptive stimulus using a cone-shaped paw-presser with a rounded tip (Analgesy-meter, Ugo-Basile, Italy) was

applied to the dorsal surface of the right hind paw. The paw pressure was defined as the pressure at which the rat voluntarily withdrew its hind paw. The test was done twice with a 1 min interval between each stimulation. A 1000 g cut-off was applied to prevent tissue injury.

2.11.3. Tail flick test (heat sensitivity)

The test was performed as previously described by D'Amour and Smith [56]. Rats were gently restrained with a cloth to position their tails toward the heat source of the tail-flick apparatus (Panlab, LE 7106, Spain). A noxious beam of light was focused on the tail about 5 cm from the tip, and the tail-flick latency (TFL), defined as the time from the onset of the heat stimulus to tail withdrawal, was recorded automatically to the nearest 0.1 s. The intensity of the radiant heat source was adjusted to yield baseline latencies between 2 and 5 s and a cut-off time was set at 10 s to avoid heat-related damage.

2.11.4. Hargreaves' test (heat sensitivity)

Heat hyperalgesia was assessed using Hargreaves' test, as previously described [57]. Rats were placed in individual cages 10 min before the assessment to acclimate. Then, a laser beam was applied to the plantar surface, and the time for paw withdrawal latency was recorded. A 20 s cut-off was established to prevent tissue damage. The final value for each rat was calculated using the average of six measurements (three per paw).

2.11.5. Acetone test (cold sensitivity)

Cold allodynia was assessed by gently applying an acetone drop to the hind paws using a syringe connected to a fine polyethylene tube, as previously described [35] and modified for rats. For the test, rats were placed in transparent plastic chambers (7 × 7 × 13 cm) with a wire mesh floor. A small mirror was also placed under the chambers to allow clear observation in a room where the temperature was kept at 22 °C. After 30 min of adaptation, acetone was applied twice to each hind paw at 1 min intervals. Using a stopwatch, the duration of licking, biting, or showing a withdrawal response or pain gesture in the paw where acetone was applied was recorded. The final value for each rat was calculated as the average of the four measurements (two per paw).

2.11.6. Formalin test (chemical sensitivity)

Following the method described by Dubuisson and Dennis [58], 5 % formalin was prepared by diluting a stock solution of 37 % formaldehyde in water. 50 μl of 5 % formalin were injected into the mid-plantar surface of the right hind paw of the rats using a 0.3 ml U-100 Insulin microsyringe (30 G needle). Formalin-induced pain was quantified by recording the number of flinches during the 1 h following injection. The formalin response has two phases (phase I and phase II).

2.11.7. Spared nerve injury (neuropathic pain model)

Rats were anesthetized with 2 % isoflurane, and spared nerve injury (SNI) surgery was performed as previously described [59], with minor modifications for rats. Briefly, an incision was made in the skin of the left thigh, followed by an incision made directly through the biceps femoris muscle, exposing the sciatic nerve and its three terminal branches (the sural, common peroneal, and tibial nerves). The tibial and common peroneal sciatic nerve branches were ligated with a silk suture and transected distally, while the sural branch was left intact.

2.12. Data processing and statistical analysis

Data were represented as mean ± S.E.M. The differences between the experimental groups were analyzed using One-Way ANOVA and Two-Way Repeated measures ANOVA, depending on the test and parameter analyzed, and followed by the Tukey post hoc test (see figure caption). Statistical analysis and graphs were performed using GraphPad Prism software (v9.0; GraphPad Software, Inc., USA). Differences in the survival curves were assessed using the Mantel-Cox test.

3. Results

3.1. Generation and validation of the σ1R KO rat

Targeted mutagenesis using CRISPR/Cas9 was performed directly in Wistar Han rat embryos by Horizon Discovery using the SAGEspeed® platform. A specific 218 base-pair deletion encompassing 46 bp of exon 1 and 34 bp of exon 2 was introduced into the σ1R gene, leading to a frameshift and predicted loss of functional protein. Several founder rats carrying distinct deletions (including 218 bp, 7 bp, and 8 bp) were identified. The colony used in this study was established by the founder with the 218 bp deletion. Genotyping was routinely performed using two primer pairs designed to distinguish WT and σ1R KO alleles. The primary primer set (1RS/2RA) amplified DNA fragments of 476 bp (WT) and 258 bp (σ1R KO), while a second set (1RS/3RA) was used for confirmation, generating 368 bp (WT) and 150 bp (σ1R KO) fragments (see representative PCR in Supplementary Fig. 3). Sequencing of the σ1R KO allele was also performed during the initial colony propagation using a third primer (4RA) to confirm the presence and integrity of the deletion (data not shown). Representative genotyping results are shown in Supplementary Fig. 3. Effective deletion of the σ1R gene in σ1R KO rats was confirmed by PCR analysis (Fig. 1A; complete gel can be found in Supplementary Fig. 3). σ1R protein expression was also quantified in the spinal cord, DRG, and liver, showing robust expression in WT rats and complete absence in σ1R KO rats (Fig. 1B and complete gel in Supplementary Fig. 4). Functional evaluation of the σ1R was conducted using [^{3}H](+)-Pentazocine as a σ1R radioligand. The binding assay indicated clear dose-dependent specific binding in brain membranes from WT rats, which was absent in samples from σ1R KO rats (Fig. 1C). Finally, immunohistochemistry assay demonstrated that σ1R is present in all afferent neurons in the DRG, with greater expression in small-sized neurons, while no labeling was observed in the DRG of σ1R KO rats (Fig. 1D). σ1R is selectively expressed in dorsal root ganglion neurons, with broad distribution in the cytoplasm and plasma membrane, but not within the neuronal nuclei.

Together, these experiments confirm the absence of the σ1R in σ1R KO rats.

3.2. σ1R expression pattern is equivalent between rats and mice but the distribution of CGRP+ and IB4+ neuronal populations differs

As shown in Fig. 1D, σ1R expression is present in all neurons, as demonstrated by its colocalization with the neuronal marker NeuN. However, a gap in the σ1R signal is frequently observed in the center of neurons, which, in many cases, is filled by NeuN labeling, suggesting that it may correspond to the nucleus. To investigate this further, we acquired higher magnification images using both a neuronal and a nuclear marker. These images confirmed that the gap corresponds to the nuclei. Further magnification showed that σ1R does not colocalize with Hoechst staining, reinforcing the conclusion that the absence of σ1R labeling corresponds to the nuclear region (Fig. 2). This observation aligns with data from mice, which exhibit a very similar staining pattern (Supplementary Fig. 5).

Although the σ1R staining pattern is comparable between rats and mice, rat neuronal cell bodies are slightly larger, which does not necessarily imply that their neuronal function is identical. When analyzing IB4 and CGRP staining, which in mice are well segregated into distinct neuronal populations (Supplementary Fig. 6), we observed colocalization of IB4 and CGRP in rats (Fig. 3).

This finding suggests that, despite structural similarities, rats exhibit neurochemical differences compared to mice.

