Expression of IGF-2 Receptor in the Auditory Cortex Improves the Precision of Recent Fear Memories and Maintains Detailed Remote Fear Memories Over Time

Title: Expression of IGF-2 receptor in the auditory cortex improves the precision of recent fear memories and maintains detailed remote fear memories over time.

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Abstract

Traumatic memories may become less precise over time and lead to the development of fear responses to novel stimuli, a process referred to as time-dependent fear generalization. The conditions that cause the growth of fear generalization over time are poorly understood. Here, we found that, in male rats, the level of discrimination at the early time point contributes to determining whether fear generalization will develop with the passage of time or not, suggesting a link between the precision of recent memory and the stability of remote engrams. We also found that the expression of insulin-like growth factor 2 receptor (IGF-2R) in layer 2/3 of the auditory cortex is linked to the precision of recent memories and to the stability of remote engrams and the development of fear generalization over time.

These findings provide new insights on the neural mechanisms that underlie the time-dependent development of fear generalization that may occur over time after a traumatic event.

Keywords

Auditory Cortex, Fear generalization, Insulin-growth Factor II, Insulin-growth Factor II Receptor, Learning and memory

Introduction

Although the original precision of some memories is maintained with the passage of time, most memories tend to become less precise, a phenomenon referred to as time-dependent generalization (Bergstrom 2016; Jasnow et al. 2017). Understanding the time-dependent nature of generalization related to threatening events is important not only for characterizing the mechanisms of long-term memory maintenance but also for studying of fear-related disorders, such as generalized phobia and PTSD, because generalized fear and avoidance after an incubation period are hallmarks of these disorders (Bergstrom 2016; Jasnow et al. 2017).

Many studies investigating the time-dependent generalization of fearful memories have focused on contextual learning, during which defensive responses spread to a novel unfamiliar context over time (Biedenkapp and Rudy 2007; Riccio and Joynes 2007; Wiltgen and Silva 2007; Wang et al. 2009; Wiltgen et al. 2010; Ruediger et al. 2011; Sauerhöfer et al. 2012; Bergstrom 2016; Poulos et al. 2016; Atucha et al. 2017; Jasnow et al. 2017; Guo et al. 2018). It has been proposed that time-dependent fear generalization of contextual memories reflects progressive loss of detailed information about the training environment (Rudy et al. 2005; Biedenkapp and Rudy 2007; Riccio and Joynes 2007; Wiltgen and Silva 2007; Wang et al. 2009; Wiltgen et al. 2010; Ruediger et al. 2011; Sauerhöfer et al. 2012; Bergstrom 2016; Poulos et al. 2016; Atucha et al. 2017; Jasnow et al. 2017) and progressive reorganization of memory traces in the hippocampal-cortical network (Biedenkapp and Rudy 2007; Riccio and Joynes 2007; Wiltgen and Silva 2007; Wiltgen et al. 2010; Atucha et al. 2017; Guo et al. 2018). A much smaller number of studies have investigated the time-dependent generalization of fear memories related to explicit sensory cues, such as auditory or visual stimuli. In 1979, Thomas and Riccio showed that rats exhibited fear-related responses to novel tones 21 days but not 1 day after

training (Thomas and Riccio 1979). More recently, it was shown that in fruit flies (König et al. 2017) and mice (Pamplona et al. 2011), the avoidance of an odor paired to a painful stimulus developed into generalized avoidance to new odors as the time between the training and testing increased. A similar result was obtained in mice conditioned to auditory cues (Pollack et al. 2018). Combined, these studies have led to the idea that fear generalization develops over time similarly in almost all animals.

All studies addressing the time-dependent generalization of contextual or cued fear memories have been performed by grouping animals without considering their individual responses to novel cues. However, recent works have shown that animals may display marked individual differences in fear responses to novel cues (Ciocchi et al. 2010; Likhtik et al. 2014; Concina et al. 2018; Grosso et al. 2018) and contexts (Wiltgen et al. 2010), with some animals showing fear-related responses to novel stimuli, indicating generalization of fear, and others responding more to the threatening stimulus than to novel ones, suggesting that they can discriminate among stimuli. Whether and how these individual differences might contribute to the onset of a time-dependent increase in fear generalization has not yet been tested.

Here, we addressed this issue by conditioning rats to a specific auditory cue (a pure tone with a frequency of 1 kHz, which acted as the conditioned stimulus (CS) and then presenting new tones of increasing frequency (7 kHz or 15 kHz) one week after learning. The animals were characterized as generalizers or discriminators on the basis of the behavior displayed in response to the CS and novel tones. Subsequently, the animals were presented similar tones at a more remote time point, i.e., 28 days after training. We found that fear generalization did not change in a similar way over time in generalizers and discriminators. Several animals that showed discrimination ability at a recent time point did not show any increase in fear generalization at a

remote time point. Conversely, in most animals that showed poorer discrimination ability at a recent time point, fear generalization grew significantly over time. We then found that insulin-like growth factor 2 receptor (IGF-2R) expressed in excitatory and inhibitory neurons in layer 2/3 of the auditory cortex acted as a cellular mechanism involved in both fear discrimination at a recent time point and long-term memory stability at remote points.

Materials and Methods

Animals

Male Wistar rats (age, 65-70 days; weight, 250-350g) were employed for all experiments. 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 o C°. All the experiments were approved by the Italian Ministry of Health (authorization no. 322/2015).

Behavioral procedures

Fear conditioning training

Rats were trained to associate a conditioned stimulus (CS) consisting in a1 kHz pure tone (80 dB, 8 s) with a painful unconditioned stimulus (US, 1 mA, 1 s), as in our previous studies (Sacco and Sacchetti 2010). The floor of the conditioning cage was made of stainless steel rods connected to a shock generator set to deliver 1 mA current. The chamber was equipped with a loudspeaker located 20 cm above the floor and connected to a tone generator set to deliver an 80

dB, 1 kHz pure tone (CS). Each animal was placed inside the chamber and left undisturbed for 2 min. Then, it was exposed to seven consecutive auditory CSs, each lasting 8 s and paired, during the last 1 s, with an electric foot shock; the auditory stimuli were separated by intervals of 22 s.

Presentation of new auditory stimuli and fear memory retention test

The presentation of novel auditory stimuli, namely, a 7 kHz tone (10 s, inter trial 40) or a 15 kHz tone (15 s, inter trial 36) and the retention of fear memory to the CS (1 kHz, 8s, 22s interval) were tested at one (recent memory) or four weeks (remote memory) after the fear conditioning procedure. Rats were handled for two consecutive days (5 min per day) and habituated to an apparatus different from that used for conditioning and placed in a different room in order to avoid conditioned fear behavior to contextual cues (Sacco and Sacchetti 2010; Concina et al. 2018). The new apparatus consisted in a transparent plastic cage enclosed within a sound-attenuating box equipped with an exhaust fan, which eliminated odorized air from the enclosure and provided background noise of 60 dB. On the third day, after two minutes of free exploration, we delivered three auditory stimuli (7 kHz or 15 kHz tones) never presented before. Two days later, animals were placed back in the same environment and exposed to three CS.

Behavioral analysis

Rats' behavior was recorded by a digital camera and the videos were analyzed to determine the duration of *freezing*. Freezing response was employed as an index of defensive behavior and expressed as the percentage of time during which there was complete absence of somatic mobility, except for respiratory movements. The assessment of freezing was done by one person blinded to the animals' assignment to an experimental group.

In order to distinguish better between generalizer and discriminator animals, we used a discrimination score (DS) obtained by dividing the percentage of freezing to CS and to the new tone employed (CS/New Tone). This procedure was employed in previous studies because it allows to identify and analyze separately the animals that discriminate against those that generalize (Ciocchi et al. 2010; Grosso et al. 2018; Likhtik et al. 2014) and to follow their behavior over time (Bergstrom 2016; Concina et al. 2018, Wiltgen et al. 2010).

Open field

Rats were tested in an open field apparatus, consisting in a plastic opaque box (50 × 50 × 50 cm). Animals were placed in the center of the apparatus at the beginning of the session. The behavior was recorded for 10 min by a digital camera. The travelled distance and the time spent by the rats in the center and periphery of the arena were analyzed offline using the Smart 3.0 software.

Surgical procedures

Recombinant mouse insulin growth factor-1 and 2 (IGF-1 and IGF-2, Tocris) were dissolved in PBS (10X) to obtain a final concentration of 25 ng/µl (Chen et al. 2011). Control rats were injected with a sterile saline solution (NaCl, 0,9%). Stereotaxic coordinates for injections were taken from the Paxinos and Watson atlas (2007). Injections were performed bilaterally at the following coordinates: Primary Auditory Cortex (Te1): AP = - 5.0, ML= ± 7.2, V= 4.2 (0.8 microliters for each side); Basolateral Amygdala (BLA): AP = -2.8, ML= ± 5.4, DV= 8.3 (0.6 microliters for each side) relative to the Bregma. A burr hole, permitting the penetration of a 28 gauge needle, was drilled over each injection site. The needle was connected to a 10 µl Hamilton syringe, connected to an infusion pump. The needle was left in place for an additional 3 min. The incision was then closed with stainless steel wound clips, and the animal was given a

subcutaneous injection of the analgesic/anti-inflammatory ketoprofen (2mg/kg body weight); it was kept warm and under observation until recovery from anesthesia. Needle track placement was verified in Nissl stained sections. The sections were histologically verified under a microscope magnified at 2x and 4x.

