Supplemental Information for **Fuccillo et al.,**
"*Autism-Associated Neuroligin-3 Mutations Commonly Impair Striatal Circuits Underlying Repetitive Behavior***"**

1. SUPPLEMENTAL FIGURE LEGENDS

Figure S1 (related to Figure 1): **Additional behavioral data for NL3-KO and NL3-R451C mutant mice documenting their shared phenotype**

(A) No differences in single-trial rotarod performance across a range of speeds for separate groups of NL3-KO mice (4-40: 22 WT & 23 KO; 6-60: 9 WT & 9 KO; 8-80: 13 WT & 10 KO) and NL3-R451C mice (4-40: 19 WT & 16 R451C; 6-60: 14 WT & 15 R451C; 8-80: 13 WT & 10 R451C). This result shows that initial motor coordination in WT and mutant mice was identical.

(B) No differences in time spent immobile during the forced swim test in NL3-KO mice (8 WT & 9 KO). Data demonstrate that NL3 mutant mice behave normally under stressful conditions.

(C) Illustration of linear regression analysis of rotarod performance. Averaged data from NL3-R451C mice and WT littermates (solid lines) are used to illustrate how lines of best fit were calculated using linear regression, yielding intercept values (dotted lines, used to estimate initial motor coordination) and slope values (hashed lines, used to estimate rate with which the repetitive motor routine is acquired) for each individual mouse.

(D-G) Rotarod performance of NL3-KO mice (upper panels, 25 WT & 27 KO) and R451C mutants (lower panels, 9 WT & 13 R451) maintained on a hybrid 129Sv x C57Bl/6 genetic background to demonstrate that the NL3 mutant phenotype is robust, and independent of genetic background. (D) Mean time to fall off the rotarod across nine trials under "standard" conditions (4 to 40 rpm over 300 s). Note that rotarod performance is decreased on this hybrid background, reducing the ceiling effect that obscured phenotypes in B6 mice under standard conditions. (E) Percentage of mice reaching maximum performance (300 s) as a function of trial. Note that fewer WT mice reach maximum on the hybrid genetic background, even after 9 trials, while almost half of NL3 mutants reach maximum by the end of 9 trials. (F) Initial coordination for each genotype, derived from the intercept value of linear regression performed on data from each individual mouse. (G) Learning rate (i.e., slope from linear regression) was significantly enhanced by both NL3 mutations.

(H-L) Video analysis of rotarod performance in WT mice to identify components of the motor routine that become less variable ('stereotyped') with training. (H & I) Vertical location and timing of hind paw steps during trial 1 (H) and trial 6 (I) at 4 to 40 rpm. Each dot marks the initiation of an individual step, with location represented vertically and time represented horizontally (upper panels, entire period of analysis (50-100s); lower panels, expansion of the bracketed epoch). (J) Average distribution of vertical step position on trials 1 and 6. Note that the distribution tightens and becomes less variable with training, as indicated by a statistically significant interaction between trial and step position. (K) Consistent decreases in the standard deviation of step position across trials for individual mice (black lines), with a significant reduction in the average standard deviation. (L) Consistent decreases in the standard deviation of step timing across trials for individual mice (black lines), with a significant reduction in the average standard deviation.

(M-P) Open field activity in NL3-KO (upper panels) and NL3-R451C mutant mice (lower panels). (M) Similar velocities of ambulatory movement. (N) No differences in percent distance traveled in the center of the open field during the first five minutes of the session, suggesting similar anxiety-like behaviors. (O) Significant increases in time spent ambulatory during the 60 min test session. (P) Significant increases in time spent performing stereotypic movements in a confined area.

(Q-R) No correlation between total distance travelled in the open field and learning rate on the rotarod for NL3 mutant mice (Q) or WT littermates (R), showing that hyperactivity and enhanced formation of repetitive motor routines are not related.

Data are means ± SEM; *significant difference between groups (ANOVA).

