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Limited Convergence of Rhinal Cortical and Dopaminergic Inputs in the Rat Basolateral Amygdala: An Ultrastructural Analysis

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Abstract

The basolateral nuclear complex of the amygdala (BLC) receives robust sensory inputs from the rhinal cortices (RCx) that are important for the generation of emotional behavior. The BLC is also one of the main targets of the mesolimbic dopamine (DA) system. DA potentiates cortical sensory inputs to the BLC, which leads to an increase in the excitability of BLC pyramidal cells. These findings suggest that there may be convergence of RCx and DA inputs onto the dendrites of pyramidal cells in the BLC. In the present study we used dual-labeling immunohistochemistry and anterograde tract-tracing at the ultrastructural level to test this hypothesis in the rat brain. RCx axons were labeled by Phaseolus vulgaris leucoagglutinin (PHA-L) injections, whereas tyrosine hydroxylase (TH) was used as a marker for DA axons. The extent of convergence of these axons was analyzed in the posterior subdivision of the basolateral nucleus (BLp), which is densely innervated by both inputs. RCx synapses were asymmetrical and mainly contacted dendritic spines (86.4%) and dendritic shafts (12.1%). TH-positive (TH+) terminals also mainly formed synapses (symmetrical) and appositions with spines and shafts of dendrites. However, ultrastructural analysis found a very low percentage of RCx terminals converging with DA terminals onto unlabeled dendrites (9.4%) and axons (7.5 %), or exhibiting direct contacts with TH+ terminals (3.8%). These findings suggest that the association of specific behaviorally-salient sensory stimuli with dopamine release in the BLC is not dependent on a point-to-point spatial relationship of cortical and DA inputs.

Keywords

electron microscopy; immunocytochemistry; tyrosine hydroxylase; PHA-L tract tracing

1. Introduction

Activation of the basolateral nuclear complex of the amygdala (BLC) by sensory cortical inputs is important for the generation of emotional behavior (Armony et al., 1997; Campeau and Davis, 1995; LeDoux et al., 1990; Uwano et al., 1995). In contrast, activation of medial

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prefrontal cortical (mPFC) inputs to the BLC suppresses many of these amygdala-dependent behaviors (Dias et al., 1996; Morgan and LeDoux, 1995). The entorhinal and perirhinal cortices (i.e. the rhinal cortices, RCx) receive highly processed sensory information from both unimodal and polymodal areas (Suzuki and Amaral, 1994; Burwell et al., 1995; Kerr et al., 2007; Furtak et al., 2007). As part of the medial temporal lobe memory system (Squire and Zola-Morgan, 1991), the RCx integrates and transfers neocortical information to the hippocampus (Burwell et al., 1995) and amygdala (Insausti et al., 1987; Russchen, 1982; Ottersen, 1982). The perirhinal cortex and the lateral entorhinal area provide robust inputs to the lateral aspect of the basolateral nucleus, including the posterior subdivision of the basolateral nucleus (BLp) (Shi and Cassell, 1999; McDonald and Mascagni, 1997; McDonald, 1998). RCx-BLC circuits are involved in the coordination of emotional responses including autonomic, endocrine and behavioral components (Aggleton, 1993; Aggleton and Mishkin, 1986) and are important in driving fast oscillatory activity during learning (Bauer et al., 2007; Collins et al., 2001). RCx-BLC connections may be important for linking declarative memory with implicit emotional memory (Suzuki, 1996).

The amygdala is also one of the main targets of the mesolimbic dopamine (DA) system (Sadikot and Parent 1990; Fallon and Ciofi 1992; Asan 1997). Dopaminergic inputs to the BLC arise in the ventral tegmental area and substantia nigra (Fallon & Ciofi, 1992). Release of DA during stress is much higher in the BLC than in other targets of the mesolimbic DA system (Coco et al., 1992; Inglis and Moghaddam, 1999), and dopaminergic projections to the BLC are critical for fear conditioning and other aversive behaviors (Pezze and Feldon, 2004; LaLumiere et al., 2004). The DA system also plays an important role in modulating cortical input into the BLC. During fear conditioning, DA reduces mPFC input and potentiates sensory cortical input to the amygdala, which subsequently leads to an increase in the activity of BLC pyramidal cells (Rosenkranz and Grace, 2001; Rosenkranz and Grace 2002a; 2002b).

The main postsynaptic targets of DA inputs to the BLC are distal dendrites and spines of pyramidal cells that often receive other asymmetrical (excitatory) inputs (Asan, 1997; Pinard et al., 2008; Muller et al., 2009). One source of excitatory inputs to the spines and distal dendrites of BLC pyramidal cells is the cerebral cortex (Hall, 1972; Smith and Paré, 1994; Brinley-Reed et al., 1995; Farb and LeDoux, 1999; Smith et al., 2000). Ultrastructural analysis of mPFC projections to the BLC has demonstrated that there is limited convergence of mPFC and DA terminals onto the same postsynaptic neuron (Pinto and Sesack, 2008). However, there have been no studies examining the convergence of sensory cortical and DA terminals onto the same neurons in the BLC. The present study combined tract-tracing and immunocytochemical methods at the ultrastructural level to characterize the patterns of synaptic connectivity between RCx and DA terminals in the BLp. The BLp was chosen because this area receives a dense innervation by both RCx and DA fibers. Given the fact that DA potentiates sensory cortical inputs, and the importance of both sensory cortical input and dopaminergic input in modulating pyramidal cell activity in the BLC (Rosenkranz and Grace, 1999), we hypothesized that RCx terminals and DA terminals would frequently converge on the same postsynaptic structure in the BLp.

2. Results

Bilateral PHA-L injections into the RCx in 4 rats produced 6 injection sites that were confined to the perirhinal and dorsolateral entorhinal cortex (Fig. 1A). The pattern of PHA-L-labeled fibers in the amygdala was very similar to that seen in previous anterograde studies of these cortical areas (Shi and Cassell, 1999; McDonald and Mascagni, 1997; McDonald, 1998). Thus, in sections processed using Very Intense Purple (PUR) as a chromogen (Fig. 1C), or in adjacent sections using nickel-enhanced DAB as a chromogen (Fig. 1B), the

density of RCx axons in the lateral nucleus and in lateral portions of the BLp was greater than the density of fibers in more medial BLp areas or in the anterior subdivision of the basolateral nucleus (BLa).

