Research Report

Effects of acute and repeated restraint stress on GABA efflux in the rat basolateral and central amygdala

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ABSTRACT

Stress can precipitate onset of multiple mood-related disorders, including depression. Examination of the neural basis of this phenomenon has highlighted the amygdala as a key component. Alterations in amygdalar activity and structure accompany various mood-related disorders, and interestingly, amygdalar morphology and behavior can be altered in animals subjected to repeated stress. Gamma-aminobutyric acid (GABA) transmission in the amygdala represents an important means by which information flow, activity, and function can be controlled; therefore, we determined the effects of acute and repeated restraint stress (RRS) on GABA efflux in the basolateral and central amygdalar complexes. In vivo microdialysis revealed that acute restraint stress increased GABA efflux in the basolateral amygdala, whereas central amygdala efflux remained unchanged. Animals subjected to prior repeated stress displayed no acute stress-mediated increases in GABA efflux in the basolateral amygdala, an event accompanied by no changes in basal GABA concentrations. Conversely, repeated restraint stress had no effect on GABA efflux or basal GABA levels in the CeA. Collectively, these data demonstrate that acute stress elicits unique and region-specific increases in GABA efflux in the rat amygdala, and that prior repeated stress differentially modifies this response.

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1. Introduction

Stress has been associated with onset or precipitation of mood-related disorders (Gold et al., 1988; 1988; Sheline, 2000). Elucidation of the neural substrates important for this association has highlighted the amygdala as key component. The amygdala is integral for the regulation of emotional behaviors (LeDoux, 2000), as well as the coordination of stress responses, as indicated by anatomical (Cullinan et al., 1995), neurochemical (Cook, 2004), behavioral (Muller et al., 2003) and physiological studies (Maroun and Richter-Levin, 2003). Alterations in amygdalar activity (Thomas et al., 2001; Hull, 2002; Drevets, 1999) and structure (Frodl et al., 2002; Brambilla et al., 2003) accompany various mood-related disorders and interestingly, amygdalar morphology and behavior can be modulated in animal models subjected to repeated stress (McEwen and Chattarji, 2004; Mitra et al., 2005; Vyas et al., 2002).

Gamman-aminobutyric acid (GABA) transmission in the amygdala represents an important means by which information flow, activity, and function can be controlled (Cassell et al., 1999; Davis et al., 1994; Woodruff et al., 2006).
For example, GABA transmission in the basolateral amygdalar complex (BLC) is postulated to modulate memory storage (Castellano et al., 1989), as well as acquisition of conditioned fear (Wilensky et al., 2000; Holahan and White, 2004; Holahan and White, 2004). Similarly, GABA transmission in the central amygdala (CeA) is also important for modulating function and activity. Unlike the BLC, however, the role GABA plays in modulating CeA function is less lucid. For example, electrophysiological data indicate that depending upon the type of receptor targeted, GABA agonists can either potentiate or block inhibitory responses in the CeA (Delaney and Sah, 1999). Thus, knowing the overall importance of GABA transmission in the amygdala, we set out to determine the effects of repeated RS on GABA efflux in the rat BLC and CeA. Further, to gain insight into more pathologically-relevant conditions, we set out to determine the effects of acute restraint stress (RS) on GABA efflux in the rat BLC and CeA. Thus, knowing the overall importance of GABA transmission in the amygdala, we set out to determine the effects of acute restraint stress (RS) on GABA efflux in these nuclei.

2. Experimental procedures

2.1. Animal protocols

Eight week old male Sprague Dawley rats (CD strain, Charles River) weighing approximately 250 g were singly housed and provided ad libitum access to food and water in accordance with all guidelines and regulations of The University of South Carolina Animal Care and Use Committee. Animals were maintained in a temperature-controlled room, with a light/dark cycle of 12/12 hours (h) (lights on at 0700 h) and handled daily for 7 days prior to experimentation.

