

Original article

Implication of sestrin3 in epilepsy and its comorbidities

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Running title

Sestrin3 in epilepsy co-morbidities

Abstract

Epilepsy is a serious neurological disorder affecting about 1% of the population worldwide. Epilepsy may arise as a result of acquired brain injury, or as a consequence of genetic predisposition. To date, genome-wide association studies and exome sequencing approaches have provided limited insights into the mechanisms of acquired brain injury. We have previously reported a pro-epileptic gene network, which is conserved across species, encoding inflammatory processes and positively regulated by sestrin 3 (SESN3). In this study, we investigated the phenotype of *SESN3* knock-out rats in terms of susceptibility to seizures and observed a significant delay in status epilepticus onset in *SESN3* knock-out compared to control rats. This finding confirms previous in vitro and in vivo evidence indicating that SESN3 may favor occurrence and/or severity of seizures. We also analyzed the phenotype of *SESN3* knock-out rats for common comorbidities of epilepsy, i.e. anxiety, depression, and cognitive impairment. *SESN3* knock-out rats proved less anxious compared to control rats in a selection of behavioral tests. Taken together, the present results suggest that SESN3 may regulate mechanisms involved in the pathogenesis of epilepsy and its comorbidities.

Keywords

sestrins, epilepsy, anxiety, depression

Abbreviations

AD: afterdischarge; AED: anti-epileptic drug; BSA: bovine serum albumin; CT: Cycle Threshold; EPM: Elevated Plus Maze; FS: Forced Swimming; GWAS: genome-wide association studies; hCG: human pregnancy urine chorionic gonadotropin; HD: head dipping; KA: kainic acid; KO: knock-out; LH-RH: Lutenizing Hormone - Releasing Hormone; mTLE: mesial temporal lobe epilepsy; mTORC1: mechanistic target of rapamycin complex 1; NOR: Novel Object Recognition; Nrf2: nuclear factor erythroid 2-related factor 2; OF: Open Field; PBS: phosphate buffered saline; PK: pharmacokinetics; PMSG: pregnant mare's serum gonadotropin; ROS: reactive oxygen species; SAP: SD: stretched-attend postures; Sprague-Dawley; SE: status epilepticus; SESN: sestrin; WT: wild-type; ZFN: zinc finger nucleases

Introduction

Epilepsies are a complex group of neurological diseases, each differing in many aspects: etiology (genetic, lesional/metabolic or unknown), localization of the epileptic focus, symptomatology. The common feature in epilepsy is the occurrence of spontaneous seizures, triggered by a paroxysmal excitatory activity in neuronal networks. However, whilst recurrent seizures are a core feature of epilepsy, people with epilepsy report additional co-morbidities (Gaitatzis et al., 2012; Pitkanen et al., 2019), including neuropsychiatric disorders such as anxiety, depression and cognitive impairment. These comorbidities have a major impact on every-day social life (Taylor et al., 2011).

The burden of epilepsy comorbidities is high: approximately 50% of adult patients suffer from at least one comorbidity (Keezer et al., 2016). Similarly in animal models of epilepsy, where models of mesial temporal lobe epilepsy (mTLE) develop a depression-like state (Steimer, 2011; Chen et al., 2016) and anxiety-related behavior (Falcicchia et al., 2018; Hesdorffer et al., 2012; Paolone et al., 2019). These observations highlight that shared pathogenic mechanisms may underlie both epileptic seizures and some of the neuropsychiatric comorbidities of epilepsy (Kanner, 2016).

Several causative mechanisms linking epilepsy and depression have been proposed, including a decrease in function of specific neurotransmitters (serotonin, noradrenaline, dopamine, GABA), tissue atrophy, morphological alterations in limbic areas and higher cytokine concentrations (IL-1beta) (Kanner, 2016; Rider et al., 2017). Comorbidities represent an important issue also for the healthcare system because they require additional, expensive medical interventions, and can also aggravate the severity of epilepsy or the side effects of anti-epileptic drugs (AEDs). The identification of pathophysiological mechanisms underlying both comorbidities and epilepsy therefore offer the potential for novel therapeutic interventions with potential health benefits beyond seizure control.

Recently, we used a systems biology approach to investigate transcriptional networks and pathways within the hippocampus of mTLE patients who underwent surgical resection of the epileptic focus, and we identified a transcription program that is overexpressed in the mTLE hippocampus and promotes expression of epileptogenic signaling pathways (Johnson et al., 2015). The sestrin-3 (*SESN3*) gene, belonging to the sestrin family of stress-inducible proteins, was identified as an activator of this transcriptional program. The pro-epileptic gene network regulated by *SESN3* was initially identified using the pilocarpine mouse model of acquired epilepsy and subsequently shown to be conserved in the human epileptic hippocampus (Johnson et al., 2015).

This observation was actually unexpected, because other sestrins may exert anti-epileptic effects. By upregulating the nuclear factor erythroid 2-related factor 2 (Nrf2) signaling, SESN1 and SESN2 are thought to inhibit the mechanistic target of rapamycin complex 1 (mTORC1), attenuating reactive oxygen species (ROS) accumulation (Rhee and Bae, 2015). mTORC1 inhibition, as obtained through injection of the prototype inhibitor rapamycin, can decrease seizure susceptibility (Citraro et al., 2016; Talos et al., 2018). However, mTORC1 activation seems to be required for ketamine-induced anti-depressant effects (Li et al., 2010), indicating that SESN1 and 2 may worsen epilepsy comorbidities.

In this work, we asked if SESN3 has a similar double-edged effect on epilepsy and epilepsy comorbidities. To explore this concept, we generated *SESN3* knock-out (*SESN3* KO) rats and studied in detail their phenotype, not only in terms of susceptibility to seizures, but also to anxiety and depression.

Materials and methods

Animals

SESN3-KO and wild type (WT) rats in a Sprague-Dawley (SD) background (Harlan, Italy) were used for all experiments. They were housed under standard conditions: constant temperature (22-24°C) and humidity (55-65%), 12h light/dark cycle, water and food ad libitum. The ARRIVE (Animal Research: Reporting *In Vivo* Experiments) guidelines have been followed. Procedures involving animals and their care were carried out in accordance with European Community, national and local guidelines, laws and policies. Experimental protocols for animal experimentation were approved by the University of Ferrara Ethical Committee and the Italian Ministry of Health (n.953/2016-PR). All animals were euthanized by anesthetic overdose.

SESN3-KO rat generation

SESN3-KO rats were generated at the Imperial College, London, exploiting a zinc finger nucleases (ZFN) engineered plasmid (Sigma Aldrich) transfected into single cell SD embryos. The ZFN technique allowed to cut the *SESN3* gene at a specific site, leading to its complete knock down (Supplementary Figure 1). Heterozygous rats were then mated to give birth to WT and *SESN3*-KO rats.

The foster females aged 8 to 12 weeks have been injected intraperitoneally (i.p.) with 40 µg (0.2 ml) of Lutenizing Hormone - Releasing Hormone (LH-RH) agonist 4 days prior to mating. On the day of mating, the recipients were individually placed with males. The following day, the females were de-mated and examined for the presence of copulation plug. The females presenting a plug have been used as embryo recipient for embryo transfer surgery.

SD females aged 4 to 6 weeks have been used as embryo donors. Two days before mating, the females were injected with 30 IU of pregnant mare's serum gonadotropin (PMSG, i.p.). On the day of mating, the females have been injected with human pregnancy urine chorionic gonadotropin (hCG, 40 IU, i.p.) and placed individually with SD males. The following day, the females were separated, killed and the oviducts removed. The embryos were then collected and left in an incubator at 37°C, 5% CO₂ until used for pronuclear injections.

