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PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS FISIOLÓGICAS: FISIOLOGIA ANIMAL COMPARADA

IMPLICAÇÕES DO HORMÔNIO DO CRESCIMENTO (GH) SOBRE ASPECTOS COMPORTAMENTAIS E COGNITIVOS EM UM MODELO DE ZEBRAFISH (*Danio rerio*) TRANSGÊNICO

Tese de conclusão de doutorado apresentado pela MSc. **Ana Lupe Motta Studzinski** como parte dos requisitos para obtenção do título de doutora em Ciências Fisiológicas: Fisiologia Animal Comparada sob orientação do Dr. Luis Fernando Marins e co-orientação da Dra. Daniela Martí Barros

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Dedico esta tese aos meus pais Ladislau e Maria Antônia e

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Sumário

Resumo Geral

A maioria dos estudos que avaliam os efeitos do eixo GH/IGF-I (hormônio do crescimento/fatores de crescimento tipo insulina I) no comportamento e memória é de mamíferos. Entretanto, para avaliar os efeitos pleiotrópicos do GH/IGF-I foi produzida uma linhagem de peixe (*Danio rerio*) geneticamente modificada que superexpressa o gene do GH denominada F0104. O objetivo deste estudo foi analisar os efeitos da superexpressão do GH sobre aspectos comportamentais e cognitivos em indivíduos machos adultos e larvas da linhagem F0104. Em relação ao comportamento reprodutivo foi observado que embora os indivíduos adultos desta linhagem (F0104) sejam mais agressivos e tenham maior atividade locomotora, eles não obtém maior sucesso no desempenho reprodutivo. Machos adultos transgênicos apresentaram vantagem no acasalamento somente quando a massa corpórea foi superior aos não-transgênicos. Quanto à receptividade das fêmeas através da análise da frequência de desovas foi demonstrado que, os cruzamentos de machos transgênicos com fêmeas não-transgênicas produziram menos desovas ($P < 0,05$). Já ao avaliar o aprendizado e a memória, os resultados mostraram que não há diferenças no comportamento exploratório ($P > 0,05$), porém foi observado um aumento significativo na memória de longa duração (LTM) dos transgênicos. Adicionalmente, machos transgênicos apresentaram um aumento generalizado na expressão dos genes dos receptores glutamatérgicos ionotrópicos AMPA (α-amino-3-hidroxi-5-metilisoxazol-4-propiônico) e NMDA (N-metil-D-aspartato), além de uma indução de três vezes na expressão do IGF-I cerebral ($P < 0,05$). Estes resultados indicam um efeito importante do GH no cérebro, intermediado pelo IGF-I, com consequente melhora na LTM em decorrência do aumento dos receptores glutamatérgicos. Em relação às larvas, a atividade locomotora não foi diferente entre transgênicos e não-transgênicos, diferentemente do que foi observado em adultos. Porém, a expressão das subunidades dos receptores AMPA e NMDA mostrou um padrão de aumento transcricional. Desta forma, pode-se concluir que a linhagem F0104 mostrou-se um modelo interessante para elucidar o papel do eixo somatotrópico sobre o comportamento, aprendizado e memória.

Palavras-chave: peixes adultos, larvas, agressividade, atividade locomotora, dominância, memória, receptores glutamatérgicos AMPA e NMDA.

Abstract

The effect of the GH/IGF-I (growth hormone/ insulin-like growth factor-I) axis on behavior and memory has been evaluated mainly in mammals. However, to evaluate the pleiotropic effects of GH/IGF-I a genetically modified zebrafish (*Danio rerio*) lineage that overexpresses the GH gene has been produced, named F0104. The aim of this study was to analyze the effects of overexpression of GH on behavioral and cognitive aspects of adult males and larvae of individuals F0104 lineage. In terms of behavior, it was observed that although adult males of this lineage (F0104) are more aggressive and increased locomotor activity, they are not more successful in reproductive performance. Transgenic males presented mating advantage only when they were larger than non-transgenic fish. Regarding female receptivity by examining the frequency of spawning demonstrated that crosses with transgenic males and non-transgenic females produced less spawn ($P < 0.05$). Learning and memory experiments showed no difference in exploratory behavior ($P > 0.05$), but an increase in long-term memory (LTM) was observed for transgenic fish. Additionally, transgenic individuals presented a general pattern of increase in the gene expression of ionotropic glutamate receptors AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) and NMDA (N-methyl-D-aspartate), as well as a three-fold induction in the expression of cerebral IGF-I ($P < 0.05$). These results indicate that GH, mediated by IGF-I, has an important effect on the brain, with consequent improvement of LTM due to increase of glutamate receptors. Differently from reported for adults, locomotor activity was not different between transgenic and non-transgenic larvae. However, the expression of subunits of AMPA and NMDA receptors displayed a generalized pattern of transcriptional increase. In this manner, it can be concluded that the F0104 lineage is an interesting model for elucidating the role of the somatotropic axis on behavior, learning and memory.

Introdução Geral

O eixo somatotrópico e seus efeitos biológicos

O hormônio do crescimento (GH) é um hormônio protéico produzido principalmente na parte anterior da hipófise, cuja principal função é estimular o crescimento somático em vertebrados. A liberação do GH é controlada principalmente pela interação de dois sinais hipotalâmicos, o hormônio liberador de GH (GHRH) e a somatostatina. Contudo, a secreção de GHRH e somatostatina está sob influência de uma complexa rede neural envolvendo múltiplos neurotransmissores como: norepinefrina, serotonina, glutamato e ácido γ-aminobutírico (GABA) (Bertherat *et al.*, 1995; González *et al*., 1999; Canosa *et al.*, 2007). Após ser sintetizado na hipófise, o GH é liberado na corrente sanguínea, atuando em determinados órgãos através da associação com receptores específicos presentes na superfície das células alvo. A identificação de receptores de GH (GHR) em uma grande variedade de tecidos indica que, possivelmente, o GH possui ações fisiológicas em vários tecidos tanto em peixes quanto em mamíferos (Kelly *et al*., 1991). No fígado, o GH induz a produção dos fatores de crescimento tipo-insulina (IGFs, "Insulin like Growth Factors") que exercerão a maioria dos efeitos biológicos deste hormônio.

Os IGFs são pequenas cadeias polipeptídicas que incluem o IGF-I e o IGF-II. Em mamíferos, estes fatores de crescimento atuam diretamente em processos de crescimento e desenvolvimento do organismo (Le Roith *et al*., 2001). Os IGFs estão presentes em uma grande variedade de tecidos, exibindo propriedades hipertróficas e hiperplásicas, sendo reguladores particularmente importantes da miogênese, exercendo um papel endócrino, parácrino e autócrino na integração entre a regulação tecido-específica e outros eventos biológicos (Hoffenberg *et al*., 1977; Shalet *et al*., 1979; Kamegai *et al*., 2005).

Além do crescimento, o chamado "eixo somatotrófico" GH/IGF, regula também o metabolismo de gorduras, proteínas e carboidratos (Davidson, 1987; Moller e Norrelund, 2003), a manutenção do sistema imune (Jeay *et al*., 2002), a resposta ao estresse, o comportamento (Yoshizato *et al*., 1998) e a função neuronal (Werther *et al.*, 1995; D'Ercole *et al.*, 1996; Le Grevès *et al.*, 2005). Estudos utilizando técnicas de perfusão em ratos demonstraram que o IGF-I circulante produzido no fígado atravessa a barreira hemato-encefálica podendo ligar-se a receptores presentes em todo cérebro (Reinhardt e Bond, 1994; Nyberg, 2000). Existem também evidências de que o cérebro produz IGF-I atuando de forma parácrina ou autócrina (Lai *et al.*, 2000). Já estudos *in vitro* descrevem as ações neurotróficas do IGF-I e IGF-II em neurônios e células gliais incluindo estimulação da síntese de DNA e de RNA (Lenoir e Honegger, 1983), indução do crescimento de neurônios (Recio-Pinto *et al.*, 1986; O'kusky *et al*., 2000; Aberg, 2010), a regulação da liberação de neurotransmissores (Kar *et al.*, 1997), sinaptogênese (Ishii, 1989; O'kusky *et al*., 2000; Aberg *et al*., 2006) e proteção contra neurotoxinas (Dore *et al.*, 1997). Camundongos transgênicos que superexpressam IGF-I no cérebro apresentam peso e volume cerebral substancialmente elevado devido ao maior número de neurônios e ao maior conteúdo de mielina total (Lee *et al.*, 1999; D'Ercole *et al*., 2002). Nogushi (1996) descreve que em camundongos deficientes de GH, o crescimento neuronal é retardado e ocorre hipomielinização. Estas disfunções podem ser amenizadas administrando GH nos estágios críticos de desenvolvimento.

Embora a principal fonte de GH seja a hipófise, outras regiões do sistema nervoso produzem pequenas quantidades do hormônio podendo atuar em receptores locais (Sonntag *et al.*, 2000; Nyberg e Hallberg, 2013). No hipocampo de humanos, por exemplo, a presença de receptores de GH sugere um efeito específico do eixo GH/IGF nas funções cognitivas (van Dam *et al*., 2000). Sabe-se que a diminuição das concentrações de GH e IGF-I com a idade está associada a mudanças que ocorrem no sistema nervoso central e, consequentemente

resultam em prejuízo na memória (Rollero *et al.*, 1998; Kinney-Forshee *et al.*, 2004). Em humanos com deficiência de GH, através de estudos clínicos, é evidenciado que a terapia de reposição do hormônio melhora a memória e as funções cognitivas, especialmente a memória de curta e longa duração (Burman *et al.*,1995; Johansson *et al.*,1995).

Memória

De um ponto de vista operacional, a memória nada mais é do que alterações estruturais de sinapses (mudanças químicas e físicas), as quais são diferentes para cada tipo de memória. Os tipos de memória conhecidos são classificados segundo a função (memória de trabalho), conteúdo (declarativas ou procedurais) e tempo de permanência (memória de curta duração, memória de longa duração e memória remota). Além disto, muitas memórias podem ser adquiridas por meio da associação de um estímulo com outro estímulo ou com uma resposta (memórias associativas e não associativas) (Izquierdo, 2011). As memórias não são adquiridas imediatamente na sua forma final. Durante os primeiros minutos ou horas após sua aquisição, elas são suscetíveis à interferência por outras memórias, por drogas ou por outros tratamentos (McGaugh, 2000; Izquierdo e McGaugh, 2000).

Vários mecanismos estão envolvidos no processo de consolidação da memória. Um dos principais mecanismos é a excitação repetida das células hipocampais, por meio da estimulação dos receptores glutamatérgicos (Izquierdo, 2011). Sabe-se que estes receptores regulam a maioria das neurotransmissões excitatórias no cérebro, desempenhando um papel importante na plasticidade neural, no desenvolvimento neural e neurodegeneração (Nakanishi e Masu, 1994). Dentre as regiões do cérebro relacionadas com a memória e aprendizado, o hipocampo tem recebido uma atenção particular nos estudos do potencial de longa duração (LTP) e da depressão de longa duração (LTD), dois mecanismos associados com a plasticidade sináptica, que envolvem a transmissão de glutamato e consequentemente os

receptores AMPA (α-amino-3-hidroxi-5-metilisoxazol-4-propiônico) e NMDA (N-metil-D-aspartato) (Collingridge e Singer, 1990).

Receptores glutamatérgicos ionotrópicos

Os receptores tipo AMPA apresentam quatro subunidades (GluR1-4 ou A-D) também conhecidas como gria1-gria4 (Rosenmund *et al.*, 1998), sendo responsáveis pela despolarização primária na neurotransmissão, exercendo um papel fundamental nas transmissões sinápticas excitatórias rápidas. O LTP e a LTD estão associados à distribuição e fosforilação dos receptores AMPA (Figura 1), demonstrando o envolvimento deste receptor com o mecanismo de aprendizagem e memória (Rumpel *et al.*, 2005; Whitlock *et al.*, 2006). Os receptores AMPAs são encontrados na forma homomérica e heterotetramérica (Bredt e Nicoll, 2003; Hollmann e Heinemann, 1994) onde a combinação das subunidades GluR1-GluR4 determinam a função do receptor canal como a propriedade de condutância de íons cátions K^+ e Na^+ sendo que, algumas composições do receptor são permeáveis ao Ca^{2+} (Figura 2) (Osawa *et al.*, 1998). Estudos com imunoprecipitação revelam que o complexo GluR2-GluR1 ou GluR2-GluR3 constituem as formações do receptor AMPA mais encontradas no hipocampo de ratos adultos (Wenthold *et al.*, 1996). Estudos eletrofisiológicos também descrevem que a maioria dos neurônios de mamíferos expressam canais AMPA heteroméricos contendo a subunidade GluR2 (Geiger *et al.*, 1995). A subunidade GLUR2 sofre uma modificação pós-traducional, onde um resíduo de glutamina presente na região do poro do receptor canal é substituído por uma arginina. Este processo é essencial para o controle do receptor AMPA. A presença do resíduo de arginina limita o fluxo de íons pelo receptor, permitindo a passagem de Na^+ e K^+ e bloqueando a passagem de íons divalentes como o Ca^{2+} (Lüscher e Malenka, 2012).

Alterações na atividade dos receptores AMPA são encontradas em patologias como isquemia (Kwak e Weiss, 2006; Liu *et al.*, 2006). Já em pesquisas realizadas com ratos que apresentam o gene da subunidade GluR2 bloqueado são evidenciadas anomalias comportamentais incluindo déficits na aprendizagem e memória (Jia *et al.*, 1996; Gerlai *et al.*, 1998; Jia *et al.*, 2001). Camundongos *knockout* para a subunidade GluR1 apresentam deficiência no LTP nas regiões CA3-CA1 do hipocampo (Zamanillo *et al.*, 1999). Experimentos utilizando agonistas e antagonistas também sugerem a participação do AMPA no desenvolvimento do sistema nervoso central (Lin *et al.*, 2006).

