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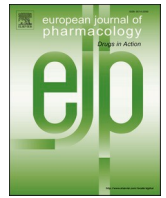
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Dopamine-neurotransmission and nociception in zebrafish: An anti-nociceptive role of dopamine receptor *drd2a*

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ABSTRACT

Dopamine (DA) is an important modulator in nociception and analgesia. Spinal DA receptors are involved in descending modulation of the nociceptive transmission. Genetic variations within DA neurotransmission have been associated with altered pain sensitivity and development of chronic pain syndromes. The variant rs6277 in dopamine receptor 2 a (*drd2a*) has been associated with a decreased D₂ receptor availability and increased nociception. The aim of this study is to further characterize the role of DA neurotransmission in nociception and the anti-nociceptive function of *drd2a*. The phenotype caused by rs6277 was modelled in zebrafish larvae using morpholino's and the effect on nociception was tested using a validated behavioural assay. The anti-nociceptive role of *drd2a* was tested using pharmacological intervention of D₂ agonist Quinpirole. The experiments demonstrate that a decrease in *drd2a* expression results in a pro-nociceptive behavioural phenotype (P = 0.016) after a heat stimulus. Furthermore, agonism of *drd2a* with agonist Quinpirole (0.2 μM) results in dose-dependent anti-nociception (P = 0.035) after a heat stimulus. From these results it is concluded that the dopamine receptor *drd2a* is involved in anti-nociceptive behaviour in zebrafish. The model allows further screening and testing of genetic variation and treatment involved in nociception.

1. Introduction

Worldwide, 1 in 5 people suffer from chronic pain (Goldberg and McGee, 2011). The treatment of pain remains a major clinical challenge, partly due to a lack of knowledge on the involvement of different neurotransmitter systems in nociception. The neurotransmitter Dopamine (DA) seems an important modulator in analgesic processes, acute and chronic pain both at spinal and supraspinal levels (Aira et al., 2014; Antypa et al., 2013; Bissonette and Roesch, 2016; Sharples et al., 2014; Stanton et al., 2018; Taniguchi et al., 2011; Taylor et al., 2019; Wood, 2008). Several genes in the DA-neurotransmission have been associated with the chronification of pain (Blanchet and Brefel-Courbon, 2018; Hoofwijk et al., 2016; Tammimaki and Mannisto, 2012). Hence, it is important to understand the exact mechanism by which DA-neurotransmission affects nociception and the involvement of genetic variation herein.

The external development of zebrafish larvae it is possible to easily manipulate the development and genetics of the organism (Kalueff et al., 2014; Lieschke and Currie, 2007). Moreover, as the zebrafish is an organism with a fully sequenced genome, it is an ideal model to study the effect of genetic variation on nociception in a time and cost-effective manner (Eijkenboom et al., 2018; Kalueff et al., 2014; Nasevicius and Ekker, 2000). The zebrafish sensory nervous system has shown to have many similarities with mammalian vertebrates including descending modulation of nociception (Correia et al., 2011; Malafoglia et al., 2013; Reinig et al., 2017; Schweitzer et al., 2012; Sneddon et al., 2003). The zebrafish model allows a direct high-throughput approach. Furthermore, it was recently shown that zebrafish could also be used as a model for assessing nociceptive processes (Curtright et al., 2015; Nguyen et al., 2014).

DA receptors are present throughout the spinal cord in humans and are expressed both in pre- and post-synaptic neurons. There are two

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classes of DA receptors, the D₁ receptor family and D₂ receptor family. The D₁ receptor family is excitatory whereas the D₂ receptor family is inhibitory (Neve et al., 2004) with D₂ receptors as the dominantly expressed receptor subtype (Lu et al., 2018; Neve et al., 2004). As zebrafish underwent an evolutionary genome duplication event, the vast majority of genes is present in duplicate (Meyer and Van de Peer, 2005). The *drd2a*, *drd2b* genes are the zebrafish orthologs of human *DRD2* whereby *drd2b* is more abundantly expressed in the brain of the zebrafish and *drd2a* is preferentially expressed in the spinal cord (Boehmler et al., 2004). Similarly to humans, zebrafish have descending dopaminergic projections from the brain to the spinal cord which can modulate nociception at the spinal cord directly (Reinig et al., 2017; van Reij et al., 2019). As *drd2a* is preferentially expressed in the spinal cord, this receptor will be the main target to study (Boehmler et al., 2004).

The single nucleotide polymorphism (SNP) rs6277 which is a non-protein-altering genetic variant, is one of the most studied genetic variants in *DRD2* in humans (Jaaskelainen et al., 2014; Zhang et al., 2007). SNP rs6277 accelerates mRNA decay and has been associated with a decreased DA D₂ receptor availability in the brain (Duan et al., 2003; Hirvonen et al., 2009). A decrease in DA D₂ receptor availability has been associated with nociception and chronic pain (Hagelberg et al., 2003; Martikainen et al., 2005; Pertovaara et al., 2004). Hence, a link between genetic variants altering the availability of DA D₂ receptors and an augmented pain response in humans is suggested.

The aim of this study is to further characterize the anti-nociceptive role of DA-neurotransmission in nociception via modulation of the *drd2a* receptor in a zebrafish model. This will be modelled via gene knockdown of *drd2a* in larval zebrafish using established morpholino oligomers and pharmacological intervention (Liu et al., 2006). Behavioural parameters will be used to quantify the effects of *drd2a* mediated neurotransmission on nociception in zebrafish. It is hypothesized that the activation of the *drd2a* receptor will be anti-nociceptive whereas as a decrease in *drd2a* receptors will result in a pro-nociceptive response.

2. Materials and methods

2.1. Zebrafish husbandry

Zebrafish (*Danio rerio*) were housed and raised at Maastricht University. Zebrafish were maintained at a 14/10h light/dark cycle, water temperature was set at 27 °C and adults were fed twice a day (Eijkenboom et al., 2018). For mating, the males and females were separated by a divider the day prior to collection. At the day of collection in the morning during the light cycle, the animals were placed in the same compartment to allow mating to take place. Eggs were collected using a fine-meshed strainer and transferred to petri dishes containing E3 medium (Nusslein-Volhard).

The zebrafish line *dat:EGFP* was developed and characterized by Xi and colleagues (Xi et al., 2011). In this line, the promoter of the dopamine transporter (*dat*) gene drives GFP expression. As a consequence, DA neurons in the brain are tagged with a fluorescent mark *in vivo*.

Experimenters (RvR and MS) were blinded for the conditions during the experiments until analysis.

2.2. Morpholino experiments

Expression of the *drd2a* gene was suppressed using a previously described translation block *drd2a* antisense morpholino (Liu et al., 2006). Antisense and control (mismatch) morpholino were ordered with the following sequences: *drd2a* morpholino 5'-AGG CAT ACG CTG TGA AGA CTT CCA T-3'; mismatch *drd2a* 5'-AGC CAT AGG GTG TGA ACA GTT CCA T-5' (Gene Tools, LLC, Philomath, OR, USA). The complementary sequence of the ATG start site is underlined in each sequence. For injection, the morpholino's were diluted in 1× Danieau and 1:10 dilution of rhodamine. Each 1–2 cell stage embryo was injected with 2 nl of morpholino solution.

