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Cytoarchitecture of the accessory olfactory bulb in the salamander *Plethodon shermani*

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ABSTRACT

Plethodontid terrestrial salamanders are emerging models in the study of the evolution of chemical communication in vertebrates. Their vomeronasal system is well defined. It comprises sensory neurons in the epithelium of the vomeronasal organ, whose axons form the vomeronasal nerve projecting to the accessory olfactory bulb (AOB), which in turn projects to the vomeronasal amygdala through the accessory olfactory tract. A detailed description of the cellular elements of the urodele AOB is lacking. Neuronal morphology in the AOB was studied by means of biocytin intracellular injections and retrograde tract tracing in the salamander *Plethodon shermani*. The AOB exhibits the characteristic lamination of olfactory bulbs, except that it displays a mixed periglomerular and mitral somata layer superficially. Mitral cells are the only AOB neurons projecting to the vomeronasal amygdala. Each mitral cell sends multiple axonal branches, generally through both dorsal and ventral portions of the accessory olfactory tract. Some mitral cells additionally send axon collaterals in the white matter immediately ventral to the AOB. AOB interneurons are divided into superficial periglomerular and deep granule cells, each category exhibiting morphological variety. Some neurons in the granule cell layer of the AOB or the region ventral to the AOB have dendritic trees that cover both regions. The present study is the first to highlight the full anatomical extent of single AOB neurons and surprisingly suggests that the ventrolateral telencephalon found below the AOB is part of the salamander vomeronasal system.

1. Introduction

Studies of the vomeronasal system (VNS, or accessory olfactory system) are mostly conducted in rodents in the context of chemical communication in mating and social behaviors. Involvement of the VNS in other aspects of olfactory behavior has rarely been investigated in mammals. However, at least one predator cue (cat odor) activates the accessory olfactory bulb (AOB) preferentially in rats suggesting that additional functions of the mammalian VNS might still await discovery (McGregor et al., 2004). Many studies suggest that the VNS mediates detection of diverse biologically relevant chemical cues in reptiles and amphibians. For example, the snake vomeronasal organ is involved in the detection of reproductive, prey and predator cues (Noble, 1937; Wilde, 1938; Kubie et al., 1978; Halpern and Frumin, 1979; Kubie and Halpern, 1979; Kirschenbaum et al., 1986; Burghardt, 1993; Alving and Kardong, 1996; Miller and Gutzke, 1999). In terrestrial salamanders, a role of the VNS in the detection of reproductive pheromones, prey cues and home areas was experimentally demonstrated (Graves, 1994; Placyk and Graves, 2002; Wirsig-Wiechmann et al., 2002; Laberge et al., 2008), and these animals can also detect predator cues on the

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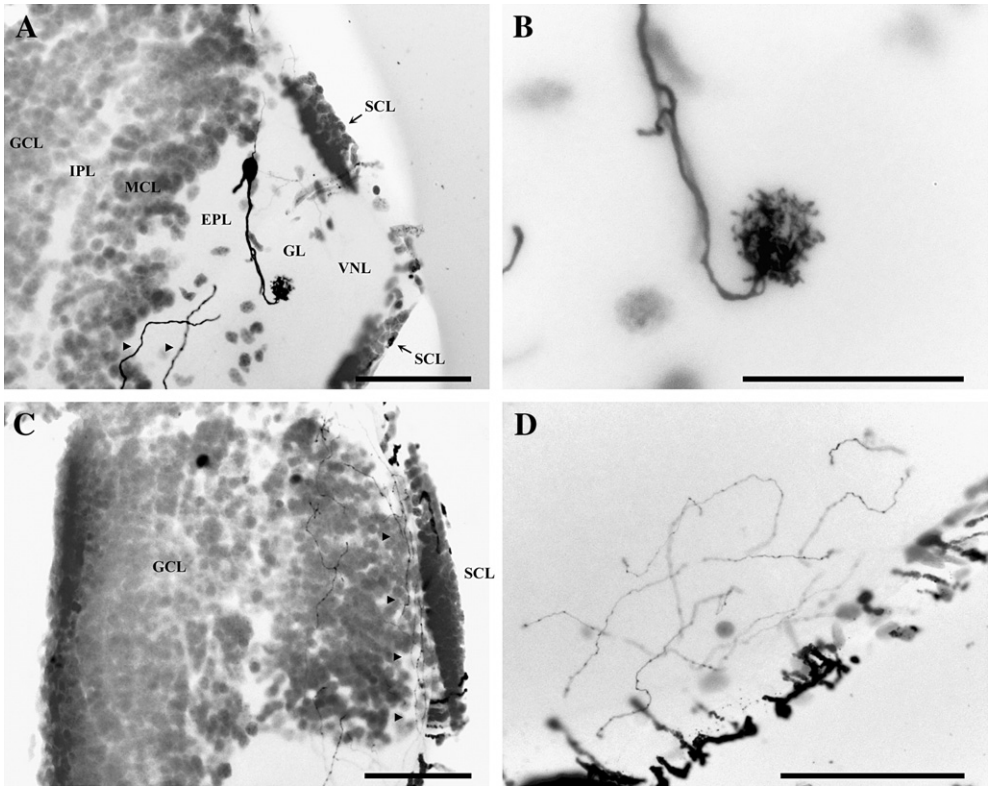

Fig. 1 – Mitral cell processes. A: Micrograph showing a mitral cell labeled by intracellular injection of biocytin in the external plexiform layer of the accessory olfactory bulb. The cell displays a glomerular arbor and two lateral dendrites (arrowheads) within this 50 μm-thick coronal section. The glomerular arbor is shown at a higher magnification in panel B. The arrowheads in panel C show the descending course of several axon branches belonging to this mitral cell in the most caudal part of the accessory olfactory bulb. D: Axon meshwork of the mitral cell in the white matter of the vomeronasal amygdala, where the accessory olfactory tract terminates. Note the abundant axonal varicosities. Scale bars are 100 μm in panels A, C and D, and 50 μm in panel B. Abbreviations: EPL: external plexiform layer; GCL: granule cell layer; GL: glomerular layer; IPL: internal plexiform layer; MCL: mitral cell layer; SCL: superficial cell layer; VNL: vomeronasal nerve layer.

substrate suggesting that the VNS could be involved in that case too (Sullivan et al., 2002).

Eisthen (1997) remarked that the presence and features of the VNS are highly variable in tetrapods. This presents an opportunity to study the selection pressures leading to elaboration or reduction of the vomeronasal organ. However, the precise role of the VNS remains unknown, and the suggestion that it is an adaptation to terrestrial life was rejected on the ground that it is present throughout the lifecycle of salamanders (aquatic and terrestrial), caecilians and frogs (Schmidt and Wake, 1990; Eisthen, 1997; Eisthen, 2000). Herrick (1921, 1924, 1931) described the urodele AOB, when present, as a very rudimentary structure compared to its counterpart in anurans. However, recent studies have shown that the VNS of some salamanders is particularly well developed (Schmidt et al., 1988; Schmidt and Roth, 1990; Laberge and Roth, 2005). This makes them good model species to elucidate VNS function. In addition, North American plethodontid salamanders are used as models for the evolution of chemical communication because they are diverse, display conserved mating sequences, and use pheromones during courtship following discrete delivery modes that are well characterized phylogenetically (see Watts et al. (2004), Palmer et al. (2005), Palmer et al. (2007)).

