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HETEROGENEOUS DOPAMINE POPULATIONS PROJECT TO SPECIFIC SUBREGIONS OF THE PRIMATE AMYGDALA

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Abstract—Amygdala dysfunction has been reported among patients with various psychiatric disorders, and dopamine is critical to the amygdala's ability to mediate fear conditioning. Recent work indicates that the midbrain dopaminergic neurons have heterogeneous receptor and membrane channel profiles, as well as differential physiologic responses to discrete stimuli. To begin understanding how dopamine affects amygdala physiology and pathology in higher primates, we mapped the inputs from the midbrain dopaminergic neurons to various amygdala nuclei in the monkey using retrograde and anterograde tracing techniques, and single and double immunofluorescence histochemistry for tracer and tyrosine hydroxylase, a dopamine marker. Our results show that the primate amygdala as a whole receives broad input, mostly from the dorsal tier of the substantia nigra, pars compacta, and the A8-retrorubral field. Input from the A10-ventral tegmental area, while present, was less prominent. These results differ from data in the rat, where the midline A10-ventral tegmental area is a major source of dopamine to the amygdala "mesolimbic" pathway. Both the "amygdala proper" and the "extended amygdala" receive the majority of their input from the dorsal tier of the substantia nigra and A8-retrorubral field, but the extended amygdala receives additional modest input from the ventral tier. In addition, the "extended amygdala" structures have a denser input than the "amygdala proper," with the exception of the lateral core of the central nucleus, which receives no input. Our anterograde studies confirm these findings, and revealed fine, diffuse terminal fibers in the amygdala proper, but a denser network of fibers in the extended amygdala outside the lateral core of the central nucleus. These results indicate that the entire extent of the dorsal tier beyond the A10-ventral tegmental area may regulate the amygdala in primates, and subsequently serve as a source of dysfunction in primate psychopathology. © 2010 Published by Elsevier Ltd on behalf of IBRO.

Key words: extended amygdala, ventral tegmental area, retrorubral field, dorsal tier, ventral tier, substantia nigra.

The limbic system is a distributed neural network of cortical and subcortical structures that coordinate emotional awareness and responses to the environment. The amygdala, a heterogeneous limbic structure residing in the medial temporal lobe, assigns emotional salience to events and coordinates with other limbic regions, including the pre-frontal cortex (PFC) and the hippocampus. Recent studies in animal models and humans indicate that functional abnormalities of the amygdala, and the limbic system as a whole, contribute to the pathophysiology of depression, as well as other psychiatric illnesses (Phillips et al., 2003a,b).

A major function of the amygdala is fear conditioning, as confirmed in animal and human studies (Phillips and LeDoux, 1992; Pitkanen et al., 1997; Alvarez et al., 2008). Fear conditioning facilitates rapid protective responses to negative stimuli, representing a key survival method for animals and humans. Yet hypersensitivity to fear conditioning may lead to misinterpretation of external stimuli as emotionally relevant or inappropriately negative. This may lead to negative thought patterns, anxiety, and ruminative thinking, all common symptoms of depressive illnesses (Wenzlaff et al., 1988; Oaksford and Stenning, 1992). Over-active amygdalar functioning has been demonstrated among patients with depression, suggesting that hyperactivity in this area correlates with depressive illnesses (Sheline et al., 2001; Siegle et al., 2002).

Dopamine (DA) is a key neurotransmitter involved in fear conditioning, and the amygdala's ability to acquire and express fear conditioning strongly depends on intact dopamine pathways. The DA neurons are now recognized as a heterogeneous cell group, and the A10-ventral tegmental area (A10-VTA) is the main source of DA to the amygdala based on studies in rodents (review; Bjorklund and Dunnett, 2007). In rats, disruption of the A10-VTA, with subsequent decreased DA release to the amygdala, impairs retrieval of previously learned fear conditioning (Nader and LeDoux, 1999). Similarly, infusion of either D1 or D2 re-

