INHIBITORY AND MODULATORY NEUROTRANSMITTER IN THE BRAIN OF SOCIALLY COMMUNICATING FISHES

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LIST OF ABBREVIATIONS

5-HT	Serotonin (5-hydroxytryptamine)
Xm	Vagal motor nucleus
ac	Anterior commissure
Ach	Acetylcholine
anc	Ansulate commissure
ANOVA	Analysis of variance
Cb	Cerebellum
CC	Cerebellar crest/central canal
CCe	Corpus cerebelli
ChAT	Choline acetyltransferase
CPG	Central pattern generator
CPr	Central pretectal region
Cx 35/36	Connexin 35/36
DAB	3,3'-diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
Dc/Dl/Dm/Dp	Central/lateral/medial/posterior zone of dorsal telecephalic area
DiV	Diencephalic ventricle
DON	Descending octovolateralis nucleus
ELISA	Enzyme linked immunosorbent assay
ELLL/MON	Electrosensory lateral line lobe/medial octavolateralis nucleus
EG	Eminentia granularis
EMN	Electromotor nucleus
FL	Facial lobe
fr	fasciculus retroflexus
G	nucleus glomerulosus
GABA	gamma-Aminobutyric acid
GCCe	Granular cell layer of the corpus cerebelli

GCVa	Granular cell layer of the valvula cerebelli
GC	Central grey
Ha	Habenula
Hc/Hd/Hv	Caudal/dorsal/ventral zone of the periventricular hypothalamus
IL	Inferior lobe of hypothalamus
IHC	Immunohistochemistry
IN	Intermediate hypothalamic nucleus
IR/iRN	Inferior raphe (nucleus)
IRc/i/r	Caudal/intermediate/rostral inferior raphe
iRF	Inferior reticular formation
kD	Kilo Dalton
lr	Lateral hypothalamic recess
MCCe	Molecular cell layer of the corpus cerebelli
MCVa	Molecular cell layer of the valvula cerebelli
MFN	Medial funicular nucleus
mlf/MLF	Medial longitudinal fasciculus
Mid	Mibrain
MO	Medulla oblongata
MON	medial octovalateralis nucleus
NInd/NInv	Dorsal/ventral interpeduncular nucleus
NLV	Nucleus lateralis valvulae
OB	Olfactory bulb
p/mTPp	Parvocellular/magnocellular periventricular posterior tuberculum
PFA	Paraformaldehyde
PB	Phosphate buffer
PBS	PB with saline
PBS-T	PBS with normal donkey serum and Triton 100
pc	Posterior commissure

PG	Preglomerular complex
PPa/PPp	Anterior/posterior parvocellular preoptic nucleus
PPr	Periventricular pretectum
pr	Posterior hypothalamic recess
PrV	Posterior ventricle
PS	Pineal stalk
PTh	Prethalamus
PTN	Posterior tuberal nucleus
PVO	Paraventricular organ
RhV	Rhombencephalic ventricle
SGN	secondary gustatory nucleus
SMN	Sonic motor nucleus
SRc/Sri/SRr	Caudal/intermediate/rostral superior raphe
SV	Saccus vasculosus
TAd	Dorsal anterior tuberal nucleus
Tel	Telencephalon
TelV	Telencephalic ventricle
TeO	Tectum opticum
TeV	Tectal ventricle
Th	Thalamus
TH	Tyrosine hydroxylase
th1	*tyrosine hydroxylase 1* gene
th2	*tyrosine hydroxylase 2* gene
TLa	Torus lateralis
TLo	Torus longitudinalis
TPp	Periventricular nucleus of the posterior tuberculum
tph 1	*tryptophan hydroxylase 1* gene
TS	Torus semicircularis

Va	Valvula cerebelli
VL	Vagal lobe
VMN	Vocal motor nucleus
VN	Vocal nerve
VPN	Vocal pacemaker
VPP	Vocal prepacemaker
Vs	Supracommissural nucleus of the ventral telencephalon

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SUMMARY

In this thesis, I investigated the neurochemical profile in socially communicating teleost fish species. By studying inhibitory and modulatory neurotransmitters in the vocal central pattern generator (CPG) in the Gulf toadfish *Opsanus beta*, I hope to reveal potential neurophysiological mechanisms that underlie the generation of vocal patterns. I additionally studied the distribution of serotonergic populations in three other socially communicating teleost species, the two catfish species *Ariopsis seemanni* and *Synodontis nigriventris*, and one toadfish species *Allenbatrachus grunniens,* to assess how social communication influences the organization of the serotonergic system.

Methodologically, I assessed the presence of GABA, glycine, acetylcholine, catecholamines and serotonin in the vocal CPG of *O. beta* by combining immunohistochemistry with tracing of the vocal CPG nuclei via neurobiotin. Furthermore, I studied the serotonergic system in the three socially communicating fish species, *A. grunniens* and the catfish species *A. seemanni* and *S. nigriventris*.

My first study showed that all neurotransmitters investigated in my thesis projected to all levels of the vocal CPG in *O. beta*. GABAergic and glycinergic neurons were present within and/or adjacent to all nuclei. A subset of glycinergic neurons in vocal pacemaker nucleus (VPN) was co-labeled with neurobiotin. In the vocal motor nucleus (VMN), neurons were cholinergic and additional cholinergic neurons were present within and adjacent to VPN and vocal pre-pacemaker nucleus (VPP). Serotonergic neurons were present caudal of the VPP and within the VMN as part of the inferior raphe nucleus, while catecholaminergic neurons were only observed in the area postrema dorsal of the VMN.

In my second study, I observed large populations of cerebrospinal fluid (CSF)-contacting serotonergic neurons in the paraventricular organ and the periventricular hypothalamus with smaller ones in the pretectum and the pineal stalk in *A. grunniens, A.*

seemanni and *S. nigriventris*. Additional serotonergic neurons were found in the preoptic area of both catfishes. Serotonergic neurons in the rhombencephalic raphe nuclei were always present. The CSF-contacting neurons of the PVO extended in the dopaminergic periventricular tuberculum but no co-expression was found with dopamine. The serotonergic input was observed within the hindbrain motor neurons innervating the swim bladder associated muscles in *A. grunniens* and *S. nigriventris*, while it was within the dendritic region in *A. seemanni*.

What I have shown in my thesis is that: First, the inhibitory profile of the vocal CPG in toadfish suggests a set of neurophysiological mechanisms to generate different vocalizations that can be adapted to context. The presence of serotonin, acetylcholine and catecholamines at all levels of the vocal CPG shows the potential of neuromodulation in shaping the motor output of the vocal CPG, but their interplay with GABA and glycine remains to be shown. Second, I showed that the general organization of serotonergic system is not influenced by social communication. Existing differences are rather species-specific than dependent on the type of social signal that is generated. Nevertheless, displaced inferior raphe (IR) neurons within the vocal motor nucleus are a feature characteristic for the highly vocal toadfishes. While the first part of my thesis provides a basis for further neurophysiological investigations of vocal pattern generation in toadfishes, my second study confirms the general organizational pattern of the serotonergic system in non-mammalian vertebrates.

1 INTRODUCTION

Vertebrates socially communicate with one another using vocalizations in various contexts such as courtship, mate choice and territorial defense (Bradbury and Vehrencamp, 2011). They evolved a diverse set of mechanisms to produce vocal sounds (recently reviewed in Ladich and Winkler, 2017). All mechanisms have in common that vocal signal generated by the underlying neuronal vocal network needs to be highly stereotypic and temporally precise to convey a meaningful signal from sender to receiver (Bradbury and Vehrencamp, 2011). Yet, the output of the vocal network also needs to be adaptable to enable the production of different vocal signals for different contexts. Neuronal inhibition and neuromodulation are two mechanism that could enable a neuronal network to achieve both, temporal precision and plasticity.

Anatomical and electrophysiological evidence suggests that inhibitory and modulatory neurotransmitters indeed play a role in shaping the output of vocal areas. Examples for such studies are the glycinergic projections that were observed to the vocal motor neurons of zebra finches, *Taeniopygia guttata* (Grisham and Arnold, 1994; Sturdy *et al.*, 2003) as well as GABAergic projections to VMN in plain fin midshipman fish, *Porichthys notatus* (Chagnaud *et al.*, 2012; Rubow, 2010). Serotonergic and catecholaminergic input was for instance found throughout the whole vocal CPG in midshipman fish (Forlano *et al.*, 2014; Forlano *et al.*, 2011; Ghahramani *et al.*, 2015a; Goebrecht *et al.*, 2014; Timothy *et al.*, 2015) and serotonergic input to respiratory motor neurons in cat controlling airflow for vocalization (Holtman, 1988), the nucleus arcopallium in zebra finches (Wood *et al.*, 2011) and the premotor and motor neurons of the African clawed frog, *Xenopus laevis* (Yu and Yamaguchi, 2010). Thus, the presence of such transmitters has been shown in a variety of different species, however, with emphasis on specific transmitter types. A full and comprehensive analysis of neurotransmitter present in vocal CPGs across vertebrates is still lacking.

Besides their presence, the effect of the variety of transmitters on vocal production has already been characterized in some animals for some transmitters: *In vitro* electrical stimulation of the medullary respiratory areas of zebra finches resulted in glycinergic inhibition

onto hindbrain motor neurons involved in vocalization (Sturdy *et al.*, 2003). In the robust nucleus of the archistriatum of zebra finches, injection of bicuculline, a $GABA_A$ antagonist, increased neuronal activity leading to lower vocalization threshold and sometimes elicited spontaneous vocalizations (Vicario and Raksin, 2000). Heavy GABAergic inhibition onto the PAG of squirrel monkey, *Saimiri sciureus*, completely inhibited vocalization, while reducing GABAergic inhibition lowered the threshold for vocalization in the PAG (Jürgens and Lu, 1993). Benzodiazepines and allopregnanolone administration, both active at $GABA_A$ receptors, reduced isolation vocalizations in rat pups (Insel *et al.*, 1986; Zimmerberg *et al.*, 1994). As these examples all show, different inhibitory and modulatory transmitter types heavily affect vocal behaviour by shaping the vocal output at the final vocal motor centers. Therefore, a comprehensive overview of these neurochemical inputs to vocal areas in one vocal model system would be helpful to fully understand how vocal behaviour is produced.

Fishes are well suited to study the basis of vocal signal generation including the neurochemical profile of vocal neuronal structures, especially at the hindbrain level, because toadfish, together with *X. laevis,* are the only vertebrate family where a hindbrain vocal central pattern generator (CPG) has been fully identified up to now. The vocal CPG can be accessed after a dorsal craniotomy (e.g., Bass and McKibben, 2003; Chagnaud *et al.*, 2011; Chagnaud *et al.*, 2012; Kittelberger *et al.*, 2006) making *in vivo* investigations easier than for instance in birds where the cerebellum covers the hindbrain vocal structures. This toadfish *in vivo* preparation allows to record so-called fictive vocalizations from the vocal nerves. The temporal and structural pattern of these fictive vocalizations correspond to a natural call (Cohen and Winn, 1967). Thus, they provide a neuronal read-out of the vocal behavior (Rubow and Bass, 2009). Furthermore, the vocal neuronal pathway of toadfish and tetrapods has a similar developmental organization (Bass and McKibben, 2003) within rhombomere 8, which suggests a common evolutionary origin of vocal pattern networks in all vertebrates (Bass, 2014; Bass *et al.*, 2008). Thus, studying vocal pattern generation in toadfish can provide a basis to understand vocal production in tetrapods.

Therefore, I studied the inhibitory and modulatory neurotransmitters in brain of four socially communicating teleost species. I assessed the neurochemical profile of the vocal CPG in the Gulf toadfish, *Opsanus beta*, as well as the serotonergic system in three socially communicating fish species, the grunting toadfish, *Allenbatrachus grunniens*, and two catfishes, *Ariopsis seemanni* and *Synodontis nigriventris*. In a pilot study, I additionally investigated the effect of inhibitory agents on the vocal motor output in the plainfin midshipman fish, *P. notatus*.

The following sections will first give a short overview of mechanism to produce vocal signal in fishes focusing on sound production via swim bladder vibrations followed by outlining the underlying vocal neuronal circuitry in toadfish.

1.1 Mechanisms for sound generation in fishes

Fishes developed a variety of mechanisms to generate social sounds (recently reviewed in Ladich and Winkler, 2017). For instance, grunts employ the pharyngeal jaw apparatus to vocalize, e.g., in the French grunt, *Haemulon flavolineatum* (Bertucci *et al.*, 2014), while seahorses have a bony articulation between the supraoccipital ridge of the neurocranium and the grooved anterior edge of the coronet to generate rapid clicking sounds similar to finger-snaps during feeding, courtship and mating (Colson *et al.*, 1998). All teleost species that I studied produce social signals via swim bladder vibrations. Therefore, I will explain this mechanism for social signal production in more detail in the following paragraph.

Swim bladder vibrations are a well-studied mechanism for vocalization in teleost fishes including the teleost fish species studied here, the grunting toadfish, the Gulf toadfish and the marine catfish *A. seemanni*. For swim bladder sounds, a single pair of striated muscles associated with the swim bladder is repetitively contracted at 100-250 Hz depending on the ambient water temperature (Bass and Baker, 1991; Brantley and Bass, 1994; Cohen and Winn, 1967; Rome, 2006; Skoglund, 1961). The muscle contractions vibrate the swim bladder, thus generating sound (Bass and Marchaterre, 1989; Rome, 2006). The swim bladder associated muscles, also referred to as drumming muscles, are either directly attached to the

wall of the swim bladder, e.g., in the toadfishes (Rice and Bass, 2009), in which case they are called intrinsic muscles. Alternatively, they are indirectly connected to the swim bladder via a bony structure, e.g., in catfishes, then called extrinsic muscles (Bass and Ladich, 2008; Carlson and Bass, 2000; Ladich, 2001; Ladich and Bass, 1998, 2005). Recent behavioral evidence showed that the upside-down catfish *S. nigriventris* which I also studied does not produce swim bladder associated sounds, but instead produces weakly electric discharges (Boyle *et al.*, 2014). This finding is in line with the known ability of other synodontids (Hagedorn *et al.*, 1990) that use the protractor muscles for electric discharge production, while others generate vocal signals or both (Baron *et al.*, 1994; Boyle *et al.*, 2014; Hagedorn *et al.*, 1990).

With these rather simple mechanisms of sound production, fishes are able to produce different types of social-context dependent vocalizations. For example, adult type I males of the midshipman fish – an established model for investigating the basis of vocal pattern generation and study object of my pilot study – can produce three call types: a grunt, a courtship call and a growl. Grunts have a short duration and are produced by both sexes during agnostic encounters. They are either emitted as a single call or a series of calls named grunt train (Bass and McKibben, 2003; Brantley and Bass, 1994; Cohen and Winn, 1967; Wang, 1999). The midshipman courtship calls are produced by type I males to attract females and are named hums (Brantley and Bass, 1994). Hums are long (some minutes to one hour), multiharmonic calls. They have a fundamental frequency of about 100 Hz and little to no amplitude modulation (Bass and Marchaterre, 1989; Brantley and Bass, 1994). The growl is a sequence of grunt- and hum-like signals that lasts from 200 ms to 5 s and is mostly produced at night with strong amplitude and frequency modulation (Brantley and Bass, 1994). The neuronal pathway that underlies for swim bladder sound generation has been especially well studied in toadfishes and will be described in the next paragraph.

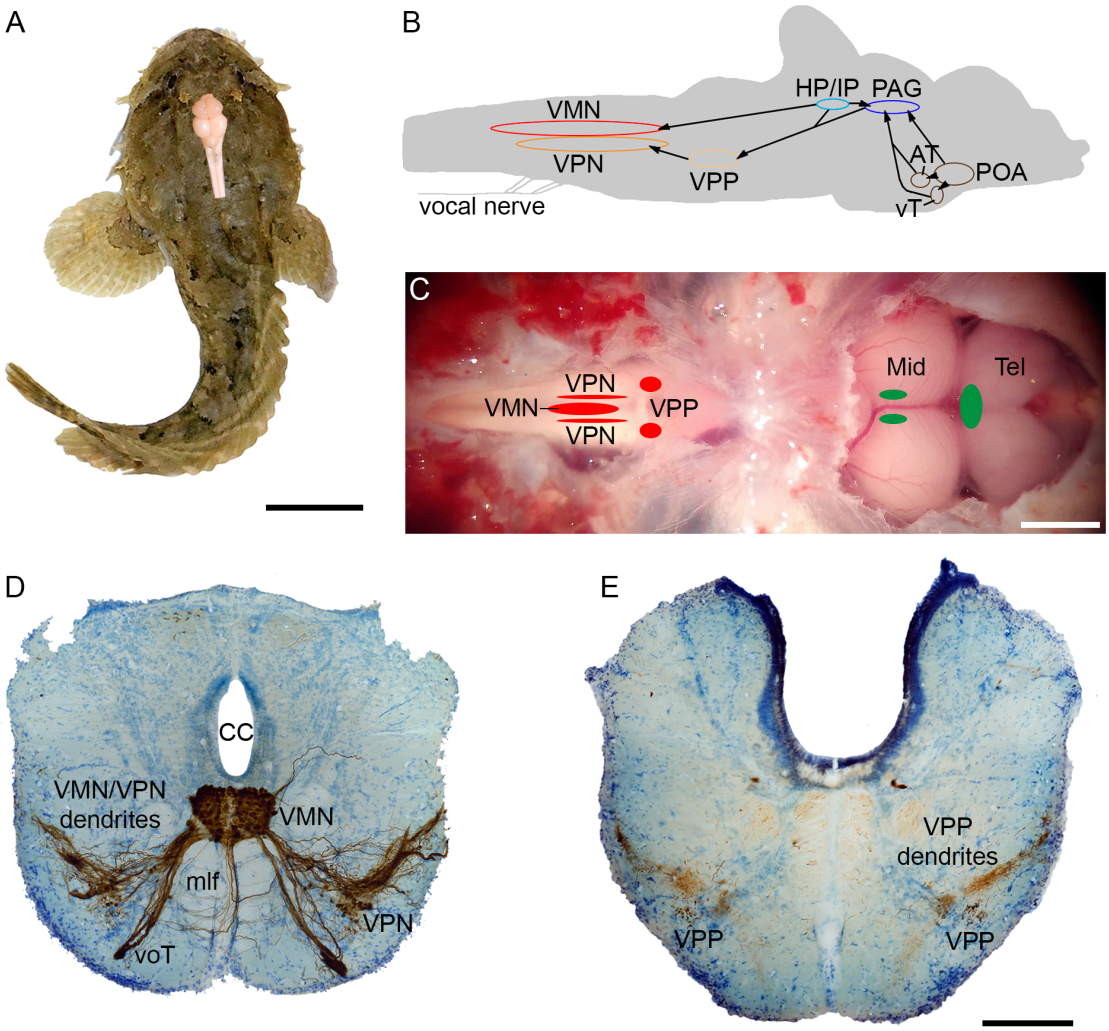
1.2 Vocal neuronal pathway underlying swim bladder sounds in toadfishes

The vocal neuronal circuitry in toadfish involves vocal areas in the forebrain, midbrain and hindbrain (Bass *et al.*, 1994; Goodson and Bass, 2002). As mentioned before, the vocal CPG of toadfish is located in the caudal hindbrain. It connects to the swim bladder associated drumming muscles via vocal nerves that originate from occipital nerve roots exiting the vocal CPG (Bass *et al.*, 1994). Labeling of the cut ends of the vocal nerve with a tracer that crosses gap junctions reveals three nuclei within the vocal CPG: the vocal motor nucleus (VMN), the vocal pacemaker nucleus (VPN) and the vocal pre-pacemaker nucleus (VPP; Bass and Baker, 1990; Bass and Marchaterre, 1989; Bass *et al.*, 1994). The VMN is a fused bilateral nucleus located medially below the rhombencephalic ventricle in the caudal hindbrain (Bass and Ladich, 2008; Bass and Marchaterre, 1989; Bass *et al.*, 1994; Chagnaud and Bass, 2014; Goodson and Bass, 2002). Among the three nuclei of the vocal CPG, it is the nucleus with the largest rostro-caudal extent, the highest number of neurons and largest sized neurons within the vocal CPG (Chagnaud and Bass, 2014; Rosner *et al.*, 2018). VMN modulates the amplitude of a call (Chagnaud *et al.*, 2012) and is innervated by the VPN (Brantley and Bass, 1994; Goodson and Bass, 2002).

The VPN is a bilateral nucleus located ventrolateral to the rhombencephalic ventricle in the hindbrain (Bass *et al.*, 1994). It has a smaller rostro-caudal extent and a smaller number of neurons and neurons of smaller size than VMN (Chagnaud and Bass, 2014; Rosner *et al.*, 2018). The VPN codes the pulse repetition rate of a call which corresponds to the fundamental

FIGURE 1: VOCAL NEURONAL CIRCUITRY IN TOADFISH.

(A) Top view of a specimen of the Gulf toadfish, *Opsanus beta,* with the location of the brain indicated. Courtesy of Midge Marchaterre. (B) Schema of the vocal neuronal network in toadfish. Redrawn after Goodson and Bass (2002) and Chagnaud and Bass (2014). (C) Top view of the *in vivo* preparation of midshipman fish used during electrophysiological recording with vocal CPG (red) and stimulation points of vocal midbrain and forebrain areas (green) indicated. (D, E) Transverse hindbrain sections at the level of the VMN/VPN (D) and VPP (E) with transneuronal neurobiotin labeling of vocal CPG and cresyl violet counterstain. Adapted from Rosner et al. (2008). The scale bar is 2 cm in A, 0.25 cm in C and 200 µm in E for D and E. AT anterior tuberal nucleus, CC central canal, CPG central pattern generator, HP hindbrain paraventricular group, IP isthmal paraventricular group, mlf medial longitudinal fasciculus, Mid midbrain, PAG periaqueductal grey, Tel telencephalon, VMN vocal motor nucleus, VPN vocal pacemaker nucleus, VPP vocal pre-pacemaker nucleus, vT ventral tuberal hypothalamus.



frequency of vocalizations in other vertebrates (Chagnaud *et al.*, 2011). The VPN is innervated by the VPP (Brantley and Bass, 1994; Goodson and Bass, 2002).

As the VPN, the VPP is a bilateral nucleus and located ventrolateral to the rhombencephalic ventricle in the hindbrain rostral to VPN. VPP has the smallest rostro-caudal extent and the lowest number of neurons, but VPP neurons are similar in size to VPN neurons (Chagnaud and Bass, 2014; Rosner *et al.*, 2018). VPP controls the duration of a call (Chagnaud *et al.*, 2011).

The vocal CPG is innervated by several hindbrain and midbrain areas among which is the periaqueductal grey (PAG; Bass *et al.*, 1994; Goodson and Bass, 2002). The PAG is established as a vocal center across vertebrates (Jürgens, 1994, 2002, 2009). In toadfish, the PAG appears throughout the whole rostro-caudal extent of the midbrain (Kittelberger and Bass, 2013). Its electrical stimulation readily initiates vocal behavior (Demski and Gerald,

1974; Fine, 1979; Goodson and Bass, 2002), its inactivation prevents vocalizations (Kittelberger *et al.*, 2006) and the activity of its neurons predict vocal output (Kittelberger *et al.*, 2006). The Pag projects to VPP (Kittelberger *et al.*, 2006).

The PAG is innervated by a complex of nuclei referred to as preoptic area-anterior hypothalamus. It is located ventrally in the forebrain at the level of the pituitary gland and consists of the anterior tuberal nucleus (AT), the ventral tuberal hypothalamus (vT) and the preoptic area (POA). Electrical stimulation of all three areas elicits vocalizations (Goodson and Bass, 2000a, b; Kittelberger *et al.*, 2006).

Although network and single neuron properties, especially of the vocal CPG, have been intensively study in toadfish (Bass and Baker, 1990; Chagnaud *et al.*, 2011; Chagnaud *et al.*, 2012), it still remains challenging to reproduce more elaborate vocal patterns using current stimulation. The application of neurotransmitters into vocal areas of the brain might be a promising approach. Knowing the inhibitory and neurochemical inputs to the vocal neuronal area in toadfish, including the vocal CPG, would provide a basis for such experiments.

1.3 Aim of this thesis

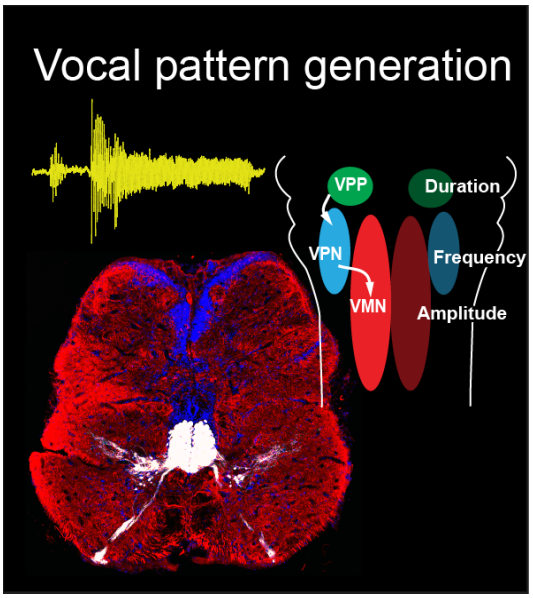
Vocal tetrapods and sonic fish produce context-dependent vocal signals ranging from short pulses, e.g., midshipman grunts, to more elaborate sounds, e.g., the hums serving as courtship calls in midshipman fish. To study the mechanisms underlying the generation of these calls, they need to be reproduced during electrophysiological experiments. Yet, this remains difficult for all types of vocalizations using current stimulations. Due to their aforementioned role in shaping the neuronal output of vocal areas, the application of neurotransmitters into vocal areas of the brain might be able to evoke different vocal signals more reliably than currently possible by current stimulation allowing the study of mechanisms underlying natural calls. In an isolated hindbrain preparation in *X. laevis* it cloud be shown that the bath application of serotonin could evoke vocal motor patterns (Yu and Yamaguchi, 2010). To guide such experiments in the intact *in vivo* preparation of the vocal CPG in toadfish, an anatomical basis is needed that shows which neurotransmitters give input to the vocal CPG.

Therefore, in chapter 1, I studied the presence of inhibitory and modulatory neurotransmitters in the vocal CPG of the Gulf toadfish (*O. beta)* by combining immunohistochemistry with tracing of the vocal CPG. This first overview of inhibitory and modulatory inputs to a vertebrate vocal CPG provides a basis for further investigations of the physiological effect these inputs have on vocal pattern generation in toadfish.

In this first study, I observed displaced serotonergic inferior raphe neurons within the hindbrain motor neurons, i.e., VMN, innervating the swim bladder musculature in *O. beta.* These neurons had been reported before in *O. tau* (Marchaterre *et al.*, 1989) raising the question if these displaced raphe neurons are a toadfish species feature and if the general organization of the serotonergic system differs in socially communicating fish species. Therefore, in chapter 2, I immunohistochemically assessed the distribution of serotonergic populations in three teleost species producing social signals with their swim bladder associated musculature: two vocal fish species, the grunting toadfish (*A. grunniens*) and a South American marine catfish (*A. seemanni)* as well the weakly electric upside-down catfish (*S. nigriventris).* I show that the general distribution of serotonergic neurons socially in these three socially communicating teleost species is similar to what has been previously reported for other fish species. Thus, observed differences are rather species specific than related to the type of social communication. Still, the displaced inferior raphe neurons within VMN seem to be a feature most likely unique to the highly vocal toadfishes.

Finally, in chapter 3, I summarize and discuss the results of the two studies presented in chapter 1 and 2. In my discussion, I incorporate results of a preliminary study added in the appendix where I show that local injection of glycine into VMN reduced call amplitude in an intact *in vivo* preparation in midshipman fish. My thesis together with previous studies on the inhibitory neurochemical profile of the vocal CPG in toadfish suggest a set of neurophysiological mechanisms within the vocal CPG to produce context-dependent vocal signals. This might guide the application of neurotransmitters to evoke different vocal signals similar to natural calls and allows to further study of the underlying neuronal mechanisms.

2 MANUSCRIPT 1



Inhibitory and modulatory inputs to a vertebrate vocal central pattern generator of a teleost fish

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Author contributions

Contribution of E.R.

- Design of study: chose the target neurotransmitters and suited antibodies, chose number of animals, developed antibody staining protocol for *Opsanus beta*, chose methods for data analysis
- Performed most of the transneuronal tracings of the vocal central pattern generator in *O. beta*
- Performed all antibody stainings
- Took all microscope images
- Performed the data analysis
- Performed the statistical analysis
- Designed all figures
- Wrote and revised the manuscript

Contribution of K.N.R.

- Performed the Western blot analysis for antibody characterization of the connexin 35/36 antibody
- Revised the manuscript

Contribution of A.H.B.

- Taught E.R. how to perform transneuronal tracing of the vocal central pattern generator in toadfish
- Performed part of the transneuronal tracings
- Provided lab space at Cornell University for E.R. to perform transneuronal tracings
- Revised the manuscript

Contribution of B.P.C.

- Design of study: suggested the studied species and study outline, gave technical advice, advised target neurotransmitter
- Revised and submitted the manuscript

B.P.C. supervised the study.

Abstract

Vocalization is a behavioral feature that is shared among multiple vertebrate lineages, including fish. The temporal patterning of vocal communication signals is set, in part, by central pattern generators (CPGs). Toadfishes are well-established models for CPG coding of vocalization at the hindbrain level. The vocal CPG comprises three topographically separate nuclei: pre-pacemaker, pacemaker, motor. While the connectivity between these nuclei is well understood, their neurochemical profile remains largely unexplored. The highly vocal Gulf toadfish, *Opsanus beta*, has been the subject of previous behavioral, neuroanatomical and neurophysiological studies. Combining transneuronal neurobiotin-labeling with immunohistochemistry, we map the distribution of inhibitory neurotransmitters and neuromodulators along with the gap junctions in the vocal CPG of this species. Dense GABAergic and glycinergic label is found throughout the CPG, with labeled somata immediately adjacent to or within CPG nuclei, including a distinct subset of pacemaker neurons co-labeled with neurobiotin and glycine. Neurobiotin-labeled motor and pacemaker neurons are densely co-labeled with the gap junction protein connexin 35/36, supporting the hypothesis that transneuronal neurobiotin-labeling occurs, at least in part, via gap junction coupling. Serotonergic and catecholaminergic label is also robust within the entire vocal CPG, with additional cholinergic label in pacemaker and prepacemaker nuclei. Likely sources of these putative modulatory inputs are neurons within or immediately adjacent to vocal CPG neurons. Together with prior neurophysiological investigations, the results reveal potential mechanisms for generating multiple classes of social context-dependent vocalizations with widely divergent temporal and spectral properties.

1 Introduction

Vocal behavior frequently plays a central role in intra- and interspecific interactions among vertebrates. Vocalizations often exhibit high temporal precision resulting in the transmission of highly stereotyped signals between sender and receiver (Bradbury and Vehrencamp, 2011). The neuronal circuitry that generates these signals includes forebrain and midbrain regions and a single or multiple hindbrain vocal central pattern generators (CPGs, e.g., Bass, 2014; Chakraborty and Jarvis, 2015).

While forebrain and midbrain regions underlying vocal behavior have received considerable attention in vertebrate lineages as diverse as birds and primates, hindbrain vocal circuits remain largely unexplored despite their undisputed role in vocal patterning (e.g., Hage and Jürgens, 2006; Jürgens, 2002; Levelt, 1989; Suthers and Goller, 1997). Evidence from electrophysiological studies in bats and monkeys, for instance, show the contribution of hindbrain neurons to the determination of the amplitude, duration and frequency of vocal signals (Jürgens and Hage, 2007; Rübsamen and Betz, 1986; Schuller and Rübsamen, 1990; Suthers and Goller, 1997; Vicario, 1991). The most comprehensive investigations of vocal CPGs, however, originate from studies of amphibians (Kelley *et al.*, 2017; Schmidt, 1992; Yamaguchi *et al.*, 2000; Zornik and Yamaguchi, 2012) and fish (Bass, 2014; Bass and Baker, 1990; Feng *et al.*, 2015).

Among vocal species of fish, toadfishes (order Batrachoidiformes, family Batrachoididae) include species commonly known as toadfish and midshipman fish (Greenfield *et al.*, 2008) that have provided tractable models for neurophysiological investigations of vocal CPGs (Ladich *et al.*, 2006). These fish produce several types of social context-dependent vocalizations (e.g., Fig. 1a) by repetitively contracting a single pair of "superfast" vocal muscles attached to the walls of the swim bladder at frequencies of about 100-250 Hz depending on the ambient water temperature (Bass and Baker, 1991; Brantley and Bass, 1994; Cohen and Winn, 1967; Rome, 2006; Skoglund, 1961). These contractions

are elicited by the activity of a hindbrain vocal motor nucleus (VMN) that innervates the vocal muscles via occipital motor nerve roots. These roots form a single vocal nerve (VN, Fig. 1b, c)

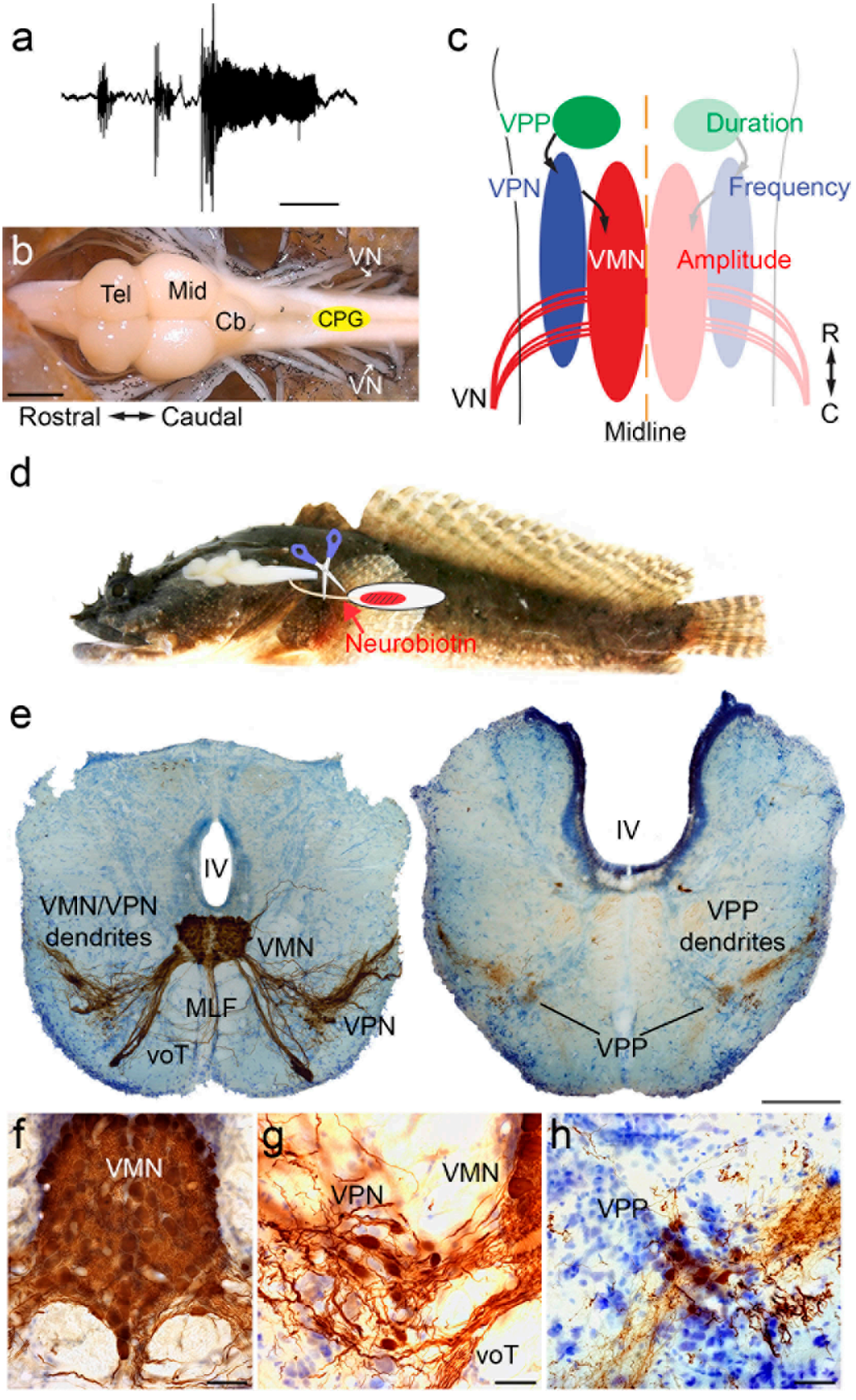


FIGURE 1: ORGANIZATION OF THE GULF TOADFISH (*OPSANUS BETA*) VOCAL CPG.

(a) Hydrophone recording of a boatwhistle, a toadfish advertisement call (L. Remage-Healy & A. H. Bass, unpublished obs.). (b) Photograph of a dorsal view of a toadfish brain showing the relative location of the vocal central pattern generator (CPG, yellow ellipse). Rostral-caudal axis is indicated. (c) Schematic (dorsal view) of the toadfish vocal CPG. The left side displays the individual nuclei (vocal pre-pacemaker nucleus, VPP; vocal pacemaker nucleus, VPN; vocal motor nucleus, VMN). The right side indicates the vocal property that is encoded by each nucleus (color coded). Rostral (R)-caudal (C) axis is indicated. (d) Side view of Gulf toadfish (modified from Bass and Rice, 2010) and brain with schematic drawing illustrating neurobiotin labeling at the cut end of the vocal nerve that innervates the vocal muscle (red) attached to the wall of the swim bladder (white). (e) Photographs of transverse hindbrain sections with transneuronal neurobiotin labeling of vocal CPG and cresyl violet counterstain at the level of the VMN/VPN circuit (left) and of the VPP (right). (f-h) Higher magnification photographs of VMN (f), VPN (g), and VPP (h). Scale bars represent 100 ms (a), 2 mm (b), 200 µm (e), 50 µm (f) and 20 µm (g, h). Abbreviations: IV, fourth ventricle; Cb, cerebellum; Mid, midbrain; MLF, medial longitudinal fasciculus; Tel, telencephalon; VN, vocal nerve; voT, vocal tract.

considered homologous to the hypoglossal nerve roots of other vertebrates (Bass *et al.*, 2008). Transneuronal tracing via labeling of the cut end of one VN with neurobiotin or biocytin (Fig. 1d, e) together with single cell electrophysiological recordings demonstrate three topographically separate vocal CPG nuclei that code for distinct call parameters: VMN for amplitude modulation, vocal pacemaker nucleus (VPN) for pulse repetition rate (corresponds to fundamental frequency) and vocal pre-pacemaker nucleus (VPP) for duration (Fig. 1f-h; Bass and Baker, 1990; Bass *et al.*, 1994; Chagnaud *et al.*, 2011; Chagnaud *et al.*, 2012).

While the organization of vocal CPGs has been investigated at network and single neuron levels for toadfishes and the African clawed frog *Xenopus laevis* (e.g., Bass and Baker, 1990; Chagnaud *et al.*, 2011; Chagnaud *et al.*, 2012; Kelley *et al.*, 2017; Yamaguchi *et al.*, 2000; Zornik and Yamaguchi, 2012), less is known about the neurochemicals that modulate their activity (e.g., Forlano *et al.*, 2014; Yu and Yamaguchi, 2010; Zornik *et al.*, 2010). The well-established neuroanatomical and neurophysiological characterization of the vocal CPG in species such as the Gulf toadfish, *Opsanus beta*, together with the ability to unambiguously identify each vocal CPG nucleus via transneuronal neurobiotin labeling, present a distinct opportunity to investigate the neurochemical profile of each component of an evolutionarily conserved vertebrate vocal CPG (Bass *et al.*, 2008; Chagnaud and Bass, 2014). Here, we report the robust distribution of inhibitory neurotransmitters and neuromodulators within the Gulf toadfish vocal CPG that likely contribute to the ability of these and other species of toadfish

to produce social context-dependent vocal behaviors with divergent temporal and spectral properties (Rice and Bass, 2009).

2 Materials and Methods

2.1 Animals

Seventeen Gulf toadfish, *Opsanus beta,* (12 males, four females, sex not reported for one fish; 3.5-18.7 cm in standard length; median = 10.5 cm; interquartile range = 10.0) were obtained from a commercial source (Gulf Specimen, Panacea, Florida, USA) and housed in aquaria at 22°C in an environmental control room on a 12:12 dark:light cycle. All surgical methods and collection of tissues were approved by the Cornell University Institutional Animal Care and Use Committee.

2.2 Vocal CPG labeling

Neurobiotin crystals (Vector Laboratories, Burlingame, CA USA) were applied to the cut end of one vocal nerve at the level of the swim bladder. A detailed description of the methods can be found in Bass et al. (1994). After a survival time of 1.5-7 days following neurobiotin application, fish were deeply anaesthetized by immersion in aquarium water with benzocaine (0.025%; Sigma Aldrich, St. Louis, MO USA) and then transcardially perfused with 4% paraformaldehyde (PFA) or 3.75% PFA and 0.25% glutaraldehyde in 0.1M phosphate buffer (PB; all: Sigma Aldrich, St. Louis, MO USA). Brains were post-fixed in the same solution for 1 h at 4°C and stored in 0.1M PB at 4°C.

2.3 Immunohistochemistry (IHC)

One day prior to sectioning, fixed brains were cryoprotected in 30% sucrose in 0.1M PB (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) at 4°C overnight. Brains were sectioned in the transverse plane with a cryostat at 25 µm (Leica microsystems, Wetzlar, Germany) and directly mounted onto microscope slides (Superfrost Ultra Plus Adhesion Slides; Thermo Fisher Scientific Inc., Braunschweig, Germany). Slides were subsequently left at room temperature for 1 h to allow the sections to dry and then either processed for IHC immediately or stored at -80°C. Each brain was sectioned into four complete series, each of which was stained with a single antibody.

