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Medial Prefrontal Cortical Innervation of the Intercalated Nuclear Region of the Amygdala

Courtney R. Pinard, Franco Mascagni, and Alexander J. McDonald*

Department of Pharmacology, Physiology and Neuroscience University of South Carolina School of Medicine Columbia, SC 29208

Abstract

The projections of the infralimbic area (IL) of the medial prefrontal cortex to the intercalated nuclei (ICNs) of the amygdala are thought to form a critical component of the forebrain circuitry for fear extinction. Despite the importance of these projections, there have been no focussed anatomical studies that have investigated the extent of IL inputs to different portions of the ICN complex. The present investigation used anterograde tract tracing in the rat to study the projections of the ventromedial PFC, including the IL, to the ICNs and surrounding amygdalar regions. Immunohistochemistry for the μ -opioid receptor (MOR) was used to identify the ICNs. At rostral levels of the amygdala there was a very dense projection to a far lateral portion of the capsular subdivision of the central nucleus (CLC) located between the main and medial ICNs, but only very light projections to these ICNs and the lateral ICNs. This distinct portion of the CLC receiving strong IL inputs was termed the capsular infralimbic target zone (CITZ), and was MOR-negative. Likewise, at more caudal levels of the amygdala, IL projections to the medial, lateral and dorsal ICNs were light to moderate compared with projections to adjacent portions of the basolateral amygdala and amygdalostriatal transitional area. These findings suggest that the putative role of the IL-to-ICN connection in fear inhibition may be mediated by light to moderate projections from the IL to the medial ICN, and that the CITZ may be an equally important amygdalar target for this function.

Keywords

anterograde tract tracing; μ -opioid receptor; infralimbic area; extinction

1. Introduction

Emotional information is integrated by distinct neuronal circuits into appropriate adaptive emotional and behavioral reactions (Pape and Paré, 2010). Fear and anxiety, which represent particularly salient emotions, have been the most extensively studied because these behaviors can be easily manipulated experimentally. One of the most powerful models for

*Correspondence to: Alexander J. McDonald Telephone: 803-216-3511 Fax: 803-216-3524 alexander.mcdonald@uscmed.sc.edu.

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studying the neural mechanisms of fear and anxiety, both in animals and humans, is classical or Pavlovian fear conditioning (Maren, 2001). During fear conditioning, animals learn to associate a normally harmless (conditioned) stimulus with an aversive (unconditioned) stimulus after these stimuli are paired. The amygdala and its connected structures represent a central fear system involved in the acquisition, expression and extinction of conditioned fear (Paré et al., 2004; Ehrlich et al., 2009; Herry et al., 2010; Pape and Paré, 2010).

Presentation of the conditioned stimulus in the absence of the aversive unconditioned stimulus will lead to extinction of the fear response in previously conditioned animals. There is evidence that extinction involves the inhibition of conditioned fear rather than erasure of the original fear memory (Rescorla and Heth, 1975). Although the exact mechanisms underlying fear extinction are not clear, recent behavioral and electrophysiological studies suggest that it may rely in part on projections from the infralimbic region (IL) of the medial prefrontal cortex (mPFC) to the intercalated nuclei (ICNs) of the amygdala (Milad and Quirk, 2002; Paré et al., 2004; Quirk and Mueller, 2008; Herry et al., 2010). The ICNs are clusters of small GABAergic neurons that are found around the periphery of the rostral half of the amygdalar basolateral nuclear complex (BLC; consisting of the lateral, basolateral, and basomedial nuclei) (Millhouse, 1986; Nitecka and Ben-Ari, 1987; McDonald and Augustine, 1993; Paré and Smith, 1993a). The ICNs, especially the medial ICNs located between the BLC and the central nucleus, receive excitatory inputs from the IL and BLC, and send GABAergic projections to the central nucleus that can inhibit amygdalar outputs to hypothalamic and brainstem regions involved in the expression of conditioned fear (Paré and Smith, 1993b; Milad and Quirk, 2002; Quirk et al., 2003; Berretta et al., 2005; Marowsky et al., 2005; Pinto and Sesack, 2008; Amano et al., 2010; Amir et al., 2011). Consistent with this candidate mechanism, behavioral studies have shown that lesions of the IL or the medial ICNs block expression of extinction (Morgan et al., 1993; Quirk et al., 2000; Likhtik et al., 2008).

The results of anatomical studies of mPFC projections to the amygdala have been inconsistent or non-definitive in regards to the extent of the innervation of the ICNs. Cassell and Wright (1986) reported that infralimbic or prelimbic (PL) projections to the rat amygdala avoided the ICNs, but projected instead to regions just outside of these nuclei. In an anatomical study conducted in our laboratory (McDonald et al., 1996), unilateral IL injections of anterograde tracer produced (bilaterally) a very dense zone of labeled fibers in a restricted lateral portion of the capsular subdivision of the central nucleus (CLC) located adjacent to the BLC (see Fig. 3 of McDonald et al., 1996), and a lower density of labeled fibers in surrounding amygdalar regions. Although the sections in this study were Nissl counterstained to identify anatomical regions, the high density of fibers in the lateral CLC obscured the Nissl staining, making it difficult to determine if the densely-targeted region was one of the ICNs, or a non-ICN portion of the CLC. Similar but lighter projections were seen with PL injections (McDonald et al., 1996). Likewise, Vertes (2004) reported bilateral IL projections to the CLC. Pinto and Sesack (2008), using GABA immunostaining to identify the ICNs in the rat, found projections to one of the caudal medial ICNs with tracer injections that involved the IL and adjacent prelimbic cortex. IL projections to the ICNs have also been observed in primates (Freedman et al., 2000).

