INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: NEUROCIÊNCIAS

CARACTERÍSTICAS MORFOLÓGICAS E ASPECTOS FUNCIONAIS DO TELENCÉFALO DE *Betta splendens* REGAN 1910

ÂNGELO CÁSSIO MAGALHÃES HORN

Porto Alegre

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UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

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Tese de doutorado apresentada ao Programa de Pós-Graduação em Ciências Biológicas: Neurociências da Universidade Federal do Rio Grande do Sul, como requisito parcial à obtenção do grau de Doutor em Neurociências.

Orientador: Prof. Dr. Alberto Antônio Rasia Filho

Porto Alegre

2017

AGRADECIMENTOS

À Alberto Antônio Rasia Filho, que foi orientador quando precisou ser, amigo quando não era obrigado a ser, mas acima de tudo por ser modelar neste difícil e competitivo mundo da produção científica. Aprendi muito contigo. Muito obrigado, irmão!

À Matilde Achaval que foi orientadora de meu orientador, como foi minha orientadora, e que semeou a ideia do “bem fazer”. Para quem aprendeu, isso não tem preço. Obrigado, professora.

Aos professores e profissionais que colaboraram diretamente com a realização desta tese por meio de seus comentários, empréstimo de materiais ou, ainda, com o seu gracioso auxílio dentro de um dos laboratórios pelos quais peregrinei. A saber: Léder Leal Xavier, Paula Rigon da Luz Soster, Sílvia Barbosa, Carmen Lúcia Andrades Rocha e Monice Santana dos Santos. Obrigado de coração.

Aos meus colegas do Instituto Federal de Educação, Ciências e Tecnologia do Rio Grande do Sul, que tudo fizeram para me auxiliar durante todo o período em que estava trabalhando e realizando o doutorado simultaneamente. Foi muito mais tempo do que eu pretendia ou gostaria nestas duas frentes. Muitíssimo obrigado!

Aos professores do Programa de Pós-graduação em Neurociências que haviam sido meus professores e colegas na época do mestrado. Vocês foram extremamente relevantes na minha formação. Obrigado.

Ao Instituto Federal de Educação, Ciências e Tecnologia do Rio Grande do Sul, representando o estado brasileiro, o povo brasileiro, e que me deu a chance desta capacitação. Obrigado.

À Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul que concedeu recursos para compra de parte do material necessário à consecução desta tese. Obrigado.

À Patrícia Thomajeski, que foi meu ponto de equilíbrio e, acima de tudo, amiga, em todos os outros momentos que não aqueles no qual o trabalho associado a esta tese me drenava as forças e a vontade. Obrigado, meu amor.

Aos meus familiares de sangue e àqueles que tenho o prazer de poder assim chamar, os quais todas as minhas ausências entenderam e perdoaram durante os anos da realização desta tese. Muito obrigado!

E, finalmente, em menção mais do que especial, pelos anos de constante incentivo à realização e pela insistência à conclusão deste curso, à minha mãe, Maria Elizabeth Dutra Magalhães. Fico feliz que veja isso acontecer. Obrigado pelo começo, pelo meio e pelo por vir.

“Somos quem podemos ser, sonhos que podemos ter... e teremos.”

Humberto Gessinger

SUMÁRIO

RESUMO

Betta splendens é um peixe actinopterígeo da subordem dos anabatídeos que apresenta marcado comportamento agonista resultante do territorialismo que o caracteriza. Esse comportamento, ao lado de uma corte e cuidado com a prole elaborados, sugere funções telencefálicas altamente desenvolvidas para este peixe. Em razão disso, e de apresentar uma série de características favoráveis quanto a sua reprodução e manutenção, ele constitui-se em um modelo potencial para as Neurociências, principalmente no que tange o estudo da agressividade. Com o objetivo de caracterizar a estrutura e possíveis funções do telencéfalo, *B. splendens* machos e fêmeas foram utilizados para (1) elaborar um mapa neuroanatômico com base na topologia, topografia e citoarquitetura dos grupos celulares presentes nessa região do encéfalo, empregando as técnicas histológicas da Hematoxilina- Eosina (HE) e de Nissl, e determinar a densidade numérica para neurônios e células gliais nos núcleos supracomissural (Vs) e pós-comissural (Vp) do telencéfalo; e, (2) identificar a atividade da enzima NADPH diaforase (NADPH-d) por método histoquímico nas diferentes áreas e grupos celulares telencefálicos deste peixe frente a um paradigma comportamental para a agressividade. A primeira abordagem revelou que o telencéfalo de machos e fêmeas de *B. splendens* apresenta uma estrutura similar, com um bulbo olfatório composto por cinco camadas celulares concêntricas e hemisférios telencefálicos constituídos por 16 e 8 grupos celulares distintos em suas regiões dorsal e ventral, respectivamente, e que não há dimorfismo sexual quanto à densidade numérica para neurônios e células gliais de Vs e Vp, áreas homólogas à amígdala medial de mamíferos. O segundo trabalho demonstou que a marcação para a atividade da enzima NADPH-d no telencéfalo de *B. splendens* é específica para cada sexo, com mais corpos celulares marcados no telencéfalo ventral do que no dorsal. Mostrou também que o paradigma comportamental voltado a promover a agressividade gerou um aumento da intensidade da marcação como do número de estruturas marcadas no telencéfalo deste peixe. Tanto a

caracterização morfológica quanto funcional do telencéfalo de machos e fêmeas de *B. splendens* aqui realizadas fornecem novos dados que vem a contribuir para a adoção dessa espécie como um modelo não mamífero para o estudo da neurobiologia da agressividade.

ABSTRACT

Betta splendens is an Actinopterygii fish of the suborder of the Anabantoidei that shows pronounced agonistic behavior resulting from its territorialism. This kind of behavior beside to an intrincate courtship and complex parental care suggest highly developed telencephalic functions to this species. Due to aforementioned and to other characteristics as an easy reproduction and simple maintenance, this species constitutes a potential model for the neuroscience area, mainly related to the study of the aggressiveness. In order to characterize the telencephalic structures relating them to their putative roles male and female *B. splendes* were used to: (1) construct a neuroanatomical map based on the topology, topography and cytoarchitecture of the cellular groups present in this brain region using the histological techniques of the Hematoxylin – Eosin (HE) and Nissl, as well as, define the numerical density for neurons and glial cells from supracomissural (Vs) and postcomissural (Vp) nuclei of the telencephalon. (2) Identify the activity of the NADPH diaphorase enzyme (NADPH-d) by its histochemical method to the different areas and structures of the telencephalon submitted to a behavioral paradigm for the aggressiveness. The first approach revealed that the telencephalon of males and females of the *B. splendens* has the same structure, with an olfactory bulb composed of five concentric cellular layers and telencephalic hemispheres constituted by 16 and 8 distinct cell groups in their dorsal and ventral regions respectively, and that there is no sexual dimorphism to the numerical density for neurons and glial cells of the Vs and Vp, homologous structures to the medial amygdala of mammals. The second work demonstrated that the activity of the enzyme NADPH-d in the telencephalon of *B. splendens* has a specific pattern for each sex, with more cellular bodies marked in ventral telencephalon than in dorsal one. It also showed that the behavioral paradigm for aggressiveness promoted an increase in the intensity as well as in the number of structures marked in the telencephalon of this fish. Both the morphological and functional characterization of the telencephalon of males and females of the *B. splendens* carried out here provide new data which contributes to the adoption of this

species as a non-mammalian model for the study of the aggressiveness in the neuroscience fields.

LISTA DE ABREVIATURAS

5-HT – Serotonina

AC – Comissura anterior

ACh - Acetilcolina

D – Telencéfalo dorsal

DA - Dopamina

Dc – Zona central do telencéfalo

Dc-1 – Divisão 1 da zona central do telencéfalo

Dc-2 – Divisão 2 da zona central do telencéfalo

Dc-3 - Divisão 3 da zona central do telencéfalo

Dc-4 – Divisão 4 da zona central do telencéfalo

Dd – Zona dorsal do telencéfalo dorsal

Dl – Zona lateral do telencéfalo dorsal

Dld – Divisão dorsal da zona lateral do telencéfalo dorsal

Dlg – Divisão granular da zona lateral do telencéfalo dorsal

Dlv-1 – Divisão ventral da zona lateral do telencéfalo dorsal, subdivisão 1

Dlv-2 – Divisão ventral da zona lateral do telencéfalo dorsal, subdivisão 2

Dm – Zona medial do telencéfalo dorsal

Dm-1 – Zona medial do telencéfalo dorsal, divisão 1

Dm-2 – Zona medial do telencéfalo dorsal, divisão 2

Dm-3 - Zona medial do telencéfalo dorsal, divisão 3

Dm-4 – Zona medial do telencéfalo dorsal, divisão 1

Dp – Zona posterior do telencéfalo dorsal

EN – Núcleo entopeduncular

eNOS- Óxido nitrico sintase endotelial

GABA – Ácido gama-aminobutírico

GLU - Glutamato

iNOS – Óxido nitrico sintase induzível

MeA – Amigdala medial

NADPH-d – Nicotinamida adenina dinucleotídio fosfato diaforase

nNOS – Óxido nitrico sintase neuronal

NO – Óxido nitrico

NOS – Óxido nitrico sintase

OB – Bulbo olfatório

SN – Sistema nervoso

Tel – Hemisfério telencefálico

Tels – Hemisférios telencefálicos

V – Telencéfalo ventral

Vc – Núcleo central do telencéfalo ventral

Vd - Núcleo dorsal do telencéfalo ventral

Vi - Núcleo intermediário do telencéfalo ventral

Vl - Núcleo lateral do telencéfalo ventral

Vp - Núcleo póscomissural do telencéfalo ventral

Vs – Núcleo supracomissural do telencéfalo ventral

Vv - Núcleo ventral do telencéfalo ventral

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1. INTRODUÇÃO

1.1 *Betta splendens* como modelo experimental nas Neurociências

Os peixes de forma geral, ao lado de anfíbios, répteis e aves, vêm sendo utilizados como modelos experimentais alternativos aos mamíferos (Tsang et al., 2017). Em razão de sua posição filogenética (são vertebrados), sua grande fecundidade e sua facilidade de manejo e baixo custo de manutenção tem se tornado uma opção viável dentro de algumas áreas da biologia experimental (Maximino et al., 2015). Neste contexto, temos entre as espécies mais comumente utilizadas *Danio rerio* (também conhecido como paulistinha ou *zebrafish*), *Carassius auratus* (peixe dourado), *Oryzias latipes* (Medaka ou *Killifsh* japonês) e *Gasterosteus aculeatus* (esgana-gata ou *threespine sticleback*) (Müller, 2005; Maximino et al., 2015). Contudo, em razão de uma ou, por vezes, mais do que uma característica específica, outras espécies de peixes vêm sendo sistematicamente aproveitadas como modelos experimentais para as Neurociências, sendo uma delas *Betta splendens*.

Betta splendens, um membro da subordem anabantoidei (Rüber et al., 2006), é oriundo do sudeste da Ásia (principalmente da Tailândia, do Camboja, da Indonésia, do Laos e do Vietnã) (Faria et al., 2006; Goldstein, 2012). É caracterizado pela presença do labirinto, órgão para respiração aérea, pela sua territorialidade, traduzida em seu marcado comportamento agressivo frente a outro indivíduo da mesma espécie, e por um comportamento reprodutivo complexo, no qual se destacam uma corte intrincada, a produção e manutenção de ninhos de bolhas e um extremo cuidado com a prole (Faria et al., 2006; Monvises, et al., 2009; Goldstein, 2012). O comportamento agressivo da espécie, que foi descrito de forma sistemática por Simpson (1968), vem sendo largamente estudado sob diferentes paradigmas, os quais envolvem a inserção dos animais em diferentes contextos sociais (exposição ao seu próprio reflexo em um espelho, a um ou mais indivíduos do mesmo gênero, a um indivíduo do gênero oposto e, por vezes, a peixes de espécies diferentes) ou a substâncias diluídas na água (como a fluoxetina e o mentol), com a descrição e quantificação dos padrões motores (por exemplo: abdução dos opérculos

branquiais, extensão das nadadeiras dorsais, mordidas, e etc...) observados (Maximino et al., 2015).

Apesar da literatura especializada quase não focar em outros aspectos da neurobiologia de *B*. *splendens* que não seu comportamento, alguns poucos trabalhos tem fornecido dados relevantes que podem ser utilizadas para melhor aproveitar o potencial desta espécie como modelo experimental (De Bruin, 1980; Marino-Neto e Sabbatini, 1983; Marino-Neto e Sabbatini, 1988). Capaz de produzir comportamentos agressivo e reprodutor, dentre outros comportamentos sociais, complexos, essa espécie deve ser possuidora de funções telencefálicas especializadas (Müller, 2005), condição atestada pelos resultados de experimentos neuroetológicos envolvendo lesões cerebrais (De Bruin, 1980) e pela evidência da existência de divisão das funções cognitivas entre ambos os lados do encéfalo, característica típica da lateralidade hemisférica (Clotfelter e Kuperberg, 2007).

1.2 Reprodução e crescimento de *Betta splendens*

A escolha de um animal como modelo experimental passa pela coleta do maior número possível de informações sobre sua biologia, permitindo que se possa, além de manipulá-lo e mantê-lo com facilidade, reproduzí-lo até a maturidade sexual.

A reprodução de *B. splendens*, assim como os métodos voltados ao seu desenvolvimento até a maturidade são descritos em uma série de trabalhos, encontrados em sitios eletrônicos na internet (Arroz, 2013; Betta Brasil, 2013; Pimenta, 2013), em livros que versam sobre aspectos biológicos da espécie (Tullock, 2006; Boruchowitz, 2009; Goldstein, 2012) e em artigos científicos (Faria et al., 2006). Contudo, em razão de uma ampla gama de variáveis envolvidas nos processos, sendo algumas de cunho genotípico e outras fenotípico, a simples aplicação de um método específico ou outro, exatamente como descritos, não se mostra sempre viável. Sabe-se que, independente do método aplicado, o desenvolvimento de *B. splendens* até a maturidade sexual, segundo informações obtidas de criadores, é de cerca de 90 a 105 dias. Um

fator de extrema relevância para o desenvolvimento normal de *B. splendens* parece ser, além da alimentação (composição e quantidade da dieta), a qualidade da água na qual este é mantido durante o seu crescimento (Parnell, 2006).

Como primeira etapa desta tese, foram desenvolvidos uma série de protocolos, com base na literatura e no método de tentativa e erro, para a reprodução, alimentação, manutenção e adaptação de espécimes de *B. splendens* às condições laboratoriais.

O método utilizado para a reprodução de *B. splendens* seguiu as orientações gerais fornecidas por Faria et al. (2006), Arroz (2013) e Pimenta (2013). A principal modificação implementada nos métodos descritos pelos autores residiu na utilização de sal iodado na concentração de 0,3% como agente bactericida. A descrição completa do método empregado encontra-se no Apêndice 1.

A manutenção e alimentação dos espécimes, desde sua eclosão até a idade adulta, foram baseadas principalmente nas informações disponíveis em Betta Brasil (2013) e modificadas segundo os dados apresentados nos Apêndices 2 e 3.

Durante o desenvolvimento dos protocolos para a manutenção e alimentação de *B. splendens* foi realizada averiguação visando aferir o grau de confiabilidade dos resultados obtidos. Ou seja, no primeiro momento aferiu-se o comprimento total das larvas/alevinos de *B. splendens* nos dias 1, 2, 3, 5, 7, 14, 21 e 28 após a eclosão e elaborou-se uma curva de crescimento (Figura 1). Adicionalmente, procedeu-se à análise morfológica dos espécimes a fim de verificar a existência de qualquer deformidade anatômica perceptível (Figura 2). A curva de crescimento, descrita pela equação $y = 2,59 + 0,17x$, onde y = ponto no eixo da ordenada e x = ponto no eixo da abscissa, apresentou uma correlação entre o comprimento total e os dias após a eclosão de 0,83 (forte) e foi significativa para um $p < 0,05$. Percebeu-se um aumento da variância no comprimento total à medida que os animais tornavam-se mais velhos, assim

como uma marcada aceleração do crescimento entre a segunda e a terceira semanas de vida após a eclosão (Figura 1).

Ao comparar nossos resultados com aqueles obtidos por Ogata e Kurokura (2012), os quais utilizam uma rotina de manutenção distinta, mas que empregaram infusório (*Paramecium sp.*) ou larvas de artêmia (*Artemia salina*) juntamente com rotíferos (*Brachionus angulares*) como fontes alimentares, observou-se crescimento semelhante entre nossos animais e aqueles que receberam apenas infusório. De maneira distinta, um comprimento total marcadamente menor foi constatado para nossos animais quando confrontados com a dieta combinada de larvas de artêmia e rotífero. Diante de tais resultados e como não observamos deformidades anatômicas nas larvas/alevinos de *B. splendens* (Figura 2), concluímos que nossos protocolos foram efetivos na manutenção e crescimento da espécie até o vigésimo oitavo dia após a eclosão (Apêndices 2 e 3).

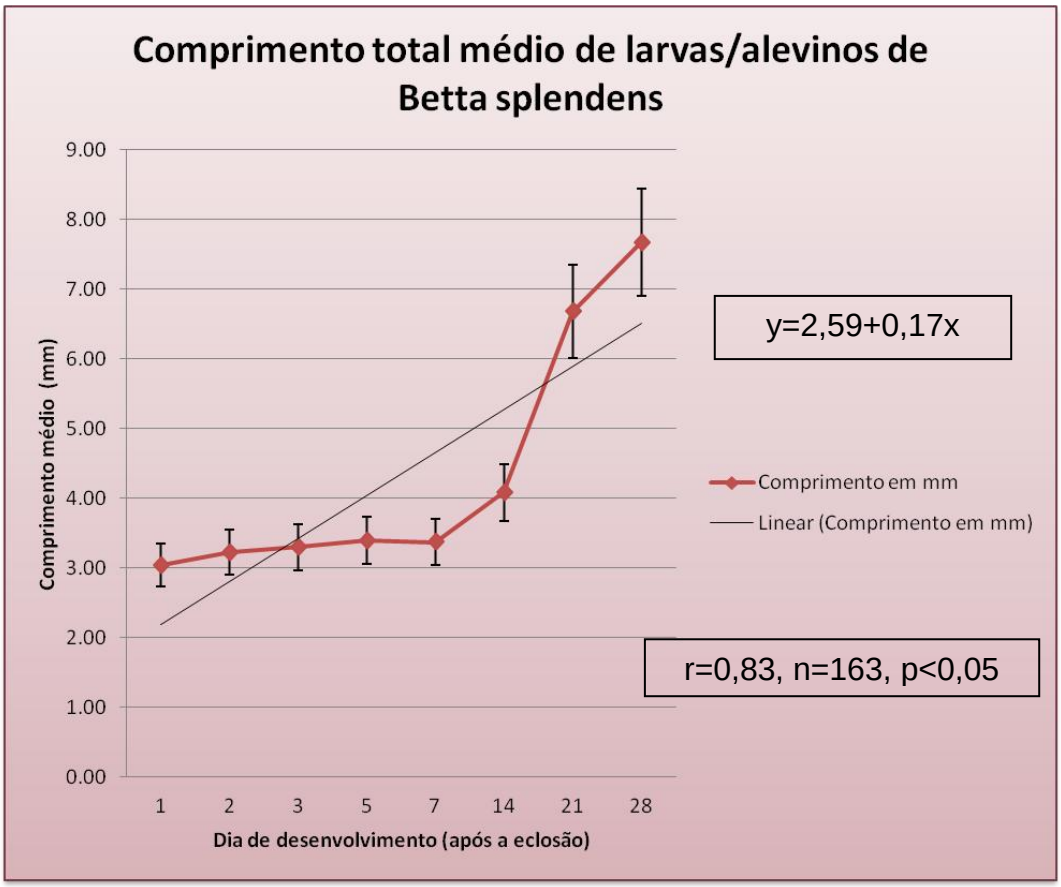

Figura 1. Curva de crescimento para o comprimento total médio das larvas/alevinos de ***B. splendens*** nos dias 1 (n=24), 2 (n=20), 3 (n=21), 5 (n=20), 7 (n=21), 14 (n=21), 21 (n=22), 28 (n=16) após a eclosão. A curva é expressa pela equação y=2,59+0,17x e mostra uma correlação de 0,83 entre o comprimento médio dos organimos e os dias de desenvolvimento, sendo esta correlação significativa para um $p < 0,05$.

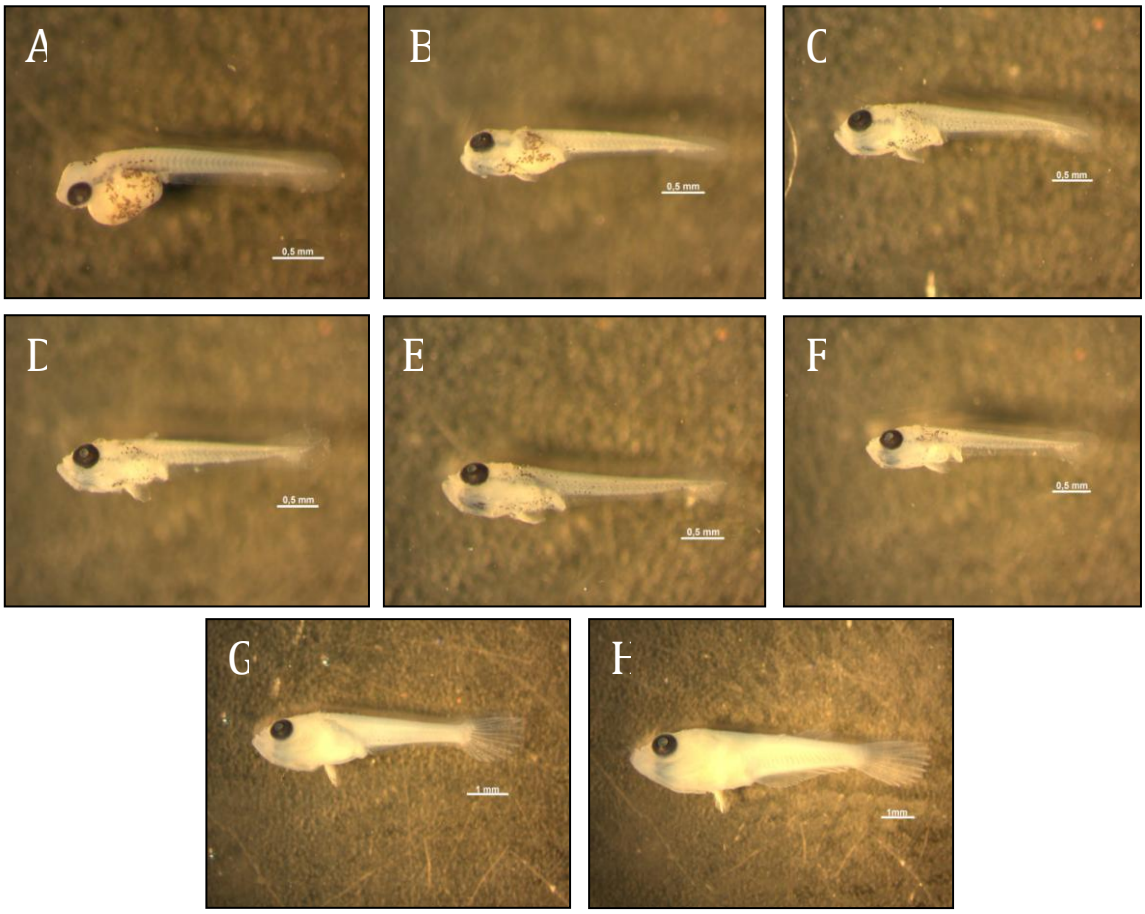

Figura 2. Larvas/alevinos de *B. splendens* no primeiro (A), segundo (B), terceiro (C), quinto (D), sétimo (E), décimo quarto (F), vigésimo primeiro (G) e vigésimo oitavo (H) dias após a eclosão. Barra de calibração de A-F = 0,5 mm e de G-H = 1 mm.

Apesar dos protocolos de manutenção e alimentação se mostrarem efetivos quanto ao desenvolvimento dos indivíduos da espécie até o vigésimo oitavo dia, avaliações posteriores apontaram uma redução na taxa de crescimento dos espécimes (dados não apresentados). Quando esses animais atingiam os 3 a 3,5 meses e tornavam-se sexualmente maduros (adultos) apresentavam dimensões muito inferiores àquelas apontadas na literatura (Arroz, 2013; Pimenta, 2013). Alguns espécimes mostravam sérias deformidades anatômicas associadas. Em razão disso, a utilização de espécimes criados no laboratório para os demais experimentos descritos nesta tese foi descartada, sendo todos os animais adquiridos de um único criador, residente na cidade de Viamão (RS). Esses últimos apresentavam dimensões correspondentes à idade, não apresentavam qualquer deformidade física e haviam sido produzidos sem a utilização de androgênios que visam aumentar o

comprimento e a massa dos espécimes, prática comum entre criadores em larga escala.

1.3 O telencéfalo dos peixes teleósteos

Atualmente, os peixes são o grupo de maior diversidade dentre os vertebrados, possuindo mais de 33.000 espécies (Fishbase, 2016). Dividem-se em três grandes grupos: (1) Agnatos, (2) Peixes cartilaginosos (Condrictes) e (3) Peixes ósseos (Osteíctes). Esses últimos dividem-se nas subordens (3.1) Actinopterygii, peixes com nadadeiras raiadas (que agregam a maioria das espécies vivas), e (3.2) Sarcopterygii, peixes com nadadeiras lobadas (Figura 3) (Nelson, 2006).

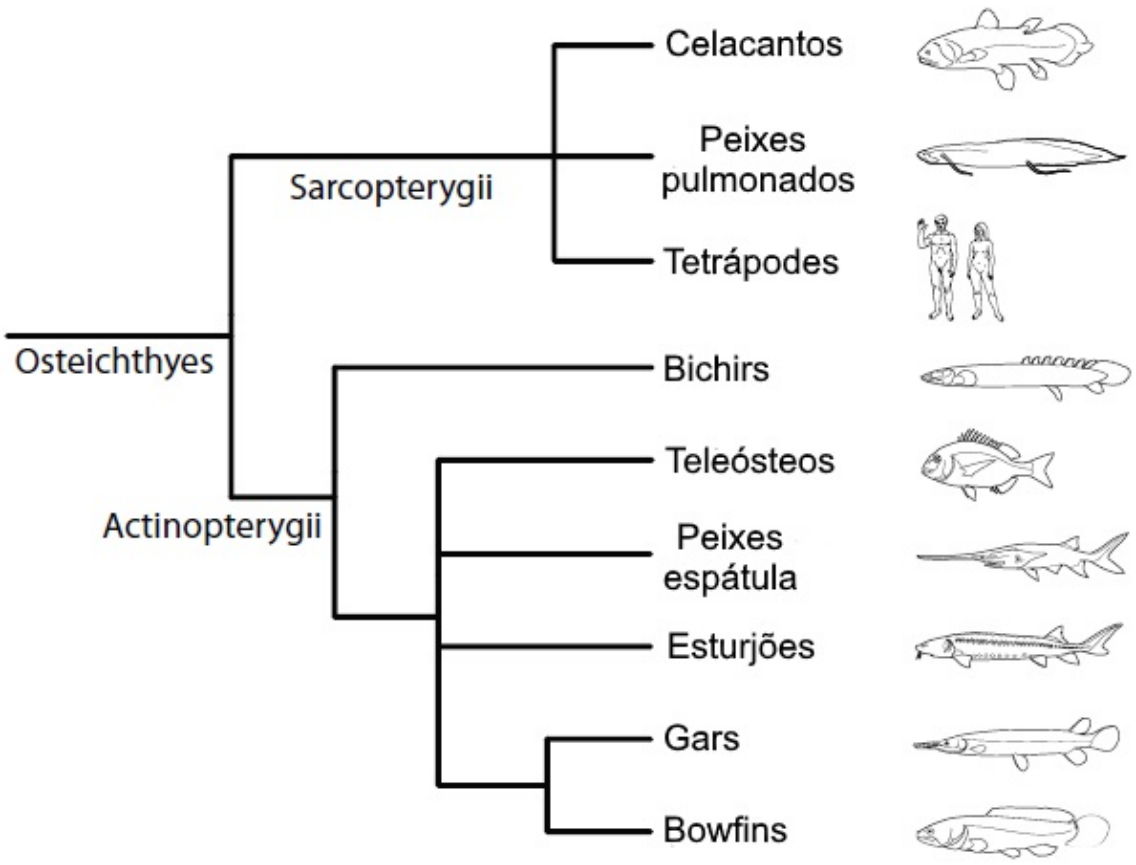

Figura 3. Subordens de peixes ósseos existentes e suas relações filogenéticas intragrupo (Modificado de Suzuki et al., 2010).

Tamanha diversidade específica apresentada pelos peixes, e notadamente pelos Actinopterygii, reflete uma capacidade de adaptação a diferentes ambientes o que, por sua vez, conduz a uma grande variação na forma do corpo, em geral, e do encéfalo, em particular, nesses animais (Kotrschal et al., 1998). Apesar da diversidade morfológica do encéfalo é possível reconhecer nos peixes um padrão de organização geral, um *Blauplan* ou *morphotype* encefálico (Wullimann e Vernier, 2007). Como parte desse padrão de organização geral observa-se, rostralmente, nos peixes Actinopterygii, um telencéfalo com dois lobos ou hemisférios cerebrais, sólidos e laterais, separados por um ventrículo, único e mediano (Buttler e Hodos, 2005). A porção dorsal destes lobos (*area dorsalis telencephali*, telencéfalo dorsal ou D) corresponde, topograficamente e é homóloga, ao pálio e a ventral (*area ventralis telencephali*, telencéfalo ventral ou V) ao subpálio dos vertebrados tetrápodes (Nieuwenhuys, 2009).

Diferente do observado para o restante dos peixes (agnatos, peixes cartilaginosos e Sarcopterygii), assim como para os vertebrados tetrápodes, nos quais o pálio é derivado de um processo de desenvolvimento por evaginação, o pálio dos peixes Actinopterygii desenvolve-se por eversão, processo pelo qual passa a extremidade rostral do tubo neural durante a formação da vesícula telencefálica (Figura 4). Apesar de não existir um consenso sobre como este processo ocorre em sua totalidade, ele parece ser o resultado da divergência (dobramento lateral) e espessamento das placas laterais dorsais do tubo neural (Yamamoto et al., 2007; Bradford Jr., 2009; Mueller e Wullimann, 2009, Nieuwenhuys, 2011).

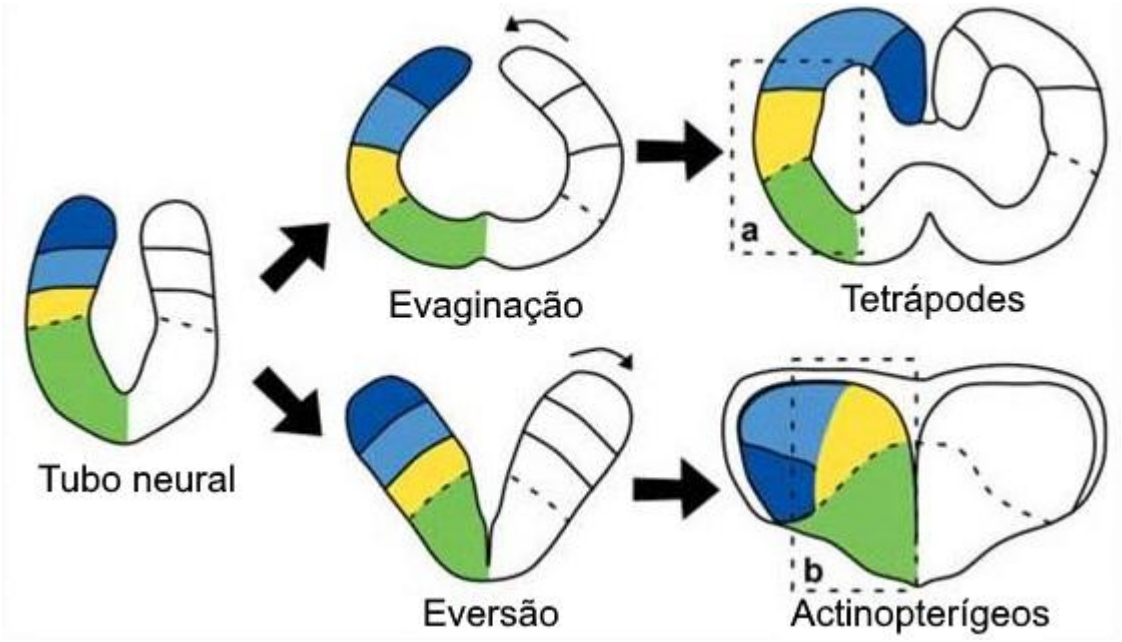

Figura 4. Esquema do desenvolvimento da extremidade rostral do tubo neural, observado em corte transversal, em tetrápodes e peixes actinopterígios (Modificado de Moreno e Gonzales, 2007).