2.2. Stress procedures

Animals were subjected to repeated RS as described previously (Reznikov et al., 2008; Reagan et al., 2004). Briefly, animals were placed in flexible wire mesh restrainers with protective rubberized edges and were restrained 6 h/day (d) for 10 d in their home cages. Restrainers were fastened using clips that allowed enough space to compensate for slight movements of the animal within the restraining apparatus. Use of this paradigm results in diminished acute RS-mediated activation of phenotypically distinct neuronal subpopulations in the rat BLC (Reznikov et al., 2008), Based upon this finding, we postulated that 6 h RS for 10 d may also provide critical knowledge regarding potential neurochemical processes that contribute to the stress-mediated alterations in amygdalar function.

2.3. Surgery

Following repeated RS or handling, animals received dual microdialysis guide cannula surgery (Bioanalytical Systems, Inc., West Lafayette, IN, USA) under sodium pentobarbital anesthesia (70 mg/kg, i.p.). Placement was targeted towards the CeA and contralateral BLC as previously described (Reznikov et al., 2007). Guide cannula placement was alternated between left and right hemispheres, so that an equal number of left and right targets for each region examined occurred. Target coordinates were calculated according to Paxinos and Watson (1998) relative to Bregma: for CeA: AP −2.0 mm, L±3.9 mm, DV −7.0 mm from skull surface; for BLC: AP −3.1 mm, L±5.0 mm; DV −7.0 mm. Guide cannulas (with stainless steel stylets to maintain the patency of the cannula) were fixed to the skull with skull screws and dental cement. Animals were allowed 2 to 3 recovery days following surgery, during which daily handling continued and habituation to microdialysis chambers occurred.

2.4. In vivo microdialysis

On the morning of microdialysis, stylets were removed and replaced with concentric microdialysis probes (Bioanalytical Systems) with a semipermeable membrane (nominal molecular weight cutoff of 30 kDa) extending 2.0 mm beyond the ventral tip of the guide cannulas. Probes were continuously perfused (2.0 μl/min) with an artificial cerebrospinal fluid (aCSF; pH 6.5) composed of the following (in mM): NaCl 150, KCl 3.0, CaCl2 1.7, MgCl2 0.9, d-glucose 4.9. Collection of dialysates (in 15 min intervals) from both probes began 3 h following probe insertion. For all groups described below, four baseline samples were collected before onset of experimental manipulations.

2.5. Experiment 1

To examine the effects of acute RS on GABA efflux, control animals (labeled ARS on graphs, n=8) and repeated RS animals (labeled RRS on graphs, n=8) were subjected to a 1 h acute RS challenge using a flat bottom clear plastic rodent restrainer as described previously (Reznikov et al., 2007). One hour after onset of acute RS, animals were released from the restrainers and four final post-stress collections occurred. Thus, a total of 12 collections were made. To control for handling effects, an additional set of animals (labeled handle on graphs, n=7) were picked up and immediately returned to the base of their bowls. This handling event occurred at the same time point coinciding with onset of acute RS challenge. A total of 12 samples were collected (four baseline, one handling, and seven post-handling collections) in order to provide comparisons across all time points.

2.6. Experiment 2

To examine whether GABA measured was derived from axonal depolarization, a separate group of control animals (n=8) received the voltage-gated sodium channel blocker tetrodotoxin (TTX; 10 μM) infused directly through each dialysis probe. This infusion began immediately after the fourth baseline collection and continued until termination of the dialysis session. Animals were subjected to a 1 h acute RS challenge as described above, commencing after the fourth TTX collection. Two post-stress/TTX samples were collected resulting in a total of 14 collections.

At the conclusion of each microdialysis experiment, rats were deeply anesthetized using isoflurane inhalation and then transcardially perfused with phosphate-buffered saline.
and 4% paraformaldehyde. Brains were removed, cryoprotected and coronally sectioned (45 μm) on a cryostat. Sections from the rostro-caudal extent of the amygdala were mounted and an acetylcholinesterase background stain was performed for assessment of probe tract location (Fig. 1, Panels A and B). Animals with probe tracts lying outside the target structures resulted in exclusion from data analysis. All dialysates were assayed for GABA utilizing HPLC with electrochemical detection and a pre-column o-phthaldialdehyde/sulfite derivatization procedure (Donzanti and Yamamoto, 1988; Rowley et al., 1995; Burrows et al., 2000). Quantification was accomplished by comparison to a 3-point standard curve bounding the expected range of dialysate concentrations. The limit of reliable detection (signal-to-noise ratio >2) using this method was approximately 0.005 μM, roughly ten-fold higher than the typical baseline dialysate concentration.

