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Electrophysiological and Morphological Identification of Neurons of the Inferior Colliculus

Master Thesis

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CONTENTS

Electrophysiological and Morphological Identification of Neurons of the Inferior Colliculus

1 Introduction

1.1 The auditory system

The auditory system is a network that includes both sensory organs and neurons. It extends from the external ear to the cerebral cortex and has a hierarchical organization. The peripheral auditory system includes the outer, middle and inner ears. Sounds travel through the outer and middle ear to the cochlea, where they are transduced into electrical signals. The auditory nerve (8th cranial nerve) transmits the action potentials through the ascending pathway to several structures in the central auditory system (Jeffery A. Winer and Schreiner 2005).

The first processing center of the ascending auditory pathway is the *Cochlear Nucleus* (CN). They are also processed in the *Superior Olivary Complex* (SOC), the *Nucleus of Lateral Lemniscus* (NLL), the *Inferior Colliculus* (IC), the *Medial Geniculate Body* (MGB), and, finally, the *Auditory Cortex* (AC). There are several principles of connectivity in the ascending auditory pathway (Figure 1). There is a divergence, i.e., the output of nearly every nucleus targets many centers, as shown in rats (Friauf and Ostwald 1988) and cats (Cant 1982; Thompson 1998). Furthermore, a convergence, the input to most nuclei derives from multiple sources, has also been shown in cats (Roth et al. 1978). There are commissural interconnections in most of the structures of the auditory pathway of the cat (L. M. Aitkin and Phillips 1984), except of the medial geniculate body, and there is an internal circuitry in every nucleus. Finally, many connections between different nuclei of the cat are reciprocal (Colwell 1975; Conlee and Kane 1982).

In addition to the ascending pathway, corticofugal descending projections from the AC control the neural processing in subcortical structures (Jeffery A. Winer and Schreiner 2005).

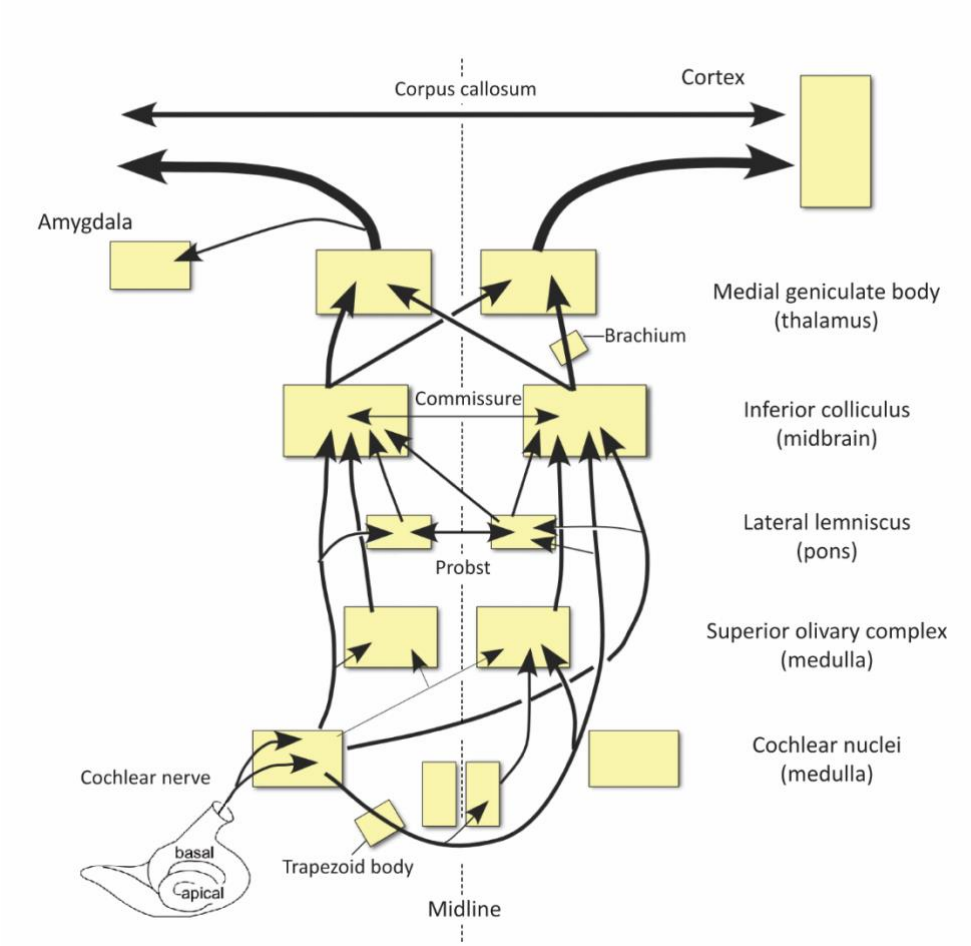

Figure 1. The central auditory system (adapted from (Jeffery A. Winer and Schreiner 2005)).

1.2 The Cochlear Nuclei

The *Cochlear Nucleus* (CN) is a structure on the lateral edge of medulla and the first nucleus of the central auditory system. It receives input from the auditory nerve (8th cranial nerve). It is subdivided into the anterior ventral division, the posterior ventral division and the dorsal division. All three subdivisions present tonotopy, preserving the topography of frequencies established in the cochlea, via the input of the auditory nerve (Jeffery A. Winer and Schreiner 2005). The CN neurons project ipsi- and contralateral to the superior olivary complex, lateral lemniscus nuclei and inferior colliculus (Cant 1982).

1.3 The Olivary Complex

The *Superior Olivary Complex* (SOC) is found ventral and medial to the CN, in the caudal portion of the pons and it consists of several nuclei. Among them, there are the lateral superior olivary nucleus (LSO), the medial superior olivary nucleus (MSO), the medial nucleus of the trapezoid body (MNTB) and the medial periolivary nuclei. All subdivisions of the SOC exhibit tonotopic organization. The monaural input of the CN is integrated in the LSO. Neurons in the MSO accomplish the phase and delay encoding from the two ears, providing the accurate spatial localization of sounds (Yin and Chan 1990). The neurons in MNTB send inhibitory projections to LSO and contribute to the creation of binaural signals (Guinan and Li 1990). The intensity-difference sensitive binaural signals target the lateral lemniscal nuclei (LLN) and the inferior colliculus (IC). Specifically, the LSO sends axons bilaterally to the IC (Tsuchitani 1977). The most lateral projections are low-frequency selective and terminate ipsilaterally, whereas the more medial projections are high-frequency selective, ending contralaterally (Elverland 1978).

1.4 The Lateral Lemniscal Nuclei

The *Lateral Lemniscus* (LL) consists of three subnuclei (dorsal, intermediate, and ventral) and is found in the lateral surface of the brain stem, near the transition from the pons to the midbrain. The LLN share projections from the CN and the SOC with the IC (Oliver et al. 1997). The dorsal nuclei of the lateral lemniscus from either side of the brain stem are interconnected by a fiber track called the commissure of Probst (Kudo 1981). The posteroventral cochlear nuclei project to the ventral nucleus of the lateral lemniscus and subsequently the VNLL sends monaural projections to the central nucleus of the inferior colliculus, whereas the DNLL sends binaural signals to the central nucleus of both ICs (Markovitz and Pollak 1994).

1.5 The Inferior Colliculus

The *Inferior Colliculus* (IC) is a midbrain structure. Its role is critical in the central auditory system in relaying and processing acoustic information. It is the main hub of ascending auditory inputs from the brainstem. The IC receives inputs from almost all parts of the CN (Oliver 1984), from a large portion of the SOC (Glendenning et al. 1992), and from both sides of LLN (Saint Marie et al. 1997). The inferior colliculus is divided into three main subdivisions: The Central Nucleus (CNIC), the External Cortex (ECIC; in older papers known as lateral nucleus), and the Dorsal Cortex (DCIC; Figure 2). Neurons of each subdivision are defined as tectal or tegmental, depending on the pattern of dendritic branching. Tectal neurons are mostly multipolar cells, with four or more primary dendrites and several higher-order arborizations, with prominent dendritic appendages (Morest and Oliver 1984). Tegmental neurons usually possess two or three primary tapering dendrites that follow straight trajectories and have few secondary branches with sparse appendages (Morest and Oliver 1984).

There is evidence that all fields of the AC send fibers to the IC, mainly bilaterally targeting all the three IC subdivisions (Bajo and King 2012). The IC is also considered as a prominent source of GABAergic projections to thalamocortical neurons of the MGB, evoking short-latency monosynaptic IPSPs in the thalamus (Peruzzi et al. 1997). Beside the extrinsic projections to the IC, many intrinsic and commissural projections have been found (Adams 1980; L. M. Aitkin and Phillips 1984). Furthermore, the IC emerges to be an important source of auditory information to the *Superior Colliculus* (SC) (Garcia Del Cano et al. 2006).

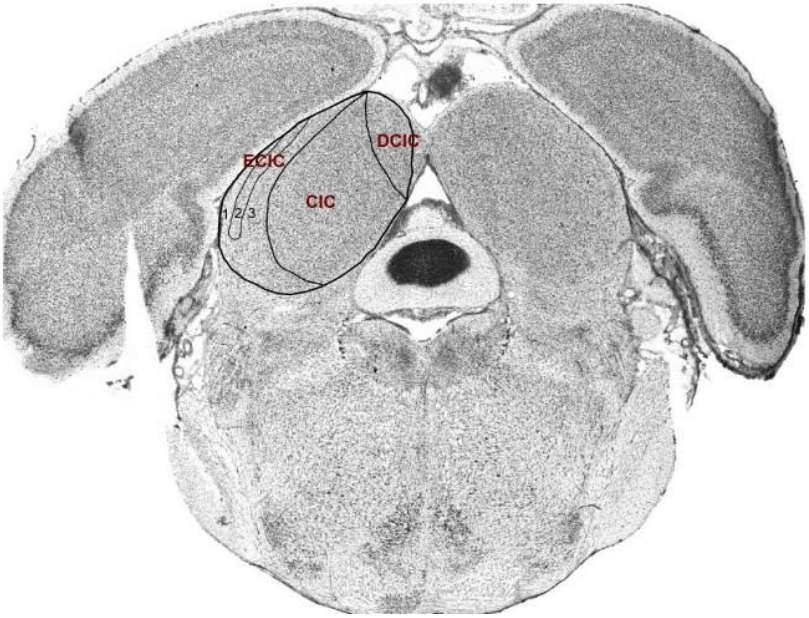

Figure 2. The subdivisions of the IC. ECIC: External Cortex, CIC: Central Nucleus, DCIC: Dorsal Cortex Adapted from (Paxinos and Watson 2009).