Tanto o pálio, mais variável estruturalmente em razão do maior grau de alteração ao qual é submetido durante o processo de eversão, quanto o subpálio, mais constante, podem ser divididos e subdivididos em grupos de massas celulares mais ou menos identificáveis, sendo o número dessas divisões distinto para os diferentes representantes dos peixes Actinopterygii (Bradford Jr., 2009). Contudo, para uma ampla gama de espécies de teleósteos, pode-se identificar uma estrutura comum em ambas as áreas, na qual se reconhece um pálio dividido em cinco partes (medial, Dm; dorsal, Dd; lateral, Dl; central, Dc e posterior, Dp) e um subpálio dividido em seis porções (Vv, ventral; Vd, dorsal, Vl, lateral; Vs, núcleo supracommissural; Vp, núcleo pós-commissural e E, núcleo entopeduncular) (Nieuwenhuys, 2009; Northcutt, 2011; González et al., 2014) (Figura 5).

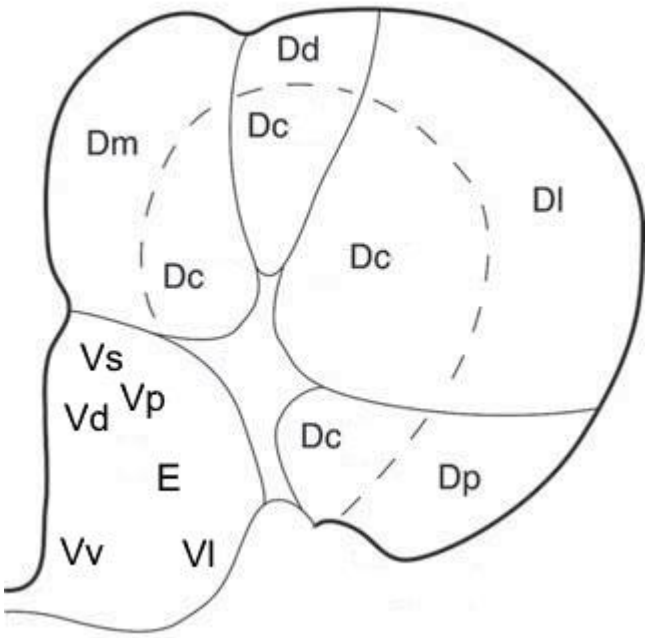

Figura 5. Representação do corte transversal do telencéfalo de teleósteos onde se identificam áreas e núcleos comuns a diferentes espécies de peixes. Dc – Zona central do telencéfalo dorsal, Dd – Zona dorsal do telencéfalo dorsal, Dl - Zona lateral do telencéfalo dorsal, Dm - Zona medial do telencéfalo dorsal, Dp - Zona posterior do telencéfalo dorsal, E – Núcleo entopeduncular, Vd – Núcleo dorsal do telencéfalo ventral, Vl - Núcleo lateral do telencéfalo ventral, Vp - Núcleo pós-comissural do telencéfalo ventral, Vs - Núcleo supracomissural do telencéfalo ventral, Núcleo ventral do telencéfalo ventral (Modificado de Northcutt, 2011).

A tentativa de estabelecer homologias entre partes do pálio e do subpálio dos tetrápodes com a de peixes Actinopterygii, baseada na organização neuroanatômica e celular, na hodologia, no desenvolvimento e na imunomarcação, tem sido alvo de uma série de trabalhos, conforme revisados por Bradford Jr. (2009), Nieuwenhuys (2009) e Mueller e Wullimann (2009). Uma dessas estruturas é a amígdala medial (MeA), em razão da ausência de um sistema vomeronasal evidente em peixes Actinopterigii.

1.4. Amigdala medial nos peixes teleósteos

O complexo amigdalóide de tetrápodes é o resultado da junção de componentes paliais e subpaliais, sendo organizado funcionalmente em três sistemas: (1) Vomeronasal, representado pela MeA; (2) Integração multimodal

(onde está o núcleo lateral da amígdala) e “Autônomo” (com o núcleo central da amígdala) (Martínez-García et al., 2002; Moreno e González, 2006; para argumentação em contrário ao uso do termo “autônomo” veja Rasia-Filho, 2006). Em peixes Actinopterygii, com base em critérios topológicos, hodológicos, de desenvolvimento e funcionais, componentes paliais e subpaliais homólogos aos sistemas de integração multimodal e “autônomo” são identificados (Maximino et al., 2013). A existência de um sistema vomeronasal, e da MeA é ainda matéria de debate. Por um lado, a ausência de um órgão vomeronasal identificável e a falta de uma combinação específica de marcadores genéticos típicos para a MeA de tetrápodes (otp^{+}, isl^{+}, $sim1^{+}$, $brn2^{+}$, $nkx2.1^{+}$, $dlx5^{-}$ and lmo^{-}) (Moreno e Gonzales, 2007; Maximino et al., 2013) afastam a possibilidade da existência da MeA nestes peixes. Não obstante, a presença de receptores olfatórios com microvilosidades no epitélio olfatório, morfologicamente similares às células receptoras do órgão vomeronasal de vertebrados tetrápodes, juntamente à expressão de genes da família de receptores vomeronasais neste tipo celular (Hansen et al., 2004; Sato et al., 2005; Grus e Zhang, 2006) e a existência de projeções olfatórias primárias e secundárias para uma região do subpálio e sua eferência para outras áreas subpaliais, hipotálamo e tubérculo posterior (Matz, 1995; Folgueira et al., 2004), como acontece na MeA de anfíbios anuros (Moreno e González, 2006), indica sua posssível presença no encéfalo dos peixos Actinopterygii.

Considerando válida esta última possibilidade, a MeA de tetrápodes, pelo menos em parte, seria homóloga aos Vs e Vp da área ventral do telencéfalo dos peixes com nadadeiras raiadas (Bradford, 2009; Connell e Hoffman, 2011; Bruce, 2012; Maximino et al., 2013) ou ainda ao Vi (Bielchl et al., 2017). Para localizar precisamente essas estruturas, dentre várias outras, e testar a ideia da homologia com a MeA no *B. splendens*, foi elaborado o primeiro artigo desta tese.

1.5. NADPH-diaforase

O óxido nitrico (NO) é um gás que quando produzido no sistema nervoso (SN) age como mensageiro em eventos intracelulares e/ou de forma parácrina (Prast e Philippu, 2001; Guix et al., 2005). Sua ação parácrina caracteriza-o como neurotransmissor, o qual, diferente de outros neurotransmissores não gasosos, notabiliza-se por não ser armazenado em vesículas sinápticas, não ser liberado por exocitose e por não possuir um receptor sináptico extracellular (Holz e Fisher, 2012).

O NO é produzido por uma classe de enzimas conhecidas como óxido nitrico sintases (NOS) em uma reação de óxido-redução que utiliza o aminoácido L-arginina e o nucleotídio nicotinamida adenina dinucleotídio fosfato (reduzido) (NADPH) como substrato e coenzima, respectivamente, produzindo L-citrulina e NO (Figura 6) (Bredt e Snyder, 1994; Wiesinger, 2001). Existem três isoformas de NOS que podem ser nominadas de forma descritiva ou numérica: (1) NOS neuronal (nNOS) ou do tipo I; (2) NOS indutível (iNOS) ou do tipo II e (3) NOS endotelial (eNOS) ou do tipo III (Nathan e Xie, 1994). Geralmente, tanto a nNOS quanto a eNOS são produzidas de forma contínua (pois são constitutivas) e possuem uma atividade dependente da participação do complexo cálcio/calmodulina. A iNOS, por outro lado, é produzida apenas quando induzida como, por exemplo, durante um processo alérgico-inflamatório, sendo sua atividade independente da presença de cálcio (Nathan e Xie, 1994; Ignarro e Jacobs, 2000).

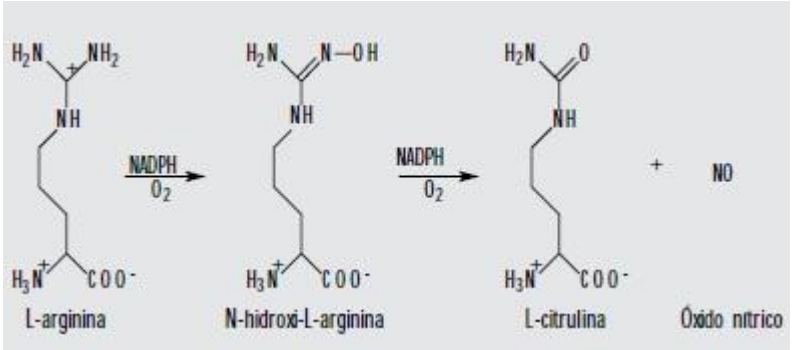

Figura 6. Esquema da produção do óxido nítrico a partir da L-arginina. NADPH – Nicotinamida adenina dinucleotídio fosfato. De acordo com Dusse et al. (2003).

Independente da forma, o principal efeito do NO é a ativação de uma forma solúvel de guanilil ciclase, a qual aumenta a concentração intracellular de cGMP. Este aumento resulta em um incremento na atividade de proteínas quinases dependentes de cGMP, o que leva a alteração do metabolismo celular. Essa sequência de eventos constitui a denominada via canônica de ação do NO (Figura 7A) (Holz e Fisher, 2012; Cossenza et al., 2014).

Alternativamente a esta forma de ação, o NO pode agir por meio de nitrosilação dos resíduos de cisteína, na qual este aminoácido, presente em proteínas G e enzimas intracelulares, recebe uma molécula de NO, tendo suas ações atenuadas após isso (Guix et al., 2005). Essa forma de ação do NO é conhecida como via da S-nitrosilação (Figura 7B) (Guix et al., 2005; Cossenza et al., 2014).

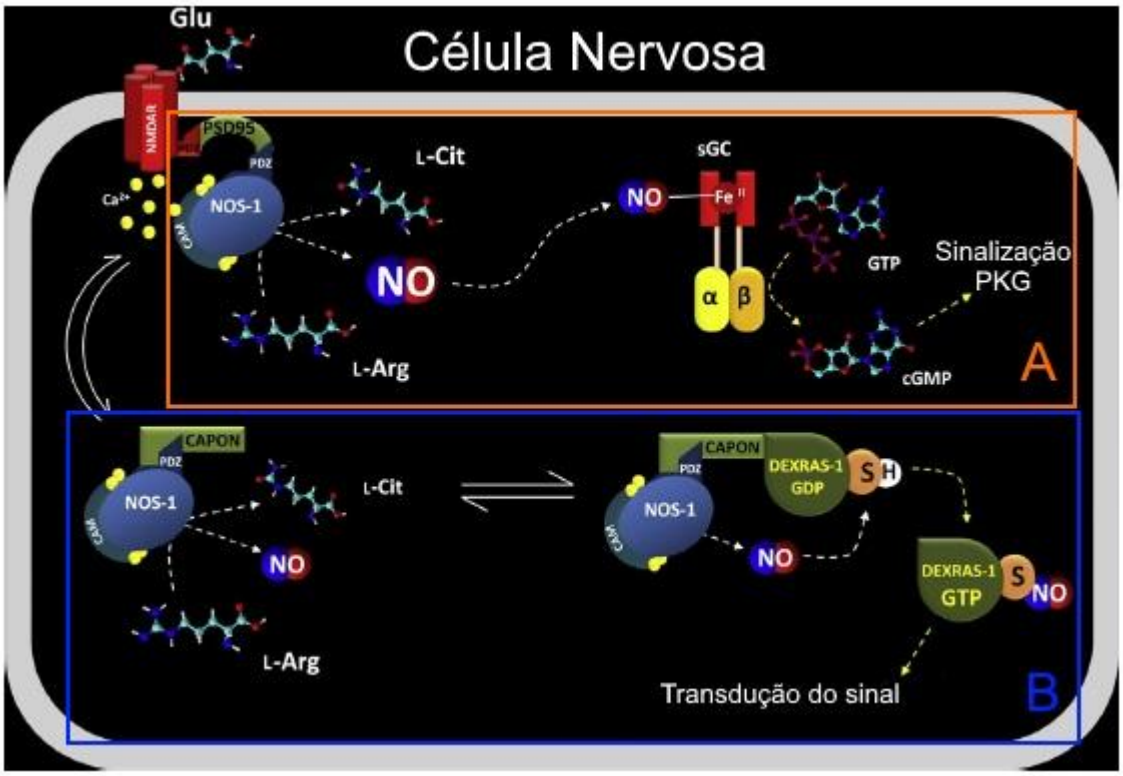

Figura 7. Representação das vias da forma de ação do óxido nítrico nas células nervosas. A. Via canônica. B. Via da S-nitrosilação. Ca^{++} - Cálcio, CAM – Calmodulina, CAPON – Proteína associada com a óxido nítrico sintase neuronal, cGMP - Guanosina monofosfato solúvel, DEXRAS-1 – Proteína G associada com a óxido nítrico sintase 1 com atividade GTPase, Fe^{++} - Ferro reduzido, Glu – Glutamato, GMP – Guanosina monofosfato, GTP- Guanosina trifosfato, H – Hidrogênio, L-Cit – L-citrulina, L-Arg – L-arginina, NMDAR- Receptor NO – óxido nítrico, NOS-1 –Óxido nítrico sintase 1, PSD95 – Proteína pós-sináptica densa 95, PDZ – Domínio PDZ, PKG – Proteína quinase dependente de cGMP, S – Enxofre, sGC – Guanilil ciclase solúvel (Modificado de Cossenza et al., 2014).

Dentre as isoformas de NOS descritas, a nNOS foi a primeira purificada e clonada. Ela é encontrada nas formas solúvel ou particulada em neurônios, astrócitos, músculos esquelético, cardíaco e liso, neutrófilos, ilhotas pancreáticas, endométrio e epitélio dos tratos respiratório e gastrintestinal (Nathan e Xie, 1994; Zhou e Zhu, 2009). As funções atribuídas a essa forma de NOS envolvem a regulação do fluxo sanguíneo por meio da promoção do relaxamento da musculatura lisa vascular; a plasticidade sináptica, facilitando a potenciação a longo prazo (LTP) e induzindo a depressão a longo prazo (LTD); a modulação da liberação de alguns neurotransmissores, como o glutamato, o ácido gama-aminobutírico (GABA), a acetilcolina (ACh), a dopamina (DA), a noradrenalina (NA), a serotonina (5-HT), a adenosina e a histamina e,

adicionalmente, a regulação da excitabilidade, sobrevivência e a produção de novos neurônios (Dawson e Dawson, 1996; Prast e Philippu, 2001; Wiesinger, 2001; Guix et al., 2005; Zhou and Zhu, 2009).

Com o objetivo de detectar direta e/ou indiretamente a localização da NOS no tecido nervoso, diferentes tipos de técnicas podem ser utilizadas como a histoquímica, a imunoistoquímica e a hibridização *in situ* (Beesley, 1995). A técnica histoquímica utilizada com tal objetivo envolve a detecção da enzima NADPH-diaforase (NADPH-d). Comparado com as outras técnicas, o método histoquímico é simples, confiável e barato (Spessert e Claassen, 1998) para identificação da atividade da NOS (Dawson et al., 1991; Hope et al., 1991). Toda NOS tem atividade NADPH-d (Tracey et al, 1993; Beesley, 1995) e, apesar da NOS representar apenas uma fração do total da atividade NADPH-d, algumas variáveis metodológicas podem ser ajustadas para distinguir a atividade de diaforase relacionada com a NOS daquela não relacionada (Blottner et al., 1995; Spessert e Claassen, 1998).

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1.6. NADPH-diaforase no telencéfalo de peixes

Como mencionado, a produção de NO no SN tem sido determinada pelo emprego de técnicas histoquímica ou imunoistoquímica, ou ainda (mas menos comumente) pela hibridização *in situ*, voltadas a localizar, direta ou indiretamente, a NOS. Para o SN de peixes, especificamente, uma série de trabalhos foram realizados utilizando a técnica histoquímica da NADPH-d (Arévalo et al., 1995; Villani e Guarnieri, 1995; Jadhao e Malz, 2004; Northcutt, 2009; Mueller et al., 2011), imunoistoquímica para a detecção da nNOS (Ferrando et al., 2012; Biswas et al., 2015) ou, ainda, ambas as técnicas combinadas (Holmqvist et al., 1994; Östholm et al., 1994; Brüning et al., 1995; Holmqvist et al., 2000; Lema e Nevitt, 2001; Cuoghi et al., 2002; Singru et al., 2003; Ando et al., 2004; Giraldez-Perez, 2008; Pushchina et al., 2012; Giraldez-Perez et al., 2013; López et al., 2016; López et al., 2017).

Em relação ao telencéfalo de peixes, região correlacionada com padrões comportamentais de maior complexidade nesses animais (Müller, 2005), a

localização da atividade da NADPH-d ou da nNOS em diferentes espécies produziu generalizações e particularidades, as quais podem ser observadas nos trabalhos de Giraldez-Perez (2008), Pushchina et al. (2012), Giraldez-Perez et al. (2013), López et al. (2016), López et al. (2017), dentre outros autores. Primeiro, o bulbo olfatório (OB) é marcado pela técnica da NADPH-d e quase nunca apresenta marcação pelo método imunoistoquímico para a nNOS. Núcleos nas regiões do D e V demonstram ser reativos a ambas as técnicas, mas em V há maior preponderância de núcleos ou agrupamentos celulares marcados. Somas neuronais, fibras (que cruzam o plano mediano, como aquelas que se projetam de V para D ou de D para V ou mesmo aquelas aferentes e eferentes em relação ao telencéfalo) e áreas do neuropilo são claramente diferenciáveis usando uma ou outra técnica. A maior parte das estruturas telencefálicas marcadas por uma técnica mostra colocalização em relação à outra, exceto para o OB.

Em razão da frequente colocalização observada entre as estruturas marcadas pela técnica da NADPH-d e da imunistoquímica para o nNOS, sugere-se que a maior parte das estruturas telencefálicas que demonstram atividade da NADPH-d sejam, em verdade, nNOS, e, assim, responsáveis pela produção do neurotransmissor NO (Lopez et al., 2016; Lopez et al., 2017).

1.7 Relação entre o óxido nítrico e agressividade

A agressividade, do ponto de vista neurobiológico e comportamental, constitui-se em padrões motores que expresam uma defesa frente a uma ameaça ou um ataque formulado com um objetivo específico (Siegel e Victoroff, 2009). Sua relação com a modulação feita pelo NO foi bem documentada no encéfalo de camundongos machos (Demas et al., 1997; Chiavegatto et al., 2001). Nesses animais, a supressão da produção de NO pela utilização do inibidor da nNOS, 7-nitroindazole (7-NI), ou pelo emprego de camundongos nocaute para a nNOS, promoveu um aumento da agressividade (Demas et al., 1997; Chiavegatto et al., 2001). Este aumento da agressividade parece ligado a uma redução na relação entre a produção e a hidrólise do neurotransmissor 5-HT e /ou uma perda de funcionalidade de receptores 5-

HT_{1A} e 5-HT_{1B} em regiões encefálicas que controlam a emoção (Chiavegatto e Nelson, 2003).

Considerando que a atividade da NADPH-d é, em grande parte, resultado da atividade da nNOS, que o produto da atividade da nNOS é o NO e que o NO modula a transmissão serotoninérgica (Prast e Philippu, 2001), parece viável supor que uma alteração na atividade da NADPH-d poderia conduzir a uma alteração na agressividade.

Apesar de não se conhecer o exato mecanismo pelo qual o NO influencia a agressividade (Bedrosian e Nelson, 2014), um recente trabalho utilizando *Danio rerio* e camundongos machos associa de forma direta a produção do NO com a atividade da enzima monoamina oxidase (Gutiérrez et al., 2017), a qual é um dos agentes responsáveis por hidrolizar a 5-HT no sistema nervoso central. Desta forma, a redução na produção de NO conduz a uma concomitante redução da agressividade (Gutiérrez et al., 2017) pelo provável aumento dos níveis de 5-HT no encéfalo. Estes dados contradizem os resultados descritos anteriormente por Demas et al (1997) e Chiavegatto et al., (2001) para camundongos machos.

2. OBJETIVOS

2.1 Objetivo geral

Caracterizar, por intermédio de uma abordagem eminentemente citoarquitetural e histoquímica, a estrutura e aspectos funcionais do telencéfalo de *B. splendens*, contribuindo assim para a utilização deste peixe como modelo experimental às Neurociências.

2.2 Objetivos específicos

1- Elaborar mapa neuroanatômico do telencéfalo de machos e fêmeas da espécie *B. splendens*, identificando os diferentes componentes (núcleos, áreas e outros componentes estruturais) com base na sua topologia, topografia e citoarquitetura.

2- Caracterizar, com base na organização citoarquitetônica, os diferentes agrupamentos celulares do telencéfalo de machos e fêmeas de *B. splendens* e definir suas extensões empregando-se as técnicas da hematoxilina-eosina (HE) e de Nissl.

3- Determinar se há dimorfismo sexual nos núcleos supracomissural e pós-comissural de *B. splendens* estabelecendo-se as densidades numéricas relativas de neurônios e células gliais pelo método estereológico do *disector* óptico.

4- Descrever a distribuição da atividade da NADPH-d por técnica histoquímica e quantificar a intensidade de sua reação para as diferentes estruturas do telencéfalo de machos e fêmeas de *B. splendens*.

5 - Avaliar as alterações na distribuição e na intensidade da marcação para NADPH-d após submeter machos e fêmeas de *B. splendens* a um paradigma comportamental para a agressividade.

3. ARTIGOS

Com base nos objetivos descritos, são apresentados dois artigos nesta tese:

Artigo 1: The cytoarchitecture of the telencephalon of Betta splendens Regan 1910 (Perciformes: Anabantoidei) with a stereological approach to the supracommissural and postcommissural nuclei.

Artigo aceito pela revista ***The Anatomical Record*** – *Advances in Integrative Anatomy and Evolutionary Biology* em agosto de 2017.

Artigo 2: NADPH-diaphorase activity in the telencephalon of male and female siamese fighting fish Betta splendens Regan 1910 after a behavioral paradigm for aggressive display.

Artigo submetido à revista ***Journal of Morphology*** em outubro de 2017.

3.1 Artigo 1

The cytoarchitecture of the telencephalon of *Betta splendens* Regan 1910 (Perciformes: Anabantoidei) with a stereological approach to the supracommissural and postcommissural nuclei

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Running title: *B. splendens* telencephalon cytoarchitecture

Grant sponsor: FAPERGS/Brazil; Grant number:12/2426-5

The Cytoarchitecture of the Telencephalon of *Betta Splendens* Regan 1910 (Perciformes: Anabantoidei) with a Stereological Approach to the Supracommissural and Postcommissural Nuclei

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ABSTRACT

Teleostean fish brains are useful models to study cellular and functional specializations along the phylogenesis. The *Betta splendens* Regan 1910 (Siamese fighting fish; Perciformes:Anabantoidei) is known for its aggressive display, courtship behavior, nest building, and offspring care. Here, we present novel and detailed data about the cytoarchitecture of the olfactory bulb and the telencephalic hemispheres of this fish. The hematoxylin-eosin and Nissl techniques served to identify brain nuclei ($n = 19$ males and $n = 21$ females) and for the stereological evaluation of the numerical density of cells and the proportion of neurons and glial cells in the ventral telencephalon supracommissural (Vs) and postcommissural (Vp) nuclei of adult males and females. These nuclei are putative homologs of the sexually dimorphic medial amygdala in mammals. The

Abbreviations: AC = anterior commissure; AOI = Area of interest; CCb = corpus cerebelli; D = dorsal telencephalon; Dc = central zone of the dorsal telencephalon; Dc-1 = central zone of the dorsal telencephalon, division 1; Dc-2 = central zone of the dorsal telencephalon, division 2; Dc-3 = central zone of the dorsal telencephalon, division 3; Dc-4 = central zone of the dorsal telencephalon, division 4; Dd = dorsal zone of the dorsal telencephalon; Dl = lateral zone of the dorsal telencephalon; Dld = dorsal division of the lateral zone of the dorsal telencephalon; Dlg = granular division of the lateral zone of the dorsal telencephalon; Dlv-1 = ventral division of the lateral zone of the dorsal telencephalon, subdivision 1; Dlv-2 = ventral division of the lateral zone of the dorsal telencephalon, subdivision 2; Dm = medial zone of the dorsal telencephalon; Dm-1 = medial zone of the dorsal telencephalon, division 1; Dm-2 = medial zone of the dorsal telencephalon, division 2; Dm-3 = medial zone of the dorsal telencephalon, division 3; Dm-4 = medial zone of the dorsal telencephalon, division 4; dDm-3 = dorsal cell group of medial zone of the dorsal telencephalon, division 3; Dp = posterior zone of the dorsal telencephalon; ECL = external cellular layer; EN = entopeduncular nucleus; GCTN = ganglion cells of the terminal nerve; GL = glomerular layer; HE = hematoxylin and eosin staining; ICL = internal cellular layer; I = olfactory nerve; II = optic nerve; IL = inferior lobe of hypothalamus; LSO = lateral septal organ; MeA = medial amygdala; MO = medulla oblongata; NT = nucleus taenia; OB = olfactory bulb; ONL = olfactory nerve fiber layer; Pit = hypophysis (pituitary gland); POA = preoptic area; PPa = anterior parvocellular preoptic nucleus; PPm = magnocellular preoptic nucleus; PPp = posterior parvocellular preoptic nucleus; Se = sulcus externus; Si = sulcus *inominatus*; Slat = sulcus lateralis; Slim = sulcus limitans; SOF = secondary olfactory fibers; Sy = sulcus ypsiloniformis; Tel = telencephalic hemisphere; TO = tectum opticum; V = ventral telencephalon; Vc = central nucleus of the ventral telencephalon; Vd = dorsal nucleus of the ventral telencephalon; Ventr = ventricle; Vi = intermediate nucleus of the ventral telencephalon; Vl = lateral nucleus of the ventral telencephalon; Vp = postcommissural nucleus of the ventral telencephalon; Vs = supracommissural nucleus of the ventral telencephalon; Vv = ventral nucleus of the ventral telencephalon

Grant sponsors: FAPERGS/Brazil; Grant number: 12/2426-5; AARF is a CNPq/Brazil researcher; Grant number: 306594/2016-1.

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Received 29 December 2016; Revised 22 May 2017; Accepted 13 July 2017.

DOI 10.1002/ar.23699
Published online 10 October 2017 in Wiley Online Library (wileyonlinelibrary.com).

olfactory bulb of *Betta splendens* consists of 5 concentrically arranged layers plus ganglion cells of the terminal nerves. The dorsal telencephalon consists of 16 different cell groups. The ventral telencephalon has 8 nuclei, plus the lateral septal organ and the nuclei of the preoptic area forming an anatomical *continuum*. The rostrocaudal extent of the Vs and Vp is not different between sexes. In both nuclei, the proportion of neurons to glial cells is approximately 2:1 and the density of neurons and glial cells is not different between sexes. These morphological findings can subserve future research on the brain function of the *Betta splendens* and the search for neural sex differences in other central areas of this same species, in other teleost species, or yet in other related vertebrate group. Anat Rec, 301:88–110, 2018.

Key words: teleost; telencephalon; cytoarchitecture; stereology; sexual dimorphism; medial amygdala

INTRODUCTION

Fishes are the most diversified vertebrates consisting about 33,000 species (FishBase, 2016). Such diversity reflects the ability of fishes to adapt to different conditions with variations in the aspect of their body and brain (Kotrschal et al., 1998). Nevertheless, it is possible to recognize a pattern of general organization of the fish brain, named a *Blauplan* or brain morphotype (Wullimann and Vernier, 2007). As part of this general pattern, the actinopterygian brain has a telencephalon with two lateral bulbous lobes or hemispheres separated by a single median ventricle (Butler and Hodos, 2005) and a pair of olfatory bulbs rostral to these lobes (Nieuwenhuys and Meek, 1990). The dorsal portion of the lateral lobes (named Area Dorsalis Telencephali, Dorsal Telencephalon) (D) corresponds to the pallium whereas the ventral portion (named Area Ventralis Telencephali, Ventral Telencephalon) (V) is equivalent to the subpallium of tetrapods (Bradford, 2009; Mueller and Wulliman, 2009; Nieuwenhuys, 2009). The number of nuclear classifications and subdivisions in these brain portions varies between the distinct groups of actinopterygian fishes (Bradford, 2009). Excellent descriptions for different teleost fishes are available elsewhere (Peter and Gill, 1975; Northcutt and Bradford, 1980; Bass, 1981; Diez et al., 1987; Nieuwenhuys and Meek, 1990; Wullimann et al., 1996; Riedel, 1997; Ischikawa et al., 1999; Cerdá-Reverter et al., 2001; Burmeister et al., 2009; Baile and Patle, 2011; Nieuwenhuys, 2011; D'Angelo, 2013; Dewan and Tricas, 2014; Ou and Yamamoto, 2016).

Topology, histochemical and immunostaining approaches, hodology, and developmental markers have been used to establish homologies between the pallium and subpallium of tetrapods and the actinopterygian fish D and V portions (Northcutt, 1995; Wullimann and Mueller, 2004; Bradford, 2009; Mueller and Wulliman, 2009; Nieuwenhuys, 2009; Northcutt, 2011 for an overview). For example, the amygdaloid complex was one of the telencephalic structure studied with this aim (Moreno and González, 2007; Northcutt, 2008; Bruce, 2012; Vargas et al., 2012). In tetrapods, the amygdaloid complex is mainly the result of the aggregation of pallial and subpallial components organized functionally in three networks: (1) "Vomeronasal" (including, for example, the medial nucleus of the amygdala, MeA); (2) "Multimodal and integrative" (including the lateral amygdala); and (3) "Modulatory of Sympathetic/Parasympathetic Responses" (including the central amygdala) (Martínez-Garcia et al., 2002; Moreno and González, 2006; adapted term according to Rasia-Filho, 2006). It is currently accepted the existence of pallial and subpallial components homologous to these above mentioned second and third networks in actinopterygian fishes (Maximino et al., 2013).

Nevertheless, the correspondence of the actinopterygian telencephalic components to the vomeronasal system or the MeA is still a matter of debate. The lack of both an evident vomeronasal organ and a combination of various genetic markers (otp^+, isl^+, $sim1^+$, $brn2^+$, $nkx2.1^+$, $dlx5^-$, and lmo^-, for example) (Moreno and González, 2007; Maximino et al., 2013) suggest the absence of a MeA in actinopterigyan fishes. Conversely, the existence of microvillar receptor cells in the olfactory epithelium, which are morphologically similar to the cells of the vomeronasal organ of tetrapods, the expression of genes of the vomeronasal receptor family (Hansen et al., 2004; Sato et al., 2005; Grus and Zhang, 2006), the connections of primary and secondary olfactory fibers within a region of the subpallium with subsequent projections to other subpallial areas, hypothalamus, and posterior tubercle (Matz, 1995; Folgueira et al., 2004) point to the possible existence of this brain structure in the actinopterygian fish. It is likely that the MeA (or at least part of it) would be homolog to the supracommissural and postcommissural nuclei of the ventral telencephalon (Vs and Vp, respectively) in fishes (Bradford, 2009; O'Connell and Hofmann, 2011; Bruce, 2012; Kress and Wullimann, 2012; Maximino et al., 2013). This is interesting because the vomeronasal pathway and the interconnected MeA subnuclei are sexually dimorphic and involved with the display of social behaviors, including reproduction and aggression, in rodents (Guillamón and Segovia, 1997; De Lorme et al., 2012; Rasia-Filho et al., 2012a,b; Greenberg and Trainor, 2016).

Betta splendens Regan, 1910 (Siamese fighting fish) is an actinopterygian fish from the suborder Anabantoidei

(Rüber et al., 2006) native to the Southeast Asia. It is characterized by its labyrinth organ and, living in groups, by stereotyped aggressive displays (Simpson, 1968), complex reproductive behavior (Rainwater and Miller, 1966), and the ability to build nests of bubbles (Faria et al., 2006; Monvises et al., 2009; Goldstein, 2012). These skills are suggestive of the existence of elaborated telencephalic functions in the *Betta splendens* (Müller, 2005). Behavioral differences between sexes are evident in this species. That is, compared to females, males show remarkable territoriality, an intricate courtship behavior, and the adoption of offspring care (Goldstein, 2012). These features are impaired after telencephalic lesions (De Bruin, 1980), indicating that sexual dimorphism can be found in this brain region. Then, the *B. splendens* can be a suitable experimental model for the study of the telencephalic structures that modulate social behaviors, such as aggression and reproduction, and the link between the neural cellular organization and the corresponding functional display (O'Connell and Hofmann, 2011).