At 24h after injection, the injection success rate and survival rate was assessed under the fluorescent microscope (DMI 4000B, Leica, Wetzlar, Germany). At 72h after injection the morphology of the developing embryos was checked using a dissection microscope as a quality control measure. Criteria for aberrant phenotypes were set as follows: heart edema: normal tail and eye growth but with a ballooned heart sack; Growth retardation: shortened tail as viewed from the lower side of the yolk to the tip of the tail; Disfigured: an aberrant phenotype not covered by the other phenotypes (e.g. a bent in the tail) possibly in combination with one of the other phenotypes; Combined Morphology: a combination of both the heart edema and growth retardation in the same zebrafish.

2.3. Zebrafish experiments

To quantify nociception in the zebrafish we measured temperature sensitivity and in particular noxious heat induced locomotion on the fifth day after fertilization (5dpf). This was assessed with an add-on, developed in-house, to the ZebraBox system (Viewpoint, Lyon, France and Maastricht Instruments BV., Maastricht, the Netherlands) (Eijkenboom et al., 2018). We used the same set-up and parameters as described and validated earlier (Eijkenboom et al., 2018). Briefly, animals were placed in 48 wells plate containing 500 µl E3 medium in the water compartment and allowed to adapt to their surroundings for 30 min in the dark. This period was followed by 10 min baseline measurement followed by the experimental phase with the temperature increase. Baseline temperature was set at 28.5 °C and was increased in the experimental phase to 41 °C. The temperature increase difference between the arena containing the water and the wells containing the fish is ±1 °C. During experiments, conditions were rotated over the 48 wells plate to reduce location bias. The ZebraBox software uses contrast differences between water and the zebrafish larvae to detect the size of the larvae. A camera records movement of the larvae and the activity of the larvae is determined by the amount pixels that change from one frame to the next.

2.4. Validation of zebrafish

To validate the sensitivity of the zebrafish quantify locomotion as a measure of nociceptive behaviour, we tested the well-known nociceptive stimulus Allyl IsoThioCyanate AITC (0.5 µM) and antinociceptive medication Paracetamol (1000 mg/L, 50 mg/L and 2.5 mg/L). Results were compared with available literature (Curtright et al., 2015).

2.5. Pharmacological experiments

To determine the effect of *drd2a* on nociception we used the *DRD2* agonist Quinpirole (QP) and *DRD2* antagonist Sulpiride (SLP). (–)-quinpirole hydrochloride (QP) and (±)-Sulpiride (SLP) was purchased from Sigma Aldrich (St. Louis, MO USA). QP was dissolved in milli-Q and diluted in E3 medium to reach the final concentration within the range of 0.2–8 µM based on previous literature (Irons et al., 2013). The optimal dose was determined in a series of pilot experiments. SLP was dissolved in DMSO and diluted in E3 medium to reach the final concentration of 75 and 150 µM based on previous literature (Li et al., 2018). All fish tested in the SLP experiments were exposed to the same concentration of DMSO as the SLP treated fish. During the pharmacological experiments, the fish were incubated 10 min prior to the start of the adaptation phase of the experiment. This period was chosen as longer periods of incubation could lead to motor effects which we wanted to avoid (Irons et al., 2013). QP and SLP was also diluted to the same concentration in the 48 wells plate as during the incubation period. Thus, the animals were exposed to QP or SLP during the experiments. E3 medium was used as vehicle control for QP experiments and E3 medium with DMSO in the SLP experiments. During experiments conditions were rotated over the 48 wells plate to reduce location bias.

2.6. Statistics

R was used to carry out the statistical analyses. For the comparison of different morphologies during the optimization of the morpholino experiments, a two-sample *t*-test was used whereby frequency of affected fish was compared with the control condition but not with other groups. For the behavioural experiments, the timeline was divided in different sets: acclimatisation phase (0–30 min), baseline phase (30–40 min), experimental phase (40–60 min). The peak activity time (45–50 min) was analysed to detect differences in nociception. Activity of the fish was standardized per fish to account for the variability between different individual larvae. Analyses compared the different conditions per experiment with each other but not between different experiments.

Analysis of behavioural data of the optimized concentrations of morpholino and QP and the experiments of SLP, AITC and paracetamol was done using a linear mixed effect (lme) model in the nlme (nlme: Linear and Nonlinear Mixed Effects Models) package (Pinheiro et al., 2019). Activity was determined in a linear regression model by group and time assuming a random intercept for each individual fish. Normality of the data was assessed by plotting the residuals of the models as histogram and QQ-plot. No obvious deviations were found. Data were considered to be significant when the calculated *P*-value < 0.05. All data are presented as average \pm standard error of the mean (S.E.M.). *P*-values shown are corrected for multiple testing.

3. Results

3.1. Optimization of the *drd2a* Morpholino

Before we can determine the effect of the *drd2a* knockdown on temperature sensitivity with our assay, the dosage of the *drd2a* morpholino had to be optimized. To determine the optimal dose of the morpholino, the morphologies of the fish had to be consistent with the literature describing this morpholino and have a significantly higher proportion of affected fish compared to the non-injected (NI) controls (Liu et al., 2006). The two morphologies described in the literature were heart edema and growth retardation.

A range of 2 ng until 10 ng was tested and morphology was assessed at 3 dpf. All the doses tested had a proportion of fish with the described phenotype (Fig. 1). The 10 ng *drd2a* morpholino had a significantly higher fraction of affected zebrafish compared to the NI control ($P = 0.037$, $t = 3.16$, 95% CI 1.42–27.20). None of the other dosages of *drd2a*

or mismatch morpholino's had a significantly higher fraction of affected zebrafish ($P > 0.05$).

3.2. Morpholino behavioural experiments

A dose of 10 ng (5 ng/nl) was determined to be the optimal dose and used in the behavioural experiments. The behavioural response of the zebrafish larvae (5 dpf) to the temperature change was assessed in the zebrafish exhibiting the normal phenotype to minimise the effect of morphology on the behavioural read-out. All three groups responded with an increase of their activity in response to the temperature increase (Fig. 2).

At baseline (30–40 min) no significant differences in activity between the three conditions is observed. During the peak activity phase of the experimental period (45–50 min) a significant increase in activity in the *drd2a* morpholino group is noted as compared to the non-injected control group (Fig. 2, $P = 0.032$, $t = 2.45$, $\beta = 1294.036$). No difference in activity between control morpholino and the non-injected control ($P = 1$, $t = -0.07$, $\beta = -36.335$) is observed. As reported in earlier studies the activity of the larvae gradually declined after reaching a maximum (Eijkenboom et al., 2018).