Immunohistochemistry

Animals were deeply anesthetized and intracardially perfused with 4% paraformaldehyde. Brains were cut with a cryostat in serial sections and immunohistochemical evaluation of IGF-2R expression was performed. Free-floating sections of Te1 and BLA cortex were pretreated with 0.3% H2O2 in PBS to reduce endogenous peroxidase activity. After several rinses, sections were then incubated in primary polyclonal rabbit anti IGF-2R (1:800 dilution, Thermofisher) antibody in the blocking solution overnight at room temperature. Subsequently, sections were washed with PBS and incubated for 1 h at room temperature with biotinylated goat anti-rabbit antibody (1:1000 dilution, Vector). The avidin-biotin peroxidase method (ABC complex, Vector, 1:100, 2 h of incubation) coupled to diaminobenzidine (0.03%, Sigma) was used to stain the sections. Sections were then rinsed in PBS and transferred to gelatin-coated slides, dehydrated and covered with a coverslip.

Immunofluorescence

In order to evaluate a putative colocalization of IGF-2R with excitatory or inhibitory neurons, we repeated IGF-2R staining in Te1 cortex in combination with antibodies against Parvalbumin (PV) (Sigma) or CamKII (Sigma). Free-floating sections, after several rinses, were incubated with rabbit anti IGF-2R (1:300 dilution) and respectively mouse anti PV (1:2000) or mouse anti CamkII (1:1000) antibodies in the blocking solution overnight at room temperature.

Subsequently, sections were washed with PBS and incubated for 1 h at room temperature with biotinylated goat anti-rabbit antibody (1:1000 dilution). The avidin-biotin complex (ABC complex 1:100, 2 h and half of incubation) was coupled to Texas Red Streptavidin (1:1000, 1 h and half, Vector) to stain IGF-2R. Alexa488 anti mouse (1:500, Jackson) were used to reveal the neuronal marker (1 h of incubation). Sections were then rinsed in PBS and transferred to gelatin-coated slides and covered with a coverslip.

Images acquisition and analysis

Images of Te1 sections with DAB staining were analyzed using Neurolucida software connected to a microscope via a color CCD camera (Sacco and Sacchetti 2010). Quantification of IGF-2R immunoreactivity was carried out at 20× magnification in a blind manner. IGF2R positive cells were counted for each slice in a fixed area (45000 microns) at the coordinates ranging the -3 to -7 anteroposterior axis (AP) of Te1 cortex and then subgrouped in a 3-5 AP interval and a 5-7 AP interval. Moreover, IGF-2R counts were performed from -2 to 3 AP in both lateral and basal regions of the amygdala (40000 microns area).

Images of Te1 sections stained with IGF-2R and different neuronal markers were acquired at a Zeiss confocal microscope at 40x magnification (230x170 microns area) using a 561 nm laser (IGF-2R) and a 488 nm laser (neuronal markers). Immunoreactive nuclei were counted as IGF-2R, PV (or CamKII) positive or double positive (IGF-2R+PV/ IGF-2R + CamKII). Percentage of double positive with respect to single markers were also computed for each slice. Mean counts for each animal obtained from all the slices were then compared.

Experimental design and statistical analysis

Experimental design (sample size, number of replicates, data exclusion) was performed according to laboratory experience and to previous published papers in the field. The effect size was measured through the partial Eta Squared (η^2) to address the power of the estimates. Statistical design and sample size are fully described in the result session and figure legends.

Shapiro-Wilk Normality test was used to determine whether the data were normally distributed.

When data were non-normally distributed, they were analyzed using a non parametric Mann-Whitney test in order to test the differences between two different groups. When data were normally distributed, we used a Student's two-tailed unpaired *t* test.

To address the between and within groups differences before and after IGF-1, IGF-2 or saline injections, we computed a 3×2 mixed-design ANOVA model with group (IGF-2, IGF-1 and saline) as between-subjects variable and condition (before and after injection) as within-subjects variable. A 2×2 mixed-design ANOVA was computed with the solely IGF-2 and saline groups. Where the group × condition interaction was significant, we performed a simple main effects analysis and we adjusted each *p* value with the Bonferroni correction. For each mixed ANOVA model we assessed the Sphericity assumption through Mauchly's Test of Sphericity.

In order to examine possible changes of behavioral groups over time, we computed a χ^2 test. Where one or more expected frequency values were under 5, we applied the Fisher's exact test.

In order to explore the relation between the amount of IGF-2R positive cells and the freezing behavior, we computed the Pearson's *r* correlation coefficient.

Data are presented as mean ± SEM. The null hypothesis was rejected at the $P < 0.05$ significance level. All statistical analyses were performed using SPSS Statistics 22 (IBM).

Results

The development of fear generalization over time depends on the physical features of the novel stimuli

We first investigated whether the individual variability in the evaluation of a new stimulus (i.e., fear discrimination or generalization) recently after fear learning influences the development of fear generalization at a remote time point. Rats were trained to associate a pure tone of a specific frequency (conditioned stimulus (CS), 1-kHz) with a painful unconditioned stimulus (US, a mild electric foot shock). We choose a non differential fear conditioning paradigm because it mimics real-life threatening experiences that occur without fine and prolonged discrimination training (Aizenberg et al. 2015; Resnik and Paz 2015; Concina et al. 2018; Grosso et al. 2018; Ito and Morozov 2019) and because this paradigm is similar to those employed in studies on fear generalization to contextual cues in which animals typically are not exposed to the novel context until testing (Biedenkapp and Rudy 2007; Wiltgen and Silva 2007; Wang et al. 2009; Wiltgen et al. 2010; Ruediger et al. 2011; Sauerhöfer et al. 2012; Poulos et al. 2016; Atucha et al. 2017; Guo et al. 2018). One week after training, we monitored the freezing response, an index of defensive behavior, to a neutral tone never perceived before. The new tone was a pure tone with a frequency of 15 kHz because in our previous study, this tone frequency was associated with discrimination ability in some rats and with generalization in others (Concina et al. 2018). The CS was presented two days later (**Fig. 1A**). As expected, some animals froze in response to the new tone as much as in response to the CS, indicating that they showed generalized fear to the new tone, and others froze much less in response to the new tone and thus display fear

discrimination (**Fig. 1B**). To classify the extent to which animals differentiated or generalized between the auditory stimuli, we defined a discrimination score (DS) obtained by dividing the percentage of time the animal froze in response to the CS by the percentage of time the animal froze in response to the new tone (CS/new tone). This procedure was employed in previous studies because it allows to identify and analyze separately the animals that discriminate against those that generalize (Ciocchi et al. 2010; Grosso et al. 2018; Likhtik et al. 2014) and to follow their behavior over time (Bergstrom 2016; Concina et al. 2018, Wiltgen et al. 2010). In our sample, animals that discriminated between stimuli froze in response to the new stimuli approximately < 50% less than in response to the CS. Therefore, we employed a DS of 2 to distinguish generalizer (G, DS <2) from discriminator (D, DS >2) animals. By applying this criterion, we found that among 13 rats, ~69% were discriminators, while ~31% were generalizers (**Fig. 1C**).

We then investigated whether and how the generalization of the 15-kHz tone may change over time. In another group of animals (n = 14), a new tone with a frequency of 15 kHz was presented at a remote time point, i.e., four weeks after training (**Fig. 1D**). Of 14 animals, 10 exhibited generalized fear (~71%), while only 4 displayed discrimination ability (~29%) (**Fig. 1E,F**). These percentages differed significantly from those obtained one week after training (**Fig. 1F**, χ^2 =4.46, p=0.03), thereby showing a development of fear generalization with the passage of time, in line with previous studies on cued (Pamplona et al. 2011; König et al. 2017; Pollack et al. 2018) and contextual (Biedenkapp and Rudy 2007; Riccio and Joynes 2007; Wiltgen and Silva 2007; Wang et al. 2009; Wiltgen et al. 2010; Ruediger et al. 2011; Sauerhöfer et al. 2012; Poulos et al. 2016; Atucha et al. 2017; Jasnow et al. 2017; Guo et al. 2018) aversive memories.

To rule out any artificial differences that may have been introduced by dividing the animals in groups, we performed an additional analysis by plotting freezing data to the new tone for all animals (discriminators and generalizers) one week after training and comparing it with freezing data to the same tone for all animals tested four weeks after training. There was an increase in freezing at the remote time point compared to the recent time point (Mann-Whitney test, U=42, p=0.016). Importantly, the strength of the response to the CS was similar at the two time points (Mann-Whitney test, U=79.50, p=0.58) (**Fig. 1G**).

Fear generalization depends on the physical features of the new stimulus, with the generalization curve shifted downward as the sensory features of the new stimulus become more different from those of the CS (Onat and Büchel 2015; Resnik and Paz 2015; Bergstrom 2016; Grosso et al. 2018; Pollack et al. 2018). We therefore investigated fear generalization in animals presented with a new tone with frequency that was much similar to that of the CS (i.e., a pure tone with a frequency of 7-kHz). For one group of rats (n = 13), the CS (1-kHz tone) was paired with the US, and the rats were presented with the new 7-kHz tone one week after training (**Fig. 1H**). Some animals displayed discrimination ability (~31%), and the others displayed generalized fear (~69%) (**Fig. 1I,J**). Another group of animals (n=13) was then presented the 7-kHz tone four weeks after training (**Fig. 1K**). Unexpectedly and in marked contrast with the data obtained for the 15 kHz tone, the percentages of rats that showed fear discrimination (~23 %) and generalization (~77 %) four weeks after training were similar to those that showed discrimination and generalization one week after training (χ^2=0.19, p=1.000) (**Fig. 1L,M**). We then analyzed freezing in response to the 7-kHz tone for all animals at recent and remote time points, and we found no significant differences between freezing at the two time points (Mann-

Whitney test, U=58, p=0.18). There was also no difference in freezing in response to the CS (Mann-Whitney test, U=73.50, p=0.58) (**Fig. 1N**).

