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Organization of Multisynaptic Circuits Within and Between the Medial and Central Extended Amygdala

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Abstract

The central and medial extended amygdala comprises the central (CEA) and medial nuclei of the amygdala (MEA), respectively, together with anatomically connected regions of the bed nucleus of the stria terminalis (BST). To reveal direct and multisynaptic connections within the central and medial extended amygdala, monosynaptic and transneuronal viral tracing experiments were performed in adult male rats. In the first set of experiments, a cocktail of anterograde and retrograde tracers was iontophoretically delivered into the medial CEA (CEAm), anterodorsal MEA (MEAd), or posterodorsal MEA (MEApd), revealing direct, topographically-organized projections between distinct amygdalar and BST subnuclei. In the second set of experiments, the retrograde transneuronal tracer pseudorabies virus (PRV) was microinjected into the CEA or MEAd. After 48 hr survival, there were no significant differences between monosynaptic and PRV cases in the subnuclear distribution or proportions of retrogradely-labeled BST neurons. However, after 60 hr survival, CEA-injected cases displayed an increased proportion of labeled neurons within the anteromedial group of BST subnuclei (amgBST) and within the posterior BST, which do not directly innervate the CEA. MEApd-injected 60 hr cases displayed a significantly increased proportion of retrograde labeling in the amgBST compared to monosynaptic and 48 hr cases, whereas MEAd-injected cases displayed no proportional changes over time. Thus, multisynaptic circuits within the medial extended amygdala overlap the direct connections comprising this anatomical unit, whereas the multisynaptic boundaries of the central extended amygdala extend into BST subnuclei previously identified as part of the medial extended amygdala.

Keywords

central amygdala; extended amygdala; medial amygdala; pseudorabies virus; bed nucleus of stria terminalis

INTRODUCTION

The bed nucleus of the stria terminalis (BST) is a heterogeneous limbic forebrain structure comprising 12 subnuclei with distinct cytoarchitectural features and anatomical connectivity. The BST has become a primary focus of research on the neural substrates of

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Statement of authors' contributions

M.S. Bienkowski designed and performed experiments, collected and analyzed data, prepared figures, and wrote the original draft manuscript. E.S. Wendel performed experiments, collected and analyzed data, and prepared figures. L. Rinaman designed experiments, analyzed data, and edited figures and manuscript text.

Conflict of Interest Statement

The authors declare no conflicts of interest.

anxiety and other stress-related disorders, due to its expression of anxiogenic neuropeptides (i.e., calcitonin gene-related peptide and corticotrophin-releasing hormone, CRH) and its neural connections with stress-responsive brain regions, particularly the amygdala (Dong et al., 2001; Duvarci et al., 2009; Hammack et al., 2009; Lungwitz et al., 2012; Sink et al., 2012; Sink et al., 2011; Walker et al., 2009; Walker et al., 2003; Yassa et al., 2012). The amygdala and BST are densely interconnected, and have been considered by some to be distributed parts of the same structure, termed the ‘extended amygdala’ (Alheid and Heimer, 1988; de Olmos and Heimer, 1999; Zahm, 2006). The concept of the extended amygdala has generated interest in unraveling the complex neuroanatomy and subnuclear connectivity of the amygdala and BST in order to better understand their structural and functional relationships.

The efferent projections of various amygdalar and BST subnuclei have been examined by Dong and colleagues in a series of PHA-L anterograde tracing studies (Dong et al., 2000; Dong et al., 2001; Dong and Swanson, 2003; Dong and Swanson, 2004a; Dong and Swanson, 2004b; Dong and Swanson, 2006a; Dong and Swanson, 2006b). In general, connections between the amygdala and BST are topographically organized such that the anterolateral group of BST subnuclei (algBST) is reciprocally connected with the central nucleus of the amygdala (CEA), comprising the “central extended amygdala” (Bienkowski and Rinaman, 2012; de Olmos and Heimer, 1999), whereas the anteromedial (amgBST) and posterior (pBST) groups of BST subnuclei are reciprocally connected with the medial nucleus of the amygdala (MEA; Fig.1), comprising the “medial extended amygdala” (de Olmos and Heimer, 1999). These topographically organized connections suggest the presence of discrete channels for information processing between specific subregions of the amygdala and BST. However, the algBST, amgBST, and pBST are interconnected with each other in a complex manner (Fig. 2), making it both possible and likely that BST subnuclei directly connected with the CEA (i.e., the central extended amygdala) exert indirect influence over BST subnuclei that are connected with the MEA (i.e., the medial extended amygdala), and vice-versa, via intra-BST circuits. Indeed, several BST subnuclei have projections that cross the topographical border separating BST components of the central and medial extended amygdala (Fig. 2, topographical border is represented by the red/blue boundary). Thus, neurons within BST subnuclear components of the central extended amygdala might contribute indirectly to the activity of the medial extended amygdala, and vice-versa. To test this hypothesis using neuroanatomical methods, a multisynaptic tracing approach is needed.

Traditional monosynaptic retrograde and anterograde tracers cannot readily define multisynaptic circuit connections. In addition, electron microscopy must be used to confirm the presence of synaptic contacts between tracer-labeled neurons. A powerful alternative approach involves the use of neurotropic viral tracers, such as pseudorabies virus (PRV), which self-amplify and transport between neurons within multisynaptic circuits across points of synaptic contact (Aston-Jones and Card, 2000; Aston-Jones et al., 2001; Aston-Jones et al., 2004; Card, 1998a; Card, 1998b; Card and Enquist, 2001; Card et al., 1999; Enquist and Card, 2003). The attenuated Bartha strain of PRV (PRV-Bartha) is especially useful for transsynaptic tracing due to its reduced virulence and its restricted transsynaptic transport in the retrograde direction.

Interpreting the synaptic connectivity of neurons within distributed circuits based on transneuronal viral labeling patterns is facilitated by examining the progression of viral infection across several post-inoculation time points (Aston-Jones and Card, 2000; Card, 1998b). In addition, previous studies have demonstrated that the retrograde tracer cholera toxin subunit B (CTB) can be successfully combined with PRV to discriminate neurons with direct (i.e., first-order) inputs to central injection sites from neurons with 2nd- or 3rd-order

projections, and the presence of CTB labeling also permits a more accurate assessment of the viral injection site (Aston-Jones and Card, 2000; Chen et al., 1999). In the present study, monosynaptic and transsynaptic tracers were used separately and in combination to quantitatively dissect the distinct multisynaptic BST circuits that provide input to the medial CEA (CEAm) vs. the anterodorsal (MEAad) or posterodorsal MEA (MEApd). In the first set of experiments, a cocktail of monosynaptic anterograde and retrograde neural tracers (i.e., biotinylated dextran amine, BDA, and Fluorogold, FG, respectively) was iontophoretically delivered into the CEA, MEAad, or MEApd of adult male rats to confirm previous reports of topographically-organized reciprocal direct connections between the amygdala and BST, and to quantify the distribution and extent of these direct connections. In the second set of experiments, the CEA, MEAad, or MEApd was targeted with injections of PRV alone, or PRV combined with CTB, to determine the distribution and number of first- or higher-order infected BST neurons.

MATERIALS AND METHODS

Animals

Adult male Sprague Dawley rats (225–250g BW; Harlan Laboratories, Indianapolis, IN, USA) were individually housed in a controlled environment (20–22°C, 12:12 hr light:dark cycle; lights off at 1900 hr) with *ad libitum* access to water and pelleted chow (Purina 5001). Viral tracing experiments were performed in an approved Biosafety Level 2+ facility. All experiments were conducted in accordance with the NIH *Guide for the Care and Use of Laboratory Animals* and were approved by the University of Pittsburgh IACUC, Recombinant DNA Committee, and Division of Environmental Health and Safety.

