Regulating anxiety with extrasynaptic inhibition

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Aversive experiences can lead to complex behavioral adaptations including increased levels of anxiety and fear generalization. The neuronal mechanisms underlying such maladaptive behavioral changes, however, are poorly understood. Here, using a combination of behavioral, physiological and optogenetic approaches in mouse, we identify a specific subpopulation of central amygdala neurons expressing protein kinase C δ (PKCδ) as key elements of the neuronal circuitry controlling anxiety. Moreover, we show that aversive experiences induce anxiety and fear generalization by regulating the activity of PKCδ+ neurons via extrasynaptic inhibition mediated by α5 subunit-containing GABAA receptors. Our findings reveal that the neuronal circuits that mediate fear and anxiety overlap at the level of defined subpopulations of central amygdala neurons and demonstrate that persistent changes in the excitability of a single cell type can orchestrate complex behavioral changes.

Anxiety disorders comprise a very prevalent and complex set of pathologies. They are associated with inappropriate fear reactions to specific stimuli, but also with less specific, more generalized states of apprehension and vigilance; that is, anxiety1. The terms fear and anxiety refer to two ethologically different defensive behavioral programs. Studies in rodents indicate that, depending on the physical distance to a predator, acute fear responses or more sustained anxiety behavior are observed1-2. When presented with an immediate, imminent and predictable threat, rodents respond with acute freezing, flight-or-fight responses. In contrast, situations providing unspecific and diffuse cues for threat predictions, such as contextual stimuli or brightly illuminated and unprotected spaces, promote sustained avoidance and risk assessment behavior1. As a consequence, in animals and in humans, anxiety states are often associated with fear generalization3,4—that is, the generation of acute fear responses to stimuli that do not predict an aversive outcome.

The neuronal circuits underlying the acquisition and expression of acute fear responses have been extensively studied using as a model classical auditory fear conditioning5, in which animals learn to associate an initially neutral conditioned stimulus (CS; a tone) with an aversive unconditioned stimulus (US; a foot shock). These studies indicate that the acquisition of conditioned fear responses depends on activity-dependent plasticity in the lateral nucleus of the amygdala and central nucleus of the amygdala (CEA). The expression of acute fear responses depends on output projections from the CEA to downstream targets in the brainstem, including the periaqueductal gray (PAG)5.

Recent studies combining mouse genetics and viral expression techniques with in vivo electrophysiological, pharmacogenetic and optogenetic approaches have begun to shed light on the exquisite anatomical and functional organization of the neuronal circuitry mediating and controlling the acquisition, expression and extinction of conditioned fear responses5,6. In the CEA, for instance, distinct cell types have been identified on the basis of functional and genetic criteria7-11. In the lateral subdivision of CEA, PKCδ+ neurons exhibit inhibitory CS responses (CSoff responses)8,9. PKCδ+ neurons receive local inhibitory inputs from CS-activated PKCδ− neurons and in turn make inhibitory synaptic connections onto PAG-projecting CEA output neurons, thereby gating acute CEA output through disinhibition8,9.

In contrast to fear circuits, the neuronal circuitry mediating sustained anxiety is poorly understood9. In vivo pharmacological and behavioral studies suggest an important role for the so-called extended amygdala, an extensively interconnected system comprising the bed nucleus of the stria terminalis (BNST), the CEA and parts of the basal forebrain12. Even though it has been suggested that acute fear and sustained anxiety responses are mediated by separate neuronal systems, strong anatomical connections between the CEA and the BNST exist, and anxiety behavior can be regulated by optogenetic manipulations of both basolateral amygdala and BNST12-14 inputs to the CEA. In vivo single-unit recordings in the CEA showed that fear conditioning induced increases in the spontaneous activity of CSoff neurons, which largely overlap with PKCδ+ neurons, strongly correlate with fear generalization8,9, a hallmark of anxiety states. This suggests that CEA PKCδ+ neurons might be involved not only in gating acute conditioned fear responses, but also in mediating sustained states of anxiety. However, the causal contribution of PKCδ+ neuron activity to fear generalization and the underlying mechanism of their change in firing behavior remains unknown.

RESULTS

PKCδ+ neurons regulate anxiety behavior

Fear generalization often correlates with states of anxiety4. Consistent with this, 24 h after differential fear conditioning, we found a negative correlation between fear generalization and time spent in the open

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Figure 1 Fear conditioning enhances anxiety. (a) Experimental protocol. Animals were exposed to five CSs or five CS–US pairings. Anxiety behavior was assessed 24 h later on the EPM, and fear generalization was quantified in a retrieval test carried out in a novel context. (b) Freezing values (percent of time) of the two experimental groups (CS only, \( n = 11 \) mice, and CS–US, \( n = 12 \) mice) during CS\(^+\) and CS\(^−\) exposure. ***\( P < 0.001, \) paired \( t \)-test for the CS–US group, \( t = 8.599, 11 \) d.f. (c) Left, example EPM trajectories of animals previously exposed to CS only or to CS–US pairings. Right, animals subjected to CS–US pairings (\( n = 12 \) mice) exhibit more anxiety behavior on the EPM than animals exposed to CS only (\( n = 11 \) mice; ***\( P < 0.001, \) Mann-Whitney rank-sum unpaired \( t \)-test). C, closed arm; O, open arm. (d) Overall EPM track length is the same for CS-only and CS–US groups (\( P = 0.865, \) unpaired \( t \)-test, \( t = 0.144, 21 \) d.f.). (e) Regression analysis reveals a significant correlation between EPM anxiety behavior and fear generalization for individual animals. White symbols represent values from individual animals. Black symbols represent binned averages of animals exhibiting different levels of fear generalization (0–0.2, 0.2–0.4 and >0.4). Linear regression: \( R = 0.6, P = 0.003, n = 12 \) mice (CS–US group). (f) There was no correlation between EPM anxiety behavior and freezing to the CS\(^+\) and CS\(^−\) for the CS-only group (\( n = 11 \) mice, CS-only group; freezing to the CS\(^−\) versus time spent in open arms: \( R = 0.298, P = 0.260; \) freezing to the CS\(^+\) versus time spent in open arms: \( R = 0.287, P = 0.335 \)). White symbols represent values from individual animals. All error bars indicate mean ± s.e.m.

arms of an elevated plus maze (EPM), a standard behavioral test of rodent anxiety (Fig. 1)\(^{13–15} \).

