Dynamic changes in neural circuitry during adolescence are associated with persistent attenuation of fear memories

Siobhan S. Pattwell1,*, Conor Liston2,3,4,*, Deqiang Jing3, Ipe Ninan5, Rui R. Yang3, Jonathan Witztum2, Mitchell H. Murdock2, Iva Dincheva3, Kevin G. Bath6, B.J. Casey4, Karl Deisseroth7 & Francis S. Lee3,4

Fear can be highly adaptive in promoting survival, yet it can also be detrimental when it persists long after a threat has passed. Flexibility of the fear response may be most advantageous during adolescence when animals are prone to explore novel, potentially threatening environments. Two opposing adolescent fear-related behaviours—diminished extinction of cued fear and suppressed expression of contextual fear—may serve this purpose, but the neural basis underlying these changes is unknown. Using microprisms to image prefrontal cortical spine maturation across development, we identify dynamic BLA-hippocampal-mPFC circuit reorganization associated with these behavioural shifts. Exploiting this sensitive period of neural development, we modified existing behavioural interventions in an age-specific manner to attenuate adolescent fear memories persistently into adulthood. These findings identify novel strategies that leverage dynamic neurodevelopmental changes during adolescence with the potential to extinguish pathological fears implicated in anxiety and stress-related disorders.

1 Fred Hutchinson Cancer Research Center, Department of Human Biology, 1100 Fairview Ave N, Seattle, Washington 98109, USA. 2 Brain and Mind Research Institute, Weill Cornell Medical College, New York, New York 10065, USA. 3 Department of Psychiatry, Weill Cornell Medical College, New York, New York 10065, USA. 4 Sackler Institute for Developmental Psychobiology, Weill Cornell Medical College, New York, New York 10065, USA. 5 Department of Psychiatry, New York University School of Medicine, New York, New York 10016, USA. 6 Department of Cognitive, Linguistic, and Psychological Sciences, Brown University, Providence, Rhode Island 02912, USA. 7 Departments of Bioengineering and Psychiatry and Behavioral Sciences, Howard Hughes Medical Institute, Stanford University, Stanford, California 94305, USA. * These authors contributed equally to this work. Correspondence and requests for materials should be addressed to F.S.L. (email: fslee@med.cornell.edu).
Fear learning emerges early in life, but the capacity to express and extinguish fear memories undergoes dynamic changes across development. The capacity for extinction depends largely on whether the fear is specific to a cue or an environmental context. Specifically during adolescence, extinction learning for cued fear memory appears to be impaired. During this same sensitive period, rodent studies have uncovered a coinciding sensitive period of suppressed expression of contextual fear. These opposing fear-related behaviours depend on distinct components of a medial prefrontal cortical circuit (the infralimbic and prelimbic cortex) that receive projections from the amygdala and ventral hippocampus, the infralimbic (IL) region of the medial prefrontal cortex (mPFC) has classically been recognized for its role in regulating fear expression via suppression of amygdala activity after extinction learning, while the prelimbic (PL) region has been recognized for its role in sustaining responses during cue-related fear expression. While this PL-amygdala input has long been known to be required for expression of a fear memory after conditioning, a novel role for the PL’s involvement in mediating extinction has recently been identified. After extinction learning, ventral hippocampal inputs to PL play a critical role in gating the expression of fear memory by enhancing local inhibition and interfering with sustained PL activity. Unlike neural circuits associated with fear to discrete cues, contextual fear requires the integration of spatial information in the environment via the hippocampus, through projections to both the amygdala and prefrontal cortex. Thus, both cued fear extinction and contextual fear expression rely on intact hippocampal projections to mPFC.

Developmental changes in the extinction of cued and expression of contextual fear memories may involve protracted development of prefrontal circuitry. Prefrontal circuits develop relatively slowly, undergoing a process of rapid synaptogenesis followed by accelerated synaptic pruning that extends into adolescence. Here, we investigate whether the protracted development of PL and connectivity with ventral hippocampus and amygdala may be contributing to the disparate phenotypes observed in cued and contextual learning. To date, no studies have directly assessed the relationship between these behaviours and synapse formation in projections from hippocampus and amygdala to the mPFC, due in part to the inaccessibility of these structures to transcranial imaging techniques.

