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Extrinsic Origins of the Somatostatin and Neuropeptide Y innervation of the Rat Basolateral Amygdala

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Abstract

The amygdalar basolateral nuclear complex (BLC) is a cortex-like structure that receives inputs from many cortical areas. It has long been assumed that cortico-amygdalar projections, as well as inter-areal intracortical connections, arise from cortical pyramidal cells. However, recent studies have shown that GABAergic long-range nonpyramidal neurons (LRNP neurons) in the cortex also contribute to inter-areal connections. The present study combined Fluorogold (FG) retrograde tract tracing with immunohistochemistry for cortical nonpyramidal neuronal markers to determine if cortical LRNP neurons project to the BLC in the rat. Injections of FG into the BLC produced widespread retrograde labeling in the cerebral hemispheres and diencephalon. Triple-labeling for FG, somatostatin (SOM), and neuropeptide Y (NPY) revealed a small number of FG+/SOM+/NPY+ neurons and FG+/SOM+/NPY– neurons in the lateral entorhinal area, amygdalopiriform transition area, and piriform cortex, but not in the prefrontal and insular cortices, or in the diencephalon. In addition, FG+/SOM+/NPY+ neurons were observed in the amygdalostriatal transition area and in a zone surrounding the intercalated nuclei. About half of the SOM+ neurons in the lateral entorhinal area labeled by FG were GABA+. FG+ neurons containing parvalbumin were only seen in the basal forebrain, and no FG+ neurons containing vasoactive intestinal peptide were observed in any brain region. Since LRNP neurons involved in corticocortical connections are critical for synchronous oscillations that allow temporal coordination between distant cortical regions, the LRNP neurons identified in this study may play a role in the synchronous oscillations of the BLC and hippocampal region that are involved in the retrieval of fear memories.

Keywords

retrograde tract tracing; long-range GABAergic neurons; amygdalostriatal transition region; entorhinal cortex; amygdalopiriform transition region; prefrontal cortex

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INTRODUCTION

The basolateral nuclear complex (BLC) of the amygdala, consisting of the lateral, basolateral, and basomedial nuclei, is a key forebrain structure involved in emotional behavior and emotional learning (Sah et al., 2003; Pape and Paré, 2010). Although it is a subcortical nuclear complex, its cell types closely resemble those of the cerebral cortex (Carlsen and Heimer, 1988; McDonald, 1992, 2003). Thus, previous studies have shown that there are two major cell classes in the BLC, pyramidal neurons and nonpyramidal neurons. Although these neurons do not exhibit a laminar or columnar organization, their morphology, synaptology, electrophysiology, and pharmacology are remarkably similar to their counterparts in the cerebral cortex (Carlsen and Heimer, 1988; McDonald, 1992, 2003; Sah et al., 2003; Pape and Paré, 2010). Thus, the principal neurons in the BLC are pyramidal-like projection neurons with spiny dendrites that utilize glutamate as an excitatory neurotransmitter, whereas most nonpyramidal neurons are spine-sparse interneurons that utilize gamma-aminobutyric acid (GABA) as an inhibitory neurotransmitter.

As in the cortex there are several distinct subpopulations of nonpyramidal neurons, including neurons that express parvalbumin (PV), somatostatin (SOM), neuropeptide Y (NPY), and vasoactive intestinal peptide (VIP), that have extensive local axonal arborizations in the nuclei of the BLC (Spampanato et al., 2011). NPY expressing neurons in the BLC, like those in the cortex, are a subpopulation of SOM+ neurons (McDonald, 1989; McDonald et al., 1995). NPY in the BLC is anxiolytic and can induce resilience to stress (Sajdyk et al., 2008). One of the interesting aspects of the NPY+ axonal plexus in the BLC is that it does not match the density of neighboring NPY+ neurons. This plexus is dense and uniform throughout the BLC, but the density of NPY+ neurons varies greatly in different nuclei. Moreover, selective lesioning of BLC NPY+ neurons does not cause a significant reduction in the density of the NPY+ axonal plexus (Truitt et al., 2009). These findings suggest that there may be an extrinsic source for many of these axons. Previous studies have demonstrated that the few NPY+ neurons in the locus ceruleus that project to the amygdala cannot account for the density of this plexus (Gustafson et al., 1986). A similar mismatch between the density of local NPY+ pyramidal neurons and the local NPY+ axonal plexus was noted in the entorhinal cortex, where the use of retrograde tract tracing demonstrated that many NPY+ inputs to this region originated from surrounding portions of the parahippocampal region and the lateral amygdalar nucleus (Köhler et al., 1986). The present study combined Fluorogold retrograde tract tracing with immunohistochemistry for cortical/amygdalar nonpyramidal neuronal markers (NPY, SOM, PV, VIP, and GABA) to determine if neurons in the cerebral hemispheres that express NPY or other nonpyramidal neuronal markers project to the BLC.

EXPERIMENTAL PROCEDURES

Injections and tissue preparation

A total of 6 adult male Sprague-Dawley rats (250–350g; Harlan, Indianapolis, IN) received unilateral injections of Fluorogold (FG) into the basolateral amygdalar nuclear complex (BLC). 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) equipped with a nose cone to maintain isoflurane inhalation during surgery. Iontophoretic injections of 2.0% FG (hydroxystilbamidine; Invitrogen, Carlsbad, CA) in saline were made into the basolateral amygdala via glass micropipettes (40 μ m inner tip diameter) using a Midgard high voltage current source set at 1.5–2.0 μ A. In some rats a single injection was made (7 s on, 7 s off, for 30 min), whereas in other rats two injections separated by 0.5 mm along the same pipette track were made (20 min for each injection). Stereotaxic coordinates were obtained from an atlas of the rat brain (Paxinos and Watson, 1997). Micropipettes were left in place for 10 minutes, and then slowly withdrawn with the current reversed to prevent FG from flowing up the pipette track. After a 5 day survival period, 4 rats (R5, R8, R10, R12) were anesthetized with a mixture of ketamine (85 mg/kg), xylazine (8 mg/kg) and acepromazine (4mg/kg) and 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). The remaining 2 rats (R14 and R15) received unilateral injections of colchicine into the lateral cerebral ventricle on the FG-injected side of the brain via a microsyringe (100 μ g colchicine dissolved in 10 μ l distilled water) 5 days after the FG injection and perfused the next day. R14 and R15 were perfused with a 4.0% paraformaldehyde/0.2% glutaraldehyde mixture to enable staining for GABA. Colchicine was used in these experiments because it inhibits axonal transport of GABA from perikarya due to its disruption of microtubules, thereby increasing perikaryal levels of GABA. Previous studies have shown that perikaryal levels of GABAergic markers in many GABAergic projection neurons are below the level of immunohistochemical detectability (Tóth and Freund, 1992; Tóth et al., 1993). Following perfusion, all brains were removed and postfixed in the perfusate for 3 hours. The cerebral hemispheres were cut into two blocks in the coronal plane at the level of the optic chiasm. Both blocks were sectioned in their entirety on a vibratome at a thickness of 50 μ m in the coronal plane and processed for immunohistochemistry in wells of tissue culture plates.

