Timing of Impulses From the Central Amygdala and Bed Nucleus of the Stria Terminalis to the Brain Stem

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INTRODUCTION

Behavioral findings indicate that the central nucleus of the amygdala (CE) and bed nuclei of the stria terminalis (BNST) subserves different functions. In particular, lesion (Campeau and Davis 1995; Hitchcock and Davis 1987 1991; LeDoux et al., 1988) and local drug infusion studies (Kim et al. 1993; Wilensky et al. 2006) have shown that the CE is critically involved in the rapid expression of conditioned fear responses to discrete sensory cues, functions that are left intact by BNST lesions (Gewirtz et al. 1998; LeDoux et al. 1988; Sullivan et al. 2004; Walker and Davis 1997). Instead, BNST lesions interfere with the development of longer “anxiety-like” states in response to more diffuse environmental contingencies, responses that often persist after termination of the threat (reviewed in Walker et al. 2003). For instance, BNST lesions were reported to disrupt corticosterone and freezing responses to contextual stimuli associated with aversive outcomes (Sullivan et al. 2004).

In contrast with these behavioral findings, however, these two structures exhibit similar anatomical properties. For instance, CE and BNST neurons send robust projections to an overlapping set of autonomic and motor brain stem nuclei thought to generate components of fear/anxiety responses (Dong and Swanson 2004 2006; Dong et al. 2000 2004; Holstege et al. 1985; Hopkins and Holstege 1978; Veening et al. 1984). Moreover, both receive strong glutamatergic inputs from the basolateral amygdala (BL) (Dong et al. 2001; Krettek and Price 1978a,b; Pare et al. 1995; Savender et al. 1995). In fact, these overlapping connections of CE and BNST, coupled to similarities in neuronal morphology and transmitter content (reviewed in McDonald 2003), have led to the proposal that the BNST and CE constitute one anatomical entity termed the extended amygdala (Alheid and Heimer 1988; deOlmos and Heimer 1999).

In further support of this idea, there are strong reciprocal connections between CE and BNST (Dong and Swanson 2006a-c; Dong et al. 2001; Krettek and Price 1978b; Price 1984). Moreover, both receive strong glutamatergic inputs from the BL (Dong et al. 2000; Holstege et al. 1985; Hopkins and Holstege 1978; Veening et al. 1984). According to these tracing studies, BNST projections to the CE mostly originate in its antero-lateral and anteromedial divisions, and the same regions receive the bulk of CE outputs. A puzzling property of amygdalo–BNST connections shown in preceding studies is that there is tremendous heterogeneity in the course taken by these axons to reach their target. Some follow a direct route, through and around the substantia innominata (ventral amygdalofugal pathway). Others follow a circuitous path, via the stria terminalis, that lengths their trajectory several-fold, raising questions as to the significance of this peculiar anatomical arrangement.

Thus this study aimed to shed light on the functional significance of BNST–amygdala connections using extracellular recordings of BNST and central amygdala (CEA) neurons in rats anesthetized with isoflurane. Our results point to an unexpected level of coordination in the timing of BNST and CE outputs relative to BL inputs.

METHODS

All procedures were approved by the Institutional Animal Care and Use committee of Rutgers State University, in compliance with the Guide for the Care and Use of Laboratory Animals (Depart-
ment of Health and Human Services). Male Sprague-Dawley rats (225–250 g) were anesthetized with a mixture of ambient air, oxygen, and isoflurane. Atropine (0.05 mg/kg, im) was administered to prevent secretions. The body temperature was maintained at 37–38°C with a heating pad. The level of anesthesia was assessed by continuously monitoring the electroencephalogram and electrocardiogram.

A local anesthetic (bupivacaine, 0.1 ml) was injected subcutaneously in the region of the scalp to be incised. Ten minutes later, the scalp was cut on the midline. The bone overlying the regions of interest was removed, and the dura mater was opened. Under stereotaxic guidance, groups of two or three tungsten stimulating electrodes (intertip spacing of ~1 mm) were inserted in the BL nucleus of the amygdala (Fig. 1A1), the stria terminalis, and just dorsal to the substantia nigra (Fig. 1A2) where CE and BNST axons en route to the brain stem form a compact bundle (Holstege et al. 1985; Hopkins and Holstege 1978).