To validate the specific effect of the morpholino we exposed the zebrafish larvae to the *drd2* antagonist SLP in order to mimic the behavioural effect in the zebrafish. Based on literature the larvae were exposed to 75 and 150 μ M SLP diluted in DMSO and E3 medium or control condition. At baseline (30–40 min) no significant differences in activity between the three conditions is observed. During the peak activity phase of the experimental period (45–50 min) a significant increase in activity in the groups exposed to SLP is noted as compared to the control group (Fig. 3, 75 μ M; $P = 0.038$, $t = 2.39$, $\beta = 1150.914$, 150 μ M, $P = 0.0028$, $t = 3.30$, $\beta = 1584.354$).

3.3. *drd2a* agonist concentration optimization

Next, to study the effect of activation of the *drd2a* receptor on nociception an agonist is used. To determine the optimal concentration a range of concentrations (0.2 μ M–8 μ M) of QP was tested based on the available literature (Irons et al., 2013). To be considered for follow-up experiments the concentrations should not lead to motor effects visible in a significantly different baseline and should have a significant effect on activity during the peak effect phase. This was tested using a two-sample *t*-test between experimental concentrations and control

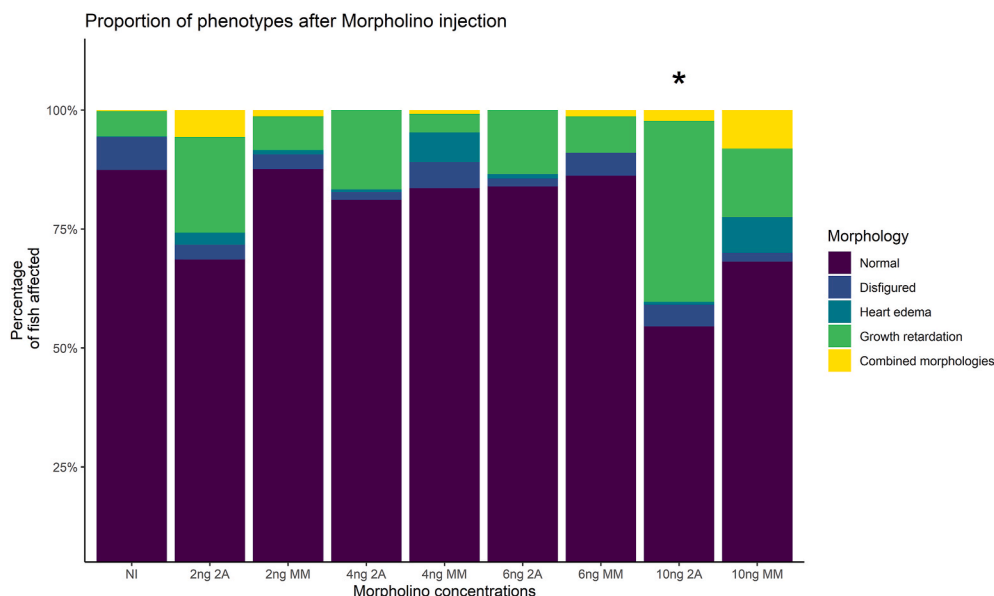


Fig. 1. Phenotype after *drd2a* morpholino injection.

The proportion of phenotypes after morpholino injection are depicted in percentages affected per condition. $N^{2ng\ 2a} = 159$, $N^{2ng\ MM} = 225$, $N^{4ng\ 2a} = 366$, $N^{4ng\ MM} = 128$, $N^{6ng\ 2a} = 230$, $N^{6ng\ MM} = 145$, $N^{10ng\ 2a} = 176$, $N^{10ng\ MM} = 160$, $N^{NI} = 1376$. NI = non-injected controls, 2A = *drd2a* morpholino, MM = mismatched morpholino, dose is presented before the type of morpholino. * = $P < 0.05$.

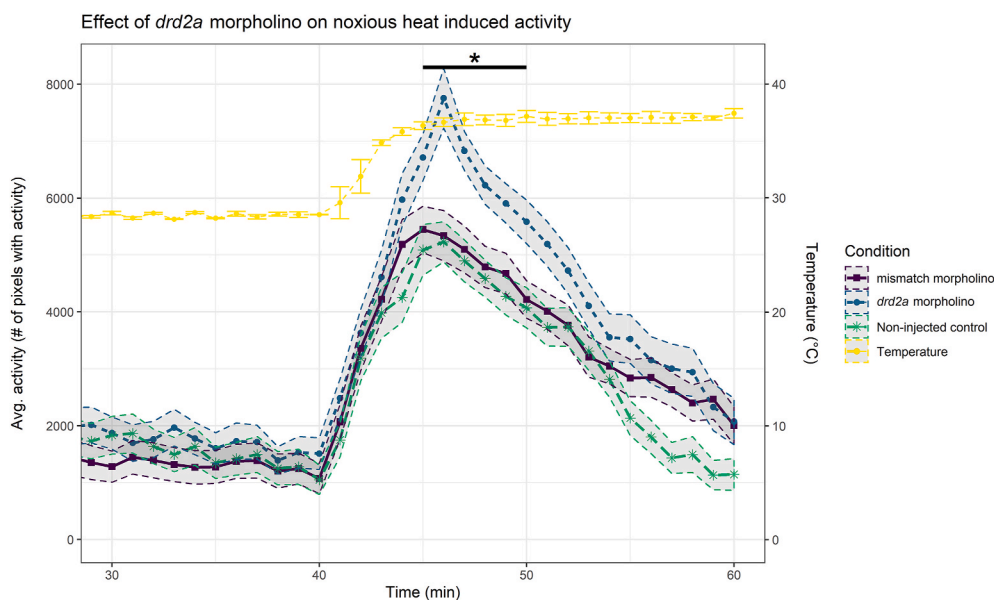


Fig. 2. Effect drd2a and control of morpholino's on noxious heat induced activity. At 5dpf, larvae injected with drd2a or mismatch morpholino were exposed to an increase in water temperature. This resulted in a significant higher activity of the zebrafish larvae injected with the drd2a morpholino. No significant differences were observed between the mismatch control and the non-injected control. $N^{\text{drd2a morpholino}} = 32$, $n^{\text{mismatch morpholino}} = 29$, $n^{\text{non-injected control}} = 32$ divided over three different experiments.