Sections through the lateral BLp from 3 animals with injections confined to the perirhinal and entorhinal cortex were used for electron microscopy (Fig. 2). At the ultrastructural level, RCx terminals in the BLp contained 4-20 large grains of particulate PUR reaction product when observed in serial sections (Figs. 3A-D). RCx terminals were round to ovoid in shape, 0.5-1.2 μm in diameter, and were characterized by small vesicles that were round and clear. In all preparations, smaller grains of PUR reaction product were sometimes observed in dendrites and glia cells adjacent to or in close proximity to the labeled terminal (Fig. 3C). This label is most likely due to transcellular transport of the tracer into these structures (Ruda and Coulter, 1982), and has also been observed in other anterograde tract-tracing investigations, including ultrastructural cortical/dopaminergic convergence studies of the striatum using wheat germ agglutinin-conjugated horseradish peroxidase as an anterograde tracer (Sesack and Pickel, 1990). When followed in a limited number of serial sections ($n = 3-7$), 67.3% (66/98) of RCx terminals formed synapses. All RCx synapses observed were asymmetrical. Out of all synaptic contacts, 86.4% (57/66) were onto spines (Figs. 3B and D) and 12.1% (8/66) were onto dendritic shafts. Most of the latter (6/8) were small-caliber ($< 1 \mu\text{m}$) distal dendrites. One RCx terminal formed a synapse with a cell body.

TH+ terminals containing diffuse DAB reaction product were easily distinguishable from RCx terminals containing particulate PUR reaction product. TH+ terminals were round to ovoid in shape, 0.4-1.0 μm in diameter, and were characterized by densely-packed, small vesicles that were round and clear (Figs. 3A-D), as described in previous studies (Asan, 1997; Pinard et al., 2008; Muller et al., 2009). Most synapses formed by TH+ terminals were with small-caliber dendritic shafts and spines, as in previous studies (Asan, 1997; Pinto and Sesack, 2008; Pinard et al., 2008; Muller et al., 2009).

Electron microscopic analysis of the BLp revealed that 54.1% [53/98] of RCx axons were within a 5.3 μm distance of TH+ axons (see Experimental Procedures; Table 1). Although in most cases RCx and TH+ axon terminals did not converge onto the same structure (Figs. 3A, B), in five cases RCx and TH+ axons contacted the same dendrite (9.4% [5/53]; Figs. 3C, D; Table 1). The targets and types of contacts formed by converging terminals in these five instances are shown in Table 2. The dendritic shafts often gave rise to spines, indicating that they belonged to pyramidal cells (Figs. 3B, C). In addition, RCx and TH+ axons sometimes contacted the same unlabeled axon or axon terminal (7.5% [4/53]), or each other (3.8% [2/53]).

3. Discussion

3.1 Rhinal cortical inputs to the BLC

The present study is the first investigation to study the possible convergence of RCx and DA terminals in the rat BLC. Our light microscopic observations are consistent with earlier studies of the RCx projections to the BLC (McDonald, 1998; Shi and Cassell, 1999). These previous investigations found that the projections to the lateral BLp arise mainly from the dorsolateral entorhinal cortex and the ventral, but not the dorsal, subregion of the perirhinal cortex. Our electron microscopic findings indicate that RCx afferents to the BLC mainly synapse with dendritic spines of presumptive pyramidal cells (86% of RCx terminals) via asymmetrical (presumptive glutamatergic) synapses. These results are consistent with previous electron microscopic studies of RCx afferents in both the cat and monkey (Smith et al., 2000), and electrophysiological studies demonstrating excitation of BLC pyramidal cells by RCx stimulation in the cat (Lang and Paré, 1997). Likewise, the predominant targets of

prefrontal afferents to the BLC in the rat and monkey (Brinley-Reed et al., 1995; Smith et al., 2000; Pinto and Sesack, 2008) and temporal cortical afferents in the cat (Hall, 1972) are spines. Thus, in all species and cortical areas examined to date, cortical inputs to the BLC mainly target dendritic spines via asymmetrical synapses. There is evidence that the great majority of dendritic spines belong to pyramidal cells (Muller et al, 2006).

3.2 Spatial relationships of dopaminergic inputs and rhinal cortical inputs in the BLC

Dendritic spines are also a prominent target of DA inputs to the BLC, but these inputs target distal dendritic shafts of identified pyramidal cells to a much greater degree than those of cortical afferents (Muller et al., 2009; see also Asan, 1997 and Pinard et al., 2008). The frequent observation (Muller et al., 2009) of TH+ inputs to spines and distal dendrites of BLC pyramidal cells that also receive asymmetrical synapses from unlabeled terminals, or terminals immunoreactive for calcium-calmodulin kinase II, an enzyme expressed by cortical pyramidal cells (and BLC pyramidal cells), suggested to us that RCx cortical inputs and DA inputs to the BLC might converge on dendrites of BLC pyramidal cells, thus accounting for the ability of DA to potentiate sensory inputs to this brain region (Rosenkranz and Grace 1999, 2001, 2002a; Grace and Rosenkranz, 2002). Converging glutamatergic and DA inputs onto spines are common in the striatum, where cortical, thalamic and BLC afferents are in a position to be influenced by dopaminergic inputs (Freund et al., 1984; Bouyer et al., 1984; Sesack and Pickel, 1990, 1992; Smith et al., 1994; Johnson et al., 1994; Moss and Bolam, 2008). These inputs often form a “triad” consisting of a spine that is postsynaptic to a DA axon terminal forming a symmetrical synapse and another (putative glutamatergic) axon terminal forming an asymmetrical synapse (Goldman-Rakic et al. 1989, 1995). It has been suggested that the formation of triads on striatonigral projection neurons in the rat selectively alters the spread of excitatory signals (Freund et al., 1984).