To test whether rapid and reversible modulation of the spontaneous activity of PKC\(^δ\) neurons in vivo is sufficient to drive changes in fear generalization, we used an optogenetic approach. PKC\(^δ\)-Cre mice were bilaterally injected with conditional recombinant adeno-associated virus (rAAV) vectors expressing either Channelrhodopsin 2 (ChR2) or Archaerhodopsin (ARCH) in a Cre-dependent manner (Fig. 2a–i and Supplementary Fig. 1). Four to 5 weeks after injection, mice underwent discriminative fear conditioning using a tone (CS\(^+\)) paired with footshock (US) and another, unreinforced tone (CS\(^−\)) (Fig. 2j,k). Twenty-four hours later, retrieval of fear memory, assessed by tone-induced freezing behavior, was tested in the presence or absence of a constant 30-s light pulse, which induced a marked and sustained increase in the firing rate of PKC\(^δ\) neurons (Fig. 2 and Supplementary Fig. 1). We found that, consistent with the previously observed correlation between fear generalization and activity of PKC\(^δ\) neurons, blue light stimulation of PKC\(^δ\) neurons increased fear generalization, calculated as the ratio between CS\(^−\) and CS\(^+\) freezing (Fig. 2k).

Next we examined whether optogenetic manipulation of PKC\(^δ\) neurons would also cause changes in EPM behavior in naive animals. In keeping with its effect on fear generalization, blue light stimulation of PKC\(^δ\) neurons decreased the relative time spent in open arms, corresponding to an anxiogenic effect, whereas yellow light stimulation was anxiolytic (Fig. 2m,n and Supplementary Fig. 2). To further support the notion that increasing or decreasing the spontaneous activity of PKC\(^δ\) neurons has anxiogenic or anxiolytic effects, respectively, we assessed light-induced behavioral changes in the open-field test (OFT), in which anxious animals tend to avoid the center area of an open-field arena\(^{15,14} \). Consistent with the effect on EPM behavior, we observed in naive animals that, even though blue light stimulation of PKC\(^δ\) neurons decreased the overall locomotion without increasing freezing behavior (Supplementary Fig. 2), this resulted in a reduction in the number of visits to the center area as a proportion of track length and to a decrease in the overall time spent in the center area (Fig. 2o and Supplementary Fig. 2). Conversely, optogenetic inhibition of PKC\(^δ\) neurons resulted in increased locomotion, an increase in the number of visits to the center area and an increase in the overall time spent in the center area (Fig. 2o,p and Supplementary Fig. 2). Taken together, these data suggest that basal activity of PKC\(^δ\) neurons in CEA exerts bidirectional control over anxiety behavior. Moreover, in the absence of any optogenetic intervention, the degree of fear generalization correlates with both the spontaneous activity of PKC\(^δ\) neurons and with anxiety behavior, suggesting that, under physiological conditions, the activity of PKC\(^δ\) neurons might be actively regulated by endogenous mechanisms.