3.3. Viability, growth curve and general status of σ1R KO rats

σ1R KO rats appear to be generally comparable to WT rats. Survival analysis over two years showed no significant differences in survival

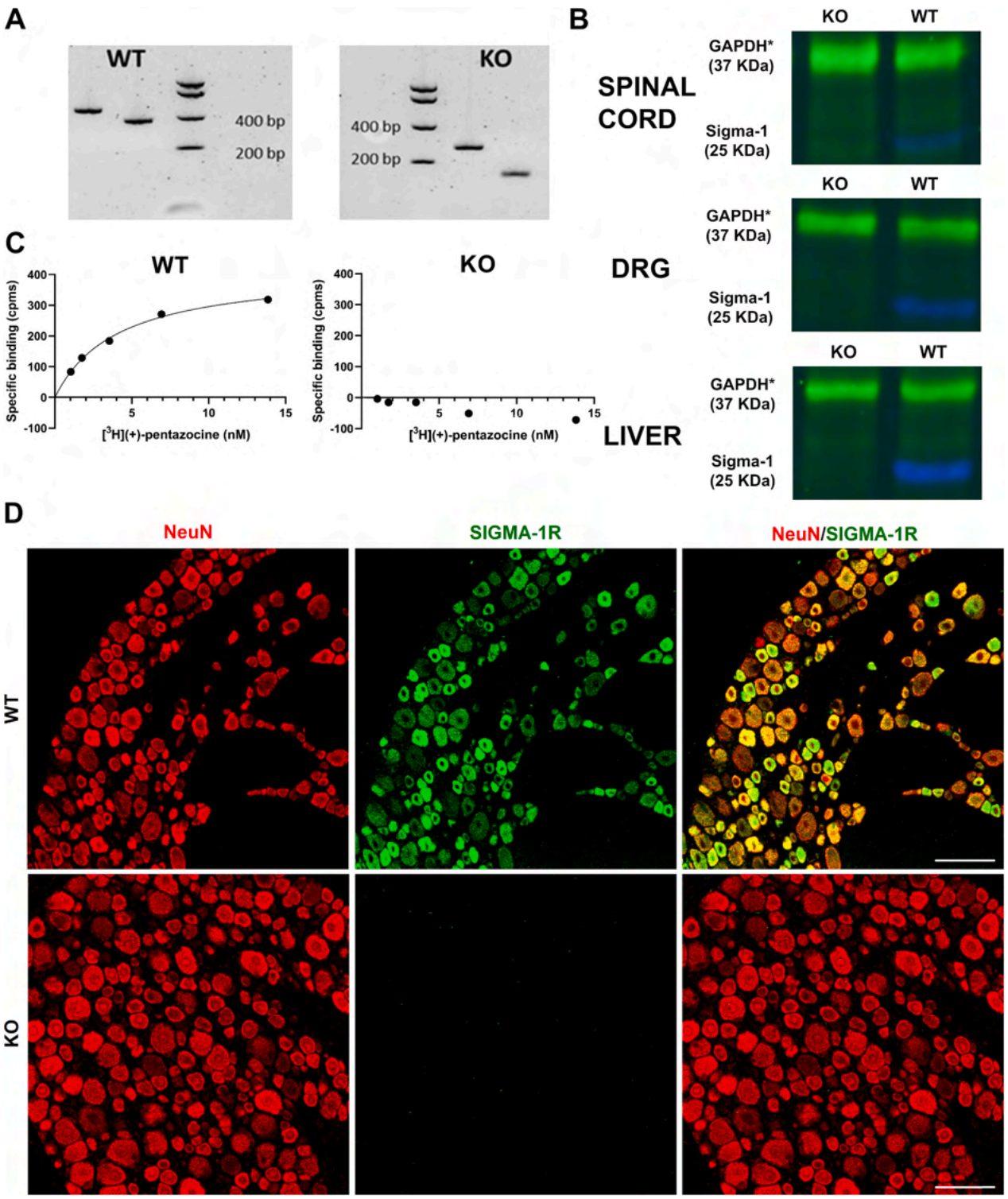

Fig. 1. Genetic and functional confirmation of the absence of the sigma-1 receptor (σ1R) by PCR, Western blot, binding assay, and immunohistochemistry. (A) Representative electrophoresis of PCR products obtained for the genotyping of wild type (WT) and σ1R knockout (KO) rats. (B) Representative immunoblots performed in spinal cord, dorsal root ganglia (DRG) and liver tissue obtained from WT and σ1R KO rats. (C) Representative binding curves of radiolabeled σ1R ligand [^{3}H](+)-Pentazocine in rat brain. Specific binding = (Total binding − total binding without membrane) − (non-specific binding − non-specific binding without membrane). (D) Representative microimages from double labeling of NeuN (red) and σ1R (green) in the L4 DRG. Top panels: samples from WT rat. Bottom panels: samples from σ1R KO rat. Scale bar, 100 μm.

curves between WT and σ1R KO rats in both males (Fig. 4A) and females (Fig. 4B). Similarly, weight gain rates were comparable between genotypes, with no differences observed at any time point in females; however, male σ1R KO rats exhibited a slightly lower weight gain rate starting from week 38 (Fig. 4C). Despite this, the weights of rats used in behavioral assays were similar between genotypes, since we used much younger rats, in which there are no differences in weight (Supplementary Fig. 2). Additionally, young rats of both sexes exhibited higher 24 h

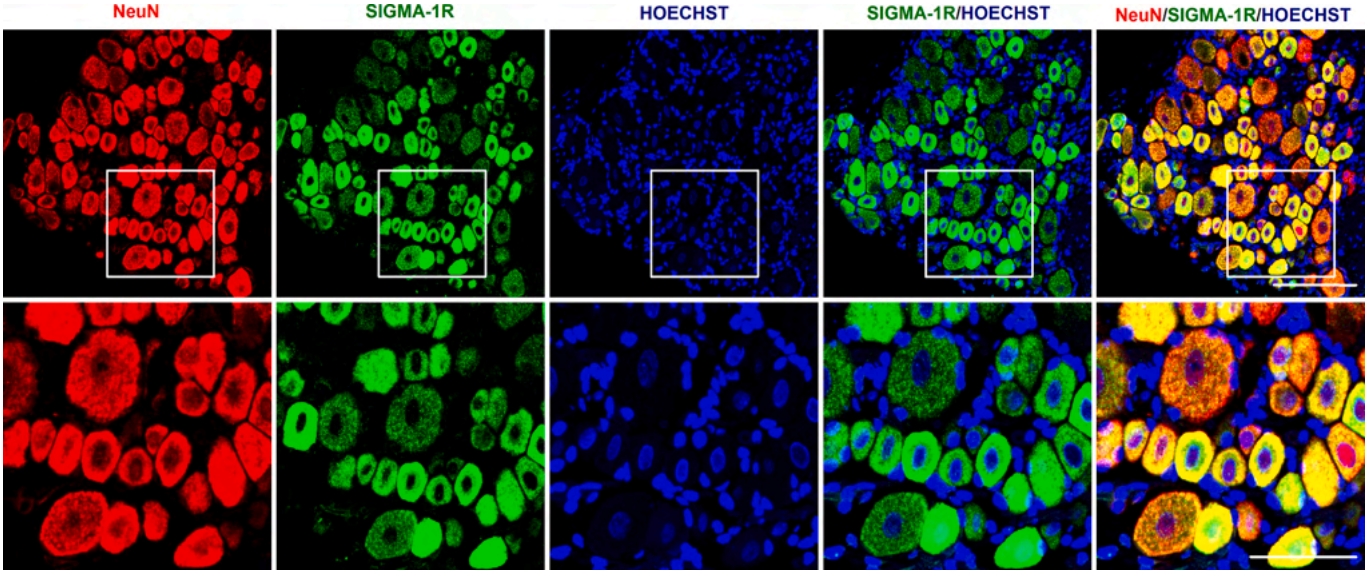

Fig. 2. Sigma-1 receptor (σ1R) is expressed selectively in dorsal root ganglion (DRG) neurons, with broad distribution in the cytoplasm and plasma membrane, but not in the neuronal nuclei. Representative images showing triple labeling of NeuN (red), σ1R (green) and Hoechst 33342 (Hoechst) (blue) in the L4 DRG from rat. Scale bar, 100 μm. The bottom panels show a higher-magnification view of the areas squared in the top panels. Scale bar is 50 μm.

