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NR2B-dependent plasticity of adult born granule cells is necessary for context discrimination

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Abstract

Adult generated granule cells (GCs) in the dentate gyrus (DG) exhibit a period of heightened plasticity 4–6 weeks post-mitosis. However, the functional contribution of this critical window of plasticity to hippocampal neurogenesis and behavior remains unknown. Here, we show that deletion of NR2B-containing NMDA receptors from adult born GCs impairs a neurogenesis-dependent form of LTP in the DG, reduces dendritic complexity of adult born GCs, but does not impact their survival. Mice in which the NR2B-containing NMDA receptor was deleted from adult born GCs did not differ from controls in baseline anxiety-like behavior or discrimination of very different contexts, but were impaired in discrimination of highly similar contexts. These results indicate that NR2B-dependent plasticity of adult born GCs is necessary for fine contextual discrimination and is consistent with their proposed role in pattern separation.

Introduction

The mammalian DG generates new neurons throughout life (Altman & Das, 1965) that functionally integrate into the local circuitry (Toni *et al.*, 2008) and can account for up to ten percent of the GC population (Imayoshi *et al.*, 2008). Studies in which adult neurogenesis has been manipulated indicate that this process contributes significantly to behavior, and it is widely hypothesized that functional differences between immature and mature GCs underlie the unique role adult-born neurons play in DG function (Zhao *et al.*, 2008; Sahay *et al.*, 2011b).

The development of adult-born GCs is a highly plastic process that recapitulates a number of aspects of early development, including the preferential expression of NMDA receptors that contain the NR2B subunit (Cull-Candy & Leszkiewicz, 2004). There is an initial critical period from 1–3 weeks post-mitosis, during which the survival and integration of adult-born GCs is dependent on both GABAergic and glutamatergic input (Ge *et al.*, 2006; Zhao *et al.*, 2008). In particular, survival of adult born GCs near the end of this time window requires NMDA receptor activation (Tashiro *et al.*, 2006). Accumulating evidence indicates the existence of a second critical window between 4–6 weeks of cell age when adult-born GCs

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Author contributions

MAK and RH conceived and designed the experiments, interpreted results and wrote the paper. MAK performed electrophysiological recordings, behavioral experiments, circuitry and data analysis. LT performed behavioral experiments and circuitry analysis.

exhibit enhanced synaptic plasticity and impact specific behavioral functions (Ge *et al.*, 2007; Denny *et al.*, 2011). Consistently, ablation of neurogenesis prevents the induction of LTP evoked by medial perforant path (MPP) stimulation in the DG of slices with intact GABAergic transmission (ACSF-LTP), suggesting that this synaptic potentiation reflects a selective effect on adult-born GCs (Snyder *et al.*, 2001; Saxe *et al.*, 2006). This form of LTP is blocked by ifenprodil or Ro25-6981, NR2B subtype specific antagonists, demonstrating that this NMDA subunit is essential for increased synaptic plasticity (Snyder *et al.*, 2001; Ge *et al.*, 2007). Together these data indicate that during this 4–6 week window adult-born GCs are hyperplastic and may as a result make unique contributions to DG circuits and hippocampal function. However, whether this period of enhanced plasticity influences the integration of young neurons into hippocampal circuitry, or influences behavior by other means remains unknown. To test this, we have selectively deleted the NR2B subunit of the NMDA receptor from adult born GCs to study the functional impact of heightened synaptic plasticity and the requirement of NR2B for adult born GC survival.

Materials and Methods

Mice

NestinCreERT2 and $NR2B^{f/f}$ mice were generated as previously described (von Engelhardt *et al.*, 2008; Dranovsky *et al.*, 2011). Experimental male mice were generated by breeding NestinCreERT2;$NR2B^{f/f}$ with $NR2B^{f/f}$ mice. For labeling adult born GCs, NestinCreERT2;$NR2B^{f/+}$;$ROSA263^{f3stopEYFP/f3stopEYFP}$ were bred with NestinCreERT2;$NR2B^{f/+}$ to generate NestinCreERT2;$NR2B^{f/f}$;$ROSA263^{f3stopEYFP/+}$and NestinCreERT2;$NR2B^{+/+}$;$ROSA263^{f3stopEYFP+}$controls. 8–10 week-old mice were injected with 3mg tamoxifen (TMX) (VEH solution of corn oil/10%ethanol) I.P./day for 5 consecutive days. Previous work with this strain revealed that a brief pulse of TMX does not affect hippocampus-dependent behavior six weeks later (Sahay *et al.*, 2011a). All experiments were approved by the IACUC at Columbia University and the New York State Psychiatric Institute.

