

Research report

Inhibition of neuronal nitric oxide reduces anxiety-like responses to pair housing

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Abstract

Many psychological disorders are characterized by anxiety and alterations in social interactions. Recent studies demonstrate that the chemical messenger nitric oxide (NO) can regulate both anxiety and social behaviours. We tested whether an enzyme that produces NO in the brain, neuronal nitric oxide synthase (nNOS), serves as an interface between social interactions and anxiety-like behaviour. Several investigators have observed that mice increase anxiety-like responses in the elevated plus-maze after pair housing. nNOS gene deletion and 3-Bromo-7-Nitroindazole were used to inhibit the production of neuronal NO. Similar to previous studies, pair housing reduced open arm exploration in the elevated plus-maze. Pair housing also increased corticotropin-releasing hormone (CRH) immunoreactive cells in the paraventricular nucleus (PVN) of the hypothalamus. Inhibition of NO production increased open arm exploration in pair-housed mice but decreased open arm exploration in individually housed mice. These results suggest that the effect of nNOS inhibition on anxiety-like responses is context dependent and that behavioural responses to social housing are altered after nNOS inhibition. This research suggests that NO may play an important role in mediating the effect social interactions have on anxiety.

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1. Introduction

Many psychological disorders are characterized by anxiety and alterations in social interactions. Social interactions influence a wide array of affective behaviours. In humans, social isolation is a risk factor for suicide [19]. In rhesus monkeys (*Macaca mulatta*), anxiety- and depressive-like behaviours increase in response to social isolation [24]. Although social isolation in primates has a negative influence on affect, individual housing has the opposite effect in male mice. Individually housed male mice have reduced anxiety-like responses compared to male mice housed in pairs [1,28,33,45]. This difference in affective responses may reflect species differences in social structure. Primates are highly social animals and isolation from

conspecifics has negative consequences in terms of affective and stress responses [18,38]. In contrast, socially housed male house mice form dominance hierarchies and territories [2,25] which may contribute to the reduced exploratory behaviour. Also, strain-specific anxiety-like responses have been reported in rodents. For example, male Wistar rats, in common with mice, increase anxiety-like responses after pair housing [41] whereas social isolation appears to be anxiogenic in male Sprague–Dawley rats [49]. The biological substrates of social interactions, anxiety, and their interaction remain unclear. One chemical system that may be of interest is nitric oxide (NO) because inhibition of neuronal nitric oxide alters social [13,29] and anxiety-like behaviours [9,11,35,46].

NO is a gas that can act as a neurotransmitter in the brain [17,30,31]. NO is labile, with a half-life of <5 s at body temperature; consequently, many studies manipulate NO indirectly by affecting its synthetic enzyme, NO synthase (NOS), that transforms arginine into NO and citrulline. Three distinct isoforms of

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NOS have been discovered: (1) eNOS (NOS-3) in the endothelial tissue of blood vessels, (2) iNOS (NOS-2) in macrophages as an inducible form, and (3) nNOS (NOS-1) in neurons, which is expressed constitutively [21].

NO has broad effects on social behaviours. Most research on the role of NO in social behaviours has focused on aggression [5,13,29,31,42] and defensive behaviours (for review see [14]). Male mice lacking the gene that encodes nNOS (nNOS^{-/-}) are highly aggressive and do not relent in response to submissive postures of wild-type (WT) males [29]. nNOS^{-/-} mice also persist in mounting anestrous WT females despite signs of disinterest [29]. The persistence of these behaviours in nNOS^{-/-} mice may reflect alterations in brain areas that are important for the processing of social cues.

Recent research suggests that the effect of nNOS inhibition on behaviour is dependent on social context. Although nNOS^{-/-} mice are more aggressive toward WT intruders, suppression of nNOS with the selective nNOS inhibitor 3-Bromo-7-Nitroindazole (3-Br-7-NI) reduces maternal aggression in response to an intruder relative to untreated dams [13]. In addition, single-housed mice treated with 3-Br-7-NI also reduce social investigation in a barrier test and a habituation–dishabituation test relative to vehicle-treated mice but this effect was eliminated when mice were pair housed [42]. Together, these results suggest that deficits in NO signaling alter behavioural responses to social interactions.

