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Working Memory Deficits in Neuronal Nitric Oxide Synthase Knockout Mice: Potential Impairments in Prefrontal Cortex Mediated Cognitive Function

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Abstract

Neuronal nitric oxide synthase (nNOS) forms nitric oxide (NO), which functions as a signaling molecule via *S*-nitrosylation of various proteins and regulation of soluble guanylate cyclase (cGC)/cyclic guanosine monophosphate (cGMP) pathway in the central nervous system. nNOS signaling regulates diverse cellular processes during brain development and molecular mechanisms required for higher brain function. Human genetics have identified *nNOS* and several downstream effectors of nNOS as risk genes for schizophrenia. Besides the disease itself, *nNOS* has also been associated with prefrontal cortical functioning, including cognition, of which disturbances are a core feature of schizophrenia. Although mice with genetic deletion of *nNOS* display various behavioral deficits, no studies have investigated prefrontal cortex-associated behaviors. Here, we report that nNOS knockout (KO) mice exhibit hyperactivity and impairments in contextual fear conditioning, results consistent with previous reports. nNOS KO mice also display mild impairments in object recognition memory. Most importantly, we report for the first time working memory deficits, potential impairments in prefrontal cortex mediated cognitive function in nNOS KO mice. Furthermore, we demonstrate Disrupted-in-Schizophrenia 1 (DISC1), another genetic risk factor for schizophrenia that plays roles for cortical development and prefrontal cortex functioning, including working memory, is a novel protein binding partner of nNOS in the developing cerebral cortex. Of note, genetic deletion of nNOS appears to increase the binding of DISC1 to NDEL1, regulating neurite outgrowth as previously reported. These results suggest that nNOS KO mice are useful tools in studying the role of nNOS signaling in cortical development and prefrontal cortical functioning.

Keywords

nNOS; DISC1; working memory; cognition; prefrontal cortex; schizophrenia

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1. Introduction

Cognition is an important higher brain function that controls behavioral outcomes. Many brain regions, including the prefrontal cortex, have been implicated in mediating key cognitive processes. Disturbances in such areas ultimately result in a wide range of cognitive deficits, which have been frequently reported as core features of neuropsychiatric disorders, such as schizophrenia. Thus, understanding cognitive function at the molecular, circuit, and behavioral levels becomes extremely valuable when studying pathophysiological mechanisms of neuropsychiatric conditions.

The association of genetic risk factors for major mental disorders and cognition has been widely examined in human studies [1,2]. For example, neuronal nitric oxide synthase (nNOS), a risk gene for schizophrenia [3,4], has been associated with prefrontal cortical functioning, including cognition, as assessed by neuropsychological testing in patients with schizophrenia as well as healthy subjects [4,5]. These results suggest the involvement of nNOS not only in the etiopathogenesis of schizophrenia, but with specific cognitive phenotypic domains. Additionally, many rare structural variants in multiple genes in NOS signaling are disrupted in patients with schizophrenia [6]. Several downstream effectors in nNOS signaling, such as CAPON and serine racemase [7,8], have also been reportedly associated with schizophrenia [9,10].

NO, a gaseous neurotransmitter produced by nNOS, is an essential messenger in diverse developmental processes involved in neural circuit formation, ranging from the refinement of axonal projections to the regulation of dendrite and spine morphologies [11,12]. Consistent with its roles during brain development, nNOS is highly expressed in the developing cerebral cortex, whereas its expression is diminished to mainly a subset of interneurons at adult stages [13]. A major mechanism of action of NO is S-nitrosylation on cysteine residues of target proteins, resulting in significant conformational changes that affect their functional activity [14]. Another critical route for NO-mediated neuronal processes is the activation of soluble guanylate cyclase (sGC) to catalyze the production of cyclic guanosine monophosphate (cGMP), a second messenger for many cellular processes, including axon/dendrite growth and synaptogenesis [15,16]. Several phosphodiesterases (PDE), enzymes that regulate the levels of cGMP, have been genetically associated with schizophrenia [17]. Nonetheless, even though such a variety of nNOS related functions have been studied at the molecular levels, their impact on behaviors remains to be elucidated.