1.5.1 The Central Nucleus of Inferior Colliculus (CIC)

The central nucleus of the Inferior Colliculus is exclusively auditory (L. Aitkin et al. 1994), and shows a tonotopic organization (Malmierca et al. 2008). In addition, it is distinguished by its laminated neuropil. Neurons in a single lamina are sensitive to similar tonal frequencies, as this subdivision of the IC is a part of the tonotopically organized ascending auditory pathway to the thalamus, and further to the AC (Jeffery A. Winer and Schreiner 2005).

Synaptic inputs to the CIC originate from neurons in the lower auditory brain stem, including mainly the cochlear nuclear complex and the superior olivary complex; fewer inputs ascend from neurons in the lateral lemniscus nuclei (Brunso-Bechtold et al. 1981). According to electron microscopy autoradiographic studies, the excitatory inputs to the CIC dominate with respect to the inhibitory ones, while it is not clear so far whether different cell types receive the same proportions of excitatory and inhibitory input (Jeffery A. Winer and Schreiner 2005).

According to studies in the cat, the dendritic fields of the principal cells are disc-shaped, actually elongated, 200 to 800 µm long and 50 to 70 µm wide, oriented in parallel arrays with the afferent axonal plexus that emerge from the lateral lemniscus (Morest and Oliver 1984).

In the rat, disc-shaped neurons are subdivided in two distinct types of neuron, the flat (F) and less flat (LF) (stellate) cells (Malmierca et al. 1993). The F cells have medium-sized, spheroidal somata (diameter 10-15μm). Their dendritic arbors show a small absolute thickness (mean about 50μm) and are denser, while the stellate (LF) cells somata are ovoid and polygonal, and vary in size. LF cells have small to medium size somata (long diameter 7-18μm) or large somata (up to 25μm), with thicker and less dense dendritic arbors (extending for about 100μm). The laminae formed by the F cells appeared to be separated by interlaminar compartments populated by the LF neurons. Also, other cell types show ovoid dendritic fields parallel to the LF cells and perpendicular to the long axis of the flat cells (Malmierca et al. 1993).

1.5.2 The Dorsal Cortex of Inferior Colliculus (DCIC)

The Dorsal Cortex of Inferior Colliculus (DCIC) occupies the dorsomedial and caudal regions of the IC. The major inputs to the dorsal cortex derive from the auditory cortex, while it receives inputs from other structures, as the MGB, the sagulum, the ipsilateral CIC and ECIC, the contralateral IC, and lower auditory brainstem structures (J. A. Winer et al. 2002).

In contrast with the CIC axonal patterns, anatomical studies showed that the cells of DCIC tend to have non-oriented axons (Oliver et al. 1991), forming layers that seem to be related to corticocollicular, commissural, and intrinsic projections rather than to specific maps (J. A. Winer et al. 1998). In the Golgi-stained mouse brain, the DCIC seems to be consisted of four layers, populated by stellate and pyramidal neurons (Barnstedt et al. 2015).

The dorsal cortex does not show tonotopic organization. Damage to the dorsal cortex in cats affects mainly attention and alertness than auditory discrimination behavior (Jane et al. 1965). Recordings of the responses of neurons in the dorsal cortex to various auditory stimuli revealed that they display stimulus-specific adaptation, as they generate strong firing in response to initial sweeps or repetitive stimulation but minimal or erratic firing thereafter. In

addition, the neurons show a strong preference to vocal stimuli than to noise (L. Aitkin et al. 1994; Syka et al. 2000). The function of DCIC in the auditory processing is ambiguous.

1.5.3 The External Cortex of Inferior Colliculus (ECIC)

The external cortex of inferior colliculus accomplishes a multisensory integration in acoustic-motor behavior (L. M. Aitkin et al. 1978). It can be divided in three layers: layer I, layer II and layer III (Figure 2). The role of the external cortex in inferior collicular processing is not well understood.

The external cortex of inferior colliculus is the target of considerable non-auditory inputs, primarily from the medullary dorsal column nuclei and the trigeminal system in the pons, which represent the somatic sensory system for the body and head, respectively (L. M. Aitkin et al. 1978; L. M. Aitkin et al. 1981; Morest and Oliver 1984). It receives also input by descending projections, mainly from layer V of the auditory and from non-auditory cortical fields (L. M. Aitkin et al. 1981; Morest and Oliver 1984; J. A. Winer et al. 1998), as well as from the contralateral IC (L. M. Aitkin and Phillips 1984). In general, it establishes strong connections with non-auditory structures, including SC, substantia nigra, periaquaductal gray, and somatosensory cortex (Druga and Syka 1984; Glendenning et al. 1992; Zhou and Shore 2006).

The ECIC has no tonotopic organization. There is a rough representation of the body surface, overlapping with the auditory representation (L. M. Aitkin et al. 1978; Zhou and Shore 2006). In addition, it is shown that there is a topographical representation of the contralateral auditory azimuth in the ECIC of the guinea pig (Binns et al. 1992). A possible role of the external cortex is the representation of the body with respect to sound stimuli.

The neurons of the ECIC have a smaller average size compared with those of the CIC, and their packing density is decreased. They are tegmental as well as tectal. The tectal cell types consist of medium-size stellate and elongate neurons, often pointing laterally toward the branchium

or ventrolaterally toward the tegmentum, and small-size stellate neurons, with variable orientation. The tegmental cell types mainly occupy the ventral and rostral regions of the ECIC. (Morest and Oliver 1984).

The neurons of the ECIC generate higher firing rates in response to broad band stimuli than pure tones, with a frequency near 10kHz in cat (L. Aitkin et al. 1994) and guinea pig (Syka et al. 2000). Furthermore, the ECIC neurons exhibit a higher average threshold and a shorter average first-spike latency than the DCIC neurons, and their maximal firing rate is lower compared to that of CIC neurons (Syka et al. 2000).

The ECIC constitutes the main source of auditory information to SC (Druga and Syka 1984; Edwards et al. 1979), a laminated structure that organizes gaze movements. The SC contains maps of visual space in the upper layers, as well as auditory and somatosensory maps in the deeper layers, so that an integrated multisensory map can localize the stimuli, and process approach and avoidance motor commands (Dean et al. 1989). Findings from anterograde and retrograde tracing analysis indicate the layer I of the ECIC as the major auditory input to the SC (Garcia Del Cano et al. 2006).

1.5.4 Immunocytochemical profiles and morphology of Inferior Collicular neurons

The inferior colliculus, as mentioned before, has a critical role in the central auditory system. The number of neurons in the rat IC is notably high, comparing with other central auditory structures; CIC contains more than 200,000 neurons, and ECIC and DCIC contain approximately 80,000 and 45,000 neurons respectively, whereas all lower structures contain 15,000-30,000 neurons (Ouda and Syka 2012).

Studies on the immunocytochemical profiles of rat IC neurons reveal that, in CIC, the less-flat neurons with polygonal or oval shapes (stellate neurons in the cat) are in majority GABAergic. Respectively, flat neurons in the rat CIC are mainly excitatory (disc-shaped cells in the cat). On

the other hand, in ECIC, GABAergic neurons are large fusiform cells (bitufted) or smaller oval neurons (observed mainly in the second layer). In DCIC, there is an ambiguous demarcation of GABAergic neurons (Ouda and Syka 2012).

Additionally, immunocytochemical studies in rats showed an organizational feature (distinct neuronal polulations - modules) in the external cortex and dorsal cortex of the inferior colliculus. These modules contain a population of GAD-positive somata and a higher local concentration of puncta. The somata in the modules are significantly smaller compared to those outside of the modules. The GAD-positive modules are absent in mice, bats, gray squirrel, cat, macaque monkey and barn owl (Chernock et al. 2004).

As mentioned before, in the central nucleus of the inferior colliculus of the cat, both disc-shaped and stellate cells are labeled with GABA antibodies. Since the same axon of these cells can project to the medium geniculate body (Peruzzi et al. 1997) and gives local collaterals, they can mediate inhibition both locally and in the MGB. The CIC is a laminated structure, so if the axon collaterals of GABAergic cells form inhibitory synapses on inhibitory cells in the same lamina, then the activity of their target neurons is enhanced in comparison with the activity of their neighbors, and in that way, they may establish a mechanism to code “place” (Oliver et al. 1991). On the other hand, if the axons make excitatory synapses, the frequency tuning may be broadened and these neurons may be sensitive to interaural time differences (Oliver et al. 1991).

Immunocytochemical studies in rats, describing the distribution of the calcium binding proteins (CBPs) Parvalbumin (PV), Calbindin (CB) and Calretinin (CR) in the IC, indicate that PV immunoreactive (PV-ir) neurons are present in all three subdivisions of the IC and their morphology resembles the less-flat cells, which is consistent with GAD immunostaining. CB immunoreactive (CB-ir) neurons are found mainly in the dorsal cortex and subsequently in the external cortex, whereas their presence decreases significantly in the central nucleus. Their somata are oval or polygonal in the DCIC, and oval, elongated or triangular in the ECIC. CR

immunoreactive (CR-ir) neurons have a significant presence in DCIC and ECIC, with a low density in CIC. Their somata in the ECIC are oval and polygonal, while their size ranges from small to large (Ouda and Syka 2012).

1.5.5 Electrophysiological features of IC neurons

Previous studies on the response properties of neurons in the central nucleus and external and dorsal cortices of the inferior colliculus revealed various types of response patterns, including onset response pattern, where the neuron fired only at the beginning of the stimulus presentation; sustained response pattern, where the neuron fired throughout the duration of the stimulus presentation; pauser units, that showed a precisely timed onset peak separated from a lower level of sustained activity by a short period of either a marked reduction or complete cessation of firing; and chopper units, with two or more clearly defined peaks near the stimulus onset. In general, the CIC neurons, compared to the respective ECIC and DCIC neurons, exhibit a sharper frequency tuning, a lower average threshold, a shorter average first-spike latency of responses to tones at the characteristic frequency (CF) and a higher rate of spontaneous activity (Syka et al. 2000).