Our analysis of the BLp found very limited convergence of RCx terminals and DA terminals onto unlabeled dendrites (9.4%) and axons (7.5 %), or direct contacts between these two types of terminals (3.8%). Although serial sections (n = 3-7) were analyzed, the low rate of convergence may under-represent the actual number of RCx and DA profiles contacting common dendrites, since complete terminal reconstruction was not conducted. This is particularly true for observing convergence onto dendritic shafts, which could easily be missed if the plane of section was not favorable for observing convergence. For example, the convergence shown in Fig. 3C would not be seen in 3-7 serial sections through the RCx terminal in a plane of section perpendicular to that depicted. Convergent inputs from DA terminals and medial prefrontal (mPFC) afferents in the BLa are also not common; only 17.3% of mPFC and TH+ profiles contact the same dendrite (Pinto and Sesack, 2008). In contrast, the extent of hippocampal/DA convergence (25.0%; Sesack and Pickel 1990) and mPFC/DA convergence (41.1%; Sesack and Pickel, 1992) in the nucleus accumbens was higher.

In the primate and rat mPFC, many spines receive both asymmetrical synaptic input and convergent symmetrical synaptic input from dopaminergic terminals, forming synaptic triads (Goldman-Rakic et al. 1989; Verney et al., 1990; Carr and Sesack, 1996). However, the source of the excitatory input has not yet been identified. Few converging synapses have been found by DA axons and the major sources of glutamatergic projections, including the hippocampus, contralateral PFC, and BLC (Sesack et al., 2003). Similarly, our findings suggest that a possible DA effect on specific excitatory inputs mediated via triads in the BLC must be restricted to a different class of excitatory afferents other than those from the RCx. The latter afferents could include glutamatergic inputs from non-RCx portions of the cerebral cortex (Hall, 1972; Smith and Paré, 1994; Brinley-Reed et al., 1995; Farb and LeDoux, 1999; Smith et al., 2000), midline/intralaminar thalamus (Carlsen and Heimer,

1988; LeDoux et al., 1991), and internuclear and intranuclear amygdalar connections arising from BLC pyramidal neurons (Stefanacci et al., 1992; Smith and Paré, 1994; Paré et al., 1995; Smith et al., 2000).

3.3 Functional Implications

The low incidence of convergence observed in the present investigation of the BLp suggests that DA modulation of RCx glutamatergic inputs might involve primarily extrasynaptic mechanisms that depend on the distribution of DA transporters and receptors (Agnati et al., 1995; Gonon, 1997). An extrasynaptic mechanism is also supported by the fact that there is low expression of the plasma membrane DA transporter (DAT) in dopaminergic neurons projecting to the BLp (Lammel et al., 2008), and a very low density of DAT immunoreactive axons in the BLC (Ciliax et al., 1995; Revay et al., 1996). In fact, in vivo voltammetric measurements of evoked extracellular DA in the BLC indicate that the DA uptake rate is 10 times less than that exhibited by the striatum (Garris and Wightman, 1994). The “sphere-of-influence” of DA diffusion to non-synaptic sites at a concentration sufficient to stimulate DA receptors in the rat striatum has been measured to be between 2-8 μm (Gonon, 1997; Arbuthnott and Wickens, 2007; Rice and Cragg, 2008). Because of the low uptake rate of DA in the basolateral nucleus, DA can diffuse 8.5 times further than in the striatum (Garris and Wightman 1995; R. Mark Wightman, personal communication) and presumably act on more distant extrasynaptic receptors. Although double-labeling electron microscopic studies of the spatial relationships of DA terminals to DA receptors might contribute to a better understanding of extrasynaptic DA/glutamatergic interactions in the BLC, these studies have yet to be performed.

The percentage of RCx terminals within 5.3 μm of TH+ terminals in the BLp (54%) was twice the percentage of mPFC terminals within 5.3 μm of TH+ terminals in the BLa (27%; Pinto and Sesack, 2008). This is consistent with light microscopic observations (data not shown) that the TH+ axonal plexus is noticeably denser in the BLp versus the BLa, and suggests that RCx synapses in the BLp would be more likely to be modulated by DA released into the extracellular space than mPFC synapses in the BLa, as long as the compartmentalization of receptors was similar in both nuclei. In pyramidal cells of the BLa, D1 dopamine receptors were found adjacent to synaptic and non-synaptic plasma membrane surfaces in dendritic shafts, at the base of dendritic spines, in spine heads near asymmetrical synapses, and in axon terminals forming asymmetrical synapses (Pickel et al., 2006; Muly et al., 2009). Interestingly, D1 immunoreactivity is sometimes found in close proximity to immunoreactivity for NMDA-type glutamate receptors in these locations. D2 receptors were found mainly in axons forming either asymmetrical or symmetrical synapses, or in spines (Pinto and Sesack, 2008). Although no mPFC axons in the BLa were observed to synapse with D2+ spines, a small percentage of these axons (7%) were themselves D2+. The presence of dopamine receptors in axon terminals forming asymmetrical synapses leaves open the possibility that the small number of TH+ terminals in direct contact with RCx terminals might be in a favorable position to modulate glutamate release from these terminals (Bamford et al., 2004).

In conclusion, the distal dendritic compartment of BLC pyramidal cells, consisting of spines and thin distal dendrites, is characterized by dopaminergic and cortical inputs, as well as dopamine and glutamate receptors in both pre- and postsynaptic locations. However, the results of anatomical investigations to date (Pinto and Sesack, 2008; this study) indicate that there is only limited opportunity for direct synaptic or appositional interactions between cortical inputs and dopaminergic axons/receptors in the BLC. These findings suggest that the association of specific behaviorally-salient sensory stimuli with dopamine release in the BLC is not dependent on a point-to-point spatial relationship of cortical and DA inputs. Although DA might modulate specific cortical afferents via extrasynaptic receptors located

on dendritic spines, it is also possible that DA inputs could nonselectively potentiate sensory inputs via receptors on dendritic shafts. Thus, studies conducted on pyramidal neurons in both the lateral and basolateral nuclei suggest that DA may postsynaptically potentiate all non-mPFC glutamatergic inputs via D1 or D2 receptors located in the somatodendritic compartment (Rosenkrantz and Grace, 2002a; Kroner et al., 2005). Although these more proximal actions would be expected to affect all glutamatergic inputs to more distal dendritic domains, the precise timing of phasic DA release in relation to cortical inputs might confer specificity (Arbuthnott and Wickens, 2007).

