Amygdaloid projections to the ventral striatum in mice: direct and indirect chemosensory inputs to the brain reward system

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INTRODUCTION

It is generally accepted that the amygdala is a key structure in the generation of emotional behaviors (LeDoux, 2000). Remarkably, in this view of the amygdala as the core of the emotional brain, rodents constitute good models for studying the neural basis of sociosexual behavior. Recent findings in mice have revealed the molecular identity of some pheromonal molecules triggering intersexual attraction. However, the neural pathways mediating this basic sociosexual behavior remain elusive. Since previous work indicates that the dopaminergic tegmento-striatal pathway is not involved in pheromone reward, the present report explores alternative pathways linking the vomeronal system with the tegmento-striatal system (the limbic basal ganglia) by means of tract-tracing experiments studying direct and indirect projections from the chemosensory amygdala to the ventral striato-pallidum. Amygdaloid projections to the nucleus accumbens, olfactory tubercle, and adjoining structures are studied by analyzing the retrograde transport in the amygdala from dextran amine and fluorogold injections in the ventral striatum, as well as the anterograde labeling found in the ventral striato-pallidum after dextran amine injections in the amygdala. This combination of anterograde and retrograde tracing experiments reveals direct projections from the vomeronasal cortex to the ventral striato-pallidum, as well as indirect projections through different nuclei of the basolateral amygdala. Direct projections innervate mainly the olfactory tubercle and the islands of Calleja, whereas indirect projections are more widespread and reach the same structures and the shell and core of nucleus accumbens. These pathways are likely to mediate innate responses to pheromones (direct projections) and conditioned responses to associated chemosensory and non-chemosensory stimuli (indirect projections). Comparative studies indicate that similar connections are present in all the studied amniote vertebrates and might constitute the basic circuitry for emotional responses to conspecifics in most vertebrates, including humans.

Keywords: chemosensory amygdala, nucleus accumbens, olfactory tubercle, islands of Calleja, striatal cell bridges, emotional brain

Abbreviations: AA, anterior amygdaloid area (AAD: dorsal; AA V: ventral); AB, accessory basal (or basomedial) amygdaloid nucleus (ABa: anterior; ABp: posterior); ac, anterior commissure (aco: anterior part; ap: posterior part); Adb, nucleus accumbens (AcbC: core; AcbSh: shell; LABsH: lateral shell; MAbChSk: medial shell); AHA, amygdalohippocampal area (AHAL: lateral division; AHAM: medial division); APir, amygdalo-piriform transition area; ASIt, amygdalo-striatal transition area; B, basal (or basolateral) amygdaloid nucleus (Bac: anterior; Rp: posterior; Bcp: ventral); BAOt, bed nucleus of the accessory olfactory tract; BST, bed nucleus of the stria terminalis; CB, cell bridges of the ventral striatum; Ce, central amygdaloid nucleus (CeC: capsular or paracapsular division; Cel.: lateral division; CeM: medial division); CI, claustrum; COAa, anterior cortical amygdaloid nucleus; COApI, posterolateral cortical amygdaloid nucleus; COApIr, posteromedial cortical amygdaloid nucleus; CPu, caudate-putamen (dorsal striatum); CXa, cortex-amygdala transition zone; DEn, dorsal endopiriform nucleus; HDB, horizontal limb of the diagonal band nucleus; I, intercalated nuclei of amygdala; IC, islands of Calleja; IC-M, major island of Calleja; IPAG, interstitial nucleus of the posterior limb of the anterior commissure; L, lateral amygdaloid nucleus (LsL: ventrolateral, LdL: dorsolateral, Lm: medial); LGp, lateral globus pallidus; LOT, nucleus of the lateral olfactory tract (1: layer 1; 2: layer 2; 3: layer 3); Me, medial amygdaloid nucleus; MeA, anterior medial amygdala; MeP, posterior medial amygdala (MePV: ventral; MePD: dorsal); Pir, piriform cortex; SI, substantia innominata; st, stria terminalis; Tu, olfactory tubercle; VEn, ventral endopiriform nucleus.
having an important emotional component. In this respect, recent findings in mice have uncovered the molecular identity of two particular pheromonal molecules triggering intersexual attraction (Roberts et al., 2010) and sexual receptivity (Haga et al., 2010). Male-soiled bedding containing some of these pheromones can be used to induce conditioned place preference (Martínez-Rícos et al., 2007) and, therefore, these sexual pheromones should be considered rewarding stimuli. Since the major pathway involved in reward processing is the dopaminergic tegmento-striatal pathway, our group has previously analyzed the role of this projection in pheromonal reward in mice. Neither systemic administration of anti-dopaminergic drugs (Agustin-Pavón et al., 2007), nor lesions of the dopaminergic neurons of the ventral tegmental area (Martínez-Hernández et al., 2006) abolish female preference for male-soiled bedding, thus suggesting that the dopaminergic tegmento-striatal pathway is not involved in pheromone reward.

In mice, at least some male sexual pheromones attractive for females are detected by the vomeronasal system (Martínez-Rícos et al., 2008), and consequently information about these sexual pheromones is processed in the vomeronasal amygdala. There is increasing evidence on the role of the amygdala on reward-related processes (Baxter and Murray, 2002; Everitt et al., 2003), but the role of the amygdala in reward driven by chemosensory stimuli has been rarely explored. The pioneering studies by Everitt, Robbins, and collaborators (Cador et al., 1989; Everitt et al., 1989, 1991; Robbins et al., 1989) indicated that basolateral amygdala projections to the nucleus accumbens mediate reward-related learning. In contrast, much less information is available regarding the projections from the chemosensory amygdala to the nucleus accumbens and other centers of the ventral striatum (McDonald, 1991b; Canteras et al., 1992; Shammah-Lagnado and Santiago, 1999).

The present report explores alternative pathways linking the chemosensory amygdala with the brain reward system by means of tract-tracing experiments studying the projections of different amygdaloid nuclei to the ventral striato-pallidum. To do so, we combine anterograde and retrograde tracing experiments to reveal amygdaloid projections to the nucleus accumbens, olfactory tubercle, and adjoining structures such as the islands of Calleja (ICj). Our results might help to understand the neural pathways mediating the behavioral responses to chemical cues, including rewarding sexual pheromones.

MATERIALS AND METHODS

ANIMALS

For this study, 35 adult, female mice (*Mus musculus*, Harlan, Barcelona, Spain; 25 of the C57BL/6j strain and 10 of the CD1 strain) were used (17.9–35.1 g). Animals were maintained in cages with food and water *ad libitum*, under natural day/night cycle, at 22–25°C. All experiments were performed in accordance with the guidelines of the European Community Council Directive 86/609/EEC on the treatment of experimental animals, and procedures were approved by the Committee of Ethics on Animal Experimentation of the University of València.

TRACT-TRACING EXPERIMENTS

To study the amygdaloid projections to the ventral striatum in the mouse, we first injected neuroanatomical tracers in different parts of the Acb (medial shell, n = 4; lateral shell, n = 1; core, n = 5; non-restricted injections, n = 2) and other regions of the ventral striatum (olfactory tubercle, n = 6; major island of calleja (ICJ), n = 1), to study the distribution of retrograde labeling in the amygdala. Then, tracers were injected in the different nuclei of the amygdala that showed retrograde labeling in the previous experiments (cortical amygdala, n = 7; deep pallial amygdala, n = 10) and the resulting anterograde labeling in the ventral striatum was analyzed. In order to study whether the associative nuclei of the deep pallial amygdala (lateral, basal and accessory basal) can indirectly relay chemosensory information to the ventral striatum, we also studied the intra-amygdaloid projections from the cortical (chemosensory) amygdaloid nuclei to the deep pallial amygdala.

In the present work, three different tracers were used: biotinylated dextran amine (BDA, 10,000 and 3,000 MW lysine fixable; Invitrogen, Carlsbad, CA, USA), tetramethylrhodamine-labeled dextran amine (RDA, 10,000 MW, lysine fixable, fluoro-ruby; Invitrogen) and fluorogold (Biotium, Hayward, CA, USA). To minimize the number of animals, each mouse usually received two tracer injections (total number of tracer injections, 46). Therefore, each injection is identified with a letter indicative of the tracer (B for BDA; R for RDA; F for fluorogold) and a number indicative of the specimen.