Figure S2 (related to Figure 2): **Detailed targeting strategy for the generation of NL3-cKO mice, verification of homologous recombination by Southern blotting, and behavioral results of genetic crosses of NL3-cKO mice with parvalbumin (PV)-Cre mice**

(A) Gene targeting strategy for Nlgn3*KI-mVenus mice. The targeting vector introduces a knock-in (KI) cassette flanked by frt sites, replicating the endogenous intron/splice acceptor upstream of exon 2, which harbors the start codon for Nlgn3. A modified NL3 cDNA (Nlgn3*) containing an mVenus tag and mutations abolishing neurexin binding (shown in yellow) is followed by a PGK-neo selection cassette flanked by F3 sites (shown in blue). The KI cassette is immediately followed by a single loxP511 site, with a second loxP511 site located in the intron downstream of exon 3. After successful homologous recombination into the endogenous locus ("KI+neo"), Flp-recombinase activity will either remove the PGK-neo selection cassette ("KI w/o neo"), or delete the entire KI cassette and leave loxP511 sites flanking exons 2 and 3 ("cKO"). The latter allele is subject to Cre-mediated recombination, allowing conditional deletion of NL3. DT: diphtheria toxin mini gene for negative selection; ScaI and EcoRV: restriction sites for Southern blot analysis.

(B) Southern blot analysis of each allelic variation leading to generation of the NL3-cKO line. Note that Nlgn3 is located on the X chromosome, and the WT-band for "KI w/o neo" represents heterozygosity in the only genomic DNA sample from a female mouse. Positive fragments can be observed after Southern blot analysis using outside probes hybridizing to the 5' homology region (after ScaI digest of genomic DNA), the 3' homology region (EcoRV digest) and mVenus (internal probe, SpeI digest, sites not depicted).

(C-F) Rotarod performance of control mice (NL3-cKO, n=11) and those carrying PV-Cre (n=13). Time to fall off is presented at 4 to 40 rpm (C) and 8 to 80 rpm (D); the terminal speed of rotation (E) was used to calculate initial coordination and learning rate (F).

(G-H) Behavior of the same mice in a test of open field activity, showing time course of activity across the entire session (G) as well as total distance travelled and number of ambulatory episodes (H).

Data are means ± SEM; *significant difference between groups (ANOVA).

Figure S3 (related to Figures 3 & 4): **Conditional deletion of mutant NL3-R451C protein has no impact on the enhanced formation of a repetitive motor routine**

(A) Illustration of the NL3-R451C allele (described in Tabuchi et al., 2007), showing that loxP sites flank the mutated exon 7 (left). Cre-recombinase deletes exon 7, removing a large part of the esterase homology domain of NL3 and causing a frame shift, thereby effectively producing a KO. Brain tissue infected with AAV-Cre virus exhibits a loss of NL3 mRNA which becomes non-detectable in R451C samples (right). Note that without cre-recombinase, the R451C-NL3 KI mRNA is expressed at wild-type levels (Tabuchi et al., 2007). This and other evidence demonstrates that NL3 is specifically expressed in neurons since Cre expression is driven by the synapsin promoter.

(B-D) Rotarod performance of control mice (n=13) and NL3-R451C mutant mice (n=4), all carrying the D1-Cre allele. Time to fall off (B) was used to calculate initial coordination (C) and learning rate (D). Note that in this experiment and the next, the rotarod protocol consisted of 9 trials at 8 to 80 rpm.

(E-G) Rotarod performance of WT mice (n=7) and NL3-R451C mutant mice (n=6) following viral injection of AAV-Cre into the NAc.

Data are means ± SEM; *significant difference between groups (ANOVA).

Figure S4 (related to Figure 3): **Quantitative RT-PCR of single-cell RNA for MSN subtype-specific probes in the dorsal striatum, and normalized mRNA abundance for neuroligins in single MSNs from both the NAc and the dorsal striatum**

(A) Illustration showing aspiration of cytoplasm from individual D1- and D2-MSNs of the dorsal striatum (top), and quantitative RT-PCR results from individual cells showing normalized mRNA expression of cell type-specific markers (bottom).

(B) Relative mRNA expression of cell type-specific markers in the dorsal striatum, quantified as the ratio between D1- and D2-MSNs.

(C-D) Normalized abundance of NL1, NL2, and NL3 mRNAs for both D1- and D2-MSNs in the NAc (C) and dorsal striatum (D), normalized to the average expression level of a panel of internal control probes (see Supplemental Methods).

Data are means ± SEM; *significant difference between cell types (ANOVA).

Figure S5 (related to Figure 7): **Additional measures of excitatory synaptic function in NL3-KO MSNs of the NAc**

(A&C) Representative traces (top) and summary graphs (bottom) for paired-pulse ratios of evoked EPSCs in D1-MSNs (A) and D2-MSNs (C).

(B&D) Representative traces (top) and summary graphs (bottom) for NMDAR/AMPAR ratios in D1-MSNs (B) and D2-MSNs (D).

(E&G) Representative traces (top) and summary graphs (bottom) for rectification of AMPAR EPSCs in D1-MSNs (E) and D2-MSNs (G).

