Functional organisation of the central complex of the grasshopper *Chorthippus biguttulus* in relation to the control of sound production

PhD Thesis

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submitted by

Michael Kunst

born in

Husum

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Herewith I declare, that I prepared the PhD-thesis "Functional organisation of the central complex of the grasshopper *Chorthippus biguttulus* in relation to the control of sound production" on my own and with no other sources and aids than quoted.

Göttingen, 20.03.2008

To my parents

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1 Introduction

One of the most fascinating questions in neuroscience is how the brain integrates sensory information from the external world with the organism's internal physiological state, to select a behavior from it's repertoire that is most appropriate to the encountered overall situation. In order to describe this interplay of different inputs in a graphic manner Konrad Lorenz created his famous psycho-hydraulic instinct model deridingly named "water-closet-model" (Lorenz (1937) Fig. 1.1A). In this model, a particular behavior is the result of an increasing drive to act. This is caused by accumulation of action specific energy (the level of fluid = motivation) in decision making parts of the nervous system. The initiation of the behavior is triggered by a so called "key-stimulus", whose efficacy is symbolized by the weight. Both, the weight and the level of fluid combine their forces against the spring of a valve that prevents the fluid to be released, or the behavior to be executed. If the sum of both forces overcomes the force of the spring to open the valve, the fluid is released and the behavior initiated. High levels of fluid combined with strong stimuli cause a stronger release of fluid leading to higher intensities of behavior. Over the years this model has not received much attention because it appeared as too simple to explain complex behavior. However, its basic principle is still valid. For behaviors that include few choices between different possibilities that, once activated, are executed in a stereotype manner it still serves as a good basic scheme to explain the selection and coordination of behavior.

To study the basic mechanisms that generate behavior, invertebrates can serve as valuble animal models. Invertebrate preparations have been extensively used in the last two centuries to investigate the neuronal circuits that govern behavior Clarac and Pearlstein (2007), with the gill withdrawal reflex of Aplysia being the most famous (Kandel 2001). Studies with invertebrates offer several technical advantages, despite the obvious ethical advantages, which becomes even more important regarding the fact that for studying the anatomical and physiological basis of behavior mainly invasive methods have to be used. Their nervous systems are easily accessible to the experimentator, and compared to vertebrates, they consist of fewer neurons that nevertheless must accomplish similar functions in the nervous system to secure survival and reproduction of the organism.

The insect nervous system is typically divided into several ganglia (Fig. 1.3A), a

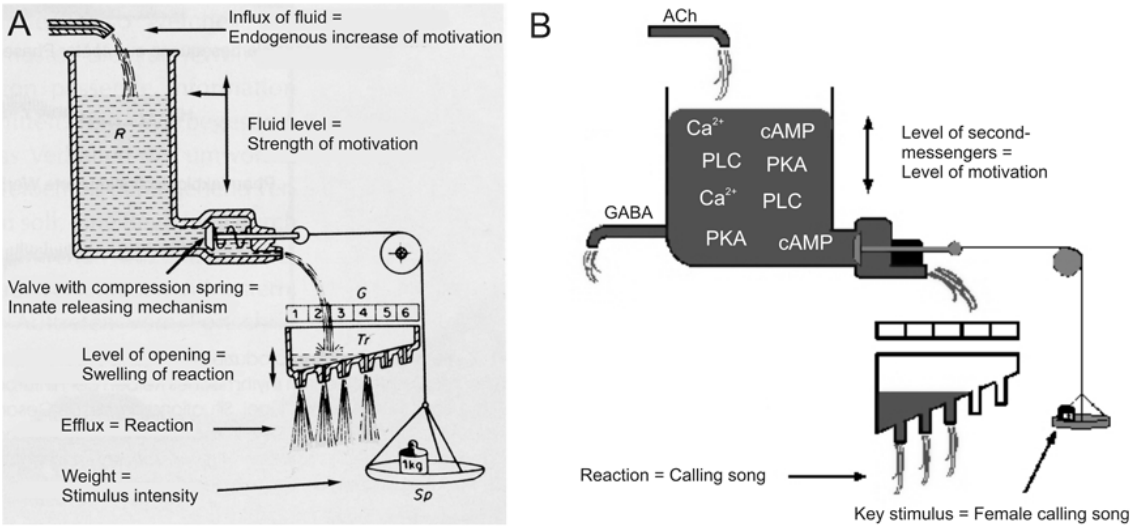


Figure 1.1: A: Psychohydraulic model after Lorenz to describe the interplay between the internal motivational state and external stimuli in creating behavior. The level of water represents the increase of action specific energy (motivation). Together with an external stimulus (the key stimulus), the compression spring gets deformed, which leads to a release of the fluid, which symbolizes the execution of a specific behavior. The intensity of the key-stimulus is represented by the weight The initiation of the triggered behavior dependends on the amount of fluid that has been accumulated, or in other words, it depends on the motivational state of the animal(Modified from Hassenstein (1983)). B: In the central complex of the grasshopper the fluid is comparable to the amount of second-messengers in specific types of neurons, which are directly influenced by the activation of mAChRs through ACh. Additionally to the original model of Lorenz, other signals may reduce the drive to perform a particular behavior, e.g. GABA release that represents unfavorable situations for the performance of stridulation. Key stimuli that may trigger sound production in grasshoppers are songs of conspecifics that signal the presence and mating readiness of potential reproductive partners.

supraoesophageal ganglion (the brain), the suboesophageal ganglion, three thoracic, and several abdominal ganglia. The different ganglia are connected to each other by paired connectives, containing the axons of ascending and descending interneurons. The ganglia consist of two main parts, the peripheral cortex where the somata of the neurons are located (Fig. 1.2A and C) and the neuropiles in the center of the ganglion (Fig. 1.2B and D). The neuropiles are regions where the fibers of neurons make synaptic contacts. It should be noted that most invertebrates neuron somata do not participate in synaptic integration since they are free of direct synaptic inputs and since dendritic- to axonal signal conduction does not involve the cell bodies.

As a model system to investigate the neuroanatomical and neurophysiological foundation of the psycho-hydraulic model of Lorenz, the control of sound production in acridic grasshoppers by the central complex was chosen. Grasshopper sound production is a stereotyped behavior with few choices of species- and situation-specific sound patterns resulting from rhythmic movements of the hind legs against the forewings, a process called stridulation. Grasshoppers use acoustic signals for mate finding, courtship and rivalry Elsner (1994). The neuromuscular excitation patterns for the sound generating hind leg movements are generated by central pattern generators in the metathoracic ganglion, which are connected to the brain via sets of stridulatory command neurons (Hedwig 1994) (Fig. 1.3B). Each of several types of these command neurons activates only one stridulatory pattern for a grasshopper

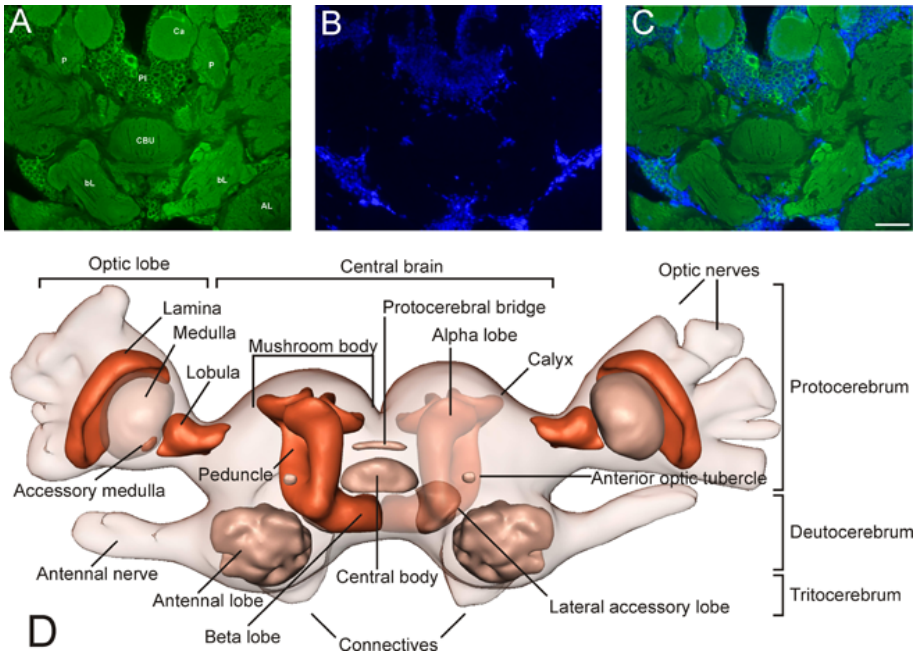


Figure 1.2: In the insect brain the somata are located in the periphery while in the center fibers contact each other in neuropiles. A-C: Frontal sections through the insect brain stained against horse-reddish peroxidase (HRP, A) and DAPI (B). Antibodies against HRP recognize glycoproteins on the surface neurons and DAPI is a fluorescent dye that intercalates between the DNA strands labeling the nucleus of all cells. While cell bodies are restricted to the periphery of the grasshopper brain, fibers can be found in the center where they contact each other in specialized regions called neuropiles. D: 3D-model of the brain of the cockroach *Leucophaea maderae* (kindly provided by Dr. Thomas Reischig) illustrating the major neuropiles and the major brain regions (protocerebrum, deutocerebrum and tritocerebrum). AL, antennal lobe; bL, β-lobe of the mushroom body; Ca, calyx; CBU, central body upper division; P, pedunculus; PI, pars intercerebralis)

species' repertoire (Hedwig and Heinrich 1997). The grasshopper *Chorthippus biguttulus* has been shown to be a suitable model to examine decision making processes through manipulation of different neurotransmitters and their respective second-messenger pathways (Heinrich et al. 2001a;b, Wenzel et al. 2002; 2005).

Recent neurophysiological data from grasshoppers suggested that the central complex (CX), a brain region in the center of the insect brain that has been assigned to motor control (Strausfeld 1999), controls the activity of command neurons that govern the central pattern generators in the third thoracic segment which are responsible for the rhythmic movements of the hindlegs during stridulation (Heinrich et al. 2001a, Wenzel et al. 2002; 2005). This principle organisation is similar in vertebrates, where circuits in the spinal cord are responsible for producing rhythmic motor patterns and modulated by higher centers like the brainstem and cortex (e.g. control of locomotor rhythm generating spinal circuits by descending activation from the mesolimbic locomotor region). Studies in grasshoppers suggest that stridulation is controlled by a balance of excitation and inhibition in the central complex (Heinrich et al. 1998b). In restrained but otherwise intact animals, injection of neuroactive substances into the central complex have been shown to modulate the threshold of grasshoppers to perform stridulation. Activation of muscarinic and nicotinic ACh-receptors promoted sound production (Heinrich et al. 1997; 2001a;b,

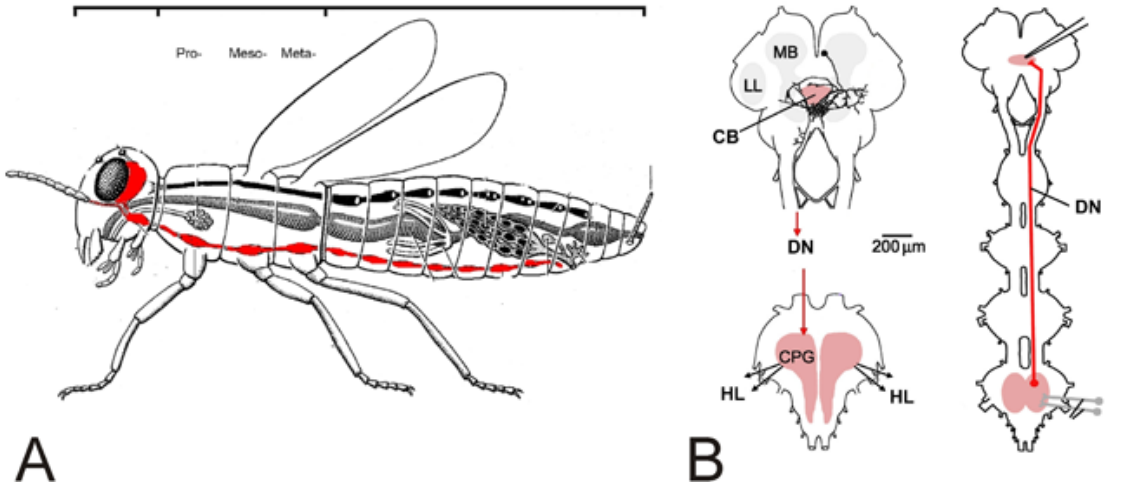


Figure 1.3: Stridulatory movements are generated by central pattern generators located in the third thoracic segment and are under the control of descending brain neurons. A: Schematic sagittal section through the body of an insect showing the location and basic organisation of the insect CNS (depicted in red). The CNS is located at the ventral part of the body and consists of several ganglia that are interconnected through connectives. B: The interneurons of the central pattern generator (CPG) that produce the rhythmic movements of the hind legs (HL) are located in the third thoracic segment. This CPG is under the control of descending interneurons of the brain (DN). These neurons have dendritic arborisations anterior and ventral to the central body (CB). MB, mushroom bodies; LL, lateral lobes.

Wenzel et al. 2002), while GABA and the NO/cGMP-signaling pathway suppressed its performance (Heinrich et al. 1998b, Wenzel et al. 2005). Furthermore, it could be shown by pharmacological experiments that the song of a female activates cholinergic projections into the central complex (Heinrich et al. 2001b, Hoffmann et al. 2007). Song of females are strong stimuli for males since they signal mating readiness of the female (Heinrich et al. 2001b). Therefore, the central body can be regarded as the neuroanatomical correlate to the "fluid storage" of the water closet-model, with the transmitters and second-messengers representing the motivational-fluid, and the calling song of the female representing the weight (Fig. 1.1B).

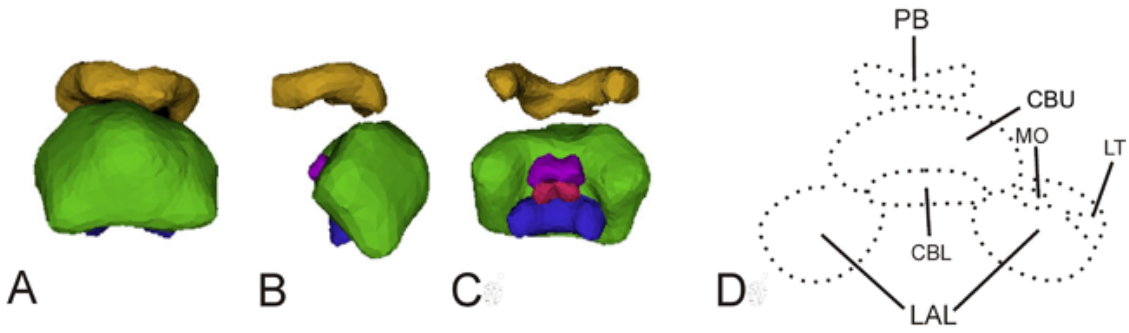


Figure 1.4: The central complex consists of several subunits. 3D-Model of the central complex showing it from ventral (A), lateral (B) and dorsal (C). The central complex consists of three subdivisions, namely the protocerebral bridge (PB, gold), the central body (CB), which is further subdivided into an upper (CBU, green) and a lower (CBL, blue) division and the paired noduli, which can be subdivided into an upper (purple) and a lower unit (red). D: Schematic representations of a frontal section through the central complex. Adjacent to the CB are the lateral accessory lobes (LALs), which are in close connection to the CB. In the LAL two distinct regions can be identified, the lateral triangle (LT) and the median olive (MO). This scheme will be used in the result part to indicate the borders of the CX on histological sections.

The central complex is a midline spanning network of highly structured neuropiles

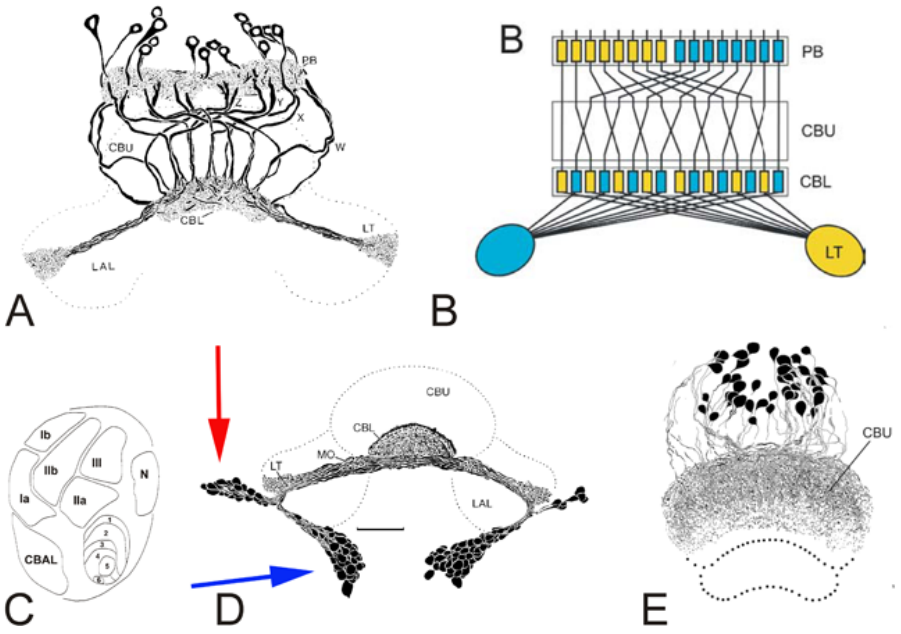


Figure 1.5: The central complex is innervated by three major types of interneurons. A: Columnar neurons intersect the PB and the CB into 16 vertical columns. Their somata are located in the pars intercerebralis. Fibers within the central complex are mainly of post-synaptic character, and in the LALs of pre-synaptic character. The fibers run through specialized fiber tracts (four in each hemisphere called w-, x-, y- and z bundles) and cross to the contralateral brain hemisphere either before entering the CB (at the posterior chiasm) or before leaving the CB to innervate the LAL (from Vitzthum et al. 1998). B: Schematic model of the connection pattern of columnar neurons (from Homberg 2004). C: Sagittal section through the central complex showing the layer like arrangement of the central body. The CBU is divided into four layers (I-III and the anterior lip, CBAL) and the CBL into six layers (layers 1-6) (modified from Homberg 1991 and Müller et al. 1997). D: Tangential neurons innervating the lower division (CBL). The somata of these tangential neurons are located in the inferior-median (blue arrow) and the inferior-lateral protocerebrum (red arrow). The fibers run through the isthmus tract and receive synaptic input in the lateral triangle and the median olive of the LAL. In the CBL they innervate all columns of particular layers. Tangential neurons have their dendrites mainly outside the CX and their axonal endings within the CX (from Homberg et al. 1999). E: Pontine neurons innervating the CBU. These neurons have their somata in the pars intercerebralis and are intrinsic to the CB, connecting different columns within the CB (from Kurylas et al. 2005).

in the insect midbrain. It occupies the center of the insect brain (Fig. 1.2D) and is composed of four interconnected subunits (Fig. 1.4A-D): the protocerebral bridge (PB), the upper (CBU) and lower divisions (CBL) of the central body (CB), and the paired noduli (Williams 1975, Homberg et al. 1987, Homberg 1994). One of the most striking features of the CX is its elaborate organisation. As shown in various insect species, the CX is arranged in fronto-horizontal layers (Fig. 1.5C), which are intersected by eight or sixteen columns (Fig. 1.5B) (Williams 1975, Strausfeld 1976, Hanesch et al. 1989, Homberg 1991, Wegerhoff and Breidbach 1992, Vitzhum et al. 1996, Vitzthum and Homberg 1998). This regular structure results from two classes of interneurons that innervate the central complex, tangential (Fig. 1.5D) and columnar neurons (Fig. 1.5A). Tangential neurons form the basis of central body layers and provide input from the median protocerebrum (mainly the lateral accessory lobes) to all columns that intersect a particular layer. The somata of these neurons are located in four distinct regions of the cortex: the ventro-median protocerebrum, the inferior-median protocerebrum, the inferior lateral protocerebrum, and the pars intercerebralis. The second type are commonly known as columnar neurons, which connect the columns of the protocerebral bridge and the central body

upper and lower division in a regular pattern of ipsi- and contralateral projections. Some send information to the contralateral lateral accessory lobes (LAL), the major input/output neuropiles of the CX. All columnar neurons have their somata in the pars intercerebralis. A third type of central complex neurons are pontine neurons (Fig. 1.5E). These are intrinsic neurons that connect different columns within the central body. Their somata are clustered together with the columnar neurons in the pars intercerebralis.

Aim of this study was: (1) To map the distribution of different neurotransmitters in the central complex that have been demonstrated in previous pharmacological studies to interfere with the cephalic control of stridulation. This should provide a better understanding of the computational operations performed in the central complex. (2) To map the distribution of other neurotransmitter/-modulator systems to provide a framework for further behavioral and physiological experiments. (3) To investigate the effect of neurotransmitters/-modulators whose contribution to the control of sound production was unknown so far.

2 Material and Methods

2.1 Animals

In sommer, adult specimen of the grasshopper *Chorthippus biguttulus* (*Ch.b.*) (L. 1758) were caught in the vicinity of Göttingen, Germany (see appendix for exact locations), and kept separately in the laboratory for up to 3 weeks at 25°C and with a light/dark cycle of 16/8. During the winter months *Ch. b.* were reared from eggs that were collected in the previous summer and kept at 4°C for at least 4 months to induce the diapause that is necessary for normal development. After this, the clutches were transferred to an incubator. The nymphs hatched after ca. 1 week at 26°C and were raised to adulthood on wheat and supplemental food for crickets (Nekton, Pforzheim, Germany) at the same conditions described above. For the experiments I used adult males whose imaginal moult was several days ago, and that sang spontaneously in their cage.

2.2 Pharmacological Brain Stimulation

2.2.1 Drugs

Dopamine, muscarine, SCH23390, flupenthixol, 6-chloro-PB, tyramine, yohimbine, Sodium nitroprusside (SNP), 3-(5′-hydroxymethyl-2′-furyl)-1-benzyl indazole (YC-1) and 3-Isobutyl-1-methylxanthine (IBMX) were obtained from Sigma-Aldrich (Hamburg, Germany). All drugs were dissolved in grasshopper saline made after Clements and May (1974)(see appendix). YC-1 was first dissolved as 100 x stock solution in dimethyl sulphoxide.

2.2.2 Setup and Preparation

For pharmacological experiments animals had to be fixed, so that neuroactive substances could be injected into the central body. Care had to be taken that the legs were still freely movable to allow stridulatory behavior. The grasshoppers were fixed at their pronotum (neckshield) to a holder by using a mixture of colophonium and

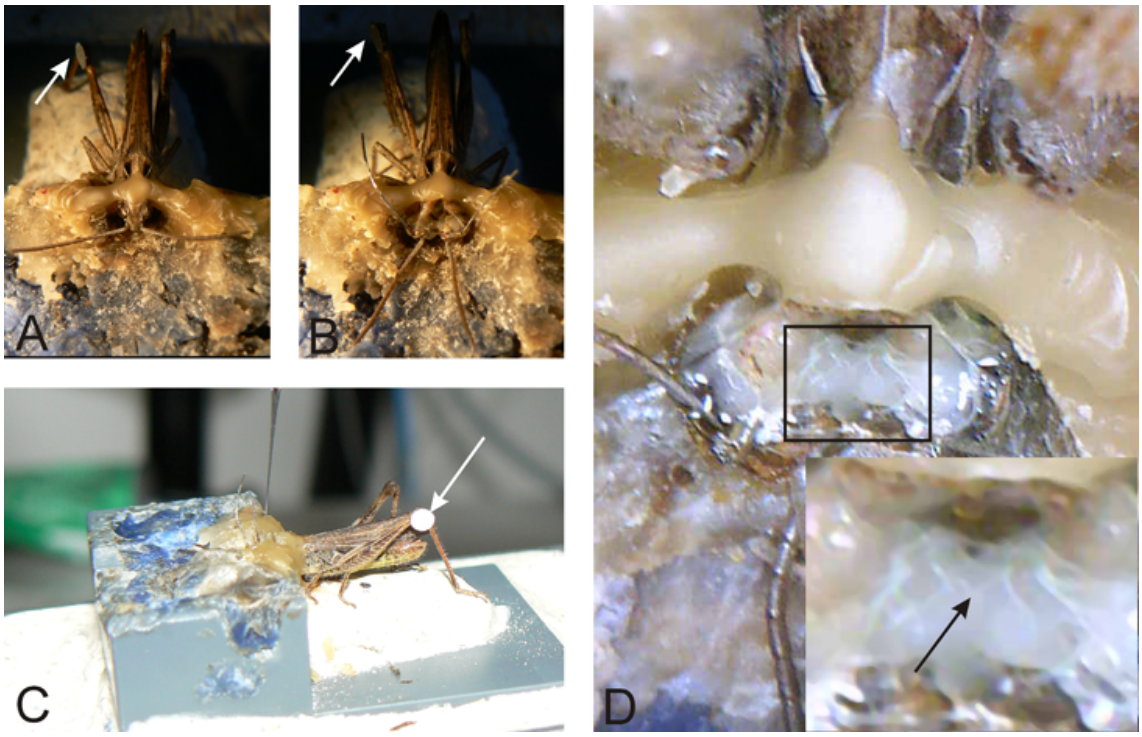


Figure 2.1: Consecutive stages during the preparation. A: The animal is fixed to the holder by using a mixture of colophonium and bee-wax. B: The head capsule has been opened with a razor blade and has been flipped to frontal and ventral. C: An injection cappillary has been inserted from the dorsal surface. White arrows in A,B and C indicate the reflective foil which is used for the recording of the hindleg movements. D: View onto the dorsal surface of the brain. Inset in the lower right corner shows an enlarged view of the region delineated by the black rectangle. The black arrow indicates the spot where the injection capillary is inserted.

bee-wax and the head was connected to the pronotum (Fig. 2.1A). All subsequent steps were performed under microscopic control (Leica MS5, Wetzlar, Germany). The exposure of the brain was achieved through V-shaped incisions of the head-cuticle between the eyes and behind each eye using a razor blade. Subsequently, the area of cuticula surrounding the compound eye was flipped to frontal and ventral (Fig. 2.1B). The cuticle surrounding the eyes was attached to the holder with fine needles. These were inserted at a region of the holder that contained sylgard. After careful removal of tracheae, airsacks and musculature, the brain was exposed. To prevent drying of the preparation, the brain was constantly covered with insect saline (see appendix).

For injections of neuroactive substances double-chamber electrodes (Harvard Instruments, Hollister, MA, USA) pulled under heat (Puller: Nashridge PE-2, London, UK) to a common tip were used. The electrodes were placed into a holder that was attached to a micromanipulator (Leitz, Wetzlar, Germany). The two chambers of the electrode were connected to an injection-pump (PV820, World Precision Instruments, Berlin, Germany), through special connectors, thin plastic tubes and a threeway cock. The injection pump itself was connected to a pneumatic source. Through the threeway cock, pressure could be applied to each chamber separately (Fig. 2.2), enabling the alternative application of two different agents to the same

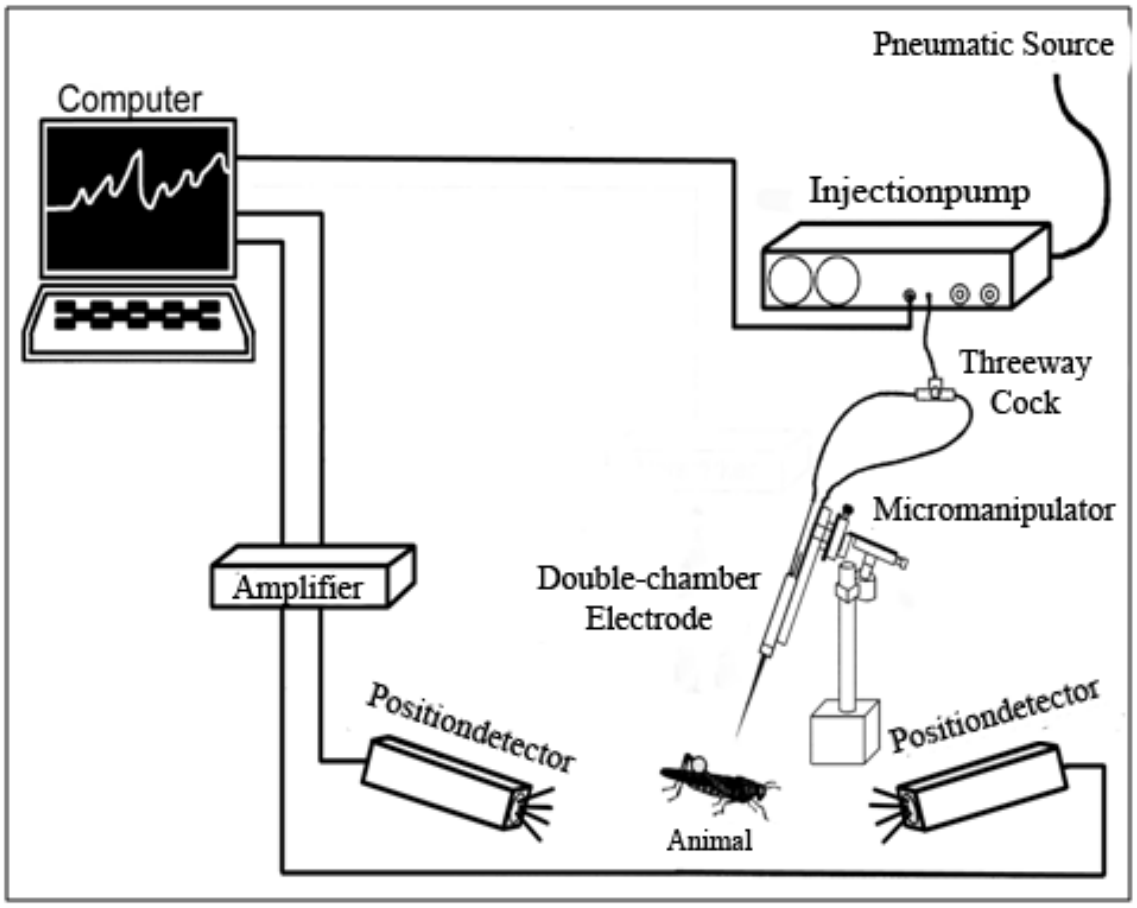


Figure 2.2: Schematic representation of the pharmaco-behavioral setup. The experimental animal is fixed to a holder and the brain is exposed (both not shown). Reflective foils are glued to the hind legs. The injection capillary is attached to a micromanipulator and connected to a pneumatic source. The hind leg movements are recorded by the position detectors and the amplified signals are sent to a PC. Additionally, the injection pulse was recorded.

spot in the brain. At the beginning of the experiment, the tip of the injection capillary had to be broken under visual control (binocular, Leica MS5, Wetzlar, Germany) to yield a diameter of approximately 10 μm. Subsequently the strength and duration of the pressure pulses had to be adjusted, such that to inject the same amount of fluid (ca. 1-3 nl, Heinrich et al. 1997) from both chambers. In order to record the movements of the legs during singing movements, optoelectronical cameras (positiondetectors) were used (Helversen and Elsner 1977). For this purpose, disks of reflective foils (Scotchlite Nr. 7610, Flextex products GmbH, Oldenburg, Germany) with a diameter of 2 mm, were glued (Fixogum, Marabu, Tamm, Germany) to the femura of the hindlegs (white arrows in Fig. 2.1A, B, and C). Light emitted from the positiondetectors was directed to the reflectors and the reflections were registered by a photosensor. The up- and downstrokes of the legs were converted to proportional voltage signal. Additionally injection pulses were registered in a separate channel. The electrical signals were amplified and sent to a personal computer. The analog signals were digitized using an A/D-transformer card (ADA Real Time Devices Inc., State College; USA) and the program TurboLab 4.3 (Stemmer Software, Puchheim, Germany) and stored as dat-files on a PC. The sampling

rate for the AD-transformation was 4 kHz

2.2.3 Pharmaco-Behavioral Experiments

After exposing the brain, the injection cappilaries were impaled into the central body, a region of the brain that coordinates grasshopper sound production (Heinrich et al. 1997; 1998b, Wenzel et al. 2002, Hoffmann et al. 2007). A conspicuous pattern of tracheae on the brain surface of all animals eased the localisation of the correct injection site (Fig. 2.1D).

To test the effect of different substances on the control of sound production I applied several protocols. (A) To see whether a substance may inhibit stridulation, a spot was identified where injections of muscarine reliably induced stridulation. Muscarine was injected at intervals of five minutes, until a uniform duration of singing was stimulated with every pulse. If this was achieved, the experiment was started. Again muscarine was applied every five minutes for several times. Two minutes after the third or fourth pulse, one single pulse from the of the test substance was applied, and its effect on subsequent stimulations with muscarine was monitored. (B) Three protocols were used to test if a substance promoted or even initiated sound production. (B_1) The procedure was very similar to (A). Again muscarine was injected at five minute intervals until a steady singing duration was achieved. The experimental protocol consisted of six injections every five minutes. Three times only muscarine was injected while during the last three pulses both, muscarine and the test substance, were applied simultaneously. (B_2) Once a spot was found at which muscarine reliably induced stridulation I waited for ten minutes and applied nine pulses of the test substance at intervals of 2 minutes. The pause of ten minutes was performed to rule out persistent excitation from previous muscarine injections. It was shown in earlier experiments that the excitatory effect of muscarine can last up to ten minutes (Wenzel et al. 2002). If the animal did not show any singing behavior during the last pulses, a single test pulse with muscarine was applied to see if the animal is still responding to the pharmacological stimulation. If the grasshopper did not sing, the experiment was discarded.

In order to trace the directly stimulated central complex neurons the capillary was positioned at a site where injections of muscarine reliably elicited stridulation, Subsequently a dextran-solution (either coupled to tetramethylrhodamine (TMR), rhodamine green or biotin), was injected to the same site every 5 minutes for three times.

After each experiment, the electrode was removed and one test pulse from each chamber was given, to ensure that none of the chambers became plugged during

the course of the experiment. If the electrode was plugged, the experiment was discarded.

2.2.4 Data Analysis

The software NEUROLAB (Hedwig and Knepper, 1992, Knepper and Hedwig, 1996) was used for visual examination and filtering of the data. The song of *Ch.b.* consists of several song sequences separated by short pauses. The sum of the duration of all individual song sequences released by one stimulation was taken as the total duration of stridulation. To compare the data between different individuals, the singing duration was normalized, setting the longest duration within one experiment to 100 % and the others in relation to this. The statistical analysis was performed using the software STATISTICA (StatSoft GmbH, Hamburg, Germany). For statistical analysis a Friedman-Test was performed, to see if the responses to different stimuli that were consecutively applied to the same site within the brain were different from each other. Given this was found, a Wilcoxon-Test was performed, to identify those responses that significantly differed from each other. The data from all experiments of the same type were pooled. The mean response was calculated and significant differences indicated by asterisks (*, $p<0.05$; **, $p<0.01$; ***, $p<0.005$).

2.3 Cell Culture

Generation of primary cell cultures was performed in a clean bench (Horizontal Laminar Flow Cabinet, Series CLF 475, Clean Air Techniek, Utrecht, Netherlands), previously irradiated with UV-light and desinfected with 70 % ethanol.

Commodities like pasteurpipettes (Brand, Wertheim, Germany), pipettes (Eppendorf, Hamburg, Germany), coverslips (10 mm, Hartenstein, Würzburg, Germany) and bikurs were autoclaved before usage (EL 2540 Benchtop Sterilizer, Tuttnauer, Breda, Netherlands).

Cell culture dishes (Coring Inc., New York, USA) and syringes (1 ml, with needle, Terumo, Leuven, Belgium and 5 ml B. Braun, Melsungen, Germany) as well as the cell culture media (L15 Leibowitz, HBBS, Invitrogen, Karlsruhe, Germany) were ordered as sterile from the distributor. The L15-medium was supplemented with 0.5 % Gentamycin (Sigma Aldrich, Hamburg, Germany) and sterile fetal calve serum (FCS, Sigma Aldrich, Hamburg, Germany) Except for the media, all materials were kept under UV-light after autoclaving until they were used.

Dissection of grasshopper brains was performed outside the clean bench. Animals were anesthetized by cooling to 4°C, decapitated and their brains dissected in a

preparation dish coated with sylgard. During dissection injury of the oesophageus had to be prevented since this usually caused strong contamination of the primary culture.

The dissected brain was transferred into a petri dish filled with modified medium (L15 with 0.5 % gentamycin). Because dissection was performed outside the clean bench the sterility of the medium could not be guaranteed, the brains in culture medium were transported to the clean bench and transferred into a culture dish with sterile medium. To facilitate later dissociation of cells, the nervous tissue was digested in a mixture of medium and collagenase (1 mg/ml, Sigma Aldrich, Hamburg) for 15 minutes at 20°C in an incubator (Hereaus UT 6060 AR Kendro, Hereaus, Hanau, Germany). To stop the reaction and facilitate dissociation of the cell, brains were transferred into calcium- and magnesium-free HBBS-medium. Next, the brains were transferred into 1.5 ml Eppendorf Safe-Lock tubes filled with 500 μl L15 medium. The mechanical dissociation was achieved by repeated suction of the brain into a 100 μl pipette. The tubes were briefly centrifuged (Quick Spin 7000, Süd-Laborbedarf, Gautling, Germany), the supernatant was discarded and the pellet resolved in medium (L15 with 0.5 % gentamycin). Subsequently the suspension was transferred to a round coverslip (10 mm ∅) that was placed in a petri-dish.

To optimize adhesion of the cells, the coverslips were coated with sterile convalin A (ConA, 1 mg/ml, Lectin from Conavalia ensiformis) for 1 h at room temperature prior to the experiments. To ensure that the cells connected to the coverslip the cell suspension was left on the coverslip for one hour, before the petri-dish was filled up with 4 ml of modified medium (L15, with 0.5 %gentamycin and 5% FCS). The culture was kept in the incubator at 29°C.

2.4 Anatomical Studies

The following primary antisera were used.

Table 2.1: Primary antisera

Antigen	Host Species	Conc.	Source
α-mAChR	rabbit	1:200	DB Sattelle
α-GABA	guinea-pig	1:1000	Protos Biotech
α-cGMP	sheep	1:5000	J deVente
α-citrulline	mouse	1:20	G Holstein
α-Dopamine	goat	1:1000	HW Steinbusch
α-ProctR	rabbit	1:200	P Taghert
α-CCAP	rabbit	1:1000	H Diercksen

α-Tyrosine Hydroxylase	mouse	1:500	Diasorin
α-allatostain	mouse	1:20	DSHB
α-allatotropin	rabbit	1:1000	D Nässel
α-LemTRP	rabbit	1:1000	D Nässel

Brains were dissected as described above. Brain tissues were fixed over night at 4°C in 4% paraformaldehyde (PFA) dissolved in 0.1 M phosphate buffer (PB). Brains were embedded in a mixture of albumine/gelatine, postfixed at 4°C in 4% PFA and sectioned with a vibratome (Leica Vibracut VT 1000, Leica, Wetzlar, Germany) into slices of 30-50 μm. Sections were rinsed over night in 0,1 M phosphate buffered saline (PBS) containing 1% Triton X-100 (PBST) to permeabilize the cell, to increase permeability of cell membranes for the antibodies. For citrulline and dopamine immunostaining, brains were fixed in 4% PFA and 1.5% glutaraldehyde for 3 hours and incubated directly after sectioning for 10 minutes in 0.1 M sodiumborohydride (in PBS) to reduce glutaraldehyde-induced autofluorescence. To decrease background staining caused by unspecific binding of the antisera, sections were blocked in a solution containing 0.25% BSA (omitted in cGMP stainings) and 5-10% normal goat/donkey (dependent of the host species of the secondary antibody) serum dissolved in PBST prior to incubation with primary antisera. Primary antisera were incubated at 4°C for 3 days on a rocking table. For cGMP immunostaining, brains were incubated prior to fixation in 10^{-2} M of the NO-donor sodium nitroprusside (SNP, Sigma) and 5 x 10^{-4} YC-1 (Sigma) dissolved in grasshopper saline to enhance NO-stimulated accumulation of cGMP via soluble guanylyl cyclase activation (for detailed protocol see Wenzel et al. 2005).

The following secondary antibodies were used:

Table 2.2: Secondary antisera

Antigen	Host Species	Conc.	Source
α-rabbit Alexa488	goat	1:300	Molecular Probes
α-rabbit Cy2	goat	1:100	Jackson Immunoresearch
α-rabbit Alexa633	goat	1:300	Molecular Probes
α-rabbit Alexa555	donkey	1:300	Molecular Probes
α-mouse Alexa488	goat	1:300	Molecular Probes
α-mouse Cy3	goat	1:100	Jackson Immunoresearch
α-guinea-pig Cy2	donkey	1:100	Jackson Immunoresearch
α-guinea-pig Alexa633	goat	1:300	Molecular Probes
α-goat Cy3	donkey	1:100	Jackson Immunoresearch
α-sheep Cy	donkey	1:50	Jackson Immunoresearch

α-sheep Alexa633	donkey	1:50	Molecular Probes

The following steps were performed in darkness to prevent bleaching of the samples. Sections were incubated over night at 4°C with the secondary antibodies and subsequently washed several times in PBST, transferred to a 1:1 mixture of PBS and glycerol (Sigmal Aldrich, München, Germany), and mounted on slides for microscopic analysis. Glycerol was taken as a mounting medium, because it has a similar refractive index as the coverslips (glycerol = 1.474; coverslips = 1.52), to avoid refractive index mismatch. For control experiments the same steps as described above were performed, but no primary antibody was added.

2.4.1 Tracing

To identify putative neurons which are involved in the control of stridulation, I co-applied biotin-coupled dextranes to the same site where muscarine reliably induced stridulation. In earlier studies it could be shown, that dextranes are taken up by post-synaptic sites in the CNS of locusts (Heinrich et al. 1998a, Lakes-Harlan et al. 1998). The brains were fixed over night in 4% PFA and the subsequent steps were performed as described above. To visualize neurons that incorporated the dextranes, the sections were labeled with streptavidin Alexa488 (1:1000 Molecular Probes, Hamburg). For double labeling with mAChR, the same antisera as described above were used. In order to label pharmacologically stimulated central complex neurons for subsequent identification in dissociated cell culture I injected a dextrane coupled to tetramethylrhodamine (TMR). In cell culture, central complex neurons could be distinguished from other brain neuron by their fluorescence.

2.4.2 Immunocytochemistry on Primary Cell Cultures

Cell cultures were fixed for 1h in 4% PFA. Cultures were afterwards washed in PBS and permeabilised in PBS containing 0.1% Triton X-100 (PBST 0.1%). To reduce non-specific background staining, cell cultures were blocked using a solution of 10% normal-serum (derived from the host species of the secondary antibody) and 0.25% bovine serum albumine (BSA) dissolved in PBST 0.1%. Staining against mAChR's was performed using the same antibodies as described above, but using different concentrations (1:500 of the primary and 1:1000 of the secondary).

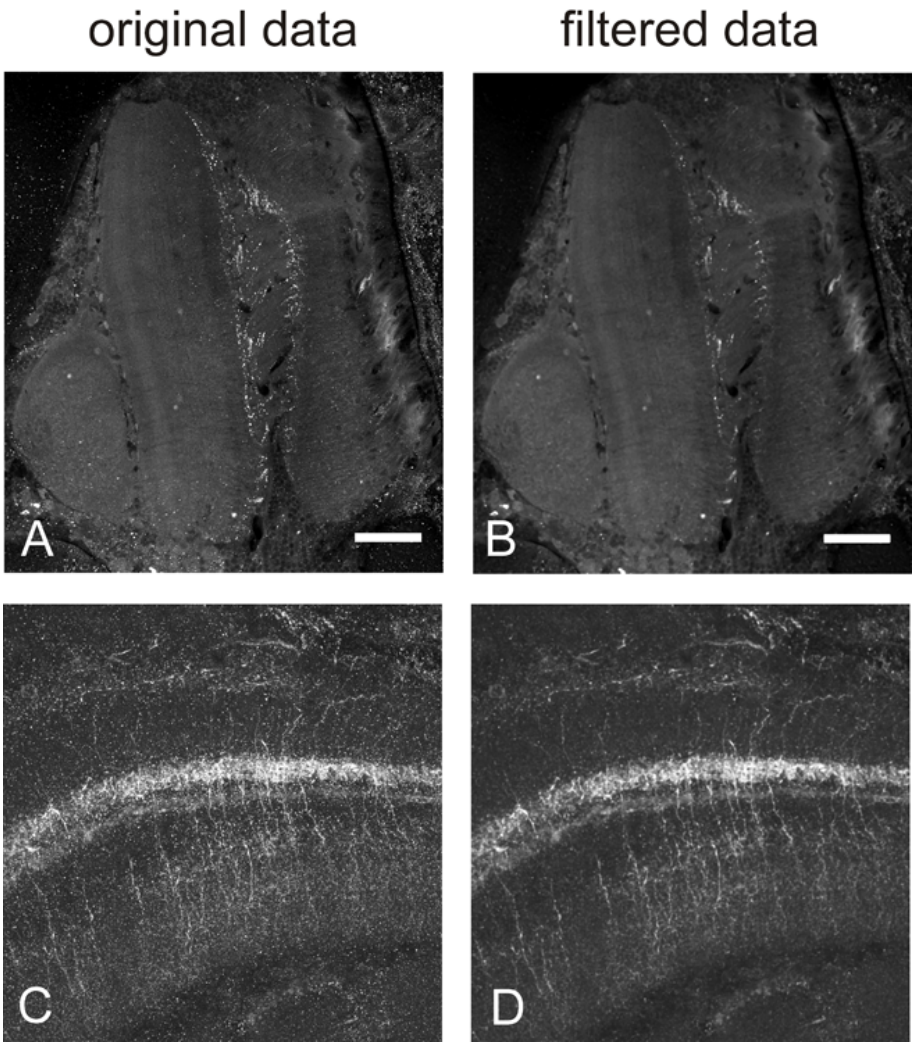


Figure 2.3: Examples for the criteria used to apply a median filter. A and B: Staining of the proctolin receptor in the optic lobes. C and D: Staining of tyrosine-hydroxylase (TH) in the optic lobe. For TH a clear improvement can be seen after application of the median filter in which a clear gain of information is visible (fibers running through the medulla be traced better). Application the filter to an image obtained for the proctolin receptor on the other hand lead to a loss of information. In this case, the punctate staining had a similar size as the noise in the image.

2.4.3 Data Analysis

The terminology for brain structures follows Strausfeld (1976). Central complex subdivisions are named according to Homberg (1991; 1994) and Müller et al. (1997). Positional information is given with respect to the body axis of the animal. Images were obtained with a Leica confocal laser scanning microscope (Leica DMRE, TCS SP2, Leica Microsystems, Heidelberg), equipped with an argon- (488 nm) and two helium/neon-lasers (543 nm and 633 nm respectively). For colocalisation studies on fibers, specimen were imaged with the objective that provided the highest lateral resolution (in our case a 40x oil immersion objective with a NA of 1.25). The lateral resolution is determined by Abbes Law and depends on the wavelength of the emitted light and the numerical aperture of the objective ($0.4\times\lambda_{em}/\mathrm{NA}$ for the lateral resolution and $1.4\times\lambda_{em}/\mathrm{NA}^2$ for the axial resolution, calculations were performed using the λ_{em} for Alexa633/Cy5, which is 648 nm, which is the longest

and therefore the limiting factor for the optical resolution). The voxel size was set to an optimal value (90 x 90 x 250 nm) according to the Nyquist theorem (Oppermann et al., 1983), meaning that the smallest resolvable unit was sampled at least twice. Subsequent image processing included first an adjustment of brightness and contrast achieved by a histogram stretching and a background substraction with a rolling ball radius of 50 pixel. Second, to reduce noise a median filter with a kernel radius of 1-2 pixels was applied. Whether this filter was applied or not was decided on the basis of visual inspection of the result (for an example of the criteria see 2.3). All images were processed with the ImageJ software (developed at the U.S. National Institutes of Health and available at http://rsb.info.nih.gov/ij/). Colocalisation of fibers (thickness of around 1 μm was measured by a distance based colocalisation analysis (Bolte and Cordelières 2006) using the JACoP-plugin. In this method, the centroids (centers of gravity) of fluorescent structures in two channels are compared. Strucures are considered as colocalised, if the distance between the centroids of the different channels are below the optical resolution. The advantage of this method is that the calculations are performed in three-dimensional space, ruling out the possibility that light emitted from structures outside the focal plane causes false positive colocalisation results. Images shown in the results part are single optical slices in which colocalised pixel are highlighted in white.

