Nitric oxide interacts with monoamine oxidase to modulate aggression and anxiety-like behaviour

Running Title: Nitric oxide and monoamine oxidase interact to modulate aggression

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Abstract

Nitric oxide (NO) is a gaseous neurotransmitter that has important behavioural functions in the vertebrate brain. In this study we compare the impact of decreased nitric NO signalling upon behaviour and neurobiology using both zebrafish and mouse. *nitric oxide synthase 1* mutant (*nos1*$^{-/-}$) zebrafish show significantly reduced aggression and an increase in anxiety-like behaviour without altered production of the stress hormone cortisol. *Nos1*$^{-/-}$ mice also exhibit decreased aggression and are hyperactive in an open field test. Upon reduction of NO signalling, monoamine neurotransmitter metabolism is reduced as a consequence of decreased Monoamine oxidase activity. Treatment of *nos1*$^{-/-}$ zebrafish with the 5-HT receptor 1A agonist 8-OH-DPAT rescues aggression and some aspects of anxiety-like behaviour. Taken together, the interplay between NO and 5-HT appears to be critical to control behaviour. Our cross-species approach challenges the previous notion that reduced neuronal NOS leads to increased aggression. Rather, *Nos1* knock-out can also decrease aggression in some situations, a finding that may have implications for future translational research.

Keywords: Nitric oxide, monoamine oxidase, zebrafish, mouse, aggression, anxiety

Introduction

Nitric oxide (NO) is a gaseous signalling molecule produced by three isoforms of the enzyme Nitric oxide synthase (NOS): NOS-I (also called neuronal NOS), NOS-II (inducible NOS, found e.g. in macrophages) or NOS-III (endothelial NOS) (Freudenberg et al., 2015). Once formed NO can diffuse across cell membranes to act as a neuromodulator in the brain, influencing multiple neurons via *en passant* synapses. In the nervous system NOS-I is located in close proximity to postsynaptic N-methyl D-aspartate receptors (NMDAR) (Kiss and Vizi, 2001). Stimulation of NMDAR with glutamate leads to an increase in intracellular calcium levels and concomitant NOS-I activation (Kiss and Vizi, 2001). Pathways downstream of NO include nitrosylation and direct binding to haemoproteins (including soluble guanylyl cyclase (sGC)) and iron-sulphur proteins (Nelson et al., 1997). Activation of sGC constitutes a major signal transduction pathway leading to production of guanosine 3',5'-cyclic monophosphate (cGMP), protein kinase G activation and phosphorylation of targets (Miller and Hoffmann, 1994). Taken together, the rapid speed of NO production, its short half-life and ability to cross cell membranes makes NO an ideal molecule to participate in volume neurotransmission (Kiss and Vizi, 2001).

Neuronal NO influences multiple behaviours by interacting with other signalling pathways. Studies in mice have uncovered a complex suite of behavioural alterations upon reduction of NO signalling although the data are conflicting. Male *Nos1* knock-out mice with a targeted deletion of exon 2 (Eliasson et al., 1997; Huang et al., 1993) exhibit increased aggression following social isolation and inappropriate mounting during sexual behaviour (Nelson et al., 1995). However, a modifier gene present in C57BL6 x 129/Sv may account for this phenotype since crossing onto a C57BL/6J background abolishes the increase in aggression (Huang et al., 1993; Le Roy et al., 2000). Other behaviours examined in male *Nos1*$^{-/-}$ mice also show high levels of variability; for example, both increases- and decreases in anxiety, learning and memory have been reported (Bilbo et al., 2003; Wultsch et al., 2007; Zhang et al., 2010). *Nos1*$^{-/-}$ also exhibit abnormal social behaviour, inattention, hyperactivity and reduced depression-like behaviour (Gao and Heldt, 2015; Tanda et al., 2009). In contrast to the

presumably hyper-aggressive males, female *Nos1*$^{-/-}$ mice show normal aggression in the resident-intruder test (Nelson et al., 1995) and reduced maternal aggression (Gammie and Nelson, 1999). Further complicating the picture, male *Nos-3* knock-out mice show *reduced* aggression levels, increased forelimb strength and enhanced fine motor control (Demas et al., 1999). Thus, the sex of the animal, genetic background and source of NO appear to influence the function of this signalling molecule. The interaction between NO and 5-HT neurotransmitter signalling appears to be particularly important. For example, the heightened aggression of C57BL6 x 129/Sv *Nos1* knock-out mice correlates with decreased 5-HT metabolism in the brain. Treatment with the 5HT receptor 1A (HTR1A) agonist 8-OH-DPAT reduces their aggression levels (Chiavegatto et al., 2001, 2003) suggesting that NO is important for normal 5-HT function and may play a significant role in psychiatric disorders with a serotonergic basis.

In humans, candidate gene studies and genome-wide approaches have linked variation in *NOS-1* to Parkinson's disease, depression, anxiety and impulsivity-related disorders (Freudenberg et al., 2015). Single nucleotide polymorphisms in *NOS-1* have also been identified in schizophrenia (Weber et al., 2014). Furthermore, a variable number tandem repeat (VNTR) that reduces gene expression in reporter gene assays has been identified in exon 1f (Exon 1f VNTR; Weber et al., 2015; Reif et al., 2009). The short (s/s) *NOS-1* VNTR genotype also interacts with environmental factors to alter impulsivity levels. In positive emotional environments s/s carriers show increased adaptive impulsivity whereas under adverse conditions (traumatic life events or familial discord) maladaptive impulsivity is triggered. Supposedly mirroring initial data from knock-out mice, the s/s genotype was found more frequently in violent prison inmates (Reif et al., 2009). However, the consequences of these polymorphisms on intracellular NO formation are still unclear. Patients carrying s/s show increased striatal activity (Hoogman et al., 2011) and decreased activation of the anterior cingulate gyrus (Reif et al., 2009), areas of the brain that are important for executive function and impulse control.