Thus, there have been no focussed anatomical studies that have investigated the extent of IL inputs to different portions of the ICN complex. The present investigation used anterograde tract tracing in the rat to study the projections of the ventromedial PFC, including the IL, to the ICNs and surrounding amygdalar regions. Immunohistochemistry for the μ -opioid receptor was used to identify the ICNs (Wilson et al., 2002; Likhtik et al., 2008). In addition, the relationship of IL inputs to D1 dopamine receptor localization in the amygdala was examined since D1 modulation of the ICNs is important for regulating ICN activity, and the ICNs express high levels of this receptor (Fuxe et al., 2003; Marowsky et al., 2005).

2. EXPERIMENTAL PROCEDURES

2.1 Injections of anterograde tracers and tissue preparation

Seven adult male Sprague-Dawley rats (250–350 gm; Harlan, Indianapolis, IN) were anesthetized with sodium pentobarbital (50 mg/kg) and placed in a stereotaxic head holder (Stoelting, Wood Dale, IL) for injections of either Phaseolus vulgaris leucoagglutinin (PHA-L; Vector Laboratories Inc., Burlingame, CA) or biotinylated dextran amine (BDA; mol. wt. 10,000; Invitrogen, Eugene, OR) into the ventral mPFC. Injection coordinates were obtained from an atlas of the rat brain (Paxinos and Watson, 2007). Bilateral iontophoretic injections of BDA (5% in 0.01 M phosphate buffer [PB], pH 7.3) or PHA-L (2.5% in 0.01 M PB, pH 7.8) were made via glass micropipettes (50 μ m inner tip diameter) using a Midgard high voltage current source set at 5.0 μ A (7 s on, 7 s off for 45 minutes). Micropipettes were left in place for 5 minutes to prevent the tracers from flowing up the pipette track and involving more dorsally located structures.

After a 10–14 day survival, rats with PHA-L injections ($n = 5$) were perfused intracardially with phosphate buffered saline (PBS; pH 7.4) containing 0.5% sodium nitrite (50 ml), followed by an acrolein/paraformaldehyde mixture (2.0% paraformaldehyde-3.75% acrolein in PB for 1 min, followed by 2.0% paraformaldehyde in PB for 30 min). Following removal, acrolein-fixed brains were postfixed in 2.0% paraformaldehyde for 1 hr. After a 5 day survival, rats with BDA injections ($n = 2$) were perfused intracardially with PBS containing 0.5% sodium nitrite (50 ml), followed by 4.0% paraformaldehyde in PBS for 20–30 min. Following perfusion, these brains were removed and postfixed for 3.5 hr in 4.0% paraformaldehyde. All brains were sectioned on a vibratome in the coronal plane at 50 μ m. Sections from acrolein-fixed brains were rinsed in 1.0% borohydride in PB for 30 min and then rinsed thoroughly in several changes of PB for 1 hr. All sections were processed for immunohistochemistry in the wells of tissue culture plates.

2.2 Dual-labeled PHA-L/MOR immunoperoxidase preparations

Dual-labeling immunoperoxidase studies combining PHA-L anterograde tract tracing (to label mPFC inputs) with μ -opioid receptor (MOR) immunohistochemistry (to label the ICNs) was performed in 5 rats. Antibodies were diluted in PBS containing 0.4% Triton X-100 and 1% normal goat serum. A one in three series of sections through the amygdala were first incubated overnight at 4°C in a rabbit anti-PHA-L antibody (1:1000; Vector Laboratories, Burlingame, CA). Sections were then rinsed in 3 changes of PBS (10 min each) and processed for the avidin-biotin immunoperoxidase technique using a rabbit ABC

kit (Vector Laboratories). Nickel-intensified DAB (3,3'-diaminobenzidine-hydrochloride; Sigma Chemical Co., St. Louis, MO) was used as a chromogen to produce a black reaction product in this first immunohistochemical sequence (Hancock, 1986). Sections were then incubated in an avidin-biotin blocking solution (Vector Laboratories) and then incubated overnight in a rabbit MOR antibody (1:1000; DiaSorin, Inc., Stillwater, MN). Sections were then processed using a rabbit ABC kit with non-intensified DAB as a chromogen to generate a brown reaction product for identification of the ICNs. Sections were mounted on gelatinized slides, dried overnight, dehydrated in ethanols, cleared in xylene, and coverslipped with Permount. Sections through the amygdala adjacent to those processed for PHA-L/MOR were processed only for MOR immunohistochemistry using non-intensified DAB as a chromogen.

