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Supporting Online Material for

Role of Secondary Sensory Cortices in Emotional Memory Storage and Retrieval in Rats

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Supporting Online Material

Materials and Methods

Subjects. Male Wistar rats were used. The animals were housed in plastic cages with food and water available ad libitum, under a 12 h light/dark cycle (lights on at 7:00 A.M.) at a constant temperature of 22 ± 1°. All experiments were conducted in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and approved by the Bioethical Committee of the University of Turin.

Surgical procedures. Excitotoxic lesions were made in seventy-day old animals (average body weight 260-330 g). Rats were mounted in a stereotaxic apparatus, an incision of the skull was made, and small burr holes were drilled to allow the penetration of a 28 gauge infusion needle. A 10 µl Hamilton syringe mounted on an infusion pump was used to deliver infusions at a rate of 0.1 µl/min. Neuronal cell loss was induced by injecting N-methyl-D-aspartic acid (NMDA) bilaterally at the coordinates taken from Paxinos and Watson (*S1*) with cortical fields referenced to Zilles atlas (*S2*). NMDA was dissolved in 100 mM PBS, pH 7.4. The needle was left in place for additional 3 min. Sham rats underwent an identical procedure, except that no infusions were made. Unoperated control groups received anesthesia and skull incision but not surgery. Following the surgical procedures, the rats were kept warm and under observation until recovery from anesthesia. All behavioral procedures were made ten days after surgery to allow for recovery.
Auditory cortices lesions. Primary auditory cortex lesion was induced by administering NMDA (20 µg/µl) at two points with the following coordinates: 4.0 mm posterior (AP) to bregma, ± 6.4 mm lateral (L) and 5.4 mm ventral (V), total volume 0.24 µl; 5.5 mm posterior to bregma, ± 6.6 mm lateral and 5.4 mm ventral, total volume 0.22 µl. Secondary auditory cortex was lesioned by injecting NMDA (20 µg/µl) at the coordinates: AP = 5.8, L= ± 6.7, V= 6.0, 0.20 µl; AP = 6.8, L= ± 6.7, V= 6.0, 0.2 µl**.** The perirhinal cortex underlying Te2 cortex was lesioned by administering NMDA at the coordinates: AP = 5.8, L= ± 6.6, V= 7.8, 0.18 µl; AP = 6.8, L= ± 6.3, V= 7.6, 0.18 µl.
Visual cortices lesions. Secondary visual cortex lesion was centered on the Oc2L by administering NMDA (20 µg/µl) at the coordinates: AP = 5.8, L= ± 6.7, V= 4.0, 0.18 µl; AP = 6.8, L= ± 6.5, V= 4.0, 0.2 µl.
Olfactory cortices lesions. The posterior piriform cortex was lesioned with 10 µg/µl NMDA at the coordinates: AP= 2.0, L= ± 6.0, V= 9.8, 0.2 µl; AP = 3.0, L= ± 6.3, V= 9.7, 0.18 µl. Stereotaxic coordinates, dosage and volumes of NMDA were chosen in order to disrupt selectively posterior piriform cortex and at the same time to spare the adjacent basolateral amygdala.
To study PKMzeta involvement in fear memories, the specific inhibitor ZIP was injected one day or one month after training. Each rat received bilateral intracranial infusions of ZIP (10 nmol/µl saline) or of the myristoylated peptide, scr-ZIP (10 nmol/µl saline), comprising a scrambled sequence of the same amino acids as ZIP (*S3, S4*). The previously reported stereotaxic coordinated were employed in order to inject ZIP or scr-ZIP into Te2 (0.6 µl/injection), Oc2L (0.4 µl/injection) or posterior piriform cortex (0.4 µl/injection). To study the diffusion of the peptide, the biotinylated version of ZIP (10 nmol/µl dimethyl sulfoxide) was injected in separate groups of rats at the volumes and the stereotaxic coordinates previously employed.

Histology. Biotinylated ZIP staining was analyzed as in a previous work (*S3*). Briefly, two hours after injection, animals were deeply anesthetized and intracardially perfused with 4% paraformaldehyde (PAF). The brain was incubated in 4% PAF overnight at 4°C and then transferred to a 30% sucrose. Free-floating coronal sections (30-µm) were cut on a cryostat. Slices were incubated with the avidin-biotin-peroxidase complex (ABC) for 1 h, and then rinsed in Tris-HCl and incubated in 3–3′ diaminobenzidine tetrahydrochloride (DAB) reaction for 5 min (0.037% DAB

in 0.05 M Tris-HCl buffer). The DAB was developed by the addition of 1% hydrogen peroxide (H_20_2), and the development stopped by washing with 0.05 M Tris-HCl.

The needle track in the case of ZIP injection or the extension of the damaged areas in the case of NMDA lesions was histologically verified at the end of the experiments with Nissl staining, using the conventional procedure. Extension of lesions was further verified with NeuN staining analysis. The rats were intracardially perfused with 4% PAF. The brain was incubated in 4% PAF overnight at 4 °C, transferred to 30% sucrose and finally sectioned at 30 μm on a cryostat. Sections were placed in NeuN antibody (1:500) at room temperature (RT) for 24 h, transferred to biotinylated anti-mouse antibody IgG (1:500) at RT for 1 h and finally placed in a solution of ABC on a shaker for 1 h. Sections were rinsed in Tris-HCl and incubated in DAB. The DAB was developed by the addition of 1% H_20_2, and the development stopped by washing with Tris-HCl. Verification of lesions was made observing NeuN-stained tissue under a microscope magnified at 2x and 4x. The lack of staining was used as an indication of neuronal loss due to lesions.

Fear conditioning paradigm. *Fear memory acquisition.* A Skinner box module was employed as a conditioning chamber as in previous work (*S5*). The floor was made of stainless steel rods (1 cm in diameter, spaced 5 cm apart) connected to a shock delivery apparatus. The apparatus was enclosed within a sound attenuating chamber. Once inside, the animals were left undisturbed for 2 min. After this time, a series of sensory stimuli acting as CSs were administered. The last 1 (auditory and olfactory fear conditioning) or 2 (visual fear conditioning) s of each CS were paired with an US consisting of a scrambled electric foot shock (intensity, 0.8 mA for remote fear memories and 0.7 mA for the recent memory). Rats were left in the chamber for an additional 1 min, and then returned to the home cage. In the fear conditioning to acoustic stimuli, seven pure tones (6 s, 85 dB, 1000 Hz, 24-s intertrial interval) or seven white noise stimuli (6 s, 88 dB, 24-s intertrial interval) were delivered as CSs by a loudspeaker located 20 cm above the grid floor. In olfactory fear conditioning, seven vinegar odors (10 s, 24-s intertrial interval) were presented using a flow-dilution olfactomer. Clean air (1.5 L/min) was directed to a solenoid valve that, when operated, passed the air to a 15-ml bottle containing 10 ml of wine vinegar. Odorized air was then directed to the conditioning chamber via ¼-in. Tygon tubing. Fear conditioning to visual stimuli was obtained by presenting seven visual stimuli (6 s, 24-s intertrial interval) produced by a fluorescent light bulb (rise-decay, 100 microsecond; 12 W) located 20 cm above the grid floor.
Fear memory retention. The animals were handled for two days (5 min per day) before memory retention. Memory was tested in a totally different apparatus located in a separate experimental room in order to avoid conditioned fear behavior to contextual cues (*S5*). The apparatus was a plastic cage with the floor and the sides made of transparent plastic and enclosed within a sound attenuating chamber equipped with an exhaust fan, which eliminated odorized air from the enclosure and provided background noise of 60 dB. Once inside, the subject was left undisturbed for 2 min. After this time, CSs were administered identical to those used during conditioning. The rat behavior was recorded by means of a digital video camera. Freezing response was taken as a fear index and measured by means of a stopwatch by one person who did not know to which experimental group each animal belonged. Freezing was defined as the complete absence of somatic mobility, except for respiratory movements.