Taken together, these results indicate that the development of fear generalization over time does not occur under all conditions but rather depends on the physical properties of the new stimulus. For a tone with a frequency that is markedly different from that of the CS (i.e., the 15-kHz tone), the percentage of animals that display generalized fear to the new tone increases significantly over time. Conversely, if the frequency of the new tone is closer to that of the CS (as in the case of the 7-kHz tone), the percentage of animals that show discrimination ability and the percentage of animals that show generalization remain unchanged over time.

The development of fear generalization at remote time points depends on the precision of recent fear memories

Our findings raise two related questions: why did the occurrence of time-dependent fear generalization differ so markedly between groups presented with 7-kHz and 15-kHz tones? Additionally, why did the evaluation of the same sensory stimulus change over time for the 15-kHz, with the stimulus being interpreted as safe at the recent time point and dangerous at the remote time point?

To answer these questions, we reasoned that because the 15-kHz tone is much more different from the CS than the 7-kHz tone, some of the animals presented the 15-kHz tone at the recent time point may show discrimination ability for the 15-kHz tone but may generalize fear to the 7-kHz tone. To test this possibility, one week after training, we presented the 15-kHz new

tone followed by the 7-kHz tone four days later (**Fig. 2A**). We found that some animals that discriminated the 15-kHz tone indeed displayed fear generalization responses to the 7-kHz tone, while other animals discriminated between the 7 kHz tone and the CS (**Fig. 2B**). These data confirmed the existence of two subpopulations of animals within the group of discriminators of the 15-kHz tone. We then tested the dynamics of time-dependent fear generalization in these subpopulations by again presenting the 15-kHz tone four weeks after training. Critically, several animals that showed discrimination ability to the 7-kHz tone at the recent time point discriminated the 15-kHz tone at the late time point (18% at the recent time point versus 11% at the remote time point). Conversely, in most rats that showed discrimination ability for the 15-kHz tone but generalization for the 7-kHz tone at a recent time point, fear generalization develops over time and spreads to the 15-kHz tone at the late time point (36% at the recent time point versus 31% at the remote time point) (**Fig. 2B**). Statistically, the percentages of animals that generalized or discriminated the 15-kHz tone were significantly different between the recent time point and the remote time point (χ^2=14.39, p=0.0001), in line with our previous data. However, the percentages of animals that discriminated or generalized the 7-kHz tone at the recent time point did not differ significantly from those of animals that discriminated or generalized the 15-kHz tone at the remote time point (χ^2=0.08, p=0.77). These data showed that the development of fear generalization over time depends on the level of discrimination ability displayed by animals at recent time points. The majority of animals that are able to discriminate both the 7-kHz and the 15-kHz tones at the early time point continue to display fear discrimination at the late time point. Conversely, the majority of animals that discriminate the 15-kHz tone but not the 7-kHz tone at the early time point display development of fear generalization over time that results in a spread of fear to the 15-kHz tone at the late time point.

Several studies have shown that the progressive erosion of memory details over time may underlie the time-dependent development of fear generalization to contextual (Rudy et al. 2005; Biedenkapp and Rudy 2007; Riccio and Joynes 2007; Wiltgen and Silva 2007; Wiltgen et al. 2010; Ruediger et al. 2011; Sauerhöfer et al. 2012; Poulos et al. 2016; Atucha et al. 2017; Jasnow et al. 2017; Guo et al. 2018) and auditory (Pollack et al. 2018) stimuli at remote time points. Our data may therefore suggest that the precision of recent memories that underlie fear discrimination at recent time points also contributes to the maintenance of detailed remote memories over time, thereby revealing a crucial link between the precision of recent memories and the stability of remote engrams.

An alternative, not mutually exclusive, interpretation of our results may rely on the fact that under certain conditions, the strength of aversive memories can intensify over time, a phenomenon termed 'fear incubation'(Diven 1937; McAllister and McAllister 1967). Recent studies suggest that this phenomenon may also be involved in the genesis of the time-dependent generalization to novel stimuli (Pickens et al. 2009) and environments (Poulos et al. 2016). We therefore investigated whether with the passage of time, the strength of fear memories becomes stronger in generalizers than in discriminators, leading to the spread of fear to novel stimuli at remote time points. To this aim, we compared the freezing in response to the CS of rats that showed fear generalization to the 15-kHz tone at the remote time point and discrimination of the 15-kHz tone at a recent time point but generalization to the 7-kHz tone with that of rats that displayed discrimination of the 15-kHz tone at a remote time point (**Fig. 2C**). No differences were detected between the two groups (Mann-Whitney test, U=27, p=0.10). In addition, we analyzed freezing to the CS at the remote time point of generalizers and discriminators presented

with the 15-kHz stimulus in the previous experiment (**Fig. 2D** and see **Fig. 1E**). Again, there were no differences between the two groups (Mann-Whitney test, U=11.50, p=0.25).

In addition to being associated with memory-related processes, the time-dependent development of fear generalization has also been associated with increases in innate fear and anxiety (Eysenck 1968; McAllister and McAllister 2006; Wiltgen and Silva 2007). To verify whether the occurrence of time-dependent fear generalization is accompanied by an increase in innate fear and anxiety in generalizers of the 15-kHz tone, we analyzed the innate defensive behaviors of generalizer and discriminator rats at the late time point. Following memory tests, a subgroup of generalizers and discriminators was subjected to the open field paradigm, a task commonly employed to analyze animals' anxiety behavior. No differences in time spent or distance traveled in the periphery or center of the arena were detected between the two groups (distance traveled in the periphery: Mann-Whitney test, U=12, p=0.27; time spent in the periphery: Mann-Whitney test, U=18, p=0.76) (**Fig. 2E**).

To further uncover the differences in innate fear behavior between generalizer and discriminator animals, we analyzed the percentage of freezing displayed by the two groups before the presentation of the new tone at the remote time point. No differences were detected between groups (Mann-Whitney test, U=19.50, p=0.97, **Fig. 2F**).

These data showed that the development of fear generalization with the passage of time that we observed in several animals is not related to the strength of the memory or innate fear or anxiety but rather may be strongly influenced by the precision of recent memories.

The expression of insulin-growth factor type 2 receptor in layer 2/3 of the auditory cortex improves fear discrimination at a recent time point.

We then sought to determine the cellular mechanism(s) that may account for the difference between discriminator and generalizer animals at the recent time point. Recently, the insulin-like growth factor 2 (IGF-2), a polypeptide expressed in the brain that has high sequence similarity to insulin, has received attention as an important molecular mechanism for learning and memory processes (Chen et al. 2011; Alberini and Chen 2012; Schmeisser et al. 2012; Pascual-Lucas et al. 2014). In particular, Alberini and colleagues demonstrated that the expression of IGF-2 receptor (IGF-2R) is essential for the formation of several hippocampal-dependent memories and that hippocampal or systemic administration of the recombinant IGF2 significantly improved long-term memory retention and persistence (Chen et al. 2011; Alberini and Chen 2012; Pascual-Lucas et al. 2014). We therefore analyzed whether IGF-2 receptor (IGF-2R) may be involved also in fear discrimination process. To this aim, we analyzed the expression of IGF-2R in the animals that demonstrated fine discrimination ability at the recent time point, namely, discriminators of the 7-kHz tone, and compared it with that in generalizer rats (**Fig. 3A**). IGF-2R immunostaining was analyzed by an experimenter blinded to the experimental conditions in the primary auditory cortex (Te1) because this region has been implicated in fear discrimination processes at recent (Aizenberg and Geffen 2013; Aizenberg et al. 2015; Wigestrand et al. 2016) and remote (Concina et al. 2018) time points (**Fig. 3B**). Immunohistochemical analysis revealed that IGF-2R expression in layers 2/3 was higher in discriminators than in generalizers (unpaired t-test, $t_{(14)}$=4.84, p=0.0003), but not in layer 4 (unpaired t-test, $t_{(14)}$=1.25, p=0.23), layers 5 (unpaired t-test, $t_{(14)}$=1.18, p=0.25) or layer 6 (unpaired t-test, $t_{(14)}$=1.08, p=0.29) (**Fig. 3A**).

Next, we analyzed whether IGF-2R was uniformly distributed across the entire auditory cortex or whether it was more abundant in a specific subregion in discriminators. We divided the auditory cortex into an anterior subregion (ranging from 3 to 5 mm anteroposterior to bregma) and a more posterior subregion (from 5 to 7 mm anteroposterior to bregma). In layers 2/3 of the more anterior region, we detected no differences in IGF-2R expression between discriminators and generalizers (unpaired t-test, $t_{(14)}$=1.68, p=0.11); however, discriminators showed more abundant IGF-2R than generalizers in the more posterior region (unpaired t-test, $t_{(13)}$=4.71, p=0.0004). In the posterior area, no differences were detected in any other cortical layers (unpaired t-test, p> 0.05 in all instances, **Fig. 3C,D**).