The embryo recipients were anesthetized with a mix of ketamine and xylazine (1:1, i.p.) and a painkiller (Carprofen, 5 mg/kg, s.c.). The female rats were placed under the microscope, the incision area between flank and ribcage was shaved and an incision was made through the skin and peritoneal wall. After the implantation of the embryos, the peritoneal wall was sutured. The female rats were then placed back in their cages on a heating pad at 37°C, until recovery from anesthesia.

After having generated the first colony of rats, animals were sent to the laboratory of the University of Ferrara, where the breeding was conducted in heterozygosis, by mating *SESN3*-KO males and SD females.

***SESN3*-KO rats genotyping**

Genotyping was conducted via PCR analysis. DNA was obtained by harvesting ear punch biopsies from 21-day-old rats, both female and male, and put into 1.5 ml tubes. Samples were then dipped in 25 µl OB protease and 180 µl lysis buffer (55°C, overnight) to enhance tissue digestion.

The day after, DNA was extracted with Tissue DNA Kit (Omega bio-tek) and the ZFN target region was PCR-amplified. After amplification, a specific nuclease was added to the DNA samples (IDT Surveyor Mutation Detection Kit), enabling to recognize the cutting sites of the DNA chain of samples from *SESN3*-KO rats. Finally, samples were run in a 2% agarose gel: WT bands were observed at 400 kb, *SESN3*-KO bands at 200 kb.

WT and *SESN3*-KO homozygous littermates were employed for all experiments.

Behavioral testing in pups

Observational screens and simple behavioral tests were performed 7 days after birth to assess general health and developmental stages. We rapidly screened rats for the appearance of the fur,

whiskers and posture and we tested them for neurological and sensory reflexes and non-complex motor activities through a battery of simple tests: the postural flexion test, the righting reflex test, the response to fear test, the negative geotaxis test and the placing responses test.

Postural flexion test. Pups were lifted by the tail at 30 cm height from a horizontal plan. If the proprioceptive system is functioning correctly, pups perceive the gravity and turn around in the natural posture. We recorded the time it took pups to turn around, with a cut-off of 60 s, and the side by which they turned.

Righting reflex test. Pups were laid supine on a horizontal plan. The time required to turn procumbent and the side by which the pup turned was noted by the experimenter.

Response to fear test. Pups were placed near the edge of a plan, facing the empty space. The time required for turning to the opposite side or for evaluating the intention to explore the empty (i.e. jumping from the plan) was recorded, with a 60 s cut off.

Negative geotaxis test. Pups were put facing down on a 30° inclined plane, and the experimenters noted the time employed to turn up, with a cut off of 60 s. Pups that failed to turn face up, or that slipped down, were assigned 60 s.

Response to stimulus test. This test evaluates the capacity of the pups to react to an external stimulus. The experimenter, leaving its paws free to move, holds the animal. Using an iron spatula, the experimenter touches both the right and the left anterior part of the rat's paw and notes if the animal moves the paw after the stimulus.

Behavioral testing in adults

A battery of behavioral tests was conducted in adult (8 weeks old) WT and *SESN3* KO male rats, to assess anxiety and depressive-related behavior, motor and cognitive functions. We employed the following tests: Elevated Plus Maze (EPM), Open Field (OF), Novel Object Recognition (NOR), Rotarod and Forced Swimming (FS). Since the FS test cause a high level of stress, only a subgroup of rats (WT n=11, *SESN3*-KO n=8) was tested and was not used in further experiments. Animals performed just one test per morning, under artificial diffused red light. Behavioral experiments were conducted in a soundproof room, into which animals were moved at least 30 min before testing. Each rat was used to be handled by researchers before being evaluated in behavioral tests.

Elevated-plus maze test. The test was performed as described by Tchekalarova and colleagues (Tchekalarova et al., 2015). The apparatus consisted of two open arms (50 x 10 cm) and two closed arms (50 x 10 x 50 cm) connected through a central platform (10 x 10 cm). The apparatus was 50 cm above floor level. Luminosity was checked using a luminometer and found to be nearly 1 in the open arms and close to zero in the closed arms. At the beginning of the test, rats were placed in the

central part of the platform, facing an open arm. The test lasted 5 min. The calculated measures were: number of entries in open arms; number of entries in closed arms; time spent in open arms; time spent in closed arms; number of stretched-attend postures (SAP), i.e. how many times the rat looked at the open arms while its body being in a closed arm; number of head dippings (HD), i.e. how many times the rat looked down from the open arms of the EPM apparatus; and number of rearings, i.e. how many times the rat looked up leaning against the walls of the closed arms. After each trial the EPM apparatus was cleaned with 0.1% ethanol solution.

Open field test. The test was performed as described before (Tchekalarova et al., 2015). The apparatus consisted of a gray box (100 x 100 cm) divided into two compartments: outer (periphery) and inner (center). The rat was placed in the center of the box and was allowed to explore it for 20 min. The calculated measures were: total distance traveled (in cm); time spent in the center; number of entries in the center; time spent in the corners; and number of entries in the corners. After each trial, the OF apparatus was thoroughly cleaned with 0.1% ethanol solution. The test was recorded using a video camera IR (DSS1000 video recording system V4.7.0041FD, AverMedia Technologies, USA), and then videos were analyzed using the Any Maze software (Ugo Basile S.R.L., Gemonio VA, Italy).

Forced swimming test. The forced swimming test was employed to investigate despair-like behavior (Porsolt, 1979). The test was performed using a plexiglass cylinder (50 x 20 cm) filled by three fourth with water kept at $25\pm 1^\circ\text{C}$. Two swim sessions were conducted: 15 min of training followed (24 h later) by a 5-min test session. After each test, the rat was wiped and kept warm by a heating device for 10 min. Two different experienced observers recorded (i) the time of immobility, which occurred when the rat floated in the water without struggling and (ii) the time of climbing or movements made by the rat to keep its head above the water. The time spent immobile has been related to a depressive behavior (D'Aquila et al., 2004).

Novel object recognition test. The NOR test was performed as described by Ennaceur and Delacour (Ennaceur and Delacour, 1988). The test consisted of three phases: habituation, acquisition and test. The OF test, conducted the day before NOR, was used as habituation phase. The day after habituation, the acquisition trial was conducted by placing the rat in the field, in which two identical objects were positioned at the corners of the arena, approximately 10 cm from the walls. Rats were free to explore the two objects for 5 min, and exploratory activity (i.e. the time spent exploring each object) was recorded. After 2 h, rats were placed again in the arena, in which one object was substituted with a novel one, not used in the acquisition phase. Again, the time that each animal spent exploring each object was measured. We considered a valid exploratory behavior when the rat directly interacted with the object. The choice of objects as novel or familiar was

carried out in a random way, and the position of each object was also alternated between trials. After each test, the arena and the objects were thoroughly cleaned with 0.1% ethanol solution.

Rotarod test. The test has been run in a three-day trial. The rotarod apparatus is composed of a rotating cylinder, divided into different compartments, one for each animal. The cylinder rotates and the speed is increased by steps of 5 rpm every three min. Maximum duration of the test was 30 min. The operator recorded every fault, i.e. when rats fell from the cylinder or hung to the cylinder without running. The test was considered finished if the animal made 4 continuous errors.

Models of epilepsy

Animals underwent seizure susceptibility tests at least two weeks after behavioral testing (i.e. at 10-14 weeks of age).