For surgery, animals were deeply anesthetized with an intraperitoneal injection of 6.5 μl of sodium pentobarbital (11 mg/ml, Sigma-Aldrich, St. Louis, MO, USA) per gram of body weight. Using stereotaxic coordinates (Paxinos and Franklin, 2001), dextran amines (5–10% in 0.01 M phosphate buffer, pH 7.6), and fluorogold (2% in saline solution) were ionophoretically injected through glass micropipettes with an inner diameter tip of 10–20 μm, using positive current (7 s on/off; 3–5 μA) during 10 min. The pipette was left in place for 5 min after injection. To avoid diffusion of the tracer along the pipette track, a mild continuous retention current (−0.9 μA) was applied during entrance and withdrawal of the micropipette. After 7–8 days of survival, animals received an overdose of sodium pentobarbital and were perfused transcardially with phosphate buffered saline (PBS; 0.9% NaCl in 0.1 M PB pH 7.6), followed by fixative (4% paraformaldehyde in 0.1 M PB, pH 7.6). Brains were postfixed for 4 h in the same fixative at 4°C and then immersed in 30% sucrose in 0.1 M PB until they sank. Frontal sections (40 μm) obtained with a freezing microtome were collected into five parallel series.

Before (immuno)histochemical processing, endogenous peroxidase was inhibited with 1% H2O2 in 0.05 M TRIS buffer (TB) pH 7.6 plus 0.9% NaCl (TRIS-buffered saline, TBS) for 30 min at room temperature. For BDA detection, sections were incubated in avidin–biotin complex (ABC Elite kit; Vector Laboratories, Burlingame, CA, USA) in TBS with 0.3% Triton X-100, either overnight at 4°C or for 2 h at room temperature, and then the peroxidase activity was revealed with 3′,3′-diaminobenzidine (DAB, Sigma; 0.02% in 0.05 M TB, pH 7.6–8.0) and 0.01% H2O2. Nickel salts (0.4% nickel ammonium sulfate) were added as an enhancing agent of the reaction product (DAB-Ni).
For immunodetection of tetramethyl rhodamine, sections were incubated in primary antibody against tetramethyl rhodamine (raised in rabbit, Molecular Probes, Cat. # A-6397, Lot: 7051-3), diluted 1:4,000 in TBS with 0.3% Triton X-100, followed by a standard peroxidase–antiperoxidase procedure (goat anti-rabbit IgG;1:100, Nordic Immunological Laboratories, Tilburg, The Netherlands; rabbit PAP, 1:800, Nordic Immunological Labs). The resulting peroxidase label was revealed as above, with or without nickel enhancement.

Fluorogold injections were observed with fluorescence microscopy. Some series were also processed for the immunohistochemical detection of the tracer. To do so, sections were postfixed with 0.1% glutaraldehyde in PBS for 4 h at 4°C, rinsed in TBS and the endogenous peroxidase activity was inhibited with 1% H2O2 (30 min). Sections were then sequentially incubated in primary antibody (rabbit anti-FG, Chemicon, Billerica, MA, USA) diluted 1:5,000 in TBS with 0.3% Triton X-100 and 2.5% normal goat serum, overnight at 4°C; secondary antibody (biotinylated goat anti-rabbit IgG, Vector Laboratories, Burlingame, CA, USA) diluted 1:200 in TBS-Tx for 2 h at room temperature; and ABC Elite (2 h, room temperature, Vector Labs) diluted in the same buffer. The resulting peroxidase labeling was visualized as detailed above.

**IMAGE ACQUISITION AND PROCESSING**

Slides were photographed in a Leitz DMRB microscope equipped with a Leica DC 300 digital camera (Leica Microsystems, Wetzlar, Germany). Digital images were imported into Adobe Photoshop (Adobe Systems, Mountain View, CA, USA), flattened by subtracting background illumination and converted to gray scale. Brightness and contrast were adjusted and resolution was set at 600 dpi. No additional filtering or manipulation of the images was performed. The final figures were composed and labeled with Adobe Photoshop.

Chartings of anterograde labeling in specific sections were drawn for illustration purposes. To do so, low-power photomicrographs of the sections were taken as described and two transparent sheets were created on top of them using Adobe Photoshop. One of them was used to delineate the cytoarchitecture of the section, whereas in the other one a detailed map of the labeling was drawn using some elements of the photograph as spatial landmarks (vessels, ventricles, Nissl staining, etc.) while observing the slides at high magnification through the microscope.

**RESULTS**

The results of our experiments are described following the nomenclature by Paxinos and Franklin (2001), except for the amygdala, where we have partially adapted the nomenclature of Pitkänen (2000). This terminology has the advantage of avoiding the confusion between the basolateral nucleus and the basolateral division of the amygdala. Briefly, the amygdala consists of a mixture of structures with pallial or subpallial origin (Puelles et al., 2000; see Figure 1). The pallial structures of the amygdala give rise to the bulk of the amygdalostriatal projections, and therefore are the focus of the present investigation. Within the pallial amygdala, we distinguish the cortical nuclei (superficial, olfacto-recipient, and layered) and several non-layered nuclei located topologically deep to them (Figure 1). The cortical nuclei include the nucleus of the lateral olfactory tract (LOT, Figure 1A,A’), the anterior cortical amygdaloid nucleus (COAa, Figure 1A–B’), the posterolateral cortical amygdaloid nucleus (COAp, Figure 1B–D’), as well as of two secondary vomeronasal centers, the bed nucleus of the accessory olfactory tract (BAOT, not shown) and the posteromedial cortical amygdaloid nucleus (COApm, Figure 1C–D’). In addition, there are several transitional areas interposed between the cortical amygdala and the piriform cortex (cortex–amygdala transition zone,
CxA, Figure 1A, A; amygdalopiriform transition area, APir, not shown). The deep pallial nuclei of the amygdala include the basal (B, or basolateral, Figure 1B–D'), the accessory basal (AB, or basomedial, Figure 1B–D'), and the lateral (L, Figure 1B–D') amygdaloid nuclei, as well as the amygdalohippocampal area (AHA, Figure 1C–D'). According to Paxinos and Franklin (2001), we consider that the B includes anterior (Ba), posterior (Bp), and ventral (Bv) divisions. Within the AB we distinguish anterior (ABa) and posterior (ABp) portions. The L shows ventrolateral (Lvl), dorsolateral (Ldl), and medial (Lm) divisions. Finally, within the AHA we consider medial (AHAm) and lateral (AHAl) subnuclei.

In the ventral striatum we consider the core (AcbC) and shell (AcbSh) of the nucleus accumbens (with its lateral and medial divisions; LAcbSh and MAcbSh respectively) following Paxinos and Franklin (2001). We also include the olfactory tubercle (Tu), the ICj, and the cell bridges of the ventral striatum (CB) since the anterograde labeling in nucleus accumbens after injections in the pallial amygdala was continuous with the one found in these structures (for previous descriptions of the striatal cell bridges in the rat see Seifert et al., 1998; Riedel et al., 2002).

