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Transient fear-induced alterations in evoked release of norepinephrine and GABA in amygdala slices

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Abstract

Presentation of a tonal cue that previously had been associated with a fearful experience (footshock) produces alterations in arousal and sleep that occur after the fearful cue is no longer presented. To begin investigating neurochemical mechanisms that may underlie the effects of fearful cue presentation, we measured release of [³H]-norepinephrine ([³H]-NE) and [¹⁴C]-γ-aminobutyric acid ([¹⁴C]-GABA) from brain regions known to regulate arousal states and REM sleep. Depolarization-evoked release of [³H]-NE from amygdalar slices of mice, which were trained to recognize a tone as a fearful cue, was suppressed at 2–3hrs after exposure of animals to the fearful cue, but recovered after 4–5 hrs. Interestingly, depolarization-evoked release of [¹⁴C]-GABA was significantly increased in the amygdala, and also showed a tendency for enhancement in the hippocampus, NPO, and DRN at 2–3 hrs after cue presentation. The changes in [¹⁴C]-GABA release were also transient; 4–5 hrs after cue presentation no significant differences were detected between samples derived from experimental groups which experienced fearful or neutral cues. The similar time course of fearful cue-induced changes in neurotransmitter release and changes in arousal and REM sleep suggests that alterations in amygdalar neurotransmission may be involved changes in arousal and sleep that occur after fear.

Keywords

amygdala; fear conditioning; GABA; noradrenaline

1. Introduction

In cued fear conditioning, a neutral cue such as a tone becomes associated with a fear-inducing stressor (typically footshock). After conditioning, the now fearful cue alone can elicit behavioral and physiological responses similar to those initially induced by the original stressor. Studies of fear conditioning have established that the presentation of fearful cues (or fearful contexts) can enhance responses such as freezing (Blanchard and Blanchard, 1969; Phillips and LeDoux, 1992; Paylor et al., 1994) and can modify other responses such as fear potentiation of startle amplitude (Davis, 1992; Davis et al., 1995; Davis et al., 1997; Walker et al., 1997).

Fear conditioning is thought to have a role in the development of fear and anxiety related pathologies (Charney and Deutch, 1996); however, what happens in the post-fear period has

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been less well studied. We and others have demonstrated that both the shock-training phase and the presentation of fearful cues and contexts alone can alter subsequent arousal including a significant suppression of rapid eye movement sleep (REM) (Sanford et al., 2003; Sanford et al., 2003; Jha et al., 2005; Pawlyk et al., 2005). The suppression of REM may be seen for 2 hr or more after presentation of fearful cues, suggesting that conditioned fear can have a relatively prolonged influence on arousal and sleep.

The amygdala is a critical region for mediating the behaviors associated with conditioned fear (Davis, 1992 ; Davis and Whalen, 2001). It also projects prominently, via its central nucleus, to a number of brainstem regions linked to arousal and to the generation and control of REM (Krettek and Price, 1978; Takeuchi et al., 1982; Price et al., 1987; Semba and Fibiger, 1992; Peyron et al., 1998), and can influence the generation of REM (Sanford et al., 1995; Calvo et al., 1996 ; Sanford et al., 2002; Tang et al., 2005; Sanford et al., 2006) and NREM (Sanford et al., 1998; Zhu et al., 1998; Sanford et al., 2006). Studies using microdialysis have demonstrated that changes in extracellular levels of neurotransmitters in the amygdala occur in association with fear conditioning and that the changes can persist following fearful stimuli (Stork et al., 2002; Macedo et al., 2005). This suggests the possibility that fear-induced changes in neurotransmitter systems in the amygdala may be associated with the longer-term changes in arousal. However, while microdialysis can provide an approximation of the time course of changes in extrasynaptic neurotransmitter levels, data collection is typically conducted in only one site, especially in small animals (Fillenz, 2005). This can limit conclusions regarding whether measured changes are specific to the recording site or whether there are more general changes that occur in a number of brain regions.

