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ENRIQUECIMENTO AMBIENTAL EM PEIXE-ZEBRA: PERFIL COMPORTAMENTAL E ENVOLVIMENTO DO SISTEMA GLUTAMATÉRGICO

PORTO ALEGRE

Fevereiro, 2018.

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Orientador: Prof. Dr. Diogo Losch de Oliveira

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“AS INDAGAÇÕES

A resposta certa, não importa nada: o essencial é que as perguntas estejam certas.”

Mario Quintana

Às duas pessoas que me encorajam todos os dias,

minha mãe e minha avó.

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SUMÁRIO

APRESENTAÇÃO

Esta dissertação está dividida em seis partes:

PARTE I: resumo, *abstract*, lista de abreviaturas, introdução e objetivos;

PARTE II: os resultados que fazem parte desta dissertação foram apresentados na forma de dois artigos científicos, **Capítulo I** e **Capítulo II**, os quais foram submetidos aos periódicos *Journal of Experimental Biology* e *Neuroscience Letters*, respectivamente. Ambos os capítulos estão subdivididos em: Introdução, Materiais e Métodos, Resultados, Discussão, Conclusão e Referências Bibliográficas;

PARTE III: discussão final;

PARTE IV: conclusão;

PARTE V: perspectivas;

PARTE VI: referências bibliográficas referentes à Parte I e Parte III.

PARTE I

RESUMO

O enriquecimento ambiental oferece estímulos sensoriais, sociais e cognitivos possibilitando ao animal bem-estar e comportamentos mais próximos ao seu natural. Alterações ambientais podem gerar mudanças de expressão gênica, bem como de comportamento. Assim, o primeiro objetivo deste trabalho foi avaliar a influência de diferentes protocolos de enriquecimento ambiental sobre o perfil comportamental do peixe-zebra (*Danio rerio*) adulto. Utilizando pedras ao fundo do aquário, plantas artificiais e os animais em cardume, foram montados três protocolos de enriquecimento ambiental. O "Protocolo 1" foi desenhado para avaliar se um ambiente enriquecido com os componentes básicos de complexidade por sete dias já induziria alterações no comportamento do peixe-zebra. Os resultados obtidos com o teste de *open tank* demonstraram que os animais do grupo ambiente enriquecido (AE) apresentaram um perfil locomotor diferenciado, com redução de distância percorrida total e velocidade média, quando comparado com animais do grupo ambiente padrão (AP). Entretanto, esta diferença se deve a uma instabilidade comportamental apresentada pelo grupo AP através do aumento de distância percorrida no fundo do aquário. Não foram observadas alterações no comportamento tipo ansiedade e preferência social entre os grupos. Desse modo, desenhamos o "Protocolo 2" a fim de avaliar se a reposta comportamental se manteria durante uma exposição mais longa (quatorze dias) ao enriquecimento ambiental. Entretanto, a diferença no perfil locomotor entre os grupos não foi apresentada neste protocolo. Com esses resultados, demonstrou-se a intensidade do estresse provocado pela simples troca entre aquários idênticos utilizados normalmente em biotérios, necessitando uma ambientação mínima de duas semanas no aquário moradia antes da realização de testes comportamentais. Por último, realizamos a avaliação do "Protocolo 3", com o intuito de estudar se a renovação de itens da complexidade do enriquecimento ambiental dentro do período quatorze dias teria alteração nos resultados já observados. Os animais do grupo AE não apresentaram diferença no perfil comportamental geral mesmo com a inserção de novos objetos, entretanto, houve uma redução de distância percorrida no fundo do aparato.

Em peixe-zebra, estudos já demonstraram a indução da plasticidade neuronal e aumento na proliferação neuronal quando submetidos a protocolos de enriquecimento. No modelo de roedores, muitos dos efeitos do enriquecimento ambiental são relacionados ao aumento de neurogênese adulta e de ramificação dendrítica, bem como alteração da funcionalidade de alguns sistemas de neurotransmissores, como o glutamatérgico. Assim, a segunda parte dessa dissertação teve como objetivo a análise da expressão proteica de um dos transportadores vesiculares de glutamato, o vGluT2, em animais expostos aos protocolos citados anteriormente. Os vGluTs são expressos em regiões dendríticas dos neurônios e quanto maior sua expressão proteica, maior é a densidade de vesículas sinápticas presentes. Os dados obtidos demonstram que essa resposta é relacionada a consolidação e habituação dos peixes a essa condição por um período mais prolongado no protocolo de enriquecimento ambiental (Protocolo 2).

Neste trabalho, foi demonstrado que o protocolo de enriquecimento ambiental altera a resposta comportamental do peixe-zebra adulto, mantendo-a mais regular do que a dos controles e isso, provavelmente, é decorrente da estimulação de processos direcionados a resposta ao estresse da novidade, bem como de vias relacionadas com a adaptabilidade. Ademais, demonstramos que a habituação por um protocolo mais longo à condição de um ambiente enriquecido é mais importante para desencadear o aumento de expressão proteica de vGluT2 em cérebro de peixe-zebra.

ABSTRACT

Environmental enrichment offers social, sensory and cognitive stimuli making possible animal welfare and behavior closer to its natural. Environmental alterations can generate changes in gene expression as well as in behavior. Thus, first aim of this study was to evaluate the influence of different environmental enrichment protocols in adult zebrafish's (*Danio rerio*) behavioral profile. Considering items more commonly described in the literature (stones at tank bottom, artificial plants and shoal), we designed three enriched environment protocols. "Protocol 1" was developed to evaluate if an enriched environment with basics components of complexity for seven days already induce behavior alterations in zebrafish. Results obtained with open tank test demonstrated that animals submitted to environmental enrichment (EE) presented differentiated locomotor profile by a reduction of total distance travelled and average speed when compared with animals in the standard environment (SE). However, this difference was due to a behavioral instability presented by animals of SE group reflected in an increase of distance travelled in last two minutes of the test. There were not alterations in anxiety-like behavior and social preference between the groups. In this way, the "Protocol 2" was designed to evaluate whether these behavioral responses in open tank test remain in a longer exposure (fourteen days) to environmental enrichment. However, the difference in locomotor profile was not significant between groups. With the results of these two first protocols, the intensity of stress provoked by a simple change of equal tanks, normally utilized in facilities, was demonstrated. This also presented the necessity of a minimum of two weeks of habituation in home tank, before conducting any behavioral test. Finally, we realized the evaluation of "Protocol 3" in order to study if a renovation of enriched environment items of complexity within the period of fourteen days would change results already observed. Animals of EE group showed no difference in general behavioral profile even with the insertion of new objects in environment, similarly to that observed in Protocol 2, however a reduction of distance travelled was presented by these animals, reflecting a little of Protocol 1.

In zebrafish, some studies have already demonstrated the induction of neuronal plasticity and increase of neuronal proliferation when animals were submitted to enriched environment protocols. In rodent models, many of effects of environmental enrichment are related to increased adult neurogenesis and dendritic branching, as well as alteration of functionality of some neurotransmitter system was known – like glutamatergic. Thus, second part of this dissertation had as objective the analyses of protein expression of one of the vesicular glutamate transporter (vGluT), the vGluT2, in those three enriched environment protocols cited previously by Western Blotting technique. The vGluTs were expressed in neuronal dendritic regions and more its expression, more density of synaptic vesicles presents in analyzed region. Data obtained demonstrated that the increase of immunocontent of vGluT2 is related with consolidation and habituation of fish to this condition for a longer period in environmental enrichment (Protocol 2).

In this study, there was demonstrated that environmental enrichment alters the adult zebrafish's behavioral response, keeping it more regular than the control group (SE) and, probably, this is due to stimulation of process directed to stress response of novelty as well as of pathways related to adaptability. In addition, we have demonstrated that habituation to the condition by a longer protocol of enriched environment is more important to trigger the increase of vGluT2 protein expression in total brain of adult zebrafish.

LISTA DE ABREVIATURAS

AE: Ambiente enriquecido

AP: Ambiente padrão

B.O.D.: *Biochemical oxygen demand*

dpf: dias pós-fertilização

GABA: Ácido ɣ-amino butírico

NT: Neurotransmissor

PVC: Policloreto de polivinila

SLC17: *Solute carrier 17 family*

SNC: Sistema nervoso central

vAChT: Transportador vesicular de acetilcolina

vGAT: Transportador vesicular de ácido ɣ-amino butírico

vGluT: Transportador vesicular de glutamato

vGluT2: Transportador vesicular de glutamato 2

VNTs: transportadores vesiculares de neurotransmissores

INTRODUÇÃO

1. PEIXE-ZEBRA

1.1. Aspectos gerais do peixe-zebra como modelo experimental

O peixe-zebra (*Danio rerio*) – também conhecido como paulistinha ou, em inglês, *zebrafish* - é um pequeno teleósteo alcançando no máximo 3 a 4 cm de comprimento na fase adulta e caracterizado pelo seu padrão de coloração de listras horizontais escuras e claras. É um peixe de água doce da família *Cyprinidae*, originário do sul da Ásia, descrito pela primeira vez por Francis Hamilton, em 1822 (Spence, Gerlach et al. 2008; Parichy 2015). A partir do estudo de Streisinger e colaboradores em 1981 - com aplicação pioneira do peixe-zebra na área de genética molecular (Streisinger, Walker et al. 1981) -, sua utilização experimental se tornou bastante ampla, alcançando diferentes áreas de estudo como biologia do desenvolvimento, produção de fármacos a teratologia e modelos de câncer (Vascotto, Beckham et al. 1997; Ali, Champagne et al. 2011; Wojciechowska, van Rooijen et al. 2016). Essa expansão é decorrente, principalmente, de algumas características biológicas e fatores favoráveis desta espécie, tais como: embriões translúcidos, rápido desenvolvimento e ciclo biológico, grande prole, baixo custo por animal e susceptibilidade à manipulação genética (Lele and Krone 1996). Ademais, estes fatores possibilitaram para o grande uso do peixe-zebra em pesquisas com a utilização de técnicas como a de Repetições Palindrômicas Curtas Agrupadas e Regularmente Interespaçadas (comumente conhecida como CRISPR/Cas9 - sigla do inglês: *Clustered Regularly Interspaced Short*

Palindromic Repeats - associated protein-9 nuclease) (Hruscha and Schmid 2015), a de morfolinos (Wyatt, Bartoszek et al. 2015) e a de optogenética (Friedrich, Jacobson et al. 2010).

1.2. Peixe-zebra como modelo experimental na área de neurociências

Um dos fatores que caracterizaram o peixe-zebra como promissor modelo translacional em diversas áreas das ciências biomédicas é a sua grande homologia genética com os seres humanos. Para cerca de 70% dos genes humanos há um correspondente em peixe-zebra (Howe, Clark et al. 2013). Na área de neurociências, o peixe-zebra também vem sendo bastante utilizado e proporciona uma nova perspectiva para o entendimento dos mecanismos envolvidos nas patologias e distúrbios que afetam o Sistema Nervoso Central (SNC) (Bandmann and Burton 2010; Stewart, Braubach et al. 2014), tais como: doença de Alzheimer (Newman, Verdile et al. 2011; Newman, Ebrahimie et al. 2014; Nada, Williams et al. 2016), crises epilépticas (Afrikanova, Serruys et al. 2013; Mussulini, Leite et al. 2013), tumores cerebrais (Li, Peng et al. 2010; Hamilton, Astell et al. 2016), esquizofrenia (Morris 2009), doença de Parkinson (Farrell, Cario et al. 2011; Sarath Babu, Murthy Ch et al. 2016), desordens do espectro autista (Kim, He et al. 2014; Stewart, Nguyen et al. 2014), entre outras (Kalueff, Stewart et al. 2014).