The extent of freezing in response to the CS was similar between generalizers and discriminators (unpaired t-test, $t_{(13)}$=0.67, p=0.51) (**Fig. 3E**). Thus, the differential expression of IGF-2R between discriminators and generalizers may be related specifically to discriminative processes rather than to the strength of the fear memory. To confirm this idea, we plotted data related to the percentage of time generalizers and discriminators froze in response to the new tone or to the CS as a function of the expression of IGF-2R in layer 2/3 of the more posterior region of the auditory cortex. We found that a higher level of IGF-2R correlated significantly with less freezing to the new tone (Pearson's correlation, p=0.0005, **Fig. 3F**) but not with the percentage of freezing in response to the CS (Pearson's correlation, p=0.71, **Fig. 3G**). These data support the idea that the differential expression of IGF-2 receptor in generalizers and discriminators is not associated with differences in the strength of fear memories but rather with differences in fear discrimination.

We then sought to identify the neural identity of cells in which IGF-2R expression differed between the two groups. Previous studies have shown that neurons expressing the

calcium-binding albumin protein parvalbumin (PV) in the auditory cortex play a key role in auditory fear discrimination (Letzkus et al. 2011; Aizenberg et al. 2015; Park et al. 2020). We therefore analyzed the expression of IGF-2R in PV-expressing neurons in discriminators and generalizers. We found that the total number of PV/IGF-2R double-positive cells was greater in discriminator rats than in generalizers (unpaired t-test, $t_{(22)}$=2.34, p=0.02) and that there were more IGF-2R+ cells in discriminators than in generalizers (unpaired t-test, $t_{(22)}$=6.25, p<0.0001) and a similar number of total PV+ neurons between the two groups (unpaired t-test, $t_{(22)}$=1.75, p=0.09) (**Fig. 3H**). Then, we used the ratio of the total number of double-positive cells and the total number of IGF-2R+ or PV+ cells to evaluate the tendency of a certain type of cell (PV+ or IGF-2R+) to coexpress the other marker regardless of the total number of these cells. The ratio of the total number of double-positive cells to the total number of PV+ cell was greater in discriminators than in generalizers (unpaired t-test, $t_{(22)}$=2.17, p=0.04) (**Fig. 3I,J**).

We performed a similar analysis by co-immunolabeling for IGF-2R and calcium-calmodulin kinase II (CamKII), a calcium-calmodulin-dependent kinase specifically expressed in excitatory neurons. A higher total number of IGF-2R-positive neurons in discriminators than in generalizers (unpaired t-test, $t_{(22)}$=7.59, p<0.0001) was accompanied by a greater number of total CamKII-positive cells (unpaired t-test, $t_{(22)}$=5.63, p<0.0001) and IGF-2R/CamkII double-positive neurons (unpaired t-test, $t_{(22)}$=6.04, p<0.0001). The percentage of double-positive cells relative to the total number of CamKII-positive cells was significantly higher in discriminators than in generalizers (unpaired t-test, $t_{(22)}$=2.17, p=0.04) (**Fig. 3K-M**).

These results showed that the difference between discriminator and generalizer rats correlated with the total proportion of PV-expressing and CamKII-expressing neurons that express IGF-2R. These data led to the hypothesis that an improvement in the IGF-2R activity

within the auditory cortex may improve fear discrimination. To test this idea, we evaluated whether the administration of the exogenous recombinant IGF-2 in this area improves fear discrimination in generalizer animals. As in the above experiments, one week after training, animals were presented a new tone with a frequency of 7 kHz and two days later with the CS to classify rats as generalizers or discriminators. Immediately after CS presentation, the generalizer rats received injection, into the auditory cortex, of saline or the recombinant IGF-2 at a dose of 25 ng/µl, which previous studies have shown to be able to enhance hippocampal-dependent memories (Chen et al. 2011) (**Fig. 4A**). Four days later, the animals were again presented with the 7-kHz tone and after two days with the CS. Moreover, because exogenous IGF-2 activates both IGF-1 and IGF-2 receptors but binds these receptors with different affinities (Nissley and Rechler 1984), we included an additional experimental group to which we administered exogenous IGF-1 (25 ng/µl) (Chen et al. 2011) instead of IGF-2 to determine whether the effects of IGF-2 on fear discrimination may be due to specific activation of IGF-2 and/or IGF-1 receptors (**Fig. 4A**). A 3 × 2 mixed-design ANOVA (main effect of group: $F_{(2,37)} = 4.63$, $p = 0.016$, main effect of condition: $F_{(1,37)} = 11.39$, $p = 0.002$, group × condition interaction $F_{(2,37)} = 4.72$, $p = 0.015$) showed that the freezing to the 7 kHz tone was similar among groups before different injections (IGF-2 vs saline, $p = 1.000$; IGF-2 vs IGF-1, $p = 0.258$; IGF-1 vs saline, $p = 0.114$) while it was different after IGF-2 administration (IGF-2 vs saline, $p = 0.048$; IGF-2 vs IGF-1, $p = 0.005$; IGF-1 vs saline, $p = 0.856$); Simple main effect within group (pre- vs post-injection): IGF-2, $p < 0.001$; IGF-1, $p = 0.182$; saline, $p = 0.856$ (**Fig. 4B**).

Critically, IGF-2-injected rats showed a similar extent of freezing to the CS as animals that received IGF-1 or saline (3 × 2 mixed-design ANOVA, main effect of group: $F_{(2,37)} = 2.78$, $p = 0.075$; main effect of condition: $F_{1,37)} = 0.061$, $p = 0.806$; group × condition interaction: $F_{(2,37)}$

= 0.87, $p = 0.426$). The absence of a difference in freezing to the CS cannot be attributed to a ceiling effect because the extent of freezing in all groups was not at the maximum level (see **Fig. 4C**). Thus, the activation of IGF-2R in the auditory cortex improves fear discrimination selectively without changing the strength of recent fear memories. These results are in line with our findings that IGF-2R expression in the auditory cortex is strictly correlated with auditory fear discrimination but not with memory strength.

Our data showed that the recombinant IGF-2 improved fear discrimination at the recent time point in generalizer animals. We then tested whether the improvement of discrimination ability observed at the recent time point can persist to a more distant time point. We administered exogenous IGF-2 or IGF-1 into the auditory cortex of animals that exhibited generalized fear for the 7-kHz tone at the recent time point and then we tested fear generalization to the same tone at the remote time point (**Fig. 4D**). A 3 × 2 mixed-design ANOVA showed significant differences among groups (main effect of group: $F_{(2,43)} = 4.11$, $p = 0.023$, main effect of condition: $F_{(1,43)} = 17.44$, $p < 0.001$, group × condition interaction $F_{(2,43)} = 5.83$, $p = 0.006$) (**Fig. 4E**). In particular, freezing was similar among groups before injections ($p = 1.00$ in all instances) while diminished after IGF-2 injections as compared to other two groups (Simple main effect between groups: IGF-2 vs saline, $p = 0.039$; IGF-2 vs IGF-1, $p = 0.003$; IGF-1 vs saline, $p = 0.725$; Simple main effect within group (pre- vs post-injection): IGF-2, $p < 0.001$; IGF-1, $p = 0.684$, saline, $p = 0.073$). Freezing to the CS was similar among groups (3 × 2 mixed-design ANOVA, main effect of group: $F_{(2,43)} = 0.047$, $p = 0.954$, main effect of condition: $F_{(1,43)} = 19.74$, $p < 0.001$, group × condition interaction $F_{(2,43)} = 0.11$, $p = 0.895$) (**Fig. 4F**).

Combined, these data established an important link between the activity of IGF-2R in the auditory cortex and fear discrimination processes.

IGF-2 receptors in the basolateral amygdala are not involved in fear discrimination.

In addition to the auditory cortex, the amygdala, particularly the lateral and basal nuclei, is a brain region essential for auditory fear discrimination (Genud-Gabai et al. 2013; Ghosh and Chattarji 2015; Rajbhandari et al. 2016; Grosso et al. 2018). Hence, in some animals in which we analyzed the expression of IGF-2R in the auditory cortex, we quantified the expression of IGF-2R in the lateral and basal nuclei of the amygdala. No differences were detected between discriminators and generalizers in the either lateral (unpaired t-test, $t_{(10)}$=0.006, p=0.99) or basal (unpaired t-test, $t_{(10)}$=0.44, p=0.66) nucleus of the amygdala (**Fig. 5A,B**).

We also tested whether the administration of the exogenous recombinant IGF-2 directly into the basolateral amygdala improves fear discrimination (**Fig. 5C**). In line with our confocal analysis, we found no effects of this treatment on fear discrimination at the recent (**Fig. 5D,E**) (2 × 2 mixed-design ANOVA, main effect of group: $F_{(1,20)} = 0.43$, $p = 0.518$, main effect of condition: $F_{(1,20)} = 0.077$, $p = 0.784$, group × condition interaction $F_{(1,20)} = 0.49$, $p = 0.492$), or at the remote time points (**Fig. 5F-H**) (2 × 2 mixed-design ANOVA, main effect of group: $F_{(1,23)} = 1.31$, $p = 0.263$, main effect of condition: $F_{(1,23)} = 0.004$, $p = 0.948$, group × condition interaction: $F_{(1,23)} = 1.50$, $p = 0.232$).

IGF-2 receptors in the auditory cortex participate in the maintenance of the precision of remote fear engrams.

