Mechanism for neurotransmitter-receptor matching

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Synaptic communication requires the expression of functional postsynaptic receptors that match the presynaptically released neurotransmitter. The ability of neurons to switch the transmitter they release is increasingly well documented, and these switches require changes in the postsynaptic receptor population. Although the activity-dependent molecular mechanism of neurotransmitter switching is increasingly well understood, the basis of specification of postsynaptic neurotransmitter receptors matching the newly expressed transmitter is unknown. Using a functional assay, we show that sustained application of glutamate to embryonic vertebrate skeletal muscle cells cultured before innervation is necessary and sufficient to up-regulate ionotropic glutamate receptors from a pool of different receptors expressed at low levels. Up-regulation of these ionotropic receptors is independent of signaling by metabotropic glutamate receptors. Both imaging of glutamate-induced calcium elevations and Western blots reveal ionotropic glutamate receptor expression prior to immunocytochemical detection. Sustained application of glutamate to skeletal myotomes in vivo is necessary and sufficient for up-regulation of membrane expression of the GluN1 NMDA receptor subunit. Pharmacological antagonists and morpholinos implicate p38 and Jun kinases and MEF2C in the signal cascade leading to ionotropic glutamate receptor expression. The results suggest a mechanism by which neuronal release of transmitter up-regulates postsynaptic expression of appropriate transmitter receptors following neurotransmitter switching and may contribute to the proper expression of receptors at the time of initial innervation.

neurotransmitter respecification | receptor specification | glutamate receptors | development | plasticity

The expression of one or more neurotransmitters in a presynaptic neuron and expression of matching transmitter receptors in the postsynaptic cells are essential features of synapse formation and function. Both initial genetic specification of transmitter identity during development (1–3) and later transmitter switching in response to sustained stimulation (4–9) raise the question: How is expression of the appropriate postsynaptic receptors achieved? The mechanisms producing transmitter receptor clustering have received extensive attention (10–14). In contrast, the way in which the choice of receptor identity is determined is less clear. Preprograming receptor identity would appear to involve a substantial burden of information storage in view of the large number of different transmitter receptors and the myriad locations and contexts in which they are expressed in the brain. Innervation-dependent specification of receptor identity, through patterns of activity or factors released presynaptically, would afford an alternative mechanism enabling plasticity of expression.

Matching changes in neurotransmitter receptor expression have been observed following neurotransmitter switching in developing and adult nervous systems, bringing this question into sharp focus. In the peripheral nervous system, neurotransmitter switching in motor neurons is accompanied by up-regulation of functional receptors that were initially present at low levels and matched the newly expressed transmitter (4, 15). Denervation and reinnervation of adult skeletal muscle by glutamatergic neurons lead to the appearance of functional neuromuscular junctions expressing GluR1 and GluR2 (alias GluA1 and GluA2) subunits, which are blocked by the AMPA receptor antagonist GYKI 52466 (16, 17). In the central nervous system, the natural developmental transmitter switch from GABA to glycine in the auditory nervous system is accompanied by alterations in the properties of postsynaptic receptors (18, 19). Changes in illumination during development or in photoperiod in the adult lead to changes in the numbers of neurons expressing dopamine in the hypothalamus that are accompanied by corresponding up- or down-regulation of dopamine receptor expression in postsynaptic neurons (5, 7).

Here, we analyze the mechanism by which changes in the class of postsynaptic neurotransmitter receptor can be regulated in the developing postsynaptic cell. We find that sustained exposure to the transmitter glutamate is both necessary and sufficient for the up-regulation of ionotropic glutamate receptors in *Xenopus* striated skeletal myocytes in culture and in vivo, investigated with calcium imaging, Western blot, and immunocytochemistry. We identify components of a signaling cascade that are necessary for expression of these receptors. Our findings suggest a process by which classes of postsynaptic transmitter receptors are initially up-regulated at newly assembling neuronal synapses and a basis for transmitter-receptor matching in response to transmitter switching.

Significance

Neurotransmitter switching generally involves replacement of an excitatory transmitter with an inhibitory transmitter or vice versa and has been linked to changes in animal behavior. There are corresponding switches in postsynaptic receptors that enable continued function of the circuit, but the mechanism by which receptor expression is regulated in this context was unknown. Sustained exposure to the neurotransmitter glutamate during development is both necessary and sufficient for the upregulation of ionotropic glutamate receptors in vertebrate striated skeletal muscle cells. This finding suggests a basis by which neurotransmitter release up-regulates expression of matching receptors at newly formed synapses during development of the nervous system and in response to neurotransmitter switching at established synapses.

