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A GABAergic projection from the central nucleus of the amygdala to the parabrachial nucleus: an ultrastructural study of anterograde tracing in combination with post-embedding immunocytochemistry in the rat

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Abstract

To determine whether axonal terminals emanating from the central nucleus of amygdala (Ce) to the parabrachial nucleus (PBN) contain gamma-aminobutyric acid (GABA) as their neurotransmitter, an electron microscopic study was performed employing the combined techniques of WGA-HRP anterograde tracing and post-embedding immunocytochemistry for GABA. Our analysis distinguished a large population of GABA immunopositive axonal terminals from the Ce that exhibited symmetrical synaptic contacts with neurons in the lateral parabrachial nucleus. Additionally, most retrogradely labeled dendrites and perikarya received synaptic contacts from GABA immunoreactive terminals, with some of them originating from the Ce. The present study provides the first direct ultrastructural evidence for a monosynaptic, GABAergic link between Ce axons and neurons of the parabrachial nucleus via classical symmetrical synapses.

Keywords: Central nucleus of amygdala; Parabrachial nucleus; Gamma-aminobutyric acid (GABA); Immunocytochemistry; Electron microscopy; Rat

Neuroanatomical and electrophysiological studies have demonstrated major synaptic connections between the central nucleus of the amygdala (Ce) and the brainstem visceral and autonomic centers [1–8,12,14,15]. In particular, the projections from the Ce to parabrachial nucleus (PBN), the nucleus of the solitary tract and the ventrolateral medulla have been implicated in the autonomic expression of responses to fear-inducing stimuli and epilepsy [9,13]. Recent quantitative studies have demonstrated that the Ce sends mostly GABAergic projections to the nucleus of the solitary tract and the ventrolateral medulla [3,14]. Since the Ce contains large populations of GABAergic neurons and extensive Ce projections to the PBN have been reported [2,8,11,15], it is likely that Ce's innervations to the PBN consist of a GABAergic component. There is as yet no ultrastructural evidence for such a GABAergic projection form the Ce to the PBN. In the present study utilizing the combined WGA-HRP anterograde tracing and post-embedding immunogold labeling for GABA technique, we aimed to determine the chemical nature of the Ce-PBN pathway and the synaptic basis of afferent GABAergic terminals impinging on post-synaptic parabrachial neurons.

Under sodium pentobarbital anesthesia (35 g/kg, i.p.), 0.05–0.1 μl of 2% Lectin-horseradish peroxidase (WGA-HRP, Sigma; dissolved in distilled water) were injected stereotaxically into the amygdaloid nucleus of rats ($n = 12$; body weight = 200–250 g) via a glass pipette coupled with a Hamilton syringe. Two or three days after the HRP injection, the rats were reanesthetized deeply and perfused with a mixture of 1.0% paraformaldehyde and 1.25% glutaraldehyde in 0.1 M phosphate buffer (PB), pH 7.3. The brains were removed and cut into 50-μm-thick transverse sections on a vibratome. The sections were reacted according to an ammonium molybdate/tetramethylbenzidine protocol to reveal the peroxidase prior to stabilization with diaminobenzidine [2,3]. The reacted sections containing the amygdala were lightly stained with Neutral Red to confirm the site of HRP injection. The sections containing the PBN were post-fixed in 1% osmium tetroxide in PB for 1 h, stained in saturated uranyl acetate, dehydrated in alcohols and finally embedded in Epon

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812. Under light microscope monitoring, the area heavily labeled with HRP was dissected from the embedded tissue for ultrathin sectioning. Ultrathin sections were mounted on uncoated nickel 150 mesh grids and immunolabeled for GABA according to protocols detailed in our previous study [3]. Briefly, grids were incubated overnight at 4 °C with a primary polyclonal GABA antibody (1:5000, Arnel) in Tris-buffered saline containing 0.1% Triton X-100, pH 7.6. They were incubated for 1 h with a goat anti-rabbit IgG conjugated to 10 or 15 nm gold particles (Amersham) at 1:25 dilution with Tris-buffered saline, pH 8.2. For controls of specificity, the primary antibody was replaced with 1% normal rabbit serum. The ultrathin sections were then counterstained with uranyl acetate and lead citrate and examined on a Hitachi H-300 electron microscope. Microscopic photographs were taken and the quantitative measurements carried out using an Analyzer image analysis system.

Under the light microscope, the spread of HRP injection were determined to be restricted in the medial and lateral divisions of the Ce. Following injections of HRP into the Ce, retrogradely labeled neurons as well as anterogradely labeled fibers were identified throughout the lateral parabrachial nucleus, especially in the external lateral and central lateral subnuclei of the PBN ipsilateral to the injection site (Fig. 1). Relatively fewer labeling was observed in the medial division of the PBN.