The initial 2 rats used in this study had FG injections into the lateral nucleus (R5) or basolateral nucleus (R8). Analysis of these cases revealed that these BLC injections produced contralateral retrograde FG labeling in very few regions: (1) prelimbic and infralimbic areas of the medial prefrontal cortex, (2) posterolateral cortical amygdalar nucleus (only with lateral nucleus injections), (3) ventral endopiriform nucleus (only with basolateral nucleus injections), and (4) layer 3 of the nucleus of the olfactory tract (only with basolateral nucleus injections). Moreover, no colocalization of FG nonpyramidal neuronal markers (SOM, NPY, PV, or VIP) was observed contralaterally. Because of these largely negative contralateral results, only the ipsilateral hemispheres in the last four rats (R10, R12, R14 and R15) were processed for immunohistochemistry.

Immunoperoxidase staining

In all brains a 1 in 3 series of sections through the amygdalar injection site, and a 1 in 5 series of sections through the remaining portions of the hemispheres, were 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). Nickel-enhanced DAB (3, 3'-diaminobenzidine-4HCl, Sigma Chemical Co., St. Louis, MO) 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. Sections were mounted on gelatinized slides, dried overnight, counterstained with pyronin Y (a pink Nissl stain), dehydrated in ethanol, cleared in xylene, and coverslipped with Permount (Fisher Scientific, Pittsburgh, PA).

FG/SOM/NPY triple-labeling experiments

In all 6 brains a 1 in 5 series of sections (i.e., at 250µm intervals) through the cerebral hemispheres was incubated in an anti-FG/SOM/NPY primary antibody cocktail overnight at 4° C. All antibodies were diluted in a solution containing 1% normal goat serum, 0.4 % Triton-X 100, and 0.1 M PBS. The following primary antibodies were used: (1) a guinea pig polyclonal FG antibody (1:2000; donated by Dr. Lothar Jennes, University of Kentucky); (2) a mouse monoclonal SOM antibody (1:4000; donated by Dr. Alison Buchan, University of British Columbia); and (3) a rabbit polyclonal NPY antibody (1:3000; Bachem Americas, Torrance, CA). After incubation in the primary antibody cocktails, sections were rinsed in 3 changes of PBS (10 min each), and then incubated in a cocktail of three secondary antibodies for 3 hrs at room temperature (1:400; Invitrogen, Eugene, OR): (1) goat anti-guinea pig AlexaFluor-488; (2) goat anti-mouse AlexaFluor-546; and (3) goat anti-rabbit AlexaFluor-633. Sections were then rinsed in 3 changes of PBS (10 min each) and mounted on glass slides using Vectashield mounting medium (Vector Laboratories).

FG/SOM/GABA triple-labeling experiments

In two of the colchicine-injected rats (R14 and R15), which were both perfused with the paraformaldehyde/glutaraldehyde mixture (see above), a 1 in 5 series of sections through the cerebral hemispheres was incubated in an anti-FG/SOM/GABA primary antibody cocktail for 48 hours at 4° C. All antibodies were diluted in a solution containing 1% normal goat serum, 0.4 % Triton-X 100, and 0.1 M PBS. The following primary antibodies were used: (1) a guinea pig polyclonal FG antibody (1:3000; donated by Dr. Lothar Jennes); (2) a mouse monoclonal SOM antibody (1:6000; donated by Dr. Alison Buchan); and (3) a rabbit polyclonal GABA antibody (1:1000; Sigma Chemical Co., St. Louis, MO). After incubation in the primary antibody cocktails, sections were incubated in the same secondary cocktail described above for the FG/SOM/NPY experiments and mounted on glass slides as described above.

FG/PV/VIP triple-labeling experiments

In two of the non-colchicine-injected rats (R5 and R8), a 1 in 5 series of sections through the cerebral hemispheres was incubated in an anti-FG/PV/VIP primary antibody cocktail

overnight at 4° C. All antibodies were diluted in a solution containing 1% normal goat serum, 0.4 % Triton-X 100, and 0.1 M PBS. The following primary antibodies were used: (1) a guinea pig polyclonal FG antibody (1:2000; donated by Dr. Lothar Jennes); (2) a mouse monoclonal PV antibody (1:5000; SWANT, Marly, Switzerland); and (3) a rabbit polyclonal VIP antibody (1:4000; ImmunoStar, Hudson, WI). After incubation in the primary antibody cocktails, sections were rinsed in 3 changes of PBS (10 min each), and then incubated in a cocktail of three secondary antibodies for 3 hrs at room temperature (1:400; Invitrogen, Eugene, OR): (1) goat anti-guinea pig AlexaFluor-488; (2) goat anti-mouse AlexaFluor-633; and (3) goat anti-rabbit AlexaFluor-546. Sections were then rinsed in 3 changes of PBS (10 min each) and mounted on glass slides using Vectashield mounting medium.

Analysis

Amygdalar injection sites in Nissl-counterstained, immunoperoxidase-stained sections were mapped onto template drawings taken from an atlas of the rat brain (Paxinos and Watson, 1997) using a drawing tube attached to an Olympus BX51 microscope under brightfield illumination. The distribution of FG+ retrogradely-labeled neurons throughout the cerebral hemispheres and diencephalon in these immunoperoxidase preparations was also studied under brightfield illumination.

Sections processed for immunofluorescence were examined with a Zeiss LSM 510 Meta confocal microscope. Fluorescence of AlexaFluor-488, AlexaFluor-546, and AlexaFluor-633 dyes was analyzed using filter configurations for sequential excitation/imaging via 488 nm, 543 nm, and 633 nm channels. Candidate FG+ double-labeled or triple-labeled neurons were initially identified at 100X magnification with an optical section thickness of 50 μ m (i.e., the thickness of the section). Each of these neurons was then examined at higher magnification (generally 200X) to verify that its apparent multiple labeling was not due to the overlap of two different neurons in close proximity. FG+ neurons that also exhibited immunostaining for one or more of the other neurochemicals investigated were plotted at 0.5 mm intervals through the cerebral hemispheres onto template drawings taken from an atlas of the rat brain (Paxinos and Watson, 1997). Since sections were stained for each combination of markers at 250 μ m intervals, the two sections that were closest to the designated level of the atlas were plotted onto each template at 0.5 mm intervals. In the GABA-stained material only neurons at the surfaces of the two sections were plotted. Amygdalar nuclei and most cortical areas were identified using the Paxinos and Watson atlas, with the exception of the lateral entorhinal cortex, which was divided into three main areas according to the description by Krettek and Price (1977): dorsolateral entorhinal area (DLEA), ventrolateral entorhinal area (VLEA), and ventromedial entorhinal area (VMEA). The locations of labeled neurons at each level were determined using anatomical cues, including the staining patterns of the neuronal markers (which differed in the various regions of the brain) and their relationship to fiber bundles (e.g., the external capsule) as in a previous study in our lab (McDonald et al., 2012). Control sections were processed with one of the antibodies omitted from the primary antibody cocktail; in all cases only the labeling with the secondary fluorescent antibodies corresponding to the non-omitted primary antibodies was observed, and only on the appropriate channel. These results

indicated that the secondary antibodies were specific for guinea pig, rabbit, or mouse immunoglobulins, and that there was no “crosstalk” between channels (Wouterlood et al., 1998). Digital images were adjusted for brightness and contrast using Photoshop 6.0 software.