The complex and sometimes contradictory role of NO in modulating aggression and anxiety prompted us to examine the function of this neurotransmitter in zebrafish, a popular model for behavioural

neuroscience. Zebrafish have a short generation time and are easy to maintain in the laboratory. The genes and neurotransmitters that control behaviour appear to be conserved across species and a large number of mutant lines have been identified. Furthermore, a combination of genetic, electrophysiological and optogenetic tools permit the neural circuits that control behaviour to be manipulated in freely swimming fish (Orger and de Polavieja, 2017; Norton and Bally-Cuif, 2010). In this study we have investigated whether mutation of zebrafish *nitric oxide synthase 1* leads to alterations in aggression and 5-HT signalling. We have compared loss of *Nos1* function in zebrafish and mouse, two translational models for human disease. We combined behavioural, neurochemical and pharmacological data to provide further evidence that the interaction between NO and 5-HT is critical to produce an appropriate behavioural response.

Experimental procedures

Zebrafish strains, care and maintenance. Adult zebrafish were maintained at the University of Leicester using standard zebrafish-keeping protocols and in accordance with institute guidelines for animal welfare. The following strains were used: *nos1*^{SH336} TALEN mutants and wild-type zebrafish generated by crossing London wild-type and *nacre*. Behavioural analyses were performed on 6- to 12-month-old adult zebrafish of both sexes. Detailed descriptions of the behavioural tests are included in the supplementary information. All zebrafish genes are written in lower case letters (e.g. *nos1*$^{-/-}$) in keeping with established nomenclature.

Generation of *nos1* zebrafish mutant line. TALENS were designed to surround the *BstXI* site in exon 1 of the *nos1* gene (bp 322-334 in ENSDART00000167834) using http://zifit.partners.org/ZiFiT/, assembled using the Golden Gate system (Cermak et al., 2011) and injected in fish with a London wild-type background (LWT). Founders were screened by amplification with the following primers ACCCTGAAGAACGTGTCACC and GCACAGGCTCGATCTCTTTC and digestion with *BstXI*. A founder that transmitted a 7 bp deletion was used to generate the mutant line by crossing to a *nacre* stock.

Mouse strains, care and maintenance. Adult male *Nos1*$^{-/-}$ mice (strain B6;129S4-Nos1^{tm1Plh}/J) were backcrossed for at least five generations onto a C57Bl/6J background (stock no 002633 Jackson Laboratories, USA). Additional wild-type male C57Bl/6J mice were obtained from Janvier Labs, France. All experiments were conducted according to the Directive of the European Communities Council of 24 November 1986 (86/609/EEC) and German animal welfare laws (TierSchG and TSchV) and were approved by the regional council in Darmstadt, Germany (FK/1055). Mice were kept on a 12:12h light/dark cycle with food and water available *ad libitum*. *Nos1*$^{-/-}$ mice and wild-type littermates were single-housed as residents in standard individually-ventilated cages for 7 days before testing. Additional wild-type C57Bl/6J males were housed in groups of five and were used as unfamiliar stimulus mice in the sociability and aggression tests. All mouse genes are written with a capital letter (e.g. *Nos1*$^{-/-}$) in keeping with established nomenclature.

Drug treatments. The MAO-B inhibitor D-(+)-Deprenyl (deprenyl) and the 5HT receptor 1A (HTR1A) agonist (±)-8-hydroxy-2-(di-n-propylamino)tetralin hydrobromide (8-OH-DPAT) were purchased from Tocris Biosciences. Drugs were dissolved in system water and applied by immersion for 3 hours before behavioural testing. Treatment duration and concentrations were chosen according to published studies (Anichtchik et al., 1996) and pilot experiments in our lab.

In situ hybridisation. In situ hybridisation was performed according to Norton et al., (2011). The following probe was used: *nitric oxide synthase 1* (*nos1*). For gene information refer to www.zfin.org. Brain sections were photographed with an optical microscope (GXM L3200B, GT Vision) and images were mounted in Adobe Photoshop version CS2 (Adobe systems).

High precision liquid chromatography analysis of monoamines and metabolites. Fish were sacrificed using a schedule 1 method. The brain was removed and divided into telencephalon (Tel), diencephalon (DI), optic tectum (TeO) and hindbrain (Hb) under a microscope. Samples were weighed, then homogenised in 80 µl of ice-cold 0.1 N perchloric acid using a 0.1 ml pestle and mortar (Fisher Thermoscientific) and centrifuged at 12.000 rcf for 15 minutes. The resulting supernatant was stored at -80°C until use. High performance liquid chromatography (HPLC) with electrochemical detection

was used to analyse dopamine (DA) and serotonin (5-HT), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) and 5-hydroxyindole acetic acid (5-HIAA). The mobile phase consisted of 75 mM sodium dihydrogen phosphate, 1 mM EDTA, 0.6 mM octane sulphonic acid (OSA) in deionised water containing 5% methanol (Sigma-Aldrich). Samples were quantified by comparison with standard solutions of known concentrations and results were expressed as femtomoles per milligram of brain. A total of 10 wild-type and 9 *nos1*$^{-/-}$ were processed for HPLC.

Monoamine oxidase assay. Monoamine oxidase (Mao) activity was analysed using the peroxidase-linked colourimetric assay described in (Anichtchik et al., 2006). This method determines the amount of a red pyridine dye formed in a chromogenic reaction driven by the oxidation of tyramine by Mao. The assay was performed in a 96-well plate (Thermo Fisher). Each well contained 100 µl 10 mM tyramine, 50 µl chromogenic solution and 5 µl brain homogenate. The assay was incubated at 28°C for 2 hours and the dye produced was quantified at different time-points using a microplate reader equipped with a 490 nm filter (iMark^{TM} BIO-RAD). Data was obtained using Microplate Manager 6 Software, version 6.2. We used 9 brains of each genotype. For drug experiments, wild-type fish were treated by immersion in 10 or 100 µM deprenyl for 3 hours before processing.

Statistics. All data were organised in Excel (Microsoft). Statistical analyses were carried out in GraphPad Prism6. All error bars denote standard error of the mean (SEM). Statistical significance was depicted as follows: (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$, (****) $p < 0.0001$. The number of animals tested is denoted by *n*.