The objective of the present study is to complete the description of the cellular substrate for olfactory processing in the vomeronasal pathway of the salamander *Plethodon shermani*. The olfactory pathways and amygdala complex of this species (previously referred to as *P. jordani*) were previously described by Schmidt et al. (1988) and Laberge and Roth (2005). The present article concerns itself with the AOB. In amphibians, the AOB forms a bulge on the lateral telencephalon caudal to the main olfactory bulb (MOB). It is found in a dorsolateral position in urodeles, as opposed to a ventrolateral position in anurans. Olfactory bulbs are crucial structures in all vertebrate olfactory pathways. They are involved in shaping olfactory signals before transmission to central regions and receive centrifugal modulation from the latter. Olfactory bulbs are laminated structures comprising projection neurons, typically mitral cells, and interneurons, mostly periglomerular and granule cells although additional neuron types are sometimes observed depending on the species (reviewed in Allison (1953), Nieuwenhuys (1967), Meisami and Bhatnagar (1998)).

The present study used intracellular injections of biocytin in order to reveal the full anatomical extent of single neurons. Cellular processes belonging to a single neuron could only be followed for a short distance in early Golgi studies of the amphibian olfactory bulbs because of simultaneous labeling of multiple neurons (Herrick (1924, 1931) and references therein; Ramón y Cajal (1995)). Further, the latter studies made few observations of the AOB itself. The aim is to lay the groundwork for a physiological investigation of the VNS in *P. shermani*.

2. Results

2.1. *Gross anatomy of the accessory olfactory bulb*

The AOB in *P. shermani* is a paired structure located in the dorsolateral telencephalon caudal to the main olfactory bulb. The AOB is of a convex semi-oval shape. Its most rostral part is almost inconspicuous in the dorsal MOB, whereas a clear caudal border is defined by a dense layer of somata reaching up to the surface of the telencephalon (see Fig. 1C). Layers are somewhat typical of olfactory bulbs as seen in Fig. 1A with a vomeronasal nerve layer (VNL), glomerular layer (GL), a cell-poor external plexiform layer (EPL), mitral cell layer (MCL), internal plexiform layer (IPL), and granule cell layer (GCL). However, there is one additional layer of superficial cell somata clearly visible with the Nissl counterstain (SCL in Fig. 1). This superficial cell layer is found in most of the AOB with the exception of the ventral part of its rostral half where the vomeronasal nerve is running superficially. The superficial cell layer appears continuous with the mitral cell layer and surrounds the white matter where the glomeruli are found. The extent of the mitral cell layer can be seen in Fig. 2, which shows neurons backfilled by an application of biocytin to the accessory olfactory tract. The compact periventricular cell mass found below the AOB is continuous with the granule cell layer of the AOB. It is unclear whether the region below the AOB is part of the AOB itself, the caudal MOB or the nucleus accumbens. It could also represent a distinct region on its own.

Intracellular injections of biocytin were used to study neuronal organization of the diverse layers of the AOB. In the following sections exemplifying the neuron types of the AOB, only intracellular labeling of high quality was selected for cytological measurements since the reconstruction of neuronal architecture is difficult or incomplete when dealing with neuron labeling of low quality. Measurements are expressed as range and mean ± standard error.

2.2. *Projection neurons*

Neurons sending axon branches in the accessory olfactory tract were considered projection neurons. Thirteen single neurons and one cluster of 2 neurons fulfilling that criterion were reconstructed (Figs. 3–7). These neurons, commonly called

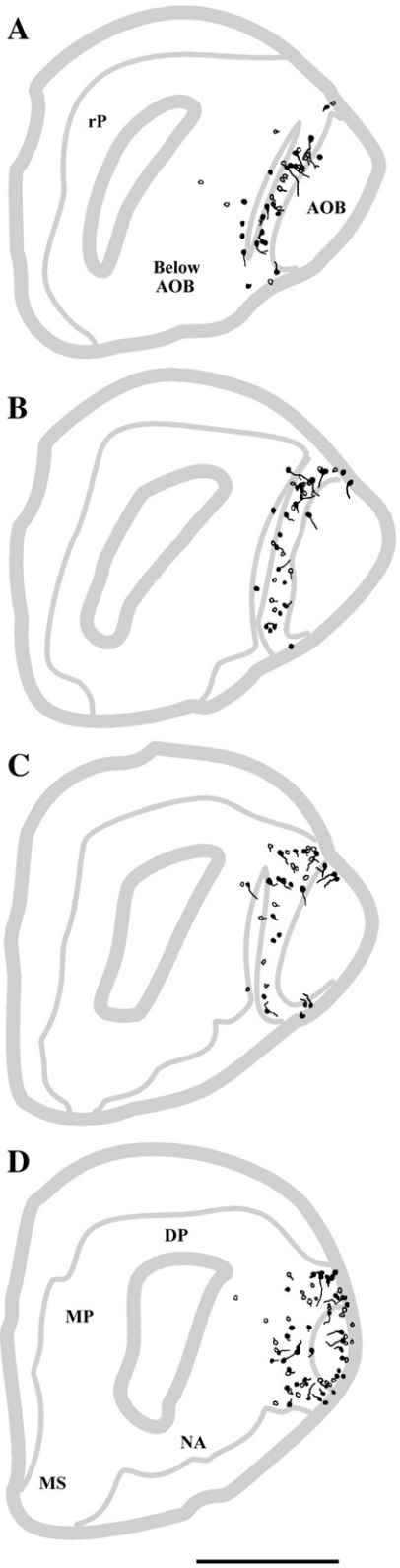

Fig. 2 – Retrograde labeling of projection neurons after application of biocytin in the accessory olfactory tract. Open cell outlines represent weakly labeled cells. Each panel represents a 50 μm-thick coronal brain section separated by 100 μm from the neighboring section. Scale bar in panel D is 1 mm. Abbreviations: AOB: accessory olfactory bulb; Below AOB: region below the accessory olfactory bulb; DP: dorsal pallium; MP: medial pallium; MS: medial septum; NA: nucleus accumbens; rP: rostral pallium.