 Extrasynaptic inhibition regulates PKC\(^δ\) neurons

Because the neuronal circuitry of the CEA consists of spontaneously active GABAergic neurons, we hypothesized that, as in the cerebellum, activation of extrasynaptic GABA\(_A\) receptors (GABA\(_A\)Rs) may be an important factor controlling the excitability and spontaneous activity of CEA neurons\(^{16,17} \). Whole-cell patch clamp recordings from identified PKC\(^δ\) neurons in acute CEA slices revealed the existence of a tonic GABA\(_A\) receptor–mediated conductance that could be blocked by picrotoxin (100 \( \mu \)M) but not by the selective competitive GABA\(_A\)R antagonist SR-95531 (2-(3-carboxypropyl)-3-amino-6-(4-methoxyphenyl)pyridazinium bromide; 1–50 \( \mu \)M)\(^{18} \) or by the selective GABA\(_B\)R antagonist CGP 52432 (1–50 \( \mu \)M) (Fig. 3a and Supplementary Fig. 3). The charge transfer mediated by tonically active, extrasynaptic GABA\(_A\)Rs, as measured by a change in the holding current, was substantially larger than the charge transfer of synaptically activated GABA\(_A\)Rs, as measured by the blockade of spontaneous inhibitory postsynaptic currents (sIPSCs) (Fig. 3b). Consistent with this marked difference in charge transfer, blocking synaptic GABA\(_A\)Rs had no significant effect on the excitability of PKC\(^δ\) neurons, whereas blocking both synaptic and tonically active extrasynaptic receptors with PTX resulted in a robust increase in spontaneous firing rate recorded in cell-attached configuration (Fig. 3c). More whole-cell recordings of PKC\(^δ\) neurons revealed that blockade of extrasynaptic, but not synaptic, GABA\(_A\)Rs led to an increase in input resistance, a slight depolarizing shift in the resting membrane potential and an increase in the input–output function as measured by depolarizing current steps or by random current fluctuations (Supplementary Fig. 3).
Bidirectional regulation of fear generalization and anxiety through optogenetic control of CEA PKCδ+ neurons. (a) Staining for cyan fluorescent protein (CFP, green) and endogenous PKCδ (red) confirms overlap (98% of CFP+ cells were PKCδ+; 100% of PKCδ+ cells were CFP+). White dashed lines denote the contours of CEA (CEI, lateral subdivision; CEm, medial subdivision). Scale bar, 30 µm. (b) Left, injection of conditional AAVs expressing ChR2-YFP or ARCH-GFP into CEA. Middle, overlap of endogenous PKCδ (red) and ChR2-YFP (green). Right, overlap of endogenous PKCδ (red) and ARCH-GFP (green). Arrows, cells stained only for PKCδ; triangles, cells with overlap. Scale bar, 20 µm. (c) Top, configuration of optrode implants; bottom, identification of light-responsive units using 10-ms blue light pulses for ChR2-expressing neurons or 300-ms yellow light pulses for ARCH-expressing neurons. Right, population analysis of identified ChR2-expressing PKCδ+ neurons before, during and after 3 min blue light stimulation. Bottom, PSTH of the z-score shows a marked increase in firing during the light pulse. Inset, z-score of the short-latency light-induced responses. (d) Rate histogram average of identified ChR2-expressing PKCδ+ neurons before, during and after 3 min light stimulation (n = 5 cells, 2 mice). (e) z-score histogram showing increased firing of ChR2-expressing PKCδ+ neurons during 3 min light stimulation (n = 5 cells, 2 mice). (g) Top, raster plot showing increased firing of an identified ChR2-expressing PKCδ+ neuron before, during and after 300 ms blue light stimulation. Bottom, PSTH of the z-score shows a marked increase in firing during the light pulse. (h) Rate histogram average of identified ARCH-expressing PKCδ+ neurons before, during and after 3 min light stimulation (n = 6 cells, 3 mice). (i) z-score histogram showing increased firing of ARCH-expressing PKCδ+ neurons during light stimulation (n = 6 cells, 3 mice). (j) Bilaterally implantation of optical fibers in a freely moving mouse after injection of DIO-AAVs expressing either ChR2 or ARCH. (k) Coronal section of a mouse brain indicating the location of CEA and the expression of a conditional rAAV expressing ChR2 and the fluorescent protein mCherry (orange). White dashed lines, anatomical boundaries of basolateral amygdala (BLA), lateral CEA (CEI) and medial CEA (CEm); blue dashed line, optical fiber insertion. Scale bar, 500 µm. (l) Top, experimental protocol. Bottom, increased fear generalization upon stimulation with blue light in PKCδ-Cre animals injected with DIO-AAV-ChR2 (n=7 mice), but not in control implanted animals subjected to the same optogenetic protocol (n=6 mice; two-way ANOVA: F1,11 = 11.83, \( P = 0.005 \) for the interaction injection \times light stimulation; post hoc Sidak’s multiple comparison test: \( P = 0.01 \) for lightoff versus lighton in DIO-AAV-ChR2 injected mice). \( *P = 0.008, \) paired t-test between lighton and lightoff in ChR2 group, \( t = -3.952, \) d.f. = 0.946, paired t-test between lightoff and lighton in control group, \( t = 0.0707, \) 5 d.f. (m) Top, protocol for analyzing optogenetic manipulations of PKCδ+ neurons during EPM behavior. EPM and OFT behavior was analyzed before fear conditioning. Bottom, example EPM trajectories of PKCδ-Cre animals injected with rAAV 2/7 EF1α::DIO-ChR2(H134R)-2A-NpHR-2A-Venus (top) or rAAV 2/5 CBA::DIO-ARCH-GFP (bottom) under lighton or lightoff conditions. (n) Enhancing the activity of PKCδ+ neurons decreases the percentage of time animals spend on open arms (n = 7 mice, \( **P = 0.006, \) paired t-test, \( t = -3.952, \) 6 d.f. between lighton and lightoff for ChR2 group), whereas decreasing the activity of PKCδ+ neurons has the opposite effect (n = 6 mice, \( **P = 0.005, \) paired t-test, \( t = -4.702, \) 5 d.f. between lightoff and lighton for ARCH group). The behavior of control animals was not altered by light stimulation (n = 9 mice, \( P = 0.214, \) paired t-test, \( t = 1.349, \) 8 d.f. between lighton and lightoff for control group). Two-way ANOVA: \( F2,19 = 26.72, \) \( *P < 0.001 \) for the interaction injection \times light stimulation; post hoc pairwise Sidak’s tests revealed significant differences between lighton in the ARCH group, lighton in the ChR2 and control group. (o) Top, protocol for optogenetic manipulations of PKCδ+ neurons during OFT. Bottom, example open-field trajectories of PKCδ-Cre animals injected with rAAV 2/7 EF1α::DIO-ChR2(H134R)-2A-NpHR-2A-Venus (top) or rAAV 2/5 CBA::DIO-ARCH-GFP (bottom) under lighton or lightoff conditions. (p) Enhancing the activity of PKCδ+ neurons decreases the number of center crossings (red square) per unit track length (n = 7 mice, \( **P = 0.006, \) paired t-test between lighton and lightoff for ChR2 group, \( t = 4.124, \) 6 d.f.), whereas decreasing the activity of PKCδ+ neurons has the opposite effect (n = 8 mice, \( **P = 0.007, \) paired t-test between lighton and lightoff for ARCH group, \( t = -3.956, \) 6 d.f.). The behavior of control animals was not altered by light stimulation (n = 7 mice, \( P = 0.408, \) paired t-test between lighton and lightoff for control group, \( t = 0.880, \) 7 d.f.). Two-way ANOVA: \( F2,19 = 8.587, \) \( *P = 0.002 \) for the interaction injection \times light stimulation; post hoc pairwise Sidak’s tests revealed significant differences between lighton in the ARCH group, lighton in the ChR2 and control group. All error bars indicate mean ± s.e.m.
These data support the idea that a tonic extrasynaptic GABA$_A$R conductance is an endogenous mechanism regulating basal PKC$\delta^+$ neuron activity.

Our observation that extrasynaptic inhibition in CEA PKC$\delta^+$ neurons was largely insensitive to the competitive GABA AR antagonist SR-95531 (1–50 µM) suggests either that extrasynaptic GABA$_A$Rs exhibit a very high affinity for GABA$\beta_1$$\gamma_2$ and/or that they can open in a spontaneous, ligand-independent manner$^{20,21}$. Consistent with the latter scenario, we found that SR-95531 (20 µM) partially antagonized the inverse agonist effect of bicuculline (20 µM) on the holding current$^{20}$ (Supplementary Fig. 4). To further address whether extrasynaptic GABA$_A$Rs in CEA PKC$\delta^+$ neurons could also report spillover of synaptically released GABA, we blocked action potential–mediated release with tetrodotoxin (TTX; 1 µM). Application of TTX reduced the sIPSC frequency in PKC$\delta^+$ neurons and resulted in a slight reduction in extrasynaptic inhibition, which correlated with the effect on sIPSC frequency (Supplementary Fig. 4). These findings suggest that both ligand-independent receptor opening and GABA spillover contribute to endogenous extrasynaptic GABA$_A$R inhibition in these neurons.