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Results

Signaling through Ionotropic Glutamate Receptors Is Necessary and Sufficient to Induce Glutamate Sensitivity of Myocytes in Cell Culture. We first investigated the basal glutamate sensitivity of embryonic trunk myocytes cocultured with neurons prior to neuronal innervation. Cultures were grown in medium containing 2 mM extracellular calcium for 18–24 h (1.5–1.7 d of development), rinsed, and loaded with Fluo-4 AM (20). The glutamate concentration in the synaptic cleft is estimated to achieve millimolar levels (21), suggesting that these were appropriate concentrations with which to test myocyte sensitivity. Twenty percent of acetylcholine-sensitive myocytes uncontacted by neurons demonstrated increased calcium fluorescence in response to local superperfusion of a test concentration of 5 mM glutamate in 2 mM calcium medium in imaging experiments (Fig. 1 *A*–*C*). Myocytes rarely responded to a second pulse of glutamate, perhaps as a result of desensitization or internalization of receptors (22–24). Because the percent of neurons expressing glutamate as a neurotransmitter increases 3-fold when neurons are cultured in the absence of extracellular calcium (4), we next grew neuron-myocyte cocultures for 18–24 h in 0 mM instead of 2 mM calcium medium. Now ∼40% of noncontacted myocytes were sensitive to glutamate when loaded with Fluo-4 AM and tested in the presence of 2 mM calcium during imaging (Fig. 1*C*; see also ref. 15). The presence of calcium increases neuronal activity that is further enhanced by veratridine (1 μM), reducing neuronal expression of glutamate (4) and the incidence of glutamate-responsive myocytes. Myocytes grown in the absence of neurons are unaffected by culture condition and have a similar baseline incidence of glutamate sensitivity as control neuron-myocyte cultures, since myocyte activity is endogenously generated in the absence of innervation and unaltered by the presence of calcium or veratridine (15, 25). These results suggested that glutamatergic neurons could be signaling to myocytes, triggering an increase in sensitivity.

To test the hypothesis that the stimulus promoting increased sensitivity to glutamate is glutamate itself, signaling through low levels of endogenous glutamate receptors, we investigated the ability of exogenously applied glutamate to affect glutamate sensitivity of myocytes. Myocytes were grown in the absence of neurons for 18–24 h in 2 mM calcium medium containing different concentrations of glutamate and tested in the presence of 2 mM calcium medium. These myocytes exhibited dose-dependent changes in the incidence of sensitivity to chronic exogenous glutamate (*SI Appendix*, Fig. S1*A*). The incidence of glutamate sensitivity increased from 20 to 60% as glutamate was raised from 0.1 to 1 or 10 μM and then decreased (Fig. 1*D* and *SI Appendix*, Fig. S1*A*). One micromolar glutamate was selected for chronic stimulation in subsequent experiments. This concentration is much lower than the test concentration and is unlikely to induce full AMPA or NMDA receptor desensitization (26, 27).

We next applied glutamate receptor antagonists together with 1 μM glutamate in 2 mM calcium medium for 18–24 h to determine which receptors are required to achieve this increase in glutamate sensitivity. Pharmacological blockade of either NMDA or AMPA receptors alone, with 50 μM AP5 or 15 μM NBQX, was ineffective in abrogating the enhanced incidence of sensitivity resulting from 1 μM glutamate exposure. However, simultaneous application of both antagonists was sufficient to fully block the increase from 20 to 60% incidence (Fig. 1*D*). The incidence of myocyte responses to 5 mM glutamate after 1 μM exposure for 18–24 h was reduced to 10% when cultures were treated with AP5 and NBQX. Application of ionotropic receptor agonists AMPA or NMDA alone or in combination (both at 1 μM) also enhanced the incidence of myocyte glutamate sensitivity (*SI Appendix*, Fig. S1*B*). These results suggest that the application of the transmitter (glutamate) alone, acting through

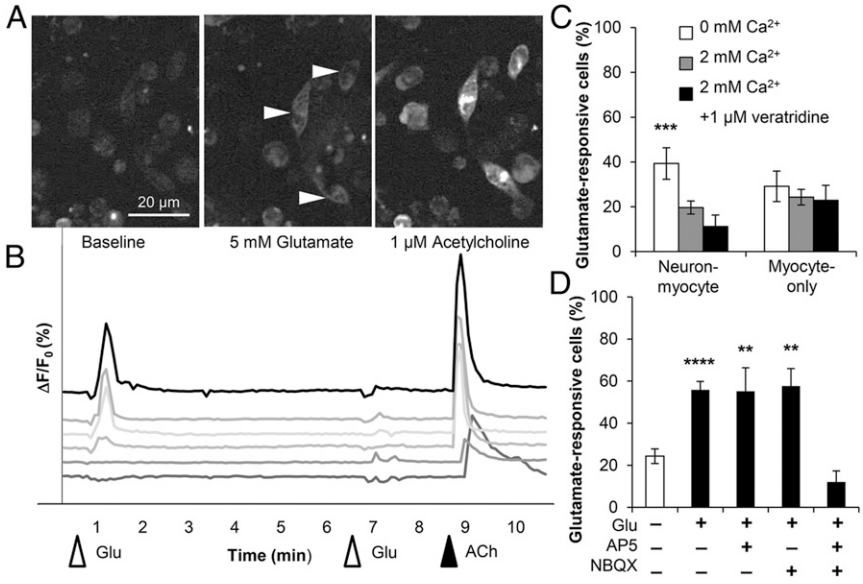

Fig. 1. Glutamate signaling through ionotropic receptors is necessary and sufficient to induce glutamate sensitivity of cultured myocytes. (*A*) Glutamate-induced Fluo-4 AM fluorescence intensity in acetylcholine-sensitive myocytes (arrowheads) in response to neurotransmitter superfusion of a neuron-myocyte coculture. (*B*) Digitized fluorescence of six myocytes in response to 5-s superfusion with 5 mM glutamate + 3 μM pancuronium or 1 μM acetylcholine following 18–24 h exposure to 1 μM glutamate in 2 mM Ca^{2+} culture medium. Traces are offset for clarity. (*C*) The incidence of glutamate sensitivity of myocytes in neuron-myocyte culture depends on the culture medium. Basal sensitivity in neuron-myocyte cultures in 2 mM Ca^{2+} increased in the absence of Ca^{2+} that increases the incidence of glutamatergic neurons but did not decrease significantly in the presence of 1 μM veratridine that decreases the incidence of glutamatergic neurons, ANOVA with Tukey's range test [$F(2, 25) = 7.947$]. Myocytes grown in the absence of neurons are unaffected by culture condition. (*D*) The combination of 50 μM AP5 and 15 μM NBQX, but not either inhibitor alone, decreased incidence of glutamate responses in myocyte-only cultures grown in the presence of 1 μM glutamate. ANOVA [$F(4, 100) = 13.37$, $P < 0.0001$] with Dunnett's post hoc test. (*C* and *D*) $n \geq 8$ cultures per group with 8–10 myocytes per culture. Values are mean ± SEM, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. See also *SI Appendix*, Figs. S1 and S2.

