

ORIGINAL ARTICLE

Altered hippocampal-dependent memory and motor function in neuropilin 2-deficient mice

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Semaphorins have an important role in synapse refinement in the mammalian nervous system. The class 3 semaphorin-3F (Sema3F) acting through neuropilin 2/plexin-A3 (Nrp2/PlexA3) holoreceptor complex signals *in vivo* to restrain apical dendritic spine morphogenesis of cortical pyramidal neurons and hippocampal neurons during postnatal development and mediates excitatory synaptic transmission. Semaphorin signaling has been implicated in the etiology of a number of neurodevelopmental disorders; however, the effects on behavior and mental function of dysregulated Sema3F-Nrp2 signaling have not been fully addressed. The present study is the first behavioral investigation of mice harboring a mutation of the *nrp2* gene. Given that loss of Nrp2 signaling alters cortical and hippocampal synaptic organization, we investigated performance of *nrp2*-deficient mice on learning and sensorimotor function that are known to depend on cortical and hippocampal circuitry. When compared with age-matched controls, *nrp2* null mice showed striking impairments in object recognition memory and preference for social novelty. In addition, *nrp2*^{-/-} mice displayed impaired motor function in the rotarod test and in observations of grooming behavior. Exploration of novel olfactory sensory stimuli and nociception were unaffected by the loss of Nrp2. Overall, loss of Nrp2 may induce aberrant processing within hippocampal and corticostriatal networks that may contribute to neurodevelopmental disease mechanisms.

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INTRODUCTION

Understanding how neurodevelopmental mechanisms establish and organize synaptic connections and ultimately impact mental function may lead to new treatments for a range of neurological and mental disorders.^{1–5} One important signaling system for navigating growing axons and their growth cones to form the appropriate connections during early nervous system,⁶ and also regulating postnatal synapse refinement⁷ involves the large, conserved protein family of the semaphorins. The semaphorin family is composed of at least 25 members across eight subclasses according to structural homology. Most of our knowledge regarding the biological roles of semaphorins in vertebrates comes from studies of the class 3 secreted semaphorins (Sema3s) in the nervous system. However, semaphorins have important roles in a wide range of physiological processes.^{8–13}

The obligate binding partner for most Sema3s, neuropilins were first identified as a neuronal cell surface protein in the *Xenopus* visual centers,¹⁴ and later as a cell adhesion molecule in the chick and mouse nervous system^{15,16} and was thought to be involved in neuronal recognition of nerve fibers and their targets. It was demonstrated that the two members of the neuropilin (Nrp) family are the receptors for Sema3s; Nrp1 binds with high affinity to Sema3A, whereas Nrp2 preferentially binds to Sema3F.^{17–20} Due to its short cytoplasmic tail, Nrps were found to be dispensable for signaling in controlling axon guidance and members of the type A plexin (Plex) family was identified as the signal transducing receptor for semaphorin-mediated axon guidance events during development.^{21–24}

Sema3F was shown to signal through Nrp2/PlexA3 receptors to serve as a strong axonal repellent and a pruning factor for

hippocampal axons in the developing nervous system.^{25–27} Indeed, Sema3F have been demonstrated, both *in vitro* and *in vivo*, to preferentially signal through the Nrp2/PlexA3 holoreceptor complex,^{19,20,28} as defects observed in both embryonic and postnatal development are recapitulated from mice deficient in the Sema3F–Nrp2/PlexA3 signaling pathway.^{25,29–31} Previously, we and others have shown that Sema3F–Nrp2/PlexA3 signals *in vivo* to restrain apical dendritic spine morphogenesis in layer 5 cortical pyramidal neurons during postnatal development and mediate excitatory synaptic transmission.^{32,33} Nrp2 expression is enriched in the molecular layer of the hippocampal formation, where the dentate gyrus granule cell dendrites reside. Sema3F is robustly expressed in the hilus, along the projection pathways of both the supra and intrapyramidal axons and the entorhinal cortex axon that innervate the dendrites of the molecular layer.²⁵ Indeed, both *Sema3F* and *Nrp2* mutants displayed an increase in dendritic spine number, distribution, size and miniature excitatory postsynaptic current frequency in both hippocampal dentate granule cells and layer 5 cortical neurons.³³ Therefore, Sema3F and Nrp2 expression pattern and function in the postnatal brain is consistent with the hypothesis that these proteins direct cortical and hippocampal neural circuit formation.