The aim of the present work was to depict the cytoarchitectonic aspect of the olfactory bulb (OB) and the telencephalic hemisphere (Tel) of *B. splendens*. Here, we present a novel and detailed map for the localization and identification of 32 different brain cell groups of this fish brain. The critical comparison with another previous study in this species is provided below. Accordingly, our morphological data are now available to address additional studies having this fish as a valuable animal model for ample research purposes. In addition, we provide a stereological evaluation of the density of neurons and glial cells in the Vs and the Vp of males and females to reveal if a sexual dimorphism would be found in these areas, which are putative homologs of the mammalian MeA.

MATERIALS AND METHODS

Animals

Forty fishes ($n = 19$ males and $n = 21$ females) of *B. splendens* with ages of at least 3–4 months were used in this work. The animals were obtained from a local supplier, free of exogenous hormone treatments, and were maintained in individual bottles (water salinity 0.3%, pH = 6.8 ± 0.4 and temperature ranging 27–30°C) for at least 2 weeks for acclimation before the beginning of the experiments. All fishes were maintained under natural light/dark cycle and were fed twice a day, six days a week, fasting for one day/week. The water was changed twice a week, preventing the accumulation of ammonia and nitrites.

All efforts were made to reduce the number of animals and their suffering. All procedures were done in compliance with the current Brazilian laws that regulate animal use for scientific purposes and according to the National Institute of Health's Guidelines for Care and Use of Laboratory Animals (NIH, 2011) and the Guidelines for the Euthanasia of Animals (American Veterinary Medical Association, 2013). This work was approved by the Ethical Committee from the Federal University of Rio Grande do Sul, Brazil (protocol no. 22625/2012).

Anatomical and Histological Procedures

The animals were anesthetized with clove oil (75mg/L) until they reach stage 4 of anesthesia identified by the absence of animal response to a mechanical stimulus applied to the caudal peduncle (Ilgenfritz et al., 2013). Fishes were weighed, measured, and euthanized by decapitation. Skullcaps were removed and the heads were kept in 4% formaldehyde for at least 72 hr at room temperature. Afterwards, brains were dissected and post-fixed in the same fixative solution until being histologically processed. Gonads were also dissected after euthanasia and fixed in 4% formaldehyde.

Twelve brains, 6 from males and 6 from females, were initially macroscopically observed using a stereomicroscope (Zeiss Stemi SV 6, Germany) and the general anatomical aspect of the brain of the *B. splendens* was determined and drawn using a camera lucida (Fig. 1A). The brains of additional 7 males and 7 females were embedded in glycol methacrylate (GMT), a procedure that reduces tissue shrinkage during histological processing. The rostral portion of the brain, comprising the telencephalon, diencephalon, and the anterior half of the midbrain, was sectioned serially (10 μm-thick each slice), either in transverse (Fig. 1B) or in sagittal planes (to confirm data), using a microtome (Leika RM 2125, Germany). Sections were stained using a hematoxylin-eosin (HE) technique adapted to material embedded in GMT (Cerri and Sasso-Cerri, 2003). That is, we put histological slides with the brain sections in (1) Gill's hematoxylin for 10 min, (2) washed the slides in tap water, (3) dried them in a hot plate, (4) used 0.5% eosin diluted in 96% ethanol for 5 min, (5) dehydrated the sections in an ascending ethanol series, (6) cleared using xylene, and (7) mounted with synthetic balsam and coverslips. The sections were observed using an optical microscope (Nikon Eclipse E200MV, Japan) to identify the areas of interest as well as to characterize the cellular groups in the telencephalon.

To compare with the HE data and to supplement the identification of brain nuclei and cell components, we also stained the OB and the Tel of 6 males and 8 females using the Nissl technique and paraffin embedding medium. The methodological procedure for this staining is the same described in details in (Bancroft and Gamble, 2008). Briefly, microtome serial sections of 10 μm-thick from the OB and the Tel were (1) dewaxed and hydrated in descending ethanol series, (2) histological slides with the brain sections were stained with a cresyl violet solution heated to 60°C for 10 min, (3) rinsed in distilled water, (4) staining was corrected using a 25% acetic alcohol solution for 5–10 sec, (5) sections were dehydrated using ascending ethanol series, (6) cleared with xylene, and (7) mounted with synthetic balsam and coverslips.

Neuroanatomical Characterization of the Telencephalon of the *B. Splendens*

The neuroanatomical details of the OB and the Tel of *B. splendens* were elaborated to serve as a map of symmetrically sectioned brain hemispheres along the rostrocaudal axis (Figs. 2 and 3). No evident difference between structures in the right and left hemispheres were observed. We arbitrarily used the right hemisphere to identify each representative area in the microscopic images. Mirror images of OB and Tel from the right hemisphere are presented in Figure 2. Data are shown

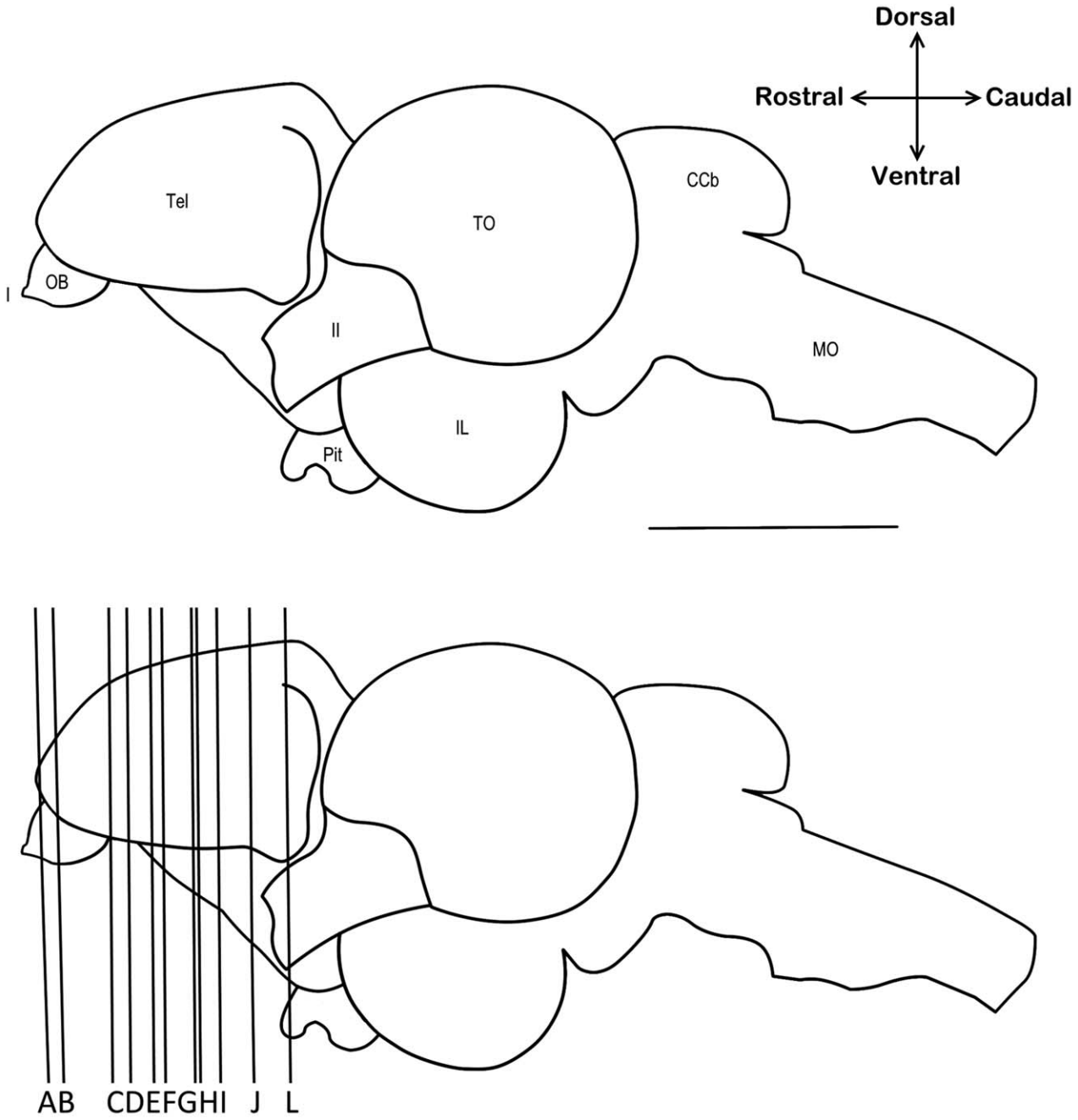


Fig. 1. Schematic image of the adult male brain of *Betta splendens*, left side view. (**A**) Major anatomical structures and (**B**) the levels (from A to L) of the transverse sections performed to study the cytoarchitectural organization of this fish telencephalon. Scale = 1 mm.

with and without overlapping the abbreviations of each studied structure.

All microscopic sections were photographed using a digital camera (Olympus D172, Japan) coupled to a light microscope (Olympus BX61, Japan) with planapochromatic objective lenses (10X and 40X). Serial images obtained under low magnification images were merged using the Adobe Photoshop CS5 software (USA). Images obtained with higher magnification served to demonstrate the cytoarchitectural organization of the selected cell nuclei. Photographs were adjusted to brightness and contrast using the same software above mentioned. Subtle differences in the color and background of the present figures are due to few technical adjustments in the images, which did not alter the biological meaning and the interpretation of the findings.

The criteria used to identify the OB and the Tel components were based on the topology and cytoarchitecture of each studied structure. Their identification was made according to the respective neuroanatomical location and

cell density, as well as the size, shape, and staining properties of local cells.

The nomenclature for the *B. splendens* telencephalic structures was based on the original work of Nieuwenhuys (1963), modified by Northcutt and Bradford (1980), and the reports of Cerdá-Reverter et al. (2001), Burmeister et al. (2009), Baile and Patle (2011), Dewan and Tricas (2014), and Ou and Yamamoto (2016). Components of the preoptic area (POA) were found as an anatomical *continuum* to the Tel. We studied them using the same criteria cited above and critically comment these findings in Discussion.

Gonadal Histology

The gonads were embedded in paraffin, sectioned (10 μm-thick, as above mentioned) and stained with HE (Bancroft and Gamble, 2008). Sections were observed under light microscopy to check the presence of sperm in testes and mature oocytes in ovaries, confirming the sexual maturity of all studied animals.

Stereological Approach

The cells located in the central portion of the Vs and Vp were classified as neurons or as glial cells according to morphological criteria of size, aspect of the chromatin, and the presence of an evident nucleolus (Fig. 4). Consecutive histological sections were analyzed reaching a total of 12–17 slices studied per animal. Cells were counted using a 100X planapochromat objective lens (1:40 NA) under oil immersion when they were within the area of interest (AOI) of the optic dissector or

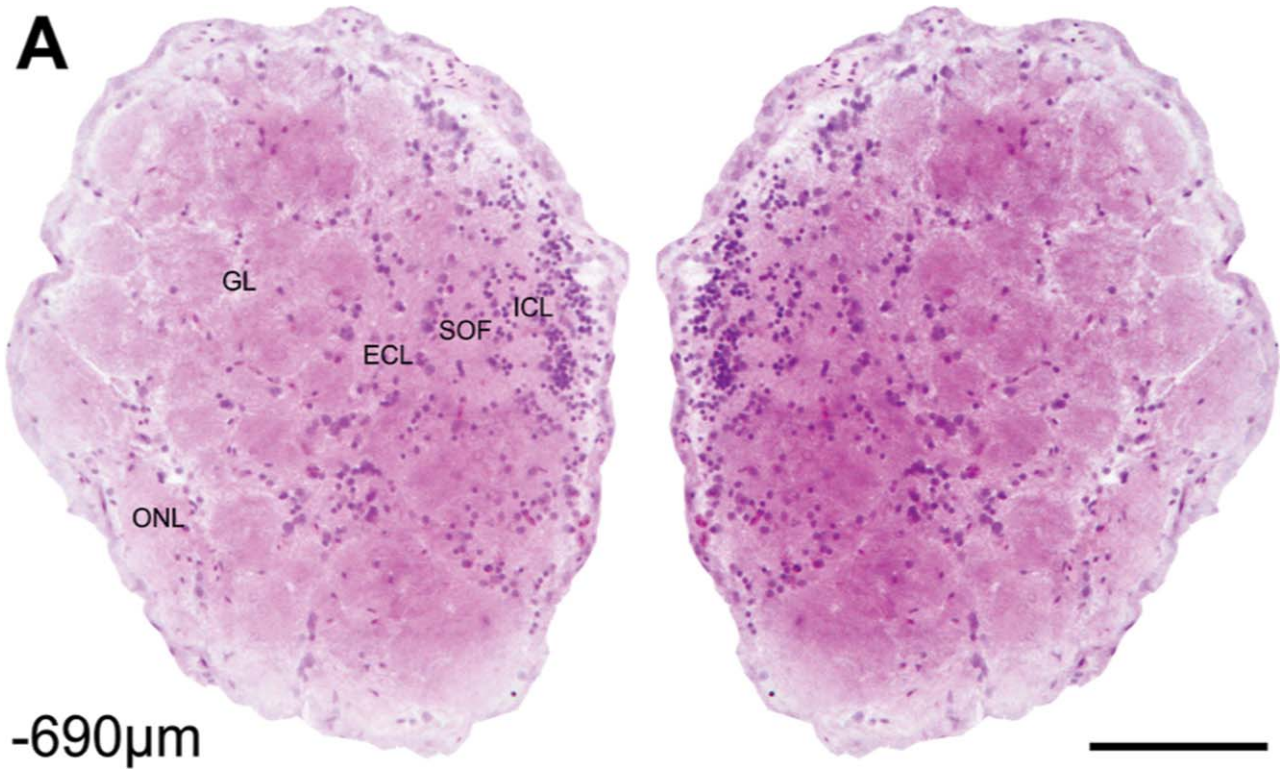
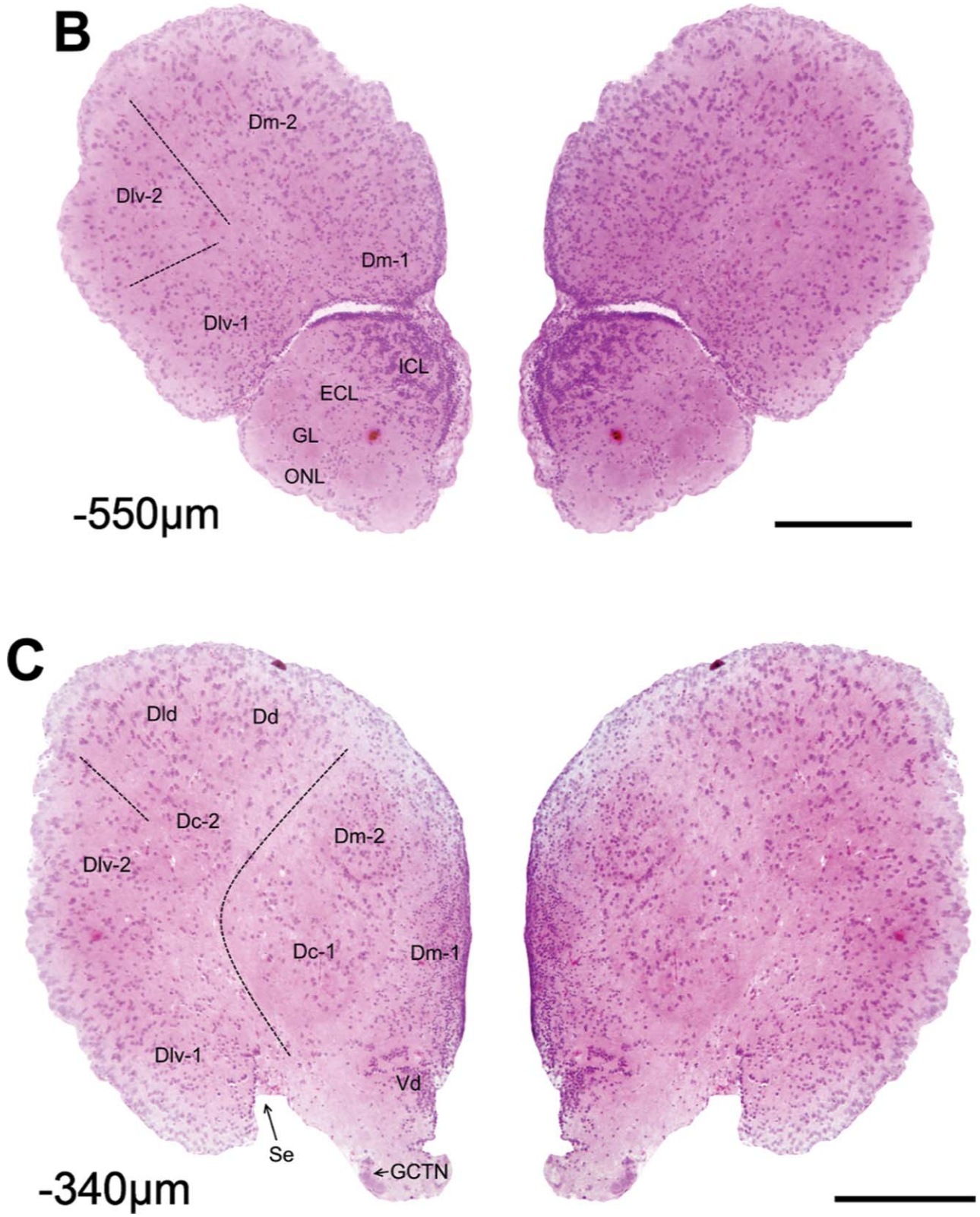


Fig. 2. (**A–C**) Photomicrographs of the *B. splendens* olfactory bulb (OB) and telencephalic hemispheres (Tel) sectioned in transverse planes from rostral to caudal levels and stained with hematoxylin and eosin. Distances from the rostral border of the anterior comissure (AC) are presented (in μm) in the left (bottom) of each image. Negative signals indicate distances rostral to the AC and positive signals are caudal to it. Dashed lines serve to separate brain structures. Images were adjusted to brightness and contrast using the Adobe Photoshop software (USA). Scale = 100 μm (in **A)** and 200 μm in the other images. (**D-E**) Photomicrographs of the *B. splendens* telencephalic hemispheres (Tel) sectioned in transverse planes from rostral to caudal levels and stained with hematoxylin and eosin. Distances from the rostral border of the anterior comissure (AC) are presented (in μm) in the left (bottom) of each image. Negative signals indicate distances rostral to the AC. Dashed lines serve to separate brain structures. Images were adjusted to brightness and contrast using the Adobe Photoshop software (USA). Scale = 200 μm. (**F-G**) Photomicrographs of the *B. splendens* telencephalic hemispheres (Tel) sectioned in transverse planes from rostral to caudal levels and stained with hematoxylin and eosin. Distances from the rostral border of the anterior comissure (AC) are presented (in μm) in the left (bottom) of each image. Negative signals indicate distances rostral to the AC. Dashed lines serve to separate brain structures. Images were adjusted to brightness and contrast using the Adobe Photoshop software (USA). Scale = 200 μm. (**H-I**) Photomicrographs of the *B. splendens* telencephalic hemispheres (Tel) sectioned in transverse planes from rostral to caudal levels and stained with hematoxylin and eosin. Distances from the rostral border of the anterior comissure (AC) are presented (in μm) in the left (bottom) of each image. Positive signal indicates the distance caudal to the AC (0, reference). Dashed lines serve to separate brain structures. Images were adjusted to brightness and contrast using the Adobe Photoshop software (USA). Scale = 200 μm. (**J-L**) Photomicrographs of the *B. splendens* telencephalic hemispheres (Tel) sectioned in transverse planes from rostral to caudal levels and stained with hematoxylin and eosin. Distances from the rostral border of the anterior comissure (AC) are presented (in μm) in the left (bottom) of each image. Positive signal indicates the distance caudal to the AC. Dashed lines serve to separate brain structures. Images were adjusted to brightness and contrast using the Adobe Photoshop software (USA). Scale = 200 μm.



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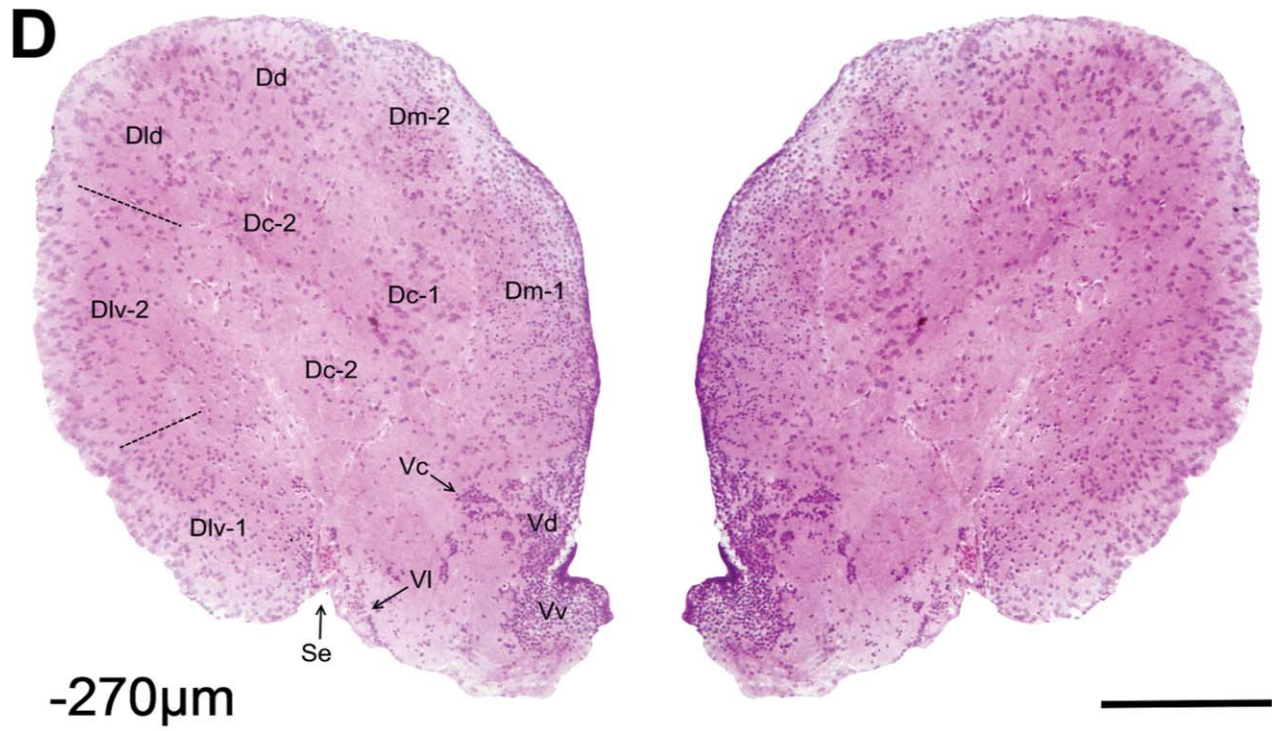

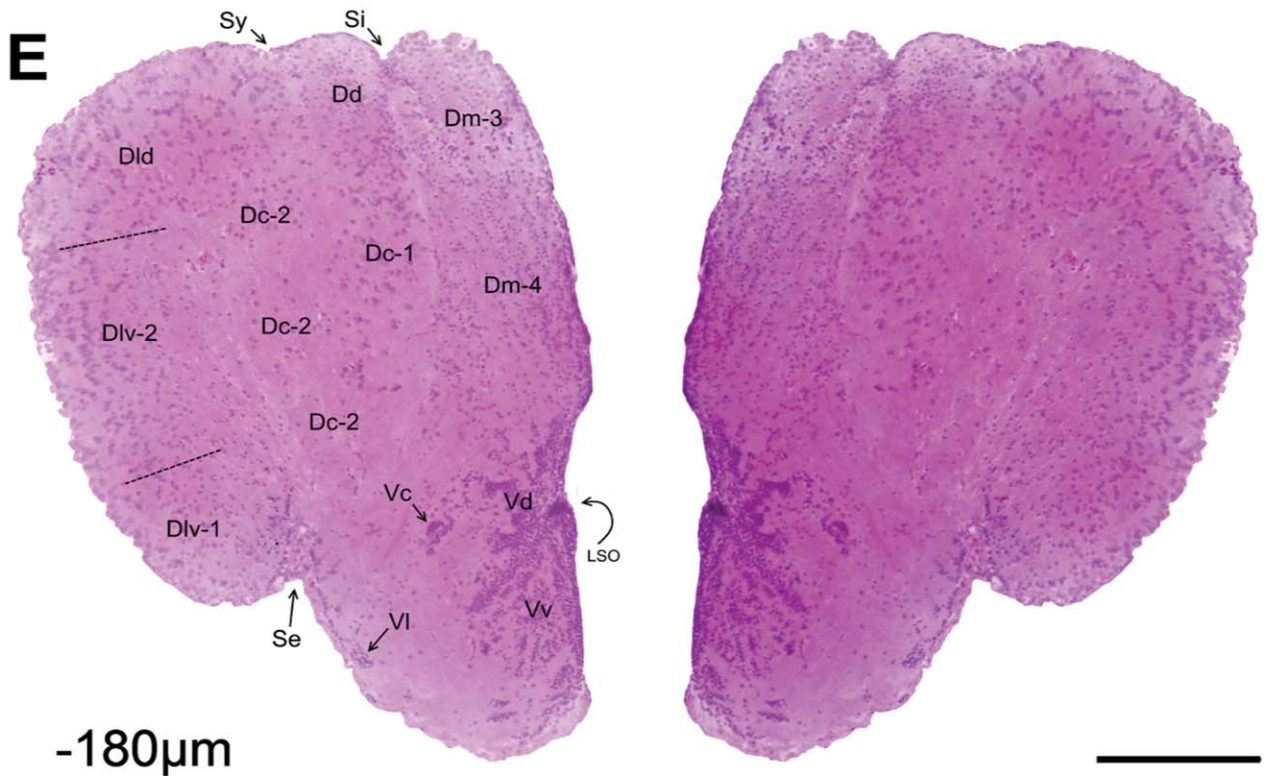

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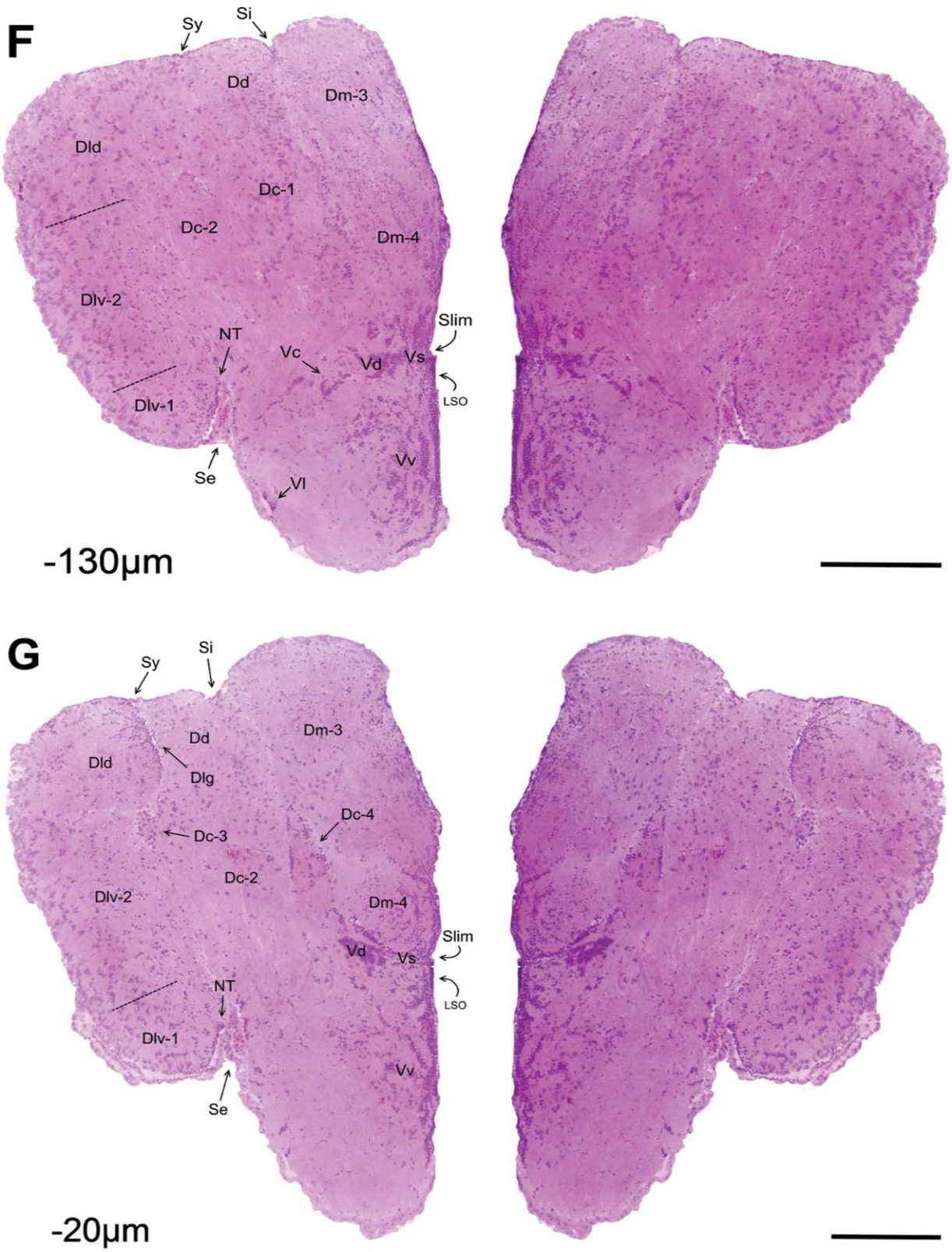

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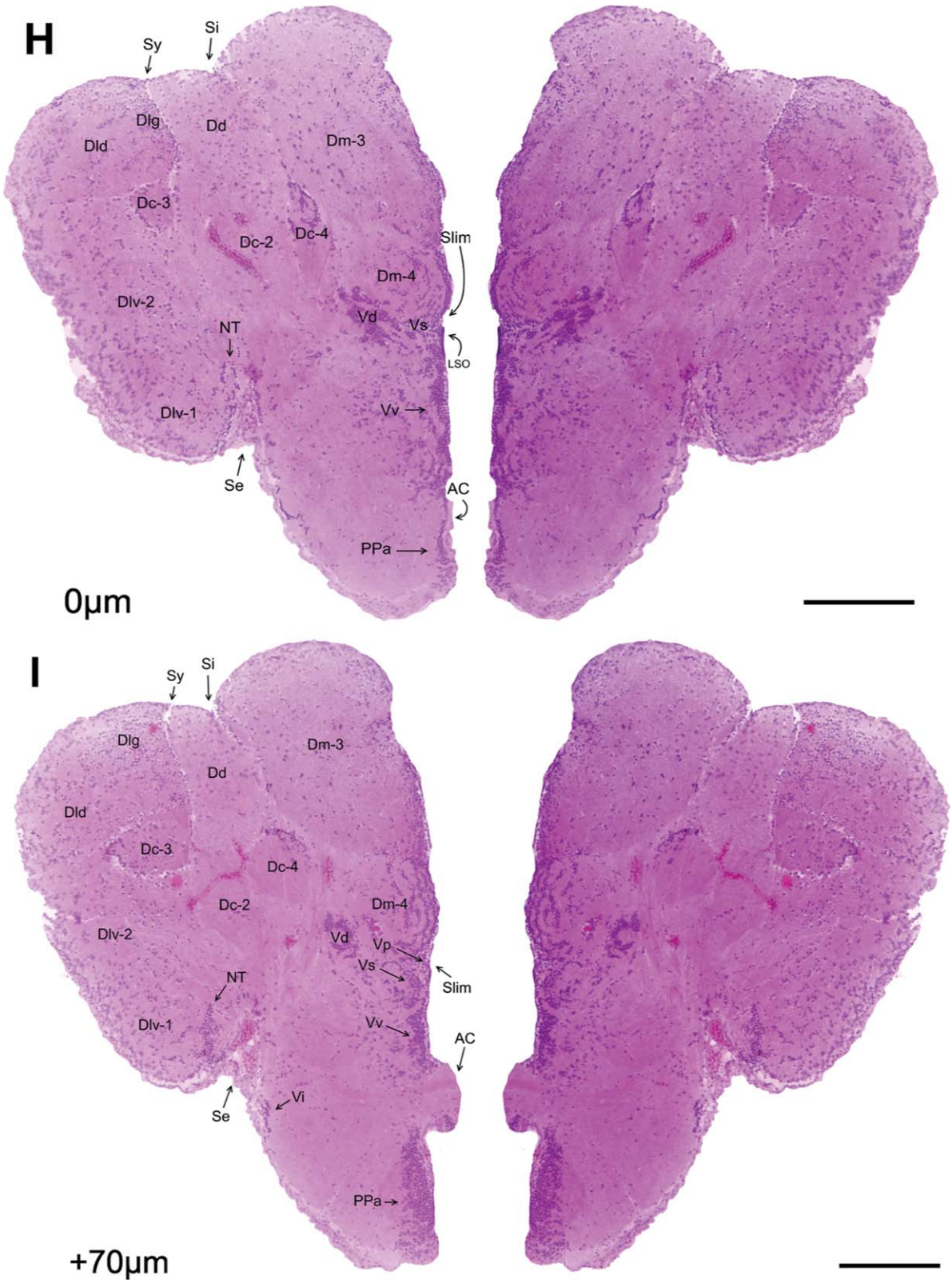