In this study, we examined the utility of an *in vitro* assay of evoked neurotransmitter release (Lonart and Johnson, 1995) for detecting fear-induced alterations in release of gamma-aminobutyric acid (GABA) and norepinephrine (NE) in the amygdala. We chose to focus on GABA and NE based on their demonstrated roles in stress and in the regulation of arousal (for reviews see (Steriade and McCarley, 1990; Bremner et al., 1996; Charney and Deutch, 1996; Herman et al., 2004; Morilak et al., 2005)). Using tissue from the same brains, we performed concurrent assays in the hippocampus, the nucleus pontis oralis (NPO) and the dorsal raphe nucleus (DRN). Each of these regions has been implicated, along with the amygdala, in conditioned fear, in stress, and in arousal and sleep (for reviews see (Steriade and McCarley, 1990; Davis, 1992 ; Charney and Deutch, 1996; Grillon et al., 1996)). Tissue for the assays was collected at 2 h and 4 h after presentation of the fearful cue to evaluate the time course of potential fear-induced alterations in neurotransmitter release, and to determine whether they may approximate the time course seen with fear-induced reductions in REM.

2. Results

Fear-induced alterations in evoked release of [³H]-NE and [¹⁴C]-GABA

Presentation of the fearful cue had significant effects on both [³H]-NE and [¹⁴C]-GABA release in the amygdala that are demonstrated in Fig. 1 and Fig. 2, respectively. Even though the animals were sacrificed 2 hrs after the presentation of the cue, and an additional 1 hr elapsed by the time we began assessing *in vitro* neurotransmitter release, depolarization-evoked release of [³H]-NE and [¹⁴C]-GABA was significantly altered at this time point. These changes were highly consistent across individual mice; amygdala samples from all mice in the fearful cue condition displayed both blunted [³H]-NE and enhanced [¹⁴C]-GABA release. However, these changes dissipated at a later time point: the same measurements on brains dissected 4 hrs after presentation of the fearful cue indicated no significant differences between experimental groups. These findings indicate that fearful cue induces a temporary change in amygdalar neurotransmitter release that can be observed 2–3 hrs after presentation even in tissue slices. Interestingly, the fearful cue induced changes were opposite for noradrenergic and GABA-

ergic release states. [^3H]-NE release showed a striking $59\pm 8\%$ depression whereas [^{14}C]-GABA release was significantly enhanced ($39\pm 14\%$) by the same experimental manipulation.

No significant differences for evoked release of either [^3H]-NE (Table 1) or [^{14}C]-GABA (Table 2) were found in the hippocampus, NPO or DRN, though there was a tendency toward a suppression of evoked release of [^3H]-NE in the hippocampus and DRN in mice that had been presented the fearful cue. Similarly, there were small enhancements of evoked [^{14}C]-GABA release in the hippocampus, NPO, and DRN of fearful mice sacrificed at 2 h after, but not 4 h. However, none of the comparisons were statistically significant.

3. Discussion

The main finding of this study was that fearful cues produced alterations in evoked release of [^3H]-NE and [^{14}C]-GABA in the amygdala that could be observed for over two h following presentation, but recovered to normal levels after 4 h following fearful cue presentation. The alterations in evoked neurotransmitter release occurred even though the fearful cues were presented to the mice in their home cage, an environment that had never been associated with shock. Also, the direction of the changes (blunted [^3H]-NE and enhanced [^{14}C]-GABA release) was the same for all samples from mice presented the fearful cue indicating consistency of fear-induced changes in neural transmission for NE and GABA.

The fear-induced changes in the amygdala contrasted to findings for the hippocampus, NPO and DRN where there were no significant differences between the shock trained and control groups in evoked release of [^3H]-NE and [^{14}C]-GABA at either of the times we examined. The relative specificity of fear-induced alterations in evoked neurotransmitter release in the amygdala are consistent with its established role in mediating the effects of cued fear (Davis, 1992 ; Davis and Whalen, 2001).

The changes in neurotransmitter release were detected using an *in vitro* technique; neurochemistry of brain slices. As this technique detects neurotransmitter release from neurons that are removed from the influence of ongoing activity of projection neurons, the observed changes reflect a change in the regulatory mechanisms of local nerve terminals and neurons. Thus, this technique is well suited to map areas which undergo changes in response to experimental manipulations, providing a measure that is independent of ongoing behavioral status, e.g., correlates of sensory processing. The observation that the changes were both regionally selective and reversible indicates that they were physiologically relevant.