### Table 1 | Retrogradely labeled neurons in the different areas of the pallial amygdala (columns) after injections in the ventral striatum (rows)

| Cortical amygdala | Deep pallial amygdala |
|-------------------|------------------------|
| COApm | LOT | COAa | CxA | COApI | APir | Ba | Bp | Bv | ABa | LAcbSh | ABp | AHA | AHAm | Lm | Lvl | Ldl |
|-------------------|------------------------|
| **ACCUMBENS SHELL** | | | | | | | | | | | | | | | | | |
| R0304 MAcbSh | X | – | X | – | – | X | XX | XXX | XX | – | X | X | – | – | – | |
| +1.34 mm | | | | | | | | | | | | | | | | | |
| B0419 MAcbSh | – | – | X | – | – | X | XXX | XXX | XX | – | XX | X | – | – | – | |
| +0.98 mm | | | | | | | | | | | | | | | | | |
| R0331 LAcbSh | – | XX | X | XX | – | X | XXX | XX | XX | – | XX | X | – | XX | – | |
| +1.10 mm | | | | | | | | | | | | | | | | | |
| **ACCUMBENS CORE** | | | | | | | | | | | | | | | | | |
| B0302 AcbC | – | X | X | X | – | – | XX | X | XX | – | XXX | X | – | – | X | XXX |
| from +1.34 to +1.10 mm | | | | | | | | | | | | | | | | | |
| B0309 dorsomedialAcbC | – | X | X | – | – | X | X | – | – | – | – | – | – | – | X |
| +1.18 mm | | | | | | | | | | | | | | | | | |
| B0331 lateralAcbC | – | X | – | – | – | – | XX | X | X | – | X | – | – | – | – | X |
| +1.18 mm | | | | | | | | | | | | | | | | | |
| **OLFACTORY TUBERCLE** | | | | | | | | | | | | | | | | | |
| F08100 ICj anteromedial | XX | XX | XX | X | XX | XX | – | XXX | X | XX | XXX | X | – | – | – | |
| +0.98 mm | | | | | | | | | | | | | | | | | |
| **NON-RESTRICTED INJECTIONS** | | | | | | | | | | | | | | | | | |
| R0301 MAcbSh | X | – | X | – | – | X | XX | XXX | XX | – | X | X | – | X | – |
| (+ medialAcbC) +1.18 mm | | | | | | | | | | | | | | | | | |
| R0335 dorsal MAcbSh | X | X | X | – | X | XX | XX | XX | – | X | XX | X | X | – | X | X |
| + AcbC +0.98 mm | | | | | | | | | | | | | | | | | |
| B0312 lateral S+ICjM | XXX | X | X | X | – | X | XX | – | XX | X | XX | – | X | X | – | |
| from +1.34 to +1.10 mm | B | B | | | | | | | | | | | | | | | |

The rostro-caudal extent of the injection site is indicated (coordinates from Bregma). For abbreviations, see list.
consider as the Bv (Figure 2B). In addition, the caudal part of the Ba (Figure 2C) and the whole Bp (Figures 2C–E) showed many labeled somata. The most rostral injection in the MacbSh (R0304), as well as those injections involving both MacbSh and AcbC (R0301 and R0338), rendered retrograde labeling in the contralateral Bp (Table 1). Moreover, retrogradely labeled cells were found in the posterior part of the AB (Figures 1C,D), where labeling was bilateral in the injections encompassing both the MacbSh and AcbC (see Table 1). A few retrograde labeled cells were also present in the medial and lateral AHA (Table 1; Figures 2D,E). Finally, injections confined to the MacbSh showed no labeled cells in the L (Table 1).
The injection in the lateral AcbSh (LAcbSh; R0331, Figure 2A) resulted in numerous labeled cells in several pallial amygdaloid centers. Within the cortical amygdala, labeled cells were abundant in the CxA but scarce in the COAa (see Table 1). At these rostral levels, the LOT showed Golgi-like labeled cells in layer 2 (Figure 2F), as well as a number of faintly labeled cell bodies in layer 3 (see inset in Figure 2F). At caudal levels, a few somata displayed granular retrograde labeling in the APir (not shown). In the deep pallial amygdala, labeled cells were abundant in all the subnuclei of the B, especially in the Ba, where labeling was bilateral with ipsilateral dominance (Table 1). The ABp also showed a moderate-to-high density of retrogradely labeled neurons (Table 1). A few labeled cells were also present in the AHAI (Table 1). Finally, a population of labeled neurons was confined to the rostral portion of the Lvl (Table 1).

Injections in the accumbens core. Two small injections restricted to the AcbC and a larger injection (B0302) including most of the dorsal AcbC plus a small portion of the adjacent caudate–putamen (Figure 2A) displayed scarce retrograde labeling (see Table 1) within the cortical amygdala. A few labeled cells were found bilaterally in layer 3 of the LOT (Table 1) and ipsilaterally in the CxA, especially in its deep portion, adjacent to the COAa (Figure 2G). On the other hand, the deep pallial amygdala showed a dense population of retrogradely labeled cells in the most anterior part of the L, especially dense in the Ldl, where labeling was bilateral. Labeled neurons were also observed in the Lvl (Table 1; Figure 2H). It is worth noting that this labeling was absent in the injections restricted to the MAcbSh. In addition, many retrogradely labeled neurons were observed in both hemispheres in the caudal aspect of the Ba (Figure 2H), in the Bv and in the AHAI. Moreover, some labeled neurons were observed in the rostralmost part of the Bp (not shown) and the ABp showed a dense population of retrogradely labeled cells (Table 1).

Injection involving the major island of Calleja. One injection centered in the lateral septum (B0312) extended ventrally to encompass the IGM, which was the only structure in the ventral striatum affected by the injection (Figure 2). This injection gave rise to a high number of retrogradely labeled cells in different amygdaloid pallial centers (Table 1). All the nuclei in the cortical amygdala, with the exception of the APir, showed retrograde labeling (Table 1), the deep portion of the COApm (mainly its caudal aspect) being the nucleus with the highest density of labeled cells (Figure 2K). The COAp showed some labeled somata in its most rostral and deep aspect (Figure 2H). Both the superficial COAa (bilaterally) and the medial CxA showed some labeled neurons (Table 1). The LOT also displayed neurons with granular labeling in the layer 2 bilaterally (not shown). In the deep pallial amygdala some neurons were present in the ventral and caudal end of the Ba and a moderate number of labeled cells in the Bp (Figures 2L,J). The AB also showed some labeled neurons in the ventral region of the caudalmost ABa (bilaterally) and in the ABp (Table 1; Figures 2L,J). In the AHA the number of labeled cells was moderate in both subdivisions (Figure 2J). Finally, some neurons in the Lvl showed granular labeling (Table 1).

Retrograde labeling in the amygdala after injections in the olfactory tubercle
Fluorogold injections involved the olfactory tubercle in six occasions. Retrograde labeling in experiment F08100 is shown as representative example. In this case, the tracer injection was centered in the ventromedial aspect of the olfactory tubercle and encompassed the anteromedial ICj (Figure 3A). Retrogradely labeled neurons in the cortical amygdala appeared mainly in the LOT (Table 1), COAa (Figure 3B), COAp and COAp, (in all three structures located in layers 2 and 3), and in APir (Figures 3C,D). A number of labeled somata appeared also in CxA. Of note, labeled cells were also observed in the medial amygdala (subpallium), but only in its anterior part (Figure 3B). In the deep pallial amygdala, fluorogold-labeled cells were abundant in the AB (both anterior and posterior parts), B (posterior and ventral parts), and AHA (Figures 3C,D). In contrast, virtually no labeling was present in the Ba and very few cells were observed in the caudal L. Outside the amygdala, but worth mentioning given its olfactory nature, dense retrograde labeling was observed in the posterior aspect of the piriform cortex (Figures 3C,D).

ANTEROGRADe LABELING ANALYSIS
Anterograde labeling in the ventral striatum after injections in the cortical amygdala
Seven injections were aimed at the cortical portion of the pallial amygdala (Figure 4), six of which were centered in the caudal cortical amygdala whereas the other one encompassed the rostral CxA and the COAa (Figure 4A). The anterograde labeling observed in the ventral striatum in representative cases (see Tables 2 and 3) is described below.

Injections in the posterior cortical amygdala. Injections B0415 and R0341 involved, to different degrees, the COApm and COAp. Case B0415 was a large BDA injection, which mainly affected the COApm but also encompassed the medialmost aspect of the COAp. On the other hand, R0341 was quite a restricted injection in the COAp, but slightly involved the lateralmost COApm (Figure 4A). In both cases, the pipette track labeled a few cells in the caudal ABp and AHAI. In addition, cases B0815, B0816, and B0817 were centered in the caudal COApm and case B0880 was centered in the COAp (not shown). In all these cases, labeled fibers arising from the injection site used three different pathways (as defined in the rat by Petrovich et al., 1996) to reach the ventral striatum, namely the stria terminalis, the ansa peduncularis (running through the interstitial nucleus of the posterior limb of the anterior commissure, IPAC, and substantia innominata, SI) and the longitudinal association bundle (which runs rostral and ventral to reach the Tu, see Johnston, 1923). These pathways gave rise to terminal fields in the midcaudal Acb and Tu.