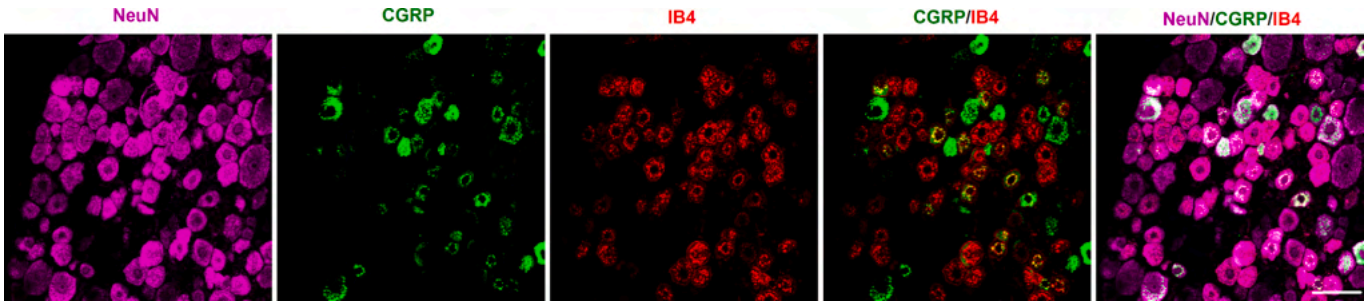

Fig. 3. Expression of CGRP and IB4 in dorsal root ganglion (DRG) neurons of rats. Representative images showing triple labeling of pan-neuronal marker NeuN (magenta), CGRP (green) and isolectin B4 (IB4, red) in the L4 dorsal root ganglion from rats. Scale bar, 50 μm.

food consumption compared to older ones ($P < 0.05$), with no significant differences between genotypes (Fig. 4D).

In the functional observation test battery (Irwin's test), no significant differences were observed in scored parameters between genotypes or between sexes within each genotype (data not shown). Among the measured parameters, females displayed slightly higher rectal temperatures regardless of genotype (Fig. 4E), while pupil diameter did not differ between groups (Fig. 4F).

In summary, no notable differences in viability or general status were observed between WT and σ1R KO rats, except for a slightly lower weight gain in male σ1R KO rats.

3.4. The absence of the σ1R does not alter any hematological parameters

Hematological parameters, including basophil count (absolute and percentage), eosinophil count, mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red blood cell (RBC) count, hematocrit, hemoglobin, white blood cell (WBC) count, lymphocyte, monocyte, neutrophil counts (absolute and percentage), platelet count, red cell distribution width (RDW), mean corpuscular volume (MCV), and mean platelet volume (MPV), were comparable across WT and σ1R KO groups with minor sex-related variations (Table 1). Similarly, biochemical parameters, such as cholesterol, triglycerides, blood urea nitrogen, creatinine, alanine aminotransferase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), bilirubin, glucose, calcium (Ca^{2+}), total protein, albumin, globulin, sodium (Na^{+}), potassium (K^{+}), chloride (Cl^{-}), and total carbon dioxide (tCO^{2}), showed no significant differences between genotypes (Table 2).

Therefore, no significant differences were observed in hematological and biochemical parameters between WT and σ1R KO rats.

3.5. Locomotor performance and functionality assessment of σ1R KO rats

No significant differences were observed between phenotypes in the rotarod test, either in the number of falls during training or in the time the rats remained on the rod during the acceleration test (Fig. 5A). Similarly, σ1R KO rats of both sexes displayed 24 h running times in the voluntary wheel running test that were comparable to WT rats (Fig. 5B), as well as comparable maximum grip strength (Fig. 5C). In contrast, σ1R KO rats of both sexes showed a dramatic reduction in the amount of gravel displaced in the burrowing test (Fig. 5D). They also demonstrated slightly lower exploratory activity, as measured by actimetry (Fig. 5E), although this reduction was only statistically significant in the parameter rearing time, between WT and σ1R KO male rats.

In summary, while σ1R KO rats did not exhibit impairments in locomotor capacity or strength, they showed reduced exploratory and burrowing behavior.

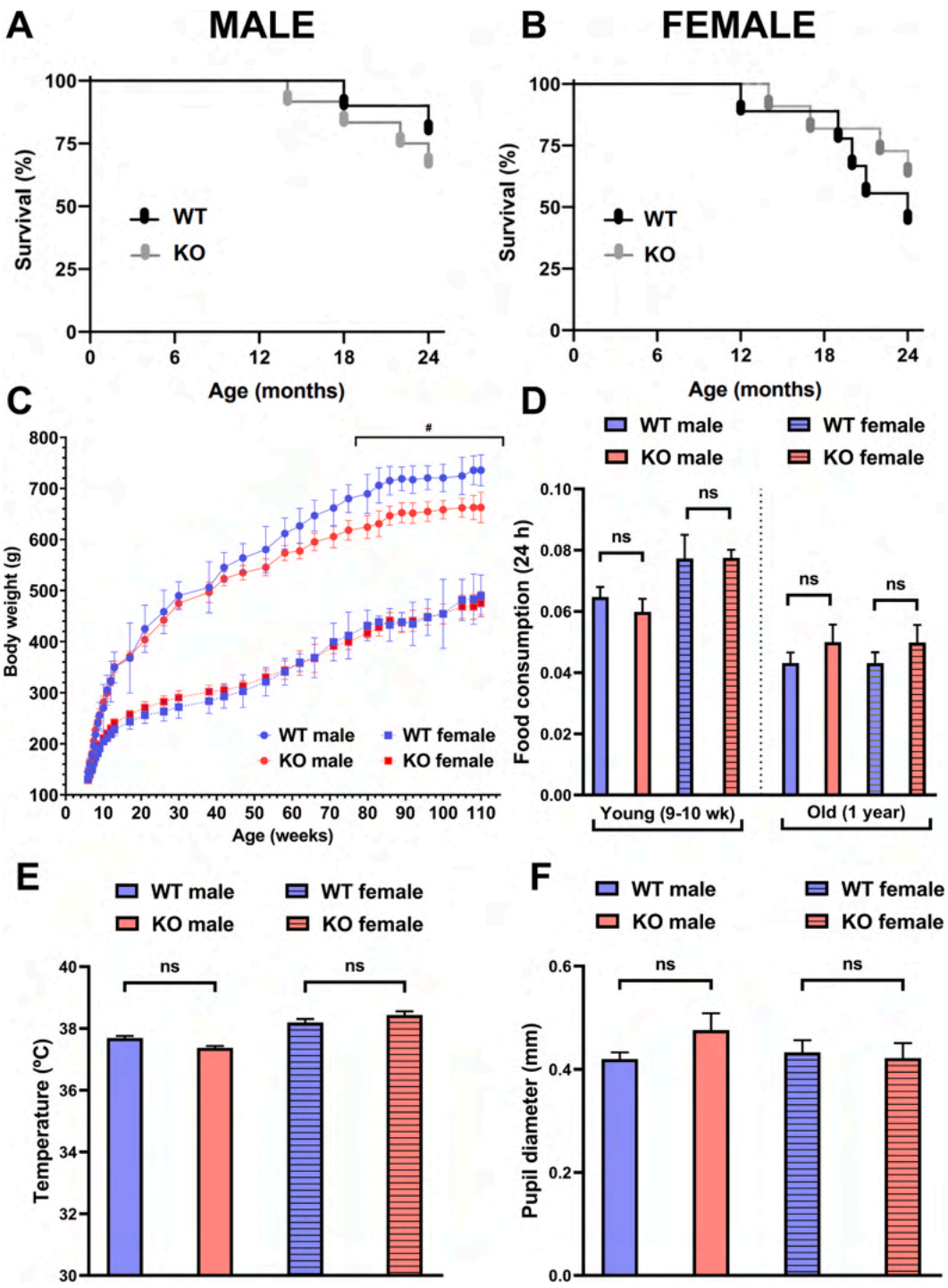

Fig. 4. Viability and evaluation of general status of wild-type (WT) and sigma-1 receptor (σ1R) knockout (KO) rats. Survival curves of male (A) and female (B) WT and σ1R KO rats over 24 months; (C) growth rate estimated as body weight gain in grams over 115 weeks; (D) food consumption was quantified during 24 h and relativized by body weight of the rats (grams eaten per body weight gram), (E) body temperature and (F) pupil diameter were measured in both genotypes as part of the Irwin test. No differences in the survival curves were found. Log-rank (Mantel-Cox) test, males: $p = 0.5968$. Females: $p = 0.4698$. Statistically significant differences between the values obtained in the σ1R KO rats and the WT rats: $^{\#}P < 0.05$ (two-way ANOVA repeated measures followed by Tukey).

