A Model to Study NMDA Receptors in Early Nervous System Development

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N-methyl-D-aspartate receptors (NMDARs) are glutamate-gated ion channels that play critical roles in neuronal development and nervous system function. Here, we developed a model to study NMDARs in early development in zebrafish, by generating CRISPR-mediated lesions in the NMDAR genes, *grin1a* and *grin1b*, which encode the obligatory GluN1 subunits. While receptors containing *grin1a* or *grin1b* show high Ca^{2+} permeability, like their mammalian counterpart, *grin1a* is expressed earlier and more broadly in development than *grin1b*. Both *grin1a*^{−/−} and *grin1b*^{−/−} zebrafish are viable. Unlike in rodents, where the *grin1* knockout is embryonic lethal, *grin1* double-mutant fish (*grin1a*^{−/−}; *grin1b*^{−/−}), which lack all NMDAR-mediated synaptic transmission, survive until ~10 d dpf (days post fertilization), providing a unique opportunity to explore NMDAR function during development and in generating behaviors. Many behavioral defects in the *grin1* double-mutant larvae, including abnormal evoked responses to light and acoustic stimuli, prey-capture deficits, and a failure to habituate to acoustic stimuli, are replicated by short-term treatment with the NMDAR antagonist MK-801, suggesting that they arise from acute effects of compromised NMDAR-mediated transmission. Other defects, however, such as periods of hyperactivity and alterations in place preference, are not phenocopied by MK-801, suggesting a developmental origin. Together, we have developed a unique model to study NMDARs in the developing vertebrate nervous system.

Key words: calcium permeability; CRISPR-Cas9; habituation; prey-capture; visual acuity; zebrafish

Significance Statement

Rapid communication between cells in the nervous system depends on ion channels that are directly activated by chemical neurotransmitters. One such ligand-gated ion channel, the NMDAR, impacts nearly all forms of nervous system function. It has been challenging, however, to study the prolonged absence of NMDARs in vertebrates, and hence their role in nervous system development, due to experimental limitations. Here, we demonstrate that zebrafish lacking all NMDAR transmission are viable through early development and are capable of a wide range of stereotypic behaviors. As such, this zebrafish model provides a unique opportunity to study the role of NMDAR in the development of the early vertebrate nervous system.

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Introduction

Nervous system function depends on the development of brain circuits that integrate appropriate cell types and establish proper connectivity. In the vertebrate nervous system, glutamate is the major excitatory neurotransmitter. At synapses that mediate rapid transmission, glutamate is converted into biological signals by ligand-gated or ionotropic glutamate receptors (iGluRs), including AMPAR, kainate, and NMDAR subtypes (Traynelis et al., 2010). Because of its unique signaling properties, including high Ca^{2+} permeability, NMDARs are central to higher brain functions, such as the plasticity underlining learning and memory (Hunt and Castillo, 2012; Paoletti et al., 2013) and brain development (Cline and Haas, 2008; Gambrill and Barria, 2011; Chakraborty et al., 2017). Because of the experimental limitations of pharmacological manipulation, especially in studies viewing

long-term outcomes as occurs for many neurodevelopmental disorders, studying NMDARs in early circuit development is challenging. Adding to this challenge, the loss of NMDAR subunits critical to early brain development in murine models is embryonic or perinatal lethal (Forrest et al., 1994; Kutsuwada et al., 1996; Sprengel et al., 1998).

Zebrafish are a powerful model to study early nervous system development as fertilization is external and larvae are transparent, which facilitates imaging of cell migration and circuit formation. Furthermore, zebrafish larvae exhibit complex behaviors that provide simple and robust readouts of higher nervous system function. Previous studies of NMDARs in zebrafish have generally used acute pharmacological manipulations, mainly MK-801, a specific antagonist of NMDARs (Huettner and Bean, 1988; Traynelis et al., 2010). These studies have highlighted the central role of NMDARs in complex brain functions and behaviors, including associative learning (Sison and Gerlai, 2011b), social interactions (Seibt et al., 2011; Dreosti et al., 2015), place preference (Swain et al., 2004), and responses to acoustic stimuli and prepulse inhibition (PPI) (Bergeron et al., 2015). Although these studies are invaluable, they do not reveal how brain or circuit development impacts specific behaviors because acute antagonist applications only change brain function for a short developmental time point and can have off-target effects.

NMDARs are heterotetramers composed of two obligate GluN1 subunits and typically two GluN2 (A–D) subunits (Paoletti et al., 2013). In zebrafish, the obligatory GluN1 subunit is encoded by two paralogous genes: *grin1a* and *grin1b*. The expression patterns of these genes have only been described before 2 days post fertilization (dpf) (Cox et al., 2005), and functional differences between paralogs are unknown. Here, we studied the developmental expression of *grin1a* and *grin1b* and the effect of their knockouts on early nervous system function. While their encoded proteins, GluN1a and GluN1b, show high Ca^{2+} permeability, like their mammalian counterparts, *grin1a* is expressed earlier in development and tends to be more widely expressed. Nevertheless, both *grin1a*^{−/−} and *grin1b*^{−/−} fish are viable as adults. Unlike rodents, *grin1* double-mutant fish (*grin1a*^{−/−}; *grin1b*^{−/−}), which lack all NMDAR-mediated synaptic transmission, survive until ~10 dpf, which is old enough to assess the impact of NMDAR deficits on larval behaviors. We took advantage of these fish to study the role of NMDARs in the development of numerous complex behaviors, including prey-capture, spontaneous and evoked movements, habituation, and PPI.

Materials and Methods

Whole-cell electrophysiology

Human embryonic kidney 293 (HEK293) cells were grown in DMEM, supplemented with 10% FBS, for 24 h before transfection (Yelshansky et al., 2004; Alsaloum et al., 2016). Zebrafish cDNA constructs were synthesized from GenScript in a pcDNA3.1(+)-p2A-eGFP vector. Zebrafish (zGluN1a/b) and rat (rGluN2A) NMDAR-encoding cDNA constructs, were cotransfected into HEK293 cells along with a separate pGFP-Cl construct at a ratio of 4:4:1 (N1:N2:eGFP) for whole-cell or macroscopic recordings using X-tremeGene HP (Roche Diagnostics, 06-366). HEK293 cells were bathed in medium containing the GluN2 competitive antagonist DL-2-amino-5-phosphopentanoic acid (100 μM , Tocris Bioscience) and Mg^{2+} (100 μM). All experiments were performed 24–48 h after transfection.

Whole-cell currents were recorded at room temperature (20°C–23°C) using an EPC-10 amplifier with PatchMaster software (HEKA), digitized at 10 kHz, and low-pass filtered at 2.9 kHz (−3 dB) using an 8 pole low

pass Bessel filter 4 (Yelshansky et al., 2004; Alsaloum et al., 2016). Patch microelectrodes were filled with an intracellular solution (in mM as follows): 140 KCl, 10 HEPES, 1 BAPTA, 4 Mg^{2+} -ATP, 0.3 Na^{+} -GTP, pH 7.3 (KOH), 297 mOsm (sucrose). Our standard extracellular solution consisted of the following (mM): 150 NaCl, 2.5 KCl, and 10 HEPES, pH 7.2 (NaOH). Currents were measured within 10 min of going whole cell.

External solutions were applied using a piezo-driven double-barrel application system. For agonist application, one barrel contained the external solution 0.1 mM glycine, whereas the other barrel contained both 0.1 mM glycine and 1 mM glutamate. For display, NMDAR currents were digitally refiltered at 500 Hz and resampled at 1 kHz.

Zebrafish maintenance and housing

Adult zebrafish strains were maintained at 28.5°C under a 13:11 h light to dark cycle and were fed a diet of artemia and GEMMA micropellets. The WT strain used for all experiments was a hybrid WT background consisting of Tubingen long-fin crossed to Brian's WT. The experiments and procedures were approved by the Stony Brook University Institutional Animal Care and Use Committee.

Whole-mount RNA in situ hybridization (ISH)

The *grin1a* probe was previously described (Cox et al., 2005). Probes for *grin1b* were generated by PCR amplification of adult brain WT cDNA using the following primers: *grin1b*: forward (GAGtatttagtgacactatag TCTGTGGACTAGCTGGCAAA) and reverse (GAGtaatacgactcacta tagggATGGACGTTGCGTGTGTA).

Lowercase letters in forward and reverse primers indicate the T7 and SP6 RNA polymerase binding sites, respectively. DIG-labeled antisense and sense probes were generated from PCR product.