The activity of the noradrenergic LC increases during stress and there is increased release and turnover of NE in projection regions (Bremner et al., 1996). Thus, the reduction in evoked [^3H]-NE release in the amygdala may reflect a rundown of release machinery in the aftermath of the robust level of activity associated with fear. However, the lack of significant fear-induced alterations in [^3H]-NE release in the other regions we examined suggests that the effect we saw in the amygdala was not simply due to an effect of fearful cues on LC causing rundown, but that it involved regulatory changes in release specific to noradrenergic projections of LC to the amygdala.

Neither basal [^3H]-NE nor basal [^{14}C]-GABA release was altered by fearful cues. As neurons of brain slices are mostly silent, the basal release measurements represent non-action potential induced but spontaneous exocytosis that is independent of $[\text{Ca}^{2+}]_o$, and that could be detected as miniature synaptic potentials in electrophysiological recordings (Capogna et al., 1996). Our observations that basal neurotransmitter effluxes were not influenced by changes in $[\text{Ca}^{2+}]_o$ indicates that basal neurotransmitter release reflected the interplay of spontaneous exocytosis and re-uptake. In addition, the finding that presentation of the fearful cue had no significant

effect on basal efflux suggests that the fearful cue induced changes are selective for Ca^{2+} -dependent exocytotic pathway.

Using microdialysis, Stork et al. (Stork et al., 2002) reported that exposure to a fearful cue was accompanied by a reduction in extracellular GABA levels in the mouse amygdala that persisted for several h after the presentation of the fearful cue. The authors suggested that their results indicated either reduced GABA release and/or increased GABA uptake from the extracellular space. The increase in evoked release we observed is consistent with their findings of reduced extracellular GABA levels, as reduced GABAergic activity may lead to increase in the size of the readily releasable pool (Murthy et al., 2001). Our data is consistent with an *in vivo* scenario where regulation of GABA release, not GABA uptake is altered, as in our experiments the extent of [^{14}C]-GABA incorporation onto brain slices was not changed after exposure to fearful cue (not shown).

Studies using microdialysis to measure footshock-induced changes in NE levels in the amygdala of rats have reported peak levels at 15 min after the footshock and a return to baseline levels within 30 min (Galvez et al., 1996; Hatfield et al., 1999). These studies used single, more intense footshocks with durations of 1 or 2 sec and are not directly comparable to our results. However, while we did not conduct measurements on mice after footshock, our data suggest that alterations in NE release can be observed for 2 or more h after the presentation of a fearful cue. Systemic administration of the GABA_A agonist, muscimol, can decrease levels of NE in amygdala (Hatfield et al., 1999) suggesting that the longer-term alterations in evoked NE release may be regulated by GABA. However, increased GABA release is not likely to explain the reduction in NE release, as Stork et al. (Stork et al., 2002) reported a decrease, not an increase in GABA levels.

Extracellular levels of NE in the amygdala of rats (Park, 2002) and cats (Shouse et al., 2000) is reduced during NREM and is lowest during REM when LC is silent. Extracellular levels of GABA also vary with arousal state and are increased in the DRN (Nitz and Siegel, 1997a) and LC (Nitz and Siegel, 1997b) during REM. Inhibition of GABAergic transmission in NPO may facilitate an increase in acetylcholine and thereby contribute to the generation of REM (Vazquez and Baghdoyan, 2004). These factors suggest that transitory changes in neurotransmitter release such as those that may occur across sleep states may have occurred without being detected with our methodology. However, though there were trends toward increased GABA release in NPO, DRN and the hippocampus at 2 h after presentation of the fearful cues, we did not find evidence of significant alterations in neurotransmitter release other than in the amygdala.

