UNIVERSIDADE FEDERAL DE SANTA MARIA – UFSM
CENTRO DE CIÊNCIAS NATURAIS E EXATAS
PROGRAMA DE PÓS GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA TOXICOLÓGICA

Fabiano Vargas da Costa

FENÓTIPOS RELACIONADOS À DOR EM PEIXE-ZEBRA: UMA CARACTERIZAÇÃO NEUROCOMPORTAMENTAL UTILIZANDO O MODELO DE DOR VISCERAL INDUZIDA PELO ÁCIDO ACÉTICO

Santa Maria, RS

2021

Fabiano Vargas da Costa

FENÓTIPOS RELACIONADOS À DOR EM PEIXE-ZEBRA: UMA CARACTERIZAÇÃO NEUROCOMPORTAMENTAL UTILIZANDO O MODELO DE DOR VISCERAL INDUZIDA PELO ÁCIDO ACÉTICO

Tese de Doutorado apresentada ao programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica do Centro de Ciências Naturais e Exatas da Universidade Federal de Santa Maria (UFSM), como requisito parcial para obtenção de título de **Doutor em Ciências Biológicas: Bioquímica Toxicológica**

Orientador: Prof. Dr. Denis Broock Rosemberg

Santa Maria, RS

2021

Fabiano Vargas da Costa

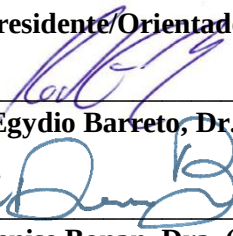
FENÓTIPOS RELACIONADOS À DOR EM PEIXE-ZEBRA: UMA CARACTERIZAÇÃO NEUROCOMPORTAMENTAL UTILIZANDO O MODELO DE DOR VISCERAL INDUZIDA PELO ÁCIDO ACÉTICO

Tese de Doutorado apresentada ao programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica do Centro de Ciências Naturais e Exatas da Universidade Federal de Santa Maria (UFSM), como requisito parcial para obtenção de título de **Doutor em Ciências Biológicas: Bioquímica Toxicológica**

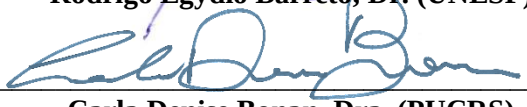
Aprovado em 26 de agosto de 2021

Denis Broock Rosemberg, Dr. (UFSM)

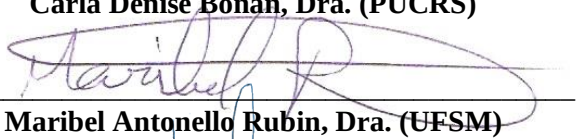
(Presidente/Orientador)



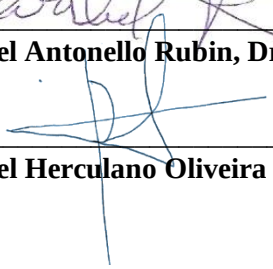
Rodrigo Egydio Barreto, Dr. (UNESP)



Carla Denise Bonan, Dra. (PUCRS)



Maribel Antonello Rubin, Dra. (UFSM)



Anderson Manoel Herculano Oliveira da Silva, Dr. (UFPA)

Santa Maria, RS

2021

AGRADECIMENTOS

Certamente, um dos maiores desafios dessa Tese, foi agradecer todas as pessoas que fizeram parte da minha trajetória acadêmica durante 12 anos. Correndo o risco de injustamente não mencionar algum dos contributos, quero deixar expresso os meus agradecimentos:

Primeiramente, agradeço a Deus, por ter colocado tantas pessoas maravilhosas na minha vida, que de uma forma positiva ou não, ajudaram a me tornar a pessoa que eu sou hoje.

À minha mãe, Ana, que verdadeiramente foi a maior mestra da minha vida que sempre acreditou no meu potencial. Obrigado por todo o carinho e dedicação, te amo.

Ao meu pai, Guto, e aos meus irmãos, Tiago e Rafael, por partilharem comigo bons e maus momentos assim como bons e maus resultados, não só na vida familiar, mas também na vida acadêmica. Obrigado por sempre estarem comigo, amo vocês.

À minha avó, Maria, e minha avó emprestada, Hélia, por todo o apoio durante a minha trajetória acadêmica.

Ao meu querido amigo/irmão e orientador, Denis Rosemberg. Obrigado por toda a sua dedicação e atenção, pelas risadas e broncas e por nunca desistir de mim. Eu só me tornei o pesquisador que eu sou hoje graças a você. Tu tens um coração do tamanho do mundo, não deixe as pessoas se aproveitarem disso. Essa Tese é um fruto do nosso trabalho, muito obrigado meu velho.

A todos os integrantes do LANE, em especial ao Luiz, Julia e Vanessa. Obrigado por todos os momentos que passamos juntos, por todas as risadas e tragos.

A todos os professores do PPGBTox, em especial a Prof. Maribel que abriu as portas da UFSM para mim. O meu muito obrigado!

Ao Professor Allan Kalueff, pela importante colaboração nos trabalhos comportamentais desta tese. Muito obrigado pela confiança, pelas discussões e ensinamentos, espero não o decepcionar.

Aos órgãos de fomento, CAPES, FAPERGS e CNPq, pelo suporte financeiro e principalmente pelo interesse nesse estudo.

A todos os membros da banca, pela leitura e exame da presente tese. Agradeço também a UFSM, pela estrutura disponibilizada e por me proporcionar um ensino gratuito e de qualidade.

Por fim, agradeço a mim mesmo. Por nunca desistir, por não desanimar nas horas mais difíceis, por trabalhar duro e por acreditar em mim mesmo!

AGRADECIMENTO ESPECIAL

Meu agradecimento mais profundo só poderia ser dedicado a duas pessoas: minha esposa, Grazi e meu filho, Miguel. Agradeço a vocês por terem caminhado ao meu lado, pela paciência, compreensão, e carinho incondicional. Nos momentos mais difíceis, que não foram raros, vocês foram a luz que me manteve no caminho certo. Sou grato por cada gesto carinhoso, por cada sorriso. Esta Tese, eu dedico a vocês! Obrigado por estarem sempre ao meu lado, AMO VOCÊS.

“A pesquisa básica é como atirar uma flecha para o ar e,

onde ela cair, pintar um alvo”.

(Homer Adkins Burton)

“A grande tragédia da ciência: o massacre de uma bela hipótese

por parte de um horrível fato”.

(Thomas Henry Huxley)

RESUMO

Tese de Doutorado
Programa de Pós-graduação em Ciências Biológicas: Bioquímica Toxicológica
Universidade Federal de Santa Maria

FENÓTIPOS RELACIONADOS À DOR EM PEIXE-ZEBRA: UMA CARACTERIZAÇÃO NEUROCOMPORTAMENTAL UTILIZANDO O MODELO DE DOR VISCERAL INDUZIDA PELO ÁCIDO ACÉTICO

AUTOR: FABIANO VARGAS DA COSTA
ORIENTADOR: DENIS BROOCK ROSEMBERG

O peixe-zebra (*Danio rerio*) é um pequeno teleósteo de água doce pertencente à família Cyprinidae, o qual vem sendo estudado em diferentes áreas científicas. Essa espécie apresenta genes evolutivamente conservados e um amplo repertório comportamental, os quais podem ser afetados por diversas modulações farmacológicas, tais como algógenos e substâncias analgésicas. Apesar de estudos recentes demonstraram que o peixe-zebra é um organismo modelo emergente para o estudo de processos relacionados à nocicepção, dados relacionados a fenótipos específicos que podem indicar dor localizada ainda carecem de informação. Portanto, a presente tese tem por objetivo caracterizar fenótipos comportamentais específicos na presença de algógenos com ênfase na validação de um novo modelo de dor visceral induzida pela administração intraperitoneal de ácido acético. No primeiro trabalho nós demonstramos que a injeção intraperitoneal de ácido acético (2,5 e 5,0%) promoveu uma resposta semelhante à contorção abdominal em roedores, que foi avaliada pela medição de um índice de curvatura abdominal. Além disso, todas as doses testadas (0,5–5,0%) reduziram a distância percorrida e a atividade vertical no teste do tanque novo. A duração do congelamento aumentou após 5,0% de ácido acético, enquanto os peixes injetados com 1,0, 2,5 e 5,0% aumentaram o tempo de permanência na área superior do tanque. Tanto a morfina (um analgésico opioide) quanto o diclofenaco (um anti-inflamatório não esteroidal, AINE) preveniram as mudanças induzidas pelo ácido acético (5,0%) no índice de curvatura corporal, enquanto a naloxona bloqueou os efeitos analgésicos da morfina. Embora a morfina atenue as respostas semelhantes à dor no peixe-zebra, não há dados mostrando se o antagonismo dos receptores opioides prolonga a duração da dor na ausência de um opioide exógeno. Portanto, em um segundo trabalho, nós investigamos se um antagonista opioide comum, naloxona, afeta a resposta semelhante a constrição abdominal. Os animais foram injetados intraperitonealmente com ácido acético (5,0%), naloxona (1,25 mg / kg; 2,5 mg / kg; 5,0 mg / kg) ou ácido acético com naloxona para investigar as mudanças na curvatura corporal por 1 h. O ácido acético provocou uma resposta semelhante a dor no peixe-zebra, conforme avaliado pelo índice de curvatura abdominal que dura aproximadamente 30 min, enquanto nenhum efeito foi observado após a injeção de PBS. Embora a naloxona sozinha não altere a frequência e a duração desse comportamento, ela prolonga de forma dependente de dose a resposta da curvatura abdominal induzida pelo ácido acético, sugerindo que opioides endógenos podem ter um papel chave na recuperação deste fenótipo específico no modelo de dor visceral aguda. Por fim, no terceiro trabalho, nós realizamos uma revisão sistemática sobre a importância dos modelos utilizando peixe-zebra para estudos relacionados à dor.

Mesmo com estruturas anatômicas distintas às dos mamíferos, ortólogos de genes envolvidos na nocicepção (ex: receptores opioides, receptores de potencial transitório, canais iônicos sensíveis ao ácido e receptores canabinoides) apresentam alto grau de similaridade genética. Em suma, esses dados corroboram o envolvimento de uma maquinaria celular evolutivamente conservada para respostas nociceptivas em peixe-zebra. De modo geral, os resultados obtidos nesta tese suportam uma nova estratégia para avaliar parâmetros comportamentais relacionados à dor no peixe-zebra com alto valor preditivo, de face e construto.

Palavras-chave: peixe-zebra; nocicepção; dor; ácido acético; curvatura abdominal

ABSTRACT

Ph.D. Thesis
Graduate Course in Biological Sciences: Toxicological Biochemistry
Federal University of Santa Maria

Pain-like behaviors-related phenotypes in zebrafish: a neurobehavioral characterization using the acetic acid model

AUTHOR: FABIANO V. COSTA
ADVISOR: DENIS B. ROSEMBERG

The zebrafish (*Danio rerio*) is a small freshwater teleost belonging to the Cyprinidae family, which has been studied in different scientific areas. This species has evolutionarily conserved genes and a relatively complex behavioral repertoire, which can be modulated by several pharmacological drugs, such as algogens and analgesic drugs. Although recent studies have shown that zebrafish is an emerging model organism for assessing nociception, specific phenotypes that may indicate local pain are still poorly understood. This thesis aimed to characterize specific behavioral phenotypes in the presence of algogens to validate a model of visceral pain induced by intraperitoneal administration of acetic acid. In a first study, we demonstrated that intraperitoneal injection of acetic acid (2.5 and 5.0%) induced a response similar to the abdominal constriction in rodents, which was assessed by measuring the abdominal curvature index. All doses tested (0.5–5.0%) reduced the distance traveled and vertical activity in the novel tank test. The duration of freezing increased after 5.0% acetic acid, while fish injected with 1.0, 2.5 and 5.0% spent more time in the top area of the tank. Both morphine (an opioid analgesic) and diclofenac (a nonsteroidal anti-inflammatory drug, NSAID) prevented acetic acid-induced changes (5.0%) in the body curvature index, while naloxone antagonized the analgesic effects of morphine. Although morphine attenuates pain-like responses in zebrafish, there are no data showing whether the opioid receptor antagonism prolongs pain duration in the absence of an exogenous opioid. In a second report, we investigated whether a common opioid antagonist, naloxone, affects the constriction-like response. Animals were injected intraperitoneally with acetic acid (5.0%), naloxone (1.25 mg / kg; 2.5 mg / kg; 5.0 mg / kg) or acetic acid with naloxone to investigate changes in the body curvature for 1 h. As expected, acetic acid elicited pain responses in zebrafish for 30 min, while no effect was observed after PBS injection. Although naloxone alone does not change the frequency and duration of this behavior, it dose-dependently prolongs the acetic acid-induced abdominal curvature response, suggesting that endogenous opioids may have a key role in recovering this specific phenotype in the acute visceral pain model. Finally, in the third study, we performed a systematic review on the importance of zebrafish models for pain-related studies. Albeit the anatomical differences between teleost fishes and mammals, orthologs of genes involved in nociception (*e.g.*, opioid receptors, transient potential receptors, acid-sensitive ion channels, and cannabinoid receptors) show a high degree of genetic similarity. These data support the involvement of an evolutionarily conserved cellular machinery for nociceptive responses in zebrafish. Overall, the results obtained here a support a new strategy to assess pain-related behavioral parameters in zebrafish with high predictive, face, and construct validity.

Keywords: zebrafish; nociception; pain; acetic acid; abdominal curvature

ABREVIAÇÕES

AB	Linhagem AB do peixe-zebra
ACTH	Hormônio adrenocorticotrófico, do inglês Adrenocorticotropic hormone
ASICs	Canais iônicos sensíveis ao ácido, do inglês - 'acid-sensing ion channels'
zASICs	Canais iônicos sensíveis ao ácido do peixe-zebra, do inglês - 'zebrafish acid-sensing ion channels'
CRF	Fator liberador de corticotropina, do inglês, 'corticotrophin release factor'
GRD	Gânglios da raiz dorsal
HPI	Eixo hipotálamo-pituitária-inter-renal
POMC	Proopiomelanocortina
SF	Barbatana curta, do inglês - 'short fin'
SNC	Sistema nervoso central
TG	Nervo trigeminal
GPCR	Receptor acoplado a proteína G, do inglês - "G protein coupled receptor"
MOP	Receptor opioide μ, do inglês – 'μ opioid receptor'
DOP	Receptor opioide δ, do inglês – 'δ opioid receptor'
KOP	Receptor opioide κ, do inglês – 'κ opioid receptor'
ME	Met-encefalina
MEI	Met-encefalina-Ile
MED	Met-encefalina-Asp
LE	Leu-encefalina
MEGY	Leu-encefalina-Gly-Tyr
y-LPH	y-lipotropina
β-MSH	β-melanotropina
α-MSH	α-melanotropina
β-END	β-endorfina
TM	Transmembrana
TRP	Receptor de potencial transitório, do inglês "Transient Receptor Potential"
TRPC	Receptor de potencial transitório canônico, do inglês "Transient Receptor Potential canonical"

TRPV	Receptor de potencial transitório vaniloide, do inglês “Transient Receptor Potential Vanilloid”
TRPA1	Receptor de potencial transitório anquirina 1, do inglês “Transient Receptor Potential Ankyrin 1”
TRPM	Receptor de potencial transitório melastatina, do inglês “Transient Receptor Potential Melastatin”
TRPP	Receptor de potencial transitório policistina, do inglês “Transient Receptor Potential Policistin”
TRPML	Receptor de potencial transitório mucolipina, do inglês “Transient Receptor Potential Mucolipin”

SUMÁRIO

1. APRESENTAÇÃO

No item **INTRODUÇÃO** consta uma revisão sucinta da literatura sobre os temas abordados nesta tese. A metodologia realizada e os resultados obtidos que compõem esta tese estão apresentados sob a forma de três artigos científicos publicados, os quais se encontram no item **DESENVOLVIMENTO**.

As seções Materiais e Métodos, Resultados e Discussão encontram-se nos próprios artigos e representam a íntegra deste estudo. Os itens **DISCUSSÃO, CONCLUSÕES, PERSPECTIVAS, REFERÊNCIAS BIBLIOGRÁFICAS** e **ANEXOS** encontram-se no final desta tese. Os itens **DISCUSSÃO, CONCLUSÕES** e **PERSPECTIVAS** visam discutir os trabalhos de forma integrada, bem como apresentar uma conclusão geral sobre os achados relacionados ao tema com as perspectivas futuras. As **REFERÊNCIAS BIBLIOGRÁFICAS** referem-se somente as citações que aparecem nos itens Introdução e Discussão desta tese.

2. INTRODUÇÃO

2.1. Dor

A dor é um sintoma debilitante de muitos distúrbios humanos e um problema biomédico urgente (MOGIL *et al.*, 2010; WOOLF, 2010). Estima-se que 80% da população mundial possui acesso limitado a medicamentos para tratamento da dor moderada a grave. Tal fato causa um profundo impacto sobre a qualidade de vida dos pacientes, acarretando consequências físicas, psicológicas, sociais e econômicas na população (FISHMAN, 2007; LOHMAN *et al.*, 2010). De acordo com a Associação Internacional para o Estudo da Dor (IASP), a dor é definida como "uma experiência sensorial e emocional desagradável associada à lesão tecidual real ou potencial, ou ainda descrita em termos que sugerem tal lesão" (LOESER *et al.*, 2008).

A percepção da dor é complexa e não envolve apenas a transmissão de um estímulo nocivo através de nociceptores localizados nas fibras nervosas aferentes primárias, mas também o processamento emocional e cognitivo, tornando-a um fenômeno subjetivo (BASBAUM *et al.*, 2009; LOESER *et al.*, 2008). Estímulos nocivos ativam o sistema nervoso central (SNC) desencadeando a percepção da dor que leva a modulação de diversas respostas comportamentais (JULIUS *et al.*, 2001; PERL, 2011; SHERRINGTON, 1906). A sensação dolorosa pode indicar uma lesão tecidual, o que se torna um importante mecanismo de defesa visando a proteção do organismo. Dessa forma, a dor aguda é de extrema importância, possuindo grande valor adaptativo em relação à sobrevivência do organismo lesado. Como descrito por Charles Darwin: 'a dor é uma "emoção homeostática", que é essencial para a sobrevivência de espécies' (BASBAUM *et al.*, 2009; DARWIN, 1872; WOOLF, 2010).

Ao contrário destes propósitos claramente protetores, a dor prolongada pode se tornar maladaptativa quando o organismo não é capaz de produzir a resolução da lesão

ou quando a plasticidade neuronal que ocorre durante a doença mantém a dor mesmo após a recuperação da lesão (KUNER, 2010). É o que acontece, por exemplo, em doenças inflamatórias crônicas (ASHBURN *et al.*, 1999). Neste contexto, o processamento sensorial é anormal e os estímulos ambientais que normalmente são inócuos, tais como leve toques ou pequenas alterações na temperatura ambiente, produzem a sensação de dor, isto é, alodinia (BASBAUM *et al.*, 2009; KUNER, 2010; SHIR *et al.*, 1990). A alodinia é descrita como uma sensação dolorosa em resposta a um estímulo não nocivo, esse termo só deve ser usado quando se sabe que o estímulo não é capaz de ativar nociceptores (JULIUS *et al.*, 2001). Considerada como uma condição patológica, a alodinia é detectada por fibras mielinizadas sensíveis ao toque do tipo Aβ (BASBAUM *et al.*, 2009; JULIUS *et al.*, 2001; KUNER, 2010). Por outro lado, os estímulos que normalmente são percebidos como dolorosos e produzem uma percepção exagerada de dor são denominados hiperalgesia. Finalmente, a dor pode ainda aparecer espontaneamente e sem necessidade de estimulação externa, podendo ser descrita como dor em queimação ou choque (LOESER *et al.*, 2008).

Existem diversos estímulos que podem induzir sensações dolorosas, tais como: calor, frio extremo, pressão e produtos químicos; através da ativação de terminações nervosas livres e periféricas de fibras aferentes sensoriais delgadas do tipo C e Aδ (mielinizadas e não mielinizadas), chamadas de nociceptores (BASBAUM *et al.*, 2009; LOESER *et al.*, 2008; WOOLF, 2010). As fibras do tipo C e Aδ são formadas por neurônios cujos corpos celulares encontram-se nos gânglios da raiz dorsal (GRD) e trigeminal (TG) e que conduzem as informações nociceptivas até o corno dorsal da medula espinhal ou o núcleo trigeminal pars caudalis na ponte, respectivamente (BESSON, 1999; RUSSO *et al.*, 1998; WOOLF, 2010).

Nas lâminas superficiais do corno dorsal da medula espinhal, as terminações dos nociceptores liberam vários neurotransmissores que podem estimular neurônios de segunda ordem (BASBAUM *et al.*, 2009). Estes neurônios formam vias que irão distribuir informações para circuitos cerebrais responsáveis pela produção das dimensões sensoriais (discriminativa) e afetivas/motivacionais (descontentamento) da dor (HUNT *et al.*, 2001). Os neurônios de segunda ordem formam vias supraespinhais, sendo as mais importantes a via espinotalâmica e a espinoparabraquial (JULIUS *et al.*, 2001). A via espinotalâmica termina no tálamo ventroposterior lateral e ventrobasal, que tem projeções para o córtex e a via espinoparabraquial se projeta ao núcleo parabraquial e tem projeções para o hipotálamo, amígdala (HUNT *et al.*, 2001). Esta percepção supraespinhal produz várias respostas autonômicas, neuroendócrinas e comportamentais relacionadas à dor (KUNER, 2010).

2.2. Dor e nocicepção relacionados a modelos animais

Estudos de dor envolvendo modelos animais datam do final do século 19 e têm sido de suma importância para a compreensão dos processos biológicos correlatos (VON FREY, 1896). Entretanto, analisar e medir com acurácia comportamentos dolorosos em animais é uma tarefa desafiadora, porque como citado acima, a dor é uma experiência complexa que envolve não apenas a transdução de estímulos nociceptivos como também o processamento cognitivo e emocional (BLACKBURN-MUNRO, 2004; PIEL *et al.*, 2014; TRACEY *et al.*, 2007). Estímulos nociceptivos ativam múltiplas áreas subcorticais e corticais, e a experiência aversiva (comumente reconhecida como dor) resulta dessa ativação conjunta e coordenada (GARCIA-LARREA *et al.*, 2013; TRACEY *et al.*, 2007; WIECH, 2016). Diferentemente dos humanos, os modelos animais de laboratório têm uma menor complexidade de rede de processamento neural, o que dificulta a correlação

da dor com a experiência individual subjetiva (FESTING *et al.*, 2005; THOMAS, 2005). Alguns cientistas afirmam que essa falta de estruturas cerebrais complexas, tais como córtex cerebral, responsáveis por interpretar o estímulo doloroso em humanos, são essenciais para o reconhecimento da dor, negando assim a capacidade animal de reconhecer e apresentar um comportamento protetor frente a um estímulo nocivo, tornando tal ação meramente reflexiva (KEY, 2016; ROSE, 2002; ROSE *et al.*, 2014). Porém, acreditar na ideia de que um certo organismo precise de um córtex humano para sentir dor é uma visão minoritária e reducionista, visto que não leva em consideração a existência de estruturas análogas no SNC em diferentes espécies.

Existe um grande número de pesquisadores que afirmam que animais de laboratório são capazes de apresentar um comportamento mais refinado e não apenas reflexivo (DAMASIO *et al.*, 2013; MERKER, 2007). Bateson (1991) sugeriu uma clara estrutura sobre a qual questões baseadas na capacidade animal de sentir dor poderiam ser aplicadas a qualquer espécie. Tais critérios foram aplicados a numerosas espécies, onde animais que preenchessem todos os critérios deveriam ser considerados capazes de sentir dor. Esses critérios são: *i*) presença de nociceptores; *ii*) vias de nociceptores convergentes para o cérebro; *iii*) estruturas cerebrais análogas ao córtex cerebral humano que processa a dor; *iv*) expressão de receptores opioides e substâncias opioides endógenas em um sistema neural nociceptivo; *v*) uma redução nos efeitos adversos comportamentais e fisiológicos após a administração de analgésicos ou anestésicos; *vi*) a esquiva de estímulos potencialmente dolorosos (BATESON, 1991). Por fim, Sneddon (2004) acrescentou que os animais devem suspender o comportamento normal por um período prolongado, ao invés de mostrar uma resposta reflexiva, com mudanças no comportamento que reflitam sinais de "desconforto" (SNEDDON *et al.*, 2014).

2.3. O uso do peixe-zebra como organismo modelo para estudos de estímulos dolorosos

Embora os roedores sejam amplamente utilizados na pesquisa translacional da dor (MEOTTI *et al.*, 2010; STEVENSON *et al.*, 2006), modelos experimentais alternativos têm ajudado a avaliar os mecanismos evolutivamente conservados subjacentes à dor e seus fenótipos comportamentais correlatos (DU *et al.*, 2017; SNEDDON *et al.*, 2003b). Modelos vertebrados não tradicionais, como o peixe-zebra, são promissores em estudos relacionados à dor (GONZALEZ-NUNEZ *et al.*, 2009), pois apresentam mecanismos evolutivamente conservados, com comportamento sensível a vários algógenos e medicamentos analgésicos (GAU *et al.*, 2013; PROBER *et al.*, 2008; SNEDDON *et al.*, 2003b). Além do envolvimento da medula espinhal e do rombencéfalo (ROSE, 2002), atividades elétricas e alterações transcricionais no mesencéfalo e no prosencéfalo (BRAITHWAITE *et al.*, 2007a; NORDGREEN *et al.*, 2014; REILLY *et al.*, 2008) foram observadas após estímulos nocivos em peixes teleósteos.

A detecção precisa de fenótipos comportamentais específicos expressos na presença de algógenos é um componente chave para avaliar adequadamente as respostas relacionadas à dor. Ao contrário dos roedores, que apresentam uma grande quantidade de fenótipos comportamentais que refletem tal comportamento (BOBINSKI *et al.*, 2018; BONIN *et al.*, 2014; DE RANTERE *et al.*, 2016; GONZALEZ-CANO *et al.*, 2017; MARTINS *et al.*, 2018), a avaliação de parâmetros específicos relacionados à dor em peixe-zebra é limitada até o momento (MAXIMINO, 2011; SCHROEDER, 2017). Por exemplo, as respostas à estímulos dolorosos do peixe-zebra, em sua grande maioria, são medidas usando alterações locomotoras e aumento da taxa de batimentos operculares como parâmetros principais (CORREIA *et al.*, 2011; CURTRIGHT *et al.*, 2015; REILLY *et al.*, 2008; TAYLOR *et al.*, 2017). Desta forma, os fenótipos mencionados acima

poderiam não representar necessariamente comportamentos nociceptivos específicos, uma vez que também indicam estados de estresse, ansiedade, sedação não específica e/ou um comportamento tipo depressivo (KALUEFF *et al.*, 2013). Entretanto, existem diferentes abordagem para induzir a dor local em espécies de peixes, como o procedimento de estimulação na nadadeira caudal (SCHROEDER, 2017) ou a administração de algógenos próximos à respectiva nadadeira (MAXIMINO, 2011). Ambos os protocolos causam movimentos erráticos e aumentam o batimento caudal, um fenótipo comportamental que reflete uma resposta nociceptiva local aguda. Alguns estudos que descrevem os principais fenótipos comportamentais do peixe-zebra adulto relacionado às respostas à dor estão esquematizados na **Tabela 1**.

2.4. Mecanismos fisiológicos e moleculares relacionadas à dor no peixe-zebra

De um modo geral, a fisiologia da resposta dolorosa em peixes teleósteos pode ser similar à encontrada em mamíferos. No homem, as fibras C estão distribuídas por todo o nervo e podem compreender 50% de fibra total (YOUNG, 1977). Entretanto, nos peixes teleósteos as fibras C são encontradas aproximadamente 4% de fibra total, as fibras A-beta são as mais comuns (53%) seguido por A-delta (33%) e A-alpha (9%) (SNEDDON, 2002). Essa grande diferença de fibras C entre humanos e peixes pode estar relacionada ao avanço na evolução dos vertebrados. Animais terrestres são expostos a drásticas mudanças climáticas, gases nocivos e maior chance de lesões mecânicas devido à gravidade. No entanto, em um ambiente aquático, as flutuações de temperatura são relativamente leves, a flutuabilidade compensa quaisquer efeitos gravitacionais e produtos químicos nocivos podem ser altamente diluídos em alguns casos (SNEDDON, 2002). Portanto, a necessidade de um sistema nociceptivo abrangente pode não ser tão

essencial quanto nos vertebrados terrestres, assim, os peixes apresentam um menos aparato neural dedicada ao processamento da dor.

Apesar das diferenças neuroanatômicas envolvidas no processamento da dor em teleósteos, o peixe-zebra (*Danio rerio*) possui o genoma evolutivamente conservado e completamente sequenciado, compartilhando um alto grau de similaridade com seus ortólogos em mamíferos (aproximadamente 70%) (HOWE *et al.*, 2013). Vários desses genes ortólogos que compõem sistemas especializados envolvidos nos processos dolorosos foram caracterizados, tais como: os canais iônicos sensíveis a ácido (ASICs, do inglês, acid sensitive ion channel), o sistema opioide e a família dos receptores de potencial transitório (TRP; do inglês, transient receptor potential), destacando o valor translacional das pesquisas com peixe-zebra nesta área de estudo (PAUKERT *et al.*, 2004; SAITO *et al.*, 2006).

2.4.1. Canais iônicos sensíveis a ácido (ASICs)

Os canais ASICs são receptores excitatórios para H^+ extracelular (LEE *et al.*, 2018). Suas funções incluem percepção periférica da dor, transmissão sináptica e mecanosensação (SCHAEFER *et al.*, 2000). Em mamíferos, existem quatro genes ASICs que codificam seis subunidades (*ASIC1a* (WALDMANN; CHAMPIGNY*; et al.*, 1997), *ASIC1b* (CHEN *et al.*, 1998), *ASIC2a* (PRICE *et al.*, 1996), *ASIC2b* (LINGUEGLIA *et al.*, 1997), *ASIC3* (WALDMANN; BASSILANA*; et al.*, 1997) e *ASIC4* (AKOPIAN *et al.*, 2000)). Em humanos, os ASICs são amplamente distribuídos nos sistemas nervosos central e periférico, mas também foram detectados em tecidos não neuronais, incluindo testículos (*ASIC3*) (BABINSKI *et al.*, 1999), glândula pituitária (*ASIC4*) (GRUNDER *et al.*, 2000), células epiteliais pulmonares (*ASIC3*) (SU *et al.*, 2006), e osso (*ASIC1–3*)

(JAHR *et al.*, 2005). No sistema nervoso periférico, os canais ASIC são encontrados predominantemente em neurônios sensoriais envolvidos na dor (CHEN *et al.*, 1998).

No peixe-zebra, seis genes ASICs já foram identificados (*asic1a, asic1b, asic1c, asic2, asic4a* e *asic4b*) (PAUKERT *et al.*, 2004). As análises filogenéticas mostram que *asic1a, asic2* e *asic4a* são ortólogos aos mamíferos, enquanto *asic1b, asic1c* e *asic4b* são variantes de splicing (PAUKERT *et al.*, 2004). Embora os ASICs do peixe-zebra sejam amplamente expressos no SNC (começando entre 24 e 48 horas após a fertilização), apenas *asic1a* é expresso no gânglio trigêmeo (região responsável por transportar informações somatossensoriais da cabeça) (PAUKERT *et al.*, 2004). Tanto *asic1a* quanto *asic1*c são expressos em neurônios sensoriais e podem contribuir para estímulos mecânicos (PAUKERT *et al.*, 2004). Além disso, *asic4a* e *asic4b* compartilham ~ 68% de similaridade genética com humanos (CHEN *et al.*, 1998). Curiosamente, apenas o *asic4a* é ativado por H^+ extracelular (pH ~ 5,8) (PAUKERT *et al.*, 2004), diferentemente do *ASIC4* mamífero, em que não é ativado por H^+ extracelular. Os efeitos promovidos pelo ácido acético, agonista dos receptores ASICs no peixe-zebra, têm sido amplamente estudados em modelos de dor. Evidencias demonstram alterações locomotoras significativas após a administração de ácido acético em diferentes vias do peixe-zebra, geralmente observados como hipolocomoção. Além disso, tal comportamento é prevenido pela administração de fármacos analgésicos, suportando a redução da locomoção como um fenótipo comportamental geral que reflete sensação dolorosa em animais adultos (TAYLOR *et al.*, 2017).

2.4.2. Sistema opioide

O sistema opioide envolve múltiplos receptores, ligantes endógenos e análogos naturais ou sintéticos, que desempenham um papel fundamental na homeostase da dor,

humor e bem-estar (CHU SIN CHUNG *et al.*, 2013; MARRON FDEZ DE VELASCO *et al.*, 2009). Em humanos, os receptores opioides pertencem à superfamília do receptor acoplado à proteína G (GPCR), e três receptores opioides clássicos foram caracterizados até agora: MOP, KOP, DOP além de seus respectivos ligantes endógenos; β-endorfina, encefalina e dinorfina (CORBETT *et al.*, 2006; RACHINGER-ADAM *et al.*, 2011; STEVENS, 2009). Adicionalmente, o receptor NOP, considerado um membro não opioide da família do receptor opioide, também foi descrito em mamíferos (LAMBERT, 2008). Os projetos de sequenciamento do genoma permitiram uma identificação abrangente dos ortólogos do peixe-zebra (DREBORG *et al.*, 2008; SUNDSTROM *et al.*, 2010), como zMOP (BARRALLO *et al.*, 2000), zKOP (ALVAREZ *et al.*, 2006), duas cópias funcionais de zDOP (BARRALLO *et al.*, 1998; PINAL-SEOANE *et al.*, 2006) e zNOP (RIVAS-BOYERO *et al.*, 2011). No peixe-zebra, os precursores de peptídeos opioides incluem dois genes de proencefalina (*penka* e *penkb*), dois genes de proopiomelanocortina (*pomca* e *pomcb*), um gene de prodinorfina (*pdyn*) e dois genes de pronociceptina (*pnoca* e *pnocb*). As propriedades farmacológicas dos receptores opioides do peixe-zebra foram amplamente caracterizadas (BAO *et al.*, 2019; DEMIN *et al.*, 2018). Enquanto zMOP, zKOP e zDOPa/b exibem alta afinidade para ambos os ligantes opioides não seletivos [H^3]-diprenorfina e [H^3]-bremazocina em ensaios de ligação de saturação (ALVAREZ *et al.*, 2006; MARRON FDEZ DE VELASCO *et al.*, 2009; PINAL-SEOANE *et al.*, 2006), eles mostram menor afinidade para ligantes altamente seletivos em mamíferos ([d-Pen^2, d-Pen^5] encefalina [H^3]-DPDPE, [H^3]-[D-Ala2, NMe-Phe4, Gly5-ol]-encefalina (DAMGO) e [H^3]-U69.593) (GONZALEZ-NUNEZ *et al.*, 2006). Estudos relatam a participação dos receptores opioides nos comportamentos nocivos induzidos pelo ácido acético. No peixe-zebra, a coadministração da morfina antagoniza a diminuição da distância percorrida causada pelo ácido acético (TAYLOR *et al.*, 2017).

Além disso, a coadministração de naloxona previne os efeitos analgésicos da morfina no modelo citado acima (TAYLOR *et al.*, 2017).

2.4.3. Receptores de potencial transitório (TRP)

A família dos canais TRP atua como sensores moleculares, respondendo a uma ampla variedade de estímulos (mudanças no pH, agentes químicos, temperatura e osmolaridade) (CLAPHAM, 2003; JULIUS, 2013). Estruturalmente, um canal TRP contém seis domínios transmembrana (TM1-6) montados como tetrâmeros para formar poros permeáveis a cátions entre TM5 e TM6 (CLAPHAM, 2003; MONTELL *et al.*, 2002). Em humanos, os canais TRP incluem 28 membros divididos em seis subfamílias, classificados como canônicos (TRPC), vaniloide (TRPV), anquirina (TRPA1), melastatina (TRPM), policistina (TRPP) e mucolipina (TRPML) (TSAGARELI *et al.*, 2019).

Interessantemente, o peixe-zebra expressa todos os receptores dos canais TRP (*trpc* (VON NIEDERHAUSERN *et al.*, 2013), *trpv* (SAITO *et al.*, 2006), *trpa* (COREY *et al.*, 2004), *trpm* (KASTENHUBER *et al.*, 2013) trpml (BENINI *et al.*, 2013) e trpp (ENGLAND *et al.*, 2017)).

Os sistemas nociceptivos periféricos e centrais do peixe-zebra são semelhantes aos de camundongos e humanos (KO *et al.*, 2019) e a função nociceptiva do TRPA1 é notavelmente conservada em espécies animais (KANG *et al.*, 2010; LAURSEN *et al.*, 2015). No entanto, ao contrário de humanos e roedores, o genoma do peixe-zebra codifica dois genes TRPA1: *trpa1a* e *trpa1b* (SAITO *et al.*, 2006). Notavelmente, *trpa1a* e *trpa1b* são ativados por agonistas de TRPA humanos (alil isotiocianato, cinamaldeído, dialil dissulfeto, acroleína e 4-hidroxinonenal) (PROBER *et al.*, 2008), e antagonizados por HC-030031, um modulador alostérico competitivo ou negativo de TRPA1 humano (KO

et al., 2019). No entanto, HC-030031 não mostra quaisquer efeitos inibitórios sobre o *trpa1b* ativado por cinamaldeído em um sistema de expressão heterólogo usando oócitos de *Xenopus laevis* (GUPTA *et al.*, 2016). No peixe-zebra o *trpa1a* medeia a detecção química, enquanto *trpa1b* responde a estímulos térmicos (ODA *et al.*, 2016; PROBER *et al.*, 2008) ao contrário de outros vertebrados (por exemplo, galinha, sapo e camundongos), onde TRPA1 é responsável por detectar produtos químicos nocivos e temperaturas prejudiciais (LAURSEN *et al.*, 2015).

Em mamíferos, o TRPV1 é um receptor polimodal, que atua como um sensor de calor nocivo (> 42° C) e contribui para distúrbios dolorosos (por exemplo, dor neuropática induzida por diabetes, dor de câncer e dor inflamatória) (VELDHUIS *et al.*, 2015). O TRPV1 também é sensível a soluções ácidas (pH <6,5), ingredientes alimentares (capsaicina) (CATERINA *et al.*, 1997), derivados de lipídios (prostaglandina E2) (MORIYAMA *et al.*, 2005), endocanabinóide, anandamida (ZYGMUNT *et al.*, 1999) e toxinas (por exemplo, vanilotoxinas e resiniferatoxina) (SZALLASI *et al.*, 1989). Curiosamente, o peixe-zebra expressa um único ortólogo ao *trpv1* que poderia ser derivado de um precursor evolucionário do tetrápode TRPV1 e TRPV2 (SAITO *et al.*, 2006). Semelhante ao ortólogo mamífero, o *trpv1* atua como um canal sensível ao calor (≥25 ° C), embora apresente uma sensibilidade menor do que a do mamífero (≥42° C) (GAU *et al.*, 2013). Evidências mostram que larvas de peixe-zebra de 5 dpf evitam temperaturas acima de 31,5° C e abaixo de 24,5° C (CURTRIGHT *et al.*, 2015), caracterizando hiperalgesia térmica. Considerando as similaridades genéticas nos sistemas descritos, além dos efeitos comportamentais robustos, o uso desta espécie em estudos translacionais relacionados à dor no peixe-zebra é bastante promissor.

De modo geral, o peixe-zebra apresenta as ferramentas moleculares necessárias para estudos neurocomportamentais de protocolos relacionados a dor. Entretanto, a

quantidade de metodologias existentes até o momento é insuficiente para demonstrar e diferenciar de maneira adequada os distintos fenótipos comportamentais relacionados a dor. Portanto, aprofundar a investigação dos comportamentos relacionados a dor através do desenvolvimento de novas metodologias mais específicas são de extrema importância.

Tabela 1. Fenótipos comportamentais tradicionalmente utilizados para medir dor em diferentes modelos experimentais que utilizam peixes-zebra adultos.

Agente nociceptivo	Animal (linhagem)	Via de administração	Fenótipo comportamental	Referências
Ácido acético (5%)	peixe-zebra (AB)	Injetado (lábios)	Diminuição na atividade locomotora, aumento na frequência respiratória	Reilly *et al.*, 2008
Ácido acético (1%)	peixe-zebra (SF)	Injetado (cauda)	Aumento dos movimentos erráticos e batida de cauda	Maximino, 2011
Ácido acético (5 and 10%)	peixe-zebra (AB)	Injetado (lábios)	Diminuição na atividade locomotora	Correia *et al.*, 2011
Clipagem de cauda (30s)	peixe-zebra (AB)	Remoção da metade posterior do tecido caudal	Aumento na frequência respiratória, batimento caudal e redução na atividade	Schroeder *et al.*, 2017
Formalina (0.1%)	peixe-zebra (AB)	Injetado (lábios ou cauda)	Diminuição na atividade locomotora	Magalhães *et al.*, 2017
Histamina (0.1, 0.5, 1, e 2 mg/kg)	peixe-zebra (AB)	Injetado (lábios)	Diminuição na atividade locomotora	Taylor *et al.*, 2017
Adjuvante de Freund Completo (CFA)	peixe-zebra (AB)	Injetado (lábios)	Diminuição na atividade locomotora	Taylor *et al.*, 2017
Cinamaldeído (10, 20, e 40 mM)	peixe-zebra (AB)	Injetado (lábios)	Diminuição na atividade locomotora	Taylor *et al.*, 2017
Isotiocianato de alila (1.02 M)	peixe-zebra (AB)	Injetado (lábios)	Diminuição na atividade locomotora	Taylor *et al.*, 2017
Ácido acético (2.5, 5, 10, e 15%)	peixe-zebra (AB)	Injetado (lábios)	Diminuição na atividade locomotora	Taylor *et al.*, 2017
Clipagem de cauda (30s)	peixe-zebra (AB)	Remoção da metade posterior do tecido caudal	Aumento do tempo gasto na parte superior do tanque, diminuição na atividade locomotora e aumento na coesão do cardume	Thomson *et al*, 2019

3. JUSTIFICATIVA E HIPÓTESE

A busca por novas abordagens relacionadas à dor em peixe-zebra, é uma estratégia que visa simplificar e eliminar redundâncias associadas à interpretação dos fenótipos relacionados à dor neste organismo modelo. Assim, é de grande necessidade que os dados coletados possam corroborar com aqueles obtidos através das metodologias existentes. Como hipótese deste trabalho, acreditamos que a administração intraperitoneal (i.p.) de ácido acético em peixe-zebra possa induzir respostas comportamentais robustas que mimetizem dor visceral com alto valor preditivo, de face e construto. Assim, este modelo poderia ser importante para a análise de um comportamento de dor localizada mais específico, sendo sensível a analgésicos classicamente utilizados na clínica. Pelo fato dos receptores opioides estarem envolvidos na atenuação e/ou recuperação da dor, a administração de naloxona como um antagonista de receptores opioides poderá prolongar o período de dor induzida pelo ácido acético no modelo proposto. Os resultados obtidos através da validação de um modelo de dor visceral agudam em peixes-zebra permitirão consolidar uma nova estratégia para avaliar parâmetros comportamentais mais específicos relacionados à dor nesta espécie. Dessa maneira, o presente estudo servirá de suporte para uma melhor compreensão das respostas comportamentais induzidas por ácido acético em modelos aquáticos relacionados à dor.

4. OBJETIVO

4.1. Objetivo geral

- Caracterizar fenótipos neurocomportamentais para validação de um novo modelo de dor visceral aguda em peixe-zebra através da administração i.p. de ácido acético.

4.2. Objetivos específicos

- Realizar uma revisão sistemática da literatura, abrangendo mecanismos moleculares relacionados a dor no peixe-zebra, bem como suas potencialidades e limitações para estudos da dor.
- Padronizar o método de indução de dor em peixes-zebra pela administração i.p. de diferentes doses de ácido acético;
- Avaliar a possível existência de fenótipos comportamentais relacionados à dor no modelo proposto;
- Verificar o efeito promovido pelo ácido acético sobre a locomoção e o padrão de nado dos peixes;
- Analisar se os comportamentos dos peixes alterados pela administração de ácido acético podem ser modulados por analgésicos;
- Verificar o envolvimento do sistema opioide nas respostas analgésicas promovidas pela morfina;
- Analisar uma possível participação dos opioides endógenos na recuperação do fenótipo doloroso induzido pela administração de ácido acético;

5. DESENVOLVIMENTO

Os tópicos "**RESULTADOS E DISCUSSÃO**" serão apresentados em forma de 3 artigos científicos. Além disso, um capítulo aceito para publicação em livro acadêmico e um protocolo disponível online podem ser encontrados nos **ANEXOS** desta tese.

Artigo 1. Artigo aceito para publicação na revista ***Current Neuropharmacology*** (Qualis referência CAPES A1, F.I. 7.363) em 2021 e se intitula *"The use of zebrafish as a non-traditional model organism in translational pain research: the knowns and the unknowns"*.

Artigo 2. Publicado na revista ***Behavioural Brain Research*** (Qualis referência CAPES A1, F.I. 3.332) em 2019 e se intitula *"Understanding nociception-related phenotypes in adult zebrafish: behavioral and pharmacological characterization using the acetic acid model"*.

Artigo 3. Publicado na revista ***Neuroscience Letters*** (Qualis referência CAPES A4 F.I. 3.046) em 2019 e se intitula *"Naloxone prolongs abdominal constriction writhing-like behavior in a zebrafish-based pain model"*.

ARTIGO 1

The use of zebrafish as a non-traditional model organism in translational pain research: the knowns and the unknowns

Fabiano V. Costa, Luiz V. Rosa, Vanessa A. Quadros, Murilo S. de Abreu, Adair R. S. Santos, Lynne U. Sneddon, Allan V. Kalueff, Denis B. Rosemberg

Current Neuropharmacology (2021). Mar 10 (no prelo);
doi: 10.2174/1570159X19666210311104408.

Send Orders for Reprints to reprints@benthamscience.net

Current Neuropharmacology, **2021**, *19*, **000-000** **1**

REVIEW ARTICLE

The use of Zebrafish as a Non-traditional Model Organism in Translational Pain Research: The Knowns and the Unknowns

Fabiano V. Costa[1,2,*], Luiz V. Rosa[1,2], Vanessa A. Quadros[1,2], Murilo S. de Abreu[3], Adair R. S. Santos[4], Lynne U. Sneddon[5], Allan V. Kalueff [6,7,8,9,10,11,12] and Denis B. Rosemberg[1,2,7,*]

[1]Laboratory of Experimental Neuropsychobiology, Department of Biochemistry and Molecular Biology, Natural and Exact Sciences Center, Federal University of Santa Maria RS, Brazil; [2]Graduate Program in Biological Sciences: Toxicological Biochemistry, Federal University of Santa Maria, RS, Brazil; [3]Bioscience Institute, University of Passo Fundo (UPF), Passo Fundo, RS, Brazil; [4]Laboratory of Neurobiology of Pain and Inflammation, Department of Physiological Sciences, Center of Biological Sciences, Federal University of Santa Catarina, Trindade, Florianópolis, SC, Brazil; Graduate Program in Neuroscience, Center of Biological Sciences, Federal University of Santa Catarina, Florianópolis, SC, Brazil; [5]University of Gothenburg, Department of Biological & Environmental Sciences, Gothenburg, Sweden; [6]School of Pharmacy, Southwest University, Chongqing, China; Ural Federal University, Ekaterinburg, Russia; [7]The International Zebrafish Neuroscience Research Consortium (ZNRC), Slidell, LA, USA; [8]Institute of Translational Biomedicine, St. Petersburg State University, St. Petersburg, Russia; [9]Neurobiology Program, Sirius University, Sochi, Russia; [10]Granov Russian Research Center of Radiology and Surgical Technologies, St. Petersburg, Russia; [11]Moscow Institute of Physics and Technology, Dolgoprudny, Russia; [12]Novosibirsk State University, Novosibirsk, Russia

ARTICLE HISTORY

Received: October 30, 2020
Revised: February 24, 2021
Accepted: February 28, 2021

DOI:
10.2174/1570159X19666210311104408

Abstract: The ability of the nervous system to detect a wide range of noxious stimuli is crucial to avoid life-threatening injury and to trigger protective behavioral and physiological responses. Pain represents a complex phenomenon, including nociception associated with cognitive and emotional processing. Animal experimental models have been developed to understand the mechanisms involved in pain response, as well as to discover novel pharmacological and non-pharmacological anti-pain therapies. Due to the genetic tractability, similar physiology, low cost, and rich behavioral repertoire, the zebrafish (*Danio rerio*) is a powerful aquatic model for modeling pain responses. Here, we summarize the molecular machinery of zebrafish responses to painful stimuli, as well as emphasize how zebrafish-based pain models have been successfully used to understand specific molecular, physiological, and behavioral changes following different algogens and/or noxious stimuli (*e.g.*, acetic acid, formalin, histamine, Complete Freund's Adjuvant, cinnamaldehyde, allyl isothiocyanate, and fin clipping). We also discuss recent advances in zebrafish-based studies and outline the potential advantages and limitations of the existing models to examine the mechanisms underlying pain responses from evolutionary and translational perspectives. Finally, we outline how zebrafish models can represent emergent tools to explore pain behaviors and pain-related mood disorders, as well as to facilitate analgesic therapy screening in translational pain research.

Keywords: Non-traditional pain models, zebrafish, noxious stimuli, nociceptors, pain-related behaviors, anti-pain medication screening.

1. INTRODUCTION

Pain is a complex process that involves the transduction of nociceptive stimuli culminating in specific behavioral and physiological responses [1]. Chronic pain is an important biomedical and social problem whose management represents a critical unmet biomedical condition [2], necessitating both novel animal experimental models and non-pharmacological and pharmacological therapies (*e.g.,* analgesic drug discovery) [3]. Thus, developing valid and sensitive animal models is a key factor to the elucidation of neural, molecular, and physiological mechanisms involved in pain response [4-6].

Measuring withdrawal thresholds to noxious stimuli has long been used to examine pain responses *in vivo* [7-9]. These approaches have markedly improved our knowledge of nociception physiology, analgesic drug properties, as well as neurotransmitters (*e.g.,* glutamate) and genes modulated in pain responses [10-13]. Although rodents are widely used in translational pain research [14, 15], developing novel alternative animal experimental models is essential to unravel evolutionarily conserved mechanisms of pain [16, 17] as

*Address correspondence to these authors at the Graduate Program in Biological Sciences: Toxicological Biochemistry, Federal University of Santa Maria, Santa Maria, RS, 97105-900, Brazil; Tel: +55 55 992196878; ORCID: 0000-0001-6218-9660; E-mail: fvcosta88@gmail.com and Laboratory of Experimental Neuropsychobiology, Department of Biochemistry and Molecular Biology, Natural and Exact Sciences Center, Federal University of Santa Maria RS, Brazil; Tel: +55 55 32208665;
E-mail: dbrosemberg@gmail.com

1570-159X/21 $65.00+.00

well as to perform high-throughput *in vivo* pharmacological screens [18]. Here, we discuss the growing utility of zebrafish models to evaluate specific molecular, physiological, and behavioral phenotypes related to pain responses. We also outline the suitability of zebrafish models to assess some debilitating pain-related mood disorders, as well as cover recent advances and potential limitations of the zebrafish as a cost-effective and translatable model organism in pain research and pharmacological screens.

