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# Impaired discrimination learning in interneuronal NMDA-GluN2B mutant mice

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## Abstract

The ability to encode new associations that lead to positive outcomes is mediated by the dorsal striatum across species. Previous studies have established a role for the *N*-methyl-D-aspartate receptor (NMDAR) and its GluN2B subunit in efficient learning behavior on a variety of tasks. Recent findings have suggested that NMDAR on GABAergic interneurons may underlie the modulation of striatal function necessary to balance efficient action with cortical excitatory input. Here we investigated how loss of GluN2B-containing NMDAR on GABAergic interneurons altered corticostriatal-mediated associative learning. Mutant mice (floxed-GluN2B x Ppp1r2-Cre) were generated to produce loss of GluN2B on forebrain interneurons and phenotyped on a touchscreen-based pairwise visual learning paradigm. We found that the mutants showed normal performance during Pavlovian and instrumental pre-training, but were significantly impaired on a discrimination learning task. Detailed analysis of the microstructure of discrimination performance revealed reduced win→stay behavior in the mutants. These results further support the role of NMDAR, and GluN2B in particular, on modulation of striatal function necessary for efficient choice behavior and suggest that NMDAR on interneurons may play a critical role in associative learning.

### Keywords

touchscreen; striatum; instrumental; knockout; NMDA; cognitive; schizophrenia; addiction; reward; prefrontal cortex

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## Introduction

*N*-methyl-D-aspartate receptors (NMDARs) are heterotetrameric complexes composed of combinations of GluN1, GluN2 and GluN3 subunits that vary with brain region and developmental age [1]. There is intense interest in elucidating the physiological, pharmacological and behavioral functions of specific NMDAR subunits.

To explore the role of GluN2A and GluN2B in learning, we and others have phenotyped mutant mice with deletions of these subunits in a touchscreen-based visual discrimination paradigm. These studies found that brain-wide GluN2A deletion impairs discrimination of complex two-dimensional shapes, but not simplified stimuli [2–4]. Brain-wide GluN2B C-terminal domain mutation, or GluN2B deletion specifically in striatal neurons, but not cortical (plus CA1 hippocampal) neurons, also produces discrimination learning deficits [4,5]. Conversely, GluN2B deletion on cortical (and dorsal CA1 hippocampal) principal neurons impairs spatial learning and trace fear memory, but leaves visual discrimination learning intact [5,6]. Taken together, these findings indicate a critical role for GluN2A and GluN2B in discrimination learning, and also identify the dorsal striatum as at least one key locus of these effects.

There is growing evidence that parvalbumin-positive (PV+) interneurons, and NMDARs expressed on these cells, exert a profound influence over neuronal network activity of brain regions, including the cortex, amygdala and hippocampus, and mediate various cognitive-behavioral processes. For example, optogenetic inhibition of PV+ interneurons in the medial prefrontal cortex disinhibits principal projection-neuron output and increases fear behavior [7]. Along similar lines, genetic deficiency of cortical PV+ interneurons disrupts reversal learning and task-related orbitofrontal cortex principal neuron activity [8]. With regards to the function of NMDAR subunits on these cells, gene deletion of the obligatory NMDAR subunit, GluN1, on a subset of (primarily, but not exclusively) PV+ forebrain interneurons impairs spatial working memory, amongst other behaviors [9]. Furthermore, hippocampal blockade of GluN2B (via the compound, Ro 25-6981) produces deficits in hippocampal-mediated maze learning, related to inhibited hippocampal interneuronal activity and augmented principal cell activity [10].

Together, these prior findings raise the question of the possible contribution of interneuronal GluN2B to other cognitive functions, including visual discrimination learning. We tested this in the current study by phenotyping mutant mice engineered to have loss of GluN2B on a subset of, mainly PV+ cells in cortex, hippocampus and lateral septum.

