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NORADRENERGIC INNERVATION OF PYRAMIDAL CELLS IN THE RAT BASOLATERAL AMYGDALA

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Abstract—The basolateral nuclear complex of the amygdala (BLC) receives dense noradrenergic/norepinephrine (NE) inputs from the locus coeruleus that play a key role in modulating emotional memory consolidation. Knowledge of the extent of synapse formation by NE inputs to the BLC, as well as the cell types innervated, would contribute to an understanding of how NE modulates the activity of the BLC. To gain a better understanding of NE circuits in the BLC, dual-label immunohistochemistry was used at the light and electron microscopic levels in the present study to analyze NE axons and their innervation of pyramidal cells in the anterior subdivision of the basolateral amygdalar nucleus (BLa). NE axons and BLa pyramidal cells were labeled using antibodies to the norepinephrine transporter (NET) and Ca^{2+}/calmodulin-dependent protein kinase (CaMK), respectively. Dual localization studies using antibodies to NET and dopamine-beta-hydroxylase (DBH) revealed that virtually all NE axons and varicosities expressed both proteins. The BLa exhibited a medium density of NET+ fibers. Ultrastructural analysis of serial section reconstructions of NET+ axons revealed that only about half of NET+ terminals formed synapses. The main postsynaptic targets were small-caliber CAMK+ dendritic shafts and spines of pyramidal cells. A smaller number of NET+ terminals formed synapses with unlabeled cell bodies and dendrites. These findings indicate that the distal dendritic domain of BLa pyramidal cells is the major target of NE terminals in the BLa, and the relatively low synaptic incidence suggests that diffusion from nonsynaptic terminals may be important for noradrenergic modulation of the BLa. **© 2012 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: electron microscopy, immunohistochemistry, norepinephrine transporter, Ca^{2+}/calmodulin-dependent protein kinase II, norepinephrine, dopamine-beta-hydroxylase.

INTRODUCTION

The amygdala is critical for emotional memory formation and the generation of appropriate behavioral responses to salient sensory stimuli and emotionally arousing events in the external world (Millan, 2003; Sah et al., 2003; McGaugh, 2004). As a heterogeneous structure, the amygdala is composed of several nuclei and has connections with numerous brain areas. The basolateral amygdalar nuclear complex (BLC) receives a dense noradrenergic input primarily originating from the locus coeruleus (LC) (Asan, 1998), which is heavily involved in stress and stress-related pathologies (Sved et al., 2002). Studies have shown that stressful stimuli such as foot shock induce norepinephrine (NE) release in the rat amygdala (Galvez et al., 1996; Quirarte et al., 1998). Also, human functional magnetic resonance imaging (fMRI) studies show that elevated NE neurotransmission enhances BLC responses to fear signals (Onur et al., 2009). Moreover, a large amount of evidence indicates that the NE system in the BLC is involved in memory modulation by stress (Cahill et al., 1995; Ferry and McGaugh, 1999, 2008; McIntyre et al., 2002; McGaugh, 2004; Roozendaal et al., 2009). Therefore, the LC is important for providing information about aversive stimuli to the BLC and generating appropriate responses to stressors, which suggests that the LC–NE circuit in the BLC could be a potential drug target for anxiety disorders.

Although there is abundant behavioral and clinical evidence for the role of NE system of the BLC in stress and anxiety, there is no clear picture about how NE affects the activity of BLC neurons. Previous studies have shown that there are two major cell classes in the BLC, pyramidal neurons and nonpyramidal neurons. Although these cells do not exhibit a laminar or columnar organization, their morphology, synaptology, electrophysiology, and pharmacology are remarkably similar to their counterparts in the cerebral cortex (McDonald, 1982, 1992a,b; Carlsen and Heimer, 1988; Washburn and Moises, 1992; Rainnie et al., 1993; Sah et al., 2003; Muller et al., 2005, 2006, 2007). Thus, pyramidal neurons in the BLC are projection neurons with spiny dendrites that utilize glutamate as an excitatory neurotransmitter, whereas most nonpyramidal neurons are spine-sparse interneurons that utilize GABA as an inhibitory neurotransmitter. Previous studies indicated that BLC neurons can be inhibited or excited by NE afferents from the LC, but in many cases it was difficult to determine whether the recorded neurons

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Abbreviations: BLa, anterior basolateral amygdalar nucleus; BLC, basolateral amygdalar nuclear complex; CaMK, Ca^{2+}/calmodulin-dependent protein kinase II; DBH, dopamine-beta-hydroxylase; LC, locus coeruleus; LTP, long-term potentiation; NE, noradrenergic/norepinephrine; NET, norepinephrine transporter; NMDARs, N-methyl-D-aspartate receptors; PB, phosphate buffer; PBS, phosphate buffered saline.

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were pyramidal cells or interneurons based on their electrophysiological properties (Buffalari and Grace, 2007, 2009; Chen and Sara, 2007). In a recent investigation in which GABAergic interneurons were identified in brain slices of genetically modified mice, it was found that some interneurons were excited by NE, but others exhibited little or no response (Kaneko et al., 2008). Thus, there is still considerable confusion regarding the differential modulation of distinct neuronal subpopulations in the BLC by NE.