Sections through the injection sites were processed for PHA-L immunohistochemistry as described above. They were then mounted on gelatinized slides, dried overnight and counter-stained with pyronin Y, a pink Nissl stain, before being coverslipped. Injection sites were mapped using a Bausch and Lomb microprojector and an Olympus BX51 microscope under bright-field illumination. Cortical areas were identified using an atlas of the rat brain (Paxinos and Watson, 2007). The effective injection site (the area from which tracer is incorporated into neurons for anterograde transport) was defined as the area that contained PHA-L labeled perikarya (Gerfen, et al., 1989).

Dual PHA-L/MOR stained sections through the amygdala were examined using an Olympus BX51 microscope. The general distribution of PHA-L+ fibers in the amygdala at 0.5 mm intervals were drawn onto templates of amygdalar sections modified from the atlas by Paxinos and Watson (2007). The fiber densities in the different ICNs were recorded using a four point scale: dense, moderate, light, or zero. "Dense" projections consisted of robust staining equal to that seen in the basolateral amygdala. "Moderate" projections consisted of staining that was substantially less than that seen in the basolateral amygdala, but substantially greater than that rated as "light". "Light" projections consisted of staining of only a few scattered fibers in the ICN examined. These evaluations were initially performed by one observer (CRP), and then checked by a second observer (AJM). The different densities of projections can be seen in the photomicrographs in the figures of this study, and can also be appreciated in camera lucida drawings of selected sections: Fig. 5A (heavy projection); Fig. 5B (moderate projection); Fig. 8 (light projection) to the ICNs. All drawings were made at 600× magnification.

In addition to the PHA-L/MOR dual-labeled preparations, we also examined the mPFC projections to the amygdalar ICNs in several single-labeled PHA-L immunoperoxidase preparations used in a previous study (McDonald et al., 1996).

2.3 Triple-labeled BDA/MOR/D1R immunofluorescence preparations

Triple-labeling immunofluorescence studies combining BDA anterograde tract tracing (to label IL inputs), MOR immunohistochemistry (to label the ICNs), and D1 dopamine receptor (D1R) immunohistochemistry was performed in 2 rats. All antibodies for these BDA/MOR/D1R preparations were diluted in a solution containing 1% normal goat serum and 0.5% Triton X-100 in PBS. Sections through the amygdala were incubated overnight at

4° C in a cocktail of rabbit anti-MOR (1:4000; DiaSorin) and rat anti-D1R antibodies (1:400; Sigma Chemical Co.). Sections were then rinsed in 3 changes of PBS (10 min each), and incubated in a cocktail of goat anti-rabbit Alexa-633 (1:400, Invitrogen, Eugene, OR) and goat anti-rat Alexa-546 (1:400, Invitrogen) antibodies for 3 hrs at room temperature. These secondary antibodies were highly cross-adsorbed by the manufacturer to ensure specificity for primary antibodies raised in each species. Sections were then rinsed in 3 changes of PBS (10 min each), and incubated in streptavidin A-488 (1:4000; Invitrogen) for 3 hrs at room temperature. Following incubation in the secondary antibodies, sections were rinsed in 3 changes of PBS (10 min each) and mounted on glass slides using Vectashield mounting medium (Vector Laboratories).

Sections were examined with a Zeiss LSM 510 Meta confocal microscope. Fluorescence of Alexa-488, Alexa-546, and Alexa-633 dyes was analyzed using filter configurations for sequential excitation/imaging via 488 nm, 543 nm, and 633 nm channels. Digital images were adjusted for brightness and contrast using Photoshop 6.0 software. An additional series of control sections from each brain was processed as described above, but with one of the primary antibodies omitted from the primary antibody cocktails in each control; no labeling was observed in the channel corresponding to the secondary antibody associated with the omitted primary antibody.

Sections through the injection sites in BDA injected rats were incubated in 0.5 % Triton X -100 in PBS for 3 hours, and ABC reagent in PBS (Vector Laboratories Inc.) for 16 hours at 4°C. Nickel-enhanced DAB was then used as a chromogen to generate a black reaction product (Hancock, 1986). Sections were then mounted on gelatinized slides, dried overnight and counter-stained with pyronin Y before being coverslipped. Injection sites were identified and mapped as described above for PHA-L injections.

3. RESULTS

3.1 Anatomy of the intercalated nuclei

MOR staining of the ICNs closely resembled that seen in previous studies (Wilson et al., 2002; Likhtik et al., 2008). These neuronal clusters could be divided into four main divisions that each extend for several mm from rostral to caudal (Marowsky et al., 2005): (1) main ICN, (2) medial ICNs, (3) lateral ICNs, and (4) dorsal ICN (Fig. 1). The main ICN is the largest intercalated nucleus. The rostral portion of this nucleus is located anterior to the basolateral nucleus, where it extends forward beneath the posterior limb of the anterior commissure. More caudally, the main ICN extends ventral to the basolateral nucleus (Fig. 1A) and splits into two parts: a lateral part that is associated with the ventral portion of the external capsule, and a medial part that extends toward the medial amygdalar nucleus, ventral to the central nucleus (Fig. 1B). The medial ICNs (mICNs) are located medial to the basolateral and lateral nuclei, and can be divided into ventral (v-mICN) and dorsal (d-mICN) portions (Fig. 1C, D). The lateral ICNs (lICNs) are located adjacent to the external capsule, lateral to the basolateral and lateral nuclei. The dorsal ICN extends along the dorsal tip of the lateral nucleus.

