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Research report

Neuronal nitric oxide synthase and NADPH oxidase interact to affect cognitive, affective, and social behaviors in mice

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HIGHLIGHTS

- Concurrent deletion of nNOS and p47phox (NOX) alters behavior in mice.
- nNOS and NOX deletions synergize to impair cognitive function.
- Deletion of nNOS or NOX alone impairs social behavior.
- Deletion of nNOS and NOX together enhances social preference.
- Current findings may provide insight into schizophrenia and autism.

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ABSTRACT

Both nitric oxide (NO) and reactive oxygen species (ROS) generated by nNOS and NADPH oxidase (NOX), respectively, in the brain have been implicated in an array of behaviors ranging from learning and memory to social interactions. Although recent work has elucidated how these separate redox pathways regulate neural function and behavior, the interaction of these two pathways in the regulation of neural function and behavior remains unspecified. Toward this end, the p47phox subunit of NOX, and nNOS were deleted to generate double knockout mice that were used to characterize the behavioral outcomes of concurrent impairment of the NO and ROS pathways in the brain. Mice were tested in a battery of behavioral tasks to evaluate learning and memory, as well as social, affective, and cognitive behaviors. p47phox deletion did not affect depressive-like behavior, whereas nNOS deletion abolished it. Both p47phox and nNOS deletion singly reduced anxiety-like behavior, increased general locomotor activity, impaired spatial learning and memory, and impaired preference for social novelty. Deletion of both genes concurrently had synergistic effects to elevate locomotor activity, impair spatial learning and memory, and disrupt prepulse inhibition of acoustic startle. Although preference for social novelty was impaired in single knockouts, double knockout mice displayed elevated levels of preference for social novelty above that of wild type littermates. These data demonstrate that, depending upon modality, deletion of p47phox and nNOS genes have dissimilar, similar, or additive effects. The current findings provide evidence that the NOX and nNOS redox signaling cascades interact in the brain to affect both cognitive function and social behavior.

1. Introduction

Nitric oxide (NO) and reactive oxygen species (ROS), such as superoxide and hydrogen peroxide (H_2O_2), at high concentrations respectively elicit nitrosative and oxidative damage in the brain [1]. Roles for these molecules in neuronal damage following insults such as hypoxia, traumatic brain injury, and ischemia have been well established. Moreover, recent evidence has implicated dysregulation of ROS and NO signaling in neurodegenerative disorders, such as Alzheimer's disease and Parkinson's disease, and in cognitive impairments associated with normal physiological aging [1–3]. Whereas ROS and NO overproduction contribute to pathology in a stressed system independently and via mutually antagonistic chemical interactions [1,4], at lower concentrations these molecules subserve normal physiological function in a coordinate

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manner and are involved in behavioral and cognitive processes [5–8].

NADPH oxidase (NOX) is a large enzymatic complex that utilizes NADPH and oxygen to produce superoxide. NOX is widely expressed in neurons throughout the brain, where it is the primary source of ROS upon stimulation of NMDA-glutamate receptors [6,9–11]. NMDA receptor-dependent synaptic plasticity is critical for learning and memory, and both pharmacological and genetic disruption of NOX subunits such as p47phox impairs learning and memory as well as hippocampal LTP [12], the form of synaptic plasticity thought to underly memory formation [13]. Indeed, superoxide production by NOX is required for NMDA receptor-dependent activation of the ERK signaling cascade and LTP in the hippocampus, while NO also contributes to ERK activation [14,15].

Neuronal nitric oxide synthase (nNOS) is widely expressed in neurons of the brain [16] where it uses oxygen, NADPH and arginine to generate NO [17]. NO signals by activating guanylyl cyclase or modifying proteins via S-nitrosylation [7,8]. Pharmacological inhibition or genetic deletion of nNOS leads to altered social behavior, impaired hippocampal-dependent learning and memory, and impaired cerebellar-dependent motor behavior [18–21].

Thus, while nNOS and NOX are separate enzymes, they are analogous, as both are NADPH- and oxygen-dependent, both are activated by NMDA receptor stimulation, and both can be controlled in a coordinate manner. Work described above has elucidated independent influences of these enzymes and their respective redox pathways upon neural function and behavior, however their neural/behavioral interactions remain unspecified. Toward this end, p47phox–nNOS double knockout mice were generated and contrasted with single knockouts in tasks to evaluate learning and memory, as well as social, affective, and cognitive behaviors.

2. Materials and methods

2.1. Animals

Homozygous double knockout mice of p47phox and nNOS genes were generated by crossing p47phox homozygous knockout mice on C57BL/6 background (The Jackson Laboratory, Bar Harbor, Maine) with nNOS homozygous knockout mice on C57BL/6 background [22]. The double heterozygotes (p47phox+/−, nNOS+/−) resulting from the first mating were bred together several times to generate homozygous double knockouts of both p47phox and nNOS (p47phox−/−, nNOS−/−). The nNOS and p47phox single knockouts used in experiments were age-matched to the wild-type and double knockout mice. All animals were housed, bred and maintained in adherence to the Johns Hopkins University Animal Care and Use Committee.