Iontophoretic delivery of monosynaptic tracers

Rats were anesthetized by isoflurane inhalation (Halocarbon Laboratories, River Edge, NJ; 1–3% in oxygen) and positioned into a Kopf stereotaxic device. A pulled glass micropipette tip (approximately 1 mm in length with 20 μ m outer tip diameter) containing a 1:1 tracer cocktail of 1% Fluorogold (FG; Fluorochrome, Denver, CO) in 0.1M cacodylic acid and 5% biotinylated dextran amine (BDA; MW 10,000; Invitrogen) in 0.9% saline was prepared and connected to a current source (Stoelting) via a copper conductance wire. The glass pipette tip was lowered into the brain at coordinates targeting either the CEA (mm from bregma: 2.1 posterior, 3.9 lateral, 8.0 ventral), MEAad (mm from bregma: 1.8 posterior, 2.8 lateral, 8.8 ventral), or MEApd (mm from bregma: 2.4 posterior, 3.5 lateral, 8.4 ventral). During the descent of the glass pipette into the brain, a -1.5μ A retaining current was used to minimize molecular diffusion of tracer. When the tip of the pipette reached the target site, the retaining current was turned off and the tracer cocktail was iontophoresed using a 7s on/off pulsed current of $+5 \mu$ A for 10 min. After tracer delivery, the pipette was withdrawn and the skin closed with stainless steel clips. Rats were injected subcutaneously with 0.5 ml of a mild analgesic (Ketofen; 2 mg/kg) and were returned to their cages after regaining consciousness and full mobility. Representative images of iontophoretic tracer delivery sites are shown in Figure 3.

Multisynaptic tracing using PRV

Rats were anesthetized by isoflurane inhalation (Halocarbon Laboratories, River Edge, NJ; 1–3% in oxygen) and positioned into a Kopf stereotaxic device. A pulled glass pipette tip was attached to the stereotaxic arm and the back end of the glass pipette was affixed to a polyethylene tube connected to a PicoPump (World Precision Instruments, Sarasota, FL). The glass pipette was back-filled with solution containing PRV-263 (3.4×10^8 pfu / mL) or a 3:1 mixture of PRV-263 and a 0.25% solution of cholera toxin subunit B (CTB; List Biological Labs, Campbell, CA, USA) diluted in deionized water. PRV-263 is a

recombinant strain of PRV that carries the Brainbow 1.0L cassette, and it displays a similar time course of infection as PRV-Bartha, its parent strain. The recombinant properties of PRV-263 (Card et al., 2011a; Card et al., 2011b) were not utilized in the present study. After loading, the glass pipette was immediately lowered into the brain targeting the same coordinates described in the preceding section (*Iontophoretic delivery of monosynaptic tracers*). The pipette tip was left in place for 3 min at the target site before 100nl of PRV-263 or PRV-263/CTB was delivered by pressure over 10 min (10 nl/min). After injection, the pipette was left in place for 3 min and then removed from the brain. The skin incision was closed with stainless steel clips and rats were injected subcutaneously with 0.5 ml of Ketofen before being returned to their home cages in the BSL-2+ facility. Representative images of PRV/CTB co-injection sites are shown in Figure 3.

Perfusion and histology

One week after tracer iontophoresis and 48 or 60 hr after PRV injections, rats were anesthetized with an overdose of sodium pentobarbital (Vortech Pharmaceuticals, Dearborn, MI) and then transcardially perfused with 0.15M NaCl followed by 500 ml of fixative solution containing 4% paraformaldehyde, 1.4% lysine, and 0.2% sodium metaperiodate in 0.1 M phosphate buffer (McLean and Nakane, 1974). Brains were post-fixed *in situ* overnight at 4°C, and then removed from the skull and cryoprotected for at least 24 hr in 20% sucrose solution before sectioning. A freezing stage microtome was used to cut coronal brain sections with a thickness of 35 µm. Sections were collected sequentially into 6 adjacent sets of sections spaced 210 µm apart, and stored in cryopreservant (Watson et al., 1986) at -20°C for later immunohistochemical processing.

Immunohistochemistry

Tissue sections from rats that received iontophoretic delivery of FG/BDA were processed for triple immunofluorescence to localize both tracers as well as calbindin 28k. Calbindin 28k is expressed by a subpopulation of GABA neurons within the BST (Paxinos et al., 1999). It is a useful marker of BST boundaries, as it is highly expressed within regions adjacent to the BST (i.e., ventrolateral septum, parastrial nucleus, ventromedial striatum) as well as within specific BST subnuclei (i.e., principal and juxtacapsular). Following treatment with 0.5% sodium borohydride, tissue sections from cases labeled with FG/BDA were incubated for 48 hr in buffer (0.1M sodium phosphate, pH 7.4) containing 0.3% Triton-X100, 1% normal donkey serum, rabbit anti-FG (1:3,000; Millipore, Temecular, CA), and mouse anti-calbindin 28k (1:250; Sigma-Aldrich, St. Louis, MO). Next, tissue sections were rinsed and then incubated for 24 hr in a mixture of Alexa Fluor 647-conjugated donkey anti-rabbit IgG to visualize FG, Alexa Fluor 488-conjugated donkey anti-mouse IgG to visualize calbindin 28k, and Cy3-conjugated streptavidin to visualize BDA (1:500 each; Jackson Immunochemicals, West Grove, PA).

Tissue sections from rats co-injected with PRV/CTB were processed for triple immunofluorescence following a protocol similar to that described above. Sections were incubated in a primary antibody cocktail comprising rabbit anti-PRV (either Rb132 or Rb133, 1:2000), goat anti-CTB (1:5,000, List Biological Labs, Campbell, CA), and mouse anti-calbindin 28k. Rb132 and Rb133 antibodies were generated against acetone-inactivated virus and specifically recognize viral epitopes present within infected neuronal nuclei and somatodendritic compartments (Card and Enquist, 2001). After rinsing, sections were incubated in a cocktail of fluorescently tagged secondary antisera (1:500 each, Jackson Immunochemicals; Cy3-conjugated donkey anti-goat IgG; Alexa Fluor 488-conjugated donkey anti-mouse IgG, and Alexa Fluor 647-conjugated donkey anti-rabbit IgG).

Tissue sections from cases injected with PRV only were processed for dual immunoperoxidase labeling of PRV and the neuroanatomical marker NeuN, in order to plot the distribution of infected (i.e., PRV-positive) BST neurons using StereoInvestigator mapping software. Sections were treated with 0.5% sodium borohydride and 30% hydrogen peroxide and subsequently incubated overnight in Rb132 or Rb133 (1:20,000). Immunoperoxidase labeling of virally-infected neurons was achieved using biotinylated donkey anti-rabbit IgG (1:500; Jackson Immunochemicals, West Grove, PA) followed by Vectastain ABC Elite reagents (Vector Laboratories, Burlingame, CA) and a nickel-intensified diaminobenzidine (DAB)-hydrogen peroxidase reaction to produce a black immunoprecipitate identifying infected neurons. NeuN immunoreactivity was subsequently revealed after sequentially incubating sections in mouse anti-NeuN (1:5000, Millipore, Temecula, CA), biotinylated donkey anti-mouse IgG (1:500; Jackson Immunochemicals, West Grove, PA), and Vectastain ABC Elite reagents, followed by a non-intensified brown DAB immunoperoxidase reaction to reveal NeuN labeling.

Immunoperoxidase- or immunofluorescence-labeled tissue sections were rinsed in buffer and mounted onto Superfrost Plus microscope slides (Fisher Scientific), allowed to dry overnight, dehydrated and defatted in graded ethanols and xylene, and coverslipped using Cytoseal 60 (VWR).

Identification of BST subnuclei

A critical aspect of this study is the identification and parcellation of BST subnuclei. The BST nomenclature used in this report is based on the Swanson rat brain atlas (Swanson, 2004). This BST nomenclature was defined based on the results of anterograde tracing studies (Dong and Swanson, 2006a) and was built upon a foundation of prior cyto- and chemoarchitectonic studies of the BST and its surrounding region (Ju and Swanson, 1989; Ju et al., 1989). However, the BST sections represented within the Swanson atlas are too infrequent (50–320 μm between sections, 40 μm section thickness) to closely match most experimental tissue sections generated in the present study, and are insufficient for identifying discrete BST subnuclei across their rostrocaudal extent. To address this issue, we generated a new BST reference atlas by using a series of Klüver-Barrera-stained, paraffin-embedded tissue sections (12 μm section thickness, 60 μm apart) that more fully reveal BST subnuclear cytoarchitecture and myelinated fiber pathways (Fig. 4). The high section frequency of this reference atlas permits close matching of experimental tissue sections with their appropriate rostrocaudal atlas level. Determination of BST subnuclear boundaries in experimental sections was guided by the atlas and adjusted as needed based on interpretation of either NeuN cytoarchitecture (in peroxidase-labeled tissue sections) or the distribution of calbindin 28k (in immunofluorescent-labeled tissue sections).

