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RELAÇÃO ENTRE O SISTEMA IMUNE E O COMPORTAMENTO EM *ZEBRAFISH*

Santa Maria, RS
2020

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Tese apresentada ao Programa de Pós-Graduação em Farmacologia, Área de Concentração em Farmacologia Aplicada à Produção Animal, da Universidade Federal de Santa Maria (UFSM, RS) como requisito parcial para a obtenção de grau de **Doutor em Farmacologia.**

Orientador: Prof. Dr. Leonardo José Gil Barcellos
Coorientador: Prof. Dr. Luiz Carlos Kreutz

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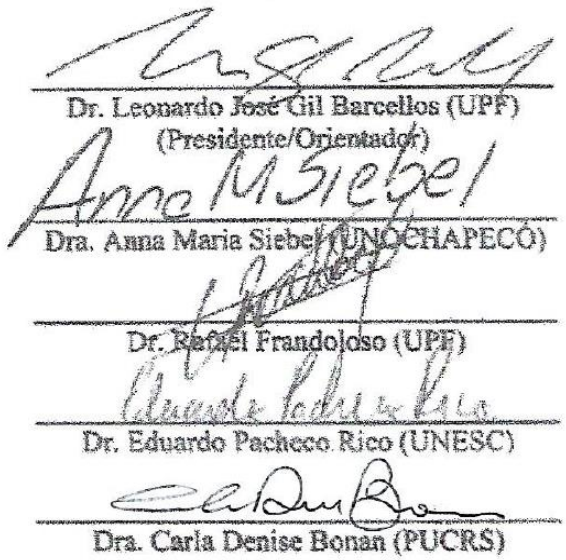

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BEIJOS E ABRAÇOS Á TODOS

“Knowledge is power”

RESUMO

RELAÇÃO ENTRE O SISTEMA IMUNE E O COMPORTAMENTO EM '*ZEBRAFISH*'.

AUTORA: Karina Schreiner Kirsten
ORIENTADOR: Leonardo José Gil Barcellos
COORIENTADOR: Luiz Carlos Kreutz

A interação entre sistema imune e comportamento já foi constatada em diversos estudos utilizando mamíferos como modelo experimental. Por outro lado, esta relação ainda não foi claramente demostrada em outras espécies, inclusive em peixes. Nesse contexto, o principal objetivo deste trabalho foi avaliar a relação entre o sistema imune e o comportamento no *zebrafish*. Para tanto, realizamos três estudos que constituíram o eixo central desta Tese de Doutorado. No primeiro estudo, separamos os peixes conforme o comportamento social e exploratório e avaliamos a expressão gênica de citocinas no cérebro. Assim verificamos que o *status* imunológico varia conforme o perfil exploratório e social do peixe. Embora demonstrando que existe uma relação entre o sistema imune e o comportamento no *zebrafish*, uma questão ficou em aberto: é o sistema imunológico que provoca a alteração de padrões comportamentais ou é o padrão comportamental que modula a resposta imune? Para responder a esta pergunta realizamos os outros dois estudos. No segundo estudo induzimos uma resposta inflamatória em um grupo de peixes e avaliamos novamente o comportamento exploratório e social, bem como a expressão gênica de citocinas no cérebro. Os peixes doentes apresentaram aumento da expressão de citocinas pró-inflamatórias no cérebro e redução da locomoção, da preferência social e da exploração de um objeto novo quando comparados ao grupo controle. Com este estudo demonstramos que o sistema imune altera o comportamento, e caracterizamos pela primeira vez o *sickness behavior* no *zebrafish*. Então, no terceiro estudo, avaliamos os efeitos do estresse agudo e crônico na expressão de citocinas e marcadores neuronais no cérebro do *zebrafish*, constatando que o estresse crônico causa importantes alterações nestes genes, enquanto um único episódio de estresse parece não ter sido suficiente para causar essas alterações. Assim demonstramos que um padrão de comportamento, como o estresse, também causa alterações na resposta imune. Desta forma, concluímos que sistema imune e comportamento estão interligados, sendo que um pode modular o outro.

Palavras-chave: citocinas, cérebro, comportamento, estresse

ABSTRACT

RELATIONSHIP BETWEEN IMMUNE SYSTEM AND BEHAVIOR IN ZEBRAFISH

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ADVISOR: Leonardo José Gil Barcellos
CO-ADVISOR: Luiz Carlos Kreutz

The interaction between immune system and behavior has already been observed in several studies using mammals as animal model. On the other hand, this relationship has not yet been clearly demonstrated in other animal species including fish. Thus, the main objective of this work is to evaluate the relationship between immune system and zebrafish behavior. To achieve our goals, we already carried out three fundamental studies that will be central to this Doctoral Thesis. In the first study, we separated fish according to their social and exploratory behavior and evaluated the expression of cytokines genes in the brain. So we verify that the immunological status varies according to the exploratory and social profile of the fish. Although we showed that there is a relationship between immune system and zebrafish behavior, a major question remains open: is the immune system that leads to behavioral pattern changes or is it the behavioral pattern that alters the immune response? To answer this question, in our second study, we induced an inflammatory response in a group of fish and reevaluated the exploratory and social behavior as well as the expression of cytokine genes in the brain. Sick fish had increased expression of proinflammatory cytokines in the brain and decreased locomotion, social preference and exploration to a new object when compared to the control group. With this study we demonstrated that the immune system changes the behavior, and we first characterized the sickness behavior in the zebrafish. Finally, in the third study we evaluated the effects of acute and chronic stress on the expression of cytokines and neuronal markers in the zebrafish brain, noting that chronic stress causes significant changes in these genes, whereas a single episode of stress did not cause changes. Thus we demonstrate that a behavioral pattern, such as stress, causes changes in the immune response. Therefore, we conclude that immune system and behavior are interconnected, and one can alter the other.

Keywords: cytokines, brain, behavior, stress

LISTA DE FIGURAS

INTRODUÇÃO

REVISÃO DE LITERATURA

ARTIGO 1

ARTIGO 2

ARTIGO 3

LISTA DE TABELAS

ARTIGO 1

ARTIGO 2

ARTIGO 3

LISTA DE ABREVIATURAS E SIGLAS

ACTH	Hormônio adenocorticotrófico
Bdnf	Fator neurotrófico derivado do cérebro
Crh	Hormônio liberador de corticotrofina
Eci	Estresse crônico imprevisível
Gr	Receptor de glicocorticóide
Hpa	Eixo hipotálamo-hipófise-adrenal
Hpi	Eixo hipotálamo-hipófise-interrenal
Hrn	High responders to novelty
Igm/igz	Imunoglobulina m/z
Il-1β	Interleucina 1 beta
Il-2	Interleucina 2
Il-4	Interleucina 4
Il-6	Interleucina 6
Il-10	Interleucina 10
Inf-γ	Interferon gama
Inf-α	Interferon alfa
Lrn	Low responders to novelty
Nk	*Natural killer*
Pamp	Padrões moleculares associados a patógenos
Pprs	Receptores de reconhecimento padrão
Tea	Transtorno do espectro autista
Th1/2	T *helper* 1/2
Tlrs	T*oll-like receptor*

SUMÁRIO

1 INTRODUÇÃO

O sistema imune e o sistema nervoso estão profundamente conectados (KIPNIS, 2018; LOUVEAU et al., 2015; OGŁODEK et al., 2014). Diversos estudos demonstram a ocorrência de alterações imunes em doenças neurológicas como autismo (ESTES; MCALLISTER, 2015; ROSS et al., 2013; SHACHAR; KARIN, 2012), esquizofrenia (MÜLLER; SCHWARZ, 2010) e depressão (DANTZER, 2009, 2008). Por outro lado, pesquisas mais recentes estudam a relação entre sistema imune e sistema nervoso com foco no papel fisiológico do sistema imune na regulação de funções neuronais, e demonstram que células e moléculas do sistema imune estão envolvidos na regulação de funções como memória (AVITAL et al., 2003; DERECKI et al., 2010), aprendizagem (DERECKI et al., 2010), ansiedade (NAUTIYAL et al., 2008) e comportamento social (FILIANO et al., 2016).

Sob outra perspectiva, quando o organismo é infectado por patógenos, a ativação da resposta imune leva a uma série de alterações comportamentais, como letargia, redução da ingestão de alimentos, do perfil exploratório e da interação social (DANTZER; KELLEY, 2007; FRENOIS et al., 2007; HABA et al., 2012; JOHNSON, 2002). Este conjunto de alterações comportamentais é chamado de comportamento de doença ou s*ickness behavior* (DANTZER; KELLEY, 2007; JOHNSON, 2002) .

Ainda, um importante fator comportamental que interfere na resposta imune é o estresse. O estresse ativa o eixo hipotálamo-hipófise-adrenal (HPA) em mamíferos (SMITH; VALE, 2006) ou hipotálamo-hipófise-interrenal (HPI) em peixes (ALDERMAN; BERNIER, 2009), culminando, em humanos e em peixes na produção do hormônio corticosteroide cortisol (PIJANOWSKI et al., 2015). Conhecido como o hormônio do estresse, o cortisol causa importante disrupção na resposta imunológica, principalmente quando é produzido de forma constante, o que acontece no estresse crônico (FRIES et al., 2015; OGŁODEK et al., 2014; TORT, 2011). Esta alteração do sistema imune pode levar a consequências como aumento da ocorrência de infecções e à neuroinflamação (JONATHAN TEA, SARAH L. ALDERMAN, 2018; LUCASSEN et al., 2014; TORT, 2011).

Figura 1 - Esquema da relação entre sistema imune e sistema nervoso

Fonte: do autor

Com base no exposto até aqui, fica evidente a profunda conexão entre os sistemas imune e nervoso, como esquematizado na figura 1. Porém, grande parte das pesquisas que evidenciam esta relação utilizam mamíferos como animal modelo (DERECKI et al., 2010; FILIANO et al., 2016; GROSSBERG et al., 2011; KELLEY et al., 2003), sendo que poucos estudos exploram o tema utilizando peixes como animal modelo (LEE et al., 2015; RAKUS et al., 2017). Assim, não há dados científicos sólidos que demonstrem que o sistema imune afeta o comportamento dos peixes.

Frente a esse cenário, nosso principal objetivo foi avaliar a relação entre o sistema imune e o comportamento no *zebrafish*. Trata-se de uma pesquisa cujos dados poderão ser extrapolados para as demais espécies aquáticas, inclusive para diferentes espécies de teleósteos usados na produção. Da mesma forma, devido ao grande potencial translacional do *zebrafish*, esse estudo também poderá ter valor para elucidar as interações entre comportamento e sistema imune em outras espécies, inclusive em humanos.

A presente Tese de Doutorado está organizada nas seguintes seções: revisão bibliográfica, objetivos, os três artigos científicos gerados no período de doutoramento, discussão, conclusão, perspectivas e referências bibliográficas.

Os artigos científicos produzidos são:

Artigo 1: *First description of behavior and immune system relationship in fish*, publicado no periódico *Scientific Reports.*

Artigo 2: *Characterization of sickness behavior in zebrafish* publicado no periódico *Brain, Behavior and Immunity*.

Artigo 3*:* *Characterization of sickness behavior in zebrafish* publicado no periódico *Stress.*

2 REVISÃO DE LITERATURA

2.1 COMPORTAMENTO

O comportamento é fundamental para diversos processos críticos que implicam na sobrevivência de um organismo, como acesso a fontes de alimentos e *habitat*, reação ao predador, e suas interações sociais ou sexuais com indivíduos da mesma espécie (BOURKE, 2014). O comportamento social é a estratégia que a maioria dos animais utiliza para lidar com o ambiente, permitindo com que sobrevivam e se reproduzam mesmo em condições desfavoráveis (WEST; GRIFFIN; GARDNER, 2007).

A variação individual de comportamento das espécies tem sido cada vez mais o foco de diversas pesquisas (MIKLÓSI; ANDEWS, 2006). Animais e seres humanos diferem na forma de como reagem ao perigo, lidam com a novidade e interagem com os seus coespecíficos. O termo mais utilizado para definir estas variações é personalidade (MACKAY; HASKELL, 2015; STAMPS; GROOTHUIS, 2010). O estudo da variação comportamental individual é complexo, pois depende de múltiplos fatores genéticos e ambientais (MACKAY; HASKELL, 2015).

Aqui, nosso foco é discutir sobre a interação entre sistema imune e comportamento no *zebrafish.*

2.2 ESTRESSE

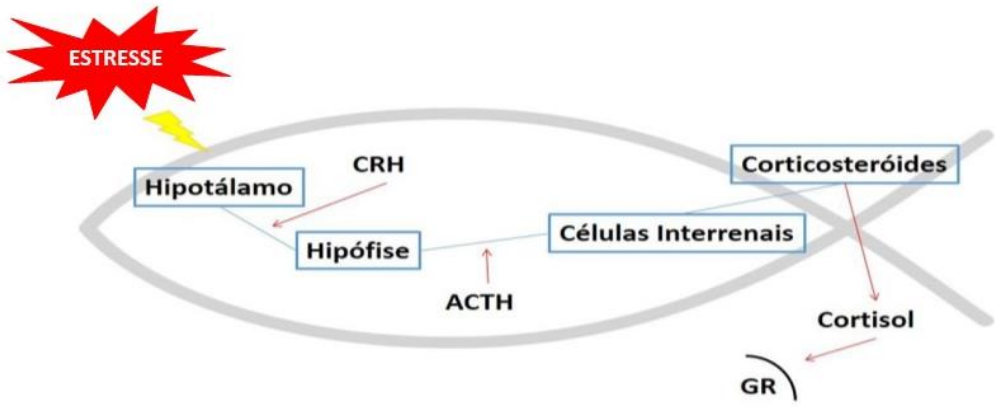
O termo estresse surgiu na área da física, para "traduzir o grau de deformidade sofrido por um material quando submetido a um esforço ou tensão". Em 1936, o endocrinologista Hans Selye, transpôs este conceito para a Medicina e Biologia, dando a ele um novo significando: "Esforço de adaptação do organismo diante de situações consideradas ameaçadoras a sua vida e a seu equilíbrio interno" (SZABO S, YOSHIDA M, FILAKOVSZKY J, 2017) .

Um importante problema de saúde pública no mundo, o estresse é um fator constante na vida moderna; uma condição em que a homeostase do organismo é perturbada, como resultado de estímulos estressores (MARIOTTI, 2015). Deve-se ponderar que embora o conceito de estresse tenha ganhado uma má reputação, é importante reconhecer que o propósito de uma resposta fisiológica ao estresse é promover a sobrevivência do organismo frente a situações de risco. Desta forma, episódios isolados de estresse são benéficos pois preparam o organismo para lidar com os desafios (DHABHAR, 2014). Por outro lado, o estresse a longo prazo ou

crônico tem inúmeros efeitos adversos à saúde, pois as alterações metabólicas causadas pelo estresse, se contínuas, podem acarretar em diversas doenças (DHABHAR, 2014).

O mecanismo biológico de resposta ao estresse é a ativação do eixo hipotálamo-hipófise-adrenal (HPA) em mamíferos ou hipotálamo-hipófise-interrenal (HPI) em peixes, como no caso do *zebrafish*. A ativação do eixo começa com um estimulo estressor, então o hipotálamo recebe este estímulo e em seguida secreta o hormônio liberador de corticotrofina (CRH), que atua no interior da hipófise induzindo a produção de outro hormônio chamado de adrenocorticotrófico (ACTH), este então atua na adrenal ou interrenal e estimula a síntese de cortisol, o hormônio do estresse (ALDERMAN; BERNIER, 2009; SMITH; VALE, 2006). A figura 2 demostra a ativação do eixo HPI no *zebrafish*.

Figura 2 - Ativação do eixo hipotálamo-hipófise-interrenal (HPI) em peixes.