(F&H) Representative traces (top, dashed red line indicates bi-exponential fit) and summary graphs (bottom) for the weighted decay of NMDAR EPSCs in D1-MSNs (F) and D2-MSNs (H).

Data are means ± SEM.

Figure S6 (related to Figure 7): **Additional measures of inhibitory synaptic function and structure in the striatum of NL3 mutant mice**

(A) Average frequency (left) and amplitude (right) of mIPSCs in D1-MSNs of the NAc, recorded from WT or NL3-R451C mutant mice. Hatched bars indicate physiology data collected from NL3-R451C mutant mice; note the significant decrease in mIPSC frequency, similar to NL3-KO mice, thus confirming a common electrophysiological phenotype at inhibitory synapses onto D1-MSNs.

(B-C) Average frequency (left) and amplitude (right) of mIPSCs in D1-MSNs of the dorsal striatum, recorded from NL3-KO (B) and NL3-R451C (C). Hatched bars indicate physiology data collected from NL3-R451C mutant mice.

(D-E) Representative traces (top) and summary graphs (bottom) for paired-pulse ratios of evoked IPSCs (D) and short-term plasticity at 10 Hz stimulation (E) in D1-MSNs.

(F) Percent suppression of evoked IPSCs by (1,2,5,6-Tetrahydropyridin-4-yl)methylphosphinic acid (TPMPA), a low-affinity antagonist of GABA receptors. This assay is sensitive to changes in the concentration of synaptically released GABA but there was no change in percent suppression in D1-MSNs from NL3-KO mice, suggesting that there is no change in synaptic GABA concentration.

(G-I) Immunohistochemical analysis of perisomatic inhibitory synapse numbers, with representative images of single confocal sections co-stained for tomato and VGAT (G), average values and individual VGAT density per cell (H), and cumulative distribution plot of VGAT density per cell for all WT and NL3-KO cells (I). Note that there is no detectable difference between WT and NL3-KO neurons.

Data are means ± SEM; *significant difference between groups (ANOVA).

Figure S7 (related to Figure 7): **Rescue of behavioral phenotypes caused by deletion of NL3 with targeted expression of DIO-Kir in D1-MSNs of the NAc, and model depicting how MSN subtype-specific activity in the NAc influences behavior**

(A) Illustration of the strategy to restore normal activity in D1-MSNs of the NAc following NL3 deletion, by viral injection of AAV-DIO-Kir2.1 in NL3-cKO mice crossed with D1-Cre.

(B-E) Rotarod performance of control mice (NL3-cKO, n=4) and those carrying D1-Cre (n=6). Time to fall off is presented at 4 to 40 rpm (B) and 8 to 80 rpm (C); the terminal speed of rotation (D) was used to calculate initial coordination and learning rate (E).

(F-G) Behavior of the same mice in a test of open field activity, showing time course of activity across the entire session (F) as well as total distance travelled and number of ambulatory episodes (G).

(H-I) Model: normal wild-type (WT) conditions involve balanced activity in D1- and D2-MSNs of the NAc (H). This balance can be disrupted by viral expression of a potassium channel subunit (Kir)

that downregulates activity in one specific cell type. Rotarod learning is diminished by downregulation of D1-MSN activity (I). Conversely, rotarod learning is enhanced by downregulation of D2-MSN activity (J). Deletion of NL3 impedes synaptic inhibition onto D1-MSNs of the NAc and thereby enhances rotarod learning (K).

Data are means ± SEM.

2. SUPPLEMENTAL TABLE

Table S1 (related to Figure 1)
Gain-of-function phenotypes on the accelerating rotarod in mice carrying ASD-associated genetic mutations

Mutant Mouse Line	Rotarod Phenotype	Human Gene Mutations Associated with ASDs
Neurexin-1α knockout (Etherton et al., 2009)	Similar coordination Enhanced learning	(Bucan et al., 2009; Ching et al., 2010; Glessner et al., 2009; Kim et al., 2008; Levy et al., 2011; Marshall et al., 2008; Sanders et al., 2011; Szatmari et al., 2007; Zahir et al., 2008)
15q11-13 duplication (Nakatani et al., 2009)	Similar coordination Enhanced learning	(Baker et al., 1994; Bolton et al., 2004; Bundey et al., 1994; Cook et al., 1997; Gurrieri et al., 1999; Keller et al., 2003; Koochek et al., 2006; Marshall et al., 2008; Sanders et al., 2011; Sebat et al., 2007; Thomas et al., 2003)
PTEN deletion (Kwon et al., 2006)	Similar coordination Enhanced learning	(Boccone et al., 2006; Butler et al., 2005; Buxbaum et al., 2007; Goffin et al., 2001; Herman et al., 2007; O'Roak et al., 2012; Orrico et al., 2009; Varga et al., 2009)
Neuroligin-3 R451C (Chadman et al., 2008)	Similar coordination Enhanced learning	(Jamain et al., 2003; Levy et al., 2011; Sanders et al., 2011; Steinberg et al., 2012; Yu et al., 2013)
CNTNAP2 knockout (Penagarikano et al., 2011)	Better performance on a single trial	(Alarcon et al., 2008; Arking et al., 2008; Bakkaloglu et al., 2008; O'Roak et al., 2011)