2. GENERAL MODEL FEATURES

The zebrafish (*Danio rerio*) is a suitable model organism in genetics, neuroscience, pharmacology, and drug discovery [18-21]. Compared to traditional rodent models, zebrafish exhibit some advantages for basic research, including lower space for maintenance and the large number of offsprings [22, 23]. These features reinforce the growing use of zebrafish as a powerful tool to perform medium-to-high throughput screens cost-effectively [24, 25]. The external fertilization also simplifies the production of transgenic lines, and the presence of translucent embryos facilitates the investigation of potential biomarkers of nociception *in vivo* (*e.g.,* using gene-expression fluorescent probes) [26-30]. Although the use of zebrafish-based pain models is relatively recent [31], this species shows high sensitivity to various nociceptive stimuli [32-35]. Notably, zebrafish respond to clinically active analgesics, representing a promising model to investigate the molecular basis of human pain related disorders [16, 36-38]. Despite the anatomical differences in the central nervous system (CNS) from humans, teleost fishes display electrical activity and transcriptional changes following noxious stimuli [39-42]. Importantly, zebrafish express genes involved in pain responses [31, 36, 37, 43-50] also presenting specific "protective" pain behaviors when exposed to noxious stimuli, suggesting the existence of central mechanisms underlying pain responses [51]. Fig. (**1**) highlights important features of zebrafish vs. other model organisms used in pain studies and the molecular correspondence between human and zebrafish orthologs involved in pain responses.

3. MOLECULAR MECHANISMS OF PAIN-RELATED RESPONSES

Pain is an individual sensation that mobilizes specific receptors to detect noxious stimuli, playing a pivotal role in survival and well-being of the species [10]. Zebrafish presents a complex physiological system that recognizes and responds to painful stimuli [13, 42, 52], including multiple subtypes of nociceptors already identified with similar organization of nociceptive circuits to those of mammals [36, 51, 53, 54]. Here, we emphasize the basic mechanisms of pain-related responses, focusing on the molecular machinery expressed in zebrafish (*e.g.,* the opioid system, the transient potential receptor family (TRP), the endocannabinoid system, as well as acid-sensitive ion channels (ASIC)) and their relevance in the study of pain.

3.1. Opioid System

The opioid system involves multiple receptors, endogenous ligands and natural or synthetic analogs, which play a key role in pain homeostasis, mood, and well-being [55, 56]. In humans, opioid receptors belong to the G protein-coupled receptor (GPCR) superfamily, and three classical opioid receptors have been characterized so far: MOP (μ = mu for morphine; encoded by the *OPRM1*), KOP (κ = kappa for ketocyclazocine; encoded by the *OPRK1*), DOP (δ = delta for vas deferens; encoded by the *OPRD1*), and their respective endogenous ligands; β-endorphin, enkephalin, and dynorphin [57-59]. Although MOP, KOP, and DOP mediate analgesic effects, they rather differ in evoking affective be-

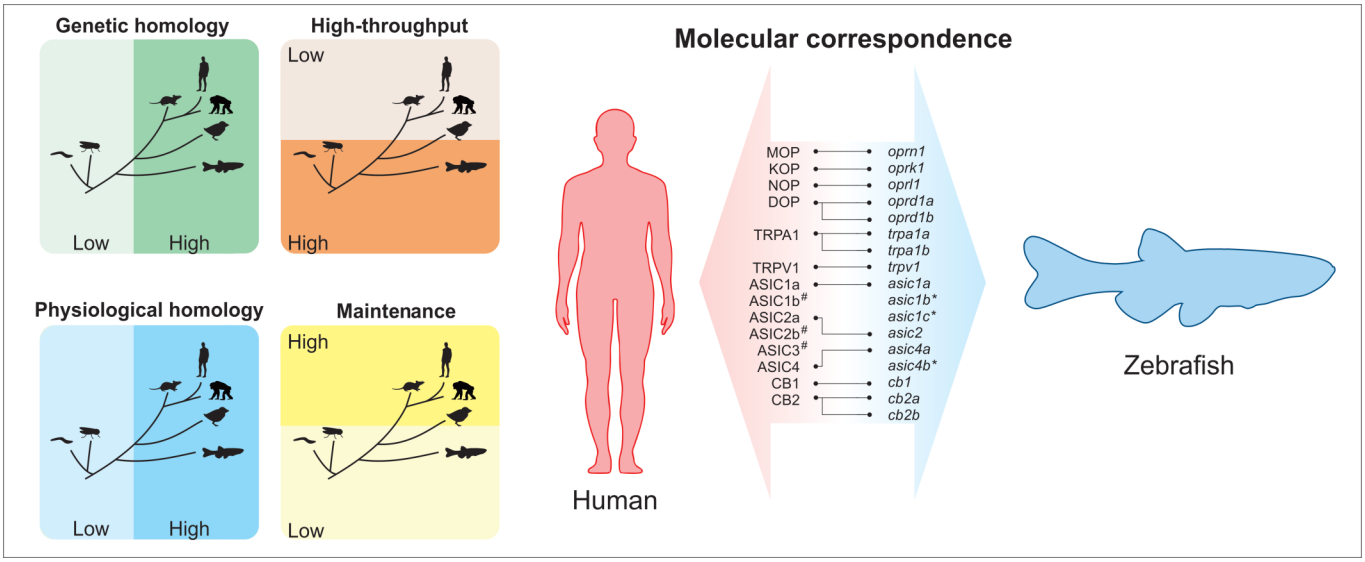


Fig. (1). General advantages and molecular features of zebrafish in translational pain research. As a vertebrate species, the zebrafish shows a high genetic and physiological homology when compared to mammals, but present a low cost of maintenance and feasibility to high-throughput screens similar to invertebrates (**left**). Molecular correspondece of the receptors involved in pain responses in humans with their respective genes in zebrafish (**right**). # are absent in zebrafish. * are splice variants. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

haviors [60, 61]. While MOP agonists produce euphoria and promote stress coping [62, 63], KOP agonists cause dysphoria and are associated with stress and negative effects [64, 65], and DOP agonists are known to elicit anxiolytic and antidepressant effects [60, 61]. Furthermore, the additional NOP (nociceptin/orphanin FQ) receptor encoded by the *OPRL1* gene, a non-opioid member of the opioid receptor family, has also been described in mammals, and its activation with specific ligands shows analgesic effects [66].

The genome sequencing projects allowed a comprehensive identification of the zebrafish orthologs [67, 68], such as zMOP (μ; encoded by the *oprm1*) [45], zKOP (κ; encoded by the *oprk1*) [44], and two functional copies of zDOP (δ; encoded by the *oprd1a* and *oprd1b*) [69, 70]. Furthermore, zNOP (nociceptin/orphanin FQ; encoded by *oprl1*) receptor has also been characterized [71]. Here, we will use MOP, DOP, KOP, and NOP for mammals' opioid receptors, as well as zMOP, zKOP, zDOPa/b, and zNOP to describe their corresponding zebrafish opioid receptors.

In zebrafish, opioid peptide precursors include two proenkephalin genes (*penka* and *penkb*), two proopiomelanocortin genes (*pomca* and *pomcb*), one prodynorphin gene (*pdyn*), and two pronociceptin genes (*pnoca* and *pnocb*). *Penka* codes four Met-enkephalins (ME), one Met-enkephalin-Ile (MEI), and one Met-enkephalin-Asp (MED) [31, 67, 72]. *Penkb* codes four ME, one Leu-enkephalin (LE), and one Met-enkephalin-Gly-Tyr (MEGY) [73]. *Pomca* codes for adrenocorticotropin (ACTH), γ-lipotropin (γ-LPH), β-melanotropin (β-MSH), and β-endorphin (β-END) while *pomcb* codes for only α-melanotropin (α-MSH) and β-END [74]. Interestingly, ACTH and α-MSH are released after hypothalamic-pituitary-interrenal axis (HPI; homologous to mammalian hypothalamic-pituitary-adrenal axis) activation following a noxious stimulus in zebrafish [75]. While ACTH stimulates the synthesis and cortisol release [76], α-MSH is responsible for camouflage response in larval zebrafish [77]. Because both molecules are released under aversive conditions, a potential involvement of camouflage in stress- and pain-responses should not be ruled out.

Pdyn codes for Ile-enkephalin, the neoendorphins (α-neoendorphin and β-neoendorphin), dynorphins A and B [78]. Furthermore, both *pnoca* and *pnocb* encode two nociceptin peptides, a nociceptin orthologous to the mammalian which presents the classical 'opioid message' (-Try-Gly-Gly-Phe-) in its N-terminus, and another 'nociceptin-like' peptide, which is lost in mammals and has low homology to mammalian nociceptin [67, 73].

The pharmacological properties of the zebrafish opioid receptors have been extensively characterized [79, 80]. While zMOP, zKOP, and zDOPa/b display high affinity toward both nonselective opioid ligands [3H]-diprenorphine and [3H]-bremazocine in saturation binding assays [44, 55, 70], they show lower affinity for highly selective ligands in mammals ([d-Pen2,d-Pen5]enkephalin [^{3}H]-DPDPE, [^{3}H]-[D-Ala^2, NMe-Phe^4, Gly^5-ol]-enkephalin (DAMGO), and [^{3}H]-U69,593) [81]. Like mammals, [^{35}S]-GTPγS binding assays demonstrate that all classical zebrafish opioid receptors act *via* G*i* (inhibitory) protein-coupled receptors after binding agonist ligands [44, 45, 69], suggesting that the information could be transduced by the same type of mammalian GPCR.

3.1.1. zMOP

Currently, MOP is considered the primary target for painkillers [82], although various adverse side effects (*e.g.,* tolerance, physical dependence, and addiction) limit their effective medical use [60]. zMOP receptors are widely distributed in the zebrafish brain, and similar to their human homologs, they are highly expressed in regions involved in analgesia and reward [83]. Although zMOP shows 74% of genetic similarity related to MOP, the genomic structure of zMOP presents a stop codon on exon 3 (and not on exon 4, as occurs in mammals) [55], suggesting some difference in behavioral effects and regulatory mechanisms mediated by zMOP [55]. For example, while naloxone alone does not affect anxiety in rodents [84] and non-human primates [85], this antagonist induces anxiety-like behaviors in adult zebrafish [86].

Similar to the mammalian ortholog, the zMOP molecular structure is well conserved in both transmembrane domain and intracellular loop [45]. However, zMOP presents low conservation in the extracellular loop as well as in the carboxyl- and amino-terminal, compared to MOP [45]. Although differences in the extracellular loop can determine the selectivity of ligands, zMOP displays similar pharmacological profile and conserved functions compared to the mammalian counterparts [33, 34, 87-89].

Binding experiments using [^{3}H]-diprenorphine demonstrate that naloxone displays highest affinity toward zMOP, followed by β-END = morphine > MEGY > ME > LE [72]. Likewise, dynorphin A and nociceptin can also bind to zMOP [73, 78]. While endomorphins exhibit partial agonistic properties at the zMOP, competition-binding assays in zebrafish brain membranes show that [^{3}H] diprenorphine binds to zMOP with a nanomolar range affinity, which is antagonized by naloxone [55, 81]. Notably, zMOP also shows analgesic properties, since morphine prevents pain behaviors in both adult and larvae zebrafish, in inflammatory and visceral pain models [32-34, 87, 89]. Like in humans, adverse effects can also be observed after zMOP activation with different agonists, and include sedation (mitragynine and morphine) [90, 91], reduced gut mobility (loperamide) [92], and addiction (morphine) [93], suggesting evolutionarily conserved biological functions related to pain and mediated by these receptors.

3.1.2. zKOP

Similar to the stimulation of other opioid receptors, the activation of KOP produces analgesia in mammals [94, 95]. However, the administration of KOP agonists into the spinal cord or intravenously antagonizes morphine-induced analgesia [96]. Clinical trials reveal psychotomimetic and hallucinogenic effects caused by KOP agonists [97]. Interestingly, a biphasic effect was observed after salvinorin A (a KOP selective agonist) injection in zebrafish; while low doses (0.1 or 0.2 μg/kg) evoke rewarding effects and excitation, high doses (5 or 10 μg/kg) cause aversive effects and hallucinogenic-like behavior [98].

zKOP presents 70% genetic similarity compared to its mammalian counterpart [44]. This receptor is well conserved in both the transmembrane domain and intracellular loop, and less conserved in the extracellular loop and the carboxyl- and amino-terminal compared to KOP [44]. Interestingly, the third extracellular loop of zKOP (determinant for the binding selectivity in mammals) displays high similarity to MOP, which may explain why morphine can bind zKOP [44]. Moreover, binding experiments using [^{3}H]-diprenorphine demonstrate that non-specific δ-κ agonist/μ antagonist bremazocine displays highest affinity toward zKOP, followed by dynorphin A = naloxone > D-Arg dynorphin A > morphine [44]. Likewise, both nociceptin and nociceptin-like peptides can also bind zKOP [73]. Because of the presence of a positive-charged residue His^{294}, which is not present in the human receptor extracellular loop, the specific KOP ligand [^{3}H]-U69,593 does not bind zKOP [44]. Zebrafish endogenous opioid MEGY is unable to bind zKOP [99], unlike the mammalian counterpart peptide MERF, which binds the three classical opioids (MOP, DOP, and KOP) [100]. While there is no direct evidence of analgesic effects following zKOP stimulation in zebrafish-based pain models, the structural similarities between zKOP and MOP could imply such a possibility.

3.1.3. zDOP

Although the most prescribed opioids (*e.g.,* morphine, fentanyl, codeine) mainly target MOP receptors, the selective activation of DOP has great potential for chronic pain treatment [101, 102]. Since anxiety and mood disorders are commonly associated with chronic pain, the ability of DOP to cause anxiolytic- and antidepressant-like side effects are desirable [103]. In humans, DOP is widely distributed in the brain, specifically in the periaqueductal gray region, the rostroventral medulla, the cerebral cortex, and the amygdala [104]. In zebrafish, two functional copies of DOP were characterized: zDOPa and zDOPb [69, 70]. Similarly, zDOPa is also widespread in the CNS, from the telencephalon to the spinal cord, including areas involved in analgesia [105]. Moreover, homology analysis revealed 66% of genetic similarity comparing zDOPa to human counterparts [69]. The zDOPa is well conserved in both the transmembrane domain and intracellular loop, being less conserved in the extracellular loop and the carboxyl- and amino-terminal than DOP [69]. In mammals, the third extracellular loop of opioid receptors is a determinant for selective binding [106]. Interestingly, two Arg residues in the third extracellular loop are substituted by a Lys, which may explain why DPDPE, the prototypical ligand for the DOP, has a low affinity for the zDOPa binding site [107]. Pharmacological characterization shows that zDOPa binds the non-selective opioid antagonist [^{3}H]diprenorphine with high affinity, followed by bremazocine > β-END > naloxone > morphine > DEDPE [108].

zDOPb is also widespread in the CNS, being more expressed in the dorsal telencephalic area, hypothalamus, reticular formation, facial lobe, and cerebellum than zDOPa [70]. zDOPb presents 65% of genetic similarity and is well conserved in both transmembrane domain and intracellular loop, as well as less conserved in the extracellular loop and the carboxyl- and amino-terminal compared to DOP [70]. Interestingly, zDOPb displays lower or no affinity toward highly selective DOP ligands due to the presence of positive and negative charges in both second and third extracellular loops [70]. While both zDOPa and zDOPb are homologs with high sequence similarity (71%) [70], they present some differences. For example, an amino acid is substituted in the protein sequence of these receptors (Glu^{112} replaces Asn^{115} in zDOPa or by Gly^{117} in zDOPb), which increases the peptide MEGY affinities for zDOPb [99]. Overall, both zDOPb and zDOPa show similar pharmacological properties and conserved functions, compared to their respective mammalian counterparts, whereas some differences in ligand selectivity also occur.

3.1.4. zNOP

Although NOP does not bind the opioid antagonist naloxone (traditionally used to discriminate opioid receptors), NOP is classified as a non-opioid member of the opioid receptor family by the International Union of Basic and Clinical Pharmacology (IUPHAR) [66]. In humans, NOP is widely distributed in both central and peripheral nervous systems [109]. Interestingly, the activation of NOP evokes distinct behavioral responses: a classical antinociceptive effect when spinally activated [110, 111], and an anti-opioid effect when NOP is supraspinally activated [112, 113].

In zebrafish, zNOP is highly expressed in the CNS and intestine, being less expressed in the peripheral nervous system [71]. Although zNOP displays 58-59% of genetic similarity related to mammalian NOP [71], their functionality may differ from the respective mammalian counterpart. Indeed, zNOP binds not only human nociceptin but also both zebrafish dynorphin A (with greater affinity) and mammalian dynorphin A, as well as naloxone (order of affinity: zebrafish dynorphin A > nociceptin > bremazocine = norbinaltorphimine = mammalian dynorphin A > naloxone) [71]. Moreover, amino acid residues that are important for ligand binding at the NOP transmembrane domains (TM) (Phe^{220} and Phe^{224} in TM5, Asp^{130} and Tyr^{131} in TM3, and Trp^{276} in TM6) are highly conserved in the zNOP [71]. Interestingly, some residues conserved in EL2 (extracellular loop 2; region responsible by interaction with ligands) of NOP are replaced by residues involved in KOP recognition [71]. This change may explain why zNOP shows a preference for the KOP-selective ligands. Ultimately, zebrafish models may help elucidate the role of NOP in the sensory perception of pain.

3.2. TRP Family

The transient receptor potential (TRP) channel family acts as molecular sensors, responding to a wide variety of stimuli (*e.g.*, changes in pH, chemical agents, temperature, and osmolarity) [114, 115]. Structurally, a TRP channel contains six putative transmembrane domains (TM1-6) assembled as tetramers to form cation-permeable pores between TM5 and TM6 [115, 116]. In humans, TRP channels include 28 members divided into six subfamilies, classified as canonical (TRPC), vanilloid (TRPV), ankyrin (TRPA1), melastatin (TRPM), polycystin (TRPP), and mucolipin (TRPML) [117]. Interestingly, zebrafish express all TRP subfamilies (trpc [118], trpv [119], trpa [120], trpm [121], trpml [122], and trpp [123]). Moreover, zebrafish have a single NompC (trpn), which is absent in humans [37]. Because TRP is considered the most important ion channel

family that detects and transmits noxious stimuli, in this topic, here we summarize key relevant findings related to the trpa and trpv subfamilies and their importance in pain responses in zebrafish.

3.2.1. Trpa1a, trpa1b, and trpv1

The peripheral and central nociceptive systems of zebrafish are similar to those of mice and humans [124] and the nociceptive function of TRPA1 is remarkably conserved across animal species [125, 126]. However, unlike in humans and rodents, the zebrafish genome encodes two TRPA1 genes (*trpa1a* and *trpa1b*) [119]. Notably, both trpa1a and trpa1b are activated by human TRPA-agonists (allyl isothiocyanate, cinnamaldehyde, diallyl disulfide, acrolein, and 4-hydroxynonenal) [37], and antagonized by HC-030031, a competitive or negative allosteric modulator of human TRPA1 [124]. However, HC-030031 failed to show any inhibitory effects on trpa1b activated by cinnamaldehyde in a heterologous expression system using *Xenopus laevis* oocytes [127]. Therefore, the hypothesis that HC-030031 antagonizes trpa1a but not trpa1b, cannot be discarded.

Unlike other vertebrates (*e.g.,* chick, frog, and mice) where TRPA1 is responsible for detecting noxious chemicals and harmful temperatures [125], mounting evidence suggests that trpa1a mediates chemical sensing, whereas trpa1b responds to thermal stimuli [37, 128], albeit a mutation on trpa1b reduces or abolishes the hyperlocomotion induced by chemical algogens [37]. Both trpa1a and trpa1b are expressed in sensory neurons and also found in different regions and structures. While trpa1a is predominantly expressed in sensory ganglia innervating visceral organs, trpa1b is expressed in sensory neurons that innervate the skin and cranial sensory ganglia [37]. These expression patterns suggest that trpa1b can be activated by both internal and external stimuli, whereas trpa1a probably detects mainly internal stimuli.

In mammals, TRPV1 is a polymodal receptor, which acts as a sensor for noxious heat (> 42°C) and contributes to painful disorders (*e.g.,* diabetic-induced neuropathic pain, cancer pain, and inflammatory pain) [129]. TRPV1 is also sensitive to acidic solutions (pH < 6.5), food ingredients (capsaicin) [130], lipid derivatives (prostaglandin E2) [131], endocannabinoid, anandamide [132], and toxins (*e.g.,* vanillotoxins and resiniferatoxin) [133]. Interestingly, zebrafish express a single trpv1-like ortholog receptor that could be derived from evolutionary precursors of tetrapod TRPV1 and TRPV2 [119]. Similar to the mammalian ortholog, trpv1 acts as a heat-sensitive channel (≥25°C), although it presents a lower sensitivity than that of the mammalian counterpart (≥42°C) [36]. Notably, 5 dpf zebrafish larvae avoid temperatures rearing above 31.5°C and below 24.5°C [134], characterizing thermal hyperalgesia. Importantly, thermal hyperalgesia is a common symptom of individuals suffering from inflammatory pain, which could support the face validity of this animal model. Also, the thermal aversion is prevented by an analgesic (ibuprofen) [134], reinforcing the predictive validity of this model.

Because amino acid residues critical to TRPV1 activation by capsaicin (Ser-512 and Thr-550) are different in trpv1 (Thr-480 and Ile-518), zebrafish larvae do not present behavioral changes after capsaicin administration *via* water immersion [36]. However, pain behaviors can be observed after a single capsaicin administration into the lips of adult zebrafish and classical TRPV1 antagonists prevent these responses [135-138]. Similar results are observed after capsaicin administration into the lips of Atlantic cod, another teleost fish [139]. In zebrafish larvae, trpv1 is expressed in the early stage (24 hours post fertilization, hpf), along the lateral line ganglion neurons, which do not have direct sensory properties [140]. In the first essay, capsaicin was administered into the water, which may explain why zebrafish larvae do not show aversive behaviors. Furthermore, a feasible interaction between trpv1 and trpa1a/trpa1b in a capsaicin-induced pain behavior cannot be discarded since, like mammals, trpv1 and trpa1a/trpa1b are co-expressed in dorsal root ganglion neurons [37], suggesting a coordinated activation of these receptors. Further studies are necessary to investigate the role of capsaicin in zebrafish-based pain models.

In general, although trpv1, trpa1a, and trpa1b have changed through vertebrate evolution, they modulate a similar behavioral repertoire compared to mammals. Moreover, differences in temperature threshold (≥ 25°C for zebrafish and ≥ 42°C in mammals) are clearly associated with the adaptation of these organisms to their respective habitats. Finally, as many aspects of the zebrafish TRPA1 and TRPV1 orthologs remain unclear, more studies are necessary to elucidate the trpv1-, trpa1a-, and trpa1b- mediated nociception.

3.3. The Endocannabinoid System

The endocannabinoid system plays a key role in various biological processes, such as energy metabolism, inflammation, pain transmission, and synaptic neurotransmission [141]. In general, the endocannabinoid system is composed of cannabinoid receptors, endogenous cannabinoid ligands (anandamide and 2-arachidonoylglycerol), and enzymes involved in the synthesis or degradation of ligands [142]. Cannabinoids exert their pharmacological effects through the activation of at least two distinct cannabinoid receptors, CB1 and CB2, although mounting evidence supports the existence of other receptors, such as GPR55 [143]. Both CB1 and CB2 receptors are presynaptic G*i* protein-coupled receptors with seven transmembrane domains that are differentially distributed in the CNS and peripheral tissues [144]. While CB1 receptor is highly expressed in the CNS (substantia nigra, globus pallidus, hippocampus, and cerebellum, with little expression in the brainstem) [145], the CB2 receptor is mainly located in immune cells peripherally [146], although it is also expressed in brain neurons (cerebellum, cortex, hippocampus, thalamus and hypothalamus, and ventral tegmental area) [147, 148]. Activation of CB1 receptors causes a robust effect on cognition, reward, and anxiety [149, 150]. On the other hand, the activation of CB2 receptors leads to immunosuppression, which limits inflammation and associated tissue damage [151]. The endocannabinoid system also modulates nociceptive responses. For example, in the peripheral system, CB1 receptors are expressed mainly in the nerve terminals and control neuronal responses [152]. Moreover, CB1 receptors are also expressed in both large and small-diameter fibers (*e.g.*, C-fibers) and inhibit the re-

lease of neurotransmitters involved in pain transmission [153-155]. Likewise, peripheral CB2 receptors reduce the release of pronociceptive molecules from immune cells and keratinocytes [152]. Additionally, cannabinoids also activate pain receptors, such as TRPV1, suggesting the use of such molecules as potential analgesics [156].

The endocannabinoid system of zebrafish is evolutionarily conserved. Basically, zebrafish possess the same receptors (cb1 and cb2 receptors share ~ 73% and 39% of genetic similarity compared to the human orthologs, respectively) [157, 158], ligands, and metabolic enzymes [159]. However, the zebrafish genome encodes two cb2 receptor genes: *cb2a* and *cb2b* (sharing a sequence identity of 98% between them) [158]. In zebrafish, cb1 mRNA has been detected in whole-body lysates by quantitative real-time reverse transcriptase polymerase chain reactions (qRT-PCRs) as early as the 3-somite stage, with the transcript exhibiting an increase through the first 15 days post-fertilization [160]. The zebrafish cb1 receptors are found in the telencephalon, hypothalamus, tegmentum, and anterior hindbrain by 2 dpf, and their expression remains similar into adulthood, paralleling the mammalian counterpart [157]. On the other hand, cb2a/b are abundant in immune system cells and their activation shows anti-inflammatory effects [158]. Moreover, cb2a/b mRNA has been detected by qRT-PCR in adult zebrafish brain, intestine, retina, gills, heart, pituitary, and spleen [158]. Although both zebrafish cb2a and cb2b do not have an amino acid residue responsible for ligand affinity to WIN55212-2 (Leu^{175} replaces Phe^{197} in zebrafish cb2a/b) [161], this radiolabeled synthetic cannabinoid (sCB) can bind to its targets in the hypothalamus, optic tectum, and telencephalon in adult zebrafish brain slices [162]. Similarly, binding assays demonstrate that the endocannabinoid (anandamide) and sCB (HU-210, WIN55212-2, and CP55940) interact with receptors in adult brain homogenates [158]. Cannabinoids also show analgesic properties, since cannabidiol, a phytocannabinoid, prevents hypolocomotion induced by acetic acid in zebrafish larvae [163].

Finally, since cb1 receptors are localized in pain-processing areas of the brain and spinal cord, and their exogenous stimulation promotes antinociception [164], zebrafish could be a powerful tool to access the interaction between opioids and cannabinoid receptors [165]. For example, low doses of cannabinoid prevented antinociceptive tolerance to morphine in animal models of neuropathic pain [166]. Additionally, pretreatment with a non-analgesic dose of tetrahydrocannabinol evokes up to a 22-fold increase in morphine-induced analgesia [167], even though the analgesic effects of cannabinoid products are relatively low [154]. Notably, CB2 receptors can also mediate antinociception and may be promising targets for pain therapy due to their relatively lower CNS expression, which can result in reduced psychotropic effects [164]. Overall, although more studies are necessary, the endocannabinoid system could be a relevant molecular pathway to study pain processes in zebrafish.

3.4. zASIC

Acid-sensing ion channels (ASICs) are excitatory receptors for extracellular H^+ [168]. Their functions include peripheral perception of pain, synaptic transmission, and mechanosensation [169]. In mammals, there are four ASICs genes encoding six subunits (*ASIC1a* [170], *ASIC1b* [171], *ASIC2a* [172], *ASIC2b* [173], *ASIC3* [174], and *ASIC4* [175]), which act as homo- or hetero-oligomeric assemblies of individual subunits [176, 177]. In humans, ASICs are widely distributed in both central and peripheral nervous systems, but have also been detected in non-neuronal tissues, including testis (*ASIC3*) [178], pituitary gland (*ASIC4*) [179], lung epithelial cells (*ASIC3*) [180], and bone (*ASIC1-3*) [181]. In the peripheral nervous system, ASIC channels are found predominantly in small-diameter sensory neurons involved in pain [171].

In zebrafish, six ASICs have already been identified (asic1a, asic1b, asic1c, asic2, asic4a, and asic4b) [182]. Phylogenetic analyses show that asic1a, asic2, and asic4a are orthologous to mammals, whereas asic1b, asic1c, and asic4b are splice variants [182]. Although, zebrafish ASICs are broadly expressed in the CNS (starting between 24 and 48 hours post fertilization), only asic1a is expressed in the trigeminal ganglion (region responsible for carrying somatosensory information from the head) [182]. Both asic1a and asic1c are expressed in sensory neurons and may contribute to mechanical stimuli [182]. Although both asic4a and asic4b share ~68% of genetic similarity [171], only asic4a is gated by extracellular H^+ (pH ~5.8) [182]. Importantly, understanding how different ASICs expressed in zebrafish contribute to specific behavioral responses still requires further scrutiny, and the use of specific transgenic lines or mutants can provide relevant findings in this field.

4. COMPARISON OF THE EXISTING ADULT ZEBRAFISH-BASED PAIN MODELS

Animal experimental models are essential to characterize the functionality of distinct classes of nociceptors across taxa [51]. Mounting evidence supports that zebrafish express the necessary cellular components to recognize nociceptive agents and display aberrant behaviors in responses to algogens [13, 42, 52], also see Fig. (**2**) describing major methodological approaches and behavioral phenotypes of adult zebrafish related to pain responses. Because pain perception in animal models is subjective, the recognition of pain-related phenotypes is a cornerstone for understanding how noxious stimuli influence animal physiology and behavior [183, 184]. Here, we summarize the main experimental approaches to access pain behavior in zebrafish.

4.1. Fin Clipping

The fin clipping is a surgical procedure that removes the caudal posterior tissue, injuring nociceptive fibers and triggering pain-like responses [185]. The fin clip procedure increases the opercular- and tail-beating, the bottom-dwelling, as well as decreases locomotion [35, 87]. These pain responses are entirely prevented after lidocaine or morphine administration [35, 87, 185], supporting a robust nociceptive response in zebrafish.

4.2. Cinnamaldehyde

Widely used in murine models, cinnamaldehyde has been recently used in zebrafish as a nociceptive agent [33]. This TRPA1 agonist acts differently on zebrafish trpa1a and

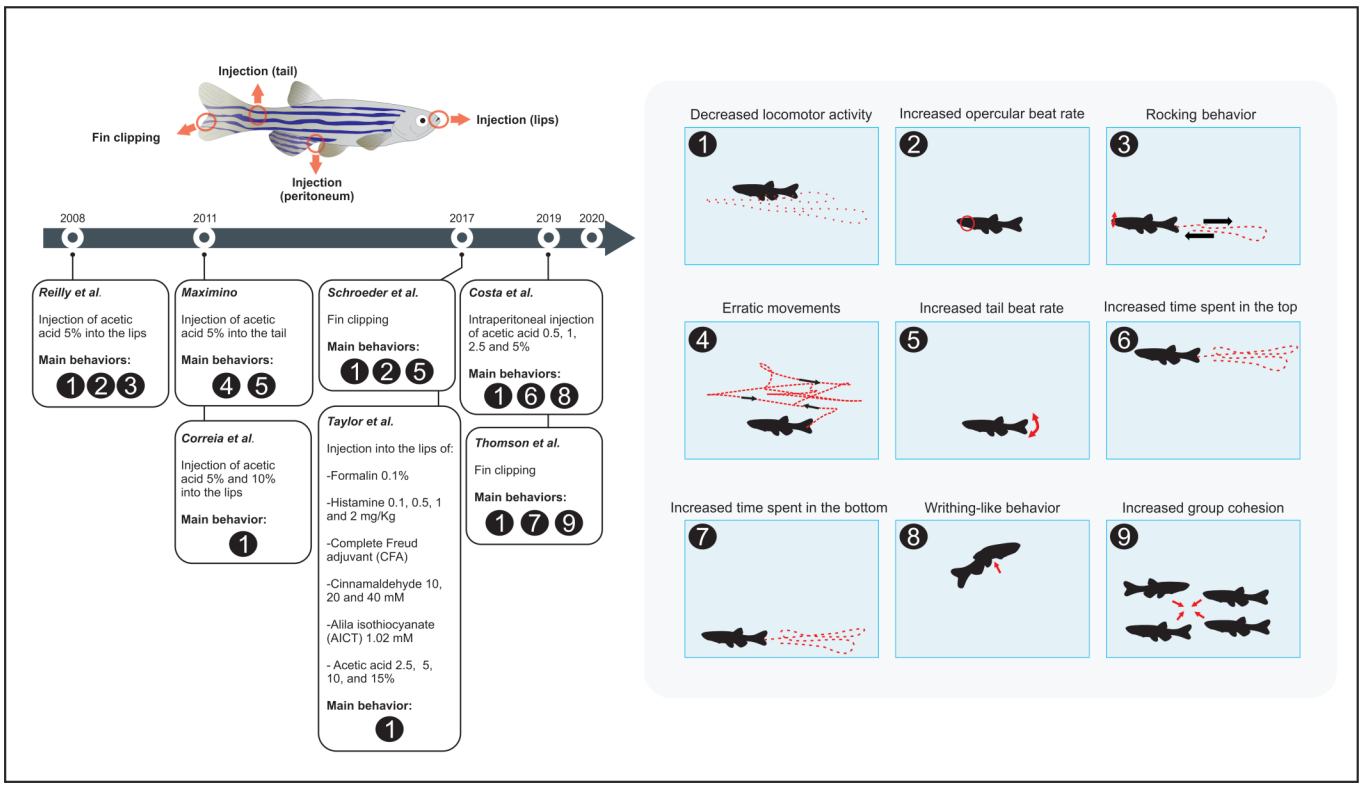


Fig. (2). Common experimental protocols used to assess pain in adult zebrafish with the respective behavioral endpoints measured in different studies depicted in timeline perspective. References: Reilly *et al.* [42], Maximino [52], Correia *et al.* [196], Schroeder *et al.* [185], Taylor *et al.* [33], Costa *et al.* [34], Thomsom *et al.* [35]. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

trpa1b receptors [127]. While trpa1a is desensitized in two applications of cinnamaldehyde (even at low concentrations), trpa1b shows uniform currents after cinnamaldehyde applications [127]. Notably, cinnamaldehyde administered into the lips (40 mM) or the tail (0.003 mM) causes hypolocomotion in zebrafish [33], supporting a well-conserved biological response even when administered in different routes and doses.

4.3. Allyl Isothiocyanate

Another TRPA1 agonist used to induce pain in zebrafish is allyl isothiocyanate (AITC; commonly known as mustard oil). While AITC consistently increases Ca^{2+} concentration in both trpa1a and trpa1b, this response is prevented by a mammalian TRPA1 antagonist (ruthenium red), suggesting that both zebrafish TRPA1 paralogs can be activated by AITC [37]. Furthermore, a single injection of AITC (1.02 M) causes a remarkable hypolocomotion in adult zebrafish [33]. Although a reduction in distance traveled has been commonly described as pain behavior in zebrafish, there are no data showing a preventive effect of analgesics in this model, therefore, further scrutiny is required.

4.4. Complete Freund's Adjuvant

Commonly used to study chronic pain in preclinical research [186], the Complete Freund's Adjuvant (CFA) is composed of inactivated mycobacteria in which are recognized by immune cells, through Toll-like receptor 4 (TRL4) [187], leading to persistent inflammation and pain-like behaviors. To date, there is a single report using CFA to access pain-like behavior in zebrafish [33]. Like in mammals, a single CFA intraperitoneal administration causes a persistent inflammation in zebrafish, starting 24 hours post-injection and persisting for at least 48 hours. CFA injection results in decreased swimming activity, which can be prevented by morphine [33]. Because depression and chronic pain often correlate in mammals [188], assessing whether the CFA administration serves as a tool to access both pain- and comorbid depression-like responses in zebrafish could be interesting in the future.

4.5. Histamine

Histamine is involved in several regulatory mechanisms, including allergic reactions and pain [189]. In general, the zebrafish histaminergic system resembles that of other vertebrates [190]. In adult zebrafish, histamine (1 and 2 mg/kg) injected into the lips causes hypolocomotion [33], similar to what occurs in zebrafish larvae [191]. Although the histaminergic system in zebrafish is well described, relatively little is known about its role in zebrafish pain responses.

4.6. Formalin Test

Unlike other approaches, the formalin test is characterized by two different stages of pain-like responses [192]. In mammals, the first stage (neurogenic) begins immediately after the injection lasting for 3-5 min, triggered by chemical stimulation of nociceptors (C fibers). The second stage (inflammatory) begins at 15-20 min and lasts for ~20-40 min,

as multiple pro-inflammatory mediators are released (*e.g.*, histamine, prostaglandins, and serotonin) [193]. Formalin (0.1 %) injected into the tail impairs the locomotion of adult zebrafish in both neurogenic (0-5min) and inflammatory (15-30 min) stages, which are prevented by analgesics (morphine and indomethacin) [32]. Although formalin is a TRPA1 agonist in mammals, there is no evidence that formalin activates the same receptor in zebrafish.

4.7. Acetic Acid

Widely used in rodents to access pain-like phenotypes [194, 195], the acetic acid administration in zebrafish evokes robust behavioral responses. For example, acid acetic injected into the lips (2.5, 5, 10, and 15%) reduces locomotion, increases the opercular beat rate, and promotes 'rocking' behavior (Fig. **2**, moving side to side) in zebrafish, as well as causes them to rub their lips against the walls or gravel [33, 42, 196]. When administered near the adipose fin, acetic acid (1 %) increases both erratic movements and tail beating [52]. Moreover, zebrafish display a writhing-like behavior following a single acetic acid (2.5 and 5%) intraperitoneal injection, analogous to writhing response in rodents [34, 197]. Importantly, all behaviors described above can be prevented by clinical analgesics, supporting their pharmacological validity to measure pain-like responses.

Indeed, zebrafish-based pain models are becoming promising strategies as first-choice tools for drug screening. However, future experiments aiming to improve the use of zebrafish in translational pain research are needed. For example, there are no methodological approaches to access allodynia, as well as neuropathic pain in zebrafish so far. These models help validate the use of zebrafish as a potential organism to test novel analgesic drugs, fostering the creation of more sensitive, complex, and objective tools in preclinical research, complementing the existent rodent approaches.

5. PAIN-RELATED MOOD DISORDERS

Chronic pain is a global problem that affects millions of people and is linked to significant morbidity, limited mobility, and social isolation [198, 199]. Moreover, chronic pain is also associated to different emotional disorders, including anxiety and depression [200-202]. Considering the complexity of these emotions, anxiety-related disorders and major depression overlap in symptoms and comorbidity, including cognitive, affective, physical, and behavioral deficits [203, 204]. Additionally, anxiety-related disorders and depression are associated with increased pain perception [205]. In humans, pharmacological agents can be used for treating both chronic pain and emotional disorders [206, 207], whereas animal models serve as important tools to understand the evolutionarily conserved mechanisms underlying these conditions [208, 209]. Evidence shows that neurotransmitters systems (*e.g.,* dopaminergic and serotonergic systems) are involved in CNS disorders and pain-regulatory processes [210, 211]. In addition to traditional rodent models, zebrafish serve as a promising tool to investigate the molecular basis of pain and emotional disorders due to high genetic homology and neurochemical conservation when compared to humans [212, 213]. Furthermore, several behavioral paradigms have been validated in zebrafish to assess anxiety- and depression-like phenotypes (*e.g.,* the novel tank and the light-dark tests), as well as pain behavior [52]. Importantly, although changes in the mean distance traveled or altered swim velocity may indicate pain-like conditions, these endpoints may also reflect other behaviors in zebrafish (*e.g.,* anxiety-, non-specific sedation- and/or depressive-like states) [214]. Indeed, locomotor-related parameters represent an overall state of "well-being" and should not be merely described as unique measures of nociception [34]. In line with this, locomotor activity should be tested in the presence of analgesics, aiming to improve predictive validity. Using complementary behavioral endpoints to assess pain responses in adult zebrafish more accurately should also be considered.

Zebrafish is sensitive to various anxiolytics and anti-pain medications [34, 215] and present well-characterized behaviors (*e.g.,* relatively complex swimming activity, as well as high social interaction). Pharmacological experiments revealed that acute morphine (2 mg/L) exposure for 20 min promotes anxiolytic-like behaviors, while 5 mg/L naloxone increases anxiety-like responses in the novel tank diving test [86]. Altogether, these data implicate opioids in the modulation of anxiety-like behaviors, reinforcing the existence of useful behavioral repertoires in zebrafish for modeling emotional disorders and pain-related phenotypes [212, 214].

6. ADVANTAGES AND LIMITATIONS OF ZEBRAFISH MODELS IN ANTI-PAIN MEDICATION SCREENING

6.1. Comparison between Larvae and Adult Zebrafish

To understand the molecular and physiological mechanisms underlying nociception in vertebrates, both larvae and adult zebrafish have become tractable systems in translational pain research. For example, adult fish show reduced activity and swimming behavior in response to a range of potentially painful laboratory procedures that are ameliorated by several drugs with analgesic properties, including local anaesthetics, non-steroidal anti-inflammatory drugs, and opioids [35, 87]. Studies utilizing < 5 dpf larvae have demonstrated the same results with a reduction in activity with selected drugs, preventing painful responses [89]. These findings confirm the validity of replacing adult zebrafish with younger animals. Moreover, a large number of larvae can be assessed simultaneously in a high-throughput manner (25-96 in well plates) compared to multiple adults being assessed at once. Furthermore, transparent larvae can be imaged in real-time to investigate CNS changes in relation to innocuous and potentially painful stimuli and whether anaesthetic or analgesic drugs can reduce or prevent pain-related brain activity. Although zebrafish larvae have less sophisticated behaviors than adults and a CNS yet to be fully matured [216], their simple and transparent brain allows for the investigation of the brain circuitry involved in pain responses during early developmental stages. The precise CNS processing of painful stimuli in zebrafish is yet to be elucidated, but their utility can help target specific brain areas involved in mammalian pain processing and assist in the search for novel analgesic compounds. However, caution should be applied to the use of < 5 dpf larvae in terms of the ethics of animal use. The 3Rs (Reduction, Replacement, Refinement)

and ethical review should apply to all animal studies, especially regarding the number of animals used in experiments [217]. Researchers should use the minimum number of larvae to achieve the research objectives, and should also apply refinement by using the least invasive techniques and safeguarding welfare.

6.2. Limitations

Zebrafish models possess some limitations in the translatability of their data due to certain differences from mammals, such as in metabolic physiology (cold-blooded fish vs. warm-blooded mammals), brain development, and anatomy (*e.g.,* zebrafish lack cortex) [218], In addition, zebrafish also present some limitations in neuroscience research. For example, the genome duplication event in teleost fishes [219] complicates genetic analyses of pain responses since some pain-related genes may exist in two copies or be missing in zebrafish. Because of its small size, the long-term monitoring of endocrine levels from blood (*e.g.,* glucose levels) is also problematic as it is difficult to obtain a sufficient amount of blood without euthanizing the animal [220].

6.3. Advantages

Zebrafish models have been used to study CNS pharmacological modulation by conventional and non-conventional therapies (*e.g.,* Traditional Chinese Medicine) [221], reinforcing its potential for the development of pharmacological and non-pharmacological therapies for CNS disorders [222] and pain. Another advantage in zebrafish compared to other animal models (*e.g.,* rodents) in pharmacological screening is its high performance. For instance, using zebrafish larvae, one may test over 10000 drugs behaviorally in a single study [223], which may help understand behavioral effects of analgesic therapies, molecular mechanisms, and neural circuits involved in pain response. Zebrafish present a high degree (~70%) of genetic homology when compared to humans [224], as well as offer several CNS genetic models [225], which reinforce this aquatic model as a powerful species to explore genetic modulation of pain and related disorders. Importantly, zebrafish have sophisticated behaviors, which can be easily assessed using automated video-tracking systems to perform 3D reconstructions of the swimming traces in adult specimens [214]. Although the development of novel automated systems to measure behavioral activities simultaneously in a group of subjects is necessary, the use of existing video-tracking technologies may increase the efficiency and speed of time-intensive manual coding to measure pain-related responses in zebrafish. This strategy not only minimizes human bias, but also allows an in-depth investigation of specific behavioral responses in fish treated with different algogens in a high-throughput and reliable manner.

7. FUTURE PERSPECTIVES AND CONCLUDING REMARKS

In conclusion, zebrafish have been considered an emergent tool to investigate the neurobehavioral basis of pain due to their numerous practical advantages over traditional (rodent) models. This species is space-efficient and enables easy experimental manipulations with a relatively lower cost of maintenance [23]. Some features such as the high reproduction rate, external fertilization, transparency of embryos, and rapid development [226], may foster studying epigenetic mechanisms of pain and probing how painful stimuli during early development affect fish as adults. Multiple transgenic lines created by gene-editing (*e.g.,* CRISPR-Cas9 or transcription activator-like effector nucleases/TALENS) methods have been generated in zebrafish [227, 228]. For exam-

Table 1. Selected open questions in zebrafish-based pain research.

Questions
• Can zebrafish present a relatively complex central processing associated with emotional responses of pain?
• Which areas of the zebrafish CNS are involved in processing painful stimuli?
• How important is the descending control of pain, given the empirical results for stress induced analgesia in zebrafish?
• Given the evidence for pain in zebrafish should we implement pain management protocols in the laboratory employing invasive techniques where pain is not the objective?
• Can we develop standard protocols for investigating pain in zebrafish to increase reproducibility within and between laboratories?
• When using non-protected or non-regulated larval zebrafish (typically <5dpf) ethical review and implementation of 3Rs frameworks should be considered as important tools to ensure humane treatment of the experimental fish. What is the best strategy to implement this aspect practically?
• How much the environment (*e.g.,* pH, temperature, salinity) can influence in zebrafish pain response?
• How much can stress (*e.g.,* experimental manipulation) influence in zebrafish pain response?
• What is the most appropriate behavioral test to assess zebrafish pain phenotypes?
• How to distinguish between a stress and a painful behavioral response in zebrafish?
• How translational to human is the zebrafish pharmacological and non-pharmacological analgesic response?
• Are there differences across strain and age in zebrafish pain response?

ple, the expression of mutant SCN9A, a voltage-gated sodium channel, represents a model to study small-fiber neuropathy and potential analgesic properties of novel compounds in zebrafish [229]. Moreover, zebrafish show high plasticity of CNS [230]. Prominent cell proliferation can occur in different CNS regions of zebrafish, such as the telencephalon and the spinal cord [231, 232], helping to elucidate how adult neurogenesis modulates pain responses, learning, and emotional behaviors, complementing the existing rodent approaches. Finally, the existence of relatively complex behavioral responses [214], as well as multiple strain-, sex- and individual differences in zebrafish, offers further important practical and conceptual advantages to elucidate the link between pain and affective disorders in this aquatic species.

Zebrafish also represent an important model organism in the discovery of novel treatments and therapeutics for pain [124, 134]. However, our present understanding of zebrafish pain-related phenotypes and their molecular mechanisms remains limited (Table **1**). One key question to be solved is the precise CNS processing of ascending nociceptive inputs and descending control of pain. This knowledge helps target specific brain areas in the pursuit of discovering new means of reducing pain in biomedical and veterinary studies. Replacing adult zebrafish with young larval forms can increase the throughput of experiments and enhance the rapid testing of pharmacological agents. This could lead to greater translatability of drug efficacy from laboratory models to clinical testing. The use of artificial intelligence monitoring systems is growing in the behavioral analysis of animals [233, 234]. Not only does this provide a means of automatically measuring behaviors that cannot be done by the human observer but it also eliminates any human error or bias and can provide a more subtle means of measuring changes in pain behavior in response to treatment and application of pain-relieving drugs. Furthermore, to combat the reproducibility crisis that we are currently experiencing in the field, standard pain assessment protocols should be developed so that all laboratories are following the same methods. For experimental studies employing invasive techniques where the pain is not the objective of that study, the development of pain management protocols for zebrafish would be a major step forward in the refinement of zebrafish use. Importantly, animal models are suitable tools to investigate the neurobehavioral mechanisms of pain, aiming to develop safer and more effective pharmacological treatments. Mounting evidence which is only briefly discussed here implicates the use of non-traditional zebrafish models as a fruitful strategy to generate important translational insights into molecular, physiological, and neurobehavioral mechanisms of pain.

CONSENT FOR PUBLICATION

Not applicable.

FUNDING

F.V.C., V.A.Q., and L.C.R. received CAPES fellowship. D.B.R. and A.R.S. are recipients of CNPq research productivity grant. D.B.R. research is also supported by PROEX/CAPES (process number 23038.005450/2020-19) and Programa PQ-Gaúcho FAPERGS (process number 19/2551-0001764-2) fellowship grants. A.V.K. is the Chair of the International Zebrafish Neuroscience Research Consortium (ZNRC). His research is supported by the Russian Science Foundation (RSF) grant 20-65-46006. L.U.S. is convenor of the FELASA working group producing a report on Pain Management in Zebrafish and is a member of the NC3Rs (UK) expert panel on zebrafish welfare. The funders did not influence writing and submission of this manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

The authors thank the financial support from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES)-Finance Code 001, and Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS). AVK collaboration is supported by Sirius University, Sochi, Russia.