A semi-quantitative analysis was performed in the two FG/SOM/GABA experiments (brains R14 and R15) to estimate the percentages of retrogradely-labeled FG+ neurons in the entorhinal cortex that were SOM+ or GABA+ (and vice versa). This analysis was done on 3 sections through the entorhinal cortex in each brain. Square areas (500µm X 500µm) in the deep layers (layers V and VI) of both the DLEA and VLEA were analyzed in each section. Because GABA immunoreactivity is only found at the surfaces of sections with the antibody used (see McDonald and Mascagni, 2001), analysis was restricted to the upper and lower surfaces of each section using a 10 µm optical section thickness. Digital photomicrographs were obtained at each surface of each area sampled and cell counts were performed while viewing the images using LSM Image Browser software. Neurons with all possible combinations of FG, SOM, and GABA immunoreactivity were counted. In addition, analysis of the upper and lower surfaces of sections through the medial prefrontal cortex, lateral prefrontal cortex, and the amygdalar region was performed to determine if FG+ or FG +/SOM+ neurons in these regions expressed GABA immunoreactivity. Because we were attempting to estimate the approximate proportion of each neuronal type rather than determine their absolute numbers, we did not use unbiased stereological counting methods (Saper, 1996).

RESULTS

Injection sites and general pattern of retrograde labeling

In most cases the centers of the FG injections were located close to the center of the BLC (Figs. 1 and 2). The cores of the injection sites, marked by dense neuropilar labeling and solid labeling of neuronal perikaryal, were confined to the nuclei of the BLC in all cases except R15 (which had slight spread into the laterally adjacent dorsal endopiriform nucleus). The dense myelinated fiber bundles in the external and intermediate capsules appeared to prevent significant spread of the injectate beyond the borders of the BLC. Surrounding the core of each injection site was a peripheral halo about 100 µm wide that mainly contained labeled glial cells. The core of each injection site was considered the effective injection site and is illustrated in Fig. 1 for each injection. In 4 cases the injection sites involved both the basolateral nucleus (anterior and/or posterior subdivisions, BL_a and BL_p respectively) and the ventromedial subdivision of the lateral nucleus (Lv) (cases R10, R12, R14, and R15) (Fig. 1). The injection sites in cases R12 and R14 also spread ventrally to involve the posterior subdivision of the basomedial nucleus (BM_p). The injection site in case R5 was restricted to the lateral nucleus, whereas in case R8 the injection site was restricted to the lateral half of the basolateral nucleus, mainly involving BL_p.

The pattern of FG retrograde labeling in the cerebral hemispheres and diencephalon was consistent with previous retrograde and anterograde tract tracing studies of the BLC (Pitkänen, 2000). Although it is possible that some of the retrogradely-labeled neurons could be labeled by uptake of tracer by fibers-of-passage (Lanciego and Wouterlood, 2011), the

iontophoretic injection of FG through narrow-diameter micropipettes should minimize uptake by these fibers (Schmued and Fallon, 1986; Pieribone and Aston-Jones, 1988; Divac and Mogensen, 1990), and the results of previous anterograde tract tracing studies are consistent with our retrograde findings. There was considerable overlap in the regions containing retrogradely-labeled FG+ neurons in the 6 brains used in this study, but also some differences depending on the nuclei injected. Briefly, neurons in the diencephalon were seen in the medial and lateral hypothalamus as well as in the midline and intralaminar nuclei of the thalamus. At rostral levels of the cerebral hemispheres, FG+ neurons were seen in the medial and lateral prefrontal cortices, anterior insular areas, endopiriform nucleus, and basal forebrain. At levels caudal to the amygdala FG+ neurons were seen in the temporal, perirhinal and entorhinal cortices, as well as in the ventral subiculum and adjacent CA1. At amygdalar levels FG+ neurons were observed mainly in the rostral portions of the medial and cortical amygdalar nuclei, as well as in the endopiriform nucleus, caudal insular cortex, and ventromedial portion of the piriform cortex. Virtually none of the small principal neurons of the intercalated nuclei (7–8 μ m in diameter) were labeled, with the exception of a small number of neurons in the lateral intercalated (paracapsular) nucleus in some cases. In contrast, it was common to observe a small number of large FG+ neurons (12–15 μ m in diameter) along the borders of the intercalated nuclei, especially surrounding the rostral intercalated nuclei located anterior to the BLA (Figs. 2B and C).

FG/SOM/NPY triple-labeling experiments

No FG+ neurons exhibiting expression of SOM or NPY were observed in any nuclei of the diencephalon (thalamus or hypothalamus) or in portions of the cerebral hemispheres rostral to the amygdala, including the prefrontal cortices and anterior insular cortices. FG+/SOM+/NPY+ triple-labeled neurons and FG+/SOM+/NPY– double-labeled neurons were found in some amygdalar nuclei and in some cortical areas lateral and caudal to the amygdala (Figs. 3–8); all FG+ neurons in these regions that expressed NPY also expressed SOM (Figs. 3, 9–11). All FG+/SOM+/NPY+ triple-labeled neurons and FG+/SOM+/NPY– double-labeled neurons in cortical areas, as well as in the basolateral and cortical amygdalar nuclei, were nonpyramidal neurons (Figs. 9–11). In each area these FG+ nonpyramidal neurons constituted a small subpopulation of the total SOM+ and NPY+ neuronal populations, and were greatly outnumbered by FG+ pyramidal cells that were not SOM+ or NPY+ (Figs. 9–11).

Amygdalar region—Clusters of large (12–15 μ m) FG+/SOM+/NPY+ triple-labeled neurons surrounding the rostral intercalated nuclei (located just anterior to the basolateral amygdala) were observed in all of the cases in this study (Figs. 3A, and 4–8 [Bregma –1.3 level]). Similar FG+/SOM+/NPY+ cells were occasionally seen along the borders of more caudal components of the intercalated nuclei as well. Figures 2B and 2C show large FG+ of similar morphology and locations in peroxidase preparations. Although some of the small (7–8 μ m) principal neurons of certain intercalated nuclei were retrogradely labeled with FG in some cases, they were never SOM+ or NPY+.

Scattered FG+/SOM+ neurons, most of which were also NPY+, were also seen in other amygdalar nuclei, especially at rostral amygdalar levels. These nuclei included the cortical

and basomedial nuclei, as well as the anterior subdivisions (but not the posterior subdivisions) of the medial nucleus, and the amygdalostriatal transition region (Figs. 3B, 4–8, 9A). In each case there were many FG+ neurons in these regions, and the peptide-positive FG+ neurons constituted only a small subpopulation of the total FG+ population. In the amygdalostriatal transition region, however, there were very few FG+ neurons and roughly equal numbers of FG+/SOM+/NPY+ and single-labeled FG+ neurons (Fig. 3B). FG+/SOM+/NPY+ triple-labeled neurons and FG+/SOM+/NPY– double-labeled neurons were also seen in the external capsule, dorsal and ventral endopiriform nuclei, ventromedial part of the posterior piriform cortex, and along the medial border of the BLA (Figs. 4–8, 9B).