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Abbreviations: III N, cranial nerve III; A8-RRF, A8-retrorubral field; A9-dt, A9-dorsal tier of substantia nigra; A9-vt, A9-ventral tier of substantia nigra; A10-VTA, A10-ventral tegmental area; AAA, anterior amygdaloid area; ABbs, accessory basal nucleus, sulcal subdivision; ABmc, accessory basal nucleus, magnocellular subdivision; ABpc, accessory basal nucleus, parvicellular subdivision; AC, anterior commissure; AChE, acetylcholinesterase; AHA, amygdalohippocampal area; Aq, aqueduct; Astr, amygdalostriatal area; Bi, basal nucleus, intermediate subdivision; Bmc, basal nucleus, magnocellular subdivision; BNST, bed nucleus of the stria terminalis; Bpc, basal nucleus, parvicellular subdivision; C, caudate nucleus; CaBP, Calbindin-D28k; CeLcn, central nucleus, lateral core subdivision; CeM, central nucleus, medial subdivision; CL, claustrum; CoA, anterior cortical nucleus; CoP, posterior cortical nucleus; DA, dopamine; DAT, dopamine transporter; EC, external capsule; GPe, globus pallidus, external segment; H, hippocampus; IC, internal capsule; IPAC, interstitial nucleus of the posterior limb of the anterior commissure; L, lateral nucleus; M, medial nucleus; ML, medial lemniscus; NGS, normal goat serum; O, optic tract; P, putamen; PAC, periamygdaloid cortex, subdivisions I-III; PAG, periaqueductal grey; PB, phosphate buffer; PFC, pre-frontal cortex; RN, red nucleus; SCP, superior cerebellar peduncle; SNpr, substantia nigra, pars reticulate; TH, tyrosine hydroxylase; TX, Triton-X; V, ventricle.

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ceptor antagonists into the amygdala of rats leads to impaired acquisition, consolidation and retrieval of learned fear conditioning (Guarraci et al., 1999, 2000; Greba and Kokkinidis, 2000; Greba et al., 2001; Fadok et al., 2009). These studies suggest that DA is necessary for different facets of fear conditioning, including the learning process of pairing an unconditional stimulus (shock) to a conditional stimulus (sound), the storage process needed to use this information for future experiences, and the ability to recall this information when necessary. Confirming the importance of DA in fear conditioning in humans, a combined positron emission tomography (PET)-ligand and functional magnetic resonance imaging (fMRI) study showed that amygdala activation in response to aversive stimuli correlated with an increase in amygdalar dopamine storage capacity (Kienast et al., 2008). The latter study points to the importance of understanding DA innervation of the amygdala in higher species.

Current knowledge of the mesolimbic projection rests on lesion and double-labeled tracer studies in rat. Tracer studies in the non-human primate have mapped midbrain inputs to the amygdala as part of larger studies examining subcortical and/or cortical afferents, but none of these studies confirmed the dopaminergic status of these inputs (Aggleton et al., 1980; Mehler, 1980; Norita and Kawamura, 1980). In addition, the heterogeneous composition and function of the amygdala nuclei lends itself to anatomic studies aimed at understanding the specific subregions of the amygdala. Therefore, our study's goal was to map the DAergic inputs to specific nuclei of the primate amygdala, with an analysis of the specific DA subpopulations involved.

EXPERIMENTAL PROCEDURES

Primate injections and surgeries

Eleven *Macaca fascicularis* monkeys, weighing between 3 and 9 kg, were used in these experiments (Labs of Virginia, Yemassee, SC, USA; Three Springs Laboratories, Pekaski, PA, USA, and Worldwide Primates, Tallahassee, FL, USATracers). Small amounts (40 nl) of either Lucifer Yellow conjugated to dextran amine (LY; 10% Molecular Probes, Eugene, OR, USA), Fluoro-Ruby conjugated to dextran amine (FR; 4% Molecular Probes) or Fluorescein conjugated to dextran amine (FS; 10% Molecular Probes) were injected into the central nucleus, amygdalostriatal area, interstitial nucleus of the posterior limbic of the anterior commissure (IPAC), medial nucleus, accessory basal (magnocellular and parvicellular subdivisions) and basal (magnocellular and parvicellular subdivisions) nuclei of the amygdala. To confirm retrograde studies, we placed bidirectional tracers at several mediolateral and rostrocaudal levels of the midbrain DA neurons to determine the pattern of anterogradely labeled fibers in the amygdala.