4. Experimental Procedures

4.1 PHA-L injection and tissue preparation

These dual-labeling EM studies combined PHA-L anterograde tract-tracing (to label RCx inputs) with immunohistochemistry for tyrosine hydroxylase (TH; to label dopaminergic terminals). Four adult, male Sprague-Dawley rats (Harlan, Indianapolis, IN) received bilateral stereotaxic injections of Phaseolus vulgaris leucoagglutinin (PHA-L; 2.5% dissolved in 10 mM phosphate buffer, pH 8.0) into the RCx. Rats were anesthetized and placed into a stereotaxic head holder. Coordinates for cortical areas were obtained from an atlas of the rat brain (Paxinos and Watson, 1997). Three injections of PHA-L at 0.5 mm intervals along the pipette track were made in order to include extensive portions of the perirhinal and dorsolateral entorhinal cortices in each case. Injections were made iontophoretically using a high-voltage current source (Stoelting, Wood Dale, IL) set at 5.0 μ A (7 s on, 7 s off for 20 min for each of the three injections on each side). After a 7 day survival period, rats were anesthetized and 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 minute, followed by 2.0% paraformaldehyde in PB for 30 minutes). Following removal, acrolein-fixed brains were postfixed in 2.0% paraformaldehyde for 30 min, and then sectioned on a vibratome in the coronal plane at 60 μ m. Sections were rinsed in 1.0% borohydride in PB for 30 minutes, then rinsed thoroughly in several changes of PB for 1 hour, and were then processed for immunocytochemistry in the wells of tissue culture plates.

4.2 Immunocytochemical procedures for electron microscopy

A mouse monoclonal anti-TH antibody (1:750-1:1000; Chemicon International Inc., Temecula, CA) was used to label dopaminergic terminals in the BLp. This antibody, which has been used in previous studies of the dopaminergic innervation of the amygdala (Pinto and Sesack, 2008; Pinard et al., 2008; Muller et al., 2009), is very specific for dopaminergic fibers and does not label the vast majority of noradrenergic fibers in the BLC (Pinard et al., 2008). Sections were incubated in a cocktail of the mouse monoclonal anti-TH antibody and a rabbit anti-PHA-L antibody (1:1000; Vector Laboratories Inc., Burlingame, CA). All antibodies were diluted in PBS containing 1% normal goat serum (NGS). In light microscopic preparations 0.4% Triton X-100 was added to the diluent to increase antibody penetration. In electron microscopic preparations Triton X-100 was omitted because the freeze/thaw technique was used to increase antibody penetration; these sections were cryoprotected in 30% sucrose in PB and freeze-thawed twice over liquid nitrogen.

Sections were then processed for the avidin-biotin immunoperoxidase technique using a rabbit Vectastain ABC kit (Vector Laboratories). PHA-L immunoreactivity was visualized first using a Vector VIP (Very Intense Purple) peroxidase substrate kit (Vector Laboratories). This procedure yields a reaction product, which we term "PUR", that appears purple in the light microscope, and granular or particulate in the electron microscope (Smiley et al., 1997; Van Haeften and Wouterlood, 2000). The PUR reaction was performed

according to the kit's instructions, but the steps in the subsequent dehydration series were shortened to minimize extraction of the purple reaction product (Lanciego et al., 1997). After rinsing, sections were incubated in an ABC blocking solution (Avidin/Biotin Blocking kit, Vector Labs), and rinsed three times (10 min each), before being incubated in biotinylated goat anti-mouse secondary antibody for one hour (1:500; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). All sections were then processed for the avidin-biotin immunoperoxidase technique using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA). DAB (3,3'-diaminobenzidinehydrochloride; Sigma Chemical Co., St. Louis, MO) was used as a chromogen to produce a brown reaction product for TH visualization.

Vibratome sections selected for electron microscopic processing were postfixed in 1–2% osmium tetroxide in 0.16 M sodium cacodylate buffer (pH 7.4) for 45 minutes, dehydrated in graded ethanols and acetone, and flat embedded in Polybed 812 (Polysciences, Inc., Warrington, PA) in slide molds, between sheets of Aclar (Ted Pella, Inc., Redding, CA). One or two vibratome sections from each of the three animals with the densest labeling of perirhinal/entorhinal afferents were chosen for thin sectioning and subsequent electron microscopic analysis. Areas in the lateral part of the BLp were remounted onto resin blanks and trimmed to an average size of 0.3–0.5 mm by 0.5–1.0 mm. Serial silver thin sections were then collected on formvar-coated slot grids, stained with uranyl acetate and lead citrate, and examined with a JEOL-200CX electron microscope. Micrographs were taken with an AMT XR40 digital camera system (Advanced Microscopy Techniques, Danvers, MA).

4.3 Electron microscopic analysis

Only areas with robust staining for both PHA-L and TH were analyzed. These areas comprised about 50–75% of the total area of each thin section used in this study. Each PUR-labeled RCx terminal was examined in 3–7 serial thin sections, and its relationship to unlabeled structures and TH-immunoreactive (TH+) axon terminals was recorded. The following relationships were quantified: 1) the percentage of RCx terminals that contacted a structure that also received a contact from a TH+ terminal; and 2) the percentage of RCx terminals that contacted a TH+ terminal. As in previous studies of cortical/dopaminergic convergence in the striatum (Sesack and Pickel, 1990, 1992) and BLC (Pinto and Sesack, 2008), only RCx axon terminals in close proximity to TH+ axon terminals were included in these counts. This prerequisite eliminates false negatives due to unequal penetration of reagents (Sesack and Pickel, 1990, 1992). In previous studies of cortical/dopaminergic convergence in the striatum and BLC, cortical and TH+ terminals were considered to be in close proximity if they were both in the same electron micrographic field when viewed at 20,000X magnification. Since these fields were approximately $14\ \mu\text{m}^2$ (13.8 or $14.5\ \mu\text{m}^2$, depending on the study), cortical and TH+ terminals were separated by no more than $5.3\ \mu\text{m}$, assuming an essentially square field. Therefore, in order to compare the extent of RCx/DA convergence seen in our study with cortical/DA convergence seen in previous studies, we only tallied the percentage of convergence for RCx terminals that were within $5.3\ \mu\text{m}$ of TH+ terminals.