Studies on NO signaling and affective behaviours have yielded mixed results. Pharmacological inhibition of NO by administration of either NG-nitro-L-arginine methyl ester (L-NAME) or NG-nitro-L-arginine (L-NOARG), two nonspecific NOS inhibitors, increases anxiety-like responses [7,8,26,35,43]. L-NOARG also reverses anxiolysis associated with benzodiazepines [36] and nitrous oxide (N₂O) [4] suggesting that NO is anxiolytic. In contrast to these results, administration of L-NAME, L-NOARG, or 7-nitroindazole (7-NI) decreases anxiety-like responses in the elevated plus-maze [9–11,15,35,46]. The nonselective properties of L-NAME and L-NOARG may be an important contributing factor in the disparate results that have been reported across studies, as these drugs influence both neuronal and endothelial NOS [48]. Furthermore, these drugs may increase anxiety-like responses through non-specific changes such as vasoconstriction or reduced locomotor activity [7,35]. However, studies using the nNOS inhibitor 7-NI report decreases in anxiety-like responses [9,11,35,46].

NO dysfunctions may alter hormones and neurotransmitters to influence anxiety-like responses. NO is intimately involved with the hypothalamic-adrenal-pituitary (HPA) axis and is located (although not exclusively) within the paraventricular nucleus (PVN) [30]. The PVN is a major source of corticotropin-releasing hormone (CRH) which induces adrenocorticotropic hormone (ACTH) release from the pituitary which stimulates cortisol (or corticosterone) release from the adrenal cortex [32]. Although cortisol has been implicated in playing a major role in the development of affective disorders, the central effects of CRH itself may be important in anxiety and anxiety-like responses [16]. CRH terminals, cell bodies, and receptors are located throughout the brain including the central nucleus of the

amygdala [44], which plays an integral role in the autonomic and behavioural responses to fearful stimuli [20,47]. Although many studies have investigated the effects of NO inhibition on anxiety-like responses, less attention has been paid to changes that occur in brain areas that are associated with fear and anxiety. In addition, many of the aforementioned studies do not consider the effect of social housing conditions on behaviour.

We examined the influence of nNOS inhibition on anxiety-like responses and CRH immunoreactivity in the PVN and central nucleus of the amygdala (CeA) of single- and pair-housed mice. We hypothesized that pair housing of WT mice would reduce open arm exploration in the elevated plus-maze. Furthermore, we expected that nNOS inhibition would eliminate the effect of pair housing on anxiety-like responses. Using nNOS^{-/-} mice and a selective nNOS inhibitor we tested whether NO function interacts with social housing conditions to influence anxiety-like responses.

2. Methods

2.1. Animals

Adult wild-type (WT) mice (B6; 129SF1/SImJ, Stock Number 002633) and nNOS^{-/-} mice (B6; 129S4/SvJae-Nos1^{tm1Pth}/J, Stock Number 101045) were obtained from Jackson Laboratory (Bar Harbor, ME, USA) for Experiment 1. Adult WT mice (B6; 129SF1/SImJ, Stock Number 002633) were again obtained from Jackson Laboratory at a later date and used in Experiment 2. The nNOS^{-/-} mutation is on a C57B6 × 129S background. The WT mice are also a C57B6 × 129S cross that is an appropriate match to the background of the nNOS^{-/-} line. Although not an exact match, this strain has been used previously as a WT control strain as well as in pharmacological manipulations [42]. Mice were housed in polypropylene cages (dimensions: 27.8 cm × 17.5 cm × 13 cm). All mice had unlimited access to food (Harlan Teklad 8640 rodent diet, Indianapolis, IN) and filtered tap water. The light cycle was 16L:8D with the dark phase beginning at 1500 h EST. All procedures were approved by the Ohio State Institutional Animal Care and Use Committee prior to the study and meet guidelines published in the National Institutes of Health (1986) *Guide for the Care and Use of Laboratory Animals*.