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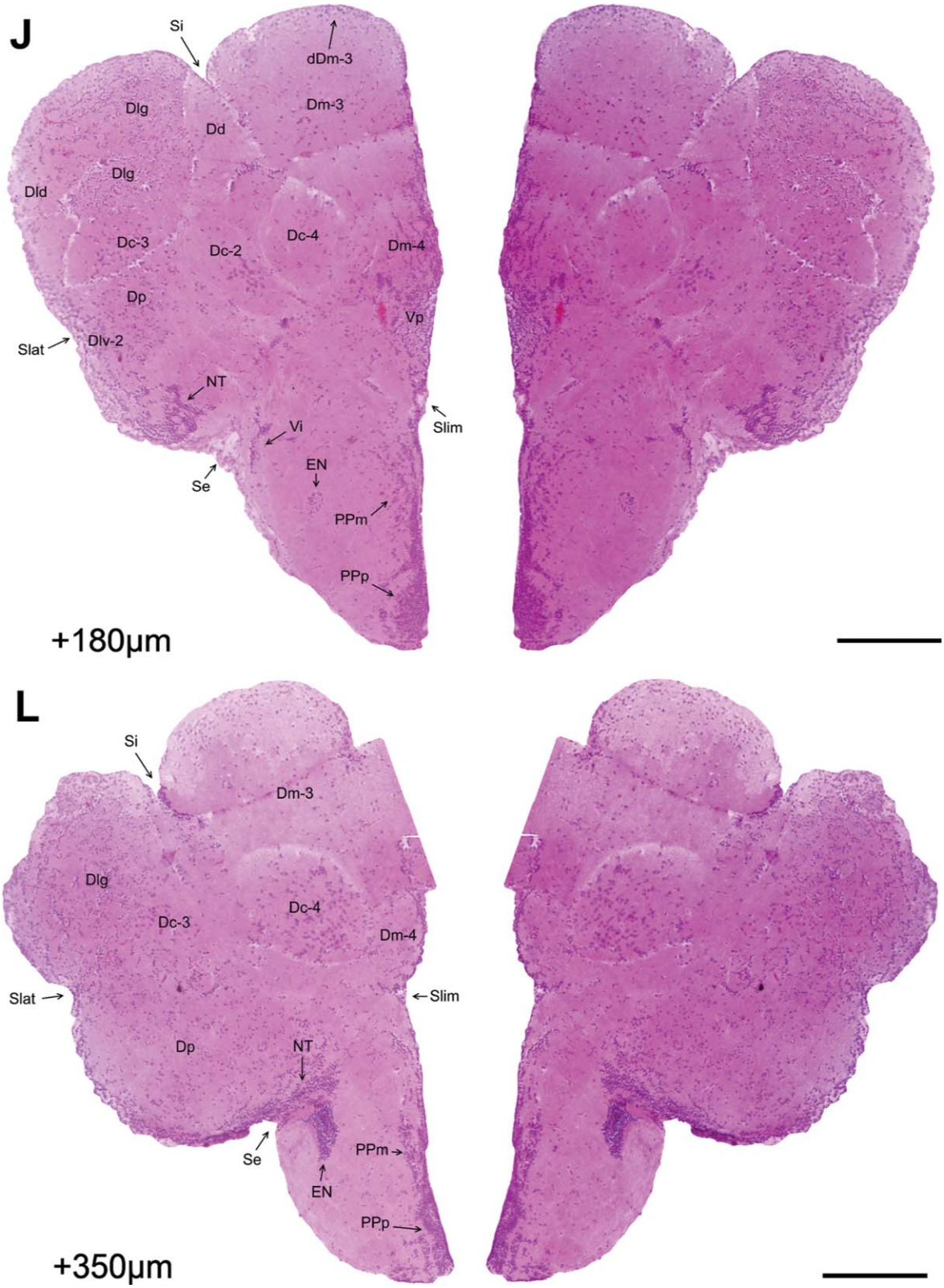

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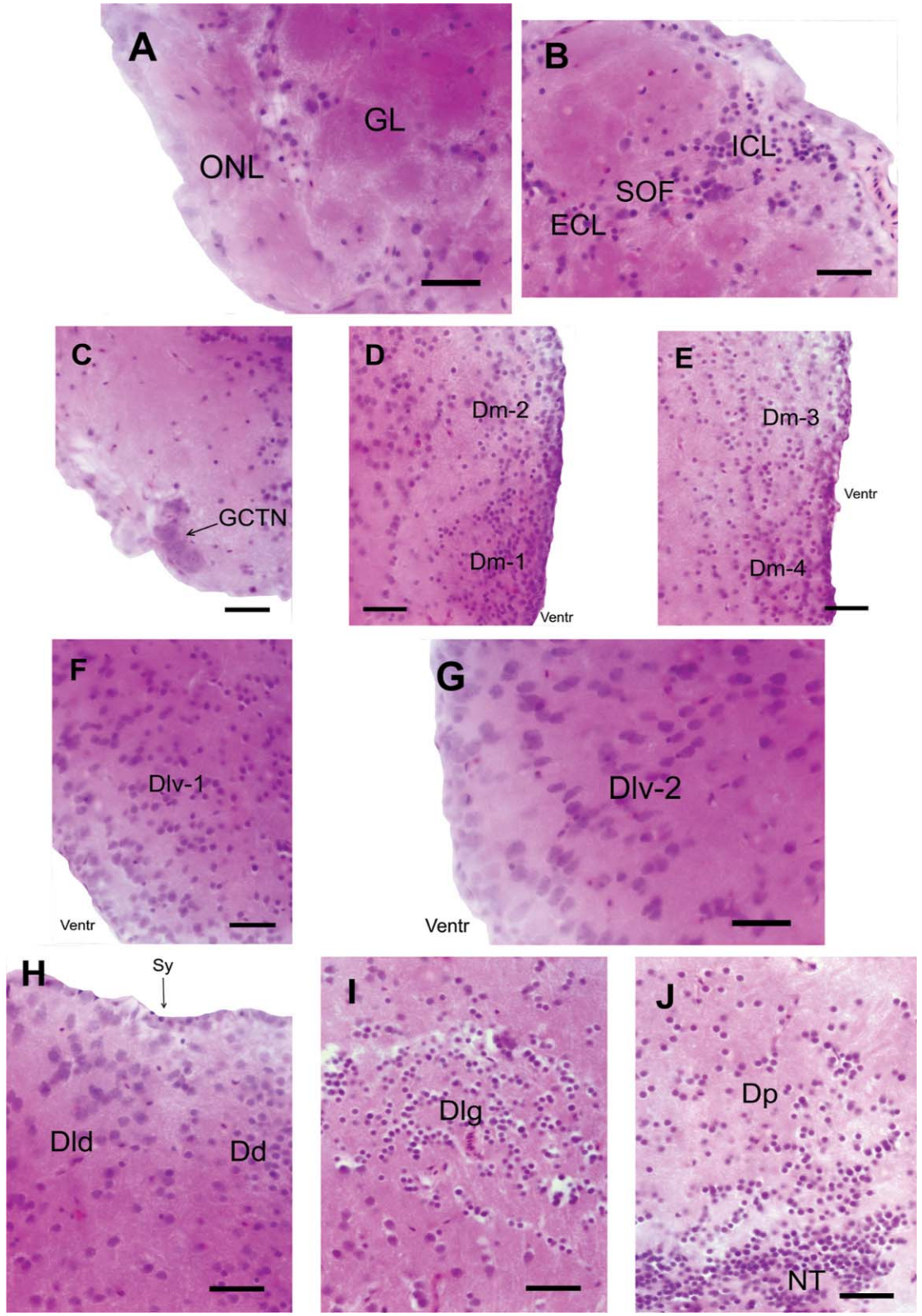
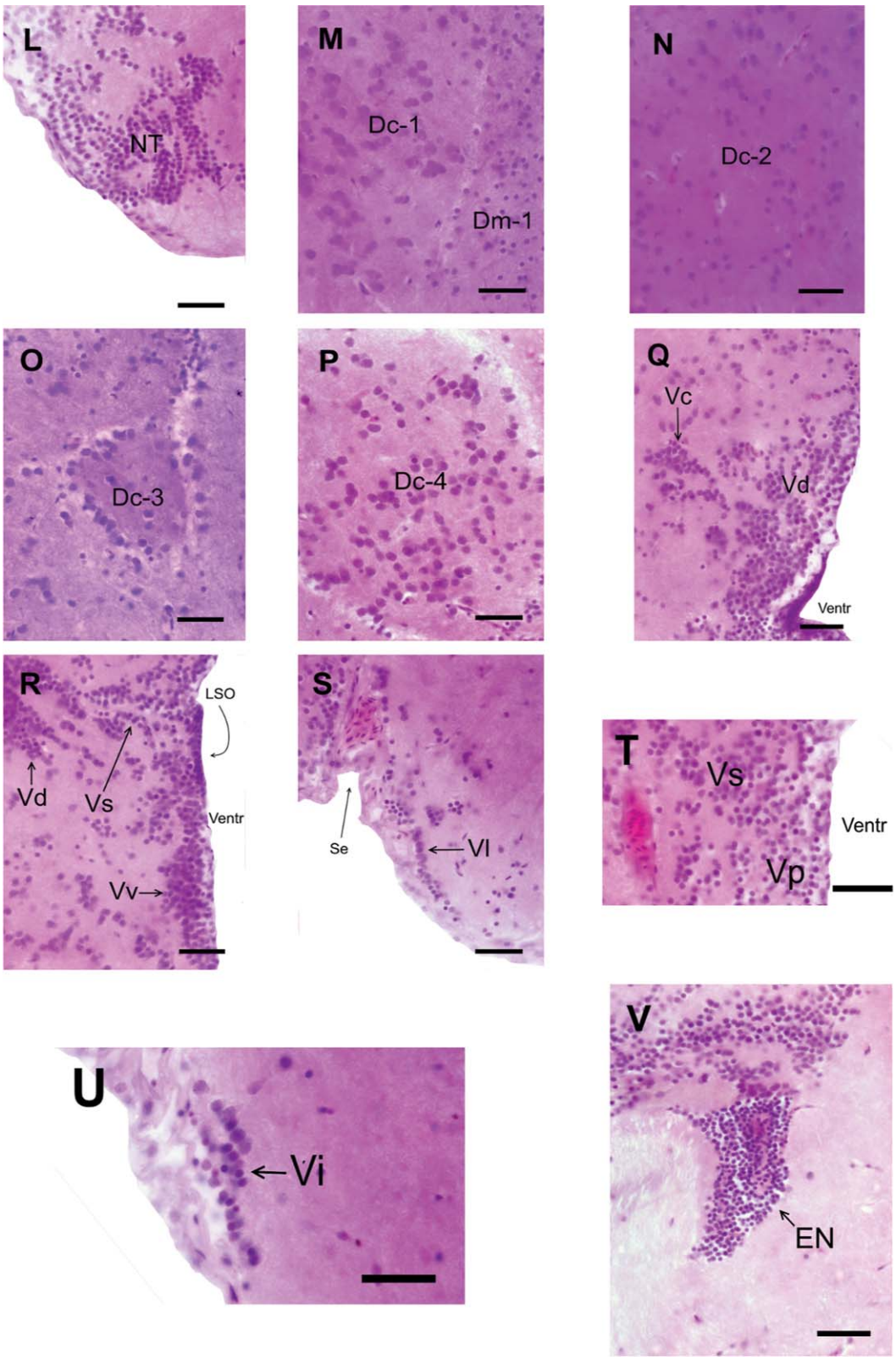


Fig. 3. (**A-J**) Photomicrographs of the *B. splendens* brain stained with hematoxylin and eosin to demonstrate the cytoarchitectural organization of the different areas and nuclei from the olfactory bulb (OB) and telencephalic hemispheres (Tel). Dorsal is up and medial is right. **A** and **B** are, respectively, sections of the ventrolateral and dorsomedial aspects from Figure 2A. **C**, Ventral area of the section shown in Figure 2C. **D**, Medial area of the section shown in Figure 2C. **E**, Medial region of the section shown in Figure 2E. **F** and **G**, Lateral and ventrolateral views, respectively, of the section shown in Figure 2C. **H**, Dorsal aspect of the section shown in Figure 2E. **I**, Dorsolateral aspect of the section shown in Figure 2J. **J**, Ventrolateral aspect of the section shown in Figure 2L. Images were adjusted to brightness and contrast using the Adobe Photoshop software (USA). Scale = 30 μm. (**L-V**) Photomicrographs of the *B. splendens* brain stained with hematoxylin and eosin to demonstrate the cytoarchitectural organization of different areas and nuclei from the olfactory bulb (OB) and telencephalic hemispheres (Tel). Dorsal is up and medial is right. **L**, Ventrolateral aspect of the section shown in Figure 2J. **M**, Centromedial area rostral to the section shown in Figure 3D. **N**, **O,** and **P**, Central view presented in Figures 2C, 2H and 2L, respectively. **Q**, Medial view from the subpallium rostral to the section shown in Figure 3D. **R**, Medial area of the section shown in Figure 2G. **S**, Ventrolateral view of the section rostral to the section in Figure 2D. **T**, Medial view of the section presented in Figure 2J. **U**, Lateral aspect of the subpallium in Figure 2I. **V**, Central view of the subpallium shown in Figure 2L. Images were adjusted to brightness and contrast using the Adobe Photoshop software (USA). Scale = 30 μm.



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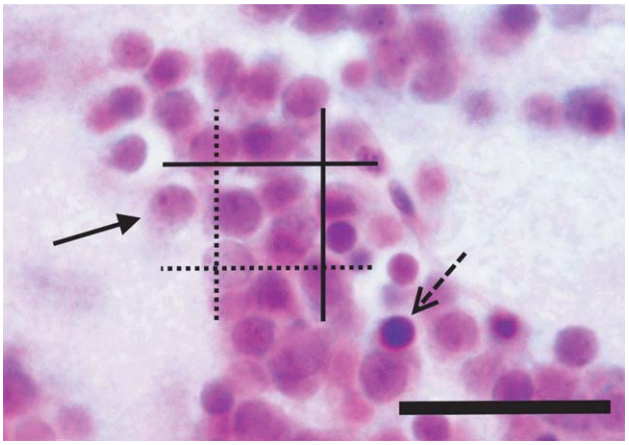

Fig. 4. Photomicrograph of hematoxylin and eosin stained cells in the supracommissural nucleus (Vs) of the *B. splendens* at the magnification used for the stereological counting. The overlaid square represents the area of interest (25 μm^2) with including (solid lines) and excluding (dashed lines) borders. A representative neuron with euchromatic chromatin and evident nucleolus is indicated by a solid arrow. Heterochromatic glial cell is indicated by a dashed arrow. Bright and contrast were adjusted using Adobe Photoshop software (USA). Scale = 10 μm.

touching the inclusion edges. The AOI consisted of a square counting frame of 5 μm × 5 μm with two inclusions and two exclusion edges (Fig. 4). This AOI was overlaid initially on the central area of the Vs or the Vp and 4 randomly chosen adjacent AOIs were studied in each brain slice. This procedure was done along the rostro-caudal axis of the Vs and Vp of both hemispheres. Mean values were calculated for each telencephalic area per animal.

The density of neurons and glial cells in the Vs and Vp of male and female fishes was estimated using the stereological optical fractionator method (cf., Salazar et al., 2014). That is, sections embedded in GMT and stained with HE were observed using a light microscope (Olympus BX61, Japan) coupled to a high resolution D172 digital camera and the Image Pro Plus 7.0 software (Media Cybernetics, USA). The relative numerical density of neurons and glial cells was estimated using the following formula:

$$Nv = (1/[a/f.h]) \cdot \left(\sum Q / \sum P\right)$$

where, Nv = estimated numerical density, a/f = area of the counting frame (25 μm^2), h = optic disector height, $\sum Q$ = sum of cells counted, and $\sum P$ = sum of analized counting frames (adapted from Salazar et al., 2014).

In addition, the proportion of neurons and glial cells was calculated dividing the number of each cell studied by the total number of cells (neurons+ glial cells) in the Vs and Vp of males and females.

Statistical Analysis

Data are presented as mean ± standard deviation (SD). The results of the numerical density of neurons and glial cells in the Vs and Vp were compared between males and females using the Student *t* test and the statistical program GraphPad Prism 6 (USA). The statistically significant level was set as $P \leq 0.05$.

RESULTS

Sexual Maturity and Body Parameters

All fishes used in this work were sexually mature and showed evident sperm and oocytes in the gonads (data not shown). The body weight and standard length of the studied animals are presented in Table 1. No statistically significant difference was found between males and females in both parameters ($P < 0.05$).

HE and Nissl Techniques Data

Both techniques provided similar results to study the *Betta splendens* telencephalon (see a representative example in Fig. 5). The results were reliable and we have chosen the HE images from GMT embedding samples to describe the components of the OB and the Tel of the studied fishes. Whenever necessary, the Nissl data served to complement the characterization of the studied nuclei concerning the cell types, cellular organization, and the aspect of the transition zones, as described below.

Cytoarchitecture of the Olfactory Bulb

The OB of the *B. splendens* has a sessile shape and is closely apposed to the cerebral hemispheres, with no evidence of an olfactory stalk or peduncle (Fig. 1A). The OB of both males and females has a similar laminar organization with 5 concentrically arranged layers, from outside to inside, in the following order: (1) Olfactory nerve fiber layer (ONL) composed of primary olfactory fibers; (2) Glomerular layer (GL); (3) External cellular layer (ECL) made up by large cells (probably mitral ones) and additional small cells (granular cells); (4) Secondary olfactory fibers (SOF) likely being the assemble of fibers from large and small cells coming from the adjacent layers; and, (5) Internal cellular layer (ICL) with a group of small cells placed dorsomedially in the OB (Figs. 2A and 3A,B). The SOF was difficult to be delineated and showed the thinnest layers. The GL was the easiest to

TABLE 1. Body weight and length of adult males and females of *B. splendens* used for the cytoarchitectural study and stereological procedure

Cytoarchitectural Study		
Sex	Weight (g)	Length (mm)
Male ($n = 13$)	0.90 ± 0.19	30.34 ± 1.54
Female ($n = 15$)	0.86 ± 0.13	31.01 ± 1.60
Stereological Procedure		
Sex	Weight (g)	Length (mm)
Male ($n = 6$)	0.97 ± 0.18	30.18 ± 2.07
Female ($n = 6$)	0.86 ± 0.15	30.67 ± 1.72

Values are expressed as mean ± SD. No statistically significant differences were found in both sexes.

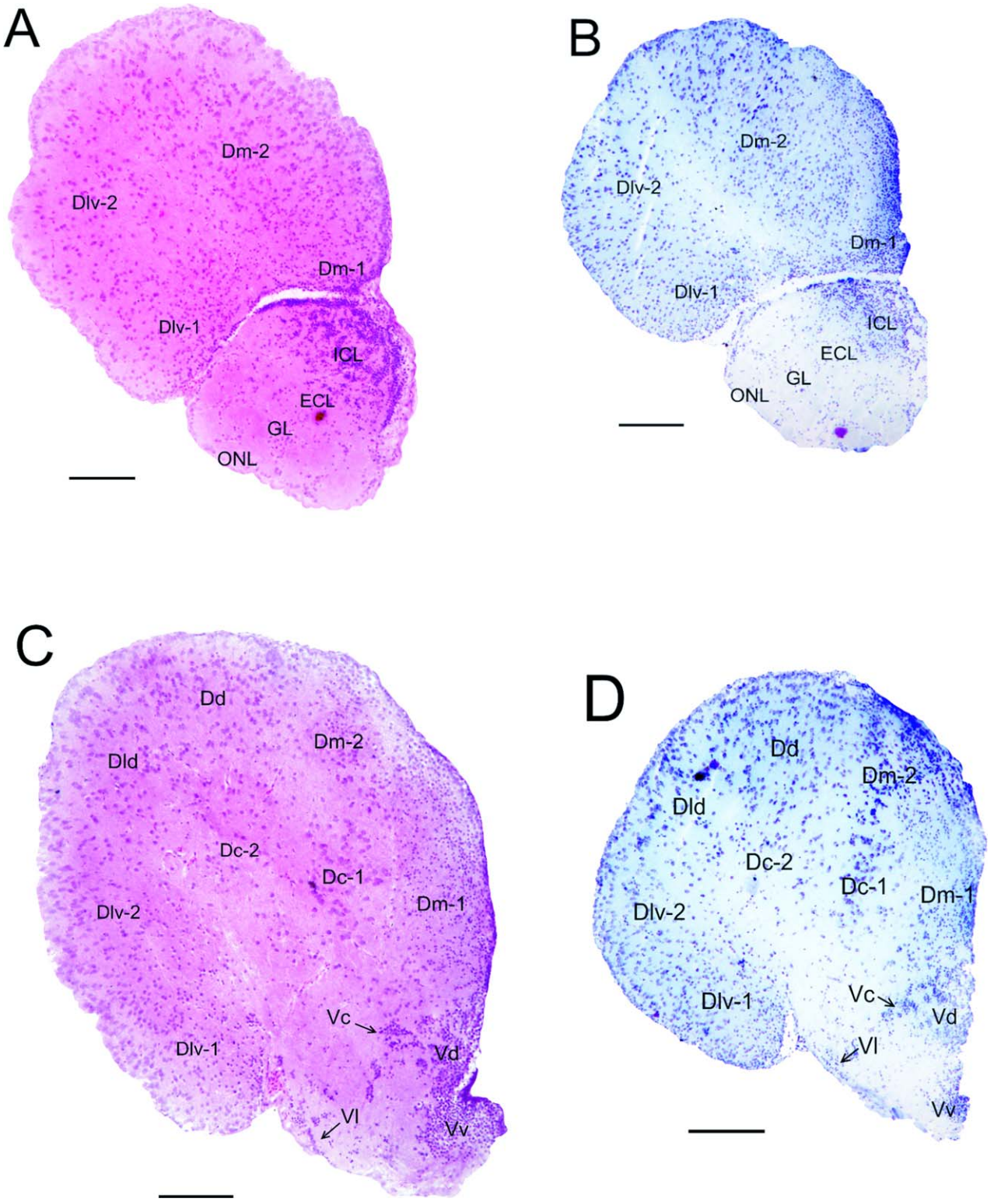

Fig. 5. Photomicrographs of the *B. splendens* olfactory bulb (OB) and telencephalic hemispheres (Tel) sectioned in transverse planes from equivalent rostral levels and stained with hematoxylin and eosin (A and C) or by the Nissl technique (B and D). Note the similar topology of the cell masses of the OB and Tel in these sections after both histological processing. Images were adjusted to brightness and contrast using the Adobe Photoshop software (USA). Scale = 100 μm.

be identified and had the thickest layers (Fig. 2A). Large and small cells showed additional features. Most large cells have a pyramidal-like cell body and an euchromatic nuclei with visible nucleolus whereas the small granular cells have invariably round shaped cell bodies with both euchromatic and heterochromatic nuclei.

Although the concentric layers are clearly separated rostrally (Fig. 2A), the layers 3 and 4 intermingle from the middle of the OB toward its caudal part and, then, it is no longer possible to identify the SOF (Fig. 2B). At the caudal end of the OB, the ONL, and the GL cannot be identified, whereas granular cells at the dorsomedial position are clearly visible composing the ICL. A small group of large cells with abundant cytoplasm was found at the lateral aspect of the most caudal aspect of the OB (Figs. 2C and 3C). These cells were identified as the ganglion cells of the terminal nerve (GCTN).

In contrast to other fishes, as *Salmo* and *Lepomis* (Northcutt and Bradford, 1980), we could not observe any ventricle inside the OB of *B. splendens*.

Cytoarchitecture of the Telencephalic Hemispheres

Males and females have a similar aspect for the telencephalic structures along the rostro-caudal axis. The telencephalon of *B. splendens* is composed of two lateral bulbous structures separated by a median single ventricle, with a "T" shape with long arms directed ventrally.

Here, we identified 5 longitudinal superficial sulci: (1) Sulcus limitans (Slim) as a ventricular sulcus in the medial wall of each telencephalon (Fig. 2F–L); (2) Sulcus *inominatus* (Si) as a dorsomedial ventricular sulcus (Fig. 2E–L); (3) Sulcus ypsiloniformis (Sy) as a dorsolateral ventricular sulcus (Fig. 2E–I); (4) Sulcus lateralis (Slat) as a lateral ventricular sulcus (Fig. 2J–L); and, (5) Sulcus externus (Se) as a ventral meningeal sulcus (Fig. 2C–L). The telencephalic hemispheres of the *B. splendens* are formed by dorsal and ventral areas. The strict boundaries of both areas are not evident along the entire rostro-caudal axis.

Dorsal Telencephalon

The D is composed of 5 cell columns or zones along its rostro-caudal axis which were named medial (Dm), lateral (Dl), dorsal (Dd), central (Dc), and posterior (Dp) ones. The Dm and Dc have 4 subdivisions each. They were numbered by the order of appearance from the rostral to the caudal axis. The Dl included the dorsal division of the lateral zone of the dorsal telencephalon (Dld), the granular division of the lateral zone of the dorsal telencephalon (Dlg), and the ventral division of the lateral zone of the dorsal telencephalon, subdivisions 1 and 2 (Dlv-1 and Dlv-2). In addition to these components, we also identified the nucleus taenia (NT) and the dorsal cell group of the medial zone of the dorsal telencephalon, division 3 (dDm-3), to compose 16 parts of the pallium in this fish species.

All Dm divisions face the ventricular or ependymal surface of the D and extend along the rostrocaudal axis, with Dm-1 and Dm-2 occupying the rostral part and Dm-3 and Dm-4 positioned at the caudal half, respectively (Fig. 2B–L). The Dm-1 is a ventral division of the Dm and can be observed dorsal to the subpallial dorsal nucleus of the ventral telencephalon (Vd) and medial to the Dc-1 by areas with a relatively lower cell density (Fig. 2B–D). The Dm-1 extends from the most rostral aspect of the Tel to a point rostral to the anterior commissure (AC) when it is substituted by the Dm-4 (Fig. 2B–E). It is composed of small, intensely stained, and packed cells (Fig. 3D,M), which became bigger and tightly organized from the lateral to the medial position in this division (Fig. 3D). The Dm-2 lies dorsal to the Dm-1 and faces laterally the Dlv-2 and Dd at rostral and caudal parts, respectively (Fig.2B–D). Compared to the Dm-1, cells in the Dm-2 are bigger, lighter, and loosely arranged (Fig. 3D). Caudally, these cells become tightly arranged, forming small groups of cells (Fig. 2D). The Dm-3 is found caudal to the Dm-2 and dorsal to the Dm-4 (Fig. 2E–L). Lateral to the Dm-3 and along most of its extent the Dd is separated superficially from this division by the Si (Fig. 2E–J). The Dm-3 has poorly stained medium-sized cells, like the Dm-2 (Fig. 3E). Rostrally, these cells show a rope-like organization (Figs. 2E and 3E) whereas, in the middle and caudal parts, they are diffusely distributed (Fig. 2F–L). An exception to this last description is observed at the most dorsal portion of the caudal Dm-3, where cells are densely arranged and identified as the dDm-3 (Fig. 2J). The Dm-4 is caudal to the Dm-1, medial to the Dc-1 rostrally and to the Dc-4 in the middle and caudal parts, ventral to the Dm-3, and dorsal to the different subpallium nuclei, as the Vd, Vs, and Vp (Fig. 2E–L). Where the AC is present, the Dm-4 lies immediately dorsal to the Slim (Fig. 2H–I). The cells of this division are smaller and intensely stained compared to those of the Dm-3 and show a laminar organization with several layers parallel to the wall of the ventricle (Figs. 2G–J and 3E).

Clearly developed in the *B. splendens*, the Dl zone has its 4 divisions directed to the ventricular surface of the D, as occurs for the Dm (Fig. 2B–L). The Dlv-1, Dlv-2, and Dld are found along almost all the extent of the rostro-caudal axis of the D, while the Dlg lies from the middle to the caudal end of this area (Fig. 2B–L). The identification of these divisions relies exclusively on their cytoarchitecture since there is not a cell-free area separating one from the other.

The Dlv-1 is ventral to the Dlv-2 throughout its extent and lateral to the NT in its caudal aspect (Fig. 2B–I). It is composed of medium sized, predominantly round and poorly stained cells arranged in small groups (Fig. 3F). The Dlv-2 is the most laterally positioned division of the Dl zone. It is located ventrally to the Dld (Fig. 2B–J) and is formed by moderately stained round or fusiform cells, bigger than those of the Dlv-1 (Fig. 3G). The main difference from the Dlv-2 to the Dlv-1 is the arrangement of the former cells, which form compact columns perpendicular to the ventricular surface (Fig. 3G). The Dld is lateral to the Dd along its first two thirds becoming flanked by Dlg from the AC level to its caudal end (Fig. 2C–J). Cells in the Dld are similar in size and staining properties to those of the Dlv-2 (Fig. 3H), but differ in their cytoarchitectural organization. In the rostral aspect of the Dld, cells are arranged in sparse perpendicular columns to the ependyma, an organizational pattern that disappears toward its caudal end, where cells do not seem to have a preferential orientation (Fig. 2C–J). The Dlg is a group of small clustered deeply stained cells that sometimes assumes a rope-like

organization (Fig. 3I). This division first appears in the rostral half of the telencephalon, near the AC, between the Dd (medially), the Dld (laterally), and deep to the Sy (Fig. 2G), turning laterally along its course to occupy the dorsolateral part of the D, close to its caudal end (Fig. 2H–L). The Dd is superficially limited by the Si medially and by the Sy laterally (Fig. 2E–I) and is composed of a strip of medium-sized and pale cells distributed diffusely (Fig. 3H). Rostrally, this zone is positioned lateral to the Dm-2 and medial to the Dld (Fig. 2C). Toward the caudal end the Dd is flanked by both the Dm-3, which replaces the Dm-2, and the Dlg, which displaces laterally the Dld (Fig. 2E–J). From the Dd to these last zone and division, an evident nuclear separation is caused by an abrupt change in the cytoarchitectonic organization (Fig. 2G–J). The Dp corresponds to a large posterior zone located caudally to the Dlv-2 and ventrally to the Dc-3 (Fig. 2J–L), which extends to the caudal end of the Tel. It is limited superficially by the Slat and the Se, at dorsal and medial positions, respectively (Fig. 2J–L). The cytoarchitecture of this zone is characterized by small cells organized in irregular strips (Fig. 3J). The NT is composed of small, deeply stained, and tightly grouped cells (Fig. 3L). This structure appears at the middle of the rostrocaudal axis, initially medial to the Dlv-1 (Fig. 2F–I) and, then, medial to the Dp (Fig. 2J–L). The NT can be found close to the superficial aspect of the Se along most of its extent (Fig. 2F–I).