1.6 The Medial Geniculate Body

The mammalian auditory thalamus, also known as the Medial Geniculate Body (MGB) is a structure that extends bilaterally from the midbrain to the forebrain and it provides auditory information to the auditory cortex and to subcortical limbic structures. The MGB contains ventral, dorsal and medial divisions. The ventral division consists, for the most part, of principal cells. It is the main sensory nucleus of the MGB (J. A. Winer 1984) and exhibits a representation of characteristic frequency (J. A. Winer and Morest 1984). The ventral division of the MGB is part of the tonotopic system of the superior auditory pathway, receiving input mainly from the central nucleus of the IC and projecting to the primary auditory cortex (AI),

the anterior auditory field (AAF) and the posterior auditory field (PAF) (Rouiller et al. 1989). The dorsal division of the MGB consists of a mixture of cell types and it has a less topographically ordered frequency representation compared to the ventral division (J. A. Winer and Morest 1984). The dorsal division of the MGB is part of the non-tonotopically organized system of the superior auditory pathway. The region of the dorsal division that receives input from the pericentral nucleus of the IC and projects reciprocally and exclusively to the secondary auditory cortex (AII) (Rouiller et al. 1989) (Andersen et al. 1980). Lastly, the medial division is the largest part of the MGB and has the most subdivisions. It receives input from non-auditory structures of the midbrain (J. A. Winer and Morest 1984), as well as from the lateral nucleus of the IC (ECIC) (Jeffery A. Winer and Schreiner 2005). The medial division has coarse tonotopic organization. It projects to the tonotopic cortical fields (AI, AAF and PAF) and to the non-tonotopically organized secondary auditory cortex (AII) (Rouiller et al. 1989) as well as to non-auditory cortex and to amygdala (Huang and Winer 2000).

1.7 The Auditory Cortex

The Auditory Cortex (AC) is found at the top of the hierarchical auditory pathway. The AC is responsible for discrimination and localization of sounds, recognition of vocalizations of specific species, comprehension and initiation of speech and auditory learning and memory (Jeffery A. Winer and Schreiner 2005). The AC is subdivided into the primary auditory cortex (AI), secondary auditory cortex (AII) and association cortex. The primary auditory cortex (AI) has a tonotopic organization and it consists of six layers. The layers III and IV receive input from the ventral division of the MGB, while the layers I and VI from the medial division (Rouiller et al. 1989) (Huang and Winer 2000). Corticofugal projections from the layers V and VI give signal to different targets, including the inferior colliculus (J. A. Winer et al. 1998), the thalamus, the olivary complex, the striatum, the amygdala and the cochlear nucleus (Jeffery A. Winer and Schreiner 2005).

2 Aim of the study

The Inferior Colliculus has a crucial role in the auditory pathway. It receives ascending auditory input from all the lower structures (Glendenning et al. 1992; Oliver and Morest 1984; Saint Marie et al. 1997), as well as descending projections of all fields of the AC (Bajo and King 2012). The GABAergic neurons of the CIC, due to their projections to the thalamocortical neurons of the MGB (Peruzzi et al. 1997) and their local collaterals, can mediate inhibition both locally and in the MGB. In that way, they may establish a mechanism to code "place". On the other hand, the excitatory synapses of neurons may define interaural time differences (Oliver et al. 1991).

The IC is also involved in the oculomotor pathway, as the ECIC, mainly, sends dense projections to the superior colliculus, mostly in the deeper layers (Garcia Del Cano et al. 2006), providing auditory information to a structure contributing to the generation of saccades.

From the three subdivisions of IC, the less well studied is the ECIC. In the present study, we tried to study neurons of the rat ECIC and describe them on the basis of their electrophysiological and morphological profiles. To this end, we have recorded the spontaneous as well as the evoked activity of single ECIC neurons *in vivo*, in order to classify them according to previous studies (see §1.5.5). After recording, we labeled them juxtacellularly with neurobiotin and fully reconstructed them in order to associate their electrophysiological and morphological features. Further electron microscopical analysis gave us insights about their excitatory or inhibitory nature.

3 Materials and Methods

3.1 Surgical procedures

Six male young adult Sprague-Dawley rats (*Rattus norvegicus*; 3-4 months-old; weighing 380 - 450g) were used for extracellular recording and juxtacellular labeling of single neurons (Magill et al. 2000; Pinault 1996). All experimental procedures were approved by the institutional committee in accordance to Presidential Decree 56/2013 that incorporates the EU directives (directive 2010/63/EU and its amendments) into the Greek law. The animals were housed in a licenced establishment (EL91 -BIObr/05) under a 12h-12h light-darkness schedule, having access to food and water, and were handled by trained individuals.

The animals were anesthetized with an intraperitoneal injection of urethane (1.25g/kg; Sigma-Aldrich Co., St. Louis, MO, USA), and, after approximately 30 minutes, an intraperitoneal injection of ketamine (30mg/Kg, Narketan-10, Vetoquinol, SA, France) and xylazine (3mg/Kg, Rompun, Bayer AG, Germany) was administrated. During the surgical and electrophysiological procedures, the animals were immobilized in a stereotaxic head holder (Kopf Instruments, Tujunga, CA, USA), and supplementary doses of ketamine and xylazine were given to maintain the anesthesia and analgesia, as needed.

After the placement of the rat in the stereotaxic head holder, a midline incision was made in the scalp and the skin was retracted laterally. A borehole was made caudally and laterally to bregma with a drill and a screw was attached to hold the ground wire. A craniotomy on the left caudal part of the cranium was made to expose the cerebral cortex above the IC. The dura was cut and retracted (*Figure 3*). A hemostatic gelatin sponge containing 5% colloid silver (Gelatamp; Roeko, Langenau, Germany) was used, when needed, to stop the bleeding. The temperature of the animal was maintained at 37^{o}C with a heating pad (Braintree Scientific, Braintree, Massachusetts, USA).

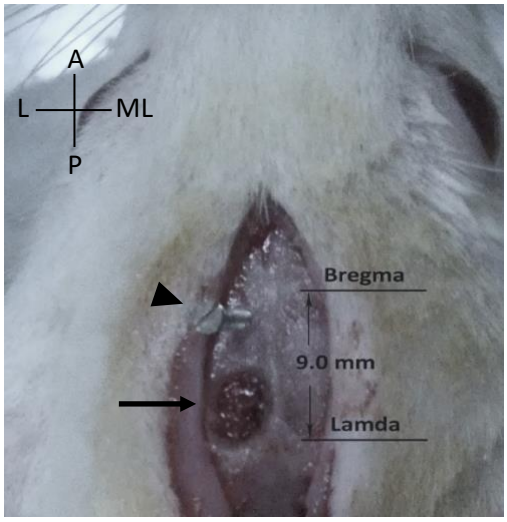

Figure 3. Photo of the surgical area on the rat skull. Anterior, the skull screw that was used to attach the ground wire is visible (arrowhead). Posterior, the craniotomy above the inferior colliculus is visible (arrow). Superimposed are shown the reference points (bregma and lambda) for stereotaxy.

3.2 *In vivo* extracellular recording of single neuron responses and juxtacellular labeling

Glass micropipettes with a microfilament fused to their inner wall (inner diameter: 0.86 mm / outer diameter: 1.5mm, Hugo Sachs Elektronik - Harvard Bioscience, GmbH, March, Germany) were pulled using the Narishige PE-2 vertical puller (Narishige, Tokyo, Japan). The tip of the micropipette was opened manually and its size was evaluated under a light microscope. The external diameter of the tips used was 1.5 to 2µm, so that the electrode resistance was between 15 and 30 MΩ, and the tips were flat to prevent the injury of the neurons.

The glass micropipette was filled with a 1.5% solution of neurobiotin (N-(2-Aminoethyl) biotinamide hydrochloride, FW 322.85 g/mol; Vector laboratories Inc., Burlingame, CA, USA), in 0.5M NaCl, and was connected to the headstage (HS-2, 1x gain; Axon Instruments Inc., Foster City, CA, USA) through a chlorinated silver wire that was inserted into the glass pipette. The headstage was connected to the Axoclamp 2A current and voltage clamp system (Axon Instruments), a preamplifier and supplier of current. Finally, the headstage was grounded through the attached screw on the animal skull, using a silver wire.

The coordinates of the targeted location were calculated with the help of an atlas of the rat brain (Paxinos and Watson 2009). The dorsoventral zero coordinate was the surface of the cortex. A computer driven micromanipulator (IVM-1000, Scientifica, East Essex, UK), was used to advance the electrode slowly (1μm/s), vertically to the exposed tissue. There was a single pipette penetration for each position and differed from other penetrations at least 400μm in the anteroposterior and/or the mediolateral axis.

Occasionally, during the electrode penetration a small intensity current pulse was applied (0.1nA, 400ms duration, 50% duty cycle), to stimulate the IC neurons. After the detection of a neuronal activity, the current was ceased immediately and the spontaneous activity of the neuron was recorded. Then, the neuron responses to acoustic stimuli were recorded. The auditory stimuli (of short or prolonged duration) included the sound of a bell at 3 different frequencies, clapping, and vocal stimuli.

The electrical signals were bandpass-filtered (0.3–5 kHz) and amplified (10x) by Axoclamp 2A and by an AC/DC amplifier (1000x, J.D., 1997, University of Crete, Greece). The signal was displayed on a 2-channel oscilloscope (HM 1008, Hameg Instruments GmbH, Mainhausen, Germany). Through a CED 1401 analogue-to-digital card (Cambridge Electronic Design Limited, CED; Science Park, Cambridge, UK) signals were digitised, interfaced by the software Spike 2 (Cambridge Electronic Design Limited), displayed online on a computer screen and saved. Additionally, an audible signal of the neuron's activity was delivered through a loudspeaker connected to a commercial audio amplifier (Crown ICISOR). The programmable 8-channel pulse generator Master-8 (A.M.P.I, Israel) was used to provide the trigger signal for the oscilloscope and the Axoclamp 2A.

After the recording of the spontaneous activity and the responses of the neuron to acoustic stimuli the procedure of juxtacellular labeling was initiated. The delivery of the tracer was achieved iontophoretically, with anodal current pulses ranged between 1 to 10nA, applied at 2.5Hz, with a 50% duty cycle (200ms ON, 200ms OFF).