2.2. Experiment 1

Upon arrival, WT and nNOS^{-/-} mice were randomly assigned to either individual or pair housing with a mouse of the same genotype (WT single housed, $n=5$, WT pair housed, $n=7$, nNOS^{-/-} single housed, $n=6$, nNOS^{-/-} pair housed, $n=6$). Two weeks after arrival, mice underwent behavioural testing in the open field chamber and elevated plus-maze on consecutive days during the dark phase.

2.2.1. Open field

The test chamber for open field was enclosed in a sound and light attenuating cabinet and consisted of a 60 cm³ clear Plexiglas arena lined with corncob bedding. The arena was surrounded by a series of infrared lights that tracked the movement of the mouse in three dimensions. Mice were allowed to acclimate to the testing room for 15 min before testing began. The test chamber was rinsed thoroughly with 70% ethanol and the bedding was changed between each test. Each test session was 30 min in duration. The results were generated online by the PAS software package (San Diego Instruments, San Diego, CA, USA). The total locomotor activity (number of beam breaks) served as the dependent measure.

2.2.2. Elevated plus-maze

The maze was ~1 m above the floor and consisted of two open arms bisected by two arms enclosed with dark-tinted acrylic. Testing began approximately

45 min after lights out and mice were allowed to acclimate to the testing room for 15 min before testing began. The room was illuminated with a dim red light. Mice were placed into a closed arm and recorded on video for 5 min. The maze was wiped with 70% ethanol between tests. An open arm entry was scored when the two forepaws had entered an open arm. Latency to enter an open arm, total time spent in the open arms, and open arm entries were scored by a blind observer using Observer software (Noldus Corp., Leesburg, VA).

2.2.3. Tissue collection

Two days after testing in the elevated plus-maze, mice were anesthetized with isoflurane and decapitated. Brains were quickly removed and fixed overnight in 5% acrolein. Brains were then transferred to 30% sucrose for 24 h and frozen at -80°C .

2.2.4. CRH immunohistochemistry

Brains were sectioned at $40\ \mu\text{m}$ on a cryostat and free-floating sections were processed for CRH immunohistochemistry as previously described [23]. Sections were washed three times in phosphate-buffered saline (PBS) and then incubated in 1% sodium borohydride in PBS for 10 min. Sections were then rinsed in 20% normal goat serum and 0.3% hydrogen peroxide in PBS for 20 min. Sections were incubated in primary CRH antibody (1:20,000, gift of Dr. Wylie Vale, Salk Institute, San Diego, CA) in 1% normal goat serum in 0.5% Triton-X PBS (PBS + TX) at 4°C for 48 h. Next, sections were rinsed three times in PBS and incubated for 90 min with biotinylated goat-anti-rabbit antibody (1:500, Vector Laboratories, Burlingame, CA) in PBS + TX. The sections were rinsed three times in PBS and then incubated for 30 min in avidin–biotin complex (ABC Elite kit, Vector Laboratories, Burlingame, CA). After three rinses in PBS, the sections were developed in diaminobenzidine for 2 min. Sections were mounted, dehydrated, and coverslipped with Permount. Sections containing the PVN and CeA were identified using a mouse brain atlas [12]. Images were captured using a Nikon E800 microscope. Images of the PVN were taken at -0.70 to -0.94 bregma. Positively stained cells in the PVN were distinct and considered positive if they showed any staining. Images of the CeA were taken at -1.46 to -1.58 bregma. Due to staining in fibers, it was difficult to quantify the number of CRH-positive cells in the CeA. To quantify staining in this area, the percentage of staining was calculated using the image analysis software ImageJ, 1.38x (NIH). Control sections in which primary antibodies were not added showed no staining.