Despite its extrasynaptic placement, microdialysis is well suited to approximate minute to minute changes in “volume neurotransmission” of monoaminergic neurotransmitters that escape the synapse (Drew et al., 2004; Fillenz, 2005). However, amino acid neurotransmitters such as GABA and glutamate are effectively cleared by synaptic uptake systems, thus extrasynaptic GABA and glutamate levels are mostly independent of neuronal activity (Korte et al., 1992). *In vivo* voltammetry and single unit recordings are useful alternatives to improve time resolution of changes in neurotransmitter levels or detect neuronal activities and highly compatible with study of ongoing behavior (Fillenz, 2005). However, none of these *in vivo* methods are easily complemented by molecular techniques. In contrast, *in vitro* techniques, including neurotransmitter release measurements by neurochemical techniques, are well suited to study both long-lasting changes in neurotransmitter release and to test the underlying molecular changes. Investigating molecular pathways that underlie regulation of neurotransmission and behavior are important for establishing genetic influences of behavior and to deepen our mechanistic understanding of brain functions.

In conclusion, our results establish the utility of this *in vitro* neurochemical assay for discriminating fear-induced alterations in NE and GABA release in multiple brain regions and demonstrate that fear-induced changes in neurotransmitter release can be detected in the amygdala. The assay may be a valuable complement to studies using microdialysis as it provides additional information regarding changes in neurotransmitter systems that occur in association with behavior. It is also amenable to molecular approaches that could be aimed at probing the cellular mechanisms of fear-induced alterations in neurotransmission.

4. Experimental Procedure

Subjects

The subjects were 7- to 9-week-old C57BL/6J mice obtained from Jackson Laboratories, Bar Harbor, ME. The mice were acclimatized to individual housing for 1 week prior to being subjected to any experimental procedures. The colony room was set on a 12-h light/dark cycle with lights on from 0700 to 1900 h, and room temperature was maintained at $24.5 \pm 0.5^\circ\text{C}$. Food and water were available *ad libitum*. All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Experimental Animals and were approved by Eastern Virginia Medical School's Animal Care and Use Committee (Protocol # 05-017).

Animal training

Training sessions were conducted outside the colony room. For shock training, the mice were placed in a chamber with a grid floor for presenting shock (E10-18RF) and allowed to explore for 5 min. Afterwards, they were presented with 15 tone-shock pairings (tone: 90 dB; 5-s duration; shock (0.2 mA); on during the last 0.5 s of the tone) followed by an additional 5 min without shock. Shock training was conducted during the third h after lights on for each of the four conditioning days. The chamber was thoroughly cleaned with diluted alcohol before each conditioning session. After shock training on each day, the mice were returned to their home cages and left undisturbed.

Auditory stimuli were produced with a frequency generator (Coulbourn model S81-06), amplified with a Harmon-Kardon amplifier (model PM655 V $\times$ 1) and routed to a speaker (Coulbourn model E12-01M) located inside the recording chamber. Stimulus parameters other than intensity (duration, rise time, interstimulus interval) were controlled by Coulbourn modular components and activated manually by the experimenter. Stimulus intensity of the tone was determined with a sound-level meter (A scale) positioned in the center of the training and recording chambers. Coulbourn Precision Regulated Animal Shockers (Model E13-14) were used to produce shock transmitted to the mice through grid floors in Coulbourn Habitest cages (Model E10-18RF). These cages were housed in Coulbourn Isolation Cubicles (Model H10-23).

Four to five days after the last training trial, the mice were presented with 15 conditioned tone stimuli over the course of 15 min. These tones were presented to the animals in their home cage without otherwise disturbing them; they could have been awake or asleep at tone onset.

To control for the effects of handling and tone presentation alone, a separate groups of animals were placed in the shock chamber and given the same number of presentations of the tone as the shock-trained animals, but never received shock. These animals were tested by presenting the tone in their home cage in the same manner as described for the shock-trained mice. All cue-presentation sessions were conducted in the home cage to avoid contextual reminders associated with shock.

Five animals in the shock training group and five in the control group were sacrificed at two h after cue presentation, and the remaining experimental and control group mice were sacrificed at 4 h after cue presentation. Thus, each time period reflects data from 5 experimental and 5 control mice. Brain slices were prepared for neurotransmitter measurements as described below.