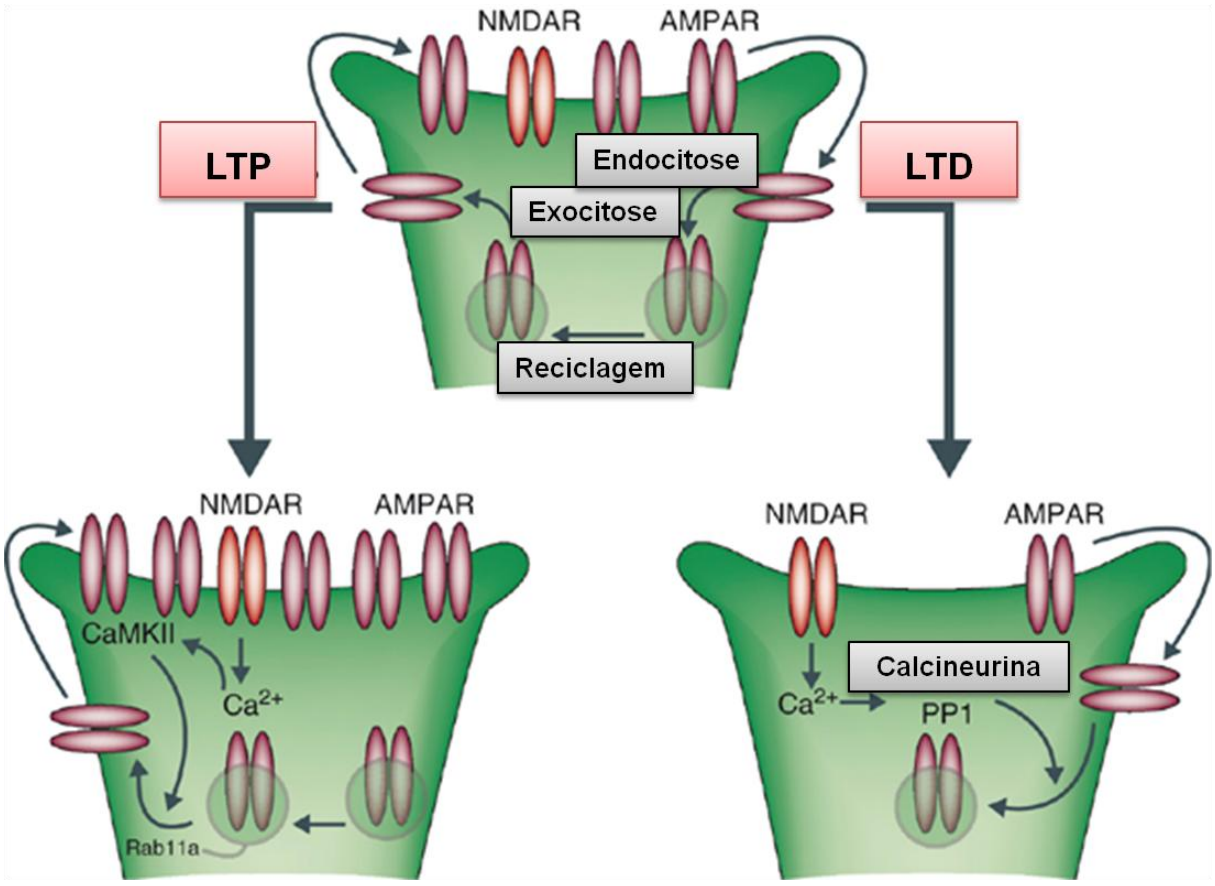

Figura 1. Modelo de tráfico de receptores AMPA durante o LTP e a LTD no neurônio pós-sináptico. No LTP o aumento do influxo de cálcio pelo receptor NMDA faz com que as proteínas CAMK II (proteína quinase Ca^{2+}/calmudulina dependente) e Rab11a ("*Ras-related protein Rab-11A*") sinalizem para que os endossomas de reciclagem se fusionem e exportem por exocitose os receptores AMPA para a membrana do neurônio. Durante a LTD os receptores AMPA são defosforilados pela fosfatase calcineurina PP1 num processo Ca^{2+}-dependente e

posteriormente são deslocados da membrana e retidos no endossoma para reciclagem. Adaptado de Citri e Malenka (2008).

Os receptores NMDA se distinguem de outros membros da família de receptores de glutamato ionotrópicos por necessitarem de glicina como co-agonista para sua ativação e serem bloqueados por Mg^{2+}-voltagem-dependente (Hollmann e Heinemann, 1994). Os NMDARs de vertebrados são heterotetraméricos, compostos por duas subunidades NR1 e duas das possíveis NR2A-D e, ocasionalmente, NR3A-B sendo permeáveis ao K^+, Na^+ e Ca^{2+} (Figura 2) (Benveniste e Mayer, 1991; Clements e Westbrook, 1991; Sucher *et al.*, 1996; Harvey-Girard e Dunn, 2003). A subunidade NR1 é essencial para o receptor exercer sua atividade de canal e confere a capacidade de atividade ligante à glicina (Kuryatov *et al*., 1994). Já a subunidade NR2 confere a atividade ligante ao glutamato governando, assim, as propriedades do receptor canal e da extensão da plasticidade sináptica. Estudos com camundongos transgênicos superexpressando a subunidade NR2B demonstram aumento na performance cognitiva (Tang *et al.*, 1999). Em indivíduos adultos, são usualmente encontradas no hipocampo e no córtex, as subunidades NR2A e NR2B e, a proporção destas subunidades diminui com a idade em diversas espécies de animais (Li e Tsien, 2009). Em cérebro de teleósteos a atividade de NMDAR parece ser importante nas transmissões sinápticas e em processos cerebrais complexos (Berman *et al.*, 2001; Nam *et al.*, 2004; Kinoshita *et al*., 2005). Em zebrafish, 10 subunidades que compreendem o complexo NMDAR já foram clonadas e suas sequências nucleotídicas determinadas incluindo *nr1.1*, *nr1.2*, *nr2a.1*, *nr2a.2*, *nr2b.1*, *nr2b.2*, *nr2c.1*, *nr2c.2*, *nr2d.1* e *nr2d.2* (Cox *et al.*, 2005).

Algumas das subunidades do receptor NMDA foram encontradas na retina de zebrafish como Grin2a.1 e Grin2a.2 em 48 horas pós-fertilização (hpf) e as subunidades Grin2d.1 e Grin2d.2 foram detectadas no cérebro posterior e no cordão espinal. Em 24 hpf e 48 hpf, os genes que expressam as subunidades Grin1.1 e Grin1.2 foram encontrados no

cordão espinal, na retina, no cérebro anterior e posterior (Cox *et al*., 2005). Em relação às subunidades do AMPA Glur1a, Glur1b, Glur2a, Glur2b, Glur3a, Glur3b, Glur4a e Glur4b Hoppmann *et al*. (2008) verificaram que em zebrafish com 24 hpf e 48 hpf todos os genes que expressam as oito subunidades são encontrados na região do telencéfalo e do cérebro posterior. Em 72 hpf com exceção das subunidades Glur3a e Glur3b, todas as subunidades se expressam no teto óptico, na medula oblongata dorsal e ventral, no cordão espinal, em células da retina, no telencéfalo e no bulbo olfatório.

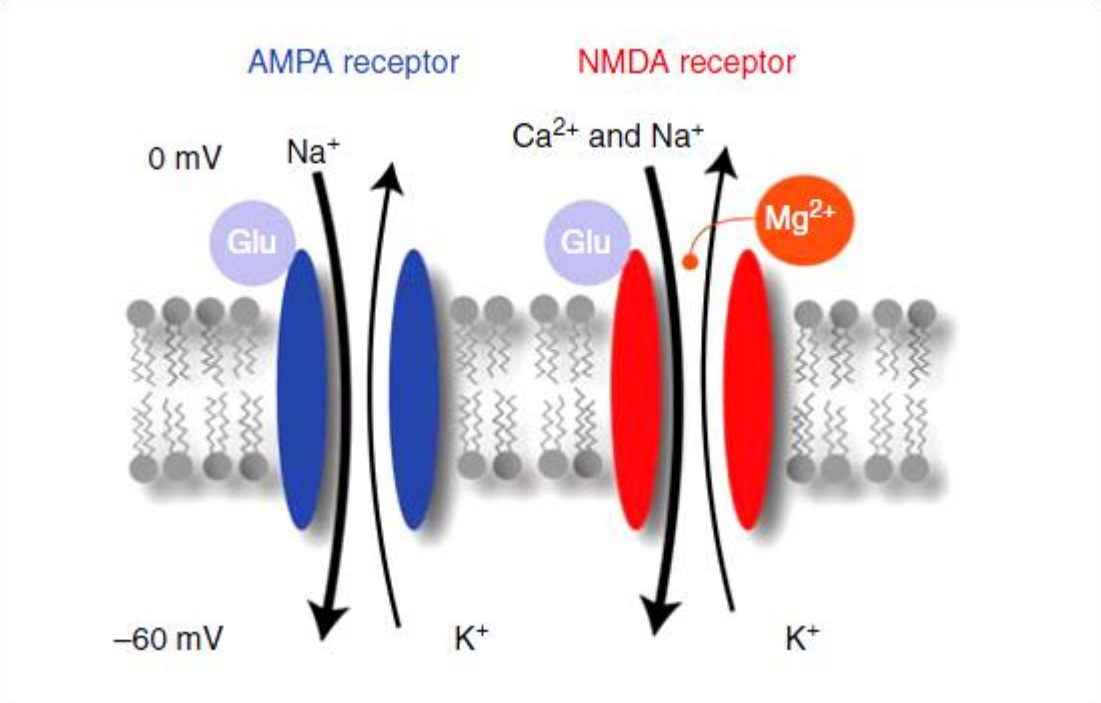

Figura 2. Receptores glutamatérgicos ionotrópicos AMPA e NMDA envolvidos na LTP e LTD. Quando o glutamato se liga ao receptor AMPA vários íons sódio fluem para dentro da célula enquanto alguns íons potássio deixam o neurônio, causando a despolarização da membrana. Os receptores NMDA também são permeáveis ao Ca^{2+}, mas apenas se o magnésio que está bloqueando o receptor é expelido após uma rápida despolarização do neurônio. Extraído de Lüscher e Malenka (2012).

Comportamento agressivo

Um tipo de comportamento social complexo que está presente em todo o reino animal é o comportamento agressivo. Enquanto na população humana a agressividade é vista como um problema, a maioria dos animais utiliza a agressividade para defender território, proteger a prole, disputar comida ou parceiro e estabelecer hierarquia (Oliveira *et al*., 2011).

O zebrafish é uma espécie de peixe utilizada como modelo de estudo neurocomportamental que tem um comportamento social de grupo, no qual estabelece hierarquias de dominância em ambos os sexos. Neste caso, o comportamento agressivo é utilizado pelos indivíduos dominantes para ocupar territórios possíveis de desovas e protegê-los de peixes subordinados (Larson *et al*., 2006; Paull *et al*., 2010).

Segundo Oliveira *et al*. (2011), quando dois machos adultos são colocados num mesmo aquário, eles iniciam um confronto, exibindo uma postura agonista caracterizada pela ereção das nadadeiras dorsal, caudal, peitorais e anal, tentativas de morder e a perseguição ao oponente nadando rapidamente. Em condições de laboratório, o comportamento agressivo pode ser mensurado de duas maneiras: avaliando a interação agonista entre dois peixes ou utilizando a estimulação induzida por espelho (Gerlai *et al*., 2000; Gerlai, 2003; Ariyomo e Watt, 2012). Recentemente, Ariyomo e Watt (2013) ao compararem os dois protocolos de comportamento agressivo evidenciaram que independente do protocolo utilizado, os peixes exibem comportamentos agressivos similares. Sabe-se que a agressividade é um componente intrassexual que pode afetar a reprodução (Spence e Smith, 2005). Para zebrafish foi evidenciado que muitas substâncias afetam o comportamento agressivo como serotonina, somatostatina, dopamina, histamina, arginina vasotocina, óxido nítrico, neuropeptídeo y e estrogênios em ambos os sexos (Larson *et al*., 2006; Filby *et al*., 2010).

Em relação ao GH, alguns estudos realizados com peixes e ratos evidenciam que este hormônio tem a capacidade de alterar o comportamento inclusive a agressividade. Em juvenis de truta arco-íris (*Oncorhynchus mykiss*) tratados com GH foi verificado aumento do apetite, da natação e da agressividade (Johnsson e Bjornsson, 1994; Jonsson *et al*., 1998). Já em ratos tratados com GH, foram observados aumento da agressividade sem afetar a atividade locomotora (Matte, 1981). Sagazio *et al.* (2011), ao estudar ratos com o gene do GHRH nocauteado apresentando deficiência de GH, observaram uma redução no comportamento agressivo.

Atividade locomotora

Outro comportamento que é importante para o *fitness* do animal e pode ser afetado pelo GH é a atividade locomotora. A locomoção permite que o peixe possa se alimentar, se defender, interagir, explorar e reproduzir ao longo da vida. Em vertebrados as atividades motoras são geradas em grande parte nas regiões do cérebro e na medula espinhal (Stein *et al*.,1997). Apesar de a locomoção ser característico de indivíduos juvenis e adultos de peixes, padrões simples de motilidade podem ser observados durante os estágios larvais (Granato *et al*., 1996). No zebrafish o primeiro movimento motor espontâneo ocorre 17 hpf (Saint-Amant e Drapeau, 1998). As larvas eclodem aproximadamente 2 ou 3 dias pós-fertilização (dpf) e ao receberem um estímulo como som, luz ou toque, expressam respostas de sobressalto (Burgess e Granato, 2007; Emran *et al*., 2008; Fetcho *et al*., 2008; McHenry *et al*., 2009; McLean e Fetcho, 2009). A bexiga natatória infla gradualmente e, a partir dos 4 ou 5 dpf, as larvas começam a nadar (Lindsey *et al*., 2010). Com 5 dpf, a visão está bem desenvolvida e as larvas começam a buscar o alimento (Neuhauss, 2003). Após os 5 dpf, as larvas são mais ativas durante o dia demonstrando ritmicidade (Prober *et al*., 2006). Larvas testadas 4, 5, 6 ou 7 dpf exibem comportamento de tigmotaxia, quando colocadas numa arena preferindo nadar nas

regiões mais externas em relação ao centro. Este comportamento é similar ao encontrado em zebrafish adultos. Na natureza, a permanência em áreas abertas proporciona uma exposição dos animais aos predadores induzindo um estado de ansiedade nesses indivíduos (Colwill e Creton, 2011).

Comportamento reprodutivo

O comportamento reprodutivo é outro tipo de comportamento que pode ser afetado pelo eixo GH/IGF já que estudos evidenciam a presença de receptores do GH e do IGF-I nas gônadas dos peixes, sugerindo a atuação em vários processos reprodutivos (Le Gac *et al*., 1996; Bereshvili *et al*., 2006). Estudos considerando os efeitos da superexpressão do GH na reprodução avaliam a fertilidade dos animais demonstrando, por exemplo, a diminuição da qualidade do esperma em *Oncorhynchus kisutch* e *Danio rerio* (Fitzpatrick *et al*., 2011; Figueiredo *et al*., 2013).

Em geral, peixes teleósteos demonstram uma grande plasticidade reprodutiva a começar pela liberação de gametas na água, defesa territorial, até o comportamento de corte. A regulação do comportamento reprodutivo ocorre pelo eixo hipotálamo-hipófise-gonadal sendo influenciado por fatores endógenos e exógenos. Entre os fatores endógenos, os hormônios esteróides gonadais são os principais reguladores do comportamento reprodutivo dos peixes podendo atuar como feromônios. Em zebrafish machos feromônios encontrados nas gônadas como glucuronídeos esteróides são conhecidos por estimular a ovulação e o comportamento de acasalamento nas fêmeas (van den Hurk e Lamber, 1987; van den Hurk e Resink, 1992). Além de fatores hormonais, peixes como o zebrafish são capazes de reconhecer seus parceiros sexuais apenas pelos caracteres sexuais secundários (Hutter *et al*., 2011).

O zebrafish apresenta o mesmo padrão de acasalamento encontrado em outros ciprinídeos. Os machos de zebrafish são territorialistas no período reprodutivo (Spence e Smith, 2005). Quando os machos se encontram eles exibem um comportamento de confronto e perseguição. Após a disputa, o macho dominante persegue a fêmea, muitas vezes empurrando-a com o focinho e nadando em círculos ou elipticamente, orientando-a até o território reprodutivo (Darrow e Harris, 2004; Sessa *et al*., 2008). Em peixes teleósteos, como o zebrafish o tamanho do corpo tende a correlaciona-se com a dominância (Wootton, 1998) e trabalhos evidenciam que as fêmeas de zebrafish preferem machos maiores (Pyron, 2003).

Uso do zebrafish como modelo neurocomportamental

Peixes exibem notável capacidade de navegação, orientação e reconhecimento do ambiente. Tais habilidades envolvem mecanismos complexos de aprendizado e memória. Em peixes teleósteos através de estudos comportamentais e neuroanatômicos foi demonstrado que a região do cérebro responsável pela aprendizagem e o processamento de memória é o telencéfalo dorsal, o maior componente do sistema nervoso de actinopterígeos, constituído pelo pálio central, medial e lateral, uma região análoga ao hipocampo e a amígdala do cérebro de mamíferos (Figura 3) (Portavella *et al.*, 2002; Rodriguez *et al.*, 2002). O aprendizado desempenha um papel chave em alguns aspectos comportamentais permitindo, por exemplo, que os peixes reajam adequadamente frente a predadores (Kelly e Magurran, 2003).

