The NMDA receptor subunit GluN3A regulates synaptic activity-induced and MEF2C-dependent transcription

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ABSTRACT

N-methyl-D-aspartate type glutamate receptors (NMDARs) are key mediators of synaptic activity-regulated gene transcription in neurons, both during development and in the adult brain. Developmental differences in the GluN2 subunit composition of NMDARs determines whether NMDARs activate the transcription factor CREB. However, whether the developmentally-regulated GluN3A subunit also modulates NMDAR-induced transcription was unknown. Here we show that knocking down GluN3A in rat hippocampal neurons promotes the inducible transcription of a subset of NMDAR-sensitive genes. This enhancement is mediated by the accumulation of phosphorylated p38 MAP kinase in the nucleus, which drives the activation of the transcription factor MEF2C and promotes the transcription of a subset of synaptic activity-induced genes including *Bdnf* and *Arc*. Our evidence that GluN3A negatively regulates MEF2C-dependent transcription reveals a novel mechanism by which NMDAR subunit composition confers specificity on the program of synaptic activity-regulated gene transcription in developing neurons.

N-methyl-D-aspartate type glutamate receptors (NMDARs) are essential for coupling sensory experience with brain development. In addition to functioning as ligand-gated ionotropic receptors, NMDARs exert long-lasting effects on neuronal biology by activating intracellular signaling cascades that subsequently impact synapse formation, maturation, and function. These inductive effects of NMDARs are mediated at least in part by the regulation of programs of gene transcription. The targets of NMDAR-regulated transcriptional signaling cascades in neurons include genes like *Bdnf* and *Arc* that directly alter synapse development and function, providing a compelling mechanism for activity-induced synaptic development and plasticity (1).

NMDARs are heterotetrameric receptors, all of which contain the obligatory GluN1 pore-forming subunit in varying stoichiometries with the glutamate-binding

GluN2 (A-D) subunits and the modulatory GluN3 (A-B) subunits (2). In addition to determining the biophysical properties of NMDA receptors (e.g. conductance, magnesium sensitivity, calcium permeability), subunit composition influences the specificity of the downstream signaling cascades initiated following receptor activation largely via protein-protein interactions with the highly variable subunit C-terminal tails (3,4). Unlike the ubiquitous expression of the GluN1 subunit, the GluN2 and GluN3 subunits show substantial regional and developmental regulation of their expression in the brain, suggesting a mechanism for NMDAR functional diversity. The ratio of GluN2A to GluN2B subunits is dynamic during postnatal development (5) and has been shown to modulate the activation of transcriptional programs by NMDARs as neurons age (6,7). Specifically, GluN2A, which predominates at synaptic receptors and in older neurons, promotes cell survival via the phosphorylation/activation of the transcription factor CREB; whereas GluN2B, which is more widely expressed in younger neurons and preferentially found at extrasynaptic receptors, activates signaling cascades that lead to CREB dephosphorylation and cell death (8,9).

Neurons in the developing postnatal brain also transiently express high levels of the GluN3A subunit, which is incorporated into functional GluN1-GluN2-GluN3 glutamate-activated NMDARs (10-13). Genetic deletion of GluN3A is associated with dysregulation of the plasticity of glutamatergic synapses in the developing brain, implicating the expression of this subunit in NMDAR-dependent aspects of brain development (14,15). Functionally, excitatory synapses show evidence of premature maturation in GluN3A knockout mice (15,16), whereas prolonging expression of GluN3A into adulthood causes excitatory synapses to persist in a juvenile state (17). Although these data indicate that GluN3A is a negative regulator of synapse maturation, the mechanism of its action in has remained to be fully understood.

We identified the transcription factor CaRF as an upstream regulator of GluN3A expression (18). CaRF knockout mice show enhanced NMDAR-induced transcription of *Bdnf* both in culture and *in vivo* in a manner that correlates with reduced GluN3A expression (18), raising the possibility that GluN3A may function to transiently inhibit the NMDAR-dependent induction of *Bdnf* and other neuronal activity-regulated genes in developing neurons. Here to determine the function of GluN3A in NMDAR-dependent transcription, we knocked down the expression of GluN3A in primary rat hippocampal neurons and determined the consequences for NMDAR-induced programs of gene expression. We find that GluN3A limits the NMDAR-activation of a select group of genes including *Bdnf* and *Arc*. GluN3A impacts downstream gene transcription by inhibiting the nuclear translocation of the p38 mitogen-activated protein kinase (MAPK), which is required for the activation of genes that depend on the transcription factor MEF2C. The net effect of this pathway is to confer specificity upon the set of synaptic signaling pathways that can induce activity-dependent transcription in developing neurons. These data bring new understanding to the mechanisms by which NMDARs regulate activity-dependent brain development.

RESULTS

Knockdown of GluN3A potentiates NMDAR-dependent induction of Bdnf

To test the requirement for GluN3A in NMDAR-inducible transcription of *Bdnf*, we characterized two independent shRNAs in lentivirus that robustly knockdown (KD) the expression of *Grin3a* mRNA and GluN3A protein in cultured embryonic rat

hippocampal neurons, and generated a GluN3A rescue construct that is resistant to shRNA1 (**Fig. 1A-C**). To activate NMDARs, we silenced neurons with TTX for 48 hours and then acutely withdrew TTX (TTX w/d), which induces rebound firing, glutamate release, and NMDAR-dependent changes in gene transcription (**Fig. S1)** (19). As we reported previously in mouse cortical neurons (18), TTX w/d from rat hippocampal neurons significantly induces the NMDAR-dependent expression of exon IV-containing forms of *Bdnf*, which are produced by the activation of *Bdnf* promoter IV (**Fig. S1**) (20). Knockdown of GluN3A with either of the two shRNAs had no significant effect on *Bdnf* IV levels in TTX silenced neurons but resulted in a significant potentiation of *Bdnf* IV mRNA transcription upon TTX w/d for each shRNA compared with its paired control infected neurons (**Fig. 1D,E**). The potentiation of *Bdnf* promoter IV activation in GluN3A KD neurons was selective for transcription induced by NMDARs, because there were no significant differences in the fold change of *Bdnf* IV mRNA induced in GluN3A KD versus control neurons following KCl-mediated membrane depolarization **(Fig. S2A**), which activates transcription selectively via the opening of L-type voltage-gated calcium channels (LVGCCs) and does not require the activation of NMDARs (18).

To determine whether the enhanced activation of *Bdnf* IV transcription in GluN3A KD neurons is a direct result of the absence of GluN3A, as opposed to an off-target effect of the shRNAs, we re-expressed the shRNA-resistant form of GluN3A in neurons that had endogenous GluN3A knocked down (**Fig. 1B,C**). Whereas GluN3A KD led to potentiation of *Bdnf* IV induction by TTX w/d relative to control, re-expression of GluN3A in KD neurons was sufficient to significantly reduce the fold change of *Bdnf* IV induction towards control levels (**Fig. 1F**). To determine whether TTX w/d induced changes in *Bdnf* mRNA induction are sufficient to result in changes in BDNF protein, we performed BDNF ELISA assays. TTX w/d induced the expression of BDNF protein in all samples (**Fig. 1G**). Similar to the effects of GluN3A KD on *Bdnf* exon IV mRNA induction, GluN3A KD neurons showed a significantly greater induction of BDNF protein after TTX w/d compared with control, whereas the coexpression of the GluN3A rescue restored BDNF protein induction to control levels.

In the context of GluN1/GluN2/GluN3 NMDARs, GluN3 has been reported to lower calcium permeability (15). To determine whether our manipulations of GluN3A expression in hippocampal neurons were affecting glutamate-induced calcium influx at synapses, we performed two-photon glutamate uncaging and dendritic spine calcium imaging in organotypic rat hippocampal slices. Local glutamate uncaging led to a transient increase in spine calcium as detected by the calcium indicator GCaMP3 **(Fig. 1H**). GluN3A KD neurons showed a significantly greater increase in GCaMP3 fluorescence upon uncaging compared with control pLKO vector transfected neurons, whereas co-expression of the GluN3A rescue plasmid with the *Grin3a* shRNA restored the calcium signal to control levels (**Fig 1I-K)**. This evidence that GluN3A levels regulate glutamate-dependent changes in spine calcium is consistent with the possibility that GluN3A modulates calcium-regulated transcriptional signaling pathways.

Knockdown of GluN3A modulates the NMDAR-induced transcriptional program

We tested other activity-regulated genes as candidate GluN3A targets and found that the potentiation of NMDAR-dependent transcription in GluN3A KD neurons was

limited to a subset of NMDAR-regulated genes. Specifically, TTX w/d-induced *Fos* expression did not differ between GluN3A KD and control neurons, however the TTX w/d induced expression of *Arc* was also potentiated in the GluN3A KD neurons compared with control, indicating that the potentiation was not limited to *Bdnf* IV (**Fig. S2B**). Thus, to determine the extent of GluN3A regulated gene expression, we performed RNA-seq on GluN3A KD and control infected neurons either in the presence of TTX silencing or 6 hr following TTX w/d. Expression of 10731 genes was detected at an FPKM>1 across triplicate replicate samples for each condition (**Table S2**).

232 genes showed differential expression (FDR adjusted P<0.05) in the presence of TTX comparing GluN3A KD with control. The expression of 196 genes, including *Grin3a*, was significantly reduced in GluN3A KD compared with control, and expression of 36 genes was significantly elevated (**Table S2, Fig. S3A**). By contrast, 815 genes were differentially expressed 6 hrs after TTX w/d comparing the GluN3A KD and control samples. 642 of these showed significantly reduced expression in the GluN3A KD samples relative to control, whereas 173, including *Bdnf,* were expressed more highly (**Fig. 2A,B**). Of the genes that were differentially expressed between the *Grin3a* KD and the control neurons after TTX w/d, only 30 genes were also differentially expressed in the presence of TTX (**Fig. S3B-C, Table S2**). These data suggest that the vast majority of differences between GluN3A KD and control samples arise as a result of TTX w/d-dependent changes in gene expression. In order to focus on these stimulus-dependent changes in gene expression, we removed the 30 stimulus-independent, GluN3A-regulated genes from subsequent analyses.