Results
Longitudinal imaging of spine dynamics in the prefrontal cortex.
To study prefrontal synaptic plasticity and development across adolescence, we adapted a prism-based preparation that has been used in other cortical areas to allow for chronic two-photon imaging in the PL region of the dorsal mPFC (Fig. 1a), overcoming several technical obstacles that are unique to the objective of chronic imaging in this structure (Methods section). Briefly, after opening a small craniotomy over the midline, a custom borosilicate glass microprism (OptoSigma, Inc.) was stereotactically implanted in the dorsal mPFC in transgenic mice expressing yellow fluorescent protein (YFP-H line; Fax) in pyramidal cells of cortical layers 2/3 and 5 (Fig. 1a,b). The microprism was chronically fixed to the skull and the craniotomy was sealed using a combination of veterinary adhesives, to allow for repeated imaging in the developing adolescent brain.

We found that the implantation procedure was well tolerated: the mice were ambulating normally, eating and drinking, and grooming appropriately within 1–2 h after the surgery. Cortical lamination patterns and neuroanatomical structures adjacent to the prism were well preserved (Supplementary Fig. 1a). Consistent with prior reports, prism implantation induced a modest but significant increase in cell density up to 50 μm from the prism face (Supplementary Fig. 1b,c). This was due in part to an increase in the density of astrocytes as detected by immunoreactivity for S100 calcium-binding protein B (S100B), a marker of mature glial cells (Supplementary Fig. 1b,d). There was no significant difference in cell density or astrocyte immunoreactivity at distances of 50–500 μm from the prism face, relative to a control area in the contralateral mPFC (Supplementary Fig. 1c,d).

After allowing 5–6 days of recovery, we used a customized two-photon laser scanning microscopy system to obtain high-resolution images of YFP-expressing pyramidal cells in the dorsal mPFC, capable of resolving postsynaptic dendritic spines (Fig. 1b,c). Using vascular landmarks and the contours of the microprism as a reference frame, we were able to return to precisely the same areas over protracted imaging intervals (Fig. 1c), allowing us to quantify the formation and pruning of postsynaptic dendritic spines on PL (but not IL; Fig. 1a) pyramidal cells in the developing dorsal mPFC. To test whether synaptic development in PL is associated with the decreased capacity for cued-fear extinction learning during adolescence, we quantified formation and pruning rates for postsynaptic dendritic spines on the apical dendrites of pyramidal cells in the dorsal mPFC over a 24-h period from postnatal day (P)30 to P31, which is within the adolescent sensitive period. Because spine turnover rates were modestly but significantly altered at distances of <50 μm from the prism face (Supplementary Fig. 1e,f), corresponding to the area of increased astrocytic reaction, all subsequent analyses focused on dendritic segments >50 μm from the prism (50–200 μm). We found that 1-day spine formation rates (Fig. 1d) in the PL region of dorsal mPFC (mean ± s.d. = 13.9 ± 1.6%) were nearly triple those observed in neighbouring frontal association (FrA) cortex (5.0 ± 0.9%) at P30, but there were no differences in spine pruning (5.8 ± 1.4% in the PL region of dorsal mPFC versus 5.4 ± 1.6% in FrA) during this period (Fig. 1e). To control for the possibility that these effects were caused by severing some long-range projections to mPFC, we used a recently published protocol to image mPFC across the midline through a prism implanted in the contralateral hemisphere (Supplementary Fig. 2a,b), preserving the integrity of ipsilateral circuits. Spine formation (12.5 ± 1.6%) and elimination rates (5.3 ± 1.3%) were statistically indistinguishable in this preparation (Supplementary Fig. 2c; P > 0.38, Mann–Whitney).

Furthermore, when the same experiment was performed in separate cohorts of mice during late adolescence (prism implantation at ~P39, imaging at ~P44–P45) and young adulthood (prism implantation at ~P84, imaging at ~P90–P91), there were no significant differences in 1-day spine formation or pruning rates between the two regions (Fig. 1d,e). Together, these data demonstrate that the onset of a developmental period of impaired fear extinction coincides with a surge in formation of excitatory postsynaptic dendritic spines that occurs specifically in mPFC.