Electron microscopic observations were made mainly from blocks containing the highest density of anterograde and retrograde labeling in the lateral parabrachial nucleus. HRP-labeled neurons and fibers were easily identified by presence of reaction product that appeared as needle-like crystalline dense structures (Figs. 2 and 3). This reaction product was largely observed within axonal terminals, proximal dendrites and perikarya, but was occasionally seen in distal small dendrites and spines. The GABA immunoreactive sections were characterized by the presence of many immunogold particles (Figs. 2 and 3) overlying some axons and terminals, whereas very few or no gold particles were present in the dendrites, perikarya and glial processes. The axonal profile was considered to be GABA immunoreactive if the number of gold particles overlying an axon was at least 10 times higher than the number of particles within an equivalent area of surrounding non-axonal structures. Upon replacing the anti-GABA antibody with the normal rabbit serum in control experiments, there was absence of immunogold particles in any profiles in the ultrathin sections.

A large number of GABA-immunoreactive terminals were observed containing round, oval and flattened synaptic vesicles and occasionally containing a few cored vesicles (Figs. 2 and 3). They formed symmetric synaptic contacts with HRP-labeled or non-HRP-labeled perikarya and dendrites. In addition, more than half (54%, 43/80) of HRP-labeled dendrites and perikarya exhibited synaptic contacts by afferent GABA immunoreactive terminals that consisted of both HRP-labeled (4/43, Fig. 3) as well as non-HRP-labeled components (39/43, Fig. 2A).

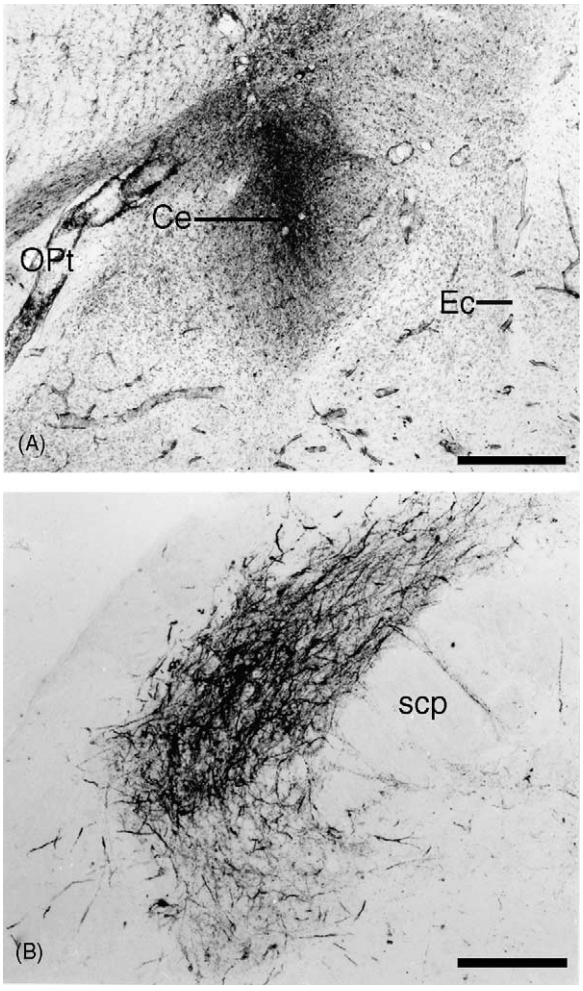

Fig. 1. Photomicrographs of WGA-HRP injection site in the Ce of case R11 (A, bar = 500 μm), and retrogradely labeled neurons and anterogradely labeled fibers in the PBN (B, bar = 100 μm). EC, external capsule; OPt, optic tract; scp, superior cerebellar peduncle.

Approximate 80% (48/60) of HRP-labeled fiber and terminal innervations from the Ce to the lateral parabrachial nucleus were GABA immunoreactive (Fig. 2B, Fig. 3). These terminals (44/48) frequently formed symmetrical synaptic contacts with non-HRP-labeled dendrites and perikarya. Some HRP/GABA dually labeled terminals (4/48) were also found to form synapses with HRP-labeled perikarya (Fig. 3). Twenty percent (12/60) of HRP-labeled axonal terminals were non-GABA immunoreactive. They were observed forming asymmetrical synaptic contacts with non-HRP-labeled profiles (Fig. 2C). No axon–axon synapses were seen in the PBN.

In this study we demonstrated that a majority of Ce's axonal terminals innervating the PBN contained GABA immunoreactivity and formed symmetrical synapses with neurons in the PBN. Some of the latter neurons in contact with GABAergic terminals from the Ce also projected to the Ce, forming a direct reciprocal neuronal circuit. A small portion of Ce-PBN projection was non-GABAergic, with axon termi-