For IHC, the slides were immersed in 0.1M PB-saline (PBS) for 30 min for rehydration. Glutaraldehyde fixed brains were additionally washed in 0.001% sodium borohydride (Sigma Aldrich Chemie GmbH, Munich, Germany) in 1 ml 0.1M PBS for 5 minutes to reduce glutaraldehyde background. A washing series (four times, 5 minutes each) in 0.5% Triton 100 (Sigma Aldrich Chemie GmbH, Munich, Germany) in 0.1M PBS (PBS-T) followed. Subsequently, each slide was washed in 10% normal donkey serum (Jackson Immunoresearch Europe Ltd., Suffolk, United Kingdom) in PBS-T for 1 h, before incubation with primary antibody (Table 1) overnight. Slides were then washed four times in PBS-T before incubation with secondary antibody and anti-biotin antibody (Table 1) for 4 h. Slides were again washed four times with PBS-T, dried and then coverslipped using a fluorescent mounting medium (Vectashield, Vector Labs Inc., Peterborough, United Kingdom) containing 4',6-diamidino-2-phenyindole (DAPI). All incubation and washing steps were performed at room temperature.

Images of brain sections were taken on a confocal laser microscope (Leica microsystems, Wetzlar, Germany) and at an epifluorescence microscope (ECLIPSE Ni, Nikon GmbH, Düsseldorf, Germany). Acquired confocal images were stacked and converted to maximum z-projections using the free software Fiji (Schindelin *et al.*, 2012). Maximum z-projections were cropped, resized and contrast and brightness were optimized for the entire image using Adobe Photoshop CS6 (Adobe Systems Software Ireland Limited, Dublin, Ireland).

2.4 Data Analysis

For three similar-sized fish (OB-15-05 (ID code): 5.3cm (standard length); OB-15-06: 5.1cm; OB-16-10: 6.1cm), we evaluated several dimensions of the neurobiotin-labeled vocal CPG nuclei (VMN, VPN, VPP): rostral-caudal extent of each nucleus, neuron number for each nucleus, individual neuron size reported as diameter, and individual neuron shape evaluated by the shape factor. Rostral-caudal extent was determined by counting all sections of each brain where neurons of the respective nuclei were labeled and then multiplied by 25 µm, i.e.,

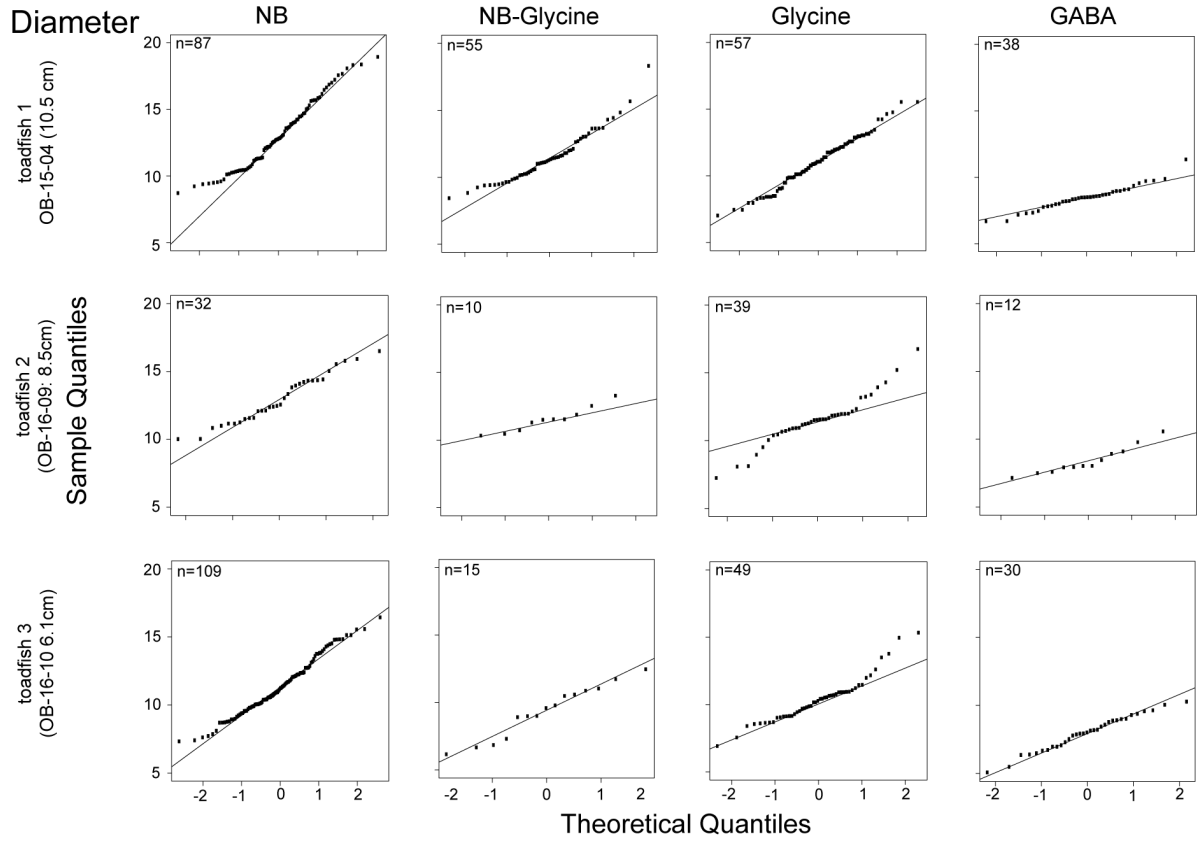
TABLE 1

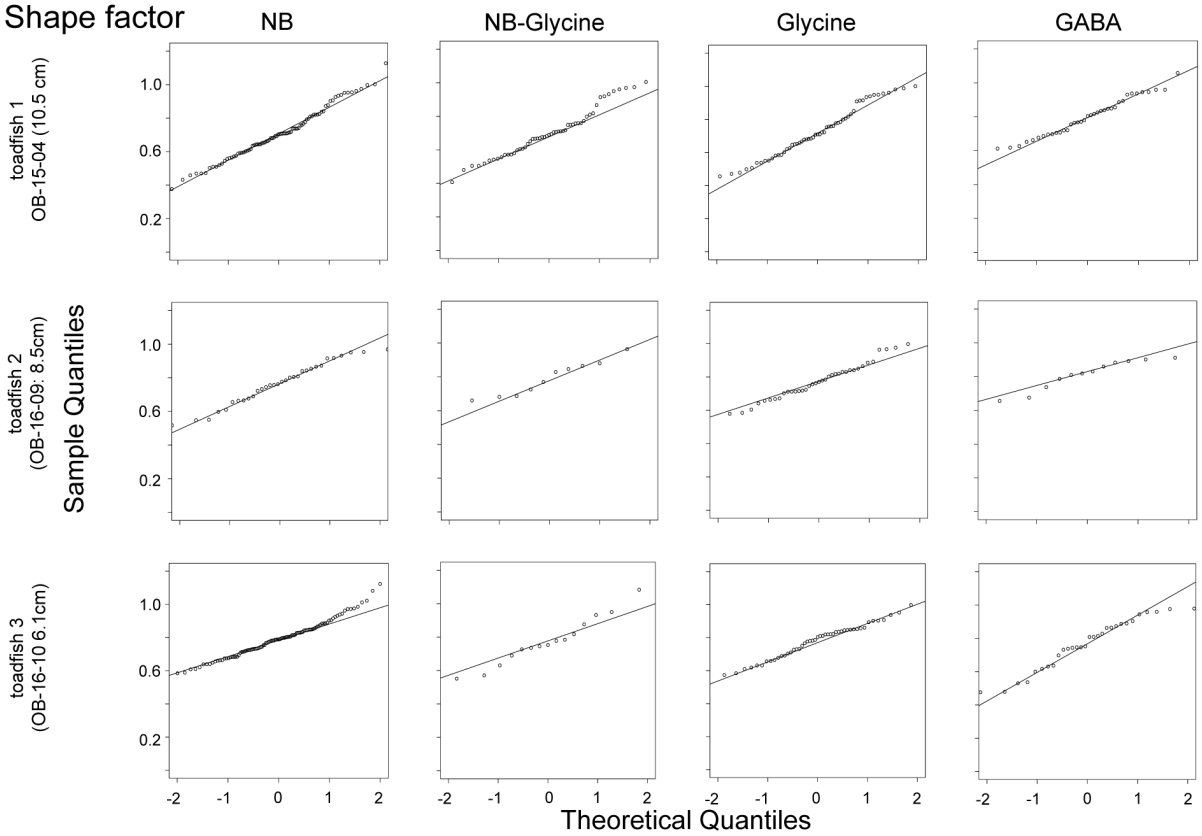
List of used primary and secondary antibodies including their name, immunogen, manufacturer, catalogue number, RRID, host, antibody type, the dilution used in this study and fixation type. Abbreviations: PFA, 4% paraformaldehyde; PFA/GLUT, 3.75% paraformaldehyde + 0.25% glutaraldehyde.

Antibody name	Immunogen	Manufacturer, Catalogue number, RRID, Host, Antibody type	Dilution	Fixation type
Primary antibodies				
ChAT	Human placental enzyme	Millipore, Ab144P, AB_2079751, goat, polyclonal	1:500	PFA
Connexin 35/36	Recombinant Perch Connexin 35	Millipore, MAB3045, AB_94632, mouse, monoclonal	1: 200	PFA or PFA/GLUT
GABA	GABA coupled to bovine serum albumin	Swant, mAB 3D5, AB_10013381, mouse, monoclonal	1:200	PFA/GLUT
Glycine	Glycine-glutaraldehyd-carriers	MoBiTec, 1015GE, AB_2560949, rabbit, polyclonal	1:200	PFA/GLUT
Serotonin	Serotonin coupled to bovine serum albumin with paraformaldehyde	Immunostar, 20080, AB_10718516, rabbit, unknown	1:500	PFA or PFA/GLUT
Tyrosine hydroxylase	TH purified from rat PC12 cells	Immunostar, 22941, AB_572268, mouse, monoclonal	1:500	PFA or PFA/GLUT
Secondary antibodies				
Alexa Fluor antibody	Mouse gamma Immunoglobins Heavy and Light chains	Jackson ImmunoResearch Labs, 715-545-150, AB_2340846, donkey, polyclonal	1:200	PFA or PFA/GLUT
Donkey Anti-goat IgG (H+L)	Goat IgG (H+L)	Molecular Probes, A11055, AB_142672, donkey, polyclonal	1:200	PFA or PFA/GLUT
Donkey Anti-Rabbit IgG (H+L) Antibody, Alexa Fluor 488 Conjugated	Rabbit gamma Immunoglobins Heavy and Light chains	Molecular Probes, A-21206, AB_141708, donkey, unknown	1:200	PFA or PFA/GLUT
Anti-biotin antibody				
Cy3-Streptavidin antibody	Streptavidin	Jackson ImmunoResearch Labs, 016-160-084, AB_2337244, donkey, unkown	1:500	PFA or PFA/GLUT

the section thickness. Neuron number was estimated using the following criteria: First, in one series (out of four, see above) of each brain all neurobiotin labeled neurons were counted in which the nucleus of a given neuron could be identified with DAPI. This value was multiplied by four to account for neurons in the four brain series (see section 2.3), and then corrected using the Abercrombie correction factor (Abercrombie, 1946). For each fish the diameter of the nucleus was measured in 10 neurons of each cell population (VMN, VPN, VPP), and the average for each population was used for the Abercrombie correction for each respective population. Neuronal soma diameter was calculated as the average of minor and major axis measurements in each fish as has been done previously in *O. beta* (Chagnaud and Bass, 2014). Shape factor was calculated as the aspect ratio of a neuron's smallest dimension in a single plane (minor axis) divided by the neuron's largest dimension (major axis). A shape factor of one represents a perfect circle with decreasing values indicating more elongated shapes. Minor and major axes were measured with the ruler tool in Adobe Photoshop CS6 software.

As we report, four groups of labeled neurons were recognized in the premotor VPN region: neurobiotin-only, GABA-only, glycine-only, and neurobiotin-glycine co-labeled. A non-neurobiotin labeled neuron (i.e. glycinergic or GABAergic) was attributed to a vocal CPG nucleus if it was surrounded by neurobiotin-labeled neurons of that nucleus or if it was located adjacent to such neurons. Due to the heterogeneity of VPN neurons, neuron number and diameter were assessed separately for those groups along with the neuron shape factor. In order to avoid size effect between fishes, neuron diameter and shape factor were compared between the VPN groups in three fish (OB-15-04: 10.5cm; OB-16-09: 8.5cm; OB-16-10: 6.1cm) that exhibited neurobiotin, GABA and glycine label. For each fish, we separately tested if neuron diameter and shape factor were significantly different between the four neuron groups. We visually assessed a normal distribution for each neuron group within each fish using normal quantile-quantile plots (Suppl. Fig. 1). We used a Kruskal-Wallis test with a Wilcoxon signed rank test as a post-hoc test only if the Kruskal-Wallis test gave a significant result. The p-value


Diameter
NB
NB-Glycine
Glycine
GABA
toadfish 1
OB-15-04 (10.5 cm)
toadfish 2
(OB-16-09: 8.5cm)
toadfish 3
(OB-16-10 6.1cm)
Sample Quantiles
Theoretical Quantiles
n=87
n=55
n=57
n=38
n=32
n=10
n=39
n=12
n=109
n=15
n=49
n=30


Shape factor
NB
NB-Glycine
Glycine
GABA
toadfish 1
OB-15-04 (10.5 cm)
toadfish 2
(OB-16-09: 8.5cm)
toadfish 3
(OB-16-10 6.1cm)
Sample Quantiles
Theoretical Quantiles

SUPPLEMENTARY FIGURE 1: NORMALITY QUANTILE-QUANTILE PLOTS FOR NEURON DIAMETER AND SHAPE FACTOR FOR EACH NEURON GROUP (NEUROBIOTIN-LABELED NEURONS - NB; GLYCINE-ONLY NEURONS - GLYCINE; NEUROBIOTIN-GLYCINE CO-LABELED NEURONS - NB-GLYCINE; GABA ONLY NEURONS - GABA).

Y-axis (sample quantiles: diameter [µm] (top) and shape factor (bottom)) refer to the quantiles within each sample (circles represent individual neurons). Theoretical quantiles refer to calculated quantiles for which a normal distribution was assumed (solid black lines).

was adjusted according to a conservative Bonferroni correction which gave 0.008 as new level of significance for posthoc test results. All values are given as median with the interquartile range as a measure of variability. All calculations and tests were performed in R (R Core Team, 2014, version 3.14).

To identify a potential topographic organization of the different neuron types in VPN, we investigated their respective distribution by overlaying all sections of a single, ipsilateral population of VPN neurons from one fish (OB-15-04). For each VPN containing hindbrain section, we marked each neuron type with an asterisk or star (see Fig. 5 a, b). Next, we formed a polygon by connecting neurobiotin-positive neurons with lines that enclose all other neurobiotin-positive neurons in one section. We restricted this step to neurobiotin-positive neurons because they form the principal neurons of VPN. For the resulting polygon, we calculated the centroid using the python script “Centroid.py” provided by Robert Fotino (https://gist.github.com/rfotino/a0fa1ef2882484e2da89#file-centroid-py). With the calculated centroids for each section, we aligned all VPN sections to construct a representation of the neurons within the VPN region by marking each neuron individually with a color coded cross and by displaying the total area covered by each neuron group (Adobe Photoshop).

2.5 DAB staining

Two reference brains from animals with transneuronal, neurobiotin-labeling of the vocal CPG were visualized with the VECTASTAIN ABC HRP Kit followed by the 3,3’-diaminobenzidine (DAB) substrate kit (both: Vector Labs Inc., Peterborough, United Kingdom). Briefly, sections were washed in distilled water for 5 min followed by incubation in 0.3% hydrogen peroxidase in 70% methanol to remove endogenous peroxidase from the tissue.

Sections were then incubated in VECTASTAIN ABC reagent for 2 h. Afterwards, the sections were washed with 0.1M PB for 5 min and incubated with DAB substrate kit working solution for 2-10 min followed by three 5 min washing steps with 0.1M PB. Slides were counterstained with cresyl violet and washed in 70% and 96% (2x) ethanol followed by 100% iso-propanol (2x) and xylol (3x). Each washing step lasted 3 min. Lastly, slides were coverslipped with Roti-Histokit II (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) and images taken in the brightfield channel of an epifluorescence microscope (ECLIPSE Ni, Nikon GmbH, Düsseldorf, Germany).

2.6 Antibody characterization

GABA. We used a monoclonal antibody (mAB 3D5; Swant, Marly, Switzerland) raised in mouse to detect gamma-aminobutyric acid (GABA). Antibody specificity was determined by the manufacturer in an enzyme linked immunosorbent assay (ELISA) by testing cross-reactivity with β-alanine, aspartate, glutamate, glycine and taurine. This revealed low cross-reactivity. This antibody was previously used to identify the presence of GABA in sea lamprey, *Petromyzon marinus* (Villar-Cerviño *et al.*, 2006).

Glycine. A polyclonal antibody (1015GE; MoBiTec, Göttingen, Germany) raised in rabbit was used to identify the amino acid glycine. Antibody specificity was tested by the manufacturer with a glycine-glutaraldehyde-protein in an ELISA test by cross-reactivity experiments with β-alanine, aspartate, GABA, glutamate and taurine. This showed low cross-reactivity. The antibody used here was previously used to identify glycinergic neurons in the auditory and vestibular system in guinea pig, *Cavia porcellus* (Peyret *et al.*, 1987) and in the brain of rats, *Rattus rattus* (Campistron *et al.*, 1986) with no trace of glial labeling. Other studies have used antibodies against glycine to identify glycinergic neurons without any sign of glial labeling in mouse (Restrepo *et al.*, 2009), rat (Downie *et al.*, 2010), sea lamprey (Villar-Cerviño *et al.*, 2008), Siberian sturgeon, *Acipenser baeri* (except for coronet cells) (Adrio *et al.*, 2011) and salamander, *Ambystoma tigrinum* (Cimini *et al.*, 2008).

Connexin. A monoclonal antibody (MAB3045; Millipore, Bedford, MA USA) raised in mouse was used to detect connexin 35/36 (Cx 35/36), a pore protein in gap junctions. The

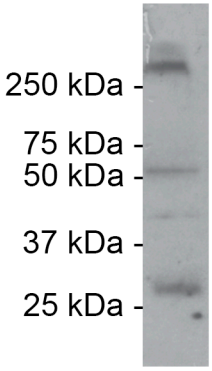


FIGURE 2: CONNEXIN 35/36 ANTIBODY LABELING IN WESTERN BLOT OF WHOLE BRAIN HOMOGENATE OF GULF TOADFISH.

Bands are between 25 and 37 kilo Dalton (kDa) ladder marks, as well as between the 50 and 75 kDa ladder marks. Results are identical to the antibody manufacturer's results in hybrid bass (exact species not provided on Millipore website for antibody MAB3045), a different fish species than the one towards which the antibody was raised. The heavy band above the 250 kDa ladder mark likely represents the six connexin protein subunits binding together to form a connexin hemichannel.

specificity assessment of the manufacturer states that this antibody reacts with fish Cx 35/36. The antibody has been used to stain Cx 35/36 in zebrafish, *Danio rerio* (Song *et al.*, 2016) where it was shown to be present in electrical synapses between motor and premotor neurons, similar to the VMN-VPN-VPP coupling in this study. We tested the antibody's specificity using a western blot of toadfish whole brain homogenates. 12% resolving, 5% stacking SDS-polyacrylamide gel electrophoresis was performed on whole brain homogenates from Gulf toadfish. Gels were transferred to polyvinylidene difluoride membrane (Bio-Rad Laboratories Inc., Hercules, CA USA), blocked overnight at 4ºC in PBS with 10% powdered milk, and then overnight at 4ºC in PBS with 10% powdered milk with 1:1000 primary antibody against Cx 35/36. Subsequently, gels were washed with PBS and incubated in secondary antibody for 2 h at room temperature. After washing in PBS, gels were treated with a chemiluminescence kit (SuperSignal West Pico; Thermo Scientific Waltham, MA USA), and exposed to film (Carestream Kodak BioMax MS; Sigma Aldrich, St. Louis, MO USA). The film was developed and scanned as 600 dpi grayscale tiff files (Epson Perfection 4990 Photo scanner; Epson, Long Beach, CA USA). The antibody manufacturer reports two bands on western blots of hybrid bass whole brain (exact species not provided on Millipore website): one between 25 and 37 kDalton (Da) and one between 50 and 75 kDa. We found similar results in Gulf toadfish whole brain homogenates on western blots (Fig. 2). An additional band >250 kDa is likely the result of six connexin protein subunits coming together to form a single connexin hemi-channel.

Serotonin. A polyclonal antibody against serotonin (20080; Immunostar, Hudson, WI USA) raised in rabbit was used to detect serotonin. According to the manufacturer, this

antibody is specific towards its target in a variety of vertebrate species and has been used in studies of the Atlantic salmon, *Salmon salar*, (Sandbakken *et al.*, 2012) and sea lamprey (Villar-Cerviño *et al.*, 2006). Additionally, the manufacturer states that the antibody serum does not react with 5-hydroxytryptophan, 5-hydroxyinodole-3-acetic acid and dopamine in cross-reactivity experiments.

Tyrosine hydroxylase. We used a monoclonal antibody that was raised in mouse against tyrosine hydroxylase (TH; 22941; Immunostar, Hudson, WI USA), the rate-limiting enzyme in catecholamine synthesis, to detect catecholaminergic input to the vocal CPG. This antibody detects the catalytic core of TH and therefore reacts with TH in a wide range of species including amphibians (Joven *et al.*, 2013) and zebrafish (manufacturer's statement). The antibody does not react with phenylalanine hydroxylase or tryptophan hydroxylase (manufacturer's statement). Western blot analysis of brain extract of midshipman, a close relative of Gulf toadfish, showed a band at 59-63 kDa as expected for TH according to the manufacturer (Goebrecht *et al.*, 2014).

Choline acetyltransferase. A polyclonal antibody raised in goat against choline acetyltransferase (ChAT, AB144P; Millipore, Bedford, MA USA), the rate limiting enzyme in the synthesis of acetylcholine (ACh), was used to assess the presence of ACh. This antibody has been tested in a wide range of species including zebrafish (Müller *et al.*, 2004), goldfish, *Carassius auratus* (Giraldez-Perez *et al.*, 2013), bichir, *Polypterus senegalus* (Lopez *et al.*, 2013) and weakly electric mormyrid fish, *Gnathonemus petersii* (Pusch *et al.*, 2013). In agreement with the manufacturer's western blot analysis in mouse brain lysate, the antibody was shown to specifically recognize bands between 68-72 kDa, e.g., in zebrafish (Coppola *et al.*, 2012), the lesser spotted dogfish, *Scyliorhinus canicula* (Anadón *et al.*, 2000), and bichir (Lopez *et al.*, 2013), corresponding to the size of ChAT protein in zebrafish (70 kDa; Volkmann *et al.*, 2010).

For all primary and secondary antibodies, we tested for nonspecific binding of the antibody to the tissue by carrying out the staining protocol as above but omitting the secondary or primary antibody, respectively. All tests revealed no staining.

3 Results

3.1 Identification of the vocal CPG nuclei

Confirming the results of a prior study of Gulf toadfish (Chagnaud and Bass, 2014), transneuronal neurobiotin transport after neurobiotin application to the VN (Fig. 1e) labels the three vocal CPG nuclei – VPP, VPN and VMN (Fig. 1e-h). In contrast, applying crystals of the much larger molecular weight biotinylated dextran (3 kDa compared to 323 Da for neurobiotin) leads only to a unilateral label of the VMN (Chagnaud and Bass, 2014). The paired VMN form a single dense column of motor neurons with the paired VPN extending alongside as ventrolateral columns. VPP appears bilaterally as ventrolateral columns just rostral to VMN and VPN. Based on measurements in three animals, the rostral-caudal extent, number, diameter, and shape factor of neurobiotin-labeled neurons in the three CPG nuclei (Table 2) is consistent with values reported in a previous study (Chagnaud and Bass, 2014).

3.2 GABAergic and glycinergic label in vocal CPG

Neurophysiological evidence supports the role of GABA in determining the temporal properties of the vocal CPG output (Chagnaud *et al.*, 2011; Chagnaud *et al.*, 2012). Dense GABAergic label is present at all levels of the vocal CPG (Fig. 3). Labeled puncta suggestive

TABLE 2

Rostro-caudal nucleus extent [µm], neuron number (Abercrombie corrected), neuron diameter [µm] and shape factor of neurobiotin-labeled neurons evaluated bilaterally in all three nuclei of the vocal CPG. All values are average and standard deviation in parentheses. Abbreviations: VMN, vocal motor nucleus; VPN, vocal pacemaker nucleus; VPP, vocal pre-pacemaker nucleus.

	VMN	VPN	VPP
Rostro-caudal extent [µm]	983 (154)	800 (20)	308 (72)
Neuron number	948 (133)	375(52)	224 (15)
Neuron diameter [µm]	17 (2)	10 (1)	10 (1)
Neuron shape factor	0.744 (0.119)	0.805 (0.105)	0.762 (0.116)

of synaptic sites occur on neurobiotin-labeled somata or within the adjacent neuropil of all three vocal nuclei (Fig. 3a, b, e, f, g, j). To identify the putative origin of these inputs, we looked for

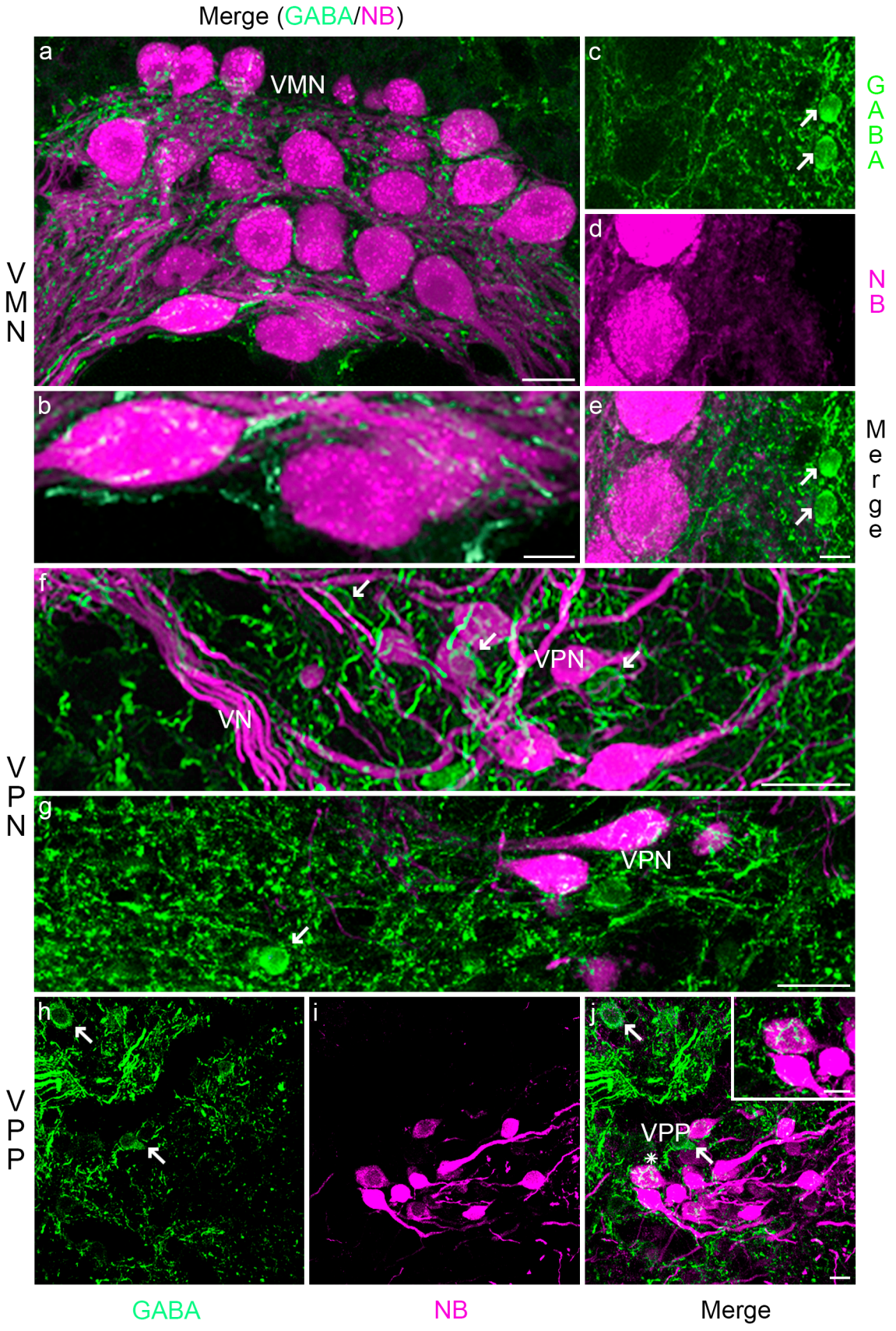


FIGURE 3: GABA IN GULF TOADFISH VOCAL CPG.

GABAergic label (green) is present in all of the neurobiotin labeled CPG nuclei (NB; magenta): a-e, vocal motor nucleus (VMN); f-g, vocal pacemaker nucleus (VPN); h-j, vocal pre-pacemaker nucleus (VPP). GABAergic somata are observed next to the VMN (arrows in c, e); next to and within the VPN (arrows in f, g) and VPP (arrows in h, j). Asterisk in j indicates somata in inset. GABAergic neurons are not co-labeled with neurobiotin (e.g., c-e; f, g; h-j). The scale bar represents 25 µm in (a), 10 µm in (b), 10 µm in (e) for (c-e), 10 µm in (f) and (g), 10 µm in (j) for (h-j) and 5 µm in inset in (j). Abbreviations: voT, vocal tract.

somata that were located immediately adjacent or close to neurobiotin-labeled neurons. Small GABAergic somata, round in shape, are lateral to the VMN (arrows, Fig. 3c, e); dorsal, ventral and lateral to VPN (e.g., arrow indicates ventrally located GABAergic neurons, Fig. 3g), as well as within VPN (arrows, Fig. 3f); and within and around VPP (arrows, Fig. 3h, j).

Similar to GABAergic label, glycinergic label is present in all vocal CPG nuclei (Fig. 4), including puncta suggestive of synapses (Fig. 4a, d, i and insets). Glycinergic neurons with round-like somata are adjacent and lateral to VMN (arrows, Fig. 4a, lower left inset), and adjacent and within VPN (e.g., arrows, Fig. 4a, b) and VPP (arrows, Fig. 4g, i). In contrast to GABAergic neurons, a subset of glycinergic neurons are co-labeled with neurobiotin within VPN (Fig. 4b-d, see asterisks in d). Neurobiotin-glycine, co-labeled fibers also enter VMN (arrow, Fig. 4f). There are no co-labeled somata or processes in VPP (Fig. 4g-i, see arrows in g, i).

3.3 Subsets of inhibitory neurons within VPN

VPN has four types of labeled neurons: neurobiotin-only, GABA-only, glycine-only and neurobiotin-glycine co-labeled. To assess if these neurons form distinct subpopulations, we compared the proportion of neurons, and the neuron diameter and shape factor in each of the four VPN groups in three fish (see section 2.4). In these fish, neurobiotin, glycine and GABA are co-stained. To avoid size effects due to different body sizes, statistical tests were performed only within individual fish. The results are summarized in table 3. Table 3a presents a summary of the results for neuron diameter and shape in each fish; tables 3b, c present the statistical results for these measures for each fish in which significant differences were

observed. The majority of neurons are only labeled with either neurobiotin or glycine. Fewer neurons are labeled with only GABA or with both neurobiotin and glycine.

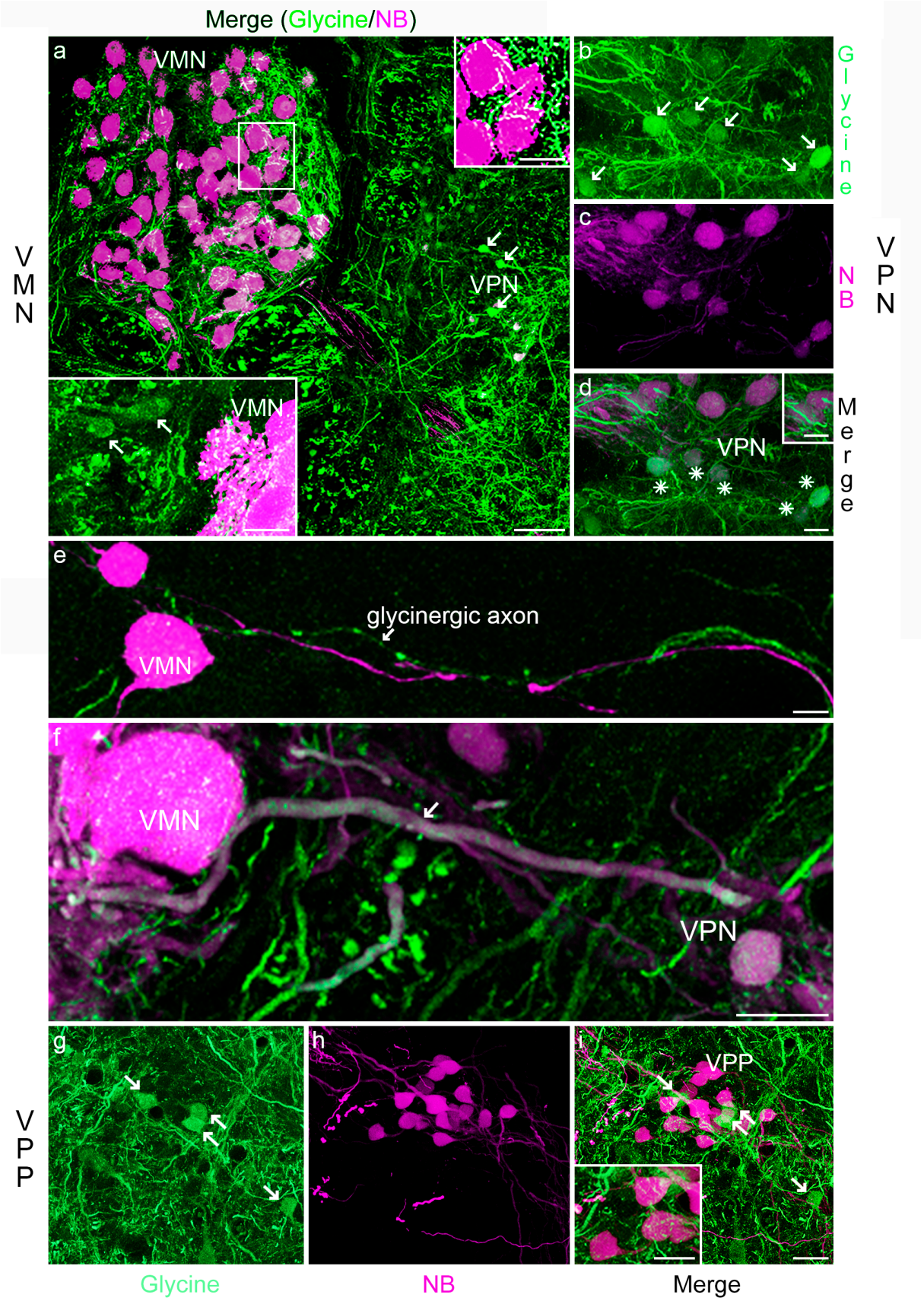


FIGURE 4: GLYCINE IN GULF TOADFISH VOCAL CPG.

Glycinergic label (green) lie within all of the neurobiotin-labeled CPG nuclei (NB; magenta): vocal motor (VMN, a), vocal pacemaker (b-d), vocal pre-pacemaker (g-i). Glycinergic somata are observed next to VMN (arrows, lower inset in a), and within VPN (arrows in a, b) and VPP (arrows in g, i). A subset of glycinergic neurons in the VPN are co-labeled with neurobiotin (asterisks in d). The upper right inset in (a) is higher magnification view of VMN. The inset in (i) highlights glycinergic label in VPP. A glycine-labeled axon in close proximity to a VMN soma is highlighted in (e). Neurobiotin-glycine co-labeled processes (f, arrow) are observed between VPN and VMN . The scale bar represents 50 μm in (a), 10 μm in (d) for (b-d), 20 μm in (e), 10 μm in (f), and 10 μm in (i) for (g-i). The scale bar is 25 μm in the upper right inset in (a), 10 μm in the lower left inset in (a) and 10 μm in insets in (d) and (i).

The neurobiotin-only labeled neurons are, in general, significantly larger in diameter than glycine-only labeled and neurobiotin-glycine co-labeled neurons ($p<0.02$ in all fish; Table 3b). Glycine-only and neurobiotin-glycine labeled neurons have a similar diameter. GABA-labeled neurons are significantly smaller in diameter than neurobiotin-only, neurobiotin-glycine co-labeled, and glycine-only labeled neurons (Table 3b; $p<0.01$ for all comparisons), except for Toadfish 3 that shows no significant difference in diameter between GABA-only labeled and neurobiotin-glycine co-labeled neurons (Table 3b). The shape factor of GABA-only neurons is significantly different from neurobiotin-only and glycine-only neurons, but not from neurobiotin-glycine co-labeled neurons in only the largest of the three toadfish examined (Table 3c). While this might suggest an effect of fish size on neuronal dimensions, a much larger sample size would be needed to assess this possibility.

To determine whether the four VPN neuron groups display a topographic organization, we assessed their distributions throughout the rostral-caudal extent of VPN in one fish (Fig. 5a, b). Neurobiotin-glycine co-labeled neurons strongly overlap the distribution of neurobiotin-only labeled neurons (Fig. 5c). The GABA-only and glycine-only labeled neurons are predominantly located along and outside the main perimeter of neurobiotin labeled VPN neurons (Fig. 5c). Thus, while a spatial separation exists between VPN neurons labeled with or without neurobiotin (neurobiotin-glycine and neurobiotin-only versus GABA-only and glycine-only, respectively), a neuron's location is not predictive of its labeling pattern.

TABLE 3

(a) Median and interquartile range (in parentheses) of neuron dimensions in subgroups of the vocal pacemaker nucleus (VPN) in three fish (animal ID code and standard length indicated) assessed as diameter [µm] and shape factor. Results of Kruskal-Wallis test and Wilcoxon signed rank test for differences in neuron diameter (b) and shape factor (c) between neuron groups in VPN.

a) Neuron dimension and shape factor

		NB-only	Glycine-only	NB-Glycine	GABA-only
Toadfish 1 OB-15-04 (10.5 cm)	diameter [µm]	13 (4)	10 (3)	11 (3)	8 (2)
	Shape factor	0.704(0.216)	0.709 (0.229)	0.690 (0.177)	0.792 (0.173)
Toadfish 2 OB-16-09 (8.5 cm)	diameter [µm]	12 (3)	11 (2)	12 (2)	8 (2)
	Shape factor	0.769 (0.183)	0.770 (0.134)	0.799 (0.2)	0.780 (0.232)
Toadfish 3 OB-16-10 (6.1cm)	diameter [µm]	11 (3)	10 (2)	9 (3)	8 (2)
	Shape factor	0.789 (0.131)	0.803 (0.157)	0.752 (0.139)	0.824 (0.232)

b) Neuron diameter

Kruskal-Wallis test	**Chi²**	**Degrees of freedom**	**p-value**
Toadfish 1 (OB-15-04)	94.883	3	**< 0.0001**
Toadfish 2 (OB-16-09)	65.576	3	**< 0.0001**
Toadfish 3 (OB-16-10)	33.811	3	**< 0.0001**

Wilcoxon signed rank test

		Gly-only		**NB-Gly**		**GABA-only**	
		W	p	W	p	W	p
Toadfish 1 (OB-15-04; 10.5cm)	NB-only	3567	**< 0.0001**	3254	**0.0003**	3240.5	**< 0.0001**
	Gly-only			1731.5	0.341	1905	**< 0.0001**
	NB-Gly					1999.5	**< 0.0001**
Toadfish 2 (OB-16-09; 8.5cm)	NB-only	905	**0.0010**	239	0.0187	956	**< 0.0001**
	Gly-only			191	0.1281	1109	**< 0.0001**
	NB-Gly					300	**< 0.0001**
Toadfish 3 (OB-16-10; 6.1cm)	NB-only	3480.5	**0.0023**	1216	**0.0023**	1185	**< 0.0001**
	Gly-only			274	0.1204	507	**< 0.0001**
	NB-Gly					118	0.1828

c) Shape factor

Kruskal-Wallis test	**Chi²**	**Degrees of freedom**	**p-value**
Toadfish 1 (OB-15-04)	12.403	3	**0.0061**
Toadfish 2 (OB-16-09)	1.8371	3	0.6069
Toadfish 3 (OB-16-10)	0.4232	3	0.9354

Wilcoxon signed rank test

		Gly-only		**NB-Gly**		**GABA-only**	
		W	p	W	p	W	p
Toadfish 1 (OB-15-04, 10.5cm)	NB-only	2367	0.5695	2484	0.7922	1101	**0.0013**
	Gly-only			1438	0.4528	648	**0.0011**
	NB-Gly					799	0.0199

Highly significant results are marked in bold. Abbreviations: NB, neurobiotin; Gly glycine.