Next, we tested whether this selective increase in spine formation, but not elimination, was associated with regionally selective, developmental changes in spine density in fixed tissue samples obtained from YFP-H line transgenic mice before, during and after this developmental surge in spine formation (that is, at P24, P31 and P45; Fig. 1f,g). Furthermore, we also characterized developmental changes in spine density in the IL region of ventral mPFC, which is inaccessible through a 1.5-mm prism. In accord with the effects we observed on spine dynamics in vivo, we found that the surge in spine formation in the IL region of dorsal mPFC was associated with a significant increase in spine density at P31 (7.4 ± 1.3%), compared with P24.
As these apical dendrites are the primary recipients of long-range projections from other cortical and subcortical areas, we hypothesized that developmental changes in projections from the ventral hippocampus and amygdala may contribute to changes in spine formation and diminished extinction learning at P30 (ref. 16). To test this hypothesis, a retrograde tracer, Fluorogold (FG), was stereotactically injected into the PL of P23, P30, P45 and adult (P60) mice to directly assess hippocampal and amygdala projections to the PL (Fig. 2). Visualization and quantification of FG-labelled cell bodies via immunohistochemistry revealed dense populations of PL-projecting cell bodies within the BLA (Fig. 2b) that increased significantly from P23 to P30, and subsequently decreased by P45 (Supplementary Figs 3–5 and Supplementary Tables 1,2) for injection site, topographic features and control regions. This transient increase in FG labelling in the BLA, may be the consequence of increased arborization of BLA inputs to PL or increased density of BLA neurons projecting to PL, and as a consequence could result in an increase in AMG influence on PL activity. These findings indicate that neurodevelopmental increases in structural connectivity in the BLA-PL circuit may contribute to diminished extinction learning previously observed during early adolescence, as BLA input to PL may activate a positive feedback loop for maintaining fear expression\(^7,17\). Subsequent synaptic pruning of the PL-BLA circuit during the
transition from adolescence to adulthood may contribute to the re-emergence of intact extinction learning in both rodents and humans4, as this PL-BLA circuit involves a highly characterized positive feedback loop implicated in fear expression and undergoes increased structural connectivity only during adolescence when cued fear is resistant to extinction.

Interestingly, these retrograde labelling experiments also revealed a significant surge in connectivity between ventral CA1 (vCA1) and PL that peaked at P30 and decreased by P45 (Fig. 2a,c,d and Supplementary Figs 3 and 4)—the same developmental period during which contextual fear expression is suppressed. FG labelling in the claustrum, thalamus, IL and other areas of hippocampus revealed no such developmental changes in PL connectivity from P23 to adulthood (Supplementary Fig. 5). In addition, FG was stereotactically injected into the IL at the same ages and hippocampal and amygdala projections to the IL assessed. Unlike the PL, there was no significant increase in vCA1 or BLA projections to the IL from P23 to P30 or P45 (Supplementary Fig. 6), suggesting further the selectivity of the increase in region-specific projections to the PL across this developmental period. Previous studies have shown that ventral hippocampal inputs serve to dampen the sustained, fear-related activity in PL, and may serve a gating function for fear-enhancing inputs from the amygdala7, particularly in the setting of ambiguous contextual information18. Thus, the selectively enhanced inputs from BLA and ventral hippocampal neurons to the PL provide structural correlates for the surge in spine formation that occurs at P30 (Fig. 1d). While increased BLA inputs to PL correlate with sustained extinction-resistant cued fear, increased ventral hippocampal inputs to PL may contribute to fearlessness for contextual elements observed during the same sensitive period3-5.