Pilocarpine. Both WT and SESN3-KO animals were randomly assigned to two groups: pilocarpine treated and controls. The former received a single injection of methyl-scopolamine (1 mg/kg, s.c.) 30 min prior to pilocarpine (370 mg/kg, i.p.), whereas the latter received a single injection of methyl-scopolamine 30 min prior to vehicle (0.9% NaCl solution). Thereafter, experienced researchers observed the animals, taking note of convulsive activity based on the Racine scoring scale (Racine et al., 1972). Usually, about 80% of WT rats develop seizures evolving into convulsive status epilepticus (SE) within the first 20-25 min after pilocarpine injection (Roncon et al., 2015). The experimenters also recorded the time required to enter convulsive SE. SE has been interrupted 3 h after its onset by administration of diazepam (20 mg/kg, i.p.).

Intrahippocampal kainic acid. We also assessed susceptibility to SE in the intra-hippocampal kainic acid (KA) model (Raedt et al., 2009). Because KA was administered in awake animals, to allow proper observation of SE, guide cannulas were implanted one week before KA administration in the right ventral hippocampus for insertion of the injecting needle.

Stereotaxic surgery was thus performed to implant 20 G guide cannulas (PlasticsOne, USA) in the ventral hippocampus of both WT and SESN3-KO rats, following Paxinos' Atlas (coordinates from bregma AP= -5.6; ML= +4.5; DV= +3.5). Anesthesia was induced with ketamine/xylazine (respectively 87 and 13 mg/kg, i.p.) and maintained with isoflurane (1.5% in oxygen). After placing the rat in the stereotaxic frame, an incision made the skull exposed and a burr hole was drilled after at the above coordinates. Six other smaller holes were drilled to position screws. The guide cannula was then inserted, dental cement was used to fix cannula and screws to the skull, and the scalp was sutured with stitches. Animals were treated with tramadol and antibiotics before and after the stereotaxic surgery and then checked daily to monitor their well-being.

After a week of recovery, rats were injected with KA (0.4 $\mu\text{g}/0.2\mu\text{l}$) using a 30 G needle connected to a 5 μl Hamilton syringe. After KA administration, rats were video-monitored and SE severity was recorded using the Racine's scale. If SE did not resolve within 2.5 h, diazepam (10 mg/kg, i.p.) was administered to stop seizure activity.

Kindling. Rats were anesthetized with mixed ketamine (Imalgene) and medetomidine (Domitor) in water (v/v; 50 mg/kg and 0.5 mg/kg, respectively; intramuscular injection, 2 ml/kg) and implanted with a bipolar stimulation/recording electrode into the right basolateral amygdala with the following coordinates: AP= -2.3, ML= -4.8, DV= +8.5, all measured from bregma. The electrode consisted of two twisted Teflon-coated stainless-steel wires. An electrode in the left occipital cortex served as the indifferent reference electrode. Bipolar, reference and ground electrodes were connected to plugs and the electrode assembly and anchor screws were held in place with dental acrylic cement (Grip Cement Liquid, Dentsply International Inc., USA) applied to the exposed skull surface. After 50 min, awakening was facilitated with an intramuscular administration (2.5 ml/kg) of atipamezole (Antisedan), 1.25 mg/kg, prepared at the final concentration of 10% in water. Following surgery, the rats were kept individually in Makrolon cages (Model 4, 480 x 375 x 210 mm) and were allowed ad libitum access to standard dry pellet food and tap water before random assignment to experimental groups.

After a postoperative period of 3 weeks, rats were placed in individual boxes and stimulated in the amygdala with increasing stimulation intensities, 1 ms monophasic square wave pulses, 50 Hz for 1 s. We started at sub-maximal electrical intensity to determine the afterdischarge (AD) seizures threshold for each animal. We used a range of stimulation intensities from 20 to 444 μA with increasing steps of 20%.

Three days after initial threshold determination, rats were stimulated once daily, 5 days per week, in the amygdala with 500 μA . Kindling was defined as the appearance of 10 consecutive stage 4 or 5 seizures according to the scale of Racine where:

- 0 = no reaction
- 1 = stereotype mouthing, eye blinking, mild facial clonus
- 2 = head nodding, severe facial clonus
- 3 = myoclonic jerks in the forelimbs
- 4 = clonic convulsions in the forelimbs with rearing
- 5 = generalized clonic convulsions associated with loss of balance

Scores 0-2 were considered to reflect the focal phase and scores 3-5 the generalized phase of the motor seizures.

Structure analysis and immunofluorescence

Rats were killed by decapitation after an anesthetic overdose. Brains were removed and immersed in 10% formalin for 48 h. They were then processed using a standard protocol (VTP 300, Bio-Optica, Milan, Italy) and paraffin-embedded. Coronal sections of 7 μm were cut across the hippocampus and mounted onto polarized slides (Superfrost slides, Diapath Martinengo, BG, Italy). Sections were dewaxed with 2 washes in xylol (10 min each), 5 min in ethanol 100%, and rehydrated in ethanol 95%, ethanol 80% and phosphate buffered saline (PBS) 1x (5 min each).

To analyze potential differences in tissue architecture, we performed a hematoxylin/eosin staining. Sections were immersed in hematoxylin for 5 min, washed in tap water, then stained with eosin for 2 min and washed again. For immunofluorescence, antigens were unmasked with a solution of citric acid and sodium citrate in a microwave oven at 750 watts (3 min), then at 350 watts (2 cycles of 5 min). Sections were incubated with 5% bovine serum albumin (BSA) and 5% serum of the species in which the secondary antibody was produced, for 30 min at room temperature. They were then incubated in a humid plastic box with primary antibodies, overnight at 4°C. The primary antibodies were: anti-IBA-1, diluted 1:200 (rabbit monoclonal, #234003, Synaptic System, Gottingen, Germany) for microglia staining; anti-GFAP 1:100 (mouse monoclonal, #MAB12029, Immunological Science, Rome, Italy) for astrocyte staining; anti-NeuN/Fox-3 1:100 (mouse monoclonal, #MAB-90228 Immunological Science, Rome, Italy) to label neuronal nuclei and cytoplasm; anti- β 3-tubulin 1:400 (mouse monoclonal, #4466X, Cell Signaling Technologies, MA, USA) for neuron staining. Two washes in 1xPBS and a 30 min incubation in 0.3% Triton X-100 were performed before applying the secondary antibody, 488 or 594 Alexa-Fluor anti-mouse or anti-rabbit (diluted 1:250 in 1x PBS) depending on the species of the primary antibody. Sections were left in a dark chamber under controlled humidity conditions for 3 h before proceeding with DAPI staining (0.0001% in 1xPBS for 15 min; Santa Cruz, Texas, USA) to label nuclei. Coverslips were mounted using an aqueous anti-fading mounting gel (Sigma).

Image analysis was performed on 6- μm paraffin-embedded coronal brain sections cut across the dorsal hippocampus (plates 21/22, Paxinos and Watson, 2007). Images were captured using a Leica microscope (DMRA2, Leica). Iba1- and GFAP-positive cells in the hippocampus were counted by automatic image analysis through a batch process in the Fiji (ImageJ) open source software (Schindelin et al., 2012), according to the following algorithm: (1) Auto-brightness/contrast to enhance signal/background ratio; (2) Reduce noise from image with mean

filter; (3) Convert image to 8-bit black and white; (4) Auto thresholding according to the MaxEntropy method (Kapur et al., 1985); (5) Analyze and count thresholded particles excluding artifacts (out of defined size-range, perfectly linear and perfectly round ones); (6) Summarize count results for each image and save in custom table. NeuN-positive pixel were measured using the same software and an algorithm tailored to measure percent of supra-threshold pixels according to the IsoData method (Ridler and Calvard, 1978). The complete macros are shown in Supplementary Figure 2. Average values of 3 sections per animal were employed for statistical analysis.

