Pattern of Distribution of Serotonergic Fibers to the Amygdala and Extended Amygdala in the Rat

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ABSTRACT

As is well recognized, serotonergic (5-HT) fibers distribute widely throughout the forebrain, including the amygdala. Although a few reports have examined the 5-HT innervation of select nuclei of the amygdala in the rat, no previous report has described overall 5-HT projections to the amygdala in the rat. Using immunostaining for the serotonin transporter, SERT, we describe the complete pattern of distribution of 5-HT fibers to the amygdala (proper) and to the extended amygdala in the rat. Based on its ontogenetic origins, the amygdala was subdivided into two major parts, pallial and subpallial components, with the pallial component further divided into superficial and deep nuclei (Olucha-Bordonau et al. 2015). SERT+ fibers were shown to distributed moderately to densely to the deep and cortical pallial nuclei, but, by contrast, lightly to the subpallial nuclei. Specifically, 1) of the deep pallial nuclei, the lateral, basolateral, and basomedial nuclei contained a very dense concentration of 5-HT fibers; 2) of the cortical pallial nuclei, the anterior cortical and amygdala–cortical transition zone rostrally and the postero medial and postero lateral nuclei caudally contained a moderate concentration of 5-HT fibers; and 3) of the subpallial nuclei, the anterior nuclei and the rostral part of the medial (Me) nuclei contained a moderate concentration of 5-HT fibers, whereas caudal regions of Me as well as the central nuclei and the intercalated nuclei contained a sparse/light concentration of 5-HT fibers. With regard to the extended amygdala (primarily the bed nucleus of stria terminalis; BST), on the whole, the BST contained moderate numbers of 5-HT fibers, spread fairly uniformly throughout BST. The findings are discussed with respect to a critical serotonergic influence on the amygdala, particularly on the basal complex, and on the extended amygdala in the control of states of fear and anxiety. J. Comp. Neurol. 525:116–139, 2017.

INDEXING TERMS: fear; anxiety; stress; basolateral complex of amygdala; central nucleus of amygdala; pallial amygdala; subpallial amygdala; bed nucleus of stria terminalis; 5-HT1A receptors; 5-HT2C receptors

The neurochemical and neuroanatomical substrates for affective behavior have been well studied. At the forefront, both serotonin, chemically, and the amygdala, anatomically, are key substrates for emotional behavior (LeDoux, 2000, 2003; Shinnick-Gallagher et al., 2003; Whalen and Phelps, 2009; Asan et al., 2013; Bauer, 2015). The amygdala (and extended amygdala) has been shown to be critically involved in affective behaviors, including fear, stress, anxiety, and reward, and is central to the processing of emotionally relevant information (LeDoux, 2000, 2003; Shinnick-Gallagher et al., 2003; Phelps, 2006; Whalen and Phelps, 2009; Duvarci and Pare, 2014; Olucha-Bordonau et al., 2015). Serotonin (5-HT) also plays a well-documented role in affective processes (Lowry et al., 2005, 2008; Hayes and Greenshaw, 2011; Asan et al., 2013; Bauer, 2015). Dysfunction of either system has been associated with a host of psychiatric disorders, including depression, chronic anxiety, and posttraumatic stress disorder (PTSD; Walker et al., 2003; Kalia, 2005; Nemeroff et al., 2006; Shin et al., 2006; Holmes, 2008; Shin and Liberzon, 2010; Hale et al., 2012; Fox and Lowry, 2013).

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Although earlier studies in the rat, using various techniques, identified 5-HT fibers within select nuclei of the amygdala, no previous report has comprehensively examined the overall distribution of 5-HT fibers in the amygdala, and the 5-HT innervation of the “extended amygdala” has been largely unexplored (Parent et al., 1981; Steinbush, 1981). Previous studies in the rat have focused mainly on 5-HT input to the central and basolateral (BLA) nuclei of the amygdala, showing that 5-HT fibers are distributed densely in BLA and by comparison lightly in the central nucleus (Ce) of the amygdala (Asan et al., 2005; Muller et al., 2007; Smith and Porrino, 2008). This is essentially the reverse of the pattern shown for (nonhuman) primates, in which Ce is densely populated, BLA relatively lightly so, with 5-HT axons (Sadikot and Parent, 1990; Freedman and Shi, 2001; Bauman and Amaral, 2005; O’Rourke and Fudge, 2006; Smith and Porrino, 2008).

Because both the amygdala and the serotonergic systems critically participate in several affective functions, notably fear and anxiety, it is important to determine the precise pattern of termination of 5-HT fibers to the amygdala and the bed nucleus of the stria terminalis (BST). Using immunohistochemical procedures for the detection of the serotonin transporter protein (SERT), we examined the distribution of 5-HT fibers in the amygdala (proper) and in BST, with attention to differential innervation of subnuclei. In brief, we show that serotonergic fibers are distributed strongly, but heterogeneously, throughout the amygdala and BST. Overall, 5-HT fibers terminate densely in the deep and cortical pallial nuclei, particularly pronounced in the basolateral complex. By contrast, the central and medial nuclei of the subpallial amygdala receive a modest serotonergic innervation. These differences in the pattern and density of 5-HT afferents in the amygdala would appear to reflect a differential serotonergic influence on discrete regions of the amygdala in various affective behaviors.

