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Stability of NMDA receptors at the plasma membrane controls synaptic and behavioral adaptations to amphetamine

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Abstract

Plastic changes in glutamatergic synapses that lead to enduring drug craving and addiction are poorly understood. By focusing on the turnover and trafficking of NMDA receptors, we found that chronic exposure to the psychostimulant amphetamine induces selective downregulation of NMDA receptor NR2B subunits in the confined surface membrane pool of rat striatal neurons at synaptic sites. Remarkably, this downregulation is a long-lived event and results from the destabilization of surface-expressed NR2B due to accelerated ubiquitination and degradation of crucial NR2B-anchoring proteins by the ubiquitin-proteasome system. The biochemical loss of synaptic NR2B further translates to the significant modulation of synaptic plasticity in the form of long-term depression at cortico-accumbal glutamatergic synapses. Behaviorally, genetic disruption of NR2B induces, whereas restoration of NR2B loss prevents, behavioral sensitization to amphetamine. Our data identify NR2B as a key regulator in the remodeling of excitatory synapses and persistent psychomotor plasticity in response to amphetamine.

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AUTHOR CONTRIBUTIONS

L.M.M., W.W., G.C.Z., X.Y.L., and M.H. conducted the biochemical and behavioral experiments and analyzed the data; W.W., X.P.C., and Y.J.Y. performed the electrophysiological experiments; C.J.P., E.E.F., and S.B. collaborated on providing expert advice, analyzing and interpreting the data, and developing and editing the manuscript; and J.G.C. oversaw the electrophysiological and other studies and co-wrote the manuscript. J.Q.W. supervised the project, designed the experiments, and wrote the manuscript.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

Keywords

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Drug addiction is a persistent mental disorder characterized by compulsive drug craving, seeking, and ingestion despite severe adverse consequences. Potential brain mechanisms underlying addiction remain elusive. Experimental animal models that mimic core features of addiction in humans have proven valuable for clarifying the underlying mechanisms. One compelling animal model for experience- and drug-dependent behavioral plasticity is the progressive and enduring augmentation of psychomotor responses to repeated psychostimulant exposure, known as behavioral sensitization1. This model corresponds to the intensification of drug craving in human addicts, and is readily elicited by many drugs, including dopamine the psychostimulants cocaine and amphetamine (AMPH).

Persistent molecular adaptations in specific neural circuits in response to repeated drug exposure are thought to lead to addiction2,3. A major neural circuit where adaptive responses are believed to occur is the mesolimbic system, a dopaminergic projection from the ventral tegmental area (VTA) to the ventral striatum/nucleus accumbens (NAc). Much of the initial work has focused on this system for defining pre- and postsynaptic plastic changes, establishing that altered dopaminergic transmission contributes to drug-related psychomotor plasticity1.

Glutamatergic inputs to the NAc are also critical for synaptic and behavioral plasticity1,4. As a major basal ganglia structure abundantly innervated with glutamatergic afferents from various cortical and subcortical areas, the NAc exhibits robust and long-lasting changes in excitatory transmission following repeated psychostimulant administration5,6, which are important for behavioral plasticity1,6. In parallel with enriched glutamate afferents, ionotropic glutamate NMDA receptors (NMDARs) are densely expressed in striatal medium spiny neurons7–10. These receptors become functional upon a heteromultimer assembly of the obligatory NR1 and the modulatory NR2 subunit. Among all modulatory subunits (A-D), NR2B is distinctly enriched in the striatum. Functionally, NR2B has been implicated in many forms of synaptic plasticity related to physiology of striatal neurons and pathogenesis of various neurological disorders11. However, whether and how NR2B adapts to AMPH remains poorly understood.

We therefore conducted a complete set of *in vivo* experiments to evaluate the role of NR2B in AMPH-dependent synaptic and behavioral plasticity. We found that chronic AMPH exposure destabilized surface-expressed NR2B in the postsynaptic density (PSD) of striatal neurons. This destabilization constitutes an enduring molecular adaptation of excitatory synapses to AMPH, leading to the concurrent development of behavioral plasticity.