Real-Time PCR

Total RNA was extracted from frozen hippocampi of WT and *SESN3*-KO rats, by using the RNeasy Lipid Tissue Mini Kit (Exiqon, #74804), following the manufacturer's instructions. RNA was quantified using Nanodrop and diluted to 150 ng/ul, then retro-transcribed to cDNA using the SuperScript IV First-Strand Synthesis System (Thermo Fisher Scientific, #18091050) and stocked at -20°C. Real-Time PCR was run to evaluate the expression of IL-1 β , TNF α , c-fos, c-jun and *Sesn1*. Data were normalized to alpha-tubulin. The reverse transcription protocol was set up following Biorad Kit indications, while the primers recognizing the gene sequencing for the final quantification were purchased from Exiqon.

Statistical analysis

All experiments were run and analyzed by blinded experimenters. Animals have been assigned randomly during the behavioral tests: only during NOR test, the researchers followed a pre-imposed order because following the correct timing and order of animals is pivotal for the test to succeed.

To test for statistical significance between two groups (i.e. *SESN3*-KO vs WT rats) a two-tailed unpaired *t*-test has been applied, and a two-tailed Mann-Whitney U-test has been used for non-normally distributed data, as indicated. The Welch's correction has been used when data showed different standard deviations between the two samples. Fold change difference analysis after Real-Time PCR was performed using the Livorak formula.

Statistical test analyses have been performed and graphs prepared with GraphPad Prism (6.0 version).

Data availability

Data supporting the findings of this study are available from the corresponding author on request.

Results

Pups behavior

To investigate the neurological development of *SESN3*-KO rats, we performed a battery of behavioral tests on KO and WT littermate pups, that provide information on proprioception, movement execution, general neurological development and fear. Seven days after birth, both male and female rats were evaluated in all tests. First, pups were tested for postural flexion and righting reflex to test proprioceptive and cognitive functions. These two tests explore the capacity of the rat to return to its normal position after being posed in an unnatural position by the operator. WT pups spent less than 10 s to complete the tasks: in the postural flexion test, they spent 9.6 ± 1.0 s to rotate by 45° when suspended by the tail (Supplementary Figure 3A), whereas in the righting reflex test they spent 5.0 ± 0.5 s to rotate from a supine to prone position (Supplementary Figure 3B). KO pups performed in a similar manner (no significant differences), as shown in Supplementary Figure 3. Next, we tested the ability to respond to an external stimulus represented by a touch with a small metal spatula to the left and the right front paw. Pups should retrieve their paw when touched. KO rats displayed a significantly higher percentage of correct answers (86.2%) compared to the WT littermates (62.8%; $P=0.003$; power= 0.77; Figure 1A).

Finally, we performed two tests to analyze fear and anxiety-related behavior. In both tests, the pup is forced to an unnatural position, i.e. positioned facing down on a 45° inclined plane in the negative geotaxis test, and on the edge of a table facing the empty space, in the response to fear test. In the negative geotaxis test, *SESN3*-KO rats were much faster in completing their task compared to WT ($P<0.001$; power=0.99; Figure 1B). Interestingly, 20 of 46 WT and 3 of 46 *SESN3*-KO pups froze and stayed immobile for more than 60 s (the cut off time). These rats were included in the analysis with a time of 60 s. We observed a similar situation in the response to the fear test. Indeed, *SESN3*-KO pups returned in the middle of the table, escaping from the empty space, significantly faster than the WT ($P=0.008$; power=0.92; Figure 1C). However, 11 KO but only 2 WT pups (out of 46 per group) attempted to jump off the table. Together, these data highlight a fearless-like attitude of *SESN3*-KO pups compared to WT littermates that, instead, displayed a passive reaction to the tests. This behavior may be linked to a less anxious phenotype.

Adult rats' behavior

Elevated Plus Maze. The EPM test aimed to evaluate anxiety. During this test, rodents normally prefer the most protected and safest areas of the apparatus, i.e. the closed arms. Thus, the more time

they spend in the open arms, the less they can be considered anxious. In this test, we observed a significant difference between the two groups both in terms of time spent ($P=0.004$) and entries ($P<0.001$; power=0.97) in the open arms, as *SESN3*-KO rats spent more time (about +30%; Figure 2A) and entered more frequently in the open arms (about +80%; Figure 2B).

During the 5-minute testing, we also explored other behavioral traits, such as the number of stretched-attended postures (SAP), the number of head dipping (HD), and the number of rearings. The comparison between *SESN3*-KO and WT rats showed a similar number of HD and rearings (Figure 2C and 2D), whereas a significantly increased number of SAP was observed in the KO group ($P=0.003$, power=0.76; Figure 2E). These findings may reflect a proactive attitude towards danger situations of *SESN3*-KO rats, in line with the behavior observed in the pups in the response to fear.

Open field. In order to better characterize such less anxious phenotype of *SESN3*-KO rats, we tested the two groups in the OF test. This test provides additional information on susceptibility to anxiety, based on the number of entries and the time spent in the central area of the arena, that is perceived as a dangerous area of the apparatus. The *SESN3*-KO rats spent slightly more time in the central area compared to WT littermates ($P=0.023$, power=0.64, Figure 3A), while entering the central area the same number of times as WT (Figure 3B). However, the average time per entry was slightly, but not significantly increased in *SESN3*-KO rats (Figure 3C). Both groups spent a similar amount of time (Figure 3E) and entered a similar number of times in the corners (Figure 3F). Importantly, the behavioral profile observed in the EPM and OF cannot be due to increased motor activity, because *SESN3*-KO and WT rats walked similar total distances in the OF arena during the test (Figure 3F).

Forced swim test. The two parameters taken into consideration were the climbing and the immobility time (Figure 4). During the 5 min testing, *SESN3*-KO rats displayed a slight, but non-significantly different tendency to keep trying to escape from the water cylinder and to stay less immobile compared to WT rats. Increasing sample size might have permitted reaching significance. However, we had to limit numbers for this highly stressful test.

Novel object recognition. We performed the novel object recognition (NOR) test to evaluate if a memory impairment was also present in *SESN3*-KO rats. During the testing trial, in which one object (familiar) was substituted by a novel one, both WT and *SESN3*-KO rats explored for a longer time the novel than the familiar object. No significant differences between the two groups could be observed (Figure 5A).

Rotarod. Finally, to further verify that changes in motor activity could account for the findings in the EPM and OF, we performed the Rotarod test. No differences in motor coordination were

observed between the two groups (Figure 5B).

Seizure susceptibility

Systemic pilocarpine. The epileptic phenotype was first explored by inducing status epilepticus (SE) by i.p. administration of pilocarpine. Eleven of 13 *SESN3*-KO rats (85%) did not develop SE within 30 min after pilocarpine injection and were administered a second half dose of the chemoconvulsant. In contrast, only 4 of 15 WT rats (27%) did not develop SE within the first 30 min and received a second half dose. Altogether, *SESN3*-KO rats needed significantly more time (and higher doses of pilocarpine) to develop the SE compared to WT ($P=0.008$; power= 0.92; Figure 6A).

Intra-hippocampal KA. Because of a progressive accumulation of fat tissue, *SESN3*-KO animals tend to have a greater body weight in adulthood compared with WT rats. This may affect the distribution of peripherally administered drugs, including pilocarpine. Therefore, we decided to verify the above findings using a SE model in which the chemoconvulsant is directly administered in the brain, namely intra-hippocampal KA. Consistent with the findings in the pilocarpine model, the onset of generalized convulsive SE was delayed in *SESN3*-KO in comparison with WT rats ($P=0.041$; power= 0.61; Figure 6B).