Our data identified IGF-2R in the auditory cortex as a cellular mechanism involved in fear discrimination at recent time points. Furthermore, we found that discrimination ability at the early time point governs the occurrence of fear generalization over time, suggesting that memories that are precise at the recent time point may be refractory to the loss of details over time. We therefore sought to determine whether IGF-2R may also be involved in the maintenance of detailed fear memories over time and therefore whether IGF-2R may be one of the cellular mechanisms that link memory precision at a recent time point to the stability of remote engrams.

To this aim, we first quantified IGF-2R expression in the auditory cortex of animals that showed a time-dependent increase in fear generalization. Because our previous experiment showed that fear generalization develops with the passage of time mostly in animals that display discrimination ability to a 15-kHz tone but generalize the 7-kHz tone at a recent time point, we compared IGF-2R expression of this group with that of rats showing generalization to 15-kHz tone at remote points. Critically, analysis of IGF-2R expression revealed a marked decrease in the expression of this receptor in animals that showed the development of fear generalization at the remote time point (unpaired t-test, $t_{(16)}=2.82$, $p=0.01$) (**Fig. 6A,B**).

We then investigated the neuronal populations in which IGF-2R expression decreased significantly over time. We analyzed the expression of IGF-2R in PV neurons and found that the total number of PV/IGF-2R double-positive cells was greater in animals tested at recent time points than in animals that showed fear generalization to the 15-kHz tone at remote time points (unpaired t-test, $t_{(16)}=2.23$, $p=0.04$) but that the total number of PV+ neurons was similar between these animals (unpaired t-test, $t_{(16)}=0.18$, $p=0.85$) (**Fig. 6C,E**). The total number of double-positive cells relative to the total number of PV+ cells was greater in the group of animals

that displayed discrimination of the 15-kHz tone at recent time points (unpaired t-test, $t_{(16)}$=2.54, p=0.02) (**Fig. 6C,E**).

We repeated the same experiment but by immunostaining for calcium-calmodulin kinase II (CamKII), and we found a greater total number of CamKII/IGF-2R double-positive cells (unpaired t-test, $t_{(16)}$=2.57, p=0.02) and a greater number of double-positive cells relative to the total number of CamKII+ cells (unpaired t-test, $t_{(16)}$=3.40, p=0.003) in the group of animals that discriminated the 15-kHz tone at the recent time point than in the animals that did not show discrimination ability. On the other hand, there was a similar number of total CamKII+ neurons between these groups (unpaired t-test, $t_{(16)}$=0.70, p=0.49) (**Fig. 6D,F**).

These data showed that the development of fear generalization with the passage of time is associated with a significant decrease in the expression of IGF-2R in both PV- and CamKII-expressing neurons in the auditory cortex, suggesting that the expression of IGF-2R in the auditory cortex may be involved in the genesis of time-dependent fear generalization and that a potentiation of IGF-2R activity would decrease the development of fear generalization over time. To test this idea, we injected the exogenous recombinant IGF-2 (25 ng/µl) (Chen et al. 2011) into the auditory cortex of animals that displayed fear generalization to the 7-kHz tone but discriminated the 15-kHz tone at the recent time interval. We then again presented the 15-kHz tone four weeks after training (**Fig. 6G**) and we compared the freezing displayed by these animals to that of animals tested in the previous experiments (see **Fig. 2**). We found that IGF-2 injection decreased freezing to the 15 kHz tone at remote time point (2 × 2 mixed-design ANOVA, main effect of group: $F_{(1,31)}$ =2.81, p =0.103; main effect of condition: $F_{(1,31)}$ = 64.24, p =0.0001; group × condition interaction: $F_{(1,31)}$ =4.95, p=0.033. Simple main effects between groups showed a difference after (IGF-2 vs control, p = 0.039) but not before injection

(IGF-2 vs control, $p = 0.934$, **Fig. 6H**). Finally, the strength of remote fear memory to the CS remained unaffected in IGF-2 injected rats ($t_{(31)}=0.71$, $p=0.478$; data not shown).

Taken together, these results revealed that with the passage of time, the expression of IGF-2R in the auditory cortex decreased significantly in animals showing an increase in fear generalization. Critically, the stimulation of IGF-2R activity through the administration of the exogenous IGF-2 directly into the auditory cortex decreased remote fear generalization. Combined with the previously described experiments, our data suggest that IGF-2R in the auditory cortex is involved in fear discrimination early after training and in the maintenance of the precision of remote fear engrams over time. Hence, IGF-2R may be a key substrate for linking memory precision to the maintenance of remote engrams over time.

Discussion

In the present work, we investigated the dynamics of fear generalization at recent and remote time points by analyzing the individual variability of animals in responding to two different tonal stimuli (7 and 15 kHz). A major finding of our study was that fear generalization did not develop uniformly with the passage of time. Several animals that effectively discriminated both tones early after learning did not display a significant increase in fear generalization over time. Conversely, animals that discriminated the 15-kHz tone but not the 7-kHz tone early after training displayed a significant broadening of generalization of fear over time, as evidenced by the higher amount of freezing displayed in response to the 15-kHz tone at the remote time point. These findings go beyond the traditional idea that fear generalization grows with the passage of time similarly in almost all animals but rather suggest that the precision of recent fear memories governs the stability of remote engrams and the development of fear generalization over time.

These results were obtained by analyzing the individual variability of animals' behavior in the presence of novel tones. Recent studies have shown that hours and days after fear learning, animals display marked different responses to new stimuli that allow them to be classified as generalizers or discriminators (Ciocchi et al. 2010; Wiltgen et al. 2010; Likhtik et al. 2014; Concina et al. 2018; Grosso et al. 2018). Strikingly, however, all studies on time-dependent fear

generalization thus far have been performed by grouping animals without considering their individual responses to novel cues. Furthermore, thus far, a very limited number of studies have addressed the issue of the time-dependent nature of fear generalization to auditory (Thomas and Riccio 1979; Pollack et al. 2018) or other sensory stimuli (Pamplona et al. 2011; König et al. 2017) paired to aversive events (Bergstrom 2016). The large majority of studies on the development of fear generalization over time have in fact been performed by conditioning animals to a training context (Biedenkapp and Rudy 2007; Riccio and Joynes 2007; Wiltgen and Silva 2007; Wang et al. 2009; Wiltgen et al. 2010; Ruediger et al. 2011; Sauerhöfer et al. 2012; Poulos et al. 2016; Atucha et al. 2017; Jasnow et al. 2017; Guo et al. 2018). If and how individual variability in discrimination or generalization of contextual cues at a recent time point governs the development of contextual fear generalization over time is unknown and should therefore be investigated in future studies.

Studies on the time-dependent fear generalization of contextual memories have led to the idea that this process relies on several factors that are not mutually exclusive (see Jasnow et al. 2017 for a recent review). Some authors have claimed that this phenomenon may rely on the progressive growth of fear-related processes that may occur with the passage of time after a traumatic event, a phenomenon called fear incubation (Diven 1937; McAllister and McAllister 1967). On the other hand, it has also been proposed that the development of fear generalization over time arises from a progressive reorganization of memory traces in the hippocampal-cortical network and a progressive loss of detailed information about the training environment (Rudy et al. 2005; Biedenkapp and Rudy 2007; Riccio and Joynes 2007; Wiltgen and Silva 2007; Wiltgen et al. 2010; Ruediger et al. 2011; Sauerhöfer et al. 2012; Poulos et al. 2016; Atucha et al. 2017; Jasnow et al. 2017; Guo et al. 2018). In line with the latter hypothesis, a recent study proposed

that in the case of auditory fear memories, the time-dependent broadening of fear generalization to novel tones can also be related to a progressive erosion of attributes of tonal conditioned stimuli (Bergstrom 2016; Pollack et al. 2018). Consistent with those studies, we found that animals that displayed time-dependent fear generalization did not differ from discriminators with regard to innate fear and anxiety or in the extent of freezing to a conditioned tone, thus supporting the idea that the time-dependent broadening of fear generalization is related to the erosion of memory details. In this framework, our data provide new evidence that details are not lost for all memories with the passage of time and that the precision of recent memories participates in governing the maintenance of the specificity of remote engrams. A memory that is precise at an early time point is refractory to the loss of details and to the development of fear generalization at a remote time point. In contrast, if a recent memory is imprecise, then it occurs a time-dependent erosion of details of remote engrams that gradually lead to the broadening of generalization of fear.

The other novel finding of our study was the identification of a cellular mechanism that may improve fear discrimination processes at both recent and remote time points. Insulin, insulin-like growth factors (IGFs), and their respective receptors constitute the IGF-related system (Alberini and Chen 2012). This system is involved in several brain functions, such as synaptic growth and plasticity, tissue repair and regeneration (Russo et al. 2005; Alberini and Chen 2012). Compared to other IGF family members, IGF-2 and its receptor (IGF-2R) are more abundantly expressed in the adult brain (Alberini and Chen 2012), and recent studies in mice and rats have provided evidence of its involvement in learning and memory processes (Chen et al. 2011; Alberini and Chen 2012; Schmeisser et al. 2012; Pascual-Lucas et al. 2014). In particular, previous studies have shown that IGF-2 is necessary for the consolidation of hippocampal-

dependent memories (Chen et al. 2011) and that injection of recombinant IGF-2 directly into the dorsal hippocampus significantly enhances long-term inhibitory avoidance and contextual fear memory retention (Chen et al. 2011; Alberini and Chen 2012; Schmeisser et al. 2012; Pascual-Lucas et al. 2014). Here, we uncovered a novel role of IGF-2R in memory processes by showing that the expression of this receptor in layer 2/3 of the auditory cortex correlates with the precise recall of recent memories as well as with the maintenance of detailed memories over time.