References for human gene mutations are representative and not exhaustive. Mouse studies that failed to detect a phenotype are not presented, as some lacked adequate sensitivity to detect improved learning phenotypes, due either to inadequate task difficulty or an insufficient number of training trials.

3. SUPPLEMENTAL EXTENDED EXPERIMENTAL PROCEDURES

Drugs. All drugs were purchased from Tocris except picrotoxin (Sigma).

Animals. Generation of the NL3-KO and NL3-R451C (Tabuchi et al., 2007), Nestin-Cre (Tronche et al., 1999), L7-Cre (Barski et al., 2000), PV-Cre (Hippenmeyer et al., 2005), D1-Cre (Gong et al., 2007), A2a-Cre (Durieux et al., 2009), and D1-tomato (Shuen et al., 2008) mouse lines have been previously described. The NL3 conditional knockout mouse line ("NL3-cKO") was generated using standard procedures (Tabuchi et al., 2007; refer to Figure S2). Briefly, using genomic DNA from an isolated phage clone comprising exons 1-4 of the mouse *Nlgn3* gene, we introduced a knock-in (KI) cassette into a unique *Mlu*I site in a non-conserved region of the intron preceding exon 2, which harbors the start codon for the NL3 reading frame. We fused an in-frame mutated cDNA of rat *Nlgn3* lacking splice site A1/2 and containing two modifications: a set of mutations that abolish NL3 binding to neurexins (Arac et al., 2007), and a reading frame for the fluorescent protein monomeric Venus (mVenus) at amino acid position 780, which is localized to the cytoplasmic portion of NL3. The cDNA was terminated by a stop codon followed by a splice donor site and a PGK-neo resistance cassette flanked by F3 sites (to allow for positive selection during ES culture). The entire KI cassette was flanked by a second set of frt sites. Immediately after the KI cassette, a pair of loxP511 sites (Branda and Dymecki, 2004) flanked wildtype exons 2 and 3, allowing for conditional deletion upon Cre-recombinase activity. Upstream of the 5' homology region, a diphtheria toxin expressing mini gene (DT) was introduced for negative selection. Electroporation into R1 embryonic stem cells, drug selection and clone isolation was performed as previously described (Nagy et al., 1993) (UT Southwestern Transgenic Facility, Dallas, TX, USA). Positive clones were verified by Southern blotting and submitted to blastocyst injection to generate Nlgn3*KI mice (Stanford Transgenic Facility, Stanford, CA, USA). Subsequent breeding to mice constitutively expressing Flp-recombinase (Rodriguez et al., 2000) resulted in deletion of either the entire KI cassette or only the PGK-neo selection cassette (due to the linked placement of F3 and frt sites). F1 offspring were chimeric for either recombination event and were backcrossed to C57BL/6 to allow proper genetic separation of both alleles. Mice carrying the floxed *Nlgn3* allele ("NL3-cKO") were bred to homozygosity and maintained by congenic breeding. The original mouse line was submitted to the Jackson Laboratory Mouse Repository for distribution (JAX Stock number: 0023398). To distinguish different allelic combinations we used oligos MX08399 (TCTTAAAGGAACAGGCCTGAAATCTCGG) and MX08401 (GATCACAGACACTGCAAT ATTCACTGGCAG), as well as TT087 (CGACCACTACCAGCAGAACA). MX08399/401 resulted in amplicon sizes of 445 bp (WT), 503 bp (KI+neo) and 589 bp (cKO); TT087/MX08401 produced a 759 bp amplicon (KI w/o neo).