Results

Reduction of NR2B: generality and specificity

To link behavior to NMDAR expression following chronic noncontingent AMPH exposure, we used an AMPH sensitization regimen (4 mg/kg, i.p., once daily for 7 consecutive days) in rats. At 14 days after AMPH injection, rats were assessed for behavioral and genomic changes. A robust behavioral sensitization to a challenge dose of AMPH (1.25 mg/kg, i.p.) was revealed in rats pretreated with AMPH (Fig. 1a). Concurrently, NR2B proteins in total striatal protein homogenates were reduced in AMPH-treated rats relative to saline-treated rats (Fig. 1b,e). This reduction was not confined to either the dorsal (caudate putamen, CPu) or ventral (NAc) striatum as both structures showed comparable reductions of NR2B (Fig. 1c—e). To determine the type of striatal output neurons displaying the NR2B loss, we conducted *in situ* hybridization and immunohistochemistry on the same sections (Fig. S1a,b). The number of neurons containing preproenkephalin (PPE) or preprodynorphin (PPD) mRNAs in the NAc remained unchanged 14 days after chronic AMPH. In PPE neurons, the number of cells immunoreactive to NR2B was reduced in AMPH-treated rats. A lesser reduction of NR2B occurred in PPD neurons. Thus, AMPH downregulates NR2B in dynorphin- and, to a greater extent, enkephalin-containing neurons.

Other NMDAR subunits were also screened for their responses to AMPH. In normal rodents, NR1 and NR2A are expressed at moderate to high levels in the striatum12. NR3A and NR3B proteins are expressed at relatively low levels13,14 whereas NR2C and NR2D proteins are almost lacking15. In response to chronic AMPH, total levels of striatal NR1, NR2A, NR3A, and NR3B proteins were not altered (Fig. S1c), indicating the insensitivity of these subunits to AMPH. Similarly, AMPH did not alter the amounts of the presynaptic proteins, including a presynaptic plasma membrane protein (syntaxin) and two synaptic vesicle membrane proteins (synaptophysin and synapsin) (Fig. S1d). Neither did AMPH affect the postsynaptic proteins, including a dendritic marker [microtubule-associated protein 2 (MAP2)], a kinase- and AMPA receptor-associated scaffold protein [A-kinase anchoring protein (AKAP)], and PSD-93/chapsyn-110 (a PSD-95 homolog of the membrane-associated guanylate kinase family) (Fig. S1e). Only PSD-95 exhibited a decrease in AMPH-treated rats (Fig. S1e), similar to results observed in the mouse striatum after repeated cocaine16. These data support that AMPH selectively modulates NR2B expression at excitatory synapses.

Reduction of NR2B: spatial and temporal characteristics

Other forebrain and midbrain structures, including the prefrontal cortex, hippocampus, amygdala, and VTA, express NR2B and are implicated in stimulant action17. However, chronic AMPH did not alter NR2B expression in these regions (Fig. S2a). Thus, AMPH-induced downregulation of NR2B appears to be selective to the striatum.

AMPH-induced behavioral plasticity is characterized by its remarkably long-lived nature. Altered receptor expression, if lasting long enough, could serve as a critical molecular mechanism for the nearly permanent behavioral modification3. We thus explored the candidacy of NR2B downregulation as a potential mechanism. We observed a long-lasting

decrease in NR2B (Fig. 1f,g). No change in NR1 and NR2A was observed at all time points. Behavioral responses to a challenge dose of AMPH (1.25 mg/kg, i.p.) were augmented at 28 and 60 days in AMPH- but not saline-treated rats. In naive rats, acute injection of AMPH (4 mg/kg, i.p.) did not alter striatal NR1, NR2A, or NR2B levels 1 day after injection (Fig. S2b). These results reveal an enduring NR2B downregulation that closely correlates with persistent behavioral plasticity.

NR2B reduction in a specific subcellular compartment

The subcellular localization of NMDARs determines the receptor property and is subject to the modulation by changing synaptic inputs12,18. After demonstrating an overall reduction of NR2B in total protein homogenates, we next investigated the response of NR2B to AMPH in different subcellular domains. Biochemical fractionation was performed to isolate proteins from distinct subcellular compartments12. The membrane-bound proteins were isolated mainly from the synaptosomal membrane (LP1) and the intracellular membrane. The latter include the membrane from two sources: 1) the intracellular light membrane (P3) containing organelles such as endoplasmic reticulum (ER)/Golgi apparatus for the synthesis and assembly of NMDARs19, and 2) the intracellular vesicular membrane (LP2) containing organelles such as vesicles for the trafficking of NMDARs (Fig. 2a). The effectiveness of fractionation was validated by confirming unique expression patterns of protein markers for distinctive compartments (Fig. 2b). The synaptic vesicular membrane protein, synaptophysin, was present in the LP2 at a high level. Calnexin, a Ca^{2+}-binding protein known to interact with glycoproteins in the ER20, was detected in the P3. α-Actinin showed a wider subcellular distribution due to its expression in both membrane and cytosolic fractions. NR2B and NR1 are membrane proteins bound to either surface or intracellular membranes12. Both proteins were present in the P2 and LP1 at high levels and in the P3 and LP2 at low levels, and were undetectable in the cytosolic S3 and LS2.