In the injections centered in the COApm, the AcbC showed labeled fibers in its ventral portion (Figures 4C–E). Sparse labeled fibers were present in most of the shell of the Acb, including its rostral tip, whereas dense terminal fields were observed in the periphery of the caudoventral LAcbSh (Figure 4E) and in the ventral aspect of the MAcbSh, next to some cell bridges of the ventral striatum (CB, Figure 4C; see below). In contrast,
FIGURE 3 | Retrograde labeling in the pallial amygdala after tracer injections in the olfactory tubercle. (A) Semi-schematic drawings of coronal sections through the ventral striatum of the mouse brain showing the extent of fluorogold injections in the Tu, with indication of the antero-posterior coordinate relative to Bregma. The photomicrograph shows the injection site in case F08100, which encompasses the antero-medial island of Calleja. The resulting retrograde labeling is mapped in (B–D). (B–D) Camera lucida drawings of frontal sections of the amygdala of a mouse mapping the retrograde labeling observed after an injection of fluorogold in the ventromedial olfactory tubercle (F08100). Note that retrogradely labeled neurons appear both in olfactory and vomeronasal nuclei and also in non-chemosensory amygdaloid structures, such as the basolateral amygdaloid complex.

in injection R0341 (centered in the COApl), the caudal AcbC showed sparse labeled fibers in its medial portion (see pattern in Figure 9A) and only a few labeled fibers were present in the AcbSh (Table 2).

The injections in COApm and COApl rendered a similar pattern of anterograde labeling in the rest of the ventral striatum, although the labeling resulting from injections in the COApm (e.g., B0415) was always denser than that observed following the injection in the COApl (R0341). After these injections, the densest terminal fields in the ventral striatum were observed in some of the CB (Figures 4C,D) and in parts of the caudomedial Tu next to them, where most of the anterograde labeling appeared in layer 3 (inset in Figure 4D) in close association with the islands of Calleja dorsal to it (ICj; Figures 4B and asterisks in Figures 4D,E). Regarding this, a relatively dense terminal field was associated with the lateral aspect of the ICjM (see inset in Figure 4C). Therefore, injections involving the caudal cortical amygdala resulted in prominent anterograde labeling in the CB and ICj (including the ICjM, see Table 3). Although much less dense, a similar distribution of anterograde labeling was found in the contralateral ventral striatum (Tables 2–3), probably connected with a compact bundle of labeled fibers located dorsally in the posterior part of the anterior commissure (acp) that appeared after large injections encompassing the COApm/pl.

Injection in the anterior cortical amygdala. Injection B0324 was centered in the caudal aspect of the CxA and the lateral edge of the COAa (Figure 4A), but the pipette track also labeled a few cells in the anterior part of the Ba and in the amygdalo-striatal transition area (AStr). Labeled fibers arising from the injection site entered the stria terminalis to end in the ventral striatum. Moreover, labeled fibers in the ansa peduncularis (running through the IPAC) also contributed to the labeling in the LAcbSh and caudal AcbC, whereas labeled fibers that ran through the longitudinal association bundle mainly innervated the olfactory tubercle.

In contrast to the injections in the posterior cortical amygdala, in this case conspicuous anterograde labeling was found throughout the antero-posterior axis of the Acb including its rostral tip (Table 2) and even reached the medial anterior olfactory nucleus (not shown). In the AcbSh labeling formed three main
terminal fields located in the medial (arrowhead and straight arrow in Figures 4F,G), ventral (Figures 4G,H), and caudolateral shell (Figure 4H). In addition, a small group of labeled fibers occupied the boundary between the MAcbSh and the rostral lateral septum (curved arrow in Figure 4F). At mid-rostral levels, the dense terminal fields that occupied the ventral portion of the MAcbSh and LAcbSh seemed to be continuous, through the CB, with labeling in the whole mediolateral extension of the Tu. In this injection, anterograde labeling in the Tu was dense in all three layers (maybe slightly denser in layer 3; see inset in Figure 4G), but showed no evident association with the IC (see Table 3).

In the caudal AcbC we observed a dense terminal field that extended ventromedially in the rostral direction, innervating the boundary with the MAcbSh (Figures 4G,H). In contrast, the ventrolateral AcbC showed less dense anterograde labeling, which appeared continuous with the terminal field found in the LAcbSh (Figure 4H).
Table 2 | Semiquantitative rating of the density of the anterograde labeling found in three rostro-caudal levels of the nucleus accumbens shell and core (columns) after different injections in the cortical and deep pallial amygdala (rows).

| MAcbSh | LAcbSh | AcbC |
|--------|--------|------|
| r (from +1.78 to +1.54 mm) | i (from +1.42 to +1.18 mm) | c (from +1.10 to +0.86 mm) | r (from +1.34 to +1.18 mm) | i (from +1.10 to +0.98 mm) | c (from +0.86 to +0.74 mm) | r (from +1.94 to +1.70 mm) | i (from +1.54 to +1.34 mm) | c (from +1.18 to +0.98 mm) |
| **CORTICAL AMYGDALA** | | | | | | | | |
| R0341 COApI | – | / | / | – | / | X | – | / | X |
| from −2.30 to −2.46 mm | b | b | b | b | b | b | b | b | b |
| B0415 COApI + COApI | – | / | / | – | / | XX | – | / | X |
| from −1.94 to −2.46 mm | b | b | b | b | b | b | b | b | b |
| B0817 COApI | / | XX | X | – | X | / | – | / | – |
| from −2.46 to −2.80 mm | b | b | b | b | b | b | b | b | b |
| B0324 CvA (+ COAa) | X | XX | XX | X | XX | XX | X | XX | XX |
| from −0.94 to −1.06 mm | b | b | b | b | b | b | b | b | b |
| **DEEP PALLIAL AMYGDALA** | | | | | | | | |
| B0310 Ba | XXX | XXX | XX | XXX | XXX | XXX | XXX | XXX | XXX |
| from −1.06 to −1.82 mm | b | b | b | b | b | b | b | b | b |
| R0336 Bp | X | XXX | XX | / | X | XX | XX | XXX | XXX |
| from −2.30 to −2.70 mm | b | B | b | B | b | b | B | B | B |
| B0311 AbI | / | X | XX | / | / | X | / | / | X |
| from −0.58 to −0.82 mm | b | b | b | b | b | b | B | B | B |
| R0324 Abp | X | XX | XX | X | X | XX | XX | XX | XX |
| from −1.82 mm | b | b | b | b | b | b | b | b | b |
| R0335 Abp | XXX | XXX | XX | XX | XXX | XXX | XXX | XXX | XXX |
| −2.18 mm | b | B | b | B | b | B | B | B | B |
| R0311 AbH | / | X | XX | / | X | X | / | X | XX |
| from −1.82 to −1.94 mm | b | B | b | B | b | B | b | B | B |
| R0305 Lvl | XX | XX | X | XX | XXX | XXX | XXX | XXX | XXX |
| −1.82 mm | B | B | B | B | B | B | B | B | B |
| R0330 Lvl (LdI + CPu) | XX | XX | XXX | X | XX | XX | XXX | XXX | XXX |
| −1.70 mm | B | B | B | B | B | B | B | B | B |
| B0334 | X | XX | / | X | XX | XX | XXX | XXX | XXX |
| Lvl (+LdI + Lm + AStr) | b | b | b | b | b | b | b | b | b |
| from −1.70 to −2.30 mm | | | | | | | | | |

The rostro-caudal extent (coordinates from Bregma) of each injection is indicated. The relative density is indicated as follows: –, no labeling; /, very few fibers; X, few fibers; XX, moderate density; XXX, dense terminal field; XXXX, very dense terminal field; XXXXX, extremely dense terminal field; B, conspicuous contralateral labeling; b, scarce contralateral labeling. For abbreviations, see list.