Leveraging connectivity findings to optimize fear extinction. Given that hippocampal inputs to PL are capable of suppressing fear expression7, might activation of this circuitry during adolescence via contextual elements ameliorate the diminished cued-fear extinction observed during this developmental window (that is, enhance extinction via hippocampal-mediated inhibition of PL neurons)? To this end, we designed a behavioural intervention that maximally targeted the contextual component of a prior conditioned cued fear19,20. While the degree of persistent attenuation of contextual fear in adults was restricted to previously described retrieval-based time windows, persistent attenuation of contextual memories in adolescents showed enhanced flexibility that correlated with a lack of synaptic potentiation in basal amygdala (Supplementary Figs 7–10; Supplementary Notes 1 and 2). Because adolescent mice show enhanced capacity for contextual extinction, but also a robust cued fear that is highly resistant to cued extinction (Supplementary Figs 11 and 12), we tested whether we could improve extinction to discrete cues by bootstrapping onto the enhanced capacity for contextual extinction during this time. Mice were conditioned at P29 and then remained in their home cages or were subjected the following day to either a contextual extinction session, a cued extinction session in a novel context, or a cued extinction session in the original fear conditioning context at P30 (Fig. 3a). Mice were then tested for freezing to the tone in a
novel context 2 weeks post-conditioning and subsequently given a re-extinction session to see if a previously acquired discrete cued fear could be attenuated during adulthood. Interestingly, mice that were cue-fear conditioned during adolescence and then left in their home cage without further manipulation maintained a heightened freezing response in adulthood that was resistant to subsequent extinction (Supplementary Fig. 13), highlighting the importance of the age at which the fear was acquired. The cohort that received cued fear extinction in a novel context and the cohort that received context-only extinction also maintained a heightened robust fear response during adulthood, likely due to the aforementioned lack of successful extinction during adolescence. The cohort that received a combination of both contextual and cued fear extinction exhibited a significant decrease in freezing to tone cues and was the only cohort that successfully re-extinguished. These findings suggest that a combination of context and cued fear extinction may offer benefits during this sensitive window of development (Fig. 3a,b, Supplementary Fig. 13).

Fear renewal, or the return of fear on experiencing a reminder cue outside of the extinction context, remains a major obstacle for clinical treatment of anxiety disorders in humans and may be the result of tipping the balance between activation-specific neuronal circuits in the hippocampus and amygdala. This clinical observation highlights the need for better treatment methods, perhaps through further investigation of hippocampally mediated techniques, such as contextual extinction combined with discrete cue reminders. The experiments presented here provide a more detailed framework for when, developmentally, contextual-based techniques can be most beneficial.

Discussion
In summary, we have identified discrete and selective surges in spine formation and increased spine density, within the PL area of dorsal mPFC, as well as enhanced structural connectivity between PL and vCA, and PL and BLA during a period in adolescence in which we observe two disparate effects on fear behaviours (heightened cued fear that is resistant to cued fear extinction and contextual fear suppression). We leveraged a new preparation for imaging mPFC through chronically implanted microprisms to identify these developmental changes. Previous studies from human and nonhuman primates have shown that the adolescent period is associated with overproduction, followed
by selective stabilization and elimination, of principally excitatory synapses in the cortex\textsuperscript{3,21}. Our anatomical findings highlight a selective and dynamic reorganization of synaptic spine circuitry within the PL, but not in the IL, during this period. In addition, as compared with other cortical regions, the PFC has been shown to have one of the highest levels overproduction of spines across postnatal developmental and it has previously been shown that during adolescence, the subsequent pruning of spines within different PFC layers (IIIC, V) occurs at different rates\textsuperscript{24–26}. The timing of elevated production of synaptic spines in PL coincides with a period of resistance to cue extinction learning and enhanced capacity for contextual fear attenuation. Curiously, during this same period we observed significantly diminished expression of contextual fear. As recent work has demonstrated that projections from BLA to PL enhance fear expression via activation of excitatory pyramidal cells within PL (ref. 7), our observed selective surge in BLA-PL connectivity could support a cyclic loop of BLA-PL and PL-BLA activity during adolescence that correlates with resistance to cued fear extinction at this age. Previous studies using anterograde tract-tracing analyses have demonstrated a significant increase in BLA connectivity to the PFC during adolescence\textsuperscript{1}. Our observation of enhanced BLA-PL at P30 is consistent with these previous findings. However, BLA inputs into the IL do not appear to undergo a similar surge in connectivity at this P30 time point (Supplementary Fig. 6), highlighting the potential for selective activation of the BLA-PL circuit during adolescence. The underlying molecular mechanism that may drive a selective enhancement of connectivity to between the BLA and PL remain to be determined. Multiple growth factors and neuromodulators have been shown to increase during this developmental window in cortex, hippocampus and amygdala, including brain-derived neurotrophic factor\textsuperscript{27}. However, we are not aware of any reports indicating that any of these factors might be modulated in a regionally selective manner to support the preferential enhancement of inputs to the PL and further studies are warranted.