Long-term habituation of the acoustic startle response. *Apparatus.* The startle response was detected using a SR-LAB startle chamber equipped with a cylindrical stabilimeter enclosed in a sound attenuating chamber. Continuous background noise (60 dB) helped mask extraneous auditory stimuli. Test stimuli were 104 dB white noise of 1 s duration with a 9-ms rise/fall time and were superimposed on the background noise. The voltage induced by the vertical displacement was rectified and sent to a computer. The startle amplitude was measured as the highest voltage peak within the first 60 ms from the stimulus onset. *Procedure.* The animals were adapted to the startle chamber for one day (two sessions per day, 3 min per session) with no stimuli presented. On the

next seven days, animals were given two sessions per day (3 min per session) with 10 presentation of the auditory stimulus on a 15-s interstimulus interval beginning 60 s after the rat was put into the chamber. Four weeks after the beginning of the training, the animals were operated. Memory retention was tested 10 days after surgery with one session per day (3 min per session). Shortly after the retention test, the subjects were given a sound never experienced before; a "click" sound produced by a keyring (0.5 s, 90 dB). Long-term habituation was assessed by comparing responsiveness across days, with only the first trial of each test session being used to prevent the effects of within-session (short-term) habituation from affecting startle amplitudes.

Long-term habituation to olfactory stimuli. *Apparatus.* Olfactory stimuli were presented in the same apparatus in which we tested fear memory retention (see above). Olfactory stimulus consists in a cheese odor dissolved in hot water in a 15-ml bottle and then delivered as described for olfactory CSs (see above). *Procedure***.** Rats were habituated to the cage for two days (two sessions per day, 5 min per session) with fresh air continuously delivered. On the next seven days, cheese odor was presented instead of fresh air. The odorant was continuously presented during each session (two 5 min sessions per day, delayed each other of one hour, 5 min per session). Four weeks after the beginning of the training, the animals were operated. Memory retention was tested 10 days after surgery with one session per day (5 min per session). After retention, the animals were given a new olfactory stimulus (a mayonnaise odor). During the first two minutes of each session, the experimenter measured how long the rat investigated the odor source with a stop watch. "Investigation" was defined as an active sniffing within 2 cm of the source of the odor.

Innate fear and anxiety analysis. The open field (90 x 90 x 35 cm) was a plastic grey box. On the bottom of the box, white lines divided the field into squares of equal size. Illumination was provided by a single 40-W bulb (3000 lx) placed 3 m above the centre of the apparatus. Rats were placed in the centre of the apparatus and observed for 5 min. The behavioral patterns were defined as follows: *Exploration*: the number of the squares crossed (all four paws) in the periphery and centre during the observation period. *Leaning*: the numbers of times the animal placed one or two paws in the contact on the wall. *Rearing*: the number of times the rat stood on its hind legs (and lifted its fore legs off the floor).

The plus maze consisted of two enclosed arms (100 x 45 cm) and two open arms forming a square cross with a square centrepiece. The apparatus was elevated 50 cm above the floor. The animal was placed in the centre of the plus-maze facing one of the open arms. The number of entries (all four paws) into, the time spent in each arm, leaning and rearing behaviours were recorded for 5 min.

Unconditioned fear responses were studied by exposing rats to the synthetic fox odor 2,4,5-trimethylthiazoline (TMT). Since Wistar rats are less sensitive to TMT, we employed Sprague-Dawley rats (*S6*). TMT was presented in a cage made of transparent plastic (40 x 20 x 16 cm). The cage was tape-marked into two equally sized sections (Area 1 contained the odor while Area 2 was farthest from it). TMT was presented by pipetting it onto one pieces of filter paper that was then taped to the corner of Area 1. Animals were habituated to the cage for two consecutive days (5 min per session). The test day, rats were first exposed for 5 min to a distilled water (6 µl per animals) put in the clean filter paper (pre-odor phase). Two hours later, animals were placed again in the cage and exposed for 5 min to TMT (6 µl per animal) put in a clean filter paper. Behavioral responses of rats were categorized as: freezing and avoidance (time spent in the Area 2).

Zif268 protein expression analysis.

Remote memory. Animals were randomly assigned to three experimental groups: "Naïve", "Familiar Tone" and "Conditioned Tone". In the "familiar tone" group, animals were exposed to 7 acoustic stimuli (6 s, 85 dB, 1000 Hz, 24-s intertrial interval). "Conditioned Tone" group consists in animals conditioned to acoustic CS as previously described. Naive rats received no stimuli. Four

weeks after training, all animals were handled and habituated to the new cage for 5 days, each day for 7 min. The cage was totally different from that employed during conditioning. The sixth day, all groups were exposed to the acoustic stimuli (see "fear memory retention" paragraph). Freezing behavior served as a control of conditioned fear.

Recent memory. Animals were randomly assigned to three experimental groups: "Naïve", "Conditioned Context" and "Conditioned Tone". "Conditioned Tone" group consists in animals conditioned to acoustic CSs as previously described. Animals conditioned to the training environment ("Context conditioned") received an identical conditioning procedure except that no CSs were presented. Naive rats received no stimuli. All animals were handled and habituated to the new cage for 3 days before training, each day for 5 min. Twenty-four hr after training, all groups were exposed to the acoustic stimuli. Freezing behavior served as a control of conditioned fear.

In both recent and remote fear memory experiments, 90 min after the completion of memory retention test, rats were deeply anaesthetized and perfused intracardially with 4% PAF. The brains were dissected, stored overnight at 4 °C, and finally transferred to 30% sucrose. Coronal sections (50-μm) were cut on a vibratome and collected in PBS. Free-floating sections were pretreated with 0.3% H_2O_2 in PBS to reduce endogenous peroxidase activity. After four rinses, sections were incubated in a blocking solution (2% bovine serum albumin (BSA), 2% normal goat serum and 0.2% Triton X-100) for 1 h at RT). Then, they were incubated in primary polyclonal rabbit anti-zif268 (1: 2000 dilution) antibodies in the blocking solution overnight al the RT. Subsequently, sections were washed with PBS and incubated for 2 h at RT with biotinylated goat antirabbit IgG (1: 2000 in PBS) followed by 1 h at RT in ABC. Sections were rinsed in PBS. The peroxidase reaction end-product was visualized by incubating sections in 0.05 M Tris (pH 7.6) containing 3.3' DAB (0.037%) as chromogen and hydrogen peroxide (0.015%) for 5 min. Finally, immunolabelled sections were washed in PBS, mounted on gelatin-coated slides, dehydrated and coverslipped. The slices were analyzed using Neurolucida software connected to a microscope via a color CCD camera. The quantification of zif268-positive cells was carried out at X 10 (Te2 cortex and amygdala) or X 4 (posterior piriform cortex) magnification. Immunoreactive nuclei were counted bilaterally using at least three serial sections for each area. The number of nuclei expressing zif268 was quantified in the area of interest at the coordinates: Te2 AP = from – 6.8 to - 7.3 mm; posterior piriform cortex: AP = from – 2.5 to - 3.3 mm; lateral and basal nuclei of the amygdala AP = from - 2.5 to - 3.3 mm; central amygdala AP = from - 2.0 to – 2.8 mm (*S2*). The mean count of each animal was divided by the mean count of the respective naïve control group in order to generate a normalized count for each animal. Data were then averaged in order to produce the mean of each group.

Statistical analysis. Student's t-test, one-way ANOVA, a mixed-design ANOVA test with groups as a between-subjects variable and retention trials as a within-subjects variable, Newman-Keuls multiple comparisons test and the Mann-Whitney test for non-parametric analysis were employed.

Supporting results

1) **Histological analysis**

From the 18 animals that were lesioned in the **Te1 area**, we excluded from analysis 4 rats due to incomplete bilateral lesions and 3 for incorrect placement of the lesions. The other rats (n = 11) sustained extensive bilateral lesions of Te1 area, with partial damage to the adjacent parietal cortex and to the Te3 cortex. In some cases, the lesioned area extended to the hippocampus. Photomicrographs of representative lesions are presented in Fig. S1A-B.

Posterior perirhinal cortex was lesioned at stereotaxic coordinates chosen in order to avoid any damage to the Area Te2. Reconstructions of the smallest and largest areas of damage are presented in Fig. S1C. From the 18 rats undergoing excitotoxic lesion of the posterior perirhinal cortex, 6 rats had partial Te2 damage, and 2 subjects had incomplete bilateral perirhinal area lesions. These animals were excluded from analyses. Photomicrographs of perirhinal cortex lesions are presented in Fig. S1D-E.