**MATERIALS AND METHODS**

Ten (five male, five female) naïve Sprague Dawley rats (Harlan, Indianapolis, IN) weighing 275–300 g on arrival were housed in pairs on a 12:12-hour light cycle for 7 days, during which food and water were given ad libitum. These experiments were approved by the Florida Atlantic University Institutional Animal Care and Use Committee and conform to all federal regulations and National Institutes of Health guidelines for the care and

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**Abbreviations**

| Acronym | Description |
|---------|-------------|
| AA | anterior amygdaloid area |
| AAD | anterior amygdaloid area, dorsal part |
| AAV | anterior amygdaloid area, ventral part |
| ac | anterior commissure |
| AC | nucleus accumbens |
| ACo | anterior cortical nucleus of amygdala |
| AHi | amygdalohippocampal area |
| AHiAL | amygdalohippocampal transition area, anterolateral part |
| AHiPL | amygdalohippocampal transition area, posteroanterolateral part |
| AHiPM | amygdalohippocampal transition area, posterolateral division |
| APir | amygdalopalliform transition area |
| ASst | amygdalostriatal transition area |
| BAC | bed nucleus of anterior commissure |
| BAOT | bed nucleus of accessory olfactory tract |
| BLA | basolateral amygdala |
| BLAa | basolateral nucleus of amygdala, anterior part |
| BLAp | basolateral nucleus of amygdala, posterior part |
| BLAv | basolateral nucleus of amygdala, ventral part |
| BMA | basomedial amygdala |
| BMaa | basomedial nucleus of amygdala, anterior part |
| BMAp | basomedial nucleus of amygdala, posterior part |
| BST | bed nucleus of the stria terminalis |
| Ce | central nucleus of amygdala |
| CeC | central nucleus of amygdala, capsular division |
| CeL | central nucleus of amygdala, lateral division |
| CeM | central nucleus of amygdala, medial division |
| CxA | cortical amygdala transition zone |
| DEn | dorsal endopiriform nucleus |
| DR | dorsal raphe nucleus |
| EA | extended amygdala |
| Fu | fusiform nucleus of BST |
| fx | fornix |
| HDB | nucleus of horizontal limb of diagonal band |
| I | intercalated nuclei of amygdala |
| IPAC | interstitial nucleus of posterior limb of anterior commissure |
| La | lateral nucleus of amygdala |
| LaDL | lateral nucleus of amygdala, dorsolateral part |
| LaVL | lateral nucleus of amygdala, ventrolateral part |
| LaVM | lateral nucleus of amygdala, ventromedial part |
| LGP | globus pallidus, lateral division |
| LOT | nucleus of lateral olfactory tract |
| LPG | lateral preoptic area |
| LSS | lateral stripe of striatum |
| LSV | lateral septal nucleus, ventral part |
| Me | medial nucleus of amygdala |
| MedA | medial nucleus of amygdala, anteroventral part |
| MeAV | medial nucleus of amygdala, anterodorsal part |
| MePD | medial nucleus of amygdala, posterodorsal part |
| MePV | medial nucleus of amygdala, posteroventral part |
| mPFC | medial prefrontal cortex |
| MR | median raphe nucleus |
| OT | olfactory tubercle |
| Pir | piriform cortex |
| PLCo | posterocortical nucleus of amygdala |
| PMCo | posteromedial cortical nucleus of amygdala |
| PS | parastriatal nucleus of BST |
| Pu | putamen |
| PVA | paraventricular nucleus of thalamus, anterior part |
| Si | substantia innominata |
| SIB | substantia innominata, basal division |
| SLM | supramammillary nucleus |
| sm | stria medullaris |
| SO | supraoptic nucleus |
| SLEA | sublenticular extended amygdala |
| STD | dorsal division of BST |
| STIA | intra-amygdala division of BST |
| STLD | lateral dorsal division of BST |
| STLI | lateral intermediate division of BST |
| STLJ | lateral juxtageniculate division of BST |
| STLP | lateral posterior division of BST |
| STLV | lateral ventral division of BST |
| STM | medial division of BST |
| STMPI | medial posterior intermediate division of BST |
| STPI | medial posterior lateral division of BST |
| STPM | medial posterior medial division of BST |
| STMV | medial ventral division of BST |
| STS | supracapsular extended amygdala |
| VEn | ventral endopiriform nucleus |
| VP | ventral pallidum |
use of laboratory animals. Rats were deeply anesthe-
tized with an intraperitoneal injection of sodium pento-
obarbital (Nembutal; 75 mg/kg). Rats were perfused
transcardially with 30–50 ml ice-cold heparinized 0.1 M
phosphate-buffered saline (PBS) followed by 200–
300 ml chilled 4% paraformaldehyde in 0.1 M phosphate
buffer (PB) at pH 7.4. The brains were removed and
postfixed for 24–48 hours in 4% paraformaldehyde in
0.1 M PB. Brains were then placed in a 30% sucrose
solution for another 48 hours. After sucrose cryoprotec-
tion, 50-μm sections were cut on a freezing sliding
microtome. Sections were collected in a six-well plate
using 0.1 M PB as a storage solution, so that every
sixth section was represented throughout the brain for
each series of sections. Sections were stored in 0.1 M
PB at 4°C until the tissue was prepared for
immunohistochemistry.

**SERT immunohistochemistry**

Immunohistochemical analysis to detect serotonergic
axons was performed with an antiserum to SERT using
an avidin-biotin complex protocol as was
described previously (Vertes et al., 2010; Linley et al.,
2013). Free-floating sections were treated with 1%
sodium borohydride (NaBH₄) in 0.1 M PB to remove
excess aldehydes. After copious 0.1 M PB washes, sections
were stored for 1 hour in 0.5% bovine serum albumin
(BSA) in 0.1 M Tris-buffered saline (TBS; pH 7.6)
containing 0.25% Triton X-100. Sections were then incu-
bated in the primary polyclonal antibody, rabbit anti-
SERT (ImmunoStar, Hudson, WI; catalog No. 24330,
RRID:AB_572209). The SERT antibody was placed in a
synthetic peptide corresponding to amino acids 579–
599 of rat SERT coupled to keyhole limpet hemocyanin
(KLH). The primary antibody was placed in a diluent of
0.1% BSA TBS containing 0.25% Triton X-100 at a con-
centration of 1:5,000 at room temperature for 24–48
hours. After further washes, sections were placed in a
secondary antibody, biotinylated goat anti-rabbit immu-
noglobulin (Vector Laboratories, Burlingame, CA; catalog
No. BA-1000, RRID:AB_2313606) in diluent at a 1:500
concentration for 2 hours. This was followed by another
series of PB washes. Sections were then incubated for
2 hours in a tertiary antibody, biotinylated horse anti-