Moreover, the injection of the exogenous recombinant IGF-2 in the auditory cortex improved fear memory discrimination but did not change the strength of fear memories, which is inconsistent with the findings of previous studies in which injection of an the exogenous IGF-2 into the hippocampus enhanced the strength of aversive memories. These findings suggest that IGF-2R plays different roles in memory processes depending on the brain structure where it is expressed. Converging evidence from studies in rodents (Sacco and Sacchetti 2010; Aizenberg and Geffen 2013; Aizenberg et al. 2015; Grosso et al. 2015; Cambiaghi et al. 2016; Wigestrand et al. 2016; Concina et al. 2018) and humans (Apergis-Schoute et al. 2014; Staib et al. 2020) suggests a role for the auditory cortex in auditory fear learning and auditory fear discrimination (for a recent review see Concina et al. 2019). In the auditory cortex, parvalbumin-positive interneurons are involved in the feedforward inhibition of pyramidal neurons, and this circuitry is essential for auditory fear discrimination (Letzkus et al. 2011; Aizenberg et al. 2015; Park et al. 2020). Our data showing that IGF-2R expression was higher in PV- and CamKII-expressing neurons in discriminator animals than in generalizers could therefore suggest that this receptor enables efficient feedforward inhibition in discriminators that in turn promotes an efficient discriminative process and the encoding of precise auditory fear memories.

Understanding the time-dependent nature of fear generalization is also important for understanding the neural mechanisms of fear-related disorders, such as generalized phobia and PTSD, as generalized fear and avoidance after an incubation period are hallmarks of these disorders. In this framework, our findings may suggest that the occurrence of generalized fear and avoidance after an incubation period also depends on the precision of the original traumatic memory and that the administration of the exogenous IGF-2 may improve memory precision over time.

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Conflict of interests

The authors declare no competing interests.

Author contributions

G.C., A.R., E.M. and F. S. devised, carried out and analyzed behavioral experiments; G.C. and L.M. performed immunohistochemical and confocal microscopy analyses; B.S. devised and analyzed the experiments and wrote the manuscript with input from G.C, A.R. and E.M. All authors discussed the results and commented on the manuscript.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

References

Aizenberg M, Geffen MN. 2013. Bidirectional effects of aversive learning on perceptual acuity are mediated by the sensory cortex. *Nat. Neurosci*.**16:** 994-996.

Aizenberg M, Mwilambwe-Tshilobo L, Briguglio JJ, Natan RG, Geffen MN. 2015. Bidirectional regulation of innate and learned behaviors that rely on frequency discrimination by cortical inhibitory neurons. *PLoS Biol*. **13.**

Alberini CM , Chen DY. 2012. Memory enhancement: consolidation, reconsolidation and insulin-like growth factor 2. *Trends Neurosci*. **35:** 274-283.

Apergis-Schoute AM, Schiller D, LeDoux, JE, Phelps EA. 2014. Extinction resistant changes in the human auditory association cortex following threat learning. *Neurobiol. Learn. Mem*. **113:** 109-114.

Atucha E, Vukojevic V, Fornari RV, Ronzoni G, Demougin P, Peter F, Atsak P, Coolen MW, Papassotiropoulos A, McGaugh JL, de Quervain DJ, Roozendaal B. 2017. Noradrenergic activation of the basolateral amygdala maintains hippocampus-dependent accuracy of remote memory. *Proc. Natl. Acad. Sci*. U S A **114:** 9176-9181.

Bergstrom H C. 2016. The neurocircuitry of remote cued fear memory. *Neurosci. Biobehav. Rev*. **71**: 409-417.

Biedenkapp JC, Rudy JW. 2007. Context preexposure prevents forgetting of a contextual fear memory: implication for regional changes in brain activation patterns associated with recent and remote memory tests. *Learn. Mem.* **14:** 201-203.

Cambiaghi M, Grosso A, Renna A, Sacchetti B. 2016. Differential recruitment of auditory cortices in the consolidation of recent auditory fearful memories. *J. Neurosci*. **36:** 8586–8597.

Chen DY, Stern SA, Garcia-Osta A, Saunier-Rebori B, Pollonini G, Bambah-Mukku D, Blitzer RD, Alberini CM. 2011. A critical role for IGF-II in memory consolidation and enhancement. *Nature* **469:** 491-497.

Ciocchi S, Herry C, Grenier F, Wolff SB, Letzkus JJ, Vlachos I, Ehrlich I, Sprengel R, Deisseroth K, Stadler MB, Müller C, Lüthi A. 2010. Encoding of conditioned fear in central amygdala inhibitory circuits. *Nature* **468:** 277-282.

Concina G, Cambiaghi M, Renna A, Sacchetti B. 2018. Coherent activity between the prelimbic and auditory cortex in the slow-gamma band underlies fear discrimination. *J. Neurosci*. **38:** 8313-8328.

Concina G, Renna A, Grosso A, Sacchetti B. 2019. The auditory cortex and the emotional valence of sounds. *Neurosci. Biobehav*. Rev. **98:** 256-264.

Diven K. 1937. Certain determinants in the conditioning of anxiety reactions. J. Psychol. Interdisciplinary and Applied. **3:** 291–308 .

Eysenck HJ. 1968. Phobias as conditioned responses. *Int. J. Psychiatry* **6:** 468-470.

Genud-Gabai R, Klavir E, Paz R. 2013. Safety signals in the primate amygdale. *J. Neurosci.* **33:** 17986-17994.

Ghosh S, Chattarji S**.** 2015. Neuronal encoding of the switch from specific to generalized fear. *Nat. Neurosci.* **18:** 112-120.

Grosso A, Cambiaghi M, Renna A, Milano L, Roberto Merlo, G. Sacco T, Sacchetti B. 2015. The higher order auditory cortex is involved in the assignment of affective value to sensory stimuli. *Nat. Commun.* **6:** 8886.

Grosso A, Santoni G, Manassero E, Renna A, Sacchetti B. 2018. The neuronal basis of fear discrimination in the lateral amygdala. *Nat. Commun*. **9:**1214.

Guo N, Soden ME, Herber C, Kim MT, Besnard A, Lin P, Ma X, Cepko CL, Zweifel LS, Sahay A. 2018. Dentate granule cell recruitment of feedforward inhibition governs engram maintenance and remote memory generalization. *Nat. Med.* **24:** 438-449.

Ito W, Morozov A. 2019. Prefrontal-amygdala plasticity enabled by observational fear. *Neuropsychopharmacology.* **44:** 1778-1787.

Jasnow AM, Lynch JF, Gilman TL, Riccio DC. 2017. Perspectives on fear generalization and its implications for emotional disorders. *J. Neurosci. Res*. **95:** 821-835.

König C, Antwi-Adjei E, Ganesan M, Kilonzo K, Viswanathan V, Durairaja A, Voigt A, Yarali A. 2017. Aversive olfactory associative memory loses odor specificity over time. J. Exp. Biol. **220:** 1548–1553.

Letzkus JJ, Wolff SB, Meyer EM, Tovote P, Courtin J, Herry C, Lüthi A. 2011. A disinhibitory microcircuit for associative fear learning in the auditory cortex. *Nature*. **480:** 331-335.

Likhtik E, Stujenske JM, Topiwala MA, Harris AZ, Gordon JA. 2014. Prefrontal entrainment of amygdala activity signals safety in learned fear and innate anxiety. *Nat. Neurosci*. **17:** 106-113.

McAllister WR, McAllister DE. 1967. Drive and reward in aversive learning. *Amer. J. Psychol.* **80:** 377-383.

McAllister WR, McAllister DE. 2006. Recovery of conditioned fear by a single postextinction shock: effect of similarity of shock contexts and of time following extinction. *Learn. Behav*. **34:** 44-49.

Nissley SP, Rechler MM. 1984. Somatomedin/insulin-like growth factor tissue receptors. *Clin. Endocrinol. Metab*. **13:** 43-67.

Onat S, Büchel C. 2015. The neuronal basis of fear generalization in humans. *Nat. Neurosci*. **18:** 1811-1818.

Pamplona FA, Henes K, Micale V, Mauch CP, Takahashi RN, Wotjak CT. 2011. Prolonged fear incubation leads to generalized avoidance behavior in mice. *J. Psychiatr. Res*. **45:** 354-360.

Park Y, Geffen MN. 2020. A circuit model of auditory cortex. *PLoS Comput. Biol*. **16.**

Pascual-Lucas, M, Viana da Silva S, Di Scala M, Garcia-Barroso C, González-Aseguinolaza G, Mulle C, Alberini CM, Cuadrado-Tejedor M, Garcia-Osta A. 2014. Insulin-like growth factor 2 reverses memory and synaptic deficits in APP transgenic mice. *EMBO. Mol. Med*. **6:** 1246-1262.

Paxinos G, Watson C. 2007. The Rat Brain in Stereotaxic Coordinates. Academic Press Elsevier, London, 2007.

Pickens CL, Golden SA, Adams-Deutsch T, Nair SG, Shaham Y. 2009. Long-lasting incubation of conditioned fear in rats. *Biol. Psychiatry* **65:** 881-886.

Pollack GA, Bezek JL, Lee SH, Scarlata MJ, Weingast LT, Bergstrom HC. 2018. Cued fear memory generalization increases over time. *Learn. Mem.* **25:** 298-308.

Poulos AM, Mehta N, Lu B, Amir D, Livingston B, Santarelli A, Zhuravka I, Fanselow MS. 2016. Conditioning- and time-dependent increases in context fear and generalization. *Learn. Mem*. **23**: 379–385.