Prefrontal, insular and parahippocampal cortices—Many FG+ retrogradely labeled neurons were observed in the medial prefrontal cortex (prelimbic and infralimbic areas) and the lateral prefrontal cortex (dorsal and ventral agranular insular areas) in the cases used in this study, but no FG+ neurons were SOM+ or NPY+ in these areas. Likewise, although numerous FG+ neurons were observed in posterior insular areas, no FG+ neurons were SOM+ or NPY+. In contrast, a small number of FG+/SOM+/NPY+ triple-labeled neurons and FG+/SOM+/NPY– double-labeled neurons was seen in parahippocampal areas caudal to the amygdala, including the amygdalopiriform transition area (APIR), perirhinal cortex (PRh), and the entorhinal cortex (Figs. 4–8 [Bregma levels –5.3, –6.3 and –7.0], 10, 11). Most FG+ single labeled neurons, as well as FG+/SOM+/NPY+ and FG+/SOM+/NPY– neurons, in the dorsolateral and ventrolateral entorhinal cortices (DLEA and VLEA, respectively) were found in the deep layers (Figs. 4–8, 10). However, occasional FG+ single labeled neurons, FG+/SOM+/NPY+, and FG+/SOM+/NPY– neurons were seen in layers II and III. Although many FG+ neurons were seen in the ventral subiculum and adjacent CA1 in all of the cases of this study, only one FG+/SOM+ neuron was observed in this region (Case R14, Fig. 5 [Bregma –5.3 level]).

APIR and the entorhinal area (EA) were the main sources of SOM and NPY inputs to the basolateral amygdala from the parahippocampal region. The percentages of FG+/SOM+ neurons in APIR and the EA that also expressed NPY varied in different animals (Figs. 4–8; Table 1), and appeared to be correlated with the extent of involvement of the basolateral nucleus (BLA and BLp) versus the lateral nucleus (LAT) in the injection site. Thus, case R5, where the injection site was confined to the LAT, had the lowest percentage of FG+/SOM+ neurons in the APIR/EA region that were also NPY+ (7%; Table 1). Case R8, where the injection site was confined to BL, had the highest percentage of FG+/SOM+ in the APIR/EA region that were also NPY+ (85%; Table 1). The other 4 cases (R10, R12, R14, and R15), where the injection sites involved both BL and LAT (and sometimes also the basomedial nucleus), had intermediate percentages of FG+/SOM+ neurons that were also NPY+ (35–52%; Table 1).

FG/SOM/GABA triple-labeling experiments

Triple labeling for FG, SOM and GABA was performed in two colchicine-injected brains (cases R14 and R15) to determine the extent to which somata of FG+/SOM+ neurons exhibited GABA immunoreactivity (i.e., were GABA+). FG+/SOM+/GABA+ neurons were found in all of the cortical areas that contained FG+/SOM+ neurons (Fig. 12). The

entorhinal cortex was one of the main sources of SOM+ inputs to the basolateral amygdala. Semi-quantitative analysis of the extent of colocalization of FG, SOM and GABA was performed in the DLEA and VLEA areas of the entorhinal cortex (Table 2). Because GABA immunoreactivity was only found at the surfaces of sections with this antibody (McDonald and Mascagni, 2001), only FG+/SOM+ neurons at the surfaces of sections were examined for GABA immunoreactivity (see Experimental Procedures). Cell counts revealed that only 2% of entorhinal FG+ neurons were SOM+, and these neurons constituted only about 5–10 % of the total population of SOM+ neurons in these areas (Table 2). Almost half (47.4%) of the FG+/SOM+ neurons were GABA+ (Table 2), which was similar to the percentage of all SOM+ neurons that were GABA+ (45.7%). It is noteworthy that no SOM-negative FG+ neurons were GABA+, indicating that SOM+ neurons were the only nonpyramidal neuronal subpopulation in the DLEA and VLEA that projected to the basolateral amygdala. FG+/GABA+ neurons, which were all SOM+, constituted 2.1% of the total population of GABA+ neurons (Table 2). SOM+ neurons constituted about one-quarter (28.5%) of the total GABA+ population. Analysis of the prelimbic and infralimbic areas of the medial prefrontal cortex, and the agranular insular areas of the lateral prefrontal cortex, revealed that no FG+ neurons in these areas exhibited expression of SOM or GABA, suggesting that only pyramidal neurons of the prefrontal cortices project to the basolateral amygdala.

Because the total number of FG+/SOM+ neurons in the rostral amygdalar nuclei, the amygdalostriatal transition area, and surrounding the rostral intercalated nucleus is small, and since even fewer such neurons were located at the surfaces of the section (which are the only zones containing GABA immunoreactivity), data regarding their extent of expression of GABA in these neurons was extremely limited. Examination of 3 FG+/SOM+ neurons in the corticomедial amygdalar nuclei, 2 FG+/SOM+ neurons in the amygdalostriatal transition area, and 3 FG+/SOM+ neurons surrounding the rostral intercalated nucleus indicated that none were GABA+. Of two FG+/SOM+ neurons in the ventromedial piriform cortex at amygdalar levels, only one was GABA+.

FG/PV/VIP triple-labeling experiments

In the two brains investigated for expression of PV and VIP in FG+ neurons (case R5: lateral nucleus injection; case R8: basolateral nucleus injection) no FG+ neurons exhibiting expression of PV or VIP were observed in any brain area extrinsic to the amygdalar nuclei injected (Fig. 13), with the exception of several FG+/PV+ neurons in the ventral pallidum and substantia innominata of the basal forebrain in case R8. Sections through the basal forebrain were not available in case R5 because they were lost due to the blocking of the brain at this level in this rat.

DISCUSSION

This study demonstrates that the basolateral nuclear complex of the amygdala (BLC) receives input from subpopulations of somatostatin-ir neurons located in a variety of structures in amygdalar and parahippocampal regions, but not from the diencephalon or rostral portions of the cerebral hemispheres, such as the medial and lateral prefrontal cortices or the insular cortex. Some of these SOM+ projection neurons express GABA, and

many express NPY, thus contributing to the dense plexus of NPY+ axons in the BLC. No extra-amygdalar VIP+ neurons project to the BLC, and only the basal forebrain contains PV + neurons that project to the BLC.

SOM+ BLC-projecting neurons in the amygdalar region

The main areas in the amygdalar region that had SOM+ neurons projecting to the BLC were rostral portions of the cortical and medial amygdalar nuclei, amygdalostratial transition region, endopiriform nucleus, posterior piriform cortex, external capsule, and a narrow zone surrounding the intercalated nuclei (IN). In contrast to the small principal neurons that comprise the INs, the FG+/SOM+ neurons that were located along the borders of the INs had large somata. All of these neurons were NPY+ and none were GABA+. They appear to correspond to the spiny peri-intercalated nuclear neurons (“SPIN neurons”) that express high levels of the m2 muscarinic cholinergic receptor (McDonald and Mascagni, 2011) since they have the same location, size, and neurotransmitter phenotype (SOM+, NPY+, and non-GABAergic). The dendritic spines of SPIN neurons are only found on distal dendrites (McDonald and Mascagni, 2011). Since only the somata and proximal dendrites of the FG +/SOM+/NPY+ neurons surrounding the INs were immunostained in the present study, possible spines extending from the dendrites of these neurons could not be observed.