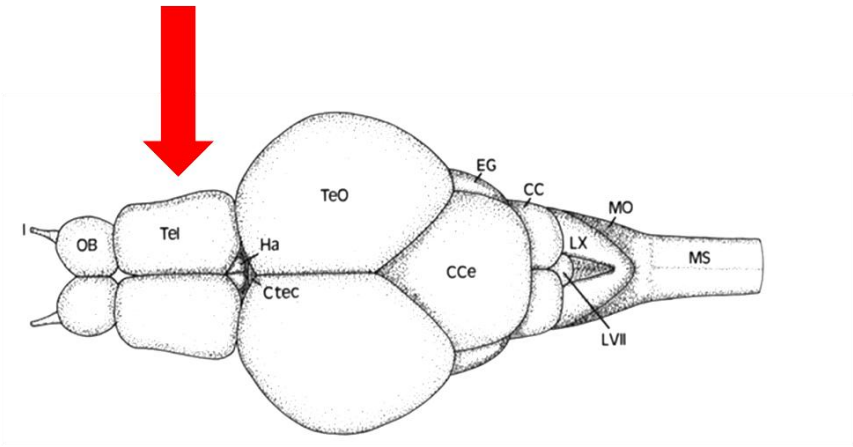

Figura 3. Áreas do cérebro do peixe teleósteo zebrafish (*Danio rerio*) indicando a região do telencéfalo - Tel (seta vermelha), OB - bulbo olfatório, Ha - habenula, Ctec - comissura tecti, TeO - teto óptico, Cce - corpus cerebelar, EG - eminência granularis, CC - crista cerebelar, LX - lobo vagal, LVII - lobo facial, MO - medula oblongata, MS - medula espinalis. Extraído de Wullimann *et al*. (1996).

Vários autores têm argumentado que o zebrafish é um modelo excelente de complexidade de sistema e simplicidade prática (Guo, 2004; Norton e Bally-Cuif, 2010; Sison e Gerlai, 2011) além do genoma sequenciado (Alsop *et al*., 2009). O cérebro do zebrafish é neuroanatomicamente e funcionalmente comparável ao de mamíferos (Guo, 2004), tendo vários sistemas de neurotransmissores já documentados nesse modelo, tais como: dopaminérgico, serotoninérgico, noradrenérgico, purinérgico e glutamatérgico (Rico *et al*., 2010; Piato *et al*., 2011). Inúmeros estudos neurocomportamentais foram realizados com o zebrafish fornecendo contribuições genéticas para o abuso de drogas e alcoolismo, para o desenvolvimento neurológico e déficits funcionais associados à síndrome alcoólica fetal, exposição à neurotoxinas, stress, autismo e epilepsia (Bilotta, 2003; Gaikwad *et al*., 2011; Stewart *et al*., 2011).

Modelo transgênico experimental

Com o objetivo de estudar os efeitos colaterais do excesso de GH, nosso grupo de pesquisa desenvolveu um modelo de zebrafish transgênico que superexpressa o gene do GH,

sendo esta a primeira linhagem de peixes transgênicos produzida no Brasil (Figueiredo *et al*., 2007a). A linhagem denominada F0104, carrega dois transgenes construídos a partir do promotor da β-actina da carpa (*Cyprinus carpio*) direcionando a expressão do gene da proteína verde-florescente (GFP) ou do cDNA do hormônio do crescimento (GH) do peixe-rei marinho *Odonthestes argentinensis* (msGH). A estratégia da transferência simultânea destas duas construções permitiu o desenvolvimento de uma linhagem de peixes transgênicos para o gene do GH que apresentam fluorescência sob luz ultravioleta, facilitando a identificação dos animais transgênicos (Figura 4).

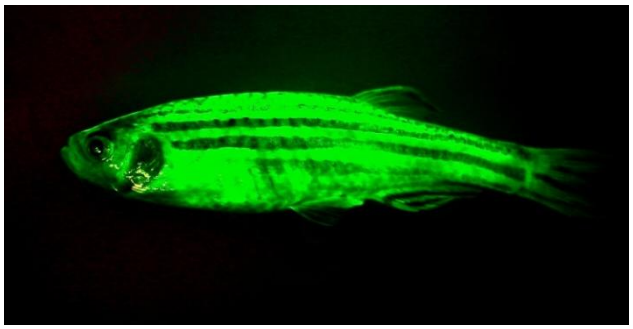

Figura 4. Peixe zebrafish transgênico para o GH e a proteína verde fluorescente (GFP) da linhagem F0104 exposto à luz ultravioleta.

Trabalhos realizados com a linhagem F0104 demonstraram alguns efeitos colaterais da transgenia. Figueiredo *et al.* (2007b) avaliaram o crescimento e a expressão de genes relacionados ao eixo somatotrófico em peixes hemizigóticos e homozigóticos da linhagem F0104. Os resultados obtidos demonstraram que, apesar dos homozigóticos terem maior expressão do GH exógeno em relação aos selvagens e aos hemizigotos, o seu crescimento não foi significativamente diferente dos selvagens, mas os hemizigotos apresentaram um crescimento significativamente superior aos outros dois grupos, sugerindo que o excesso de GH não reflete, necessariamente, um aumento direto na performance do crescimento. Corroborando estes resultados, as análises da expressão do IGF-I demonstraram que os

hemizigotos expressaram maiores níveis deste gene em relação às outras duas classes analisadas. Studzinski *et al.* (2009) verificaram que proteínas supressoras de citoquinas SOCS1 e SOCS3 regulam negativamente a via de sinalização do GH nos peixes homozigotos, bloqueando o crescimento destes indivíduos. Rosa *et al.* (2008) constataram que os indivíduos homozigotos da linhagem F0104 apresentaram um maior consumo de oxigênio e uma maior produção de espécies reativas de oxigênio em relação aos hemizigotos e aos não-transgênicos, relacionando o excesso de GH com o potencial de geração de estresse oxidativo. Com relação a parâmetros reprodutivos, Figueiredo *et al*. (2013) demonstraram que indivíduos da linhagem F0104 apresentaram uma redução nos parâmetros espermáticos como motilidade, integridade do DNA e da membrana plasmática além, da diminuição da fertilização e eclosão. Adicionalmente, outros estudos foram realizados com esta linhagem mostrando uma série de efeitos pleiotrópicos do GH sobre a estrutura muscular, o estresse osmótico e o sistema imune (Almeida *et al*., 2013; Batista *et al*., 2014; Kuradomi *et al*., 2011). Entretanto, nenhum estudo foi realizado com esta linhagem abordando os efeitos colaterais do GH sobre o comportamento reprodutivo, atividade locomotora, agressividade, cognição e memória.

Objetivo Geral

Analisar os efeitos do hormônio do crescimento (GH) sobre o comportamento reprodutivo, atividade locomotora, agressividade, aprendizado e memória em um modelo de zebrafish transgênico.

Objetivos específicos

- Avaliar o comportamento reprodutivo de machos de zebrafish transgênicos para o GH a partir de componentes intrasexuais;

- Analisar a capacidade de aprendizado e memória de zebrafish transgênicos para o GH através do teste de comportamento exploratório e esquiva inibitória;
- Avaliar a influência do GH sobre a expressão gênica de diferentes subunidades dos receptores AMPA e NMDA em larvas e no cérebro de zebrafish adultos.

Biossegurança e ética na experimentação animal

Para o desenvolvimento de pesquisa com organismos geneticamente modificados a legislação brasileira (Lei 11.105, de 24 de março de 2005) determina a necessidade de requisição de um CQB (Certificado de Qualidade em Biossegurança) da instituição interessada junto a Comissão Técnica Nacional de Biossegurança (CTNBio). A FURG possui CQB desde maio/1999 (CQB No. 0112/99), seguindo as normas determinadas pela Instituição Normativa no. 12 (DOU 28/05/98). Em relação ao segundo e ao terceiro artigo, todos os procedimentos utilizados foram revisados e aprovados pela Comissão de Ética em Uso Animal da Universidade Federal do Rio Grande - CEUA/FURG (Processo 23116.005578/2013-83).

ARTIGO I

Título: Intrasexual components of GH-transgenic zebrafish (*Danio rerio*) do not favor reproductive success

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Intrasexual components of GH-transgenic zebrafish (*Danio rerio*) do not favor reproductive success

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Highlights:

We evaluated GH-transgenic fish on intrasexuals components.

GH-transgenic male are aggressive, but they don't have higher reproductive success.

GH-transgenic dominates competitions only when they have larger size.

Transgenic males do not efficiently stimulate females.

Abstract

Growth hormone (GH), apart from inducing animal growth, exerts several effects on organisms, including on behavior and reproduction. In order to evaluate the effect of GH on reproduction, we analyzed intrasexual (male dominance in the reproductive territory, aggressiveness and locomotor activity) and intersexual (female receptiveness) components in a GH-transgenic zebrafish (*Danio rerio*) model. Results showed that although transgenic individuals increases locomotion and aggressive behavior, they did not obtain higher success in reproductive competitions. Transgenic males presented mating advantage only when they were larger than non-transgenics. In addition, crosses between transgenic males and non-transgenic females produced less spawn. These results can be explained in terms of the high energetic cost imposed by GH-trangenesis, which entails damage to other systems including reproduction.

Keywords: Growth hormone; Intrasexual competition; Spawning frequency; Locomotor activity; Aggressiveness.

1. Introduction

Growth hormone (GH) is a protein hormone produced and secreted mainly by the adenohypophysis, and acts by interacting with its specific receptor (GHR) through the GH/IGF-I (insulin-like growth factor I) axis in several systems, leading to the animal's somatic growth (Le Roith et al., 2001). Although the GH gene has been genetically manipulated for increasing growth rates in fish, with promising results for aquaculture, this manipulation can also have side effects. Behavioral and physiological alterations have been observed in GH-transgenic fish, and include increase in appetite, competitiveness for food, locomotor ability, aggressiveness, and food conversion efficiency (Abrahams and Sutterlin, 1999; Devlin et al., 1999; Duan et al., 2011; Fu et al., 2007; Jonsson et al., 1996). Considering that GH-transgenic fish will likely be commercialized in the near future (Ledford, 2013), it is necessary to investigate whether the observed behavioral alterations can lead to advantages in these fish, in case of eventual leaks into the environment (Muir and Howard, 1999).

Knowing that GH and IGF-I receptors are found in fish gonads, it is evident that GH acts in several processes that involve reproduction (Berishvili et al., 2006; Le Gac et al., 1996). However, studies on the effects of GH overexpression on reproduction have focused mainly on fertility; for example, GH-transgenic coho salmon (*Oncorhynchus kisutch*) show reduced sperm quality (Fitzpatrick et al., 2011). *In vitro* studies on the other hand have not verified this decrease in sperm quality in GH-transgenic coho salmon and carp (Bessey et al., 2004; Zhong et al., 2012). Reproductive behavior is also an important component of animal fitness, and can be divided in two phases: intrasexual selection, occurring between males of the same species when disputing reproductive territory and females, and intersexual selection, characterized by the selection of the most attractive male by the female. Aggressiveness and locomotion can influence intrasexual selection, while intersexual selection depends on criteria that vary between species, such as color, size, nest quality (in species with parental care),

kinship (in species that avoid consanguinity), resistance to parasites, aggressiveness, and ability to compete (Katsiadaki and Sebire, 2011).

In order to investigate the side effects of GH overexpression, our research group developed a transgenic zebrafish (*Danio rerio*) model that overexpresses a piscine exogenous GH gene (Figueiredo et al., 2007a). Apart from increased growth rates, studies with this transgenic lineage (named F0104) have demonstrated a series of pleiotropic effects on intracellular regulation of GH, muscle structure, susceptibility to osmotic stress, metabolic rates, production of reactive oxygen species, aging, and the immune system (Almeida et al., 2013; Batista et al., 2014; Figueiredo et al., 2007b; Kuradomi et al, 2011; Rosa et al., 2008; Rosa et al., 2010; Rosa et al., 2011; Studzinski et al., 2009). In terms of reproductive parameters, a study with F0104 fish demonstrated a decrease in motility, DNA integrity, and sperm membrane, as well as reduced fertilization and eclosion (Figueiredo et al., 2013).

Considering that GH interferes with reproductive and behavioral parameters, the present studied aimed towards evaluating important fitness components of F0104 lineage fish, in order to gain a better understanding of the reproductive behavior of animals that overexpress GH. This was achieved by evaluating intrasexual components such as male dominance capacity in the reproductive territory, aggressiveness, locomotion, and intersexual components such as female receptivity.

2. Material and methods

2.1. Transgenic fish

In this study transgenic fish of the F0104 lineage were used, produced by co-injecting two constructions under the transcriptional control of ß-actin of carp *Cyprinus carpio*. The first construction drives gene expression of the marine silverside *Odonthestes argentinensis* growth hormone, and the second regulates expression of green fluorescent protein (GFP) (Figueiredo et al., 2007a). Transgenic and non-transgenic fish were obtained by crossing

hemizygous transgenic males of lineage F0104 with non-transgenic females. The Mendelian proportion between transgenic and non-transgenic was observed in all progenies.

For reproductive behavior experiments, non-transgenic females were kept isolated from males in 25 L aquariums in a closed system with continuous water flow and total water renovation every 30 min. Individual transgenic (T) and non-transgenic (NT) males were maintained in 3 L aquariums under controlled conditions for one week. For evaluation of aggressive behavior, fish were individualized in 25 L aquariums for 72 h in the closed system. All fish were kept under the following conditions: 28°C, oxygenation near saturation, pH around 7, and a 14 h L: 10 h D photoperiod. Animals were fed twice daily with commercial feed (ColorBits, Tetra). The experiments were conducted in accordance to Brazilian legislation about ethics on animal experimentation.

2.2. Male dominance

For the male dominance experiment, males and females were kept in separate aquariums. The reproductive behavior of domesticated zebrafish is influenced by the photoperiod, with reproduction occurring over the first hours of light after a dark cycle (Darrow and Harris, 2004). In this manner, the experiment began as soon as the lights turned on. Males were simultaneously placed in the aquarium of non-transgenic females, at a 2:1 male:female ratio. We then placed a trap for egg collection, which is considered the reproductive territory of the aquarium (Spence and Smith, 2005). This trap consists of an open plastic box covered with mesh that permits only the passage of eggs, with artificial vegetation on top that stimulates spawning. This reproductive territory is invariably dominated by one of the males after competing for the female.

The time elapsed until one of the males dominated the reproductive area, preventing the approach of the other male, was established. Time was measured with a chronometer from

the moment males were placed into the female's aquarium, and reproductive behavior was monitored for 90 min. In order to differentiate fish, morphological characteristics such as fin size and head and operculum format were observed. Additionally, transgenesis was confirmed in males by identification of the green fluorescent marking of GFP, under ultraviolet light.

During mating we identified three distinct phases of male competition described in Table 1.

2.2.1. Experiment 1: non-transgenic versus transgenic fish, same age and different weights

Twenty reproductive competitions were performed between non-transgenic (NT) and transgenic (T) fish of same age (5-6 months) and different weights. Four transgenic and five non-transgenic males were used, with each transgenic competing once with each non-transgenic male. Mean weight (mean ± SEM) of non-transgenic individuals was 295 ± 26 mg, while transgenic fish weighed 455 ± 35 mg.

2.2.2. Experiment 2: transgenic and non-transgenic fish, same weight and different age

Mating competition between five transgenic (6 months) 13 non-transgenic males (8-9 months) of same sizes and different age was evaluated through 19 tests. Only individuals with similar weight were used in competitions, with some non-transgenic individuals used no more than twice. Mean weights of transgenic and non-transgenic fish were 435 ± 85 mg and 432 ± 84 mg, respectively. This experiment aimed to verify if any factors apart from size offer an advantage to transgenic fish.