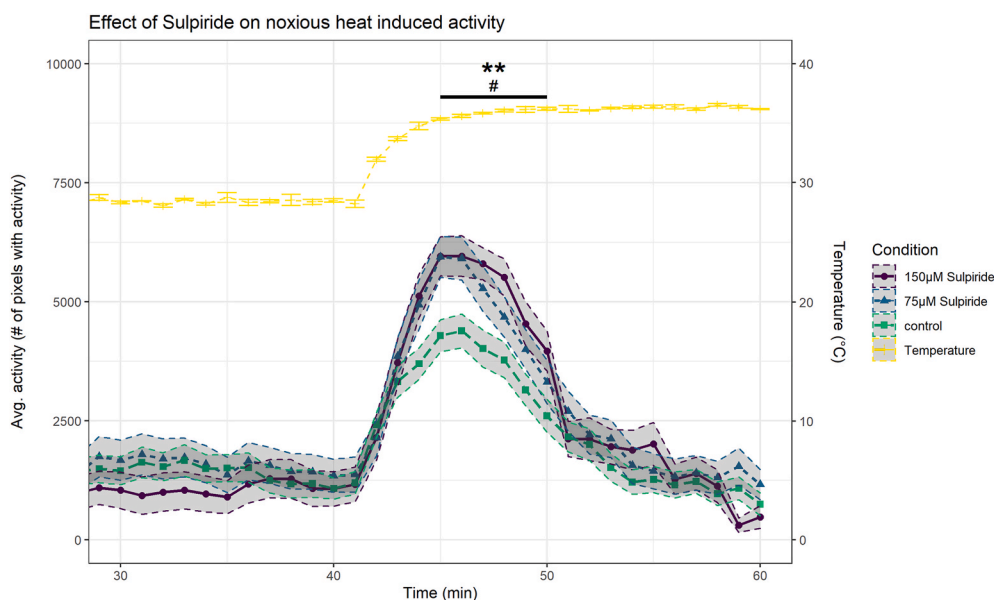


Fig. 3. Comparison of average baseline and peak activity of zebrafish larvae at 5dpf exposed to quinpirole and control condition. A significant difference in baseline activity was observed in the concentrations 4 and 8 μM ($P < 0.0001$) compared to control. No significant change was observed in the baseline activity of 0.2 and 1 μM ($P = 0.18$) compared to control. There was a significant decrease in the average peak activity of larvae exposed to 0.2 μM ($P < 0.05$). No significant differences compared to control were found for the other concentrations. * $P < 0.05$, *** $P < 0.0001$. $n^{0.2\mu\text{M}} = 40$, $n^{1\mu\text{M}} = 47$, $n^{4\mu\text{M}} = 31$, $n^{8\mu\text{M}} = 29$, $n^{\text{control}} = 71$.

group.

There were significant differences in average baseline activity compared to control for the concentrations 4 μM ($P = 7.52 \cdot 10^{-13}$) and 8 μM ($P = 2.16 \cdot 10^{-15}$) (Fig. 4, left side). There was no significant difference between control and 0.2 μM ($P = 0.72$) and 1 μM ($P = 0.296$). No significant effect on average peak activity was found for the concentrations 4 μM ($P = 1$) and 8 μM ($P = 1$). There was a significant decrease in average peak activity for the concentration of 0.2 μM ($P = 5.6 \cdot 10^{-5}$) and 1 μM ($P = 0.084$) (Fig. 4, right hand side).

3.4. Pharmacological behavioural experiments

The concentration of 0.2 μM QP was determined to be optimal and used in the behavioural experiments. The behavioural response of the zebrafish larvae (5dpf) to the temperature change was assessed in the zebrafish exhibiting the normal phenotype to minimise the effect of morphology on the behavioural read-out. Both groups responded with an increase of their activity in response to the temperature increase

(Fig. 4).

At baseline (30–40 min) there were no significant differences in activity between the two conditions. However, during the peak activity phase of the experimental phase (45–50min) a significant decrease in activity is noted in the QP group as compared to the control group (Fig. 5, $P = 0.035$, $t = -2.13$, $\beta = -958.178$). As observed in earlier studies the activity of the larvae gradually declined after reaching a maximum (Eijkenboom et al., 2018).

3.5. Zebrafish validation

To determine the sensitivity of the zebrafish assay, we exposed the zebrafish to similar conditions as described by Curtright et al. (2015). AITC exposure (0.5 μM) showed an increase in baseline activity of zebrafish larvae at 5 dpf ($P = 0.000$, $t = 6.50$, $\beta = 1950.94$, Supplemental Fig. 1) but no significantly altered peak activity in the experimental phase ($P = 0.19$, $t = -1.32$, $\beta = -510.215$). Paracetamol exposure did not lead to an increased baseline activity but did lead to a

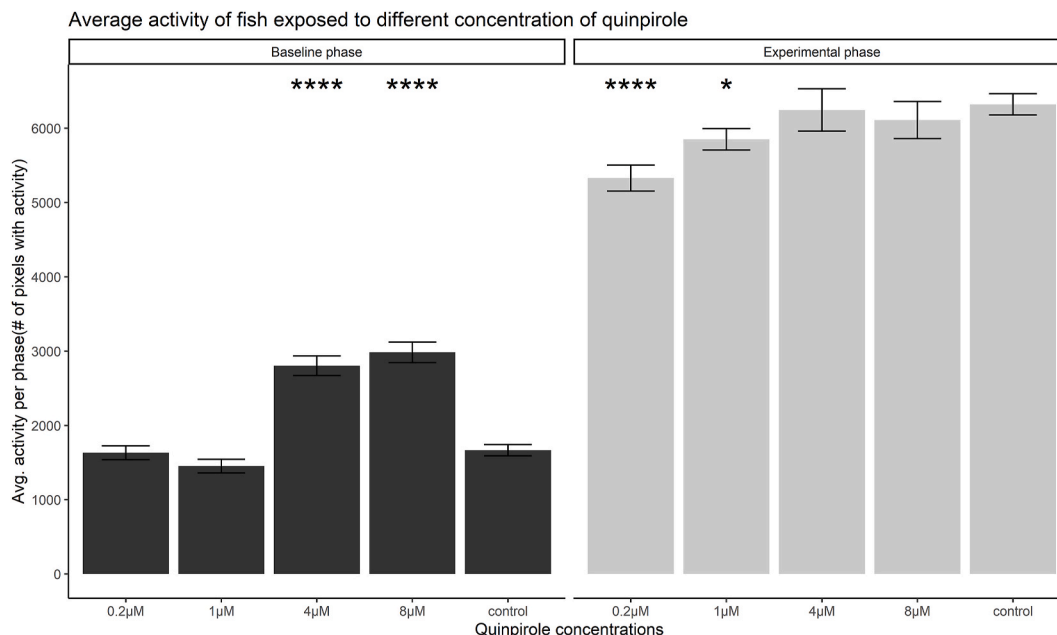


Fig. 4. Effect of Quinpirole on noxious heat induced activity increased.

At 5dpf, larvae were exposed to either quinpirole or control E3 medium followed by an increase in water temperature. This resulted in a significantly lower activity of the zebrafish larvae exposed to Quinpirole. No significant differences were observed between the mismatch control and the non-injected control. $N^{\text{quinpirole}} = 70$, $n^{\text{control}} = 74$.