Neurotransmitter release measurements from brain slices

I. General procedures—Animals were decapitated under halothane anesthesia and the brains then rapidly dissected and placed into ice-cold phosphate buffered saline, pH 7.4. One mm thick coronal slices were made by the aid of a mouse brain matrix (Model BS-5000C, Braintree Scientific, Braintree, MA). The amygdala, NPO, and DRN were dissected using visual landmarks with custom made tissue punchers. The hippocampus was dissected using a scalpel. Tissues were further cut into 0.05 mm by 0.2 mm pieces to increase surface area for neurotransmitter uptake and oxygen/glucose supply. After washing, to label the endogenous neurotransmitter pools, slices were co-incubated with 60 nM [^3H]-NE (1-[7,8- ^3H] Noradrenaline, 40.0 Ci/mmol specific activity, Amersham Biosciences) and 200 nM [^{14}C]-GABA (γ -aminobutyric acid, 43.1 mCi/mmol specific activity, Sigma-Aldrich, St. Louis, MO) for 30 min at 35 °C in freshly bubbled (95% O_2 /5% CO_2) Krebs-bicarbonate-HEPES buffer (KBH) composed of the following (in mM): NaCl, 118; KCl, 3.5; CaCl_2 , 1.25; MgSO_4 , 1.2; KH_2PO_4 , 1.2; NaCO_3 , 25; HEPES-NaOH (pH 7.4), D-glucose, 11.5; pargyline, 10 μM ; ascorbic acid, 0.6 mM; and EDTA (ethylenediaminetetraacetic acid), 0.1 mM. Ca^{2+} -free KBH buffer contained 0.1 mM EGTA (ethylene-glycol-bis(oxyethylenitrilo)-tetraacetic acid) instead of 1.25 mM CaCl_2 . To evaluate influence of ^{14}C -GABA metabolism on our measurements, we compared [^{14}C]-GABA release properties with or without γ -vinyl-GABA (100-300 μM , Tocris Biochemicals, Ellisville, MO), a GABA transaminase inhibitor, in pilot experiments. As no significant differences were observed, we did not include γ -vinyl-GABA in the experimental buffer. After double-labeling with the radioactive tracers, slices were transferred to a superfusion chamber (0.1 ml chamber volume) and superfused with continuously bubbled KBH (warmed to 35 °C, 0.2 ml/min superfusion rate) for 45 min to remove un-incorporated radioactivity. Three 3-min fractions of the superfusate were collected to determine basal (resting) neurotransmitter efflux. To probe the state of the release machinery, we induced neurotransmitter release by depolarization, by rapid switching of superfusion lines from normal KBH to a KBH containing 50 mM KCl, for 1.5 min. In the depolarizing buffer the NaCl concentration was reduced from 118 mM to 72.7 mM in order to maintain iso-osmolality. ^3H and ^{14}C content of the superfusate and the activity remaining in the slices at the end of the experiment were determined by liquid scintillation counting (Beckman Instruments Inc, Fullerton, CA), using a double-label method, which was calibrated and validated in the range of expected radioactivity efflux values. Release was expressed as the fractional release rate, calculated as the fraction of radioactivity released, divided by the amount remaining in slices at that particular time point. Amount of depolarization-evoked release was calculated as the area under the peak. Basal efflux under control conditions was set to 100%, the amount of depolarization-evoked release was calculated as the area under the peak and was expressed as % of basal release. Fractional release value of the last fraction before stimulus application was used as basal release value in these calculations. Comparisons across conditions were conducted using Student's t-test.

II. Extracellular Ca^{2+} concentration- ($[\text{Ca}^{2+}]_o$) dependence of neurotransmitter release—Under physiological conditions, invasion of action potentials into the noradrenergic nerve terminals of the hippocampus evokes neurotransmitter release by activation P/Q and N-type calcium channels followed by the influx of extracellular Ca^{2+} (Kimura et al., 1995; Lonart et al., 1998; Khvotchev et al., 2000) leading to vesicular exocytosis. In contrast, GABA release may contain both $[\text{Ca}^{2+}]_o$ -dependent and independent components (Bernath, 1992; During et

al., 1995; Wang et al., 1996; Sciancalepore et al., 1998). To assess the extent to which $[Ca^{2+}]_o$ -dependent processes contribute to $[^3H]$ -NE and $[^{14}C]$ -GABA efflux in our experimental system; in three mice, we carried out release measurements in each of the four brain regions with or without the presence of Ca^{2+} in the experimental buffer. This approach has been used in studies of the hippocampus (Lonart and Johnson, 1995; Katona et al., 1999), but was especially important for measurements from the amygdala, NPO and DRN, where *in vitro* neurochemistry of $[^3H]$ -NE and $[^{14}C]$ -GABA release has not been established.

