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2 Protein kinase C-gamma knockout mice show impaired hippocampal short-term memory
3 while preserved long-term memory

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1 **Abstract**
2
3 The brain encodes, stores, and retrieves relevant information in the form of memories that are

4 classified as short-term (STM) and long-term memories (LTM) depending on the interval

5 between acquisition and retrieval. It is classically accepted that STM undergo a consolidation

6 process to form LTM, but the molecular determinants involved are not well understood. Among

7 the molecular components relevant for memory formation, we focused our attention on the

8 protein kinase C (PKC) family of enzymes since they control key aspects of the synaptic

9 plasticity and memory. Within the different PKC isoforms, PKC-gamma has been specifically

10 associated with learning and memory since mice lacking this isoform (PKC-gamma KO mice)

11 showed mild cognitive impairment and deficits in hippocampal synaptic plasticity. We now

12 reveal that PKC-gamma KO mice present a severe impairment in hippocampal-dependent STM

13 using different memory tests including the novel object-recognition and novel place-recognition,

14 context fear conditioning and trace fear conditioning. In contrast, no differences between

15 genotypes were observed in an amygdala-dependent test, the delay fear conditioning. Strikingly,

16 all LTM tasks that could be assessed 24 h after acquisition were not perturbed in the KO mice.

17 The analysis of c-Fos expression in several brain areas after trace fear conditioning acquisition

18 showed a blunted response in the dentate gyrus of PKC-gamma KO mice compared to WT mice,

19 but such differences between genotypes were absent when the amygdala or the prefrontal cortex

20 were examined. In the hippocampus, PKC-gamma was found to translocate to the membrane

21 after auditory trace, but not after delay fear conditioning. Together, these results indicate that

22 PKC-gamma dysfunction affects specifically hippocampal-dependent STM performance and

23 disclose PKC-gamma as a molecular player differentially involved in STM and LTM processes.

24

25 **Keywords:** short-term memory, long-term memory, PKC-gamma, memory acquisition, c-Fos

26 expression

1   **Introduction**

2   Memory is a brain function crucial for adaptive behavior characterized by the encoding, storage,

3   and retrieval of relevant information [1]. Memories can be classified according to their

4   permanence as short- and long-term memory (STM and LTM, respectively) [2]. STM encoding

5   occurs rapidly in the hippocampus, while the consolidation process to form LTM involves other

6   brain areas including the neocortex [3–5].

7   The cellular and molecular mechanisms underlying STM consolidation into LTM are largely

8   unknown, although specific players have been proposed [1, 6]. Among those, the protein kinase

9   C (PKC) family of enzymes, composed of serine/threonine protein kinases with a highly

10   conserved catalytic domain, plays a relevant role in learning and memory [7–9]. This family

11   includes at least 12 isoforms involved in multiple physiological functions beyond learning and

12   memory, such as cell growth and proliferation, differentiation, immune responses, apoptosis and

13   angiogenesis, among others [10, 11], but their isoform specialization, if any, has not been yet

14   clarified. PKC isoforms are classified in three groups based on their structure and activation

15   mechanism [8, 10]: i) the conventional/classical isoforms (alpha, beta-I, beta-II and gamma) are

16   activated by both calcium and diacylglycerol (DAG), ii) the novel isoforms (delta, epsilon, eta,

17   theta and mu) are activated by DAG in a calcium-independent manner, and iii) the atypical

18   isoforms (zeta and lambda) do not require neither calcium nor DAG to be activated, but they are

19   sensitive to phospholipids [11, 12].

20   The PKC-gamma isoform is highly expressed in the hippocampus, the cortex and the amygdala

21   [13, 14], primarily in the dendrites and neuronal cell bodies [15, 16]. At the cellular level, PKC-

22   gamma was described to translocate to the cell membrane after a single learning episode in the

23   context fear conditioning [17]. PKC-gamma phosphorylates, among other preferential substrates,

24   the N-methyl-D-aspartate receptor (NMDAR) subunit NR1, the functionally essential subunits

25   in NMDAR tetramers [18]. Phosphorylation of NR1 plays a key role in synaptic plasticity,

26   memory and learning [19, 20]. Interestingly, the genetic deletion of the *Prkcg* gene encoding

1   PKC-gamma in mice revealed a mild alteration in spatial memory using the Morris water maze

2   task, and in repetitive-pairing context-dependent fear conditioning when LTM was assessed [21],

3   while STM performance was not explored. In addition, hippocampal long-term potentiation

4   (LTP) was found affected in PKC-gamma knockout (KO) mice, while other forms of synaptic

5   plasticity, such as long-term depression and paired-pulse facilitation were similar to wild-type

6   (WT) controls [15], overall suggesting that PKC-gamma may be a regulatory component of LTP.

7   The objective of this work was to further investigate the relevance of PKC-gamma deletion in

8   cognitive performance and on the associated cellular responses during memory acquisition.

9

10

1 **Material and methods**

2 *Animals*

3 Male C57BL/6J wild-type mice (WT) and male constitutive PKC-gamma KO mice, B6;129P-

4 Pkcctm1St1 [15] backcrossed to C57BL/6J for at least 10 generations were used at 8-16 weeks

5 of age (approx. 25-30 g). Animals were housed at controlled environmental conditions (21 ± 1ºC

6 temperature and $55\pm10\%$ humidity). Food and water were available *ad libitum*. Lighting was

7 maintained at 12 h cycles (on at 8 am and off at 8 pm). All the experiments were performed

8 during the light phase of the dark/light cycle. Animals were habituated to the experimental room

9 and handled for one week before starting the experiments. All animal procedures were conducted

10 in accordance with the standard ethical guidelines (European Communities Directive

11 2010/63/EU) and approved by the local ethical committee (Comitè Ètic d'Experimentació

12 Animal, CEEA-PRBB). All behavioral analyses were performed blind to the experimental

13 conditions.

14

15 *Spontaneous alternation task*

16 <u>*T-maze spontaneous alternation:*</u> Spatial working memory was assayed in a T-shaped maze

17 composed by two opposed arms and a perpendicular arm (T-shaped; 6 cm width x 30 cm length

18 x 15 cm height). Animals were allowed to freely explore the maze for 9 min, starting from the

19 end of the same arm in the maze facing the wall. Entries into all arms of the maze were counted

20 (traversing the head and two front paws was considered a valid entry) and the percentage of

21 spontaneous alternation was calculated taking into account the sequential entries in all three arms

22 divided by the total number of possible alternations (total number of entries -2).

23 <u>*Y-maze spontaneous alternation:*</u> Additionally, spatial working memory was assayed in a second

24 cohort of PKC-gamma KO and WT mice using a Y-shaped maze. This maze is composed by

25 three identical arms that intersected at 120º (Y-shaped; 6.5 cm width x 30 cm length x 15 cm

26 height). Animal behavior was analyzed as described for the T-maze task.

1   *Barnes maze*

2   Spatial learning and reference memory were assessed using the Barnes maze, as previously

3   described [22]. The maze consists of a circular platform (90 cm in diameter) with 20 equally

4   spaced holes through which mice may escape from a bright light (300 lx). Only one hole allows

5   the escape to a dark/target box. Visual cues were placed surrounding the maze for navigational

6   reference (Supp. Fig. 1). Smart v3.0 software was used to control the video-tracking system.

7   Briefly, mice were first habituated to the maze. In this phase, animals were placed in the center

8   of the maze covered by an opaque cylinder for 10 sec. After removal of the opaque cylinder,

9   mice were gently guided to the target hole by surrounding them within a cylinder with transparent

10   walls so mice could see where the scape hole was located. Then, they were left inside the target

11   box for 2 min and then taken to the homecage. 1 h later, the first training phase was carried out.

12   During training, each mouse performed 2 trials per day on 4 consecutive days. Each training trial

13   started with the mouse placed in the center of the Barnes maze covered by an opaque cylinder

14   for 10 sec. Then, animals were allowed to explore the maze for 3 min. During this period, the

15   number of primary errors to find the target hole were measured. Each training trial ended when

16   the mouse entered the target box or after 3 min of exploration. The mouse was allowed to stay in

17   the target box for 1 min. When mice did not reach the target box within 3 min, the experimenter

18   guided the mouse gently to the escape box using a transparent cylinder. On day 5, the first test

19   trial was conducted 24 h after the last training day. During the test trial, the target hole was closed.

20   Animals were placed in the center of the maze covered by an opaque cylinder for 10 sec. Next,

21   exploration was analyzed during 90 sec to reveal the number of pokes in each hole. Using the

22   tracking system, the time spent in each quadrant (target, opposite, left and right quadrants) and

23   the distance travelled were measured. A second test trial was conducted to assess remote memory

24   on day 12, 7 days after the last training session.

25

26

1   *Novel object-recognition (NOR) test*

2   Object-recognition memory was assayed in a V-shaped maze (30 cm long x 4.5 cm wide x 15

3   cm height each corridor), as previously described [23, 24]. On day 1, mice were habituated to the

4   empty maze for 9 min. On day 2, mice were introduced in the maze for 9 min, where 2 identical

5   objects were presented, one at the end of each corridor. Memory was then tested at different times

6   after acquisition to assess STM (10 min and 3 h) or LTM (6 h, 24 h or 48 h) (see diagram in

7   Supp. Fig. 2a). For the memory test, mice were placed again in the V-maze for a period of 9 min,

8   where a novel object replaced one of the familiar objects. The total time spent exploring each of

9   the 2 objects (novel and familiar) was recorded, defining exploration as the orientation of the

10   nose towards the object. A discrimination index (DI) was calculated as the difference between

11   the time spent exploring either the novel (Tn) or familiar (Tf) object divided by the total time

12   exploring both objects (Tn+Tf) (DI=(Tn–Tf)/(Tn+Tf)). A positive discrimination index reflects

13   memory retention for the familiar object as mice explore the novel object for longer than the

14   familiar object. Mice exploring less than 10 sec both objects, or less than 3 sec one of the objects

15   on the memory test were excluded from the analysis.

16

17   *Novel place-recognition (NPR) test*

18   Mice were first habituated to an empty open field (25 cm long x 25 cm wide x 15 cm height) for

19   10 min during 2 consecutive days. On day 3, mice were trained for 10 min with 2 identical objects

20   (training phase) located at 2 opposite corners of the open field. The test phase was performed 3

21   h (STM assay) or 24 h (LTM assay) after the last habituation session, where one of the objects

22   was located in a different corner (novel location) (see diagram in Supp. Fig. 2b). The time spent

23   exploring both objects in the novel and familiar locations were computed to calculate the

24   discrimination index similarly to the NOR test. Total exploration time during the test was

25   considered as a measure of general activity. Mice that explored objects for less than 5 sec were

26   excluded from the analysis.

1   *Consecutive NOR/NPR and NPR/NOR*

2   In order to assess STM and LTM in the same mouse, we combined NOR and NPR tests at 3 h

3   and 24 h, both of them performed in an empty open field (25 cm long x 25 cm wide x 15 cm

4   height). First, we used a set of animals that performed 2 training sessions with the same objects

5   located at the same place. Then, mice were evaluated for STM in the NOR test (3 h after the last

6   training session) and for LTM in the NPR test (24 h after the last training session). In addition,

7   another set of mice were evaluated first for STM in the NPR test and for LTM in the NOR test

8   after the training sessions (see diagram in Supp. Fig. 3).

9

10 *Fear conditioning*

11 <u>*Context fear conditioning:*</u> Context recognition memory was assayed in a conditioning chamber

12 with an electrifiable floor, as previously described [23]. On day 1, mice were placed in the shuttle

13 box and after a period of free exploration, mice received a footshock (Unconditioned stimulus

14 (US): 2 sec, 0.6 mA intensity). Freezing behavior (lack of movement except for respiration) due

15 to context re-exposure was assessed in the same conditioning chamber 1 h, 3 h or 24 h after the

16 conditioning session. For testing, mice were placed again in the conditioning chamber for 5 min

17 in absence of the shock and the freezing behavior was recorded (see diagram in Supp. Fig. 2c).