Kindling. Because both the pilocarpine and the KA model are chemically induced, we decided to investigate also the electrical kindling model. Contrary to the expectations, we did not find any difference in AD threshold or kindling development between *SESN3*-KO and WT rats (Figure 6C and 6D).

Morphology and immunohistochemistry

Histological analysis was focused on the hippocampus, the region where *SESN3* was originally identified to regulate a proconvulsive module of genes (Johnson et al., 2015). Based on hematoxylin-eosin staining, no differences in the structure of the hippocampus were detected (data not shown). The number and morphology of neurons, astrocytes and microglial cells were also not obviously altered (Figure 7 and Supplementary Figure 4).

Real-Time PCR

Expression levels of selected Module-1 genes (*IL-1 β* , *TNF α* , *c-fos*, *c-jun*), which we previously show to be regulated by *SESN3* in the hippocampus (Johnson et al., 2015), has been quantified as Cycle Threshold (CT) number using the Bio-Rad software. No significant differences between the

two groups was observed. We therefore hypothesized that compensatory mechanisms may be set in motion by knocking-out *SESN3*. In line with this idea, although not a demonstration of it, *SESN1* expression levels were slightly higher in *SESN3*-KO animals than in WT controls, even if not in a significant manner (data not shown).

Discussion

The extensive analysis of the phenotype of *SESN3*-KO rats presented here supports the notion that *SESN3* not only promotes seizures as previously reported (Johnson et al., 2015), but may also impact the appearance of certain comorbidities of epilepsy (e.g. anxiety), while not interfering with others (e.g. cognitive impairment).

SESN3-KO rats and their WT littermates were subject to a battery of behavioral tests assessing anxiety, depression, cognition and motor function. The most striking result was that both pups and adult *SESN3*-KO rats manifested increased ability to cope with novelty and stress, such as the exploration of the empty space at the edge of a table, or when being placed into the open space of an arena. These findings indicate that these animals are less prone to the fear and anxiety that normally guide animals to stay away from situations that are perceived as potentially dangerous. Importantly, this is not due to an unspecific stimulation of motor activity, because the distance walked in the open field during a test session and the performance on the Rotarod test were identical in KO and WT rats. Moreover, because behavioral analyses were made in the same animals, when pups and then as adults, these behavioral traits do not appear to undergo compensation in the course of life.

Based on these findings, we hypothesized that *SESN3*-KO rats would have been also less prone to depression. This idea was tested using the FST, a rather stressful test that induces a state of desperation due to the impossibility to escape a life-threatening situation. While a lower tendency to desperation (i.e. continued attempts to escape and less immobility) was observed in *SESN3*-KO rats, this was not statistically significant. In addition, we did not observe any improvement in cognitive tests like novel object recognition.

Next, we thought to confirm in mammals the observation previously made in a Zebrafish model, that knocking down *SESN3* can reduce seizure susceptibility (Johnson et al., 2015). As a first study test we employed the pilocarpine model, and found that, in keeping with the previous results, susceptibility to SE was reduced in *SESN3*-KO rats, because they required higher doses and

more time than WT to develop SE. In our WT group, these parameters were identical to those previously reported in the literature (Curia et al., 2008). However, a limitation in this experiment is that the pharmacokinetics (PK) of pilocarpine may be altered in adult *SESN3*-KO rats, because these animals tend to gain weight and, in particular, accumulate fatty tissue (Narasimhan et al., 2009a; Narasimhan et al., 2009b; Dalina et al., 2018). In addition, other authors have reported the opposite result that knocking down *SESN3* using a different approach (namely, intra-hippocampal injection of lentivectors expressing a small interfering RNA against *SESN3*) aggravates pilocarpine-induced acute seizures (Huang et al., 2018). Therefore, we thought to employ a model in which a convulsant agent (KA) is directly injected in the brain, thus avoiding the PK confounding factor (Bragin et al., 2004). This approach confirmed our data from the pilocarpine model. In addition, we also decided to test an electrical model of epilepsy, amygdala kindling. However, we did not observe any difference in AD threshold or kindling development between the two groups, suggesting a prominent role of *SESN3* on seizure generation and generalization, rather than on epileptogenesis.

Finally, similar to *SESN2* KOs (Kallenborn-Gerhardt et al., 2013), we did not find differences in morphology or number of neuronal cells populations nor significant activation of astrocytes or microglia between *SESN3*-KO and WT rats.

Overall, these data suggest the presence of a phenotype characterized by reduced anxiety and fear, as well as lower susceptibility to seizures. However, only some of these traits are clearly evident in *SESN3* KO rats, while others are more subtle and not confirmed when using additional tests. One possible interpretation of these inconsistencies may be the constitutive gene knock out, which could trigger compensatory mechanisms during development.

As stated in the Introduction, the finding that *SESN1* and *2* inhibit mTORC1 suggest an anti-epileptic but also a pro-depressant effect (Li et al., 2010; Citraro et al., 2016; Talos et al., 2018). On the other hand, our present data support the notion that increased *SESN3* activity may have both a pro-epileptic effect (confirming our previous findings (Johnson et al., 2015)), and an anxiogenic effect. Whereas the former effect is likely due to activation of expression of a module of pro-epileptic genes (Johnson et al., 2015), the second remains to be investigated. *SESN3* is primarily regulated by *FOXO1* and *FOXO3* (Budanov, 2011). *FOXO* proteins are expressed in the limbic system, in particular in the hippocampus, amygdala and nucleus accumbens, areas implicated in the regulation of mood and emotions (Wang et al., 2015). Indeed, *FOXO3* was hypothesized to be a pro-depression gene, because *FOXO3*-deficient mice are less prone to develop depressive-like behavior (Polter et al., 2009). These effects of *FOXO3* may be mediated by activation of *SESN3* expression that, in turn, will increase expression of the genes in the pro-epileptic module, many of

which (for example IL-1b may exert anxiogenic and/or depressant effect (Pineda et al., 2012). In neurons, FoxO3a activation is involved in protection against excitotoxic and proteotoxic insults (Mojsilovic-Petrovic et al., 2009). Furthermore, activation of FoxO3a has been associated with increased neuronal excitability (Howlett et al., 2008). Differential role of FoxO3a has been suggested depending on the duration of seizures, meaning brief and repetitive or prolonged and harmful (Caballero-Caballero et al., 2013); this may explain why we did not highlight an impact of *SESN3* KO in the kindling model which induces limited neuronal death in comparison to SE models.

In conclusion, the present data suggest that *SESN3* may represent a common mechanism of epilepsy and anxiety (maybe depression), i.e. some comorbidities of epilepsy. Identifying common mechanisms of epilepsy and its comorbidities would be important to develop therapies that are not purely anti-seizure and, in some cases, may even worsen comorbidities (Kanner, 2016). The present findings are informative in that direction and will prompt more detailed investigations. However, the limitations of this study should be also carefully considered. First, behavioral testing was performed in normal (naïve) animals. It will be important in the future to extend it to chronically epileptic animals in which anxiety, depression and cognitive impairment are already present (Hesdorffer et al., 2012; Falcicchia et al., 2018; Paolone et al., 2019). Second, part of the phenotype may have been obscured in our experiments by compensatory mechanisms that occurred during development in constitutive KO. Therefore, another important future goal will be to modulate *SESN3* expression in key brain areas and at specific time points. These limitations notwithstanding, the present study identifies a possible target for the treatments of both epilepsy and its psychiatric comorbidities. Strategies to pursue this aim may include antisense oligonucleotides, gene therapy vectors expressing small interfering RNAs (siRNAs) against *SESN3*, or even small molecules. Molecules activating other sestrins have been developed already (Kato et al., 2019), suggesting that it may also be possible in the future to identify *SESN3*-selective antagonists.