Young adult mice were transferred from The Johns Hopkins University to The Ohio State University and, after clearing import quarantine, were acclimated to the vivarium room for at least 2 weeks prior to behavioral testing. Mice were group housed in standard laboratory cages (32 cm × 18 cm × 14 cm), at 21 ± 4 °C, 50 ± 10% relative humidity, and given ad libitum access to filtered tap water and rodent chow (Harlan Teklad 8640, Indianapolis, IN). Mice were held in a 14:10 L:D photoperiod, with lights on at 00:00 h, and off at 14:00 h Eastern Standard Time. All care was provided by University Laboratory Animal Resource staff and all procedures were approved by the OSU IACUC and conform to guidelines established by the National Institutes of Health [23].

2.2. Verification of gene deletion

Gene deletion was verified using PCR genotyping procedures published by The Jackson Laboratory (Bar Harbor, Maine) for both p47phox and nNOS. Additionally, genotyping was re-verified by sending tail-clips of mice to Transnetyx Inc. (Cordova, TN) where real-time PCR was used to genotype (Fig. 1).

2.3. Behavioral testing

A total of 56 mice were used in separate cohorts. The first cohort comprised mixed sex and genotype and was used for affective behavior and prepulse inhibition testing. The second cohort comprised all males representing all four genotypes and was used for spatial learning and memory and social behavior testing. Barnes maze testing occurred during the middle of the light phase. However, because circadian rhythms in mice affect behavior [19,24], all other behavioral testing occurred early in the dark (active) phase and tests were conducted under very dim red background lighting. Mice were allowed to habituate to the respective behavioral testing areas for a minimum of 30 min prior to testing.

2.4. Activity in an open field

Mice were individually placed into a novel open field arena (40.5 cm × 40.5 cm), contained within sound- and light-attenuating chambers, surrounded by horizontal 16 × 16 infrared beam array (PAS, San Diego Instruments, CA). Mice were allowed to freely explore the novel arena for 30 min before being returned to their home cage. Total activity, activity in the center, and activity in the periphery (the outer 5 × 5 beams) were recorded.

2.5. Elevated plus maze

Mice were introduced to the center of the elevated plus maze consisting of 2 open arms (10 cm × 50 cm) and two closed arms (10 cm × 50 cm × 40 cm) elevated 40 cm above the floor (Stoelting). Mice were allowed to freely explore the maze for 5 min. Behavior was recorded to video and scored later for time in each arm by an observer, blinded to genotype of the mice, using commercially available software (The Observer, Noldus, Leesburg, VA).

2.6. Light/dark preference

Early in the dark phase, mice were placed into the lighted area of a light/dark box (Stoelting, Wood Dale, IL), allowed to freely explore the apparatus for a 5 min testing period, which was recorded on video. Video was later scored for time in light, time in dark, and transitions by an observer using The Observer software as above.

2.7. Forced swim

Depressive-like behavior was evaluated using a single forced swim trial [25] as previously described [26]. Briefly, mice were placed into a cylinder filled 30 cm with water (29 °C) for 5 min. Video recordings of behavior were later scored for time swimming and time floating using The Observer software as above.

2.8. Acoustic startle and prepulse inhibition

Startle reactivity was measured using a single startle chamber (SR-Lab, San Diego Instruments, San Diego, CA). Early in the dark phase, mice were exposed to a continuous background noise of 65 dB for a 10 min acclimation period. A series of acoustic stimuli were given in semi-random order consisting of startle trials (pulse alone), pre-pulse trials (pre-pulse + pulse), and no-stimulus trials (no-stim), with 12–30 s inter-trial intervals. The acoustic pre-pulse intensities were 69, 73, and 81 dB. Pre-pulse inhibition was measured by pre-pulse + pulse trials that consist of a 20 ms pre-pulse, 100 ms delay, then a 40 ms 120 dB startle pulse. Percent of pre-pulse inhibition (PPI) was determined using the equation: %PPI = [(pulse only startle amplitude − prepulse startle amplitude)/pulse only startle amplitude] × 100.

2.9. Barnes maze

Evaluation of hippocampus-dependent spatial learning and memory was evaluated using the Barnes maze [27] as previously described [28]. Briefly, in the middle of the light phase each mouse was trained on the Barnes maze (for mouse, San Diego Instruments, CA) across six days (3 trials/day, 120 s max/trial) to find the location of the escape box using conspicuous extramaze visual cues placed around the testing room. Latency to escape, error, and path length data were collected using commercially available video tracking software (HVS Image 2100, HVS Image Labs, Buckingham, UK).

2.10. Three chamber social test

Prior to social testing, all mice were individually housed for a minimum of 10 days. The three chamber social test was performed using male mice as previously described [29]. Briefly, the test consists of three phases: habituation, sociability, and preference for social novelty [30]. The apparatus consisted of a 3-chambered polycarbonate box (62 cm *L* × 40 cm *W* × 20 cm *H*) divided into 3 equal chambers, with small cages (8.5 cm diameter × 10 cm *H*) placed into the two outer chambers. Early in the dark phase, under dim red light, mice were introduced into the center chamber and allowed to freely explore the three chambers for a 10 min habituation period. A same-sex wild type (WT) stimulus was placed in the cage in one of the outer chambers, and the experimental mouse was allowed to freely explore the apparatus and approach the stimulus mouse (sociability). After 10 min, another stimulus mouse was introduced into the other outer chamber, and the experimental mouse was allowed to interact with both the newly introduced stimulus (novel mouse) and previously introduced stimulus (familiar mouse) for an additional 10 min for the preference for social novelty test. For the sociability test, a greater amount of time interacting with the cage containing the stimulus mouse than the empty cage is interpreted as prosocial behavior, and in the latter test, greater interaction time with the novel stimulus compared to the familiar stimulus is interpreted as a preference for social novelty.