Fonte: Adaptado de IDALENCIO, 2019

O cortisol age ligando-se a receptores de glicocorticoides (GR) presentes em diversas células e tecidos. As funções sistêmicas do cortisol incluem a modulação da abertura e fechamento de canais iônicos e, dessa forma, o desencadeamento das respostas fisiológicas rápidas como, por exemplo, alteração da liberação de neurotransmissores na fenda sináptica e, consequentemente, alteração da excitabilidade neuronal. Ainda, o cortisol atua aumentando os níveis de glicose, a pressão sanguínea, a frequência cardíaca e alterando a resposta imune (ILIAS PEROGAMVROS, 2012; OGŁODEK et al., 2014) .

A desregulação do eixo HPA/HPI, e a produção excessiva de cortisol estão intimamente relacionados aos efeitos deletérios do estresse crônico a saúde. A intensidade do estresse pode ser avaliada pelos níveis séricos de cortisol e outras alterações fisiológicas. O cortisol provoca efeitos sistêmicos como o aumento da frequência cardíaca, da pressão arterial e disrupção do sistema imune (DHABHAR, 2014).

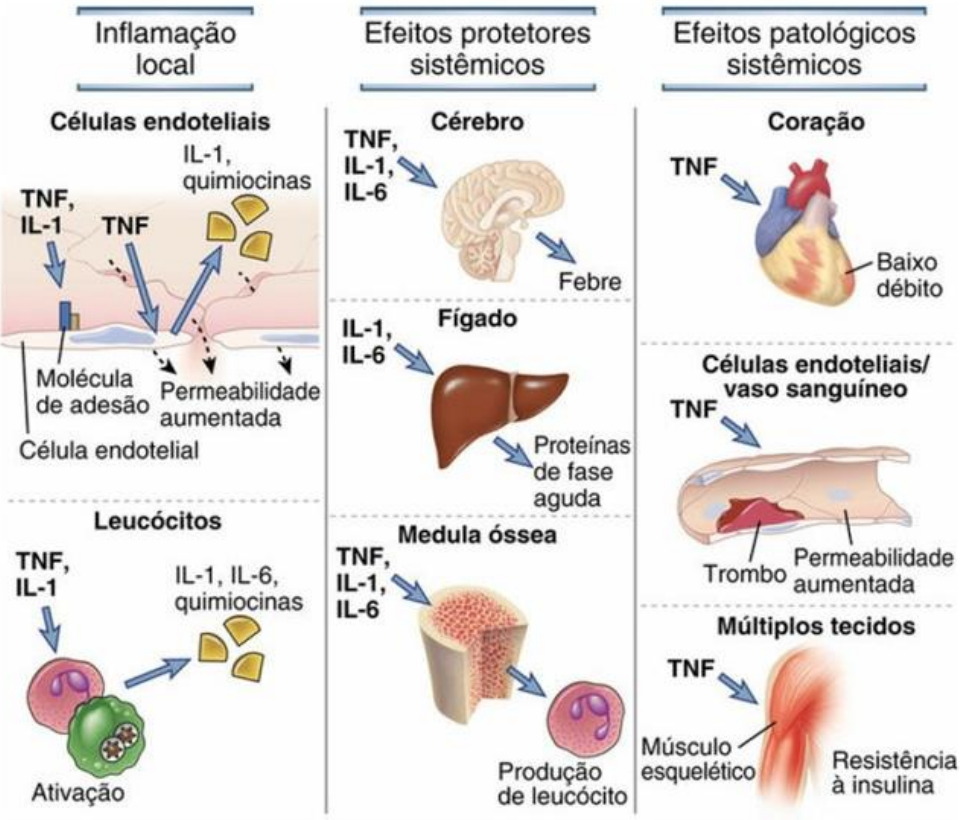
2.3 SISTEMA IMUNE

A função fisiológica do sistema imune em todas as espécies é defender o organismo contra moléculas estranhas, sejam elas microrganismos ou macromoléculas como proteínas e polissacarídeos (YATIM; LAKKIS, 2015). A resposta imune é subdividida em resposta imune inata, que apresenta uma resposta rápida e constante contra patógenos, e a resposta imune adaptativa, que ocorre de forma mais lenta, porém é altamente específica e gera memória imunológica (BEN-SHAANAN et al., 2016). Tanto a resposta imune inata quanto a adaptativa são orquestradas por células e moléculas, que produzem uma resposta coletiva e coordenada para eliminação dos agentes agressores (LIN; SCOTT, 2012).

As citocinas são proteínas produzidas em resposta à estimulação do sistema imune, e são uma das formas de comunicação das células imunológicas (DINARELLO, 2007). As citocinas possuem um papel central na regulação da resposta imune inata e adquirida e podem atuar de forma local ou sistêmica. Além disso, algumas citocinas influenciam a atividade de diversos tipos celulares e são denominadas pleiotrópicas; por outro lado a ação sobre as células alvo pode ser redundante, quando diferentes citocinas possuem os mesmos efeitos funcionais (WANG; SECOMBES, 2013). Os peixes produzem citocinas com estrutura e função muito similares a dos mamíferos (ZHU et al., 2012).

As citocinas atuam em diferentes fases da resposta imune, através da ligação com receptores específicos presentes na membrana das células alvo (SHACHAR; KARIN, 2012). Na resposta imune inata, para o início do processo inflamatório, as mais importantes são o fator de necrose tumoral α (TNF-α), interleucina 1β (IL- 1β) e a interleucina 6 (IL-6), produzidas principalmente por macrófagos e células dendríticas (ZOU et al., 2003). As principais ações destas citocinas são o recrutamento de leucócitos para os locais de inflamação, através da estimulação da produção de quimiocinas e da expressão de moléculas de adesão nos leucócitos (integrinas) e no endotélio (selectinas), promovendo a diapedese, ou seja, a migração dos leucócitos da circulação sanguínea para o tecido inflamado (ABBAS; LICHTMAN; PILLAI, 2015). Essas citocinas também possuem efeitos sistêmicos e, atuando sobre o hipotálamo, induzem a febre, e no fígado, estimulam a produção de proteínas de fase aguda. Na figura 3 estão resumidas as principais ações locais e sistêmicas das citocinas pró-inflamatórias TNF-α, IL- 1β e IL-6.

Figura 3 - Ações locais e sistêmicas das citocinas durante o processo inflamatório.



Fonte: ABBAS; LICHTMAN; PILLAI, 2015

A interleucina 12 (IL-12) é outra citocina pró-inflamatória também produzida por células dendríticas e macrófagos, principalmente em resposta a infecções por vírus e bactérias intracelulares (ABBAS; LICHTMAN; PILLAI, 2015). A IL-12 age sobre os linfócitos T *helper* (Th) *naives*, estimulando sua diferenciação em linfócitos T *helper* 1 (Th1), que são células efetoras da resposta imune celular e importante para o combate de infecções intracelulares. Ainda, a IL-12 atua sobre as células *natural killers* (NK), aumentando sua atividade citotóxica e estimulando a secreção de interferon gama (INF-γ) (TRINCHIERI, 2003).

O INF-γ, por sua vez, desempenha papel importante na imunidade inata e adquirida. O IFN-γ é secretado principalmente por células NK e por linfócitos Th1, e é a principal citocina ativadora de macrófagos, pois induz a síntese de intermediários reativos de oxigênio de óxido nítrico, estimulando a destruição dos patógenos fagocitados (SCHRODER et al., 2004). Além disso, também estimula o aumento do processamento e a capacidade de apresentação de antígenos e a indução da expressão de outras citocinas e quimiocinas necessárias para o recrutamento de leucócitos ao local da inflamação. Na resposta imune adaptativa, o INF-γ estimula a resposta imune celular de forma similar a IL-12 (SHACHAR; KARIN, 2012).

Na resposta imune adquirida, as citocinas medeiam à proliferação e a diferenciação de linfócitos após o reconhecimento do antígeno, sendo que a produção de citocinas é uma das principais respostas dos linfócitos Th após ativados (BEN-SHAANAN et al., 2016). Dentre as citocinas envolvidas na resposta imune adquirida, a interleucina 2 (IL-2) é secretada por linfócitos Th1 ativados, e estimula a proliferação e a diferenciação destas células, exercendo também um papel central sobre as células T reguladoras (BONILLA; OETTGEN, 2010). A interleucina 4 (IL-4), produzida pelo linfócito Th2, atua sobre os linfócitos B, com ação reguladora na secreção de anticorpos (ABBAS; LICHTMAN; PILLAI, 2015).

O processo inflamatório é fundamental para defender o organismo contra a ação de microrganismos. No entanto, a inflamação pode causar disturbios metabólicos e danos teciduais (LIN; SCOTT, 2012). Assim, o sistema imune possui mecaninsmos anti-inflamatórios que reduzem a produção de moléculas pró-inflamatórias para limitar os danos teciduais e restaurar a homeostase (LYER; CHENG, 2013). A interleucina 10 (IL-10) é uma potente citocina com efeitos anti-inflamatórios, produzida por macrófagos e células dendríticas e age sobre estas células inibindo a produção de citocinas inflamatórias como IL-1β, TNF-α e IL-12, inibindo o processo inflamatório (LYER; CHENG, 2013).

O *zebrafish* possui um sistema imune bem desenvolvido, no qual a resposta imune inata é a forma primária de defesa contra agentes agressores (MEEKER; TREDE, 2008; RENSHAW; TREDE, 2012). Células imunológicas, receptores de reconhecimento padrão (PPRs) como os *toll-like receptors* (TLRs), defensinas e citocinas possuem estrutura e função muito parecidas com os mamíferos (MEEKER; TREDE, 2008; SECOMBES; WANG; BIRD, 2011; SUAREZ-CARMONA et al., 2015). Já a resposta imune adaptativa dos peixes teleósteos é menos desenvolvida quando comparada a dos mamíferos. É composta por linfócitos B que secretam pelo menos duas classes de imunoglobulinas (IgM, IgZ) (LAGOS et al., 2017), e por linfócitos T com os subconjuntos T *helper*, T citotóxico e T regulatório (CASTRO et al., 2011; FISCHER; KOPPANG; NAKANISHI, 2013; NAKANISHI et al., 2011; RENSHAW; TREDE, 2012). Devido a similariade entre o sistema imune do *zebrafish* e dos mamíferos, seu uso como modelo em estudos translacionais em doenças imunológicas e neuroimunológicas humanas vem crescendo constantemente nos últimos anos (KALUEFF et al., 2014; NOVOA; FIGUERAS, 2012).

2.4 SISTEMA NERVOSO X SISTEMA IMUNE

Até recentemente, o cérebro era considerado um sítio imune privilegiado, isto é, acreditava-se que o sistema imune tinha acesso restrito a este órgão, e a interação entre sistema imune e sistema nervoso quando acontecia, era considerada patológica (RANSOHOFF; ENGELHARDT, 2012). Pesquisas recentes identificaram a presença vasos linfáticos no cérebro, confirmando que células do sistema imune tem amplo acesso a este órgão. Além da micróglia, que são os macrófagos residentes do sistema nervoso central, outras células imunes, incluindo linfócitos T, monócitos e mastócitos residem no cérebro e circulam no liquido cefalorraquidiano (LOUVEAU et al., 2015). Estas descobertas abriram um amplo campo de investigação a respeito das interações entre sistema nervoso e sistema imune.

2.4.1 Sistema Imune X Doenças Neurológicas

Classicamente o estudo da relação entre sistema imune e sistema nervoso teve como foco a ocorrência de disfunções imunes em pacientes com doenças neurológicas como esquizofrenia (MÜLLER; SCHWARZ, 2010), autismo (ESTES; MCALLISTER, 2015) e depressão (DANTZER, 2008). Estas patologias muitas vezes estão relacionadas com a ocorrência de infecções maternas ou neonatais, além disso os pacientes apresentam um aumento dos níveis séricos de citocinas pró-inflamatórias, moléculas fundamentais para a resposta imune e que estão envolvidas na regulação de diversas funções neuronais (DANTZER, 2009; ESTES; MCALLISTER, 2015).

O envolvimento de agentes infecciosos e do sistema imune na fisiopatologia da esquizofrenia é discutido a décadas (KESTEREN et al., 2017). Infecções durante a gravidez ou nos primeiros meses de vida aumentam a prevalência de esquizofrenia; porém, não é um patógeno específico que desencadeia a patologia mas sim, é a resposta imune da mãe que aumenta o risco de esquizofrenia nos descendentes (MÜLLER; SCHWARZ, 2010). Dados genéticos recentes mostram que mutações que elevam a susceptibilidade para a esquizofrenia estão relacionadas a genes com importantes funções imunes (KESTEREN et al., 2017). Ainda, pacientes esquizofrênicos tem um aumento das citocinas pró-inflamatórias (GIBNEY; DREXHAGE, 2013). Diversas pesquisas estão sendo realizadas para desvendar os mecanismos moleculares pelos quais as citocinas afetam o sistema dopaminérgico e glutamatérgico (KESTEREN et al., 2017; MÜLLER; SCHWARZ, 2010).

O sistema imune também está envolvido na patogênese do transtorno do espectro autista (TEA) (ESTES; MCALLISTER, 2015; GIBNEY; DREXHAGE, 2013; ROSS et al., 2013). Embora existam diversos fatores ambientais que contribuam para o TEA, a maioria deles está relacionado a alterações na resposta imune durante a gestação ou logo após o nascimento (CAREAGA; MURAI; BAUMAN, 2016). Indivíduos com TEA tem níveis sanguíneos aumentados de citocinas pró-inflamatórias como IL-1β, IL-6, TNF-α, INF-γ, entre outras (ESTES; MCALLISTER, 2015). Ainda, vários estudos recentes mostram que o TEA está associado com alterações da microbiota intestinal. Crianças autistas tem níveis elevados de *Clostridium* spp. e *Desulfovibrio* spp. em sua microbiota intestinal, e este desequilíbrio pode ser uma das causas da disfunção imune observada (ESTES; MCALLISTER, 2015). Terapias experimentais com substituição da microbiota intestinal em indivíduos autistas apresentam resultados promissores (KANG et al., 2017).

A depressão é outra patologia que envolve alterações imunológicas (MAES et al., 2012). Em resposta a uma infecção o sistema imune produz citocinas pró-inflamatórias que atuam no cérebro causando o *Sickness Behavior* (seção 3.3) (DANTZER, 2009). A ativação crônica do sistema imunológico, por patologias ou estresse crônico, pode levar a uma exacerbação do comportamento de doença e o desenvolvimento de depressão em indivíduos vulneráveis (DANTZER, 2008). Estudos demonstram a ocorrência de disfunção imune em indivíduos depressivos, com níveis aumentados de citocinas pró-inflamatórias (DANTZER, 2009, 2008; GIBNEY; DREXHAGE, 2013). Além disso, é muito comum pacientes em tratamento imunoterápico com IL-2 e INF-α desenvolverem depressão, bem como, transtornos depressivos são mais prevalentes em pessoas com doenças crônicas como *Diabetes Melitus* tipo 2, artrite reumatoide e doença cardiovascular (OGŁODEK et al., 2014). Os possíveis mecanismos moleculares pelo qual o sistema imune pode levar a depressão já foram comprovados cientificamente (CAPURON et al., 2002; OGŁODEK et al., 2014; SWAAB; BAO; LUCASSEN, 2005). A inflamação reduz os níveis plasmáticos de triptofano, um aminoácido essencial para síntese de serotonina, além de interferir nos mecanismos de neurotransmissão da serotonina (CAPURON et al., 2002). Ainda, as citocinas levam a hiperatividade do eixo HPA, que é frequentemente associado a depressão clínica (SWAAB; BAO; LUCASSEN, 2005).