Remarkably, AMPH, at 14 days of withdrawal, reduced NR2B levels in the homogenate, P2, and LP1 (Fig. 2b,c). However, AMPH slightly increased NR2B in the P3 and LP2 (although $p > 0.05$). As a result, the ratio of NR2B in the LP1 versus the P3/LP2 was reduced (0.58 ± 0.09-fold saline, $p < 0.05$). Meanwhile, AMPH did not alter NR1, α-actinin, synaptophysin, or calnexin in any fraction (Fig. 2b,c). A further attempt was made to isolate the PSD from excitatory synapses in striatal tissue. Abundant NR2B and NR1 proteins were displayed in the isolated PSD (Fig. 2d)21. The purity of PSD was confirmed by the lack of PKCε, a PKC isozyme exclusively present in the presynaptic compartment (Fig. 2d). AMPH reduced NR2B, but not NR1 or NR2A, proteins in the PSD (Fig. 2e). These results demonstrate selective NR2B downregulation in a specific subcellular compartment (synaptosomal membranes).

To substantiate and confirm this compartment-specific effect, we employed two other biochemical techniques to isolate native receptors from surface and intracellular pools in intact neurons22,23. We first treated striatal tissue with the membrane-impermeable bis(sulfosuccinimidyl)suberate (BS^3). This reagent selectively cross-links surface membrane-bound receptors to form high molecular weight aggregates, which can be readily separated from unlinked intracellular receptors by gel electrophoresis. In the second

procedure, we treated striatal tissue with the protease chymotrypsin to cleave extracellular N-terminal domains of surface receptors. This generates receptor fragments with a molecular weight lower than intact intracellular receptors. As shown in Fig. 3a, in BS^3-treated tissue, NR1 and NR2B displayed a high molecular weight band (surface subunits) and a monomeric molecular weight band (intracellular subunits) as compared to a single monomeric molecular weight band (surface + intracellular subunits) in noncross-linked tissue. The proteolysis with chymotrypsin revealed a smaller molecular weight band (surface) and a monomeric molecular weight band (intracellular) as detected with an antibody against the intracellular C-terminus. The selectivity of BS^3 and chymotrypsin was confirmed by the lack of their effects on α-actinin, an intracellular protein (Fig. 3a). Moreover, treatment of striatal homogenates with either BS^3 or chymotrypsin completely eliminated the monomeric bands of NR1 and NR2B (data not shown). Both assays yielded nearly identical estimates of the percentage of NR1 and NR2B in the surface pool (Fig. 3b) and the intracellular pool (approximately 20–40%).

We next examined effects of AMPH on NR2B in the surface (S) and intracellular (I) pools. At a 14-day withdrawal time, a robust decrease in NR2B in the surface pool of AMPH-treated rats was observed in BS^3 cross-linking experiments (Fig. 3c). This was accompanied by a slight increase in the intracellular pool of NR2B (although $p>0.05$). Due mostly to the decrease in surface NR2B, the total protein level (S+I; Fig. 3c) and the S:I ratio of NR2B (0.56 ± 0.1-fold saline, $p< 0.05$) were reduced. Similar results were observed in NR2B assays with chymotrypsin in the intracellular pool (Fig. 3d). No changes were seen in NR1 or α-actinin (Fig. 3c,d). These results demonstrate a selective decrease in NR2B in the confined surface pool.