To determine if there are common biological processes among genes dysregulated in the absence of GluN3A, we subjected the differentially regulated gene lists to gene ontology (GO) analysis. The program of gene expression induced by TTX w/d in control neurons is enriched for a number of categories suggesting inflammatory and oxidative stress responses (**Table S3**). Genes that showed significantly lower expression in GluN3A KD neurons compared with control after TTX w/d were enriched for categories that include inflammatory responses and signaling cascades (**Table S3**). Two of these GO categories (GO:0007568~aging, GO:0071222~cellular response to lipopolysaccharide) are shared with the ontologies of genes that are induced by TTX w/d in control neurons. Furthermore, a substantial fraction (~18%) of the overall set of TTX w/d-inducible genes show impaired post-stimulus expression in the absence of GluN3A (**Fig. 2B**). These data suggest that GluN3A positively contributes to the regulation of a subset of the transcriptional response induced upon TTX w/d.

We next turned to the set of 162 genes that, like *Bdnf*, showed elevated expression in GluN3A KD compared with control neurons after TTX wd. These genes were enriched for two GO categories, one of which, GO:0007268~chemical synaptic transmission, contains a set of genes that mediate synaptic communication between neurons (*Grm5, Hcrtr2, Gabrg2, Gabra1, Grm6, P2rx2, Gabra5, Snca, Grin2a, Tac1, Slitrk5)*. Mirroring the inverse relationship we discussed above between GluN3A KD and TTX w/d, about 30% (49/162) of the genes that show elevated expression in GluN3A KD neurons are among the large set of genes significantly repressed by TTX wd in the control (**Fig. 2C**, **Table S2**). Overall the set of genes that show reduced expression in control neurons after TTX w/d include

many categories with synaptic functions, suggesting homeostatic repression of synaptic function by TTX w/d-induced neuronal firing (**Table S3**). These data suggest that GluN3A also positively contributes to the regulation of a subset of the transcriptional response repressed upon TTX w/d.

GluN3A inhibits NMDAR-dependent activation of MEF2C

Given that TTX w/d-induced gene expression is mediated by the activation of NMDARs, it is not surprising that loss of the NMDAR subunit GluN3A would blunt the cellular response to this stimulus. However, 70% (113/162) of genes that show elevated expression in *Grin3a* KD neurons after TTX w/d are either not regulated by TTX w/d in control neurons or they are inducible in control neurons but more strongly induced by TTX w/d in the GluN3A KD neurons (**Fig. 2D**). These data suggest that GluN3A may actively oppose a mechanism of gene expression that can be induced by NMDAR activation only in the absence of this subunit. In addition to *Bdnf*, the set of genes that are "superinduced" in GluN3A KD neurons includes the metalloproteinase *Tll1,* the endoglucuronidase enzyme *Hpse,* the neuropeptide *Tac1,* the extracellular matrix protein *Dcn,* the calcium-stimulated adenylate cyclase *Adcy8,* the leucine rich repeat kinase *Lrrk2,* the regulator of calcineurin *Rcan2,* and the synaptic adhesion protein *Cntnap4*. Although we cannot rule out that some of the differentially expressed genes in GluN3A KD neurons arise from off-target effects of the shRNA, the fact that we found other genes co-regulated with *Bdnf* encouraged us to pursue possible transcriptional mechanisms of this effect.

Prior studies have shown that the incorporation of GluN2B subunits in NMDARs represses NMDAR-dependent activation of the pro-survival transcription factor CREB (9). To determine whether GluN3A might have a similar repressive effect on specific NMDAR-regulated transcription factors, we first used Gene Set Enrichment Analysis (GSEA) to identify common transcription factor binding sites found among the promoters of the 113 genes that show elevated induction after TTX wd in GluN3A KD neurons compared with control. Among known TF motifs, these data identified enriched binding sites for the MEF2 and NFAT transcription factors **(Table 1)**. Both of these transcription factors are known to mediate synaptic activity-regulated gene transcription in neurons, although NFAT is thought to be selectively dependent on somatic L-type calcium channels for its activation even following direct synaptic glutamate receptor stimulation (21,22).

Both *Bdnf* promoter IV and *Arc* are targets of regulation by MEF2, which works in collaboration on these genes with the activity-dependent transcription factor CREB (23,24). To monitor the activity of these transcription factor families, we transfected GluN3A KD or control infected neurons with luciferase reporter plasmids in which the expression of luciferase is under the control of binding sites for either the CREB (CREB response elements; CRE) or MEF2 (MEF2 response elements; MRE) families of transcription factors.

In control infected neurons TTX w/d induced a significant increase in luciferase expression from both the CRE and MRE reporters **(Fig. 3A,B)**, demonstrating NMDAR-dependent activation of CREB and MEF2 family transcription factors. However, in GluN3A KD neurons, we detected significantly more luciferase expression from the MRE reporter after 6hr and 8hr of TTX w/d compared with the expression in control infected neurons. By contrast CRE reporter activity was not different in GluN3A KD neurons as compared with controls at any

time point (**Fig. 3A,B**). These data suggest that the potentiation of NMDAR-dependent transcription in GluN3A KD neurons could arise from enhanced activation of MEF2 transcription factors.

To determine whether MEF2 family transcription factors are required for the potentiation of NMDAR-induced gene transcription in GluN3A KD neurons, we used lentiviral shRNAs to knockdown expression of MEF2A, MEF2C, or MEF2D, the three major MEF2 family members expressed in cultured hippocampal neurons (23). Previously we showed that knockdown of MEF2C but not MEF2A or MEF2D impairs the LVGCC-dependent induction of *Bdnf* exon IV (23). Here we found that, whereas knocking down any of the three MEF2 family members (**Fig. S4A-C)** did not affect TTX w/d-induced *Bdnf* IV expression in control neurons, knockdown of MEF2C, but not MEF2A or MEF2D, eliminated the potentiation of *Bdnf* IV expression upon TTX w/d in GluN3A KD neurons (**Fig. 3C**).

To test whether knockdown of GluN3A potentiates the transcriptional activity of MEF2C, we cotransfected neurons with plasmids encoding a Gal4-MEF2C fusion protein and a UAS-luciferase reporter along with either *Grin3a* shRNA1 or the paired control vector. Neuronal MEF2C is comprised of two major splice variants: both isoforms contain exons encoding the α1 and β domains, but the alternatively spliced exon encoding the γ domain is present in only about 50% of *Mef2c* mRNA transcripts in hippocampus and cortex (23,25). MEF2C splice variants lacking the γ-domain are the most highly activated of the MEF2C isoforms following LVGCC activation (23). Here we found that only Gal4-MEF2C fusion proteins that lack the γ domain are activated upon TTX w/d (**Fig. 3D**). Furthermore, only the γ-lacking isoform of MEF2C (MEF2Cα1β) showed potentiation upon TTX w/d in GluN3A KD neurons compared with control neurons (**Fig. 3D**). Importantly, because these studies were done with co-transfection of the knockdown and the Gal4 reporter plasmids, which leads to expression of both constructs in only about ~1% of the neurons in our cultures, these experiments demonstrate that the effects of GluN3A knockdown on the activation of MEF2C are cell autonomous.

To test whether expression of the MEF2Cα1β isoform is sufficient to drive NMDAR-dependent *Bdnf* IV transcription, we re-expressed human MEF2Cα1β, which is resistant to the rat *Mef2c* shRNA, in GluN3A and MEF2C double KD neurons (**Fig. S4D**). Whereas the knockdown of MEF2C eliminated the potentiation of both *Bdnf* IV and *Arc* transcription in GluN3A KD neurons, replacement of human MEF2Cα1β in GluN3A and MEF2C double KD neurons was sufficient to restore induction of expression to the levels seen in GluN3A KD neurons (**Fig. 3E,F).**

Enhanced NMDAR-dependent nuclear activation of p38 MAPK in GluN3A knockdown neurons

To identify the signaling mechanisms underlying the potentiation of NMDAR-induced transcription in GluN3A KD neurons, we first asked whether pharmacological blockade of known MEF2 regulatory pathways (26) would inhibit the potentiation of Gal4-MEF2Cα1β activity upon TTX w/d. Although calcineurin-dependent dephosphorylation of MEF2s is required for the membrane depolarization-dependent activation of MEF2-dependent transcription in cultured hippocampal neurons (27), we found that inhibition of calcineurin with CSA and FK506 had no effect on Gal4- MEF2Cα1β activation following TTX w/d in either control or GluN3A KD neurons (**Fig. 4A)**. By contrast pretreatment of neurons with the p38 MAPK inhibitor SB203580 inhibited the induction of

Gal4- MEF2Cα1β activity in GluN3A KD neurons (**Fig. 4A**). Interestingly, p38 MAPK blockade had no significant effect on TTX w/d-induced Gal4-MEF2Cα1β activity in control infected neurons indicating a selective requirement for the p38 pathway in MEF2C activation in GluN3A KD neurons.

p38 MAPK activates MEF2C by inducing its phosphorylation at three sites: Thr293, Thr 300, and Ser387, with the numbering referring to amino acid positions in human MEF2C$\alpha_1\beta$ (28). To test the functional importance of these phosphorylation sites in neurons, we mutated each of these residues to Ala in the context of the Gal4-MEF2Cα1β fusion protein and assessed the effects on TTX w/d-induced luciferase expression in neurons cotransfected with the GluN3A KD shRNA plasmid. Mutating MEF2C at either Thr293/Thr300 or Ser387 rendered the Gal4 fusion protein unresponsive to TTX w/d in GluN3A knockdown neurons (**Fig. 4B**). These data suggest that p38 MAPK-dependent phosphorylation of MEF2C is required for the activation of MEF2C in GluN3A knockdown neurons.

If p38 MAPK-dependent phosphorylation of MEF2C is selectively mediating the potentiation of NMDAR-dependent transcription in GluN3A KD neurons, then pharmacological inhibition of the p38 MAPK pathway in GluN3A KD neurons should restore transcriptional activation following TTX w/d to the levels observed in control neurons. Indeed, pretreatment of neurons with the p38 MAPK inhibitor SB203580 had no effect on the magnitude of TTX w/d-induced *Bdnf* IV expression in control neurons, but it abolished the potentiation of *Bdnf* IV induction in GluN3A KD neurons (**Fig. 4C**). Taken together, these data suggest that GluN3A inhibits NMDAR-dependent transcription by opposing the p38 MAPK-dependent phosphorylation and activation of MEF2C.