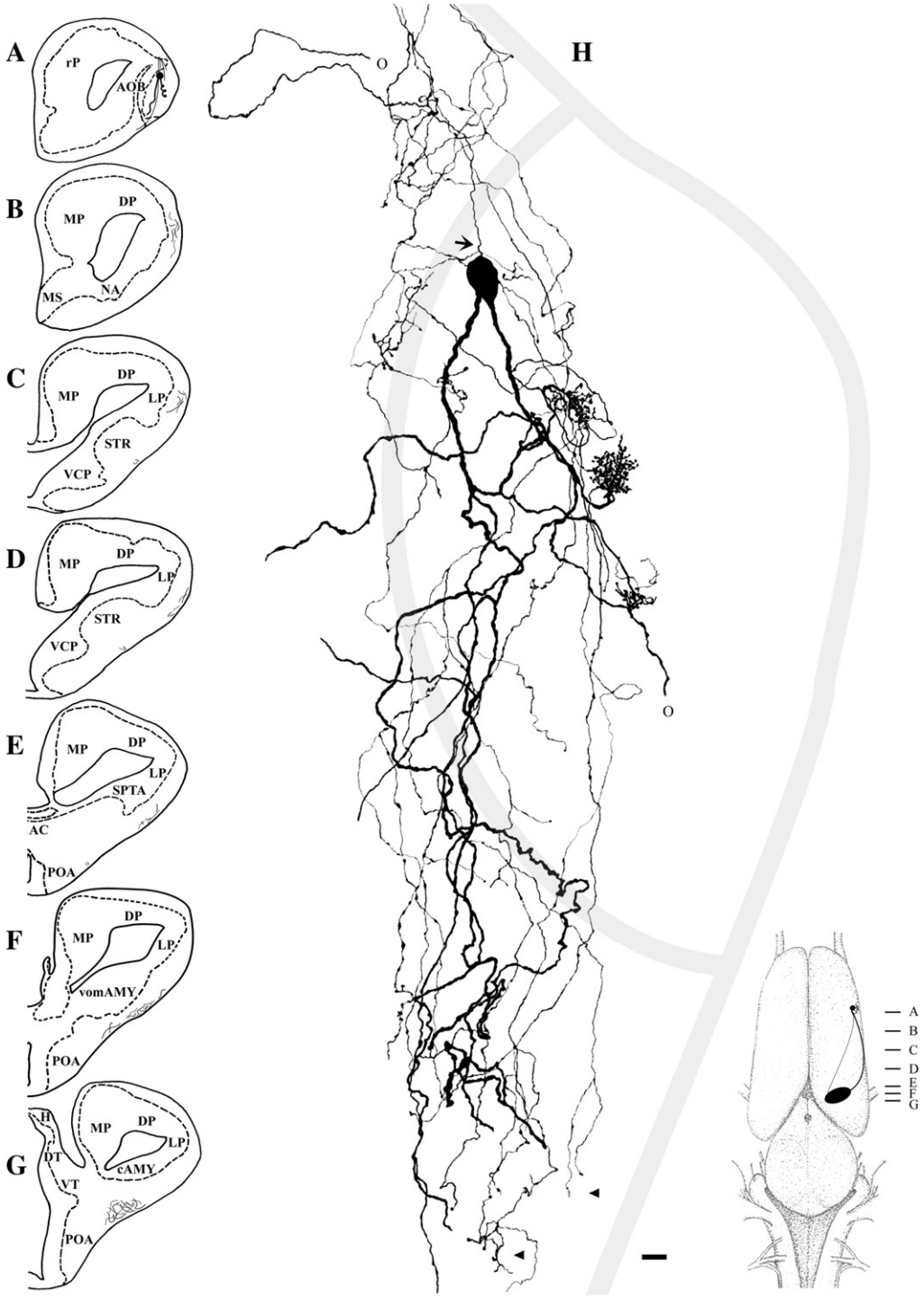

Fig. 3 – Reconstruction of the mitral cell shown in Fig. 1. A–G: the right half of selected coronal brain sections is illustrated showing soma location and axonal processes. Levels of brain section and a schema of axon paths are illustrated on a schematic dorsal view of the salamander brain in the bottom right corner. Panel H (middle) shows the cellular reconstruction, which, for clarity, includes only processes found within the accessory olfactory bulb. The light gray outline represents the outside surface of the brain and the limit between the mitral and external plexiform layers at the level of the soma. The arrow indicates the origin of one axon, whereas circles indicate the cut end of an axon branch that projects to the vomeronasal amygdala. Lateral dendrites are the thick processes extending ventrally, whereas most axon branches are very thin and display varicosities. Some axon branches of this mitral cell terminate in the white matter below the accessory olfactory bulb (arrowheads). Scale bar in H is 10 μm. Abbreviations: AC: anterior commissure; AOB: accessory olfactory bulb; cAMY: caudal amygdala; DP: dorsal pallium; DT: dorsal thalamus; H: habenula; LP: lateral pallium; MP: medial pallium; MS: medial septum; NA: nucleus accumbens; POA: preoptic area; rP: rostral pallium; SPTA: striato-pallial transition area; STR: striatum; VCP: ventral cellular prominence; vomAMY: vomeronasal amygdala; VT: ventral thalamus.

mitral cells, have their cell bodies in the rostral or caudal superficial cell layer (n=3), the mitral cell layer (n=9), or the external plexiform layer (n=2). Depth of soma is 184±23.7 μm for the mitral cells not located superficially. Fig. 2 also shows that some mitral cells could be present close to the internal plexiform layer, although none were injected intracellularly. The lightly labeled cell outlines seen in Fig. 2 are assumed to result from poor tracer uptake or transcellular labeling (see Discussion).

The soma of mitral cells is usually piriform or round and ranged in dimension from 11–20 μm (15.8±0.7) for diameter of the major axis and 10–16 μm (12.7±0.5) for diameter of the minor axis. Reconstructions of mitral cells with their soma in the dorsal AOB are shown in Fig. 5, those with their soma in the mid- and ventral AOB in Fig. 6, and those with their soma located in the superficial or caudal AOB in Fig. 7. These figures display the great variability in mitral cell anatomy in each part of the AOB. Most mitral cells have one thick (1–4 μm) primary dendrite giving rise to dendritic branches and axons. Only two mitral cells displayed two primary dendrites originating from the soma. Two types of dendrites were observed: 1) dendrites bearing glomerular arbors in the glomerular layer and 2) lateral (basal) dendrites invading the external plexiform layer (see Figs. 1A–B for examples). Both types have a smooth surface without spines. Dendritic thickenings in mitral cells were only seen in the glomerular arbors. The number of dendrites varies greatly across mitral cells. The number of lateral dendrites ranges from 0–5 (2.4±0.5) with a diameter between 0.5 and 0.8 μm, whereas the number of glomerular arbors ranges from 1–5 (3.2±0.3) displaying a size between 7 and 36 μm (17.5±1.1). Only one superficial mitral cell had a single glomerular arbor (Fig. 7C). Some dendrites bearing glomerular arbors converge onto the same site forming a structure apparently belonging to the same glomerulus (e.g. Fig. 6A). Unfortunately, the material did not permit the delineation of glomeruli. Some of the dendrites ending in the glomerular layer are better described as terminal fields since they cover a large portion of the glomerular layer without forming the dense rounded processes characteristic of mitral cell glomerular arbors (Fig. 5B). Glomerular arbors belonging to the same mitral cell could be found in close vicinity within the glomerular layer or far apart, sometimes dispersed about the whole extent of the AOB.

Each mitral cell has 1 to 3 primary axons originating from the soma (8), primary dendrite (15), dendrite forming a glomerular arbor (2), or lateral dendrite (3). These primary axons sometimes branch profusely shortly after their origin in the AOB. They vary in diameter between approximately 0.1 and 0.5 μm and are easily recognized by the presence of varicosities. The many axonal branches present at the level of the AOB do not appear to terminate locally. They either join the dorsal and ventral portions of the accessory olfactory tract (n=10 cells; see Laberge and Roth (2005) for a description of the accessory olfactory tract in *P. shermani*), the dorsal accessory olfactory tract only (n=3 cells) or they go around the caudal AOB in the ventral direction and curve back rostrally to terminate in the region below the AOB (n=2 cells). This axonal projection to the region below the AOB could be ascertained without doubt in only two mitral cells (Figs. 3 and 5A), but could be more abundant because its demonstration was difficult. Fig. 1C shows axon branches running in the peripheral region of the caudal AOB where they take a ventral course; some of the branches then curve rostrally to end in the region below the AOB, others join the accessory olfactory tract.