3 Results

This study was intended to provide detailed information about the neurochemical organisation of a decision making neuropile in the brain of an invertebrate in order to complement and understand the mechanisms that underly the selection and coordination of situation specific behavior. As a model system we used the acoustic communication of the grasshopper *Chorthippus biguttulus*, which is controlled by the central complex in the brain. Immunocytochemistry was performed to describe the distribution of transmitters, receptors and intracellular signals in the central complex whose contribution to the control of sound production has been determined in previous pharmaco-behavioral studies (Heinrich et al. 2001a;b, Wenzel et al. 2002; 2005, Hoffmann et al. 2007). In addition it was also stained against neuropeptides, to see if they are coexpressed with previously identified transmitters and therefore may modulate their impact on information processing in the central complex. Furthermore, additional pharmaco-behavioral experiments were performed, to test other transmitter systems for potential contribution to the control of gasshopper sound production. And, at last, I conducted tracing experiments, to identify the neurons that are directly affected during pharmaco-behavioral studies by injections of pharmacological agents.

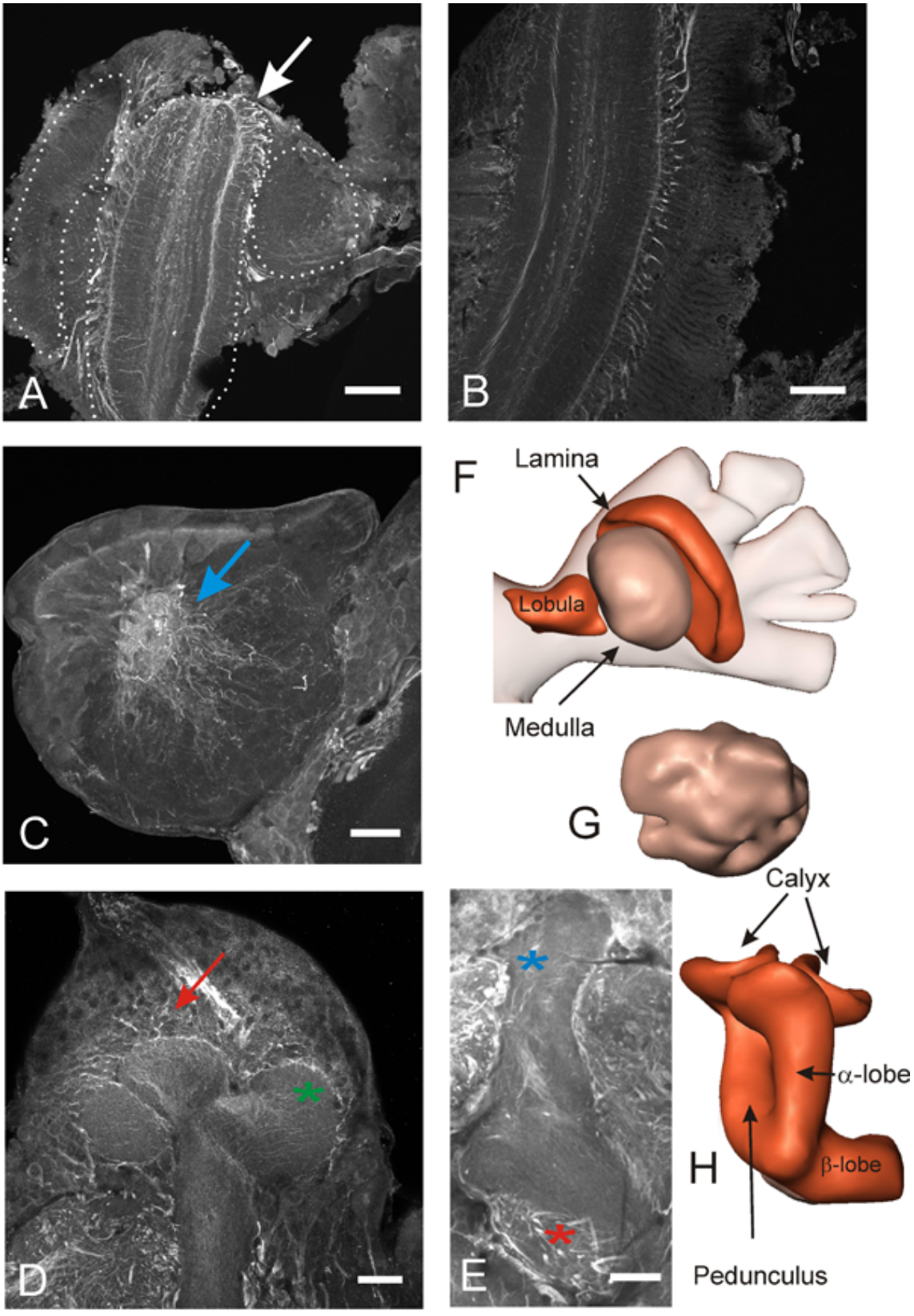
3.1 Immunocytochemistry

3.1.1 Muscarinic Acetylcholine-Receptors (mAChRs)

Acetylcholine is the principle excitatory transmitter in the insect CNS. It acts through two types of receptors, the nicotinic receptor (nAChR), which is ionotropic and the muscarinic receptor (mAChR), which is a G-protein coupled receptor that activates a second-messenger cascade. Repeated injections of the mAChR agonist muscarine into the central body have been shown to reliably induce singing behavior.

3.1.1.1 General distribution of mAChRs in the grasshopper brain

For detecting mAChRs in the brain of *Ch.b.*, a polyclonal antibody generated against a mAChR cloned from was used *D. melanogaster* (Blake et al. 1993). The specificity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Medulla
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Calyx
α-lobe
β-lobe
H
Pedunculus

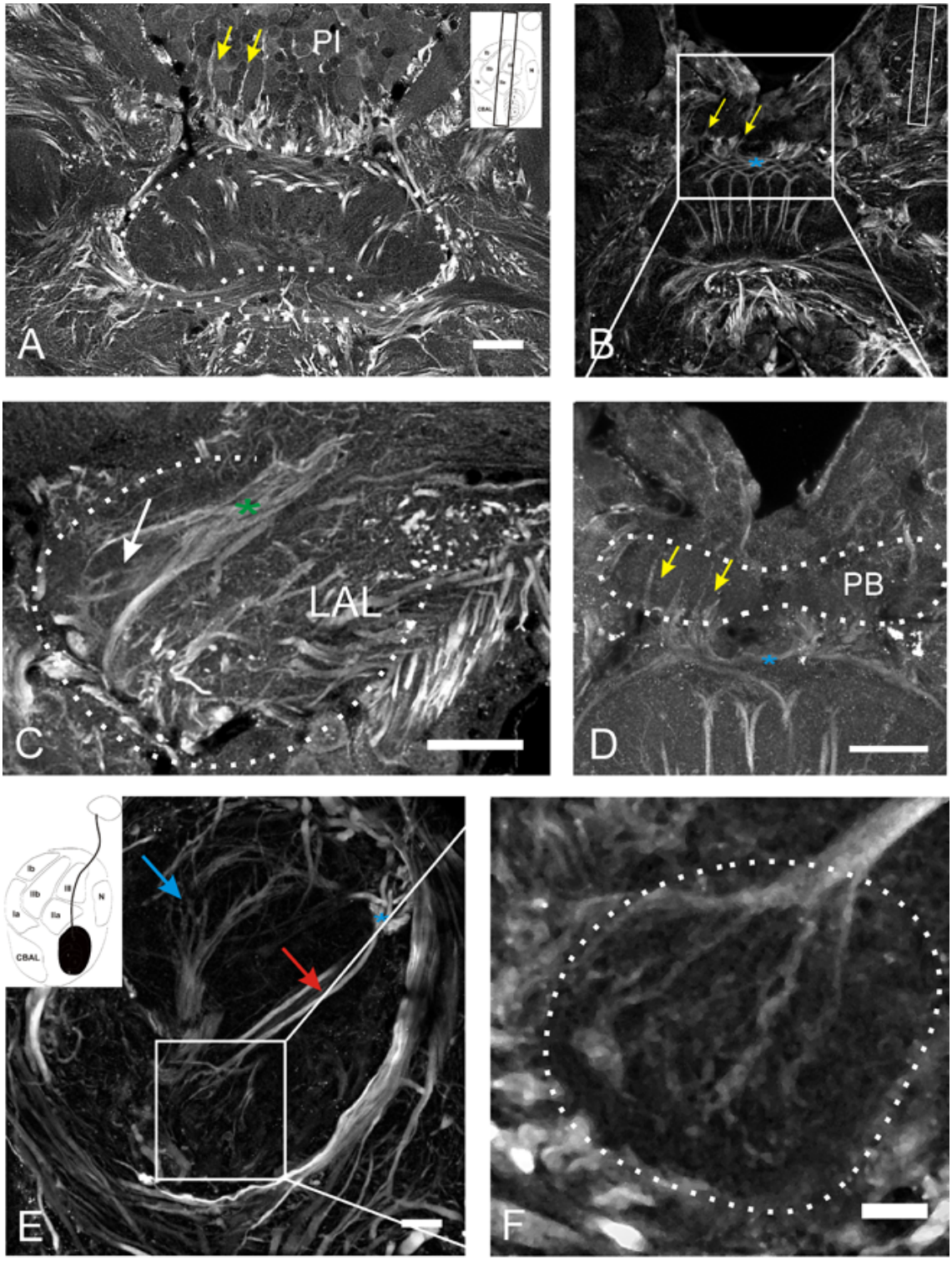
of this antibody and its applicability in *Ch.b.* has been demonstrated by western blot analysis (Hoffmann et al. 2007). To further validate the specificity of this antiserum, the staining pattern in the grasshopper brain (Fig. 3.1) was compared with the expression in the mAChRs in the brain of *D. melanogaster*. Strong immunostaining was detected in antennal lobes (AL) and optic lobes (OL), staining of less intensity could be observed in the mushroom bodies (MB).

The AL (Fig. 3.1G for a schematic representation) is a sphere shaped part of the insect brain which receives sensory input from antennal olfactory receptor neurons (ORNs). The AL consists of several glomeruli, spheroidal neuropilar structures housing the synaptic contacts between the ORNs and the AL interneurons. These glomeruli are arranged in one or two layers around a central fibrous core. In locusts, these glomeruli are not individually distinguishable. Staining in the AL was found in local neurons, whose somata were located around the AL (Fig. 3.1C). The staining was most prominent in fibers making up the central fibrous core (indicated by blue arrow in Fig. 3.1C).

The mushroom bodies are paired structures in the midbrain (protocerebrum) of insects (Fig. 3.1H). They consist of three main parts, the calyx, the pedunculus and two lobes (α and β), which are made up by the intrinsic Kenyon cells (KC). These cells have their dendritic regions in the calyx, where they receive their input mainly from projection neurons of the AL. The axons of the KCs run through the pedunculus and terminate in the lobes (either α or β), where they make synaptic contacts with extrinsic neurons that connect the MBs with surrounding brain areas. Weak staining could be detected in the somata of the KCs (red arrow in Fig. 3.1D) as well as in the fibers that innervate the calyces (green asterisk in Fig. 3.1D) and run down the pedunculus (blue asterisk in Fig. 3.1D) where they terminate in the α-lobe (red asterisk in Fig. 3.1E).

The optic lobes are the visual centers of the insect brain. They consists of three major neuropiles, the lamina (1st order visual neuropile), the medulla (2nd order) and the lobula (3rd order) (Fig. 3.1F). Staining could be detected in all parts of the optic lobe, but most strongly in the medulla (Fig. 3.1A and B). The medulla consists of several layers which are innervated by monopolar cells of the visual system and mAChRs can be detected in monopolar cells innervating the medulla.

Figure 3.1: General distribution of mAChR in the grasshopper brain. A and B: Frontal section through the optic lobe. Staining could be detected in several layers of the medulla (white arrow). Only sparse staining could be found in the lobula while lamina was free of mAChR-ir. C: Frontal section through the antennal lobe. Intense immunostaining could be detected in the inner core neuropile (blue arrow). D and E: Frontal section through the mushroom bodies. Faint immunoreactivity could be detected in the calyx (green asterisk in D) as well as in somata of the kenyon cells (red arrow in D). Additional staining could be detected in the pedunculus (blue asterisk in E) and one column of the β-lobe(red asterisk in E). F-G Schematic 3D-representation of the described brain regions (courtesy of Dr. Thomas Reischig).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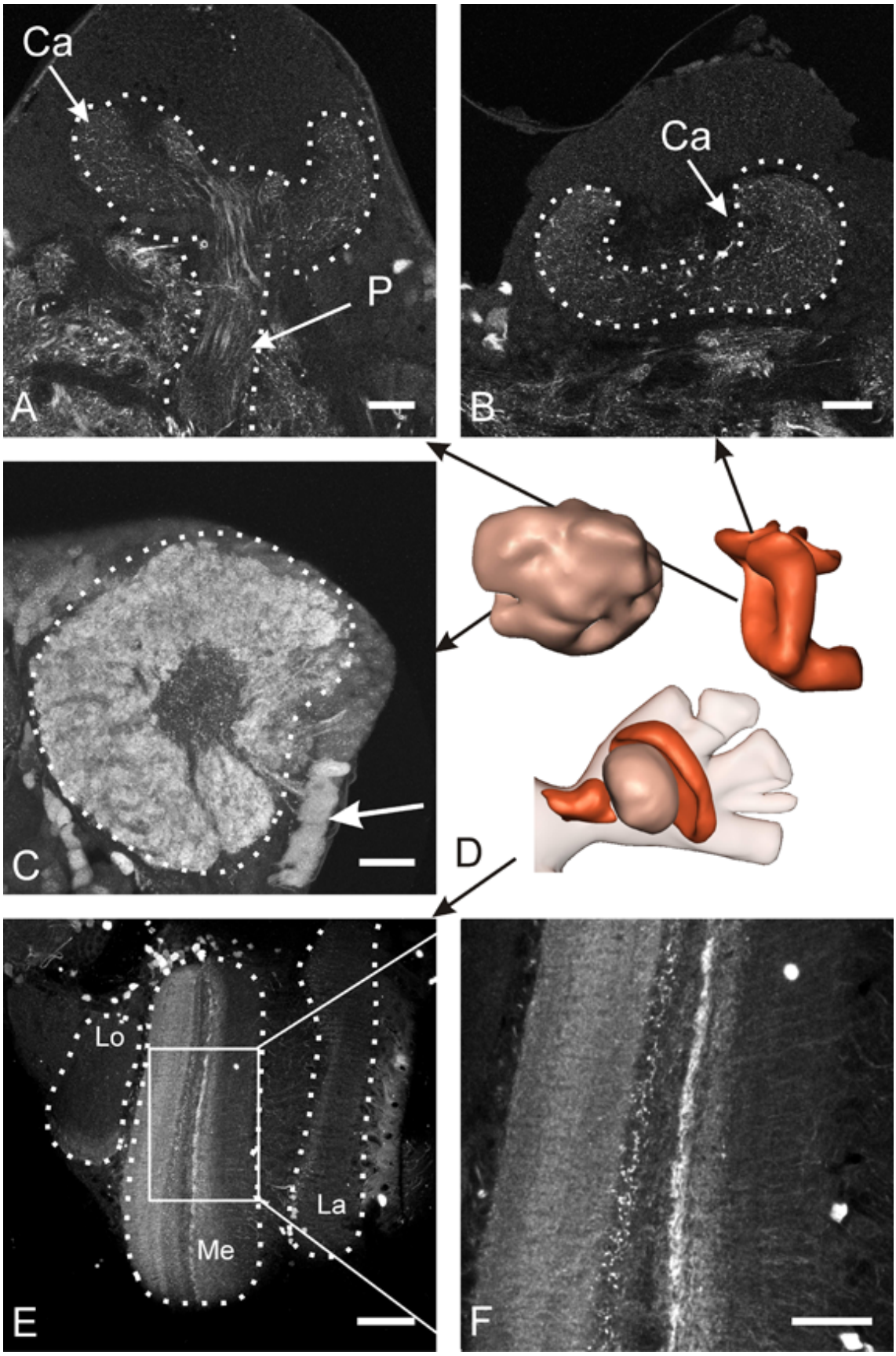
3.1.1.2 Distribution of mAChRs in the central complex

mAChR immunoreactivity in the central complex (Fig 3.2) was mainly observed in two types of columnar neurons that connected the central complex with the lateral accessory lobes. The weakly stained somata of both types of neurons were located in the pars intercerebralis (PI) (Fig. 3.2A) a cortex region that lies dorsal to the protocerebral bridge (PB). MAChR expressing neurons gave rise to a number of thin neurites within the protocerebral bridge (yellow arrows in Fig. 3.2A, B and D). From the bridge, the neurons projected as large diameter fibers via four pairs of fiber bundles, the w-, x-, y- and z-bundles (Williams 1975) through the posterior chiasm (indicated as blue asterisk in Fig. 3.2B, D and E), at which half of the fibers crossed to the contralateral hemisphere before entering the central body. One fiber type (MR1, blue arrow in Fig. 3.2E) was passing through layer I of the upper division and projected dorsally along the anterior border of the CBL. As visualized in sagittal sections through the central complex (Fig. 3.2E and F), the other type of mAChR immunopositive fibers (MR2) passed as part of the posterior vertical bundles (indicated by red arrow in Fig. 3.2E) (Williams 1972) through layer III of the central body upper division and formed arborizations that innervated the lower division with smooth endings (Fig. 3.2F, for a schematic representation of the projection pattern see inset in Fig. 3.2E). From the central body, the fibers were projecting to the contralateral LAL. The fibers projected through the LAL as part of the isthmus tract (green asterisk in Fig. 3.2C) and terminated in the lateral triangle of the LAL (indicated by white arrow in Fig. 3.2C).

Control experiments in which the primary antibody was omitted showed no staining in the described regions (see appendix).

Figure 3.2: Distribution of mAChR in the central complex. A-D: Frontal sections through the central complex, insets in A and B and indicate the section plane through the central complex. MAChR-ir is restricted to columnar fibers whose somata are located in the pars intercerebralis (PI). These neurons sent their small neurites into the protocerebral bridge (yellow arrows in A, B and D). The main fibers ran as large diameter neurites via four pairs of fiber bundles, the w-, x-, y- and z-bundles through the posterior chiasm (PCh, indicated by blue asterisk in A, B, D and E) between the protocerebral bridge and the central body and innervated single columns of the lower division. The fibers run to the contralateral lateral accessory lobe via the isthmus tract (indicated by green asterisk in C) and seem to terminate in the lateral triangle (indicated by white arrow in C) E: Sagittal section of the central body. Two types of columnar fibers can be distinguished. One type (indicated by blue arrow) runs through layer I of the CBU and passes along the anterior border of the CBL , while the other type (indicated by red arrow) runs through layer III as part of the posterior vertical bundle and innervates the CBL (inset describes the projection and innervation pattern of the second type). F: Sagittal section of the CBL. The arborization pattern in the lower division does not seem to be restricted to a certain layer but rather extends diffusely throughout the entire CBL. The smooth appearance of arborisation indicates that they are of post-synaptic character. LAL, lateral accessory lobe; PB, protocerebral bridge; PI, pars intercerebralis. Scale bars = 50 μm in A, B, C and D; 20 μm in E; 10 μm in F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3.1.2 GABA

GABA is the principle inhibitory neurotransmitter in the insect brain. Its distribution in the brain has been studied in various insect species. When injected into the central body of a grasshopper during stridulation it causes a fast and short lasting inhibition of this behavior (Heinrich et al. 1998b).

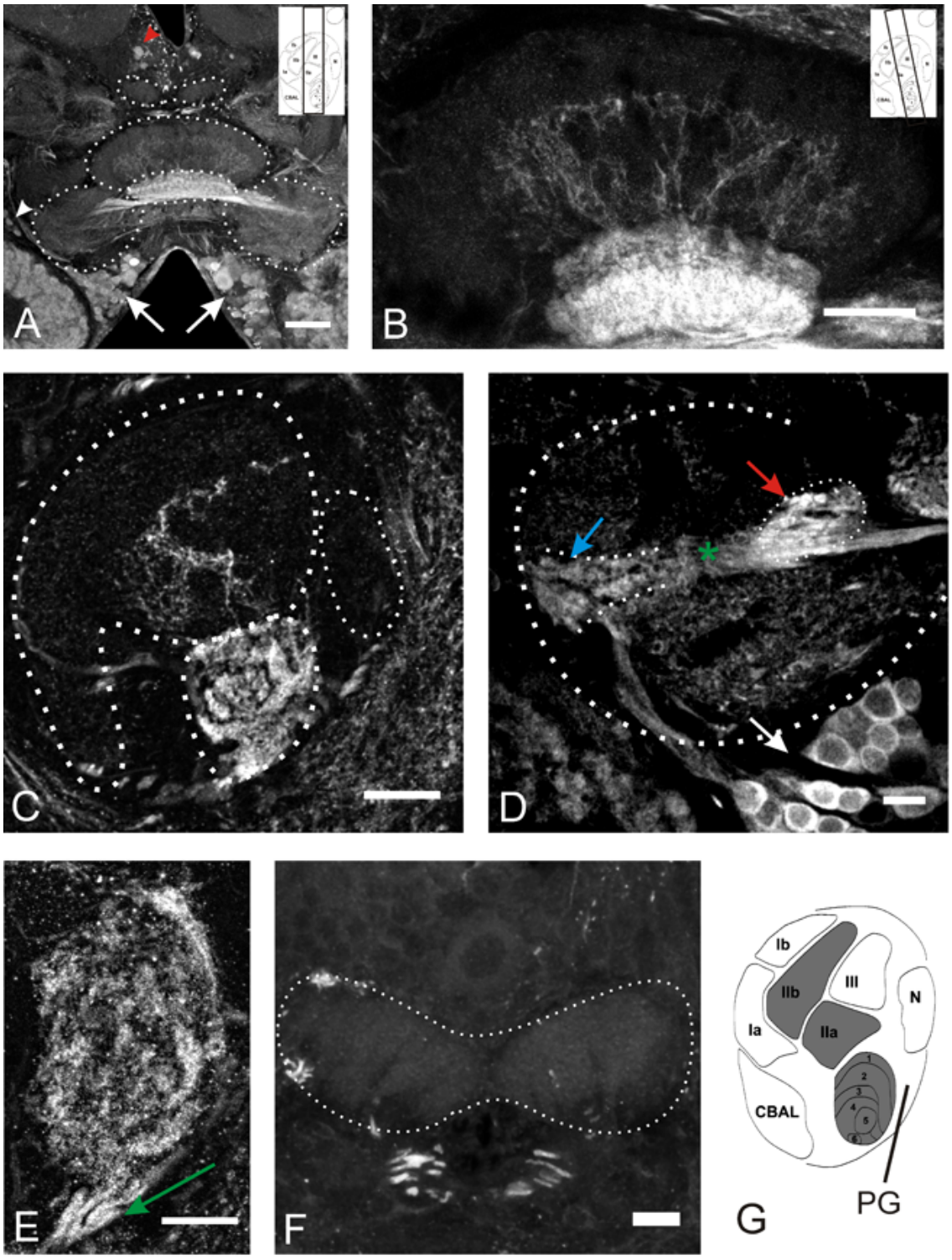
3.1.2.1 General distribution of GABA in the brain

The distribution of GABAergic cells in the brain of *Ch.b.* (Fig. 3.3), is very similar to that of other insect species. (Schäfer and Bicker 1986, Homberg et al. 1987, Meyer et al. 1986, Hanesch et al. 1989, Blechschmidt et al. 1990, Becker and Breidbach 1993, Strambi et al. 1998, Homberg et al. 1999). GABA can be found in all neuropiles of the optic lobes (Fig. 3.3E and F). Most intensive staining was observed in the medulla (Fig. 3.3F), while lamina and lobula showed weaker staining. Strong immunostaining was detected in local neurons of the AL (Fig. 3.3C) that innervate the glomeruli. Additionally, strong and distinct staining was found in the calyces of the mushroom bodies (Fig. 3.3A and B).

3.1.2.2 GABA in the central complex

Strong staining against GABA could be detected in the central complex (Fig. 3.4). The CX is strongly innervated by bilateral pairs of GABA immunoreactive tangential neurons, with their somata in the inferior median protocerebrum (white arrows in Fig. 3.4A and D). Additionally, a small number of neurons is located more laterally at the border to the inferior lateral protocerebrum (white arrowheads in Fig. 3.4A). The entire lower division of the central body is densely innervated with GABAergic arborisations, while in the upper division only layer II contains sparse GABAergic neurites (Fig. 3.4A, B and C). The fibers of these neurons run through the isthmus tract (green asterisk in Fig. 3.4D) and enter the CB via the posterior groove (PG, indicated by green arrow in Fig. 3.4E). The staining pattern is virtually the same as already described for the *S. gregaria*, with the exception that in *Ch. biguttulus* only layer II of the CBU is supplied with GABAergic fibers and not also layer I (Homberg et al., 1999). Homberg et al. (1999) described sidebranches with knob-like appearance in the lateral triangle and the median olive of the LAL in the locust

Figure 3.3: General distribution of GABA in the grasshopper brain. A and B: Frontal brain section showing GABAergic fibers in the calyces (Ca) of the mushroom bodies. Strong immunoreactivity was also be detected in fibers innervating the pedunculus (P in A). Strong immunoreactivity could be found in local interneurons of the antennal lobe (cell bodies indicated by arrow), that innervated all glomeruli of the antennal lobe. D: Schematic 3D-models of the described brain structures. Arrows point to the section of the respective structure. E and F: Frontal section through the optic lobe showing strong immunoreactivity in the medulla (Me), while the lamina (La) and the lobula (Lo) exhibit only weak immunostaining.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S.gregaria, which can detected in our preparations too (Fig. 3.4D, LT indicated by blue arrow, MO indicated by red arrow). No GABA immunoreactive neurites could be detected in the protocerebral bridge and the noduli (Fig. 3.4C and F). Müller et al. (1997) distinguished five different types of tangential neurons in *S. gregaria* that innervated the lower division on the basis of soma position and innervation of the CBL. In comparison to that study, GABA immunoreactive neurons of *Ch. biguttulus* most likely belong to the types TL2, TL3 and TL4. The two other types of tangential neurons described in that study had their somata in the ventro-median protocerebrum and the pars intercerebralis (PI). Although we also detected GABA positive neurons in the PI (red arrowheads in Fig. 3.4A), their fibers did not enter the central body, but seemed to pass posteriorly to it.

Control experiments in which the primary antibody was omitted showed no staining in the described regions (see appendix).

3.1.3 The NO/cGMP-system

3.1.3.1 Nitric oxide

Since its discovery as a neurotransmitter, nitric oxide has been mapped in various insect species (Elphick et al. 1993; 1995; 1996a, Elphick 1997, Bicker and Hähnlein 1995, Müller 1994, Müller et al. 1997, O'Shea et al. 1998, Ott and Burrows 1998; 1999, Bullerjahn and Pflüger 2003, Bullerjahn et al. 2006, Kurylas et al. 2005). Injections of the NO-donor SNP into the central body of *Ch.b.* have been shown to inhibit singing behavior (Wenzel et al. 2005). To label neurons of the central complex that pontially mediate this inhibition, antibody stainings against citrulline were performed. Citrulline is generated as a side-product during nitric oxide (NO) formation (Fig. 3.5) and its accumulation in neurons is regarded as a correlate for recent activity connected to NO release.

Figure 3.4: Distribution of GABA in the central complex. A and B: Frontal sections showing the midbrain (A) and the central body (B). The most prominent staining can be seen in the central body (CB). While the entire lower division is GABA positive, only parts of the upper division contain GABA. The somata of these fibers are located in the inferior-median protocerebrum (white arrows in A and D) and in the inferior lateral protocerebrum (arrowheads in A). C and E: Sagittal section of the central body. Staining in the upper division is restricted to layer II, while the other layers contain no GABA. The lower division is evenly stained. GABA positive fibers entering the central body could be detected in the posterior groove (green arrow in E) D: Frontal section showing the lateral accessory lobe. Fibers, originating from cells in the inferior median protocerebrum run through the isthmus tract (indicated by green asterisk) before they enter the central body. Knob-like shaped staining could be found in the lateral triangle (blue arrow). Additionally, ramifications could be found in the median olive (red arrow). F: Frontal section through the protocerebral bridge , showing that it was free of label. G: Schematic drawing of a sagittal section through the CB. Regions highlighted in gray contain GABA positive fibers (modified from Homberg 1991 and Müller et al. 1997). Scale bars = 100 μm in A; 50 μm in B; 40 μm in C; 20 μm in D, E and F

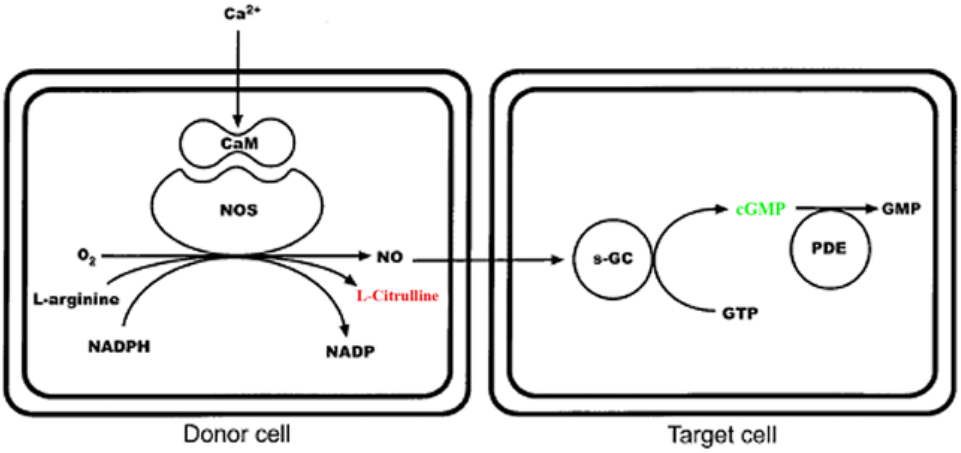


Figure 3.5: Neuronal activity in the NO donor cell leads to the influx of Ca^{2+}, which stimulates via calmodulin (CaM) the nitric oxide synthase (NOS) enzyme. NOS catalyzes the conversion of arginine into citrulline, which is formed stoichiometrically with NO and can therefore be regarded as a specific marker for neurons which have actively produced NO prior to fixation. In the target cell NO binds to a heme moiety in soluble guanylyl cyclase (s-GC), resulting in the stimulation of the enzyme and consequent elevation of cGMP concentration. cGMP is hydolyzed by phosphodiesterases (PDE). sGC-expressing target cells can be identified by immunocytochemistry with specific antisera against cGMP. Modified from Bicker (2001)

3.1.3.2 General distribution of citrulline in the brain

Anti-citrulline immunocytochemistry in *Ch. biguttulus* brains (Fig. 3.6) labeled subsets of nitric oxide synthase expressing and NADPH diaphorase positive neurons previously described in the locust *S. gregaria* (Kurylas et al. 2005) and *Ch. biguttulus* (Wenzel et al. 2005). For example, strong immunostaining could be detected in monopolar cells of the visual system (Fig. 3.6B) and in local neurons of the AL (white arrow in Fig. 3.6A). In contrast, immunostaining in the mushroom bodies (Fig. 3.6C and D) was either faint or not existing.

3.1.3.3 Citrulline in the central complex

Prominent citrulline-ir could be detected in the CBU (Fig. 3.7). Citrulline immunoreactive fibers emerged from somata in the anterior pars intercerebralis (white arrows in Fig. 3.7A, B, D and E) and the ventro-median protocerebrum (red arrows in Fig. 3.7A) to innervate the upper division of the central body. All other central complex neuropiles were entirely free of citrulline-associated labeling. Sagittal sections (Fig. 3.7E) revealed that citrulline accumulation was restricted to layers II and III of the upper division, whereas layer I contained no detectable immunofluorescence. Citrulline immunopositive neurons included pontine (CT1) and probably also tangential neurons (CT2).

Fibers of pontine neurons run through the posterior chiasm (red asterisk in Fig. 3.7B, D, and E) to innervate columns of other CBU layers. Fibers connecting different columns of CBU run through the posterior face (blue arrowheads in Fig. 3.7E) (Boyan et al. 1993). We were not able to distinguish if either of these neurons

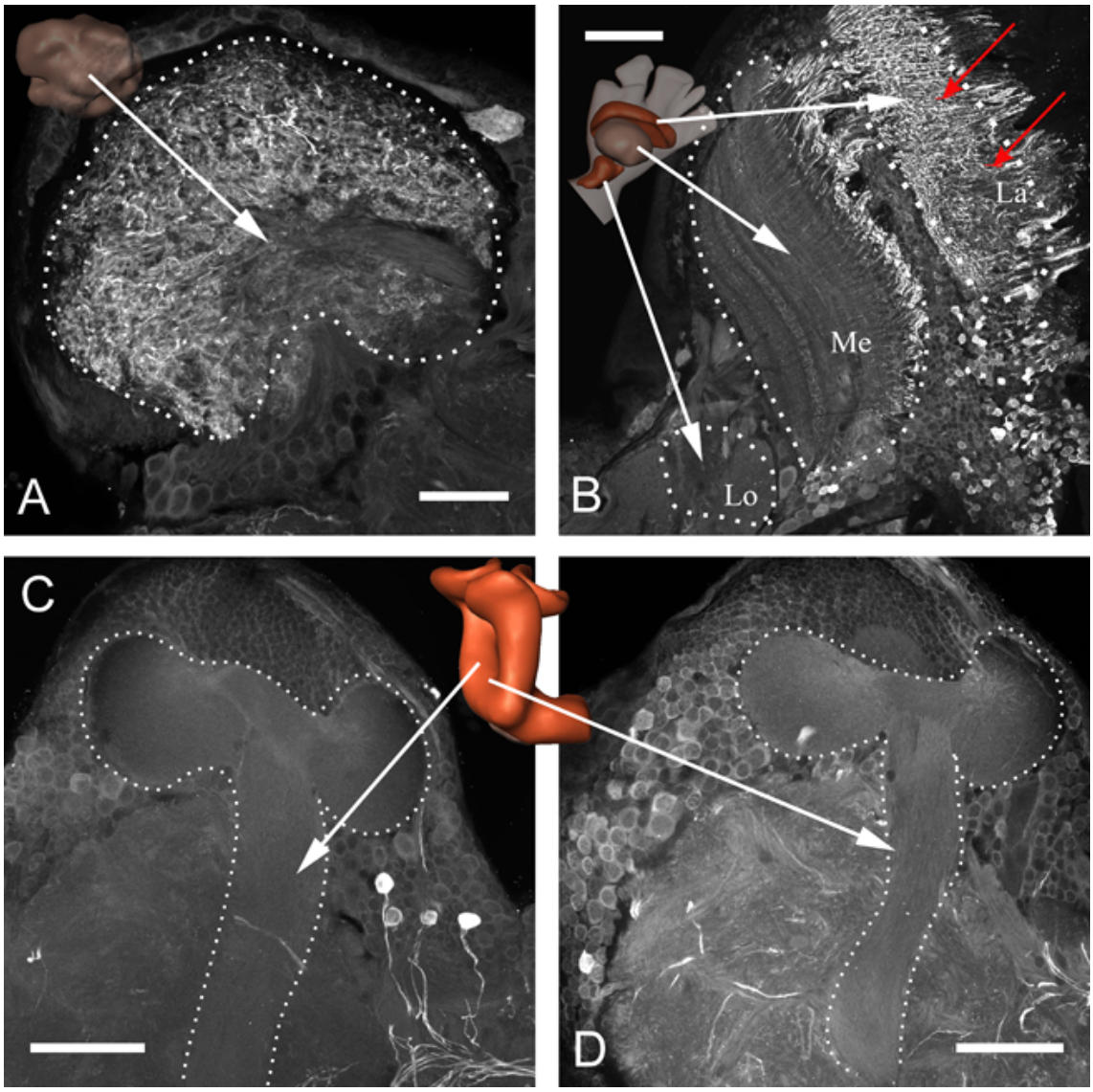
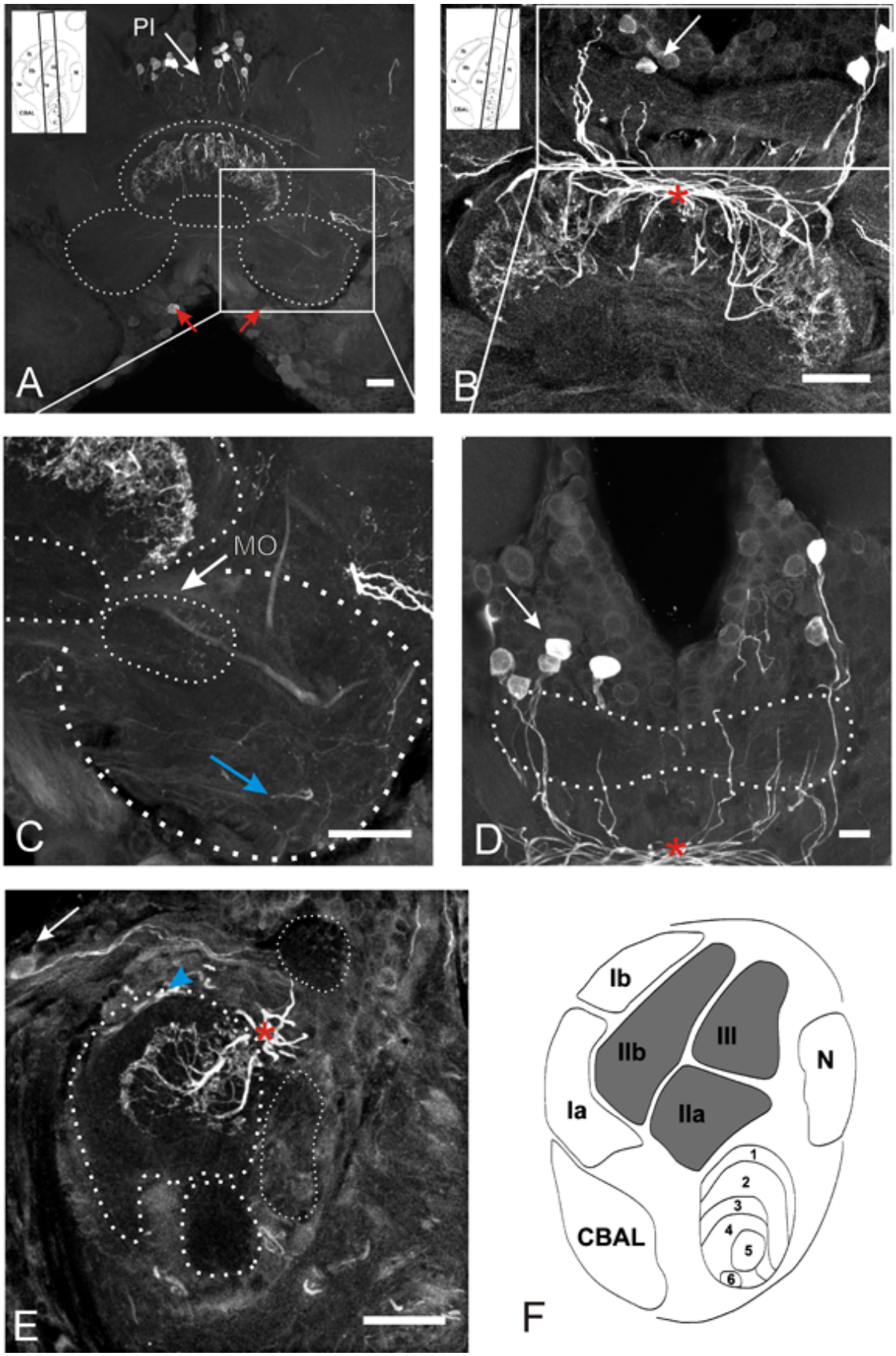


Figure 3.6: General distribution of citrulline in the grasshopper brain. A: Frontal section showing citrulline immunoreactivity in the antennal lobe (AL). Strong labeling could be detected in fibers innervating all glomeruli of the AL. B: Frontal section through an optic lobe. Strong immunostaining could be detected in monopolar cell that innervate the lamina (indicated by red arrow) and outer layers of the medulla. Only faint labeling could be detected in the inner layers of the medulla, while the lobula is devoid of staining. C and D: Frontal sections showing the mushroom bodies (MBs). The MBs are nearly free of citrulline. Only very weak citrulline-ir could be detected in lateral parts of the calyces. Insets showing schematic 3D-representations of the brain structures in all images are courtesy of Dr. Thomas Reischig. Scale bars = 100 μm in B; 50 μm in A, C and D

innervates only specific parts of layers II and III (layer II can be further distinguished into IIa/IIb) or both layers entirely. Citrulline immunoreactive tangential neurons entered the CX within the tract IT2 and through the posterior groove. These fibers also seemed to innervate the upper division of the CB.

In line with earlier studies that described the distribution of nitric oxide synthase in locusts (*S.gregria*: Kurylas et al. 2005, *Ch.b.*: Wenzel et al. 2005), we were also able to detect a bilateral pair of immunoreactive somata in the ventro median protocerebrum (red arrows in Fig 3.7A) which was described as TL-1 neuron by Kurylas et al. (2005). These fibers have been described to innervate the CBL but not the CBU, but we could not see any citrulline-IR in the CBL in our preparations, which raises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the question if these neurons do not innervate the CBU in *Ch.b.* or whether these neurons were just not active enough to accumulate detectable amounts of citrulline in the lower division. Citrulline-IR-fibers of unknown origin (either tangential or columnar) seemed to leave the CX via the IT1-tract and formed arborizations in the median olive (MO, Fig. 3.7C) and the ventral shell of the lateral accessory lobes (blue arrow in Fig. 3.7C). This also contrasts reports from *S. gregaria* (Kurylas et al. 2005), where the median olive was free of NO-producing fibers.

Control experiments in which the primary antibody was omitted showed no staining in the described regions (see appendix).

3.1.3.4 cGMP

The main target of NO in the central nervous system is the soluble guanylyl cyclase (Wykes and Garthwaite 2004), which, once activated by NO converts GTP to cyclic GMP, thereby increasing cytosolic concentration of cGMP (Fig. 3.5). To label neurons that respond to NO, I incubated the brains with a NO-donor and subsequently used an antiserum against cGMP.

3.1.3.5 General distribution of cGMP in the brain

Strong immunostaining against cGMP (Fig. 3.8) could be found in regions where also citrulline was detected, namely the optic lobes (Fig. 3.8A) and the AL (Fig. 3.8B). In the OL immunostaining was restricted to photoreceptor cells that innervate the lamina (white arrow in Fig. 3.8A). Staining in the antennal lobes was primarily detected in somata (blue arrow in Fig. 3.8B) but not in the fibers innervating the glomeruli (blue arrowhead in Fig. 3.8B). A similar situation was found in the mushroom bodies (MBs), where strong immunostaining in the somata of the kenyon cells (red arrow in Fig. 3.8C) but no immunopositive fibers in the calyces (red arrowhead in Fig. 3.8C) were detected. Very intense staining could be found in a group of

Figure 3.7: Distribution of citrulline in the central complex. A-D: Frontal sections showing citrulline in the midbrain and central complex E: Sagittal section through the central complex. The most prominent staining against citrulline can be seen in the central complex, while other parts known to produce NO (e.g. the mushroom bodies compare 3.6C and D) are not stained with this method. Two groups of somata can be located. One is found in the pars intercerebralis (PI, indicated by white arrows in A, B and D), while the other is located in the inferior median protocerebrum (indicated by red arrows in A). Citrulline-ir is restricted to the upper division of the central body, while the lower division is completely free of immunostaining. Citrulline could be detected only in the layers II and III of the CBU, while layer I was free of staining. Two main fiber types can be distinguished. The most prominent staining could be seen in the posterior chiasm (PCh, red asterisks in B, D and E), which is typical for pontine neurons. Additionally faint labeling could be seen in tracts entering the CB through the dorsal and posterior face (blue arrowhead in E), which is another indication that citrulline positive fibers belong to the pontine type. Staining could also be detected in the posterior groove which is typical for tangential neurons. Staining of weaker intensity could be detected in the LAL. Citrulline-ir was seen in the median olive (MO in C) and the ventral shell (blue arrow in C) of the LAL. F: Schematic drawing of a sagittal section through the CB. Regions highlighted in gray contain citrulline positive fibers (modified from Homberg 1991 and Müller et al. 1997). Note the absence of citrulline-ir from structures of the central complex having shown to contain high activity of NADPHd-activity in the locust S.gregaria, namely the CBL and the noduli. Scale bars = 50 μm in A, B, C and E; 20 μm in D

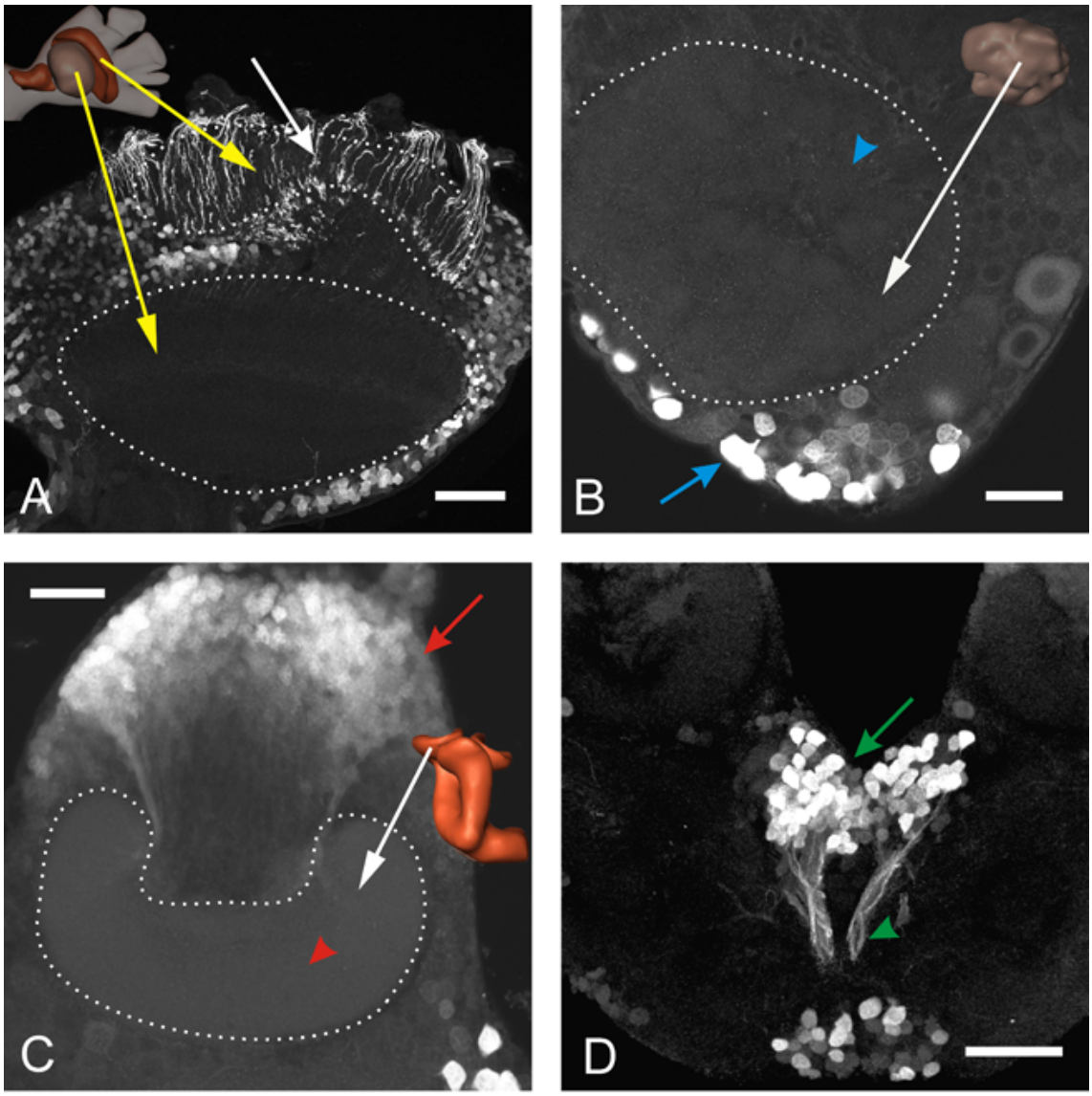


Figure 3.8: General distribution of cGMP in the grasshopper brain. A: Frontal section through the optic lobe. Strong immunostaining could be detected in photoreceptor cells that terminate in the lamina (white arrow). B: Frontal section of the antennal lobe. cGMP could be found in the somata of local interneuron (blue arrow), but not in fibers innervating the glomeruli of the AL (blue arrowhead). C: Frontal section showing the calyx of the mushroom body (MB). As for the antennal lobe, cGMP could only be detected in the somata of the MB-intrinsic Kenyon-cells (red arrow), while no labeled fibers could be detected in the MB itself (red arrowhead). D: Fontal section of the posterior protocerebrum. High concentrations of cGMP are found in neurosecretory cells of the posterior pars intercerebralis (green arrow). The fibers of these somata project through the NCC2 (green arrowhead) to the corpora allata / corpora cardiaca, two structures, that release neurohormones into the hemolymph. Scale bars = 100 in μm A and D; 50 μm in B and C

neurosecretory cells of the posterior pars intercerebralis (PI) (green arrow in Fig. 3.8D). These neurons project to the so called corpora allata (CA) / corpora cardiaca (CC) via the nerve NCC2, which shows also strong cGMP-ir (green arrowhead in Fig. 3.8D). The CC/CA are two closely associated neurosecretory organs of the brain, that release hormones into the hemolymph.