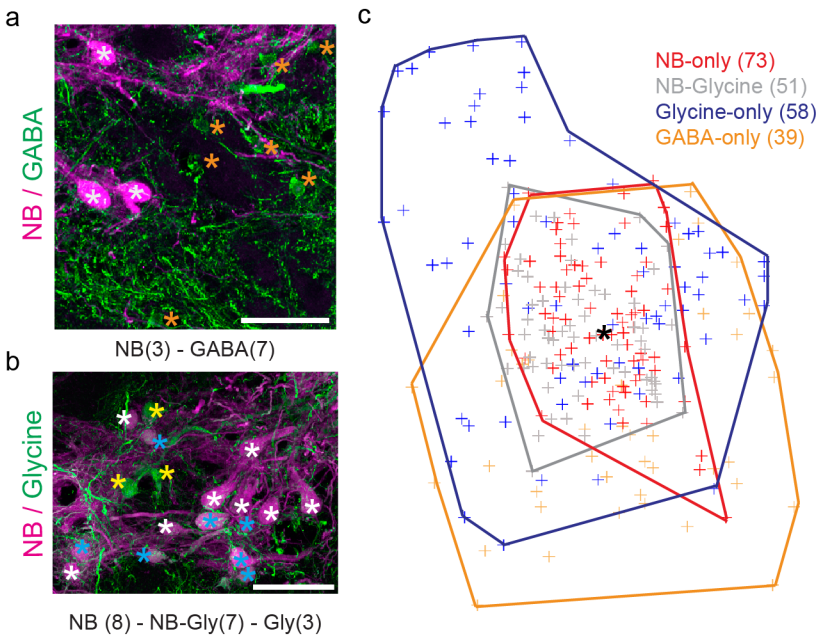


FIGURE 5: DISTRIBUTION OF GABAERGIC AND GLYCINERGIC NEURONS IN VOCAL PACEMAKER NUCLEUS (VPN) OF GULF TOADFISH.

Representative photomicrographs (a, b) of a single, ipsilateral VPN population in which the position (asterisks) of neurobiotin (NB; white asterisks in (a) and (b)), GABA (orange asterisks in (a)), glycine-only (yellow asterisks in (b)) and neurobiotin-glycine co-labeled (light blue stars in (b)) neurons are indicated. Sample size is given in parentheses for each group. Color-coded representations of the distribution of the four neuron groups in the VPN region is shown in (c). Each neuron's location is represented by a color coded cross and the outline of the population by a color-matched polygon. Sample size is given in parentheses for each group. Black asterisk marks the center of VPN.

3.4 Gap junction label in vocal CPG

Anatomical and neurophysiological evidence has led to the proposal that the robust transneuronal transport of neurobiotin throughout the vocal CPG depends, in whole or in part, on electrotonic coupling within the VPP-VPN-VMN network (Bass and Marchaterre, 1989; Bass *et al.*, 1994; Chagnaud *et al.*, 2011; Chagnaud *et al.*, 2012). We investigated the distribution of gap junction proteins throughout the vocal CPG using an antibody against Cx 35/36. All neurobiotin-labeled VMN (Fig. 6a-i) and many VPN (Fig. 6j-l) somata appear co-labeled with Cx 35/36. Label in VMN is mainly along the perimeter of somata and processes (Fig. 6a-f), except at its rostral end where it is dense over somata (Fig. 6g-i). Label in VPN (Fig. 6 j-l) is also dense over somata (arrow, Fig. 6l), though extensive label is also present in regions surrounding these somata (Fig. 6l). VPP exhibits diffuse Cx 35/36 label (Fig. 6m-o)

mainly adjacent to neurobiotin-labeled somata (Fig. 6o). As in VMN (see above), label in VPN (e.g., inset, Fig. 6l) and VPP (e.g., inset Fig. 6o) has a punctate-like appearance. A few somata immediately adjacent to VMN that are not labeled with neurobiotin also have a ring of punctate, Cx 35/36-like immunoreactivity along the outer margin (e.g., arrow in Fig. 6a-c).

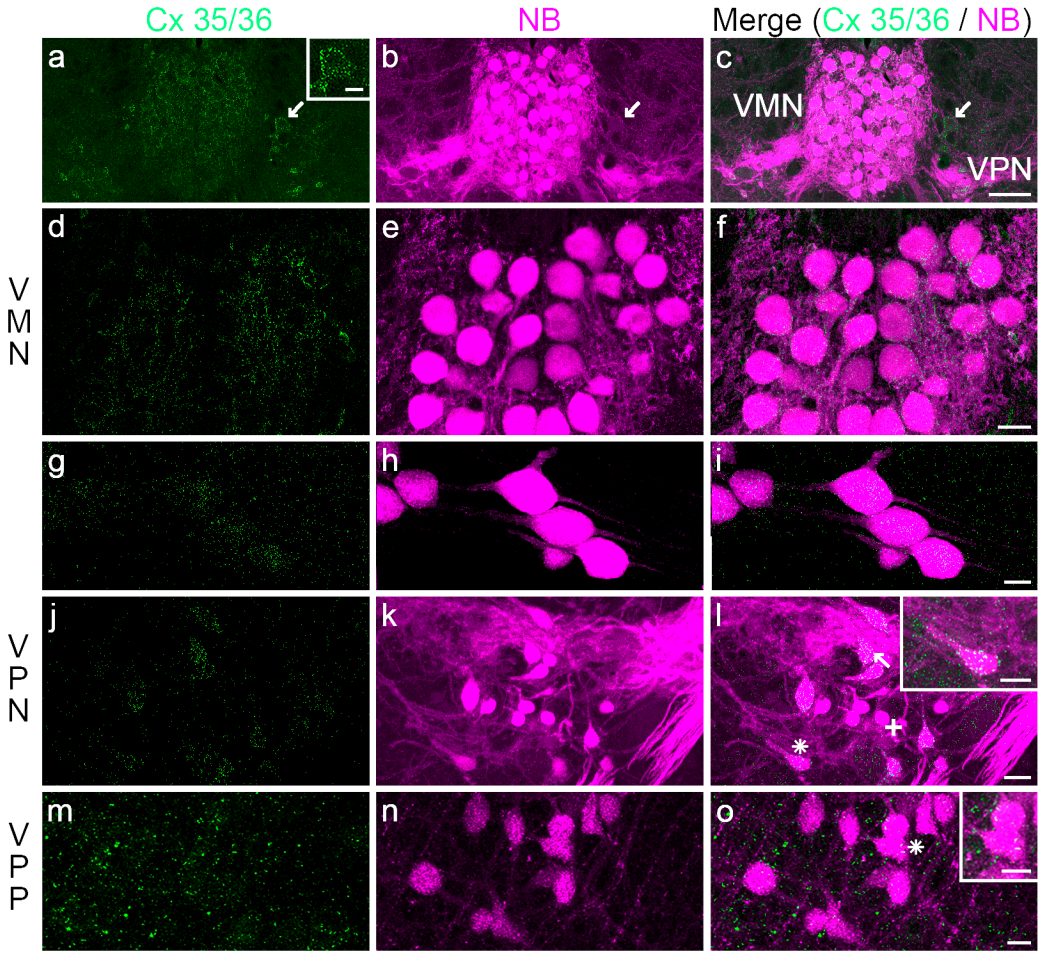


FIGURE 6: CONNEXIN 35/36 DISTRIBUTION IN GULF TOADFISH VOCAL CPG.

Connexin 35/36 (Cx 35/36; green) as a marker of gap junctions is present in all CPG nuclei, labeled with neurobiotin (NB; magenta). Cx 35/36 label is prominent on all somata of the vocal motor nucleus (VMN; a-i). Punctate-like Cx 35/36 label also outlined the perimeter of cells adjacent to VMN that are not co-labeled with neurobiotin, but similar in shape to VMN neurons (arrow in c). Cx 35/36 label is also prominent on somata of the vocal pacemaker nucleus (VPN; j-l, arrow and cross in l indicate examples of labeled and unlabeled somata, respectively). Asterisk in (l) indicates neuron in inset. Cx 35/36 is also present in the vocal pre-pacemaker (VPP, m-o and inset in o). Asterisk in (o) indicates neurons in inset. Scale bar represents 100 µm in (c) for (a-c), 25 µm in (f) for (d-f), 10 µm in (i) for (g-i), 10 µm in (l) for (j-l), 10 µm in (o) for (m-o), 20 µm for the inset in (a) and 10 µm for the insets in (l) and (o).

3.5 Serotonergic label in vocal CPG

Serotonin modulates the activity of a variety of motor systems including those responsible for vocal behavior (e.g., Wood *et al.*, 2011; Yu and Yamaguchi, 2010). Serotonergic innervation occurs throughout the vocal CPG (Fig. 7). Label in the VMN is minimal (Fig. 7a), except for the most rostral pole where it is prominent (Fig. 7b). Serotonergic label is abundant lateral to the VMN, amongst the VMN and VPN dendrites (e.g., arrow Fig. 7c; see Fig. 1f for orientation). In contrast to most of the VMN, label is found throughout VPN and VPP; in some cases, the label is directly over somata (insets, Fig. 7f, g), although it is most prominent in adjacent regions (Fig. 7f, g).

A few serotonergic cells lie within the central region of VMN (Fig. 7d) and along its ventral margin where neurites that branch within VMN are especially apparent (Fig. 7e). No serotonergic cells are found within VPN or VPP. None of the serotonergic neurons are co-labeled with neurobiotin, suggesting that they are not coupled via gap junctions within the vocal CPG. The origin of the serotonergic input to VMN arises, at least in part, from axons of serotonergic cells within and along the perimeter of VMN (arrows, Fig. 7d, e). The origin of the input to VPN and VPP is less obvious though some input to VPN may come from nearby VMN-associated serotonergic neurons. Other serotonergic neurons lie further ventral to VMN, VPN and VPP, or ventral to the fourth ventricle at caudal levels of the cerebellum in the location of the caudal component of the inferior raphe nucleus (iRN, Fig. 7g).

3.6 Catecholamine label in vocal CPG

Given the evidence for a role of catecholamines in vocal mechanisms in several vertebrate taxa (Appeltants *et al.*, 2003; Creighton *et al.*, 2013; Forlano *et al.*, 2014; Goebrecht *et al.*, 2014), we investigated the presence of catecholaminergic input to the vocal CPG using an antibody generated against TH, the rate-limiting enzyme in the synthesis of dopamine and noradrenaline (Fig. 8). All three vocal CPG nuclei exhibit dense TH label (Fig. 8a, d, g, j). In contrast to serotonin, catecholaminergic label is abundant both within and lateral to the VMN, VPN and VPP (Fig. 8a-j). Unlike serotonin, there is no apparent rostral-caudal difference in the

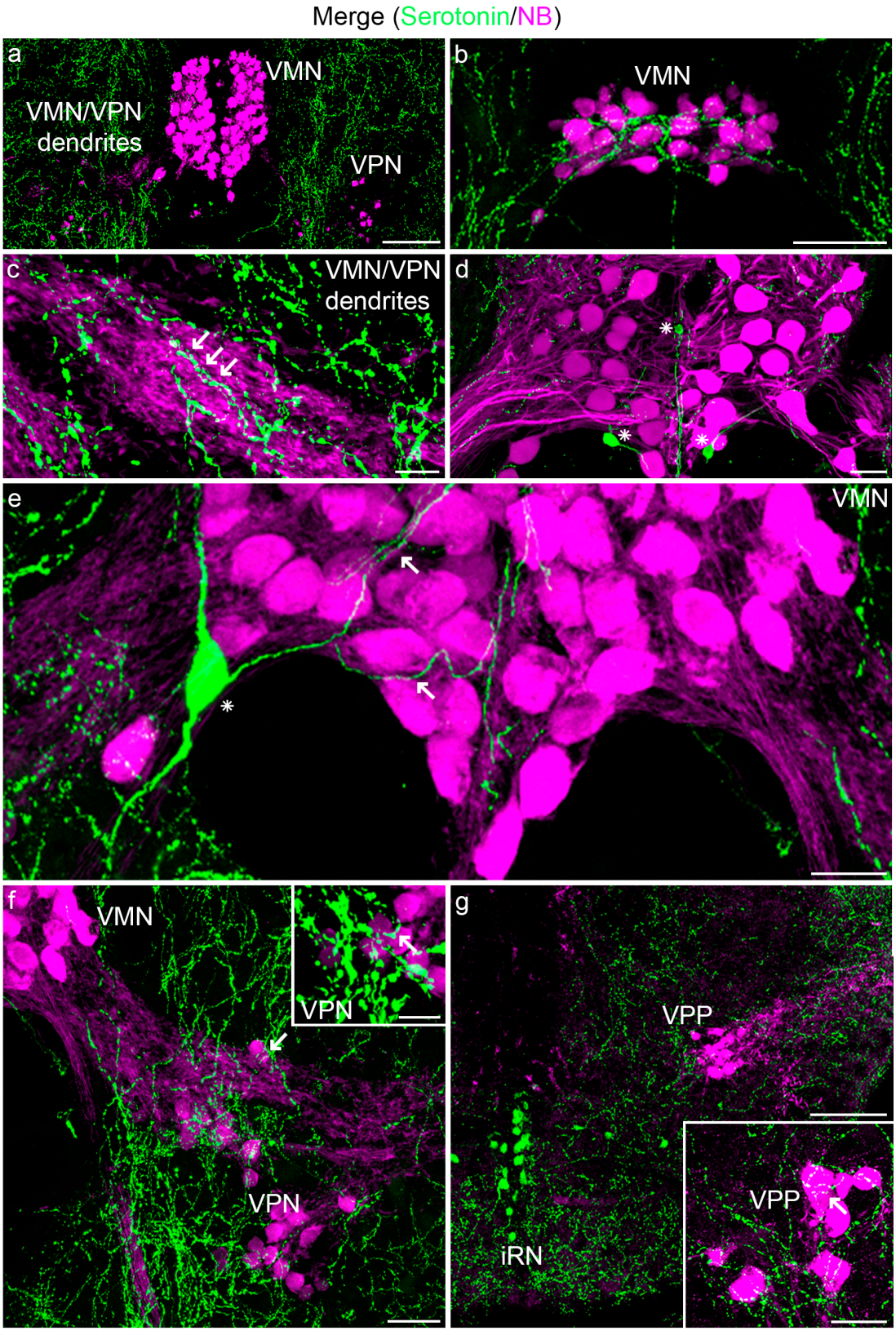


FIGURE 7: SEROTONIN IN GULF TOADFISH VOCAL CPG.

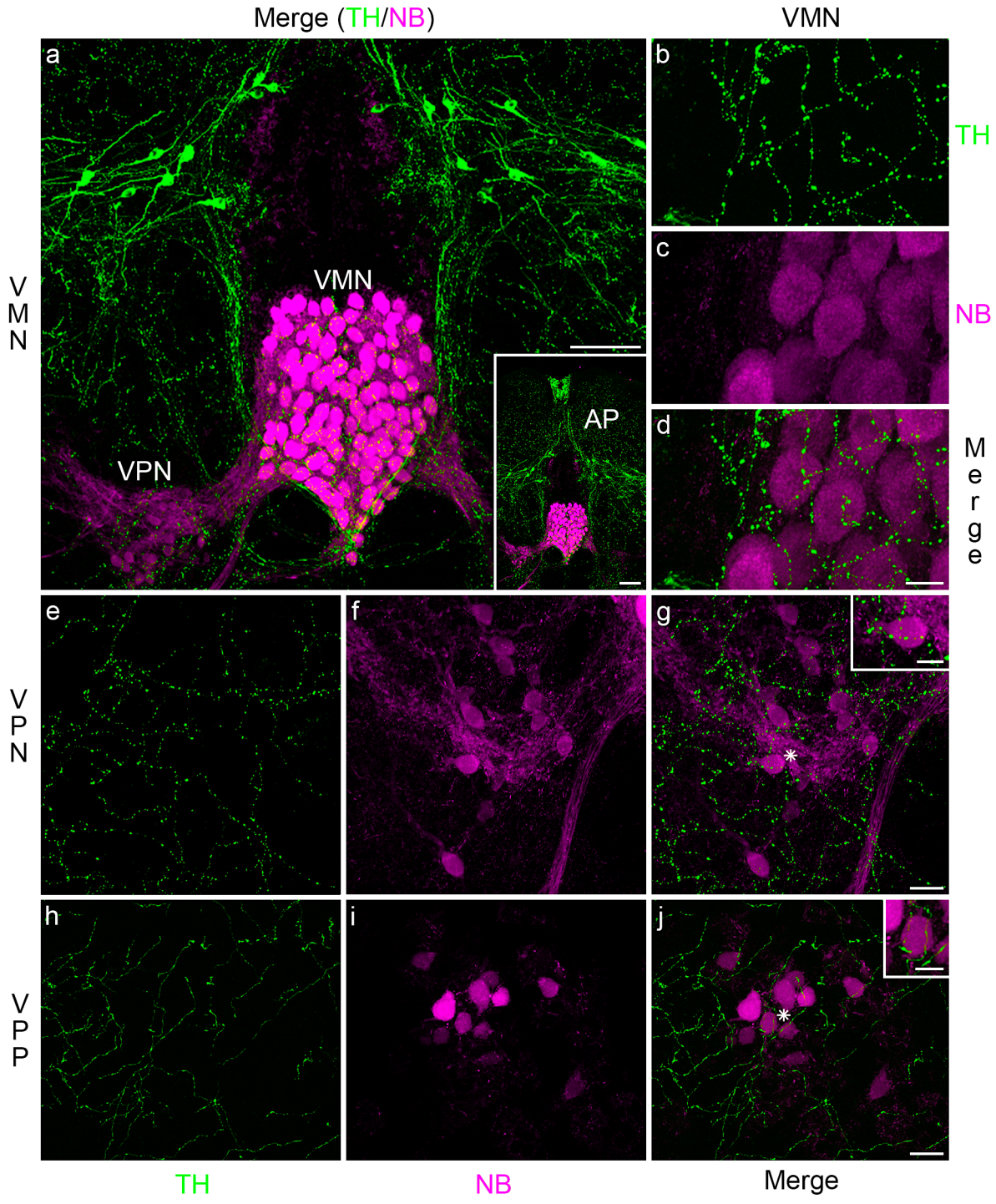


FIGURE 8: CATECHOLAMINES IN GULF TOADFISH VOCAL CPG

FIGURE 7: SEROTONIN IN GULF TOADFIHS VOCAL CPG. All CPG nuclei, labeled with neurobiotin (NB; magenta), exhibit serotonergic label (green). Weak label is found within most of the VMN (a), except for the rostral pole where it is prominent (b). Serotonergic label is abundant lateral to the VMN, i.e., amongst VMN dendrites (arrows in c). Serotonergic somata are within and adjacent to VMN (asterisks in d and e). Serotonergic label in vocal pacemaker (VPN) and pre-pacemaker (VPP) nuclei is within the region where somata cluster (e.g., arrows in f and insets in f-g). Serotonergic cells are also within the inferior raphe nucleus (iRN) that had a rostral-caudal extent spanning all levels of the vocal CPG (e.g., at the level of VPP in g). Scale bar represents 100 μm in (a), (b) and (g); 5 μm in (c); 20 μm in (d) and (e); 25 μm in (f); 10 μm in the insets in (f) and (g).

FIGURE 8: CATECHOLAMINES IN GULF TOADFISH VOCAL CPG. Dense tyrosine hydroxylase (TH)-like immunoreactivity indicative of catecholamines (green) occurs within the neurobiotin-labeled (NB, magenta) vocal motor (VMN, a-d), vocal pacemaker (VPN, e-g) and vocal pre-pacemaker (VPP, h-j) nuclei. Insets in g and j highlight catecholaminergic label at sites indicated by asterisks in VPN and VPP, respectively. Catecholaminergic processes likely originate from somata dorsal to the vocal CPG (a) and the area postrema (AP, inset in a). Scale bar represents 100 μm in (a) and its inset, 20 μm in (d) for (b-d), 20 μm in (g) for (e-g), 10 μm in (j) for (h-j), and 5 μm in the insets in (g) and (j).

intensity of label in VMN. Catecholaminergic neurons are not located within any of the vocal CPG nuclei, although some are dorsolateral to the VMN-VPN circuit (Fig. 8a) immediately ventral to the tightly clustered TH cells of the area postrema (Fig. 8 inset in a, see also Forlano et al., 2014 for detailed description in midshipman fish). As with serotonin, none of the catecholaminergic neurons are transneuronally labeled with neurobiotin.

3.7 Cholinergic label in vocal CPG nuclei

In addition to serotonin and catecholamines, we investigated cholinergic input to the vocal nuclei, a third potential source of neuromodulation. Not surprisingly, neurobiotin-labeled VMN somata and axons are positively labeled for ChAT, the enzyme that catalyzes the synthesis of acetylcholine (Fig. 9a-f). By contrast, neurobiotin-labeled VPN (Fig. 9g-i) and VPP (Fig. 9j-l) neurons are not co-labeled with ChAT. Ellipsoid-shaped cholinergic somata were, however, within and in close proximity to larger neurobiotin-labeled VPN neurons (arrow, Fig. 9g, i). At the level of VPP, there is less robust cholinergic label in neurons adjacent to similarly sized neurobiotin-labeled neurons (asterisk and arrow, Fig. 9j, l; inset, Fig. 9j of cell indicated by asterisk). Cholinergic puncta within VPN and VPP (insets, Fig.9i, l) suggest cholinergic inputs to these nuclei. Due to the extensive overlap of VMN and VPN dendrites (e.g., Fig. 1e,

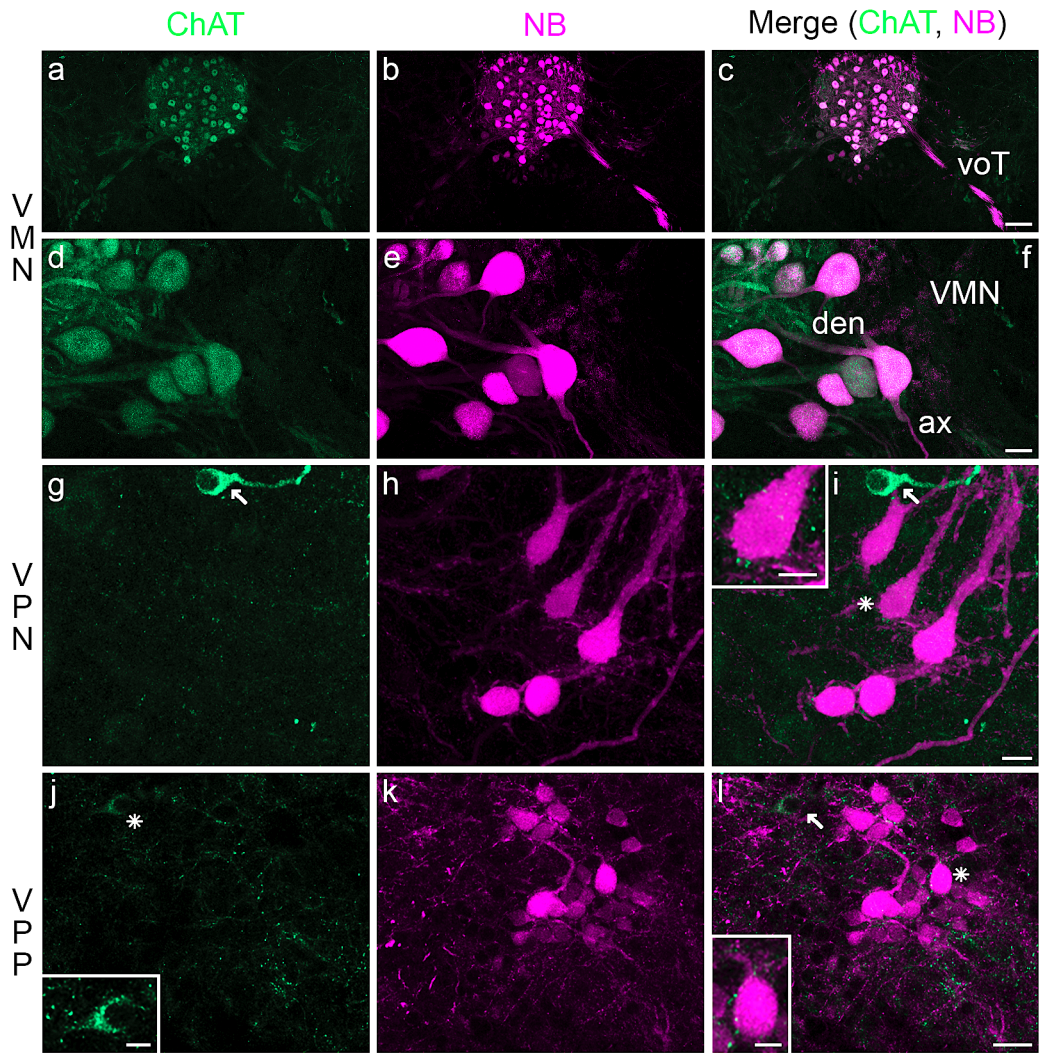


FIGURE 9: CHOLINE ACETYLTRANSFERASE IN GULF TOADFISH VOCAL CPG.

Vocal motor nucleus (VMN, a-f) labeled with neurobiotin (NB; magenta) as well as the vocal tract (voT) show choline acetyltransferase (ChAT; green)-like immunoreactivity (a-f). ChAT label is also present in the vocal pacemaker (VPN, g-i) and vocal pre-pacemaker (VPP, j-l) nuclei. Asterisks in (i) and (l) indicate neuron shown in respective insets that highlight punctate-like label. Small, ellipsoid-shaped ChAT positive somata are adjacent to VPN neurons (arrow in g, i). Cells adjacent to VPP show weak label (asterisk and arrow in j, l; inset in j highlights labeled cell adjacent to asterisk). The scale bar represents 50 µm in (c) for (a-c), 10 µm in (f) for (d-f), 10 µm in (i) for (g-i), 10 µm in (l) for (j-l), and 5 µm in insets in (i), (j) and (l).

g; also see Chagnaud & Bass, 2014) and dense labeling with ChAT, we cannot distinguish cholinergic input to VMN somata or VMN and VPN dendrites.

3.8 Vocal CPG-auditory hindbrain pathway

A prominent neuroanatomical and neurophysiological feature of the vocal CPG is its link to rostral hindbrain auditory nuclei, in particular a medial division of the descending

octovolateralis nucleus (DON, Fig. 10a) that is a part of the ascending auditory system (Bass *et al.*, 2000; Bass *et al.*, 1994). Although transneuronally labeled DON neurons are not co-labeled with any of the neurotransmitters and

modulators studied here, weakly labeled glycinergic neurons are immediately adjacent to transneuronally labeled DON neurons (arrows, Fig. 10b-d).

Unlike prior studies in midshipman fish and toadfish (Bass *et al.*, 1994; Chagnaud and Bass, 2014), transneuronally labeled somata also lie within the rostral hindbrain inferior reticular formation (iRF; Fig. 10a shows hindbrain level and example of neurobiotin-filled neuron in the inset). These neurons are not co-labeled for any of the neurotransmitters and modulators studied here, although weakly labeled glycinergic neurons are nearby (arrows, Fig. 10e-g). This finding is particularly intriguing because of the known involvement of the reticular formation in vocal mechanisms (e.g., Jürgens and Hage, 2007).

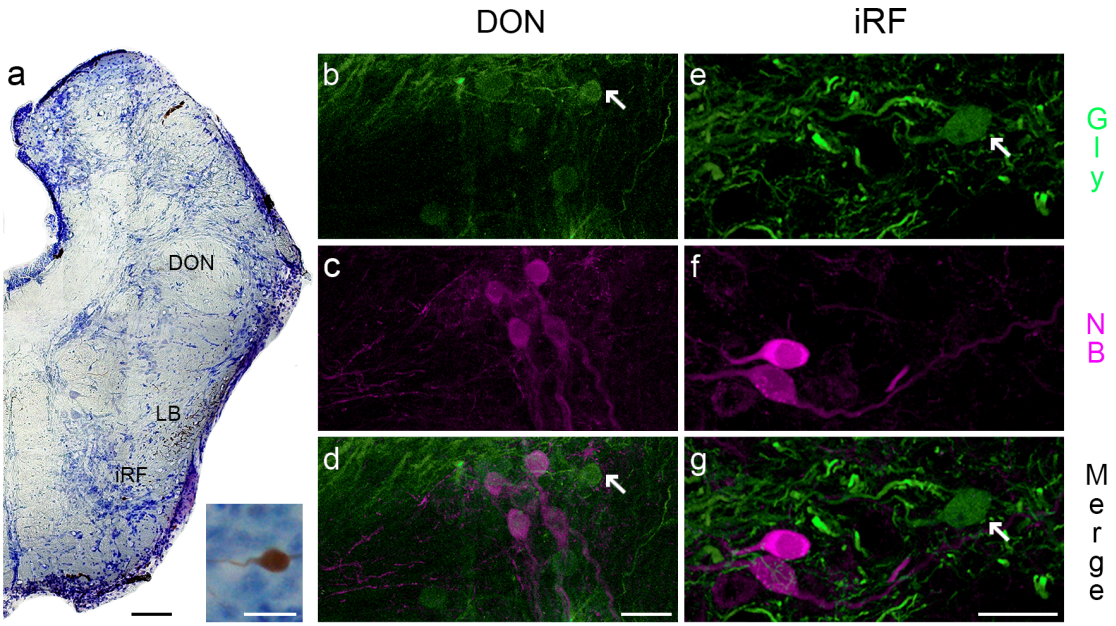


FIGURE 10: GLYCINERGIC NEURONS IN AUDITORY-RECIPIENT DORSAL OCTOVOLATERALIS NUCLEUS (DON), AND IN THE INFERIOR RETICULAR FORMATION (IRF) OF THE GULF TOADFISH.

Cresyl violet stained transverse section of the rostral hindbrain shows location of DON and iRF neurons (a; LB indicates lateral brainstem bundle). The inset in (a) (bottom right) shows a close-up of a neurobiotin-labeled iRF neuron. Glycinergic somata (green, Gly) lie close to neurobiotin-labeled DON and iRF neurons (NB; magenta; DON: b-d; iRF: e-g). Scale bar is 50 µm in (a) and 20 µm in inset in (a), 20 µm in (d) for (b-d) and 10 µm in (g) for (e-g).

4 Discussion

By combining robust transneuronal neurobiotin-labeling of all nuclei comprising the vocal CPG of toadfish with immunohistochemical identification of inhibitory neurotransmitters and putative neuromodulators as well as a gap junction protein, we provide anatomical evidence for robust inhibitory and modulatory inputs within the vocal CPG of a teleost fish. These results complement prior neurophysiological investigations of the intrinsic and network properties of this vertebrate model for vocal-acoustic communication. We interpret the punctate-like label often observed as indicative of synaptic inputs, although we recognize the need to verify this assumption by combining neurobiotin-labeling of vocal neurons with immuno-electron microscopy. All CPG nuclei exhibit prominent GABAergic and glycinergic input. Surprisingly, and in contrast to GABAergic neurons, a subset of premotor neurobiotin-labeled pacemaker neurons (VPN) are co-labeled with glycine, suggesting that they are coupled to other vocal CPG neurons via gap junctions. This is consistent with extensive gap junction protein (connexin) labeling in VPN. Gap junctions are also abundant in the motor neuron population (VMN), supporting the hypothesis that gap junction coupling is prominent in the VPN-VMN circuit (Bass and Marchaterre, 1989; see also Bass *et al.*, 1994; Chagnaud *et al.*, 2011; Chagnaud *et al.*, 2012). The weaker connexin label on premotor VPP somata suggests that transneuronal labeling of VPP is due to gap junction coupling between VPP axons and the somata and/or dendrites of VMN and/or VPN neurons. As discussed below, the evidence for catecholaminergic, serotonergic and cholinergic label suggests a role for these neuromodulators along with inhibitory neurotransmitters and gap junctions in establishing the rhythmic, oscillatory-like output of the vocal CPG that is translated directly into the temporal features of vocal behavior.

4.1 GABAergic and glycinergic neurons within the vocal CPG

4.1.1 GABA. In line with a previous study of midshipman fish that used a GABA antibody shown to be specific in oyster toadfish*, Opsanus tau* (Holstein *et al.*, 2004), GABAergic neurons are positioned lateral to VMN in the Gulf toadfish. These neurons are the

likely source of the strong GABAergic input to VMN (Chagnaud *et al.*, 2012). GABAergic neurons are also found within VPN and adjacent to VPP. None of the GABAergic neurons are co-labeled with neurobiotin, suggesting a lack of gap junction coupling to other vocal CPG neurons. Neurophysiological studies in midshipman fish are consistent with these findings. Injection of bicuculline, a competitive $GABA_A$ receptor antagonist, into VPP leads to an increase in call duration, the vocal parameter coded by VPP (Chagnaud *et al.*, 2011). Intracellular recordings of VMN neurons together with local bicuculline injections into VMN show that GABAergic action at first distorts and then eliminates VMN activity, revealing that GABAergic inhibition is essential to generate vocal signals (Chagnaud *et al.*, 2012).

Activation of GABAergic neurons that inhibit VMN might originate from within or outside of the vocal CPG. VPP is a well-suited candidate from within the vocal CPG, as VPP neurons fire just before and for the duration of the vocal behavior (Chagnaud *et al.*, 2011). A candidate from outside of the vocal CPG would be the midbrain periaqueductal gray (PAG). The PAG activates the vocal CPG via direct input to the duration coding VPP neurons (Chagnaud *et al.*, 2011; Goodson and Bass, 2002; Kittelberger *et al.*, 2006) and itself may influence call duration (Kittelberger *et al.*, 2006). Axon collaterals from the PAG might activate GABAergic neurons adjacent to VMN and VPN to prime the vocal system and to sculpt vocal activity. If GABAergic neurons tonically inhibit VMN during vocal behavior, this prolonged GABAergic action would facilitate a de-inactivation of voltage dependent sodium channels of the weakly excitable VMN motor neurons (Chagnaud *et al.*, 2012).

4.1.2 Glycine. Glycinergic neurons are found at all levels of the vocal CPG. Together with GABA, there are three types of inhibitory neurons in VPN: those labeled only for either glycine or GABA, and glycinergic neurons co-labeled with neurobiotin. All three types may provide input to VMN and/or other VPN neurons that are only labeled with neurobiotin. The glycine-neurobiotin population is especially interesting as the co-label suggests that these neurons are coupled electrically via gap junctions to other vocal CPG neurons; in support, there is strong Cx 35/36 label in VPN. Due to extensive gap junction coupling in the vocal

network, the location of the electrotonic coupling (dendro-dendritic, dendro-axonic, axo-axonic) of neurobiotin-glycine neurons to VMN and/or other VPN neurons remains elusive.

There are three possible, non-exclusive functions for the neurobiotin-glycine co-labeled VPN neurons. The first is that VPP's direct activation of VPN neurobiotin-only neurons would co-activate neurobiotin-glycinergic neurons via electrotonic coupling. This co-activation could, in turn, inhibit non-glycinergic VPN neurons via a chemical (glycinergic) synapse. This form of recurrent inhibition could lead to the oscillatory-like firing pattern that characterizes VPN neurons during vocal activity (Bass and Baker, 1990; Chagnaud *et al.*, 2011). The oscillatory properties of VPN neurons would then be a network property of the neurobiotin-only labeled VPN population rather than an intrinsic property of these neurons. Electrophysiological recordings from VPN in midshipman fish strengthen this hypothesis as current injection into VPN neurons does not induce the characteristic firing frequency displayed during vocal activity (Chagnaud *et al.*, 2012).

Second, gap junction coupling between VMN motor neurons and neurobiotin glycine co-labeled VPN neurons could lead to a direct modulation of motor neuron activity by (VPN) premotor neurons. VMN activity could depolarize neurobiotin-glycine co-labeled VPN neurons via electrical synapses and thereby activate them. The activation of these VPN neurons could, in turn, modify the firing pattern of neurobiotin-only labeled VPN neurons via a chemical glycinergic synapse. As described above, this would lead to a change in the firing frequency of VPN neurons and thus modify VMN activity. Similar motor-premotor coupling has recently been shown in zebrafish and *Drosophila* locomotor networks in regard to motor rhythm control (Matsunaga *et al.*, 2017), as well as in the *Xenopus* vocal pattern generator (Lawton *et al.*, 2017).

A third possible function of the glycine-neurobiotin co-labeled VPN neurons is that they contribute to the synchronous firing of the VMN neurons during vocal behavior (Chagnaud *et al.*, 2012). Depolarization of the neurobiotin-glycine co-labeled neurons in VPN via electrical coupling could inhibit VMN neurons via chemical glycinergic synapses. This would result in the suppression of VMN activity directly after VMN neurons are activated, thus leading to a precise

and rapid suppression of VMN activity and repolarization of its motor neurons. This VMN activity dependent inhibition would foster synchronized firing of vocal motor neurons by preventing sustained action potential firing. The coupling of glycinergic neurons to the VPN, VMN or both nuclei, could thus contribute to the extreme synchrony and temporal precision that characterizes the VPN-VMN circuit in toadfishes (Chagnaud *et al.*, 2011; Chagnaud *et al.*, 2012). Glycinergic innervation of hindbrain motor neurons involved in vocal production has also been shown in zebra finches, *Taeniopygia guttata* (Sturdy *et al.*, 2003). Whether a subset of these glycinergic neurons are also gap junction coupled to vocal motor neurons, as in toadfish, remains unknown.

4.2 Neuromodulators in the vocal CPG

4.2.1 Serotonin. In agreement with reports for midshipman fish and the oyster toadfish that used different antibodies from the one used here (Forlano *et al.*, 2011; Marchaterre *et al.*, 1989; Timothy *et al.*, 2015), we report serotonergic input throughout the vocal CPG. Serotonergic neurons within the VMN clearly provide one source of serotonergic innervation to the VMN. Potentially, these serotonergic neurons could also innervate adjacent VPN neurons and the more rostral VPP population; the dense serotonergic input to VPN, in particular, makes it impossible to assess this here. Another potential source of serotonergic input to the vocal CPG are serotonergic neurons within the inferior raphe nucleus that have projections throughout the brainstem of fish (e.g., Corio *et al.*, 1991; Grant *et al.*, 1989; Kah and Chambolle, 1983).

Serotonergic input to all three vocal CPG nuclei suggests that serotonin can modulate one or more of the three main vocal parameters - amplitude, frequency and duration, all of which remains to be tested using electrophysiology and behavioral assays. Serotonin is known to initiate vocal motor patterns in an isolated brain preparation of *Xenopus laevis* (Rhodes *et al.*, 2007). In contrast, systemically administered serotonin agonists terminate territorial calling in the Puerto Rican coquí frog, *Eleutherodactylus coqui* (Ten Eyck, 2008). While the influence of serotonin on vocalization could be species-dependent, these contrasting effects of serotonin

in frogs might reflect methodological differences using bath application of serotonin in an isolated brain preparation versus a systemic injection in intact animals.

Serotonergic projections to vocal populations have also been observed for cat respiratory motor neurons involved in controlling airflow for vocalization (Holtman, 1988) as well as to the nucleus of the arcopallium in zebra finch (Wood *et al.*, 2011). Similarly, *X. laevis* laryngeal premotor and motor neurons receive serotonergic input (Yu and Yamaguchi, 2010). Thus, serotoninergic input to vocal motor areas appears to be a conserved pattern among vocal vertebrates.

4.2.2 Catecholamines. In accordance with previous studies in midshipman fish using different antibodies (Forlano *et al.*, 2014; Goebrecht *et al.*, 2014), we show dense catecholaminergic input to the vocal CPG in Gulf toadfish. Like midshipman fish (Forlano *et al.*, 2014), neurons located dorsolateral to the VMN and within the area postrema likely provide catecholaminergic input to VMN neurons in Gulf toadfish. Additional catecholaminergic inputs from other neurons within the central nervous system, e.g., spinal projecting dopaminergic neurons in the diencephalon as suggested for midshipman fish (Forlano *et al.*, 2014), can, however, not be excluded.

Catecholamines might alter vocal production in toadfishes at forebrain levels such as the anterior hypothalamus (Forlano and Bass, 2011; Forlano *et al.*, 2014; Kittelberger *et al.*, 2006) as well as at the level of the hindbrain vocal CPG and midbrain (Heisler and Kittelberger, 2012). Consistent with the anatomical evidence for catecholaminergic input to VMN, dopamine receptor subtypes are upregulated in midshipman VMN compared to the surrounding hindbrain (Feng *et al.*, 2015). Catecholamine innervation of VMN is also denser in sneaker type II male compared to highly vocal, advertisement calling type I male midshipman fish, suggesting an inhibitory role of catecholamines (Ghahramani *et al.*, 2015b; Goebrecht *et al.*, 2014; for behavior see also Brantley & Bass, 1994). A preliminary report shows that injection of dopamine into the PAG of midshipman suppresses vocal motor output, while dopamine receptor antagonists partially block this inhibition (Heisler and Kittelberger, 2012). Similarly,

dopamine-like receptor activity negatively influences advertisement calling in green tree frogs, *Hyla cinerea,* after intraperitoneal injection of dopamine agonists (Creighton *et al.*, 2013). Noradrenaline reduces activity in a brain slice preparation of the robust nucleus of the arcopallium, the premotor song control nucleus in songbirds, after bath application (Solis and Perkel, 2006). Perhaps contrary to this general inhibitory role for catecholamines, catecholaminergic innervation of vocal areas is more abundant in singing male canaries, *Serinus canaria*, compared to non-singing females (Appeltants *et al.*, 2003).

4.2.3 Acetylcholine. The pattern of ChAT label observed for the Gulf toadfish matches that previously shown for the VMN of midshipman fish using a different antibody (Brantley and Bass, 1988). Like serotonin and catecholamines, there is cholinergic input to VPN and VPP. While injections of cholinergic antagonists into vocal midbrain sites of squirrel monkeys, *Saimiri sciureus* show no effect on vocal production (Jürgens and Lu, 1993), *in vivo* and *in vitro* application of acetylcholine and its agonists to respiratory pacemaker-like neurons of the pre-Bötzinger complex show increased duration, frequency and amplitude of spontaneous inspiratory bursts (Burton *et al.*, 1997; Monteau *et al.*, 1990; Murakoshi *et al.*, 1985; Shao and Feldman, 2000). Perhaps acetylcholine plays a more widespread role in enhancing premotor activity in hindbrain pattern generators, including the vocal CPG of toadfish.