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Competing interests

None.

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Figure legends

Figure 1. Behavioral testing on pups. (A) Response to stimulus. Scatter dot plot of all responses. Shown are the correct (1) or wrong (0) responses following a stimulus to the right and left anterior paw of WT (open circles) and of the *SESN3*-KO rats (solid circles) (n=46 rats). Horizontal lines indicate the percentage of correct responses. Statistical analysis: Mann-Whitney U test. (B) Negative geotaxis test. Time spent by WT and *SESN3*-KO rats to turn from down to up on the 45° inclined plan (n=46 rats). (C) Response to fear. Time spent by WT and *SESN3*-KO rats to turn from the edge to the center of the plan (n=46 rats). Statistical analysis in B and C: unpaired *t* test with Welch's correction. * P<0.05; ** P<0.01; *** P<0.001.

Figure 2. Elevated plus maze test. (A) Percent of time spent exploring the open arms over the total time of exploration. (B) Percent of entries in the open arms over the total number of entries. (C) Number of HD during the 5 min of testing. (D) Number of rearings during the 5 min of testing. (E) Number of SAP during the 5 min of testing. Statistical significance has been calculated with the Unpaired T test with the Welch's correction. In all panels, open circles represent WT (n=23 rats) and solid circles represent *SESN3*-KO rats (n=37 rats). Statistical analysis in A and B: Mann-Whitney U test. Statistical analysis in B and C: unpaired *t* test with Welch's correction. ** P<0.01; *** P<0.001.

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Figure 4. Forced swimming test. The plot shows the time spent by the rats in trying to climb the cylinder or swimming in an attempt to find an escape (climbing) and the time spent floating immobile (immobility). Open circles represent WT (n=13 rats) and solid circles represent *SESN3*-KO rats (n=8 rats).

Figure 5. Novel object recognition and rotarod tests. (A) Novel object recognition. Percentage of time spent interacting with the familiar and the novel object in 30 WT and 33 *SESN3*-KO rats. (B) Rotarod. Time spent on the rods during in 3 trials of testing. Open circles represent WT (n=10 rats) and solid circles represent *SESN3*-KO rats (n=19 rats).

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Figure 7. Immunohistochemical analysis. Neurons (A-C), labelled in red with a NeuN antibody), astrocytes (D-F, labelled in red with a GFAP antibody), microglial cells (G-I, labeled in green with an Iba-1 antibody). Shown are representative sections of the dentate gyrus area from naïve WT (A,D,G) and *SESN3*-KO rats (B,E,H). DAPI staining in blue. Data quantification in C,F,I. In all panels, open circles represent WT and solid circles represent *SESN3*-KO rats. Horizontal bar in panel B (for all panels) = 100 μm .

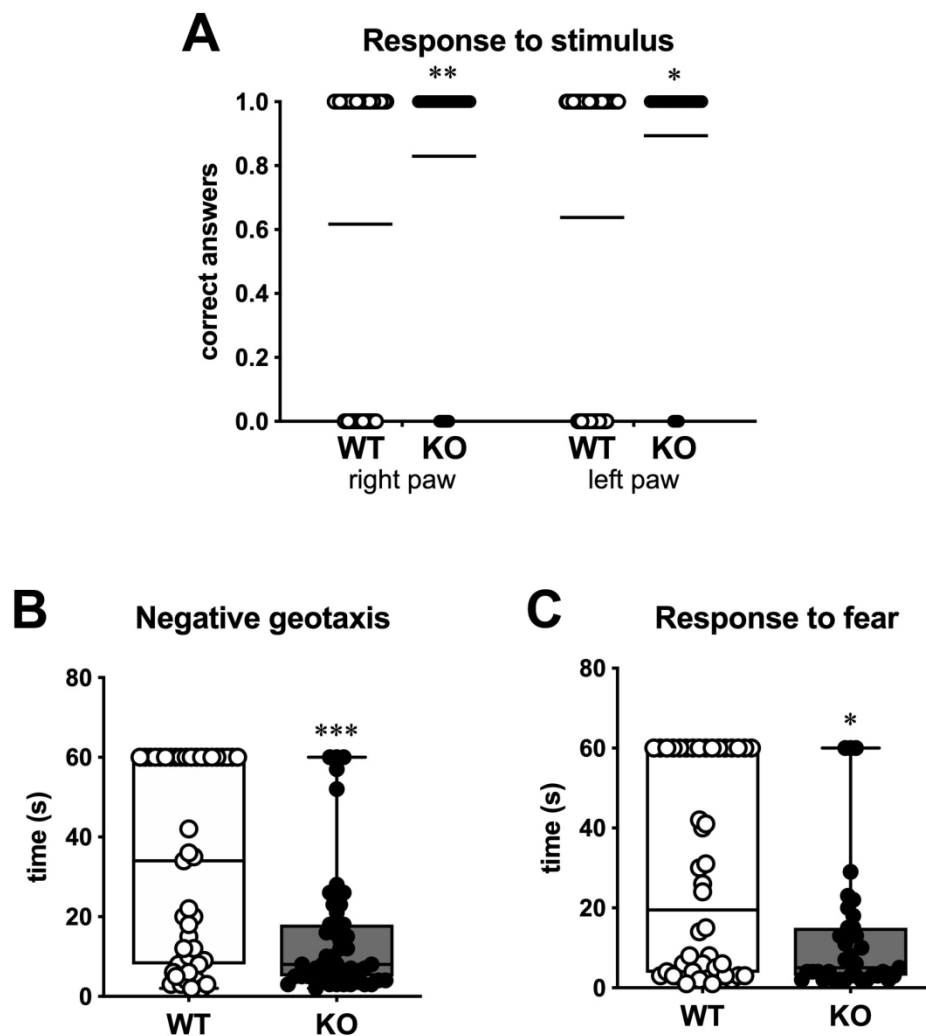


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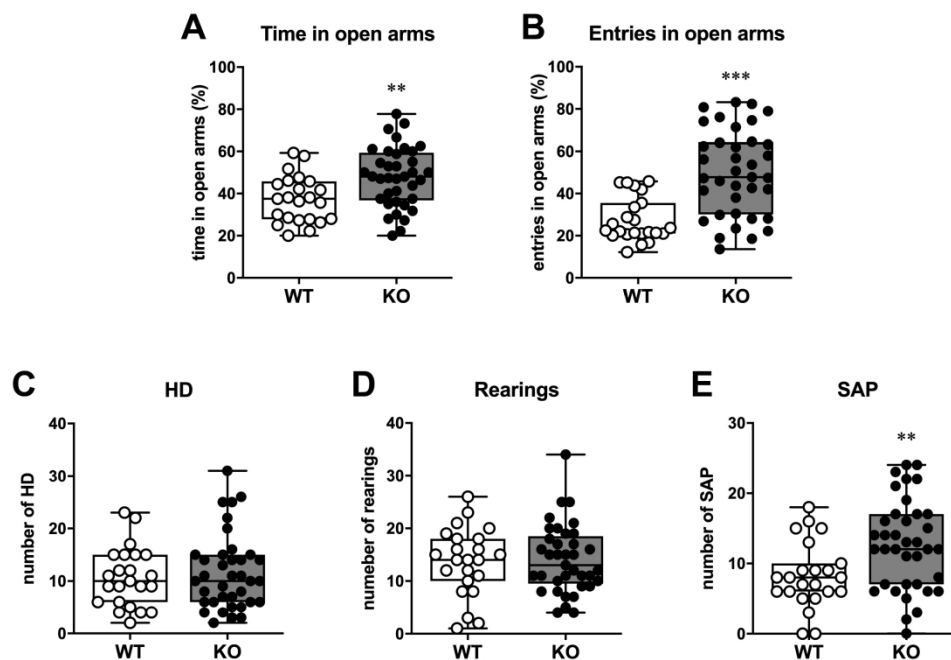