3.1.3.6 cGMP in the central complex

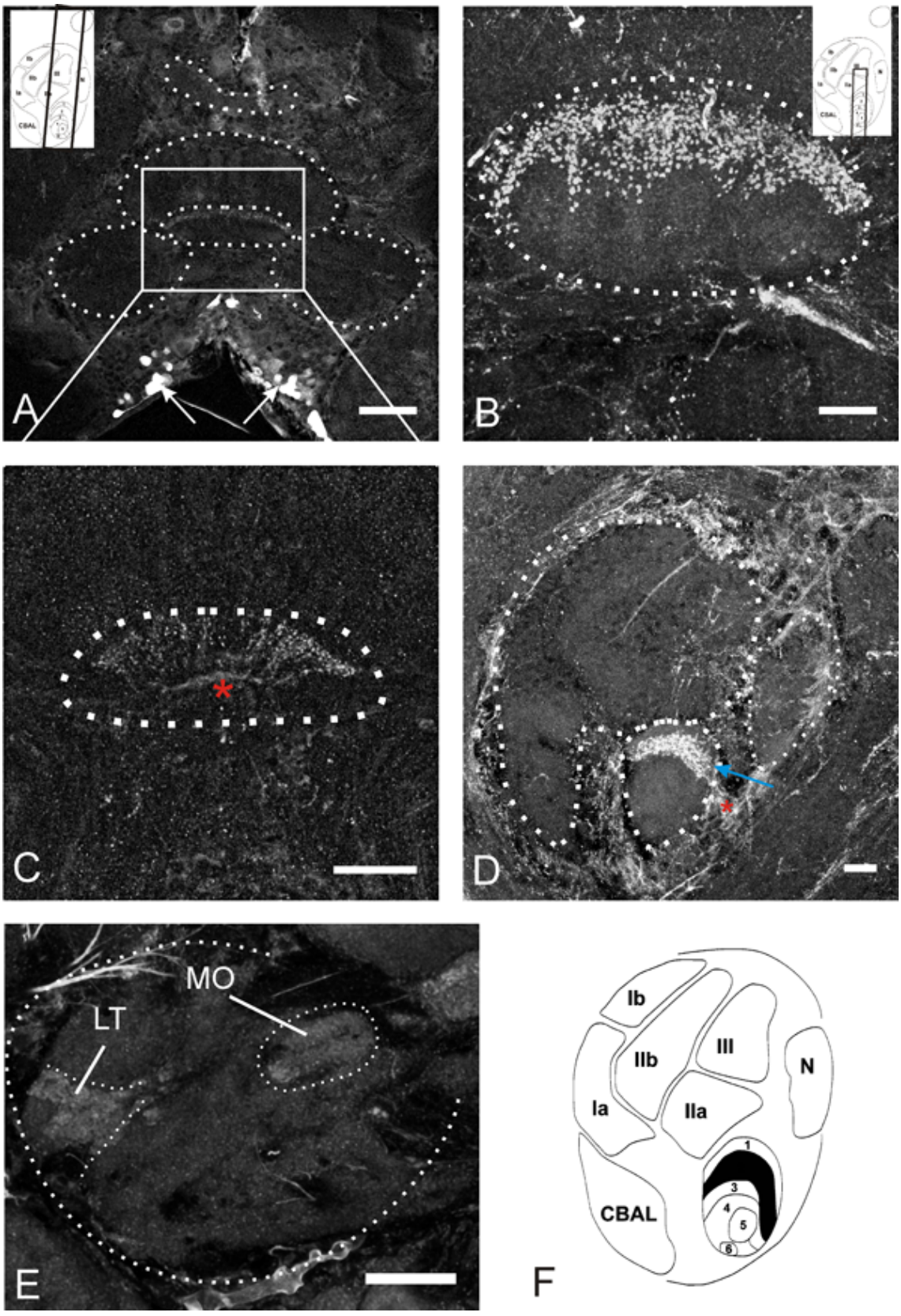
Cyclic GMP immunoreactivity in the central complex (Fig. 3.9) was exclusively observed in tangential neurons (CG1-neurons) innervating the lower division of the central body (Fig. 3.9A-D). As it is typical for this type of tangential neurons, their cell bodies were located as bilateral pairs in the infero-median protocerebrum (white arrows in Fig. 3.9A). Sagittal sections (Fig. 3.9D) revealed that accumulation of cGMP was restricted to neurites in layer 2 of the CBL (blue arrow in Fig. 3.9D) and that these fibers entered the CBL via the posterior groove (red asterisk in Fig. 3.9C and D). This staining pattern closely resembles that of TL-2 and TL-3 neurons previously described in *S. gregaria* (Müller et al., 1997). TL-2 and TL-3 neurons can be distinguished by their branching patterns within the lateral accessory lobes. Labeling in the LAL of *Ch.b.* was generally weak but faint immunostaining could be detected in both, the median olive (MO in Fig. 3.9E) and the lateral triangle (LT in Fig. 3.9E).

No immunoreactivity could be detected in the other subdivisions of the central complex including the central body upper division that has been shown provide the only source of NO in the central complex. NO-stimulated cGMP accumulation in brain neuropiles surrounding the CB was essentially absent. One possible explanation could be, that cGMP upregulation occurred only in neuronal compartments where high amounts of the sGC are localized, either in synaptic regions or at their production site in the somata.

Control experiments in which the primary antibody was omitted showed no staining in the described regions (see appendix).

3.1.4 GABA and cGMP

Both, GABA and NO-stimulated accumulation of cGMP in the central complex have been demonstrated to suppress grasshopper sound production (?Wenzel et al. 2005). Since both signaling molecules, GABA and cGMP could be detected in the same types of tangential neurons that innervate similar regions of the lower division of the central body, I investigated the possibility of their colocalisation in the central complex (Fig. 3.10A_1-B_3). Double labeling experiments showed that cGMP is upregulated upon NO-stimulation in GABAergic neurons of the CBL, linking these two inhibitory transmitter systems, which both suppress stridulation. Distance based colocalisation analysis reveals that cGMP is primarily upregulated in GABAergic fibers (Fig. 3.10A_{1-3}), but only in a subset of all GABAergic fibers innervating the CBL (96 % of cGMP positive fibers are also positive to GABA, while only 21 % of the GABAergic fibers accumulated cGMP). Fibers are restricted to a dorsal layer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of the CBL, presumably layer 2 (Fig. 3.10A_{1-3}, colocalised fibers are highlighted in white). We performed colocalisation analysis on fibers, because cGMP staining of the somata varied greatly between different preparations, while labeling of the fibers was constant. Nevertheless, we were also able to detect colocalisation in somata located in the inferior-median protocerebrum (white arrows in Fig. 3.10B_{1-3}), a region in which tangential neurons that innervate the lower division of the CB have their somata.

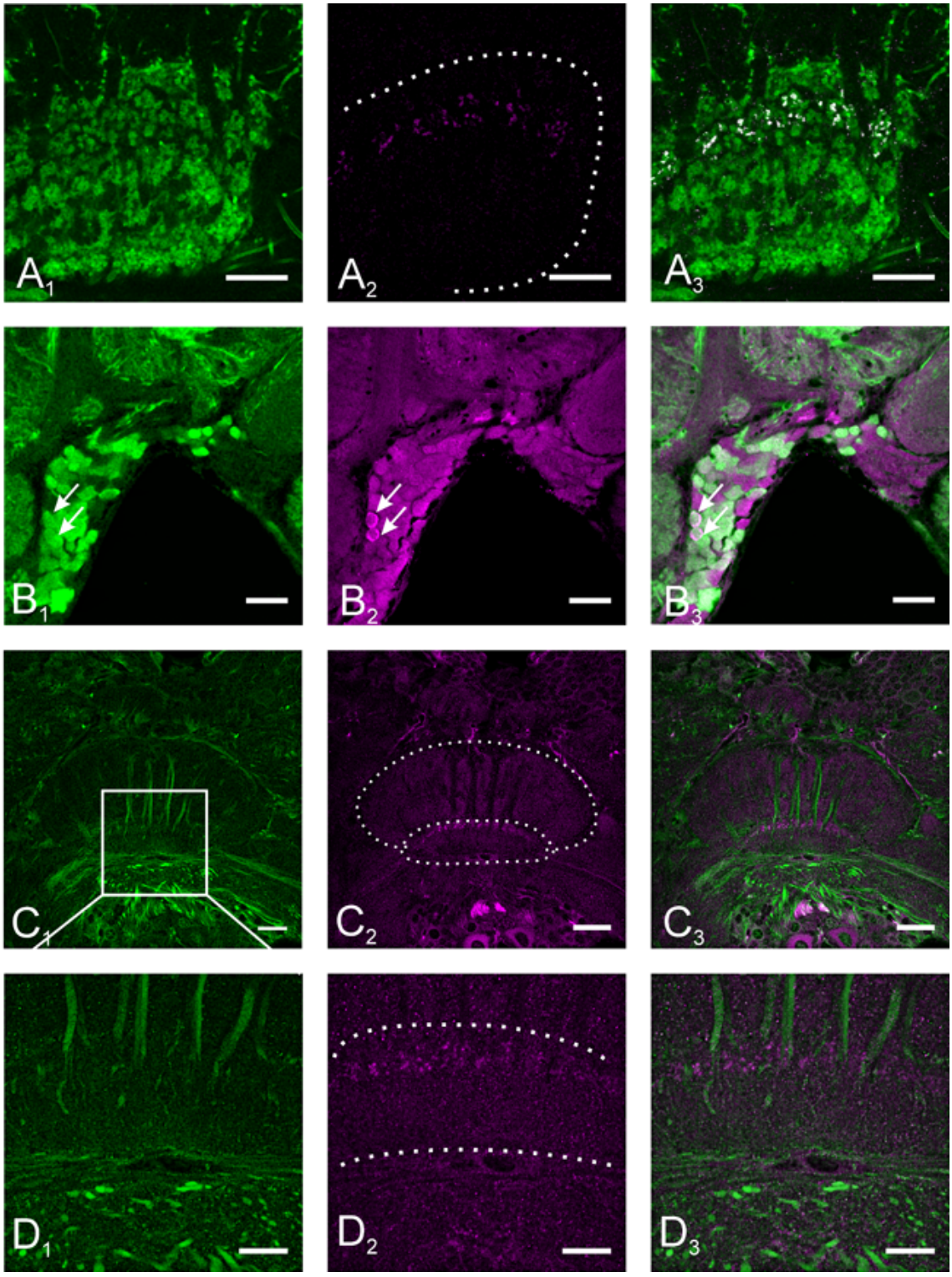
3.1.5 mAChR and cGMP

Activation of the NO/cGMP signaling pathway in the central complex has been demonstrated to suppress muscarine-stimulated sound production in restrained *Ch. biguttulus* (Wenzel et al. 2005). Since both, mAChR-expressing columnar neurons and cGMP-accumulating tangential neurons extensively arborize in the lower division of the central body, the possibility was investigated that NO may stimulate the production of cGMP in these columnar output neurons of the central complex. Double labeling of mAChRs and NO-stimulated cGMP revealed no evidence of colocalization of the two antigens although both were expressed in overlapping regions and in closely associated neurites in the lower division of the central body (Fig. 3.10C_1-D_3). The lack of colocalization indicates that muscarinic excitation-dependent output of the central complex, which stimulates sound production, is not a direct target of NO-induced inhibition of the behavior.

3.1.6 Tyrosine-Hydroxylase/Dopamine

The catecholamine dopamine belongs to the group of biogenic amines. Its distribution in the nervous system has been studied in a variety of insect species (Mercer et al. 1983, Schäfer and Rehder 1989, Nässel and Elekes 1992, Wendt and Homberg 1992, Bicker 1999, Mesce et al. 2001). Like other biogenic amines, dopamine has been implicated in the modulation of endocrine activities and various behaviors Murdock

Figure 3.9: Distribution of cGMP in the central complex. A: Frontal section, showing the central complex and surrounding midbrain structures. CGMP-ir is restricted to a specific layer of the lower division. The somata of these neurons are located in the inferior median protocerebrum (indicated by white arrows). One reason could be cGMP upregulation occurred only in neuronal compartments where high amounts of the sGC are localized, either in synaptic regions or at their production site in the somata. B and C: Frontal sections showing the lower division. Strong cGMP-ir could be detected in tangential neurons running close to the anterior border of the CBL. The neurites seem to enter the CBL from posterior (red asterisks in C and D) direction and innervate the CBL in a fan-shaped fashion (best seen in C). The appearance of neurites in the CBL is beaded-like (best seen in B) which indicates that they are of pre-synaptic character in this region. D: Sagittal section of the central body: Staining in the CBL is restricted to layer 2 (indicated by blue arrow), while the other layers are completely devoid of staining. E: Frontal section of the lateral accessory lobe (LAL). Only faint labeling can be detected in the LAL. This staining is restricted to the lateral triangle (LT) and the median olive (MO). Striking is the absence of cGMP in regions such as the CBU where NO-production could be shown through citrulline-ir (CBU). F: Schematic drawing of a sagittal section through the CB. Regions highlighted in black contain cGMP positive fibers (modified from Homberg 1991 and Müller et al. 1997). Scale bars = 100 μm in A; 20 μm in B, D and E; 10 μm in C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(1971), Bicker and Menzel (1989), Mustard et al. (2005). Increasing dopaminergic transmission in the fruit fly *D. melanogaster* increases sexual arousal (Andretic et al. 2005) and injections of dopamine into the central body of *Ch.b.* induces stridulation (see below). Dopamine is produced from tyrosine by the enzyme tyrosine-hydroxylase. To label dopaminergic neurons, two different antibodies were used. One was directed against tyrosine-hydroxylase (TH), while the other was directed against dopamine itself.

3.1.6.1 Comparison between Dopamine- and Tyrosine-Hydroxylase-IR

Both antisera stained virtually the same neurons so that in the subsequent result and discussion part no difference is made between the two antibodies.

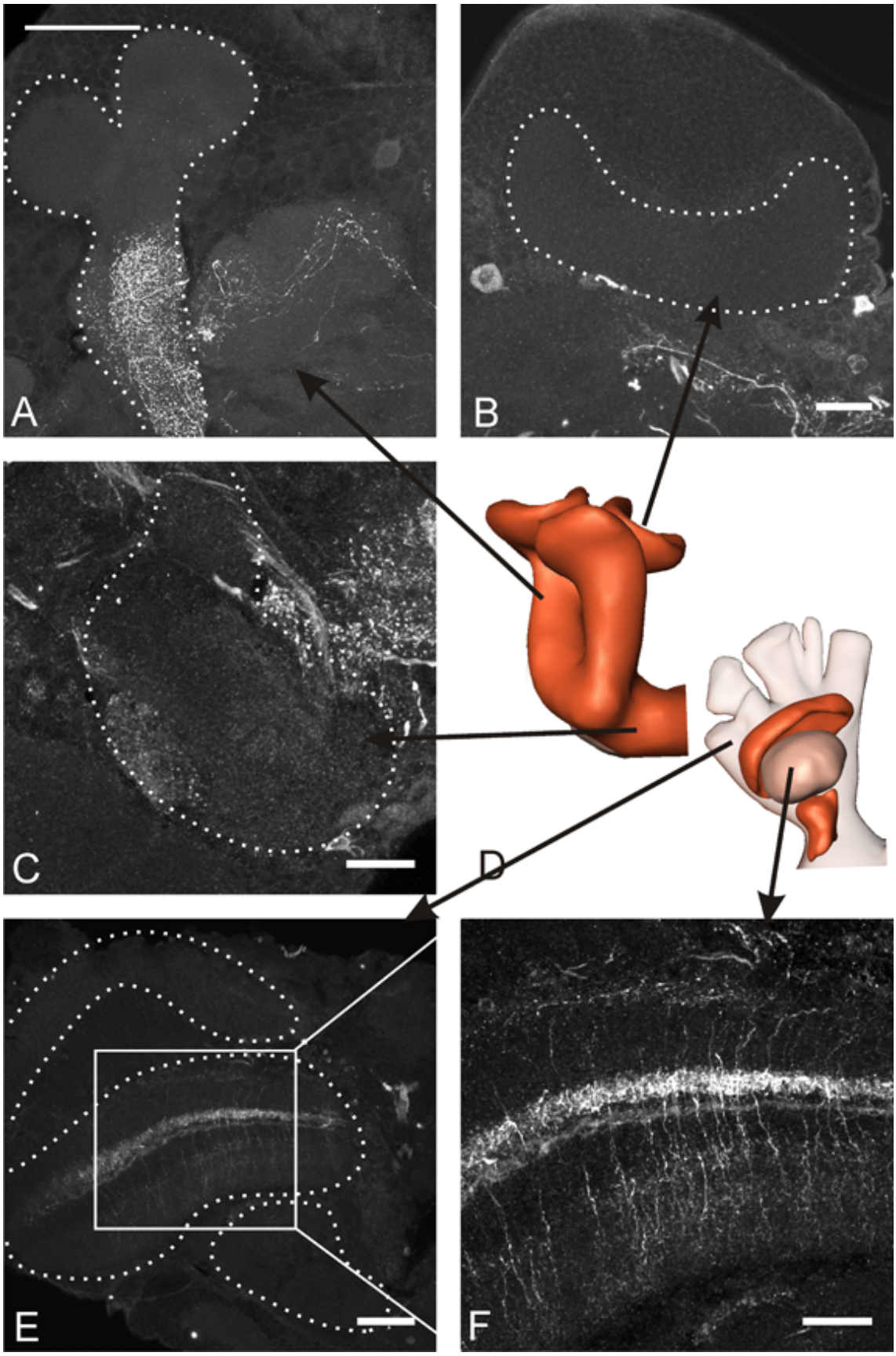
3.1.6.2 General distribution of Tyrosine-Hydroxylase/Dopamine in the brain

The staining pattern for dopaminergic neurons in the brain (Fig. 3.11) is very similar to the one already described for the locust *S. gregaria* (Wendt and Homberg 1992). Dopaminergic neurons are found throughout the entire brain with only two exceptions, the calyces of the mushroom bodies (Fig. 3.11A and B) and the antennal lobes. Most of the dopaminergic somata were located in the optic lobes (Fig. 3.11E and F). Strong staining could be found in the medulla while the lobula contained only sparse staining. Staining in the mushroom bodies was restricted to the lobes (Fig. 3.11C) and the pedunculus (Fig. 3.11A).

3.1.6.3 Tyrosine-Hydroxylae/Dopamine in the central complex

The highest concentration of dopamine in the brain could be found in the central body (Fig. 3.12 and 3.13) and staining pattern appeared similar to the staining already described for the locust *Schistocerca gregaria* (Wendt and Homberg 1992). Staining in the CB stems from tangential neurons that innervate both subdivisions of the CB, with stronger staining in the CBU compared to the CBL. The staining in the CBL originated from three different clusters of neurons. One type had its somata located in the lateral pars intercerebralis (blue arrowheads in Fig. 3.12E).

Figure 3.10: Colocalisation of cGMP with GABA and mAChR. A_1-B_3 Double labeling of GABA (green) and cGMP (magenta) in the central body. Colocalisation (highlighted in white) could be seen in tangential neurons of the lower division. While cGMP seems to be exclusively upregulated in GABAergic fibers, only a subset of GABA-ir fibers is also positive to cGMP. This indicates that the inhibitory effect of NO on grasshopper sound production is mediated through influencing GABAergic signaling. Additionally, colocalisation could be found in somata of the ventro-median protocerebrum (arrows in B_1-B_3), the region where tangential neurons innervating the lower division have their somata. C_1-D_3 Double labeling of mAChR (green) and cGMP (magenta) in the central body. No Colocalisation could be detected, indicating, that NO does not exert its effect directly on mAChR-ir fibers. Scale bars 50 μm in B_1-C_3; 20 μm D_1-D_3; 10 μm in A_1-A_3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These neurons sent their fibers as part of the w-bundle (blue arrows in Fig. 3.12E) to the lateral accessory lobes (LAL). There, they sent off numerous sidebranches into the dorsal and ventral shell (Fig. 3.12B, dorsal shell is indicated by a yellow asterisk and ventral shell by a red asterisk). The neurons fasciculated and ran as part of the isthmus tract (indicated as blue asterisk in Fig. 3.12B and C) towards the central body, which they entered through the posterior groove (green asterisks in Fig 3.12C and in Fig. 3.13A-C). These fibers were innervating the entire lower division and the layers II and III of the upper division. According to the terminology for dopaminergic neurons in the brain of the locust *S. gregaria* this neuron type can be described as DP2-like.

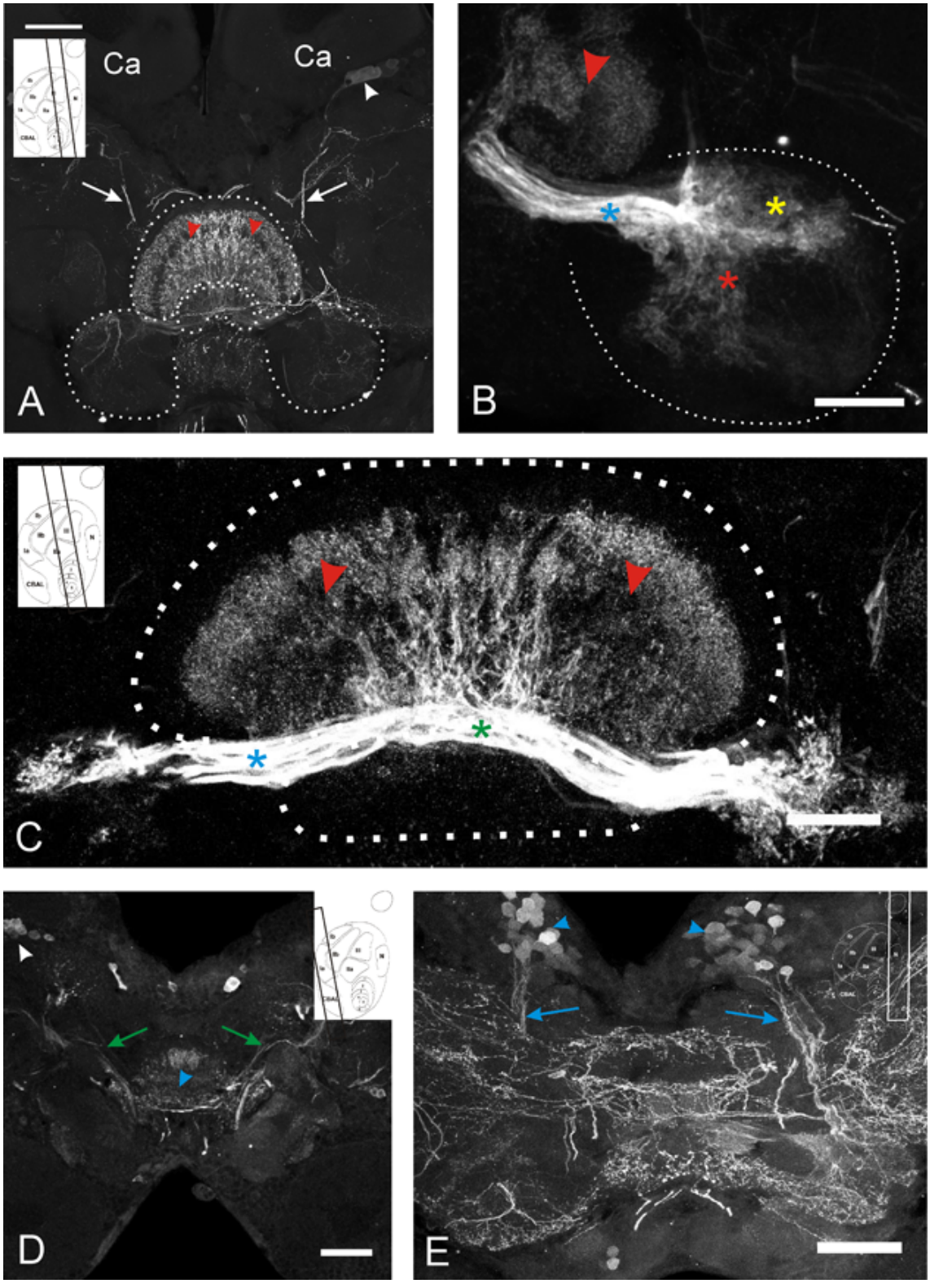
The two other neuron-types had their somata in the lateral protocerebrum, close to the calyces (white arrowheads in Fig. 3.12A and D). They sent their neurites as part of the anterior bundle (AB) towards the CB (green and white arrows in Fig. 3.12A and D and in Fig. 3.13B). The AB runs along the pedunculus of the mushroom bodies, where these neurons seem to have additional ramifications. These neurons differed in their arborization pattern within the central complex. While one type (DC-2-like) is innervating only the anterior lip of the CBU (CBAL, blue arrowheads in Fig. 3.12E and Fig. 3.13C, greens arrows indicate the respective fibers of the AB), the other (DC-1-like) innervates the inner layers of the CBU (layers II and III, red arrowheads in Fig. 3.12A-D and Fig. 3.13A-C, white arrows indicate the respective fibers of the AB).

No staining could be detected in the noduli and the protocerebral bridge. Control experiments in which the primary antibodies were omitted showed no staining in the described regions (see appendix).

3.1.7 Neuropeptides in the central complex

A large number of neuropeptides has been identified in the insect brain (at least 35 neuropeptide genes have been identified in *Drosophila melanogaster* (Nässel 2002, Nässel and Homberg 2006). Neuropeptides have also been identified in insect neurons by immunocytochemistry (Homberg 2002, Nässel 2002, Nässel and Homberg 2006). From these studies, it is known that most insect neuropeptides are present in both, interneurons and neurosecretory or endocrine cells. In insects, neuropeptides have been most extensively studied with respect to their roles as circulating hor-

Figure 3.11: General distribution of dopaminergic neurons in the grasshopper brain. A-C: Frontal sections showing dopaminergic neurons in the mushroom bodies. Dopamine could be detected in the pedunculus (A) and single columns of the β-lobes (C), while the calyces are free of dopaminergic innervation (B). D: Schematic 3D-models of the described brain structures (Courtesy of Dr. Thomas Reischig). Arrows point to the section of the respective structure. E and F: Frontal sections through one optic lobe. Dopaminergic neurons are found in the inner layers of the medulla. Scale bars = 100 μm in A and D; 50 μm in B, C and F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mones (Ewer and Reynolds 2002, Claeys et al. 2005, Ewer 2005), while their function in interneurons has not been extensively studied so far. The aim of the following studies is, (A) to map peptidergic circuits in the central complex whose function in the control of acoustic communication is known and (B) to examine the distribution of other peptidergic systems in the CX to provide a framework for further behavioral and physiological studies.

3.1.8 Proctolin-receptor

Proctolin is a pentapeptide (Arg-Tyr-Leu-Pro-Thr) that holds a special position in the field of arthropod neurophysiology. It was the first bioactive peptide to be isolated from insect tissue and to be structurally characterized (Sturrat and Brown, 1975). Since its discovery, it has been found in a variety of other invertebrate and even vertebrate species (Bishop and O'Shea 1982, Keshishian and O'Shea 1985, Siwicki et al. 1985, Nässel and O'shea 1987, Breidbach and Dircksen 1989, Orchard et al. 1989). Proctolin displays potent myotropic activity not only in visceral muscles but also when applied to skeletal and heart muscles of a range of insect species (Orchard et al. 1989). Apart from its function in the peripheral nervous system it has been also found in brain interneurons of invertebrates (Orchard et al. 1989), but its function as a central nervous signal remained elusive. A role for proctolin in modifying a motor network was demonstrated in the stomatogastric ganglion of the crab *Cancer borealis* (Marder et al. 1986, Nusbaum and Marder 1989). In the grasshopper *Chorthippus biguttulus*, it could be shown, that injections of proctolin into the central body induce singing behavior (Vezenkov 2004). To label potential targets of proctolin-stimulation in the central complex, immunostainings against the proctolin-receptor were performed. For this, an antibody generated against the proctolin-receptor of *Drosophila melanogaster* was used (Johnson et al. 2003). Activation of this proctolin receptor lead to an increase in the intracellular levels of calcium by an IP3-mediated mechanism and by promoting the entry of extracellular

Figure 3.12: Frontal sections showing dopaminergic neurons in the central complex. A: Frontal section through the median protocerebrum showing DA/TH-immunoreactivity in the central complex and surrounding brain structures. Somata of DC1-like neurons can be seen lateral to the calyx of the mushroom bodies (white arrowhead). The fibers of these neurons run as part of the anterior bundle (white arrows) and innervate the inner layers of the upper division (red arrowheads) and the entire lower division. B: Frontal section of the lateral accessory lobes (LAL) showing the arborization pattern of DP2-like neurons. DP2-like neurons give off sidebranches into the dorsal (yellow asterisk) and ventral shell (red asterisk) of the LAL. From the LAL the fibers continue through the isthmus tract (blue asterisk) and innervate the inner layers of the upper division (red arrowhead). C: Frontal section through the central body showing DP2-like-neurons. Fibers run from the LAL towards the CB as part of the isthmus tract (blue asterisk) and enter the CB via the posterior groove (green asterisk) to innervate inner layers of the upper division (red arrowheads) and the entire lower division. D: Frontal section through the median protocerebrum, showing DC2-like neurons. The somata are located lateral to the calyces (white arrowhead) and sent their fibers via the anterior bundle (green arrows) towards the central body, where they innervate the anterior lip (blue arrowhead). E: Frontal section through the median protocerebrum showing DP2-like neurons. The somata of these neurons are located in the lateral pars intercerebralis (blue arrowheads) join the w-bundle and run along the lateral edges of the central body (blue arrowheads) to innervate the LAL. Scale bars = 100 μm in A, D and E; 50 μm in B and C

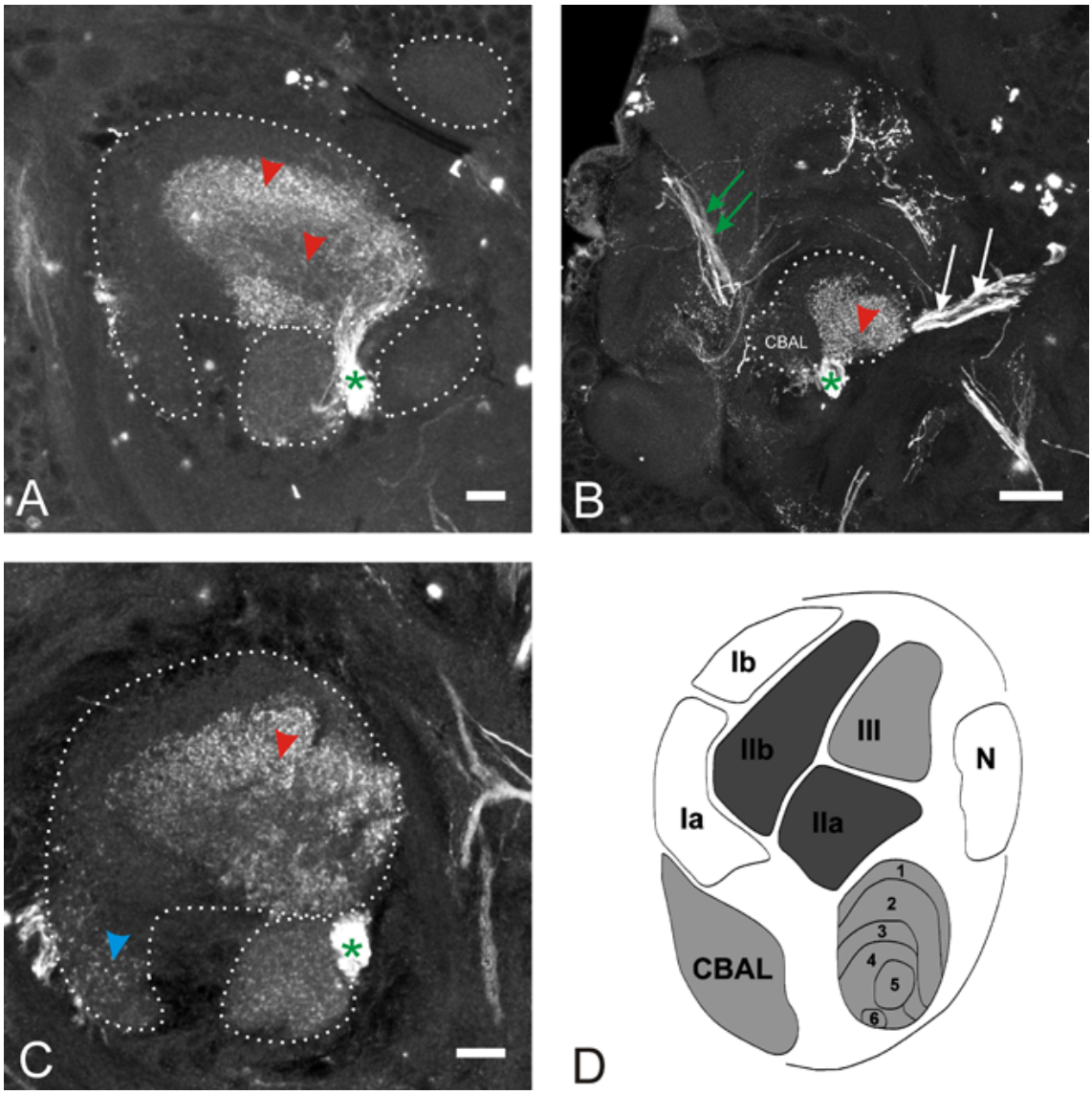


Figure 3.13: A-C: Sagittal sections through the central complex. The entire lower division is positive for dopamine, but is less intensively stained compared to the upper division (red arrowheads in A-C). Staining in the upper division is most prominent in layer II, while the anterior lip (CBAL blue arrowhead in C) and layer III exhibit weaker staining intensity and layer I is completely devoid of labeling. Additional staining could be detected in the posterior groove (green asterisks in A-C) and the anterior bundle. Immunostaining originating from DC2-like neurons that innervate the CBAL is depicted by green arrows, while fibers of DC1-like neurons that innervate the upper division are indicated by white arrows. D: Schematic drawing of a sagittal section through the CB. Regions highlighted in gray contain dopaminergic fibers, darker shades of gray indicate stronger staining intensities (modified from Homberg 1991 and Müller et al. 1997). Scale bars = 50 μm in B; 20 μm in A and C

calcium (Baines et al. 1990; 1996).

3.1.8.1 General distribution of the proctolin-receptor in the brain

The proctolin receptor could be detected in several brain regions (Fig. 3.14). As for the staining against mAChRs, the somata were only weakly stained. Staining against the proctolin-receptor (PR) was found in the optic lobes (Fig. 3.14B) and the antennal lobes (Fig. 3.14A). The optic lobes contained punctuate staining in the medulla (white arrowheads in Fig. 3.14B) and the lamina (blue arrowheads in Fig. 3.14B), while the lobula was free of label. Staining in the antennal lobe seemed

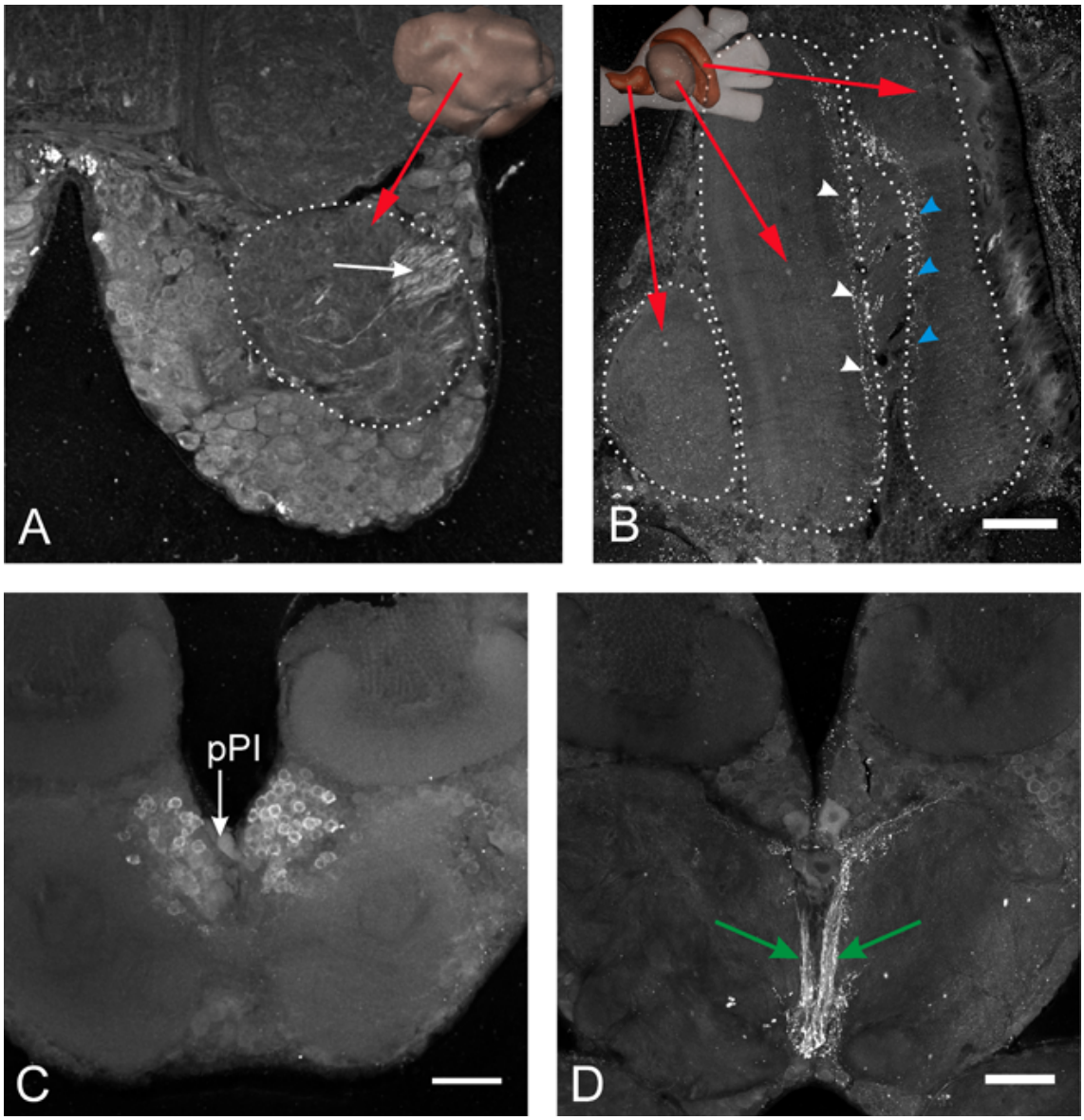


Figure 3.14: General distribution of the proctolin-receptor in the grasshopper brain. A: Frontal section through the antennal lobe. Proctolin-receptor- immunoreactivity (ProcR-ir) could be detected in particular lateral part of the antennal lobe (white arrow). This immunoreactivity seems to stem from one single fiber that arborizes extensively. B: Frontal section through the optic lobes. Punctate immunostaining could be detected in monopolar cells, that connect outer layers of the lamina (blue arrowheads) with proximal layers of the medulla (white arrowheads). C: Section through the posterior protocerebrum. Strong signals could be detected in neurosecretory cells of the posterior pars intercerebralis (pPI). D: Section through the posterior protocerebrum showing ProcR-ir in the NCC2 (green arrows). All scale bars = 100 μm

to be restricted to a particular lateral region (white arrow in Fig. 3.14A). Very prominent staining could be found in a group of neurosecretory cells of the posterior pars intercerebralis (pPI, Fig. 3.14C) including their axons forming the the nerve NCC2 (green arrows in Fig. 3.14D), indicating a role of proctolin in the control of hormone release from the corpora cardiaca/corpora allata.

3.1.8.2 Proctolin-receptors in the central complex

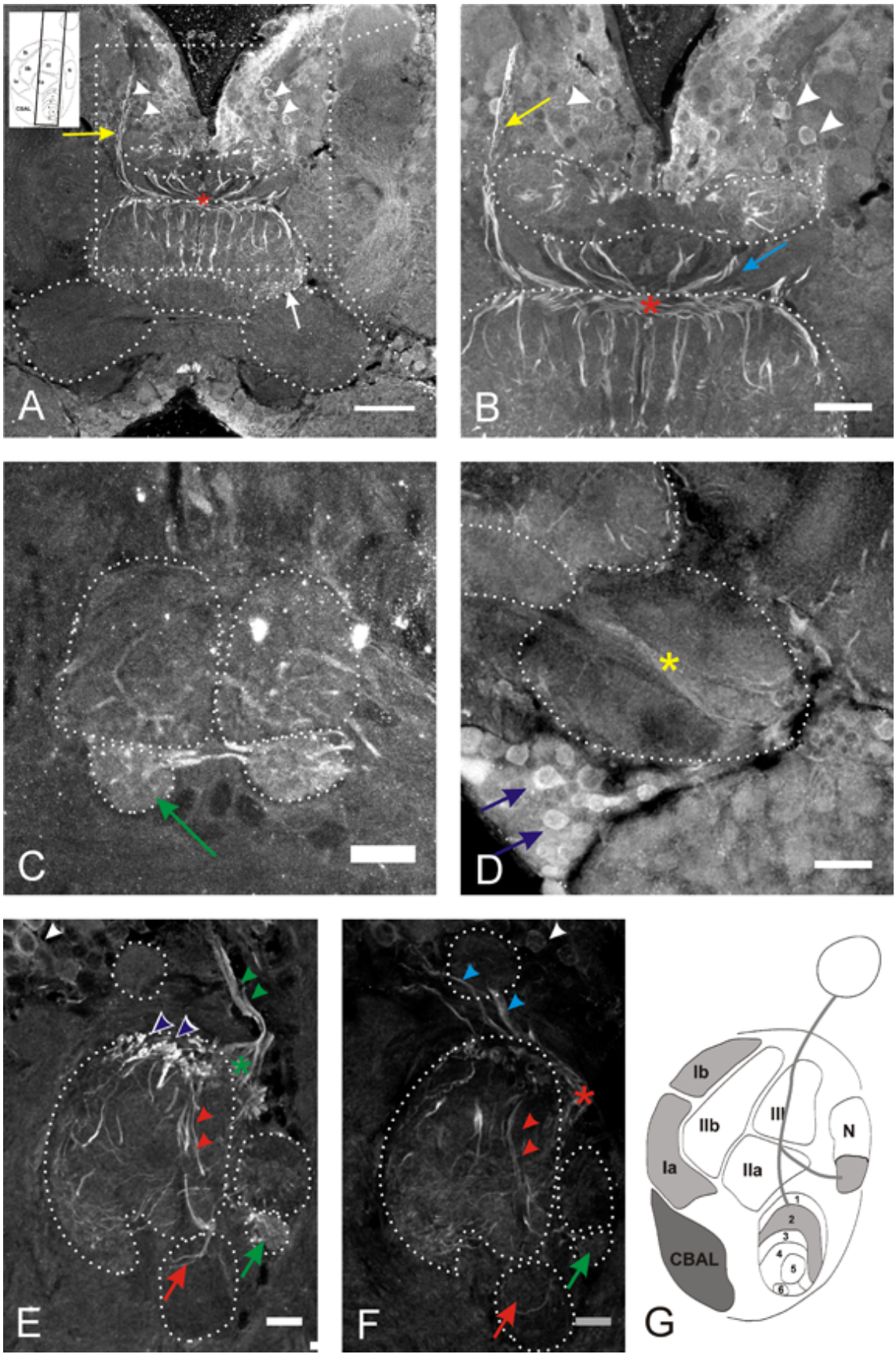
PR-ir could be found in tangential, columnar and pontine neurons innervating the central complex (Fig. 4.5). Columnar and pontine neurons had their somata located

in the anterior PI (white arrowheads in Fig. 4.5A, B, C, F and E). The columnar neurons sent thin neurites (yellow arrows in Fig. 4.5A and D) into the protocerebral bridge where they gave off numerous sidebranches (best seen in Fig. 4.5B). From the PB, they ran as large diameter fibers (blue arrows in Fig. 4.5A and B) via the w, x, y, z-bundles towards the CB, which they entered through the posterior chiasm (PCh, red asterisks in Fig. 4.5A, B and F). They ran through layer III of the CBU as part of the posterior vertical bundle (red arrowheads in Fig. 4.5E and F). The neurons bifurcated, while one branch was innervating the lower unit of the noduli (green arrows in Fig. 4.5C, E and F), the other branch was innervating the CBL. Staining in the CBL was weak, but seemed to be restricted to layer 2 of the CBL (red arrow in Fig. 4.5E).

Somata of pontine neurons were intermingled with those of columnar neurons and also had similar arborization patterns (green arrowheads in Fig. 4.5E). Both neuron types run through the w, x, y, z-bundles and enter the CB through the PCh. The main difference is that pontine neurons do not gave off sidebranches into the PB, but rather pass the PB posteriorly (Fig. 4.5E). Also, pontine neurons enter the CB through the PCh more dorsal, compared to columnar neurons (green asterisk in Fig. 4.5E). Pontine neurons restrict their innervations of the CBU to layer I and the anterior lip (CBAL). They connect the different columns within particular layers. Fibers connecting the different columns could be detected in the dorsal face (dark blue arrowheads in Fig. 4.5E).

Additionally, I was able to detect staining from tangential neurons that seemed to innervate layer I of the upper division. The staining originated from somata located in the inferior median protocerebrum (IMP) (dark blue arrows in Fig. 4.5D). These neurons sent fibers through the isthmus tract (IT, yellow asterisk in Fig. 4.5D) that entered the CB through the posterior groove. Unfortunately I was not able to detect

Figure 3.15: Distribution of the proctolin receptor in the central complex. A: Frontal section through the median protocerebrum. Proctolin-receptors (ProcRs) could be detected on columnar and pontine fibers whose somata are located in the pars intercerebralis (white arrowheads). The columnar neurons sent their small neurites into the protocerebral bridge (yellow arrows). The main fibers ran as large diameter neurites via four pairs of fiber bundles, the w-, x-, y- and z-bundles (blue arrows in B) through the posterior chiasm (red asterisks) between the protocerebral bridge and the central body (CB) and innervate the upper division. C: Frontal section of the noduli. Immunoreactivity could be detected in the lower unit of the noduli. D: Frontal section of the lateral accessory lobes. ProcR-positive fibers could be located in the isthmus tract (yellow asterisk). These fibers presumably originated from tangential neurons located in the inferior median protocerebrum (dark blue arrows). E and F: Sagittal sections through the central complex. E shows the arborization pattern of pontine neurons. These neurons have their somata in the anterior pars intercerebralis (white arrowhead). The fibers of these neurons pass the protocerebral bridge (PB) posteriorly (green arrows) and enter the central body through the posterior chiasm (PCh, green arrowheads) to terminate in the outer layers of the upper division. Fibers of the pontine neurons that connect different columns run through the dorsal face (dark blue arrow). Additional fibers of columnar neurons could be detected in the posterior vertical bundle (PVB, red arrowheads) that terminate in the lower division (red arrow) and the lower unit of the noduli (green arrow). F shows the arborization pattern of a columnar neurons. The somata of these neurons intermingle with those of pontine neurons (white arrowhead). The fibers of the neurons passed the protocerebral bridge anteriorly and gave off fine sidebranches into the PB. The fibers enter the CB through the PCh, but at a more ventral position compared to pontine neurones. In the CB the fibers terminate as part of the PVB in the lower division (red arrow) and the lower unit of the noduli (green arrow). G: Schematic drawing of a sagittal section through the CB. Regions highlighted in gray contain dopaminergic fibers (modified from Homberg 1991 and Müller et al. 1997). Scale bars = 100 μm in A; 50 μm in B, C and D; 20 μm in E and F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PR-ir in the posterior grove. PR-ir positive neurons in the IMP, the faint staining in the IT and the staining pattern in layer I of the CBU (white arrow in Fig. 4.5A) strongly suggest that immunoreactive tangential neurons innervate the CB. Apart from the staining in the IT, no proctolin-receptors could be detected in the LAL.