AMPH increases ubiquitination of synaptic proteins

The regulated degradation of proteins by the ubiquitin proteasome pathway is emerging as an important modulator of synaptic plasticity[24,25]. We therefore investigated whether chronic AMPH regulates ubiquitin conjugation of striatal synaptosomal proteins after a 14-day withdrawal. Ubiquitin conjugates were abundant in saline-treated rats, and increased in AMPH- treated rats ($126.1 \pm 0.7\%$ of saline, $p < 0.05$; Fig. 4a). To directly examine *de novo* ubiquitin conjugation, we conducted ubiquitin conjugation assays. Strong ATP-dependent ubiquitin conjugation was revealed in both cytosol and synaptosomes of striatal neurons from normal rats (Fig. 4b), indicating the existence of all necessary elements for an effective ubiquitin incorporation machinery in these biochemically enriched subcellular fractions. Also, AMPH enhanced ubiquitin conjugation in synaptosomes and the PSD, but not the cytosol (Figs. 4c and S3). These results provide direct evidence for the ability of AMPH to regulate ubiquitin conjugation at synaptic sites and to enhance susceptibility of postsynaptic proteins for the proteasome-mediated degradation.

We next set to identify specific substrates for the ubiquitin conjugation at excitatory synapses. We first purified polyubiquitinated proteins from striatal synaptosomes in pull-down assays with a glutathione-S-transferase (GST) fusion protein, i.e., high-affinity polyubiquitin binding proteasome subunit S5a (GST-S5a). Abundant ubiquitinated proteins were readily precipitated with GST-S5a, but not GST alone (Fig. 5a). The de-ubiquitinating

enzymes, isopeptidase-T and UCH-L3, abolished the precipitation of ubiquitinated proteins (Fig. 5a), confirming a ubiquitin-mediated purification. We then identified specific polyubiquitination substrates in GST-S5a precipitates using a panel of antibodies in subsequent immunoblots. Three key multivalent postsynaptic scaffolds, Shank/ProSAP, guanylate kinase-associated protein (GKAP), and AKAP, were present at high molecular weight ubiquitinated species (Fig. 5b). In contrast, other PSD proteins surveyed, including PSD-95, NR1, and NR2B, were not detected. Thus, similar to cultured cortical neurons24, polyubiquitination normally occurs in a selective subset of scaffold proteins in striatal neurons *in vivo*.

To directly test whether AMPH affects the ubiquitination of Shank, GKAP, and/or AKAP, GST-S5a pull-down assays were performed with striatal synaptosomes from saline- or AMPH-treated rats at day 14 of withdrawal. A significant increase in the ubiquitination of Shank and GKAP, but not AKAP, was observed in AMPH-treated rats (Fig. 5c,d), identifying Shank and GKAP as two substrates sensitive to AMPH. It is considered that the ubiquitination and degradation of these two scaffold proteins may affect the stability of proteins that require Shank and/or GKAP to anchor them to the PSD. Shank and GKAP are known to anchor PSD-95 and thus PSD-95-linked NR2B. Both PSD-95 and NR2B showed parallel reduction in their abundance after AMPH. Thus, PSD-95 and NR2B, even though they are not direct ubiquitin targets, could be affected in their stability in the PSD by the ubiquitination of their crucial anchoring proteins. Indeed, this concept was supported by further pharmacological studies. Treatment of striatal slices with either of two structurally distinct cell-permeable proteasome inhibitors, MG132 (5 μM, 3 h) or lactacystin (2 μM, 3 h), markedly rescued the reduction of synaptosomal Shank and GKAP in AMPH-treated rats (Figs. 5e and S4). This rescue was mediated through the selective suppression of proteasome-mediated degradation since inhibition of the lysosome with leupeptin (50 μg/ml, 3 h) had no effect (data not shown). Remarkably, the reduction of NR2B and PSD-95 was reversed simultaneously by MG132 and lactacystin in AMPH-treated rats (Figs. 5e and S4). Thus, Shank and GKAP are master regulators that stabilize PSD-95 and NR2B in the PSD. Their accelerated removal destabilizes PSD-95 and NR2B, leading to the concurrent loss of these protein cohorts.

Destabilization of NR2B affects synaptic plasticity

Whole-cell voltage-clamp recordings of synaptically evoked NMDAR-mediated excitatory postsynaptic currents (EPSCs) were conducted in NAc slices to explore whether the biochemical destabilization of NR2B can translate to the modulation of NR2B-NMDARs. The relative contribution of NR2B to EPSCs was determined by measuring the sensitivity to ifenprodil, an NR2B-selective inhibitor. Ifenprodil-sensitive EPSCs were substantially reduced in AMPH- treated rats at a 14-day withdrawal period (30.6 ± 6.4% of saline, $p < 0.05$; Fig. 6a). Consistent with this, the decay constant for NMDA EPSCs was shorter (58.7 ± 3.5 ms for saline; 39.3 ± 3.3 ms for AMPH, $p < 0.05$). Thus, the loss of surface NR2B impairs ifenprodil-sensitive NR2B-NMDAR-mediated currents.