Contacts of RCx and TH+ terminals with neuronal structures were classified as either synapses or appositions. Synaptic contacts were identified based on: 1) parallel spacing of apposed membranes exhibiting membrane thickenings, 2) the presence of dense filaments in the synaptic cleft; and 3) clustered synaptic vesicles associated with the presynaptic membrane (Peters et al., 1991). Contacts that did not appear to be synaptic were termed “appositions.” Labeled profiles receiving synapses or appositions were identified as perikarya, larger-caliber ($>1\ \mu\text{m}$) and small-caliber ($<1\ \mu\text{m}$) dendrites, dendritic spines, axons, or axon terminals using established morphological criteria (Peters et al., 1991).

4.4 Light microscopic mapping of injection sites

For analysis at the light microscopy level, sections through the injection site were processed for PHA-L immunohistochemistry as described above, but nickel-enhanced DAB was used as a chromogen to produce a black reaction product (Hancock, 1986). These sections were mounted on gelatinized slides, dried overnight, counterstained with pyronin Y (a pink Nissl stain) to label neuronal perikarya, and coverslipped with Permount (Fisher Scientific, Fair Lawn, NJ). Injection sites were mapped using a Bausch and Lomb microprojector and an Olympus BX51 microscope using bright-field illumination. Cortical areas were identified using an atlas of the rat brain (Paxinos and Watson, 1997). The effective injection site (the area from which PHA-L is incorporated into neurons for anterograde transport) was defined as the area that contained PHA-L labeled perikarya (Gerfen, et al., 1989). In addition, some sections through the amygdala at regular intervals were processed for light microscopic PHA-L labeling in order to survey levels of the amygdala to be used for electron microscopic analysis.

Acknowledgments

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Abbreviations

BLC	Basolateral nuclear complex of the amygdala
DA	Dopamine
mPFC	Medial prefrontal cortex
PHA-L	Phaseolus vulgaris leucoagglutinin
PUR	Very Intense Purple reaction product
RCx	Rhinal cortices
TH	Tyrosine hydroxylase

References

- Aggleton JP. The contribution of the amygdala to normal and abnormal emotional states. *Trends Neurosci.* 1993; 19:328–333. [PubMed: 7691009]
- Aggleton, JP.; Mishkin, M. The amygdala: sensory gateway to the emotions.. In: Plutchik, R.; Kellerman, H., editors. *Emotion: Theory, Research and Experience*. Vol. 3. Academic; New York: 1986. p. 281-299.
- Agnati LF, Zoli M, Stromberg I, Fuxe K. Intercellular communication in the brain: wiring versus volume transmission. *Neuroscience.* 1995; 69:711–726. [PubMed: 8596642]
- Arbuthnott GW, Wickens J. Space, time and dopamine. *Trends Neurosci.* 2007; 30:62–69. [PubMed: 17173981]
- Armony JL, Servan-Schreiber D, Romanski LM, Cohen JD, LeDoux JE. Stimulus generalization of fear responses: effects of auditory cortex lesions in a computational model and in rats. *Cereb Cortex.* 1997; 7:157–165. [PubMed: 9087823]
- Asan E. Ultrastructural features of tyrosine-hydroxylase-immunoreactive afferents and their targets in the rat amygdala. *Cell Tissue Res.* 1997; 288:449–469. [PubMed: 9134859]
- Bamford NS, Zhang H, Schmitz Y, Wu NP, Cepeda C, Levine MS, Schmauss C, Zakharenko SS, Zablow L, Sulzer D. Heterosynaptic dopamine neurotransmission selects sets of corticostriatal terminals. *Neuron.* 2004; 42:653–663. [PubMed: 15157425]
- Bauer EP, Paz R, Paré D. Gamma oscillations coordinate amygdalo-rhinal interactions during learning. *J Neurosci.* 2007; 27:9369–9379. [PubMed: 17728450]

- Bouyer JJ, Park DH, Joh TH, Pickel VM. Chemical and structural analysis of the relation between cortical inputs and tyrosine hydroxylase-containing terminals in rat neostriatum. *Brain Res.* 1984; 302:267–275. [PubMed: 6145508]
- Brinley-Reed M, Mascagni F, McDonald AJ. Synaptology of prefrontal cortical projections to the basolateral amygdala: an electron microscopic study in the rat. *Neurosci Lett.* 1995; 202:45–48. [PubMed: 8787827]
- Burwell RD, Witter MP, Amaral DG. Perirhinal and postrhinal cortices of the rat: a review of the neuroanatomical literature and comparison with findings from the monkey brain. *Hippocampus.* 1995; 5:390–408. [PubMed: 8773253]
- Campeau S, Davis M. Involvement of subcortical and cortical afferents to the lateral nucleus of the amygdala in fear conditioning measured with fear-potentiated startle in rats trained concurrently with auditory and visual conditioned stimuli. *J Neurosci.* 1995; 15:2312–2327. [PubMed: 7891169]
- Carlsen J, Heimer L. The basolateral amygdaloid complex as a cortical-like structure. *Brain Res.* 1988; 441:377–380. [PubMed: 2451985]
- Carr DB, Sesack SR. Hippocampal afferents to the rat prefrontal cortex: synaptic targets and relation to dopamine terminals. *J Comp. Neurol.* 1996; 369:1–15. [PubMed: 8723699]
- Ciliax BJ, Heilman C, Demchyshyn LL, Pristupa ZB, Ince E, Hersch SM, Niznik HB, Levey AI. The dopamine transporter: immunochemical characterization and localization in brain. *J Neurosci.* 1995; 15:1714–1723. [PubMed: 7534339]
- Coco ML, Kuhn CM, Ely TD, Kilts CD. Selective activation of mesoamygdaloid dopamine neurons by conditioned stress: attenuation by diazepam. *Brain Res.* 1992; 590:39–47. [PubMed: 1422845]
- Collins DR, Pelletier JG, Paré D. Slow and fast (gamma) neuronal oscillations in the perirhinal cortex and lateral amygdala. *J Neurophysiol.* 2001; 85:1661–1672. [PubMed: 11287489]
- Dias R, Robbins TW, Roberts AC. Dissociation in prefrontal cortex of affective and attentional shifts. *Nature.* 1996; 380:69–72. [PubMed: 8598908]
- Fallon, JH.; Ciofi, P. Distribution of monoamines within the amygdala. In: Aggleton, JP., editor. *The Amygdala.* Academic Press; New York: 1992. p. 97-114.
- Farb CR, LeDoux JE. Afferents from rat temporal cortex synapse on lateral amygdala neurons that express NMDA and AMPA receptors. *Synapse.* 1999; 33:218–229. [PubMed: 10420169]
- Freund TF, Powell JF, Smith AD. Tyrosine hydroxylase- immunoreactive boutons in synaptic contact with identified striatonigral neurons, with particular reference to dendritic spines. *Neuroscience.* 1984; 13:1189–1215. [PubMed: 6152036]
- Furtak SC, Wei SM, Agster KL, Burwell RD. Functional neuroanatomy of the parahippocampal region in the rat: the perirhinal and postrhinal cortices. *Hippocampus.* 2007; 17:709–722. [PubMed: 17604355]
- Garris PA, Wightman RM. In vivo voltammetric measurement of evoked extracellular dopamine in the rat basolateral amygdaloid nucleus. *J Physiol.* 1994; 478:239–249. [PubMed: 7965845]
- Garris, PA.; Wightman, RM. Regional differences in dopamine release, uptake, and diffusion measured by fast-scan cyclic voltammetry. In: Boulton, AA.; Baker, GB.; Adams, RN., editors. *Voltammetric Methods.* Humana Press; New York: 1995. p. 179-212.
- Gerfen, CR.; Sawchenko, PE.; Carlsen, J. The PHA-L anterograde axonal tracing method.. In: Heimer, L.; Zaborsky, L., editors. *Neuroanatomical tract-tracing methods 2: Recent progress.* Plenum Press; New York: 1989. p. 19-47.
- Goldman-Rakic P. Cellular basis of working memory. *Neuron.* 1995; 14:477–485. [PubMed: 7695894]
- Goldman-Rakic PS, Leranth C, Williams SM, Mons N, Geffard M. Dopamine synaptic complex with pyramidal neurons in primate cerebral cortex. *Proceedings of the National Academy of Sciences of the USA.* 1989; 86:9015–9019. [PubMed: 2573073]
- Gonon F. Prolonged and extrasynaptic excitatory action of dopamine mediated by D1 receptors in the rat striatum in vivo. *J Neurosci.* 1997; 17:5972–5978. [PubMed: 9221793]
- Grace AA, Rosenkranz JA. Regulation of conditioned responses of basolateral amygdala neurons. *Physiol Behav.* 2002; 77:489–493. [PubMed: 12526988]
- Hall, E. Some aspects of the structural organization of the amygdala. Eleftheriou, BE., editor. *Plenum Press; New York: 1972. p. 95-119.*