### Methods

**Subjects—**GluN2B mutant mice were generated by crossing a *loxP*-flanked GluN2B mutant mouse [5,6,11] with a mutant mouse in which Cre recombinase is driven under the control of the Ppp1r2 promoter [9]. This Cre line has previously been shown to delete GluN1 (in GluN1-floxed mutants) in ~40% of GABAergic interneurons (primarily PV+) in forebrain regions including cortex, hippocampus and lateral septum, while other regions including the striatal and amygdala are largely spared [9]. The GluN2B-floxed line was repeatedly backcrossed onto the C57BL/6J background and when crossed with a CaMKII-

Cre line has a ~95% C57BL/6J background, as estimated from analysis of 150 SNP makers [6]. The Cre line was backcrossed 6 times to the C57BL/6N background. Mutants (GluN2B-floxed/Cre-positive) and non-mutant (GluN2B-floxed/Cre-negative) mice were littermates bred from GluN2B-floxed/Cre-positive dams x GluN2B-floxed/Cre-negative sires. Male and female mice, (6–7 per genotype) aged 8–14 weeks at the start of testing, were housed with same-sex littermates in a temperature and humidity controlled vivarium under a 12 h light/dark cycle (lights on 0600 h). Procedures were approved by NIAAA Animal Care and Use Committee and strictly followed the NIH guidelines 'Using Animals in Intramural Research.

## Behavioral testing

**Apparatus—**Behavior was tested using previously described methods [12] and involved 3 stages of pre-training followed by discrimination learning (Figure 1a). An operant chamber measuring 21.6 × 17.8 × 12.7 cm (model # ENV-307W; Med Associates, St. Albans, VT, USA) was housed within a sound and light attenuating box. The grid floor of the chamber was covered with solid Plexiglas to aid ambulation. A pellet dispenser at one end of the chamber delivered a 14-mg reward pellet (#F05684; BioServ, Flemington, NJ, USA) into a food magazine. A house light and tone generator was located on the same side as the magazine. At the opposite end the chamber was a touch-sensitive screen (Light Industrial Metal Cased TFT LCD Monitor; Craft Data Limited, Aylesbury, UK). A black Plexiglas panel with 5 × 5 cm windows separated by 0.5 cm and located at a height of 6.5 cm from the chamber floor permitted touches to two response windows. Custom-made software ('MouseCat', written by L.M. Saksida) controlled stimulus presentation and recorded touchscreen responses and magazine entries.

**Pre-training—**Body weights were gradually reduced and maintained at 85% of free-feeding weight throughout testing. To reduce neophagia, mice were fed ~10 reward pellets/mouse in the home cage prior to testing and then acclimated to the operant chambers for 30 minutes with 10 pellets available in the food magazine.

**Stage 1:** In stage 1 of pre-training, a randomly-shaped stimulus was presented in one of the two touchscreen windows (pseudorandomly) for 10 seconds. On disappearance of the stimulus, a pellet reward was delivered in the magazine, coincident with illumination of light in the magazine and a 2-sec 65-dB tone presentation. Stage 1 criterion was the consumption of 30 pellets within 30 minutes.

**Stage 2:** In stage 2, a randomly-shaped stimulus was presented in a touchscreen window for an unlimited period, and the mouse were required to make a response (nosepoke) at the window to receive a reward. Stage 2 criterion was the consumption of 30 pellets within 30 minutes.

**Stage 3:** During stage 3, a randomly-shaped stimulus was again presented in a touchscreen window (left/right position randomized) for an unlimited period, but only after the mouse made a head entry into the magazine. A response at the stimulus-containing window produced a reward. A response to the window not containing the stimulus produced a 15-second timeout period in which the house light was extinguished and head entries could not

lead to new stimulus presentation. Following blank window responses, the stimulus was presented in the same window on the next trial until a correct response was made – after which the stimulus-containing window was randomly selected. Stage 3 criterion was making ≥75% of responses at the stimulus-containing window over a 30-trial session.

**Discrimination learning—**For discrimination learning, 2 novel stimuli were concurrently presented: 1 per response window. The designation of rewarded and non-rewarded stimulus for each mouse was randomized across groups. The stimuli were presented following a head entry in the magazine. A response at the rewarded stimulus produced a reward (= 'correct'), while a response at the non-rewarded stimulus (= 'error') led to a 15-second timeout period in which the house light was extinguished and head entries could not lead to new stimuli being presented for 15-seconds. After an error, the 2 stimuli were presented in the same left/right configuration window on the next trial (correction trial) until a correct response was made – after which the configuration was randomly determined. Errors made during correction trials were calculated separately (= 'correction errors'). The time to make a response after stimulus presentation and the time to retrieve the reward following a correct response was also recorded. Performance criterion was making an average of ≥85% correct responses over 2 consecutive 30-trial sessions.