SPIN neurons appear to correspond to the large spiny neurons of the INs described by Millhouse (1986) in Golgi preparations. Although Millhouse described them as located within the INs, the cell body of the one neuron depicted (see Fig. 16 in Millhouse, 1986) appears to be located at the ventral border of the main IN in the rostral amygdala. Millhouse reported that axons of Golgi-stained large spiny neurons of the INs project away from the INs in lateral or ventral directions, but beaded collaterals of these axons arborize both within and just outside the INs (Millhouse, 1986). The present study indicates that the BLC is also a major target of these neurons; SPIN neurons were densely labeled with FG in all of the animals in this study. It is also of interest that there are large GABAergic *aspiny* neurons associated with the intercalated nuclei that are SOM-negative and innervate interneurons in the BLC (Bienvenu et al., 2015). Establishing the neuronal targets of SPIN cells within the BLC, as well as their transmitter, will be helpful in determining their functional role in amygdalar circuitry.

The finding of a small number of FG+/SOM+/NPY+ neurons in the amygdalostratial transition (ASt) region agrees with a previous study which reported the presence of FG +/NPY+ neurons in this region with FG injections into the BLC (Urban et al., 2011). This is an exception to the general rule that the striatum and striatal-like areas such as the ASt and central nucleus (which all contain medium-sized spiny principal neurons) do not project to the cortex or to nuclei with cortex-like cell types such as the BLC (McDonald, 1992; McDonald et al., 2003). However, studies using the anterograde tracer PHA-L have shown that whereas injections into the BLC produce a very dense axonal plexus in the ASt, injections into the ASt produce only a moderate number of labeled axons in the BLC (Jolkkonen et al., 2001a). Moreover, the latter axons are morphologically distinct from those seen in other ASt targets. These data suggest that the ASt projections to the BLC may arise from one or more subpopulations of ASt neurons that are distinct from the medium spiny

principal neurons. Jolkkonen and co-workers suggested that cholinergic neurons in the ASt are the origins of this projection to the BLC, but the present study indicates that a separate subpopulation of FG+/SOM+/NPY+ neurons is also involved. There is evidence that release of NPY into the BLC from the axons of these ASt neurons may participate in the regulation of anxiety and fear responses to conditioned stimuli (Urban et al., 2011).

A small number of SOM+/NPY+ neurons and SOM+/NPY– neurons in the anterior portions of the medial amygdalar nucleus were retrogradely-labeled by BLC injections of FG. The high density of SOM+ and SOM+/NPY+ neurons in the medial nucleus, and the fact that many of these cells take part in its projections to the medial preoptic-hypothalamic region, suggests that most of these cells are a subpopulation of the principal neurons in this nucleus (McDonald, 1987, 1989; Rowniak et al., 2008).

In the present study a small number of nonpyramidal FG+/SOM+/NPY+ neurons were observed in the posterior piriform cortex and the underlying endopiriform nuclei, but these cells were greatly outnumbered by FG+/SOM–/NPY– pyramidal neurons. This is consistent with previous studies which have shown that these regions project to the BLC (McDonald, 1998; Pitkänen, 2000), that SOM+ and NPY+ neurons in these regions, like other cortical and cortex-like regions, are nonpyramidal, and that NPY+ neurons make up a subpopulation of SOM+ neurons (Kowianski et al., 2004; Suzuki and Bekkers, 2010). To the authors' knowledge, this is the first report indicating that some of these neurons are projection neurons.

The cortical and basomedial amygdalar nuclei, like the BLC, contain cortex-like cell types (McDonald 1992, 2003), including nonpyramidal neurons exhibiting colocalization of SOM and NPY (McDonald 1989). Although previous studies have shown that the anterior cortical and basomedial nuclei project to the basal and lateral nuclei (Pitkänen, 2000), the present study is the first investigation to show that a small number of SOM+ and SOM+/NPY+ nonpyramidal neurons contribute to this projection. It will be of interest to determine if these SOM+ neurons innervate the distal dendrites and spines of BLC pyramidal neurons like local SOM+ interneurons of the BLC (Muller et al., 2007). Such connections may play a role in gating excitatory inputs to these dendrites (Muller et al., 2007).

The present study demonstrated that many of the SOM+/NPY+ neurons in the ventral portion of the external capsule located between the endopiriform nucleus and BLC project to the BLC. These neurons appear to be similar to the SOM+/NPY+ nonpyramidal neurons located in or adjacent to the white matter of the neocortex that project to neighboring or distant areas of the cortex (Tomioka et al., 2005; Tomioka and Rockland, 2007). Since the BLC is a cortex-like structure, it is not surprising that it receives a projection from similar SOM+/NPY+ nonpyramidal neurons associated with the white matter. It will be of interest to determine if these neurons receive cortical inputs, but not thalamic inputs, like their counterparts of the subneocortical white matter (Tamamakei and Tomioka, 2010).

SOM+ BLC-projecting neurons in the parahippocampal region

Consistent with previous anterograde and retrograde tract tracing studies in rats (McDonald and Mascagni, 1997; Shi and Cassell, 1999; Pitkänen, 2000; Jolkkonen et al., 2001b; Kishi

et al., 2006) injections of FG into the BLC in the present investigation produced dense retrograde labeling in the ventral subiculum and adjacent CA1, and in the parahippocampal region (perirhinal cortex, entorhinal cortex, and amygdalopiriform transition area). However, with the exception of one neuron in the ventral subiculum, all retrogradely-labeled SOM+ and SOM+/NPY+ neurons were observed in the parahippocampal region rather than the hippocampal formation, and all were of nonpyramidal morphology. These results in rat differ from a previous study in the cat where large injections of the retrograde tracer horseradish peroxidase into the BLC labeled small numbers of nonpyramidal neurons in the cornu ammonis of the hippocampus (Ino et al., 1990). Müller and coworkers (Müller et al., 2011) found a small number of retrogradely labeled GABAergic nonpyramidal neurons in the ventral subiculum and adjacent CA1 with injections that involved the entire rat amygdala, suggesting that hippocampal nonpyramidal neurons may project to portions of the amygdala outside of the BLC regions injected in the present study.

Although the great majority of nonpyramidal neurons in cortical areas are thought to be GABAergic, only about half of the long-range nonpyramidal neurons (LRNP neurons) in the entorhinal cortex projecting to the BLC were GABA+. However, GABAergic neurons with long projections typically have low levels of somatic GABA, since GABA is rapidly transported from the soma to the distant axon terminals of these neurons (Onteniente et al., 1986; Tóth and Freund, 1992). Thus, the GABA-negative SOM+ LRNPs in the parahippocampal region in the present study may be GABAergic but have levels of somatic GABA that are below the threshold for immunohistochemical detection using immunofluorescence, even when colchicine is used to increase somatic levels by inhibiting axonal transport. This interpretation is consistent with previous studies performed in GAD67-GFP transgenic mice which have shown that all SOM+ neurons in both neocortical and paleocortical areas are GABAergic (Tamamaki et al., 2003; Suzuki and Bekkers, 2010). In fact, the finding that only about half of all SOM+ neurons in the entorhinal cortex (both FG+/SOM+ and FG-/SOM+) are GABA+ could indicate that many SOM+ entorhinal neurons are LRNP neurons, but some may have projections to areas located outside of the BLC regions injected with FG in the present study. Our finding of limited GABA expression in SOM+ neurons in the entorhinal cortex agrees with the results of a previous study (Wouterlood and Pothuizen, 2000). Our finding of colocalization of SOM and NPY in many nonpyramidal neurons in parahippocampal regions is also consistent with previous reports (Köhler et al., 1987).