3.3 Tissue preparation methods

After the juxtacellular labeling, the animal remained *in situ* for one to two hours, to allow for the diffusion of the tracer to the most distal parts of the labeled neurons. Thereafter, the animal received a lethal dose (0.7ml/Kg) of sodium pentobarbitone (200 mg/ml, Vétoquinol, Lure, France). Analgesia and loss of consciousness were assured by checking the pedal reflex and when these were reached the animal was tied in the supine position. Subsequently, the skin below the diaphragm was incised and the diaphragm was cut. Through a long feeding needle, a cold (4^{o}C) 0.9% NaCl solution was administrated via the left ventricle of the heart, for 1-2min. On the side, the right atrium was incised to allow for blood removal. Immediately after the saline, cold fixative [4% paraformaldehyde (MERCK), 15% saturated picric acid (saturated, 1.3% in H_2O, SIGMA), and 0.05% glutaraldehyde (EM Grade, TAAB) in 0.1M phosphate buffer (pH 7.2; PB)] was allowed pass for 15 minutes.

After fixation, the brain was removed and cut in three blocks, taking care that the areas of interest were in a single block and they were postfixed at 4^{o}C in fixative overnight. The block of interest was cut coronally in 70μm thick serial sections (*Figure 4*), with a vibrating blade microtome (Leica Biosystems Richmond, LLC, USA). The sections were stored in tissue culture plates, in 0.1M PB containing 0.05% sodium azide, to prevent fungal contamination, at 4^{o}C.

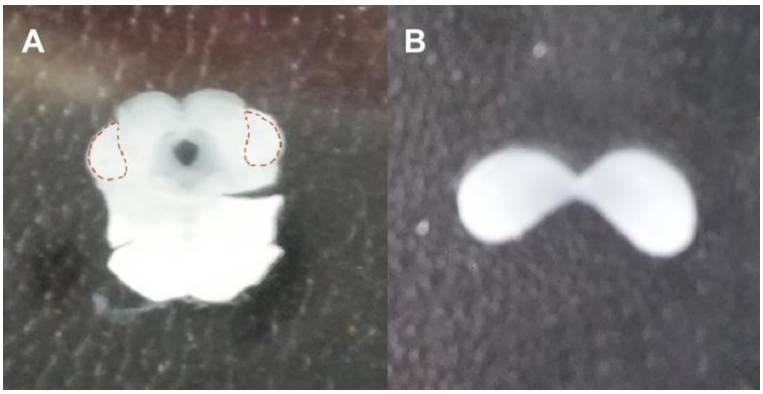

Figure 4. 70μm thick coronal sections at two different rostro-caudal planes. (A) A midbrain section, where both the inferior and superior colliculus appear (dashed line indicates the rostral IC). (B) A posterior section of Inferior colliculus. The cerebral cortices are not shown.

3.4 Visualization of labeled neurons and immunocytochemistry

The methods for visualization of the labeled neurons, combined with immunocytochemistry have been described earlier (Dalezios et al. 2002; Ferraguti et al. 2005; Klausberger et al. 2005). These methods fit for the observation of the tissue under a light microscope and permit for the subsequent analysis of regions of interest with a transmission electron microscope.

3.4.1 Visualization and reconstruction of labeled neurons

To visualize the tracer, the avidin – biotin peroxidase complex (ABC) method was used. Sections containing labeled structures (as estimated by the injection coordinates) were washed thoroughly in 0.1M PB to remove residues of sodium azide and fixative. Subsequently the sections were cryoprotected in 10% and 20% sucrose in 0.1M PB, at 4^{o}C and freeze-thawed in a liquid nitrogen atmosphere. The freeze-thawing allows for a better penetration of the reagents into the tissue.

After a few washes in 0.1M PB, the sections were transferred in the incubation buffer (50mM Tris Buffer Saline, pH 7.4 [TBS]) for ~10min. Then, they were incubated in avidin-biotin horseradish peroxidase complex (VECTASTAIN® Elite® ABC-HRP Kit [Peroxidase, Standard], Vector Laboratories, Burlingame, CA, U.S.A.; ABC, diluted 1:200 in TBS) under gentle agitation (~48 hours at 4°C). After washes in TBS and TB, the sections were incubated in a DAB solution (0.5mg/ml TB; Sigma-Aldrich, Poole, UK) for at least 10min and, subsequently, hydrogen peroxide (Merck, Darmstadt, Germany) was added to the solution (final concentration 0.01–0.02%) and the sections left for the color to develop for another 7-10 minutes. The reaction was stopped by several washes of the sections in TB followed by washes in 0.1M PB.

The sections were contrasted with 1-1.5% OsO_4 (TAAB, Aldermaston, Berkshire, UK) in 0.1M PB, for 40 to 50 minutes and counterstained in an aqueous solution of 1% uranyl acetate (TAAB). Finally, they were dehydrated in a series of increasing ethanol concentrations (50%, 70%, 90%, 95% and 100%) in ddH_2O, and propylene oxide (Sigma-Aldrich, St. Louis, MO, USA).

After dehydration, the sections were immersed in epoxy resin (Durcupan™ ACM, Sigma) for at least 2h, flat mounted on slides and left to polymerize at 60°C overnight.

The labeled structures were drawn using a light microscope (Zeiss Axioskop) equipped with a drawing tube, under the 100x oil immersion objectives. For the neuron reconstruction, the drawings from the consecutive sections were scanned, digitized using the Canvas drawing software (ACD Systems International Inc., Victoria, BC, Canada), and combined in a single figure.

3.4.2 Immunocytochemistry for Electron Microscopy

Immunocytochemistry was performed before the ABC reactions to test for the expression of specific proteins by the labeled neurons, their inputs or targets using gold-labeled antibodies. Briefly, the sections were cryoprotected, freeze-thawed twice and preincubated at room temperature (R/T) in 20% normal goat serum (NGS, Vector) dissolved in TBS for an hour. Then they were transferred in a solution of a primary antibody (Swant, Bellinzona, Switzerland; see *Table 1* for details*)* and under gentle agitation incubated at 4^{o}C for at least 36h. Subsequently, the sections were washed in TBS to remove unbound antibodies and transferred to a solution of a secondary antibody (Nanoprobes, Yaphank, NY, USA; *Table1*), labeled with 1.4nm gold particles in TBS, at 4^{o}C for at least 36h, under gentle agitation.

After washing the sections to remove the unbound secondary antibody, they were postfixed with 0.5% Glutaraldehyde in TBS for ~10 minutes and the gold particles were enhanced with colloidal silver for 5-10min, following the instructions of the commercially available kit (HQ Silver, Nanoprobes). The reaction was terminated by washes in ddH_2O, followed by washes in TBS, and then the sections were incubated in ABC reagent (diluted 1:150) and developed for the neurobiotin detection, as previously described.

Table 1. Primary and secondary antibodies used in the study.

Primary Antibody	**Host**	**Concentration**	**Source**
Monoclonal anti-Parvalbumin	Mouse	1:5000	Swant
Monoclonal anti-Calbindin D-28K	Mouse	1:5000	Swant
Monoclonal anti-Calretinin	Mouse	1:5000	Swant
Anti-Calbindin D-28K	Rabbit	1:5000	Swant
Anti-Calretinin	Rabbit	1:5000	Swant
Secondary Antibody			
Nanogold anti-mouse-Fab	Goat	1:100	Nanoprobes
Nanogold anti-rabbit-Fab	Goat	1:100	Nanoprobes

3.4.3 Procedures for electron microscopy

After the reconstruction of the labeled neuron, the coverslips were removed and areas of interest (approximately 1x1mm) were re-embedded in a Durcupan block, which was left to polymerize at 60^{o}C overnight. This method permits for the inspection of the tissue in the block under a light microscope and the restriction of its dimensions to fit around the areas of interest. The block edge containing the tissue was manually trimmed to a pyramid and its surface restricted to a trapezoid of approximately 500x200µm. Consecutive ultra-thin sections (70nm) were cut with a 45° diamond knife (Diatome Ltd, Nidau, Switzerland) in an ultramicrotome (EM UC6, Leica) and collected on copper, pioloform (Agar Scientific Ltd, Stansted, Essex, UK) coated, slot grids (TAAB or Agar Scientific).

The ultrathin sections were stained with lead for 2-3 minutes, washed in ddH_2O, dried and stored in a gridbox (Leica). The observation of the sections was performed at 80kV at a JEM-2100 transmission electron microscope (JEOL Ltd, Akishima, Tokyo, JAPAN).

4 Results

In the present study, we recorded the spontaneous and evoked (by acoustic stimuli) activity of seven IC neurons. Two of these neurons (PS35 and PS41), located in the ECIC, appeared to be well labeled and were fully reconstructed from serial 70µm sections. In the other cases, the somata were lost but, nevertheless, we managed to reconstruct some structures, most likely axons.

Both reconstructed neurons project through the intercollicular commissure to the contralateral IC, in agreement with previous studies, which showed that several cells of the ECIC project contralaterally (Adams 1980; L. M. Aitkin and Phillips 1984).

Both neurons showed an irregular low frequency spontaneous activity and responded to auditory stimuli, although with a different response pattern. Their firing rate was increased after cessation of the current pulses, which indicated their proper labeling.

4.1 Neuron PS35

Neuron PS35 shows an irregular spontaneous activity of low frequency (mean±SD: 0.78±1.80 spikes/s, N=60; Figure 5A). During the sound stimulation, the neuron's firing rate increases (3.45±1.03 spikes/s, N=11, $p<0.001$ Student's *t*-test; Figure 5B), as well as for several seconds after the end of the sound stimulation (4.88±1.55 spikes/s, N=8, $p<0.001$; Figure 5B), compared to spontaneous activity. During juxtacellular labeling the cell fires at a high frequency (Figure 5C) and shows an increased firing rate after cessation of the current pulses (16.25±17.98 spikes/s, N=20, Figure 5D), a sign of successful labeling.