ionotropic receptors, is both necessary and sufficient to induce a significant increase in the incidence of myocyte sensitivity to glutamate. The results indicate that glutamate recruits glutamate sensitivity and that the responses that have been recruited are mediated by ionotropic glutamate receptors. Analysis of Western blots for GluN1 and GluA1 confirms up-regulation of NMDA receptors at 1.5 d in vitro. (*SI Appendix*, Fig. S2 *A* and *B*).

Metabotropic Glutamate Receptors Do Not Stimulate Glutamate-Induced Sensitivity of Myocytes In Vitro. The activity or surface expression of ionotropic glutamate receptors (iGluRs) can be modulated by activation of metabotropic glutamate receptors (mGluRs) in conjunction with or independent of iGluR activation (28–31), and mGluRs have been reported on skeletal muscle (32, 33). Therefore, mGluRs could mediate the effects of exogenous glutamate on the glutamate sensitivity of myocytes together with iGluRs. To determine whether the effects of glutamate on myocyte glutamate sensitivity were mediated by metabotropic receptors, we tested the contributions of mGluRs to myocyte glutamate sensitivity using class-specific agonists and antagonists. Inhibition of group I mGluRs was sufficient to elevate baseline but not the induced incidence of myocyte glutamate sensitivity. Inhibition of group II or III mGluRs had no effect on baseline or induced incidence of glutamate responses, suggesting their downstream effectors do not mediate agonist-induced glutamate sensitivity. Activation of each of the three classes of mGluRs abrogated the incidence of glutamate-induced myocyte sensitivity (Fig. 2), despite differences in downstream effectors, indicating that metabotropic glutamate receptors do not contribute to the increased myocyte responses to glutamate.

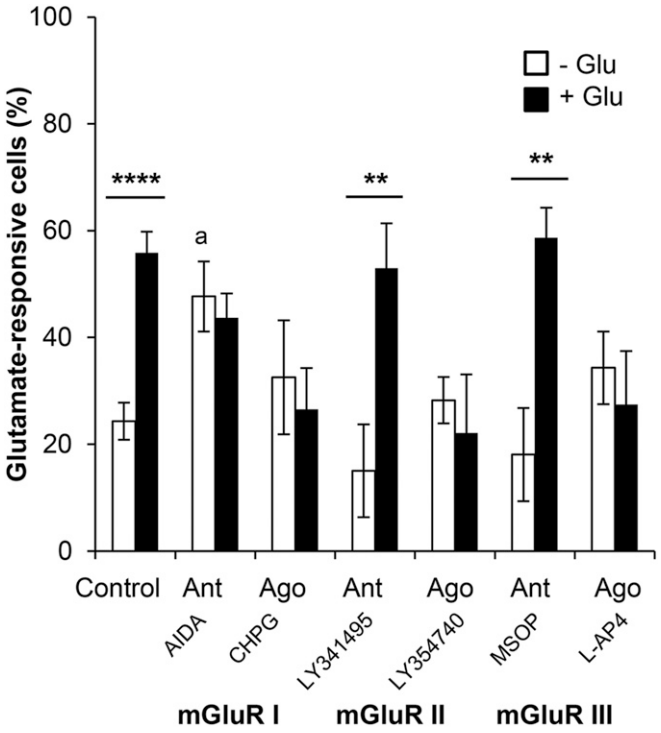

Fig. 2. Metabotropic glutamate receptors do not stimulate glutamate-induced sensitivity of myocytes. Antagonists (Ant) to type I mGluRs (100 μM AIDA) but not type II mGluRs (100 μM LY341495) or type III mGluRs (100 μM MSOP) enhanced the incidence of glutamate sensitivity in the absence of exogenous glutamate (ANOVA with Dunnett's post hoc test compared to control $F(6, 80) = 2.207$; a, $P < 0.05$), with no additional gain in sensitivity in the presence of glutamate (ANOVA $F(6, 103) = 5.091$; $P < 0.05$). The increased incidence of glutamate sensitivity in response to glutamate was blocked in myocytes cultured in the presence of agonists (Ago) for each class of mGluR (I, 100 μM CHPG; II, 100 μM LY354740; III, 20 μM L-AP4) (two-tailed unpaired *t* tests between glu+ and glu- pairs). $n \geq 8$ cultures per group with 8–10 myocytes per culture. Values are mean ± SEM. $^{**}P < 0.01$, $^{****}P < 0.0001$.

Immunocytochemistry Does Not Detect Up-Regulated Glutamate Receptors at Early Stages In Vitro. Immunohistochemically detectable AMPA and NMDA receptor subunits GluA1 and GluN1 are expressed in *Xenopus* myocytes as early as 1.3 d of development in vivo, before significant levels of clustered nAChRs appear (15). Strikingly, we did not detect increases in immunocytochemically detected GluN1 NMDA and GluA1 AMPA receptor subunits in fixed and permeabilized noncontacted myocytes in neuron-myocyte cocultures in the absence vs. presence of 2 mM calcium after 20 h in vitro (1.6 d of development) (*SI Appendix*, Fig. S2 *C–F*), despite the changes in myocyte glutamate sensitivity at this time. Small clusters were observed adjacent to nuclei or at the extremities of the cells. Changes in receptor expression could be due to increases in other glutamate receptor subunits; alternatively changes in GluN1 and GluA1 expression may be detected optically with sensitive calcium imaging before changes are sufficiently large to be observed by immunocytochemical examination, perhaps because the newly expressed receptors are not clustered.