The Dc is observed near the posterior level of the OB until the caudal pole of the Tel (Fig. 2C–L). The Dc is flanked medially by the Dm, laterally by the Dl, dorsally by the Dd, and ventrally by the Dp, the NT, and the nuclei of the V. Here, we found the largest cells in the *B. splendens* telencephalon, whose characteristic aspect allows the clear identification of this zone. The Dc-1 is the most rostral division and extends approximately to the middle part of the Tel (Fig. 2C–F). The Dc-1 is flanked medially by the Dm-1 and by the Dm-4, and dorsomedially by the Dm-2 and by the Dm-3 (Fig. 2C–F). The Dc-1 has large fusiform and moderately-stained cells often arranged in small groups of cells (Fig. 3M). The Dc-2 extends from approximately the rostral end of Dc-1 to the caudal level beyond the AC (Fig. 2C–J). Medially, there are the Dc-1 and the Dc-4. Laterally, there are the Dld, the Dlv-1, and the Dlv-2 along the rostro-caudal axis (Fig. 2C–J). The cytoarchitecture of this zone differs from that of the Dc-1 by having relatively small, round, intensely stained, and diffusely organized cells (Fig. 3N). The Dc-3 and the Dc-4 are both identified at the caudal third of the Tel as two well-delineated zones (Fig. 2G–L). The Dc-3 arises between the Dld and the Dlv-2 laterally and the Dc-2 medially and throughout the caudal end of the Tel (Fig. 2G–J). Its cells are large, as those of the Dc-2, and round shaped, often forming small strings (Fig. 3O). The Dc-4 lies immediately caudal to the Dc-1, lateral to the Dm-3 and the Dm-4, and medial to the Dc-2 in most of its extent (Fig. 2G–J). The Dc-4 is narrow in its rostral end, becoming gradually wide toward its caudal aspect (Fig. 2G–J). In the rostral area of this zone, the cells are medium-sized and grouped close to its boundaries. Toward the caudal end, the cells of the Dc-4 become large, as those of the Dc-2, and form various small groups with a diffuse organization (Fig. 3P).

Ventral Telencephalon

The V has 8 nuclei plus the lateral septal organ (LSO) and the POA nuclei forming an anatomical *continuum*. These V nuclei are the dorsal (Vd), ventral (Vv), central (Vc), lateral (Vl), Vs, Vp, intermediate (Vi), and entopeduncular (EN) nuclei. In the *B. splendens*, the Vd, Vv, Vc, and Vl are rostral to the AC; the Vs lies at the same level as the AC and the Vp, and the Vi and EN are caudal to the AC (Fig. 2C–L). The LSO is found rostral to the AC (Fig. 2E–H). The POA begins at the level of the AC and extends toward the diencephalon (Fig. 2H–L).

Contrasting to the D, all cells in the V have a round and small shape. There are only 3 groups of cells that differ from this pattern: the EN, the LSO, and the preoptic nucleus (PPm). The EN and LSO cells are the smallest ones whereas the PPm cells are the biggest ones in the V. The Vd is the most rostral nucleus of the V. It lies caudal to the ICL of the OB and extends until the AC level (Fig. 2C–I). Along its extent, the Vd is turned from medial to lateral and from ventral to dorsal, from the lower ventricular wall to the center of the V (Fig. 2C–I). The Vd is formed mostly by clustered cells (Fig. 3Q,R). The Vv appears caudal and ventral to the Vd, extending until the level of the AC (Fig. 2D–I). Local cells are bigger, but stained similarly to the Vd (Fig. 3R). They are organized in compact laminae near the ventricular wall, becoming more diffuse laterally (Fig. 2D–I). The Vc is a small group of cells, lateral to the Vd, and in the rostral half of the Tel (Fig. 2D–F). The cells of this nucleus are densely arranged and markedly stained (Fig. 3Q). The Vl is a lateral and laminar nucleus parallel to the meningeal surface of the Tel, medial to the Se, and extending by the same distance than Vc (Fig. 2D–F). Its cells are lightly stained when compared to those in the Vd, Vv, and Vc (Fig. 3S).

The Vs appears slightly rostral to the AC, immediately ventral to the Dm-4, medial to the Vd, near the LSO, and close to the Slim at the ventricular surface of the V (Fig. 2F–I). Compared to the Vd, this nucleus is composed of lighter and less densely packed cells arranged in groups (Fig. 3R). The Vp appears at the caudal third of the Tel, ventral to the Dm-4, and close to the Slim, displacing the Vs laterally (Fig. 2I–J). Compared to the Vs cells, the Vp ones are lightly stained and more diffusely distributed (Fig. 3T). In spite that both of these last nuclei are anatomically continuous, it is still possible to differentiate one from another. No reliable condition allowed the identification of additional group arrangements.

The Vi is a nucleus composed of highly stained cells forming an irregular column parallel to the meningeal surface of the lateral V and medial to the Se (Fig. 3U), showing a more dorsal position toward the caudal end of the Tel (Fig. 2I–J).

The EN is an evident nucleus located at the most caudal aspect of the Tel of the *B. splendens* (Fig. 2J–L). It is composed of very small, deeply stained, and densely grouped cells (Fig. 3V). The LSO is a column of tiny, tight, and strongly stained cells limiting the ventricular surface of the V, ventral to the Slim (Figs. 2E–H and 3R).

Betta splendens shows a POA associated with the V. In this area, three nuclei can be identified: the anterior parvocellular preoptic nucleus (PPa), the posterior

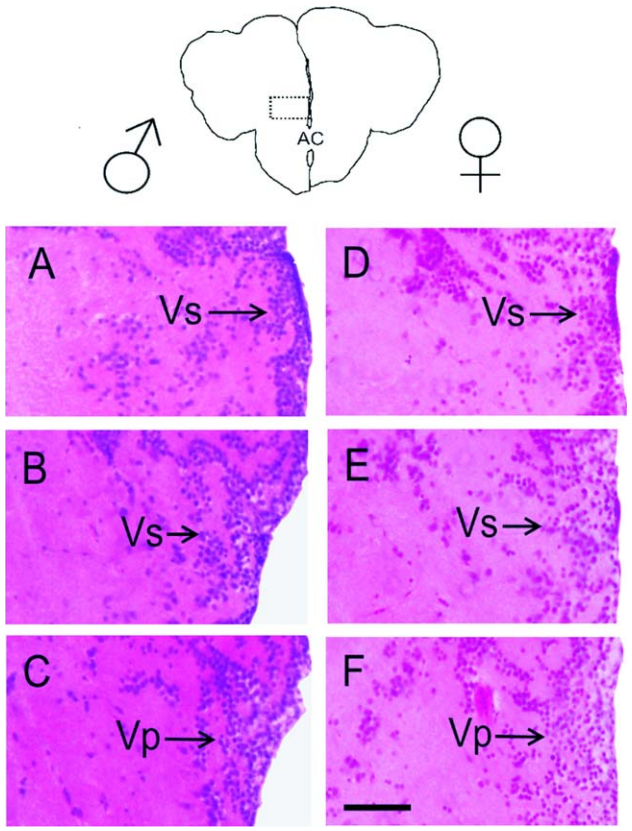

Fig. 6. Schematic image of coronal section of the adult male brain of *Betta splendens* at the level of the anterior commissure (AC). Dashed rectangle indicates the sampled areas to identify the supracommissural (Vs) and the postcommissural (Vp) nuclei along the rostrocaudal axis. The rostro-caudal extent of the Vs has approximately 310 μm whereas the Vp has near 110 μm and a similar extent and aspect in both sexes. Photomicrographs of the Vs and Vp from males (A, B, and C) and females (D, E, F) correspond to rostral (A and D), middle (B and E) and caudal (C and F) levels in relation to the AC position. Images were adjusted to brightness and contrast using the Adobe Photoshop software (USA). Hematoxylin and eosin staining. Scale = 50 μm.

parvocellular preoptic nucleus (PPp), and the PPm. The PPa is located immediately ventral to the AC and close to ventricular surface (Fig. 2H–I). The PPa is formed only by relatively small cells which are loosely packed and poorly stained (Fig. 2I). Caudal to the AC and caudoventral to the PPa, the PPp extends toward to the end of the Tel (Fig. 2J–L). The PPp has smaller, but more stained and densely grouped cells than the PPa (Fig. 2J). The PPm arises dorsal to the PPp and caudal to the AC. The PPm has relatively large and packed cells when compared to the PPa and the PPp, showing a laminar organization parallel to the ventricular wall (Fig. 2L).

Stereological Data

We looked for the overall size of the Vs and Vp in males and females prior to estimating cell number in these regions. We did not observe any evident sexual dimorphism in the overall size of these nuclei using both HE and Nissl staining techniques. The rostro-caudal length of the Vs has approximately 310 μm whereas the Vp has near 110 μm with a similar extent and aspect in both sexes (Fig. 6). We did not find evident cellular heterogeneities within both nuclei that would lead to a significant variability in the data of males and females.

Neurons and Glial Cells

The use of GMT embedding reduced the tissue shrinkage and the staining allowed the identification of neurons and glial cells in the telencephalon of the *B. splendens*. Neurons were identified as cells with an euchromatic nucleus, an evident nucleolus, and an acidophilus pale cytoplasm. Cell bodies were round or fusiform with variable sizes (Fig. 4). Glial cells had a heterochromatic nuclei with no visible nucleolus and the cytoplasm, when visible, formed a dense eosinophilus narrow ring. As a rule, glia showed a small cell body size (Fig. 4).

Percentage and Numerical Density of Cells in the Vs and Vp

Data for the Vs of males and females are shown in Figure 7. The percentage of neurons and glial cells in males and females were 63% and 37% and 72% and 28%, respectively. The total density of cells (neurons + glia), neurons or glial cells did not show statistically significant differences between sexes ($P > 0.05$; Fig. 7A–C).

Data for the Vp of males and females are shown in Figure 8. Similar values as in the Vs were found for the percentage of neurons and glial cells in the Vp of males and females. Also, the total density of cells (neurons + glia), neurons or glial cells did not show statistically significant differences between sexes ($P > 0.05$; Fig. 8A–C).

DISCUSSION

Our results provide novel data about the cytoarchitectonic of the OB and the Tel of the *B. splendens* in both male and female fishes. We found more neurons than glial cells in the Vs and the Vp, but no evidence for a sexual dimorphism in these areas. These important issues will be discussed and compared with the current literature in the following paragraphs.

The Delineation of Nuclei in the Fish Brain

The identification and delineation of telencephalic nuclei has been reported for different species of fishes (Cerdá-Reverter et al., 2001; Burmeister et al., 2009; Baile and Patle, 2011; D'Angelo, 2013; Dewan and Tricas, 2014; Ou and Yamamoto, 2016). However, it is still problematic to establish the precise borders for all nuclei and zones in the brain. It would be relatively easy to establish the border between adjacent cell groups when a rim of cell-free zone or a bundle of axons are evident. Unfortunately, this is not the most common condition for the brain areas in various species. Usually the transition between different cell groups occurs through an intermediate zone with merged cells of different types. For example, the OB layers do not always have sharply defined boundaries and one layer can be intermingled with the elements of the adjacent layers (Northcutt and Bradford, 1980; Nieuwenhuys and Meek, 1990). The problem of delineating the boundaries of each nucleus

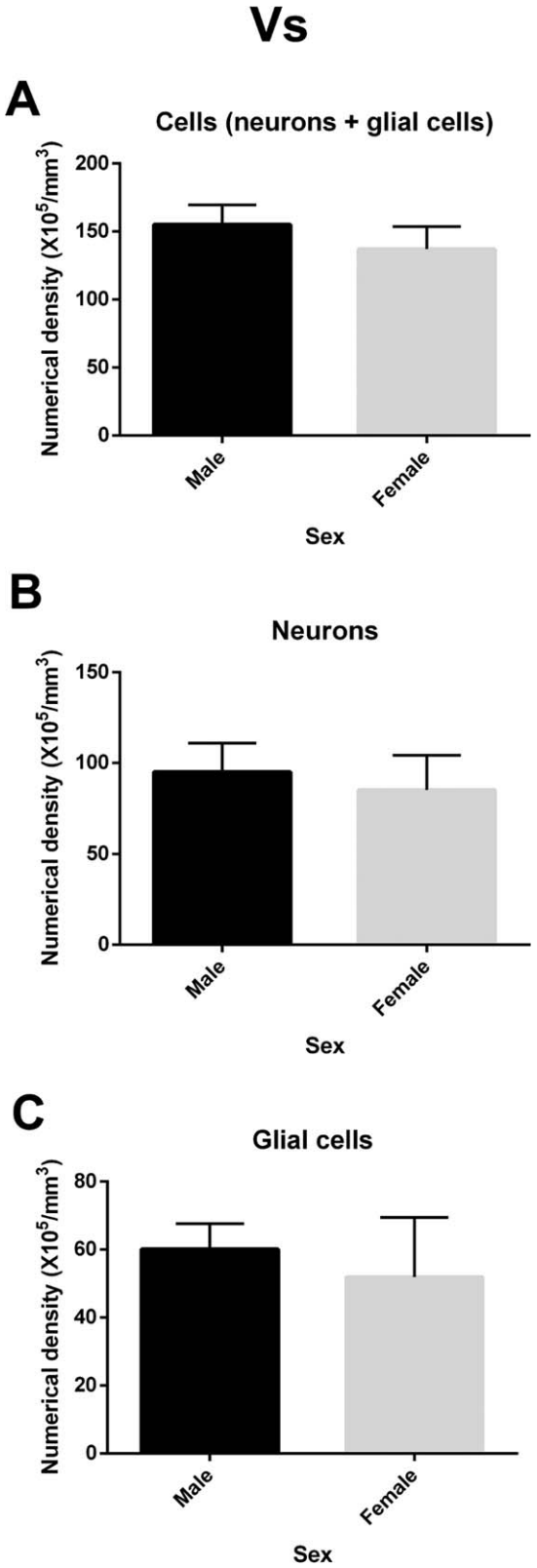

Fig. 7. Estimation of numerical density of cells (neurons + glial cells in **A**), only neurons (**B**) or only glial cells (**C**) from the supracommissural nucleus (Vs) of adult male and female *B. splendens*. Values are mean ± standard deviation. No statistically significant difference was found between sexes.

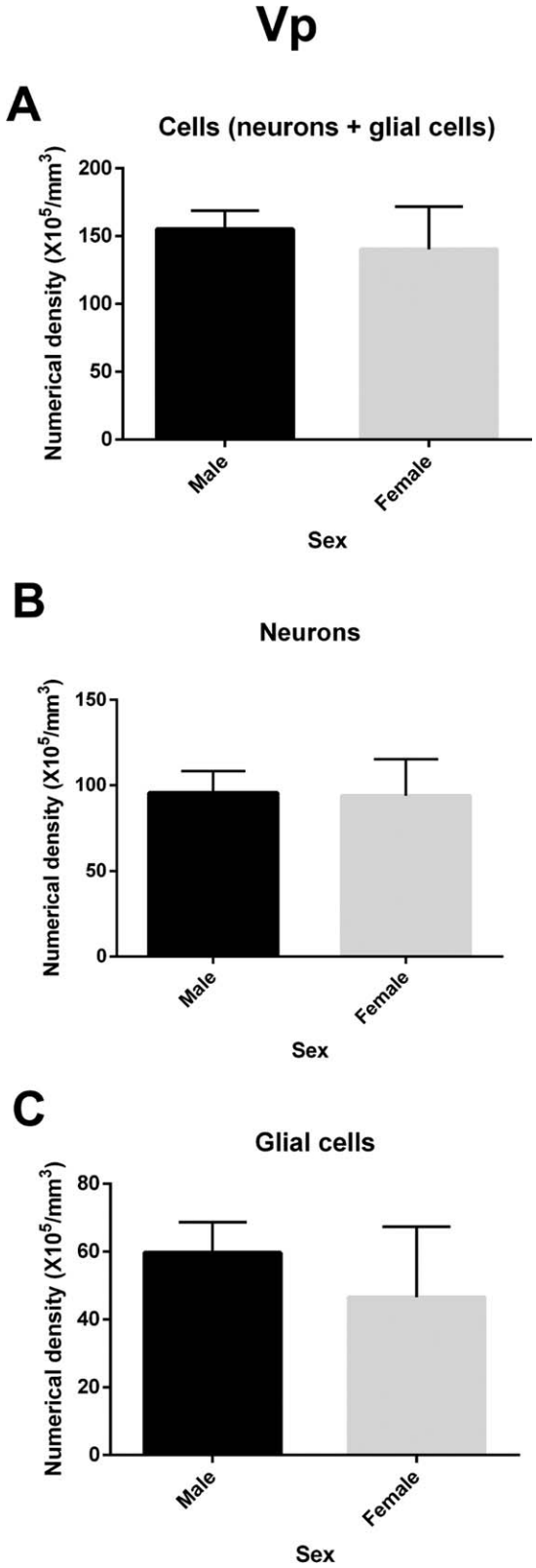

Fig. 8. Estimation of numerical density of cells (neurons + glial cells in **A**), only neurons (**B**) or only glial cells (**C**) from the postcommissural nucleus (Vp) of adult male and female *B. splendens*. Values are mean ± standard deviation. No statistically significant difference was found between sexes.

and its subdivisions was properly discussed in the work of Gahr (1997). Accordingly, it is necessary the combination of different histological and functional techniques to determine the various features of the neural cells.

Therefore, we decided to identify each component of the OB and Tel in the *B. splendens*, but we did not include strict borders to all the nuclei or subnuclei studied here. The same precautionary procedure was done previously for the delineation of the subnuclei of the MeA in rats and humans (Dall'Oglio et al., 2013; Salazar et al., 2014). Nevertheless, other authors have the inherent difficulties for doing so was critically stated in the rat brain atlas of Kruger et al. (1995; also compare the delineation of the borders of the rat MeA subnuclei in the atlas of Paxinos and Watson, 1998 and 2006). That is, "... many of the outlines of nuclei are apparently discrepant with the opinions of other scientists; in some instances, there is a forced, artificial recognition of distinctions that are not recognizable as separate nuclei. This occurs whenever a cell group becomes scattered in development and is interspersed with other functional entities. There are numerous examples of nuclei consisting partly of "straggling" small neuronal aggregates that can be misleadingly outlined... The limits of many structures are often striking at low magnification, but there are numerous circumstances where high magnification cannot resolve the problems inherent in attempting to impose boundaries in regions of gradual gradients of change or, in the case of fiber architecture, where there is no way of recognizing functionally distinct contiguous tracts. The criteria for drawing lines between neural structures often differ for individual investigators..." (Kruger et al., 1995).

Therefore, we think that additional experimental data, such as those from the Golgi technique and the extensive study of cellular components, anterograde and retrograde connectional tracers, cytochemical and/or immunocytochemical approaches, would help to determine the ultimate borders of each nucleus, if they are not really a *continuum* of cell shapes and arrangements in the brain parenchyma. It has also to be considered the rigorous recommendations about antibody characterization and appropriate controls needed for immunohistochemistry, which also includes studies in fishes and that are not fully available for the *B. splendens*. That is, it must be determined that the antibody is actually recognizing its specific target in the species and tissue examined, the pattern of bands stained, and the molecular weight in Western blot, radioimmunoassay or ELISA, controls including preadsorption against the original antigen, the lack of staining in knockout animals (if they already exist), to check for cross-reactions, and comparisons with the *in situ* hybridization pattern or previously reported data (Saper, 2005).

The Cytoarchitectonic Organization of the Telencephalon in the *B. Splendens*

This is the first description of the OB of the *B. splendens*. As would be expected for most of the teleost fishes (Nieuwenhuys, 1967; Nieuwenhuys and Meek, 1990), the OB of the *B. splendens* lacks bulbar ventricles and is composed of 5 concentric laminar layers. However, different OB cellular organization were reported in the *C. gachua* (Baile and Patle, 2011), *N. furzeri* (D'Angelo, 2013), and *T. japonicus* (Ou and Yamamoto, 2016). These fishes have 4 concentric laminar layers and the SOF absent. The OB of the *B. splendens* displays a similar aspect, but only from its middle aspect to the caudal pole.

The GCTN were identified in the lateral aspect of the caudal OB of the *B. splendens* based on their topology and characteristic features including shape, size, and how these cells are grouped to form a ganglion. Morphological aspects differ the GCTN of the *B. splendens* from the GCTN of other teleost fishes where data are available. That is, in the *Lepomis cyanellus*, the GCTN are scattered along the ventromedial surface of the OB (Northcutt and Bradford, 1980) and, in the *Ictalurus punctatus*, they can be identified in a ventromedial position in the olfactory nerve fiber layer, rostrally in the OB (Bass, 1981). In the *Dicentrarchus labrax*, *Channa gachua*, and *Trachurus japonicas*, the GCTN occupy a caudomedial position in the OB (Cerdá-Reverter et al., 2001; Baile and Patle, 2011; Ou and Yamamoto, 2016). In the *Carassius auratus*, the GCTN send both peripheral terminals to the olfactory epithelium and central projections to the Vv, Vs, POA, TO, hypothalamus, and retina, which suggests the participation of these OB cells in a network controlling the reproductive behavior (Demski and Northcutt, 1983; von Bartheld and Meyer, 1986; Fujita et al., 1993).

A general pattern of organization and cytoarchitecture can be found in the Tel of actinopterygian fishes (Nieuwenhuys and Meek, 1990; Northcutt, 1995; Bradford, 2009; Nieuwenhuys, 2009). Then, various Tel cell groups were assumed as homolog even in teleost fishes from different ecological niches and phylogenetic relationships (see examples in Northcutt and Bradford, 1980; Bass, 1981; Diez et al., 1987; Riedel, 1997; Cerdá-Reverter et al., 2001; Burmeister et al., 2009; Baile and Patle, 2011; D'Angelo, 2013; Dewan and Tricas, 2014; Ou and Yamamoto, 2016). Our results indicate that the Tel of the *B. splendens* is similar to that of other teleosts.

Marino-Neto and Sabbatini (1988) previously studied the telencephalon and the POA of the male B. splendens, although with different aims and details. For example, there was not available the descriptive analysis of the telencephalon and the POA or the characterization of all cell groups and sulci, except by some information about a restrict number of structures. More detailed neuroanatomic data are essential to support future studies because the *B. splendens* has a great potential as an experimental model to reveal different aspects of social behaviors (Müller, 2005) that can be directly associated with both telencephalon and POA functions (De Bruin, 1980).

Here, we describe the existence of 16 different cell groups in the D of both male and female *B. splendens*. Differently, Marino-Neto and Sabbatini (1988) identified 13 different cell masses in the D, with differences found in the description of the Dm and Dl zones. In this regard, the Dm zone reported in this previous work contains the Dmd and Dmv divisions and the dDmd cell group. Respectively, the Dmv and Dmd, based on topological, topographic, and cytoarchitectonic criteria, are the same as the Dm-1 and Dm-2 divisions observed by us. Considering its position, it is possible that the presently reported Dm-3 division corresponds to the more caudal Dmd of Marino-Neto and Sabbatini (1988).

However, despite Dm-2 and Dm-3 have cells with similar sizes, the organization of them are completely different in both divisions (see results and Fig. 3D,E), justifying their classification in two separated parts. The dDmd cell group of Marino-Neto and Sabbatini (1988) was described as a group of small, dense-packed, subependimal cells at the caudal region of the Dmd. Such small, dense-packed, subventricular cells were observed by us in the Dm-3 division. Previous works reported a medial zone of the teleostean telencephalon divided in 4 divisions (Riedel, 1997; Cerdá-Reverter et al., 2001; Baile and Patle, 2011; Dewan and Tricas, 2014). Our identification of the Dm-4, the fourth division of the Dm, is the first report of this kind in the *B. splendens*.

Marino-Neto and Sabbatini (1988) described the Dl zone with the Dlv, Dld, and Dlp subdivisions. The Dlv corresponds topologically and topographically to both Dlv-1 and Dlv-2 subdivisions in our description. The separation of the Dlv proposed by us is based on the different size and arrangement of the cells present in this subdivision (see results and Fig. 3F,G), as also evident in Sea bass (Cerdá-Reverter et al., 2001) and in Tilapia (Burmeister et al., 2009). The Dld is the same subdivision identified by us. Finally, the Dlp identified by Marino-Neto and Sabbatini (1988) was not characterized in our material. In their work, the Dlp arises between the Dld and Dlv, and occupies the most lateral aspect of the telencephalon. Caudally, this cell group would have migrated dorsally, assuming the position of the Dld. In our material there is not a cell group resembling that. However, the caudal part of our Dlg shows a topology and topography similar to that of the Dlp, although the topology of its cranial region is distinct and the Dlg appears between the Dd and the Dld. There is no information about the cytoarchitecture of the Dlp of the *B. splendens* in the original work of Marino-Neto and Sabbatini (1988) and the cellular identity of this division should be revised with additional studies.

Our work also identified two important discrepancies in the V part of the Tel of *B. splendens*, i.e., the presence of the LSO and the position of the Vp. Here, the LSO was identified and characterized by the first time in the *B. splendens*. This structure was described before in the *D. labrax* (Cerdá-Reverter et al., 2001) and the *C. gachua* (Baile and Patle, 2011). That name was used because it appears to be similar to the corresponding nucleus in the bird telencephalon, where the LSO is a circumventricular organ that may serve basic functions associated with photoreception (Kuenzel et al., 1997). Moreover, Marino-Neto and Sabbatini (1988) identified the Vp as a cell group arising immediately above the AC and that migrate dorsally toward the caudal telencephalon. In our samples, the region immediately above the AC is occupied by cells with a position, aspect (medium-sized), and organization similar to that of the Vv. The Vp cells in other teleostean fishes (Cerdá-Reverter et al., 2001; Burmeister et al., 2009; Baile and Patle, 2011; Ou and Yamamoto, 2016), independently of the exact position adopted by them, are identified as small and pale ones, which match with the cells described by us as the Vp cell group.

Although still controversial, the POA has been considered as a part of the telencephalic subpallium on the basis of shared molecular profiles (Moreno and González, 2011; González et al., 2014). That is further supported by the finding that the POA contributes tangentially migrating neurons to the telencephalic pallium, similar to the other parts of the subpallium, but not the hypothalamus (Puelles et al., 2013). Different from the work of Marino-Neto and Sabbatini (1988), we identified 3 nuclei in the POA of the *B. splendens*: PPa, PPp, and PPm. Based on the cytoarchitectonic characteristics of the PPp shown in this previous study, we further separated it in an anterior and posterior regions since differences between the PPa and PPp can be observed in the size, staining pattern, and arrangement of local cells (shown in Fig. 2H–L).

No evidence for a Sexual Dimorphism in the Numerical Density of Cells in the Vs and Vp of the *B. Splendens*

Our initial prediction was that the Vs and Vp might be sexually dimorphic in the *B. splendens*. Indeed, there are evident differences between males and females in the fish brain. Examples of such differences between sexes were already reported for the motor control in the medulla oblongata, for the modulation of the pituitary secretion as well as in the density of galanin and substance P cells, in the distribution and size of GnRH cells, and in the number of vasotocin and isotocin neurons (Conklin and Polemics, 1997; Jadhao et al., 2001; Ohya and Hayashi, 2006; Costa et al., 2011; Kotrschal et al., 2012).

The homology of the Vs and Vp of actinopterygian fishes with specific counterparts in the tetrapods is not completely settled until the moment (for a discussion on different lines of evidence see Maximino et al., 2013). If the Vs and the Vp (or, at least, part of them) are homologous to the tetrapod MeA (Bradford, 2009; O'Connell and Hofmann, 2011; Bruce, 2012; Maximino et al., 2013), that would imply that olfactory and vomeronasal information could arrive to these nuclei to modulate reproductive behavior. In this sense, teleost fishes have olfactory afferents from the OB to the Vs and/or to the Vp (Davis et al., 1981; Murakami et al., 1983; Levine and Dethier, 1985; Matz, 1995; Riedel and Krug, 1997; Folgueira et al., 2004; Yamamoto et al., 2007). Also, teleosts have a terminal nerve (related to the GCNT) that projects to the Vs (Demski and Northcutt, 1983; von Bartheld and Meyer, 1986). As the Vs projects to the Vp (Shiga et al., 1985; Folgueira et al., 2004; Rink and Wullimann, 2004), both cell groups could respond to the same terminal nerve stimulation. The exact nature of the information coded by this terminal nerve pathway is controversial, but could involve chemical (pheromone), visual, magnetic, thermal and/or auditory stimuli (Demski and Northcutt, 1983; Dulka et al., 1987; Fujita et al., 1993). If there really exists a vomeronasal system in teleost fishes, the Vs and Vp cell groups and their connections could be included as part of this functional network (Dulka, 1993). This possibility is partly supported by two interesting findings: in goldfish, lesions in the Vs and the adjacent ventral telencephalic areas, besides the POA, resulted in the impairment of the male and female sexual behavior (Kyle and Peter, 1982; Kyle et al., 1982; Koyama et al., 1984) and, in male and female red salmons, electrical stimulation in both the Vs and the POA were able to elicit sexual behavior (Satou et al., 1984). Interestingly, these results agree with the

functions described for the rat MeA (reviewed in Rasia-Filho et al., 2012b).

The vomeronasal system and the MeA show a remarkable sexual dimorphism in mammals, including differences in the volume of different nuclei, the number of neurons and the complexity of glial cells (Guillamón and Segovia, 1997; Rasia-Filho et al., 2012a,b and references therein; Greenberg and Trainor, 2016). Considering the fish Vs and Vp an homologous to the mammalian MeA with similar hodology and function (O'Connell and Hofmann, 2011, Teles et al., 2015; Perathoner et al., 2016), we tested the presence of a putative sexual dimorphism in these fish telencephalic areas using a stereological approach for estimating the number of neurons and glial cells (Salazar et al., 2014). In rats, it was previously reported that prepubertal males have more neurons in the right posterodorsal MeA (MePD) than females (Cooke and Woolley, 2009). At adulthood, the MePD of male rats have more neurons and glial cells than females (Morris et al., 2008). Here, in both the Vs and the Vp, the numerical density for total cells (neuron + glial cells), neurons, glia or their relative proportion did not show any sex differences in the adult, sexually mature *B. splendens*. We studied groups of animals with equivalent body weight and length as well as overall size of these studied regions.

These results are intriguing because they were obtained from a species with notable differences in aggressive and sexual behaviors displayed by males and females. It is possible that a proposed homology of the Vs and the Vp with the MeA in the *B. splendens* does not involve numerical density of cells in these regions, but, rather, local connections and functional display. If so, sexual dimorphism would occur in other morphological parameters, such as the ultrastructural synaptic organization and/or astrocytic shape, or in other interconnected telencephalic regions of the social behavior network in this fish brain. These results also rise further important working hypotheses: If the vomeronasal system exists in teleostean fishes (Dulka, 1993; Grus and Zhang, 2006; Ubeda-Bañon et al., 2011), evident sexual dimorphism occurs in other fishes than the *B. splendens* or in other structures than the Vs and the Vp? Could the sexual dimorphism in the vomeronasal network be considered a new evolutionary trait raised after the appearance of teleosts? These important questions will benefit from the present morphological data to be answered in the future.

In conclusion, our present work provides both novel and comparative data for the study of the *B. splendens* and the teleost fishes. We present the localization of cell groups in the OB and the equivalent pallium and subpallium telencephalic areas in this fish brain. We did not find a sexual dimorphism in the cellular density of the assumed homologous of the MeA, the Vs and the Vp, in the *B. splendens*. Then, it is possible that such male to female cellular specialization occurs in other brain structures, teleost species or related vertebrate group.

ACKNOWLEDGMENTS

The authors are grateful to Prof. Léder L. Xavier (Laboratório de Biologia Celular e Tecidual, Depto. Ciências Morfofisiológicas, PUCRS, Brazil) for skilful comments on the stereological procedure used in this work. AARF is a CNPq/Brazil researcher.

CONFLICT OF INTEREST

Authors declare no actual or potential conflict of interest.

AUTHORS CONTRIBUTIONS

ACMH: research design, all procedures for data acquisition and interpretation, manuscript elaboration.

AARF: material and methods procedures, results interpretation, manuscript elaboration.