3.2 PHA-L injections into the ventromedial prefrontal cortex

Five rats received bilateral PHA-L injections into the infralimbic cortex (IL), with variable amounts of spread into the ventrally adjacent dorsal peduncular cortex (DP), and/or dorsally adjacent prelimbic (PL) cortex (Figs. 2, 3). Injections on the left and right sides of each animal were fairly congruent in regards to anterior-posterior level and dorsal-ventral extent. One exception was case R38, where the injection on the left side was anterior to that on the right (R38Rt) and involved extensive amounts of the medial orbital cortex (not illustrated). In all of the injections numerous neurons filled by uptake of PHA-L were found in the deep cortical layers, and in most cases many labeled neurons were also found in the superficial cortical layers as well (Fig. 3). Since previous studies have shown that IL and PL projections to the intercalated nuclear amygdalar region and basolateral amygdala are bilateral (McDonald et al., 1996; Vertes, 2004), the amygdalar projections seen on each side of the brain with the bilateral injections of the present study represent the total projections from the injection sites into both the right and left mPFC.

The projections of the mPFC to the intercalated nuclear region of the amygdala were very similar in all of the rats used in this study. Case R36 will be used as a representative case to illustrate the projections of the IL. The injection sites involved the IL on each side, with little spread into the prelimbic or dorsal peduncular cortices (Figs. 2 and 3). The amygdalar projections seen on both sides of the brain were virtually identical. The great majority of PHA-L labeled axons in the amygdala were varicose, but some were smooth. Axonal varicosities were between 0.5–2.0 μm in diameter. Since these varicosities were of the same size and shape as mPFC axon terminals forming synapses in the ICNs and basolateral amygdala (Pinto and Sesack, 2008), varicose axons were interpreted as terminal axonal segments innervating postsynaptic amygdalar neurons. Smooth axons were interpreted as nonterminal axons. The distribution of labeled axons in the amygdala closely resembled that seen in previous studies of IL projections (Hurley et al., 1991; McDonald et al., 1996; Vertes, 2004). Only the projections to the ICNs and surrounding regions will be considered in this study.

Figure 4 shows the projections to rostral portions of the amygdala (bregma -2.2 level) that were seen on the right side of brain R36. At rostral levels of the anterior basolateral nucleus (BLa), from approximately bregma -1.8 to bregma -2.4 levels, there was a dense projection to the far lateral part of the capsular subdivision of the central nucleus (CLC), along the medial border of BLa (see Figs. 4A, 4D, 5A). This capsular infralimbic target zone (CITZ) was located between the main and medial ICNs at these levels, and exhibited little if any of the MOR-ir that characterizes the ICNs (see also Fig. 1B, which shows a section that was adjacent to that shown in Fig. 4A, but stained only for MOR). There was also a moderate projection to the amygdalostratial transition area (AStr; located between the central nucleus and caudate putamen) and the adjacent ventromedial subdivision of the lateral nucleus (Lvm; Fig. 4A) at these levels. Projections to rostral portions of the main ICN (located rostral to the BLa) and caudal portions of the main ICN (located ventromedial to the BLa; Fig. 4C) were light. Likewise, very few labeled fibers were observed in the medial ICN, lateral ICNs, or dorsal ICN at this level (Figs. 4A, 4B, 5A). The projections seen on the left

side, including the projection to the CITZ, were very similar to those seen on the right side of the brain with these bilateral injections.

Figure 6 shows the projections to more caudal portions of the amygdala (bregma -2.6 level) on the right side of brain R36. The same pattern of projections was seen on the left side of this brain. There were dense projections to a continuous zone that stretched across the AStr and the adjacent ventromedial subdivision of the lateral nucleus (Lvm; Fig. 6A). The density of labeled axons dropped off precipitously at the borders of the neighboring ICNs. Thus, the density of labeled axons was light in the dorsal part of the medial ICN (Figs. 6A, 6B) and the lateral ICN (Figs. 6A, 6D), and of moderate density in the ventral part of the medial ICN that is located just lateral to the central nucleus (Figs. 6A, 6C). The moderate innervation of the ventral medial ICN is depicted in a camera lucida drawing in Fig. 5B.

As expected from previous studies of prefrontal-amygdalar projections (McDonald et al., 1996), injections of the IL that also involved significant portions of the dorsally adjacent prelimbic cortex (e.g. case R40, see Fig. 2) produced projections to the dorsal portion of the BLA, in addition to the projections to the AStr and Lvm seen with IL injections (Fig. 7B). However, as with injections confined to the IL, these projections tended to avoid entering the adjacent ICNs. Thus, at rostral levels there was a dense projection to the CITZ that largely avoided adjacent portions of the main and medial ICNs (Fig. 7A). At more caudal levels there were dense projections to the AStr and medial portions of the lateral and basolateral nuclei that largely avoided the adjacent ventromedial and dorsomedial ICNs (Fig. 7B). Very few fibers were seen in the lateral ICNs at any rostrocaudal level (Fig. 7). The dense projections of the mPFC to the CITZ, and the light to moderate projections to the medial ICNs, were also seen in the other 3 rats analysed in this study (R37, R38, and R-39; Fig. 8).