All experiments were conducted on male littermates at least 4 weeks old (for physiology and *in vivo* stereotactic injection) and at least 6 weeks old (for behavior). All lines were maintained by backcrossing to C57Bl/6J, except when noted. Breeding strategies were

dictated by the X-chromosomal localization of *Nlgn3*. For experiments involving NL3-KO and NL3-R451C mutant mice, female heterozygotes were crossed with males carrying the D1-tomato reporter transgene. Male offspring lacking D1-tomato were used for behavioral experiments, while male offspring carrying D1-tomato were used for slice physiology experiments. D1-Cre and A2a-Cre lines were maintained by breeding hemizygous males with wildtype females, as available evidence suggests BAC transgenes themselves do not affect behavior in the hemizygous state on a C57Bl/6J genetic background (Bateup et al., 2010; Chan et al., 2012; Nelson et al., 2012). The NL3-cKO mouse line was bred to homozygosity, and female NL3-cKO homozygotes were crossed to males carrying one copy of a Cre transgene, generating male offspring that were all hemizygous for the floxed *Nlgn3* allele and either expressed or lacked Cre. The only exception was the cross between NL3-cKO and L7-Cre (Figures 2F-L), where approximately half the mice were generated using the aforementioned strategy, while the remaining mice were generated by crossing L7-Cre homozygous males with female NL3-cKO heterozygotes. Similar results were obtained with both breeding strategies, so these data have been combined for presentation. Mice were weaned at ~21 days of age and housed in groups of 2-5 on a 12-hour light/dark cycle (lights on 0700-1900h), with free access to food and water except during behavioral testing. All procedures conformed to National Institutes of Health *Guidelines for the Care and Use of Laboratory Animals* and were approved by the Stanford University Administrative Panel on Laboratory Animal Care.

Behavioral Assays. All behavioral testing occurred between 0800-1800h.

Rotarod performance (Etherton et al., 2009) was tested using a five-station rotarod treadmill (ENV-575M, Med Associates). The standard range of acceleration (ending at 4 to 40 rpm) was expanded using custom hardware purchased from the vendor, allowing us to test speeds up to 8 to 80 rpm, while maintaining a constant rate of acceleration over 300s. Testing consisted of three trials per day, separated by at least 5 min each, over the course of 4 days (12 total trials). On the first day of testing, mice were acclimated to the apparatus by being placed on the stationary rotarod for 60s. Each trial was terminated when a mouse fell off, made one complete backwards revolution while hanging on, or after 300s (maximum speed, no further acceleration). During a trial, mice that turned 180˚ to face backward (same direction as rotation) were gently guided to turn around and face forward (opposite direction of rotation). Digital videos of wildtype and NL3-KO mice on the rotarod were recorded from behind to visualize the location of rear paws. Videos were imported to ImageJ and analyzed using the “Manual Tracking” plugin (Fabrice Cordele, Institut Curie, Orsay) to determine three components of the motor routine: the vertical location of each hind paw when a step was initiated; the time when each step was iniated; and the length of each step.

Open field activity tests (Grueter et al., 2010) were conducted in an arena (ENV-510, Med Associates) housed within a sound-attenuating chamber, equipped with a ventilation fan and illuminated by a single overhead light. The location of the mouse within the arena was

monitored in three dimensions by arrays of infrared beams connected to a computer running Activity Monitor software (Med Associates). This software package calculated the distance travelled during 5 min epochs, which were summed together to calculate total distance travelled throughout the entire 60 min test session. The entire session was also subdivided on the basis of time spent ambulatory, stereotypic, and resting. Software parameters for this analysis included a box size of 4, a resting delay of 2000, and an ambulatory trigger of 3. The percentage of distance traveled through the center of the arena was calculated during the first 5 minutes of the session, defining the center zone based on the central 75% in each dimension. The number of ambulatory episodes and movement velocity while ambulatory were analyzed throughout the entire session.

Force plate actometer assays (Fowler et al., 2001) and the forced swim test (Crowley et al., 2004) were conducted as previously described. For the force plate analysis, 25 min sessions were performed at similar times of the day with concomitant video recording throughout the sessions. Videos were used to correlate the recorded force waveforms with particular aspects of the mouse behavioral repertoire. For the forced swim test, behavior was recorded using an overhead camera and analyzed using Viewer software (BIOBSERVE), with immobility defined by a movement velocity less than 1 cm/s.

Virus Generation and In Vivo Stereotactic Injection. The adeno-associated viruses (AAVs) used in this study were produced by the Stanford Neuroscience Gene Vector and Virus Core. In brief, AAV-DJ (Grimm et al., 2008) was produced by transfection of HEK 293 cells (ATCC) with three plasmids: an AAV vector expressing the target construct (Cre-GFP, ΔCre-GFP, DIO-NL3, or DIO-Kir2.1); AAV helper plasmid (pHELPER; Agilent); and AAV rep-cap helper plasmid (pRC-DJ, gift from M. Kay). At 72h after transfection, the cells were collected and lysed by a freeze-thaw procedure. Viral particles were then purified by an iodixanol step gradient ultracentrifugation method. The iodixanol was diluted and the AAV was concentrated using a 100-kDa molecular weight cut-off ultrafiltration device. The genomic titer was determined by quantitative PCR.