Results

Expression of *nos1* in the adult zebrafish brain and generation of a TALEN mutant line

We first characterised *nos1* expression in the adult zebrafish brain complementing the original studies by Holmqvist and colleagues (Holmqvist et al., 2000). In the telencephalon, *nos1* expression is seen in the dorsal, ventral and posterior ventral telencephalon (Vd, Vv, Vp), the medial, lateral (Fig. 1a-c), and

posterior dorsal telencephalon (Dm, Dl, Dp) and the anterior and posterior part of the preoptic area of the anterior hypothalamus (PPa and PPp; Fig. 1d,e). *nos1* is also expressed in the ventral part of periventricular pretectal nucleus (PPv), the dorsal (DP) and central posterior (CP) thalamic nucleus, the posterior nucleus of the posterior tuberculum (TPp), the ventral zone of the periventricular hypothalamus (Hv) and the posterior thalamic nucleus (P) (Fig. 1f,g). Other hypothalamic regions that express *nos1* include the paraventricular organ (PVO), the posterior tuberal nucleus (PTN), the lateral hypothalamic nucleus (LH), the subglomerular nucleus (SG), and the dorsal and caudal zones of the periventricular hypothalamus (Hd, Hc) (Fig. 1g,h). Sparse expression is also seen in the superior raphe formation (SRF) and the nucleus interpeduncularis (Nln), the griseum central (GC), the nucleus isthmi (NI) and the corpus mammilare (CM) (Fig. 1j-l). We next generated a mutant line that harbours a seven base pair deletion in the first exon of *nos1* using TALEN genome engineering (*nos1*^{SH336}; Fig. 1m). The mutation led to a premature stop codon that truncates Nos1 protein at amino acid 109 deleting a *BstXI* restriction site (Fig. 1n). We confirmed the reduction of NOS1 by Western blot using a NOS1 specific antibody (Fig. 1o) (Robertson et al., 2014).

Mutation of *nos1* causes a reduction of NO signalling

We next investigated the impact of reduced *nos1* activity on NO signalling. Expression of the *nos1* gene was severely decreased in the brain of mutants compared to wild-type fish (Fig. 2a-h). In agreement with this, qPCR analysis revealed a strong reduction of *nos1* gene expression in *nos1*$^{-/-}$ (Fig. 2i). Conversely, there was no difference in expression of the gene *nos2a* between genotypes whereas *nos2b* showed increased expression in the mutant brain (Fig. 2i). We assessed NO signalling using the Griess assay that measures nitrite levels in the brain. Comparison of *nos1*$^{-/-}$ and wildtype revealed a significant reduction of NO signalling in the mutant fish (Fig. 2j). However, even when *nos1* activity is reduced some NO signalling is maintained in the brain.

Loss of *nos1* function alters aggression and anxiety-like behaviour

In mice, loss of *Nos1* function triggers a number of behavioural alterations that also includes increased aggression (*Nos1*$^{-/-}$ on the C57BL6 x DBA/2 background; Chiavegatto and Nelson, 2003; Nelson et al., 1995). We assessed the agonistic behaviour of *nos1*$^{-/-}$ zebrafish in two different tests: dyadic interaction between two zebrafish and mirror-induced aggression (Gerlai et al., 2000; Norton et al., 2011). Surprisingly, *nos1*$^{-/-}$ mutants showed a strong reduction of aggression compared to wild-types in both paradigms. In the mirror test, *nos1*$^{-/-}$ only exhibited a few short bouts of aggression (Fig. 3a) although they swam the same distance as wild-types in this test (Fig. 3b). In the dyadic test, aggression was reduced and *nos1*$^{-/-}$ spent more time freezing (Fig. 3c-f). The heightened aggression of *Nos1*$^{-/-}$ mice was reported to be triggered by social isolation (Nelson et al., 1995). We isolated by zebrafish for one week (removing olfactory and visual cues) and measured their agonistic behaviour. Isolated *nos1*$^{-/-}$ also exhibited reduced aggression and increased time spent freezing compared to similarly treated wild-type zebrafish (Fig. 3g,h). In zebrafish, freezing on the bottom of the tank is indicative of anxiety-like behaviour. We examined this in more detail using the novel tank test (Egan et al., 2009). *nos1*$^{-/-}$ avoided the top of the tank, spending more time at the bottom and alternating between freezing and bouts of erratic swimming (increased angular velocity), further read-outs of anxiety-like behaviour (Fig. 3i-l). We next measured behaviour in a large tank (the open field test). *nos1*$^{-/-}$ showed a preference for the centre of the tank suggesting reduced anxiety. However, there was also a reduction in the total distance swum and an increase in the time spent freezing suggesting that zebrafish are more anxious (Fig. 3m-o). We also recorded choice behaviour in a two-sided black/white tank (Lau et al., 2011). Both genotypes spent a similar amount of time on the non-preferred white side of the tank (data not shown). However, *nos1*$^{-/-}$ showed fewer transitions between compartments demonstrating decreased locomotion (Fig. 3p). Thus *nos1*$^{-/-}$ shows similar anxiety-like behaviour as wild-types in the black/white tank when taking into account locomotor abnormalities.

Nos1$^{-/-}$ mice also exhibit reduced aggression

The aggression phenotype of *nos1*$^{-/-}$ zebrafish contrasts with the initial descriptions of *Nos1*$^{-/-}$ mice (Chiavegatto et al., 2001; Nelson et al., 1995) suggesting that NO may control behaviour differently in these species. We next measured aggression in *Nos1*$^{-/-}$ mice (harbouring the same mutation as the original *Nos1*$^{-/-}$ but backcrossed onto C57Bl6 for at least 5 generations) using the resident-intruder paradigm. In agreement with our zebrafish data, *Nos1*$^{-/-}$ mice showed reduced agonistic behaviour compared to wild-types. There was a decrease in the number- and duration of attacks (Fig. 4a,b) and an increase in attack latency (Fig. 4c). In the open field test, *Nos1*$^{-/-}$ mice were hyperactive (Fig. 4d) but spent a similar amount of time in the centre as wild-types (Fig. 4e). Thus, murine anxiety does not appear to be altered by reduced *Nos1* function. We also investigated the preference for social novelty. Both genotypes showed a similar initial level of interest when interacting with a novel mouse, although the effect was stronger for wild-types than *Nos1*$^{-/-}$ (Fig. 4f). However, when a second unfamiliar mouse was introduced wild-types showed a preference for the novel mouse whereas *Nos1*$^{-/-}$ did not, indicating impaired processing of emotional stimuli (Fig. 4g).