18 <u>*Cued fear conditioning:*</u> On day 1, mice were placed in a conditioning chamber with an

19 electrifiable floor and after 2 min of free exploration, they were exposed to an auditory stimulus

20 (conditioned stimulus, CS) for 1 min. Just at the end of the auditory stimulus (delay fear

21 conditioning) or 15 sec after the end of the auditory stimulus (trace fear conditioning), the animal

22 received a footshock (US: 2 sec, 0.35 mA intensity). Different groups of mice were tested 3 h

23 (for STM) or 24 h (for LTM) later by placing them in a new environment (glass cylinder for

24 observation in a new experimental room) with the presence of the CS. Freezing behavior due to

25 CS re-exposure was recorded for 1 min (see diagram in Supp. Fig. 2d).

26 *Locomotor activity test*

1 Spontaneous locomotor responses of PKC-gamma KO and WT mice were evaluated in

2 individual locomotor activity boxes (10.8 cm width × 20.3 cm length × 18.6 cm high), (Imetronic,

3 Pessac, France) under dim light (20-25 lx). The total activity (number of beam breaks) and the

4 total number of rearings was detected during 10 min by infrared sensors.

5

6 *Plantar test*

7 Heat hyperalgesia was evaluated by measuring paw withdrawal latency in response to radiant

8 heat with a plantar test apparatus (Ugo Basile, Italy). Mice were placed in Plexiglas boxes (9 cm

9 diameter, 20 cm height) positioned on a glass surface and habituated to the environment for 30

10 min before testing. The mean paw withdrawal latencies for left and right hind paws were

11 determined from the average of 3 separate trials, taken at 5-10 min intervals to prevent thermal

12 sensitization. A cut-off time of 20 sec was used to prevent tissue damage.

13

14 *Brain samples for immunoblot analysis*

15 Brain samples were obtained from PKC-gamma KO and WT mice in basal conditions or 30 min

16 after being exposed to the trace or the delay fear conditioning protocol. Hippocampal tissues

17 were dissected from brains, frozen on dry ice and stored at −80 °C until used, as previously

18 reported [23]. Samples from all animal groups, in each experiment, were processed in parallel,

19 to minimize inter-assay variations.

20

21 *Sample preparation of total solubilized fraction*

22 From total solubilized fraction brain areas were processed as described previously [25]. Frozen

23 brain areas were dounce homogenized in 30 volumes of lysis buffer (50 mmol/L Tris HCl pH

24 7.4, 150 mmol/L NaCl, 10% glycerol, 1 mmol/L EDTA, 10 µg/mL aprotinin, 1 µg/mL leupeptine,

25 1 µg/mL pepstatin, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L sodium orthovanadate,

26 100 mmol/L sodium fluoride, 5 mmol/L sodium pyrophosphate, and 40 mmol/L beta-

1 glycerolphosphate) plus 1% Triton X-100. After 10 min incubation at 4°C, samples were

2 centrifuged at 16,000 g for 20 min to remove insoluble debris. Protein contents in the

3 supernatants were determined by DC-micro plate assay (Bio-Rad, Madrid, Spain), following

4 manufacturer's instructions.

5

6 *Preparation of membrane and cytosolic fractions*

7 Frozen brain areas were dounze-homogenized in 30 volumes of cytosol buffer (50 mmol/L Tris-

8 HCl pH 7.4, 0.32 mol/L saccharose, 1 mmol/L EDTA, 10 µg/mL aprotinin, 1 µg/mL leupeptine,

9 1 µg/mL pepstatin, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L sodium orthovanadate,

10 100 mmol/L sodium fluoride, 5 mmol/L sodium pyrophosphate, and 40 mmol/L beta-

11 glycerolphosphate) and immediately centrifuged at low speed (1,000 g) for 10 min at 4°C. The

12 supernatant (S1) was recovered. The pellet was resuspended with 600 ul of cytosol buffer and

13 immediately centrifuged at low speed (1,000 g) for 10 min at 4°C. The supernatant (S2) was

14 recovered. Both supernatant (S1 + S2) were centrifuged at 16,000 g for 30 min at 4°C. The

15 supernatant was recovered and used as cytosolic fraction. The pellet was resuspended in 5

16 volumes of membrane buffer (50 mmol/L Tris-HCl pH 7.4, 1 mmol/L EDTA, 150 mmol/L NaCl,

17 10% glycerol, 10 µg/mL aprotinin, 1 µg/mL leupeptine, 1 µg/mL pepstatin, 1 mmol/L

18 phenylmethylsulfonyl fluoride, 1 mmol/L sodium orthovanadate, 100 mmol/L sodium fluoride,

19 5 mmol/L sodium pyrophosphate, and 40 mmol/L beta-glycerolphosphate) and used as

20 membrane fraction. Protein content in both the cytosolic and the membrane fraction were

21 determined by DC-micro plate assay (Bio-Rad), following manufacturer's instructions.

22

23 *Immunoblot analysis*

24 Hippocampal protein samples were prepared as described previously [25]. The antibodies used

25 for immunoblot were: PKC-alpha (1:500), PKC-beta I (1:500), PKC-beta II (1:500), PKC-delta

26 (1:500), PKC-epsilon (1:500), PKC-eta (1:500) and PKC-theta (1:500) (Santa Cruz

1   Biotechnology, Santa Cruz, CA, USA), PKC-gamma (1:500) (Abcam, UK), pNR1 (Ser890)

2   (1:200) (Cell Signaling Technologies, The Netherlands) and NR1 (1:500) (Novus Biologicals,

3   Spain). Primary antibodies were detected with horseradish peroxidase-conjugated anti-rabbit

4   (1:15,000) or anti-mouse (1:15,000) antibodies (Cell Signaling Technologies) and visualized by

5   enhanced chemiluminiescence detection (Luminata Forte Western HRP substrate,

6   MerckMillipore). Digital images were acquired on ChemiDoc XRS System (Bio-Rad) and

7   quantified by The Quantity One software v4.6.3 (Bio-Rad). Optical density values for target

8   proteins were normalized to GAPDH (1:15,000) (Santa Cruz Biotechnology) or NR1 as loading

9   control in the same sample and expressed as a percentage of the control group (WT).

10

11   *Tissue preparation for immunofluorescence*

12   Mice were perfused 120 min after being exposed to a training session of the trace or the delay

13   fear conditioning. Mice from the control group were perfused 120 minutes after going through

14   the same protocol (handling, and exposure to the fear conditioning box and auditory stimulus)

15   but without receiving the footshock. Mice were deeply anesthetized by intraperitoneal injection

16   (0.2 ml/10 g of body weight) of a mixture of ketamine (100 mg/kg) and xylazine (20 mg/kg)

17   prior to intracardiac perfusion with 4% paraformaldehyde in 0.1 M Na_2HPO_4/0.1 M NaH_2PO_4

18   buffer (PB), pH 7.5, delivered with a peristaltic pump at 19 ml/min flow for 3 min. Subsequently,

19   brains were extracted and post-fixed in the same fixative solution for 24 h and transferred to a

20   solution of 30 % sucrose in PB overnight at 4 ºC. Coronal frozen sections (30 μm) of the dorsal

21   hippocampus (coordinates relative to Bregma: −1.22 mm to −1.82 mm), basolateral amygdala

22   (from Bregma: −1.22 mm to −1.82 mm) and the prelimbic prefrontal cortex (from Bregma: 1.98

23   mm to 1.54 mm) were obtained on a freezing microtome and stored in a solution of 5% sucrose

24   at 4 ºC until used.

25

26   *Immunofluorescence*

1 Free-floating brain slices were rinsed in PB, blocked in a solution containing 3% donkey serum

2 (DS) (Sigma-Aldrich, Madrid, Spain) and 0.3 % Triton X-100 (T) in PB (DS-T-PB) at room

3 temperature for 2 h, and incubated overnight in the same solution with the primary antibody to

4 c-Fos (sc-7202, 1:1000, rabbit, Santa Cruz Biotechnology), PKC-gamma (1:1000, rabbit,

5 Abcam, UK) and neuronal nuclei (NeuN) (1:1000, mouse, MerckMillipore), at 4 ºC. The next

6 day, after 3 rinses in PB, sections were incubated at room temperature with the secondary

7 antibody AlexaFluor-555 donkey anti-rabbit (c-Fos and PKC-gamma) (1:500, Life

8 Technologies, Thermo Fisher Scientific, MA, USA) and secondary antibody AlexaFluor-488

9 goat anti-mouse (NeuN) (1:1000, Jackson ImmunoResearch, UK) in DS-T-PB for 2 h. After

10 incubation, sections were rinsed and mounted immediately after onto glass slides coated with

11 gelatin in Fluoromont-G with 4′,6-diamidino-2-phenylindole (DAPI) (Invitrogen, Thermo Fisher

12 Scientific, MA, USA) as counterstaining.

13

14 *Image analysis.*

15 Immunostained brain sections were analyzed with a 10× objective using a Leica DMR

16 microscope (Leica Microsystems, Wetzlar, Germany) equipped with a digital camera Leica DFC

17 300FX (Leica Microsystems). For prelimbic cortex analysis, a 430 μm sided square region of

18 interest (ROI) was delimited for quantification. For basolateral amygdala and dentate gyrus

19 analysis the DAPI signal was used for the delimitation of this area in each image for

20 quantification. The images were processed using the ImageJ analysis software. c-Fos positive

21 neurons in each brain area were quantified using the automatic 'particle counting' option with a

22 fixed configuration that solely detected c-Fos positive cell bodies matching common criteria of

23 size and circularity. A fixed threshold interval was set to distinguish the c-Fos positive neurons

24 from the background. In addition, all quantifications were individually checked by an expert

25 observer blind to the experimental conditions. Six representative brain sections of each mouse

26 were quantified, and the average number of c-Fos-positive neurons was calculated for each

1 mouse. The data are expressed as the mean number of c-Fos-positive cells per squared mm (n =

2 4–6 mice per experimental group). For the c-Fos data the displayed images were flipped for

3 orientation consistency and transformed to grey scale for display. Images for PKC-gamma

4 detection were adjusted for brightness and contrast for display.

5

6 *Statistical analysis*

7 Comparisons between groups were performed by one-way or two-way analysis of variance

8 (ANOVA) for multiple-group comparisons, depending on the factors involved. *Post hoc*

9 comparisons were performed by Bonferroni test only when significant main effect of one-way

10 ANOVA or significant effect of factors or interaction between factors of two-way ANOVA were

11 revealed. The statistical analysis was performed using STATISTICA (StatSoft) software. The

12 artwork was designed using GraphPad Prism 7. All results were expressed as mean ± s.e.m.

13 Differences were considered statistically significant when $p < 0.05$.

14

15

16

1   **Results**

2   *Lack of PKC isoform compensation in hippocampal PKC-gamma KO samples*

3   In order to discard any compensatory effect in the PKC-gamma KO mice, the expression of all

4   the PKC isoforms bearing a Ser residue at the hydrophobic domain, similar to PKC-gamma, was

5   studied in hippocampal homogenates. As expected, the immunodetection of PKC-gamma

6   confirmed the absence of this isoform in the hippocampus of PKC-gamma KO mice compared

7   to WT controls (Fig. 1a). Then, immunodetection of PKC-alpha, -beta I, -beta II, -epsilon, -eta,

8   -delta and -theta isoforms (Fig. 1b-h) revealed no significant differences between WT and PKC-

9   gamma KO mice. The immunofluorescence detection of PKC-gamma protein in WT mice was

10   clearly specific in all subregions of the hippocampus, including CA1 and dentate gyrus,

11   compared to the background levels detected in the PKC-gamma KO mice (Fig. 1i)

12

13   *PKC-gamma KO mice show specific spatial memory deficits*

14   PKC-gamma KO and WT mice were analyzed in the spontaneous alternation task to assess

15   working memory. The T-maze task (Fig. 2a) revealed a significant decrease in the number of

16   spontaneous alternations performed by the PKC-gamma KO mice compared to the WT control

17   mice, pointing to a significant deficit in spatial working memory. This deficit was also observed

18   with the Y-maze task (Fig. 2b). No differences in any of the tasks were observed between

19   genotypes in the total number of corridor entries, discarding an effect on general exploration or

20   locomotion as a confounding factor (Supp. Fig. 4a, b).