goglobulin (Vector Laboratories; catalog No.
BA9500, RRID:AB_2313580) in diluent at a 1:500
concentration. After washing of the tissue in 0.1 M PB, sections
were incubated for 1 hour in an avidin-biotin
complex (ABC) using the Vectastain Elite ABC-
Peroxidase kit (Vector Laboratories; catalog No. PK-
7100, RRID:AB_2336827) in a diluent at a 1:200
concentration. After final 0.1 M PB washes, serotonin
fibers expressing the serotonin transporter protein were
visualized with the chromagen 0.022% 3,3′-diaminobenzi-
dine (DAB; Aldrich, Milwaukee, WI) and 0.003% hydrogen
peroxide in TBS for approximately 4–6 minutes. Sections
were stored in 0.1 M PB at 4°C until mounted onto
chrome-alum gelatin-coated slides, dehydrated using
graded methanols, and coverslipped with Permount. The
specificity of the primary and secondary antibodies have
been verified in previous studies (Vertes et al., 2010;
Linley et al., 2013). With the present antiserum to SERT,
immunostained sections through the pons and mesen-
cephalon displayed identical patterns of cell and fiber
SERT⁺ labeling as shown previously for these regions of
the upper brainstem (Sur et al., 1996; Vertes and Crane,
1997; Yamamoto et al., 1998). Additionally, sections
reacted without the primary or secondary antibodies did
not show immunoreactivity (data not shown).

**Nissl staining**

To reference the cytoarchitectonic borders of nuclei of the
amygdala and BST, sections throughout the amygdala
and extended amygdala were visualized with a cresyl vio-
let stain. Briefly, sections were mounted on chrome-alum
coated slides, rinsed for 2 × 10 minutes in xylene, rehy-
drated in graded methanols, and immersed in a 0.1% cre-
syl violet (Acros Organics, Thermo Fisher Scientific,
Waltham, MA) in an acetic acid buffer for 15–20 minutes.
Slides were then rinsed briefly with deionized water, dehy-
drated in graded methanols (to 100% methanol), cleared
with xylene, and coverslipped with Permount.

**Photomicroscopy**

Lightfield photomicrographs at 100× magnification
were taken for visualization of SERT⁺ fibers throughout
the rostrocaudal extent of the amygdala (proper) and the
extended amygdala. Photomicrographs were cap-
tured with a Q Imaging (QICAM) camera mounted on a
Nikon Eclipse E600 microscope. Digital images were
captured and reconstructed in Nikon Elements and
then imported into Adobe Illustrator and Photoshop CC
2014 (Adobe, Mountain View, CA) to adjust brightness
and contrast and to outline borders of nuclei of the
amygdala/BST. Representative Nissl-stained sections
throughout the extent of the amygdala/BST were cap-
tured and imported into Adobe Illustrator to map the
subdivisions of nuclear groups.

**Note on nomenclature for the amygdala and extended amygdala**

The nomenclature and nuclear demarcations used for
nuclei of the amygdala and extended amygdala are
those of Olucha-Bordonau et al. (2015), as follows. The
amygdaloid complex has recently been subdivided

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according to its ontogenetic origin into two major divisions, pallial and subpallial components, with the pallial component further divided into superficial and deep nuclei (Medina et al., 2004; García-López et al., 2008; Martínez-García et al., 2008; Bupesh et al., 2011; Hertel et al., 2012; Olucha-Bordonau et al., 2015). This

Figure 1. A: Low-magnification Nissl-stained transverse section at the transition between the amygdala (proper) and the extended amygdala showing the locations of nuclei at this level. B: Pattern of distribution of 5-HT fibers to nuclei of the amygdala at the same level, or to the sublenticular region of the amygdala. For abbreviations see list. Scale bar = 500 μm in B; 1.2 mm for A. [Color figure can be viewed at wileyonlinelibrary.com.]
demarcation is analogous to a cortical/subcortical differentiation, with the cortical component showing a distinct lamination and with a few exceptions the subcortical sector exhibiting no layering. Furthermore, the pallial amygdala, like the cortex, contains a greater proportion of glutamatergic to GABAergic cells; whereas the subpallial amygdala, like subcortical telencephalic structures, contains significantly more GABAergic relative to glutamatergic cells. The superficial division of the pallial amygdala (or the cortical pallial amygdala) consists of the anterior (ACo), posterolateral (PLCo), and posteromedial (PMCo) cortical nuclei as well as transition zones between the ACo and piriform cortex; that is, rostrally, the cortical–amygdala transition zone (CxA) and caudally the amygdalo–piriform transition area (APir). The deep (or nuclear) division of the pallial amygdala consists of the oft-referred to “basolateral complex” of the amygdala, comprising the lateral (La), basolateral (BLA), and basomedial (BMA) nuclei as well as the amygdalo–hippocampal area (AHi). The subpallial division of the amygdala consists primarily of the medial (Me), central (Ce) and anterior nuclei and the intercalated cell masses.

The subpallial region of the ‘extended amygdala’ (EA) consists of two components, 1) the BST, which includes the intra-amygdala area (STIA), the supracapular EA (STS), and the BST (proper) divisions, and 2) the...
The sublenticular extended amygdala (SLEA) composed of the sublenticular part of the substantia innominata (SI) and the interstitial nucleus of the posterior limb of the anterior commissure (IPAC), lying dorsolateral to the anterior amygdaloid area (Olucha-Bordonau et al., 2015). The septal sector of BST (or BST proper) surrounds the anterior commissure and is composed of several subnuclei, with those of the anterior division

Figure 3. A: Low-magnification Nissl-stained transverse section through the anterior amygdala showing the locations of nuclei at this level. B: Pattern of distribution of 5-HT fibers to nuclei of the amygdala at the same level. For abbreviations see list. Scale bar = 500 μm in B; 800 μm for A. [Color figure can be viewed at wileyonlinelibrary.com.]
associated with the central nucleus of amygdala and those of the posterior division associated with the medial nucleus of amygdala (Dong et al., 2001; Alheid, 2003; Olucha-Bordonau et al., 2015).