Imaging and quantitative analysis

Cases with accurate tracer delivery sites were analyzed to determine the distribution and number of retrogradely-labeled neurons within individual BST subnuclei. Tissue series labeled for NeuN and PRV immunoperoxidase were used to create maps of the rostrocaudal distribution of infected BST neurons. BST subnuclear boundaries were determined by comparing cytoarchitecture revealed by NeuN labeling to the Klüver-Barrera-stained reference atlas described in the preceding section (*Identification of BST subnuclei*). The distribution of infected neurons across 6 BST sections was plotted at 40 $\times$ magnification using a Nikon light microscope connected to a computerized data acquisition system (StereoInvestigator; MBF Bioscience).

Tissue sections processed for triple immunofluorescent labeling, including tracer iontophoresis and PRV experiments, were photographed using an Olympus

photomicroscope equipped with a Hamamatsu digital camera (Hamamatsu Photonics, Hamamatsu, Japan) and filters to visualize Cy2, Cy3, and Cy5 fluorescence in the green, red, and blue color channels, respectively. Using a 10× objective, 6–21 overlapping photographic images were obtained in each of 6 tissue sections through the rostrocaudal extent of the BST. Images were transferred to a computer and merged into one high-resolution panoramic image per tissue section using Adobe Photoshop software. The borders of the BST and its constituent subnuclei were drawn onto the Photoshop image with reference to the Klüver-Barrera-stained set of atlas sections. Single- (either FG- or PRV-positive) and double-labeled (PRV+CTB-positive) neurons within each BST subnucleus were visualized in separate color channels and quantified using Photoshop's count tool.

The number of FG-, PRV-, or PRV+CT-positive neurons within each individual BST subnucleus was totaled across rostrocaudal sections through each subnucleus. Within each experimental case, the proportion of labeling within a given BST subnucleus was calculated using the formula [(number of labeled neurons within a given BST subnucleus) / (total number of labeled BST neurons across subnuclei) × 100] to normalize between-subject variability in injection site size and tracer uptake. The distribution of retrogradely-labeled neurons generated by iontophoretic delivery of FG was indistinguishable from the distribution of CTB-labeled neurons produced by co-injections of PRV/CTB (see Results), and so first-order retrograde labeling data from these cases were combined into a single 'monosynaptic labeling' group. For each amygdalar injection site (i.e., CEAm, MEAad, and MEApd), and for each independent BST subnucleus, statistical comparisons of the proportion of total BST labeling confined to that specific BST subnucleus were performed using one-way ANOVA, with tracer injection group (i.e., monosynaptic CTB or FG vs. 48 hr PRV vs. 60 hr. PRV) as the independent variable, followed by post-hoc *t* tests when the ANOVA indicated a significant effect of tracer injection group. Within each amygdalar injection site and tracer injection group, cross correlation analyses were performed to determine the relationship of retrograde labeling between individual BST subnuclei. Effects, differences, and correlations were considered significant when $P < 0.05$.

RESULTS

Iontophoretic delivery of FG/BDA tracer cocktail into amygdalar subnuclei (Fig. 3) generated retrogradely-labeled (i.e., FG-positive) neurons overlapping anterogradely-labeled (i.e., BDA-positive) terminal fields within the BST (Figs. 5–7). BST labeling was topographically and differentially distributed after tracer cocktail delivery into the CEAm (Fig. 5), MEAad (Fig. 6), and MEApd (Fig. 7). CEAm-targeted delivery sites generated dense anterograde and retrograde labeling within the algBST (Fig. 5), while MEAad- and MEApd-targeted sites generated varying densities of anterograde and retrograde labeling within specific subregions of the amgBST and pBST (Figs. 6 and 7). In cases with MEAad tracer delivery, anterograde and retrograde labeling was localized within more lateral regions of the BSTpr, and also was distributed within the BSTif, BSTtr, and BSTam (Fig. 6). Conversely, in cases with MEApd tracer delivery, retrograde and anterograde labeling was concentrated within the most medial aspect of the BSTpr, with additional light labeling observed within the BSTam (Fig. 7).

Statistical analyses were performed to quantitatively compare retrograde labeling patterns among the three amygdalar tracer delivery sites (i.e., CEAm vs. MEAad vs. MEApd). First, we examined whether mixing CTB with PRV altered the pattern of first-order retrograde labeling achieved within the BST compared to retrograde labeling achieved after iontophoretic delivery of FG/BDA. An independent samples *t*-test determined that there was no significant difference between the number of PRV+CTB-labeled neurons after 48 hr ($n=7$) vs. 60 hr survival time ($n=14$; 135 ± 53 PRV+CTB-labeled neurons after 48 hr vs. 108 ± 10

PRV+CTB-labeled neurons after 60 hr, $P > 0.05$), consistent with PRV+CTB-labeled neurons as 1st-order amygdala-projecting BST neurons. Iontophoretic delivery of FG/BDA ($n = 7$) resulted in significantly more first-order FG-positive BST projection neurons compared to the number of BST neurons that were double-labeled for both CTB and virus after co-injection of PRV/CTB ($n = 21$; 577 ± 62 FG-labeled neurons vs. 117 ± 27 PRV+CTB-labeled neurons, $P < 0.001$). However, the distribution of first-order BST projection neurons in both tracer groups was similar, with no significant tracer group differences in the proportion of total BST neurons that occupied the amgBST ($P = 0.06$), algBST ($P = 0.83$), or pBST ($P = 0.30$). Thus, proportional labeling data from both tracer groups were combined into a single monosynaptic tracer group (Table 1). There also were no significant differences in the total number of PRV-positive BST neurons counted in rats that received amygdalar injections of PRV/CTB cocktail vs. those injected with PRV alone ($P = 0.98$ for the 48 hr post-inoculation group; $P = 0.26$ for the 60 hr post-inoculation group).

Retrograde BST labeling after tracer delivery into the CEAm

Quantification of monosynaptic retrograde labeling across BST subnuclear groups after CEAm tracer delivery revealed that approximately 20% of retrogradely-labeled BST neurons were located within the pBST, ~16% were located within the amgBST, and ~64% were located within the algBST (Fig. 9). One-way ANOVA confirmed that these BST subnuclear labeling distribution patterns were significantly different than patterns achieved in rats after tracer delivery into the MEAd or MEApd, as described below [pBST: $F(2,25) = 53.37$, $P < 0.001$; amgBST: $F(2,25) = 15.60$, $P < 0.001$; algBST: $F(2,25) = 131.15$, $P < 0.001$].

More BST neurons were labeled 48 and 60 hr after PRV injections into the CEAm compared to the number of neurons labeled after monosynaptic CEAm tracer delivery, with more PRV labeling present at 60 hr vs. 48 hr post-inoculation (Fig. 8). One-way ANOVA confirmed a significant effect of tracer group (i.e., monosynaptic vs. 48 hr PRV vs. 60 hr PRV) on the total number of labeled BST neurons [$F(2,22) = 18.02$, $P < 0.001$] (see Fig. 8 for post-hoc comparisons). However, despite increased numbers of PRV-positive BST neurons in the 48 hr post-inoculation group compared to the number of retrogradely-labeled neurons in rats with monosynaptic tracer delivery, the subnuclear distribution of labeled neurons within the pBST, amgBST, and algBST was similar between groups ($P > 0.05$ for each subnuclear comparison; Fig. 9). The proportion of total PRV retrograde labeling located within the pBST and amgBST was significantly higher in rats killed 60 hr post-PRV ($P < 0.05$ for between-group comparisons in both regions; Fig. 9), whereas the proportion of total retrograde labeling located within the algBST was significantly lower in 60 hr PRV cases ($P < 0.05$ for both between-group comparisons; Fig. 9).

