Research

Differential regulation of glutamic acid decarboxylase gene expression after extinction of a recent memory vs. intermediate memory

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Extinction reduces fear to stimuli that were once associated with an aversive event by no longer coupling the stimulus with the aversive event. Extinction learning is supported by a network comprising the amygdala, hippocampus, and prefrontal cortex. Previous studies implicate a critical role of GABA in extinction learning, specifically the GAD65 isoform of the GABA synthesizing enzyme glutamic acid decarboxylase (GAD). However, a detailed analysis of changes in gene expression of GAD in the subregions comprising the extinction network has not been undertaken. Here, we report changes in gene expression of the GAD65 and GAD67 isoforms of GAD, as measured by relative quantitative real-time RT-PCR, in subregions of the amygdala, hippocampus, and prefrontal cortex 24–26 h after extinction of a recent (1-d) or intermediate (14-d) fear memory. Our results show that extinction of a recent memory induces a down-regulation of *Gad65* gene expression in the hippocampus (CA1, dentate gyrus) and an up-regulation of *Gad67* gene expression in the infralimbic cortex. Extinguishing an intermediate memory increased *Gad65* gene expression in the central amygdala. These results indicate a differential regulation of *Gad* gene expression after extinction of a recent memory vs. intermediate memory.

[Supplemental material is available for this article.]

Learning to fear and accurately predict which environmental stimuli are potentially dangerous is an important survival skill. However, learned fear that persists into situations that are no longer dangerous is not beneficial and can lead to anxiety disorders, such as Post-Traumatic Stress Disorder (PTSD). Learning to fear requires the amygdala (LeDoux 2000). The hippocampus and prefrontal cortex (PFC) modulate amygdala-dependent fear memories, and the three structures together form a network central to extinction (Quirk and Mueller 2008).

Exposure to stress induces long-term adaptations in the brain that involve persistent alterations in gene expression (Briand and Blendy 2010). Most studies investigate how extinction affects a recently formed fear memory, usually administering extinction training 24 h after fear learning. Since PTSD patients do not typically receive treatment within the first 24 h post-trauma, more studies should be directed to how extinction affects older fear memories. Extinguishing an older, maintained memory may initiate different mechanisms than when extinguishing a recently formed memory. In fact, administering extinction immediately after fear learning involves a mechanism that is unique to when extinction is 24 h after fear learning (Myers et al. 2006).

Several studies have demonstrated a role for GABA in extinction learning (for review, see Myers and Davis 2007; Ehrlich et al. 2009). GABA is produced by the enzyme glutamic acid decarboxylase (GAD) which exists in two isoforms: GAD65 and GAD67. GAD67 maintains basal GABA levels, whereas GAD65 is rapidly activated in times of high GABA demand (Martin and Rimvall 1993). Deleting the *Gad67* gene is a lethal mutation (Asada et al. 1997), highlighting its importance in development and survival. Using in situ hybridization, Heldt and Ressler (2007) showed that *Gad67* gene expression decreases in the lateral amygdala

2.5 h after fear conditioning and increases in the basolateral amygdala 1.5 h after extinction training. Mice lacking the GAD65 isoform display elevated anxiety levels (Kash et al. 1999; Stork et al. 2000, 2003), generalization of fear to neutral auditory cues (Bergado-Acosta et al. 2008), and impaired extinction of cued fear (Sangha et al. 2009). Fear conditioning also decreases *Gad65* gene expression in the basolateral complex of the amygdala 24 h after fear learning (Bergado-Acosta et al. 2008). However, a quantitative analysis of changes in *Gad65* and *Gad67* gene expression in the subregions comprising the extinction network has not been undertaken. Here, we use relative quantitative real-time RT-PCR to measure changes in *Gad65* and *Gad67* mRNA levels in specific subregions of the amygdala, hippocampus, and prefrontal cortex 24 h after extinction of a recent (1-d) or intermediate (14-d) fear memory. Since *Gad65* gene expression was decreased in the BL complex 24 h post-fear conditioning (Bergado-Acosta et al. 2008), we chose 24 h post-conditioning as our time point to investigate changes in gene expression of *Gad65* and *Gad67*.

Results

Extinction of recent and intermediate memory

In total, eight groups of mice were conditioned (total of 85 animals) (Fig. 1A). The two “conditioning groups” received either paired ($n = 9$) or unpaired ($n = 8$) fear conditioning over the course of two days. These mice were then sacrificed 24–26 h later. The “extinction-recent” groups received extinction training in a neutral context 1 d after fear training and were subsequently sacrificed 24–26 h later (recent:paired, $n = 10$; recent:unpaired, $n = 9$). Some of the paired mice did not receive this extinction training and served as “no extinction” controls (recent:no extinction, $n = 10$). In addition, some paired mice that also received extinction training ($n = 6$) were not sacrificed but instead tested for extinction memory. The “extinction-intermediate” groups

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Figure 1. Experimental protocol. (A) After either paired or unpaired fear training, some mice were sacrificed 1 d later (Conditioning group). Remaining mice received extinction training either 1 d later (Extinction-recent group) or 14 d later (Extinction-intermediate group) and were sacrificed 1 d after extinction training. Some mice did not receive extinction training and served as “no extinction” controls. X indicates time of sacrifice. (B) Mean % time spent freezing (± SEM) during the 1st CS+ during extinction training and recall for recent and intermediate groups. The conditioning groups were sacrificed (arrow “1”), while the recent and intermediate groups underwent extinction training (R1–R6). Recent:paired and intermediate:paired groups displayed successful extinction of fear across R1–R6 (* $P < 0.05$, recent:paired R1 vs. R3 and intermediate:paired R1 vs. R4). Recent:unpaired and intermediate:unpaired groups did not display significant freezing compared to the respective paired groups (* $P < 0.05$, R1 recent:paired vs. R1 recent:unpaired and R1 intermediate:paired vs. R1 intermediate:unpaired). Some mice from the recent:paired, recent:no extinction, intermediate:paired, and intermediate:no extinction groups were tested for extinction recall (E) 1 d later. Remaining mice were instead sacrificed (arrow “2”). Mice that did not receive extinction froze significantly more than mice that did receive extinction training (* $P < 0.05$, E recent:no extinction vs. E recent:paired and E intermediate:no extinction vs. E intermediate:paired). (C) Tissue was laser microdissected from specific areas of the amygdala (i), prefrontal cortex (ii), and hippocampus (iii). (LA) lateral amygdala, (CE) central amygdala, (BA) basolateral amygdala, (CC) corpus callosum, (CPu) caudate putamen, (LV) lateral ventricle, (PL) prelimbic, (IL) infralimbic, (DG) dentate gyrus. Scale bars, 350 μ m.