For the placement of stimulating and recording electrodes, the following stereotaxic coordinates were used (all relative to the bregma and in mm). For BL, antero-posterior (AP) −2.3, medio-lateral (ML) 5.0, dorso-ventral (DV) 8.7, and AP −2.8, ML 4.8, DV 7.8. For CE, the coordinates were AP −2.6, ML 4.1, DV 8.0 and AP −1.8, ML 3.6, DV 7.8. For BNST, the coordinates were AP −0.8, ML 1.7, DV 6−7.5 and −0.2, ML 1.6, DV 6−7.5. For brain stem, three electrodes were inserted at the same AP level (−6.0), three different ML levels 1.6, 2.1, 2.6, and DV positions 7.7, 7.3, 6.9, respectively.

Evoked responses were recorded in CE and BNST with high-impedance (10–12 MΩ) tungsten microelectrodes (FHC, Bowdoin, ME). The positions of the microelectrodes were adjusted independently with micromanipulators. A subset of rats was prepared with electrolytic lesions (0.6 mA for 5–10 s). The brains were extracted from the skull, fixed in 2% paraformaldehyde and 1% glutaraldehyde, sectioned on a vibrating microtome (at 100 μm), and stained with cresyl violet to show electrode placements, as shown in Fig. 1. The microelectrode tracks were reconstructed by combining micrometer readings and histology. To be included in the analysis, cells had to be histologically confirmed as being located in the regions of interest. Analyses were performed off-line with commercial software (IGOR, WaveMetrics, Lake Oswego, OR; Matlab, Natick, MA) and custom-designed software running on personal computers. Spikes were detected using a window discriminator after digital filtering of the raw waves. All values are expressed as means ± SE.

RESULTS

Database

A total of 130 CE and 96 BNST neurons that were spontaneously active and/or responsive to electrical stimuli delivered in the BL or brain stem were recorded from 48 intact rats in this study. Histological controls (Fig. 1B2) showed that our sample of CE cells included 102 and 28 neurons recorded in the medial (CEm) and lateral (CEl) parts of the CE, respectively. For BNST cells (Fig. 1B1), most were recorded in the anterolateral region (n = 83), as defined by Ju and Swanson (1989), with the rest in the posterior (n = 13) region.

Consistent with earlier anatomical findings indicating that CEm has more extensive brain stem projections than CEl (Hopkins and Holstege 1978; Petrovich and Swanson 1997;
Veening et al. 1984), the incidence of brain stem–projecting cells, as identified by their antidromic responses to brain stem stimuli, was significantly higher in CEm than CEi (Fisher exact test, \( P < 0.001 \)). Indeed, as many as 76% of CEm cells (or 78 of 102) were antidromically responsive to brain stem stimuli compared with 32% of CEi cells (or 9 of 28). In the BNST, all antidromically responsive neurons to brain stem stimuli (30% or 29 of 96) were located in the anterolateral region. Thus we focused on these anterolateral BNST neurons.

**Latency of brain stem–evoked antidromic responses in CE and BNST neurons**

Figure 2 shows representative examples of CE (Fig. 2A) and BNST (Fig. 2B) neurons that generated antidromic spikes in response to brain stem stimulation. As shown in the superimpositions of evoked responses (Fig. 2, A1 and B1), antidromic action potentials could easily be distinguished from synaptically evoked spikes because they had a fixed latency. Moreover, antidromic spikes failed when spontaneous action potentials occurred in the collision interval (Fig. 2, A2 and B2, Collision). Another property common the CE and BNST cells was that the transition between the initial segment and somatodendritic components of antidromic spikes was slower than seen in spontaneously occurring action potentials (Fig. 2, A1 and B1, insets), often giving rise to clear break between the initial segment and somatodendritic components of the spikes (Fig. 2, A1 and B1, arrowheads in insets).

Consistent with previous findings in rats (Quirk et al. 2003) and rabbits (Pascoe and Kapp 1985), antidromic response latencies to brain stem stimuli were distributed bimodally in CE neurons with an early mode at 9.7 ± 0.7 ms and a late one at 29.4 ± 0.7 ms (Fig. 2A3). Computing the Kolmogorov-Smirnov test for goodness of fit confirmed that the antidromic response latencies of CE neurons were not normally distributed \( (P < 0.01) \). In contrast, the frequency distribution of brain stem-evoked antidromic response latencies was unimodal in BNST neurons (average of 10.6 ± 0.8 ms; Fig. 2B3).