2.3. Experiment 2

Mice were randomly assigned to receive Silastic implants containing the selective neuronal nitric oxide synthase inhibitor 3-Bromo-7-Nitroindazole (3-Br-7-NI) [13,42]. Silastic tubing (1.98 mm i.d. \times 3.18 mm o.d.; Dow Corning, Midland, MI) was packed with 2 mm of 3-Br-7-NI and then implanted subcutaneously between the scapulae (empty capsule single-housed, $n=5$, empty capsule pair-housed, $n=9$, 3-Br-7-NI capsule single-housed, $n=5$, 3-Br-7-NI capsule pair-housed, $n=5$). The optimal length of the capsule was determined in a pilot study of the effects of 1, 2, and 3 mm of 3-Br-7-NI on citrulline staining. One week after surgery, mice underwent behavioural testing as previously described in Experiment 1.

2.4. Statistical analysis

Data from Experiment 1 were analysed with two-way ANOVAs, testing for the effects of genotype, housing environment, and their interaction. Data from Experiment 2 were analysed with two-way ANOVAs, testing for the effects of 3-Br-7-NI, housing environment, and their interaction. In both experiments, planned comparisons were used to compare the effects of genotype or implant type within housing environment and the effects of housing environment within either genotype or implant type. In the elevated plus-maze data of Experiment 1, two mice were removed from the single-housed, WT condition because they entered an open arm and did not move for the remainder of the test. ANOVAs and planned comparisons were considered statistically significant if $p < 0.05$.

3. Results

3.1. Experiment 1

nNOS $-/-$ mice entered the open arms sooner than WT mice regardless of housing environment ($F_{1,18} = 25.428$, $p < 0.05$). There was a significant interaction between genotype and housing for time spent in the open arm ($F_{1,18} = 5.6$, $p < 0.05$; Fig. 1A). Among WT mice, pair housing decreased time spent in the open arms ($p < 0.05$; Fig. 1A). This effect was reversed, however, in the nNOS $-/-$ mice; pair-housed nNOS $-/-$ mice spent more time in the open arms compared with single-housed nNOS $-/-$ mice ($p < 0.05$; Fig. 1A). Among single-housed mice, nNOS $-/-$ mice significantly reduced the amount of time in the open arm compared with WT mice ($p < 0.05$; Fig. 1A). Genotype and housing interacted to affect number of open arm entries ($F_{1,18} = 13.621$, $p < 0.05$; Fig. 1B). Pair-housed nNOS $-/-$ mice entered the open arms significantly more than pair-housed WT and single-housed nNOS $-/-$ mice ($p < 0.05$ in both cases; Fig. 1B). There was a significant genotype by housing environment interaction on the number of CRH-positive cells in the PVN ($F_{1,20} = 5.433$; $p < 0.05$; Figs. 2 and 3). In WT mice, pair housing significantly increased the number of CRH-positive cells ($p < 0.05$; Fig. 2). In nNOS $-/-$ mice, housing did not alter CRH-positive cells in the PVN ($p > 0.05$; Fig. 2). Among single-housed mice, nNOS $-/-$ mice had significantly more CRH-positive cells in the PVN compared with WT mice ($p < 0.05$; Fig. 2). There was no effect of housing or genotype on CRH staining in the CeA ($p > 0.05$).

Genotype and housing did not interact to affect total locomotor activity in the open field ($F_{1,20} = 0.122$, $p > 0.05$; Table 1). Neither housing environment nor genotype alone affected total locomotor activity in the open field chamber ($p > 0.05$ in both cases; Table 1).