21   Spatial learning and long-term memory (LTM) were also studied using the Barnes maze (Fig. 2c,

22   d). Both genotypes showed similar spatial learning during the spatial training sessions (Supp.

23   Fig. 5a). Then, recent (24 h after the last training) (Fig. 2c) and remote (7 d after the last training)

24   (Fig. 2d) spatial LTM assessment showed a significant recall of the target quadrant in both

25   genotypes, although PKC-gamma KO mice showed decreased accuracy compared to WT mice

1   (Supp. Fig. 5b-e). No differences between genotypes were observed in the locomotor activity

2   during the Barnes test performance (Supp. Fig. 5f, g) discarding a motility bias in this test.

3

4   *Short-term non-emotional memory performance is impaired in mice lacking PKC-gamma*

5   Non-emotional memory performance was evaluated in PKC-gamma KO and WT mice using 2

6   memory tasks: the novel object-recognition (NOR) test (Fig. 3a) and the novel place-recognition

7   (NPR) test (Fig. 3b). STM performance in these tasks (10 min and 3 h after training) was

8   significantly impaired in PKC-gamma KO mice. However, no differences between genotypes

9   were observed in the same tasks when memory was assessed beyond 6 h of the training session

10 (Fig. 3a, b). Such specific differences were not related to exploratory behavior or motility since

11 total exploration times were similar between genotypes in the memory tests (Supp. Fig. 6a-g),

12 and locomotor activity assessment did not reveal genotype divergences in overall activity (Fig.

13 3c). To further discard differences between animal batches, both STM and LTM were tested in

14 the same animal combining the two non-emotional memory tasks previously described. One set

15 of animals performed consecutively the NOR test to study STM, and the NPR test to study LTM.

16 Another set of animals performed the NPR test to study STM followed by the NOR test to study

17 LTM. Such approach further demonstrated that PKC-gamma KO mice present significant non-

18 emotional STM, but not LTM deficits, independent of the memory task performed in the first

19 place (Supp. Fig. 7 a,b).

20

21 *PKC-gamma KO mice present deficits in hippocampal-dependent short-term fear memory.*

22 Fear memory was assessed using the context recognition test (Fig. 4a), the trace fear conditioning

23 (Fig. 4b) and the delay fear conditioning (Fig. 4c) paradigms in PKC-gamma KO and WT mice.

24 Memory performance was similar between both genotypes when LTM was studied in all three

25 paradigms (Fig. 4a-c). In contrast, significant differences were observed in STM performance

1 depending on the memory task assessed. While PKC-gamma KO mice displayed a significant

2 STM impairment in hippocampal-dependent tasks, such as the context recognition at 1h and 3h

3 (Fig. 4a) and the trace fear conditioning (Fig. 4b), no deficits were observed in the delay fear

4 conditioning (Fig. 4c), an hippocampal-independent task [26]. A potential bias between

5 genotypes in STM performance due to footshock sensitivity was disregarded based on the similar

6 performance of both genotypes observed when LTM was assessed, and on the similar nociceptive

7 sensitivity displayed by both genotypes in response to plantar stimulation (Fig. 4d).

8

9 *PKC-gamma KO mice differentially express c-Fos in the dentate gyrus following the trace, but*

10 *not the delay fear conditioning schedule*

11 Cued fear conditioned memory encoding involves the expression of the immediate-early gene c-

12 Fos in a subpopulation of hippocampal cells [27]. Thus, we hypothesized that PKC-gamma KO

13 and WT mice may differently encode hippocampal-dependent fear memories. For this reason,

14 independent cohorts of mice were exposed to trace or delay fear conditioning paradigms, using

15 mice handled similarly but not exposed to the footshock as control group. Then, we analyzed the

16 expression of c-Fos in memory relevant brain areas. Quantification of c-Fos positive cells in the

17 dentate gyrus of the hippocampus, a sub-region where memory-associated cells have been

18 allocated [28], revealed a sharp increase in c-Fos positive cells after trace fear conditioning

19 training in WT mice, but not in PKC-gamma KO mice (Fig. 5a). Interestingly, c-Fos expression

20 was comparable between genotypes after delay fear conditioning training (Fig. 5a), a non-

21 hippocampal-dependent task that had not shown differences between genotypes at STM (Fig.

22 4c). No differences between genotypes were found when other brain regions were studied for c-

23 Fos expression including the basolateral amygdala (Fig. 5b) and the prelimbic cortex (Fig. 5c),

24 pointing to a region-specific impairment in c-Fos expression in PKC-gamma KO mice. Reference

1   mouse brain atlas pictures are presented to show the exact region where the cellular analysis was

2   performed (Supp. Fig. 8).

3

4   *PKC-gamma translocates to the membrane preferentially after trace but not delay fear*

5   *conditioning*

6   Hippocampal PKC-gamma has been described to translocate from the cytosol to the membrane

7   after spatial learning [29] and context fear conditioning training [17]. We focused in the detection

8   of hippocampal PKC-gamma translocation after trace and delay fear conditioning, following the

9   same training paradigms as in Figure 5. For this purpose, membrane-enriched and cytosolic

10   hippocampal fractions were prepared from samples obtained 30 min after training. Membrane-

11   enriched fraction analysis revealed a significant amount of PKC-gamma only in mice that went

12   through the trace fear conditioning, but not after delay fear conditioning training or in control

13   mice, exposed to the same environment (conditioning chamber) and auditory stimulus, but

14   without unconditioned stimulus (footshock) (Fig. 6a). No significant differences were observed

15   in the detection of PKC-gamma between all three experimental conditions in the cytosolic

16   fraction (Fig. 6b), indicating that only a small fraction of hippocampal PKC-gamma pool is

17   recruited to the membrane after the trace fear conditioning training. This membrane targeting of

18   PKC-gamma could be related to alterations in NR1 signaling since we found that in hippocampal

19   whole solubilized samples there is a significant difference in the phosphorylation level of the

20   NR1 subunit at Ser890 (Supp. Fig. 9), a residue preferentially phosphorylated by PKC activity

21   [19] when we compared PKC-gamma KO mice to WT controls. This result indicates that PKC-

22   gamma removal partially jeopardizes the overall phosphorylation of this residue and may

23   compromise NR1-dependent plasticity.

24

1 **Discussion**

2 Our study reveals that the PKC-gamma KO mice show significant deficits in short-term forms of

3 memory, for which the long-term forms are completely preserved pointing to an independent

4 mechanistic regulation of both memory ranges derived from the same training episode. The

5 hippocampal STM disturbances in PKC-gamma KO mice were concomitant with alterations in

6 c-Fos expression in the hippocampus induced by the encoding of a trace, but not by a delay, fear

7 conditioned memory.

8 The PKC family of kinases is a relevant group of enzymes important for learning and memory

9 [8]. There is much interest in the identification of specific functions associated to otherwise

10 apparently redundant PKC isoforms, which in many occasions have overlapping expression

11 patterns [14, 30]. Isoform specification has been previously studied through the use of genetic

12 deletion of a single isoform gene. Indeed, mice lacking the gene encoding PKC-beta showed

13 significant deficits in fear conditioning paradigms, which were not accompanied by detectable

14 alterations in synaptic plasticity [31]. The genetic deletion of PKC-gamma resulted in mild

15 alterations in spatial learning and fear conditioning [21] with significant reductions in

16 hippocampal LTP, although other forms of hippocampal synaptic plasticity (including

17 hippocampal long-term depression and paired-pulse facilitation) remained comparable to those

18 in WT mice [15]. Notably, our study shows that PKC-gamma deletion did not lead to

19 compensation through the expression of other functionally homologous isoforms in the

20 hippocampus including alpha, beta I, beta II, epsilon, eta, delta and theta.

21 Our behavioral data clearly reveals that PKC-gamma KO mice present specific STM deficits in

22 tasks where there is a significant contribution of the hippocampal activity. A significant deficit

23 in spatial working memory compared to WT mice was detected in two conformations, the T-

24 maze and the Y-maze. Performance of correct alternations in this task is associated to specific

25 temporal patterns of theta oscillations between the prefrontal cortex and the hippocampus [32],

26 pointing to potential deficits in such interaction between brain areas in PKC-gamma KO mice,

1 since they showed close to chance alternation values. In addition, our evaluation of long-term

2 spatial learning in the Barnes maze only revealed mild differences between genotypes, much

3 similar to the findings previously obtained by others with the same mouse model using the Morris

4 water maze [21, 33]. When fear memory was assessed in one of these previous studies, they

5 found a mild deficit in context-dependent fear conditioning LTM using a paradigm with several

6 footshock pairings [21]. This result is in contrast with the proper LTM performance we found

7 after a single pairing in our context recognition paradigm. Indeed, we found that our training

8 paradigm using one single pairing was clearly impaired in PKC-gamma KO mice when memory

9 was assessed 1 h and 3 h after conditioning. To further characterize the relevance of PKC-gamma

10 deletion in fear memory performance, we took advantage of the different neurobiological

11 substrates underlying the trace and delay fear conditioning paradigms [26]. These are similar

12 tasks, just differing in the trace period between the conditioned (auditory stimulus) and

13 unconditioned stimuli (single footshock). Previous studies have described a particular role of the

14 amygdala and the hippocampus in these fear conditioning paradigms. Indeed, the inactivation of

15 the amygdala produced deficits in delay, but not in trace fear conditioning, while the hippocampal

16 inactivation produced the opposite effect [26, 34, 35]. Moreover, other brain regions would also

17 be involved since the inactivation of the prelimbic cortex abolished fear expression [36]. Given

18 that PKC-gamma KO mice had a significant deficit in short-term trace fear conditioning, but no

19 alteration in short-term delay fear conditioning, the differences in the cellular outcome of both

20 training protocols were explored in specific brain areas relevant for STM performance

21 specifically during the acquisition phase. We studied c-Fos expression, a marker of neuronal

22 activity [37] that predicts memory acquisition [38, 39] and is a commonly used approach for the

23 study of memory engram cells [40] since its promoter can be used to tag neurons active during a

24 learning process [41]. These engrams or neuronal ensembles for fear memory have been

25 identified in the dentate gyrus of the hippocampus [28], the basolateral amygdala [41] and the

26 prefrontal cortex [42], where the modulation of the activity of these cells expressing c-Fos may

1   alter memory-associated behaviors [43, 44]. Our results showed that PKC-gamma KO mice

2   exposed to the hippocampal-dependent trace fear conditioning protocol presented a lower

3   number of c-Fos-positive cells than WT mice in the dentate gyrus, but not in the basolateral

4   amygdala or the prelimbic cortex. Although in another timeframe, and limited to the behavioral

5   paradigm we used, these data are parsimonious with a model previously proposed suggesting that

6   memory engram cells are rapidly formed in the hippocampus, the basolateral amygdala and

7   prefrontal cortex during the acquisition of a fear conditioning memory [45]. Then, in recent

8   recall, engrams in the basolateral amygdala and hippocampus drive the behavior, while those of

9   the prefrontal cortex remain less involved. However, in remote recall, engrams in the

10   hippocampus become less relevant while prefrontal cortex engrams gain relevance, driving the

11   behavior together with those of the basolateral amygdala. Therefore, although our cellular study

12   of c-Fos is limited to the acquisition phase of the cued fear conditioning paradigms, our results

13   on the dentate gyrus of the hippocampus suggest that PKC-gamma signaling would be key for

14   proper hippocampal encoding of memory. In this regard, the study of c-Fos expression after

15   context fear conditioning or after memory retrieval could be relevant next steps to further assess

16   the role of PKC-gamma signaling for recent or remote recall on hippocampal-dependent

17   memories.