Atualmente, diversos testes comportamentais foram desenvolvidos para peixe-zebra com fins semelhantes aos já amplamente utilizados em roedores: *open tank* (Rosemberg, Rico et al. 2011), teste claro/escuro (Serra, Medalha et al. 1999; Maximino, Marques de Brito et al. 2010; Cordova, Dos Santos et al. 2016) e interação social (também conhecido como: preferência social); bem como *shoaling* e *mirror*

biting (Pham, Raymond et al. 2012). Estes testes são baseados em comportamentos intrínsecos do peixe-zebra, como preferência por locais profundos e escuros e nado em cardume. Estes testes permitem avaliar diversos aspectos, tais como comportamento tipo ansiedade e perfil exploratório e locomotor.

Outra característica importante que reforça a utilização do peixe-zebra nesta área de conhecimento é que, assim como em outras espécies de peixes da família *Cyprinidae,* o peixe-zebra apresenta uma extensiva neurogênese na vida adulta (Zupanc, Hinsch et al. 2005). Atualmente já foram descritas 16 regiões encefálicas proliferativas (Grandel, Kaslin et al. 2006). Com isto, o peixe-zebra também é considerado como um bom modelo para o estudo da neurogênese tanto em fase larval quanto na vida adulta. Ademais, neste modelo já foram identificados diversos sistemas de neurotransmissores tais como histaminérgico (Kaslin and Panula 2001), colinérgico (Behra, Cousin et al. 2002), purinérgico (Kucenas, Li et al. 2003), serotoninérgico (Rink and Guo 2004), dopaminérgico (Boehmler, Obrecht-Pflumio et al. 2004), GABAérgico (Kim, Nam et al. 2004) e glutamatérgico (Edwards and Michel 2002). Apesar de alguns destes sistemas citados ainda não terem seus mecanismos celulares e moleculares totalmente elucidados em peixe-zebra, já representa um relevante aspecto para a pesquisa na área biomédica.

2. TRANSPORTADORES VESICULARES DE GLUTAMATO (vGluTs)

2.1. Características gerais dos vGluTs

Os transportadores vesiculares de neurotransmissores (VNTs, sigla do inglês *vesicular neurotransmitter transportes*) são proteínas presentes na membrana de vesículas sinápticas responsáveis pela captação de neurotransmissores (NT) específicos, determinando a quantidade de NT que será liberada na fenda sináptica. Os VNTs são considerados marcadores específicos de células neuronais contendo o respectivo NT. Os tipos de VNTs, normalmente, diferem-se de acordo com o NT que transportam para o interior das vesículas sinápticas, tais como: VGAT, o transportador vesicular de ácido ɣ-amino butírico (GABA); VAChT, transportador vesicular de acetilcolina; vGluT, transportador vesicular de glutamato; entre outros (Van Liefferinge, Massie et al. 2013).

Os transportadores vesiculares de glutamato, também conhecidos pela sigla vGluT, são um tipo de VNTs responsáveis pelo transporte e posterior armazenamento de glutamato para no interior das vesículas sinápticas, o qual, através de exocitose, é liberado posteriormente na fenda sináptica. O transporte vesicular de glutamato possui doze regiões transmembranas preditas e é atrelado a um tipo de bomba de prótons ATPase vacuolar, visto que é necessária a geração de um gradiente eletroquímico como força motriz juntamente com o componente químico de alteração de pH para a captação desse NT. (Ueda 2016; Takeda and Ueda 2017)

As três isoformas de vGluTs (vGluT1, vGluT2 e vGluT3) são pertencentes a família gênica SLC17, juntamente com o transportador vesicular de aminoácidos excitatórios (VEAT) e o transportador vesicular de nucleotídeo (VNUT). As diferentes isoformas de vGluT diferem consideravelmente na sequência de aminoácidos nas

regiões N- e C-terminal, entretanto, divergem pouco na região de domínio central. Os vGluT1 e vGluT2 são co-expressos nos estágios iniciais do desenvolvimento, sendo que primeiramente o vGluT2 é expresso em grande quantidade e, em seguida, transientemente, o vGluT1 assume a maior expressão. Em geral, essas duas isoformas também possuem distribuição complementar no cérebro adulto: vGluT1 predomina no córtex cerebral e hipocampo, enquanto o vGluT2, no diencéfalo e regiões inferiores do tronco encefálico. Já o vGluT3 é conhecido pela presença em terminal de células fotorreceptoras e em células auditivas, bem como pela co-localização com outros tipos de transportadores vesiculares (VGAT e VAChT, por exemplo). (Liguz-Lecznar and Skangiel-Kramska 2007; Van Liefferinge, Massie et al. 2013; Ueda 2016; Takeda and Ueda 2017)

2.2. vGluTs no peixe-zebra

No *Zebrafish Information Network* (ZFIN), já é possível encontrar informações sobre a identificação de sequências gênicas e proteicas de duas isoformas de vGluT em peixe-zebra. Em 2004, Higashijima e colaboradores identificaram três diferentes sequências gênicas, pertencentes à família gênica *slc17*, similares às observadas em mamíferos (Higashijima, Mandel et al. 2004). Uma dessas sequências caracterizadas se apresentou homologas à de vGluT1 descrita em roedores, e as outras duas, à de vGluT2 de roedores. Assim, as isoformas encontradas no peixe-zebra foram nomeadas como: vGluT1, vGluT2.1 (ou vGluT2a) e vGluT2.2 (ou vGluT2b). Entretanto, o que há descrito de localização encefálica na literatura provém deste estudo em fase embrionária e larval do peixe-zebra, demonstrando a presença do vGluT1 em limitadas regiões cerebrais e não é expresso até pelo menos cinco dias de vida. Já tanto vGluT2.1 e

vGluT2.2 são expressas no cérebro e medula espinhal. Em outro estudo, foi demonstrado a presença de vGluT3 em células auditivas da larva do peixe-zebra (Einhorn, Trapani et al. 2012).

3. ENRIQUECIMENTO AMBIENTAL

Alterações ambientais levam a diversas mudanças, as quais vão desde a expressão gênica até alterações em padrões comportamentais (Pryce, Mohammed et al. 2002). Os animais que são utilizados em pesquisa, normalmente, são mantidos em um ambiente padrão de biotério, no qual não há nenhum estímulo adicional a caixa moradia. O enriquecimento ambiental é uma estratégia que visa o fornecimento de estímulos social, sensorial e cognitivo ao animal, proporcionando comportamentos mais próximos ao seu natural e uma melhora do seu bem-estar (Bezzina, Verret et al. 2015). Nos protocolos de ambiente enriquecido descritos para roedores, os animais são colocados em grupo em caixas moradia maiores do que as convencionais de padrão de biotério, utilizando de diversos utensílios, tais como: rodas de correr, labirintos em tubo de plástico e objetos de diferentes cores e tamanhos com suas localizações e configurações alteradas ao longo do protocolo para que os estímulos do ambiente continuem constantes (van Praag, Kempermann et al. 2000; Sztainberg and Chen 2010; Garthe, Roeder et al. 2015).

A exposição de roedores ao enriquecimento ambiental produz diversos efeitos encefálicos tanto a nível molecular quanto a nível celular (Kotloski and Sutula 2015). Além disso, altera o perfil comportamental desses animais em vários testes comportamentais (Birch, McGarry et al. 2013; Grinan-Ferre, Perez-Caceres et al. 2015; Zerwas, Trouche et al. 2015). Trabalhos mostram que o ambiente enriquecido provoca

uma melhora em alguns mecanismos envolvidos na cognição em diferentes modelos animais de doenças neurológicas, tais como epilepsia (Fares, Belmeguenai et al. 2013; Morelli, Ghiglieri et al. 2014; Kotloski and Sutula 2015), doença de Alzheimer (Costa, Cracchiolo et al. 2007; Polito, Chierchia et al. 2014; Ziegler-Waldkirch, P et al. 2018), transtorno do espectro autista (Favre, La Mendola et al. 2015; Yamaguchi, Hara et al. 2017) e doença de Huntington (Mo, Renoir et al. 2015; Kreilaus, Spiro et al. 2016), bem como em distúrbios psiquiátricos (Laviola, Hannan et al. 2008), como esquizofrenia (Burrows, McOmish et al. 2015; Bator, Latusz et al. 2018), e no comportamento tipo ansiedade (Grippo, Ihm et al. 2014; Brenes, Lackinger et al. 2015; Manuel, Gorissen et al. 2015; Ragu Varman and Rajan 2015; Soares, Rorato et al. 2015; Aujnarain, Luo et al. 2018).

Além disso, já foi demonstrado que o protocolo de enriquecimento ambiental aumenta o volume cerebral de camundongos adultos aproximadamente 24 horas após a exposição ao ambiente (Scholz, Allemang-Grand et al. 2015). Uma relação entre o ambiente enriquecido e um aumento na neurogênese já foi demonstrado em roedores (Clemenson, Deng et al. 2015; Clemenson, Lee et al. 2015; Gualtieri, Bregere et al. 2017; Yagishita, Suzuki et al. 2017).

3.1. Enriquecimento ambiental no modelo experimental de peixe-zebra

Como em modelos animais de roedores, o enriquecimento ambiental e seus benefícios já se tornaram também um interesse de estudo no modelo do peixe-zebra. Os protocolos de ambiente enriquecido mais utilizados usam um contexto inanimado com pedras ao fundo do aquário e/ou plantas artificiais, e em grupo que serve como o estímulo social. Entretanto, muitos destes trabalhos possuem uma grande variabilidade nos componentes de enriquecimento, bem como no próprio tempo de permanência dos animais neste tipo de ambiente (von Krogh, Sorensen et al. 2010; Schroeder, Jones et al. 2014; Collymore, Tolwani et al. 2015; Keck, Edgerton et al. 2015; Manuel, Gorissen et al. 2015; DePasquale, Neuberger et al. 2016; Giacomini, Abreu et al. 2016; Marcon, Mocelin et al. 2018).

O estudo do enriquecimento ambiental em peixe-zebra já obteve resultados relacionados à melhora na fertilidade e fecundidade dos animais (Wafer, Jensen et al. 2016), bem como na indução da plasticidade neuronal em cérebro de peixe-zebra por um ambiente socialmente enriquecido (Lindsey and Tropepe 2014), e um aumento na proliferação de neurônios de peixes que permaneceram em um aquário com plantas artificiais e substratos (von Krogh, Sorensen et al. 2010). Ademais, protocolo de ambiente enriquecido com grande complexidade - contendo cano de PVC, pedras e plantas de cores diferenciadas – induziu um aumento do tamanho do telencéfalo dos animais quando comparados com animais controles (DePasquale, Neuberger et al.). O enriquecimento ambiental também se mostrou como uma ótima possibilidade de tratamento complementar ou, até mesmo, alternativo para a redução dos níveis de estresse (Giacomini, Abreu et al. 2016).

OBJETIVO

1. OBJETIVO GERAL

O objetivo geral deste trabalho foi avaliar se o enriquecimento ambiental modula o comportamento e o sistema glutamatérgico em peixe-zebra adulto.

2. OBJETIVOS ESPECÍFICOS

Os objetivos específicos estão apresentados de acordo com os respectivos capítulos:

2.1. Capítulo I

Analisar se diferentes protocolos de enriquecimento ambiental afetam o desempenho do peixe-zebra adulto em testes comportamentais.

2.2. Capítulo II

Avaliar se diferentes protocolos de enriquecimento ambiental alteram o imunoconteúdo de vGluT2 em cérebro de peixe-zebra adulto.

PARTE II

CAPÍTULO I

Artigo em preparação para submissão

Título: *Environmental enrichment affects adult zebrafish (Danio rerio) behavior in the open tank test*

Autores: Thainá Garbino dos Santos, Luca Frangipani Araújo, Ben Hur Marins Mussulini e Diogo Losch de Oliveira.

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Justificativa: Estudos dos efeitos de diferentes protocolos de enriquecimento ambiental em peixe-zebra já vem sendo realizados na literatura. Entretanto, ainda há uma necessidade de descrição do perfil comportamental desses protocolos neste modelo animal.