Extrasynaptic GABA$_A$Rs in other brain areas have been shown to contain $\alpha_2/\delta$, $\alpha_3/\delta$ or $\alpha_5$ subunits$^{22}$. Because $\alpha_5$-containing GABA$_A$Rs ($\alpha_5$-GABA$_A$Rs) are abundant in CEA$^{23,24}$ and their expression decreases both after fear conditioning$^{24}$ and in a mouse model of increased trait anxiety$^{25}$, we suspected that $\alpha_5$-GABA$_A$Rs might contribute to extrasynaptic inhibition in CEA PKC$\delta^+$ neurons. Immunohistochemical
double labeling of CEA sections from PKCδ-Cre-CFP mice with an anti-α5 subunit antibody and an anti-CFP antibody\(^2\) revealed that about 70% of PKCδ neurons expressed the α5 subunit (Supplementary Fig. 5). No α5 immunohistochemical labeling could be detected in CEA sections obtained from mice lacking the gene encoding the α5 subunit (Gabra5\(^{-/-}\) mice) (Supplementary Fig. 5). Next, to test for the presence of functional α5-GABA\(_A\)Rs in CEA PKCδ neurons, we applied two distinct α5 inverse agonists. Both L-655,708 (50 nM–50 μM) and the more specific inverse agonist methyl-[8-chloro-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5-α][1,4]benzodiazepin-3-yl]methyl ether, or PWZ-029 (100 nM–1 μM)\(^2\) reduced the extracellular GABA\(_A\)R conductance (Fig. 3d and Supplementary Fig. 6). In contrast, the neurosteroid 30,50α-tetrahydrodeoxycorticosterone (10–100 nM), a modulator of δ-containing GABA\(_A\)Rs, and zolpidem (20–300 nM), an agonist of α1-containing GABA\(_A\)Rs, had no effect (Supplementary Fig. 6). The effect of 1 μM PWZ-029 was specific for α5-GABA\(_A\)Rs, as it was absent in PKCδ neurons recorded in slices obtained from mice lacking α5 receptors in PKCδ neurons (α5\(^{-/-}\) mice; Prkcd-cre × Gabra5\(^{loxP/loxE}\)) (Fig. 3d). Like application of PTX, application of PWZ-029 increased the spontaneous activity of PKCδ neurons recorded in the cell-attached configuration in acute CEA slices (Fig. 3e).

Consistent with the notion that α5-GABA\(_A\)Rs underlie a tonically active conductance mediated by extrasynaptic receptors, two different inverse agonists of α5-GABA\(_A\)Rs, L-655,708 and PWZ-029, had no effect on sIPSC frequency or amplitude in PKCδ neurons (Supplementary Fig. 6). To unequivocally demonstrate the extrasynaptic localization of α5-GABA\(_A\)Rs in PKCδ neurons, we performed pre-embedding electron microscopy. Consistent with the electrophysiological data, this electron microscopy revealed that immunometal particles labeling α5-GABA\(_A\)Rs in identified PKCδ neurons localized predominantly to extrasynaptic dendritic regions, with very little synaptic labeling (Fig. 3f). Compared to PKCδ neurons, PKCδ neurons exhibited higher levels of extrasynaptic α5-GABA\(_A\)Rs as measured with electrophysiological or electron microscopy approaches (Supplementary Fig. 7).

Figure 4 Experience-dependent reduction of extrasynaptic inhibition predicts fear generalization. (a) Freezing before (baseline) and during the presentation of the CS\(^-\) and CS\(^+\) 24 h after training for animals exposed to CS only (n = 8 mice) and animals subjected to CS–US pairings (n = 8 mice; ***P < 0.001 for CS\(^+\) in CS–US group, H = 81.715, 2 d.f.; P = 0.412 for CS only group, H = 1.771, 2 d.f.; Kruskal-Wallis one-way ANOVA followed by Dunn’s pairwise multiple-comparisons test). (b) Representative current traces recorded in vitro from PKCδ neurons in slices obtained from control (CS only) and fear conditioned (CS–US) animals. Traces illustrate the sequential application of L-655,708 (50 nM) and PTX (100 μM). Scale bar: 50 pA, 10 s. (c) Left, to test for the presence of functional α5-GABA\(_A\)Rs in CEA PKCδ neurons, we applied two distinct α5 inverse agonists. Both L-655,708 (50 nM–50 μM) and the more specific inverse agonist methyl-[8-chloro-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5-α][1,4]benzodiazepin-3-yl]methyl ether, or PWZ-029 (100 nM–1 μM)\(^2\) reduced the extracellular GABA\(_A\)R conductance (Fig. 3d and Supplementary Fig. 6). In contrast, the neurosteroid 30,50α-tetrahydrodeoxycorticosterone (10–100 nM), a modulator of δ-containing GABA\(_A\)Rs, and zolpidem (20–300 nM), an agonist of α1-containing GABA\(_A\)Rs, had no effect (Supplementary Fig. 6). The effect of 1 μM PWZ-029 was specific for α5-GABA\(_A\)Rs, as it was absent in PKCδ neurons recorded in slices obtained from mice lacking α5 receptors in PKCδ neurons (α5\(^{-/-}\) mice; Prkcd-cre × Gabra5\(^{loxP/loxE}\)) (Fig. 3d). Like application of PTX, application of PWZ-029 increased the spontaneous activity of PKCδ neurons recorded in the cell-attached configuration in acute CEA slices (Fig. 3e).

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Finally, we performed extracellular single-unit recordings in the CEA of freely moving animals and locally applied PWZ-029 using glass micropipettes. Local application of PWZ-029 increased the spontaneous firing rate of CEA units compared to that of vehicle controls (Supplementary Fig. 8), providing further evidence that endogenous α5 GABAR-mediated inhibition controls spontaneous activity of CEA neurons in vivo.

**Experience-dependent reduction of extrasynaptic inhibition**

To test whether the previously described, fear conditioning–induced increases in PKCδ neuron firing rate are paralleled by changes in endogenous, functional extrasynaptic GABAAR-mediated inhibition, we compared extrasynaptic GABAAR conductances in PKCδ+ neurons recorded ex vivo in slices obtained from fear-conditioned animals or from control animals that were only exposed to unreinforced CSs (Fig. 4a). PKCδ+ neurons recorded from animals subjected to CS–US pairings exhibited a marked reduction in the total extrasynaptic GABAAR conductance and in the tonic conductance mediated by α5-containing receptors, with no changes in sIPSC frequency or amplitude, nor in the tonic conductance mediated by δ-containing receptors (Fig. 4b,c and Supplementary Fig. 9). In addition, this reduction in α5-GABAAR-mediated extrasynaptic inhibition was cell type specific, as PKCδ− neurons exhibited the opposite effect (Supplementary Fig. 9).