Two forms of synaptic plasticity at cortico-accumbal glutamatergic synapses, paired-pulse facilitation (PPF) and long-term depression (LTD), were examined in NAc slices to

determine the modulation of synaptic plasticity. PPF is a sensitive measure of the probability of transmitter release and a common form of short-term presynaptic plasticity. AMPH had no effect on PPF after 14 days of withdrawal (Fig. 6b), suggesting the lack of gross change in presynaptic function. We then assessed an NMDAR-dependent form of synaptic plasticity (LTD). We found that LTD was reduced in AMPH-treated rats compared to saline-treated rats (Fig. 6c,d). To explore if the loss of NR2B contributes to the reduction of LTD, we treated NAc slices from AMPH-treated rats with the proteasome inhibitor MG132, previously demonstrated to reverse the loss of NR2B (Fig. 5e). We then compared the LTD recorded in these slices with the LTD recorded in slices treated with vehicle control (DMSO). We found that MG132 (5 μM, 3 h), but not DMSO, markedly restored LTD (Fig. 6e,f). MG132 did not alter normal LTD in saline-treated rats (Fig. S5). Thus, the NR2B loss appears to contribute to the impairment of cortico-accumbal LTD in response to AMPH.

Role of NR2B in regulating behavioral sensitivity to AMPH

Both pharmacological and genetic approaches were utilized to determine if the biochemical and electrophysiological remodeling of NR2B-NMDARs leads to the regulation of behavioral sensitivity to acute and chronic AMPH. The systemically active NR2B antagonist RO25–6981 (IC_{50} of 9 nM for NR1/NR2B and 52 μM for NR1/NR2A)26 did not alter spontaneous motor activity (Fig. 7a), whereas at 0.4 mg/kg, it significantly augmented the locomotor response to acute injection of either AMPH (1.25 mg/kg, i.p.; Fig. 7b) or a D1 receptor agonist SKF81297 (0.5 mg/kg, i.p.; Fig. S6a) in naive rats. Another NR2B antagonist ifenprodil (1–2 mg/kg) also augmented motor responses to AMPH (Fig. S6b). To determine whether the effect of systemic RO25–6981 occurs locally in the striatum, a microinjection study was performed. Injections of this agent into the NAc produced the same effect as that seen after systemic injections (Fig. 7c). These data indicate that NR2B-NMDARs inhibit behavioral sensitivity to acute AMPH.

We next explored the role of NR2B in behavioral sensitization. At 14 days, challenge injections of saline + AMPH (1.25 or 2 mg/kg) induced an augmented behavioral response in AMPH-pretreated rats compared to saline-pretreated rats (Figs. 7f and S6c). In contrast, challenge treatments with RO25–6981 (0.4 mg/kg) + AMPH (1.25 or 2 mg/kg) induced nearly identical locomotor activities in saline- and AMPH-treated rats (Figs. 7g and S6c). Similar results were seen in the experiments with ifenprodil (Fig. S6d). These data demonstrate that antagonism of NR2B induces sensitization-like behavioral responses to challenge injections of AMPH in saline-pretreated rats, which is comparable to that seen in AMPH-pretreated rats. It is therefore possible that the downregulation of NR2B after chronic AMPH constitutes a critical molecular step leading to behavioral sensitization.

The role of NR2B was then evaluated using a region-specific genetic approach with small interfering RNAs (siRNAs) *in vivo*. We targeted the NAc for this study because the NAc is a central site critical for the expression of behavioral sensitization1 and is also a key limbic structure for molecular studies linking glutamate receptors and scaffolds to sensitized behavior5,16,27. Intra-accumbal infusions of NR2B siRNAs at a higher dose (5 but not 0.5 μg/side, once daily for 3 days; 1 day after final injection) reduced NR2B in total proteins (Fig. 8a,b) and in the LP1 (data not shown) in the injected site. The reduction was only

partial or insignificant 3 or 5 days after the final siRNA treatment, respectively. No effect of NR2B siRNAs was seen on total NR2A and NR1 proteins. Additionally, in NR2B siRNA- and control siRNA-treated rats, there was no difference in the number of apoptotic cells as detected by fragment-end labeling and the number of pyknotic cells in striatal slices. These data demonstrate the selective, reversible, and non-toxic knockdown of NR2B.