Whole-mount RNA ISH was performed as described by Thisse et al. (1993). Embryos were stained with NBT/BCIP. Before photography, embryos were cleared with 2:1 benzyl benzoate to benzyl alcohol and then mounted in Canada Balsam with 2.5% methyl salicylate. Embryos were imaged on a Zeiss Axioplan 2 microscope with AxioCam HRC mounted camera and AxioVision Software.

CRISPR-Cas9 gene targeting and zebrafish microinjections

gRNAs were designed through IDT custom gRNA Design Tool. gRNAs were complexed to Cas9 protein, forming a ribonucleoprotein, using the Alt-R CRISPR-Cas9 System (IDT); 0.5 nl of ribonucleoprotein (25 pg of gRNA and 125 pg of Cas9 protein) was injected into the cell of pronased (1 mg/ml) 1-cell embryos. Ten to 20 embryos from each injection were assayed (by PCR) for lesions using primers flanking the gRNA target site. If insertions or deletions were detected when screened on a conventional agarose gel, siblings were grown to adulthood and screened for germline transmission. All mutations were outcrossed at least twice to minimize the effect of off-target endonuclease activity on behavioral phenotypes.

Zebrafish genotyping and size comparisons

Adult zebrafish viability was assayed after 2 months post-fertilization. The following primers were used to screen the CRISPR mutations by PCR and for subsequent genotyping: *grin1a*: forward (ATTAGGAATG GTGTGGGCTGGC) and reverse (GGTGATGCGCTCCTCAGGCC); and *grin1b*: forward (GGTGCCCTCGGAGCTTTTC) and reverse (GGAAGGCTGCCAAATTGGCAGT).

For size comparisons of adult zebrafish, sibling WT and mutant fish were generated from a heterozygous intercross and raised together in the same tanks, with equal densities across the different tanks. Size measurements were reported as length from the snout tip to the fork of the caudal fin. Genotyping and establishment of sex were not performed until after measurements at 2 months. Results were reported as a combination of both male and female fish, as separation of fish by sex did not alter the reported results.

RT-PCR

RNA was extracted from pools of four larvae at 3 dpf from homozygous mutant or homozygous WT intercrosses using Trizol (Invitrogen) and Direct-zol RNA Minipreps (Zymo Research). cDNA was synthesized from 0.2 μg of RNA using Superscript II Reverse Transcriptase

Figure 1. Zebrafish paralogs show functional currents and high Ca^{2+} permeability. **A**, Whole-cell glutamate-activated currents recorded in HEK293 cells in response to sustained (2.5 s) glutamate applications (1 mM, gray bar) to measure NMDAR currents for various GluN1 constructs: rat GluN1 (rGluN1) (left); and zebrafish GluN1a (zGluN1a) (middle) or GluN1b (zGluN1b) (right). All GluN1 constructs were coexpressed with rat GluN2A (rGluN2A). Peak current amplitudes (I_{peak}) were measured at the beginning of the glutamate application. Holding potential, -70 mV. Left, Scale bar values. **B**, Bar graph (mean $\pm$ SEM) of peak current amplitudes (I_{peak}) for the various constructs (rGluN1, $n = 10$; zGluN1a, $n = 6$; zGluN1b, $n = 7$). None of the values was significantly different ($F_{(2,20)} = 0.84$, $p = 0.445$, ANOVA). **C**, Measuring Ca^{2+} permeability. Current-voltage (I - V) relationships in an external solution containing high Na^{+} (140 mM) and 0 added Ca^{2+} (open circles) or 10 mM Ca^{2+} (solid circles) to determine reversal potentials (E_{rev}), the point where currents cross the 0 current axis. The 0 Ca^{2+} I - V is the average of that recorded before and after the 10 mM Ca^{2+} recording. We used changes in reversal potentials (ΔE_{rev}) to calculate P_{Ca}/P_{Na} (Jatzke et al., 2002). **D**, Relative calcium permeability (P_{Ca}/P_{Na}) (mean $\pm$ SEM) calculated from ΔE_{rev} s for rat rGluN1/rGluN2A (8.3 ± 0.4 mV, $n = 8$), zGluN1a/rGluN2A (7.9 ± 0.7 mV, $n = 9$), or zGluN1b/rGluN2A (8.6 ± 0.5 mV, $n = 8$). None of the values was significantly different ($F_{(2,22)} = 0.99$, $p = 0.39$, ANOVA).

(Invitrogen). The following primers were used to screen the cDNA by PCR: *grin1a*: forward (ATTAGGAATGGTGTGGGCTGGC) and reverse (ATGAATTTGTCTGATGGGTTCT); and *grin1b*: forward (GTGCCCTCGGAGCTTTTCG) and reverse (AGGAAGGCTGCCA AATTGGCA).

qPCR

RNA was extracted as stated above. Pools of four larvae were collected at 1 and 3 dpf, while pools of 2 larvae were collected at 5 dpf. qPCR was conducted on a LightCycler 480 (Roche Diagnostics) using PerfeCTa SYBR GreenFastMix (QuantaBio). Transcript levels from each sample were normalized to β -actin. In each experiment, three pools of cDNA from biological replicates were run in duplicate for each genotype. The following primers were used to screen the cDNA by PCR (Menezes et al., 2015): *grin1a*: forward (ATAAAGACGCCCGCAGGAAGC) and reverse (CGTGCTGACAGACGGGTCCGAC); and *grin1b*: forward (AATGCAGCTGGCCTTTGCAGC) and reverse (CTCTTGATGTTGG AGGCCAGTTG).

Live imaging

Zebrafish larvae were immersed in 3% methylcellulose, after anesthetization in ice water, and images were taken on a Zeiss Discovery version 20.

Western blot

Total protein was isolated from 7 dpf zebrafish heads from WT and *grin1* double mutants, and HEK293 cells transfected with plasmids encoding zGluN1a and zGluN1b, along with rat GluN2A. *grin1* double-

mutant numbers were enhanced through a behavior screen at 6 dpf, isolating $\sim 85\%$ *grin1* double mutants. *Post hoc* genotyping confirmed this recovery rate. Twenty-four heads were isolated from each genotype and placed into 80 μ l of lysis buffer consisting of 1 $\times$ Cell Lysis Buffer (Cell Signaling Technology, #9803), 1 mM PMSF, and 1 tablet of cComplete protease inhibitor cocktail tablet (Sigma Millipore, #11836170001). For each HEK293 sample, a confluent 60 mm plate of HEK293 cells was isolated with 500 μ l of lysis buffer. Samples were sonicated (model: Lab Supplies, G112SPIT, 80 kHz, 600 V) for 2 min on ice-cold water and subsequently were incubated for 15 min at 4°C, and centrifuged at 14,000 rpm, 15 min at 4°C (model: Eppendorf Centrifuge 5417R; rotor: 45-30-11). HEK293 protein sample concentrations were quantified using a DC Lowry Assay kit (Bio-Rad, #500-0116). Zebrafish protein samples could not be quantified since the absorbance was below the detection threshold; 20 μ l of zebrafish lysate or 50 μ g of HEK293 total protein was boiled in 2 $\times$ Laemmli's buffer with 10% β -mercaptoethanol for 10 min at 95°C. Samples and Chameleon Duo IR Dye ladder (Li-Cor, #928-60000) were loaded onto Mini-PROTEAN TGX 7% Precast Gel (Bio-Rad) and electrophoresed at 120 V for 60 min. Gel was transferred to a methanol-activated PVDF membrane using the wet transfer system Mini Trans-Blot Cell (Bio-Rad).

PVDF membrane was washed with dH_2O , reactivated with methanol, then washed with 1 $\times$ TBS. Membrane was incubated in Intercept blocking buffer (Li-Cor, #927-70001) for 60 min at room temperature in dark. Membrane was incubated with primary antibodies in blocking buffer

with 0.2% Tween-20: 1:500 mouse anti-GluN1 (Synaptic Systems, #114-001), 1:500 rabbit anti-GAPDH (Abcam, #ab210113) for 60 min at room temperature in dark. Membrane was washed with 1× TBS with 0.1% Tween-20, then incubated with secondary antibodies in blocking buffer with 0.2% Tween-20, 0.02% SDS: 1:5000 donkey anti-mouse-IR800CW (Li-Cor, #926-32212), 1:5000 goat anti-rabbit-IR800CW (Li-Cor, #926-32211) for 60 min at room temperature in dark. Membrane was then washed with 1× TBS and imaged using Odyssey infrared imaging system (Li-Cor).

Behavioral assays

Behavioral assays were performed on multiple clutches from different parents to minimize the effects of genetic background. Embryos were kept in 150 mm Petri dishes in egg water (6 × g of synthetic sea salt, 20 ml of methylene blue (1 × g/L) solution in 20 L of water, pH 7, at concentrations <100 larvae per plate).