4.4 Vocal CPG projections to brainstem areas

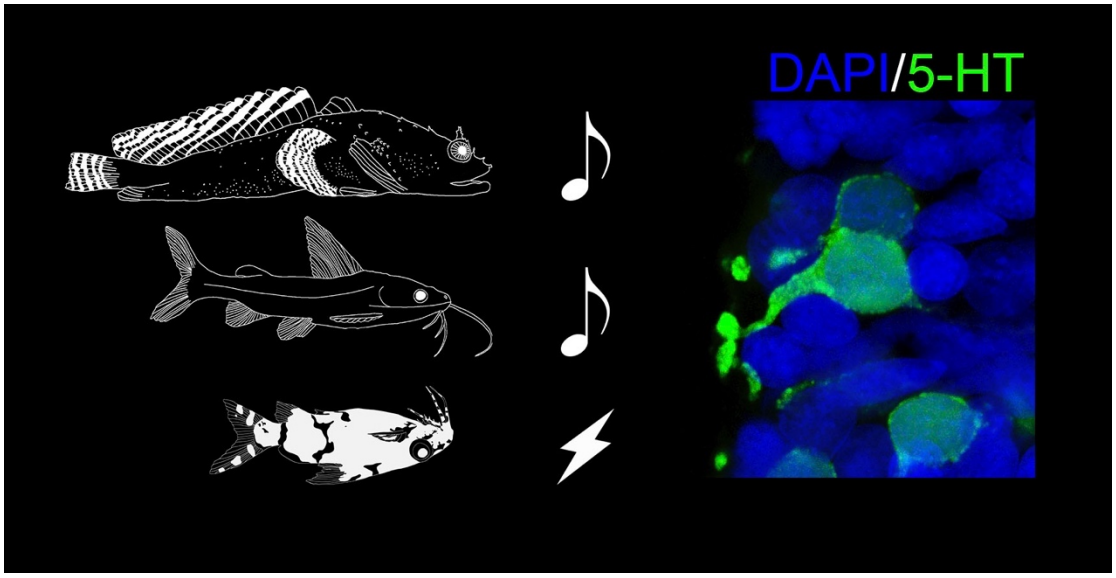
Like previous studies in midshipman fish and Gulf toadfish (Bass *et al.*, 1994; Chagnaud and Bass, 2014; Weeg *et al.*, 2005), the vocal CPG is connected to auditory hindbrain neurons with evidence for nearby glycinergic neurons. Although previous electrophysiological experiments reveal that inhibition is important in determining vocal CPG output (earlier section), it is also known to play an important role in brainstem mechanisms of vocal-acoustic integration in mammals (Smotherman, 2007). Unexpectedly, neurobiotin-labeled cells are also located in the iRF in close proximity to glycinergic neurons. After carefully revisiting the material used by Chagnaud and Bass (2014), neurobiotin-labeled iRF neurons

are also observed in that material, although the iRF labeling in that study was quite weak compared to the robust labeling that we observe here and so was easily missed. GABAergic neurons are also found in the iRF of the African cichlid fish, *Astatotilapia burtoni*, that is also sonic (Maruska *et al.*, 2017). Studies of squirrel monkeys demonstrate the reticular formation's involvement in vocal production (Jürgens and Hage, 2007). The PAG and other vocal midbrain sites in midshipman are also connected to the reticular formation (Goodson and Bass, 2002; Kittelberger and Bass, 2013). While suggestive, the involvement of the reticular formation in vocal patterning or production in toadfishes awaits neurophysiological investigation.

Concluding remarks

In this study, we show prominent inhibitory and neuromodulatory input at all levels of the toadfish vocal CPG that suggest a suite of neurophysiological mechanisms to achieve a variety of motor programs (Gjorgjieva *et al.*, 2016; Harris-Warrick and Marder, 1991), with a single set of topographically separate nuclei – VMN, VPN, VPP - resulting in context-dependent vocal signals. The presence of inhibitory transmitters such as GABA and glycine, along with gap junction coupling within each of the CPG nuclei, likely contribute to determining two predominant features of the vocal CPG – extreme temporal precision and synchrony (Chagnaud *et al.*, 2012). How serotonin, catecholamines and acetylcholine interact with these transmitters to shape vocal production remains to be shown. The proposed evolutionarily conserved organization of vocal CPGs (Bass *et al.*, 2008; Bass *et al.*, 1994), together with the available neurophysiological evidence in toadfishes and other sonic species of vertebrates, suggests that comparable neurochemically-dependent mechanisms are present in other vertebrate vocal CPGs.

3 MANUSCRIPT 2



Serotonin systems in three socially communicating teleost species, the grunting toadfish (*Allenbatrachus grunniens*), a South American marine catfish (*Ariopsis seemanni*), and the upside-down catfish (*Synodontis nigriventris*)

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Author contributions

Contribution of E.R.

- Design of study: chose antibody against serotonin and MAP2 with corresponding secondary antibodies, number of animals, developed antibody staining protocol for studied fish species
- Performed part of the perfusions of fish specimen
- Performed antibody stainings
- Took all microscope images
- Designed all figures
- Wrote and revised the manuscript

Contribution of B.P.C.

- Design of study: suggested the studied fish species and study outline, gave technical advice
- Performed part of the perfusions of fish specimen
- Revised and submitted the manuscript

Contribution of M.F.W.

- Gave extensive advice on anatomical determination of brain structures
- Suggested antibody against tyrosine hydroxylase to determine character of PVO neurons in *S. nigriventris*
- Wrote and revised the manuscript

M.F.W. and B.P.C. supervised the study.

Abstract

We investigated immunohistochemically the distribution of serotonergic cell populations in three teleost species (one toadfish, *Allenbatrachus grunniens*, and two catfishes, *Synodontis nigriventris* and *Ariopsis seemanni*). All three species exhibited large populations of 5-HT positive neurons in the paraventricular organ (PVO) and the dorsal (Hd) and caudal (Hc) periventricular hypothalamic zones, plus a smaller one in the periventricular pretectum, a few cells in the pineal stalk, and – only in catfishes – in the preoptic region. Furthermore, the rhombencephalic superior and inferior raphe always contained ample serotonergic cells. In each species, a neuronal mass extended into the hypothalamic lateral recess. Only in the toadfish did this intraventricular structure contain serotonergic cells and arise from Hd, whereas in the catfishes it emerged from medially and represents the dorsal tuberal nucleus seen in other catfishes as well. Serotonergic cells in PVO, Hd and Hc were liquor-contacting. Those of the PVO extended into the midline area of the periventricular posterior tubercular nucleus in both catfishes. Dopaminergic, liquor-contacting neurons were additionally investigated using an antibody against tyrosine hydroxylase (TH) in *S. nigriventris* showing that TH was never co-localized with serotonin. Because TH antibodies are known to reveal mostly or only the TH1 enzyme, we hypothesize that *th1*-expressing dopamine cells (unlike *th2*-expressing ones) do not co-localize with serotonin.

Since the three investigated species engage in social communication using swim bladder associated musculature, we investigated the serotonergic innervation of the hindbrain vocal or electromotor nuclei initiating the social signal. We found in all three species serotonergic fibers seemingly originating from close-by serotonergic neurons of inferior raphe or anterior spinal cord. Minor differences appear to be rather species-specific than dependent on the type of social communication.

1 Introduction

Sound or vocal production for social communication is a common feature across animals, from invertebrates to vertebrates. While humans readily perceive acoustic communication on land, for example that of songbirds (e.g., Hoffmann *et al.*, 2019) or of insects (e.g., Michelson and Nocke, 1974) and in the aquatic medium e.g. the sounds of whales (Ladich and Winkler, 2017), the fact that many teleost fish produce sounds for social communication (Ladich and Winkler, 2017) remains somewhat more inconspicuous. Vocal fishes occur in various taxa, including species as diverse as mormyrids/elephantfishes (e.g., Crawford, 1992), catfishes (e.g., Pruzsinszky and Ladich, 1998), piranhas (e.g., Melotte *et al.*, 2016), sea-robins (e.g., Fish and Mowbray, 1970) and toadfishes (e.g., Maruska and Mensinger, 2009; Fig. 1; for more details see below).

Swim bladder vibrations represent one of various mechanisms how ray-finned fish produce vocalizations (reviewed in Amorim, 2006; Bass *et al.*, 2015; Ladich and Winkler, 2017). The underlying neuronal control of these swim bladder sounds have been well studied in the family Batrachoididae including toadfishes and midshipman (reviewed in Bass and Ladich, 2008). In batrachoidids, the swim bladder musculature produces swim bladder vibrations via repetitive muscle contractions (Cohen and Winn, 1967). This musculature is innervated by motor neurons of the vocal motor nucleus (VMN) located in the ventral caudal hindbrain as part of the vocal central pattern generator in toadfish (Bass, 1985; Bass and Marchaterre, 1989; Bass *et al.*, 1994; Chagnaud and Bass, 2014). Recently, studies on inhibitory and modulatory neurotransmitters involved within the VMN of the Gulf toadfish, *Opsanus beta*, revealed the complexity of the premotor circuitry within the vocal central pattern generator. Among others, serotonin (5-hydroxytryptamine, 5-HT) was investigated and sparse serotonergic neurons were unexpectedly observed within VMN (Rosner *et al.*, 2018), as was previously reported in the VMN of another batrachoidid fish, the oyster toadfish, *Opsanus tau*, (Marchaterre *et al.*, 1989). Typically in ray-finned fish, serotonin-containing cells are found in the pretectum, the paraventricular organ (PVO), the periventricular hypothalamus, the pineal organ and the superior and inferior raphe located in the caudal rhombencephalon, and some

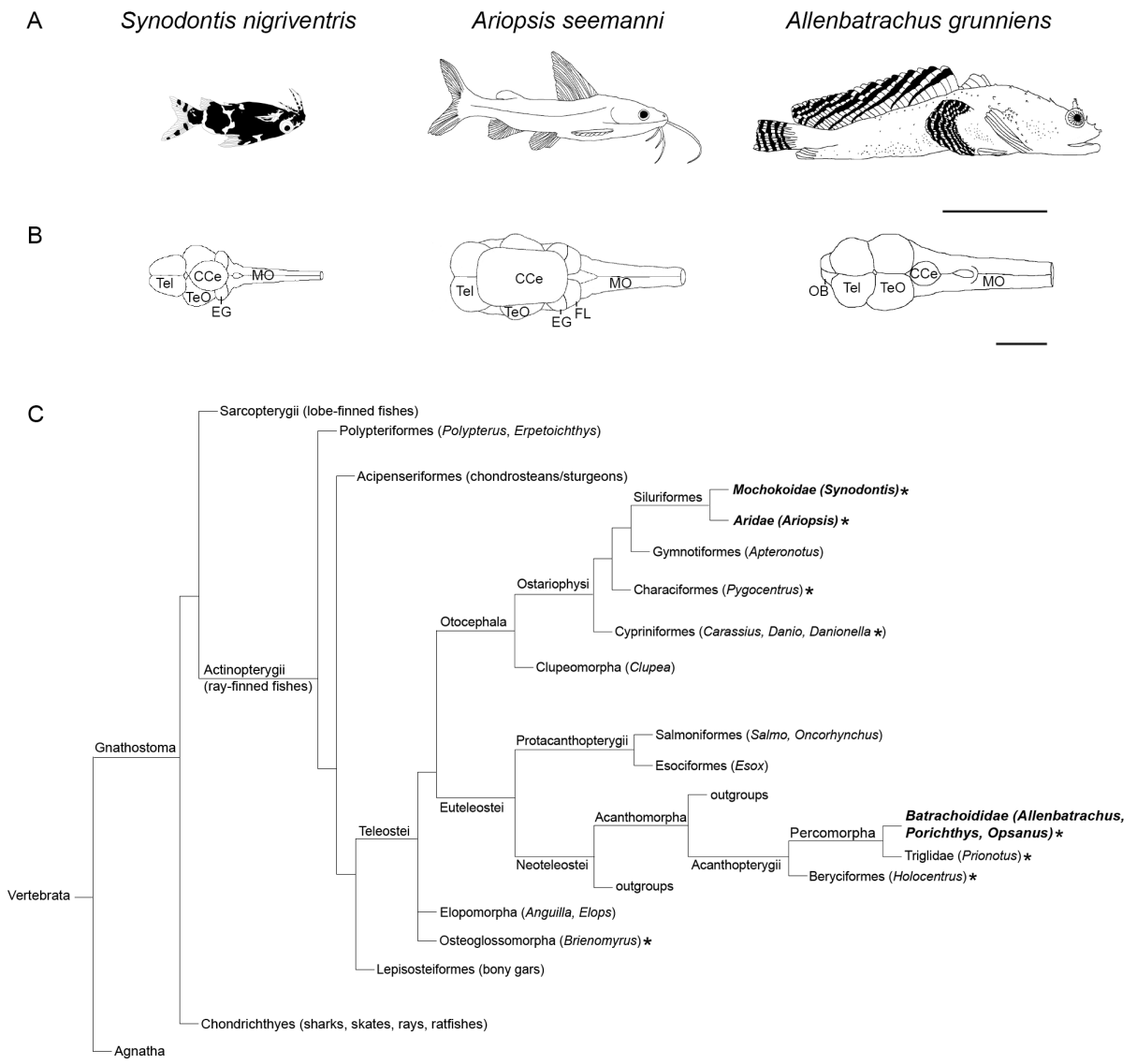

FIGURE 1: BODY AND BRAIN OUTLINES OF STUDY SPECIES *SYNODONTIS NIGRIVENTRIS*, *ARIOPSIS SEEMANNI* AND *ALLENBATRACHUS GRUNNIENS* AND SIMPLIFIED CLADOGRAM DISPLAYING THE RELATIONSHIP BETWEEN THE STUDIED SPECIES WITHIN GNATHOSTOMES.

(A) Ink drawings of the three studied teleost species. S. nigriventris is shown in its usual up-side down posture during swimming. (B) Schematic drawings of the corresponding brains viewed from dorsal. (C) Simplified cladogram depicting the phylogenetic relationship between the studied species within teleosts (systematics based on Hughes et al. 2018, Diogo et al. 2008 and Near et al., 2012) and in the wider context of vertebrates. Note that clades with an asterisk include species where swim bladder dependent vocal signaling has been described. Scale bar is 5 cm in (A), 0.2 cm in (B). CCe corpus cerebelli, EG eminentia granularis, FL facial lobe, MO medulla oblongata, OB, olfactory bulb, Tel telencephalon, TeO tectum opticum.

species show additional serotonergic neurons in the olfactory bulb, sparsely in other parts of the telencephalon and in the pre-optic area (reviewed in Lillesaar, 2011). Although, the observed serotonergic neurons within the VMN in the genus *Opsanus* were unexpected, these

neurons most likely are misplaced inferior raphe neurons, as this is the only serotonergic population commonly described in this area. Nevertheless, this observation raised the question whether serotonergic neurons among the hindbrain motor neurons innervating the swim bladder associated muscles are a unique feature for the genus *Opsanus* within toadfishes or a common pattern among fish using their swim bladder for social communication.

To resolve this question, we investigated the serotonergic populations in the brain of three teleost fish species that socially communicate with swim bladder signals. We chose the grunting toadfish, *Allenbatrachus grunniens*, because it represents a different genus to the formerly studied toadfish species. We further investigated two catfish species which are remotely related to toadfishes, the South American marine catfish, *Ariopsis seemanni*, and the upside-down catfish, *Synodontis nigriventris*, to resolve whether serotonergic neurons in the involved motor neurons occur outside of the genus *Opsanus* and toadfishes. All three species use their swim bladder for social communication, the first two with vocal signals, the third one with electric signaling (Boyle *et al.*, 2014). Misplaced inferior raphe neurons were found in VMN of *A. grunniens* but no 5-HT containing cells were present in the swim bladder musculature innervating nuclei in both catfishes, probably making this a toadfish specific feature. However, anterior spinal 5-HT neurons were seen close to the swim bladder innervating neurons in both catfishes. The observed differences in serotonergic innervation and presence of serotonergic neurons within the swim bladder musculature innervating nuclei are minor and appear to be rather species-specific than dependent on the type of social communication. Other serotonin-containing brain populations found in the studied three teleost species are generally in line with previous publications on serotonergic populations in fish (reviewed in Lillesaar, 2011; see Discussion).

2 Material and Methods

2.1 Animals

In this study, we investigated the serotonergic system in the brain of three teleost fish species: two catfishes, the blotched upside-down catfish, *S. nigriventris*, and the Columbian shark catfish, *A. seemanni*, as well as a toadfish species, the grunting toadfish, *A. grunniens*. As the common name suggests, adult nocturnal *S. nigriventris* (body outline and brain Fig. 1A and 1B left), native to the Congo Basin of Cameroon, usually swim upside-down but rests upside-up at the bottom or at objects (Ohnishi *et al.*, 1996). The second studied catfish, *A. seemanni* (body outline and brain Fig. 1A and 1B middle), is a South American marine catfish, that lives in schools in the brackish water of river mouths emptying into the Pacific Ocean (Schmidtke *et al.*, 2013). The studied toadfish, *A. grunniens* (body outline and brain Fig. 1A and 1B right), is a coastal species of the Indo-West Pacific and usually hovers close to the ground or hides under rocks.

All three fish species are teleosts (simplified cladogram in Fig. 1C; based on Diogo *et al.*, 2008; Hughes *et al.*, 2018b; Near *et al.*, 2012b). Both catfish species belong to the order Siluriformes in the superorder Ostariophysi and the clade Otocephala. Within the Siluriformes, *S. nigriventris* is a member of the family Mochokoidae, while *A. seemanni* belongs to the family Aridae. The grunting toadfish is part of the clade Euteleostei and the superorder Acanthopterygii. Within the Acanthopterygii, *A. grunniens* belongs to the family Batrachoididae nested in the taxon Percomorpha.

As well known for members of the family Batrachoididae, *A. grunniens* contracts its muscles associated to the swim bladder to produce sounds for communication with conspecifics. Both studied siluriform catfishes also socially communicate with conspecifics. While *A. seemanni* also makes swim bladder sounds (Schmidtke *et al.*, 2013), *S. nigriventris* generates weakly electric signals with its swim bladder associated muscles and additionally generates sounds via pectoral fin movement (Parmentier *et al.*, 2010). Fish taxa included in the cladogram in Fig. 1C which contain species that are known to socially communicate using

swim bladder musculature are indicated with an asterisk. Except for catfishes and toadfishes (see discussion), these are osteoglossomorphs, like the small elephantfish *Brienomyrus spec*. (Bass, 1985; Ladich and Bass, 1998), the characiform red-bellied piranha, *Pygocentrus nattereri* (Ladich and Myrberg, 2006; Millot *et al.*, 2011)), the beryciform squirrelfish (Bass, 1985) and percomorph sea robins, *Prionotus evolans* and *carolinus,* (Bass, 1985; Bass *et al.*, 1986; Ladich and Bass, 1998) and possibly the dracula fish, *Danionella Dracula*, (Britz and Conway, 2015).

Specimens of the three studied teleost fish species were purchased from a commercial fish distributor (EFS, Sonnefeld, Germany) and were housed at the LMU Biocenter at 25-27°C in an environmental control room on a 12:12 dark:light cycle.

2.2 Tissue collection

To preserve the brains of adults of undetermined age and sex for anatomical processing (*S. nigriventris*: 2, *A. seemanni*: 2, *A. grunniens*: 3), fish were first deeply anaesthetized by immersion in aquarium water with benzocaine (0.025%; Sigma Aldrich, Taufkirchen, Germany) and then transcardially perfused with 4% paraformaldehyde (PFA; Carl Roth GmbH, Karlsruhe, Germany) in 0.1M phosphate buffer (PB; pH 7,4) containing KH_2PO_4 (20 mM) and Na_2HPO_4 x 2 H_2O (80 mM; all: Carl Roth GmbH, Karlsruhe, Germany). Brains were post-fixed in the same solution for 1 h at 4°C and stored in 0.1M PB at 4°C until further processing. Collection of tissues was conducted in accordance with the *Guide for care and use of laboratory animals* (2011) of the National Institute of Health and the EU Directive 2010/63/EU for animal experiments.

2.3. Immunohistochemistry (IHC)

Immunohistochemical processing followed the protocol published in Rosner et al. (2018) with the following modifications: Continuous series of brain sections for two specimens of each studied species were generated in transverse plane with a cryostat (Leica microsystems, Wetzlar, Germany) at 25 µm to 40 µm. For a third specimen of *A. grunniens,*

four compartments throughout this brain were collected of which one was stained. One of these brain series was used in this study. Brain sections collected from the studied species were incubated with an antibody against serotonin (5-HT) to assess the distribution of serotonergic cells along with a fluorescent mounting medium containing 4',6-Diamidino-2-Phenylindole (DAPI; Vectashield with DAPI, Vector Labs Inc., Peterborough, United Kingdom), a nuclear stain that visualizes gross anatomical organization. In one specimen of each species, we additionally used an antibody against microtubule associated protein 2 (MAP2) to visualize neuronal soma structure. In one *S. nigriventris* specimen, we additionally stained with an antibody against tyrosine hydroxylase (TH) as a marker for catecholamines (see results and discussion) to assess the distribution of dopaminergic neurons at the level of the paraventricular organ. Details on primary and secondary antibodies used are listed in Table 1.

Nonspecific binding of all primary and secondary antibodies to tissue of each studied species was tested by performing our staining protocol but omitting the secondary or primary antibody, respectively. All tests revealed no staining.

2.4. Image editing

Images were acquired with an epifluorescence microscope (ECLIPSE Ni, Nikon GmbH, Düsseldorf, Germany) with a Nikon Digital Sight DSU1 Photomicrographic Camera (Nikon Instruments Inc.) and NIS-Elements F4.60.00 software. The microscope was equipped with Nikon Plan UW 0.06 (2x), Plan Fluor 109/0.30 (10x) and Plan Fluor 209/.0.50 (20x) objectives. Additionally, a confocal laser-scanning microscope Leica TCS SP-5 (Leica microsystems, Wetzlar, Germany) was used. Epifluorescence images were used to create overviews of the brain sections in which serotonergic cells were observed and to document the serotonergic populations. For the overviews, one half (right side of panels shown in left columns of respective figures) of the brain section shows the DAPI channel of the epifluorescence microscope image displaying cell nuclei for gross anatomical organization. The other half (left side of panels shown in left columns of respective figures) is a stylized drawing of the DAPI side of the respective brain section in Adobe Photoshop (CS6; Adobe Systems Software

Ireland Limited, Dublin, Ireland). The DAPI images were converted to high-contrast black and white pictures and stitched together if more than one image had to be photographed to display the whole brain section. Moderate corrections for artefacts, e.g., small dust grains, were performed on DAPI images if necessary. In some cases, the contralateral part of the section was taken for the DAPI documentation.

Confocal images were used to show close-ups of serotonergic neurons and to investigate co-labelling of 5-HT and TH. Confocal images were stacked and converted to maximum z-projections using ImageJ (Schneider *et al.*, 2012). Maximum z-projections were cropped and resized and contrast and brightness were optimized for the whole image using Adobe Photoshop.

TABLE 1

List of primary and secondary antibodies used in this study.

Antibody	Catalogue number	Host	Clonality	Dilution	Supplier
Primary antibodies					
MAP2	CH22103	Chicken	monoclonal	1:1000	Neuromics
Serotonin	20080	Rabbit	unknown	1:500	Immunostar
Tyrosine hydroxylase 2	MAB318	Mouse	monoclonal	1:200	Millipore
Secondary antibodies					
Donkey F(ab')2 anti-chicken IgY (H+L)-Cy3	703-166-155	Donkey	polyclonal	1:200	Dianova
Donkey F(ab')2 anti-mouse IgG (H+L)-Cy3	715-116-151	Donkey	polyclonal	1:200	Dianova
Donkey Anti-Rabbit IgG (H+L) Antibody, Alexa Fluor 488 Conjugated	A-21206	Donkey	unknown	1:200	Molecular Probes

The table includes the name, catalogue number, host, clonality, dilution and supplier of antibodies used in this study.

3 Results

We refer to teleostean brain nuclei and neural structures in this study based on the nomenclature of Braford and Northcutt (1983) as adapted for zebrafish by Wullimann et al. (1996), with some modifications explained in Baeuml et al. (2019). For group specific terms, Striedter (1990; catfish) and Karoubi et al. (2016; acanthopterygii) were additionally consulted.

In the following, we present a description of the serotonergic systems in each of the three studied teleost species from preoptic region to hindbrain.

3.1 Anterior parvocellular preoptic nucleus (PPa)

Serotonergic neurons were present in the PPa below the anterior commissure in both catfish species, *S. nigriventris* (Fig. 2B) and *A. seemanni* (Fig. 3B). While few serotonergic neurons were found in *S. nigriventris* (Fig. 2E, I, M*)*, serotonergic neurons were more numerous in *A. seemanni* (Fig. 3E, I, M*)*. We observed no serotonergic neurons in the PPa of *A. grunniens*.

3.2 Pineal stalk (PS)

Caudal of the PPa in the diencephalon, the next serotonin (5-HT) positive neurons were present in the PS which is positioned dorsal to the prethalamus in all three studied fish species. 5-HT positive neurons in PS were observed dorsal to the habenula in both catfishes (*S. nigriventris* Fig 2C, *A. seemanni* Fig. 3C) and ventral to the habenula at the origin of PS in *A. grunniens* (Fig. 4B). These 5-HT positive neurons were numerous in *S. nigriventris* (Fig. 2F, J, N) and *A. seemanni* (Fig. 3F, J, N). Fewer serotonergic neurons were present in *A. grunniens* (Fig. 4E, F, H).

3.3 Periventricular pretectum (PPr)

Further caudal in the diencephalon, we observed serotonergic neurons in the PPr of all three studied fish species (*S. nigriventris* Fig. 2D, *A. seemanni* Fig. 3D, *A. grunniens* Fig. 8B).

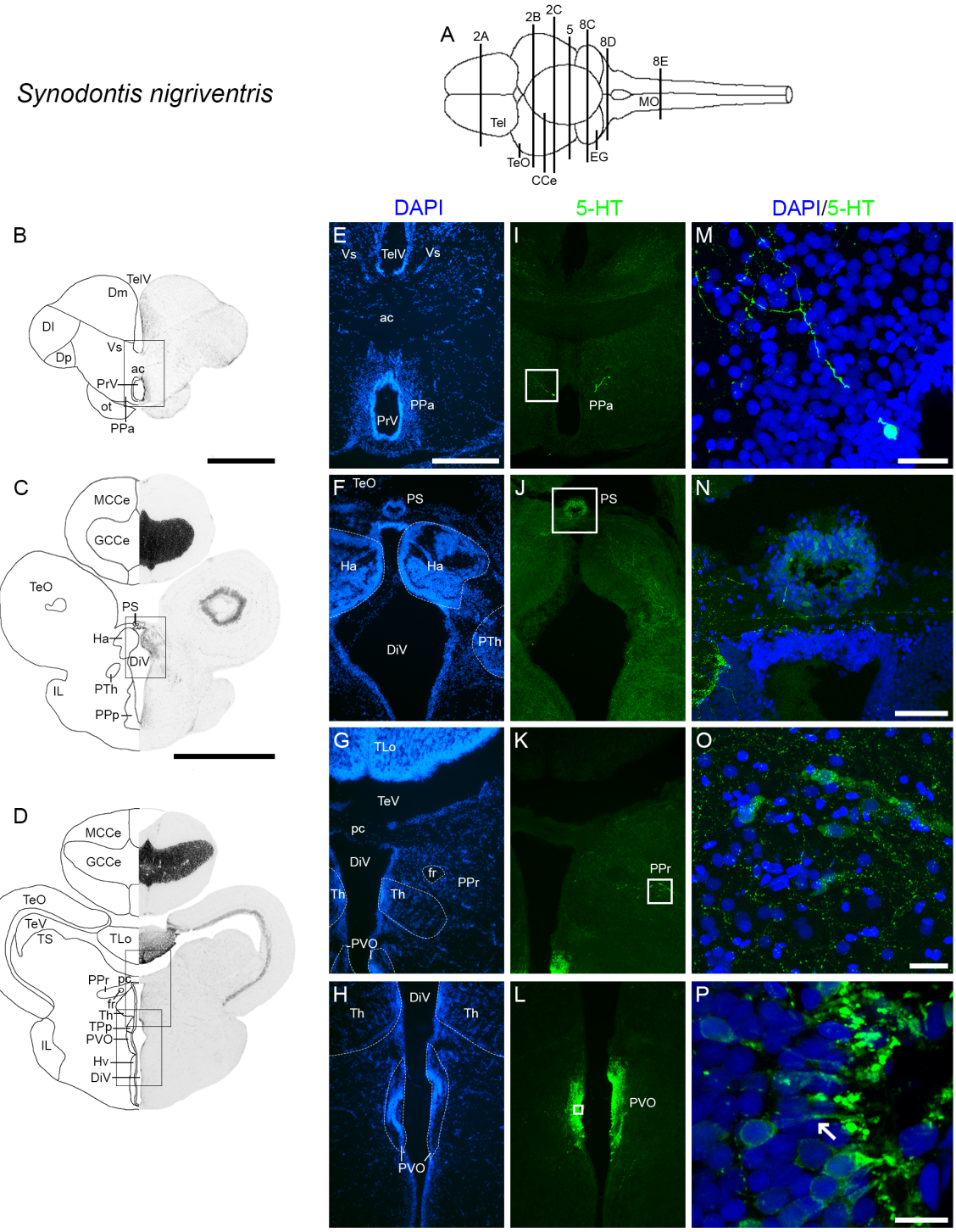

FIGURE 2: SEROTONERGIC CELL POPULATIONS IN THE ANTERIOR PARVOCELLULAR PREOPTIC NUCLEUS (PPA), THE PINEAL STALK (PS), THE PERIVENTRICULAR PRETECTUM (PPR) AND PARAVENTRICULAR ORGAN (PVO) IN SYNODONTIS NIGRIVENTRIS.

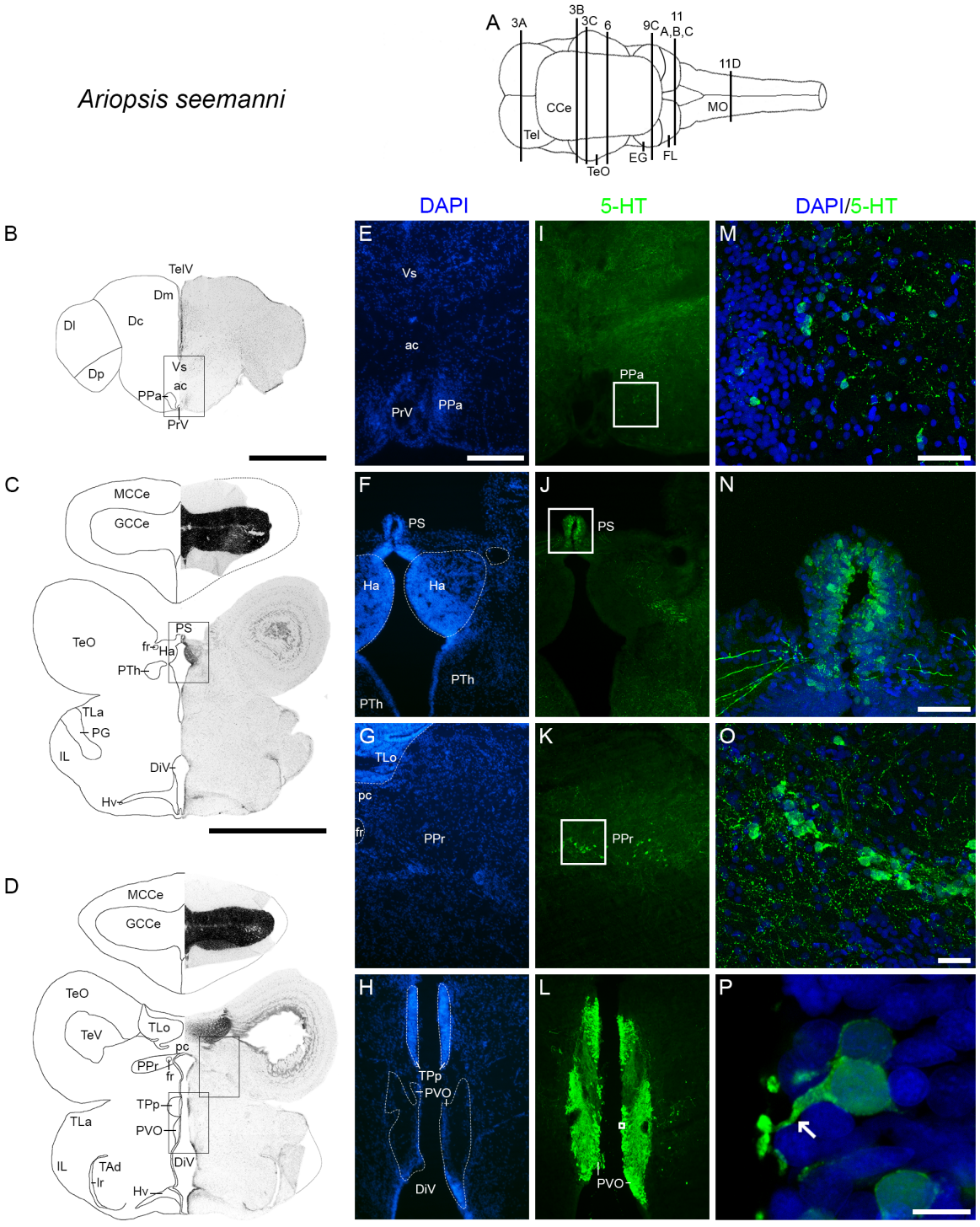

FIGURE 3: SEROTONERGIC CELL POPULATIONS IN THE ANTERIOR PARVOCELLULAR PREOPTIC NUCLEUS (PPA), THE PINEAL STALK (PS), THE PERIVENTRICULAR PRETECTUM (PPR) AND PARAVENTRICULAR ORGAN (PVO) IN *ARIOPSIS SEEMANNI.*

FIGURE 2: SEROTONERGIC CELL POPULATIONS IN THE ANTERIOR PARVOCALLULAR PREOPTIC NUCLEUS (PPA), THE PINEAL STALK (PS), THE PREIVENTRICULAR PRETECTUM (PPR) AND THE PARAVENTRICULAR ORGAN IN *SYNODONTIS NIGRIVENTRIS*.

(A) Schematic drawing of the dorsal view of brain of *Synodontis nigriventris*. Vertical lines indicate representative levels with corresponding figure number for each serotonergic population shown in plates. Left column: Transverse brain sections show line drawings (left side of B-D) and anatomical organization with DAPI (right side of B-D) illustrating the location of serotonergic neurons in PPa (B), PS (C), PPr and PVO (D). Middle columns (E-L): Epifluorescence images of the framed rectangles indicated in B-D show DAPI (blue, E-H) and serotonin (5-HT, green, I-L) stains. Right column (M-P): Confocal images show magnifications of serotonergic neurons. The small rectangles in I-L indicate the position of neurons shown in the magnifications. The arrow in P indicates a neuronal process contacting the liquor in the diencephalic ventricle. Scale bar is 500 µm in B, 500, µm in C for C-D, 100 µm in E for E-L, 20 µm in M and O, 50 µm in N and 10 µm in P. Abbreviations: ac anterior commissure, CCe corpus cerebelli, Dc/Dl/Dm/Dp central/lateral/medial/posterior zone of the dorsal telencephalon, DiV diencephalic ventricle, EG eminentia granularis, FL facial lobe, fr fasciculus retroflexus, GCCe granular cell layer of the corpus cerebelli, Ha habenula, Hv ventral zone of periventricular hypothalamus, IL inferior lobe of hypothalamus, lr lateral hypothalamic recess, MCCe molecular layer of the corpus cerebelli, MO medulla oblongata, pc posterior commissure, PPa/PPp, anterior/posterior parvocellular preoptic nucleus, PPr periventricular pretectum, PrV preoptic ventricle, PS pineal stalk, PTh prethalamus, PVO paraventricular organ, TeO tectum opticum, Tel telencephalon, TeV tectal ventricle, TelV telencephalic ventricle, TLa torus lateralis, TLo torus longitudinalis, TPp periventricular nucleus of posterior tuberculum, TS torus semicircularis, Vs supracommissural nucleus of the ventral telencephalon.

FIGURE 3: SEROTONERGIC CELL POPULATIONS IN THE ANTERIOR PARVOCALLULAR PREOPTIC NUCLEUS (PPA), THE PINEAL STALK (PS), THE PERIVENTRICULAR PRETECTUM (PPR) AND THE PARAVENTRICULAR ORGAN IN *ARIOPSIS SEEMANNI.*

(A) Schematic drawing of the dorsal view of brain of *Ariopsis seemanni*. Vertical lines indicate representative levels with corresponding figure number for each serotonergic population shown in plates. Left column: Transverse brain sections show line drawings (left side of B-D) and anatomical organization with DAPI (right side of B-D) illustrating the location of serotonergic neurons in PPa (B), PS (C) and PPr and PVO (D). Middle columns (E-L): Epifluorescence images of the framed rectangles indicated in B-D show DAPI (blue, E-H) and serotonin (5-HT, green, I-L) stains. Right column (M-P): Confocal images show magnifications of serotonergic neurons. The small rectangles in I-L indicate the position of neurons shown in the magnifications. The arrow in P indicates a neuronal process contacting the liquor in the diencephalic ventricle. Scale bar is 500 µm in B, 500 µm in C for C-D, 100 µm in E for E-L, 50 µm in M and N and 10 µm in O and P. Abbreviations: ac anterior commissure, CCe corpus cerebelli, Dc/Dl/Dm/Dp central/lateral/medial/posterior zone of the dorsal telencephalon, DiV diencephalic ventricle, EG eminentia granularis, FL facial lobe, fr fasciculus retroflexus, GCCe granular cell layer of the cerebellum, Hd/Hv dorsal/ventral zone of periventricular hypothalamus, Ha habenula, IL inferior lobe of hypothalamus, lr lateral hypothalamic recess, MCCe molecular layer of the corpus cerebelli, MO medulla oblongata, pc posterior commissure, PG preglomerular complex, PPa anterior parvocellular preoptic nucleus, PPr periventricular pretectum, PrV preoptic ventricle, PS pineal stalk, PTh prethalamus, PVO paraventricular organ, TAd dorsal anterior tuberal nucleus (Striedter, 1990), Tel telencephalon, TeO tectum opticum, TeV tectal ventricle, TelV telencephalic ventricle, TLa torus lateralis, TLo torus longitudinalis, TPp periventricular nucleus of posterior tuberculum, TS torus semicircularis, Vs supracommissural nucleus of the ventral telencephalon.

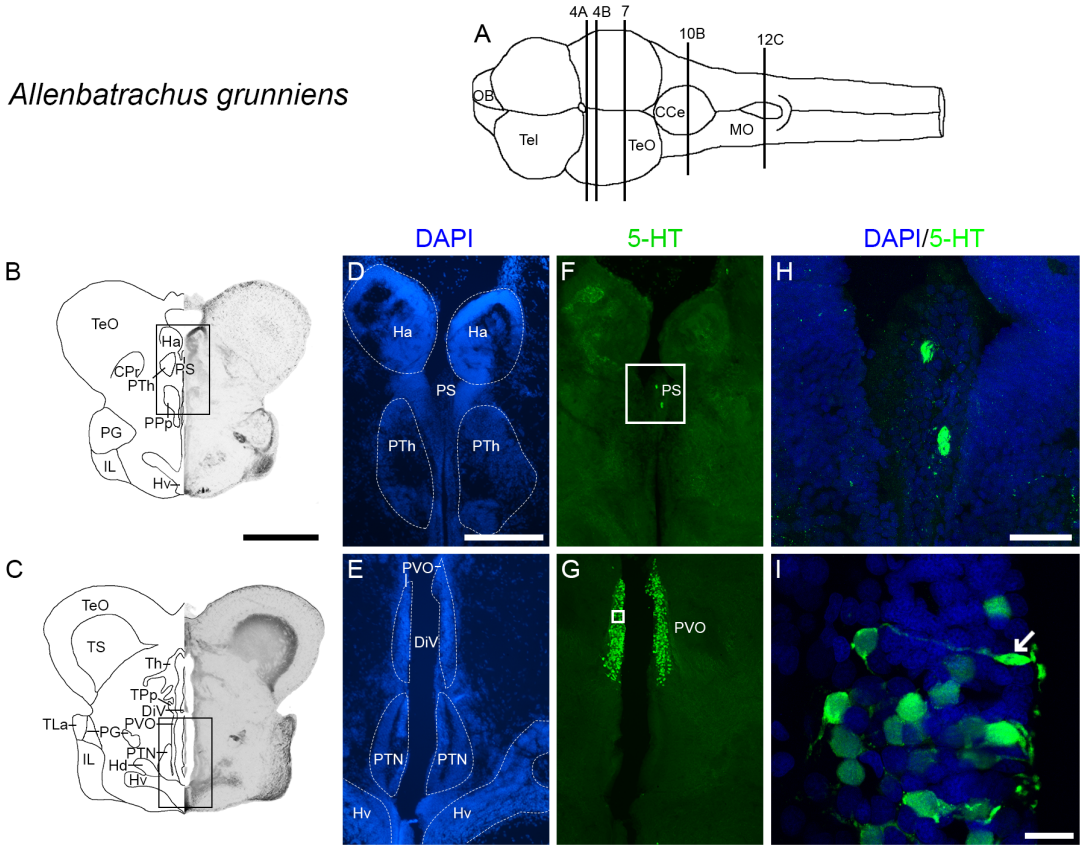

FIGURE 4: SEROTONERGIC CELL POPULATIONS IN THE PINEAL STALK (PS) AND PARAVENTRICULAR ORGAN (PVO) IN *ALLENBATRACHUS GRUNNIENS*. (A) SCHEMATIC DRAWING OF THE DORSAL VIEW OF BRAIN OF *ALLENBATRACHUS GRUNNIENS*.