Hypothalamic-pituitary interrenal axis functions normally in *nos1*$^{-/-}$ zebrafish

NO signalling has been linked to activation of the hypothalamic-pituitary-adrenal axis (HPA), a set of interacting pathways that help mediate an organism's stress response. Dysregulation of the HPA can lead to anxiety suggesting a possible mechanism underlying the behavioural phenotype of *nos1*$^{-/-}$. We measured the stress hormone cortisol using an enzyme-linked immunosorbent assay (ELISA). Wild-type and *nos1*$^{-/-}$ zebrafish showed similar basal cortisol levels (Fig. 5). Furthermore, exposure to air for 30-seconds increased cortisol levels in both genotypes suggesting that the hypothalamic-pituitary interrenal (HPI) axis, the teleostean equivalent of the HPA does not influence the behavioural phenotype of *nos1*$^{-/-}$ (Fig. 5).

Reduced breakdown of monoamine neurotransmitters in *nos1*$^{-/-}$ mutants

The control of aggression and anxiety has been linked to monoamine neurotransmitter signalling, and aggressive *Nos1*$^{-/-}$ mice exhibit decreased breakdown of 5-HT in the brain (Chiavegatto et al., 2001). We used high precision liquid chromatography (HPLC) to assess the basal levels of 5-HT, noradrenaline, DA and their metabolites in wild-type and mutant zebrafish. Using HPLC we uncovered a reduction of the DA metabolite DOPAC in the telencephalon, diencephalon, optic tectum and hypothalamus of *nos1*$^{-/-}$ (Fig. 6a-d). There was also an increase of 5-HT and a decrease of noradrenaline in the hindbrain (Fig. 6d). Analysis of neurotransmitter turnover revealed further alterations. The DOPAC/DA ratio was decreased in the optic tectum and hindbrain of *nos1*$^{-/-}$ (Fig. 6e) without changes to HVA/DA (Fig. 6f). There was also a strong decrease in the 5-HIAA/5-HT ratio in the telencephalon of *nos1*$^{-/-}$ (Fig 6g). In the vertebrate brain 5-HT and DA are metabolised by the monoamine oxidase enzymes (MAOA and MAOB). Loss of *MAOA* function leads to increased impulsive aggression in both humans and mice (Brunner et al., 1993a, 1993b; Cases et al., 1995; Dorfman et al., 2014). Thus Mao, the zebrafish homologue of MAOA and MAOB, is a promising candidate to underpin the phenotype of *nos1*$^{-/-}$, especially since an interaction between MAO and NOS-I has already been demonstrated (Laas et al., 2010). Using an enzyme activity assay we detected reduced Mao activity in *nos1*$^{-/-}$ compared to wild-types (Fig. 6h). The decrease in Mao activity could be due to reduced gene expression following life-long abrogation of NO signalling. We investigated this issue using quantitative real-time PCR. In contrast to the diminished enzyme activity, *nos1*$^{-/-}$ zebrafish exhibited increased expression of *monoamine oxidase*, whereas *Nos1*$^{-/-}$ mice showed heightened *Mao* expression in the frontal cortex and decreased expression in the amygdala and raphe nucleus (Fig. 7a,b). This suggests that there is a compensatory up-regulation of Mao activity in some parts of the brain. To investigate the link between Mao and behaviour we treated wild-type zebrafish with deprenyl, a drug that inhibits Mao in zebrafish and MAOB in other vertebrates (Anichtchik et al., 1996). We hypothesised that deprenyl treatment would mimic the phenotype of *nos1*$^{-/-}$, decreasing wild-type aggression to the level seen in mutant zebrafish. Immersion in 10 µM or 100 µM deprenyl for 3 hours reduced enzyme activity in line with published data (Fig. 8a; Anichtchik et al., 1996). Drug treated zebrafish also showed a strong decrease

in mirror-induced aggression (Fig. 8b,c) and increased anxiety-like behaviour in the novel tank test (Fig. 8d,e). Thus, even though *nos1*$^{-/-}$ harbour a life-long reduction of NO signalling, acute treatment of wild-type zebrafish with deprenyl is sufficient to mimic their behavioural phenotype.

5-HT signalling underlies the behavioural phenotype of *nos1*$^{-/-}$

The most dramatic change to neurotransmitter signalling in *nos1*$^{-/-}$ was decreased 5-HT turnover in the telencephalon. We investigated the connection between 5-HT and behaviour by applying the 5-HT receptor 1A (Htr1A) agonist 8-OH-DPAT to mutants reasoning that an increase in 5-HT levels should rescue their phenotype. Treatment with 1 mg/L 8-OH-DPAT rescued aggression in *nos1*$^{-/-}$ increasing agonistic levels to those of wild-type zebrafish (Fig. 8f). However, anxiety-like behaviour in the novel tank test was not rescued by drug application. Although the time spent in the top of the novel tank increased for both genotypes (Fig. 8g) there was still a significant difference between them. The time spent freezing and angular velocity of *nos1*$^{-/-}$ was rescued by 8-OH-DPAT treatment (Fig. 8h,i).

Discussion

In this study we have characterised *nos1*$^{-/-}$ mutant zebrafish with decreased NO signalling in the brain. Loss of *Nos1* caused behavioural alterations including reduced aggression in both zebrafish and mice, increased anxiety-like behaviour in zebrafish and hyperactivity in mice. The zebrafish *nos1*$^{-/-}$ phenotype correlates with reduced breakdown of 5-HT and DA and decreased Mao activity. Pharmacological stimulation of 5-HT signalling using the Htr1A agonist 8-OH-DPAT was able to rescue most of these phenotypes highlighting the interaction between NO and 5-HT in controlling behaviour as previously shown in *Nos1*$^{-/-}$ mice (Chiavegatto et al., 2001).