2.4.2 Sistema imune x Comportamento

Por muitos anos os estudos da interação entre o sistema imune e sistema nervoso focaram apenas nas patologias causadas por disfunções imunológicas (RANSOHOFF;

ENGELHARDT, 2012). Atualmente, este foco tem mudado, pois agora sabe-se que o sistema imune exerce papel fisiológico na regulação de funções neurológicas (KIPNIS, 2018) . Células e moléculas do sistema imune atuam na regulação de funções como aprendizagem, ansiedade e comportamento (DERECKI et al., 2010; FILIANO et al., 2016; NAUTIYAL et al., 2008).

Os mastócitos, células do sistema imune conhecidas por seu papel em respostas alérgicas nas vias aéreas superiores, pele e trato gastrointestinal, foram encontrados nas meninges e no cérebro, onde secretam várias moléculas no hipocampo, incluindo histamina, citocinas, quimiocinas e o neurotransmissor serotonina, atuando na neurogênese, no aprendizado e na memória, e também regulando a ansiedade. Ratos deficientes em mastócitos apresentam ansiedade e déficits de aprendizagem e memória (NAUTIYAL et al., 2008).

A IL-4 produzida por linfócitos T das meninges demonstrou ter papel fundamental em funções neuronais. Camundongos submetidos a testes de memória apresentam acumulo de linfócitos T nas meninges. Ainda, camundongos *knockout* tanto para linfócitos T, quanto para IL-4 apresentam importantes déficits de memória e aprendizagem (DERECKI et al., 2010).

A citocina pró-inflamatória INF-γ, também secretada de linfócitos T presentes nas meninges, está profundamente envolvida no controle do comportamento social. Uma pesquisa recente demonstrou que camundongos *knockout* para o gene do INF-γ apresentam importante déficit comportamental em comparação a camundongos normais. Além disso, sem o INF- γ os camundongos apresentam hiperconectividade neuronal, um distúrbio frequentemente encontrado em pacientes com disfunções neurológicas, como o transtorno do espectro autista. Uma injeção de INF-γ no fluido cérebro-espinhal é suficiente para normalizar a conectividade neuronal e acabar com o distúrbio de comportamento social, demonstrando que esta citocina desempenha um papel chave na regulação na conectividade neuronal (FILIANO et al., 2016).

2.4.3 Sickness behavior

A ativação da resposta imune ocorre quando receptores TLRs de macrófagos e células dendríticas se ligam a moléculas conservadas na superfície de microrganismos, genericamente denominadas de padrões moleculares associados a patógenos (PAMPs) (YATIM; LAKKIS, 2015). Assim, as células imunes são ativadas e liberam citocinas pró-inflamatórias, principalmente IL-1β, IL-6 e TNF-α, que promovem o recrutamento de células imunes para o local da inflamação (DINARELLO, 2007), e também enviam sinais para o cérebro, através de vias neuronais e endócrinas, informando a ocorrência de um processo infeccioso (DANTZER;

KELLEY, 2007). Em resposta, o cérebro induz alterações metabólicas, hormonais e comportamentais (KELLEY et al., 2003).

O *sickness behavior* é caracterizado por uma série de alterações comportamentais que incluem letargia, ansiedade, redução da atividade locomotora, ingestão de água e comida, reprodução, atividade exploratória e interação social (FRENOIS et al., 2007; GAYKEMA; GOEHLER, 2011; GROSSBERG et al., 2011; HENNESSY; TERRENCE; SCHILLER, 2014). Este padrão comportamental é tipicamente induzido por infecções agudas ou injuria tecidual e funciona como um mecanismo de resposta adicional do sistema imune, pois esta mudança de comportamento previne que o organismo gaste energia e se exponha a perigos, facilitando o processo de cura (JOHNSON, 2002).

Uma vez que os neurônios não possuem receptores como os TLRs para identificar patógenos, o comportamento de doença é desencadeado por moléculas do sistema imune (DANTZER; KELLEY, 2007). As citocinas pró-inflamatórias, como IL-1β, IL-6 e TNF-α são moléculas chave nesse processo, são produzidas pelo cérebro ou em outro lugar do corpo e chegam ao cérebro para promover estas alterações comportamentais (KELLEY et al., 2003).

2.4.4 Estresse x sistema imune

O estresse possui uma entrelaçada relação com o sistema imune, que pode acarretar tanto a ativação exacerbada quanto a supressão da resposta imune, dependendo do tempo e da persistência do agente estressor (DHABHAR, 2014; TORT, 2011).

A ação imunossupressora do estresse ocorre principalmente devido a ação do cortisol sobre as células imunológicas. Algumas das ações do hormônio do estresse sobre a resposta imune intata incluem a inibição da migração de granulócitos, e sob resposta imune adaptativa a diminuição da proliferação de linfócitos, da produção de anticorpos, diminuição da resposta de Th1 em relação à Th2 (BRAUN et al., 1997; DUMBELL et al., 2016). Estes efeitos levam a redução da capacidade o sistema imunológico de responder adequadamente a agentes agressores, aumentando assim o risco de infecções. Ainda, o aumento da resposta de Th2 está associada a reações alérgicas, asma, urticária e alergia a alimentos (BRAUN et al., 1997)

Além do aumento da incidência de doenças infecciosas, a ativação exacerbada do sistema imune causada pelo estresse está associada ao aumento de citocinas pró-inflamatórias, principalmente IL-1β, IL-6 e TNF-α (CALCIA et al., 2016). Níveis elevados destas citocinas causam alterações no sistema nervoso central, incluindo modificações na função da micróglia e de astrócitos, culminando em neuroinflamação. A longo prazo, como o que ocorre no estresse

crônico, a neuroinflamação pode predispor a patologias neurológicas como depressão e ansiedade (FRIES et al., 2015).

Em peixes, a resposta ao estresse ocorre através da ativação do eixo HPI (item 2.2), que também culmina a produção de cortisol (ALSOP; VIJAYAN, 2009). Pesquisas utilizando o peixes como modelo animal demonstram a ocorrência dos efeitos imunossupressores do estresse, como redução do número de linfócitos circulantes em *zebrafish*, e da atividade da lisozima, do sistema do complemento e da resposta de anticorpos e outras espécies de teleósteos (GRZELAK et al., 2017; SUNYER, J.O., TORT, 1995; VERBURG-VANKEMENADE, B.M.L., STOLTE, E.H., METZ, J.R., CHADZINSKA, 2009). O efeito pró-inflamatório do estresse também é evidenciado em pesquisas com o *zebrafish*, como aumento de citocinas após protocolo de estresse crônico imprevisível (ECI) (MARCON et al., 2016; SONG et al., 2018).

2.5 O *ZEBRAFISH* COMO MODELO ANIMAL

O *zebrafish* (*Danio rerio*) é um pequeno peixe teleósteo muito utilizado como modelo animal em pesquisa científica (RENSHAW; TREDE, 2012). É um organismo que possui desenvolvimento embrionário rápido, e alcança a fase adulta em aproximadamente três meses (KIMMEL et al., 1995). Trata-se de uma espécie de pequeno porte, o que permite com que sejam armazenados em grandes quantidades com baixo custo de manutenção (DAHM; GEISLER, 2006). Além disso, o genoma do *zebrafish* foi totalmente sequenciado e apresenta 70% de similaridade com o genoma humano (HOWE et al., 2013). Devido a estas características favoráveis, o uso desta espécie em estudos científicos é amplamente aceita (DAHM; GEISLER, 2006; MIKLÓSI; ANDEWS, 2006; RENSHAW; TREDE, 2012; VARGAS; SIGURGEIRSSON, 2011) .

O *zebrafish* vive normalmente em cardumes e apresenta um comportamento social desde o início da sua vida (SAVERINO; GERLAI, 2008a). Além disso, existem vários testes comportamentais padronizados para uso científico no *zebrafish*. Assim, ele é considerado um ótimo modelo para estudos comportamentais, como avaliação de estresse (ABREU et al., 2018; JENNIFER M. RAMSAY, GRANT W. FEISTA, ZOLTÁN M. VARGAB, MONTE WESTERFIELDB, L.; KENTD, 2009; PIATO et al., 2011), comportamento exploratório (AHMAD; RICHARDSON, 2013; SACKERMAN et al., 2010), ansiedade (BARCELLOS et al., 2018) e interação social (FILBY et al., 2010; SAVERINO; GERLAI, 2008b).

A relação entre sistema imune e comportamento é bastante estudada em mamíferos (DERECKI et al., 2010; FILIANO et al., 2016; GROSSBERG et al., 2011; LOUVEAU et al.,

2015; MOON et al., 2015), porém existem poucas pesquisas que investigam esta interação em peixes. Assim, há uma carência de estudos que demonstrem a relação entre sistema imune e comportamento no *zebrafish* (LEE et al., 2015; NOVOA; FIGUERAS, 2012).

3 OBJETIVOS

3.1 OBJETIVO GERAL

Avaliar se o sistema imune e o comportamento estão inter-relacionados no *zebrafish*.

3.2 OBJETIVOS ESPECÍFICOS

Avaliar se a expressão gênica de citocinas no cérebro varia de acordo o perfil de comportamento exploratório e preferência social do *zebrafish*.

Avaliar se a ativação da resposta imune provoca alterações no comportamento dos peixes compatíveis com as descritas para o s*ickness behavior* em mamíferos.

Avaliar se a ativação da resposta imune tecidual induz alterações na expressão de citocinas e marcadores de função neuronal no cérebro.

Avaliar se a ocorrência de estresse agudo e crônico induzem alterações na expressão gênica de citocinas no cérebro marcadores de função neuronal do *zebrafish*.

4 ARTIGOS

Neste capítulo serão apresentados três artigos. O primeiro artigo, intitulado *First description of behavior and immune system relationship in fish*, foi publicado no periódico *Scientific Reports*, em janeiro de 2018, sob DOI:10.1038/s41598-018-19276-3 ISSN 2045-2322 (Qualis A1 nas Ciências Biológicas II e fator de impacto de 4,01).

O segundo artigo, intitulado *Characterization of sickness behavior in zebrafish*, foi publicado no periódico *Brain, Behavior and Immunity*, em julho de 2018 sob o DOI:10.1016/j.bbi.2018.07.004 (Qualis A1 nas Ciências Biológicas II e fator de impacto de 6,1).

O terceiro artigo, intitulado *Acute and chronic stress differently alter the expression of cytokine and neuronal markers genes in zebrafish brain* foi publicado no periódico *Stress* em fevereiro de 2020 sob o DOI: 10.1080/10253890.2020.1724947 (Qualis B1 nas Ciências Biológicas II e fator de impacto 2,1)

4.1 ARTIGO 1 - *FIRST DESCRIPTION OF BEHAVIOR AND IMMUNE SYSTEM RELATIONSHIP IN FISH*

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First description of behavior and immune system relationship in fish

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Considering the intriguing relationship between immune system and behavior recently described in mammals, and the lack of information of this relationship in fish, here we describe for the first time the interaction between the immune system and social and exploratory behavior in zebrafish. Fish high responders to novelty (HRN) presented a proinflammatory profile, with increased IL-1β and reduced IL-10 expression compared to fish low responders to novelty (LRN). Likewise, fish less responsive to social stimuli have a reduced expression of INF-γ. We show that fish with different behavior patterns have differences in the immune response. Our findings indicate that the interplay between immune system and behavior in zebrafish is similar to that found in mammalian models and that zebrafish should be considered as a potential model organism to study the relationship between immune system and behavior.

The immune system and behavior are deeply connected. Human behavioral disorders such as autism, schizophrenia and depression are associated with dysfunction of the immune system, with altered levels of cytokines, which in turn might directly affect neuronal function[1–3]. While the capacity of neurological diseases to interfere with immune system is widely studied[4] but, in contrast, investigations on the role of the immune system in regulating neuronal physiology are only emerging recently[5,6]. Studies in rodents demonstranted that cells and molecules of the immune system are involved in neurological functions such as learning, memory, anxiety and social behavior[5–9]. Mast cells, which are involved in allergic responses, are present in meninges and brain and are key cells during neurogenesis: a deficiency in mast cells increases anxiety, and causes learning and memory deficits[7]. Learning and memory are also affected by Interleukin 4 (IL-4), a cytokine produced by mast cells during inflammation, and by T lymphocytes, which are found on meninges and central to adaptive immune responses[8]. Furthermore, Interferon gamma (INF-γ), a pleiotropic cytokine also produced by T lymphocytes is involved in controlling social behavior[9].

Although the interaction of nervous and immune system has been investigated using mammals as animal models, there is a potential to exploit other species as model organisms. The zebrafish is one of the main animals species used in scientific research[10], widely employed in behavioral studies[11], because it presents behavior characteristics of other vertebrates[12], clear individual differences of behavior[13], besides there are several behavioral tests standardized for scientific use in the species[14]. Considering the intriguing relationship between immune system and behavior[3,5–9,15], and the lack of information of this relationship in fish, here we describe the interaction between the immune system and social and exploratory behavior in zebrafish.

Results

Novel object test - immune system x exploratory behavior. The time spent in the presence of a novel object was significantly lower in fish classified as high responders to novelty (HRN) (Wald-Wolfowitz rank test, $P < 0.0001$, Fig. 1A). These HRN fish also presented differences in locomotor parameters as decreased distance traveled (1B), line crossings (1C) and rotations (1D), and spent less time at the tank bottom (1E). In the HRN fish, the mRNA levels of IL-1β were upregulated while the levels of IL-10 mRNA were downregulated indicating a proinflammatory profile. No significant differences were found in the mRNA levels of the other cytokines tested (1F). The raw data and statistics are presented as Supplementary Information.

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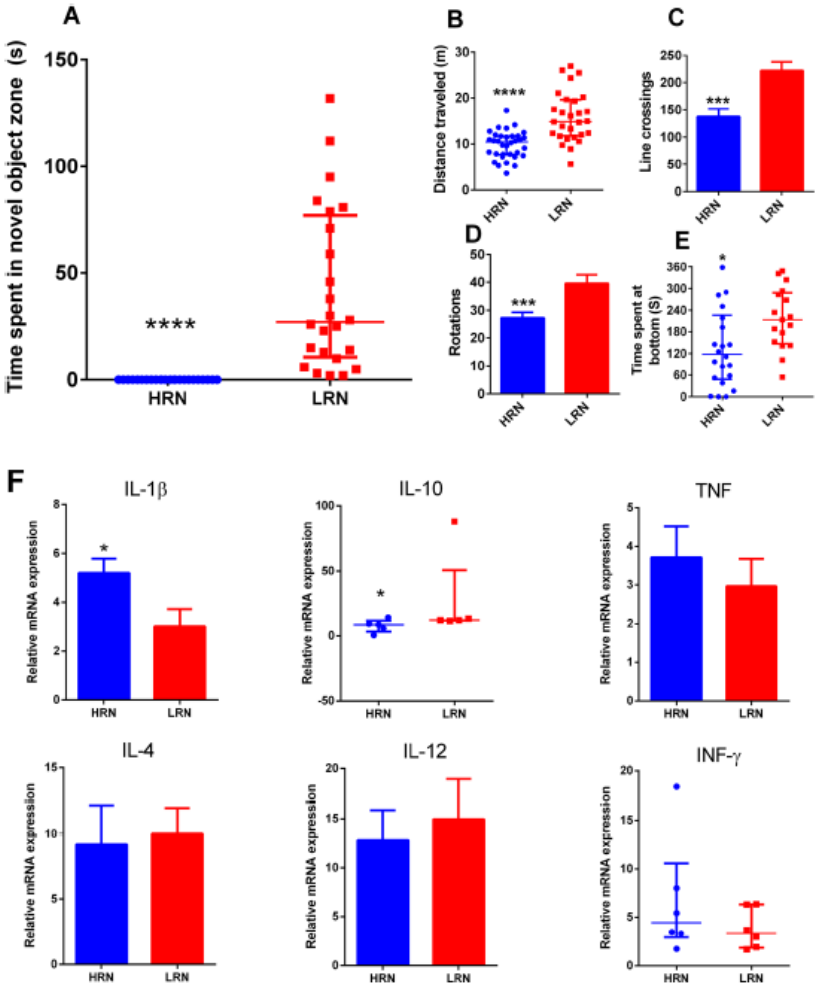

Figure 1. Novel object test. Time spent in the novel object zone (**A**), distance traveled (**B**), line crossings (**C**), rotations (**D**), time spent at the bottom (**E**) and expression of cytokine genes (**F**). Each data represents the mean ± SEM or median ± interquartile range, depending on the data normality assessed by the Bartlett's test. In panels A to E data represents the mean or median of 24 fish. In panel F, data represents the mean or median of 6 pooled samples. Significant differences are indicated by asterisk (*$p < 0.05$; ****$p < 0.0001$).