3.2. Experiment 2

Housing condition and 3-Br-7-NI-treatment interacted to affect latency to enter an open arm ($F_{1,20} = 6.294$; $p < 0.05$; Fig. 4). Among mice implanted with empty capsules, pair-housed mice significantly increased the latency to enter an open arm compared with single-housed mice ($p < 0.05$; Fig. 4). Among pair-housed mice, treatment with 3-Br-7-NI significantly reduced the latency to enter an open arm compared with empty capsules ($p < 0.05$; Fig. 4). Pair housing reduced time in the open arms of the mice implanted with empty capsules ($p < 0.05$; Fig. 1C). Treatment with 3-Br-7-NI reversed this effect: pair-housed mice treated with 3-Br-7-NI spent signifi-

Table 1
Average beam breaks \pm S.E.M. after 30 min in the locomotor chamber

Treatment	Single	Paired
WT	4366 \pm 169	4918 \pm 473
nNOS $-/-$	4813 \pm 474	5738 \pm 669
Empty implant	4388 \pm 579	3519 \pm 290
3-Br-7-NI implant	4142 \pm 413	4318 \pm 525

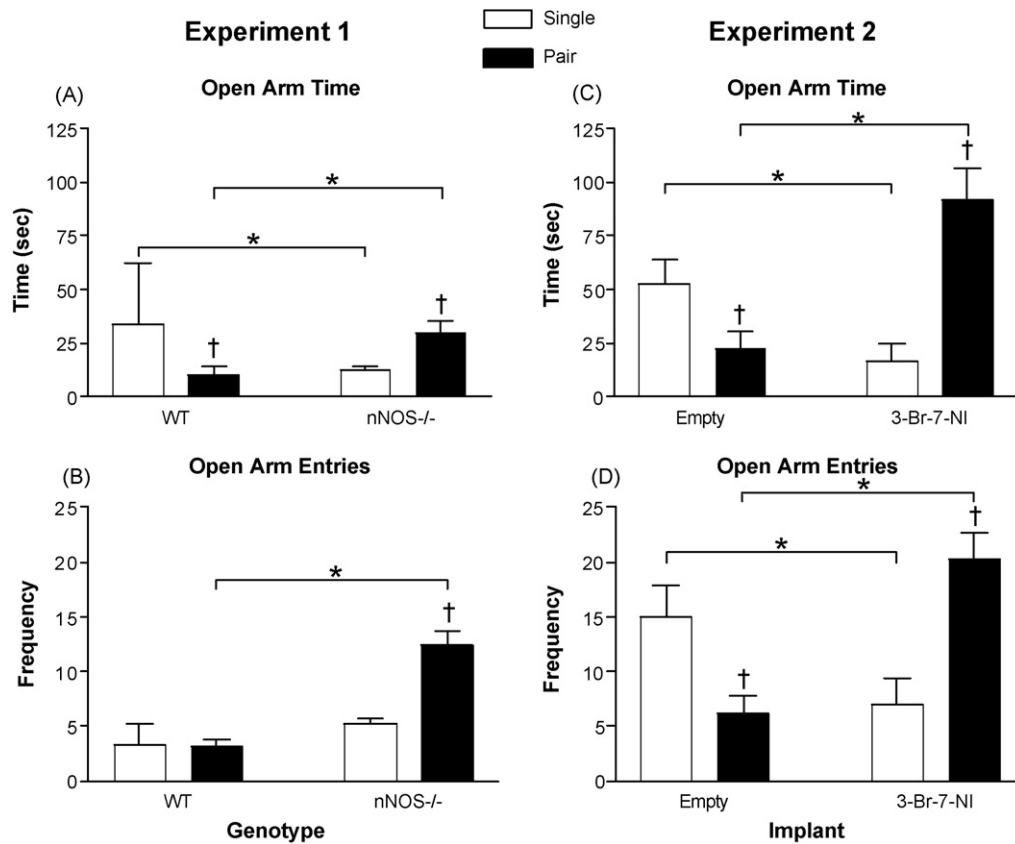


Fig. 1. Mean \pm S.E.M. time (A and C) spent in the open arms and frequency (B and D) of entries into the open arms of an elevated plus maze in nNOS $^{-/-}$ or 3-Br-7-NI-treated mice compared to wild-type or empty-capsule control animals, respectively. Open bars represent single-housed mice. Black bars represent pair-housed mice. *(Comparison within housing environment) = $p < 0.05$; †(Comparison within genotype or implant) = $p < 0.05$.