Semaphorin dysregulation has been linked to a range of neurological disorders,^{34–37} and may have a key role in learning and memory by modulating synaptic plasticity in the adult hippocampus.^{32,38} Nevertheless, the effects on behavior of dysregulated Sema3F/Nrp2 signaling remain unknown. Here, we tested mice on a range of tasks, including those that depend on hippocampal and corticostriatal circuits that we have shown to be altered by *nrp2* knockout. Dysregulation of these circuits have

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been implicated in a number of mental disorders including autism and schizophrenia; therefore, understanding their function is of particular relevance to understanding disease mechanisms. Specifically, we used recognition memory tasks that depend on the dentate gyrus and its projections into the CA3 subregion of the hippocampus. This circuit has a key role in pattern separation—the process of transforming similar representations or memories into highly dissimilar, nonoverlapping representations.^{39–41} In addition, we examined the acquisition of a repetitive motor behavior that relies on corticostriatal circuitry.^{42–44} We show that *Nrp2*-deficient animals are impaired in object and social recognition memory and repetitive motor behavior, but display normal sensory processing. Taken together, our results reveal novel functions of *Sema3F*–*Nrp2* signaling in complex behavior output.

MATERIALS AND METHODS

Mice

The *Nrp2* knockout mice have been previously described in detail, both its expression patterns and developmental phenotypes.²⁹ Mice used in this study have been backcrossed for 10 plus generations to the C57BL/6NTac background strain, and only males ($n=5–7$ per group) were used for all behavioral testing. We used wild-type (+/+) littermates as controls, heterozygous (+/-), and homozygous (-/-) *nrp2* mice. However, heterozygous *nrp2* mice display a normal neural anatomical and electrophysiological phenotype.^{29,33,45–47} In addition, we observed in our mouse-breeding colony that the *nrp2* locus does not follow the Mendelian 3:1 ratio of distribution; in fact, the ratio of inheritance for the homozygous mutant is much less. Thus to circumvent this hurdle, we have optimized the number of homozygous progenies by setting up heterozygous and homozygous crosses. The heterozygous was used as controls for the rotarod and olfactory tests, and, in the majority of the heterozygous data obtained for all other tests, followed the same pattern as the wild type. All procedures were approved by the Rutgers Institutional Animal Care and Use Committee.

Equipment and tests

Novel object recognition test. Novel object recognition testing was based on previously described procedures.⁴⁸ Mice were tested with two objects in a 40 cm × 40 cm (w/d) open-field arena. During the sample phase, two identical objects were placed in opposite corners of the arena 10 cm from the nearest walls. Mice were placed in the center of the arena and allowed to freely investigate both objects for 10 min after which they were returned to their home cage for 30 min. During the 5-min test phase, mice encountered one 'familiar' object from the sample phase and a novel object. The number of sniffs to the familiar and novel object was assessed and quantified from video footage by observers that were blind to the genotype of the animals. The objects consisted of a white plastic funnel and a white and yellow bird-shaped toy and were wiped down with isopropyl alcohol between phases.

Social novelty test. Preference for social novelty was tested in a three-chambered arena, modified from that previously described.⁴⁹ Each of the three chambers of the arena were equally sized and separated from each other by a plexi-glass barrier. A small hole allowed passage between the chambers. Mice were first habituated to the empty arena for 30 min. During the 10-min sample phase, an unfamiliar male mouse was confined to one of the chambers by a small wire cage placed over it and the test mouse was allowed to freely roam the apparatus for 10 min. The opposite chamber contained a wire cage with no mouse. During the test phase, the mouse from the sample phase was returned to the apparatus and confined to one chamber. This mouse was designated the 'familiar' mouse. An unfamiliar mouse was confined to the opposite chamber. The location of the familiar and novel mice was randomized across subjects. The test mouse was placed in the center of the arena and allowed to freely roam the apparatus for 10 min. The amount of time spent in each chamber was assessed and quantified using tracking analysis from Noldus Observer software (Noldus, Leesburg, VA, USA).

