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GABAERGIC SOMATOSTATIN-IMMUNOREACTIVE NEURONS IN THE AMYGDALA PROJECT TO THE ENTORHINAL CORTEX

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Abstract—The entorhinal cortex and other hippocampal and parahippocampal cortices are interconnected by a small number of GABAergic nonpyramidal neurons in addition to glutamatergic pyramidal cells. Since the cortical and basolateral amygdalar nuclei have cortex-like cell types and have robust projections to the entorhinal cortex, we hypothesized that a small number of amygdalar GABAergic nonpyramidal neurons might participate in amygdalo-entorhinal projections. To test this hypothesis we combined Fluorogold (FG) retrograde tract tracing with immunohistochemistry for the amygdalar nonpyramidal cell markers glutamic acid decarboxylase (GAD), parvalbumin (PV), somatostatin (SOM), neuropeptide Y (NPY), vasoactive intestinal peptide (VIP), and the m2 muscarinic cholinergic receptor (M2R). Injections of FG into the rat entorhinal cortex labeled numerous neurons that were mainly located in the cortical and basolateral nuclei of the amygdala. Although most of these amygdalar FG+ neurons labeled by entorhinal injections were large pyramidal cells, 1–5% were smaller long-range nonpyramidal neurons (LRNP neurons) that expressed SOM, or both SOM and NPY. No amygdalar FG+ neurons in these cases were PV+ or VIP+. Cell counts revealed that LRNP neurons labeled by injections into the entorhinal cortex constituted about 10–20% of the total SOM+ population, and 20–40% of the total NPY population in portions of the lateral amygdalar nucleus that exhibited a high density of FG+ neurons. Sixty-two percent of amygdalar FG+/SOM+ neurons were GAD+, and 51% were M2R+. Since GABAergic projection neurons typically have low perikaryal levels of GABAergic markers, it is actually possible that most or all of the amygdalar LRNP neurons are GABAergic. Like GABAergic LRNP neurons in hippocampal/parahippocampal regions, amygdalar LRNP neurons that project to the entorhinal cortex are most likely involved in synchronizing oscillatory activity between the two regions. These oscillations could entrain synchronous firing of amygdalar and entorhinal pyramidal neurons, thus facilitating functional interactions between them, including synaptic plasticity. © **2015 Published by Elsevier Ltd. on behalf of IBRO.**

Key words: retrograde tract tracing, long-range GABAergic neurons, parasubiculum, entorhinal cortex, nonpyramidal neurons, prefrontal cortex.

INTRODUCTION

The parahippocampal region, consisting of the entorhinal and perirhinal cortices, is an important part of the medial temporal lobe memory system (Squire and Zola-Morgan, 1991). It relays sensory information from the neocortex to the hippocampus and then transmits hippocampal outputs back to the neocortex for memory storage. The entorhinal cortex, which projects directly to the hippocampus, receives robust inputs from the amygdala that have been shown to be involved in fear conditioning and the facilitation of long-term memory consolidation by emotional arousal (Roeslor et al., 2002; Majak and Pitkanen, 2003; McIntyre et al., 2012). Various aspects of fear learning and memory involve synchronization of theta activity in the basolateral amygdala and dorsal hippocampus (Seidenbecher et al., 2003; Pape et al., 2005; Narayanan et al., 2007a,b). Since the dorsal hippocampus and basolateral amygdala are not directly interconnected, synchronization of theta activity between these structures may involve a relay in the entorhinal cortex (Mizuseki et al., 2009). These coherent oscillations produce recurring time windows that facilitate synaptic interactions, including synaptic plasticity involved in mnemonic function (Paré et al., 2002). In fact, in vivo electrophysiological studies have shown that pyramidal projection neurons and interneurons in the basolateral amygdala fire at opposite phases of entorhinal theta (Paré and Gaudreau, 1996).

Although glutamatergic pyramidal cells are the main cell type involved in the interconnections of the various cortical structures of the medial temporal lobe memory system, recent studies suggest that a small number of GABAergic nonpyramidal neurons participating in these interconnections are critical for synchronizing oscillatory activity in these structures (Jinno et al., 2007; Meltzer

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Abbreviations: AHA, amygdalohippocampal area; BLa, anterior basolateral nucleus; CBL, corticobasolateral amygdala; CCK, cholecystokinin; DLEA, dorsolateral entorhinal area; ec, external capsule; FG, Fluorogold; GAD, glutamic acid decarboxylase; IN, intercalated nuclei; Lv, ventromedial lateral nucleus; LRNP, long-range nonpyramidal; M2R, m2 muscarinic receptor; Mpd, posterodorsal medial nucleus; mPFC, medial prefrontal cortex; NPY, neuropeptide Y; PaS, parasubiculum; PBS, phosphate-buffered saline; PC, piriform cortex; PV, parvalbumin; SOM, somatostatin; TLE, temporal lobe epilepsy; VIP, vasoactive intestinal peptide; VLEA, ventrolateral entorhinal area; VMEA, ventromedial entorhinal area; VSub or VS, ventral subiculum.