The microstructure of behavior was examined by calculating the length of strings of consecutive responses correct or error/correction errors [10]. These data were expressed as the session-wise average correct and error string length, as well as the percent frequency distribution of string-blocks of a specific length (ranging from 1 through 10 or more). In addition, data were analyzed as pairs of consecutive responses to determine the percentage of correct responses followed by another correct response (='win →stay') and the percentage of errors responses followed by a correct response (='lose→shift'). The total number of each pair-type was tallied across discrimination sessions.

**Statistical analysis—**Genotype differences in behavioral measures were analyzed using Student's t-tests or 2-factor analysis of variance followed by Newman Keuls post hoc tests. The threshold for statistical significance was $P<.05$.

## Results

### Pre-training

Analysis of pre-training performance showed that the mutants did not significantly differ from controls on any of the 3 pre-training stages (Figure 1b). These data demonstrate that the mutant mice were fully able to form an association between a visual stimulus and reward and execute a reinforced instrumental response.

### Discrimination learning

In the discrimination learning task, mutant mice required significantly more trials to reach criterion than controls ($t(11)=-2.47$, $P<.05$) (Figure 1c). Two mutants and one control mouse failed to attain criterion with extensive training - 60-session cutoff and their scores were included in the analysis. The mutants also made significantly more errors ($t(11)=-2.48$, $P<$.

05) (Figure 1d) and correction errors t(11)=-2.43, *P*<.05) (Figure 1e) than control mice. By contrast, neither the time to respond to the stimulus (*P*>.05), nor retrieve the reward from the magazine (*P*>.05) differed between genotypes (Figure 1f). Together, these data show that the mutant mice exhibited a major deficit in visual discrimination learning that was not, as indicated by the latency measures, related to gross impairments in motor functions or appetitive motivation.

### Microstructure of discrimination performance

Analysis of the microstructure of performance on the discrimination task found that the length of strings of consecutive correct responses or errors [10] did not differ between genotypes, whether calculated as a task-average (*P*>.05) (Figure 2a) or the percent frequency distribution of string-blocks of a specific length (block-size effect for errors: F1,11=197.21, *P*<.01; block-size effect for corrects: F1,11=74.55, *P*<.01; genotype effect for either measure: *P*>.05) (Figure 2b).

Marked genotype differences were evident after segregating performance during discrimination into types of successive response-pairs. The mutants made significantly fewer win→stay (mutant= 61.94 ±2.3; control=71.63 ±2.4; t(11)=2.87, *P*<.05, d=.66), but similar lose→shift (mutant= 47.86 ±2.8; control=53.88 ±2.8; *P*>.05), response-pairs than controls (Figure 2c). These microstructural data reveal how that impairments in the discrimination learning task in the mutants were associated with an inability to properly utilize outcome information from correct trials to guide responding.

## Discussion

To test the role of interneuronal GluN2B-containing NMDARs in cognition, we engineered a novel mutant mouse to delete GluN2B specifically on GABAergic interneurons. We found that these mutants exhibited marked deficits in rewarded visual discrimination learning, assayed on a touchscreen-based task. The majority of the mutants tested was able to learn the task to criterion, but required more trials than non-mutant controls, and also made more errors and correction errors. In addition, the mutants were significantly less likely than controls to adopt a successful win→stay strategy. These deficits in learning were not due to abnormal motor function or motivation during the discrimination task. Moreover, the mutants were unimpaired during multiple stages of pre-training that taxed that ability to form visual stimulus-reward associations, as well the execution of instrumental responses. Thus, the impairment in the GluN2B mutant mice was specific to the learning of the visual discrimination task.

### New insights into the role of interneuronal GluN2B in learning

A recent study found that GluN1 deletion on the same population of PV+ interneurons targeted in the current GluN2B mutant line leads to impaired spatial working memory [9]. Additionally, pharmacological antagonism of GluN2B (using Ro 25-6981) produces impairments in hippocampal-mediated maze learning that are associated with decreased interneuronal activity and elevated principal cell activity in the hippocampus [10]. The current data extend these prior findings by demonstrating marked discrimination learning

deficits following mutation of GluN2B on forebrain interneurons. Moreover, they provide insight into the nature of this learning impairment, as evidenced from detailed microstructural analysis of performance. Specifically, the predominant performance strategy adopted by non-mutant control mice during discrimination learning was win→stay (i.e., to follow a correct response with another response at the same, rewarded, stimulus). The significant reduction in use of this strategy suggest that the mutants had difficulty utilizing positive feedback following correct responses to instruct future choices, which could be due, for instance, to a deficit in working memory or outcome-updating.