Reduced NO signalling in *nos1* mutants

Mutation of *nos1* led to a reduction of gene activity as shown by both in situ hybridization and qPCR (Fig. 2a-i). This suggests that non-sense mediated decay of the mutated mRNA may have occurred. However, the Griess assay revealed the presence of nitrites in the *nos1*$^{-/-}$ brain (Fig. 2j) suggesting that residual NO synthesis still occurs. This observation can be explained by the presence of other sources of nitrites in the fish brain, as well as compensation by *nos2b* in agreement with previous studies (Diaz et al., 2015). Although we have only characterised one *nos1* mutant allele, several lines of evidence point to reduced nitric oxide signalling including the absence of Nos1 in the Western blot (Fig. 1o); the decrease in *nos1* expression detected by qPCR (Fig. 2i); and the reduced nitrite levels (Fig. 2j). We are therefore confident that we the behavioural phenotype is due to mutation of *nos1*. In this study we have focussed on the behaviour of adult zebrafish. Injection of capped mRNA at the single cell stage could be used to rescue some of the phenotypes shown here if they are triggered during embryonic development. However, it would be hard to interpret this experiment if we do not see a rescue, since capped mRNA is only stable for a few hours and we have characterised mature fish from 3 months onwards. Future studies would benefit from generating a second *nos1* mutant line – perhaps in a different genetic background to investigate the effect of modifier genes on the behavioural phenotype (Le Roy et al., 2000).

Behavioural differences between zebrafish *nos1*$^{-/-}$ and several sub-strains of *Nos1*$^{-/-}$ mice

The highly cited original description of *Nos1*$^{-/-}$ reported heightened aggression following social isolation (Nelson et al., 1995) and either increases or decreases in anxiety (Bilbo et al., 2003; Wultsch et al., 2007). In contrast to this, mutation of zebrafish *nos1* leads to a pronounced reduction of aggression coupled to increased anxiety-like behaviour. Blunted aggression was evident in both mirror-induced stimulation and dyadic fights (Fig. 3a-f) and social isolation of *nos1*$^{-/-}$ prior to testing did not alter this phenotype (Fig. 3g,h). Therefore, an influence of the social environment cannot explain the presumed difference between *nos1*$^{-/-}$ zebrafish and *Nos1*$^{-/-}$ mice. The heightened aggression of *Nos1*$^{-/-}$ disappears when it is crossed onto a different genetic background indicating that

a modifier gene is necessary to elicit this phenotype (Le Roy et al., 2000). To clarify this issue, we carried out detailed behavioural analysis of *Nos1*$^{-/-}$ mice backcrossed onto a Bl6 background. These experiments revealed a stable decrease of resident-intruder aggression in agreement with our zebrafish data (Fig. 4a-c).

NOS-I also has an important role in controlling anxiety. *nos1*$^{-/-}$ zebrafish show increased anxiety-like behaviour in the novel tank test and open field test whereas this behaviour was not modified in the black-white tank. There was a decrease in locomotion in the open field test (Fig. 3n) but not the mirror-induced aggression test (Fig. 3b). The novel tank and black-white tests have already been dissociated behaviourally and pharmacologically (Blaser and Rosemberg, 2012). The novel tank may measure the response to novelty whereas the black-white test could examine the motivational conflict between fear and exploration. In contrast to this, *Nos1*$^{-/-}$ mice exhibited hyperactivity in the open field test without changes to time in the centre suggesting that anxiety is not altered. This result agrees with a previous study of *Nos1*$^{-/-}$ mice backcrossed onto the C57BL/6J background. *Nos1*$^{-/-}$ were found to be hyperactive in the open field test, elevated plus maze and light/dark transition test without showing other anxiety phenotypes (Tanda et al., 2009). Taken together, this suggests that the increased time spent in the centre of the open field may be secondary to changes in locomotion. Social interactions can also modify the role of NO signalling in anxiety perhaps explaining these discrepancies. For example, pharmacological inhibition of NOS-I can be either anxiolytic or anxiogenic depending upon whether mice are single- or grouped housed before testing (Workman et al., 2008). Further studies comparing zebrafish housing conditions to levels of anxiety-like behaviour will be required in order to resolve this difference between species.

Two splice variants of *Nos1* have been described in mice and one of these, *NOS-Iβ*, is upregulated in the striatum and cortex of *Nos1*$^{-/-}$ meaning that gene activity is not completely abolished (Eliasson et al., 1997). The maintenance of *NOS-Iβ* expression in some brain areas but not others could explain the hyperactivity of knock-out mice in the OFT. The zebrafish genome also contains splice variants that are predicted to code for alternative NOS1 proteins. Although we have not examined the expression

of these variants in *nos1*$^{-/-}$ it is unlikely that they influence behaviour, since the mutant allele leads to a stop codon upstream of the predicted amino acid changes. The differences in anxiety levels could thus be explained by a difference in the severity of NO signalling reduction in zebrafish compared to mice. However, we favour the hypothesis that the behavioural function of NO is dependent upon either interaction with other genes or environmental factors, the ethological relevance of the tests used for each species, or the activity of NOS-I in different brain circuits in zebrafish and mouse. Future studies will be required to address this issue.

Similarities to human psychiatric disorders

The behavioural phenotype of *nos1*$^{-/-}$ zebrafish is reminiscent of several psychiatric disorders linked to nitric oxide. *NOS1* has been connected to depression and anxiety as well as impulsivity-related diseases such as schizophrenia and ADHD (Freudenberg et al., 2015). The human *NOS1* gene is complex with multiple splice variants and alternative coding first exons (Bros et al., 2006). Of particular relevance to this study, an association has been reported between a polymorphism in the promoter region of *NOS1* exon 1f and violent aggression (Reif et al., 2009). This study appears to contradict our results since the Exon 1f VNTR reduces gene expression. However, only one alternative first exon is driven by the affected promoter and the impact upon overall NOS-I expression is unknown; alternative first exons might be upregulated in a compensatory manner. This could lead to altered intracellular distribution of NOS-I without decreasing NO production. Furthermore, the association could be accounted for by a broader increase in impulsivity rather than aggression, suggesting that the connection between NOS isoforms and aggression needs to be analysed in more detail.