In this regard, genetic deletion of nNOS in animal models is a useful tool in studying the role of nNOS in brain function and behavior [18]. nNOS knockout (KO) mice have been characterized by an increase in aggressive behavior and sexual behavior, as well as hyperactivity and abnormal social behavior [19,20]. No consistent results have been reported in anxiety-related behaviors as some studies suggest increased anxiety levels [21], while others stress anxiolytic-like phenotypes [22]. Contextual fear conditioning has been reported to be significantly impaired [23]. Impairments in cognitive functions, such as spatial reference and spatial working memory, have been reported [20,21,24]. Nonetheless, cognitive functions dependent upon the integrity of prefrontal cortical regions remain to be studied.

In this study, we characterized cognition, including prefrontal cortex mediated behaviors in nNOS KO mice. Most importantly, we found working memory deficits in nNOS KO mice. Furthermore, we demonstrated Disrupted-in-Schizophrenia 1 (DISC1), another genetic risk factor for schizophrenia that plays roles for cortical development and prefrontal cortex functioning [25–27], including working memory, is a novel protein binding partner of nNOS

in the developing cerebral cortex. Moreover, we found that nNOS influences the interaction of DISC1 and nuclear distribution element-like 1 (NDEL1), interaction which regulates neurite outgrowth as previously reported [28,29]. Our findings implicate nNOS signaling in cortical development and potential prefrontal cortical functioning.

2. Materials and Methods

2.1. Animals

Mice with a deletion of the *nNOS* gene (nNOS KO mice), were generated and backcrossed onto the C57BL/6 background as previously described [18,30]. Mice were housed in groups of five under controlled conditions of temperature and humidity and access to food and water available ad libitum. Genotyping was done by polymerase chain reaction (PCR) of genomic DNA from tail using specific primers for the *nNOS* gene. Homozygous nNOS KO and wild-type male mice were raised to the age of 6 months and subjected to behavioral analysis. Behavioral experiments were performed in the Behavioral Facility at Johns Hopkins University and were conducted according to the University's Animal Care and Use Committee's guidelines.

2.2. Behavioral tests

Behavioral tests were conducted on male mice housed with a 12 hr light/dark cycle. Tests were conducted during the light period of the cycle. All male mice were subjected to the behavioral tests in the following order: open field test, novel object recognition test, fear conditioning test, Y-maze test, and delayed non-matching to place T maze test. The interval between different behavioral tests was one week. Each apparatus was cleaned with 70% ethanol between individual animals to control for odor cues. For detailed procedures of Open-field test, Y-maze test, Novel object recognition (NOR) test, and Delayed non-matching to place (DNMTP) task, see supplementary information.

NOR test—The NOR test was performed following our published protocol [25]. Briefly, mice were placed in an open field arena and allowed to explore testing environment for 3 consecutive days. On day 4, mice were placed in the same apparatus and allowed to explore two objects. On day 5, mice were placed in the apparatus containing the now familiar object as well as a novel object and the time spent exploring each object was recorded. After 30 min, mice were tested in the spatial component of novel object recognition test where one of the objects now occupied a novel location in the arena. The amount of time spent exploring novel location was recorded.

DNMTP task in T maze—The DNMTP test was performed in the T maze following our published method [25] with modifications. Briefly, 10% sucrose loaded in 2 opposing goal arms was used as reward. Each mouse was habituated to T-maze (2 discrete 10 min trials per day for 3 consecutive days). For training, each mouse was given a forced run (the sample) to one arm and then, after a 5 s delay, given access to both opposing arms (the choice). Correct choices were reinforced with 10% sucrose solution reward at the end of the correct arm (10 discrete trials per day). After mice made at least 80% correct choices on 2 consecutive days, the testing phase began, where delays of 5, 30, 60, or 120 s were randomly introduced between the sample run and choice run (3 trials per delay on 4 consecutive days). Performance during training and % correct choices during testing were used as a measure of cognitive function.