**FIG. 2.** Physiological identification of brain stem–projecting CE (A) and BNST (B) neurons by antidromic invasion from the brain stem. In A and B, panel 1 shows superimposed antidromic responses to brain stem stimulation, whereas panel 2 shows cases where the antidromic spikes failed because of collision with spontaneous action potentials. The insets in panel 1 show superimpositions of antidromic (black) and spontaneous (red) spikes. Note that the transition between the initial segment and somato-dendritic components of the spikes is longer for antidromic action potentials. Panel 3 shows a frequency distribution of antidromic response latencies evoked from the brain stem in samples of 87 CE and 29 BNST cells.
As mentioned in the introduction, previous tract-tracing studies have shown that CE axons can reach the brain stem directly, via the ventral amygdalofugal pathway, or through a longer round-about path, the stria terminalis. Thus these findings led us to suspect that the axons of CE cells with longer conduction times to the brain stem might course through the stria terminalis.

To test this idea, 26 rats were prepared with electrolytic lesions of the stria terminalis. Post hoc histological controls showed that in 12 of these cases, the stria was successfully lesioned with minimal damage to adjacent structures (Fig. 3A). An additional sample of CE neurons \((n = 42)\) was recorded in these rats and the distribution of brain stem–evoked antidromic response latencies was compared with that seen in intact rats (Fig. 3B). For the purpose of statistical comparisons, we used a cut-off of 20 ms to define cells with short versus long conduction times. In intact rats (Fig. 3B, solid line), our sample of antidromically responsive CE cells \((n = 87)\) was divided equally between cells with short \((47\%)\) versus long \((53\%)\) conduction times. In contrast, in rats prepared with lesions of the stria terminalis (Fig. 3B, dashed line), our sample of antidromically responsive CE cells \((n = 15)\) was mostly comprised of cells with short conduction times \((80\%)\) of cells). Using a Fisher exact test, the differing incidence of CE neurons with short versus long conduction times to the brain stem in intact versus stria terminalis lesioned rats was found to be statistically significant \(P = 0.034\).

**Latency of BL-evoked orthodromic responses in CE and BNST neurons**

Compared with the ventral amygdalofugal pathway, the stria terminalis lengthens the path of CE axons to the brain stem several-fold, raising questions as to the significance of this apparently disadvantageous arrangement. Because the BNST and CE both receive major excitatory inputs from the BL nucleus, we reasoned that lengthening the axonal path of some CE neurons might allow synchronization of BNST and CE impulses to the brain stem when they are both activated by BL inputs. To test this idea, we applied electrical stimuli in the BL nucleus and compared orthodromic response latencies in CE and BNST neurons.

Figure 4 shows representative examples of BL-evoked orthodromic responses in CE (Fig. 4A1) and BNST (Fig. 4B1) neurons (note different time base) and the corresponding peri-stimulus histograms of neuronal discharges (Fig. 4, A2 and B2). The incidence of such orthodromic responses to BL stimuli was significantly higher among CE than BNST neurons (CE, 45% or 46 of 102; BNST, 31% or 26 of 83; Fisher exact test, \(P < 0.02\)). However, the likelihood of observing BL-evoked orthodromic responses was similar for CE neurons with short versus long conduction times to the brain stem (Fisher exact test, \(P > 0.15\)).

As shown in the representative examples of Fig. 4, A1 and A2, CE cells generally responded with a pronounced, but brief, period of increased firing probability, lasting 3–6 ms. In contrast, the responses of BNST cells were more distributed in time, lasting 10–17 ms (Fig. 4, B1 and B2; the origin of this difference is considered in the DISCUSSION). The contrasting temporal profile of CE and BNST responses to BL stimuli led us to use two different measures to analyze response latencies: response onset versus response peak. The latency to response onset was defined as the average of the first two consecutive 1-ms bins of poststimulus time histograms with counts three times higher than seen in the 10-ms period preceding the BL stimulus. In neurons showing no spontaneous activity during the prestimulus period, the latency to response onset was defined as the average of the first two poststimulus bins with counts.