2.7. Statistical analysis

No hemispheric differences were observed and therefore data from each hemisphere were combined for each region. For every animal, each sample collection was expressed as a percent of baseline efflux (i.e. each sample time point was divided by the average of the first four baseline collections). This was done to control for variability between subjects. Data were then subjected to analysis of variance (ANOVA) with TIME as a repeated measure and TREATMENT as a between-subjects factor. Fisher’s LSD post-hoc comparisons were employed to determine the source of significance (P<0.05) revealed by ANOVA. For TTX experiments, data were subjected to an ANOVA to assess for significant differences among treatment groups (P<0.05). All n values on graph represent total number included for statistical analysis following application of exclusion criteria (i.e. mistargeted probe placement).

3. Results

3.1. Effects of stress on GABA efflux in the rat basolateral and central amygdala

3.1.1. Experiment 1

No differences in basal BLC GABA levels were observed across treatment groups (F2,17=0.185; P=0.8331) (Fig. 2, Panel A). However, a rapid increase in BLC GABA efflux was observed upon onset of acute RS in control animals (Fig. 3, Panel A), an event succeeded by oscillatory fluctuations resembling a wave-like pattern. An acute handling event did not change GABA efflux in the BLC. Examination of animals subjected to repeated RS revealed markedly attenuated acute RS-mediated increases in GABA efflux, with values remaining near baseline levels for the entirety of the microdialysis session. Statistical analysis revealed a significant main effect of TIME (F11,187 = 3.113; P = 0.001) and TREATMENT (F1,17 = 4.279; P = 0.031). A significant TREATMENT×TIME interaction (F22,187 = 4.422; P<0.0001) was also observed.

Post-hoc analysis revealed significant increases in GABA efflux in control animals challenged with acute RS relative to baseline during the first and last stress collections, as well as during the final post-stress collection. A significant decrease in GABA efflux was also noted in control animals subjected to acute RS during the second post-stress collection relative to baseline. Significant differences were observed between control animals challenged with acute RS and animals experiencing a handling event during the first and second stress collections, as well as during the final stress and post-stress collections. Finally, significant differences were also observed.

![Fig. 1 – Representative photomicrograph of acetylcholinesterase-stained sections demonstrating probe placement of targeted regions. (Panel A) Demonstration of probe tract (dark red/brown staining) targeted towards the CeA. (Panel B) Demonstration of probe tract targeted towards the BLC. Scale bar equals approximately 1.2 mm. Abbreviations: BLA, basolateral amygdaloid nucleus anterior; BLP, basolateral amygdaloid nucleus posterior; BLV, basolateral amygdaloid nucleus ventral; BMP, basomedial amygdaloid nucleus posterior; BMA, basomedial amygdaloid nucleus anterior LaVL, lateral amygdaloid nucleus ventrolateral; LaVM, lateral amygdaloid nucleus ventromedial; LaDL, lateral amygdaloid nucleus dorsolateral; CeC, central amygdaloid nucleus capsular; CeL, central amygdaloid nucleus lateral division; CeM, central amygdaloid nucleus medial division; opt, optic tract; Pir, piriform cortex. Image source overlay: Paxinos and Watson, 1998.](image-url)
between repeated RS animals and control animals subjected to acute RS during the first and last stress collection, as well as during the second and final post stress collection. No significant differences were observed between repeated RS animals and those that experienced a handling event.

In the CeA, no differences in basal GABA levels were observed among treatment groups \( (F_{2,18} = 2.478; P = 0.1120) \) (Fig. 2, Panel B). In addition, no alterations in GABA efflux were observed in animals subjected to acute RS or a handling event (Fig. 3, Panel B). Similarly, no changes in GABA efflux were observed in the CeA of repeated RS animals challenged with acute RS, although efflux appeared slightly above baseline values. Statistical analysis revealed no main effects of TIME \( (F_{11,198} = 1.169; P = 0.311) \) or TREATMENT \( (F_{1,18} = 1.703; P = 0.210) \), indicating GABA profiles did not differ across treatment groups. Additionally, no TREATMENT×TIME interactions \( (F_{22,198} = 1.167; P = 0.281) \) were observed.