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et al., 2012; Caputi et al., 2013). In fact, all major portions of the medial temporal lobe memory system are interconnected by various subpopulations of nonpyramidal GABAergic projection neurons (Jinno, 2009; Caputi et al., 2013). The main amygdalar nuclei projecting to the entorhinal cortex and other cortices of the medial temporal lobe memory system are the basolateral and cortical nuclei (the corticobasolateral nuclear complex of the amygdala; CBL) which contain neurons that resemble those of the cerebral cortex (McDonald, 1992a, 2003; Sah et al., 2003). The principal neurons in the CBL are pyramidal-like projection neurons that utilize glutamate as an excitatory neurotransmitter, whereas most nonpyramidal neurons in the CBL are interneurons that utilize GABA as an inhibitory neurotransmitter (McDonald, 1982, 1985, 1992a,b, 1996, 2003; Millhouse and deOlmos, 1983; Fuller et al, 1987; Carlsen and Heimer, 1988; McDonald and Augustine, 1993). Like the cortex, the CBL contains at least four distinct subpopulations of GABAergic nonpyramidal neurons that can be distinguished on the basis of their content of calcium-binding proteins and peptides, including: (1) parvalbumin (PV), (2) somatostatin (SOM), (3) vasoactive intestinal peptide (VIP), and (4) cholecystokinin (CCK) (Kemppainen and Pitkänen, 2000; McDonald and Betette, 2001; McDonald and Mascagni, 2001, 2002; Mascagni and McDonald, 2003; Mascagni et al., 2009). In addition, it has been demonstrated that the expression of neuropeptide Y (NPY) defines a distinct subpopulation of SOM + neurons in the CBL (McDonald, 1989; McDonald et al., 1995). A previous study that mainly focused on the interconnections of the parahippocampal region, but also included the lateral amygdalar nucleus in the same horizontal sections, demonstrated that some NPY neurons were involved in the interconnections of these areas, including nonpyramidal NPY + neurons in the lateral nucleus (L) that had projections to the entorhinal cortex (Köhler et al., 1986). The present study combined Fluorogold (FG) retrograde tract tracing with immunohistochemistry for nonpyramidal cell markers to more thoroughly investigate the involvement of nonpyramidal neurons in the projections of the amygdala to the entorhinal region.

EXPERIMENTAL PROCEDURES

Injections and tissue preparation

A total of 11 adult male Sprague–Dawley rats (250–350 g; Harlan, Indianapolis, IN, USA) received either unilateral (n = 9) or bilateral (n = 2) injections of FG into some of the main cortical areas targeted by the basolateral amygdala (Pitkänen et al., 2002). The bilateral injections (all into the entorhinal/subicular region) made after an analysis of rats with unilateral injections of this region revealed that the average number of retrogradely labeled neurons in the contralateral amygdala was less than one per section, consistent with previous anterograde and retrograde tract tracing studies of these projections (Pikkarainen et al., 1999; Majak and Pitkanen, 2003). All experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Use and Care Committee (IACUC) of the University of South Carolina. All experiments were conducted in a manner that minimized suffering and the number of animals used.

Rats were anesthetized with inhaled isoflurane and placed in a stereotaxic head holder (Stoelting, Wood Dale, IL, USA) equipped with a nosecone to maintain isoflurane inhalation during surgery. Iontophoretic injections of 2% FG (hydroxystilbamidine; Invitrogen, Carlsbad, CA, USA) in saline were made into the cortex via glass micropipettes (40-μm inner tip diameter) using a Midgard high voltage current source set at 1.0–2.0 μA (7 s on, 7 s off, for 20–40 min). Stereotaxic coordinates were obtained from an atlas of the rat brain (Paxinos and Watson, 1997). In some cases (e.g., R39; Fig. 1) two injections 0.5 mm apart were made along the same pipette track to produce an oval injection site. In one case (R51, Fig. 1) two injections were made with two different pipettes spaced 0.5 mm apart along the mediolateral axis to produce a large flattened injection site. The cortical areas injected were in the lateral entorhinal cortex and/or adjacent ventral subicular region (eight rats), medial prefrontal cortex (mPFC) (one rat), lateral prefrontal cortex (one rat) or insular cortex (one rat). Micropipettes were left in place for 10 min, and then slowly withdrawn with the current reversed to prevent FG from flowing up the pipette track. After a 5-day survival period, eight rats were anesthetized with a mixture of ketamine (85 mg/kg), xylazine (8 mg/kg) and acepromazine (4 mg/kg) and then perfused intracardially with phosphate-buffered saline (PBS; pH 7.4) containing 1.0% sodium nitrite (50 ml), followed by 4.0% paraformaldehyde in 0.1 M phosphate buffer at pH 7.4 (1 liter). Three rats with unilateral injections of FG received unilateral injections of colchicine into the lateral cerebral ventricle on the FG-injected side via a microsyringe (100-μg colchicine dissolved in 10-μl distilled water) after a 5-day survival period; one day later these rats were perfused as described above. Following perfusion, all brains were removed and postfixed for 3 h in 4.0% paraformaldehyde. Brains were sectioned on a vibratome at a thickness of 50-μm in the coronal plane and processed for immunohistochemistry in wells of tissue culture plates.

Immunoperoxidase staining

In all brains a series of sections through the injection site and basolateral amygdala (bregma levels −1.8 to −4.6) at 150–300-μm intervals was incubated in a guinea-pig FG antibody (1:4000; donated by Dr. Lothar Jennes, University of Kentucky) overnight at 4 °C. These sections were then processed for avidin–biotin peroxidase immunohistochemistry using a guinea-pig Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA). Nickel-enhanced DAB (3,3′-diaminobenzidine-4HCl, Sigma Chemical Co., St. Louis, MO, USA) was used as a chromogen to generate a black reaction product (Hancock, 1986). All antibodies were diluted in a solution containing 1% normal goat serum, 0.4% Triton-X 100, and 0.1 M PBS. These sections were mounted on gelatinized slides, dried overnight, counterstained with pyronin Y (a pink Nissl stain), dehydrated in ethanol, cleared in

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