REFERENCES

[1] Garcia-Larrea, L.; Bastuji, H. Pain and consciousness. *Prog Neuropsychopharmacol Biol Psychiatry,* **2018**, *87*(Pt B), 193-199. http://dx.doi.org/10.1016/j.pnpbp.2017.10.007

[2] Mogil, J.S.; Davis, K.D.; Derbyshire, S.W. The necessity of animal models in pain research. *Pain,* **2010**, *151*(1), 12-17. http://dx.doi.org/10.1016/j.pain.2010.07.015 PMID: 20696526

[3] Yekkirala, A.S.; Roberson, D.P.; Bean, B.P.; Woolf, C.J. Breaking barriers to novel analgesic drug development. *Nat. Rev. Drug Discov.,* **2017**, *16*(8), 545-564. http://dx.doi.org/10.1038/nrd.2017.87 PMID: 28596533

[4] Piel, M.J.; Kroin, J.S.; van Wijnen, A.J.; Kc, R.; Im, H.J. Pain assessment in animal models of osteoarthritis. *Gene,* **2014**, *537*(2), 184-188. http://dx.doi.org/10.1016/j.gene.2013.11.091 PMID: 24333346

[5] Blackburn-Munro, G. Pain-like behaviours in animals-how human are they? *Trends Pharmacol. Sci.,* **2004**, *25*(6), 299-305. http://dx.doi.org/10.1016/j.tips.2004.04.008 PMID: 15165744

[6] Tracey, I.; Mantyh, P.W. The cerebral signature for pain perception and its modulation. *Neuron,* **2007**, *55*(3), 377-391. http://dx.doi.org/10.1016/j.neuron.2007.07.012 PMID: 17678852

[7] Gregory, N.S.; Harris, A.L.; Robinson, C.R.; Dougherty, P.M.; Fuchs, P.N.; Sluka, K.A. An overview of animal models of pain: disease models and outcome measures. *J. Pain,* **2013**, *14*(11), 1255-1269. http://dx.doi.org/10.1016/j.jpain.2013.06.008 PMID: 24035349

[8] Bobinski, F.; Teixeira, J.M.; Sluka, K.A.; Santos, A.R.S. Interleukin-4 mediates the analgesia produced by low-intensity exercise in mice with neuropathic pain. *Pain,* **2018**, *159*(3), 437-450. http://dx.doi.org/10.1097/j.pain.0000000000001109 PMID: 29140923

[9] Martins, D.F.; Martins, T.C.; Batisti, A.P.; Dos Santos Leonel, L.; Bobinski, F.; Belmonte, L.A.O.; Mazzardo-Martins, L.; Cargnin-Ferreira, E.; Santos, A.R.S. Long-Term Regular Eccentric Exercise Decreases Neuropathic Pain-like Behavior and Improves Motor Functional Recovery in an Axonotmesis Mouse Model: the Role of Insulin-like Growth Factor-1. *Mol. Neurobiol.,* **2018**, *55*(7), 6155-6168. http://dx.doi.org/10.1007/s12035-017-0829-3 PMID: 29250715

[10] Basbaum, A.I.; Bautista, D.M.; Scherrer, G.; Julius, D. Cellular and molecular mechanisms of pain. *Cell,* **2009**, *139*(2), 267-284. http://dx.doi.org/10.1016/j.cell.2009.09.028 PMID: 19837031

[11] Coderre, T.J.; Melzack, R. The contribution of excitatory amino acids to central sensitization and persistent nociception after formalin-induced tissue injury. *J. Neurosci.,* **1992**, *12*(9), 3665-3670.

http://dx.doi.org/10.1523/JNEUROSCI.12-09-03665.1992 PMID: 1326610

[12] Woolf, C.J. What is this thing called pain? *J. Clin. Invest.,* **2010**, *120*(11), 3742-3744. http://dx.doi.org/10.1172/JCI45178 PMID: 21041955

[13] Malafoglia, V.; Bryant, B.; Raffaeli, W.; Giordano, A.; Bellipanni, G. The zebrafish as a model for nociception studies. *J. Cell. Physiol.,* **2013**, *228*(10), 1956-1966. http://dx.doi.org/10.1002/jcp.24379 PMID: 23559073

[14] Meotti, F.C.; Coelho, I dos S.; Santos, A.R. The nociception induced by glutamate in mice is potentiated by protons released into the solution. *J. Pain,* **2010**, *11*(6), 570-578. http://dx.doi.org/10.1016/j.jpain.2009.09.012 PMID: 20338819

[15] Stevenson, G.W.; Bilsky, E.J.; Negus, S.S. Targeting pain-suppressed behaviors in preclinical assays of pain and analgesia: effects of morphine on acetic acid-suppressed feeding in C57BL/6J mice. *J. Pain,* **2006**, *7*(6), 408-416. http://dx.doi.org/10.1016/j.jpain.2006.01.447 PMID: 16750797

[16] Sneddon, L.U.; Braithwaite, V.A.; Gentle, M.J. Novel object test: examining nociception and fear in the rainbow trout. *J. Pain,* **2003**, *4*(8), 431-440. http://dx.doi.org/10.1067/S1526-5900(03)00717-X PMID: 14622663

[17] Du, X.; Yuan, B.; Wang, J.; Zhang, X.; Tian, L.; Zhang, T.; Li, X.; Zhang, F. [Effect of heat-reinforcing needling on serum metabolite profiles in rheumatoid arthritis rabbits with cold syndrome]. *Zhongguo Zhenjiu,* **2017**, *37*(9), 977-983. PMID: 29354920

[18] MacRae, C.A.; Peterson, R.T. Zebrafish as tools for drug discovery. *Nat. Rev. Drug Discov.,* **2015**, *14*(10), 721-731. http://dx.doi.org/10.1038/nrd4627 PMID: 26361349

[19] Stewart, A.M.; Braubach, O.; Spitsbergen, J.; Gerlai, R.; Kalueff, A.V. Zebrafish models for translational neuroscience research: from tank to bedside. *Trends Neurosci.,* **2014**, *37*(5), 264-278. http://dx.doi.org/10.1016/j.tins.2014.02.011 PMID: 24726051

[20] Fontana, B. D.; Mezzomo, N. J.; Kalueff, A. V.; Rosemberg, D. B. The developing utility of zebrafish models of neurological and neuropsychiatric disorders: A critical review. *Exp Neurol,* **2018**, *299*(Pt A), 157-171. http://dx.doi.org/10.1016/j.expneurol.2017.10.004

[21] Kalueff, A.V.; Stewart, A.M.; Gerlai, R. Zebrafish as an emerging model for studying complex brain disorders. *Trends Pharmacol. Sci.,* **2014**, *35*(2), 63-75. http://dx.doi.org/10.1016/j.tips.2013.12.002 PMID: 24412421

[22] Hoo, J.Y.; Kumari, Y.; Shaikh, M.F.; Hue, S.M.; Goh, B.H. Zebrafish: A Versatile Animal Model for Fertility Research. *BioMed Res. Int.,* **2016**, *2016*, 9732780. http://dx.doi.org/10.1155/2016/9732780 PMID: 27556045

[23] Avdesh, A.; Chen, M.; Martin-Iverson, M.T.; Mondal, A.; Ong, D.; Rainey-Smith, S.; Taddei, K.; Lardelli, M.; Groth, D.M.; Verdile, G.; Martins, R.N. Regular care and maintenance of a zebrafish (*Danio rerio*) laboratory: an introduction. *J. Vis. Exp.,* **2012**, (69), e4196. http://dx.doi.org/10.3791/4196 PMID: 23183629

[24] Stewart, A.M.; Gerlai, R.; Kalueff, A.V. Developing highERthroughput zebrafish screens for in-vivo CNS drug discovery. *Front. Behav. Neurosci.,* **2015**, *9*, 14. http://dx.doi.org/10.3389/fnbeh.2015.00014 PMID: 25729356

[25] Kithcart, A.; MacRae, C.A. Using Zebrafish for High-Throughput Screening of Novel Cardiovascular Drugs. *JACC Basic Transl. Sci.,* **2017**, *2*(1), 1-12. http://dx.doi.org/10.1016/j.jacbts.2017.01.004 PMID: 30167552

[26] Chen, X.; Gays, D.; Santoro, M.M. Transgenic Zebrafish. *Methods Mol. Biol.,* **2016**, *1464*, 107-114. http://dx.doi.org/10.1007/978-1-4939-3999-2_10 PMID: 27858360

[27] Kawakami, K.; Asakawa, K.; Hibi, M.; Itoh, M.; Muto, A.; Wada, H. Gal4 Driver Transgenic Zebrafish: Powerful Tools to Study Developmental Biology, Organogenesis, and Neuroscience. *Adv. Genet.,* **2016**, *95*, 65-87. http://dx.doi.org/10.1016/bs.adgen.2016.04.002 PMID: 27503354

[28] Rougeot, J.; Torraca, V.; Zakrzewska, A.; Kanwal, Z.; Jansen, H.J.; Sommer, F.; Spaink, H.P.; Meijer, A.H. RNAseq Profiling of Leukocyte Populations in Zebrafish Larvae Reveals a *cxcl11* Chemokine Gene as a Marker of Macrophage Polarization During Mycobacterial Infection. *Front. Immunol.,* **2019**, *10*, 832. http://dx.doi.org/10.3389/fimmu.2019.00832 PMID: 31110502

[29] Gu, G.E.; Park, C.S.; Cho, H.J.; Ha, T.H.; Bae, J.; Kwon, O.S.; Lee, J.S.; Lee, C.S. Fluorescent polydopamine nanoparticles as a probe for zebrafish sensory hair cells targeted *in vivo* imaging. *Sci. Rep.,* **2018**, *8*(1), 4393. http://dx.doi.org/10.1038/s41598-018-22828-2 PMID: 29535354

[30] Liu, C.X.; Li, C.Y.; Hu, C.C.; Wang, Y.; Lin, J.; Jiang, Y.H.; Li, Q.; Xu, X. CRISPR/Cas9-induced *shank3b* mutant zebrafish display autism-like behaviors. *Mol. Autism,* **2018**, *9*, 23. http://dx.doi.org/10.1186/s13229-018-0204-x PMID: 29619162

[31] Gonzalez-Nunez, V.; Rodríguez, R.E. The zebrafish: a model to study the endogenous mechanisms of pain. *ILAR J.,* **2009**, *50*(4), 373-386. http://dx.doi.org/10.1093/ilar.50.4.373 PMID: 19949253

[32] Magalhães, F.E.A.; de Sousa, C.Á.P.B.; Santos, S.A.A.R.; Menezes, R.B.; Batista, F.L.A.; Abreu, A.O.; de Oliveira, M.V.; Moura, L.F.W.G.; Raposo, R.D.S.; Campos, A.R. Adult Zebrafish (*Danio rerio*): An Alternative Behavioral Model of Formalin-Induced Nociception. *Zebrafish,* **2017**, *14*(5), 422-429. http://dx.doi.org/10.1089/zeb.2017.1436 PMID: 28704145

[33] Taylor, J.C.; Dewberry, L.S.; Totsch, S.K.; Yessick, L.R.; DeBerry, J.J.; Watts, S.A.; Sorge, R.E. A novel zebrafish-based model of nociception. *Physiol. Behav.,* **2017**, *174*, 83-88. http://dx.doi.org/10.1016/j.physbeh.2017.03.009 PMID: 28288793

[34] Costa, F.V.; Rosa, L.V.; Quadros, V.A.; Santos, A.R.S.; Kalueff, A.V.; Rosemberg, D.B. Understanding nociception-related phenotypes in adult zebrafish: Behavioral and pharmacological characterization using a new acetic acid model. *Behav. Brain Res.,* **2019**, *359*, 570-578. http://dx.doi.org/10.1016/j.bbr.2018.10.009 PMID: 30296529

[35] Thomson, J.S.; Al-Temeemy, A.A.; Isted, H.; Spencer, J.W.; Sneddon, L.U. Assessment of behaviour in groups of zebrafish (*Danio rerio*) using an intelligent software monitoring tool, the chromatic fish analyser. *J. Neurosci. Methods,* **2019**, *328*, 108433. http://dx.doi.org/10.1016/j.jneumeth.2019.108433 PMID: 31520651

[36] Gau, P.; Poon, J.; Ufret-Vincenty, C.; Snelson, C.D.; Gordon, S.E.; Raible, D.W.; Dhaka, A. The zebrafish ortholog of TRPV1 is required for heat-induced locomotion. *J. Neurosci.,* **2013**, *33*(12), 5249-5260. http://dx.doi.org/10.1523/JNEUROSCI.5403-12.2013 PMID: 23516290

[37] Prober, D.A.; Zimmerman, S.; Myers, B.R.; McDermott, B.M., Jr; Kim, S.H.; Caron, S.; Rihel, J.; Solnica-Krezel, L.; Julius, D.; Hudspeth, A.J.; Schier, A.F. Zebrafish TRPA1 channels are required for chemosensation but not for thermosensation or mechanosensory hair cell function. *J. Neurosci.,* **2008**, *28*(40), 10102-10110. http://dx.doi.org/10.1523/JNEUROSCI.2740-08.2008 PMID: 18829968

[38] Dooley, K.; Zon, L.I. Zebrafish: a model system for the study of human disease. *Curr. Opin. Genet. Dev.,* **2000**, *10*(3), 252-256. http://dx.doi.org/10.1016/S0959-437X(00)00074-5 PMID: 10826982

[39] Rose, J.D. The Neurobehavioral Nature of Fishes and the Question of Awareness and Pain. *Fish. Sci.,* **2002**, *10*(1), 1-38.

[40] Braithwaite, V.A.; Boulcott, P. Pain perception, aversion and fear in fish. *Dis. Aquat. Organ.,* **2007**, *75*(2), 131-138. http://dx.doi.org/10.3354/dao075131 PMID: 17578252

[41] Nordgreen, J.; Horsberg, T.E.; Ranheim, B.; Chen, A.C. Somatosensory evoked potentials in the telencephalon of Atlantic salmon (Salmo salar) following galvanic stimulation of the tail. *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.,* **2007**, *193*(12), 1235-1242. http://dx.doi.org/10.1007/s00359-007-0283-1 PMID: 17987296

[42] Reilly, S.C.; Quinn, J.P.; Cossins, A.R.; Sneddon, L.U. Behavioural analysis of a nociceptive event in fish: Comparisons between three species demonstrate specific responses. *Appl. Anim. Behav. Sci.,* **2008**, *114*, 248-259. http://dx.doi.org/10.1016/j.applanim.2008.01.016

[43] Sanchez-Simon, F.M.; Rodriguez, R.E. Developmental expression and distribution of opioid receptors in zebrafish. *Neuroscience,* **2008**, *151*(1), 129-137. http://dx.doi.org/10.1016/j.neuroscience.2007.09.086 PMID: 18082336

[44] Alvarez, F.A.; Rodriguez-Martin, I.; Gonzalez-Nuñez, V.; Marrón Fernández de Velasco, E.; Gonzalez Sarmiento, R.; Rodríguez, R.E. New kappa opioid receptor from zebrafish *Danio rerio*. *Neurosci. Lett.,* **2006**, *405*(1-2), 94-99. http://dx.doi.org/10.1016/j.neulet.2006.06.028 PMID: 16842913

[45] Barrallo, A.; González-Sarmiento, R.; Alvar, F.; Rodríguez, R.E. ZFOR2, a new opioid receptor-like gene from the teleost zebrafish (*Danio rerio*). *Brain Res. Mol. Brain Res.,* **2000**, *84*(1-2), 1-6. http://dx.doi.org/10.1016/S0169-328X(00)00152-2 PMID: 11113526

[46] Dymowska, A.K.; Boyle, D.; Schultz, A.G.; Goss, G.G. The role of acid-sensing ion channels in epithelial Na+ uptake in adult zebrafish (*Danio rerio*). *J. Exp. Biol.,* **2015**, *218*(Pt 8), 1244-1251. http://dx.doi.org/10.1242/jeb.113118 PMID: 25722005

[47] Viña, E.; Parisi, V.; Abbate, F.; Cabo, R.; Guerrera, M.C.; Laurà, R.; Quirós, L.M.; Pérez-Varela, J.C.; Cobo, T.; Germanà, A.; Vega, J.A.; García-Suárez, O. Acid-sensing ion channel 2 (ASIC2) is selectively localized in the cilia of the non-sensory olfactory epithelium of adult zebrafish. *Histochem. Cell Biol.,* **2015**, *143*(1), 59-68. http://dx.doi.org/10.1007/s00418-014-1264-4 PMID: 25161120

[48] van der Vaart, M.; Spaink, H.P.; Meijer, A.H. Pathogen recognition and activation of the innate immune response in zebrafish. *Adv. Hematol.,* **2012**, *2012*, 159807. http://dx.doi.org/10.1155/2012/159807 PMID: 22811714

[49] Watzke, J.; Schirmer, K.; Scholz, S. Bacterial lipopolysaccharides induce genes involved in the innate immune response in embryos of the zebrafish (*Danio rerio*). *Fish Shellfish Immunol.,* **2007**, *23*(4), 901-905. http://dx.doi.org/10.1016/j.fsi.2007.03.004 PMID: 17442590

[50] Levanti, M.; Randazzo, B.; Viña, E.; Montalbano, G.; Garcia-Suarez, O.; Germanà, A.; Vega, J.A.; Abbate, F. Acid-sensing ion channels and transient-receptor potential ion channels in zebrafish taste buds. *Ann. Anat.,* **2016**, *207*, 32-37. http://dx.doi.org/10.1016/j.aanat.2016.06.006 PMID: 27513962

[51] Sneddon, L.U.; Braithwaite, V.A.; Gentle, M.J. Do fishes have nociceptors? Evidence for the evolution of a vertebrate sensory system. *Proc. Biol. Sci.,* **2003**, *270*(1520), 1115-1121. http://dx.doi.org/10.1098/rspb.2003.2349 PMID: 12816648

[52] Maximino, C. Modulation of nociceptive-like behavior in zebrafish (*Danio rerio*) by environmental stressors. *Psychol. Neurosci.,* **2011**, *4*(1), 149-155. http://dx.doi.org/10.3922/j.psns.2011.1.017

[53] Caron, S.J.; Prober, D.; Choy, M.; Schier, A.F. *In vivo* birthdating by BAPTISM reveals that trigeminal sensory neuron diversity depends on early neurogenesis. *Development,* **2008**, *135*(19), 3259-3269. http://dx.doi.org/10.1242/dev.023200 PMID: 18755773

[54] Pan, Y.A.; Choy, M.; Prober, D.A.; Schier, A.F. Robo2 determines subtype-specific axonal projections of trigeminal sensory neurons. *Development,* **2012**, *139*(3), 591-600. http://dx.doi.org/10.1242/dev.076588 PMID: 22190641

[55] Marron Fdez de Velasco, E.; Law, P.Y.; Rodríguez, R.E. Mu opioid receptor from the zebrafish exhibits functional characteristics as those of mammalian mu opioid receptor. *Zebrafish,* **2009**, *6*(3), 259-268. http://dx.doi.org/10.1089/zeb.2009.0594 PMID: 19761379

[56] Chu Sin Chung, P.; Kieffer, B.L. Delta opioid receptors in brain function and diseases. *Pharmacol. Ther.,* **2013**, *140*(1), 112-120. http://dx.doi.org/10.1016/j.pharmthera.2013.06.003 PMID: 23764370

[57] Rachinger-Adam, B.; Conzen, P.; Azad, S.C. Pharmacology of peripheral opioid receptors. *Curr. Opin. Anaesthesiol.,* **2011**, *24*(4), 408-413. http://dx.doi.org/10.1097/ACO.0b013e32834873e5 PMID: 21659869

[58] Corbett, A.D.; Henderson, G.; McKnight, A.T.; Paterson, S.J. 75 years of opioid research: the exciting but vain quest for the Holy Grail. *Br. J. Pharmacol.,* **2006**, *147*(Suppl. 1), S153-S162. http://dx.doi.org/10.1038/sj.bjp.0706435 PMID: 16402099

[59] Stevens, C.W. The evolution of vertebrate opioid receptors. *Front. Biosci.,* **2009**, *14*, 1247-1269. http://dx.doi.org/10.2741/3306 PMID: 19273128

[60] Valentino, R.J.; Volkow, N.D. Untangling the complexity of opioid receptor function. *Neuropsychopharmacology,* **2018**, *43*(13), 2514-2520. http://dx.doi.org/10.1038/s41386-018-0225-3 PMID: 30250308

[61] Lutz, P.E.; Kieffer, B.L. Opioid receptors: distinct roles in mood disorders. *Trends Neurosci.,* **2013**, *36*(3), 195-206. http://dx.doi.org/10.1016/j.tins.2012.11.002 PMID: 23219016

[62] Loh, H.H.; Liu, H.C.; Cavalli, A.; Yang, W.; Chen, Y.F.; Wei, L.N. mu Opioid receptor knockout in mice: effects on ligand-induced analgesia and morphine lethality. *Brain Res. Mol. Brain Res.,* **1998**, *54*(2), 321-326. http://dx.doi.org/10.1016/S0169-328X(97)00353-7 PMID: 9555078

[63] Matthes, H.W.; Maldonado, R.; Simonin, F.; Valverde, O.; Slowe, S.; Kitchen, I.; Befort, K.; Dierich, A.; Le Meur, M.; Dollé, P.; Tzavara, E.; Hanoune, J.; Roques, B.P.; Kieffer, B.L. Loss of morphine-induced analgesia, reward effect and withdrawal symptoms in mice lacking the mu-opioid-receptor gene. *Nature,* **1996**, *383*(6603), 819-823. http://dx.doi.org/10.1038/383819a0 PMID: 8893006

[64] Ma, J.; Zhang, Y.; Kalyuzhny, A.E.; Pan, Z.Z. Emergence of functional delta-opioid receptors induced by long-term treatment with morphine. *Mol. Pharmacol.,* **2006**, *69*(4), 1137-1145. http://dx.doi.org/10.1124/mol.105.019109 PMID: 16399848

[65] Zhu, Y.; King, M.A.; Schuller, A.G.; Nitsche, J.F.; Reidl, M.; Elde, R.P.; Unterwald, E.; Pasternak, G.W.; Pintar, J.E. Retention of supraspinal delta-like analgesia and loss of morphine tolerance in delta opioid receptor knockout mice. *Neuron,* **1999**, *24*(1), 243-252. http://dx.doi.org/10.1016/S0896-6273(00)80836-3 PMID: 10677041

[66] Lambert, D.G. The nociceptin/orphanin FQ receptor: a target with broad therapeutic potential. *Nat. Rev. Drug Discov.,* **2008**, *7*(8), 694-710. http://dx.doi.org/10.1038/nrd2572 PMID: 18670432

[67] Sundström, G.; Dreborg, S.; Larhammar, D. Concomitant duplications of opioid peptide and receptor genes before the origin of jawed vertebrates. *PLoS One,* **2010**, *5*(5), e10512. http://dx.doi.org/10.1371/journal.pone.0010512 PMID: 20463905

[68] Dreborg, S.; Sundström, G.; Larsson, T.A.; Larhammar, D. Evolution of vertebrate opioid receptors. *Proc. Natl. Acad. Sci. USA,* **2008**, *105*(40), 15487-15492. http://dx.doi.org/10.1073/pnas.0805590105 PMID: 18832151

[69] Barrallo, A.; González-Sarmiento, R.; Porteros, A.; García-Isidoro, M.; Rodríguez, R.E. Cloning, molecular characterization, and distribution of a gene homologous to delta opioid receptor from zebrafish (*Danio rerio*). *Biochem. Biophys. Res. Commun.,* **1998**, *245*(2), 544-548. http://dx.doi.org/10.1006/bbrc.1998.8496 PMID: 9571192

[70] Pinal-Seoane, N.; Martin, I.R.; Gonzalez-Nuñez, V.; Marron Fernandez de Velasco, E.; Alvarez, F.A.; Sarmiento, R.G.; Rodriguez, R.E. Characterization of a new duplicate delta-opioid receptor from zebrafish. *J. Mol. Endocrinol.,* **2006**, *37*(3), 391-403. http://dx.doi.org/10.1677/jme.1.02136 PMID: 17170080

[71] Rivas-Boyero, A.A.; Herrero-Turrión, M.J.; Gonzalez-Nunez, V.; Sánchez-Simón, F.M.; Barreto-Valer, K.; Rodríguez, R.E. Pharmacological characterization of a nociceptin receptor from zebrafish (*Danio rerio*). *J. Mol. Endocrinol.,* **2011**, *46*(2), 111-123. http://dx.doi.org/10.1530/JME-10-0130 PMID: 21247980

[72] Gonzalez-Nunez, V.; Jimenez González, A.; Barreto-Valer, K.; Rodríguez, R.E. *In vivo* regulation of the μ opioid receptor: role of the endogenous opioid agents. *Mol. Med.,* **2013**, *19*, 7-17. http://dx.doi.org/10.2119/molmed.2012.00318 PMID: 23348513

[73] Gonzalez-Nunez, V.; Gonzalez-Sarmiento, R.; Rodríguez, R.E. Cloning and characterization of a full-length pronociceptin in zebrafish: evidence of the existence of two different nociceptin sequences in the same precursor. *Biochim. Biophys. Acta,* **2003**, *1629*(1-3), 114-118. http://dx.doi.org/10.1016/j.bbaexp.2003.08.001 PMID: 14522087

[74] Gonzalez-Nunez, V.; Gonzalez-Sarmiento, R.; Rodríguez, R.E. Identification of two proopiomelanocortin genes in zebrafish (*Danio rerio*). *Brain Res. Mol. Brain Res.,* **2003**, *120*(1), 1-8. http://dx.doi.org/10.1016/j.molbrainres.2003.09.012 PMID: 14667571

[75] Wagle, M.; Mathur, P.; Guo, S. Corticotropin-releasing factor critical for zebrafish camouflage behavior is regulated by light and sensitive to ethanol. *J. Neurosci.,* **2011**, *31*(1), 214-224.

http://dx.doi.org/10.1523/JNEUROSCI.3339-10.2011 PMID: 21209207

[76] Demin, K.A.; Taranov, A.S.; Ilyin, N.P.; Lakstygal, A.M.; Volgin, A.D.; de Abreu, M.S.; Strekalova, T.; Kalueff, A.V. Understanding neurobehavioral effects of acute and chronic stress in zebrafish. *Stress,* **2021**, *24*(1), 1-18. http://dx.doi.org/10.1080/10253890.2020.1724948 PMID: 32036720

[77] Sheets, L.; Ransom, D.G.; Mellgren, E.M.; Johnson, S.L.; Schnapp, B.J. Zebrafish melanophilin facilitates melanosome dispersion by regulating dynein. *Curr. Biol.,* **2007**, *17*(20), 1721-1734. http://dx.doi.org/10.1016/j.cub.2007.09.028 PMID: 17919909

[78] Gonzalez-Nuñez, V.; Marrón Fernández de Velasco, E.; Arsequell, G.; Valencia, G.; Rodríguez, R.E. Identification of dynorphin a from zebrafish: a comparative study with mammalian dynorphin A. *Neuroscience,* **2007**, *144*(2), 675-684. http://dx.doi.org/10.1016/j.neuroscience.2006.09.028 PMID: 17069980

[79] Bao, W.; Volgin, A.D.; Alpyshov, E.T.; Friend, A.J.; Strekalova, T.V.; de Abreu, M.S.; Collins, C.; Amstislavskaya, T.G.; Demin, K.A.; Kalueff, A.V. Opioid neurobiology, neurogenetics and neuropharmacology in zebrafish. *Neuroscience,* **2019**, *404*, 218-232. http://dx.doi.org/10.1016/j.neuroscience.2019.01.045 PMID: 30710667

[80] Demin, K.A.; Meshalkina, D.A.; Kysil, E.V.; Antonova, K.A.; Volgin, A.D.; Yakovlev, O.A.; Alekseeva, P.A.; Firuleva, M.M.; Lakstygal, A.M.; de Abreu, M.S.; Barcellos, L.J.G.; Bao, W.; Friend, A.J.; Amstislavskaya, T.G.; Rosemberg, D.B.; Musienko, P.E.; Song, C.; Kalueff, A.V. Zebrafish models relevant to studying central opioid and endocannabinoid systems. *Prog. Neuropsychopharmacol. Biol. Psychiatry,* **2018**, *86*, 301-312. http://dx.doi.org/10.1016/j.pnpbp.2018.03.024 PMID: 29604314

[81] González-Núñez, V.; Barrallo, A.; Traynor, J.R.; Rodríguez, R.E. Characterization of opioid-binding sites in zebrafish brain. *J. Pharmacol. Exp. Ther.,* **2006**, *316*(2), 900-904. http://dx.doi.org/10.1124/jpet.105.093492 PMID: 16207834

[82] Chan, H.C.S.; McCarthy, D.; Li, J.; Palczewski, K.; Yuan, S. Designing Safer Analgesics *via* μ-Opioid Receptor Pathways. *Trends Pharmacol. Sci.,* **2017**, *38*(11), 1016-1037. http://dx.doi.org/10.1016/j.tips.2017.08.004 PMID: 28935293

[83] Sivalingam, M.; Ogawa, S.; Parhar, I.S. Mapping of Morphine-Induced *OPRM1* Gene Expression Pattern in the Adult Zebrafish Brain. *Front. Neuroanat.,* **2020**, *14*, 5. http://dx.doi.org/10.3389/fnana.2020.00005 PMID: 32153369

[84] Belzung, C.; Agmo, A. Naloxone potentiates the effects of subeffective doses of anxiolytic agents in mice. *Eur. J. Pharmacol.,* **1997**, *323*(2-3), 133-136. http://dx.doi.org/10.1016/S0014-2999(97)00142-8 PMID: 9128831

[85] Kalin, N.H.; Shelton, S.E.; Barksdale, C.M. Opiate modulation of separation-induced distress in non-human primates. *Brain Res.,* **1988**, *440*(2), 285-292. http://dx.doi.org/10.1016/0006-8993(88)90997-3 PMID: 3359215

[86] Stewart, A.; Wu, N.; Cachat, J.; Hart, P.; Gaikwad, S.; Wong, K.; Utterback, E.; Gilder, T.; Kyzar, E.; Newman, A.; Carlos, D.; Chang, K.; Hook, M.; Rhymes, C.; Caffery, M.; Greenberg, M.; Zadina, J.; Kalueff, A.V. Pharmacological modulation of anxiety-like phenotypes in adult zebrafish behavioral models. *Prog. Neuropsychopharmacol. Biol. Psychiatry,* **2011**, *35*(6), 1421-1431. http://dx.doi.org/10.1016/j.pnpbp.2010.11.035 PMID: 21122812

[87] Deakin, A.G.; Buckley, J.; AlZu'bi, H.S.; Cossins, A.R.; Spencer, J.W.; Al'Nuaimy, W.; Young, I.S.; Thomson, J.S.; Sneddon, L.U. Automated monitoring of behaviour in zebrafish after invasive procedures. *Sci. Rep.,* **2019**, *9*(1), 9042. http://dx.doi.org/10.1038/s41598-019-45464-w PMID: 31227751

[88] Magalhães, F.E.A.; Batista, F.L.A.; Lima, L.M.G.; Abrante, I.A.; Batista, F.L.A.; Abrante, I.A.; de Araújo, J.I.F.; Santos, S.A.A.R.; de Oliveira, B.A.; Raposo, R.D.S.; Campos, A.R. Adult Zebrafish (*Danio rerio*) As a Model for the Study of Corneal Antinociceptive Compounds. *Zebrafish,* **2018**, *15*(6), 566-574. http://dx.doi.org/10.1089/zeb.2018.1633 PMID: 30153094

[89] Lopez-Luna, J.; Al-Jubouri, Q.; Al-Nuaimy, W.; Sneddon, L.U. Reduction in activity by noxious chemical stimulation is ameliorated by immersion in analgesic drugs in zebrafish. *J. Exp. Biol.,* **2017**, *220*(Pt 8), 1451-1458. http://dx.doi.org/10.1242/jeb.146969 PMID: 28424313

[90] Khor, B.S.; Jamil, M.F.; Adenan, M.I.; Shu-Chien, A.C. Mitragynine attenuates withdrawal syndrome in morphine-withdrawn zebrafish. *PLoS One,* **2011**, *6*(12), e28340. http://dx.doi.org/10.1371/journal.pone.0028340 PMID: 22205946

[91] Cachat, J.; Canavello, P.; Elegante, M.; Bartels, B.; Hart, P.; Bergner, C.; Egan, R.; Duncan, A.; Tien, D.; Chung, A.; Wong, K.; Goodspeed, J.; Tan, J.; Grimes, C.; Elkhayat, S.; Suciu, C.; Rosenberg, M.; Chung, K.M.; Kadri, F.; Roy, S.; Gaikwad, S.; Stewart, A.; Zapolsky, I.; Gilder, T.; Mohnot, S.; Beeson, E.; Amri, H.; Zukowska, Z.; Soignier, R.D.; Kalueff, A.V. Modeling withdrawal syndrome in zebrafish. *Behav. Brain Res.,* **2010**, *208*(2), 371-376. http://dx.doi.org/10.1016/j.bbr.2009.12.004 PMID: 20006651

[92] Shi, Y.; Zhang, Y.; Zhao, F.; Ruan, H.; Huang, H.; Luo, L.; Li, L. Acetylcholine serves as a derepressor in Loperamide-induced Opioid-Induced Bowel Dysfunction (OIBD) in zebrafish. *Sci. Rep.,* **2014**, *4*, 5602. http://dx.doi.org/10.1038/srep05602 PMID: 24998697

[93] Bretaud, S.; Li, Q.; Lockwood, B.L.; Kobayashi, K.; Lin, E.; Guo, S. A choice behavior for morphine reveals experience-dependent drug preference and underlying neural substrates in developing larval zebrafish. *Neuroscience,* **2007**, *146*(3), 1109-1116. http://dx.doi.org/10.1016/j.neuroscience.2006.12.073 PMID: 17428610

[94] Bhargava, H.N.; Thorat, S.N. Effect of dizocilpine (MK-801) on analgesia and tolerance induced by U-50,488H, a kappa-opioid receptor agonist, in the mouse. *Brain Res.,* **1994**, *649*(1-2), 111-116. http://dx.doi.org/10.1016/0006-8993(94)91053-7 PMID: 7953621

[95] Yamada, H.; Shimoyama, N.; Sora, I.; Uhl, G.R.; Fukuda, Y.; Moriya, H.; Shimoyama, M. Morphine can produce analgesia *via* spinal kappa opioid receptors in the absence of mu opioid receptors. *Brain Res.,* **2006**, *1083*(1), 61-69. http://dx.doi.org/10.1016/j.brainres.2006.01.095 PMID: 16530171

[96] McDonald, J.; Lambert, D. Opioid receptors. *Contin. Educ. Anaesth. Crit. Care Pain,* **2005**, *5*(1), 22-25. http://dx.doi.org/10.1093/bjaceaccp/mki004

[97] Liu-Chen, L.Y. Agonist-induced regulation and trafficking of kappa opioid receptors. *Life Sci.,* **2004**, *75*(5), 511-536. http://dx.doi.org/10.1016/j.lfs.2003.10.041 PMID: 15158363

[98] Braida, D.; Limonta, V.; Pegorini, S.; Zani, A.; Guerini-Rocco, C.; Gori, E.; Sala, M. Hallucinatory and rewarding effect of salvinorin A in zebrafish: kappa-opioid and CB1-cannabinoid receptor involvement. *Psychopharmacology (Berl.),* **2007**, *190*(4), 441-448. http://dx.doi.org/10.1007/s00213-006-0639-1 PMID: 17219220

[99] Gonzalez-Nuñez, V.; Toth, G.; Rodríguez, R.E. Endogenous heptapeptide Met-enkephalin-Gly-Tyr binds differentially to duplicate delta opioid receptors from zebrafish. *Peptides,* **2007**, *28*(12), 2340-2347. http://dx.doi.org/10.1016/j.peptides.2007.10.002 PMID: 18022288

[100] Mansour, A.; Hoversten, M.T.; Taylor, L.P.; Watson, S.J.; Akil, H. The cloned mu, delta and kappa receptors and their endogenous ligands: evidence for two opioid peptide recognition cores. *Brain Res.,* **1995**, *700*(1-2), 89-98. http://dx.doi.org/10.1016/0006-8993(95)00928-J PMID: 8624732

[101] Kieffer, B.L.; Gavériaux-Ruff, C. Exploring the opioid system by gene knockout. *Prog. Neurobiol.,* **2002**, *66*(5), 285-306. http://dx.doi.org/10.1016/S0301-0082(02)00008-4 PMID: 12015197

[102] Gavériaux-Ruff, C.; Kieffer, B.L. Delta opioid receptor analgesia: recent contributions from pharmacology and molecular approaches. *Behav. Pharmacol.,* **2011**, *22*(5-6), 405-414. http://dx.doi.org/10.1097/FBP.0b013e32834a1f2c PMID: 21836459

[103] Goldenberg, D.L. The interface of pain and mood disturbances in the rheumatic diseases. *Semin. Arthritis Rheum.,* **2010**, *40*(1), 15-31. http://dx.doi.org/10.1016/j.semarthrit.2008.11.005 PMID: 19217649

[104] Sharif, N.A.; Hughes, J. Discrete mapping of brain Mu and delta opioid receptors using selective peptides: quantitative autoradiography, species differences and comparison with kappa receptors. *Peptides,* **1989**, *10*(3), 499-522. http://dx.doi.org/10.1016/0196-9781(89)90135-6 PMID: 2550910

[105] Porteros, A.; García-Isidoro, M.; Barrallo, A.; González-Sarmiento, R.; Rodríguez, R.E. Expression of ZFOR1, a delta-opioid receptor, in the central nervous system of the zebrafish (Danio rerio). *J. Comp. Neurol.,* **1999**, *412*(3), 429-438.

http://dx.doi.org/10.1002/(SICI)1096-9861(19990927)412:3<429::AID-CNE4>3.0.CO;2-L PMID: 10441231

[106] Valiquette, M.; Vu, H.K.; Yue, S.Y.; Wahlestedt, C.; Walker, P. Involvement of Trp-284, Val-296, and Val-297 of the human delta-opioid receptor in binding of delta-selective ligands. *J. Biol. Chem.,* **1996**, *271*(31), 18789-18796. http://dx.doi.org/10.1074/jbc.271.31.18789 PMID: 8702536

[107] Meng, F.; Ueda, Y.; Hoversten, M.T.; Thompson, R.C.; Taylor, L.; Watson, S.J.; Akil, H. Mapping the receptor domains critical for the binding selectivity of delta-opioid receptor ligands. *Eur. J. Pharmacol.,* **1996**, *311*(2-3), 285-292. http://dx.doi.org/10.1016/0014-2999(96)00431-1 PMID: 8891611

[108] Rodriguez, R.E.; Barrallo, A.; Garcia-Malvar, F.; McFadyen, I.J.; Gonzalez-Sarmiento, R.; Traynor, J.R. Characterization of ZFOR1, a putative delta-opioid receptor from the teleost zebrafish (Danio rerio). *Neurosci. Lett.,* **2000**, *288*(3), 207-210. http://dx.doi.org/10.1016/S0304-3940(00)01239-8 PMID: 10889344

[109] Mollereau, C.; Mouledous, L. Tissue distribution of the opioid receptor-like (ORL1) receptor. *Peptides,* **2000**, *21*(7), 907-917. http://dx.doi.org/10.1016/S0196-9781(00)00227-8 PMID: 10998524

[110] King, M.A.; Rossi, G.C.; Chang, A.H.; Williams, L.; Pasternak, G.W. Spinal analgesic activity of orphanin FQ/nociceptin and its fragments. *Neurosci. Lett.,* **1997**, *223*(2), 113-116. http://dx.doi.org/10.1016/S0304-3940(97)13414-0 PMID: 9089686

[111] Ko, M.C.; Wei, H.; Woods, J.H.; Kennedy, R.T. Effects of intrathecally administered nociceptin/orphanin FQ in monkeys: behavioral and mass spectrometric studies. *J. Pharmacol. Exp. Ther.,* **2006**, *318*(3), 1257-1264. http://dx.doi.org/10.1124/jpet.106.106120 PMID: 16766718

[112] Zeilhofer, H.U.; Calò, G. Nociceptin/orphanin FQ and its receptor--potential targets for pain therapy? *J. Pharmacol. Exp. Ther.,* **2003**, *306*(2), 423-429. http://dx.doi.org/10.1124/jpet.102.046979 PMID: 12721334

[113] Pan, Z.; Hirakawa, N.; Fields, H.L. A cellular mechanism for the bidirectional pain-modulating actions of orphanin FQ/nociceptin. *Neuron,* **2000**, *26*(2), 515-522. http://dx.doi.org/10.1016/S0896-6273(00)81183-6 PMID: 10839369

[114] Julius, D. TRP channels and pain. *Annu. Rev. Cell Dev. Biol.,* **2013**, *29*, 355-384. http://dx.doi.org/10.1146/annurev-cellbio-101011-155833 PMID: 24099085

[115] Clapham, D.E. TRP channels as cellular sensors. *Nature,* **2003**, *426*(6966), 517-524. http://dx.doi.org/10.1038/nature02196 PMID: 14654832

[116] Montell, C.; Birnbaumer, L.; Flockerzi, V. The TRP channels, a remarkably functional family. *Cell,* **2002**, *108*(5), 595-598. http://dx.doi.org/10.1016/S0092-8674(02)00670-0 PMID: 11893331

[117] Tsagareli, M.G.; Nozadze, I. An overview on transient receptor potential channels superfamily. *Behav. Pharmacol.,* **2020**, *31*(5), 413-434. http://dx.doi.org/10.1097/FBP.0000000000000524 PMID: 31833970

[118] Von Niederhäusern, V.; Kastenhuber, E.; Stäuble, A.; Gesemann, M.; Neuhauss, S.C. Phylogeny and expression of canonical transient receptor potential (TRPC) genes in developing zebrafish. *Dev. Dyn.,* **2013**, *242*(12), 1427-1441. http://dx.doi.org/10.1002/dvdy.24041 PMID: 24038627

[119] Saito, S.; Shingai, R. Evolution of thermoTRP ion channel homologs in vertebrates. *Physiol. Genomics,* **2006**, *27*(3), 219-230. http://dx.doi.org/10.1152/physiolgenomics.00322.2005 PMID: 16926268

[120] Corey, D.P.; García-Añoveros, J.; Holt, J.R.; Kwan, K.Y.; Lin, S.Y.; Vollrath, M.A.; Amalfitano, A.; Cheung, E.L.; Derfler, B.H.; Duggan, A.; Géléoc, G.S.; Gray, P.A.; Hoffman, M.P.; Rehm, H.L.; Tamasauskas, D.; Zhang, D.S. TRPA1 is a candidate for the mechanosensitive transduction channel of vertebrate hair cells. *Nature,* **2004**, *432*(7018), 723-730. http://dx.doi.org/10.1038/nature03066 PMID: 15483558

[121] Kastenhuber, E.; Gesemann, M.; Mickoleit, M.; Neuhauss, S.C. Phylogenetic analysis and expression of zebrafish transient receptor potential melastatin family genes. *Dev. Dyn.,* **2013**, *242*(11), 1236-1249. http://dx.doi.org/10.1002/dvdy.24020 PMID: 23908157

[122] Benini, A.; Bozzato, A.; Mantovanelli, S.; Calvarini, L.; Giacopuzzi, E.; Bresciani, R.; Moleri, S.; Zizioli, D.; Beltrame, M.; Borsani, G. Characterization and expression analysis of mcoln1.1 and mcoln1.2, the putative zebrafish co-orthologs of the gene responsible for human mucolipidosis type IV. *Int. J. Dev. Biol.,* **2013**, *57*(1), 85-93. http://dx.doi.org/10.1387/ijdb.120033gb PMID: 23585356

[123] England, S.J.; Campbell, P.C.; Banerjee, S.; Swanson, A.J.; Lewis, K.E. Identification and Expression Analysis of the Complete Family of Zebrafish *pkd* Genes. *Front. Cell Dev. Biol.,* **2017**, *5*, 5. http://dx.doi.org/10.3389/fcell.2017.00005 PMID: 28271061

[124] Ko, M.J.; Ganzen, L.C.; Coskun, E.; Mukadam, A.A.; Leung, Y.F.; van Rijn, R.M. A critical evaluation of TRPA1-mediated locomotor behavior in zebrafish as a screening tool for novel anti-nociceptive drug discovery. *Sci. Rep.,* **2019**, *9*(1), 2430. http://dx.doi.org/10.1038/s41598-019-38852-9 PMID: 30787340

[125] Laursen, W.J.; Anderson, E.O.; Hoffstaetter, L.J.; Bagriantsev, S.N.; Gracheva, E.O. Species-specific temperature sensitivity of TRPA1. *Temperature (Austin),* **2015**, *2*(2), 214-226. http://dx.doi.org/10.1080/23328940.2014.1000702 PMID: 27227025

[126] Kang, K.; Pulver, S.R.; Panzano, V.C.; Chang, E.C.; Griffith, L.C.; Theobald, D.L.; Garrity, P.A. Analysis of Drosophila TRPA1 reveals an ancient origin for human chemical nociception. *Nature,* **2010**, *464*(7288), 597-600. http://dx.doi.org/10.1038/nature08848 PMID: 20237474

[127] Gupta, R.; Saito, S.; Mori, Y.; Itoh, S.G.; Okumura, H.; Tominaga, M. Structural basis of TRPA1 inhibition by HC-030031 utilizing species-specific differences. *Sci. Rep.,* **2016**, *6*, 37460. http://dx.doi.org/10.1038/srep37460 PMID: 27874100

[128] Oda, M.; Kurogi, M.; Kubo, Y.; Saitoh, O. Sensitivities of Two Zebrafish TRPA1 Paralogs to Chemical and Thermal Stimuli Analyzed in Heterologous Expression Systems. *Chem. Senses,* **2016**, *41*(3), 261-272. http://dx.doi.org/10.1093/chemse/bjv091 PMID: 26826723

[129] Veldhuis, N.A.; Poole, D.P.; Grace, M.; McIntyre, P.; Bunnett, N.W. The G protein-coupled receptor-transient receptor potential channel axis: molecular insights for targeting disorders of sensation and inflammation. *Pharmacol. Rev.,* **2015**, *67*(1), 36-73. http://dx.doi.org/10.1124/pr.114.009555 PMID: 25361914

[130] Caterina, M.J.; Schumacher, M.A.; Tominaga, M.; Rosen, T.A.; Levine, J.D.; Julius, D. The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature,* **1997**, *389*(6653), 816-824. http://dx.doi.org/10.1038/39807 PMID: 9349813

[131] Moriyama, T.; Higashi, T.; Togashi, K.; Iida, T.; Segi, E.; Sugimoto, Y.; Tominaga, T.; Narumiya, S.; Tominaga, M. Sensitization of TRPV1 by EP1 and IP reveals peripheral nociceptive mechanism of prostaglandins. *Mol. Pain,* **2005**, *1*, 3. http://dx.doi.org/10.1186/1744-8069-1-3 PMID: 15813989

[132] Zygmunt, P.M.; Petersson, J.; Andersson, D.A.; Chuang, H.; Sørgård, M.; Di Marzo, V.; Julius, D.; Högestätt, E.D. Vanilloid receptors on sensory nerves mediate the vasodilator action of anandamide. *Nature,* **1999**, *400*(6743), 452-457. http://dx.doi.org/10.1038/22761 PMID: 10440374

[133] Szallasi, A.; Blumberg, P.M. Resiniferatoxin, a phorbol-related diterpene, acts as an ultrapotent analog of capsaicin, the irritant constituent in red pepper. *Neuroscience,* **1989**, *30*(2), 515-520. http://dx.doi.org/10.1016/0306-4522(89)90269-8 PMID: 2747924

[134] Curtright, A.; Rosser, M.; Goh, S.; Keown, B.; Wagner, E.; Sharifi, J.; Raible, D.W.; Dhaka, A. Modeling nociception in zebrafish: a way forward for unbiased analgesic discovery. *PLoS One,* **2015**, *10*(1), e0116766. http://dx.doi.org/10.1371/journal.pone.0116766 PMID: 25587718

[135] Santos, A.L.E.; Leite, G.O.; Carneiro, R.F.; Roma, R.R.; Santos, V.F.; Santos, M.H.C.; Pereira, R.O.; Silva, R.C.; Nagano, C.S.; Sampaio, A.H.; Rocha, B.A.M.; Delatorre, P.; Campos, A.R.; Teixeira, C.S. Purification and biophysical characterization of a mannose/N-acetyl-d-glucosamine-specific lectin from Machaerium acutifolium and its effect on inhibition of orofacial pain *via* TRPV1 receptor. *Arch. Biochem. Biophys.,* **2019**, *664*, 149-156. http://dx.doi.org/10.1016/j.abb.2019.02.009 PMID: 30772259

[136] do Nascimento, J.E.T.; de Morais, S.M.; de Lisboa, D.S.; de Oliveira Sousa, M.; Santos, S.A.A.R.; Magalhães, F.E.A.; Campos, A.R. The orofacial antinociceptive effect of Kaempferol-3-O-rutinoside, isolated from the plant Ouratea fieldingiana, on adult zebrafish (Danio rerio). *Biomed. Pharmacother.,* **2018**, *107*, 1030-1036.
http://dx.doi.org/10.1016/j.biopha.2018.08.089 PMID: 30257314

[137] Soares, I.C.R.; Santos, S.A.A.R.; Coelho, R.F.; Alves, Y.A.; Vieira-Neto, A.E.; Tavares, K.C.S.; Magalhaes, F.E.A.; Campos, A.R. Oleanolic acid promotes orofacial antinociception in adult zebrafish (Danio rerio) through TRPV1 receptors. *Chem. Biol. Interact.,* **2019**, *299*, 37-43.
http://dx.doi.org/10.1016/j.cbi.2018.11.018 PMID: 30496739

[138] Batista, F.L.A.; Lima, L.M.G.; Abrante, I.A.; de Araújo, J.I.F.; Batista, F.L.A.; Abrante, I.A.; Magalhães, E.A.; de Lima, D.R.; Lima, M.D.C.L.; do Prado, B.S.; Moura, L.F.W.G.; Guedes, M.I.F.; Ferreira, M.K.A.; de Menezes, J.E.S.A.; Santos, S.A.A.R.; Mendes, F.R.S.; Moreira, R.A.; Monteiro-Moreira, A.C.O.; Campos, A.R.; Magalhães, F.E.A. Antinociceptive activity of ethanolic extract of Azadirachta indica A. Juss (Neem, Meliaceae) fruit through opioid, glutamatergic and acid-sensitive ion pathways in adult zebrafish (Danio rerio). *Biomed. Pharmacother.,* **2018**, *108*, 408-416.
http://dx.doi.org/10.1016/j.biopha.2018.08.160 PMID: 30236850

[139] Ghysen, A.; Dambly-Chaudière, C. The lateral line microcosmos. *Genes Dev.,* **2007**, *21*(17), 2118-2130.
http://dx.doi.org/10.1101/gad.1568407 PMID: 17785522

[140] Eckroth, J.R.; Aas-Hansen, Ø.; Sneddon, L.U.; Bichão, H.; Døving, K.B. Physiological and behavioural responses to noxious stimuli in the Atlantic cod (Gadus morhua). *PLoS One,* **2014**, *9*(6), e100150.
http://dx.doi.org/10.1371/journal.pone.0100150 PMID: 24936652

[141] Basavarajappa, B.S.; Shivakumar, M.; Joshi, V.; Subbanna, S. Endocannabinoid system in neurodegenerative disorders. *J. Neurochem.,* **2017**, *142*(5), 624-648.
http://dx.doi.org/10.1111/jnc.14098 PMID: 28608560