Objetivo geral: O objetivo deste trabalho foi avaliar o perfil comportamental do peixe-zebra adulto em três diferentes protocolos de enriquecimento ambiental.

Objetivo específico: Avaliar o perfil do comportamento tipo ansiedade e social dos peixes submetidos ao protocolo de enriquecimento ambiental de 7 dias, bem como analisar o perfil locomotor e exploratório dos animais quando expostos aos três protocolos.

TITLE PAGE

RESEARCH PAPER

TITLE

Environmental enrichment affects adult zebrafish (Danio rerio) behavior in the open tank test

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Environmental enrichment affects adult zebrafish (*Danio rerio*) behavior in the open tank test

ABSTRACT

Zebrafish has become a widely used animal model in biomedical research. Diverse studies have used zebrafish to study the effects of enriched environment on behavioral and neurochemical parameters. However, the composition and time duration of environmental enrichment protocols used are very variable. The aim of present study was to investigate the effect of different environmental enrichment protocols on behavioral profile of adult zebrafish. Two hundred and seventy adult wild-type zebrafish from both sexes were used. Animals were submitted to three different environmental enrichment protocols. For Protocol 1, a reduction in the total distance travelled and average speed was observed in EE group when compared to SE group in the open tank test. In Protocol 2, animals of both SE and EE groups did not demonstrate difference in all open tank parameters. In Protocol 3, the EE group reduced the total distance travelled at bottom zone when compared with SE group. These results can bring one question about enriched environments: the change to an environmental enrichment to be more comfortable for animals than the routine change for similar aquariums by the environmental complexity instigates the natural fast adaptability.

KEYWORDS

environmental enrichment; zebrafish; *Danio rerio*; behavior; open tank test; black/white test; social preference test.

1. INTRODUCTION

Zebrafish has become an widely used animal model in biomedical research (Vascotto et al., 1997). It is small tropical freshwater fish (3-4 cm) from *Cyprinidae* family originating from south of Asia(Parichy, 2015; Spence et al., 2008). In addition, zebrafish is known for its particular and favorable characteristics like translucent embryos, great offspring and susceptibility to genetic manipulation. (Lele and Krone, 1996) On the other hand, this animal model is also utilized for neuroscience studies (Sumbre and de Polavieja, 2014) what influenced some authors to describe behavioral tests (Blaser et al., 2010; Maximino et al., 2010; Pham et al., 2012; Rosemberg et al., 2011).

Increasing in the use of zebrafish in several areas of biomedical research has caused the growth of demand of these animals. Major part of these fish request is supplied by diverse local commercial suppliers. At the same time, the emergence of zebrafish facilities around the world brought a trouble about a necessity of standardization for feeding, water conditions, animal maintenance, care and housing protocols by a union of the different research groups worldwide, like a better description of these items in articles. In relation to this, there is also a concern about zebrafish welfare relating to a poor presentation of complexity to animals in tanks and in what is more appropriate for this specific aquatic animal model. Environmental enrichment

appears as important factor by providing a reality closer to the natural habitat reflecting inherent behavior of specie in this context. (Osborne et al., 2016)

Diverse studies present design of enrichment environment for zebrafish, however the composition and time duration are many variables (Collymore et al., 2015; DePasquale et al., 2016; Giacomini et al., 2016; Keck et al., 2015; Manuel et al., 2015; Schroeder et al., 2014). These protocols difference of formation range from quantity, size, composition (artificial or natural) and model (underwater or floating) of plants until the presence or not of gravel or stones and/or sand (real or picture placed on the tank bottom), as well as have or not social stimulus. Furthermore, the residence time of fish in these designs vary according to several factors since study of the enriched environment's influence during growth of animals (Manuel et al., 2015) until minimum time required for pharmacological treatment used concomitantly or for determined analyses (Giacomini et al., 2016), for example.

Large part of these works focus on the behavior evaluations in adult zebrafish, meanwhile any of them deliberate about pure ethology over this enriched condition and daily condition used in most facilities. For this reason, the aim of this present study is to observe the baseline behavioral response of adult zebrafish submitted to environmental enrichment protocols when compared with animals in normally conditions used, making an analysis of behavioral profile, against novelty.

2. METHODS

2.1. MATERIAL

Artemia nauplii was purchased from Artêmia Salina do RN, Brazil, and commercial flake fish food from Alcon (BASIC®), Brazil. Methylene blue was purchased from Dinâmica® Química Contemporânea Ltda., Brazil. Instant Ocean® Sea Salt was purchased from Pentair Aquatic Eco-Systems Inc. (PAES), USA. Artificial green plants were purchased from Vigo Ar (those with 25 cm of height), Global Flex, Brazil, and from Soma Aquatic Plants Economy (those with 10 cm of height), China. Other chemicals and stones were purchased from local commercial suppliers.

2.2. ETHICAL NOTE

All experimental procedures were performed accordingly Brazilian's Law for Care and Use of Laboratory Animals (Law 11794/2008) and were previously approved by the Research Ethical Committee for Animal Care and Use of Universidade Federal do Rio Grande do Sul (protocol number: #31576). All experimental designs were developed to minimize the number of fish used and its discomfort or suffering.

2.3. ANIMALS

Two hundred and seventy adult (8 months old) wild-type zebrafish (*Danio rerio*) from both sexes were used. All fish were obtained by local breeding through pair-wise crossings and kept at 14h/10h dark/light cycle. Embryos were maintained at embryo

medium (NaCl 5.03 mM, KCl 0.17 mM, $CaCl_2$ 0.33 mM, $MgSO_4$ 0.33 mM, pH 7.5±0.5, 28±1° C) and housed in Biochemical Oxygen Demand (B.O.D.) incubator (28±1° C) at a density of 1 animal/2,5 mL. At 5 days post fertilization (dpf), larval zebrafish were fed with *Paramecium* infusoria *ad libitum*, which was gradually replaced by *Artemia* nauplii at 13 dpf. Young fish (21 dpf) were transferred to a recirculating housing system and kept at following conditions:28±1° C, pH and of 7.5±0.5, conductivity of 500 µS/cm, and density of 3 animals/L. Adult animals were fed four times a day with *Artemia* nauplii (09:00 a.m. and 05:00 p.m.) and commercial flake fish food (11:00 a.m. and 03:00 p.m.) at 4-5% of the total biomass. All animals were experimentally naive, healthy and free of any signs of disease.

2.4. ENVIRONMENTAL ENRICHMENT

Environmental enrichment was performed inside of recirculating housing system tanks with the same water quality parameters described above. Animals were divided into two major groups as follow: Standard Environment (SE), which consisted in an empty tank with only recirculation water; and Enriched Environment (EE), which consisted into aquarium with stones at the bottom of the tank and three artificial green plants with 25 cm of height (see supplementary Fig. 1 for more details). Three different environmental enrichment protocols were tested.

2.4.1. Protocol 1

In protocol 1 animals were exposed to the enriched environment by 7 consecutive days. Control groups were exposed to the empty tank for the same period. This protocol was referred throughout the text as “SE or EE 7 days”.

2.4.2. Protocol 2

The protocol 2 was similar to Protocol 1, except for the exposition to the enriched environment that was 14 days. Control groups were exposed to the empty tank for the same period. This protocol was named “SE or EE 14 days”.

2.4.3. Protocol 3

Protocol 3 lasted 14 days, which were divided in two equal segments of 7 days. In the first segment, compositions of the tanks for both control and treated groups were equal to the description for Protocol 1. On the eighth day, animals were moved to the new other tanks. For control group, these new tanks were empty. For treated group, new tanks had stones at bottom, one brown cylindrical PolyVinyl Chloride (PVC) pipe (25 mm of diameter), one brown PVC pipe T format (25 mm of diameter) and four distinct green artificial plants with 10 cm of height. This protocol was named “SE or EE 7+7 days”.

2.5. BEHAVIORAL PROCEDURES

One hour before beginning of behavioral tests, all animals were fed with commercial flake fish food (4-5% of biomass). After 30 min, both SE and EE groups were removed from housing tanks and placed into 8 L tanks (each one free of any substrate or plants). This procedure was performed in order to reduce the putative novelty-induced stress caused by the removal of animals from their housing tanks and placement them directly in behavioral testing tanks. All animals were allowed to habituate for 30 min in experimental room. Only 10 animals of each tank were tested,

remaining always 3 fish in tank at the end of the test to avoid stress responses resulting from animal isolation. In all behavioral tests, animals were individually introduced in the center of the apparatus and each one was used for a single testing. Behavioral procedures were conducted between 9:00 a.m. and 12:00 p.m. and were analyzed by ANY-maze® video-tracking system (Stoelting CO, EUA).

2.5.1. Open tank test

Open tank test were performed accordingly to Rosemberg *et al* (2011). Briefly, a trapezoidal apparatus with dimensions of 28 x 23 x 16 x 7 cm (top length x bottom length x height x width) filled with 1.5 L with water was used. Fish were observed and video-tracked during 360 s. Light intensity above the apparatus was 210-215 lux. Apparatus was virtually divided in three major areas identified as top, middle and bottom. *N* was 15-17 per group.

2.5.2. Black/white test

Black/white test were performed accordingly to Córdova *et al.* (Cordova et al., 2016). Briefly, apparatus consisted of a tank (30 x 10 x 15 cm; length x width x height) divided in two equal compartments (one covered white and other covered black). The water column depth was 4 cm and light intensity inside of apparatus was 210-215 lux (for both compartments). Fish were observed and video-tracked during 360 s. *N* was 16-20 animals/group.

2.5.3. Social preference test

Social preference test was adapted from Pham *et al.* (Pham et al., 2012). Each testing fish was placed in the central of the experimental tank (28,9 x 10 x 15cm; length x width x height). On one side of the experimental tank, an empty tank was placed (named opposite tank) with dimensions of 10 x 10 x 15 cm (length x width x height); on the other side, a tank of identical size held 3 zebrafish (named conspecific tank). To quantify fish preference between conspecific tank in detriment of the apposed one, the experimental fish tank was divided in three equal zones. Conspecific zone corresponded to the segment closer to the conspecific tank; opposite zone corresponded to the segment closer to the opposed tank; and center zone corresponded to the zone between conspecific and opposite zones. Fish were observed and video-tracked during 360 s and total amount of time spent in each zone was measured. Water column was 6 cm for all tanks. Light intensity was 210-215 lux above the apparatus. N = 19-20 animals/group.

2.6. STATISTICAL ANALYSES

All data were expressed as mean+S.E.M. Time spent in each zone of social preference test was analyzed by two-way ANOVA followed by Tukey *post hoc* test. Time spent in white compartment of black/white test was analyzed by one-sample t test. All other data were analyzed by *Student* t test for unpaired sample. Distance travelled across time was expressed as mean$\pm$S.E.M. and analyzed by ANOVA of repeated measures followed by Tukey *post hoc* test. $P < 0.05$ was considered significant.

3. RESULTS

3.1. PROTOCOL 1

In open tank test, animals of EE group presented no changes in the total time mobile when compared to SE group ($t31= 0.3878$, $P=0.7008$). However, a reduction in the total distance travelled and average speed was observed in EE group when compared to SE group ($t31= 2.586$, $P=0.0146$; $t31= 3.137$, $P=0.0037$; respectively; Fig. 1A). Decrease in total distance travelled of EE animals occurred at the bottom of the apparatus, since EE group displayed a reduction in total distance travelled at bottom zone ($t31= 3.274$, $P=0.0026$; Fig. 1B) and presented in the last two minutes of testing (5 min: $P =0.0036$; 6 min: $P =0.0068$; Fig. 6). For all others open tank parameters, there were no differences between EE and SE groups (Fig. 1B).