Glutamate Exposure Is Necessary and Sufficient To Induce Expression of GluN1 in Myocytes In Vivo. To determine whether glutamate is effective in increasing myocyte glutamate receptor expression in vivo, we implanted agarose beads impregnated with 2 mM glutamate or vehicle medium at 1 d of development to achieve sustained local diffusion of agonist or control (4, 5, 34). Glutamate exposure induced up-regulation of the GluN1 (NR1) NMDA receptor subunit at 2.8 d of development compared to levels achieved with control beads loaded with medium, visualized immunohistochemically (Fig. 3 *A* and *B* and *SI Appendix*, Fig. S3). Myocytes were a day older when assayed in vivo than when assayed in culture, likely contributing to the detection of receptors. Beads loaded with 10 μM AMPA and NMDA produced similar up-regulation of GluN1 (Fig. 3 *B* and *C*). These increases were blocked when NMDA and AMPA receptor blockers (0.5 mM AP5 and 0.15 mM NBQX) were included with glutamate in the beads (Fig. 3 *B* and *D*).

Pharmacological Screening Suggests Components of the Signal Transduction Cascade Required for Glutamate-Induced Glutamate Sensitivity of Myocytes In Vitro. We returned to imaging calcium elevations in cultured myocytes for pharmacological tests of the role of activity-dependent kinases in increasing the percent of glutamate-sensitive myocytes. Using specific antagonists, we identified adenylate cyclase (AC), mitogen-activated protein kinase kinase MEK1/2 (MAP2K1/MAP2K2), p38, and JNK as downstream effectors of glutamate signaling that mediate agonist-induced glutamate sensitivity (Fig. 4). Activation of group II and III mGluRs typically leads to inhibition of AC (35, 36) and suppresses glutamate-induced increased sensitivity to glutamate (Fig. 2), suggesting that activation of AC may be downstream of ionotropic receptor activation in glutamate-induced sensitivity. Addition of 8-Br-cAMP (300 μM) to cultures increased myocyte sensitivity to glutamate without sustained application of glutamate, indicating that it may be part of the induction pathway that is not additive with the effect of sustained glutamate application. Inhibition of PKA, mTOR, or PKC significantly elevated baseline glutamate sensitivity with no additional increase in the presence of glutamate.

Morpholino Gene Knockdown Identifies Roles for p38 and JNK1 in Glutamate-Induced Glutamate Sensitivity of Myocytes In Vitro and Up-Regulation of GluN1 In Vivo. Morpholinos (MOs) are useful tools for reducing expression of genes in vertebrate embryos (37, 38). We used MOs previously demonstrated to efficiently reduce

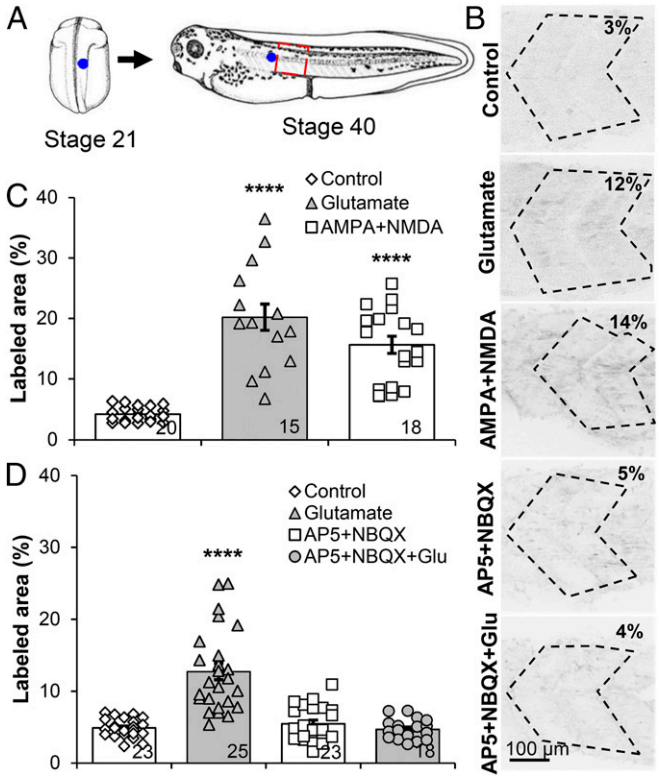

Fig. 3. Glutamate signaling to ionotropic receptors is necessary and sufficient to induce NMDA receptor up-regulation in myocytes in vivo. (*A*) Agarose beads loaded with drugs were implanted at stage 21 (22.5 h) and animals raised to stage 40 (2.8 d). (*B*) Glutamate delivered by agarose beads stimulated an increase in GluN1 immunoreactivity (% of labeled area within indicated borders) in trunk myocytes. (*C*) Bead delivery of 10 μM AMPA and 10 μM NMDA also induced expression of GluN1 in myocytes, ANOVA [$F(2, 51) = 26.8$] with Dunnett's post hoc test. (*D*) Bead delivery of 0.5 mM AP5 plus 0.15 mM NBQX abrogated the glutamate-induced increase in GluN1 expression, ANOVA [$F(3, 86) = 31.47$]. $n = 4$ independent experiments for each graph. The lower right number on each bar is the number of embryos examined. Values are mean ± SEM. ****$P < 0.0001$. See also *SI Appendix*, Fig. S3.