Link between NOS-I, 5-HT and Monoamine oxidase

One novel finding of our study is that the behavioural phenotype of *nos1*$^{-/-}$ zebrafish correlates with decreased breakdown of 5-HT in the forebrain due to a reduction of Mao activity (Fig. 6c,d). Treatment of rats with the NO donor molsidomine increases monoamine metabolism (Lorenc-Koci et al., 2013)

379 whereas NOS-I inhibition with N3-nitro-L-arginine decreases neurotransmitter turnover in mouse
380 (Karolewicz et al., 2001) further linking NO to Mao. In wild-type zebrafish the Mao inhibitor deprenyl
381 mimicked some aspects of the *nos1*$^{-/-}$ phenotype (Fig. 8b-e). Zebrafish only have one Mao orthologue
382 compared to two in humans and mice (Anichtchik et al., 2006). In most species, MAO-A degrades 5-
383 HT and NA whereas DA is metabolised by both MAO-A and MAO-B (Dorfman et al., 2014; Bortolato et
384 al., 2011). The presence of a single isozyme in zebrafish may disrupt monoamine signalling more
385 severely than in other species. In agreement with this, MAO-A/B double knock-out mice show
386 decreased 5-HT breakdown, reduced exploration and increased anxiety (Chen et al., 2004). MAO-A/B
387 knock-outs also display brief aggressive contact. Therefore, the anxiety phenotype of MAO-A/B mice
388 may shape their agonistic behaviour (Chen et al., 2004). Mice with a hypomorphic reduction of MAO-
389 A show context-dependant neophobia and increased perseverative behaviour without changes to
390 aggression or locomotion (Bortolato et al., 2011) whereas in humans, loss of MAO-A leads to
391 heightened impulsive aggression (Brunner et al., 1993a; 1993b). Similar to NO, alteration to MAO
392 function can lead to a variety of behavioural outcomes depending upon the molecular lesion and
393 behavioural test. Although enzyme activity is reduced, the level of *mao* expression was increased in
394 *nos1*$^{-/-}$ zebrafish compared to wild-types suggesting that changes may occur at the post-translational
395 level. NO has already been shown to modulate monoamine reuptake by indirect phosphorylation- or
396 direct S-nitrosylation of SERT, NET and DAT (Chanrion et al., 2007, Miller and Hoffman, 1994).
397 Similarly, Mao activity could be reduced by phosphorylation or S-nitrosylation of the protein. Negative
398 feedback could then lead to heightened levels of *mao* gene expression in compensation for reduced
399 enzyme activity. NO can also alter neurotransmitter release via phosphorylation of synaptosomal
400 proteins (Hirsch et al., 1993) thereby altering the amount of time in which neurotransmitters interact
401 with their receptors.
402 5-HT has been linked to anxiety and aggression in a number of species. In mice, reducing 5-HT in the
403 forebrain during postnatal stages provokes anxiety-like behaviour (Gross et al., 2002; Gingrich and
404 Hen, 2001). Conversely, acute inhibition of 5-HT neuron activity (by overexpressing Htr1A or applying

8-OH-DPAT to mice with Htr1A restricted to presynaptic raphe neurons) increases aggression (Audero et al., 2013). Furthermore, infusion of 8-OH-DPAT into the raphe nucleus and hippocampus is anxiolytic (Menard and Treit, 1999). The interaction between 5-HT and NO signalling is complex involving reciprocal modulation of release and reuptake (Chanrion et al., 2007). Htr1A activation can tonically inhibit NOS-I function (Herculano et al., 2015) and NO also acts downstream of Htr1A by altering CREB phosphorylation (Zhang et al., 2010). Importantly, NOS-I is co-expressed with Htr1A in ascending dorsal raphe 5-HT neurons that project to the cortex (Lu et al., 2010). The neural circuits that co-express both Nos1 and Htr1a are well-placed to control aggression and anxiety. Treatment of *nos1*$^{-/-}$ zebrafish with 8-OH-DPAT rescued aggression similar to the selective stimulation of presynaptic Htr1A autoreceptors described above. The discrepancies between our data and the studies of Chiavegatto and colleagues are likely to be due to the mixed genetic background of the mice used in their research. The inability of 8-OH-DPAT to rescue the time spent at the bottom of the novel tank (Fig. 8g) does not rule out a role for 5-HT in this behaviour since multiple different receptors may influence anxiety. Additionally, time at the bottom could be less sensitive to 5-HT levels than freezing, requiring a higher dose of 8-OH-DPAT to alter its expression.

In summary, our analysis of *nos1*$^{-/-}$ zebrafish provides further evidence that NO signalling plays a critical role in modulating aggression and anxiety-like behaviour in the vertebrate brain. We show that the interaction between NO and 5-HT is mediated by monoamine oxidase and confirm that manipulation of NO can lead to either increases or decreases in aggression and anxiety levels, most likely due to modifier genes in the genetic background (Le Roy et al 2000) or individual differences in Mao activity or 5-HT signalling.

Author disclosures

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HCG and WHJN designed the zebrafish experiments. HCG performed and analysed all zebrafish experiments. AOL and FF designed, performed and analysed all mouse experiments. GF carried out the Western blot. RW, EM and FvE designed and created the zebrafish TALEN lines. AR and WHJN analysed data and wrote the manuscript. All authors approved the final version of the manuscript. The authors declare no conflict of interest.