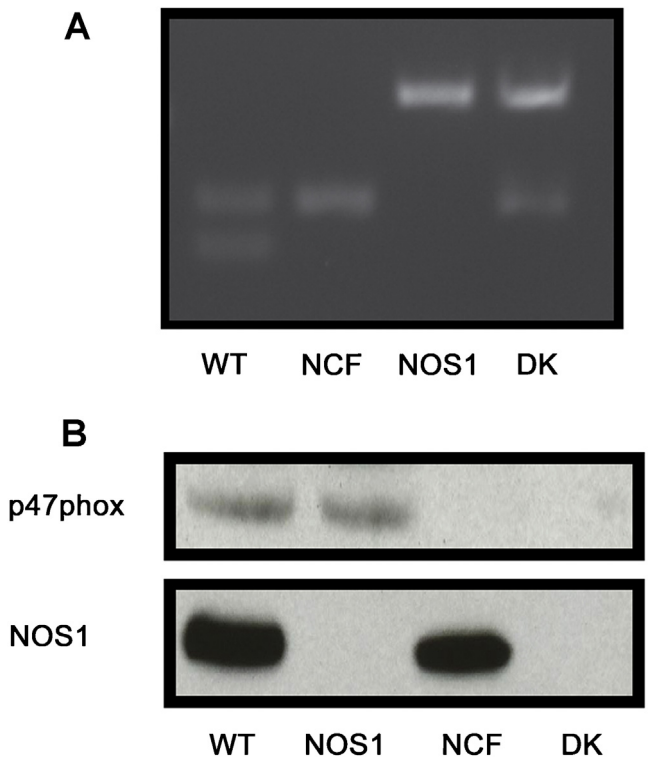

Fig. 1. Verification of gene deletion. Representative image of PCR-based genotyping using DNA samples isolated from tail-clips of animals as marked (A). Representative image of western-blot derived from protein extracts of brain homogenates analyzed using antibodies against p47phox and neuronal NOS (B). WT, NCF, NOS1, and DK refer to wild-type, p47phox knockout, neuronal NOS knockout, and double knockout of p47phox and neuronal NOS, respectively.

2.11. Social behavior in a neutral arena

After completion of three-chamber test, mice were tested for social interaction with a novel untethered same-sex stimulus mouse in a neutral arena. The experimental and the stimulus mouse were simultaneously introduced into opposite corners of a novel enclosure (42 cm L × 22 cm W × 20 cm H) and allowed to freely interact for 5 min. Video recordings were later scored for social behaviors (direct interaction and ano-genital investigation) using The Observer software as above.

2.12. Statistics

All data were analyzed with SPSS statistics software (v18, IBM, Armonk, NY) using 2 × 2 ANOVA with genotype and sex as factors. Significant main effects or interactions were followed by LSD post hoc analyses. For tests containing data from both sexes, no main effects of sex were found, or interactions of sex with gene deletion, thus sexes were collapsed for analysis to increase statistical power. Data with unequal variance were log transformed prior to analysis. Statistical outliers (>2.5 SD from the overall mean) were excluded from analysis ($n = 2$ for PPI, $n = 2$ for Barnes maze). A $p \leq 0.05$ was considered statistically significant [31].

3. Results

3.1. Sensorimotor

Sensorimotor screening of the mice prior to behavioral testing identified no deficits in sensory ability (visual, olfactory, tactile), or reflexive responses to stimuli (data not shown). Auditory testing is integral to the PPI test detailed below.

3.2. Open field

Deletion of either nNOS ($F_{1,3} = 14.731$, $p < 0.05$) or p47phox ($F_{1,3} = 8.376$, $p < 0.05$) increased general activity in the open field, and deletion of both genes had an additive effect, with p47phox-nNOS KO mice having increased activity over both p47phox ($p < 0.05$) and nNOS ($p = 0.06$) konckouts (Fig. 2). Deletion of p47phox, nNOS, or both together, decreased rearing activity, with the nNOS knockouts showing the lowest levels (Fig. 2B). Deletion of p47phox did not affect anxiety as measured by central tendency in the open field ($F_{1,3} = 1.093$, $p > 0.05$), whereas deletion of nNOS reduced anxiety-like behavior ($F_{1,3} = 12.623$, $p < 0.05$; Fig. 2C).

3.3. Elevated plus maze

Deletion of the p47phox gene decreased anxiety-like behavior (time in open arms) in the elevated plus maze (EPM, $F_{1,3} = 7.458$, $p < 0.05$), and there was an interaction of p47phox and nNOS deletion on this behavior ($F_{1,3} = 9.288$, $p < 0.05$). Compared to WT mice, all genotypes had decreased anxiety-like behavior in the EPM (Fig. 2D).