From the 124 rats undergoing **secondary auditory cortex Te2 lesions**, we excluded 27 because they showed incomplete bilateral damage. The others (n = 97) sustained extensive bilateral cell loss in all layers of Area Te2, with partial damage also to the caudal portion of Te3 and of Te1 cortices. Additional cell loss in the occipital cortex above Te2 region was observed in many rats. The damage to Te1 or to Oc2L cortices was presented in some, but not all, the animals that display amnesia following Te2 lesion. In some cases, the lesioned area extends to the hippocampal region CA1 or it included a portion of the posterior perirhinal cortex. In all cases, the anterior perirhinal cortex was intact (Fig. S2). This is at variance with a previous study (*S7*), in which both perirhinal and Te2 cortices were disrupted.

From the 70 rats undergoing lesion in the **secondary occipital visual cortex Oc2L**, 10 were excluded owing to incomplete bilateral damage and 12 due to the extension of lesions to the Te2 cortex. In the remaining rats (n = 48), lesions also included parts of primary visual cortex, Oc1B (Fig. S3).

From the 87 rats that received lesions in the **posterior piriform cortex**, 17 were excluded because the lesion affected the adjacent basolateral amygdala and 12 for the incomplete bilateral posterior piriform cortex damage. In the remaining animals (n = 58), the damaged area included the posterior piriform cortex, and in 20 the lesion extended to the anterior perirhinal cortex (Fig. S4). In these cases, the disruption of perirhinal cortex was most often unilateral and not complete, with the dorsal portion of perirhinal cortex spared by the lesion.

The combined histological and behavioral data support the assertion that amnesia, when present, is strictly related to secondary sensory cortices lesions and not due to unintended disruption of adjacent brain sites. In more detail, secondary auditory cortical lesions aimed at the Te2 area of Zilles (*2*) impaired auditory fear memories. In some animals, the lesion partially extends to the Te1 cortex and/or to the Oc2L cortex. Amnesia, however, cannot be due to damage of these sites since we found that Te1- or Oc2L-lesions did not affect auditory fear conditioned responses. In some cases, Te2 lesions also affect the posterior perirhinal cortex. However, previous (*S7*) and present results showed that lesions restricted to posterior perirhinal cortex did not hamper auditory fear memories. Finally, in some (but not in all) animals, Te2 lesions also induced partial hippocampal damage. Indeed, similar partial hippocampal damage was also present in some of the animals that were lesioned in Te1 cortex and that did not show amnesia.

In rats lesioned in the secondary visual cortex, the damage includes the Oc2 lateral cortex (*2*) and in some cases (but not in all) also extends to Oc1B cortex. There was no damage to other brain regions. Posterior piriform cortical lesions were restricted to this site. In some rats, damage also extends to the anterior perirhinal cortex. Such a perirhinal cortex lesion was frequently unilateral and incomplete. Indeed, it has been previously reported (*S8*) that a complete lesion of the anterior perirhinal cortex impairs the association between olfactory cues and footshock, an effect that we did not observe in the present study.

2) Freezing analysis in NMDA-lesioned animals

In addition to measuring freezing displayed by animals during each CS presentation, we also examined freezing during the time interval that elapses between CSs administration. Total cumulated freezing time (i.e., total seconds spent freezing both during, and between, CSs administration) is presented in fig. S5.

Remote fear memories. Fig. S5A depicts total time freezing in animals lesioned in the primary auditory cortex and in those sham-operated. Student's t-test indicated no differences between the two groups (t_{19} = 1,17, P > 0.05). Fig. S5B shows total time freezing in sham-operated animals, in those lesioned in Te2 or in posterior perirhinal cortex and in those retrained. The one-way ANOVA test revealed differences among groups ($F_{(3,50)}$ = 25,49, P < 0.05). Newman-Keuls test showed differences between Te2-lesioned subjects and the other groups (P < 0.05). Similarly, one-way ANOVA showed differences among sham-operated, Oc2L lesioned and retrained animals ($F_{(2,34)}$ = 63,68, P < 0.05) (Fig. S5C). Post hoc Newman-Keuls test revealed differences between the Oc2L-lesioned group and the other ones (P < 0.05). Finally, total time freezing in animals lesioned in the posterior piriform cortex was statistically reduced with respect to sham-operated and retrained animals ($F_{(2,29)}$ = 8,01, P < 0.05) (Fig. S5D).

Recent fear memories. Total freezing time displayed by animals lesioned in Te2 cortex one day after training is presented in fig. S5E. Student's t-test indicated no difference between lesioned and sham-operated rats (t_{23} = 0,99, P > 0.05). Similarly, there was no difference between Oc2L- and sham-operated animals (t_{20} = 0,63, P > 0.05) (fig. S5F) and between rats lesioned in the posterior piriform cortex and those sham-operated (t_{21} = 0,94, P > 0.05) (fig. S5G).

Memory saving. As described above, the animals that showed amnesia following cortical lesions were retrained. During the re-training session, rats were put in the original conditioning chamber and after 2 min of free exploration CSs were administered in association with the US. During the presentation of the first CS, we measured freezing displayed by animals before shock delivery. Such a measurement may provide information on the possible sparing of memory (or "saving") after cortical lesions. The results are presented in fig. S5H-J. In comparison with naïve animals, Te2 lesioned rats showed increased freezing to CS presentation (Mann-Whitney test, T = 160,0, P < 0.05) (Fig. S5H). Oc2L-lesioned rats froze similarly to naïve animals (Mann-Whitney test, T = 97,0, P > 0.05) (Fig. S5I). Posterior piriform lesioned animals showed increased freezing compared to naïve animals (Mann-Whitney test, T = 231,0, P < 0.05) (Fig. S5J). The results might indicate that there was a partial memory saving in animals lesioned in Te2 or posterior piriform cortex. However, since rats were retrained in the same apparatus in which they were originally conditioned, the presence of contextual cues may be responsible for the observed freezing. To better clarify this issue, we examined contextual freezing during the two minutes that precede CS administration in naïve and lesioned animals. In comparison with naïve animals, Student's t-test revealed increased freezing in animals lesioned in Te2 (t_{27} = - 6,30, P < 0.05) (Fig. S5H) or in posterior piriform cortex (t_{23} = - 3,46, P < 0.05) (Fig. S5J). Such a contextual freezing may be at least in part responsible for freezing observed during the subsequent CS presentation.

3) Freezing analysis in ZIP-injected animals

Recent fear memories. The total time freezing displayed by rats that received ZIP into Te2 cortex one day after conditioning is shown in fig. S6A. Student's t-test indicated no difference between ZIP-injected and control rats (t_{14} = - 0,35, P > 0.05). Similarly, there were no differences between Oc2L-injected and control animals (t_{12} = 0,76, P > 0.05) (fig. S6B) and between posterior piriform cortex-injected rats and control subjects (t_{12} = 1,16, P > 0.05) (fig. S6C).

Remote fear memories. Animals that received ZIP into Te2 cortex one month after training showed a significant decrease in cumulated freezing both two days and two weeks after injection (Fig. S6D). A mixed-design ANOVA test revealed differences between groups ($F_{(1,19)}$ = 21,96, P < 0.05) but not between trials ($F_{(1,19)}$ = 3,66, P > 0.05). The group x trial interaction was not significant ($F_{(1,19)}$ = 0,89, P > 0.05). Rats that received ZIP into Oc2L cortex froze significantly less than the control ones at both time intervals ($F_{(1,14)}$ = 121,97, P < 0.05; group x trial interaction, $F_{(1,14)}$ = 15,32, P < 0.05) (Fig. S6E). Finally, ZIP administration into posterior piriform cortex elicited amnesia both two days and two weeks after injection ($F_{(1,14)}$ = 13,48, P < 0.05; group x trial interaction, $F_{(1,14)}$ = 5,37, P < 0.05) (Fig. S6F).

4) Freezing analysis in the modality-specificity experiments

Auditory fear memory. Total time spent freezing is provided in fig. S6G. One-way ANOVA showed no differences between groups ($F_{(2,25)}$ = 0,99, P > 0.05).