3.6. Evaluation of anxiodepressive behaviors in σ1R KO rats

We used the open field and the startle tests to assess anxiety in rodents. No significant differences between genotypes were observed in any of the parameters evaluated in the open field test, although σ1R KO rats showed a slight, non-significant reduction in the time spent in the center (Fig. 6A). Similarly, no genotype differences were detected in the startle response test. Considering that the sensor was not adjusted to the different weights, males (mainly σ1R KO) exhibited significantly higher startle response values due to their size (Fig. 6B). No differences were observed between genotypes in the percentage of prepulse inhibition, though males showed higher but non-significantly different values, consistent with their startle response results (Fig. 6B). Regarding depression-like behavior, immobility times during the forced swim test did not differ between groups in either the last 5 min of the training session or the test session (Fig. 6C).

Table 1
Hematological parameters for WT and σ1R KO rats.

Parameter		WT male	WT female	KO male	KO female
Basophil (10^9/L)	Mean	0.002	0.003	0.005	0.008
	s.e.m.	0.001	0.001	0.002	0.002
Basophil (%)	Mean	0.05	0.118	0.09	0.236
	s.e.m.	0.04	0.045	0.03	0.086
MCHC (g/dL)	Mean	33.90	34.04	33.60	34.13
	s.e.m.	0.14	0.15	0.16	0.14
Eosinophil (10^{12}/L)	Mean	0.005	0.005	0.003	0.01
	s.e.m.	0.002	0.002	0.001	0.004
Eosinophil (%)	Mean	0.084	0.15	0.059	0.24
	s.e.m.	0.035	0.053	0.011	0.08
MCH (pg)	Mean	18.10	18.65	17.90	19.14
	s.e.m.	0.17	0.22	0.16	0.25
RBC (10^{12}/L)	Mean	8.80	8.10	9.20	7.80
	s.e.m.	0.14	0.25	0.14	0.13
Hematocrit (%)	Mean	47.00	44.35	48.90***	43.71
	s.e.m.	0.62	1.07	0.73	0.49
Hemoglobin (g/dL)	Mean	16.00	15.11	16.40	14.94
	s.e.m.	0.18	0.34	0.21	0.16
WBC 10^9/L	Mean	6.20****	3.13	6.00**	3.75
	s.e.m.	0.60	0.28	0.30	0.29
Lymphocyte (g/dL)	Mean	4.30**	2.25	4.5*	2.66
	s.e.m.	0.68	0.25	0.34	0.26
Lymphocyte (%)	Mean	68.90	71.03	73.80	70.25
	s.e.m.	7.10	2.80	2.48	2.01
Monocyte (10^9/L)	Mean	0.90	0.43	0.90	0.45
	s.e.m.	0.19	0.09	0.12	0.07
Monocyte (%)	Mean	13.80	14.09	15.90	12.54
	s.e.m.	3.33	2.41	2.18	2.22
Neutrophil (10^9/L)	Mean	1.00	0.44	0.60	0.62
	s.e.m.	0.28	0.09	0.09	0.09
Neutrophil (%)	Mean	17.20	14.61	10.10	16.73
	s.e.m.	4.97	2.98	1.81	2.38
Platelet (10^9/L)	Mean	865.60	628.50	847.90	835.00
	s.e.m.	72.48	118.13	64.62	22.36
Platelet (%)	Mean	0.50	0.37	0.50	0.47
	s.e.m.	0.04	0.07	0.04	0.01
RDW (%)	Mean	10.90	10.86	11.00	10.96
	s.e.m.	0.16	0.19	0.17	0.15
MCV (fL)	Mean	53.60	54.89	53.30	56.16
	s.e.m.	0.43	0.49	0.39	0.60
MPV (fL)	Mean	5.60	6.01	5.60	5.65
	s.e.m.	0.11	0.10	0.07	0.12

Abbreviations: MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; MPV, mean platelet volume; RBC, red blood cell; RDW, red blood cell volume distribution; WBC, white blood cell. No significant differences between genotypes. Statistically significant differences between the values obtained in male and female groups: $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$ (One-way ANOVA followed by Tukey; n = 8 per group).

Table 2
Biochemical parameters for WT and σ1R KO rats.

Parameter		Male WT	Female WT	Male KO	Female KO
Cholesterol (mg/dL)	Mean	76.55	81.50	60.81	81.91
	s.e.m.	2.80	11.39	2.50	6.30
Triglycerides (mg/dL)	Mean	163.13	125.88	195.88	168.29
	s.e.m.	14.29	23.17	12.31	23.48
Blood urea nitrogen (mg/dL)	Mean	14.13	16.38	13.63*	16.14
	s.e.m.	0.37	0.86	0.43	0.65
Creatinine (mg/dL)	Mean	0.44	0.48	0.44	0.36
	s.e.m.	0.03	0.03	0.03	0.04
Alanine aminotransferase (U/L)	Mean	41.50**	30.50	37.38	30.29
	s.e.m.	2.54	0.94	2.02	2.25
Alkaline phosphatase (U/L)	Mean	200.38****	94.00	163.38***	86.00
	s.e.m.	17.07	7.68	10.60	6.94
Aspartate transaminase (U/L)	Mean	67.25	68.13	64.63	60.14
	s.e.m.	2.32	3.22	1.84	1.13
Bilirubin (mg/dL)	Mean	0.30	0.25	0.31	0.30
	s.e.m.	0.00	0.03	0.01	0.00
Glucose (mg/dL)	Mean	127.75	142.50	133.75	134.14
	s.e.m.	3.69	8.68	2.21	5.04
Ca^{2+} (mg/dL)	Mean	11.84	11.34	11.56	11.66
	s.e.m.	0.12	0.17	0.18	0.16
Total protein (g/dL)	Mean	7.18	6.96	7.05	7.54
	s.e.m.	0.15	0.07	0.13	0.15
Albumin (g/dL)	Mean	5.94	6.30	5.94	>6.5
	s.e.m.	0.06	0.07	0.06	
Globulin (g/dL)	Mean	1.13	0.50	1.15	n.a.
	s.e.m.	0.10	0.07	0.09	
Na^+ (mmol/L)	Mean	141.50	138.00	141.13	142.14
	s.e.m.	1.19	1.22	0.65	1.50
K^+ (mmol/L)	Mean	5.18	5.16	5.40	5.01
	s.e.m.	0.10	0.26	0.15	0.09
Cl^- (mmol/L)	Mean	101.38	102.88	101.38	101.29
	s.e.m.	0.88	1.60	0.81	0.72
tCO_2 (mmol/L)	Mean	26.00	21.50	26.63	25.29
	s.e.m.	0.77	0.31	0.68	0.26

Abbreviations: n.a., not available. No significant differences between genotypes. Statistically significant differences between the values obtained in male and female groups: $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$ (One-way ANOVA followed by Tukey; n = 8 per group).

Overall, these findings suggest that σ1R KO rats do not exhibit exacerbated anxious or depressive behaviors compared to WT rats.