expression of their respective target in *Xenopus* embryos (*SI Appendix*). Gene knockdown of p38 using MOs delivered in vitro blocks agonist-induced sensitivity (Fig. 5*A*), confirming specificity of the pharmacological inhibitor and implicating p38β (MAPK11) and p38γ (MAPK12), but not p38α (MAPK14), as effectors. Because pharmacological inhibition of p38 suppressed agonist-induced myocyte glutamate sensitivity, we tested the effect of glutamate exposure on its phosphorylation status, which is indicative of its activation state. Glutamate exposure induces a transient increase in p38 phosphorylation after several hours (Fig. 5 *B* and *C*). Consistent with this result, glutamate-mediated up-regulation of GluN1 receptor subunit in vivo is blocked by simultaneous inhibition of p38β (Fig. 5 *D* and *E*). Calcium elevation in response to glutamate receptor activation may activate p38, which then activates the MEF2 transcription factor that binds to the GluN1 promoter (39, 40).

Targeted knockdown of JNK1 (MAPK8) using MOs delivered in vitro blocks agonist-induced glutamate sensitivity (Fig. 6*A*), confirming specificity of the pharmacological inhibitor and identifying JNK1 as a critical effector. Because pharmacological inhibition of JNK also abolished agonist-induced myocyte glutamate sensitivity in vitro (Fig. 4*A*), we then examined its activation. Glutamate exposure induces a transient decrease in JNK phosphorylation that returns to baseline after several hours (Fig. 6 *B* and *C*). Deactivation of JNK1 downstream of glutamate receptor activation presumably reduces phosphorylation of factors that can inhibit transcription of GluN1. Glutamate-mediated up-regulation of the GluN1 receptor subunit in vivo is blocked by MO knockdown of JNK1 (Fig. 6 *D* and *E*).

Expression of the MEF2C Transcription Factor Is Necessary for Up-Regulation of GluN1 In Vivo. The promoter for the NMDA receptor subunit GluN1 (NR1) contains a functional MEF2 recognition sequence (40, 41). Because MEF2C is a calcium-sensitive transcription factor and a p38 target (39), MEF2C was a likely candidate to regulate glutamate-mediated up-regulation of the GluN1 receptor subunit. We tested the requirement for MEF2C in glutamate-mediated GluN1 up-regulation in vivo. Targeting MEF2C with MOs via agarose bead delivery abrogated the up-regulation of GluN1 in response to glutamate (Fig. 7) supporting transcription-dependent glutamate-mediated up-regulation of NMDA receptor subunits.

Discussion

Our results demonstrate that sustained exposure to the neurotransmitter glutamate during development is both necessary and sufficient for the up-regulation of ionotropic glutamate receptors in striated skeletal myocytes in vitro and in vivo. Calcium imaging of cultured myocytes provides functional evidence that is substantiated by Western blots. Immunohistochemistry reveals an increase in GluN1 in vivo. Myocyte-only cultures facilitated calcium imaging that enabled detection of glutamate-induced enhancement of glutamate sensitivity at 1 d in vitro and increased expression of GluN1 by Western blot at 1.5 d in vitro. These assays were both more sensitive than immunocytochemistry. The expression of calcium permeable AMPAR could also contribute to early increases in glutamate sensitivity. Our findings identify components of a signaling cascade that are necessary for expression of glutamate receptors in developing myocytes and suggest a mechanism by which up-regulation occurs. The results

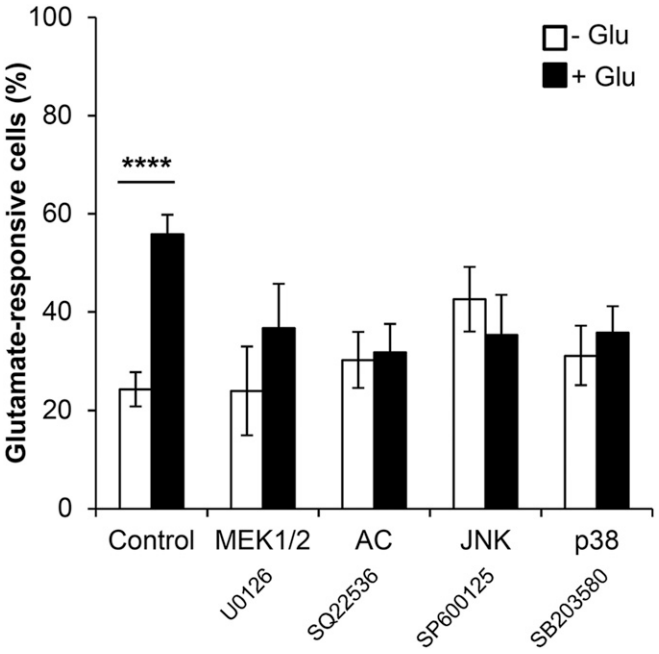

Fig. 4. Inhibitors of adenylate cyclase and MAP kinases suppress glutamate-induced glutamate sensitivity of myocytes. Coincubation of glutamate with pharmacological inhibitors identified adenylate cyclase (AC; 5 μM SQ22536), MEK1/2 (10 μM U0126), JNK (5 μM SP600125), and p38 (10 μM SB203580) as mediators of the increase in glutamate sensitivity, ANOVA [$F(9, 131) = 5.265$] with Tukey's post hoc test. $n \geq 8$ cultures per group and 8–10 myocytes per culture. Values are mean ± SEM. ****$P < 0.0001$.