First, slices were incubated with radiolabeled neurotransmitter in normal KBH buffer containing 1.2 mM Ca^{2+} to label the endogenous neurotransmitter pools, then loaded to superfusion chambers. Next, to directly compare release amounts in normal and Ca^{2+} -free buffers, half of the chambers were superfused with normal buffer and half of the chambers were superfused with a buffer where $CaCl_2$ was replaced with 100 μM EGTA, to chelate residual Ca^{2+} . Neither basal $[^3H]$ -NE nor basal $[^{14}C]$ -GABA efflux was dependent on extracellular Ca^{2+} (not shown) suggesting that under basal conditions $[^3H]$ -NE and $[^{14}C]$ -GABA effluxes are determined by $[Ca^{2+}]_o$ -independent processes, e.g., miniature synaptic potentials and re-uptake. To the contrary, high potassium-depolarization evoked hippocampal $[^3H]$ -NE release was more than 99% dependent on the presence of extracellular Ca^{2+} , as reported previously (Kimura et al., 1995; Lonart et al., 1998). Similarly, measurements of evoked $[^3H]$ -NE release from the amygdala (97%, Fig. 3.), NPO (97%), and DRN (99%) were also highly $[Ca^{2+}]_o$ -dependent, indicating a release mechanism that uses vesicular exocytosis triggered by influx of extracellular Ca^{2+} .

$[Ca^{2+}]_o$ -dependence of $[^{14}C]$ -GABA release was quite different. Only about 64% of the evoked $[^{14}C]$ -GABA release from the hippocampus, 58% from the amygdala (Fig. 3), and 71% from the NPO was dependent on $[Ca^{2+}]_o$, suggesting that in these areas GABA is released from both $[Ca^{2+}]_o$ -dependent vesicular stores, and $[Ca^{2+}]_o$ -independent cytoplasmic pools (Bernath, 1992; Canas et al., 2004). Surprisingly, release of $[^{14}C]$ -GABA from the DRN was almost completely (96%) dependent on $[Ca^{2+}]_o$.

These findings validated our methods, and suggest that the release assays we employed replicate salient physiological features of hippocampal NE and GABA release. In addition, these measurements established baseline parameters of $[^3H]$ -NE and $[^{14}C]$ -GABA release from the amygdala, NPO, and the DRN.

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Figure 1. Presentation of a fearful cue suppresses depolarization-evoked [^3H]-NE release from slices of the mouse amygdala

Four to five days after the last exposure to tone-shock pairing (fear conditioned (FC)), or to tone alone (neutral cue (NC)), the mice were presented with the tone in their home cages, and sacrificed 2 h (panels A and C) or 4 h later (panels B and D). Amygdala slices were dissected and labeled with [^3H]-NE and [^{14}C]-GABA, transferred into superfusion chambers, washed for 45 min, and depolarized with the introduction of a stimulatory buffer (50 mM KCl in KBH buffer, for 90 sec pulse, indicated by an arrow) as described in the Methods section.

Representative experiments (A and B) and summary data are shown (C and D). Basal efflux under control conditions was set to 100%, the amount of depolarization-evoked release was calculated as the area under the peak and is expressed as % of basal release under control conditions. Summary data are mean $\pm$ SEM values determined from 5 animals measured in duplicates. Asterisks (**) indicate statistically significant differences (Student's t-test, $p < 0.01$).

Figure 2. Presentation of a fearful cue enhances depolarization-evoked [^{14}C]-GABA release from slices of the mouse amygdala

Four to five days after the last exposure to tone-shock pairing (fear conditioned (FC)), or to tone alone (neutral cue (NC)), mice were presented with the tone in their home cages, and sacrificed 2 h (panels A and C) or 4 h later (panels B and D) for *in vitro* neurotransmitter measurements as described in Fig. 1 and the Methods section. Representative experiments (A and B) and summary data are shown (C and D). Introduction of the depolarizing buffer is indicated by an arrow. Data are mean $\pm$ SEM values determined from 5 animals measured in duplicates. Asterisk (*) indicates statistically significant difference (Student's t-test, $p < 0.05$).