3.7. Sensory parameters and nociception of σ1R KO rats

WT and σ1R KO rats showed a very similar response pattern to mechanical stimuli, since the tactile thresholds in response to von Frey filaments (Fig. 7A) or the withdrawal latencies in the Randall-Selitto test (Fig. 7B) were practically identical between genotypes, with significantly higher values in the von Frey thresholds in males due to their larger body size. Regarding sensitivity to thermal stimuli, both WT and σ1R KO rats also showed a similar pattern, displaying comparable reactions to cold in the acetone test (Fig. 7C), and to heat stimuli in the tail flick test (Fig. 7D) or Hargreaves' test (Fig. 7E) across genotypes. Finally,

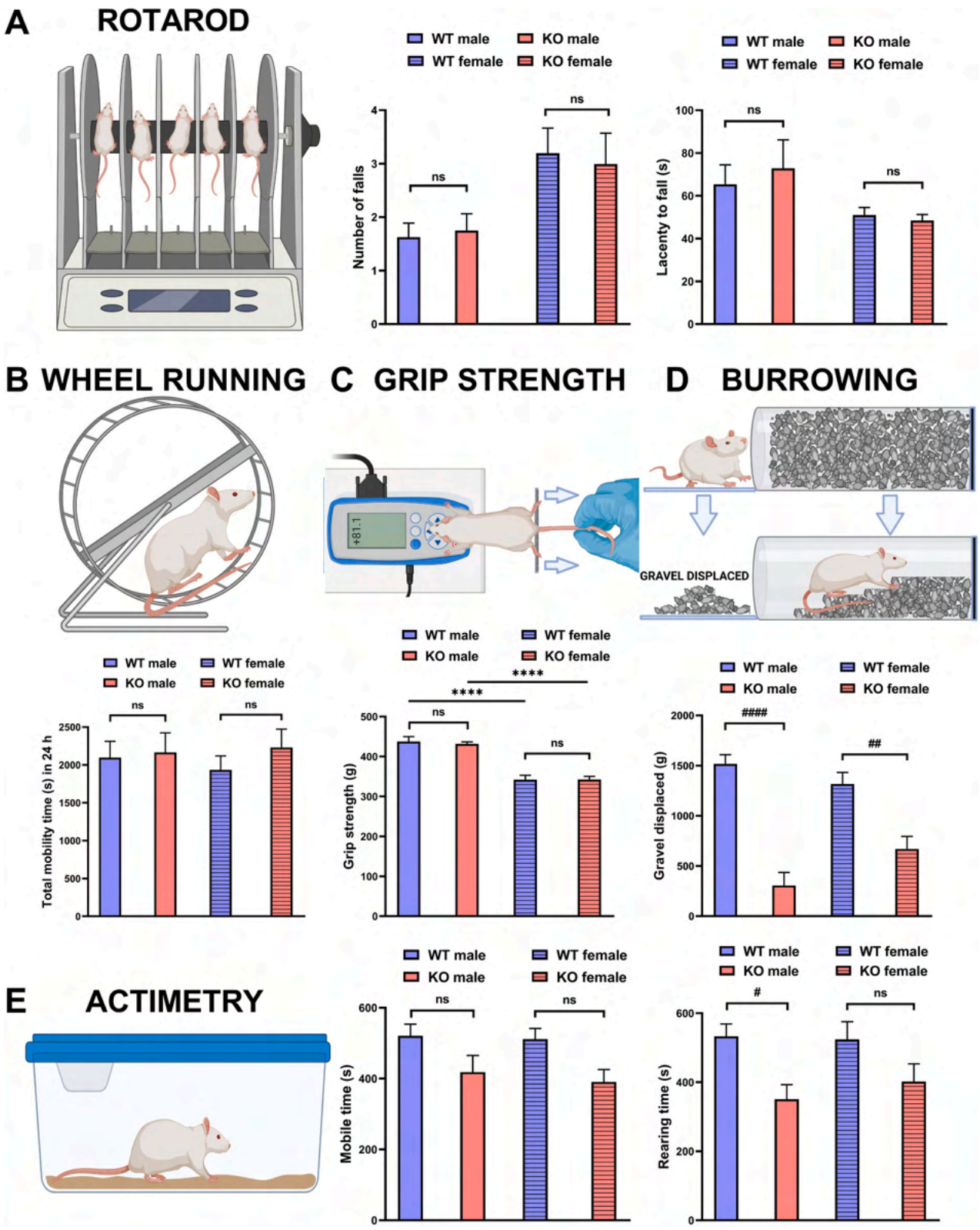

Fig. 5. Sigma-1 receptor (σ1R) knockout (KO) rats exhibited normal locomotor capacity and strength but demonstrated reduced exploratory and burrowing behavior. (A) Number of falls during the training session and latency to fall (s) during the test were compared between genotypes and sexes. (B) Total mobility time (s) values during 24 h in voluntary wheel running were compared between genotypes and sexes. (C) Maximum grip strength values in grams were recorded and compared between genotypes and sexes. (D) The total amount of gravel displaced during 30 min of the burrowing test was weighed and compared between genotypes and sexes. (E) Total mobility and rearing time were recorded by an infrared actimeter during 1 h of evaluation. Each bar and vertical line represent the mean ± SEM of the values obtained in 8–12 rats. Statistically significant differences between the values obtained in the σ1R KO rats and the WT rats: $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#\#}P < 0.0001$; statistically significant differences between males and females of the same genotype: $^{****}P < 0.0001$ (one-way ANOVA followed by Tukey).

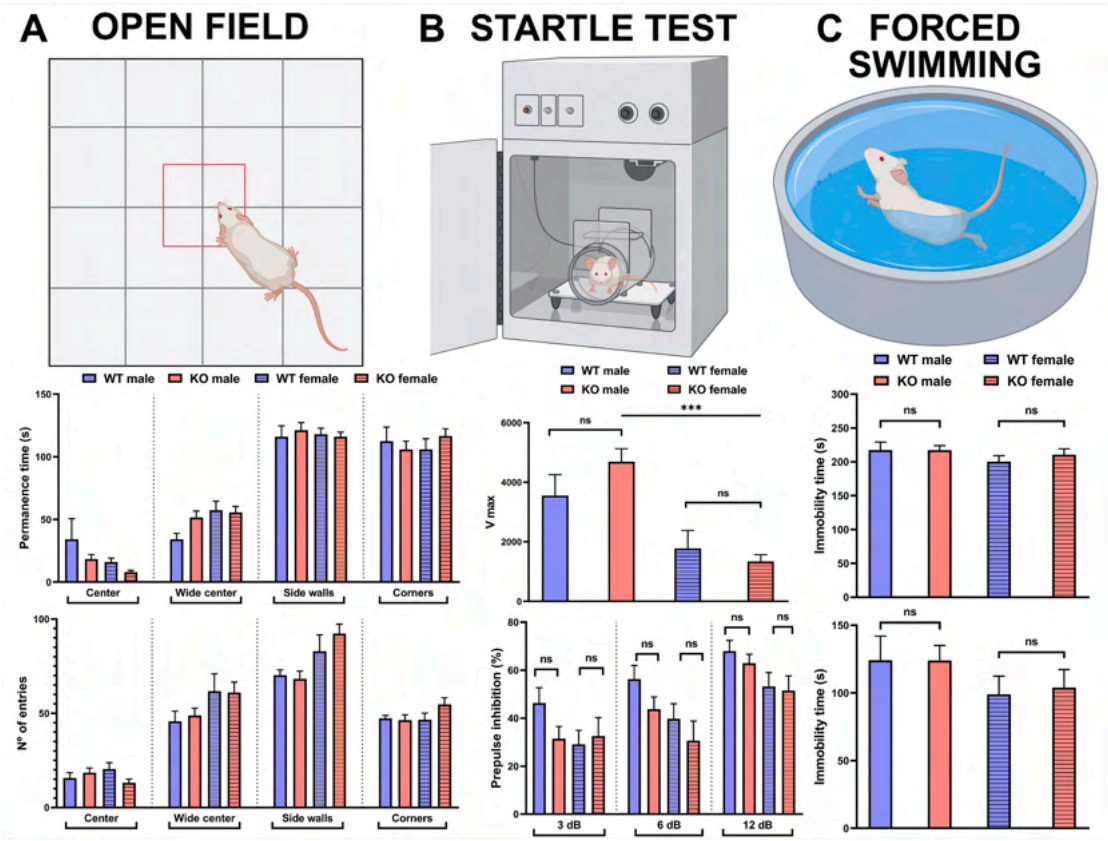

Fig. 6. Behavioral assessment in wild type (WT) and sigma-1 receptor (σ1R) knockout (KO) rats revealed no significant differences in anxiodepressive-like behaviors. (A) Open field test: time spent (s) in different zones (center, wide center, side walls, and corners) and the number of entries into these zones were recorded to assess exploratory and anxiety-like behavior. (B) Startle response was estimated as the maximum force of the reaction of the animal to a sudden noise (Vmax) and prepulse inhibition as the reduction in % of the initial startle response caused by the addition of three lower intensity prepulses (3 dB, 6 dB, 12 dB). They were measured to evaluate sensorimotor gating and anxiety. (C) Forced swim test: immobility time (s) during training (15 min) and test (5 min) sessions was analyzed to assess depressive-like behavior. Each bar and vertical line represent the mean ± SEM of the values obtained in 8–12 rats per group. No statistically significant differences (ns) were observed between genotypes in any of the behavioral tests performed. Statistically significant differences between males and females of the same genotype: $^{***}P < 0.001$ (one-way ANOVA followed by Tukey).

female σ1R KO rats displayed non-significant reduced pain-like behaviors in the second phase of the formalin test compared to female WT rats, whereas no differences were observed in males (Fig. 7F).