Immunohistochemistry

For survival experiments, 48hr after the last TMX or VEH injection, BrdU was injected i.p. (150 mg/kg). For all experiments, mice were perfused (4% paraformaldehyde), brains postfixed, cryoprotected, and sections (353m) of the entire DG were labeled for BrdU, GFAP, NeuN, DCX, Ki67 or GFP as previously described (Sahay *et al.*, 2011a)(Rat-anti-BRDU 1:100 Serotec, Rabbit-anti-GFAP 1:1500, DAKO, mouse-anti-NeuN, 1:500, Chemicon, goat-anti-DCX, 1:500, SantaCruz; rabbit-anti-Ki67, 1:100, Vector). Experimenters blind to treatment counted (at 20X) BrdU, DCX and Ki67 cells in every 6^{th} section throughout the DG, and DCX cells exhibiting tertiary dendrites were counted. For co-labeling of BRDU/NeuN/GFAP, confocal scans (FluoView1000, Olympus) at 40X, were taken of 60 BrdU cells/treatment across the AP axis of the DG. For EYFP/DCX counts, 120 DCX+ cells were imaged across the AP axis of the DG to determine co-labeling with EYFP, excluding type-1 cells determined by their radial-glia morphology. Sholl analysis was conducted as previously described (Sahay *et al.*, 2011a), analyzed with the ImageJ Sholl Analysis plug-in (http://www-biology.ucsd.edu/labs/ghosh/software/).

Slice electrophysiology

400um transverse hippocampal slices were incubated in an interface chamber at 32°C and perfused with oxygenated ACSF (in mM: 11NaCl, 2.5KCl, $1.3MgSO_4$, $2.5CaCl_2$, $26.2NaHCO_3$, $1NaH_2PO_4$, 11glucose). Slices equilibrated for 2h before a bipolar stimulating electrode was placed in the MPP and evoked field potentials were recorded in the molecular layer using a glass capillary microelectrode (1–3MΩ) filled with ACSF. Isolation of the

MPP was confirmed by assessing paired-pulse depression (PPD) of the MPP/DG synaptic connection at 50ms, which generated the highest level of depression (McNaughton, 1980). Input–output curves were obtained after 10min of stable recordings. The stimulation intensity that produced 1/3 of the maximal response was used for the test pulses and tetanus. After 15min of stable baseline (once every 20s), LTP was induced with a four trains of 1s each, 100Hz within the train, repeated every 15s. Responses were recorded every 20s for 60min after LTP induction. The same protocol was used to elicit and record LTP of mature GCs except that 103M bicuculline was added to the ACSF to block $GABA_A$-receptor-mediated inhibition.

Behavioral experiments

All behavioral experiments were conduced in male mice 14–16 weeks of age. For each test, time after TMX or VEH injection is noted in the figures or text.

Open field—Mice were placed in an acrylic open-field chamber 40cm long×40cm wide×37cm high with illumination set to 16-20lux (low lux) or 600lux (high lux). Data were collected in 5 min bins during 60 min trials and analyzed for total distance traveled as well as time and distance traveled in center.

Forced swim test, novelty suppressed feeding and elevated plus maze—FST, NSF and EPM were conducted as previously described (Dulawa *et al.*, 2004; David *et al.*, 2009; Sahay *et al.*, 2011a)

Novel object recognition—Naive mice were placed in a storage container (45×30×30 cm) with woodchip bedding, and videotaped from above at a light intensity of 16-20lux. Two objects were placed at either end of the arena (a slide box, a clear funnel or a white ceramic shoe, randomized presentation). Mice explored two objects for 4 trials of 5min, with a 3min inter-trial interval (during which the box would be wiped clean and the bedding changed), then one object was replaced with a novel object. An experimenter blind to the treatment condition analyzed videotapes, and total approaches, investigation time and grid crossings were measured for each of the trials.

Fear conditioning—Conditioning took place in Coulbourn fear conditioning boxes that contained one clear plexiglass wall, three aluminum walls and a stainless steel grid floor. In the one-trial contextual fear conditioning, the training context (A) included a houselight and fan, and anise scent was placed under the grid floor. 180s later, mice received single 2s foot shock of 0.75mA. Mice were taken out 15s after termination of the foot shock and returned to their home cage. The box was cleaned with Sanicloths between runs. Digital video cameras recorded the session, FreezeFrame and Freeze View software (Actimetrics) were used for recording and analyzing freezing behavior. For the dissimilar context, C, the grid floor was covered with a plastic panel and cage bedding. The chamber walls were covered and made circular using plastic inserts, the house fan and lights were turned off, and a mild lemon scent was placed below the grid floor. The chamber door was left ajar during testing. 70% ethanol used to clean the chamber between runs.