We next examined if NR2B siRNAs affect behavioral responses to acute AMPH in naive rats. Intra-accumbal injection of NR2B siRNAs dose-dependently augmented horizontal locomotor responses to AMPH (Fig. 8c). However, injection of NR2B siRNAs into the dorsolateral striatum had little effect on locomotor activity induced by AMPH, even though the siRNA still reduced NR2B in the injected site (Fig. S7a–c).

We then assessed the effect of NR2B siRNAs in our chronic model (Fig. 8d). At day 14 (1 day after final siRNA injection), a challenge AMPH injection (1.25 mg/kg) induced the typical behavioral sensitization in rats pretreated with AMPH followed by intra-accumbal injections of control siRNAs. However, in rats treated with NR2B siRNAs, AMPH produced no further behavioral sensitization in AMPH-pretreated rats compared to saline-pretreated rats. At day 20 (7 days after the final siRNA injection), a time when the knockdown of NR2B by siRNAs recovered to normal levels, AMPH resumed its ability to elicit greater behavioral responses in AMPH-pretreated rats than that in saline-pretreated rats. These results complement those from aforementioned pharmacological experiments with RO25–6981 and support the requirement of downregulated NR2B for the full expression of motor sensitization.

Further efforts were made to evaluate the importance of NR2B. At 14 days after 7 daily AMPH, intra-accumbal injection of the proteasome inhibitor MG132 (0.2 μg) reversed the loss of NR2B in the injected NAc, and reduced the sensitized motor response to a challenge dose of AMPH, whereas DMSO had no effect (Fig. 8e). Intra-accumbal injection of lactacystin (0.2 μg) produced similar results (Fig. 8f). Neither MG132 nor lactacystin affected behavioral responses to acute AMPH injection (Fig. S8). In another approach, bilateral intra-accumbal injections of lactacystin (0.2 μg) were given 5 min prior to each daily saline or AMPH injection (Fig. S9a). At 7 days of withdrawal, lactacystin reduced behavioral sensitization to a challenge dose of AMPH (Fig. S9b) and the accumbal loss of NR2B (Fig. S9c). These data provide further evidence for the model that the loss of NR2B is required for behavioral sensitization to AMPH.

Discussion

Accumbal NR1 proteins remain stable after chronic cocaine exposure in most early studies28–30. Unlike NR1, NR2B proteins were reduced after chronic AMPH administration (this study). This reduction seems to be a general adaptation of the entire striatum (both the NAc and CPu), as seen in the case of the NR2B-anchoring protein PSD-9516. However, in the light of well-documented differences between the ventral and dorsal striatum31, the loss of NR2B in the two classical divisions (NAc and CPu) or in the different NAc regions (shell and core) may play distinct roles in synaptic and behavioral plasticity. At the cellular level, NR2B was reduced in a greater number of D2 receptor-

bearing striatopallidal output neurons. This seems to imply a greater impact of downregulated NR2B-NMDARs on the D2-dependent striatopallidal indirect pathway relative to the D1-dependent striatonigral direct pathway in the basal ganglia. Future studies need to clarify the region- and cell-type-specificity of NR2B adaptations to AMPH in terms of expression and function of the subunit.

Our results show a selective loss of NR2B in the surface membrane at synaptic sites. In contrast to the surface section, NR2B in the intracellular pool involving synthesis, assembly, and secretion of proteins was insensitive to AMPH. Of note, striatal NR2B mRNAs were not altered after repeated cocaine administration32. Thus, it is less likely that an overall inhibition of new NR2B protein synthesis could account for the NR2B reduction. Suppressed trafficking or delivery of NR2B from the intracellular pool to the surface membrane is also less likely. This is because impaired NR2B externalization of this kind should cause a redistribution phenomenon that is characterized by the subtraction of NR2B in the surface pool in combination with the proportional addition of NR2B in the intracellular pool, while total NR2B proteins remain unchanged12,23. The fact that the NR2B downregulation selectively occurred in the surface pool implies a scenario, in which the removal of existing NR2B from the surface pool is accelerated, followed by enhanced degradation of the protein12,24,33. The evidence from this study, indeed, supports this scenario (see below).