Pharmacology. MK-801 (20 mM) was diluted in DMSO. For all experiments, unless otherwise noted, final concentration used was 20 μM in 0.1% DMSO. The dosage of MK-801 (20 μM) used throughout this study was an intermediate concentration from a previously performed dose-response analysis of MK-801 on zebrafish (Sison and Gerlai, 2011a).

Prey-capture paradigm. *Paramecium multimicronucleatum* were obtained from Carolina Biological Supply and were maintained at room temperature with new cultures generated every 3 weeks. *Paramecia* from cultures of 2–5 weeks of age were filtered and moved to a 35 mm Petri dish on the day of the assay.

Individual larvae were assayed at 7 dpf; 20–60 *paramecia* were added to individual 35 mm Petri dishes filled with 3 ml of either system or egg water, yielding a water depth of ~3 mm. Fifty frames (~2.5 s) of *paramecia* movement were recorded using an Excelis MPX5C-PRO camera with Mosaic version 2.0 software at the beginning and end of each trial, which lasted 1.5 h. These videos were processed using ImageJ as previously described (Gahtan et al., 2005). *Paramecia*-trace images were manually counted by a blind viewer. Multiple control plates containing only *paramecia* were used in each assay to account for any decrease in *paramecia* viability. Results were expressed as a ratio of the proportion of *paramecia* eaten, normalized to their respective control.

Dark flash behavior paradigm. Dark-flash experiments were performed in 9-well gridded plates. Each plate of fish was light-adapted for 20 min before testing. Ten dark flash stimuli were administered with an interstimulus interval (ISI) of 1 s and a dark-flash consisting of a white-spectrum light (~500 μW/cm²) turning off for 1 s. Larvae were tracked for 600 ms at 1000 frames/s using a DRS Lightning high speed camera (DEL Imaging) to measure O-bend responsiveness. Kinematics were analyzed using FLOTE software (Burgess and Granato, 2007a).

Spontaneous and evoked movement paradigm. At 48 h post fertilization (hpf), embryos were transitioned to 24-well behavior plates, 1 embryo per well in 1.5 ml of egg water. Behavior was recorded between 4 and 6 dpf using a Zebrafish imaging system (Viewpoint Life Sciences) and tracked with automated video-tracking software (Zebrafish;

Figure 2. Expression of *grin1a* and *grin1b* in the zebrafish nervous system. Whole-mount ISH of *grin1a* and *grin1b* at 24 hpf (A–D), 80 hpf (E–H), and 122 hpf (I–L) showing dorsal (A, B, E, F, I, J) and lateral (C, D, G, H, K, L) views. B, D, F, H, J, L, Insets, Sense probes of *grin1b*. A, C, Arrows indicate limited bilateral expression. M, N, Dorsal view of the retina at 80 hpf for *grin1a* (M) and *grin1b* (N). O–R, Dorsal view of the spinal cord at 24 hpf (O, P) and 120 hpf (Q, R) for *grin1a* and *grin1b*. S, Relative expression levels of *grin1a* and *grin1b* at 24 hpf ($n=9$), 72 hpf ($n=9$, $**p=0.0022$, ratio paired t test), and 120 hpf ($n=6$) in WT larvae from qPCR. ND, Not detected. Results are normalized to *grin1a* expression at each individual time point.

Viewpoint Life Sciences). Gross motor movements were reported as movement count, the number of distinct movements generated, averaged per genotype.

Acoustic startle behavior paradigm. Individual fish were placed in 9-well ~1 cm² gridded plates and tested for changes in short latency startle responses (SLC) to acoustic/vibrational stimuli and habituation of SLC. Pulsed acoustic stimuli were delivered using a Type 3810 minishaker (Bruel-Kjaer) and controlled using a BNC-2110 DAQ board (National Instruments). Behavioral responses were imaged at 1000 frames/s using a DRS Lightning high speed camera (DEL Imaging) and analyzed using FLOTE software (Burgess and Granato, 2007a). SLC responses were assigned based on response latency and kinematics. Control larvae were WT and heterozygous siblings from the same clutch as mutants. We assigned larvae to each group by genotyping after behavioral analysis.

For SLC and PPI experiments, each plate of fish was presented with 20 presentations each of (1) high-intensity startle stimuli (37 ± 1 dB re. m/s²), (2) low-intensity startle stimuli (19 ± 1 dB re. m/s²), and prepulse stimuli of 19 ± 1 dB dB re. m/s² followed by high-intensity startle stimuli at (3) 50 ms ISI and (4) 500 ms ISI. These 80 stimuli were delivered in

Figure 3. Generation of loss-of-function lesions in *grin1a* and *grin1b* using CRISPR-Cas9. **A**, Schematic depiction of membrane topology of two NMDAR subunits (functional NMDARs are tetramers). Blue and red arrows indicate approximate sites for gRNA targets for *grin1a* and *grin1b*, respectively. NMDARs are composed of four modular domains: the extracellular ATD and LBD; the membrane-embedded TMD; and the intracellular CTD. Each individual subunit contributes three transmembrane segments (M1, M3, and M4) and an M2 pore loop to form the ion channel. The most highly conserved motif in iGluRs, the SYTANLA motif (labeled in yellow), is within the M3 segment. gRNAs were designed to prevent generation of this motif and downstream elements. **B**, Linear representation of the NMDAR subunit protein sequence, highlighting elements downstream of the gRNA lesion sites in the *grin1* mutants that are predicted to be absent, including half of the LBD (S2) and all of the M4 and CTD. In the linear representation, the two lobes of the LBD are represented as S1 and S2; whereas in the 3D representation of the structure,

pseudorandom order with a 30 s interval between consecutive stimulus presentations. PPI was calculated as the percent decrease in SLC responsiveness in the prepulse trials compared with no prepulse trials. To control for baseline SLC responsiveness, PPI analysis was only conducted on fish that responded in 30%–95% of trials in the high-intensity startle stimulus alone condition.

For habituation experiments, larvae were presented with high-intensity startle stimuli with 1 s interval between stimulus presentations. This paradigm has demonstrated rapid habituation, with SLC decreases of up to 80% after 20 stimulus presentations (Wolman et al., 2011). Because of the stochasticity of individual-level data, we analyzed responsiveness in 4 epochs of 10 stimuli. Habituation was quantified as the percent decrease in SLC responsiveness in the last epoch relative to the first epoch.

Experimental design and statistical analysis

Data analysis was performed using IgorPro, Excel, and MiniTab 18. All average values are presented as mean $\pm$ SEM. The number of replicates is indicated in the figure legends. In instances where we were only interested in whether outcomes were statistically different from WT or an appropriate control, we used an unpaired two-tailed Student's *t* test to test for significance. In instances where we were interested in how multiple groups varied from each other, we used a one-factor ANOVA and followed with a *post hoc* Tukey's test. Statistical significance was set at $p < 0.05$.

Sex in zebrafish is indeterminate at the age of our larval studies and was thus not a factor in our experimental design.

Results

Functional NMDARs are heterotetramers composed of two obligatory GluN1 subunits and typically some combination of GluN2 (A–D) subunits. Initially, we studied the functional properties of the zebrafish *grin1* (GluN1) paralogs, *grin1a* and *grin1b*.