18   We cannot totally discard the possibility that the behavioral responses in PKC-gamma KO mice

19   are related to more broad alterations in brain circuits due to the absence of PKC-gamma during

20   neurodevelopment. Nevertheless, the fact that the PKC-gamma KO phenotype was restricted to

21   alterations in specific STM tasks, indicates that the differences in STM performance were

22   probably related to specific roles of PKC-gamma signaling than caused by general

23   neurodevelopmental variations. In addition, the PKC-gamma KO phenotype was somewhat

24   reminiscent of the differential effect of acute low doses of the non-specific PKC inhibitor

25   chelerythrine on novel object-recognition memory described elsewhere [46]. When chelerythrine

26   effect was assessed shortly after familiarization (3 h), there was a significant deficit in memory

1   performance that was not observed when the effect was assessed 24 h later. Indeed, other

2   signaling pathways have been specifically associated to LTM, such as the

3   mammalian/mechanistic target of rapamycin (mTOR) pathway, or the translation machinery, as

4   novel object-recognition LTM, but not STM, is sensitive to the inhibition of both mechanisms

5   with temsirolimus or anisomycin, respectively [46].

6   We also observed that PKC-gamma translocates to the membrane preferentially after trace fear

7   conditioning training. This result reinforces previous data observed after context fear

8   conditioning [17], and further strengthens the role PKC-gamma activity may play in hippocampal

9   dependent tasks. Notably, we also found that context fear conditioning performance was also

10 affected in PKC-gamma KO mice when STM was assessed. The lack of PKC-gamma activity in

11 the membrane compartment in memory tasks with a strong hippocampal engagement could affect

12 the phosphorylation of key elements for synaptic plasticity such as NMDA receptors [47]. While

13 other phosphorylation-sensitive residues on NR1, such as Ser896 and Ser897, were similarly

14 phosphorylated in WT and PKC-gamma KO samples (data not shown), we found that the NR1

15 Ser890 phosphorylation was diminished, in basal conditions, in the PKC-gamma KO mice. The

16 phosphorylation of Ser890 has been previously related to PKC-gamma [19, 48]. This residue was

17 previously found to affect NR1 intracellular and membrane localization, as exemplified in a

18 heterologous expression system [49]. Notably, NMDA receptors have been consistently involved

19 in memory formation, including fear learning [19, 50, 51], and the diminished phosphorylation

20 of Ser890 in NR1 subunits in the hippocampus of PKC-gamma KO mice could be related to the

21 impaired hippocampal encoding observed in different memory tasks in PKC-gamma KO

22 animals.

23 Together, our data reveal the differential effect of PKC-gamma genetic deletion in memory

24 performance related to the time of recall in hippocampal-dependent memory tests. Notably, we

25 found a significant dysfunction of PKC-gamma KO mice in hippocampal-dependent memory

26 tests when recall was performed at short times (before 3 h of training) but not when recall was

1   assessed at later times. This result strongly suggests parallel encoding of memory for short-term

2   and long-term usage, where PKC-gamma activity would be specifically involved in the

3   mechanisms for hippocampus-engaging short-lived memory forms.

4

5   **Ethical approval:**

6   "All applicable international, national, and/or institutional guidelines for the care and use of

7   animals were followed."

20

21   **Conflict of interests:**

22   All authors report no biomedical financial interests or potential conflicts of interest.

23

24

22

1   **Figure legends**

2   Figure 1. **PKC isoform expression in the hippocampus of PKC-gamma KO mice.** Replicates

3   of hippocampal samples (20 μg protein/well) from WT and PKC-gamma KO animals were run

4   in parallel and membrane replicates were incubated with PKC isoform-specific antibodies to

5   independently detect the conventional isoforms The detection of PKC-gamma was performed as

6   a control. No differences were found between WT and KO mice in the other PKC isoforms. (**a**)

7   PKC gamma (n = 6-7) $[F_{(1,11)} = 49.493, p < 0.001]$, (**b**) alpha (n = 6-7) $[F_{(1,11)} = 2.409, p$

8     $= 0.149]$, (**c**) beta I (n = 6-7) $[F_{(1,11)} = 3.096, p = 0.106]$, (**d**) beta II (n = 6-7) $[F_{(1,11)} =$

9     $0.748, p = 0.405]$ and the novel isoforms (**e**) PKC epsilon (n = 6-7) $[F_{(1,11)} = 0.242, p =$

10    $0.632]$, (**f**) eta (n = 6-7) $[F_{(1,11)} = 0.033, p = 0.859]$, (**g**) delta (n = 6-7) $[F_{(1,11)} = 0.240, p$

11    $= 0.634]$ and (**h**) theta (n = 6-7) $[F_{(1,11)} = 1.036, p = 0.331]$. (**i**) Immunofluorescence

12   detection of PKC-gamma protein in CA1 (*stratum radiatum* and *stratum oriens*) and dentate

13   gyrus (especially hilus and inner molecular layer) of the hippocampus was notably higher in WT

14   mice compared to PKC-gamma KO mice. Scale bar 150 μm. Data are expressed as mean ± s.e.m.

15   *** p < 0.001 (PKC-gamma KO compared to WT).

16

17   Figure 2. **Spatial memory performance was evaluated in the PKC-gamma KO mice**. Spatial

18   STM was studied in the PKC-gamma KO mice (**a-b**) compared to WT controls in two different

19   shaped mazes, (**a**) the T-maze (n = 11-16) $[F_{(1,25)} = 6.144, p < 0.05]$ and (**b**) the Y-maze (n =

20   7-11) $[F_{(1,16)} = 22.933, p < 0.001]$. (**c-d**) Spatial memory was studied using the Barnes Maze

21   task (n = 7-11). The percentage of time spent in the target zone was analyzed, demonstrating

22   differences between genotypes [(**c**) 24h: $F_{(1,16)} = 6.882, p < 0.05$; (**d**) 7d: $F_{(1,16)} = 5.022$,

23    $p < 0.05]$. (**c**) At spatial LTM test (24 h), the % of time in the target quadrant in WT (Target vs

24   -1 $[F_{(1,12)} = 31.03, p < 0.001]$, Target vs 1 $[F_{(1,12)} = 34.953, p < 0.001]$, Target vs Opposite

25    $[F_{(1,12)} = 34.331, p < 0.001]$) and PKC-gamma KO (Target vs -1 $[F_{(1,20)} = 27.248, p <$

26    $0.001]$, Target vs 1 $[F_{(1,20)} = 32.081, p < 0.001]$, Target vs Opposite $[F_{(1,20)} = 22.603, p <$

1   0.001]) was higher than in the other quadrants. (**d**) In remote test (7 d), the % of time was also

2   higher in the target quadrat for WT (Target vs -1 [$F(1,12) = 14.613$, $p < 0.01$], Target vs 1

3   [$F(1,12) = 17.825$, $p < 0.01$], Target vs Opposite [$F(1,12) = 13.932$, $p < 0.01$]) and PKC-

4   gamma mice (Target vs -1 [$F(1,20) = 4.48$, $p < 0.05$], Target vs 1 [$F(1,20) = 27.872$, $p <$

5   0.001], Target vs Opposite [$F(1,20) = 31.604$, $p < 0.001$]). Data are expressed as mean $\pm$ s.e.m.

6   * $p < 0.05$, *** $p < 0.001$ (PKC-gamma KO compared to WT). && $p < 0.01$, &&& $p < 0.001$

7   (in WT compared with target). # $p < 0.05$, ### $p < 0.001$ (in PKC-gamma KO compared with

8   target).

9   Figure 3. **Short-term, but not long-term non-emotional memories are affected in PKC-**

10 **gamma KO mice.** (**a**) Short-term (10 min (n = 9-10) [$F(1,17) = 24.115$, $p < 0.001$] and/or 3 h

11 (n = 7-8) [$F(1,13) = 6.787$, $p < 0.05$]) and long-term memory (6 h (n = 7-8) [$F(1,13) =$

12 2.539, $p = 0.135$], 24 h (n = 7-8) [$F(1,20) = 0.0001$, $p = 0.992$] and 48 h (n = 7-11) [$F(1,16) =$

13 0.115, $p = 0.739$]) were studied in the novel object-recognition test. (**b**) Short-term (3 h (n = 9-

14 10) [$F(1,16) = 23.749$, $p < 0.001$]) and long-term (24 h (n = 9-10) [$F(1,15) = 1.759$, $p =$

15 0.205]) memory were also studied in the novel place-recognition test comparing the PKC-gamma

16 KO mice with WT mice. (**c**) No differences were observed between the PKC-gamma KO and

17 wild-type mice when locomotion was studied (n = 7-11) [Number of beam breaks: $F(1,16) =$

18 0.063, p = 0.805; Number of rearings $F(1,16) = 1.135$, p = 0.305]. Data are expressed as mean

19 $\pm$ s.e.m. * $p < 0.05$, *** $p < 0.001$ (PKC-gamma KO compared to WT).

20 Figure 4. **Hippocampal-dependent short-term emotional memories are impaired in the**

21 **PKC-gamma KO mice.** Short-term and long-term memories were studied in the (**a**) context

22 recognition test [1 h (n = 7-8): $F(1,13) = 5.236$, $p < 0.05$; 3 h (n = 14): $F(1,26) = 5.314$, $p <$

23 0.05; 24 h (n = 12-14): $F(1,24) = 0.372$, $p = 0.548$], the (**b**) trace fear conditioning test [3 h (n

24 = 8-10): $F(1,16) = 13.385$, $p < 0.01$; 24 h (n = 8): $F(1,14) = 0.892$, $p = 0.361$] and the (**c**)

25 delay fear conditioning test [3 h (n = 6): $F(1,10) = 0.013$, $p = 0.910$; 24 h (n = 7-13): $F(1,18)$

26 $= 0.203$, $p = 0.657$] comparing the PKC-gamma KO mice with the WT controls. (**d**) No

1 differences were observed between the PKC-gamma KO and wild-type mice when nociception

2 was studied (n = 10) [paw: $F(1, 36) = 1.122$, p = 0.297; genotype: $F(1, 36) = 1.106$, p = 0.30;

3 interaction: $F(1, 36) = 0.107$, p = 0.745]. Data are expressed as mean ± s.e.m. * p < 0.05, *** p

4 < 0.001 (PKC-gamma KO compared to WT).

5 Figure 5. **c-Fos expression in the dentate gyrus of the hippocampus was altered in PKC-**

6 **gamma KO mice after trace fear conditioning.** c-Fos quantification was performed in brain

7 samples obtained 2 h after mice exposure to the trace or the delay fear conditioning protocol.

8 This analysis was performed in different brain regions including (**a**) the dentate gyrus (DG) of

9 the hippocampus (n = 3-6) [conditioning protocol: $F(2, 22) = 6.786$, $p < 0.01$; genotype: $F(1, 22)$

10 $= 0.309$, $p = 0.584$; interaction: $F(2, 22) = 6.668$, $p < 0.01$], (**b**) the basolateral amygdala (BLA)

11 (n = 3-6) [conditioning protocol: $F(2, 22) = 33.36$, $p < 0.001$; genotype: $F(1, 22) = 0.851$, $p =$

12 0.366; interaction: $F(2, 22) = 0.934$, $p = 0.408$] and (**c**) the prelimbic cortex (PL) (n = 2-6)

13 [conditioning protocol: $F(2, 22) = 28.093$, $p < 0.001$; genotype: $F(1, 22) = 2.514$, $p = 0.127$;

14 interaction: $F(2, 22) = 1.143$, $p = 0.337$]. Data are expressed as mean ± s.e.m. * p < 0.05, ** p <

15 0.01, *** p < 0.001, (Trace or delay fear conditioning compared to control in WT mice), # p <

16 0.05, ### p < 0.001 (Trace or delay fear conditioning compared to control in PKC-gamma KO

17 mice), & p < 0.05 (WT vs. PKC-gamma KO mice). Scale bar 200 μm.

18 Figure 6. **Recruitment of PKC-gamma to the membrane compartment in the hippocampus**

19 **after fear conditioning training.** (**a**) In the membrane fraction, PKC-gamma isoform is

20 significantly increased 30 min after trace fear conditioning compared to control subunit (n = 4)

21 and delay groups subunit (n = 4) [conditioning protocol: $F(2, 9) = 21.048$, $p < 0.001$]. (**b**) No

22 differences were found between conditioning protocols in the cytosolic fraction neither after trace

23 (n = 4) or delay (n = 4) [conditioning protocol: $F(2, 9) = 1.098$, $p = 0.374$] fear conditioning.