All experiments were carried out according to National Institutes of Health guidelines, and reviewed by the University of Rochester Committee on Animal Research. Animals were given i.m. injections of ketamine hydrochloride (10 mg/kg) (Hospira, Inc., Lake Forest, IL, USA), intubated, and deeply anesthetized with i.v. pentobarbital (initial dose 20 mg/kg), which was maintained as needed during surgery. We performed a craniotomy to visualize cortical surface landmarks, and electrophysiologic mapping to locate internal landmarks such as the anterior commissure, striatum and amygdala (Fudge et al., 2004; Fudge and Tucker, 2009). Stereotaxic coordinates for these boundaries were determined, and the locations of the nuclei were estimated. The retrograde tracer was pressure-injected over 10–15 min into individual nuclei using a 0.5 μl Hamilton syringe (Hamilton Company, Reno, NV, USA), and the syringe was left in place for 20 min to prevent leakage of tracer up the syringe track. Only one injection of each tracer was used per animal. After injections were placed, the bone flap was replaced and the overlying musculature and skin sutured. Prophylactic antibiotics and pain medication were given for 7–10 days post-operatively.

Ten to thirteen days after surgery, animals were deeply anesthetized and killed by perfusion through the heart with 0.9% saline containing 0.5 ml of heparin sulfate (200 ml/min for 10 min), followed by cold 4% paraformaldehyde in 0.1 M phosphate buffer/30% sucrose solution (100 ml/min for 1 h). The brain was removed, placed in fixative overnight, and sunk in increasing gradients of sucrose (10%, 20%, 30%). Brains were cut on a freezing microtome (50 μm sections) and saved in cryoprotectant solution (30% ethylene glycol and 30% sucrose in 0.1 M phosphate buffer) at −20 °C (Rosene et al., 1986). Every 24th slice was used in our studies, and adjacent sections were used for determining anatomical landmarks.

Single-labeling immunocytochemistry

Tracers. Sections were thoroughly rinsed in 0.1 M phosphate buffer (pH 7.2) with 0.3% Triton-X (PB-TX). After treatment with endogenous peroxidase inhibitor for 5 min, followed by more rinses, sections were pre-incubated in a blocking solution of 10% normal goat serum in 0.1 M PB-TX (NGS-PB-TX) for 30 min. Tissue was then placed in primary antisera to LY (1:2000, Molecular Probes, rabbit), FS (1:2000, Molecular Probes, rabbit), or FR (1:1000, Molecular Probes, rabbit) for approximately 96 h at 4 °C. After thorough rinsing with 0.1 M PB-TX, and pre-incubation with 10% NGS-PB-TX, sections were incubated in biotinylated secondary anti-rabbit antibody. Tracers were visualized using the avidin-biotin reaction (Vector ABC Standard kit, Burlingame, CA, USA). Additional compartments for each case were also processed for tracer enhanced with nickel intensification (3, 3′-diaminobenzidine tetrahydrochloride with 1% nickel ammonium sulfate and 1% cobalt chloride, catalyzed by 0.03% hydrogen peroxide for 1–2 min) and counterstained with acetylcholinesterase (AChE) (Geneser-Jensen and Blackstad, 1971) or cresyl violet.

Calbindin-D28k (CaBP). Adjacent or near adjacent sections through the ventral midbrain were used to demarcate the dorsal and ventral tiers of the SNpc according to CABP immunoreactivity (Lavoie and Parent, 1991; German et al., 1992). Sections were thoroughly rinsed, and preincubated in 10% NGS-PB-TX as described above, and then incubated for 96 h in CaBP (Chemicon, 1:10,000, mouse) antisera. Sections were then rinsed, blocked and incubated in secondary anti-mouse biotinylated antibody. Following thorough rinsing, CaBP protein was visualized using the avidin–biotin reaction described above.

Double-labeling fluorescent immunocytochemistry

Optimal dilutions for primary antibodies used in immunofluorescent studies were established in advance using single-label fluorescent labeling. In addition, control tissue from animals without tracer injections and immunohistochemical processing was examined for autofluorescence. The primary antibodies used were (1) tyrosine hydroxylase (TH; mouse, Millipore, Temecula, CA, USA) diluted to 1:5000, and antiserum to one of the following: (2) Lucifer Yellow tracer (LY; rabbit, Molecular Probes, Eugene, OR, USA) diluted to 1:3000; Fluorescein (FS; rabbit, Molecular Probes) diluted to 1:750; or Fluoro-Ruby (FR; rabbit, Molecular Probes) diluted to 1:2000. Fluorescent immunocytochemistry was run on coronal sections from the level of the mammillary bodies rostrally to the emergence of the brainstem A5 catecholamine group caudally. For each injection site, every twenty-fourth slice was pulled from storage in a −20 °C freezer and rinsed for 15 min in four