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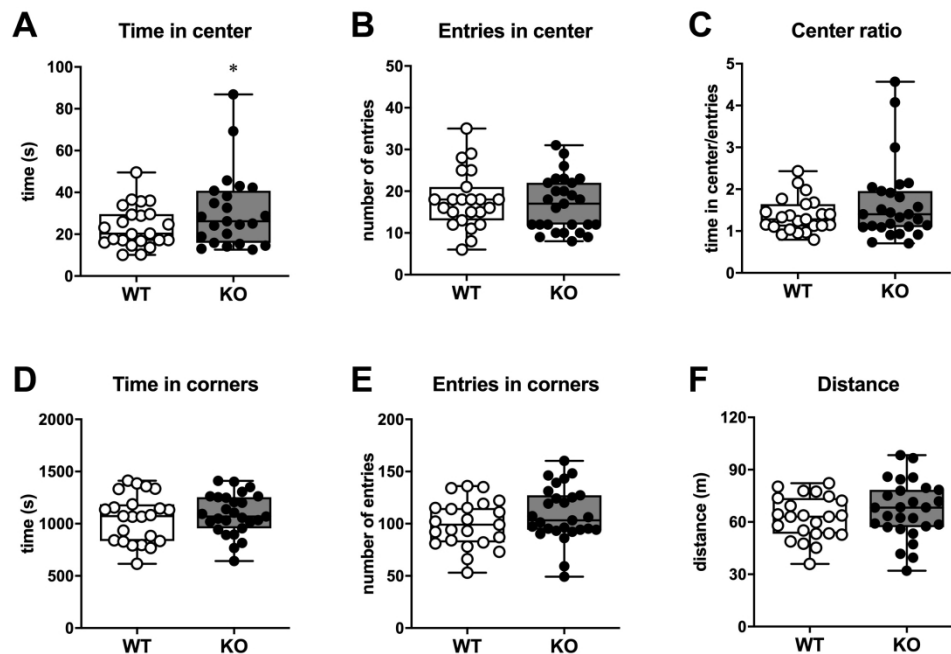


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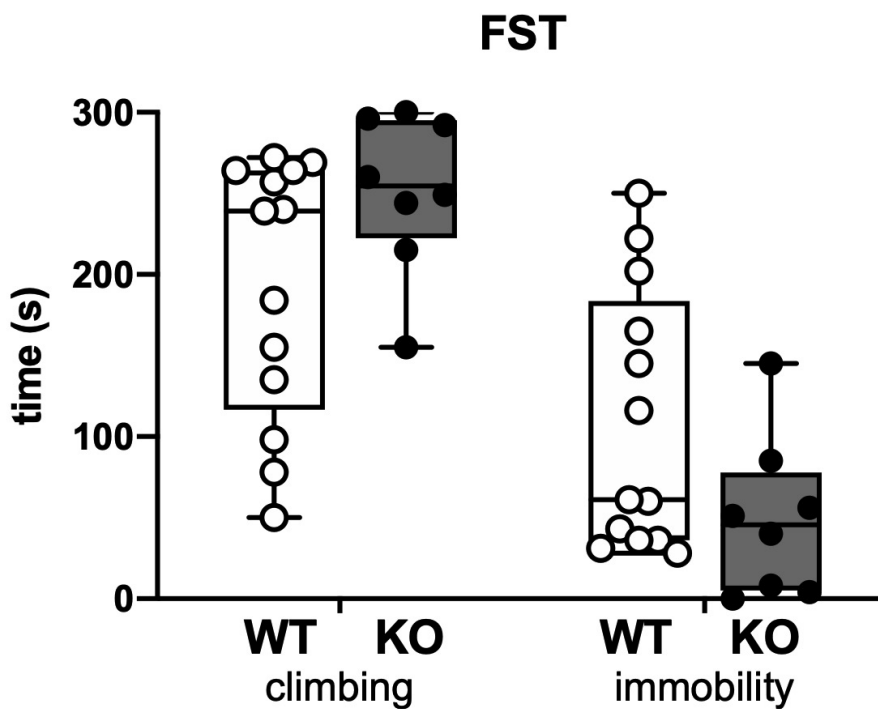


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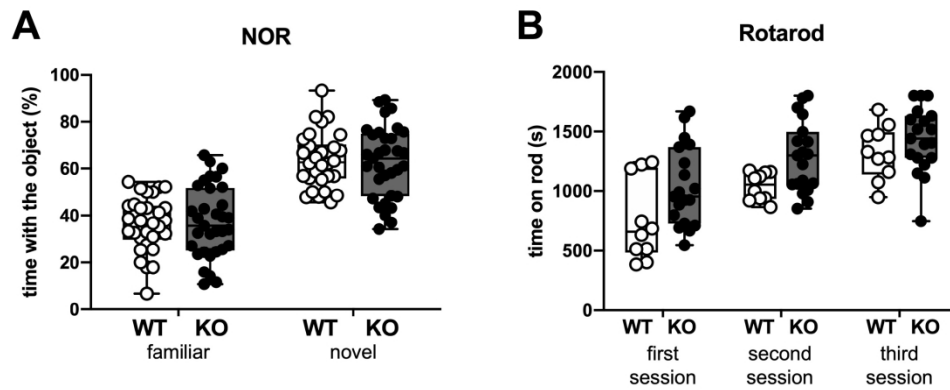


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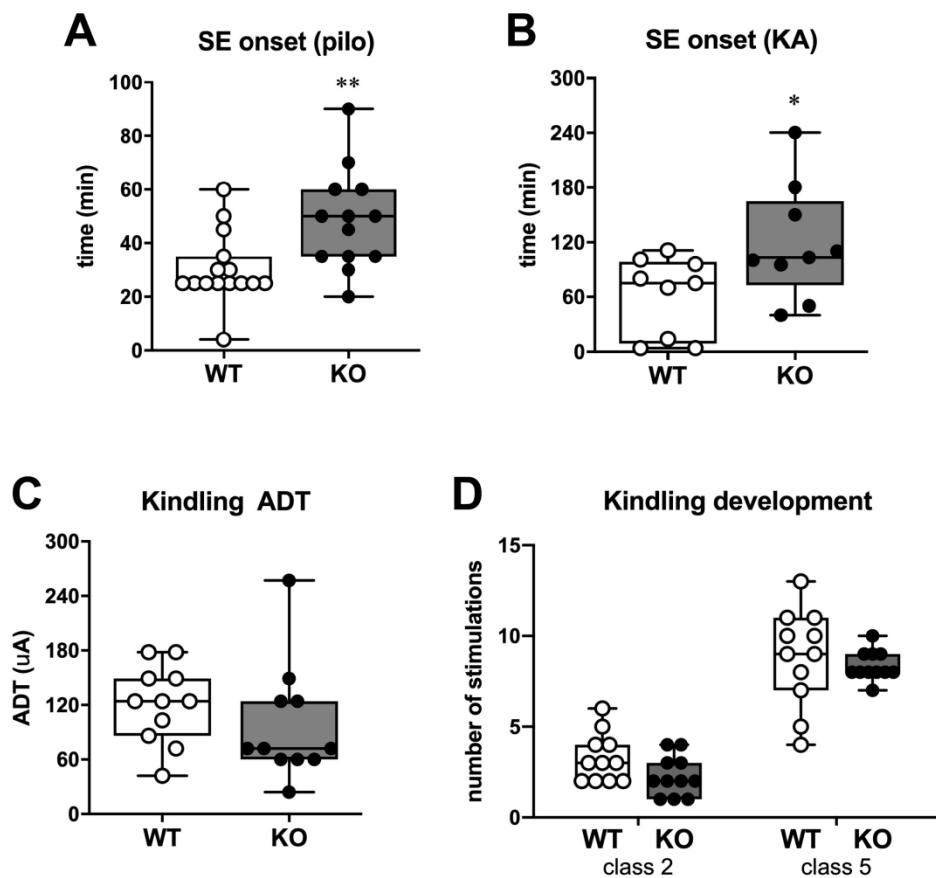