24 Data are expressed as mean ± s.e.m. ** p < 0.01,*** p < 0.001 (compared to control fear

25 conditioning protocol).

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Conventional Isoforms

Novel Isoforms

☐ Wild-type   ■ PKC-gamma KO

MOLN-D-19-01111-R3
Gomis-González et al. Figure 1

a T-maze

b Y-maze

c Barnes-maze 24 h

d Barnes-maze 7 d

☐ Wild-type   ■ PKC-gamma KO
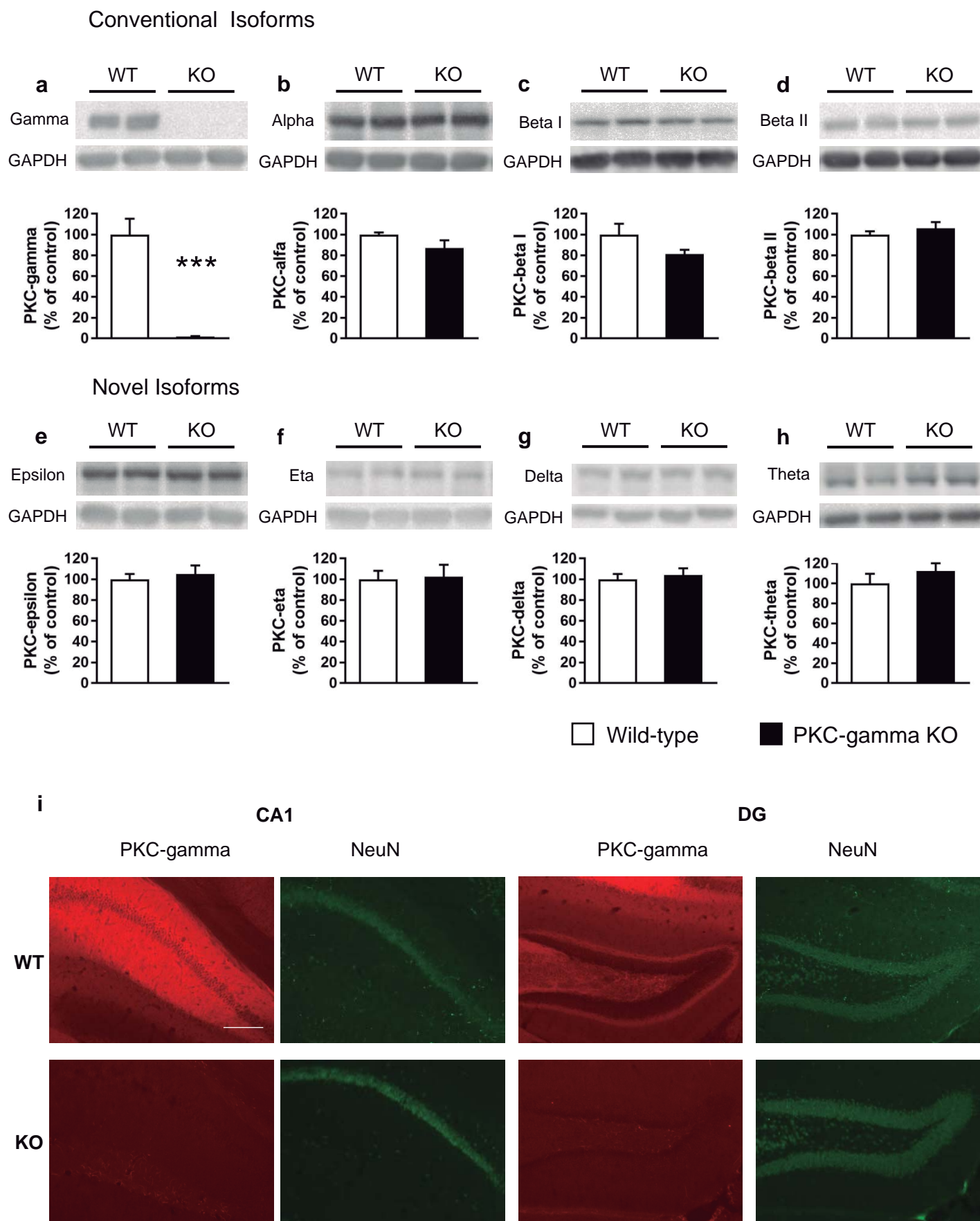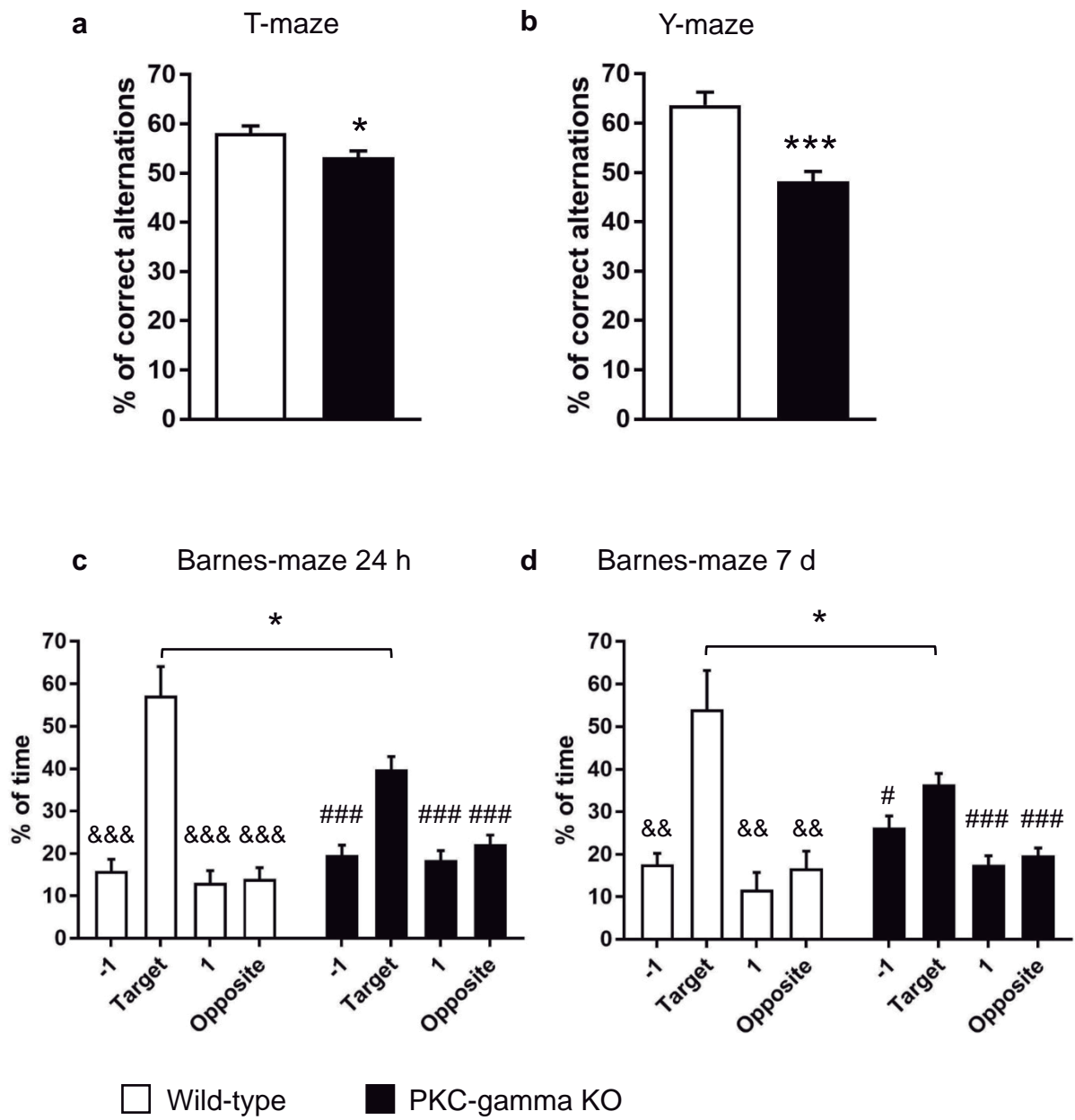
MOLN-D-19-01111-R3
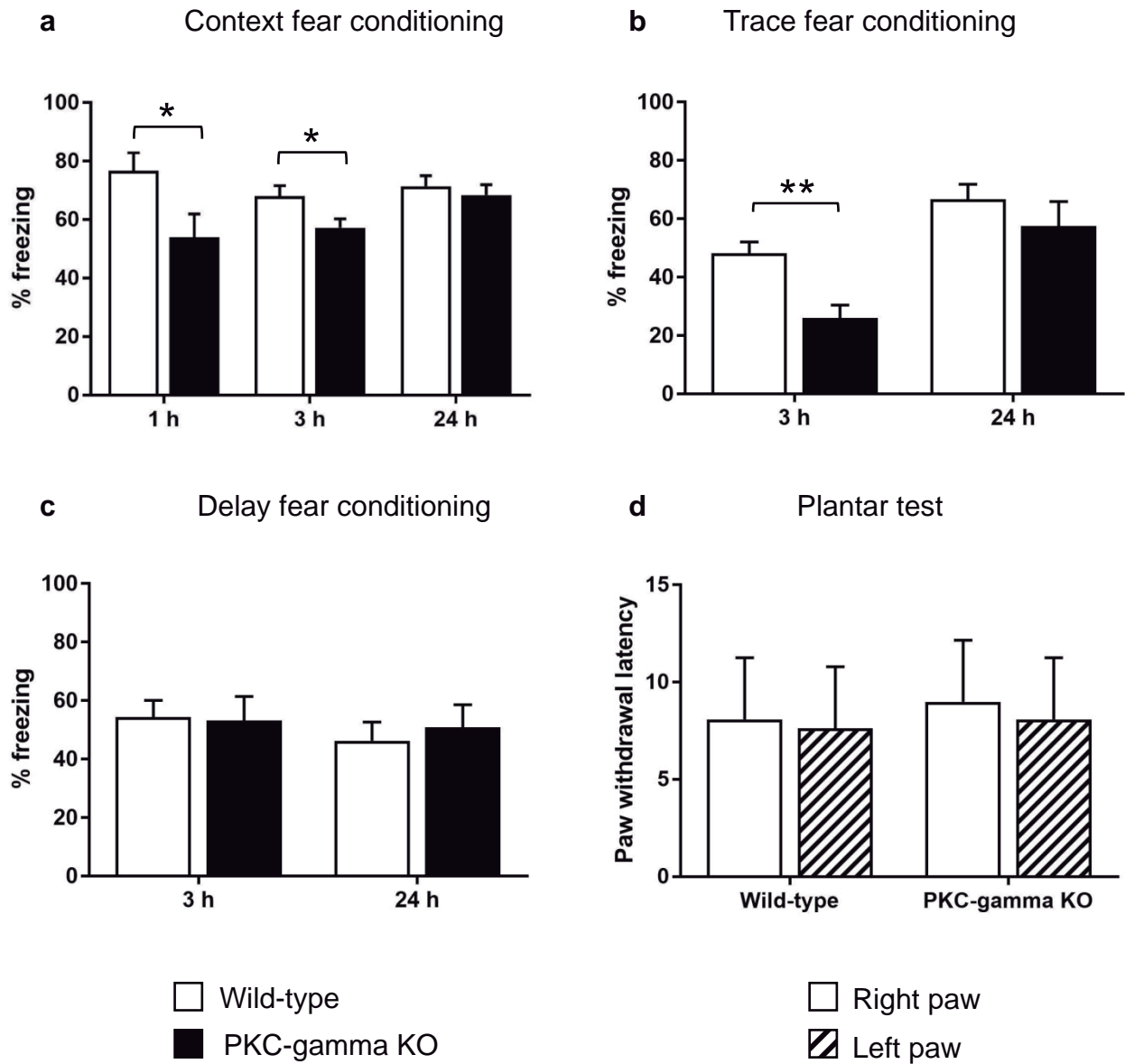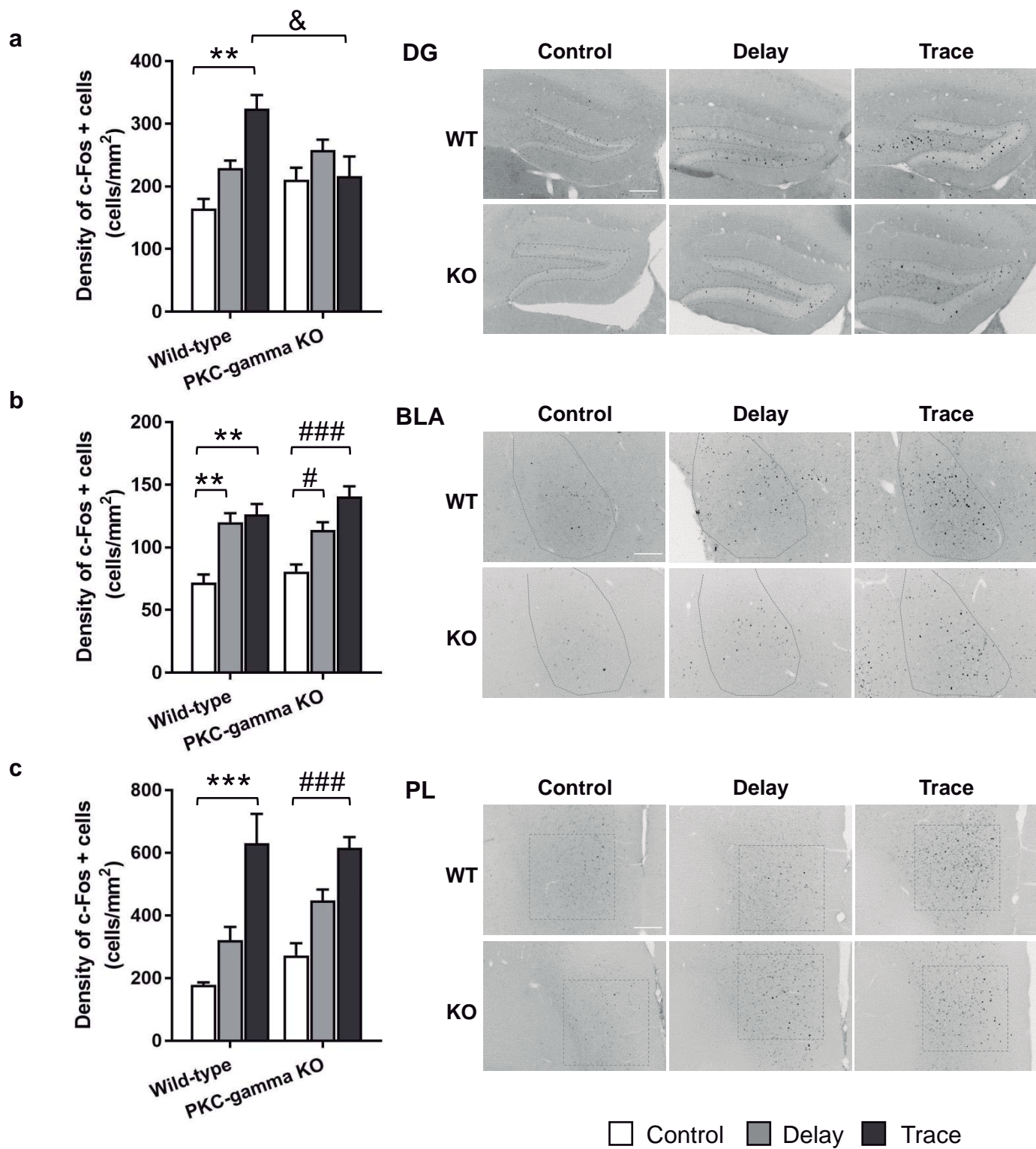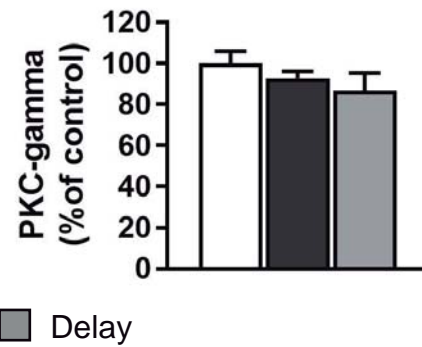Gomis-González et al. Figure 2