2.2.3. Experiment 3: non-transgenic males, different age and sizes

Twenty reproductive competitions were performed between non-transgenic fish of distinct age and sizes. In this experiment we used four males of the larger size (9 months)

class against five males (5 months) of the smaller size class, and each male of one class competed once against each male of the other class. Mean weight (± SEM) of the larger non-transgenic males was 491 ± 82 mg, and the five smaller males weighed 274 ± 41 mg. The goal of this experiment was to verify if size is an important characteristic in competition between non-transgenic males for a female.

2.3. Spawning frequency and locomotor activity

We monitored spawning resulting from standard mating (two females for each male) that involved crosses only between non-transgenic fish (n = 6), and non-transgenic females x hemizygous transgenic males (n = 6). Females had used sexual maturity and approximate body weight to males tested. Animals were kept in aquariums with traps simulating the reproductive territory, which were removed 2 h after the lights came on for egg collection. Spawning frequency was calculated as the number of spawning events for each group during 30 days of experiment.

Locomotion of adult non-transgenic (n = 17) and transgenic male fishes (6-8 months) (n = 20) was evaluated using an open field test. These experiments were conducted in a tank (25 x 40 cm) with 5 x 5 cm quadrant subdivisions on its floor and 2.5 cm water depth, allowing only horizontal swimming. Water temperature was maintained at around 27ºC. Fish were placed individually into the tank and acclimated for 30 s, and locomotor activity was measured by quantifying the number of quadrants crossed over 180 s.

2.4. Aggressiveness

Aggressive behavior was evaluated through mirror stimulus tests (Ariyomo and Watt, 2012, 2013, Desjardins and Fernald, 2010; Gerlai et al., 2000). Before tests, fish remained in isolated aquariums for 72 h. Transgenic (n = 15) and non-transgenic males (n = 14) with ages

between 6-8 months were individually placed into a small aquarium (30 cm length x 15 cm height x 10 cm width). A mirror was positioned with an angular tilt of 22.5° from the back wall of the aquarium, in such a way that the left vertical end of the aquarium was in contact with the mirror. Therefore, when the fish swam at the left side of the aquarium, its reflected image seemed closer. Fish were filmed for 1 min after 30 s acclimation, followed by a second round of 1 min after 10 min habituation (Gerlai et al., 2000). For image analysis the aquarium was divided with vertical lines into four sections (SE1, SE2, SE3 and SE4), allowing quantification of the number of times fish entered each sector. Behavior was considered aggressive when fish remained at the segment to the far left (SE1) fighting with its own image, or its "opponent".

2.5. Statistical analyses

Differences in dominance times were tested through ANOVA, followed by Tukey's test for multiple comparisons. In order to test for differences between observed and expected frequencies in mating competitions of differently sized males, a Qui-squared test was used. In this case, the conservative hypothesis was that the tested classes presented the same chance of success. In the spawning frequency, locomotor activity and aggressiveness tests, ANOVA followed by paired T tests was used. Data are expressed as mean ± SEM. All tests were performed with a significance level set at 5%.

3. Results

3.1. Reproductive behavior

In the mating competition between transgenic and non-transgenic males of same age and different sizes, transgenic fish won 100% of crosses. In the second experiment, larger non-transgenic males dominated 100% of crosses. When competing similarly sized transgenic and non-transgenic males, non-transgenic individuals won 89% of the time (17/19), while

transgenic fish won only 11% of crosses (2/19) ($P < 0.01$, Qui-squared) (Figure 1). Mean to acquire of dominance times were not significantly different between transgenic (17.6 ± 4.4 min – experiment 1) and non-transgenic (17.1 ± 5.8 min – experiment 2 and 23.5 ± 5.8 min – experiment 3) fish ($P > 0.05$).

3.2. Spawning frequency and locomotor activity

Monthly spawning frequency of non-transgenic fish was 9.66 ± 2.33, while reproduction of transgenic males with non-transgenic females presented frequency of 3.66 ± 2.33 ($P < 0.05$) (Figure 2a). Non-transgenic fish crossed a mean (± SEM) of 87.06 ± 14.06 quadrants, while transgenic fish crossed 149.3 ± 13.8 throughout 180 s ($P < 0.05$) (Figure 2b).

3.3. Aggressive behavior

In the first minute of the aggressive behavior test, no difference between the two groups was observed (NT 10.14 ± 2.24 s and T 11.47 ± 1.76 s) ($P > 0.05$). After 10 min of habituation, the transgenic group confronted its own image for more time at SE1 (27.47 ± 3.12 s) while the non-transgenic group remained 14.14 ± 1.95 s ($P < 0.05$). It was also verified that the transgenic group presented higher confrontation time 10 min after habituation, when compared to the first minute of analysis (Figure 2).

4. Discussion

In the present study, adult hemizygous males of the F0104 lineage demonstrated increased aggressiveness and locomotion, but success in reproductive competitions occurred only when they were larger than non-transgenics. Also, larger non-transgenic males beat smaller ones; an expected result when considering that size is a determinant factor in teleost mating (Wootton, 1998). However, female zebrafish can also display courting behaviors with submissive males (Watt et al., 2011). These results agree with the GH-transgenic Atlantic

salmon (*Salmo salar*), even when larger than farmed fish have not been shown to obtain mating advantage (Moreau et al., 2011). This disadvantage has been related to the exaggerated size of GH-transgenic salmon, which hinders the approximation of females and allows smaller individuals to court females more easily (Bessey et al., 2004).

The increased locomotion observed in F0104 individuals has also been shown in GH-transgenic Atlantic salmon (*Salmo salar*) (Abrahams and Sutterlin, 1999) and rainbow trout (*Oncorhynchus mykiss*) treated with GH (Johansson et al., 2004; Johansson et al., 2005; Jonsson et al., 1996; Jonsson et al., 2003). However, GH-transgenesis in carp *Cyprinus carpio* has been shown to decrease swimming capacity (Li et al., 2007). Studies with GH-transgenic *Oncorhynchus kisutch* have also shown reduction in locomotor activity (Farrell et al., 1997; Lee et al., 2005). These authors raised the hypothesis that this reduction occurs due to excessively rapid growth in locomotor muscles, which leads to deficiency in these muscles and other associated systems, such as respiration and circulation. It is possible that these contradictory observations are result of the inherent random events associated to transgene integration into host genome. In addition, the transgene effect can also be species-specific, which makes difficult any comparison among transgenic lines.

Based on the results obtained in the reproductive behavior test, it seemed that transgenic fish did not present any behavioral alteration besides increased locomotion that could favor it in competition. However, when evaluating aggressive behavior through the mirror test, it was observed that transgenic fish presented higher confrontation time after 10 min of habituation than non-transgenic individuals. The mirror test has been used by several authors and is efficient for quantifying the aggressive behavior of fish (Ariyomo and Watt, 2012, 2013; Desjardins and Fernald, 2010; Gerlai et al., 2000). Several studies report that GH transgenesis causes an increase in aggressive behavior, locomotion, appetite and ability to compete for food (Abrahams and Sutterlin, 1999; Devlin et al., 1999; Duan et al., 2011;

Sundström et al., 2003). In GH-transgenic carps, aggressiveness levels were 2.7 times higher than non-transgenic individuals (Duan et al., 2011). Similar results have been found for juvenile rainbow trout treated with GH (Johnsson and Bjornsson, 1994; Jonsson et al., 1998) and GH-transgenic coho salmon (*Oncorhynchus kisutch*) (Sundström et al., 2004). However, Bessey et al. (2004) evidenced that GH-transgenic coho salmon are less aggressive than non-transgenics. The authors attribute this reduced aggressiveness to a combination of genetic and environmental factors. Aggressiveness between individuals of the same species can be a stress factor with negative impacts on reproduction (Le Galliard et al., 2005; Marchlewska-Koj, 1997). Studies on the reproductive behavior of zebrafish suggest that very aggressive males display agonistic postures for a longer period of time, impairing interaction with females and leading to lower reproductive success (Sih and Watters, 2005; Spence and Smith, 2005). The mean to acquire of dominance time of transgenic males of the F0104 lineage in the first experiment was not significantly different from non-transgenic fish in the remaining experiments, discarding the possibility of lower reproductive success caused by the increase in confrontation time in transgenic males.

If locomotor activity and aggressiveness were crucial for intrasexual selection, as revealed by some studies that report a tendency towards dominance in more aggressive and active animals (Spence and Smith, 2005; Paull et al., 2010), transgenic fish would have dominated all experiments. However, when F0104 transgenic males were compared to non-transgenics of same weight, transgenic fish presented disadvantage in competitions for reproductive territory. GH-transgenic coho salmon (*Oncorhynchus kisutch*) have been shown to reduce chasing and biting behavior, as well as nest fidelity, when compared to non-transgenics (Bessey et al., 2004). Additionally, Fitzpatrick et al. (2011) observed reduced courting behavior in GH-transgenic fish of this species, as well as impaired spermatic parameters. Figueiredo et al. (2013) also described deficient spermatic parameters for

individuals of the F0104 lineage. In the same manner, GH-transgenesis in mice leads to negative effects on sperm and fertility (Bartke et al., 1992). A possible explanation for these effects could be the energetic cost of rapid growth caused by GH excess. GH-transgenic fish display accelerated growth and even gigantism as observed in salmon and carp (Devlin et al., 1994; Nam et al., 2001), which has a high cost for the animal and can affect other systems. Individuals of the F0104 lineage show increased metabolic rates and higher oxygen consumption (Rosa et al., 2008; Rosa et al., 2010), as well as immune system problems (Batista et al., 2014). Alterations in the respiratory metabolism have also been observed in salmon (Cook et al., 2000; Deitch et al., 2006; Herbert et al., 2001; Seddiki et al., 1996; Stevens et al., 1998) and tilapias (McKenzie et al., 2003) that overexpress GH.

Although there are some studies on the reproductive parameters of GH-transgenic individuals, studies on spawning frequency are rare. In the present study, spawning frequency of crosses between transgenic males and non-transgenic females was lower (3.66 ± 2.33) than non-transgenic individuals (9.66 ± 2.33) over 30 days ($P < 0.05$). Previous studies on the spawning of F0104 individuals showed decrease in fertilization rates and eclosion frequency (Figueiredo et al., 2013). Moreau et al. (2011) also observed reduced fertilization in GH-transgenic salmon (*Salmo salar*). However, *in vitro* analyses of GH-transgenic homozygous *Cyprinus carpio* did not show reduction in fertility and eclosion (Lian et al., 2013; Zhong et al., 2012), but this could be due to the controlled environment of *in vitro* experiments that lead to different responses. *In vivo* studies, on the other hand, are more faithful to natural reproductions.

The results obtained here show that GH overexpression in zebrafish of the F0104 lineage increases locomotion and aggressive behavior, but does not lead to higher reproductive success. GH-transgenic males presented mating advantage only when they were larger than non-transgenic individuals. Since these animals presented accelerated growth with

high energetic costs, it becomes clear that even under controlled conditions there is not enough energy to meet the individual's energetic demand. The behavioral alterations of higher aggressiveness and locomotor activity also represent a cost for the organism. Individuals are likely directing energy towards growth, which entails damage to other systems including reproduction.

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Table

Table I. Ethogram of dominance phases of non-transgenic and transgenic male zebrafish (*Danio rerio*) during reproductive behavior.

Phases	Description
Chase	Males chase the female swimming rapidly for a relatively short period.
Dispute	Males initiate aggressive interactions with chase, try to bite, and erected fins.
Dominance	Dominant male subdues another male and tends to prevent their entry into reproductive territory (trap).

Figure captions

Figure 1. Winning percentage in mating competition between transgenic and non-transgenic zebrafish males. **(a)** The same age (5-6 months) and different size: small non-transgenic - NT (S) (295 ± 26 mg) and larger transgenic - T (L) (455 ± 35 mg). **(b)** Different age and approximately same size: non-transgenic – NT (8-9 months) (432 ± 84 mg) and transgenic - T (6 months) (435 ± 85 mg). **(c)** Different age and different size: small non-transgenic – NT (S) (5 months) (274 ± 41 mg) and larger non-transgenic –NT (L) (9 months) (491 ± 82 mg) ($P < 0.01$).

Figure 2. (a) Spawning frequency, calculated as the number of spawning for each zebrafish group (non-transgenic – NT and transgenic – T) during 30 days (* $P < 0.05$) ($n = 6$ per group). (b) The number of quadrants crossed in 180 s by non-transgenic - NT ($n = 17$) and transgenic - T ($n = 20$) male zebrafish, after 30 s of habituation (** $P < 0.01$). Date expressed mean ± SEM.

Figure 3. Time of aggressive interactions of non-transgenic - NT ($n = 14$) and transgenic - T ($n = 15$) male zebrafish (*Danio rerio*) with their own image, in the nearst mirror segment, during the first minute after 30 s of habituation and at the tenth minute, after 10 min habituation. Date expressed mean ± SEM (* $P < 0.05$).

Figure 1.

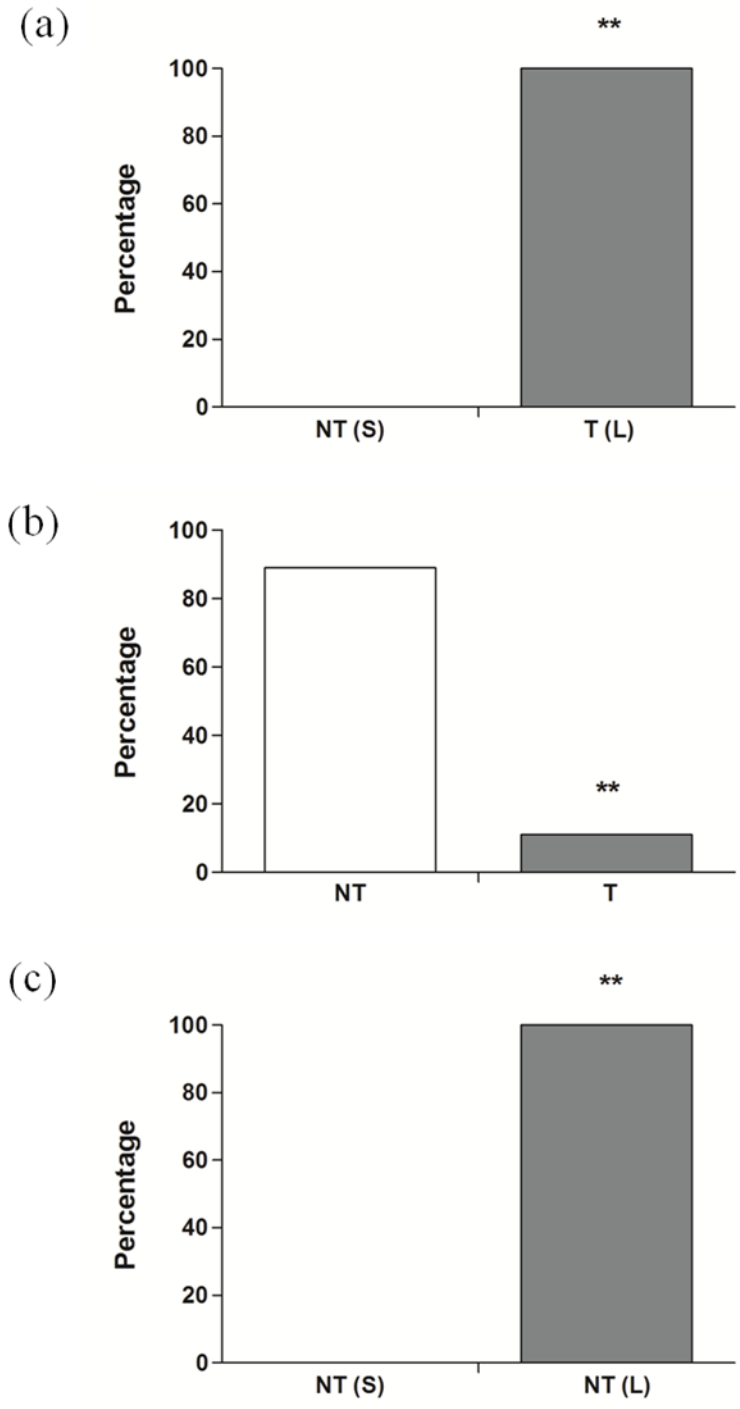

(a)
**
100
80
60
40
20
0
Percentage
NT (S)
T (L)
(b)
100
80
60
40
20
0
Percentage
**
NT
T
(c)
**
100
80
60
40
20
0
Percentage
NT (S)
NT (L)

Figure 2.