To determine whether the differential p38 MAPK-dependent activation of MEF2C we observed in GluN3A KD neurons reflects differential NMDAR-dependent activation of the p38 MAPK pathway, we subjected GluN3A KD or control infected neurons to TTX w/d and measured the activation of p38 MAPK by western blotting with an antibody selective for the MKK3/6 phosphorylation sites on p38 at Thr180/Tyr18. When we assayed cytoplasmic fractions from our neuronal cultures, we found that p38 MAPK was phosphorylated within five minutes after TTX w/d in both control and GluN3A KD neurons, and we saw that the magnitude and time course of activation of p38 MAPK did not differ between these cultures (**Fig. 5A,B**). Thus GluN3A does not inhibit the activation of cytoplasmic p38 MAPK.

However many signaling proteins, including those in the p38 MAPK pathway, must undergo rapid nuclear translocation to induce gene transcription following extracellular stimulation (29,30). To determine whether the nuclear activation of p38 MAPK is affected by the presence of GluN3A in NMDARs, we harvested nuclei from GluN3A KD and control neurons and ran them for western analysis. In contrast to the activation of p38 MAPK in the cytoplasm, we saw TTX w/d-induced phosphorylation of p38 MAPK only in the nuclei of GluN3A KD neurons, with no significant increase in phosphorylation in the control-infected neurons (**Fig. 5C,D**). To confirm the differential localization of phospho-p38 MAPK after TTX w/d in GluN3A KD and control neurons, we used immunostaining with the phospho-p38 MAPK antibody to localize the activated kinase in dissociated hippocampal neurons (**Fig. 5E**). Confocal images through control-infected cells show a robust TTX w/d-induced signal in the cytoplasm that is absent

from the nucleus. By contrast in GluN3A KD neurons the phospho-p38 MAPK signal fills the cell including the nucleus. Quantification of the relative distributions of phospho-p38 MAPK in these two compartments demonstrates that there is a significant increase in the nuclear:cytoplasmic ratio of phospho-p38 MAPK immunostaining signal in neurons lacking GluN3A (**Fig. 5F).** This difference in the localization of activated p38 MAPK in the GluN3A knockdown neurons is due to the lack of GluN3A because we were able to restore the control distribution by re-expressing shRNA resistant GluN3A in the GluN3A KD neurons (**Fig. 5E,F**). Taken together, these data suggest that GluN3A inhibits the subset of NMDAR-dependent transcription that is driven by MEF2C by blocking the activation of nuclear p38 MAPK. Our model is shown in **Fig. S5.**

DISCUSSION

Our data describe a novel mechanism that regulates the specificity of NMDAR-dependent gene expression in the developing brain. We show that the NMDAR subunit GluN3A inhibits the ability of NMDARs to induce activity-dependent gene transcription. Because GluN3A is directly incorporated into NMDARs, it provides a means to selectively inhibit the ability of NMDARs to induce activity-regulated genes, while leaving LVGCC-induced transcription intact. This is important because many activity-regulated neuronal genes have pleiotropic functions that are differentially coupled to the activation of distinct upstream calcium signals. For example, though *Bdnf* is induced by both LVGCCs and NMDARs, LVGGC-induced BDNF expression is strongly linked to neuronal survival pathways (31), where NMDAR-induced BDNF is implicated in GABAergic synapse maturation (32). It is likely that the consequences of BDNF expression are defined by the context in which it is induced. Thus by inhibiting the coupling of activity-regulated genes to NMDAR activation, GluN3A has the ability to limit NMDAR-dependent effects on synapse development without impairing the activation of these genes by stimuli such as spontaneous action potentials that promote neuronal survival and neurite outgrowth (33).

GluN3A is highly expressed only during a brief period of early postnatal forebrain development (3,11) and downregulation of GluN3A prior to the onset of the critical period for sensory-driven cortical plasticity is required for the maturation of excitatory synapse development (17). However, the mechanisms that allow GluN3A to exert this inhibitory effect on cortical plasticity have been unknown. Our evidence that GluN3A limits NMDAR dependent *Bdnf* and *Arc* transcription suggest that the GluN3A dependent inhibition of NMDAR-dependent gene transcription inhibits premature synaptic maturation by restricting the activation of plasticity-inducing gene products. In the visual cortex, Arc is required for the experience-dependent establishment of normal ocular dominance (34) and BDNF promotes the maturation of GABAergic inhibition in the developing cortex, which drives closure of the critical period (32). The developmental downregulation of GluN3A would permit the robust induction of this transcriptional program, promoting the onset of the later stages of critical period plasticity.

Although NMDARs pass current and contribute to synaptic potentials, it is primarily the ability of these channels to function as synaptic activity-regulated signaling receptors that underlies their unique roles in brain development and synaptic plasticity. All functional glutamate-sensing NMDARs are comprised of both GluN1 and GluN2 subunits, with some receptors also containing GluN3s, and the biophysical and biochemical properties of specific subtypes of these receptors vary based on their subunit

composition. Notably, GluN3A can form excitatory glycine receptors with GluN1 in the absence of the glutamate binding GluN2 subunits (2). The contribution of these receptors to stimulus-regulated transcription remains unknown and it is possible that the loss of GluN1/GluN3A receptors contribute to the regulation of genes that show impaired TTX w/d induction in the GluN3A KD neurons (**Fig. 2B**). However, with respect to the potentiation of NMDAR-inducible gene expression in GluN3A KD neurons, we suggest that it is more likely that GluN3A is modulating calcium influx (**Fig. 1K**) or protein-protein interactions to influence the activation of transcriptional signaling cascades.

GluN2A and GluN2B-containing NMDARs have been particularly highly studied given the evidence linking these two subtypes of receptors to distinct biological functions (7). The distinct signaling consequences of activating these two classes of NMDARs has been shown to depend on the ability of their intracellular C-terminal tails to differentially recruit signaling complexes to the NMDAR (9). Similar to the GluN2s, the long C-tail of GluN3A provides unique NMDAR docking sites for intracellular signaling proteins that can influence the functional impact of NMDAR activation. For example, GluN3A-dependent association with the endocytic protein Pacsin1 (Syndapin1) enhances the membrane trafficking of NMDARs (35), and the association of GluN3A with the phosphatase PP2A promotes dephosphorylation of the GluN1 subunit at Ser897 (36). In addition to effects on the NMDAR itself, protein associations with the GluN3A C-terminal tail regulate local synaptic signaling in dendritic spines (37). Our data now further indicate that incorporation of GluN3A can modulate the ability of NMDARs to signal to the nucleus. Interestingly whereas GluN2A and GluN2B differentially regulate NMDAR-dependent activation of the transcription factor CREB, we find that GluN3A selectively inhibits the activation of MEF2 transcription factors. Thus in addition to controlling the likelihood of transcriptional activation, differential use of NMDAR subunits can confer specificity on the set of downstream transcription factors that are activated by synaptic stimulation.

Although activation of any single activity-regulated transcription factor is sufficient to induce transcription of a reporter gene (e.g. **Fig. 3A,B**) the regulation of most neuronal IEGs is under the control of multiple activity-regulated transcription factors (38). The presence of multiple transcription factor binding sites in the promoters and enhancers of neuronal activity-dependent genes may allow for specificity in the coupling of subsets of activity-responsive genes to distinct sources of upstream activation (39). Consistent with this model we find that GluN3A selectively inhibits the component of NMDAR-induced transcription that depends on MEF2C while leaving LVGCC and CREB-dependent transcription intact. The selective regulation of these pathways results in the activation of MEF2C/CREB co-regulated genes like *Bdnf* and *Arc* being driven to two different levels – either high levels of transcription through the coordinate activation of MEF2C and CREB, or a lower level of activation, presumably mediated by CREB in the absence of MEF2C activation. Importantly, substantial data support the hypothesis that the levels to which these stimulus-regulated genes are induced is important for their function in the brain. The spatial and temporal induction of BDNF is particularly tightly regulated in the brain, and even mild increases or decreases in BDNF levels are associated with developmental and functional neuronal abnormalities (20,40-42). More recently we have shown that modulation of the bursting properties of *Fos* transcription are sufficient

to change membrane properties of hippocampal neurons demonstrating a direct link between the magnitude of activity-inducible gene expression and neuronal function (43).

One of the most interesting questions raised by this study is how incorporation of GluN3A into NMDARs at the cell surface leads to the regulation of gene transcription in the nucleus. Our data indicate that a key step in this process is the ability of GluN3A to inhibit the activation of nuclear p38 MAPK. The p38 MAPK is rapidly activated by glutamate-induced calcium influx through NMDARs in neurons (44), and in this context p38 has attracted interest for its involvement in the recycling of AMPA-type glutamate receptors that underlies activity-dependent changes in synaptic strength (45). NMDARs activate p38 MAPK via the regulation of the small GTPases Ras, Rac1, and/or Rap1, and the differential association of GTPase exchange factors (GEFs) and GTPase activating proteins (GAPs) with NMDARs that have distinct GluN2 subunit composition has been suggested to contribute to the ability of NMDARs to induce LTP versus LTD (46). Like the GluN2 subunits, GluN3A regulates the NMDAR-dependent activation of small GTPases by binding directly to the small GTPase Rheb and indirectly inhibiting the activation of Rac1 (37). However, our data indicate that GluN3A has no effect on the time course or magnitude of p38 MAPK activation; instead the primary consequence of GluN3A is to restrict the subcellular distribution of activated p38 MAPK to the cytoplasm, whereas NMDARs lacking GluN3A can also drive the appearance of activated p38 MAPK in neuronal nuclei.