[142] Luchtenburg, F.J.; Schaaf, M.J.M.; Richardson, M.K. Functional characterization of the cannabinoid receptors 1 and 2 in zebrafish larvae using behavioral analysis. *Psychopharmacology (Berl.),* **2019**, *236*(7), 2049-2058.
http://dx.doi.org/10.1007/s00213-019-05193-4 PMID: 30820632

[143] Lauckner, J.E.; Jensen, J.B.; Chen, H.Y.; Lu, H.C.; Hille, B.; Mackie, K. GPR55 is a cannabinoid receptor that increases intracellular calcium and inhibits M current. *Proc. Natl. Acad. Sci. USA,* **2008**, *105*(7), 2699-2704.
http://dx.doi.org/10.1073/pnas.0711278105 PMID: 18263732

[144] Pertwee, R.G.; Howlett, A.C.; Abood, M.E.; Alexander, S.P.; Di Marzo, V.; Elphick, M.R.; Greasley, P.J.; Hansen, H.S.; Kunos, G.; Mackie, K.; Mechoulam, R.; Ross, R.A. International Union of Basic and Clinical Pharmacology. LXXIX. Cannabinoid receptors and their ligands: beyond CB□ and CB□. *Pharmacol. Rev.,* **2010**, *62*(4), 588-631.
http://dx.doi.org/10.1124/pr.110.003004 PMID: 21079038

[145] Herkenham, M.; Lynn, A.B.; Little, M.D.; Johnson, M.R.; Melvin, L.S.; de Costa, B.R.; Rice, K.C. Cannabinoid receptor localization in brain. *Proc. Natl. Acad. Sci. USA,* **1990**, *87*(5), 1932-1936.
http://dx.doi.org/10.1073/pnas.87.5.1932 PMID: 2308954

[146] Munro, S.; Thomas, K.L.; Abu-Shaar, M. Molecular characterization of a peripheral receptor for cannabinoids. *Nature,* **1993**, *365*(6441), 61-65.
http://dx.doi.org/10.1038/365061a0 PMID: 7689702

[147] Van Sickle, M.D.; Duncan, M.; Kingsley, P.J.; Mouihate, A.; Urbani, P.; Mackie, K.; Stella, N.; Makriyannis, A.; Piomelli, D.; Davison, J.S.; Marnett, L.J.; Di Marzo, V.; Pittman, Q.J.; Patel, K.D.; Sharkey, K.A. Identification and functional characterization of brainstem cannabinoid CB2 receptors. *Science,* **2005**, *310*(5746), 329-332.
http://dx.doi.org/10.1126/science.1115740 PMID: 16224028

[148] Zhang, H.Y.; Gao, M.; Shen, H.; Bi, G.H.; Yang, H.J.; Liu, Q.R.; Wu, J.; Gardner, E.L.; Bonci, A.; Xi, Z.X. Expression of functional cannabinoid CB_2 receptor in VTA dopamine neurons in rats. *Addict. Biol.,* **2017**, *22*(3), 752-765.
http://dx.doi.org/10.1111/adb.12367 PMID: 26833913

[149] Oleson, E.B.; Beckert, M.V.; Morra, J.T.; Lansink, C.S.; Cachope, R.; Abdullah, R.A.; Loriaux, A.L.; Schetters, D.; Pattij, T.; Roitman, M.F.; Lichtman, A.H.; Cheer, J.F. Endocannabinoids shape accumbal encoding of cue-motivated behavior *via* CB1 receptor activation in the ventral tegmentum. *Neuron,* **2012**, *73*(2), 360-373.
http://dx.doi.org/10.1016/j.neuron.2011.11.018 PMID: 22284189

[150] Rey, A.A.; Purrio, M.; Viveros, M.P.; Lutz, B. Biphasic effects of cannabinoids in anxiety responses: CB1 and GABA(B) receptors in the balance of GABAergic and glutamatergic neurotransmission. *Neuropsychopharmacology,* **2012**, *37*(12), 2624-2634.
http://dx.doi.org/10.1038/npp.2012.123 PMID: 22850737

[151] Muccioli, G.G.; Lambert, D.M. Current knowledge on the antagonists and inverse agonists of cannabinoid receptors. *Curr. Med. Chem.,* **2005**, *12*(12), 1361-1394.
http://dx.doi.org/10.2174/0929867054020891 PMID: 15974990

[152] Ibrahim, M.M.; Porreca, F.; Lai, J.; Albrecht, P.J.; Rice, F.L.; Khodorova, A.; Davar, G.; Makriyannis, A.; Vanderah, T.W.; Mata, H.P.; Malan, T.P., Jr CB2 cannabinoid receptor activation produces antinociception by stimulating peripheral release of endogenous opioids. *Proc. Natl. Acad. Sci. USA,* **2005**, *102*(8), 3093-3098.
http://dx.doi.org/10.1073/pnas.0409888102 PMID: 15705714

[153] Wilson, R.I.; Nicoll, R.A. Endocannabinoid signaling in the brain. *Science,* **2002**, *296*(5568), 678-682.
http://dx.doi.org/10.1126/science.1063545 PMID: 11976437

[154] Hohmann, A.G.; Herkenham, M. Localization of central cannabinoid CB1 receptor messenger RNA in neuronal subpopulations of rat dorsal root ganglia: a double-label in situ hybridization study. *Neuroscience,* **1999**, *90*(3), 923-931.
http://dx.doi.org/10.1016/S0306-4522(98)00524-7 PMID: 10218792

[155] Drew, G.M.; Lau, B.K.; Vaughan, C.W. Substance P drives endocannabinoid-mediated disinhibition in a midbrain descending analgesic pathway. *J. Neurosci.,* **2009**, *29*(22), 7220-7229.
http://dx.doi.org/10.1523/JNEUROSCI.4362-08.2009 PMID: 19494144

[156] Starkus, J.; Jansen, C.; Shimoda, L.M.N.; Stokes, A.J.; Small-Howard, A.L.; Turner, H. Diverse TRPV1 responses to cannabinoids. *Channels (Austin),* **2019**, *13*(1), 172-191.
http://dx.doi.org/10.1080/19336950.2019.1619436 PMID: 31096838

[157] Lam, C.S.; Rastegar, S.; Strähle, U. Distribution of cannabinoid receptor 1 in the CNS of zebrafish. *Neuroscience,* **2006**, *138*(1), 83-95.
http://dx.doi.org/10.1016/j.neuroscience.2005.10.069 PMID: 16368195

[158] Rodriguez-Martin, I.; Herrero-Turrion, M.J.; Marron Fdez de Velasco, E.; Gonzalez-Sarmiento, R.; Rodriguez, R.E. Characterization of two duplicate zebrafish Cb2-like cannabinoid receptors. *Gene,* **2007**, *389*(1), 36-44.
http://dx.doi.org/10.1016/j.gene.2006.09.016 PMID: 17126498

[159] Krug, R.G., II; Clark, K.J. Elucidating cannabinoid biology in zebrafish (Danio rerio). *Gene,* **2015**, *570*(2), 168-179.
http://dx.doi.org/10.1016/j.gene.2015.07.036 PMID: 26192460

[160] Migliarini, B.; Carnevali, O. A novel role for the endocannabinoid system during zebrafish development. *Mol. Cell. Endocrinol.,* **2009**, *299*(2), 172-177.
http://dx.doi.org/10.1016/j.mce.2008.11.014 PMID: 19071191

[161] Song, Z.H.; Slowey, C.A.; Hurst, D.P.; Reggio, P.H. The difference between the CB(1) and CB(2) cannabinoid receptors at position 5.46 is crucial for the selectivity of WIN55212-2 for CB(2). *Mol. Pharmacol.,* **1999**, *56*(4), 834-840.
PMID: 10496968

[162] Connors, K.A.; Valenti, T.W.; Lawless, K.; Sackerman, J.; Onaivi, E.S.; Brooks, B.W.; Gould, G.G. Similar anxiolytic effects of agonists targeting serotonin 5-HT1A or cannabinoid CB receptors on zebrafish behavior in novel environments. *Aquat. Toxicol.,* **2014**, *151*, 105-113.
http://dx.doi.org/10.1016/j.aquatox.2013.12.005 PMID: 24411165

[163] Ellis, L.D.; Berrue, F.; Morash, M.; Achenbach, J.C.; Hill, J.; McDougall, J.J. Comparison of cannabinoids with known analgesics using a novel high throughput zebrafish larval model of nociception. *Behav. Brain Res.,* **2018**, *337*, 151-159.
http://dx.doi.org/10.1016/j.bbr.2017.09.028 PMID: 28935439

[164] Walker, J.M.; Hohmann, A.G. Cannabinoid mechanisms of pain suppression. *Handb. Exp. Pharmacol.,* **2005**, (168), 509-554.
http://dx.doi.org/10.1007/3-540-26573-2_17 PMID: 16596786

[165] Crombie, K.M.; Brellenthin, A.G.; Hillard, C.J.; Koltyn, K.F. Endocannabinoid and Opioid System Interactions in Exercise-Induced Hypoalgesia. *Pain Med.,* **2018**, *19*(1), 118-123. http://dx.doi.org/10.1093/pm/pnx058 PMID: 28387833

[166] Slivicki, R.A.; Iyer, V.; Mali, S.S.; Garai, S.; Thakur, G.A.; Crystal, J.D.; Hohmann, A.G. Positive Allosteric Modulation of CB_1 Cannabinoid Receptor Signaling Enhances Morphine Antinociception and Attenuates Morphine Tolerance Without Enhancing Morphine- Induced Dependence or Reward. *Front. Mol. Neurosci.,* **2020**, *13*, 54. http://dx.doi.org/10.3389/fnmol.2020.00054 PMID: 32410959

[167] Smith, F.L.; Cichewicz, D.; Martin, Z.L.; Welch, S.P. The enhancement of morphine antinociception in mice by delta9-tetrahydrocannabinol. *Pharmacol. Biochem. Behav.,* **1998**, *60*(2), 559-566. http://dx.doi.org/10.1016/S0091-3057(98)00012-4 PMID: 9632241

[168] Lee, C.H.; Chen, C.C. Roles of ASICs in Nociception and Proprioception. *Adv. Exp. Med. Biol.,* **2018**, *1099*, 37-47. http://dx.doi.org/10.1007/978-981-13-1756-9_4 PMID: 30306513

[169] Schaefer, L.; Sakai, H.; Mattei, M.; Lazdunski, M.; Lingueglia, E. Molecular cloning, functional expression and chromosomal localization of an amiloride-sensitive Na(+) channel from human small intestine. *FEBS Lett.,* **2000**, *471*(2-3), 205-210. http://dx.doi.org/10.1016/S0014-5793(00)01403-4 PMID: 10767424

[170] Waldmann, R.; Champigny, G.; Bassilana, F.; Heurteaux, C.; Lazdunski, M. A proton-gated cation channel involved in acid-sensing. *Nature,* **1997**, *386*(6621), 173-177. http://dx.doi.org/10.1038/386173a0 PMID: 9062189

[171] Chen, C.C.; England, S.; Akopian, A.N.; Wood, J.N. A sensory neuron-specific, proton-gated ion channel. *Proc. Natl. Acad. Sci. USA,* **1998**, *95*(17), 10240-10245. http://dx.doi.org/10.1073/pnas.95.17.10240 PMID: 9707631

[172] Price, M.P.; Snyder, P.M.; Welsh, M.J. Cloning and expression of a novel human brain Na+ channel. *J. Biol. Chem.,* **1996**, *271*(14), 7879-7882. http://dx.doi.org/10.1074/jbc.271.14.7879 PMID: 8626462

[173] Lingueglia, E.; de Weille, J.R.; Bassilana, F.; Heurteaux, C.; Sakai, H.; Waldmann, R.; Lazdunski, M. A modulatory subunit of acid sensing ion channels in brain and dorsal root ganglion cells. *J. Biol. Chem.,* **1997**, *272*(47), 29778-29783. http://dx.doi.org/10.1074/jbc.272.47.29778 PMID: 9368048

[174] Waldmann, R.; Bassilana, F.; de Weille, J.; Champigny, G.; Heurteaux, C.; Lazdunski, M. Molecular cloning of a non-inactivating proton-gated Na+ channel specific for sensory neurons. *J. Biol. Chem.,* **1997**, *272*(34), 20975-20978. http://dx.doi.org/10.1074/jbc.272.34.20975 PMID: 9261094

[175] Akopian, A.N.; Chen, C.C.; Ding, Y.; Cesare, P.; Wood, J.N. A new member of the acid-sensing ion channel family. *Neuroreport,* **2000**, *11*(10), 2217-2222. http://dx.doi.org/10.1097/00001756-200007140-00031 PMID: 10923674

[176] Vukicevic, M.; Kellenberger, S. Modulatory effects of acid-sensing ion channels on action potential generation in hippocampal neurons. *Am. J. Physiol. Cell Physiol.,* **2004**, *287*(3), C682-C690. http://dx.doi.org/10.1152/ajpcell.00127.2004 PMID: 15115705

[177] Hesselager, M.; Timmermann, D.B.; Ahring, P.K. pH Dependency and desensitization kinetics of heterologously expressed combinations of acid-sensing ion channel subunits. *J. Biol. Chem.,* **2004**, *279*(12), 11006-11015. http://dx.doi.org/10.1074/jbc.M313507200 PMID: 14701823

[178] Babinski, K.; Lê, K.T.; Séguéla, P. Molecular cloning and regional distribution of a human proton receptor subunit with biphasic functional properties. *J. Neurochem.,* **1999**, *72*(1), 51-57. http://dx.doi.org/10.1046/j.1471-4159.1999.0720051.x PMID: 9886053

[179] Gründer, S.; Geissler, H.S.; Bässler, E.L.; Ruppersberg, J.P. A new member of acid-sensing ion channels from pituitary gland. *Neuroreport,* **2000**, *11*(8), 1607-1611. http://dx.doi.org/10.1097/00001756-200006050-00003 PMID: 10852210

[180] Su, X.; Li, Q.; Shrestha, K.; Cormet-Boyaka, E.; Chen, L.; Smith, P.R.; Sorscher, E.J.; Benos, D.J.; Matalon, S.; Ji, H.L. Interregulation of proton-gated Na(+) channel 3 and cystic fibrosis transmembrane conductance regulator. *J. Biol. Chem.,* **2006**, *281*(48), 36960-36968. http://dx.doi.org/10.1074/jbc.M608002200 PMID: 17012229

[181] Jahr, H.; van Driel, M.; van Osch, G.J.; Weinans, H.; van Leeuwen, J.P. Identification of acid-sensing ion channels in bone. *Biochem. Biophys. Res. Commun.,* **2005**, *337*(1), 349-354. http://dx.doi.org/10.1016/j.bbrc.2005.09.054 PMID: 16185661

[182] Paukert, M.; Sidi, S.; Russell, C.; Siba, M.; Wilson, S.W.; Nicolson, T.; Gründer, S. A family of acid-sensing ion channels from the zebrafish: widespread expression in the central nervous system suggests a conserved role in neuronal communication. *J. Biol. Chem.,* **2004**, *279*(18), 18783-18791. http://dx.doi.org/10.1074/jbc.M401477200 PMID: 14970195

[183] Vierck, C.J.; Mauderli, A.P.; Wiley, R.G. Assessment of pain in laboratory animals: a comment on Mogil and Crager (2004). *Pain,* **2005**, *114*(3), 520-523. http://dx.doi.org/10.1016/j.pain.2005.01.021 PMID: 15777880

[184] Treede, R.D. Assessment of pain as an emotion in animals and in humans. *Exp. Neurol.,* **2006**, *197*(1), 1-3. http://dx.doi.org/10.1016/j.expneurol.2005.10.009 PMID: 16297914

[185] Schroeder, P.G.S. L. U., Exploring the efficacy of immersion analgesics in zebrafish using an integrative approach. *Appl. Anim. Behav. Sci.,* **2017**, *187*, 93-102. http://dx.doi.org/10.1016/j.applanim.2016.12.003

[186] Larson, A.A.; Brown, D.R.; el-Atrash, S.; Walser, M.M. Pain threshold changes in adjuvant-induced inflammation: a possible model of chronic pain in the mouse. *Pharmacol. Biochem. Behav.,* **1986**, *24*(1), 49-53. http://dx.doi.org/10.1016/0091-3057(86)90043-2 PMID: 3945666

[187] Beggs, S.; Salter, M.W. Microglia-neuronal signalling in neuropathic pain hypersensitivity 2.0. *Curr. Opin. Neurobiol.,* **2010**, *20*(4), 474-480. http://dx.doi.org/10.1016/j.conb.2010.08.005 PMID: 20817512

[188] Sheng, J.; Liu, S.; Wang, Y.; Cui, R.; Zhang, X. The Link between Depression and Chronic Pain: Neural Mechanisms in the Brain. *Neural Plast.,* **2017**, *2017*, 9724371. http://dx.doi.org/10.1155/2017/9724371 PMID: 28706741

[189] Brown, R.E.; Stevens, D.R.; Haas, H.L. The physiology of brain histamine. *Prog. Neurobiol.,* **2001**, *63*(6), 637-672. http://dx.doi.org/10.1016/S0301-0082(00)00039-3 PMID: 11164999

[190] Kaslin, J.; Panula, P. Comparative anatomy of the histaminergic and other aminergic systems in zebrafish (Danio rerio). *J. Comp. Neurol.,* **2001**, *440*(4), 342-377. http://dx.doi.org/10.1002/cne.1390 PMID: 11745628

[191] Peitsaro, N.; Sundvik, M.; Anichtchik, O.V.; Kaslin, J.; Panula, P. Identification of zebrafish histamine H1, H2 and H3 receptors and effects of histaminergic ligands on behavior. *Biochem. Pharmacol.,* **2007**, *73*(8), 1205-1214. http://dx.doi.org/10.1016/j.bcp.2007.01.014 PMID: 17266939

[192] McNamara, C.R.; Mandel-Brehm, J.; Bautista, D.M.; Siemens, J.; Deranian, K.L.; Zhao, M.; Hayward, N.J.; Chong, J.A.; Julius, D.; Moran, M.M.; Fanger, C.M. TRPA1 mediates formalin-induced pain. *Proc. Natl. Acad. Sci. USA,* **2007**, *104*(33), 13525-13530. http://dx.doi.org/10.1073/pnas.0705924104 PMID: 17686976

[193] Dubuisson, D.; Dennis, S.G. The formalin test: a quantitative study of the analgesic effects of morphine, meperidine, and brain stem stimulation in rats and cats. *Pain,* **1977**, *4*(2), 161-174. http://dx.doi.org/10.1016/0304-3959(77)90130-0 PMID: 564014

[194] Noriega, V.; Sierralta, F.; Poblete, P.; Aranda, N.; Sotomayor-Zarate, R.; Prieto, J.C.; Miranda, H.F. Receptors involved in dexketoprofen analgesia in murine visceral pain. *J. Biosci.,* **2020**, *45*, 94. http://dx.doi.org/10.1007/s12038-020-00064-z PMID: 32713857

[195] Siegmund, E.; Cadmus, R.; Lu, G. A method for evaluating both non-narcotic and narcotic analgesics. *Proc. Soc. Exp. Biol. Med.,* **1957**, *95*(4), 729-731. http://dx.doi.org/10.3181/00379727-95-23345 PMID: 13465785

[196] Correia, A.D.; Cunha, S.R.; Scholze, M.; Stevens, E.D. A Novel Behavioral Fish Model of Nociception for Testing Analgesics. *Pharmaceuticals,* **2011**, *4*(4), 665-680. http://dx.doi.org/10.3390/ph4040665

[197] Costa, F.V.; Canzian, J.; Stefanello, F.V.; Kalueff, A.V.; Rosemberg, D.B. Naloxone prolongs abdominal constriction writhing-like

behavior in a zebrafish-based pain model. *Neurosci. Lett.,* **2019**, *708*, 134336.
http://dx.doi.org/10.1016/j.neulet.2019.134336 PMID: 31220523

[198] Murray, C.J.; Lopez, A.D. Measuring the global burden of disease. *N. Engl. J. Med.,* **2013**, *369*(5), 448-457.
http://dx.doi.org/10.1056/NEJMra1201534 PMID: 23902484

[199] Treede, R.D.; Rief, W.; Barke, A.; Aziz, Q.; Bennett, M.I.; Benoliel, R.; Cohen, M.; Evers, S.; Finnerup, N.B.; First, M.B.; Giamberardino, M.A.; Kaasa, S.; Korwisi, B.; Kosek, E.; Lavand'homme, P.; Nicholas, M.; Perrot, S.; Scholz, J.; Schug, S.; Smith, B.H.; Svensson, P.; Vlaeyen, J.W.S.; Wang, S.J. Chronic pain as a symptom or a disease: the IASP Classification of Chronic Pain for the International Classification of Diseases (ICD-11). *Pain,* **2019**, *160*(1), 19-27.
http://dx.doi.org/10.1097/j.pain.0000000000001384 PMID: 30586067

[200] Gatchel, R.J.; Peng, Y.B.; Peters, M.L.; Fuchs, P.N.; Turk, D.C. The biopsychosocial approach to chronic pain: scientific advances and future directions. *Psychol. Bull.,* **2007**, *133*(4), 581-624.
http://dx.doi.org/10.1037/0033-2909.133.4.581 PMID: 17592957

[201] Li, Q.; Liu, S.; Zhu, X.; Mi, W.; Maoying, Q.; Wang, J.; Yu, J.; Wang, Y. Hippocampal PKR/NLRP1 Inflammasome Pathway Is Required for the Depression-Like Behaviors in Rats with Neuropathic Pain. *Neuroscience,* **2019**, *412*, 16-28.
http://dx.doi.org/10.1016/j.neuroscience.2019.05.025 PMID: 31125603

[202] Parent, A.J.; Beaudet, N.; Beaudry, H.; Bergeron, J.; Bérubé, P.; Drolet, G.; Sarret, P.; Gendron, L. Increased anxiety-like behaviors in rats experiencing chronic inflammatory pain. *Behav. Brain Res.,* **2012**, *229*(1), 160-167.
http://dx.doi.org/10.1016/j.bbr.2012.01.001 PMID: 22245257

[203] Craske, M.G.; Rauch, S.L.; Ursano, R.; Prenoveau, J.; Pine, D.S.; Zinbarg, R.E. What is an anxiety disorder? *Depress. Anxiety,* **2009**, *26*(12), 1066-1085.
http://dx.doi.org/10.1002/da.20633 PMID: 19957279

[204] Malhi, G.S.; Mann, J.J. Depression. *Lancet,* **2018**, *392*(10161), 2299-2312.
http://dx.doi.org/10.1016/S0140-6736(18)31948-2 PMID: 30396512

[205] Guimarães, F.S.C.; Graeff, A.P. Modulation of anxiety behaviors by 5-HT-interacting drugs. In: *Handbook of Behavioral Neuroscience*; Robert, J.; Blanchard, D. C. B.; Griebel, G.; Nutt, D., Eds.; Elsevier, **2008**; 17, pp. 241-268.

[206] Ferjan, I.; Lipnik-Štangelj, M. Chronic pain treatment: the influence of tricyclic antidepressants on serotonin release and uptake in mast cells. *Mediators Inflamm.,* **2013**, *2013*, 340473.
http://dx.doi.org/10.1155/2013/340473 PMID: 23710115

[207] Dale, R.; Stacey, B. Multimodal Treatment of Chronic Pain. *Med. Clin. North Am.,* **2016**, *100*(1), 55-64.
http://dx.doi.org/10.1016/j.mcna.2015.08.012 PMID: 26614719

[208] Humo, M.; Lu, H.; Yalcin, I. The molecular neurobiology of chronic pain-induced depression. *Cell Tissue Res.,* **2019**, *377*(1), 21-43.
http://dx.doi.org/10.1007/s00441-019-03003-z PMID: 30778732

[209] Descalzi, G.; Mitsi, V.; Purushothaman, I.; Gaspari, S.; Avrampou, K.; Loh, Y.E.; Shen, L.; Zachariou, V. Neuropathic pain promotes adaptive changes in gene expression in brain networks involved in stress and depression. *Sci. Signal.,* **2017**, *10*(471), eaaj1549.
http://dx.doi.org/10.1126/scisignal.aaj1549 PMID: 28325815

[210] Chesselet, M.F.; Carmichael, S.T. Animal models of neurological disorders. *Neurotherapeutics,* **2012**, *9*(2), 241-244.
http://dx.doi.org/10.1007/s13311-012-0118-9 PMID: 22460561

[211] Richards, E.M.; Mathews, D.C.; Luckenbaugh, D.A.; Ionescu, D.F.; Machado-Vieira, R.; Niciu, M.J.; Duncan, W.C.; Nolan, N.M.; Franco-Chaves, J.A.; Hudzik, T.; Maciag, C.; Li, S.; Cross, A.; Smith, M.A.; Zarate, C.A., Jr A randomized, placebo-controlled pilot trial of the delta opioid receptor agonist AZD2327 in anxious depression. *Psychopharmacology (Berl.),* **2016**, *233*(6), 1119-1130.
http://dx.doi.org/10.1007/s00213-015-4195-4 PMID: 26728893

[212] Stewart, A.M.; Grossman, L.; Nguyen, M.; Maximino, C.; Rosemberg, D.B.; Echevarria, D.J.; Kalueff, A.V. Aquatic toxicology of fluoxetine: understanding the knowns and the unknowns. *Aquat. Toxicol.,* **2014**, *156*, 269-273.
http://dx.doi.org/10.1016/j.aquatox.2014.08.014 PMID: 25245382

[213] Wyatt, C.; Bartoszek, E.M.; Yaksi, E. Methods for studying the zebrafish brain: past, present and future. *Eur. J. Neurosci.,* **2015**, *42*(2), 1746-1763.
http://dx.doi.org/10.1111/ejn.12932 PMID: 25900095

[214] Kalueff, A.V.; Gebhardt, M.; Stewart, A.M.; Cachat, J.M.; Brimmer, M.; Chawla, J.S.; Craddock, C.; Kyzar, E.J.; Roth, A.; Landsman, S.; Gaikwad, S.; Robinson, K.; Baatrup, E.; Tierney, K.; Shamchuk, A.; Norton, W.; Miller, N.; Nicolson, T.; Braubach, O.; Gilman, C.P.; Pittman, J.; Rosemberg, D.B.; Gerlai, R.; Echevarria, D.; Lamb, E.; Neuhauss, S.C.; Weng, W.; Bally-Cuif, L.; Schneider, H. Towards a comprehensive catalog of zebrafish behavior 1.0 and beyond. *Zebrafish,* **2013**, *10*(1), 70-86.
http://dx.doi.org/10.1089/zeb.2012.0861 PMID: 23590400

[215] Maximino, C.; Puty, B.; Benzecry, R.; Araújo, J.; Lima, M.G.; de Jesus Oliveira Batista, E.; Renata de Matos Oliveira, K.; Crespo-Lopez, M.E.; Herculano, A.M. Role of serotonin in zebrafish (Danio rerio) anxiety: relationship with serotonin levels and effect of buspirone, WAY 100635, SB 224289, fluoxetine and para-chlorophenylalanine (pCPA) in two behavioral models. *Neuropharmacology,* **2013**, *71*, 83-97.
http://dx.doi.org/10.1016/j.neuropharm.2013.03.006 PMID: 23541719

[216] Singleman, C.; Holtzman, N.G. Growth and maturation in the zebrafish, Danio rerio: a staging tool for teaching and research. *Zebrafish,* **2014**, *11*(4), 396-406.
http://dx.doi.org/10.1089/zeb.2014.0976 PMID: 24979389

[217] Sneddon, L.U.; Halsey, L.G.; Bury, N.R. Considering aspects of the 3Rs principles within experimental animal biology. *J. Exp. Biol.,* **2017**, *220*(Pt 17), 3007-3016.
http://dx.doi.org/10.1242/jeb.147058 PMID: 28855318

[218] Parker, M.O.; Brock, A.J.; Walton, R.T.; Brennan, C.H. The role of zebrafish (*Danio rerio*) in dissecting the genetics and neural circuits of executive function. *Front. Neural Circuits,* **2013**, *7*, 63.
http://dx.doi.org/10.3389/fncir.2013.00063 PMID: 23580329

[219] Lu, J.; Peatman, E.; Tang, H.; Lewis, J.; Liu, Z. Profiling of gene duplication patterns of sequenced teleost genomes: evidence for rapid lineage-specific genome expansion mediated by recent tandem duplications. *BMC Genomics,* **2012**, *13*, 246.
http://dx.doi.org/10.1186/1471-2164-13-246 PMID: 22702965

[220] Lakstygal, A.M.; de Abreu, M.S.; Lifanov, D.; Wappler-Guzetta, E.A.; Serikuly, N.; Alpsychov, E.T.; Wang, D.; Wang, M.; Tang, Z.; Yan, N. Zebrafish models of diabetes-related CNS pathogenesis. *Prog. Neuropsychopharmacol. Biol. Psychiatry,* **2019**, *98*, 48-58.
PMID: 30476525

[221] Wang, D.; Hu, G.; Wang, J.; Yan, D.; Wang, M.; Yang, L.; Serikuly, N.; Alpyshov, E.; Demin, K.A.; Galstyan, D.S.; Amstislavskaya, T.G.; de Abreu, M.S.; Kalueff, A.V. Studying CNS effects of Traditional Chinese Medicine using zebrafish models. *J. Ethnopharmacol.,* **2021**, *267*, 113383.
http://dx.doi.org/10.1016/j.jep.2020.113383 PMID: 32918992

[222] de Abreu, M.S.; Giacomini, A.C.V.V.; Genario, R.; Rech, N.; Carboni, J.; Lakstygal, A.M.; Amstislavskaya, T.G.; Demin, K.A.; Leonard, B.E.; Vlok, M.; Harvey, B.H.; Piato, A.; Barcellos, L.J.G.; Kalueff, A.V. Non-pharmacological and pharmacological approaches for psychiatric disorders: Re-appraisal and insights from zebrafish models. *Pharmacol. Biochem. Behav.,* **2020**, *193*, 172928.
http://dx.doi.org/10.1016/j.pbb.2020.172928 PMID: 32289330

[223] Jordi, J.; Guggiana-Nilo, D.; Bolton, A.D.; Prabha, S.; Ballotti, K.; Herrera, K.; Rennekamp, A.J.; Peterson, R.T.; Lutz, T.A.; Engert, F. High-throughput screening for selective appetite modulators: A multibehavioral and translational drug discovery strategy. *Sci. Adv.,* **2018**, *4*(10), eaav1966.
http://dx.doi.org/10.1126/sciadv.aav1966 PMID: 30402545

[224] Howe, K.; Clark, M.D.; Torroja, C.F.; Torrance, J.; Berthelot, C.; Muffato, M.; Collins, J.E.; Humphray, S.; McLaren, K.; Matthews, L.; McLaren, S.; Sealy, I.; Caccamo, M.; Churcher, C.; Scott, C.; Barrett, J.C.; Koch, R.; Rauch, G.J.; White, S.; Chow, W.; Kilian, B.; Quintais, L.T.; Guerra-Assunção, J.A.; Zhou, Y.; Gu, Y.; Yen, J.; Vogel, J.H.; Eyre, T.; Redmond, S.; Banerjee, R.; Chi, J.; Fu, B.; Langley, E.; Maguire, S.F.; Laird, G.K.; Lloyd, D.; Kenyon, E.; Donaldson, S.; Sehra, H.; Almeida-King, J.; Loveland, J.; Trevanion, S.; Jones, M.; Quail, M.; Willey, D.; Hunt, A.; Burton, J.; Sims, S.; McLay, K.; Plumb, B.; Davis, J.; Clee, C.; Oliver, K.; Clark, R.; Riddle, C.; Elliot, D.; Threadgold, G.; Harden, G.; Ware, D.; Begum, S.; Mortimore, B.; Kerry, G.; Heath, P.; Phillimore, B.; Tracey, A.; Corby, N.; Dunn, M.; Johnson, C.; Wood, J.; Clark, S.; Pelan, S.; Griffiths, G.; Smith, M.; Glithero, R.; Howden, P.;

Barker, N.; Lloyd, C.; Stevens, C.; Harley, J.; Holt, K.; Panagiotidis, G.; Lovell, J.; Beasley, H.; Henderson, C.; Gordon, D.; Auger, K.; Wright, D.; Collins, J.; Raisen, C.; Dyer, L.; Leung, K.; Robertson, L.; Ambridge, K.; Leongamornlert, D.; McGuire, S.; Gilderthorp, R.; Griffiths, C.; Manthravadi, D.; Nichol, S.; Barker, G.; Whitehead, S.; Kay, M.; Brown, J.; Murnane, C.; Gray, E.; Humphries, M.; Sycamore, N.; Barker, D.; Saunders, D.; Wallis, J.; Babbage, A.; Hammond, S.; Mashreghi-Mohammadi, M.; Barr, L.; Martin, S.; Wray, P.; Ellington, A.; Matthews, N.; Ellwood, M.; Woodmansey, R.; Clark, G.; Cooper, J.; Tromans, A.; Grafham, D.; Skuce, C.; Pandian, R.; Andrews, R.; Harrison, E.; Kimberley, A.; Garnett, J.; Fosker, N.; Hall, R.; Garner, P.; Kelly, D.; Bird, C.; Palmer, S.; Gehring, I.; Berger, A.; Dooley, C.M.; Ersan-Ürün, Z.; Eser, C.; Geiger, H.; Geisler, M.; Karotki, L.; Kirn, A.; Konantz, J.; Konantz, M.; Oberländer, M.; Rudolph-Geiger, S.; Teucke, M.; Lanz, C.; Raddatz, G.; Osoegawa, K.; Zhu, B.; Rapp, A.; Widaa, S.; Langford, C.; Yang, F.; Schuster, S.C.; Carter, N.P.; Harrow, J.; Ning, Z.; Herrero, J.; Searle, S.M.; Enright, A.; Geisler, R.; Plasterk, R.H.; Lee, C.; Westerfield, M.; de Jong, P.J.; Zon, L.I.; Postlethwait, J.H.; Nüsslein-Volhard, C.; Hubbard, T.J.; Roest Crollius, H.; Rogers, J.; Stemple, D.L.; Rogers, J.; Stemple, D.L. The zebrafish reference genome sequence and its relationship to the human genome. *Nature,* **2013**, *496*(7446), 498-503. http://dx.doi.org/10.1038/nature12111 PMID: 23594743

[225] Cheresiz, S.V.; Volgin, A.D.; Kokorina Evsyukova, A.; Bashirzade, A.A.O.; Demin, K.A.; de Abreu, M.S.; Amstislavskaya, T.G.; Kalueff, A.V. Understanding neurobehavioral genetics of zebrafish. *J. Neurogenet.,* **2020**, *34*(2), 203-215. http://dx.doi.org/10.1080/01677063.2019.1698565 PMID: 31902276

[226] Kimmel, C.B. Genetics and early development of zebrafish. *Trends Genet.,* **1989**, *5*(8), 283-288. http://dx.doi.org/10.1016/0168-9525(89)90103-0 PMID: 2686119

[227] Ata, H.; Clark, K.J.; Ekker, S.C. The zebrafish genome editing toolkit. *Methods Cell Biol.,* **2016**, *135*, 149-170. http://dx.doi.org/10.1016/bs.mcb.2016.04.023 PMID: 27443924

[228] Varshney, G.K.; Sood, R.; Burgess, S.M. Understanding and Editing the Zebrafish Genome. *Adv. Genet.,* **2015**, *92*, 1-52. http://dx.doi.org/10.1016/bs.adgen.2015.09.002 PMID: 26639914

[229] Eijkenboom, I.; Sopacua, M.; Otten, A.B.C.; Gerrits, M.M.; Hoeijmakers, J.G.J.; Waxman, S.G.; Lombardi, R.; Lauria, G.; Merkies, I.S.J.; Smeets, H.J.M.; Faber, C.G.; Vanoevelen, J.M.; Group, P.S. Expression of pathogenic SCN9A mutations in the zebrafish: A model to study small-fiber neuropathy. *Exp. Neurol.,* **2019**, *311*, 257-264. http://dx.doi.org/10.1016/j.expneurol.2018.10.008 PMID: 30316835

[230] Labusch, M.; Mancini, L.; Morizet, D.; Bally-Cuif, L. Conserved and Divergent Features of Adult Neurogenesis in Zebrafish. *Front. Cell Dev. Biol.,* **2020**, *8*, 525. http://dx.doi.org/10.3389/fcell.2020.00525 PMID: 32695781

[231] McIntosh, R.; Norris, J.; Clarke, J.D.; Alexandre, P. Spatial distribution and characterization of non-apical progenitors in the zebrafish embryo central nervous system. *Open Biol.,* **2017**, *7*(2), 160312. http://dx.doi.org/10.1098/rsob.160312 PMID: 28148823

[232] Than-Trong, E.; Bally-Cuif, L. Radial glia and neural progenitors in the adult zebrafish central nervous system. *Glia,* **2015**, *63*(8), 1406-1428. http://dx.doi.org/10.1002/glia.22856 PMID: 25976648

[233] Pylatiuk, C.; Zhao, H.; Gursky, E.; Reischl, M.; Peravali, R.; Foulkes, N.; Loosli, F. DIY Automated Feeding and Motion Recording System for the Analysis of Fish Behavior. *SLAS Technol.,* **2019**, *24*(4), 394-398. http://dx.doi.org/10.1177/2472630319841412 PMID: 31013465

[234] Franco-Restrepo, J.E.; Forero, D.A.; Vargas, R.A. A Review of Freely Available, Open-Source Software for the Automated Analysis of the Behavior of Adult Zebrafish. *Zebrafish,* **2019**, *16*(3), 223-232. http://dx.doi.org/10.1089/zeb.2018.1662 PMID: 30625048

ARTIGO 2

Understanding nociception-related phenotypes in adult zebrafish: behavioral and pharmacological characterization using the acetic acid model

Fabiano V. Costa, Luiz V. Rosa, Vanessa A. Quadros, Adair R. S. Santos
Allan V. Kalueff, Denis B. Rosemberg

Behavioural Brain Research (2019). Feb 1; 359:570-578.
DOI: 10.1016/j.bbr.2018.10.009

Behavioural Brain Research 359 (2019) 570–578

Contents lists available at ScienceDirect

Behavioural Brain Research

journal homepage: www.elsevier.com/locate/bbr

Understanding nociception-related phenotypes in adult zebrafish: Behavioral and pharmacological characterization using a new acetic acid model

Fabiano V. Costa[a,b,*], Luiz V. Rosa[a,b], Vanessa A. Quadros[a,b], Adair R.S. Santos[c,d], Allan V. Kalueff[e,f,g,h,i,j,k,l], Denis B. Rosemberg[a,b,l,**]

[a] Laboratory of Experimental Neuropsychobiology, Department of Biochemistry and Molecular Biology, Natural and Exact Sciences Center, Federal University of Santa Maria, Santa Maria, RS, 97105-900, Brazil
[b] Graduate Program in Biological Sciences: Toxicological Biochemistry, Federal University of Santa Maria, Santa Maria, RS, 97105-900, Brazil
[c] Laboratory of Neurobiology of Pain and Inflammation, Department of Physiological Sciences, Center of Biological Sciences, Federal University of Santa Catarina, Florianópolis, SC, 88040-900, Brazil
[d] Graduate Program in Neuroscience, Center of Biological Sciences, Federal University of Santa Catarina, Florianópolis, SC, 88040-900, Brazil
[e] School of Pharmaceutical Sciences, Southwest University, Chongqing 400716, China
[f] Laboratory of Biological Psychiatry, Institute of Translational Biomedicine, St. Petersburg State University, St. Petersburg 199034, Russia
[g] Ural Federal University, Ekaterinburg 620002, Russia
[h] Institute of Experimental Medicine, Almazov National Medical Research Centre, St. Petersburg 197341, Russia
[i] Research Institute of Physiology and Basic Medicine, Novosibirsk 630117, Russia
[j] Granov Russian Research Center of Radiology and Surgical Technologies, Pesochny 197758, Russia
[k] ZENEREI Research Center, Slidell 70458, LA, USA
[l] The International Zebrafish Neuroscience Research Consortium (ZNRC), Slidell, LA 70458, USA

ARTICLE INFO

Keywords:
Nociception
Body curvature index
Acetic acid
Zebrafish
Behavioral responses

ABSTRACT

Pain, a severely debilitating symptom of many human disorders, is a growing, unmet biomedical problem. Although the use of zebrafish (*Danio rerio*) to investigate both behavioral and physiological nociception-related responses is expanding rapidly, the characterization of behavioral phenotypes that reflect injury location is limited, making the results of such studies difficult to interpret. Here, we characterize putative nociception-related behavioral phenotypes in adult zebrafish following an intraperitoneal (i.p.) administration of acetic acid, a well-established protocol for visceral pain in rodents. Acetic acid (2.5 and 5.0%) induced an abdominal constriction-like response, which was assessed by measuring a body curvature index. Moreover, all doses tested (0.5–5.0%) reduced distance traveled and vertical activity in the novel tank test. Freezing duration increased following 5.0% acetic acid, whereas fish injected with 1.0, 2.5, and 5.0% spent more time in top area of the tank. Both morphine (an opioid analgesic) and diclofenac (a nonsteroidal anti-inflammatory drug, NSAID) prevented the 5.0% acetic acid-induced changes in body curvature index, whereas naloxone blocked these effects of morphine. Overall, zebrafish exposed to a single acetic acid i.p. injection display abnormal body curvature and specific changes in behavioral parameters sensitive to anti-nociceptive pharmacological modulation. We suggest that the abdominal constriction-like response represents a novel specific nociceptive-related phenotype in zebrafish. In general, our findings support the growing utility of zebrafish in translational pain research and antinociceptive drug discovery.

1. Introduction

Pain is a severely debilitating symptom of many human disorders, representing an urgent unmet biomedical problem [1,2]. Nociception involves transmission of painful signals by nociceptors in the primary afferent nerve fibers, in response to mechanical, thermal or chemical

* Corresponding author at: Graduate Program in Biological Sciences: Toxicological Biochemistry, Federal University of Santa Maria, Santa Maria, RS, 97105-900, Brazil.
** Corresponding author at: Department of Biochemistry and Molecular Biology, Natural and Exact Sciences Center, Federal University of Santa Maria, Santa Maria, RS, 97105-900, Brazil.
E-mail addresses: fvcosta88@gmail.com (F.V. Costa), dbrosemberg@gmail.com (D.B. Rosemberg).

https://doi.org/10.1016/j.bbr.2018.10.009
Received 22 August 2018; Received in revised form 19 September 2018; Accepted 4 October 2018
Available online 05 October 2018
0166-4328/

noxious stimuli [3,4]. This stimulus then activates the central nervous system (CNS), triggering pain perception and behavioral responses to pain [5–7]. Although rodent models are widely used in translational pain research [8,9], other alternative experimental models help assess evolutionarily conserved mechanisms underlying nociception and their associated behavioral phenotypes [10,11].

The zebrafish (*Danio rerio*) is rapidly emerging as a promising model organism to study nociceptive-like responses [12]. Homologous to human nociceptors, Transient Receptor Potential (TRP) ion channels (e.g., TRPV1 and TRPA1), acid-sensing ion channels (ASICs), and toll-like receptors (TLRs) have already been characterized in zebrafish [13–18]. Although various studies use locomotor changes and opercular beat rate as major endpoints of fish nociception [19–22], many of them focus on behavioral parameters affected by algogens, and do not specifically measure nociception per se. For example, changes in distance traveled or altered swim velocity do not necessarily indicate a specific nociception-related phenotype, and may also reflect fish stress, anxiety-, nonspecific sedation- and/or depressive-like behaviors [23]. Therefore, these endpoints may not offer a proper specificity for adult fish nociception, making the results obtained somewhat difficult to interpret.

Zebrafish injected with acetic acid near the adipose fin [24] or submitted to the fin clipping procedure [25] show a higher frequency of tail beating, suggesting a local nociceptive effect. However, there is limited empirical evidence of specific nociception-related behaviors that may result from direct activation of nociceptors. To further characterize potential nociception-related phenotypes in a new model organism (zebrafish), we describe a novel protocol using the intraperitoneal acetic acid administration, conceptually adapted from a well-established nociception rodent model [26]. Our goal was not to transfer the effective model from mice to fish, but to develop a novel protocol that is efficient in zebrafish and is based on specific nociceptive behavioral responses of this aquatic species. Additionally, to explore potential pharmacological mechanisms involved in nociception-related behaviors, we further validate this method using two clinically active medications with distinct analgesic properties: a non-selective opioid receptor agonist morphine, and a nonsteroidal anti-inflammatory drug (NSAID) diclofenac.

2. Methods

2.1. Animals

Subjects were 85 animals (~50:50 male:female ratio) adult zebrafish (*Danio rerio*) (4–6 months-old, weighing 0.250–0.300 g) of short-fin wild type outbred strain obtained from a local commercial distributor (Hobby Aquarios, RS, Brazil). Animals were acclimated in 50-L thermostatic aerated housing tanks (50 × 35 x 30 cm, length x height x width) equipped with filters containing activated carbon for at least two weeks prior to the experiments. The water was pretreated with AquaSafe™ (Tetra, USA) and the temperature was set at 27 ± 1 °C, pH 7.2 ± 0.15, conductivity at 1300–1500 $\mu S.cm^{-1}$, dissolved oxygen at 6.0 ± 0.1 mg/L, total ammonia at < 0.01 mg/L, nitrate < 50 mg/L, nitrite < 0.1 mg/L, alkalinity and hardness at 75 mg/L $CaCO_3$ [27]. Water conditions were monitored daily and 50% water was changed twice a week. Illumination was provided by ceiling-mounted fluorescent light tubes on a 14/10 light/dark photoperiod cycle (lights on at 7:00 am and off at 9:00 pm). Fish were fed thrice daily with commercial flake fish food (Alcon BASIC™, Alcon, Brazil). All animals were experimentally naive and maintained in accordance with the US National Institutes of Health Guide for Care and Use of Laboratory Animals. All protocols were approved by the Ethics Committee on Animal Use of the Federal University of Santa Maria (Protocol 5438310817).

2.2. Drugs

Acetic acid (99%), morphine sulfate, naloxone methiodide, and phosphate buffered saline (PBS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Diclofenac sodium was purchased from Novartis (São Paulo, Brazil).

2.3. Acetic acid administration protocol and behavioral analyses

Zebrafish (n = 5 per experimental group) were randomly selected from at least three housing tanks and placed individually for 6 min in the observation tanks (15 × 13 x 10 cm, length x height x width) with a 10-cm water depth. Water conditions were similar to those used in the housing tanks and the baseline behavioral activity of fish was recorded for 6 min (Fig. 1A). Then, PBS (control group) or acetic acid (using PBS as vehicle) were administered intraperitoneally (i.p.). Animals were gently handled, anesthetized in cold water [28], and briefly immobilized using a small wet net. Acetic acid (0.5, 1.0, 2.5, or 5.0%) was quickly injected into the midline between the pelvic fins through the net. The i.p. injection occurred in a relatively short period (less than 10 s) and this method was preferred over other known methods of fish i.p. treatment (e.g., chemical anesthesia with tricaine or other similar drugs) in order to prevent any additional anesthesia from concomitantly factoring into the drug effects examined here. Importantly, this protocol allows a fast evaluation of the swimming activity after fish return to the water, minimizing potential pharmacological interference on complex behaviors (e.g., depth preference, immobility, and locomotion). Behavioral recordings started as soon as the fish regains equilibrium (± 1 min post-injection), thus allowing a more precise observation of acute pain responses [28]. No differences in body curvature indexes were observed between male and female after 5.0% acetic acid injection (see Supplementary Fig. S1) and thus, male and female were used in a ~50:50 male:female ratio per group. Because no differences were observed in body curvature indexes when 5 vs. 10 animals were treated with PBS or 5.0% acetic acid in pilot experiments (see Supplementary Fig. S2), we chose to use n = 5 for behavioral assays, adhering to the 3Rs principles of ethical animal experimentation. Two separate batches of fish were tested in this study, and the results were generally consistent between the batches. All injections were performed using a BD Ultra-fine™ 30U syringe (needle size 6 mm x 0.25 mm) with a maximum volume of 10 µL (which is shown not to impair normal zebrafish behaviors [28,29]). The doses of acetic acid used here were selected based on our pilot studies and on previous reports that assessed nociception in fish species, including zebrafish [20,30]. Then, fish were returned to their observation tanks and behavioral activities were recorded for 6 min (Fig. 1A) using a digital camera (Nikon Coolpix P900, Tokyo, Japan). All behavioral tests in this study were performed between 09:00 am and 4:00 pm. After the experimental procedures, animals were immediately anesthetized in cold water (4 °C) and then euthanized by decapitation.

2.4. Effects of morphine, naloxone, and diclofenac on behavior

Following the baseline recordings for 6 min, animals were gently handled using a small net and the 6 fish cohorts were tested in parallel after i.p. injections of PBS (control), acetic acid (5.0%), morphine (2.5 mg/kg), acetic acid plus morphine, naloxone (5.0 mg/kg), and acetic acid plus morphine plus naloxone. Behavioral activities of these cohorts (n = 5 per experimental group) were recorded for 6 min (Fig. 1A), as described above. Morphine treatment for this study was chosen because as a classical, clinically approved and potent opioid analgesic drug [31] widely used in experimental models of pain [32,33], with a known sensitivity in zebrafish [20]. Naloxone was chosen for this study as a classical opioid antagonist [31], commonly used to antagonize morphine antinociception in various experimental model organisms [34,35], including zebrafish [20]. The doses of

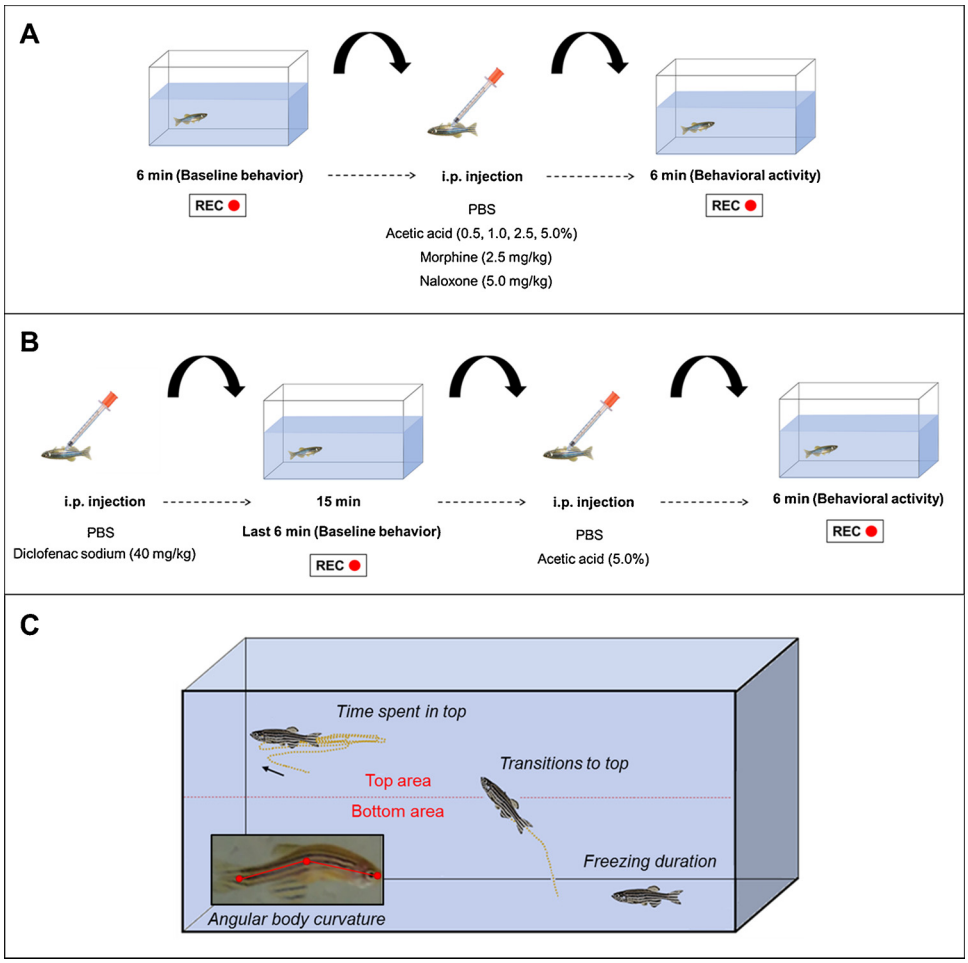


Fig. 1. A brief summary of the experimental protocol and behavioral analyses performed in the study. (A) Nociception-like behavioral phenotyping following PBS, acetic acid, morphine, and naloxone intraperitoneal (i.p.) injections. (B) Protocol used for assessing the behavioral effects of zebrafish pretreated with diclofenac sodium 15 min prior to PBS and acetic acid i.p. injections. Baseline behavioral values and body curvatures were measured for each animal tested. (C) Behaviors of zebrafish were analyzed before and after acetic acid administration. Inset: the three body points (frontal, central and posterior, denoted by red dots) used here to estimate the angular body curvature, calculated as area under curve (AUC, see text for details).

morphine and naloxone used here were similar to those described in a previous report using zebrafish [20].