RESULTS

We describe the pattern of distribution of serotonergic fibers to the amygdala (proper) as well as that to the EA (or BST complex), as illustrated by a
Figure 5. A: Low-magnification Nissl-stained transverse section through the midportion of the amygdala showing the locations of nuclei at this level. B: Pattern of distribution of 5-HT fibers to nuclei of the amygdala at the same level. Note the dense labeling of nuclei of the basolateral complex (La, BMA, BLA), particularly BLA, and the sparse labeling of divisions of the central nucleus (Ce). For abbreviations see list. Scale bar = 500 μm in B; 1 mm for A. [Color figure can be viewed at wileyonlinelibrary.com.]
Figure 6. A: Low-magnification Nissl-stained transverse section through the midportion of the amygdala showing the locations of nuclei at this level. B: Pattern of distribution of 5-HT fibers to nuclei of the amygdala at the same level. Note the dense labeling of nuclei of the basolateral complex (La, BMA, BLA), particularly BLA, and the sparse labeling of divisions of the central nucleus (Ce). For abbreviations see list. Scale bar = 500 μm in B; 1 mm for A. [Color figure can be viewed at wileyonlinelibrary.com.]
representative case (case 14). The findings of nonillustrated cases directly correspond to those of the illustrated case.

**Amygdala**

Figures 1–11 depict the pattern of distribution of 5-HT fibers rostrocaudally throughout the amygdala and BST.
Figure 8. A: Low-magnification Nissl-stained transverse section through the posterior amygdala showing the locations of nuclei at this level. B: Pattern of distribution of 5-HT fibers to nuclei of the amygdala at the same level. Note the dense labeling of nuclei of the basolateral complex (La, BMA, BLA), particularly BLA, and the sparse to light labeling of divisions of the central (Ce) and medial nuclei (Me). For abbreviations see list. Scale bar = 500 μm in B; 700 μm for A. [Color figure can be viewed at wileyonlinelibrary.com.]
Figure 9. A: Low-magnification Nissl-stained transverse section through the posterior amygdala showing the locations of nuclei at this level. B: Pattern of distribution of 5-HT fibers to nuclei of the amygdala at the same level. Note the dense labeling of nuclei of the basolateral complex (La, BMA, BLA), particularly BLA, and the light labeling of the medial nucleus. For abbreviations see list. Scale bar = 500 µm in B; 670 µm for A. [Color figure can be viewed at wileyonlinelibrary.com.]
consist of a series of Nissl-stained sections (Figs. 1A–11A) showing the locations of nuclei of the amygdala and corresponding SERT-immunostained sections (Figs. 1B–11B) depicting patterns of labeling at these levels of the amygdala. Figure 1B shows the pattern of distribution 5-HT fibers at a caudal level of the extended amygdala (EA) as it merges with the amygdala proper. As depicted (Fig. 1B), the interstitial nucleus of the posterior limb of the anterior commissure (IPAC), surrounding the anterior commissure (ac), was moderately

Figure 10. A: Low-magnification Nissl-stained transverse section through the posterior amygdala showing the locations of nuclei at this level. B: Pattern of distribution of 5-HT fibers to nuclei of the amygdala at the same level. Note the dense labeling of the posterior part of the basolateral nucleus, setting it apart from surrounding structures. For abbreviations see list. Scale bar = 500 µm in B; 1 mm for A. [Color figure can be viewed at wileyonlinelibrary.com.]
Figure 11. A: Low-magnification Nissl-stained transverse section through the posterior amygdala showing the locations of nuclei at this level. B: Pattern of distribution of 5-HT fibers to nuclei of the amygdala at the same level. Note the dense labeling of the posterior part of the basomedial and basolateral nuclei. For abbreviations see list. Scale bar = 500 µm in B; 750 µm for A. [Color figure can be viewed at wileyonlinelibrary.com.]
labeled. This contrasts with considerably lighter labeling of IPAC at a more anterior level (see Fig. 14). At the rostral pole of the amygdala (Fig. 2B), the anterior dorsal (AAD) and anterior ventral (AAV) nuclei as well as the rostral extent of the basomedial nucleus (BMA) were moderately to densely labeled. SERT fibers spread moderately throughout cortical pallial nuclei, distributing nonhomogeneously to the anterior cortical nucleus (ACo) and the cortical amygdala transition zone (CxA) but evenly within the nucleus of the lateral olfactory tract (LOT). With regard to ACo and CxA, labeling was dense in superficial layers (layer 1) and tapered in deeper layers. This same pattern continued laterally from CxA to the piriform cortex. With the emergence of the central nucleus (Ce) at this level (Fig. 2B), the capsular division of Ce (CeC) was lightly labeled; the medial division (CeM) moderately labeled.

More caudally in the anterior amygdala (Fig. 3B), 5-HT fibers distributed heavily to the anterior basolateral (BLAa) and anterior basomedial (BMAa) nuclei and significantly but less densely to AAD and the anterodorsal part of the medial nucleus (MeAD), lying ventral to AAD. By contrast, labeling was less pronounced (or moderate) in CeM and in the amygdalostradiatal transition area (AST), lateral to CeC. The pattern of labeling of ACo and CxA was similar to that at rostral levels, with a decrease in density from superficial to deep layers, and was stronger in ACo and CxA than in the medially adjacent bed nucleus of the accessory olfactory tract (BAOT). As seen rostrally, the CeC was lightly labeled, whereas the intercalated nuclei (I) were lightly to moderately labeled.

Progressing posteriorly (Fig. 4B), marked differences began to emerge in the relative density of labeling across nuclei of the amygdala. More precisely, the very dense labeling of the lateral nucleus (La) and the anterior (BLAa) and posterior (BLAp) divisions of the basolateral nucleus (BLA), strongly contrasted with the light labeling of the medially adjacent central nucleus and AST. This trend would continue more caudally. The ventral basolateral nucleus (BLAv) and BMAa contained slightly fewer labeled fibers than did BLA, whereas AAD, the dorsal (MeAD) and ventral (MeAV) medial nuclei, and the cortical nuclei (CxA, ACo, and BAOT) were less densely labeled than the basal groups. Layer 1 of cortical areas ACo and CxA was heavily labeled, layers 2/3 moderately labeled, as seen rostrally.

