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POSTSYNAPTIC TARGETS OF GABAERGIC BASAL FOREBRAIN PROJECTIONS TO THE BASOLATERAL AMYGDALA

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Abstract—Recent studies indicate that the basolateral amygdala, like the neocortex and hippocampus, receives GABAergic inputs from the basal forebrain in addition to the well-established cholinergic inputs. Since the neuronal targets of these inputs have yet to be determined, it is difficult to predict the functional significance of this innervation. The present study addressed this question in the rat by employing anterograde tract tracing combined with immunohistochemistry at the light and electron microscopic levels of analysis. Amygdalopetal axons from the basal forebrain mainly targeted the basolateral nucleus (BL) of the amygdala. The morphology of these axons was heterogeneous and included GABAergic axons that contained vesicular GABA transporter protein (VGAT). These axons, designated type 1, exhibited distinctive large axonal varicosities that were typically clustered along the length of the axon. Type 1 axons formed multiple contacts with the cell bodies and dendrites of parvalbumin-containing (PV+) interneurons, but relatively few contacts with calretinin-containing and somatostatin-containing interneurons. At the ultrastructural level of analysis, the large terminals of type 1 axons exhibited numerous mitochondria and were densely packed with synaptic vesicles. Individual terminals formed broad symmetrical synapses with BL PV+ interneurons, and often formed additional symmetrical synapses with BL pyramidal cells. Some solitary type 1 terminals formed symmetrical synapses solely with BL pyramidal cells. These results suggest that GABAergic neurons of the basal forebrain provide indirect disinhibition, as well as direct inhibition, of BL pyramidal neurons. The possible involvement of these circuits in rhythmic oscillations related to emotional learning, attention, and arousal is discussed.

Key words: substantia innominata, anterograde tract tracing, immunohistochemistry, electron microscopy, disinhibition, vesicular GABA transporter.

The basal forebrain (BF) contains a diffuse array of cholinergic and noncholinergic neurons that extend through a continuous region which includes the medial septal area, diagonal band of Broca, ventral pallidum, and substantia innominata (SI) (Mesulam et al., 1983; Woolf, 1991; Gritti et al., 1997, 2003). This complex has topographically organized connections with different forebrain regions including the hippocampus, neocortex, and basolateral amygdala (Mesulam et al., 1983; Záborszky et al., 1999). The BF is important for attention and learning, and has been implicated in several neurological diseases, including Alzheimer's disease (Everitt and Robbins, 1997).

One important subtype of noncholinergic BF neuron that has been extensively studied is the GABAergic corticopetal neuronal population (Freund and Buzsáki, 1996; Semba, 2000; Sarter and Bruno, 2002). These BF cells, which can be identified using either GABAergic markers or antibodies to the calcium-binding protein parvalbumin (Freund, 1989; Gritti et al., 2003), innervate primarily interneurons in both the hippocampus and neocortex (Freund and Gulyás, 1991; Freund and Meskenaite, 1992; Freund and Buzsáki, 1996). The axons of BF GABAergic corticopetal neurons (type 1 axons) have a very distinctive morphology. In contrast to the cholinergic axons (type 2 axons), which have small axon terminals, type 1 axons have very large, clustered terminals that form multiple synapses with cortical interneurons. Via this innervation BF GABAergic neurons indirectly disinhibit assemblies of cortical pyramidal cells and act together with cholinergic BF neurons to generate rhythmic oscillatory activity (Freund and Buzsáki, 1996; Tóth et al., 1997; Buzsáki, 2002; Borhegyi et al., 2004).

A recent study utilizing retrograde tract tracing combined with immunohistochemistry demonstrated that the basolateral amygdala, like cortical regions, receives projections from parvalbumin-positive GABAergic neurons in the BF that are mainly located in the ventral pallidum and SI (Mascagni and McDonald, 2009). However, since the neuronal targets of these inputs have yet to be determined, it is difficult to predict the functional significance of this innervation. The present study addressed this question by employing anterograde tract tracing combined with double- and triple-labeling immunohistochemistry at the light and electron microscopic levels of analysis. These studies indicate that the BF GABAergic inputs to the amygdala provide a particularly robust innervation of parvalbumin interneurons in the basolateral nucleus, but a much weaker innervation of other interneuronal subpopulations. Unlike the innervation of the hippocampus by BF GABAergic neurons, there was also a moderate innervation of pyramidal projection neurons in the basolateral amygdala. Surprisingly, many of the inputs to pyramidal cells were made