Vertical lines indicate representative levels with corresponding figure number for each serotonergic population shown in plates. Left column: Transverse brain sections show line drawings (left side of B-C) and anatomical organization with DAPI (right side of B-C) illustrating the location of serotonergic neurons in PS (B) and PVO (C). Middle columns (D-G): Epifluorescence images of the framed rectangles indicated in A and B show DAPI (blue, D, E) and serotonin (5-HT, green, F, G) stains. Right column (H-I): Confocal images show magnifications of serotonergic neurons. The small rectangles in F and G indicate the position of the neurons shown in the magnifications. The arrow in I indicates a neuronal process contacting the liquor in the diencephalic ventricle. Scale bar is 500 µm in B for B-C, 100 µm in D for D-G, 50 µm in H and 10 µm in I. Abbreviations: CCe corpus cerebelli, CPr central pretectal region, DiV diencephalic ventricle, Hv ventral zone of periventricular hypothalamus, Ha habenula, IL inferior lobe of hypothalamus, lr lateral hypothalamic recess, pc posterior commissure, OB olfactory bulb, PG preglomerular complex, MO medulla oblongata, PS pineal stalk, PTh prethalamus, PTN posterior tuberal nucleus, PVO paraventricular organ, SV saccus vasculosus, TeO tectum opticum, TeV tectal ventricle, TLa torus lateralis, TLo torus longitudinalis, TPp periventricular nucleus of posterior tuberculum, TS torus semicircularis.

The PPr was located lateral to the posterior commissure and the fasciculus retroflexus and dorsal to the thalamus in all studied species (*S. nigriventris* Fig. 2G, K, O; *A. seemanni* Fig.

3G, K, O; *A. grunniens* Fig. 8F, J, N). Due to the ventral bending of the anteroposterior brain axis, the PPr, although being part of the most posterior diencephalon, is in all three species seen at section levels together with the hypothalamus. The hypothalamus constitutes, together with the telencephalon dorsal to it, the most anterior brain division.

3.4 Paraventricular organ (PVO)

The PVO, located in the posterior tuberculum, contained serotonergic neurons in all three studied fish species (*S. nigriventris* Fig. 2D, *A. seemanni* Fig. 3D, *A. grunniens* Fig. 4C). The neurons of the PVO were positioned directly adjacent bilaterally of the diencephalic ventricle (*S. nigriventris* Fig. 2H, L; *A. seemanni* Fig. 3H, L; *A. grunniens* Fig. 4E, G). They were bipolar cerebrospinal-fluid (CSF) -contacting neurons with one process contacting the diencephalic liquor cerebrospinalis (called liquor or CSF in the following; arrow in Fig. 2P (*S. nigriventris*), Fig. 3P (*A. seemanni*), Fig. 4I (*A. grunniens*)).

Surprisingly, we observed in both catfish species that serotonergic neurons at the level of the PVO extended further dorsally than expected into the periventricular region of the TPp. Usually in teleost fish, the periventricular nucleus of the posterior tuberculum (TPp) is found dorsal to the PVO (see discussion). In zebrafish, for example, the TPp is characterized by its dopaminergic magno- and parvocellular neurons (Kaslin and Paula, 2001; Rink and Wullimann, 2001) and only the PVO additionally by serotonergic cells (Kaslin and Panula, 2001). Catecholaminergic neurons that are positioned rostral of the midbrain-hindbrain boundary are always dopaminergic (Ma, 1997; Moore and Bloom, 1978; Parent *et al.*, 1984; Smeets and Reiner, 1994). Therefore, we used tyrosine hydroxylase (TH) as a marker for dopamine and co-stained brain sections in *S. nigriventris* with 5-HT (Fig. 5) to clarify if the dorsal 5-HT neurons observed here are part of the TPp and if 5-HT and TH are co-localized in PVO neurons as previously described by *in situ* hybridization (Fig. 5; Xavier *et al.*, 2017). We indeed observed that the dorsal population of serotonergic neurons at anterior levels of the PVO in *S. nigriventris* extends into the anterior part of the TPp (surrounded by white lines and marked with a plus in Fig. 5A-D), while magno- and additional parvocellular dopaminergic TPp

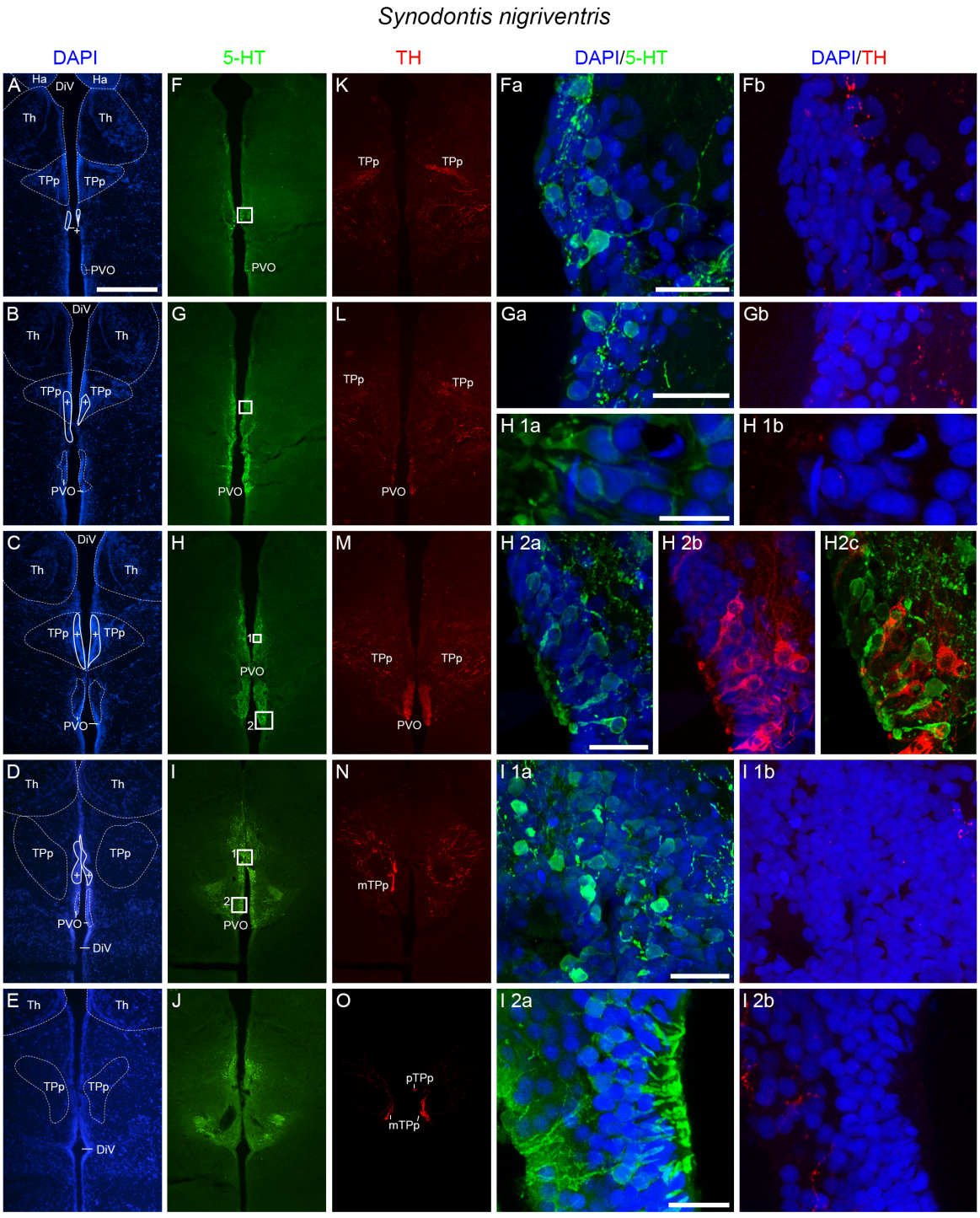

FIGURE 5: SEROTONERGIC AND DOPAMINERGIC NEURONS AT THE LEVEL OF THE PERIVENTRICULAR POSTERIOR TUBERCULUM (TPP) AND THE PARAVENTRICULAR ORGAN (PVO) IN *SYNODONTIS NIGRIVENTRIS*.

FIGURE 5: SEROTONERGIC AND DOPAMINERGIC NEURONS AT THE LEVEL OF THE PERIVENTRICULAR POSTERIOR TUBERCULUM (TPP) AND THE PARAVENTRICULAR ORGAN (PVO) IN SYNODONTIS NIGRIVENTRIS.

(A-O): Epifluorescence images of DAPI (blue, A-E), serotonin (green, 5-HT, F-J) and tyrosine hydroxylase (red, TH, K-O), as marker for dopamine. The periventricular serotonergic cells extending into TPp are surrounded by white lines and marked with a plus (H 1-2, I 1-2): Confocal images zoom into the framed rectangles in F-I. Fa-b, Ga-b, H1 and I1 show that there exists no co-localization of 5-HT and TH in the periventricular serotonergic population within TPp. Although having the same distribution and being densely packed in the ventral PVO at intermediate levels, no co-localization of 5-HT and TH could be observed (H2). H2c shows an overlay of the red 5-HT channel and the green TH channel from H2a and H2b, respectively. F-G and I1 illustrate that serotonergic neurons at anterior (Fa-Ga) and posterior levels (I1a) of the periventricular serotonergic cells within TPp are not liquor-contacting, while they are liquor-contacting at intermediate levels (H2a). Serotonergic neurons in the PVO were liquor-contacting at all levels (H2a, I2a). Scale bar is 100 µm in A for A-O, 20 µm in Fa (also for Fb), in Ga (also for Gb), in H1a (also for H1b), in H2a (also for H2b-c), in I1a (also for I1b), in I2a (also for I2b). Abbreviations: PVO paraventricular organ, p/mTPp parvocellular/magnocellular periventricular posterior tuberculum, Th thalamus.

cells begin at more posterior levels of the PVO and extend posteriorly beyond it (Fig. 5D-E; N-O; note that in E no more 5-HT cell bodies, but only fibers are present). As expected, the serotonergic neurons in the proper PVO are liquor-contacting over their entire extent (Fig. H2a, I2a). The dorsal population of serotonergic neurons extending into TPp at their most rostral and caudal extent were mostly not liquor-contacting (Fig. 5Fa, Ga, I1a), while the serotonergic neurons of this population at intermediate levels were CSF-contacting (Fig. 5H1a). Serotonergic and TH-positive cells did not occur in the same location within TPp (e. g., Fig. 5Fa-b, Ga-b, H1a-1b), but their distribution largely overlapped in the proper PVO (Fig. 5H2a, H2b). Although, serotonergic and TH-positive neurons in the proper PVO were densely packed and located next to each other, sometimes in different layers, they showed no co-localization, as was evident in confocal analysis (Fig. 5H2c).

3.5 Hypothalamus

Because of the anteroposterior axis bending mentioned already, the hypothalamus is seen at the same section levels as the PVO and the TPp despite its more anterior position. The dorsal hypothalamus (Hd) is characterized by a periventricular cell zone around the lateral hypothalamic recess in all teleosts including the species investigated here, that is in *S. nigriventris* (Fig. 6A, B), *A. seemanni* (Fig. 7A, B) and *A. grunniens* (Fig. 8A). Serotonergic

neurons were present in the dorsal zone of the periventricular hypothalamus (Hd) positioned around the medial beginning – but not around the lateral extent – of the lateral hypothalamic recess (lr; *S. nigriventris* Fig. 6A-B; *A. seemanni* Fig. 7A-B; *A. grunniens* Fig. 8A, C) and in the caudal zone of the periventricular hypothalamus (Hc) located around the posterior hypothalamic recess in all three studied fish species (*S. nigriventris* Fig. 6C-D, *A. seemanni* Fig. 7B-C, *A. grunniens* Fig. 8C-D). Particularly large populations of serotonergic neurons were found dorsal of lr in the intermediate hypothalamic nucleus (IN; after Rink and Wullimann, 2001) as well as ventral to the lateral hypothalamic recess. This predominance of the serotonergic population in IN within Hd was prominent in *S. nigriventris* (Fig. 6E, I, M), while it was less distinct in *A. grunniens* (Fig. 8E, I, G, K) and only slightly visible in *A. seemanni* (Fig. 7D, H, L), where we observed a cell group associated with the medial part of Hd that most likely corresponds to IN (Fig. 7F, J, N). In the more posterior parts of the periventricular zone of the dorsal hypothalamus, the serotonergic cells were more evenly distributed (*S. nigriventris* Fig. 6F, J; *A. seemanni* Fig. 7F, J; *A. grunniens* Fig. 8G, K).

Both in the two catfishes and in the toadfish, neuronal tissue was present within lr where it partially (asterisk in *A. grunniens* Fig. 8A) or almost completely filled the ventricular space (TAd in *S. nigriventris* Fig. 6A-B, *A. seemanni* Fig. 7A-B). This neuronal structure in catfishes corresponds to the ventral part of the dorsal tuberal nucleus (TAd) as described in the channel catfish (*Ictalurus punctatus*; (Striedter, 1990)) and appears to invade the lr from medial. In the toadfish, this intraventricular mass was concentrated at the rostral beginning of the lr and appeared to invade the ventricular space from the lining of the anterior Hd itself as can be seen in Figure 8E-I. Moreover, these neurons were serotonergic in toadfish (Fig. 8E, I, M), while this was not the case in the catfishes (*S. nigriventris* Fig. 6E-F, I-J; Fig. *A. seemanni* Fig. 7D-F, H-J), a fact which also reflects on their different origins.

In the Hc of all studied fish species, serotonergic neurons were more or less evenly distributed as a ring around the posterior recess (*S. nigriventris* Fig. 6H, L; *A. seemanni* Fig. 7F-G, J-K; *A. grunniens* Fig. 8H, L). The ring-like formation was lost in the most rostral part of

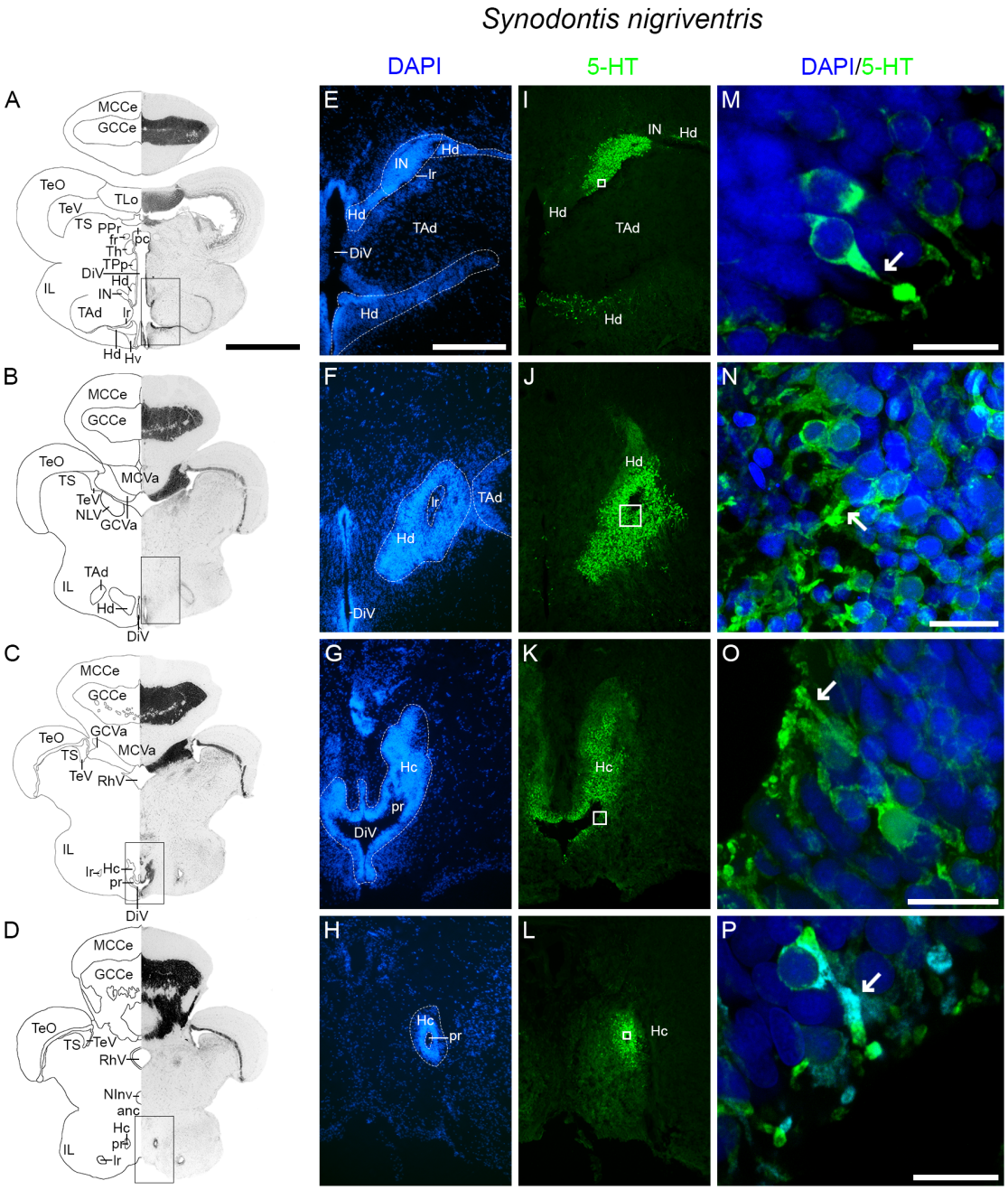

FIGURE 6: SERONERGIC CELL POPULATIONS IN THE HYPOTHALAMUS IN SYNODONTIS NIGRIVENTRIS.

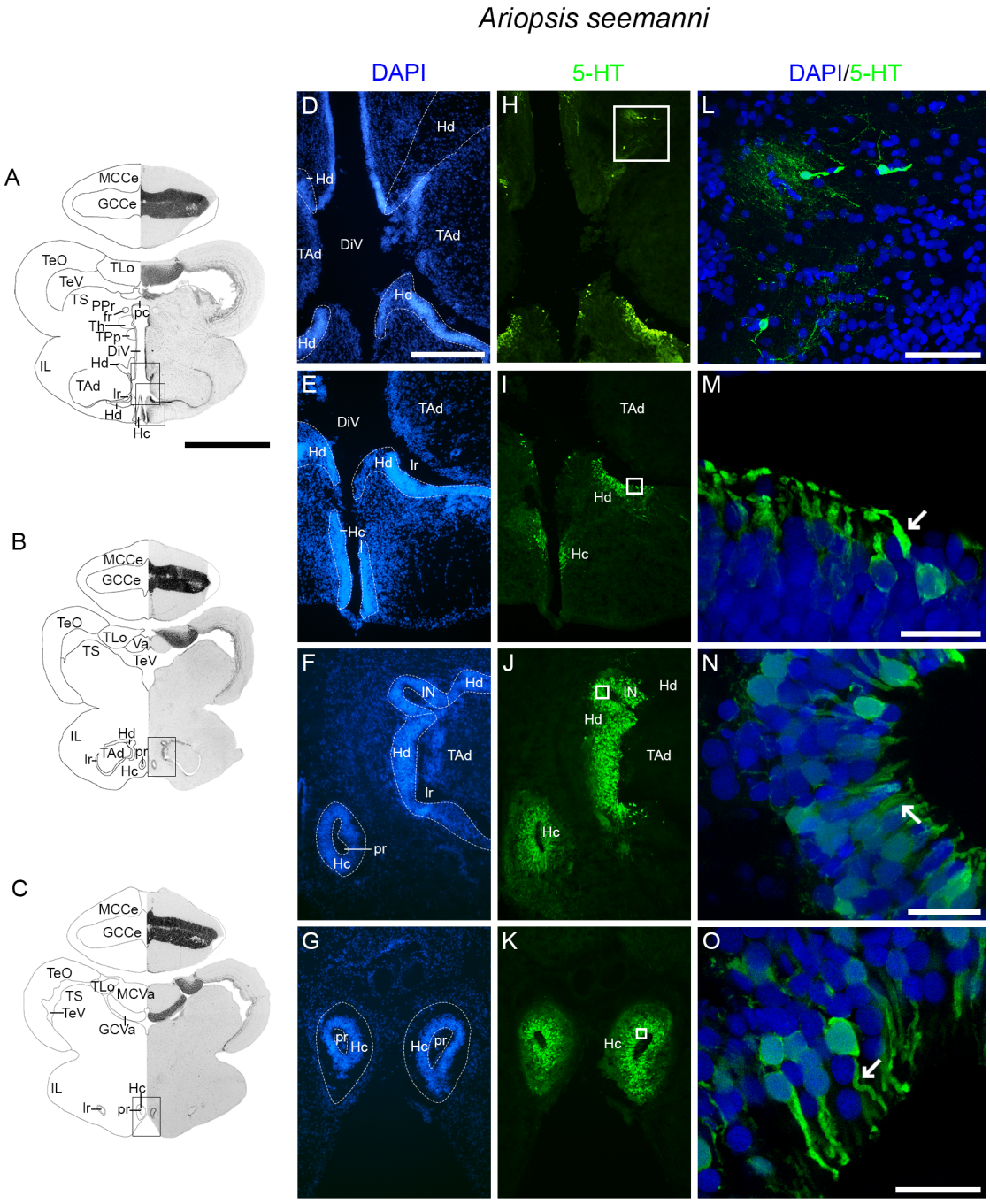

FIGURE 7: SEROTONERGIC CELL POPULATIONS IN THE HYPOTHALAMUS IN *ARIOPSIS SEEMANNI.*

FIGURE 6: SEROTONERGIC CELL POPULATIONS IN THE HYPOTHALAMUS IN *SYNODONTIS NIGRIVENTRIS*.

Left column: Transverse brain sections show line drawings (left side of A-D) and anatomical organization with DAPI (right side of A-D) illustrating the location of serotonergic cells in the dorsal hypothalamus (A, B) and the caudal hypothalamus (C, D). Middle columns (E-L): Epifluorescence images of the framed rectangles indicated in A-D show DAPI (blue, E-H) and serotonin (5-HT, green, I-L) stains). Right column (M-P): Confocal images show magnifications of serotonergic neurons. The small rectangles in I-L indicate the position of the neurons shown in the magnifications. Since the confocal images in M and P show a small area, respective rectangles in I and L are displayed slightly larger than actual picture size. The arrows in M-P indicate a liquor-contacting neuronal process. TAd in A and B indicates a neural mass within the ventricle of the lateral hypothalamic recess which corresponds to the ventral part of the dorsal tuberal nucleus of Striedter (1990). Scale bar is 500 µm in A for A-D, 100 µm in E for E-L, 10 µm in M and P and 20 µm in N and O. Abbreviations: anc ansulate commissure, DiV diencephalic ventricle, fr fasciculus retroflexus, GCCe granular layer of the corpus cerebelli, GCVa granular layer of the valvula cerebelli, Hc/Hd/Hv caudal/dorsal/ventral zone of periventricular hypothalamus, IL inferior lobe of hypothalamus, IN intermediate hypothalamic nucleus (of Rink and Wullimann, 2001; Baeuml et al., 2019), lr lateral hypothalamic recess, MCCe molecular layer of the corpus cerebelli, MCVa, molecular layer of the valvula cerebelli, NInv ventral part of interpeduncular nucleus, NLV nucleus lateralis valvulae, pc posterior commissure, PPr periventricular pretectum, pr posterior hypothalamic recess, RhV rhombencephalic ventricle, SRr rostral part of superior raphe, TAd dorsal anterior tuberal nucleus (Striedter, 1990), TeO tectum opticum, TeV tectal ventricle, TLo torus longitudinalis, TS torus semicircularis.

FIGURE 7: SEROTONERGIC CELL POPULATIONS IN THE HYPOTHALAMUS IN *ARIOPSIS SEEMANNI*.

Left column: Transverse brain sections show line drawings (left side of A-C) and anatomical organization with DAPI (right side of A-C) illustrating the location of serotonergic cells in the dorsal hypothalamus (A), the caudal part of the dorsal hypothalamus and rostral end of the caudal hypothalamus (B) and the caudal hypothalamus (C). Middle columns (D-K): Epifluorescence images of the framed rectangles indicated in A-C show DAPI (blue, D-G) and serotonin (5-HT, green, H-K) stains. Right column (L-O): Confocal images show magnifications of serotonergic neurons. The small rectangles in H-K indicate the position of the neurons shown in the magnifications. The arrows in M to O indicate liquor-contacting neuronal processes. TAd in A and B indicates a neural mass within the ventricle of the lateral hypothalamic recess which corresponds to the ventral part of the dorsal tuberal nucleus of Striedter (1990). Scale bar is 500 µm in A for A-C, 100 µm in D for D-I, 20 µm in J-L. Abbreviations: DiV diencephalic ventricle, fr fasciculus retroflexus, GCCe granular layer of the corpus cerebelli, GCVa granular layer of the valvula cerebelli, Hc/Hd caudal/dorsal zone of periventricular hypothalamus, IL inferior lobe of hypothalamus, IN intermediate hypothalamic nucleus (of Rink and Wullimann, 2001; Baeuml et al., 2019), lr lateral hypothalamic recess, MCCe molecular layer of the corpus cerebelli, MCVa molecular layer of the valvula cerebelli, pc posterior commissure, PPr periventricular pretectum, pr posterior hypothalamic recess, TAd dorsal anterior tuberal nucleus (Striedter, 1990), TeO tectum opticum, TeV tectal ventricle, TLo torus longitudinalis, TS torus semicircularis, Va valvula cerebelli.

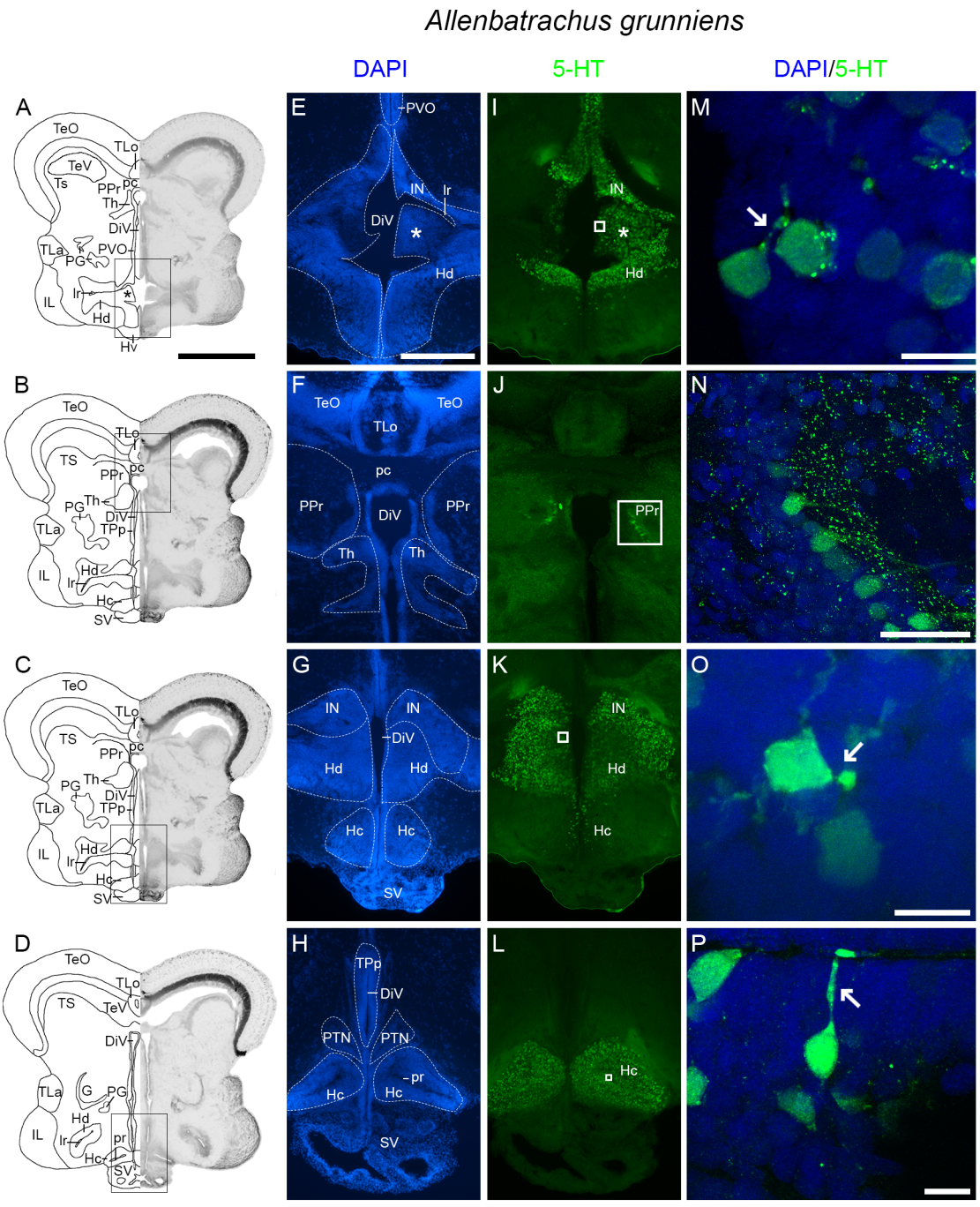

FIGURE 8: SEROTONERGIC CELL POPULATIONS IN THE HYPOTHALAMUS AND PERIVENTRICULAR PRETECTUM IN *ALLENBATRACHUS GRUNNIENS*.

FIGURE 8: SEROTONERGIC CELL POPULATIONS IN THE HYPOTHALAMUS AND PERIVENTRICULAR PRETECTUM IN *ALLENBATRACHUS GRUNNIENS*.

Left column: Transverse brain sections show line drawings (left side of A-D) and anatomical organization with DAPI (right side of A-D) illustrating the location of serotonergic cells in the anterior part of dorsal hypothalamus (A), the periventricular pretectum (B), the intermediate and dorsal hypothalamus (C) and the caudal hypothalamus (D). Middle columns (E-L): Epifluorescence images of the framed rectangles indicated in A-D show DAPI (blue, E-H) and serotonin (5-HT, green, I-L) stains. F and J is dorsal of G and K in the same section. Right column (M-P): Confocal images show magnifications of serotonergic neurons. The small rectangles in I-L indicate the position of the neurons shown in the magnifications. The arrows in M, O and P indicate liquor-contacting neuronal processes. Asterisks in A/E/I indicate an intraventricular neural mass apparently emerging from the dorsal zone of the periventricular hypothalamus. Scale bar is 500 µm in A for A, E, I and M, 200 µm in B for B-C, F-G, J-K and N-O, 10 µm in D, L and P and 50 µm in H. Abbreviations: DiV diencephalic ventricle, Hc/Hd/Hv caudal/dorsal/ventral zone of periventricular hypothalamus, G nucleus glomerulosus, IL inferior lobe of hypothalamus, IN intermediate hypothalamic nucleus (of Rink and Wullimann, 2001; Baeuml et al., 2019), lr lateral hypothalamic recess, pc posterior commissure, PG preglomerular complex, PPr periventricular pretectum, pr posterior recess, PTN posterior tuberal nucleus, PVO paraventricular organ, SV saccus vasculosus, TeO tectum opticum, TeV tectal ventricle, Th thalamus, TLa torus lateralis, TLo torus longitudinalis, TPp periventricular nucleus of posterior tuberculum, TS torus semicircularis.

Hc (Fig. 6G, K; Fig. 8G, K). Here, serotonergic neurons were found lining the midline diencephalic ventricle shortly before the posterior recess emerges in *S. nigriventris* (Fig. 6G, K), *A. seemanni* (Fig. 7E, I) and *A. grunniens* (Fig. 8G, K).

As noted for the serotonergic neurons in PVO, those in Hd and Hc of all three studied fish species as well as the serotonergic neurons in the neuronal mass in lr of *A. grunniens* were bipolar CSF-contacting neurons with one process contacting the liquor of the lateral or posterior recess (Hd: arrow in Fig. 6M (*S. nigriventris*); Fig, 7M, N (*A. seemanni*); Fig. 8M, O (*A. grunniens*); Hc: arrow in Fig. 6P (*S. nigriventris*), Fig. 7O (*A. seemanni*), Fig. 8P (*A. grunniens*)). In contrast to the PVO, no TH-positive neurons were observed in Hd and Hc of *S. nigriventris*.

3.6 Superior raphe (SR)

Further caudal in the anterior rhombencephalon, the superior raphe nucleus or complex was found in all three fish species (*S. nigriventris* Fig. 9A-C, *A. seemanni* Fig. 10, *A. grunniens* Fig. 11). The most rostral cells of SR were seen medioventrally to the rhombencephalic ventricle lining the interpeduncular nucleus (NIn). While serotonergic neurons were present only around

the dorsal part of the NIn in *S. nigriventris* (Fig. 9F, K) and *A. seemanni* (Fig. 10D, G), they extended to the ventral part of NIn in *A. grunniens* (Fig. 11D, G). Serotonergic neurons of this rostral part of SR seemed to be more numerous and more distinctly segregated in toadfish (Fig. 11D, G) than in both catfishes (*S. nigriventris* Fig. 9F, K; *A. seemanni* Fig. 10D, G). At the rostral level of SR, serotonergic neurons were observed between the dorsal and ventral interpeduncular nucleus in *A. grunniens* (Fig. 11G). At the intermediate level of SR, serotonergic neurons were found dorsal to the still present NIn and medioventrally to the medial longitudinal fascicle (mlf) in *A. grunniens* (Fig. 11E, H) and *S. nigriventris* (Fig. 9G, L) and ventral to the mlf in *A. s* (Fig. 10E, H) where the NIn had already ended. While neurons stayed close to the midline in *S. nigriventris* (Fig. 9G, L), some neurons segregated more laterally in *A. seemanni* (Fig. 10E, H) and *A. grunniens* (Fig. 11E, H). Serotonergic neurons of the caudal extent of SR were observed at the level of the caudal part of the cerebellum in *S. nigriventris* (Fig. 9C) and *A. seemanni* (Fig. 10C) and at the level of the caudal part of the torus semicircularis in *A. grunniens* (Fig. 11C). The most caudal SR neurons were concentrated at the midline in all three fish species (*S. nigriventris* Fig. 9H, M; *A. seemanni* Fig. 10F, I; *A. grunniens* Fig. 11F, I). Neurons appeared more numerous in *A. grunniens* and *A. seemanni* compared to *S. nigriventris.* Posterior to the caudal part of SR, we observed a distinct gap with only few, scattered serotonergic neurons before the inferior raphe started.

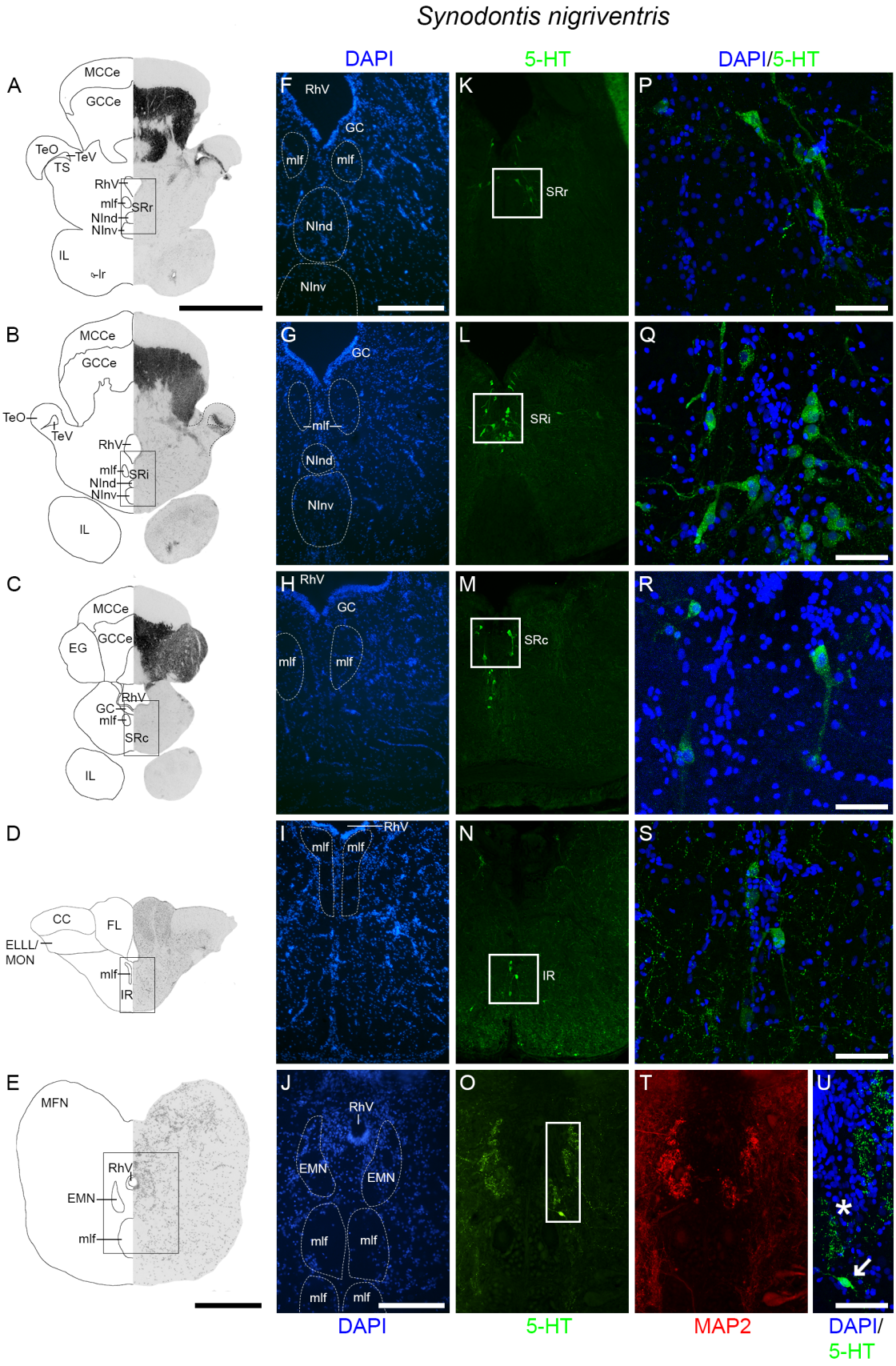

FIGURE 9: SEROTONERGIC CELL POPULATIONS IN THE RAPHE NUCLEI AND CLOSE TO THE ELECTROMOTOR NUCLEUS IN *SYNODONTIS NIGRIVENTRIS*.

FIGURE 9: SEROTONERGIC CELL POPULATIONS IN THE RAPHE NUCLEI AND CLOSE TO THE ELECTROMOTOR NUCLEUS IN SYNODONTIS NIGRIVENTRIS.

Left column: Transverse brain sections show line drawings (left side of A-E) and anatomical organization with DAPI (right side of A-E) illustrating the location of the rostral (A), intermediate (B) and caudal (C) superior raphe, the inferior raphe (D) and the electromotor nucleus (E). Middle columns (F-O): Epifluorescence images of the framed rectangles indicated in A-E show DAPI (blue, F-J) and serotonin (5-HT, green, K-O) stains. T displays the same section as J and O but stained against microtubule associated protein 2 (MAP2). Right column (P-S, U): Confocal images show magnifications of serotonergic neurons. The small rectangles in K-O indicate the position of the neurons shown in the magnifications. (U) The arrow indicates a serotonergic neuron at the anterior spinal level close to the electromotor nucleus and the asterisk indicates potential serotonergic projections to EMN. Scale bar is 500 µm in A for A-D, 500 µm in E, 100 µm in F for F-I and K-N, 50 µm in J for J-T and for P-S and U. Abbreviations: CC cerebellar crest, ELLL/MON electrosensory lateral line lobe/medial octavolateralis nucleus, EG eminentia granularis, EMN electromotor nucleus, FL facial lobe, GCCe granular cell laver of the corpus cerebelli, IL inferior lobe of hypothalamus, IR inferior raphe, lr lateral hypothalamic recess, MCCe molecular cell layer of the corpus cerebelli, MFN medial funicular nucleus, mlf medial longitudinal fascicle, NInd/NInv, dorsal/ventral interpeduncular nucleus, RhV rhombencephalic ventricle, SRc/SRi/SRr caudal/intermediate/rostral part of superior raphe, TeO optic tectum, TeV tectal ventricle.