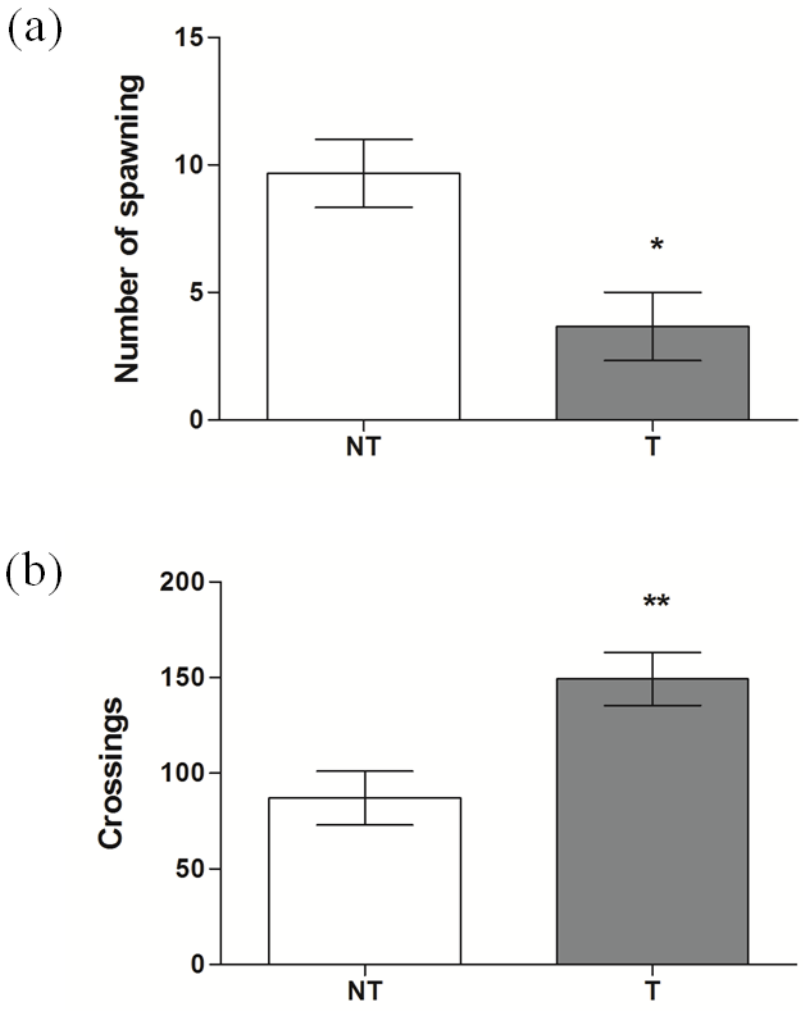
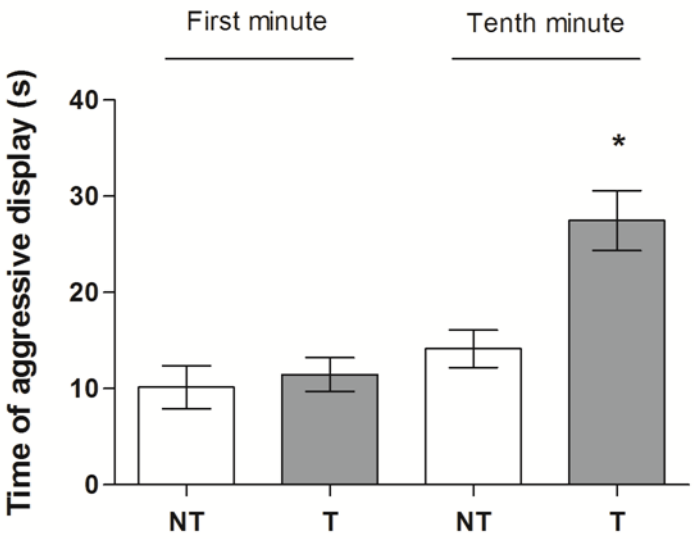

(a)
Number of spawning
15
10
5
0
*
NT
T
(b)
Crossings
200
150
100
50
0
**
NT
T

Figure 3.


First minute
Tenth minute
Time of aggressive display (s)
40
30
20
10
0
*
NT
T
NT
T

1

2

3

ARTIGO II

Título: Growth hormone (GH) increases cognition and expression of ionotropic glutamate receptors (AMPA and NMDA) in transgenic zebrafish (*Danio rerio*)

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Growth hormone (GH) increases cognition and expression of ionotropic glutamate receptors (AMPA and NMDA) in transgenic zebrafish (*Danio rerio*)

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Abstract

The GH/IGF-I somatotropic axis is responsible for somatic growth in vertebrates, and has important functions on the nervous system. Among these, learning and memory functions related to the neural expression of ionotropic glutamate receptors, mainly types AMPA and NMDA, can be highlighted. Studies on these mechanisms have been almost exclusively conducted on mammal models, with little information available on fish. In this manner, this study aimed at evaluating the effects of the somatotropic axis on learning and memory of a GH-transgenic zebrafish (*Danio rerio*) model (F0104 lineage). Exploratory behavior and long-term memory (LTM) were tested in an inhibitory avoidance apparatus, and cerebral expression of *igf-I* and genes that code for the main subunits of the AMPA and NMDA receptors were evaluated. Results showed no exploratory differences, but a significant increase in LTM was noted for transgenic fish. Transgenic animals also showed a generalized pattern of increase in the expression of AMPA and NMDA genes, as well as a three-fold induction in *igf-I* expression in the brain. When analyzed together, these results indicate that GH, mediated by IGF-I, has an important effect on the brain, with improvement in LTM as a result of increased glutamate receptors. The transgenic lineage F0104 was shown to be an interesting model for elucidating the intricate mechanisms related to the effect of the somatotropic axis on learning and memory in vertebrates.

Keywords: Growth hormone overexpression, Transgenic zebrafish, F0104 line, Brain, Long-term memory, Inhibitory avoidance, Ionotropic receptors, AMPA, NMDA, Insulin-like growth factor I.

Introduction

Growth hormone (GH), or somatotropin, affects growth and differentiation of vertebrates through mechanisms that depend on the activation of GH receptors (GHR) and an intracellular signaling cascade that culminates with synthesis of growth factors, especially insulin-like growth factor I (IGF-I) (Le Roith *et al*. 2001). The GH/IGF-I axis also acts upon the central nervous system, affecting growth, neurogenesis and neuroprotection (Nyberg 2000; O'Kusky & Ye 2012; Ramsey *et al*. 2004; Schneider-Rivas *et al.* 1995). Although the main source of GH is the hypophysis, other tissues of the nervous system also produce small quantities of this hormone, acting in a paracrine or autocrine manner (Aberg *et al*. 2006; Sonntag *et al*. 2005). In the human hippocampus, for example, the presence of GH receptors suggests a specific effect of the GH/IGF axis on cognitive functions (van Dam *et al*. 2000). In non-human mammals, the concentration of GH/IGF-I in the brain changes with age (Le Grevès *et al*. 2005; Sun *et al*. 2005). The use of supplementary IGF-I in older rodents has been shown to revert cognitive deficit (Markowska *et al*. 1998). In clinical trials conducted with GH-deficient humans, it was demonstrated that GH reposition improves memory and cognitive functions, especially short- and long-term memory (STM and LTM, respectively) (Arwert *et al*. 2005; Burman *et al.* 1995; Johansson *et al.* 1995).

The effects of GH on cognitive functions alter ionotropic glutamate receptors AMPA (α-amino-3hydroxy-5methylisoxazole-4propionic) and NMDA (N-methyl-D-aspartate) (Le Grevès *et al*. 2006; Mahmoud & Grover 2006; Molina *et al*. 2012; Park *et al*. 2010). AMPA and NMDA receptors are involved in two important mechanisms associated with synaptic plasticity, long-term potential (LTP) and long-term depression (LTD), which act in memory formation and involve transmission of glutamate, the main central nervous system excitatory neurotransmitter (Collingridge & Singer 1990). The induction of hippocampal LTP requires NMDA receptors and agonistic effects on this receptor increase cognitive potentiality, while

antagonistic effects decrease abilities such as locomotor activity, learning and memory in zebrafish (*Danio rerio*) (Blank *et al*. 2009; Swain *et al*. 2004). Although studies have revealed that hippocampal LTP requires activity of NMDA receptors, synaptic efficiency increase depends partly on recruitment of AMPA receptors (Malinow & Malenka 2002). In mammals, AMPA receptors present four genes that express *glur1-4* or *A-D* subunits, also known as *gria1-gria4a* (Rosenmund *et al*. 1998), and can be found in homomeric and heterotetrameric forms (Bredt & Nicoll 2003; Hollmann & Heinemann 1994). NMDA are heterotetrameric, present five genes expressing *nr1-nr2* subunits (Ishii *et al*. 1993; Moriyoshi *et al*. 1991), and unlike other members of the ionotropic glutamate receptor family, require glycine as a co-agonist for activation, high calcium permeability and a voltage-dependent magnesium block (Hollmann & Heinemann 1994). In zebrafish, due to the duplication of the genome, eight genes express AMPA receptor subunits and ten genes express NMDA subunits (Cox *et al*. 2005; Hoppmann *et al*. 2008).

Regarding AMPA subunits Glur1a, Glur1b, Glur2a, Glur2b, Glur3a, Glur3b, Glur4a and Glur4b, Hoppmann *et al*. (2008) verified that in zebrafish at 24 hours post fertilization (hpf) and 48 hpf all genes that express the eight subunits are found in the telencephalon and posterior brain. At 72 hpf, with the exception Glur3a and Glur3b, all subunits are expressed in the optic tectum, dorsal and ventral medulla oblongata, spinal chord, retinal cells, telencephalon and olfactory bulb. Some NMDA subunits are found in zebrafish retinae, such as Grin2a.1 and Grin2a.2 at 48 hpf, and Grin2d.1 e Grin2d.2 are detected in the posterior brain and spinal chord. At 24 hpf and 48 hpf, the genes expressing Grin1.1 and Grin1.2 subunits are found in the spinal chord, retina, and the anterior and posterior brain (Cox *et al*. 2005).

The majority of studies that evaluate the effects of GH on AMPA and NMDA receptors and cognitive functions use hormone administration in rodent models. Depending

on the administration pathway, dosage, and invasiveness, different responses are observed (Le Grevès *et al*. 2006; Park *et al*. 2010; Ramis *et al*. 2013). In order to evaluate the side effects of excess GH, our research group developed a GH-transgenic zebrafish lineage (F0104 lineage) that overexpresses the GH gene in a constitutive manner (Figueiredo *et al*. 2007). Considering that zebrafish have been widely used as a vertebrate model to study genetic, developmental and neurobehavioral processes (Guo 2004; Norton & Bally-Cuif 2010; Swain *et al*. 2004), the F0104 lineage represents an interesting model for evaluating how GH affects memory and cognitive functions. No studies have yet assessed this influence, and in this context, the present study aims to evaluate the effect of GH overexpression on learning and memory through behavioral tests and expression of genes that encode ionotropic glutamate receptors AMPA and NMDA in zebrafish brain.

Materials and Methods

Transgenic fish

Transgenic fish of the F0104 lineage were produced by co-injecting two genetic constructions under transcriptional control of the ß-actin promoter of *Cyprinus carpio*. The first construction directs growth hormone gene expression of the marine silverside *Odonthestes argentinensis*, and the second regulates green fluorescent protein (GFP) expression (Figueiredo *et al*. 2007). Fluorescence is used as a marker and allows *in vivo* identification of transgenesis. Transgenic and non-transgenic fish were obtained by crossing F0104 hemizygous transgenic males with non-transgenic females, resulting in Mendelian proportions of transgenic and non-transgenic offspring.

Fish were kept in 15 L aquariums in a closed cultivation system with continuous water flow, total water renovation every 30 min, temperature of 28 $^{\circ}$C, oxygenation near saturation, pH around 7 and 14 hours light/10 hours dark photoperiod. All animals were fed twice daily with commercial feed containing 47% protein (ColorBits, Tetra). All procedures were

analyzed and approved by the Animal Ethics Committee of the Federal University of Rio Grande - CEUA/FURG (Process #23116.005578/2013-83).

Inhibitory avoidance apparatus

The inhibitory avoidance apparatus was developed by the Biology Institute especially for fish (Castro *et al*. 2009), and involves the association of a harmless stimulus (dark zone) with an aversive stimulus (an electrical shock). The apparatus consists of a PVC tube (33 cm X 7.3 cm X 8 cm) separated into two zones (light and dark) and filled with water. This device contains a continuous 5 mA power source that generates a potential difference of 5-6 V. The source feeds a parallel-plate capacitor at the dark zone, where the aversive stimulus is applied. A switch that opens and closes the circuit instantly, applying only an electrical pulse that does not cause additional damage to the fish, triggers the power source.

Exploratory behavior

For the exploratory behavior test we used transgenic (n = 10) and non-transgenic (n = 10) zebrafish males at the same age (7-months-old). Fish were gently placed into the light zone of the apparatus while the partition between zones remained shut. After 30 seconds of familiarization, the partition was removed and fish were allowed to explore the dark zone. Latency period for the first entrance into the dark zone, transitions from light to dark zones, and permanence time in the light zone were measured for five minutes. All behavioral experiments were recorded for posterior video analysis.

Inhibitory avoidance

After the exploratory behavior experiment, the same individuals were trained in inhibitory avoidance. Immediately after habituation the partition was removed and fish received two 5-6 V shocks upon entering the dark zone (training session), and were then removed from the apparatus and transferred to temporary aquariums. For evaluating long-term memory (LTM), animals were tested 48 h after training. Fish were habituated for 30 s

after removing the partition, and the latency of entrance into the dark zone was quantified up to a maximum 180 s. In the test session no shock was applied. Following the inhibitory avoidance test, fish were euthanized and brains were removed and stocked at -80 °C for gene expression analysis.

Gene expression

For gene expression analysis, total RNA was extracted from six whole brains of each non-transgenic and transgenic group, through TRIZol method (Invitrogen, Brazil) according to the manufacturer's instructions. RNA was quantified using a QuBit fluorometer (Invitrogen, Brazil) and sample integrity was confirmed by agarose gel electrophoresis (1%). cDNA was synthesized by reverse transcriptase of RNA using High Capacity cDNA Reverse Transcription kits (Applied Biosystems, Brazil). Gene expression was analyzed quantitatively through Real Time PCR (qPCR), with each sample analyzed in triplicate on an ABI 7500 platform Real Time System (Applied Biosystems, Brazil) using Platinum SYBR Green qPCR SuperMix – UDG (Invitrogen, Brazil). Specific primers for each gene were designed using Primer Express 3.0 software (Applied Biosystems, Brazil) based on sequences available in GenBank (http://www.ncbi.nlm.nih.gov) (Table 1). All used primers displayed efficiency close to 100% (data not shown). qPCR cycling conditions were 50 °C for 2 min, 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s. Tested reference genes were *β-actin* (beta-actin), *b2m* (beta-2-microglobuline) and *ef1α* (elongation factor 1 alpha). The *ef1α* was selected as a reference gene using the *geNorm* software (Vandesompele *et al*. 2002). Target gene expression was normalized by *ef1α*, using the $2^{-\Delta Ct}$ equation: $\Delta Ct = Ct_{target\ gene} - Ct_{ef1\alpha}$ (Livak & Schmittgen 2001).