While the surge in connectivity between vCA1 and PL may be insufficient to override the PL-BLA circuit during cue extinction, enhanced vCA1-PL circuit activation during contextual extinction may persistently attenuate the contextual fear (Supplementary Fig. 8), likely due to vCA1 projections to inhibitory GABAergic neurons\textsuperscript{28} within PL. Utilizing the enhanced capacity for contextual fear attenuation during adolescence, we showed that contextual fear can be persistently attenuated with a single extinction session during this adolescent period. However, once outside this ‘sensitive period’ between P29 and P43, this form of extinction has minimal effects in reducing contextual fears acquired in adulthood. Finally, exploiting this capacity for contextual extinction with a combined context-cue extinction session offers a significant benefit to cued fear extinction in the adolescent time frame. These findings also demonstrate that fear memories can be blocked and persistently suppressed without ever being expressed during adolescence in mice. This unexpected discovery implies that prophylactic behavioural treatments aimed at preventing the emergence of anxiety-like symptoms after traumatic or stressful experience through contextual means may prove more beneficial than waiting for symptoms to emerge before intervening. Previous studies have shown that acute or chronic stress in adolescence leads to long-lasting anatomical and behavioural consequences, especially with regard to anxiety and fear-related behaviours\textsuperscript{28–30}. Our findings provide one of the first indications for developmentally informed treatments for preventing the emergence of anxiety disorders following stressful or traumatic events, even if they are asymptomatic, as a form of prophylactic treatment. These findings also highlight the potential importance of a contextual extinction-based therapy and preventive interventions for youth. Such non-invasive behavioural interventions, as part of restorative training, may help to inoculate against fear memories and improve future outcomes in children and adolescents exposed to stressful or traumatic events, warranting the need for future studies to explore the mechanistic link between the changes in prefrontal cortical development and contextual fear learning discussed here.

**Methods**

**Surgical implantation of an intracranial microprism.** Custom glass microprisms (OptoSigma, Santa Ana, CA) were intracranially implanted in transgenic mice expressing YFP in layers 2/3 and 5 pyramidal cells (Thy1/YFP-H line, Jackson Labs, Bar Harbor, ME). The microprisms used in this study were square, right-angle prisms, 1.5 mm on a side and made of (BK7) borosilicate glass with a reflective aluminium coating on the hypotenuse. Because the aluminium coating was not always stable over long-imaging intervals, an additional silicon dioxide protective coating was applied on the external surface of the hypotenuse.

The implantation procedure was performed 5–6 days before imaging. Thus, separate cohorts of mice were used for each developmental time point, for example, in one cohort, the microprism was implanted on ~ P24 for imaging at P30–P31, and in a second cohort, the microprism was implanted on ~ P39 for imaging at P44–P45. Buprenorphine (0.5 mg kg\textsuperscript{−1} IP) and dexamethasone (1 mg kg\textsuperscript{−1} IP) were administered before surgery for prophylactic analgesia and to reduce perioperative inflammation, respectively. Surgical-plane anaesthesia was achieved using isoflurane (5% for induction, 2–3% for maintenance), monitoring for depth of anaesthesia every 10 min throughout the procedure. A sterile eye lubricant was applied to both eyes to prevent corneal drying, and normothermia was maintained using a heating pad. After removing the fur on the scalp using a scissors, the animal’s head was stabilized in a stereotaxic device using ear bars and a nose restrainer (David Kopf Instruments, Tujunga, CA). The scalp was then prepped for surgery with a Betadine scrub.