To identify which BST subnuclei accounted for the altered labeling distribution patterns in PRV 60 hr cases, separate one-way ANOVAs were performed to assess the proportion of total BST labeling present within each of 12 individual BST subnuclei (Figs. 10–12). Tracer group (i.e., monosynaptic vs. PRV 48 hr vs. PRV 60 hr) had a significant effect on the proportion of labeled neurons within only 3 of the 12 BST subnuclei, i.e., the BSTal [$F(2,22) = 14.39$, $P < 0.001$; Fig. 10], the BSTam [$F(2,22) = 16.46$, $P < 0.001$; Fig. 11], and the BSTpr [$F(2,22) = 8.70$, $P = 0.02$; Fig. 12]. Post-hoc tests determined that a significantly higher proportion of retrograde labeling was present within the BSTpr (Fig. 12) and BSTam (Fig. 11) of PRV 60 hr cases compared to both monosynaptic and PRV 48 hr cases. Conversely, significantly lower proportions of total retrograde labeling were present within the BSTal (Fig. 10) of PRV 48 and 60 hr cases compared to labeling in monosynaptic cases.

If PRV virions are transporting across two nodes of a circuit, the amount of labeling within these nodes should be more highly correlated than nodes within distinct multisynaptic

circuits. Correlation analyses were performed to determine whether the significantly-changed proportion of BST labeling present within individual BST subnuclei (for CEAm-injected cases, BSTpr, BSTam, BSTal, and BSTov) was associated with the proportion of labeling within other BST subnuclei. In CEAm-injected cases, BSTpr and BSTam labeling proportions were not significantly correlated with each other ($R=0.233$, $P > 0.05$), but labeling proportions within both the BSTpr and BSTam were negatively correlated with labeling proportions in the BSTal (vs. BSTpr, $R=-0.58$, $P < 0.005$; vs. BSTam, $R=-0.41$, $P < 0.05$) and BSTov (vs. BSTpr, $R=-0.53$, $P < 0.05$; vs. BSTam, $R=-0.47$, $P < 0.05$). Labeling proportions within the BSTov were also negatively correlated with labeling proportions in the BSTif ($R=-0.57$, $P < 0.005$) and BSTtr ($R=-0.51$, $P < 0.05$), but were positively correlated with labeling proportions within the BSTfu ($R=0.40$, $P < 0.05$).

Retrograde BST labeling after tracer delivery into the MEAad

Quantification of monosynaptic retrograde labeling within BST subnuclear groups in rats after MEAad-targeted tracer delivery revealed that approximately 56%, ~33%, and ~11% of retrogradely-labeled neurons were located within the pBST, amgBST, and algBST, respectively (Fig. 9). One-way ANOVA revealed no significant effect of tracer group on the proportion of retrograde labeling within the pBST [$F(2,22) = 0.29$, $P = 0.75$], amgBST [$F(2,22) = 1.41$, $P = 0.27$], or algBST [$F(2,22) = 0.20$, $P = 0.82$].

Similar to results in CEAm-injected rats, one-way ANOVA confirmed a significant effect of tracer group on the total number of retrogradely-labeled BST neurons in rats after MEAad injection [$F(2,22) = 36.39$, $P < 0.001$] (Fig. 8). Post-hoc tests revealed significantly greater numbers of retrogradely-labeled BST neurons within the 48 hr and 60 hr PRV groups compared to BST labeling in the monosynaptic tracer group (see Fig. 8). The number of PRV-labeled BST neurons 60 hr post-inoculation was also significantly greater than in 48 hr PRV cases (Fig. 8).

Despite no significant differences in the overall pattern of labeling across BST subnuclear groups, separate one-way ANOVAs revealed a small but significant effect of tracer group on the proportion of retrograde labeling within the BSTrh [$F(2,22) = 3.51$, $P < 0.05$], and a near-significant effect of tracer group within the BSTov [$F(2,22) = 3.451$, $P = 0.05$] (Fig. 10). Post-hoc tests determined that the small proportion of retrograde labeling within the BSTrh was significantly increased in PRV 48 hr cases compared to the smaller proportion of labeling in monosynaptic cases (Fig. 10), and the proportion of labeling in PRV 60 hr cases was significantly decreased compared to PRV 48 hr labeling (Fig. 10). Correlation analyses of proportional labeling within the BST subnuclei revealed no significant associations between labeling within the BSTrh and labeling within any other BST subnucleus.

Retrograde BST labeling after tracer delivery into the MEApd

Quantification of monosynaptic BST retrograde labeling in rats that received tracer delivery into the MEApd revealed that approximately 84%, ~13%, and ~4% of retrogradely-labeled neurons were located within the pBST, amgBST, and algBST, respectively (Fig. 9). Separate one-way ANOVAs confirmed a significant effect of tracer group on the proportion of retrogradely labeled neurons within the pBST [$F(2,13) = 4.08$, $P = 0.04$] and amgBST [$F(2,13) = 5.08$, $P = 0.02$]. Post-hoc tests revealed significantly lower proportions of retrograde labeling within the pBST of PRV 60 hr cases compared to PRV 48 hr and monosynaptic labeling cases (Fig. 9). In addition, a significantly higher proportion of labeling was present within the amgBST in PRV 60 hr cases compared to PRV 48 hr and monosynaptic labeling cases (Fig. 9).

Similar to results in CEAm- and MEAAd-injected cases, PRV injections into the MEApd resulted in more total BST labeling at 48 and 60 hr post-inoculation times compared to BST labeling in rats injected with monosynaptic tracer (Fig. 8). One-way ANOVA confirmed a significant effect of tracer group on the total number of retrogradely-labeled BST neurons after MEApd injection [$F(2,13) = 9.54, P = 0.003$]. Post-hoc tests revealed significantly larger numbers of retrogradely-labeled BST neurons in the PRV 48 hr and 60 hr groups compared to monosynaptic retrograde labeling (Fig. 8). Despite the trend towards increased PRV labeling within the BST in PRV 60 hr cases compared to PRV 48 hr cases, the difference was not significant ($P = 0.10$).

Separate one-way ANOVAs within individual BST subnuclei revealed a significant effect of tracer group on the proportion of retrograde labeling within the BSTdm [$F(2,13) = 4.17, P = 0.04$; Fig. 11], and nonsignificant trends within the BSTpr [$F(2,13) = 3.40, P = 0.07$; Fig. 12] and BSTam [$F(2,13) = 3.60, P = 0.06$; Fig. 11]. Post-hoc tests determined that the low proportion of retrograde labeling within the BSTdm in PRV 60 hr cases was significantly increased compared to the proportion of labeling within the BSTdm in monosynaptic cases (Fig. 11).

Correlation analyses of the proportional retrograde labeling within individual BST subnuclei in MEApd-injected cases were performed to determine whether labeling in other subnuclei was correlated with the decreased BSTpr labeling and/or the increased BSTam and BSTdm labeling. Retrograde labeling with the BSTdm was positively correlated with labeling in the BSTv ($R=0.65, P < 0.05$) and BSTfu ($R=0.61, P < 0.05$), and was negatively correlated with labeling in the BSTpr ($R=-0.64, P < 0.05$). The proportion of retrograde labeling within the BSTpr was negatively correlated with labeling in the BSTif ($R=-0.57, P < 0.05$), BSTrh ($R=-0.69, P < 0.05$), BSTal ($R=-0.75, P < 0.05$), BSTov ($R=-0.73, P < 0.05$), BSTfu ($R=-0.59, P < 0.05$), BSTam ($R=-0.77, P < 0.05$), and BSTdm ($R=-0.64, P < 0.05$). In contrast, BSTam labeling was negatively correlated with labeling within the BSTpr ($R=-0.77, P < 0.05$), and was positively correlated with labeling in the BSTrh ($R=0.82, P < 0.05$), BSTov ($R=0.79, P < 0.005$), BSTfu ($R=0.71, P < 0.05$), and BSTal ($R=0.79, P < 0.005$).