Rotarod test. Mice were tested on a standard rotarod device (Med Associates, St Albans, VT, USA). Mice were placed on the spindle, which linearly accelerated from 4 to 40 r.p.m. The trial ended when the mouse fell

off the spindle, made one complete revolution while gripping the spindle, or 5 min had elapsed. Each mouse received five trials per day over 2 days with ~10 min between trials.

Hot-plate test. Mice were placed in a standard hot-plate apparatus. The hot plate measured 10 cm × 10 cm and was surrounded by a plastic enclosure. Mice were initially habituated to the apparatus for 5 min. During the test, the temperature was set to 55 °C. Mice were placed in the center of the apparatus for 2 min. Videotape footage was scored by two trained observers, who were blind to the genotype of the animals. The number of hind limb withdrawals was recorded and quantified.

Grooming, rearing and bed-pushing behavior. Mice were videotaped for 20 min in a cage filled with cedar bedding that was identical to their home cage. Two trained observers, who were blind to the genotype of the animals, scored and quantified the frequency of rearing and grooming events and displacement of bedding material over the 20-min time period.

Olfactory habituation/dishabituation. Mice were sequentially presented with scented cotton swabs and investigatory behavior was quantified from video footage by observers that were blind to the genotype of the animals. Each cotton swab was inserted into the cage for 2 min and affixed to the cage lid. Sniffs were counted and quantified when the nose was oriented toward and within 2 cm of the swab. Any bouts that involved physical contact with the swab was not counted. Mice received three consecutive presentations of swabs soaked in distilled water, followed by three presentations of swabs soaked in vanilla or banana extract that was diluted to 1:100 concentration. Mice then received presentations of swabs wiped in the bedding of conspecific male mice.

Data analysis

Data analysis was conducted in SPSS (IBM, Chicago, IL, USA). Data from behavioral observations were averaged from two observers and subjected to analyses of variance (ANOVAs) and *t*-tests with Bonferroni *post hoc* correction for multiple comparisons. Data from the novel object and social novelty tests were converted into ratios ('familiar' and 'novel' as a percent of total behavior). These data were subjected to two-factor ANOVAs with genotype as a between-subjects factor and novelty as a within-subject factor.

RESULTS

Nrp2 knockout alters novel object recognition and social novelty behavior

We hypothesized that *nrp2*^{-/-} mice would show a deficit in tasks that depend on hippocampal function, particularly those mediated by the dentate gyrus mossy fiber pathway.^{39–41} To test this hypothesis, we evaluated performance in novel object and social novelty tests that are known to rely on the functions of these subregions of the hippocampus.^{50,51} Counting the number of sniffs directed at novel and familiar objects during the recognition test, we found a significant preference to investigate novel objects among wild-type and *nrp*^{+/-} mice. Data were normalized by dividing the number of sniffs for each object by the total number of sniffs during the test. The normalized data depicted in Figure 1a shows a preference for novelty among wild-type (68% novel object; 32% familiar object normalized number of sniffs per object) and *nrp*^{+/-} mice (68% novel object; 32% familiar object normalized number of sniffs per object). Group comparisons using paired-samples *t*-tests revealed that preference for novelty was significant for both groups (wild-type $t_5 = 3.42$; *nrp*^{+/-} $t_5 = 3.47$, $P < 0.05$) (Figure 1a). In contrast, no difference in investigatory behavior among novel and familiar objects was observed among *nrp2*^{-/-} mice (51% novel object; 49% familiar object normalized number of sniffs per object). An analysis of the total number of investigatory behaviors directed at the two objects revealed no effect of genotype on sniffing frequency (Figure 1b). Wild-type, heterozygous and null mice made similar amounts of object-directed sniffs during the test (average sniffs per minute: wild-type 9.1 novel object, 4.6 familiar object; *nrp*^{+/-}