received extinction training in a neutral context 14 d after fear training and were subsequently sacrificed 24–26 h later (intermediate:paired, $n = 9$; intermediate:unpaired, $n = 9$). Again, some of the paired mice did not receive this extinction training and served as “no extinction” controls (intermediate:no extinction, $n = 9$). And, additional paired mice that received extinction training ($n = 6$) were not sacrificed but tested for extinction memory.

A two-way repeated measures ANOVA revealed a significant interaction between group and session ($F_{(15,225)} = 5.759$, $P < 0.001$), as well as a main effect of both group ($F_{(3,45)} = 20.800$, $P < 0.001$) and session ($F_{(5,45)} = 32.077$, $P < 0.001$) across extinction training (R1 to R6). During the first retrieval session (R1) (Fig. 1B), only mice that received paired training showed increased freezing to the cue compared to unpaired controls. The recent:paired group froze significantly more than the recent:unpaired

group ($P < 0.05$), and, in a likewise fashion, the intermediate:paired group froze significantly more than the intermediate:unpaired group ($P < 0.001$). As extinction progressed (R1–R6), freezing levels decreased. The recent:paired group showed a significant reduction in freezing levels beginning at R3 onward (one-way repeated measures ANOVA, $F_{(5,75)} = 24.423$, $P < 0.001$; R1 vs. R3, R4, R5, and R6, $P < 0.001$ for each). The intermediate:paired group showed a significant reduction in freezing levels beginning at R4 onward (one-way repeated measures ANOVA, $F_{(5,70)} = 23.287$, $P < 0.001$; R1 vs. R4, R5, and R6, $P < 0.001$ for each). During the extinction recall test (E) (Fig. 1B), only mice that received extinction training showed a reduction in freezing compared to no extinction controls. Specifically, recent:paired animals ($n = 6$) showed reduced freezing compared to recent:no extinction ($n = 4$) animals, and intermediate:paired animals ($n = 6$) showed reduced freezing compared to intermediate:no extinction ($n = 3$) animals (one-way ANOVA, $F_{(3,13)} = 6.642$, $P < 0.05$; $P < 0.05$ for each comparison).

No significant changes in gene expression after cued fear conditioning

Gene expression was measured by relative quantitative real-time RT-PCR on microdissected tissue collected from lateral amygdala (LA), basolateral amygdala (BA), central amygdala (CE), prelimbic area of the PFC (PL), infralimbic area of the PFC (IL), CA1 area of the hippocampus (CA1), and the dentate gyrus (DG) (Fig. 1C). *Gad67* and *Gad65* gene expression were measured in paired and unpaired mice that were sacrificed 24–26 h after 2 d of fear training. Significant changes between paired and unpaired groups were not found within any region in either *Gad67* or *Gad65* gene expression (unpaired t -tests, $P > 0.05$ in each case) (Table 1). Thus, 1 d after our 2-d training paradigm, cue specific fear conditioning does not result in significant changes in *Gad67* or *Gad65* in the amygdala, hippocampus, or prefrontal cortex.

Reduction in *Gad65* gene expression in the hippocampus after extinction of a recent memory

Recent memory

Gad67 gene expression levels did not significantly differ among recent:no extinction, recent:paired, and recent:unpaired groups: DG, one-way ANOVA $F_{(2,15)} = 0.273$, $P > 0.05$ (recent:no extinction $n = 6$, recent:paired $n = 6$, recent:unpaired $n = 6$); CA1, one-way ANOVA $F_{(2,15)} = 0.697$, $P > 0.05$ (recent:no extinction $n = 6$, recent:paired $n = 6$, recent:unpaired $n = 6$) (Fig. 2A,B).

Table 1. No significant changes in *Gad67* or *Gad65* gene expression 1 d after cued fear conditioning

		Paired			Unpaired			
		Mean	SEM	N	Mean	SEM	N	
		(% naive)			(% naive)			
<i>Gad67</i>	DG	94.891	9.775	9	110.763	24.149	8	$P > 0.05$
	CA1	153.206	17.985	8	190.448	30.061	6	$P > 0.05$
	IL	127.310	11.666	8	172.156	26.282	7	$P > 0.05$
	PL	156.535	26.612	8	176.182	18.114	8	$P > 0.05$
	LA	221.246	40.031	8	183.598	40.687	7	$P > 0.05$
	BA	135.052	30.756	8	98.698	32.744	8	$P > 0.05$
<i>Gad65</i>	CE	184.593	30.722	9	169.369	27.787	8	$P > 0.05$
	DG	134.378	13.011	9	143.199	20.237	8	$P > 0.05$
	CA1	134.932	15.567	8	182.350	31.778	7	$P > 0.05$
	IL	118.128	16.113	7	186.287	39.611	7	$P > 0.05$
	PL	99.687	21.516	8	148.763	17.163	8	$P > 0.05$
	LA	268.369	33.745	8	211.538	39.648	6	$P > 0.05$
	BA	86.678	13.396	8	87.668	30.610	8	$P > 0.05$
	CE	182.567	26.527	9	156.718	15.626	8	$P > 0.05$

Figure 2. Reduced *Gad65* gene expression in the hippocampus after extinction of a recent memory. Relative gene expression changes of *Gad67* and *Gad65* expressed as % naive ($\pm$ SEM) 1 d after extinction of a recent or intermediate memory. A significant down-regulation of *Gad65* gene expression occurred in the DG (A) and CA1 (B) in the recent:paired + extinction group as compared to recent:no extinction and recent:unpaired + extinction (* $P < 0.05$).