- Hancock MB. Two color immunoperoxidase staining: visualization of anatomic relationships between immunoreactive neural elements. *Am J Anat.* 1986; 175:343–352. [PubMed: 2422916]
- Inglis FM, Moghaddam B. Dopaminergic innervation of the amygdala is highly responsive to stress. *J Neurochem.* 1999; 72:1088–1094. [PubMed: 10037480]
- Insausti R, Amaral DG, Cowan WM. The entorhinal cortex of the monkey: II. Cortical afferents. *J. comp. Neurol.* 1987; 264:356–395. [PubMed: 2445796]
- Johnson LR, Aylward RL, Hussain Z, Totterdell S. Input from the amygdala to the rat nucleus accumbens: its relationship with tyrosine hydroxylase immunoreactivity and identified neurons. *Neuroscience.* 1994; 61:851–65. [PubMed: 7530817]
- Kerr KM, Agster KL, Furtak SC, Burwell RD. Functional neuroanatomy of the parahippocampal region: the lateral and medial entorhinal areas. *Hippocampus.* 2007; 17:697–708. [PubMed: 17607757]
- Kröner S, Rosenkranz JA, Grace AA, Barrionuevo G. Dopamine modulates excitability of basolateral amygdala neurons in vitro. *J Neurophysiol.* 2005; 93:1598–1610. [PubMed: 15537813]
- Lalumiere RT, Nguyen LT, McGaugh JL. Post-training intrabasolateral amygdala infusions of dopamine modulate consolidation of inhibitory avoidance memory: involvement of noradrenergic and cholinergic systems. *Eur J Neurosci.* 2004; 20:2804–2810. [PubMed: 15548223]
- Lammel S, Hetzel A, Häckel O, Jones I, Liss B, Roeper J. Unique properties of mesoprefrontal neurons within a dual mesocorticolimbic dopamine system. *Neuron.* 2008; 57:760–773. [PubMed: 18341995]
- Lanciego JL, Goede PH, Witter MP, Wouterlood FG. Use of peroxidase substrate Vector VIP for multiple staining in light microscopy. *J Neurosci Methods.* 1997; 74:1–7. [PubMed: 9210569]
- Lang EJ, Paré D. Similar inhibitory processes dominate the responses of cat lateral amygdaloid projection neurons to their various afferents. *J Neurophysiol.* 1997; 77:341–352. [PubMed: 9120575]
- LeDoux JE, Cicchetti P, Xagoraris A, Romanski LM. The lateral amygdaloid nucleus: sensory interface of the amygdala in fear conditioning. *J Neurosci.* 1990; 10:1062–1069. [PubMed: 2329367]
- LeDoux JE, Farb CR, Milner TA. Ultrastructure and synaptic associations of auditory thalamo-amygdala projections in the rat. *Exp Brain Res.* 1991; 85:577–586. [PubMed: 1717305]
- McDonald AJ. Cortical pathways to the mammalian amygdala. *Progress in Neurobiology.* 1998; 55:257–331. [PubMed: 9643556]
- McDonald AJ, Mascagni F. Projections of the lateral entorhinal cortex to the amygdala: a Phaseolus vulgaris leucoagglutinin study in the rat. *Neuroscience.* 1997; 77:445–59. [PubMed: 9472403]
- Morgan MA, LeDoux JE. Differential contribution of dorsal and ventral medial prefrontal cortex to the acquisition and extinction of conditioned fear in rats. *Behav Neurosci.* 1995; 109:681–688. [PubMed: 7576212]
- Moss J, Bolam JP. A dopaminergic axon lattice in the striatum and its relationship with cortical and thalamic terminals. *J Neurosci.* 2008; 28:11221–11230. [PubMed: 18971464]
- Muller JF, Mascagni F, McDonald AJ. Pyramidal cells of the rat basolateral amygdala: synaptology and innervation by parvalbumin immunoreactive interneurons. *J Comp Neurol.* 2006; 494:635–650. [PubMed: 16374802]
- Muller JF, Mascagni F, McDonald AJ. Dopaminergic innervation of pyramidal cells in the rat basolateral amygdala. *Brain Struct Funct.* 2009; 213:275–88. [PubMed: 18839210]
- Muly EC, Senyuz M, Khan ZU, Guo JD, Hazra R, Rainnie DG. Distribution of D1 and D5 dopamine receptors in the primate and rat basolateral amygdala. *Brain Struct Funct.* 2009; 213:375–393. [PubMed: 19669160]
- Ottersen OP. Connections of the amygdala of the rat. IV. Corticoamygdaloid and intraamygdaloid connections as studied with axonal transport of horseradish peroxidase. *J. comp. Neurol.* 1982; 205:30–48. [PubMed: 7068948]
- Paré D, Smith Y, Paré JF. Intra-amygdaloid projections of the basolateral and basomedial nuclei in the cat: Phaseolus vulgaris-leucoagglutinin anterograde tracing at the light and electron microscopic level. *Neuroscience.* 1995; 69:567–583. [PubMed: 8552250]
- Paxinos, G.; Watson, C. *The Rat Brain in Stereotaxic Coordinates.* Academic Press; New York: 1997.