Nuclear translocation of p38 requires the phosphorylation-dependent association of p38 with β-like importins in the cytoplasm followed by active transport through the nuclear pore that is mediated by the small GTPase Ran (29). The nuclear export of p38 MAPK is also regulated, either as a consequence of dephosphorylation in the nucleus (47) or via the physical association of p38 MAPK with a downstream substrate bearing a nuclear export signal (48). One mechanism that controls the localization of phospho-p38 MAPK is the mechanism of its upstream activation. In addition to phosphorylation by the canonical upstream MAPK kinases MKK3/6, p38 MAPK can be activated by autophosphorylation in complex with the TAB-1 scaffold protein (49). In cardiomyocytes, when TAB-1 binds to and induces the autophosphorylation of p38 it prevents the nuclear translocation of the activated kinase and antagonizes the activation of gene transcription driven by MKK-dependent p38 activation (50). Future investigation of the mechanisms of NMDAR-induced p38 MAPK activation in neurons may expand our understanding of the regulatory processes that control this important kinase signaling cascade and regulate its impact on activity-dependent brain development

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Experimental Procedures

Plasmids: Two independent shRNAs were used to knockdown rat *Grin3a* and each was paired with a vector-matched control. *Grin3a* shRNA1 (TRCN0000100220; 5'-GCTCCATGACAAGTGGTACAA-3') was purchased from Thermo Scientific and the empty pLKO.1 vector was used as Ctrl1. *Grin3a* shRNA2 (5'-GTATCCGGCAGATATTTGAAA-3') was cloned into the pLLx3.8 vector and a scrambled version of the shRNA2 sequence (5′-GCCTGCAGTATGACTCAGTAA-3′) in pLLx3.8 was used as Ctrl2. The shRNA-resistant GluN3A expression plasmid was constructed by using PCR to introduce silent mutations (GCT**a**CATGACAAGT**t**GTACAA) into the region of rat *Grin3a* targeted by *Grin3a* shRNA1, and then placing the *Grin3a* coding

sequence under control of the ubiquitin promoter in the lentiviral expression vector pFUIGW (51). The Gal4 DNA binding domain fusions with human MEF2Cα1β and MEF2Cα1βγ were described (25). The TT293/300AA and S387A mutations of Gal4-MEF2Cα1β were generated by site-directed mutagenesis PCR. The viral expression plasmid for the rescue of MEF2 expression in neurons infected with the shRNA targeting rat *Mef2c* was generated by cloning the full coding sequence of human MEF2Cα1β, which is not targeted by the rat *Mef2c* shRNA used, into the vector pFUIGW. We purchased the CRE luciferase reporter (Agilent, 219075) and TK-renilla luciferase plasmid (Promega, E2231). The following plasmids were reported previously: MRE luciferase reporter plasmid (27), the UAS luciferase reporter plasmid (52), and shRNAs targeting rat *Mef2a*, *Mef2c*, and *Mef2d* in the vector pLKO.1 (TRCN0000095959, TRCN0000012068, and TRCN0000085268 respectively, Thermo Scientific) (23).

Antibodies. Primary antibodies used in this study for western blotting were mouse anti-Actin, 1:5000 (Millipore Cat# MAB1501, RRID:AB_2223041); rabbit anti-GluN3A, 1:1000 (Millipore Cat# 07-356, RRID:AB_2112620); mouse anti-Transferrin Receptor, 1:2000 (Thermo Fisher Scientific Cat# 13-6800, RRID:AB_2533029); rabbit anti-phospho-Thr180/Tyr182 p38 MAPK, 1:1000 (Cell Signaling Technology Cat# 4511, RRID:AB_2139682); and rabbit-anti-MEF2C 1:1000 (Abcam Cat# ab64644, RRID:AB_2142861). Primary antibodies used in this study for immunocytochemistry were mouse anti-phospho-Tyr182 p38 MAPK, 1:50 (Santa Cruz Biotechnology Cat# sc-7973, RRID:AB_670359).

Dissociated Neuron Cultures: Neuron-enriched cultures were generated from hippocampus of male and female E18.5 CD IGS rat embryos (Charles River Laboratories) and cultured as previously described (40,52). Activation of NMDAR-dependent transcription by withdrawal from tetrodotoxin (TTX w/d) (18,19) was done by treating neurons starting on the fifth day in vitro (DIV5) for 48 hrs with 1μM TTX (Tocris) prior either to harvesting cells (for the control condition) or washing out the TTX with Neurobasal medium with B27 supplements (Invitrogen) for 6hrs unless otherwise indicated in the text on DIV7. Isotonic membrane depolarization with 55mM extracellular KCl was done as previously described for 6hrs on DIV7 (23). Pharmacological blockers were added 2 min prior to TTX w/d or KCl addition and maintained throughout the period of stimulation. TTX (1μM), FK506 (1μM), cyclosporin A (1μM), SB203580 (2.5μM or 25μM), CNQX (10μM) were purchased from Tocris. Nimodipine (5μM) was purchased from Sigma and and APV (100μM) was purchased from Fisher scientific. All experiments were conducted in accordance with an animal protocol approved by the Duke University Institutional Animal Care and Use Committee.

Lentiviral Infection: For viral infection of neurons, shRNA or viral expression constructs were packaged as lentivirus in HEK 293T cells following standard procedures. Concentrated viruses were titered on cultured neurons, and rat hippocampal neurons were infected for 6 hrs on day in vitro (DIV) 1 or DIV2 at a multiplicity of infection of 1 in BME medium (Sigma) with 0.4μg/mL added polybrene (Sigma).

Multi-electrode array (MEA) recordings
Rat hippocampal neurons were plated in a 48-well MEA plate (Lumos 48, Axion biosystems) coated with poly-D-lysine and

laminin, at a density of 60,000 cells/well. Extracellular recordings were performed at 37°C with 5% CO2 using a Maestro MEA system and AxIS software (Axion Biosystems). Data was acquired at 12.5 kHz and filtered with a Butterworth bandpass filter at 200Hz-3kHz. A spike detector was used to detect action potentials with a 6x standard deviation. TTX w/d was conducted as described. Recordings were conducted immediately prior to TTX washout, 30 min after TTX washes, and immediately after the addition of 10 µM CNQX (Tocris, 1045) and 100 µM APV (Fisher scientific, 01-051-0). Data were displayed and analyzed using Axion Biosystem's Neural Metrics Tool.

Quantitative PCR. RNA was harvested on DIV7 following 90min or 6hrs of KCl-mediated membrane depolarization or TTX w/d as described in the text. RNA was harvested using the Absolutely RNA Miniprep Kit (Agilent) and cDNA was synthesized by Superscript II (Invitrogen). Quantitative SYBR green PCR was performed on an ABI 7300 real-time PCR machine (Applied Biosystems) using intron-spanning primers (IDT) listed in **Table S1**. Data were all normalized to expression of the housekeeping gene *Gapdh* to control for sample size and processing. In some cases, as described in the text, we reported mRNA levels as relative expression or relative fold induction in neurons infected with any single independent shRNA compared with its paired control shRNA (Ctrl1 or Ctrl2).

Western Blotting. Cells were homogenized in homogenization buffer (320 mM Sucrose, 10mM HEPES pH 7.4, 2mM EDTA, 1mM DTT and protease inhibitors). Nuclear pellet was pelleted by centrifuging at 1500xg for 15min. Cytosolic and membrane fractions were separated from the supernatant by centrifuging at 200,000xg for 20min. Membrane, cytoplasmic, and nuclear extracts were run for SDS-PAGE and transferred to Nitrocellulose for western blotting following standard procedures. Bands were visualized with fluorescent secondary antibodies (Biotium) using the Odyssey imaging system (LI-COR Bioscience) and quantified using ImageJ. Actin was used as a loading control.

BDNF ELISA. Two-site BDNF ELISA was performed as previously described using acid extraction of BDNF from cultured neurons (40,42). Total protein concentration in the lysate was measured by BCA protein assay kit (Pierce) and BDNF protein concentrations were measured by the BDNF Emax ImmunoAssay System (Promega) or the mature BDNF rapid ELISA kit (Biosensis). Neurons were infected with lentiviral constructs and treated with TTX as described for RNA studies, then harvested for ELISA 6 hrs after TTX w/d.

Two-photon glutamate uncaging and calcium imaging: Hippocampal slices were prepared from postnatal day 5–7 rats and cut into 350-µm sections using a McIlwain tissue chopper. Slices were plated on tissue culture inserts (Millicell) fed by tissue medium (for 2.5 l: 20.95 g MEM, 17.9 g HEPES, 1.1 g $NaHCO_3$, 5.8 g D-glucose, 120 µl 25% ascorbic acid, 12.5 ml L-glutamine, 2.5 ml insulin, 500 ml horse serum, 5 ml 1 M $MgSO_4$, 2.5 ml 1 M $CaCl_2$) and incubated at 35 °C in 3% CO_2. After 1–2 weeks in culture, CA1 pyramidal neurons were transfected with the control, Grin3a shRNA KD or KD+ GluN3A rescue plasmids as well as the cell fill mCherry and the calcium indicator GCaMP3 with ballistic gene transfer using gold beads (8–12 mg) coated with plasmids containing 30 µg of total cDNA. Cells were imaged 2–5 days after transfection. Two-photon imaging was performed using a Ti-sapphire laser (MaiTai, Spectraphysics) tuned to a wavelength of 920 nm, allowing simultaneous excitation of GCaMP3 and

mCh. Imaging experiments were performed at 8Hz collection speed (frames/second). All samples were imaged using <2 mW laser power measured at the objective. Fluorescence emission was collected using an immersion objective (60×, numerical aperture 0.9, Olympus), divided with a dichroic mirror (565 nm), and detected with two separate photoelectron multiplier tubes (PMTs) placed downstream of two wavelength filters (Chroma, HQ510-2p to select for green and HQ620/90-2p to select for red). The green channel was fitted with a PMT having a low transfer time spread (H7422-40p; Hamamatsu), while the red channel was fitted with a wide-aperture PMT (R3896; Hamamatsu). The green and red channel signals were acquired using a data acquisition board (PCI-6110) controlled with ScanImage software. A second Ti-sapphire laser tuned at a wavelength of 720 nm was used to uncage 4-methoxy-7-nitroindolinyl-caged-L-glutamate (MNI-caged glutamate) in extracellular solution with a train of 4–6 ms, 4–5 mW pulses (5 times at 0.5 Hz) near a spine of interest. Experiments were performed in Mg^{2+} fee artificial cerebral spinal fluid (ACSF; 127 mM NaCl, 2.5 mM KCl, 4 mM $CaCl_2$, 25 mM $NaHCO_3$, 1.25 mM NaH_2PO_4 and 25 mM glucose) containing 1 μM tetrodotoxin (TTX) and 4 mM MNI-caged L-glutamate aerated with 95% O_2 and 5% CO_2 at 30 °C, as described previously. Anywhere from 1–10 spines were stimulated per cell.

Calcium Trace Analysis: GCaMP3 intensity was calculated as the background-subtracted integrated fluorescence intensity over a region of interest around the dendritic spine head (fluorescence, F). Changes in the intensity of GCaMP3 were calculated as $\Delta F/F_0$, where F_0 is the average fluorescence intensity before stimulation. To account for potential drift in the focal plane during imaging, the GCaMP3 signal was normalized to the red channel signal, smoothed by a factor of 16 frames (2 seconds). Uncaging-triggered averages were calculated by first averaging the GCaMP3 traces from all spines from a particular condition together. Stimulation periods were then extracted according to uncaging onset timing, resulting in two-second blocks corresponding to the time point immediately before uncaging through the frame immediately before the next stimulus, for each of the five stimuli delivered. The fluorescence intensity value at the beginning of each of the five 2-second blocks was subtracted from that block to account for the incomplete decay of GCaMP3. Blocks were then averaged together to generate an average response to the uncaging stimulus, here referred to as the uncaging-triggered average. As such, error in this measurement is indicative of the variability between the successive uncaging-evoked GCaMP3 responses. All analysis was performed using custom MATLAB software.