Statistical analyses

Exploratory behavior data of groups were evaluated through paired t-test. The long-term memory results of the inhibitory avoidance test were analyzed by KrusKall-Wallis and U

Mann-Whitney tests. In gene expression analysis, assumptions of normality and homogeneity of variance were previously tested, data transformed when necessary, and comparisons conducted through paired t-tests. Data were expressed as mean ± standard error. For all tests significance level was $P < 0.05$.

RESULTS

Exploratory behavior

Latency time (seconds) of first entry into the dark zone was not significantly different between non-transgenic (17.0 ± 1.0 s) and transgenic (25.8 ± 10.3 s) fish ($P > 0.05$). The number of crossings from the light to dark zone was 13.6 ± 1.1 times for non-transgenic and 11.4 ± 2.4 for transgenic fish ($P > 0.05$). Latency in the light zone was 156.5 ± 19.2 s for non-transgenic and 196.4 ± 25.2 s for transgenic fish ($P > 0.05$) (Figure 1a, 1b and 1c).

Inhibitory avoidance

In terms of long-term memory, it was verified that after the practice session the latency time of first entry into the dark zone was significantly higher in transgenic (121.5 ± 30.5 s) than non-transgenic fish (27.3 ± 6.8 s) ($P < 0.05$) (Figure 1d).

Gene expression

Gene expression of AMPA subunits (*glur1a*, *glur1b*, *glur2a*, *glur2b*, *glur3a*, *glur3b*, *glur4a*, *glur4b*) in non-transgenic and transgenic zebrafish brains is shown in Figure 2. Significant induction was observed in transgenic fish for all tested genes except the *glur2b* subunit. Transgenic individuals presented a 20% increase in transcriptional levels of genes that express *glur1a* and *glur2a* subunits. Expression of *glur1b*, *glur3b* and *glur4a* genes increased 30%, while *glur3a* and *glur4b* showed 40% induction in transgenic when compared to non-transgenic fish ($P < 0.05$). NMDA receptor subunits *grin1b*, *grin2ab*, *grin2da* and *grin3a* showed induction in expression levels of 70, 60, 30 and 80%, respectively, in

transgenic animals ($P < 0.05$) (Figure 3). Transgenic fish brains presented three-fold induction of *igf-I* expression levels when compared to non-transgenics ($P < 0.05$) (Figure 4).

Discussion

In terms of exploratory behavior, there was no significant difference between non-transgenic and transgenic fish ($P > 0.05$). This is a valuable finding, since it excludes a variable that can interfere with LTM. Evaluation of behavior in the inhibitory avoidance apparatus evidenced that this equipment can be used to test not only different types of memory but also exploratory behavior.

When analyzing long-term memory (LTM) 48 h after training, it was observed that excess GH improved the LTM of transgenic when compared to non-transgenic individuals ($P < 0.05$) (Figure 1d). Similar results have been found in humans using GH replacement therapy, where both LTM and STM were improved (Arwert *et al*. 2005; Burman *et al.* 1995; Johansson *et al.* 1995). On the other hand, Schneider-Rivas *et al*. (1995) propose that GH replacement modulates LTM in three-month-old, but not in 24-month-old, mice. These different responses in mice are related to the natural decrease with age in GH and IGF-I concentration in the nervous system, and consequently result in memory impairment (Kinney-Forshee *et al*. 2004; Rollero *et al*. 1998).

Several studies state that IGF-I mediates action of GH (Deak & Sonntag 2012; Sonntag *et al*. 2005), and along with this hormone, increases proliferation of progenitor cells and formation of new neurons of the hippocampus (Aberg *et al*. 2006; Aberg 2010). If new nerve cells are being formed at regions where the acquisition, consolidation and recovery of memory occur, additional or optimized synaptic connections are necessary, and LTP and LTD are essential for these processes. LTP and LTD require excitation of hippocampal cells, which correspond to telencephalon in fish, by means of stimulation of ionotropic glutamate receptors such as AMPA and NMDA (Izquierdo 2011). As shown in Figure 3, analysis of genes that

express AMPA and NMDA subunits showed induction of all genes except one AMPA subunit, *glur2b*. Le Grevès *et al*. (2005) demonstrated that subcutaneous injections of IGF-I induced increase in expression of the *nr2b* subunit in young rat hippocampus. In the case of the transgenic fish used in this study, hormones are likely being produced constitutively, which could explain the systemic responses of the analyzed receptors.

The function of IGF-I on cognition and expression of ionotropic receptors is evident, and GH also directly affects these cognitive parameters. The presence of GH in the hippocampus and pre-frontal cortex of mammals suggests that it has an important function in cognition and memory mediation (Enhamre-Brolin *et al*. 2012; Le Grevès *et al*. 2006). Increase in expression levels of *nr1* and *nr2a* subunits has been found in hypophysectomized mice that received GH, with concomitant improvement in learning (Le Grevès *et al*. 2006). Additionally, mice that received inhaled and injected GH increased expression of *nr1* and *nr2b* subunits and enhanced memory acquisition (Park *et al*. 2010). Treatments with acute doses of GH in mice have evidenced its positive effects on working memory, as well as the involvement of glutamate receptors AMPA and NMDA (Ramis *et al*. 2013). *In vitro* studies demonstrated that chronic GH treatment elevates excitatory post-synaptic potential (EPSP) via AMPA and NMDA in pyramidal neurons of the CA1 region of mice hippocampus (Mahmoud & Grover 2006). Additionally, Molina *et al*. (2012) verified that GH restores synaptic transmission levels in the hippocampus in an NMDA and AMPA-dependent manner, with subsequent LTP increase. Therefore, it is clear that GH and IGF-I have direct effects on the subunits of AMPA and NMDA receptors, which are known to participate in memory formation and consolidation (Nyberg & Hallberg 2013).

Many functions of GH are attributed to circulating IGF-I produced in the liver, while others are due to IGF-I produced locally in the brain (Aberg *et al*. 2006; Deak & Sonntag 2012; Sonntag *et al*. 2005). By analyzing IGF-I gene expression in zebrafish brain, we

demonstrated that transgenic fish presented expression levels almost three times as high as non-transgenics. Increased IGF-I expression levels have also been observed in the brains of GH-transgenic Nile tilapia (*Oreochromis niloticus*) (Eppler *et al*. 2010). In mice that overexpress IGF-I in the brain, an increase in neurogenesis and synaptogenesis has been observed in the hippocampus, an important memory-forming region in mammals (O´Kusky *et al*. 2000; Ye *et al*. 2004). Humans with mutations in the *igf-I* gene or receptor (*igf-IR*) present severe growth issues, microcephaly and mental retardation (Abuzzahab *et al*. 2003; Woods *et al*. 1997). Furthermore, IGF-I infusions in rodents have been shown to cause attenuation in cognitive deficits related to the aging process (Markowska *et al*. 1998). Mitschelen *et al*. (2011) evaluated mice with the IGF-I gene knocked out in the liver and hippocampus, and observed important alterations in tissue morphology with consequent behavioral alterations such as depression. This information corroborates with the results obtained in this work, which reinforce the paracrine and autocrine effect of IGF-I on the brain, including on cognitive processes.

Although we did not analyze the specific region of the fish telencephalon that is homologous to the mammalian hippocampus (Salas *et al*. 2006), the use of a GH-transgenic lineage allowed us to evidence the biological response of excess GH on memory acquisition and consolidation. This is the first study to evaluate the effects of GH overexpression on cognitive factors such as LTM, and on genes that express the AMPA and NMDA subunits. Transgenic individuals, besides presenting improved LTM, increased expression of *igf-I* and AMPA and NMDA receptor subunits in the brain.

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Table

Table 1. Gene-especific primers designed using sequences available at GenBank (www.ncbi.nlm.nih.gov)

Gene	Primers	*GenBank*	Amplicon (bp)
glur1a	**F: 5'-CAACGACGCCATGCACTGCTCC-3'**	**NM_205598**	**74**
	R: 5'-CGATCCGCCCGTTCTCCCCAC-3'		
glur1b	**F: 5'-GGTGAGAATGGTCGTGTGATGGCC-3'**	**NM_205730**	**72**
	R: 5'-CGCCTGGCCCATACATGGCAG-3'		
glur2a	**F: 5'-CTCGGCGGATCTCCAAGCGTC-3'**	**NM_131894**	**85**
	R: 5'-CCATTCCGATCCGAAACGCGC-3'		
glur2b	**F: 5'-GGGATGACAACTGGAGGCTCTCCA-3'**	**NM_1318952**	**90**
	R: 5'-TCCGATCCGAAACGCGCTGT-3'		
glur3a	**F: 5'-ACCCAGGCTGGCTTTCCAAACCA-3'**	**NM_198339**	**96**
	R: 5'-GAGCTGAACGGCGAAGCGGAA-3'		
glur3b	**F: 5'-CGTCTCTCGCTGGCTTCCCC-3'**	**NM_198360**	**94**
	R: 5'-TGCACAGCGAATCTGAATGCGC-3'		
glur4a	**F: 5'-GCGGATCCTGGTGTTGATTCCGC-3'**	**NM_214806**	**75**
	R: 5'-CCACCGATTTGGACACTGCTGGG-3'		
glur4b	**F: 5'-ACGGCCCCAGAAGGATCGGAT-3'**	**NM_212752**	**73**
	R: 5'-GGAAGCAAGGCGTGATCCTGGG-3'		
grin1b	**F: 5'-GCGCCTGGTTCTGTTCGCCT-3'**	**NM_001144131**	**70**
	R: 5'-CACAGTCTTGGGTTCGCATCCGC-3'		
grin2ab	**F: 5'-TGCGTCCGGCTCGTGGGATT-3'**	**XM_693978**	**82**
	R: 5'-ATGCTCCCTGGGTCCGTCTGA-3'		
grin2da	**F: 5'-CCGCCACTGTTTAGGCCGGA-3'**	**XM_001921123**	**74**
	R: 5'-TGCAGCAGCTGTACATTCCCCG-3'		
grin3a	**F: 5'-GTGGCTGGGAAGAGAGCATGGC-3'**	**XM_694977**	**100**
	R: 5'- GAAGGTCCCCAGCCAAAGCGG-3'		
igf-I	**F: 5'-CAGGCAAATCTCCACGATCTC-3'**	**NM_131825**	**60**
	R: 5'-TTTGGTGTCCTGGAATATCTGT-3'		
ef1α	**F: 5'-GGGCAAGGGCTCCTTCAA-3'**	**NM_131263**	**54**
	R: 5'-CGCTCGGCCTTCAGTTTG-3'		

Figure captions

Figure 1. Exploratory behavior: (a) latency period for the first entrance into the dark zone (s), (b) crossings - transitions from light to dark zones, (c) permanence time in the light zone (s) ($P > 0.05$). Long-term memory – LTM: Latency to enter the dark zone (d) * ($P < 0.05$). Non-transgenic (NT) (n = 10) and transgenic zebrafish (T) (n = 10) Date are expressed as mean ± SEM.

Figure 2. Relative expression of genes (a) *glur1a*, (b) *glur1b*, (c) *glur2a*, (d) *glur2b*, (e) *glur3a*, (f) *glur3b*, (g) *glur4a* and (h) *glur4b* – AMPA receptor in non-transgenic (NT) (n = 6) and transgenic (T) (n = 6) zebrafish brain. Date are expressed as mean ± SEM. * $P < 0.05$;** $P < 0.01$.

Figure 3. Relative expression of genes (a) *grin1b*, (b) *grin2ab*, (c) *grin2da* and (d) *grin3a* – NMDA receptor in non-transgenic (NT) (n = 6) and transgenic (T) (n = 6) zebrafish brain. Date are expressed as mean ± SEM. * $P < 0.05$;** $P < 0.01$.

Figure 4. Relative expression *igf-I* gene in non-transgenic (NT) and transgenic (T) zebrafish brain. Date are expressed as mean ± SEM. ** $P < 0.01$.

Figure 1.

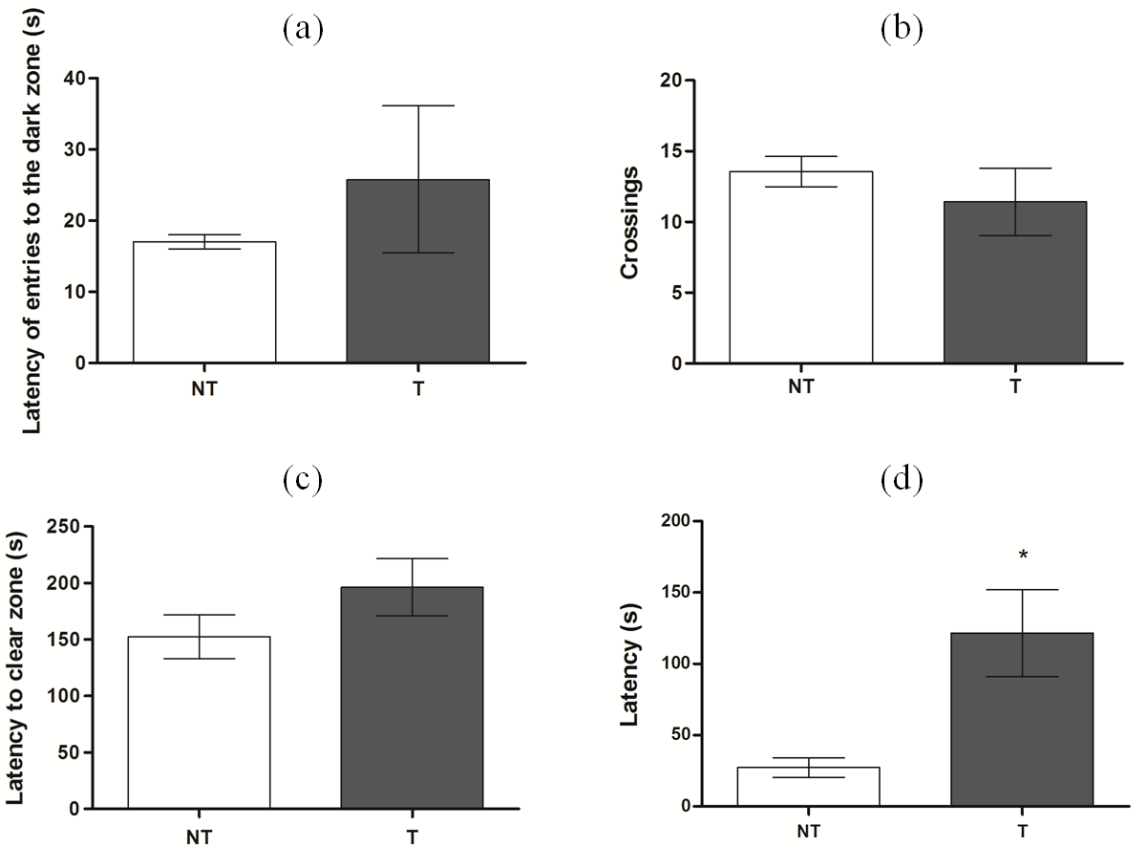

(a)
Latency of entries to the dark zone (s)
40
30
20
10
0
NT
T
(b)
Crossings
20
15
10
5
0
NT
T
(c)
Latency to clear zone (s)
250
200
150
100
50
0
NT
T
(d)
Latency (s)
200
150
100
50
0
*
NT
T

Figure 2.