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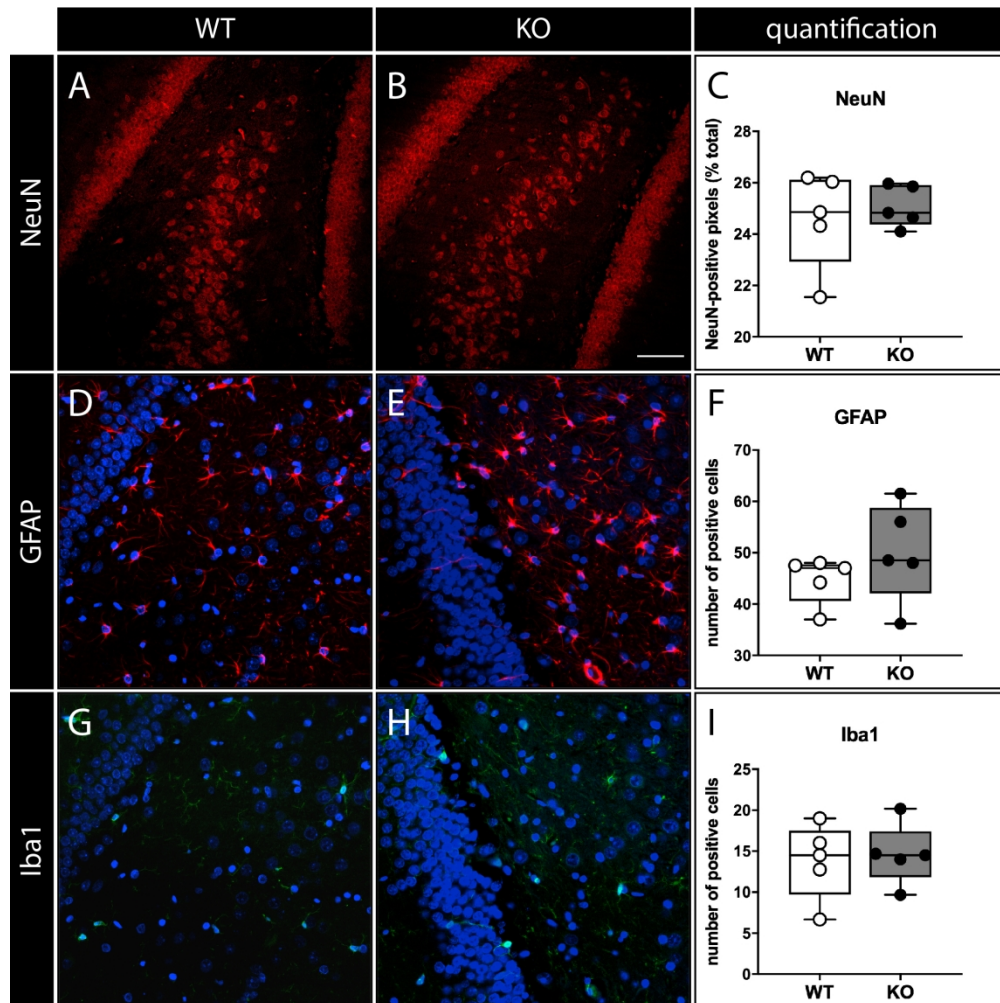
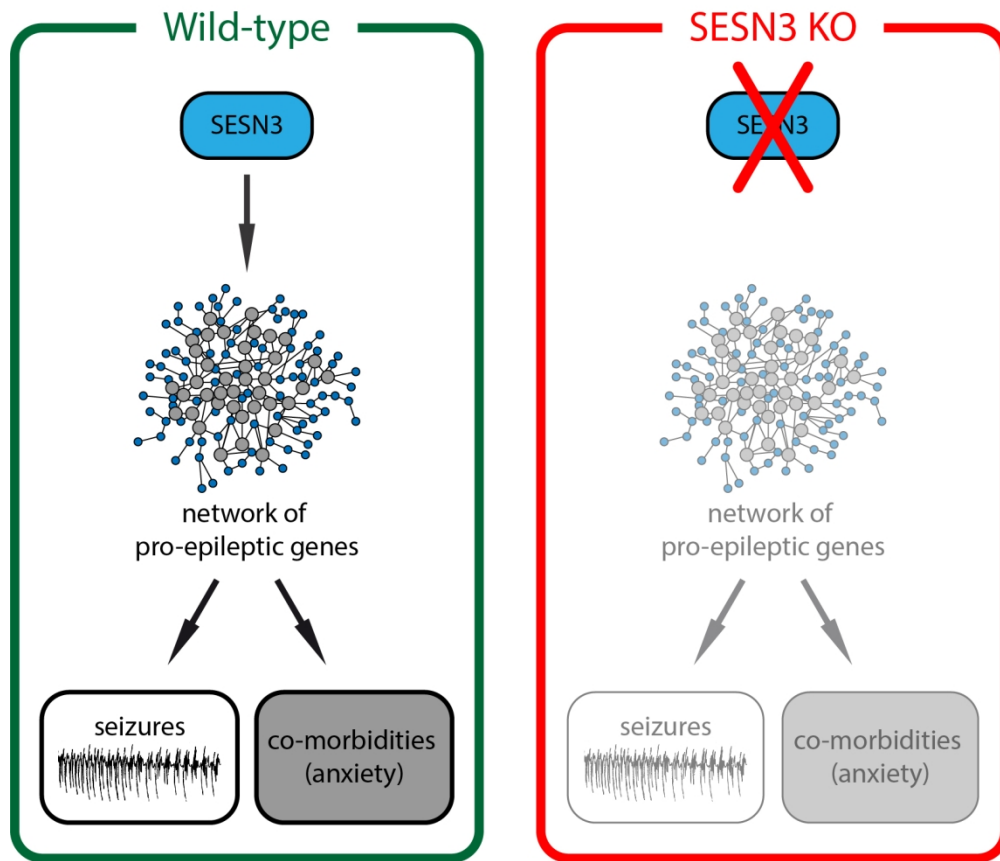


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Graphical abstract

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Abbreviated summary

We previously identified a pro-epileptic gene network positively regulated by sestrin 3 (SESN3). Here, we found that SESN3 knock out rats have decreased susceptibility to seizures and reduced propensity to anxiety, suggesting that SESN3 may regulate mechanisms causally involved in the pathogenesis of both epilepsy and its comorbidities.