Consistent with the fact that the distance between the stimulation and recording sites is shorter for CE than BNST neurons, both measures yielded shorter latencies for CE than BNST neurons. Indeed, using 1.5 times the threshold BL stimulation intensity (usually \(-0.3\) mA), the average latency to response onset was \(7.6 \pm 0.4\) ms for CE neurons \((n = 46;\) Fig. 4A3) compared with \(16.5 \pm 0.7\) ms for BNST neurons \((n = 26;\) Fig. 4B3). The difference between the latency to response onset of CE and BNST neurons was statistically significant \((t\text{-test}, P < 0.001)\). It should be noted that further increases in stimulation intensity did not appreciably reduce the latency to response onset of CE and BNST neurons.

Similarly, as shown in the average peristimulus histograms of Fig. 4C, the latency of the response peak was significantly shorter for CE \((8.1 \pm 0.4\) ms; Fig. 4C, thick line) than BNST neurons \((23.6 \pm 1.1\) ms; Fig. 4C, thin line; \(t\text{-test}, P < 0.001)\). However, the difference between the two cell groups was much larger with this estimate of response latency. In fact, consistent with our timing hypothesis, the difference in latency to peak was of the same order as that seen between the antidromic responses of BNST cells and CE neurons with long conduction times. In closing, it should be mentioned that separate analyses of the latency to peak of BL-evoked responses in BNST neurons with \((n = 8)\) versus without \((n = 15)\) physiologically identified projections to the brain stem yielded qualitatively identical results \((25.1 \pm 3.8\) and \(22.8 \pm 2.1\) ms latencies, respectively; \(t\text{-test}, P > 0.05)\).
This study was undertaken to shed light on the functional interactions between CE and BNST neurons with a particular emphasis on the relative timing of their outputs to the brain stem. The interest of this question stems from earlier findings suggesting that despite having similar connections and anatomical properties, CE and BNST play different roles in regulating behavior. Our results point to an unexpected level of coordination in the timing of BNST and CE outputs to the brain stem, relative to BL inputs. Below, we consider the significance of these findings in light of previous anatomical and behavioral studies on the role of the extended amygdala.

Path heterogeneity in CE projections to brain stem

Prior tract-tracing studies have shown puzzling variations in the path followed by CE axons to the brain stem (Dong et al. 2001; Krettek and Price 1978b; Price and Amaral 1981; Sun and Cassell 1993; Veinante and Freund-Mercier 2003). Although many CE axons reach the brain stem directly, via the ventral amygdalofugal pathway, others follow the stria terminalis over its entire course. Thus they first course caudally, then arch dorsally and rostrally along the lateral aspect of the thalamus, and later curve ventrally and caudally to merge with axons of the ventral amygdalofugal pathway.

Consistent with this, our analysis of brain stem–evoked antidromic response latencies has shown that CE output neurons fall into two classes, with short or long conduction times to the brain stem. The rarity of CE cells with long antidromic response latencies in rats prepared with lesions of the stria terminalis strongly suggests that the neurons with long conduction times correspond to the subset of CE cells whose axons reach the brain stem via the stria terminalis. The net consequence of this path heterogeneity is that some CE impulses reach their brain stem targets quickly, in \(<10\) ms, whereas others take around three times longer.

What could be the significance of this peculiar arrangement? One possibility is that it serves no special purpose. According to this view, the path heterogeneity would reflect a developmental oddity where some CE cells, subjected to conflicting chemotaxic cues, would be lured into the stria terminalis, whereas others would merge with the ventral amygdalofugal pathway. However, a second possibility, the one we favor, is that this arrangement serves to synchronize CE and BNST impulses to the brain stem (Fig. 5). Synchronization of CE and BNST impulses to the brain stem would likely enhance the postsynaptic impact of each input.

Consistent with this possibility, we observed that the latency of peak BL-evoked responses was longer in BNST cells, by \(<20\) ms, than seen in CE cells. This difference closely approximated the conduction delay introduced by lengthening the path of CE axons to the brain stem via the stria terminalis. Thus by lengthening the path of some CE axons to the brain stem, the arrival of BL-driven CE impulses would be delayed, allowing for synchronization of BNST and CE impulses on their targets. This idea is further supported by a previous anatomical study showing that the same BL neurons that project to CE also contribute axon collaterals to the BNST (Smith and Millhouse 1985).