Since several works have been reported that distance travelled in bottom of the open tank test is an index of anxiety in zebrafish, we performed black/white test to verify if EE animals could present low levels of anxiety. Animals from both groups did not display preference for black or white compartments (SE: $t15 = 1.129$, $P=0.2766$; EE: $t19 = 2.016$, $P= 0.0581$; Fig. 2A). In addition, there was no difference between groups in number of entries in black compartment ($t34 = 1.129$, $P=0.2670$; Fig. 2B).

In social preference test, SE and EE did not show any alterations in social preference. Both groups presented similar time spent in each zone of apparatus ($F2,111 = 286.6$, $P<0.0001$; Fig. 3A). On the other hand, like those observed in the open tank, EE group presented a reduced total distance travelled ($t37 = 2.332$, $P=0.0252$) and lower average speed ($t37 = 2.802$, $P=0.0080$) on apparatus when compared to SE group

(Fig. 3B). There was no difference in time mobile between groups ($t37 = 0.2920$, $P=0.7704$).

3.2. PROTOCOL 2

Animals of both SE and EE groups did not demonstrate difference in total distance travelled, time mobile and average speed in open tank test (respectively: $t29 = 1.255$, $P=0.2196$; $t29 = 0.1890$, $P=0.8514$; $t29 = 1.157$, $P=0.2566$; Fig. 4). Also, there were no changes in all parameters of open tank zones (Fig. 5) and on data across time analyzed (Fig. 6).

3.3. PROTOCOL 3

SE and EE groups did not present difference in the total distance travelled, time mobile or average speed in open tank test (respectively: $t29 = 1.521$, $P=0.1390$; $t29 = 0.3740$, $P=0.7111$; $t29 = 1.589$, $P=0.1230$; Fig. 4). There were no changes in any parameters in the top zone analyzed and, in the middle zone of apparatus, animals of EE group only spent more time ($t29 = 2.081$, $P=0.0463$; Fig. 5). However, similar to observed in Protocol 1, EE group reduced the total distance travelled at bottom zone when compared with SE group ($t29 = 2.171$; $P=0.03882$; Fig. 5). This reduction was in the first minute of test ($P=0.0097$; Fig. 6).

4. DISCUSSION

Large variation in the composition elements of environmental enrichment designs for adult zebrafish exists in the literature. However, the majority of these studies contain an inanimate context with stones at tank bottom with a shoal (as social stimulus) and/or artificial plants. In this way, we elaborate the three protocols described above utilizing these most common items in its basic formation: stones, shoal and artificial plants (with addition of other complex substrate in second fragment of Protocol 3, like described more detailed above).

In order to analyze if a shorter period of maintenance of animals into environmental enrichment was enough to observe some difference on behavioral parameters with SE group, the first protocol proposed in this study was designed. Fish kept during one week of enriched environment presented a differential locomotor profile characterized by lower speed and decreased total distance travelled in open tank test compared to SE group. Our results also demonstrated that this alteration in general characteristics of locomotion was reflected in reduction in distance travelled on the apparatus bottom area in the last fifth and sixth minutes of this behavioral procedure. In addition, the fifth minute presented change in the maximum speed. Figure 6 (line 7 days) evidence that these alterations observed of EE group were due to instability on SE group which increase its distance travelled on bottom area in last two minutes of the test, while animals of EE group maintained constant profile in all parameters analyzed during whole time. Zebrafish is known to live naturally in a complex place with various substrates and high changes in local temperature (Spence et al., 2008), for example. The complexity of environment where this specie remains can overcome its natural

adaptability to diverse expositions a new condition – like a behavioral apparatus – and these animals responds better to the stress related with novelty than those of SE group.

Previous studies demonstrated that zebrafish has preference for dark environments (Maximino et al., 2010; Serra et al., 1999). This characteristic, named scottotaxis, was purposed as an index of anxiety in this model. Other articles related alterations in anxiety of rodents which stayed for a time on enriched environment (Brenes et al., 2015; Grippo et al., 2014; Ragu Varman and Rajan, 2015; Soares et al., 2015). Despite of it, we did not observe any preference of both groups in white/black test. Zebrafish is also described as a social animal (Spence et al., 2008), however when we evaluated its social preference, the EE group presented a social profile but not different from SE group. Meanwhile, enriched environment demonstrated alters the locomotor profile of animals by a lower speed and decrease on total distance travelled in social preference test. This is the first time that results of both open tank and social preference tests present same locomotor profile of animals submitted in some condition - in this case, to environmental enrichment protocol - when compared with control group. Hereupon, all results of protocol 1 demonstrated that environmental enrichment presents its own behavioral profile, probably based on natural mechanism triggered by a complexity of environment.

To verify whether this different locomotor profile would be maintained for a longer period of duration (14 days), we designed the protocol 2 and realized only the open tank test. Although we did not observe differences in all parameters analyzed, both groups present lower brute values when compared to protocol 1. In addition, the SE group demonstrated that this duration of protocol is enough to animals of this group present constant distance travelled at bottom tank over testing time, unlike that seen in the results of protocol 1. As the results of this protocol did not demonstrate the same

behavioral profile observed in the procotol 1, like it was hypothesized, we evaluated if a renovation of elements of enriched environment over longer time duration could present it. In this way, we design the Protocol 3 with duration of fourteen days, however with one change of standard / enriched environment when one week of protocol completed.

However, our results demonstrated an association between data of both protocols already observed. EE did not present difference in locomotor profile by a reduction in total distance travelled - like in protocol 2 -, but presented distance reduced at apparatus bottom - such as in protocol 1. This can suggest that the presentation to a more complex system during first 7 days, made the animals more adapted to new complex substrates on enriched environment - fish responding to the test of a third manner.

5. CONCLUSIONS

These results can bring one question to discussion: the stress caused in animals by routine exchange for different aquariums despite these being similar. Results of SE group in protocol 1 demonstrated instability over test duration when, after 14 days in the same aquarium (Protocol 2), animals of this group presented more stability of parameters analyzed. Maybe, the change to an environmental enrichment be more comfortable for animals than the routine change for similar aquariums by the environmental complexity instigates the natural fast adaptability.

6. ABBREVIATIONS

B.O.D., biochemical oxygen demand; dpf, days post fertilization; EE, enriched environment; PVC, polyvinyl chloride; SE, standard environment.

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8. DISCLOSURES

The authors of this paper have no conflict of interest.

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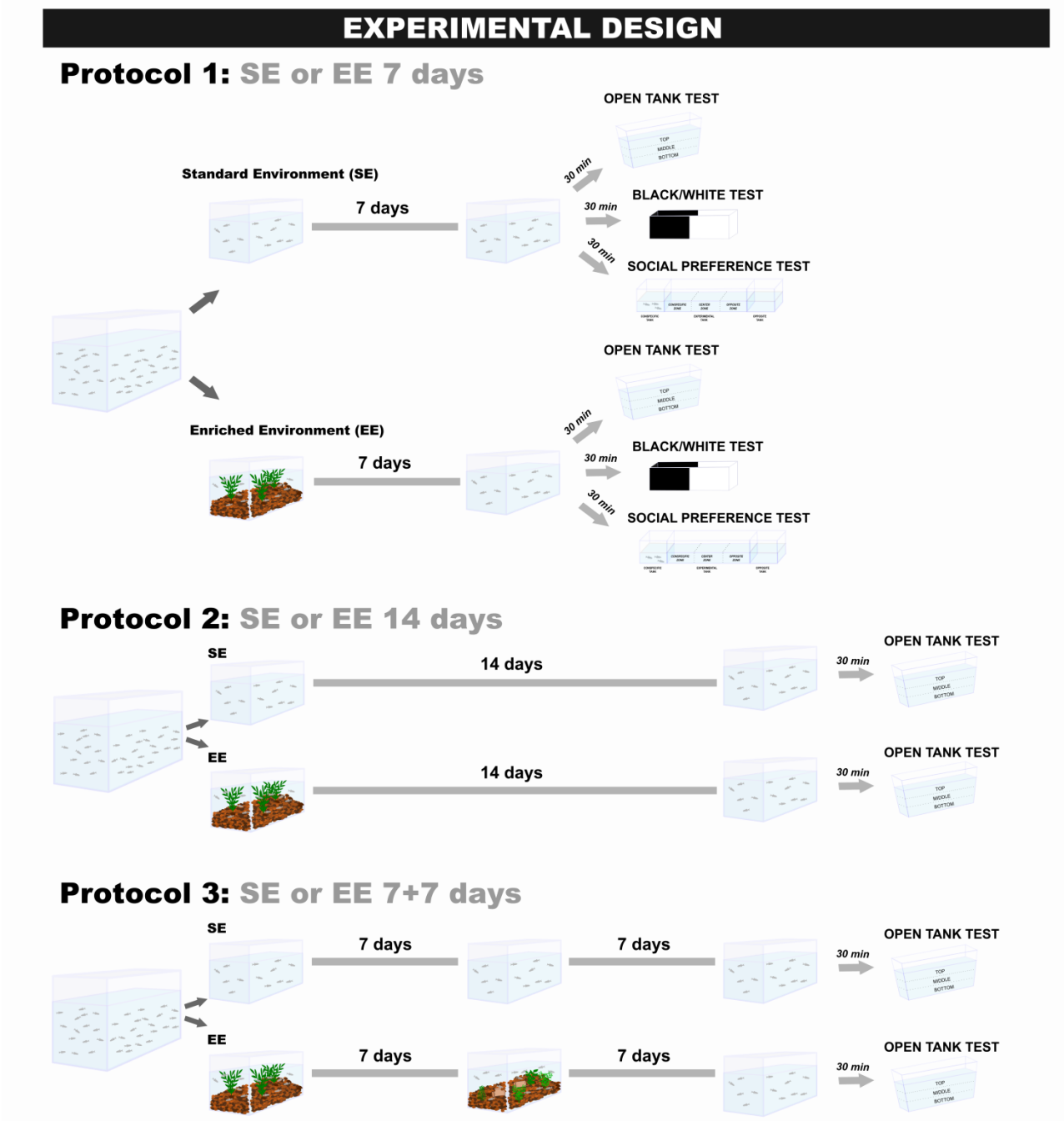
10. FIGURE AND LEGENDS



Supplementary figure 1. Experimental design of 3 environmental enrichment protocols for adult zebrafish used in this study. Protocol 1: Standard Environment (SE), which consisted in an empty tank with only recirculation water; and Enriched Environment (EE), which consisted into aquarium with stones at the bottom of the tank and three artificial green plants (25 cm of height) during 7 days. Protocol 2: similar to

Protocol 1, but with 14 days of duration. Protocol 3: divided in two segments of 7 days; first segment equal to Protocol 1; in the begging of second segment, animals were moved to the new other tanks: for SE group, new tanks were empty, and for EE group, new tanks had stones at bottom, one brown cylindrical PolyVinyl Chloride (PVC) pipe (25 mm of diameter), one brown PVC pipe T format (25 mm of diameter) and four distinct green artificial plants with 10 cm of height. Thirty minutes before the begging of behavioral tests, all groups were removed from housing tanks and placed into 8 L tanks (each one free of any substrate or plants). For protocol 1, open tank, black/white test and social preference test were performed. For Protocol 2 and Protocol 3, only open tank test was realized.