Naïve mice were used for contextual fear discrimination. The training context, A, was the same as used above for one-trial fear conditioning. The dissimilar context, C, was as described above, and the similar (no-shock) context, B, shared many features of the training context including an exposed stainless steel grid floor, but differed in that two plastic inserts were used to cover the walls and make them circular, the house fan and lights were turned off, and the chamber door was left ajar during testing. A lemon scent was used as an olfactory cue, and 70% ethanol was used to clean the grids between runs. Mice were brought

into the testing room in transport buckets by the same experimenter who had handled the mice for the training context. After 2h, mice were placed in the similar context, in which they were left for 180s and were never shocked. Measurement of the freezing levels in both the training context (3-min pre-shock) and the similar context (3min) each day allowed the assessment of freezing in the two contexts. The order of presentation of contexts was randomized across days.

Results

Mice in which exon 9 of the NR2B gene was flanked by two loxP sites ($NR2B^{2lox}$) (von Engelhardt *et al.*, 2008) were bred with $NestinCreER^{T2}$ mice in which the TMX regulated Cre-recombinase ($CreER^{T2}$) is expressed under the control of a nestin promoter fragment (Dranovsky *et al.*, 2011). TMX injection in adult mice excises sequences between the loxP sites selectively in neural stem cells and transit-amplifying progenitors to generate adult-born GCs lacking NR2B ($iNR2B^{Nes}$). Using enhanced yellow fluorescent protein (EYFP), as a surrogate for NR2B recombination and marker for adult born GCs (Fig 1A–B), we found that 71.9 +/− 7.2% of doublecortin (DCX)-positive neurons expressed EYFP six weeks after TMX induction.

We next assessed whether NR2B is required for young GC survival. In animals expressing the EYFP reporter, young GC numbers did not significantly differ between $NR2B^{f/f}$ and $NR2B^{+/+}$ littermates (Fig 1C, unpaired t-test, n=3/geno, t_4=0.79, p=0.49). In addition, BrdU pulse-chase experiments indicated that NR2B deletion did not affect survival of 2, 4, 6 or 8 week-old neurons, as no difference was seen in number of BrdU positive cells, or phenotype of cells born after NR2B deletion (Figure 1E, F, n=3–7/treatment, unpaired t-test, 2wk, t_4=1.3, p=0.3, 4wk, t_4=0.6, p=0.6, 6wk t_7=−1.4, p=0.2, 8wk, t_{11}=−1.7, p=0.13, percent BRDU/NeuN t_4=1.87, p=0.14). There was also no difference between $iNR2B^{Nes}$ and controls in progenitor proliferation as measured by Ki67+ cells (Figure 1E, n=5 TAM, 4 VEH, unpaired t-test, t_7=−1.5, p=0.18), or in generation of immature DCX-positive neurons (Figure 1F&I, n=5 TAM, 4 VEH, unpaired t-test, t_7=−0.7, p=0.52). We next assessed dendritic complexity of adult born GCs lacking NR2B. Although within the DCX population the number of cells that exhibited tertiary dendrites did not differ between TMX and VEH treatment (Figure 1J, n=5 TAM, 4 VEH, unpaired t-test, t_7=−0.8, p=0.44), Sholl analysis on the dendrites of EYFP+ neurons from $NR2B^{f/f}$ and $NR2B^{+/+}$ littermates six weeks after TMX injection revealed a decrease in dendritic complexity after deletion of NR2B (Figure 1K, N=12–15 cells/3 mice/treatment, repeated measure ANOVA, genotype X distance interaction $F_{(28,700)}$=1.5, P=0.03)

To determine the consequence of NR2B deletion in adult-born neurons on synaptic plasticity in the DG, we measured neurogenesis-dependent ACSF-LTP in slices taken 6–7 weeks after VEH or TMX treatment. Deletion of NR2B did not disrupt the input-output relationship (Fig 2B, n=12 slices/genotype, repeated measures ANOVA, treatment effect $F_{(1,22)}$=0.85, p=0.37, treatment X intensity interaction $F_{(19,418)}$=1.1, p=0.38) or paired pulse depression of fEPSPs evoked by MPP stimulation (Fig 2C, t_{21}=1.08, p=0.29). However, ACSF-LTP induction by high frequency stimulation (four 1 s, 100 Hz trains every 15 s) was absent in $iNR2B^{Nes}$ slices (Figure 2D, repeated measures ANOVA, last 10 minutes treatment effect $F_{(1,22)}$=25.5, p<0.001). In contrast, LTP obtained in the presence of the $GABA_A$ receptor antagonist bicuculline did not differ between groups (Figure 2E, n=4 VEH, 7 TMX, repeated measures ANOVA, last 10 minutes treatment effect $F_{(1,9)}$=0.9, p=0.37). These results suggest that the mature GCs exhibit normal LTP and that the deficit in ACSF-LTP is due to loss of NR2B in the immature neuron population. These $iNR2B^{Nes}$ mice provide therefore a model to test the contribution of the enhanced synaptic plasticity of immature neurons to behavior.