3.4. Light/dark box

Deletion of p47phox similarly decreased anxiety-like behavior in the light/dark box ($F_{1,3} = 5.558$, $p < 0.05$), whereas nNOS deletion had a marginal effect on anxiety-like behavior ($F_{1,3} = 2.878$, $p = 0.10$; Fig. 2E).

3.5. Porsolt forced swim

Deletion of nNOS abolished depressive-like responses in the forced swim test ($F_{1,3} = 18.504$, $p < 0.05$), whereas deletion of p47phox had no effect on this behavior ($F_{1,3} = 0.329$, $p > 0.05$; Fig. 2F).

3.6. Prepulse inhibition of acoustic startle

There were no differences due to genotype on startle responses during the 120 dB pulse alone trials, indicating that all mice had similar hearing sensitivity and an intact acoustic startle reflex ($p > 0.05$, data not shown). Deletion of nNOS impaired PPI at all intensities (69 dB, $F_{1,3} = 16.337$, $p < 0.05$; 73 dB, $F_{1,3} = 25.439$, $p < 0.05$; 81 dB, $F_{1,3} = 55.938$, $p < 0.05$), whereas p47phox deletion only impaired PPI at the two highest intensities (69 dB, $F_{1,3} = 0.374$, $p > 0.05$; 73 dB, $F_{1,3} = 7.093$, $p < 0.05$; 81 dB, $F_{1,3} = 8.877$, $p < 0.05$; Fig. 3). Additionally, at the two highest prepulse intensities, nNOS and p47phox had an additive effect on PPI impairment (Fig. 3).

3.7. Spatial learining and memory

Deletion of either nNOS or p47phox impaired spatial learning and memory in the Barnes maze, as measured by increased latency to learn the location of the escape box (nNOS $F_{1,12} = 21.978$, $p < 0.05$; p47phox $F_{1,12} = 5.889$, $p < 0.05$), and follow-up within-day comparisons showed the effects of these genes on spatial memory impairment were additive (Fig. 4A). Deletion of nNOS had no effects on errors ($F_{1,12} = 0.263$, $p > 0.05$; Fig. 4B) or path length ($F_{1,12} = 0.066$, $p > 0.05$; Fig. 4C), whereas deletion of p47phox increased both errors ($F_{1,12} = 12.096$, $p < 0.05$; Fig. 4B) and path length ($F_{1,12} = 12.096$, $p < 0.05$; Fig. 4C).

3.8. Social behaviors

Regardless of genotype, all mice displayed positive sociability by spending more time in the chamber containing the stimulus mouse (Fig. 5A) and by spending more time directly investigating (sniffing) the cage containing the stimulus mouse (Fig. 5B) in the three-chamber social test ($p < 0.05$ for all measures). There were main effects of both nNOS ($F_{1,3} = 5.034$, $p < 0.05$) and p47phox ($F_{1,3} = 4.908$, $p < 0.05$), and an interaction of nNOS and p47phox ($F_{1,3} = 12.966$, $p < 0.05$; Fig. 5C), on chamber preference during the