Social preference test - immune system x social behavior. Zebrafish exhibiting a "no preference" profile spent less time in the segment close to their conspecifics ($p < 0.0001$) (Fig. 2A). In these fish without social preference, the INF-γ gene expression was significantly reduced ($p < 0.01$). No differences in the mRNA levels were detected in the other genes evaluated (Fig. 2B). The raw data and statistics are provided as Supplementary Information.

Discussion

Here we show a clear relationship between the immune response of zebrafish and their exploratory and social behavior. Fish high responders to novelty (HRN) express cytokines with a proinflammatory profile, with increased IL-1β and reduced IL-10 expression compared to fish low responders to novelty (LRN). Likewise, fish less responsive to social stimuli have a reduced expression of INF-γ. Despite the clear relationship between the expression of selective immune-related genes and behavior, an intriguing question arises: is the immune system

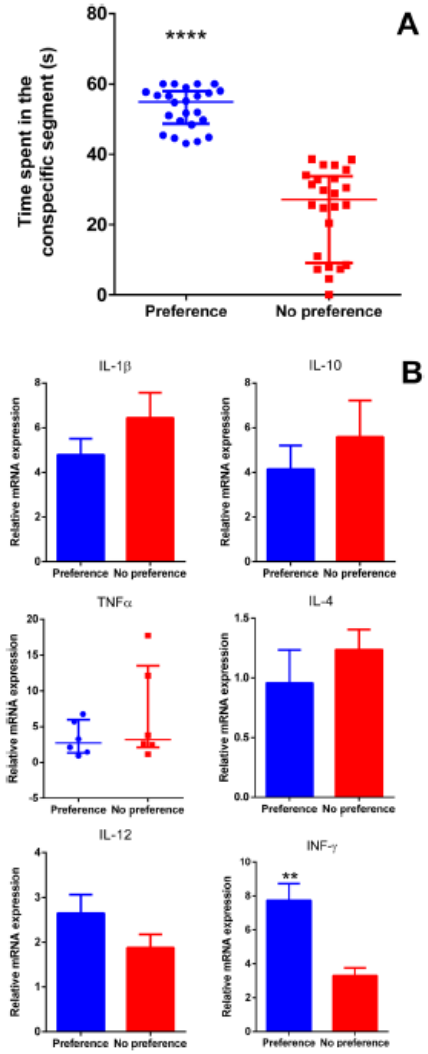

Figure 2. Social preference test- time spent in the conspecific segment. (**A**), and gene expression of cytokines (**B**). Each data represents the mean ± SEM or median ± interquartile range, depending on the data normality assessed by the Bartlett's test. In panel A data represents the median of 24 fish and in panel B data represents the mean or median of 6 pooled samples. Significant differences are represented by asterisk (**$p < 0.01$; ****$p < 0.0001$).

that leads to different behavioral patterns or is the behavioral pattern that alters cytokine profile and the outcome of the immune response?

Our main hypothesis is that the immune system modulates fish behavior. Challenging pathogens trigger a complex set of innate and adaptive immune mechanisms interconnected by cytokines secreted by several immune cells[16]. Besides promoting resistance to pathogens these cytokines modulate behavior to minimize the risk of new infections[4,17]. This cytokine-driven "sickness behavior", that is a set of behavioral changes described in mammals, characterized by reduces social interaction, water and food intake and exploration of new environments and new objects[2,18]. This proinflammatory profile supports previous work suggesting that neophobia (HRN fish) may be a behavioural defense mechanism, lowering both risk of predation and exposure to infection through reduced exposure[19–21].

HRN fish presented lower locomotor activity and remained less time at the bottom of the tank compared to LRN fish. At a simplistic view, LRN fish seems to exhibit an anxiety-like behavior[14]. However, we hypothesized that the increased locomotor activity observed with the LRN fish reflects their response to the new object. As they are exploring, they spend more time at the bottom of the tank where the new object is located. On the other hand, HRN fish may be avoiding the new object segment moving less and staying longer in the top of the tank, keeping a greater distance from the new object.

In the social behavior test, fish that spent less time close to their conspecific, had reduced levels of INF-γ mRNA. Interestingly, a recent study demonstrated that INF-γ knockout mice displayed reduced social behavior, indicating the central role of this cytokine on social interaction[6]. That study also demonstrated a relationship between the expression of IFN- γ and the social context in zebrafish[6]. Although social behaviour is crucial for survival and reproduction in many species[19], it is also well documented that increasing exposure to conspecifics increases the spread of pathogens[6].

Thus, it would be expected that increased social interactions would be indicative of a robust expression of immune related genes, mainly INF-γ, which appears as a key molecule in this interplay. Nonetheless, although we demonstrated the immune-behavioral relationship, we cannot ascertain weather the reduction of INF-γ leads the fish to respond less to social stimuli, or its lower interaction with the school reflects lower expression of INF-γ. Likewise, HRN behavior in fish results from the expression of proinflammatory cytokine genes or it is their fearful, stressing-type of behavior that leads to the expression IL-1B? There are evidences supporting both theories. Behavioral changes were observed in animals knockout for specific immune-related genes: for instance, lack of IL-4 expression might cause learning and memory deficits, and anxiolytic disorders[5,8]; IL-1β receptor knockouts have memory deficits[9], and social dysfunction was observed in mice knockout for INF-γ[6]. Thus, at infection, the sudden rise on cytokine expression needed to mount an immune response to overcome threatening pathogens leads to behavioral changes known as sickness behavior[17]. These data support the hypothesis that cytokines, mastering communication between cells and tissues, act also upon the nervous system causing behavioral changes.

On the other hand, behavioral changes may also alter the immune response. It has been shown that social status (dominant vs. subordinate) leads to different patterns of immune response[20,21]. Subordinated animals have a profile of proinflammatory immune cells compared to their dominant cohorts. Furthermore, when the social hierarchy is changed, the immune system alters equally, so it appears that behaviour can also modulate immune response in some situations[20]. Thus, it is likely that subordinated animals are more stressed and, consequently, prone to have increased inflammatory response. Furthermore, several studies in humans showed an imbalance of cytokines concomitantly with pathologies such as depression and autism[3,15,22–24].

Thus, neurological disturbances might alter functioning of the immune system, and immune dysfunctions can lead to behavioral changes. There is not a relationship of cause-effect, but an intimate interaction between the immune and nervous systems and little is known about the molecular pathways involved in this relationship.

Here, the relationship between behavior and immune system is described for the first time in fish. We show that the expression of specific cytokine genes in the brain of fishes varies according to behavior pattern. Our findings show that neuro-immunological interplay in fish is similar to current mammalian models highlighting the potential of zebrafish as model organism to study immune-behavioral relationship. In an ecological perspective, fish with an immune suppression cytokine profile would reduce interaction with conspecifics in several aspects of

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Materials and Methods

Study strategy. To evaluate the relationship between behavior and immune systems, we firstly discriminated zebrafish according to their exploratory behavior (high and low responders to novelty, HRN and LRN respectively) and according to their social behavior (preference and no preference for conspecifics). After, we evaluated the gene expression of selected cytokines in the brain to verify if there were differences in the immunological status in fish presenting different behaviors.

Animals and Maintenance. A stock population of 195 180-day old adult wild-type zebrafish (*Danio rerio*), mixed sex (50:50) from a single brood, were held in a tank equipped with biological filters, with a natural photoperiod (14 h light:10 h dark) and under constant aeration. The fish were fed twice a day with commercial flaked food provided *ad libidum*. Water temperature was maintained at 26 ± 2 C°, dissolved oxygen concentrations at 6.5 ± 0.4 mg/L, pH 7.0 ± 0.25, and the total ammonia concentration was less than 0.5 mg/L. Fish used in the study were in perfect health and were not subjected to any procedure or exposure to drugs prior to the experiments.

Behavioral evaluation. Two behavioral tests were performed in different zebrafish groups: the new object test, to classify fish into HRN and LRN in relationship to their exploratory behavior, and the social preference test, to discriminate fish according to their response to social stimuli. In both tests, fish behavior was recorded by a Logitech Quickcam PRO 9000 camera located in front of the tank, and the videos analyzed using ANY-maze® software (Stoelting CO, USA), which tracked animal behavior throughout testing. After testing, the water of the tank was completely changed prior to testing a new fish.

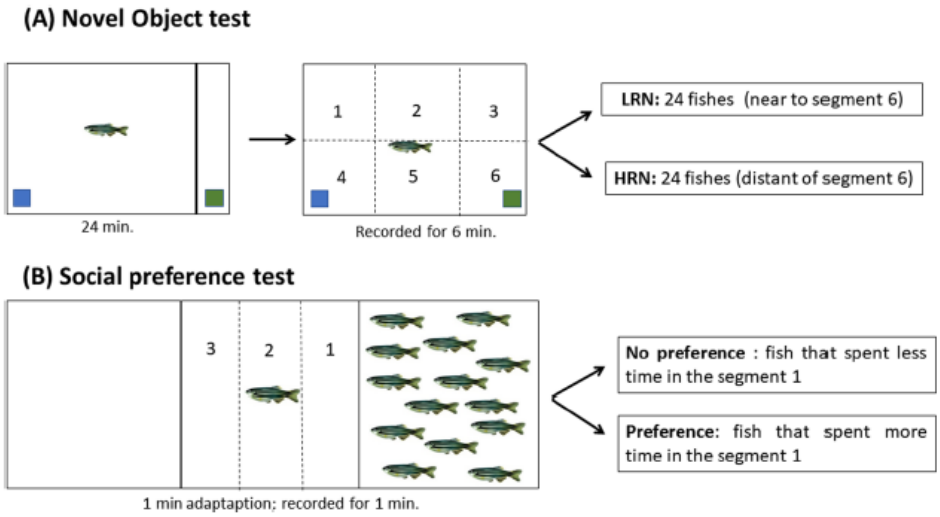

Figure 3. Schematic representation of the methodology used to discriminate exploratory (**A**) and social (**B**) behavior of zebrafish. The drawings in the panels A and B were drawn by KK.

Novel object test. A total of 120 fish were tested to determine their responsiveness to novelty. The protocol was adapted from Braida *et al.*[33] and May *et al.*[34]. Each fish was individually transferred to the test tank (24 × 8 × 20 cm; width × depth × height), which contained one object at each extremity (blue and green plastic squares measuring 4 × 4 cm). One of the object was covered by an opaque partition close to the extremity and could not be seen by the fish. The fish was introduced into the tank and for 24 minutes (based in the time to memory acquisition[35]) it was in contact with only one object. Then, the partition was removed and fish had access to the "new object". After partition removal, the behavior was recorded for 6 minutes. The color of the new object (blue or green) varied in each test. For the analysis, the test tank was virtually divided into six segments and the fish were classified by the time spent in the segment of the new object. We considered as HRN the 24 fish that did not enter once in the segment of the new object and as LRN the 24 fish that stayed longer next to the new object (Fig. 3A). We analyzed also the total distance traveled (m), number of line crossings, number of rotations and time in the bottom part of the tank (s).

Social preference test. A total of 60 fish were tested to determine their preference for conspecifics. In this test, fish were transferred individually to the test tank (30 × 15 × 10 cm; width × depth × height). The test tank was positioned between two equal sized tanks, one without fish and the other containing 15 conspecifics[36]. After transferring to the test tank, fish were acclimated for 60 seconds and their behavior was recorded for 60 seconds. For the image analysis, the test tank was virtually divided into three vertical segments. The first segment was the one nearest to conspecifics, while the third segment was next to the empty tank. The relative time zebrafish spent in the first segment was calculated as response to social stimuli (Fig. 3B).

Euthanasia and brain extraction. Immediately after performing the behavioral tests, each fish was anesthetized with Eugenol (Sigma Aldrich, Brazil, 50 mg/L) and euthanized by sectioning the spinal cord. Then, each fish was separately packed in microtubes, frozen in liquid nitrogen for 30 seconds and stored at −80 °C. For brain extraction, the skull was opened, the cranial nerves severed, and the brain carefully removed[37].

RNA extraction and cDNA synthesis. Due to the small size, the brains of four fish with the same behavioral profile were randomly pooled and used for total RNA extraction, totaling six RNA samples per group (24 fish) (6 samples per group). Tissue lysis was done using the Tissuelyser LT® (Qiagen, Brazil). RNA was extracted using RNeasy® Mini Kit (Qiagen, Brazil) and submitted to a DNAse I amplification grade treatment (Invitrogen, EUA) to eliminate genomic DNA. The RNA quality and concentration was measured by spectrophotometry (Nanophotometer Pearl®, IMPLEN, Germany), and stored at −80 °C. For cDNA synthesis, 500 ng of total RNA was used for the reverse transcription assay, using SuperScript® III Reverse Transcriptase (Invitrogen, EUA) and random primers.

Gene expression analysis by real-time quantitative PCR (qPCR). Real time PCR (qPCR) was performed in 48-well plates (MicroAmp Fast optical 48 well plate reaction, 0.1 ml. Applied Biosystems, USA) in a final volume of 10 µl. The mix consisted of 400 nM of each primer, 5 µl of SYBR Select Master Mix (Applied Biosystems, USA) and 1 µl of cDNA (diluted 1:10). The reaction was carried out in a Step One equipment Applied

Gene	Primer (5′-3′)	Efficiency	Accession number
TNF-α	F: GACCACAGCACTTCTACCG R: ACATTTTCCTCACTTTCGTTCAC	98,3%	NM_212859
IL-1β	F: GCTGGAGATGTGGACTTC R: ACTCTGTGGATTGGGGTTTG	102%	NM_212844
INF-γ	F: TGCCTCAAAATGGTGCTACTC R: AATCGGGTTCTCGCTCCTG	97,1%	AB158361.1
IL-4	F: TCTCTGCCAAGCAGGAATG R: CAGTTTCCAGTCCCGGTATATG	99,4%	AM403245.2
IL-12	F: CTGTAGGATCCATCCAAACATCT R: CACTGGCACTTCTACCCTATTT	96,8%	AB183002.1
IL-10	F: CTCTGCTCACGCTTCTTCTT R: GCTCCCTCAGTCTTAAAGGAAA	101,4%	BC163038.1
β-Actin	F: GCAAAGGGAGGTAGTTGTCTAA R: GAGGAGGGCAAAGTGGTAAA	97,7%	AF057040.1

Table 1. Immune genes, primers nucleotide sequence and qPCR efficiency.