We next tested iNR2B^{Nes} mice in a number of depression/anxiety-related behavioral assays. In neither low or high lux open field testing did the iNR2B^{Nes} mice differ from controls in locomotor activity, habituation, or percent time in center, but in the low lux condition they showed a trend to less distance traveled in the center of the arena (Fig 3A–F, low lux, n=13/treatment, total distance traveled: treatment effect unpaired t-test, t_{24}=0.15, p=0.9, treatment X time interaction $F_{11,264}$=1.19, p=0.29, percent time in center, t_{24}=−1.5, p=0.15, percent center distance, t_{24}=−2.03, p=0.05, high lux, total distance traveled: treatment effect, t_{10}=0.14, p=0.9, treatment X time interaction $F_{10,110}$=0.89, p=0.55, percent time in center, treatment effect, t_{10}=−0.5, p=0.63, percent center distance, t_{10}=−0.7, p=0.9). In addition, as seen in irradiated animals at baseline (Santarelli *et al.*, 2003; David *et al.*, 2009), iNR2B^{Nes} mice did not significantly differ from control mice in immobility time in the forced swim test (FST), in open arm time in the elevated plus maze (EPM) or latency to feed in the novelty suppressed feeding task (NSF) (Figure 3G–I, n=6/treatment, FST, treatment effect $F_{(1,10)}$=0.01, p=0.9, treatment X time interaction $F_{(5,50)}$=0.6, p=0.7, EPM, $t_{10=}$1.9, p=0.08, NSF n=12–13/treatment, Kaplan-Meier Survival analysis, Mantel-Cox log-rank test, p=0.6, unpaired t-test t_{23}=1.2, p=0.26).

Recently, arresting adult neurogenesis has been shown to impact novelty exploration (Jessberger *et al.*, 2009; Denny *et al.*, 2011). To test whether impaired DG plasticity in iNR2B^{Nes} mice influences novelty exploration, mice explored two objects for 5 minutes for 4 consecutive trials, then one object was replaced with a novel object, and number of approaches to the novel object were measured (Fig 4A). iNR2B^{Nes} mice explored the objects and the arena less than control mice during early training trials, yet they habituated to arena and objects similar to control mice (Figure 4B, N=12/treatment, total object approaches: repeated measures ANOVA genotype effect $F_{(1,22)}$=6.03, p=0.02, genotype X trial interaction $F_{(3,66)}$=0.34, p=0.8, arena grid crossings,, ANOVA genotype effect $F_{(1,22)}$=6.23, p=0.021, geno X trial interaction $F_{(4,88)}$=0.75, p=0.56). When the familiar object was replaced with a novel object, iNR2BNes did not discriminate between the two objects. (Figure 4C, repeated measures ANOVA genotype effect $F_{(1,22)}$=5.6, p=0.03, genotype X object interaction $F_{(2,22)}$=12.2, p=0.002, discrimination ratio (novel-familiar/total) VEH=0.495+/−.08, TMX=0.023 +/−.13, p=0.005), further highlighting a deficit in exploration in iNR2B^{Nes} mice.

We next tested iNR2B^{Nes} in a one-trial contextual fear conditioning. iNR2B^{Nes} mice did not differ from control mice in time spent freezing in the shock context “A” 24 hours after training, or in a significantly different context presented 48 hours after training suggesting that rapid encoding of contextual information was unaffected (Figure 4E, n=25 VEH, 24 TMX, repeated measures ANOVA, treatment effect, $F_{(1,47)}$=0.8, p=0.4, treatment X training effect $F_{(1,47)}$=0.54, p=0.46). To assess contextual discrimination, iNR2B^{Nes} mice were tested in two fear discrimination tasks that requires mice to either distinguish between two very different contexts (Figure 4F–G), or two highly similar contexts (Figure HI). Both groups of mice could efficiently discriminate between the two different contexts (Figure 4G, n=6/group, treatment effect, $F_{(1,20)}$=0.9, p=0.4, , context effect, $F_{(1,20)}$=40.8, p<0.001, genotype X day X context interaction $F_{(2,40)}$=0.04, p=0.96). Yet, when tested to discriminate between two highly similar contexts, freezing scores revealed that iNR2B^{Nes} mice took longer to learn the task, suggesting impaired fine discrimination in iNR2B^{Nes} mice (Figure 4G, n=15–17/treatment, repeated measures ANOVA, day X geno X context interaction $F_{(7,210)}$=2.39, p=0.023, VEH: context effect $F_{(1,28)}$=7.68, p=0.01, day X context interaction $F_{(7,196)}$=7.23, p<0.0001, TMX: context effect $F_{(1,32)}$=2.6, p=0.12, day X context interaction $F_{(7,224)}$=2.13, p=0.04). By day 4, control mice could efficiently discriminate between the contexts, while iNR2B^{Nes} required 8 days to significantly discriminate between the contexts (Fig 4H, day 4, repeated measures ANOVA, treatment X context interaction $F_{(1,30)}$=5.6, p=0.02).