Next, the skin superficial to the skull was incised medially and reflected away using sterile surgical instruments, and a small circular section of skin (~ 0.75 cm in diameter) was excised. The periosteum was bluntly dissected away. Using the digital stereotaxic device, the skull overlying the prefrontal cortical area to be imaged was located in stereotaxic coordinates (0–1.5 mm lateral to midline; ~ 1.7–1.8 mm anterior to Bregma). The precise location of the area to be imaged along the anterior-posterior axis was selected to avoid transecting large blood vessels, which could be visualized by thinning the skull in phosphate buffered saline using a compact dental drill and sterile drill bit. A circular head plate was then centered over the area of interest and fixed to the skull using Metabond dental cement (C&B Metabond, www.parkell.com). Next, a rectangular craniotomy slightly larger than the prism (~ 1.7 × 1.7 mm, flush with the midline and the animal’s head) was performed using a dental drill. To minimize the risk of damaging the prism into the brain, a small incision (~ 1.6 mm) was made in the dura using a fresh sterile scalpel blade, and the dura beneath the craniotomy was gently dissected away, taking care not to compress the underlying brain tissue or puncture the sagittal venous sinus. This resulted in no more than trace bleeding that could be controlled using a sterile Surgicel sponge (Johnson & Johnson). Throughout the procedure, the area was irrigated regularly with artificial cerebrospinal fluid. At this stage in the procedure, the area was ready for implantation of the microprism.

Relative to other cortical areas, we found that the prefrontal cortex was more sensitive to damage due to compression during the insertion process and more prone to bleeding due to the presence of the midline venous sinus and large blood vessels emanating from this structure. To mitigate the former problem, we used a digital stereotaxic micromanipulator (David Kopf Instruments) to aid in the insertion process. The microprism was attached to a syringe with a 25G, 0.5″ hypodermic needle using a carbon vacuum line. The syringe was attached to a standard electrode manipulator and digital display console (Kopf Model 940) to achieve micrometre-precision manipulation of the position of the microprism. In this way, the microprism was lowered slowly into the brain over the course of ~ 5 min in increments of ~ 100 μm, allowing time for the tissue to adjust and accommodate the volume of the prism. This process was continued until the upper surface of the prism was nearly flush with the dorsal surface of the cortex. We also took two measures to reduce the risk of bleeding during the insertion process. First, the face of the prism was positioned such that it did not bisect major vasculature to the extent this was possible. Second, the prism was initially inserted ~ 100 μm lateral to midline to avoid contact with the midline venous sinus during the insertion process. After lowering the prism to the desired location (flush with the dorsal surface of the cortex), the prism was gently moved medially using the micromanipulator until it was flush with the midline. Using these measures, intraoperative bleeding was minimal. When the microprism was successfully positioned in the desired location, the central vacuum line was turned off, allowing the blunt needle to disengage from the surface of the prism without disturbing it or the adjacent cortex.
After inserting the prism and dispensing the syringe needle, we applied a thin layer of 1% agarose to the surface of the brain along the perimeter of the implant to protect the underlying tissue. Next, a veterinary adhesive (Vetbond, 3M Inc., St. Paul, MN) was applied to adhere the prism to the skull. Finally, a layer of Metabond was applied for added durability in the fixation of the prism to the skull. At the end of the procedure, this layer of Metabond adhered to the prism, skull and head plate, and covered all areas of exposed skull. We found that this preparation was typically highly stable over imaging intervals of many months.

To control for the possibility that spine turnover rates may be altered by severing long-range projections to and from mPFC on implantation of the prism, we used a preparation inspired by a recently published protocol for severing long-range projections to and from mPFC on implantation of the head plate, and covered all areas of exposed skull. We found that this preparation was typical highly stable over imaging intervals of many months. At the end of the procedure, this layer of Metabond adhered to the prism, skull and head plate, and covered all areas of exposed skull. We found that this preparation was typically highly stable over imaging intervals of many months. After inserting the prism and disengaging the syringe needle, we applied a thin layer of 1% agarose to the surface of the brain along the perimeter of the implant to protect the underlying tissue. Next, a veterinary adhesive (Vetbond, 3M Inc., St. Paul, MN) was applied to adhere the prism to the skull. Finally, a layer of Metabond was applied for added durability in the fixation of the prism to the skull. At the end of the procedure, this layer of Metabond adhered to the prism, skull and head plate, and covered all areas of exposed skull. We found that this preparation was typically highly stable over imaging intervals of many months.