Visual fear memory. Fig. S6H depicts total time freezing in animals conditioned to visual CSs. One-way ANOVA revealed significant differences among groups ($F_{(2,24)}$ = 8,96, P < 0.05). Post-hoc Newman-Keuls test showed a significant difference between Te2-lesioned rats and the other groups (P < 0.05).

Olfactory fear memory. Fig. S6I shows cumulated freezing after conditioning to olfactory CSs. There were no differences among groups ($F_{(2,26)}$ = 0,97, P > 0.05).

5) Innate fear and anxiety analysis

Open field test. Exploratory and fear-related spontaneous behaviors were analyzed in Te2- (n = 8), Oc2L- (n = 10) and posterior piriform- (n = 10) lesioned animals and compared with those of naïve rats (n = 10). During 5 min of free exploration in the open field apparatus, the numbers of squares crossed by the subjects in the centre did not differ among the lesioned- and the control rats, as indicated by the one-way ANOVA test ($F_{(3,34)}$ = 0,308, P > 0.05) (Fig. S8A). The time spent in the centre of the apparatus was also similar among groups ($F_{(3,34)}$ = 1,069, P > 0.05) (Fig. S8B). We also measured two additional spontaneous behaviors that animals displayed during the exploration of a new environment, e.g. rearing and leaning behaviors. Again, there were no difference among groups in the number of rearing ($F_{(3,34)}$ = 1,022, P > 0.05) and of leaning ($F_{(3,34)}$ = 0,465, P > 0.05) instances (Fig. S8C, D).

Elevated plus maze test. Five days after the open field analysis, the animals were submitted to an additional paradigm aimed at studying spontaneous fear-related and exploratory behaviors, i.e. the elevated plus maze test. The one-way ANOVA test showed no difference among the lesioned and the naïve rats for all the parameters analyzed, i.e. the number of crossing in the open arms ($F_{(3,34)}$ = 0,76, P > 0.05) and in the closed arms ($F_{(3,34)}$ = 0,528, P > 0.05) (Fig. S8E), the time spent in the open arms ($F_{(3,34)}$ = 0,244, P > 0.05) (Fig. S8F), the number of rearing ($F_{(3,34)}$ = 2,293, P > 0.05) (Fig. S8G) and of leaning ($F_{(3,34)}$ = 1,804, P > 0.05) (Fig. S8H**)**.

Unconditioned fear responses to a predator odor. Several studies demonstrated that the 2,4,5 trimethylthiazoline (TMT), a synthetic predator odor originally extracted from fox feces induces species-specific unconditioned fear responses in naive rats. Therefore, we employed TMT in order to test if cortical lesions impair unconditioned fear responses towards a specific sensory stimulus. Since Wistar rats are less sensitive to TMT, we employed Sprague-Dawley rats (*S6*). Two different unconditioned fear-related behaviors (i.e., freezing and avoidance) were studied before and during TMT presentation in naïve animals (n = 9), in Te2- (n = 7), Oc2L- (n = 8) and posterior piriform- (n = 8) lesioned rats. A mixed-design ANOVA revealed significant differences in the freezing response before and during TMT presentation ($F_{(1,28)}$ = 129,8, P < 0.05) but not between groups ($F_{(3,28)}$ = 0,55, P > 0.05) and a non-significant group x trial interaction ($F_{(3,28)}$ = 0,20, P > 0.05), thus indicating that TMT elicits a significant freezing response in all tested animals (Fig. S8I). Finally, all groups showed a similar avoidance of TMT ($F_{(3,28)}$ = 180,6, P > 0.05) in respect to the non-odor presentation ($F_{(1,28)}$ = 1,80, P < 0.05) (Fig. S8J).

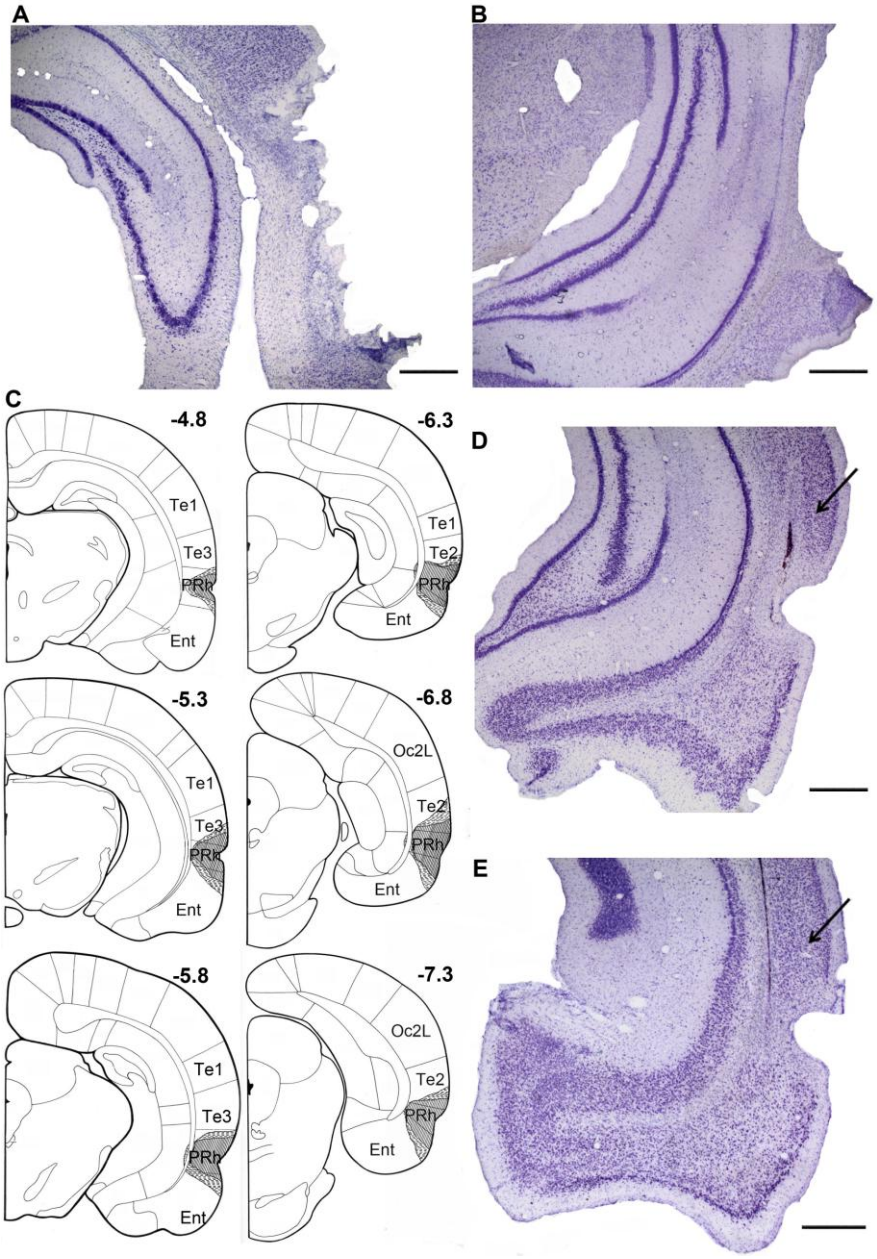

Fig. S1. Lesions of Te1 cortex and of posterior perirhinal cortex.

(**A-B**) Photomicrographs of Nissl-staining of Te1 lesion (2x magnification). Scale bars, 500 μm.
(**C**) Histological reconstructions of the smallest (*black*) and largest (*hatched*) excitotoxic lesions aimed at the posterior perirhinal cortex below Te2 cortex. Negative numbers indicate posterior distance from bregma. Plates adapted from the atlas of Zilles (*S2*).
(**D-E**) Photomicrographs of posterior perirhinal cortex lesions with relative sparing of Te2 cortex (solid arrows). Scale bars, 500 μm.