At the midrostrocaudal amygdala (Fig. 5B), the basal group continued to be strongly labeled, which, as rostrally, differed significantly from the sparse to light labeling of Ce. Labeling was slightly heavier in CeM than in CeC or in the lateral division of Ce (CeL). Among the basal group, labeling was densest in BLA (BLAa and BLAp), followed by La, BMAa, and BLAv, which were similarly labeled. With the exception of the intercalated group, which was lightly labeled, remaining
nuclei at this level were moderately labeled. This includes the ASt, MeAD, MeAV, ACo, CxA, and the intra-amygdalar component of BST (STIA). As rostrally, labeling gradually weakened from superficial to deep layers of ACo and CxA.

More caudally (Fig. 6B), labeled fibers were densely concentrated in La and bordering regions of BLAa and BLAp but tapered slightly in ventral parts of the basal complex, that is, in the anterior (BMAa) and posterior (BMAP) divisions of BMA and in BLAv. All divisions of the Ce were sparsely labeled, with a few more fibers in CeM than in the other divisions of Ce. The ASt component of Ce was moderately labeled. Labeling thinned from that of anterior levels in MeAD and MeAV such that at this level both divisions of Me were lightly labeled. The ACo and the posterolateral cortical nucleus (PLCo; which now emerged) and the STIA were moderately labeled.

Proceeding caudally (Fig. 7B), labeled fibers continued to innervate pallial structures densely, particularly BLA. Labeling was very pronounced in BLAa and BLAp as well as in the dorsolateral part of La (LaDL). By comparison, the ventrolateral (LaVL) and ventromedial (LaVM) divisions of La were moderately labeled, as were BLAv, BMAP, and PLCo. For subpallial structures, ASt and STIA were moderately labeled, whereas MePD and MePV, all divisions of Ce, and the intercalated nuclei were lightly labeled.

More posteriorly (Fig. 8B), labeling remained dense in the basal complex, heaviest in BLAa and BLAp, slightly less so in LaDL, and strong but somewhat weaker still in BLAv, BMAP, and medial (LaVM) and lateral (LaVL) divisions of La. As with the anterior cortical nuclei, labeling was denser in superficial than deep layers of PLCo. Subpallial structures, including STIA, MePD, and MePV, were lightly to moderately labeled. Similar to the central and medial nuclei (see above), labeling gradual thinned in STIA from rostral to caudal levels.
Figure 14. A: Low-magnification Nissl-stained transverse section through the posterior part of the extended amygdala showing the locations of nuclei at this level. B: Pattern of distribution of 5-HT fibers to nuclei of the extended amygdala at the same level. For abbreviations see list. Scale bar = 250 µm in B; 375 µm for A. [Color figure can be viewed at wileyonlinelibrary.com.]

Figure 15. A: Low-magnification Nissl-stained transverse section through the posterior part of the extended amygdala showing the locations of nuclei at this level. B: Pattern of distribution of 5-HT fibers to nuclei of the extended amygdala at the same level. For abbreviations see list. Scale bar = 250 µm in B; 275 µm for A. [Color figure can be viewed at wileyonlinelibrary.com.]
At the posterior amygdala (Fig. 10B), BLAp was densely (or massively) labeled, clearly differentiating it from surrounding structures. Labeling was also pronounced in other deep pallial structures, including La (LaDL, LaVM, LaVL) and BMAp and within the anterolateral amygdalohippocampal transition area (AHIAL). Of the cortical pallial structures, the postero medial cortical nucleus (PMCo), the PLCo, and the amygdalopiriform transition area (APIp) were moderately to densely labeled. Unlike the laminar differences in labeling of PMCo and PLCo, labeled fibers spread quite uniformly throughout APIp.

At the caudal aspect of the amygdala (Fig. 11B), deep and cortical pallial nuclei contained moderate to dense concentrations of labeled fibers, with labeling heaviest in medial aspects of BLAp and BMAp. Although less pronounced, La was also strongly labeled with a dorsoventral gradient such that LaDL was more heavily labeled than either LaVM or LaVL. The medial (AHiPL) and lateral (AHiPL) nuclei of the amygdalohippocampal transition area were moderately labeled, AHiPM slightly more heavily than AHiPL. PMCo and APIp contained moderate numbers of labeled fibers, with labeling heaviest in layer 1 of PMCo.

**BST**

As indicated (see Introduction), the subpallial EA consists of two components, BST and the sublenticular EA (SLEA). The BST contains three subdivisions, the main one being BST proper. The BST (proper), which at rostral levels straddles the anterior commissure (ac), is composed of several subnuclei, which, as will be described, contain differing concentrations of 5-HT fibers. Figures 12–15 depict the pattern of distribution of 5-HT fibers rostrocaudally throughout BST, and consist of a series of Nissl-stained sections (Figs. 12A–15A) showing the locations of nuclei of BST and corresponding SERT-immunostained sections (Figs. 12B–15B) depicting the patterns of labeling at these levels of BST.

At the rostral pole of BST (Fig. 12B), labeled fibers spread quite homogeneously throughout BST, being distributed moderately to three subnuclei, the anterior medial (STMA), the lateral intermediate (STLI), and the lateral ventral (STLV) nuclei, and sparsely to the lateral dorsal nucleus (STLD). A similar pattern was observed more caudally (Fig. 13B). Specifically, with exception of STLD which, as rostrally, was sparsely labeled, each of the subnuclei surrounding the anterior commissure was moderately labeled. The dorsal subnuclei, including STMA, STLI, and lateral posterior (STLP) nuclei, were slightly more densely labeled than the ventral group, which included the medial ventral nucleus (STMV), fusiform nucleus (Fu), and parastrial nucleus (PS).

The pattern of labeling more caudally in BST (Figs. 14B, 15B) largely mirrored that at rostral levels. As shown (Fig. 14B), the STLD was lightly labeled, whereas neighboring regions, medially and ventrally, were moderately labeled. They included STMA, STLV, STLP, STLI, PS, and Fu. Among these nuclei, labeling was heaviest in Fu and lightest in STMA. At the caudal extent of BST (Fig. 15B), labeling was stronger ventrally/ventrolaterally than dorsomedially. The medial part of the medial posterior nucleus (STMPM) was lightly labeled, whereas adjacent nuclei dorsally and ventrolaterally were moderately labeled. This included the dorsal subgroup (STD), the intermediate (STMPI) and lateral (STMPI) parts of the medial posterior nucleus, the STLP, the lateral intermediate nucleus (STLI), and the bed nucleus of the anterior commissure (BAC).