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fixative at 4°C overnight and transferred to a sucrose solution (30% sucrose in 0.1 M phosphate buffer) at 4°C for 48 h. Coronal sections (40 μm) were cut on a freezing microtome. One section of every three was mounted in the correct order and the direction. The other sections (between every third) were collected in an antifreeze solution (30% Glycerol, 30% ethylene glycol and 40% 0.25 M phosphate buffer) and stored at –20°C, such that three total sets of series representative sections were taken and for each set sections were 120 μm apart. The mounted set slides were air dried for 3 h and cover-slipped by using a novel mounting solution (containing 10% SiO2, 0.1 M Tris, pH = 11). The use of 10% SiO2 in high pH in the mounting solution enhanced the fluorescence of FG significantly, thus avoiding the need for standard immunohistochemistry to visualize FG labelling. This method also allowed for more accurate quantification of data by avoiding issues often associated with immunohistochemistry, such as variable staining due to uneven penetration of IgG (15 μm). Previous immunohistochemistry techniques often use 40 μm tissue sections, but usually leave 10 μm in the middle un-stained such that the section forms ‘sandwich-like’ staining pattern that can interfere with the accuracy of quantification. Since there is no need for secondary antibody staining of floating sections using this method that allows for direct measurement of FG fluorescence, sections could be mounted immediately after slicing, which allowed for intact serial sections avoiding any section loss, and facilitating systemic sampling and stereological analysis. Serial sections were imaged with a Nikon 80i fluorescent microscope with a 60×/1.42 NA Plan APO objective (Nikon Instruments). The basal amygdala principal neurons were visualized using video- or video-enhanced videos. For each set 120 sections were acquired using a MicroFire digital camera and FireFrame software (Optiphoton, Princeton, NJ, USA) and stereological estimation of cell density was performed using StereoInvestigator Software (MicroBrightfield, VA, USA). Stereological estimation of area of injection sites and retro-labelled cell density in brain regions strictly followed stereological rules. Briefly, systemic random sampling by choosing one of three sections that includes PL as starting sections, then take sections 120 μm until ventral hippocampus disappears. By referring to Allen Brain Atlas, 6–7 regions were traced with a 4x lens, then stereological estimation was performed by using different probes. Injection sites were screened and judged, and only mice with injections mainly limited to the rostral 2/3 of PL and well limited in IL were included in the final analysis. The volume of injection was calculated by Cavalieri estimator and calculated by divided volume of injection site by total PL volume. Contours of basolateral nucleus (BLA) and ventral hippocampus, claustrum and medial dorsal thalamic nucleus were made, and systemically random sampling grid was applied to drawn of brain region. The volume of brain region was then calculated by using Cavalieri estimator and total cell numbers were estimated by Fractionator with counting frame size 25 × 25 × 40 μm and sampling grid size 100 × 100 μm. We used a one-way ANOVA to test for main effects of age for each region along with post hoc analyses (Tukey) to test for differences between select groups.

Electrophysiology. Brains were quickly removed after pentobarbital anesthetization and placed in ice-cold artificial cerebrospinal fluid of the following composition (in mM): NaCl (138), KCl (2.5), glucose (10), NaHP2O4 (1), CaCl2 (1), MgSO4 (2) and NaHCO3 (25) bubbled with 95% O2/5% CO2 (pH 7.4). Coronal slices (400 μm) containing amygdala were cut with a vibratome and maintained at room temperature for 90 min in a brain-slice keeper to allow recovery. A single slice was then transferred to a recording chamber perfused with artificial cerebrospinal fluid as described above. For recording 2 mM CaCl2 was used. The temperature of the solution was maintained at 32°C with a TC324B in-line solution heater and controller (Warner Instruments). The basal amygdala principal neurons were visualized using video-enhanced differential interference contrast microscopy (Hamamatsu C4505), with an Olympus RX50WI upright microscope fitted with a × 40 long-working distance water immersion objective (0.70 NA). Field potentials (4–6 MΩ) were recorded with an intracellular electrode as described before in the presence of bicuculline (10 μM). Spontaneous postictic currents (EPSCs) were recorded at ~60 mV in the basal amygdala neurons by electrical stimulation of the perirhinal cortex using an extracellular electrode as described before in the presence of bicuculine (10 μM). Spontaneous EPSCs (sEPSCs) were recorded at ~60 mV, in the presence of bicuculine. Recordings were made using an Axopatch 200B amplifier (Molecular Devices) and digitized by Digidata 1322A (Molecular Devices). Synaptic stimulation was induced through extracellular stimulation using a Fisher Scientific Stimulator A065 (World Precision Instruments). Recordings were rejected when series resistance or holding current changed by 10%. Electrophysiology data are presented as mean ± s.e.m. Given the possibility of variation, sEPSCs were recorded in multiple slices from a minimum of five mice in each group and pooled together for analysis using t-test. Repeated measure ANOVA followed by Bonferroni-corrected post hoc tests were used for comparing EPSCs. Greenhouse-Geisser correction was applied when sphericity was violated. P < 0.05 was considered statistically significant.