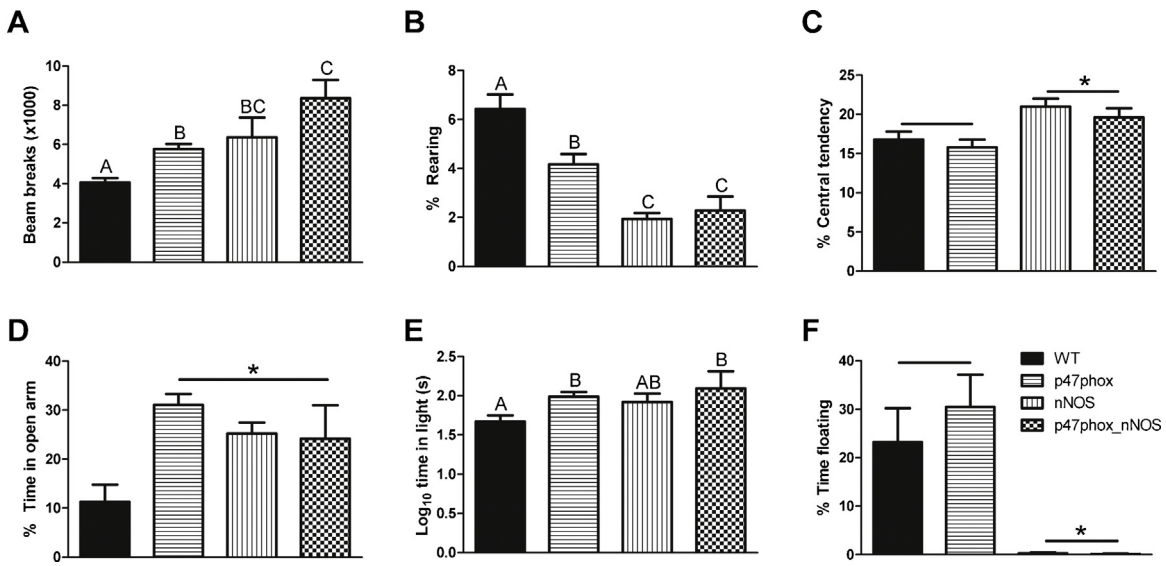

Fig. 2. The effects of nNOS and p47phox knockout on affective behaviors. Deletion of nNOS and p47phox increased locomotor activity in an additive manner in an open field arena (A). Deletion of nNOS and p47phox reduced rearing in the open field, with nNOS having the greatest effect (B). nNOS deletion reduced anxiety-like behavior in the open field (C), elevated plus maze (D), and light–dark box. Deletion of p47phox reduced anxiety-like behavior in the elevated plus maze (D) and light–dark box (E), but did not affect anxiety in an open field (C). p47phox deletion did not affect depressive-like behavior in the Porsolt test, whereas nNOS deletion abolished depressive-like floating behavior (F). Shared letters indicate no significant difference in LSD post hoc analysis. $^{*}p < 0.05$, 2 × 2 ANOVA. WT $n = 9$, p47phox $n = 8$, nNOS $n = 10$, p47phox_nNOS $n = 4$.

preference for social novelty test. Both nNOS and p47phox knockout mice did not show a preference for social novelty, whereas double knockout mice had an enhanced preference for social novelty compared to WT mice ($p < 0.05$; Fig. 5C). A similar pattern (interaction of nNOS and p47phox: $F_{1,3} = 16.624$, $p < 0.05$) was found for direct investigation of the cage containing the stimulus mouse, where only WT and double knockout mice showed a preference for the cage containing the novel stimulus mouse ($p < 0.05$; Fig. 5D). During the neutral arena test, no attacks, biting, or boxing were observed in any genotype. Deletion of nNOS reduced total time of direct investigation of the stimulus mouse ($F_{1,3} = 6.406$, $p < 0.05$; not shown) and ano-genital sniffing ($F_{1,3} = 8.487$, $p < 0.05$; not shown), whereas deletion of p47phox increased investigation time ($F_{1,3} = 6.233$, $p < 0.05$; not shown) without affecting anogenital sniffing ($F_{1,3} = 0.065$, $p > 0.05$; not shown).

4. Discussion

Reactive oxygen species and nitric oxide at physiological levels have important roles in both neuronal signaling and physiological function more broadly. Selective disruption of NOX and nNOS signaling by targeted gene mutations alters behavior; however, concurrent disruption of both of these redox pathways affects an array of behaviors in either a synergistic or diametric manner. Concurrent deletion of nNOS and p47phox synergized to increase impairments in cognitive function, whereas concurrent deletion of these genes had a diametric effect on social behavior when compared to deletion of each gene alone. These findings may provide insight into how the crosstalk between these two pathways in the brain influences cognitive and social disorders, as detailed below.

4.1. General activity and affective behaviors

Consistent with previous findings [12,21,32], in a novel open field, both nNOS and p47phox knockout mice display increased horizontal locomotor activity, with a decrease in vertical activity. In double knockouts both nNOS and NOX deletion similarly impaired rearing, whereas the gene deletions had an additive effect on horizontal activity, with p47phox–nNOS double knockouts showing the highest activity levels (Fig. 2A). Also in alignment with previous reports [21,32,33], nNOS deletion reduced anxiety-like behavior in the open field (Fig. 2C). The anxiolytic effects of this gene deletion were also present in two other measures of anxiety: the elevated plus maze (Fig. 2D) and the light–dark box (Fig. 2E). Similarly,

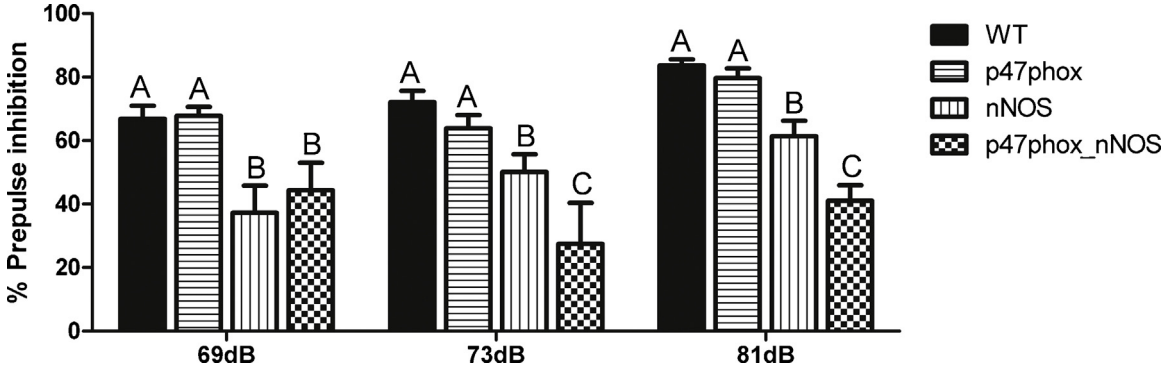

Fig. 3. Prepulse inhibition of acoustic startle. Deletion of nNOS impaired prepulse inhibition of acoustic startle at all prepulse intensities tested. p47phox deletion alone did not affect PPI at any intensity, however at the highest intensities, p47phox deletion had an additive effect with nNOS on inhibition of PPI. Shared letters indicate no significant difference. $^{*}p < 0.05$. WT $n = 9$, p47phox $n = 8$, nNOS $n = 10$, p47phox_nNOS $n = 4$.