Anatomical knowledge of NE circuits in the BLC should help to clarify the uncertainties of these physiological studies. A previous electron microscopic study using dopamine-beta-hydroxylase (DBH) as a marker for NE axons suggested that only 11% of DBH+ terminals in BLC form synapses (Asan, 1998). This suggests that the NE innervation of the BLC may be mainly mediated by non-junctional, diffuse release of NE into the extracellular space (i.e., ‘‘volume transmission’’). However, a later study indicated that 31% of DBH+ terminals in the lateral amygdalar nucleus formed synaptic junctions (Farb et al., 2010). The discrepancies in the two studies may be the result of different criteria for synapse identification, the nucleus studied, or differences in tissue fixation that could affect synaptic morphology. Because these studies analyzed single thin sections, rather than serial section reconstruction of synapses, it seems likely that the synaptic incidence may actually be higher than 31%. This is an important issue to resolve for the BLC since ‘‘wired’’ synaptic transmission and volume transmission have distinct functional correlates in terms of specificity and spatiotemporal aspects of neurotransmission (Agnati et al., 1995).

There is also confusion regarding the postsynaptic targets of NE axon terminals in the BLC. Single-label electron microscopic studies found that the main postsynaptic targets of DBH+ terminals were dendritic shafts and spines, but the cell types of origin of these structures could not be determined (Asan, 1998; Farb et al., 2010). Double-labeling studies using GABA as a marker for interneurons suggested that only about 4% of DBH+ terminals formed synapses with interneurons in the BLC (Li et al., 2002). However, another double-labeling study of the BLC reported that about 30% of DBH+ terminals contacted interneurons that exhibited immunoreactivity for choline acetyltransferase (Li et al., 2001). Thus, it is still not clear to what extent NE terminals synapse with pyramidal projection neurons versus interneurons in the nuclei of the BLC.

The purpose of the present study was to determine the synaptic incidence and postsynaptic targets of NE axon terminals in the anterior subdivision of the basolateral amygdalar nucleus (BLa), the main nucleus responsible for NE-mediated memory consolidation (McGaugh, 2004). An antibody to the norepinephrine transporter protein (NET) was used to label NE axons since a previous study of this nucleus found that visualization of DBH immunoreactivity at the ultrastructural level required immunohistochemical procedures that hindered morphological preservation of neuronal processes and synapses (Asan, 1998). This is the first electron microscopic study of the amygdala to use NET as a marker for noradrenergic axons. An antibody to the alpha subunit of calcium/calmodulin-dependent protein kinase II (CaMK) was used to selectively label pyramidal cells (McDonald et al., 2002) in these dual-labeling studies. To obtain a more precise estimation of the synaptic incidence of the NE innervations of the BLa, a serial section analysis of NET+ terminals and their contacts was performed.

EXPERIMENTAL PROCEDURES

Tissue preparation

Male Sprague–Dawley rats weighing 250–350 g were used in these experiments. Rats were handled in accordance with the principles of laboratory animal care and protocols approved by the University of South Carolina Institutional Animal Care and Use Committee. Brain tissue was prepared as described in previous studies of our lab (Muller et al., 2011). For light microscopy, rats were anesthetized with chloral hydrate (350 mg/kg) and perfused intracardially with phosphate-buffered saline (PBS; pH 7.4) containing 1.0% sodium nitrite (50 ml) followed by 4% paraformaldehyde in phosphate buffer (PB; pH 7.4) for 20 min. Then brains were removed and postfixed in the perfusate for three hours. For electron microscopy, rats were anesthetized and perfused intracardially with PBS containing 0.5% sodium nitrite (50 ml) followed by 2% paraformaldehyde-3.75% acrolein in PB for 1 min, followed by 2% paraformaldehyde in PB for 30 min. Brains were removed, postfixed in 2% paraformaldehyde for 1 h, and sectioned on a vibratome in the coronal plane at 50 μm.

Light microscopic immunocytochemistry

Single-label localization of NET+ fibers was performed in two rats using a rabbit antibody to NET (1:2000, antibody 43411, obtained from Dr. Randy D. Blakely, Vanderbilt University School of Medicine). All antibodies were diluted in PBS containing 0.4% Triton X-100 and 1% normal goat serum. Sections were incubated in the primary antibody overnight at 4 °C and then processed for the avidin–biotin immunoperoxidase technique using a biotinylated goat anti-rabbit secondary antibody (1:500, Jackson Immunoresearch Laboratories, West Grove, PA) and a Vectastain standard ABC kit (Vector laboratories, Burlingame, CA) with nickel-intensified 3,3′-diaminobenzidine 4HCl (DAB, Sigma Chemical Co., St. Louis, MO, USA) as a chromogen to generate a black reaction product (Hancock, 1986). After the reactions, sections were mounted on gelatinized slides, dried overnight, dehydrated in ethanol, cleared in xylene, and coverslipped in Permount (Fisher Scientific, Pittsburgh, PA, USA). Sections were analyzed with a Nikon E600 microscopy system and digital light micrographs were taken with a Micropublisher digital camera.

Confocal microscopic fluorescence immunocytochemistry

Dual localization studies were performed in two rats using the rabbit NET antibody (1:1000; see above) and a mouse monoclonal antibody to dopamine-beta-hydroxylase (DBH; 1:600; catalog #MAB308, EMD Millipore, Billerica, MA), the synthetic enzyme for norepinephrine, to determine the overlapping extent of NET immunoreactivity and DBH

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