NMDARs containing zebrafish GluN1 paralogs are highly Ca^{2+} -permeable

NMDARs are ligand-gated ion channels (Paoletti et al., 2013). To define potential functional differences between zebrafish paralogs, we expressed *grin1a* and *grin1b* in a heterologous expression system (see Materials and Methods). To reduce potential variations with the GluN2 subunit, transfected constructs to express fish GluN1a (zGluN1a) or GluN1b (zGluN1b) with rat GluN2A (rGluN2A) (Fig. 1). As a reference, we compared these recordings with those for rat GluN1 (rGluN1) coexpressed with rGluN2A (rGluN1/rGluN2A).rGluN1/rGluN2A

Figure 4. *grin1a*^{-/-} but not *grin1b*^{-/-} fish have decreased growth into adulthood. **A**, **B**, Representative photographs of *grin1a*^{+/+} and *grin1a*^{-/-} fish (**A**) or of *grin1b*^{+/+} and *grin1b*^{-/-} fish (**B**) at 2 months after fertilization. Scale bars, 1 mm. **C**, **D**, Standard length comparison (mean $\pm$ SEM) of (**C**) *grin1a*^{+/+} ($n = 17$) and *grin1a*^{-/-} ($n = 5$) ($**p = 0.0014$, *t* test) or (**D**) of *grin1b*^{+/+} ($n = 17$) and *grin1b*^{-/-} ($n = 31$) at 2 months after fertilization. As different conditions during development may contribute to size variations between crosses (see *grin1a*^{+/+} [**A**] vs *grin1b*^{+/+} [**B**] controls), we only made size comparisons between within-cross controls. **E**, Standard length comparison from 8 weeks (56 dpf, $**p = 0.0014$, *t* test), 10 weeks (70 dpf, $***p = 6.5 \times 10^{-4}$, *t* test), 12 weeks (84 dpf, $**p = 0.0054$, *t* test), and 14 weeks (98 dpf, $**p = 0.0067$, *t* test) for *grin1a*^{+/+} ($n = 17$) and *grin1a*^{-/-} ($n = 5$). After initial measurement at 8 weeks, fish were moved to individual tanks.

shows strong expression in HEK293 cells (Alsouloum et al., 2016; Amin et al., 2017) (Fig. 1A, left). NMDARs containing zGluN1a (Fig. 1A, middle) or zGluN1b (Fig. 1A, right) also showed robust current amplitudes that were indistinguishable from rGluN1 (Fig. 1B). Hence, both zGluN1a and zGluN1b form functional channels with rat GluN2A subunits.

A critical physiological property of NMDARs is their high Ca^{2+} permeability (Paoletti et al., 2013), which is dominated by GluN1 (Burnashev et al., 1992; Watanabe et al., 2002). We therefore assayed the Ca^{2+} permeability (P_{Ca}), relative to Na^{+} ($P_{\text{Ca}}/P_{\text{Na}}$), of NMDARs containing zGluN1a or zGluN1b (Fig. 1C,D). To measure $P_{\text{Ca}}/P_{\text{Na}}$, we measured changes in reversal potentials going from a high Na^{+} solution without Ca^{2+} to the same solution containing 10 mM Ca^{2+} (see Materials and Methods) (Jatzke et al., 2002; Amin et al., 2018). NMDARs containing zGluN1a or zGluN1b showed a high Ca^{2+} permeability that is indistinguishable from that of NMDARs containing rGluN1 (Fig. 1D). Thus, zebrafish NMDARs, regardless of the GluN1 paralog, are highly Ca^{2+} -permeable, like their mammalian counterpart (Wollmuth, 2018).

they are designated D1 (mainly composed of S1) and D2 (mainly composed of S2). **C**, **D**, Schematic of gRNA target sites for *grin1a* (blue arrow) (**C**) and *grin1b* (red arrow) (**D**) as well as alignments of nucleotide (top) and amino acid (bottom) sequences. Induced mutations within the nucleotide sequences are denoted as either dashes (deletions) or highlighted blue (insertions). gRNA target site on the nucleotide sequence (gray highlight) and PAM sites (bolded) are adjacent to generated mutations. Altered amino acid sequence (bolded) and early stop codons (red STOP) are generated in all alleles, disrupting the SYTANLAAF motif (yellow highlight) as well as removing the D2 (S2) lobe of the LBD, which would make the receptor nonfunctional. **E**, **F**, Lesions altered mRNA size in expected fashions. cDNA amplification of the following: (**E**) *grin1a*^{+/+} and *grin1a*^{-/-} (unless otherwise noted, *grin1a*^{-/-} indicates the sbu90 allele) producing expected product sizes of 147 and 140 bp, respectively; and (**F**) *grin1b*^{+/+} and *grin1b*^{-/-} (unless otherwise noted, *grin1b*^{-/-} indicates the sbu94 allele) producing expected product sizes of 109 and 126 bp, respectively. M, Marker. For all genotypes, RNA was collected from homozygous intercrosses at 3 dpf. **G**, **H**, qPCR of *grin1a* (**G**) and *grin1b* (**H**) expression at 5 dpf in *grin1* control ($n = 6$), *grin1a*^{-/-} ($n = 6$), and *grin1b*^{-/-} ($n = 6$) fish. **G**, **H**, None of the values was significantly different.

Figure 5. *grin1* double mutants can survive through early larvae development. **A**, Viability of *grin1* double mutants at 6 dpf and >2 months after fertilization generated from double heterozygous intercrosses using the *sbu90* and *sbu94* alleles for *grin1a* and *grin1b*, respectively. Expected recovery of *grin1* double mutants is 1/16 (6.25%). **B**, Lateral views of live images of WT (top) and *grin1* double mutants (middle, bottom) at 6 dpf. *grin1* double mutants often exhibited uninflated swim bladders (bottom). **C**, Western blot analysis of GluN1 from HEK293 cells expressing zGluN1a (lane 2) or zGluN1b (lane 3) (along with rat GluN2A), or from zebrafish lysate from WT (lane 4) or *grin1* double mutant (lane 5) larvae. GAPDH was used as a protein reference.

Differential expression of zebrafish *GRIN1* paralogs in early development

grin1a is expressed in the brain, retina, and spinal cord at 1 dpf with expression becoming more robust by 2 dpf (Cox et al., 2005). Over the same period, *grin1b* expression is much weaker (Cox et al., 2005). To further define their expression, we studied *grin1a* and *grin1b* expression using whole-mount RNA ISH over a wider developmental range (1, 3, and 5 dpf) (Fig. 2). For these experiments, we used the published *grin1a* probe (Cox et al., 2005) but redesigned the *grin1b* probe to reduce background (see Materials and Methods).

Consistent with earlier results, we observed expression of *grin1a* at 24 hpf in the telencephalon (Fig. 2A,C, arrows) and in the spinal cord (Fig. 2O). We could not detect any *grin1b* transcripts at this developmental stage (Fig. 2B,D,P). By 80 hpf, *grin1a* and *grin1b* are expressed more broadly throughout the brain and in distinct domains in the retina (*grin1a*: Fig. 2E,G,M; *grin1b*: Fig. 2F,H,N). At 122 hpf, *grin1a* and *grin1b* are expressed in the telencephalon, optic tectum, hindbrain (*grin1a*: Fig. 2I,K; *grin1b*: Fig. 2J,L) and in the retina (data not shown).

Although both *grin1a* and *grin1b* have overlapping expression, there are notable differences. In the spinal cord, *grin1a* is robustly expressed between 24 and 122 hpf, but we could not detect any *grin1b* expression at these stages (Fig. 2O–R). In the telencephalon and optic tectum, *grin1a* is broadly expressed at 122 hpf, whereas *grin1b* has a more discrete expression pattern (*grin1a*: Fig. 2I,K; *grin1b*: Fig. 2J,L).

The RNA ISH experiments suggest that *grin1a* is expressed earlier and at higher levels than *grin1b*. To directly test this idea, we conducted qPCR (Fig. 2S). As expected, *grin1a* was readily detected at 24 hpf, whereas *grin1b* could not be detected (Fig. 2S, left). In addition, at 72 hpf, the expression of *grin1a* was significantly greater than that of *grin1b* (Fig. 2S, middle), but by 120 hpf, expression levels were comparable (Fig. 2S, right). Hence, *grin1a* is expressed earlier than *grin1b* and shows a more robust expression pattern through 3 dpf.

Generation of *grin1a* and *grin1b* mutations

To study the requirements for NMDAR-mediated transmission in the developing zebrafish nervous system, we disrupted the

grin1a and *grin1b* genes using CRISPR-Cas9 (Chang et al., 2013; Hwang et al., 2013). We targeted the highly conserved SYTANLAFF motif within the M3 transmembrane segment (Fig. 3A (Wollmuth and Sobolevsky, 2004; Traynelis et al., 2010)). Frameshift mutations in this region will prevent formation of the ion channel, the ligand-binding domain (LBD), the last transmembrane domain (M4), and the C-terminal domain (CTD) (Fig. 3B). Any protein generated from these alleles would be unable to bind ligand, and iGluRs lacking the M4 segment do not tetramerize nor reach the membrane (Salussolia et al., 2011, 2013; Gan et al., 2015). We analyzed three germline mutations for each paralog. Sequencing of each lesion revealed a predicted frameshift mutation and a nearby stop codon (Fig. 3C, D). For the majority of our subsequent analysis, we used alleles of *grin1a* and

grin1b that yielded the earliest predicted stop codon: a 7-nucleotide deletion in *grin1a* (*grin1a*^{sbu90}) and a 17-nucleotide insertion in *grin1b* (*grin1b*^{sbu94}). RT-PCR only revealed *grin1a* or *grin1b* mRNA species containing the indel in the respective mutants (Fig. 3E,F). The M3 segment including the SYTANLAFF is integral to iGluR function; any disruption of the M3 segment would by itself ablate receptor function (Kazi et al., 2014; Hansen et al., 2018). Given the functional importance of the M3 segment, it is improbable that any cryptic splicing event that bypassed the lesion site in this region could produce a functional receptor. Based on the anticipated consequences of the mutations on NMDAR structure, any protein produced from the *grin1a* and *grin1b* mutant alleles would not form a functional receptor.