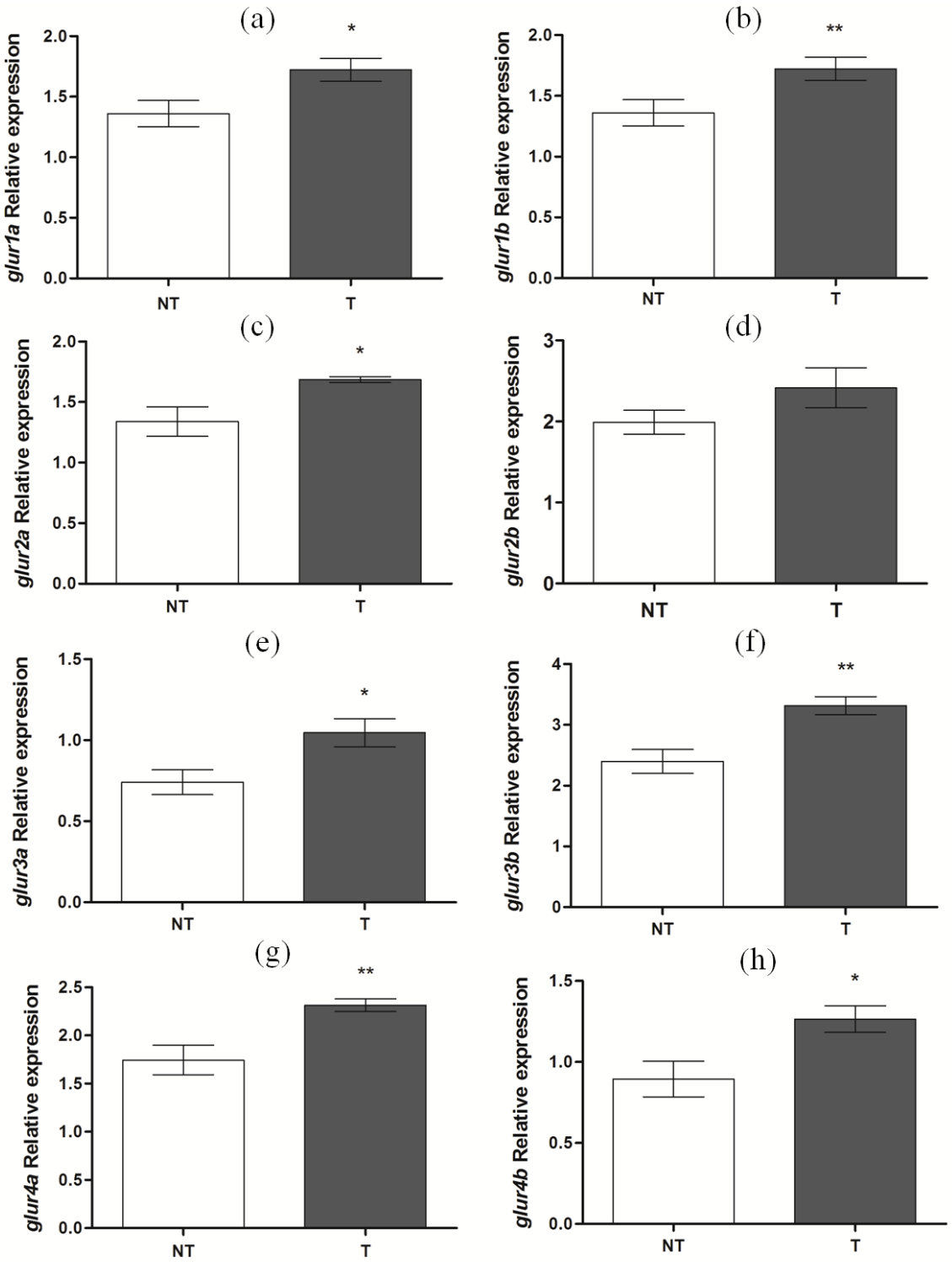

(a)
glur1a Relative expression
2.0
1.5
1.0
0.5
0.0
*
NT
T
(b)
glur1b Relative expression
**
(c)
glur2a Relative expression
*
(d)
glur2b Relative expression
3
2
1
0
(e)
glur3a Relative expression
1.5
1.0
0.5
(f)
glur3b Relative expression
4
3
2
1
(g)
glur4a Relative expression
2.5
(h)
glur4b Relative expression

Figure 3.

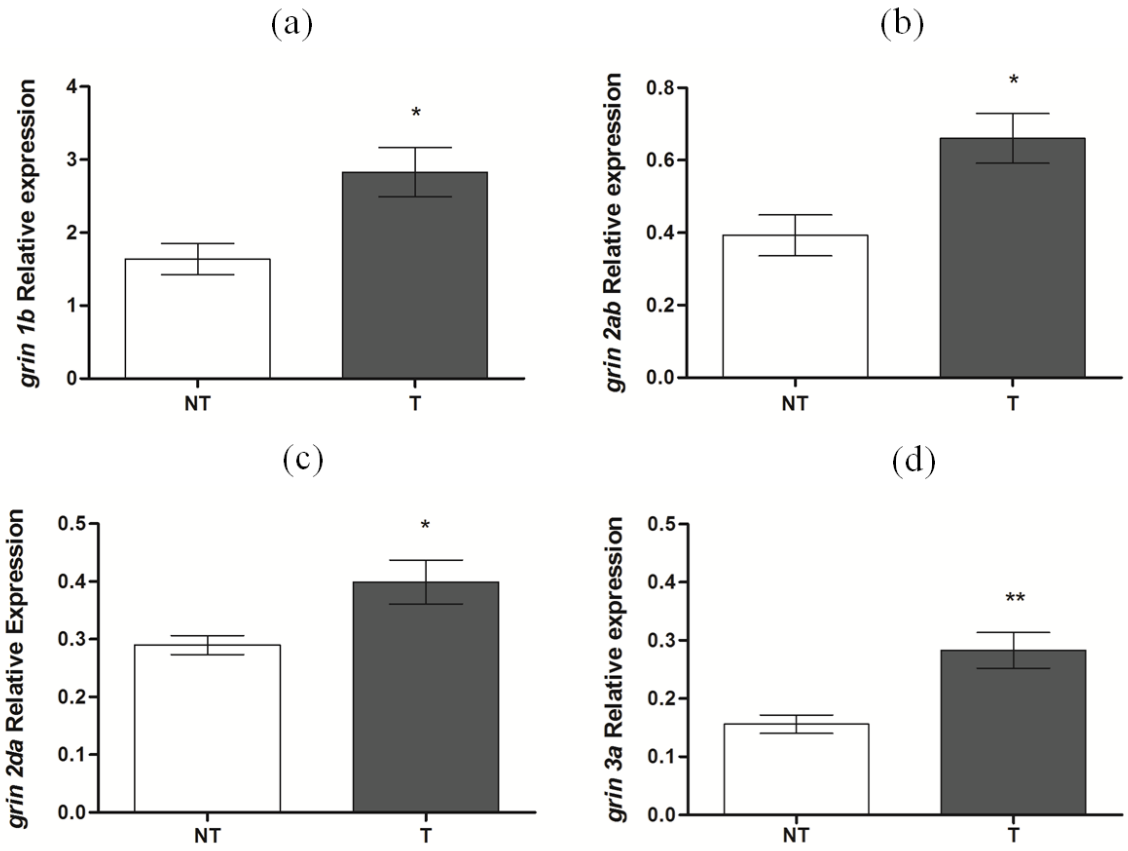
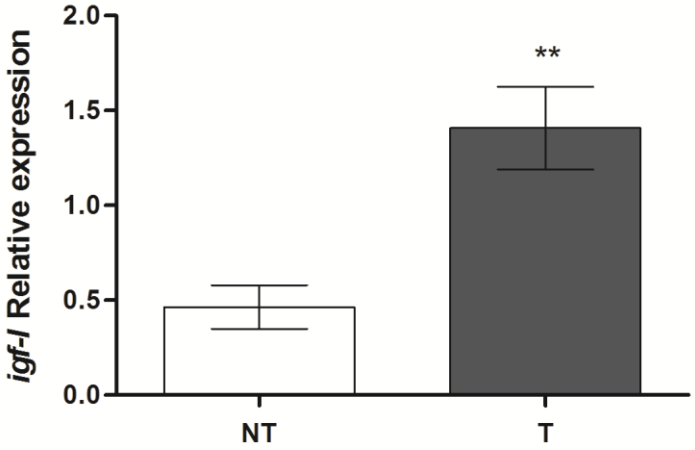

(a)
grin 1b Relative expression
4
3
2
1
0
*
NT
T
(b)
grin 2ab Relative expression
0.8
0.6
0.4
0.2
0.0
*
NT
T
(c)
grin 2da Relative Expression
0.5
0.4
0.3
0.2
0.1
0.0
*
NT
T
(d)
grin 3a Relative expression
0.5
0.4
0.3
0.2
0.1
0.0
**
NT
T

Figure 4.



ARTIGO III

Título: Effects of GH transgenesis on the locomotor activity and gene expression of AMPA and NMDA receptors in zebrafish larvae (*Danio rerio*)

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Effects of GH transgenesis on the locomotor activity and gene expression of AMPA and NMDA receptors in zebrafish larvae (*Danio rerio*)

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Highlights:

First study on the effect of GH overexpression in locomotor activity of larval fish.

The effect of GH overexpression in expression of ionotropic glutamate receptors.

Keywords: Transgenic zebrafish, Glutamate receptors,AMPA, NMDA.

Abstract

The zebrafish (*Danio rerio*) has been recognized as an important model for genetic-physiological studies, given the similarity of its main biological mechanisms to other vertebrates, mainly mammals. The ease of cultivating this fish, along with determined biological characteristics, makes it an attractive model for behavioral and cognitive studies. The use of larval phases increases even more the possibility of analyses, since a large number of individuals can be used in already-established behavioral tests. In this manner, the present work aimed to evaluate the effects of growth hormone (GH) on the locomotor activity and expression of ionotropic glutamate receptors (AMPA and NMDA) of transgenic zebrafish (lineage F0104) larvae. Locomotor activity was not different between transgenic and non-transgenic animals, differently from observed in previous studies with adult zebrafish. However, the expression of subunits of AMPA and NMDA receptors showed a general pattern of transcriptional increase. These results indicate the effect of GH on locomotor activity is development-dependent. However the effect on expression of the subunits of AMPA e NMDA receptors appears to be independent of the developmental stage. In this manner, the transgenic larval model can be considered an interesting tool in the investigation of how GH affects behavioral and cognitive aspects of vertebrates.

Introduction

Despite growth hormone (GH) is known as the main responsible for somatic growth in vertebrate, other functions are attributed to this peptide hormone which is produced and secreted by the anterior hypophysis. Neurotrophic function has been already described, when GH is associated with its main biological effector, IGF-I (*Insulin-like growth factor I*), such as neurogenesis, neurotransmitters release and synaptogenesis, among other [2,1]. Additionally, studies employing GH-deficient humans has been shown that the hormone replacement improves cognitive functions and memory, especially short and long-term memory [5,13]. Regarding fish species, genetic manipulation of GH has demonstrated alterations concerning aggressiveness, appetite, food conversion and locomotor activity [14,3,6,9,7]. The locomotor activity can be influenced by the GH/IGF-I axis through alterations in the central nervous system mediated by dopamine and serotonin, as previously demonstrated in GH-transgenic mice [4].

The way GH affects the cognitive and locomotor functions can be directly related to ionotropic glutamatergic receptors activity at the neuronal membrane. Within this group, there are the AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor) and NMDA (N-methyl-D-aspartate) receptors. The AMPA receptors of mammals are presented into four subunits (GLUR1-4), which present a fundamental role in fast excitatory synaptic transmissions [20]. On the other hand, vertebrate NMDA receptors are heterotetrameric, composed by a combination of two NR1 subunits and two 2A-D (NR2A-D) subunits and, in some cases, also may present NR3 subunits. Besides, these receptors can be distinguished from the other members of the family once, besides glutamate, they need glycine as a co-agonist for its activation [12]. Both, AMPA and NMDA receptors can be found involved in synaptic plasticity phenomena at the central nervous system, as well as related to other cellular mechanisms such as those occurring in the neuromotor junctions [10]. Although

many works reported relations between the GH/IGF-I axis and the ionotropic glutamatergic receptors, most of these studies are realized using mammals and only a few use adult transgenic biological models.

Few studies approach the relation between the somatotropic axis and the AMPA/NMDA receptors in fish species, and there is still missing a transgenic fish model developed specifically for this purpose. Additionally, studies concerning AMPA and NMDA receptors in zebrafish larvae are basically focused on electrophysiology [21,16,22]. Within this context, zebrafish (*Danio rerio*) rises as an important model due to important biological characteristics allowing easy genetic manipulation, rapid development and easy farming at small spaces. Besides, the brain neuroanatomy of zebrafish is similar to that observed in mammals under a functional perspective [11]. Many neurotransmitters systems have been already documented in the mentioned model, such as dopaminergic, serotonergic, purinergic and glutamatergic [19,18]. The transgenic zebrafish lineage named F0104 was developed by our research group aiming to evaluate the collateral effects of GH overexpression [8]. The lineage caries two transgenes designed based on the β-actin promoter from carp (*Cyprinus carpio*) directing the expression of the green fluorescent protein (GFP) or the cDNA of growth hormone from *Odonthestes argentinensis* (msGH). Therefore, the aim of the present study was to investigate the effects of GH overexpression on the locomotor activity and the expression of AMPA and NMDA ionotropic glutamatergic receptors in transgenic zebrafish larvae.

Material and Methods

Transgenic larvae were obtained by the breeding of transgenic males from F0104 lineage and non-transgenic females. The transgenic larvae were identified using a fluorescence microscope by GFP expression. The Mendelian proportion between transgenic and non-transgenic (1:1) was observed in all progenies. The larvae were maintained in 1L

aquaria under constant oxygenation, temperature of 26 ºC and fed twice a day with commercial ration. All applied procedures were reviewed and approved by the Ethics Committee for Animal Use from Universidade Federal do Rio Grande - CEUA/FURG (Process number 23116.005578/2013-83).

Larvae with 13 days post fertilization (dpf) (n = 30 per group) were used, which were individually placed into circular arenas measuring 3.3 cm of diameter which were recorded during 30 minutes. The amount of water inside the arenas allowed only horizontal swimming. The video analysis was processed by the software specifically developed for this purpose (Popiolek, 2014). The distances covered in centimeters along time were considered.

Following the locomotor activity experiments, the larvae were euthanized for further gene expression analysis. Six samples per group were used, each sample composed by a pool of five larvae. Total RNA was extracted using TRIzol reagent (Invitrogen, Brazil), according to manufacturer's instructions. RNA was quantified using a QuBit fluorometer (Invitrogen, Brazil) and samples integrity was confirmed by electrophoresis in 1% agarose gel. The cDNA was synthesized by RNA reverse transcription using *High Capacity cDNA Reverse Transcription kit* (Applied Biosystems, Brazil). Gene expression was evaluated quantitatively by Real Time PCR (qPCR), and each sample was analyzed in triplicate in a ABI *7500 platform Real Time System* (Applied Biosystems, Brazil), using *Platinum SYBR Green qPCR SuperMix - UDG* (Invitrogen, Brazil). Specific primers for each AMPA, *glur1a*, *glur1b*, *glur2a*, *glur3a*, *glur3b*, and NMDA gene, *grin1b*, *grin2ab* e *grin2da* (Table 1) were designed based on gene sequences available at GenBank (http://www.ncbi.nlm.nih.gov), using the software *Primer Express 3.0* (Applied Biosystems, Brazil). All primers employed presented efficiencies near 100% (data not-shown). The qPCR reactions conditions were 50 °C for 2 min, 95 °C for 2 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 30 s. The *ef1α* (elongation factor 1 alpha) and *ß-actin* (beta-actin) were chosen as reference genes for

constitutive expression, which presented no variation between the experimental groups. The expression of the target gene was normalized to a geometric mean of *ef1α* and *ß-actin*, using the equation of $2^{-\Delta Ct}$ (Livak and Schmittgen, 2001) as it follows: $\Delta Ct = Ct_{\text{target gene}} - Ct_{\text{mean } ef1\alpha\text{-}ß\text{-}actina}$.