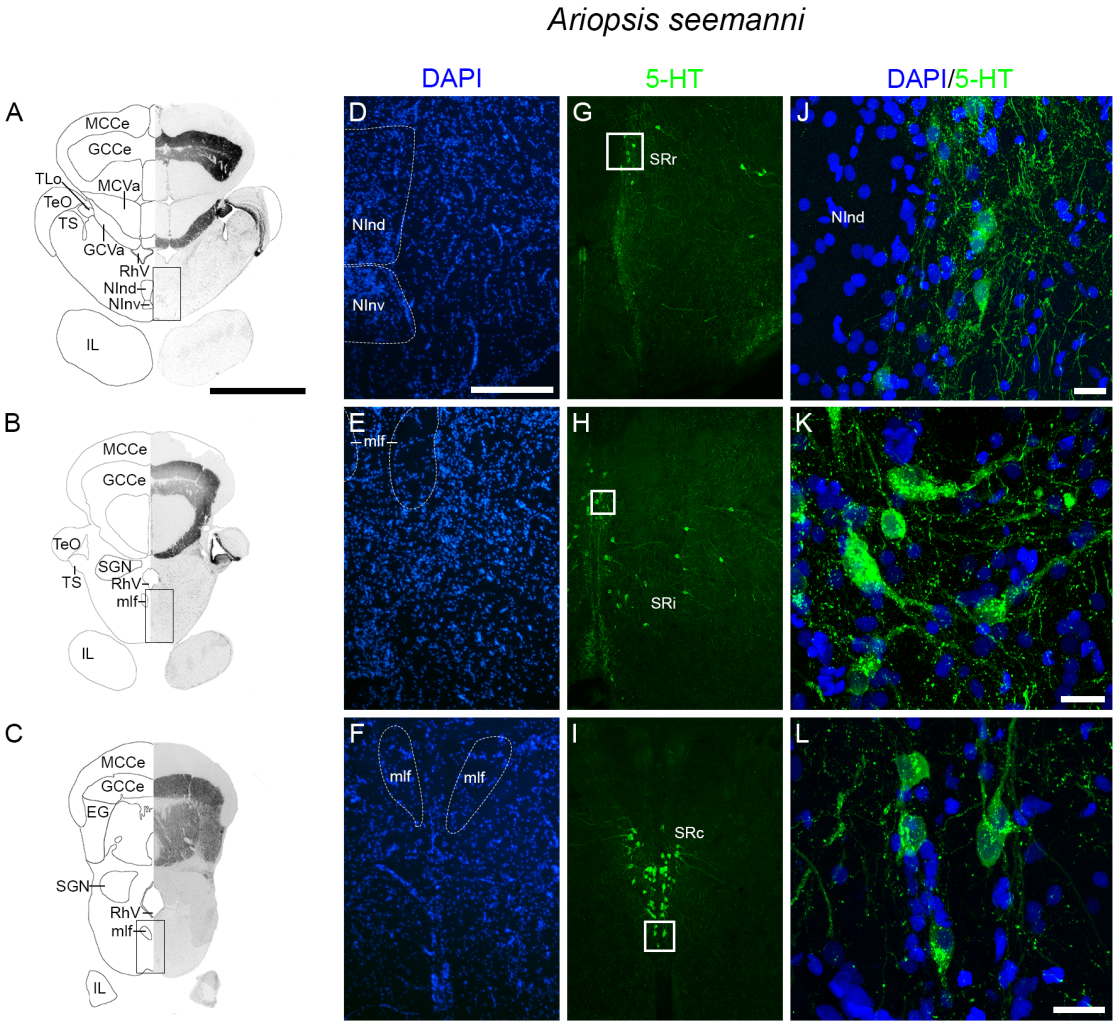

FIGURE 10: SEROTONERGIC CELL POPULATIONS IN THE SUPERIOR RAPHE NUCLEUS IN *ARIOPSIS SEEMANNI*.

Left column: Transverse brain sections show line drawings (left side of A-C) and anatomical organization with DAPI (right side of A-C) illustrating the location of rostral (A), intermediate (B) and caudal (C) superior raphe. Middle columns (D-I): Epifluorescence images of the framed rectangles indicated in A-C show DAPI (blue, D-F) and serotonin (5-HT, green, G-I) stains. Right column (J-L): Confocal images show magnifications of serotonergic neurons. The small rectangles in G-I indicate the position of the neurons shown in the magnifications. Scale bar is 500 in A µm for A-C, 200 µm in D for D-I, 20 µm in J-L. Abbreviations: EG eminentia granularis, GCCe granular cell layer of the corpus cerebelli, GCVa granular cell layer of the valvula cerebelli, IL inferior lobe of hypothalamus, MCCe molecular cell layer of the corpus cerebelli, MCVa molecular cell layer of the valvula cerebelli, mlf medial longitudinal fascicle, NInd/NInv dorsal/ventral interpeduncular nucleus, RhV rhombencephalic ventricle, SGN secondary gustatory nucleus, SRc/SRi/SRr caudal/intermediate/rostral superior raphe, SV saccus vasculosus, TeO tectum opticum, TLo torus longitudinalis, TS torus semicircularis.

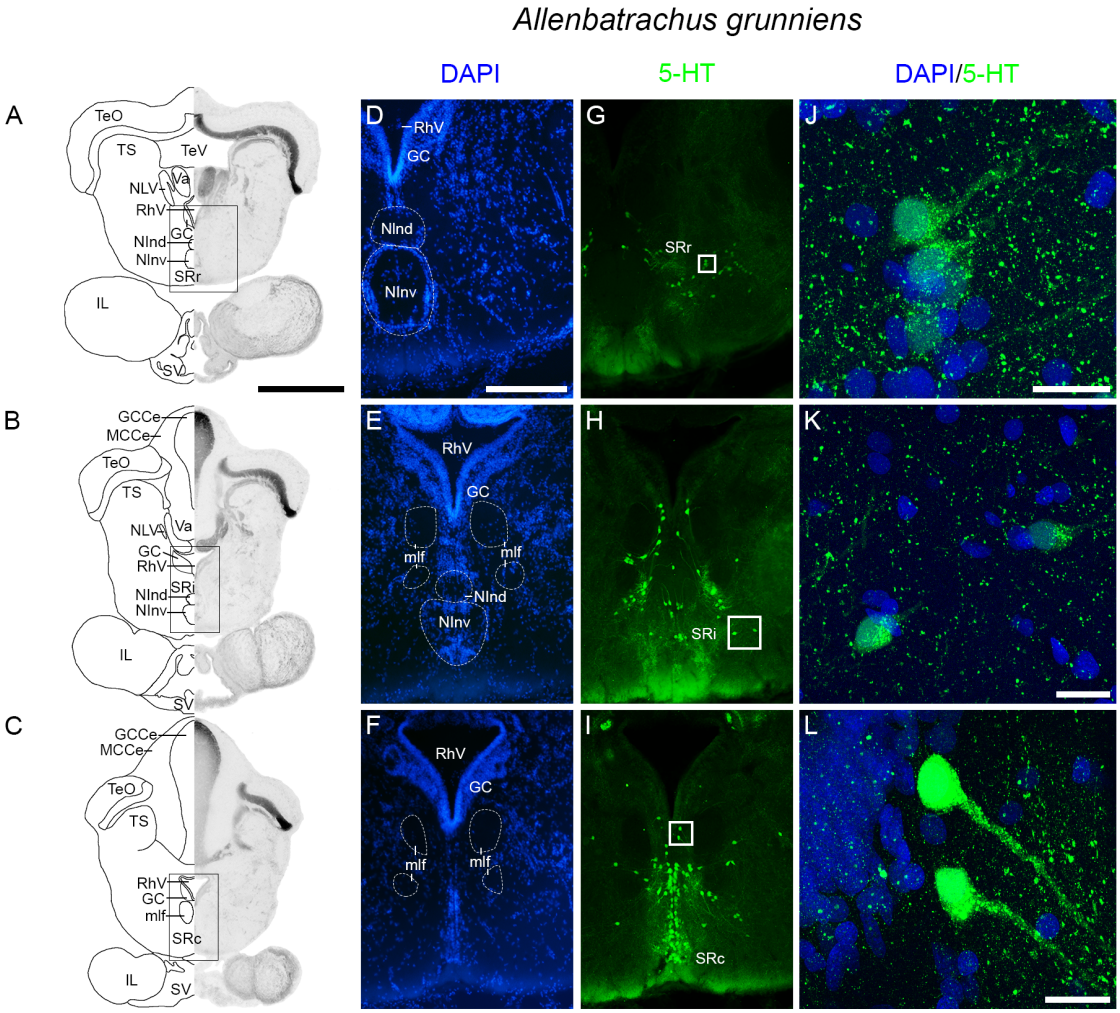

FIGURE 11: SEROTONERGIC CELL POPULATIONS IN THE SUPERIOR RAPHE NUCLEUS IN *ALLENBATRACHUS GRUNNIENS*.

Left column: Transverse brain sections show line drawings (left side of A-C) and anatomical organization with DAPI (right side of A-C) illustrating the location of rostral (A), intermediate (B) and caudal (C) superior raphe. Middle columns (D-I): Epifluorescence images of the framed rectangles indicated in A-C show DAPI (blue, D-F) and serotonin (5-HT, green, G-I) stains. Right column (J-L): Confocal images show magnifications of serotonergic neurons. The small rectangles in G-I indicate the position of the neurons shown in the magnifications. Scale bar is 500 µm in A for A-C, 100 µm in D for D-I, 20 µm in J-L. Abbreviations: GC central grey, GCCe granular cell layer of the corpus cerebelli, IL inferior lobe of hypothalamus, MCCe molecular cell layer of the corpus cerebelli, mlf medial longitudinal fascicle, NInd/NInv dorsal/ventral interpeduncular nucleus, NLV nucleus lateralis valvulae, RhV rhombencephalic ventricle, SRc/SRi/SRr caudal/intermediate/rostral superior raphe, SV sacclus vasculosus, TeO tectum opticum, TS torus semicircularis, Va valvula.

3.7 Inferior raphe (IR)

The IR was located in the ventral part of the posterior rhombencephalon in all three studied species (*S. nigriventris* Fig. 9D, *A. seemanni* Fig. 12A-C, *A. grunniens* Fig. 13). In *A. seemanni* and *S. nigriventris,* the IR was located ventral of the mlf at the level of the facial lobe, the electrosensory part of the lateral line lobe/ medial octavolateralis nucleus and the caudal part of the cerebellar crest. A separation in rostral (Fig. 12E, I, M), intermediate (Fig. 12F, J, N) and caudal level of IR (Fig. 12G, K, O) could be observed in *A. seemanni* with neurons becoming more numerous at the rostral pole. In *S. nigriventris,* IR had a narrower anteroposterior extent and no clear separation could be distinguished (Fig. 9I, N, S). In *A. grunniens*, the IR started rostrally at the level of the vagal lobe (Fig. 13A). Few serotonergic neurons were located in a column at the midline (Fig. 13D, G, J). The intermediate part of IR was at the level with the toadfish vocal motor nucleus (VMN, Fig. 13B) which innervates the muscles attached to the swim bladder. Here, serotonergic neurons were more numerous and more ventrally scattered than in the rostral extent of IR (Fig. 13E, H). The caudal level of IR in *A. grunniens* was observed in the most caudal part of the VMN (Fig. 13C). Here, only few serotonergic neurons were found (Fig. 13F, I, L). VMN received serotonergic projections (asterisk, Fig. 13K) and, occasionally, serotonergic neurons were observed within the VMN (arrows, Fig. 13K). These serotonergic neurons are most likely displaced neurons of the IR.

In contrast to what we observed in toadfish, the IR in both catfishes was not at the same level with the motor neurons innervating the muscles associated with the swim bladder. The vocal motor (*A. seemanni*) and electromotor neurons (*S. nigriventris*) projecting to the swim bladder associated muscles were found caudally to the IR at the spino-occipital or most anterior spinal level in both catfishes (*S. nigriventris* Fig. 9E, *A. seemanni* Fig. 12D). In both catfish species, lateral spinal serotonergic neurons were observed at the level of the EMN/VMN (arrow in Fig. 9U (*S. nigriventris)*, Fig. 12L (*A. seemanni*). In both catfishes, we also observed serotonergic projections next to EMN/VMN (asterisk in Fig. 9U (*S. nigriventris),* Fig. 12L *(A. seemanni)*). In *A. grunniens,* we also observed these serotonergic neurons scattered in the ventral part at the anterior spinal level caudal of IR (not shown).

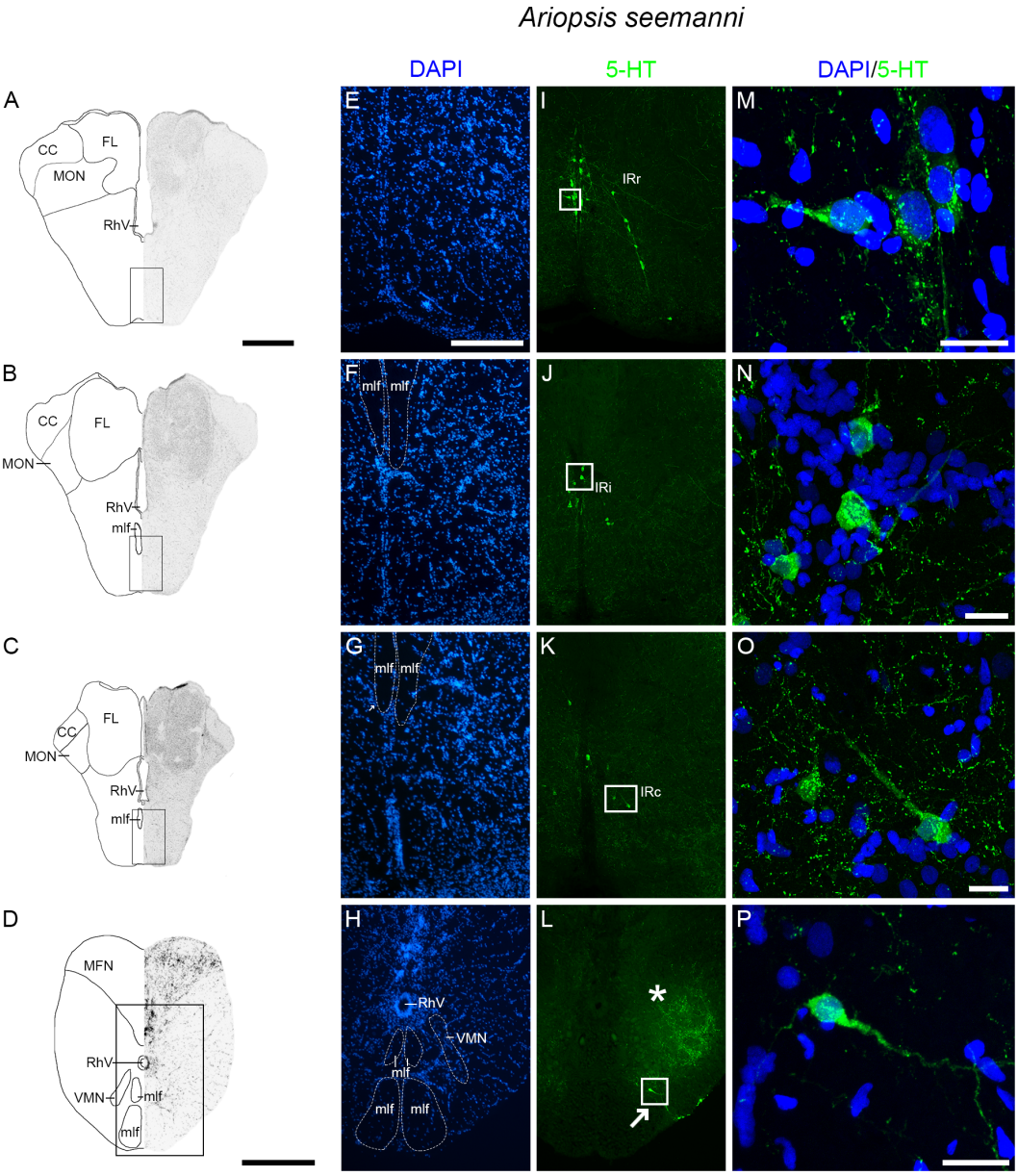

FIGURE 12: SEROTONERGIC CELL POPULATIONS IN THE INFERIOR RAPHE NUCLEUS AND CLOSE TO THE VOCAL MOTOR NUCLEUS IN *ARIOPSIS SEEMANNI*.

Left column: Transverse brain sections showing line drawings (left side of A-D) and anatomical organization with DAPI (right side of A-D) illustrating the location of rostral (A), intermediate (B) and caudal (C) inferior raphe as well as the (D) vocal motor nucleus (VMN). Middle columns (E-L): Epifluorescence images of the framed rectangles indicated in A-D show DAPI (blue; E-H) and serotonin (5-HT, green, I-L). (L) The arrow indicates serotonergic neurons at the anterior spinal level close to the VMN and the asterisk indicates potential serotonergic projections to the VMN dendritic field. Right column (M-P): Confocal images show magnifications of serotonergic neurons. The small rectangles in I-L indicate the position of the neurons shown in the magnifications. Scale bar is 500 µm in A for A-C, 500 µm in D, 100 µm in E for E-L, 20 µm in M-P. Abbreviations: CC cerebellar crest, FL facial lobe, IRc/IRi/IRr caudal/intermediate/rostral inferior raphe, MFN medial funicular nucleus, mlf medial longitudinal fasicle, MON medial octovalateralis nucleus, RhV rhombencephalic ventricle, VMN vocal motor nucleus.

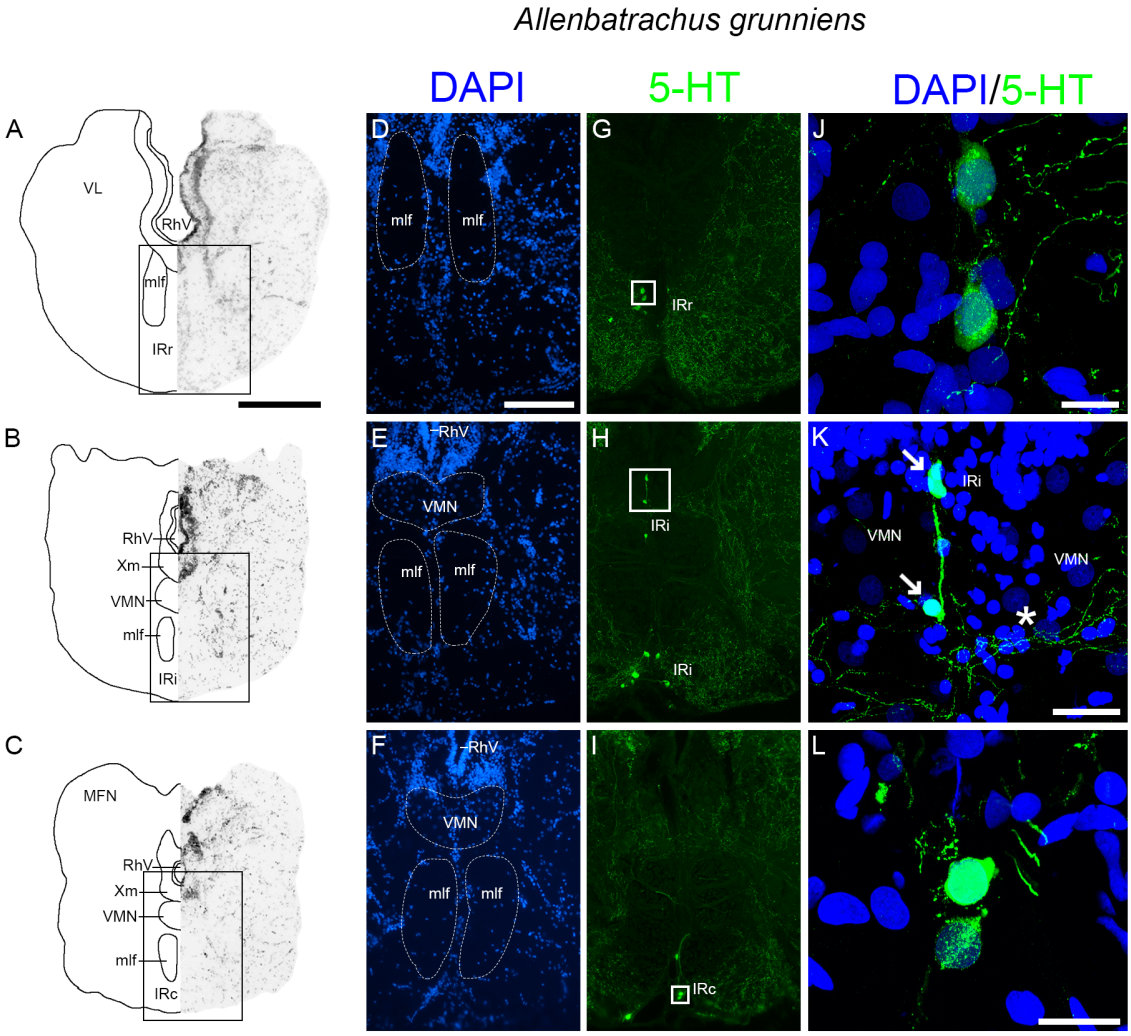

FIGURE 13: SEROTONERGIC CELL POPULATIONS IN THE INFERIOR RAPHE NUCLEUS IN *ALLENBATRACHUS GRUNNIENS*.

Left column: Transverse brain sections showing line drawings (left side of A-C) and anatomical organization with DAPI (right side of A-C) illustrating the location of rostral (A), intermediate (B) and caudal (C) inferior raphe. Middle columns (D-I): Epifluorescence images of the framed rectangles indicated in A-C show DAPI (blue, D-F) and serotonin (5-HT, green, G-I). Right column (J-L): Confocal images show magnifications of serotonergic neurons. The small rectangles in G-I indicate the position of the neurons shown in magnifications. (K) The arrows indicate IR neurons within VMN and the asterisk indicates potential serotonergic projections to VMN. Scale bar is 500 µm in A for A-C, 100 µm in D for D-I, 20 µm in J-L. Abbreviations: Xm vagal motor nucleus, IRc/IRi/IRr caudal/intermediate/rostral inferior raphe, MFN medial funicular nucleus, mlf medial longitudinal fascicle, RhV rhombencephalic ventricle, VL vagal lobe, VMN vocal motor nucleus.

4 Discussion

We compared the distribution of serotonergic neurons in the brains of three teleost fish species that communicate socially using swim bladder related musculature, i.e., two vocalizing (*A. seemanni*, *A. grunniens*) and one weakly electric fish (*S. nigriventris*). Serotonin-containing neurons were found in the pineal stalk, pretectum, paraventricular organ and hypothalamus in the diencephalon, in the raphe nuclei in the rhombencephalon and at anterior spinal levels in all three species. The major difference in serotonergic populations between the studied species was the presence of serotonergic neurons in the anterior part of the preoptic area in both catfish species (*A. seemanni* and *S. nigriventris*), but absence thereof in the toadfish (*A. grunniens*). In all three fish species, we also investigated the serotonergic system at the level of the vocal (VMN) or electromotor neurons (EMN) to assess potential differences between vocal and electric communication. The observed differences might be more species related than dependent on the type of communication.

In the following, we discuss our results in the light of the hypothesis of increasing restriction of serotonergic systems to the raphe during vertebrate phylogeny (Herculano and Maximino, 2014; Lillesaar, 2011; Parent *et al.*, 1984); which we will call restriction hypothesis in the following) according to what has been reported previously about serotonergic systems in other ray-finned fish species and in other vertebrates. We will not consider the retina and focus our discussion on fish taxa, but refer for sarcopterygian taxa (incl. amniotes) to the recent comparative analysis of López and González (2014).

4.1 Telencephalon

In the telencephalon of ray-finned fishes, serotonergic neurons are sometimes observed in the olfactory bulb, for example in acipenseriforms (chondrosteans; the sturgeons *Acipenser baeri* and *Huso huso*; Adrio *et al.*, 1999)), or in teleosts, such as the Atlantic croaker, *Micropogonias undulates*, (Khan and Thomas, 1993). However, in most teleosts investigated, only 5-HT fibers, not cell bodies, are seen in the olfactory bulb, such as in the goldfish *Carassius auratus* (Kah and Chambolle, 1983), zebrafish *Danio rerio* (Kaslin and Panula,

2001), the African catfish *Clarias gariepinus* (Corio et al., 1991), the three-spined stickleback *Gasterosteus aculeatus* (Ekström and Veen, 1984), the European bass *Dicentrarchus labrax* (Batten *et al.*, 1993), the cichlid *Astatotilapia burtoni* (Loveland et al., 2014), the Senegalese sole *Solea senegalensis* (Rodríguez-Gómez et al., 2000) and the South-American and African weakly electric fishes *Apteronotus leptorhynchus* (Johnston *et al.*, 1990) and *Gnathonemus petersii* (Meek and Joosten, 1989), respectively. Additional serotonergic cell populations occur in the ventral (subpallial) telencephalon rostral to the anterior commissure in acipenseriforms (chondrosteans, *A. baeri, H. huso;* Adrio *et al.*, 1999).

Because chondrosteans show serotonergic cells in the olfactory bulb and ventral telencephalon which are absent in but one teleost species (olfactory bulb, see above; Khan and Thomas, 1993) at first sight, these data appear to confirm the restriction hypothesis (see above) which assumes that serotonergic populations exist in all major brain parts in primitive serotonergic systems and become concentrated in the raphe region in mammals (Herculano and Maximino, 2014; Lillesaar, 2011; Parent *et al.*, 1984). However, telencephalic serotonergic cells are also absent in other ancestral ray-finned fishes, i.e., polypteriforms, *Polypterus senegalus* and *Erpethoichthys calabaricus* (Chiba, 1999; López and González, 2014; Reiner and Northcutt, 1992), or lepisosteiforms, *Lepisosteus osseus* (Parent and Northcutt, 1982) and *Lepisosteus productus* (Chiba and Oka, 1999). The telencephalon of cartilaginous fishes also contains no serotonergic cells, for example in the stingray *Dasyatis sabina;* (Ritchie *et al.*, 1983), the spiny dogfish *Squalus acanthias* (Northcutt *et al.*, 1988; Stuesse and Cruce, 1992), the thornback guitarfish *Platyrhinoidis triseriata* (Stuesse *et al.*, 1990), the horn shark *Heterodontus francisi* (Stuesse *et al.*, 1991), the lesser dogfish *Scyliorhinus canicula* (Carrera *et al.*, 2008) and the ratfish *Hydrolagus colliei* (Stuesse and Cruce, 1991). Since also agnathans and all sarcopterygians lack telencephalic 5-HT cells (reviewed in López and González, 2014), a cladistic analysis suggests that sturgeons evolved them newly (compare Fig. 1). Rich serotonergic fibers, however, reach both olfactory bulb and ventral and dorsal telencephalon in all fish species investigated. Thus, the absence of serotonergic neurons in the olfactory bulb and telencephalon in the toadfish and two catfishes studied here suggests

that the serotonergic system of these three species conforms to the picture seen in most teleosts and other vertebrates.

4.2 Diencephalon

The teleostean forebrain's anteroposterior divisions include three posteriorly positioned prosomeres (i.e., pretectal P1, thalamic P2 and prethalamic P3) followed by the anteriorly located secondary prosencephalon (i.e., dorsally the telencephalon and ventrally the hypothalamus; Wullimann and Puelles, 1999) with the preoptic region representing an alar plate derived intermediate region between telencephalon and hypothalamus. However, to simplify the discussion here we use the term 'diencephalon' for the entire forebrain excluding the telencephalon. In all three teleost fish species studied here, massive populations of serotonergic neurons were found in the diencephalic populations of the paraventricular organ in the posterior tuberculum and in the hypothalamus. Smaller serotonergic populations were present in the preoptic area and the periventricular pretectum. Serotonin-containing neurons were also found in the pineal stalk of the studied toadfish and catfishes.

Preoptic area. A few serotonergic neurons were found in the anterior periventricular preoptic nucleus (PPa) in both studied catfishes. Serotonergic neurons were also described in the preoptic area of various other teleosts, such as *M. undulatus* (Khan and Thomas, 1993), three weakly electric fishes, that is, *S. nigriventris* (this study), the elephant-nose fish (Grant *et al.*, 1989) and the brown ghost knifefish (Johnston *et al.*, 1990), in acipenseriforms (chondrosteans; Adrio *et al.*, 1999) and in polypteriforms (Chiba, 1999; López and González, 2014) as well as in various cartilaginous fishes (Carrera *et al.*, 2008; Ritchie *et al.*, 1983; Stuesse *et al.*, 1991; Stuesse and Cruce, 1991) and agnathans (reviewed in López and González, 2014). However, serotonergic preoptic cells are absent in lepisosteiforms (Chiba and Oka, 1999; Parent and Northcutt, 1982) and many teleosts, e. g., *A. grunniens*, and cartilaginous fishes, as well as in all amniotes, although they are present in sarcopterygians, i.e. lungfishes and amphibians (reviewed in López and González, 2014). This phylogenetic distribution (compare with Fig. 1) suggests that teleosts, such as *A. grunniens*, (and various

other teleosts and various cartilaginous fishes) lost serotonergic preoptic cells independently of amniotes and that the two studied catfishes share preoptic serotonergic cells as an ancestral feature with other anamniotes.

Epiphysis. While only few serotonin-containing neurons were present in the pineal stalk of the toadfish, these were numerous in the pineal stalk in both catfish species studied. Serotonin-containing neurons in the pineal stalk and pineal vesicle have also been observed in other teleosts (Corio *et al.*, 1991; Ekström and Ebbeson, 1989; Ekström and Veen, 1984; Frankenhuis-van den Heuvel and Nieuwenhuys, 1984; Kaslin and Paula, 2001; Margolis-Kazan *et al.*, 1985; for species identification see 4.1), in polypteriforms (López and González, 2014) and cartilaginous fishes (Carrera *et al.*, 2008). Among others, the function of the pineal organ is the production of melatonin (Tordjman *et al.*, 2017). Melatonin is a hormone whose synthesis is linked to the control of the circadian cycle. While light inhibits melatonin production, darkness facilitates it. Therefore, serotonin-containing cells are universally present in the pineal organ of vertebrates (López and González, 2014). But because melatonin is a hormone derived from 5-HT (Tordjman *et al.*, 2017), the occurrence of cells containing 5-HT in the epiphysis is linked to the circadian synthesis of melatonin and may be dependent on the time point brains were collected for analysis in all studies.

Pretectum. Serotonergic neurons exist in the periventricular pretectum (PPr) positioned around and lateral of the fasciculus retroflexus in the toadfish and the two catfish species studied. Previously it was debated if this population is part of the dorsal thalamus ((e.g., Rink and Guo, 2004); (see also discussion in López and González, 2014)). Today, it is regarded as part of the pretectum supported by the fact that periventricular pretectal neurons include separate serotonergic and dopaminergic populations which project to the optic tectum (Kress and Wullimann, 2012; Lillesaar *et al.*, 2009). Accordingly, a bilateral pretectal serotonergic population was also observed in other teleosts (Corio *et al.*, 1991; Ekström and Ebbeson, 1989; Ekström and Veen, 1984; Frankenhuis-van den Heuvel and Nieuwenhuys, 1984; Grant *et al.*, 1989; Johnston *et al.*, 1990; Kah and Chambolle, 1983; Kaslin and Paula, 2001; Khan and Thomas, 1993; Margolis-Kazan *et al.*, 1985; Meek and Joosten, 1989; Reiner and

Northcutt, 1992), sturgeons (Adrio *et al.*, 1999) and polypteriforms (López and González, 2014). Because also lampreys and cartilaginous fishes have serotonergic pretectal cells, these seem to be ancestrally present in vertebrates, but are lost in tetrapods (strangely enough, the Nile crocodile appears to re-evolve them; see López and González, 2014)).

Paraventricular organ (PVO). The PVO is considered here part of the posterior tuberculum which is defined as the basal plate division of thalamic (P2) and prethalamic prosomeres (P3; Vernier and Wullimann, 2009) and, thus, treated separately from the periventricular hypothalamic serotonergic liquor-contacting cell populations discussed later. Another posterior tubercular nucleus is the periventricular nucleus of the posterior tuberculum (TPp; see below) which is located dorsal to the PVO. Both nuclei are positioned close to the diencephalic ventricle. More anteriorly (in transverse sections this appears ventrally because of the earlier mentioned bending of the forebrain axis), the ventral (Hv) and dorsal (Hd) periventricular zones of the hypothalamus follow. The extent of the PVO is characterized by the presence of serotonergic neurons which are typically CSF-contacting (see citations below). We confirmed this cytological pattern with the presence of a dense population of bipolar liquor-contacting serotonergic neurons in the PVO of all three studied species. The PVO was also found in other fish species studied for the serotonergic system. The PVO was called nucleus posterioris periventricularis in the goldfish brain atlas of Peter and Gill (1975) and in a revised goldfish atlas by Braford and Northcutt (1983), described as zona limitans, a term which was also used initially for the zebrafish brain (Wullimann *et al.*, 1996). Also confusing is that another nucleus within the dorsal periventricular hypothalamus (the intermediate hypothalamic nucleus here, see below) had been called PVO in the latter two publications. Later, this old terminology was changed and the zona limitans renamed PVO (see Baeuml *et al.*, 2019; Rink and Wullimann, 2001 for further discussion). Furthermore, Meek and Nieuwenhuys (1998) also identified a PVO in a similar midline posterior tubercular position in the trout brain (*Salmo gairdneri*).

Thus, various papers identify the serotonergic PVO in a midline posterior tubercular position with either of these two older names, e.g., in the Senegal bichir (*Polypterus senegalus*;

(Reiner and Northcutt, 1992)), in the goldfish (Kah and Chambolle, 1983), three-spined stickleback (Ekström and Veen, 1984), common platyfish *Xiphophorus maculatus* (Margolis-Kazan *et al.*, 1985), African catfish (Corio *et al.*, 1991), trout (Frankenhuis-van den Heuvel and Nieuwenhuys, 1984; where the cells representing the PVO remain unnamed but are clearly depicted) and Atlantic croaker (Khan and Thomas, 1993), while it was called PVO in other teleosts (Batten *et al.*, 1993; Kaslin and Paula, 2001; Rodríguez-Gómez *et al.*, 2000), but also in acipenseriforms ((chondrosteans; (Adrio *et al.*, 1999)) and polypteriforms (Chiba, 1999; López and González, 2014). In cartilaginous fishes, serotonergic cells corresponding to the PVO were assigned to the ventral aspect of the posterior tuberculum (Stuesse and Cruce, 1992; Stuesse *et al.*, 1991; Stuesse and Cruce, 1991) or called organum vasculosum (Ritchie *et al.*, 1983). Lampreys have an uninterrupted series of liquor-contacting cells stretching from the posterior tuberculum into the hypothalamus (Abalo *et al.*, 2007; Cornide-Petronio *et al.*, 2013; Pierre *et al.*, 1992). In sarcopterygians such as lungfishes, an extended PVO, including hypothalamic divisions, is recognized (NPv of López and González, 2015). However, a midline posterior tubercular subdivision of this extended lungfish PVO - located between hypothalamus and alar prethalamus – can be recognized in lungfishes (López and González, 2015; the part of NPv shown in their Fig. 1h) and a similar situation exists in amphibians (Beltramo *et al.*, 1998; Corio *et al.*, 1992; Ueda *et al.*, 1984). Thus, liquor-contacting serotonergic posterior tubercular-hypothalamic populations (see below) remain phylogenetically present in sarcopterygians, including monotremes, but are finally lost in marsupial and placental mammals (Manger *et al.*, 2002). This loss is the main event that had led to the restriction hypothesis (Herculano and Maximino, 2014; Lillesaar, 2011; Parent *et al.*, 1984). Because a clear functional role for diencephalic monoaminergic CSF-contacting cells is still elusive, so is the evolutionary explanation for their loss in marsupials and placental mammals.

In both catfish species studied here, serotonergic cells at the periventricular lining of the PVO extended dorsally into the area where the TPp was located. We confirmed this by co-staining of 5-HT and TH in *S. nigriventris*. A similar situation was reported previously in a few

other teleosts (Corio *et al.*, 1991; Johnston *et al.*, 1990; Khan and Thomas, 1993; Margolis-Kazan *et al.*, 1985; Meek and Joosten, 1989). For example, in *A. leptorhynchus,* various populations of liquor-contacting neurons are described along the diencephalic ventricle from posterior tuberculum into the hypothalamus. These populations were collectively called PVO cells, but various neuroanatomical terms were used in addition to describe the nuclei containing these cells (Johnston *et al.*, 1990). Interestingly, there are serotonergic cells at the ventricular lining of their TPp leading over into the proper PVO and hypothalamic serotonergic cells. Such a dorsal expansion of the PVO into TPp is definitely not seen in other teleost species, for example in *C. auratus* (Kah and Chambolle, 1983), *G. aculeatus* (Ekström and Veen, 1984), *D. labrax* (Batten *et al.*, 1993) and *D. rerio* (Kaslin and Paula, 2001; Rink and Guo, 2004).

In line with the nomenclature used in this paper, we use PVO only for the conventionally recognized structure ventral to TPp and refer additionally to 5-HT cells extending into TPp. In *S. nigriventris*, both areas were found to contain CSF-contacting neurons which, for the classical PVO, has been reported before repeatedly in teleosts (Corio *et al.*, 1991; Johnston *et al.*, 1990) and it is likely that in cases where this cytological feature for PVO cells was not reported is not a true species difference.

Recently, an *in situ* hybridization study showed that transcripts of the two genes *th2 and tph1*, coding for the synthetic enzymes tyrosine hydroxylase 2 (see below for explanation) and tryptophan hydroxylase 1, are co-localized in neurons of the PVO in chicken, *Gallus gallus*, African clawed frog, *Xenopus laevis,* and zebrafish (Xavier *et al.*, 2017). Additionally, these authors demonstrated immunohistochemical co-localization of 5-HT and dopamine in the zebrafish brain. This is in contrast to the earlier conclusion that 5-HT and TH are not co-localized in neurons of the PVO also using immunohistochemical stainings in zebrafish (Kaslin and Paula, 2001). The absence of 5-HT and TH co-localization in the proper PVO of *S. nigriventris* observed in the present study is in line with the immunohistochemical study in zebrafish (Kaslin and Paula, 2001), but both are seemingly in conflict with the results by Xavier and colleagues (Xavier *et al.*, 2017). However, this is only an apparent conflict. Two *tyrosine*

hydroxylase genes – *th1* and *th2* – were shown to exist (Yamamoto *et al.*, 2010, 2011) and *th2* was found to be expressed exclusively in many CSF-contacting PVO neurons of chicken, *X. laevis* and zebrafish (Chen *et al.*, 2009; Xavier *et al.*, 2017). The zebrafish PVO also expresses high levels of the dopamine transporter gene (Holzschuh *et al.*, 2001). Furthermore, available antibodies against TH reveal the TH1 enzyme, but fail to visualize TH2 (Xavier *et al.*, 2017; Yamamoto *et al.*, 2011). Therefore, those few dopamine cells that have been revealed by immunohistochemistry in the PVO and dorsal (Hd) and caudal (Hc) periventricular hypothalamus (Kaslin and Paula, 2001; Rink and Wullimann, 2001) likely represent *th1*-dopamine cells (Xavier *et al.*, 2017). We did not observe dopaminergic neurons in Hd and Hc of *S. nigriventris* as Kaslin and Panula (2001) did in *D. rerio*, probably indicating that our used antibody against tyrosine hydroxylase only detected *th1* and that potential dopaminergic neurons in Hd and Hc of *S. nigriventris* must be *th2*-dopamine cells. Thus, both Kaslin and Panula's analysis (2001) in *D. rerio* and our analysis in *S. nigriventris* of missing co-localization of dopamine with 5-HT and that of existing co-localization of *th2* and *tph1* or 5-HT and dopamine in chicken, frog an zebrafish (Xavier *et al.*, 2017) are all consistent and likely correct. This leads to the hypothesis that *th1*-dopamine cells do not co-localize with 5-HT.

Hypothalamus. The dorsal (Hd) and caudal (Hc) zones of the periventricular hypothalamus (after Braford and Northcutt, 1983; Wullimann *et al.*, 1996) line the lateral (lr) and posterior (pr) hypothalamic recesses, respectively. They partially contain CSF-contacting serotonergic neurons positioned in Hd around the medial beginning of the lateral recess – but not around its lateral extent – and in Hc surrounding the posterior recess, as seen in *S. nigriventris*, *A. seemanni* and *A. grunniens*.

This nomenclature was used in the Senegal bichir where serotonergic neurons were also found in Hd and fewer in Hc (Reiner and Northcutt, 1992), a finding later confirmed for polypteriforms (Chiba, 1999; López and González, 2014), although there the hypothalamic serotonergic cells were included terminologically into a larger PVO. In acipenseriforms, serotonergic cells of the Hd were described as a posterior PVO and those in Hc as being around the posterior (i.e. caudal) recess (Adrio *et al.*, 1999). Regarding the hypothalamus,

nucleus recessi lateralis and nucleus recessi posterioris are often used as alternative terms for Hd and Hc, respectively. Both contain CSF-contacting serotonergic neurons in lepisosteiforms (Chiba and Oka, 1999; Parent and Northcutt, 1982), and various teleosts (Adrio *et al.*, 1999; Batten *et al.*, 1993; Corio *et al.*, 1991; Ekström and Veen, 1984; Frankenhuis-van den Heuvel and Nieuwenhuys, 1984; Grant *et al.*, 1989; Johnston *et al.*, 1990; Kah and Chambolle, 1983; Khan and Thomas, 1993; Margolis-Kazan *et al.*, 1985; Meek and Joosten, 1989; Rodríguez-Gómez *et al.*, 2000). In several of these papers, the serotonergic cells at the lateral and posterior hypothalamic recesses were additionally included into a larger PVO.

Four recesses were described in *G. petersii,* that is, anterior, intermediate, lateral and posterior ones (Meek *et al.*, 1989). Serotonergic neurons were found around the anterior recess (corresponding to the proper PVO), the intermediate and lateral recesses, both being part of Hd, and around the posterior recess corresponding to Hc (Grant *et al.*, 1989; Meek and Joosten, 1989). Because these serotonergic cells were CSF-contacting neurons around the recesses they were all additionally included into one larger PVO in *G. petersii,* while in zebrafish the serotonergic cells in Hd and Hc were seen as intermediate and posterior parts of the PVO, respectively (Kaslin and Paula, 2001; with their anterior PVO representing the classical PVO). Alternatively, the serotonergic part of Hd was called ‘intermediate hypothalamic nucleus’ (IN; Rink and Guo, 2004; Rink and Wullimann, 2001).