Nonsense-mediated decay of mutant mRNA can cause transcriptional adaptation and upregulation of related genes (El-Brolosy et al., 2019). We therefore assessed levels of the lesioned mRNA and the corresponding paralog in *grin1a*^{−/−} and *grin1b*^{−/−} fish at 5 dpf (Fig. 3G,H). We detected a trending, but not significant, decrease in *grin1a* expression in *grin1a*^{−/−} larvae at 5 dpf ($p = 0.06$, t test; Fig. 3G), suggesting possible non-sense-mediated decay. However, for *grin1b*^{−/−} larvae, we detected no corresponding difference in *grin1b* expression (Fig. 3H). To determine whether transcriptional adaptation enhanced expression of the corresponding paralog, we assayed the expression levels of *grin1b* in *grin1a*^{−/−} (Fig. 3H) and of *grin1a* in *grin1b*^{−/−} (Fig. 3G) at 5 dpf but did not detect compensation. *grin1a*, but not *grin1b*, is expressed in the spinal cord at 5 dpf (Fig. 2Q,R). We therefore also assayed *grin1b* expression in the spinal cord of 5 dpf *grin1a*^{−/−} larvae by RNA ISH of intact larvae and by qPCR of spinalized larvae. We did not detect compensation by either method (data not shown). Together, transcriptional upregulation of the corresponding paralog is not a prominent feature of the *grin1* alleles at larval stages.

grin1a and *grin1b* single-mutant fish are viable, but *grin1a* mutants display growth defects

grin1a and *grin1b* single-mutant fish appear morphologically normal at 6 dpf. Although both survive to adulthood and are fertile, *grin1a*^{−/−} adults are not recovered at expected Mendelian ratios. Of the 292 adult fish generated from *grin1a*^{+/−}

intercrosses, only 35 were *grin1a*^{-/-} fish, resulting in 11.9% recovery instead of the predicted 25.0% ($p = 1.6 \times 10^{-6}$, χ^2 test). Adult *grin1a*^{-/-} fish that are recovered are smaller than their WT siblings (Fig. 4A,C). In contrast, *grin1b*^{-/-} fish show no growth nor viability deficits (Fig. 4B,D). For *grin1a*^{-/-} fish, a size deficit compared with their WT siblings is apparent between 56 and 98 dpf, although this narrows later in development (Fig. 4E). The same reduced viability and growth deficit observed in *grin1a*^{sbu90/sbu90} was also present in a second allele, *grin1a*^{sbu92} (data not shown). These phenotypic differences between *grin1a*^{-/-} and *grin1b*^{-/-} fish indicate that GluN1b is unable to fully compensate for the loss of GluN1a in *grin1a*^{-/-}.

Zebrafish lacking *grin1* survive until 10 dpf

Knockout of *grin1* in mice is embryonic lethal (Forrest et al., 1994). Both *grin1a* and *grin1b* single-mutant fish survive to adulthood and produce viable offspring. Zebrafish *grin1* double mutants (*grin1a*^{-/-}; *grin1b*^{-/-}) are recovered at expected Mendelian ratios at 6 dpf (Fig. 5A) but do not survive past 10 dpf (Fig. 5A; and data not shown). The majority of *grin1* double mutants fail to fully inflate their swim bladders (Fig. 5B) but grow at comparable rates through early larval development (data not shown).

To confirm the absence of GluN1 in the *grin1* double mutants, we performed Western blot analysis (Fig. 5C). As a control, we extracted lysates from HEK293 cells expressing either *grin1a* or *grin1b*, coexpressed with rat GluN2A. The GluN1 antibody binds to both zGluN1a and zGluN1b (Fig. 5C, lanes 2 and 3) and showed a band of the expected size in protein lysates extracted from the heads of 7 dpf WT fish (Fig. 5C, lane 4). In contrast, we were unable to detect GluN1 immunoreactivity in 7 dpf *grin1* double-mutant larvae (Fig. 5C, lane 5). These results are consistent with our RT-PCR data (Fig. 3D,E), indicating an absence of WT *grin1* transcripts and protein in mutant fish.

NMDARs are required for normal feeding behavior

Fish lacking NMDARs can survive until 10 dpf (Fig. 5). Given the importance of NMDARs to brain function, we wanted to understand the complexity of behaviors that the *grin1* double-mutant larvae could generate. To begin to address this question, we assayed prey-capture, which is a complex behavior requiring visual and motor coordination (Sammelhack et al., 2014) and is an essential behavior for survival. We used a larval feeding assay to assess the ability of individual larvae to capture paramecia (Fig. 6A) (Gahtan et al., 2005). Because of variation in the number of paramecia eaten by control larvae from day to day, we normalized the proportion of paramecia eaten by the experimental group to their same-day control (Fig. 6B–D).

Figure 6. *grin1* mutations cause feeding and visually evoked startle deficits. **A**, Representative traces of paramecium movement used to assay feeding. Traces generated by analyzing 2.5 s of paramecium movement. **B–D**, Proportion of paramecia eaten over the trial period (mean $\pm$ SEM) normalized to control for the following: (**B**) *grin1* control (*grin1a*^{+/+}; *grin1b*^{-/-}, $n = 18$) or *grin1a*^{-/-}; *grin1b*^{-/-} ($n = 33$) fish ($***p = 1.8 \times 10^{-11}$, t test); (**C**) *grin1a*^{+/+} ($n = 25$) or *grin1a*^{-/-} ($n = 23$) fish ($***p = 8.9 \times 10^{-6}$, t test); and (**D**) *grin1b*^{+/+} ($n = 14$) or *grin1b*^{-/-} ($n = 10$) fish. **E**, Representative response to dark flash stimulus from resting (**Ei**) to characteristic O-bend (**Eiii**) and reorientation (**Eiv**) of a WT zebrafish larva. **F**, Percentage of larvae responding to a dark flash stimulus with an O-bend for *grin1* control (*grin1a*^{+/+}; *grin1b*^{-/-} or *grin1a*^{-/-}; *grin1b*^{-/-}, $n = 45$) or *grin1a*^{-/-}; *grin1b*^{-/-} ($n = 16$) fish ($***p = 9.6 \times 10^{-5}$, t test). **G**, **H**, Kinetics of dark flash O-bend response: (**G**) latency until O-bend response ($***p = 4.6 \times 10^{-13}$, t test); and (**H**) bend angle of response for O-bends ($n = 104$, $n = 14$) generated by *grin1* control (*grin1a*^{+/+}; *grin1b*^{-/-} or *grin1a*^{-/-}; *grin1b*^{-/-}) or *grin1a*^{-/-}; *grin1b*^{-/-}, respectively.

Compared with their controls, *grin1* double-mutant larvae showed a significant deficit in prey-capture (Fig. 6B). Nevertheless, these fish could successfully perform this complex behavior. We also tested the *grin1* single mutants in this assay. Like the *grin1* double mutant, *grin1a*^{-/-} also showed a significant deficit in prey-capture (Fig. 6C), a phenotype recapitulated in the *grin1a*^{sbu92} allele (data not shown). No deficits were observed in *grin1b*^{-/-} larvae (Fig. 6D). Hence, the *grin1* double-mutant and *grin1a*^{-/-} fish show a notable deficit in prey-capture. This inability to efficiently capture prey may contribute to the early death of *grin1* double mutants (Fig. 5) and reduced growth rate of *grin1a* single mutants (Fig. 4).

Loss of NMDAR results in visual deficits

Zebrafish are highly visual animals (Gestri et al., 2012). One possible explanation for the reduced prey-capture is a disruption in vision. Indeed, both *grin1* paralogs are expressed in the retina (Fig. 2M,N) and NMDAR-mediated transmission is necessary for proper development of the visual system (Schmidt et al., 2000). To assay vision, we used a stereotyped behavioral response, the O-bend, which is a visually evoked response to a dark flash stimulus (Fig. 6E) (Burgess and Granato, 2007a; Wolman et al., 2011).