A similar reduction in α5-GABAAR-mediated extrasynaptic inhibition in PKCδ+ neurons along with increased EPM anxiety was observed in animals exposed to a contextual fear conditioning protocol (US only: Supplementary Fig. 9), indicating that these changes are not specifically induced by auditory CS–US associations acquired during cued fear conditioning, but are rather a consequence of US exposure. Notably, in animals subjected to discriminative cued or contextual fear conditioning, we observed a striking negative correlation between fear generalization, as measured by CS+/CS− or context discrimination, and the amplitude of the extrasynaptic GABAAR conductance (Fig. 4d and Supplementary Fig. 9). These data suggest that an experience-dependent reduction in extrasynaptic inhibition of PKCδ+ neurons may contribute to fear generalization at the behavioral level.

**Reducing α5-GABAAR expression is anxiogenic**

We therefore directly examined whether interfering with the expression of the α5 subunit in CEA would be causally related to fear generalization. First we used a brain area–specific approach by injecting an AAV expressing Cre recombinase bilaterally into CEA of conditional α5-floxed animals (α5+/−, Gabra5loxP/loxP) (Fig. 5a). In whole-cell recordings from AAV-Cre-infected neurons in slices obtained from α5+/− animals, α5-GABAAR-mediated extrasynaptic inhibition was completely abolished, whereas it was normal in neighboring uninfected...
neurons or in infected neurons in slices from α5+/− animals (Fig. 5b).
Behavioral analysis of pAAV-Cre infected α5+/+ animals showed that
 genetic deletion of α5-GABAARs in CEA was anxiogenic as measured
by increased EPM anxiety and enhanced fear generalization (Fig. 5c.d).
Even though these data are consistent with the notion that α5-
GABAARs regulate anxiety by controlling the activity of PKCδ
neurons, an obvious caveat to this genetic approach is that it is not
cell type specific. Given that, upon fear conditioning, the α5-GABAAR-mediated
electrophysiological conductance changes in opposite directions in PKCδ
and PKCθ cells, and consistent with the notion that PKCθ cells are
thought to be largely downstream of PKCδ cells8,9, this also suggests
that the conductance decrease in PKCθ neurons is dominant with
regard to the manipulation of PKCθ cells and may be sufficient to
induce anxiety behavior.

To manipulate α5 subunit expression in a brain area– and cell
type–specific manner, we devised a Cre-conditional short hairpin
RNA construct targeting Gabra5 mRNA (Supplementary Fig. 10).
In HEK293T cells, expression of Gabra5 shRNA strongly reduced
α5-GABAAR protein levels (Supplementary Fig. 10). We then
specifically expressed this shRNA construct in CEA PKCδ neurons by
local injection of a DIO (double-floxed inverted open reading frame)-
AAV expressing the Gabra5 shRNA or scrambled control shRNA into the
CEA of PKCδ-Cre mice (Fig. 6a). Comparison of the remaining
extrasynaptic GABAAR conductance in PKCδ neurons infected with
Gabra5 shRNA or with scrambled control shRNA revealed that
expression of the Gabra5 shRNA strongly decreased both total and
α5-mediated extrasynaptic inhibition (Fig. 6b–d), further indicating
that extrasynaptic GABAAR currents are predominantly mediated by
α5-containing GABAARs in these neurons.

To test whether reducing the expression of α5-GABAAR subunits
specifically in CEA PKCδ neurons influences anxiety behavior and
fear generalization, we bilaterally injected PKCδ-Cre animals with
DIO-AAV Gabra5 shRNA or scrambled control shRNA. Four weeks
after injection, mice underwent EPM and OFT behavior studies and
were subsequently subjected to discriminative fear conditioning.
Animals injected with Gabra5 shRNA spent significantly less time on
the open arms of the EPM, exhibited fewer center crossings and spent
less time in the center area in the OFT, and showed enhanced fear
generalization compared to animals injected with a vector expressing
control shRNA (Fig. 6e–g and Supplementary Fig. 2), thus indicating
that reducing α5-GABAAR expression in PKCδ neurons was suffi-
cient to cause increased anxiety behavior.

DISCUSSION
Taken together, our results identify the regulation of cellular excit-
ability through extrasynaptic inhibition mediated by α5-GABAARs in
CEA PKCδ neurons as an important mechanism for the orches-
tration of a behavioral program associated with generalized fear and
sustained anxiety. In principle, regulation of extrasynaptic inhibition
might be achieved by changes in the numbers and/or properties of
α5-GABAARs expressed by CEA PKCδ neurons. Alternatively,
alterations in ambient GABA concentration might also contribute.
However, given our observations that part of the tonic conductance
was mediated by spontaneous ligand-independent receptor open-
ings, that fear conditioning induced opposite changes in distinct cell
types, and that there are α5-GABAARs associated with intracellular
compartments, an experience-dependent change in the expression
and/or trafficking of α5-GABAARs is the most parsimonious explana-
tion. Previous studies involving genetic or systemic pharmacological
manipulations leading to reduced or completely absent α5-mediated
extrasynaptic inhibition revealed an improved performance in cognitive
learning tasks and mild anxiety-like behavior22,26–28. Given that fear
conditioning and trait anxiety are associated with decreased Gabra5
mRNA24,25, this suggests that the cell type–specific regulation of
α5-GABAARs controls the excitability of CEA PKCδ neurons in an
experience-dependent manner.

Our present findings demonstrate that PKCδ neurons are a
central gateway not only to the circuitry controlling acute fear
responses8,9 but also to the circuitry underlying sustained
anxiety. Moreover, our results suggest that regulation of the cellular
excitability of PKCδ neurons, or upstream CEA neurons, could be
a general mechanism by which external or internal stimuli, such as
aversive experience, stress, nausea, satiety or drugs of abuse such as
ethanol, modulate complex behavioral states including anxiety and
feeding behavior.23,29,30 This might involve additional, α5-GABAAR-R
independent mechanisms, such as the regulation of potassium
channels through neuromodulatory– or neuromodulator-activated
G protein–coupled receptors39.