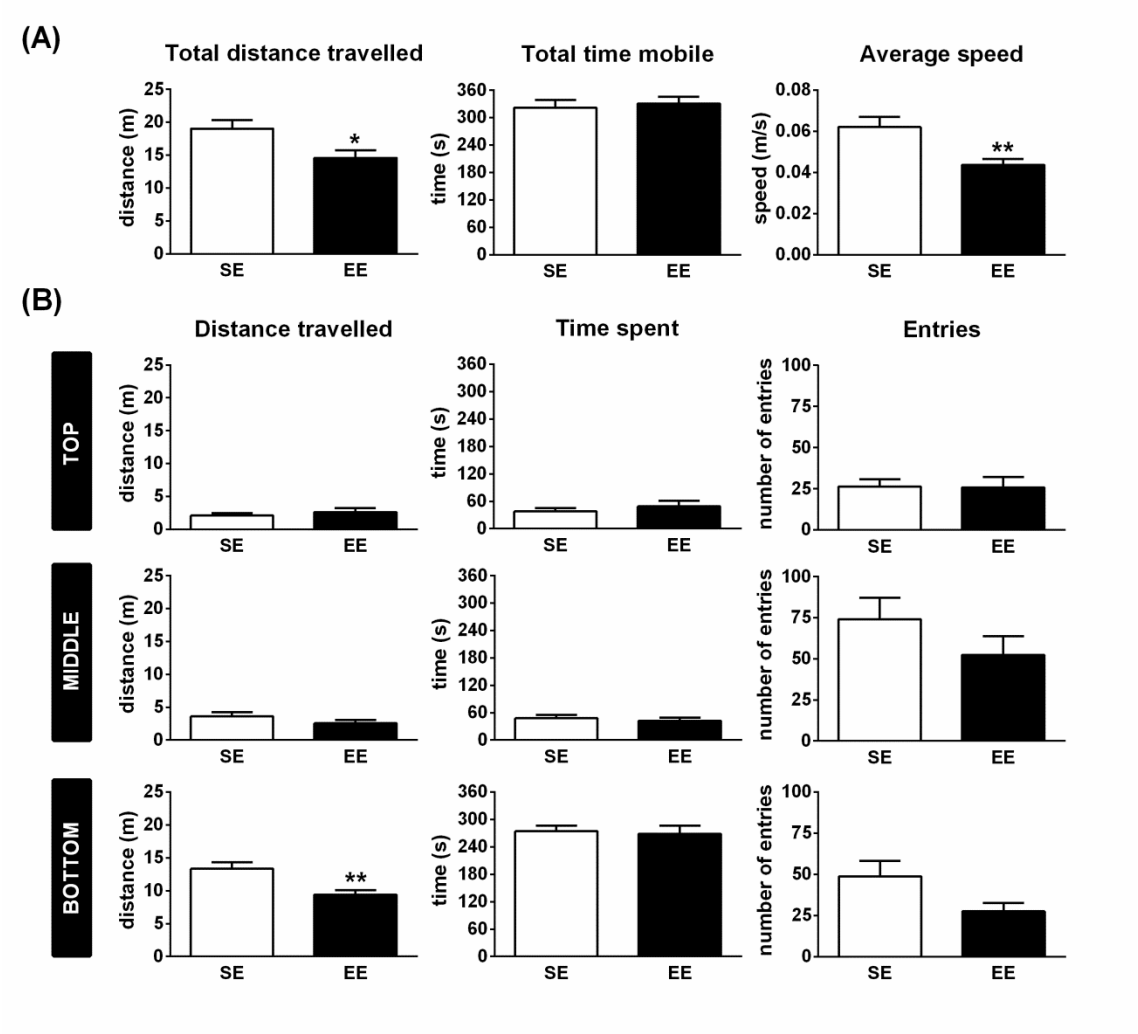


Figure 1. Locomotor and exploratory profile of SE and EE 7 days groups in open tank test. (A) Total distance travelled (m), total time mobile (s) and average speed (m/s) presented by both groups during whole testing. **(B)** Distance travelled, time spent and number of entries in three different apparatus zones: top, middle and bottom. Data were expressed as mean±S.E.M. and analyzed by *Student* t test for unpaired sample. * $p< 0.05$; ** $p < 0.001$.

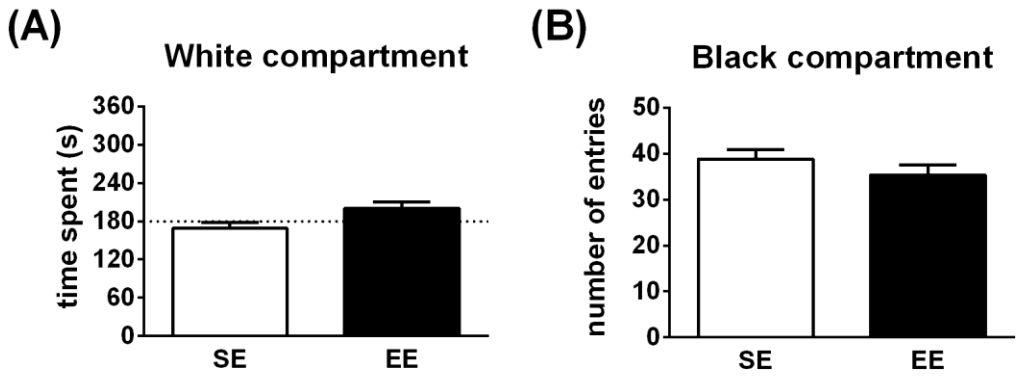


Figure 2. Evaluation of anxiety-like behavior of SE and EE 7 days groups in black/white test. (A) Time spent in white compartment (s). **(B)** Number of entries in black and white compartments of the apparatus. Data were expressed as mean±S.E.M. and in (A) analyzed by One-Sample t test and those in (B) by *Student* t test for unpaired sample.

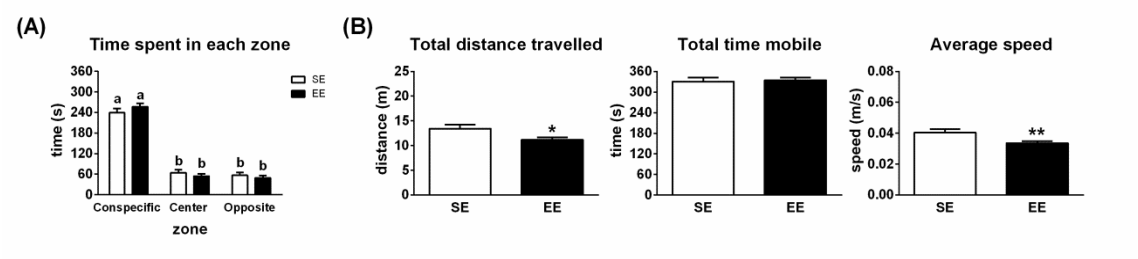


Figure 3. Social interaction profile of SE and EE 7 days groups in social preference test. (A) Time spent (s) in conspecific, center and opposite zones of experimental apparatus. **(B)** Representation of locomotor parameters: total distance travelled (m), total time mobile (s) and average speed (m/s) in the behavioral test. Data were expressed as mean±S.E.M. and in (A) analyzed by two-was ANOVA followed by Tukey *post hoc* test and in (B) by *Student* t test for unpaired sample. * $p < 0.05$; ** $p < 0.001$.

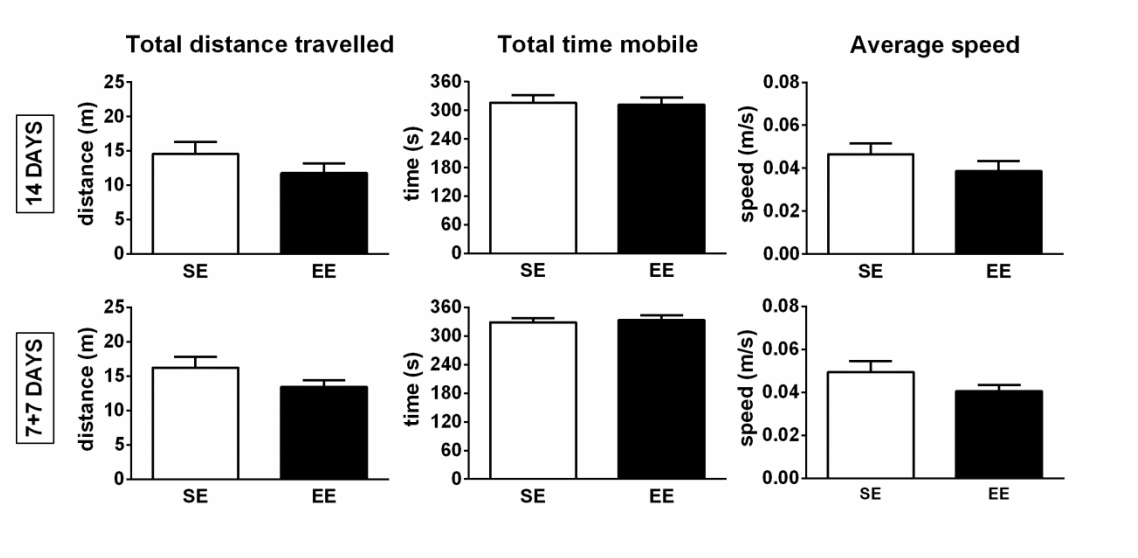


Figure 4. Locomotor profile of animals submitted in the Protocol 2 (SE and EE 14 days) and the Protocol 3 (SE and EE 7+7 days) in open tank test. Total distance travelled (m), total time mobile (s) and average speed (m/s) presented by groups during whole testing. Data were expressed as mean±S.E.M. and analyzed by *Student* t test for unpaired sample.

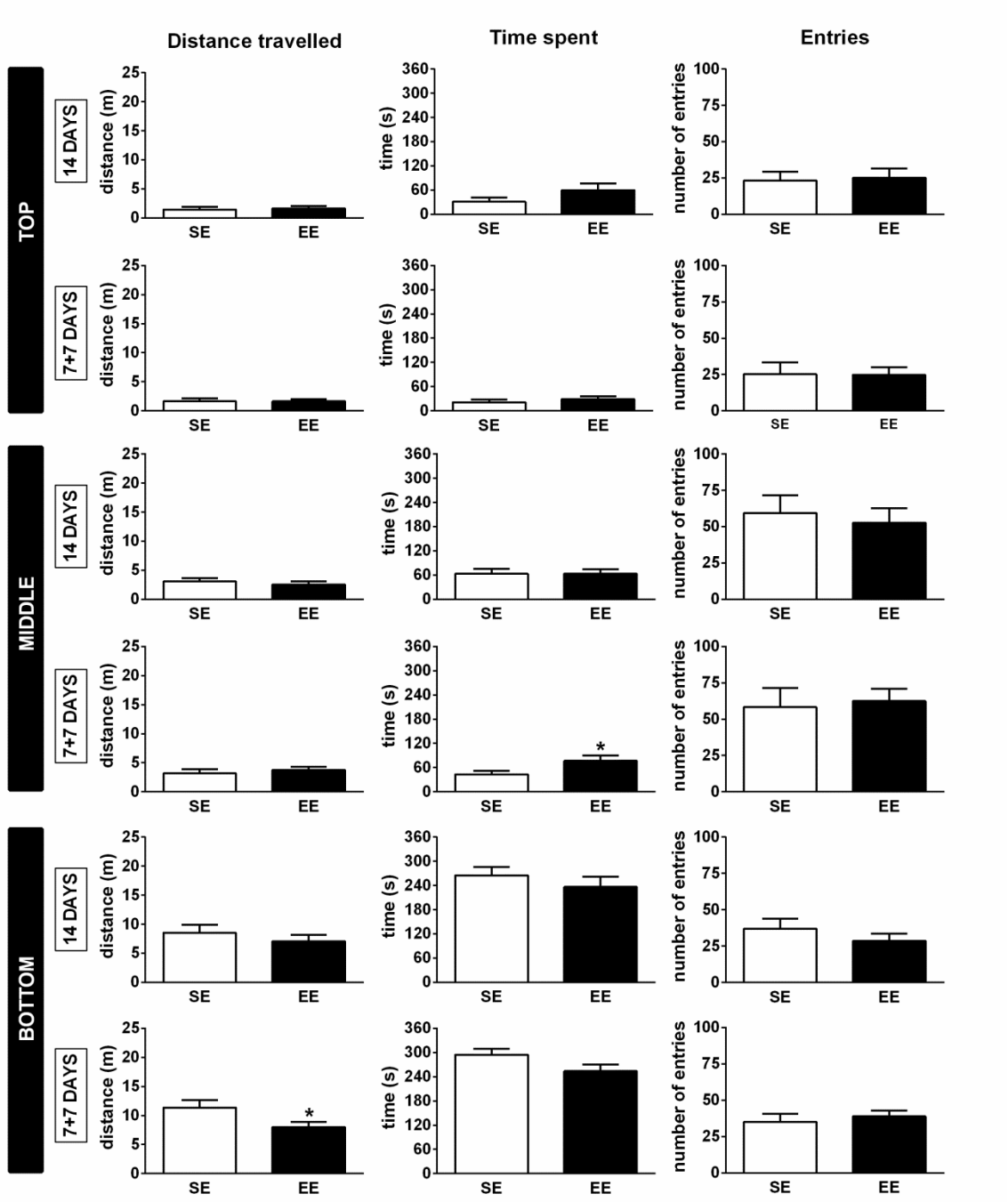


Figure 5. Locomotor and exploratory profile of SE and EE 14 days and 7+7 days groups in three zones of open tank test. Distance travelled, time spent and number of entries in three different apparatus zones: top, middle and bottom. Data were expressed as mean±S.E.M. and analyzed by *Student* t test for unpaired sample. * $p < 0.05$.