### 3.1.2. Experiment 2

In control rats (i.e. rats with no prior stress history) infusion of TTX did not change basal GABA levels in the BLC (Fig. 4). Significantly and importantly, however, acute RS did not increase BLC GABA efflux under TTX conditions. This event was succeeded by fluctuating levels resembling a wave-like pattern. Handling did not increase GABA efflux (closed gray circles). Animals subjected to repeated restraint stress displayed dramatic impairments in acute restraint stress-mediated alterations in GABA efflux (open circles). This event was succeeded by fluctuating levels resembling a wave-like pattern. Handling did not increase GABA efflux (closed gray circles). Animals subjected to repeated restraint stress displayed dramatic impairments in acute restraint stress-mediated alterations in GABA efflux (open circles). The results of the current study demonstrate that acute RS increases GABA efflux in the BLC, but not CeA of control rats.

### 4. Discussion

The results of the current study demonstrate that acute RS increases GABA efflux in the BLC, but not CeA of control rats. Fig. 2 – Examination of basal GABA levels. Repeated restraint stress did not change basal GABA levels in the BLC or CeA. Data are shown as concentration (μM). Abbreviations: ARS, acute restraint stress; BLC, basolateral amygdala; CeA, central amygdala; RRS, repeated restraint stress.

Fig. 3 – Effects of acute stress on GABA efflux in the rat basolateral and central amygdala. (Panel A) Acute restraint stress increased GABA efflux in the BLC during the initial stress collection in control animals (closed black circles). This event was succeeded by fluctuating levels resembling a wave-like pattern. Handling did not increase GABA efflux (closed gray circles). Animals subjected to repeated restraint stress displayed dramatic impairments in acute restraint stress-mediated alterations in GABA efflux (open circles). (Panel B) GABA efflux was not altered in the CeA of animals subjected to acute restraint stress (closed black circles). Similarly, handling did not change GABA efflux in this region (closed gray circles). Prior repeated restraint stress had no effect of GABA efflux in the CeA (open circles). Symbols: \(^*\) compared to baseline, \(P < 0.05\); \(^#\) compared to handled controls, \(P < 0.05\); \(@\) compared to RRS animals, \(P < 0.05\).

Abbreviations: ARS, acute restraint stress; BLC, basolateral amygdala; CeA, central amygdala; RRS, repeated restraint stress.
animals. This effect was absent in animals subjected to repeated RS and then challenged with acute RS. Lastly, repeated RS had no effect on CeA GABA efflux or basal GABA levels in either region. These results illustrate that acute RS elicits region-specific alterations in GABA transmission, and that repeated RS modifies this response.

When using microdialysis approaches to assess amino acid transmission it is essential to consider whether efflux measured represents true synaptic release or is related instead to nonsynaptic sources (Timmerman and Westerink, 1997). To address this concern, we used the sodium channel blocker TTX thereby inhibiting the rising phase of an action potential and presumably stimulus-induced vesicular release. While infusion of TTX did not change basal GABA efflux in either the BLC or CeA, acute RS-mediated changes in the BLC were abolished under TTX conditions. Based upon these results, it is probable that acute RS-mediated changes in BLC GABA efflux are due to local (and fibers of passage) axonal depolarization, although changes in glial metabolism as well as nonneuronal pools may also contribute to these neurochemical changes (Timmerman and Westerink, 1997).

It is also possible that GABA measured in our study reflects volume transmission (Fuxe et al., 2005). Interest regarding volume transmission as an important means of communication in the CNS originated from observations of transmitter-receptor mismatches, meaning that the extent of terminal innervation for a given transmitter did not correlate with the regional distribution of receptors for that transmitter (Agnati et al., 1986). Using this model, it is possible that under stress conditions changes in pressure gradients generated by pulsatility in brain vessels and cerebral blood flow (Agnati et al., 2005) influence GABA diffusion and subsequent sampling in the amygdala. This phenomenon may explain why a main effect of TIME was observed in the CeA under acute RS+TTX conditions. It is possible that TTX infusion unmasked a nonclassical neurotransmission-mediated phenomenon that is obscured under normal physiological conditions. Indeed, recent evidence suggests that GABA release from the intercalated cell (ITC) masses of the amygdaloïd complex can be induced by application of the peptide cholecystokinin, an event postulated to occur through volume transmission (Perez et al., 2007). Therefore, it remains to be determined whether any of these possibilities contribute to GABA measured in our study.