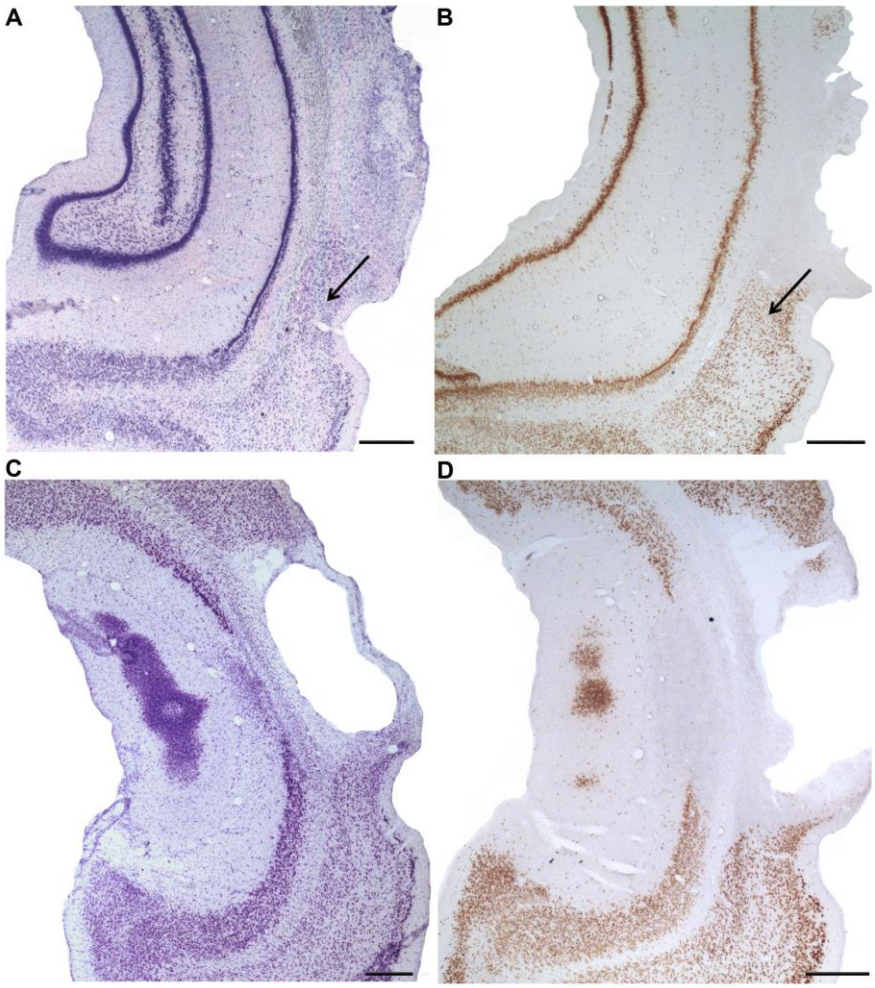

Fig. S2. Lesions of Te2 cortex.

(**A**, **C**) Representative photomicrographs of Nissl-staining of Te2 lesion (2x magnification) with relative sparing of the adjacent perirhinal cortex (solid arrows). Scale bars, 500 μm.
(**B, D**) Coronal sections through Te2 cortex stained with NeuN antibody (2x magnification). Scale bars, 500 μm.

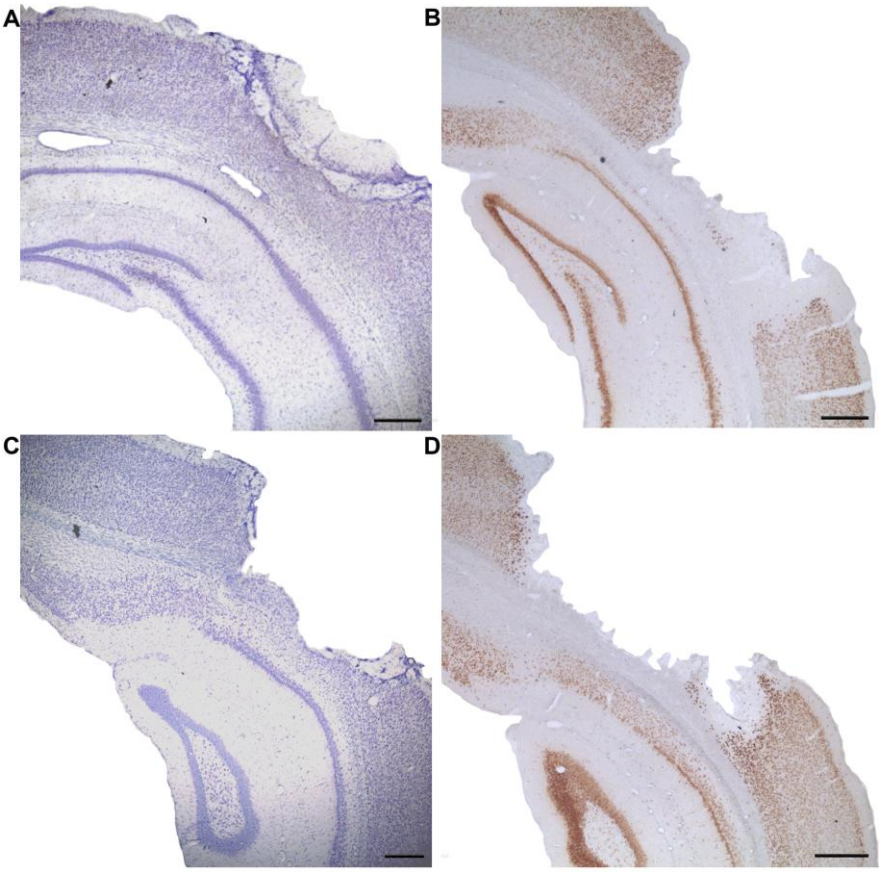

Fig. S3. Lesions of Oc2L cortex.

(**A, C**) Photomicrographs of Nissl-staining of Oc2L lesions (2x magnification), with relative sparing of the adjacent Te2 cortex. Scale bars, 500 μm.
(**B, D**) NeuN staining of Oc2L cortical damage. Scale bars, 500 μm.

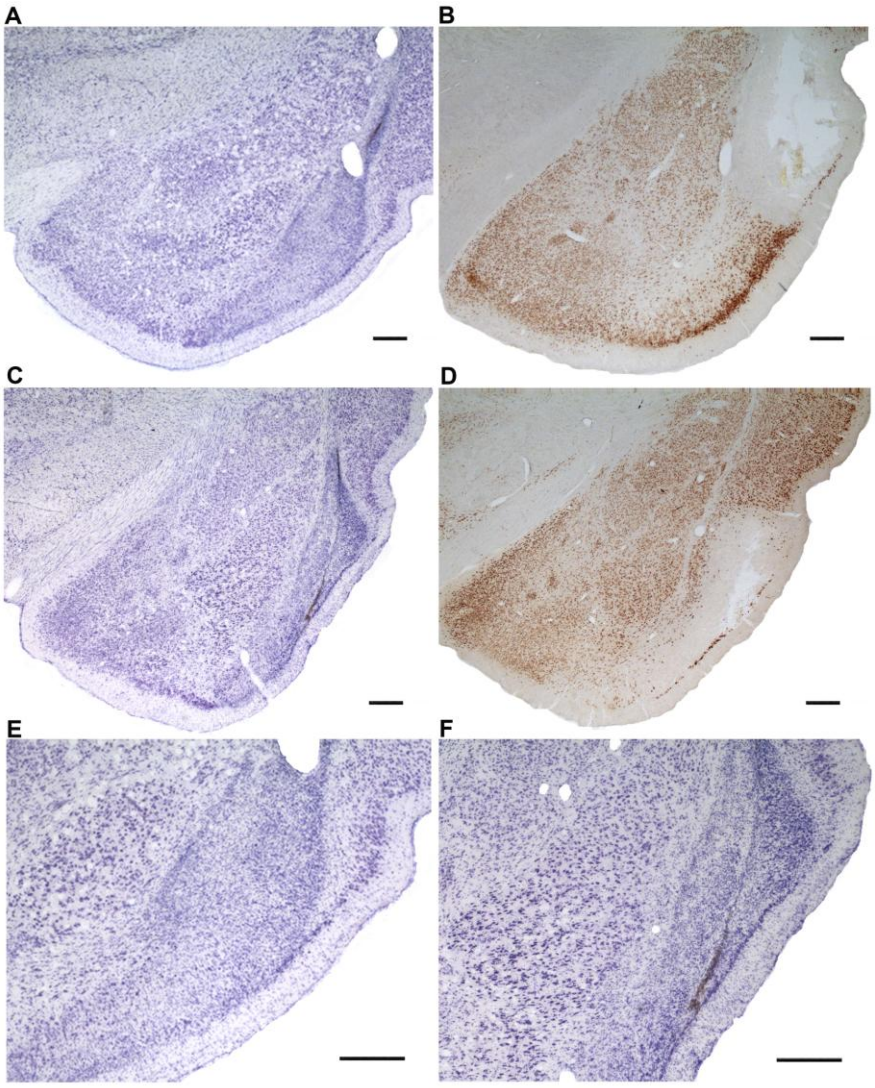

Fig. S4. Lesions of posterior piriform cortex.