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630

631 **Figure legends**

632 **Figure 1.** *nos1* expression and the *nos1*^{SH336} TALEN mutant line. (**a-l**) Images of coronal sections of the
633 adult zebrafish brain, showing *nos1* in situ hybridisation expression in the dorsal, ventral and posterior
634 ventral telencephalon (Vd, Vv, Vp), the medial, lateral, and posterior dorsal telencephalon (Dm, Dl,
635 Dp) and the anterior and posterior part of the preoptic area of the anterior hypothalamus (PPa and
636 PPp). *nos1* is also expressed in the ventral part of periventricular pretectal nucleus (PPv), the dorsal
637 (DP) and central posterior (CP) thalamic nucleus, the posterior nucleus of the posterior tuberculum
638 (TPp), the ventral zone of the periventricular hypothalamus (Hv), the posterior thalamic nucleus (P).
639 Other hypothalamic regions that express *nos1* include the paraventricular organ (PVO), the posterior
640 tuberal nucleus (PTN), the lateral hypothalamic nucleus (LH), the subglomerular nucleus (SG), and the
641 dorsal and caudal zones of the periventricular hypothalamus (Hd, Hc). Sparse expression is also seen
642 in the superior raphe formation (SRF) and the nucleus interpeduncularis (Nln), the griseum central
643 (GC) and the nucleus isthmi (NI) and the corpus mammilare (CM). (**m**) Cartoon depicting the 7 base-
644 pair deletion in exon 1 of *nos1*. (**n**) Polymerase chain reaction (PCR) genotyping of wild-type, *nos1*$^{+/-}$
645 and *nos1*$^{-/-}$ before- and after *BstXI* digestion. PCR amplification of the region flanking the mutated site
646 in wild-types generated a 373 bp product that gave two fragments of 303 bp and 70 bp after digestion
647 with *BstXI*. (**o**) Western blot showing reduced protein levels in *nos1*$^{-/-}$.
648
649 **Figure 2.** Reduction of NO signalling in *nos1*$^{-/-}$ mutants. *nos1* expression is reduced in the brain of
650 *nos1*$^{-/-}$ mutants (**e-h**) compared to (**a-d**) wild-type. (**i**) qPCR expression analysis of *nos1*, *nos2a* and
651 *nos2b* in the brain. Unpaired *t*-test n=8 each genotype: *nos1* $p < 0.0001$, *nos2a* non-significant; *nos2b*
652 $p = 0.0416$. (**j**) The concentration of NO metabolites measured by the Griess assay is reduced in *nos1*$^{-}$
653 $^{/-}$ compared to wild-types. Unpaired *t*-test $n = 5$ each genotype: $p = 0.0070$. ****$p < 0.0001$; **$p <$
654 0.01; *$p < 0.05$.
655

Figure 3. Behaviour of *nos1*$^{-/-}$ zebrafish. (**a-h**) *nos1*$^{-/-}$ show reduced aggression compared to wild-types. This includes (**a**) reduced time spent in aggressive display in the mirror test (p = 0.0036), and (**b**) less time spent in the mirror zone (p = 0.0016; n = 12 wild-type, n = 11 *nos1*$^{-/-}$), (**c**) fewer bites in a dyadic test (p < 0.0001), (**d**) fewer chases (p < 0.0001), (**e**) fewer circling events (p = 0.0006) and (**f**) more time spent freezing (p = 0.0394; n = 11 wild-type pairs, n = 14 *nos1*$^{-/-}$ pairs), and (**g**) reduced mirror aggression following social isolation (p = 0.0029), and (**h**) more time freezing (p = 0.0323; n = 10 wild type, n = 10 *nos1*$^{-/-}$). (**i-l**) *nos1*$^{-/-}$ exhibit increased anxiety-like behaviour including (**i,j**) decreased time at the top of a novel tank (p = 0.0001), (**k**) increased time spent freezing (p = 0.0076), (**l**) and increased angular velocity (p = 0.0068; n = 12 wild-type, n = 13 *nos1*$^{-/-}$). Unpaired t-test with Welch correction or Mann Whitney U test. (**m-o**) Open field test. (**m**) *nos1*$^{-/-}$ swim less distance in the open field (p = < 0.0001); (**n**) show decreased thigmotaxis (p = 0.0007) and (**o**) spend increased-time freezing (p < 0.0001; n = 14 wild-type, n = 14 *nos1*$^{-/-}$; t-test with Welch correction or Mann Whitney U test was performed). (**p**) Black-white preference test. *nos1*$^{-/-}$ show significantly fewer transitions between black and white (p = < 0.0001; n = 14 wild-types, n = 16 *nos1*$^{-/-}$; Mann Whitney U test). (*) p < 0.05, (**) p < 0.01, (***) p < 0.001, (****) p < 0.0001.

Figure 4. Behaviour of *Nos1*$^{-/-}$ knock-out mice. (**a-c**) *Nos1*$^{-/-}$ mice show reduced aggression in the resident-intruder test including (**a**) decreased number of attacks (F (1, 70) = 5.549, p = 0.0213), (**b**) decreased attack duration (F (1, 70) = 7.642, p = 0.0073) and (**c**) increased attack latency (F (1, 70) = 16.84, p = 0.0001; n = 8 each, two-way ANOVA). (**d**,**e**) *Nos1*$^{-/-}$ exhibit (**d**) hyperactivity in the open field test (p = 0.0003), whereas (**e**) time in the centre was similar for both wild-types and knock-outs (n = 8 each, unpaired t-test with Welch correction). (**f**,**g**) *Nos1*$^{-/-}$ mice exhibit impaired processing of social stimuli. (**f**) Both genotypes interact more time interacting with a novel mouse (stranger 1) introduced in the open field (empty vs stranger 1: WT, p <0.0001; *Nos1*$^{-/-}$, p = 0.0009) but the preference is increased in wild-types compared to knock-outs (p = 0.0297). **(g)** When a second novel mouse (stranger 2) is placed in the open field the wild-type mice spend more time interacting with it than