The infralimbic cortex extends from bregma 3.7 to bregma 2.5 (Paxinos and Watson, 2007). The five rats that were stained for PHA-L and MOR had PHA-L injections that collectively involved most of the rostrocaudal extent of the IL, but not its caudal pole. To determine if the caudal pole of the IL had a substantial projection to the ICNs, we examined case P-39 from a previous PHA-L study of prefrontal-amygdalar projections performed in this lab (McDonald et al., 1996). This rat received a unilateral PHA-L injection that was confined to the caudal pole of the IL (see Fig. 1A from McDonald et al., 1996). The projections to the amygdala were similar to those seen in the five PHA-L/MOR brains, but were lighter. On the ipsilateral side, there were projections to the CITZ, but relatively few fibers entered the ICNs (Fig. 9 herein). The same pattern was seen on the contralateral side, but the density of projections to the CITZ was slightly higher. Since the fiber density in the CITZ in this particular Nissl-stained brain was not extremely high, it was also possible to examine the cytoarchitecture of the CITZ (Fig. 9). It is noteworthy that neurons in the CITZ are not clustered like those of the adjacent ICNs. This non-clustered pattern was also seen in several normal (non-PHA-injected) Nissl-stained brains that were examined.

In cases that received unilateral injections into the prelimbic cortex in this previous study (e.g., case P-9, Fig. 1A of McDonald et al., 1996), there were bilateral projections to the

CITZ, but the fiber density was less than that seen with infralimbic injections (see Fig. 6, bregma levels -1.8 and -2.3 , of McDonald et al., 1996 for the ipsilateral projection).

3.3 BDA injections into the infralimbic cortex

Bilateral injections of BDA were made into the infralimbic cortex in two rats to determine if the capsular infralimbic target zone (CITZ), identified by its robust IL innervation, expressed D1 dopamine receptor (D1R) immunoreactivity. MOR-ir was used to mark the ICNs. The results were very similar on both sides of the brain in both rats. BDA-labeled axons were seen in the CITZ between the medial and main ICNs, but very few axons were seen in the adjacent ICNs. Dense D1R immunoreactivity was observed in the ICNs, but only moderate D1R immunoreactivity was observed in the CITZ (Fig. 10). This differential density of D1R immunoreactivity was also observed in several normal (non-injected) brains that were stained for D1R using immunofluorescence or immunoperoxidase techniques, and has been depicted in a previous study (see Fig. 1 of Fuxe et al., 2003).

4. DISCUSSION

This is the first tract tracing study to investigate projections from the medial prefrontal cortex to the ICNs of the amygdala using MOR as a marker to definitively identify the ICNs. The projections to the amygdala appeared to be identical to that seen in previous tract tracing studies (McDonald et al., 1996; Vertes, 2004; McDonald 1998). The most surprising finding of this investigation was that the most robust projection to the amygdala from the IL was to a MOR-negative portion of the CLC located between the main and medial ICNs at rostral levels of the BLA. This distinct portion of the CLC was termed the capsular infralimbic target zone (CITZ). This projection was seen in a previous study (see Fig. 3, levels -1.8 and -2.3 , of McDonald, 1996) but its relationship to the ICNs in this region could not be determined. In contrast to the robust projection to the CITZ, IL projections to the ICNs were relatively weak compared to projections to neighboring regions, including the CITZ, basolateral amygdala, and amygdalostratial transition area. However, projections to the caudal part of the ventral medial ICN were somewhat more robust than to other ICNs. Since the projections to the CITZ and ICNs were not noticeably different in rats that received IL injections that also involved the ventral portion of the prelimbic area, the great majority of the projections to the CITZ and ICNs appear to originate from the IL.

The CITZ of the rat amygdala appears to correspond to the anterior part of a region of the mouse amygdala termed the intramedullary gray (IMG; Busti et al., 2011). Using a variety of markers for the ICNs, Busti and coworkers reported that the IMG contained very few neurons expressing markers that characterize the ICNs. For example, Figure 1G of Busti and coworkers (2011) shows a section through the mouse amygdala with over a hundred neurons in the main and medial ICNs that express *Foxp2* (a transcription factor protein), but only two *Foxp2*⁺ neurons can be seen in the IMG. Likewise, *in situ* hybridization studies have shown a high density of neurons in the main and medial ICNs that express MOR mRNA, but none in the CITZ which lies between these ICNs (see Fig. 3E of Poulin et al., 2006). The present study also indicates that levels of D1R distinguish the CITZ from the ICNs and the non-CITZ portion of the CLC (see also Fig. 1 of Fuxe et al., 2003). The ICNs, CITZ, and

non-CITZ portion of the CLC have high, moderate, or very low levels of D1R immunoreactivity, respectively.