Compared with its sibling *grin1* controls, *grin1* double-mutant fish showed a significant decrease in the number of O-bends

Figure 7. The *grin1* double mutant and MK-801-treated, but not *grin1a*^{-/-} or *grin1b*^{-/-}, alter spontaneous movement. **A–D**, Spontaneous movements at 6 dpf and evoked movements after a transition from light to dark. Line graphs of average number of movement counts per minute (mean ± SEM) for the following: (**A**) *grin1a*^{+/+} (*n* = 64) or *grin1a*^{-/-} (*n* = 79) fish; (**B**) *grin1b*^{+/+} (*n* = 49) or *grin1b*^{-/-} (*n* = 50) fish; (**C**) *grin1* control (*grin1a*^{+/+}; *grin1b*^{+/+}, *grin1a*^{+/+}; *grin1b*^{+/-}, and *grin1a*^{-/-}; *grin1b*^{+/-}, *n* = 148) or *grin1a*^{-/-}; *grin1b*^{-/-} (*n* = 32); and (**D**) fish treated with vehicle (0.1% DMSO) (*n* = 26) or the NMDAR antagonist MK-801 (20 μM in 0.1% DMSO) (*n* = 27). Vehicle or MK-801 was acutely administered 1 h before the start of the spontaneous movement trial and was included in the bath solution throughout the trial. **E–H**, Bar graphs, of associated spontaneous movement trials, depicting average movements (mean ± SEM) during the early acclimation (first 5 min of acclimation), late acclimation (last 15 min of acclimation), and light and dark periods for the following: (**E**) *grin1a*^{+/+} or *grin1a*^{-/-}; (**F**) *grin1b*^{+/+} or *grin1b*^{-/-}; (**G**) *grin1* control or *grin1* double mutant (***p* = 1.9e-13 for early acclimation; **p* = 0.025 for light; ****p* = 6.7e-10 for dark, *t* test); and (**H**) vehicle or MK-801-treated fish (***p* = 0.0012 for early acclimation; ****p* = 2.1e-6 for late acclimation; ****p* = 2.6e-6 for light; ****p* = 1.4e-14 for dark, *t* test).

in response to a dark flash (Fig. 6F). When an O-bend was evoked, their latency to initiation was also significantly slower (Fig. 6G). Neither the *grin1a* nor *grin1b* single mutants showed any alteration in O-bend responsiveness or latency (data not shown). Of the O-bends generated by the *grin1* double-mutant

fish, there were no changes in the kinematic parameters of bend angle (Fig. 6H) or angular velocity (data not shown), suggesting that the O-bends, although infrequently generated, are normal. These findings suggest the motor circuit underlying this behavior is largely intact.

In summary, the significantly reduced dark-stimulus evoked response suggests that *grin1* double-mutant fish have strongly impaired, but not entirely absent, visual function. In contrast, the visual function of the *grin1* single mutants is sufficient to respond normally to dark flash stimuli. Although both *grin1a* single and *grin1* double-mutant fish have a prey-capture deficit, only the *grin1* double-mutant fish have a robust visual deficit. Hence, the basis for prey-capture deficits is complex and likely to involve defects outside the visual system.

Spontaneous and evoked movements are disrupted in *grin1* double mutant and MK-801-treated fish

To further understand the requirement of NMDARs in larval zebrafish behavior, we used a spontaneous and light-to-dark evoked swimming protocol. This protocol provides an overview of motor control and response to a photic stimulus, which is driven by both deep brain photoreceptors and retinal signaling (Fernandes et al., 2012). In addition to assaying locomotor behavior of *grin1* mutants, we also tested WT larvae acutely treated with MK-801, an NMDAR channel blocker (Traynelis et al., 2010), to begin distinguishing acute and developmental roles of NMDAR-mediated transmission in generating these behaviors. MK-801 is highly specific for NMDARs over non-NMDAR subtypes (Huettner and Bean, 1988) and does not show any preference for different GluN2 subunits (Traynelis et al., 2010).

We monitored spontaneous movements of individual 6 dpf *grin1a* and *grin1b* single mutants and *grin1* double-mutant larvae in 24-well plates during a 50 min paradigm with periods in light (acclimation and light periods) and dark. Turning off the illumination evokes behavior referred to as a visual motor response (Burgess and Granato, 2007a; Emran et al., 2008). In this paradigm, WT zebrafish display stereotyped behaviors (Fig. 7A–D, gray traces): their number of movements stabilizes during the acclimation period and then transiently increases following the transition to darkness. *grin1a*^{−/−} (Fig. 7A,E) and *grin1b*^{−/−} (Fig. 7B,F) fish replicate this behavior. In contrast, although *grin1* double-mutant larvae achieve coordinated swimming, they exhibit a different behavioral profile (Fig. 7C): they are hyperactive during the initial acclimation period, have reduced levels of activity during the light period, and show a decrease in activity on the change to the dark period (Fig. 7C,G). Other swim parameters in the *grin1* double-mutant larvae, such as duration of movements and distance traveled, showed the same alterations as observed in the movement count (data not shown). These behavioral alterations were not dependent on swim bladder inflation (data not shown).

This altered phenotype in *grin1* double-mutant fish could reflect that a behavior requires NMDAR-mediated transmission or that an underlying circuit failed to develop properly in the

Figure 8. *grin1* double mutants display comparable behaviors to MK-801-treated larvae in multiple swim parameters. **A**, Box plot and individual responses (dots) for photic response (average distance traveled in response to the light change) for *grin1* control (*grin1a*^{+/+}; *grin1b*^{+/+}, *grin1a*^{+/+}; *grin1b*^{+/+} and *grin1a*^{+/+}; *grin1b*^{+/+}, *n* = 149) or *grin1* double mutant (*grin1a*^{−/−}; *grin1b*^{−/−}, *n* = 31, ****p* = 4.3e-5, *t* test) (left); and vehicle (0.1% DMSO, *n* = 27) or MK-801-treated (20 μM in 0.1% DMSO, *n* = 27, ****p* = 4.1e-7, *t* test) (right). **B**, **C**, Bar graphs (mean ± SEM) for the following: (**B**) swim length (ratio of distance per movement to control) (****p* = 1.2e-10 for *grin1* double; ***p* = 0.0056 for MK-801, *t* test); and (**C**) swim speed (ratio of speed during movements to control) (****p* = 2.0e-13 for *grin1* double; ****p* = 2.7e-4 for MK-801, *t* test) during the spontaneous locomotion assay at 6 dpf. For calculating swim length and swim speed, only large movements (speeds > 8 mm/s) were used, to exclude drifting movement between bursts. Same groups as from **A**.

absence of NMDAR-mediated transmission. To distinguish these possibilities, we applied MK-801, an NMDAR channel blocker, to WT fish 1 h before behavioral testing (Fig. 7D) to acutely antagonize NMDAR-mediated transmission. Any phenotype present in the *grin1* double mutants that is recapitulated by MK-801 treatment suggests that the phenotype is generated by the lack of NMDAR-mediated transmission, whereas if MK-801 treatment does not replicate the phenotype, it suggests a developmental effect of the long-term absence of NMDAR-mediated signaling. In the spontaneous and photic-evoked movement paradigm, we observed both outcomes. In parallel with the *grin1* double-mutant fish, acute MK-801 treatment significantly reduced spontaneous activity during the light period and eliminates the stereotypic response to the light change (Fig. 7H). In contrast to the *grin1* double-mutant fish, acute MK-801 treatment did not induce hyperactivity during the initial acclimation period (Fig. 7H).

MK-801 treatment phenocopies multiple behaviors observed in the *grin1* double mutants

grin1 double mutants both showed decreases in gross movement counts throughout the trial and lacked the stereotypical visual motor response found in WT larvae (Fig. 7G), outcomes phenocopied by MK-801 treatment (Fig. 7H). Nevertheless, how robust this overlap in behavior is between *grin1* double-mutant and MK-801-treated fish is unclear given the gross nature of our movement measurements. We therefore characterized additional swim parameters, as well as the instantaneous response to the transition from light to dark in more detail (Fig. 8).