Discussion

Adult hippocampal neurogenesis, a unique form of DG plasticity, is regulated by behavioral experience, and when manipulated can have specific effects on behavior. Recent hypotheses have suggested that the enhanced synaptic plasticity exhibited by 4–6 week old GCs allows them to uniquely contribute to hippocampal circuit function, and thus behavior, yet this has been difficult to test. Here we have examined the impact of this critical window of heightened plasticity on the function of young GCs. Deletion of the NR2B subunit from adult-born GCs resulted in an absence of ACSF-LTP similar to what is observed in neurogenesis ablation models confirming that adult born GCs are necessary for this form of plasticity via synaptic NR2B containing NMDA receptors. Surprisingly, unlike NR1 deletion (Tashiro *et al.*, 2006), deletion of NR2B did not affect cell survival, possibly due of residual non NR2B-containing NMDA receptors. Deletion of NR2B did reduce dendritic complexity, highlighting the differential role of NR2B in dendritic patterning of developmentally and adult born GCs (Espinosa *et al.*, 2009).

Lesion and genetic studies have documented a role for the DG in novelty exploration (Hunsaker *et al.*, 2008). The DG may play a role in both novelty encoding and context discrimination by contributing to the extraction of novel features of an environment to facilitate the storage of similar experiences as distinct representations. iNR2B^{Nes} mice showed a deficit in novelty exploration, which manifested as a decrease in novel object exploration. While arrest of adult neurogenesis has been shown to impact novelty recognition in mice (Denny *et al.*, 2011) and rats (Jessberger *et al.*, 2009), a number of studies have specified a role for the perirhinal cortex in object recognition memory (Winters & Bussey, 2005), suggesting that hippocampus may only play a role in object memory when contextual cues are used to support recognition (Winters *et al.*, 2004). As iNR2B^{Nes} mice were tested in the presence of extra-maze cues, this may have contributed to their decrease in novelty recognition. Alternatively, the decrease in object exploration during training may also have been responsible for their deficit in object recognition.

Multiple studies indicate that the DG, and specifically young GCs, play a role in pattern separation (Clelland *et al.*, 2009; Sahay *et al.*, 2011a; Nakashiba *et al.*, 2012). Ablation of adult neurogenesis impairs appetitive spatial pattern separation as measured in a two choice touch-screen task and a nonmatching to place radial arm maze task (Clelland *et al.*, 2009). More recently, it has been shown that either increasing or decreasing levels of adult neurogenesis has bidirectional effects on the ability to distinguish between similar contextual representations in a fear discrimination task (Sahay *et al.*, 2011a; Nakashiba *et al.*, 2012). Here, we show that iNR2B^{Nes} mice are impaired in this task, similar to what was found in irradiated mice (Sahay *et al.*, 2011a; Nakashiba *et al.*, 2012). These results suggest that the critical window of heightened plasticity of young neurons is necessary for their contribution to fine contextual discrimination. Thus, these studies indicate that the main contribution of adult–born neurons to hippocampal function occurs during their critical 4–6 week period of heightened plasticity and that this heightened plasticity modulates the DG/CA3 circuit to facilitate pattern separation.

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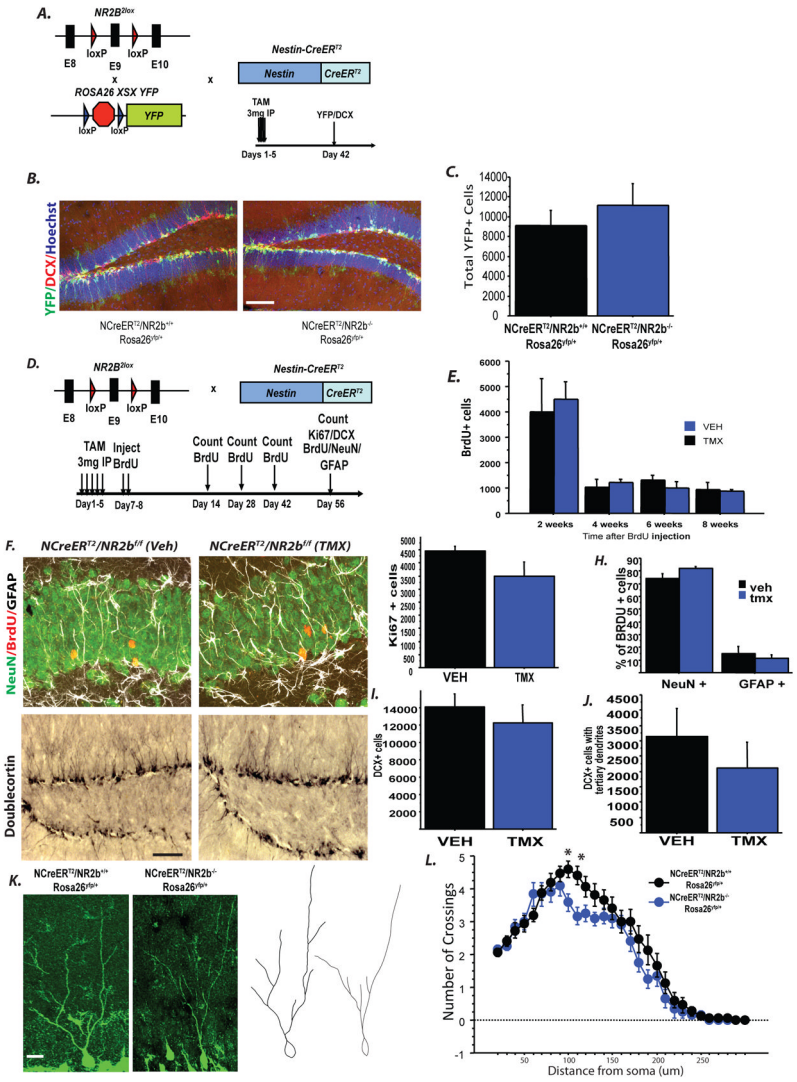