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3.2 Artigo 2

NADPH-diaphorase activity in the telencephalon of male and female siamese fighting fish *Betta splendens* Regan 1910 after a behavioral paradigm for aggressive display

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NADPH-diaphorase in the telencephalon of male and female Siamese fighting fish *Betta splendens* Regan 1910 after a behavioral paradigm for aggressive display

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Short title: *Betta splendens* telencephalic NADPH-d

Grant sponsor: FAPERGS/Brazil (12/2426-5)

Abstract

Nitric oxide (NO) is produced in the brain by the nitric oxide synthase (NOS) and acts as a gaseous transmitter on the genesis, excitability and survival of neurons, in synaptic plasticity and regulation of the release of neurotransmitters relevant for social behavior display. Here, we aim to detect putative nitrergic structures (cell somata and neuropil) in the telencephalon of male and female Siamese fighting fish *Betta splendens* submitted to a behavioral paradigm of aggression. Twenty five specimens, assigned to one of four groups based on their sex and exposure/non-exposure to a mirror, had their aggressive behavior assessed and their telencephalon submitted to the NADPH-diaphorase (NADPH-d) histochemical procedure. Our results showed that animals exposed to the mirror had a characteristic aggressive display, with gill cover abduction and gill flaring. Such behavior was absent in those fishes not subjected to the same condition. The NADPH-d procedure stained both cell somata and neuropil. NADPH-d activity was more intense in the challenged animals than in the controls. Basically, the same telencephalic structures were stained in both males and females, but a sex-specific pattern was found, whereby stained somata (in Vd, Vp, Vs, and Vv in males and Vp in females) and neuropil (in Dlg, Dlv-1, and Vv in males and NT in females) were identified in the stimulated groups compared to the controls. These data suggest that the *B. splendens* might be a useful animal model for the study of aggression and, in addition, that different telencephalic areas might be suitable for revealing sex differences and homologies with mammalian counterparts for this behavioral display.

Keywords: NADPH-d; NO; Brain; Aggressive behavior; Fish

Abbreviations

I -Olfactory Nerve

II - Optic Nerve

5-HT - Serotonin

AC - Anterior commissure

CCb - Corpus cerebella

CNS – Central nervous system

D - Dorsal telencephalon

Dc - Central zone of the dorsal telencephalon

Dc-1 - Central zone of the dorsal telencephalon - division 1

Dc-2 - Central zone of the dorsal telencephalon - division 2

Dc-3 - Central zone of the dorsal telencephalon - division 3

Dc-4 - Central zone of the dorsal telencephalon - division 4

Dd - Dorsal zone of the dorsal telencephalon

Dl - Lateral zone of the dorsal telencephalon

Dld - Dorsal division of the lateral zone of the dorsal telencephalon

Dlg - Granular division of the lateral zone of the dorsal telencephalon

Dlv-1 - Ventral division of the lateral zone of the dorsal telencephalon - subdivision 1

Dlv-2 - Ventral division of the lateral zone of the dorsal telencephalon - subdivision 2

Dm - Medial zone of the dorsal telencephalon

Dm-1 - Medial zone of the dorsal telencephalon - division 1

Dm-2 - Medial zone of the dorsal telencephalon - division 2

Dm-3 - Medial zone of the dorsal telencephalon - division 3

Dm-4 - Medial zone of the dorsal telencephalon - division 4

Dp - Posterior zone of the dorsal telencephalon

ECL - External cellular layer

EN - Entopeduncular nucleus

eNOS- Endothelial nitric oxide synthase

GCTN - Ganglion cells of the terminal nerve

GL - Glomerular layer

ICL - Internal cellular layer

IL - Inferior lobe of hypothalamus

iNOS – Inducible nitric oxide synthase

LSO - Lateral septal organ

MO - Medula oblongata

NADPH-d – NADPH-diaphorase

nNOS – Neuronal nitric oxide synthase

NO – Nitric Oxide

NOS – Nitric oxide synthase

NT - Nucleus taenia

OB - Olfactory bulb

ONL - Olfactory nerve fiber layer

Pit - Hypophysis (pituitary gland)

POAm - Magnocellular cells of preoptic area

POAp - Parvocellular cells of preoptic area

SOF - Secondary olfactory fibers

Tels - Telencephalic hemispheres

TO - Tectum opticum

V - Ventral telencephalon

Vc - Central nucleus of the ventral telencephalon

Vd - Dorsal nucleus of the ventral telencephalon

Vi - Intermediate nucleus of the ventral telencephalon

Vl - Lateral nucleus of the ventral telencephalon

Vp - Postcommissural nucleus of the ventral telencephalon

Vs - Supracommissural nucleus of the ventral telencephalon

Vv - Ventral nucleus of the ventral telencephalon

1. Introduction

In the central nervous system (CNS), nitric oxide (NO) acts as an intracellular and/or an intercellular messenger (Prast & Philippu, 2001; Guix et al., 2005). In the latter condition it is one of the so-called gasotransmitters, i.e. gaseous molecules that are not present in the synaptic vesicles, are not released by exocytosis and have no specific synaptic extracellular receptor (Holz & Fisher, 2012).

NO is produced by a class of enzymes known as nitric oxide synthase (NOS) in a redox reaction involving L-arginine and NADPH which results in L-citrulline and NO (Bredt & Snyder, 1994; Wiesinger, 2001). There are three NOS isoforms which are referred to by descriptive or numerical designations: neuronal NOS (nNOS) or type I; endothelial NOS (eNOS) or type III; and inducible NOS (iNOS) or type II (Nathan & Xi, 1994). In general, the first two are expressed constitutively and have a calcium/calmodulin-dependent activity, whereas the third is inducible (as the name indicates) and is calcium insensitive (Nathan & Xi, 1994; Ignarro & Jacobs, 2000). The main cellular effect of NO is the activation of a soluble form of guanylyl cyclase that increases the intracellular cGMP concentration. This increase is followed by a rise in the activity of cGMP-dependent protein kinases with a consequent modification of the cell metabolism, constituting a ‘canonical’ pathway (Holz & Fisher, 2012; Cossenza et al., 2014). Other NO pathway/action mechanism involves the nitrosylation of the cysteine residues of an array of intracellular proteins

(enzymes and G proteins), known as S-nitrosylation (Guix et al., 2005; Cossenza et al., 2014), which usually decreases the activity of the target proteins (Guix et al., 2005).

Different techniques can be used to directly or indirectly detect the location of NOS (and NO synthesis), such as histochemistry, immunohistochemistry and in situ hybridization (Beesley, 1995). Compared to other approaches, the NADPH-diaphorase (NADPH-d) histochemical technique employed in the present research provides a simple, robust and economic method (Spessert & Claassen, 1998) for visualizing NOS activity (Dawson et al., 1991; Hope et al., 1991). Furthermore, all known NOS involve NADPH-d activity (Tracey et al, 1993; Beesley, 1995) and although NOS represents only a fraction of the total NADPH-d activity, some methodological variables can be adjusted to distinguish NOS-related from NOS-unrelated diaphorase activity (Blottner, Grozdanovic, & Gossrau, 1995; Spessert & Claassen, 1998).

nNOS, the first isoform of NOS to be purified and cloned, is found in soluble and/or particulate forms in neurons, astrocytes, skeletal, cardiac and smooth muscle, neutrophils, pancreatic islets, endometrium and the epithelia of the respiratory and gastrointestinal tracts (Nathan & Xie, 1994; Zhou & Zhu, 2009). Its proposed physiological actions encompass regulation of local blood flow by vascular smooth muscle relaxation, synaptic plasticity (for example, facilitating LTP and inducing LTD); modulation of the release of some neurotransmitters, such as glutamate, gamma-aminobutyric acid, acetylcholine, dopamine, noradrenaline, serotonin (5-HT), adenosine and histamine; and neuron genesis, excitability and survival (Dawson & Dawson, 1996; Prast & Philippu, 2001; Wiesinger, 2001; Guix et al., 2005; Zhou & Zhu, 2009). The

relationship between NO and aggressive behavior has been well documented in male mice. Pharmacological or genetic inhibition of nNOS has been shown to evoke increased aggression (Demas et al., 1997; Chiavegatto et al., 2001). This heightened aggressive behavior appears to be linked to a reduction in 5-HT turnover and to 5-HT_{1A} and 5-HT_{1H} receptor dysfunction in regions of the brain regulating emotion (Chiavegatto & Nelson, 2003).

Although the findings regarding NO in the CNS were obtained mainly from mammals, additional evidence points to the importance of this molecule throughout the animal kingdom (Toda & Ayajiki, 2006). In fish, several studies of NO have sought to detect NOS activity using the NADPH-d technique (Arévalo et al., 1995; Villani & Guarnieri, 1995; Jadhao & Malz, 2004; Northcutt, 2009; Mueller et al., 2011), nNOS using immunohistochemistry (Ferrando et al., 2012; Biswas et al., 2015) or combined the use of both techniques (Holmqvist et al., 1994; Östholm et al., 1994; Brüning et al., 1995; Holmqvist et al., 2000; Lema and Nevitt, 2001; Coughi et al., 2002; Singru et al., 2003; Ando et al., 2004; Giraldez-Perez, 2008; Pushchina et al., 2012; Giraldez-Perez et al., 2013; López *et al*, 2016; López *et al*, 2017).

Betta splendens Regan (1910) is an *Actinopterygii* fish of the *anabantoidei* suborder (Rüber, Britz & Zardoya, 2006) originated in the Southeast Asia (Thailand, Cambodia, Indonesia, Laos and Vietnam, among others). It is characterized by its labyrinth organ, the building of nests of bubbles and by a marked aggressive behavior towards conspecifics (Faria et al., 2006; Monvises, et al., 2009; Goldstein, 2012). The aggressive behavior, first systematically described by Simpson (1968), is well known in male specimens, but also observed in females, and directed against males or other females

(Braddock & Braddock, 1955; Goldstein, 1975; Robertson, 1979). In *B. splendens*, a link between NO and aggressiveness has not yet been established. However, the action of 5-HT on aggression in this fish has been reported in studies with fluoxetine, in which the administration of this drug reduced agonistic behavior in different experimental conditions (Dzieweczynski & Herbert, 2012; Forsatkar et al., 2014).

The aim of the present research was to identify, for the first time, the presence and distribution of NADPH-d in the telencephalon of the fish *B. splendens*. Additionally, we compared the presence and pattern of activity of NADPH-d in males and females when animals were submitted to a paradigm for aggressive behavior display.

2. Material and Methods

2.1 Animals

We used 25 *Betta splendens* Regan, 1910 (Perciformes: Anabantoidei) sexually mature fish (n = 12 males and n = 13 females, 5 months-old). The animals were obtained from a local supplier and maintained in individual bottles containing 1.5 L of water (pH = 6.8 ± 0.4, temperature ranging from 27 to 30°C and salinity 0.15%) for at least two weeks for acclimation. The fish were free of any exogenous hormone treatments, fed commercial food twice a day/six days a week and maintained under a natural light/dark cycle. To prevent the accumulation of ammonia and nitrites, water was changed twice a week.

All procedures were made conducted according to the Brazilian laws that regulate animal use for scientific purposes, according to the National Institute of Health Guidelines for Care and Use of Laboratory Animals (NIH, 2011), and the Guidelines for the Euthanasia of Animals (American Veterinary Medical Association, 2013). The number of animals and their suffering were reduced to the minimum. The present research was approved by the local Ethical Committee (Federal University of Rio Grande do Sul, Brazil; protocol no. 22625).

2.2 Behavioral Recordings

After acclimation, males and females were randomly assigned to the control (n = 6 each) or stimulated groups (n = 6 and 7, respectively). The fish were transferred to coated bottles (volume of water and environment conditions as described above) that did not allow the visualization of other animals along 14 consecutive days. On the 15th day, the animals were individually transferred to an aquarium (200 mm length x 90 mm width x 150 mm height, 1L of water; Figure 1) illuminated by a 9W lamp, for a period of 30 min for acclimation. The left and back walls of the aquarium were coated in order to avoid giving the fish undesired visual stimuli. A mirror, initially hidden by a coated partition, was placed against the right wall (Figure 1). The front wall of the aquarium was uncoated to allow behavioral observation and recordings made using a digital camera (SONY Cyber-shot model DSC-W7, Japan) for 20 min. For the control group, the aquarium partition was kept in its original position and the fish were left undisturbed during all the recording period, whereas for the stimulated

group, the partition was removed and fish were exposed to the mirror. After the recordings, all the animals were returned to their original bottles where they remained for additional 2 h before euthanasia.

2.3. Behavioral analysis

The occurrence of gill cover abduction with gill flaring was considered indicative of agonistic behavior since it is a reliable motor pattern predictor of aggression in fish species (Karino & Someya, 2007; Verbeek, Iwamoto, & Murakami, 2007). Gill cover abduction with gill flaring was based on the description provided by Simpson (1968) and is shown in Figure 2A and 2B. This motor pattern is characterized by a simultaneous deviation (abduction) of the opercula from the median plane of the fish body and an enlargement of the branchiostegal membranes. We evaluated the occurrence of this behavior by measuring the total time (in seconds) during which the animals displayed the behavior and calculated time (as a percentage) of the total time of the recording. The time spent by each fish displaying this behavior was counted only when the animal was at a distance equal to or less than twice the total length of its body from the mirror.

2.4 Euthanasia and Fixation

All animals were anaesthetized with MS-222 (Sigma-Aldrich, USA) 0.9 g/L until a deep stage of anesthesia was attained. This condition (classified as ‘stage 4’) was identified by the absence of an animal motor response after a

strong mechanical stimulus applied to the caudal peduncle (Ilgenfritz et al., 2013). The fish were weighed, measured, and euthanized by decapitation between 11 am to 6 pm. The skullcaps were removed and the heads were kept in 4% paraformaldehyde (Reagen, Brazil) diluted in 0.1 M phosphate buffer solution (PB, pH 7.4) at 4º C for 2 h. The brains were dissected and post-fixed in the same fixative solution for an additional 2 h period and stored in 30% sucrose solution diluted in PB until the NADPH-d procedure was conducted (after 23-32 days).

2.5 NADPH-diaphorase histochemical procedure

The present approach was adapted from de Castilhos et al. (2009) and Rigon et al. (2009). NADPH was used as a substrate to reduce nitro blue tetrazolium salt (NBT, chromogen) producing an identifiable dark blue precipitate (formazan), as described by Hope and Vincent (1989) and improved by Spessert & Claassen (1998).

The brains were embedded in 3.5% agarose gel solution diluted in PB. The entire telencephalon was sectioned (30 µm-thick) in the coronal plane along the rostro-caudal axis using a vibratome (Leitz, Germany). Sections were collected in cold PB, cleaned of remaining agarose gel, and immersed in 0.1 M phosphate buffer saline solution (PBS, pH 7.4) at 4ºC for 12 hours. Afterwards, sections were pre-incubated with 200 µl of Triton X-100 (Sigma-Aldrich, USA) diluted in 100 mL of PBS at room temperature for 10 minutes, and incubated at 37°C in a solution containing NADPH (0.5 mg/mL; Sigma-Aldrich, USA), NBT (0.2 mg/mL; Sigma-Aldrich, USA) and Triton X-100 (2 µl/mL; Sigma-Aldrich,

USA) for 2 h. Sections were rinsed in PBS, placed on gelatinized microscopic slides, dehydrated in ethanol, cleared in xylene, and covered with synthetic balsam and coverslips. All sections were pre-incubated and incubated at the same time and in parallel to avoid variations in the reaction during the procedure. The negative control for the reaction of NADPH and NBT consisted of sections incubated without the substrate.

2.6 NADPH-diaphorase reaction

The sections were observed using an optical microscope (Nikon Eclipse E200MV, China) to identify cell somata and the neuropil in the telencephalon of male and female *B. splendens* from the control and stimulated groups. The intensity of the reaction was verified by direct observation and quantified, based on the intensity of the color generated by the reaction, using the following relative scale: (-) absence, (+) weakly stained, (++) moderately stained, and (+++) intensely stained, based in the intensity of the color generated by the reaction. This kind of measurement was only possible because all sections were processed at the same time, as previously mentioned.

The identification and nomenclature adopted for the telencephalic structures of *B. splendens* was based on the work of Horn & Rasia-Filho (2017). A map for the telencephalic areas was made using a digital camera (Olympus D172, Japan) coupled to a microscope (Olympus BX61, Japan) with 4X and 10X objective lens. Photomicrographs were employed to elaborate schematic drawings to highlight the topographic location of reactive cell somata in all the studied groups (Figure 3).

2.7. Statistical Analysis

Data are shown as mean and standard deviation. The Kruskal-Wallis test and the Dunn *post hoc* test were used to compare the body weight and standard length among the four groups (control and stimulated males and females) used in the behavioral paradigm. The Mann-Whitney test was adopted to compare the time spent displaying gill cover abduction with gill flaring between the male and female challenged groups. GraphPad Prism 6 (USA) statistical software was used in the analysis and the level of statistical significance was set at $p < 0.05$.

3. Results

3.1 Animals

The body weight and standard length of all studied male and female fish are shown in Table 1. No statistical difference was found for these two morphological parameters between the experimental groups (H = 2.72, P = 0.44 and H = 2.15, P = 0.54, respectively).

3.2 Behavioral data

Neither the control males nor the control females showed agonistic behavior as evidenced by spontaneous abduction of the gill cover accompanied by gill flaring (Table 2). Rather, the fish in these groups spent the entire

recording time exploring the environment, sometimes close to the front wall of the aquarium.

On the other hand, males and females in the stimulated groups display notably displayed agonistic behavior along the recording time (Table 2). The proportion of the time spent by males and females exhibiting gill cover abduction with gill flaring was 54.8% and 41.8% of the recording time, respectively (Table 2). Finally, comparing the time spent by these two groups displaying agonistic behavior was not statistically different (U = 13, P = 0.29) (Table 2).

3.3 NADPH-diaphorase

3.3.1 General characteristics

The NADPH-d histochemical reaction in the telencephalon of *B. splendens* evidenced areas with different neuropil background intensities (Figure 4A). In some of these areas we identified groups of cells with well-defined somata (Figure 4B) and processes (Figure 4C), some of which showed varicosities (Figure 4D). These processes were commonly caudal to the level of the anterior commissure where they form tract-like structures connecting the telencephalic hemispheres (Tels) as well as the D and V (Figure 4C). In other cases, these processes could not be traced far from their cell bodies, which meant we were unable to define their projection fields.

3.3.2 Location of the NADPH-diaphorase activity in the control groups

3.3.2.1 Olfactory bulb

In the olfactory bulb (OB), NADPH-d activity was observed in the glomerular layer (GL) and olfactory nerve fiber layer (ONL) in both male and female specimens (Table 3). The intensity of the stain generated by the enzymatic reaction in these structures was classified as moderate to intense (Table 3).

3.3.2.2 Telencephalic hemispheres

The D and V of both male and female showed several neuropil areas reactive to NADPH-d. Cell somata were identified in fewer areas (Table 3, Figure 5).

In D, stained neuropil was observed in the central zone of the dorsal telencephalon - division 2 (Dc-2), central zone of the dorsal telencephalon - division 3 (Dc-3), central zone of the dorsal telencephalon - division 4 (Dc-4), dorsal zone of the dorsal telencephalon (Dd), dorsal division of the lateral zone of the dorsal telencephalon (Dld), granular division of the lateral zone of the dorsal telencephalon (Dlg), ventral division of the lateral zone of the dorsal telencephalon - subdivision 1 (Dlv-1), ventral division of the lateral zone of the dorsal telencephalon - subdivision 2 (Dlv-2), medial zone of the dorsal telencephalon - division 2 (Dm-2), and medial zone of the dorsal telencephalon - division 4 (Dm-4). Among these areas, NADPH-d reaction in the Dld was exclusive to males whereas the reaction in the Dlv-1, Dlv-2, Dlg and Dm-1 were exclusive to females (Table 3).

In V, stained neuropil was observed in the entopeduncular nucleus (EN), dorsal nucleus of the ventral telencephalon (Vd), intermediate nucleus of the

ventral telencephalon (Vi), postcommissural nucleus of the ventral telencephalon (Vp), supracommissural nucleus of the ventral telencephalon (Vs) and ventral nucleus of the ventral telencephalon (Vv). Among them, Vv was only stained in females (Table 3).

Cell somata were only identified in the Dm-4, EN, Vd, Vi and Vs. In males, they were observed in the Dm-4, EN and Vi whereas in females they were found in the Vd, Vs and Vi (Table 3, Figure 5).

3.3.3 Differences in the NADPH-diaphorase activity between groups

The male and female control groups showed a similar location and intensity of the NADPH-d staining in the cell somata and in the neuropil. However, when the control and stimulated groups were compared, differences between animals of the same sex were apparent. In the animals from the stimulated groups the reaction to NADPH-d was more widespread in the D and V, with more areas stained and more intense staining than in the control groups (Table 3, Figure 5).

In the OB, while no additional stained area was identified, the intensity of the reaction was greater in the GL and in the ONL of behaviorally challenged males (Table 3). In the Tels, further stained somata (in the Vd, Vp, Vs and Vv in males and in the Vp in females) and neuropil (in the Dlg, Dlv-1, and Vv in males and in the NT in females) were identified in the stimulated groups compared to the control groups (Table 3, Figure 5). In addition, the intensity of the NADPH-d activity was higher in the somata (in the Dm-4, EN and Vi in males and in the Vd and Vs in females) and neuropil (Dc-3, Dm-4, EN, Vd, Vi, Vp, and Vs in

males and Dc-4, EN, and Vd in females) in the stimulated groups compared to the control groups (Table 3, Figure 5).

4. Discussion

4.1 Animal choice

Here, we report novel data for the distribution of the NADPH-d activity in the telencephalon of the *B. splendens* and compared it between males and females after a behavioral paradigm for aggressive display. In contrast to most other species of fish, *B. splendens* is territorial and presents evident aggressive behavior when faced by a member of the same species (Monvises, et al., 2009; Goldstein, 2012). Agonistic behavior evoked by an aggressive stimulus is easily identified by motor patterns such as gill cover abduction with gill flaring (Alyan, 2010; HedayatiRad et al., 2017). These characteristics make *B. splendens* a valuable animal model for the kind of experimental approach employed here.

4.2 NADPH-diaphorase activity

Specific cells and neuropil areas in the telencephalon were found after the NADPH-d histochemical technique. Reactive cells showed evident somata, sometimes with short processes originating from them. In the neuropil, the intensity of the NADPH-d reaction was uneven in all regions, giving rise to clearly circumscribed areas. This characteristic has been used by some authors to anatomically identify telencephalic nuclei of fishes (Northcutt, 2009; Mueller et al., 2011).

Although not all NADPH-d reactivity corresponds to NOS activity, various studies have shown that the location of NADPH-d reactivity overlaps with NOS enzymes in the nervous tissue of fishes (Holmqvist et al., 1994; Holmqvist et al., 2000; Östholm et al., 1994; Brüning et al., 1995; Lema and Nevitt, 2001; Coughi et al., 2002; Singru et al., 2003; Ando et al., 2004; Giraldez-Perez, 2008; Pushchina et al., 2012; Giraldez-Perez et al., 2013; López et al., 2016; López et al., 2017). The actual presence of NOS can only be confirmed by immunohistochemistry or hybridization *in situ*, but correlations such as those highlighted above allow us to assume that the NADPH-d activity described here indicates the presence of NOS in the telencephalon of *B. splendens*.

4.3 NADPH-diaphorase localization in control groups

NADPH-d activity was observed in all the major regions of the telencephalon (i.e. OB and Tels) in *B. splendens*. In the telencephalon, groups of stained cells and reactive neuropil were identified in the D and in V. Regarding the localization of NADPH-d staining in the telencephalon, despite some differences between males and females in the localization of NADPH-d staining in the telencephalon, generally, the reactive telencephalic regions appear to be similar in both sexes.

In the OB, NADPH-d activity was detected, with moderate to intense staining, in the GL and ONL of both sexes. These structures are also reactive in the OB of the *Carassius auratus* (Giraldez-Perez et al., 2008; Giraldez-Perez, Gaytan, & Pasaro, 2013), *Lepisosteus oculatus* (López et al., 2017), *Oncorhynchus kisutch* (Lema & Nevitt, 2001), *Oncorhynchus nerka* (Ando et al.,

2004), *Oreochromis mossabicus* (Singru, Skaharkar, & Subhedar, 2003), *Polypterus senagalus* and *Erpetoichthys calabaricus* (Lopez et al., 2016), *Protopterus dolloi* (Northcutt, 2009), *Salmo salar* (Holmqvist et al., 1994) and *Tinca tinca* (Arévalo et al., 1995). In the OB of most of these above mentioned fish species, there was no nNOS labeling colocalization with NADPH-d activity (Holmqvist et al., 1994; Lema & Nevitt, 2001; Giraldez-Perez et al., 2008, Giraldez-Perez, Gaytan, & Pasaro, 2013; López et al., 2016; López et al., 2017). This discrepancy would occur because the olfactory system of vertebrates displays a cytochrome P-450 reductase with NADPH-d activity (López et al., 2017). Thus, the local NADPH-d staining in the OB would reflect the activity of this other kind of enzyme, an issue that could instigate further studies directed towards the GL and ONL parts of the OB of the *B. splendens.*

The Tels of the *B. splendens* showed only few stained cell somata, contrasting with the large number of neuropil areas marked by the NADPH-d histochemical reaction. In the Tels of *B. splendens*, considering male and female specimens together, NADPH-d-reactive cell somata were stained in Dm4, EN, Vd, Vi and Vs. In other actinopterygian fishes, brain cell somatas marked by NADPH-d histochemistry, nNOS immunohistochemistry or hybridization *in situ* provided variable results. That is to say, they occurred mainly in the Dc and Dl in *Carassius auratus* (Villani & Guarnieri, 1995); in the Dm, Dld, Dc, Vv, Vs, Vp and Vi in *Carassius auratus* (Brüning, Katzbach, & Mayer, 1995); in the Dc, Vv, Vl, Vs, Vi and CA in *Tinca tinca* (Arévalo et al., 1995); in the Dc, Dd, Dl, Dm, Dp, Vd, Vs, Vl and Vv in *Oncorhynchus* nerka (Ando et al., 2004); in the EN in *Haplochromis burtoni* (Jadhao & Malz, 2004); in the Dm, Dd, Dl, Dc, Dp, Vd, Vv, Vl, Vs, Vp and ENT in *Carassius auratus*

(Giraldez-Perez et al., 2008, Giraldez-Perez, Gaytan, & Pasaro, 2013); in the Dc, Dl, Dm, Dd, Vd and Vv in *Oncorhynchus masou* (Pushchina, Varaksin, & Obukhov, 2012); in the Dc, Dd, Dm, Dl, Vd, Vc, Vv and En in *Labeorohita* (Biswas et al., 2015); in the P1, P2, P3, Vn, Vd, Vv, Vs and Vp in *Polypterus senegalus* and *Erpetoichthys calabaricus* (López et al., 2016) and in the Dl, Vd, Vv, Vn, VL, Vs and Vp in *Lepisosteus plathyrhincus and Lepisosteus oculatus* (López et al., 2017). However, these results show that putative or true nitrergic cells are distributed in a species-specific pattern in the fish brain, which is also the case for the *B. splendens*, as reported here.

Another noteworthy characteristic observed by us regarding the NADPH-d stained cell bodies in the *B. splendens* Tels is that the locations of the reactive cell somata clusters were different when comparing males to females. With the exception of Dm-4, all the clusters of cell somata were located in the V of both sexes. The presence of nitrergic cells in the V of the fish, as well as in the subpallium of other vertebrates (homologous to the V of the fish) is a common characteristic in vertebrates (Lopes et al., 2017). In males, clusters of cell somata were observed in Dm4, EN and Vi. Whereas in females clusters of cells were stained in Vd, Vi and Vs. In the neuropil of the *B. splendens* Tels, there was considerable overlap of the NADPH-d activity in some areas (Dc-2, Dc-3, Dc-4, Dd, Dm-4, EN, Vd, Vi, Vp and Vs), but some staining patterns were exclusive to males (in the Dld) or to females (in the Dlg, Dlv-1, Dlv-2, Dm-2 and Vv).

These distinct results for the NADPH-d activity in cell bodies and neuropils in the Tels of male and female specimens of *B. splendens* represent a sexual dimorphism in this species. Considering that the presence of nNOs cell

groups in the Tels of actinopterygian fish varies along the phases of the post-embryonic development (Biswas et al., 2015), it is plausible to consider the existence of a sex-dependent pattern of NADPH-d activity in the Tels of *B. splendens*. In line with this hypothesis, it has previously been reported in rodents that gonadal hormones (testosterone and estrogen) affect the central NO producing systems within regions relevant for the control of sexual behavior (Panzica et al., 2006; de Castilhos et al., 2009), of which the aggressiveness is one of the components.

4.4 Effects of the aggressive stimulation

In the *B. splendens*, the aggressive stimulus (mirror image) provided to the challenged groups caused a strong agonistic behavior with abduction of the gill covers accompanied by gill flaring. This kind of behavior was not observed in the control groups. In the stimulated groups, the intensity of the staining of NADPH-d reactive nuclei was increased and additional reactive areas were identified in the telencephalon. These additional areas were the cell somata in the Vd, Vs, Vp and Vv and the neuropil in the Dlg, Dlv-1 and Vv in males and the NT neuropil in females.

These data suggest that NO synthesis in the telencephalic structures of *B. splendens* in the stimulated groups might modulate sensory, motor and/or cognitive functions for the display of aggressive behavior. In this regard, it is noteworthy the homology that some of the reactive cell somata in the groups exposed to the aggressive stimulus have with structures that modulate social behaviors in the tetrapod brain. That is, the EN is homologous to

entopeduncular nucleus proper/globus pallidus and to the bed nucleus of the stria medullaris (Mueller & Guo, 2009, Ganz et al., 2012). The Vd and Vv are considered homologous to the striatum and septo/pallidum, respectively (Ganz et al., 2012; Gonzales et al., 2014; López *et al*, 2017). The Vi was recently suggested as homologous to the medial amygdala (Biechl et al., 2017). The Vs and Vp are both considered homologous to the bed nucleus of stria terminalis and the central amygdala (Ganz et al., 2012, Maximino et al., 2013), as well as to the medial amygdala (O'Connell & Hofmann, 2011). Finally, Vp is thought to be homologous to central amygdala (Ganz et al., 2012).

One mechanism that could explain the relationship between the NADPH-d activity (and the expected NO synthesis) and the aggressive display in *B. splendens* would involve 5-HT. In mice, depressed 5-HT metabolism and/or 5-HT_{1A} and 5-HT_{1B} receptor activity elevates the aggressive behavior (Chiavegatto & Nelson, 2003; Nelson et al., 2006). As NO modulates serotonergic transmission (Prast & Philippu, 2001) and since the absence of NO elevates aggressive behavior (Bedrosian & Nelson, 2014), the relationship between the levels of NO and aggression would be inverse. By contrast, Gutiérrez et al. (2017), found significantly reduced aggression in mutant knockout nNOS zebrafish. This reduction was a consequence of decreased monoamine oxidase activity NO mediated (Gutiérrez et al., 2017). This result regarding zebrafish aggressive behavior is in line with our results showing the increased NADPH-d enzyme activity, which could, as in the case of zebrafish, challenge previous thinking suggesting reduced nNOS increases aggression (Gutiérrez *et al*., 2017).

As the mechanism whereby NO influences aggression is not fully understood (Bedrosian & Nelson, 2014), it would be necessary firstly to determine how NO signaling is related to aggressive behavior: in a direct (Clotfelter *et al*., 2007; Dzieweczynski & Herbert, 2012; Forsatkar *et al*., 2014) or inverse (Gutiérrez *et al*., 2017) way. In this context, *B. splendens* would seem to be a useful model to test these causal effects in both males and females.

ACKNOWLEDGMENTS

This research was supported by grants from *Fundação de Pesquisa do Estado do Rio Grande do Sul* (FAPERGS) [Grant number: 12/2426-5]. We are grateful to Dra.Paula Rigon da Luz for the reagents used to perform the NADPH-diaphorase histochemical technique. AARF is a CNPq researcher.

CONFLICT OF INTERESTS

The authors have no conflict of interests to declare.

AUTHOR CONTRIBUTIONS

ACMH conceived and designed the study, performed the histochemical and microscopic analyses and wrote the manuscript. AARF conceived and designed the study and wrote the manuscript.

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Figure 1. Photograph of the aquarium used in the behavioral recordings. P, Partition; M, Mirror.

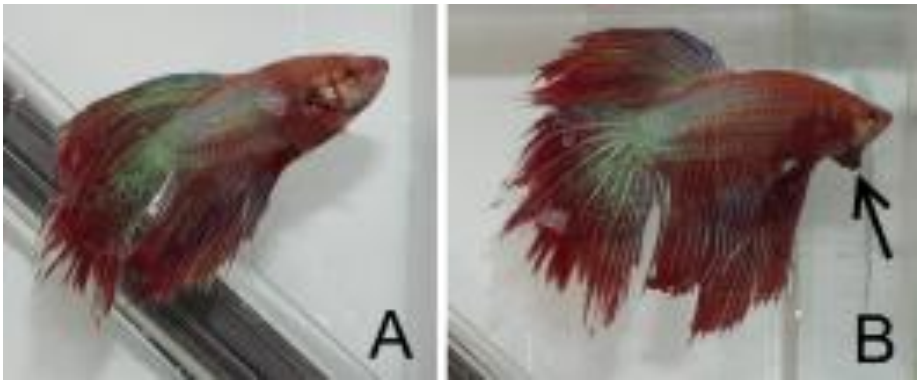


Figure 2. Photographs of the *B. splendens* under testing. **A**. Male from the control group. **B**. Male from the stimulated group in front of a mirror. Arrow shows gill cover abduction with gill flaring.