In common platyfish (Margolis-Kazan *et al.*, 1985), African catfish (Corio *et al.*, 1991), brown ghost knifefish (Johnston *et al.*, 1990), three-spined stickleback (Ekström and Veen, 1984) and zebrafish (Kaslin and Paula, 2001), serotonergic neurons are limited to the part of Hd dorsal to the lateral recess**.** As explained above, the term IN was introduced for this nucleus in zebrafish (Rink and Guo, 2004; Rink and Wullimann, 2001). In the present study, we adopted this nomenclature and identified an IN located dorsally to the lateral recess in Hd in all three species investigated. Additional serotonergic neurons were observed dorsal to the lateral recess in Hd beyond the IN, but even more so in Hd ventral to the lateral recess in the three species studied. Serotonergic neurons of Hd were also observed dorsal and ventral to the lateral recess in the Senegalese sole (Rodríguez-Gómez *et al.*, 2000), European bass (Batten

et al., 1993), goldfish (Kah and Chambolle, 1983), Atlantic croaker (Khan and Thomas, 1993), elephant-nose fish (Meek and Joosten, 1989) as well as in primitive actinopterygians, such as polypteriforms (Reiner and Northcutt, 1992), acipenseriforms (Adrio *et al.*, 1999) and lepisosteiforms (Parent and Northcutt, 1982).

In all three fish species studied here, we observed neuronal tissue that grew into the lateral recess. This intraventricular mass seemed to be part of the anterior Hd in *A. grunniens*, which was consistent with the fact that this mass contained CSF-contacting serotonergic neurons. However, in the two catfish species studied, the intraventricular mass most likely corresponds to the dorsal part of the tuberal nucleus (TAd) previously described in the channel catfish (Striedter, 1990). While the TAd in the investigated catfishes was devoid of serotonergic neurons, some serotonergic neurons were described in the lateral tuberal nucleus in elephant-nose fish (Meek and Joosten, 1989) and African catfish (Corio *et al.*, 1991) as well as in the anterior and lateral tuberal nucleus in European bass (Batten *et al.*, 1993), but these might represent the most anterior beginning of serotonergic cells of Hd.

As in all telelosts investigated, the diencephalic serotonergic system in the three studied species is concentrated in the PVO, Hd and Hc, with smaller populations present in the pretectum and (in catfishes) the preoptic region. Additionally, in the three-spined stickleback and the common platyfish, serotonergic neurons were present in the pituitary gland (Ekström and Veen, 1984; Margolis-Kazan *et al.*, 1985), and in sockeye salmon fry, *Oncorhyhchus nekra*, serotonergic neurons were reported in the habenula located next to the pineal organ (Ekström and Ebbeson, 1989).

4.3 Mesencephalon

No serotonergic neurons were observed in the mesencephalon of the studied toadfish and catfish species, which is in line with most studies in teleosts (see above). However, a few 5-HT cell somata were observed in the optic tectum of. *D. labrax* (Batten *et al.*, 1993), as well as in both torus semicircularis and midbrain tegmentum in *M. undulatus* (Khan and Thomas, 1993).

4.4 Rhombencephalon

In the present study, we observed serotonergic neurons in the superior raphe in the anterior rhombencephalon and in the inferior raphe in the posterior rhombencephalon in two catfish (*S. nigriventris*, *A. seemanni*) and one toadfish (*A. grunniens*) species, with the two populations well separated by a considerable gap. Serotonergic neurons in the raphe region as observed in our study were described in all fish species investigated.

Superior Raphe (SR) nuclei. Inspired by mammalian nomenclature, sometimes at least dorsal and medial divisions of the superior raphe (SR) were described in teleosts, for example in the three-spined stickleback (Ekström and Veen, 1984), sockeye salmon fry (Ekström and Ebbeson, 1989), the brown ghost knifefish (Johnston *et al.*, 1990), the African catfish (Corio *et al.*, 1991), the Atlantic croaker (Khan and Thomas, 1993), the European bass (Batten *et al.*, 1993), the zebrafish (Kaslin and Paula, 2001), as well as in sturgeons (Adrio *et al.*, 1999). However, in the zebrafish a strongly overlapping dorsoventral SR organization with a tendency for anterior projection sites originating more dorsally than posterior ones within the SR was reported (Lillesaar *et al.*, 2009). A lateral neuronal cluster of the SR projected specifically to the preglomerular region (Lillesaar *et al.*, 2009). Similarly, a rough clustering of serotonergic SR neurons in *A. grunniens* was observed, especially at intermediate levels. Here, a mediodorsal, medial, medioventral and lateral cluster of serotonergic neurons might be recognized. Nevertheless, there are no distinct gaps between these neuronal clusters and no tracing studies are available to resolve potential differential targets of these neuronal clusters. Thus, we did not further subdivide the SR of *A. grunniens*. A SR without subdivisions was also described in the trout (Frankenhuis-van den Heuvel and Nieuwenhuys, 1984), the elephant-nose fish (Grant *et al.*, 1989; Meek and Joosten, 1989), and the Senegalese sole (Rodríguez-Gómez *et al.*, 2000).

Inferior Raphe (IR) nuclei. An inferior raphe (IR), sometimes also named raphe posterioris, was described in the three-spined stickleback (Ekström and Veen, 1984), elephant-nose fish (Grant *et al.*, 1989; Meek and Joosten, 1989), brown ghost knifefish (Johnston *et al.*,

1990), Atlantic croaker (Batten *et al.*, 1993), Senegalese sole (Rodríguez-Gómez *et al.*, 2000) and zebrafish (Kaslin and Paula, 2001), as well as in sturgeons (Adrio *et al.*, 1999). No IR was reported in *C. gariepinus* (Corio *et al.*, 1991), which might, however, be due to methodological reasons as a pretreatment with monoamine-oxidase inhibitor is needed to visualize the inferior raphe nucleus in the three-spined stickleback (Ekström and Veen, 1984). In goldfish and the common platyfish, serotonergic raphe neurons were not divided into superior and inferior raphe (Kah and Chambolle, 1983; Margolis-Kazan *et al.*, 1985).

Finally, in the elephant-nose fish an intermediate raphe nucleus between the superior and inferior raphe was found (Grant *et al.*, 1989; Meek and Joosten, 1989). Additional raphe nuclei were also observed in the brown ghost knifefish where a third subnucleus of the superior raphe, the raphe centralis (Johnston *et al.*, 1990), was present. In the European bass (Batten *et al.*, 1993) and in sockeye salmon fry, a raphe magnus was described (Ekström and Ebbeson, 1989). In Senegalese sole, serotonergic neurons were observed in the interpeduncular nucleus at the level of the superior raphe (Rodríguez-Gómez *et al.*, 2000). We also observed serotonergic neurons between the dorsal and ventral part of the interpeduncular nucleus in *A. grunniens* but interpreted these to be part of the superior raphe.

In addition, the valvula was reported to have some serotonergic neurons in the common platyfish (Margolis-Kazan *et al.*, 1985) and the Atlantic croaker (Khan and Thomas, 1993). Moreover, in all actinopterygians, there are ventral serotonergic cells in the spinal cord.

Reticular formation and medullary spinal cord junction. In addition to the concentration of serotonergic neurons in superior and inferior raphe, few serotonergic neurons were observed scattered in the ventral medulla/anterior spinal level caudal to the inferior raphe in the toadfish and two catfishes studied here, as well as the in European bass (Batten *et al.*, 1993). Similar serotonergic neurons were also observed to be scattered in the ventral reticular formation in sea bass (Batten *et al.*, 1993) and Senegalese sole (Rodríguez-Gómez *et al.*, 2000), close to the midline in zebrafish (Kaslin and Paula, 2001), at one level with the inferior olive in the elephant-nose fish and in the ventrolateral hindbrain between the superior and

inferior raphe nucleus in zebrafish (Lillesaar *et al.*, 2009). In Atlantic croaker, serotonergic neurons were observed near the obex (Khan and Thomas, 1993). Also in sturgeons, serotonergic neurons were found in the reticular formation named dorsal, medial, superior and inferior reticular nucleus named after the raphe nucleus next to which the respective reticular nucleus was located laterally (Adrio *et al.*, 1999).

4.5 Serotonergic system at the level of VMN/EMN

In this study, we also investigated potential differences between vocal and electric swim bladder related communication in the three studied fish species. All studied species have a motor nucleus located in the ventral hindbrain that projects to muscles attached to the swim bladder. However, this motor nucleus is located at a different anteroposterior position. In *A. grunniens*, the vocal motor nucleus (VMN) is a fused, heart-shaped midline nucleus located directly medioventrally to the rhombencephalic ventricle and vagal motor nucleus in the posterior hindbrain (Bass *et al.*, 1994; Chagnaud and Bass, 2014; Marchaterre *et al.*, 1989). In *A. seemanni*, the VMN is a bilateral nucleus that is located ventrolaterally to the rhombencephalic ventricle at an anterior spinal level (whereas dorsal at this level the rhombencephalic medial funicular nucleus is present; Schlichtholz, 2015). In *S. nigriventris*, the nucleus innervating the swim bladder muscles is similarly positioned as in *A. seemanni* somewhat ventrolaterally to the rhombencephalic ventricle at the level of the medial funicular nucleus (Ladich and Bass, 1996). This nucleus was previously referred to as sonic motor nucleus (SMN) in *S. nigriventris* (Ladich and Bass, 1996). However, recent behavioral evidence showed that *S. nigriventris* does not produce swim bladder associated sounds, but instead generates weakly electric discharges (Boyle *et al.*, 2014). This finding is in line with the known ability of other synodontids (Hagedorn *et al.*, 1990) that use the protractor muscles for electric discharge production, while others generate vocal signals or both (Baron *et al.*, 1994; Boyle *et al.*, 2014; Hagedorn *et al.*, 1990). We thus refer to the nucleus innervating the swim bladder associated muscle in *S. nigriventris* as electromotor nucleus (EMN) instead of SMN.

Serotonergic fibers were observed adjacent to VMN in *A. seemanni,* while they were located within EMN in *S. nigriventris* and VMN in *A. grunniens*. Anterior spinal serotonergic neurons were located close to VMN and EMN in *A. seemanni* and *S. nigriventris*, respectively, while displaced IR neurons were observed within VMN in *A. grunniens*. Serotonergic fibers were also observed within the VMN in plainfin midshipman fish, *Porichthys notatus* (Forlano *et al.*, 2011), and the Gulf toadfish, *O. beta* (Rosner *et al.*, 2018)), both belonging to Batrachoididae as *A. grunniens*. In addition, serotonergic neurons within VMN were also observed in the Gulf toadfish (Rosner *et al.*, 2018) and the oyster toadfish (Marchaterre *et al.*, 1989). This indicates a ubiquitous pattern for displaced IR neurons in Batrachoididae. It would be interesting to see if the sister clade midshipman, also has serotonergic neurons within VMN confirming this pattern for other toadfishes. Overall, we did observe differences in the presence of serotonergic fibers between vocal motor and electromotor nuclei within siluriforms, but similarities between one silurid (*S nigriventris*) and the toadfish (*A. grunniens*). Thus, these differences and similarities appear to be species-specific rather than related to electric or acoustic function.

5 Conclusions

In the present study, we found serotonergic cell populations in three teleost species (one toadfish and two catfish) highly concentrated in the diencephalon and the rhombencephalic raphe region with an additional small population in the anterior nucleus of the preoptic area in both catfish species. Displaced IR neurons in VMN of *A. grunniens* but no 5-HT containing cells in the nuclei innervating the swim bladder musculature in both catfishes probably makes this a toadfish specific feature. All three species had a neuronal mass extending into the lateral recess corresponding to the dorsal tuberal nucleus in catfishes but likely representing an extension of the dorsal periventricular hypothalamus in toadfish. Serotonergic, liquor-contacting neurons at the level of the proper PVO extended into the midline area of the periventricular posterior tubercular nucleus in both catfishes. We also found

that dopaminergic, liquor-contacting neurons (most likely containing *th1*) in the PVO were not co-localized with 5-HT in *S. nigriventris*. Because TH antibodies are known to show mostly or only the TH1 enzyme, we hypothesize that *th1*-expressing dopamine cells (unlike *th2*-expressing ones) do not co-localize with 5-HT.

Since all three species engage in social communication, we investigated if serotonergic innervation of the involved hindbrain motor nuclei that produce the vocal or electromotor signal is present and possibly different. The observed serotonergic fibers and close-by serotonergic neurons showed only minor differences between the three species which seemed to be rather species-specific than dependent on the type of social communication.

4 DISCUSSION

Anatomical and electrophysiological evidence suggest different inhibitory and modulatory neurotransmitters heavily affect the output of vocal areas in various vertebrate species. So far most present studies focused on specific transmitter types including inhibitory and modulatory neurotransmitters. However, a comprehensive overview of the inhibitory and modulatory neurochemical profile of vocal networks in one vocal system would be helpful in order to gain a complete understanding of the mechanisms underlying adaptive vocal behavior. Due to the direct relationship between vocal neuronal readout, the identification of a hindbrain vocal CPG and the established *in vivo* preparation, the highly vocal toadfishes are an ideal model to study the basis of vocal signal generation.

Therefore, in the first part of my thesis, I analysed the inhibitory and modulatory inputs to the hindbrain vocal CPG in the toadfish *O. beta* by combing immunohistochemistry with labeling of the vocal CPG with a tracer that crosses gap junctions. Together with the immunohistochemical identification of a gap junction protein, my findings complement prior electrophysiological studies of the intrinsic and network features of the hindbrain vocal system. These findings allow to suggest mechanisms to generate multiple types of social context-dependent vocalizations with different temporal and spectral properties. Additionally, I investigated the serotonergic system in another toadfish, *A. grunniens*, the vocal catfishes, *A. seemanni* and the weakly electric catfish *S. nigriventris* to see if social communication influences the organization of the serotonergic system. I show that the general organization of the serotonergic system of these socially communicating teleosts is similar to that in other ray-finned fishes. Observed differences in the serotonergic fibers present within or close to the hindbrain motor nuclei involved in social signalling appear to be species-specific rather than related to the type of social communication signals that are produced. However, displaced inferior raphe cells within the hindbrain motor neurons involved in social signalling appear to be a prominent feature of the highly vocal toadfishes.

The next sections will give a detailed discussion of the inhibitory and modulatory neurotransmitters, observed in the brain of these four teleost species studied here.

4.1 Inhibitory neurotransmitter in vocal areas of vertebrates

4.1.1 GABAergic innervation influences vocal output

Chapter 1 showed a dense GABAergic label to VMN, VPN and VPP with GABAergic neurons within and close by to vocal nuclei in the Gulf toadfish *O. beta*. GABAergic input was previously shown in the VMN of midshipman fish, another toadfish species, with GABAergic neurons positioned laterally to the nucleus (pilot study in appendix; Chagnaud *et al.*, 2012; Rubow, 2010). The GABAergic input to VMN of midshipman was shown to provide a tonic hyperpolarization to VMN. Local injections of GABA into VMN of midshipman fish decreased call amplitude – the call parameter modulated by VMN – and call duration – controlled by VPP (Chagnaud *et al.*, 2011) – as did local injection of the two $GABA_A$ antagonists muscimol and gabazine (Rubow, 2010). Single neuron recordings of VMN neurons together with injection of bicuculine, another $GABA_A$ antagonist, into VMN showed that a lack of $GABA_A$ mediated GABAergic inhibition first distorts and then eliminates vocal VMN activity (Chagnaud *et al.*, 2012). This shows three things: First, the diminished vocal VMN activity observed in intracellular neuron recordings explains the aforementioned decrease in call duration in response to altering GABAergic inhibition without spread of an $GABA_A$ antagonist to VPP. Second, GABAergic inhibition is crucial to sustain the tonic hyperpolarization characteristic for VMN neurons. This tonic hyperpolarization prevents spontaneous firing of VMN neurons and thus facilitates temporal precision and neuronal synchrony which are crucial features of vocal motor pattern generation. Lastly, tonic hyperpolarization in VMN is mediated, at least in part, via $GABA_A$ receptors.

The role of $GABA_B$ receptors in GABAergic inhibition at the VMN is less clear. In an electrophysiological pilot study that I conducted in midshipman fish, I investigated the effect of the $GABA_B$ antagonist saclofen (Fig. 1). I locally injected saclofen into the VMN of midshipman fish and recorded the vocal motor output on the occipital nerve roots exiting the hindbrain (see appendix for detailed description of material and methods and results). Preliminary results showed no significant change in the vocal motor output in response to local injection of saclofen, while a similar study also in midshipman fish showed that call duration and call

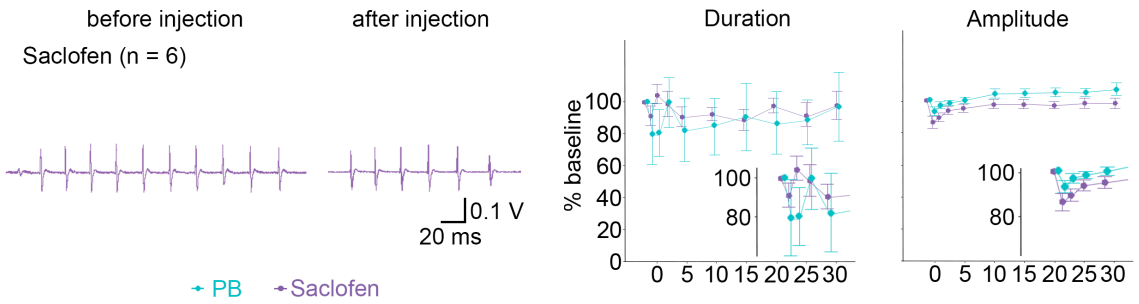

FIGURE 1: RESPONSE IN CALL DURATION AND CALL AMPLITUDE TO INJECTION OF SACLOFEN IN MIDSHIPMAN FISH.

Examples of vocal nerve recording illustrating changes in vocal pattern before and after injection are given on the left, response of call duration is shown in the middle and response of call amplitude is shown on the right. Purple trace represents saclofen (10 mM). Turquoise trace represents phosphate buffer (PB). Inserts in line graph for duration and amplitude show measurements before injection and 0, 1, 2.5 and 5 min after injection. Single points represent mean values for all animals tested (n = 6) with standard error. Mean values are given as proportion to the baseline measurement.

amplitude were reduced in response to local injection of baclofen, a second $GABA_B$ antagonist (Rubow, 2010). Baclofen injection experiments were conducted during breeding season of midshipman fish with an electrical stimulation of 75 µA (Rubow, 2010), while I performed my pilot study after the breeding season stimulating at call threshold which was lower than 75 µA. I stimulated at call threshold which is the minimum current needed to evoke a vocal response to avoid exhausting the vocal site during the experimental procedure. Since type I males of midshipman fish are more prone to vocalize during breeding season (Rubow and Bass, 2009), this might make vocal response more robust even at higher electrical stimulation. Thus, one possible, yet unlikely, reason for the diverting response to injection of $GABA_B$ antagonists might be these different stimulation currents. The seasonal changes in gene expression known from these fishes (Fergus *et al.*, 2015; Forlano and Bass, 2005; Rohmann *et al.*, 2013) including GABA receptor subunits within the VMN (Feng *et al.*, 2015) might also have contributed to the differences seen between these two sets of experiments. To test for a seasonal dependence, saclofen injection experiments should be repeated under the same experimental conditions as for baclofen and with threshold activation currents.

As in VMN, local injection of GABA into VPP of midshipman fish decreased call duration – the call parameter that is controlled by VPP (Rubow, 2010). While small amounts of the $GABA_A$ antagonist muscimol and gabazine also reduced call duration after local injection into

VPP, injection of greater amounts of gabazine disinhibited VPP resulting in irregular, buzz-like vocalizations with a long duration (Rubow, 2010).

GABA was also shown to affect vocal motor patterns in other vertebrate species. Heavy GABAergic inhibition onto the periaqueductal gray (PAG) of squirrel monkeys completely inhibited vocalization, while lowering GABAergic inhibition via $GABA_A$ antagonists lowered the threshold for vocalization in the PAG (Jürgens and Lu, 1993). Benzodiazepines and allopregnanolone administration, both active at $GABA_A$ receptors, reduced isolation vocalizations in rat pups (Insel *et al.*, 1986; Zimmerberg *et al.*, 1994). In accordance with GABAergic projections that were observed to the song control system of zebra finches (Grisham and Arnold, 1994), injection of bicuculline into the robust nucleus of the archostriatum of zebra finches increased neuronal activity leading to lower vocalization thresholds and sometimes elicited spontaneous vocalizations. Thus, GABAergic inhibition appears to be of tremendous importance in producing vocal motor patterns not only in the vocal CPG of toadfish but also in other vocal vertebrates.

4.1.2 Glycinergic Innervation and its implications for vocal motor output generation

Glycine is another inhibitory neurotransmitter active in the brain of vertebrates. Chapter 1 showed - for the first time - glycinergic label in all nuclei of a vocal CPG in toadfish (*O. beta*). In my pilot study, I show glycinergic projections in the VMN of midshipman fish (appendix). Glycinergic neurons were observed within and close by the VMN, VPN and VPP. While none of the aforementioned GABAergic neurons were labeled with neurobiotin, that due to its small molecular weight is able to cross gap junctions, a subset of the glycinergic neurons in VPN were co-labeled with neurobiotin indicating electrical coupling to the vocal CPG.

Since the vocal CPG of toadfish is usually identified using a tracer able to cross gap junction, i.e., neurobiotin (Bass and Baker, 1990), this method has served as a means to define the boundaries of the vocal CPG nuclei assigning neurons that were labeled with such a tracer as part of the vocal CPG. Chapter 1, however, revealed that there are neurons either within or

surrounding vocal CPG nuclei that are not labeled with neurobiotin but with an inhibitory or modulatory neurotransmitter. For example, glycinergic neurons in VPN did not differ in size or shape from neurons that were glycinergic and neurobiotin-positive. Thus, they morphologically form a continuous neuron group within VPN and would be indistinguishable without the neurobiotin tracer. These glycine-neurobiotin co-labeled neurons within VPN thus raised the question if the classical definition of the vocal CPG nuclei should be rethought. The definition of a neuron being part of the vocal CPG, should thus in the future also include neurons that are immediately surrounded or next to labeled vocal CPG neurons.

In my aforementioned pilot study, I also investigated the effect of glycinergic agents on the vocal output generated by VMN (Fig. 2). In response to injections of glycine, call amplitude was significantly reduced shortly after injection of glycine (Fig. 2A right), while the glycine antagonist strychnine had no effect (Fig. 2B). The missing response to injection of strychnine might again be related to the fact that experiments were performed outside of the breeding season of midshipman fish, or that the concentration of strychnine was low (10 µM) for a bulk injection. Test injections showed that a higher concentration of strychnine led to complete failure of VMN neuronal output for the first time points of the recording period. A higher stimulation current (75 µA) and shifting experiments into breeding season would potentially enable the use of a higher concentration of strychnine.

Nevertheless, the glycinergic inhibition had a significant effect in modulating call amplitude probably working via a different mechanism than GABAergic inhibition. Three mutually not exclusive putative circuits of how the glycine-neurobiotin co-labeled neurons might influence vocal activity can be imagined (Fig. 2C-E). The first circuit starts with a direct activation of VPN neurobiotin-only neurons via VPP that at the same time activates the glycine-neurobiotin VPN neurons via gap junctional coupling (Fig. 2C). The co-activated glycine-neurobiotin VPN neurons would then - via a chemical (glycinergic) synapse - inhibit the neurobiotin-only VPN neurons. This kind of recurrent inhibition might provide the oscillatory-like firing pattern that is characteristic for VPN neurons observed during vocal activity (Bass and Baker, 1990; Chagnaud *et al.*, 2011) making this not an intrinsic property of the

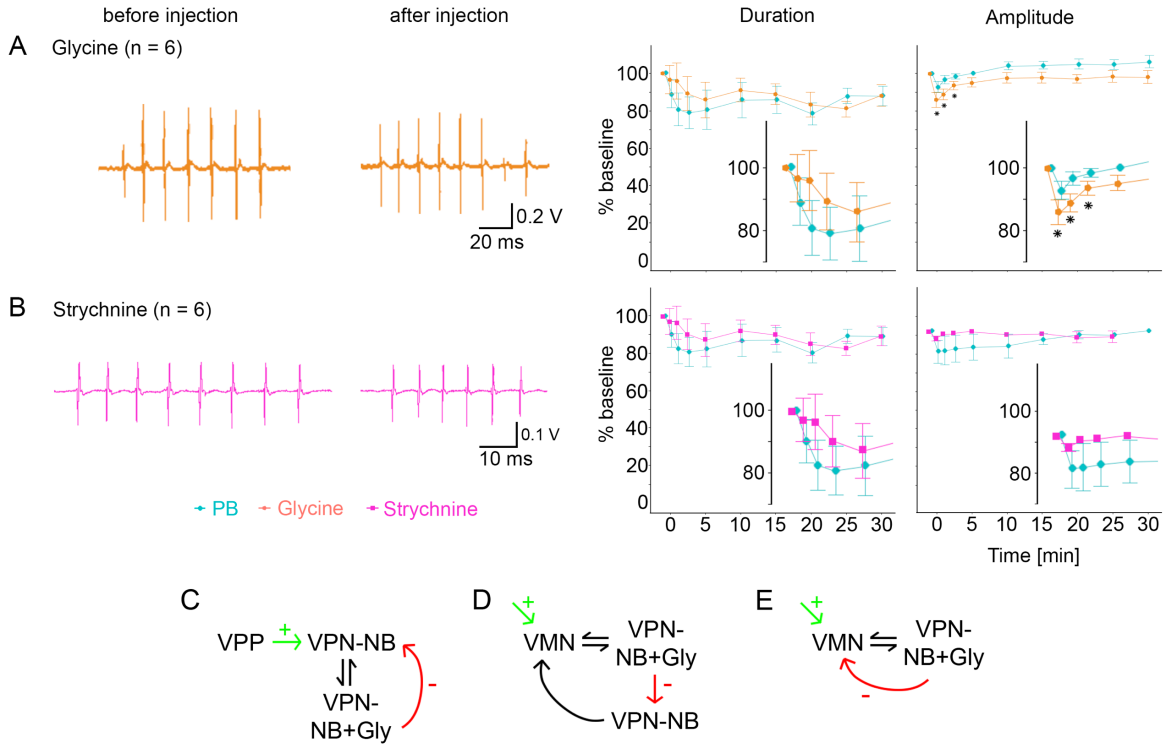

FIGURE 2: EFFECT OF GLYCINERGIC INHIBITION ON VOCAL MOTOR OUTPUT AND POTENTIAL VPN/VMN CIRCUITS. (A, B) Response in call duration and call amplitude to injection of glycine (A; orange; concentration = 0.2 M) and strychnine (B; pink; concentration = 10 µm) in midshipman fish. For each panel, examples of vocal nerve recording illustrating changes in vocal pattern before and after injection are given on the left, response of call duration in the middle and response to call amplitude on the right. Turquoise represents response to sham injection of phosphate buffer (PB). Inserts in line graph for duration and amplitude show measurements before injection and 0, 1, 2.5 and 5 min after injection. Call amplitude was significantly reduced in response to injection of glycine from directly after until 2.5 min (A, middle). Single points represent mean values with standard error for all animals tested with one agent (n = 6). Mean values are given as proportion to the baseline measurement. Stars represent significant changes ($p < 0.05$) of call parameters. (C-E) Potential circuits between neurobiotin (NB)-glycine (Gly) VPN neurons and other components of the toadfish vocal CPG. Plus/green arrows indicate a depolarizing, synaptic input. Minus/red arrows indicate a glycinergic, synaptic inhibitory input. Double arrows symbolize a gap junction. Abbreviations: VMN vocal motor nucleus, VPN vocal pacemaker nucleus, VPP vocal pre-pacemaker nucleus.

neurobiotin-only VPN neurons but a network property. In favor of this hypothesis, current injection into VPN neurons during vocal activity does not induce the characteristic oscillatory-like firing pattern (Chagnaud *et al.*, 2011).

Second, gap junctional coupling between VMN neurons and the glycine-neurobiotin co-labelled VPN neurons would depolarize glycine-neurobiotin VPN neurons (Fig. 2D). The now activated glycine-neurobiotin VPN neurons could then modify the activity of neurobiotin-only VPN changing the firing frequency of VPN and thus modify VMN activity. Recently, such

coupling between motor and premotor neurons has been shown in the vocal central pattern generator of *Xenopus* (Lawton *et al.*, 2017) and in the locomotor networks of zebrafish and *Drosophila* affecting the motor rhythm (Matsunaga *et al.*, 2017).

Lastly, electric coupling of glycine-neurobiotin VPN and VMN neurons and depolarization of the first could also result in inhibition of VMN neurons via a glycinergic synapse (Fig. 2E). This inhibition of VMN neurons would suppress VMN activity directly after activation preventing sustained VMN activity and thus supporting synchronous firing of VMN neurons. Glycinergic projections were also observed to the vocal motor neurons of zebra finches which, as in toadfish, are located in the hindbrain (Sturdy *et al.*, 2003) and glycinergic inhibition of the same was observed after *in vitro* electrical stimulation of the medullary respiratory areas (Sturdy *et al.*, 2003). If these glycinergic neurons are electrically coupled to the vocal motor neurons remains, to this date, unknown.

Thus, as does GABAergic inhibition, glycinergic inhibition also contributes to generating the extreme synchrony and temporal precision characterizing the VMN-VPN circuit. The recurrent inhibition and the VMN dependent activity of glycine-neurobiotin VPN neurons described here give a possible explanation how multiple vocal signals with different temporal and spectral properties might be generated within the vocal CPG of toadfish.

4.2 Modulatory neurotransmitters in the brain of vocal vertebrates

In addition to inhibition, neuromodulation is another mechanism altering neuronal activity, hence has the potential to produce different types of signals for social communication. Classic neuromodulators are catecholamines and serotonin, but acetylcholine can also act as a neuromodulator in the brain of vertebrates (Picciotto *et al.*, 2012). The next sections will describe their presence in vocal neuronal areas and their effect on the generated vocal output.

4.2.1 Catecholamine innervation and its potential inhibitory influence on vocal output generation

Catecholaminergic input was found at all levels of the vocal CPG in *O. beta* (chapter 1) as was previously reported in midshipman fish using different antibodies (Forlano *et al.*, 2014; Goebrecht *et al.*, 2014). Catecholaminergic neurons were observed in the area postrema dorsal of the VMN, likely providing input to the vocal CPG of *O. beta* as is the case in midshipman fish (Forlano *et al.*, 2014). Nevertheless, it cannot be excluded that also diencephalic dopaminergic regions, e.g., spinal projections from dopaminergic diencephalic neurons (Forlano *et al.*, 2014), provide catecholaminergic input to the vocal CPG of *O. beta*.

Catecholamines might have a suppressing effect on vocal activity. In a preliminary report, the injection of dopamine into the periaqueductal grey of midshipman fish inhibited vocal motor output, while injection of dopamine antagonists partly prevented this inhibition (Heisler and Kittelberger, 2012). Consistently in midshipman fish, the VMN of the highly vocal type I males that produce long courtship calls has a weaker catecholaminergic innervation compared to the less vocal type II males that sneak fertilization (Ghahramani *et al.*, 2015b; Goebrecht *et al.*, 2014; for behavior see also Brantley & Bass, 1994). Additionally, the expression of dopamine subtypes is upregulated in the VMN and surrounding areas in midshipman fish (Feng *et al.*, 2015). Catecholamines probably also alter vocal activity in forebrain vocal areas of toadfishes, i.e., the anterior hypothalamus (Forlano and Bass, 2011; Forlano *et al.*, 2014; Kittelberger *et al.*, 2006). Similar effects were observed *in vivo* in the green tree frogs *Hyla cinerea*, where intraperitoneal injection of dopamine agonists decreased advertisement calling by affecting dopamine-like receptor activity (Creighton *et al.*, 2013), and in *in vitro* brain slice preparations in zebra finches where noradrenaline decreased activity in the robust nucleus of the arcopallium (Solis and Perkel, 2006). Seemingly in contrast, vocal areas of singing male canaries, *Serinus canaria,* received stronger catecholaminergic input compared to non-singing females (Appeltants *et al.*, 2003). The physiological consequences of this stronger input need to be investigated.

4.2.2 Cholinergic innervation at (vocal) motor centers

Acetylcholine is the neurotransmitter active at the neuromuscular junction of vertebrates and is therefore found in their motor neurons. As expected, cholinergic label was present within motor neurons of VMN in *O. beta* (chapter 1), in line with previous results for the VMN of midshipman fish using a different antibody (Brantley and Bass, 1988).

Somewhat unexpectedly, cholinergic projections were also observed in VPN and VPP of *O. beta* along with cholinergic neurons within and adjacent to the nuclei (chapter 1), indicating that acetylcholine could also modulate VPN activity (e.g., Picciotto *et al.*, 2012). While no effect on vocal production was observed after injection of acetylcholine antagonists into vocal midbrain sites of squirrel monkeys (Jürgens and Lu, 1993), it affected duration, frequency and amplitude of spontaneous inspiratory bursts after *in vivo* and *in vitro* application of acetylcholine and its agonists to respiratory pacemaker-like neurons of the pre-Bötzinger complex in rats (Burton *et al.*, 1997; Monteau *et al.*, 1990; Murakoshi *et al.*, 1985; Shao and Feldman, 2000). Thus, acetylcholine might enhance activity of premotor centers in hindbrain pattern generators, including the toadfish vocal CPG.

4.2.3 Serotoninergic system in ray-finned fishes and its role in vocal output generation

4.2.3.1 Serotoninergic innervation at vocal areas

Serotonin is another classical neuromodulator in the brain of vertebrates. In chapter 1, serotonergic projections were observed in VMN, VPN and VPP of *O. beta*. Serotonergic label throughout the whole vocal CPG was also reported in midshipman fish (Forlano *et al.*, 2011; Timothy *et al.*, 2015), the oyster toadfish (Marchaterre *et al.*, 1989) and the grunting toadfish (chapter 2). Serotonergic projections were also observed to respiratory motor neurons that control airflow in vocalizations in cats (Holtman, 1988), to laryngeal premotor and motor neurons in *X. laevis* (Yamaguchi *et al.*, 2000) and to the nucleus of the arcopallium in zebra finches (Wood *et al.*, 2011). Thus, serotonergic input to vocal motor areas might be a conserved pattern in vocal vertebrates.

Since serotonergic input was observed at all levels of the vocal CPG in toadfish, it holds the potential to modulate all three call parameters – duration, frequency and amplitude. Injection of serotonin in the robust nucleus of the arcopallium (RA) in slice preparations of zebra finch brains (*Taeniopygia guttata*) enhanced endogenous firing of RA neurons (Wood *et al.*, 2011) and single unit *in vivo* recordings in RA revealed serotonergic modulation of spectral song properties (Wood *et al.*, 2013). In an isolated hindbrain preparation of *X. laevis*, vocal motor patterns could be initiated after bath application of serotonin (Yu and Yamaguchi, 2010). In *in vivo* and *in vitro* recordings on periaqueductal gray cells of rats, injection of 8-OH-DPAT, a serotonin agonist, had an inhibitory effect (Behbehani *et al.*, 1993), while Injection of serotonin antagonists into the periaqueductal gray of squirrel monkeys did not alter vocalizations (Lu and Fay, 1993). Thus, serotonin has an activating effect on motor and premotor neurons, while it might has an inhibitory effect on higher vocal control centers.

Serotonergic innervation of the vocal CPG likely originates from the serotonergic neurons that were observed close to the VPP, as part of the inferior raphe, and within VMN in *O. beta* (chapter 1). Serotonergic neurons were also observed within VMN in *O. tau* (Marchaterre *et al.*, 1989) and *A. grunniens* (chapter 2). The serotonergic neurons within VMN are most likely displaced inferior raphe cells since this is the serotonergic populations typically described close by. While serotonergic neurons were also reported close by the hindbrain motor neurons innervating the swim bladder associated musculature in the socially communicating catfishes *A. seemanni* and *S. nigriventris* (chapter 2), none were reported within the motor neuronal group. Thus, displaced inferior raphe neurons into motor nuclei involved in social signal production might be a feature unique to toadfish.

Chapter 2 also showed serotonergic fibers to hindbrain motor neurons involved in social signaling in *S. nigriventris* and to their dendritic region in *A. seemanni*. Serotonergic neurons were observed close by these hindbrain motor neurons in both studied catfishes. While these hindbrain motor neurons were previously regarded as sonic motor neurons in catfishes (Ladich and Bass, 1998), it was recently shown that they are used for electric signaling in the catfish *S. nigriventris* (Boyle *et al.*, 2014). The above described differences in

the serotonergic input to vocal hindbrain motor neurons in *A. seemanni* and electromotor hindbrain motor neurons in *S. nigriventris*, are however likely to reflect species-specific differences, rather than relating to the type of social signal production because serotonergic projections were also present in the VMN of the vocal teleost fishes, i.e., toadfishes (chapter 1; Forlano *et al.*, 2011; Marchaterre *et al.*, 1989; Timothy *et al.*, 2015).

4.2.3.2 SEROTONINERGIC POPULATIONS IN RAY-FINNED FISHES

In addition to serotonergic neurons close by VMN and EMN and within VMN, chapter 2 showed that serotonin-containing neurons were also present in the diencephalon and rhombencephalon of *A. grunniens*, *A. seemanni* and *S. nigriventris*, but none in the telencephalon and mesencephalon. The next sections will compare the serotonergic populations observed in these teleost species to serotonergic populations described in other fishes to see if social communication influences the general organization of the serotonergic system.

Serotonergic populations in the telencephalon are not ancestral

While the absence of serotonergic neurons in the telencephalon of *A. grunniens*, *A. seemanni* and *S. nigriventris* reflects the picture in most other studied teleost species (Batten *et al.*, 1993; Corio *et al.*, 1991; Ekström and Veen, 1984; Frankenhuis-van den Heuvel and Nieuwenhuys, 1984; Grant *et al.*, 1989; Johnston *et al.*, 1990; Kah and Chambolle, 1983; Margolis-Kazan *et al.*, 1985; Meek and Joosten, 1989; Rodríguez-Gómez *et al.*, 2000), serotonergic neurons were described in the ventral (subpallial) telencephalon rostral to the anterior commissure and the olfactory bulb in the sturgeons *Acipenser baeri* and *Huso huso* (Adrio *et al.*, 2011), and in olfactory bulb of the Atlantic croaker, *M. undulates* (Khan and Thomas, 1993). This data at first glance seems to align with the restriction hypothesis which proposes that in the ancestral condition serotonergic neurons were present in all major parts of the brain but got concentrated within the rhombencephalic raphe in the mammalian brain (Herculano and Maximino, 2014; Lillesaar, 2011; Parent *et al.*, 1984). Yet, serotonergic

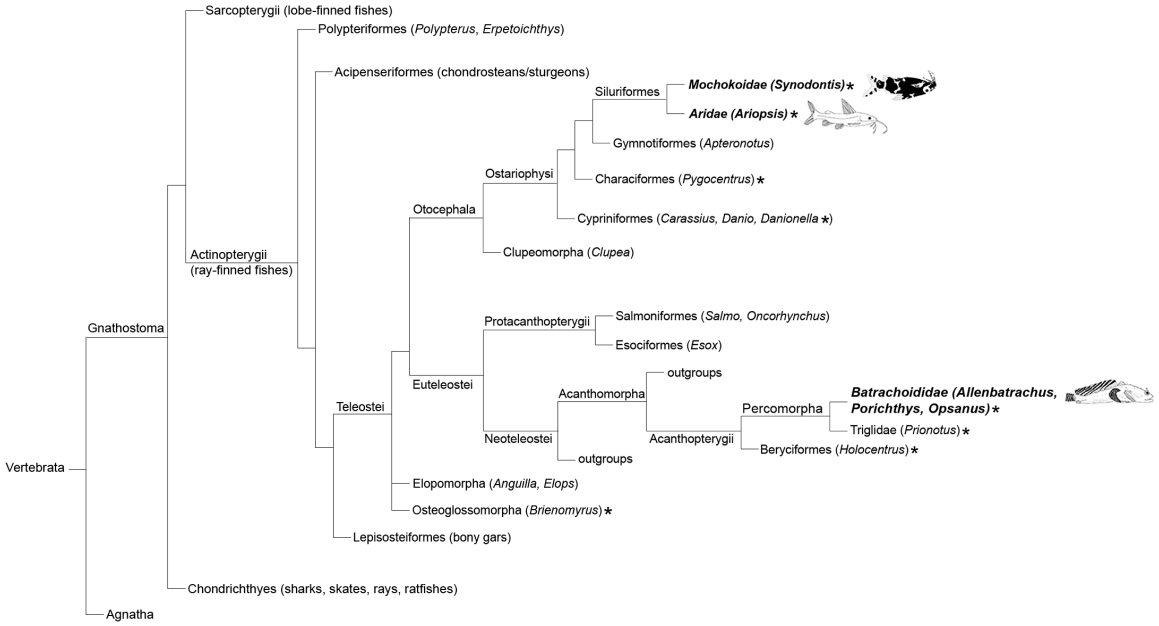

FIGURE 3: SIMPLIFIED CLADOGRAM DESCRIBING THE PHYLOGENETIC RELATIONSHIP OF TELESOSTS IN THE BROADER CONTEXT OF VERTEBRATES.

Systematics are based on Hughes et al. (2018), Diogo et al. (2008) and Near et al. (2012). An asterisk identifies clades where species producing swim bladder dependent vocal signalling are found. Body outlines of the three species studied in chapter 2 are displayed behind their respective family. Adapted from Rosner et al. (2019).

neurons are also missing in the telencephalon of ancestral ray-finned fishes (Chiba, 1999; Chiba and Oka, 1999; López and González, 2014; Parent and Northcutt, 1982), cartilaginous fishes (Carrera *et al.*, 2008; Northcutt *et al.*, 1988; Ritchie *et al.*, 1983; Stuesse *et al.*, 1991; Stuesse and Cruce, 1991; Stuesse *et al.*, 1990), agnathans and all sarcopterygians (reviewed in López and González, 2014) suggesting that telencephalic serotonergic neurons newly evolved in sturgeons (see Fig. 3 for simplified cladogram).