a Novel Object Recognition
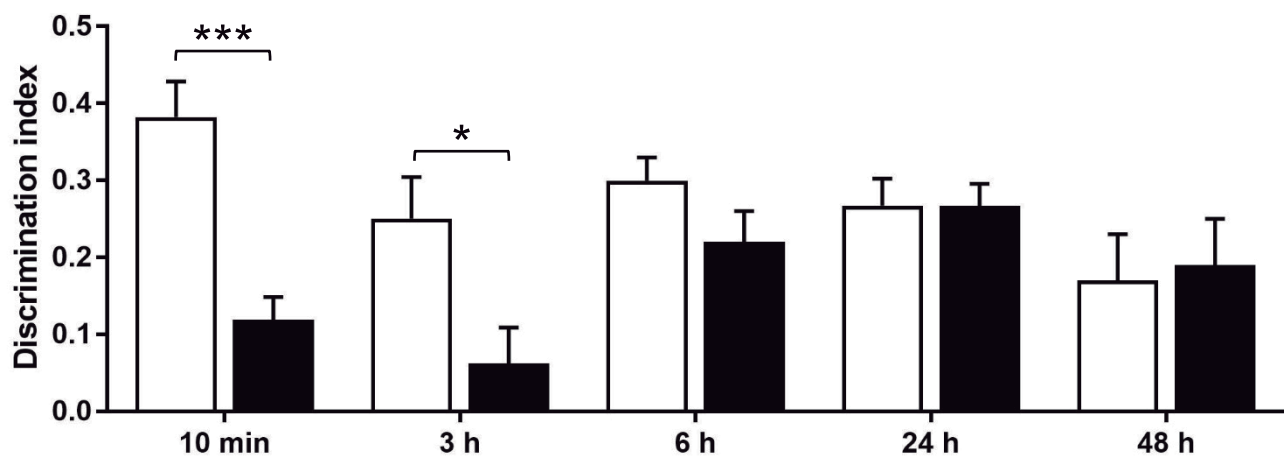
b Novel Place Recognition
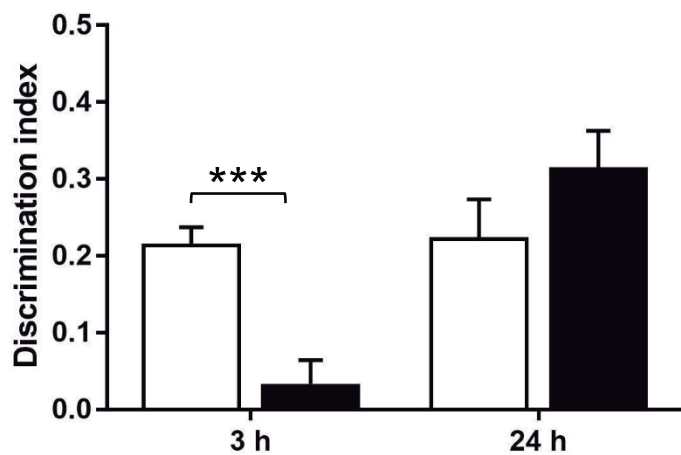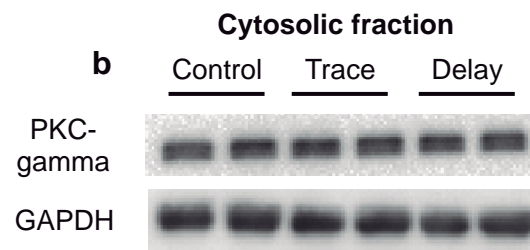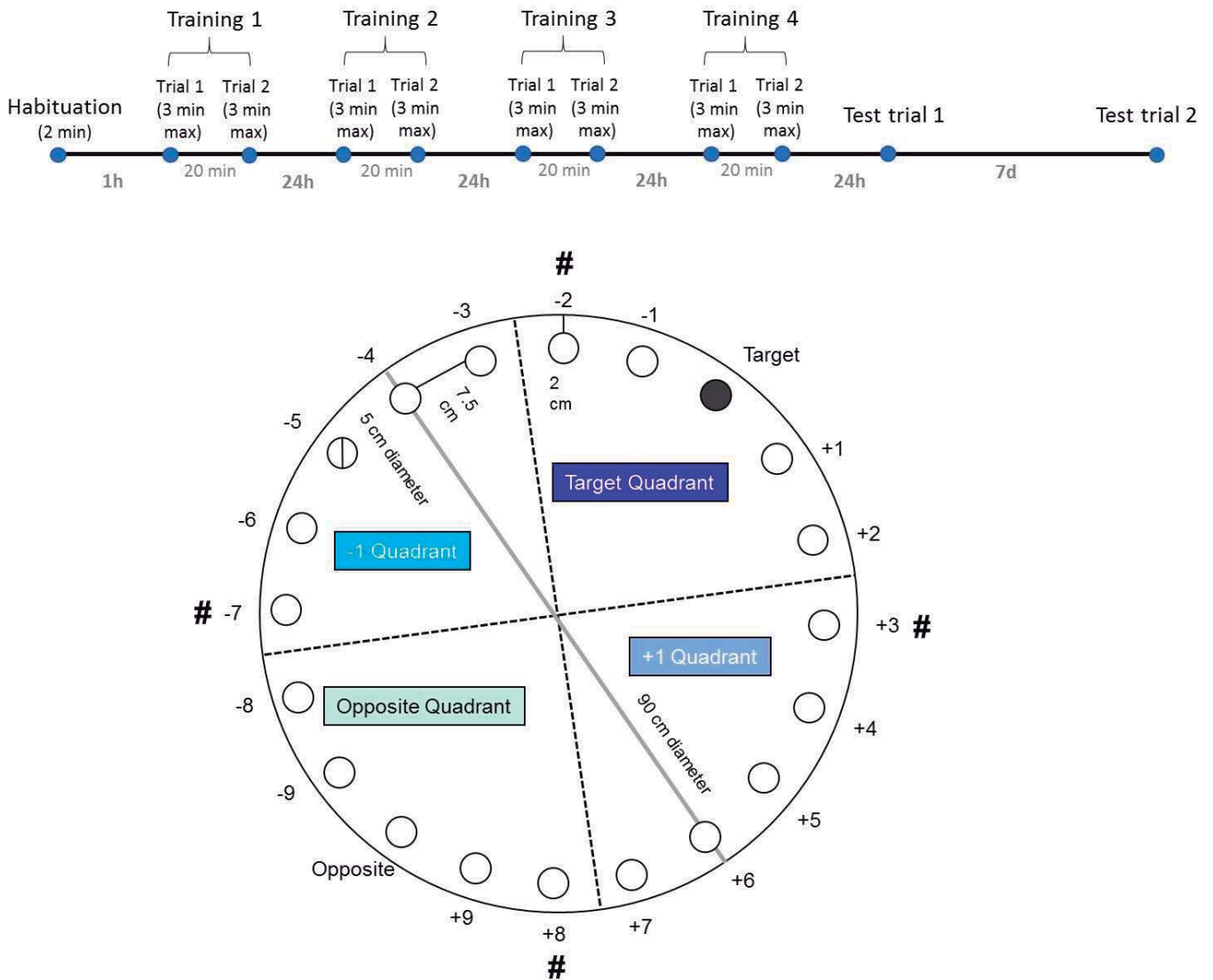
c Locomotor activity
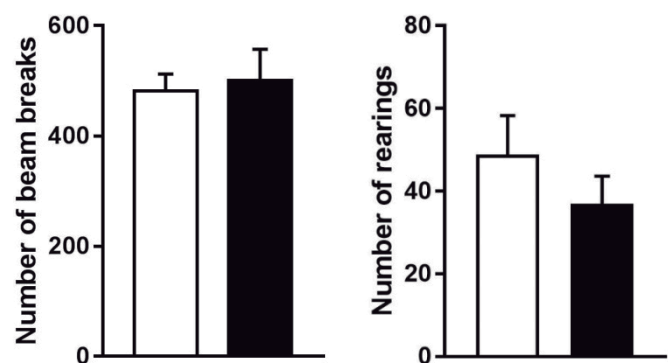
☐ Wild-type
■ PKC-gamma KO