Intracranial injection of virus in vivo was performed using a stereotaxic instrument (David Kopf) under general ketamine-medetomidine anesthesia. A small volume (~1uL) of concentrated virus solution was injected bilaterally into NAc (AP +1.50, ML +/-1.10, DV –4.40) or dorsal striatum (AP +0.70, ML +/-2.50, DV –2.40) at a slow rate (100nL per min) using a syringe pump (Harvard Apparatus). The injection needle was withdrawn 5 min after the end of the infusion. Behavioral testing began ~7 days after injection (for AAV-DIO-Kir2.1), or 4-6 weeks after injection (for AAV-Cre and AAV-DIO-NL3). Injection sites were confirmed in all animals by fixing brain tissue and preparing coronal sections (50-100um) containing dorsal striatum or NAc. The percentage of cells labelled with DAPI that also expressed GFP was similar after injection of AAV-GFPCre into the dorsal striatum (70.4 $\pm$ 3.5%) and the NAc (81.3 $\pm$ 3.7%), indicating a comparable degree of infection. The decrement in rotarod performance observed after injection of AAV-DIO-Kir2.1 into the

dorsal striatum (Figures 6L-6N) confirms that our injection protocol infected a sufficient number of neurons in this brain region to generate a behavioral effect.

Quantitative RT-PCR and immunoblotting. The brains of adult male mice were dissected, with one hemisphere subjected to RNA extraction and the other used for protein analysis. RNA extraction was performed using TRIzol reagent according to manufacturer's protocol (Invitrogen, Carlsbad, CA, USA) and quantified using a ND-1000 spectrophotometer (NanoDrop, ThermoScientific, Wilmington, DE, USA). To determine transcript levels of Nlgn1, Nlgn2, Nlgn3 and ACTB we performed quantitative RT-PCR using the LightCycler480 Master Hydrolysis Probes Kit, RNase Inhibitor and ROX Reference Dye (Roche Diagnostics, Indianapolis, IN, USA) as previously described (Pang et al., 2010). FAM-dye coupled detection assays for mouse neuroligins (20x) were purchased from Integrated DNA Technologies (IDT, Coralville, IO, USA): Nlgn1 (forward TTGGGCAATAAACTCTCCTGG, reverse TTCCAAGGGCAATACAGTCTC and probe CCAAGGTTTAACGGTTCGCCACT), Nlgn2 (Mm.PT.49a.10816796) Nlgn3 (forward CACTGTCTCGGATGTCTTCA, reverse CCTCTATCTGAATGTGTATGTGC and probe CCTGTTTCTTAGCGCCGGATCCAT), and mouse ACTB (4352933E) was purchased from Applied Biosystems (Foster City, CA, USA).

Single cells for transcriptional analysis were aspirated under DIC optics from D1-tomato BAC transgenic mice. Pipette tips for cellular aspiration were backfilled with 0.5ul of 2X Cells Direct buffer (Cells Direct One-Step qRT-PCR kit, Invitrogen, Carlsbad, CA) and cytosolic contents were deposited in 0.5ul RNase/DNase free PCR tubes containing 5ul of 2x buffer. Reverse transcription and 18 cycles of target specific PCR amplification was performed within the same tube, after which 4ul of ExoSAP-IT (Affymetrix, Cleveland, OH) was added to degrade remaining primers. Approximately 2.5ul of amplified single cell material was added to each well of a standard 96x96 microfluidics PCR chip (Fluidigm, South San Francisco, CA) and these cells were probed with both custom and pre-designed Taqman qRT-PCR assays from Integrated DNA Technologies (IDT, Coralville, IO). Probe quality was assessed by efficiency calculations and sample quality was determined by a panel of housekeeping and neuronal control genes. Differences in single cell input was offset by normalizing all Ct values to the average of three control probes. Data analysis and generation of heat maps was done in Mathematica 9.0 (Wolfram Research, Champaign, IL).