Control experiments in which the primary antibody was omitted showed no staining in the described regions (see appendix).

3.1.9 Tachykinin related peptides

Tachykinin-like peptides (TKLPs) constitute a large and diverse family, found in vertebrates and invertebrates. TKLPs can be divided into two distinct groups based on their C-terminal sequence motif. All known vertebrate tachykinins share the C-terminal pentapeptide FXGLM, whereas the tachykinin related peptides (TKRPs), that appear exclusively in invertebrates, contain the somewhat different pentapeptide FX_1GX_2R (Broeck et al. 1999, Nachman et al. 1999, Nässel 1999). Both groups, however are ancestrally related (Schoofs et al. 1990, Broeck et al. 1999, Nässel 2002). To stain neurons positive to TRPs, an antibody generated against the TRP of the cockroach *Leucophaea maderae* (LemTRP) was used, whose specificity to TRPs of other insect species has been confirmed in previous studies (Sliwowska et al. 2001, Nässel and Winther 2002, Isaac and Nässel 2003, Pascual et al. 2008).

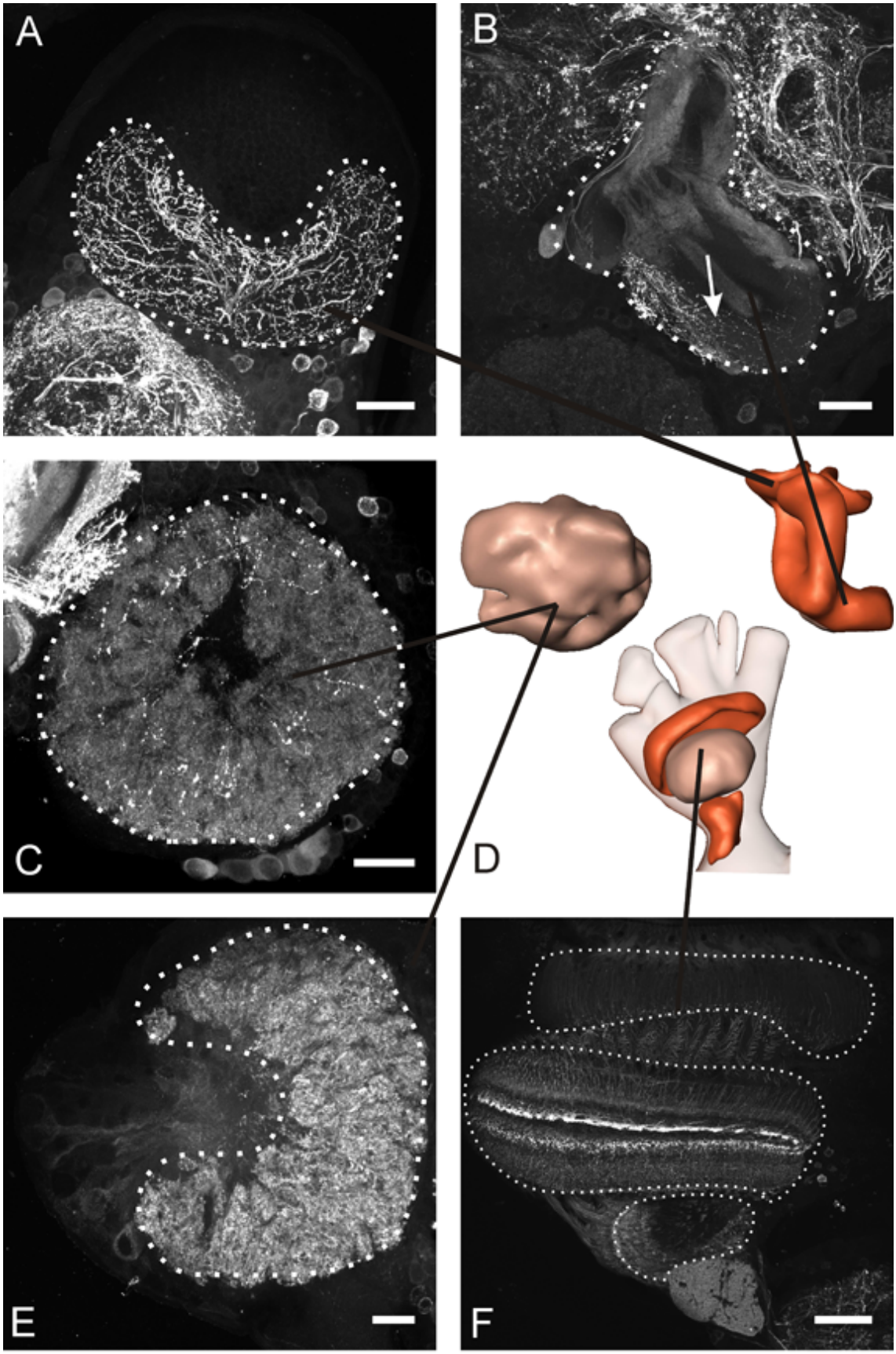
3.1.9.1 General distribution of TRP in the brain

TRP-ir could be found in all optic lobe neuropiles (Fig. 3.16F), especially in the medulla, while staining in the lamina was much weaker. Additionally, strong immunoreactivity was detected in the calyces of the mushroom bodies (Fig. 3.16A), and in columns of the β-lobe (Fig. 3.16B). Only sparse staining could be found the AL (Fig. 3.16C and E).

3.1.9.2 TRPs in the central complex

TRP related immunofluorescence was detected in all subdivisions of the central complex (Fig. 3.17) and the staining pattern is very similar to the one described

Figure 3.16: General distribution of tachykinin related peptides (TRPs) in the grasshopper brain. A: Frontal section of the mushroom body calyx. Intense immunoreactive fibers could be detected in fibers innervating the calyx. B: Fontal section of the mushroom body β-lobe. Immunostaining was restricted to a specific column of the β-lobe (white arrowhead). C and E: Frontal sections of the antennal lobes (AL). Dense immunostaining can be detected in all glomeruli of the AL. D: Schematic 3D-models of the described brain structures (Courtesy of Dr. Thomas Reischig). Arrows point to the section of the respective structure. E: Frontal section through the optic lobes (OL). TRPs could be detected in the inner layers of the medulla. Staining in the lamina and medulla was rather weak compared to the medulla. Scale bars = 100 μm in F; 50 μm in A, B, C and E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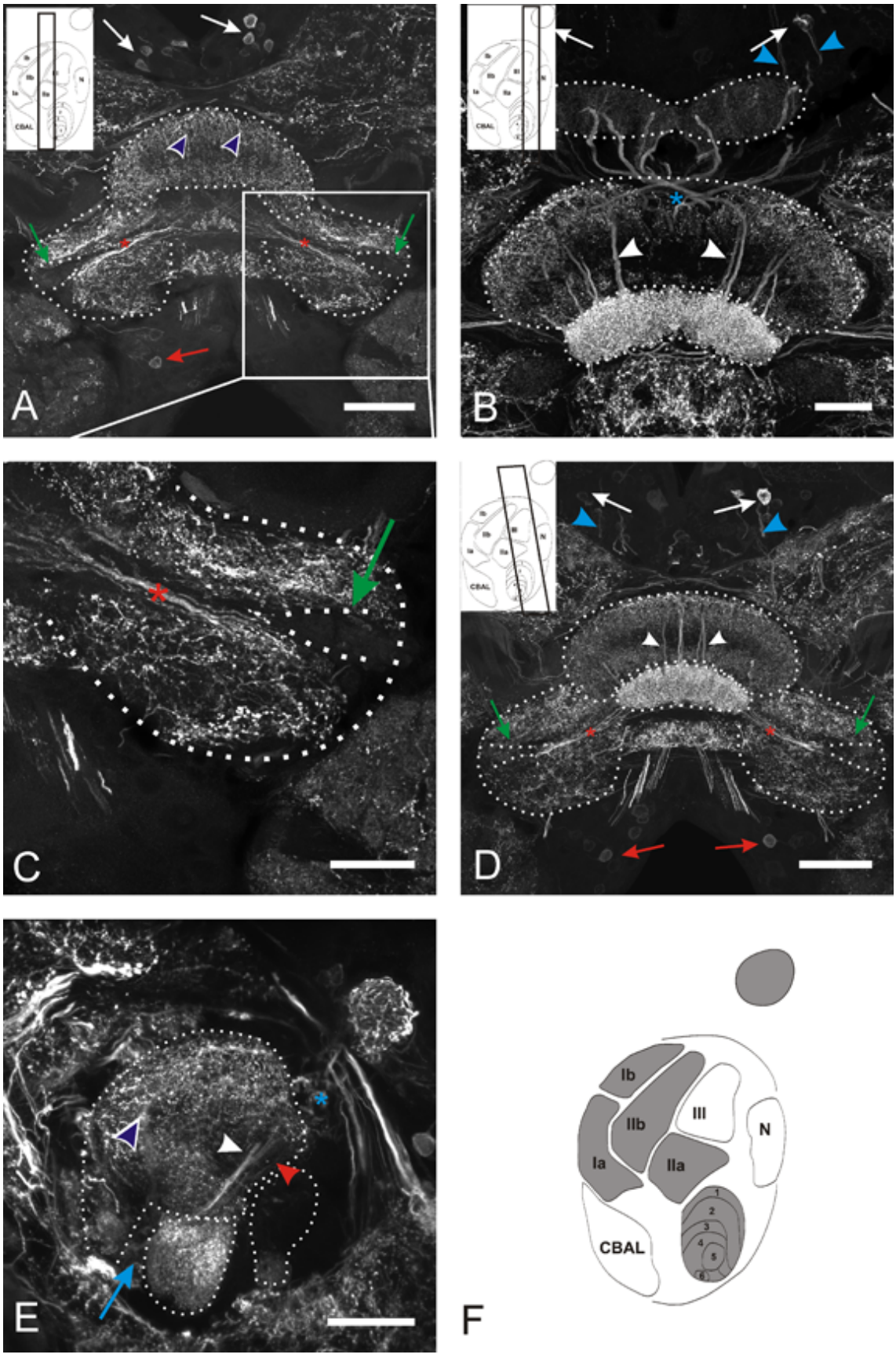
for the locust *Schistocerca gregaria* (Vitzthum and Homberg 1998). Several types of tangential and columnar neurons could be identified neurons. There were two main types of columnar neurons, all of them had their somata in the anterior pars intercerebralis (white arrows in Fig. 3.17A, B and C). Staining of columnar neurons in the CB was much weaker compared to tangential neurons.

The projection pattern of the columnar neurons is similar to the one described earlier positice for the mAChR. These neurons innervate the PB with thin diameter neurites (blue arrowheads in Fig. 3.17B and D). The main fibers left the bridge ventrally, joined the w, x, y, and z-bundles and entered the CB via the PCh (indicated by blue asterisks in Fig. 3.17B and E) (Williams 1975). In each bundle two large immunoreactive fibers were detected. All except for the most lateral fibers of the w-bundle continued trough the posterior vertical fiber bundles (PVB) of the central body (white arrowheads in Fig. 3.17B, D and E) (Williams 1972) and entered the lower division of the central, where they innervated all layers. The fibers left the CB through the ventral groove complex, joined the isthmus tract (red asterisks in Fig. 3.17A, C and D) and terminated in the lateral triangle of the LAL (green arrows in Fig. 3.17A, C and D). Except for the difference that in *S. gregaria* layer 5 of the CBL was free of immunostaining, labeling in *Ch.b.* resembled the projection of LTC-1 neurons in the locust *S.gregaria* (Vitzthum and Homberg 1998). Therefore this neuron type can will be termed as LTC1-like and subsequent nomenclature of the other TRP-positive neurons will be done with respect to the terminology introduced by Vitzthum et al. (1998).

Vitzthum and Homberg (1998) reported a second system of columnar neurons, with a similar projection pattern, but smaller somata and thinner neurites (LTC2-like). It was also possible to detect a neuronal population similar to that (red arrowhead in Fig. 3.17E), but due to the superposition of the two fiber system and the rather weak staining intensity of the smaller neurons, the projection pattern of these neurons within the central body could not be followed.

Figure 3.17: Distribution of tachykinin related peptides (TRPs) in the central complex. A, B and D: Frontal sections through the median protocerebrum showing LemTRP-positive somata of columnar neurons in the pars intercerebralis (white arrows) and tangential neurons in the inferior-median protocerebrum (red arrows). Dark blue arrowheads in A show fibers of LTC4-like neurons that innervate layer I of the upper division. In the central body LTC1-like neurons projected through layer III of the upper division (white arrowheads in B and D). The fibers of these neurons have their origin in the PI and sent thin neurites to the PB (blue arrowheads in B and D). Fibers connecting the CB with the LAL could be detected in the isthmus tract (red asterisks in A and D). These fibers gave of fine sidebranches within the lateral triangle of the LAL (green arrows). C: Frontal section showing the LAL from part A at higher magnification. Red asterisks indicate the isthmus tract and the green arrow the lateral triangle. E: Sagittal section through the central complex. Most intense immunostaining can be detected in the CBL and layer I of the CBU. Layer II of the CBU exhibits only weak staining whereas layer III is devoid of labeling. Columnar neurons that innervate the CBL (LTC1-like) run through the posterior vertical bundle (PVB, white arrowhead). Additionally, less intensively stained fibers could be detected in the PVB (LTC2-like, indicated by red arrow). Staining in the CBU stems largely from columnar neuron that enter the CB anterior to the CBL (blue arrow) and run through layer I of the CBU (dark blue arrowhead). F: Schematic drawing of a sagittal section through the CB. Regions highlighted in gray contain LemTRP positive fibers (modified from Homberg 1991 and Müller et al. 1997).Scale bars = 100 μm in A and D; 50 μm in B, C and E.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Two other types of columnar neurons that innervated the upper division were also recognized. One type (LTC3-like) had a projection pattern similar to the ones described above, meaning that they entered the CB through the PCh and ran as part of the PVB. Instead of entering the CBL it arborised within the CBU, but it could not be determined in which layer specifically.

From the other type (LTC4-like) it was not possible to determine the location of the somata. The neurites of this cell-type entered the CB through the ventral groove complex (blue arrows in Fig. 3.17E) and innervated the layer I and IIa of the CBU (dark blue arrowheads in Fig. 3.17E).

One type of tangential neuron could be found, that innervated the CBL (LTT1-like). The somata of these neurons were located in the inferior median protocerebrum (red arrows in Fig. 3.17A and D). The fibers of these neurons ran through the isthmus tract (red asterisks in Fig. 3.17A, C and D), gave off fine sidebranches in the LT of the LAL and innervated the inner layers of the CBL. No staining could be detected in the noduli.

Control experiments in which the primary antibody was omitted showed no staining in the described regions (see appendix).

3.1.10 Crustacean cardioactive peptide

The crustacean cardioactive peptide (CCAP) was originally described as a potent cardioexcitatory peptide in crustaceans (Stangier et al. 1987). But soon after its discovery it could be characterized in different insect species (Stangier et al. 1989, Cheung et al. 1992, Furuya et al. 1993, Lehman et al. 1993). Studies on insects showed that CCAP display a variety of excitatory actions on visceral and skeletal muscles Dircksen (1998), Vullings et al. (1998). To label neurons of the CX that contain CCAP, I performed immunocytochemistry by using a polyclonal antibody which has already been shown to reliably detect CCAP in the locust *S. gregaria* (Dircksen and Homberg 1995).

3.1.10.1 General distribution of CCAP in the brain

The distribution of CCAP in the brain of *Ch. b.* is comparable to the one already described for *S. gregaria* (Dircksen and Homberg 1995). CCAP can be found in most brain regions (Fig. 3.18) including the optic lobes (Fig. 3.18C and D), where it is found in several layers of the medulla (Fig. 3.18C) as well as in the most distal layer of the lamina (white arrows in Fig. 3.18D). The AL is only sparsely invaded by three to four neurons, that innervate only the dorsal part of the AL (Fig. 3.18A).

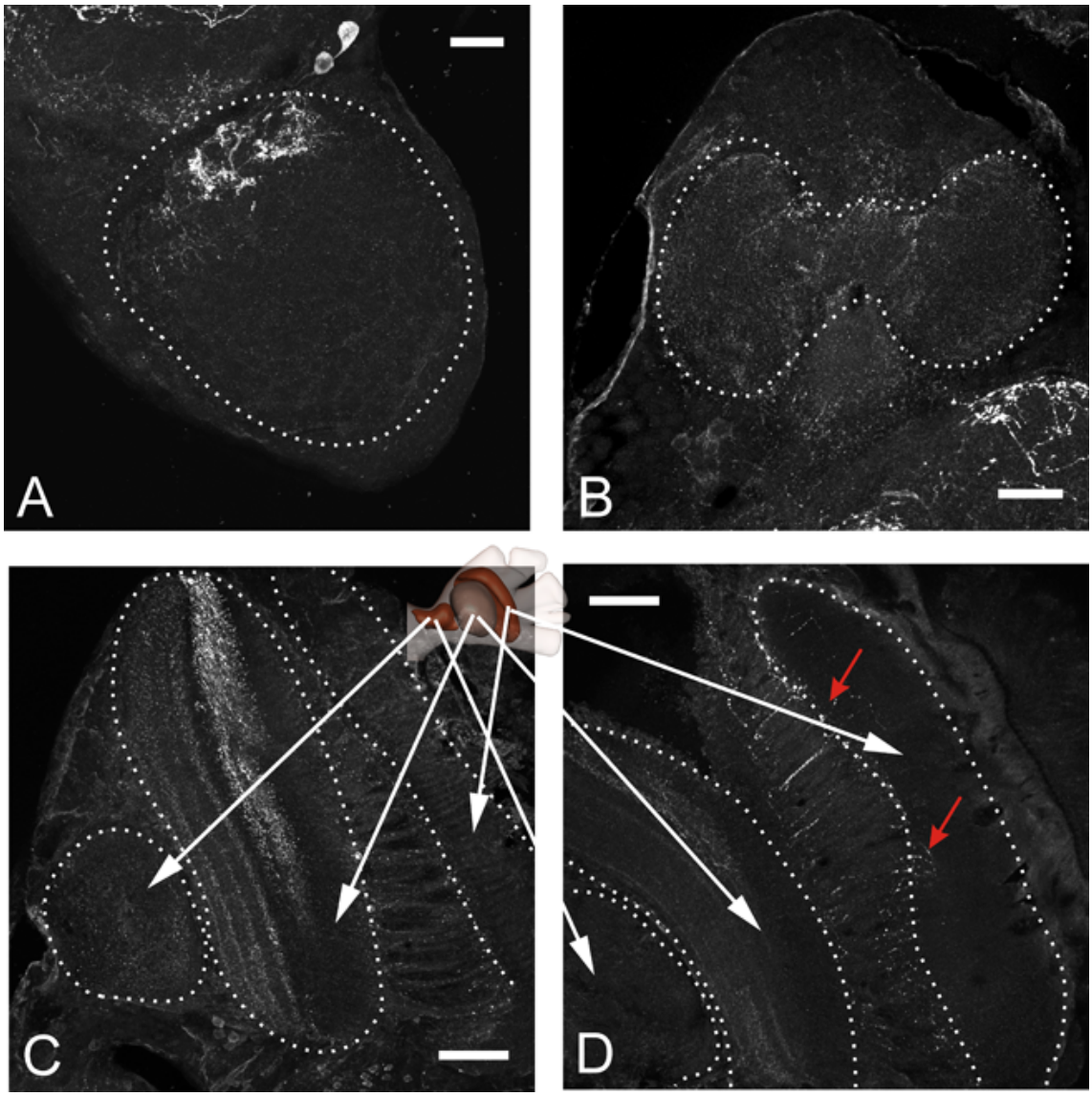


Figure 3.18: General distribution of crustacean cardioactive peptide in the grasshopper brain. A: Frontal section through the antennal lobe (AL). Only sparse staining could be detected in the AL. This staining originates from three to four neurons on the dorsal part of the AL. B: Frontal section through the calyx of the mushroom body. No staining could be detected in this brain structure. C and D: Frontal sections through the optic lobes (OL). Most of the detected CCAP-ir was found in the medulla, while the lobula and the lamina (white arrows in D indicate monopolar cells that connect lamina and medulla) exhibit only faint labeling. Scale bars = 100 μm in C and D; 50 μm in A and B

No staining could be detected in the mushroom body (Fig. 3.18B), which seems to be the only brain region that is completely free of CCAP immunostaining.

3.1.10.2 CCAP in the central complex

CCAP-ir in the central complex (Fig. 3.19 and Fig. 3.20) resulted from two different types of tangential neurons and one type of pontine neurons. One type of tangential neurons had its cell bodies in a cortex region lateral to the calyces (white arrowheads in Fig. 3.19C and D). They sent of fibers that projected to the central body through the anterior bundle (blue arrowheads in Fig. 3.19C and D) and innervated the anterior lip of the CBU (white arrows in Fig. 3.19A and C and Fig

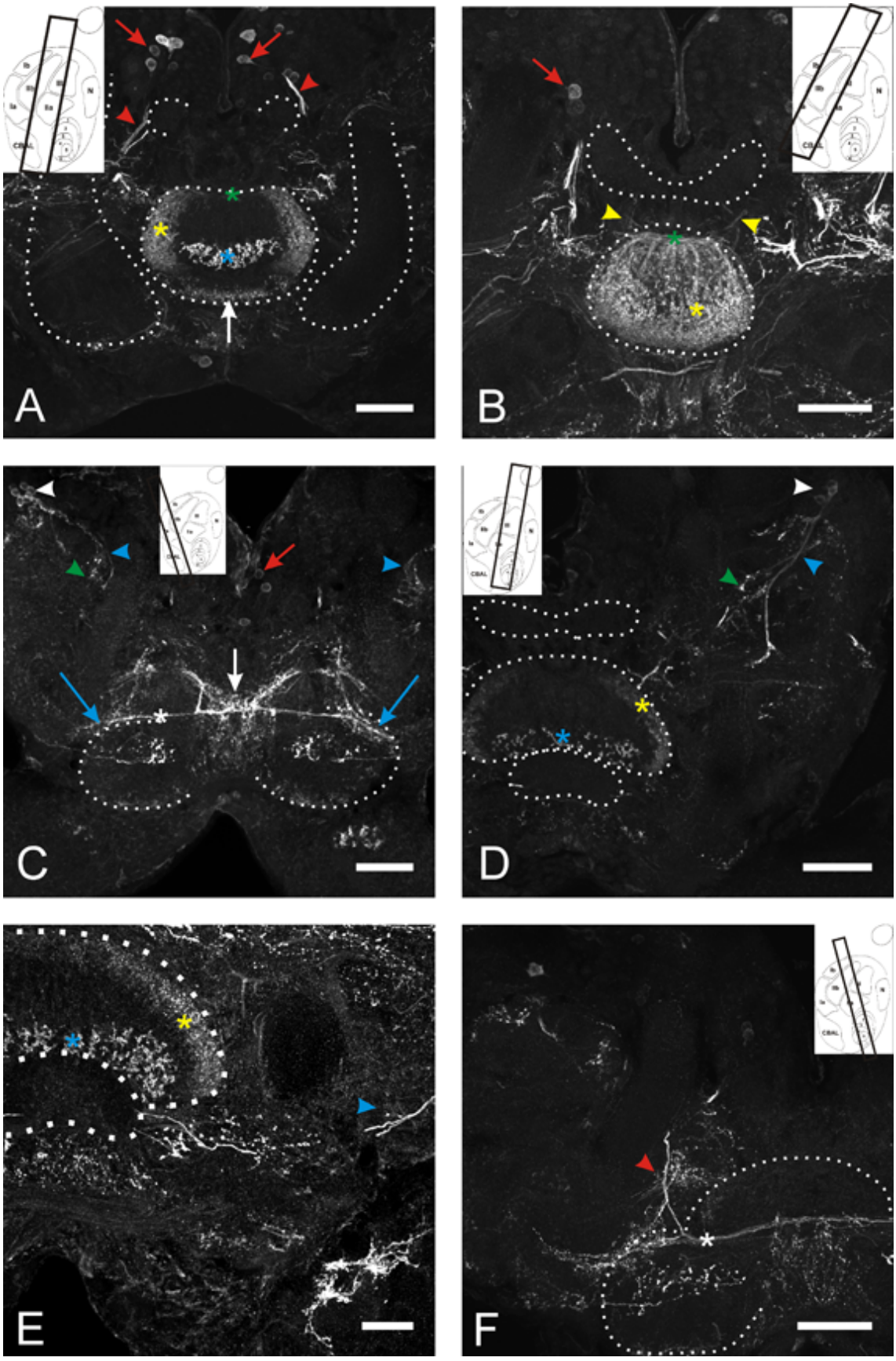
3.20A and B). These neurons seemed to give off fine sidebranches in the superior median protocerebrum (green arrowheads in Fig. 3.19C and D). From the anterior lip, fibers continued through the isthmus tract to innervate the LAL (blue arrows in Fig. 3.19C). According to the terminology of CCAP-neurons in *S.gregaria* introduced by Dircksen and Homberg (1995), these neurons can be regarded as cp-7-like neurons.

The second type of neuron (cp-8-like) had its somata in the anterior pars intercerebralis (red arrows in Fig. 3.19A-C and Fig. 3.20B). The fibers of these neurons left the PI as part of the w-bundle and passed the CB laterally (red asterisks in Fig. 3.19A, B and F) to innervate the LAL where they gave rise to fine sidebranches which could not be traced further. The main neurite continued through the IT (white asterisks in Fig. 3.19C and F) to the CBU, which it entered through the posterior groove (dark blue arrowhead in Fig. 3.19B). In the CBU the fibers gave rise to fan-shaped varicose processes throughout layer IIa (blue asterisks in Fig. 3.19A, D and E and Fig. 3.20A and B).

A third type of CCAP-ir neurons that could be detected belonged to the pontine type (cp-9-like). The somata of these neurons were clustered in the PI together with the somata of the cp-7-like neurons. The fibers of these neurons joined the w-, x-, y-, and z-bundles (only faintly labeled, yellow arrowheads in Fig. 3.19B) and entered the CB through the PCh (green asterisks in Fig. 3.19A and B and Fig. 3.20A) and terminated in layer I of the CBU (yellow asterisks in Fig. 3.19A, B, D and E and Fig. 3.20A and B). Fibers connecting the different columns of layer I could be detected in the dorsal face (green arrows in Fig. 3.20A). No staining could be detected in the PB the CBL or the noduli.

Control experiments in which the primary antibody was omitted showed no staining in the described regions (see appendix).

Figure 3.19: Frontal sections showing the distribition of crustacean cardioactive peptide-immunoreactivity in the central complex. A: Frontal section of the median protocerebrum with cp7-like neurons innervating the central body. The somata of cp7-like neurons are located in the lateral pars intercerebralis (PI, red arrows). Fibers leave the PI and join the w-bundle to pass the CB at the lateral edges (red arrowheads). In the central body these neurons innervate layer IIa (blue asterisk). The greens asterisk indicates the posterior chiasm (PCh) through which cp9-like neurons enter the CB to innervate layer I (yellow asterisk). B: Frontal section through the median protocerebrum showing CCAP-ir in layer I of the CB (yellow asterisk). The fibers enter the CB through the PCh (green asterisk). The red arrow indicates a CCAP-positive cp7-like soma in lateral PI. C-E: Frontal section of the median protocerebrum showing the arborization pattern of cp8-like neurons. The somata of these neurons are located lateral to the calyces of the mushroom bodies (white arrowhead). The fibers of these neurons ran as part of the anterior bundle towards the CB (blue arrowheads) and the gave off fine sidebranches in the superior median protocerebrum (SMP, indicated by green arrowhead). Cp8-like neurons innervate the anterior lip of the CBU (CBAL). From the CBAL the neurons run through the isthmus tract (white asterisk) to terminate in the lateral accessory lobes (LAL, blue arrows). Red arrows indicate somata of cp9-like neurons in the PI that innervate layer I of the CBU (yellow asterisks in D and E). F: Frontal section through the median protocerebrum showing the arborization pattern of cp7-like neurons in the LAL and CB. Fibers that passed at the lateral edge of central body the innervated the LAL where they gave rise to sidebranches that could not be traced further. From the LAL the fibers run through the isthmus tract to innervate layer IIa of the CBU. Scale bars = 100 μm in A, B, C, D and F; 50 μm in E.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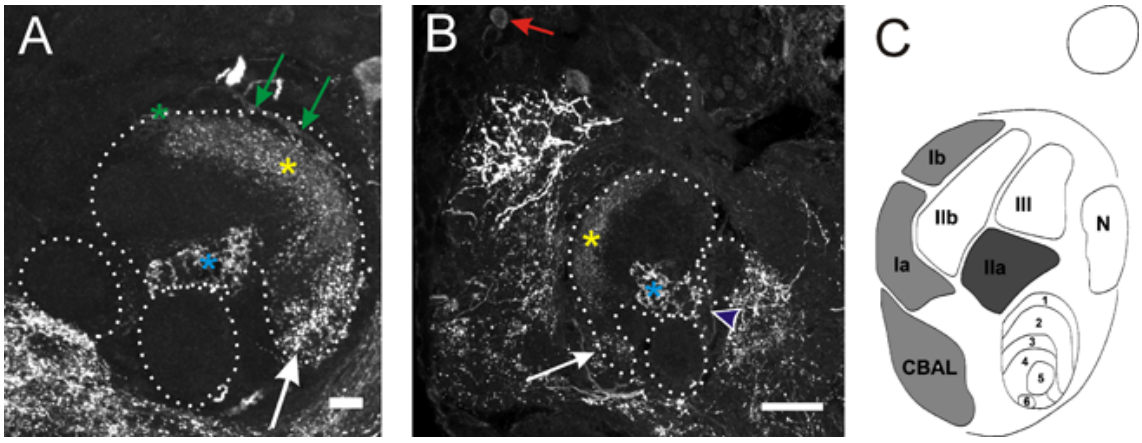

Figure 3.20: Sagittal sections showing the distribution of crustacean cardioactive peptide-immunoreactivity (CCAP) in the central complex. A and B show the distribution of CCAP in the different layers of the upper division. Most intense staining could be detected in layer IIa (blue asterisks) originating from cp8-like neurons. This type of neurons enters the CBU via the posterior groove (indicated by dark blue arrowhead in B). Labeling of layer I (yellow asterisks) stems from cp9-like neurons. These type of neuron has its somata in the anterior pars intercerebralis (red arrow in B) and enter the CB via the posterior chiasm (green asterisk in A). Fibers connecting the different columns of layer I could be detected in the dorsal face (green arrows in A). Staining in the anterior lip (white arrows) results from cp7-like neurons. C: Schematic drawing of a sagittal section through the CB. Regions highlighted in gray contain dopaminergic fibers, darker shades of gray indicate stronger staining intensities (modified from Homberg 1991 and Müller et al. 1997). Scale bars = 50 μm in B; 20 μm in A

3.1.11 Allatostatin

Allatostatins (ASTs) are structurally diverse peptides that were originally shown to inhibit biosynthesis of juvenile hormone in the corpora allata of a variety of insect species (Woodhead et al. 1989, Kramer et al. 1991, Lorenz et al. 1995, Bellés et al. 1999). In locusts, allatostatins (AS) belong to a family of ten neuropeptides, that share the common carboxyterminus Y/FXFGL/Iamide (Bendena et al. 1999). The ten peptides of this family are encoded by a single gene (Schoofs et al., 1998). Allatostatins (sometimes also called schistostatins) have a myoinhibitory effect on the locust oviduct. Unlike in other invertebrates, they exert no effect on the biosynthesis of juvenile hormones from the corpora allata (CA). This is interesting with respect to the fact that juvenile hormones released from the CA modulate the receptivity and reproduction related sound production of female grasshoppers. For detecting allatostatins in the brain of the grasshopper, a monoclonal antibody raised against allatostatins from the cockroach *Diploptera punctata* was used, which has been used as a marker for AS-positive cells in a variety of invertebrate species (Stay et al. 1992, Yoon and Stay 1995, Utting et al. 2000, Loesel et al. 2002).

3.1.11.1 General distribution of Allatostatin in the brain

The distribution of allatostatin in the brain of *Ch.b.* (Fig. 3.21) is very similar to the one already described for the locust *S. gregaria* (Vitzthum et al., 1996). AS-ir could be found in the OL (Fig. 3.21B), the AL (Fig. 3.21A) and the MBs Fig. 3.21C and D). Staining in the OL was strongest in particular layers of the medulla

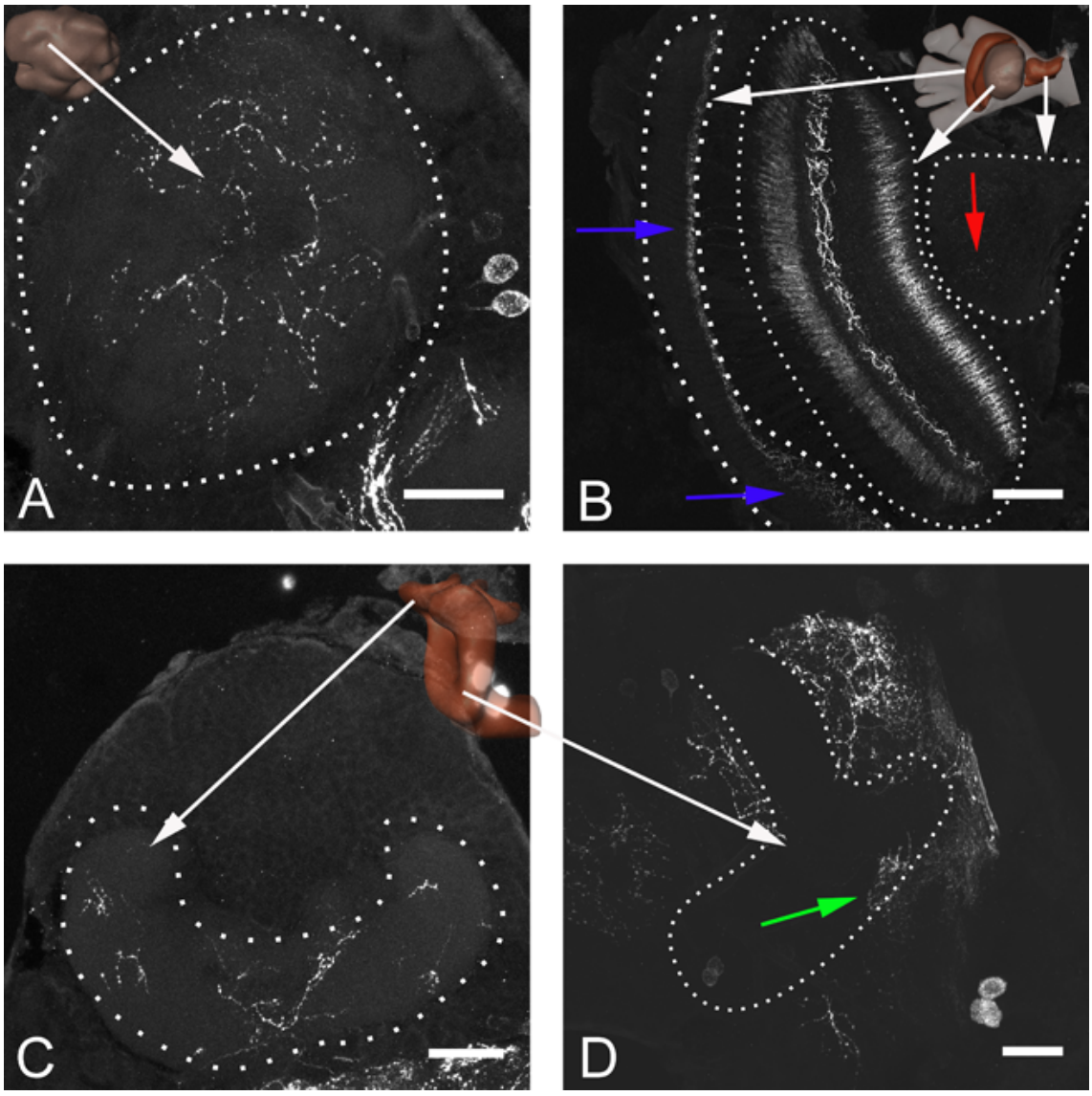

Figure 3.21: General distribution of allatostatin in the grasshopper brain. A: Frontal section through the antennal lobe showing sparse but distinct immunolabeling. B: Frontal section through an optic lobe. Most intense labeling appeared in inner layers of the medulla, while only weak immunofluorescence could be detected in the lamina (blue arrow) and the lobula (red arrow). C and D: Frontal sections through the mushroom body calyx (C) and β-lobe (D). Both regions exhibit AS-ir, extending throughout the entire calyx while only certain parts of the β-lobe are invaded by AS-containing processes (green arrow).

and also in a more distal layer of the lamina (blue arrows in Fig. 3.21B). Only faint immunostaining could be detected in the lobula (red arrow in Fig. 3.21B). While immunostaining in the OL was quite strong, only weak AS-ir could be found the AL and the MB. Staining in the MB seemed to be restricted to the calyces (Fig. 3.21C) and one specific column of the mushroom bodies (green arrow in Fig. 3.21D).

3.1.11.2 Allatostatin in the central complex

Allatostatin was found in three main types of tangential neurons innervating the upper division of the central body (Fig. 3.22). The fibers of all three types had their origin in somata located in the inferior-median protocerebrum (white arrows

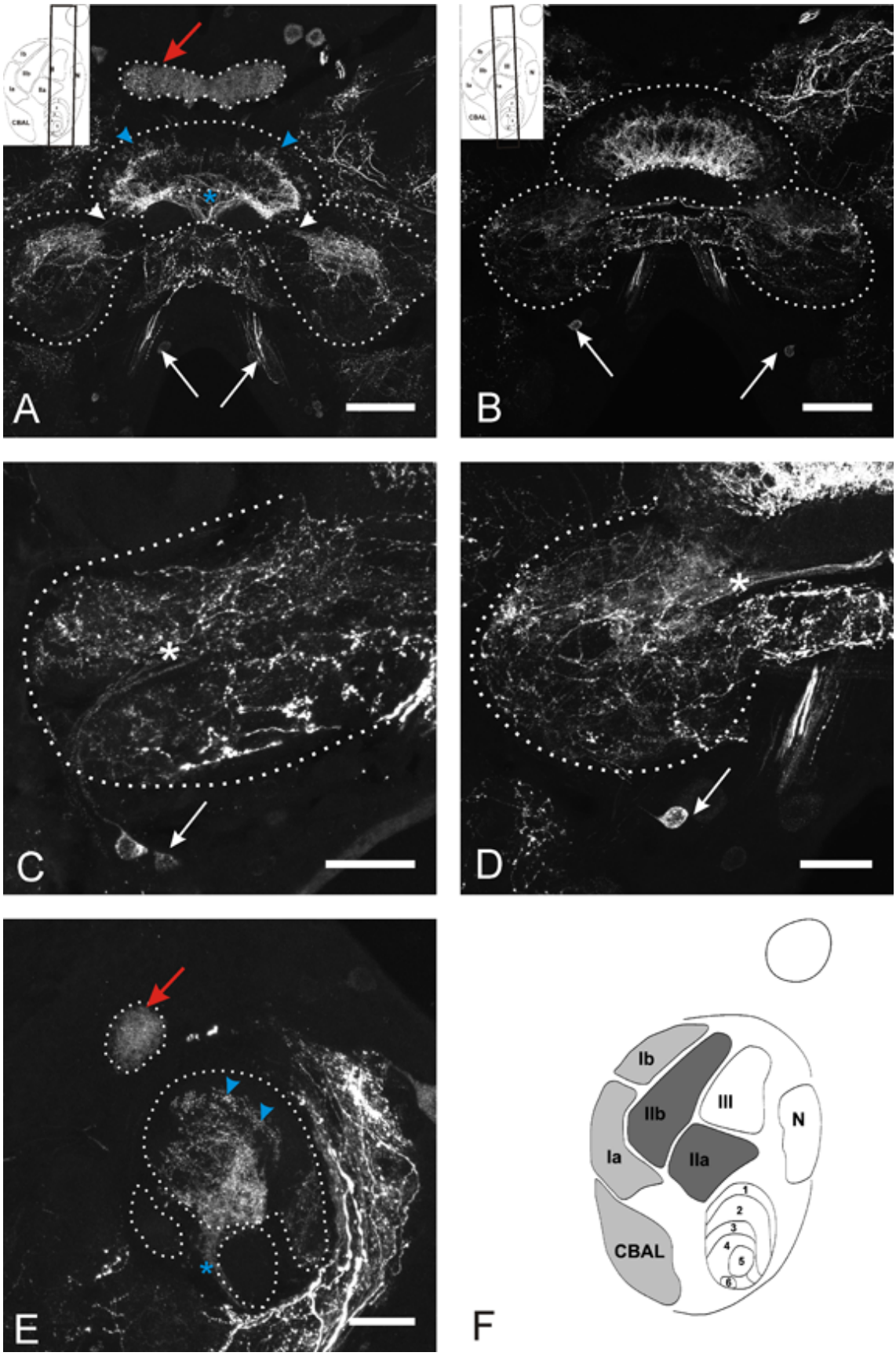
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Fig. 3.22A-D).

The first type of tangential neuron sent their fibers through the isthmus tract (white asterisks in Fig. 3.22C and D) to the central body, entered via the posterior groove (blue asterisks in Fig. 3.22A and E) and innervated layer IIb of the CBU. These neurons gave off fine sidebranches within the LAL (Fig. 3.22C and D), but these could not be traced in detail because they intermingled with other fibers. According to the terminology for AS-positive neurons in the CX introduced by Vitzthum et al. (1996), these neurons are most likely homologous to AST-2 neurons.

The second type of AS-ir neuron (AST1-like) had a similar projection pattern but did not enter the CB through the posterior groove. Instead it entered at a more dorsal position, called the isthmus (white arrowheads in Fig. 3.22A). The fibers run dorsal to the noduli and innervate layer IIa of the CBU.

A third type of neuron (AST3-like), whose somata could not be traced, was identified on the basis of its labeled projections. The neurites of these fibers entered the CBU at a similar position as the AST1-like fiber type but innervated parts of layer I (blue arrows in Fig. 3.22A and E). Additional strong staining could be detected in the PB (red arrowheads in Fig. 3.22A and E), but this staining did not originate from fibers that also innervated the CB. No staining could be detected in the noduli.

Control experiments in which the primary antibody was omitted showed no staining in the described regions (see appendix).

3.1.12 Allatotropin

Allatotropins (ATs) are a family of highly conserved insect neuropeptides, named for their stimulating effect on the corpora allata in certain insect species (Elekonich and Horodyski 2003). The hormonal and peripheral effects of these peptides are diverse and seem to vary in different insect species (Elekonich and Horodyski 2003). Apart from their high abundance in peripherally projecting neurons, immunocytochem-

Figure 3.22: Distribution of allatostatin in the central complex. A and B: Frontal section through the median protocerebrum showing the posterior central complex. Strong immunostaining could be detected in the protocerebral bridge (red arrow in A and E), but this staining does not originate from neurons which also innervate the CB. Staining in the CBU originates from somata in the inferior median protocerebrum (white arrows). AST2-like neurons enter the CBU through the posterior groove to give rise to fan-shaped arborization in inner layers, while AST1-like neurons entered through the isthmus (white arrowheads). Labeling in parts of layer I (blue arrowheads) stems from AST3-like neurons. C and D: Frontal sections through the lateral accessory lobe. Fibers of tangential neurons innervating the CB have their somata in the inferior median protocerebrum (white arrows), project through the isthmus tract (white asterisks) and give off fine sidebranches in the dorsal and ventral shell that could not be traced further. E: Sagittal section through the central complex. Labeling could be found in the entire layer II, while only parts of layer I exhibit AS-ir (blue arrowheads). Labeling in layer III is also detectable but its origin is unknown. AST2-like fibers entering the CB through the posterior groove can be detected (blue asterisk). Additionally, staining could be detected in neurons innervating the CB, but these neurons did not arborize in the CB. F: Schematic drawing of a sagittal section through the CB. Regions highlighted in gray contain AS Darker shades of gray indicate stronger staining intensities (modified from Homberg 1991 and Müller et al. 1997).Scale bars = 100 μm in A and B; 50 μm in C, D and E

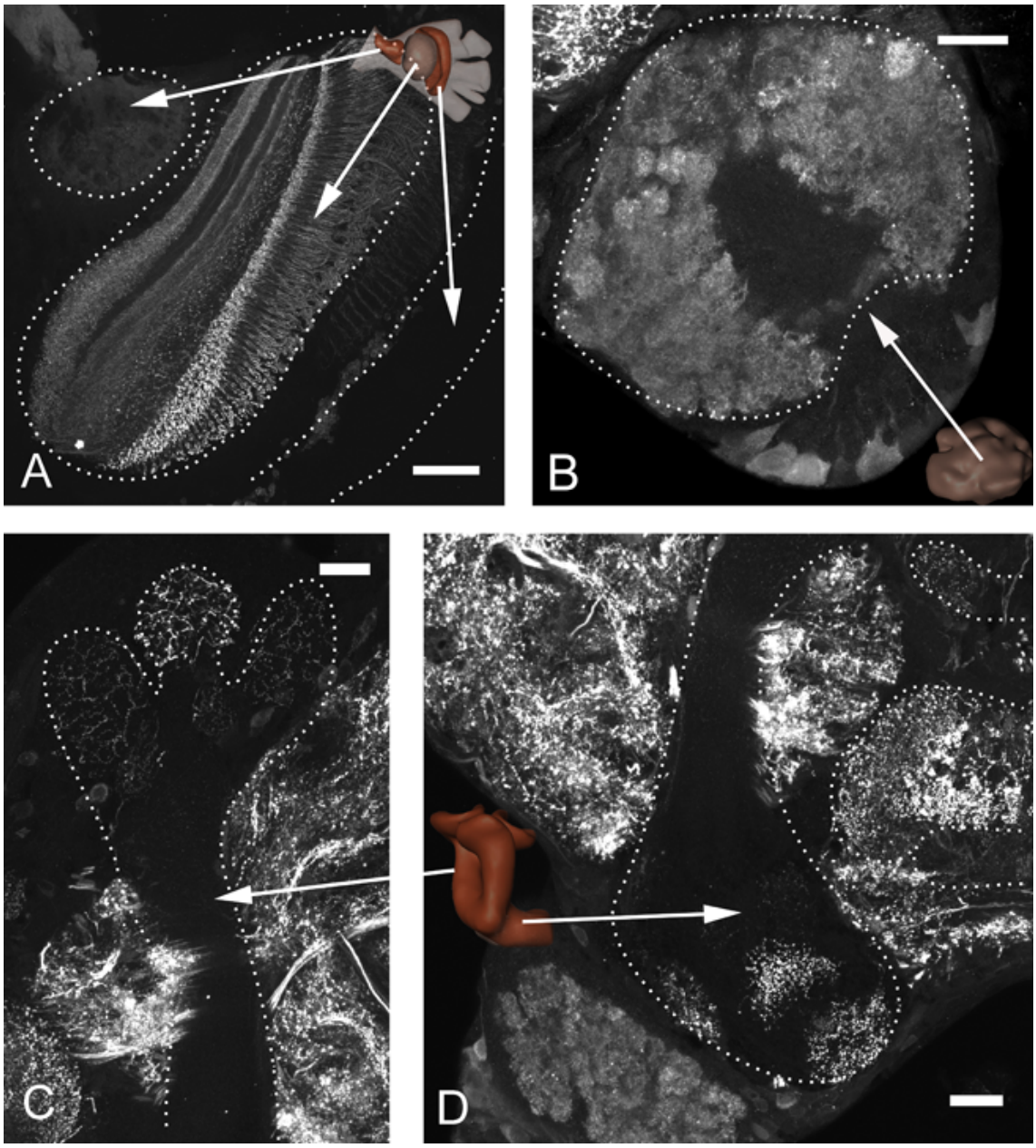


Figure 3.23: General distribution of allatotropin in the grasshopper brain. A: Frontal section through the optic lobe. Strong allatotropin-immunoreactivity (AT-ir) could be detected in the medulla, while the lamina and the medulla showed only weak staining. B: Frontal section of the antennal lobe. AT-ir could be found in local interneurons that innervated all glomeruli. C and D: Frontal sections through the mushroom bodies. While intense labeling could be seen in the calyx and specific parts of the β-lobe the pedunculus was free of staining. Scale bars = 100 μm in A; 50 μm in B-D.

istry and gene expression studies reported the presence of allatotropin containing interneurons in the brain and ventral nerve cord of various insect species from different taxa (Zitnan et al. 1993; 1995, Rudwall et al. 2000, Truesdell et al. 2000, Tu et al. 2001, Park et al. 2002, Homberg et al. 2004). To label neurons of the central body that contain allatotropin, I used an antibody that has been demonstrated to reliably detect allatotropin in locusts (Homberg et al. 2004).