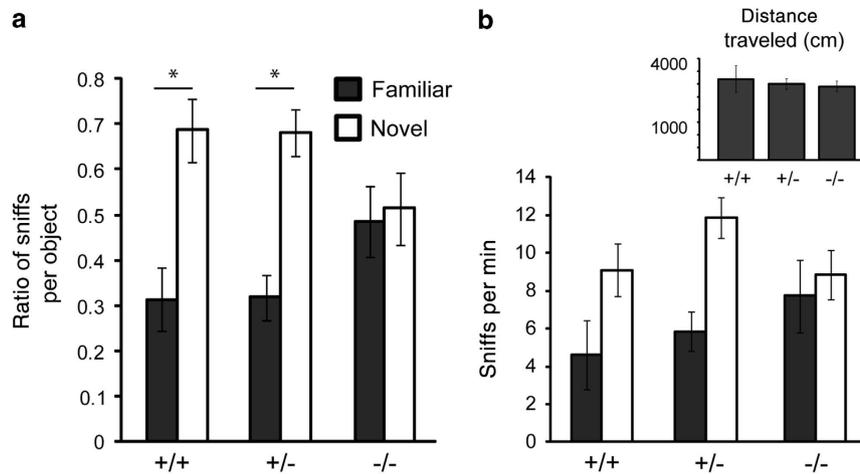


Figure 1. *Nrp2*^{-/-} mice show impairments in tests of novel object recognition memory. Mice were placed in an open arena containing a familiar and novel object and the number of sniffs directed at these objects was recorded. (a) *Nrp2*^{+/-} and wild-type mice showed a significant novelty preference on the basis of normalized data, which was calculated by dividing the number of sniffs directed at familiar and novel objects by the total number of sniffs. *Nrp2*^{-/-} showed no novelty preference. (b) Number of sniffs per minute directed at familiar and novel objects for the three mouse strains tested. No significant difference between genotypes for total distance traveled (inset; $P = 0.67$). * $P < 0.01$ paired-samples t -test; error bars, ± 1 s.e.m. Nrp, neuropilin.

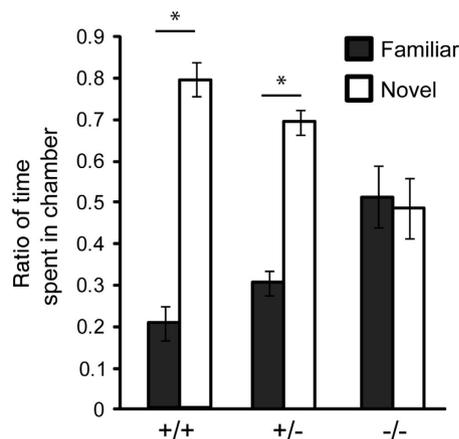


Figure 2. *Nrp2*^{-/-} mice show impairments in tests of preference for social novelty. Mice were placed in the center of a three-chambered arena—the left and right chambers contained a familiar and novel conspecific mouse under an enclosure. The amount of time each mouse spent in the chambers containing the familiar and novel mouse was recorded. On the basis of normalized data, which was calculated by dividing the amount of time spent in the chambers containing familiar and novel mice by the total amount of time, *Nrp2*^{+/-} and wild-type mice showed a significant preference for the novel mouse. *Nrp2*^{-/-} mice showed no preference. * $P < 0.01$ paired-samples t -test; error bars, ± 1 s.e.m. Nrp, neuropilin.

11.5 novel object, 4.8 familiar object; *Nrp2*^{-/-} 8.8 novel object; 7.7 familiar object). In addition, an analysis of locomotion (total distance traveled) during the first test phase found no effects between genotypes (ANOVA $P = 0.67$; Figure 1b-inset). Overall these data reveal that all mouse strains were equally engaged in the task, but the *Nrp2* knockout strain alone failed to show a significant object preference.