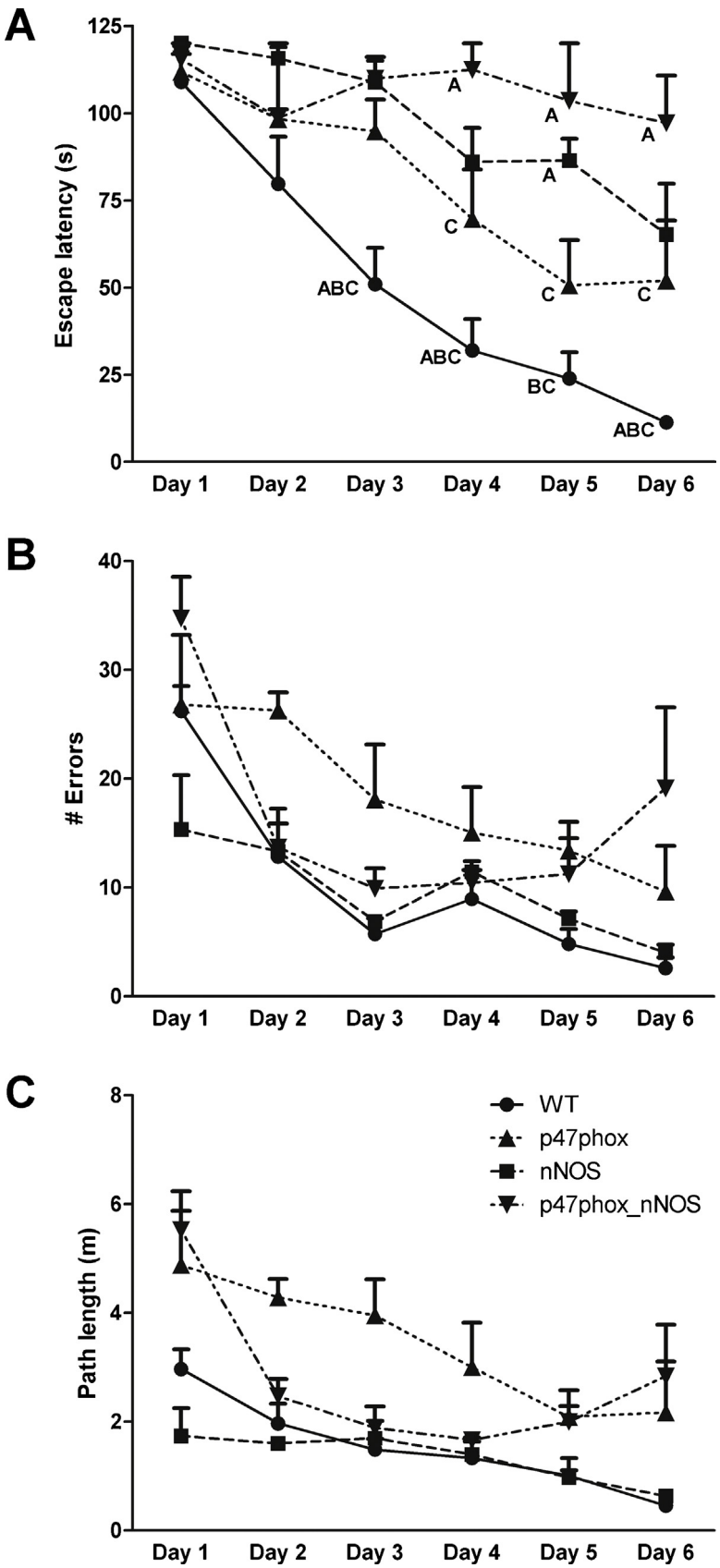

Fig. 4. Spatial learning and memory in the Barnes maze. p47phox and nNOS deletion impair spatial learning and memory in an additive manner by increasing escape latency across training days in the Barnes maze (A). Only p47phox deletion increased the number of errors (B) and path length (C) prior to escape in the Barnes maze. Letters indicate: A – different from p47phox, B – different from nNOS, C – different from p47phox_nNOS. WT $n = 5$, p47phox $n = 5$, nNOS $n = 3$, p47phox_nNOS $n = 3$.