Figure 9. MK-801 cannot phenocopy all altered behaviors in *grin1* double mutants. **A**, Spontaneous movements during the acclimation period at 4 and 5 dpf. Line graphs of average number of movement counts per minute (mean $\pm$ SEM) for *grin1* control (*grin1^{+/+}; grin1b^{+/+}*, *grin1^{+/+}; grin1b^{+/-}* and *grin1^{+/+}; grin1b^{-/-}*, $n = 100$, $n = 101$) or *grin1^{-/-}; grin1b^{+/+}* ($n = 17$, $n = 17$) for 4 and 5 dpf, respectively. **B**, Bar graphs of associated spontaneous movement trials, depicting average movements (mean $\pm$ SEM) during the early acclimation (first 5 min of acclimation) for 4 dpf ($***p = 4.4 \times 10^{-9}$, t test), 5 dpf ($***p = 5.2 \times 10^{-8}$, t test), and 6 dpf ($***p = 1.9 \times 10^{-12}$, t test). Bar graph for 6 dpf is for reference and is the same data as in Figure 7G. **C**, Schematic representation of the inner and outer portions of a behavior well. **D**, **E**, Number of entries into the inner section of behavior well (mean $\pm$ SEM) (**D**) and percentage of time spent in the inner behavior well (mean $\pm$ SEM) (**E**) during the spontaneous movement paradigm (Fig. 7) ($***p = 4.1 \times 10^{-4}$ for *grin1* double, t test). Different conditions/genotypes tested include the following: *grin1* control (*grin1^{+/+}; grin1b^{+/+}*, *grin1^{+/+}; grin1b^{+/-}* and *grin1^{+/+}; grin1b^{-/-}*, $n = 148$) or *grin1^{-/-}; grin1b^{+/+}* ($n = 32$); and vehicle (0.1% DMSO) ($n = 26$) or MK-801 (20 μ M in 0.1% DMSO) ($n = 27$) treated.

To further assess the light-to-dark behavioral response, we measured the distance traveled in the second after the light change. Because of the reduced responsiveness to a dark flash stimulus (Fig. 6F), we suspected the *grin1* double mutants would show deficits in responding to the extinguishing of the light. Indeed, *grin1* double mutant showed significant decreases in this photic-evoked response compared with controls, a phenotype recapitulated by MK-801 treatment (Fig. 8A). Paralleling the O-bend results, *grin1a* and *grin1b* single mutants showed no altered response (data not shown).

In terms of additional swim parameters, we found that both the swim length (Fig. 8B) and swim speed (Fig. 8C) were significantly increased in both the *grin1* double-mutant and the MK-801-treated fish compared with controls. *grin1a* and *grin1b* single mutants showed no alteration in these swim kinetics (data not shown). Thus, MK-801 phenocopies both gross and fine movement parameters of the *grin1* double mutants, suggesting, at minimum, that these deficits reflect a lack of NMDAR-mediated transmission. In addition to these spontaneous swim parameters, MK-801 treatment of WT fish also phenocopied the prey-capture deficit found in *grin1* double-mutant fish (Fig. 5B): MK-801-treated fish (0.28 ± 0.12 , $n = 15$; mean $\pm$ SEM, n = number of fish tested) captured a significantly lower percentage of paramecia compared and standardized to their vehicle control (1.00 ± 0.12 , $n = 14$) ($p = 9.4 \times 10^{-6}$, t test).

Behavioral deficits in the *grin1* double mutants with a likely developmental origin

The *grin1* double-mutant (Fig. 7C,G), but not MK-801-treated (Fig. 7D,H), fish exhibited hyperactivity immediately after being

placed in the testing apparatus. Because acute NMDAR antagonism does not replicate this hyperactivity, it is presumably developmental in origin. Consistent with this idea, *grin1* double-mutant fish at earlier developmental time points (4 and 5 dpf) also show hyperactivity during the early acclimation period (Fig. 9A,B). The lack of hyperactivity in the MK-801-treated fish is unlikely due to off-target effects of MK-801, since applying MK-801 to the *grin1* double-mutant fish did not abolish this hyperactivity (data not shown). Hence, the circuit underlying the hyperactivity during the early acclimation period is most likely due to the lack of NMDAR-mediated signaling during early development.

Zebrafish larvae spend a greater duration of time in the outer portions of a circular well than would be expected by random chance (Fig. 9C; and data not shown) (Schnorr et al., 2012). Changes in place preference in larval zebrafish may reflect levels of anxiety (Schnorr et al., 2012) and/or altered search strategy (Horstick et al., 2017). While *grin1* double mutants enter the center portion of the well as often as their control siblings (Fig. 9D, left), they exit more rapidly and do not remain in the center as long as controls (Fig. 9E, left). In contrast, fish treated with MK-801 showed no difference compared with control in either their entrances into the center of the well or the time spent there (Fig. 9D,E, right panels). Hence, variations in place preference in the *grin1* double-mutant fish presumably have a developmental origin.

grin1 mutants fail to habituate to acoustic stimuli

The circuit underlying escape responses to acoustic stimuli in fish is well studied and NMDAR-dependent (Lopez-Schier,

2019). We therefore assayed acoustic responsiveness, habituation, and sensorimotor gating to further define the breadth of behaviors *grin1* mutant fish can generate.

Intense auditory stimuli induce rapid escape responses in larvae, including Mauthner-cell-initiated Short latency C-bend (SLC) responses (Burgess and Granato, 2007b). SLC responsiveness was increased in the *grin1* double mutants compared with controls (Fig. 10A). Additional kinematics of this startle response in *grin1* double mutants, including the initial bend angle, angular velocity, and duration and latency of response, were unchanged (data not shown), suggesting that the *grin1* double-mutant startle kinematics were unaltered. Paralleling the result in the *grin1* double mutant, SLC responsiveness was increased in *grin1a*^{-/-} larvae compared with sibling controls at low- and high-stimulus intensities (Fig. 10B), whereas *grin1b*^{-/-} larvae showed no differences (Fig. 10C).

To test for changes in SLC habituation, we presented startling stimuli at 1 s intervals and measured habituation as the percent decrease in SLC responsiveness (see Materials and Methods). *grin1* double mutants fish showed a decrease in habituation compared with controls (Fig. 10D). Even at a lower stimulus intensity, *grin1* double-mutant fish still showed a habituation deficit (data not shown), confirming that habituation was not being obscured by the high SLC response percentage. *grin1a*^{-/-} (Fig. 10E), but not *grin1b*^{-/-} (Fig. 10F), larvae also exhibited decreases in habituation compared with WT siblings.

In summary, these results are consistent with previous work, which has shown that MK-801 treatment leads to both increased response to acoustic startle and decreased habituation (Wolman et al., 2011). They also suggest that acoustic startle responses and nonassociative learning are primarily mediated by GluN1a.

grin1 mutants show sensorimotor gating deficits

Despite its simplicity, the SLC response circuit exhibits a form of sensorimotor gating, PPI, which results in the attenuation of the SLC response (Burgess and Granato, 2007b). The extent of PPI depends on the ISI between the prepulse and pulse stimuli, and pharmacological blockade of NMDAR signaling selectively affects long-interval (>300 ms) PPI (Burgess and Granato, 2007b; Bergeron et al., 2015). We therefore tested PPI at 50 and 500 ms intervals and found that *grin1* double mutants had deficits at 500 ms ISI, but not at 50 ms ISI (Fig. 10G), consistent with previous pharmacological experiments.

Figure 10. Disruption of sensorimotor gating and nonassociative learning in *grin1* mutant fish. **A–C**, Responsiveness of larvae to a low- and high-intensity acoustic stimuli measured as percentage of larvae responding with a SLC for the following: (**A**) *grin1* control (*grin1a*^{+/+}; *grin1b*^{-/-}, *n* = 22) or *grin1* double mutant (*n* = 20) (**p* = 0.019 for low intensity, *t* test); (**B**) *grin1a*^{+/+} (*n* = 18) or *grin1a*^{-/-} (*n* = 20) (***p* = 0.0091 for low intensity; ***p* = 0.0044 for high intensity, *t* test); and (**C**) *grin1b*^{+/+} (*n* = 13) or *grin1b*^{-/-} (*n* = 15). **D–F**, Habituation to a high-intensity acoustic stimuli measured as a habituation index for the following: (**D**) *grin1* control (*grin1a*^{+/+}; *grin1b*^{-/-} and *grin1a*^{+/+}; *grin1b*^{-/-}, *n* = 12) or *grin1* double mutant (*n* = 4) (****p* = 5.4e-4, *t* test); (**E**) *grin1a*^{+/+} (*n* = 22) or *grin1a*^{-/-} (*n* = 21) (***p* = 0.0034, *t* test); and (**F**) *grin1b*^{+/+} (*n* = 12) or *grin1b*^{-/-} (*n* = 10). **G–I**, PPI percentage for ISIs of 50 and 500 ms for the following: (**G**) *grin1* control (*n* = 6, *n* = 17) or *grin1* double mutant (*n* = 8, *n* = 12) (****p* = 4.0e-5 for long ISI, *t* test); (**H**) *grin1a*^{+/+} (*n* = 15, *n* = 15) or *grin1a*^{-/-} (*n* = 10, *n* = 11) (***p* = 0.002 for long ISI, *t* test); (**I**) *grin1b*^{+/+} (*n* = 9, *n* = 9) or *grin1b*^{-/-} (*n* = 10, *n* = 12). We used (*grin1a*^{+/+}; *grin1b*^{-/-}) intercrosses to generate an enhanced number of *grin1* double mutants for **A**, **D**, and **G** in this assay. In these crosses, *grin1a*^{+/+}; *grin1b*^{-/-} and *grin1a*^{+/+}; *grin1b*^{-/-} were used as a control, as no deficits were observed in *grin1b*^{-/-} fish.