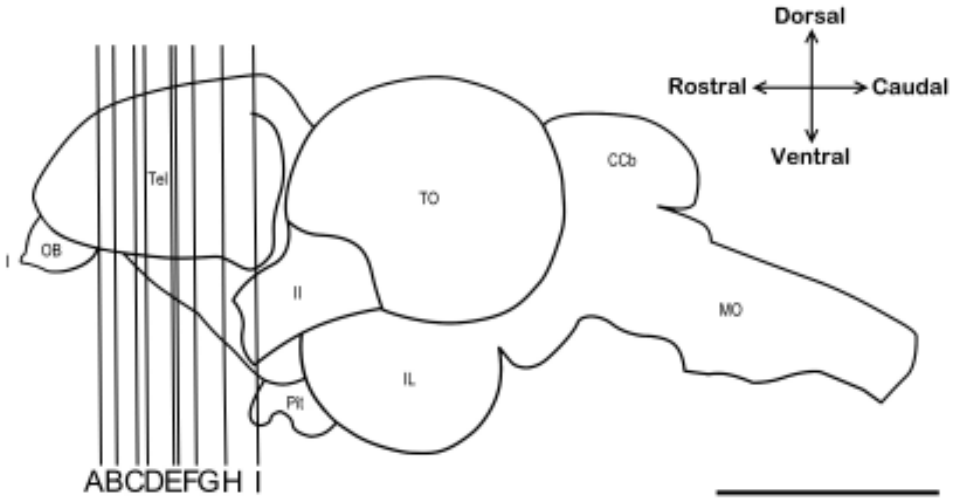


Figure 3. Schematic image of the left side of the brain of an adult male *B. splendens* showing the major anatomical structures and the levels of the transverse sections where the NADPH-d activity was evaluated in the telencephalon (from **A** to **I**). Scale bar = 1 mm.

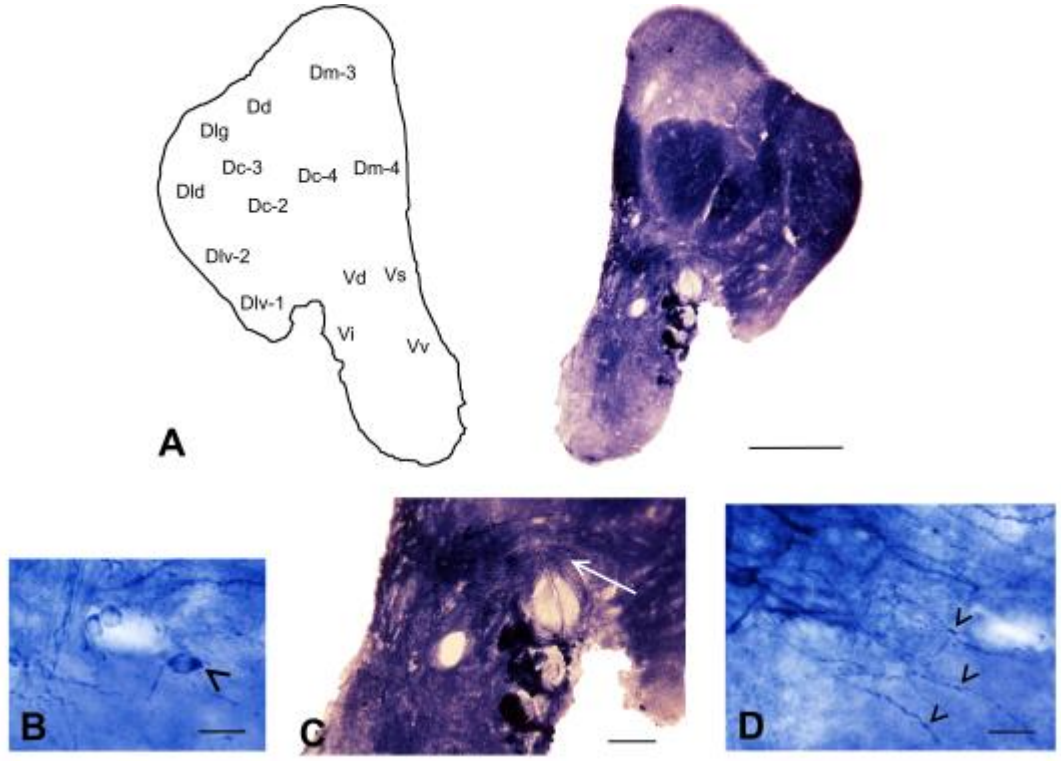
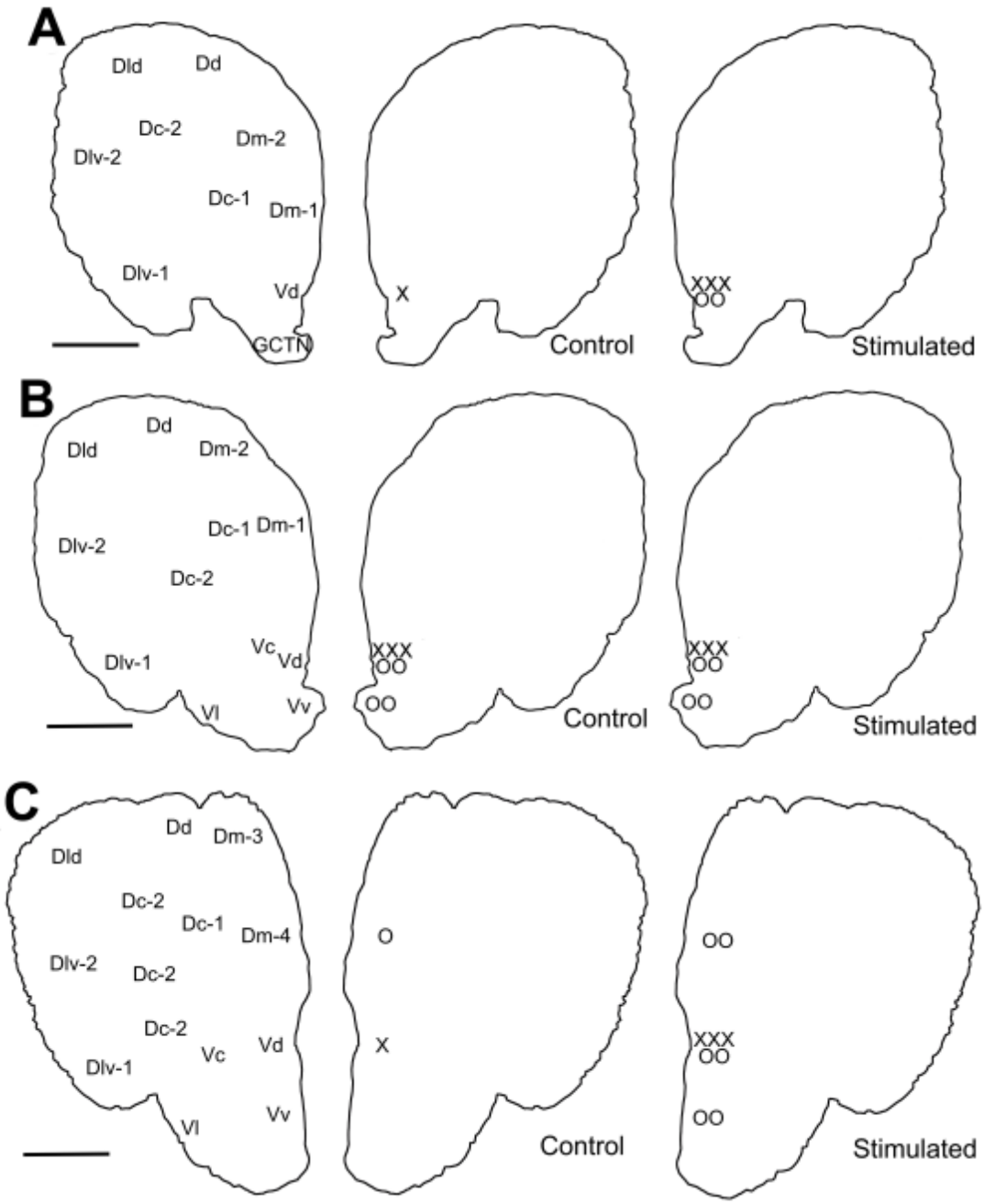


Figure 4. Transverse sections of the *B. splendens* telencephalon. **A**. The right telencephalic hemisphere, caudal to anterior commissure (AC), shows different NADPH-d background intensities in the neuropil. The structures of the telencephalon are identified by their abbreviations on the left. **B**. Cell from the central zone of the dorsal telencephalon - division 3 (Dc-3) with its somata clearly identified. The arrow head indicates the cytoplasm of the cell. **C**. Detail of the Figure 3A image showing numerous processes projecting from the ventral telencephalon (V) to the dorsal telencephalon (D). Arrow shows fibers projecting from the dorsal nucleus of the ventral telencephalon (Vd) to the central zone of the dorsal telencephalon - division 2 (Dc-2). **D**. Neuronal processes with varicosities (arrow heads) projecting from V to D. Dorsal and medial are to the top and left, respectively. Images were adjusted for brightness and contrast using the Adobe Photoshop software (USA). Dc-2 = Central zone of the dorsal telencephalon - division 2, Dc-3 = Central zone of the dorsal telencephalon - division 3, Dc-4 = Central zone of the dorsal telencephalon - division 4, Dd = Dorsal zone of the dorsal telencephalon, Dld = Dorsal division of the lateral zone of the dorsal telencephalon , Dlg = Granular division of the lateral zone of the dorsal telencephalon, Dlv-1 = Ventral division of the lateral zone of the dorsal telencephalon -subdivision 1, Dlv-2 = Ventral division of the lateral zone of the dorsal telencephalon – subdivision 2, Dm-3 = Medial zone of the dorsal telencephalon - division 3, Dm4 = Medial zone of the dorsal telencephalon - division 4, Vd = Dorsal nucleus of the ventral telencephalon, Vi = Intermediate nucleus of the ventral telencephalon, Vs = Supracommissural nucleus of the ventral telencephalon, Vv = Ventral nucleus of the ventral telencephalon. Scale bars = 200 µm (**A**), 10 µm (**B** and **D**) and 50 µm (**C**).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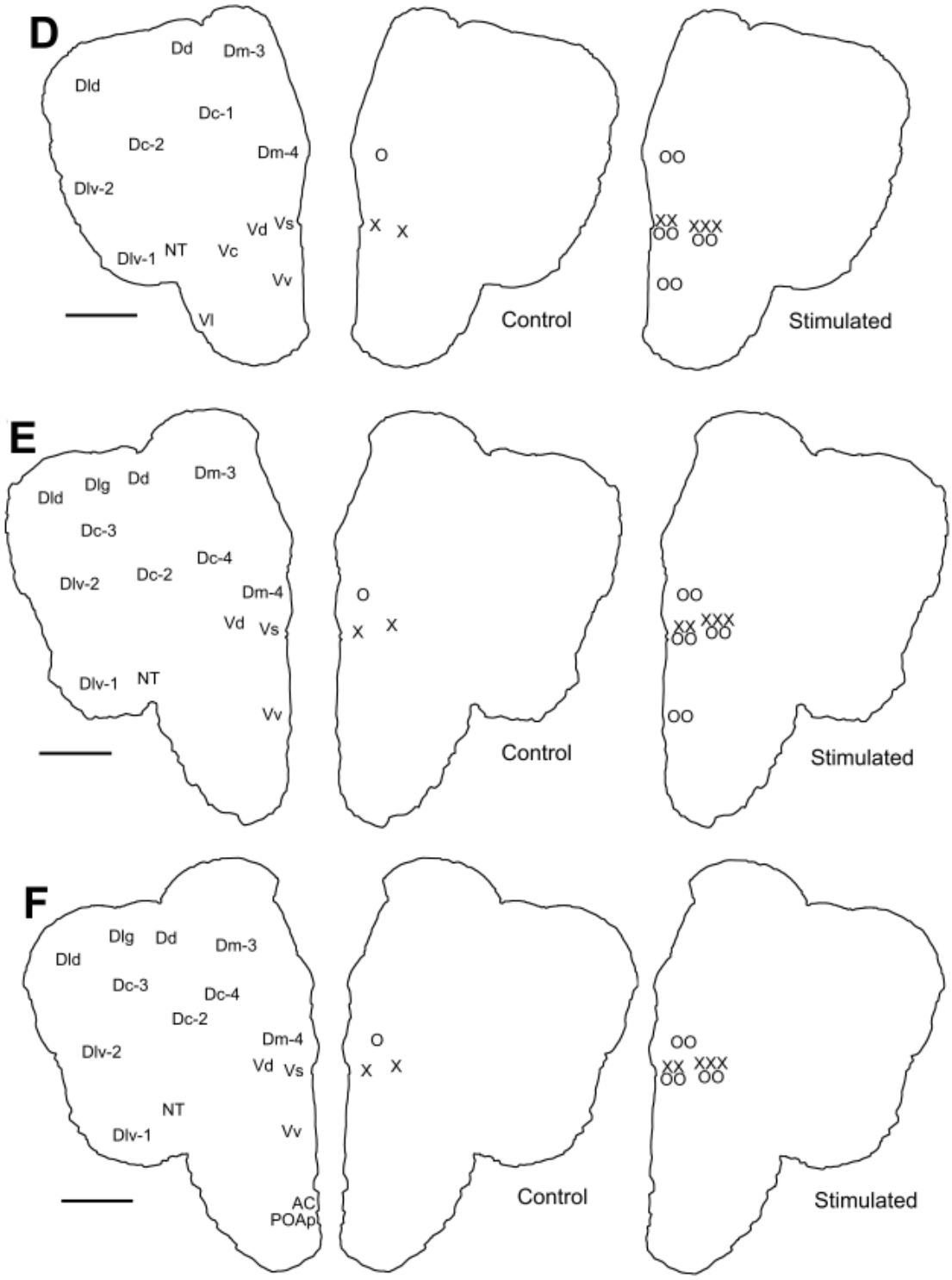
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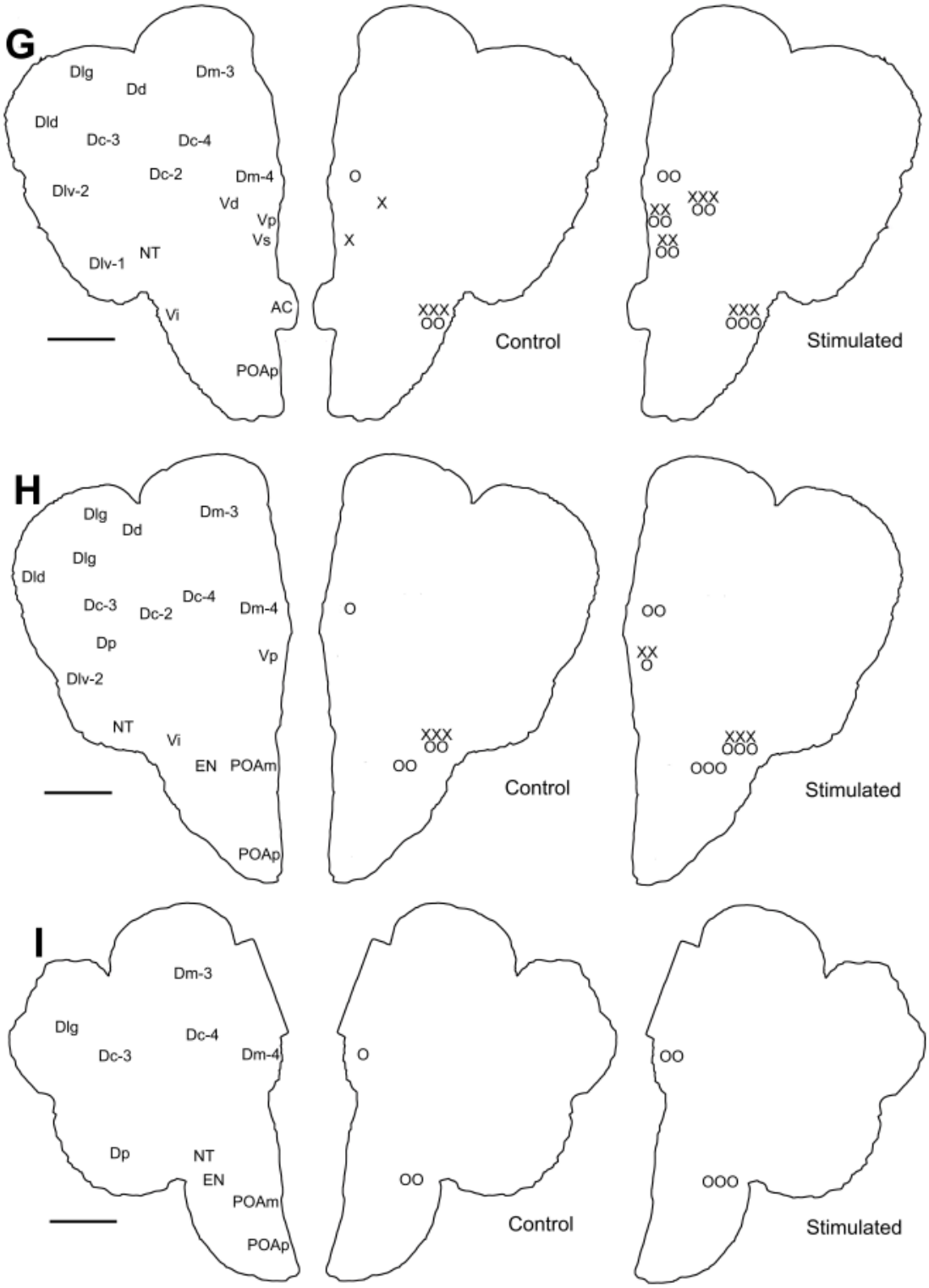
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Figure 5. Schematic drawings of the localization of NADPH-d reactivity in the cell somata in the control and stimulated groups of male and female *B. splendens*. Telencephalic hemispheres sectioned in the transverse plane from rostral to caudal levels (from **A** to **I**) as represented in Figure 3. In the right halves of each stained section, somata from males are represented by open dots, while reactive somata from females are represented by 'x' in the control and stimulated groups. In the left half, the structures of the telencephalon are identified by their abbreviations. AC = Anterior commissure, Dc- 1 = Central zone of the dorsal telencephalon - division 1, Dc-2 = Central zone of the dorsal telencephalon - division 2, Dc-3 = Central zone of the dorsal telencephalon - division 3, Dc-4 = Central zone of the dorsal telencephalon - division 4, Dd = Dorsal zone of the dorsal telencephalon, Dld = Dorsal division of the lateral zone of the dorsal telencephalon , Dlg = Granular division of the lateral zone of the dorsal telencephalon, Dlv-1 = Ventral division of the lateral zone of the dorsal telencephalon -subdivision 1, Dlv-2 = Ventral division of the lateral zone of the dorsal telencephalon – subdivision 2, Dm-1 = Medial zone of the dorsal telencephalon - division 1, Dm-2 = Medial zone of the dorsal telencephalon - division 2, Dm-3 = Medial zone of the dorsal telencephalon - division 3, Dm4 = Medial zone of the dorsal telencephalon - division 4, Dp = Posterior zone of the dorsal telencephalon, EN = Entopeduncular nucleus, GCTN = Ganglion cells of the terminal nerve, NT = Nucleus taenia, POAm = Magnocellular cells of preoptic area, POAp = Parvocellular cells of preoptic area, Vc = Central nucleus of the ventral telencephalon, Vd = Dorsal nucleus of the ventral telencephalon, Vi = Intermediate nucleus of the ventral telencephalon, Vl = Lateral nucleus of the ventral telencephalon, Vs = Supracommissural nucleus of the ventral telencephalon, Vv = Ventral nucleus of the ventral telencephalon. Scale = 200 µm.

Table 1- Comparison of body weight (BW) and standard length (SL) of male and female *B. splendens* from the control and stimulated groups used in the behavioral study. Values are expressed as mean ± SD.

BW	**Control**	**Stimulated**
Male	0.88 ± 0.10 [a] (n=6)	0.95 ± 0.14[a] (n=6)
Female	0.94 ± 0.07 [a] (n=6)	0.86 ± 0.12[a] (n=7)
SL	**Control**	**Stimulated**
Male	34.20 ± 2.05[a] (n=6)	34.23 ± 1.74[a] (n=6)
Female	34.78 ± 0.66[a] (n=6)	33.27 ± 1.60[a] (n=7)

[a] Values with the same letter for the same physical characteristic among the groups are statistically equal to a p≤0.05

Table 2- Mean±SD of the time spent (s) and proportion of the time spent (%) displaying gill cover abduction with gill flaring in male and female of *B. splendens* from the control and stimulated groups.

Male				Female			
Control (n= 6)		Stimulated (n= 6)		Control (n= 6)		Stimulated (n= 7)	
Time spent	Proportion	Time spent	Proportion	Time spent	Proportion	Time spent	Proportion
0±0	0	657.5±375.4[a]	54.8	0±0	0	501.7±214.6[a]	41.8

*Values with the same letter to the same variable between sexes are statistically equal to a p≤0.05

Table 3- Staining intensity of NADPH-diaphorase activity in the somata and neuropil of telencephalic structures from males and females of *B. splendens* **subjected to a control condition or after a behavioral paradigm for aggressive display ("stimulated").**

Telencephalic structures	NADPH-diaphorase activity							
	Male				Female			
	Control (n= 6)		Stimulated (n= 6)		Control (n=6)		Stimulated (n=7)	
	Somata	Neuropil	Somata	Neuropil	Somata	Neuropil	Somata	Neuropil
Anterior commissure (AC)	-	-	-	-	-	-	-	-
Central zone of the dorsal telencephalon, division 1 (Dc-1)	-	-	-	-	-	-	-	-
Central zone of the dorsal telencephalon, division 2 (Dc-2)	-	++	-	++	-	++	-	++
Central zone of the dorsal telencephalon, division 3 (Dc-3)	-	+	-	++	-	++	-	++
Central zone of the dorsal telencephalon, division 4 (Dc-4)	-	+	-	+	-	+	-	++
Dorsal zone of the dorsal telencephalon (Dd)	-	++	-	+	-	++	-	++
Dorsal division of the lateral zone of the dorsal telencephalon (Dld)	-	++	-	++	-	-	-	-
Granular division of the lateral zone of the dorsal telencephalon (Dlg)	-	-	-	++	-	++	-	++
Ventral division of the lateral zone of the dorsal telencephalon, subdivision 1 (Dlv-1)	-	-	-	++	-	++	-	++
Ventral division of the lateral zone of the dorsal telencephalon, subdivision 2 (Dlv-2)	-	-	-	-	-	++	-	++
Medial zone of the dorsal telencephalon, division 1 (Dm-1)	-	-	-	-	-	-	-	-
Medial zone of the dorsal telencephalon, division 2 (Dm-2)	-	-	-	-	-	++	-	-
Medial zone of the dorsal telencephalon, division 3 (Dm-3)	-	-	-	-	-	-	-	-
Medial zone of the dorsal telencephalon, division 4 (Dm-4)	+	++	++	+++	-	++	-	++
Posterior zone of the dorsal telencephalon (Dp)	-	-	-	-	-	-	-	-
External cellular layer (ECL)	-	-	-	-	-	-	-	-
Entopeduncular nucleus (EN)	++	++	+++	+++	-	++	-	+++
Ganglion cells of the terminal nerve (GCTN)	-	-	-	-	-	-	-	-
Glomerular layer (GL)	-	++	-	+++	-	+++	-	+++
Internal cellular layer (ICL)	-	-	-	-	-	-	-	-
Lateral septal organ (LSO)	-	-	-	-	-	-	-	-
Nucleus taenia (NT)	-	-	-	-	-	-	-	+++
Olfactory nerve fiber layer (ONL)	-	++	-	+++	-	+++	-	+++
Secondary olfactory fibers (SOF)	-	-	-	-	-	-	-	-
Central nucleus of the ventral telencephalon (Vc)	-	-	-	-	-	-	-	-
Dorsal nucleus of the ventral telencephalon (Vd)	-	+	++	++	+	++	+++	+++
Intermediate nucleus of the ventral telencephalon (Vi)	++	++	+++	+++	+++	+++	+++	+++
Lateral nucleus of the ventral telencephalon (Vl)	-	-	-	-	-	-	-	-
Postcommissural nucleus of the ventral telencephalon (Vp)	-	+	+	++	-	++	++	++
Supracommissural nucleus of the ventral telencephalon (Vs)	-	+	++	++	+	++	++	++
Ventral nucleus of the ventral telencephalon (Vv)	-	-	++	++	-	++	-	++

-, absence of activity; +, weakly stained; ++, moderately stained and; +++, intensely stained.

4. DISCUSSÃO GERAL

Em razão da maior proximidade filogenética com o homem e, portanto, da grande quantidade de semelhanças morfológicas e funcionais, as Neurociências utilizam mamíferos como modelo experimental preferencial, e, dentre esses, dos ratos e camundongos, se valendo de algumas características específicas vantajosas (Maximino et al., 2015; Remage-Healey et al., 2017). Apesar disso, outros modelos, por apresentarem características únicas e/ou serem economicamente mais viáveis, vem se tornando comuns nos laboratórios. Dentre esses está o peixe paulistinha (*Zebrafish, Danio rerio*), o qual possui alta fecundidade, é de fácil manutenção e é portador de um considerável índice de homologia genética com o homem (Tsang et al., 2017). Possuidores das mesmas vantagens apontadas para o paulistinha, mas donos de traços próprios e mais favoráveis a uma ou outra abordagem dentro das Neurociências, outras espécies de peixes como o *Astyanax mexicanus* e o *Gasterosteus aculeatus* são também, mas mais raramente utilizadas (Müller, 2005). Neste contexto encontra-se o *B. splendens*.

Betta splendens, um peixe altamente territorialista e que busca, invariavelmente, estabelecer relações de dominância frente a um congênere, vem sendo utilizado em paradigmas comportamentais envolvendo agressividade (Alyan, 2010; Dzieweczynski et al., 2012; Forsatkar et al., 2014). Por apresentar um intrincado repertório de comportamentos sociais que vão além daquele utilizado para o estabelecimento de dominância, como os comportamentos de corte e cuidado parental, acredita-se ser esta espécie possuidora de funções telencefálicas elaboradas (Müller, 2005) que podem ser exploradas em estudos comparativos com os demais vertebrados, em geral, e com os próprios mamíferos, em particular.

A construção de tal quadro, contudo, necessita de uma maior quantidade de informações sobre os diferentes aspectos morfológicos, funcionais e do desenvolvimento do telencéfalo da espécie em questão. Dados como esses foram obtidos raras vezes até o presente momento. De fato, somente se

conhecem os trabalhos neuroetológicos de De Bruin (1980), que provocava lesões ou estimulava áreas telencefálicas específicas e verificava como tais manipulações se refletiam nos comportamentos reprodutor e agressivo, e de Marino-Neto e Sabbatini (1983), que geravam lesões especificamente na Dm e verificaram as possíveis alterações da agressividade dos peixes, além do trabalho de Marino-Neto e Sabbatini (1988), que pruduziram um atlas estereotáxico do telencéfalo, diencéfalo e parte do mesencéfalo de machos da espécie. Além desses, nenhum outro trabalho com viés morfológico e/ou funcional foi desenvolvido até agora.

Neste contexto, esta tese procura fornecer dados morfológicos, alguns apresentando íntima relação com aspectos funcionais, que visam caracterizar o telencéfalo de *B. splendens*. Para tanto, machos e fêmeas tiveram a organização citoarquitetural de seus telencéfalos descritos e a atividade da enzima NADPH-d localizada e mensurada em uma escala relativa, comparando espécimes sujeitos a um estímulo agressivo com animais controle.

Apesar de dados sobre a organização do telencéfalo de machos de *B. splendens* já ter sido originalmente publicada no trabalho de Marino-Neto e Sabbatini (1988), detalhes desta organização, assim como a citoarquitetura das diferentes áreas permaneceram sem a devida atenção. Por outro lado, o acúmulo de informações sobre a morfologia do telencéfalo de outras espécies, desde a data da publicação do referido trabalho até os dias de hoje, permitiram novas e, em alguns casos, divergentes interpretações dos dados morfológicos obtidos. Ainda, fêmeas nunca tiveram o seu telencéfalo examinado e, portanto, potenciais dimorfismos sexuais na estrutura do telencéfalo que pudessem redundar nas marcadas diferenças comportamentais entre os sexos, no que se refere ao comportamento social desses peixes, jamais haviam sido examinadas.

O primeiro artigo demostrou que a organização morfológica do telencéfalo de machos e fêmeas de *B. splendens* é similar entre os sexos. Também demonstrou que o telencéfalo (aqui considerados o OB e o Tel), comparado com o de outros peixes teleósteos como *Salmo gairdneri* (Salmoniformes), *Lepomis cyanellus* (Perciformes) (Northcutt e Bradford Jr.,

1980), *Ictalurus punctatus* (Siluriformes) (Bass, 1981), *Barbus meridionalis* (Cypriniformes) (Diez et al., 1987), *Astyanax hubbsi* (Characiformes) (Riedel, 1997), *Dicentrarchus labrax* (Perciformes) (Cerdá-Reverter, 2001), *Astatotilapia burtoni* (Perciformes) (Burmeister et al., 2009), *Channa gachua* (Perciformes) (Baile e Patle, 2011), *Nothobranchius furzeri* (Cyprinodontiformes) (D'Angelo, 2013), *Chaetodon multicinctus* (Perciformes) (Dewan e Tricas, 2014) e *Trachurus japonicus* (Perciformes) (Ou e Yamamoto, 2016) possui uma série de estruturas (núcleos e áreas) homólogas, independente da posição filogenética ou hábito de vida adotado pela espécie.

Este trabalho encontrou discrepâncias neuroanatômicas quando comparado com o trabalho de Marino-Neto e Sabbatini (1988), uma vez que identificamos 16 núcleos em D, contra 13 originalmente apontados por esses autores. Alguns núcleos foram identificados pela primeira vez aqui, como é o caso da divisão granular da zona lateral do telencéfalo dorsal (Dlg), as subdivisões 1 e 2 da divisão ventral da zona lateral do telencéfalo dorsal (Dlv-1 e Dlv-2, respectivamente) e a divisão 4 da zona medial do telencéfalo dorsal (Dm-4). Em V identificou-se pela primeira vez o órgão septal lateral e foi reinterpretado, com base nos dados topológicos e citoarquiteturais de outras espécies, a identidade de Vp.

No primeiro artigo, em razão da homologia sugerida entre a MeA dos tetrápodes e Vs e Vp dos peixes actinopterígeos (Bradford, 2009; Connell e Hoffman, 2011; Bruce, 2012; Maximino et al., 2013) e do fato da MeA modular comportamentos sociais nos mamíferos, procurou-se a existência de dimorfismo sexual nesses núcleos em *B. splendens* utilizando o mesmo critério que serviu para identificar diferenças na MeA de ratos machos e fêmeas (Cooke et al., 2009), quer seja, a densidade numérica relativa de neurônios e células gliais. Não houve contagens diferentes nem em Vs e nem em Vp na espécie de peixe estudada. Tal resultado pode significar que o dimorfismo sexual pode não existir neste nível de organização celular ou pode estar ligado a outras estruturas telencefálicas neste peixe. Por exemplo, recentemente uma nova proposta de homologia entre a MeA de tetrápodes e um núcleo do telencéfalo (Vi) de *Danio rerio* foi proposta por Bielchl et al. (2017). Isso sugere

que o Vi também deva ser estudado futuramente em *Betta splendens* para verificar a existência de dimorfismo sexual.