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Abbreviations: BDA, biotinylated dextran amine; BF, basal forebrain; BFGN, basal forebrain GABAergic neuron; BL, basolateral nucleus; BLa, anterior subdivision of the basolateral nucleus; BLp, posterior subdivision of the basolateral nucleus; CB, calbindin; CR, calretinin; DAB, 3,3'-diaminobenzidine hydrochloride; PB, phosphate buffer; PBS, phosphate buffered saline; PC, pyramidal cell; PHA-L, *Phaseolus vulgaris* leucoagglutinin; PUR, very intense purple (VIP) reaction product; PV, parvalbumin; SI, substantia innominata; SOM, somatostatin; VGAT, vesicular GABA transporter; VP, ventral pallidum.

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by the same axon terminals that innervated parvalbumin interneurons.

EXPERIMENTAL PROCEDURES

Injections of anterograde tracers and tissue preparation

Adult male Sprague-Dawley rats (250–350 g; Harlan, Indianapolis, IN, USA) were anesthetized with sodium pentobarbital (50 mg/kg) and placed in a stereotaxic head holder (Stoelting, Wood Dale, IL, USA) for injections of either biotinylated dextran amine (BDA; mol.wt. 10,000; Invitrogen, Eugene, OR, USA) or *Phaseolus vulgaris* leucoagglutinin (PHA-L; Vector Laboratories Inc., Burlingame, CA, USA) into the SI and/or ventral pallidum (VP) of the BF. Injection coordinates were obtained from an atlas of the rat brain (Paxinos and Watson, 1997). Unilateral or bilateral iontophoretic injections of BDA (4–5% in 0.01 M phosphate buffer [PB], pH 7.3) or PHA-L (2.5% in 0.01 M PB, pH 7.8) were made via glass micropipettes (50 μm inner tip diameter) using a Midgard high voltage current source set at 5.0 μA (7 s on, 7 s off, for 20–30 min for each injection). Injections were made using either one pipette aimed at the center of the BF, or two pipettes at different mediolateral coordinates. Two injections were made along each pipette track at different dorsoventral coordinates separated by 0.5–1.0 mm. Micropipettes were left in place for 5 min following the last injection along each pipette track to prevent the tracers from flowing up the track and involving more dorsally located structures.

After a 5-day survival for BDA injections, or a 10–14-day survival for PHA-L injections, rats for light or confocal microscopy were perfused intracardially with phosphate buffered saline (PBS; pH, 7.4) containing 0.5% sodium nitrite (50 ml), followed by 4.0% paraformaldehyde in PBS for 20–30 min. Following perfusion, these brains were removed and postfixed for 3.5 h in 4.0% paraformaldehyde. Rats to be used for electron microscopy were perfused intracardially with PBS containing 0.5% sodium nitrite (50 ml), followed by an acrolein/paraformaldehyde mixture (2.0% paraformaldehyde-3.75% acrolein in PB for 1 min, followed by 2.0% paraformaldehyde in PB for 30 min). Following removal, acrolein-fixed brains were postfixed in 2.0% paraformaldehyde for 1 h. All brains were sectioned on a vibratome in the coronal plane at either 50 μm (for light microscopy) or 60 μm (for electron microscopy). Sections from acrolein-fixed brains were rinsed in 1.0% borohydride in PB for 30 min and then rinsed thoroughly in several changes of PB for 1 h. All sections were processed for immunohistochemistry in the wells of tissue culture plates.