cantly more time in the open arms compared with pair-housed mice with empty capsules ($p < 0.05$; Fig. 1C). Among single-housed mice, 3-Br-7-NI-treatment reduced the time spent in the open arms ($p < 0.05$; Fig. 1C). Housing condition and treatment with 3-Br-7-NI interacted to affect number of open arm entries ($F_{1,20} = 9.858$, $p < 0.05$; Fig. 1D). Of mice with empty cap-

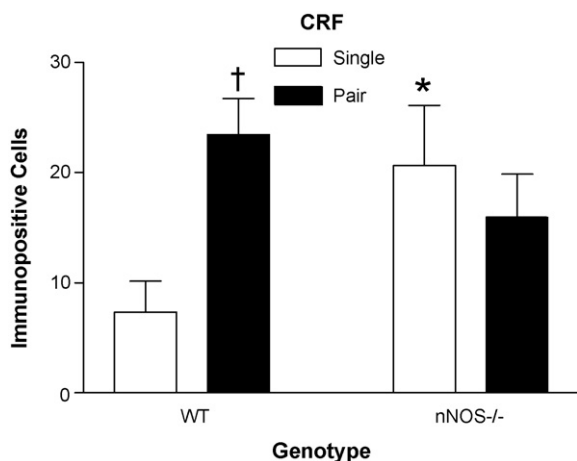


Fig. 2. Mean \pm S.E.M. of number of cells stained positively for corticotropin-releasing factor (CRH) in the paraventricular nucleus (PVN). Open bars represent single housed mice. Black bars represent pair-housed mice. *(Comparison within housing environment) = $p < 0.05$; †(Comparison within genotype) = $p < 0.05$.

sules, pair housing significantly reduced the number of open arm entries ($p < 0.05$; Fig. 1D). Treatment with 3-Br-7-NI reversed this effect: pair-housed mice with 3-Br-7-NI capsules significantly increased open arm entries compared with paired mice implanted with empty capsules ($p < 0.05$; Fig. 1D). Among single-housed mice, 3-Br-7-NI-treatment reduced the number of open arm entries ($p < 0.05$; Fig. 1D). There was a significant interaction between 3-Br-7-NI-treatment and housing environment on the number of closed arm entries ($F_{1,20} = 6.489$, $p < 0.05$). This effect was largely attributable to the difference between pair- and single-housed mice within the control group: pair-housed mice with empty capsules reduced entries into closed arms compared with single-housed mice with empty capsules ($p < 0.05$). The amount of time spent in the center of the elevated plus-maze was unaffected by 3-Br-7-NI-treatment and housing environment ($F_{1,20} = 0.975$, $p > 0.05$).

In the open field, housing environment and 3-Br-7-NI-treatment did not interact to affect total locomotor activity ($F_{1,20} = 1.413$, $p > 0.05$; Table 1). Neither housing environment nor 3-Br-7-NI-treatment alone affected total locomotor activity in the open field chamber ($p > 0.05$ in both cases; Table 1).