Golgi studies seem to indicate that the rat CITZ contains medium-sized neurons with spiny dendrites that run tangential to the medial border of the BLC (see Fig. 6 of Millhouse, 1986). In situ hybridization studies of the amygdala suggest that these CITZ neurons are GABAergic, just like the neurons of the ICNs and the non-CITZ portions of the CLC (see Fig. 2J of Poulin et al., 2008). Activation of GABAergic CITZ neurons by IL inputs, therefore, should result in the indirect inhibition of the postsynaptic targets of these cells by the IL. Other possible targets of IL axons in the CITZ are the distal dendritic segments of pyramidal cells of the BLA. Many of these dendrites extend into the most lateral portion of the CLC for 50–75 μm (see Figs. 8 and 9 of McDonald, 1984).

Since numerous studies using a variety of methodologies have provided evidence that IL-to-ICN projections are critical for extinction (Morgan et al., 1993; Quirk et al., 2000; Milad and Quirk, 2002; Likhtik et al., 2008; Knapska and Maren, 2009; Amano et al., 2010; Amir et al., 2011), an unexpected finding of the present investigation was that IL projections to the ICNs were relatively light compared to projections to surrounding amygdalar structures such as the CITZ and BLC. Despite the fact that the medial ICN receives only a moderate projection from the IL, a previous study demonstrated robust bilateral induction of c-Fos expression in the medial ICNs with unilateral picrotoxin-induced activation of the IL (Berretta et al., 2005). A possible explanation for these somewhat discrepant results may lie in the unique physiology of medial ICN neurons. These neurons are fast-spiking and respond to IL stimulation by firing high frequency spike bursts (Collins and Paré, 1999; Amir et al., 2011). Moreover, they exhibit an unusual slowly inactivating voltage-dependent potassium conductance (I_{SD}) which activates at subthreshold voltages and inactivates in response to depolarization (Royer et al., 2000). Because I_{SD} deinactivates very slowly, depolarization of medial ICN neurons by excitatory inputs can produce a self-sustained state of increased excitability which is associated with increased input resistance and membrane depolarization (Royer et al., 2000; Li et al., 2011). Thus, burst firing in response to IL inputs combined with the properties of I_{SD} may permit a robust and sustained activation of medial ICN neurons to occur despite a relatively light innervation by the IL.

Marowsky and coworkers (2005) found that stimulation of cortical efferents in the external capsule produced feedforward inhibition of the BLC via activation of inhibitory neurons in the lateral ICNs. Likewise, stimulation of fibers in the intermediate capsule located between the BLC and the central nucleus produced feedforward inhibition of the central nucleus via activation of the medial ICNs. Although these investigators suggested that the fibers in both capsules might originate from the medial PFC, the results of the present study indicate that very few fibers from the medial PFC enter the external capsule and terminate in the lateral ICNs. This implies that most of the fibers in the external capsule that innervate the lateral ICNs originate from lateral cortical areas. Indeed, in preliminary studies of the innervation of MOR+ ICNs by the temporal and perirhinal cortices, we found that these temporal/perirhinal projections coursed through the external capsule and mainly innervated the lateral ICNs versus the medial ICNs (Pinard and McDonald, personal observations). Similar to the

IL projection to the medial ICNs, the temporal/perirhinal projections to the lateral ICNs were relatively light compared with their innervation of the adjacent BLC.

Another important finding of Marowsky and coworkers (2005) was that dopamine, via a D1 receptor mechanism, hyperpolarized neurons in both the medial and lateral ICNs, thus reducing the feedforward inhibition to the central nucleus and BLC, respectively. These disinhibitory mechanisms may be important for the ability of dopamine to facilitate fear and anxiety, and to attenuate extinction, via D1 receptors in the amygdala (Lamont and Kokkinidis, 1998; Borowsky and Kokkinidis, 1998; Guaracci et al., 1999; Greba and Kokkinidis, 2000). Light microscopic studies have shown that the ICNs exhibit robust, diffuse, neuropilar D1 immunoreactivity (D1R-ir; Fuxe et al., 2003). Ultrastructural analysis has demonstrated that this D1R-ir is associated with shafts and spines of ICN dendrites (Pinto and Sesack, 2008). In their Figure 1, Fuxe and coworkers (2003) show sections at the bregma -2.1 and -2.2 levels that have robust D1R-ir in the ICNs, but also exhibit moderate D1R levels in a zone between the main and medial ICNs that clearly corresponds to the CITZ. These results match the pattern of D1R-ir seen in the BDA/MOR/D1 triple labeling studies of the present investigation (Fig. 10); our results also demonstrate that the IL projections to the CITZ are exactly congruent with the distribution of D1R-ir in this MOR-negative region, but that very few fibers enter the D1/MOR-ir medial or main ICNs at these levels. These findings suggest the possibility that dopamine, via D1 receptors, may hyperpolarize CITZ neurons, similar to its action in the ICNs. This could block the ability of the IL to indirectly inhibit the postsynaptic targets of CITZ neurons, which might result in an enhancement of fear and anxiety, and an attenuation of extinction. It will be of obvious interest to determine these targets of CITZ neurons and to examine whether this presumptive inhibitory projection plays a role in fear expression and extinction.