To assess the potential antinociceptive effects of diclofenac, animals ($n = 5$ per experimental group) were handled and injected i.p. with PBS or diclofenac (40 mg/kg), as described above. Then, animals were kept for 15 min in the tank, and their activity was recorded for 6 min before and after acetic acid (5.0%) or PBS injection (**Fig. 1B**). Diclofenac was chosen as a common, clinically approved non-steroidal anti-inflammatory analgesic drug [36] widely used in experimental models to assess nociceptive-related phenotypes [37,38].

2.5. Behavioral parameters

To establish potential nociception-related behaviors of zebrafish, their swimming was recorded 6 min prior to (baseline values) and after a single acetic acid i.p. injection. All behaviors were video-recorded (as described above) and analyzed by automated video-tracking software (Any-Maze™, Stoelting, CO, USA) at 30 frames/s, to quantify the distance traveled, freezing duration, transitions to top area, and time spent in top area of the tank (**Fig. 1C**). Freezing was defined as a complete immobility (≥ 2 s) except by gills and eyes accompanied by fast opercular beat rates, as described elsewhere [23]. The local effects of acetic acid were assessed by measuring the body curvature index, which represents an abdominal constriction-like response. Frontal digital pictures were taken every 30 s (totaling 24 photos per fish). Afterward, the body curvature was measured with ImageJ 1.45 software for Windows (NIH, Bethesda, MD, USA). We selected three points to estimate the fish body curvature: a frontal (in the front of the head), a central (in the middle of the animal's body – between the anal and dorsal fins), and a posterior point (at the caudal fin) (**Fig. 1C, inset**). Results were subtracted from 180° to calculate a value representing a body curvature index. Temporal variations in the body curvature index were measured by two trained observers blinded to the experimental condition (inter-rater reliability > 0.90) and expressed as the area under curve (AUC). Importantly, both observers and data analysts were blinded to the treatment groups. Only person who treated the animals knew their conditions, but did not analyze the data, and only compiled the results after analyses.

2.6. Statistics

Data normality and homogeneity of variances were analyzed by Kolmogorov–Smirnov and Bartlett's tests, respectively. Changes in body curvature index and behavioral activity were analyzed by paired Student's *t*-test, one-way analysis of variance (ANOVA, factor: treatment), or two-way ANOVA (factors: acetic acid and diclofenac). Differences among groups were further assessed by Dunnett's post hoc test (acetic acid vs. PBS) or by Student-Newman-Keuls multiple comparison test for significant ANOVA data. Results were expressed as means ± standard error of the mean (S.E.M.) and non-parametric data (freezing duration) was previously log-transformed. Two trained observers blinded to the experimental conditions selected the fish images used here as representative examples for illustrations on a consensus basis. The inter-rater reliability was calculated using Spearman correlation. The level of significance was set at $p \leq 0.05$ in all analyses.

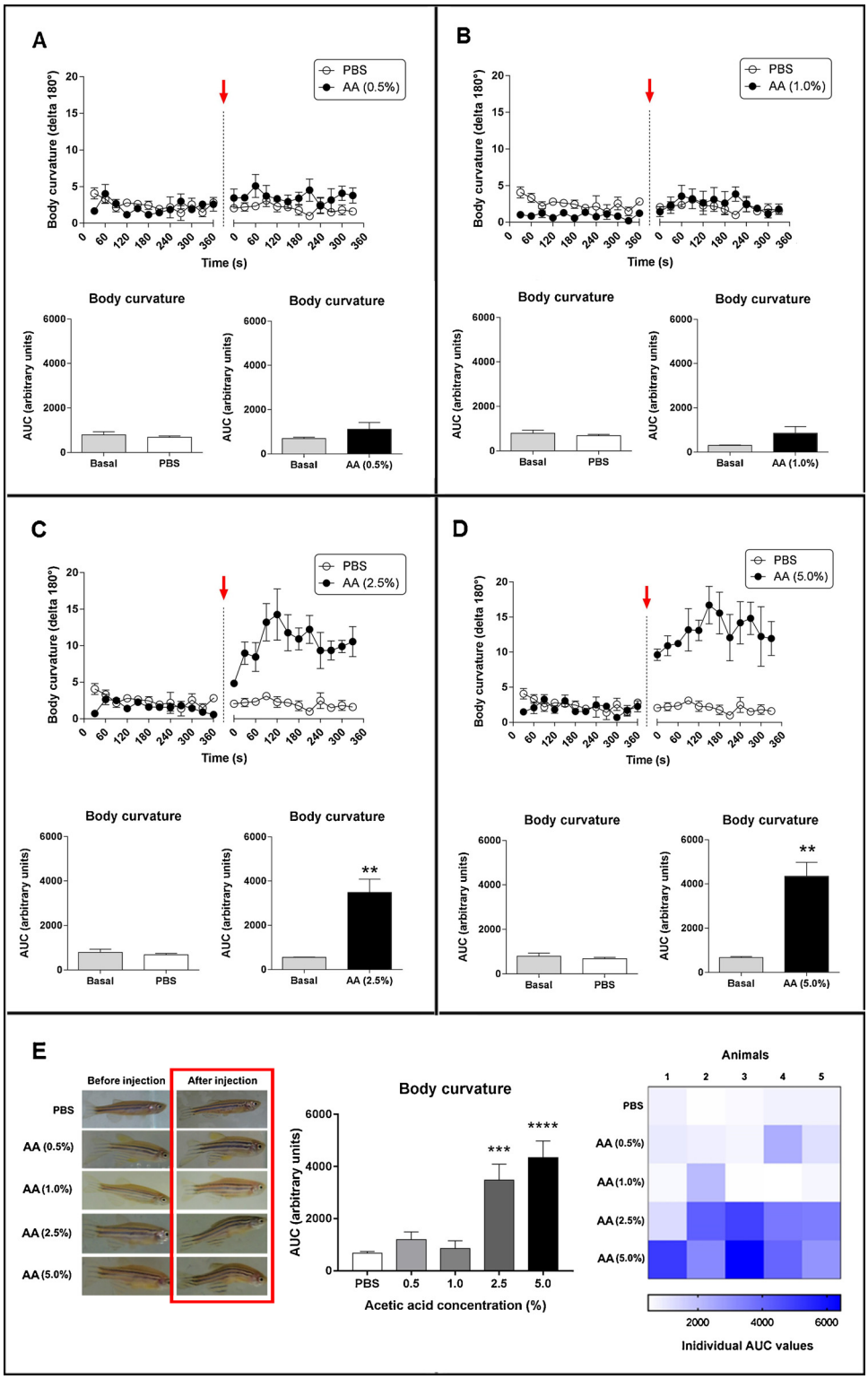


Fig. 2. Intraperitoneal acetic acid injection markedly affects the body curvature index. (A–D) Changes in body curvature index in PBS and acetic acid groups (AA 0.5, 1.0, 2.5, and 5.0%) across time. Red arrows indicate the moment of injection. Area under curve (AUC) values were calculated and expressed as arbitrary units. (E) Representative images displaying the zebrafish phenotypes before and after acetic acid injection, as well as the effects of acetic acid at different doses in relation to PBS group, and the heat map diagram showing the values of AUC obtained for each fish tested. Data are expressed as means ± S.E.M. and analyzed by paired Student's *t* test or one-way ANOVA (factor: treatment), followed by Dunnett's post-hoc test for significant ANOVA data . (**$p < 0.01$; ***$p < 0.005$; **** $p < 0.001$, AA vs. basal (C-D), AA vs. PBS (E)).

3. Results

3.1. Intraperitoneal acetic acid injection changes zebrafish behavioral phenotypes

Acetic acid (0.5 and 1.0%, i.p.) injection did not modify zebrafish body curvature index ($t_{(df = 4)} = 1.47$, $p = 0.2154$ and $t_{(df = 4)} = 1.855$, $p = 0.1371$, respectively) (Fig. 2A and B). However, temporal analyses revealed an increased body curvature index following 2.5 ($t_{(df = 4)} = 4.877$, $p = 0.0082$) and 5.0% acetic acid ($t_{(df = 4)} = 5.734$, $p = 0.0046$) (red arrow, 360 s) compared to their baseline conditions (Fig. 2C and D). Importantly, PBS alone did not change the body curvature index ($t_{(df = 4)} = 0.6246$, $p = 0.5661$). The effects of different doses of acetic acid on body curvature index in comparison to control (PBS), as well as representative images of fish from each experimental condition, and the heat map for individual AUC values, are shown in Fig. 2E. Moreover, the abdominal constriction-like behavior measured here is highly reproducible, showing similar responses when

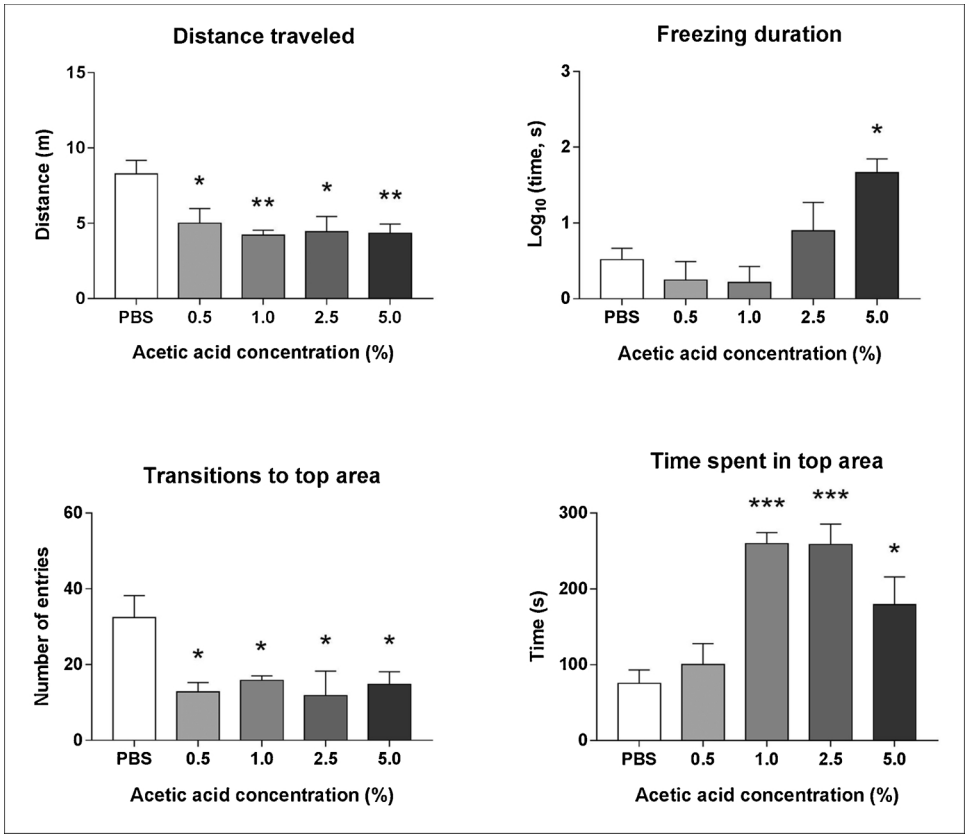


Fig. 3. Intraperitoneal acetic acid injection potently affects zebrafish behavior. Locomotor and exploratory endpoints were assessed by distance traveled, freezing duration, number of entries and time spent in top area. Data are expressed as means ± S.E.M. and analyzed by one-way ANOVA (factor: treatment), followed by Dunnett's post-hoc test for significant ANOVA data (*$p <$ 0.05; **$p <$ 0.01; ***$p <$ 0.005 vs. PBS; $n = 5$ per group).

5 or 10 fish per group were tested previously (Supplementary Fig. S2). Figure 3 shows the influence of acetic acid on zebrafish behavior, as it reduced locomotion ($F_(4,20_) = 4.487$, $p =$ 0.0095) and transitions to top area ($F_{(4,20)} = 3.784$, $p =$ 0.0189) compared to control (PBS). Freezing duration increased at 5.0% ($F_{(4,20)} = 6.005$, $p =$ 0.0024), whereas fish injected with 1.0, 2.5, and 5.0% acetic acid spent more time in top ($F_{(4,20)} = 11.17$, $p <$ 0.0001). A representative video showing the main baseline behaviors of zebrafish, as well as the behavioral responses following PBS and 5% acetic acid i.p. injections is provided as a Supplementary Video S1 online.

3.2. Opioid system modulates acetic acid-induced changes in body curvature index and behavior

A non-selective opioid receptor agonist morphine co-administration completely prevented changes in body curvature index following 5.0% acetic acid i.p. injection. Additionally, naloxone antagonized the preventive effects of morphine ($F_{(5,24)} = 33.83$, $p <$ 0.0001, Fig. 4A). Representative images of fish from different experimental cohorts and the heat map of individual AUC values are shown in Fig. 4B and C.

Fig. 5 illustrates the involvement of the opioid system in modulating zebrafish behaviors following acetic acid i.p. administration. While morphine abolished acetic acid-induced effects on freezing ($F_{(5,24)} = 10.9$, $p <$ 0.0001) and time spent in top of the tank ($F_{(5,24)} = 4{,}216$, $p <$ 0.0068), naloxone antagonized the effects of morphine only for time spent in top ($F_{(5,24)} = 4{,}216$, $p <$ 0.0068). In contrast, neither morphine nor naloxone injected alone affected zebrafish behavior at doses given (Fig. 5).

3.3. Diclofenac prevents acetic acid-induced changes in body curvature index

As shown in Fig. 6A, an NSAID diclofenac pretreatment completely prevented changes in body curvature index following 5.0% acetic acid i.p. injection. Furthermore, diclofenac alone did not change fish body curvature index ($F_{(3,16)} = 33.19$, $p <$ 0.0001). Representative images of fish at different experimental conditions and the heat map of individual AUC values are provided in Fig. 6B and C, respectively. Figure 6D shows modulatory effects of diclofenac on fish behavior following acetic acid i.p. administration. Diclofenac pretreatment did not prevent acetic acid-induced changes in fish locomotor and vertical activity. Moreover, although diclofenac alone reduced vertical transitions, it did not affect other zebrafish behaviors (Fig. 6D).

4. Discussion

The present study combined an acute acetic acid i.p injection with in-depth behavioral analyses as a novel model to characterize potential nociception-related behaviors in zebrafish. Here, we showed that acetic acid acutely decreases locomotion and induces a characteristic abdominal constriction-like phenotype (measured as body curvature index in zebrafish), analogous to writhing response in rodents. Morphine co-administration prevented acetic acid-induced variations in the body curvature index and corrected some other behaviors. While naloxone antagonized the antinociceptive effects of morphine on body curvature index, zebrafish pretreated with an NSAID diclofenac showed no abnormal body curvature following acetic acid administration (albeit not rescuing other behavioral responses). Based on known pharmacological actions of morphine and diclofenac, we suggest that the abdominal constriction-like behavior represents a general nociception-related response to acetic acid i.p. injection, corrected by both clinically active analgesics with distinct antinociceptive mechanisms.

Studies involving nociception in teleost fish are important to characterize the functionality of distinct classes of nociceptors across taxa and species [30]. Mounting evidence suggests that zebrafish express necessary cellular components to recognize nociceptive agents, and display complex abnormal behaviors in responses to algogens [22,24,39]. Since pain perception in animal models is subjective, the

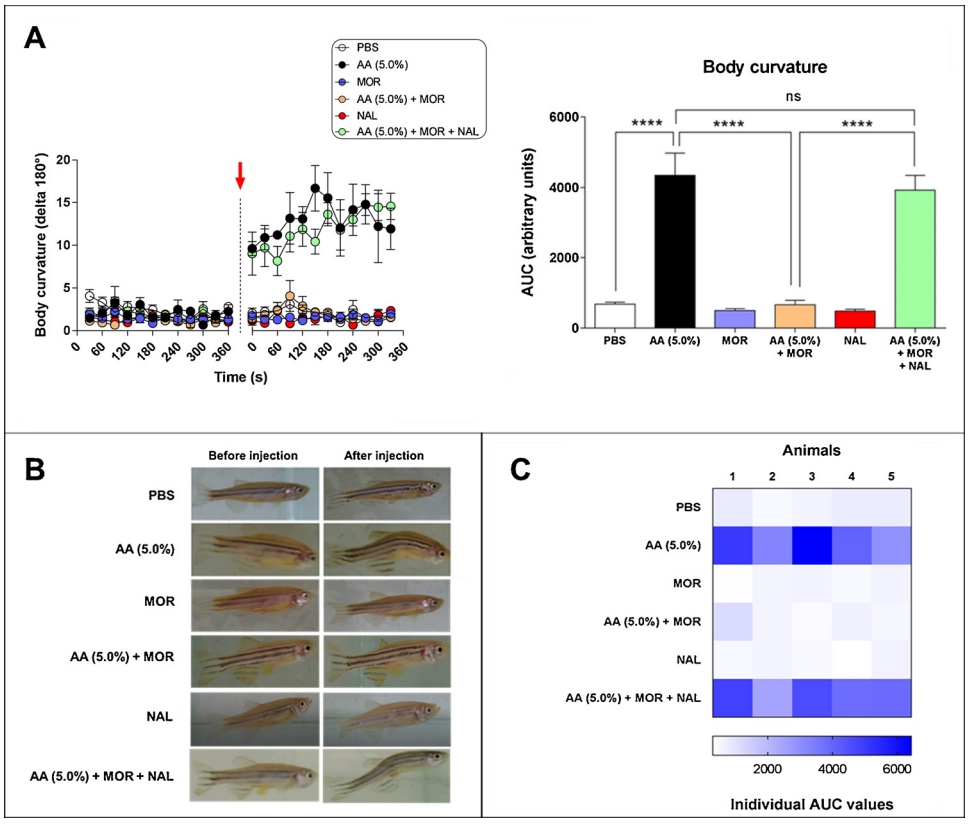


Fig. 4. Effects of morphine (MOR, 2.5 mg/kg, i.p.) and naloxone (NAL, 5.0 mg/kg, i.p.) on 5.0% acetic acid (AA)-induced changes in zebrafish body curvature index. (A) Changes in body curvature index in PBS, AA, MOR, AA + MOR, NAL, and AA + MOR + NAL groups across time. The red arrow indicates the moment of injection. Area under curve (AUC) values were calculated and expressed as arbitrary units. Data are expressed as means ± S.E.M. and analyzed by one-way ANOVA (factor: treatment), followed by Student-Newman-Keuls post-hoc test for significant ANOVA data (ns = no significance; **** $p < 0.001$; $n = 5$ per group). (B) Representative images displaying the zebrafish phenotypes before and after the treatments. (C) Heat map diagram showing the values of AUC obtained for each fish tested.

recognition of pain- and nociception-related phenotypes is key for understanding how noxious stimuli influence animal physiology and behavior [40,41]. In the present study, acetic acid increased the body curvature index, which was sustained for 25–30 min after the injection (Supplementary Fig. S3). Because the maximal response was observed within the first 6 min after injection, all behaviors were analyzed using this respective period. Moreover, as we did not detect overt sex differences in zebrafish responses to 5.0% acetic acid i.p. administration (see Supplementary Fig. S1), male and female data were pooled in our experiments. Although neurochemical mechanisms underlying pain perception can differ between sexes in rodents and humans [42,43], previous other data also showed no sex differences in zebrafish behavioral responses following 5.0% acetic acid administration [44], corroborating our present results.

Furthermore, we observed that acetic acid at various doses consistently reduced locomotion and vertical activity in zebrafish in the novel tank test. However, only 5.0% acetic acid decreased freezing duration (inducing hypolocomotion), while 1.0, 2.5, and 5.0% reduced

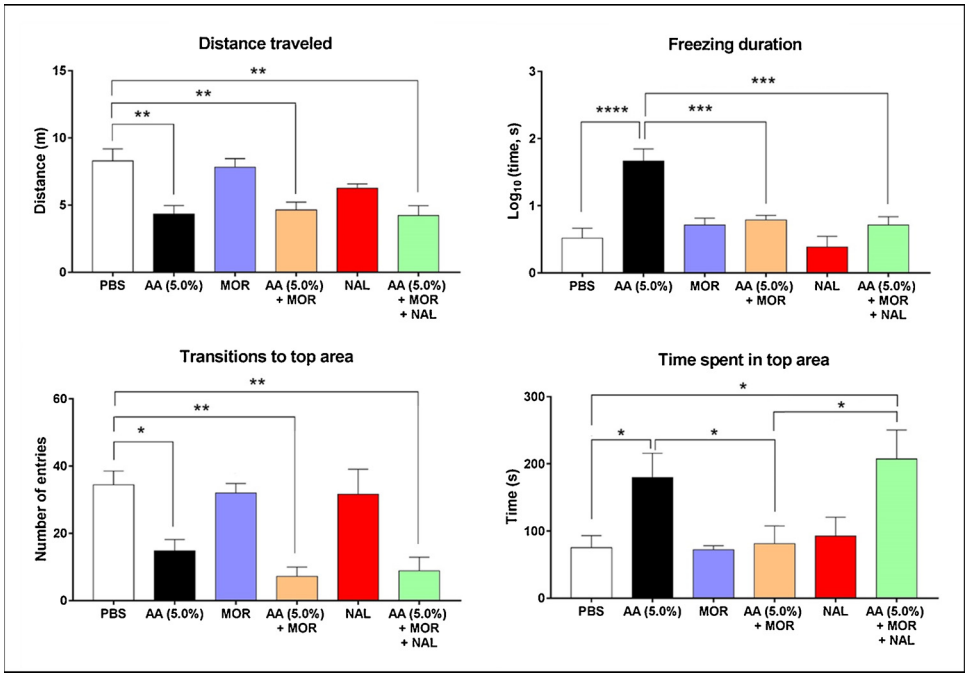


Fig. 5. Effects of morphine (MOR, 2.5 mg/kg, i.p.) and naloxone (NAL, 5.0 mg/kg, i.p.) on 5.0% acetic acid (AA)-induced changes in zebrafish behavior. Locomotor and exploratory endpoints were assessed by distance traveled, freezing duration, number of entries and time spent in top area. Data are expressed as means ± S.E.M. and analyzed by one-way ANOVA (factor: treatment), followed by Student-Newman-Keuls post-hoc test for significant ANOVA data (*$p < 0.05$; **$p < 0.01$; ***$p < 0.005$, ****$p < 0.001$; $n = 5$ per group).

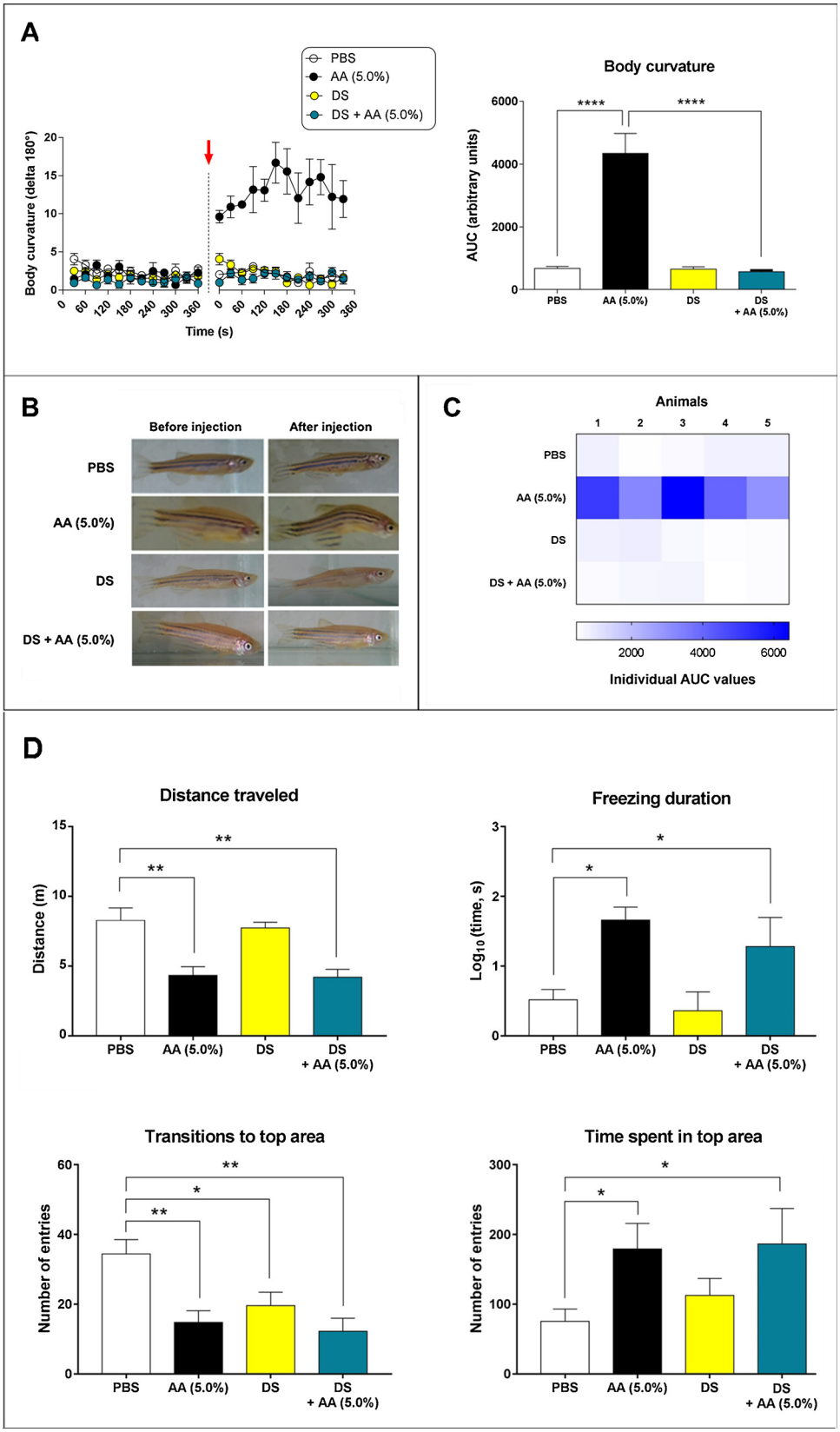


Fig. 6. Diclofenac (DS, 40 mg/kg, i.p.) pretreatment prevents 5.0% acetic acid (AA)-induced changes in zebrafish body curvature index (A-C), but does not affect other behavioral endpoints (D). (A) Changes in body curvature index in PBS, AA, DS, and DS + AA groups across time. The red arrow indicates the moment of injection. Area under curve (AUC) values were calculated and expressed as arbitrary units. Data are expressed as means ± S.E.M. and analyzed by two-way ANOVA (factors: acetic acid and diclofenac), followed by Student-Newman-Keuls post-hoc test for significant ANOVA data (****$p < 0.001$). (B) Representative images displaying the zebrafish phenotypes before and after the treatments. (C) Heat map diagram showing the values of AUC obtained for each fish tested. (D) Brief summary of the effects of diclofenac pretreatment on AA-induced changes in zebrafish locomotor and exploratory endpoints. Data are expressed as means ± S.E.M. and analyzed by two-way ANOVA (factors: acetic acid and diclofenac), followed by Student-Newman-Keuls post-hoc test for significant ANOVA data (*$p < 0.05$; **$p < 0.01$; $n = 5$ per group).

time spent in top (reflecting lower exploratory activity in this model) [45]. Notably, locomotor deficits are commonly linked to poorer animal welfare in both rodents and zebrafish [19,21,46]. Despite the relatively simpler organization of CNS in teleosts than in mammals, zebrafish have complex and well-described behaviors that extends far beyond simple instinctive/reflective reactions to various stimuli [23]. For example, multiple drugs that acts on the CNS without effects on nociception can modulate the vertical activity of zebrafish [45,47,48]. Thus, while vertical positioning in the tank involves central processing beyond the reflex responses to peripheral nociceptive input, locomotion

and exploration behavioral endpoints may reflect affected animal physiology when exposed to a noxious stimulus [49–51].

Activating the ASIC receptors, acetic acid i.p. administration in rodents is considered a well-established model for analgesic drug research [52]. In zebrafish, six ASICs have already been identified (zASIC1.1, zASIC1.2, zASIC1.3, zASIC2, zASIC4.1, and zASIC4.2) [53], and their phylogenetic analyses show that zASIC1.1, zASIC2, and zASIC4.1 are orthologous to mammals [53], while zASIC2 and zASIC 4.2 are not activated by acids [54]. Although more studies are needed to clarify whether zASICs activation plays a role in the abdominal constriction-like phenotype reported here, measuring the changes in body curvature index represents a simple, low-cost, and fast protocol to screen potential antinociceptive drugs using the acetic acid model reported here. Moreover, this behavioral endpoint closely parallels the writhing response of rodents that reflects direct activation of nociceptors following acetic acid administration.

While the writhing response in mice is observed for 0.6% acetic acid [26,55], the abdominal constriction-like phenotype in zebrafish occurs following 2.5 and 5.0% acetic acid i.p. administration. Since this may reflect different sensitivity to acetic acid in rodents and fish, in order to better pharmacologically dissect zebrafish nociceptive responses, we administered two traditional analgesics, morphine and diclofenac. Both drugs predictably abolished acetic acid-induced abnormal body curvature, thereby confirming the relation of this endpoint to nociception and its good predictive validity, given their efficacy in preventing this response. In rodents, morphine prevents the writhing phenotype at doses ranging from 0.18 to 5.0 mg/kg [56]. Because the zebrafish opioid system is generally similar (both pharmacologically and biochemically) to its mammalian counterpart [12], and since naloxone counteracted the effects of morphine observed here, it is possible that activation of opioid receptors negatively modulates the abdominal constriction-like response in the acid acetic i.p. model of nociception developed here.

5. Conclusion

In summary, our data show that zebrafish display an aberrant abdominal phenotype following a single acetic acid i.p. injection, analogous to writhing response described in rodents, accompanied by other behavioral changes (e.g., reduced locomotion, increased freezing, and changes on vertical activity). We also suggest that variations in body curvature may reflect a specific nociception-related phenotype, which is simple, easy-to-quantify and prevented by clinically active analgesics. Collectively, this indicates that acetic acid i.p. administration offers an excellent opportunity for assessing nociception-related behavioral phenotypes in zebrafish pain models. However, although the opioid system mediates antinociception in the acetic acid i.p. injection model, future studies of other relevant pain-related biomarkers (i.e., modulation of specific genes, receptors, as well as cortisol levels) may provide further insights into the mechanisms underlying acetic acid-mediated nociceptive responses in zebrafish.

Conflict of interest

The authors declare no competing interests.

Acknowledgments

The authors recognize financial support and fellowships from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Finance Code 001. F.V.C. (process number 88882.182161/2018-01), L.V.R. (process 88882.182163/2018-01), and V.A.Q. (process 88882.182127/2018-01) are recipients of fellowship from CAPES. D.B.R. and A.R.S. are recipients of the CNPq research productivity grant. D.B.R. is also supported by PROEX/CAPES fellowship grant (process 23038.005848/2018-31; grant 0737/2018) A.V.K. is the Chair of the International Zebrafish Neuroscience Research Consortium (ZNRC). His research is supported by the Russian Foundation for Basic Research (RFBR) grant 16-04-00851. All authors contributed to the preparation of the manuscript and approved its final version. The funders had no influence on the study design, collection, analysis, and interpretation of data, as well as on writing and submission of this manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.bbr.2018.10.009.

References

[1] J.S. Mogil, K.D. Davis, S.W. Derbyshire, The necessity of animal models in pain research, Pain 151 (1) (2010) 12–17.
[2] C.J. Woolf, What is this thing called pain? J. Clin. Invest. 120 (11) (2010) 3742–3744.
[3] J.D. Loeser, R.D. Treede, The Kyoto protocol of IASP basic pain terminology, Pain 137 (3) (2008) 473–477.
[4] A.I. Basbaum, D.M. Bautista, G. Scherrer, D. Julius, Cellular and molecular mechanisms of pain, Cell 139 (2) (2009) 267–284.
[5] E.R. Perl, Pain mechanisms: a commentary on concepts and issues, Prog. Neurobiol. 94 (1) (2011) 20–38.
[6] C.S. Sherrington, Observations on the scratch-reflex in the spinal dog, J. Physiol. 34 (1-2) (1906) 1–50.
[7] D. Julius, A.I. Basbaum, Molecular mechanisms of nociception, Nature 413 (6852) (2001) 203–210.
[8] G.W. Stevenson, E.J. Bilsky, S.S. Negus, Targeting pain-suppressed behaviors in preclinical assays of pain and analgesia: effects of morphine on acetic acid-suppressed feeding in C57BL/6J mice, J. Pain 7 (6) (2006) 408–416.
[9] F.C.C. Meotti, S. I dos, A.R. Santos, The nociception induced by glutamate in mice is potentiated by protons released into the solution, J. Pain 11 (6) (2010) 570–578.
[10] L.U. Sneddon, V.A. Braithwaite, M.J. Gentle, Novel object test: examining nociception and fear in the rainbow trout, J. Pain 4 (8) (2003) 431–440.
[11] X. Du, B. Yuan, J. Wang, X. Zhang, L. Tian, T. Zhang, X. Li, F. Zhang, Effect of heat-reinforcing needling on serum metabolite profiles in rheumatoid arthritis rabbits with cold syndrome, Zhongguo Zhen Jiu 37 (9) (2017) 977–983.
[12] V. Gonzalez-Nunez, R.E. Rodriguez, The zebrafish: a model to study the endogenous mechanisms of pain, ILAR J. 50 (4) (2009) 373–386.
[13] P. Gau, J. Poon, C. Ufret-Vincenty, C.D. Snelson, S.E. Gordon, D.W. Raible, A. Dhaka, The zebrafish ortholog of TRPV1 is required for heat-induced locomotion, J. Neurosci. 33 (12) (2013) 5249–5260.
[14] D.A. Prober, S. Zimmerman, B.R. Myers, B.M. McDermott, S.H. Kim, S. Caron, J. Rihel, L. Solnica-Krezel, D. Julius, A.J. Hudspeth, A.F. Schier, Zebrafish TRPA1 channels are required for chemosensation but not for thermosensation or mechanosensory hair cell function, J. Neurosci. 28 (40) (2008) 10102–10110.
[15] A.K. Dymowska, D. Boyle, A.G. Schultz, G.G. Goss, The role of acid-sensing ion channels in epithelial Na+ uptake in adult zebrafish (*Danio rerio*), J. Exp. Biol. 218 (Pt 8) (2015) 1244–1251.
[16] E. Viña, V. Parisi, F. Abbate, R. Cabo, M.C. Guerrera, R. Laura, L.M. Quiros, J.C. Perez-Varela, T. Cobo, A. Germana, J.A. Vega, O. Garcia-Suarez, Acid-sensing ion channel 2 (ASIC2) is selectively localized in the cilia of the non-sensory olfactory epithelium of adult zebrafish, Histochem. Cell Biol. 143 (1) (2015) 59–68.
[17] M. van der Vaart, H.P. Spaink, A.H. Meijer, Pathogen recognition and activation of the innate immune response in zebrafish, Adv. Hematol. 2012 (2012) 159807.
[18] J. Watzke, K. Schirmer, S. Scholz, Bacterial lipopolysaccharides induce genes involved in the innate immune response in embryos of the zebrafish (*Danio rerio*), Fish Shellfish Immunol. 23 (4) (2007) 901–905.
[19] A. Curtright, M. Rosser, S. Goh, B. Keown, E. Wagner, J. Sharifi, D.W. Raible, A. Dhaka, Modeling nociception in zebrafish: a way forward for unbiased analgesic discovery, PLoS One 10 (1) (2015) e0116766.
[20] J.C. Taylor, L.S. Dewberry, S.K. Totsch, L.R. Yessick, J.J. DeBerry, S.A. Watts, R.E. Sorge, A novel zebrafish-based model of nociception, Physiol. Behav. 174 (2017) 83–88.
[21] A.D. Correia, S.R. Cunha, M. Scholze, E.D. Stevens, A novel behavioral fish model of nociception for testing analgesics, Pharmaceuticals (Basel) 4 (4) (2011) 665–680.
[22] S.C. Reilly, J.P. Quinn, A.R. Cossins, L.U. Sneddon, Behavioural analysis of a nociceptive event in fish: comparisons between three species demonstrate specific responses, Appl. Anim. Behav. Sci. 114 (2008) 248–259.
[23] A.V. Kalueff, M. Gebhardt, A.M. Stewart, J.M. Cachat, M. Brimmer, J.S. Chawla, C. Craddock, E.J. Kyzar, A. Roth, S. Landsman, S. Gaikwad, K. Robinson, E. Baatrup, K. Tierney, A. Shamchuk, W. Norton, N. Miller, T. Nicolson, O. Braubach, C.P. Gilman, J. Pittman, D.B. Rosemberg, R. Gerlai, D. Echevarria, E. Lamb, S.C. Neuhauss, W. Weng, L. Bally-Cuif, H. Schneider, C. Zebrafish neuroscience research, towards a comprehensive catalog of zebrafish behavior 1.0 and beyond, Zebrafish 10 (1) (2013) 70–86.
[24] C. Maximino, Modulation of nociceptive-like behavior in zebrafish (*Danio rerio*) by environmental stressors, Psychol. Neurosci. 4 (1) (2011) 149–155.

[25] P.G.S. Schroeder, L.U. Sneddon, Exploring the efficacy of immersion analgesics in zebrafish using an integrative approach, Appl. Anim. Behav. Sci. 187 (2017) 93–102.
[26] H.O. Collier, L.C. Dinneen, C.A. Johnson, C. Schneider, The abdominal constriction response and its suppression by analgesic drugs in the mouse, Br. J. Pharmacol. Chemother. 32 (2) (1968) 295–310.
[27] A. Avdesh, M. Chen, M.T. Martin-Iverson, A. Mondal, D. Ong, S. Rainey-Smith, K. Taddei, M. Lardelli, D.M. Groth, G. Verdile, R.N. Martins, Regular care and maintenance of a zebrafish (*danio rerio*) laboratory: an introduction, J. Vis. Exp (69) (2012) e4196.
[28] M.D. Kinkel, S.C. Eames, L.H. Philipson, V.E. Prince, Intraperitoneal injection into adult zebrafish, J. Vis. Exp. (42) (2010).
[29] S.K. Richetti, M. Blank, K.M. Capiotti, A.L. Piato, M.R. Bogo, M.R. Vianna, C.D. Bonan, Quercetin and rutin prevent scopolamine-induced memory impairment in zebrafish, Behav. Brain Res. 217 (1) (2011) 10–15.
[30] L.U. Sneddon, V.A. Braithwaite, M.J. Gentle, Do fishes have nociceptors? Evidence for the evolution of a vertebrate sensory system, Proc. Biol. Sci. 270 (1520) (2003) 1115–1121.
[31] H. Pathan, J. Williams, Basic opioid pharmacology: an update, Br. J. Pain. 6 (1) (2012) 11–16.
[32] F.R. Hamann, A.M. Zago, M.F. Rossato, V.R. Beck, C.F. Mello, T.F. de Brum, L.M. de Carvalho, H. Faccin, S.M. Oliveira, M.A. Rubin, Antinociceptive and antidepressant-like effects of the crude extract of *Vitex megapotamica* in rats, J. Ethnopharmacol. 192 (2016) 210–216.
[33] M. Llorca-Torralba, J.A. Mico, E. Berrocoso, Behavioral effects of combined morphine and MK-801 administration to the locus coeruleus of a rat neuropathic pain model, Prog. Neuropsychopharmacol. Biol. Psychiatry 84 (Pt A) (2018) 257–266.
[34] C.I.B. Walker, S.M. Oliveira, R. Tonello, M.F. Rossato, E. da Silva Brum, J. Ferreira, G. Trevisan, Anti-nociceptive effect of stigmasterol in mouse models of acute and chronic pain, Naunyn. Schmiedebergs Arch. Pharmacol. 390 (11) (2017) 1163–1172.
[35] J. Ferreira, A.E. Floriani, V.C. Filho, F. Delle Monache, R.A. Yunes, J.B. Calixto, A.R. Santos, Antinociceptive properties of the methanolic extract and two triterpenes isolated from Epidendrum mosenii stems (orchidaceae), Life Sci. 66 (9) (2000) 791–802.
[36] R. Altman, B. Bosch, K. Brune, P. Patrignani, C. Young, Advances in NSAID development: evolution of diclofenac products using pharmaceutical technology, Drugs 75 (8) (2015) 859–877.
[37] R. Rodrigues-Filho, M.M. Campos, J. Ferreira, A.R. Santos, J.A. Bertelli, J.B. Calixto, Pharmacological characterisation of the rat brachial plexus avulsion model of neuropathic pain, Brain Res. 1018 (2) (2004) 159–170.
[38] A.S. Hasani, M. Soljakova, M.H. Jakupi, S.Z. Ustalar-Ozgen, Preemptive analgesic effect of diclofenac: experimental study in rats, Middle East J. Anaesthesiol. 21 (3) (2011) 355–360.
[39] V. Malafoglia, B. Bryant, W. Raffaeli, A. Giordano, G. Bellipanni, The zebrafish as a model for nociception studies, J. Cell. Physiol. 228 (10) (2013) 1956–1966.
[40] C.J. Vierck, A.P. Mauderli, R.G. Wiley, Assessment of pain in laboratory animals: a comment on mogil and crager (2004), Pain 114 (3) (2005) 520–523 author reply 523-4.
[41] R.D. Treede, Assessment of pain as an emotion in animals and in humans, Exp. Neurol. 197 (1) (2006) 1–3.
[42] J. Etherton, M. Lawson, R. Graham, Individual and gender differences in subjective and objective indices of pain: gender, fear of pain, pain catastrophizing and cardiovascular reactivity, Appl. Psychophysiol. Biofeedback 39 (2) (2014) 89–97.
[43] B. Hellstrom, U. Lundberg, Pain perception to the cold pressor test during the menstrual cycle in relation to estrogen levels and a comparison with men, Integr. Physiol. Behav. Sci. 35 (2) (2000) 132–141.
[44] S. Dewberry, C. Taylor, L. Yessick, S. Totsch, S. Watts, R. Sorge, Development and validation of a partially automated model of inflammatory chronic pain in zebrafish, J. Pain 17 (4) (2016) S50.
[45] R.J. Egan, C.L. Bergner, P.C. Hart, J.M. Cachat, P.R. Canavello, M.F. Elegante, S.I. Elkhayat, B.K. Bartels, A.K. Tien, D.H. Tien, S. Mohnot, E. Beeson, E. Glasgow, H. Amri, Z. Zukowska, A.V. Kalueff, Understanding behavioral and physiological phenotypes of stress and anxiety in zebrafish, Behav. Brain Res. 205 (1) (2009) 38–44.
[46] M.G. Rahbardar, B. Amin, S. Mehri, S.J. Mirnajafi-Zadeh, H. Hosseinzadeh, Rosmarinic acid attenuates development and existing pain in a rat model of neuropathic pain: an evidence of anti-oxidative and anti-inflammatory effects, Phytomedicine 40 (2018) 59–67.
[47] P.R. Ziani, T.E. Muller, F.V. Stefanello, B.D. Fontana, T. Duarte, J. Canzian, D.B. Rosemberg, Nicotine increases fear responses and brain acetylcholinesterase activity in a context-dependent manner in zebrafish, Pharmacol. Biochem. Behav. 170 (2018) 36–43.
[48] L.V. Rosa, A.P. Ardais, F.V. Costa, B.D. Fontana, V.A. Quadros, L.O. Porciuncula, D.B. Rosemberg, Different effects of caffeine on behavioral neurophenotypes of two zebrafish populations, Pharmacol. Biochem. Behav. 165 (2018) 1–8.
[49] T.S. Kristiansen, A. Ferno, J.C. Holm, L. Privitera, S. Bakke, J.E. Fosseidengen, Swimming behaviour as an indicator of low growth rate and impaired welfare in Atlantic halibut (*Hippoglossus hippoglossus L.*) Reared at three stocking densities, Aquaculture 230 (2004) 137–151.
[50] M.J. Gentle, Pain in birds, Anim. Welf. 1 (1992) 235–247.
[51] G.W. Stevenson, J. Cormier, H. Mercer, C. Adams, C. Dunbar, S.S. Negus, E.J. Bilsky, Targeting pain-depressed behaviors in preclinical assays of pain and analgesia: drug effects on acetic acid-depressed locomotor activity in ICR mice, Life Sci. 85 (7-8) (2009) 309–315.
[52] E. Siegmund, R. Cadmus, G. Lu, A method for evaluating both non-narcotic and narcotic analgesics, Proc. Soc. Exp. Biol. Med. 95 (4) (1957) 729–731.
[53] M. Levantia, B. Randazzoa, E. Viña, G. Montalbanoa, O. Garcia-Suarezb, A. Germanàa, J.A. Vega, F. Abbatea, Acid-sensing ion channels and transient-receptor potential ionchannels in zebrafish taste buds, Ann. Anatomy 207 (2016) 32–37.
[54] M. Paukert, S. Sidi, C. Russell, M. Siba, S.W. Wilson, T. Nicolson, S. Grunder, A family of acid-sensing ion channels from the zebrafish: widespread expression in the central nervous system suggests a conserved role in neuronal communication, J. Biol. Chem. 279 (18) (2004) 18783–18791.
[55] R.I. Taber, D.D. Greenhouse, J.K. Rendell, S. Irwin, Agonist and antagonist interactions of opioids on acetic acid-induced abdominal stretching in mice, J. Pharmacol. Exp. Ther. 169 (1) (1969) 29–38.
[56] M. Shamsi Meymandi, F. Keyhanfar, Assessment of the antinociceptive effects of pregabalin alone or in combination with morphine during acetic acid-induced writhing in mice, Pharmacol. Biochem. Behav. 110 (2013) 249–254.

ARTIGO 3

Naloxone prolongs abdominal constriction writhing-like behavior in a zebrafish-based pain model

Fabiano V. Costa, Julia Canzian, Flavia V. Stefanello, Allan V. Kalueff, Denis B. Rosemberg

Neuroscience Letters (2019). Aug 24; 708:134336
DOI: 10.1016/j.neulet.2019.134336

Neuroscience Letters 708 (2019) 134336

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Research article

Naloxone prolongs abdominal constriction writhing-like behavior in a zebrafish-based pain model

Fabiano V. Costa[a,b,*,1], Julia Canzian[a,b,1], Flavia V. Stefanello[a], Allan V. Kalueff[c,d,e,f], Denis B. Rosemberg[a,b,f,*]

[a] Laboratory of Experimental Neuropsychobiology, Department of Biochemistry and Molecular Biology, Natural and Exact Sciences Center, Federal University of Santa Maria, 1000 Roraima Avenue, Santa Maria, RS, 97105-900, Brazil
[b] Graduate Program in Biological Sciences: Toxicological Biochemistry, Federal University of Santa Maria. 1000 Roraima Avenue, Santa Maria, RS, 97105-900, Brazil
[c] School of Pharmaceutical Sciences, Southwest University, Chongqing 400716, China
[d] Institute of Experimental Medicine, Almazov National Medical Research Center, St. Petersburg 197341, Russia
[e] Ural Federal University, 19 Mira street, Ekaterinburg, 620002, Russia
[f] The International Zebrafish Neuroscience Research Consortium (ZNRC), 309 Palmer Court, Slidell, LA 70458, USA

ARTICLE INFO

Keywords:
Nociception
Abdominal constriction writhing-like behavior
Acetic acid
Naloxone
Zebrafish

ABSTRACT

The ability to detect noxious stimuli is essential to survival. However, pathological pain is maladaptive and severely debilitating. Endogenous and exogenous opioids modulate pain responses *via* opioid receptors, reducing pain sensibility. Due to the high genetic and physiological similarities to rodents and humans, the zebrafish is a valuable tool to assess pain responses and the underlying mechanisms involved in nociception. Although morphine attenuates pain-like responses of zebrafish, there are no data showing if the antagonism of opioid receptors prolongs pain duration in the absence of an exogenous opioid. Here, we investigated whether a common opioid antagonist naloxone affects the abdominal constriction writhing-like response, recently characterized as a zebrafish-based pain behavior. Animals were injected intraperitoneally with acetic acid (5.0%), naloxone (1.25 mg/kg; 2.5 mg/kg; 5.0 mg/kg) or acetic acid with naloxone to investigate the changes in their body curvature for 1 h. Acetic acid elicited a robust pain-like response in zebrafish, as assessed by aberrant abdominal body curvature, while no effects were observed following PBS injection. Although naloxone alone did not alter the frequency and duration of this behavior, it dose-dependently prolonged acetic acid-induced abdominal curvature response. Besides reinforcing the use of the abdominal writhing-like phenotype as a behavioral endpoint to measure acute pain responses in zebrafish models, our novel data suggest a putative role of endogenous opioids in modulating the recovery from pain stimulation in zebrafish.