Studies on cerebellar granule cells indicate that the level of tonic
inhibition modulates the gain, and hence the signal–to–noise ratio, of
sensory-evoked phasic responses16,17,31. Future experiments will have
to address how tonic inhibition mediated by extrasynaptic GABAARs in
CEA interacts with phasic responses elicited by acute conditioned
or unconditioned sensory stimuli. A further important open question
is whether the phasic and sustained signals are read out by the same
population of downstream neurons or by different populations of
neurons, possibly even located in different downstream brain regions.
In conclusion, our study not only identifies a molecular mechanism
underlying the regulation of an entire repertoire of concerted behav-
ioral changes associated with anxiety states, but also provides a defined
cellular entry point into the neuronal circuitry underlying a complex
behavioral state such as anxiety.

METHODS
Methods and any associated references are available in the online
version of the paper.

Note: Any Supplementary Information and Source Data files are available in the
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AUTHOR CONTRIBUTIONS
The manuscript was prepared by A.L. and P.B. Electrophysiological recordings in vitro, behavioral experiments and viral injections were performed by P.B.
Pharmacology combined with single-unit recordings in freely moving animals
were designed and performed by L.D. In vivo recordings from optogenetically
identified neurons were performed by M.M. and J.P.F. Immunohistochemistry
was accomplished by C.X. T.L. cloned the conditional shRNA plasmid. M.M.P.
and J.M.C. provided the PWZ-029. L.X. tested the shRNA efficacy on
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U.R. provided perfused brains of α5−/− mice and of α5+/+ mice. All electron microscopy experiments were performed and analyzed by Y.K. and F.F.

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The authors declare competing financial interests: details are available in the online version of the paper.

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Behavior. Auditory and contextual fear conditioning. Fear conditioning and fear retrieval took place in two different contexts, A and B. The conditioning and retrieval boxes and the floor were cleaned before and after each session with 70% ethanol or 1% acetic acid before and after each session, respectively. To score freezing behavior, an automatic infrared beam detection system placed on the bottom of the experimenter's chambers (Coulbourn Instruments) was used. Mice were considered to be freezing if no movement was detected for 2 s. The measure was expressed as a percentage of time spent freezing. To ensure that our automatic system scores freezing rather than just immobility, we previously compared the values obtained with those measured using a classical time-sampling procedure during which an experimenter blind to the experimental conditions determined the mice to be freezing or not freezing every 2 s (defined as the complete absence of movement except for respiratory movements). The values obtained were 95% identical and the automatic detection system was therefore used throughout the experimental sessions. Tones were presented as CSs and the CS− (total CS duration of 30 s, consisting of 50-mips pips at 0.9 Hz, 2-ms rise and fall; pip frequency 7.5 kHz or white noise, 80 dB sound pressure level). Discriminative fear conditioning was performed on day 1 by pairing the CS+ with a US (1-s foot shock, 0.6 mA, five CS+/US pairings; intertrial interval 20–180 s) (CS− US group). The onset of the US coincided with the offset of the CS+. The CS− was presented after each CS+/US association but was never reinforced (five CS− presentations, inter-trial interval 20–180 s). The frequencies used for CS+ and CS− were counterbalanced across animals. On day 2, conditioned mice were submitted to fear retrieval in context B, during which they received four presentations of the CS− and the CS+ each. Control animals (CS only) were treated in the same manner but were not exposed to the US, and they did not freeze during exposure to the tones (Fig. 4a,b). The US-only group was exposed to five USs. Auditory fear generalization was quantified by calculating the ratio between the freezing values during the CS+ and CS− presentations. Contextual fear generalization was quantified by calculating the ratio between the freezing values during exposure to a novel context, CCTX, and to the conditioning context CTX.

Open-field test. All anxiety tests (open field and elevated plus maze) shown in Figures 1 and 4 were performed before subjecting animals to fear conditioning procedures. Mice were always placed at the periphery of an open field arena (50 cm × 50 cm) placed in a larger, sound-attenuated box. Light intensity inside the open field arena was 1.2 lux. Movements were monitored by a camera (Logitech) located on top of the arena. AVI files were analyzed using ViewerII 5.1 software (Bioserve GmbH). The open field was divided in center area (20 cm × 20 cm) and peripheral area. Total track length was calculated with the center of the animal’s body as the reference; the number of visits to the center area was scored when all four paws were located in the center area. Behavior was scored and statistically analyzed during the first 5 min.

Elevated plus maze (EPM). The EPM was composed of two arms enclosed by light gray walls and two open arms (length 230 mm each, elevated 300 mm above ground). Mice were placed in the center area between the arms and their behavior was monitored for 10 min with a camera (Logitech, Newark, CA, USA) placed on the top of the maze. After every behavioral session, the maze was cleaned with an ethanol-based disinfectant solution (Fugaten Spray; Lysoform Dr. Hans Rosemann GmbH, Germany). This solution has a different smell than the ethanol or acetic acid used for cleaning the fear-conditioning context. Video tracking software (ViewerII 5.1 software, Bioserve GmbH) was used to track the animal’s location. Compartment visits were scored only when the animal had all four paws in one area. For all the behavioral sessions, the experimental groups were tested in an interleaved manner to avoid batch effects.
prior drug applications. Whole-cell patch-clamp recordings were excluded if the access resistance exceeded 13 MΩ or changed more than 20% during the recordings. Seal resistance, for cell-attached recordings, was around 20 to 50 MΩ and data were excluded if it changed more that 20% from the initial value. Data were recorded with a MultiClamp 700B (Molecular Devices) amplifier, filtered at 0.2 kHz and digitized at 10 kHz. Data were acquired and analyzed with Clampex 10.0, Clampfit 10.0 (Molecular Devices) and the Mini Analysis Program (Synaptosoft, Decatur, GA). All chemicals for the internal and external solutions were purchased from FluKoSigma (Buchs, Switzerland). Glutamatergic blockers were purchased from Tocris Bioscience (Bristol, UK). TTX was from Latoxan (Valence, France). Firing frequency elicited by injection of square current pulses shown to be normalized by the maximum value evoked by the maximum current step for each cell.