Rajbhandari AK, Zhu R, Adling C, Fanselow MS, Waschek JA. 2016. Graded fear generalization enhances the level of cfos-positive neurons specifically in the basolateral amygdala. *J. Neurosci. Res.* **94:** 1393-1399.

Resnik J, Paz R. 2015. Fear generalization in the primate amygdala. *Nat. Neurosci*.**18**: 188-190.

Riccio DC, Joynes RL. 2007. Forgetting of stimulus attributes: some implications for hippocampal models of memory. *Learn. Mem*. **14:** 430-432.

Rudy JW, Biedenkapp JC, O'Reilly RC. 2005. Prefrontal cortex and the organization of recent and remote memories: an alternative view. *Learn. Mem.* **12:** 445-446.

Ruediger S, Vittori C, Bednarek E, Genoud C, Strata P, Sacchetti B, Caroni P. 2011. Learning-related feedforward inhibitory connectivity growth required for memory precision. *Nature* **473:** 514-518.

Russo VC, Gluckman, PD, Feldman EL, Werther GA. 2005. The insulin-like growth factor system and its pleiotropic functions in the brain. *Endocr. Rev.* **26:** 916–943.

Sacco T, Sacchetti B. 2010. Role of secondary sensory cortices in emotional memory storage and retrieval in rats. *Science* **329:** 649-656.

Sauerhöfer E, Pamplona FA, Bedenk B, Moll GH, Dawirs RR, von Hörsten S, Wotjak CT, Golub Y. 2012. Generalization of contextual fear depends on associative rather than non-associative memory components. *Behav. Brain. Res.* **233:** 483-493.

Schmeisser MJ, Baumann B, Johannsen S, Vindedal GF, Jensen V, Hvalby ØC, Sprengel R, Seither J, Maqbool A, Magnutzki A, Lattke M, Oswald F, Boeckers TM, Wirth T. 2012. IkappaB kinase/nuclear factor kappaB-dependent insulin-like growth factor 2 (Igf2) expression regulates synapse formation and spine maturation via Igf2 receptor signaling. *J. Neurosci.* **32**: 5688–5703.

Staib M, Abivardi A, Bach DR. 2020. Primary auditory cortex representation of fear conditioned musical sounds. *Human Brain Mapping*.

Thomas DA, Riccio DC. 1979. Forgetting of a CS attribute in a conditioned suppression paradigm. *Animal. Learn. Behav*. **7:** 191–195.

Wang SH, Teixeira CM, Wheeler AL, Frankland PW. 2009. The precision of remote context memories does not require the hippocampus. *Nat. Neurosci.* **12:** 253-255.

Wigestrand MB, Schiff HC, Fyhn M, LeDoux JE, Sears RM. 2016. Primary auditory cortex regulates threat memory specificity. *Learn. Mem.* **24:** 55-58.

Wiltgen BJ, Silva AJ. 2007. Memory for context becomes less specific with time. *Learn. Mem.* **14:** 313-317.

Wiltgen BJ, Zhou M, Cai Y, Balaji J, Karlsson MG, Parivash SN, Li W, Silva AJ. 2010. The hippocampus plays a selective role in the retrieval of detailed contextual memories. *Curr. Biol.* **20:** 1336-1344.

Figure Legends

Figure 1. Fear generalization increases over time to the 15 kHz tone but not to the 7 kHz tone. (A) Scheme of the experiment: animals were tested with the novel 15 kHz tone one week after training. **(B)** Freezing of each animal (n=13) to the new tone (15 kHz) and to the CS (1 kHz) was employed to classify discriminators (DS>2) and generalizers (DS<2). **(C)** Proportion of discriminators (69%) and generalizers (31%) to the 15 kHz tone at the one-week interval. **(D)** Another group of animals was tested to the new 15 kHz tone four weeks after training. **(E)** Freezing of individual rats (n=14) to the 15 kHz tone and to the CS four weeks after training. **(F)** The proportion of discriminators (29%) and generalizers (71%) tested with the 15 kHz tone at four weeks after training was different from that obtained at one week (χ^2 =4.46, p=0.03). **(G)** The amount of freezing to the 15 kHz of all animals (discriminators + generalizers) was higher at the remote versus recent time interval (U=42, p=0.016). Importantly, the strength of the response to the CS was similar between the two time points (U=79.50, p=0.58). **(H)** Scheme of the experiment: animals (n=13) were tested with the 7 kHz tone one week after training. (**I)** Freezing of discriminators (DS>2) and generalizers (DS<2) to the 7 kHz tone and to the CS (1 kHz). **(J)** Proportion of discriminators (31%) and generalizers (69%) tested with the 7 kHz tone at one week from conditioning. **(K)** Animals were tested to the 7 kHz tone four weeks after training. **(L)** Freezing of individual rats (n=13) to the 7 kHz tone and to the CS four weeks after training. **(M)** The proportion of discriminators (23%) and generalizers (77%) to the 7 kHz tone four weeks after training remained similar to that obtained at one-week (χ^2=0.19, p=1.000). **(N)** Freezing of all animals (discriminators + generalizers) tested to the 7 kHz tone was similar between the recent and the remote time intervals (U=58, p=0.18), similarly to the strength of the

response to the CS (U=73.50, p=0.58). *P < 0.05, **P < 0.01, ***P < 0.001. All data are mean and SEM. Chi Square test (F, M); Mann-Whitney test (G, N).

Figure 2. The growth of fear generalization over time depends on the precision rather than to the strength of recent fear memories. (A) Scheme of the experiment: animals were tested to the novel 15 kHz tone one week after learning. Four days later, animals (n=44) were presented with the 7 kHz tone, and two weeks later again with the 15 kHz tone and the CS. **(B)** The proportion of discriminators to the 15 kHz tone at the recent time interval (n=24) significantly decreased at the remote time interval (n=7) (χ^2=14.39, p=0.0001), while the percentage of rats discriminating the 7 kHz tone at the recent interval (n=8) remained constant at the remote time point (n=7) (χ^2=0.08, p=0.77). **(C)** Freezing to the CS of rats that showed fear generalization to the 15 kHz tone at the remote time point and that had shown at the recent time point discrimination to the 15 kHz tone but generalization to the 7 kHz tone (DGG, n=14) was similar to that displayed by rats that continued to discriminate the 15 kHz tone at the remote time point (DDD, n=5 and DGD, n=2, U=27, p=0.10). **(D)** Freezing to the CS displayed by generalizers (n=10) and discriminators (n=4) to the 15 kHz at the remote time point was similar between the two groups (U=11.50, p=0.25). **(E)** Rats that showed fear generalization (n=10) or discrimination (n=4) to the 15 kHz tone at the remote time point displayed a similar behavior in the exploration of the open field arena (time spent in the periphery: U=18, p=0.76; travelled distance in the periphery: U=12, p=0.27). **(F)** The two groups showed a similar behavior also before tone delivery (U=19.50, p=0.97). *P < 0.05, **P < 0.01, ***P < 0.001. All data are mean and SEM. Chi Square test (B); Mann-Whitney test (C, D, E, F).

Figure 3. The expression of the insulin-growth factor receptor type 2 (IGF-2R) in the layer 2/3 of the auditory cortex underlies fear discrimination at the recent time point. (A) IGF-2R positive cells in the primary auditory cortex (Te1, ranging from -3 to -7 AP from bregma) were higher in discriminators (n=8) with respect to generalizers (n=8) in the layer 2/3 ($t_{(14)}$=4.84, p=0.0003), while no between-groups differences were detected in other layers (p>0.05). **(B)** Te1 was divided in an anterior (ranging from antero-posterior coordinates of 3 to 5 mm as respect to the bregma) and a more posterior (from 5 to 7 mm) subregion. **(C)** In the layer 2/3 of the anterior region no differences were detected between discriminators and generalizers (3-5 AP, $t_{(14)}$=1.68, p=0.11), while in the more posterior region discriminators showed more abundant IGF-2R positive cells (5-7 AP, $t_{(13)}$=4.71, p=0.0004). No differences were detected in all other cortical layers (p> 0.05 in all instances). **(D)** Representative photos of IGF-2R immunostaining in discriminator and generalizer animals (scale bar: 200 μm). **(E)** Freezing to the new tone was lower in discriminators with respect to generalizers ($t_{(13)}$=5.36, p=0.0001), while the freezing to the CS was similar between groups ($t_{(13)}$=0.67, p=0.51). **(F,G)** The higher level of IGF-2R+ cells correlated significantly with the lower freezing to the new tone (r = -0.78, p=0.0005) but not with the percentage of freezing to the CS (r = 0.10, p=0.71). **(H)** The total number of PV-IGF-2R double positive cells was greater in discriminator rats (n=12) with respect to generalizers (n=12) ($t_{(22)}$=2.34, p=0.0287), along a constant majority of IGF-2R+ cells in discriminators ($t_{(22)}$=6.25, p<0.0001) and a similar number of total PV+ neurons ($t_{(22)}$=1.75, p=0.09). **(I)** The ratio between the total number of double positive cells and the total number of PV+ was greater in discriminators ($t_{(22)}$=2.17, p=0.040). **(J)** Example of immunofluorescent staining of IGF-2R (red) and PV (green) in the Te1 of a discriminator and a generalizer rat (scale bar: 20 μm). **(K,L)** Co-immunolabeling of IGF-2R with CamKII revealed, together with the

higher total number of IGF-2R+ cells in discriminators ($t_{(22)}$=7.59, p<0.0001), a greater number of the total CamKII+ cells ($t_{(22)}$=5.63, p<0.0001) and double positive IGF-2R+CamkII neurons ($t_{(22)}$=6.04, p<0.0001). The percentage of double positive cells with respect to the total number of CamKII+ was significantly higher in discriminators with respect to generalizers ($t_{(22)}$=2.17, p=0.040). **(M)** Example of immunofluorescent staining of IGF-2R (red) and CamKII (green) in the Te1 cortex of discriminator and generalizer animals (scale bar: 20 μm). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. All data are mean and SEM. Unpaired *t* test (A, C, E, H, I, K, L); Pearson's *r* correlation coefficient (F, G).