Tendo sido elaborado um mapa neuroanatômico para o telencéfalo da espécie em estudo, o segundo trabalho procurou estabelecer a distribuição da atividade da enzima NADPH-d em todos os núcleos previamente identificados. Adicionalmente, machos e fêmeas de *B. splendens* tiveram os resultados histoquímicos para detecção da NADPH-d testados em situações onde os animais eram ou não expostos a sua própria imagem no espelho (paradigma comportamental para a agressividade). Com isso, descrevemos que o padrão de distribuição da enzima ocorre no *B. splendens* como no telencéfalo (tanto para o OB quanto para o Tel) de outros peixes actinopterígeos (Holmqvist et al., 1994; Östholm et al., 1994; Arévalo et al., 1995; Brüning et al., 1995; Villani e Guarnieri, 1995; Holmqvist et al., 2000; Lema e Nevitt, 2001; Cuoghi et al., 2002; Singru et al., 2003; Ando et al., 2004; Jadhao e Malz, 2004; Giraldez-Perez, 2008; Northcutt, 2009; Mueller et al., 2011; Pushchina et al., 2012; Giraldez-Perez et al., 2013; López et al., 2016; López et al., 2017). Ou seja, há reação identificada em corpos celulares e em fibras localizadas no neurópilo, os corpos celulares localizados em V são mais comumente e intensamente marcados do que aqueles em D. No entanto, como discutido nesse artigo, apesar de encontrarmos muitas semelhanças na identidade das estruturas marcadas, como ocorre com os corpos celulares localizados em Vd, Vs e EN de outros peixes comparativamente ao *B. splendens*, o padrão geral de marcação parece ser espécie-específico. Ademais, quando comparados os diferentes sexos de *B. splendens* nas condições descritas acima, surge um padrão de marcação específico para cada sexo.

Chama a atenção que o paradigma comportamental empregado foi capaz de alterar marcadamente o padrão de marcação neural observado, ou seja, há reação identificada em estruturas adicionais no telencéfalo, além de tornar a marcação mais intensa naquelas anteriormente detectadas na técnica histoquímica empregada. Isso sugere que há ativação neural envolvida na percepção sensorial e na expressão do comportamento agressivo. E, dentre as áreas que passam a ter maior atividade, encontraram-se corpos celulares que tornaram-se marcados ou tiveram aumento da intensidade de sua marcação no

EN, Vd, Vi, Vv, Vs e Vp. O EN é homólogo ao núcleo entopeduncular próprio/globo pálido e ao núcleo do leito da estria medular (Mueller e Guo, 2009; Ganz et al., 2012). O Vd e o Vv são considerados homólogos ao estriado e ao septo/pálido, respectivamente (Ganz et al., 2012; Gonzales et al., 2014; López et al, 2017). O Vi foi recentemente proposto como homólogo da MeA (Biechl et al., 2017). O Vs e o Vp são considerados homólogos à MeA (O'Connell and Hofmann, 2011), ou alternativamete ao leito do núcleo da estria medular e à amigdala central (Ganz et al., 2012; Maximino et al., 2013).

É muito interessante dizer que no encéfalo dos tetrápodes estas estruturas têm como um de seus papeis mediar aspectos motores e sociais/reprodutivos do comportamento (Rasia-Filho et al., 2000; Connell e Hoffman, 2011). Como a marcação discutida acima corresponde em grande parte (para os Tels) à ação da nNOS, que o NO modula uma série de neurotransmissores, e dentre eles a 5-HT como explicado anteriormente, e que o 5-HT modula a agressividade, podemos supor que a produção de NO e, assim, a atividade da NADPH-d, estão relacionadas com o comportamento agressivo. Apesar de atribuir-se em camundongos machos a redução da produção de NO ao aumento da agressividade (Demas et al., 1997; Chiavegatto et al., 2001), o que supostamente contraria nossos resultados, um trabalho recente com o peixe *Danio rerio* e camundongos machos demonstrou que o NO interage de maneira direta (o aumento de um, leva ao aumento do outro) com a atividade da enzima monoamina oxidase (Gutiérrez et al., 2017). Desta forma, pelas razões discutidas na introdução desta tese, uma redução da produção do NO conduz os organismos citados acima a uma concomitante redução da agressividade (Gutiérrez et al., 2017), fato que se alinha com os resultados obtidos por nós para o *B. splendens*.

Por fim, o conjunto de resultados apresentados nesta tese abre caminho para futuros trabalhos dentro das Neurociências que venham a abordar de forma comparada diferentes aspectos da agressividade. Isso torna o *B. splendens* uma nova alternativa de modelo experimental não mamífero que pode ser empregado com esta finalidade.

5. CONCLUSÕES

Os resultados desta tese permitem concluir que:

1. A caracterização morfológica e histoquímica aqui realizadas contribuem de modo referencial para a futura utilização do telencéfalo de *B. splendens* em diferentes abordagens metodológicas neuroanatômicas e do estudo do comportamento agonista desses peixes em diferentes contextos experimentais.

2. Machos e fêmeas de *B. splendens* possuem um telencéfalo semelhante entre si e similar a de outros peixes teleósteos, considerando-se critérios topológicos, topográficos e citoarquiteturais.

3. Os agrupamentos celulares, apesar de claramente distintos entre si em razão de sua estrutura citoarquitetural própria, não apresentam limites claros em todas as suas dimensões, podendo ter suas extensões aproximadamente definidas por técnicas histológicas (H.E. e Nissl) que permitem observar a estrutura tecidual.

4. Não há dimorfismo sexual nas densidades numéricas relativas de neurônios e células gliais em Vs e Vp do telecéfalo de *B. splendens*, estruturas homólogas à MeA de mamíferos.

5. A atividade NADPH-d no telencéfalo de *B. splendens* apresenta diferença entre os sexos, fato ainda não demostrado para outras espécies; havendo predominância de marcação de corpos celulares em V em detrimento de D, característica filogeneticamente conservada entre os vertebrados.

6. O estímulo agressivo fornecido durante o paradigma comportamental (imagem no espelho), aplicado para machos e fêmeas de B. splendens, promoveu o aumento da atividade da

enzima NADPH-d em diversas estruturas telencefálicas, atestando a provável participação do NO na modulação da agressividade, como observado em *Danio rerio* e em camundongos.

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7. APÊNDICES

7.1 Apêndice 1

Metodologia empregada na reprodução de *Betta splendens*

a- Colocar o macho no aquário de reprodução já preparado (nível da água em 9cm, temperatura de 28ºC, refúgio para a fêmea e um pedaço de isopor) e a fêmea, dentro de um frasco, onde a mesma possa observar o macho e o macho possa observa-la.
b- Vinte e quatro horas após, observar se o macho construiu o ninho de bolhas junto ao isopor. Se sim, liberar a fêmea no aquário; se não, esperar mais 24 horas; se ainda não, trocar o macho.
c- Acender luz do ambiente e mante-la acesa até a retirada do macho do aquário de reprodução.
d- Vinte e quatro horas após, retirar a fêmea do aquário retornando-a ao seu betário. Está fêmea estará apta a reproduzir novamente 21 dias após a postura.
e- Verificar, com uma lanterna, a existência de ovos no ninho de bolhas.
f- Se não houver ovos recomeçar o processo com um novo casal (retira-se também o macho).
g- Se houver ovos, esperar a eclosão, que deverá ocorrer em até 48 horas.
h- Se não houver ovos retira-se o macho, limpa-se o aquário e o prepara para um novo casal.
i- Após a eclosão evitar abrir a tampa do aquário com perda de umidade e calor.
j- No início do 3º dia após a eclosão (larvas já orientadas horizontalmente) retirar o macho do aquário de reprodução, retornando-o ao seu betário, ligar a bomba de aeração com pedra porosa e alimentar as larvas segundo tabela desenvolvida no laboratório. Neste momento também se retira o isopor e o esconderijo da fêmea.
k- Limpar o aquário com a sifonagem do fundo e trocas parciais de água de acordo com tabela desenvolvida no laboratório.
l- Após 2-3 meses remover os animais para seus betários individuais.

Observações:

a- Adicionar sal grosso na água na concentração de 0,3% (3g/L).

7.2 Apêndice 2

Protocolo para manutenção de *Betta splendens*

a- Manter a temperatura da água em 27ºC.
b- Manter o nível da água do aquário, no máximo, em 10cm.
c- Ligar o aerador após a retirada do macho do aquário, no terceiro dia, e desliga-lo após a completa formação do labirinto, no dia 60 (recomendado entre 40 e 90 dias).
d- Não realizar trocas de água durante a primeira e a segunda semanas após a eclosão. Deve-se adicionar água durante a segunda semana, cuidando para não ultrapassar os 25% do volume inicial de água no aquário.
e- Durante as duas primeiras semanas, enquanto os animais estiverem se alimentando de infusório e náuplios de artêmia, retirar material precipitado e as cascas e cistos de artêmia que sobrarem após a sua alimentação.
f- As trocas iniciam no final da segunda semana e terminam no dia 60 (recomendado entre o dia 60 e 90).
g- Durante a terceira semana proceder trocas de 10% do volume da água (salina a 0,3%) de 24 em 24 horas até o início da quarta semana.
h- Proceder a trocas de 25% do volume de água (salina a 0,3%) de 24 em 24 horas do início da quarta semana até o dia 60.
i- Após transferir os peixes para seus betários, no dia 60, proceder a trocas completas de água uma vez por semana, até o dia 90, e depois disso duas vezes por semana.
j- Suspender a alimentação por 24 a 48 horas se perceber dificuldade do animal manter o equilíbrio (Distúrbio da bexiga natatória).
k- Aferir o pH (entre 6,6 e 7,0) e os conteúdos de amônia e nitritos uma vez por semana.

7.3 Apêndice 3

Tabela de alimentação para *Betta splendens*

Etapa	Alimentação	Quantidade	Observação
Eclosão – 2º dia	Saco vitelínico	-	
3º dia – 5º dia	Infusório salino (0,3%)	10 gotas	3 vezes ao dia
	Náuplios de artêmia	5 gotas	Total de 2mL
6º dia – 15º dia	Infusório salino (0,3%)	5 gotas	3 vezes ao dia
	Náuplios de artêmia	10 gotas	Total de 2mL
16º dia – 30º dia	Náuplios de artêmia	1/2 concha rasa	2 vezes ao dia Total de 2,5mL
31º dia – 60º dia	Náuplios de artêmia	1/2 concha rasa	2 vezes ao dia
	Ração comercial triturada	1 porção pequena	Total de 2,5mL + Ração
61º dia – 90º dia	Ração comercial triturada	1 porção média	2 vezes ao dia
91º dia em diante	Ração comercial triturada e depois inteira	1 porção média e depois de 3 a 5 pelotas	2 vezes ao dia

Indução à anestesia e recuperação de *Betta splendens* (Regan, 1910): uma proposta de utilização de um anestésico de baixo custo para uma espécie de peixe ornamental de grande apelo comercial

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Resumo: A manipulação sofrida por peixes destinados à comercialização gera um estado de estresse que favorece a contração de doenças e/ou a morte. Uma alternativa é a sedação/anestesia. Neste contexto, o óleo de cravo surge como uma alternativa promissora, pois induz a anestesia e propicia uma rápida recuperação. O objetivo do presente trabalho foi propor a concentração de óleo de cravo que melhor se adequa à indução da anestesia em *Betta splendens* e a sua recuperação. Para isto exemplares de *B. splendens* foram submetidos a soluções de óleo de cravo nas concentrações de 10, 25, 50, 75, 100 ou 150mg/L ou a uma solução de álcool etílico com concentração de 1.075mg/L. Os resultados demostraram não haver indução à anestesia quando utilizada a concentração de 10mg/L ou a exposição dos espécimes ao álcool etílico. Para outras concentrações de óleo de cravo percebeu-se uma redução na latência da anestesia com o aumento da concentração. O tempo de recuperação mostrou aumento quando concentrações de 25mg/L e 50mg/L foram comparadas. Conclui-se que a concentração de 25mg/L é a melhor escolha para a anestesia de *B. splendens* em razão de sua latência e do maior tempo para que o último estágio de anestesia seja alcançado.

Palavras-chave: *Betta splendens*; Anestesia; Óleo de cravo.

Induction to anesthesia and recovery of Betta splendens (Regan, 1910): a proposal for the use of a low-cost anesthetics for an ornamental fish species of great commercial appeal

Abstract: The manipulation suffered by commercial fish generates a state of stress that favors the contraction of diseases and/or death. An alternative is sedation/anesthesia. In this context, clove oil appears as a promising alternative, as it induces anesthesia and provides a rapid recovery. The aim of the present study was to propose the concentration of clove oil that is best suited to the induction of anesthesia in *Betta splendens* and its recovery. For this, *B. splendens* samples were submitted to solutions of clove oil at the concentrations of 10, 25, 50, 75, 100 or 150mg/L or to a solution of ethyl alcohol with concentration of 1.075mg/L. The results showed no induction to anesthesia when the concentration of 10mg/L is used or the exposure of the specimens to ethyl alcohol occurs. For other concentrations of clove oil, a decrease in the latency of the anesthesia was observed with increasing concentrations. The recovery time showed an increase when concentrations of 25mg/L and 50mg/L were compared between them. It is concluded that the concentration of 25mg/L is the best choice for the anesthesia of *B. splendens* because of its latency and the longer time for the last stage of anesthesia to be reached.

Keywords: *Betta splendens*; Anesthesia; Clove oil.

INTRODUÇÃO

A manipulação, invariavelmente, submete os animais que não estão habituados com esta prática a um estado de estresse. Para os peixes destinados a ornamentação ou ao consumo esta é uma condição extremamente prejudicial, uma vez que os conduz a aquisição de doenças ou a morte.

Uma alternativa visando reduzir o estresse causado pela manipulação é a sedação ou a anestesia. Há uma série de substâncias que atuam com esta finalidade, sendo a mais utilizada para peixes, por recomendação de diferentes órgãos que tratam de questões éticas referentes ao uso de animais nas mais diferentes áreas como o Conselho Federal de Medicina Veterinária do Brasil e a Associação Americana de Medicina Veterinária, os barbitúricos e seus derivados, (CFMV, 2002; AVMA, 2007). No Brasil, o anestésico mais utilizado com este fim é a benzocaína (ethyl-p-aminobenzoato) (GOMES *et al*., 2001). Apesar de ser um anestésico eficiente, apresentando uma série de aspectos favoráveis (NEIFFER e

STAMPER, 2009), não é fácil de ser obtido, além de não possuir o melhor custo-benefício quando comparado a outras alternativas (SOUZA, *et al*., 2012).

É consenso que o anestésico ideal deve produzir uma rápida indução à anestesia e à recuperação, gerando o mínimo de estresse; deve resultar em imobilização por tempo suficiente para o procedimento desejado e deve ter grande margem de segurança para o animal, para quem o manipula e para o ambiente (GOODMAN, 2012). Neste contexto, o óleo de cravo da índia vem sendo cada vez mais utilizado. Além de atender aos critérios citados acima, contudo, com um tempo de recuperação um pouco mais longo quando comparado a outros anestésicos, é de fácil obtenção e possui um excelente custo-benefício (NEIFFER e STAMPER, 2009).

O oléo de cravo é um produto natural extraído do cravo da índia (*Syzygium aromaticum*; sinônimo *Cariophyllus aromaticus*), sendo utilizado por seres humanos como anestésico tópico para dores de dente, de cabeça e nas articulações, desde a antiguidade (ROSS e ROSS, 2008). O efeito anestésico do óleo de cravo deve-se ao eugenol (4-allyl-2-methoxyphenol), isoeugenol e metileugenol, mas principalmente ao primeiro, que corresponde a aproximadamente 84% do conteúdo da substância vendida comercialmente (NEIFFER e STAMPER, 2009; JAVAHERY *et al*., 2012).

Muitos são os trabalhos experimentais que mais recentemente descreveram e/ou mensuraram os efeitos do óleo de cravo ou do eugenol como anestésico para diferentes espécies de peixes (HISANO *et al*., 2008; PEREIRA-DA-SILVA *et al*. 2009; SIMÕES *et al*., 2010; DELBON e RANZANI PAIVA, 2012; DIMER *et al*., 2012; ROTILI *et al*. 2012), com excelentes resultados, quanto a indução da anestesia e manutenção dos valores normais das variáveis fisiológicas, como os níveis de hormônios plasmáticos, a quantidade de células sanguíneas e a glicemia, dos animais testados (HOLLOWAY *et al*., 2004; VELISEK *et al*., 2006; MOREIRA *et al*., 2011). Seus efeitos como anestésico acabaram, inclusive, superando os efeitos obtidos por outras substâncias como a tricaína metano sulfonato (TMS ou MS-222), um derivado da benzocaína, amplamente utilizado e recomendado como anestésico na pesquisa com peixes, e a própria benzocaína (GRUSH et. al., 2004; HOLLOWAY *et al*., 2004; OLIVEIRA *et al*., 2009, SOUZA et. al. 2012).

Apesar de eficiente, de fácil obtenção e reduzido valor frente a outros anestésicos, a dosagem a ser utilizada para se obter o efeito anestésico varia com a espécie, a massa e a fase de desenvolvimento do peixe, assim como, o valor das

variáveis ambientais e a temperatura (BORSKI e HODSON, 2003; NEIFFER e STAMPER, 2009; GOODMAN, 2012; JAVAHERY *et al*., 2012).

Betta splendens (Regan 1910) (Actinopterygii, Anabantoidei), também chamado de “a joia do oriente” ou “Betta esplêndido” por alguns criadores, é um peixe da subordem anabantoidei (ITIS, 2006), oriundo de diversos países da Ásia, como a Tailândia, a Indonésia, o Vietnã e a China, e que apresenta um comportamento tipicamente territorialista, demonstrando grande agressividade frente a um potencial invasor/competidor da mesma espécie (FARIA *et al*., 2006; KARINO e SOMEYA, 2007; VERBEEK *et al*., 2007). Em razão de constituírem-se em peixes ornamentais de fácil manutenção, possuem grande apelo comercial e, em decorrência disso, um número significativo de informações referentes a aspectos de sua biologia e criação (BORUCHOWITZ, 2006; FARIA *et al*., 2006; GOLDSTEIN, 2012; TULLOCK, 2006). Por outro lado, estudos propositivos tentando reduzir o estresse (e também, a morte, direta ou indiretamente associada ao estresse) causado pela manipulação, com fins comerciais ou científicos, são limitados para esta espécie (SOMMANI *et al*., 1999; PATTANASIRI *et al*., 2008; PATTANASIRI *et al*., 2016).

O objetivo deste trabalho foi propor a concentração de óleo de cravo que melhor se adequa à indução da anestesia e ao tempo de recuperação em exemplares adultos de *B. splendens*, visando reduzir o estresse dos animais durante a sua manipulação.

MATERIAIS E MÉTODOS

Animais

Trinta e seis espécimes adultos de *B. splendens*, de ambos os sexos, foram obtidos em um comércio local e levados ao Laboratório de Histologia do Instituto Federal de Educação, Ciência e Tecnologia do Rio Grande do Sul (IFRS) – Campus Porto Alegre, onde foram mantidos por uma semana em recipientes individuais, nos quais não era permitido que mantivessem contato visual com outros animais, para aclimatação. Os recipientes possuíam 0,5L de água, com salinidade de 0,3%. Todos os indivíduos foram submetidos à iluminação natural e temperatura em torno dos 28ºC, sendo alimentados, duas vezes ao dia com ração comercial. Duas vezes

durante a semana foram medidos o pH e os níveis de amônia e nitritos da água, sendo esta parcialmente (25% do volume total) substituída.

Todos os procedimentos adotados nesta pesquisa com os espécimes respeitaram a Lei de Procedimentos para o Uso Científico de Animais (BRASIL, 2008), de 8 de outubro de 2008, regulamentada pelo Decreto nº 6.899, de 15 de julho de 2009 (BRASIL, 2009) e a Resolução 714, de 20 de junho de 2002, do Conselho Federal de Medicina Veterinária, que dispõe sobre procedimentos e métodos de eutanásia em animais (CFMV, 2002)

Este trabalho foi aprovado pelo Comité de Ética da Universidade Federal do Rio Grande do Sul (UFRGS), Brasil (Protocolo nº 22625).

Desenho experimental

Os animais foram divididos em sete grupos de 3 a 4 animais e, após jejum de 40 horas, colocados em contato com uma solução de óleo de cravo nas concentrações de 5, 10, 25, 50, 100 e 150mg/L, assim como com uma solução de álcool etílico na concentração de 1.075mg/L (que corresponde a maior concentração de álcool etílico encontrada entre as soluções de óleo de cravo).

Foram utilizados dois aquários de igual tamanho (200mm comprimento X 90mm profundidade X 150mm de altura) e com igual volume de água (1,5L). A água utilizada em ambos possuía as mesmas temperatura e salinidade utilizadas nos recipientes destinados à aclimatação. No primeiro aquário (aquário para anestesia, Figura 1) eram misturadas à água uma solução de óleo de cravo previamente solubilizada em etanol 95% (solução estoque) ou apenas etanol, até que se atingissem as concentrações descritas anteriormente. No segundo, apenas água (aquário para recuperação). Cada indivíduo, então, era introduzido no primeiro aquário, depois de intensa oxigenação da água por 5 minutos, e o tempo de indução à anestesia (em minutos), cronometrado e anotado. Após, os animais eram transferidos para o segundo aquário, iniciando a contagem do tempo de recuperação (em min).

Figura 1 - Aquário para anestesia.

Fonte: os autores.

O tempo de permanência no aquário para anestesia se estendia até o animal atingir o estágio 4 de anestesia (Quadro 1) ou ter completado uma hora no mesmo. Ao contrário da anestesia, não foi estabelecido um tempo máximo para a permanência no aquário para a recuperação.

Após recuperados, os animais tiveram o seu comprimento padrão e massa aferidos, utilizando-se um paquímetro analógico série 125 (Starrett, Brasil), em milímetros, e uma balança semianalítica BK300 (Gehaka, Brasil), em gramas, respectivamente.

Passada a manipulação experimental os animais foram observados por uma semana e a fim de registrar possíveis mortes.

Anestésico

O óleo de cravo extraído de *Cariophyllus aromaticus* (densidade de 1,04 g/ml) foi obtido em farmácia de manipulação e diluído em etanol 95% a 10%, para facilitar sua solubilização em água (solução estoque). Esta solução estoque foi, então, diluída na água do aquário para anestesia até obter-se as concentrações desejadas para os grupos de animais utilizados, sendo intensamente oxigenada por 5 minutos antes da introdução do animal em seu interior, como descrito anteriormente.

Estágios de anestesia e recuperação

Foram identificados para os peixes submetidos ao óleo de cravo, pelas características comportamentais, quatro estágios de anestesia e mais a recuperação. O tempo (em minutos) que cada animal levou para atingir cada um dos estágios de anestesia foi anotado e uma tabela representando a relação entre a concentração do anestésico, o estágio de anestesia e este tempo, construída (Tabela 1). Da mesma forma o tempo (em minutos) necessário para que cada animal se recuperasse da anestesia foi registrado e expresso nesta mesma tabela (Tabela 1).

Os estágios de anestesia e a recuperação utilizados foram aqueles descritos por WOODY *et al*., (2002) e caracterizados no Quadro 1.

Quadro 1 - Características comportamentais para peixes durante diferentes estágios de anestesia e recuperação.

Estágio de anestesia	Características comportamentais
1	Movimentos operculares lentos ou erráticos
2	Dificuldade de manter a posição normal de nado enquanto parado e perda esporádica do equilíbrio.
3	Perda completa do equilíbrio com incapacidade de assumir a posição normal de nado.
4	Sem reação à manipulação ou a um forte estímulo no pedúnculo caudal.
Recuperação	Recuperação da posição normal de nado e da capacidade de nadar horizontalmente.

Fonte: WOODY *et al*. (2002).

RESULTADOS

Nossos resultados demonstraram que os exemplares de *B. splendens*, de ambos os sexos, com comprimento padrão entre 27,2mm e 44,8mm e massa variando de 0,61g a 2,29g, apresentaram uma latência menor para ingressar nos estágios de anestesia com valores mais altos a medida que a concentração do anestésico ao qual foram expostos aumentou (Tabela 1). Por exemplo, animais expostos a concentrações de anestésico de 25mg/L atingiram o estágio 3 apenas depois de transcorridos 7 minutos do início da exposição ao óleo de cravo, enquanto

animais submetidos a uma concentração de 100mg/L atingiram o mesmo estágio já no primeiro minuto de exposição (Tabela 1).

Não houve indução à anestesia quando se utilizou a concentração de 10mg/L de óleo de cravo, mesmo depois de transcorrida uma hora da exposição ao anestésico (Tabela 1).

Por outro lado, o tempo de recuperação dos animais apresentou grande variação entre as diferentes concentrações e entre os diferentes animais submetidos a uma mesma concentração do anestésico (Tabela 1). Cabe atentar que, como exemplo, ao comparar animais expostos à uma concentração de óleo de cravo de 25mg/L com aqueles submetidos a uma concentração equivalente a 100mg/L, o tempo máximo necessário à recuperação dos representantes do segundo grupo é inferior ao tempo máximo de recuperação obtido pelos animais do primeiro grupo, não fazendo qualquer sentido a não ser sob a lógica da variabilidade.

O álcool etílico, em uma concentração equivalente a 1.075mg/L, valor igual à maior concentração desta substância misturada ao óleo de cravo (encontrada na solução de óleo de cravo de 150mg/L) e com o papel de aumentar a solubilidade do anestésico à água, não apresentou qualquer efeito anestésico.

Transcorrida uma semana da exposição à solução de óleo de cravo ou a de álcool etílico não houve morte de qualquer espécime testado.

Tabela 1 - Tempo para indução aos estágios de anestesia e para a recuperação em *B. splendens* expostos a soluções de óleo de cravo com diferentes concentrações.

Concentração (mg/L)	Anestesia (min)																Recuperação (min)
	10										20		30	40	50	60	
	1	2	3	4	5	6	7	8	9	10	15	20					
10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	N
25	0,1	0,1	0,1	0,1	0,1	0,1	1,2,3	1,2,3	1,2,3	1,2,3	1,2,3	2,3	2,4	2	2,3	2,3	2-8
50	1	1,2	1,2,3	2,3	2,3	3	3	3	3	3	3,4	3,4	4	-	-	-	9-12
75	1,2	2,3	2,3	3	3,4	3	3	3	3,4	3	4	-	-	-	-	-	5-13
100	1,2,3	3	3	3	3,4	3,4	4	-	-	-	-	-	-	-	-	-	5-7
150	1,2,3	3	3,4	3,4	3	4	-	-	-	-	-	-	-	-	-	-	5-10

0, Ausência de anestesia; 1, Estágio 1; 2, Estágio 2; 3, Estágio 3 e 4, Estágio 4. N, Não houve necessidade de recuperação.

DISCUSSÃO

Apesar do efeito anestésico do óleo de cravo já ter sido aferido em *B. splendens* (PATTANASIRI *et al*., 2008), tanto as concentrações utilizadas quanto os tempos de permanência dos animais em contato com a substância foram diferentes daqueles propostos em nosso trabalho. Enquanto, no trabalho de 2008 as concentrações variavam de 5 a 100ppm e o tempo de exposição máximo era de 48 horas em nosso trabalho a concentração máxima foi de 150mg/L e o tempo máximo de 60min.

Diferente do trabalho de PATTANASIRI *et al*., (2008), concentrações iguais a 10mg/L, na presente pesquisa, não induziram à anestesia, nem mesmo após transcorrida uma hora de exposição. Por outro lado, todas as concentrações de óleo de cravo iguais ou superiores a 25mg/L conduziram à anestesia os exemplares de *B. splendens* já no primeiro minuto dos testes. No trabalho de 2008, a uma concentração de 20ppm, o óleo de cravo induziu a anestesia apenas após transcorridos 30min de exposição, ocorrendo o mesmo com uma concentração ligeiramente inferior (15ppm). Concentração capaz de induzir a anestesia depois de transcorridos 5 minutos de contato dos peixes com o anestésico foi observada apenas em concentrações superiores a 50ppm (PATTANASIRI *et al*., 2008). Tais discrepâncias observadas entre os dois trabalhos podem ser explicadas pela concentração de eugenol encontrada nas amostras de óleo de cravo utilizadas, uma vez que os autores citados acima obtiveram o óleo de *Syzigium aromaticum*.

Adicionalmente, a presente investigação mostrou que a latência para se atingir estágios de anestesia cada vez mais altos (do 1 ao 4) tornou-se cada vez menor a medida que havia um aumento na concentração do anestésico, concluindo-se que a ação do óleo de cravo na indução à anestesia, mostrou um padrão dose-dependente. Tal dado concorda com aquele já expresso para *B. splendens* (PATTANASIRI *et al*., 2008) e para outras espécies de peixes que utilizaram o óleo de cravo como anestésico (WOODY *et al*., 2002; HISANO *et al*., 2008; NEIFFER e STAMPER, 2009; OLIVEIRA *et al*., 2009; PEREIRA-DA-SILVA *et al*., 2009; SIMÕES *et al*., 2010). Assim, utilizando-se a concentração de 25mg/L, os estágios 2 e 3 são obtidos após transcorridos 7 minutos de exposição, enquanto o estágio 4 só advêm aos 20 minutos de contato do animal com o anestésico. Para todas as demais concentrações os estágios 2 ou 3 são obtidos já nos primeiros 2 minutos de

exposição, enquanto o estágio 4 é observado após 15minutos, para uma concentração de 50mg/L, 5 minutos, para concentrações de 75mg/L e 100mg/L e 3 minutos para a concentração de 150mg/L.

Desta forma, com base na latência observada, a concentração de óleo de cravo de 25mg/L parece ser a que melhor se adequa a reduzir o estresse causado pela manipulação com certa margem de segurança, uma vez que exige um tempo para a indução à anestesia (estágio 1) reduzido (1 a 15min) e cerca de trinta minutos para que o estágio 4 seja alcançado, estágio este que poderia conduzir o animal à morte pela interrupção da ventilação e redução do débito cardíaco (AVMA, 2007).

Além da concentração indicada acima outras concentrações poderiam vir a ser testadas com a mesma finalidade, como concentrações intermediárias entre os 10mg/L e 25mg/L.

Quanto ao tempo de recuperação, surpreendentemente, os dados não mostraram a existência de uma clara relação dose-dependente (quanto maior a concentração do anestésico maior o tempo de recuperação), como ocorre com outros peixes (PEREIRA-DA-SILVA *et al*., 2009; SIMÕES *et al*., 2010) e mesmo com *B. splendens* (PATTANASIRI *et al*., 2008). Apesar de se observar um padrão de dose-dependência quando comparadas as concentrações de 25mg/L e 50mg/L, percebe-se, que o tempo máximo de recuperação de animais expostos a concentrações de 100mg/L e 150mg/L são menores do que daqueles exemplares submetidos a concentrações de 25mg/L e 50mg/L, respectivamente. Esta discrepância parece ser resultado de fatores intrínsecos, próprios dos animais testados, e está calcada na variabilidade intraespecífica da população amostrada. Uma maneira de reduzir esta variabilidade seria aumentando o número de animais testados em cada uma das concentrações propostas.

Concluindo, este trabalho objetivou testar a ação de diferentes concentrações de óleo de cravo na indução da anestesia e no tempo de recuperação de exemplares adultos de *B. splendens*, com a finalidade de propor sua utilização na redução do estresse causado pela manipulação. Os dados apontam que a concentração de 25mg/L de óleo de cravo é a que melhor se adequa ao objetivo proposto, em razão de sua latência reduzida e do longo tempo necessário para conduzir o animal ao estágio mais profundo de anestesia.

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8. ANEXOS

8.1 Anexo 1

U F R G S
UNIVERSIDADE FEDERAL
DO RIO GRANDE DO SUL

PRÓ-REITORIA DE PESQUISA

Comissão De Ética No Uso De Animais

PROPESQ

CARTA DE APROVAÇÃO

Comissão De Ética No Uso De Animais analisou o projeto:

Número: 22625
Título: Localização da expressão de vasotocina e isotocina na região ventral do telencéfalo e, especialmente, na área/núcleo supracomissural de Betta splendens (Regan 1910) (Actinopterygii, Anabantoidei) su

Pesquisadores:

Equipe UFRGS:

ANGELO CASSIO MAGALHAES HORN - coordenador desde 01/05/2012
ALBERTO ANTONIO RASIA FILHO - pesquisador desde 01/05/2012

Comissão De Ética No Uso De Animais aprovou o mesmo , em reunião realizada em 14/05/2012 - Sala de reuniões do 2º andar do prédio da reitoria, em seus aspectos éticos e metolodológicos, para a utilização de doze espécimes de peixes B. splendens de ambos os gêneros (6 casais), de acordo com as Diretrizes e Normas Nacionais e Internacionais, especialmente a Lei 11.794 de 08 de novembro de 2008 que disciplina a criação e utilização de animais em atividades de ensino e pesquisa.

Porto Alegre, Quinta-Feira, 24 de Maio de 2012

p/FLAVIO ANTONIO PACHECO DE ARAUJO
Coordenador da comissão de ética

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