DISCUSSION

Iontophoretic delivery of FG/BDA tracer cocktail revealed that the majority of FG-labeled BST neurons were located within BDA-labeled amygdala terminal fields, confirming the interpretation of several anterograde tracing studies in rats (Canteras et al., 1995; Dong et al., 2000; Dong et al., 2001; Dong and Swanson, 2003; Dong and Swanson, 2004a; Dong and Swanson, 2004b; Dong and Swanson, 2006a; Dong and Swanson, 2006b). Our results also are consistent with a previous study using cocktail tracer injections in hamsters (Coolen and Wood, 1998), indicating that specific amygdala subnuclei are connected to specific BST subnuclei via topographically-organized bidirectional projection systems. The current analysis of monosynaptic and transsynaptic retrograde labeling in FG/BDA-, PRV-, and PRV/CTB-injected cases provides the first quantitative report of the distribution of CEAm-, MEAAd-, and MEApd-projecting BST circuits. As expected, CEAm-projecting BST neurons were located primarily within the algBST, while MEAAd- and MEApd-projecting BST neurons were distributed within complementary regions of the pBST and amgBST (Fig. 13). Analysis of labeling data from rats that were sacrificed 48 hours after receiving PRV injections into amygdalar subnuclei revealed no change in the distribution pattern of retrograde labeling compared to the distribution of labeling revealed by monosynaptic tracer transport, despite an approximately 10-fold increase in the number of PRV-positive BST neurons. These similar subnuclear patterns of retrograde BST labeling indicate that 2nd-order, transsynaptically-infected neurons are contained within the same BST subnuclei and/

or subnuclear groups as 1st-order neurons that project directly to the tracer-targeted amygdalar subregion (Fig. 13).

In contrast to the similar subnuclear distribution of BST labeling observed in both monosynaptic and 48 hr PRV cases, labeling distribution patterns were shifted in 60 hr PRV cases after CEAm- and MEApd-targeted injections, whereas the distribution of BST labeling 60 hr after MEAad-targeted PRV injections remained similar to that observed in 48 hr MEAad-targeted cases. In CEAm 60 hr cases, a significantly greater proportion of retrograde labeling was present within amgBST and pBST (specifically the BSTam and BSTpr subnuclei) compared to monosynaptic retrograde tracer-injected CEAm cases, evidence that the CEAm receives multisynaptic input from BST subnuclei beyond those that directly innervate (and receive input from) the CEA. Further correlation analysis of retrograde labeling within CEAm-injected cases revealed that the BSTpr and BSTam were not significantly correlated, suggesting that the BSTpr and BSTam multisynaptically project to the CEAm via distinct pathways, rather than as two nodes along the same multisynaptic circuit. MEApd 60 hr cases displayed a significantly greater proportion of retrograde labeling in the amgBST, including increased labeling within the BSTdm and a non-significant but strong trend towards increased labeling within the BSTam. Additional correlation analyses determined that BSTpr retrograde labeling was negatively correlated to many other BST subnuclei, suggesting that, in MEApd-injected cases, the BSTpr is isolated from much of the BST subnuclei, and changes in retrograde labeling within the amgBST reflect a separate subnetwork of amgBST subnuclei. In both MEAad and MEApd injection cases, retrograde labeling within the algBST was relatively light, particularly within the BSTov. These findings support the view that both monosynaptic and polysynaptic BST inputs to the MEA are confined to the amgBST and pBST.

Methodological considerations

Despite differences in the number of FG- and PRV+CTB-labeled BST neurons in rats that received amygdalar injections of either FG/BDA or PRV/CTB, there were no significant tracer-related differences in the proportional distribution of monosynaptic retrograde labeling across BST subnuclear groups. Differences in the total number of FG- vs. PRV +CTB-labeled neurons are likely due to differences in tracer concentration, uptake affinity, and local diffusion at the injection site. There were no significant differences in the number of PRV-positive BST neurons in rats injected with a 3:1 mixture of PRV/CTB compared to labeling in rats injected with PRV alone, consistent with previous reports indicating that CTB does not reduce PRV invasiveness, transport, or replication (Aston-Jones and Card, 2000; Chen et al., 1999).

Interestingly, the total number of retrogradely-labeled BST neurons was similar within monosynaptic cases, within PRV 48 hr cases, and within PRV 60 hr cases regardless of amygdala injection site (Fig. 8). For each amygdala injection group, monosynaptic retrograde tracers typically labeled an average of 100–200 neurons, while viral labeling in PRV 48 hr and 60 hr cases totaled approximately 1000–2000 and 3000–4000 neurons, respectively (Fig. 8). While these consistent labeling results are partially explained by the use of consistent injection parameters for each amygdala subnucleus, they also reveal strong similarities in the architecture of amygdala-projecting BST circuits. Previous studies indicate that PRV retrograde transport and infection of neurons within multisynaptic circuits depends on the number and variety of axonal inputs received by each neuron in the circuit (Card et al., 1999). In the present study, the similar total numbers of BST neurons infected, regardless of amygdalar injection site, suggests similarities in the synaptic wiring of BST neurons within CEAm-, MEAad-, and MEApd-projecting circuits. In other words, first- and second-order BST projection neurons may receive a similar number of axonal inputs from

second- and third-order BST neurons, respectively, regardless of whether the multisynaptic BST circuits are targeting the CEA or the MEA.

An important technical consideration is the possibility that some BST retrograde labeling was produced by uptake by fibers of passage within the tracer-targeted amygdala nuclei. Varying degrees of retrograde transport via fibers of passage have been demonstrated in experiments using FG, CTB, and PRV (Aston-Jones and Card, 2000; Lanciego and Wouterlood, 2006). Due to the high affinity of PRV for extracellular matrix proteins located at synapses, viral uptake by fibers of passage is relatively limited compared to uptake of FG or CTB (Aston-Jones and Card, 2000). In rats injected with a mixture of PRV/CTB, only double-labeled neurons were counted as monosynaptic labeling, although many more single-labeled CTB neurons also were present within the BST. CTB-labeled BST neurons that were not PRV-positive likely represent neurons that were labeled due to uptake by fibers of passage within the tracer injection site, and/or due to greater diffusion of CTB from the injection site relative to PRV. Thus, quantification of the distribution of only double-labeled PRV+CTB neurons should have reduced the potential impact of including BST neurons labeled only via fibers of passage. We also observed no significant difference in the distribution pattern of labeling produced by FG iontophoresis vs. PRV/CTB injections, supporting the view that BST labeling distributions were not significantly affected by potential tracer uptake via fibers of passage within amygdalar tracer delivery sites.

The shifting distribution of viral labeling between 48 and 60 hr post-injection suggests the presence of intra-BST multisynaptic inputs to BST neurons that directly target the amygdala. However, alternative route of transsynaptic PRV transport from amygdalar injection sites to the BST are possible. Such alternative routes might include intra-amygdalar connections as well as hypothalamic or brainstem circuit nodes. In this study, however, we interpret our findings within the context of previous reports of intra-BST pathways, and we hypothesize that these pathways are the most plausible routes for increased PRV labeling within the BST at the 60 hr survival time. Additional experiments would be necessary needed to test the involvement of other hypothesized circuit nodes, e.g., by using lesion approaches (Card and Enquist, 2012; Jasmin et al., 1997; Luo et al., 2011), or by isolating PRV transport to specific circuits by using a cre-dependent PRV tracing approach (Card et al., 2011b).