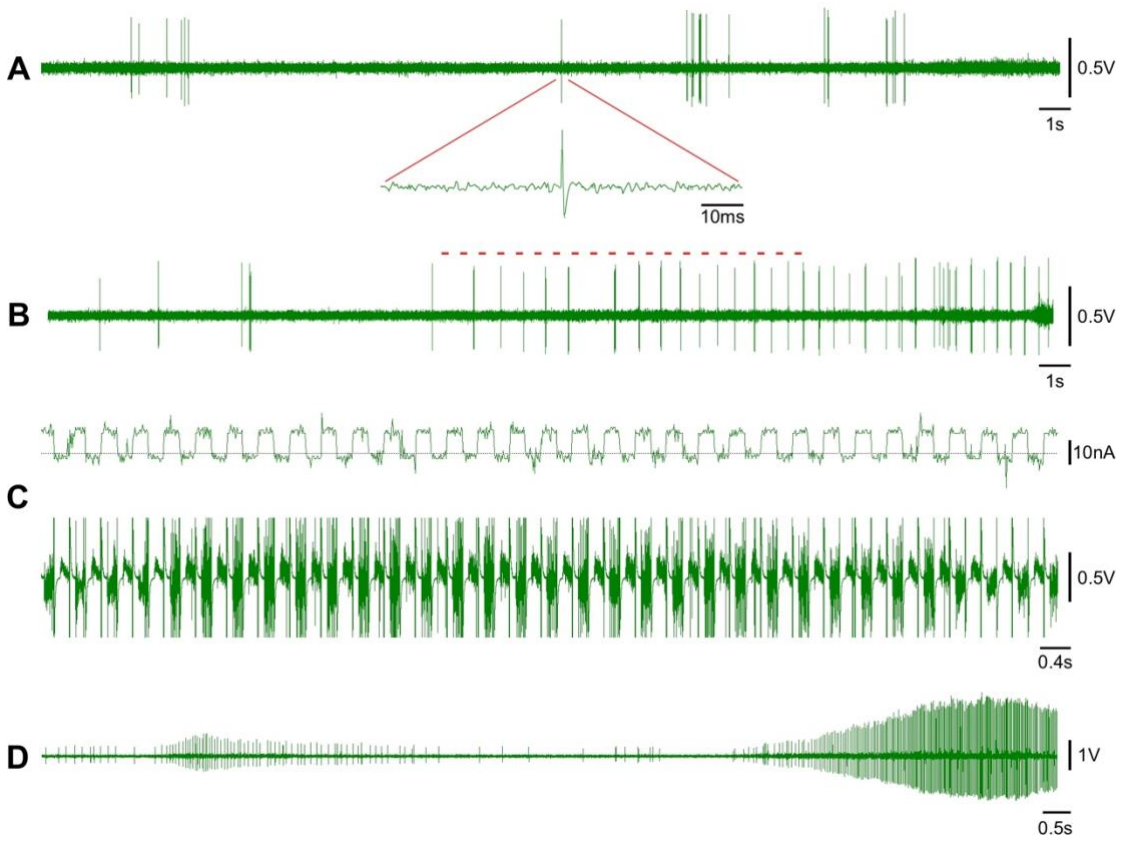

Figure 5. (A) Neuron PS35 shows irregular low frequency spontaneous activity. A spike is magnified to show its shape. (B) The neuron's response to sound stimuli (dashed line). After the sound stimulus the firing rate increases. (C) *Upper trace:* Current pulses for juxtacellular labeling (200ms ON, 200ms OFF). *Lower trace:* During juxtacellular labeling the neuron fires repeatedly synchronized to current pulses. (D) Firing rate increases after the cessation of the current pulses.

Neuron PS35 has a large (long axis diameter 36.8µm) multipolar horizontally oriented soma (Figure 6, Figure 8A) that lies in the ECIC (Figure 7, *Inset*) and six primary dendrites emerged from it. The dendrites are beaded, with few dendritic appendages (Figure 8). The order of dendritic bifurcations reaches 5 to 6 and the dendritic tree extends 140µm rostrally, 490µm caudally, 360µm dorsally, 220µm ventrally, 340µm medially and 250µm laterally.

The axon starts from a primary dendrite (red asterisk in Figure 6) immediately bifurcates and wends medially and laterally giving numerous local collaterals with many boutons in the somatic vicinity (Figure 7). A main axonal branch heads medially, crosses the midline and enters the contralateral IC, without drifting away at the somatic rostrocaudal level. Unfortunately, this axon labeling fades gradually and we were not able to detect its terminals.

The rostral axon ends at the medial geniculate body (MGB) giving numerous boutons. The whole axonal field extends 4.57mm mediolaterally, 2.15mm dorsoventrally and 2.1mm rostrocaudally.

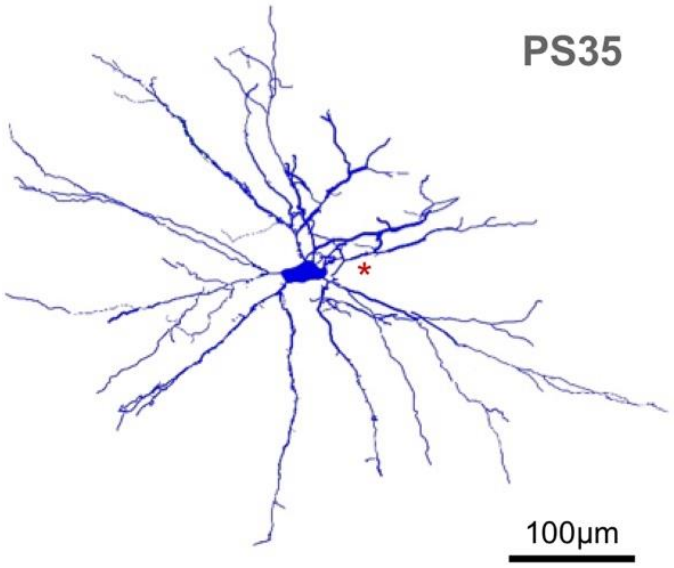

Figure 6. Reconstruction of the PS35 soma and dendrites from 9 consecutive 70µm thick coronal sections. The cell has a giant multipolar soma. Six primary dendrites sprout and extend to several directions. The dendrites are beaded with a few dendritic appendages. The axon arises from a primary dendrite (red asterisk).

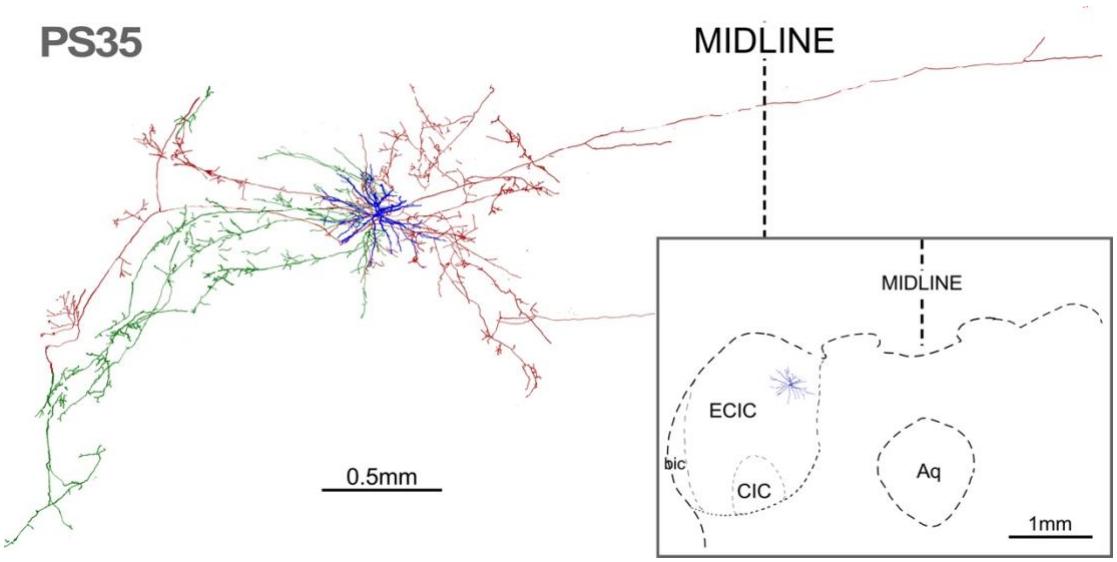

Figure 7. Reconstruction of PS35 from 30 consecutive 70µm coronal sections. Soma and dendrites are shown in blue. The neuron extends parallel to the IC surface. The axon emits an axonal branch that projects through the commissure to the contralateral IC. It arborizes locally around the soma and extends mainly rostrally (green line), while a part of it travels caudally (red line) and forms numerous boutons. The mediolateral axonal extent is more than 4mm. *Inset*: Position of the PS35 soma and dendrites in the ECIC. Aq: Aqueduct; bic: brachium of the inferior colliculus; CIC: central nucleus of inferior colliculus; ECIC: external cortex of inferior colliculus.

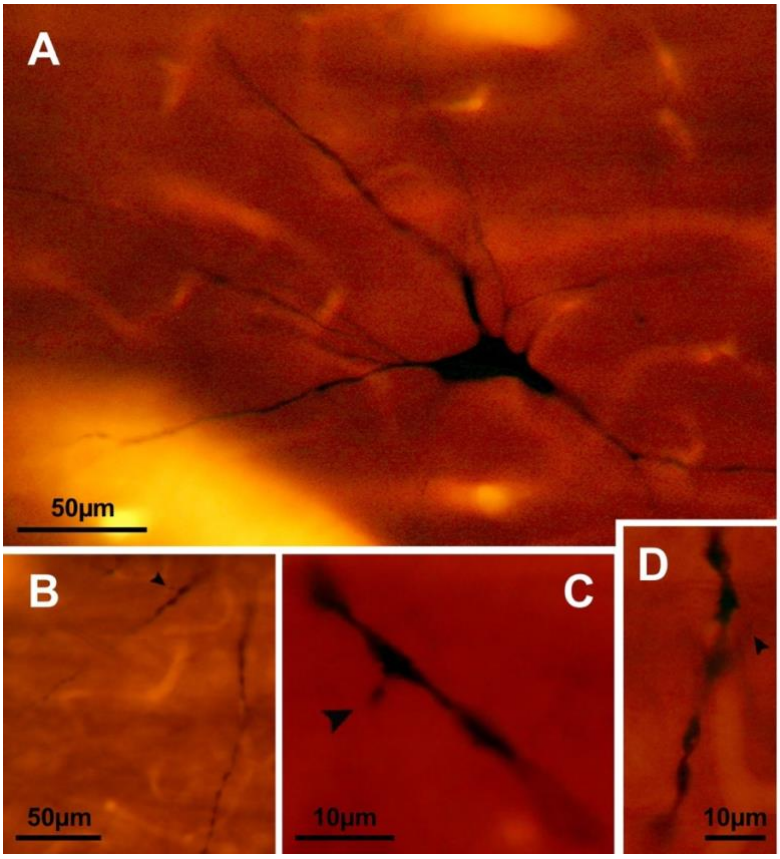

Figure 8. Light microscope photographs of PS35 soma and dendrites from individual 70µm sections. (A) Soma and primary dendrites. (B-D) Photographs showing the beaded dendrites. Arrowheads indicate dendritic appendages.