Diencephalic serotonergic neurons are mostly cerebrospinal-fluid contacting

In the diencephalon, large bipolar, cerebrospinal-fluid (CSF)-contacting serotonergic neurons were observed in the paraventricular organ (PVO) and the dorsal and caudal zone of the periventricular hypothalamus in *A. grunniens*, *A. seemanni* and *S. nigriventris* (chapter 2). The extent of the PVO is characterized by the presence of serotonergic neurons, while serotonergic neurons of the dorsal and caudal periventricular hypothalamus line the lateral and posterior recess, respectively. In case of *A. grunniens,* serotonergic neurons were observed in

neuronal tissue that invaded the lateral recess most likely originating from the dorsal zone of the hypothalamus (chapter 2). This intraventricular mass was also present in both catfishes *A. seemanni* and *S. nigriventris* (chapter 2) corresponding to the lateral tuberal nucleus described in channel catfish (Striedter, 1990).

CSF-contacting serotonergic neurons were also found in various teleost fish species in the PVO and hypothalamus (Batten *et al.*, 1993; Corio *et al.*, 1991; Ekström and Veen, 1984; Frankenhuis-van den Heuvel and Nieuwenhuys, 1984; Grant *et al.*, 1989; Johnston *et al.*, 1990; Kah and Chambolle, 1983; Khan and Thomas, 1993; Margolis-Kazan *et al.*, 1985; Meek and Joosten, 1989; Rodríguez-Gómez *et al.*, 2000), in chondrostreans (Adrio *et al.*, 1999), polypteriforms (Chiba, 1999; López and González, 2014) and lepisosteiforms (Chiba and Oka, 1999; Northcutt *et al.*, 1988), although sometimes using a different terminology or, in case of the PVO, being regarded as part of the posterior tuberculum. Lampreys, on the other hand, have an uninterrupted population of CSF-contacting serotonergic neurons from posterior tuberculum to hypothalamus (Abalo *et al.*, 2007; Cornide-Petronio *et al.*, 2013; Pierre *et al.*, 1992). In sarcopterygians, i.e., lungfishes, and similarly amphibians, the extended PVO includes hypothalamic divisions where a midline posterior tubercular subdivision between hypothalamus and prethalamus can be distinguished (López and González, 2015). In some teleost species including the three-spined stickleback (Ekström and Veen, 1984), serotonergic neurons were limited to a section dorsal of the lateral recess which was termed intermediate hypothalamus in zebrafish (Rink and Guo, 2004; Rink and Wullimann, 2001) and could also be identified in *A. grunniens, A. seemanni* and *S. nigriventris* (chapter 2). Thus, CSF-contacting serotonergic neurons in PVO and hypothalamus are common in vertebrates, but are finally lost in mammals (López and González, 2014; see Fig. 3 for simplified cladogram).

In *A. seemanni* and *S. nigriventris*, midline CSF-contacting serotonergic neurons of the PVO extended dorsal into the periventricular nucleus of the posterior tuberculum (TPp, chapter 2). The same situation is found in few other teleost species (Corio *et al.*, 1991; Johnston *et al.*, 1990; Khan and Thomas, 1993; Margolis-Kazan *et al.*, 1985; Meek and Joosten, 1989). Typically, the PVO is characterized by serotonergic neurons, while TPp is characterized by

magno- and parvocellular dopaminergic neurons (Kaslin and Paula, 2001; Rink and Wullimann, 2001). The dopaminergic character of TPp was confirmed in *S. nigriventris* by co-staining with antibodies against serotonin and tyrosine hydroxylase (TH) as a marker for catecholamines (chapter 2). This co-staining showed no co-localization of serotonin and TH which agrees with another immunohistochemical study in zebrafish (Kaslin and Paula, 2001). Contrary, a recent *in situ* hybridization study revealed co-localization of the genes *th2* and *tph1*, coding for the synthetic enzymes tyrosine hydroxylase 2 and tryptophan hydroxylase 1, respectively, in the PVO of chicken, *X. laevis* and zebrafish (Xavier *et al.*, 2017). Additionally, immunohistochemical analysis showed co-localization of serotonin and dopamine in the zebrafish brain (Xavier *et al.*, 2017). These contrary observations can be resolved when considering two facts. First, two TH genes exist – *th1* and *th2* (Yamamoto *et al.*, 2010, 2011) – with *th2* being exclusively expressed in most CSF-contacting neurons in the PVO of chicken, *X. laevis* and zebrafish. Second, commercial antibodies against TH often only detect the TH1 enzyme while failing to reveal TH2 (Xavier *et al.*, 2017). Thus, the few dopaminergic neurons that were found in the PVO and dorsal and caudal periventricular hypothalamus might be *th1*-expressing neurons only (Kaslin and Paula, 2001; Rink and Wullimann, 2001). The lack of dopaminergic neurons in the dorsal and caudal periventricular hypothalamus in *S. nigriventris* points to the fact the antibody used against TH only detects *th1* and potential dopaminergic neurons in the periventricular hypothalamus of *S. nigriventris* must be *th2*-expressing cells. Thus, the observations of both immunohistochemical studies on zebrafish (Kaslin and Paula, 2001) and *S. nigriventris* (chapter 2) as well as those of the *in situ* hybridization study (Xavier *et al.*, 2017) are presumably correct indicating that *th1*-dopamine neurons do not co-localize with serotonin.

In addition to PVO and hypothalamus, smaller, non-CSF-contacting serotonergic populations were observed in the pre-optic area of the two catfishes *A. seemanni* and *S. nigriventris* but not in toadfish and in the pretectum of both catfishes and of the toadfish *A. grunniens* (chapter 2). Serotonergic neurons were also observed in the pre-optic area of few other teleost fishes (Grant *et al.*, 1989; Johnston *et al.*, 1990; Khan and Thomas, 1993), in

cartilaginous fishes (Carrera *et al.*, 2008; Ritchie *et al.*, 1983; Stuesse *et al.*, 1991; Stuesse and Cruce, 1991), chondrosteans (Adrio *et al.*, 1999), polypteriforms (Chiba, 1999; López and González, 2014) and agnathans (reviewed in López and González, 2014). But they are absent in numerous other teleost fish and cartilaginous fishes, lepisosteiforms and all amniotes except in the lungfish and amphibians (reviewed in López and González, 2014). Thus, the two catfishes share the ancestral situation in other anamniotes, while teleosts such as *A. grunniens* might have lost the pre-optic serotonergic population independently of amniotes (see Fig. 3 for simplified cladogram).

The periventricular serotonergic neurons next to the fasciculus retroflexus has been previously regarded as part of the thalamus (e.g., Rink and Wullimann, 2001). Today, they are seen as part of the pretectum supported by the presence of separate populations of periventricular serotonergic and dopaminergic neurons which project to the optic tectum (Kress and Wullimann, 2012; Lillesaar *et al.*, 2009). Bilateral serotonergic populations were also described in the pretectum of other teleosts (Corio *et al.*, 1991; Ekström and Ebbeson, 1989; Ekström and Veen, 1984; Frankenhuis-van den Heuvel and Nieuwenhuys, 1984; Grant *et al.*, 1989; Johnston *et al.*, 1990; Kah and Chambolle, 1983; Kaslin and Paula, 2001; Khan and Thomas, 1993; Margolis-Kazan *et al.*, 1985; Meek and Joosten, 1989; Reiner and Northcutt, 1992), sturgeons (Adrio *et al.*, 1999) and polypteriforms (López and González, 2014), but are lost in most tetrapods (López and González, 2014). This suggest that a pretectal serotonergic population is the ancestral condition in vertebrates (see Fig. 3 for simplified cladogram).

Besides the diencephalic serotonergic populations concentrated in PVO and hypothalamus with the smaller populations in pretectum and pre-optic area, serotonergic neurons were found in the pituitary gland of three-spined stickleback (Ekström and Veen, 1984) and common platyfish (Margolis-Kazan *et al.*, 1985) as well as in the habenula next to the pineal organ in sockeye salmon fry (Ekström and Ebbeson, 1989).

Serotonin-positive neurons were observed in the pineal organ of *A. grunniens*, *A. seemanni* and *S. nigriventris* which might be linked to the synthesis of melatonin. Melatonin is a hormone derived from serotonin. Therefore, serotonin-containing neurons are universally

present in the pineal organ of vertebrates (López and González, 2014) including teleosts (Corio *et al.*, 1991; Ekström and Ebbeson, 1989; Ekström and Veen, 1984; Frankenhuis-van den Heuvel and Nieuwenhuys, 1984; Kaslin and Paula, 2001; Margolis-Kazan *et al.*, 1985).

No mesencephalic serotonergic populations described in socially communicating fishes

No mesencephalic serotonergic neurons were found in *A. grunniens*, *A. seemanni* and *S. nigriventris* which is in line with the situation in most teleosts (see above), but few serotonergic cells were observed in the optic tectum of the European bass, *D. labrax,* (Batten *et al.*, 1993) as well as in the torus semicircularis and midbrain tegmentum in *M. undulates* (Khan and Thomas, 1993).

Rhombencephalic serotonergic populations are concentrated in the raphe nuclei

In the rhombencephalon, the grunting toadfish *A. grunniens* and both catfishes *A. seemanni* and *S. nigriventris* showed serotonergic neurons in the superior and inferior raphe nucleus (chapter 2). Both raphe nuclei are described in most studied fish species (Frankenhuis-van den Heuvel and Nieuwenhuys, 1984; Grant *et al.*, 1989; Kah and Chambolle, 1983; Margolis-Kazan *et al.*, 1985; Meek and Joosten, 1989; Rodríguez-Gómez *et al.*, 2000)

In some fish species, a dorsal and medial superior raphe can be recognized according to the nomenclature in mammals (Adrio *et al.*, 1999; Batten *et al.*, 1993; Corio *et al.*, 1991; Ekström and Ebbeson, 1989; Ekström and Veen, 1984; Johnston *et al.*, 1990; Kaslin and Paula, 2001; Khan and Thomas, 1993). A rough clustering in mediodorsal, medial, medioventral and lateral appeared in the superior raphe of *A. grunniens* especially at an intermediate level (chapter 2). Due to the lack of distinct gaps and missing tract tracing of these potential clusters, the superior raphe was chosen not to be further subdivided in *A. grunniens*. In the elephant-nose fish, an intermediate raphe was described between superior and inferior raphe (Grant *et al.*, 1989; Meek and Joosten, 1989) and a raphe magnus was observed in Sockeye salmon fry (Ekström and Ebbeson, 1989). No division in superior and inferior raphe was found for serotonergic raphe cells in goldfish and the common platyfish (Kah and

Chambolle, 1983; Margolis-Kazan *et al.*, 1985), while no inferior raphe was described in African catfish (Corio *et al.*, 1991). However, this lack might has methodological reasons since the inferior raphe nucleus of three-spined stickleback can only be visualized after as pre-treatment with monoamine-oxidase inhibitor (Ekström and Veen, 1984).

At one level with the raphe region, serotonergic neurons were also observed in the interpeduncular nucleus in Senegalese sole (Rodríguez-Gómez *et al.*, 2000). In *A. grunniens*, serotonergic neurons were present between the dorsal and ventral division of the interpeduncular nucleus, but regarded as part of the superior raphe (chapter 2). Additionally, serotonergic neurons were reported in the valvula of common platyfish (Margolis-Kazan *et al.*, 1985) and Atlantic croaker (Khan and Thomas, 1993).

Serotonergic neurons commonly occur in the medullary spinal cord junction of ray-finned fishes as well as in the reticular formation

Caudal of the raphe region, all actinopterygians have ventral serotonergic neurons scattered throughout the spinal cord. Additionally, few serotonergic neurons are found in the ventral medulla/anterior spinal cord level in *A. grunniens*, *A. seemanni*, *S. nigriventris* (chapter 2) and in European bass (Batten *et al.*, 1993), in the ventrolateral hindbrain between superior and inferior raphe in zebrafish (Lillesaar *et al.*, 2009), at one level with the inferior olive in elephant-nose fish (Meek and Joosten, 1989) and near the obex in Atlantic croaker (Khan and Thomas, 1993). In the reticular formation, serotonergic neurons were observed in European bass (Batten *et al.*, 1993), Senegalese sole (Rodríguez-Gómez *et al.*, 2000) and sturgeons (Adrio *et al.*, 1999). In sturgeons, four nuclei were described in the reticular formation named after the raphe nucleus that was positioned medial to them (Adrio *et al.*, 1999).

As shown in the previous sections, the serotonergic populations observed in the two vocal and one weakly electric teleost species investigated in chapter 2 is similar to that of other fishes. Thus, it seems that social communication does affect the general organization of the serotonergic system and observed differences are species-specific and rather reflect the

ancentral relationship to the serotonergic system in other fishes. Nevertheless, displaced serotonergic neurons within the VMN seem to be a feature characteristic for toadfishes.

4.3 CONCLUDING REMARKS

In my thesis, I investigated inhibitory and modulatory neurotransmitter in the brains of socially communicating teleost fishes. I showed pronounced GABAergic, glycinergic, serotonergic and catecholaminergic input to all levels of the vocal CPG, cholinergic input to VPN and VPP in the Gulf toadfish *O. beta* as well as serotonergic input to the VMN in the grunting toadfish *A. grunniens* and the catfish species *A. seemanni* and the to EMN in *S. nigriventris*.

The inhibitory inputs to the vocal CPG of toadfish together with my preliminary electrophysiological results on the effect of glycine and previous electrophysiological investigations indicate that a set of neurophysiological/neurochemical mechanisms within the vocal CPG that could allow to generate different vocal motor patterns. This generation of different vocalizations is essential for context-dependent vocal signal production. The observed gap junctional coupling between vocal CPG nuclei together with the presence of the neurotransmitters GABA and glycine might, in addition, contribute to the extreme temporal precision and synchrony, two essential properties of the toadfish vocal CPG. Due to the proposed evolutionary conserved organization of vocal CPGs and the available neurophysiological evidence in toadfishes, comparable neurochemically-dependent mechanisms might be present in other vertebrate vocal CPGs.

While the physiological interactions of GABA and glycine with serotonin, catecholamines and acetylcholine remain to be shown, chapter two of my thesis, focusing on the distribution of serotonergic populations in the brains of *A. grunniens, A. seemanni* and *S. nigriventris,* indicates that the extent of serotonergic innervation of hindbrain motor neurons involved in social signaling might be rather species-related than dependent on the type of social signals that is being generated. Nevertheless, the displaced inferior raphe cells observed in VMN of *O. beta* and *A. grunniens* appear, in this context, to be a distinguishing feature for toadfishes. Overall, the serotonergic system was similar between the three socially communicators *A. grunniens, A. seemanni* and *S. nigriventris* and in line with previous reports

indicating that ancestral relationship rather than social communication governs the general organization of the serotonergic system in these teleost species. However, serotonergic, liquor-contacting neurons of the paraventricular organ in both catfishes extended further dorsal into the periventricular nucleus of the paraventricular organ than expected. These were not co-localized with dopaminergic (most likely *th1*-expressing), liquor-contacting neurons of the PVO. Since TH antibodies were shown to primarily detecting the TH1 enzyme, this suggest that *th2*- but not *th1*-expressing cells co-localize with serotonin.

6 APPENDIX

Injection of inhibitory agents into the VMN of midshipman fish

Material and Methods

Animals

Plainfin midshipman fish (*Porichtyus notatus)* have two male morphs. Large, territorial type I males build nests and acoustically court females. Smaller, female-like type II steal fertilizations from type I males by sneaking into their nests or by satellite-spawning (Brantley and Bass, 1994). 18 adult type I midshipman fish (standard length = 12-20 cm, median = 14.4 ± 2.1 cm) were used in this study. Fish were collected by hand and housed alone or in groups of two (if small in body size) in tanks filled with salt water (salinity 30 ppm) in an environmental control room at 17 ± 2°C on a 14:10 h light:dark cycle. All surgical and experimental procedures were approved by the Cornell University Institutional Animal Care and Use Committee.

Surgery

Surgery followed procedures as previously described in Bass & Baker (1990) and Kittelberger & Bass (2013). In short, fish were deeply anaesthetized by immersion in 0.025% benzocaine (Sigma, St. Louis, MO, USA) in tank water. Bupivacaine hydrochloride (0.01 g/ml, 0.2 ml; Sigma, St. Louis, MO, USA) and pancuronium bromide (0.5 mg/kg body weight; Sigma, St. Louis, MO, USA) were additionally injected for long term anesthesia/analgesia and muscle relaxation/immobilization, respectively. A dorsal craniotomy exposed midbrain, hindbrain, rostral spinal cord and occipital nerve roots, hereafter referred to as vocal nerve. After craniotomy, fish were transferred to an experimental tank resting on a vibration isolation table (TMC, Peabody, MA, USA) where recirculated, chilled salt water (17 ± 2°C) was pumped across their gills. Fish were allowed to recover for 1 h after the surgery.

Stimulation

An extracellular ball electrode was positioned on the vocal nerve root to monitor fictive vocal output. Fictive vocalizations were amplified 1,000 times and band pass filtered from 300 Hz to 5 kHz (Model 1700, Differential AC amplifier, A-M Systems, Sequin, WA USA). Fictive vocalizations were evoked by current stimulation in previously identified vocal midbrain regions (Bass *et al.*, 1994; Goodson and Bass, 2002; Kittelberger and Bass, 2013). Current stimulations were delivered with a tungsten stimulation electrode. Sequences of stimulation bursts were provided by a stimulus isolator driven by a stimulus generator (Model A310 Accupluser, World Precision Instruments, Sarasota, FL USA) generating 5 transistor-transistor logic pulses at 200 Hz with intersequence intervals of 1 sec. Stimulation was at the call threshold, the minimum current stimulation that is needed to evoke vocal activity.

Injections and recording

Injection electrodes (glass microelectrodes, 1/0.58 OD/ID mm; World Precision Instruments, Sarasota, FL USA) pulled on a horizontal puller (Model P-97; Sutter Instruments Co., Novato, CA) with a diameter of ~20 µm were fabricated and filled with either one of the effectors – the $GABA_B$ antagonist saclofen, the neurotransmitter glycine or its antagonist strychnine – all dissolved in 0.1 M phosphate buffer (PB), or only 0.1 M PB (sham injection; all chemicals: Sigma, St. Louis, MO, USA). In all animals (six animals for each effector), two injections were performed: one with a neurotransmitter or antagonist and a second one with PB alone (control). The order of injections was randomized between animals with at least 1 h between end of first recording and second injection. Neurobiotin (5%; Vector Laboratories Inc., Burlingame, CA USA) was added to the injected solution to later confirm the injection sites.

Injections were delivered with a picospritzer (Biomedical Engineering, Thornwood, NY USA). Injection volume per ejection was defined by ejecting 10 times from an injection electrode to form a droplet on the tip of the electrode. This droplet was taken up into a microcapillary and the travelling distance of the fluid in the capillary was measured. Then, the injection volume was calculated using the following formula:

$$injection\ volume\ [in\ nL] = \frac{\frac{fluid\ len * cap\ vol}{cap\ len}}{nr\ ejections} \quad (1)$$

The length of the capillary (fluid len) is multiplied with the total volume of the microcapillary (cap vol; 100 nL), divided by the total length of the capillary (cap len; 32 mm). The result is divided by the number of ejections (nr ejections; 10) to get the injected volume of one ejection. The picospritzer was configured to deliver 25 nL per ejection. For each injection, I had the picospritzer eject 4 times. Thus, a total volume of 100 nL was injected.

The concentration of the effectors was determined in preliminary results. I determined the effector's concentration where I could observe a vocalization reaction to almost every stimulus during recording after injection and no or few failures. Thus, I determined 2 mM for glycine, 10 µm for strychnine and 10 mM for saclofen.

Recordings were performed directly before (baseline) and in defined time periods (0, 1, 2.5, 5, 10, 15, 20, 25 and 30 min) after injection. All recordings were 30 s long with a stimulus train each second. Recordings were digitized using the software Signal (Cambridge Electronic Design Limited, Cambridge, UK) and values for each time point extracted using custom-written scripts programmed in the software IGOR Pro 6 (WaveMetrics, Portland, OR USA).

Analysis

The vocal recordings consist of a series of spikes (vocal burst) which allow the estimation of vocal burst duration, frequency and amplitude (Rubow and Bass, 2009). Vocal burst duration is the time between the first and last spike. Vocal burst frequency is the number of spikes divided by the duration. Vocal burst amplitude is the distance between the peak amplitude of each spike from baseline levels. Means of these estimates were calculated to infer call duration, fundamental frequency and call amplitude of each animal at each recording time point for 30 sec. The values are given as proportion of the baseline measurement taken directly before injection.

A repeated measures ANOVA was performed to assess the change in call duration, fundamental frequency and call amplitude in response to effector or sham injection over time. First, normal distribution of residuals and homogeneity of variance was checked in each effector condition for each vocal parameter. If one or both assumptions were violated, data was rank transformed. Pairwise t-tests were performed as post-hoc test with Bonferroni correction for multiple comparisons. Data were considered to be significantly different when $p < 0.05$. All values are given as means with standard error of the mean. Data analysis was performed in R (version 3.4.3; R Core Team, 2014).

Labeling single neurons

To intracellularly label single VMN neurons, intracellular electrodes (resistance 35–60 MΩ) were pulled on a horizontal puller and filled with 5% neurobiotin in 0.5 M KCOOH (Sigma, St. Louis, MO, USA). Neuronal signals were amplified 100-fold (Biomedical Engineering, Thornwood, NY USA) and digitized at a rate of 20 kHz (Digidata 1322A; Axon instruments, Sunnyvale CA USA) using the software pCLAMP 9 (Axon instruments). An external clock (Biomedical Engineering, Thornwood, NY USA) sending transistor-transistor logic pulses synchronized stimulus delivery and data acquisition. A current step applied to the recording electrode was used to monitor electrode resistance during the search for neurons. To label a single neuron, a positive current (4 - 10 nA) with a duty cycle of 50% at 2 to 4 Hz was passed through a neurobiotin-filled recording electrode for 3 to 30 min. Multiple neurons were injected. After a 2 to 6 h survival, fish were deeply anesthetized (0.025% benzocaine) and perfused with ice-cold teleost Ringer solution (Cavanaugh, 1956) with 10 U/ml heparin (Elkins-Sinn, Cherry Hill, NJ USA), followed by 3.5% paraformaldehyde and 0.25% glutaraldehyde in 0.1 M PB. Brains were removed from the skull, postfixated in the same solution for 1 h and stored in 0.1 M PB at 4°C.

Immunohistochemistry (IHC)

Brains with intracellularly labelled neurons were immunohistochemically stained for GABA and glycine. First, brains were cryoprotected by incubation in 30% sucrose in 0.1 M PB (CARL ROTH GmbH + Co. KG, Karlsruhe, Germany) at 4°C overnight. The next day, brains were sectioned at a cryostat (Leica microsystems, Wetzlar, Germany) in the transverse plane at 25 µm and directly mounted onto microscope slides (Superfrost Ultra Plus Adhesion Slides; Thermo Fisher Scientific Inc., Braunschweig, Germany). Subsequently, slides were left at room temperature for 1 h to allow the sections to dry and then either immediately processed for IHC or stored at -80°C.

Antibody staining followed the protocol for toadfish described previously (Rosner *et al.*, 2018). In short, slides were immersed in 0.1M PB-saline (PBS; Sigma Aldrich Chemie GmbH, Munich, Germany) for 30 min to rehydrate, followed by three washing steps in 0.001% sodium borohydride (Sigma Aldrich Chemie GmbH, Munich, Germany) diluted in 1 ml 0.1 M PBS to reduce glutaraldehyde background. A washing series (four times for 5 min) in 0.5% Triton 100 (Sigma Aldrich Chemie GmbH, Munich, Germany) in 0.1 M PBS (PBS-T) and incubation in 10% normal donkey serum (NDS; Jackson Immunoresearch Europe Ltd., Suffolk, United Kingdom) in PBS-T for 1h followed afterwards. Subsequently, slides were incubated in the primary antibody (Table 1) overnight. The next day, a washing series (four times for 5 min) followed in PBS-T before incubation in the secondary antibody (Table 1) for 4h. After secondary antibody incubation, slides were again washed four times for 5 min with PBS-T, dried and then coverslipped using a fluorescent mounting medium containing 4',6-Diamidino-2-Phenyindole (DAPI; Vectashield with DAPI, Vector Labs Inc., Peterborough, United Kingdom). All incubation and washing steps were performed at room temperature.

Images of brain sections were acquired on a confocal laser microscope (model: SP5, Leica microsystems, Wetzlar, Germany). Images were further processed in Fiji (stacked and maximum z projected; Schindelin *et al.*, 2012) and Adobe Photoshop CS6 (cropped, resized, contrast and brightness optimization; Adobe Systems Software Ireland Limited, Dublin, Ireland).

TABLE 1

List of used primary and secondary antibodies including their name, immunogen, manufacturer, catalogue number, RRID, host, antibody type, the dilution used in this study and fixation type. Abbreviations: PFA/GLUT, 3.75% paraformaldehyde, 0.25% glutaraldehyde.

Antibody name	Immunogen	Manufacturer, Catalogue number, RRID, Host, Antibody type	Dilution	Fixation type
Primary antibodies				
GABA	GABA coupled to bovine serum albumin	Swant, mAB 3D5, AB_10013381, mouse, monoclonal	1:200	PFA/GLUT
Glycine	Glycine-glutaraldehyd-carriers	MoBiTec, 1015GE, AB_2560949, rabbit, polyclonal	1:200	PFA/GLUT
Secondary antibodies				
Alexa Fluor antibody	Mouse gamma Immunoglobins Heavy and Light chains	Jackson ImmunoResearch Labs, 715-545-150, AB_2340846, donkey, polyclonal	1:200	PFA/GLUT
Donkey Anti-Rabbit IgG (H+L) Antibody, Alexa Fluor 488 Conjugated	Rabbit gamma Immunoglobins Heavy and Light chains	Molecular Probes, A-21206, AB_141708, donkey, unknown	1:200	PFA/GLUT
Anti-biotin antibody				
Cy3-Streptavidin antibody	Streptavidin	Jackson ImmunoResearch Labs, 016-160-084, AB_2337244, donkey, unkown	1:500	PFA/GLUT

Results

GABAergic and glycinergic input to midshipman fish VMN

I investigated GABAergic and glycinergic projections to the vocal motor nucleus (VMN) in two brains of midshipman fish by immunohistochemically staining transversal hindbrain sections where single neurons were intracellulary labeled with neurobiotin (see Material and Methods; Fig. 2). I confirmed GABAergic input to VMN (Fig. 1A, D, E) which has been shown in previous studies (Chagnaud *et al.*, 2010; Chagnaud *et al.*, 2012). In addition, I found glycinergic input to VMN in midshipman fish (Fig. 1A, B, C). Glycinergic input to VMN was recently described in *O. beta*, a close relative of midshipman fish (Rosner *et al.*, 2018).

In the following, I will report the response to injections of saclofen ($GABA_B$ antagonist) as well as increased or decreased glycinergic inhibition by injection of glycine or its antagonist strychnine into VMN.

Glycine injections into VMN

Glycine injections (0.2 M) into VMN affected call amplitude but not call duration (Fig. 2A). Call amplitude was significantly reduced by about 15% compared to baseline levels directly after injection, lasting until 2.5 min after injection (ANOVA: $F(9, 50) = 2.642$, $p = 0.0138$; paired t-test: base to 0 min $p = 0.0019$, base to 1 min $p = 0.0011$, base to 2.5 min $p = 0.0017$; Fig. 2A, right, orange trace). Call amplitude dropped slightly, but not significantly, after injection of saline solution (ANOVA: $F(9, 50) = 2.875$, $p = 0.0081$; paired t-tests: all not significant; Fig. 2A, right, turquoise trace). Although call amplitude increased again to baseline level 25 min after glycine injection, it increased above baseline levels after sham injections in the same time period (Fig. 2A, right). Thus, there seemed to be an upward drift of baseline during recordings. If correcting for potential upward drift, call amplitude stayed roughly 7% below baseline for the rest of the recording. Call duration was slightly diminished directly after glycine injection up to 5 min post injection.

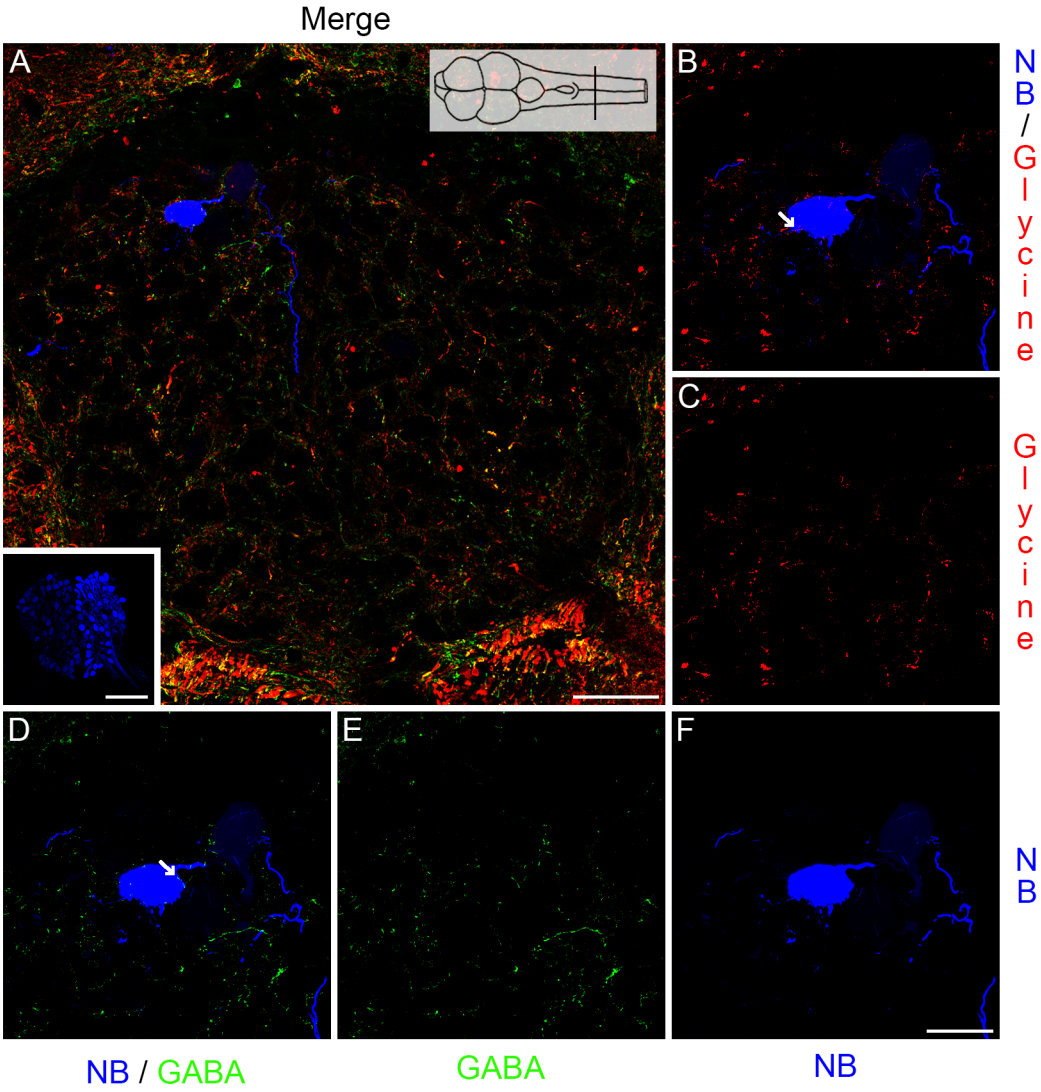

FIGURE 1: GLYCINERGIC AND GABAERGIC INPUT TO VMN IN MIDSHIPMAN FISH (*PORICHTHYS NOTATUS*).

(A) A single neuron of the vocal motor nucleus (VMN, blue) traced with neurobiotin (NB) receiving glycinergic (red) and GABAergic (green) input. The upper right inset shows the level where VMN is located in the hindbrain. The lower left inset shows a tracing of the whole VMN. (B-C) Glycinergic input to the traced VMN neuron (arrow B). (D-E) GABAergic input to the traced VMN neuron (arrow D). The input overlaps with the VMN neuron's somata and the fiber originating from the neuron. Scale bar is 100 µm in A and inset in A and 20 µm in F for B-E.

This reduction was not significant, neither for duration (ANOVA: F (9, 50) = 0.883, p = 0.546) nor for frequency (ANOVA: F (9, 50) = 0.988). Call frequency was not affected (not shown).

Strychnine injections into VMN

Injection of the glycine antagonist strychnine (10 µM) had no significant effect on call duration (ANOVA: F (9, 50) = 0.986, p = 0.464, Fig. 2B, middle), fundamental frequency

(ANOVA: F ((9, 50) = 0.986, p = 0.464, not shown) or call amplitude (ANOVA: F (9, 50) = 0.842, p = 0.582; Fig. 2B, right). Yet, call duration and call amplitude could be observed to decrease compared to baseline directly following injection (Fig. 2B: duration: middle; amplitude: right). For call duration, decrease was maximal with 20 % lower than baseline 2.5 min after injection (Fig. 2B, middle, pink trace). Call amplitude was maximally decreased at 12 % lower than baseline directly after injection (Fig. 2B, right, pink trace).

Saclofen injections into VMN

Injections of saclofen into VMN (10 mM) did not affect fundamental frequency (ANOVA: F (9, 40) = 1.822, p = 0.0942) or call amplitude (ANOVA: F (9, 35) = 0.751, p = 0.661) but showed a trend in reducing call duration (Fig. 2C, middle). Although, call duration was lowered by about 20% of baseline levels 2.5 min after injection of saclofen and stayed at that level for the remaining recording time (Fig. 2C, middle, purple trace), this effect was not significantly different from baseline measurements (ANOVA: F (9, 40) = 0.62, p = 0.773).

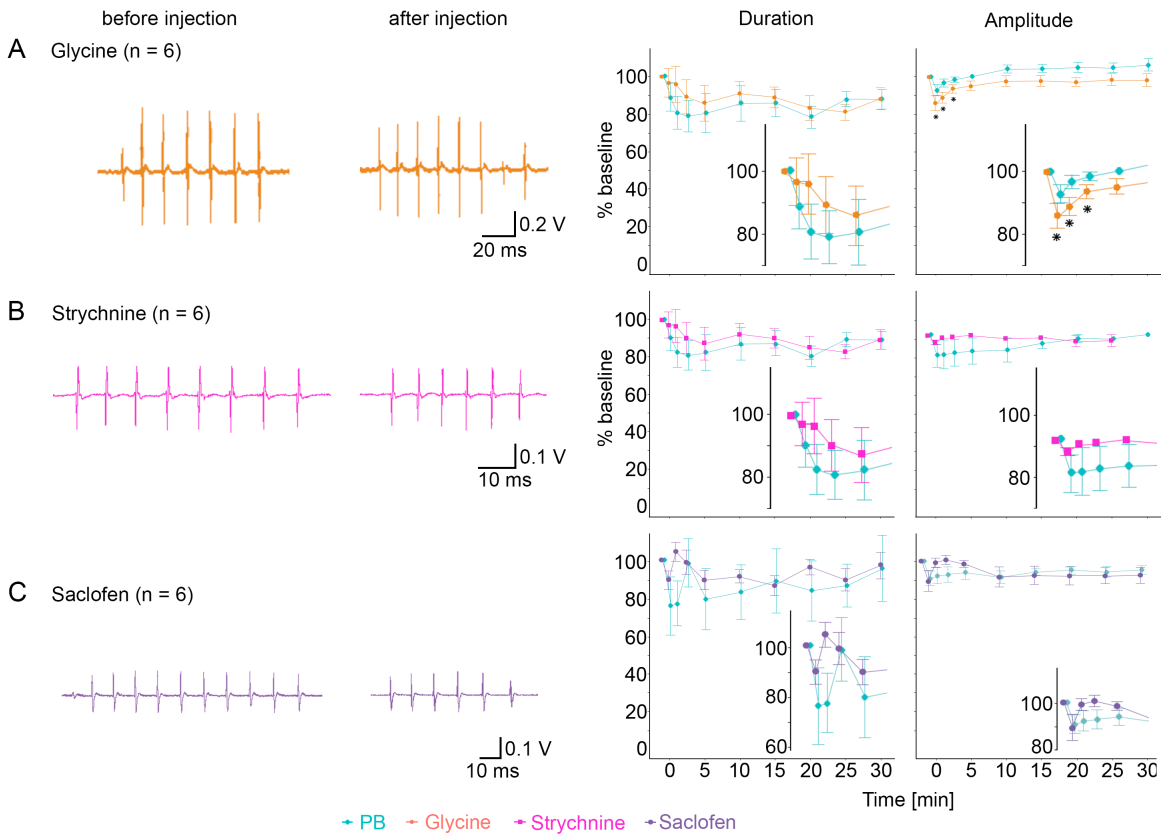

FIGURE 2: RESPONSE IN CALL DURATION AND CALL AMPLITUDE TO INJECTION OF GLYCINE (A; ORGANGE; 0.2 M), STRYCHNINE (B; PINK; 10 µM) AND SACLOFEN (C; PURPLE; 10 MM).

For each panel, examples of vocal nerve recording illustrating changes in vocal pattern before and after injection are given on the left, response of call duration in the middle and response to call amplitude on the right. Turquoise represents response to sham injection of phosphate buffer (PB). Inserts in line graph for duration and amplitude show measurements before injection and 0, 1, 2.5 and 5 min after injection. Single points represent mean values for all animals (n = 6) tested with one agent with standard error. Mean values are given as proportion to the baseline measurement.

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9 LIST OF PUBLICATIONS

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Rosner, E., und Voigt, C. C.: "Selective Oxidation of saturated and unsaturated fatty acids in hibernating Common Noctule Bats (Nyctalus noctula)", J Exp Biol, 221(Pt 4), doi: 10.1242/jeb.168096 (published: online 19/02/2018, no printed version).

Rosner, E., Rohman, K. N., Bass, A. H. and Chagnaud, B. P.: "Inhibitory and modulatory inputs to the vocal central pattern generator of a teleost fish", J Comp Neurol, 526 (8), 1368-1388, doi: 10.1002/cne.24411 (published: online 28/02/2018, in print: 01/06/2018).

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10 EIDESSTATTLICHE VERSICHERUNG/AFFIDATIV

Hiermit versichere ich an Eides statt, dass ich die vorliegende Dissertation „Inhibitory and modulatory neurotransmitter in the brain of socially communicating fishes“ selbstständig angefertigt habe, mich außer der angegebenen keiner weiteren Hilfsmittel bedient und alle Erkenntnisse, die aus dem Schrifttum ganz oder annähernd übernommen sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen habe.

I hereby confirm that the dissertation “Inhibitory and modulatory neurotransmitter in the brain of socially communicating fishes“ is the result of my own work and that I have only used sources or materials listed and specified in the dissertation.

München, den / Munich, date 13/02/2020

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Elisabeth Rosner

12 AUTHOR CONTRIBUTIONS

The authors contributed to the manuscripts as follows:

Manuscript 1) Elisabeth Rosner, Kevin N. Rohman, Andrew H. Bass and Boris P. Chagnaud, “Inhibitory and modulatory inputs to the vocal central pattern generator of a teleost fish“

Contribution of E.R.

- Design of study: chose the target neurotransmitters and suited antibodies, chose number of animals, developed antibody staining protocol for *Opsanus beta*, chose methods for data analysis
- Performed most of the transneuronal tracings of the vocal central pattern generator in *O. beta*
- Performed all antibody stainings
- Took all microscope images
- Performed the data analysis
- Performed the statistical analysis
- Designed all figures
- Wrote and revised the manuscript

Contribution of K.N.R.

- Performed the Western blot analysis for antibody characterization of the connexin 35/36 antibody
- Revised the manuscript

Contribution of A.H.B.

- Taught E.R. how to perform transneuronal tracing of the vocal central pattern generator in toadfish
- Performed part of the transneuronal tracings
- Provided lab space at Cornell University for E.R. to perform transneuronal tracings
- Revised the manuscript

Contribution of B.P.C.

- Design of study: suggested the studied species and study outline, gave technical advice, advised target neurotransmitter
- Revised and submitted the manuscript

B.P.C. supervised the study.

Manuscript 2) Elisabeth Rosner, Boris P. Chagnaud and Mario F. Wullimann, ”Serotonin systems in three socially communicating teleost species, the grunting toadfish (*Allenbatrachus grunniens*), a South American marine catfish (*Ariopsis seemannii*), and the upside-down catfish (*Synodontis nigriventris*)“

Contribution of E.R.

- Design of study: chose antibody against serotonin and MAP2 with corresponding secondary antibodies, number of animals, developed antibody staining protocol for studied fish species
- Performed part of the perfusions of fish specimen
- Performed antibody stainings
- Took all microscope images
- Designed all figures, wrote and revised the manuscript

Contribution of B.P.C.

- Design of study: suggested the studied fish species and study outline, gave technical advice
- Performed part of the perfusions of fish specimen
- Revised and submitted the manuscript

Contribution of M.F.W.

- Gave extensive advice on anatomical determination of brain structures
- Suggested antibody against tyrosine hydroxylase to determine character of PVO neurons in *S. nigriventris*
- Wrote and revised the manuscript

M.F.W. and B.P.C. supervised the study.

Hiermit bestätige ich die angegebenen Beiträge zur Erstellung der Manuskripte.

I hereby confirm the stated contribution to the manuscripts

München, den / Munich, date _____

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Elisabeth Rosner