4.1. Acute stress-mediated increases in GABA efflux in the basolateral amygdala

Studies suggest that GABA transmission is fundamental for acquisition and expression of fear-related memory (Stork et al., 2002; Bauer and LeDoux, 2004; Venton et al., 2006). Although we cannot directly say that the changes in GABA efflux measured in our study contribute to fear-related memory without a behavioral correlate, it is interesting to speculate that increases in GABA efflux upon acute RS challenge may be related to this phenomenon.

The oscillatory pattern observed in the BLC is rather striking. The mechanism underlying this phenomenon remains unknown. However, oscillatory GABA release has been documented in the ventromedial hypothalamus during both glucoprivic (Spector et al., 1996) and insulin-induced hypoglycemia states (Beverly et al., 2001), metabolic challenges that may be considered stressful and thereby activate the hypothalamic-pituitary axis. Moreover, evidence suggests that the activity of the synthetic enzyme for GABA, glutamic acid decarboxylase, is influenced by glucose availability in the ventromedial hypothalamus (Beverly and Martin, 1990; 1991). Thus, it is possible that glucose availability in the BLC modulates the availability of releasable pools of GABA. Alternatively, it has been suggested that acute serotonin release activates GABA neurons in the BLC, but under chronic release or high levels, excitation of this cell population is reduced (Rainnie, 1999). Since serotonin is released under states of stress in the BLC (Kawahara et al., 1993), and is postulated to have divergent effects on BLC GABAergic neurons (Rainnie, 1999), it is possible that acute RS-mediated alterations in serotonin release contribute to the oscillatory pattern we observe.

4.2. Lack of acute restraint stress-mediated effect on GABA efflux in the central amygdala

Changes in GABA levels in the CeA in response to acute RS were not observed, a finding paralleling that noted in animals subjected to the forced swim test (Ebner et al., 2005). The CeA receives GABAergic input from ITC masses located within the amygdaloïd complex (Pare and Smith, 1993). It has been suggested that activation of the lateral amygdala during fear-related experiences excites ITC neurons located at the same lateromedial level, causing an inhibition of more medially located ITC cells, leading to eventual disinhibition of the cells in the medial capsular division of the CeA (Pare et al., 2004). The final consequence of this proposed cascade is facilitation of CeA output to brainstem effector sites, thereby reinforcing
appropriate autonomic responses. Neurochemically, this model predicts increased GABA release onto medially located ITC cells coupled to a decreased GABA release onto cells located in the medial capsular division of the CeA. Since these events may contribute to the extracellular GABA efflux measured in this study, it is possible that the overall net sum of GABA release is reflected as no change. Accordingly, this sequence of events may provide a functional correlate to the neurochemical profile reported here.

4.3. Effects of repeated restraint stress on GABA efflux in the basolateral but not central amygdala

Reduced inhibitory neurotransmission in the BLC is involved in the enhancement of conditioned fear behavior in animals subjected to previous stress (Rodriguez Manzanares et al., 2005). Repeated stimulation of corticotropin releasing factor receptors in the BLC also results in enhanced anxiety-like behaviors, an event associated with decreased GABAergic inhibition (Rainnie et al., 2004). Similarly, Truitt et al. describe increased anxiety-like behavior following disruption of GABAergic neuronal subpopulations in the BLC (Truitt et al., 2007). Our data may provide a neurochemical correlate for these observed phenomena since repeated RS leads to significant impairments in GABA efflux in the BLC relative to control animals. Altered GABA efflux in the BLC following repeated RS may also reflect reduced activation of inhibitory neurons (Reznikov et al., 2008), which in turn may reflect a fundamental change in the relay of converging sensory information. If true, then possible outcomes may include altered assessment of emotional valence in conjunction with altered behavioral responses (Truitt et al., 2007; Bachevalier and Malkova, 2006), events congruent with the aforementioned observations.