A significant down-regulation of *Gad65* gene expression was detected in the recent:paired compared to the recent:no extinction and recent:unpaired groups: DG, one-way ANOVA $F_{(2,19)} = 5.873$, $P < 0.05$; $P < 0.05$ each comparison (recent:no extinction $n = 8$, recent:paired $n = 6$, recent:unpaired $n = 8$); CA1, one-way ANOVA $F_{(2,15)} = 23.894$, $P < 0.01$; $P < 0.05$ each comparison (recent:no extinction $n = 6$, recent:paired $n = 6$, recent:unpaired $n = 6$) (Fig. 2A,B). Thus, extinction of a recent memory resulted in reduced *Gad65* gene expression in both the DG and CA1.

Intermediate memory

Gad67 gene expression levels did not significantly differ among intermediate:no extinction, intermediate:paired, and intermediate:unpaired groups: DG, one-way ANOVA $F_{(2,20)} = 1.826$, $P > 0.05$ (intermediate:no extinction $n = 6$, intermediate:paired $n = 9$, intermediate:unpaired $n = 8$); CA1, one-way ANOVA $F_{(2,19)} = 0.128$, $P > 0.05$ (intermediate:no extinction $n = 6$, intermediate:paired $n = 8$, intermediate:unpaired $n = 8$) (Fig. 2A,B). Similarly, *Gad65* gene expression levels were not significantly different between recent:no extinction, recent:paired, and recent:unpaired groups: DG, one-way ANOVA $F_{(2,18)} = 0.14$, $P > 0.05$ (intermediate:no extinction $n = 6$, intermediate:paired $n = 8$, intermediate:unpaired $n = 7$); CA1, one-way ANOVA $F_{(2,19)} = 0.714$, $P > 0.05$ (intermediate:no extinction $n = 6$, intermediate:paired $n = 8$, intermediate:unpaired $n = 8$) (Fig. 2A,B).

Increased *Gad67* gene expression in the infralimbic cortex after extinction of a recent memory

Recent memory

A significant up-regulation of *Gad67* gene expression in the IL was detected in the recent:paired group ($n = 6$) compared to the recent:no extinction group ($n = 6$) and recent:unpaired group ($n = 6$) (one-way ANOVA $F_{(2,15)} = 10.705$, $P < 0.05$; $P < 0.05$

each comparison) (Fig. 3A). A similar up-regulation of *Gad67* was not seen in the PL among the recent groups (one-way ANOVA $F_{(2,15)} = 0.077$, $P > 0.05$; recent:no extinction $n = 6$, recent:paired $n = 6$, recent:unpaired $n = 6$) (Fig. 3B). No observable differences were seen for *Gad65* expression among the recent groups in the IL (one-way ANOVA $F_{(2,15)} = 0.276$, $P > 0.05$; recent:no extinction $n = 6$, recent:paired $n = 6$, recent:unpaired $n = 6$) or PL (one-way ANOVA $F_{(2,15)} = 1.090$, $P > 0.05$; recent:no extinction $n = 6$, recent:paired $n = 6$, recent:unpaired $n = 6$) (Fig. 3A,B). Thus, extinction of a recent memory resulted in increased *Gad67* gene expression in the IL but not the PL.

Intermediate memory

Gad67 gene expression levels did not significantly differ among intermediate:no extinction, intermediate:paired, and intermediate:unpaired groups: IL, one-way ANOVA $F_{(2,19)} = 1.123$, $P > 0.05$ (intermediate:no extinction $n = 6$, intermediate:paired $n = 9$, intermediate:unpaired $n = 7$); PL, one-way ANOVA $F_{(2,20)} = 0.133$, $P > 0.05$ (intermediate:no extinction $n = 6$, intermediate:paired $n = 8$, intermediate:unpaired $n = 9$) (Fig. 3A,B). Similarly, *Gad65* gene expression levels were not significantly different between intermediate:no extinction, intermediate:paired, and intermediate:unpaired groups: IL, one-way ANOVA $F_{(2,20)} = 0.563$, $P > 0.05$ (intermediate:no extinction $n = 6$, intermediate:paired $n = 9$, intermediate:unpaired $n = 8$); PL, one-way ANOVA $F_{(2,18)} = 0.298$, $P > 0.05$ (intermediate:no extinction $n = 6$, intermediate:paired $n = 6$, intermediate:unpaired $n = 9$) (Fig. 3A,B).

Increased *Gad65* gene expression in the central amygdala after extinction of an intermediate memory

Recent memory

Gad67 gene expression levels did not significantly differ among recent:no extinction, recent:paired, and recent:unpaired groups: LA, one-way ANOVA $F_{(2,15)} = 0.381$, $P > 0.05$ (recent:no extinction

Figure 3. Increased *Gad67* gene expression in the infralimbic cortex after extinction of a recent memory. Relative gene expression changes of *Gad67* and *Gad65* expressed as % naive ($\pm$ SEM) 1 d after extinction of a recent or intermediate memory. A significant up-regulation of *Gad67* gene expression occurred in the IL (A) in the recent:paired + extinction group as compared to recent:no extinction and recent:unpaired + extinction (* $P < 0.05$). (B) No significant changes were detected in the PL.