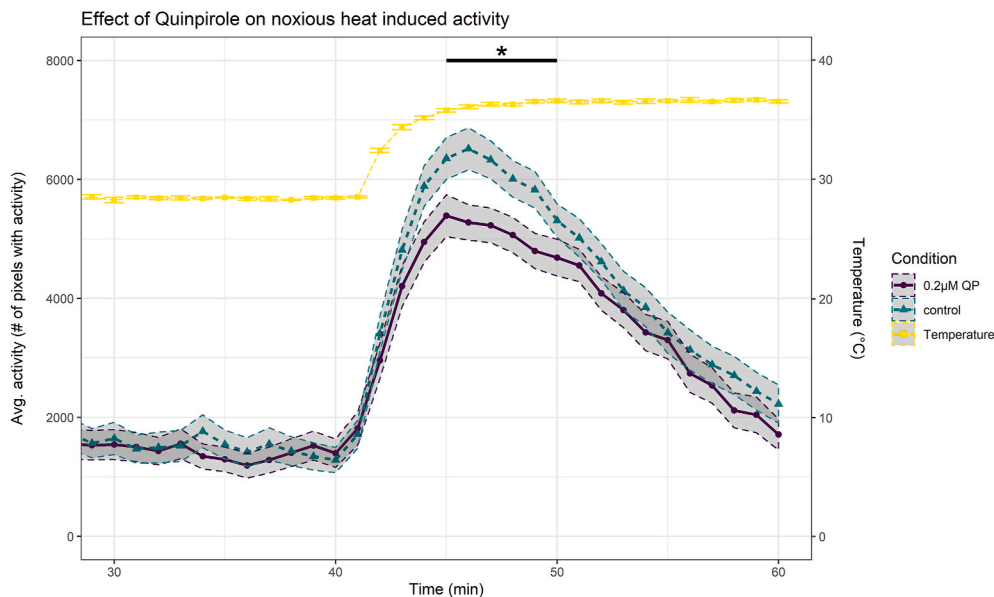


Fig. 5. Effect of Quinpirole on noxious heat induced activity increase.

At 5dpf, larvae were exposed to either quinpirole or control E3 medium followed by an increase in water temperature. This resulted in a significantly lower activity of the zebrafish larvae exposed to Quinpirole. No significant differences were observed between the mismatch control and the non-injected control. $N^{\text{quinpirole}} = 70$, $n^{\text{control}} = 74$.

significant decreased peak activity in the experimental phase (1000 mg/L; $P = 0.039$, $t = -2.52$, $\beta = -1582.407$, 50 mg/L; $P = 0.0036$, $t = -3.30$, $\beta = -2056.524$, 2.5 mg/L; $P = 0.0192$, $t = -2.77$, $\beta = -1708.308$, Supplemental Fig. 2).

4. Discussion

The aim of this study was to further characterize the anti-nociceptive function of dopamine receptor 2 a (*drd2a*). The results of our experiments indicate that knockdown of the inhibitory *drd2a* gene leads to pronociceptive behaviour in zebrafish which was confirmed by applying a *drd2a* antagonist. Furthermore, the activation of the *drd2a* receptor via application of agonist QP leads to anti-nociceptive behaviour. From this,

we conclude that the *drd2a* is involved in modulation of nociception in zebrafish providing further insight in the role of the DA receptor 2 in pain in humans.

Pharmacological modulation of the D_2 receptor in rodents clearly demonstrated a role for DA in nociception and specifically the anti-nociceptive effects of the D_2 receptor (Cobacho et al., 2014; Dai et al., 2016; Qing-Song et al., 1992). Furthermore, genetic variants have been associated with a decrease in D_2 receptor availability and pain phenotypes (Duan et al., 2003; Hagelberg et al., 2003; Hirvonen et al., 2009; Martikainen et al., 2005; Pertovaara et al., 2004). The present study is the first to show a causative link between a decrease in D_2 receptor expression and pro-nociceptive behaviour. The present findings not only validate prior studies but also provides a framework for future genetic

screening in zebrafish. Several techniques are available (incl. morpholino's, CRISPR and mRNA overexpression) to test the functional effects of known and unknown variants associated with pain phenotypes (Ata et al., 2016; Cobacho et al., 2014; Irion et al., 2014; Jaaskelainen et al., 2014). In this study, we modelled the phenotype associated with a variant with known functional effects to proof the causation between variant and behaviour. In order to confirm validity of the *drd2* knock-down SLP (dosage 75–150 μ M) was used. This DRD2 antagonist increased the average peak activity similar to that observed with the stop morpholino (Figs. 2 and 3). Hence, sulpiride does phenocopy the effects of *drd2a* knockdown.

It has been shown that dopamine plays an important role in pain and nociception in different clinical phenotypes (van Reij et al., 2019; Wood, 2008). This study provides further evidence for the role of a dopamine receptor expression and a pharmacological intervention on nociception. Pharmacological intervention using QP is an interesting option in the clinical phenotypes which show an altered nociceptive threshold due to an aberrant dopaminergic neurotransmission (e.g. pain in Parkinson's disease) (Blanchet and Brefel-Courbon, 2018; Brefel-Courbon et al., 2005; Chaudhuri and Schapira, 2009). Specifically QP could provide relief in "OFF" periods when administered in subclinical dosages to counteract the decreased nociceptive threshold and central pain symptoms associated with the decrease in dopamine levels (Chaudhuri and Schapira, 2009). However, patients carrying the SNP rs6277 would most likely not benefit from QP, as the decrease in receptor availability could prevent the QP from exerting its effects on the dopaminergic neurotransmission (Cherubini et al., 2016).

The zebrafish is an excellent model to assess the functional effects of genetic variations. In the pain field, a zebrafish model of small-fibre neuropathy (SFN) in which the pathogenicity of certain known pathological variants was tested, has recently been described (Eijkenboom et al., 2018). In the present study, an established antisense morpholino is used to downregulate the expression of the DA receptor 2A (Liu et al., 2006). This knockdown of the DA receptor 2A leads to decreased expression of DA receptor 2A, and models the human phenotype of genetic variation based on SNP rs6277. In humans, this SNP results in a decrease in DA₂ receptor availability and is associated with chronic pain disorders (Duan et al., 2003; Hagelberg et al., 2003; Hirvonen et al., 2009; Martikainen et al., 2005; Pertovaara et al., 2004).

An important aspect in this study is the modelling of nociception in zebrafish. Zebrafish have a functional nociceptive sensory system from 16 h post fertilization (hpf) onwards (Malafoglia et al., 2013; Sneddon et al., 2003). In addition, zebrafish possess a functional opioid system, nociceptors, descending neuronal control and brain structures to process and respond to potentially noxious external stimuli (Gonzalez-Nunez and Rodríguez, 2009; Sneddon, 2009; Sneddon et al., 2003; Tay et al., 2011). So far, zebrafish have been shown to respond to thermal and chemical nociceptive signals, as well as analgesics (Correia et al., 2011; Curtright et al., 2015; Eijkenboom et al., 2018; Malafoglia et al., 2014; Maximino, 2011; Prober et al., 2008; Sneddon et al., 2003; Taylor et al., 2017).

These approaches can be beneficial to research pipelines as they provide easy and fast screening and are easily translated to other model organisms or humans. This robust response of zebrafish larvae to nociceptive stimuli and attenuation of the effect in response to pharmacological intervention indicates the validity of zebrafish as model for nociception. The customized zebrafish behavioural testing system Zebibox has been extensively validated for thermal nociception as described by Eijkenboom and colleagues (Eijkenboom et al., 2018). It is important to note that in this zebrafish nociception model, the altered activity of the zebrafish larvae in response to an increase in temperature is robust, providing a decent window to see effects. Furthermore, the zebibox was validated as the application of a known nociceptive agent (AITC) and a known analgesic (paracetamol) showed similar results in line with use of other validated tests such as a place-preference test (Curtright et al., 2015). Therefore, the Zebibox is the ideal method to

assess thermal nociception in zebrafish.