(**A, C**) Representative photomicrographs of Nissl-staining of posterior piriform cortical lesion (2x magnification). Scale bars, 350 μm.
(**B, D**) Coronal sections through piriform cortex stained with NeuN antibody. Scale bars, 350 μm.
(**E-F**) Substantial cell loss in lesioned rats was apparent throughout the posterior piriform with no damage to the basolateral amygdala (4x magnification). Scale bars, 350 μm.

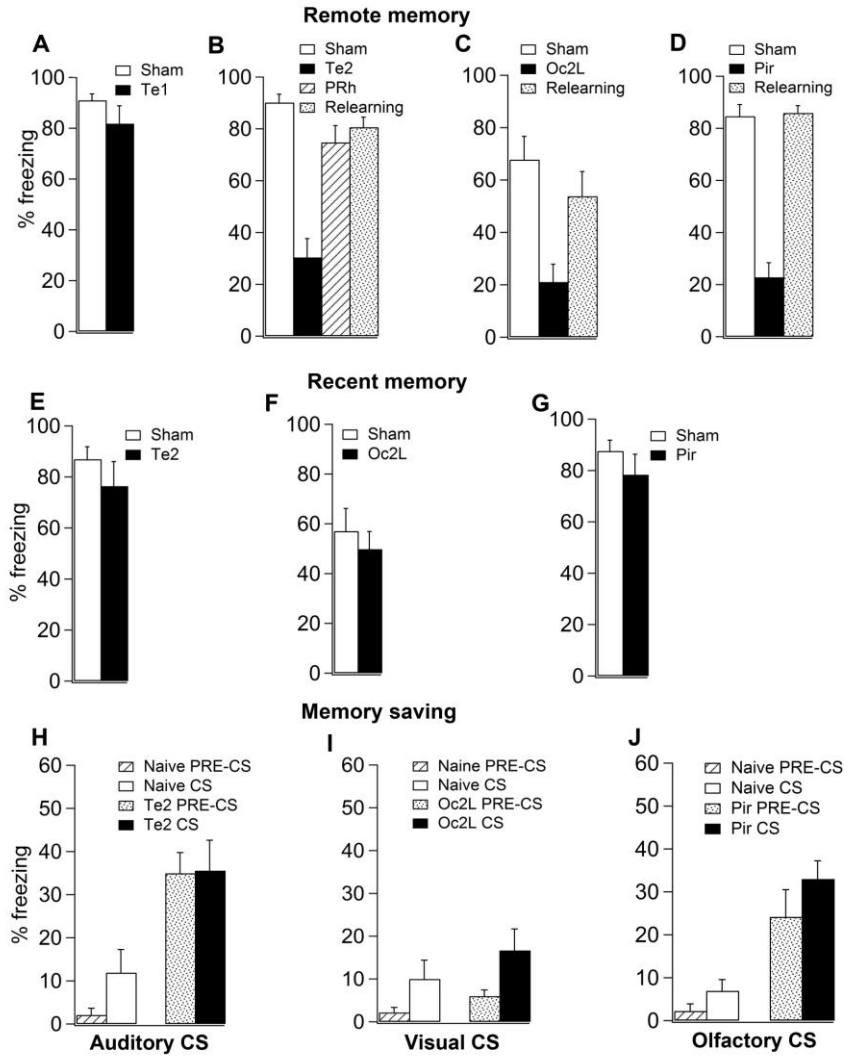

Fig. S5. Total time spent freezing in animals with cortical lesions.

(**A-D**) Remote fear memories were assessed by measuring total time freezing displayed during CSs presentation and during the time interval among CSs in rats lesioned in (**A**) primary auditory (Te1) cortex; (**B**) secondary (Te2) or posterior perirhinal (PRh) cortices; (**C**) secondary visual (Oc2L) cortex and (**D**) posterior piriform (Pir) cortex and in animals retrained.
(**E-G**) Recent fear memories. Total time freezing in animals lesioned in (**E**) Te2 cortex, (**F**) Oc2L cortex and (**G**) posterior piriform (Pir) cortex and in sham-operated rats.
(**H-J**) Memory saving. During the re-acquisition trial, we measured freezing during the two minutes that precede CS presentation (PRE-CS) and during the administration of the first CS (CS) in naïve animals and in those lesioned in (**H**) Te2 cortex, (**I**) Oc2L cortex and (**J**) posterior piriform (Pir) cortex. All values are mean ± SEM.

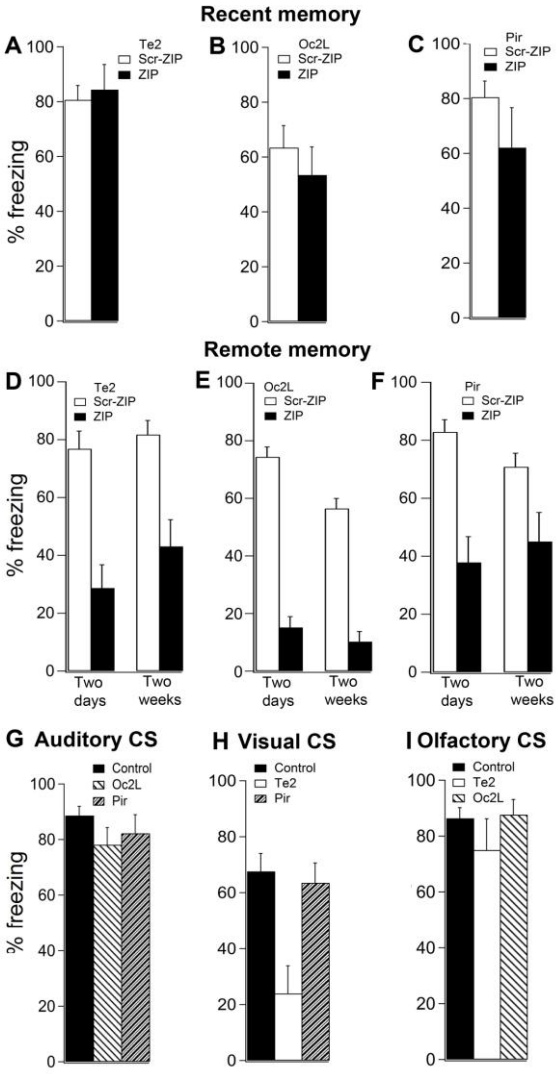

Fig. S6. Total time spent freezing in animals treated with the PKMzeta inhibitor ZIP and in the modality-specificity experiments.

(**A-C**) Recent fear memories. Freezing was measured for the entire retention trial (i.e., during CSs presentation and during the time interval among CSs) in animals that received ZIP or the scrambled inactive version of the peptide (src-ZIP) in (**A**) Te2 cortex, (**B**) Oc2L cortex and (**C**) posterior piriform (Pir) cortex.
(**D-F**) Remote fear memories. Cumulated freezing in rats that received ZIP in (**D**) Te2 cortex, (**E**) Oc2L cortex and (**F**) posterior piriform (Pir) cortex. Memory was tested two days and two weeks after the peptide injection.
(**G-I**) Total time spent freezing measured during (**G**) acoustic, (**H**) visual or (**I**) olfactory fear memory retention tests. All data points show mean ± SEM.