Immunocytochemistry for electron microscopy, using primary antibodies against calcium binding proteins (visualized with silver-enhanced gold conjugated secondary antibodies), was performed in some of 70µm thick sections prior to ABC-DAB reactions for the neurobiotin visualization. Electron microscopic analysis of neurobiotin labeled dendrites and axons in ultrathin (70nm thick) sections revealed that neuron PS35 does not express the calcium binding protein calretinin (Figure 9), whereas it expresses the calcium binding protein parvalbumin (Figure 10).

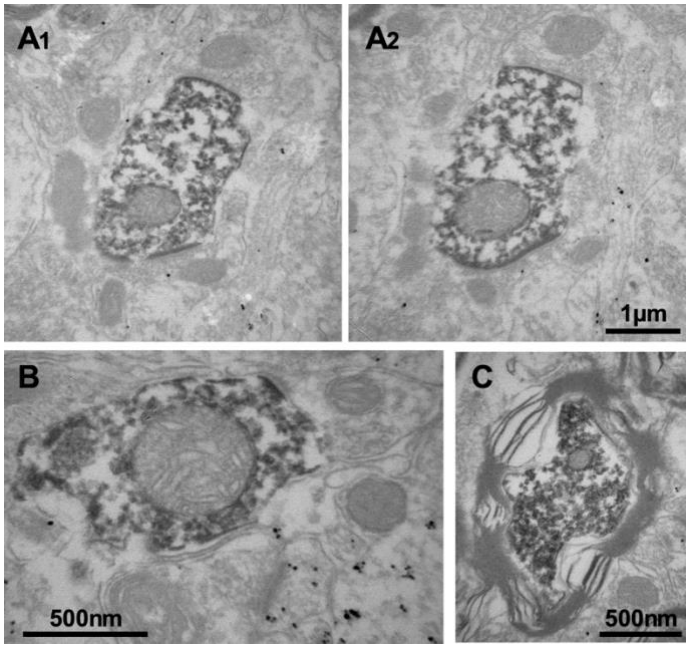

Figure 9. Microphotographs of ultrathin (70nm thick) sections of neurobiotin labeled PS35 dendrites (A1-A2, B) and axon (C). Gold particles label calretinin immunoreactive profiles. There is no colocalization of the DAB product and gold particles, showing that PS35 does not express calretinin.

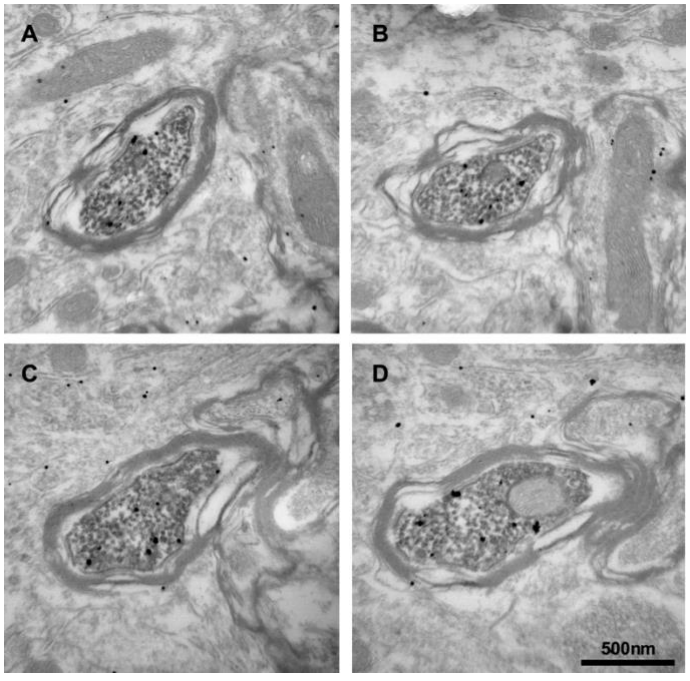

Figure 10. Microphotographs of ultrathin (70nm thick) sections of the same PS35 axonal profile. Numerous gold particles, labeling for parvalbumin, are present in the neurobiotin labeled axon, showing that neuron PS35 expresses parvalbumin.

4.2 Neuron PS41

Neuron PS41 also shows an irregular low frequency spontaneous activity (mean±SD: 0.83±1.01 spikes/s, N=59; Figure 11A). During auditory stimulation, the neuron decreases its activity (0.06±0.24 spikes/s, N=17 p<0.005 Student's *t*-test; Figure 11B), and fires right after the cessation of the stimulus (1.33±1.07 spikes/s, N=12). During juxtacellular labeling neuron fires repeatedly (Figure 11C), increasing its firing rate after cessation of the current pulse (8.05±3.22 spikes/s, N=19; Figure 11D).

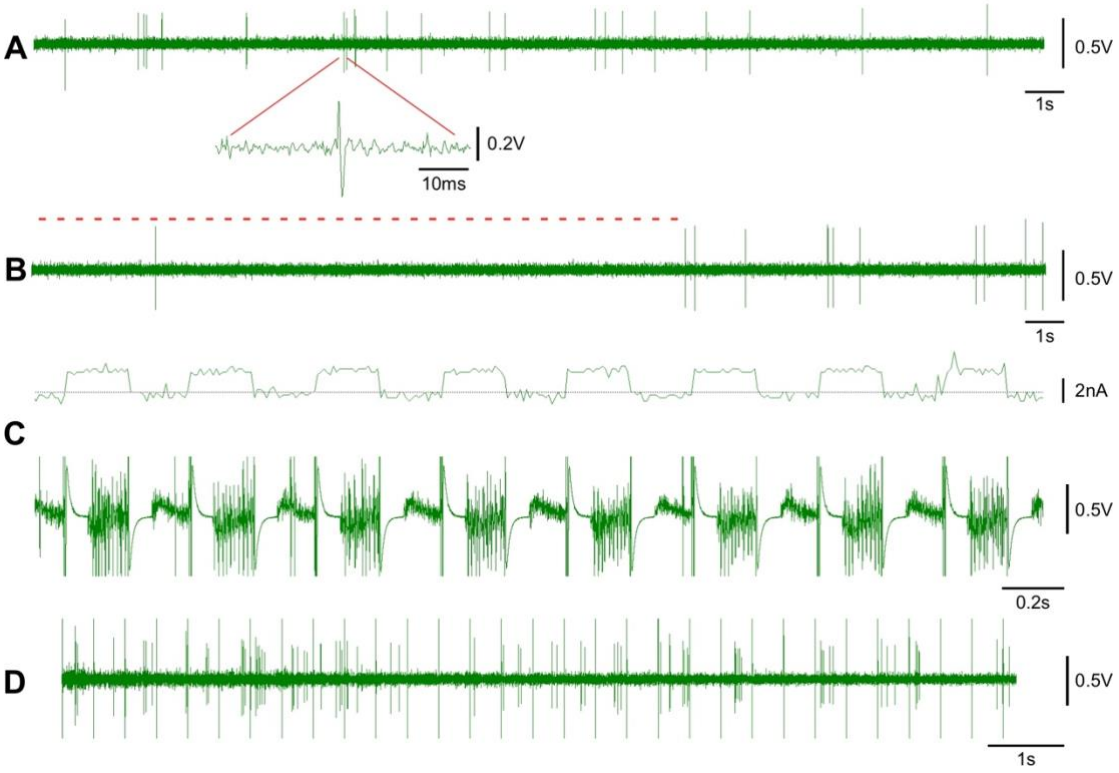

Figure 11. (A) Neuron PS41 shows irregular low frequency spontaneous activity. A spike is magnified to show its shape. (B) During sound stimulation (dashed line) the neuronal activity decreases. After the auditory stimulus, the firing rate increases. (C) *Upper trace:* Current pulses for juxtacellular labeling (200ms ON, 200ms OFF). *Lower trace:* During juxtacellular labeling the neuron fires repeatedly synchronized to current pulses. (D) Firing rate increases after the cessation of the current pulses.

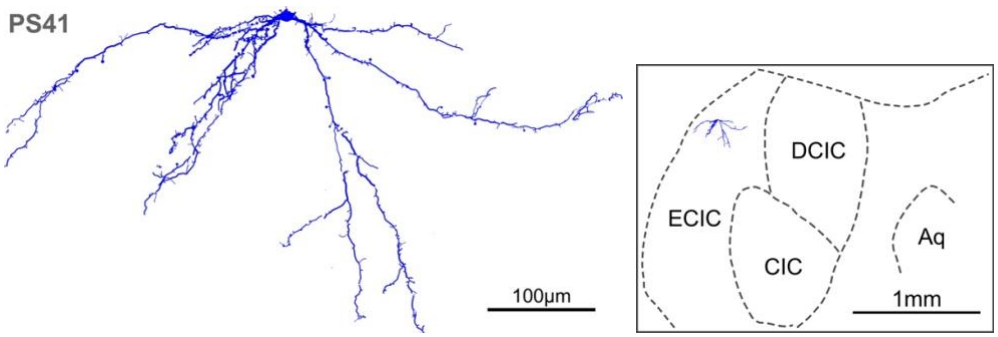

Figure 12. Reconstruction of the PS41 soma and dendrites from 8 concecutive 70µm thick coronal sections. Soma is bipolar and the dendrites are oriented ventrally, each one emitting numerous spines. The neuron lies in the dorsolateral region of the ECIC, near the IC surface (*Inset*). Aq: Aqueduct; CIC: central nucleus of inferior colliculus; DCIC: dorsal cortex of inferior colliculus; ECIC: external cortex of inferior colliculus.

