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REVIEW ARTICLE

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

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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.

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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

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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

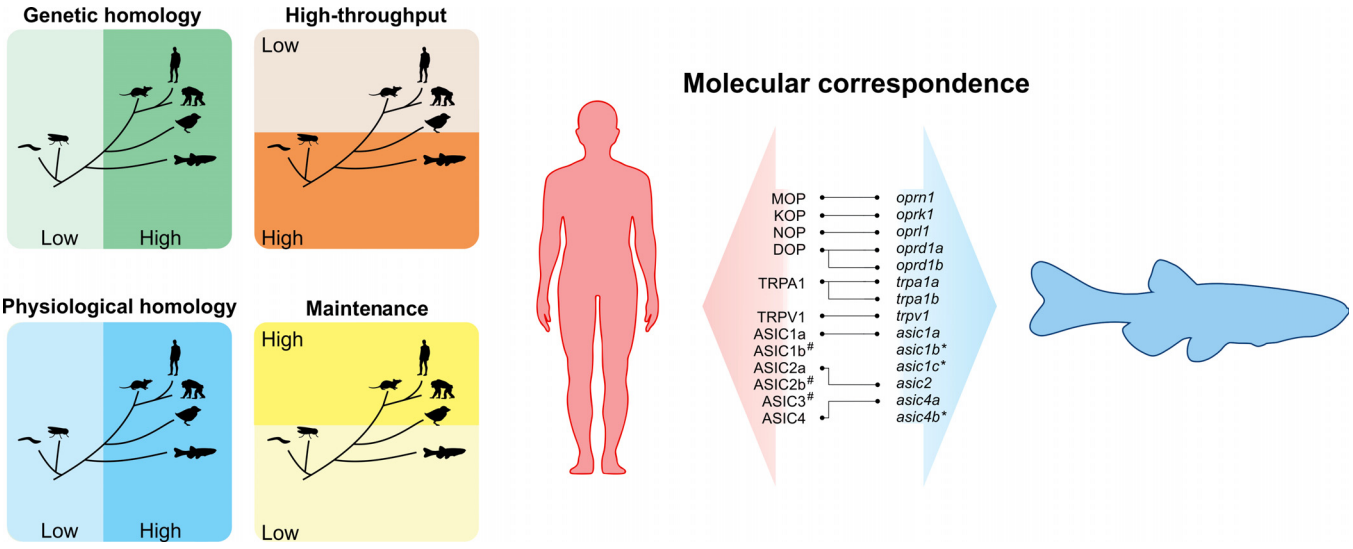

Fig. (1). General advantages and molecular features of zebrafish in translational pain research. As a vertebrate species, the zebrafish shows a high genetic and physiological homology when compared to mammals, but present a low cost of maintenance and feasibility to high-throughput screens similar to 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*).

analgesic effects, they rather differ in evoking affective behaviors [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]. Be-

cause 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 release 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

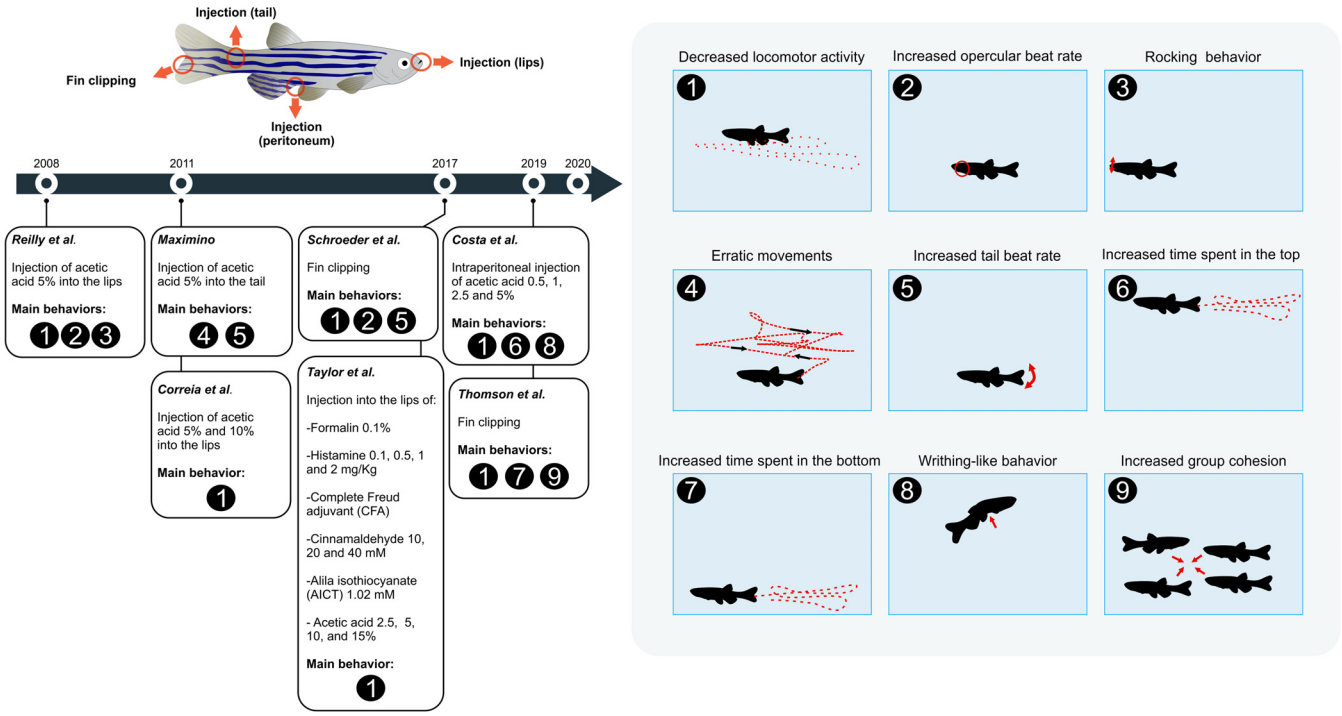

Fig. (2). Common experimental protocols used to assess pain in adult zebrafish with the respective behavioral endpoints measured in different studies depicted in timeline perspective. References: Reilly *et al.* [42], Maximino [52], Correia *et al.* [196], Schroeder *et al.* [185], Taylor *et al.* [33], 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*).

TRPA1 agonist acts differently on zebrafish trpa1a and 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 (in-

flammatory) 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.

CONCLUSION

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 example, the expression of mutant SCN9A, a voltage-gated sodi-

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?

um 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

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CONFLICT OF INTEREST

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

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