RNA-seq: Rat hippocampal neurons were infected with the Ctrl1 or *Grin3a* shRNA1 lentiviruses on DIV1, treated with 1μM TTX on DIV5 and then on DIV7 cells were either harvested for RNA (Ctrl TTX and KD TTX conditions) or TTX was withdrawn for 6hr prior to RNA harvest (Ctrl TTX w/d and KD TTX w/d). Three independent triplicate replicate samples were obtained for each condition. RNA was submitted to the Duke University sequencing core facility for polyA selection, library generation, and single-end 50bp sequencing on the Illumina HiSeq2500. Following quality score-based trimming and adaptor filtering, reads were aligned to the UCSC rn5 reference transcriptome using Tophat2.0. Cuffdiff was used for pairwise tests of differential expression between normalized gene read counts of Fragments Per Kilobase of exon per Million fragments mapped (FPKM) with the default significance threshold of FDR < 0.05 used for

all analyses. Heat maps and volcano plots were generated using R biostatistics packages. We used the Database for Annotation, Visualization, and Integrated Discovery (DAVID) (53) to find enriched Biological Process gene ontologies and the Molecular Signatures Database v6.2 (MSigDB) of Gene Set Enrichment Analysis (54) to search a database of transcription factor binding sites (55) with our gene sets. RNAseq data were deposited at GEO with the accession number GSE133917.

Neuronal Transfection for Luciferase Assays. Neuron cultures were transfected with calcium phosphate on DIV3-5 (52). Cotransfection of pTK-renilla luciferase (Promega) was used to control for transfection efficiency and sample handling. For the MRE-luc and CRE-luc reporters, luciferase reporter plasmid were transfected into cultured embryonic rat hippocampal neurons that had previously been infected with either *Grin3a* shRNA1 or Ctrl1 lentiviruses. Lysates were harvested on DIV9-10 after 0, 4, 6, 8, 14, or 22 hrs of TTX w/d. For the UAS-luc reporter, luciferase reporter and Gal4 fusions were cotransfected with either *Grin3a* shRNA1 or Ctrl1 plasmid. Lysates were harvested on DIV7 + 6 hrs TTX w/d as described above. Luciferase activity was determined using the Dual-Luciferase Reporter Assay System (Promega).

Immunofluorescence. Embryonic rat hippocampal neurons were cultured on PDL/laminin coated glass coverslips (Bellco) and fixed in 4% paraformaldehyde at room temperature for 10 mins. Neurons were blocked in 10% normal goat serum and permeabilized in 0.3% Triton X-100 prior to antibody incubation. Coverslips were incubated in primary antibodies overnight at 4°C. Secondary antibodies (1:500) were incubated at room temperature for 1 hr. Hoechst dye (0.1µg/ml, Sigma) was used to label nuclei. Images were captured on a Leica SP8 confocal microscope with 1µm thickness optical section. Nuclear and cytoplasmic total pixel intensities were quantified by the ImageJ macro Intensity Ratio Nuclei Cytoplasm Tool (http://dev.mri.cnrs.fr/projects/imagej-macros/wiki/Intensity_Ratio_Nuclei_Cytoplasm_Tool). Briefly, this plugin first identifies cells based on their diameter and threshold signal above background. Once set these parameters are held constant for all images in the set. ~300 cells in 15 images per treatment group were quantified.

Statistical Analyses. Unless otherwise indicated, all data presented are the average of at least three biological replicates from each of at least two independent experiments. Also unless otherwise indicated, data were analyzed in GraphPad Prism by one or two-way ANOVA as appropriate to the data structure followed by post-hoc comparisions with Tukey's or Sidak's multiple comparison tests. For the calcium imaging data, multiple comparisons between the control, shRNA, and rescue conditions were performed using ANOVA followed by Fisher's Least Significant Difference test. A $p<0.05$ was considered significant. Bar and line graphs show mean values and all error bars show standard deviation.

Data Availability: Raw data used to generate the figures in this paper are stored on a secure Duke University School of Medicine server and are available upon request to the corresponding author, Anne E. West, Department of Neurobiology, Duke University Medical Center, 311 Research Dr., DUMC Box 3209, Bryan Research 301D, Durham, NC 27710. 919-681-1909,

west@neuro.duke.edu. RNAseq data were deposited at GEO with the accession number GSE133917.

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Abbreviations: N-methyl-D-aspartate type glutamate receptors (NMDARs), L-type voltage gated calcium channels (LVGCCs), p38 mitogen-activated protein kinase (p38 MAPK), withdrawal (w/d), knockdown (KD), amino-5-phosphonovaleric acid (APV), Tetrodotoxin (TTX), Nimodipine (Nim), potassium chloride (KCl), enzyme-linked immunosorbent assay (ELISA), Mef2 response element (MRE), Creb response element (CRE), myocyte enhancer factor 2 (MEF2), short hairpin RNA (shRNA)

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Gene Set Name	# Genes in Gene Set (K)	# Genes in Overlap (k)	k/K	p-value	FDR q-value
AACTTT_UNKNOWN	1890	33	0.0175	1.15E-20	7.05E-18
CAGGTG_E12_Q6	2485	31	0.0125	2.57E-15	7.90E-13
YTATTTTNR_**MEF2**_02	697	17	0.0244	3.96E-13	8.12E-11
TGGAAA_**NFAT**_Q4_01	1896	24	0.0127	4.93E-12	7.57E-10
TAATTA_CHX10_01	810	16	0.0198	4.71E-11	5.79E-09
TTGTTT_FOXO4_01	2061	23	0.0112	1.78E-10	1.83E-08
GGGAGGRR_MAZ_Q6	2274	21	0.0092	3.40E-08	2.65E-06
TTANTCA_UNKNOWN	952	14	0.0147	3.45E-08	2.65E-06
AMEF2_Q6	259	8	0.0309	1.48E-07	9.39E-06
CTGCAGY_UNKNOWN	765	12	0.0157	1.78E-07	9.39E-06

Table 1. GSEA top transcription factor motifs identified in the set of 113 genes from **Fig. 2D**.

Figure Legends:

Figure 1: **GluN3A knockdown selectively enhances NMDAR-dependent *Bdnf* transcription.** **(A)** Levels of *Grin3a* mRNA in rat hippocampal neurons infected with lentiviruses encoding

shRNAs targeting *Grin3a* (shRNA1, shRNA2) or their paired control vectors (pLKO or pLLx3.8). *Grin3a* mRNA levels are scaled to levels in cells infected with the paired control viruses. Two-way ANOVA for vector F(1,45)=1.033, p=0.32, and shRNA F(1,45)=425.6, p<0.0001. For pLKO shRNA1 vs Ctrl1, n=16/virus, p<0.0001. For pLLx3.8 shRNA2 vs Ctrl2, n=9 Ctrl 2, 8 shRNA2, p<0.0001. **(B)** Expression of *Grin3a* mRNA in neurons infected with the *Grin3a* KD and shRNA-resistant GluN3A rescue viruses. **(C)** Membrane fraction from cultured hippocampal neurons infected with the indicated lentiviruses analyzed by Western blot using an antibody that detects the GluN3A protein (green). Transferrin receptor is shown as a loading control (red). Predicted MW of GluN3A is 130kDa. **(D, E)** Levels of *Bdnf* IV mRNA in hippocampal neurons infected with the indicated lentiviruses in the presence of TTX or 6hrs following TTX w/d. Values are scaled to Ctrl TTX for comparison. **D,** pLKO, Two-way ANOVA for treatment F(1, 41) = 49.53 p<0.0001, for virus F(1, 41) = 172.5 p<0.0001, and for treatment x virus interaction F(1, 41) = 36.12, p<0.0001. Ctrl TTX n=11, Ctrl TTX w/d n=13, shRNA TTX=9, shRNA TTX w/d=12. Ctrl TTX vs TTX w/d p<0.0001, shRNA TTX vs TTX w/d p<0.0001. Ctrl TTX w/d vs shRNA TTX w/d p<0.0001. **E,** pLLx3.8. Two-way ANOVA for treatment F(1, 18) = 32.97 p<0.0001, for virus F(1, 18) = 97.67 p<0.0001, and for treatment x virus interaction F(1, 18) = 16.98, p=0.0006. Ctrl TTX n=6, Ctrl TTX w/d n=6, shRNA TTX=6, shRNA TTX w/d=5. Ctrl TTX vs TTX w/d p=0.0036, shRNA TTX vs TTX w/d p<0.0001. Ctrl TTX w/d vs shRNA TTX w/d p<0.0001. **(F)** Levels of TTX w/d-induced *Bdnf* IV mRNA in hippocampal neurons infected with the indicated lentiviruses relative to the control condition (Ctrl), whose induced average is scaled to 100%. Two-way ANOVA for treatment F (1, 30) = 160.5, p<0.0001, for virus F (2, 30) = 13.33, p=0.0001, and treatment x virus interaction F (2, 30) = 12.25, p=0.0001. n=6/condition. For TTX w/d, Ctrl vs KD p<0.0001, KD vs rescue p=0.0064, Ctrl vs rescue p=0.0019. **(G)** Levels of BDNF protein in hippocampal neurons infected with the indicated lentiviruses in the presence of TTX or 6hrs following TTX w/d. Control condition (Ctrl) TTX w/d induced average is scaled to 100%. Two-way ANOVA for treatment F (1, 54) = 144.8, p<0.0001, for virus F (2, 54) = 4.103 p=0.022, and treatment x virus interaction F (2, 54) = 5.547 p=0.0064. n=12/condition for Ctrl and KD, 6/condition for rescue. For TTX w/d Ctrl vs *Grin3a* KD p=0.0016, KD vs rescue p=0.012. **(H)** Representative 2-photon images of hippocampal CA1 neurons expressing GCaMP3 (green) and mCherry (red). (left) Baseline images corresponding to the average projection the first 31 frames of imaging, prior to uncaging. (right) Average projection images of the first two frames after each of the five uncaging pulses (10 total imaging frames). White circles indicate the uncaging point**.** **(I)** Average $\Delta F/F_0$ traces showing response to glutamate uncaging (black triangles) for control (n = 29 spines; 3 cells), *Grin3a* KD (n = 32 spines, 3 cells), and KD+GluN3A rescue (n = 32 spines, 3 cells) conditions. Data corresponds to mean +/- SEM. **(J)** Uncaging-triggered average of the responses shown in **(I)** Traces correspond to the average baseline-subtracted response to each glutamate uncaging pulse, where baseline is the frame immediately before each uncaging stimulus. Error corresponds to SEM of the five uncaging pulses for each condition. **(K)** Average integrated GCaMP3 signal for each condition. The average area under the curve was calculated for each spine, then averaged for a given condition. Asterisks indicated statistical significance as determined by ANOVA and a posthoc test using Fisher's Least Significant Difference. Ctrl vs. KD: p = 0.003, ctrl vs. rescue: p = 0.6, KD vs. rescue: p = 0.009. *p<0.05 for *Grin3a* KD vs. Ctrl, ^p<0.05 GluN3A rescue vs *Grin3a* KD.