Figure 3. $[\text{Ca}^{2+}]_o$ -dependence of depolarization-evoked $[\text{}^3\text{H}]$ -norepinephrine ($[\text{}^3\text{H}]$ -NE) and $[\text{}^{14}\text{C}]$ - γ -amino-butyric acid ($[\text{}^{14}\text{C}]$ -GABA) release from slices of the mouse amygdala

Slices of naïve animals were dissected, labeled with $[\text{}^3\text{H}]$ -NE and $[\text{}^{14}\text{C}]$ -GABA, and superfused as described in Fig. 1, with the exception that the 45 min washing period was carried out either with normal or Ca^{2+} -free KBH buffer, which was supplemented with 100 μM EGTA to chelate residual Ca^{2+} . Release was stimulated with the introduction of a stimulatory buffer (50 mM KCl in either normal or Ca^{2+} -free KBH buffer with normal osmolality, for 90 sec pulse, indicated by an arrow). Line figures (A and B) show fractional release data from representative experiments. Bar figures (C and D) present summaries of basal and high potassium depolarization evoked release from three experiments. Basal efflux under control conditions was set to 100%, the amount of depolarization-evoked release was calculated as the area under the peak and is expressed as % of basal release. Data presented are measurements from 3 animals (mean $\pm$ SEM).

Table 1**Depolarization-evoked [³H]-NE release from slices of the mouse amygdala, hippocampus, NPO and DRN**

Four to five days after the last exposure to tone-shock pairing (fear conditioned (FC)) or to tone alone (neutral cue (NC)), the mice were presented with the tone in their home cages, and sacrificed 2 hrs or 4 hrs later for *in vitro* neurotransmitter measurements. Data are mean $\pm$ SEM values determined from 5 animals measured in duplicates. Statistically significant differences were determined by Student's t-test.

Brain Region	Sample Time	NC	FC	p value
Amygdala	2h	15.30 $\pm$ 1.78	6.23 $\pm$ 0.76	<0.01
	4h	10.23 $\pm$ 1.94	9.11 $\pm$ 1.27	0.65
Hippocampus	2 h	10.90 $\pm$ 1.80	8.57 $\pm$ 0.44	0.26
	4 h	13.61 $\pm$ 2.72	9.15 $\pm$ 0.69	0.16
NPO	2 h	12.25 $\pm$ 2.61	13.55 $\pm$ 2.97	0.78
	4 h	12.37 $\pm$ 1.89	9.47 $\pm$ 1.46	0.27
DRN	2 h	13.90 $\pm$ 1.50	12.31 $\pm$ 2.26	0.63
	4 h	10.30 $\pm$ 1.49	10.18 $\pm$ 1.85	0.96

Table 2**Depolarization-evoked [^{14}C]-GABA release from slices of the mouse amygdala, hippocampus, NPO and DRN**

Four to five days after the last exposure to tone-shock pairing (fear conditioned (FC)) or to tone alone (neutral cue (NC)), the mice were presented with the tone in their home cages, and sacrificed 2 hrs or 4 hrs later for *in vitro* neurotransmitter measurements. Data are mean $\pm$ SEM values determined from 5 animals measured in duplicates. Statistically significant differences were determined by Student's t-test.

Brain Region	Sample Time	NC	FC	p value
Amygdala	2h	5.13 $\pm$ 1.15	8.61 $\pm$ 0.50	<0.05
	4h	5.93 $\pm$ 1.30	4.78 $\pm$ 0.61	0.45
Hippocampus	2 h	4.91 $\pm$ 1.48	6.66 $\pm$ 1.41	0.47
	4 h	6.65 $\pm$ 0.61	5.65 $\pm$ 1.33	0.52
NPO	2 h	7.24 $\pm$ 1.68	10.24 $\pm$ 1.97	0.35
	4 h	5.74 $\pm$ 1.98	7.06 $\pm$ 0.75	0.56
DRN	2 h	6.59 $\pm$ 1.56	8.35 $\pm$ 2.08	0.58
	4 h	5.17 $\pm$ 0.59	5.96 $\pm$ 1.42	0.63