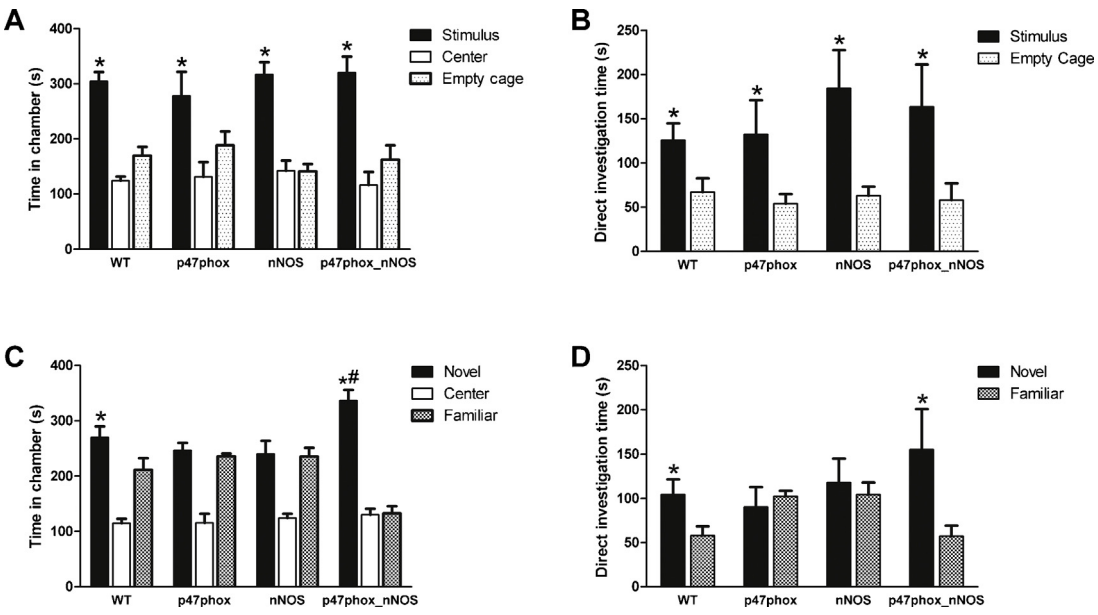

Fig. 5. Sociability and preference for social novelty. Gene deletion had no effect on sociability (A, B) in the three chamber social test. However, deletion of p47phox or nNOS singly abolished preference for social novelty (C, D), whereas deletion of both nNOS and p47phox together not only recovered preference for social novelty (C,D), the gene deletions interacted to increase the preference for social novelty above that of WT mice (C). $^{*}p < 0.05$. WT $n = 6$, p47phox $n = 5$, nNOS $n = 6$, p47phox_nNOS $n = 5$.

p47phox deletion was anxiolytic in both the plus maze (Fig. 2D) and the light–dark box (Fig. 2E). Consistent with a previous report [12], p47phox deletion did not affect anxiety-like behavior in the open field (Fig. 2C). Double knockout mice displayed fewer anxiety like responses than WT mice in all three tests of anxiety (Fig. 2C–E).

Whereas p47phox deletion influenced anxiety-like behavior, it did not affect depressive-like behavior in the Porsolt test (Fig. 2F). Kishida et al. (2006) also reported that depressive-like responses in the tail suspension test were not altered upon loss of NOX activity. A role for NOX in depressive-like behavior has been identified; however, it is driven by stress-induced upregulation of NOX [34], and we did not stress mice via restraint in the current study. Independent of NOX genotype, nNOS deletion abolished all depressive-like behavior (floating), inducing a hyperactive manic-like state in the Porsolt test (Fig. 2F). Although NO has been implicated in bipolar disorder (BD), especially in the manic phase (ME), its specific role is unclear. Plasma NO levels generally are elevated in BD–ME patients [35,36] but see [37], however plasma NO measurements do not accurately reflect central NO levels [38], and nNOS neuron numbers are reduced in post-mortem human brains from BD and schizophrenic patients [39].

Both nNOS and NOX can interact with dopaminergic (DA) and serotonergic (5HT) systems to affect depressive-like and anxiety-like behaviors. Nitric oxide alters both DA and 5HT signaling [40], NOX is expressed in mesolimbic DA neurons [41], and nNOS is expressed in dorsal raphe 5HT neurons [42]. Increased striatal dopamine D1 signaling in nNOS KO mice is associated with decreased depressive- and anxiety-like behavior [21], and 5HT interacts with hippocampal nNOS to alter anxiolytic behavior [33]. However, the specific roles of NOX in anxiety-like behavior and nNOS in depressive-like behavior remain largely undescribed.

4.2. Prepulse inhibition of acoustic startle

Deficits in sensorimotor gating measured via PPI are a hallmark of schizophrenia [43,44], and both nNOS and dopamine have roles in this disease [45,46]. Although no baseline differences were reported in previous studies using nNOS knockout mice, dopamine receptor agonists such as phencyclidine [47] and SKF81297 [21] impair PPI in nNOS knockout mice but not in WT mice. Our current findings, that nNOS knockout mice have impaired PPI (Fig. 3), appear to contradict these previous reports. However, both the Klamer and Tanda studies performed their behavioral experiments during the light phase, whereas our behavior was performed in the dark phase. Testing during the dark (active) phase instead of the light phase has previously unmasked behavioral differences in nNOS mutant mice [19]. Furthermore, mesolimbic DA levels fluctuate in a circadian manner, with highest levels occurring during the dark phase [48,49]. Thus, elevated endogenous DA levels, associated with circadian phase changes, may have driven the PPI impairment found in the current study, whereas the previous studies required exogenous DA, in the form of agonists, to reveal the PPI impairment in nNOS knockouts.

Similar to the effects of nNOS knockout, deletion of p47phox impaired PPI at the two highest prepulse intensities, and concurrent deletion of p47phox and nNOS had additive effects on PPI impairment at all prepulse intensities (Fig. 3). How these two genes interact in an additive manner to affect PPI responses is currently unknown. However, these schizophrenia-like impairments could arise via convergent influence of both signaling pathways upon the DA system in brain regions implicated in the pathophysiology of schizophrenia, such as the medial prefrontal cortex (mPFC), amygdala, striatum, nucleus accumbens, and hippocampus [46].