Protein analysis was performed as described previously (Tabuchi et al., 2007). Briefly, brain hemispheres were homogenized in phosphate buffered saline with 0.3% TritonX-100 supplemented with protease inhibitor cocktail Complete (Roche Diagnostics). Brain homogenate was incubated for 2h at 4˚C by rotation to extract proteins, and insoluble membrane fractions were removed after 15 min centrifugation at 4˚C and 13,000 rpm. 20 µl of supernatant was diluted in SDS-containing sample buffer and separated on an 8% SDS-containing polyacrylamide gel. Proteins on the gel were blotted onto nitrocellulose membrane (Protran BA 85, 0.45µm, GE Healthcare, Pittsburg, PA, USA) and incubated in

blocking solution containing 5% milk powder. Incubation with primary antibody (689 affinity-purified from rabbit, 1:2000; Tabuchi et al., 2007) was performed overnight at 4˚C, and secondary antibody incubation (peroxidase-coupled goat anti-rabbit, 1:5000; MP Biomedicals, Solon OH, USA) occurred for 2h at room temperature. ECL reaction was performed using Lumi-Light Western blotting substrate (Roche) according to manufacturer's protocol.

Electrophysiology. Parasagittal slices (250 μm) containing NAc or dorsal striatum were prepared using standard procedures (Grueter et al., 2010). Briefly, mice were anesthetized with isoflurane and decapitated, brains were quickly removed and placed in ice-cold low-sodium/high-sucrose dissecting solution. Slices were cut by adhering the lateral surface of the brain to the stage of a Leica vibroslicer, and allowed to recover for a minimum of 60 min in a submerged holding chamber (~25˚C) containing artificial cerebrospinal fluid (aCSF) containing (in mM) 119 NaCl, 2.5 KCl, 2.5 $CaCl_2$,1.3 $MgSO_4$, 1 NaH_2PO_4, 11 glucose and 26.2 $NaHCO_3$.Slices were then removed from the holding chamber and placed in the recording chamber where they were continuously perfused with oxygenated (95% O_2/5% CO_2) aCSF at a rate of 2mL per min at ~30˚C. To monitor EPSCs, picrotoxin (50uM) was added to the aCSF to block $GABA_A$ receptors. To monitor IPSCs, D-APV (50uM) and NBQX (10uM) were added to block NMDARs and AMPARs, respectively.

Whole-cell recordings from MSNs in the NAc core (identified by the presence of the anterior commissure) were obtained under visual control using IR-DIC optics. D1- and D2-MSNs were distinguished by the presence or absence of red tomato fluorescence, respectively, which was excited using a mercury arc lamp with a bandpass filter (HQ545/30X). Our single-cell transcriptional analysis confirmed that MSNs lacking red fluorescence in fact expressed prototypical D2-MSN markers. Voltage-clamp recordings were made with electrodes (2-5 MΩ) filled with (in mM) 120 $CsMeSO_4$, 15 CsCl, 10 TEA-Cl, 8 NaCl, 10 HEPES, 0.2-5 EGTA, 5 QX-314, 4 MgATP, and 0.3 NaGTP. Spermine (0.1mM) was included when measuring the current-voltage relationship of AMPAR currents, and all reported holding potentials are corrected for a liquid junction potential of ~10mV. Excitatory and inhibitory afferents were stimulated at 0.1Hz (unless otherwise noted) with a bipolar nichrome wire electrode placed at the border between NAc core and the cortex dorsal to the anterior commissure. MSNs were voltage-clamped at -80mV unless otherwise noted. For current-clamp recordings, electrodes were filled with (in mM) 130 $KMeSO_4$, 10 NaCl, 2 $MgCl_2$, 0.16 $CaCl_2$, 10 HEPES, and 0.5 EGTA. Recordings were performed using a MultiClamp 700B (Molecular Devices), filtered at 2 kHz and digitized at 10 kHz. Data acquisition and analysis were performed on-line using the Recording Artist package (Dr. Rick Gerkin) written in Igor Pro (Wavemetrics). Input and series resistance were monitored continuously and experiments were discarded if either parameter changed by >20%.

For current-clamp experiments, we monitored the number of spikes fired during a fixed current injection (500 ms). Miniature synaptic currents were recorded in the presence of

tetrodotoxin (0.5 uM) at holding potentials of -80 mV (for mEPSCs) or 0 mV (for mIPSCs). At least 200 events per cell were acquired in 15 s blocks and detected using a threshold of 5 pA; all events included in the final data analysis were verified by eye. Paired pulse ratios were acquired by applying a second afferent stimulus of equal intensity at specified times (20-800 ms) after the initial stimulus, and then calculating the ratio between peak amplitudes of the second and first stimulus. NMDAR/AMPAR ratios were determined by evoking dual-component EPSCs at +40 mV, then applying D-APV (50 uM) to isolate the AMPAR EPSC. The NMDAR EPSC was obtained by digital subtraction and peak amplitudes were used to calculate the ratio. The pharmacologically isolated NMDAR EPSC was fit with a bi-exponential function to calculate weighted NMDAR decay kinetics. We measured the rectification of AMPAR currents by evoking a pharmacologically isolated AMPAR EPSC at holding potentials ranging from -80 to +40 mV, and normalizing peak amplitude to the current at -80 mV. LTD induced by bath application of R,S-DHPG (100 uM) was monitored while evoking 50ms paired pulses at 0.05 Hz.