Next, we asked whether Nrp2 signaling influences social recognition memory. Recording the amount of time spent in the chamber containing a novel or familiar mouse during the preference test, we found a significant preference for novelty among wild-type and *Nrp2*^{+/-} mice. Data were normalized by dividing the amount of time spent in each chamber by the total amount of time spent in both chambers. The normalized data depicted in Figure 2 (and also see Supplementary Video) show a

significant preference for novelty among wild-type (79% novel mouse; 21% familiar mouse normalized time spent in each chamber) and *Nrp2*^{+/-} (69% novel mouse; 31% familiar mouse normalized time spent in each chamber) mice. Group comparisons using paired-samples t -tests revealed significant differences in novelty preferences for both the groups (wild-type $t_5 = 7.32$; *Nrp2*^{+/-} $t_4 = 6.49$, $P < 0.01$). No preference was observed in *Nrp2*^{-/-} mice (51% novel mouse; 49% familiar mouse normalized time spent in each chamber). The lack of preference for a novel mouse among knockout mice was not due to a lack of sociability. During the first familiarization phase of the test, *Nrp2*^{-/-} mice spent significantly longer time with a mouse compared with a chamber containing an empty enclosure ($t_6 = 6.48$, $P < 0.01$, data not shown).

Nrp2 knockout impairs repetitive motor behavior but preserves sensory processing

Nrp2 knockout alters cortical layer V neurons, which form the primary cortical input to the striatum. We hypothesized that tasks, such as the rotarod, that rely on corticostriatal circuitry would be impaired in *Nrp2*^{-/-} mice. We acknowledge that while it is customary to use wild type as controls, we believe it is justifiable to use heterozygous here as controls for the following reasons. On the basis of previous published neural developmental results for Nrp2 signaling, all characterized phenotypes observed are recessive and heterozygous animals display normal neural anatomical and electrophysiological phenotypes identical to the wild-type littermates.^{29,33,45–47} Thus, we believe that in most cases the heterozygous is a better control group as they are most similar genetically to the homozygous null, but do not show the neural defects reported in the null animals.

We found a significant impairment in rotarod performance among *Nrp2* mice, with the latency to fall significantly greater among the knockout strain compared with their control heterozygous counterparts (ANOVA $F_{1,10} = 4.78$, $P = 0.05$, Figure 3). The latency to fall was significantly shorter in *Nrp2*^{-/-} compared with controls (+/-) beginning at trial 8. To further examine motor behavior, we made detailed observations of home-cage behavior. We found no effects on bed-pushing behavior (Figure 4a). However, we found that *Nrp2*^{-/-} mice made significantly longer grooming bouts compared with wild-type ($P = 0.03$) and *Nrp2*^{+/-} mice ($P = 0.02$, Figure 4b). In addition, knockout mice made significantly more frequent grooming bouts ($P < 0.01$) and rearing

behavior ($P=0.03$) compared with wild-type mice (Figures 4b and d). Overall, these data suggest that acquisition or performance of repetitive motor behavior in $nrp2^{-/-}$ mice is impaired by lack of Nrp2 function.

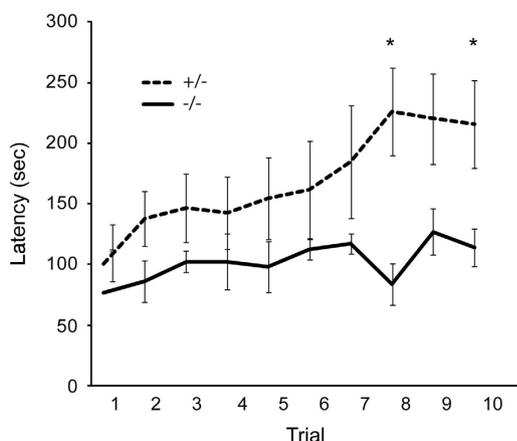


Figure 3. $Nrp2^{-/-}$ mice show impaired rotarod performance. Mice were given 10 rotarod trials over 2 days. Although the latency to fall increased with training, $nrp2^{-/-}$ mice had an overall significantly shorter latency to fall compared with control mice. Comparisons at individual trials revealed significantly shorter latencies to fall for $nrp2^{-/-}$ at trials 8 and 10. * $P < 0.05$ independent samples *t*-test; error bars, ± 1 s.e.m. Nrp, neuropilin.