MOLN-D-19-01111-R3
Gomis-González et al. Figure 3

a Context fear conditioning

b Trace fear conditioning

c Delay fear conditioning

d Plantar test

☐ Wild-type
■ PKC-gamma KO

☐ Right paw
▨ Left paw

MOLN-D-19-01111-R3
Gomis-González et al. Figure 4

a

DG

| | Control | Delay | Trace |

b

BLA

| | Control | Delay | Trace |

c

PL

| | Control | Delay | Trace |

□ Control ▨ Delay ■ Trace

MOLN-D-19-01111-R3
Gomis-González et al. Figure 5

Membrane fraction

a   Control   Trace   Delay

PKC-gamma

Ponceau

Cytosolic fraction

b   Control   Trace   Delay

PKC-gamma

GAPDH

☐ Control ■ Trace ■ Delay
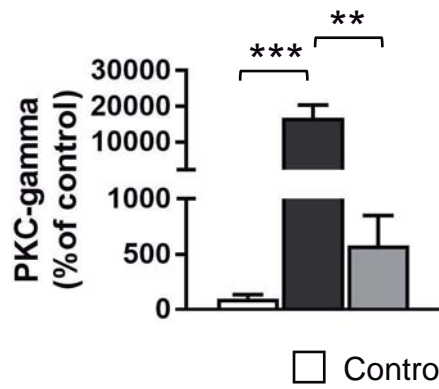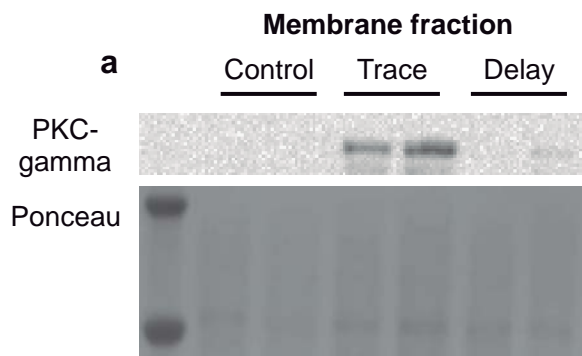
MOLN-D-19-01111-R3
Gomis-González et al. Figure 6

Supplementary Figure 1. **Timeline and scheme of the Barnes maze task.** Timeline of Barnes maze test indicating the training sessions and the time points when the tests were performed (Test Trial 1-recent spatial long-term memory; Test Trial 2-remote spatial long-term memory). The maze consists of a circular platform (90 cm diameter) with 20 equally spaced holes of 5 cm diameter separated by 7.5 cm. Holes have a distance of 2 cm from the perimeter of the circular maze and numbered from +1 to +9 (on the right side of the target hole) and from -1 to -9 (on the left). There is also an opposite hole to the target hole. The maze is divided in 4 quadrants containing 5 holes each one: target quadrant (from -2 to +2), +1 quadrant (from +3 to +7), opposite quadrant (from +8 to -8) and -1 quadrant (from -7 to -3). In the limits of the quadrants there are different visual cues surrounding the maze (represented as #)
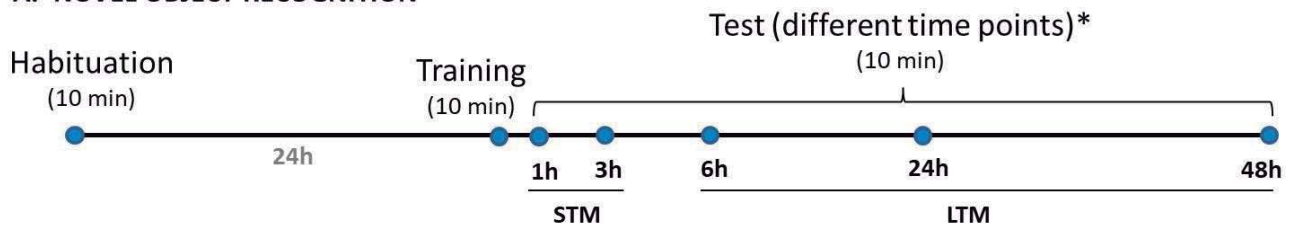
Supplementary Figure 2. **Timeline scheme of the behavioral tests performed in the study.** Scheme showing the time intervals and time points when the different behavioral tests were performed, including: Novel object recognition test, novel place recognition test, context recognition test and trace/delay fear conditioning. For every test, each time-point was performed in a different cohort of mice.

*Tests done at different time points were performed by different sets of animals
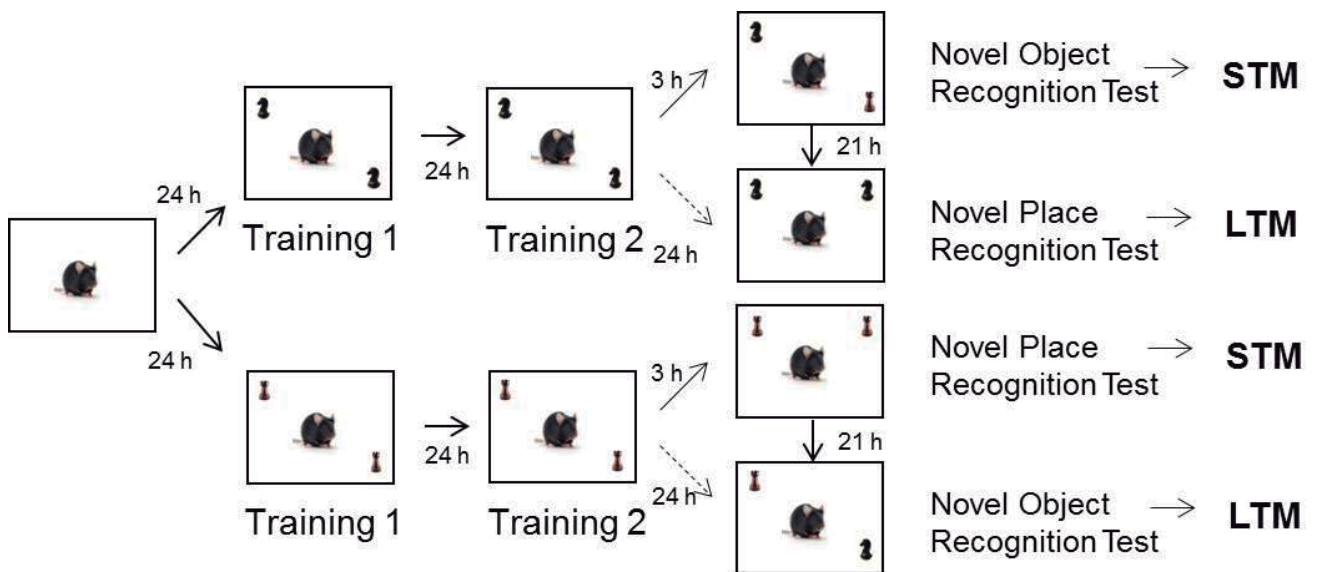
Supplementary Figure 3. **Scheme for combined NOR/NPR tasks**. Protocol used that combined the novel object-recognition (NOR) test and the novel place-recognition (NPR) test in order to study short-term and long-term memory in the same animal (see Material and methods section for details).
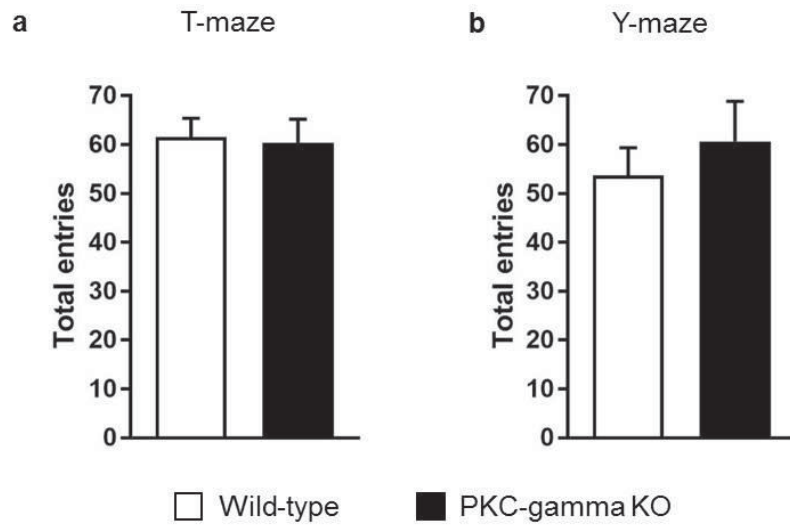
Supplementary Figure 4. **Locomotor activity measurements in spatial memory performance tests.** No significant differences in total entries were observed between PKC-gamma KO mice compared with WT neither in the T-maze [$F_{(1,25)}$ = 0.031, p=0.861] (**a**) or the Y-maze [$F_{(1,16)}$ = 0.373 , p = 0.55] (**b**). Data are expressed as mean ± s.e.m.
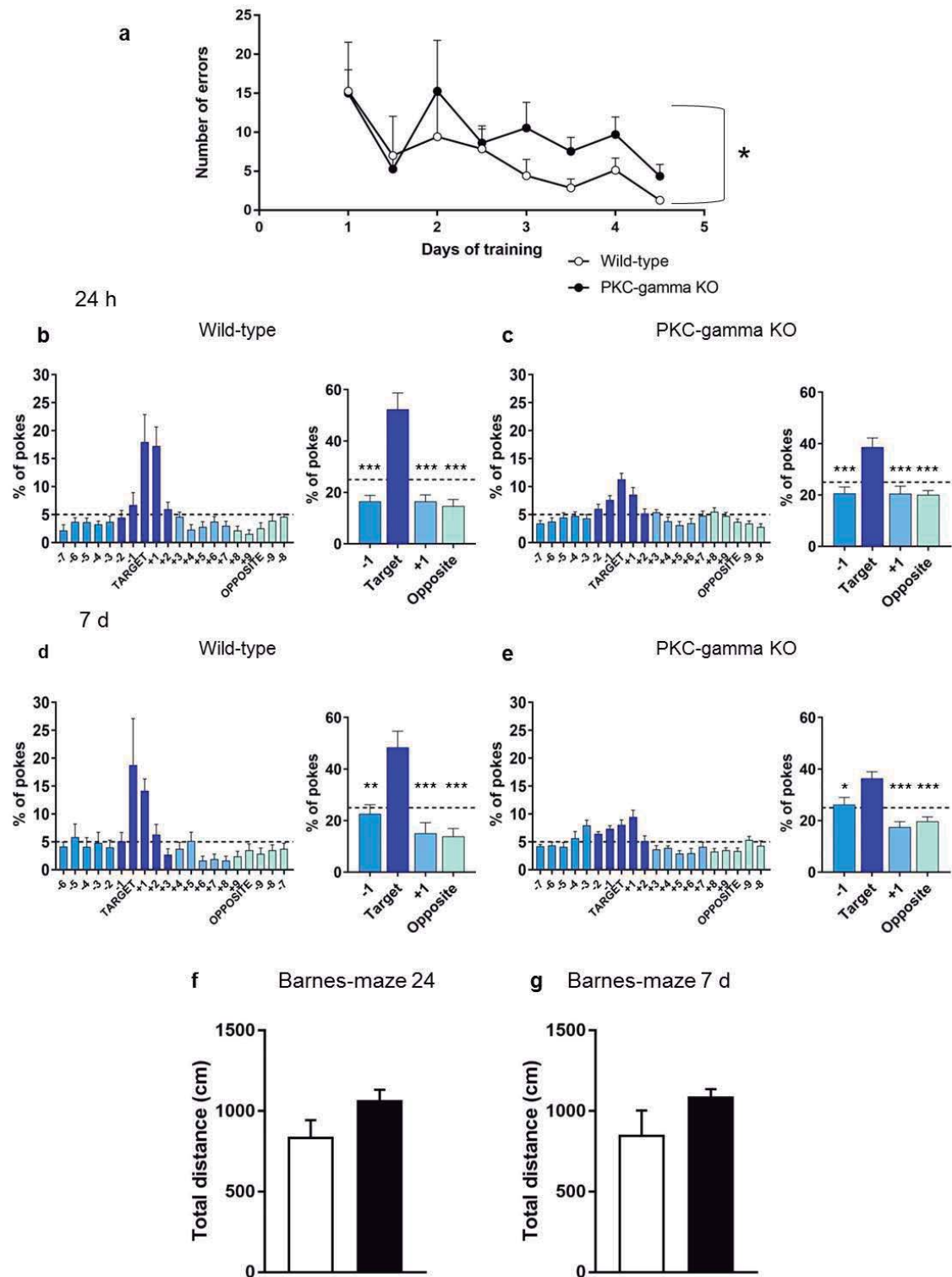
Supplementary Figure 5. **Spatial preference memory was evaluated in the PKC-gamma KO mice.** Learning and spatial memory were studied using the Barnes Maze task. (**a**) No differences were observed between the PKC-gamma KO and wild-type mice during training sessions [genotype: $F_{(1, 16)} = 1.897$, $p = 0.187$; time (trial): $F_{(7, 112)} = 2.237$, $p < 0.05$; interaction: $F_{(7, 112)} = 0.315$, $p = 0.946$]. 24 h (**b-c**) and 7 d (**d-e**) after the last training sessions memory retention was assessed. At the first trial test, the % of pokes in the target quadrant in WT (**b**) and PKC-