Short-term plasticity of IPSCs was assessed using 1s trains of 10Hz stimulation, evoked every 60s. The percent suppression of evoked IPSCs by (1,2,5,6-Tetrahydropyridin-4-yl)methylphosphinic acid (TPMPA, 50 µM), a low-affinity antagonist of GABA receptors (Barberis et al., 2005), was determined by bath application. The inhibition/excitation ratio was determined by first evoking AMPAR-mediated currents at a holding potential of -40 mV, the reversal potential for GABAR-mediated current under our experimental conditions. Cells were subsequently depolarized to 0 mV and NBQX was applied, blocking AMPARs and leaving a residual current mediated by monosynaptic inhibition through GABARs. D-APV was present throughout the experiment to block NMDARs. The inhibition/excitation ratio was calculated by dividing peak GABAR current by peak AMPAR current.

Immunohistochemistry. WT and NL3-KO mice carrying the D1-tomato reporter transgene were transcardially perfused with 4% PFA and the brains were postfixed overnight at 4˚C. 50 uM sections were cut in PBS on a Leica vibratome and subsequently blocked in 10% horse serum and 0.1% Triton X-100 for 2 hrs. After washing, the following primary antibodies were applied overnight in 1% horse serum and 0.1%Triton X-100: VGAT (1:500 dilution; rabbit polyclonal, Synaptic Systems, Goettingen, Germany) and RFP (1:500 dilution; rat monoclonal, Allele Biotechnology, USA). After washing, the following secondary antibodies were incubated for 2 hours at RT in 1% horse serum and 0.1% Triton X-100: Alexa488 goat anti-mouse and Alexa594 goat anti-rat (1:750 dilution; Molecular Probes, Life Technologies, USA). Slices were mounted following the final wash in Fluoromount-G (SouthernBiotech, USA) and imaged on a Zeiss confocal microscope. Single optical sections of ~1.5uM thickness were imaged and analyzed in Image-J. The number of VGAT positive puncta immediately adjacent to tomato-positive neurons were counted by hand with analysis restricted to cells that had distinct borders and were clearly being imaged in cross-section. A minimum of 50 cells were counted in 4 WT mice and 4 NL3-KO mice.

Data Analysis. All data were collected and analyzed by experimenters blind to treatment condition. Experimental data were always compared to control data from littermate animals collected during the same time period. The rotarod learning and open field hyperactivity phenotypes initially characterized in NL3-KO mice (Figure 1) were used to conduct a power analysis to determine appropriate sample sizes for subsequent experiments. Using the means and standard deviation of learning slope on the rotarod, as well as a Type I error rate (α) of 0.05, we found that 9 mice/group were necessary to achieve 80% power to detect an increase in this parameter. A similar calculation based on open field data yielded a sample size of 7 mice/group to achieve 80% power.

Behavioral data were analyzed using factorial analysis of variance (ANOVA), with genotype or virus as a between-subject factor, and time or trial as a repeated measure. For rotarod data sets, the interaction term provided a quantitative measure of statistical confidence that learning rate across trials differed between groups (Nieuwenhuis et al., 2011). Significant interactions were decomposed using tests of simple effects. Rotarod data were also analyzed by performing linear regression on data from each individual animal, with the slope used as an index of learning rate, and the intercept used to estimate initial motor coordination. These measures, as well as total distance travelled in the open field as well as the number of ambulatory episodes, were analyzed by one-way ANOVA followed by Bonferroni-corrected post-hoc tests. Physiology data were analyzed using one-way ANOVA and the Kolmogorov-Smirnoff (KS) test. Type I error rate was set at $\alpha = 0.05$ (two-tailed) for all statistical comparisons, and all data are presented as mean +/- standard error of the mean (SEM). When ANOVA results are presented in figures, significant effects are highlighted by bold and italic font. Individual comparisons that are not significant are not identified.

4. SUPPLEMENTAL REFERENCES

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