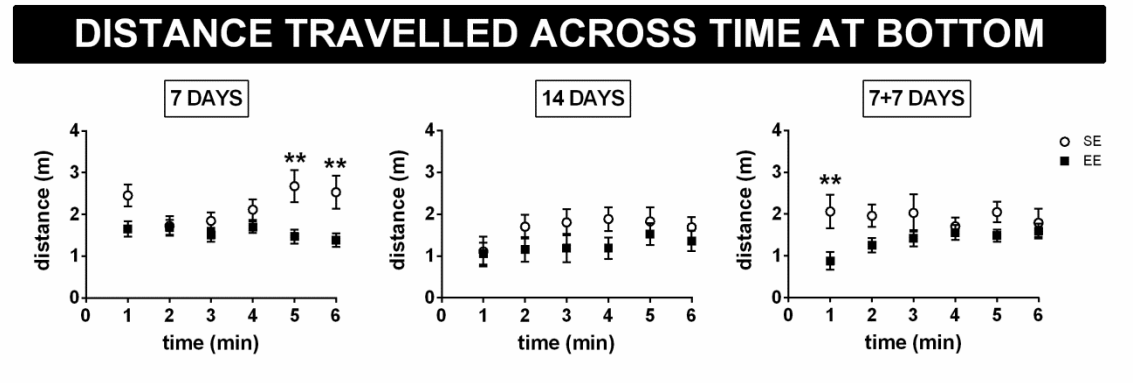


Figure 6. Analyses across time at bottom apparatus of three protocols used in the open tank test. Distance travelled (m) at bottom zone per minute of behavioral testing. Data were expressed as mean±S.E.M. and analyzed by ANOVA of repeated measures followed by Tukey *post hoc* test. ** $p < 0.001$.

CAPÍTULO II

Artigo em preparação para submissão

Título: *Adaptation time in environmental enrichment and possible relation with dendritic branching through immunocontent of vGluT in adult zebrafish*

Autores: Thainá Garbino dos Santos e Diogo Losch de Oliveira.

Revista: *Neuroscience Letters*

Fator de impacto: 2,180

Qualis-CAPES-CBII: B2

Justificativa: Na literatura, ainda não há a descrição sobre arborização dendrítica e como o sistema glutamatérgico respondem a diferenças ambientais no cérebro do peixe-zebra adulto.

Objetivo geral: Objetivo deste trabalho foi relacionar o vGluT2 com o aumento de ramificação dendrítica nos neurônios no cérebro de peixe-zebra adulto.

Objetivo específico: Analisar o aumento de imunoconteúdo de vGluT2 em amostras de cérebro de peixe-zebra adulto e induzir o aumento de ramificação dendrítica estimuladas por diferentes protocolos de enriquecimento ambiental.

TITLE PAGE

RESEARCH ARTICLE

TITLE

Adaptation time in environmental enrichment and possible relation with dendritic branching through immunocontent of vGluT in adult zebrafish

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TITLE

Adaptation time in environmental enrichment and possible relation with dendritic branching through immunocontent of vGluT in adult zebrafish

ABSTRACT

Environmental enrichment protocols generate increased neuronal proliferation and plasticity in zebrafish. However, a little is known about possible brain process and mechanisms involved in the environmental enrichment protocols used in adult zebrafish, besides these already cited. Considering that vesicular glutamate transporter (vGluT) is a component located in dendritic regions, we analyzed whether there are more synaptic connection in animals which stayed enriched environment in three different protocols. vGluT2 immunocontent on total brain samples was determined by Western Blotting. Sixty-five adult (8 months old) wild-type zebrafish (*Danio rerio*) from both sexes were used. Results showed that only in Protocol 2, which fish stayed for 14 days in environment enriched without changes during protocol time duration, the animals in this condition demonstrated an increase of vGluT2 band intensity (P = 0.0332) when compared with SE group. These data demonstrated that longer adaptation time (fourteen days) in the same enriched environment is necessary for increasing of vGluT2 immunocontent, possibly indicating an enhanced dendritic branching in adult zebrafish.

KEYWORDS

Zebrafish; Environmental enrichment; Vesicular glutamate transporter; Glutamate; Western blotting.

ABBREVIATIONS

B.O.D., Biochemical Oxygen Demand; dpf, days post fertilization; EE, enriched environment; PVC, PolyVinyl Chloride; SE, standard environment; vGluT, vesicular glutamate transporter.

1. INTRODUCTION

Many studies about environmental enrichment in animal experimental models has been published lately. This increased interest is due to the search for better understanding of mechanisms and pathways involved of this condition. In rodent models, environments enriched have been demonstrated that ambience which offers social, sensory and cognitive stimulus for animals [2], also bringing cognitive improvements observed in several disease experimental models [1, 6, 7, 13, 15]. These benefits have been related to the increased plasticity, neuronal proliferation and dendritic branching provided by environmental enrichment incentive.

Some of these features noted in rodent have also been demonstrated in zebrafish (*Danio rerio*) experimental model. It is known that a social enriched environment induces neuronal plasticity in zebrafish brain [8]. Besides this, an environment with artificial plants and substrates at tank bottom also increased neuronal proliferation when compared with fish which stayed in tank without any stimulus [12]. However, in this

context, the studies with environmental enrichment and zebrafish are limited to evaluate the proliferative rate of new neuronal cells. Until present moment, there is nothing indicating about synaptic connections. At the same time, several neurotransmitter systems already have been identified in a zebrafish. Among them, the glutamatergic system with its ionotropic and metabotropic receptors, excitatory amino acid transporters (EAAT) and vesicular glutamate transporter (vGluT) [5, 10].

Vesicular glutamate transporter (vGluT) is a brain-specific isoform of sodium-dependent inorganic phosphate transporter and has three isoforms already described - named as vGluT1, vGluT2 and vGluT3 in mammal (for review see [11]) and, respectively, vGluT1, vGluT2.1/vGluT2.2, and vGluT3 in zebrafish [3, 5]. This type of glutamate transporter is presenting in membrane of synaptic vesicles, being responsible for glutamate accumulating into these vesicles, and the vesicular glutamate uptake is driven by a difference of membrane potential in (positive) and outside of vesicles generate by a vacuolar-type H^+ ATPase. Therefore, vGluTs are present in terminal nerves participating of glutamatergic synaptic connections being an important component of this process.

A little is known about possible brain process and mechanisms involved with environmental enrichment protocols used in adult zebrafish, besides those already mentioned. Basing in rodent studies, which demonstrated that has increased dendritic arbor and considering that vGluT as a component presenting in this neuronal region, we would analyze if there are more synaptic connection in fish which stayed enriched environment. Aiming to understand more details about it, which can occur due to the period time of duration and renovation of composition elements of enriched environment in this animal model, we evaluated the vGluT immunocontent on zebrafish brain samples.

2. MATERIAL AND METHODS

2.1. MATERIAL

Artemia nauplii was purchased from Artêmia Salina do RN, Brazil, and commercial flake fish food alconBASIC® from Alcon, Brazil. Methylene blue was purchased from Dinâmica® Química Contemporânea Ltda., Brazil. Instant Ocean® Sea Salt was purchased from Pentair Aquatic Eco-Systems Inc. (PAES), USA. Artificial green plants of 25 cm of height were purchased from Vigo Ar, Global Flex, Brazil, and those of 10 cm of height from Soma Aquatic Plants Economy, China. Rabbit polyclonal antibody anti-VGLUT2 (HY-19) IgG fraction (catalog number: V 2514) was purchased from Sigma-Aldrich® Inc., USA. Mouse monoclonal antibody anti-β-actin (ACTBD11B7) IgG_1 fraction (catalog number: sc-81178) was purchased from Santa Cruz Biotechnology Inc., USA. Secondary antibodies polyclonal goat anti-mouse immunoglobulins/horseradish peroxidase-conjugated (HRP) and polyclonal goat anti-rabbit immunoglobulins/HRP were purchased from Dako, Agilent Technologies, USA. Precision Plus Protein™ Standards Dual Color (catalog number: #161-0374) was purchased from Bio-Rad Laboratories Inc., USA. SuperSignal® West Pico Chemiluminescent Substrate was purchased from Thermo Fisher Scientific Inc., USA. Amersham™ Hybond ECL Nitrocellulose Blotting Membrane 0.45 μm was purchased GE Healthcare Life Science, USA. All other western blot assay reagents were purchased from Sigma-Aldrich® Inc., USA, and other general chemicals were purchased from local commercial suppliers.

2.2. ETHICAL NOTE

All experimental procedures were performed accordingly Brazilian's Law for Care and Use of Laboratory Animals (Law 11794/2008) and were previously approved by the Research Ethical Committee for Animal Care and Use of Universidade Federal do Rio Grande do Sul (protocol number: #31576). All experimental designs were developed to minimize the number of fish used and its discomfort or suffering.

2.3. ANIMALS

Sixty-five adult (8 months old) wild-type zebrafish (*Danio rerio*) from both sexes were used. All fish were obtained by local breeding through pair-wise crossings and kept at 14h/10h dark/light cycle. Embryos were maintained at embryo medium (NaCl 5.03 mM, KCl 0.17 mM, $CaCl_2$ 0.33 mM, $MgSO_4$ 0.33 mM, pH 7.5+0.5, 28+1° C) and housed in Biochemical Oxygen Demand (B.O.D.) incubator (28+1° C) at a density of 1 animal/2,5 mL. At 5 days post fertilization (dpf), larval zebrafish were fed with Paramecium infusoria ad libitum, which was gradually replaced by *Artemia nauplii* at 13 dpf. Young fish (21 dpf) were transferred to a recirculating housing system and kept at following conditions: 28+1° C, pH and of 7.5+0.5, conductivity of 500 µS/cm, and density of 3 animals/L. Adult animals were fed four times a day with *Artemia nauplii* (09:00 a.m. and 05:00 p.m.) and commercial flake fish food (11:00 a.m. and 03:00 p.m.) at 4-5 % of the total biomass. All animals were experimentally naive, healthy and free of any signs of disease. N = 8-9 for each one of the groups was used in this study.

2.4. ENVIRONMENTAL ENRICHMENT

Environmental enrichment was performed inside of recirculating housing system tanks with the same water quality parameters described above. Animals were divided into two major groups as follow: Standard Environment (SE), which consisted in an empty tank with only recirculation water; and Enriched Environment (EE), which consisted into aquarium with stones at the bottom of the tank and three artificial green plants with 25 cm of height (see supplementary Fig. 1 for more details). Three different environmental enrichment protocols were tested.