5. CONCLUSIONS

This investigation demonstrates that a discrete portion of the capsular subdivision of the central nucleus bordering the BLA, the capsular infralimbic target zone (CITZ), receives a very dense innervation by the IL, whereas the projection to the ICNs is relatively light. The CITZ is very different from the ICNs in several ways in addition to its greater innervation by the IL. Unlike the ICNs it is: (1) MOR-negative, (2) exhibits substantially less D1 dopamine receptor expression, and (3) does not consist of a compact cluster of neurons. However, the location of the CITZ, interposed between the medial and main ICNs, suggests the possibility that the intercalated nuclear complex may be anatomically and neurochemically heterogeneous, and that the CITZ may be a distinct portion of this complex that is functionally related to the ICNs.

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Abbreviations

AStr	amygdalostratial transition area
BDA	biotinylated dextran amine
BL	basolateral nucleus
BLa	anterior subdivision of the basolateral nucleus
BLC	basolateral nuclear complex
BLp	posterior subdivision of the basolateral nucleus
CITZ	capsular infralimbic target zone
CL	lateral subdivision of the central nucleus
CLC	capsular subdivision of the central nucleus
D1R	D1 dopamine receptor
dICN	dorsal intercalated nucleus
DP	dorsal peduncular cortex
EC	external capsule
ICN	intercalated nucleus
IL	infralimbic cortex
La	lateral nucleus
Ldl	dorsolateral subdivision of the lateral nucleus
lICN	lateral intercalated nucleus
Lvm	ventrolateral subdivision of the lateral nucleus
mICN	medial intercalated nucleus
MOR	μ -opioid receptor
mPFC	medial prefrontal cortex
PHA-L	Phaseolus vulgaris leucoagglutinin
PL	prelimbic cortex

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Research Highlights

- The infralimbic cortex (IL) has projections to the intercalated nuclei (ICN) of the amygdala
- The strongest projection to the ICN region is to a zone located between the main and medial ICNs
- This zone is termed the “capsular infralimbic target zone” (CITZ)
- The CITZ is distinguished from the ICNs due to its lack of μ -opioid receptor immunoreactivity
- The IL projection to the ICNs is much lighter than to the CITZ and neighboring basolateral amygdala

Fig. 1.

Photomicrographs of coronal amygdalar sections immunostained for the μ -opioid receptor (lateral is to the right). Sections are arranged from rostral to caudal (A: bregma level -1.8 , B: bregma level -2.2 , C: bregma level -2.6 , D: bregma level -2.9). Abbreviations: BL, basolateral nucleus; CL, lateral subdivision of the central nucleus; CLC, capsular subdivision of the central nucleus; dICN, dorsal intercalated nucleus; d-mICN, dorsal portion of the medial intercalated nucleus; EC, external capsule; IICN, lateral intercalated nucleus; La, lateral nucleus; mICN, medial intercalated nucleus; v-mICN, ventral portion of the medial intercalated nucleus. Scale bar = $100\ \mu\text{m}$.

Fig. 2.

Coronal sections, modified from the atlas by Paxinos and Watson (2007) and arranged from rostral to caudal, illustrating injection sites into the mPFC (cases R36–R40). All injections are plotted as though they were on the right side of the brain, but the abbreviations “Lf” (left) and “Rt” (right) indicate the side of the brain injected in each rat. All injections included the infralimbic cortex (IL), but some injections had variable amounts of spread into the ventrally adjacent dorsal peduncular cortex (DP), and/or dorsally adjacent prelimbic cortex (PL).

Fig. 3.

Gray scale digital micrographs of a PHA-L injection into the infralimbic cortex (IL) in a section that was lightly Nissl-stained with pyronin Y (injection R36Rt; compare with Fig. 2B). (A) Low power photomicrograph. (B) Higher power photomicrograph of the injection site in A. Note that labeled neurons were found both in the superficial layers (layers II–III) and the deeper layers (layers V–VI) of the infralimbic cortex. Scale bar = 500 μ m in A, 150 μ m in B.

Fig. 4.

Photomicrographs illustrating PHA-L-labeled infralimbic projections (black) to the right amygdala in case R36 at bregma level -2.2 (lateral is to the right). MOR-labeled ICNs are brown. (A) Low power photomicrograph showing a moderate innervation of the lateral nucleus (La) and amygdalostriatal transition area (AStr), and a dense projection to the capsular infralimbic target zone (CITZ) adjacent to the medial border of the BLA. The CITZ is a MOR-negative region located between the main ICN and the medial ICN (mICN). See Fig. 5A for a drawing of the axons in the region of the CITZ. (B) Higher power photomicrograph of the light innervation of the medial ICN shown in A. (C) Higher power photomicrograph of the light innervation of the main ICN, the medial portion of which is shown in A. (D) Higher power photomicrograph of the dense innervation of the CITZ shown in A. Scale bar in A = $100\ \mu\text{m}$. Scale bar in D (for panels B–D) = $50\ \mu\text{m}$.

Fig. 5.