It is now well established that all major portions of the medial temporal lobe memory system are interconnected by various populations of GABAergic LRNP neurons, including many that are SOM+ (see Jinno, 2009 for a review). Thus, LRNP neurons have been shown to contribute to the following projections: (1) neocortex to perirhinal cortex (Pinto et al., 2006); (2) perirhinal cortex to entorhinal cortex (Pinto et al., 2006; Apergis-Shoute et al., 2007); (3) entorhinal cortex to hippocampus (Germroth et al., 1989; Melzer et al., 2012); (4) hippocampus to subicular and retrosplenial regions (Jinno et al., 2007; Miyashita and Rockland, 2007); and (5) subicular region to entorhinal cortex (Van Haeften et al., 1997). The finding of the present study that GABAergic LRNP neurons in the entorhinal cortex, and to a lesser extent the perirhinal cortex, project to the BLC indicates that this temporal lobe GABAergic “supernetwork” (Buzsaki and Chrobak, 1995) includes the basolateral

amygdala (Fig. 14). In fact, recent studies in our lab have shown that these connections are reciprocal; injections of FG into the entorhinal cortex label SOM+/GABA+ LRNP neurons in the BLC (McDonald and Zaric, 2015).

Only a small number of LRNPs are involved in most of the interconnections of the temporal lobe GABAergic supernetwork. In the present study we found that only 2% of entorhinal projection neurons were LRNP neurons, and they constituted only 2% of the total GABAergic population. These percentages are very similar to those exhibited by the perirhinal to entorhinal cortex projection where 3% of perirhinal projection neurons were LRNP neurons, and they constituted 2% of the total GABAergic population (Apergis-Shoute et al., 2007). However, electron microscopic studies found that 12% of the axons involved in the latter projection formed symmetrical synapses typical of inhibitory inputs, which suggests that these perirhinal LRNP neurons have axons that branch extensively (Pinto et al., 2006). It will be of interest to perform similar quantitative ultrastructural analyses of entorhinal inputs to the BLC, as well as electrophysiological studies of these projections, to assess their ability to inhibit amygdalar activity. It will also be important to determine the neuronal targets of parahippocampal LRNP inputs to the BLC. Either pyramidal neurons (Pinto et al., 2006; Jinno et al., 2007) or nonpyramidal neurons (Van Haefen et al., 1997; Melzer et al., 2012) have been identified as targets of LRNP neurons in different hippocampal/parahippocampal circuits.

Like other GABAergic LRNP neurons in hippocampal/parahippocampal regions, the parahippocampal LRNP neurons that project to the BLC are most likely involved in synchronizing oscillatory activity between the two regions (Buzsaki and Chrobak, 1995; Jinno, 2009; Caputi et al., 2013). It is of interest in this regard that the axons of hippocampal GABAergic LRNP neurons have a larger diameter and thicker myelin sheath than pyramidal cell axons, which suggests that these faster inhibitory inputs may reset the excitability and oscillatory phase of their targets just before the slower excitatory pyramidal cell inputs arrive (Jinno et al., 2007). Thus it seems possible that GABAergic entorhinal and perirhinal LRNP neurons projecting to the BLC could be involved in the synchronization of a variety of oscillations, including delta, theta, gamma, and slow oscillations between the BLC and rhinal cortices (Paré and Gaudreau, 1996; Paré et al., 2002; Bauer et al., 2007). These oscillations could entrain synchronous firing of rhinal cortical and BLC pyramidal neurons, thus facilitating functional interactions between them, including synaptic plasticity. In fact, various aspects of fear learning involve synchronization of theta activity in the lateral amygdalar nucleus and dorsal hippocampus (Seidenbacher et al. 2003; Pape et al., 2005; Narayanan et al., 2007a, b). Since the dorsal hippocampus and lateral nucleus are not directly interconnected, the synchronization of theta activity between these structures may involve a relay in the entorhinal cortex (Mizuseki et al., 2009). Connections between the BLC, hippocampus, and the medial prefrontal cortex (mPFC) are critical for contextual regulation of fear learning and extinction (Orsini et al., 2011). Because coupled theta activity increases in all three regions during fear retrieval (Lesting et al., 2011), it is surprising that no LRNP neurons were observed in the mPFC with FG injections into the BLC in the present study. It is possible that synchronization between the BLC and mPFC depends on a mechanism that does not involve LRNPs, or depends on a relay in the rhinal cortices. However, to the authors' knowledge there have been no studies that have

investigated whether connections between the mPFC and hippocampal/parahippocampal regions involve GABAergic LRNP neurons.

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Abbreviations used in the text and figures

ACo	anterior cortical nucleus
AHA	amygdalohippocampal area
APir	amygdalopiriform transition area
AStr	amygdalostratial transition area
BLa	anterior basolateral nucleus
BLp	posterior basolateral nucleus
BLv	ventral basolateral nucleus
BMa	anterior basomedial nucleus
BMp	posterior basomedial nucleus
CL	lateral central nucleus
CLC	lateral capsular central nucleus
CM	medial central nucleus
CP	caudate-putamen
DLEA	dorsolateral entorhinal area
ec	external capsule
EN	endopiriform nucleus
FG	Fluorogold
IN	intercalated nucleus
L	lateral nucleus
LAT	lateral nucleus
Ld	dorsolateral lateral nucleus
Lv	ventromedial lateral nucleus
Lvm	ventromedial lateral nucleus
LOT	nucleus of the lateral olfactory tract
LRNP	long-range nonpyramidal
Mad	anterodorsal medial nucleus

Mav	anteroventral medial nucleus
Mpd	posterodorsal medial nucleus
Mpv	posteroventral medial nucleus
NPY	neuropeptide Y
PC	piriform cortex
PLCo	posterolateral cortical nucleus
PMCo	posteromedial cortical nucleus
PRh	perirhinal cortex
SOM	somatostatin
st	stria terminalis
VLEA	ventrolateral entorhinal area
VMEA	ventromedial entorhinal area
VSub	ventral subiculum

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Highlights

- 2% of cells in entorhinal cortex that project to the BLC contain somatostatin (SOM)
- Some of these nonpyramidal neurons also express NPY and GABA
- None of these nonpyramidal neurons express parvalbumin or VIP
- SOM+/NPY+ neurons projecting to the BLC are also seen in other amygdalar nuclei
- No neurons in prefrontal cortex that project to the BLC contain SOM, NPY or GABA

Figure 1.

Locations of Fluorogold injection sites in the amygdala. The extent of each injection site is plotted at multiple levels of the amygdala at 0.5 mm intervals from rostral (Bregma -1.8) to caudal (Bregma -4.3). A) Location of injections in cases R5, R8 and R14. B) Location of injections in cases R10, R12, and R15. Scale bar (lower left corner of B) = 0.5mm.