Therefore, these findings suggest that rats lacking σ1R exhibit a very similar nociceptive response in comparison with WT rats, in response to mechanical, thermal or chemical stimuli.

3.8. *Evaluation of nerve injury-induced neuropathic pain in σ1R KO rats*

We investigated the role of σ1R in neuropathic pain following spared nerve injury (SNI) by comparing responses to mechanical, heat, and cold stimuli in WT and σ1R KO female rats. Baseline responses to the von Frey, Hargreaves, and acetone tests prior to surgery were comparable between genotypes across all groups tested (Fig. 8B–D). We then studied sensory sensitivity seven days after SNI surgery. We found that the surgical procedure induced a strong and significant reduction in the mechanical threshold in both WT and σ1R KO rats, indicating that both groups developed marked mechanical allodynia. However, this reduction was more pronounced in WT rats, as shown by the significant difference between genotypes after SNI (Fig. 8B). Both WT and σ1R KO rats developed heat hypersensitivity after SNI, as evidenced by significantly reduced paw withdrawal latencies in the Hargreaves test, although the heat hypersensitivity was significantly less pronounced in σ1R KO rats (Fig. 8C). Finally, WT rats subjected to SNI also displayed pronounced cold allodynia, characterized by a significantly prolonged duration of paw licking/shaking in response to acetone (Fig. 8D). By contrast, σ1R KO rats showed a dramatic reduction in cold allodynia following SNI surgery (Fig. 8D).

Based on the above findings, σ1R KO rats exhibited attenuated neuropathic pain-like behaviors following peripheral nerve injury.

4. Discussion

In this study, we developed a σ1R KO rat model using CRISPR/Cas9 gene-editing technology. The effective depletion of the σ1R was validated by different experimental approaches, ensuring that the rats lacked σ1R expression. We also carried out a comprehensive phenotypic characterization, aimed at determining whether the absence of σ1R affects general viability, health status, or motor and behavioral performance. Moreover, given the role of σ1R in neuropsychiatric disorders and pain modulation, we specifically examined anxiodepressive-like behaviors as well as nociceptive and neuropathic pain responses.

We demonstrated the absence of σ1R in our model using a comprehensive set of techniques. Western blot analysis showed a robust σ1R expression in the spinal cord, dorsal root ganglia (DRG), and liver of WT rats, consistent with previous findings in mice [34,60]. In addition, the σ1R expression pattern observed via immunofluorescence closely mirrors what has been previously described in mice, showing a robust and selective expression in all neuronal cell bodies of the DRG, with a more pronounced presence in small-sized neurons [33,61,62]. Furthermore, the observed expression is localized exclusively in the neuronal cell body (absent in the nuclei), as σ1R is known to be a resident protein of the

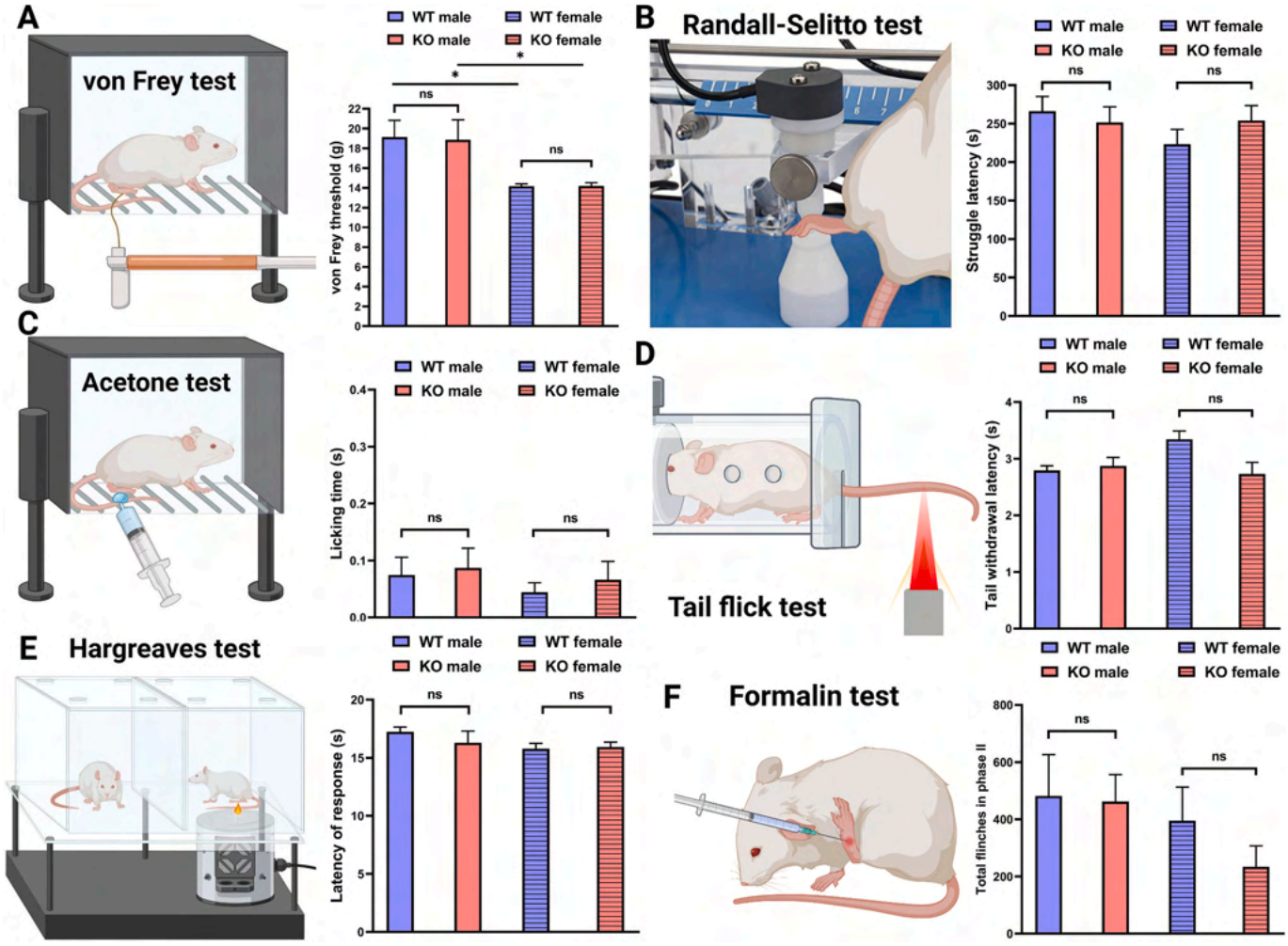

Fig. 7. Sigma-1 receptor (σ1R) knockout (KO) rats do not show alterations in mechanical or thermal sensitivity or nociception. Mechanical sensitivity was assessed using the von Frey test as mechanical pain thresholds (g) (A) and in the Randall-Selitto test as the struggle latency (s) in response to pressure applied to the paw (B). (C) Cold sensitivity was measured with the acetone test by recording licking time (s) following the application of a cold stimulus. Heat sensitivity was assessed using the tail flick test as the latency to tail withdrawal (s) in response to a heat stimulus (D) and in the Hargreaves' test as the latency to paw withdrawal (s) in response to radiant heat (E). (F) Formalin Test: total flinches in Phase II were counted following formalin injection to assess persistent inflammatory pain. Each bar and vertical line represent the mean ± SEM of the values obtained in 8–12 rats per group. No statistically significant differences (ns) were observed between genotypes across all tests performed. Statistically significant differences between males and females of the same genotype: $^{*}P < 0.05$ (one-way ANOVA followed by Tukey).