The BSTpr and BSTam provide multisynaptic input to the CEAm

A major outcome of our study is that the BSTpr appears to provide multisynaptic input to the CEA (Fig. 13). Together with the MEAd and MEApd, the BSTpr is a constituent member of the brain's social and reproductive behavior network in rodent species (Newman, 1999). In male hamsters, electrolytic lesions of the BSTpr increase ejaculation latency, and decrease chemoinvestigatory behavior towards females (Newman, 1999). Studies analyzing neuronal Fos expression have identified specific neuronal subpopulations within the BSTpr and MEApd that are activated in response to discrete chemosensory stimuli and mating events (Veening and Coolen, 1998). Our finding that the BSTpr provides multisynaptic input to the CEAm suggests an anatomical substrate through which anxiety-related behaviors mediated by the CEA could be modulated by inputs from the BSTpr that are sensitive to reproductive status and context. The BSTpr and MEApd display sexual dimorphism with regards to nuclear volume (Hines et al., 1992; Mizukami et al., 1983), synaptic organization (Nishizuka and Arai, 1981; Nishizuka and Arai, 1983), and neurotransmitter phenotype (Malsbury and McKay, 1987; Miller et al., 1989), effects that are mediated by circulating androgens (Cooke et al., 1999). It remains to be determined whether the BSTpr provides multisynaptic input to the CEAm in female rats, as in males (present study). However, the BST is proposed to participate in sex-specific processes of stress-altered learning, based on evidence that a masculinized, but not a feminized, BST is necessary for stress to enhance classical eyeblink conditioning (Bangasser et al., 2005;

Bangasser and Shors, 2008; Bangasser and Shors, 2010). Sex differences in fear conditioning also have been reported (Baran et al., 2009; Pryce et al., 1999), in which the CEAm plays a critical role (Duvarci et al., 2011). Most studies on sex differences focus on the ability of sex hormones to modulate central neural signaling pathways. However, potential sex differences in the structure of multisynaptic circuits from the BSTpr to the CEAm would provide an anatomical substrate through which these differences become manifest.

Relatively little is known regarding the role of the BSTam, perhaps because its potential functions are so broad. The BSTam is involved in diverse and relatively diffuse neural circuits, including direct connections with regions implicated in neuroendocrine, autonomic, and somatomotor behavioral outputs. In addition, the BSTam is the only BST subnucleus to receive input from all other BST subnuclei (Fig. 2). Based on its diverse and widespread connectivity, Dong and Swanson hypothesized that the BSTam performs an integrative role within a differentiated striatopallidal circuit that regulates body energy homeostasis (Dong and Swanson, 2006a). Our viral tracing results demonstrate that the BSTam participates directly or indirectly in CEAm-, MEAd-, and MEApd-projecting circuits, supporting the view that the BSTam serves a broad integrative function within the BST (Fig. 13).

Patterns of monosynaptic anterograde and retrograde labeling produced by iontophoretic delivery of FG/BDA into amygdalar subregions suggest that the BSTpr and BSTam contain synaptically-distinct subdivisions. Tracer iontophoresis into the MEAd produced weak FG/BDA labeling within the most caudal level of the BSTpr (Fig. 6A), but produced robust labeling within lateral parts of the BSTpr at more rostral levels (Fig. 6B, C). Conversely, FG/BDA delivered into the MEApd produced robust anterograde/retrograde labeling within the caudal BSTpr (Fig. 7A) and within more medial aspects of the rostral BSTpr (Fig. 7B,C). Interestingly, PRV injections into either the MEAd or the MEApd produced more PRV labeling in both BSTpr subregions after 60 hr vs. 48 hr survival times (e.g., compare Fig. 6G–I with Fig. 7M–O), suggesting that the two BSTpr subregions are interconnected, despite differential direct inputs to the amygdala. Further, while PRV tracing results indicate that the CEAm receives multisynaptic (i.e., both direct and indirect) inputs from the BSTam, most of the transsynaptically-labeled BSTam neurons present in 60 hr cases are located in close proximity to the algBST and anterior commissure, with smaller numbers of neurons scattered within more medial aspects of the BSTam (Fig. 5N–R). A segregated distribution of PRV-infected neurons was not observed within the BSTam of MEAd- and MEApd-injected cases. Together, these data suggest that multisynaptic input to the CEAm may arise from a localized subregion of BSTam, whereas MEA subnuclei receive a more distributed input from the entire BSTam.

Neuroendocrine vs. autonomic BST circuits

Previous studies have suggested that MEA/BST circuits are more likely than CEA/BST circuits to be involved in the regulation of neuroendocrine function, based on the greater overlap of MEA projections with those of neuroendocrine-related BST neurons (Prewitt and Herman, 1998) and evidence that the MEA and pBST/amgBST directly innervate regions of the hypothalamic visceromotor pattern generator network that regulates pituitary output (Thompson and Swanson, 2003). In addition, viral tracing experiments have revealed the distribution of multisynaptic circuits that project to autonomic vs. neuroendocrine systems. After PRV injection into the wall of the stomach, transsynaptically-labeled neurons eventually appear within the algBST (Rinaman et al., 2000). In contrast, after PRV injections into the medial parvocellular subregion of the paraventricular nucleus of the hypothalamus (PVN), which contains neurohypophyseal endocrine neurons, retrogradely- and transsynaptically-labeled neurons appear within the amgBST and pBST (A.C. Khandai and J.P. Card, Univ. of Pittsburgh, unpublished observations). Considering the distribution of

pre-gastric/autonomic and pre-neuroendocrine BST circuits vs. the distribution of amygdala-projecting BST circuits, the pattern of viral labeling observed after PRV injections into the MEApd and MEAad is most similar to the distribution of pre-neuroendocrine BST circuits. Conversely, the CEAm receives direct projections from BST subnuclei that maintain pre-autonomic connections, and which may be modulated by inputs from the BST subnuclei that regulate neuroendocrine output.

The extended amygdala as a striatopallidal circuit

Lennart Heimer, Larry Swanson, and their colleagues have published compelling arguments for two different models describing the anatomical relationship between the amygdala and BST, i.e., Heimer's "extended amygdala" concept vs. Swanson's striatopallidal circuit concept (Alheid and Heimer, 1988; de Olmos and Heimer, 1999; Swanson, 2000; Swanson and Petrovich, 1998; Thompson and Swanson, 2003). The "extended amygdala" model posits that the BST is a continuous rostral extension of the central and medial amygdala, bridged by cellular columns within the interposing substantia innominate (SI). From this perspective, neural circuits that link amygdalar and BST subregions are considered internal, local associative connections. However, the apparent complexity of such internal circuits (e.g., see Dong and Swanson, 2006a,b) has complicated our understanding of how specific individual subregions exert control over functional outputs of either the amygdala or the BST. Conversely, results from the present study provide new evidence that specific subregions of the amygdala and BST are synaptically linked through discrete channels of connectivity that course within as well as between the medial and central divisions, rather than through a dense matrix of interconnectivity.

Swanson's striatopallidal model emphasizes the similarities as well as the differences between striatopallidal and amygdala/BST circuits. In a previous report, we suggested that the primary difference between amygdala/BST and striatopallidal circuits is the existence of an enhanced bottom-up interoceptive visceral feedback pathway for amygdala/BST circuits (Bienkowski and Rinaman, 2012). Compared to striatopallidal circuits that control voluntary somatomotor behavior, interoceptive feedback may be more important for amygdala/BST circuits that regulate autonomic and neuroendocrine output. This hypothesis is consistent with evidence that changes in visceral motor outflow and hormone release can occur with little or no cortical involvement.

The presence of topographically-organized amygdala and BST projection systems suggests that each amygdalar subnucleus and respective component of BST inputs comprise distinct parallel striatopallidal channels, with little integration between channels. In this manner, BST projections to the amygdala would be similar to pallidostriatal circuits (Kita and Kita, 2001; Rajakumar et al., 1994; Walker et al., 1989). However, results from the present study indicate that the CEAm receives multisynaptic input from BST subnuclei that would not be considered to occupy the BST-CEAm striatopallidal channel, providing a substrate for integration of information across amygdala/BST channels. It is unknown whether multisynaptic pallidostriatal circuits are maintained within distinct topographic pallidal boundaries, but this could be examined by applying the viral tracing approach used in the present study. Overall, our findings lead us to view the amygdala and BST subnuclei as differentiated parts of a striatopallidal circuitry, in which anatomical differences are hypothesized to reflect special adaptations of amygdala/BST circuits for the control of largely subconscious homeostatic functions.

Conclusions

While previous studies have defined the efferent projections of amygdalar and BST subnuclei, BST subnuclear interconnections have complicated our understanding of how

multisynaptic amygdala-projecting BST circuits are organized. Results from the present study reveal several organizational principles for multisynaptic BST circuit outputs to specific functional groups of amygdala subnuclei. We also present new evidence that the BSTpr and BSTam provide multisynaptic input to the CEAm, suggesting that the CEAm receives multimodal inputs related to diverse reproductive, autonomic, and neuroendocrine BST functions.