Figure 2: GluN3A contributes to the program of NMDAR-inducible transcription. (A) Volcano plot showing genes significantly different at an FDR-adjusted P<0.05 (red dots)

comparing Ctrl TTX w/d with *Grin3a* KD TTX w/d. **(B)** Venn diagram showing genes significantly induced by TTX w/d in Ctrl neurons (red) compared with genes reduced in *Grin3a* KD vs Ctrl after TTX w/d (blue). **(C)** Venn diagram showing genes significantly repressed by TTX w/d in Ctrl neurons (blue) compared with genes with higher expression in *Grin3a* KD compared with Ctrl after TTX w/d (red). **(D)** Heat map showing the relative expression of the 113 genes higher after TTX w/d in *Grin3a* KD compared with Ctrl that are either unchanged or induced by TTX w/d in Ctrl. n=3 biological replicates per sample.

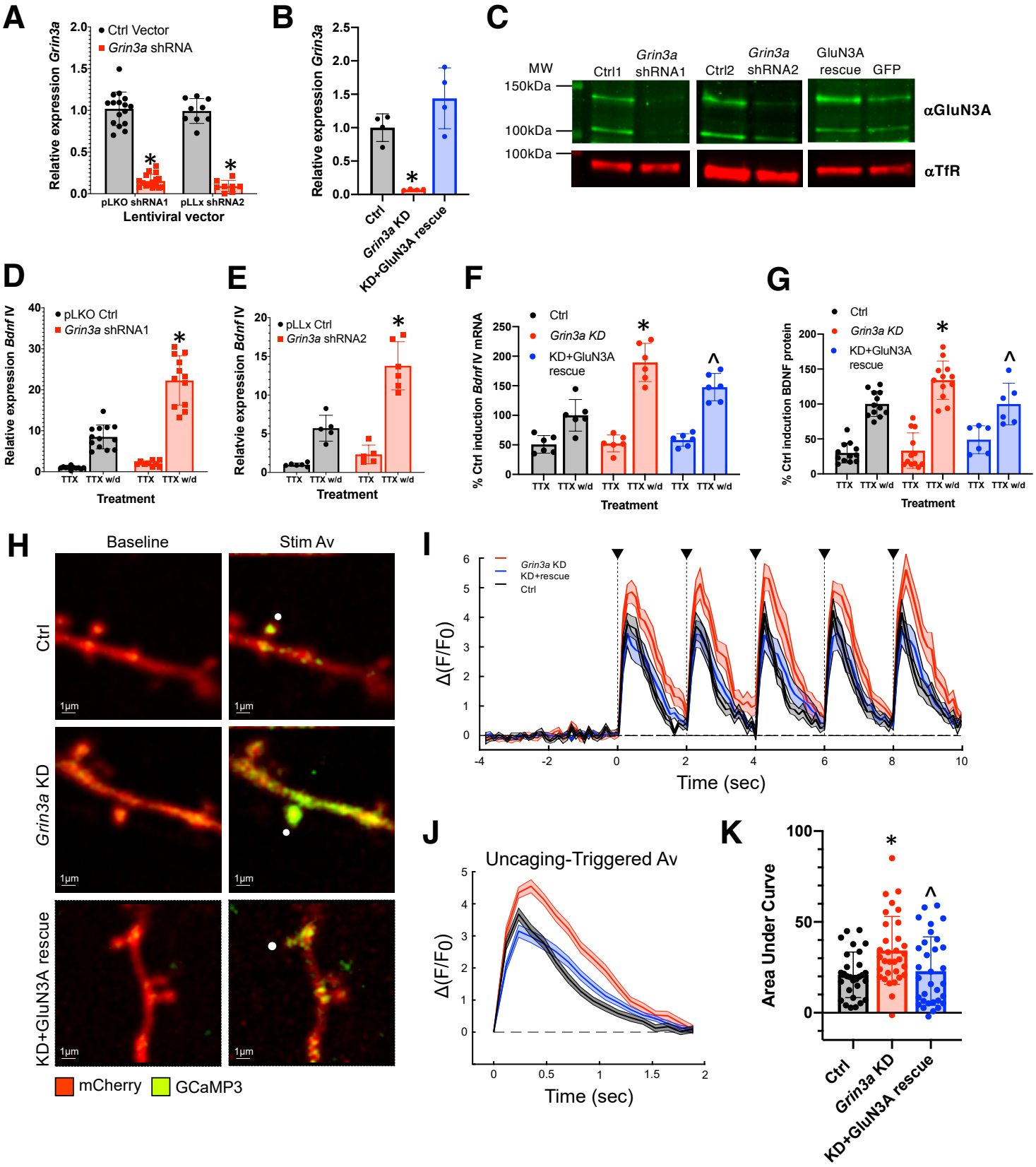
Figure 3: **GluN3A knockdown enhances MEF2C-dependent transcription. (A)** Luciferase expression in hippocampal neurons transfected with MRE-Luc plasmid and infected with either *Grin3a* shRNA1 or control (Ctrl) lentiviruses. Neurons were stimulated with TTX w/d for the indicated amounts of time prior to lysis. Data expressed as fold induction over TTX (0 hrs). Two-way ANOVA for time $F(4,62)=34.65$, $p<0.0001$, for virus $F(1, 62) = 32.16$, $p<0.0001$, and for virus x time interaction $F(4, 62) = 7.602$, $p<0.001$. n=11 at 0hr, 6 at 4hr, 7 at 6hr, 7 at 8hr, and 6 at 22hr. *Grin3a* shRNA vs Ctrl at 6hr and 8 hr, $p<0.0001$. **(B)** Luciferase expression in hippocampal neurons transfected with CRE-Luc plasmid and infected with either *Grin3a* shRNA1 or Ctrl lentiviruses. Neurons were stimulated as described in (A). Two-way ANOVA for time $F(5, 24) = 23.00$, $p<0.0001$ and virus $F(1, 24) = 1.407$, $p=0.2471$. n=4 at 0hr, 4 at 4hr, 2 at 6hr, 4 at 8hr, 2 at 14 hr, and 2 at 22hr. **(C)** Levels of *Bdnf* IV mRNA in hippocampal neurons infected both with lentiviruses containing *Grin3a* shRNA1 or its paired control and lentiviruses expressing shRNAs targeting individual MEF2 family members. Two-way ANOVA for treatment $F(1, 77) = 68.53$, $p<0.001$, for virus $F(7, 77) = 2.520$, $p=0.022$, and for treatment x virus interaction $F(7, 77) = 2.254$, $p=0.039$. n=6/condition except Ctrl TTX=4 and *Mef2c+Grin3a* dKD TTX w/d=5. For each viral condition TTX w/d was significantly different from TTX, and for TTX there were no significant differences between viral conditions. Significant TTX w/d *posthoc* comparisons: Ctrl vs *Grin3a* KD $p=0.0093$, *Grin3a* KD vs *Mef2c+Grin3a* dKD $p=0.041$. **(D)** Luciferase expression in hippocampal neurons cotransfected with the pUAS-Luc plasmid, expression vectors for Gal4 fusions of the indicated MEF2C splice variants, and *Grin3a* shRNA1 or its paired Ctrl1 vector. Data are shown as relative luciferase expression compared to control condition (Ctrl1, TTX). Two-way ANOVA for vector $F(2, 31) = 12.67$, $p<0.0001$, for virus/treatment $F(3, 31) = 6.803$, $p=0.0012$, and for vector x virus/treatment interaction $F(6, 31) = 3.318$, $p=0.012$. For Gal4-MEF2Cα1β, Grin3a KD TTX vs TTX w/d. $p=0.0003$, *Grin3a* KD TTX w/d vs Ctrl TTX w/d $p=0.0027$, n=4 Ctrl TTX w/d and *Grin3a* KD TTX, 3 *Grin3a* KD TTX w/d . **(E,F)** Levels of TTX w/d-induced *Bdnf* IV mRNA (E) or *Arc* mRNA (F) in hippocampal neurons infected with the indicated lentiviruses. *Arc* mRNA was measured following 30min TTX w/d. Induced mRNA levels are shown as percentage of induction relative to control condition (Ctrl). *Bdnf* IV ANOVA for virus $F (3, 20) = 25.85$, $p<0.0001$. n=6/sample. Ctrl vs KD $p<0.0001$, KD vs dKD $p=0.0007$, dKD vs rescue $p=0.0002$. *Arc* ANOVA for virus $F (3, 19) = 9.641$, $p=0.0004$. n=6/sample except dKD=5. Ctrl vs KD = 0.0066, KD vs dKD $p=0.0024$, dKD vs rescue $p=0.006$. *$p<0.05$ *Grin3a* KD vs. Ctrl, #$p<0.05$ dKD vs *Grin3a* KD, ^$p<0.05$ rescue vs dKD.