When comparing human dopamine receptor families to their zebrafish counterparts, a sequence similarity of $\pm 70\%$ is observed whereby the transmembrane segments are conserved (Boehmler et al., 2004). The zebrafish genome is duplicated in comparison to the human genome leading to a duplication of the genes available. While some of these duplications have been lost in evolution, other orthologs developed new functionality (Meyer and Van de Peer, 2005). The zebrafish genome contains two genes for the dopamine D₂ receptor (*drd2a* and *drd2b*). The expression of *drd2a* and *drd2b* differs with respect to both location and developmental stage of expression in the zebrafish CNS (Maximino and Herculano, 2010). The expression of *drd2a* in zebrafish has been shown to be detectable from 8 hpf onwards, while spinal cord shows expression of *drd2a* at 36 hpf (Barreto-Valer et al., 2012; Shontz et al., 2018). *Drd2b* receptor is detectable from 24 hpf and mainly expressed in tectum, tegmentum and telencephalon (Shontz et al., 2018). Large similarity has been reported between zebrafish *drd2a* and *drd2b* expression patterns and mammalian D₂ receptors (Maximino and Herculano, 2010). This makes the zebrafish an excellent model to study the effect of DA neurotransmission on different processes, including nociception. The differential distribution and targeting of *drd2a* and *drd2b* allows to investigate the effect of DA in specific CNS structures.

During development, the DA system in zebrafish larvae is sensitive to external influences. With respect to the use of *drd2a* morpholino it is important to note that growth retardation and heart edema have been described (Liu et al., 2006). With our morphological examinations, we observed both growth retardation and heart edema phenotypes developing in the zebrafish larvae (see Fig. 1). At the optimal morpholino concentration of 10 ng (5 ng/nl) the proportion of fish with these phenotypes was significantly higher than in the control groups as seen previously (see Fig. 1). This confirms that we have the same phenotype in the zebrafish as the phenotypes mentioned in the study where the morpholino was originally described (Liu et al., 2006).

In addition, studies have shown that (ant-)agonism of the dopaminergic receptors can specifically affect locomotion (Barreto-Valer et al., 2012; Boehmler et al., 2007; Irons et al., 2013; Shontz et al., 2018). The effect of DA on locomotion is a crucial aspect as the behavioural assay in this study is based on locomotion of the zebrafish larvae and it could interfere with the effect on nociception. In our study we noted a significant increase in baseline locomotion (at 40–50 min of exposure) during exposure to the intermediate dosages of 4 μ M and 8 μ M, as reported previously (Irons et al., 2013). No effect of the lower dosages (0.2 μ M and 1 μ M) on baseline locomotion was noted. Therefore, we conclude that the results of QP on nociception in the temperature nociception assay are not confounded by a locomotion effect of QP. Note that similar results were found for DRD2 antagonist SLP and paracetamol in dosages as used. This further supports the conclusion that the observed QP effect is related to nociception and not related to locomotion. AITC exposure at baseline temperature of 28.5 °C has been shown to result in an increased activity of zebrafish at baseline but no effect was shown in this study on the activity after temperature increase.

There are some limitations to this study. The first issue is the indirect modelling of the functional effects of rs6277. This SNP normally promotes mRNA decay leading to a decreased expression of the receptor. Overexpressing mRNA with rs6277 in zebrafish would therefore not lead to the matching phenotype as more DA receptor mRNA is present next to the already transcribed *drd2* mRNA. Therefore, the morpholino approach was chosen to model the clinical phenotype as closely as possible. Although there is significant overlap between the human and the zebrafish genome, there are differences between zebrafish and human genomes including two different orthologs of the D₂ receptor (Boehmler et al., 2004; Klee et al., 2012). *Drd2a* is the gene mostly expressed in the spinal cord here is the first modulation relay station in the transduction of the nociceptive signal (the spinal pain-gate) (Melzack and Wall, 1965). The nociceptive system and spinal pain-gate seems well conserved between fish and vertebrates (Braithwaite and

Boulcott, 2007; Gonzalez-Nunez and Rodríguez, 2009; Malafoglia et al., 2013; Reinig et al., 2017). The spinal pain-gate modulates the nociceptive signal transduction, making spinally expressed *drd2a* the best candidate to study in nociception (Boehmler et al., 2004; Maximino and Herculano, 2010). Second issue is the large variability seen in the behaviour of the zebrafish. Zebrafish are outbred animals, which already indicates genetic variability, and they respond differently to the conditions in the experiments. To account for this variability all experiments were executed using multiple runs.

In conclusion, we report a causative link between D₂ receptor expression and nociception in zebrafish. With these experiments the genetic variants in humans phenotype and DA neurotransmission characterized by SNP rs6277 was modelled *in vivo* in a zebrafish using a morpholino targeted at *drd2a*. Future studies could use this established zebrafish nociception assay to functionally assess the effect of genetic variations on morphology, behaviour and pharmacological intervention screening.

Credit author statement

Roel R.I. van Reij: Investigation, Writing, Formal analysis, Visualization; Maud M.A. Salmans: Investigation, Formal analysis; Ivo Eijkenboom: Resources, Methodology; Nynke J. van den Hoogen: Supervision, Investigation, Writing; Elbert A.J. Joosten: Supervision; Conceptualization, Funding acquisition, Writing - Review & Editing; Jo M. Vanoevelen: Supervision, Conceptualization, Resources, Writing - Review & Editing.

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Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ejphar.2021.174517>.

References

- Aira, Z., Barrenetxea, T., Buesa, I., Gómez-Esteban, J.C., Azkue, J.J., 2014. Synaptic upregulation and superadditive interaction of dopamine D₂-and μ -opioid receptors after peripheral nerve injury. *PAIN*® 155, 2526–2533.
- Antypa, N., Drago, A., Serretti, A., 2013. The role of COMT gene variants in depression: bridging neuropsychological, behavioral and clinical phenotypes. *Neurosci. Biobehav. Rev.* 37, 1597–1610.
- Ata, H., Clark, K.J., Ekker, S.C., 2016. The zebrafish genome editing toolkit. *Methods Cell Biol.* 135, 149–170.
- Barreto-Valer, K., Lopez-Bellido, R., Macho Sanchez-Simon, F., Rodriguez, R.E., 2012. Modulation by cocaine of dopamine receptors through miRNA-133b in zebrafish embryos. *PLoS One* 7, e52701.
- Bissonette, G.B., Roesch, M.R., 2016. Development and function of the midbrain dopamine system: what we know and what we need to. *Gene Brain Behav.* 15, 62–73.
- Blanchet, P.J., Brefel-Courbon, C., 2018. Chronic pain and pain processing in Parkinson's disease. *Prog. Neuro-psychopharmacol. Biol. Psychiatr.* 87, 200–206.