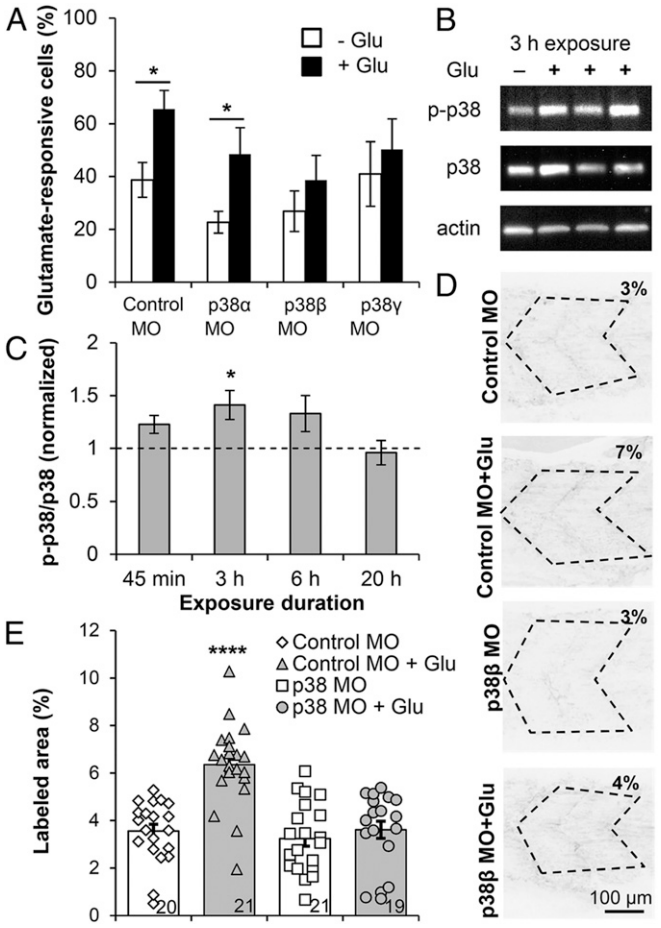

Fig. 5. p38 is required for glutamate-induced glutamate sensitivity of myocytes and up-regulation of NMDA receptors. (*A*) MO-mediated knockdown of p38β or p38γ in vitro abolished the glutamate-mediated increased sensitivity of myocytes to glutamate without affecting baseline sensitivity, unpaired *t* tests between –glu and +glu pairs. $n \geq 5$ cultures per group with 8–10 myocytes per culture. (*B*) Western blots of cultured myocytes exposed to glutamate were probed for p-p38, p38, and actin. (*C*) The ratio of phosphorylated versus total p38 increased briefly after glutamate exposure. Values are from three experimental replicates, one-sample *t* tests to expected mean of 1 (control). (*D*) MO-mediated p38β knockdown in vivo eliminated the glutamate-induced increase in GluN1 expression, ANOVA [$F(3, 77) = 18.98$] with Tukey's post hoc test. (*E*) Quantification of *D*. $n = 4$ independent experiments for each bar. Values are mean ± SEM. $^{*}P < 0.05$, $^{****}P < 0.0001$.

provide a basis for neurotransmitter-receptor matching during neurotransmitter switching at neuronal synapses during development (5) and in the adult nervous system (7), as well as following damage to mature neuromuscular synapses (9, 16, 17). The findings may also be relevant to initial pairing of transmitter receptors to neurotransmitters released at newly formed synapses.

Pharmacological and MO experiments suggest a model by which glutamate acting through ionotropic receptors triggers several converging pathways (Fig. 8). In this model, deactivation of JNK alters phosphorylation of jun/ATF transcription factors that bind to the AP-1 site in the GluN1 promoter (42). There appears to be no reported direct connection between JNK deactivation and NMDAR signaling, and JNK may act through an unknown intermediary signal. Calcium influx through NMDARs can activate MEF2 (43). Activation of AC generates cAMP that combines with metabotropic actions of NMDARs and calcium to activate p38 (44, 45). p38 in turn increases MEF2 DNA binding and transactivation (39). MEF2 requires the ubiquitous transcription factor SP1/3 for efficient DNA binding to the GluN1 promoter (40). We propose that the combined actions of these pathways create a feed forward cycle that allows sustained exposure to glutamate to up-regulate and maintain NMDA receptor subunit GluN1 expression. While this model specifically addresses GluN1, the obligatory NMDA receptor subunit, there may be a similar mechanism to up-regulate other subunits to form functional receptors that these experiments detected by increases in glutamate sensitivity.