In this case, labeling in the Acb, Tu, and CB was bilateral with clear ipsilateral dominance (Tables 2 and 3, more evident in the MAcbSh and caudal AcbC, see Table 2). Labeled fibers were seen to cross the midline through the acp but did not conform conspicuous bundles.

**Anterograde labeling in the deep pallial amygdala after injections in the cortical amygdala**

To check whether the associative nuclei of the deep pallial amygdala (L, B, and AB) can indirectly relay chemosensory information to the ventral striatum, we studied the intra-amygdaloid projections from the cortical (chemosensory) amygdaloid nuclei to the deep pallial amygdala. Injections in the vomeronasal-recipient COApI gave rise to anterograde labeling in the AB (Figure 5A), particularly dense in the posterior part of this nucleus, with sparse fibers observed in the B and L. In contrast, injections in the olfactory-recipient nuclei COApI rendered moderate-to-dense anterograde labeling in the B, mainly in its lateral part, extending into the ventrolateral L (Figure 5B). Finally, the injection placed in the Cx (which receives olfactory projections, but also a minor vomeronasal input, Gutiérrez-Castellanos et al., 2010), resulted in abundant anterograde labeling in both the AB and B, which was especially dense in their lateral aspect (Figure 5C). In this case, moderately dense anterograde labeling was also observed in the Lvl (Figure 5C).
Table 3 | Semiquantitative rating of the density of the anterograde labeling found in three rostro-caudal levels of the olfactory tubercle (Tu; the antero-posterior coordinates to Bregma are indicated), the cell bridges of the ventral striatum (CB), and in association with the islands of Calleja (ICj) and the major island of Calleja (ICjM) (columns), after different injections in the cortical and deep pallial amygdala (rows).

| CORTICAL AMYGDALA | Tu | CB | ICj | ICjM |
|-------------------|----|----|-----|------|
|                   | r (from +1.98 to +1.42 mm) | i (from +1.34 to +0.74 mm) | c (from +0.62 to +0.02 mm) |
| R0341 COApl       | /  | X  | XX  | XX   | X   |
| from –2.30 to –2.46 mm | b  | b  | b   | b    |
| R0415 COApm + COApl | X | XX | XXX | XXX | XXX |
| from –1.94 to –2.46 mm | B | b  | B   | B    |
| R0817 COApm       | XX | XXX | /   | XXX  | XXX |
| from –2.46 to –2.80 mm | /  | /  | /   | /    |
| R0324 Cx(A(+ COAa) | XX | XX | XXX | XXX | /   |
| from –0.94 to –1.06 mm | b  | b  | b   | b    |
| R0310 Ba          | XXXX| XXXX| XXX | XXXX| X   |
| from –1.06 to –1.82 mm | B  | B  | B   | B    |
| R0336 Bp          | XX | XXX | XX  | XXX | X   |
| from –2.30 to –2.70 mm | b  | B  | B   | b    |

| DEEP PALLIAL AMYGDALA |
|-----------------------|
| B0311 ABa             | X  | X  | X   | XX  | X   | X   |
| from –0.68 to –0.82 mm | b  | b  | b   | b   | b   | b   |
| R0324 Ahp             | XX | XX | XX  | XX  | /   | –   |
| –1.82 mm              | B  | B  | B   | B   | b   | b   |
| R0335 Ahp             | XXX| XXX| XXX | XXX | X   | /   |
| –2.18 mm              | B  | B  | B   | B   | b   | b   |
| R0311 AHAi            | X  | XX | XX  | X   | XX  | XX  |
| from –1.82 to –1.94 mm | b  | B  | B   | B   | b   | B   |
| R0305 Lvl             | XXX| XXX| XXX | XXX | X   | /   |
| –1.82 mm              | B  | B  | B   | B   | b   | b   |
| R0330 Lvl (LdL + CPUs) | XXX| XX | XX  | XXX | X   | /   |
| –1.70 mm              | B  | B  | B   | B   | b   | b   |
| B0334 Lvl(+LdL + Lm + ASir) | /  | X  | X   | X   | /   | /   |
| from –1.70 to –2.30 mm | /  | /  | /   | /    | /    | /   |

The rostro-caudal extent (coordinates from Bregma) of each injection is indicated. The density of labeling is scored according to the following code: –, no labeling; /, very few fibers; X, few fibers; XX, moderate density; XXX, dense terminal field; XXXX, very dense terminal field; XXXXX, extremely dense terminal field; B, conspicuous contralateral labeling; b, scarce contralateral labeling. For abbreviations, see list.

Injections in the basal nucleus. Injection B0310 was restricted to the medial aspect of the Ba, although a few cells in the anterior edge of the AStr were apparently labeled by the pipette track (Figure 6A). A bundle of thick, labeled fibers entered the stria terminalis (through the IPAC and SI) and seemed to be continuous with the labeling in the causal LAcSh. Moreover, labeled fibers left the injection site and run rostralwards (through the longitudinal association bundle) to innervate the LOT and, after crossing the ventral anterior amygdala (AAV) gave rise to a dense terminal field in the Tu (Figures 6B,C).

The Acb displayed dense fields of anterograde labeling in all its divisions (Table 2). In its lateral half, labeling was very dense and showed a complex patchy distribution (Figure 6C), whereas in the medial AcbC labeling was mainly composed of sparse labeled fibers except for dense terminal field in its rostral aspect (Figure 6B). The MAcbSh displayed patchy, dense fiber labeling mainly confined to its ventral aspect (Figures 6B,C). This labeling showed continuity (through the CB, Figure 6C) with even denser terminal fields in the medial Tu (Figures 6B,C). In contrast, in the dorsal aspect of the MAcbSh only sparse labeled fibers were visible that did not make up well-defined terminal fields (Figures 6B,C). The LAcSh showed a dense terminal field that was also continuous with the extremely dense labeling found in the lateral CB and Tu (Figure 6C). In the Tu, the labeling was dense in layers 3 and very dense in layer 2 (Figures 6B,C and inset in Figure 6C), but showed no clear association with the deep ICj or ICjM. On the contrary, the anterograde labeling in the Tu showed a gap where a superficial ICj was present (Figure 6C and inset). However, a few, thick, labeled fibers always reached these superficial ICj (see inset in Figure 6C).
Injections in the accessory basal nucleus. Injection B0311 was centered in the rostral tip of ABa, and also affected the deepest aspect of the COAa and the intercalated cell masses of the amygdala (I), as well as a few cells in the AAD and the SI (Figure 7). In contrast to injections in B, this case displayed scarce labeling in the Acb and the Tu (Table 2; Figure 7B). Three groups of fibers could be tracked from the injection to the ventral striatum. First, thick, labeled fibers ran through the stria terminalis up to terminal fields in the BST and dorsolateral, caudal AcbC (not shown). Some labeled fibers followed the ansa peduncularis (through the IPAC) to the caudalmost LacbSh and the ventral pallidum (Figure 7B). Finally, fibers in the longitudinal association bundle gave rise to a dense terminal meshwork in the horizontal limb of the diagonal band nucleus (HDB) that extended into the medialmost CB (inset in Figure 7B) and the medial Tu, where it innervated all three layers. A few labeled fibers apparently entered some ICj (inset in Figure 7B), and surrounded the ICJM (Figure 7B). Moreover, the ventral MACSh displayed scarce fiber labeling apparently connected with the bundles innervating the HDB and/or VP (Figure 7B). The contralateral, caudal ventral striatum also showed few labeled fibers (see Tables 2 and 3), which were observed to cross the midline through the acp.

Two small injections (R0324 and R0335) located in the ABp (with a few labeled cells in the AHAl in case R0335; Figure 7A) revealed a pattern of fiber labeling in the ventral striatum substantially more extensive than the one observed following the injection in the ABa (compare the Figures 7B–D). The amygdalofugal fibers followed the three pathways described above. First, thin labeled fibers of the stria terminalis apparently gave rise to a moderately dense terminal field in the whole AcbC and the ventral MACSh (Figures 7C,D). Second, labeled fibers in the ansa peduncularis gave rise to a terminal field extending from the IPAC to the caudal LacbSh, where labeling was less dense (Table 2; Figures 7C,D). Finally, labeled fibers in the longitudinal association bundle reached the Tu, where they preferentially innervated layers 2,3, and 1b of its central aspect (inset in Figure 7D). Labeling in the Tu was continuous, through the CB, with the one observed in the ventral AcbSh (see arrows in Figures 7C,D). Few labeled fibers were associated to the outer edge of most of the ICj (inset in Figure 7C) and, to a lesser extent, to the inner margin of the ICJM (arrowhead in Figure 7C). In both injections, the contralateral ventral striatum also showed a moderate density of anterograde labeling (Tables 2 and 3).