endoplasmic reticulum [63,64]. As expected, σ1R expression was completely absent in σ1R KO rats, in line with previous findings in σ1R KO mice [35,65]. In addition, we tested [^{3}H](+)-pentazocine binding in both WT and σ1R KO rats, and found that specific [^{3}H](+)-pentazocine binding was robust in WT rats but abolished in the mutant rats, a finding consistent with previous results found in σ1R KO mice [40], which indicates the inability of the mutated receptors to bind a prototypic σ1R drug. The absence of specific [^{3}H](+)-pentazocine binding in our mutant rats provides strong evidence that the radioligand is selective for σ1R and confirms that the specific [^{3}H](+)-pentazocine binding observed in WT rats is entirely attributable to σ1R.

The neurochemistry of sensory neurons is heterogeneous, particularly among unmyelinated C fibers, which are typically classified as peptidergic neurons that express neuropeptides such as calcitonin-gene related peptide (CGRP), and non-peptidergic neurons identified by isolectin B4 (IB4) binding [66]. Here we show that the expression pattern of the σ1R in DRG neurons is very similar in rats and mice. However, we demonstrated that there is a substantial overlap between CGRP-expressing neurons and neurons that bind IB4 in the rat DRG, whereas these two populations of sensory neurons (peptidergic and non-peptidergic) are well differentiated in mice, consistent with previous studies [36,67,68]. Thus, the neurochemistry of sensory neurons differs between rats and mice, which increases the interest in the development of σ1R KO rats.

As observed in σ1R KO mice [9], the absence of this receptor does not affect the viability of σ1R KO rats, with mortality curves equivalent to those of WT rats. Additionally, weight gain was also similar, except for a slightly significant reduction in weight gain in older male rats (starting at 38 weeks). This reduction cannot be attributed to an eating disorder, since we demonstrated that food intake was similar between both genotypes. Interestingly, a previous study reported that σ1R KO mice that were fed a high-fat diet also showed a significant reduction in body weight, but only in male mice [69]. The same study showed that σ1R blockade inhibited adipocyte-like differentiation of mouse embryonic fibroblasts. Therefore, the significant reduction in weight gain we observed in older male σ1R KO rats, likely with a higher percentage of body fat due to age, may be explained by the modulation of adipocyte differentiation by σ1R described previously [69]. Why this finding only occurs in male rats is unknown. The role of σ1R in adipocyte function and sex-specific weight regulation warrants further investigation. Nevertheless, the weight differences we observed in older rats are unlikely to affect the phenotypic experiments carried out in this work or by

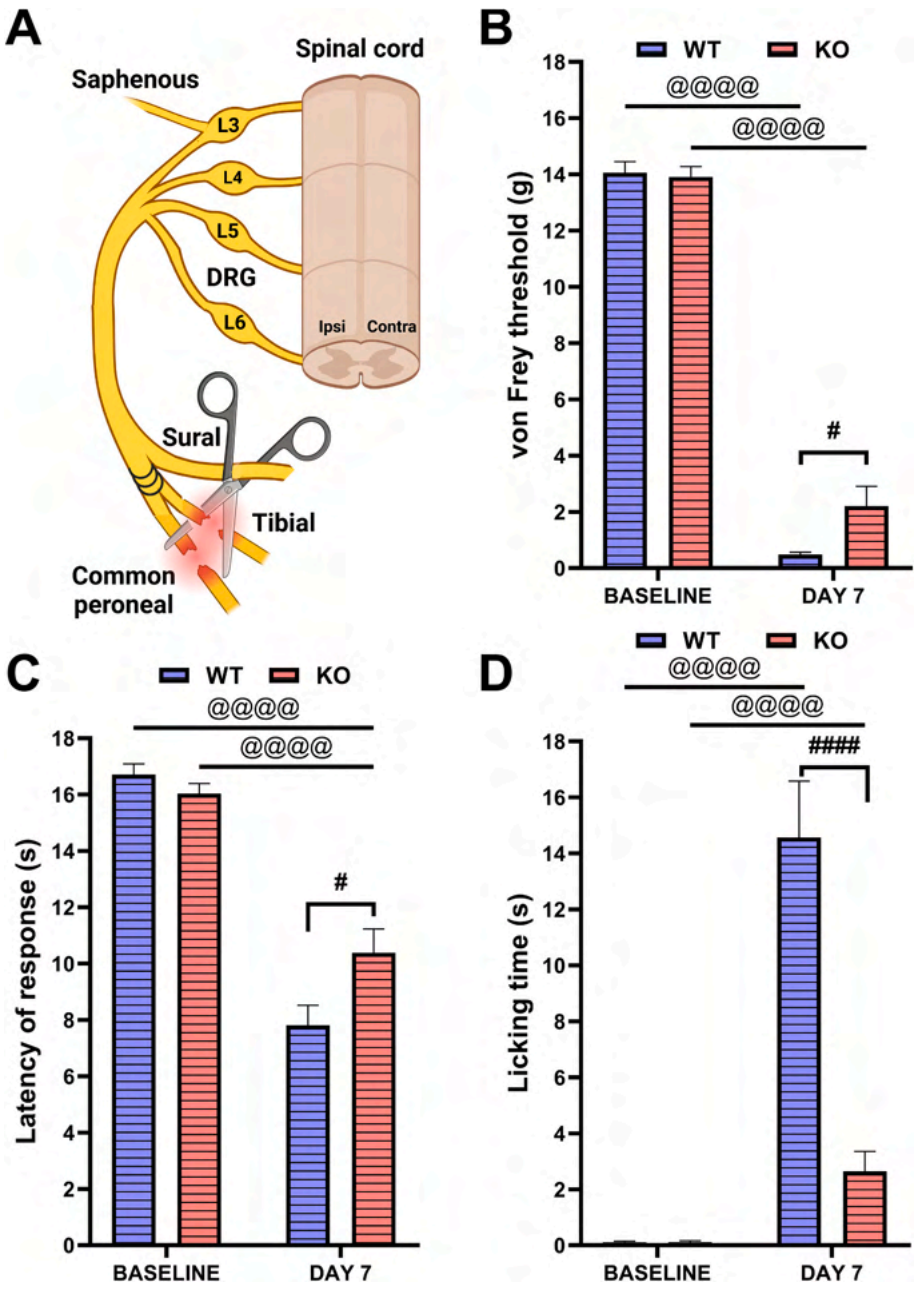

Fig. 8. Comparison of spared nerve injury (SNI)-induced neuropathic pain behaviors in female wild type (WT) and sigma-1 receptor (σ1R) knockout (KO) rats. (A) Schematic representation of spared nerve injury where common peroneal and tibial nerves are sectioned while the sural nerve is left intact. The von Frey threshold (B), latency to hind paw withdrawal in the Hargreaves test (C), and duration of hind paw licking or shaking in the acetone test (D), were recorded 1 day before baseline and on day 7 post-surgery in the paw ipsilateral to the surgery. Each point and vertical line represent the mean ± SEM of the values obtained in 8 rats. Statistically significant differences between the values obtained before surgery (baseline) and 7 days after SNI: $^{@@@@}P < 0.0001$; and between WT and σ1R KO rats: $^{\#}P < 0.05$; $^{\#\#\#\#}P < 0.0001$ (one-way ANOVA followed by Tukey test).

other researchers, since behavioral experiments are usually conducted on younger rats [70]. We used the Irwin test to evaluate possible alterations in behavior and physiological functions associated with the deletion of the σ1R gene [41]. No alterations were observed in the σ1R KO rats in the Irwin test, nor in other general status parameters such as feeding, temperature or pupil size. Likewise, we did not find any significant differences in hematological and biochemical parameters between WT and σ1R KO rats, which indicates that the absence of the σ1R does not induce notable alterations in hematological or biochemical profiles in either sex.