2.3. Co-immunoprecipitation

For antibodies and plasmid information, see supplementary information.

Immunoprecipitation (IP) was performed as previously described [28,31]. Briefly, cells or

tissue from mice developing cerebral cortex were lysed in a RIPA buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM MgCl₂, 5 mM DTT, 1 mM PMSF, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, and protease inhibitor mixture). Supernatant fractions obtained after centrifugation at 10,000 g for 15 min were incubated with primary antibodies and protein G plus/Protein A agarose (Calbiochem, Germany). The immunoprecipitates were analyzed with SDS-PAGE, followed by western blotting. 5% of total protein lysates for IP were used as input.

2.4. Cell culture and transfection

For detailed procedures, see supplementary information.

2.5. Statistical analysis

Data are presented as means $\pm$ standard error of the mean (S.E.M.). Statistical comparisons between nNOS KO and wild-type mice were performed using two-tailed unpaired Student T-test. For DNMT1 test, repeated measures analysis of variance (ANOVA) was also used with delay interval as repeated measure factor and genotype as between subject factor. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Evaluation of body weight in nNOS KO mice

Body weight differences may reflect atypical development and could affect behavior nonspecifically. We therefore examined differences in body weight between nNOS KO and wild-type mice at 6 months of age, but observed no difference (Fig. S1, $p = 0.4336$).

3.2. Hyperactivity in nNOS KO mice

The open field test was first used to monitor locomotor activity in both a horizontal and vertical plane. nNOS KO mice were significantly more active than wild-types (Fig. 1A, $p = 0.0075$). No differences were found in rearing (Fig. 1B, $p = 0.7855$). Anxiety related behavior was also evaluated in the open field test. Since mice with reduced anxiety-like behavior tend to prefer center areas in the open field apparatus, the percentage of time spent in the center of the open field was measured. There was no significant difference in the percentage of time spent in the central area between both groups of mice (Fig. 1C, $p=0.1149$).

3.3. Impairment of recognition memory in nNOS KO mice

In order to examine cognitive functions in nNOS KO mice, we first assessed object and spatial recognition memory by the NOR test. During the training session, no difference was observed between nNOS KO and wild-type mice in total exploration time (Fig. 2A, $p = 0.9878$), suggesting no significant effect of hyperactivity on general exploratory behavior and comparable levels of motivation for the two objects used. We also found no significant difference in exploratory preference (Fig. 2B, $p = 0.7381$), indicating that both groups of mice displayed equal preference for the two objects. During the retention session, when one of the two objects was replaced by a new one, a trend was observed for reduced level of exploratory preference for the novel object in nNOS KO mice with respect to wild-types (Fig. 2B, $p=0.0738$), suggesting that nNOS KO mice exhibited a lack of net preference between novel and familiar object.

Another measure of the NOR test was used to assess spatial recognition memory. During the retention session, the level of exploratory preference for the novel location in nNOS KO

mice was significantly reduced with respect to wild-types (Fig. 2C, $p=0.0020$), revealing impairment in spatial recognition memory in nNOS KO mice.

3.4. Impairment of context-dependent fear conditioning in nNOS KO mice

We next evaluated emotional memory by conducting the fear conditioning test. During training, similar levels of freezing were displayed by nNOS KO and wild-type mice before shock exposure (Fig. S2A, $p = 0.3571$). nNOS KO mice showed significantly less freezing relative to wild-types when returned to the same environment in which they were exposed to the electrical shock (Fig. S2A, $p = 0.0185$), suggesting that context-dependent emotional information processing may be impaired. Nonetheless, hyperactivity in nNOS KO mice may influence the difference displayed by both groups. Cued version of fear conditioning was also conducted to evaluate the ability of mice to remember an association between a tone and an electrical shock. Similar levels of freezing were displayed by nNOS KO and wild-type mice before tone exposure (Fig. S2B, $p = 0.4666$). During auditory cue re-exposure in a different context, there was no statistically significant difference in freezing behavior between the two groups (Fig. S2B, $p = 0.1484$), suggesting no impairments in cue fear conditioning.