2.4.1. Protocol 1

In protocol 1, animals were exposed to the enriched environment by 7 consecutive days. Control groups were exposed to the empty tank for the same period. This protocol was referred throughout the text as “SE or EE 7 days”.

2.4.2. Protocol 2

The protocol 2 was similar to Protocol 1, except for the exposition to the enriched environment that was 14 days. Control groups were exposed to the empty tank for the same period. This protocol was named “SE or EE 14 days”.

2.4.3. Protocol 3

Protocol 3 lasted 14 days, which were divided in two equal segments of 7 days. In the first segment, compositions of the tanks for both control and treated groups were equal to the description for Protocol 1. On the eighth day, animals were moved to the new other tanks. For control group, these new tanks were empty. For treated group, new tanks had stones at bottom, one brown cylindrical PolyVinyl Chloride (PVC) pipe (25 mm of diameter), one brown PVC pipe T format (25 mm of diameter) and four distinct green artificial plants with 10 cm of height. This protocol was named “SE or EE 7+7 days”.

2.5. WESTERN BLOTTING

Animals were decapitated and its total brains (with olfactory bulb, telencephalon, optic tectum and cerebellum) were dissected. Brain samples were homogenized in lysis buffer (5 mM Tris base, 1 mM EDTA, 0.1 % SDS and protease inhibitor cocktail; pH 7.4), heated at 95° C for 10 min, and protein content was normalized to 2 μg/μL according adaptation of protein assay method of Lowry modified by Peterson [9] described by Zenki *et al* (2014) [14]. Samples were diluted 1:1 in sample buffer (0.01 g % Bromophenol Blue, 60 mM Tris base, 20 % glycerol, 2 % SDS and 5 % 2-β-mercaptoethanol; pH 6.8). 20μg protein quantity was pipetted for each sample, with final protein concentration 1 μg/μL, and it was resolved by 8 % SDS-

PAGE (Mini- PROTEAN® Tetra System; Bio-Rad Laboratories Inc., USA). Proteins were electro transferred to nitrocellulose membranes using semi-dry transfer apparatus (Trans-Blot® SD Semi-Dry Transfer Cell; Bio-Rad Laboratories Inc., USA). After 1 h of incubation in blocking solution containing powered 3% bovine serum albumin (for anti-VGLUT2) or 5 % milk (for anti-β-actin) and 0.1 % Tween-20 in Tris-buffered saline (TBS; 10 mM Tris base and 30 mM NaCl; pH 7.4), membranes were incubated with primary antibody anti- vGluT2 (HY-19) IgG (1:3,000) at 4° C overnight or with anti-β-actin (1:3,000) at 4° C for 2 h. Membranes were exposed to secondary antibody HRP goat anti-rabbit diluted 1:1,000 or goat anti-mouse diluted 1:1,000 for 2 h at 4° C. The chemiluminescence was detect using ImageQuant™ LAS 4000 (GE Healthcare, USA). Band intensity was analyzed by Image Lab™ software 6.0 (Bio-Rad Laboratories Inc., USA).

β-actin was used as protein loading control. Rat brain sample (5 μg/μL) was used as positive control of antibodies and zebrafish liver sample (0.5 μg/μL) was used as negative control for unspecific reaction of antibody anti-vGluT2 using in zebrafish samples (data not shown).

2.6. STATISTICAL ANALYSES

All data were expressed as mean ± standard error of mean (S.E.M.) and were analyzed by Student t test for unpaired sample. $P < 0.05$ was considered statically significantly.

3. RESULTS

In this study, we analyzed the vesicular glutamate transporter 2 (vGluT2) immunocontent in brain samples of adult zebrafish exposed at three different environmental enrichment protocols by western blot assay. Results showed that only in Protocol 2, which fish stayed for 14 days in environment enriched without changes during protocol time duration, the animals demonstrated an increase of vGluT2 band intensity ($P = 0.0332$) when compared with SE group (Fig. 1C). On the other hand, Protocols 1 and 3 did not present difference of vGluT2 immunocontent *(*$P = 0.5371$ and $P = 0.5029$; Fig. 1A and 1C, respectively) between groups.

4. DISCUSSION

The present study demonstrated that adult zebrafish exposed in different model of environmental enrichment protocols had also distinct vGluT2 immunocontent as answer to condition depending of time duration and composition of enrichment. According with previous studies in rodent models, environmental enrichment protocols are capable to stimulated beneficial process in diverse situations – like an increased dendritic branching or arbor. We performed this experimental design in order to analyze a possible relation of glutamatergic system and increased synaptic connection in zebrafish. Here, it was observed that both duration and type of environmental enrichment protocol have an influence on the way how the adult zebrafish responds neurochemically through glutamatergic system. Protocol 1, which represents a short-term enriched environment with two components of complexity (stones and artificial

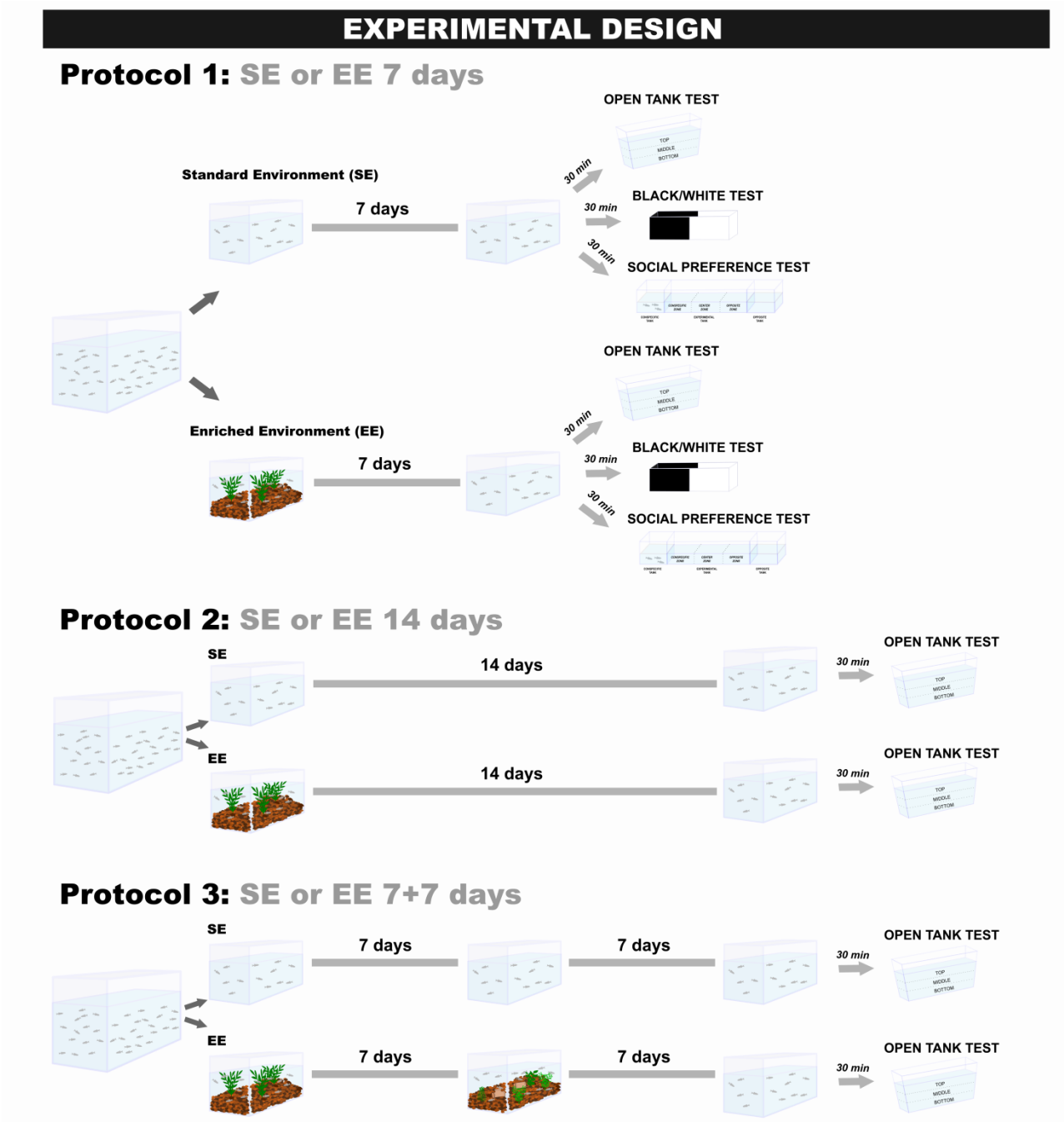
plants), demonstrated any alteration in vGluT2 immunocontent. However, in the Protocol 2, with longer duration than Protocol 1, we observed an increased in vGluT2 immunocontent in animals of EE group than those of SE group. Contrary to what we hypothesize, adult zebrafish exposed in a long-term protocol as Protocol 2, but with a renovation of elements of complexity in eighty day (named Protocol 3), did not present difference in protein expression between groups.

In zebrafish model and environmental enrichment, increased neuronal proliferation has been already described, but other process and mechanisms need to start studying. In this work, we initiated this discussion becoming that zebrafish have alterations in protein expression of one type of transporter involved in glutamatergic system. However, this alteration did not seem to have a direct relation with the maintenance of novity of environment and its complexity over time of environmental enrichment, but have with a consolation and habituation of animals in this different condition. Heinla *et al.* (2015) demonstrated that genic expression of Lsamp (limbic system associated membrane protein) is increased in rodents in enriched environments conditions and it is related with adaptability in changing environments [4]. This study, although in mouse model, can bring interesting information for our results, corroborating for our hypothesis that animals also need a time period for adaptation in a different ambiance and so, start to present neurochemical characteristics evoked by a distinct environment.

5. CONCLUSION

These findings demonstrated that a longer adaptation time in enriched environment without renovation of elements of enrichment over period (Protocol 2) is required to increase the protein expression of vGluT2 in adult zebrafish, possibly indicating an enhanced dendritic branching in this animal model.

6. FIGURE AND LEGEND



Supplementary figure 1. Experimental design of 3 environmental enrichment protocols for adult zebrafish used in this study. Protocol 1: Standard Environment (SE), which consisted in an empty tank with only recirculation water; and Enriched Environment (EE), which consisted into aquarium with stones at the bottom of the tank and three artificial green plants (25 cm of height) during 7 days. Protocol 2: similar to

Protocol 1, but with 14 days of duration. Protocol 3: divided in two segments of 7 days; first segment equal to Protocol 1; in the begging of second segment, animals were moved to the new other tanks: for SE group, new tanks were empty, and for EE group, new tanks had stones at bottom, one brown cylindrical PolyVinyl Chloride (PVC) pipe (25 mm of diameter), one brown PVC pipe T format (25 mm of diameter) and four distinct green artificial plants with 10 cm of height. Thirty minutes before the begging of behavioral tests, all groups were removed from housing tanks and placed into 8 L tanks (each one free of any substrate or plants). For protocol 1, open tank, black/white test and social preference test were performed. For Protocol 2 and Protocol 3, only open tank test was realized.