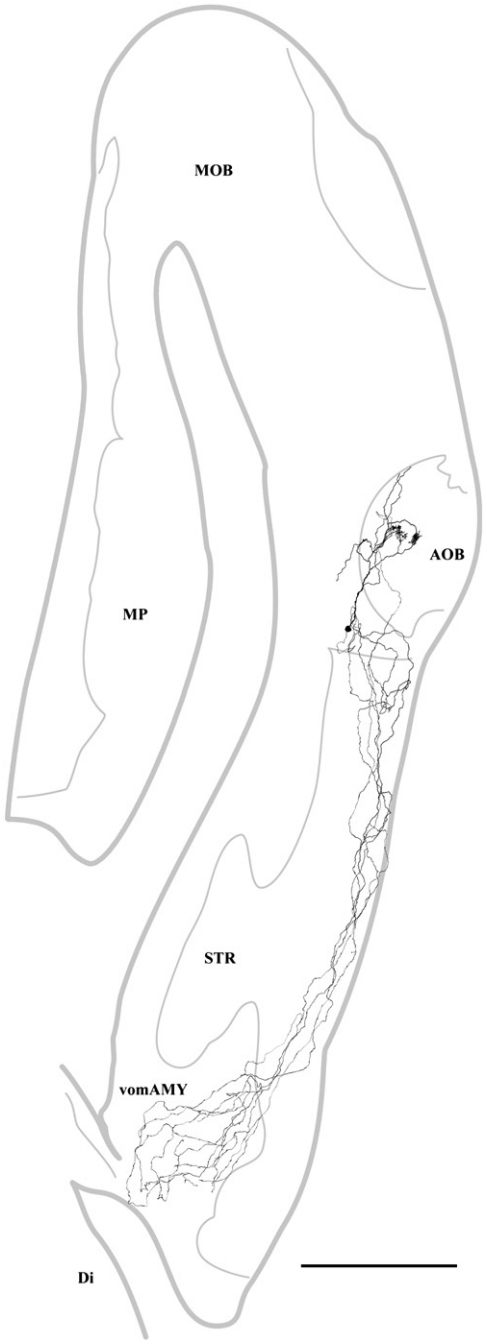

Fig. 4 – Reconstruction of a mitral cell from horizontal brain sections. The soma is located in the caudal part of the mitral cell layer. This plane of section enabled reconstruction of the whole axonal projection to the vomeronasal amygdala. Finer gray lines indicate the border between gray and white matter. Scale bar is 1 mm. Abbreviations: AOB: accessory olfactory bulb; Di: diencephalon; MOB: main olfactory bulb; MP: medial pallium; STR: striatum; vomAMY: vomeronasal amygdala.

Fig. 1D shows a micrograph of terminal axonal meshwork of the accessory olfactory tract in the caudal vomeronasal amygdala belonging to the neuron reconstructed in Fig. 3. Fig. 4 shows the whole dendritic and axonal extent of a mitral cell reconstructed from horizontal brain sections. It is important to note that axonal varicosities were present along the whole course of the axon branches in the AOB, the region caudal to the AOB, the striato-pallial transition area and the vomeronasal amygdala. Axon varicosities appeared more abundant in the latter two regions.

2.3. *Interneurons*

2.3.1. *Periglomerular cells*

Six single neurons and two clusters of two neurons were reconstructed (Fig. 8). The soma of periglomerular cells was located in the superficial cell layer, with the exception of two neurons situated in the shallow part of the glomerular layer. Unlike mitral cells, the soma of periglomerular cells could be found throughout the whole extent of the superficial cell layer.

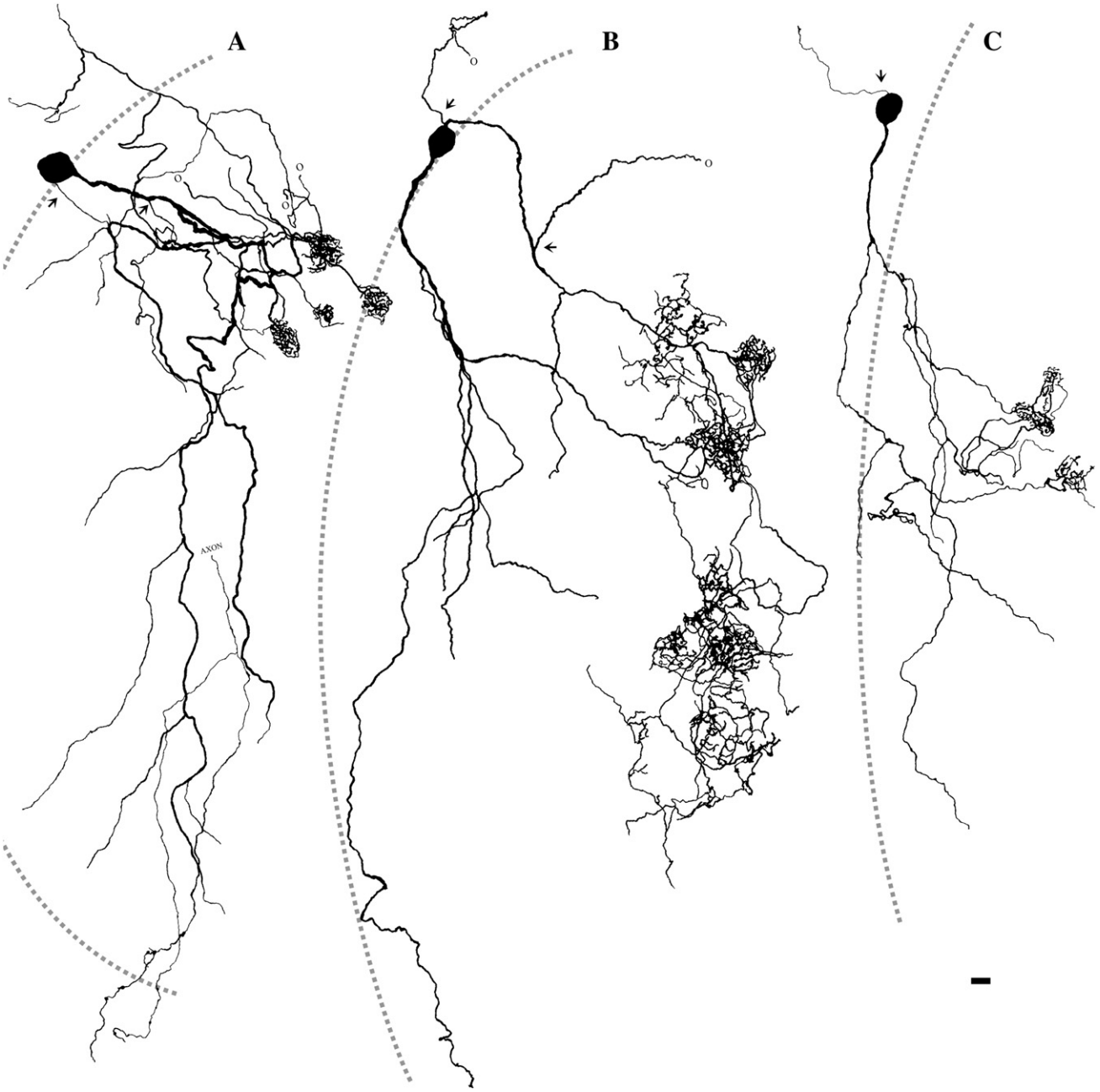

Fig. 5 – Reconstruction of mitral cells situated in the dorsal accessory olfactory bulb. The brains were sectioned in the coronal plane and only cell processes found within the accessory olfactory bulb are included. The hatched gray lines represent the limit between the mitral and external plexiform layers at the level of the soma. Arrows indicate the sites of axon origin. Circles indicate interrupted axon branches, which demonstrably innervated the vomeronasal amygdala. Scale bar is 10 μm.