682 with stranger 1 (p = 0.0153) and also more time than the knock-outs (p = 0.0249). n = 8 each; two-way
683 ANOVA followed by Sidak's post hoc). (*) $p < 0.05$, (***) $p < 0.001$, (****) $p < 0.0001$.
684
685 **Figure 5.** Cortisol levels in *nos1*$^{-/-}$ zebrafish. Cortisol levels are similar in wild-type and *nos1*$^{-/-}$ before-
686 and after a stressful episode. Cortisol increases in wild-types and *nos1*$^{-/-}$ after stressful episode (basal
687 levels vs stress levels: wild-type, p = 0.0008; *nos1*$^{-/-}$, p = 0.0005; n = 11 per group; two-way ANOVA
688 followed by Sidak's multiple comparisons tests). (***) $p < 0.001$.
689
690 **Figure 6.** Neurochemical analysis of *nos1*$^{-/-}$ zebrafish. (**a**-**d**) High precision liquid chromatography
691 analysis of wild-type and *nos1*$^{-/-}$ (**a**) telencephalon, (**b**) diencephalon, (**c**) optic tectum and (**d**)
692 hindbrain. There is a statistically significant reduction of DOPAC levels in the telencephalon (p =
693 0.0310), diencephalon (p = 0.0070), hindbrain (p = 0.0117) and optic tectum (p = 0.0169), a decrease
694 in NA in the hindbrain (p = 0.049) and an increase in 5-HT levels in the hindbrain (p = 0.0352) of *nos1*$^{-}$
695 $^{/-}$ (n = 10 wild-type, n = 9 *nos1*$^{-/-}$; multiple t-tests with Holm-Sidak correction for multiple comparisons).
696 (**e**) *nos1*$^{-/-}$ shows reduced breakdown of DA to DOPAC in the TeO (p = 0.0397) and Hb (p = 0.0168), (**f**)
697 but there is no change in breakdown of DA to HVA. (**g**) *nos1*$^{-/-}$ show reduced breakdown of 5-HT to
698 5HIAA in the TeO (p = 0.0332; n = 10 wild-type, n = 9 *nos1*$^{-/-}$; t-tests with Holm-Sidak correction for
699 multiple comparisons). (**h**) Monoamine oxidase activity is reduced in the brain of *nos1*$^{-/-}$ (60 min p =
700 0.0019, 90 min p = 0.0006, 120 min 0.0021; n = 7 wild-type, n = 9 *nos1*$^{-/-}$; two-way ANOVA followed by
701 Sidak's post hoc). Abbreviations: DA, dopamine; Di, diencephalon; DOPAC, 3,4-Dihydroxyphenylacetic
702 acid; Hb, Hindbrain; HVA, homovanillic acid; NA, noradrenaline; Tel, telencephalon, TeO, optic tectum;
703 5HIAA, 5-hydroxyindoleacetic acid; 5-HT, 5-hydroxytryptamine. (*) $p < 0.05$, (**) $p < 0.01$, (***) $p <$
704 0.001.
705
706 **Figure 7.** Relative expression of *Mao* in the brain. (**a**) In zebrafish, *mao* expression is increased in *nos1*$^{-}$
707 $^{/-}$ compared to wild-type (p = 0.0050; n = 8 each; t-test with Welch correction). (**b**) In mouse, *Mao*

expression is increased in the frontal cortex (p = 0.0102) and is decreased in the amygdala (p = 0.0401) and raphe nucleus (p = 0.0053) of *Nos1*$^{-/-}$ knock-out compared to wild-type (n = 19 wild-type, n = 12 *Nos1*$^{-/-}$ knock-out; t-tests with Holm-Sidak correction for multiple comparisons). Abbreviations: FC, frontal cortex; Amz, amygdala; Str, striatum; NAcc, nucleus accumbens; Hc, hippocampus; Hy, hypothalamus; R, raphe nucleus. (*) $p < 0.05$, (**) $p < 0.01$.

Figure 8. Pharmacological manipulation of *nos1*$^{-/-}$ zebrafish. (**a**) Acute treatment of wild-type fish with deprenyl decreases monoamine oxidase activity at the time points indicated ($p < 0.0001$; n = 10 each; two-way ANOVA followed by Dunnett's post hoc). (**b,c**) Deprenyl treatment decreases aggression in the mirror setup including (**b**) decreased aggressive display (wild-type vs 10 µM deprenyl: p = 0.0007, wild-type vs 100 µM deprenyl: $p < 0.0001$; n = 11 each; Kruskal-Wallis test followed by Dunn's post hoc) and (**c**) increased freezing (wild-type vs 10 µM deprenyl: p = 0.0026, wild-type vs 100 µM deprenyl: p = 0.0203; n = 11 each, one-way ANOVA followed by Dunnett's post hoc). (**d,e**) Deprenyl increases anxiety-like behaviour in the novel tank test, including (**d**) reduced the time spent at the top of a novel tank (wild-type vs 10 µM deprenyl: p = 0.0255, wild-type vs 100 µM deprenyl: p = 0.0006; n = 11 each; Kruskal-Wallis test followed by Dunn's post hoc) and (**e**) increased freezing (wild-type vs 100 µM deprenyl: $p < 0.0001$; n = 11 each; one-way ANOVA followed by Dunnett's post hoc). (**f**) Treatment with the Htr1A agonist 8-OH-DPAT rescues the reduced aggression of *nos1*$^{-/-}$ (p = 0.0286; n = 11 per group; two-way ANOVA followed by Sidak's post hoc). (**g**) Treatment with 8-OH-DPAT also increases the time spent in the top of a novel tank by both genotypes (control versus treatment: wild-type, $p < 0.0001$; unpaired t-test with Welch correction; *nos1*$^{-/-}$, p = 0.0017; Mann Whitney U test) without rescuing the phenotype since there is a significant difference between wild-type and *nos1*$^{-/-}$ either in the control groups (p = 0.0002; Mann Whitney U test) or after treatment (p = 0.0001, unpaired t-test with Welch correction). (**h**) 8-OH-DPAT treatment rescues the increased time spent freezing (wild-type versus *nos1*$^{-/-}$, $p < 0.0001$; Mann Whitney U test; control versus treatment, $p < 0.0001$; Mann Whitney U test) and (**i**) the increase in angular velocity observed in *nos1*$^{-/-}$ (wild-type versus *nos1*$^{-/-}$, p

734 < 0.0001; control versus 8-OH-DPAT, $p < 0.0001$; two-way ANOVA followed by Sidak's post hoc; $n = 19$

735 wild-type control, $n = 10$ wild-type treated, $n = 8$ *nos1*$^{-/-}$ control, $n = 10$ *nos1*$^{-/-}$ treated). (*) $p < 0.05$,

736 (**) $p < 0.01$, (***) $p < 0.001$, (****) $p < 0.0001$.

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