4.3. Spatial learning and memory in the Barnes maze

Confirming previous reports, deletion of nNOS [21,50] or p47phox [12] in our study impaired visually-cued spatial learning and memory (Fig. 4). Moreover, concurrent knockout of nNOS and p47phox exerted an additive effect on the impairment of hippocampal learning and memory (Fig. 4). Spatial learning and memory in the hippocampus is dependent upon synaptic plasticity [51–53]. NOX is expressed in the hippocampus [10,11], and both pharmacological inhibition and genetic interruption of NOX signaling in the hippocampus disrupt LTP [12]. Pharmacological inhibition of NO production in the hippocampus, where nNOS is widely expressed [16] also impairs LTP [54]. However, both nNOS and eNOS contribute to the role of NO in hippocampal LTP

[55]. Deletion of nNOS may impair LTP by depressing protein S-nitrosylation pathways that regulate AMPA receptor (AMPAR) function [56,57]. Loss of NOX might similarly impair AMPAR function. Because nNOS and NOX pathways can signal in a coordinate manner [5], the regulation of AMPARs by NMDA-mediated activation of NOS and NOX affords a mechanism whereby NOS and NOX additively impact synaptic plasticity.

4.4. Social behaviors relevant to autism

The relationship between nNOS and aggressive behavior in male mice is well established [18,20,58]. However, aggression can be dissociated from other social interactions by tethering the stimulus mouse behind a barrier to limit direct interactions and to give greater control of the interaction to the experimental mouse [59], which is inherent to the design of the three-chamber social test [30] utilized in the current study. Independent of genotype, all mice demonstrated similar (normal) levels of sociability as measured by time in chamber and time investigating the stimulus mouse (Fig. 5A and B). However, deletion of either p47phox or nNOS singly impaired the preference for social novelty (Fig. 5C and D). A recent study [21] showed that, when compared to WT, nNOS−/− mice had reduced sociability (yet remained sociable) and had no preference for social novelty. Although the current results are roughly similar, the expected preference for social novelty in WT mice was not seen in the Tanda study [30], which may reflect differences in testing environment or circadian variations described above as their tests were performed in the light phase. However, it has been reported that circadian phase does not impact social behaviors in some strains of mice [60].

To our knowledge, the current data are the first to report social behaviors relevant to autism in p47phox knockout mice. Deletion of p47phox did not affect sociability but diminished the preference for social novelty. By contrast, deletion of both genes concurrently restored the preference for social novelty with the double knockout mice displaying enhanced preference for social novelty compared to WT mice (Fig. 5C and D). Among the main clinical symptoms of autism are impairments in reciprocal social interaction and social communication [61]. Based on recent behavioral studies, the BTBR mouse strain has emerged as the leading candidate to model autism [62]. Although the underlying neuroanatomical and neurochemical bases of autism spectrum disorder remain elusive, it is likely polygenic and results from complex gene–environment interactions in multiple brain regions [63,64]. The current results identify two candidate genes, nNOS and p47phox, which affect social behaviors relevant to autism. More importantly, because p47phox–nNOS double knockout mice display social behaviors diametrically opposed to the respective single knockouts, they may provide a unique opportunity to investigate the neural and biochemical substrates of autism-like behavior.

4.5. Conclusion and future directions

Targeted gene mutations concurrently disrupting both NOX and nNOS redox signaling alter behavior in either a synergistic or diametric manner. Concurrent deletion of nNOS and p47phox synergize to impair cognitive function relevant to schizophrenia (PPI and hippocampal function). On the other hand, social behaviors relevant to autism (preference for social novelty) in double knockouts are altered diametrically when compared to deletion of each gene alone. The present findings may provide insight into how the crosstalk between these two pathways influences cognitive and social disorders.

Whereas the current study was designed to behaviorally assess the interaction of these two pathways, a biochemical analysis of the various brain regions implicated in the behaviors described above will be necessary to understand the interaction of these two pathways at the molecular level. As mentioned, NO can affect cell signaling by either activating the guanylyl cyclase cascade or via altering S-nitrosylation of target proteins [7,8,56,57,65], and NOX can affect cell signaling by creating superoxide, which upon rapid dismutation to hydrogen peroxide by superoxide dismutase, can directly and reversibly modify cysteine residues of target proteins (S-sulfenylation). While S-sulfenyl groups in proteins are unstable, they can rapidly form disulfides or sulfenamides or at higher concentrations of peroxide might be further oxidized to the irreversible sulfinic and sulfonic acid derivatives [66]. Alternatively, NOX can be regulated by S-nitrosylation [67,68]. Both NO and ROS can signal independently or in a coordinate manner dependent on the temporal dynamics of NOS and NOX activation, and dependent on whether they act on the same or different cysteine residue of target proteins [5]. The ability of NO and ROS to activate or inactivate a protein adds to the complexity of determining the physiological outcome of the signaling cascades they regulate [69,70]. Thus, it will be important to determine brain region-specific protein nitrosylation and sulfenylation states, and their corresponding functional consequences in order to assess how these two pathways may interact at the molecular level to affect behavior in either a convergent (additive) or diametric manner as described in this study.

Conflict of interest

The authors declare no conflicts of interest.

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