FIG. 4. BL stimulation orthodromically activates CE (A) and BNST (B) neurons. In A and B, panel 1 shows orthodromic responses to BL stimuli, panel 2 shows the corresponding peristimulus histogram of unit discharges, and panel 3 shows the frequency distribution of onset response latencies in samples CE (A3) and BNST (B3) neurons. C: average peristimulus histogram of neuronal discharges for CE (thick line) and BNST neurons (thin line). Before averaging, the data of each cell were normalized by dividing the number of spikes in each bin by the number of stimuli. Note that there was a much larger difference between the timing of the response peaks (16-ms difference) than between response onsets (6 ms).
However, there is a third possible interpretation for our findings. This view assumes that BNST and CE neurons with slow versus fast conduction times to the brain stem do not converge on the same brain stem neurons. Although the tract-tracing data indicates that the brain stem targets of CE and BNST neurons overlap extensively at a macroscopic level (Dong and Swanson 2004, 2006a–c; Dong et al. 2000; Holstege et al. 1985; Hopkins and Holstege 1978; Veening et al. 1984), it remains to be shown, at the single cell level, whether convergence occurs for inputs originating from all three cell groups. For instance, it is conceivable that fast-conducting CE neurons contact brain stem neurons involved in the rapid mediation of short-lived fear responses. In contrast, slow conducting CE neurons and BNST cells might contact brain stem targets that are involved in more persistent fear responses. An important challenge for future studies will be to compare the brain stem projection sites of BNST and CE neurons with slow or fast conduction times to the brain stem.

A puzzling difference between CE and BNST neurons evidenced in this study was that BL stimuli evoked a much longer period of increased firing probability in BNST than CE cells. Although differences in the electroresponsive properties of CE and BNST neurons might have contributed to this effect, it is also possible that BL stimuli engaged contrasting polysynaptic influences. In particular, CE neurons receive a strong GABAergic input from intercalated amygdala neurons (Pare and Smith 1993) that also receive inputs from BL (Royer et al. 1999). The excitation of ITC neurons by BL inputs was previously shown to generate a rapid feed-forward inhibition in CE neurons, limiting the duration of BL-evoked excitatory postsynaptic potentials (EPSPs) (Royer et al. 1999). In addition, BL projects to the medial prefrontal cortex (Krettek and Price 1977) that in turn projects to BNST (Vertes 2004). Thus the excitation of BNST neurons by BL inputs may have been prolonged via the activation of the medial prefrontal cortex.

**Behavioral significance of path heterogeneity**

Our analyses of BL-evoked response latencies and conduction times to the brain stem argue for a tight temporal coordination between CE and BNST outputs. However, this view does not fit with the lesion and pharmaco-behavioral studies reviewed in the introduction that stress the different functions of CE and BNST. However, it remains that some effects of BNST and CE lesions overlap. For instance, ibotenic acid lesions of BNST (Gray et al. 1993) and CE (Van de Kar et al. 1991) attenuate the increase in corticosterone associated with the expression of contextually conditioned fear. More relevant to the theme of this paper are studies where CE and BNST lesions were reported to attenuate behaviors that are thought to depend on parallel projections of these structures to the brain stem. For instance, it was observed that the expression of contextual conditioned freezing responses is attenuated by both CE and BNST lesions (Goosens and Maren 2001; Gray et al. 1993; Sullivan et al. 2004; Van de Kar et al. 1991). Moreover, CE and anterior BNST lesions prevent the pain-induced increase in vocalization seen following exposure to noxious electrical stimuli (Crown et al. 2000). Both of these effects are thought to depend on parallel projections of CE and BNST to the periaqueductal gray (PAG): the ventral PAG for freezing responses (LeDoux et al. 1988) and the dorsal PAG for the pain-induced increase in vocalization (Crown et al. 2000; McLemore et al. 1999).
Conclusions

Although recent behavioral studies have stressed the differing functions of CE and BNST, others point to overlapping roles of these two structures via their common brainstem projection sites. Consistent with the latter view, our results indicate that the path of BNST and CE axons is such that BL-driven CE and BNST impulses can reach the brainstem simultaneously, maximizing their impact on common targets.

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