Figure 4: **p38 MAP kinase activity is required for *Bdnf* IV potentiation. (A)** Luciferase expression in hippocampal neurons cotransfected with the UAS-Luc plasmid, a Gal4-MEF2Cα1β expression vector and either the *Grin3a* shRNA1 plasmid or its paired control. Hippocampal neurons were stimulated for 6 hrs with TTX w/d in the absence of presence of Cyclosporin A +FK 506(CsA/FSK) or SB203580 at the doses indicated. Induced luciferase levels are shown as

percentages of induction relative to the control at 100%. Two-way ANOVA for virus F (1, 40) = 25.07, p<0.0001, for treatment F (3, 40) = 8.724, p=0.0001, and for virus x treatment interaction F (3, 40) = 3.620, p=0.021. n=6/condition. Grin3a KD vs Ctrl for TTX w/d p=0.0005, for TTX w/d+CsA/FSK p=0.0021. **(B)** Luciferase expression in hippocampal neurons cotransfected with the pUAS-Luc plasmid, expression plasmids encoding the indicated Gal4-MEF2Cα1β vectors and the *Grin3a* shRNA1 plasmid. Data are shown as fold change in luciferase levels compared to the WT MEF2Cα1β in TTX. Two way ANOVA for vector F (2, 27) = 10.53, p=0.0004, for treatment, F (1, 27) = 10.41, p=0.0033 and treatment x vector interaction F (2, 27) = 5.590, p=0.0093. n=8/group for WT plasmid, 4 for TT293,300AA, and 5 for S387A. For Gal4-MEF2Cα1β TTX vs TTX w/d p=0.0002. (**C**) Levels of *Bdnf* IV mRNA in hippocampal neurons infected with the indicated lentiviruses then stimulated with TTX w/d. During TTX w/d neurons were either left untreated (Ctrl) or SB203580 was added. mRNA levels are reported as percentage of induction relative to control. Two-way ANOVA for virus F(1,15)=3.09, p=0.01, for treatment F(2,15)=36.48 p<0.0001, and treatment x virus interaction F(2,15)=12.88, p=0.0006. n=3 except 4 Ctrl TTX w/d. Ctrl vs *Grin3a* KD for TTX w/d p=0.0014 **p*<0.05 *Grin3a* KD vs. Ctrl, #p<0.05 TTXw/d vs TTX.

Figure 5: **Nuclear p38 MAPK phosphorylation is selectively enhanced upon NMDAR activation in GluN3A knockdown neurons. (A)** Western blot of phosphorylated p38/MAPK (p-p38) in the cytoplasm fraction of hippocampal neurons infected with the indicated shRNA virus or paired control virus then stimulated with TTX w/d for the indicated amounts of time prior to lysis. Actin is shown as a loading control. **(B)** Quantification of two independent experiments as described in (A). Band density in each lane was quantified using ImageJ. Expression was normalized to actin for each sample. p-p38 levels are reported as fold induction relative to unstimulated (0 min) condition. Two-way ANOVA for time F(5, 24) = 30.85, p<0.0001, for virus F(1, 24) = 6.8, p=0.015, and for time x virus interaction F(5, 24) = 1.6, p=0.20. **(C)** Western blot of phosphorylated p38/MAPK (p-p38) in the nuclear fraction of hippocampal neurons after TTX w/d. **(D)** Quantification of three independent experiments as described in (C). Two-way ANOVA for time F(5, 22) = 6.2, p=0.001, for virus F(1, 22) = 6.7, p=0.017 and for time x virus interaction F(5, 22) = 3.8, p=0.012. For *Grin3a* KD values differed from basal at 15 min (p=0.031) and 30 min (p=0.012). **(E)** Representative images of hippocampal neurons (DIV7) immunostained with antibodies against p-p38 (red) and Hoechst nuclear dye (blue). Neurons were infected with the indicated lentiviruses then stimulated with TTX w/d for 30mins. **(F)** Quantification of the fraction of total p-p38 in the nucleus from (E). ANOVA for virus F(2, 42) = 11.31, p=0.0001. n=15/virus. Ctrl vs KD p<0.001, KD vs. rescue p=0.014. **p*<0.05 *Grin3a* KD vs Ctrl, ^*p*<0.05 rescue vs *Grin3a* KD.

Figure 1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Lentiviral vector
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Relative expression Grin3a
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KD+GluN3A rescue
C
MW
Ctrl1
Grin3a shRNA1
Ctrl2
Grin3a shRNA2
GluN3A rescue
GFP
150kDa
100kDa
100kDa
αGluN3A
αTfR
D
Relative expression Bdnf IV
pLKO Ctrl
Grin3a shRNA1
TTX
TTX w/d
Treatment
E
Relatvie expression Bdnf IV
pLLx Ctrl
Grin3a shRNA2
Treatment
F
% Ctrl induction Bdnf IV mRNA
Ctrl
Grin3a KD
KD+GluN3A rescue
Treatment
G
% Ctrl indcution BDNF protein
Ctrl
Grin3a KD
KD+GluN3A rescue
Treatment
H
Baseline
Stim Av
Ctrl
Grin3a KD
KD+GluN3A rescue
1μm
mCherry
GCaMP3
I
Grin3a KD
KD+rescue
Ctrl
Δ(F/F0)
Time (sec)
J
Uncaging-Triggered Av
Δ(F/F0)
Time (sec)
K
Area Under Curve
Ctrl
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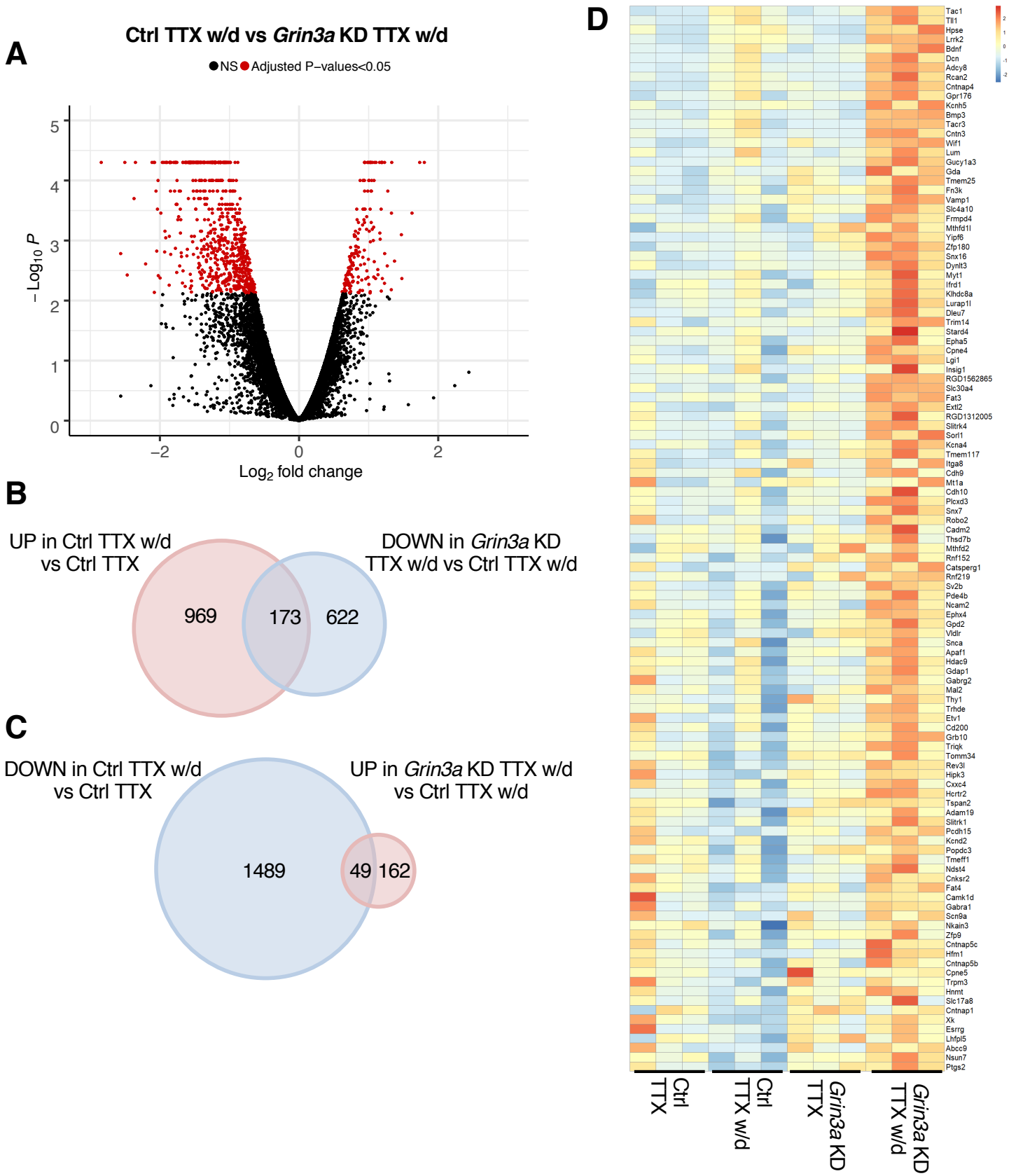
KD+GluN3A rescue