$n = 6$, recent:paired $n = 6$, recent:unpaired $n = 6$); BA, one-way ANOVA $F_{(2,15)} = 0.0995$, $P > 0.05$ (recent:no extinction $n = 6$, recent:paired $n = 6$, recent:unpaired $n = 6$); CE, one-way ANOVA $F_{(2,15)} = 1.136$, $P > 0.05$ (recent:no extinction $n = 6$, recent:paired $n = 6$, recent:unpaired $n = 6$) (Fig. 4A–C). Similarly, *Gad65* gene expression levels were not significantly different between recent:no extinction, recent:paired, and recent:unpaired groups: LA, one-way ANOVA $F_{(2,15)} = 0.965$, $P > 0.05$ (recent:no extinction $n = 6$, recent:paired $n = 6$, recent:unpaired $n = 6$); BA, one-way ANOVA $F_{(2,15)} = 0.370$, $P > 0.05$ (recent:no extinction $n = 6$, recent:paired $n = 6$, recent:unpaired $n = 6$); CE, one-way ANOVA $F_{(2,15)} = 0.085$, $P > 0.05$ (recent:no extinction $n = 6$, recent:paired $n = 6$, recent:unpaired $n = 6$) (Fig. 4A–C).

Intermediate memory

Gad67 gene expression levels did not significantly differ among intermediate:no extinction, intermediate:paired, and intermediate:unpaired groups: LA, one-way ANOVA $F_{(2,18)} = 4.039$, $P > 0.05$ (intermediate:no extinction $n = 6$, intermediate:paired $n = 7$, intermediate:unpaired $n = 8$); BA, one-way ANOVA $F_{(2,19)} = 1.427$, $P > 0.05$ (intermediate:no extinction $n = 6$, intermediate:paired $n = 7$, intermediate:unpaired $n = 9$); CE, one-way ANOVA $F_{(2,20)} = 0.390$, $P > 0.05$ (intermediate:no extinction $n = 6$, intermediate:paired $n = 9$, intermediate:unpaired $n = 8$) (Fig. 4A–C). Similarly, *Gad65* gene expression levels were also not significantly different between intermediate:no extinction, intermediate:paired, and

intermediate:unpaired groups in the LA (one-way ANOVA $F_{(2,17)} = 1.551$, $P > 0.05$; intermediate:no extinction $n = 6$, intermediate:paired $n = 7$, intermediate:unpaired $n = 7$) or BA (one-way ANOVA $F_{(2,21)} = 1.354$, $P > 0.05$; intermediate:no extinction $n = 6$, intermediate:paired $n = 9$, intermediate:unpaired $n = 9$) (Fig. 4A,B). However, a significant up-regulation of *Gad65* gene expression was found in the CE: the intermediate:paired group ($n = 7$) was significantly higher than both the intermediate:no extinction group ($n = 6$) and intermediate:unpaired group ($n = 7$) (one-way ANOVA $F_{(2,17)} = 6.045$, $P < 0.05$; $P < 0.05$ each comparison) (Fig. 4C). Thus, extinction of an intermediate memory induces *Gad65* gene expression in CE but not LA or BA.

Discussion

Here, relative quantitative real-time RT-PCR was used to measure changes in *Gad65* and *Gad67* mRNA levels in the DG, CA1, IL, PL, LA, BA, and CE 24–26 h after extinction of a recent (1-d) or intermediate (14-d) fear memory. Our results show that extinction of a recent memory induces a down-regulation of *Gad65* gene expression in the hippocampus (CA1 and DG) and an up-regulation of *Gad67* gene expression in the infralimbic cortex. Extinguishing an intermediate memory resulted in increased *Gad65* gene expression in the central amygdala. These data indicate a differential regulation of *Gad* gene expression after extinction of a recent memory vs. intermediate memory. These changes, if confirmed at the protein level, suggest that extinction of a recent or intermediate fear memory is capable of altering GAD levels in an isoform and regionally specific manner. The changes likely result from neural processes involving synaptic plasticity during erasure of fear memory and/or inhibitory learning in the relevant circuits (Pape and Pare 2010). For fear extinction, associated changes in protein levels would likely support an alteration in basal and/or activity-dependent GABAergic inhibition.

Possible role of decreased *Gad65* expression in the hippocampus after extinguishing a recent fear memory

There is evidence that contextual learning occurs during extinction of cued fear (Bouton et al. 2006). Studies with *GAD65*-deficient mice have shown they are normal in hippocampal-dependent tasks (Asada et al. 1996; Sangha et al. 2009). This indicates that *GAD65*-mediated signaling is not induced during hippocampal-dependent tasks. However, these results do not negate the possibility that extinction of fear reduces hippocampal *Gad65* gene expression, as seen in the present study. A reduction in hippocampal *Gad65* expression may be a reflection of contextual learning during extinction training. Reduced *Gad65* expression would result in less hippocampal inhibition and may promote synaptic plasticity within the hippocampus during fear extinction. The change in *Gad65* expression we observed was only seen after extinguishing a 1-d fear memory, not a 14-d fear memory. This may imply that GABAergic signaling in the hippocampus has greater influence on newer fear memories, and other brain regions or pathways are recruited to extinguish older fear memories.

Possible role of increased *Gad67* expression in the infralimbic cortex after extinguishing a recent fear memory

The PFC is strongly implicated in influencing the expression of extinction (Quirk and Mueller 2008). Recent evidence suggests a division of labor between the IL and PL, with the IL associated with suppressing fear and the PL linked to expressing fear (Burgos-Robles et al. 2009; Laurent and Westbrook 2009). The S1 mouse strain, which is profoundly impaired in fear extinction, shows PL hyperactivity as measured by c-Fos and Zif268 activation

Figure 4. Increased *Gad65* gene expression in the central amygdala after extinction of an intermediate memory. Relative gene expression changes of *Gad67* and *Gad65* expressed as % naive ($\pm$ SEM) 1 d after extinction of a recent or intermediate memory. No significant changes were detected in the LA (A) or BA (B). A significant up-regulation of *Gad65* gene expression occurred in the CE (C) in the intermediate: paired + extinction group compared to intermediate:no extinction and intermediate:unpaired + extinction (* $P < 0.05$).