Increasing evidence supports that ubiquitination and degradation of synaptic proteins serve as an underlying mechanism for the enduring modification of protein expression and function at excitatory synapses25,34. To date, a subset of synaptic scaffolds, including Shank, GKAP, and AKAP, were found to be polyubiquitinated in hippocampal and cortical neurons24,35. The data regarding the PSD-95 and NR1 ubiquitination were less consistent24,35–37. In this study, we screened a number of synaptic proteins for ubiquitination in striatal neurons *in vivo*. We identified that Shank, GKAP, and AKAP, but not NR1, NR2B, or PSD-95, were among direct targets of ubiquitin. More importantly, two out of these three scaffold proteins (Shank and GKAP) were upregulated in ubiquitination by AMPH. AKAP is a scaffold linking PSD-95 to various kinases, whereas GKAP, a much more abundant multi-domain scaffold, links PSD-95 to Shank, which further anchors to the predominant filamentous actin (F-actin) cytoskeleton via actin-interacting proteins38. Thus, the degradation of the two master PSD-95-anchoring proteins (Shank and GKAP) can impair the stability of PSD-95. Since PSD-95 is well established to inhibit NR2B internalization and stabilize surface NR2B at synaptic sites39,40, impaired PSD-95 may cause destabilization of NR2B, despite that neither PSD-95 nor NR2B are direct substrates of ubiquitination. This model is supported by our pharmacological studies in which the proteasome inhibitors, which blocked the Shank and GKAP degradation, reversed the loss of PSD-95 and NR2B abundance in AMPH-treated rats.

The findings from this study may reveal a metaplastic basis for the AMPH-associated learning and memory3,41. Striatal neurons are among those that readily express LTD27,42, a cellular model of synaptic plasticity for learning and memory. This homosynaptic plasticity is NMDAR-dependent42 and is subject to the modification by addictive drugs. For instance, a challenge injection of cocaine reduced LTD in the NAc in chronic cocaine-

treated mice5. Enduring inhibition of LTD occurred in the NAc of rats trained to self-administer cocaine (contingent administration), although not in yoked rats treated with repeated noncontingent administration (experimenter administered) of cocaine43. Blunted LTD in the striatum was also seen after chronic exposure to other psychoactive drugs (methamphetamine, cannabinoid, and ethanol)44–46. These data support an inhibitory modulation of LTD by these drugs, which is consistent with our findings in rats exposed to AMPH. This inhibited LTD, together with the reduced basal intrinsic excitability of postsynaptic NAc neurons and the augmented long-term potentiation (LTP) in the NAc16, could be among the cardinal features of abnormal synaptic plasticity derived from chronic stimulant exposure41. These plastic changes likely occur in a subset of excitatory synapses on spiny neurons and are critical for the expression of behavioral plasticity5,16. The loss of surface NR2B at synaptic sites seems to confer an attractive metaplastic mechanism for these changes. It is possible, although it requires further experimental clarification, that the subtraction of surface NR2B contributes to the development of a relatively depressed state of spiny neurons, which permits lesser induction of LTD and greater induction of LTP in reward circuits41.

The AMPA receptor is another target of stimulants. Increased AMPA receptor transmission in the NAc promoted cocaine-seeking47 and psychomotor sensitization23,29, while other studies also showed no or inverse link between AMPA receptor expression levels and these behaviors48–50. In either case, weakened NR2B-NMDARs with or without relatively strengthened NR2A-NMDARs can contribute to modulating AMPA receptors at defined subsets of synapses. As such, the two receptors act in concert to determine behavioral sensitivity to stimulants. Future studies need to elucidate how, when, and where they interact to control synaptic and behavioral plasticity.

Methods

Animals

Adult male Wistar rats weighting 175–200 g (Charles River, New York, NY) were individually housed in a controlled environment at a constant temperature of 23°C and humidity of 50 ± 10% with food and water available *ad libitum*. The animal room was on a 12/12 h light/dark cycle. Rats were allowed 6–7 days of habituation to the animal colony. All animal use procedures were in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee.