Injection in the amygdalo-hippocampal area. A small injection (R0311) was restricted to the rostral tip of the AHAl (Figure 7A). Labeled fibers in the stria terminalis apparently innervated the AcbC (where labeling decreased from caudal to rostral, see Table 2), especially its ventral half (Figures 7E,F) and the midcaudal MACSh, where labeled fibers were preferentially found in the medial edge of the nucleus (arrow in Figure 7F) and next to the core-shell boundary (Figures 7E,F). A dense terminal field was closely associated to the inner ICJM (see inset in Figure 7F). The labeled bundles in the IPAC/SI could be followed rostrally up to the midcaudal LacbSh, where sparse anterograde labeling was observed (Figure 7F). Finally, labeled fibers running in...
FIGURE 6 | Anterograde labeling in the ventral striatum after tracer injections in the basal nucleus of the amygdala. (A) Semi-schematic drawings of frontal sections showing the extent of the tracer injections in the basal nucleus of the amygdala, with indication of the antero-posterior coordinate relative to Bregma. (B, C) Anterograde labeling found in the ventral striatum after a tracer injection in the Ba (B0310). Although the ICj are nearly devoid of labeling, a few thick, labeled fibers enter the clusters of granule cells (see inset in (C)). Labeling in the AcbSh shows continuity with the one in the Tu through the CB. Labeling in the Tu occupies layers 2 and 3, whereas layer 1 is virtually free of labeling (inset in (C)). (D, E) Anterograde labeling in the ventral striatum after injection R0336, which involved a large portion of the Bp. Labeling in the core of the Acb apparently shows continuity with the one in MAcbSh (see arrowheads in (E)) and, through it, with the labeling in the CB and medial Tu. labeling in the Tu occupies not only layers 2 and 3 but also deep layer 1 (inset in (E)). For abbreviations, see list. Scale bar in (E) = 400 μm, valid for the whole figure.

the longitudinal association bundle innervated the HDB and all three layers of Tu, preferentially in its medial half (Figure 7F). This terminal field, which was connected with the one in the AcbSh through the CB (see asterisk in Figures 7E, F), also included dense labeled fibers in close association with the medial ICj (Figure 7F).

Labeled fibers crossed the midline through the acp to innervated some structures of the contralateral, caudal ventral striatum (Tables 2 and 3).

Injections in the lateral nucleus. Three injections were aimed at the L (see Figure 8). Injection R0330 was centered in the rostral Lvl, although it also encompassed the ventral part of the Ldl and involved a few cells in the caudoventral CPu. Injection R0305 consisted of a small injection restricted to the Lvl at intermediate antero-posterior levels. Finally, a large BDA injection encompassed the whole caudal L (Ldl, Lvl, and Lm) and the AStr (B0334). In these three injections, labeled fibers ran in the stria terminalis up to the AcbC, where they gave rise to the densest terminal field in the ventral striatum, which extended to the adjacent, ventralmost CPu (Table 2; Figures 8B–E). From the AcbC, anterogradely labeled fibers extended to the ventral AchSh, where they formed a conspicuous terminal field (Figures 8B–D), whereas the dorsal MAcbSh (interposed between the core and the medial
FIGURE 7 | Anterograde transport in the ventral striatum after tracer injections in the accessory basal amygdaloid nucleus and amygdalo-hippocampal area. (A) Semi-schematic drawings of frontal sections showing the extent of the tracer injections in the accessory basal nucleus of the amygdala. The antero-posterior coordinate relative to Bregma is indicated in each section. (B) Anterograde transport in the MAcbSh and medialmost caudal Tu after a tracer injection in the ABa (B0311). Scattered labeled fibers are present in the ICj and in all three layers of the Tu (inset). (C,D) Frontal sections through the ventral striatum of a mouse that received an RDA injection in the ABp (R0335; no counterstaining). Besides extensive labeling in the Acb core and shell (especially in the LAcbSh), a labeled terminal field is found next to some of the ICj [inset in (C)] and in the ICjM (arrowhead in (C)]. In the Tu, anterograde labeling is found in layers 2 and 3 and in deep layer 1 [inset in (D)]. (E,F) Anterograde labeling in the ventral striatum after an RDA injection restricted to the lateral AHA (R0311) (no counterstaining). Moderately dense labeling occupies the ventral core as well as parts of the medial and lateral shell of the Acb. Scattered labeled fibers are also found in the neighboring CB (asterisks) and in the Tu. In addition, conspicuous terminal fields are found in the medial ICj and, especially, next to the ICjM [see inset in (F)]. For abbreviations, see list. Scale bar in (F) = 400 μm, valid for the whole figure.

septum), was virtually devoid of labeling (Figures 8B–D). Labeling in the ventral AcbSh was continuous, through the CB, with labeling in the Tu (Figures 8C–E). Anterogradely labeled fibers displayed a clear association with the most medial ICj (see inset in Figure 8D), but not with the ICjM (see Figure 8D). After the small injections in the rostral L (R0305, R0330), labeling in the Tu was particularly dense, and preferentially occupied layer 3 and (to a lesser extent) layer 2 (Figures 8B–D and inset in Figure 8B).

In addition, some labeled fibers ran through the ansa peduncularis (through the SI and the IPAC) and gave rise to a moderate-to-dense terminal field in the midcaudal LAcbSh (Figure 8E). Finally, a small number of labeled fibers were seen in longitudinal association bundle, apparently giving rise to anterograde labeling in the Tu.

In all the injections encompassing the L, anterograde labeling in the ventral striatum was bilateral with ipsilateral dominance (see Tables 2 and 3).

DISCUSSION

Our work combines anterograde and retrograde tracing to analyze the projections of the amygdala to the ventral striatum, with emphasis on the putative pathways allowing the transfer of chemosensory information to the reward system of the brain.
To properly interpret our results we should first discuss some limitations of the tract-tracing techniques we have used.

**METHODODOLOGICAL CONSIDERATIONS**

In the first part of the study we have used the retrograde transport of fluorogold and biotin- and rhodamine-labeled dextran amines to label the amygdaloid afferents to the ventral striatum. Although dextran amines are generally used as anterograde tracers, in certain conditions they also reliably render retrograde transport (Rajakumar et al., 1993; Reiner et al., 2000; Schofield, 2008) and they have been used as such in mammals (Schofield and Cant, 1996). In fact, our retrograde tracing experiments replicate the results of similar experiments in rats using fluorogold (Brog et al., 1993). In addition, our results of retrograde tracing of the amygdal-o-striatal pathways are consistent with those of anterograde tracing of the same projections (see below).

In the second part of the study, we have injected dextran amines in the amygdala to analyze the anterograde transport to the ventral striatum and within the amygdala itself. Since in our experimental conditions dextran amines are transported both retrogradely and anterogradely, in the interpretation of the results we take into account the possibility of fiber labeling due to axon collaterals from retrogradely labeled cells. In addition, in every injection anterogradely labeled fibers can be tracked through previously defined
tracts (see below) from the injection site to the terminal fields in the ventral striatum. This indicates that the bulk of the labeling found in the striatum originates in cells located in the injection site in the amygdala. On the other hand, the projections revealed by anterograde labeling in the amygdala have been confirmed by means of retrograde labeling from injections in the striatum, except for a single case, the projection from the lateral nucleus of the amygdala to the olfactory tubercle, as discussed below.