To examine the possibility of sensory dysfunction caused by $nrp2$ knockout, we tested mice on a hot-plate test and an olfactory habituation/dishabituation task. We found no effects of genotype on hot-plate performance. The frequency of hind-foot movements during exposure to the hot plate did not differ among genotypes ($P > 0.87$, Figure 5a). Similarly, we found no effect of $nrp2$ knockout in olfactory investigatory behavior. Habituation to sensory stimuli does not require hippocampal engagement when stimuli are sequentially presented without delay, therefore performance in this task primarily assesses sensory function. All mice showed investigatory behavior directed toward odor stimuli that was enhanced by novelty. Mice, independent of genotype, sniffed more frequently to a novel odor compared with a familiar odor as shown by a main effect of odor novelty on sniffing frequency independent of other factors (repeated-measures ANOVA $F_{1,14} = 6.62$, $P = 0.02$, Figure 5b). Although there was no effect of genotype on sniffing frequency, there were significant interactions involving genotype and odor type (social versus nonsocial, ANOVA $F_{2,14} = 4.84$, $P = 0.02$). Heterozygous and knockout mice made significantly more sniffs to nonsocial odors compared with wild-type mice, whereas no difference in sniffing frequency among genotypes was observed for social odors. Overall, these data indicate intact olfactory sensory processing in $nrp2^{-/-}$ mice. Therefore, the deficits in recognition memory that were observed are not likely a consequence of impaired sensory function.

DISCUSSION

We believe our findings are the first detailed behavioral investigation of $nrp2$ -deficient mice. Given that loss of Nrp2 signaling alters