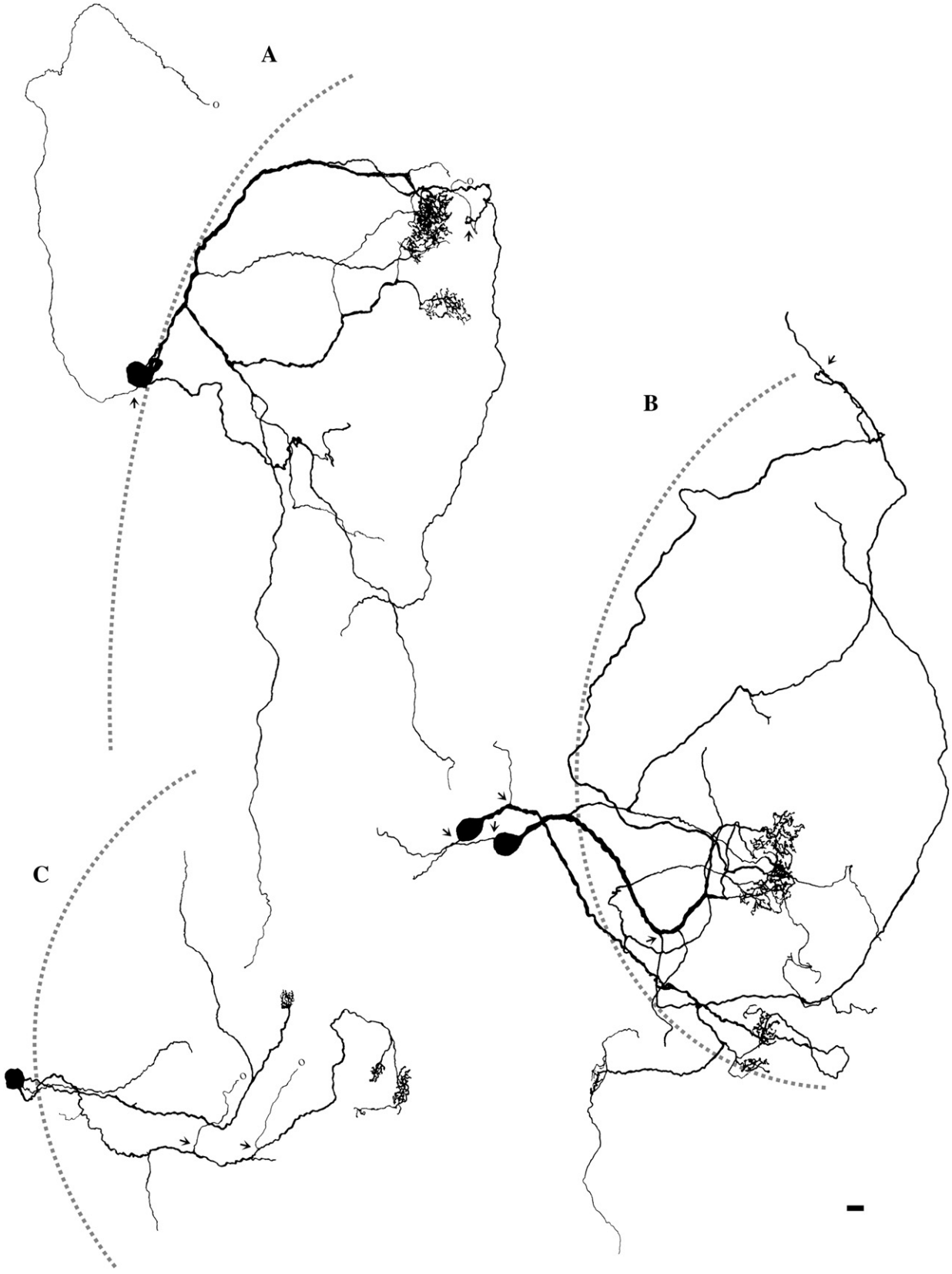

Fig. 6 – Reconstruction of mitral cells situated in the mid- and ventral accessory olfactory bulb. Panel B shows a cluster of two mitral cells simultaneously labeled by one intracellular injection of biocytin. The brains were sectioned in the coronal plane and only cell processes found within the accessory olfactory bulb are included. The hatched gray lines represent the limit between the mitral and external plexiform layers at the level of the soma. Arrows indicate the sites of axon origin. Circles indicate interrupted axon branches, which demonstrably innervated the vomeronasal amygdala. Scale bar is 10 μm.

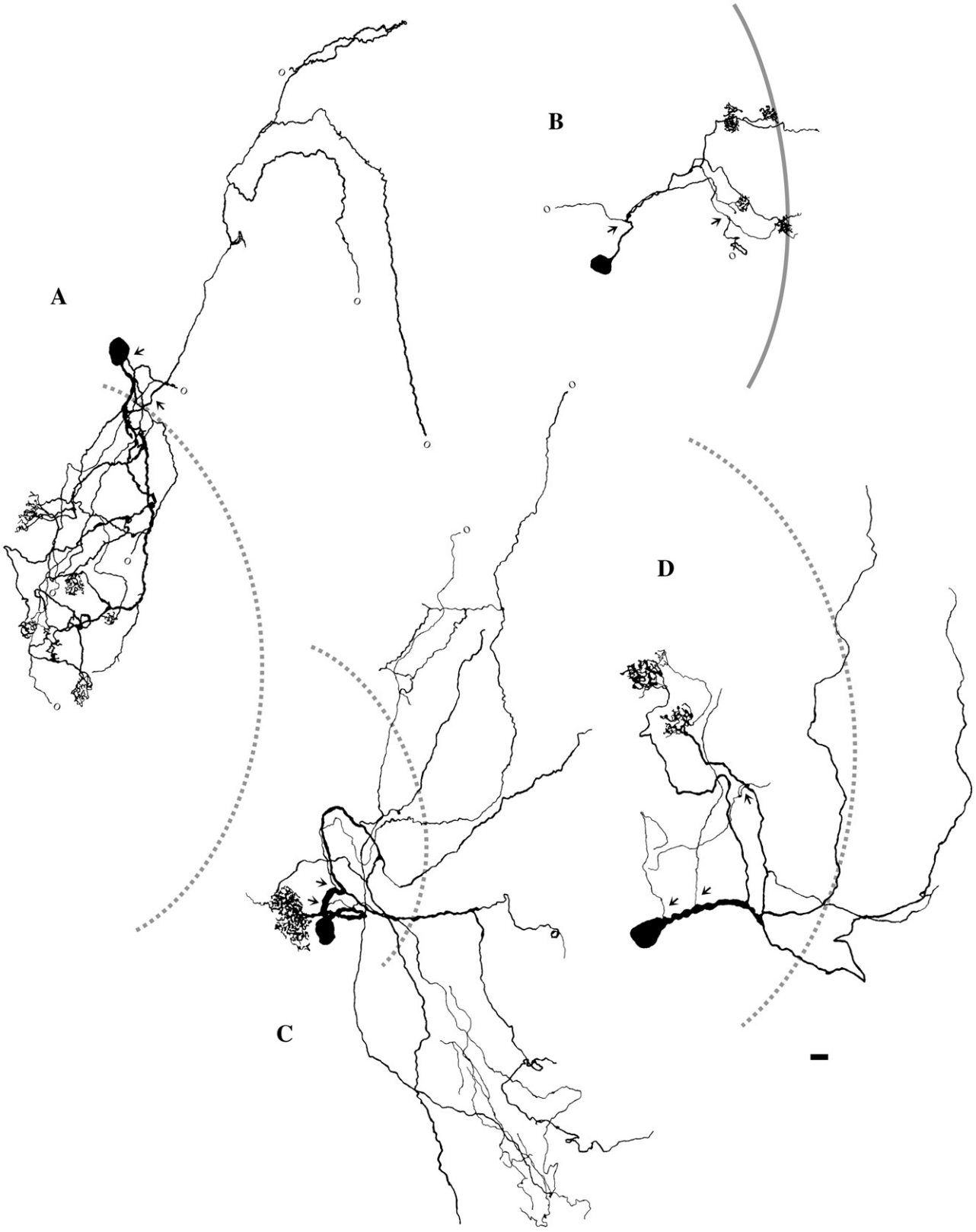

Fig. 7 – Reconstruction of mitral cells situated in the superficial or very caudal accessory olfactory bulb. A: superficial rostral dorsal; B: very caudal; C–D: superficial caudal. The brains were sectioned in the coronal plane and only cell processes found within the accessory olfactory bulb are included. In panels A, C and D, the hatched gray lines represent the limit between the mitral and external plexiform layers at the level of the soma, whereas the gray line in B represents the outside surface of the brain because the mitral and plexiform layers are absent at that caudal level. Arrows indicate the sites of axon origin. Circles indicate interrupted axon branches, which demonstrably innervated the vomeronasal amygdala. Scale bar is 10 μm.