Previous rodent studies have implicated cortical and striatal brain regions in successful win→stay performance tested in various rewarded-choice settings. For example, lesion and inactivation studies have shown that the orbitofrontal cortex, dorsal striatum and nucleus accumbens contribute to the adoption of win→stay behavior in rats performing probabilistic-discounting and reversal tasks [13–17]. To our knowledge, there is currently no direct evidence connecting GluN2B to win→stay behavior in a task analogous to the one used here. Interestingly, however, preliminary evidence from human subjects has linked common gene variants in the GluN2B gene (*GRIN2B*) with differences in the likelihood of adopting a win→stay strategy in a rewarded decision-making task [18].

## Comparison with mutants with GluN2B deletion on principal cells

The findings of the current study extend our previous observation that gene deletion of GluN2B on striatal neurons but not cortical principal neurons, impairs learning in the same visual discrimination task employed here [5]. The absence of effects of cortical principal GluN2B loss, in particular, provides an interesting contrast with the impairments seen in the interneuronal mutants. This may be explained by different regional patterns of GluN2B deletion in the two mutant lines. The interneuronal line likely has more extensive deletion, that encompasses not only cortex but also septo-hippocampal regions [9], than the more restricted deletion in cortex and dorsal CA1 hippocampal seen in the principal-cell line [6].

Alternatively, the different mutant phenotypes could reflect differential roles for GluN2B on cortical interneurons and cortical principal neurons. The class of largely PV+ cortical interneurons preferentially targeted in the GluN2B mutant has a profound influence on cortical output and cortex-dependent behaviors [7,8]. We have previously shown that touchscreen reversal (discrimination was not tested) learning is sensitive to lesions of the medial prefrontal cortex [19]. As such, loss of interneuronal GluN2B could have disrupted cortical processing to impact learning, possibly via functional effects on subcortical areas critical to discrimination learning, such as the dorsal striatum. However, further studies will be needed to clarify the roles of GluN2B on different cells-types in cortex, for example by comparing the effects of Cre-viral-mediated knockdown of GluN2B in the same cortical regions in the interneuronal and principal-cell line.

## Comparison with pharmacological studies of GluN2B

In contrast to the learning deficits found in the GluN2B mutants, prior studies have found that systemic treatment with GluN2B antagonists (which presumably target GluN2B on principal neurons and interneurons throughout the brain) selectively disrupt measures of

cognitive flexibility (e.g. reversal, extinction), whilst leaving initial learning intact [20,21]. However, it should be noted, firstly, that the functional effect of these compounds on the NMDAR is concentration-dependent (with agonist-like activity at low concentrations) and, secondly, that the drugs antagonize not only diheteromeric GluN1-GluN2B NMDARs, but also, albeit with lower potency, GluN1-GluN2A-GluN2B triheteromers [22] (whereas GluN2A function appears to be intact following GluN2B deletion, at least on principal neurons [6,23]). These or other factors could contribute to differences in the profile of behavioral effects produced by GluN2B antagonists and genetic deletions. Further complicating the comparison is the potential for genetic mutation of GluN2B caused adaptations in other molecules that are not produced by acute drug treatment, and these changes contributed to the discrimination learning deficits found in the mutants. In this context, deletion of GluN2B in principal neurons leads to a reduction in expression of the GluN1 subunit, albeit without general alterations in NMDAR physiological function [6,23].

### Conclusions

In summary, the current study reveals a previously undocumented role for interneuronal GluN2B-containing NMDARs in a measure of learning. Mutant mice generated to have loss of GluN2B on forebrain interneurons showed profound, but behaviorally selective, impairments on a touchscreen-based visual discrimination task. These findings add to growing body of evidence linking interneuronal NMDARs with cognitive functions, and may have potential implications for understanding mnemonic dysfunction in psychiatric diseases, ranging from alcohol use disorder and schizophrenia, that are associated with cortical and NMDAR abnormalities [24–27].