Single-unit recordings and in vivo pharmacology. Single-unit recordings and in vivo pharmacology were performed in chronically implanted animals. Three-to-4-month-old mice were anesthetized with isoflurane (induction: 4%, maintenance: 1.5%, Attane, Minrad Inc., Buffalo, NY, USA) in oxygen-enriched air (Oxymat 3, Weimann, Hamburg, Germany) and fixed in a stereotaxic frame (Kopf Instruments, Tujunga, California, USA). Core body temperature was maintained at 36.5 °C by a feedback-controlled heating pad (FHC, Bowdoinham, ME, USA). Analgesia was provided by local injection of ropivacain (200 µl of 2 mg/ml, s.c., Naropin, AstraZeneca, Switzerland) and systemic injection of meloxicam (100 µl of 5 mg/ml, i.p., Metacam, Boehringer-Ingelheim, Ingelheim, Germany). Mice were unilaterally implanted in the CEA with a custom-built injector consisting of a multi-wire electrode attached to a guide cannula (26 gauge, with dummy screw caps, Plastics One, Roanoke, USA) and aimed at the injection site, 80-µm coronal slices on a vibratome (Leica Microsystems, Heerbrugg, Switzerland). Free-floating sections were rinsed in PBS. Subsequently, sections were incubated in blocking solution (20% bovine serum albumin (BSA) and 0.5% Triton X-100 in PBS (PBST)) for 2 h. Sections were then incubated in blocking solution (3% BSA and 0.5% PBST) containing the primary polyclonal rabbit anti-α-tubulin antibody (1:500, gift from W. Sieghart, Medical University of Vienna, Vienna, Austria) and goat anti-GFP antibody for PKCδ-Cre-CFP (1:1,000, Abcam) for 48 h at 4 °C. Subsequently, sections were washed with PBS three times (5 min each) and incubated for 4 h at room temperature with fluorescent donkey anti-rabbit–Alexa Fluor 594 and donkey anti-goat–Alexa Fluor 488 (Invitrogen; both 1:500 in 3% BSA and 0.5% PBST). Finally, immunolabeled sections were rinsed three times with PBS, mounted on gelatin-coated slides, dehydrated and coverslipped. The brains from wild-type and Gabra5−/− mice were treated with the same staining procedures and imaged with the same settings using a LSM 700 confocal microscope (Carl Zeiss AG, Germany). For the other stainings, the brains were postfixed in PFA for 4 h at 4 °C and then cut into 80-µm-thick coronal slices in PBS. Sections were then incubated in blocking solution (3% BSA and 0.5% PBST). The immunostaining for PKCδ-Cre-CFP was performed on free-floating brain sections by overnight incubation at 4 °C with goat anti-GFP antibody (1:1,000, Abcam) and then 2 h incubation at room temperature with anti-goat–Alexa Fluor 488. The endogenous PKCδ+ samples were stained by overnight incubation with mouse anti-PKCδ+ antibody (1:500, BD Transduction Laboratories) at 4 °C and then overnight incubation at 4 °C with goat anti-mouse–Alexa 568. The NeuN samples were stained by overnight incubation at 4 °C with mouse anti-NeuN antibody (1:500, Millipore) and then overnight incubation at 4 °C with goat anti-mouse–Alexa 568. Finally, slices were imaged with a LSM 700 confocal microscope (Carl Zeiss AG, Germany). The fluorescence intensity of AAV samples is strong enough to allow clear pictures with the confocal microscope without specific immunostaining. Antibodies used: goat anti-GFP antibody for PKCδ-Cre-CFP (1:1,000, Abcam), catalog number 194017-1; mouse anti-PKCδ, BD Transduction Laboratories, catalog number 610398; GABAAR α1 antibody, kindly provided by W. Sieghart (as in electron microscopy section, below); anti-neuN (Millipore), catalog number MAB377; donkey anti-goat–Alexa Fluor 488, Invitrogen, catalog number A11055; donkey anti-rabbit–Alexa Fluor 594, Invitrogen, catalog number A-12107; donkey anti–mouse IgG–Alexa Fluor 688, Invitrogen, catalog number A10037.

Electron microscopy. Analysis was carried out on adult male C57Bl/6 mice (n = 3; 25–30 g; Charles River, Sulzfeld, Germany). Before use, the animals were...
housed in groups of four or five under controlled laboratory conditions (12:12 h light/dark cycle with lights on at 07:00 h; 21 ± 1 °C; 60% humidity) with food and water ad libitum for at least 2 weeks after delivery from the supplier. Animals were deeply anesthetized by intraperitoneal injection of thiopental (100 mg/kg, i.p.) and perfused transcardially with PBS (0.9% NaCl, pH 7.4) followed by ice-cold fixative made of 4% w/v paraformaldehyde, 0.05% w/v glutaraldehyde and 15% v/v of a saturated solution of picric acid in phosphate buffer (PB; 0.1 M, pH 7.4) for 10 min. Brains were immediately removed from the skull, washed in 0.1 M PB and stored in 0.1 M PB containing 0.05% sodium azide at 6 °C until immunohistochemical experiments were performed. Tissue blocks were cryo-
protected in 20% sucrose in 0.1 M PB overnight at 4 °C and were freeze-thawed once to allow antibody penetration. The brain tissue blocks were sliced coronally into 70-μm-thick sections on a Leica VT1000S vibratome (Leica Microsystems, Vienna, Austria). The free-floating sections were incubated in mouse Ig Blocking Reagent (diluted 1:27.7; Vector Laboratories, Burlingame, CA, USA) in Tris-buff-
ered saline (TBS; 50 mM, 0.9% NaCl, pH 7.4) for 1 h at room temperature (RT) and then incubated in a blocking solution containing 20% normal goat serum (NGS) in TBS for 1 h at RT. The sections were incubated for three overnights at 6 °C in primary antibodies diluted in TBS containing 2% normal goat serum. The primary antibodies were a rabbit antibody against the α5-GABAAR subunit (kindly provided by W. Sieghart, Medical University of Vienna, Austria) and mouse monoclonal antibody against PKCβ (catalog number 610397, BD Biosciences, Franklin Lakes, NJ, USA). They were diluted 1:200 and 1:200, respectively. After three 10-min washes with TBS, 1.4-mm nanogold-conjugated goat anti-rabbit (Nanoprobes, Yaphank, NY, USA; catalog number 2004) and biotinylated goat anti-mouse antibodies (Vector Laboratories; catalog number BA-9200) were applied overnight at 6 °C at a dilution of 1:100 in a buffer with the same composition as for the primary antibody. The sections were then washed and incubated in 1% glutaraldehyde and 4% PFA in PB 0.1 M for 10 min at RT. After the additional fixation, the sections were rinsed with ultra-pure water. Nanogold particles were amplified with silver using the HQ Silver Enhancement kit (Nanoprobes) for 8 min and then for another 5 min with fresh solution at RT under light microscopy control. The sections were washed extensively in MilliQ water and then with TB and were reacted with 0.015% 3,3′-diaminobenzidine (DAB; Sigma, Munich, Germany) and 0.4% nickel (II) sulfate hexahydrate (Sigma) as chromogen and 0.01% H2O2 as the electron donor. The sections were subsequently washed with 0.1 M PB and treated with 2% OsO4 in 0.1 M PB for 40 min at RT. After several rinses with 0.1 M PB and then with MilliQ water, the sections were contrasted with 1% uranyl acetate in 50% ethanol for 30 min at RT protected from light. The dehydration steps were done with graded ethanol (50, 70, 90 and 100%) and propylene oxide at RT before embedding in epoxy resin. The sections were transferred into weighting boats containing epoxy resin (Durecupan ACM-Fluka, Sigma) and kept overnight at RT. The sections were then transferred onto siliconized slides, coveredlipped with ACLA film coverslips (Ted Pella, Inc., Redding, CA, USA) and incubated for 3 d at 60 °C. Pieces containing CEA were trimmed and reembedded in the resin for 2 d at 60 °C. Ultrathin sections at 60 nm were examined in a Philips CM120 TEM, equipped with a Morada (Nanoprobes) as chromogen and 0.01% H2O2 as the electron donor. The sections were