Normality and homogeneity criteria were previously tested for variance, and gene expression data were transformed when necessary. The data from locomotor activity and gene expression were evaluated by t-test. Data were expressed as mean ± standard error. The significance level considered was $p < 0.05$.

Results and Discussion

The results from locomotor activity tests presented no significant differences between the analyzed groups (NT = 447.9 ± 222.9 cm and T = 416.2 ± 221.3 cm). Therefore, GH transgenesis apparently does not affect larvae locomotor activity. This result diverges from that observed for transgenic adults of the same lineage, when transgenic animals evinced a locomotor activity 3 times higher when compared with non-transgenic. These differences might be related to GH effects on distinct development stages of zebrafish. Additionally, the manner as locomotor activity was evaluated in adult individuals and larvae was different, and it could have affected the response. The method considered for adults was the number of quadrants crossed along the time while for larvae it was considered the distance covered in centimeters along the time, resulting in more accurate data. Possibly, the increased locomotor activity observed for transgenic adults might be, also, related to the presence of other hormones mutually acting with GH. The sexual hormones are strong candidates, once sexual maturation is also influenced by GH and alters males and female behavior. Also, the metabolic demand imposed by GH excess, caused by transgenesis, should be considered. Evidently, a larva is able to attend this demand better than an adult, once the sexual maturation involves a greater energetic cost. Considering this scenario, the locomotor activity

from adults could be much more related to the feeding behavior than to a direct effect of GH on motor neurons. Supporting this hypothesis, there are the results from the expression of AMPA and NMDA genes. Previously data using adult transgenic zebrafish demonstrated a generalized increasing expression pattern. From the 12 analyzed subunits, only one presented no significant increase. Regarding the results of gene expression shown in the present work, the pattern was very similar to that observed in adults. From the eight analyzed subunits in larvae, the expression was increased in six of these (NMDA: *grin1b*, *grin2ab* – AMPA: *glur1a*, *glur1b*, *glur2a*, *glur3b*). The effect on the expression of AMPA and NMDA receptors subunits appears to be independent from the development phase, while it is development-dependent for the locomotor activity. Therefore, the transgenic larval model can be considered an interesting tool to study GH effects on the behavioral and cognitive aspects in vertebrate. Further studies, employing the larval transgenic model in cognitive tests, could help to better understand the complex mechanisms of learning and memory involving GH in vertebrates.

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Table

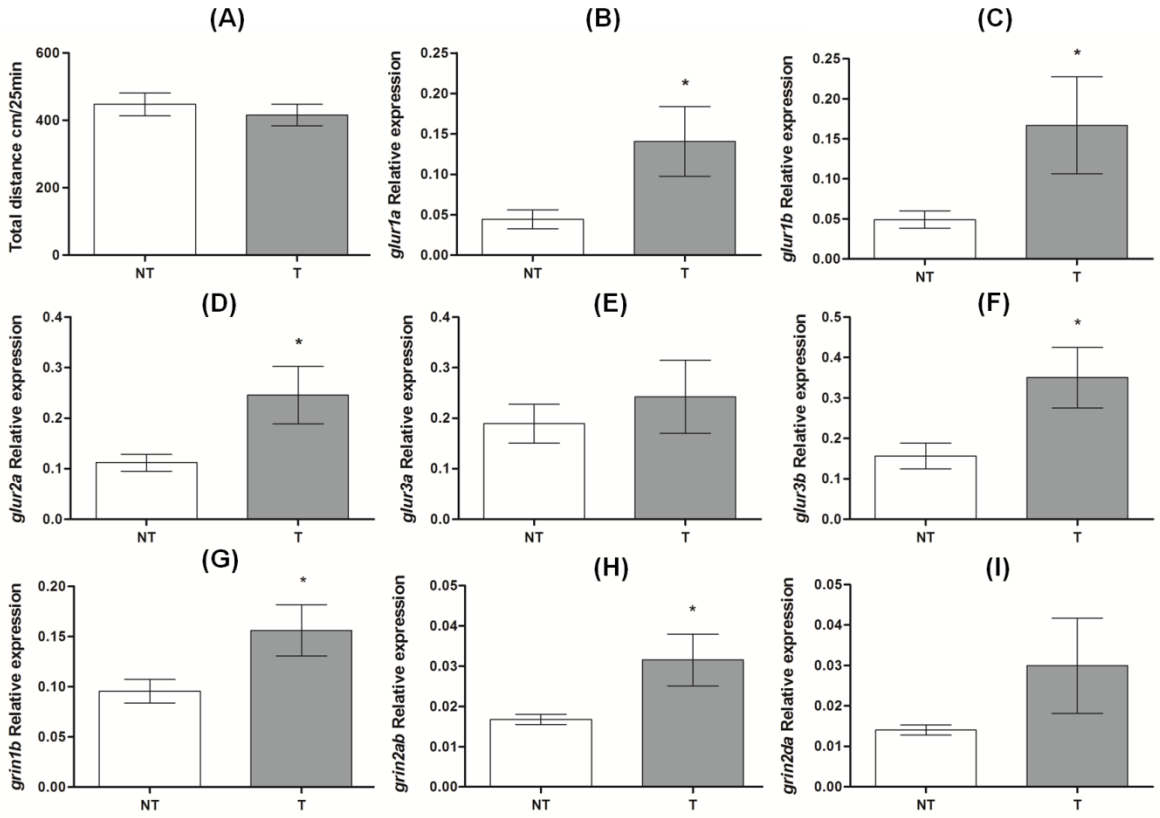
Table 1. Gene-especific primers designed using sequences available at GenBank (www.ncbi.nlm.nih.gov)

Gene	Primers	*GenBank*
glur1a	**F: 5'-CAACGACGCCATGCACTGCTCC-3'**	**NM_205598**
	R: 5'-CGATCCGCCCGTTCTCCCCAC-3'	
glur1b	**F: 5'-GGTGAGAATGGTCGTGTGATGGCC-3'**	**NM_205730**
	R: 5'-CGCCTGGCCCATACATGGCAG-3'	
glur2a	**F: 5'-CTCGGCGGATCTCCAAGCGTC-3'**	**NM_131894**
	R: 5'-CCATTCCGATCCGAAACGCGC-3'	
glur3a	**F: 5'-ACCCAGGCTGGCTTTCCAAACCA-3'**	**NM_198339**
	R: 5'-GAGCTGAACGGCGAAGCGGAA-3'	
glur3b	**F: 5'-CGTCTCTCGCTGGCTTCCCC-3'**	**NM_198360**
	R: 5'-TGCACAGCGAATCTGAATGCGC-3'	
grin1b	**F: 5'-GCGCCTGGTTCTGTTCGCCT-3'**	**NM_001144131**
	R: 5'-CACAGTCTTGGGTTCGCATCCGC-3'	
grin2ab	**F: 5'-TGCGTCCGGCTCGTGGGATT-3'**	**XM_693978**
	R: 5'-ATGCTCCCTGGGTCCGTCTGA-3'	
grin2da	**F: 5'-CCGCCACTGTTTAGGCCGGA-3'**	**XM_001921123**
	R: 5'-TGCAGCAGCTGTACATTCCCCG-3'	
ef1α	**F: 5'-GGGCAAGGGCTCCTTCAA-3'**	**NM_131263**
	R: 5'-CGCTCGGCCTTCAGTTTG-3'	
ß-actina	**F:5'-CTGTCCACCTTCCAGCAGAT-3'**	**AF_180817**
	R:5' –GATGGACCTGCCTCGTCGTA-3'	

Figure caption

Figure 1. Locomotor activity: total distance moved in cm/25min (n = 30) (A). Relative expression of genes *glur1a*, *glur1b*, *glur2a*, *glur3a* e *grin3b* - AMPA receptor (B, C, D, E, F) and genes *grin1b*, *grin2ab*, *grin2da* - NMDA receptor (G, H, I) in non-transgenic (NT) and transgenic (T) zebrafish larvae. Date are expressed as mean ± SEM (n = 6). * $p < 0.05$.

Figure 1.


(A)
Total distance cm/25min
600
400
200
0
NT
T
(B)
glur1a Relative expression
0.25
0.20
0.15
0.10
0.05
0.00
*
NT
T
(C)
glur1b Relative expression
0.25
0.20
0.15
0.10
0.05
0.00
*
NT
T
(D)
glur2a Relative expression
0.4
0.3
0.2
0.1
0.0
*
NT
T
(E)
glur3a Relative expression
0.4
0.3
0.2
0.1
0.0
NT
T
(F)
glur3b Relative expression
0.5
0.4
0.3
0.2
0.1
0.0
*
NT
T
(G)
grin1b Relative expression
0.20
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(H)
grin2ab Relative expression
0.05
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0.01
0.00
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NT
T
(I)
grin2da Relative expression
0.05
0.04
0.03
0.02
0.01
0.00
NT
T

Discussão Geral

Os resultados apresentados neste estudo demonstram que a superexpressão do GH altera parâmetros comportamentais e cognitivos em peixes. Indivíduos machos adultos hemizigotos da linhagem F0104 apresentam aumento da atividade locomotora e da agressividade, mas o sucesso nas competições reprodutivas ocorre somente quando transgênicos apresentam o peso superior aos machos não-transgênicos.

O aumento da atividade locomotora dos indivíduos transgênicos também foi descrito para salmonídeos transgênicos para o GH (Abrahams & Sutterlin, 1999) e em trutas arco-íris tratadas com GH (Jonsson *et al*., 1996; Jonsson *et al*., 2003; Johanssson *et al*., 2005). Já o aumento da agressividade foi encontrado em carpas (*Cyprinus carpio*) transgênicas para o GH (Duan *et al*., 2011). Resultados semelhantes foram encontrados em truta arco-íris juvenis tratadas com GH (Jonsson *et al*., 1998). Provavelmente o aumento da agressividade e da atividade locomotora seja destinado para a busca pelo alimento já que animais transgênicos necessitam de uma alta demanda energética para suprir o crescimento acelerado.

Ao avaliar a atividade locomotora das larvas transgênicas não foi encontrado o mesmo padrão dos adultos. As larvas não apresentaram diferenças entre grupos transgênicos e não-transgênicos testados. Duas possíveis explicações: os efeitos do GH serem distintos dependendo do estágio de desenvolvimento e o método empregado para avaliar a atividade locomotora. Nos adultos, o método considerado foi o número de quadrantes cruzados ao longo de um tempo enquanto que, nas larvas foi considerada a distância percorrida em centímetros ao longo do tempo gerando um dado mais preciso. É possível que o aumento observado na locomoção dos adultos transgênicos esteja relacionado, também, com a presença de outros hormônios que possam atuar em conjunto com o GH.

Quanto às reproduções, a redução da frequência de desovas de cruzamentos de fêmeas não-trangênicas com machos transgênicos comparando com cruzamentos de indivíduos não-transgênicos podem ser resultado da deficiência de estímulo dos machos transgênicos. Machos transgênicos podem não ser reconhecidos pelas fêmeas pelo formato do corpo ou provavelmente por prejuízos nos feromônios. Bessey *et al.* (2004) ao avaliar salmão transgênico para o GH observaram que machos transgênicos podem não ser escolhidos pelas fêmeas por apresentarem redução no comportamento de cortejo e diminuição nas características sexuais secundárias. Em relação aos feromônios um estudo realizado com carpas transgênicas para o GH evidenciou que os danos observados nas gônadas destes peixes estavam relacionadas à diminuição da síntese e liberação do hormônio luteinizante (LH) que é responsável pelo desenvolvimento e maturação das gônadas acarretando em danos nos feromônios Cao *et al.* (2014). Provavelmente isto também pode estar ocorrendo com os indivíduos da linhagem F0104 dificultando o reconhecimento e estímulo das fêmeas.

Em relação ao comportamento exploratório analisado no aparato de esquiva inibitória, os peixes transgênicos adultos não apresentaram diferenças significativas. Ao avaliar a memória de longa duração (LTM) 48 h após o treino, foi observado que o excesso de GH claramente melhora a LTM dos indivíduos transgênicos comparando com os não-transgênicos ($P < 0,05$). A melhora da LTM também foi encontrada em humanos tratados com o GH (Burman *et al.*,1995; Johansson *et al.*,1995; Arwert *et al.*, 2005).

Ao analisar os genes que expressam as subunidades do receptor AMPA e NMDA que estão envolvidos em processos de consolidação da memória, foi observada uma indução praticamente em todos os genes. Estudos que administram o GH em roedores evidenciam os efeitos positivos sobre esses receptores (Park *et al.*, 2010; Ramis *et al.*, 2013). Adicionalmente, muitos das ações do GH são atribuídas ao IGF-I circulante produzido no fígado enquanto outras são oriundas do IGF-I produzido localmente no cérebro (Aberg *et al.*,

2006; Sonntag *et al*., 2005; Deak e Sonntag, 2012). Ao analisar a expressão do gene do IGF-I no cérebro do zebrafish foi demonstrado que indivíduos transgênicos apresentam níveis de expressão quase três vezes superiores em relação aos não-transgênicos. Roedores que superexpressam o IGF-I no cérebro apresentaram aumento da neurogênese e sinaptogênese no hipocampo, uma região que é importante para a formação da memória em mamíferos (O´Kusky *et al*., 2000; Ye *et al*., 2004). Já estudos utilizando injeções de IGF-I evidenciam o aumento dos níveis da subunidade *nr2* do NMDA (Le Grevès *et al*., 2006). Tanto o GH quanto o IGF-I exercem efeitos diretos sobre as subunidades dos receptores AMPA e NMDA, os quais reconhecidamente participam de mecanismos de formação e consolidação da memória (Nyberg e Hallberg, 2013). Provavelmente a melhora na cognição dos transgênicos seja pelo o aumento da expressão desses receptores no cérebro.

Estudos sobre os receptores AMPA e NMDA em larvas de zebrafish são basicamente de eletrofisiologia (Todd *et al*., 2004; McDearmid e Drapeau, 2006; Wiggin *et al*., 2012). Quando avaliamos a expressão dos genes das subunidades dos receptores AMPA e NMDA nas larvas transgênicas para o GH, também foi observado um aumento da expressão com exceção de duas subunidades *glur3a* e *grin2da*. O efeito do GH na expressão das subunidades dos receptores AMPA e NMDA parece ser independente da fase de desenvolvimento.

Conclusões Gerais

1. A superexpressão do GH aumenta o comportamento agressivo e a atividade locomotora em indivíduos machos adultos.
2. Machos transgênicos da linhagem F0104 apresentam vantagem na disputa pelas fêmeas quando o peso corporal for superior ao do oponente.
3. A superexpressão do GH beneficia a LTM e aumenta a expressão gênica das subunidades dos receptores glutamatérgicos ionotrópicos AMPA e NMDA.
4. A superexpressão do GH aumenta a expressão do IGF-I no cérebro.
5. A superexpressão do GH não altera a atividade locomotora em larvas.
6. Larvas transgênicas apresentam aumento nos níveis de expressão gênica das subunidades dos receptores glutamatérgicos ionotrópicos AMPA e NMDA.

Perspectivas

1. Mapear, através de técnicas imunohistoquímicas, a distribuição dos receptores AMPA e NMDA no cérebro de transgênicos e não-transgênicos.
2. Avaliar os efeitos da expressão aumentada do IGF-I cerebral observada nos transgênicos sobre a neurogênese e a sinaptogênese.
3. Utilizar antagonistas específicos dos receptores AMPA e NMDA para estimar a participação de cada um na LTM aumentada dos transgênicos.
4. Produzir novas linhagens transgênicas expressando proteínas fluorescentes sob controle de promotores gênicos relacionados aos receptores AMPA e NMDA, como ferramentas para o estudo do aprendizado e memória em vertebrados.

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