Biosystems) with the following conditions: initial denaturing at 95 °C for 10 min followed by 40 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. At the end, a standard melting curve was included to confirm the specificity of the amplified product. Samples were performed in triplicate. The genes analyzed, their respective primers and the efficiency of each reaction are indicated in Table 1. Non template controls and the expression of a housekeeping gene (β-actin) were also analysed for comparison purposes. For the calibration curve, each gene was cloned into the pGEM-TEasy Vector System (Promega) and transformed into competent One Shot TOP10 E. coli (Promega) and cultured in LB supplemented with ampicillin. Cloning was confirmed by PCR and the resulting plasmid was extracted using the Wizard Plus SV minipreps DNA purification system (Promega). Then, the calibration curve consisted of decimal dilutions (1:10) of each cloned gene. To better compare the results from different groups the same threshold value (0.080) was used. The relative quantification of gene expression, was carried out by the $2^{-\Delta\Delta ct}$ formula[38].

Ethical Note. This study was approved by the Ethics Commission for Animal Use (CEUA) at Universidade de Passo Fundo, UPF, Passo Fundo, RS, Brazil (Protocol 008/2017) and met the guidelines of the National Council for the Control of Animal Experimentation (CONCEA).

Statistical analysis. Data of time spent in the new object segment exhibited unequal variance between treatment groups and was compared using a Wald-Wolfowits rank test. All other data of behavioral phenotypes were compared by Student's t test or by Mann-Whitney test depending on the data normality (assessed by the Bartlett's test). In all experiments, P was set at <0.05.

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Author Contributions

K.K., L.C.K. and L.J.G.B. conceptualize the experiments, analyzed the results, wrote the manuscript and prepare the figures. K.K. and D.F. conducted experimental procedures. All authors have read and approved the manuscript for publication.

Additional Information

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4.2 ARTIGO 2 - *CHARACTERIZATION OF SICKNESS BEHAVIOR IN ZEBRAFISH*

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Full-length Article

Characterization of sickness behavior in zebrafish

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ABSTRACT

In a previous study we showed a clear relationship between immune system and behavior in zebrafish and we hypothesized that the immune system is capable of inducing behavioral changes. To further investigate this subject and to address our main question, here we induced an inflammatory response in one group of fish by the inoculation of formalin-inactivated *Aeromonoas hydrophila* bacterin and compared their social and exploratory behavior with control groups. After the behavioral tests, we also analyzed the expression of cytokines genes and markers of neuronal activity in fish brain. In the bacterin-inoculated fish, the locomotor activity, social preference and exploratory behavior towards a new object were reduced compared to the control fish while the expression of proinflammatory cytokines in the brain was upregulated. With this study we demonstrated for the first time that the immune system is capable of causing behavioral changes that are consistent with the sickness behavior observed in mammals.

1. Introduction

Zebrafish has a fully developed immune system in which the innate immune response is the primary form of defense against pathogens (Meeker and Trede, 2008; Renshaw and Nikolaus, 2012). Immune cells, pattern recognition receptors (PRR) such as toll-like receptors (TLRs), defensins and several cytokines are similar in structure and functions to mammals (Meeker and Trede, 2008; Secombes et al., 2011; Suarez-carmona et al., 2015). Teleost adaptive immune system, although primitive when compared to mammals, is composed of B lymphocytes that secrete at least two classes of immunoglobulins (IgM, IgZ) (Lagos et al., 2017), and T lymphocytes with subsets as helper, cytotoxic, and regulatory T cells (Castro et al., 2011; Fischer et al., 2013; Nakanishi et al., 2011; Renshaw and Nikolaus, 2012). Because of this, the use of zebrafish as a model of translational studies of human and animal diseases has been growing steadily over the last few years.

Recent studies refuted the dogma of the central nervous system being an immunological privileged organ and indicated a possible relationship between immune system and behavior in mammals (Kipnis, 2018) and fish (Lee et al., 2015; Rakus et al., 2017). Indeed, neurological disorders such as depression, autism and schizophrenia are associated with dysfunction of the immune system (Dantzer, 2009; Dantzer et al., 2008; Estes and McAllister, 2015; Gibney and Drexhage, 2013; Müller and Schwarz, 2010; Ross et al., 2013). Furthermore, recent studies indicated that cells and molecules of the immune system are involved in neurological functions such as learning, memory, anxiety and social behavior (Derecki et al., 2010; Filiano et al., 2016; Moon et al., 2015; Nautiyal et al., 2008). In addition, when the organism is infected by a pathogen, the activation of the immune response leads to a pattern of behavioral alterations known as "sickness behavior" (Kelley et al., 2003).

Sickness behavior is a set of behavioral changes characterized by lethargy, anxiety, reduced physiological function such as locomotor activity, food intake, reproductive performance, exploratory activity and social interaction (Dantzer and Kelley, 2007; Grossberg et al., 2011; Haba et al., 2012; Hennesey et al., 2014; Kelley et al., 2003). This pattern of behavior is typically induced by acute infections and/or tissue injury, aiming to prevent the organism from expending energy and exposing itself to other pathogens, facilitating the healing process (Dantzer et al., 2008; Kelley et al., 2003). Since neurons do not have receptors to identify pathogens, sickness behavior is triggered by molecules derived from immune cells, which are implicated as potential

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modulators of brain function (Ashley and Demas, 2017; Dunn, 2006). Proinflammatory cytokines such as IL-1β, IL-6 and TNF-α are key molecules in this process (Kelley et al., 2003) and during systemic infection might reach the brain where they promote these behavior changes (Dantzer et al., 2008). Sickness behavior is well described in mammals, including humans, but in fish there is not enough data yet to characterize this phenomenon (Johnson, 2002).

In a previous study we showed the relationship between immune system and behavior in zebrafish (Kirsten et al., 2018). According to their exploratory behavior, fish high responders to novelty (HRN), that do not explore a new object in the test tank (i.e. neophobic), express cytokines with a proinflammatory profile (IL-1β upregulated and IL-10 down regulated) compared to fish low responders to novelty (LRN) that explore a new object in the test tank. In relation to their social behavior, fish less responsive to social stimuli have a reduced expression of INF-γ (Kirsten et al., 2018). Despite this, a fundamental question remained unanswered: is the immune system that leads to different behavioral patterns or is the behavioral pattern that alters the immune response? Here we show that systemic activation of immune cell can leads to a pattern of behavioral changes that may be characterized as a sickness behavior, well described in mammals (Johnson, 2002), but not yet fully characterized in zebrafish.

2. Materials and methods

2.1. Animals and maintenance

We used 72 mixed sex, 180-day old, wild-type zebrafish (*Danio rerio*) from our stock population, which results from mating of different males and females. Fishes were kept in one larger tank in our aquarium system and then transferred to 24 smaller tanks (3 fish per tank) for a seven day acclimatization period. All tanks were equipped with biological filters and kept under natural photoperiod (14 h light:10 h dark). Water temperature was maintained at 27 ± 2C°, dissolved oxygen concentrations at 6.0 ± 0.4 mg/L, pH 7.0 ± 0.25, and the total ammonia concentration was less than 0.5 mg/L. The fish were fed twice a day with commercial flaked food, and water was under constant aeration. This study was approved by the Ethics Commission for Animal Use (CEUA) of Universidade de Passo Fundo, Rio Grande do Sul, Brazil (Protocol #008/2017-CEUA) and met the guidelines of the National Council for the Control of Animal Experimentation (CONCEA).

2.2. Study strategy

To assess the behavioral changes caused by systemic activation of the immune response, we separated the zebrafish into three groups The experimental group called stimulated immune system (SIS), in which an inflammatory response was induced by the inoculation of formalin inactivated *Aeromonas hydrophila* bacterin, and two control groups: one inoculated with sterile PBS (Inoculated control – IC) and another group that did not undergo any procedure (Negative Control – NC). Behavioral changes were assessed by the novel tank test, novel object test, and social preference test, 24 h after the immune challenge (SIS group) and PBS inoculation (IC group). After the behavioral tests, we analyzed the expression of cytokines genes and markers of neuronal activity in fish brain. For a better understanding, a scheme of the methodology is represented in Fig. 1.

2.3. Stimulation of the immune system

To stimulate the immune response, fish were anesthetized in Eugenol (Sigma Aldrich, Brazil, 50 mg/L) and immediately inoculated with 50 µl of 4×10^5 formalin-inactivaded *Aeromonas hydrophila* bacterin (SIS group) mixed to 10% Montanide adjuvant (Seppic, France), or 50 µl of sterile PBS (IC group) using a ultra-Fine BD insulin syringe 0.25 mm. The NC group did not undergo any procedure before the behavioral tests.

2.4. Evaluation of behavior parameters

The behavioral tests were performed in all groups of zebrafish 24 h after the injection procedure. The tests performed were: novel tank test, in which parameters of locomotion and anxiety were evaluated; social preference test, to discriminate fish according to their response to social stimuli; and the novel object test, to evaluate their exploratory behavior. The fish performance during the tests were recorded by a Logitech Quickcam PRO 9000 camera located in front of the tank, and the videos analyzed using ANY-maze® software (Stoelting CO, USA). After each test, the water of the tank was completely changed prior to testing a new fish.

2.4.1. Novel tank test and social preference test

The novel tank test and the social preference test were performed together, with twelve fish from each group. The novel tank test was carried out first and each fish was placed in the test tank (24 × 8 × 20 cm; width × depth × height) that had both lateral sides closed by an opaque partition positioned in between the tanks, as indicated in Fig. 1, and its behavior was recorded for 6 min. The following parameters were analyzed: distance traveled (m), mean swimming speed (m/s), number of line crossing, absolute turn angle, and time spent at the bottom and at the top of the test tank. After that, the partitions were carefully removed for the social preference test: one side of the test tank had no fish and the other side had 15 mixed sex conspecifics. The fish behavior was recorded for 60 s to assess its preference for the empty side or conspecifics. For the image analysis, the test tank was virtually divided into three vertical segments, 8 cm width and 6,5 cm height each. The first segment was the one nearest to conspecifics, while the third segment was next to the empty tank. The relative time zebrafish spent in the first segment was calculated as response to social stimuli.

2.4.2. Novel object test

Twelve fish from each group were tested to determine their responsiveness to novelty, according to the protocol we published recently (Kirsten et al., 2018). Each fish was individually transferred to the test tank (24 × 8 × 20 cm; width × depth × height), which contained one object at each extremity (blue and green plastic squares measuring 4 × 4 cm). One of the objects was covered by an opaque partition close to the extremity and could not be seen by the fish. The fish was introduced into the tank and for 24 min it was in contact with one object only. Then, the partition was removed and fish had access to the "new object", and its behavior was recorded for 6 min. The color of the new object (blue or green) varied in each test. For the analysis, the test tank was virtually divided into six segments and the time spent in the segment of the new object was evaluated.

2.5. Euthanasia and brain extraction

Immediately after performing the behavioral tests, each fish was anesthetized with Eugenol and euthanized by sectioning the spinal cord. Then, each fish was separately packed in micro tubes, frozen in liquid nitrogen for 30 s and stored at −80 °C. For brain extraction, the skull was opened, the cranial nerves severed, and the brain carefully removed (Vargas et al., 2011).

2.6. RNA extraction, DNA synthesis and gene expression analyses

Since in the behavioral tests the IC and NC groups did not present differences, we evaluated the gene expression of the SIS and IC groups.

Due the small size of zebrafish, three fish brains from the same group were randomly pooled and used for total RNA extraction, totalizing six RNA samples per group. Tissue lysis was done using the

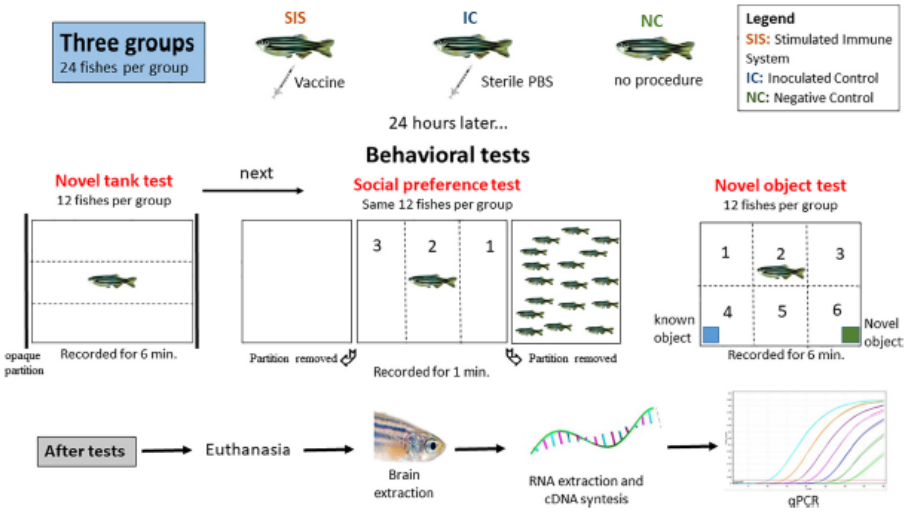

Fig. 1. Schematic representation of the methodology used to evaluate behavioral changes in fish.

Tissuelyser LT® (Qiagen, Brazil). RNA was extracted using RNeasy® Mini Kit (Qiagen, Brazil) and genomic DNA was eliminated by DNAse I amplification grade treatment (Invitrogen, EUA) The RNA concentration and quality was measured by spectrophotometry (Nanophotometer Pearl®, Implen, Germany), and stored at −80 °C. The cDNA synthesis was done using 500 ng of total RNA, using NextGeneration MMLV RNAse H Minus First-Strand cDNA Synthesis Kit (DNA express) and random primers.

For gene expression analysis, real time PCR (qPCR) was performed in a Rotor-gene Q equipment (Qiagen) using NextGeneration ECO SYBR Green HotStart qPCR Kit (DNA express), specific primers and cDNA (diluted 1:10). The genes analyzed, their respective primers and the efficiency of each reaction are indicated in Table 1. Non template controls and the expression of a housekeeping gene (β-actin) were also analyzed for comparison purposes. All samples were performed in triplicate. For the calibration curve, each gene was cloned into the pGEM-TEasy Vector System (Promega) and transformed into competent One Shot TOP10 *E. coli* (Promega) and cultured in LB supplemented with ampicillin. Cloning was confirmed by PCR and the resulting plasmid was extracted using the Wizard Plus SV minipreps DNA purification system (Promega). Then, the calibration curve consisted of decimal dilutions (1:10) of each cloned gene. To better compare the results from different groups the same threshold value (0.100) was used. The relative quantification of gene expression was carried out by the $2-\Delta\Delta ct$ formula (Rao et al., 2013).

Table1
qPCR primers.

Gene	Primer (5′-3′)	Efficiency	Accession number
β-Actin	F: GCAAAGGGAGGTAGTTGTCTAA R: GAGGAGGGCAAAGTGGTAAA	104%	AF057040.1
cFOS	F: CAGCTCCACCACAGTGAAGA R: GCTCCAGGTCAGTGTTAGCC	104%	DQ003339.2
BDNF	F: CGCCGTTACTCTTTCTCTTGG R: CCATTAGTCACGGGGACCTTC	96%	NM_001308648.1
TNF-α	F: GACCACAGCACTTCTACCG R: ACATTTTCCTCACTTTCGTTCAC	95%	NM_212859
IL-1β	F: GCTGGAGATGTGGACTTC R: ACTCTGTGGATTGGGGTTTG	101%	NM_212844
INF-γ	F: TGCCTCAAAATGGTGCTACTC R: AATCGGGTTCTCGCTCCTG	98%	AB158361.1
IL-4	F: TCTCTGCCAAGCAGGAATG R: CAGTTTCCAGTCCGGTATATG	100%	AM403245.2
IL-10	F: CTCTGCTCACGCTTCTTCTT R: GCTCCCTCAGTCTTAAAGGAAA	105%	BC163038.1
IL-6	F: GCGTCCTGACGTGGTATAAAG R: GTCGTTTGGTGCTGTGTTTG	96%	NM_001261449.1
IL-12	F: CTGTAGGATCCATCCAAACATCT R: CACTGGCACTTCTACCCTATTT	104%	AB183002.1

2.7. Statistical analyses

For the analysis of behavioral phenotypes we first compared the data of the IC and NC groups by Student's *t* test or by Mann-Whitney test (depending on the data normality assessed by the Bartlett's test) and we found that there was no statistical difference between the groups in any of the behavioral tests. Then we compared the group SIS with the IC by the same tests. The gene expression analyses also were performed by Student's *t* test or by Mann-Whitney. In all experiments, P was set at < 0.05.