After confirming that the absence of σ1R does not significantly affect viability or general well-being, we evaluated its possible impact on motor function and physical performance. No deficits were observed in rotarod, voluntary wheel running, or grip strength tests, consistent with previous findings in σ1R KO mice [11,71,72]. However, subtle differences were seen in exploratory behavior, with σ1R KO males exhibiting reduced rearing time in the actimeter. Additionally, a more striking difference we observed was a dramatic reduction in burrowing behavior in σ1R KO rats of both sexes, which displaced less than half the amount of gravel compared to WT rats. This reduction in actimetry and burrowing cannot be attributed to motor impairments, since the σ1R KO rats showed normal motor function in the grip strength, rotarod and wheel running tests. However, burrowing behavior is a common innate activity conserved in laboratory rodents that appears to constitute a

'behavioral need', and it is highly motivating and reinforcing [73]. It has been reported that σ1R modulates reward-driven behavior in response to different reinforcers (psychostimulants, alcohol, food), and σ1R antagonists or σ1R KO mice generally reduce behavioral responses to these rewards [74]. Therefore, it could be hypothesized that σ1R KO rats have the reinforcement that produces the burrowing behavior inhibited.

A possible depressive phenotype could also contribute to the reduction in burrowing behavior [46,75], and several reports have shown that the absence of the σ1R receptor results in depressive-like behaviors in mice [11,72,76–80]. To further investigate a potential depressive trait in σ1R KO rats, we used the forced swim test, a widely-used method for assessing depressive-like behavior in rodents [51], which has been successfully used in all previously mentioned studies on σ1R KO mice. However, no differences in depressive-like behavior were detected in our mutant rats. Since the experimental approach does not appear to explain the discrepancy between the results obtained with σ1R KO rats and mice, we hypothesize that biological differences between species are probably key in these divergent outcomes. It is worth noting that not all studies using σ1R KO mice have reported depressive-like behavior [81], with conflicting results often attributed to differences in the strains used [81]. Both strain and rodent species are known to influence brain neurochemistry, gene expression, and behavior [31]. Altogether, these findings indicate that genetic background is of utmost importance for the modulatory effect of the absence of functional σ1Rs in depressive-like behaviors.

On the other hand, discrepancies between studies regarding the anxiolytic phenotype of σ1R KO mice have also been reported. In this study, we used the open field and startle tests to assess possible anxiety-like behaviors in σ1R KO rats and did not find any significant effects. In line with our results, previous studies in σ1R KO mice also failed to detect anxiety-related behaviors across different tests [11,77,79]. However, some studies have shown that σ1R KO mice displayed anxiogenic-like behaviors mainly based on the observation that mice spent less time in the center of the open field [72,81]. Interestingly, our data also showed that σ1R KO rats tended to spend less time in the center of the open field, although statistical significance was not reached. Differences between species, mouse strains, or methodologies across laboratories could explain this lack of consistency. Pharmacological studies with σ1R ligands have shown that, whereas σ1R agonists show antidepressant- and anxiolytic-like properties [82–85], σ1R antagonists are able to reverse such effects [85], but they are not able to trigger the opposite behavior, i.e., to induce depressive- or anxiogenic-like behavior [82,86], in line with our data in σ1R KO rats.

Extensive literature highlights the crucial role of σ1R in pathological pain, with its genetic or pharmacological blockade producing analgesic effects in different pain models [1,10,61,87]. In mice, σ1R KO significantly reduced pain-related behaviors across multiple pain models, including formalin injection [9,88], neuropathic pain of different etiologies [59,71,89], and visceral pain [90]. By contrast, σ1R KO mice showed normal nociception in the absence of an injury or when the somatosensory system is not sensitized [40]. To determine whether σ1R deletion affects sensory processing and nociception in rats, we evaluated several sensory parameters. In naïve σ1R KO rats, mechanical and thermal sensitivity were unchanged compared to WT controls, consistent with previous findings in σ1R KO mice, where no genotypic differences were observed across various sensory tests (von Frey [71], Hargreaves', Randall-Selitto [91], or acetone [71]). However, our formalin-induced nociception test revealed mild, non-significant differences, particularly in females, partially mirroring prior studies that reported about a 55 % reduction in formalin-induced pain-related behaviors in female σ1R KO mice [88].

As previously mentioned, σ1R inhibition reduces pathological pain and has been studied extensively in neuropathic pain, even at the clinical level, with the σ1R antagonist S1RA currently being tested in clinical trials with promising results [25,26]. Therefore, we examined pain-related behaviors in σ1R KO rats using the spared nerve injury (SNI) model, which is one of the most widely used and standardized models of traumatic nerve damage [92]. Our results partially resemble those obtained in mice in the model of SNI, with a markedly stronger effect on cold allodynia and a significant yet modest effect on mechanical allodynia [59]. However, we found that σ1R KO rats exhibited reduced thermal hyperalgesia after nerve injury, in contrast to previous findings in which σ1R KO mice developed thermal hyperalgesia in a similar way to WT mice [59]. Another study using a different model of nerve injury (partial sciatic nerve ligation), also reported that σ1R KO mice displayed reduced cold and mechanical allodynia, but they normally developed heat hyperalgesia [89]. Although we have shown that σ1R is present in every single sensory neuron in rats, as it is in mice [61], there are important differences in the neurochemistry of sensory neurons between the two species that could explain these differences in the pain phenotype in neuropathic pain [68,93]. Nevertheless, numerous studies have shown that pharmacological inhibition of σ1R can reduce thermal hyperalgesia in various pain models, both in WT mice and rats. The conflicting results between σ1R KO mice and pharmacological antagonism could be due to compensatory mechanisms [59,94], which could be less relevant in rats. Further research is needed to elucidate this discordance.

5. Conclusion

The σ1R KO rat model developed in this study confirms that the absence of σ1R does not affect viability, general health, or motor function but it does reveal reduced exploratory behaviors and minor sex-specific nociceptive differences. Consistent with findings from mouse studies, σ1R KO rats exhibit antiallodynic effects in a neuropathic pain model, reinforcing the role of σ1R in pain modulation and its potential as a therapeutic target. The σ1R KO rat model described in this study represents a valuable tool for investigating the role of σ1R in the physiology and pathophysiology of biological processes, as well as for the development of new drugs with affinity for the σ1R.

CRediT authorship contribution statement

Miguel Á. Huerta: Writing – review & editing, Writing – original draft, Methodology, Investigation. **Xavier Codony:** Writing – review & editing, Writing – original draft, Methodology, Investigation. **M. Carmen Ruiz-Cantero:** Writing – review & editing, Investigation. **Mónica Porras:** Investigation. **Miguel Á. Tejada:** Writing – review & editing, Investigation. **Aitana Rickert-Llàcer:** Investigation. **Antonia Artacho-Cordón:** Investigation. **Daniel Zamanillo:** Writing – review & editing, Writing – original draft, Supervision, Software, Methodology. **Enrique J. Cobos:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Funding acquisition. **Francisco R. Nieto:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition.

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Declaration of competing interest

Xavier Codony, Monica Porras, and Daniel Zamanillo report a relationship with WeLab that includes employment. All other authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Xavier Codony, Monica Porras, and Daniel Zamanillo reports a relationship with WeLab that includes: employment. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2025.123953.

Data availability

Data will be made available on request.

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