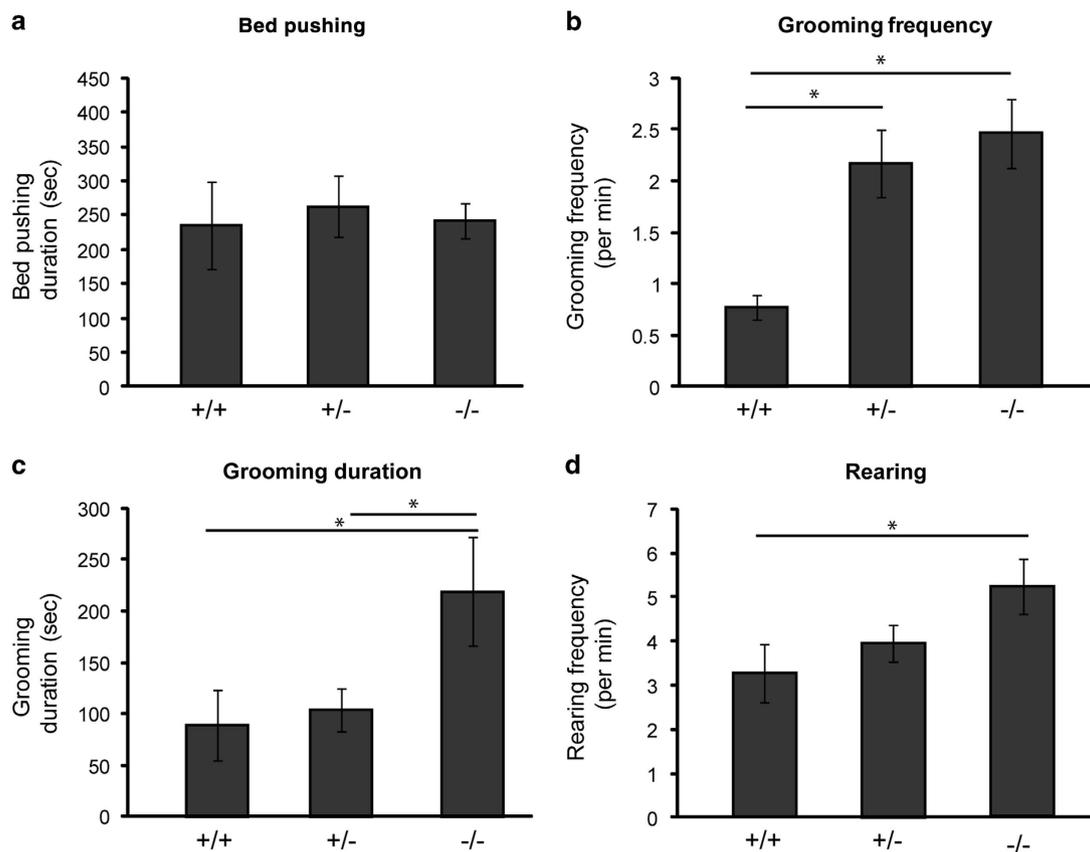


Figure 4. Grooming behaviors of $Nrp2$ mice. Mice were placed in an empty cage and two trained observers scored the frequency and duration of different behaviors. (a) No difference in bed-pushing behavior was observed among strains. (b) $Nrp2^{-/-}$ and $nrp2^{+/-}$ mice made more frequent grooming bouts compared with wild-type mice (c) $Nrp2^{-/-}$ mice display longer grooming bouts compared with $nrp2^{+/-}$ and wild-type mice. (d) $Nrp2^{-/-}$ mice made more frequent rearing behaviors compared with wild-type mice. * $P < 0.05$, paired-samples *t*-test with Bonferroni correction; error bars, ± 1 s.e.m. Nrp, neuropilin.

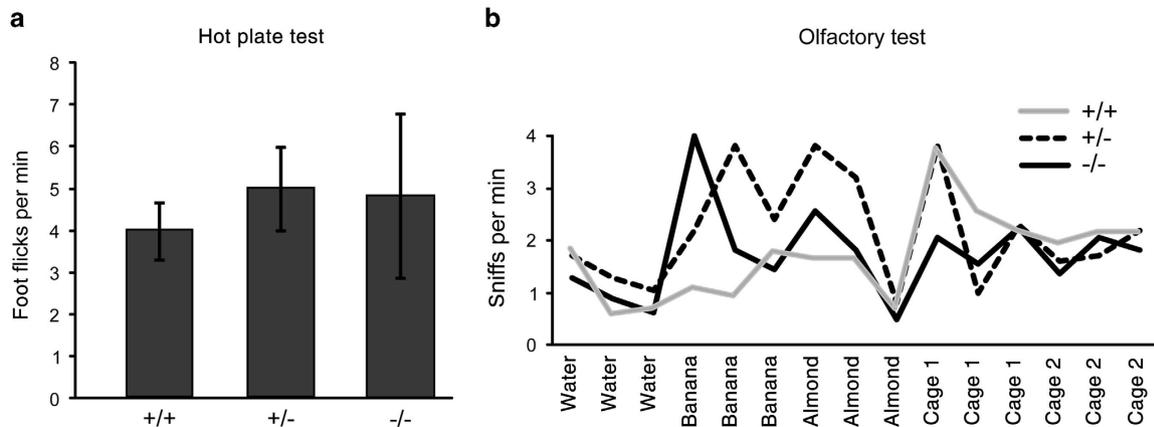


Figure 5. *Nrp2*^{-/-} mice have normal hot-plate performance and olfactory habituation. (a) Mice were placed in a hot-plate apparatus and the number of hind-foot flicks in a 2-min interval was tallied. No effect of genotype was observed. (b) Mice were presented with a series of scented cotton swabs and the number of sniffs directed at the swabs was recorded. We presented both nonsocial odors (vanilla and banana extract) and social odors taken from used mouse bedding. Each odor sample was presented three times. We found an overall effect of novelty: mice sniffed more frequently to novel odors compared with familiar odors. Knockout and heterozygous mice sniffed more frequently to nonsocial odors compared with wild-type mice. *Nrp*, neuropilin.

hippocampal and cortical synaptic organization, we hypothesized that these mice would display deficits in tasks that depend on these circuits. In support of this hypothesis, *nrp2*^{-/-} mice were significantly impaired in tests of object and social recognition memory. We additionally found impaired rotarod performance, suggesting that *nrp2* knockout impacts the acquisition or performance of repetitive motor behavior. Overall, we found that *Sema3F-Nrp2* signaling fundamentally influences behavior, either through their actions on neural circuit formation during embryonic brain development or postnatal synaptic refinement and plasticity.