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List of Abbreviations

aco	anterior commissure
BAC	bed nucleus of the anterior commissure
BDA	biotinylated dextran amine
BST	bed nucleus of stria terminalis
algBST	anterolateral subnuclear group of the bed nucleus of stria terminalis
amgBST	anteromedial subnuclear group of the bed nucleus of stria terminalis
pBST	posterior subnuclear group of the bed nucleus of stria terminalis
BSTal	bed nucleus of stria terminalis, anterolateral subnucleus
BSTam	bed nucleus of stria terminalis, anteromedial subnucleus
BSTdm	bed nucleus of stria terminalis, dorsomedial subnucleus
BSTfu	bed nucleus of stria terminalis, fusiform subnucleus
BSTif	bed nucleus of stria terminalis, interfascicular subnucleus
BSTju	bed nucleus of stria terminalis, juxtacapsular subnucleus
BSTmg	bed nucleus of stria terminalis, magnocellular subnucleus
BSTov	bed nucleus of stria terminalis, oval subnucleus
BSTpr	bed nucleus of stria terminalis, principal subnucleus
BSTrh	bed nucleus of stria terminalis, rhomboid subnucleus
BSTtr	bed nucleus of stria terminalis, transverse subnucleus
BSTv	bed nucleus of stria terminalis, ventral subnucleus
CEA	central amygdalar nucleus
CEAm	central amygdalar nucleus, medial part
CEAl	central amygdalar nucleus, lateral part
CRH	corticotrophin-releasing hormone
CTB	cholera toxin subunit B
FG	Fluorogold
fx	fornix
int	internal capsule

MEA	medial nucleus of the amygdala
MEAad	medial nucleus of the amygdala, anterodorsal subnucleus
MEAav	medial nucleus of the amygdala, anteroventral subnucleus
MEApd	medial nucleus of the amygdala, posterodorsal subnucleus
mfb	medial forebrain bundle
mct	medial corticohypothalamic tract
NA	nucleus accumbens
oc	optic chiasm
opt	optic tract
PRV	pseudorabies virus
PS	parastrial nucleus of the hypothalamus
PVN	paraventricular hypothalamic nucleus
SI	substantia innominata
sm	stria medullaris
st	stria terminalis
V3	third ventricle
VL	lateral ventricle

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Figure 1.

The amygdala and BST coordinate autonomic, neuroendocrine, and somatomotor responses to internal and external stimuli and events (left). Results from anterograde tracing studies support the existence of topographically-organized reciprocal connections between amygdalar and BST subnuclei (right). The CEA (red) is reciprocally connected to the anterolateral group of BST subnuclei (algBST; red, including al, rh, fu, ov, and ju), while the MEAad (green) and MEApd (blue) are reciprocally connected to complementary subregions of the posterior BST (pBST; including pr, tr, and if) and the anteromedial BST subnuclei group (amgBST; including am, mg, v, and dm). Schematics adapted from (Swanson, 2004). See list for abbreviations.

Figure 2.

Anterograde tracing summary for efferent projections from individual BST subnuclei, revealing robust subnuclear interconnectivity (data summarized from multiple Dong and Swanson publications, 2000–2006; see text). BST subnuclei with direct inputs to the CEA are shown on the darker background (i.e., comprising portions of the central extended amygdala), whereas BST subnuclei with inputs to the MEA are shown on the lighter background (comprising portions of the medial extended amygdala). The discrete topographic connections of distinct BST subnuclei and amygdalar subregions suggests the presence of separate pathways for information processing (see Fig. 1). However, the presence of rich intra-BST connectivity suggests that integration between the central and medial extended amygdala may occur, as indicated by connection lines that cross the light/dark background borders. Grey dashed lines indicate connections described by Dong and colleagues as sparse, or for which the presence of terminal boutons along anterogradely-labeled fibers was unclear, suggesting that the fibers might only be passing through without forming synaptic connections. See list for abbreviations.

Figure 3. Examples of tracer delivery sites for FG/BDA (A–C) or PRV/CTB (D–F, 48 hr survival; G–I, 60 hr survival) within the CEAm (first column; A,D,G), MEAad (middle column; B,E,H), and MEApd (right column; C,F,I). Coronal schematic insets within each panel (adapted from (Swanson, 2004)) indicate the location and boundaries of each targeted amygdala subnucleus. FG/BDA iontophoresis (A–C) in each region produced somewhat spherical FG (green) delivery sites, with some FG labeling in adjacent regions reflecting local retrograde transport. BDA labeling (white/magenta) is much more limited to the central core of the iontophoretic delivery site, with anterograde fiber labeling often extending away from the site (e.g., panel C). PRV/CTB pressure co-injections (D–I) produced teardrop-shaped tracer delivery sites, with CTB anterograde and retrograde labeling (magenta) extending into larger areas compared to PRV labeling (green/white). CTB labeling often involved the optic tract, through which the injecting pipette traveled (e.g., panels E,F,G,I). The presence of PRV-infected neurons within and near the injection site is likely due to retrograde labeling of neurons with local axon terminals, which account for the majority of PRV uptake. PRV-positive neurons also may become labeled via their axonal projections and synaptic inputs to infected neurons in the BST and other brain regions, especially at the longer (60 hr) survival time. Scale bar = 500 μ m. See list for abbreviations.

Figure 4.

Sections from our Klüver-Barrera-stained BST reference atlas, revealing neurons in pink and myelinated fibers in blue. Tissue sections are 20 μm thick, with a represented sectioning frequency of 60 μm . Rostrocaudal levels (lower left corner in each image) refer to distance from the most rostral BST level, depicted in panel B (0 μm). BST subnuclei, along with the hypothalamic parastrial nucleus (ps) and bed nucleus of the anterior commissure (BAC), are labeled in black, while fiber tracts and ventricles are labeled in red. See list for abbreviations.

Figure 5.

Retrograde and anterograde labeling in the BST after monosynaptic FG/BDA iontophoresis or PRV/CTB co-injection into the CEAm. Sections representing six rostrocaudal levels were used to quantify retrograde labeling (caudal left, rostral right). A–F: Iontophoretic delivery of FG/BDA cocktail into the CEAm produced dense BDA anterograde labeling (magenta) and FG retrograde labeling (green) in the algBST. G–L: Co-injection of PRV/CTB into the mCEA revealed a CTB monosynaptic retrograde labeling (magenta) and PRV-labeling (green) 48 hr post-injection that was similar to the distribution of labeling produced by FG. M–R: The proportion of total PRV labeling located within the amgBST and pBST was significantly increased 60 hr post-injection (for quantification, see Figs. 8 and 9). Scale bars = 100 μ m. See list for abbreviations.

Figure 6.

Retrograde and anterograde BST labeling after monosynaptic FG/BDA iontophoresis or PRV/CTB co-injection into the MEAad. Sections representing six rostrocaudal levels were used to quantify retrograde labeling (caudal top left, rostral bottom right right). A–F: Iontophoretic delivery of FG/BDA cocktail into the MEAad produced dense BDA anterograde labeling (magenta) and FG retrograde labeling (green) in the amgBST and pBST. Anterograde and retrograde algBST labeling was sparse, especially within the BSTov. The distribution of CTB monosynaptic retrograde labeling (magenta) and PRV-labeling (green) at 48 hr (G–L) and 60 hr (M–R) after co-injection of PRV/CTB into the MEAad was similar to the distribution of FG-labeled neurons (for quantification, see Figs. 8 and 9). Scale bars = 100 μ m. See list for abbreviations.

Figure 7.

Retrograde and anterograde BST labeling after monosynaptic FG/BDA iontophoresis or PRV/CTB co-injection into the MEApd. Sections representing six rostrocaudal levels were used to quantify retrograde labeling (caudal left, rostral right). A–F: Iontophoretic delivery of FG/BDA cocktail into the MEApd produced dense BDA anterograde labeling (magenta) and FG retrograde labeling (green) in the pBST, specifically the BSTpr, with lighter labeling in the BSTam. This labeling distribution was distinct from labeling in more lateral aspects of the BSTpr and BSTam after MEAd injections (see Fig. 5). G–L: Co-injection of PRV/CTB into the MEApd produced CTB monosynaptic retrograde labeling (magenta) and PRV labeling (green) that at 48 hr post-injection was similar to the distribution of FG labeling. M–R: After 60 hr survival, the proportion of total PRV labeling present within the amgBST was significantly increased, specifically within the BSTam (for quantification, see Figs. 8 and 9). Scale bars = 100 μ m. See list for abbreviations.