- Boehmler, W., Carr, T., Thisse, C., Thisse, B., Canfield, V.A., Levenson, R., 2007. D₄ Dopamine receptor genes of zebrafish and effects of the antipsychotic clozapine on larval swimming behaviour. *Gene Brain Behav.* 6, 155–166.
- Boehmler, W., Obrecht-Pflumio, S., Canfield, V., Thisse, C., Thisse, B., Levenson, R., 2004. Evolution and expression of D₂ and D₃ dopamine receptor genes in zebrafish. *Dev. Dynam.* 230, 481–493.
- Braithwaite, V.A., Boulcott, P., 2007. Pain perception, aversion and fear in fish. *Dis. Aquat. Org.* 75, 131–138.
- Brefel-Courbon, C., Payoux, P., Thalamas, C., Ory, F., Quelven, I., Chollet, F., Montastruc, J.L., Rascol, O., 2005. Effect of levodopa on pain threshold in Parkinson's disease: a clinical and positron emission tomography study. *Mov. Disord.* 20, 1557–1563.
- Chaudhuri, K.R., Schapira, A.H., 2009. Non-motor symptoms of Parkinson's disease: dopaminergic pathophysiology and treatment. *Lancet Neurol.* 8, 464–474.
- Cherubini, E., Di Napoli, A., Noto, A., Osman, G.A., Esposito, M.C., Mariotta, S., Sellitri, R., Rucco, L., Cardillo, G., Ciliberto, G., Mancini, R., Ricci, A., 2016. Genetic and functional analysis of polymorphisms in the human dopamine receptor and transporter genes in small cell lung cancer. *J. Cell. Physiol.* 231, 345–356.
- Cobacho, N., de la Calle, J.L., Paino, C.L., 2014. Dopaminergic modulation of neuropathic pain: analgesia in rats by a D₂-type receptor agonist. *Brain Res. Bull.* 106, 62–71.
- Correia, A.D., Cunha, S.R., Scholze, M., Stevens, E.D., 2011. A novel behavioral fish model of nociception for testing analgesics. *Pharmaceuticals* 4, 665.
- Curtright, A., Rosser, M., Goh, S., Keown, B., Wagner, E., Sharifi, J., Raible, D.W., Dhaka, A., 2015. Modeling nociception in zebrafish: a way forward for unbiased analgesic discovery. *PLoS One* 10, e0116766.
- Dai, W.L., Xiong, F., Yan, B., Cao, Z.Y., Liu, W.T., Liu, J.H., Yu, B.Y., 2016. Blockade of neuronal dopamine D₂ receptor attenuates morphine tolerance in mice spinal cord. *Sci. Rep.* 6, 38746.
- Duan, J., Wainwright, M.S., Comeron, J.M., Saitou, N., Sanders, A.R., Gelernter, J., Gejman, R.V., 2003. Synonymous mutations in the human dopamine receptor D₂ (DRD2) affect mRNA stability and synthesis of the receptor. *Hum. Mol. Genet.* 12, 205–216.
- Eijkenboom, I., Sopacua, M., Otten, A.B.C., Gerrits, M.M., Hoesjmakers, J.G.J., Waxman, S.G., Lombardi, R., Lauria, G., Merkies, I.S.J., Smeets, H.J.M., Faber, C.G., Vanoevelen, J.M., 2018. Expression of pathogenic SCN9A mutations in the zebrafish: a model to study small fiber neuropathy. *Exp. Neurol.* 311, 257–264.
- Goldberg, D.S., McGee, S.J., 2011. Pain as a global public health priority. *BMC Publ. Health* 11, 770.
- Gonzalez-Nunez, V., Rodríguez, R.E., 2009. The zebrafish: a model to study the endogenous mechanisms of pain. *ILAR J.* 50, 373–386.
- Hagelberg, N., Forssell, H., Aalto, S., Rinne, J.O., Scheinin, H., Taiminen, T., Nagren, K., Eskola, O., Jaaskelainen, S.K., 2003. Altered dopamine D₂ receptor binding in atypical facial pain. *Pain* 106, 43–48.
- Hirvonen, M.M., Lumme, V., Hirvonen, J., Pesonen, U., Nägren, K., Vahlberg, T., Scheinin, H., Hietala, J., 2009. C957T polymorphism of the human dopamine D₂ receptor gene predicts extrastriatal dopamine receptor availability in vivo. *Prog. Neuro Psychopharmacol. Biol. Psychiatr.* 33, 630–636.
- Hoofwijk, D.M.N., van Reij, R.R.I., Rutten, B.P., Kenis, G., Buhre, W.F., Joosten, E.A., 2016. Genetic polymorphisms and their association with the prevalence and severity of chronic postsurgical pain: a systematic review. *Br. J. Anaesth.* 117, 708–719.
- Irion, U., Krauss, J., Nusslein-Volhard, C., 2014. Precise and efficient genome editing in zebrafish using the CRISPR/Cas9 system. *Development (Cambridge, England)* 141, 4827–4830.
- Irons, T.D., Kelly, P.E., Hunter, D.L., MacPhail, R.C., Padilla, S., 2013. Acute administration of dopaminergic drugs has differential effects on locomotion in larval zebrafish. *Pharmacol. Biochem. Behav.* 103, 792–813.
- Jaaskelainen, S.K., Lindholm, P., Valmunen, T., Pesonen, U., Taiminen, T., Virtanen, A., Lamusuo, S., Forsell, H., Hagelberg, N., Hietala, J., Pertovaara, A., 2014. Variation in the dopamine D₂ receptor gene plays a key role in human pain and its modulation by transcranial magnetic stimulation. *Pain* 155, 2180–2187.
- Kalueff, A.V., Echevarria, D.J., Stewart, A.M., 2014. Gaining translational momentum: more zebrafish models for neuroscience research. *Prog. Neuro Psychopharmacol. Biol. Psychiatr.* 55, 1–6.
- Klee, E.W., Schneider, H., Clark, K.J., Cousin, M.A., Ebbert, J.O., Hooten, W.M., Karpayak, V.M., Warner, D.O., Ekker, S.C., 2012. Zebrafish: a model for the study of addiction genetics. *Hum. Genet.* 131, 977–1008.
- Li, F., Lin, J., Liu, X., Li, W., Ding, Y., Zhang, Y., Zhou, S., Guo, N., Li, Q., 2018. Characterization of the locomotor activities of zebrafish larvae under the influence of various neuroactive drugs. *Ann. Transl. Med.* 6, 173.
- Lieschke, G.J., Currie, P.D., 2007. Animal models of human disease: zebrafish swim into view. *Nat. Rev. Genet.* 8, 353–367.
- Liu, N.-A., Liu, Q., Wawrowsky, K., Yang, Z., Lin, S., Melmed, S., 2006. Prolactin receptor signaling mediates the osmotic response of embryonic zebrafish lactotrophs. *Mol. Endocrinol.* 20, 871–880.
- Lu, Y., Doroshenko, M., Lauzadis, J., Kanjiya, M.P., Rebecchi, M.J., Kaczocha, M., Puopolo, M., 2018. Presynaptic inhibition of primary nociceptive signals to dorsal horn lamina I neurons by dopamine. *J. Neurosci.* 38, 8809.
- Malafoglia, V., Bryant, B., Raffaelli, W., Giordano, A., Bellipanni, G., 2013. The zebrafish as a model for nociception studies. *J. Cell. Physiol.* 228, 1956–1966.
- Malafoglia, V., Colasanti, M., Raffaelli, W., Balciunas, D., Giordano, A., Bellipanni, G., 2014. Extreme thermal noxious stimuli induce pain responses in zebrafish larvae. *J. Cell. Physiol.* 229, 300–308.
- Martikainen, I.K., Hagelberg, N., Mansikka, H., Hietala, J., Nagren, K., Scheinin, H., Pertovaara, A., 2005. Association of striatal dopamine D₂/D₃ receptor binding