**DIRECT AND INDIRECT PATHWAYS THROUGH THE AMYGDALA RELAY CHEMOSENSORY INFORMATION TO THE VENTRAL STRIATUM**

Our results demonstrate that both cortical and deep amygdaloid nuclei extensively innervate the ventral striatum. Given that the cortical amygdaloid nuclei (COAa, CxA, COApI, and COApM) receive olfactory and vomeronasal projections from the olfactory bulbs, it is likely that the amygdalo-striatal projections originated in these nuclei convey chemosensory information. In addition, our results show that intra-amygdaloid projections originated from these cortical amygdaloid nuclei innervate the lateral, basal, and accessory basal nuclei of the basolateral complex, which are considered associative nuclei (they receive important projections from unimodal and multimodal areas from the thalamus and cortex, see Turner and Herkenham, 1991; McDonald, 1998; Swanson and Petrovich, 1998). These projections from the chemosensory cortical amygdala to the deep associative nuclei are consistent with previous data reported in rats. The projection from the vomeronasal-recipient COApM to the AB was observed by Canteras et al. (1992) and Kemppainen et al. (2002). Regarding the COApI, it is included as part of the periamygdaloid cortex by several authors (see Pitkänen, 2000), and a number of studies show projections from the periamygdaloid cortex to the L (Ottersen, 1982; Luskin and Price, 1983; Majak and Pitkänen, 2003) and to the B (Ottersen, 1982; Luskin and Price, 1983). Finally, to our knowledge the connections of the CxA have not been previously analyzed, but a previous study using the retrograde transport of HRP in the rat showed retrogradely labeled cells in this area following injections in the L and B (Ottersen, 1982; see his Figures 2D and 5F). None of these intra-amygdaloid projections had been previously shown in mice. Therefore, both the direct projections from the chemosensory cortical amygdala and the indirect projections from the associative amygdaloid nuclei might convey chemosensory information to the ventral striatum.

**ORGANIZATION OF AMYGDALO-STRIATAL PATHWAYS IN THE MOUSE**

These amygdalo-striatal projections course through three pathways, namely the *stria terminalis*, the *ansa peduncularis*, and the longitudinal association bundle, in agreement with previous descriptions (Johnston, 1923; Kelley et al., 1982; Petrovich et al., 1996). In general, the amygdaloid fibers terminating in the AcbC and dorsomedial AcbSh course through the *stria terminalis* and represent a rostral extension of the terminal fields in the BST. A second amygdalo-striatal pathway courses through the *ansa peduncularis* crossing the SI/IPAC and entering the lateral AcbSh, and maybe the caudalateral AcbC. A third contingent of amygdalo-striatal fibers runs rostrallywards through the longitudinal association bundle (Johnston, 1923) thus reaching directly the caudal Tu and adjacent structures. Fibers running in all three pathways might contribute to the innervation of the ventral aspect of the AcbSh and the adjoining CB.

The termination fields of the main amygdaloid projections to the ventral striatum are summarized in Figure 9. As it can be observed, virtually all the divisions of the ventral striatum receive inputs from one or several amygdaloid nuclei, thus depicting a complex pattern of amygdalo-striatal projections.

**Ventral striatal projections of the cortical amygdaloid nuclei**

Our experiments reveal important amygdalo-striatal projections arising from several cortical amygdaloid nuclei which, according to the present results, were probably underestimated in previous studies. The anterior aspect of the cortical amygdala displays substantial projections to the Acb and Tu/CB apparently originated from deep cells in the Cx/COAa boundary (Figures 2B and 3B). This projection was also suggested by previous observations in rats (McDonald, 1991b) and monkeys (Friedman et al., 2002). Regarding the posterior cortical amygdala, our results show that a number of deep cells of the COApM and COApI are retrogradely labeled by injections in the Tu. These projections are confirmed by the anterograde labeling following injections in the COApM and COApI, which reveal a conspicuous projection to the deep Tu, CB, and some ICg (see below; Ubeda-Bañón et al., 2008), as well as sparse projections to the caudal AcbC and AcbSh (see Figure 9), in agreement with previous results in rats (Canteras et al., 1992).

Our injections in the core and lateral shell of the Acb, also showed retrogradely labeled cells in the LOT. The location of these labeled cells depended on the injection site: whereas tracer injections in the L/AcbSh rendered retrograde labeling in layers 2 and 3 of the LOT, those in the AcbC only showed labeled cells in layer 3. These data fully fit the results of anterograde tracing in the rat reported by Santiago and Shammah-Lagnado (2004).

Finally, in agreement with the findings by Brog et al. (1993) in the rat, our results of retrograde labeling demonstrate the presence of important projections to the M/AcbSh and Tu arising from the APir. Although we have not performed tracer injections in the APir, experiments of anterograde transport carried out in the rat by Shammah-Lagnado and Santiago (1999) confirm this projection. As suggested previously (Luskin and Price, 1983), these projections might be association fibers, originating from pyramidal cells located in layers II and III.

**Ventral striatal projections of the deep nuclei of the pallial amygdala**

This pattern of amygdalo-striatal projections originated by the deep pallial amygdala displays three main similarities with the results of previous studies in other mammalian species.

Firstly, as described in rats and cats (Krettek and Price, 1978; Groenewegen et al., 1980; Kelley et al., 1982; Russchen and Price, 1984; McDonald, 1991a,b; Brog et al., 1993; Wright et al., 1996) and in monkeys (Russchen et al., 1985; Price et al., 1987; Friedman et al., 2002; Fudge et al., 2002, 2004), the bulk of the amygdalo-Acb projections arises from deep pallial nuclei (B and AB). Secondly, our results indicate that in mice, like in rats and cats (Krettek and Price, 1978; Wright et al., 1996) the projections from the anterior and posterior parts of the B to the Acb display a rough topography according to which the Ba projects mostly to the anterior...
Novejarque et al. Amygdalo-striatal projections in mice

**FIGURE 9 | Summary of the projections from pallial amygdala to the ventral striatum in the mouse.** Schematic drawings of frontal sections through the ventral striatum of the mouse (rostral on top, caudal bottom), showing the main terminal fields of the projections from the cortical (A,B) and deep pallial amygdala (C–H) in the ventral striatum. The relative density of the terminal fields, as inferred from our injections, is represented as different gray levels (the darker the denser). The patterned area in the medial AcbC in (A) indicates a terminal field that is labeled after the injection in the COApm but not in the injection centered in the COApl. For abbreviations, see list.

and posterolateral Acb/Tu, whereas the Bp projects mainly to the medial posterior Acb/Tu (Figures 9C,D). And finally, anterograde transport after injections in B and AB reveal a patchy distribution of labeled fibers in the medial AcbSh (see Figures 6C,E and 7B,D) that recalls the one described in the rat and cat by Krettek and Price (1978). Whether or not these patchy projections are related to inhomogeneities in calbindin-immunoreactivity as reported in the rat (Wright et al., 1996), the similarities in the amygdalo-Acb projections between mice and other mammals are noticeable. This similarity also makes very unlikely that the fibers observed in our material be due to labeling of axonic collaterals from retrogradely labeled cells.

An additional conclusion drawn from our findings is that the AHA and L substantially contribute to the projections from the deep pallial amygdala to the Acb and Tu. Experiments in rats have also demonstrated substantial anterograde transport to the caudal Acb after tracer injections in the AHA (Canteras et al., 1992, posterior amygdala using their nomenclature) and dense retrograde labeling in the AHA following injections in the MAcbSh (McDonald, 1991b; Brog et al., 1993).

Finally, our results clearly indicate that the L, a nucleus usually viewed as the sensory interface of the amygdala (at least in the context of fear conditioning, LeDoux et al., 1990; Pitkänen et al., 1997), shows important projections to the ventral striatum. The existence of this connection has been previously suggested in rats (Kelley et al., 1982; McDonald, 1991b; Brog et al., 1993) and primates (Friedman et al., 2002; Fudge et al., 2002). Our results indicate that these projections mainly arise from the dorsolateral (Ldl) and ventrolateral subnuclei (Lvl, see Table 1). This is confirmed by anterograde transport after injections in the L (involving the Lvl and/or Ldl), which display labeled fibers in the midcaudal AcbC (from where they extend into the rostromedial CPu), as well as in the ventral aspect of the MAcbSh in and the LAcbSh (Figure 9H). This projection apparently extends into the Tu/CB complex and the Icj, although we could not confirm this observation with retrograde labeling.