Figure 2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173
622
C
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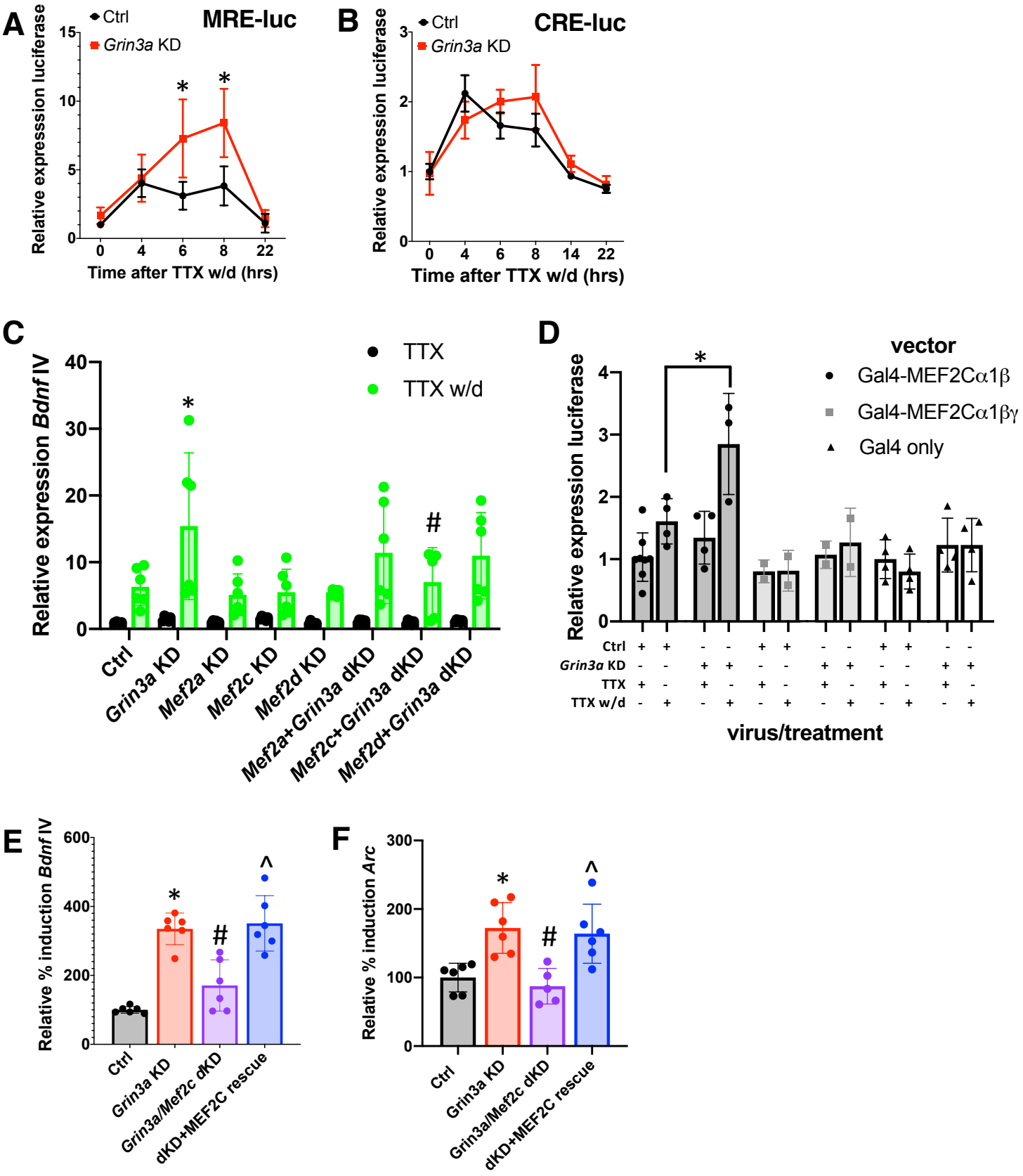
Figure 3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Time after TTX w/d (hrs)
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CRE-luc
Ctrl
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Relative expression luciferase
Time after TTX w/d (hrs)
C
TTX
TTX w/d
Relative expression Bdnf IV
Ctrl
Grin3a KD
Mef2a KD
Mef2c KD
Mef2d KD
Mef2a+Grin3a dKD
Mef2c+Grin3a dKD
Mef2d+Grin3a dKD
D
vector
Gal4-MEF2Cα1β
Gal4-MEF2Cα1βγ
Gal4 only
Relative expression luciferase
Ctrl
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TTX
TTX w/d
virus/treatment
E
Relative % induction Bdnf IV
Ctrl
Grin3a KD
Grin3a/Mef2c dKD
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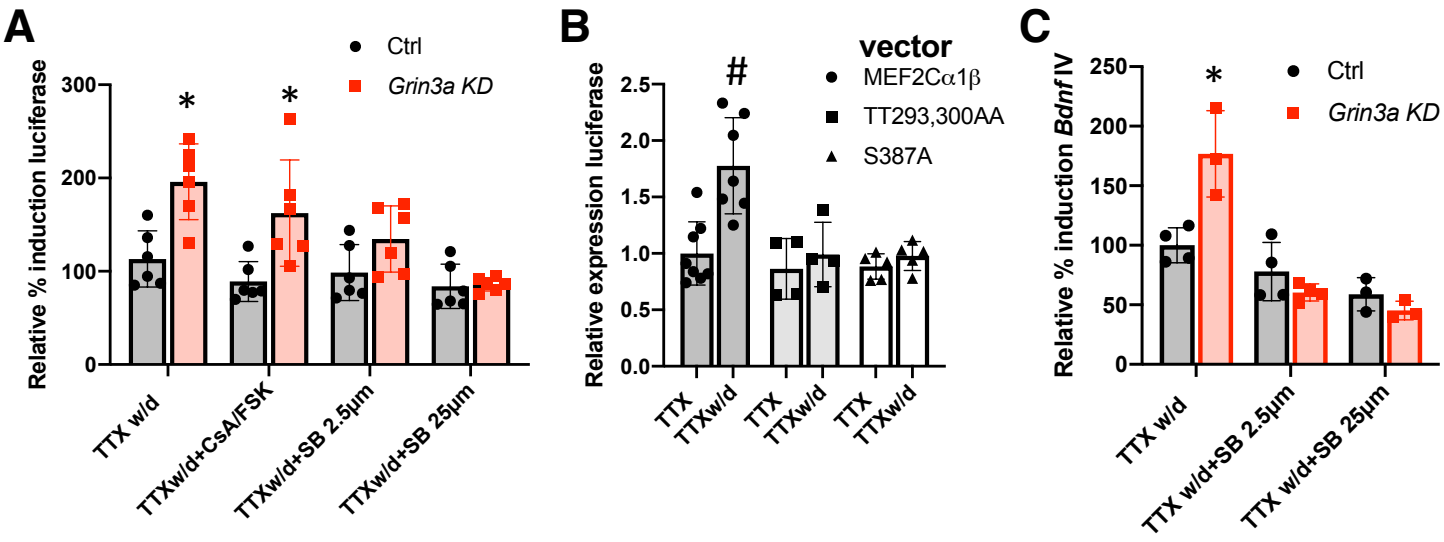
Figure 4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*
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TTXw/d+SB 25μm
B
Relative expression luciferase
2.5
2.0
1.5
1.0
0.5
0.0
#
vector
MEF2Cα1β
TT293,300AA
S387A
TTX
TTXw/d
TTX
TTXw/d
TTX
TTXw/d
C
Relative % induction Bdnf IV
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100
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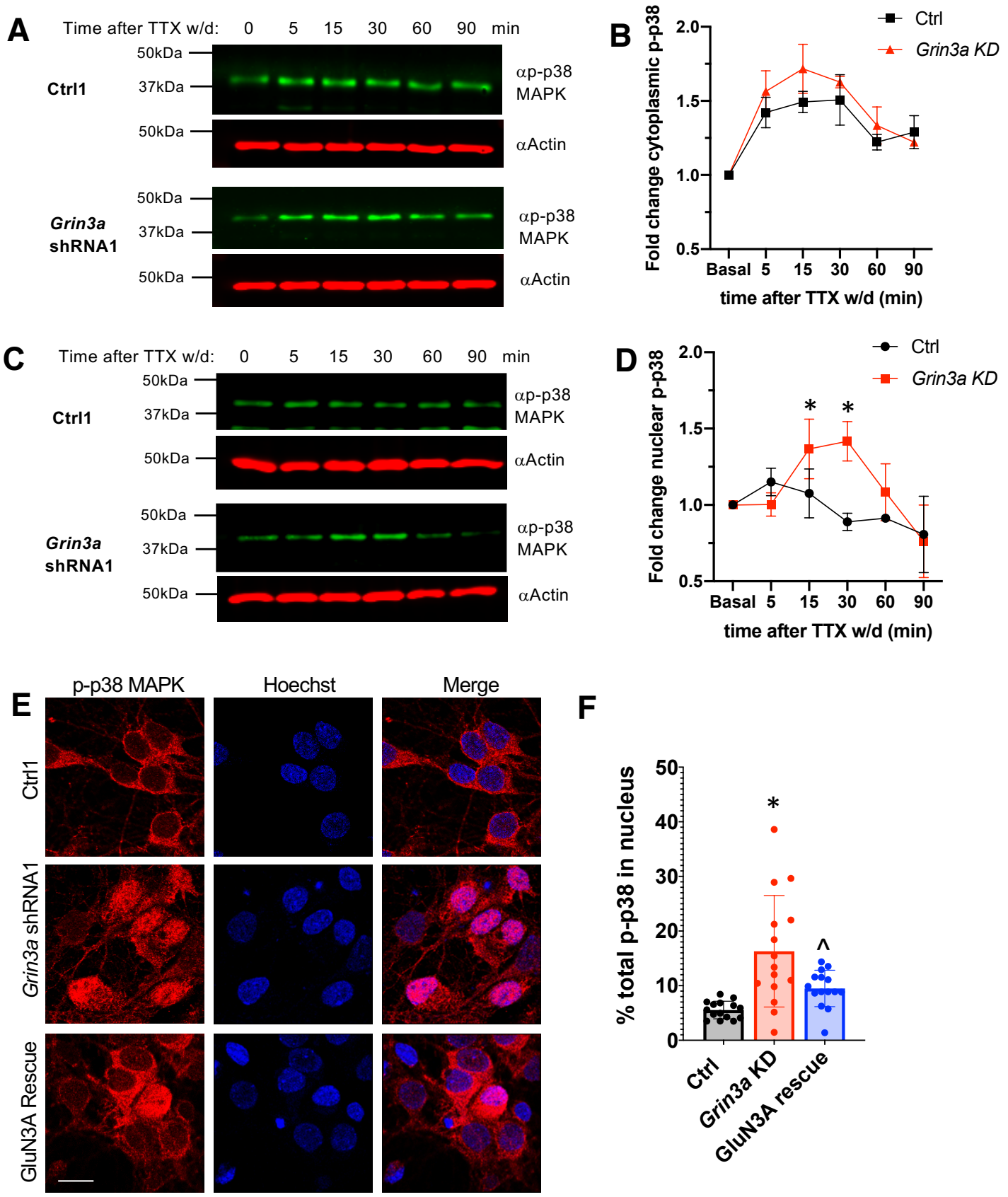
TTX w/d+SB 2.5μm
TTX w/d+SB 25μm

Figure 5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αActin
B
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Grin3a KD
Fold change cytoplasmic p-p38
2.0
1.5
1.0
0.5
Basal 5 15 30 60 90
time after TTX w/d (min)
C
Time after TTX w/d: 0 5 15 30 60 90 min
Ctrl1
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37kDa
αp-p38 MAPK
αActin
D
Ctrl
Grin3a KD
Fold change nuclear p-p38
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1.5
1.0
0.5
*
*
Basal 5 15 30 60 90
time after TTX w/d (min)
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p-p38 MAPK
Hoechst
Merge
Ctrl1
Grin3a shRNA1
GluN3A Rescue
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50
40
30
20
10
0
*
^
Ctrl
Grin3a KD
GluN3A rescue