Repeated RS had no effect on GABA efflux or basal GABA levels in the CeA. This lack of effect may reflect the phylogenetic and evolutionary differences of the BLC and CeA. Since the CeA represents a conserved evolutionary brain structure of survival heritage (Moreno and Gonzalez, 2007), its overall ability to withstand or adapt to homeostatic challenges may be more rigorous or encompass different mechanisms than that of the more evolutionarily recent BLC. Indeed, it has been noted that the BLC is under tonic GABAergic inhibition (Shekhar et al., 2003); whether this is the case for the CeA still remains under debate. Moreover, many of the GABAergic neurons within the CeA are projection neurons (Sun et al., 1994), as opposed to the local projecting GABAergic interneurons of the BLC. Thus, the apparent immunity of CeA GABA efflux from stress-mediated effects likely reflects the neurochemical and functional differences of these two major subdivisions.

4.4. Implications for disease and considerations

It is important to consider the pathology of interest when discussing the effects of stress. Repeated exposure to stress in animal models, including stress-resistress and time-dependent sensitization, produces behavioral and neuroendocrine responses similar to those described in individuals with posttraumatic stress disorder (Harvey et al., 2003, 2004; Khan and Liberzon, 2004; Faure et al., 2007). Yet other studies describe that repeated stress produces depressive-like symptoms (Reagan et al., 2008), as well as increases vulnerability to anxiety-like behaviors (Rodriguez Manzanares et al., 2005). The repeated RS paradigm used in the current study has been proposed to represent a potential transitory period during which stress-mediated effects on hippocampal function are just manifesting, an observation not noted with 10 d of 2 h daily RS (McLaughlin et al., 2007). Knowing that the extension of repeated RS to 21 d results in hippocampal atrophy (Vyas et al., 2002) paralleling that observed in depressive illness and posttraumatic stress disorder (McEwen, 2003), suggests that changes occurring in the amygdala at 10 d may serve as key contributors to these endpoints. Therefore, it is possible that stress-mediated alterations in amygdalar GABA efflux may possess relevance for human conditions such as depressive illness and anxiety-related disorders, including posttraumatic stress disorder.

We have recently shown that acute RS increases c-Fos expression in specific neuronal subpopulations in the BLC (Reznikov et al., 2008), including GABAergic interneurons. This finding coincides with the current neurochemical data demonstrating an acute increase in GABA release in the BLC upon acute RS challenge. Further, the repeated RS paradigm used in the current study significantly decreases c-Fos expression in BLC GABAergic neurons, again paralleling the neurochemical findings reported here. Therefore, acute and repeated RS conditions elicit unique changes in the cellular and neurochemical endpoints we have examined. However, it is important to note evidence suggesting that acute corticosterone treatment in rats produced amygdalar dendritic hypertrophy and elevated anxiety-like behavior (Mitra and Sapolsky, 2008), an effect not potentiated by 10 d of corticosterone treatment. Duvvari and Pare also suggest that a single glucocorticoid application to BLC slices increases the excitability of amygdalar neurons by reducing inhibitory GABA currents (Duvvari and Pare, 2007). Thus, it is possible that 10 d of RS is not necessary to promote marked effects on GABA efflux in the BLC and that perhaps the initial 6 h RS session on day one initiated a cascade of events that lead to impairments in acute RS-mediated increases in BLC GABA efflux. Therefore, future studies are necessary to more thoroughly examine these possibilities.

5. Conclusions

The results of current study demonstrate that GABA transmission is modulated differentially during and following exposure to an acute stressful stimulus in two major subdivisions of the rat amygdala. Moreover, exposure to repeated RS modified GABA efflux in a region-specific fashion, suggesting that GABA transmission in the BLC and CeA may differ in their functional significance and/or regulating mechanisms. Finally, considering that such marked effects were observed on GABA efflux in the BLC under conditions with potential clinical relevance strengthens the hypothesis that altered GABA transmission in the amygdala likely contributes to altered emotionality and subsequent manifestation of pathological states. Therefore, examination of whether stress-mediated alterations in GABA transmission
are important targets of certain mood-stabilizing agents represents an important future direction.

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