3.5. Working memory deficits in nNOS KO mice

We further investigated cognitive function in nNOS KO mice by evaluating spatial working memory in a Y- maze test. nNOS KO mice exhibited significantly less spontaneous alternation relative to wild-types (Fig. 3A, $p=0.0467$). There was no significant difference in total number of arm entries (Fig. 3B, $p = 0.8598$), indicating the results were not affected by hyperactivity of nNOS KO mice.

A DNMTTP task, a task highly dependent on the integrity of prefrontal cortical areas [32,33], was performed to examine working memory in more detail. Mice were trained in the DNMTTP task to a criterion of 80% correct choices, after which they were tested using 5, 30, 60, and 120 s delay intervals. nNOS KO mice learned and performed the task as well as wild-type mice during training (Fig. 3C, $p = 0.6628$). In the working memory test, performance, averaged over 4 testing days, decreased in both nNOS KO and wild-type mice as a function of delay [$F_{(3,54)} = 33.64$, $p < 0.001$] (Fig. 3D) and a repeated measures ANOVA revealed a significant effect of genotype [$F_{(1,18)} = 4.75$, $p < 0.05$] (Fig. 3D). Although genotype x delay interaction was not significant [$F_{(3,54)} = 2.02$, $p = 0.1226$], the performance of nNOS KO mice was significantly lower relative to wild-types at 30 s (Fig. 3D, $p = 0.0316$) and 60 s (Fig. 3D, $p = 0.0096$) delay intervals. These results suggest impairment in working memory in nNOS KO mice.

3.6. nNOS-DISC1 interaction and influence of nNOS on DISC1-NDEL1 interaction

DISC1, another genetic risk factor for schizophrenia, plays roles in the development of cerebral cortex [25,26,34]. Evidences from human and animal studies suggest that DISC1 is also associated with cognitive function, including working memory [27,34]. To examine the possibility of convergence of nNOS signaling and DISC1 pathways, we tested whether nNOS and DISC1 interact with each other. Myc-tagged DISC1 was exogenously expressed together with nNOS in HEK 293 cells. We found that nNOS was co-precipitated with DISC1, and reciprocal co-immunoprecipitation was also observed (Fig. 4A). Consistent with this result, we also found endogenous protein interaction of nNOS and DISC1 in the developing cerebral cortex of wild-type, but not nNOS KO mice (Fig. 4B). Of interest, genetic deletion of nNOS appears to increase the binding of DISC1 to NDEL1 of which interaction is important for regulating neurite outgrowth [28,29] (Fig. 4B).

4. Discussion

nNOS KO mice have reportedly displayed various behavioral abnormalities, including hyperactivity, aggressive behavior, abnormal social behavior, impaired spatial memory, impairments in contextual fear conditioning, and abnormal pre-pulse inhibition [19–21,23,24,35]. In this study, we also reproduced that nNOS KO mice exhibit hyperactivity. Of note, it has been reported that nNOS KO mice exhibit no difference in the amount of time spent in the center of open field apparatus, but enter the center with a higher frequency than wild-type mice [21]. Our results are consistent in that no differences were found between nNOS KO mice and wild-type mice in the amount of time spent in the center of open field apparatus. Increase in amount of time spent in center of apparatus later reported by Lubec and Miyakawa's group [20,24] as well as higher frequency of entries into the center of apparatus exhibited by nNOS KO mice [21] is likely a reflection of hyperactivity rather than reduced anxiety-like behavior as lack of nNOS has not consistently affected other measures of anxiety, as some studies suggest increased anxiety levels [21], while others stress anxiolytic-like phenotypes [22].