3. Results

It is important to note that all fish in the SIS group presented skin lesions at the inoculation site, confirming the occurrence of an inflammatory response. The IC group did not showed lesion after inoculation.

3.1. Behavior parameters

There was no statistical difference between the NC and IC groups in any of the behavioral tests (table 2). Thus, we compared the results of the behavioral tests and the gene expression of the IC and SIS groups.

In the novel tank test fish from the SIS group travelled a smaller distance ($p = 0.03$), had lower mean speed ($p = 0.01$), and lower number of angle turns ($p = 0.02$). The other parameters evaluated did not differ significantly between the groups (Fig. 2.A).

The social preference test demonstrated that fish from the SIS group spent less time near their conspecific ($p < 0.0001$) and more time in the opposite segment ($p = 0.002$) (Fig. 2.B).

Table 2
Behavioral tests of NC and IC groups. The data are expressed as the mean ± SEM. In the first column: 1. Novel tank test; 2. Social preference test; 3. Novel object test.

Parameters	NC	IC	*p*
Distance Traveled (m)[1]	18.06 ± 2.401	16.43 ± 1.897	0.59
Mean speed (m/s)[1]	0.005 ± 0.0006	0.0045 ± 0.0005	0.43
Line crossing[1]	127.3 ± 28.14	117.4 ± 18.33	0.77
Turn angle[1]	48764 ± 7362	54619 ± 6465	0.55
Time at the bottom (s)[1]	230.3 ± 20.86	251.8 ± 16.91	0.43
Time at the top (s)[1]	46.65 ± 9.054	28.30 ± 6.645	0.11
Time in the conspecific segment (s)[2]	41.38 ± 5.03	43.52 ± 5.78	0.78
Time in the opposite conspecific segment(s)[2]	5.382 ± 1.93	3.318 ± 1.827	0.44
Time in the novel object zone(s)[3]	38.39 ± 11.56	27.69 ± 11.53	0.52
Latency to enter in the novel object zone(s)[3]	69.67 ± 28.14	85.23 ± 32.44	0.72

In the novel object test fish from the SIS group spent less time exploring the new object ($p = 0{,}004$) and took a longer time to enter the new object zone ($p = 0{,}04$) (Fig. 2.C).

3.2. Gene expression analyses

The mRNA levels of the proinflammatory cytokines IL-1β, IL-6 and TNF-α were upregulated in the brain of fishes from the SIS group ($p < 0{,}01$). No significant differences were found in the mRNA levels of the other cytokines tested, as well as in the expression of BDNF and cFOS genes (Fig. 3).

4. Discussion

We recently described the relationship between behavior and immune system in zebrafish (Kirsten et al., 2018). From that study we wondered whether it is the immune system that leads to different behavioral patterns or is the behavioral pattern that alters the immune response? To approach this subject we carried out studies with bacterin injected zebrafish that were then used on behavior tests. We used male and female fish randomly selected from our stock that were not priorly evaluated regarding acclimatization or behavior characteristics. In our hands (unpublished data), social and exploratory behavior are similar amongst males and females; thus, the results we found are no influenced by fish gender. In addition, because of zebrafish small size and the difficulties to obtain enough blood to evaluate circulating cytokines we opted to evaluate cytokine mRNA levels by qPCR. Nonetheless, this does not lessen the strength of our data in that the measurement of cytokines mRNA levels has been widely used to evaluate immune system activation in several species including fish (Grossberg et al., 2011; Kelley et al., 2003; Krishnananthasivam et al., 2017; Vojtech et al., 2009) Our data indicate that fish behavior changes parallel to activation of a systemic immune response, strengthening our previous hypothesis that the immune system modulates behavior (Kirsten et al., 2018).

To stimulate an inflammatory immune response we used a formalin-inactivated gram-negative bacterium (*A. hydrophila*) usually associated with hemorrhagic septicemia in fish (Rodríguez et al., 2008). The amount of bacteria used *per* fish was equivalent to the LC_{50} of *A. hydrophila* for zebrafish (Rodríguez et al., 2008). Furthermore, to potentiate the immune response the bacteria was mixed with Montanide (10% v/v) which is widely used as vaccine adjuvant in aquaculture species (Håstein et al., 2005; Lagos et al., 2017; Pavan et al., 2016). Because this is a pioneering study aiming to investigate possible behavioral changes induced by activation of immune cells, we opted to evaluate all parameters (behavioral and immunological) at a single time point (24 h post inoculation) in which immune activation, here determined by expression of cytokine genes, would be at higher level, as reported previously (Lagos et al., 2017). Accordingly, zebrafish inoculated intraperitoneally with the bacterin had a systemic inflammatory response that altered the expression of cytokines gene in the brain and this led to alterations in behavioral parameters such as reduction of distance traveled, mean speed and number of angle turns in the novel tank test, social preference and exploratory behavior to a new object. Similar pattern of behavioral changes characterizes the sickness behavior, well described in mammals, mainly rodents (Frenois et al., 2007; Haba et al., 2012; Layé et al., 2000) and humans (Dantzer et al., 2008; Johnson, 2002; Kipnis, 2018), but not fully characterized in fish (Lee et al., 2015; Rakus et al., 2017).

Sickness behavior is a well-organized adaptive response to enhance disease resistance and facilitate recovery (Johnson, 2002). The behavioral changes found in this study, such as reduction of locomotor activity, reduction of social preference and exploration of a new object are characteristic of rodents sickness behavior (Gaykema and Goehler, 2011; Grossberg et al., 2011; Kelley et al., 2003). Reduction of locomotor activity occurs to redistribute physiological priorities to combat infection and to minimize energy expenditure (Grossberg et al., 2011). Reinforcing our hypothesis, zebrafish showed a reduction in locomotor activity a day after bacterial infection (Lee et al., 2015).

Immune-stimulated fish presented a large reduction of social preference, spent much less time in the segment near the conspecific fish when compared to the healthy animals. Social interaction and comingling with conspecifics potentiates the spread of pathogens (Filby et al., 2010; Kelley et al., 2003). However, the detection of pathogens by immune cells alerts the central nervous system that in turn reduces social behavior and responsive to conspecific most likely as a self-protection mechanism and as an evolutionary tool to preserve the shoal. Zebrafish is a social species exhibiting a high preference for being close to conspecifics (Saverino and Gerlai, 2008). Based in this highly social behavior of zebrafish, the reduction of social interaction is a sensitive tool to evaluate sickness behavior.

Regarding exploratory behavior, SIS fish presented a significant reduction in the time spent exploring a new object. Reduced exploratory activity is a characteristic of sickness behavior in mammals, and act as a defense mechanism to reduce the risk of predation, energy expenditure and exposure to other pathogens (Bourke, 2014). In fact, fish classified as high responders to novelty (i.e. neophobic) presented a higher expression of proinflammatory cytokines (Kirsten et al., 2018), corroborating the hypothesis that the inflammatory response reduces the exploratory profile in the zebrafish.

The immune system communicates with the brain by secreting molecules known as cytokines (Dunn, 2006). In the absence of pathogens, the peripheral injection of proinflammatory cytokines causes the classic behavioral changes of sickness behavior in rodents (Dantzer, 2009). Here, the increased expression of IL-1β, TNF-α and IL-6 in fish from the SIS group, a clear demonstration that an inflammatory process is ongoing, lead to alterations in the expression of immune genes in the brain, as demonstrated also in mammals (Dunn, 2006). Among the cytokines, the IL-1β alone can trigger sickness behavior, while IL-6 and TNF- α act to potentiate the IL-1β effects (Dantzer, 2009; Layé et al., 2000).

Once it had been accepted that the immune system can induce behavioral alterations by using cytokines as communicating molecules, the question is how does it happen? We evaluated the expression of the nerve growth factor brain-derived neurotrophic factor (BDNF) and neuronal marker cFOS genes, but they were similar in both groups. Some studies reported that increases of IL-1β into the hippocampus decreases BDNF in the brain of aging rats and leads to a deficit of long-lasting synaptic plasticity (Cortese et al., 2011; Prieto et al., 2015); in zebrafish, mRNA levels of BDNF and cFOS were not altered by the systemic immune activation.

Furthermore, the interaction of immune system and behavior is being investigated in several neurological dysfunctions such as

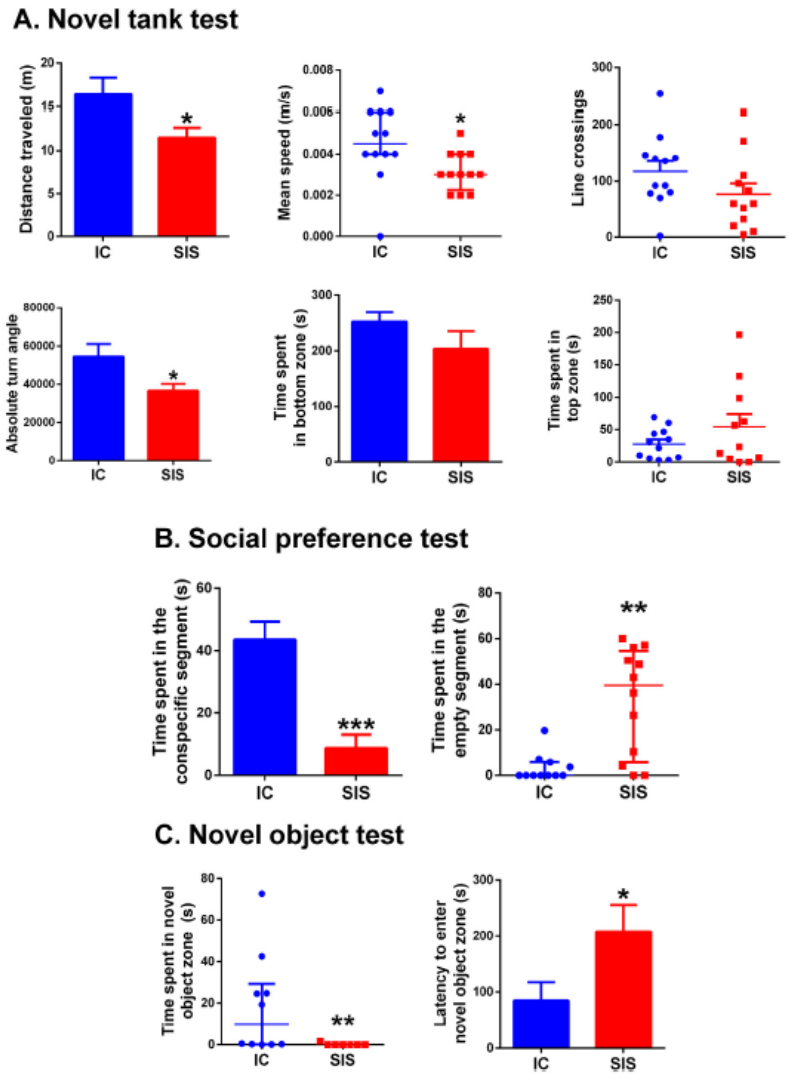

Fig. 2. Behavior parameters tests of fish from the IC and SIS groups. (A) Novel tank test (B), social preference test, and (C) novel object test. Each data represents the mean ± SEM or median ± interquartile range, depending on the data normality assessed by the Bartlett's test. Significant differences are indicated by asterisk (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). Means were compared using Student's t test while medians using Mann-Whitney test.

depression and autism (Dantzer, 2009; Estes and McAllister, 2015; Gibney and Drexhage, 2013; Meshalkina et al., 2018; Ogłodek et al., 2014). Thus, the characterization of sickness behavior in zebrafish may constitute a potential animal model for studies involving immune system and neurological disorders. Even though specific behavior varies from species to species, the sickness behavior seems to be conserved among all vertebrates (Hart, 1988).

To the best of our knowledge, here we demonstrate for the first time sickness behavior in zebrafish, with characteristic reduced locomotor activity, social preference and exploration to a new object after activation of the immune response. Sickness behavior is classically caused by pathogens, but other conditions such as stress and environmental contaminants that cause immune disruption can lead to this behavioral pattern. In an ecological perspective, the sickness behavior has a negative impact on fish individuals, because changes such as reduced social interaction makes fish more susceptible to predators and has a negative impact on reproduction, reduces exploratory activity and the search for food. However, in a population perspective, the sickness behavior occurs to improve survival by reducing/blocking the pathogen spread amongst the population.

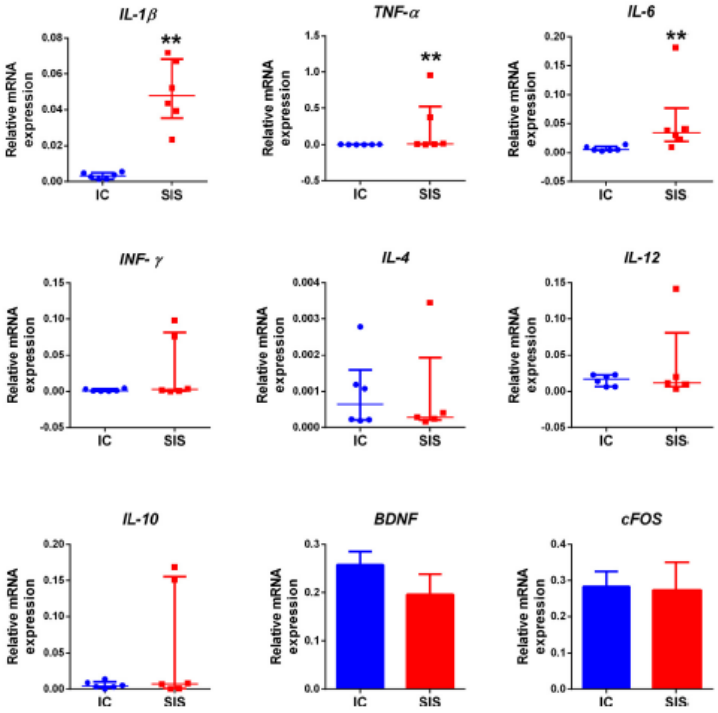

Fig. 3. Gene expression analyses on the brain of fish from the IC and SIS groups. Each data represents the mean ± SEM or median ± interquartile range, depending on the data normality assessed by the Bartlett's test. Significant differences are indicated by asterisk (**$p < 0.01$). Means were compared using Student's t test while medians using Mann-Whitney test.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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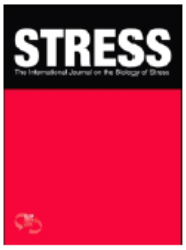
4.3 ARTIGO 3 - *ACUTE AND CHRONIC STRESS DIFFERENTLY ALTER THE EXPRESSION OF CYTOKINE AND NEURONAL MARKERS GENES IN ZEBRAFISH BRAIN*



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Acute and chronic stress differently alter the expression of cytokine and neuronal markers genes in zebrafish brain

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SHORT COMMUNICATION

Acute and chronic stress differently alter the expression of cytokine and neuronal markers genes in zebrafish brain

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ABSTRACT

We report the effects of acute and chronic stress on the expression of selective immune-related genes and markers of neuronal function in the brain of the zebrafish (*Danio rerio*). Fish were distributed into three groups: the non-stressed control group; the acute stress (AS) group, submitted to a single stressing episode; and the unpredictable chronic stress (UCS) group, submitted to two daily stressing episodes of alternating times and types of stress. The stressing protocols were applied for a period of 14 days. The UCS protocol triggered the expression of the pro-inflammatory cytokine genes IL-1β and TNF-α, the anti-inflammatory cytokine IL-10 (negative feedback from the immune system), reduction in cFOS gene expression, and caused neuro-inflammation. The AS protocol had no effect on gene expression. Altered expression of cytokine genes, as observed in our study, correlates with several pathologies associated with neuro-inflammation, and the reduction of cFOS gene expression may indicate the occurrence of reduced neuronal plasticity. Our study further extends our knowledge about the interaction of the immune system and the different forms of stress.