3.1.12.1 General distribution of allatotropin in the brain

Allatotropin (AT) could be detected in various brain neuropiles (Fig. 3.23) and showed a similar distribution compared to the locust *S. gregaria* (Homberg et al. 2004). Strong immunostaining appeared in the optic lobes (Fig. 3.23A), namely the medulla while the lamina as well as the lobula contained weak AT-ir. In the mushroom bodies the calyces (Fig. 3.23C) and the β-lobe (Fig. 3.23D) are invaded by AT-positive processes. Only diffuse staining could be detected in the AL (Fig. 3.23B).

3.1.12.2 Allatotropin in the central complex

All areas of the central complex except for the lower division show AT-immunostaining (Fig. 3.24). One striking feature of the staining was that labeling of somata and fibers outside the neuropiles was very weak, while fibers within the neuropiles were stained quite strong. This made statements about the exact projection pattern of the neurons difficult. Immunostaining in the central body largely originates from two types of bilaterally symmetric tangential neurons.

One type had its somata in the anterior pars intercerebralis (white arrowheads in Fig. 3.24A, C, E and F). These fibers had a similar projection pattern as one of the dopamine/TH- (DP2-like) and CCAP-positive neuron type (cp8-like). They ran as part of the w-bundle along the lateral edges of the CB and innervated the LAL (blue arrowheads in Fig. 3.24A and C). From the LAL the neurites projected towards the CB entered it dorsal to the noduli (green arrowheads in Fig. 3.24E and F) and finally give rise to fan-shaped arborizations in the CBU (white arrows in Fig. 3.24A and D). According to the terminology of AT-cells in the CX (Homberg et al. 2004), this neuron type can be regarded as MT1-like.

The other type of AT-ir neuron resembled MT2-neurons described in the locust brain ((Homberg et al. 2004) although the location of the cell bodies could not be determined. The neurites of these cells innervate the superior median protocerebrum, run along the α-lobes of the mushroom bodies (red arrowheads in Fig. 3.24B and D) and enter the CB trough the posterior groove (blue asterisks in Fig. 3.24D-F) to innervate the layers of the upper division. Weak immunostaining could be detected in the PB (green arrows in Fig. 3.24A, D, E and F), but this labeling did not originate from fibers connecting the PB to the CB. No staining could be detected in the noduli.

Control experiments in which the primary antibody was omitted showed no staining in the described regions (see appendix).

3.1.13 LemTRP and GABA

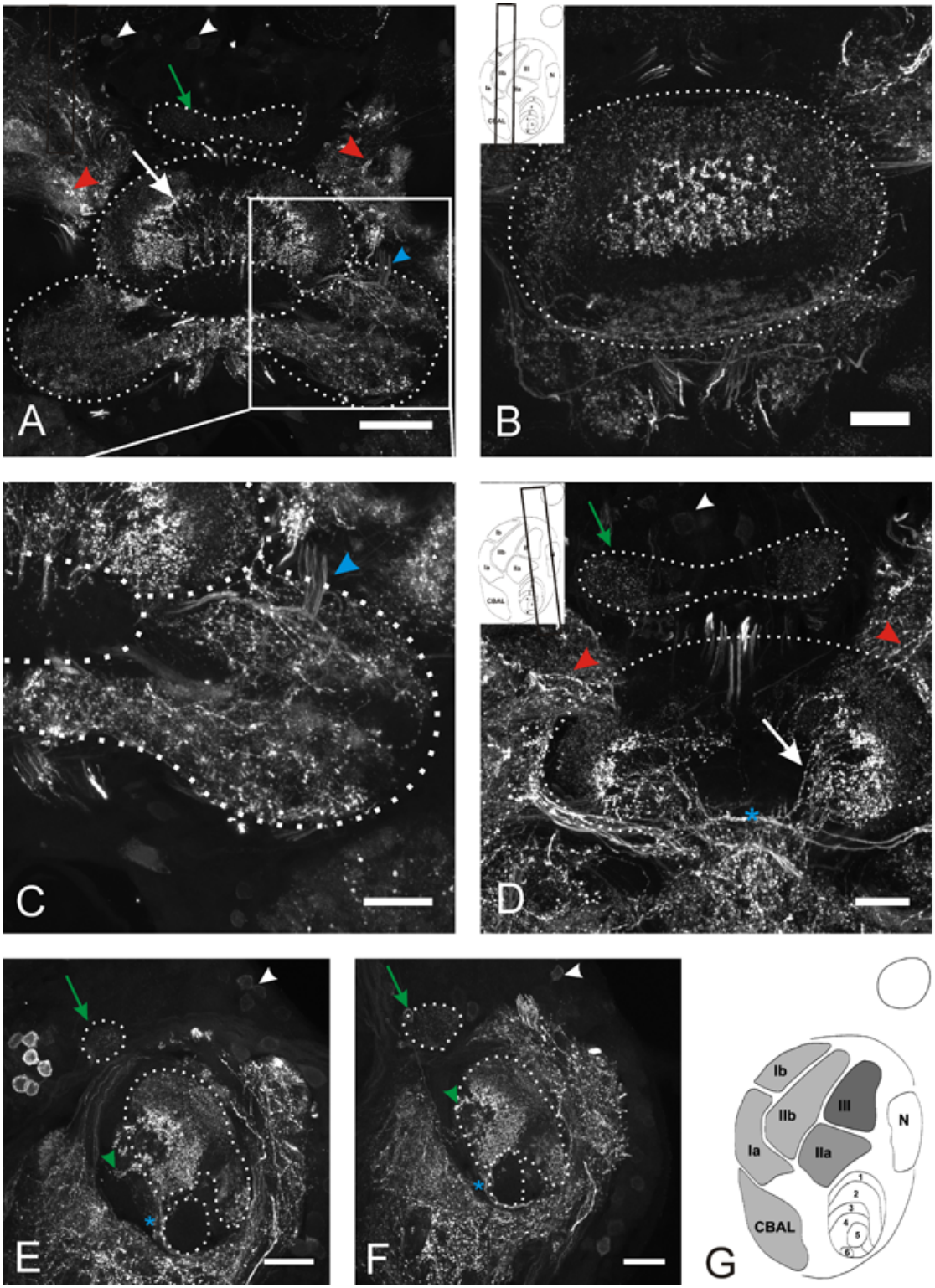
One of the main reasons to stain against neuropeptides was, to look for overlapping patterns with neurotransmitters that have been shown to influence the performance of grasshopper sound production. The antisera against LemTRP provided a very similar pattern of immunostaining as anti-GABA labeling, especially in the lower division. Colocalisation of LemTRP and GABA has been confirmed in the locust *S. gregaria* (Vitzthum and Homberg 1998). Similar labeling patterns could be detected in the grasshopper *Ch.b.* (Fig. 3.25). Strong colocalisation could be detected in all layers of the CBL (white arrowheads in Fig. 3.25A_3-C_3). Colocalised fibers originated from somata in the inferior median protocerebrum (white arrows in Fig.3.25A_3 and D_3). The fibers ran through the IT (yellow arrowheads in Fig. 3.25A_3 and D_3) and gave off sidebranches in the LT (red arrowheads in Fig. 3.25A_3 and D_3) and the MO (blue arrowheads in Fig. 3.25A_3 and D_3). They entered the CB through the posterior groove and innervated all layers of the CBL (Fig. 3.25C_3). According to the terminology for tangential neurons of the CBL in *S. gregaria* (Müller et al. 1997), these neurons can be regarded as homolog to TL2- and TL3-neurons.

3.2 Tracing Studies

3.2.1 Incorporation of dextranes injected into the central body

To gain further insight into the information flow within the central body that mediates the control of grasshopper sound production, tracing studies were performed (Fig. 3.26). For this, fluorescently-coupled dextranes were co-injected at spots where muscarine reliably induced stridulation. In locusts, it could be shown that dextranes are primarily incorporated by post-synaptic sites (Heinrich et al. 1998a,

Figure 3.24: Distribution of allatotropin in the central complex. A: Frontal section through the median protocerebrum showing the posterior central complex. Weakly stained allatotropin-immunoreactive (AT-ir) somata of MT1-like neurons could be detected in the pars intercerebralis (white arrowheads). The fibers of these neurons pass the CB laterally and enter the LAL (blue arrowheads). Fibers of MT2-like neurons entering the CB from the superior median protocerebrum (SMP) can be seen (red arrowheds). Only faint staining was detectable in the PB (green arrow). B: Frontal section through the anterior CB displaying AT-ir in layer IIa of the CBU. C: Frontal section showing the LAL of A at higher magnification. Fibers of MT1-like neurons that enter the LAL can be seen (blue arrowheads). These fibers gave off fine sidebranches that could not be traced further. D: Frontal section through the posterior CX showing the noduli. Fibers of MT1-like neurons could be detected that enter the CB through the posterior groove (blue asterisk) and gave rise to fan-shaped arborisations in layer I and II of the CBU. White arrowhead indicates weak AT-ir positive somata of MT1-like neurons in the PI. Red arrowheads point to MT2-like neurons, coming from the SMP. Only faint labeling could be detected in the PB. E and F: Sagittal sections through the central complex. Most intense labeling was detected in layer II of the CBU, while layer I and the CBAL exhibited only weak staining. MT1-like neurons enter the central body via the posterior groove, while fibers of MT2-like run dorsal to the noduli. Green arrows point to the PB, which shows only weak AT-ir and white arrowheads to MT1-like somata in the PI. F: Schematic drawing of a sagittal section through the CB. Regions highlighted in gray contain AS, darker shades of gray indicate stronger staining intensities (modified from Homberg 1991 and Müller et al. 1997). Scale bars = 100 μm in A; 50 μm in B-F.


A
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C
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G
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IIb
III
N
Ia
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6

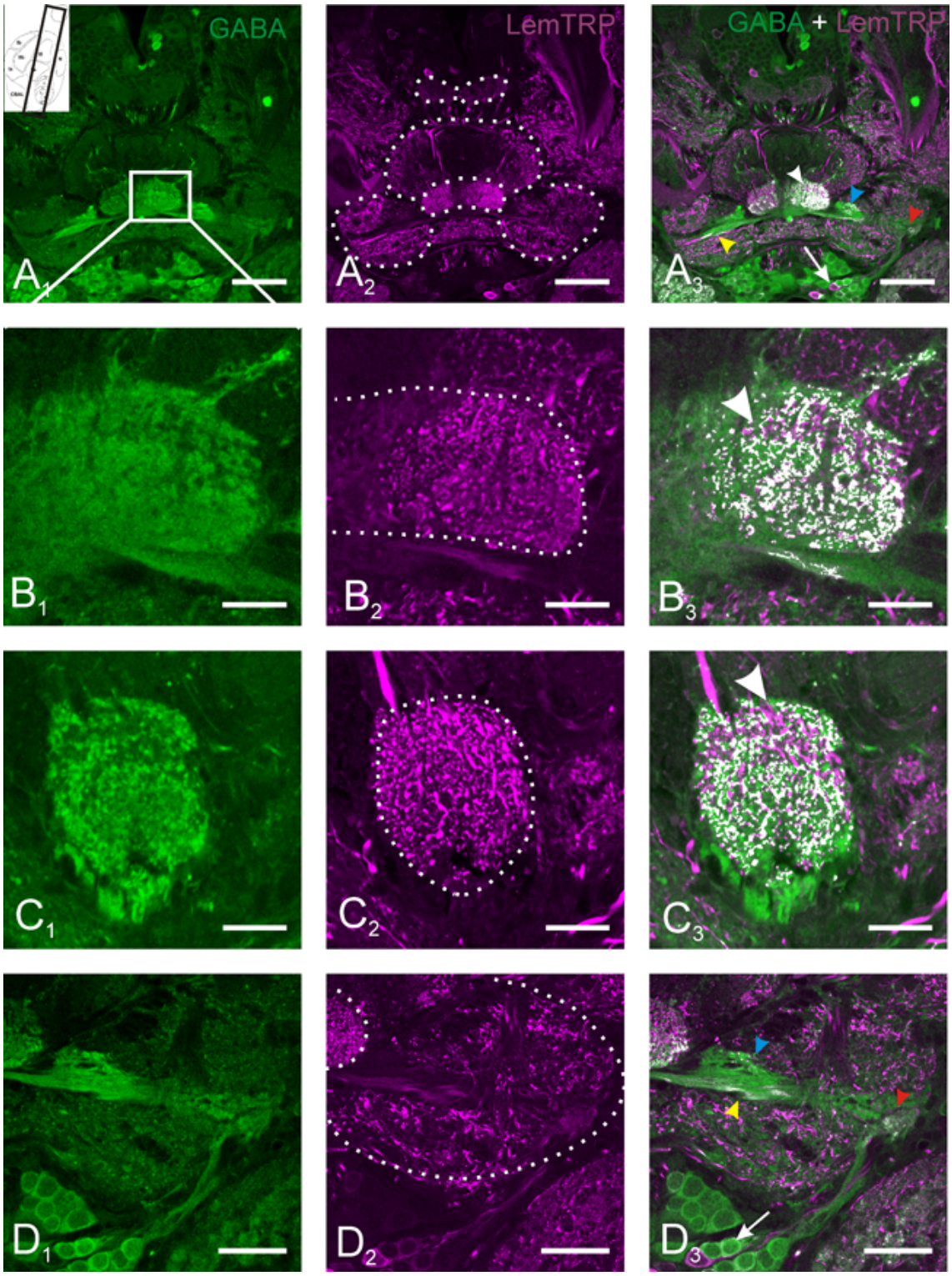


Figure 3.25: Double staining of GABA (green) and LemTRP (magenta) in the central complex. Figure displays frontal sections through the (A), the lower division of the central body (B) and the lateral accessory lobe (C) and a sagittal section through the lower division. Colocalisation was found in tangential neurons with somata in the inferior-median-protocerebrum (white arrows) innervating the lower division (white arrowheads). Colocalisation could be detected in all layers of the CBL. Outside the CB colocalisation could be detected in the median olive (blue arrowheads) the isthmus tract (yellow arrowheads) and the lateral triangle (red arrowheads). Scale bars = 100 μm in A; 50 μm in D; 20 μm in B and C.

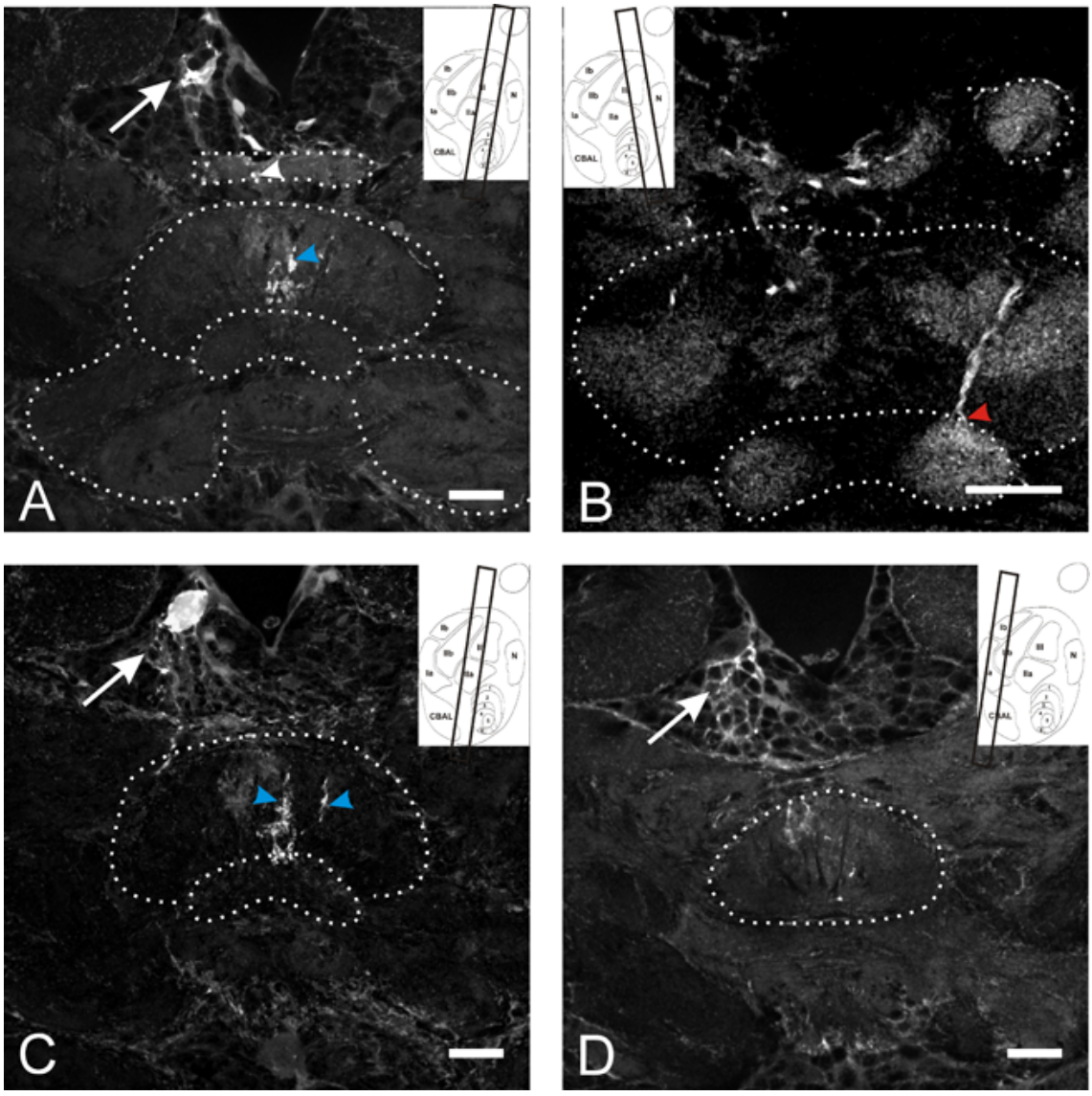


Figure 3.26: Labeled neurons incorporated dextranes that were co-injected to a site within the central complex, where muscarine stimulated sound production. A-D: Frontal sections through the central complex at different planes. Stained somata could be detected in the pars intercerebralis (PI, white arrows in A and C). Columnar fibers running through the posterior vertical bundle were labeled in the central body (indicated by blue arrowheads in A and C). These neurons seemed to terminate in columns of the lower division (red arrowhead in B) Additionally, columnar fibers terminating in layer I of the upper division incorporated the dextrane (D).

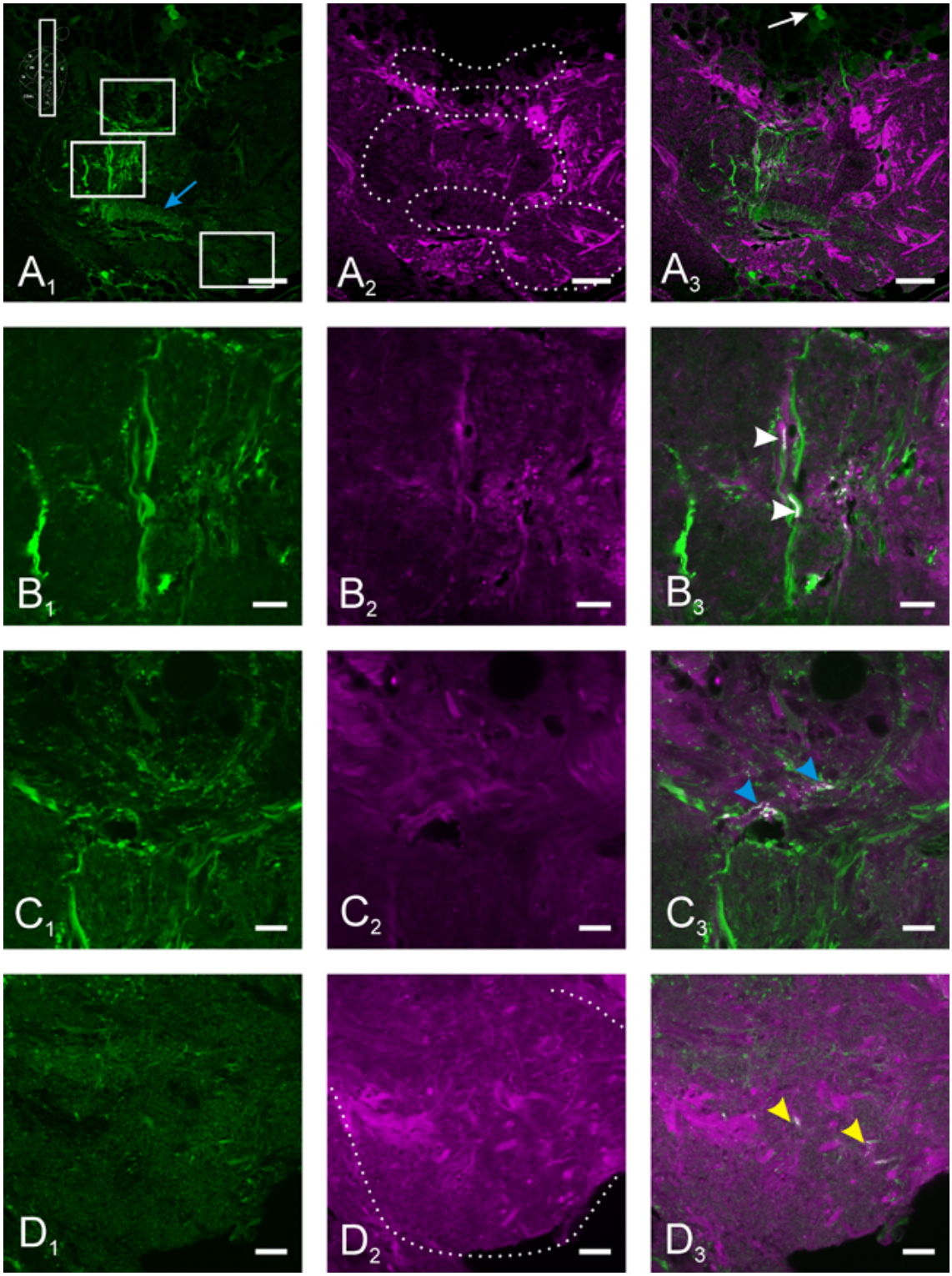
Lakes-Harlan et al. 1998). Injections of dextranes labeled exclusively neurons with projections in the central body. These experiments support the assumption that by injections of muscarine, neurons of central body are directly activated. Unfortunately, the staining intensity was not equally distributed throughout the entire neuron. Incorporated dextranes rather accumulated in specific compartments of neurons especially in fibers within the central body and in the respective cell bodies. This is probably due to the fact that the dye has been diluted to strong in thinner regions of the neurites provide a strong fluorescent signal. The number of labeled neurons varied between 3 to 14. Most of the neurons labeled by this method belonged to the columnar type, similar to the ones described earlier to be mAChR-ir positive. The somata of these neurons were located in the pars intercerebralis (white

arrows in Fig. 3.26A, C and D). These Neurons sent off neurites that have small sidebranches in the PB (white arrowhead in Fig. 3.26A). From the PB, the neurites leave ventrally and enter the CB via the posterior chiasm passed through inner layers of the CBU (blue arrowheads in Fig. 3.26A and C). Faintly stained terminals of the neurons could be detected in single columns of the CBL (red arrowhead in Fig. 3.26B). Staining within the lateral accessory lobe was very weak and could not be followed. In some experiments tangential neurons could also be stained. This happened very rarely and was probably due to injury of the neurons.

3.2.2 Colocalisation of mAChR and incorporated dextranes

To confirm that the neurons which incorporated the dextranes may have mediated stimulating effect of muscarine on sound production, double staining of mAChRs incorporated dextranes was performed (Fig. 3.27). These labeling studies revealed that dextranes were incorporated by mAChR-expressing columnar neurons, suggesting that these neurons indeed mediate the initiation of sound production upon muscarine stimulation. These neurons belong to the type of mAChR containing neurons (MR1) that have been described in (Fig. 3.1). Unfortunately the somata of mAChR-expressing neurons were stained in the preparations for colocalisation, but somata of neurons that incorporated the dextranes could be detected in the PI (white arrows in Fig. 3.27 A_3), the region were the somata mAChR containing neurons are located. The fibers of the neurons that contained both labels ran through the PVB of the CBU and arborized in the CBL. Colocalisation could be detected in the CBU (white arrowheads in Fig. 3.27B_3), the PCh (blue arrowheads in Fig. 3.27C_3) and also in the LAL (yellow arrowheads in Fig. 3.27D_3). Additionally, staining was also detected in tangential neurons of the CBL (indicated by blue arrows in 3.27A_1), but this is probably due to injury of those neurons and no colocalisation with mAChR could be detected in these neurons. Most interesting in these staining is, that I was able to detect staining in the LAL. This staining was found in parts of the dorsal shell of the LAL and not, as it would be expected for this type of neurons, in the lateral triangle.

Figure 3.27: Frontal sections through the central complex labeled for intravitally taken up dextranes (green) and mAChR (magenta). Neuron that incorporated the dye have their somata in the pars intercerebralis (white arrow in C3). Colocalisation could be detected in columnar fibers that pass through the upper division (white arrowheads in B3) and enter the central body through the posterior chiasm (blue arrowheads in C3). Staining outside the central complex could be detected in the lateral accessory lobes, but no statement about their specific arborisation pattern can be made. Additionally the dye also seems to be taken up by an tangential neuron innervating the CBL, probably due to injury during impalement of the electrode into the CB. Scale bars = 100 μm in A; 10 μm in B, C and D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3.3 Cell culture

In order to enable in vitro studies on cultured central complex neurons that contribute to the control of sound production, fluorescently labeled dextranes were injected to sites where sound production could be stimulated. After dissociation and culturing of grasshopper brain neurons, the fluorescent label was used to identify those neurons that had post-synaptic structures at the site of pharmacological stimulation and probably were directly stimulated by the injected drug. To establish this method, one has first to demonstrate, that (1) neurons in primary culture express the mAChRs, (2) intravitally labeled neurons can be detected in primary cell culture and (3) that dextrane-labeled neurons in culture also express mAChRs. The following results from studies on primary cell cultures of grasshopper brains were generously provided by Christian Heck.

3.3.1 MAChRs in cell culture

As a basis for further functional studies in cell cultures of grasshopper brain neurons, one has to show that neurons in culture express the mAChR, to rule out the possibility that responses of the cell to muscarine are mediated through unspecific side-effects by activation of other G-protein coupled receptors. For this I performed antibody staining against the mAChR (Fig. 3.28) with the same antiserum that was used to label mAChRs on brain sections (Fig. 3.1, 3.2 and 3.27). It was possible to detect mAChRs on the surface of neurons in primary cell culture (Fig. 3.28B, C and D). Initially, the receptor is expressed in cell body membranes, but with increasing time of cultivation, muscarinic receptors are contained in the membrane of regenerating neurites (white arrowheads in Fig. 3.28D). Cell counts revealed, that on average 22% of the neurons in culture express mAChRs.

3.3.2 Detection of intravitally labeled neurons in cell culture

The next step was to show that neurons that incorporated the fluorescently coupled dextrane can be identified in primary cultures. For detection of intravitally labeled neurons in culture the dextrane-injected brain had to be incubated in a humid chamber for 24 to make sure that some dye is accumulated the soma. This procedure markedly decreased the sterility of the primary culture. Cell cultures obtained from brains that were subjected to this labeling procedure could only be maintained for up to three days. Nevertheless, neurons that incorporated the dextranes in a preceeding pharmaco-behavioral experiment could clearly be identified in dissociated cell cultures (Fig. 3.29). Most cultures made from brains that were injected with

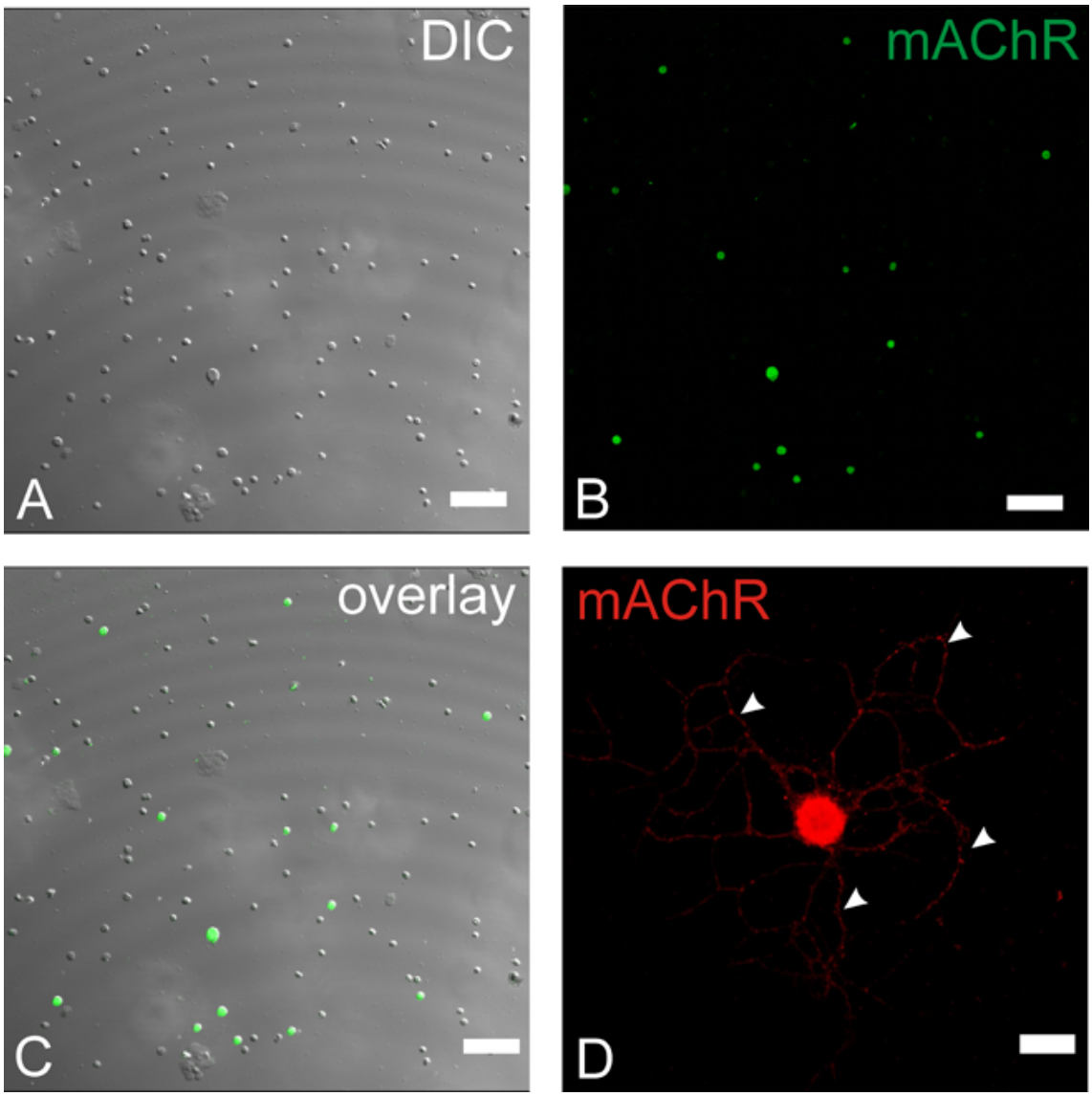


Figure 3.28: Neurons in primary cell culture express the mAChR. A: Differential-interference-contrast (DIC) image of primary cell culture derived from the brain of *Ch.b.*. B: Immunostaining against mAChR. Neurons that express the receptor are stained green primary cell culture. Neurons positive for the receptor are stained green. C: Overlay of A and B, showing that only a subset of the neurons in primary cell culture express the muscarinic receptor. D: Confocal image of a grasshopper brain neuron after 17 days in culture. Presence of mAChRs is visualzed by red fluorescence. Compared to younger cultures in A-C which express the receptor only on the soma surface, mAChR-ir could also be detected on regenerated neurites (white arrowheads). Scale bars = 80 μm in A-C; 20 μn in D

either tetramethyrhodamine- or rhodamine-green-dextrane contained few fluorescent cells. The number of labeled cells in culture varied between 0 to 7.

3.3.3 Colocalisation of mAChRs with incorporated dextranes in cell cultures of grasshopper brain neurons

As a last crucial step, it had to be demonstrated that the intravitally labeled neurons in culture also express mAChRs. For this, an antibody staining was performed on primary cultures made from brains in which fluorescently labeled dextranes were injected at effective stimulation sites (Fig. 3.30). In all cultures tested (n=3), TMR-

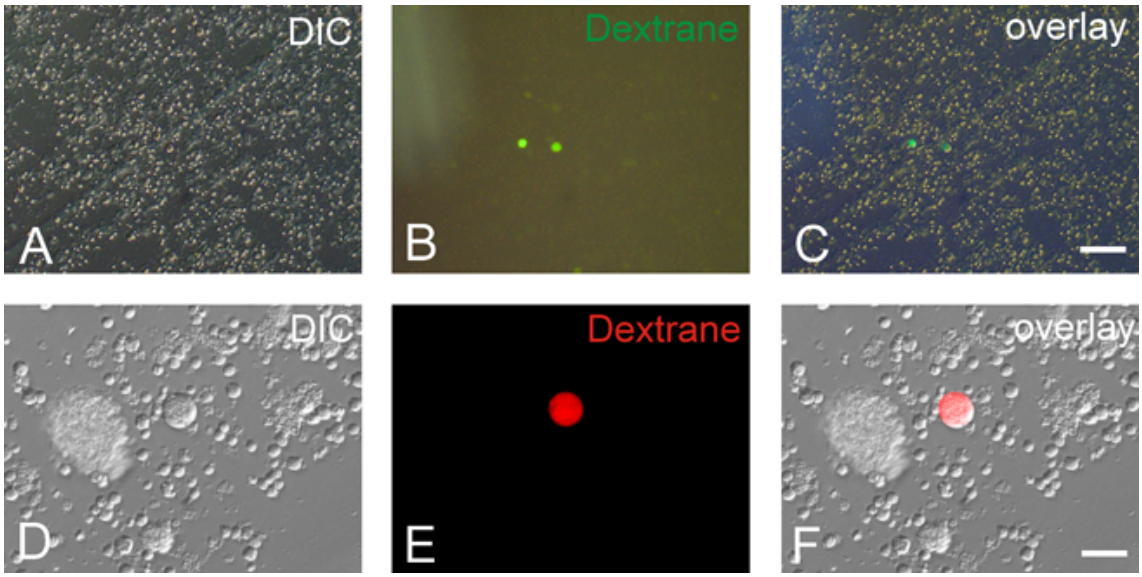


Figure 3.29: Central complex neurons in primary cell culture that incorporate the dextrane after successful stimulation of sound production through muscarine. A and D: Differential-interference-contrast (DIC) image of neurons in primary cell cultures at different magnifications. B and E: Fluorescent images of the same culture containing neurons that incorporated fluorescent dextranes (dextrane-rhodamine-green in B and tetramethylrhodamine-dextrane in E) following injections to effective sites in the central body. C and F: Overlay, showing that only a small fraction of the neurons in culture contained the fluorescent dextrane. Scale bars = 80 μm in C; 20 μm in F. bars

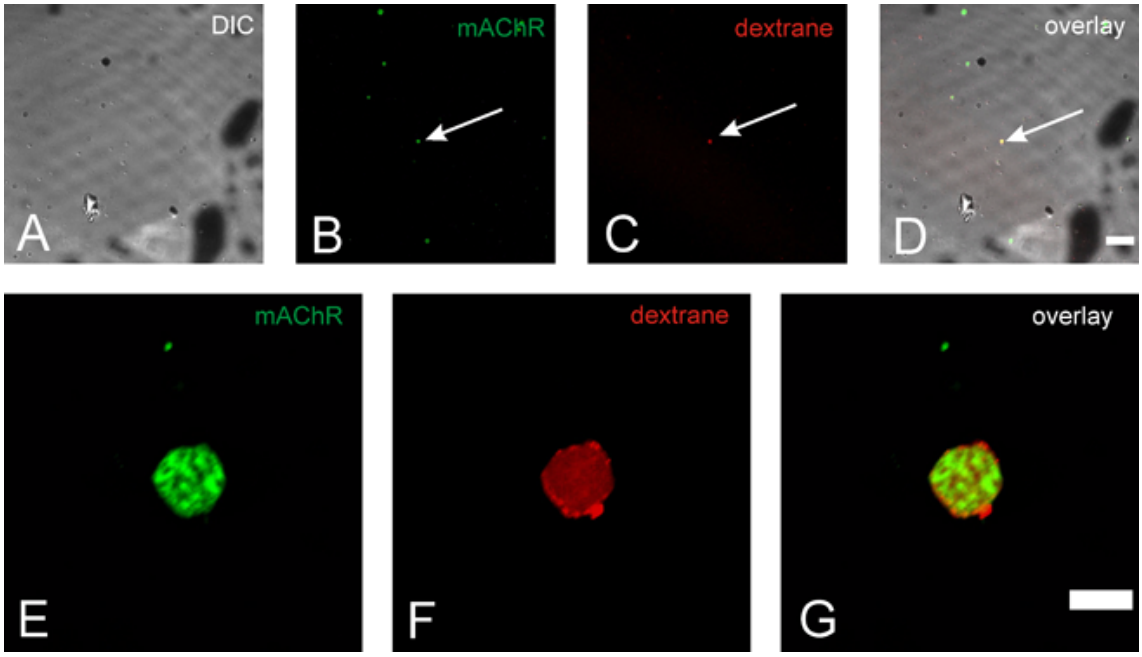


Figure 3.30: Colocalisation of mAChR and central complex neuron that incorporated the fluorescent dye after successful stimulation of sound production with muscarine. A: Differential interference contrast (DIC) image of a primary cell culture. B: Fluorescent staining against mAChRs in primary cell culture. mAChR-positive neurons are shown in green. C: Fluorescent image of an intravitally labeled neuron in the same culture. D: Overlay of A-C showing that the intravitally labeled neuron expressed the mAChR (white arrow). E-G: Confocal image of a single cell that incorporated the fluorescent dextrane (E) that shows colocalisation (G) with the mAChR (F). Note that the intravitally labeled cells show an evenly distribution of the dye, while mAChR occurs in patches. Scale bars = 80 μm in D; 10 μm in G.

labeled neurons also exhibited mAChR-ir. Staining of mAChR could be detected on a subset of TMR-labeled neurons, similar to the situation already found in brain sections. Another finding was, that staining of mAChR on the cell surface seemed to occur in patches (Fig. 3.30E), while the fluorescently labeled dextrane was evenly distributed throughout the entire soma (Fig. 3.30F).

3.4 Pharmacology

Injections of neuroactive substances into the central body have been proven to be a valuable tool to decipher their role in the control of acoustic communication (Heinrich et al. 1997; 1998b; 2001a;b, Wenzel et al. 2002, Hoffmann et al. 2007). So far the main attention has been focused on classical neurotransmitters (ACh and GABA) as well as one unconventional transmitter (NO). To gain a further understanding I wanted to examine the modulatory role of biogenic amines. Biogenic amines have been shown to modulate a variety of different behaviors (Neckameyer 1998, Bainton et al. 2000, Li et al. 2000, Rothenfluh and Heberlein 2002, Kume et al. 2005, Chang et al. 2006,) and to influence activity states of entire networks (Ayali and Harris-Warrick 1999, Bucher et al. 2003, Christie et al. 2004, Goaillard et al. 2004)

3.4.1 Tyramine

Figure 3.31: Synthesis of TA and OA. The amino acid tyrosine is the starting point for the synthesis of both compounds, TA and OA. TA is the direct decarboxylation product of tyrosine. This is achieved through the tyrosine decarboxylase (TDC). OA is produced from TA through the tyramine-β-hydroxylase (TβH). Modified from Roeder, 2005

Tyramine (TA) is the invertebrate analogue to epinephrine and the precursor of octopamine (OA), the invertebrate counterpart to norepinephrine. It is generated from tyrosine by the tyrosine-decarboxylase (TDC) (Fig. 3.31). For a long time TA was not regarded as a transmitter but just as the precursor of OA. Only recently a role for TA as a neural transmitter has been established (Saudou et al. 1990). The function of TA is not fully understood so far, but emerging evidences point to a role as functional antagonist to OA (Roeder 2005), which is supported by the fact that the two transmitters activate opposing second-messenger cascades in all systems studied so far (Roeder 2005). While OA is coupled positively to the adenylyl-cyclase (AC) and releases Ca^{2+} from internal stores (Battelle and Kravitz 1978, Han et al.

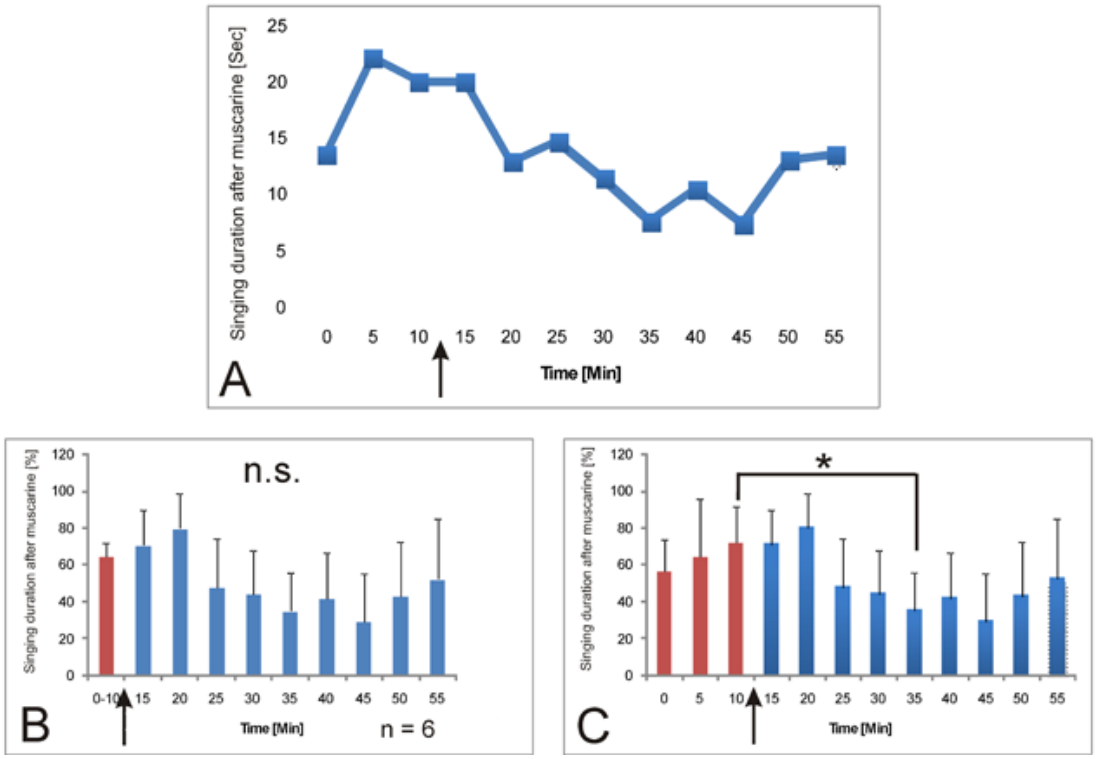


Figure 3.32: Tyraminergic influence on muscarine dependent stridulation. A: A typical result from a single experiments is shown. In this experiment repeated injections of muscarine ($10^{-}3$) are given at intervals of five minutes. The subsequent singing duration was calculated and plotted in seconds on the y-axis. After 12 minutes (between the third and fourth muscarine injection, indicated by black arrow) a single pulse of tyramine (TA, $10^{-}3$) was applied to the same site. After the injection of TA the singing duration in response to muscarine clearly decreases with a minimum around 40 minute. At the end of the experiments, the singing duration recovers but stays still slightly under the duration before the application of TA. B and C: For statistical analysis the singing duration was normalized for each experiments (see Material and Methods) and and data from different experiments were pooled. Responses to muscarine are shown as red bars, while responses to muscarine after TA application are shown as blue bars B: The mean responses before the application of TA were compared to the responses to muscarine after TA application. This test revealed no significant differences. C: If the same analysis was performed by using not the mean response but instead comparing the single responses before and after the application of TA, a significant difference could be detected in the singing duration between the injection of muscarine at minute 10 compared to minute 35 ($p<0.05$, indicated by *)

1998, Bischof and Enan 2004, Balfanz et al. 2005), TA inhibits the AC (Blenau et al. 2000, Cazzamali et al. 2005).

3.4.1.1 Pharmacological effects of tyramine on muscarine induced sound production

Stimulation of muscarinic AChRs in the central complex promotes sound production via activation of adenylyl cyclase and accumulation of cAMP. Because all characterized TA-receptors studied so far reduced intracellular levels of cAMP, it was tested whether application of TA could reduce the duration of muscarine-stimulated sound production in *Ch.b.*. For this muscarine was injected in regular intervals of five minutes and the time the animal spent singing in response to the pulse was measured.

Between the 3rd and the 4th pulse of muscarine (minute 12, indicated by black arrows in Fig. 3.32A, B and C) a single pulse of tyramine was applied. Fig. 3.32A shows the typical result of one of these experiments. Directly after the application of tyramine the muscarine stimulated singing duration decreased. This decrease has its maximum between 15 and 25 minutes after tyramine was injected. At the end of the experiment, the singing duration recovered and reached similar values as before TA-injection. For statistical analysis, the results of all experiments were pooled, normalized to 100% (n=6) and a Friedmann-Test was performed ($p>0.05$). For detecting an effect of TA on muscarine dependent stridulation, two criteria were used. (1) the mean response to muscarine before the application of TA was calculated and compared with every response to muscarine after the TA injection (3.32B). In this case no statistical significance could be found. (2) The responses at each time interval before and after the injection of TA were compared to each other (3.32). This was only performed if the three responses to muscarine before the application of TA were not different from each other (Friedmann-Test $p>0.1$). Through this test it could be shown that on average, the response at minute 35 (23 minutes after the TA injection) showed a significant decrease in singing duration compared to the last muscarine injection before the tyramine application at minute ten ($p<0.05$, Friedmann-Test followed by an Wilcoxon-test). From the two criteria described above (1) was regarded as the stronger. Significant differences obtained by (2) have to be considered as weak.

To further prove that this effect is specific for tyramine and not mediated by unspecific binding of TA to other biogenic amine receptors, blocking experiments were performed, in which a mixture of TA and the TA-antagonist yohimbine were injected. The idea was that by injections of a mixture of the natural agonist (TA) and an antagonist (yohimbine), the effect mediated by the activation of TA-receptors should be blocked, or at least diminished by the antagonist. The same protocol as already described for tyramine experiments were used. Fig. 3.33A shows the typical result of one of these experiments. Muscarine induced singing duration remained the same after the application of TA/yohimbine. After normalizing and pooling, the results of all experiments ($n = 5$, Fig.3.33B) statistical analysis showed that the responses at the different time points were not significantly different from each other (Friedman-Test $p>0.1$). Therefore it seems likely, that the influence of tyramine on muscarine dependent stridulation is mediated by activation of TA-receptors.