Receptor-transmitter matching requires a low level of expression of multiple classes of postsynaptic receptors, for which evidence has been obtained at neuromuscular junctions (15, 46). This mechanism for ensuring matching of transmitters with their cognate receptors avoids the need for prior specification of receptor localization, which would require encoding of large amounts of information and likely be unresponsive to changes in presynaptic neurotransmitter identity that occur during transmitter switching. Cell adhesion molecules are known to play

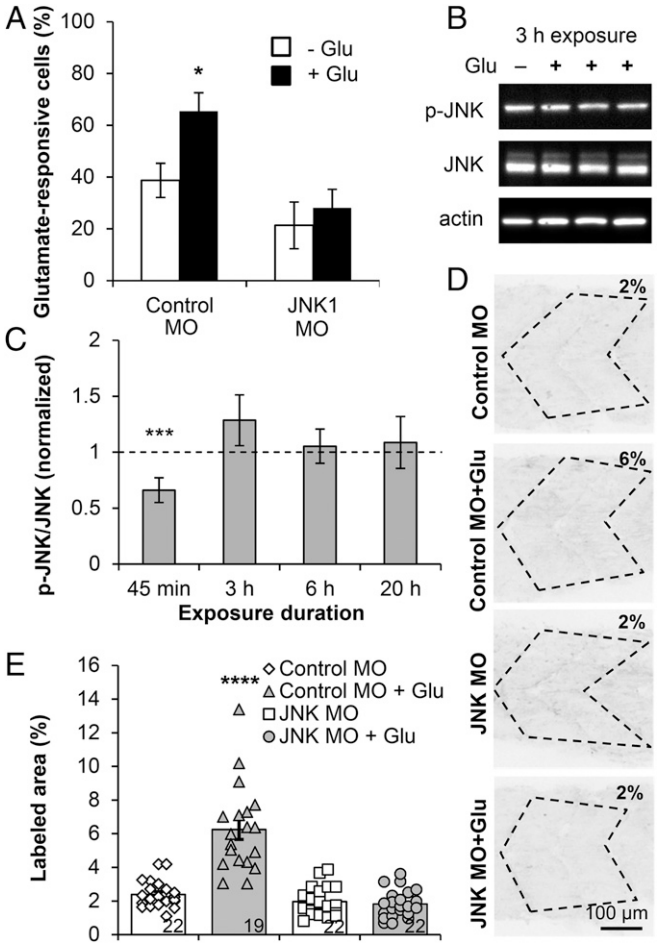

Fig. 6. JNK is required for glutamate-induced myocyte glutamate sensitivity and NMDA receptor up-regulation. (*A*) MO-mediated knockdown of JNK1 in vitro abolished the glutamate-mediated increased sensitivity of myocytes to glutamate (ANOVA $F(3, 35) = 6.87$ with Tukey's post hoc test). $n \geq 8$ cultures per group with 8–10 myocytes per culture. (*B*) Western blots of cultured myocytes exposed to glutamate were probed for p-JNK, JNK, and actin. (*C*) The ratio of phosphorylated to total JNK decreased temporarily after glutamate exposure. Values are from three experimental replicates; one-sample *t* tests to expected mean of 1 (control). (*D*) MO-mediated JNK knockdown in vivo eliminated the effect of glutamate on increased GluN1 expression, ANOVA [$F(3, 81) = 43.74$] with Tukey's post hoc test. (*E*) Quantification of *D*. $n = 4$ independent experiments. Values are mean ± SEM. $^{*}P < 0.05$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

roles in synaptogenesis at the neuromuscular junction (47). Our results suggest that secreted factors are involved in postsynaptic receptor expression and function. We predict that neurons express a reserve pool of transmitter receptors, normally expressed at low density, the levels of which can be up-regulated in response to release of transmitters by innervating nerve terminals.

Myocytes appear to constitutively express cholinergic receptors, with glutamate up-regulating glutamate receptors under certain circumstances (16, 17). Consistent with this picture, ACh receptors are expressed even when the gene encoding choline acetyltransferase has been knocked out (48) and are required to maintain the appropriate postsynaptic specializations of the motor endplates (47). Glutamatergic signaling does not lead to down-regulation of ACh receptors (Fig. 1 and *SI Appendix*, Fig. S1; refs. 9 and 17). How many classes of transmitter receptors are myocytes capable of expressing? Future work will determine whether the repertoire is limited to a small group of receptors for classical transmitters such as glutamate, GABA, and ACh, or whether receptors for biogenic amines and peptides are also inducible by their ligands.

Materials and Methods

Animals. All animal procedures were performed in accordance with institutional guidelines and approved by the University of California San Diego Institutional Animal Care and Use Committee. See *SI Appendix* for further details.

Cell Culture and Ca^{2+} Imaging. Myocytes were cultured for 18–24 h and loaded with 1 μM Fluo-4 AM Ca^{2+} indicator (Invitrogen) and 0.01% Pluronic F-127 detergent (Molecular Probes). Images were acquired at 0.2 Hz for 10.5 min. During imaging, cells were continuously superfused at 5 mL/min with 2 mM Ca^{2+} medium. Five-second pulses of 5 mM glutamate dissolved in 2 mM Ca^{2+} medium in the absence of exogenous glycine and presence of 3 μM pancuronium or 1 μM acetylcholine were applied to test myocyte sensitivity (15). Cells were considered glutamate- or ACh-sensitive when fluorescence amplitude met or exceeded 20% of $\Delta F/F_0$, more than twice the SD of baseline (Fig. 1*B*). Stock concentrations of drugs were added to culture medium and washed out prior to Fluo-4 AM loading. See *SI Appendix* for details.

Western Blotting. Western blots were carried out on protein extracts from myocytes cultured for 6, 20, or 38 h. For pMAPK time courses with glutamate exposures <6 h, myocytes were allowed to differentiate morphologically for 6 h and exposed to glutamate only during the final 45 min or 3 h before lysis. Antibodies used were as follows: rabbit anti-GluR1 (GluA1) and mouse anti-NMDAR1 (GluN1) (Millipore), rabbit anti-p-p38, rabbit anti-p38 (Cell Signaling), rabbit anti-pJNK (Promega), rabbit anti-JNK (Santa Cruz Biotechnology), and rabbit anti-actin (Sigma-Aldrich). Blots were incubated with peroxidase-conjugated secondary antibodies and bands were visualized using horseradish peroxidase chemiluminescence. See *SI Appendix*.