- Peters, A.; Palay, S.L.; Webster, H.D. The fine structure of the nervous system. Oxford University Press; New York: 1991.
- Pezze MA, Feldon J. Mesolimbic dopaminergic pathways in fear conditioning. Mesolimbic dopaminergic pathways in fear conditioning. *Prog Neurobiol.* 2004; 74:301–320. [PubMed: 15582224]
- Pickel VM, Colago EE, Mania I, Molosh AI, Rainnie DG. Dopamine D1 receptors co-distribute with N-methyl-D-aspartic acid type-1 subunits and modulate synaptically-evoked N-methyl-D-aspartic acid currents in rat basolateral amygdala. *Neuroscience.* 2006; 142:671–690. [PubMed: 16905271]
- Pinard CR, Muller JF, Mascagni F, McDonald AJ. Dopaminergic innervation of interneurons in the rat basolateral amygdala. *Neuroscience.* 2008; 157:850–863. [PubMed: 18948174]
- Pinto A, Sesack SR. Ultrastructural analysis of prefrontal cortical inputs to the rat amygdala: spatial relationships to presumed dopamine axons and D1 and D2 receptors. *Brain Struct Funct.* 2008; 213:159–175. [PubMed: 18340460]
- Revay R, Vaughan R, Grant S, Kuhar MJ. Dopamine transporter immunohistochemistry in median eminence, amygdala, and other areas of the rat brain. *Synapse.* 1996; 22:93–99. [PubMed: 8787132]
- Rice ME, Cragg SJ. Dopamine spillover after quantal release: rethink-ing dopamine transmission in the nigrostriatal pathway. *Brain Res Rev.* 2008; 58:303–313. [PubMed: 18433875]
- Rosenkranz JA, Grace AA. Modulation of basolateral amygdala neuronal firing and afferent drive by dopamine receptor activation in vivo. *J Neurosci.* 1999; 19:11027–11039. [PubMed: 10594083]
- Rosenkranz JA, Grace AA. Dopamine attenuates prefrontal cortical suppression of sensory inputs to the basolateral amygdala of rats. *J Neurosci.* 2001; 21:4090–4103. [PubMed: 11356897]
- Rosenkranz JA, Grace AA. Cellular mechanisms of infralimbic and prelimbic prefrontal cortical inhibition and dopaminergic modulation of basolateral amygdala neurons in vivo. *J Neurosci.* 2002a; 22:324–337. [PubMed: 11756516]
- Rosenkranz JA, Grace AA. Dopamine-mediated modulation of odour-evoked amygdala potentials during pavlovian conditioning. *Nature.* 2002b; 417:282–287. [PubMed: 12015602]
- Ruda M, Coulter JD. Axonal and transneuronal transport of wheat germ agglutinin demonstrated by immunocytochemistry. *Brain Res.* 1982; 249:237–246. [PubMed: 6182945]
- Russchen FT. Amygdalopetal projections in the cat: cortical afferent connections. A study with retrograde and anterograde tracing techniques. *J. comp. Neurol.* 1982; 206:159–179. [PubMed: 7085926]
- Sadikot AF, Parent A. The monoaminergic innervation of the amygdala in the squirrel monkey: an immunohistochemical study. *Neuroscience.* 1990; 36:431–447. [PubMed: 1977101]
- Sesack SR, Pickel VM. In the rat medial nucleus accumbens, hippocampal and catecholaminergic terminals converge on spiny neurons and are in apposition to each other. *Brain Res.* 1990; 527:266–279. [PubMed: 1701338]
- Sesack SR, Pickel VM. Prefrontal cortical efferents in the rat synapse on unlabeled neuronal targets of catecholamine terminals in the nucleus accumbens septi and on dopamine neurons in the ventral tegmental area. *J Comp Neurol.* 1992; 320:145–160. [PubMed: 1377716]
- Sesack SR, Carr DB, Omelchenko N, Pinto A. Anatomical substrates for glutamatedopamine interactions: evidence for specificity of connections and extrasynaptic actions. *Ann N Y Acad Sci.* 2003; 1003:36–52. [PubMed: 14684434]
- Shi CJ, Cassell MD. Perirhinal cortex projections to the amygdaloid complex and hippocampal formation in the rat. *J Comp Neurol.* 1999; 406:299–328. [PubMed: 10102498]
- Smiley JF, Morrell F, Mesulam MM. Cholinergic synapses in human cerebral cortex: an ultrastructural study in serial sections. *Exp Neurol.* 1997; 144:361–368. [PubMed: 9168836]
- Smith Y, Bennett BD, Bolam JP, Parent A, Sadikot AF. Synaptic relationships between dopaminergic afferents and cortical or thalamic input in the sensorimotor territory of the striatum in monkey. *J Comp Neurol.* 1994; 344:1–19. [PubMed: 7914894]
- Smith Y, Paré D. Intra-amygdaloid projections of the lateral nucleus in the cat: PHA-L anterograde labeling combined with postembedding GABA and glutamate immunocytochemistry. *J. Comp. Neurol.* 1994; 342:232–248. [PubMed: 7911130]