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KEYWORDS
Brain; cytokines; immune system; unpredictable chronic stress; acute stress; neuronal function markers

1. Introduction

Stress is a growing condition of significant concern to public health, mainly in developed countries. Stressing episodes induce metabolic changes that allow individuals to appropriately respond to stressor stimuli (Dhabhar, 2014). However, intensive and/or chronic stressing episodes may exceed the responding capacity and trigger several pathologies (Calcia et al., 2016). Cardiovascular diseases (Steptoe & Kivimäki, 2012) and pathologies that affect the central nervous system, like anxiety and depression, may be associated with stress (Baxter et al., 2013). The role of chronic stress on disease development includes deregulation of the hypothalamic-pituitary-adrenal (HPA) axis, activation of neuro-inflammation, and neuronal apoptosis (Pijanowski et al., 2015).

Stress is associated with elevated pro-inflammatory cytokines (Calcia et al., 2016). The immune system responds to stressors and communicates with the central nervous system through different mechanisms including cytokine signaling, vagal innervation, and the lymphatic system (Louveau et al., 2015). High circulating levels of cytokines induce changes in microglia and astrocyte functions, leading to neuro-inflammation and neurodegeneration (Calcia et al., 2016) which, in turn, might be associated with structural and functional changes in the brain that predispose humans to mental illness (Réus et al., 2015). To better understand the biological effects of stress, animal models such as the zebrafish (*Danio rerio*) are widely used in translational research (Piato et al., 2011; Glovatchcka, Ennes, & Mayer, 2012). The zebrafish brain is neuroanatomically and functionally comparable to the mammal brain (Vargas, Jóhannesdóttir, Sigurgeirsson, Torsteinsson, & Karlsson, 2011). In addition, zebrafish stress response is mediated by the hypothalamus–pituitary–interrenal (HPI) axis, which is similar to the mammalian hypothalamus–pituitary–adrenal (HPA) axis (Abreu et al., 2018) and culminates with the production of cortisol.

The deleterious effects of stress have been intensively studied using zebrafish and, for that, different stressing protocols were developed, like the unpredictable chronic stress (UCS) (Piato et al., 2011) and several acute stress (AS) protocols (Abreu et al., 2018). Previous studies related to the effect of stressing analyzed behavioral alterations, cortisol levels, and inflammatory response by measuring levels of selected cytokines (Marcon et al., 2016; Piato et al., 2011); however, there is a lack of information on a wider range of cytokine gene expression in the brain during acute and chronic stress. Thus, here we evaluated the expression of cytokine genes

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In this study we show that the brain reacts to acute and to chronic stresses in a divergent way. While chronic stress enhanced the expression of proinflammatory cytokines and decrease cFOS gene in the zebrafish brain, acute stress did not caused any changes in the expression of these genes.

and biomarkers of neuronal function in the brain of zebrafish undergoing acute and chronic stress.

2. Methods

2.1. Ethical and legal note

This study was approved by the Ethics Committee on the Use of Animals (CEUA), Universidade de Passo Fundo, Rio Grande do Sul, Brazil (Protocol #019/2018-CEUA), and abided by the guidelines of the National Council for the Control of Animal Experimentation (CONCEA). In addition, this research was registered in the SisGen (Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado) and complied with their guidelines (registration code A14E252).

2.2. Animals and maintenance

We used wild type zebrafish (*Danio rerio*) from our stock population; fish were kept in a glass aquarium (14 × 40 × 30 cm; width × depth × height) under constant water flux and a 14/10 h light/dark cycle. The experiment was carried out using 96 mixed sex adults that were transferred to the experimental tanks (24 glass tanks: 20 × 15 × 15 cm; width × depth × height; 3 fish per tank) and acclimatized for seven days prior to stressing.

Water temperature was maintained at 28 ± 2 °C, dissolved oxygen concentration was 6.0 ± 0.4 mg/L, pH was 7.0 ± 0.25, and the total ammonia concentration was less than 0.5 mg/L.

2.3. Study strategy

After acclimatizing, zebrafish were allocated into three groups of 24, as follows: a non-stressed control group (CG); an acute stress group (AS) that was submitted to a single episode of stress; an unpredictable chronic stress group (UCS) that was submitted to a 14-day stressing protocol. After the stressing protocols, we analyzed the expression of selective cytokine genes and markers of neuronal activity in the fish brain.

2.4. Acute stress protocol

The single stress episode consisted of exposing the fish to the air for one minute (Song et al., 2018). The fish was then returned to the aquarium and sacrificed one hour after the stressing episode to evaluate gene expression. This time point was chosen because it is the estimated time for a possible alteration of gene expression after stimulation (Vojtech et al., 2009).

2.5 Unpredictable chronic stress protocol

The UCS protocol was adapted from Piato et al. (Piato et al., 2011) and consisted of two daily stressing episodes over 14 days. The complete UCS protocol, with the time and sequence of each stressor, is shown in Table 1. Fish were sacrificed 12 hours after the last stress episode to assure that the analysis of gene expression represented the cumulative effect of the UCS and not the last stress episode.

Table 1. Procedure of the unpredictable chronic stress protocol.

Day	Stress
1	11:00 am – **Heating** tank water up to 33 °C for 30 min
	15:30 pm – **Chasing** for 4 min with a net
2	08:00 am – **Low water level** for 2 min
	16:00 pm – **Exposure to predator** (*Astronotus ocellatus*) in close proximity for 50 min
3	10:30 am – **Social isolation**, for 45 min in a 250 ml beaker
	18:00 pm – **Exposure to air** for 1 min/3 times with 10 min interval
4	09:00 am – **Tank change**, three consecutive times
	15:00 pm – **Cooling** tank water to 23 °C for 30 min
5	10:00 am – **Crowding** of 10 animals for 50 min in a 250 ml beaker
	15:00 pm –**Heating**
6	11:30 am – **Predator**
	17:00 pm – **Chasing**
7	10:00 am – **Social Isolation**
	15:00 pm – **Tank Change**
8	08:00 am – **Cooling**
	18:00 pm – **Crowding**
9	10:00 am – **Air exposure**
	17:00 pm – **Predator**
10	09:00 am – **Tank change**
	15:00 pm – **Heating**
11	10:30 am – **Low water level**
	16:30 pm – **Social Isolation**
12	08:00 am – **Crowding**
	14:30 pm – **Cooling**
13	10:00 am – **Chasing**
	15:30 pm – **Low water level**
14	09:30 am – **Tank change**
	18:00 pm – **Air exposure**

2.6. Euthanasia, brain, and RNA extraction

We anesthetized fish by immersion in eugenol (50 mg/L) prior to killing by sectioning the spinal cord. Then, the skull was opened and the cranial nerves severed to carefully remove the brain (Vargas et al., 2011). For RNA extraction, the brains of three fish were pooled per sample (total of 6 samples per 18-fish group).

Pooled brain samples were lysed using the Tissuelyser LT® (Qiagen, Hilden, Germany). RNA extraction was then carried out using the RNeasy® Mini Kit (Qiagen, Hilden, Germany) and treated with DNAse I amplification grade® (Invitrogen, Carlsbad, CA, USA) to eliminate genomic DNA. The RNA quality and concentration were measured by spectrophotometry (Nanophotometer Pearl®; IMPLEN, Munich, Germany).

2.7. cDNA synthesis and gene expression analysis

Total RNA (500 ng) was used for cDNA synthesis in the reverse transcription assay (QuantiTect® III Reverse Transcription kit, Qiagen, Hilden, Germany). A real time PCR (qPCR) was performed on the Rotor-Gene Q (Qiagen, Hilden, Germany), using GoTaq® qPCR Master Mix (Promega, Madison, Wisconsin, USA), cDNA (diluted 1:10), and specific primers. The genes analyzed, their respective primers, and the efficiency of each reaction are described in Table 2. All samples were analyzed in triplicate. Non-template controls and the expression of a housekeeping gene (β-actin) were also analyzed for comparison purposes. The relative

Table 2. Genes amplified, qPCR primers, and efficiency of amplification.

Gene	Primer (5'- 3')	Efficiency (%)	Accession number
β-Actin	F: GCAAAGGGAGGTAGTTGTCTAA R: GAGGAGGGCAAAGTGGTAAA	98	AF057040.1
TNF-α	F: GACCACAGCACTTCTACCG R: ACATTTTCCTCACTTTCGTTCAC	101	NM_212859
IL-1β	F: GCTGGAGATGTGGACTTC R: ACTCTGTGGATTGGGGTTTG	97	NM_212844
IL-6	F: GCGTCCTGACGTGGTATAAAG R: GTCGTTTGGTGCTGTGTTTG	102	NM_001261449.1
INF-γ	F: TGCCTCAAAATGGTGCTACTC R: AATCGGGTTCTCGCTCCTG	96	AB158361.1
IL-4	F: TCTCTGCCAAGCAGGAATG R: CAGTTTCCAGTCCCGGTATATG	96	AM403245.2
IL-10	F: CTCTGCTCACGCTTCTTCTT R: GCTCCCTCAGTCTTAAAGGAAA	100	BC163038.1
cFOS	F: CAGCTCCACCACAGTGAAGA; R: GCTCCAGGTCAGTGTTAGCC	99	DQ003339.2
BDNF	F: CGCCGTTACTCTTTCTCTTGG R: CCATTAGTCACGGGGACCTTC	101	NM_001308648.1

quantification of gene expression was carried out using the $2^{-\Delta\Delta ct}$ formula.

2.8. Statistical analysis

Data were analyzed using ordinary one-way ANOVA followed by Tukey's multiple comparison test or using the Kruskal-Wallis test followed by Dunn's multiple comparison test, depending on data normality, as assessed by Brown-Forsythe and Bartlett's tests. In all comparisons, significance was set at <0.05.

3. Results

The mRNA levels of the pro-inflammatory cytokines IL-1β and TNF-α, and the anti-inflammatory cytokine IL-10, were highest in the brains of fish from the UCS group (Figure 1). The mRNA levels of cFOS were lowest in the UCS group (Figure 1). No significant differences were found in the mRNA levels of the other genes tested, nor for any of the genes tested in the AS group.

4. Discussion

Here we show that acute and chronic stressing differently affect the expression of cytokine genes and neuronal markers in the zebrafish brain. The UCS increased the expression of the pro-inflammatory cytokine genes (IL-1β and TNF-α) and the anti-inflammatory cytokine gene IL-10. In addition, UCS induced a reduction in the expression of the cFOS gene, which may indicate reduction of neuronal plasticity (Jaworski, Kalita, & Knapska, 2018). On the other hand, a single isolated stressing episode (AS) had no effect on the expression of the evaluated genes.

In the UCS group of fish, the increased expression of pro-inflammatory cytokine genes in the brain is consistent with an inflammatory reaction. As commented above, fish submitted to the UCS protocol had increased levels of IL-1β and TNF-α mRNA, both pro-inflammatory cytokines produced by microglia in response to chronic stress (Calcia et al., 2016). Previous studies using the UCS protocol in zebrafish reported increased levels of some pro-inflammatory cytokines in the body (Song et al., 2018) and brain (Marcon et al., 2016). However, here we measured a wider range of cytokines in the brain that allowed us to better assess the neuroimmunological phenomena after chronic stress.

Interestingly, in the UCS group, we also observed increased expression of the gene encoding the anti-inflammatory cytokine IL-10, which is produced by astrocytes and has the potential to act by protecting neurons against the potential neurodegeneration caused by stress (Palmer & Ousman, 2018). The increase of IL-10 is a response to inflammation and a mechanism of neuroprotection (Garcia, Stillings, Leclerc, & Phillips, 2017).

In contrast to the findings in the UCS group, the expression of pro-inflammatory cytokine genes was not altered in fish from the AS group, indicating that an isolated stressing episode might not be enough to cause alterations in pro-inflammatory cytokines, or that the post-stress evaluation time (one hour) chosen to collect samples did not overlap with the alteration in cytokine expression (Vojtech et al., 2009).

Chronic stress can promote microglia activation and cytokine production in human and rodent models (Glovatchcka et al., 2012). Microglia are immune cells present in the brain and represent the main line of defense in the central nervous system; additionally, these cells modulate neuronal function in illness and health (Calcia et al., 2016). Chronic neuroinflammation causes changes in microglia, which in turn may be associated with structural and functional changes (CITAR) in the brain, predisposing individuals to mental illnesses (Réus et al., 2015) like depression, anxiety, schizophrenia and autism spectrum disorders (Calcia et al., 2016). The upregulation of cytokine gene expression, as demonstrated in the present study, and their role in neuro-inflammation, reinforces the translational potential of the zebrafish for studying the effects of stress in the brain.

In a neuroimmunological context, each cytokine has unique characteristics, and several were altered in our study. In rodents, increased expression of TNF-α is involved in fear memory (Yu et al., 2017). Similarly, in our study, the repeated

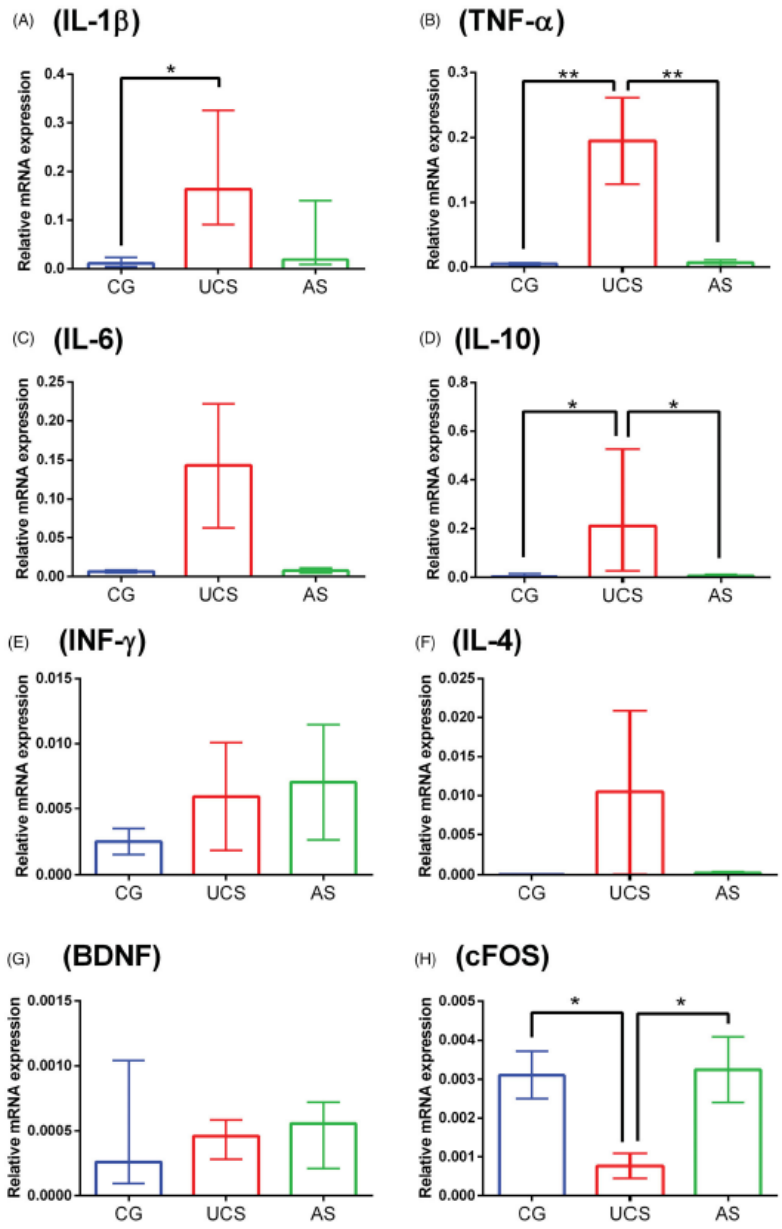

Figure 1. Gene expression in the brains of fish from the control (CG), unpredictable chronic stress (UCS) and acute stress (AS) groups. Data are expressed as mean ± SEM (panels B, C, E, F and H) or median ± interquartile range (panels A, D and G), depending on the data normality, which was assessed using Bartlett's test. The asterisks indicate significant differences (*$p = < 0.05$, **$p = < 0.01$).

episodes of stress might have induced a fear memory and upregulation of TNF-α (Yu et al., 2017). Furthermore, increased levels of IL-β in the brain can cause impairments to memory and learning, as it has a deleterious effect on neuronal plasticity (Avital et al., 2003). Here, we also found reduced levels of cFOS expression which, as discussed below, is involved in memory and learning processes (Velazquez, Caputto, & Boussin, 2015). Behavioral tests have shown that

stress impairs memory and learning in zebrafish (Manuel et al., 2014), which corroborates the molecular findings of our study.