3.4.2 Dopamine

As already mentioned above, dopamine is a potent neuromodulator that affects various behaviors in invertebrates Murdock (1971), Bicker and Menzel (1989), Mustard

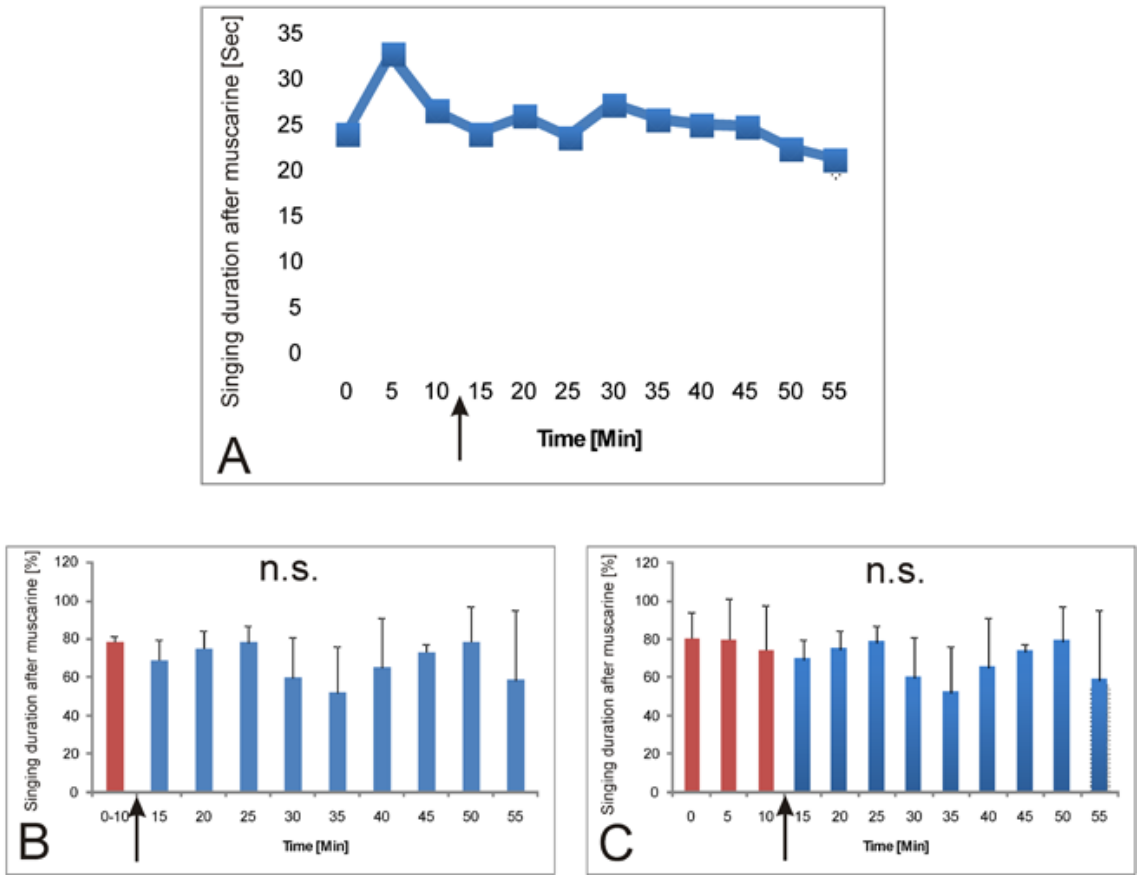


Figure 3.33: Block of tyraminergic effects through yohimbine. A: A typical result from a single experiment is shown. After the application of the mixture of tyramine and yohimbine (both (10^{-3}), indicated by black arrow) no obvious reduction of muscarine (10^{-3}) stimulated singing duration could be detected. If statistical analysis were performed on the normalized and pooled data no significant differences could be detected, neither between the mean response of muscarine (B) nor between the single pulses (C) and the response after the application of TA and yohimbine.

et al. (2005) as well as in vertebrates (Ikemoto and Panksepp 1999, Floresco 2007, Hoebel et al. 2007). Due to its high abundance in the central complex (Fig. 3.12 and 3.13) and the fact that increasing dopaminergic transmission in fruit fly, leads to increased courtship behavior (Andretic et al. 2005), we investigated how injections of dopamine into the central body affect the control of sound production.

3.4.2.1 Pharmacological effects of dopamine on the control of sound production

In insects it could be shown, that dopamine binds to two major types of receptors, D1-like and D2-like, named after their vertebrate counterparts. D1-like receptors are positively coupled to both the AC- and PLC-second-messenger pathway. D2-like receptors on the other hand inhibit the AC (Mustard et al. 2005). Dopamine could have influenced the performance of sound production in both direction, either suppressing or promoting it. To test for a potential inhibitory influence of dopamine,

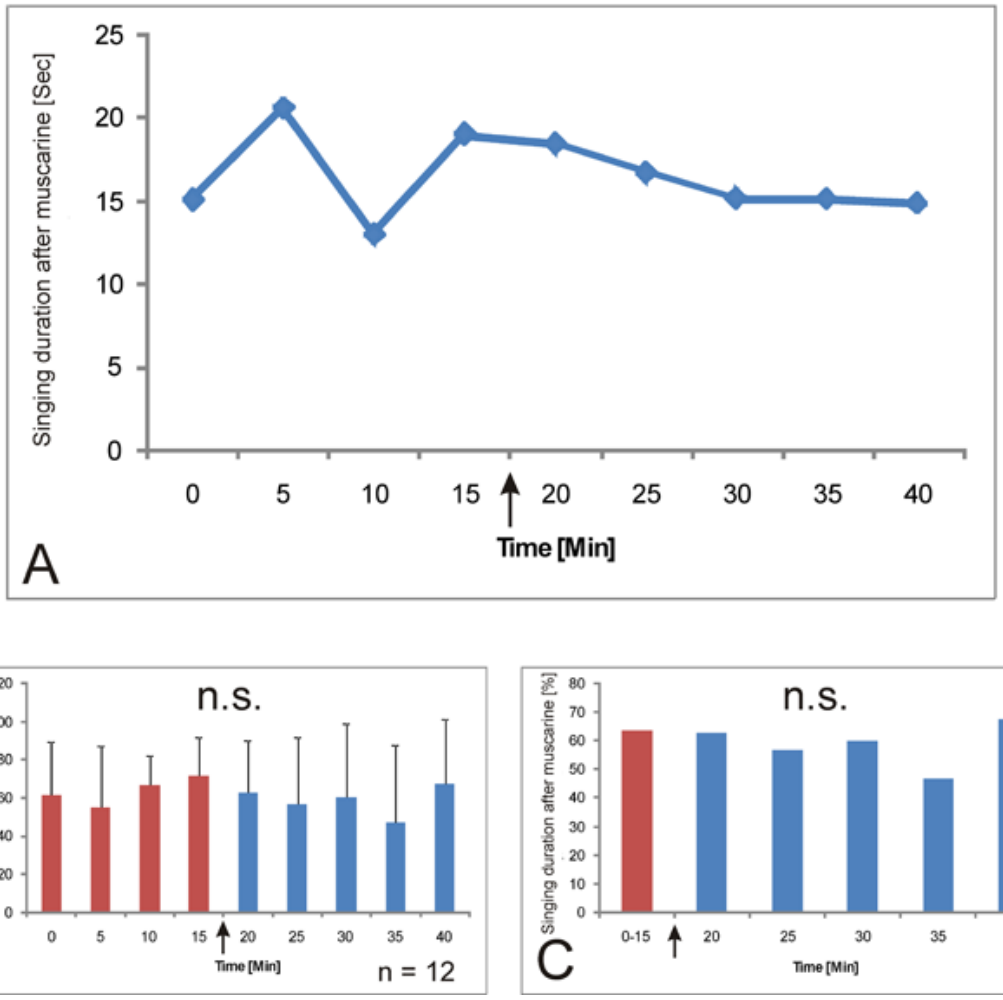


Figure 3.34: Dopamine has no inhibitory influence on muscarine stimulated sound production. To test for an potential inhibitory influence of dopamine (10^-3) on the duration of muscarine (10^-3) dependent sound production, a similar protocol as for tyramine was applied. A shows the typical result of one of those experiments. Application of dopamine did not change the duration of muscarine dependent sound production at any time after its application. Statistical analysis of normalized and pooled data from 12 experiments also revealed no significant differences (B and C, responses to muscarine before application of TA are depicted by red bars and after by blue bars)

a similar protocol as for TA was used. Fig.3.34 shows the typical result of one of these experiments. No obvious difference can be seen in the responses to muscarine before and after the dopamine injection. Statistical analysis of the averaged data also revealed no significant differences (Friedmann-Test $p>0.1$).

To test for a possible excitatory effect of DA I used two protocols. In the first protocol I searched for a stimulation site where muscarine reliably induced stridulation. Once such a spot was found, I waited for ten minutes and applied eight/nine injections of DA from the other chamber with a time interval of two/five minutes. Fig. 3.35A and B show two typical results from those experiments with either two (A) or five minutes (B) time intervals between DA injections. I waited for ten minutes before starting the series of DA-stimuli to ensure complete decay of muscarine-induced

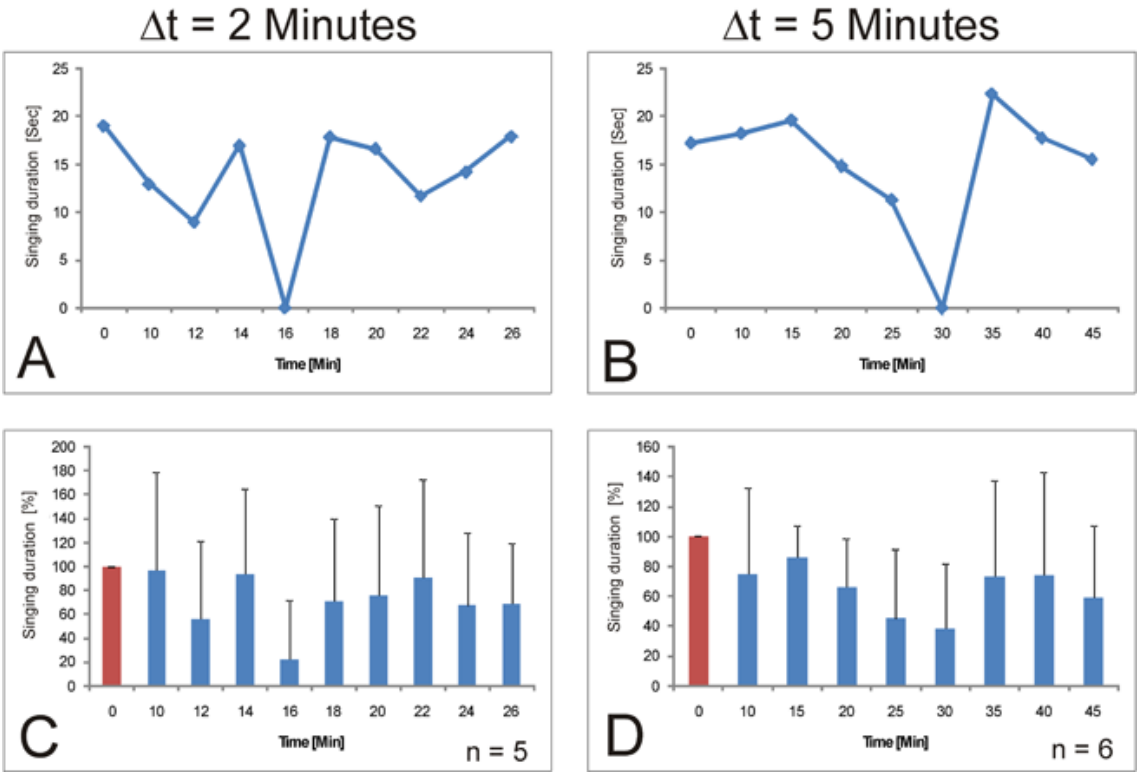
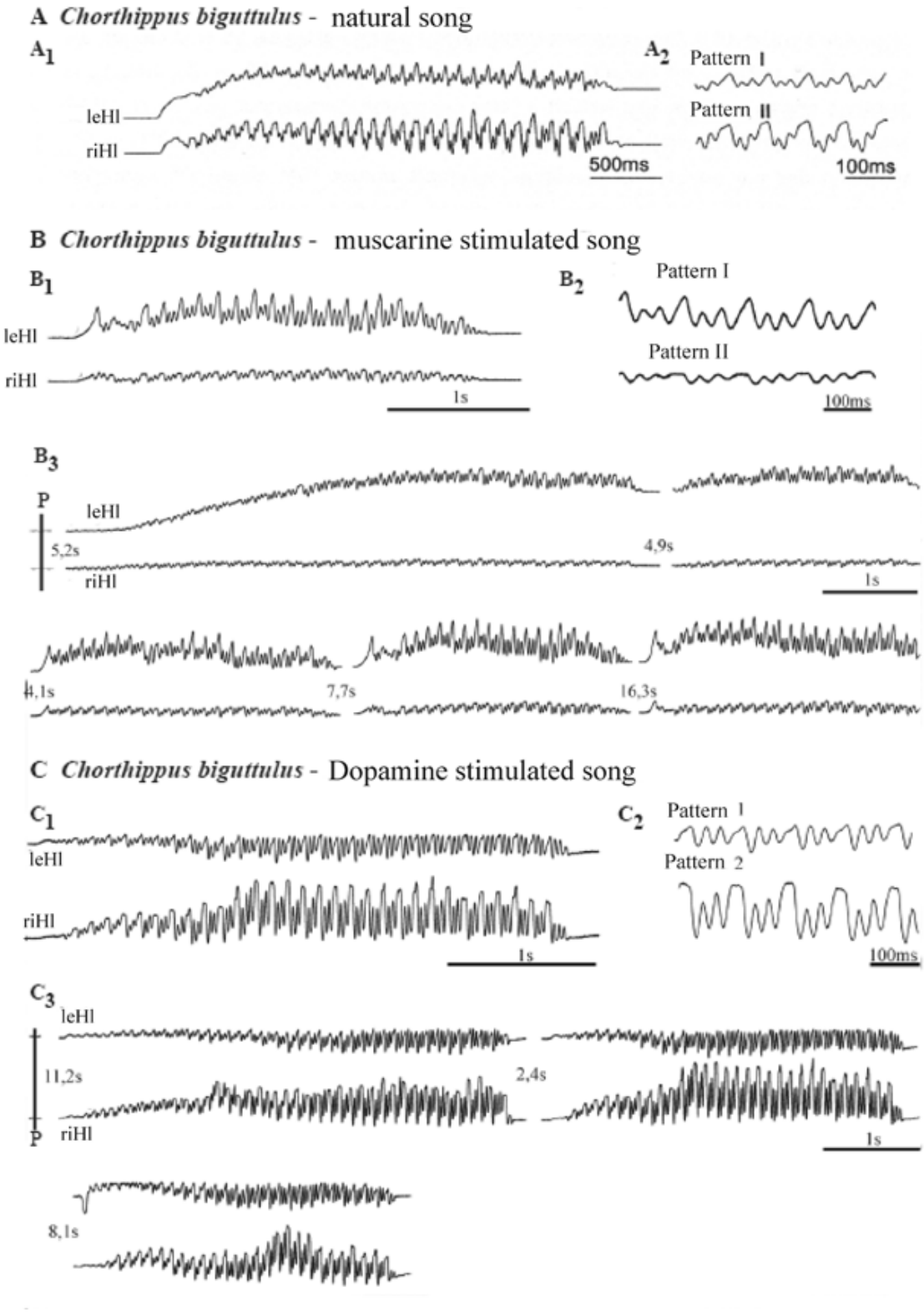


Figure 3.35: Dopamine induces stridulation at the same site as muscarine. Repeated injections of dopamine ($10^{-}3$) to the same site where muscarine ($10^{-}3$) induced stridulation also activated singing behavior. Dopamine injections were performed at intervals of either 2 minute (A) or 5 minutes (B). In both cases dopamine reliably stimulated stridulation, although not each dopamine injection in every experiment lead to a specific response

excitation from the preceding pulse. Because it could be also shown that after one switches between the chambers of the glass electrode the first three injections also contain small amounts from the other chambers at least four injections have to be made to be sure that only dopamine is applied. Injections of DA reliably induced stridulation even at 40 minutes after the last muscarine pulse. It should be noted, that dopamine injections not always induced stridulation.

In the second protocol, the glass electrode contained only dopamine, to rule out the possibility that stimulating effects could be caused by muscarine in the other cham-

Figure 3.36: Injections of dopamine into the central complex elicit sound production. Movement patterns of the right (riHL) and the left (leHL) hind leg during sound production of a male *Chorthippus biguttulus* (from Wenzel (2000). A_1: The natural stridulation pattern of *Ch.b.* consists of song sequences composed of repeating units of typically 3-4 up-and-down movements of the hind legs. A_2: Detailed image of the hind leg movements. Both hindlegs perform coordinated but slightly different pattern. B_1: Injections of muscarine ($10^{-}3$) into the central body elicits species-typical stridulation after 6-68 seconds. B_2: The same difference in movement patterns as in natural songs was detectable. B_3: Entire stridulation pattern (recording time = one minute) with five syllables. Periods in which no sound production occurred were cut out for clarity and the duration indicated at the respective position. C_1: Injections of dopamine ($10^{-}3$) into the central body elicits species-typical stridulation after 4-52 seconds. C_2: The same difference in movement patterns as in natural songs was detectable. C_3: Entire stridulation pattern (recording time = one minute) with three syllables. P = pulse (application of muscarine/dopamine)


A Chorthippus biguttulus - natural song
A1
leHl
riHl
500ms
A2
Pattern I
Pattern II
100ms
B Chorthippus biguttulus - muscarine stimulated song
B1
leHl
riHl
1s
B2
Pattern I
Pattern II
100ms
B3
P
leHl
5,2s
4,9s
riHl
1s
4,1s
7,7s
16,3s
C Chorthippus biguttulus - Dopamine stimulated song
C1
leHl
riHl
1s
C2
Pattern 1
Pattern 2
100ms
C3
leHl
11,2s
2,4s
P
riHl
1s
8,1s

ber. The natural song of *Ch.b.* is composed of individual sequences with a duration of 2-6 seconds. Each sequence consist of 20-50 repeated units of sound-generating movements of the hindlegs, usually one large amplitude up- and down-movement followed by 1-3 smaller amplitude movements (Elsner 1974) (Fig. 3.36A). The two hindlegs perform slightly different movement patterns (pattern I and pattern II).

The calling song of *Ch.b.* is very similar to the courtship song. The are only minor differences in the loudness and the up-stroke of the hindlegs during a sequence (Reis, 1995). Because both characteristics merge seemingly seamlessly and especially the loudness is very variable, distinguishing both song types is not attempted in this study.

Stridulatory behavior could be elicited through injections of dopamine into the central body (Fig. 3.36B). The duration of song sequences varied between 1-7 s and the latency between application of DA and the first sequence was 4-52 s.

Unlike for muscarine, which reliably induced stridulation after each injection, the response to dopamine was more variable, meaning that not every injection elicited a response.

After proving that dopamine applied to the central complex can stimulate stridulation, it was investigated whether muscarine- and dopamine dependent stridulation could act in an additive mode on the duration of sound production when both pathways are simultaneously activated. The experimental protocol consisted of six injections with a time interval of five minutes. The first three stimuli consisted of muscarine alone while with the second three pulses both substances (muscarine and dopamine) were co-injected to the same site in the brain. The same protocol was used with the D1-receptor agonist 6-chloro-PB instead of dopamine. The results are shown in Fig 3.37. To test for statistical differences several analysis were performed. First the average responses of the two different treatments (muscarine alone and muscarine and DA/6-Chloro-PB) were calculated and compared. Second, the mean responses from each treatment were calculated and compared and finally the means of muscarine responses at each stimulation site were calculated and compared with the responses to single pulses of the combined stimulus. None of these evaluations indicated a significant increase in singing duration by co-activation of the DA signaling pathway (Friedmann-Test $p>0.1$).

Dopamine may contribute to the control of sound production as a tonically released neuromodulator that, directly or indirectly, permanently increases the excitability of neurons expressing the mAChR. To test this Possibility I used the same protocol as for testing the inhibitory effect of DA but this time I used DA-receptor antagonists. If constantly released dopamine increases the excitability of mAChR expressing neurons, blocking dopaminergic signaling should also decreases the response to muscarine stimuli. These experiments were performed using two different

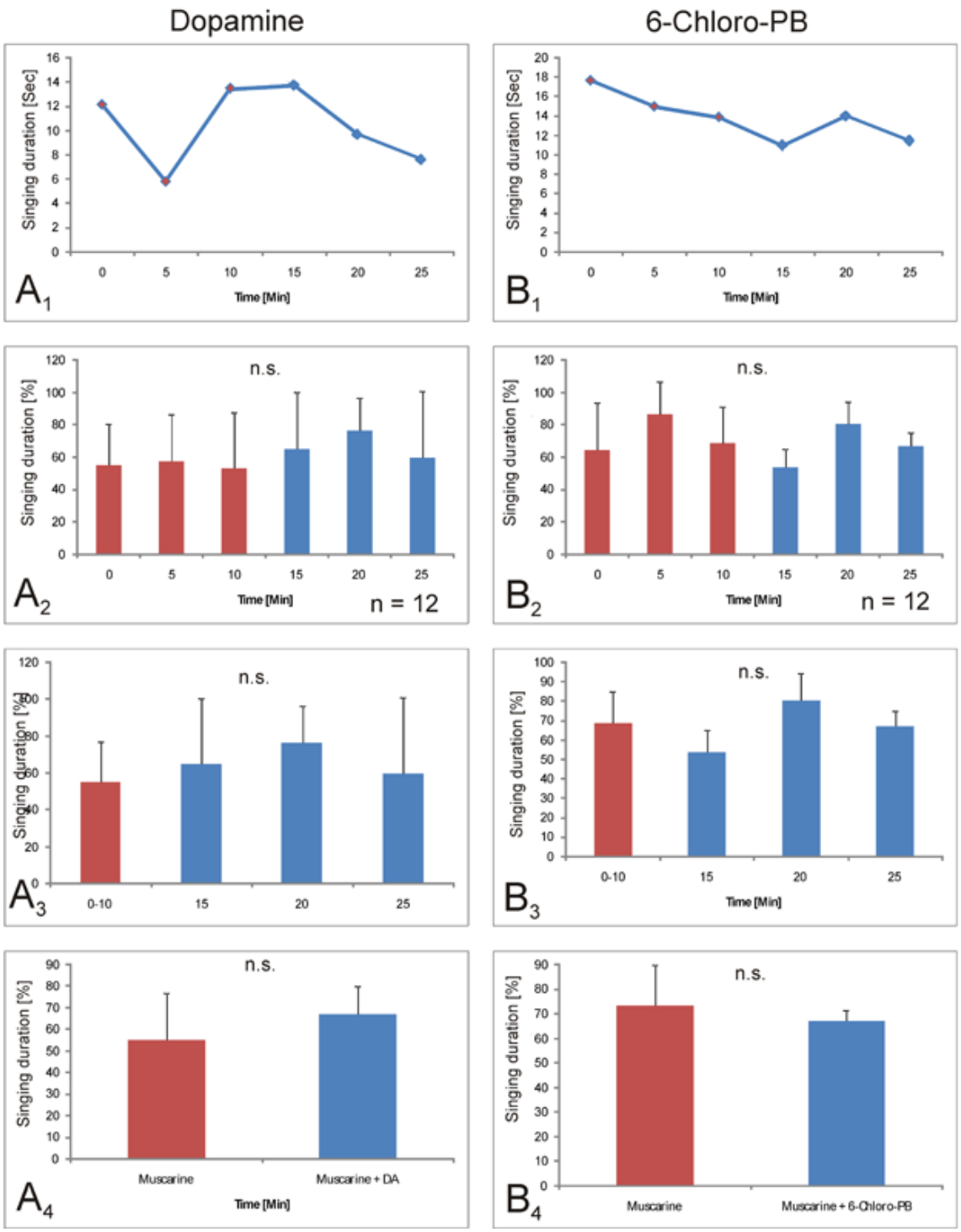


Figure 3.37: Dopamine does not increase muscarine stimulated stridulation. Neither dopamine ($10^{-}3$) (A) or the D1-receptor agonist 6-chloro-PB ($10^{-}3$)(B) show an effect on the singing duration when coapplied with muscarine (blue bars) compared to muscarine alone (red bars). Statistical analysis included a comparison between the single responses (A_2 and B_2), between the mean of the muscarine responses compared to the responses to the mixture (A_3 and B_3) and between the means responses to muscarine and the mean responses to the mixture (A_3 and B_3).

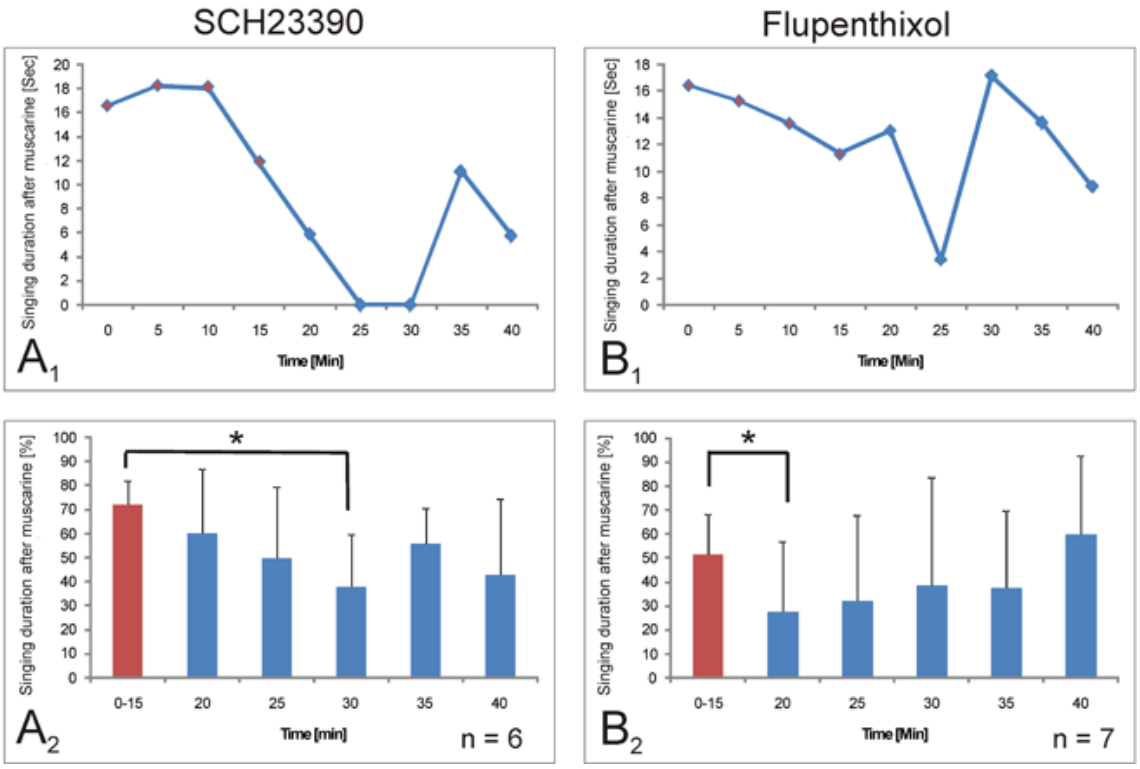


Figure 3.38: The effect of the two dopamine antagonists (both $10^{-}3$) SCH23390 (D1-specific, A) and flupenthixol (D1/D2-antagonist, B) are examined. Both substances cause a significant decrease of singing duration in response to muscarine. While the effect for SCH23390 needed some tine to develop (significant reduction at minute 30, $p<0.05$, indicated by *) the effect of flupenthixol was already recognizable at the first muscarine injection that followed the application of flupenthixol. Red bars indicate the response to muscarine before injection of antagonists and blue bars after.

DA-receptor antagonists, SCH233390 (a specific D1-antagonist) and flupenthixol (an unspecific D1/D2-antagonist). Statistical analysis was the same as described for TA.

SCH23390 (Fig. 3.38A_{1-3}) significantly decreased the response to muscarine 15 minutes after its application is significantly different ($p<0.05$, Wilcoxon-test) compared to the mean difference before the application of SCH233390 (minutes 0-15). In the results it can be clearly seen that this inhibitory effect gradually increased over time till it reached a significant value.

Flupenthixol (Fig. 3.38B_{1-3}) significantly decreased ($p<0.05$, Wilcoxon-test) muscarine dependent stridulation singing duration already five minutes after its application compared to the mean response to muscarine before application of the D1/D2-antagonist. The results show a gradual increase of muscarine dependent sound production with increased time after flupenthixol application.

This results suggest a tonic release of DA in the CB since since blocking of dopaminergic transmission results in a decrease of overall excitation that promotes sound production.

4 Discussion

The central complex has been noticed in early anatomical studies as one of the most regularly organized neuropiles in the insect brain (Strausfeld 1976, Hanesch et al. 1989, Strauss 2002) but its functional role remained elusive for a long time. Recent studies implicated that the central complex is responsible for processing spatial information (Vitzthum et al. 2002, Liu et al. 2006, Heinze and Homberg 2007) and as a pre-motor control center in the insect brain (Homberg et al. 1987, Strauss and Heisenberg 1993, Strausfeld 1999, Strauss 2002, Wessnitzer and Webb 2006). In particular neural substrates that select and initiate behaviors or stereotype behavioral components seem to reside in the central complex neuropiles (Popov et al. 2005, Ridgel et al. 2007, Wenzel et al. 2002). In acoustically communicating grasshoppers, such as the species *Ch. biguttulus* used in this study, the central complex constitutes the major central nervous neuropil responsible for the situation-specific selection and coordination of sound patterns in contexts of reproduction and intermale competition. Pharmacological studies showed that this behavior is controlled by a balance of excitation and inhibition within this neuropile (Heinrich et al. 1997; 1998b, Wenzel et al. 2002). Both, increasing excitation and decreasing inhibition promote the performance of sound production. Until now several neurotransmitters and neuromodulators have been identified that promote (ACh, proctolin) or suppress (NO/cGMP, GABA) both, spontaneous and conspecific song-stimulated stridulation. One aim of the present study was to complement previous results from pharmaco-behavioral experiments with neuroanatomical data about the expression of components of signaling pathways in the central complex which contribute to the control of sound production and to deduce the flow of information in this neuropile. For this immunostainings were performed against several transmitters and receptor systems from which it is known that they affect acoustic communication when injected into the central complex (For an overview see Table 4.1). (B) Moreover, I tried to identify additional transmitters that modulate arousal in the central complex and hence contribute to the control of grasshopper sound production.

Table 4.1: Distribution of transmitter/modulator systems in the CB

Neuron	*Type*	*Transmitter/ Receptor*	*Cell body location*	*Regions innervated within the CB*	*Regions innervated outside the CB*
MR1	*columnar*	*mAChR*	*pars intercerebralis*	*all layers of the CBL*	*lateral triangle of the LAL*
GT	*tangential*	*GABA*	*ventro-median / inferior-median and inferior lateral protocerebrum* [1]	*all layers of the CBL and layer II of the CBU*	*median olive and lateral triangle of the LAL*
CT1	*pontine*	*NO (citrulline)*	*pars intercerebralis*	*layers II and III of the CBU*	*none*
CT2	*tangential*	*NO (citrulline)*	*inferior median protocerebrum*	*not detectable*	*median olive of the LAL*
CG1	*tangential*	*cGMP*	*inferior median protocerebrum*	*layer 2 of the CBL*	*lateral triangle and median olive of the LAL* [2]
DP2-like	*tangential*	*dopamine*	*lateral pars intercerebralis*	*all layers of the CBL and layers II and III of the CBL*	*dorsal and ventral shell of the LAL*
DC1-like	*tangential*	*dopamine*	*lateral to the calyces*	*layers II and III of CBU*	*SMP and α-lobe of the MB*
DC2-like	*tangential*	*dopamine*	*lateral to the calyces*	*anterior lip of the CBU*	*SMP and α-lobe of the MB*
PR1	*columnar*	*ProcR*	*anterior pars intercerebralis*	*CBL*	*lower unit of the noduli*
PR2	*pontine*	*ProcR*	*anterior pars intercerebralis*	*layer I and anterior lip of the CBU*	*none*
PR2	*tangential*	*ProcR*	*inferior median protocerebrum*	*layer I of the CBU*	*could not be determined*
LTC1-like	*columnar*	*TRP*	*pars intercerebralis*	*all layers of the CBL*	*lateral triangle of the LAL*
LTC2-like	*columnar*	*TRP*	*pars intercerebralis*	*all layers of the CBL*	*lateral triangle of the LAL*

[1] Several different types of tangential neurons could be described. Reconstruction of single cell types from such an enormous mass of neurons is difficult. Because all neurons seemed to connect the CB with the lateral accessory lobes, no further distinctions are made

[2] Because tangential neurons normally innervate either the median olive OR the lateral triangle it cannot be ruled out that labeling in both regions comes from distinct neuron types

LTC3-like	*columnar*	*TRP*	*pars intercerebralis*	*CBU*	*could not be determined*
LTC4-like	*columnar*	*TRP*	*unknown*	*layers I and IIa of the CBU*	*unknown*
LTT1-like	*tangential*	*TRP*	*inferior median protocerebrum*	*inner layers of the CBL*	*lateral triangle of the LAL*
cp7-like	*tangential*	*CCAP*	*lateral to the calyces*	*anterior lip of the CBU*	*LAL*
cp8-like	*tangential*	*CCAP*	*anterior pars intercerebralis*	*layer IIa of the CBU*	*LAL*
cp9-like	*pontine*	*CCAP*	*anterior pars intercerebralis*	*layer I the CBU*	*none*
AST1-like	*tangential*	*allatostatin*	*inferior median protocerebrum*	*layer IIa of the CBU*	*LAL*
AST2-like	*tangential*	*allatostatin*	*inferior median protocerebrum*	*layer IIb of the CBU*	*LAL*
AST3-like	*tangential*	*allatostatin*	*unknown*	*layer I of the CBU*	*unknown*
MT1-like	*tangential*	*allatotropin*	*pars intercerebralis*	*CBU*	*LAL*
MT2-like	*tangential*	*allatotropin*	*unknown*	*CBU*	*SMP and α-lobe of the MBs*

4.1 Immunocytochemistry

4.1.1 mAChRs the Central Complex

Activation of mAChRs in the central complex of grasshoppers has been shown to stimulate sound production by activation of both, adenylyl-cyclase and phospholipase C-initiated second messenger pathways (Heinrich et al. 2001b, Wenzel et al. 2002). Immunocytochemistry with an antibody raised against a *D. melanogaster* mAChR (Blake et al. 1993), whose specificity for *Ch.biguttulus* has been proven by western blotting (Hoffmann et al. 2007), labeled two types of columnar neurons (Fig. 3.2). One type innervated the lower division of the central body, while the other projected directly into the contralateral lateral accessory lobe passing along the anterior border of the lower division. Columnar neurons with arborizations in the CBL closely resemble the CL1-fiber type described for the locust *S. gregaria* (Müller et al. 1997). Projections of this type of neuron pass through layer III of the upper division within the posterior vertical bundles (Williams 1972). This type of columnar neuron is the only central complex neuron that expresses mAChRs and forms arborizations in a central body neuropil. Since songs of conspecific females have been demonstrated to initiate response songs via activation of muscarinic re-

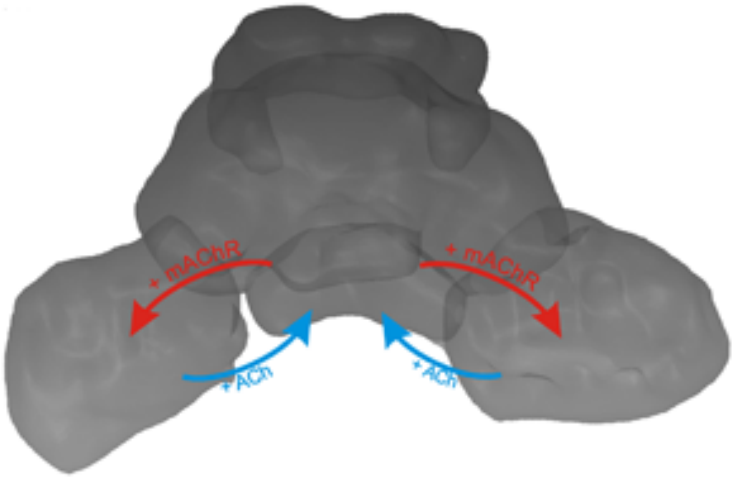


Figure 4.1: Schematic representation of the cholinergic information flow in the central complex promoting sound production. ACh (blue arrow) is released into the lower division of the central body where it binds to mAChRs located on columnar output neurons. The columnar become excited and sent this excitatory information (red arrow) back to the lateral accessory lobes.

ceptors in the central complex (Hoffmann et al. 2007) we speculate that mAChR expressing columnar neurons most likely receive auditory sensory input. Electrophysiological recordings will be necessary to validate this assumption. The other kind of mAChR expressing columnar neuron projects through layer I of the upper division and lacks any arborizations in the CBL. Neurons with similar anatomical features have been described in *S. gregaria* by Homberg and coworkers (Vitzthum et al. 2002). These CP-neurons are sensitive to polarized light. In *S. gregaria*, the neurons restrict their dendritic arborizations to single columns of the protocerebral bridge and send axonal projections through the CB to the contralateral LAL. It seems unlikely, that these neurons are activated by injections of muscarine that stimulates singing in *Ch.b.*, since they have no ramifications in the central body.

Columnar neurons are the presumed output neurons of the central complex that, among other targets, connect to premotor interneurons in the lateral accessory lobes. Since columnar neurons are the only central complex neurons that express mAChRs, stridulation-inducing injections of muscarine into the central body may directly stimulate excitatory output of the central complex (Fig. 4.1). This hypothesis is supported by the fact that staining outside the central complex is rather weak, assuming that receptor density is lower in axonal (located in the LAL) than in dendritic regions (located in the central complex).

4.1.2 GABA in the Central Complex

GABA is the major fast inhibitory transmitter in the insect brain. Injections of GABA into the central complex have been shown to inhibit stridulation induced

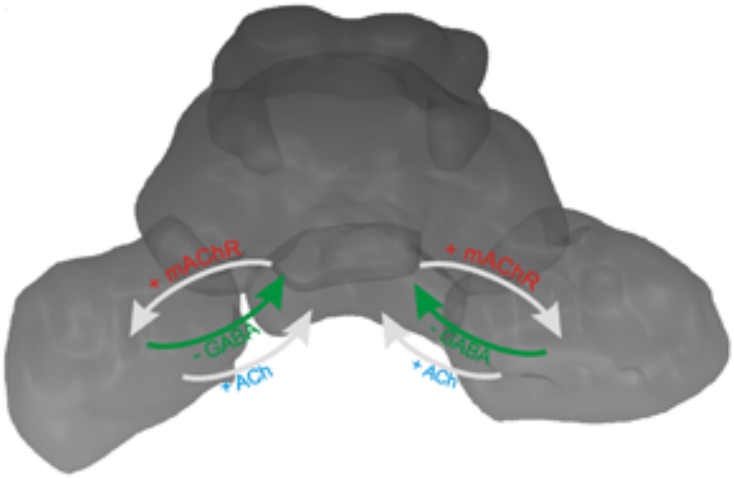


Figure 4.2: Schematic representation of the GABAergic information flow in the central complex inhibiting sound production. GABA is released from tangential neurons into the lower division of the central body (green arrow) where it most likely directly binds to GABA-receptor on mAChR-expressing neurons to decrease their activity.

by cholinergic agonists after short latency and with short duration (Heinrich et al. 1998b) suggesting that $GABA_A$ receptors mediate this effect.

The distribution of GABA in the CX of *Ch. biguttulus* (Fig. 3.4) is very similar to that of other insect species (Schäfer and Bicker 1986, Homberg et al. 1987, Meyer et al. 1986, Hanesch et al. 1989, Blechschmidt et al. 1990, Becker and Breidbach 1993, Strambi et al. 1998, Homberg et al. 1999) implicating that the functional role of GABA in the CX may also be conserved. The CX is strongly innervated by tangential neurons having their somata in the inferior median and inferior lateral protocerebrum. The entire lower division is densely supplied with GABAergic fibers, while only parts of the upper division, namely layer II, contain GABAergic fibers. A dense meshwork of GABA-containing neurites intermingles with the neurites of mAChR expressing columnar neurons in the lower division of the central body. It is therefore conceivable that the columnar output neurons of the central complex may represent direct targets for GABAergic inhibition. The short-lived suppression of muscarine-stimulated sound production, that was observed in pharmacological studies may therefore results from GABA mediated inhibition of stridulation-promoting central complex output neurons (Fig. 4.2).

4.1.3 The NO-cGMP signaling pathway

NO has been shown to inhibit muscarine-induced stridulation, when injected into the central body at the same site as muscarine. This effect is mediated by activation of soluble guanylyl-cyclase and subsequent accumulation of cytosolic cGMP levels (Wenzel et al. 2005). Antisera against citrulline (a side product during the formation of NO) and cGMP are valuable tools to label the functional status of neurons

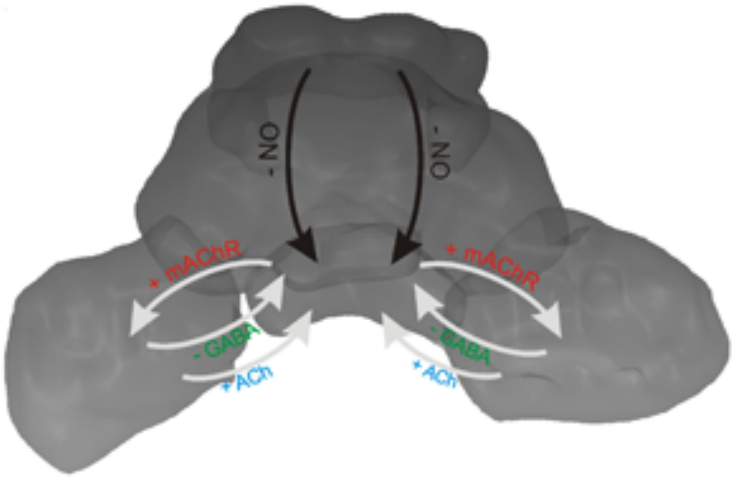


Figure 4.3: Schematic representation of the NO-mediated information flow in the central complex inhibiting sound production. NO is produced by pontine neurons in the upper division of the central from where it diffuses to the lower division (black arrow). In the lower division NO modulates inhibitory input through upregulation of cGMP in GABAergic neurons.

that actively produce (citrulline) or respond to NO (cGMP) in various vertebrate and invertebrate preparations (DeVente et al. 1987, Bicker et al. 1996, Bicker and Schmachtenberg 1997, Jones and Elphick 1999, Cayre et al. 2005).

4.1.3.1 Citrulline in the central complex

Anti-citrulline immunocytochemistry labeled a subset of NO producing neurons, previously described by anti universal NOS immunocytochemistry and NADPH diaphorase staining in *Ch. biguttulus* (Wenzel et al. 2005) and *S. gregaria* (Kurylas et al. 2005). This appeared most obvious in the mushroom bodies which display a high level of NADPH diaphorase activity but no detectable accumulation of citrulline (Fig. 3.6C and D). This discrepancy most likely resulted from a lack of appropriate olfactory input during dissection of the brain and preceding tissue fixation and suggested that citrulline immunopositive neurons in other brain regions had actively produced considerable amounts of NO prior to fixation. Citrulline accumulation in central complex neurons may therefore reflect a behavioral situation that is unfavorable for sound production (like being restrained for brain dissection) that suppresses singing by NO release in the central body (Fig. 4.3). This hypothesis was recently supported by pharmaco-behavioral studies with *Ch. biguttulus* females in which systemic application of the NOS inhibitor aminoguanidine caused both, a substantial reduction of citrulline accumulation in central complex neurons and an increased sound production in response to male calling songs (Weinrich et al. 2008). Citrulline was exclusively detected in layers II and III of the CBU. Previous histological studies in *S. gregaria* (Kurylas et al. 2005) indicated that three types of neurons in the central complex, pontine, columnar, and tangential neurons, contain

NOS and may release NO upon activation. These results were confirmed in my thesis on *Ch. biguttulus* by citrulline-immunocytochemistry, which shows citrulline-accumulation in pontine and probably also tangential neurons. Accumulation of citrulline was detected in the upper division of the central body, which is in line with previous studies in *Ch.b.* that detected NOS by immunocytochemistry and NADPH diaphorase staining (Wenzel et al. 2005) but contrasts with studies on *S. gregaria*, where NOS was also present in the lower division (Kurylas et al. 2005).

4.1.3.2 cGMP in GABAergic neurons of the central complex

NO-stimulated accumulation of cGMP in the central complex was exclusively detected in GABA-containing tangential neurons innervating layer 2 of the lower division of the central body. These fibers represent the only possible targets for NO released within the central body. Cyclic GMP positive neurites belonged to neurons that appeared similar to TL2 and TL3 neurons described in *S. gregaria* by Müller and coworkers (Müller et al. 1997). These neurons are suggested to provide input from LAL to the lower division of the central body. Both, GABA and NO-mediated production of cGMP in the central body have been demonstrated to suppress muscarine-stimulated sound production. In the lower division of the central body both, cGMP accumulating GABAergic fibers and mAChR-expressing columnar output neurons of the central complex were located in close vicinity. I therefore hypothesize that NO-mediated production of cGMP may suppress stridulation through increase of GABA release, that directly or via another interneuron inhibits the muscarine-sensitive columnar output neurons. Colocalization of NO-sensitive soluble guanylyl cyclase or NO-stimulated cGMP with GABA has previously been described in the antennal lobes of locusts and moths (Bicker et al. 1996, Collmann et al. 2004), but the physiological effect of cGMP on GABAergic neurons in unknown.

4.1.4 Dopamine/Tyrosine-Hydroxylase in the central complex

Dopamine has been shown to modulate several different types of behavior in both invertebrates and vertebrates. In invertebrates most of the research on dopamine has been focused on its role in learning and memory, where it could be shown that dopamine is important for the formation of aversive memory (Schwaerzel et al. 2003, Schroll et al. 2006, Unoki et al. 2005; 2006). Recently it was reported that up-regulation of dopaminergic transmission increases sexual arousal in *D.melanogaster* (Andretic et al. 2005, Kume et al. 2005).

In the grasshopper *Ch.b.* injections of dopamine into the central body induce stridulation (Fig. 3.36). Antisera against dopamine and the dopamine producing enzyme

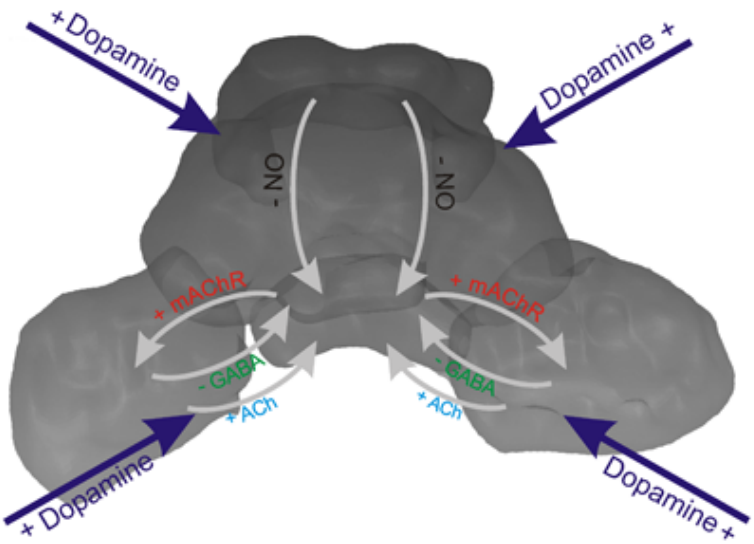


Figure 4.4: Schematic representation of the dopaminergic information flow in the central complex promoting sound production. Dopamine is released into the central body by tangential neurons (dark blue arrow) that receive their input from surrounding brain regions. If dopamine directly or indirectly activate output neurons of the central body cannot be said.

tyrosine-hydroxylase label three types of tangential neuron in the central body (DP2-, DC1- and DC2-like) that innervate all parts of the CBU except for layer I (Fig. 3.12E and Fig. 3.13). This staining pattern is very similar to the one from *S. gregaria* (Wendt and Homberg 1992), with the exception that dopamine was also detected in layer I of the CBU. Although dopamine can be found in various other brain neuropiles, its highest concentration can be found in the central body. This is not a specialty of grasshoppers, or orthopteriod insect, but can also be found in *D.melanogaster* and other flies (Nässel and Elekes 1992).

The abundance of high levels of dopamine in the central body of various insect species makes it very unlikely that dopamine serves a specific role for the control of acoustic communication, but implies a more general role as a gain setter that increases arousal in premotor centers. The branching pattern of dopaminergic neurons outside the central complex suggests that they may integrate information from various brain regions and/or mediate arousal to different brain regions that contribute to the control of behaviors related to the same context (Fig. 4.4). While all other central complex neurons described in this study so far exclusively target the lateral accessory lobes, dopaminergic central complex neurons additionally connect with other brain neuropiles including the superior median protocerebrum and the mushroom bodies. Although the mushroom bodies are mainly implied in the formation of memory (Heisenberg 1998; 2003, Menzel 2001), there are a few reports about their function on the control of motor behavior (Martin et al. 1998) and their electrical stimulation in crickets elicited natural stridulation patterns (Huber 1955; 1960; 1963; 1965, Wadepuhl and Huber 1979, Wadepuhl 1983).