- Smith Y, Paré JF, Paré D. Differential innervation of parvalbumin-immunoreactive interneurons of the basolateral amygdaloid complex by cortical and intrinsic inputs. *J Comp Neurol.* 2000; 416:496–508. [PubMed: 10660880]
- Squire LR, Zola-Morgan S. The medial temporal lobe memory system. *Science.* 1991; 253:1380–1386. [PubMed: 1896849]
- Stefanacci L, Farb CR, Pitkänen A, Go G, LeDoux JE, Amaral DG. Projections from the lateral nucleus to the basal nucleus of the amygdala: a light and electron microscopic PHA-L study in the rat. *J Comp Neurol.* 1992; 323:586–601. [PubMed: 1430325]
- Suzuki WA. The anatomy, physiology and functions of the perirhinal cortex. *Curr Opin Neurobiol.* 1996; 6:179–186. [PubMed: 8725959]
- Suzuki WA, Amaral DG. Perirhinal and parahippocampal cortices of the macaque monkey: cortical afferents. *J Comp Neurol.* 1994; 350:497–533. [PubMed: 7890828]
- Uwano T, Nishijo H, Ono T, Tamura R. Neuronal responsiveness to various sensory stimuli, and associative learning in the rat amygdala. *Neuroscience.* 1995; 68:339–361. [PubMed: 7477945]
- Van Haeften T, Wouterlood FG. Neuroanatomical tracing at high resolution. *J Neurosci Methods.* 2000; 103:107–116. [PubMed: 11074100]
- Verney C, Alvarez C, Geffard M, Berger B. Ultrastructural double-labelling study of dopamine terminals and GABA-containing neurons in rat anteromedial cerebral cortex. *Eur J Neurosci.* 1990; 2:960–972. [PubMed: 12106083]

Fig. 1.

Photomicrographs depicting the results of PHA-L injections into the rhinal cortices (RCx). A) Photomicrograph of a representative injection into the perirhinal (PR) and dorsolateral entorhinal (DLEnt) cortices in case R33Rt. The effective injection site in this case was limited to these areas (compare with Fig. 2C), but diffuse neuropilar reaction product extends into the temporal association cortex (TeA) and amygdalopiriform transition area (APir). B) Fiber terminations in the lateral nucleus (La) and posterior subdivision of the basolateral amygdala (BLp), and fibers of passage in the external capsule (EC), resulting from the RCx injection shown in A (Bregma -3.3 level, left is medial). PHA-L labeled fibers were stained using the ABC peroxidase procedure using nickel-enhanced DAB as a chromogen. This section was not stained for TH. C) Higher power (grayscale) photomicrograph of the BLp in an osmicated resin-embedded section stained for both PHA-L and TH (Bregma -3.3 level). Scale bars = 100 μ m in B, 10 μ m in C.

Fig. 2.

Locations of the effective injection sites in the three cases analyzed by electron microscopy:

A) Case R33Lf; B) Case R34Lf; C) Case R33Rt (Lf: left side injection; Rt: right side injection). All injections were confined to the perirhinal (PR) and dorsolateral entorhinal (DLEnt) cortices. Images were modified from the atlas by Paxinos and Watson (2007).

Fig. 3.

Electron micrographs of the posterior subdivision of the basolateral amygdalar nucleus (BLp) immunostained for PHA-L (to identify RCx terminals) and TH (to identify DA terminals). TH⁺ terminals containing diffuse DAB reaction product are frequently found in the same field as RCx terminals containing particulate PUR reaction product. A) A RCx (RCx-t) and a TH⁺ (TH-t) axon terminal are in the same field, but are not apposed to the same dendrite (D). B) A RCx axon terminal forms an asymmetrical synapse with an unlabeled spine (Sp1) and a TH⁺ axon terminal forms a symmetrical synapse with a separate dendrite (D) that gives rise to a spine (Sp2). The synaptic nature of the contacts is more convincing in neighboring serial sections (arrows in inserts). C) A RCx axon terminal and a TH⁺ axon terminal contact the same dendrite (D). The RCx axon terminal forms a contact with the dendritic shaft which exhibited characteristics of a synapse in a neighboring serial section. The TH⁺ axon terminal forms an apposition with the base of a spine (Sp). The plasma membrane of the dendrite has a serrated appearance, suggesting that it actually gives rise to several spines. Note that this dendrite contains a sparse array of smaller grains of PUR reaction product in close proximity to the PUR-labeled RCx axon terminal that may have resulted from transneuronal transport of PHA-L. D) An RCx axon terminal forms an asymmetrical synapse with a spine (Sp) that also receives an apposition from a TH⁺ axon terminal. Scale bars = 500 nm.

Table 1

Rhinal cortical (RCx) axons in the rat BLp: association with tyrosine hydroxylase immunoreactive (TH+) axons

Total number of RCx axon terminals	98
Number of RCx axon terminals forming synapses	66/98 (67.3%)
Number of RCx axon terminals observed within 5.3 μ m of TH+ axons	53/98 (54.1%)
Number of RCx and TH+ axons contacting common unlabeled dendrites	5/53 (9.4%)
Number of RCx and TH+ axons contacting common unlabeled axons	3/53 (7.5%)
Number of RCx and TH+ axons directly apposed to each other	2/53 (3.8%)

Table 2

Targets and types of contacts formed by converging terminals of rhinal cortical (RCx) and tyrosine hydroxylase immunoreactive (TH+) axons.

RCx Terminal	Contacted Structure	RCx Contact	TH+ Contact
T-4	Dendritic shaft	Apposition	Synapse (n=2) ¹
T-5	Dendritic shaft	Apposition	Synapse (n=2) ¹
T-8	Dendritic shaft	Apposition	Apposition
T-12	Dendrite ²	Synapse w/shaft	Apposition w/spine
T-14	Spine ³	Synapse	Apposition

¹ In each case two TH+ terminals formed synapses with a dendritic shaft that was also contacted by an RCx terminal forming an apposition.

² Contacts with this dendrite are shown in Fig. 3C.

³ Contacts with this spine are shown in Fig. 3D.