**Cre-regulated knockdown of α5 subunits.** Four pairs of DNA oligonucleotides targeting the mouse α5-GABAAR were designed using RNAi Explorer and tested in HEK293T cells by cotransfacing the rat α5 subunit with the knockdown constructs. The HEK293T cell line was Mycoplasma negative. Sequence no. 2 (TCCATTGCACACACATACTCATCAGNM; NM_176942.4, 765–784) showed the best knockdown (**Supplementary Fig. 10**). For conditional expression, the RNAi construct was inserted into a modified lentivox 3.7 (pLL3.7) dsRed (pSICO) vector that containsloxP sites within the TATAbox sequence35. The oligonucleotide for the shRNA was cloned into pSICO digested with XhoI and Hpal. A scrambled control oligonucleotide (CATACGGTCAATCCTCAGAACA) was also synthesized and constructed in the same vector. All constructs were verified by sequencing. To test conditional expression, HEK293T cells were plated into 24-well plate with a density of 8.0 × 104 cells per well and were transfected with constructs expressing the rat α5 subunit, α5-GABAAR, knockdown or the scrambled control shRNA and Cre at a ratio of 1:1. The cells were washed with PB and lysed in 200 μl sample buffer. Twenty microilters of each sample were used for SDS-PAGE and western blots. The α5 antibody (Novus, NB300–195) was diluted at a ratio of 1:1000.

**Generation of conditional AAV Gabra5 iRNA constructs.** The AAV shRNA constructs allow conditional (Cre-loxP), stable expression of both shRNA for RNA interference under the promoter U6, and reporter protein tdTomato driven by the EF1α (EF1α) promoter. The sequences of both Gabra5 shRNA and control shRNA were first synthesized with EcoRI/EcoRV restriction sites at each end and inserted into pBMH vector (Biomatik USA, LLC). Gabra5 shRNA: TGTCATTGCACACACACTCATCTCAAGAGGATCGTTGTTGGCAGATGGTCTTTTTC. Control shRNA: TGCATAACGTCAATCTCATAACTACGGTCAATCCTCAG.

The pBMH shRNA constructs were digested with EcoRI/EcoRV initially, and the shRNA-containing segments were recycled and purified for ligation with pAAV-EF1α-DIO-hChR2YFP (Deisseroth laboratory, Stanford University) to insert the shRNA sequences after the secondlox271/loxP site. Mouse U6 promoter was synthesized with EcoRI and EcoRV sites for inserting into pAAV-EF1α-tdTomato-WPRE-pA to generate the segment mU6-TdTomato, which later replaced the hChR2YFP of the pAAV-EF1α-DIO-hChR2YFP-shRNA-WPRE-pA backbone designed with Ascl and NheI restriction sites. The expression of tdTomato driven by the EF1α promoter and shRNA driven by the mU6 promoter is achieved at the same time upon Cre recombination34 (**Supplementary Fig. 10**). The constructs were sequenced before being amplified with an endonuclease-free column (Macherey-nagel; Germany) and were further validated in cultured cells by co-transfecting a Cre construct (data not shown). Two AAVs (serotype9; Vector Core; University of Pennsylvania) for the expression of Gabra5 shRNA (pAAV-EF1α-DIO-tdTomato-U6-Gabra5.iRNA) and control shRNA (pAAV-EF1α-DIO-tdTomato-U6-control.shRNA) were injected into the CEA of the PKCβ-Cre-EYFP transgenic animals to ensure cell type–specific knockdown of the α5 subunit in PKCβ cells in CEI.

**Statistics.** Normal data distribution and equal variance was formally tested using SigmaPlot 13.0 or GraphPad Prism 6 for all statistical analyses. No statistical methods were used to predetermine sample sizes, but our sample sizes are similar to those reported in previous publications18,30,31. Data collection and analysis were not performed blind to the conditions of the experiments. Data were collected and processed randomly apart from the data collected from grouped animals. SigmaPlot does not allow one to extract precise P values if P < 0.001 for every statistical analysis reported. GraphPad Prism does not allow one to extract precise P values when performing post hoc multiple-comparison tests. A **Supplementary Methods Checklist** is available.

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Corrigendum: Regulating anxiety with extrasynaptic inhibition

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