4.1.5 Neuropeptides

Neuropeptides compose a huge amount of neuromodulatory substances. Despite their abundance in interneurons of the insect brain a particular function in the CNS has only been assigned to a few of them (Nässel 2002, Nässel and Homberg 2006). Most of our knowledge about neuropeptides derives from studies on the peripheral nervous system and its influences on the functions of effectors such as muscles, organs and epithelial tissues. In general, neuropeptides are not considered as genuine neurotransmitters but more as neuromodulators, which are usually released as cotransmitters together with a conventional transmitter (Nusbaum et al. 2001). Very interesting work comes from the stomatogastric nervous system of crustaceans. This is a very simple network which controls the movements of the foregut and the oesophagus (Simmers et al. 1995, Clarac and Pearlstein 2007). In this system "cocktails" of neuropeptides determine the rhythm produced by pattern generating circuit (Skiebe 2001, Nusbaum et al. 2001). The central complex is far more complicated compared to the stomatogastric nervous system but the general function is the same, namely to coordinate different motor patterns and select the most appropriate according to the situation encountered. From other insects and especially the locust *S. gregaria* it is known that the central complex contains a variety of neuropeptides (Nässel 2002, Nässel and Homberg 2006). The finding that only small subsets of neurons that innervate the CX contain neuropeptides makes it very likely that these peptides are specific in modulating a certain behavior. A first indication comes from proctolin, which could be shown to promote singing behavior when injected into the CB (Vezenkov 2004). Testing out the effect on the control sound production of every neuropeptide that can be found in the central complex is a quite laborious and daunting task. To perform a kind of "pre-screening", one can focus on the peptides which are co-expressed with transmitters that have been already shown to alter the motivation to perform stridulation when injected into the central body (ACh, GABA, NO/cGMP, tyramine and dopamine). For this, one has to map the distribution of the different neuropeptides in the CX and compare it with the distribution of signaling molecules known to contribute to the control of grasshopper sound production.

4.1.5.1 Proctolin-receptors in the central complex

The neuropeptide proctolin has been detected in the nervous system of various insects (Bishop and O'Shea 1982, Keshishian and O'Shea 1985, Nässel and O'shea 1987, Orchard et al. 1989, Breidbach and Dircksen 1991), but its function so far remains elusive. Pharmacological studies have shown that activation of the proctolinergic signaling system in the central complex can induce stridulatory behavior

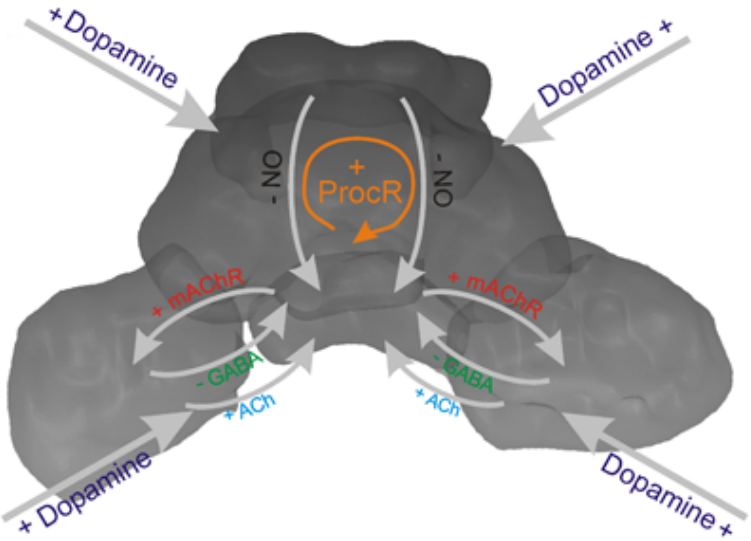


Figure 4.5: Schematic representation of the dopaminergic information flow in the central complex promoting sound production. Proctolin released into the binds to receptors that are located on neurons whose fibers are restricted to the central complex (orange arrow). Most of them are located in the upper division of the central. How this neurons lead to increased excitatory output of the central body is unknown.

(**?**). To see which neurons are affected by injections of proctolin, antibody staining against the proctolin-receptor (ProcR) were performed. The antibody has been generated against the ProcR from *D. melanogaster* (Johnson et al. 2003) and has not been used in studies on other insect species. The staining pattern outside the CX (Fig. 3.14) is very similar to the one described for *Drosophila*. Johnson et al. (2003) described punctate staining in the optic lobe similar to the one detected in *Ch.b.* (Fig. 3.14B). Furthermore, strong immunostaining could be detected in neurons of the posterior pars intercerebralis (Fig. 3.14C and D) which project through the NCC2 to the corpora cardiaca and corpora allata, a structure that also shows ProcR-ir in *D. melanogaster*. This distribution supports the assumption that the antisera may specifically label ProcRs also in *Ch.b.*.

Staining in the central body was most prominent in the upper division (CBU), a region that also shows strong labeling in *Drosophila* (CBU is called fan-shaped body in flies). Unfortunately, no data are available about the distribution of proctolin in the CX of *Ch.b.*. Overlapping pattern of proctolin- and proctolin receptor-ir would further support the specificity of the antisera against the proctolin-receptor. Staining in the CBU of *Ch.b.* stems from columnar and pontine neurons that have their somata in the anterior pars intercerebralis. While the pontine neurons seem to predominantly innervate the outer layers of the CBU (layer I), columnar neurons could also be detected in the CBL and the noduli. Especially the finding that columnar neurons are stained is interesting because mAChRs, which have a key role in initiating sound production, could also be located on columnar neurons. Unlike mAChR-positive neurons, which connect the PB and the CB with the lateral accessory lobes, columnar neurons expressing the ProcR seem to be intrinsic to the

central complex, indicating that activation of ProcR in the central complex does not directly increase output of the central complex as it is expected for mAChR. Proctolinergic signaling on the other hand seems to modulate information processing within the central complex (Fig. 4.5), like it has been shown for NO.

To date, only one receptor specific for proctolin could be cloned (CG6986) which is the original antigen of the antibody used in this study (Johnson et al. 2003). Expression of this receptor in HEK-cells revealed that the application of proctolin leads to an increase of intracellular calcium, probably due to the coupling of the receptor to G_q and subsequent activation of the PLC-pathway. There is compelling evidence from pharmacological studies, that proctolin receptors also activate the PLC-pathway in various peripheral targets of other insects (Baines et al. 1990, Lange et al. 1988, Mazzocco-Manneval et al. 1998), but also for other signaling systems (Baines and Downer 1992, Swales and Evans 1988, Wegener and Nässel 2000).

4.1.5.2 LemTRPs in the central body

Tachykinin related peptides constitute a conserved family that is structurally related to mammalian tachykinins. TRPs are strongly expressed in all subdivisions of the central complex by output (columnar) as well as input (tangential) neurons. The distribution is very similar to the one already described for the locust *S. gregaria* (Vitzthum and Homberg 1998) and the terminology introduced in that publication was adapted for *Ch.b.*. Like for dopamine, the high abundance of TRPs in the central complex implicates a more general role in controlling motor behavior. The expression pattern in the CBL is very similar to GABA and colocalisation studies revealed a strong overlap of both signaling molecules in this brain region. Functional data that support the role of TRPs as a cotransmitter to GABA derived from the visual system of the crayfish *Pacifsastacus leniusculus* (Glantz et al. 2000). In this animal, TRP is coexpressed in GABAergic amacrine cells. Electrophysiological recordings showed that TRP potentiated GABAergic effects on photoreceptor cells. These results and the finding that TRPs colocalise with GABA not only in insects (another species in which TRPs and GABA are colocalised in the central complex is *S. gregaria*, Vitzthum et al. 1998) may implicate a evolutionary conserved role for TRPs as cotransmitter at GABAergic synapses. Another finding that supports the hypothesis that TRPs are a more general modulator for motor behavior resulted from *D. melanogaster* (Winther et al. 2006). Flies in which the gene for TRPs (dtk) is functionally silenced using RNAi show general hyperactivity. Taken together, TRPs are highly abundant in the central complex, a structure generally believed to control motor behavior (Strausfeld 1999) and are coexpressed with GABA, the main inhibitory transmitter in the insect CNS. Removal of TRPs from the brain through silencing the dtk-gene leads to hyperactivity, which can be explained as a

potential disinhibition due to reduced GABAergic inhibitory signaling. A similar neurochemical organisation seems to be established in the central complex of *Ch.b.*, were pharmacological disinhibition by picrotoxin-mediated inactivation of chloride channel associated receptors (e.g. the $GABA_A$-receptor) releases sound production (Heinrich et al. 1998b). This treatment also causes specific hyperactivity with a strong impact, since GABA-mediated inhibition was completely removed. So far, the nature of the signal transduction machinery mediating the modulatory action of TRPs on GABAergic transmission is highly speculative. Only two TRP-receptors could be cloned from *Drosophila* (NKD and DTKR). Studies on HEK-cells which heterologously expressed one of those receptors (DTKR) Birse et al. (2006) showed that it increases both intracellular cAMP and calcium levels. If this is also the case in locusts, it would make a direct inhibitory effect of TRP on mAChR-expressing cells very unlikely, because these are same intracellular intracellular signals that mediate the promotion of sound production upon mAChR activation (Heinrich et al. 2001a, Wenzel et al. 2002), but they can be expressed as autoreceptors on GABAergic synapses regulating the synaptic release of GABA. Another possibility is that the potentiation of GABAergic signaling is mediated via the other receptor type (NKD), but this has not been investigated with respect to the G-protein coupled to it and the distribution of NKD in the CNS has not been studied yet, due to the lack of appropriate antibodies.

Immunocytochemistry against DTKR showed that this receptor type is primarily expressed in the fan-shaped body (the *Drosophila* homologue to the CBU) and not in the ellipsoid body (the *Drosophila* homologue to the CBL) of *D.m.* (Birse et al. 2006). Colocalisation of GABA and TRP on the other hand was only detected in the CBL (at least in locusts, for *drosophila* no colocalisation studies have been performed).

4.1.5.3 CCAP in the central body

Crustacean cardioactive peptide (CCAP) was first identified in the crab *Carcinus maenas* where it is released from pericardial organs to accelerate heart frquency (Stangier et al. 1987). In later studies CCAP was identified in peripheral and central nervous systems of insects (Stangier et al. 1989, Cheung et al. 1992, Furuya et al. 1993, Lehman et al. 1993, Dircksen and Homberg 1995).

In the central complex of *Ch.b.* CCAP could be detected in two types of tangential neurons and one type of pontine neurons. The expression of CCAP is restricted to neurons innervating the CBU. As for most other neuropeptides, knowledge about the function of CCAP in the CNS of insects is very limited. Recently it could be shown that CCAP plays an important role in development, controlling the sequential acti-

vation of specific motor programs during the ecdysis cycle of *Drosophila* (Kim et al. 2006a;b). Till now, only one receptor for CCAP could be cloned from *Drosophila* (CG6111), but the intracellular signaling pathways associated with it have not been examined. CCAP expression appears to be quite variable between different insect species. While the brain of locusts contains about 250 pairs of CCAP-ir neurons (Dircksen and Homberg 1995) in the brains of flies and the beetle Tenebrio molitor, there are only two to five pairs (Breidbach and Dircksen 1991, Dircksen 1998), which leads to the speculation that CCAP is associated with certain species-specific behaviors. The distribution of CCAP in the central complex is very similar to the one already described for the locust *S. gregaria* implicating a similar role for this neuropeptide in the two species. Unfortunately CCAP has not been mapped in the CX of other insect species, so that no more general assumptions can be made about the function of CCAP in the central complex of insects (one exception is the CX of the cockroach *L. madeara*, where CCAP is found in the CBL, T. Reischig, pers. communication).

Comparison of CCAP-ir with the distribution of transmitter systems that affect acoustic communication shows, that the distributions of at least two of the neuronal types that express CCAP (cp7- and cp8-like) are very similar to dopaminergic neurons in the central body (DP2-like and DC-2-like). Two further confirm that these neurons have CCAP as a cotransmitter colocalisation studies have to be performed. If CCAP has an influence of the control of sound production, it would be presumably also modulatory, because it only can be found in the CBU and may therefore not directly impact the activity of central complex output neurons that receive their input in the lower division. Nevertheless, the results obtained from CCAP immunostaining implicate a possible role as modulator of acoustic communication.

4.1.5.4 Allatostatin in the central body

Allatostatins are pleiotropic neuropeptides that have been shown to inhibit the synthesis of juvenile hormones in the corpora allata in various insect species (Stay and Tobe 2007).

Allatostatin immunoreactivity (AS-ir) was detected in three types of tangential neurons which have similar projection patterns compared to *S. gregaria* (AST1-3-like) (Vitzthum et al. 1996). A striking difference between the locust and *Ch.b.* is the lack of stained columnar neuron, which were described in *S. gregaria*. One possible explanation for these different results could be that different antisera were used in both studies (polyclonal, instead of a monoclonal that was used in this thesis). Nevertheless, both antibodies were raised against the same subtype of AS (AST-A, or cockroach like), assuming that labeled tangential neurons in *Ch.b.* are homologous

to the ones from *S. gregaria*. If the lack of labeled columnar neurons truly reflects that these neurons contain no AS or is just an effect of different specificity of the antibody can be only shown, if the experiments on *Ch.b.* are repeated using the polyclonal antibody of Vitzthum et al. (1996). On the other hand it could be also possible that staining of columnar neurons outside the CX was simply to weak to be detected. Sagittal sections revealed staining in the CBAL, whose origin could not be reconstructed (Fig. 3.22E). In *S. gregaria*, staining of the CBAL resulted from columnar neurons. It is therefore likely that this labeling stems from columnar neurons. Stainings against LemTRP already showed that staining in columnar neurons is less intense compared to tangential neurons (Fig. 3.22A-D).

As already described for other neuropeptides, AS-ir in the central body is mainly found in the upper division. The staining patterns closely resemble those of GABA. Studies on the locust *S. gregaria* already showed, that AS and GABA colocalise in the CBU (Homberg et al. 1999), but to show that this is also the case in *Ch.b.*, double labeling experiments have to be performed.

So far two receptors for AST-A could be isolated from *D.m.* (AlstR-1 and -2), but functional studies have not been conducted to investigate the possible second-messenger pathways associated with these receptors.

A study with *C. elegans* demonstrated an effect of AS on motor behavior (Bendena et al. 2008). In this study it could be shown, that AS seems to inhibit foraging behavior. With respect to the finding that AS is coexpressed in GABAergic neurons of the locust central complex (Homberg et al. 1999) it is tempting to speculate that AS may facilitate GABAergic transmission, like it is the case for TRPs in the visual system of crayfish (Glantz et al. 2000).

Another interesting aspect is the fact that allatotropins may be coexpressed with dopamine, a transmitter that promotes stridulation. Studies on the peripheral system have already shown that AS and AT have antagonistic effect. While AT increases the biosynthesis of juvenile hormones in the corpora allata, AS inhibits this process (Stay and Tobe 2007, Lungchukiet et al. 2008). A similar situation could be found in the foregut of a moth, where AT increases foregut contraction while AS has an inhibitory effect (Matthews et al. 2007). To validate if this antagonistic function of AS and AT may also be established in the song control of grasshoppers, pharmaco-behavioral studies need to be performed.

4.1.5.5 Allatotropin in the central body

Allatotropins (AT) compose a family of highly conserved insect neuropeptides named for their stimulating effect of juvenile hormone synthesis in the corpora allata of certain insect species (Elekonich and Horodyski 2003). Its distribution could be mapped

in various insect species (Zitnan et al. 1993, Rudwall et al. 2000, Tu et al. 2001, Petri et al. 2002, Homberg et al. 2004). The distribution of AT in the grasshopper *Ch.b.* is very similar to the one described for the locust *S. gregaria* (Homberg et al. 2004) and therefore the terminology introduced for these neurons has been adapted.

In grasshopper, allatotropins are found in two types of tangential neurons innervating the central body (MT1- and MT2-like). Both types solely innervated the upper division while the lower division is completely devoid of AT. The distribution pattern of the MT1-like neurons is very similar to the one of the dopaminergic type (DP2-like) innervating the upper division. It is therefore possible that a subset of dopaminergic neurons uses allatotropin as a cotransmitter. To validate this assumption colocalisation studies have to be performed.

Other peptidergic neurons with an innervation pattern of the central complex similar to MT1-like neurons are the CCAP-positive cp-7-like neurons. While MT1-like neurons seem to innervate all layers of the CBU (although differences can be seen, with the highest expression in layer II), cp-7-like are restricted to layer IIa. It would be interesting to investigate, whether CCAP and AT are coexpressed in the same or different subsets of central complex neurons. No AT-receptor has been identified in any insect species, so that no statements can be made how AT can influence the second-messenger systems that affect acoustic communication.

Functional evidence for a modulatory role of AT on motor behavior comes from the cockroach *Leucophaea maderae* (Petri et al. 1995; 2002), where it could be shown that anAT-related peptide is involved in the photic entrainment of the circadian clock. It was demonstrated that AT and GABA have a similar role, but morphological examinations indicated that it is very unlikely that these two system are coexpressed in the same neuron (T. Reischig, pers. communication). Colocalisation of GABA and AT were described in antennal lobe neurons of the locust *S. gregaria.* Comparison of the distribution pattern of GABA and AT in the central complex of *Ch.b.* makes its very unlikely, that these two substances are found in the same neurons.

4.2 Tracing studies

To gain a better understanding of the information flow within the central complex tracing experiments were performed using co-injections of dextranes at sites where muscarine induced stridulation. In earlier studies it could be shown that dextranes are primarily incorporated from post-synapses in locusts (Heinrich et al. 1998a, Lakes-Harlan et al. 1998). Injections of dextranes into the well known auditory neuropile of *Locusta migratoria* exclusively labeled those auditory interneurons that

had their post-synaptic (input) compartments in the injected half of the ganglion. Injections of dextranes therefore should label neurons that are functionally downstream to the injection site and for example, can be used to clarify which neurons in the central body are directly activated by injection of muscarine.

These tracing studies labeled columnar neurons of the central body (Fig. 3.26) with their somata in the pars intercerebralis. Although the site of injection (recognizable from the increased background) sometimes seemed to be located in the upper division, the fibers labeled were in most cases arborising in the CBL. One disadvantage of this tracing method is, that it relies on active retrograde transport (Köbbert et al. 2000). This leads to incomplete staining of the neurons with axonal structures not labeled, which makes full reconstruction of the neuron difficult. Nevertheless, this method reliably labeled dendritic regions and somata of neurons within the central body. The finding that columnar neurons are stained by this method further supports the hypothesis that columnar neurons have their post-synapses in the CB.

Double labeling experiments showed that neurons labeled by injected dextranes also expressed the mAChR. Not all neurons labeled by this method are also positive for the mAChR, but this was not expected because the dye is just randomly incorporated by post-synaptic sites and does not differentiate between synapses using different transmitters.

4.3 Cell culture

One of the major disadvantages of our pharmaco-behavioral setup is the limited control over the concentration of injected substances at their cellular targets, meaning that dose response curves are difficult to obtain. This makes it very difficult to draw conclusions about the efficacy of particular chemical signals. Furthermore, one most likely always activates populations of neurons by injection of drugs into brain neuropiles. Among these may also be neurons that are not involved in the control of sound production, and may activate behaviors whose performance is incompatible with acoustic communication (e.g. escape responses) and therefore inhibit sound production indirectly.

To overcome these problems, pharmacological and physiological experiments can be performed on primary cell culture. To characterise central complex neurons that control grasshopper sound production in dissociated primary cell culture, they have to be distinguished from other brain neurons. Since dextranes that have been injected to effective stimulation sites within the central complex are incorporated via post-synaptic compartments and accumulate in cell bodies, neurons with post-synapses in control circuits for stridulation can be identified by dextrane-coupled

fluorescent label after dissociation of brain cells. On average, 3.2 labeled cells per culture could be found of which a subpopulation also expressed the mAChR on the soma. The finding that neurons in culture express functional receptors in cell body membranes is somehow surprising, regarding the fact that the somata of insect neurons are regarded as electrically passive, but it could be shown through electrophysiological experiments that neurons in the ventral nerve cord of locusts responded to application of neurotransmitters (Burrows 1996) indicating that they express the appropriate receptor.

Numerous studies identified specific phrmacological responses to transmitter stimulation on isolated neuronal cell bodies, that lost their neurites during dissociation of neural tissue (Kreissl and Bicker 1992, Bicker 1996, Goldberg et al. 1999, Grünewald 2003, Grünewald et al. 2004, Wüstenberg and Grünewald 2004, Barbara et al. 2005; 2008). One way to measure the response of a culture neuron is calcium-imaging. Earlier studies on grasshopper brains showed that not only the cAMP-signaling pathway but also the PLC-second-messenger pathway, which results in a release of Ca^{2+} from internal stores, is activated by activation of the mAChR (Wenzel et al. 2002).

Neurons that were labeled with dextrane at a site where muscarine injections stimulated sound production could be identified in primary cell culture. When stimulated with in muscarine these neurons responded with an increase in cytosolic Ca^{2+}-concentration (Fig. 4.6). Future experiments could include testing the effects of different biogenic amines or neuropeptides on these neurons.

This approach can be extended to study any neuron in vitro that can be labeled via dextranes at sites where a particular signaling substance has an effect on sound production and whose identity could be confirmed by immunocytochemistry after physiological experiments in vitro. One example would be proctolin, using the available antibody against the proctolin-receptor to characterize first neurons on histological section and in culture and later on perform physiological experiments.

4.4 Pharmacology

Despite the disadvantages just described, pharmaco-behavioral studies with intact grasshoppers offers a great opportunity to examine the effect of various signaling systems in one specific brain region on the performance of particular behaviors (Heinrich et al. 1997; 1998b; 2001a;b, Wenzel et al. 2005, Hoffmann et al. 2007). The role of biogenic amines for modulation of general behavioral states and thresholds for particular behaviors is of special interest and has been addressed in a number of reviews (Bicker and Menzel 1989, Edwards and Kravitz 1997, Huber et al.

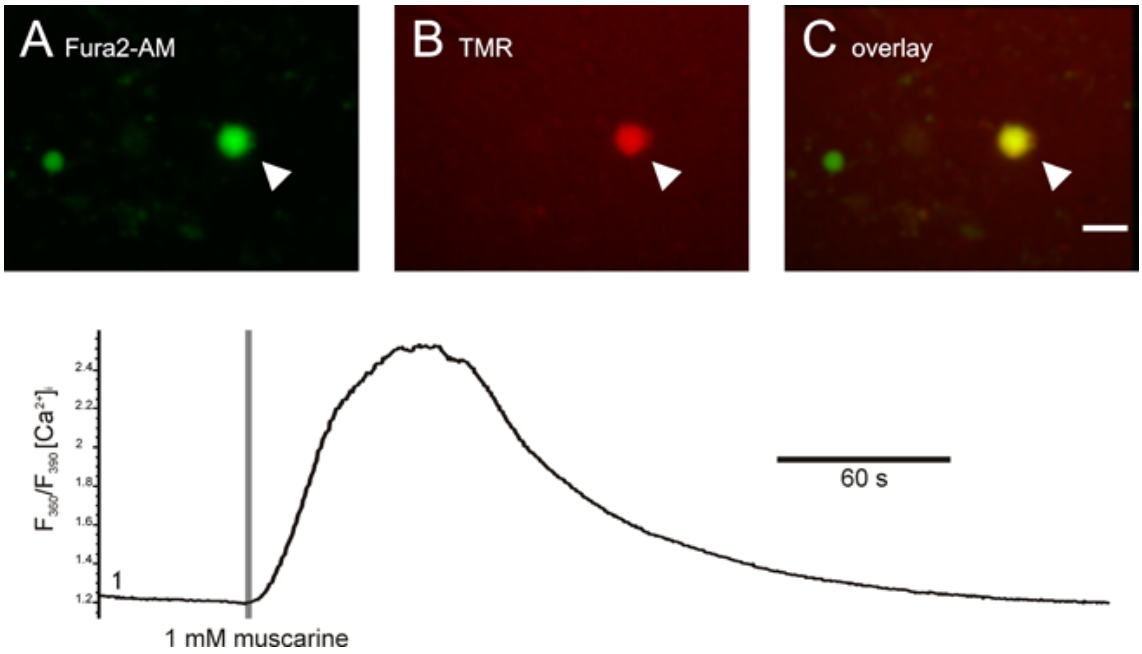


Figure 4.6: Example showing that neurons of the central complex respond to muscarine with an increase in intracellular calcium. A-C: Images of neurons in primary culture that are labeled with tetramethylrhodamine-dextrane (TMR) injected to a site in the central complex where muscarine stimulated sound production and the fluorescent calcium-indicator Fura2 applied to the culture medium. C shows the overlay indicating that the intravitally labeled neurons is loaded with the calcium-indicator. D: Calcium-imaging from the neuron shown in A-C. Application of muscarine caused a strong increase in $[Ca^{2+}]_i$.

1997, Kravitz 2000). One of the main ideas how biogenic amines regulate behavior is that they act as neuromodulators. According to this, the theory of "orchestration of behavior" has been formulated (Hoyle 1985). This hypothesis states, that neuromodulators (biogenic amines or peptides) are released into specific neuropiles to configure distinct neuronal assemblies to produce coordinated neuronal activity. The following section will focus on two biogenic amines (tyramine and dopamine) for which it was possible to assign a function in the control of acoustic communication by the central body.

4.4.1 Tyramine

Tyramine (TA) is the invertebrate counterpart of adrenaline. It is a decarboxylation product of the amino acid tyrosine and a precursor of octopamine (OA) (Roeder 2005). Only recently, a transmitter function was assigned to TA acting through G-protein coupled receptors (Saudou et al. 1990). Because TA, together with OA, represent the only nonpeptidergic transmitter system that is restricted to invertebrates, pharmacologists have focused their attention on the corresponding receptors, which are still believed to represent promising targets for new insecticides. Not much is known about the effect of TA on behavior. TA is generally believed to act as a functional antagonist to OA, which is supported by the fact that both transmitters activate opposing second-messenger cascades. Recent studies demonstrated that

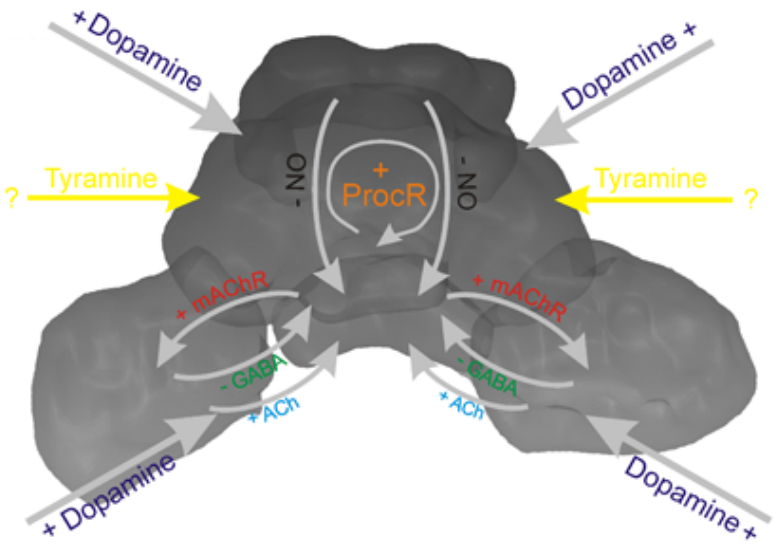


Figure 4.7: Schematic representation of the tyraminergic information flow in the central complex inhibiting sound production. Tyramine released into the central body (yellow arrow) leads to a decrease of sound production. Neither the origin nor the target of tyramine in the central body are known, but pharmacological data indicate that the inhibitory effect is probably mediated via an indirect increase of excitatory output.

tyramine and octopamine differentially regulate flight behavior in *D. m.* (Brembs et al. 2007), but this difference did not result from antagonistic effects of TA and OA on particular neurons of the flight control circuits. The authors concluded that antagonistic actions of OA and TA may not be a general feature of invertebrate locomotor behaviors but specifically affect distinct aspects of different motor behaviors (in their case flight initiation and maintenance).

Injections of tyramine into the central body of grasshoppers decreased the duration of muscarine stimulated singing (Fig. 3.32). This effect seems to be specifically mediated by tyramine receptors, because injection of a mixture of TA and yohimbine (antagonist for TA-receptors) did not alter muscarine stimulated stridulation. One possible explanation for this could be that activation of TA-receptors decreased cAMP-levels in mAChR expressing cells. The only characterized TA receptor so far (Saudou et al. 1990) has been shown to be negatively coupled to AC, leading to a decrease of intracellular levels of cAMP. Although this could generally fit well with the described observation two things have to be considered. (1) The detected effect was rather weak and (2) it took a rather long time to develop (23 minutes). To determine the neurons that release and react to TA and actively suppress stridulation, the distribution of tyramine and (even more important) of its receptor within the CX has to be determined.

4.4.2 Dopamine

In both vertebrates and invertebrates, the biogenic amine dopamine is implicated in many functions including locomotion, cognition, and development. Furthermore,

misregulation of dopamine signaling is believed to play a role in a number of human disorders including Schizophrenia, Parkinson's disease, Tourette's syndrome, and drug addiction. A recent report showed the effect of increasing dopaminergic transmission in *D.m.* (Andretic et al. 2005) through feeding of the DA-reuptake inhibitor methamphetamine, which causes an increase in behavioral arousal leading to decreased sleep and increased courtship behavior. The connection between dopamine and courtship behavior is of special relevance to this study because the acoustic communication examined in this study is part of the courtship ritual of grasshoppers. It is worth to mention that dopamine not generally increased all kinds of behavior but seemed to be specific to certain behaviors, because although the methamphetamine fed flies spent more time courting, their reproductive success was lower compared to control animals.

The results presented in this thesis indicate that dopamine signaling in the central complex promotes sound production as a major component of grasshopper courtship behavior. The strongest evidences for this came from experiments where injections of dopamine into the CB elicited stridulation in *Ch.b.* (Fig. 3.35and 3.36). Experiments testing for a modulatory influence of dopamine (either inhibitory or excitatory) on muscarine-stimulated stridulation revealed no significant effects (Fig. 3.36 and 3.37). The lack of an inhibitory modulatory influence of DA is in line with its capability to elicit stridulation. Nevertheless potential inhibitory influences at particular sites within the song control circuit cannot be excluded per se, because one of the two types of invertebrate DA-receptors (D2-like) is negatively coupled to the AC (Hearn et al. 2002, Suo et al. 2003, Beggs et al. 2005). The lack of excitatory influence is more difficult to explain. According to the theory that dopamine acts as a neuromodulator, one would expect that joined application of dopamine and muscarine should lead to longer singing duration compared to muscarine alone. This was not the case (Fig. 3.37). A potential explanation would be that through the injections of muscarine, the stridulation activity output of the central complex is already maximally activated. Muscarine stimulated duration of sound production increased over the first three to five applications in regular intervals until each further muscarine pulse elicited similar behavioral responses. Only after reaching the experimental phase with similar responses to individual muscarine stimuli, dopamine was co-applied to the same injection site. By this time persisting muscarine mediated excitation may have reaches such high levels, that additional dopamine mediated excitation did not result in further increased physiological and behavioral responses.

Experiments with antagonists to DA-receptor showed a clear effect on muscarine stimulated stridulation (Fig. 3.38). Application of the antagonist lead to a clear reduction in singing duration, indicating a tonic dopamine release into central body neuropiles. This could also partly explain why no positive modulatory effects of

dopamine could be detected. Biogenic amines and dopamine in particular have been demonstrated to need optimal concentration mediate their effects. Deviations from this concentration (either more or less) can diminish the effect or even reverse it (inverted U-hypothesis, Hebb (1955). Stimulation of grasshopper sound production with different dopamine concentrations may therefore exert different behavioral responses. The results of my experiments suggests that both known dopamine-receptor subtypes may be involved in increasing the motivation to perform singing. When using the unspecific antagonist flupenthixol, the inhibitory effect of muscarine stimulated stridulation appeared earlier (at the first muscarine stimulus after the application of flupenthixol), while the effect for the specific D1-antagonist SCH23390 took longer to develop (12 minutes after application). These results imply a similar function of dopamine on the control of motor behavior in insects and vertebrates (for detailed discussion see functional implications). To further prove this assumption one has to define which neurons in the central complex express the different types of dopamine receptors. Immunocytochemical experiments on *D.m.* showed that a D1-like receptor is strongly expressed in all subdivisions of the CX except for the PB (Kim et al. 2003). So far no attempts have been published to map the distribution of D2-like receptors in the central complex of insects, although suitable antibodies may available that have been shown to label D2-receptor in the CNS of *D.m.* (Draper et al. 2007).

4.5 Functional implications

In connection with results from earlier pharmacological studies the anatomical data presented in this study provide a first crude framework for the flow of information within the central complex. Our conclusions are largely based on the expression of some of the transmitters and their receptors known to contribute to the cephalic control of grasshopper sound production in particular types of central complex neurons that serve as inputs or outputs for information processed in this set of neuropiles. Our results indicate that the lower division of the central body may play a particularly important role for the initiation of sound production since various signaling pathways known to promote (ACh) or to suppress (GABA and NO-stimulated cGMP) sound production converge in this neuropil. Columnar output neurons that express mAChRs have their dendritic arborizations in this central complex neuropil that intermingle with neurites of GABAergic tangential neurons, part of which also upregulate cGMP upon NO stimulation. Therefore, GABA- and NO/cGMP-mediated inhibition of sound production may result from direct synaptic inhibition of those neurons that initiate the behavior upon cholinergic excitation.

The finding that expression of mAChRs and NO-stimulated upregulation of cGMP

are restricted to certain types of neuron supports that these neurons indeed mediate the effects on the performance of sound production that have been described in previous pharmacological studies. Supporting evidence that decision making for motor behaviors takes place in the CBL derived from *D.m.*. Martin et al. (2001) expressed tetanus toxin in neurons of the ellipsoid body to block their synaptic output. As a result of this inactivation the temporal coordination of spontaneous walking bouts was altered.

Recent studies on fruit flies indicated that the CBU and the PB may have a modulatory role in the control of locomotor activity (Martin et al. 1999), being responsible for the maintenance of walking behavior. The histological data presented in this study support this theory, because all signaling substances (biogenic amines and neuropeptides) that were only capable of altering the efficacy of muscarine stimulation, instead of providing sufficient excitation to trigger or completely suppress stridulation, were predominantly abundant in the central body upper division.

The role of proctolin in this system is a little bit elusive. Although the receptor could also be detected in columnar neurons just like the mAChR, the fibers of the neurons expressing the proctolin-receptor are mainly found in the CBU. These neurons restrict their connections to the central complex and do not sent information surrounding brain structures, while neurons expressing the mAChR sent their fibers to the contralateral LAL. Injections of proctolin should not directly increase excitatory output of the central complex like injections of muscarine do, but rather modulate information processing within the CBU (Fig. 4.5) that in turn leads to a stronger activation or weaker inhibition of the mAChR-expressing neurons in the CBL. One way to achieve this would be through decreasing NO-production in the CBU. Columnar neuron with similar morphology as the the proctolin receptor expressing neurons in *Ch.b.* (with respect to location of cell body and the innervation pattern with the central complex) have been described in *D.m.* (Martin et al. 1999). If the synaptic output of this neuron type was blocked by expression tetanus toxin the animal displayed decreased locomotor activity.

The psycho-hydraulic model states that behavior is a results of an increasing drive to act which is caused by a "action-specific energy", which is accumulated in distinct parts of the nervous system (Lorenz 1937). As a refinement resulting from this study, mAChR expressing columnar output neurons of the central complex seem to represent the final point of convergence of neural information related to the control of sound production and determine whether a behavior is executed or not. Therefore, these neurons can be regarded as the fluid storage for the "action-specific energy" in the model of Konrad Lorenz. Excitatory and inhibitory input seems to be integrated in these central complex output neurons and the motivation to sing has its correlate in the level of second messengers that accumulate upon muscarinic excita-

tion. Previous studies (Heinrich et al. 2001b, Wenzel et al. 2002) demonstrated that muscarinic excitation is mediated by activation of both the phospholipase C and the adenylyl cyclase second messenger pathways. Whether both pathways may be expressed in the same or different neurons and whether both pathways are activated by the same or different types of mAChR is still unknown. The antibody used in this study was raised against a *D. melanogaster* mAChR, which so far remains the only molecularly identified muscarinic receptor in insects (Blake et al. 1993). Studies on frog oocytes , Cos-7 and S2-cell lines that heterologously expressed this receptor demonstrated its positive coupling to the phospholipase C signaling pathway (Blake et al. 1993, Millar et al. 1995). Since data from various pharmacological studies indicated that insects express multiple types of pre- and postsynaptic mAChRs that, alternatively to activating phospholipase C, can activate or inhibit adenylyl cyclase-dependent second messenger cascades (Knipper and Breer 1989, Wenzel et al. 2002), expression of a second type of mAChR in the central complex that is not detected by this antibody cannot be excluded. On the other hand it is also possible that one receptor can activate two different second-messenger cascades (dual coupling) or activate one pathway that connects to another through one of the individual signaling molecules and enzymes. One example from insects showing dual-coupling is the D1-like-receptor from *D.m.* (Reale et al. 1997). Stimulation of this receptor leads to the activation of both, the cAMP- and the PLC-second-messenger cascade, with the cAMP-cascade activated by the α-subunit and the PLC-pathway by the $\beta\gamma$-subunit of the G-protein. Whether the same holds true for the mAChR in the central complex of grasshoppers remains to be open for further investigations.

This study also identified new transmitter systems that affect the control of sound production. Both, tyramine and dopamine, belong to the group of biogenic amines. Unfortunately it was not possible to determine the distribution of TA in the central complex making it impossible to identify which neurons in the central complex release or react to tyramine. Results from the locust *L. migratoria* showed that tyraminergic fibers globally invade all parts of the CB (Pflüger et al. 2007). Although TA-receptors in other insects inhibit second-messenger pathways whose activation is connected to an increase in the motivation to sing, the long latency of the tyramine effect make a direct influence on the mAChR-expressing neurons unlikely. TA, together with octopamine, has been implicated in the control of fight or flight behavior in locusts (Roeder 2005), inducing metabolic and behavioral adaptation, leading to enhanced energy supply, increased muscle performance, increased sensory perception, and a matched behavior. One possible mode of action how TA decreases motivation to sing would be the activation of other motor program involved in this behavior (e.g. escape responses), whose activity could suppress sound production. Another recent report showed that TA has similar of effects on flight behavior of

D.melanogaster. (Brembs et al. 2007), namely that it inhibits the initiation of flight. If this is the case TA could act at two stages in this system. It could either modulate by decreasing excitatory input (through ACh) or decreasing excitatory output (through mAChR-expressing cells) of the CB. Because so far TA-receptors have only been shown to inhibit the activity of the target neurons by inhibiting the AC and increasing Cl^--conductance (Cazzamali et al. 2005) it seems unlikely that direct inhibitory input is increased. Recently an antibody against the TA-receptor from the honeybee *Apis mellifera* has been generated and this antibody shows strong signals in the central body (Mustard et al. 2007). Future experiments should include the mapping of this receptor in the CB of *Ch.b.*. Furthermore calcium-imaging experiments on primary culture neurons can be performed, to see if TA decreases the calcium response to muscarine.

Another interesting aspect of the work from Brembs et al. (2007) on *D. melanogaster* is that OA increases the duration of spontaneous flight bouts. Due to the fact that high amounts of OA can be detected in the CB of *Ch.b.* (data not shown) it would be interesting to see if a similar effect of OA, that counteracts TA, on the control of sound production can be found.

The other transmitter to which a function in the control of acoustic communication could be assigned is dopamine. Most of the research performed so far focused on the role of dopamine in relation to learning and memory and only few studies dealt with the control of locomotor activity (Feany and Bender 2000, Haywood and Staveley 2004, Draper et al. 2007, Liu et al. 2008). Dopaminergic transmission in relation to motor behavior is of special interest because disturbances of this transmitter system are implicated in motor dysfunctions like Parkinson or chorea Huntington. Comparison of mammals with invertebrates has demonstrated that key elements of dopaminergic neurotransmission are evolutionary conserved (Eveleth et al. 1986, Neckameyer and Quinn 1989, Gotzes et al. 1994, Sugamori et al. 1995, Feng et al. 1996, Han et al. 1996, Pörzgen et al. 2001, Hearn et al. 2002, Greer et al. 2005). Insects with their comparable simpler structured nervous systems can be valuable models to dissect the basic principles that underlie the control of motor behavior by dopamine. The value of insect preparations is also represented by the fact that *D.m.* is one of the most persuasive animal models for Parkinson's disease. (Feany and Bender 2000).

This study shows a clear stimulatory effect on sound production for dopamine in the central body of the grasshopper *Ch.b.*. This is most strikingly seen by the fact that injections of DA into the central body elicit stridulatory behavior. This stimulatory effect must be mediated by one or all three types of tangential (input) neurons to the CB that connect the central body with various surrounding brain structures (LAL, SMP, MB). Special notion should be addressed to DC1-like neurons, because a

neuron with a similar morphology affected locomotor activity in *D.m.* (Martin et al. 1999). Genetical silencing of these neurons by expression of tetanus toxin caused a decrease in locomotor activity. Unfortunately the authors did not investigate the main transmitter of these neurons. Another interesting aspect of DC1-like neurons is that they may directly link the mushroom bodies with the CB. Although the main function of the mushroom bodies is believed to be formation of memory there is also evidence that they have a function in motor control. Early experiments with crickets and grasshoppers showed that MB activation dampen the general motor activity (Huber 1955; 1960; 1963; 1965, Otto 1971, Wadepuhl and Huber 1979, Wadepuhl 1983, Homberg 1987) and the same seems to be the case in *D.m.* (Martin et al. 1998). These data, together with the finding that dopaminergic signaling in the central body increase the motivation to perform stridulation lead to the speculation that the inhibitory function of the MB is maybe at least partly mediated by inhibiting dopaminergic DC1-like neurons.

Dopamine seems to be constantly released into the central body, which was demonstrated by coapplications of DA-antagonists leading to a reduction of muscarine-stimulated sound production (Fig. 3.38). This effect is stronger when both dopamine receptor subtypes are blocked compared to inhibiting only the D1-like type. Because both receptors activate opposing second-messenger cascades (D1-like are coupled to an increase in cAMP and/or intracellular calcium, while D2-like receptors inhibit the AC) in other insect species, it is unlikely that they are expressed on the same neuron. A similar situation exists in the basal ganglia of vertebrates (DeLong 2000) where two pathways are present that control motion. One is called the direct pathway that facilitates movement while the other (the indirect pathway) inhibits movements. Dopamine, released from neurons of the substantia nigra into the basal ganglia activates the direct pathway through binding to D1-receptors and inhibits the indirect pathway by binding to D2-receptors. Whether a similar mechanism is present in the central complex of grasshoppers cannot be derived from the pharmacological results presented here. One way of proving this theory would include the mapping of the different receptor subtypes within the central complex. Additional experiments could be performed to test if receptor subtype specific agonists are also able to induce stridulation or if both receptor systems have to be activated.

Various studies on locusts and other insects implicated that the central complex is involved in processing of spatial information (Homberg 2004, Liu et al. 2006, Heinze and Homberg 2007). In a recent paper it was shown, that single columns of the protocerebral bridge respond to specific e-vector orientations of dorsally presented polarized light, resulting in a maplike representation of this visual cue (Heinze and Homberg 2007). Work from our lab indicates that in male grasshoppers of the species *Ch. biguttulus*, the song of a conspecific female activates cholinergic path-

ways that project to the central body (Heinrich et al. 2001b, Hoffmann et al. 2007). Both types of stimuli, e-vector and conspecific songs, are processed by similar types of columnar neurons. Whether individual neurons of this type are multimodal or different functional subgroups of columnar neurons exist remains to be shown. Multimodality of these neurons would makes also sense, because both stimuli (polarized light and female songs) are used for orientation (Mappes and Homberg 2004, Helversen 1972, Helversen and Helversen 1983). Desert locusts for example use the polarized sky-pattern for spatial navigation during their migratory phases, while grasshoppers perform sound based localization of mating partners. It is tempting to speculate that direction of a sound source may also be encoded by activity in columnar neurons. It was demonstrated in earlier studies that muscarinic excitation in the central body increases the responsiveness of a male to female calling songs. Since insect muscarinic receptors have been shown to either increase postsynaptic excitability or decrease presynaptic transmitter release (Breer and Sattelle 1987, LeCorronc and Hue 1993), it was not clear whether muscarinic effect in the grasshopper central complex were mediated by pre- or postsynaptic (or both) mechanism. This thesis now showed that the increase of responsiveness is mediated by an excitation of postsynaptic mAChR located on output-neurons of the CX which mediate sound production, instead of presynaptic feedback autoreceptors, a function that is served by muscarinic receptors in other insect neuropiles.

In contrast, unfavorable situations that suppress sound production, like being restrained in an experimental setup or being handled by the experimentor seem to activate NO release in the upper division of the CB from those neurons that were detected by citrulline immunocytochemistry. Supporting evidence from pharmacobehavioral studies with *Ch. biguttulus* females in which suppression of citrulline accumulation in these neurons by NOS inhibitor was correlated with enhanced responsiveness to male song stimulation (Weinrich et al. 2008).

NO synthesized by NOS-expressing neurons diffuses through rather large volumes of neuropile and causes the accumulation of cGMP in cells containing soluble guanylyl cyclase (Bredt and Snyder 1992, Garthwaite and Boulton 1995). The finding that cGMP upregulation occurs in tangential neurons containing GABA suggests that the inhibitory effect of NO is probably mediated via an increased release of GABA, which is known to inhibit pharmacologically induced stridulation (Heinrich et al. 1998b), linking these two transmitter systems with similar function also anatomically. Furthermore both substances (GABA and NO) are tonically inhibiting sound production in the central body, which could be shown by experiments in which blocking GABA and NO-transmission induced stridulation (Heinrich et al. 1998b, Weinrich et al. 2008). The finding that NO-stimulated cGMP upregulation is found in tangential neurons indicates that NO acts as retrograde transmitter in the cen-

tral body. NO mediated retrograde transmission has previously been reported in the visual system and at the neuromuscular junction of insects (Elphick et al. 1996b, Bicker and Schmachtenberg 1997, Elphick and Jones 1998, Jones and Elphick 1999, Schmachtenberg and Bicker 1999).

While NO-producing cells are only found in the upper division, NO responsive neurons were exclusively located in the lower division. This represents the first evidence of direct information flow between the CBU and the CBL in a locust and adds a new level complexity to the central body connectivity. A direct information transfer between the CBU and the CBL could so far only be described in *D. melanogaster* (Hanesch et al. 1989) In contrast histological investigations in *S. gregaria* revealed no tracts that could mediate such a direct information transfer (Homberg 2004). NO signaling appears to be a rather unconventional way of communication between the two subdivisions, but the finding that none of three different methods applied to label NO-producing cells (NADPH diaphorase, anti-NOS- and anti-citrulline-immunocytochemistry) stained neurons in the CBL, and that the distance between NO-producing and cGMP accumulating fibers (in our case 40-60 μm) lies within functional range of NO in other nervous tissues (90 μm: Kasai and Petersen (1994), 300 μm: Gonzales-Zulueta, 1997; 100 μm: Madison and Schumann, 1995; $>$200 μm: (Wood and Garthwaite 1994) support this hypothesis.

Neuropeptides represent a huge variety neuromodulators that coexpressed along with other transmitter systems. Compared to other neuromodulators like biogenic amines, neuropeptides seem to be restricted to limited numbers of neurons. Work on the stomatogastric nervous system of crustaceans showed that various neuropeptides specifically induce different rhythms in this network. It is tempting to speculate if a similar situation may holds true for the central complex. The histological examinations presented in this study identified three possible candidates that may positively (allatotropin, CCAP) or negatively (allatostatin) modulate the decision to sing in the central complex.