1. Introduction

Pain is a serious health problem that affects 20% of adult population worldwide [18]. Thus, understanding the basic physiological mechanisms underlying pain is a cornerstone for developing new therapeutic strategies [18]. Pain is frequently comorbid with other disorders, including depression [2–4], anxiety [13,30], and other neuropsychiatric conditions [17]. Acute pain plays an adaptive role, eliciting fast physiological and behavioral response against tissue damage [23,24,28,39].

Pain activates synaptic terminals located on peripheral fibers, carrying the information to the brain which triggers descending pathways of pain [7,15,33,40]. Collectively, these biochemical events also release endogenous opioid peptides (β-endorphins, enkephalins, and dynorphins), evoking adaptive analgesic effects in vertebrates [11,40]. As important regulators of nociception, these peptides are of great interest in drug addiction research [14,29], including functional studies of a key role of opioid system in modulating pain-like responses in novel experimental models, such as fish [5,13,14,31].

The zebrafish (*Danio rerio*) is a promising alternative model organism in translational pain research [10,22]. This species possess the classic μ (ZfMOP), δ (ZfDOP1 and ZfDOP2), and κ (ZfKOP) opioid receptors, as well as agonist opioid peptide precursors with high similarity to their human orthologs [1,5,6,13,22,29]. Mounting evidence shows that fish are sentient species and discriminate pain caused by different stimuli [10,12,32]. For example, the abdominal constriction

* Corresponding authors.
E-mail addresses: fvcosta88@gmail.com (F.V. Costa), dbrosemberg@gmail.com (D.B. Rosemberg).
[1] Both first authors.

https://doi.org/10.1016/j.neulet.2019.134336
Received 6 May 2019; Received in revised form 7 June 2019; Accepted 16 June 2019
Available online 18 June 2019

writhing-like behavior following acetic acid intraperitoneal (i.p.) injection has been recently described in adult zebrafish [10]. Because this phenotype is pharmacologically sensitive to classical analgesics (*e.g.*, morphine and diclofenac) this model shows a high degree of predictive validity to assess pain-like responses in zebrafish [10,37]. Although naloxone (a non-selective opioid antagonist) antagonizes morphine-induced analgesia [10,12], there is no evidence suggesting a putative role of endogenous opioid system in modulating pain-like behaviors in zebrafish so far. To further test this hypothesis, the goal of this study was to investigate whether naloxone can dose-dependently prolong the recovery of abdominal constriction writhing-like responses following acetic acid i.p. injection in zebrafish.

2. Materials and methods

2.1. Animals

Wild-type adult zebrafish (*Danio rerio*) (~50:50 male:female ratio, 4–6 months-old, weighing 0.25–0.3 g, short-fin phenotype) were obtained from a local commercial supplier (Hobby Aquários, RS, Brazil) and kept for two weeks in a 50-L thermostatic aquarium filled with aerated non-chlorinated water at 27 ± 1 °C, pH 7.0–7.2. Fluorescent ceiling-mounted light tubes provided room illumination on a 14/10 light/dark photoperiod cycle (lights on at 7:00 am and off at 9:00 pm). Animals were fed thrice daily with commercial flake food (Alcon BASIC™, Alcon, Brazil). All protocols were approved by the Ethic Commission on Animal Use of The Federal University of Santa Maria (Protocol 5438310817) and animal experimentation fully adhered the National Institute of Health Guide for Care and Use of Laboratory.

2.2. Drugs

Acetic acid (99%), naloxone methiodide, and phosphate buffered saline (PBS) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.3. Experimental protocol

All behavioral tests were performed between 09:00 am and 4:00 pm. Experiments were performed using acetic acid (5.0%, i.p.) to induce pain responses, and PBS as vehicle [10]. Injections were performed with a BD Ultra-fine™ 30U syringe (needle size 6 mm × 0.25 mm) at a volume of 10 µL. First, animals were anesthetized in cold water [25] and injected i.p. with PBS (control), acetic acid alone, naloxone alone, or acetic acid plus naloxone at three doses (1.25, 2.5, and 5.0 mg/kg). The doses of naloxone used here were chosen based on previous reports [10,37]. After injection, animals were individually transferred to observation tanks (15 × 13 × 10 cm, length × height × width) with a 10 cm water column height, and their behaviors were recorded for 1 h using a digital camera (Nikon Coolpix P900, Tokyo, Japan). Animals were further anesthetized in cold water (4 °C) and then euthanized by decapitation. Because no sex differences are observed in fish behavior following acetic acid i.p. administration [10], both male and female zebrafish were separated from their home tanks and divided randomly for each cohort by a computerized random number generator (www.random.org).

The abdominal constriction writhing-like phenotype was used as a behavioral endpoint to measure pain-like responses, as described elsewhere [10]. Briefly, frontal photos were taken every 30 s and later analyzed using ImageJ 1.45 for Windows. We selected three positions: a frontal (in the front of head), a central (middle of the animal body – between anal and dorsal fins) and posterior (at the dorsal fin) to estimate the fish body curvature. Results were subtracted from 180° and multiplied by (-1) to estimate the body curvature index. Temporal variations in the body curvature index were measured by two trained observers blinded to the experimental condition (inter-rater reliability > 0.90). Fig. 1 outlines the experimental design of the present study.

2.4. Statistics

Data normality and homogeneity of variances were analyzed by Kolmogorov–Smirnov and Bartlett's tests, respectively. Changes in the body curvature index were analyzed by unpaired Student's *t*-test and latencies to recover pain-like behavior were analyzed using one-way analysis of variance (ANOVA) (treatment as factor). Temporal variations in the behavior were analyzed using repeated measures ANOVA (treatment as factor and time as repeated measure). *Post hoc* comparisons were made using Tukey's test when necessary. Results were expressed as means ± standard error of the mean (S.E.M.), and statistical significance was set at $p \leq 0.05$.

3. Results

To assess whether the antagonism of opioid receptors prolongs the abdominal constriction writhing-like response in a dose-dependent manner, animals were injected with a single concentration of acetic acid (5.0%) in the presence or absence naloxone (1.25, 2.5 and 5.0 mg/kg). Fig. 2 shows the temporal effects of naloxone and acetic acid on the body curvature index for 1 h. Repeated measures ANOVA yielded significant effects of treatment x time interaction ($F_{(595,\ 2856)} = 21.97$, $p < 0.0001$), time ($F_{(119,\ 2856)} = 92.24$, $p < 0.0001$), and treatment ($F_{(5,\ 24)} = 1375$, $p < 0.0001$). Acetic acid induced a robust abdominal constriction writhing-like behavior, with maximal response observed 120 s after the injection. The area under curve (AUC) analyses showed that naloxone alone (5.0 mg/kg, i.p.) did not modify the abdominal curvature compared to the PBS group ($t_{(df\ =\ 8)} = 1.03$, $p = 0.3333$) (Fig. 3). Similarly, both lower doses of naloxone alone (1.25 and 2.5 mg/kg) did not alter the body curvature index (data not shown). However, fish cotreated with naloxone (1.25, 2.5, and 5.0 mg/kg) and 5.0% acetic acid showed longer latency to return to the baseline normal curvature than fish injected with acetic acid alone ($F_{(3,16)} = 1577$, $p < 0.0001$) (Fig. 4).

4. Discussion

The present study examined whether the non-selective blockade of opioid receptors modulates selected pain-like responses in zebrafish injected with acetic acid. Our novel findings show that naloxone markedly prolongs the recovery of abdominal constriction writhing-like behavior, suggesting that the activation of opioid receptors by endogenous ligands may play a role in adaptive antinociception in zebrafish.

Zebrafish are responsive to acetic acid administration, which activates acid-sensing ion channels (zASIC) [26] and elicits pain-like responses [9]. While morphine induces analgesia in the acetic acid model described here, the co-administration of naloxone blocked such effect, supporting the involvement of opioid system in zebrafish antinociception [10,37]. Likewise, both zebrafish µ-opioid receptor and peptide precursors share a high molecular homology with their respective human counterparts [21,27].

Interestingly, naloxone alone (0.5–5 mg/L) elicits anxiogenic-like effects in adult zebrafish by reducing the time spent in top area of the novel tank and increasing the number of erratic movements [35]. Like rodents [16,34], zebrafish display robust behavioral responses to morphine and naloxone, which modulate rewarding properties and the development of withdrawal-like symptoms [8]. For example, naloxone precipitates withdrawal-like syndrome in zebrafish, implicating the opioidergic system in such response [34]. Here, in line with these findings, we showed that naloxone (1.25–5.0 mg/kg) dose-dependently prolonged pain-like responses in adult zebrafish induced by acetic acid.

Notably, the opioid peptide precursors reported in zebrafish include two proenkephalin genes (PENKa and PENKb), two

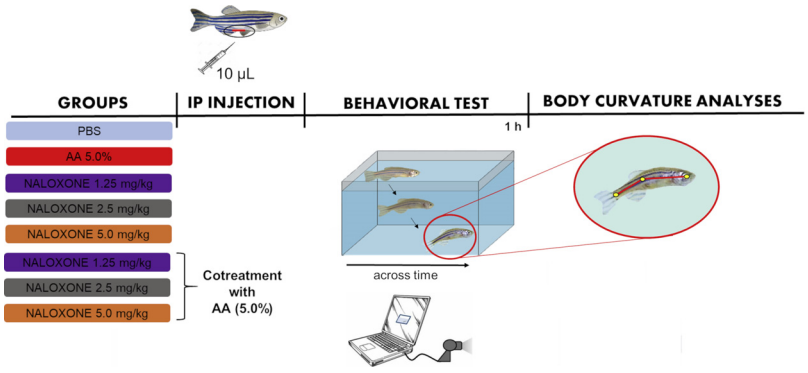


Fig. 1. A brief summary of the experimental protocol. Pain-like responses were measured following vehicle (PBS), acetic acid (AA), naloxone, and AA plus naloxone intraperitoneal (i.p.) injections. Behaviors were analyzed for 1 h after treatments for three body points (frontal, central and posterior, denoted by yellow dots) used to estimate the angular body curvature.

proopiomelanocortin genes (POMCa and POMCb), one prodynorphin gene (PDYN), and one pronociceptin gene (PNOC). PENKa codes four Met-ENKs (ME), one Met-enkephalin-Ile (MEI), and one Met-enkephalin-Asp (MED) [21,22,36]. PENKb codes four Met-enkephalins (ME) one Leu-enkephalin (LE), and one Met-enkephalin-Gly-Tyr (MEGY) [19]. POMCa codes for adenocorticotropin (ACTH), γ-lipotropin (γ-LPH), β-melanotropin (β-MSH) and β-endorphin (β-END), while POMCb codes for only α-MSH and β-END [20]. Interestingly, naloxone shows a highest affinity toward zebrafish μ-opioid receptor (followed by β-END = morphine > MEGY > ME > LE) and this species also has some duplicated genes of opioid peptide precursors [22,29]. Thus, it is difficult to compare results across species, as well as establish a specific mechanism of action, since possible differences in binding affinities may also exist. Although we cannot rule out the involvement of endogenous opioids in the results described here, the activation of opioid receptors play a role in the recovery of abdominal constriction writhing-like behavior following acetic acid i.p. injection because naloxone, a potent opioid receptor antagonist, prolongs this response. Nevertheless, further studies are needed to probe the pharmacological properties of zebrafish opioid receptors and ligands in both pain- and pain-unrelated neurobehavioral models.

In conclusion, zebrafish treated with naloxone show delayed recovery of abdominal constriction writhing-like behaviors evoked by acetic acid i.p. administration. Although the involvement of human endogenous opioid system in pain-responses has long been known [14,33,38], the putative similar role for fish endogenous opioids in the

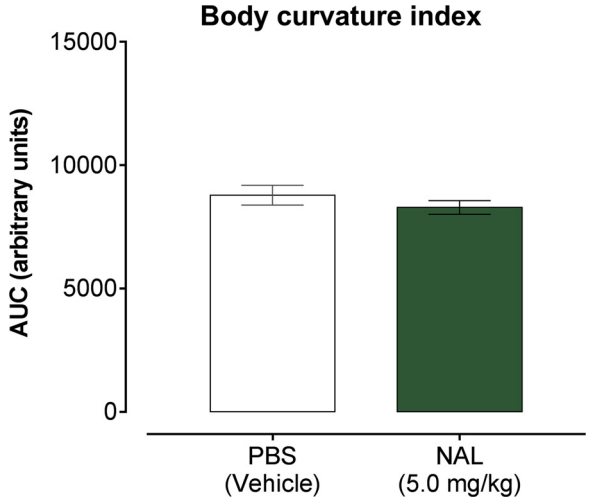


Fig. 3. Intraperitoneal naloxone injection does not change the body curvature index following vehicle (PBS) and naloxone (NAL) i.p. injection. Area under the curve (AUC) values were calculated and expressed as arbitrary units. Data are expressed as means ± S.E.M. and analyzed by unpaired Student's *t* test ($n = 5$ per group).

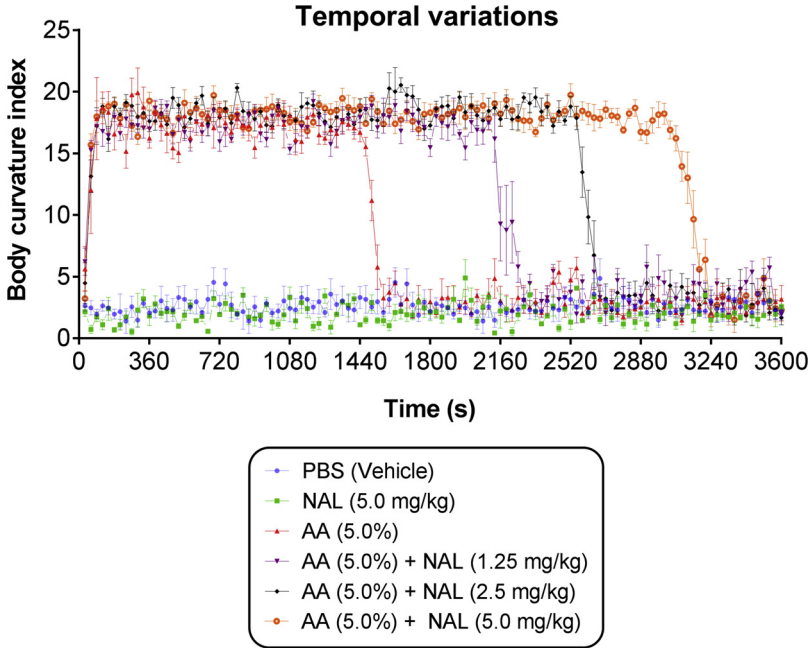


Fig. 2. Naloxone delays the recovery of abdominal constriction writhing-like phenotypes in adult zebrafish evoked by 5.0% acetic acid (AA) i.p. injection. Changes in the body curvature index (assessed as area under the curve, AUC) in vehicle (PBS), AA, naloxone (NAL), and AA + NAL groups are presented across time for the entire duration of 1-h testing. Data are expressed as means ± S.E.M. ($n = 5$ per group) and analyzed by repeated measured ANOVA followed by Tukey's test, considering $p < 0.05$ as significant.

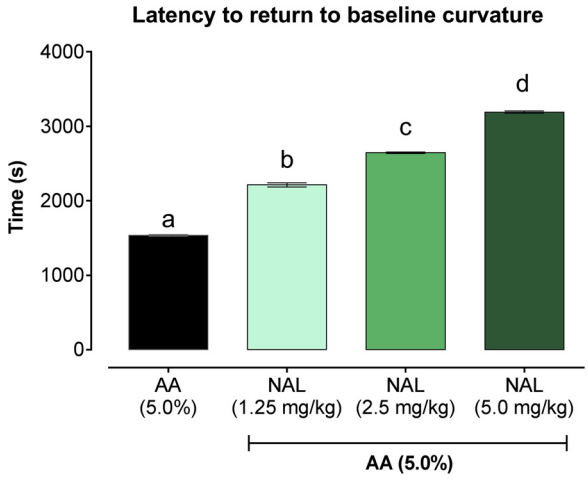


Fig. 4. Naloxone (NAL) dose-dependently prolongs writhing-like pain response induced by acetic acid (AA) in adult zebrafish, assessed as the latency to return to baseline (normal) abdominal curvature following AA (5.0%; control), NAL (1.25, 2.5 and 5 mg/kg) + AA (5.0%) i.p. injection. Data are expressed as means ± S.E.M. and analyzed by one-way ANOVA, followed by Tukey's *post hoc* test when necessary. Different letters indicate statistical differences among groups ($p < 0.05$, $n = 5$ per group).

recovery of pain-like phenotypes is novel, and merits further scrutiny. The present study may also have several additional implications. For example, despite some species differences in the opioidergic system between zebrafish and humans [5,13], such similarity in recovery phenotypes may support a higher (than previously recognized) degree of evolutionarily conserved pain biology and pathobiology across taxa. Suggesting the putative role of endogenous opioids in modulating the recovery from pain stimulation in adult zebrafish, these findings also corroborate the use of the abdominal writhing-like profiling as a new promising assay for further nociceptive and anti-nociceptive drug screening in this aquatic species. Finally, although the present study focused on adult specimens, the possibility of developing similar pain models for larval fish may also be critical.

Declaration of Competing Interest

The authors declare that no competing interests exist.

Acknowledgements

We recognize the financial support and fellowships from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS), and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). F.V.C. was recipient of CAPES fellowship. J.C. and F.V.S. receive the CNPq fellowship. D.B.R. is a recipient of CNPq research productivity grant (305051/2018-0) and his work is also supported by the PROEX/CAPES (process number 23038.005848/2018-31) and PRONEM/FAPERGS (process number 16/2551-0000248-7) fellowship grants. A.V.K. is the Chair of the International Zebrafish Neuroscience Research Consortium (ZNRC). His research is supported by the Russian Science Foundation (RSF) grant 19-15-00053. All authors contributed to the preparation of the manuscript and approved its final version. The funders had no influence on the study design, collection, analysis, and interpretation of data, as well as on writing and submission of this manuscript.

References

[1] F.A. Alvarez, I. Rodriguez-Martin, V. Gonzalez-Nunez, E. Marron Fernandez de Velasco, R. Gonzalez Sarmiento, R.E. Rodriguez, New kappa opioid receptor from zebrafish *Danio rerio*, Neurosci. Lett. 405 (2006) 94–99.

[2] B.A. Arnow, E.M. Hunkeler, C.M. Blasey, J. Lee, M.J. Constantino, B. Fireman, H.C. Kraemer, R. Dea, R. Robinson, C. Hayward, Comorbid depression, chronic pain, and disability in primary care, Psychosom. Med. 68 (2006) 262–268.

[3] M.J. Bair, R.L. Robinson, G.J. Eckert, P.E. Stang, T.W. Croghan, K. Kroenke, Impact of pain on depression treatment response in primary care, Psychosom. Med. 66 (2004) 17–22.

[4] M.J. Bair, R.L. Robinson, W. Katon, K. Kroenke, Depression and pain comorbidity: a literature review, Arch. Intern. Med. 163 (2003) 2433–2445.

[5] W. Bao, A.D. Volgin, E.T. Alpyshov, A.J. Friend, T.V. Strekalova, M.S. de Abreu, C. Collins, T.G. Amstislavskaya, K.A. Demin, A.V. Kalueff, Opioid Neurobiology, Neurogenetics and neuropharmacology in zebrafish, Neuroscience 404 (2019) 218–232.

[6] A. Barrallo, R. Gonzalez-Sarmiento, F. Alvar, R.E. Rodriguez, ZFOR2, a new opioid receptor-like gene from the teleost zebrafish (*Danio rerio*), Brain Res. Mol. Brain Res. 84 (2000) 1–6.

[7] R.J. Bodnar, Endogenous opiates and behavior: 2013, Peptides 62 (2014) 67–136.

[8] J. Cachat, P. Canavello, M. Elegante, B. Bartels, P. Hart, C. Bergner, R. Egan, A. Duncan, D. Tien, A. Chung, K. Wong, J. Goodspeed, J. Tan, C. Grimes, S. Elkhayat, C. Suciu, M. Rosenberg, K.M. Chung, F. Kadri, S. Roy, S. Gaikwad, A. Stewart, I. Zapolsky, T. Gilder, S. Mohnot, E. Beeson, H. Amri, Z. Zukowska, R.D. Soignier, A.V. Kalueff, Modeling withdrawal syndrome in zebrafish, Behav. Brain Res. 208 (2010) 371–376.

[9] X. Chen, G. Polleichtner, I. Kadurin, S. Grunder, Zebrafish acid-sensing ion channel (ASIC) 4, characterization of homo- and heteromeric channels, and identification of regions important for activation by H+, J. Biol. Chem. 282 (2007) 30406–30413.

[10] F.V. Costa, L.V. Rosa, V.A. Quadros, A.R.S. Santos, A.V. Kalueff, D.B. Rosemberg, Understanding nociception-related phenotypes in adult zebrafish: behavioral and pharmacological characterization using a new acetic acid model, Behav. Brain Res. 359 (2019) 570–578.

[11] B.M. Cox, M.J. Christie, L. Devi, L. Toll, J.R. Traynor, Challenges for opioid receptor nomenclature: IUPHAR Review 9, Br. J. Pharmacol. 172 (2015) 317–323.

[12] A. Curtright, M. Rosser, S. Goh, B. Keown, E. Wagner, J. Sharifi, D.W. Raible, A. Dhaka, Modeling nociception in zebrafish: a way forward for unbiased analgesic discovery, PLoS One 10 (2015) e0116766.

[13] K.A. Demin, D.A. Meshalkina, E.V. Kysil, K.A. Antonova, A.D. Volgin, O.A. Yakovlev, P.A. Alekseeva, M.M. Firuleva, A.M. Lakstygal, M.S. de Abreu, L.J.G. Barcellos, W. Bao, A.J. Friend, T.G. Amstislavskaya, D.B. Rosemberg, P.E. Musienko, C. Song, A.V. Kalueff, Zebrafish models relevant to studying central opioid and endocannabinoid systems, Prog. Neuropsychopharmacol. Biol. Psychiatry 86 (2018) 301–312.

[14] S. Dreborg, G. Sundstrom, T.A. Larsson, D. Larhammar, Evolution of vertebrate opioid receptors, Proc Natl Acad Sci U S A 105 (2008) 15487–15492.

[15] L. Garcia-Larrea, R. Peyron, Pain matrices and neuropathic pain matrices: a review, Pain 154 (Suppl 1) (2013) S29–43.

[16] C. Garcia-Lecumberri, I. Torres, S. Martin, J.A. Crespo, M. Miguens, C. Nicanor, A. Higuera-Matas, E. Ambrosio, Strain differences in the dose-response relationship for morphine self-administration and impulsive choice between Lewis and Fischer 344 rats, J. Psychopharmacol. (Oxford) 25 (2011) 783–791.

[17] B. Gerdle, J. Bjork, C. Henriksson, A. Bengtsson, Prevalence of current and chronic pain and their influences upon work and health care-seeking: a population study, J. Rheumatol. 31 (2004) 1399–1406.

[18] D.S. Goldberg, S.J. McGee, Pain as a global public health priority, BMC Public Health 11 (2011) 770.

[19] V. Gonzalez-Nunez, R. Gonzalez-Sarmiento, R.E. Rodriguez, Cloning and characterization of a full-length pronociceptin in zebrafish: evidence of the existence of two different nociceptin sequences in the same precursor, Biochim. Biophys. Acta 1629 (2003) 114–118.

[20] V. Gonzalez-Nunez, R. Gonzalez-Sarmiento, R.E. Rodriguez, Identification of two proopiomelanocortin genes in zebrafish (*Danio rerio)*, Brain Res. Mol. Brain Res. 120 (2003) 1–8.

[21] V. Gonzalez-Nunez, A. Jimenez Gonzalez, K. Barreto-Valer, R.E. Rodriguez, In vivo regulation of the mu opioid receptor: role of the endogenous opioid agents, Mol Med 19 (2013) 7–17.

[22] V. Gonzalez-Nunez, R.E. Rodriguez, The zebrafish: a model to study the endogenous mechanisms of pain, ILAR J. 50 (2009) 373–386.

[23] S. Hestehave, K.S.P. Abelson, T. Bronnum Pedersen, G. Munro, The analgesic efficacy of morphine varies with rat strain and experimental pain model; implications for target validation efforts in pain drug discovery, Eur. J. Pain (2018).

[24] D. Julius, A.I. Basbaum, Molecular mechanisms of nociception, Nature 413 (2001) 203–210.

[25] M.D. Kinkel, S.C. Eames, L.H. Philipson, V.E. Prince, Intraperitoneal injection into adult zebrafish, J. Vis. Exp. (2010).

[26] M. Levantia, B. Randazzoa, E. Viña, G. Montalbanoa, O. Garcia-Suarezb, A. Germanàa, J.A. Vega, F. Abbatea, Acid-sensing ion channels and transient-receptor potential ionchannels in zebrafish taste buds, Annals of Anatomy 207 (2016) 32–37.

[27] E. Marron Fdez de Velasco, P.Y. Law, R.E. Rodriguez, Mu opioid receptor from the zebrafish exhibits functional characteristics as those of mammalian mu opioid receptor, Zebrafish 6 (2009) 259–268.

[28] M.H. Ossipov, The perception and endogenous modulation of pain, Scientifica (Cairo) (2012) 561761.

[29] F.M. Sanchez-Simon, R.E. Rodriguez, Developmental expression and distribution of opioid receptors in zebrafish, Neuroscience 151 (2008) 129–137.

[30] J. Sareen, T. Houlahan, B.J. Cox, G.J. Asmundson, Anxiety disorders associated with suicidal ideation and suicide attempts in the National Comorbidity Survey, J.

Nerv. Ment. Dis. 193 (2005) 450–454.
[31] L.U. Sneddon, Evolution of nociception in vertebrates: comparative analysis of lower vertebrates, Brain Res. Brain Res. Rev. 46 (2004) 123–130.
[32] L.U. Sneddon, Pain perception in fish: indicators and endpoints, ILAR J. 50 (2009) 338–342.
[33] C. Stein, M. Schafer, H. Machelska, Attacking pain at its source: new perspectives on opioids, Nat. Med. 9 (2003) 1003–1008.
[34] A. Stewart, K. Wong, J. Cachat, S. Gaikwad, E. Kyzar, N. Wu, P. Hart, V. Piet, E. Utterback, M. Elegante, D. Tien, A.V. Kalueff, Zebrafish models to study drug abuse-related phenotypes, Rev. Neurosci. 22 (2011) 95–105.
[35] A. Stewart, N. Wu, J. Cachat, P. Hart, S. Gaikwad, K. Wong, E. Utterback, T. Gilder, E. Kyzar, A. Newman, D. Carlos, K. Chang, M. Hook, C. Rhymes, M. Caffery, M. Greenberg, J. Zadina, A.V. Kalueff, Pharmacological modulation of anxiety-like phenotypes in adult zebrafish behavioral models, Prog. Neuropsychopharmacol. Biol. Psychiatry 35 (2011) 1421–1431.
[36] G. Sundstrom, S. Dreborg, D. Larhammar, Concomitant duplications of opioid peptide and receptor genes before the origin of jawed vertebrates, PLoS One 5 (2010) e10512.
[37] J.C. Taylor, L.S. Dewberry, S.K. Totsch, L.R. Yessick, J.J. DeBerry, S.A. Watts, R.E. Sorge, A novel zebrafish-based model of nociception, Physiol. Behav. 174 (2017) 83–88.
[38] R.J. Valentino, N.D. Volkow, Untangling the complexity of opioid receptor function, Neuropsychopharmacology 43 (2018) 2514–2520.
[39] C.J. Woolf, What is this thing called pain? J. Clin. Invest. 120 (2010) 3742–3744.
[40] D. Yarnitsky, Role of endogenous pain modulation in chronic pain mechanisms and treatment, Pain 156 (Suppl 1) (2015) S24–S31.

6. DISCUSSÃO

Durante décadas, a mensuração dos limiares de retirada de estímulos nocivos era utilizada para examinar as respostas à dor *in vivo* (BOBINSKI *et al.*, 2018; GREGORY *et al.*, 2013; MARTINS *et al.*, 2018). Essas abordagens melhoraram significativamente nosso conhecimento acerca da fisiologia da dor, propriedades de drogas analgésicas, bem como neurotransmissores e genes envolvidos nas respostas à dor. Embora os roedores sejam amplamente usados na pesquisa da dor translacional (MEOTTI *et al.*, 2010; STEVENSON *et al.*, 2006), o desenvolvimento de novos modelos de animais experimentais alternativos é essencial para desvendar mecanismos evolutivamente conservados, bem como realizar triagens farmacológicas *in vivo* de alto rendimento (DU *et al.*, 2017; SNEDDON *et al.*, 2003a). Assim, nesta tese, discutimos a crescente utilização do modelo experimental do peixe-zebra para avaliar fenótipos moleculares, fisiológicos e comportamentais específicos relacionados às respostas dolorosas. Também delineamos os recentes avanços e limitações potenciais do peixe-zebra como organismo modelo de baixo custo, traduzível em pesquisa de dor e *screens* farmacológicos. Além disso, caracterizamos os distintos fenótipos comportamentais relacionados à dor em peixe-zebra após a administração i.p. de ácido acético.

A utilização de modelos de dor baseados no peixe-zebra vem crescendo muito na última década (GONZALEZ-NUNEZ *et al.*, 2009). Esta espécie mostra alta sensibilidade a vários estímulos nociceptivos (COSTA *et al.*, 2019; MAGALHAES *et al.*, 2017; TAYLOR *et al.*, 2017; THOMSON *et al.*, 2019), além de responder a analgésicos clinicamente ativos, representando assim um organismo modelo promissor para investigar as bases moleculares de distúrbios relacionados à dor humana (GAU *et al.*, 2013; PROBER *et al.*, 2008). Apesar das diferenças anatômicas no sistema nervoso central (SNC) em relação aos humanos, os peixes teleósteos (por exemplo, peixe-zebra)

exibem atividades elétricas e alterações transcricionais após estímulos nocivos (BRAITHWAITE *et al.*, 2007b; NORDGREEN *et al.*, 2007; REILLY *et al.*, 2008; ROSE, 2002). É importante ressaltar que o peixe-zebra expressa genes envolvidos nas respostas à dor (ALVAREZ *et al.*, 2006; GAU *et al.*, 2013; GONZALEZ-NUNEZ *et al.*, 2009; LEVANTIA *et al.*, 2016; PROBER *et al.*, 2008; SANCHEZ-SIMON *et al.*, 2008), e também apresenta comportamentos específicos de "proteção" quando expostos a estímulos nocivos, sugerindo a existência de mecanismos centrais subjacentes às respostas dolorosas (SNEDDON *et al.*, 2003a).

Deste modo, **no primeiro estudo** abordado nesta tese, realizamos uma revisão sistemática visando discutir as vantagens e limitações do uso do peixe-zebra como organismo modelo para estudos relacionados à dor. Comparado aos modelos tradicionais de roedores, o peixe-zebra demonstra algumas vantagens na pesquisa básica, incluindo o menor espaço para manutenção e a grande prole (AVDESH *et al.*, 2012; HOO *et al.*, 2016). A fertilização externa também simplifica a produção de linhagens transgênicas, e a presença de embriões translúcidos facilita a investigação de potenciais biomarcadores de nocicepção *in vivo* (por exemplo, usando sondas fluorescentes de expressão gênica). Além disso, um alto grau (~ 70%) de homologia genética quando comparado aos humanos (HOWE *et al.*, 2013), bem como vários modelos genéticos do SNC (CHERESIZ *et al.*, 2020), reforçam este modelo aquático como uma ótima espécie para explorar a modulação genética da dor e desordens relacionadas. Esses recursos robustecem o uso crescente de peixe-zebra como uma ferramenta robusta para realizar triagens de rendimento médio a alto de maneira econômica (KITHCART *et al.*, 2017; STEWART *et al.*, 2015). Entretanto, o peixe-zebra possui algumas limitações às representações de seus dados, devido a certas diferenças em relação aos mamíferos, como na fisiologia metabólica (peixes de sangue frio vs. mamíferos de sangue quente), desenvolvimento do

cérebro e anatomia (por exemplo, o peixe-zebra não possui córtex) (PARKER *et al.*, 2013). Além disso, o peixe-zebra também apresenta algumas limitações na pesquisa em neurociência. Por exemplo, o evento de duplicação do genoma em peixes teleósteos (LU *et al.*, 2012) complica as análises genéticas das respostas à dor, uma vez que alguns genes relacionados à dor podem existir em duas cópias ou estar ausentes no peixe-zebra. Devido ao seu pequeno tamanho, o monitoramento de longo prazo dos níveis endócrinos do sangue (por exemplo, níveis de glicose) também é problemático, pois é difícil obter uma quantidade suficiente de sangue sem realizar a eutanásia do animal (LAKSTYGAL *et al.*, 2019).

O **segundo estudo** desta tese teve como objetivo caracterizar um modelo de dor visceral aguda em peixes-zebra através da administração i.p. de ácido acético. Para isso, os animais foram injetados com ácido acético (0.5, 1.0, 2.5, e 5.0%), e posteriormente observados os comportamentos exploratórios bem como fenótipos comportamentais relacionados à dor. Os animais tratados com ácido acético (1.0, 2.5, e 5.0%) apresentaram alterações locomotoras, tais como uma menor distância percorrida e aumento no tempo gasto na parte superior do aquário. Vários estudos utilizam alterações locomotoras como principais endpoints de dor em peixes (CORREIA *et al.*, 2011; CURTRIGHT *et al.*, 2015; REILLY *et al.*, 2008; TAYLOR *et al.*, 2017). A maioria deles é projetada para investigar parâmetros comportamentais afetados por algógenos, e não para avaliar especificamente a dor *per se*. Neste modelo, nós caracterizamos um novo fenótipo comportamental que até então não havia sido descrito para a espécie. Após a administração i.p. de ácido acético (2.5, e 5.0%) os animais apresentaram um fenótipo semelhante à contorção abdominal (medido como índice de curvatura corporal), análogo à resposta de contorção em roedores submetidos ao modelo de dor visceral. Além disso, este comportamento é facilmente reproduzido utilizando um pequeno número de animais, o que atende o critério dos 3Rs

de experimentação animal. Cabe ressaltar que não detectamos diferença de sexo nas respostas de curvatura do peixe-zebra ao ácido acético (2.5, e 5.0%), mostrando que este parâmetro não é influenciado pelo sexo dos animais. A resposta dolorosa foi completamente prevenida pela coadministração de analgésicos clássicos, como a morfina (agonista μ opioide), bem como pelo pré-tratamento com um anti-inflamatório amplamente utilizados na clínica, o diclofenaco sódico. Além disso, a naloxona (inibidor não seletivo opioide) antagonizou os efeitos analgésicos da morfina no índice de curvatura corporal. Portanto, como o sistema opioide do peixe-zebra é semelhante (tanto farmacológica quanto bioquimicamente) ao seu homólogo mamífero (GONZALEZ-NUNEZ *et al.*, 2009), e, uma vez que a naloxona neutralizou os efeitos da morfina, sugerimos que a ativação dos receptores opioides modula negativamente o comportamento semelhante a contorção abdominal induzido pelo ácido acético. No geral, esses achados nos mostram que o modelo de dor visceral aguda em peixe-zebra possui um alto valor de face (respostas comportamentais análogas a de outras espécies) e preditivo (sensibilidade farmacológica a moléculas moduladoras da dor).

Em humanos, o sistema opioide está envolvido no sistema de inibição endógena da dor, onde a liberação de opioides endógenos (encefalinas) promovido pela ativação das vias descendentes da dor gera um processo analgésico (PASTERNAK *et al.*, 2013). O projeto de sequenciamento do genoma permitiu uma identificação abrangente dos ortólogos do peixe-zebra, tais como as encefalinas (Met-encefalinas, Met-encefalina-Ile e Met-encefalina-Asp) (GONZALEZ-NUNEZ *et al.*, 2013; GONZALEZ-NUNEZ *et al.*, 2009; SUNDSTROM *et al.*, 2010). Assim, um possível mecanismo de inibição endógena da dor no zebrafish não poderia ser descartado. Portanto, o **terceiro estudo** desta tese teve como objetivo investigar se a naloxona prolonga a recuperação de respostas semelhantes a contorções abdominais após a injeção i.p. de ácido acético em peixes-zebra. Para isso,

os animais foram tratados com ácido acético (5.0%; resposta máxima observada no estudo anterior) e ácido acético (5.0%) coadministrado com diferentes doses de naloxona (1.25, 2.5 e 5 mg/kg). A coadministração de naloxona (1.25, 2.5 e 5 mg/kg) prolongou de forma dose dependente as respostas dolorosas em peixes-zebra induzidas por ácido acético (a qual possui duração aproximada de 30 min). Embora tanto o receptor μ opioide do peixe-zebra quanto os precursores de peptídeos endógenos compartilhem uma homologia molecular elevada com seus respectivos homólogos humanos (MARRON FDEZ DE VELASCO *et al.*, 2009), é difícil comparar resultados entre espécies, bem como estabelecer um mecanismo de ação específico, uma vez que também podem existir possíveis diferenças nas afinidades de ligação. Entretanto, não podemos descartar o envolvimento de opioides endógenos nos resultados descritos aqui, visto que a ativação dos receptores opioides desempenham um papel na recuperação do comportamento semelhante a contorções abdominal após a administração de ácido acético, uma vez que a naloxona, um potente antagonista do receptor de opioides, prolonga essa resposta. Assim, um papel putativo dos opioides endógenos na modulação da recuperação da dor em peixes-zebra, não pode ser descartado.

De modo geral, destacamos que a conservação da maquinaria celular relacionada aos processos nociceptivos bem como o rico repertório comportamental, tornam o peixe-zebra um organismo emergente para estudos translacionais relacionados à dor e na busca futura de potenciais fármacos analgésicos.

7. CONCLUSÃO

A partir dos resultados apresentados, podemos observar uma robusta evolução no conhecimento sobre fenótipos comportamentais relacionados à dor no peixe-zebra, utilizando o modelo de contorção abdominal. Assim, como conclusões parciais da presente Tese, podemos afirmar que:

- O peixe-zebra é um organismo modelo emergente para estudos relacionados à dor em função do robusto repertório comportamental e da maquinaria celular responsável pela nocicepção;
- O comportamento de contorção abdominal observado após a administração intraperitoneal de ácido acético é análogo ao fenótipo apresentado por roedores e caracteriza-se como uma medida comportamental de fácil mensuração;
- A administração de ácido acético alterou parâmetros exploratórios, tais como redução na distância percorrida bem como aumentou o tempo gasto na área superior do aquário;
- O comportamento de contorção abdominal observado após a administração intraperitoneal de ácido acético mostrou-se sensível a analgésicos tradicionalmente utilizados na clínica;
- O sistema opioide possui um papel modulador na resposta de curvatura corporal, uma vez que a naloxona bloqueou o efeito analgésico da morfina;
- A coadministração de naloxona atrasou a recuperação do animal na alteração da curvatura corporal induzida pelo ácido acético, sugerindo o papel putativo de opioides endógenos na modulação da recuperação da estimulação da dor em peixes-zebra adultos;

Em suma, verificamos que após uma única injeção i.p., o peixe-zebra exibe um fenótipo comportamental distinto, análogo à resposta de contorção descrita em roedores, acompanhada por outras mudanças comportamentais (por exemplo, locomoção reduzida,

congelamento aumentado e mudanças na atividade vertical). Tal comportamento pode refletir um fenótipo específico relacionado à dor, que é simples, fácil de quantificar e prevenido por analgésicos clinicamente ativos. Portanto, os resultados observados nesta tese possibilitarão uma melhor validação a nível de construto do modelo proposto neste projeto, e, assim, um maior conhecimento das bases mecanísticas subjacentes à dor. Por fim, nossos achados permitirão a expansão de estudos utilizando este organismo modelo em pesquisas científicas relacionadas à busca de potenciais estratégias farmacológicas para prevenção da dor.

8. PERSPECTIVAS

Os resultados apresentados nesta tese contribuem para a consolidação do peixe-zebra como um organismo modelo para estudos relacionados às respostas dolorosas. Entretanto, futuras investigações ainda devem ser realizadas com intuito de elucidar os mecanismos subjacentes a dor no peixe-zebra. Por exemplo, em peixes teleósteos, estímulos estressores ativam o eixo hipotalâmico-pituitária-inter-renal (HPI), causando o aumento dos níveis do fator liberador de corticotropina (CRF) e a liberação do cortisol pelas células inter-renais (função semelhante à glândula adrenal de mamíferos) (MEZZOMO *et al.*, 2018). Assim, diferentemente dos roedores que possuem a corticosterona como o principal hormônio do estresse, os peixes expressam o cortisol de modo similar ao que ocorre em humanos. Além disso, estudos demonstraram que o CRF é um componente crítico para as respostas de camuflagem do peixe-zebra (WAGLE *et al.*, 2011), onde o aumento da pigmentação via ativação dos melanossomos é ausente em larvas que possuem este gene silenciado através da técnica de morfolino. Dessa maneira, tanto respostas pigmentares como respostas fisiológicas associadas ao estresse podem possuir uma relação direta com vias da modulação da dor. O modelo parece interessante também para investigar os mecanismos relacionados com a alodínia mecânica no peixe-zebra. Em peixes teleósteos, 53% das fibras sensoriais são do tipo A-beta (SNEDDON, 2002), responsáveis pela indução da alodínia mecânica. Da mesma forma, um modelo bem utilizado para indução da alodínia mecânica em camundongos já foi descrito no peixe-zebra para avaliar edema (MAGALHAES *et al.*, 2017). Assim, o desafio seria a criação de um aparato capaz de mensurar tal resposta.

Finalmente, em uma perspectiva mais ampla e de longo prazo, fica o planejamento da consolidação de uma linha consistente de pesquisa dentro de nossa Universidade, a qual vise fazer novos estudos avaliando aspectos relacionados à dor em peixe-zebra.

Assim a implementação deste campo de pesquisa certamente trará benefícios não apenas na utilização do peixe-zebra como um modelo alternativo/complementar para estudos da dor, mas também, contribuirá para a formação de recursos humanos.

REFERÊNCIAS

AKOPIAN, A. N.; CHEN, C. C.; DING, Y.; CESARE, P.; WOOD, J. N. A new member of the acid-sensing ion channel family. **Neuroreport**, 11, n. 10, p. 2217-2222, Jul 14 2000.

ALVAREZ, F. A.; RODRIGUEZ-MARTIN, I.; GONZALEZ-NUNEZ, V.; MARRON FERNANDEZ DE VELASCO, E.; GONZALEZ SARMIENTO, R. *et al.* New kappa opioid receptor from zebrafish *Danio rerio*. **Neurosci Lett**, 405, n. 1-2, p. 94-99, Sep 11 2006.

ASHBURN, M. A.; STAATS, P. S. Management of chronic pain. **Lancet**, 353, n. 9167, p. 1865-1869, May 29 1999.

AVDESH, A.; CHEN, M.; MARTIN-IVERSON, M. T.; MONDAL, A.; ONG, D. *et al.* Regular care and maintenance of a zebrafish (*Danio rerio*) laboratory: an introduction. **J Vis Exp**, n. 69, p. e4196, Nov 18 2012.

BABINSKI, K.; LE, K. T.; SEGUELA, P. Molecular cloning and regional distribution of a human proton receptor subunit with biphasic functional properties. **J Neurochem**, 72, n. 1, p. 51-57, Jan 1999.

BAO, W.; VOLGIN, A. D.; ALPYSHOV, E. T.; FRIEND, A. J.; STREKALOVA, T. V. *et al.* Opioid neurobiology, neurogenetics and neuropharmacology in zebrafish. **Neuroscience**, Jan 30 2019.

BARRALLO, A.; GONZALEZ-SARMIENTO, R.; ALVAR, F.; RODRIGUEZ, R. E. ZFOR2, a new opioid receptor-like gene from the teleost zebrafish (*Danio rerio*). **Brain Res Mol Brain Res**, 84, n. 1-2, p. 1-6, Dec 8 2000.

BARRALLO, A.; GONZALEZ-SARMIENTO, R.; PORTEROS, A.; GARCIA-ISIDORO, M.; RODRIGUEZ, R. E. Cloning, molecular characterization, and distribution of a gene homologous to delta opioid receptor from zebrafish (*Danio rerio*). **Biochem Biophys Res Commun**, 245, n. 2, p. 544-548, Apr 17 1998.

BASBAUM, A. I.; BAUTISTA, D. M.; SCHERRER, G.; JULIUS, D. Cellular and molecular mechanisms of pain. **Cell**, 139, n. 2, p. 267-284, Oct 16 2009.

BATESON, P. Assessment of pain in animals. **Animal Behaviour**, 42, p. 827-839, 1991.

BENINI, A.; BOZZATO, A.; MANTOVANELLI, S.; CALVARINI, L.; GIACOPUZZI, E. *et al.* Characterization and expression analysis of *mcoln1.1* and *mcoln1.2*, the putative zebrafish co-orthologs of the gene responsible for human mucolipidosis type IV. **Int J Dev Biol**, 57, n. 1, p. 85-93, 2013.

BESSON, J. M. The neurobiology of pain. **Lancet**, 353, n. 9164, p. 1610-1615, May 8 1999.

BLACKBURN-MUNRO, G. Pain-like behaviours in animals - how human are they? **Trends Pharmacol Sci**, 25, n. 6, p. 299-305, Jun 2004.

BOBINSKI, F.; TEIXEIRA, J. M.; SLUKA, K. A.; SANTOS, A. R. S. Interleukin-4 mediates the analgesia produced by low-intensity exercise in mice with neuropathic pain. **Pain**, 159, n. 3, p. 437-450, Mar 2018.

BONIN, R. P.; BORIES, C.; DE KONINCK, Y. A simplified up-down method (SUDO) for measuring mechanical nociception in rodents using von Frey filaments. **Mol Pain**, 10, p. 26, Apr 16 2014.

BRAITHWAITE, V. A.; BOULCOTT, P. Pain perception, aversion and fear in fish. **Dis Aquat Organ**, 75, n. 2, p. 131-138, May 4 2007a.

BRAITHWAITE, V. A.; BOULCOTT, P. Pain perception, aversion and fear in fish. **Diseases of Aquatic Organisms**, 75, n. 2, p. 131-138, May 4 2007b.

CATERINA, M. J.; SCHUMACHER, M. A.; TOMINAGA, M.; ROSEN, T. A.; LEVINE, J. D. *et al.* The capsaicin receptor: a heat-activated ion channel in the pain pathway. **Nature**, 389, n. 6653, p. 816-824, Oct 23 1997.

CHEN, C. C.; ENGLAND, S.; AKOPIAN, A. N.; WOOD, J. N. A sensory neuron-specific, proton-gated ion channel. **Proc Natl Acad Sci U S A**, 95, n. 17, p. 10240-10245, Aug 18 1998.

CHERESIZ, S. V.; VOLGIN, A. D.; KOKORINA EVSYUKOVA, A.; BASHIRZADE, A. A. O.; DEMIN, K. A. *et al.* Understanding neurobehavioral genetics of zebrafish. **J Neurogenet**, 34, n. 2, p. 203-215, Mar - Jun 2020.

CHU SIN CHUNG, P.; KIEFFER, B. L. Delta opioid receptors in brain function and diseases. **Pharmacol Ther**, 140, n. 1, p. 112-120, Oct 2013.

CLAPHAM, D. E. TRP channels as cellular sensors. **Nature**, 426, n. 6966, p. 517-524, Dec 4 2003.

CORBETT, A. D.; HENDERSON, G.; MCKNIGHT, A. T.; PATERSON, S. J. 75 years of opioid research: the exciting but vain quest for the Holy Grail. **Br J Pharmacol**, 147 Suppl 1, p. S153-162, Jan 2006.

COREY, D. P.; GARCIA-ANOVEROS, J.; HOLT, J. R.; KWAN, K. Y.; LIN, S. Y. *et al.* TRPA1 is a candidate for the mechanosensitive transduction channel of vertebrate hair cells. **Nature**, 432, n. 7018, p. 723-730, Dec 9 2004.

CORREIA, A. D.; CUNHA, S. R.; SCHOLZE, M.; STEVENS, E. D. A Novel Behavioral Fish Model of Nociception for Testing Analgesics. **Pharmaceuticals**, 4, n. 4, p. 665-680, Apr 18 2011.

COSTA, F. V.; ROSA, L. V.; QUADROS, V. A.; SANTOS, A. R. S.; KALUEFF, A. V. *et al.* Understanding nociception-related phenotypes in adult zebrafish: Behavioral and pharmacological characterization using a new acetic acid model. **Behav Brain Res**, 359, p. 570-578, Feb 1 2019.

CURTRIGHT, A.; ROSSER, M.; GOH, S.; KEOWN, B.; WAGNER, E. *et al.* Modeling nociception in zebrafish: a way forward for unbiased analgesic discovery. **PLoS One**, 10, n. 1, p. e0116766, 2015.

DAMASIO, A.; CARVALHO, G. B. The nature of feelings: evolutionary and neurobiological origins. **Nat Rev Neurosci**, 14, n. 2, p. 143-152, Feb 2013.

DARWIN, C. R. The expression of the emotions in man and animals. **London: John Murray**, 1st edition, 1872.

DE RANTERE, D.; SCHUSTER, C. J.; REIMER, J. N.; PANG, D. S. The relationship between the Rat Grimace Scale and mechanical hypersensitivity testing in three experimental pain models. **Eur J Pain**, 20, n. 3, p. 417-426, Mar 2016.

DEMIN, K. A.; MESHALKINA, D. A.; KYSIL, E. V.; ANTONOVA, K. A.; VOLGIN, A. D. *et al.* Zebrafish models relevant to studying central opioid and endocannabinoid systems. **Prog Neuropsychopharmacol Biol Psychiatry**, 86, p. 301-312, Aug 30 2018.

DREBORG, S.; SUNDSTROM, G.; LARSSON, T. A.; LARHAMMAR, D. Evolution of vertebrate opioid receptors. **Proc Natl Acad Sci U S A**, 105, n. 40, p. 15487-15492, Oct 7 2008.

DU, X.; YUAN, B.; WANG, J.; ZHANG, X.; TIAN, L. *et al.* Effect of heat-reinforcing needling on serum metabolite profiles in rheumatoid arthritis rabbits with cold syndrome. **Zhongguo Zhen Jiu**, 37, n. 9, p. 977-983, Sep 12 2017.

ENGLAND, S. J.; CAMPBELL, P. C.; BANERJEE, S.; SWANSON, A. J.; LEWIS, K. E. Identification and Expression Analysis of the Complete Family of Zebrafish pkd Genes. **Front Cell Dev Biol**, 5, p. 5, 2017.

FESTING, S.; PATEL, T. The ethics of research involving animals: a review of the Nuffield Council on Bioethics report from a research perspective. **Altern Lab Anim**, 33, n. 6, p. 654-658, Dec 2005.