Figure 1. Deletion of NR2B in adult born GCs does not affect neurogenesis in the DG

A–B 3-mouse design using an inducible reporter, EYFP, to measure GC survival and TMX-induced recombination with (**B)** representative images of YFP and DCX expression six weeks after TMX injection (scale bar represents 100um). **C.** Total number of adult born GCs did not differ between NR2B deficient and WT littermate controls six weeks after TMX injection as measured by total numbers of EYFP cells. **D.** Genetic and experimental timeline for neurogenesis measurement. **E.** No effect on cell survival as measured by BRDU number at specified times post-injection. **F.** Representative images for BRDU (NeuN-green, BrdU-red, GFAP- white) and DCX experiments (scale bar: *top* 30um, *bottom* 100um). **G.** No effect of treatment on total Ki67 immunoreactive cells. **H.** No significant difference in phenotype of BRDU cells 8 weeks after induction. **I&J.** No significant difference in total number of DCX cells, or DCX+ cells with tertiary dendrites. **K&L** Sholl analysis revealed a decrease in dendritic complexity in adult born GCs lacking NR2B (left: representative images and tracings, scale bar 20um). Data are mean +/− SEM.

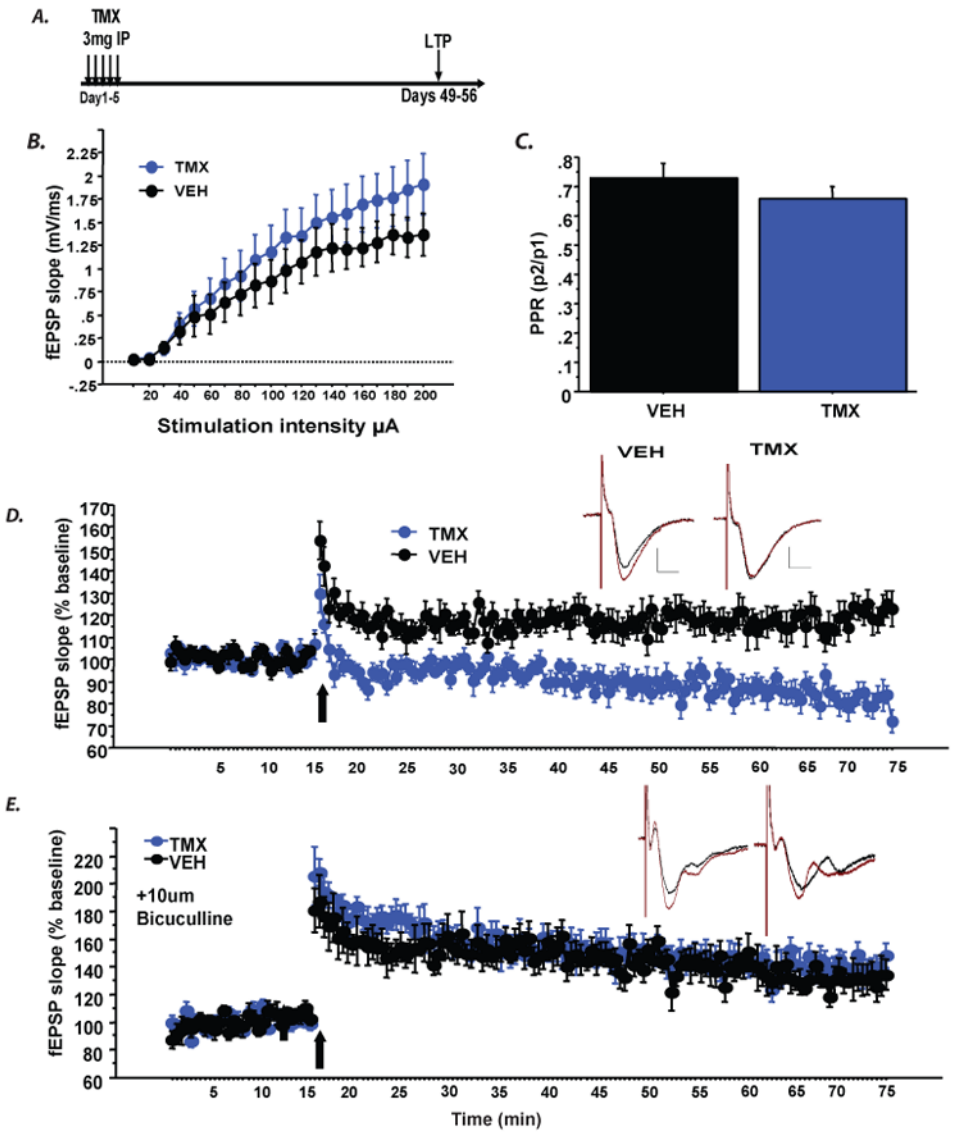

Figure 2. Impaired ACSF-LTP in the DG of iNR2B^{Nes} mice

A. Experimental timeline for electrophysiology experiments. No differences in **(B)** input-output relationship or **(C)** paired pulse depression (50ms ISI) of MPP inputs to the DG after deletion of NR2B. **D.** Significantly impaired ACSF-LTP in slices from iNR2B^{Nes} mice as compared to controls. Inset: representative average traces before and after HFS, scale bars represent 0.5mV and 5ms. **E**. No difference in magnitude of LTP in slices in the presence of 10um bicuculline. Data are mean +/− SEM.

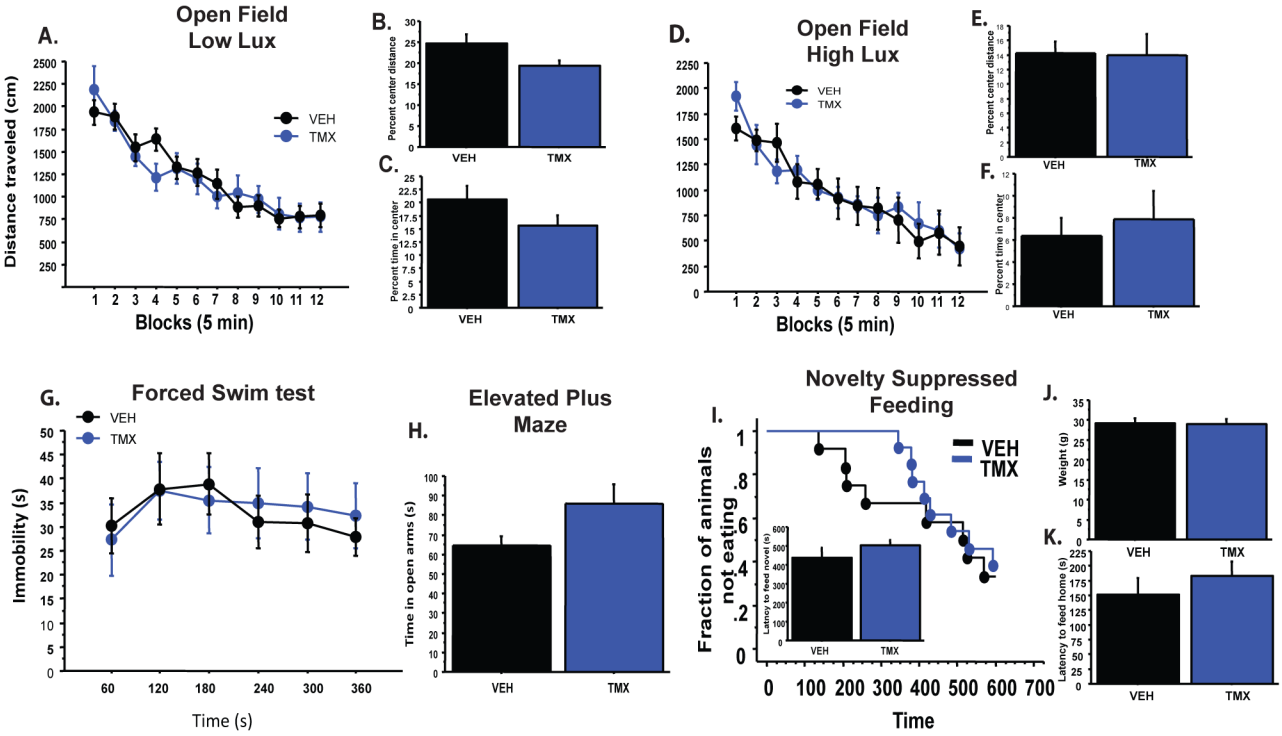

Figure 3. iNR2B^{Nes} mice in depression/anxiety-related behavioral assays

A–F. Open field test under low and high lux conditions with total distance traveled (A,D), percent center distance (B,E) and percent center time (C,F). **G.** Forced swim test. **H.** Elevated Plus Maze. **I.** Novelty suppressed feeding task. iNR2B^{Nes} mice did not differ from controls in FST, EPM or NSF. Data are mean +/− SEM.