Figure 2.

(A) Photomicrograph of the FG injection site in case R10 in a Nissl-counterstained immunoperoxidase preparation. The core of the injection site is indicated by the white border. At this level the injection mainly involved the anterior and posterior subdivisions of the basolateral nucleus and the ventromedial subdivision of the lateral nucleus (compare with Fig. 1B). The core of the injection was entirely confined to these nuclei, but the peripheral halo extended into the external capsule laterally and the intermediate capsule medially. (B) Higher power view of the region dorsal to the injection site in R10 in a section just caudal to that shown in A. Saturation of the red color has been slightly reduced to enable better distinction between black retrogradely-labeled neurons and pink unlabeled Nissl-stained neurons. Note black retrogradely-labeled neurons in the dorsolateral subdivision of the lateral nucleus (Ld), including a cluster of retrogradely-labeled neurons (arrow) adjacent to the external capsule (ec). This cluster is suggestive of the dorsolateral intercalated nucleus, but closer examination revealed that the labeled neurons were medium-sized pyramidal neurons rather than the small principal neurons of the intercalated nuclei. None of the small principal neurons of the dorsomedial intercalated nucleus (outlined by asterisks) are labeled, although one large retrogradely labeled neuron is located along its dorsal border (arrowhead); one of the dendrites of this neuron extends medially along the

dorsal border of this intercalated nucleus. The dorsomedial intercalated nucleus is known to project to the ventromedial intercalated nucleus located between the BLA and the central nucleus (Royer et al., 2000), which was in the peripheral halo of the injection site. The lack of retrograde labeling in the dorsomedial intercalated nucleus is consistent with our suggestion that only the core of the injection site should be considered the effective injection site. (C) Large retrogradely labeled neurons (arrows) associated with the rostral intercalated nuclei (IN) located just anterior to the BLA were seen in all of the cases in this study (Case R14 depicted). The numerous small principal neurons of the IN are not retrogradely labeled, but do exhibit a light non-specific nuclear label in this particular section. Scale bars = 300 μm in A, 100 μm in C (B is the same magnification as C).

Figure 3.

Confocal immunofluorescence photomicrographs of FG+ retrogradely-labeled neurons (green), SOM immunoreactive neurons (red), and NPY immunoreactive neurons (blue) in the region surrounding the rostral intercalated nucleus (located just anterior to the BLA) in case R14 (A; Bregma -1.3 level), and in the amygdaloatrial transition area in case R10 (B; Bregma -3.3 level). FG+/SOM+/NPY+ triple-labeling is white. A1) White triple-labeled FG+/SOM+/NPY+ neurons (arrows) located along the border and dorsal to the rostral intercalated nucleus (IN), which has a high density of NPY+ axons. Magenta SOM+/NPY+ neurons that are not FG+ (arrowheads) are also seen in this location. A2) The same field as A1, but with the green channel deleted. B1) Three white triple-labeled FG+/SOM+/NPY+ neurons (arrows) located in the amygdaloatrial transitional area dorsomedial to the BLA and dorsolateral to the lateral subdivision of the central nucleus (CL). Note single-labeled FG+ neurons in BLA (green) and single-labeled SOM+ neurons in CL (red). B2) The same field as B1, but with the green channel deleted. Scale bar (in A2) = 50 μ m for all photomicrographs.

Figure 4.

Sections arranged from rostral (upper left, Bregma -1.3 level) to caudal (lower right, Bregma -7.0 level) depicting the locations of retrogradely labeled FG+ neurons expressing both SOM and NPY (red dots) or just SOM (blue dots) in case R10. The center of the injection site is shown in green (see Fig. 1 to see the full rostrocaudal extent of the injection site). Each dot represents one neuron. Neurons are plotted from two sections at most levels; asterisks in the lower left corner of a pane indicate levels at which only one section was available for plotting. See abbreviation list for abbreviations. Templates are modified from the atlas by Paxinos and Watson (1997).

Figure 5.

Sections arranged from rostral (upper left, Bregma -1.3 level) to caudal (lower right, Bregma -7.0 level) depicting the locations of retrogradely labeled FG+ neurons expressing both SOM and NPY (red dots) or just SOM (blue dots) in case R14. The center of the injection site is shown in green (see Fig. 1 to see the full rostrocaudal extent of the injection site). Each dot represents one neuron. Neurons are plotted from two sections at most levels; asterisks in the lower left corner of a pane indicate levels at which only one section was available for plotting. See abbreviation list for abbreviations. Templates are modified from the atlas by Paxinos and Watson (1997).

Figure 6.

Sections arranged from rostral (upper left, Bregma -1.3 level) to caudal (lower right, Bregma -7.0 level) depicting the locations of retrogradely labeled FG+ neurons expressing both SOM and NPY (red dots) or just SOM (blue dots) in case R15. The center of the injection site is shown in green (see Fig. 1 to see the full rostrocaudal extent of the injection site). Each dot represents one neuron. Neurons are plotted from two sections at most levels; asterisks in the lower left corner of a pane indicate levels at which only one section was available for plotting. See abbreviation list for abbreviations. Templates are modified from the atlas by Paxinos and Watson (1997).

Figure 7. Sections arranged from rostral (upper left, Bregma -1.3 level) to caudal (lower right, Bregma -7.0 level) depicting the locations of retrogradely labeled FG+ neurons expressing both SOM and NPY (red dots) or just SOM (blue dots) in case R8. The center of the injection site is shown in green (see Fig. 1 to see the full rostrocaudal extent of the injection site). Each dot represents one neuron. Neurons are plotted from two sections at most levels; asterisks in the lower left corner of a pane indicate levels at which only one section was available for plotting. See abbreviation list for abbreviations. Templates are modified from the atlas by Paxinos and Watson (1997).

Figure 8. Sections arranged from rostral (upper left, Bregma -1.3 level) to caudal (lower right, Bregma -7.0 level) depicting the locations of retrogradely labeled FG+ neurons expressing both SOM and NPY (red dots) or just SOM (blue dots) in case R5. The center of the injection site is shown in green (see Fig. 1 to see the full rostrocaudal extent of the injection site). Each dot represents one neuron. Neurons are plotted from two sections at most levels; asterisks in the lower left corner of a pane indicate levels at which only one section was available for plotting. See abbreviation list for abbreviations. Templates are modified from the atlas by Paxinos and Watson (1997).

Figure 9.

Confocal immunofluorescence photomicrographs of FG+ retrogradely-labeled neurons (green), SOM immunoreactive neurons (red), and NPY immunoreactive neurons (blue) in the PLCo in case R10 (A, Bregma -2.3 level) and the piriform cortex in case R8 (B; Bregma -3.3 level). FG+/SOM+/NPY+ triple-labeling is white. A1) A single white triple-labeled FG+/SOM+/NPY+ neuron (arrow) in the PLCo is surrounded by numerous non-FG-labeled SOM+ neurons (red). A2) The same field as A1, but with the green channel deleted. Neurons with SOM/NPY colocalization are magenta. B1) A single white triple-labeled FG+/SOM+/NPY+ neuron (arrow) in the piriform cortex. B2) The same field as B1, but with the green channel deleted. Neurons with SOM/NPY colocalization are magenta. Scale bar (in B2) = 50 μm for all photomicrographs.