5

gamma KO (**c**) was higher than in the other quadrants (**b**: Target vs -1 [$F(1,12) = 27.773$, $p < 0.001$], Target vs 1 [$F(1,12) = 27.148$, $p < 0.001$], Target vs Opposite [$F(1,12) = 29.919$, $p < 0.001$]; **c**: Target vs -1 [$F(1,20) = 17.289$, $p < 0.001$], Target vs 1 [$F(1,20) = 15.686$, $p < 0.001$], Target vs Opposite [$F(1,20) = 22.740$, $p < 0.001$]). In the second trial test, the % of pokes were also higher in the target quadrat for WT (**d**) and PKC-gamma mice (**e**) (**d**: Target vs -1 [$F(1,12) = 12.643$, $p < 0.01$], Target vs 1 [$F(1,12) = 19.351$, $p < 0.001$], Target vs Opposite [$F(1,12) = 24.014$, $p < 0.001$]; **e**: Target vs -1 [$F(1,20) = 7.322$, $p < 0.05$], Target vs 1 [$F(1,20) = 32.857$, $p < 0.001$], Target vs Opposite [$F(1,20) = 29.88$, $p < 0.001$]). (**f-g**). In Barnes Maze task no significant differences in total distance were observed in the test trial [24 h: $F(1,16) = 3.981$, $p = 0.063$; 7 d: $F(1,16) = 3.305$, $p = 0.087$] revealing that differences between groups described in Figure 1 are not due to alterations in exploratory behavior or locomotor activity]. Data are expressed as mean ± s.e.m. * $p < 0.05$, ** $p < 0.01$ *** $p < 0.001$ (compared to target quadrant).

a 10 min

b 3 h

c 6 h

d 24 h

e 48 h

☐ Wild-type
■ PKC-gamma KO

f 3 h

g 24 h
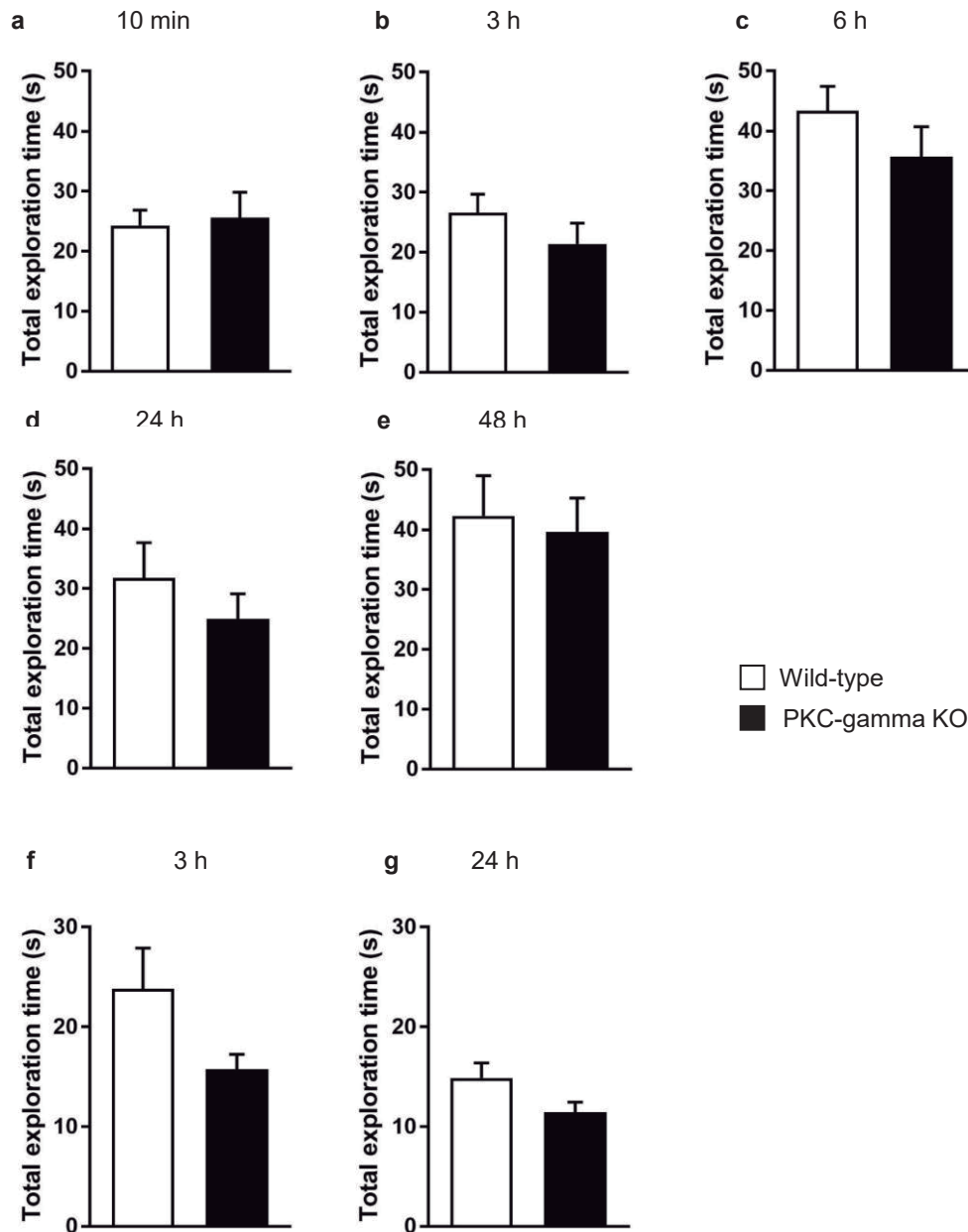
Supplementary Figure 6. **Exploration time on the test session for short-term and long-term novel object-recognition test and novel place-recognition test**. No significant differences in total exploration time were observed between PKC-gamma KO mice compared with WT in short-term ((**a**) 10 min [$F_{(1,17)} = 0.066$, $p = 0.80$] and (**b**) 3 h [$F_{(1,13)} = 1.268$, $p = 0.28$]) and long-term memory ((**c**) 6 h [$F_{(1,13)} = 1.451$, $p = 0.25$], (**d**) 24 h [$F_{(1,20)} = 0.903$, $p = 0.353$] and (**e**) 48 h [$F_{(1,16)} = 0.088$, $p = 0.77$]) in the novel object-recognition test neither in short-term (**f**) 3 h [$F_{(1,16)} = 3.467$, $p = 0.081$]) and long-term (**g**) 24 h [$F_{(1,15)} = 50.09$, $p = 0.070$]) in the novel place-recognition test. These data reveals that differences between groups described in Figure 2 are not due to alterations in exploratory behavior or locomotor activity. Data are expressed as mean ± s.e.m.
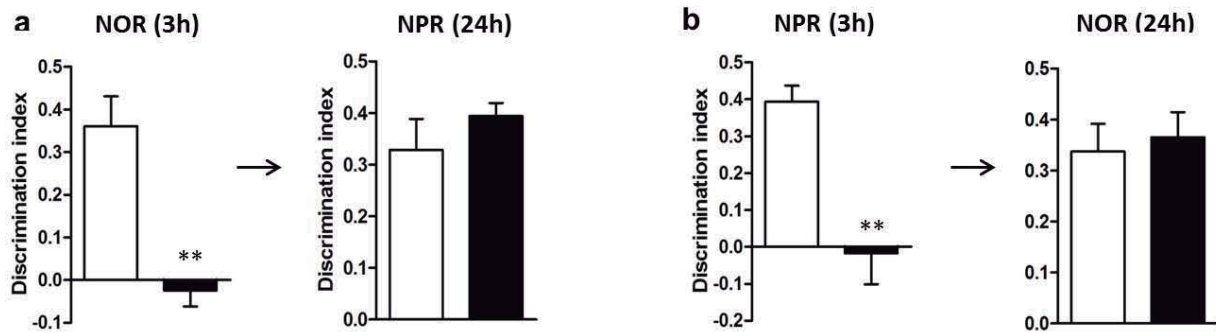
Supplementary Figure 7. **Short-term and long-term memories were studied in the same animal by using two different non-emotional memory tasks. (a)** One set of animals performed the NOR test to study STM $[F(1,6) = 23.058, p < 0.01]$ and the NPR test to study LTM $[F(1,6) = 0.63, p = 0.458]$ while another set (**b**) performed the NPR test to study STM $[F(1,7) = 15.948, p < 0.01]$ and the NOR test to study LTM $[F(1,8) = 0.144, p = 0.714]$. In both cases, PKC-gamma KO animals only present memory impairments when STM was studied, while LTM was preserved. Data are expressed as mean ± s.e.m. ** p < 0.01 (PKC-gamma KO mice compared to WT).
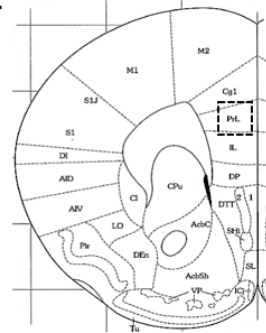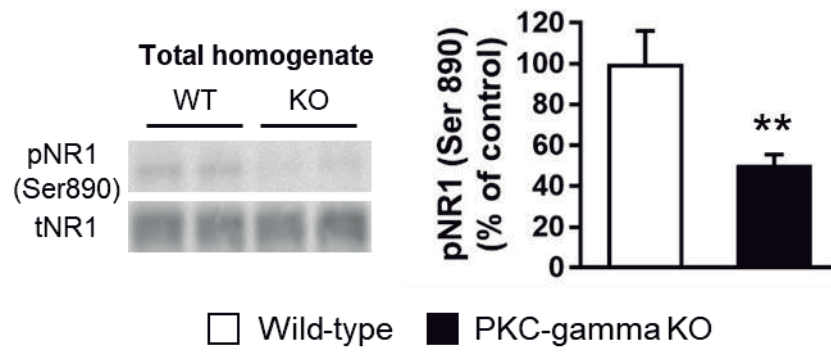
Supplementary Figure 8. **Reference atlas pictures indicating the exact region where the cellular analysis was performed.**

Supplementary Figure 9. **PKC-gamma KO mice presented a diminished phosphorylation of serine 890 in NR1 subunit**. a significant difference in the phosphorylation level of the NR1 subunit at Ser890 was observed between WT and PKC-gamma KO (n=6-7) [$F_{(1,11)} = 9.846$, $p < 0.01$]. Data are expressed as mean ± s.e.m. ** $p < 0.01$ (PKC-gamma KO compared to WT)