Neuron PS41 has an ovoid small soma (mediolateral diameter approximately 10µm; Figure 12, Figure 14) that lies in the dorsolateral region of ECIC (Layer I), near the IC surface (Figure 12, *Inset*). The neuron is bipolar but its dendrites extend and bifurcate ventrally to soma (564µm mediolaterally, 298µm dorsoventrally and 560µm rostrocaudally; Figure 12). The dendrites bear numerous spines (Figure 12, Figure 14).

The main axon starts from the primary dendrite medially to soma, arborizes near soma and its branches travel rostrally (following ventral and medial directions) and caudally (mainly to the dorsal; Figure 13). The rostral branch of the medial axon (Figure 13, *green*; *Inset*) enters the intermediate layers of the SC giving a few axon terminals, in accordance with previous studies, which showed that neurons located in the dorsolateral region of the ECIC project to the SC (Garcia Del Cano et al. 2006). The dorsal branch of the caudal axon, follows the IC surface, travels through the commissural nucleus of the the inferior colliculus and projects to the contralateral IC, but we could not detect its terminal zone, due to an apparent loss of labeling (Figure 13).

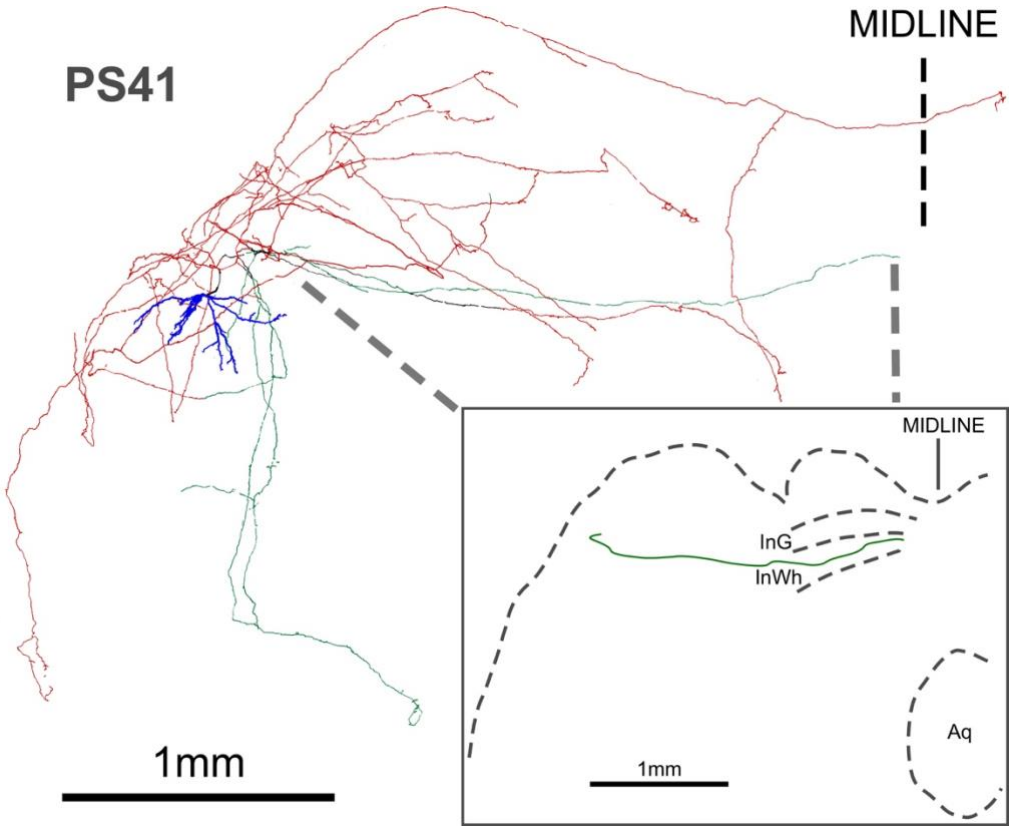

Figure 13. Full reconstruction of neuron PS41 from 32 concecutive 70µm thick coronal sections. The axon travels caudally (red), passes through the commissural nucleus of the IC and projects to the contralateral IC. *Inset:* The medial branch of the rostral axon (green) enters the intermediate layers of the superior colliculus. Aq: Aqueduct; InG: Intermediate Gray layer of superior colliculus; InWh: Intermediate White layer of superior colliculus.

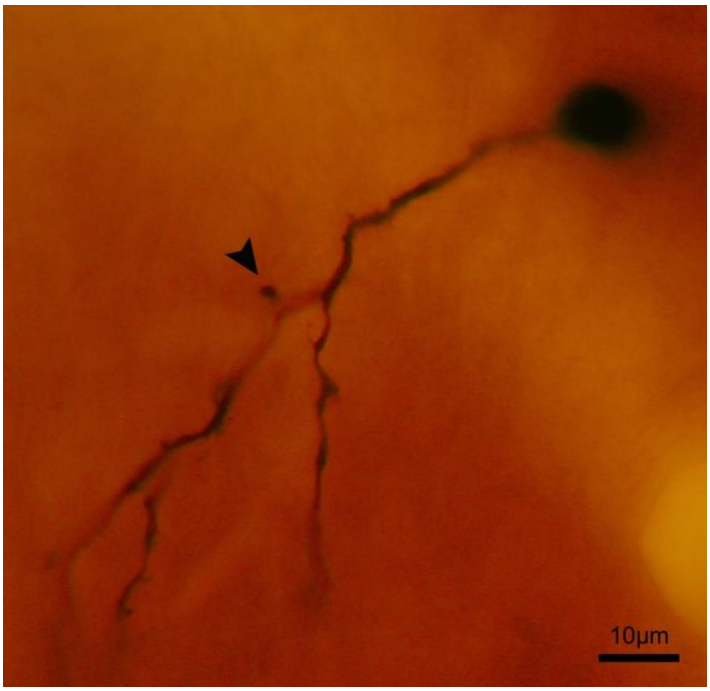

Figure 14. Light microscope photograph of neuron PS41 soma and primary dendrites from a 70µm thick section. The neuron has a small ovoid soma (diameter ~10µm). The dendrites are oriented ventrally of the soma and they are spiny (arrowhead).

The axonal arborizations extend 3.69mm mediolaterally, 2.64mm dorsoventrally, and 2.24mm rostrocaudally. Most of the axon is extending caudally to soma forming numerous boutons, as well as *en passant* boutons (Figure 15). Another feature of the caudal axon is the formation of several axonal loops (Figure 15B).

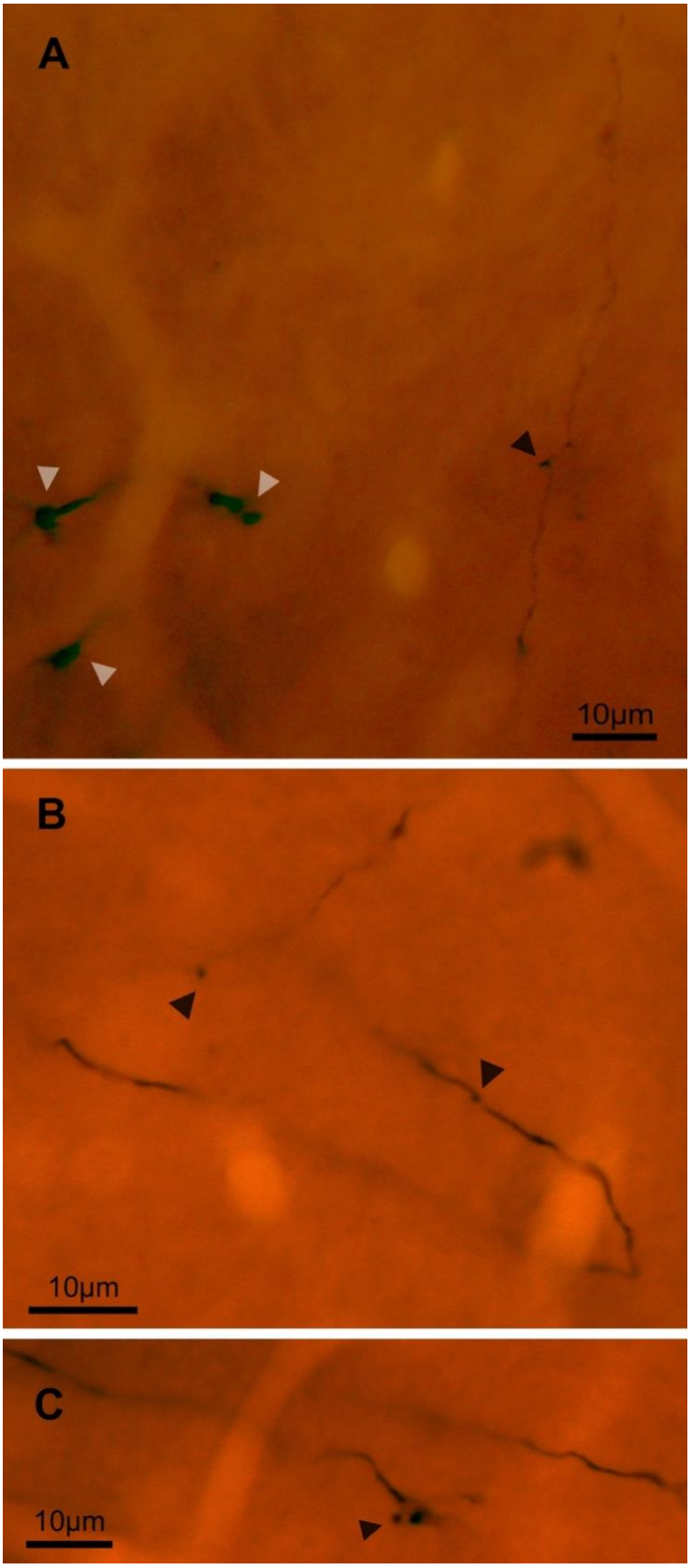

Figure 15. Light microscope photographs of the PS41 axon from 70µm thick coronal sections. The axon forms numerous *en passant* boutons (black arrowheads). (A) The white arrowheads indicate dendritic profiles. (B, C) The caudal axon forms several loops. The same axonal segment is shown in 2 different focal planes.

An axonal segment of neuron PS41 (part of this is shown in Figure 15A) was reembedded in epoxy resin and consecutive ultrathin (70nm thick) sections were examined in a transmission electron microscope. As shown in Figure 16, the labelled axon *en passant* boutons characteristically bypass dendritic shafts (d) and form Type I (presumably excitatory) synapses with dendritic spines (sp) in ECIC.