Figure 4. IGF-2 improves fear discrimination at recent e remote times. (A) Scheme of the experiments: animals that generalized fear to the new tone (7 kHz tone) one week after learning were injected with IGF-2 (n=16), IGF-1 (n=10) or saline (n=14) shortly after fear memory recall. The new tone and the CS were presented again four days later. **(B)** Freezing to the 7 kHz tone consistently decreased following IGF-2 injection (3 × 2 mixed-design ANOVA, main effect of group: $F_{(2,37)} = 4.63$, $p = 0.016$, $\eta^2 = 0.200$; main effect of condition: $F_{(1,37)} = 11.39$, $p = 0.002$, $\eta^2 = 0.235$; group × condition interaction $F_{(2,37)} = 4.72$, $p = 0.015$, $\eta^2 = 0.203$). Simple main-effect analysis indicated a significant difference between IGF-2 injected rats with respect to IGF-1 and saline groups after the injections (IGF-2 vs saline, $p = 0.048$; IGF-2 vs IGF-1, $p = 0.005$; IGF-1 vs saline , $p = 0.856$) but not before (IGF-2 vs saline, p =1.000; IGF-2 vs IGF-1, p =0.258; IGF-1 vs saline, $p = 0.114$), and a significant difference only in the IGF2-injected group before and after injection (IGF-2, p <0.0001; IGF-1, $p = 0.182$; saline, $p = 0.856$). **(C)** CS memory retention was similar among three groups (3 × 2 mixed-design ANOVA, main effect of group: $F_{(2,37)} = 2.78$, $p = 0.075$, $\eta^2 = 0.131$; main effect of condition: $F_{(1,37)} = 0.061$, p =

0.806, $\eta^2 = 0.002$; group × condition interaction: $F_{(2,37)} = 0.87$, $p = 0.426$, $\eta^2 = 0.045$). **(D)** A similar experiment but with animals tested three weeks after injections. **(E)** Freezing to the 7 kHz tone diminished after IGF-2 injections (n=19) while remained similar after IGF-1 (n=11) or saline (n=16) (3 × 2 mixed-design ANOVA, main effect of group: $F_{(2,43)} = 4.11$, $p = 0.023$, $\eta^2 = 0.161$; main effect of condition: $F_{(1,43)} = 17.44$, $p < 0.0001$, $\eta^2 = 0.289$; group × condition interaction $F_{(2,43)} = 5.83$, $p = 0.006$, $\eta^2 = 0.213$). Simple main-effect analysis indicated a significant difference between groups after different injections (IGF-2 vs saline, $p = 0.039$; IGF-2 vs IGF-1, $p = 0.003$; IGF-1 vs saline, $p = 0.725$) but not before ($p = 1.00$ in all instances), and a significant difference within the same IGF-2-injected group before and after injection (IGF-2, $p < 0.0001$; IGF-1, $p = 0.684$, saline, $p = 0.073$). **(F)** Fear memory to the CS was similar among groups before and after injections (3 × 2 mixed-design ANOVA, main effect of group: $F_{(2,43)} = 0.047$, $p = 0.954$, $\eta^2 = 0.002$; main effect of condition: $F_{(1,43)} = 19.74$, $p < 0.001$, $\eta^2 = 0.315$; group × condition interaction $F_{(2,43)} = 0.11$, $p = 0.895$, $\eta^2 = 0.005$). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. All data are mean and SEM. 3×2 mixed-design ANOVA (B, C, E, F).

Figure 5. IGF-2 receptors in the basolateral amygdala are not involved in fear discrimination. (A) The number of IGF-2R positive cells was similar between discriminators (n=6) and generalizers (n=6) in both lateral ($t_{(10)}=0.006$, $p=0.99$) and basal ($t_{(10)}=0.44$, $p=0.66$) nuclei. **(B)** Immunostaining of IGF-2R+ cells in LA and BA in discriminator and generalizer animals (scale bar: 200 μm) **(C)** Scheme of the experiments. **(D)** A 2 × 2 mixed-design ANOVA did not show any difference between saline (n=9) and IGF-2 (n=13) injected rats. Main effect of group: $F_{(1,20)} = 0.43$, $p = 0.518$, $\eta^2 = 0.021$; main effect of condition: $F_{(1,20)} = 0.077$, $p = 0.784$, $\eta^2 = 0.004$; group × condition interaction $F_{(1,20)} = 0.49$, $p = 0.492$, $\eta^2 = 0.024$). **(E)** The strength of

fear memory was similar between groups (2 × 2 mixed-design ANOVA: Main effect of group: $F_{(1,20)} = 0.60$, $p = 0.445$, $\eta^2 = 0.030$; main effect of condition: $F_{(1,20)} = 0.11$, $p = 0.738$, $\eta^2 = 0.006$; group × condition interaction: $F_{(1,20)} = 0.28$, $p = 0.599$, $\eta^2 = 0.014$). **(F)** A similar experiment but with IGF-2 (n=14) or saline (n=11) injected animals tested at remote time point. **(G)** No difference were found between groups in freezing to the 7 kHz tone (2 × 2 mixed-design ANOVA, main effect of group: $F_{(1,23)} = 1.31$, $p = 0.263$, $\eta^2 = 0.054$; main effect of condition: $F_{(1,23)} = 0.004$, $p = 0.948$, $\eta^2 < 0.001$; group × condition interaction: $F_{(1,23)} = 1.50$, $p = 0.232$, $\eta^2 = 0.062$). **(H)** Fear memory was similar between groups before and after injections (2 × 2 mixed-design ANOVA, main effect of group: $F_{(1,23)} = 0.50$, $p = 0.483$, $\eta^2 = 0.022$; main effect of condition: $F_{(1,23)} = 10.59$, $p = 0.003$, $\eta^2 = 0.315$; group × condition interaction $F_{(1,23)} = 0.32$, $p = 0.576$, $\eta^2 = 0.014$). *P < 0.05, **P < 0.01, ***P < 0.001. All data are mean and SEM. Unpaired *t* test (A); 2×2 mixed-design ANOVA (D, E, G, H).

Figure 6. IGF-2 receptors in the auditory cortex improves the precision of remote fear engrams over time. (A) The total number of IGF-2R+ cells decreased in animals that generalized fear to 15 kHz tone at the late time point (DGG, n=9) with respect to animals that had discriminated the 15 kHz tone at recent times (DG, n=9) ($t_{(16)}$=2.82, p=0.01). **(B)** Representative images of IGF-2R expression in the two groups (scale bar: 20 μm). **(C)** The total number of PV-IGF-2R double positive cells was greater in animals that discriminated the 15 kHz tone (DG) at the recent time point with respect to animals that generalized fear to 15 kHz tone (DGG) at the remote time point ($t_{(16)}$=2.23, p=0.040). The two groups showed a similar number of total PV+ neurons ($t_{(16)}$=0.18, p=0.85). The total number of double positive cells with respect to the total PV+ was greater in DG than in DGG animals ($t_{(16)}$=2.54, p=0.02). **(D)** Animals that

discriminated the 15 kHz tone at the recent time point (DG, n=9) showed a greater number of CamKII-IGF-2R double positive cells when compared with animals that generalized fear at remote time (DGG, n=9) ($t_{(16)}$=2.57, p=0.020). The two groups did not differ in the number of total CamKII+ neurons ($t_{(16)}$=0.70, p=0.49) whilst DG displayed a greater number of double positive cells with respect to the total CamKII ($t_{(16)}$=3.40, p=0.003). **(E)** Immunofluorescence of IGF-2R and PV in representative DG and DGG rats (scale bar: 20 μm). **(F)** Immunofluorescence of IGF-2R and CamKII in DG and DGG animals (scale bar: 20 μm). **(G)** A additional group of DG rats (n=17) underwent IGF-2 injection in Te1 cortex after CS presentation. Animals were then tested at remote time point. **(H)** Freezing to the 15 kHz tone tested one month after training was significantly decreased in DG rats injected with IGF-2 peptide if compared with DG rats that in vast majority generalized fear to the same tone at the remote time point (see Figure 2) (2 × 2 mixed-design ANOVA, main effect of group: $F_{(1,31)}$ =2.81, p =0.103, η^2 =0.083; main effect of condition: $F_{(1,31)}$ = 64.24, p =0.0001, η^2 =0.675; group × condition interaction: $F_{(1,31)}$ =4.95, p=0.033, η^2 =0.138. Simple main effects between groups showed a difference in freezing to the 15 kHz tone after (IGF-2 vs control, p = 0.039) but not before injection (IGF-2 vs control, p = 0.934). *P < 0.05, **P < 0.01, ***P < 0.001. All data are mean and SEM. Unpaired *t* test (A, C, E); 2x2 mixed-design ANOVA (H).