FISHMAN, S. M. Recognizing pain management as a human right: a first step. **Anesth Analg**, 105, n. 1, p. 8-9, Jul 2007.

GARCIA-LARREA, L.; PEYRON, R. Pain matrices and neuropathic pain matrices: a review. **Pain**, 154 Suppl 1, p. S29-43, Dec 2013.

GAU, P.; POON, J.; UFRET-VINCENTY, C.; SNELSON, C. D.; GORDON, S. E. *et al.* The zebrafish ortholog of TRPV1 is required for heat-induced locomotion. **J Neurosci**, 33, n. 12, p. 5249-5260, Mar 20 2013.

GONZALEZ-CANO, R.; TEJADA, M. A.; ARTACHO-CORDON, A.; NIETO, F. R.; ENTRENA, J. M. *et al.* Effects of Tetrodotoxin in Mouse Models of Visceral Pain. **Mar Drugs**, 15, n. 6, Jun 21 2017.

GONZALEZ-NUNEZ, V.; BARRALLO, A.; TRAYNOR, J. R.; RODRIGUEZ, R. E. Characterization of opioid-binding sites in zebrafish brain. **J Pharmacol Exp Ther**, 316, n. 2, p. 900-904, Feb 2006.

GONZALEZ-NUNEZ, V.; JIMENEZ GONZALEZ, A.; BARRETO-VALER, K.; RODRIGUEZ, R. E. In vivo regulation of the mu opioid receptor: role of the endogenous opioid agents. **Mol Med**, 19, p. 7-17, Mar 5 2013.

GONZALEZ-NUNEZ, V.; RODRIGUEZ, R. E. The zebrafish: a model to study the endogenous mechanisms of pain. **ILAR J**, 50, n. 4, p. 373-386, 2009.

GREGORY, N. S.; HARRIS, A. L.; ROBINSON, C. R.; DOUGHERTY, P. M.; FUCHS, P. N. *et al.* An overview of animal models of pain: disease models and outcome measures. **J Pain**, 14, n. 11, p. 1255-1269, Nov 2013.

GRUNDER, S.; GEISSLER, H. S.; BASSLER, E. L.; RUPPERSBERG, J. P. A new member of acid-sensing ion channels from pituitary gland. **Neuroreport**, 11, n. 8, p. 1607-1611, Jun 5 2000.

GUPTA, R.; SAITO, S.; MORI, Y.; ITOH, S. G.; OKUMURA, H. *et al.* Structural basis of TRPA1 inhibition by HC-030031 utilizing species-specific differences. **Sci Rep**, 6, p. 37460, Nov 22 2016.

HOO, J. Y.; KUMARI, Y.; SHAIKH, M. F.; HUE, S. M.; GOH, B. H. Zebrafish: A Versatile Animal Model for Fertility Research. **Biomed Res Int**, 2016, p. 9732780, 2016.

HOWE, K.; CLARK, M. D.; TORROJA, C. F.; TORRANCE, J.; BERTHELOT, C. *et al.* The zebrafish reference genome sequence and its relationship to the human genome. **Nature**, 496, n. 7446, p. 498-503, Apr 25 2013.

HUNT, S. P.; MANTYH, P. W. The molecular dynamics of pain control. **Nat Rev Neurosci**, 2, n. 2, p. 83-91, Feb 2001.

JAHR, H.; VAN DRIEL, M.; VAN OSCH, G. J.; WEINANS, H.; VAN LEEUWEN, J. P. Identification of acid-sensing ion channels in bone. **Biochem Biophys Res Commun**, 337, n. 1, p. 349-354, Nov 11 2005.

JULIUS, D. TRP channels and pain. **Annu Rev Cell Dev Biol**, 29, p. 355-384, 2013.

JULIUS, D.; BASBAUM, A. I. Molecular mechanisms of nociception. **Nature**, 413, n. 6852, p. 203-210, Sep 13 2001.

KALUEFF, A. V.; GEBHARDT, M.; STEWART, A. M.; CACHAT, J. M.; BRIMMER, M. *et al.* Towards a comprehensive catalog of zebrafish behavior 1.0 and beyond. **Zebrafish**, 10, n. 1, p. 70-86, Mar 2013.

KANG, K.; PULVER, S. R.; PANZANO, V. C.; CHANG, E. C.; GRIFFITH, L. C. *et al.* Analysis of Drosophila TRPA1 reveals an ancient origin for human chemical nociception. **Nature**, 464, n. 7288, p. 597-600, Mar 25 2010.

KASTENHUBER, E.; GESEMANN, M.; MICKOLEIT, M.; NEUHAUSS, S. C. Phylogenetic analysis and expression of zebrafish transient receptor potential melastatin family genes. **Dev Dyn**, 242, n. 11, p. 1236-1249, Nov 2013.

KEY, B. Why fish do not feel pain. **Animal Sentience**, 3, p. 1-17, 2016.

KITHCART, A.; MACRAE, C. A. Using Zebrafish for High-Throughput Screening of Novel Cardiovascular Drugs. **JACC Basic Transl Sci**, 2, n. 1, p. 1-12, Feb 2017.

KO, M. J.; GANZEN, L. C.; COSKUN, E.; MUKADAM, A. A.; LEUNG, Y. F. *et al.* A critical evaluation of TRPA1-mediated locomotor behavior in zebrafish as a screening tool for novel anti-nociceptive drug discovery. **Sci Rep**, 9, n. 1, p. 2430, Feb 20 2019.

KUNER, R. Central mechanisms of pathological pain. **Nat Med**, 16, n. 11, p. 1258-1266, Nov 2010.

LAKSTYGAL, A. M.; DE ABREU, M. S.; LIFANOV, D. A.; WAPPLER-GUZZETTA, E. A.; SERIKULY, N. *et al.* Zebrafish models of diabetes-related CNS

pathogenesis. **Prog Neuropsychopharmacol Biol Psychiatry**, 92, p. 48-58, Jun 8 2019.

LAMBERT, D. G. The nociceptin/orphanin FQ receptor: a target with broad therapeutic potential. **Nat Rev Drug Discov**, 7, n. 8, p. 694-710, Aug 2008.

LAURSEN, W. J.; ANDERSON, E. O.; HOFFSTAETTER, L. J.; BAGRIANTSEV, S. N.; GRACHEVA, E. O. Species-specific temperature sensitivity of TRPA1. **Temperature (Austin)**, 2, n. 2, p. 214-226, Apr-Jun 2015.

LEE, C. H.; CHEN, C. C. Roles of ASICs in Nociception and Proprioception. **Adv Exp Med Biol**, 1099, p. 37-47, 2018.

LEVANTIA, M.; RANDAZZOA, B.; VIÑA, E.; MONTALBANOA, G.; GARCIA-SUAREZB, O. *et al.* Acid-sensing ion channels and transient-receptor potential ionchannels in zebrafish taste buds. **Annals of Anatomy**, 207, p. 32-37, 2016.

LINGUEGLIA, E.; DE WEILLE, J. R.; BASSILANA, F.; HEURTEAUX, C.; SAKAI, H. *et al.* A modulatory subunit of acid sensing ion channels in brain and dorsal root ganglion cells. **J Biol Chem**, 272, n. 47, p. 29778-29783, Nov 21 1997.

LOESER, J. D.; TREEDE, R. D. The Kyoto protocol of IASP Basic Pain Terminology. **Pain**, 137, n. 3, p. 473-477, Jul 31 2008.

LOHMAN, D.; SCHLEIFER, R.; AMON, J. J. Access to pain treatment as a human right. **BMC Med**, 8, p. 8, Jan 20 2010.

LU, J.; PEATMAN, E.; TANG, H.; LEWIS, J.; LIU, Z. Profiling of gene duplication patterns of sequenced teleost genomes: evidence for rapid lineage-specific genome expansion mediated by recent tandem duplications. **BMC Genomics**, 13, p. 246, Jun 15 2012.

MAGALHAES, F. E. A.; DE SOUSA, C.; SANTOS, S.; MENEZES, R. B.; BATISTA, F. L. A. *et al.* Adult Zebrafish (*Danio rerio*): An Alternative Behavioral Model of Formalin-Induced Nociception. **Zebrafish**, 14, n. 5, p. 422-429, Oct 2017.

MARRON FDEZ DE VELASCO, E.; LAW, P. Y.; RODRIGUEZ, R. E. Mu opioid receptor from the zebrafish exhibits functional characteristics as those of mammalian mu opioid receptor. **Zebrafish**, 6, n. 3, p. 259-268, Sep 2009.

MARTINS, D. F.; MARTINS, T. C.; BATISTI, A. P.; DOS SANTOS LEONEL, L.; BOBINSKI, F. *et al.* Long-Term Regular Eccentric Exercise Decreases Neuropathic Pain-like Behavior and Improves Motor Functional Recovery in an Axonotmesis Mouse

Model: the Role of Insulin-like Growth Factor-1. **Mol Neurobiol**, 55, n. 7, p. 6155-6168, Jul 2018.

MAXIMINO, C. Modulation of nociceptive-like behavior in zebrafish (*Danio rerio*) by environmental stressors. **Psychology & Neuroscience**, 4, n. 1, p. 149 - 155, Jun 15 2011.

MEOTTI, F. C., Coelho, I dos S.; SANTOS, A. R. The nociception induced by glutamate in mice is potentiated by protons released into the solution. **J Pain**, 11, n. 6, p. 570-578, 2010.

MERKER, B. Consciousness without a cerebral cortex: a challenge for neuroscience and medicine. **Behav Brain Sci**, 30, n. 1, p. 63-81; discussion 81-134, Feb 2007.

MEZZOMO, N. J.; FONTANA, B. D.; KALUEFF, A. V.; BARCELLOS, L. J. G.; ROSEMBERG, D. B. Understanding taurine CNS activity using alternative zebrafish models. **Neurosci Biobehav Rev**, 90, p. 471-485, Jul 2018.

MOGIL, J. S.; DAVIS, K. D.; DERBYSHIRE, S. W. The necessity of animal models in pain research. **Pain**, 151, n. 1, p. 12-17, Oct 2010.

MONTELL, C.; BIRNBAUMER, L.; FLOCKERZI, V. The TRP channels, a remarkably functional family. **Cell**, 108, n. 5, p. 595-598, Mar 8 2002.

MORIYAMA, T.; HIGASHI, T.; TOGASHI, K.; IIDA, T.; SEGI, E. *et al.* Sensitization of TRPV1 by EP1 and IP reveals peripheral nociceptive mechanism of prostaglandins. **Mol Pain**, 1, p. 3, Jan 17 2005.

NORDGREEN, J.; HORSBERG, T. E.; RANHEIM, B.; CHEN, A. C. Somatosensory evoked potentials in the telencephalon of Atlantic salmon (Salmo salar) following galvanic stimulation of the tail. **J Comp Physiol A Neuroethol Sens Neural Behav Physiol**, 193, n. 12, p. 1235-1242, Dec 2007.

NORDGREEN, J.; TAHAMTANI, F. M.; JANCZAK, A. M.; HORSBERG, T. E. Behavioural effects of the commonly used fish anaesthetic tricaine methanesulfonate (MS-222) on zebrafish (*Danio rerio*) and its relevance for the acetic acid pain test. **PLoS One**, 9, n. 3, p. e92116, 2014.

ODA, M.; KUROGI, M.; KUBO, Y.; SAITOH, O. Sensitivities of Two Zebrafish TRPA1 Paralogs to Chemical and Thermal Stimuli Analyzed in Heterologous Expression Systems. **Chem Senses**, 41, n. 3, p. 261-272, Mar 2016.

PARKER, M. O.; BROCK, A. J.; WALTON, R. T.; BRENNAN, C. H. The role of zebrafish (Danio rerio) in dissecting the genetics and neural circuits of executive function. **Front Neural Circuits**, 7, p. 63, 2013.

PASTERNAK, G. W.; PAN, Y. X. Mu opioids and their receptors: evolution of a concept. **Pharmacol Rev**, 65, n. 4, p. 1257-1317, 2013.

PAUKERT, M.; SIDI, S.; RUSSELL, C.; SIBA, M.; WILSON, S. W. *et al.* A family of acid-sensing ion channels from the zebrafish: widespread expression in the central nervous system suggests a conserved role in neuronal communication. **J Biol Chem**, 279, n. 18, p. 18783-18791, Apr 30 2004.

PERL, E. R. Pain mechanisms: a commentary on concepts and issues. **Prog Neurobiol**, 94, n. 1, p. 20-38, Jun 2011.

PIEL, M. J.; KROIN, J. S.; VAN WIJNEN, A. J.; KC, R.; IM, H. J. Pain assessment in animal models of osteoarthritis. **Gene**, 537, n. 2, p. 184-188, Mar 10 2014.

PINAL-SEOANE, N.; MARTIN, I. R.; GONZALEZ-NUNEZ, V.; MARRON FERNANDEZ DE VELASCO, E.; ALVAREZ, F. A. *et al.* Characterization of a new duplicate delta-opioid receptor from zebrafish. **J Mol Endocrinol**, 37, n. 3, p. 391-403, Dec 2006.

PRICE, M. P.; SNYDER, P. M.; WELSH, M. J. Cloning and expression of a novel human brain Na+ channel. **J Biol Chem**, 271, n. 14, p. 7879-7882, Apr 5 1996.

PROBER, D. A.; ZIMMERMAN, S.; MYERS, B. R.; MCDERMOTT, B. M., Jr.; KIM, S. H. *et al.* Zebrafish TRPA1 channels are required for chemosensation but not for thermosensation or mechanosensory hair cell function. **J Neurosci**, 28, n. 40, p. 10102-10110, Oct 1 2008.

RACHINGER-ADAM, B.; CONZEN, P.; AZAD, S. C. Pharmacology of peripheral opioid receptors. **Curr Opin Anaesthesiol**, 24, n. 4, p. 408-413, Aug 2011.

REILLY, S. C.; QUINN, J. P.; COSSINS, A. R.; SNEDDON, L. U. Behavioural analysis of a nociceptive event in fish: Comparisons between three species demonstrate specific responses. **Applied Animal Behaviour Science**, 114, p. 248–259, Jan 21 2008.

RIVAS-BOYERO, A. A.; HERRERO-TURRION, M. J.; GONZALEZ-NUNEZ, V.; SANCHEZ-SIMON, F. M.; BARRETO-VALER, K. *et al.* Pharmacological characterization of a nociceptin receptor from zebrafish (*Danio rerio*). **J Mol Endocrinol**, 46, n. 2, p. 111-123, Apr 2011.

ROSE, J. D. The Neurobehavioral Nature of Fishes and the Question of Awareness and Pain. **Fisheries Science**, 10, n. 1, p. 1-38, 2002. Review.

ROSE, J. D.; ARLINGHAUS, R.; COOKE, S. J.; DIGGLES, B. K.; SAWYNOK, W. *et al.* Can fish really feel pain? **FISH and FISHERIES**, 15, p. 97–133, 2014.

RUSSO, C. M.; BROSE, W. G. Chronic pain. **Annu Rev Med**, 49, p. 123-133, 1998.

SAITO, S.; SHINGAI, R. Evolution of thermoTRP ion channel homologs in vertebrates. **Physiol Genomics**, 27, n. 3, p. 219-230, Nov 27 2006.

SANCHEZ-SIMON, F. M.; RODRIGUEZ, R. E. Developmental expression and distribution of opioid receptors in zebrafish. **Neuroscience**, 151, n. 1, p. 129-137, Jan 2 2008.

SCHAEFER, L.; SAKAI, H.; MATTEI, M.; LAZDUNSKI, M.; LINGUEGLIA, E. Molecular cloning, functional expression and chromosomal localization of an amiloride-sensitive Na(+) channel from human small intestine. **FEBS Lett**, 471, n. 2-3, p. 205-210, Apr 14 2000.

SCHROEDER, P. G. S., L. U. Exploring the efficacy of immersion analgesics in zebrafish using an integrative approach. **Applied Animal Behaviour Science**, 187, p. 93-102, Feb 2017.

SHERRINGTON, C. S. Observations on the scratch-reflex in the spinal dog. **J Physiol**, 34, n. 1-2, p. 1-50, Mar 13 1906.

SHIR, Y.; SELTZER, Z. A-fibers mediate mechanical hyperesthesia and allodynia and C-fibers mediate thermal hyperalgesia in a new model of causalgiform pain disorders in rats. **Neurosci Lett**, 115, n. 1, p. 62-67, Jul 17 1990.

SNEDDON, L. U. Anatomical and electrophysiological analysis of the trigeminal nerve in a teleost fish, Oncorhynchus mykiss. **Neurosci Lett**, 319, n. 3, p. 167-171, Feb 22 2002.

SNEDDON, L. U.; BRAITHWAITE, V. A.; GENTLE, M. J. Do fishes have nociceptors? Evidence for the evolution of a vertebrate sensory system. **Proc Biol Sci**, 270, n. 1520, p. 1115-1121, Jun 07 2003a.

SNEDDON, L. U.; BRAITHWAITE, V. A.; GENTLE, M. J. Novel object test: examining nociception and fear in the rainbow trout. **J Pain**, 4, n. 8, p. 431-440, Oct 2003b.

SNEDDON, L. U.; ELWOOD, R. W.; ADAMO, S. A.; LEACH, M. C. Defining and assessing animal pain. **Animal Behaviour**, 97, p. 201-212, 2014.

STEVENS, C. W. The evolution of vertebrate opioid receptors. **Front Biosci (Landmark Ed)**, 14, p. 1247-1269, Jan 1 2009.

STEVENSON, G. W.; BILSKY, E. J.; NEGUS, S. S. Targeting pain-suppressed behaviors in preclinical assays of pain and analgesia: effects of morphine on acetic acid-suppressed feeding in C57BL/6J mice. **J Pain**, 7, n. 6, p. 408-416, Jun 2006.

STEWART, A. M.; GERLAI, R.; KALUEFF, A. V. Developing highER-throughput zebrafish screens for in-vivo CNS drug discovery. **Front Behav Neurosci**, 9, p. 14, 2015.

SU, X.; LI, Q.; SHRESTHA, K.; CORMET-BOYAKA, E.; CHEN, L. *et al.* Interregulation of proton-gated Na(+) channel 3 and cystic fibrosis transmembrane conductance regulator. **J Biol Chem**, 281, n. 48, p. 36960-36968, Dec 1 2006.

SUNDSTROM, G.; DREBORG, S.; LARHAMMAR, D. Concomitant duplications of opioid peptide and receptor genes before the origin of jawed vertebrates. **PLoS One**, 5, n. 5, p. e10512, May 6 2010.

SZALLASI, A.; BLUMBERG, P. M. Resiniferatoxin, a phorbol-related diterpene, acts as an ultrapotent analog of capsaicin, the irritant constituent in red pepper. **Neuroscience**, 30, n. 2, p. 515-520, 1989.

TAYLOR, J. C.; DEWBERRY, L. S.; TOTSCH, S. K.; YESSICK, L. R.; DEBERRY, J. J. *et al.* A novel zebrafish-based model of nociception. **Physiol Behav**, 174, p. 83-88, May 15 2017.

THOMAS, D. Laboratory animals and the art of empathy. **J Med Ethics**, 31, n. 4, p. 197-202, Apr 2005.

THOMSON, J. S.; AL-TEMEEMY, A. A.; ISTED, H.; SPENCER, J. W.; SNEDDON, L. U. Assessment of behaviour in groups of zebrafish (*Danio rerio*) using an intelligent software monitoring tool, the chromatic fish analyser. **J Neurosci Methods**, 328, p. 108433, Dec 1 2019.

TRACEY, I.; MANTYH, P. W. The cerebral signature for pain perception and its modulation. **Neuron**, 55, n. 3, p. 377-391, Aug 2 2007.

TSAGARELI, M. G.; NOZADZE, I. An overview on transient receptor potential channels superfamily. **Behav Pharmacol**, 31, n. 22, p. 413-434, Dec 10 2019.

VELDHUIS, N. A.; POOLE, D. P.; GRACE, M.; MCINTYRE, P.; BUNNETT, N. W. The G protein-coupled receptor-transient receptor potential channel axis: molecular insights for targeting disorders of sensation and inflammation. **Pharmacol Rev**, 67, n. 1, p. 36-73, 2015.

VON FREY, M. On the Use of Stimulus Hairs. p. 71-131, 1896.

VON NIEDERHAUSERN, V.; KASTENHUBER, E.; STAUBLE, A.; GESEMANN, M.; NEUHAUSS, S. C. Phylogeny and expression of canonical transient receptor potential (TRPC) genes in developing zebrafish. **Dev Dyn**, 242, n. 12, p. 1427-1441, Dec 2013.

WAGLE, M.; MATHUR, P.; GUO, S. Corticotropin-releasing factor critical for zebrafish camouflage behavior is regulated by light and sensitive to ethanol. **J Neurosci**, 31, n. 1, p. 214-224, Jan 5 2011.

WALDMANN, R.; BASSILANA, F.; DE WEILLE, J.; CHAMPIGNY, G.; HEURTEAUX, C. *et al.* Molecular cloning of a non-inactivating proton-gated Na+ channel specific for sensory neurons. **J Biol Chem**, 272, n. 34, p. 20975-20978, Aug 22 1997.

WALDMANN, R.; CHAMPIGNY, G.; BASSILANA, F.; HEURTEAUX, C.; LAZDUNSKI, M. A proton-gated cation channel involved in acid-sensing. **Nature**, 386, n. 6621, p. 173-177, Mar 13 1997.

WIECH, K. Deconstructing the sensation of pain: The influence of cognitive processes on pain perception. **Science**, 354, n. 6312, p. 584-587, Nov 4 2016.

WOOLF, C. J. What is this thing called pain? **J Clin Invest**, 120, n. 11, p. 3742-3744, Nov 2010.

YOUNG, R. Fiber spectrum of the trigeminal sensory root of frog, cat and man determined by electron microscopy. **Pain in the trigeminal region**, p. 137-160, 1977.

ZYGMUNT, P. M.; PETERSSON, J.; ANDERSSON, D. A.; CHUANG, H.; SORGARD, M. *et al.* Vanilloid receptors on sensory nerves mediate the vasodilator action of anandamide. **Nature**, 400, n. 6743, p. 452-457, Jul 29 1999.

ANEXO A - CAPÍTULO ACEITO PARA PUBLICAÇÃO EM LIVRO ACADÊMICO

Nociception-related behavioral phenotypes in adult zebrafish

Fabiano V. Costa, Luiz V. Rosa, Allan V. Kalueff, and Denis B. Rosemberg

The neurobiology, physiology, and behavior of pain. Volume 3. Chapter 34. Psychology and Behaviour – Nociception-related behavioral phenotypes in adult zebrafish. 2021

Chapter 34

Nociception-related behavioral phenotypes in adult zebrafish

Fabiano V. Costa[a], Luiz V. Rosa[a], Allan V. Kalueff[b,c] and Denis B. Rosemberg[a]

[a]*Department of Biochemistry and Molecular Biology School of Life Sciences, Federal University of Santa Maria, Santa Maria, Rio Grande do Sul, Brazil*, [b]*School of Pharmacy, Southwest University, Chongqing, China*,

[c]*Ural Federal University, Yekaterinburg, Russia*

zebrafish; pain; animal models; nociception

Abbreviations

TRP	**transient receptor potential ion channels**
TRPA1a	**transient receptor potential ankyrin1a**
TRPA1b	**transient receptor potential ankyrin1b**
TRPV1	**transient receptor potential vaniloid-1**
zDOPa/b	**zebrafish δ opioid a/b receptor**
zKOP	**zebrafish κ opioid receptor**
zMOP	**zebrafish μ opioid receptor**
zNOP	**zebrafish nociceptin opioid receptor**

Introduction

Pain evokes various responses in vivo that serve to communicate unpleasant experiences at various levels (Keefe & Pryor, 2007). Nociception is the neural processes of encoding and processing noxious stimuli, whereas pain is the conscious perception of nociception, and suffering is the negative emotional responses to nociception and pain (Fig. 1) (Fordyce et al., 1984). However, accurate analyses and measurement of pain-related behaviors in animal models can be challenging, since pain is a complex experience that involves not only transduction of nociceptive stimuli but also cognitive and emotional processing (Blackburn-Munro, 2004; Piel, Kroin, van Wijnen, Ranjan, & Im, 2014; Tracey & Mantyh, 2007). Together, this raises the question of how do we measure pain-related behaviors in subjects that cannot express their feelings? Moreover, can we verify if our observations match with the subjective experience of these individuals? To address this knowledge gap, here we discuss various behaviors related to pain, focusing on a novel experimental model organism, such as the zebrafish (*Danio rerio*), in translational pain research.

The Neurobiology, Physiology, and Behavior of Pain. https://doi.org/10.1016/B978-0-12-820589-1.00034-8

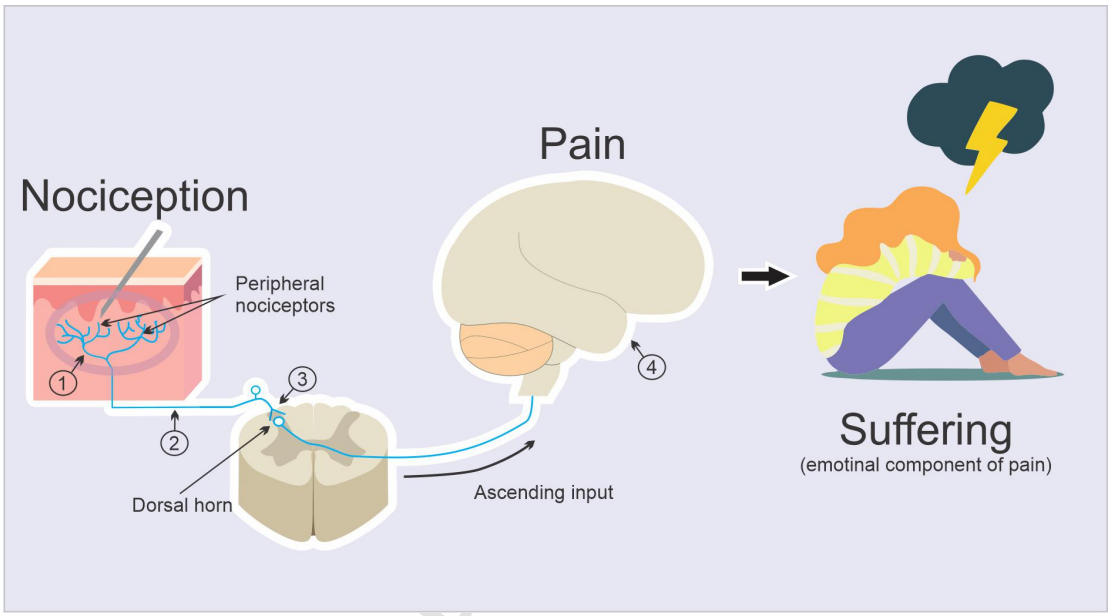


FIG. 1 Simplified diagram of the ascending pain pathway. Nociceptive stimulus is recognized by nociceptors. This signal travels to the dorsal horn of the spinal cord where it is sent to the brain for conscious perception of nociceptive stimuli. Finally, subjects exhibit negative emotional responses to nociception and pain, thereby causing suffering. 1—Transduction; 2—Transmission; 3—Modulation; 4—Perception (Basbaum, Bautista, Scherrer, & Julius, 2009).

Nociception and pain in animal models

Unlike humans, laboratory animal models have less complex neural processing networks, making it difficult to correlate nociception with the individual subjective experience (Festing & Patel, 2005; Thomas, 2005). Bateson (1991) has suggested several clear criteria for the ability to feel pain in various species, noting that only animals that met all these criteria should be considered sentient organisms. Briefly, they should possess (i) nociceptors; (ii) converging nociceptor pathways to the brain; (iii) brain structures analogous to the human cerebral cortex that processes pain; as well as (iv) express opioid receptors and endogenous opioid substances in a nociceptive neural system; (v) display lower adverse behavioral and physiological effects after administration of analgesics or anesthetics; (vi) avoid potentially painful stimuli. Sneddon and colleagues have suggested an additional criterion for animal sentience, namely that animals should suspend normal behavior for a prolonged period, instead of showing a reflexive response, with changes in behavior that reflect signs of 'discomfort' (Sneddon, Elwood, Adamo, & Leach, 2014).

Zebrafish as animal model for translational pain research

Although rodents are widely used in pain research (Meotti, Coelho, & Santos, 2010; Stevenson, Bilsky, & Negus, 2006), novel alternative experimental models, such as zebrafish, are also becoming important. For example, they can increase our understanding of evolutionarily conserved, "core" mechanisms underlying nociception and related behaviors (Du et al., 2017; Sneddon, Braithwaite, & Gentle, 2003b). Indeed, zebrafish are becoming critical in probing pain-related mechanisms and behaviors (Gonzalez-Nunez & Rodriguez, 2009), based on their evolutionarily conserved mechanisms involved in nociception, and robust, well-characterized behaviors (Fig. 2) that are bidirectionally sensitive to various algogens and analgesic drugs (Bao et al., 2019; Costa et al., 2019; Kalueff et al., 2013; Sneddon et al., 2003b; Taylor et al., 2017). Orthologs to human receptors involved in nociception have already been characterized in zebrafish, including the μ opioid receptor (zMOP; encoded by *oprm1*) (Barrallo, Gonzalez-Sarmiento, Alvar, & Rodriguez, 2000), the κ opioid receptor (zKOP; encoded by *oprk1*) (Alvarez et al., 2006), the two functional copies of zebrafish δ opioid receptor (zDOP; encoded by *oprd1a* and *oprd1b*) (Barrallo, Gonzalez-Sarmiento, Porteros, Garcia-Isidoro, & Rodriguez, 1998; Pinal-Seoane et al., 2006), the nociceptin opioid receptors (zNOP, encoded by *oprl1*) (Rivas-Boyero et al., 2011), as well as the transient receptor potential ion channels (TRPA1a, TRPA1b, and TRPV1) (Prober et al., 2008; Saito & Shingai, 2006) and acid-sensing ion channels (Paukert et al., 2004). Overt electrical activity and transcriptional changes in the midbrain and forebrain occur after harmful stimuli in teleost fishes, including zebrafish (Braithwaite & Boulcott, 2007; Nordgreen, Tahamtani, Janczak, & Horsberg, 2014; Reilly, Quinn, Cossins, &

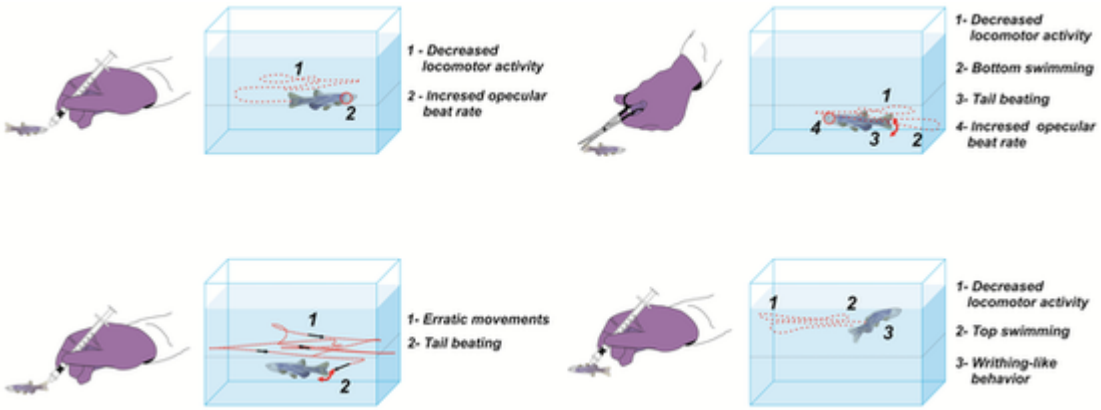


FIG. 2 Behavioral neurophenotyping of pain in zebrafish models. A brief summary of the main experimental protocols to assess pain in adult zebrafish and their respective behavioral phenotypes.

Sneddon, 2008). Moreover, while structural anatomy of nociceptive fibers in zebrafish has not been examined in detail, other teleost species possess typical nociceptors (*e.g.*, A-delta and C-fibers) (Sneddon, 2002), collectively suggesting that this taxon fully adheres to the Bateson-Sneddon's criteria of sentience listed above.

Analyses of specific behavioral phenotypes expressed in the presence of algogens is a cornerstone for an adequate assessment of nociception-related responses in vivo. Unlike rodents, which have multiple well-characterized pain-like behaviors (Bobinski, Teixeira, Sluka, & Santos, 2018; Bonin, Bories, & De Koninck, 2014; De Rantere, Schuster, Reimer, & Pang, 2016; Gonzalez-Cano et al., 2017; Martins et al., 2018), such phenotypes in zebrafish remain poorly understood (Table 1), necessitating further translational research in this organism.

Zebrafish-based pain models

Following the characterization of polymodal nociceptors in teleost fish (Sneddon, Braithwaite, & Gentle, 2003a), the search for new approaches to assess pain responses in zebrafish has begun. The first protocol reported in zebrafish involved the application of 5 μL of 5% acetic acid onto the fish lips, causing hypolocomotion, increased opercular beat rate, and an aberrant "rocking" behavior (Reilly et al., 2008), as briefly summarized in Fig. 2.

Altered locomotion is an important behavioral endpoint which indicates poor welfare (Kristiansen et al., 2004), and is similar to the immobility observed in other animals (e.g., chickens) after a noxious stimulation (Gentle, 1992). Albeit useful for evaluating pain-like behavior in adult zebrafish (Correia et al., 2011; Curtright et al., 2015; Taylor et al., 2017), this endpoint does not necessarily represent specific pain-like behavior, since it may indicate states of stress, anxiety, nonspecific sedation, toxicity and/or a pro-depressant behavior (Kalueff et al., 2013).

Thus, different approaches to induce local nociception in fish species, such as administration of algogens close to the fins (Maximino, 2011), or the caudal fin stimulation procedure (Schroeder & Sneddon, 2017), have been proposed. Both protocols induce erratic movements (swimming in a zig-zag unpredictable pattern) and increased tail beating (tail-beat movements that do not lead to propulsion in the water, Fig. 2), suggesting a local nociceptive effect. Additionally, the writhing-like phenotype has been recently reported as a novel behavioral endpoint to measure local pain (Costa, Canzian, Stefanello, Kalueff, & Rosemberg, 2019; Costa, Rosa, et al., 2019). Such behavior can be elicited by a single intraperitoneal injection of 5 μL 5% acetic acid, producing marked changes in body posture (resembling the writhing phenotype in rodents), as well as hypolocomotion. Interestingly, the writhing-like behavior of zebrafish (Fig. 2) reflects a direct activation of nociceptors following acetic acid administration, and is also sensitive to classical anti-pain medications (e.g., morphine and diclofenac). Furthermore, nociceptive effects following the administration of different algogenic agents have also been reported in zebrafish. For example, administration of histamine, complete Freund's adjuvant, cinnamaldehyde, alila isothiocyanate, or formalin, lowers zebrafish locomotor activity (strikingly similar to effects in rodents) (Magalhaes et al., 2017; Taylor et al., 2017). However, unlike pain caused by these substances, there are no current protocols that measure mechanical allodynia (pain to nonnociceptive stimuli) or neuropathic pain in adult zebrafish, thus meriting further scrutiny. Finally, although measuring a single pain-related behavioral endpoint may yield conflicting or nonspecific results, the parallel assessment of several different behaviors and using alternative animal models and/or species can improve face and predictive validity of such models of pain.

Table 1

TABLE 1 Selected examples of zebrafish-based pain models in the AB strain (see Fig. 2 for a graphic summary).

Noxious agents	Treatment	Behavioral endpoints	References
Acetic acid (5 %)	Injected (lips)	Decreased locomotor activity, increased opercular beat rate, and rocking behavior	Reilly et al. (2008)
Acetic acid (1 %)[a]	Injected (tail)	Increased both erratic movements and tail beating	Maximino (2011)
Acetic acid (5 and 10%)	Injected (lips)	Decreased locomotor activity	Correia, Cunha, Scholze, and Stevens (2011)
Fin clipping (30 s)	Removal of caudal posterior tissue	Increased opercular beat rate, increased tail beating and decreased locomotor activity	Schroeder and Sneddon (2017)
Formalin (0.1 %)	Injected (lips and tail)	Decreased locomotor activity	Magalhaes et al. (2017)
Histamine (0.1, 0.5, 1 and 2 mg/kg)	Injected (lips)	Decreased locomotor activity	Taylor et al. (2017)
Complete Freund's adjuvant	Injected (lips)	Decreased locomotor activity	Taylor et al. (2017)
Cinnamaldehyde (10, 20 and 40 mM)	Injected (lips)	Decreased locomotor activity	Taylor et al. (2017)
Alila isothiocyanate (1.02 M)	Injected (lips)	Decreased locomotor activity	Taylor et al. (2017)
Acetic acid (2.5, 5, 10 and 15 %)	Injected (lips)	Decreased locomotor activity	Taylor et al. (2017)
Acetic acid (0.5, 1, 2.5 and 5 %)	Injected (peritoneum)	Decreased locomotor activity, increased time spent on top area and altered body curvature	Costa, Rosa, et al. (2019)
Fin clipping	Removal of caudal posterior tissue	Increased time at the bottom, decreased locomotor activity and more clustered shoals	Thomson, Al-Temeemy, Isted, Spencer, and Sneddon (2019)

[a] *In the SF strain.*

Applications to other areas

Moreover, because zebrafish have sophisticated behaviors, automated video-tracking tools can be used to perform 3D reconstructions of the swimming traces (Cachat et al., 2011; Rosa et al., 2018; Thomson et al., 2019) to assess pain-related behaviors. The expression of all major opioid receptors, as well as the presence of well-characterized neurotransmitter systems, makes zebrafish a valuable tool to investigate central mechanisms underlying their pain pathobiology. Given high fertility rate of zebrafish and the transparency of embryos (Antinucci & Hindges, 2016; Sakai, Ijaz, & Hoffman, 2018), the analysis of molecular brain markers using whole-mount in situ hybridization and fluorescent probes can help determine how genes and proteins exert genetic and epigenetic regulation of pain. Finally, high genetic homology (> 70%) with humans, evolutionarily conserved molecular targets, as well as small size and easy/inexpensive maintenance (Fontana, Mezzomo, Kalueff, & Rosemberg, 2018; Howe et al., 2013), make zebrafish models a powerful tool in translational pain research.

Other agents of interest

Furthermore, assessing specific behaviors can be important for elucidating neural circuitry and specific brain areas activated when zebrafish are exposed to noxious stimuli. Measuring the relative expression of *C-fos* (Chatterjee, Tran, Shams, & Gerlai, 2015), an immediate early gene that reflects neuronal activation (e.g., using whole-mount in situ hybridization and/or immunoistochemistry), can be promising to investigate how specific brain regions contribute to pain-like responses. Moreover, the spatiotemporal measurements of behavior not only in adult fish, but also using larval fish, may enhance probing how locomotion can be affected during and after the exposure to algogens.

Examining how genes influence nociception is still in its early stages in various organisms, including zebrafish (Bao et al., 2019; Barrallo et al., 2000). However, because zebrafish have a high degree of genetic conservation, this species offers a promising forward-genetics system to explore evolutionarily conserved candidate genes in nociception (Howe et al., 2013). Analyzing physiological parameters is a key strategy in elucidating mechanisms underlying pain responses. Likewise, because zebrafish express well-characterized aversive behaviors (e.g., bottom dwelling, freezing, erratic movements) (Kalueff et al., 2013), the correlation of fear/anxiety-like responses with specific pain-like behaviors can elucidate how stress influence pain in zebrafish and vice-versa.

The use of pharmacological tools also facilitates the discovery of novel mechanisms involved in zebrafish nociception. For example, both agonists (morphine) and antagonists (naloxone) of opioid receptors play a key role in opioidergic modulation of analgesia in zebrafish, regulating local pain (Bao et al., 2019; Demin et al., 2018; Taylor et al., 2017). Moreover, the suitability of zebrafish for medium-to-high throughput pharmacological screening (Stewart, Gerlai, & Kalueff, 2015) may accelerate preclinical discovery of novel drugs with analgesic properties.

Finally, using systems biology approaches to investigate transcriptome, proteome, and metabolome can be strategically incorporated into zebrafish-based pain models. Such omics-based approaches can also facilitate the translatability of zebrafish studies across species aiming to identify evolutionarily conserved pathways related to pain and nociception.

Mini-dictionary of terms

Nociceptors. Sensory receptors transducing and encoding noxious stimuli.
Teleosts. The largest infraclass of *Actinopterygii*, the ray-finned fish, comprising around 96% of the existing fish species.
Formalin. Injection of formalin induces a concentration-dependent long-lasting (at least 14 days) mechanical allodynia (first stage) and hyperalgesia (second stage). At the former, formalin activates C-fibers, releasing substance P and bradykinin. At the latter, various pro-inflammatory mediators are released (*e.g.*, histamine, prostaglandins, and serotonin).
Histamine. An important mediator of the inflammatory response.
Cinnamaldehyde. Organic compound found in cinnamon, an agonist of the TRPA1 receptor.
Alila isothiocyanate. An organic compound found in mustard oil, and agonist of the TRPA1 receptor.
Erratic movements. Fast left-right swimming with abrupt changes in velocity and direction of movement. This phenotype indicates increased fear/anxiety-like states.
Bottom dwelling. Movement to/preference toward the bottom of the tank, often in response to threat. This phenotype indicates increased fear/anxiety-like states.
Freezing. A complete cessation of movement (except for gills and eyes) by the fish while at the bottom of the tank. This phenotype indicates increased fear/anxiety-like states.

Key facts

Key facts of zebrafish

- The complete genome sequence of the zebrafish was published in 2013.
- The zebrafish is a tropical fish native to the Southeast Asia.
- The use of zebrafish as a model organism in biomedicine has begun in the 1960s.
- Zebrafish have a high (>70%) genetic homology to humans.
- Nearly 85% of genes known to be associated with human diseases have a zebrafish counterpart.

Summary points

- Unlike humans, laboratory animal models have less complex neural processing networks.
- The zebrafish has evolutionarily conserved mechanisms involved in nociception, and its behaviors are bidirectionally sensitive to various algogens and analgesic drugs.
- Overt electrical activity and transcriptional changes in the midbrain and forebrain occur after harmful stimuli in teleosts.
- Analyses of specific behavioral phenotypes expressed in the presence of algogens is key to adequately assess nociceptive responses in vivo.

- Because zebrafish have complex behaviors, automated video-tracking tools can be used, including 3D reconstructions of swimming traces, to assess pain-like behaviors.
- Zebrafish represent an important, translationally relevant model system to investigate the evolutionarily conserved neural bases of pain responses in vertebrates.

References

Alvarez, F.A., Rodriguez-Martin, I., Gonzalez-Nunez, V., Marron Fernandez de Velasco, E., Gonzalez Sarmiento, R., & Rodriguez, R.E. (2006). New kappa opioid receptor from zebrafish *(Danio rerio)*. Neuroscience Letters, 405(1–2), 94–99. doi:10.1016/j.neulet.2006.06.028.

Antinucci, P., & Hindges, R. (2016). A crystal-clear zebrafish for in vivo imaging. Scientific Reports, 6, 29490. doi:10.1038/srep29490.

W. Bao A.D. Volgin E.T. Alpyshov A.J. Friend T.V. Strekalova M.S. de Abreu ...A.V. Kalueff Opioid neurobiology, neurogenetics and neuropharmacology in zebrafish Neuroscience 404 2019 218 232 10.1016/j.neuroscience.2019.01.045

Barrallo, A., Gonzalez-Sarmiento, R., Alvar, F., & Rodriguez, R.E. (2000). ZFOR2, a new opioid receptor-like gene from the teleost zebrafish (*Danio rerio*). Brain Research. Molecular Brain Research, 84(1–2), 1–6. doi:10.1016/S0169-328X(00)00152-2.

Barrallo, A., Gonzalez-Sarmiento, R., Porteros, A., Garcia-Isidoro, M., & Rodriguez, R.E. (1998). Cloning, molecular characterization, and distribution of a gene homologous to delta opioid receptor from zebrafish (*Danio rerio*). Biochemical and Biophysical Research Communications, 245(2), 544–548. doi:10.1006/bbrc.1998.8496.

Basbaum, A.I., Bautista, D.M., Scherrer, G., & Julius, D. (2009). Cellular and molecular mechanisms of pain. Cell, 139(2), 267–284. doi:10.1016/j.cell.2009.09.028.

Bateson, P. (1991). Assessment of pain in animals. Animal Behaviour, 42, 827–839. doi:10.1016/S0003-3472(05)80127-7.

Blackburn-Munro, G. (2004). Pain-like behaviours in animals – How human are they? Trends in Pharmacological Sciences, 25(6), 299–305. doi:10.1016/j.tips.2004.04.008.

Bobinski, F., Teixeira, J.M., Sluka, K.A., & Santos, A.R.S. (2018). Interleukin-4 mediates the analgesia produced by low-intensity exercise in mice with neuropathic pain. Pain, 159(3), 437–450. doi:10.1097/j.pain.0000000000001109.

Bonin, R.P., Bories, C., & De Koninck, Y. (2014). A simplified up-down method (SUDO) for measuring mechanical nociception in rodents using von Frey filaments. Molecular Pain, 10, 26. doi:10.1186/1744-8069-10-26.

Braithwaite, V.A., & Boulcott, P. (2007). Pain perception, aversion and fear in fish. Diseases of Aquatic Organisms, 75(2), 131–138. doi:10.3354/dao075131.

J. Cachat A. Stewart E. Utterback P. Hart S. Gaikwad K. Wong ...A.V. Kalueff Three-dimensional neurophenotyping of adult zebrafish behavior PLoS One 6 3 2011 e17597 10.1371/journal.pone.0017597

Chatterjee, D., Tran, S., Shams, S., & Gerlai, R. (2015). A simple method for immunohistochemical staining of zebrafish brain sections for c-fos protein expression. Zebrafish, 12(6), 414–420. doi:10.1089/zeb.2015.1147.

Correia, A.D., Cunha, S.R., Scholze, M., & Stevens, E.D. (2011). A novel behavioral fish model of nociception for testing analgesics. Pharmaceuticals (Basel), 4(4), 665–680. doi:10.3390/ph4040665.

Costa, F.V., Canzian, J., Stefanello, F.V., Kalueff, A.V., & Rosemberg, D.B. (2019). Naloxone prolongs abdominal constriction writhing-like behavior in a zebrafish-based pain model. Neuroscience Letters, 708, 134336. doi:10.1016/j.neulet.2019.134336.

Costa, F.V., Rosa, L.V., Quadros, V.A., Santos, A.R.S., Kalueff, A.V., & Rosemberg, D.B. (2019). Understanding nociception-related phenotypes in adult zebrafish: Behavioral and pharmacological characterization using a new acetic acid model. Behavioural Brain Research, 359, 570–578. doi:10.1016/j.bbr.2018.10.009.

A. Curtright M. Rosser S. Goh B. Keown E. Wagner J. Sharifi ...A. Dhaka Modeling nociception in zebrafish: A way forward for unbiased analgesic discovery PLoS One 10 1 2015 e0116766 10.1371/journal.pone.0116766

De Rantere, D., Schuster, C.J., Reimer, J.N., & Pang, D.S. (2016). The relationship between the rat grimace scale and mechanical hypersensitivity testing in three experimental pain models. European Journal of Pain, 20(3), 417–426. doi:10.1002/ejp.742.

K.A. Demin D.A. Meshalkina E.V. Kysil K.A. Antonova A.D. Volgin O.A. Yakovlev ...A.V. Kalueff Zebrafish models relevant to studying central opioid and endocannabinoid systems Progress in Neuro-Psychopharmacology & Biological Psychiatry 86 2018 301 312 10.1016/j.pnpbp.2018.03.024

X. Du B. Yuan J. Wang X. Zhang L. Tian T. Zhang ...F. Zhang Effect of heat-reinforcing needling on serum metabolite profiles in rheumatoid arthritis rabbits with cold syndrome Zhongguo Zhen Jiu 37 9 2017 977 983 10.13703/j.0255-2930.2017.09.018

Festing, S., & Patel, T. (2005). The ethics of research involving animals: A review of the Nuffield Council on bioethics report from a research perspective. Alternatives to Laboratory Animals, 33(6), 654–658. doi:10.1177/026119290503300603.

Fontana, B.D., Mezzomo, N.J., Kalueff, A.V., & Rosemberg, D.B. (2018). The developing utility of zebrafish models of neurological and neuropsychiatric disorders: A critical review. Experimental Neurology, 299(Pt A), 157–171. doi:10.1016/j.expneurol.2017.10.004.

Fordyce, W.E., Lansky, D., Calsyn, D.A., Shelton, J.L., Stolov, W.C., & Rock, D.L. (1984). Pain measurement and pain behavior. Pain, 18(1), 53–69. doi:10.1016/0304-3959(84)90126-x.

Gentle, M.J. (1992). Pain in birds. Animal Welfare, 1, 235–247.

Gonzalez-Cano, R., Tejada, M.A., Artacho-Cordon, A., Nieto, F.R., Entrena, J.M., Wood, J.N., & Cendan, C.M. (2017). Effects of tetrodotoxin in mouse models of visceral pain. Marine Drugs, 15(6). doi:10.3390/md15060188.

Gonzalez-Nunez, V., & Rodriguez, R.E. (2009). The zebrafish: A model to study the endogenous mechanisms of pain. ILAR Journal, 50(4), 373–386. doi:10.1093/ilar.50.4.373.

K. Howe M.D. Clark C.F. Torroja J. Torrance C. Berthelot M. Muffato ...D.L. Stemple The zebrafish reference genome sequence and its relationship to the human genome Nature 496 7446 2013 498 503 10.1038/nature12111

A.V. Kalueff M. Gebhardt A.M. Stewart J.M. Cachat M. Brimmer J.S. Chawla ...C. Zebrafish Neuroscience Research Towards a comprehensive catalog of zebrafish behavior 1.0 and beyond Zebrafish 10 1 2013 70 86 10.1089/zeb.2012.0861

Keefe, F.J., & Pryor, R.W. (2007). Assessment of pain behaviors. In Schmidt, R.F., & Willis, W.D. (Eds.), Encyclopedia of pain (pp. 136–138). Berlin, Heidelberg: Springer Berlin Heidelberg.

Kristiansen, T.S., Ferno, A., Holm, J.C., Privitera, L., Bakke, S., & Fosseidengen, J.E. (2004). Swimming behaviour as an indicator of low growth rate and impaired welfare in Atlantic halibut (*Hippoglossus hippoglossus L.*) reared at three stocking densities. Aquaculture, 230, 137–151. doi:10.1016/S0044-8486(03)00436-8.