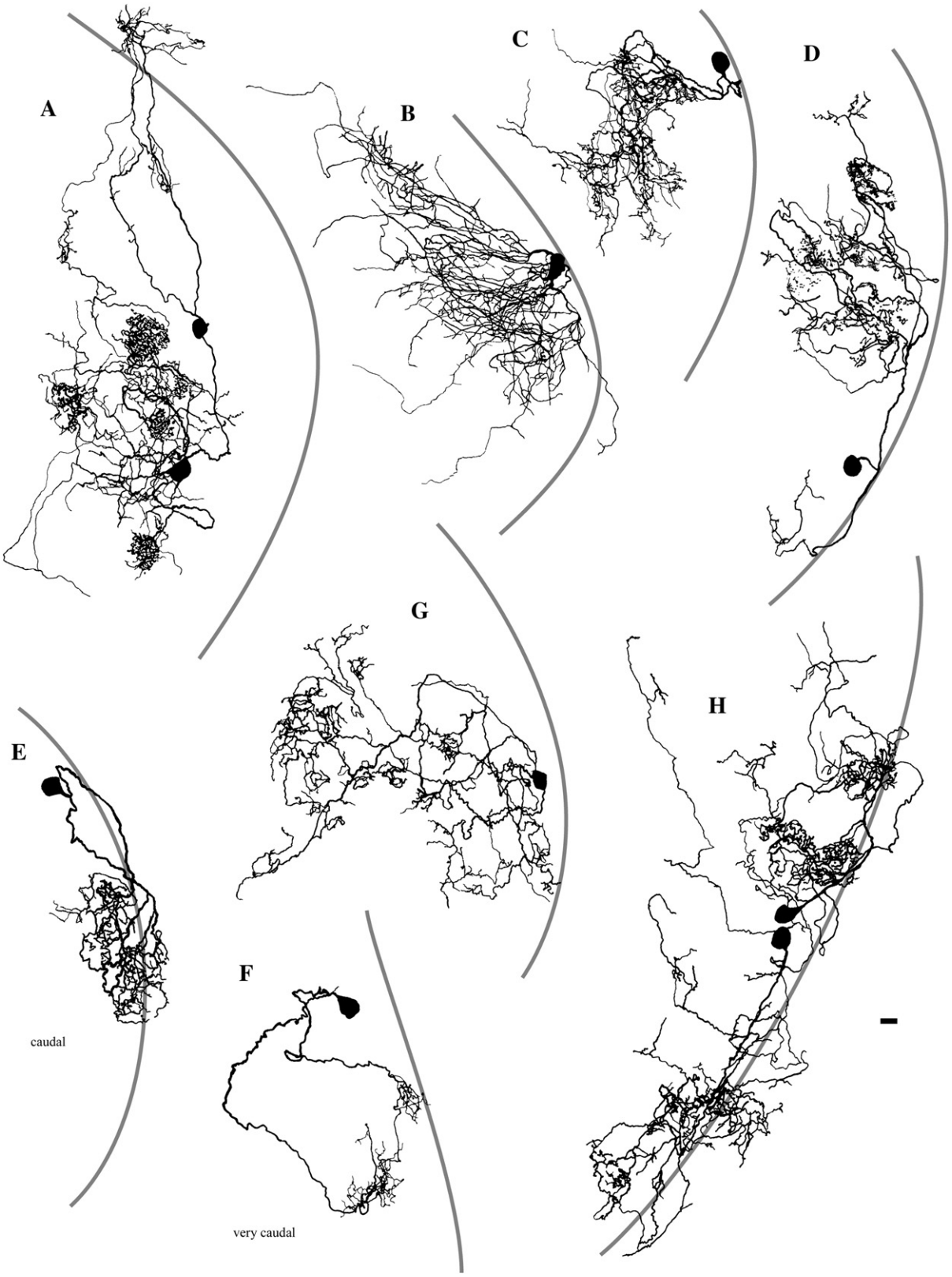

Fig. 8 – Reconstruction of periglomerular cells of the accessory olfactory bulb. A: periglomerular cells situated in the glomerular layer. B–H: periglomerular cells situated in the superficial cell layer. Panels A and H show clusters of two periglomerular cells simultaneously labeled by one intracellular injection of biocytin. The soma of neurons shown in panels E and F are situated in the caudal part of the accessory olfactory bulb and send processes rostrally. For the sake of clarity, the neurons found in the left half of the accessory olfactory bulb were flipped horizontally. The gray lines represent the outside surface of the brain at the level of the soma. Sections were cut in the coronal plane. Scale bar is 10 μm.