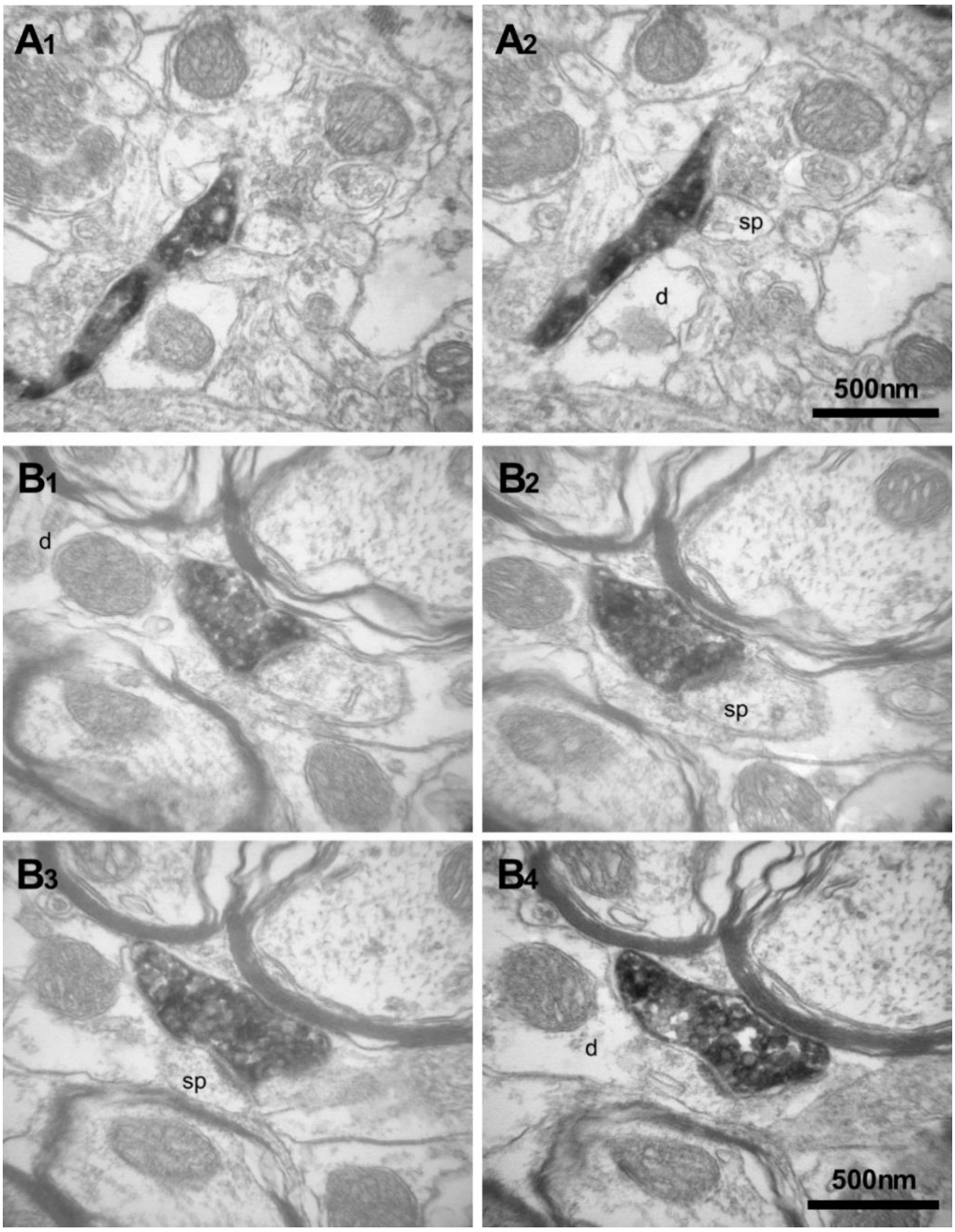

Figure 16. Microphotographs of consecutive ultrathin (70nm thick) sections (A1-A2, B1-B4) of the PS41 axon. In both cases, the labeled *en passant* boutons bypass the dendritic shafts (d) and form Type I synapses with spines (sp). (B1-B4) The same bouton forms synapses with two different spines (B1-B2, B3-B4).

4.3 Partially recovered neuronal structures

Besides the two neurons described above, some structures showing axonal morphological features were reconstructed (Figure 17). It looks like that soma (Figure 17, arrowhead) lies in the ventromedial part of the ECIC (Figure 17, *Inset)*, according to the morphology of the axons and the stereotaxic coordinates of the electrode penetration. The main axon is thick and the axonal field extends rostrally, and enters the SC, forming several boutons.

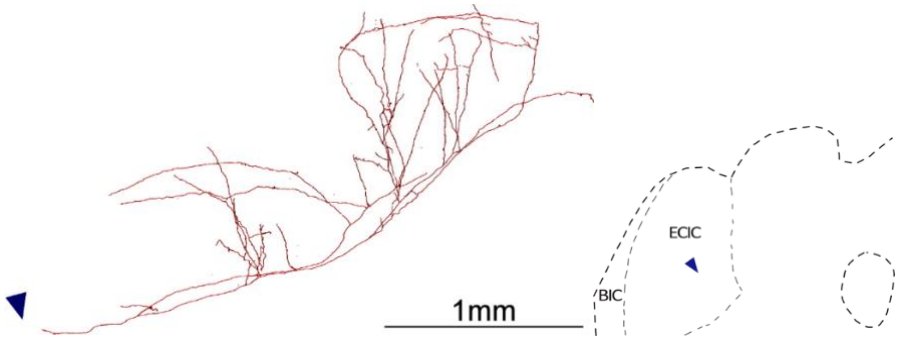

Figure 17. Reconstruction of neuronal structures, considered as axon, from consecutive 70µm thick coronal sections. The likely position of soma is indicated by the blue arrowhead. The axonal field is dense, extents rostrally, and inserts the SC, forming several boutons.

5 Discussion

In the present study, we tried to identify neurons of the inferior colliculus in terms of their electrophysiological and morphological features. Two neurons of the ECIC have been labeled with neurobiotin and fully reconstructed from serial sections. Somata of both neurons were found to be in the external cortex of the inferior colliculus and their axons projected to the contralateral IC, consistent with previous studies (Adams 1980; L. M. Aitkin and Phillips 1984), which showed that many ECIC neurons project through the intercollicular commissure to the contralateral IC.

Neuron PS35 is a multipolar giant neuron (soma diameter >22µm) that responds to auditory stimuli by rapidly increasing its firing rate and keeping it increased for several seconds after the cessation of the stimulus. This behavior suggests that this neuron may receive both monosynaptic and polysynaptic (through other nuclei) cochlear input and (at least during an auditory stimulus) it is similar with sustained neurons reported in the IC of cat and guinea pig (L. Aitkin et al. 1994; Syka et al. 2000). These neurons are suited for direct and rapid transfer of acoustic information to MGB.

In terms of its somatodendritic morphology, it resembles neurons that have previously been reported in the IC of the rat and receive converging inputs (Gonzalez-Hernandez et al. 1991).

GABAergic neurons of the IC have also been reported to be larger than the non-GABAergic ones and may project to both contralateral IC and ipsilateral MGB (Ito et al. 2009). Furthermore, the clusters of PV neurons of the ECIC are very similar to the clusters of GABAergic neurons (Ouda and Syka 2012). Our results for PS35 (Figure 10) show that this neuron expresses the calcium binding protein parvalbumin. Thus, neuron PS35 may be a GABAergic one and a part of the monosynaptic GABAergic input of the IC to the MGB (that was also shown in the rat) and modulates the firing pattern of thalamocortical projection neurons (Peruzzi et al. 1997). It remains to examine the boutons of PS35 to find out, if this neuron is GABAergic, too.

Neuron PS41 has a small ovoid soma assigned to small to medium size neurons (diameter <13μm) found in the mouse (Gonzalez Hernandez et al. 1986). The neuron is bipolar and its peculiar characteristic is a dendritic tree that extends and bifurcates only ventrally to soma. As far as we know, neurons with somatodendritic morphology resembling that of neuron PS41 have not been reported in any species.

Its response to auditory stimuli suggests that it is a pauser (inhibition) neuron, similar to those previously recorded in guinea pig (Syka et al. 2000). These neurons are extremely rare in the ECIC and are under a prolonged influence of local GABAergic neurons.

PS41 neuron is found in Layer I of the ECIC, sends axon collaterals to the intermediate layers of the SC and, as far as we know, it is the first such neuron that has been described in this way. Its soma localization in layer I of the ECIC agrees with a previous study (Garcia Del Cano et al. 2006), showing that this specific area of the ECIC is the main auditory input to the SC. The auditory stimuli are coded in head centered coordinates, while visual targets and saccades are coded in retinotopic coordinates in the SC. The visual inputs from the superficial SC layers are transformed into motor coordinates in the deeper layers that have the ability of integration of multisensory inputs. Thus, our finding that the axon enters the intermediate layers of the SC is consistent with previous physiological studies in the monkey (Jay and Sparks 1984) (Jay and Sparks 1987a) (Jay and Sparks 1987b). These studies imply that IC axons send their auditory signals in the intermediate and deep layers of the SC. This way, auditory and visual maps share a reference system (motor error) converging to a common motor pathway that generates saccades. Therefore, neuron PS41 could be part of the auditory input of the IC to the SC, which participates in the generation of gaze movements and which processes approach and avoidance motor commands (Dean et al. 1989).

The examination of a small number of neuron's PS41 terminals under the electron microscope showed that this neuron is most likely excitatory, since it establishes type I synapses with dendritic spines (Nakamoto et al. 2013). A striking feature (at least for the local axon

examined) is that there is a preference for dendritic spines as its targets. Further analysis of its terminals is needed to establish if this is the case and, also, if this preference applies to its superior collicular targets. Of course, more experiments will give us information of how many types of IC neurons contribute auditory information to the SC.

In conclusion, this was a first attempt to identify inferior collicular neurons using extracellular recordings and subsequent juxtacellular labeling to consistently correlate their morphological and physiological features. We consider that the results of the present study are promising and demonstrate that this painstaking task will contribute to a richer and deeper knowledge of how neuronal circuits process sensory information and control our behavior.

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