(Whittle et al. 2010). Further, expression of freezing behavior is correlated with sustained PL responses; these responses decrease after extinction of a recent memory (Burgos-Robles et al. 2009). Also, inactivating the PL impairs fear expression, with no effect on extinction memory (Sierra-Mercado et al. 2011). In contrast, IL inactivation has no effect on fear expression but does impair within-session extinction learning and extinction memory (Sierra-Mercado et al. 2011). Neurons expressing high levels of GAD67 may provide tonic inhibition (Miner and Sarter 1999). The increase in *Gad67* expression we observed in IL may thus represent an increase in basal activity of GABA neurons and increased inhibition of the PL by the IL. In fact, there are reciprocal projections between the IL and PL (Hoover and Vertes 2007), although their neurochemical nature remains unknown. An alternative possibility is that there is increased GABAergic inhibition of inhibitory inputs to the IL resulting in an overall disinhibitory influence. The increased disinhibition in the IL would be associated with inhibition of fear during extinction. Long term changes in *Gad67* expression may be induced by extinction training to promote IL-associated inhibition of fear. This is consistent with the results of Akirav et al. (2006) in which a GABA_A agonist microinjected into the IL before extinction training facilitates the extinction of conditioned fear for at least 48 h.

In addition to innervating pyramidal neurons within the mPFC, hippocampal afferents also exert a direct excitatory influence on GABAergic interneurons within the mPFC (Gabbott et al. 2002; Tierney et al. 2004). These inhibitory circuits could provide feed-forward inhibition and aid in the temporal control of the prefrontal neural network (Kawashima et al. 2006). It could also facilitate synchronization of PFC neurons with hippocampal activity (Tierney et al. 2004). In fact, increased synchronization at theta frequency between the dorsal hippocampal CA1 area and infralimbic area has been previously reported by our group during fear extinction (Sangha et al. 2009; Lesting et al. 2011). Like the hippocampus, the change in *Gad* expression was only seen after extinguishing a recent memory and not an intermediate memory. Again, this may imply that GABAergic signaling in the IL has greater influence on newer fear memories, and other mechanisms are initiated to extinguish older fear memories.

Possible role of increased *Gad65* expression in the central amygdala after extinguishing an intermediate fear memory

The CE is the major output of the amygdala and is composed of mostly GABAergic neurons (Ehrlich et al. 2009; Pape and Pare 2010). The majority of CE neurons contain both GAD65 and GAD67 mRNA (Pitkanen and Amaral 1994; Poulin et al. 2008). In the present study, we did not attempt to separate the medial and lateral divisions of the central amygdala. Recent evidence suggests that GABAergic signaling in the lateral division dictates the output of the medial division, which is the ultimate output of the amygdala (Cicocchi et al. 2010; Duvarci et al. 2011; Tye et al. 2011). Furthermore, there are reciprocal interactions between types of GABAergic neurons within the lateral division, which result in both inhibitory and disinhibitory influences on the output neurons of the medial division and thereby contribute to fear acquisition and generalization (Cicocchi et al. 2010). Because GAD65 is generally found in apoenzyme form, it may serve as a reserve supply of inactive enzyme that can become activated in response to increased demands on GABAergic neurons, and therefore GAD65 may be elevated in neurons that are highly sensitive to regulation by afferent input (Miner and Sarter 1999). An increase in GAD65-mediated GABAergic signaling in the CE might indicate a functional reorganization of these intricate circuits, leading to reduced amygdalar output and fear expression. The changes we observed in *Gad65* gene expression were only seen after extin-

guishing an older 14-d fear memory, indicating that the longer a fear memory is stored and maintained, the more likely that GABAergic circuits within the CE are recruited to successfully reduce that fear.

Role of GABA after extinction learning

Several studies have investigated the role of GABA in fear extinction which have yielded mixed and, at times, contradictory results (for review, see Makkar et al. 2010). GABAergic influences are dependent on the time point of manipulation as well as on the brain region manipulated. Most of the evidence indicates that extinction is facilitated when GABAergic transmission is either decreased during extinction training and consolidation or increased before extinction testing (for review, see Makkar et al. 2010). Molecular studies demonstrate that several hours after the start of extinction training, molecules and receptors that facilitate GABA transmission are up-regulated in the amygdala (Chhatwal et al. 2005; Heldt and Ressler 2007). Extinction of fear involves the acquisition of a context-gated inhibitory association between the conditioned stimulus and noxious unconditioned stimulus, and this inhibitory association has been shown to be mediated by GABA binding to GABA_A receptors (Harris and Westbrook 1998). All of this suggests that the expression of fear inhibition after extinction training is dependent upon functional GABAergic transmission. Our results are consistent with this observation.

One molecular study (Heldt and Ressler 2007) found decreased *Gad67* gene expression 2.5 h after fear conditioning in the LA and increased *Gad67* gene expression 1.5 h after extinction in the BA. In contrast, we found no change in *Gad67* anywhere in the amygdala 1 d after fear conditioning or 1 d after extinction of a 1-d fear memory. One possibility for the discrepancy in results is that the changes found by Heldt and Ressler (2007) are, perhaps, temporary. Heldt and Ressler (2007) looked at *Gad67* expression 2.5 h after fear training, whereas our study measured levels 24–26 h after fear conditioning. Plus, their extinction training utilized the same context as the fear conditioning, whereas we used a neutral context for extinction. What is consistent are the *Gad65* data: Like Heldt and Ressler (2007), we also did not observe changes in *Gad65* gene expression in the amygdala after fear conditioning or after extinction of a recent memory.