4. Discussion

These data indicate that nNOS inhibition alters the effect of pair housing on anxiety-like responses. WT, pair-housed

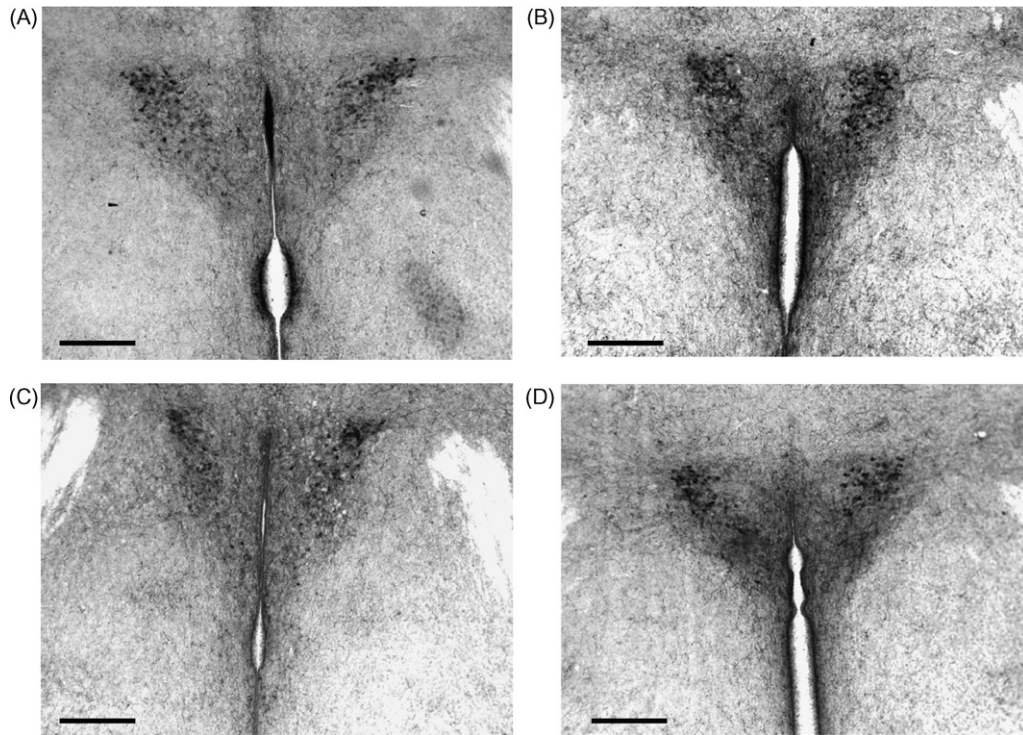


Fig. 3. Corticotropin-releasing hormone staining in the paraventricular nucleus: (A) wild-type single housed; (B) wild-type pair-housed; (C) nNOS^{-/-} single housed; (D) nNOS^{-/-} pair housed. Scale bars = 180 μm.

mice reduced open arm exploration in the elevated plus-maze. nNOS inhibition, however, reversed this effect. Further, nNOS inhibition (either by genetic or pharmacological manipulation) decreased open arm exploration of single-housed mice in the elevated plus-maze but increased open arm exploration in pair-housed mice.

In this study, pair housing increased anxiety-like responses in male mice in the elevated plus-maze without altering basal locomotor activity. This confirms previous research establishing that, in mice, social housing increases anxiety-like

responses [1,28,33,45]. This is a unique phenomenon that may be attributable to the social structure of mice. Socially housed male mice tend to form dominance hierarchies [22] which may explain the decreased exploratory behaviour of pair-housed mice. Subordinate mice tend to reduce exploratory behaviour when compared with dominant mice [34]. More study is warranted, however, to determine if reduced exploratory behaviour in paired mice is a result of social dominance hierarchies or simply a result of fighting between cage mates. One possible mechanism for this effect is an increase in CRH production, as we observed that pair housing increased the number of CRH-positive cells in the PVN (Figs. 2 and 3).