Camera lucida drawings of PHA-L labeled axons in the right amygdala in case R36. (A) Drawing of the PHA-L labeled axons in the section shown in Fig. 4A (lateral is to the right). Although almost all of these axons exhibited varicosities, they are not illustrated in this low magnification drawing. Note the high density of axons in the CITZ, located in the far lateral portion of the capsular subdivision of the central nucleus (CLC). The mICN and main ICN contain very few labeled axons. (B) Higher power drawing of the PHA-L labeled axons in the ventral medial ICN in the section shown in Fig. 6A. Axonal varicosities are illustrated in this drawing. Labeled axons that were located in the adjacent lateral central (CL) and basolateral (BL) nuclei are not drawn. Note that the density of labeled axons in the caudal ventromedial ICN (B) is much less than in the CITZ (A), but greater than in the main ICN or rostral portions of the medial ICN (A). Scale bars = 50 μ m for A, 25 μ m in B.

Fig. 6.

Photomicrographs illustrating PHA-L-labeled infralimbic projections (black) to the right amygdala in case R36 at bregma level -2.6 . MOR-labeled ICNs are brown. (A) Low power photomicrograph showing a dense concentration of IL fibers in the amygdalostriatal transition area (AStr) and the ventromedial subdivision of the lateral nucleus (Lvm). As shown at higher magnification in B–D, the density of fibers drops off precipitously at the borders of the dorsomedial (d-mICN), ventromedial (v-mICN), and lateral (IICN) ICNs. (B) Higher power photomicrograph of the d-mICN region shown in A. (C) Higher power photomicrograph of the v-mICN region shown in A (see Fig. 5B for a drawing of the axons in the v-mICN in this same section). (D) Higher power photomicrograph of the IICN region shown in A. Scale bar in (A) = 100 μm . Scale bar in D (for panels B–D) = 50 μm .

Fig. 7.

Photomicrographs illustrating PHA-L-labeled infralimbic/prelimbic projections (black) to the right amygdala in a case with an injection site that involved both IL and PL (R40). MOR-labeled ICNs are brown. (A) Low power photomicrograph showing a dense concentration of IL fibers in the CITZ at bregma level -2.2. Few fibers are seen in the adjacent main ICN, medial ICN (mICN), or lateral ICN (lICN). (B) Low power photomicrograph of a more caudal section showing a dense concentration of fibers in the medial portions of Lvm and BLA, and a much lighter innervation of the ventromedial ICN (v-mICN) and dorsomedial ICN (d-mICN). Scale bar = 200 μ m.

Fig. 8.

Photomicrographs of PHA-L-labeled mPFC projections (black) to the left amygdala in cases R37 (A1 and A2), R38 (B1 and B2), and R39 (C1 and C2) at two rostrocaudal (bregma -2.2 : A1, B1, C1; bregma -2.6 : A2, B2, C2) (lateral is to the right). MOR-labeled ICNs are brown. In all three cases there were dense projections to the MOR-negative CITZ, and light to moderate projections to the MOR-positive medial ICNs. In cases R37 and R39 the same pattern of labeled axons was also seen in the right amygdala due to the similarity of the placement of the injection sites on the two sides of the brain. This was also seen in case R38, with the exception of the projection to the CITZ, which was more dense on the left side (B1) than the right side (not shown). Since the injection site on the right side of R38 had greater involvement of the IL (see above), these findings suggest that the projection of the IL to the contralateral CITZ is more dense than the ipsilateral projection. Scale bar (in A1) = 100 μ m and is valid for all six photomicrographs.

Fig. 9. Photomicrograph (left panel) and camera lucida drawing (right panel) of PHA-L-labeled infralimbic projections (black) to the ipsilateral amygdala in case P-39 (lateral is to the left). The section in the left panel was counterstained with pyronin Y. Asterisks indicate two ICNs located along the medial border of the BLA: the dorsally situated ICN is part of the medial ICN, and the ventrally situated ICN is a dorsal extension of the main ICN. Arrows in left panel indicate axons in the CITZ, located along the medial border of the BLA. These axons are seen more clearly in the drawing. Although almost all of these axons exhibited varicosities, they are not illustrated in this drawing (see inset which shows a photomicrograph of the varicose axons in the boxed area in the right panel). Note that there is no substantial projection to the main ICN located ventral to the CITZ. Likewise, axons are found medial to the more dorsally situated medial ICN, but few axons enter this nucleus. Also note that the neurons in the CITZ are not clustered like those of the adjacent ICNs. Scale bar = 100 μ m.

Fig. 10.

Photomontages of a section through the amygdala in a brain that received bilateral BDA injections into the infralimbic cortex. A) BDA labeled axons (green) are found in highest density in the CITZ (arrows). This section was also stained for D1R (red) and MOR (blue). D1R immunoreactivity was moderate in the amygdalostriatal area (AStr) and CITZ, and very dense in the ICNs. Colocalization of D1R (red) and MOR (blue) in the ICNs is indicated by the light magenta coloring in these two nuclei. Note that the zone of BDA-labeled axons in the CITZ is congruent with the extent of the D1R-ir. Only one BDA+ axon was seen in each of the ICNs in this section. B) Photomicrograph of the section shown in A, but with the green BDA axonal labeling and the blue MOR labeling deleted so that the difference in the density of red D1 labeling in the CITZ and ICNs is more clearly seen. Scale bar = 100 μ m.