At first glance, our data seem at odds with our previous findings of decreased *Gad65* gene expression 1 d after fear conditioning in the amygdala (Bergado-Acosta et al. 2008). In contrast to Bergado-Acosta et al., the current experiment utilized two fear conditioning sessions instead of one in order to produce a robust memory before extinction and match our most recent publication on GAD65 knock-out mice and extinction (Sangha et al. 2009). Thus, the results presented here are 1 d after reconditioning. In addition, our previous report showed that the decrease in *Gad65* gene expression occurred 24 h after one fear conditioning session but not 14 d afterward. Expression was not measured 2 d after one fear conditioning session which would correspond to the time point used in the present study.

Extinguishing recent vs. intermediate fear memories

Extinction within 1 h after fear learning involves a mechanism that is consistent with an unlearning/erasure mechanism and may depend upon depotentiation as its cellular mode of action (Myers et al. 2006). However, extinction initiated at longer delays recruits inhibitory learning mechanisms (Myers and Davis 2007). As a memory ages, it becomes increasingly stable and more resilient to disruption (Frankland et al. 2006). We saw evidence for this in the present study. The extinction curve for mice with an intermediate fear memory was shifted to the right compared to mice with a recent fear memory (Fig. 1B). Freezing did not significantly

decrease until the 4th extinction session in mice with an intermediate fear memory, whereas mice with a recent fear memory significantly decreased their freezing by the 3rd extinction session. Upon examination, the changes in *Gad* expression after extinguishing a recent memory seemed to occur upstream in the extinction circuit, in the IL, DG, and CA1. Extinction of an intermediate memory, on the other hand, was associated with changes downstream in the fear extinction circuit, the CE. These results are consistent with the idea that an older memory is more stably incorporated into the network, and extinguishing such a memory may require different mechanisms.

Materials and Methods

Subjects

Eight- to 12-wk-old C57BL/6J male mice (Harlan Laboratories, AN Venray, The Netherlands) were moved to single housing at least 7 d prior to starting any experiment. They were in a 12 h:12 h light-dark cycle (lights on at 7 am) and provided food and water ad libitum. All procedures were performed under strict observance of the European Communities Council Directive (86/609/EEC) for experimentation on animals and were approved by the Bezirksregierung Münster (AZ 50.0835.1.0, G 53/2005).

Behavioral procedure

Mice were first adapted to the fear conditioning apparatus (TSE) and six neutral tone presentations (CS−, 2.5 kHz, 85 dB, 10 sec, 20-sec inter-stimulus interval [ISI]) twice (2–3 h apart) (Supplemental Fig. S1). Fear training commenced the next day and consisted of three tones (CS+, 10 kHz, 85 dB, 10 sec, randomized 10- to 30-sec ISI) which were each coterminated with a 1-sec foot shock (scrambled 0.4 mA); this was repeated the following day. The two tones were counter-conditioned, and control mice received foot shocks unpaired with the tones. One subset of mice was sacrificed 24–26 h after paired or unpaired training (Day 3). Remaining mice received extinction training in a neutral context (clear, plastic 19-cm length × 19-cm width × 13-cm height box, filled with sawdust bedding) either 1 d (recent) or 14 d (intermediate) after fear training. Extinction training consisted of six retrieval sessions (R1–R6, 6 min each) 30 min apart in which both the CS− and CS+ were presented without foot shocks (four 10-sec CS− at 20-sec ISI, 40-sec silence, four 10-sec CS+ at 20 sec ISI); mice remained in the neutral context between retrieval sessions. For some mice, retention of this extinction training was assessed the following day in the extinction context (E; identical to the retrieval sessions); remaining mice were sacrificed 24–26 h post-extinction training (Day 4 or 17). Naive mice ($n = 6$) were home-cage controls.

Freezing, i.e., complete immobility with the exception of respiratory movements, is an innate defensive behavior and was taken as a behavioral measurement of fear (Blanchard and Blanchard 1969; Fendt and Fanselow 1999). The total time spent freezing was quantified during the entire 10 sec of the first CS+ presentation for each session and reported as a percentage of time spent freezing. Group and session differences during extinction training were detected by repeated measures two-way ANOVA, while group differences during the extinction recall test were detected via a one-way ANOVA. All post hoc analyses were made with Tukey's test. P values < 0.05 were considered statistically significant.

Upon termination of behavioral experiments, mice received an overdose of isoflurane, and brains were removed, placed in room temperature tissue embedding medium, frozen at -20°C , and subsequently stored at -70°C .

Laser capture microdissection

Frozen brain sections of 30- μm thickness were cut on a cryostat at -20°C and placed on RNase-free PALM Membrane Slides (Carl Zeiss) coated with Poly-L-Lysine (Sigma-Aldrich). Sections were stained with hematoxylin and eosin (Carl Roth). Via laser micro-

dissection (PALM MicroBeam system, Carl Zeiss), tissue from the following areas was isolated: central amygdala (CE) (capsular and lateral areas) (-1.22 - to -1.7 -mm AP, ± 2.0 - to 2.6 -mm ML, -4.0 - to -4.75 -mm DV), basolateral amygdala (BA) (major: BA anterior area, minor: basomedial posterior area) (-1.22 - to -1.7 -mm AP, ± 2.5 - to 3.0 -mm ML, -4.1 - to -4.85 -mm DV), lateral amygdala (LA) (major: dorsolateral area, minor: ventrolateral area) (-1.22 - to -1.7 -mm AP, ± 2.8 - to 3.1 -mm ML, -3.8 - to -4.35 -mm DV), dentate gyrus (DG) (granular layer) (-1.82 - to -2.06 -mm AP, ± 0.4 - to 1.7 -mm ML, -1.6 - to -2.0 -mm DV), CA1 area of the hippocampus (pyramidal tract and oriens layer) (-1.82 - to -2.06 -mm AP, ± 0.5 - to 1.7 -mm ML, -0.9 - to -1.1 -mm DV), infralimbic (IL) area of the prefrontal cortex (PFC) (1.54 - to 1.7 -mm AP, 0 - to ± 0.7 -mm ML, -1.9 - to -2.2 -mm DV), and the prelimbic (PL) area of the PFC (1.54 - to 1.7 -mm AP, 0 - to ± 0.7 -mm ML, -1.75 - to -1.4 -mm DV) (Fig. 1C). Isolated tissue was stored in a lysis buffer (RNeasy Micro Kit; Qiagen), and RNA isolation was performed with DNase treatment using the Qiagen RNeasy Micro Kit.

cDNA synthesis

Single-strand cDNA synthesis was performed in thin-walled PCR tubes containing reverse transcriptase buffer (Qiagen), deoxyribonucleotide triphosphates (dNTPs, 5 mM each; Qiagen), random hexanucleotide primer (50 μM ; Invitrogen), 20 U RNasin (Promega), 1 μL Sensiscript reverse transcriptase (Qiagen), and 6 μL isolated RNA (final volume 20 μL) at 37°C for 1 h.