**DISCUSSION**

The present report describes the pattern of distribution of 5-HT fibers to the amygdala (proper) and to the extended amygdala, or the bed nucleus of the stria terminalis complex.

**Amygdala**

Overall, serotonergic fibers are distributed densely but differentially throughout nuclei of the amygdala. SERT⁺ fibers heavily innervate most of the deep and cortical pallial nuclei of amygdala, which contrasts with minimal innervation of subpallial nuclei. Among the deep pallial structures, the nuclei of the basal complex, consisting of the lateral, basolateral, and basomedial nuclei (and their subregions), contained the densest concentration of 5-HT fibers, particularly posterior aspects of the basolateral complex. In general, cortical pallial structures contained fewer SERT⁺ fibers than the deep pallial nuclei.

Among the subpallial amygdalar nuclei, there is a rostrocaudal gradient, whereby SERT⁺ fibers are distributed more heavily to anterior than to posterior divisions of subpallial structures. 5-HT fibers terminate moderately in the anterior dorsal and anterior ventral nuclei of the anterior amygdala, in the amygdalostriatal transition area, and in the anterodorsal and anteroventral parts of the medial nucleus, lightly in the posterodorsal and posteroverentral sectors of Me and in the intercalated nuclei and generally sparsely in the central nucleus.
BST

The subpallial extended amygdala consists of two components: 1) BST, including the intra-amygdalar (STIA) and BST (proper) divisions, and 2) the sublenticular EA consisting of SI and IPAC (Olucha-Bordonau et al., 2015). The BST proper contained a moderate collection of SERT+ fibers, which on the whole was of lesser magnitude than those in the amygdala. With the exception of a light innervation of lateral dorsal division of BST (STLD) and the medial posterior nucleus (STMPM), 5-HT fibers terminate moderately within structures of BST. These included STMA, STMV, STLI, STLV, STLP, Fu, and PS rostrally and the STD, STMPI, ST MPL, STLI, and the lateral STLP caudally. The anterior pole of IPAC contained moderate numbers of 5-HT fibers, as did the STIA, lying ventral to Ce and coextensive with it.

Comparison with previous examinations of serotonergic innervation of the amygdala

As discussed, no previous report has described the overall pattern of distribution of 5-HT fibers to the amygdala (or to BST) in the rat. Nonetheless, some studies have examined the 5-HT innervation of select nuclei of the amygdala, concentrating on the basal group and the central nucleus (Sur et al., 1996; Commons et al., 2003; Muller et al., 2007; Smith and Porrino, 2008; Bonn et al., 2013). With minor differences among reports, the common finding was that 5-HT fibers distribute densely to the basolateral nucleus, strongly but less heavily to the lateral nucleus, and sparsely to the central nucleus. The 5-HT innervation of the basomedial and medial nuclei was intermediate to that of BLA/La and Ce. The present results are generally consistent with these findings, with the exception that we observed 1) denser labeling of the posterior than the anterior BLA, 2) stronger labeling of the medial than lateral or capsular divisions of Ce, and 3) light labeling of the posterodorsal and posteroverentral divisions of the medial nucleus.

Correspondence between 5-HT innervation of the amygdala/BST and midbrain raphe projections to the amygdala/BST

The serotonergic input to the forebrain originates almost entirely from midbrain raphe nuclei, namely, the dorsal (DR) and median raphe (MR) nuclei and the B9 group (Vertes and Martin, 1988; Vertes, 1991; Vertes and Crane, 1997; Vertes et al., 1999; Morin and Meyer-Bernstein, 1999; Vertes and Linley, 2007, 2008; Muzerelle et al., 2015). Early reports on the rat (Vertes, 1991; Vertes et al., 1999) and hamster (Morin and Meyer-Bernstein, 1999), using anterograde tracers, described pronounced DR projections (mainly from the rostral DR) to the amygdala, with minor projections from MR. In accordance with the present findings, the DR was shown to target strongly the lateral, basolateral, and cortical nuclei of amygdala and lightly the medial nucleus (Vertes, 1991; Morin and Meyer-Bernstein, 1999; Muller et al., 2007; Bonn et al., 2013). Unlike the present demonstration of sparse 5-HT labeling of the central nucleus, moderate DR-Ce projections were described by Bienkowski and Rinaman (2013). Some of the DR input to Ce, however, undoubtedly originated from nonserotonergic DR neurons.

Similar to the amygdala (proper), midbrain raphe projections to BST arise predominantly from DR (or the rostral DR) and minimally from MR (Vertes, 1991; Vertes et al., 1999; Morin and Meyer-Bernstein, 1999; Vertes and Linley, 2008; Muzerelle et al., 2015). Consistent with present findings, DR fibers were shown to distribute moderately, and quite uniformly, throughout BST, with a preference for the anterior ventrolateral sector of BST, which includes the fusiform, parastrial, and medial and lateral ventral nuclei of BST (Vertes, 1991; Morin and Meyer-Bernstein, 1999; Vertes and Linley, 2008; Bienkowski and Rinaman, 2013; Muzerelle et al., 2015).

Functional role of serotonergic input to the amygdala, with a focus on the basolateral nucleus

As discussed above, both the amygdala and the serotonergic system serve direct, and likely complementary, roles in emotional behavior, prominently including fear and anxiety (Davis, 2000). For instance, selective serotonin reuptake inhibitors (SSRIs) have been described as the “gold standard” in the treatment of anxiety disorders (Inoue et al., 2011; Burghardt and Bauer, 2013), possibly in large part involving their actions on the amygdala.