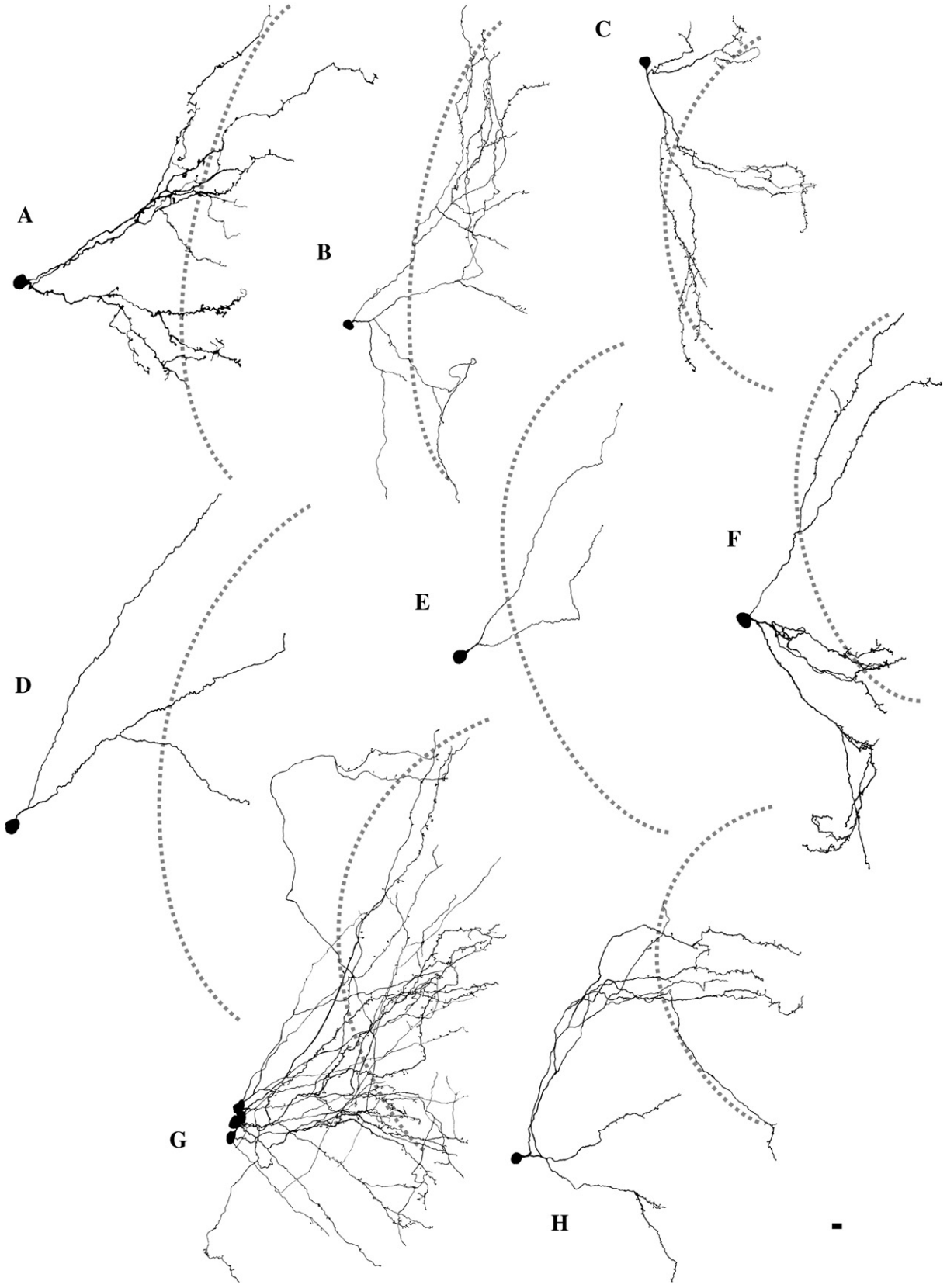

Fig. 9 – Reconstruction of granule cells. A–F: cells situated in the granule cell layer of the accessory olfactory bulb. G–H: cells situated in the region below the accessory olfactory bulb. Panel G is a cluster of four neurons simultaneously labeled by one intracellular injection of biocytin. For the sake of clarity, the neurons found in the left half of the accessory olfactory bulb were flipped horizontally. The hatched gray lines represent the full extent of the limit between the mitral and external plexiform layers at the level of the soma. Sections were cut in the coronal plane. Scale bar is 10 μm.

Cell bodies were mostly round or piriform with a major diameter ranging between 11.5 and 17 μm (13.5±0.6) and a minor diameter of 8–14 μm (10.7±0.5). Most periglomerular cells extend two smooth main processes into the glomerular layer where they branch profusely, sometimes forming arborizations reminiscent of the glomerular arbors of mitral cells, but generally more loosely organized. These arborizations number 1–8 (3.3±1) per single cell and are larger than the glomerular arbors of mitral cells (range between 23 and 63 μm, mean 39.8±6.8 for the arborizations that could be easily measured). Periglomerular cells also display processes that do not form glomerular-like structures. One periglomerular cell illustrated in Fig. 8B possesses atypical fine processes lacking thickenings. Periglomerular cells do not appear to possess axons.

2.3.2. *Granule cells*

Six single neurons with their cell body located in the granule cell layer of the AOB were reconstructed (Figs. 9A–F) along with a single neuron and a cluster of 4 neurons located below the AOB in the compact cell mass that is continuous with the granule cell layer of the AOB (Figs. 9G–H).

Their soma was found between 179 and 346 (263±20.1) μm deep from the surface, of a mostly piriform or round shape with a major diameter ranging between 11.5 and 14 μm (13.1±0.3) and a minor diameter of 9–11 μm (10.6±0.3). Granule cells have dendrites almost exclusively oriented toward the brain surface reaching up to the external plexiform layer and, occasionally, the glomerular layer. Some neurons have dendrites displaying a spiny surface (Figs. 9A–C, F–H) particularly on their distal parts, while others have smooth dendrites (Figs. 9D–E). Each smooth dendrite branch displays a slight thickening at its extremity resembling a terminal bouton. Granule cells have no axon.

Interestingly, the neuron shown in Fig. 9F belongs to the AOB, but it sends dendrite branches in both the AOB and the region found below it. Similarly, the neurons shown in Fig. 9G–H are located ventrally and send dendrite branches both laterally in the region below the AOB and dorsolaterally in the AOB proper. Only one neuron was labeled in the peripheral part of the region below the AOB (not shown). Its dendrites covered the white matter of the whole region below the AOB, while its axon branches distributed widely, ascending to all parts of the rostral pallium and descending all the way to the caudal pole of the telencephalon, with many branches apparently targeting the dorsolateral telencephalon in addition. It did not contact the AOB itself.

3. Discussion

Just as in mammals, the urodele AOB can be well developed, rudimentary or absent depending on the species (Herrick, 1921, 1924, 1931; Meisami and Bhatnagar, 1998; Eisthen, 2000). In *P. shermani*, the large AOB displays an atypical layer; the superficial cell layer comprising mixed cellular types, which include mitral cells in its rostral and caudal parts and periglomerular cells in its entire extent. Each mitral cell typically sends multiple axon branches to the vomeronasal amygdala. It could also be demonstrated that some of the axon branches of mitral cells terminate in the region below the AOB. Periglomerular and granule cells are the two main interneuron types in the salamander AOB. They are distinguished by the location of their somata and the territories covered by their dendrites. Dendrites of periglomerular cells distribute in the glomerular layer, whereas dendrites of granule cells distribute in the granule, mitral, external plexiform and glomerular layers. It could also be observed that interneurons in the region below the AOB send dendrites into the AOB proper, possibly to participate in the modulation of vomeronasal information.

3.1. *Methodological considerations*

Single intracellular injections of biocytin sometimes produced multiple labeling of neurons. This phenomenon might be due to leakage of biocytin at the injection site, transcellular biocytin transport via gap junctions present in the olfactory bulb (Christie et al., 2005) or via chemical synapses (Luo and Dessem, 1996). Interestingly, a much smaller proportion of neuron clusters was obtained in the AOB in comparison to biocytin injections made in the ventral telencephalon in the same salamander species using the same method (Laberge and Roth, 2005). This suggests that the potential for coupling between neurons varies across brain regions and is lower in the AOB. Considering the above, it appears that some of the lightly labeled cells shown in Fig. 2 could result from transcellular transport of biocytin, and thus would not represent projection neurons. The amount of such transcellular labeling, or its presence, is unfortunately undetermined and the alternative explanation of a lower uptake of the tracer by projection neurons cannot be rejected.

In the present study, measurements were made indirectly on the cellular reconstructions. This method is obviously not ideal, especially when dealing with fine cellular processes (e.g. axons of mitral cells), but should prove precise enough for comparisons between studies of the olfactory bulb.

3.2. *Comparisons with other vertebrates*

3.2.1. *Mitral cells*

No clear axonal or dendritic characteristic of mitral cells could be associated with the layer position of the cell body; thus, the present study offers no evidence for the presence of tufted cells in the salamander AOB. This is in agreement with the observation of Nieuwenhuys (1967) that tufted cells have not been described in olfactory bulbs of non-mammalian forms. Mitral cells projecting to the region below the AOB could possibly represent a distinct type, but the difficulty in ascertaining these projections make a classification attempt unreliable when based on the present data.