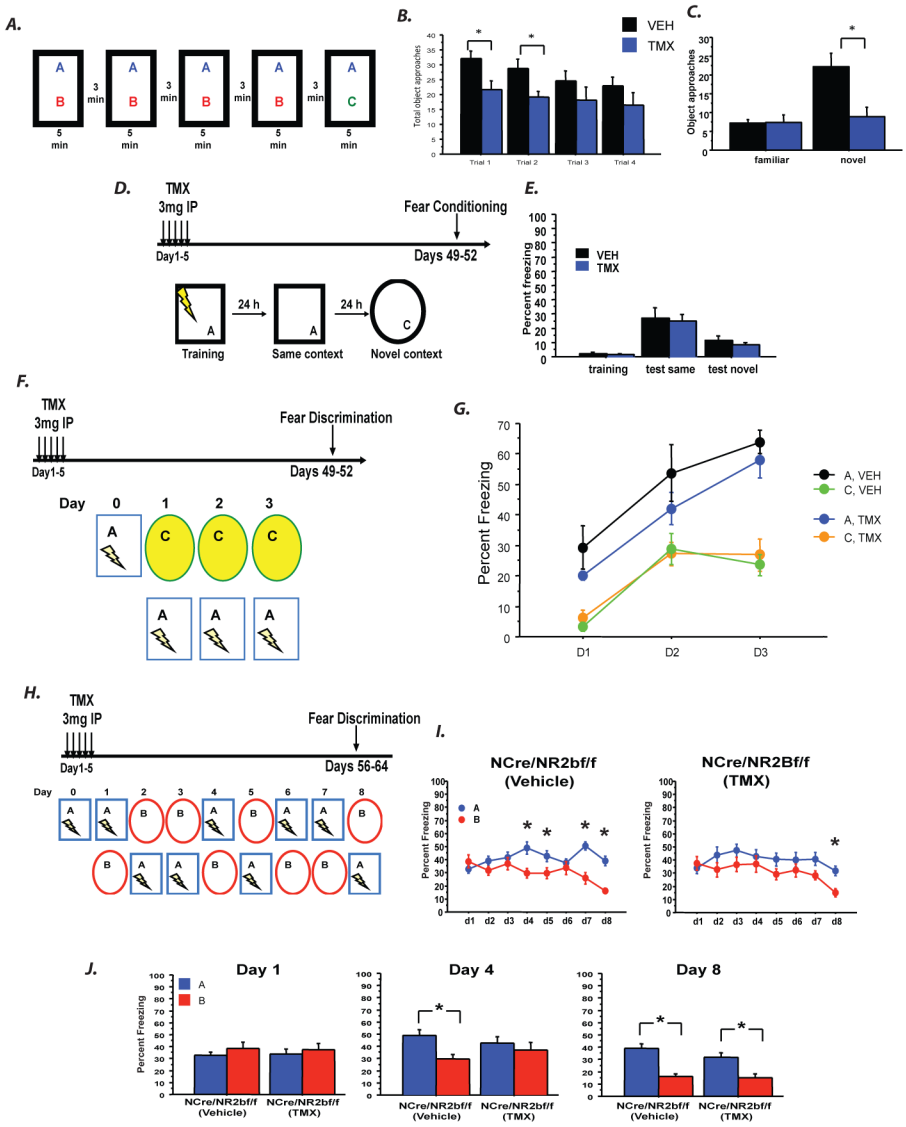

Figure 4. Impaired object exploration and contextual fear discrimination in iNR2B^{Nes} mice

A. Experimental design for novelty exploration. **B–C** Total approaches to the objects during training trials and testing reveal iNR2B^{Nes} mice explored the novel objects significantly less than control mice. **D–E.** iNR2B^{Nes} mice did not differ from controls in an one-shock contextual fear conditioning protocol, or did not generalize freezing to a novel context. **F–G.** iNR2B^{Nes} mice did not differ from controls in discriminating between two different contexts (experimental design in F). **H–I.** iNR2B^{Nes} mice were slower to discriminate two similar contexts than VEH treated controls (experimental design in H). **J.** VEH treated controls could discriminate by the 4th day of training, while iNR2B^{Nes} could not discriminate until the 8th day of training. Asterisks represent $p<0.05$, data are mean +/− SEM.