F.E.A. Magalhaes C. de Sousa S. Santos R.B. Menezes F.L.A. Batista A.O. Abreu ...A.R. Campos Adult Zebrafish (*Danio rerio*): An alternative behavioral model of formalin-induced nociception Zebrafish 14 5 2017 422 429 10.1089/zeb.2017.1436

D.F. Martins T.C. Martins A.P. Batisti L. Dos Santos Leonel F. Bobinski L.A.O. Belmonte ...A.R.S. Santos Long-term regular eccentric exercise decreases neuropathic pain-like behavior and improves motor functional recovery in an axonotmesis mouse model: The role of insulin-like growth Factor-1 Molecular Neurobiology 55 7 2018 6155 6168 10.1007/s12035-017-0829-3

Maximino, C. (2011). Modulation of nociceptive-like behavior in zebrafish (*Danio rerio*) by environmental stressors. Psychology & Neuroscience, 4(1), 149–155. doi:10.3922/j.psns.2011.1.017.

Meotti, F.C., Coelho, I.D.S., & Santos, A.R. (2010). The nociception induced by glutamate in mice is potentiated by protons released into the solution. The Journal of Pain, 11(6), 570–578. doi:10.1016/j.jpain.2009.09.012.

Nordgreen, J., Tahamtani, F.M., Janczak, A.M., & Horsberg, T.E. (2014). Behavioural effects of the commonly used fish anaesthetic tricaine methanesulfonate (MS-222) on zebrafish (*Danio rerio*) and its relevance for the acetic acid pain test. PLoS One, 9(3), e92116. doi:10.1371/journal.pone.0092116.

Paukert, M., Sidi, S., Russell, C., Siba, M., Wilson, S.W., Nicolson, T., & Grunder, S. (2004). A family of acid-sensing ion channels from the zebrafish: Widespread expression in the central nervous system suggests a conserved role in neuronal communication. The Journal of Biological Chemistry, 279(18), 18783–18791. doi:10.1074/jbc.M401477200.

Piel, M.J., Kroin, J.S., van Wijnen, A.J., Ranjan, K.C., & Im, H.J. (2014). Pain assessment in animal models of osteoarthritis. Gene, 537(2), 184–188. doi:10.1016/j.gene.2013.11.091.

Pinal-Seoane, N., Martin, I.R., Gonzalez-Nunez, V., Marron Fernandez de Velasco, E., Alvarez, F.A., Sarmiento, R.G., & Rodriguez, R.E. (2006). Characterization of a new duplicate delta-opioid receptor from zebrafish. Journal of Molecular Endocrinology, 37(3), 391–403. doi:10.1677/jme.1.02136.

D.A. Prober S. Zimmerman B.R. Myers B.M. McDermott Jr. S.H. Kim S. Caron ...A.F. Schier Zebrafish TRPA1 channels are required for chemosensation but not for thermosensation or mechanosensory hair cell function The Journal of Neuroscience 28 40 2008 10102 10110 10.1523/JNEUROSCI.2740-08.2008

Reilly, S.C., Quinn, J.P., Cossins, A.R., & Sneddon, L.U. (2008). Behavioural analysis of a nociceptive event in fish: Comparisons between three species demonstrate specific responses. Applied Animal Behaviour Science, 114, 248–259. doi:10.1016/j.applanim.2008.01.016.

Rivas-Boyero, A.A., Herrero-Turrion, M.J., Gonzalez-Nunez, V., Sanchez-Simon, F.M., Barreto-Valer, K., & Rodriguez, R.E. (2011). Pharmacological characterization of a nociceptin receptor from zebrafish (*Danio rerio*). Journal of Molecular Endocrinology, 46(2), 111–123. doi:10.1530/JME-10-0130.

Rosa, L.V., Ardais, A.P., Costa, F.V., Fontana, B.D., Quadros, V.A., Porciuncula, L.O., & Rosemberg, D.B. (2018). Different effects of caffeine on behavioral neurophenotypes of two zebrafish populations. Pharmacology, Biochemistry, and Behavior, 165, 1–8. doi:10.1016/j.pbb.2017.12.002.

Saito, S., & Shingai, R. (2006). Evolution of thermoTRP ion channel homologs in vertebrates. Physiological Genomics, 27(3), 219–230. doi:10.1152/physiolgenomics.00322.2005.

Sakai, C., Ijaz, S., & Hoffman, E.J. (2018). Zebrafish models of neurodevelopmental disorders: Past, present, and future. Frontiers in Molecular Neuroscience, 11, 294. doi:10.3389/fnmol.2018.00294.

Schroeder, P.G., & Sneddon, L.U. (2017). Exploring the efficacy of immersion analgesics in zebrafish using an integrative approach. Applied Animal Behaviour Science, 187, 93–102. doi:10.1016/j.applanim.2016.12.003.

Sneddon, L.U. (2002). Anatomical and electrophysiological analysis of the trigeminal nerve in a teleost fish, *Oncorhynchus mykiss*. Neuroscience Letters, 319(3), 167–171. doi:10.1016/s0304-3940(01)02584-8.

Sneddon, L.U., Braithwaite, V.A., & Gentle, M.J. (2003a). Do fishes have nociceptors? Evidence for the evolution of a vertebrate sensory system. Proceedings of the Biological Sciences, 270(1520), 1115–1121. doi:10.1098/rspb.2003.2349.

Sneddon, L.U., Braithwaite, V.A., & Gentle, M.J. (2003b). Novel object test: Examining nociception and fear in the rainbow trout. The Journal of Pain, 4(8), 431–440. doi:10.1067/S1526-5900(03)00717-X.

Sneddon, L.U., Elwood, R.W., Adamo, S.A., & Leach, M.C. (2014). Defining and assessing animal pain. Animal Behaviour, 97, 201–212. doi:10.1016/j.anbehav.2014.09.007.

Stevenson, G.W., Bilsky, E.J., & Negus, S.S. (2006). Targeting pain-suppressed behaviors in preclinical assays of pain and analgesia: Effects of morphine on acetic acid-suppressed feeding in C57BL/6J mice. The Journal of Pain, 7(6), 408–416. doi:10.1016/j.jpain.2006.01.447.

Stewart, A.M., Gerlai, R., & Kalueff, A.V. (2015). Developing highER-throughput zebrafish screens for in-vivo CNS drug discovery. Frontiers in Behavioral Neuroscience, 9, 14. doi:10.3389/fnbeh.2015.00014.

Taylor, J.C., Dewberry, L.S., Totsch, S.K., Yessick, L.R., DeBerry, J.J., Watts, S.A., & Sorge, R.E. (2017). A novel zebrafish-based model of nociception. Physiology & Behavior, 174, 83–88. doi:10.1016/j.physbeh.2017.03.009.

Thomas, D. (2005). Laboratory animals and the art of empathy. Journal of Medical Ethics, 31(4), 197–202. doi:10.1136/jme.2003.006387.

Thomson, J.S., Al-Temeemy, A.A., Isted, H., Spencer, J.W., & Sneddon, L.U. (2019). Assessment of behaviour in groups of zebrafish (*Danio rerio*) using an intelligent software monitoring tool, the chromatic fish analyser. Journal of Neuroscience Methods, 328, 108433. doi:10.1016/j.jneumeth.2019.108433.

Tracey, I., & Mantyh, P.W. (2007). The cerebral signature for pain perception and its modulation. Neuron, 55(3), 377–391. doi:10.1016/j.neuron.2007.07.012.

ANEXO B - LISTA DE TRABALHOS COLABORATIVOS DESENVOLVIDOS DURANTE O DOUTORADO

1. Canzian, J.; Muller, T.E.; Franscescon, F.; Michelotti, P.; Fontana, B.D.; **Costa, F.V**.; Rosemberg, D.B. Modeling psychiatric comorbid symptoms of epileptic seizures in zebrafish. **J Psychiatr Res**, 2019, 119, 14-22.

2. Demin, K.A.; Lakstygal, A.M.; Volgin, A.D.; de Abreu, M.S.; Genario, R.; Alpyshov, E.T.; Serikuly, N.; Wang, D.; Wang, J.; Yan, D.; Wang, M.; Yang, L.; Hu, G.; Bytov, M.; Zabegalov, K.N.; Zhdanov, A.; Harvey, B.H.; **Costa, F**.; Rosemberg, D.B.; Leonard, B.E.; Fontana, B.D.; Cleal, M.; Parker, M.O.; Wang, J.; Song, C.; Amstislavskaya, T.G.; Kalueff, A.V. Cross-species Analyses of Intra-species Behavioral Differences in Mammals and Fish. **Neuroscience**, 2020, 429, 33-45.

3. Quadros, V.A.; **Costa, F.V**.; Canzian, J.; Nogueira, C.W.; Rosemberg, D.B. Modulatory role of conspecific alarm substance on aggression and brain monoamine oxidase activity in two zebrafish populations. **Prog Neuropsychopharmacol Biol Psychiatry**, 2018, 86, 322-330.

4. Quadros, V.A.; Rosa, L.V.; **Costa, F.V**.; Muller, T.E.; Stefanello, F.V.; Loro, V.L.; Rosemberg, D.B. Involvement of anxiety-like behaviors and brain oxidative stress in the chronic effects of alarm reaction in zebrafish populations. **Neurochem Int**, 2019, 129, 104488.

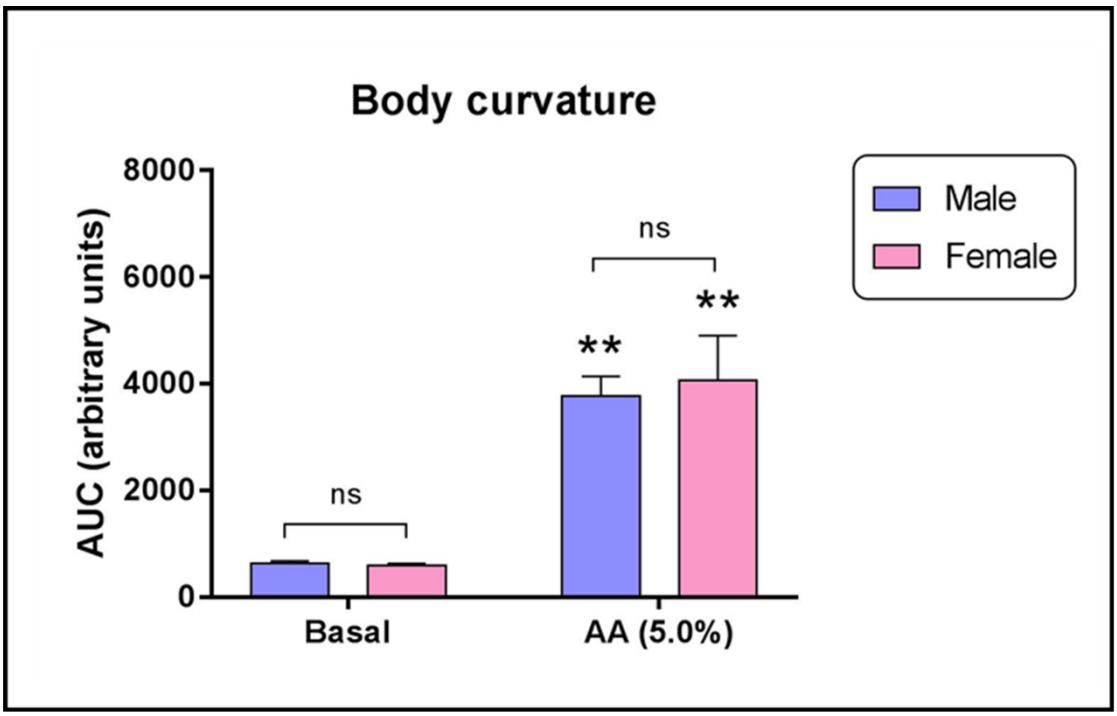
5. Rosa, L.V.; Ardais, A.P.; **Costa, F.V**.; Fontana, B.D.; Quadros, V.A.; Porciuncula, L.O.; Rosemberg, D.B. Different effects of caffeine on behavioral neurophenotypes of two zebrafish populations. **Pharmacol Biochem Behav**, 2018, 165, 1-8.

6. Rosa, L.V.; **Costa, F.V**.; Canzian, J.; Borba, J.V.; Quadros, V.A.; Rosemberg, D.B. Three- and bi-dimensional analyses of the shoaling behavior in zebrafish: Influence of modulators of anxiety-like responses. **Prog Neuropsychopharmacol Biol Psychiatry**, 2020, 102, 109957.

7. Quadros, V.A.; Rosa, L.V.; **Costa, F.V**.; Koakoski, G.; Barcellos L. J.G.; Rosemberg, D.B. Predictable chronic stress modulates behavioral and neuroendocrine phenotypes of zebrafish: Influence of two homotypic stressors on stress-mediated responses. **Comp Biochem Physiol C Toxicol Pharmacol.** 2021, 247, 109030.

8. Abreu, M. S.; **Costa, F.V**.; Giacomini A. C. V. V.; Demin, K. A.; Petersen, E. V.; Rosemberg, D.B., Kalueff, A.V. Exploring CNS effects of American traditional medicines using zebrafish models. **Curr. Neuropharmacol**. DOI: 10.2174/1570159X19666210712153329.

ANEXO C – FIGURA SUPLEMENTAR DO ARTIGO 1, MOSTRANDO A SEMELHANÇA DAS RESPOSTAS DE CURVATURA ABDOMINAL EM PEIXES MACHOS E FÊMEAS



ANEXO D. LINK PARA ACESSO AO VÍDEO SUPLEMENTAR QUE MOSTRA OS FENÓTIPOS COMPORTAMENTAIS EM PEIXE-ZEBRA INJETADOS COM PBS E ÁCIDO ACÉTICO (5.0%) PUBLICADO NO **ARTIGO 1.**

https://ars.els-cdn.com/content/image/1-s2.0-S0166432818312099-mmc4.mp4

ANEXO E. PROTOCOLO DA METODOLOGIA UTILIZADA NESTA TESE DISPONÍVEL ONLINE (PROTOCOLS.IO).

https://www.protocols.io/view/modeling-acute-visceral-pain-in-adult-zebrafish-bwjkpckw

Modeling acute visceral pain in adult zebrafish

Fabiano Costa[1], Denis Rosemberg[1]
[1]Federal University of Santa Maria

Jul 16, 2021
1 Works for me
Share
dx.doi.org/10.17504/protocols.io.bwjkpckw

Fabiano Denis Rosemberg

Denis Rosemberg

ABSTRACT

This protocol describes a reliable procedure for assessing acute pain responses in adult zebrafish (*Danio rerio*) based on the abdominal writhing-like phenotype following a single intraperitoneal injection of acetic acid. The method is an inexpensive, fast, and easy-to-use protocol for measuring pain-like responses in adult zebrafish. The protocol involves five steps: analyses of baseline behavior, drug injection, post-injection recordings of behavior, euthanasia, and data analyses/interpretation. Intraperitoneal injection of 2.5–5.0% acetic acid elicits a robust pain-like behavior by changing zebrafish body curvature, which can be easily quantified using freely available imaging software. This response is sensitive to pharmacological manipulations, as morphine prevents altered body curvature, while naloxone blocks these effects. Pretreatment with diclofenac sodium (a non-steroidal anti-inflammatory drug commonly used as an analgesic) also prevents writhing-like behavior. Demonstrating high predictive and face validity, this unbiased protocol can be performed over the course of ~2 days, enabling a reliable assessment of acute pain-like responses in zebrafish, thus fostering in-depth analyses of complex pain-related mechanisms and anti-pain drug discovery.

DOI

dx.doi.org/10.17504/protocols.io.bwjkpckw

PROTOCOL CITATION

Fabiano Costa, Denis Rosemberg 2021. Modeling acute visceral pain in adult zebrafish. **protocols.io** https://dx.doi.org/10.17504/protocols.io.bwjkpckw

FUNDERS ACKNOWLEDGEMENT

Conselho Nacional de Desenvolvimento Científico e Tecnológico 305051/2018-0

MANUSCRIPT CITATION please remember to cite the following publication along with this protocol

Costa FV, Rosa LV, Quadros VA, Santos ARS, Kalueff AV, Rosemberg DB. (2019) Understanding nociception-related phenotypes in adult zebrafish: Behavioral and pharmacological characterization using a new acetic acid model. Behav Brain Res. 359, 570-578. https://doi.org/10.1016/j.bbr.2018.10.009

KEYWORDS

zebrafish, nociception, acute abdominal pain, abnormal body curvature, acetic acid, pharmacological manipulations, opioid system, analgesic properties

CREATED

Jul 13, 2021

LAST MODIFIED

Jul 16, 2021

PROTOCOL INTEGER ID

51532

GUIDELINES

Strikingly paralleling the abdominal constriction writhing-like response observed in rodents, the present protocol **(Fig. 1)** is easy-to-use, inexpensive, and does not require complex trials or sophisticated automated tools. Specifically, a single acetic acid administration (2.5–5.0%, i.p.) elicits overt changes in zebrafish body posture that persist for 30 min after injection. This phenotype can be easily analyzed using freely available software (*e.g.*, Image J) for Windows, Mac OS X, and Linux systems, and can also be complemented with automated video-tracking tools (*e.g.*, Any-Maze^{TM}, Stoelting, CO and/or NOLDUS EthoVision XT, Wageningen, Netherlands) to assess other relevant zebrafish behaviors, as locomotion and exploration. Albeit sensitive and unbiased, the protocol has limitations to be considered for further refining and model development. For example, the present protocol is based on a relatively narrow (2.5–5.0%) range of algogenic (acetic acid) response that affects the body curvature. Our pilot experiments using 7.0% of acetic acid unaltered body curvature (vs. 5.0%) but caused a high (~50%) mortality rate. Furthermore, a highly trained researcher is needed to perform an i.p. injection, because if poorly performed, it may induce unwanted side-effects, including such non-specific phenotypes as hyperactivity, corkscrew swimming or and/or the loss of posture (ataxia). The ability of other chemicals (beyond acetic acid) to evoke the observed writhing-like responses also merits further testing. Finally, potential individual, and/or strain differences in nociception merits further scrutiny in zebrafish.

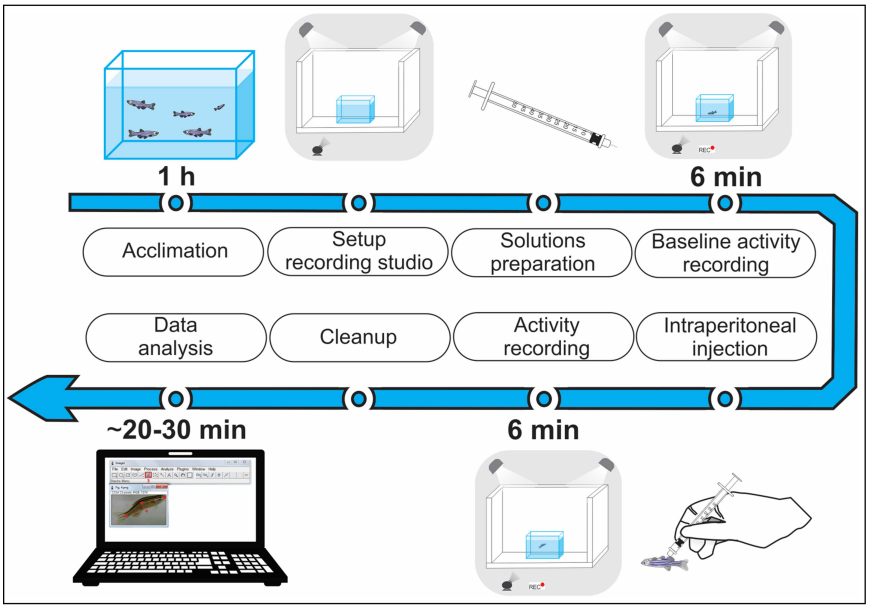


Fig. 1. Schematic diagram showing the experimental procedures to model abdominal constriction writhing-like responses in adult zebrafish and time required for the main steps of the present protocol.

MATERIALS TEXT

Acetic acid glacial **Sigma Aldrich Catalog #ARK2183**

Phosphate Buffered Saline **Thermo Fisher Scientific Catalog #28374**

Sodium chloride **P212121**

Morphine sulfate salt pentahydrate **Sigma Aldrich Catalog #M8777**

Citation: Fabiano Costa, Denis Rosemberg (07/16/2021). Modeling acute visceral pain in adult zebrafish. https://dx.doi.org/10.17504/protocols.io.bwjkpckw

Naloxone methiodide **Sigma Aldrich Catalog #N129**

Diclofenac sodium salt **Sigma Aldrich Catalog #D6899**

Experimental tanks (3.5 L)
Aquarium
Tecniplast **ZB30TK**
3.5 L tank made of blue polycarbonate

Insulin gauge needle (BD Ultra-fineTM II, NJ, USA)
(8 mm x 0.3 mm)
BD **328838**
0.3 mL

Computer
Computer
Dell **v3681w206w**
Desktop or notebook with necessary basic software able to acquire digital video recordings, run appropriate imaging and/or video tracking software, as well as statistical packages (e.g., Excel, GraphPad Prism, SPSS, or similar).

C505e HD BUSINESS WEBCAM
Webcam
Logitech **no number**
High-resolution video cameras (e.g., HD webcams connected to a computer through a USB) or similar.

Citation: Fabiano Costa, Denis Rosemberg (07/16/2021). Modeling acute visceral pain in adult zebrafish. https://dx.doi.org/10.17504/protocols.io.bwjkpckw

SAFETY WARNINGS

Acetic acid is a severe irritant agent. In contact with the skin or eyes, an 80% solution can be corrosive, causing severe burns. Use personal protective equipment (*e.g.*, gloves, lab coat, safety glasses, and respirator) to prevent contamination and limit exposure.

BEFORE STARTING

As subjects, use adult zebrafish (~50% male and female ratio, 4–6 months old). Heterogeneous wild-type (*e.g.*, short-fin), specific (*e.g.*, AB, TU) or genetically modified zebrafish strains can be used. While using the same-batch fish cohorts is desirable, fish from different batches still provide highly consistent data. For testing, animals must be randomly separated from their housing tanks and assigned to specific experimental groups using a computerized random number generator (*e.g.*, www.random.org). Both male and female zebrafish can be used for the experiments since no gender difference in writhing-like responses to acetic acid was observed in the reference paper. Note, however, that if using additional behavioral endpoints and/or treatments, animals from both sexes must be tested separately to avoid false positive or negative findings, and consistent with recent NIH guidelines on the inclusion of both males and females in biomedical experimentation. All animal experimentation should be performed in accordance with the Institutional Animal Care and Use Committee (IACUC) following national guidelines and standards.

ACCLIMATIZATION AND BASELINE BEHAVIORAL RECORDINGS ●**TIMING** ~ 1 h for acclimatization; 6 min per animal 1h 6m

1 ⚠

Transport animals from their holding facility to the experimental room for acclimation 1 h prior to the experiments. Avoid low- or high density of fish to prevent social isolation or crowding stress (tanks must have 1–2 fish per 1 L of water). Fish must acclimate to the facility before testing, and the water used must have similar physicochemical characteristics to those of housing tanks. The use of home tank or holding tank water (with properly adjusted temperature and salinity) is required. **!CAUTION:** Ensure that the experimental tank is filled with non-chlorinated water before use set at optimal temperature (27-28°C).

2 ⚠

Mount a camera on the frontal side of tank test and connect video recorder to the power in a switch-on mode. Provide adequate illumination (fluorescent light bulbs), to ensure that the enlightenment is proper to differentiate the subject from the apparatus, allowing a precise detection of fish. However, avoid excessive brightly lit environments (stressful for zebrafish) and dark areas (poor fish recognition and video-tracking detection of locomotion if desirable).
? TROUBLESHOOTING

? TROUBLESHOOTING

Problems: (1) Inappropriate video lighting.

Possible reasons: (1) Lack or excess of brightness, heterogeneous background.

Solutions: (1) Measure the light intensity above the experimental tank with a lux meter or open Android or IOS application (*e.g.*, Lux meter, Crunchy ByteBox, Germany, or similar) to ensure adequate illumination. Use a styrofoam background to improve contrast.

3 Prepare the acetic acid solutions in 1.5 mL microcentrifuge tubes (2.5% or 5.0% diluted in PBS or 0.9% NaCl).

4 ⚠

Citation: Fabiano Costa, Denis Rosemberg (07/16/2021). Modeling acute visceral pain in adult zebrafish. https://dx.doi.org/10.17504/protocols.io.bwjkpckw

Transfer the fish from the holding tank to the experimental tank and start recording for 6 minutes to measure the baseline behavioral phenotypes. Animals should be transferred individually to the experimental tank. Ensure adequate transport to minimize handling stress. **!CAUTION:** If more than a single injection is applied, the baseline behavior should be recorded after the first injection to avoid false positive/negative results. **?TROUBLESHOOTING**

? TROUBLESHOOTING

Problem: (1) Pretreatment alters the normal zebrafish behavior.

Possible reasons: (1) The drug used affects the swimming activity of fish and/or elicits abnormal phenotypes. Incorrect injection.

Solutions: (1) Run a pilot experiment using different concentrations of the drug. Analyze zebrafish behavior at different time intervals after pretreatment. Use highly-trained researchers to perform the injections.

INTRAPERITONEAL INJECTIONS ●**TIMING** ~ 3 min per animal 3m

5

Before i.p. injections, video recordings must be stopped.

6

Remove the fish from the tank with a fishing net and proceed with the i.p. injection using a BD Ultra-fine™ 30U syringe (needle size 6 x 0.25 mm) with a volume of 10 µL (which does not impair normal zebrafish behaviors). Animals must be gently handled, briefly anesthetized, and later immobilized using a small wet fishing net (< 5 s). The i.p. injections should be quickly performed into the midline between the pelvic fins. To minimize potential interference of drugs in the behavioral endpoints measured and to allow a fast evaluation of the swimming activity, avoid using anesthetics (tricaine or other similar drugs). Injections can be quickly performed through the net in fish previously anesthetized in cold water without affecting complex behaviors (*e.g.*, depth preference, immobility, and locomotion). **?TROUBLESHOOTING**

? TROUBLESHOOTING

Problem: (1) Unconventional responses (*e.g.*, fish do not exhibit curvature, fish die).

Possible reasons: (1) Incorrect injection, incorrect acetic acid concentration.

Solutions: (1) Use highly-trained researchers to perform the injections. Ensure the appropriate acetic acid concentration is used.

FINAL BEHAVIOR RECORDING ●**TIMING** 6 min per animal 6m

7

Relocate the fish into the experimental tank to record the behavioral phenotypes following i.p. injections for 6 minutes.

Citation: Fabiano Costa, Denis Rosemberg (07/16/2021). Modeling acute visceral pain in adult zebrafish. https://dx.doi.org/10.17504/protocols.io.bwjkpckw

CLEANUP ●**TIMING** 1–2 min per animal 2m

8 Stop the behavioral recording and remove the fish from the experimental tank. Animals must be euthanized following national guidelines and standard protocols.

9 Discard the water used previously, wash the tank thoroughly with tap water and refill it with non-chlorinated water before testing another fish.

10 Repeat steps 4–9 for the next subject tested.

VIDEO ANALYSES ●**TIMING** 20–30 min per animal 30m

11

Digital pictures of fish at sagittal plane **(Fig. 2)** must be taken every 30 s, totaling 24 photos per fish. We strongly recommend the use of "Prt Sc" (*print screen*) Windows tool.

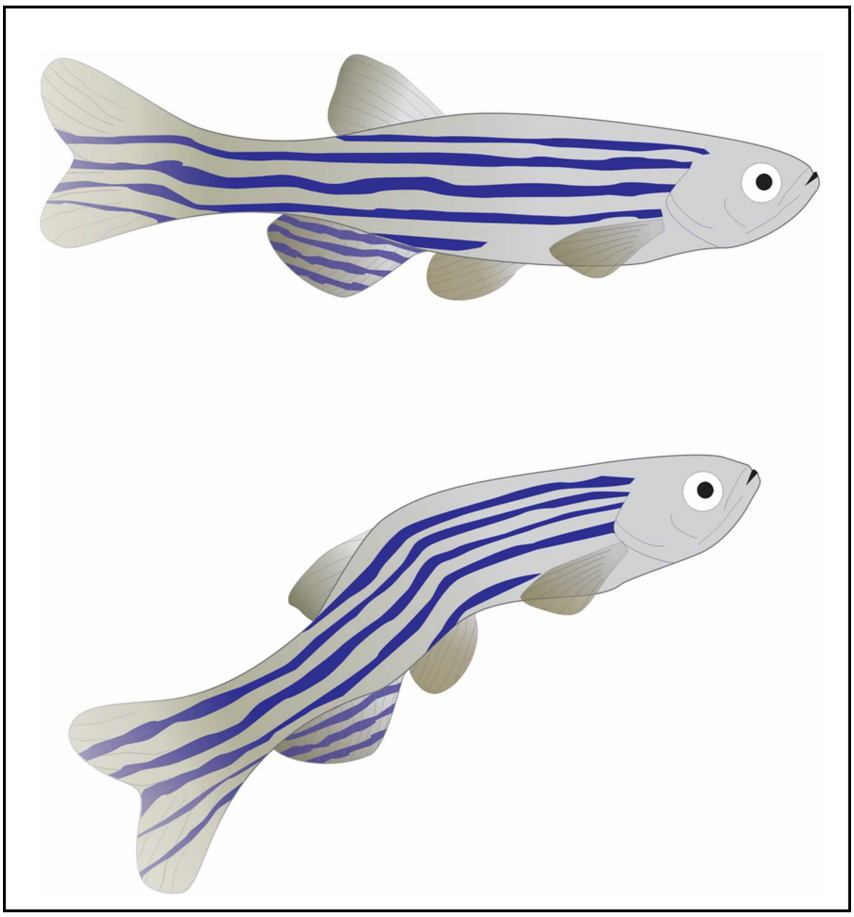


Fig. 2. Representative phenotypes of zebrafish following vehicle (PBS, upper fish) or acetic acid (5.0% AA, lower fish) i.p. administration.

12

Citation: Fabiano Costa, Denis Rosemberg (07/16/2021). Modeling acute visceral pain in adult zebrafish. https://dx.doi.org/10.17504/protocols.io.bwjkpckw

With ImageJ software (available for download at https://imagej.nih.gov/ij/download.html), open the digital pictures of fish sagittal plane that represent the first 30 s **(1-2)**, and using the angle tool **(3)**, select three positions to estimate the fish body angulation: a frontal (in the front of the head), a central (in the middle of the animal's body – between the anal and dorsal fins), and a posterior one (at the caudal fin) **(4)**. Then, analyze and measure the fish angular value **(5-7)**.
!CAUTION: Results must be subtracted from 180° to calculate a value representing the body curvature index **(Fig. 3)**.
?TROUBLESHOOTING

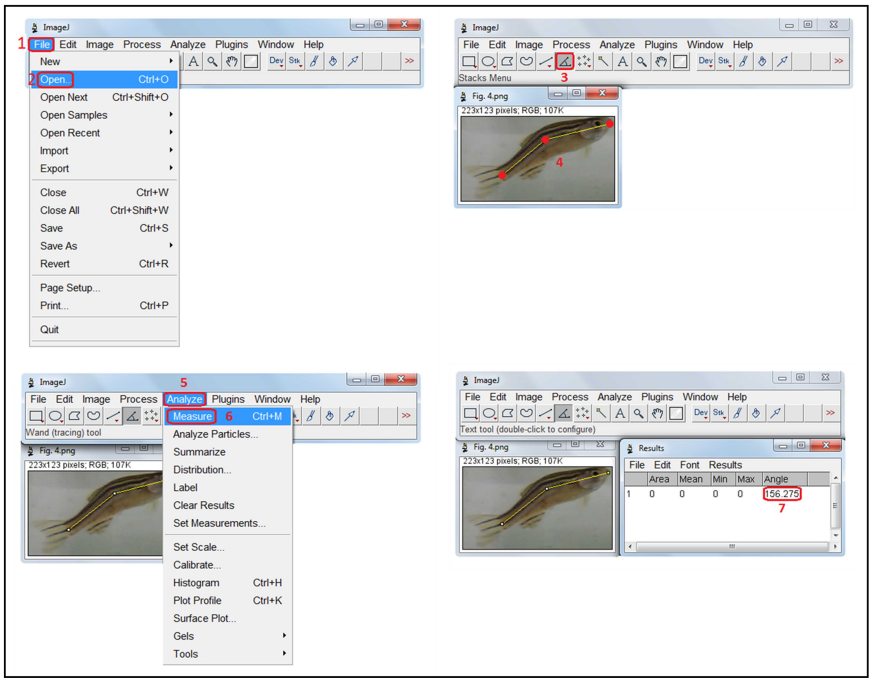


Fig. 3. Main steps to analyze the abdominal writhing-like phenotype using the Image J software. After opening, click on "file" **(1)** and "open" **(2)** to analyze the sagittal pictures taken. Using the angle tool **(3)**, select three points (frontal, central, and posterior) **(4)** to estimate the body angulation. Then, select "analyze" **(5)** and "measure" **(6)** to obtain the angle value **(7)**, which must be subtracted from 180°.

?TROUBLESHOOTING

Problem: (1) Fish do not swim at the sagittal plane. **(2)** The image is not appropriate to measure the body curvature.

Possible reasons: (1) Fish freeze during the entire test period due to increased stress responses; **(2)** The quality of video and/or image acquisition makes the analysis difficult. High-speed swimming in the experimental tank due to increased erratic movements.

Solutions: (1) Ensure adequate manipulation to minimize handling and/or other stressful conditions. Use a narrow experimental tank to restrict the lateral swimming activity. Alternatively, record videos from frontal- and top-view positions to generate 3D traces; **(2)** Ensure a proper manipulation and/or injection to minimize stress. Improve video/image resolution. Use a high-resolution camera to record the behaviors. Select a more appropriate image to quantify the writhing-like response at the respective time interval by using the Prt Sc" (*print screen*) Windows tool (to obtain high definition screenshots).

STATISTICAL ANALYSES ●**TIMING** 20–30 min, depending on amount of data collected 30m

13

Citation: Fabiano Costa, Denis Rosemberg (07/16/2021). Modeling acute visceral pain in adult zebrafish. https://dx.doi.org/10.17504/protocols.io.bwjkpckw

Several options exist for analyzing the data, based on the specific experimental design. Use the nonparametric Wilcoxon-Mann-Whitney U-test or parametric Student's *t*-test (if data are homoscedastic or normally distributed) for comparing two groups. For more than two groups, use analysis of variance (ANOVA), followed by appropriate post-hoc comparison (*e.g.*, Tukey, Dunn, Student-Newman-Keuls or Dunnet tests). Depending on the study design, an *n*-way ANOVA can generally be applied to assess various factors (*e.g.*, drug/treatment, dose, sex, strain, time, trial, age). Even if a small number of cohorts is used ($n = 5$), robust differences can be detected in acetic acid-treated fish vs. PBS (control group) (*e.g.*, effect size calculated using Cohen's $d = 3.646$). Thus, the use of adult zebrafish to assess pain-like responses following a single i.p. acetic acid injection brings direct "3R's" benefits (refinement, replacement, reduction) of animal experimentation. Use ANOVA with repeated measures to assess time-dependent modulation of the observed phenotypes, if necessary. To facilitate data analysis, export data from every 30 s into separate Excel spreadsheets (one spreadsheet per fish tested in a 6-min trial). The area under curve (AUC) from each individual can be further calculated to express the specific behavioral endpoint using specific statistical packages (*e.g.*, GraphPad Prism).

ANTICIPATED RESULTS

14 The anticipated results described below show robust pain-like responses in zebrafish as reported elsewhere.

Costa FV, Rosa LV, Quadros VA, Santos ARS, Kalueff AV, Rosemberg DB (2019). Understanding nociception-related phenotypes in adult zebrafish: Behavioral and pharmacological characterization using a new acetic acid model.. Behavioural brain research. https://doi.org/10.1016/j.bbr.2018.10.009

Intraperitoneal injection of 5.0% acetic acid (AA) induces a typical, well-defined abdominal constriction writhing-like phenotype (measured as body curvature index) in zebrafish, analogous to writhing response observed in rodents. **Fig. 4** illustrates typical results obtained using the present protocol. Notably, these results are robust and replicable even with a small number of subjects per cohort (*e.g.*, $n = 5$), obtained by trained researchers blinded to the experimental condition with inter-rater reliability > 0.85–0.90. Acetic acid-treated fish show a prominent increase in the body curvature index when compared to control (PBS). Interestingly, intraperitoneal injections do not affect the baseline curvature since no differences are observed in PBS-treated group.

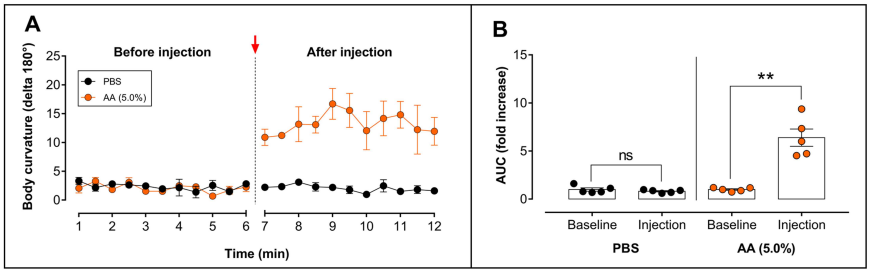


Fig. 4. Intraperitoneal AA injection elicits abdominal constriction writhing-like behavior in adult zebrafish. **(A)** Temporal variations in the body curvature in the vehicle (PBS) and 5.0% AA groups before and after i.p. injection. The red arrow indicates the moment of injection. **(B)** Changes in the area under curve (AUC) following i.p. injections. Results are expressed as means ± S.E.M. and analyzed by paired Student's *t*-test. (**$p < 0.01$; $n = 5$ per group). Data are similar to those reported in the previously published reference paper (Costa et al., 2019).

Our protocol also enables studying the involvement of the opioid system in zebrafish pain-like behaviors, as well as the effects of potential pro- and anti-pain drugs. For example, co-administration of 2.5 mg/kg morphine (MOR), an opioid agonist classically used as an analgesic drug, inhibits the effects of acetic acid on the body curvature index, while a common 'reference' non-specific opioid antagonist naloxone (NAL, 5.0 mg/kg) predictably antagonizes morphine-evoked analgesic responses **(Fig. 5)**.

Citation: Fabiano Costa, Denis Rosemberg (07/16/2021). Modeling acute visceral pain in adult zebrafish. https://dx.doi.org/10.17504/protocols.io.bwjkpckw

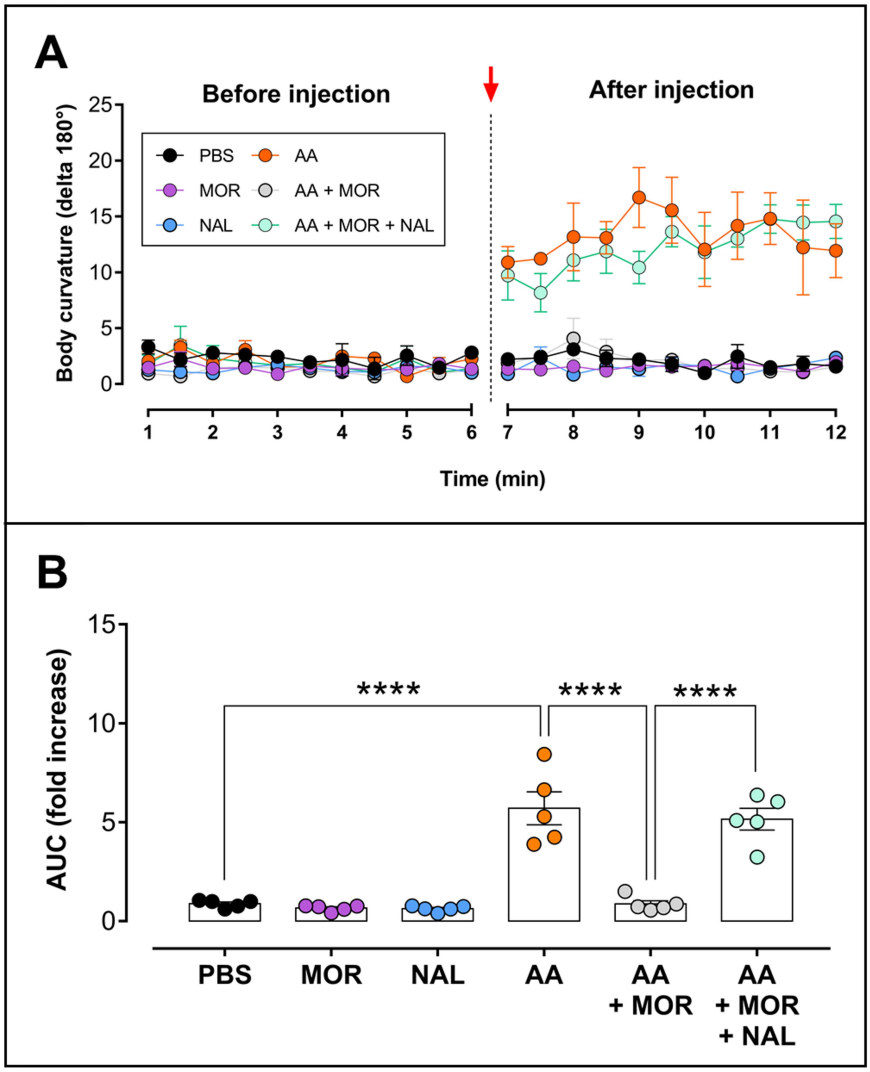


Fig. 5. Influence of opioidergic system in the 5.0% AA-induced abdominal constriction writhing-like responses. **(A)** Changes in the body curvature in vehicle (PBS), AA, 2.5 mg/kg MOR, AA+MOR, 5.0 mg/kg NAL, and AA+MOR+NAL groups across time. The red arrow indicates the moment of injection. **(B)** Area under curve (AUC) values following i.p. injections, expressed as fold increase in relation to PBS group. Results are shown as means ± S.E.M. and analyzed by one-way ANOVA (factor: treatment), followed by Tukey's post-hoc test for significant ANOVA data (**** $p < 0.001$; $n = 5$ per group). Data are similar to those reported in the previously published reference paper (Costa et al., 2019).

Pretreatment with the non-steroidal anti-inflammatory drug, diclofenac (40 mg/kg, for 15 min), another commonly used analgesic agent that does not target the opioidergic system, prevents acetic acid-induced changes in the body curvature index **(Fig. 6)**. These findings suggest that assessing writhing-like responses in the present protocol may be a sensitive and specific approach to evaluate pain-related phenotypes in adult zebrafish.

Citation: Fabiano Costa, Denis Rosemberg (07/16/2021). Modeling acute visceral pain in adult zebrafish. https://dx.doi.org/10.17504/protocols.io.bwjkpckw

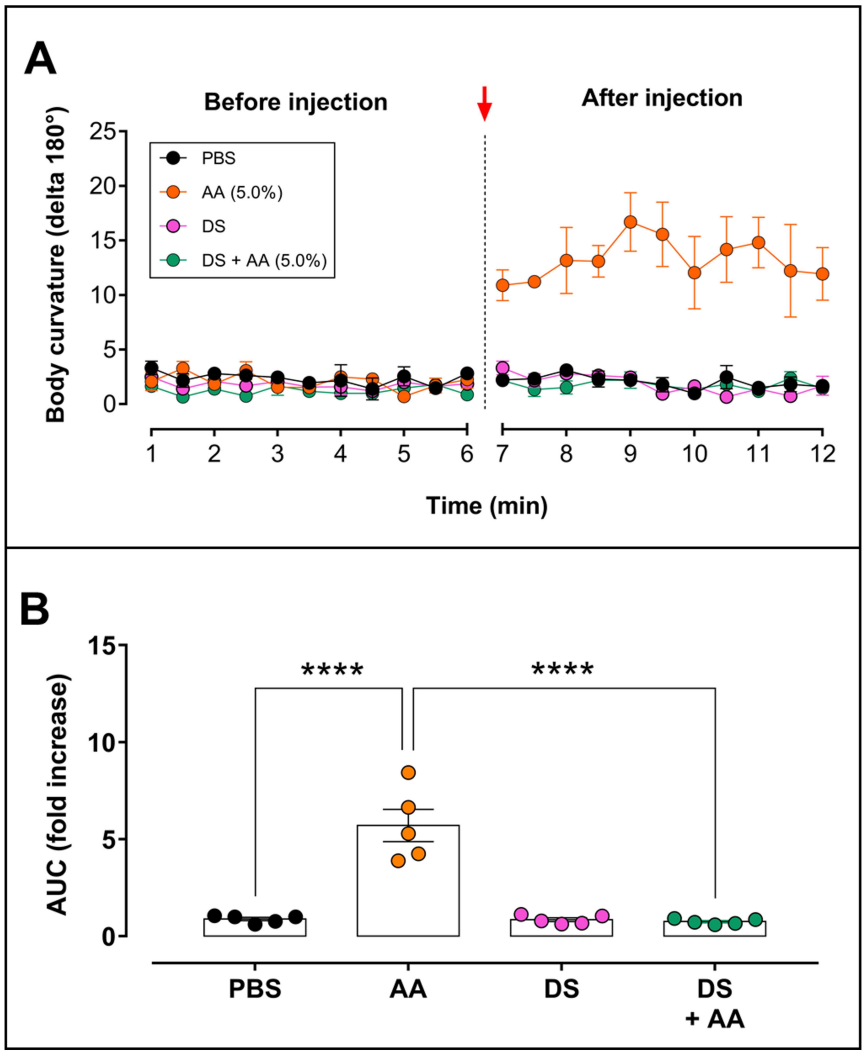


Fig. 6. Diclofenac (DS, 40 mg/kg, i.p.) pretreatment prevents 5.0% acetic acid (AA)-induced abdominal constriction writhing-like behavior. **(A)** Temporal variations in the body curvature index in vehicle (PBS), AA, DS, and DS+AA groups across time. The red arrow indicates the moment of AA or PBS injection. **(B)** Area under curve (AUC) values, expressed as fold increase in relation to PBS group. Baseline recordings were performed 15 min after PBS or DS i.p. injections. Results are expressed as means ± S.E.M. and analyzed by one-way ANOVA (factor: treatment), followed by Tukey's post-hoc test for significant ANOVA data (**** $p < 0.001$; $n = 5$ per group). Data are similar to those reported in the previously published reference paper (Costa et al., 2019).

Overall, these robust behavioral and pharmacological responses show high predictive and face validity of the present zebrafish-based pain model. The protocol can be run using animals selected randomly from their home tanks since no differences in the body curvature index was observed when different genders are tested **(Fig. 7)**. Collectively, these data show that the protocol described here enables measuring specific acute pain responses in adult zebrafish.

Citation: Fabiano Costa, Denis Rosemberg (07/16/2021). Modeling acute visceral pain in adult zebrafish. https://dx.doi.org/10.17504/protocols.io.bwjkpckw

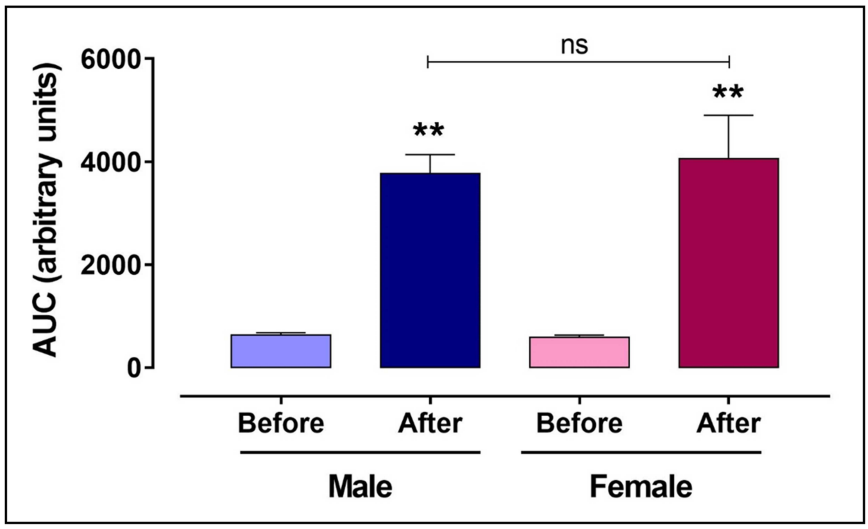


Fig. 9. Male and female show similar effects of 5.0% acetic acid (AA) in the abdominal constriction writhing-like phenotype. Body curvature indexes (before, and after i.p. AA injections) are shown as the area under curve (AUC), expressed as arbitrary units. Data are expressed as means ± S.E.M. and analyzed by repeated measures ANOVA (factors: gender and time), followed by Bonferroni's post-hoc test (** $p < 0.01$ when compared to their respective baseline values; ns = non-significant; $n = 5$ per group). Data are similar to those reported in the previously published reference paper (Costa et al., 2019).

Citation: Fabiano Costa, Denis Rosemberg (07/16/2021). Modeling acute visceral pain in adult zebrafish. https://dx.doi.org/10.17504/protocols.io.bwjkpckw

ANEXO F - CARTA DE APROVAÇÃO DA COMISSÃO DE ÉTICA NO USO DE ANIMAIS – CEUA/UFSM

Comissão de Ética no Uso de Animais
da
Universidade Federal de Santa Maria

CERTIFICADO

Certificamos que a proposta intitulada "Endofenótipos comportamentais relacionados à nocicepção em peixe-zebra (Danio rerio): uma caracterização comportamental utilizando o modelo do ácido acético", protocolada sob o CEUA nº 5438310817 (ID 001660), sob a responsabilidade de **Denis Broock Rosemberg** *e equipe; Fabiano Vargas da Costa; Vanessa Andreatta de Quadros* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Universidade Federal de Santa Maria (CEUA/UFSM) na reunião de 28/09/2017.

We certify that the proposal "Nociception-related endophenotypes in zebrafish: a behavioral characterization using the acetic acid model ", utilizing 180 Fishes (males and females), protocol number CEUA 5438310817 (ID 001660), under the responsibility of **Denis Broock Rosemberg** *and team; Fabiano Vargas da Costa; Vanessa Andreatta de Quadros* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the Federal University of Santa Maria (CEUA/UFSM) in the meeting of 09/28/2017.

Finalidade da Proposta: Pesquisa

Vigência da Proposta: de 10/2017 a 09/2019 Área: Bioquímica E Biologia Molecular

Origem:	Não aplicável biotério						
Espécie:	Peixes	sexo:	Machos e Fêmeas	idade:	2 a 4 meses	N:	180
Linhagem:	Heterogênea			Peso:	0250 a 0280 g		

Local do experimento: Laboratório de Fisiologia de Peixes (LAFIPE), UFSM, CCS - Departamento de Farmacologia e Fisiologia.

Santa Maria, 05 de agosto de 2021

Profa. Dra. Patrícia Severo do Nascimento
Presidente da Comissão de Ética no Uso de Animais
Universidade Federal de Santa Maria

Prof. Dr. Saulo Tadeu Lemos Pinto Filho
Vice-Presidente da Comissão de Ética no Uso de Animais
Universidade Federal de Santa Maria