4.6 Conclusions

For the sound production of grasshoppers, several transmitter systems could be shown to be involved in this integration process (promoting: ACh, proctolin; inhibiting GABA, glycine and NO). The present study gives insight on the information flow within the central complex (Fig. 4.8). ACh is released into the central body and acts on muscarinic receptors located on outputs neurons in the lower division. The same neurons are inhibited by GABA, while GABAergic neurons are modulated by NO produced in the upper division. We therefore propose that the

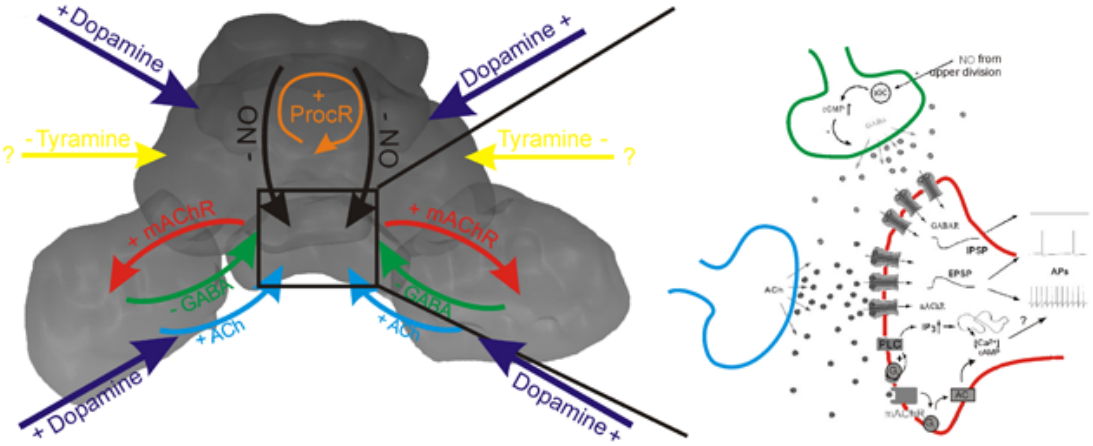


Figure 4.8: A: Scheme of the information flow within the central body mediating sound production within the central complex. Excitatory (through ACh, blue arrow) and inhibitory information (through GABA, green arrow) is sent from the LAL to the CBL where it is integrated from mAChR-positive cells, which relay excitatory information back to the LAL (red arrow). The integration process within the CBL is inhibitory modulated through NO from the upper division (black arrow), which acts on GABAergic transmission. Additional excitatory modulation comes from various surrounding brain through dopamine that is released globally into the central body (dark blue arrow) and proctolin, which modulates information processing intrinsic to the CB (orange arrow). Tyraminergic signaling (yellow arrow) plays an inhibitory role in this systems, but it can not be determined in which part of the CB it exerts its role and to which other brain regions tyramine is connected. B: Scheme of information flow within the lower division of the central body. Excitatory and inhibitory information converges onto the same kind of neuron. Excitation is mediated via ACh acting on nicotinic ACh receptors (nAChR), leading to rapid mediated excitation, and on muscarinic ACh-receptors leading to longer lasting excitation through the activation of second-messenger cascades (PLC- and cAMP-signaling pathway), which adds on the nicotinic activation and leads to a stronger excitation of the neuron. Inhibition is mediated through GABAergic signaling, acting on PTX-sensitive ionotropic GABA-receptors. Both transmitters are tonically released, and the decision whether to sing or not is determined by which transmitters system is stronger activated. Additionally, this decision is also modulated by the upper division, which influences synaptic transmission through action of NO, which diffuses from the upper division and leads to accumulation of cGMP in GABAergic neurons, and therefore facilitates inhibition. (modified from Trimmer, 1995).

mAChR-positive output neurons of the lower division of the CB are the neural correlate of the fluid storage in the psycho-hydraulic model by Lorenz. Furthermore, this works represents a framework that should facilitate the examination of other neurotransmitter/-modulators/-peptides systems in this preparation. It was possible to identify new transmitters systems that seem to have either promoting (dopamine) or inhibiting effects (tyramine). At last several neuropeptides could be identified in the CX. Double stainings of these neuromodulators with the transmitter-/receptor-systems of which a function in the control of acoustic communication may complete our picture of information flow within the central complex.

5 Summary

This thesis examined the neurochemical architecture of the central complex in the brain of the grasshopper Chorthippus biguttulus in relation to the control of sound production. In order to increase knowledge about information processing in the central complex, I used three methodic approaches: (A) Immunocytochemistry using antisera against signaling molecules that have been shown to either promote or inhibit singing behavior when injected into the central complex. In addition the distribution of neuropeptides in the central complex has been mapped to provide a framework for further physiological studies. (B) Pharmaco-behavioral experiments in which signaling molecules were injected into the central complex of intact grasshoppers to evaluate their impact on sound production. (C) Tracing experiments to label pharmacologically stimulated central complex neurons for anatomical identification and physiological characterization in dissociated primary cell culture.

The anatomical studies lead to the following results:

- mAChRs are exclusively expressed by columnar neurons that receive synaptic input in the lower division and connect the central complex with the contralateral lateral accessory lobes. Muscarinic-cholinergic excitation of postsynaptic receptors directly increases excitatory output of the central complex that promotes sound production.
- GABA is found in tangential neurons that innervate the entire lower division. GABAergic terminals are closely associated with mAChR-positive fibers. GABA-mediated inhibition of grasshopper sound production in the central complex most likely results from direct activation of GABA-receptors on mAChR-expressing columnar output neurons of the central complex.
- A subset of nitric oxide synthase-expressing central complex neurons is activated in situations (e.g. being restrained) where sound production is inappropriate. Nitric oxide is produced by neurons of the upper division of the central body and suppresses sound production through activation of soluble guanylyl cyclase in tangential neurons of the lower division. This result provides the first evidence for information transfer between the upper and lower division of the central body in orthopteran insects.
- NO stimulates the accumulation of cGMP in tangential neurons of the central body lower division. All NO-responsive neurons express GABA as primary transmitter, indicating that NO exerts its inhibitory effect on sound production indirectly through modulation of GABA release from tangential neurons onto columnar output neurons of the central complex.

- Dopamine is found in three types of tangential neurons that innervate all layers, except layer I, of the central body upper division. The global presence of dopamine in the central body may hint to a role as a modulator of general arousal rather than being a specific regulatory signal for acoustic communication. Two types of dopaminergic neurons link the central complex with the mushroom bodies.
- Proctolin receptors are expressed by pontine intrinsic to the central body and by columnar neurons intrinsic to the central complex. Proctolin may therefore mediate its promoting effect on sound production by contributing to intrinsic information processing within the central complex that indirectly increases excitatory output of the central complex.
- Tachykinin-related peptides are expressed by tangential and columnar neurons in all subdivisions of the central complex. Prominent colocalisation with GABA in the lower division suggests a role in the control of sound production through modulation of GABAergic inhibition of columnar output neurons. A similar role could be the case for allatostatin because the staining patters of AS in the upper division is similar to GABA. CCAP and allatotropin were detected in tangential neurons with similar morphology to dopaminergic neurons innervating the central body upper division

The pharmaco-behavioral experiments lead to the following conclusions:

- Injections of tyramine into the central complex inhibited muscarine-stimulated sound production. The inhibitory effect was weak and appeared after long latencies, but its suppression by the tyramine receptor antagonist yohimbine suggested a specific involvement of tyramine receptors.
- Dopamine injections into the central complex induced species specific sound production. Stimulatory effects of muscarine where not enhanced by dopamine, suggesting that both signals serially contribute to the flow of information within the central complex, instead of acting within parallel pathways that later converge onto central complex output neurons.
- Injections of dopamine receptor antagonists into the central complex decreased muscarine-stimulated singing, suggesting that dopamine released into the central complex tonically promotes sound production.

The tracing experiments lead to the following conclusion:

- Dextranes injected at sites in the central complex where muscarine stimulated sound production are incorporated by mAChR-expressing columnar neurons, demonstrating that this method is capable of labeling neurons that are directly stimulated by pressure injection of drugs.
- Neurons that incorporated fluorescently labeled dextranes can be identified in dissociated primary cell culture and physiologically characterized under defined experimental conditions. Immunocytochemistry can be used to confirm the type of neuron studied in vitro.

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Appendix

Insect Saline (Clemens and May 1974)

140 mM	NaCl
10 mM	KCl
4 mM	$NaH_2PO_4*2H_2O$
5 mM	Na_2HPO_4
2 mM	$CaCl_2*2H_2O$

Dissolved in 1l distilled water. The pH was adjusted with NaOH or HCl to 7.2

Phosphat Buffered Saline (PBS)

8 g	NaCl
0.2 g	KCl
1.44 g	Na_2HPO_4
0.24 g	NaH_2PO_4

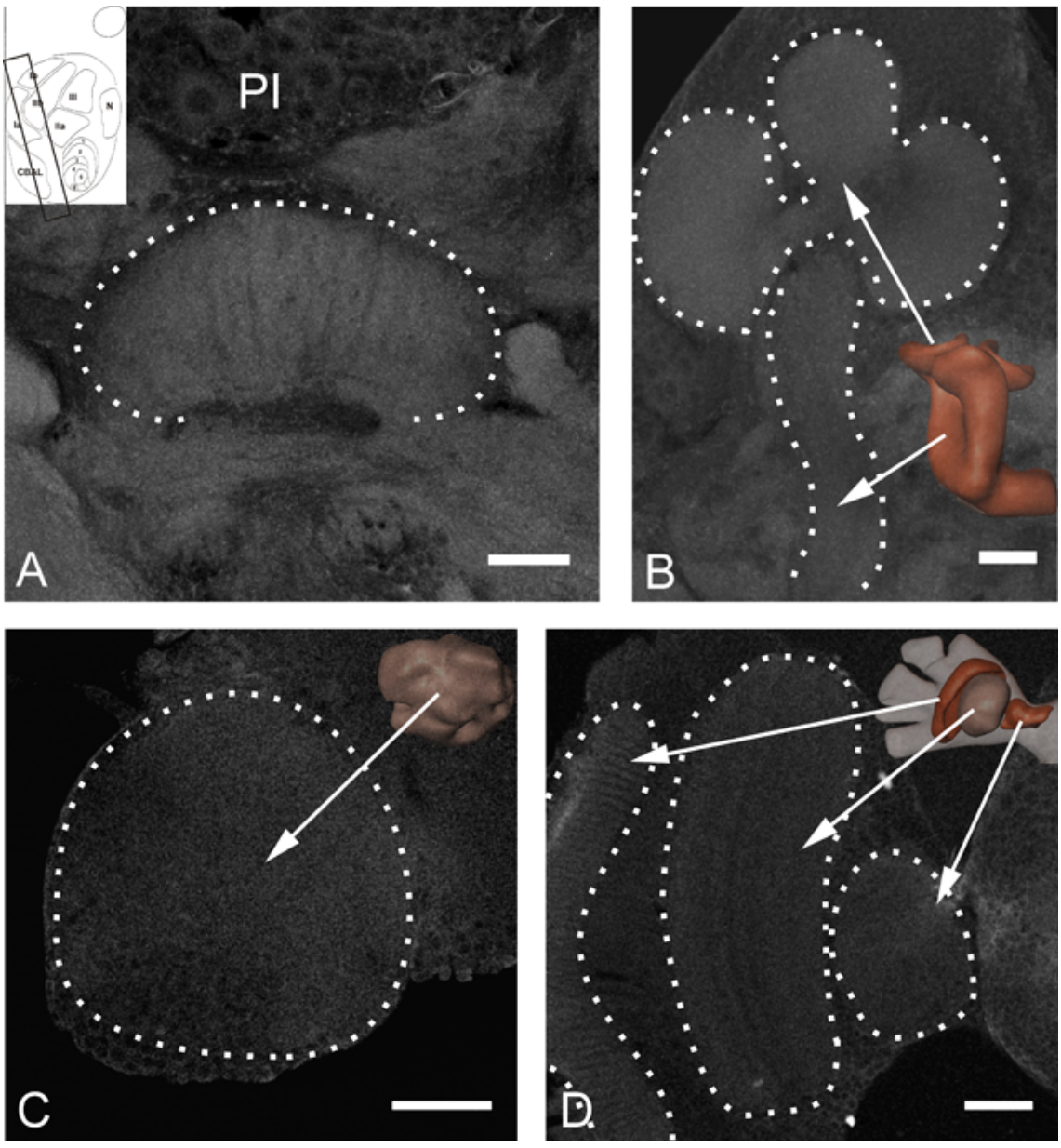
Dissolved in 1l distilled water. The pH was adjusted to 7.2 through adding of HCl and NaOH

4% Paraformaldehyde (PFA)

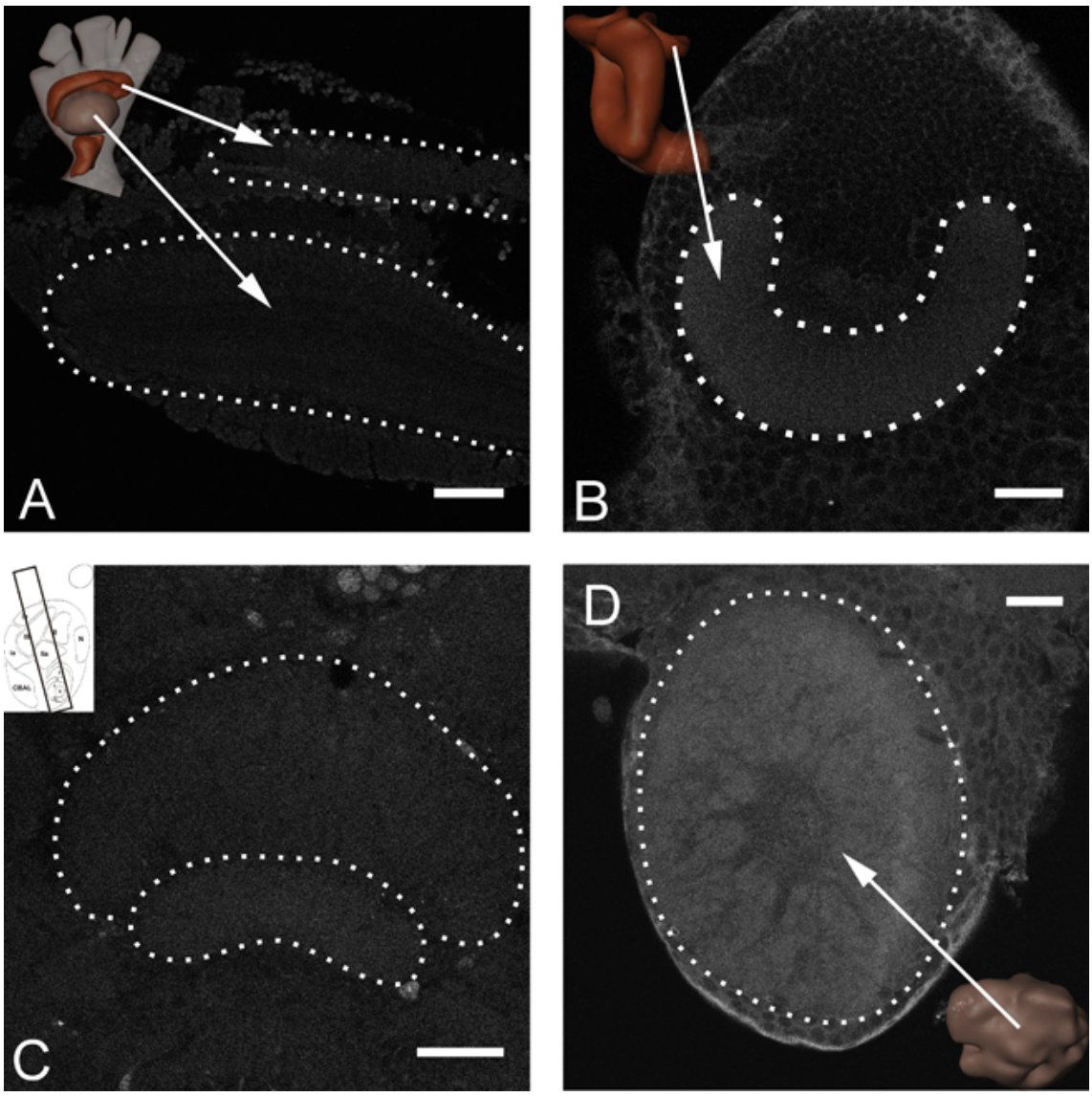
4 g	Paraformaldehyde
0.58 g	Na_2HPO_4
0.15 g	NaH_2PO_4

Dissolved at 60°C in 50 ml of distilled water.After cool down the pH was adjusted to 7.4 with Na_2HPO_4-solution and NaH_2PO_4-solution. Subsequently the solution was filled up with distilled water to a final volume of 100 ml

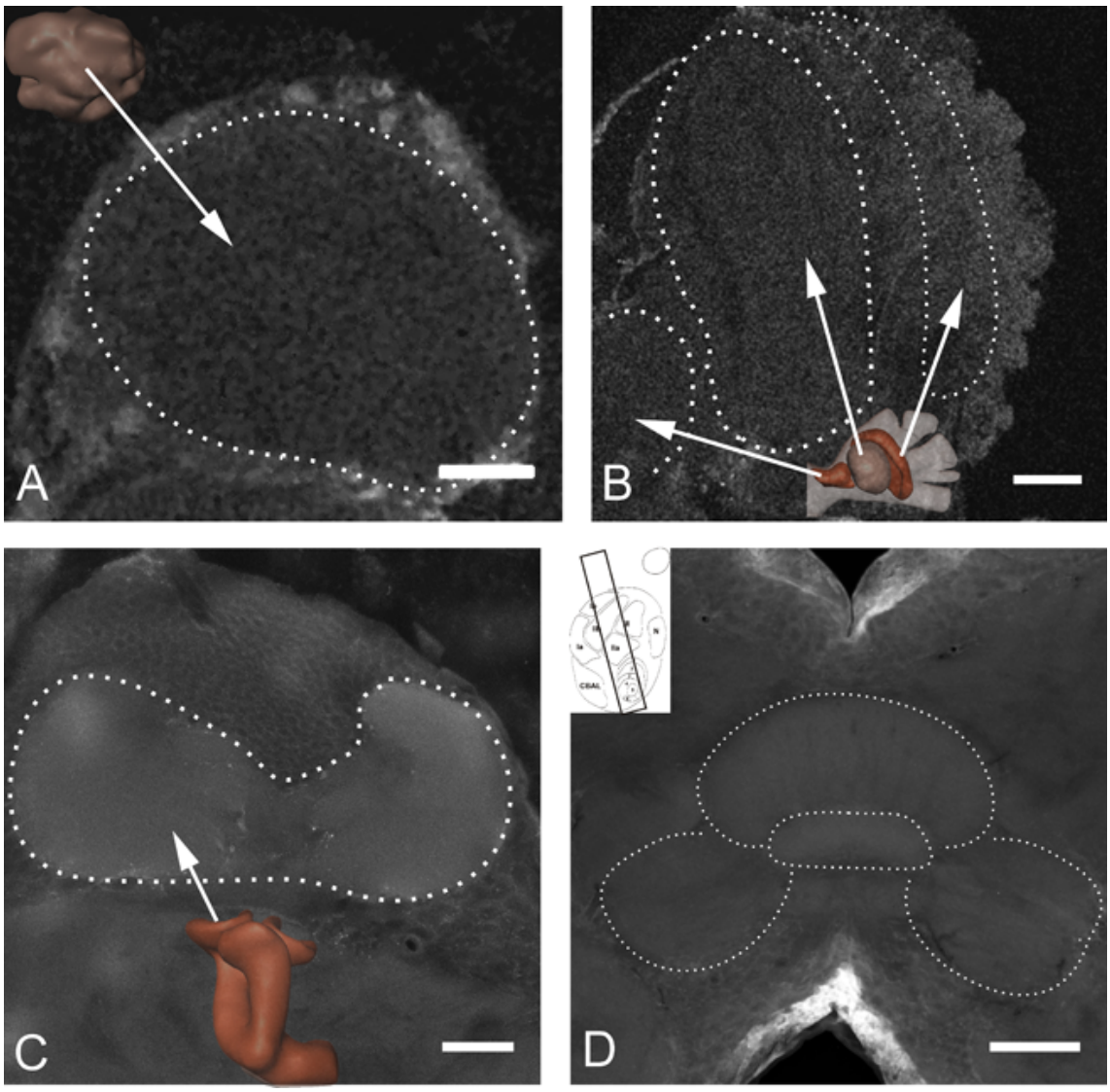
Exact locations of the collections spot for *Ch.b.*. A: Overview of Göttingen and the surrounding area. B: Collection spot in the city of Göttingen close to the institute. C: Collection spot "Einzelberg" close to Gross-Schneen. D: Collection spot "Wendebachstausee", close to Niedernjesa. Scale bars in B-D = 100 m.



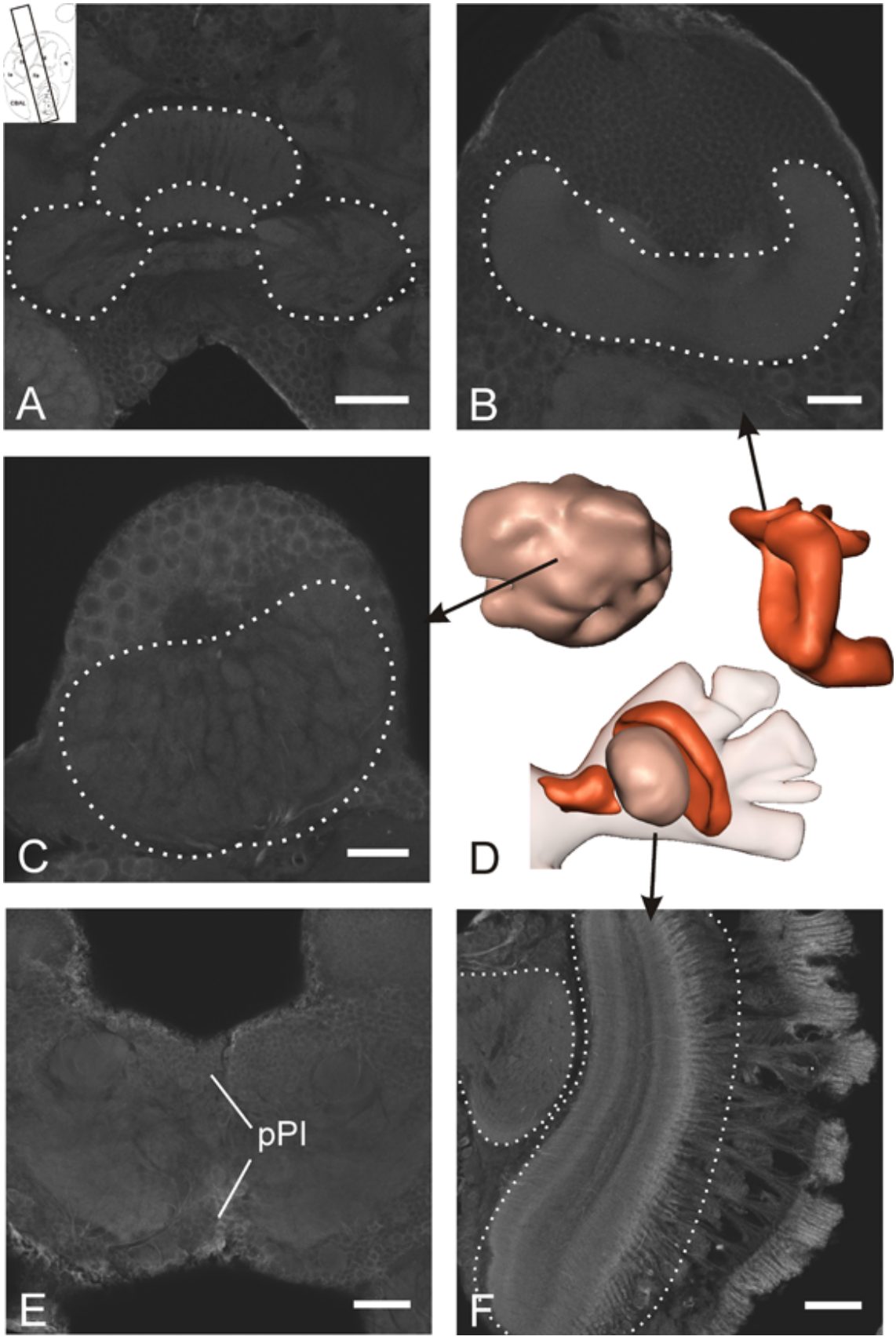
Control section for mAChR-immunocytochemistry in which the primary antibody was omitted. Frontal sections through the central complex (A), the mushroom body (B), the antennal lobe (C) and the optic lobe (D) displaying no mAChR-ir. Scale bars = 100 μm in C and D; 50 μm in A and B.



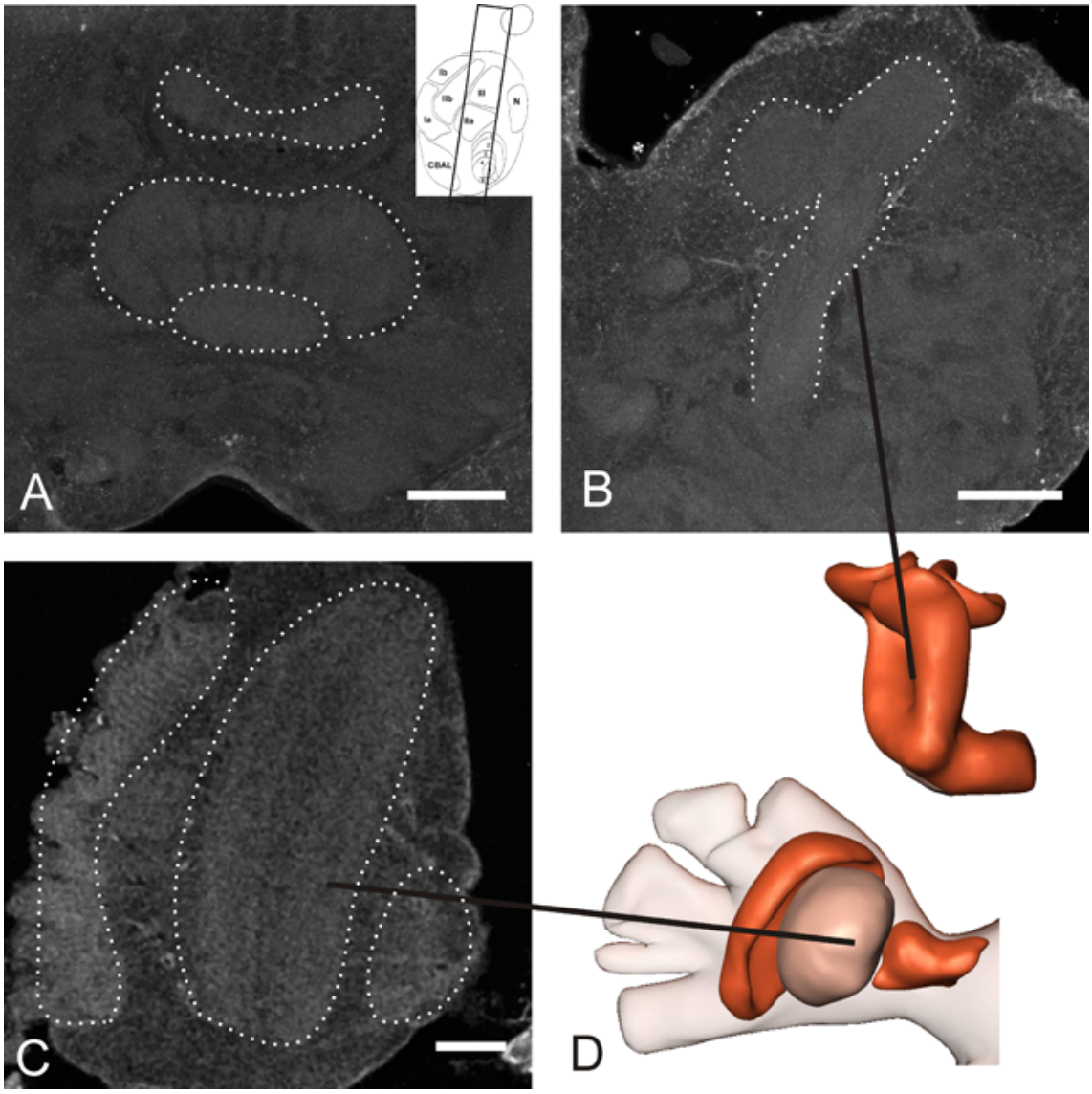
Control section for GABA-immunocytochemistry in which the primary antibody was omitted. Frontal sections through the optic lobe (A), the mushroom body (B), the central complex (C) and the antennal lobe (D) displaying no GABA-ir. Scale bars = 100 μm in A; 50 μm in B-D.



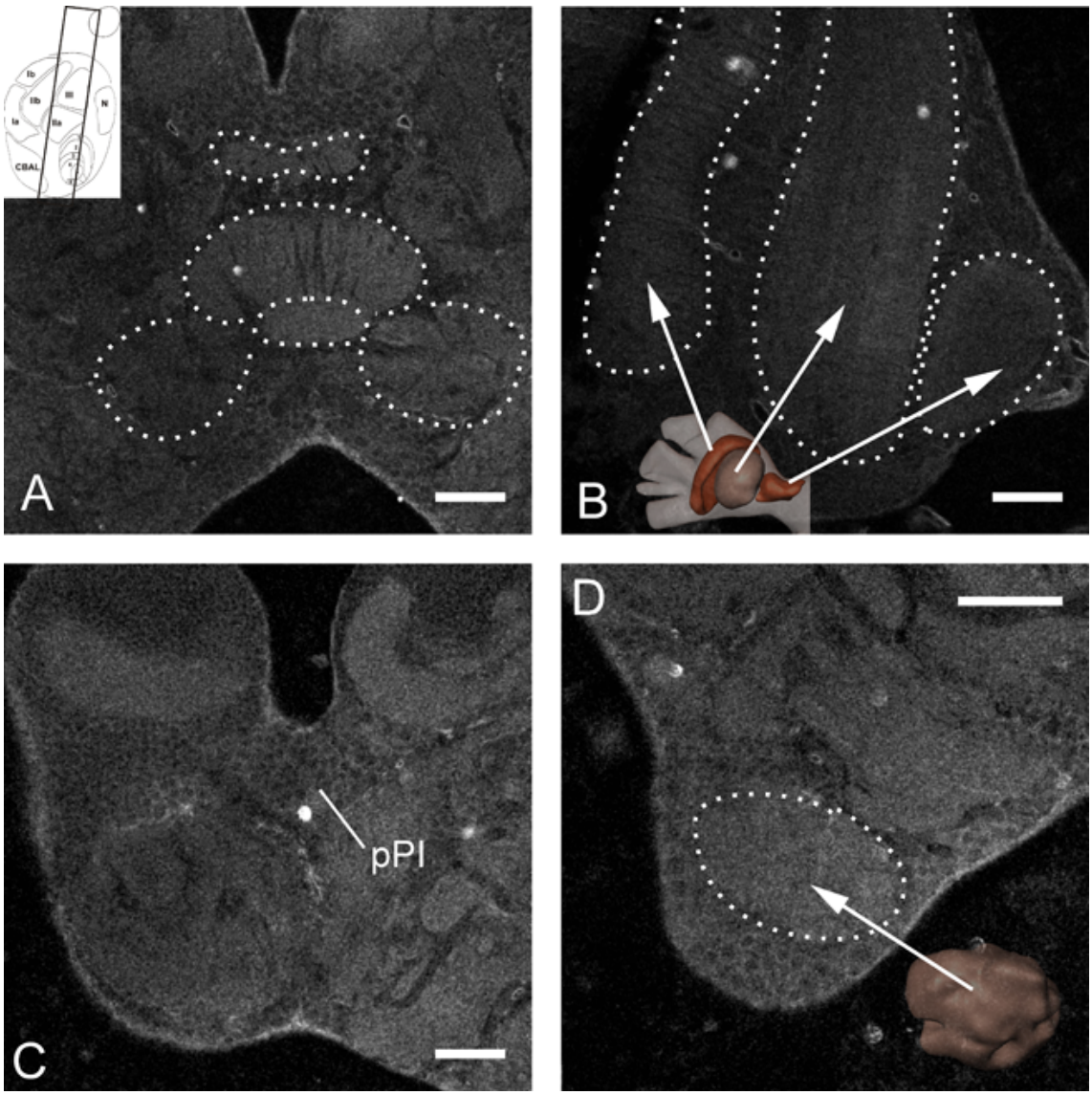
Control section for citrulline-immunocytochemistry in which the primary antibody was omitted. Frontal sections through the antennal lobe (A), the optic lobe (B), the mushroom body (C) and the central complex (D) displaying no citrulline-ir. Scale bars = 100 μm in A and C; 50 μm in B and D.



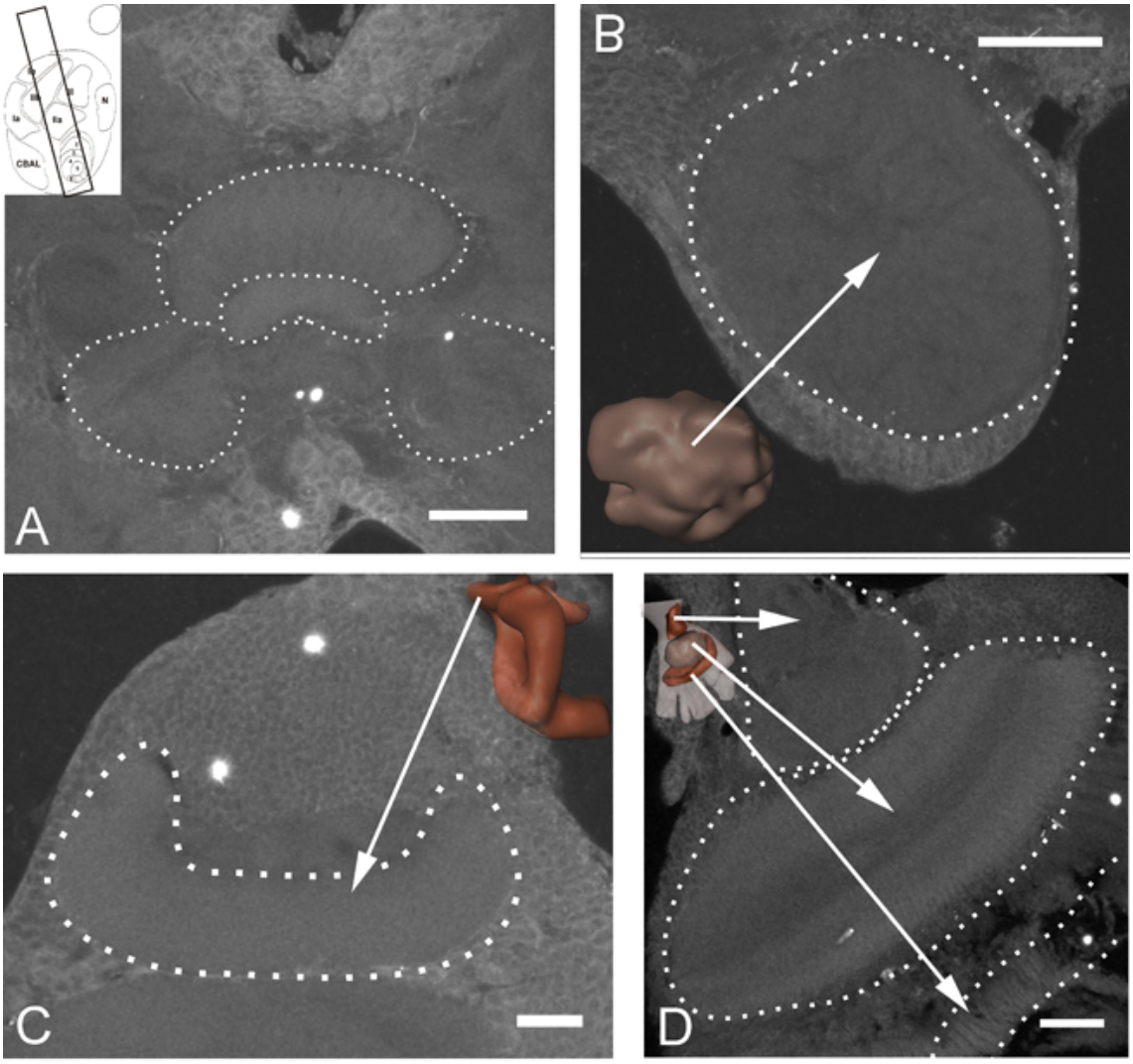
Control section for cGMP-immunocytochemistry in which the primary antibody was omitted. Frontal sections trough the central complex (A), the mushroom body (B), the antennal lobe (C), the posterior protocerebrum (E) and the optic lobe (F) displayed no cGMP-ir. D: 3D-models of the described brain region, arrows point to the respective brain structure. Scale bars = 100 μm in A, E and F; 50 μm in B and C.



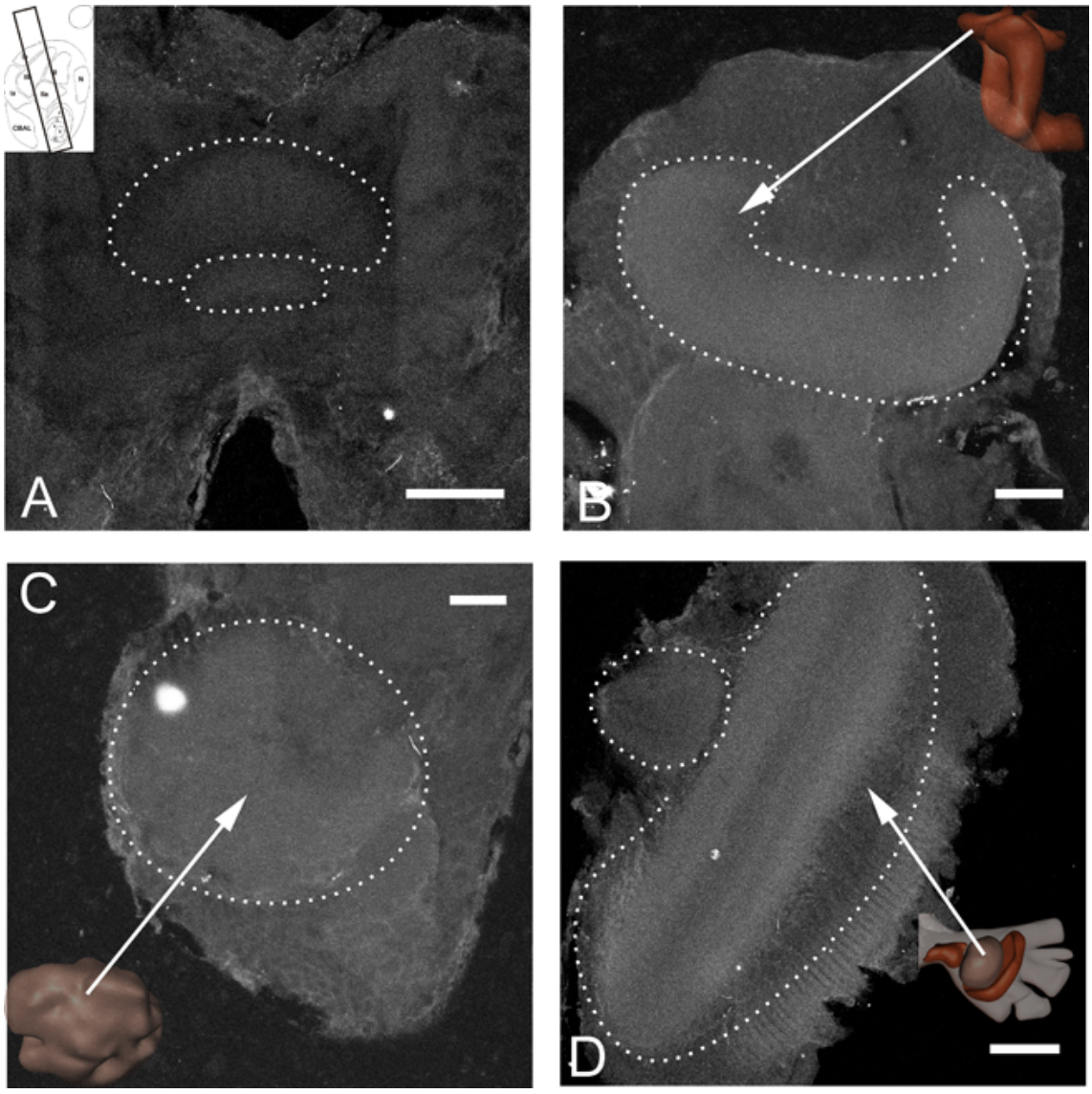
Control section for tyrosine-hydroxylase-immunocytochemistry in which the primary antibody was omitted. Frontal sections through the central complex (A), the mushroom body (B) and the optic lobe (C) displaying no tyrosine-hydroxylase-ir. D: 3D-models of the described brain region, arrows point to the respective brain structure. All scale bars 100 μm.



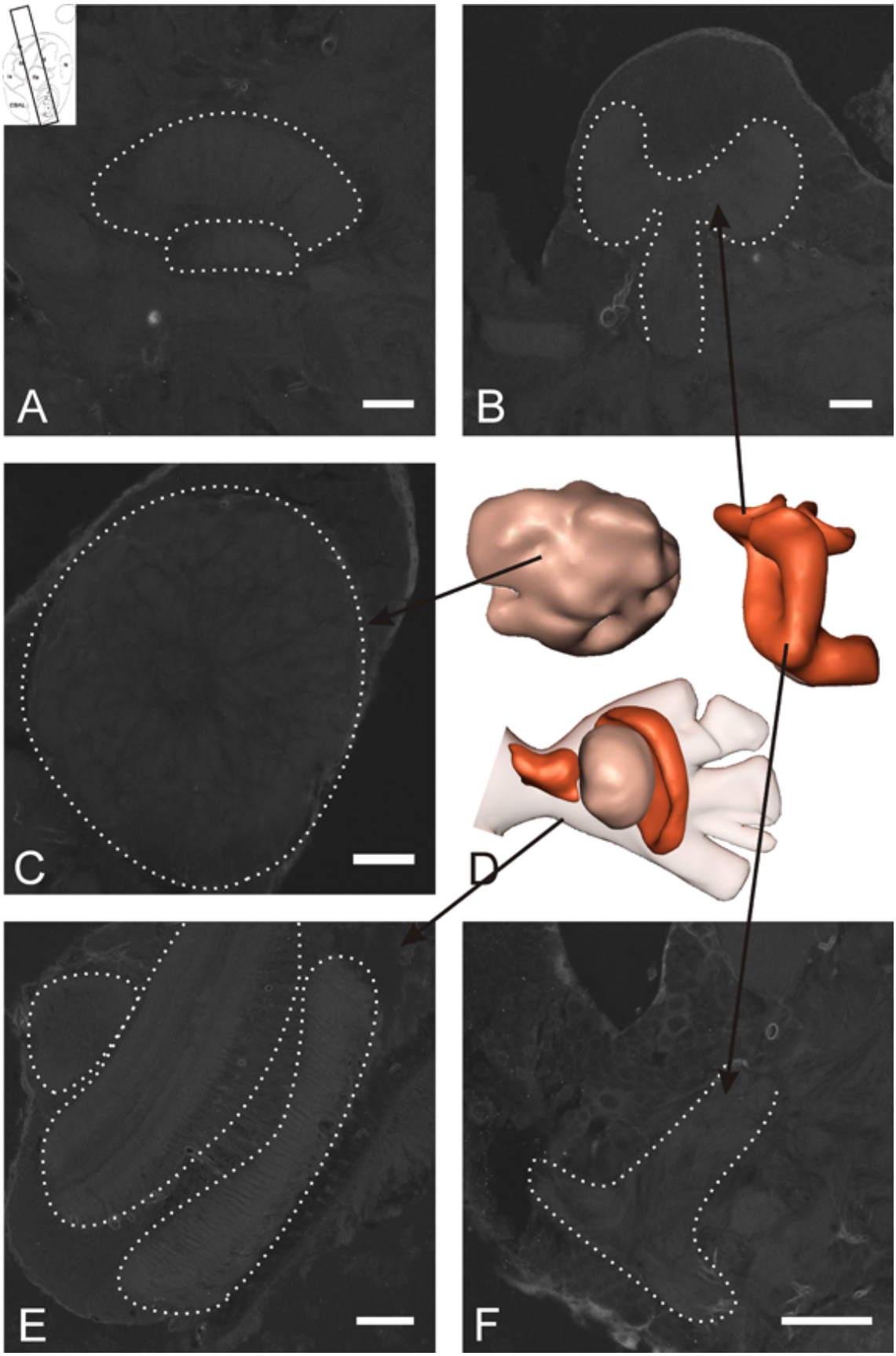
Control section for proctolin-receptor-immunocytochemistry in which the primary antibody was omitted. Frontal sections through the central complex (A), the optic lobe (B), the posterior protocerebrum (C) and the antennal lobe (D) displaying no proctolin-receptor-ir. All scale bars 100 μm.



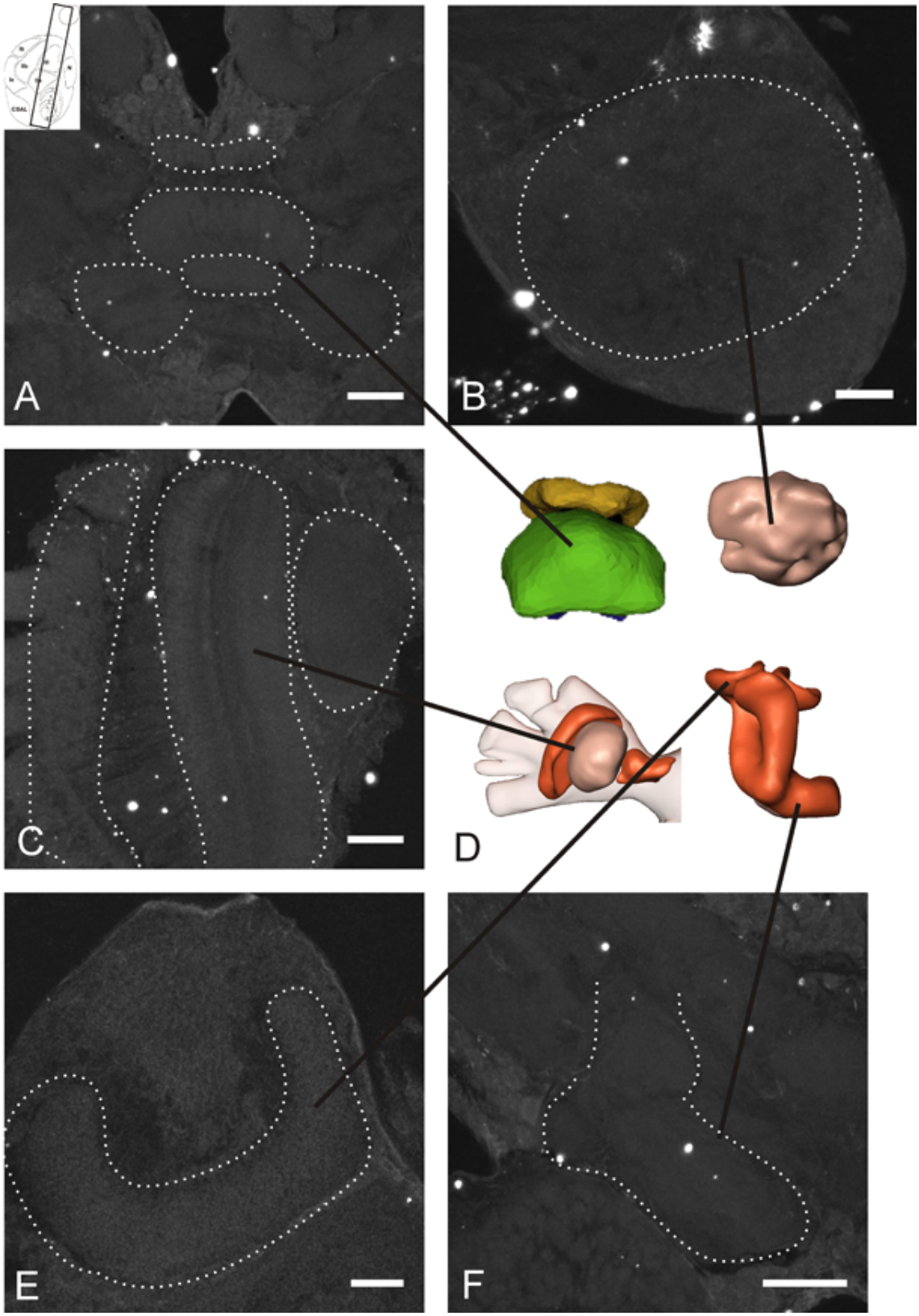
Control section for LemTRP-immunocytochemistry in which the primary antibody was omitted. Frontal sections through the central complex (A), the antennal lobe (B), the mushroom body (C) and the optic lobe (D) displaying no LemTRP-ir. Scale bars = 100 μm in A, B and D; 50 μm in C.



Control section for CCAP-immunocytochemistry in which the primary antibody was omitted. Frontal sections through the central complex (A), the mushroom body (B), the antennal lobe (C) and the optic lobe (D) displayed no CCAP-ir. Scale bars = 100 μm in A and D; 50 μm in B and C.



Control section for allatostatin-immunocytochemistry in which the primary antibody was omitted. Frontal sections through the central complex (A), the mushroom body (B and F), the antennal lobe (C) and the optic lobe (E) displaying no allatostatin-ir. D: 3D-models of the described brain region, arrows point to the respective brain structure. Scale bars = 100 μm in E and F; 50 μm in A-C.



Control section for allatotropin-immunocytochemistry in which the primary antibody was omitted. Frontal sections through the central complex (A), the antennal lobe (B) , the optic lobe (C) and the mushroom body (E and F) displaying no allatotropin-ir. D: 3D-models of the described brain region, arrows point to the respective brain structure. Scale bars = 100 μm in A, C and F; 50 μm in B and E.

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Curriculum Vita

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