Inhibition of nNOS either by gene deletion or treatment with 3-Br-7-NI reduced open arm exploration in single-housed mice; an effect that was more pronounced in 3-Br-7-NI-treated mice than nNOS^{-/-} mice (Fig. 1). This observation suggests that NO reduces anxiety in single-housed mice. However, nNOS inhibition in pair-housed mice increases open arm exploration. Given these results, nNOS inhibition appears to play a role in the ability to respond behaviourally to social stimuli. Research on aggressive behaviours and nNOS inhibition also supports this conclusion. Typically, a supine posture establishes dominance in an agonistic encounter and will cause the attacker to discontinue. nNOS^{-/-} mice, however, do not cease attacks when the WT counterpart submits [29]. In addition, nNOS inhibition via 3-Br-7-NI reduces maternal aggression in response to an intruder [13]. Lastly, nNOS inhibition does not seem to impair the ability to identify social cues (evident in a habituation–dishabituation test). Instead, nNOS-deficient mice appear less motivated to investigate social stimuli [42]. Collectively, these data suggest

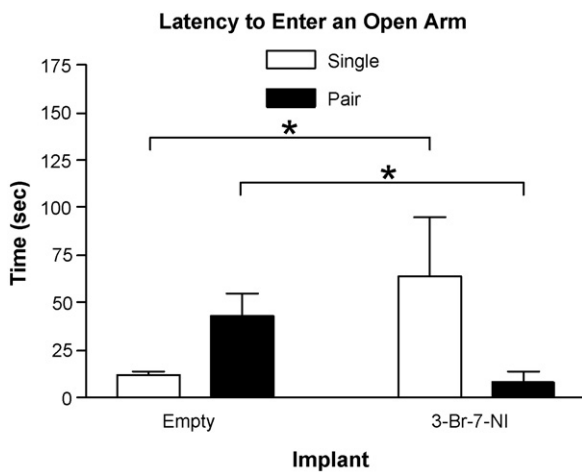


Fig. 4. Mean ± S.E.M. of latency (s) to enter an open arm in the elevated plus-maze. Open bars represent single-housed mice. Black bars represent pair-housed mice. *(Comparison within housing environment) = $p < 0.05$; †(Comparison within implant type) = $p < 0.05$.

that behavioural responses to social stimuli are impaired after nNOS inhibition.

In our study, nNOS inhibition did not simply impair the behavioural response to pair housing but had the opposite effect on behaviour. We observed that single-housed, nNOS^{-/-} mice and pair-housed, WT mice had more CRH-positive cells in the PVN relative to WT, single-housed mice (Figs. 2 and 3). This increase in CRH staining is associated with a reduction in open arm exploration. Nitric oxide is colocalized with CRH in a subset of cells in the PVN [40,50] and also modulates the release of CRH in vitro [6]. Further evidence indicates that NO can modulate the effects of CRH on exploratory behaviour. Pharmacological inhibition of nNOS reverses the behavioural effects of exogenous CRH [37]. If pair housing alters behaviour through CRH activity, then it is possible that deficient NO signaling can reverse these behaviours by altering CRH activity. Pair housing, however, did not significantly reduce CRH staining of nNOS^{-/-} mice but did significantly increase open arm exploration, indicating that CRH may not be solely responsible for altered anxiety-like responses in nNOS^{-/-} mice. Alternatively, pair housing and nNOS disruption may interact to effect serotonin which ultimately can affect anxiety-like responses. Both social housing [39] and nNOS gene deletion [5] alter serotonin turnover.

Our results from pair-housed mice are consistent with previous research indicating that nNOS inhibition is anxiolytic in group-housed male mice [35,46]. These studies used 7-NI, which is an inhibitor of nNOS and is less selective than the nNOS inhibitor we used (3-Br-7-NI). Although substantial variability exists in reports of the effects of NOS inhibitors on anxiety-like responses, the type of NOS inhibitor may explain much of the variability in the effects of NOS inhibition on anxiety-like responses. Both L-NAME and L-NOARG may alter anxiety-like behaviours though vasoconstriction or hypertension by affecting eNOS [48], whereas NOS inhibitors selective for the neuronal isoform only affect NO signaling through nNOS [3,27].

Our results suggest that future studies on the effects of nNOS on affective behaviours should carefully consider the impact of social environment. The results of this research may also provide insight into psychological disorders that are characterized by anxiety.

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