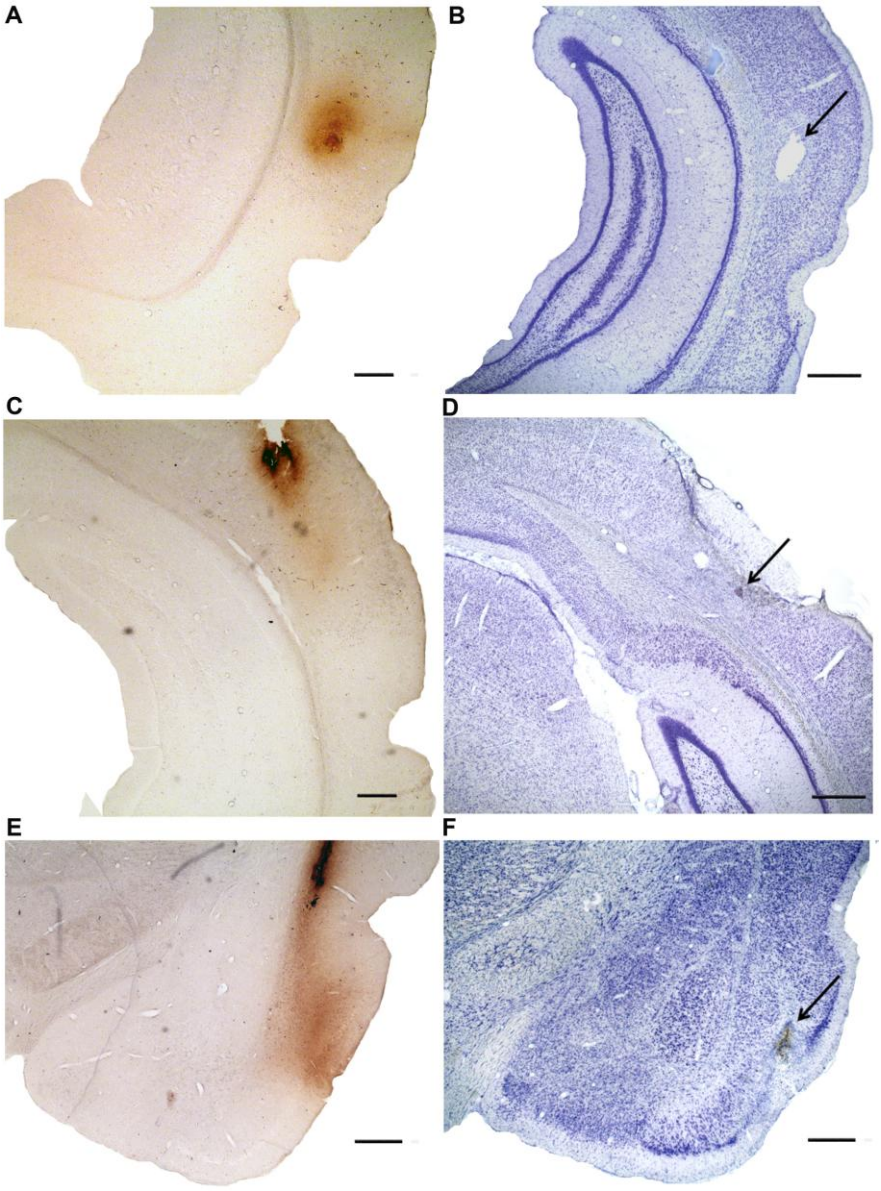

Fig. S7. Immunocytochemistry analysis of biotin-labeled ZIP diffusion.

(**A, C, E**) Immunocytochemistry performed 2 hours after injection of 10 nmol biotin-labeled ZIP shows the diffusion of the peptide (brown) in (**A**) Te2, (**C**) Oc2L and (**E**) posterior piriform cortex. Biotinylated ZIP diffusion is largely restricted to the target sites. Scale bars, 500 μm.
(**B, D, F**) Histological analysis carried out at the end of the behavioral experiments. Photomicrographs showed the position of the needle track (solid arrows) in (**B**) Te2, (**D**) Oc2L and (**F**) posterior piriform cortex. Scale bars, 500 μm.

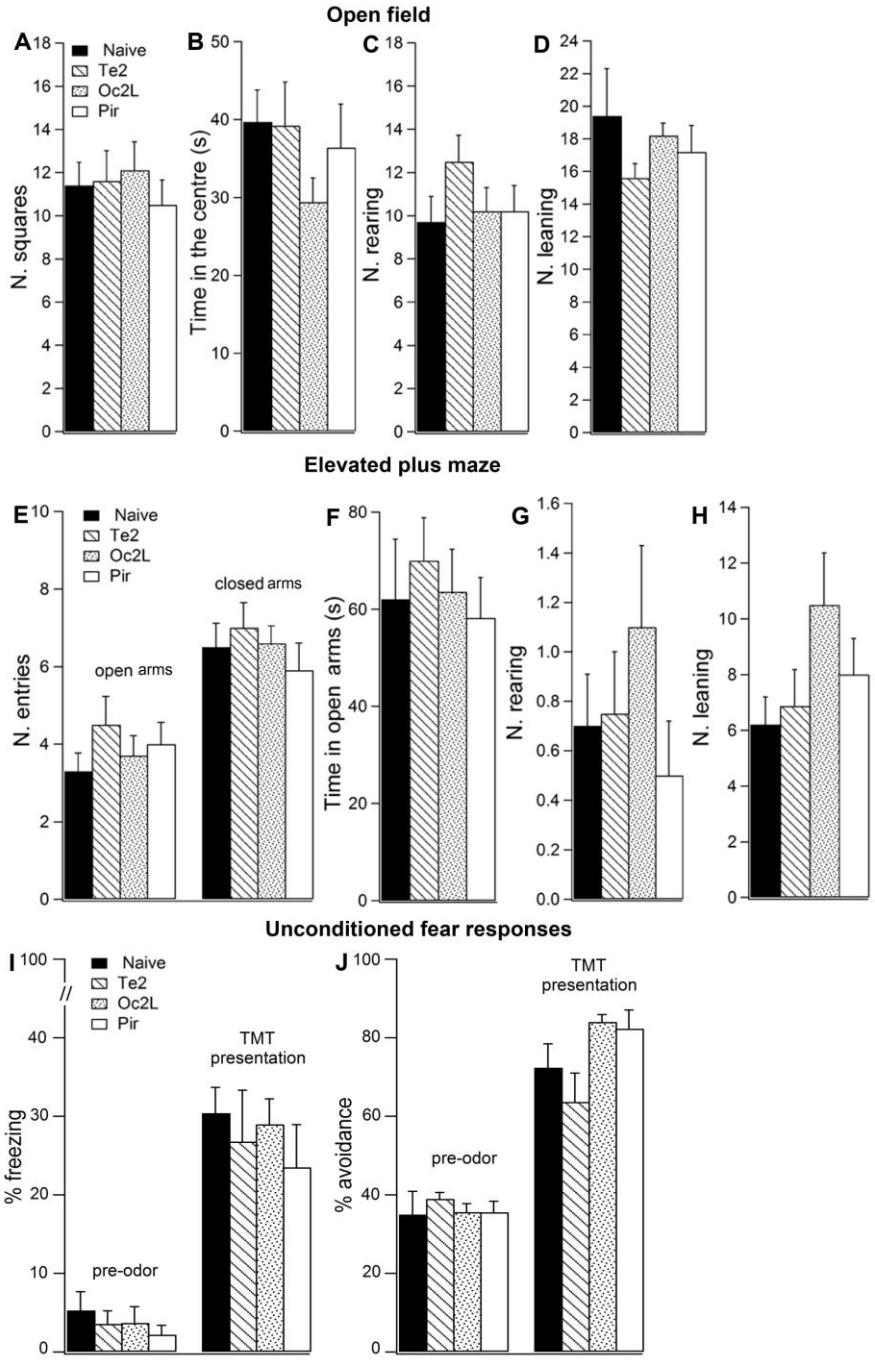

Fig. S8. Innate fear and anxiety.

(**A-D**) In the open field, we analyzed the number of square crossed and the time spent in the centre of the apparatus by naïve, Te2-, Oc2L- and posterior piriform-(Pir) lesioned animals as well as their rearing and leaning behaviors.
(**E-H**) In the elevated plus maze paradigm, we measured the number of entries in the open and closed arms, the time spent in the open arms, rearing and leaning behaviors.
(**I-J**) Freezing (**I**) and avoidance (**J**) were measured both before (pre-odor) and during the presentation of the synthetic fox odor 2,4,5-trimethylthiazoline (TMT). All values are mean ± SEM.