Paralleling these results, *grin1a*^{-/-} larvae also showed deficits at 500 ms ISI, but not at 50 ms ISI (Fig. 10H), whereas *grin1b*^{-/-} larvae showed no differences in PPI (Fig. 10I), indicating a requirement for GluN1a in PPI at long ISI.

Discussion

NMDAR-mediated signaling underlies a variety of higher-order brain functions, including neural plasticity, learning, memory,

and neurodevelopment and correspondingly modulates a variety of behaviors (Paoletti et al., 2013). In the present study, we took advantage of zebrafish to generate knockouts of the obligatory GluN1 subunit. Surprisingly, we find fish completely lacking NMDAR-mediated signaling can survive through early development, until ~10 dpf. These larvae lacking NMDAR can initiate a wide variety of behaviors, including prey-capture, burst swimming, and stereotypic responses to visual and acoustic stimuli, although generally fish lacking NMDARs showed deficits relative to WT. In addition, using acute treatment with MK-801 as a reference, we find that some of these behavioral deficits arise from a lack of NMDAR-mediated transmission, whereas others appear to have a developmental component. Given that these NMDAR-lacking fish can perform an array of behaviors, they will be an invaluable tool to study how NMDAR signaling affects early brain development.

A variety of lines of evidence support the idea that *grin1* double-mutant fish have no residual GluN1 function. First, we introduced CRISPR-Cas9 lesions in the highly conserved M3 pore lining helix (Fig. 3A–D), such that any alternative splicing event that circumvented the mutations would render the channel non-functional. Second, we detected no WT *grin1* mRNA in the individual mutants (Fig. 3E,F), nor could we detect zGluN1a or zGluN1b protein in lysate from larval *grin1* double mutants (Fig. 5C). Finally, many behavioral deficits of the *grin1* double mutant were phenocopied by MK-801, including prey-capture (data not shown), the response to a photic stimulus (Figs. 7, 8), and acoustic startle modulation (Fig. 10).

Zebrafish underwent an ancient genome duplication (Amores et al., 1998), leading to two *grin1* paralogs, *grin1a* and *grin1b*. These zebrafish GluN1 paralogs, when coexpressed with rat GluN2A in a heterologous expression system, show glutamate-gated currents and are highly Ca^{2+} -permeable with zebrafish GluN1a and GluN1b similar to each other and to the rat GluN1 subunit (Fig. 1). Nevertheless, *grin1a* and *grin1b* showed notable differences in expression (Fig. 2). *grin1a* is expressed more robustly in early development than *grin1b*, and it is expressed in the developing spinal cord, where *grin1b* is absent, suggesting differential spatiotemporal requirements for the two paralogs.

Despite sequence and functional similarity, zebrafish lacking either *grin1a* or *grin1b* show stark phenotypic and behavioral differences. *grin1a*^{−/−} fish have reduced viability and growth deficits, whereas *grin1b*^{−/−} adults do not (Fig. 4). Similarly, *grin1a*^{−/−} fish have a prey-capture deficit (Fig. 6C) and an altered responsiveness to acoustic stimuli (Fig. 10), whereas *grin1b*^{−/−} do not. Still, while we did not identify any phenotype for *grin1b*^{−/−} mutant alone, there were instances where the *grin1* double-mutant fish showed distinct phenotypes to the single mutants, including responses to a dark flash (Fig. 6E–H) and spontaneous and evoked behavior (Figs. 7, 8), highlighting that *grin1b* plays an important role in brain function. Overall, these results suggest that *grin1a* and *grin1b* have unique functions, likely due to their spatiotemporal expression differences, but future experiments will be needed to fully explain these differences and to better understand their distinct roles in the circuits generating these behaviors.

Genetic compensation occurs in response to non-sense-mediated decay (El-Brolosy et al., 2019), but we did not detect any compensatory increases in the other paralog (Fig. 3G,H). While NMDARs have distinct functional properties, other classes of iGluRs, including AMPARs and kainate receptors, could provide some compensatory and/or redundancy of function either at the

synaptic or circuit level. Consistent with this idea, activation of either AMPAR or NMDAR is sufficient to generate organized locomotive activity in spinalized zebrafish, but only AMPARs are necessary for generation of this movement (Wahlstrom-Helgren et al., 2019). This observation may account for the finding that *grin1a* single mutants and *grin1* double mutants generate coordinated spontaneous locomotor activity despite an apparent lack of NMDAR in their spinal cord. Nevertheless, future studies will be needed to explore compensatory mechanisms in the *grin1* mutant fish.

The *grin1* double mutants were able to generate many behaviors, including sufficiently coordinated sensory and locomotor activity for prey-capture (Fig. 6B), O-bend responses to visual stimuli (Fig. 6F–H), burst swimming (Fig. 7C), and SLC responses to acoustic stimuli (Fig. 10A). Relative to WT, however, the *grin1* double mutants display a variety of alterations in their behavioral repertoire. Many of these alterations are replicated by administration of MK-801, including prey-capture deficits (data not shown), gross motor movement responses to photic stimuli (Fig. 7D), abnormal startle responsiveness (Fig. 8A), swim kinetics (Fig. 8B,C), and SLC habituation (Wolman et al., 2011). We did not determine the status of the swim bladder in all experiments involving *grin1* double-mutant fish (e.g., prey-capture) and hence cannot entirely rule out the possibility that the underinflation of the swim bladder in *grin1* double mutants is contributing to some of these phenotypes. Nevertheless, WT larvae treated with MK-801 retain a fully inflated bladder yet replicate many of the *grin1* double-mutant behavioral alterations. This suggests that the observed alterations are a result of the lack of NMDAR-mediated transmission, rather than caused by underinflation of the swim bladder. It is also possible that short-term block of NMDAR-mediated transmission by MK-801 may be overshadowing additional developmental roles of NMDAR, as is likely the case for more complex behaviors.

We found evidence that certain behavior alterations stem from developmental effects of loss of NMDAR-mediated transmission. Some examples are the hyperactivity at the onset of experimentation (Fig. 9A,B) and a reduced time spent in the center of the well (Fig. 9E). It is interesting to note that the alteration in visual motor response in the *grin1* double mutant, notably a decrease in movements generated, has also been previously reported in WT larvae in larger behavior chambers (Horstick et al., 2017). This similarity may suggest either an alteration in photic-stimuli-induced search strategy or an alteration in the perception of their environment for the *grin1* double mutant.

In response to acoustic stimuli, the *grin1* double mutant displayed multiple phenotypes previously observed in pharmacological inhibition of NMDAR in larvae: an increase in SLC responsiveness (Fig. 10A) (Bergeron et al., 2015), a decrease in habituation to an acoustic stimuli (Fig. 10D) (Roberts et al., 2011; Wolman et al., 2011), and a deficit in PPI at long ISIs (Fig. 10G) (Burgess and Granato, 2007b; Bergeron et al., 2015). Interestingly, these phenotypes are also all observed in the *grin1a* single mutant, suggesting a greater role of zGluN1a in the generation of these behaviors (Fig. 10B,E,H). The acoustic startle circuit generation of SLC responses is well characterized (Lopez-Schier, 2019), and these mutants may be an ideal resource to elucidate cell specific requirements for NMDAR transmission within the circuit.

We developed a model that provides a unique opportunity to explore NMDAR functions in an early vertebrate nervous system. Given the similarity in sequence and measured

physiological properties of the *grin1* paralogs to their mammalian orthologs, and the wide array of behaviors larval zebrafish lacking all NMDAR-mediated transmission are capable of (Wahlstrom-Helgren et al., 2019), zebrafish are an ideal organism to study the developmental roles of NMDAR. We found that removing GluN1, and by extension removing NMDAR-mediated transmission, altered multiple behaviors. Remarkably, many complex behaviors, such as coordinated swimming and responses to stimuli, were retained in mutants lacking GluN1. This set of behaviors, as well as the viability through early larval stages, allows for future studies in defining the role of NMDAR in cell fate decisions and circuit formation in the early vertebrate nervous system.

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