Light microscopic dual-labeling immunoperoxidase preparations

A sequential two-color immunoperoxidase technique was used in nine rats (four injected with BDA and five injected with PHA-L) to analyze possible contacts of BF afferents with different interneuronal subpopulations in the basolateral amygdala. Previous studies have shown that there are two major cell classes in the basolateral amygdala, 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, 1984, 1992; Carlsen and Heimer, 1988; Washburn and Moises, 1992; Rainnie et al., 1993; Paré et al., 2003; Sah et al., 2003; Muller et al., 2005, 2006, 2007). Thus, pyramidal neurons in the basolateral amygdala 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. Recent dual-labeling immunohistochemical studies suggest that the basolateral amygdala, like the neocortex (Kubota et al., 1994; Kubota and Kawaguchi, 1997), contains at least four distinct subpopulations of GABAergic interneurons that can be distinguished on the basis of their content of calcium-binding proteins and peptides (Kemppainen and Pitkänen, 2000; McDonald and Betette, 2001; McDonald and Mascagni, 2001, 2002; Mascagni and McDonald, 2003). The innervation of the three main interneuronal subpopulations (parvalbumin (PV+), somatostatin (SOM+), and calretinin (CR+) neurons) by BF axons was investigated in the present study.

Sections through the injection sites and amygdala in BDA injected rats were incubated in 0.5% Triton X-100 in PBS for 3 h, and ABC reagent in PBS (Vector Laboratories Inc.) for 16 h at 4 °C. Nickel-enhanced DAB (3,3′-diaminobenzidine hydrochloride; Sigma Chemical Co., St. Louis, MO, USA) was then used as a chromogen to generate a black reaction product (Hancock, 1986). Three series of sections through the amygdala were subsequently rinsed in PBS and incubated overnight at 4 °C in one of the following three primary antibodies for distinct interneuronal subpopulations (McDonald and Mascagni, 2001, 2002; Mascagni and McDonald, 2003): (1) mouse anti-parvalbumin (1:2000; Sigma Chemical Co.), (2) mouse anti-calretinin (1:4000; Millipore, Billerica, MA, USA), or (3) mouse anti-somatostatin (1:1000, SOMA-8 monoclonal antibody obtained from Alison Buchan, University of British Columbia). All antibodies were diluted in 1% normal goat serum in PBS with 0.5% Triton X-100. Sections were then incubated in goat anti-mouse IgG (1:100; Sternberger Monoclonals, Inc., Lutherville, MD, USA) for 1 h, rinsed three times (10 min each) in PBS, followed by mouse peroxidase-antiperoxidase (PAP) complex (1:100; Sternberger Monoclonals, Inc.) for 1 h, and then reacted with nonintensified DAB to produce a brown reaction product.

Sections through the injection sites and amygdala in PHA-L injected rats were incubated in a rabbit anti-PHA-L antibody (1:1000; Vector Laboratories Inc.) for 16 h at 4 °C and then processed for the avidin-biotin immunoperoxidase technique using a rabbit Vectastain ABC kit (Vector Laboratories Inc.). Nickel-enhanced DAB was used as a chromogen to generate a black reaction product. Three series of sections through the amygdala were subsequently rinsed in PBS and incubated overnight at 4 °C in one of the three primary antibodies for different interneuronal subpopulations (see above). After rinsing, sections were incubated in an ABC blocking solution (avidin/biotin blocking kit, Vector Laboratories, Inc.), and rinsed three times (10 min each), before being incubated in biotinylated goat anti-mouse secondary antibody (1:500; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) for 1 h. All sections were then processed for the avidin-biotin immunoperoxidase technique using a Vectastain ABC reagent (Vector Laboratories Inc.), and then reacted with nonintensified DAB to produce a brown reaction product.

In both BDA and PHA-L injected brains, sections through the amygdala were mounted on gelatinized slides, dried overnight, dehydrated in alcohols, cleared in xylene, and coverslipped with Permount (Fisher Scientific, Fair Lawn, NJ, USA). Sections through injection sites were counterstained with Cresyl Violet or Pyronin Y (a pink Nissl stain) before coverslipping. Injection sites were mapped using an Olympus BX51 microscope under bright-field illumination. Basal forebrain areas were identified using an atlas of the rat brain (Paxinos and Watson, 1997). The effective injection site (the area from which tracer is incorporated into neurons for anterograde transport) was defined as the area that contained BDA or PHA-L labeled perikarya (Gerfen et al., 1989).

Triple-labeling immunofluorescence preparations

Light and electron microscopy of immunoperoxidase preparations revealed that BF afferents to the basolateral amygdala contacted mainly PV+ interneurons, and to a lesser extent pyramidal cells. A triple-labeling immunofluorescence technique was used in four