In the AOB of vertebrates each mitral cell typically supplies several main dendrites to more than one glomerulus. This pattern is also present in the MOB of teleosts, amphibians, reptiles and birds, but a mitral cell usually contacts only one glomerulus in mammals, agnathans and some cartilaginous fishes (Allison, 1953; Nieuwenhuys, 1967; Kosaka and Hama, 1982; Oka, 1983; Fujita et al., 1988; Dryer and Graziadei, 1994). However, some variability was observed in the frog MOB and the zebrafish olfactory bulb, where both uniglomerular and multiglomerular mitral cells exist (Jiang and Holley, 1992;

Fuller et al., 2006). Such variability, although to a lesser extent, was also highlighted here in the *P. shermani* AOB where the pattern of multiple glomerular arbors did not apply to all mitral cells. The basket-like glomerular arbors and lateral dendrites of AOB mitral cells are similar in salamander, lizard and mammals, but less so when compared to the mitral cells in the snake AOB, which possess more tuft-like glomerular arbors (Mori, 1987; Iwahori et al., 1989a; Llahi and Garcia-Verdugo, 1989; Takami and Graziadei, 1991; Wagner et al., 2006; Yonekura and Yokoi, 2008). Tuft-like arbors characterize MOB mitral cells in reptiles and mammals, whereas mitral cell arbors in the amphibian MOB are more similar to AOB arbors (Iwahori et al., 1989b; Scalia et al., 1991; Jiang and Holley, 1992; Dryer and Graziadei, 1994).

3.2.2. *Periglomerular cells*

Different types of periglomerular cells could be identified showing qualitatively different cellular processes or a distinct cell body location. The most common type found in the superficial cell layer showed processes resembling the dendrites of mammalian periglomerular cells, but in contrast to the latter, had no obvious axon (Pinching and Powell, 1971; Schneider and Macrides, 1978; López-Mascaraque et al., 1990). The periglomerular cell with atypical processes illustrated in Fig. 8B is similar to the periglomerular cells described in the MOB of *Xenopus* tadpoles (see Fig. 2B in Nezlin et al. (2003)), which also lack axons. Amphibian periglomerular cells could thus lack axons. However, Herrick (1924) described periglomerular cells with axons in the MOB of *Ambystoma*. In the mammalian AOB, many periglomerular cells are interneurons; they are unipolar, bipolar or multipolar (Takami et al., 1992). Some of the salamander periglomerular cells (e.g. Fig. 8H) also appear unipolar. In reptiles, descriptions of periglomerular cells are also consistent with the present description in salamander, except that they possess axons (Iwahori et al., 1989a; Llahi and Garcia-Verdugo, 1989). Excepting the axon, the cellular processes of periglomerular cells appear conserved throughout vertebrate phylogeny.

3.2.3. *Granule cells*

Two distinct types in the granule cell layer could also be recognized: neurons with either spiny or smooth processes. Further, some neurons had dendrites that covered both the AOB and the region below it. The cells with spiny processes are typical granule cells. As described by Herrick (1924) in *Ambystoma*, granule cells in *P. shermani* lack basal dendrites and this is also the case in reptilian olfactory bulbs (Iwahori et al., 1989a,b; deep granule cells in Llahi and García-Verdugo, (1989)). This stands in contrast to the presence of basal dendrites in granule cells of the frog and mammalian MOB (Price and Powell, 1970b; López-Mascaraque et al., 1986; Scalia et al., 1991). The cells with smooth dendrites display one terminal structure per dendrite branch and are clearly distinct from the spiny granule cells. Further, they show no clear similarities with Golgi or Cajal cells (Price and Powell, 1970a). They possibly represent a distinct type of granule cells with smooth dendrites in the salamander AOB. Interestingly, tyrosine hydroxylase-positive cells in the granule cell layer of the frog MOB and AOB share similarities with the smooth dendrite cells described here (Boyd and Delaney, 2002). Thus, these cells possibly represent a distinct type of interneuron in the granule cell layer expressing catecholamines. The present study provides no evidence of Herrick's transitional cells possessing axons in the granule cell layer (Herrick, 1924). In amphibians, such transitional cells are possibly found only in the MOB. One last remark concerns the internal plexiform layer of the AOB. Nissl staining revealed that this layer is present in *P. shermani* contrary to the claim of Allison (1953) that it is lacking in the amphibian AOB. However, its fiber content could not be revealed in the present study.

3.3. *Functional considerations*

The present study showed that some neurons in the AOB and the region below the AOB have dendrites that cover both regions. Thus, the region below the AOB is in a position to modulate the salamander VNS. It could belong to the AOB itself, but a migrated neuron found below the AOB displayed connections distinct from any AOB neuron type suggesting related but distinct functions for the two brain regions. Further investigation is needed in order to characterize precisely the region below the AOB in salamander.

A previous work in *P. shermani* suggested the existence of an extended vomeronasal amygdala in the dorsolateral telencephalon between the lateral pallium and the striatum (Laberge and Roth, 2005; Laberge et al., 2006), which further sends strong axonal projections to the region just caudal to the AOB suggesting that the latter could also be part of the salamander VNS. These findings highlight the fact that the VNS, at least in the salamander, appears more elaborate than previously thought. In support of the latter, recent experiments in the same salamander species showed that stimulation of the vomeronasal nerve induces intracellular responses in most of the ipsilateral telencephalon supporting widespread convergence between the main and vomeronasal olfactory systems (F.C. Roth and F. Laberge, unpublished observations). The above and the recent observations of anatomical convergence between the two olfactory systems in the rat telencephalon by Pro-Systiaga et al. (2007) suggest that the importance of VNS influence in the vertebrate telencephalon might have been greatly underestimated.

4. Experimental procedures

4.1. *Animals*

The experiments were carried out in 35 adult specimens of the salamander *P. shermani*, previously known as *P. jordani* (Highton and Peabody, 2000). Twenty-eight salamanders (23 females, 5 males) were used for intracellular labeling, whereas 7 salamanders (4 females, 3 males) were used for tract tracing. The animals were wild-caught from a single locality in Macon Co., North Carolina, U.S.A. (35°10′48″N, 83°33′38″W; collecting permit Dr. Lynne Houck, Oregon State University). All experiments were approved by the veterinary office of the Ministry of Health of the state of Bremen, Germany. The animals were held by groups of 10 in 80 liter terraria equipped with soil bedding, several hiding covers and water. They were fed once a week with crickets.

4.2. *Biocytin labeling*

All experiments were carried out *in vitro* in isolated brain preparations. Animals were anesthetized by exposure to carbon dioxide gas for 10 min in a closed plastic box, put in a Petri dish and perfused transcardially with 20 ml of cold oxygenated Ringer's solution (Na^+ 129 mM, K^+ 4 mM, Ca^{2+} 2.4 mM, Mg^{2+} 1.4 mM, Cl^- 115 mM, HCO_3^- 25 mM, glucose 10 mM, pH 7.4). A stream of carbon dioxide was maintained over the animals' skin throughout that procedure. The animals were quickly decapitated, the lower jaw was removed and the skull was opened from the roof of the mouth to enable brain dissection. Intracellular biocytin labeling and tract tracing were performed as described by Laberge and Roth (2005), except that the brain was approached from the lateral surface for intracellular AOB injections. One or two injections were made in each half of the brain. In case two injections were made, they were at different rostrocaudal levels. For tract tracing, applications of crystalline biocytin were made in the early portion of the accessory olfactory tract in order to backfill as many projection neurons as possible. Brains were sectioned in the coronal or, in a few cases, horizontal plane. All brain sections were counterstained with 0.1% cresyl violet. Labeled neurons were reconstructed by hand with the help of a camera lucida (Carl Zeiss Inc., Jena, Germany), scanned and graphically processed in Photoshop 6.0 (Adobe Systems Inc., San Jose, CA). Each neuron reconstruction comprised many or all brain sections displaying part of the accessory olfactory bulb. A microscope slide with reticle graduated at 10 mm intervals (Carl Zeiss Inc.) was used to establish a scale bar on the neuron reconstructions, which yielded the equivalence factor: 1 mm on the reconstruction equals 1.923 μm on the microscopic preparation (using objective 40×). Measurements were then made directly on the reconstructions applying the equivalence factor. The micrographs were scanned with a digital camera (AxioCam HR, Carl Zeiss Inc.).

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