Analyzing the gene expression of neuronal markers, the nerve growth factor, brain-derived neurotrophic factor (BDNF) and cFOS gene, we found a reduction in cFOS gene expression in the UCS group. The cFOS is an early expression gene that is a well-known marker of neuronal activity (Velazquez et al., 2015). The cFOS protein is involved in key cellular events, including cell proliferation, differentiation, and survival. Also, it is involved in the processes of memory and learning (Velazquez et al., 2015). Previous studies in zebrafish showed that the expression of cFOS can be induced by acute stress (Pavlidis, Theodoridi, & Tsalafouta, 2015). However, the expression of the cFOS gene in our AS group of fish was not altered, probably because we evaluated the expression one hour after the stress episode and, as cFOS is an early expression gene, its value probably returned to basal level during this time (Jaworski et al., 2018; Pavlidis et al., 2015). On the other hand, the UCS group showed a down regulation of the cFOS gene, indicating a possible reduction of neuronal plasticity in fish exposed to chronic stress (Jaworski et al., 2018).

BDNF is a neurotrophic factor involved in maturation, differentiation and survival of neurons in the nervous system, and depicts a neuroprotective effect under adverse conditions, such as chronic stress (Lucini, Angelo, Cacialli, Palladino, & Girolamo, 2018). Chronic stress might alter BDNF (Manuel et al., 2014; Pavlidis et al., 2015) but, conversely, here we could not find any alterations in the expression of the BDNF gene in the stressed groups. The fact that we analyzed gene expression rather than protein concentration may have been a limitation for detecting possible alterations. Moreover, in fish submitted to UCS, cFOS gene expression was reduced but BDNF gene expression was not. Although correlated, BDNF and cFOS may have different gene expression patterns, which may also vary according to the post-stress assessment time (Pavlidis et al., 2015).

In conclusion, our most important finding is the different pattern of expression of cytokine genes and neuronal markers caused by acute and chronic stress in the zebrafish brain. Chronic stress caused significant alterations in the expression of genes linked to inflammation and neuronal plasticity, while acute stress did not change the expression of these genes, suggesting that a single stressing episode does not impair cerebral homeostasis.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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5 DISCUSSÃO

A principal conclusão que podemos chegar com os resultados descritos nos três artigos que compõem essa tese de doutorado, é de que o sistema imune e o comportamento dos peixes se inter-relacionam. Além disso, essa relação parece ser bidirecional onde o sistema imunológico é capaz de modular o comportamento e o padrão comportamental também pode modular a resposta imune no cérebro. No primeiro artigo mostramos a ocorrência da interação entre o sistema imune e os comportamentos exploratório e social do *zebrafish*. No segundo, demonstramos que a ativação da resposta imune é capaz de causar alterações comportamentais, características do comportamento de doença. Já no terceiro artigo, evidenciamos que o estresse crônico, e suas consequentes alterações no padrão normal de comportamento, aumenta a expressão gênica de citocinas pró-inflamatórias no cérebro, o que não ocorre em casos de estresses agudos.

Como primeiro passo para alcançar o objetivo principal desta tese de doutorado, avaliamos se peixes com diferentes personalidades apresentavam diferenças na expressão gênica de citocinas a nível cerebral. Encontramos que, quanto ao perfil exploratório, peixes classificados como *high responders to novelty* (HRN), ou seja, que não exploram um objeto novo no tanque teste, expressavam citocinas com um perfil pró-inflamatório, com aumento de IL-1β e redução de IL-10, comparados aos peixes classificados como *low responders to novelty* (LRN), que exploram o objeto novo. Da mesma forma, peixes com menor resposta à estímulos sociais apresentaram expressão reduzida de INF-γ no cérebro.

Estudos prévios, realizados em mamíferos, demonstraram que o comportamento de neofobia, correspondente aos nossos peixes HRN, pode ser desencadeado pela resposta inflamatória após infecção por patógenos ou pela injeção periférica de citocinas, como a IL-1β (DUNN; ANTOON; CHAPMAN, 1991; HABA et al., 2012). Nesse primeiro estudo não provocamos a ativação da resposta imune, mas peixes com maior expressão de IL-1β no cérebro apresentaram comportamento semelhante ao de neofobia demonstrado em mamíferos. Esta redução da exploração de um novo objeto pode ser interpretada como um mecanismo de defesa, pois reduz o risco de predação e de exposição a patógenos (BOURKE, 2014; HABA et al., 2012).

Já no teste de comportamento social, peixes que passaram menos tempo no segmento próximo aos seus coespecíficos tiveram expressão reduzida de INF-γ. Um estudo recente mostrou que camundongos *knockout* para o INF-γ apresentam déficit no comportamento social, mostrando que esta citocina tem papel crucial na interação social (FILIANO et al., 2016).

Embora o comportamento social seja essencial para a sobrevivência das espécies, a agregação tende a aumentar a disseminação de patógenos. Assim, o aumento da interação social poderia levar ao aumento da expressão de genes imunes relacionados, e neste aspecto, o INF-γ parece ser uma molécula chave nesta interação (FILIANO et al., 2016).

Com este primeiro artigo demonstramos que existe uma clara relação imuno-comportamental; porém não podemos afirmar se é a redução do INF-γ que leva os peixes a responderem menos aos estímulos sociais, ou sua menor interação com os coespecíficos causa menor expressão de INF-γ. Da mesma forma, é o menor comportamento exploratório que resulta na expressão aumentada de IL-1β ou é o seu comportamento de medo e/ou estresse que leva ao aumento da expressão desta citocina? Então, surge a pergunta: é o sistema imune que modula alterações comportamentais, ou são os padrões de comportamento que levam a alterações da resposta imune?

Para responder esta pergunta, executamos os experimentos relatados nos nossos outros dois artigos. No segundo *paper*, demonstramos que a partir da ativação da resposta imune o comportamento dos peixes muda, reforçando a hipótese de que o sistema imune é capaz de modular o comportamento. Após uma resposta inflamatória, os peixes apresentaram redução da locomoção, da preferência social e do perfil exploratório quando em contato com um objeto novo. Esses padrões de alterações comportamentais caracterizaram o *sickness behavior* nos peixes, já bem descrito em mamíferos, porém até o momento, ainda não caracterizado em peixes.

O *sickness behavior* consta de um padrão de alterações comportamentais, que atua como uma resposta adicional do sistema imune (DANTZER; KELLEY, 2007). Dentre as alterações comportamentais apresentadas por peixes doentes, a redução da locomoção consequentemente reduz à exposição aos patógenos e os gastos de energia, facilitando o processo de cura (GROSSBERG et al., 2011). A redução do comportamento exploratório também é um mecanismo de defesa, pois reduz o risco de predação, de exposição a outros patógenos e também poupa energia (HABA et al., 2012). Corroborando com este resultado, em nosso primeiro estudo, peixes neofóbicos apresentaram uma maior expressão de IL-β no cérebro, reforçando a hipótese de que a resposta inflamatória reduz o comportamento exploratório no *zebrafish*. Ainda, peixes doentes apresentaram uma importante redução do comportamento social, perdendo a preferência inata do *zebrafish* por ficar próximo aos seus coespecíficos (SAVERINO; GERLAI, 2008a). A busca por isolamento na doença é uma característica marcante de diversas espécies, uma vez que a agregação aumenta o potencial de disseminação

de patógenos (HENNESSY; TERRENCE; SCHILLER, 2014; KELLEY et al., 2003; KENNEDY; ADOLPHS, 2012).

O sistema imune se comunica com o cérebro para causar as alterações comportamentais através de citocinas (DANTZER; KELLEY, 2007). Aqui, um processo inflamatório tecidual na periferia levou a alterações das citocinas pró-inflamatórias IL-1β, IL-6 e TNF-α no cérebro; e, estas moléculas são responsáveis pelo padrão comportamental observado (KELLEY et al., 2003). Uma vez bem estabelecido que as citocinas levam a alterações de comportamento, a questão é: como elas fazem isso? Em nossos estudos analisamos a expressão cerebral de dois marcadores de função neuronal, o fator neurotrófico derivado do cérebro (BDNF) e cFOS, e a expressão foi similar nos grupos experimentais, embora alguns estudos em mamíferos já tenham demonstrado que o aumento de IL-1β pode levar a redução do BDNF (CORTESE et al., 2011) . Existem diversas vias pelas quais as citocinas podem alterar a função neuronal, como através das conecções neuronais, da ativação do eixo HPI e agindo diretamente sobre neurotransmissores (ASHLEY; DEMAS, 2017; DUNN, 2006). Mais pesquisas serão necessárias para demonstrar em quais destas vias as citocinas atuam e como isto ocorre.

Em nosso terceiro artigo, com a finalidade de avaliar se a alteração no padrão de comportamento é capaz de alterar a resposta imune a nível cerebral, submetemos diferentes grupos de peixes a um único episódio de estresse (estresse agudo) e a um protocolo de estresse crônico imprevisível (ECI) por 14 dias. O grupo exposto ao estresse agudo não apresentou alterações na expressão dos genes avaliados no cérebro, já o grupo submetido a estresse crônico apresentou aumento da expressão das citocinas pró-inflamatórias IL-1β e TNF-α, da citocina anti-inflamatória IL-10 e redução da expressão do marcador de função neuronal cFOS.

A novidade desse estudo foi demonstrar que o estresse agudo e crônico afetam de forma diferente a expressão gênica no cérebro. No grupo submetido a ECI, houve o aumento da expressão de citocinas no cérebro compatível com um processo inflamatório. Pesquisas anteriores relatam níveis aumentados de citocinas no corpo e no cérebro de *zebrafish* (MARCON et al., 2016; SONG et al., 2018). Diversos estudos clínicos e em roedores apontam que o estresse pode causar a ativação da micróglia, células imunes residentes no sistema nervoso, aumentando a produção de citocinas (HIMMERICH et al., 2013; MARIOTTI, 2015; SWAAB; BAO; LUCASSEN, 2005; VIKTORIYA GLOVATCHCKA, HELENA ENNES, EMERAN A MAYER, 2012). Alterações na micróglia causadas pela neuroinflamação podem estar associadas a alterações estruturais e funcionais no cérebro que predispõe a patologias neurológicas como depressão e ansiedade (CALCIA et al., 2016; FRIES et al., 2015).

Analisando a expressão gênica de marcadores neuronais, encontramos uma redução na expressão do gene cFOS no grupo ECI, enquanto o gene BDNF não sofreu alterações. A proteína cFOS está envolvida nos principais eventos celulares, incluindo proliferação, diferenciação e sobrevivência celular, além de estar envolvida nos processos de memória e aprendizado (VELAZQUEZ; CAPUTTO, 2015). Estudos anteriores com *zebrafish*, mostraram que a expressão de cFOS pode ser induzida por estresse agudo (PAVLIDIS; THEODORIDI; TSALAFOUTA, 2015) . No entanto, no nosso estudo, a expressão do gene cFOS não foi alterada após um único episódio de estresse, provavelmente porque avaliamos a expressão uma hora após a ocorrência do estressor e, como a cFOS é um gene de expressão precoce, seu valor provavelmente já tenha retornado ao nível basal após esse período (JAWORSKI; KALITA; KNAPSKA, 2018; PAVLIDIS; THEODORIDI; TSALAFOUTA, 2015). Por outro lado, o grupo ECI mostrou uma redução do gene cFOS, indicando uma possível redução da plasticidade neuronal em peixes expostos ao estresse crônico (JAWORSKI; KALITA; KNAPSKA, 2018).

Considerando o exposto, podemos afirmar que não existe uma relação de causa-efeito, mas uma interação íntima entre os sistemas imunológico e nervoso. Neste contexto, tanto a ativação da resposta imune pode acarretar em alterações comportamentais, como a alteração de padrões de comportamento, como o que acontece no estresse, pode levar a uma modulação do sistema imunológico.

Os dados gerados nestes três estudos trazem importantes informações para o uso do *zebrafish* como modelo no campo na neuroimunologia, uma vez que muitas pesquisas recentes procuram modelos alternativos para avaliar a interação entre sistema imune e sistema nervoso em várias patologias neurológicas, como depressão e autismo.

6 CONCLUSÃO

A conclusão geral desta tese de doutorado é de que existe uma clara inter-relação entre o sistema imune e o comportamento no *zebrafish* e foi baseada nas seguintes conclusões preliminares:

A expressão gênica de citocinas no cérebro varia de acordo co perfil de comportamento exploratório e preferência social do *zebrafish.*

A ativação do sistema imunológico provoca um conjunto de alterações comportamentais no *zebrafish* que pode ser caracterizado como *sickness behavior.*

A resposta imune periférica induz alterações na expressão de citocinas e marcadores de função neuronal no cérebro.

O estresse crônico induz alterações na expressão gênica de citocinas e de marcadores de função neuronal no cérebro do *zebrafish*.

7 PERSPECTIVAS

Devido aos resultados obtidos em nossos estudos sobre o eixo neuroimune no *zebrafish*, e considerando que as informações a respeito da interação entre sistema imune e comportamento em peixes são escassas, continuaremos aprofundando nossos estudos nesta área.

O nosso próximo passo é aprofundar a pesquisa na área de sistema imune comportamental, o quanto o sistema imune interfere no comportamento social do *zebrafish*? Em nosso segundo estudo, onde caracterizamos o s*ickness behavior* no *zebrafish*, evidenciamos que a presença de resposta inflamatória ativa reduz significativamente a preferência social no *zebrafish*.

O isolamento na doença está bem descrito em mamíferos e é característica de preservação das espécies, uma vez que o convívio social aumenta a transmissão de patógenos (SHAKHAR, 2019). Queremos aprofundar pesquisas nessa área em peixes, para tanto, pretendemos analisar se há modificações no agrupamento do cardume ao se inserir um peixe com uma resposta inflamatória ativa. Ainda, pretendemos verificar se o peixe doente se isola do grupo ou se os peixes saudáveis identificam o coespecífico doente e o excluem do cardume.

Com esta e diversas outras ideias daremos continuidade as pesquisas na área da neuroimunologia utilizando como animal modelo *o zebrafish*. Uma área fascinante e um amplo campo para novas descobertas.

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