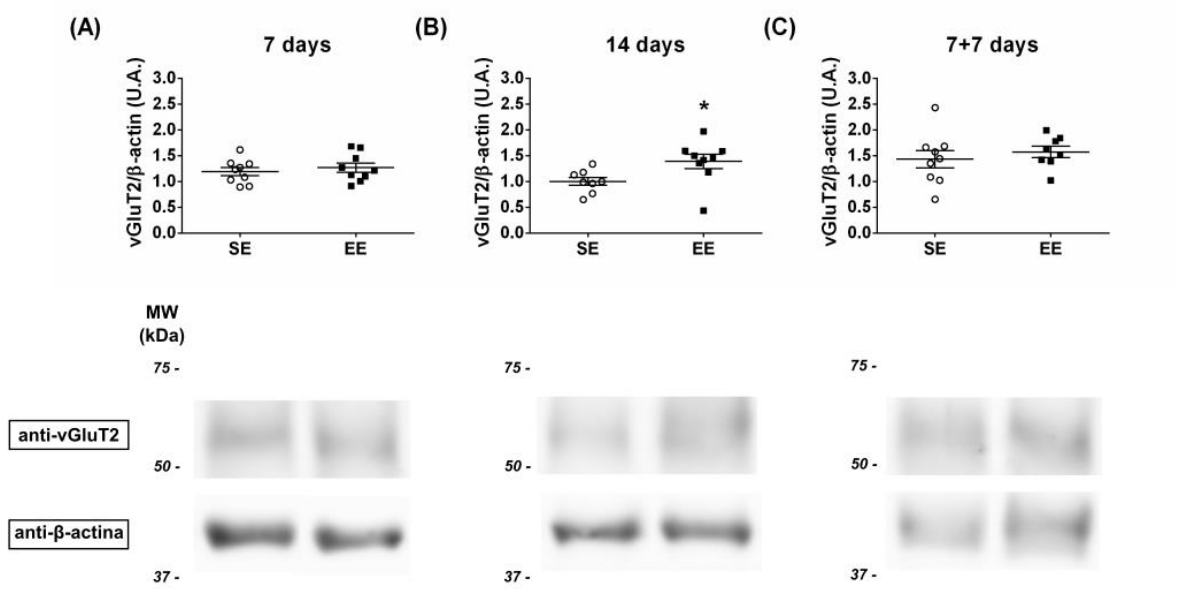


Figure 1. vGluT2 immunocontent in brain samples of adult zebrafish submitted to three different protocols of environmental enrichment. Quantification of ratio between vGluT2 and β-actin band intensities of animals of SE and EE groups exposed to Protocol 1 (7 days) **(A)**, Protocol 2 (14 days) **(B)**, and Protocol 3 (7+7 days) **(C)** and respective representative western blotting bands of groups below each graphic. Data were expressed in mean ± S.E.M. and analyzed by Student t test for unpaired sample. * = $P < 0.05$.

7. ACKNOWLEDGMENTS

This work was financed by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS) and Pró-Reitoria de Pesquisa da Universidade Federal do Rio Grande do Sul (PROPESQ-UFRGS).

8. DISCLOSURES

The authors of this paper have no conflict of interest.

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PARTE III

DISCUSSÃO

O enriquecimento ambiental oferece diversos estímulos, sendo eles sensoriais, sociais e cognitivos (Bezzina, Verret et al. 2015). Muitos autores defendem que um ambiente mais enriquecido proporciona comportamentos mais próximos ao natural para os animais usados como modelos experimentais na pesquisa biomédica (Osborne, Paull et al. 2016). Sabe-se que grande parte dos biotérios não oferta tal complexidade ambiental para os mesmos, entretanto o enriquecimento ambiental possibilita uma ampliação de aspectos intrínsecos ao animal, promovendo um melhor bem-estar animal.

Com o aumento da demanda de utilização global do peixe-zebra como modelo experimental em diversas áreas da pesquisa biomédica, a necessidade da padronização das condições de reprodução, criação, manutenção, moradia e cuidado nos locais que alojam esses animais se tornou mais explícita. Normalmente, os peixes são mantidos em aquários estáticos ou em sistema automatizados de recirculação de água sem nenhum estímulo nesses ambientes. Sabe-se que o peixe-zebra é um animal comumente encontrado em canais de irrigação em plantações de arroz do sul da Ásia, locais normalmente de movimentação lenta, com vegetação aquática e não sombreados, apresentando também bastante resistência a amplas variações de temperatura e diferentes profundidades de coluna de água (Spence, Gerlach et al. 2008). Neste contexto, encontra-se a discussão sobre a melhoria na complexidade ambiental oferecida nos aquários-moradia desses animais quando são utilizados como modelo experimental; encaixando-se a proposta da inserção de protocolos de enriquecimento ambiental.

No peixe-zebra, já foram descritos diversos protocolos de enriquecimento ambiental, contudo, há uma grande variabilidade no modo como é realizada a

composição desses protocolos. Por este fato, há certa dificuldade para a comparação entre os resultados obtidos por estudos já existentes da literatura. Todavia, a maioria dos autores utiliza um contexto inanimado com pedras ao fundo do aquário, plantas artificiais e/ou os animais em cardume. Considerando esses itens como os mais comumente descritos, montamos três protocolos de enriquecimento ambiental para peixe-zebra adulto.

Assim, o "Protocolo 1" – também nomeado de "7 dias" - foi desenhado com o intuito de avaliar se um ambiente com os componentes básicos de complexidade (pedras, plantas artificiais e cardume), por um tempo curto (7 dias), seria suficiente para induzir alterações no perfil comportamental do peixe-zebra. Primeiramente, os resultados obtidos com o teste de *open tank* demonstraram que os animais submetidos ao ambiente enriquecido (AE) apresentaram um perfil locomotor diferenciado dos animais do grupo ambiente padrão (AP). Entretanto, como demonstrado na **Figura 6** do Capítulo I, observa-se que esta diferença se deve a uma instabilidade comportamental apresentada pelos animais do grupo AP nos dois últimos minutos de teste no fundo do aparato. Como comentado anteriormente, é conhecido que o peixe-zebra, naturalmente, vive em lugares com certa complexidade e também com grandes variações nas condições ambientais, como na temperatura, por exemplo. Assim, com estes resultados, demonstramos como a complexidade do ambiente pode induzir a manifestação da adaptabilidade natural desta espécie a alterações, visto que os animais que permaneceram durante setes dias no protocolo de enriquecimento ambiental responderam de forma comportamental estável ao longo do teste - ao contrário do que observamos com o grupo AP. Na literatura, já existem dados que apresentam a relação do enriquecimento ambiental e a redução do estresse induzido em peixe-zebra em protocolos de estresse diferenciados (Giacomini, Abreu et al. 2016; Marcon, Mocelin et

al. 2018), o que pode então ser responsável pela resposta comportamental constante dos animais do grupo AE no nosso estudo.

Adicionalmente, observamos que não há alteração entre os grupos em relação ao comportamento tipo ansiedade e ao de preferência social. Entretanto, conseguimos relacionar os resultados do teste de *open tank* com o de preferência social pela primeira vez, visto que neste segundo teste, observamos também um perfil locomotor mais lento nos animais do grupo AE quando comparados com os do AP. Isto reforça nossa hipótese de desencadeamento de mecanismos da adaptabilidade natural dos animais quando submetidos ao ambiente enriquecido, possibilitando uma resposta mais estável a ambientes novos.

Baseado nestes resultados, questionou-se se essa reposta comportamental se manteria numa exposição mais longa ao enriquecimento ambiental (14 dias). Por esta razão, desenhamos o "Protocolo 2" - também nomeado de "14 dias" - e observamos que a diferença apresentada no perfil comportamental entre os grupos não era mais significativa. Quando considerados os dados brutos desses resultados, percebe-se que, na realidade, o que ocorre é a estabilidade da resposta comportamental dos animais do grupo AP ao longo do tempo de teste *open tank* – o que não ocorria nos resultados com o protocolo anterior. Isto corrobora com a explicação dos resultados encontrados pelo Protocolo 1, trazendo um ponto bastante importante para a discussão: o quão intenso é o estresse provocado pela simples troca entre aquários idênticos utilizados em biotérios, necessitando uma ambientação mínima de duas semanas no aquário moradia antes da realização de testes comportamentais – a fim de não ser observado um resultado instigado pelo estresse decorrente da alteração de aquário.

Por último, realizamos a avaliação do Protocolo 3 - também nomeado de "7+7 dias" - a fim de estudar se a mudança de itens da complexidade do enriquecimento

ambiental dentro do período mais prolongado que utilizamos (14 dias) teria alguma influência nos resultados já observados. Neste protocolo, a exposição a um ambiente com componentes de enriquecimento ambiental, durante os primeiros sete dias, favoreceu para que os animais se adaptassem mais facilmente um novo ambiente com diferentes itens de complexidade nos dias subsequentes do protocolo através da combinação dos dados dos protocolos anteriores. Os animais do grupo AE não apresentaram diferença no perfil comportamental geral mesmo com a inserção de novos objetos, similarmente ao observado no Protocolo 2, entretanto foi apresentada uma redução de distância percorrida no fundo do aparato por esses animais, refletindo um pouco do Protocolo 1.

Muitos dos efeitos do enriquecimento ambiental são relacionados com diversos fatores cerebrais. Entre esses, em modelos de roedores, já foram descritas melhorias cognitivas em diferentes modelos experimentais de algumas doenças. Isto é bastante relacionado a aumento de neurogênese adulta e de ramificação dendrítica. Além disso, o enriquecimento ambiental também altera a funcionalidade de alguns sistemas de neurotransmissores, como por exemplo o glutamatérgico e GABAérgico (Segovia, Yague et al. 2006; Hullinger, O'Riordan et al. 2015). Assim, a segunda parte dessa dissertação (Capítulo II) teve como objetivo a análise da expressão proteica de um dos transportadores vesiculares de glutamato, o vGluT2. Os vGluTs são expressos em regiões dendríticas e quanto maior sua expressão proteica, maior é a densidade de vesículas sinápticas presentes na região analisada.

Nesta parte do trabalho, observamos somente um aumento na quantidade de expressão proteica do vGluT2 nos animais do grupo AE quando submetidos a um ambiente enriquecido durante 14 dias (Protocolo 2). Esses resultados demonstram que a duração do protocolo e a complexidade do ambiente influenciam na resposta

neuroquímica através do sistema glutamatérgico. Entretanto, esses dados demonstram que essa resposta não é diretamente relacionada à mudança da complexidade ambiental (Protocolo 3) ou a um protocolo com duração curta (Protocolo 1), mas sim à consolidação e habituação dos peixes a uma condição diferente por um período mais prolongado de permanência neste ambiente (Protocolo 2). Estudos em roedores já demonstraram que o enriquecimento ambiental aumenta a expressão gênica de uma proteína relacionada a adaptação a ambientes novos, no caso ao ambiente enriquecido (Heinla, Leidmaa et al. 2015). Assim, mesmo este estudo sendo em roedor, a nossa hipótese é de que o peixe-zebra adulto precisa de um tempo considerável para realizar uma adaptação a esta nova condição e, então, apresentar um possível aumento de conexões sinápticas com características neuroquímicas envolvidas ao sistema glutamatérgico por este ambiente novo.

PARTE IV

CONCLUSÃO

Neste trabalho, demonstramos que peixe-zebra adulto submetido ao enriquecimento ambiental possui uma resposta comportamental mais regular do que os controles e isso, provavelmente, é decorrente a estimulação de processos direcionados a resposta ao estresse da novidade, bem como de vias relacionadas com a adaptabilidade. Além disso, também demonstramos que, aparentemente, a habituação por um protocolo mais longo à condição de um ambiente enriquecido é mais importante para desencadear o aumento de expressão proteica de vGluT2.

PARTE V

PERSPECTIVAS

Como perspectivas deste estudo, primeiramente, segue-se um melhor entendimento do modelo experimental de peixe-zebra adulto para avaliação do processo de neurogênese adulta relacionando o enriquecimento ambiental. Também, há o interesse de analisar o perfil de diferenciação neuronal subsequente destas células em proliferação com o enfoque na característica glutamatérgica, visto que este neurotransmissor está relacionado com plasticidade sináptica, manutenção da citoarquitetura neuronal e atividade trófica no desenvolvimento.

Entretanto, para isto, também é necessária uma melhor compreensão dos vGluTs no cérebro do peixe-zebra adulto. Assim, outra perspectiva deste estudo é a avaliação da distribuição das diferentes isoformas do vGluT nas distintas regiões encefálicas do peixe-zebra adulto, tanto em análise de expressão gênica e proteica – bem como de localização cerebral.

PARTE VI

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