Amygdaloid projections to the islands of Calleja and associated territories of the olfactory tubercle

Our tracing experiments reveal not only important amygdaloid projections to the Tu, but also very important connections with other structures including Icj and the CB (see Figure 9). The Icj are densely packed groups of small cells that occupy diverse positions within the ventral striatum. Most of them are placed within the Tu, where they are located in layers 2–3 and even impinge upon layer 1 reaching the pial surface. In contrast, the IcjM separates the medial AcbSh from the ventral aspect of the lateral septum. The COApm/COApl and AHA display strong projections to the medial Icj and IcjM (see Table 3). In contrast, the remaining amygdaloid nuclei give rise to limited projections to the Icj, which are relatively rare in the case of the IcjM (see Table 3). These results partially agree with previous studies on
the ICj connections indicating that the bulk of the amygdaloid projections to the ICj arose from the cortical nuclei (Krettek and Price, 1978; Fallon, 1983). Recent reports have also described in some detail these projections in rats and mice (Úbeda-Bañón et al., 2007, 2008).

The projections to the CB have received minor attention. In this respect, labeling in a portion of the Tu was always accompanied by labeling of those CB adjacent to it (see Table 3) suggesting that the Tu and CB are functionally related.

FUNCTIONAL CONSIDERATIONS: ROLE OF AMYGDALO-STRIATAL PATHWAYS IN EMOTIONAL EVALUATION OF CHEMSENSORY STIMULI

As we have seen, the pallial amygdala projects heavily to the core and shell of the nucleus accumbens, the olfactory tubercle, and the striatal cell bridges linking the ventral Acb with the Tu, and the ICj. The amygdala-accumbens projections are considered to be involved in reward-related processes (Everitt et al., 1999; Baxter and Murray, 2002; see below). In this respect, our findings lead to two main conclusions.

First of all, the Tu receives massive projections from the pallial amygdala, conveying olfactory and vomeronasal information (from the cortical amygdala) and non-chemosensory information (from the basolateral amygdala). Since these projections mainly terminate in layers 2 and 3, they likely target the cell bodies and proximal dendrites of the principal cells and thus probably have a strong influence on the activity of the Tu. Therefore, the Tu, CB and ICj constitute a portion of the ventral striatum mediating responses to a variety of chemo- and non-chemosensory stimuli, as suggested by Talbot et al. (1988a,b) on the basis of anatomical and neurochemical studies in different mammals. In this respect, several nuclei of the pallial amygdala project to the ICj. However, the projection from the COApm (the main cortical target of the accessory olfactory bulb, Scala and Winans, 1975) apparently terminates, in a fairly specific way, in the neuropil-rich core of the ICj, and in the lateral aspect of the ICJM. Although the inner structure and cell composition of the ICj are very complex (Fallon et al., 1978, 1983; Fallon, 1983), the specific input from a vomeronasal relay suggest that some ICj might be specialized structures of the ventral striatum devoted to processing vomeronasal information.

The second conclusion drawn from our findings is that the medial Tu and adjoining structures (CB and ICj) are very likely involved in mediating (afforded) reward-related responses to chemical signals. This is supported by the fact that both electrical stimulation in the medial Tu (Prado-Alcala and Wise, 1984) and local administration of addictive drugs (Kornetsky et al., 1991; Ikekimo et al., 2005) are rewarding. Since the medial aspect of the Tu and neighboring CB and ICj, including the ICJM, receive distinct projections from the vomeronasal amygdala (COApm and AHA) and stimulation of the vomeronasal amygdala is rewarding (Kane et al., 1991), it is likely that these amygdalo-striatal projections mediate reward responses to (among other stimuli) vomeronasal and/or olfactory-detected chemosignals (see also Úbeda-Bañón et al., 2007, 2008).

Regarding the projections from the basolateral amygdala to the ventral striatum, they are involved in the expression of reward-related behaviors toward secondary reinforcers (Cador et al., 1989; Everitt et al., 1989). In fact, Pavlovian second-order conditioning is impaired by lesions of either the accumbens (Parkinson et al., 1999) or the basolateral amygdala (Hatfield et al., 1996; Blundell et al., 2001). These findings indicate that the learning processes occurring in the basolateral amygdala endow neutral stimuli with appetitive properties by means of their association with primary reinforcers. Through its projections to the ventral striatum, the basolateral amygdala would mediate goal-directed responses to these newly attractive stimuli.

In this respect, it has been shown in female mice that odors are not innately attractive but become so after their association with non-volatile (e.g., vomeronasal), innately rewarding male pheromones (Moncho-Bogani et al., 2002; Martinez-Ricós et al., 2007, 2008; Roberts et al., 2010). This association of olfactory and vomeronasal stimuli might occur in the basolateral amygdala, since the cortical amygdala, and piriform cortex (present results; McDonald, 1998) display convergent projections to deep amygdaloid nuclei (Canteras et al., 1992; Pitkänen, 2000; Lanuza et al., 2008). This hypothesis is consistent with the expression of c-fos in the basolateral amygdala of female mice induced by male odors before and after olfactory–vomeronasal associative learning (Moncho-Bogani et al., 2005). A similar role has been proposed for the basolateral amygdala in olfactory–gustatory associative learning mediating go–no go appetitive responses (Schoenbaum et al., 1999).

EVOLUTION OF THE EMOTIONAL BRAIN

Comparative studies (Bruce and Neary, 1995; Martínez-García et al., 2002, 2007) indicate that reptiles possess a well-developed pallial amygdala composed of chemo- and non-chemosensory cortical structures (superficial, layered) and multimodal deep nuclei. Anatomical and neurochemical data in different reptiles and in birds strongly suggest that the projections from the pallial amygdala to the ventral striatum were already present in the ancestral amniote and underwent a conservative evolution (Novejarque et al., 2004; Martinez-García et al., 2007). In this respect, our finding that parts of the cortical amygdala of mammals display substantial projections to the ventral striatum fit the results of similar studies in non-mammals. Thus, the vomeronasal cortex of squamate reptiles (the nucleus sphericus), like its mammalian counterpart, the COApm, projects massively to a portion of the ventral striatum located between the nucleus accumbens and the olfactory tubercle. In snakes this portion of the ventral striatum is called the olfactostriatum (Lanuza and Halpern, 1997), and displays characteristic neurochemical features (Martínez-Marcos et al., 2005). Together with recent data in rats (Úbeda-Bañón et al., 2008), the present results suggest that the “olfactostriatum” would be represented in the mammalian brain by the anteromedial cell bridges and ICj, plus portions of the adjacent Tu that receive the projection from the COApm. In fact, like the ophidian olfactostriatum (Martínez-Marcos et al., 2005) the medial portions of the CB, the ICj, and the Tu display a moderate-to-dense innervation of neupeptide Y-immunoreactive fibers (Riedel et al., 2002; Úbeda-Bañón et al., 2008). In addition, cells in the Tu and the ICj express 5-hydroxytryptamine-2A receptors (Mijnster et al., 1997; Jansson et al., 2001). Although these data support this homology, it is noteworthy that the CB, the ICj, and the Tu show a
high density of TH-immunoreactive axons (Seifert et al., 1998; Riedel et al., 2002; Ubeda-Bañón et al., 2008), whereas the ophidian olfactostriatum displays a comparatively sparser innervation of TH-immunoreactive fibers (Martínez-Marcos et al., 2005).

Anatomical, neurochemical, and functional data suggest that the telencephalon of the ancestral amniote possessed an amygdalo-striatal system. The fact that this circuitry has remained well conserved indicates that its function is crucial for survival and reproduction. Indeed, the amygdala receives multimodal sensory information and controls the expression of basic emotional behaviors, namely reward/attraction (throughout its projections to ventral striatum) or fear/anxiety/aversion (throughout its projections to the central extended amygdala). Therefore, in the ancestral amniote, the amygdalo-striatal system constituted part of the primal emotional brain, since it probably allowed not only the appropriate behavioral response toward unconditioned attractive (rewarding) stimuli, but also learning from these events by means of the emotional tagging of neutral sensory stimuli.

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