The detailed circuitry of the amygdala responsible for the acquisition and expression of fear/anxiety has been well described (see Duvarci and Pare, 2014; Herry and Johansen, 2014; Tovote et al. 2015). The primary cell groups of the amygdala involved in fear/anxiety are the basal group (La, BLA and BMA), the central nucleus (or CeL and CeM), and the intercalated nuclei. For the most part, information flows unidirectionally from La to BLA to CeL and then to CeM, such that La is the principal afferent node and CeM the output site in fear/anxiety. In general, associations between neutral sensory stimuli and aversive events (tone–foot shock pairings) are made in La, transferred with modifications through
BLA/BMA and the intercalated nuclei to CeL and then to CeM to affect behavior. Accordingly, the BLA (and BMA) is pivotal positioned to modify information transferred from La to CeM. This coupled with the present demonstration that the BLA receives a dense 5-HT input suggests that BLA may be a primary (amygdalar) target for 5-HT’s actions on fear/anxiety (Amano et al., 2011). Consistent with this, serotonin has been shown to exert significant modulatory effects at the BLA in anxiety-like behaviors (Asan et al., 2013; Burghardt and Bauer, 2013; Bauer, 2015).

Anxiety-producing stimuli/conditions release serotonin to the amygdala/BLA (Hale et al., 2012; Fox and Lowry, 2013), which is initially (or acutely) anxiogenic but with time (or chronically) becomes anxiolytic (Burghardt et al., 2004; Burghardt and Bauer, 2013). In a parallel manner, various SSRIs initially elicit fear/anxiety-like behaviors (Burghardt et al., 2004, 2007; Grillon et al., 2007; Ravinder et al., 2011) but with long-term use become anxiolytic (Zhang et al., 2000; Li et al., 2001; Burghardt et al., 2004; Hashimoto et al., 2009; Deschaux et al., 2011; Inoue et al., 2011).

Although the effects of SSRIs on fear/anxiety are not limited to the amygdala or BLA, the BLA appears to be a principal site for the early anxiogenic and later anxiolytic effects of SSRIs (Inoue et al., 2004, 2011; Kitaichi et al., 2014). In addition, the acute anxiogenic actions of SSRIs reportedly involve 5-HT2C receptors (mainly of BLA), whereas the chronic anxiolytic effects involve 5-HT1A receptors of BLA (Burghardt et al., 2007; Christianson et al., 2010; Vicente and Zangrossi, 2012, 2014; de Andrade Strauss et al., 2013). For instance, with regard to 5-HT2C receptors, Vicente and Zangrossi (2012) showed that 1) intra-BLA injections of the 5-HT2C receptor agonist MK-212 and the 5-HT2C receptor antagonist SB-242084 enhanced or suppressed anxiety-like behaviors, respectively, on the elevated T-maze, and 2) SB-242084 blocked the (acute) anxiogenic effect elicited by the systemic administration of the antidepressants imipramine or fluoxetine. Vicente and Zangrossi (2014) subsequently confirmed that MK-212 injected into BLA was anxiogenic and further showed that anxiogenic actions were attenuated with chronic treatment with imipramine or fluoxetine. Taken together the findings support the view that the acute anxiogenic, as well as the chronic anxiolytic, effects of antidepressants are in part mediated through 5-HT2C receptors of BLA. Specifically, a 5-HT2C-mediated activation of BLA neurons is anxiogenic, whereas the gradual (chronic) desensitization of 5-HT2C receptors of these cells contributes to anxiolysis.

By contrast with the anxiogenic effects of 5-HT2C receptors, several studies have shown that 5-HT1A receptors produce anxiolytic actions at the BLA (Zangrossi et al., 1999; Li et al., 2012; de Andrade Strauss et al., 2013; Vicente and Zangrossi, 2014). For example, de Andrade Strauss et al. (2013) showed that BLA injections of the 5HT1A receptor agonist 8-OH-DPAT suppressed anxiety-like behavior on three tests of anxiety, with effects blocked by the prior administration of the 5-HT1A antagonist WAY-100635. Moreover, Vicente and Zangrossi (2014) reported that the anxiogenic actions produced by intra-BLA injections of the 5-HT2C receptor agonist MK-212 were blocked with chronic antidepressant administration but were reinstated with intra-BLA injections of the 5-HT1A antagonist WAY-100635. Accordingly, the authors (Vicente and Zangrossi, 2014) concluded that “both a reduction in 5-HT2C-R and a facilitation of 5-HT1A-R-mediated neurotransmission in the BLA are involved in the anxiolytic effect of antidepressant drugs.” Consistently with this, Li et al. (2012), using recombinant adenovirus-induced alterations in 5-HT receptor expression in the amygdala, demonstrated that decreases in 5-HT1A, or increases in the 5-HT2C, receptor expression produced anxiogenic actions on two tests of anxiety-like behavior in mice.

Although the precise mechanism(s) whereby 5-HT1A and 5-HT2C receptors of BLA cells affect anxiety-like states remains to be fully determined, the process understandably involves interneurons and principal cells. With some exceptions (see below) increases in BLA pyramidal cell activity appear to be anxiogenic, decreases anxiolytic. With respect to 5-HT1A receptors, an early report in anesthetized rats (Stein et al., 2000) showed that the 5-HT1A agonist 8-OH-DPAT suppressed the activity of most pyramidal cells (PCs) of BLA. In accordance with this, Cheng et al. (1998) showed that serotonin, acting via 5-HT1A receptors, suppressed the activity of principal BLA cells and reduced a depolarization-evoked influx of calcium into these cells.