Cognitive deficits associated with the prefrontal cortex have been frequently reported in schizophrenia and nNOS has been genetically associated with prefrontal cortical functioning [2,4]. Although deficits in memory functions have been implied in animals administered nNOS inhibitors [36], behavioral deficits on tasks highly dependent on prefrontal cortex have not been studied yet in nNOS KO mice. In this study, we report for the first time working memory deficits in nNOS KO mice. In the DNMTTP test, nNOS KO mice exhibited worse performance compared to wild-type mice, indicating that working memory is impaired in nNOS KO mice. Consistently, nNOS KO mice exhibited a lack of net preference between novel and familiar object in the NOR test, a test which is, in part, mediated by prefrontal cortex [37]. Nonetheless, the data should be evaluated carefully, because phenotypes in cognition might be confounded by differences in basic sensorimotor functions relevant to the tasks. Given that differences observed in the DNMTTP task were moderate and performance in this task may be modulated by other brain regions, such as hippocampus, investigation using behavioral paradigms more specifically associated with prefrontal cortex function should be conducted in future studies.

nNOS plays important roles for regulation of many molecular signaling pathways via S-nitrosylation of diverse downstream effectors, contributing to brain development and adult brain function. Although Nelson and colleagues [38] have proposed that alterations in serotonergic neuronal transmission result in aggressive behaviors in nNOS KO mice, the link between nNOS-mediated functional processing and resultant behaviors largely remains to be elucidated. In this study, we found DISC1 is a novel protein binding partner for nNOS. Of interest, augmentation of DISC1-NDEL1 interaction was also observed in nNOS KO mice. Since proper regulation of DISC1-NDEL1 interaction is important for neurite outgrowth [28,29], nNOS may regulate neuronal development via DISC1-NDEL1 interaction. Given that DISC1 plays roles for cortical development and prefrontal cortex functioning, including working memory [25–27], further investigation of the molecular interaction between nNOS, DISC1, and NDEL1 is important in addressing how genetic disturbances in nNOS-mediated signaling contribute to the underlying mechanisms of impaired cognition in schizophrenia.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Hyperactivity and anxiety-related behavior of nNOS KO mice

(A) In the open field test, nNOS KO mice were significantly more active in the 30 min session. (B) No differences were found in rearing. (C) No differences were found in percentage of time spent in central area ($n = 9$ per group). Data are shown as mean $\pm$ SEM. ** $p < 0.01$.

Fig. 2. Impairment of recognition memory in nNOS KO mice

(A) In the NOR test, no differences were exhibited in total exploration time during training. (B) No differences were exhibited in exploratory preference during training. nNOS KO mice displayed mild impairment in object recognition memory as shown by the reduced exploratory preference. (C) In the spatial component of the NOR test, nNOS KO mice displayed significant impairment in spatial recognition memory as shown by the reduced exploratory preference ($n = 7-8$ per group). Data are shown as mean $\pm$ SEM. ** $p < 0.01$.

Fig. 3. Working memory deficits in nNOS KO mice

(A) In the Y-maze test, nNOS KO mice exhibited significantly less percent alternations. (B) No differences were found in total number of arm entries ($n = 8$ per group). (C) In the DNMTTP task, no difference was observed during training. There was no delay between the sample-run and choice-run. (D) nNOS KO had a significantly lower number of percent correct responses during trials with 30 s and 60 s delay ($n = 10$ per group). Data are shown as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$.

Fig. 4. nNOS-DISC1 interaction and influence of nNOS on DISC1-NDEL1 interaction

(A) Myc-tagged DISC1 was co-transfected with nNOS into HEK293 cells. Lysates were immunoprecipitated with antibodies against myc or nNOS. Immunoprecipitates (IPs) were analyzed by western blotting with antibodies against nNOS or myc to analyze nNOS-DISC1 interaction. The input of each protein is also shown. (B) Endogenous interaction of DISC1 with nNOS and NDEL1 was assessed by co-immunoprecipitation in the developing cerebral cortex at postnatal day 3 (P3). Endogenous nNOS-DISC1 interaction in wild-type mice, but not in nNOS KO mice, and augmentation of DISC1-NDEL1 interaction by genetic deletion of nNOS was observed. The input of each protein is also shown. IB indicates an antibody used for Western blotting.