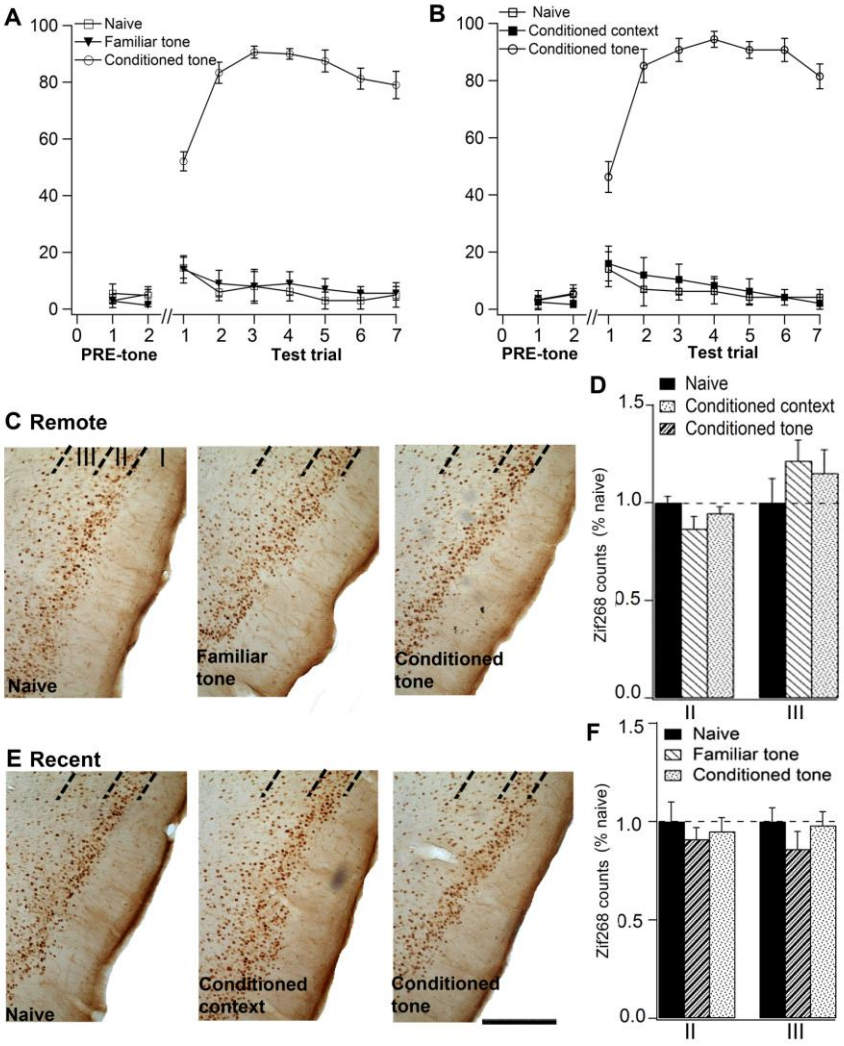

Fig. S9. Zif268 expression in the posterior piriform cortex after remote or recent fear memory test.

(A) Fear behavior was analyzed one month after training in three different groups of animals: "naïve", "familiar tone" and "conditioned tone". Freezing was measured both two min before (PRE-tone) and during (Test trial) the presentation of seven tones. Mixed ANOVA showed significant differences among groups ($F_{(2,41)}$ = 707,9, P < 0.05). Newman-Keuls test indicated that the "conditioned tone" group differed from the other two groups (P < 0.05).

(**B**) One day after training, freezing response was measured in "naïve", "conditioned context" and "conditioned tone" groups. Mixed ANOVA showed difference among groups ($F_{(2,22)}$ = 485,2 , P < 0.05). Newman-Keuls test revealed that "conditioned tone" group differed from the other two groups (P < 0.05). Note that fear behavior was measured in a totally different apparatus from that used for conditioning.

(C) Representative photomicrographs (4x magnification) within posterior piriform cortical layer I-III after remote memory test. (**D**) Zif268 counts are similar in the three behavioral groups in layer II ($F_{(2,41)}$ = 2,49, P > 0.05), and III ($F_{(2,41)}$ = 0,81, P > 0.05).

(E-F) There were no difference among the groups in layer II ($F_{(2,22)}$ = 0,32, P > 0.05), and III ($F_{(2,22)}$ = 0,40, P > 0.05) after recent memory test. Scale bars, 350 μm. All values are mean ± SEM.

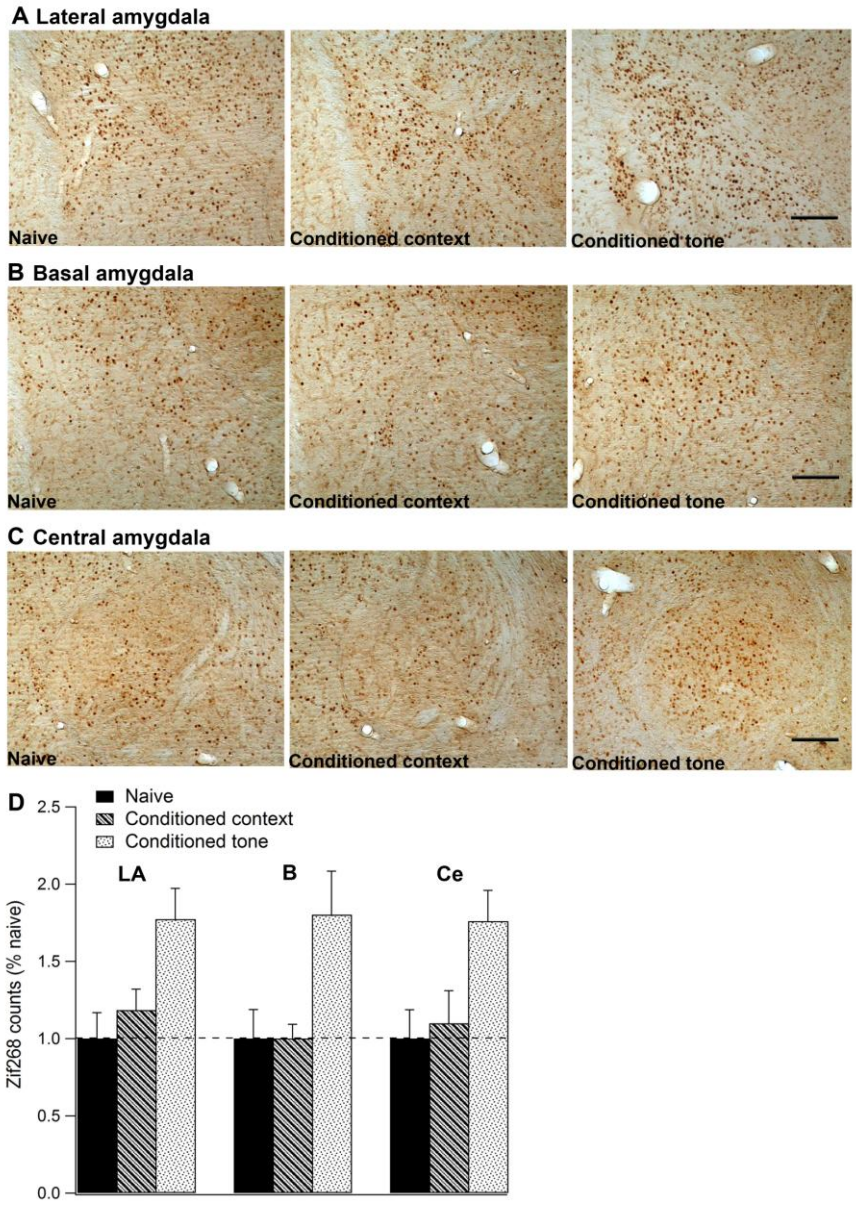

Fig. S10. Expression of zif268 protein in the amygdala after recent fear memory test.
(A-C) Representative photomicrographs (10x magnification) of zif268 expression within lateral (LA), basal (B) and central (Ce) amygdala regions after recent memory test. Scale bars, 130 μm.
(D) Lateral, basal and central amygdala activity was significantly increased after recent fear memory test mainly in the "conditioned tone" group. All data points show means ± SEM.

Supporting references

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