Nrp2^{-/-} mice were impaired in tests of novel object recognition and social novelty. Both tests rely on the animal acquiring and retaining an episodic-like memory of the sensory features of an object or mouse and using this information during the test phase to guide their investigatory behavior. Performance of *nrp2*^{-/-} mice in both tests may therefore reflect impaired acquisition or retention of long-lasting episodic-like memory. Accurate performance on these tasks is known to depend on the hippocampal formation.^{50,51} Previously, we have shown altered synapse structure and physiology in the hippocampus of *nrp2*^{-/-} mice.³³ Therefore, impaired performance that was observed in *nrp2*^{-/-} mice on these tasks may represent a defect in synaptic plasticity in the hippocampus. Further investigation using complex mouse genetics such as conditional knockout animals crossed to specific CreER-lines, which is beyond the scope of this study, will determine whether the deficits in behavior that were observed are due to the effects of loss of function in embryonic, postnatal or adult animals.

Although our study is not a comprehensive behavioral phenotype and the possibility remains that factors such as anxiety or impaired sensory processing could influence performance on the novelty tests, additional evidence we gathered would argue against this possibility. For example, we found no significant difference between *nrp2*^{-/-} mice and matched controls in exploratory activity during the learning phases of these tests. If anxiety prevented these mice from learning then presumably it would have significantly altered behavior during the learning phase. Likewise, we found no differences between strains in bed-pushing behavior, an indicator of anxiety in mice. We found no impairment in responses to nociceptive or olfactory stimuli suggesting intact sensory processing in *nrp2*^{-/-} mice. Another possible explanation for our findings is that *nrp2*^{-/-} mice may have intact hippocampal processing but show no novelty

preference. This interpretation is unlikely given that we found *nrp2*^{-/-} mice investigated objects and mice during the learning phase and during the olfactory habituation task, suggesting an intact interest in these animals in exploring novel environmental stimuli.

We found impairments in repetitive motor behavior in *nrp2*^{-/-} mice. Mice were impaired in rotarod performance and made longer grooming bouts. *Nrp2* deletion increases excitability and spine density in layer 5 cortical pyramidal neurons, which form the cortical projection to the basal ganglia. Corticobasal ganglia circuitry is responsible for action control and alterations to this circuit can increase motor stereotypy.^{52,53} Therefore, the present findings may be explained in the context of impaired corticobasal ganglia function. However, additional experiments beyond the scope of this study will be necessary to fully test this hypothesis.

Our results are consistent with other behavioral studies in animals deficient in semaphorin signaling. For example, *Sema6A*-deficient animals possess alterations in cortical and limbic organization and show impaired object recognition memory and hyper-exploratory behavior,³⁷ similar to the pattern of behavior that was observed in *nrp2*^{-/-} mice, whereas loss of *Sema4D* enhances motor behavior.⁵⁴ More broadly, our findings are consistent with behavioral deficits observed in mice with neurodevelopmental defects affecting synaptic organization.^{55,56}

Overall, our results provide novel evidence of behavioral modification in *Sema3F-Nrp2* signaling. *Nrp2* null mice showed striking impairments in both hippocampal-dependent memory tasks and repetitive motor behavior. The changes in behavior that were observed may reflect aberrant processing within hippocampal and cortical networks known to be impacted by *nrp2* knockout. Finally, our findings are relevant to understanding neurodevelopmental diseases such as ASD: the behavioral phenotype matches some of the core features of ASD (learning impairments, repetitive behavior) and the neural phenotype of altered cortical microcircuitry is consistent with contemporary theories of ASD pathophysiology.^{57,58}

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

MWS and TST designed the study, analyzed the data and wrote the manuscript. MG carried out the majority of the experiments and MWS assisted in some of the behavioral testing procedures.

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