Behaviour. Animals. Male, C57BL6/J mice were used for all experiments. To eliminate potential developmentally sensitive, shipping-induced stress effects, breeding pairs of C57BL6/J wild-type mice from Charles River (Wilmington, MA) were sent in the cold and monitored daily. Litters were weaned at P21 and males from various litters were randomly combined to eliminate any litter-driven effects on behaviour. Mice were housed five per cage in a temperature and humidity-controlled vivarium maintained on a 12 h light/dark cycle. Mice had ad libitum access to food and water. Separate cohorts of mice (aged P29–P90) were used for all fear conditioning and electrophysiology experiments. All procedures regarding animal care and treatment were in compliance with guidelines established by Weill Cornell Medical College’s Institutional Animal Care and Use Committee and the National Institutes of Health.

Fear conditioning. Mice were fear conditioned in mouse test cage (Coulbourn Instruments, Allentown, PA) inside a sound-attenuated box. The chamber (Context A) was cleaned in between each mouse and scented by peppermint-scented (0.1%) ethanol (EtOH; 70%). On conditioning day, (day 1), following a 2-min acclimation period, mice were conditioned with three trials consisting of a 30-s tone cue (5kHz, 70dB) that co-terminated with a 1-s, 0.7-mA foot shock delivered through the electrified floor grid. Each trial was separated by a 30-s intertrial interval. After the final tone-shock pairing, mice remained in the conditioning chamber for 1 min before being returned to their home cages. Trials were digitally recorded and freezing during the initial 2 min acclimation/expansion period was scored as a measure of baseline freezing to the conditioning context.

For contextual reconsolidation update experiments, mice were returned to Context A for either a long-term memory test (LTM Test #1), or a retrieval session, 24 h post-conditioning, where freezing behaviour was scored during the last 3.5 min of the total 5.5 min spent in the chamber for contextual experiments. After this fear retrieval, mice were returned to their home cages and then returned to Context A for a contextual extinction session after a specified amount of time (10 min, 1 h, 24 h and so on. based on experimental group) and remained in the context for 1 h. During the post-extinction long-term memory (LTM Test #2) test, mice were returned to Context A again and freezing behaviour was scored during the last 3.5 min of a 5.5-min trial. To test for fear erasure by examining fear extinction of conditioned fear in adolescent rats.

Cue reconsolidation update experiments followed a similar format, except involved tone cue presentations. Following fear conditioning, mice were returned to their home cages. 24 h post-conditioning, mice were placed in Context B for a retrieval session during which a single tone cue was played. After this fear retrieval, mice were returned to their home cages and then returned to Context B for a cue extinction session after a specified amount of time (10 min, 1 h, 24 h and so on. based on experimental group) and were presented with 18 tones (unless noted otherwise in figures), with a 3-min intertrial interval. During the post-extinction long-term memory (LTM Test #2) test, mice were returned to Context A again and freezing to a tone cue was scored and calculated after a percentage of time spent freezing to tone. To test for fear erasure by examining retrieval, mice were returned in their home cages for over a month and re-tested for cue fear. To test for fear erasure by reinstatement, mice were introduced to a novel context (Context C; a red cylinder, cleaned and scented with lemon scented (0.1%) EtOH (70%), with electrified floor grid), and after a 1-min acclimation period, were given a single, unsignaled (no tone cue) 1 s, 0.7-mA foot shock and remained in Context C for 1 min post-shock before being returned to their home cages. A final LTM test (LTM Test #3) was given in which mice were tested for freezing to either tone (Context B; three 30 s tone cues) or context (Context A; last 3.5 min of a 5.5-min trial). Experiments that tested for erasure by examining fear renewal (day 4), examined freezing to Context A after a 1-month interval and measure freezing behaviour for the last 3.5 min of a 5.5-min trial.

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