Figure 8.

Average total number of retrogradely-labeled BST neurons following tracer delivery into the CEAm, MEAd, and MEApd. There was a significant effect of tracer group on the number of retrogradely-labeled BST neurons (see Results). In CEAm- and MEAd-injected cases, the 48 hr and 60 hr PRV groups displayed significantly larger numbers of retrogradely-labeled BST neurons compared to BST retrograde labeling in the monosynaptic tracer group (*, $P < 0.05$ compared to monosynaptic), and labeling in PRV 60 hr cases was significantly increased compared to labeling in PRV 48 hr cases (#, $P < 0.05$ compared to PRV 48 hr cases). Within the MEApd injection group, the number of retrogradely-labeled neurons in PRV 48 hr and 60 hr cases was significantly greater than in monosynaptic cases (*, $P < 0.05$ compared to monosynaptic), whereas the difference between PRV 48 hr and 60 hr cases was not significant. See list for abbreviations.

Figure 9.

Distribution of retrograde labeling within BST subnuclear groups. Iontophoretic delivery of monosynaptic tracers into the CEA, MEAd, or MEApd produced 3 distinct patterns of retrograde labeling within BST subnuclear groups. There was a significant effect of tracer group on the distribution of retrograde labeling in rats with CEA- and MEApd-targeted injections, but not in rats with MEAd-targeted injections (see Results). For all three amygdalar injection sites, the proportional distribution of labeling across the algBST, amgBST, and pBST was not significantly different between monosynaptic and PRV 48 hr cases ($P > 0.05$ for each subnuclear comparison), despite the almost 10-fold higher number of retrogradely labeled neurons in PRV 48 hr cases (see Fig. 7 for total counts). In CEA-targeted cases, the proportions of total BST retrograde labeling present within the amgBST and pBST were significantly increased, and proportions within the algBST decreased, in PRV 60 hr vs. 48 hr and monosynaptic cases (*, $P < 0.05$ compared to monosynaptic; #, $P < 0.05$ compared to PRV 48 hr). In MEApd-targeted cases, the proportion of labeling was significantly increased within the amgBST and decreased within the pBST in PRV 60 hr cases compared to labeling in PRV 48 hr and monosynaptic cases (*, $P < 0.05$ compared to monosynaptic; #, $P < 0.05$ compared to PRV 48 hr). No proportional distribution differences were observed as a function of tracer group in MEAd-targeted cases. See list for abbreviations.

Figure 10.

Distribution of retrograde labeling within individual subnuclei of the algBST. Generally, higher proportions of retrograde labeling were observed within algBST subnuclei in CEA-injected cases compared to MEAad- or MEApd-injected cases. In CEA-injected cases, there was a significant effect of tracer group on the proportional distribution of labeling within the BSTal (top left) and BSTov (top right). In CEA-injected cases, the proportion of retrograde labeling in the BSTal was significantly lower in PRV 48 and 60 hr groups compared to the monosynaptic group (*, $P < 0.05$ compared to monosynaptic.). CEA-injected PRV 60 hr cases also displayed a lower proportion of labeling within the BSTov compared to monosynaptic and PRV 48 hr cases (*, $P < 0.05$ for both comparisons). In MEAad-injected cases, tracer group had a significant effect on the proportion of labeling within the BSTrh (left center), with a larger proportion of retrograde labeling present in the BSTrh in PRV 48 hr cases compared to monosynaptic or PRV 60 hr cases (a, $P < 0.05$ compared to PRV 60 hr cases). See list for abbreviations.

Figure 11.

Distribution of retrograde labeling within individual subnuclei of the amgBST. In CEA-injected cases, there was a significant effect of tracer group on the proportion of retrograde labeling in the BSTam. PRV 60 hr cases displayed a significantly larger proportion of retrograde labeling in the BSTam compared to monosynaptic cases (*, $P < 0.05$ compared to monosynaptic). In MEApd-injected cases, there was a significant effect of tracer group on the proportion of labeling in the BSTdm, and a nonsignificant trend towards an effect of tracer group on labeling within the BSTam. A significantly greater proportion of labeling was found within the BSTdm in PRV 60 hr cases compared to monosynaptic cases (*, $P < 0.05$ compared to monosynaptic). See list for abbreviations.

Figure 12.

Distribution of retrograde labeling within individual pBST subnuclei. There was a significant effect of tracer group on the proportion of retrograde labeling within the BSTpr. In CEA-injected cases, the PRV 60 hr group displayed a significantly larger proportion of retrograde labeling in the BSTpr compared to both PRV 48 hr and monosynaptic cases (*, $P < 0.05$ compared to monosynaptic; #, $P < 0.05$ compared to PRV 48 hr). In MEApd cases, there was a nonsignificant trend towards reduced labeling in PRV 60 hr cases compared to labeling in monosynaptic and 48 hr cases. See list for abbreviations.

Figure 13.

Summary diagram for proposed multisynaptic BST pathways targeting the CEAm, MEAad, and MEApd. The CEAm (top) receives robust direct input from algBST projection neurons (1st-order connections). Results from PRV labeling assessed 48 hr post-injection supports the view that CEAm-projecting algBST neurons receive input from additional neurons within the algBST (2nd-order connections) that do not directly innervate the CEAm. An increase in the proportion of total viral labeling present within the BSTam and BSTpr 60 hr post-PRV injection suggests that BSTam and BSTpr neurons are multisynaptically connected to algBST projection neurons and/or their presynaptic inputs. MEAad neurons (middle) receive robust direct input from subnuclei of the amgBST subnuclei, as well as from the BSTif and BSTtr. Since the proportional distribution of viral labeling was not significantly different at 48 vs. 60 hr survival times, these data suggest that 2nd- and 3rd-order multisynaptic pathways are likely isolated as a distributed subnetwork within the amgBST, BSTif, and BSTtr. The MEApd (bottom) receives substantial direct input from the BSTpr. In 48 hr PRV cases, the proportion of total viral labeling present within the BSTpr was not significantly different from FG (monosynaptic) labeling results, despite significantly larger numbers of infected vs. FG-labeled neurons, evidence that MEApd-projecting BSTpr neurons receive direct input primarily from BSTpr interneurons. At the longer 60 hr survival interval, significant proportional increases of viral labeling within the BSTdm and BSTam suggest that these BST subnuclei provide 3rd-order input to the MEApd.

Table 1

Experimental animals divided into groups based on injection site and tracing approach. Case numbers are listed in the columns. Twenty-one rats were injected with PRV/CTB mixture, generating both monosynaptic and viral labeling data. These cases (shaded cells) are listed in both the monosynaptic labeling group and in one of the PRV groups (see Results). Final group sizes for each tracer group and injection site are indicated at the bottom of each column. Results from 45 rats produced 66 sets of data analyzed in this study.

Monosynaptic Tracer Group				PRV 48 hr Group				PRV 60 hr Group			
CEA	MEAd	MEApd	CEA	MEAd	MEApd	CEA	MEAd	MEApd	MEAd	MEApd	MEAd
11-449	11-450	12-34	11-291	11-314	11-313	12-15	12-46	12-48			
11-473	11-537	12-35	11-293	12-168	11-108	12-16	12-47	12-234			
12-170	12-168	12-37	11-311	12-169	12-171	12-17	12-195	12-290			
12-240	12-169	12-171	11-185	12-324	12-172	12-240	12-252				
12-289	12-324	12-172	11-186		12-173	12-289	12-253				
12-291	12-195	12-173	11-187			12-291	12-254				
12-292	12-252	12-234	11-188			12-292	12-255				
12-294	12-253	12-290	11-312			12-294	12-256				
	12-254		12-170				12-235				
	12-255										
	12-235										
	12-256										
n = 8	n = 12	n = 8	n = 9	n = 4	n = 5	n = 8	n = 9	n = 3			