Relative quantitative real-time RT-PCR

Relative quantitative real-time RT-PCR was performed on cDNA prepared from isolated RNA using commercial FAM-labeled detection assays (TaqMan; Applied Biosystems) for the reference gene *Pgk1* and the genes of interest, *Gad1* (glutamic acid decarboxylase 67) and *Gad2* (glutamic acid decarboxylase 65). All assays were assured to detect cDNA derived from mRNA, and not genomic DNA, by performing appropriate reverse transcriptase minus controls. PCRs were done in conjunction with the Real Master Mix (5-Prime) in an ABI Prism 7000 Sequence Detection System (Applied Biosystems; 50 cycles of 15 sec at 95°C and 1 min at 60°C , preceded by a 2-min decontamination step at 50°C and 10-min denaturation step at 95°C). Threshold cycle values ($C(t)$) obtained from the real-time RT-PCR were normalized to a reference gene (*Pgk1*) for each target gene of interest (Livak and Schmittgen 2001). Only $C(t)$ values with a standard error of < 0.16 were included in the study as per the standards set forth by Applied Biosystems. As a result, this criterion has yielded uneven sample sizes. These values for each experimental group were again normalized to the average of naive controls ($n = 6$) using the $2^{-\Delta\Delta C(t)}$ method (Livak and Schmittgen 2001), where $\Delta\Delta C(t) = \text{experimental } [C(t)_{\text{target}} - C(t)_{\text{reference}}] - \text{naive } [C(t)_{\text{target}} - C(t)_{\text{reference}}]$. Data are expressed as % naive $[(2^{-\Delta\Delta C(t)} \times 100\%) \pm \text{SEM}]$. Values $> 100\%$ represent an up-regulation compared to naive and, conversely, values $< 100\%$ represent a down-regulation compared to naive. One-way ANOVAs were performed on % naive values between conditioned groups for each gene of interest, with the criterion for significance being $P < 0.05$ (two-tailed). All post hoc analyses were made with Tukey's test.

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References

- Akavir I, Raizel H, Maroun M. 2006. Enhancement of conditioned fear extinction by infusion of the GABA_A agonist muscimol into the rat prefrontal cortex and amygdala. *Eur J Neurosci* **23**: 758–764.