Figure 10.

(A) Photomicrograph showing the locations of FG+ retrogradely-labeled neurons (black) at the Bregma -6.3 level in case R10 in a Nissl-counterstained immunoperoxidase preparation. Note dense retrograde labeling in the perirhinal cortex (PRh; mainly layers II and VI), the deep layers (layers V and VI) of the dorsolateral and ventrolateral entorhinal cortex (DLEA and VLEA, respectively), and the ventral subiculum (VSub). Fewer FG+ neurons are seen in the ventromedial entorhinal cortex (VMEA) and layer III of the DLEA and PRh. (B1) Confocal micrograph of the VLEA in case R10 showing labeling for FG (green), SOM (red) and NPY (blue). Note dense FG retrograde labeling confined to layers V and VI. One FG+ neuron expresses SOM (yellow; arrow). Also in the field is a double-labeled SOM+/NPY+ neuron that does not contain FG (magenta; arrowhead). (B2) The same field shown in B1, but with the green channel deleted to enable easier appreciation of SOM and NPY staining. Note SOM expression, but not NPY expression, in the FG+/SOM+ neuron shown in B1 (arrow). Scale bar = 300 μ m in A, 50 μ m in B.

Figure 11.

Confocal immunofluorescence photomicrographs of FG+ retrogradely-labeled neurons (green), SOM immunoreactive neurons (red), and NPY immunoreactive neurons (blue) in the amygdalopiriform transition area (APIR) in cases R15 (A) and R12 (B), and in the VLEA in case R10 (C). FG+/SOM+/NPY+ triple-labeling is white. FG+/SOM+ structures that are not NPY+ are yellow. (A1) A single FG+/SOM+ neuron (arrow) in APIR. (A2) The same field as A1, but with the green channel deleted. (B1) A single FG+/SOM+/NPY+ neuron (arrow) in APIR. (B2) The same field as B1, but with the green channel deleted. (C1) A single FG+/SOM+ neuron (arrow) in the VLEA. (C2) The same field as A1, but with the green channel deleted. Scale bar (in C2) = 50 μ m for all photomicrographs.

Figure 12.

Confocal immunofluorescence photomicrographs of FG+ retrogradely-labeled neurons (green), SOM immunoreactive neurons (red), and GABA immunoreactive neurons (blue) in the DLEA in case R15 (A) and in the VLEA in case R14 (B). FG+/SOM+/GABA+ triple-labeling is white. FG+/SOM+ structures that are not GABA+ are yellow. (A1) A single FG+/SOM+/GABA+ neuron (arrow) in DLEA. (A2) The same field as A1, but only showing SOM+ structures. (A3) The same field as A1, but only showing GABA+ structures. (B1) A FG+/SOM+/GABA+ neuron (arrow) and a FG+/SOM+/GABA- neuron (arrowhead) in the VLEA. (B2) The same field as B1, but only showing SOM+ structures. (B3) The same field as B1, but only showing GABA+ structures. Scale bar (in B3) = 20 μ m for all photomicrographs.

Figure 13. Confocal immunofluorescence photomicrograph of FG+ retrogradely-labeled neurons (green), PV immunoreactive neurons (red), and VIP immunoreactive neurons (cyan) in the DLEA in case R8. All immunoreactive neurons are single-labeled. Scale bar = 20 μ m.

Medial Temporal Lobe GABAergic Network and its Connections with the Basal Forebrain

Figure 14.

Schematic diagram illustrating interconnections between the amygdala, hippocampal/parahippocampal areas, and basal forebrain that are mediated in part by GABAergic projection neurons, most of which also express SOM (shown in red). The series of glutamatergic projections starting at the entorhinal cortex and extending through the hippocampal formation, including the “trisynaptic circuit”, are indicated by black arrows (all other glutamatergic projections are not shown). GABAergic projections from the basal forebrain to the amygdala (from the substantia innominate and ventral pallidal regions; SI/VP) and hippocampus (from the medial septum) are shown in blue; these neurons also express PV. The SOM+ projection to the basolateral amygdala seen in the present study is indicated by an asterisk. Numbers in other connections of the amygdala refer to the following studies: (1) McDonald and Zaric, 2015; (2) McDonald et al., 2012; (3) Mascagni and McDonald, 2009 and Muller et al., 2011; (4) Müller et al., 2011. See reviews by Jinno (2009) and Caputi and coworkers (2013) for the studies demonstrating the connections that are not designated by numbers.

Table 1

Percentages of Fluorogold-labeled somatostatin-positive nonpyramidal neurons in the amygdalopiriform transition area (APIR) and entorhinal area (EA) that were also NPY-positive.

Case	Nuclei injected ²	APIR	EA	APIR+EA Combined
R5	LAT	0% (0/5)	10% (1/10)	7% (1/15)
R8	BLp, BLa	100% (5/5)	75% (6/8)	85% (11/13)
R10	BLa, LAT, BLp	50% (5/10)	30% (3/10)	40% (8/20)
R12	BLp, BMp, LAT	9% (1/11)	80% (8/10)	43% (9/21)
R14 ^I	BLp, BLa, LAT, BMp	67% (4/6)	22% (3/14)	35% (7/20)
R15 ^I	BLa, BLp, LAT	75% (3/4)	47% (9/19)	52% (12/23)
Total		44% (18/41)	42% (30/71)	43% (48/112)

^I Cases that received colchicine injections

² Nuclei are listed in the order of their extent of involvement (from greatest to least, see Fig. 1)

Table 2

Percentages of neurons in the DLEA and VLEA that exhibited various combinations of Fluorogold (FG), somatostatin (SOM), and GABA immunoreactivity in cases R14 and R15.

Area	Brain	% FG+ neurons that were SOM+	% FG+/SOM+ neurons that were GABA+	% SOM+ neurons that were FG+	% GABA+ neurons that were FG+	% SOM+ neurons that were GABA+ (both FG+ and FG-)	% GABA+ neurons that were SOM+ (both FG+ and FG-)
DLEA	R14	2.3% (6/263)	50.0% (3/6)	8.3% (6/72)	2.9% (3/103)	40.3% (29/72)	28.6% (29/103)
	R15	2.4% (6/253)	33.3% (2/6)	10.5% (6/57)	1.7% (2/120)	45.6% (26/57)	21.7% (26/120)
	Total	2.3% (12/516)	41.7% (5/12)	9.3% (12/129)	2.2% (5/223)	42.6% (55/129)	24.7% (55/223)
VLEA	R14	0.9% (2/224)	50.0% (1/2)	3.2% (2/63)	1.1% (1/91)	44.4% (28/63)	30.8% (28/91)
	R15	2.6% (5/195)	60.0% (3/5)	6.9% (5/73)	2.7% (3/111)	52.1% (38/73)	34.2% (38/111)
	Total	1.7% (7/419)	57.1% (4/7)	5.2% (7/136)	2.0% (4/202)	48.5% (66/136)	32.7% (66/202)
	Combined	2.0% (19/935)	47.4% (9/19)	7.2% (19/265)	2.1% (9/425)	45.7% (121/265)	28.5% (121/425)