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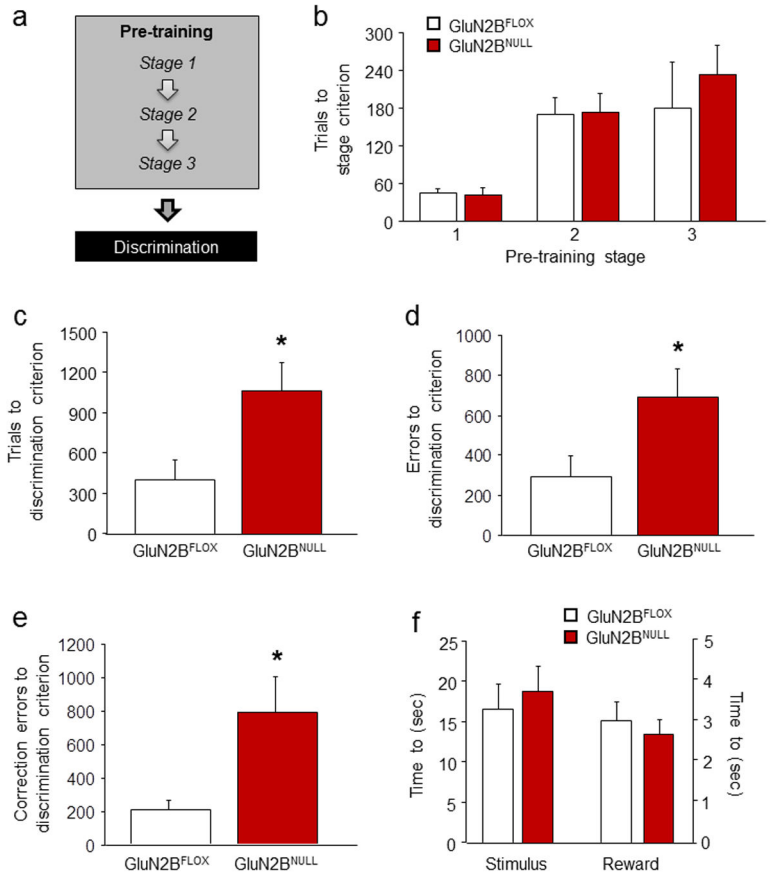


**Figure 1. GluN2B mutant mice exhibit impaired visual discrimination learning**

GluN2B$^{NULL}$ mice were trained on 3 sub-stages of pre-training and then pairwise discrimination (**A**). GluN2B$^{NULL}$ mice took a similar number of sessions to attain pre-training criteria as floxed controls (**B**), but required significantly more trials (**C**) to learn a pairwise discrimination to criterion than floxed controls. GluN2B$^{NULL}$ made significantly more first presentation errors (**D**) and correction errors (**E**) during discrimination, as compared to floxed controls. GluN2B$^{NULL}$ did not differ from floxed controls on motivation to respond to stimuli or retrieve food reward (**F**) as measured by stimulus and reward response latencies. n=6–7 mice per genotype. Data are means ±SEM. **P*<.05 mutant vs floxed control.

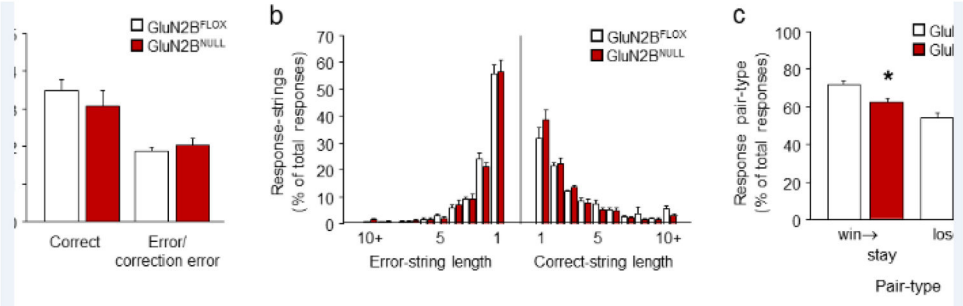


**Figure 2. GluN2B mutant mice show less win-stay performance**

On average, GluN2B$^{NULL}$ mice showed similar lengths of correct and error strings as floxed controls as measured by average string length (**A**) or examined by string-length blocks as percent of total responses made (**B**). GluN2B$^{NULL}$ control mice made significantly less win→stay response-pairs than floxed controls (**C**). n=6–7 mice per genotype. Data are means ±SEM. *$P$<.05 mutant vs floxed control.