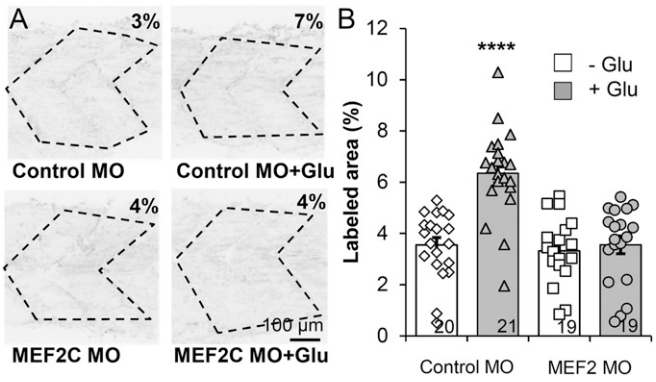

Fig. 7. MEF2 is required for NMDA receptor subunit up-regulation in vivo. (*A*) GluN1 up-regulation is abolished by MEF2C MO-mediated knockdown. (*B*) Quantification of *A*, $n = 4$ independent experiments. Values are mean ± SEM, ANOVA [$F(3, 75) = 21.67$] with Tukey's post hoc test. ****$P < 0.0001$.

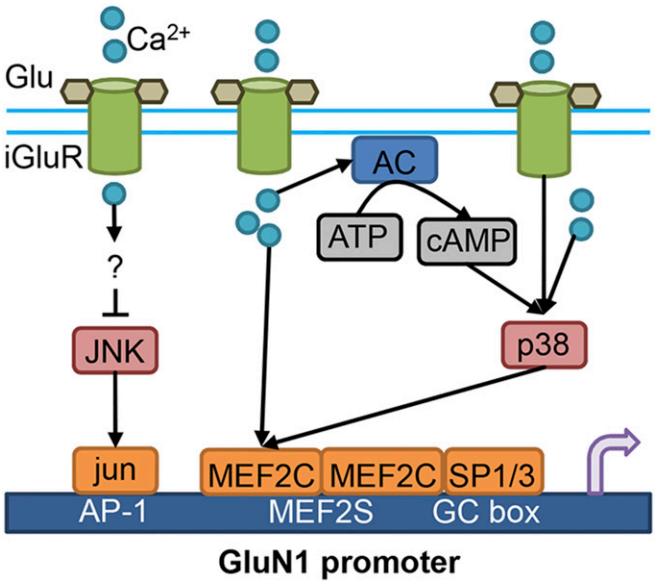

Fig. 8. Model of neurotransmitter-receptor matching. Glutamate activation of ionotropic receptors (iGluR) deactivates JNK through an unknown intermediary to regulate jun/ATF transcription factors that bind to the AP-1 site in the GluN1 promoter. In parallel, iGluR stimulation leads to activation of adenylate cyclase (AC) to generate cAMP that activates p38 in combination with metabotropic actions of NMDARs. Ca^{2+} binding Ca^{2+}-mediated activation of PI3K, and p38-mediated phosphorylation of MEF2 dimers facilitate DNA binding and transactivation. MEF2 requires SP1/3 for efficient DNA binding to the GluN1 promoter. The combined actions of these pathways create a feed-forward cycle that allows sustained exposure to glutamate to up-regulate and maintain NMDA receptor subunit GluN1 expression.

Bead Implantation. Spatial and temporal control of delivery of pharmacological agents was achieved using agarose beads loaded with 2 mM Ca^{2+} medium with or without drugs or vivo-MOs and implanted at stage 21 (5). Embryos were processed for immunohistochemistry at stage 40. See *SI Appendix*.

Immunostaining. Culture immunocytochemistry and whole mount immunohistochemistry were performed as previously described (4, 15) using mouse anti-NR1-CT (Millipore) or rabbit anti-GluR1 and fluorescent secondary antibodies. Puncta larger than 0.75 μm, ~1.5× the size of extracellular debris, were counted as receptor clusters. The longest linear dimension of each receptor cluster was measured. Skinned stage 40 larvae were incubated with anti-NMDAR1 and imaged on a Leica SP5 confocal microscope. The percent of GluN1-labeled area was determined by measuring the fraction occupied by pixels of intensity at or below an empirically determined constant threshold. See *SI Appendix*.

MOs. Cell membrane-permeable vivo-MOs from Gene Tools were delivered in vitro and in vivo. MOs targeting JNK1/MAPK8 [5′-TGCTGTCACGCTTGCTTCGGCTCAT-3′ (49)], p38α/MAPK14 [5′-GACGTAAGATTGATTGGATGACATA-3′ (50)], and MEF2C [5′-CCATAGTCCCCGTTTTTCTGTCTTC-3′ (51)] were previously described. MOs targeting p38β/MAPK11 (5′-CGCCCGCTCATCTTGCCCCGACCGG-3′) and p38γ/MAPK12 (5′-CGAGTTCCCGGCAGGCTCCT-3′) were designed by GeneTools. A standard control MO (5′-CCTCTTACCTCAGTTACAATTTATA-3′) and a 5-base JNK1 mismatch (5′-TGCTGTGACCCTTCCTTCCGCTGAT-3′) were used to test the specificity of MO effects.

Quantification and Statistical Analysis. Statistical tests used and number of replicates are provided in each figure legend. Values were considered significantly different at $P < 0.05$. See *SI Appendix*.

Data Availability Statement. All data for the paper are contained in the article.

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