Whereas the effects of serotonin on 5-HT1A-R-containing cells of BLA are fairly straightforward, specific 5-HT actions on 5-HT2C-R cells of BLA are not fully resolved. For instance, Rainnie (1999) demonstrated in a slice preparation that the application of 5-HT or 5-HT2 agonists to BLA activated GABAergic interneurons, with consequent inhibition of pyramidal cells. Although this finding appears inconsistent with a proposed role for 5-HT2C receptors in anxiogenesis (i.e., the activation, not the suppression, of principal BLA activity is thought to be anxiogenic), it should be noted that 1) a nonspecific 5-HT2 agonist was used (α-methyl-5-HT); results may differ with a more specific 5-HT2C agonist; and 2) high concentrations or the prolonged application of 5-HT suppressed the excitatory action of 5-HT on BLA interneurons, and hence their inhibitory effect on PCs.
Furthermore, in contrast to these results, Stein et al. (2000) showed, in intact rats, that the 5-HT$_{2C}$ agonist DOI increased the activity of virtually all principal BLA neurons, whereas Reznikov et al. (2008) reported that restraint stress produced elevated levels of c-fos expression in the vast majority of PCs of BLA. Taken together, these findings would indicate that 5-HT$_{2/2C}$ receptor cell activation excites both interneurons and principal cells of BLA, but, as noted by Campbell and Merchant (2003), the likely net effect would be an “augmentation of BLA output, which could underlie the anxiogenic responses of 5-HT$_{2C}$ agonists at behaviorally relevant doses.”

Whereas a reduction of principal cell activity of BLA is generally thought to contribute to anxiolysis, Tye et al. (2011) recently identified a subset of BLA cells whose activation suppressed anxiety-like behavior in mice. Specifically, using optogenetic techniques, they demonstrated that stimulation of a select population of BLA cells projecting to CeL produced anxiolytic actions, presumably via a BLA-induced activation of GABAergic CeL cells and the consequent inhibition of CeM output neurons. Interestingly, however, the overall (or nonspecific) activation of BLA neurons was found to be anxiogenic, which is consistent with previously discussed reports (Vicente and Zangrossi, 2012; Zangrossi and Graeff, 2014).

Finally, given the pronounced 5-HT input to the basal nuclei of the amygdala, we focused on the role of 5-HT afferents to the BLA complex in fear/anxiety, but it is certainly well recognized that the amygdala participates in a wide range of emotional behaviors, many of which have been linked to particular subnuclei. For instance, Ferguson et al. (2001) showed that oxytocin acts on the medial nucleus of the amygdala to facilitate social recognition in mice. Possibly directly related to this, it has recently been found that SSRIs taken during the second or third trimester, for the treatment for depression, increase the risk for autism-spectrum disorders (Boukhris et al., 2015). Although we showed that 5-HT fibers distribute moderately to the medial nucleus, it is nevertheless possible that an SSRI-induced developmental disruption (or reorganization) of serotonergic projections to the medial nucleus could contribute to the social deficits associated with autism-spectrum disorders.

**Functional role of serotonergic input to the BST**

Similar to the amygdala proper, the BST also plays a prominent role in affective behaviors, prominently stress and anxiety (Davis et al., 2010). In general, serotonin exerts anxiolytic effects at the BST (Levita et al., 2004; Gomes et al., 2011, 2012). This partially involves a 5-HT-mediated suppression of excitatory glutamatergic and corticotrophin-releasing factor (CRF) inputs to the BST as well as intrinsic CRF-mediated excitation at BST. For instance, CRF infused into the anterolateral BST elicits anxiety-like behaviors, including increased startle and reduced exploratory behavior in the elevated plus maze (Lee and Davis, 1997; Sahuque et al., 2006).

Rainnie and colleagues (Hammack et al. 2009; Daniel and Rainnie, 2015) recently proposed a model for the interactive roles of the DR and BST in stress/anxiety. Specifically, stressors produce an increase in the release of CRF to both the DR and the BST that initially is anxiogenic but with time becomes anxiolytic. In effect, at low doses, CRF binds to high-affinity CRF$_1$ receptors of the DR to inhibit the firing of 5-HT DR cells, whereas, at higher doses, CRF also binds to CRF$_2$ receptors to increase the discharge of DR neurons and thereby releases 5-HT to the BST in the suppression of anxiety (Hammack et al., 2003).

Three types of neurons have been identified in the lateral BST of rodents, type I–III cells. Type I and III neurons discharge at regular rates, with the threshold to activation higher for type III than for type I cells, whereas type II cells display a bursting pattern of discharge that has been associated with the release of various peptides from the cells (Egli and Winder, 2003; Hammack et al., 2007). Type I neurons constitute 29%, type II neurons 55%, and type III neurons 16% of the population of lateral BST neurons (Hammack et al., 2007). The BST contains a rich array of 5-HT receptors that differentially populate the three cell types and thus determine their roles in stress/anxiety (Daniel and Rainnie, 2015). The main 5-HT receptor types are 5-HT$_{1A}$, 5-HT$_{1B}$, 5-HT$_{2C}$, and 5-HT$_{7}$ receptors and, of those most abundantly 5-HT$_{1A}$ receptors. The 5-HT$_{1A}$ receptor is expressed on all three types of BST cells (Hazra et al., 2012). Because 5-HT$_{1A}$ receptor activation hyperpolarizes neurons, the predominant (or net) effect of 5-HT applied to BST is a hyperpolarization of BST neurons (Guo et al., 2009; Hazra et al., 2012) and anxiolytic actions at BST (Levita et al., 2004; Gomes et al., 2011, 2012). In contrast to 5-HT$_{1A}$ receptors, 5-HT depolarizes 5-HT$_{2C}$ and 5-HT$_{7}$ receptor-containing neurons of the BST. 5-HT$_{2C}$ receptors are located almost entirely on type III cells, which are mainly CRF neurons and reportedly form a feed-forward loop with the DR in the elicitation of anxiety-like behaviors. Specifically, a DR release of 5-HT to the BST activates type III cells, which in turn releases CRF to the DR, drives DR neurons, and thus maintains the cycle. However, the release of 5-HT to BST also activates 5-HT$_{7}$-receptor-containing BST neurons.
neurons, present on type I and type II neurons, which suppress the activity of type III CRF neurons to dampen anxiety-like states. In sum, the actions of 5-HT at the BST are complex, with ultimate effects on stress/anxiety dependent on the relative recruitment of various types of 5-HT receptors, favoring 5-HT_{1A} receptors and anxiolysis.

**CONFLICT OF INTEREST STATEMENT**

The authors declare no conflicts of interest.

**ROLE OF AUTHORS**

SBL collected the data; SBL and FO-B played major roles in the preparation of the figures; RPV played a major role in writing the manuscript. All authors, however, were involved in all phases of the planning and execution of the research.

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