- Asada H, Kawamura Y, Maruyama K, Kume H, Ding R, Ji FY, Kanbara N, Kuzume H, Sanbo M, Yagi T, Obata K. 1996. Mice lacking the 65-kDa isoform of glutamic acid decarboxylase (GAD65) maintain normal levels of GAD67 and GABA in their brains but are susceptible to seizures. *Biochem Biophys Res Commun* **229**: 891–895.
- Asada H, Kawamura Y, Maruyama K, Kume H, Ding R-G, Kanbara N, Kuzume H, Sanbo M, Yagi T, Obata K. 1997. Cleft palate and decreased brain γ -aminobutyric acid in mice lacking the 67-kDa isoform of glutamic acid decarboxylase. *Proc Natl Acad Sci* **94**: 6496–6499.
- Bergado-Acosta JR, Sangha S, Narayanan RT, Obata K, Pape H-C, Stork O. 2008. Critical role of the 65-kDa isoform of glutamic acid decarboxylase in consolidation and generalization of Pavlovian fear memory. *Learn Mem* **15**: 163–171.
- Blanchard RJ, Blanchard DC. 1969. Crouching as an index of fear. *J Comp Physiol Psychol* **67**: 370–375.
- Bouton ME, Westbrook RF, Corcoran KA, Maren S. 2006. Contextual and temporal modulation of extinction: Behavioral and biological mechanisms. *Biol Psychiatry* **60**: 352–360.
- Briand LA, Blendy JA. 2010. Molecular and genetic substrates linking stress and addiction. *Brain Res* **1314**: 219–234.
- Burgos-Robles A, Vidal-Gonzalez I, Quirk GJ. 2009. Sustained conditioned responses in prelimbic prefrontal neurons are correlated with fear expression and extinction failure. *J Neurosci* **29**: 8474–8482.
- Chhatwal JP, Myers KM, Ressler KJ, Davis M. 2005. Regulation of gephyrin and GABA_A receptor binding within the amygdala after fear acquisition and extinction. *J Neurosci* **25**: 502–506.
- Ciocchi S, Herry C, Grenier F, Wolff SB, Letzkus JJ, Vlachos I, Ehrlich I, Sprengel R, Deisseroth K, Stadler MB, et al. 2010. Encoding of conditioned fear in central amygdala inhibitory circuits. *Nature* **468**: 277–282.
- Duvarci S, Popa D, Pare D. 2011. Central amygdala activity during fear conditioning. *J Neurosci* **31**: 289–294.
- Ehrlich I, Humeau Y, Grenier F, Ciocchi S, Herry C, Luthi A. 2009. Amygdala inhibitory circuits and the control of fear memory. *Neuron* **62**: 757–771.
- Fendt M, Fanselow MS. 1999. The neuroanatomical and neurochemical basis of conditioned fear. *Neurosci Biobehav Rev* **23**: 743–760.
- Frankland PW, Ding H-K, Takahashi E, Suzuki A, Kida S, Silva AJ. 2006. Stability of recent and remote contextual fear memory. *Learn Mem* **13**: 451–457.
- Gabbott P, Headlam A, Busby S. 2002. Morphological evidence that CA1 hippocampal afferents monosynaptically innervate PV-containing neurons and NADPH-diaphorase reactive cells in the medial prefrontal cortex (Areas 25/32) of the rat. *Brain Res* **946**: 314–322.
- Harris JA, Westbrook RF. 1998. Evidence that GABA transmission mediates context-specific extinction of learned fear. *Psychopharmacology (Berl)* **140**: 105–115.
- Heldt SA, Ressler KJ. 2007. Training-induced changes in the expression of GABA_A-associated genes in the amygdala after the acquisition and extinction of Pavlovian fear. *Eur J Neurosci* **26**: 3631–3644.
- Hoover WB, Vertes RP. 2007. Anatomical analysis of afferent projections to the medial prefrontal cortex in the rat. *Brain Struct Funct* **212**: 149–179.
- Kash SF, Tecott LH, Hodge C, Baekkeskov S. 1999. Increased anxiety and altered responses to anxiolytics in mice deficient in the 65-kDa isoform of glutamic acid decarboxylase. *Proc Natl Acad Sci* **96**: 1698–1703.
- Kawashima H, Izaki Y, Grace AA, Takita M. 2006. Cooperativity between hippocampal-prefrontal short-term plasticity through associative long-term potentiation. *Brain Res* **1109**: 37–44.
- Laurent V, Westbrook RF. 2009. Inactivation of the infralimbic but not the prelimbic cortex impairs consolidation and retrieval of fear extinction. *Learn Mem* **16**: 520–529.
- LeDoux JE. 2000. Emotion circuits in the brain. *Annu Rev Neurosci* **23**: 155–184.
- Lesting J, Narayanan RT, Kluge C, Sangha S, Seidenbecher T, Pape HC. 2011. Patterns of coupled θ activity in amygdala-hippocampal-prefrontal cortical circuits during fear extinction. *PLoS One* **6**: 21714. doi: 10.1371/journal.pone.0021714.
- Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2(-\Delta\Delta C(T))$. *Methods* **25**: 402–408.
- Makkar SR, Zhang SQ, Cranney J. 2010. Behavioral and neural analysis of GABA in the acquisition, consolidation, reconsolidation, and extinction of fear memory. *Neuropsychopharmacology* **35**: 1625–1652.
- Martin DL, Rimvall K. 1993. Regulation of γ -aminobutyric acid synthesis in the brain. *J Neurochem* **60**: 395–407.
- Miner LA, Sarter M. 1999. Intra-accumbens infusions of antisense oligodeoxynucleotides to one isoform of glutamic acid decarboxylase mRNA, GAD65, but not to GAD67 mRNA, impairs sustained attention performance in the rat. *Brain Res Cogn Brain Res* **7**: 269–283.
- Myers KM, Davis M. 2007. Mechanisms of fear extinction. *Mol Psychiatry* **12**: 120–150.
- Myers KM, Ressler KJ, Davis M. 2006. Different mechanisms of fear extinction dependent on length of time since fear acquisition. *Learn Mem* **13**: 216–223.
- Pape H-C, Pare D. 2010. Plastic synaptic networks of the amygdala for the acquisition, expression, and extinction of conditioned fear. *Physiol Rev* **90**: 419–463.
- Pitkanen A, Amaral DG. 1994. The distribution of GABAergic cells, fibers, and terminals in the monkey amygdaloid complex: An immunohistochemical and in situ hybridization study. *J Neurosci* **14**: 2200–2224.
- Poulin JF, Castonguay-Lebel Z, Laforest S, Drolet G. 2008. Enkephalin co-expression with classic neurotransmitters in the amygdaloid complex of the rat. *J Comp Neurol* **506**: 943–959.
- Quirk GJ, Mueller D. 2008. Neural mechanisms of extinction learning and retrieval. *Neuropsychopharmacology* **33**: 56–72.
- Sangha S, Narayanan RT, Bergado-Acosta JR, Stork O, Seidenbecher T, Pape H-C. 2009. Deficiency of the 65-kDa isoform of glutamic acid decarboxylase impairs extinction of cued but not contextual fear memory. *J Neurosci* **29**: 15713–15720.
- Sierra-Mercado D, Padilla-Coreano N, Quirk GJ. 2011. Dissociable roles of prelimbic and infralimbic cortices, ventral hippocampus, and basolateral amygdala in the expression and extinction of conditioned fear. *Neuropsychopharmacology* **36**: 529–538.
- Stork O, Ji F-Y, Kaneko K, Stork S, Yoshinobu Y, Moriya T, Shibata S, Obata K. 2000. Postnatal development of a GABA deficit and disturbance of neural functions in mice lacking GAD65. *Brain Res* **865**: 45–58.
- Stork O, Yamanaka H, Stork S, Kume N, Obata K. 2003. Altered conditioned fear behavior in glutamate decarboxylase 65 null mutant mice. *Genes Brain Behav* **2**: 65–70.
- Tierney P, Dégénétais E, Thierry AM, Glowinski J, Gioanni Y. 2004. Influence of the hippocampus on interneurons of the rat prefrontal cortex. *Eur J Neurosci* **20**: 514–524.
- Tye KM, Prakash R, Kim SY, Fenno LE, Grosenick L, Zarabi H, Thompson KR, Gradinaru V, Ramakrishnan C, Deisseroth K. 2011. Amygdala circuitry mediating reversible and bidirectional control of anxiety. *Nature* **471**: 358–362.
- Whittle N, Hauschild M, Lubec G, Holmes A, Singewald N. 2010. Rescue of impaired fear extinction and normalization of cortico-amygdala circuit dysfunction in a genetic mouse model by dietary zinc restriction. *J Neurosci* **30**: 13586–13596.

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