- potential with pain but not tactile sensitivity or placebo analgesia. *Neurosci. Lett.* 376, 149–153.
- Maximino, C., 2011. Modulation of nociceptive-like behavior in zebrafish (*Danio rerio*) by environmental stressors. *Psycholo. Neurosci.* 4, 149.
- Maximino, C., Herculano, A.M., 2010. A review of monoaminergic neuropsychopharmacology in zebrafish. *Zebrafish* 7, 359–378.
- Melzack, R., Wall, P.D., 1965. Pain mechanisms: a new theory. *Science* 150, 971–979.
- Meyer, A., Van de Peer, Y., 2005. From 2R to 3R: evidence for a fish-specific genome duplication (FSGD). *Bioessays* 27, 937–945.
- Nasevicius, A., Ekker, S.C., 2000. Effective targeted gene/“knockdown/” in zebrafish. *Nat. Genet.* 26, 216–220.
- Neve, K.A., Seamans, J.K., Trantham-Davidson, H., 2004. Dopamine receptor signaling. *J. Recept. Signal Transduct.* 24, 165–205.
- Nguyen, M., Stewart, A.M., Kalueff, A.V., 2014. Aquatic blues: modeling depression and antidepressant action in zebrafish. *Prog. Neuro Psychopharmacol. Biol. Psychiatr.* 55, 26–39.
- Nusslein-Volhard, C.D., 2002. *A Practical Approach. R Zebrafish*. New York, USA.
- Pertovaara, A., Martikainen, I.K., Hagelberg, N., Mansikka, H., Nägren, K., Hietala, J., Scheinin, H., 2004. Striatal dopamine D2/D3 receptor availability correlates with individual response characteristics to pain. *Eur. J. Neurosci.* 20, 1587–1592.
- Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D., 2019. *Nlme : Linear and Nonlinear Mixed Effects Models*.
- Prober, D.A., Zimmerman, S., Myers, B.R., McDermott, B.M., Kim, S.-H., Caron, S., Rihel, J., Solnica-Krezel, L., Julius, D., Hudspeth, A.J., Schier, A.F., 2008. Zebrafish TRPA1 channels are required for chemosensation but not for thermosensation or mechanosensory hair cell function. *J. Neurosci.* 28, 10102.
- Qing-Song, L., Jian-Tian, Q., Dafny, N., 1992. D2 dopamine receptor involvement in spinal dopamine-produced antinociception. *Life Sci.* 51, 1485–1492.
- Reinig, S., Driever, W., Arrenberg, A.B., 2017. The descending diencephalic dopamine system is tuned to sensory stimuli. *Curr. Biol.* 27, 318–333.
- Schweitzer, J., Löhr, H., Filippi, A., Driever, W., 2012. Dopaminergic and noradrenergic circuit development in zebrafish. *Dev. Neurobiol.* 72, 256–268.
- Sharples, S.A., Koblinger, K., Humphreys, J.M., Whelan, P.J., 2014. Dopamine: a parallel pathway for the modulation of spinal locomotor networks. *Front. Neural Circ.* 8, 55.
- Shontz, E.C., Souders II, C.L., Schmidt, J.T., Martyniuk, C.J., 2018. Domperidone upregulates dopamine receptor expression and stimulates locomotor activity in larval zebrafish (*Danio rerio*). *Gene Brain Behav.* 17, e12460.
- Sneddon, L.U., 2009. Pain perception in fish: indicators and endpoints. *ILAR J.* 50, 338–342.
- Sneddon, L.U., Braithwaite, V.A., Gentle, M.J., 2003. Do fishes have nociceptors? Evidence for the evolution of a vertebrate sensory system. *Proc. Roy. Soc. Lond. B Biol. Sci.* 270, 1115.
- Stanton, C.H., Holmes, A.J., Chang, S.W.-C., Joormann, J., 2018. From stress to Anhedonia: molecular processes through functional circuits. *Trends Neurosci.* 42, 23–42.
- Tammimäki, A., Mannisto, P.T., 2012. Catechol-O-methyltransferase gene polymorphism and chronic human pain: a systematic review and meta-analysis. *Pharmacogenetics Genom.* 22, 673–691.
- Taniguchi, W., Nakatsuka, T., Miyazaki, N., Yamada, H., Takeda, D., Fujita, T., Kumamoto, E., Yoshida, M., 2011. In vivo patch-clamp analysis of dopaminergic antinociceptive actions on substantia gelatinosa neurons in the spinal cord. *Pain* 152, 95–105.
- Tay, T.L., Ronneberger, O., Ryu, S., Nitschke, R., Driever, W., 2011. Comprehensive catecholaminergic projectome analysis reveals single-neuron integration of zebrafish ascending and descending dopaminergic systems. *Nat. Commun.* 2, 171.
- 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. *Physiol. Behav.* 174, 83–88.
- Taylor, N.E., Pei, J., Zhang, J., Vlasov, K.Y., Davis, T., Taylor, E., Weng, F.J., Van Dort, C. J., Solt, K., Brown, E.N., 2019. The role of Glutamatergic and Dopaminergic neurons in the periaqueductal Gray/Dorsal Raphe: separating analgesia and anxiety. *eNeuro* 6.
- van Reij, R.R.I., Joosten, E.A.J., van den Hoogen, N.J., 2019. Dopaminergic neurotransmission and genetic variation in chronification of post-surgical pain. *Br. J. Anaesth.* 123, 853–864.
- Wood, P.B., 2008. Role of central dopamine in pain and analgesia. *Expert Rev. Neurother.* 8, 781–797.
- Xi, Y., Yu, M., Godoy, R., Hatch, G., Poitras, L., Ekker, M., 2011. Transgenic zebrafish expressing green fluorescent protein in dopaminergic neurons of the ventral diencephalon. *Dev. Dyn. Off. Publ. Am. Assoc. Anatomists* 240, 2539–2547.
- Zhang, Y., Bertolino, A., Fazio, L., Blasi, G., Rampino, A., Romano, R., Lee, M.L., Xiao, T., Papp, A., Wang, D., Sadec, W., 2007. Polymorphisms in human dopamine D2 receptor gene affect gene expression, splicing, and neuronal activity during working memory. *Proc. Natl. Acad. Sci. U. S. A.* 104, 20552–20557.