Surface receptor cross-linking assays

Surface expression of NR2B was assayed using a membrane-impermeable cross-linking reagent BS^3, which only cross-links proteins on the surface of live cells23. Brains were removed following anesthesia and sliced (400 μm). The striatum was dissected and added to eppendorf tubes containing ice-cold artificial cerebrospinal fluid (ACSF). BS^3 (Pierce, Rockford, IL) was added to 2 mM and incubated with gentle agitation for 30 min at 4°C. The cross-linking reaction was terminated by quenching with 20 mM of glycine (10 min, 4°C). The tissue was then washed four times in cold ACSF (10 min each), homogenized to

obtain total protein homogenate, and analyzed directly by SDS-PAGE (4–12% Tris-glycine gels, Invitrogen).

Chymotrypsin assays

The striatal slices were added to eppendorf tubes containing ice-cold ACSF. Chymotrypsin (Worthington Biochemical Corporation, Lakewood, New Jersey) was added to 1 mg/ml and incubated at 32°C for 45 min. Proteolysis was stopped by three 1-min washes with cold ACSF containing 1 mM of an irreversible chymotrypsin inhibitor 4-(2-aminoethyl)benzenesulphonyl fluoride (AEBSF). The tissue was then homogenized to obtain total protein homogenate, and analyzed directly by SDS-PAGE (4–12% Tris-glycine gels, Invitrogen).

AMPH treatments and behavioral assessments

In all experiments involving AMPH sensitization, animals were treated with a single daily i.p. injection of AMPH (4 mg/kg) for 7 consecutive days in home cages. Age-matched animals received 7 daily i.p. injection of saline (1 ml/kg) and served as controls. Before the 7-day treatment regimen, all animals were routinely injected with saline (once daily for two days) to accommodate them to injection procedures. Behavioral responses to a challenging dose of AMPH (1.25 mg/kg, i.p.) were analyzed 14 or indicated days after the last injection. Locomotion in an open field in a sound-attenuated room was evaluated with an infrared photocell-based, automated Opto-Varimex-Micro apparatus (Columbus Instruments, Columbus, OH) under illuminated conditions21. Rats were placed in an activity chamber and were habituated to the chamber environment for 2 h before each experiment. Three sensor pairs positioned in X, Y (horizontal) and Z (vertical) directions were assigned to each cage and status about infrared beam interruptions by presence of animals was transferred from all sensors to a computer with operating software. Information about horizontal and vertical activities was recorded at 5 min intervals before and after a dose of saline or AMPH.

Statistics

The data were evaluated using a one- or two-way ANOVA, as appropriate, followed by a Bonferroni (Dunn) comparison of groups using least squares-adjusted means. Probability levels of < 0.05 were considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

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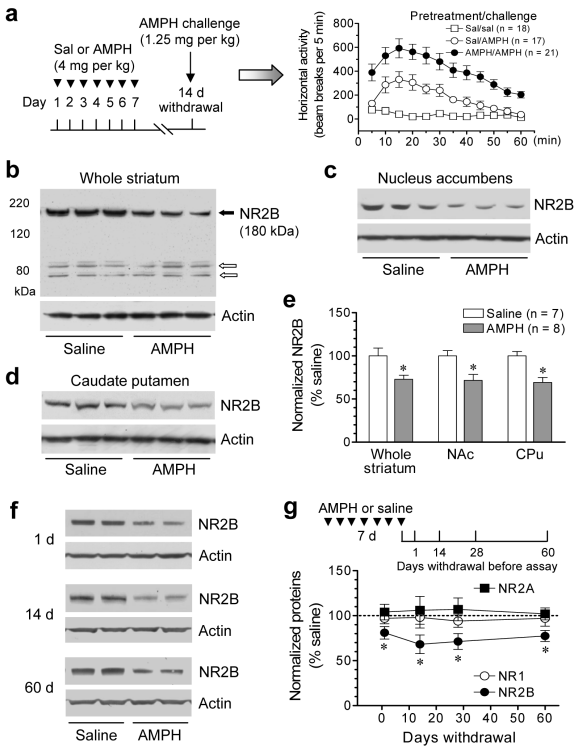

Figure 1. Repeated AMPH administration causes behavioral sensitization and reduces NR2B protein expression in the rat striatum

(a) A behavioral sensitization model in response to repeated AMPH administration (4 mg/kg, i.p., once daily for 7 days). Locomotor responses to a challenge dose of AMPH (1.25 mg/kg, i.p.) were tested 14 days after the discontinuation of drug injections. **(b—d)** Effects of repeated AMPH administration on total NR2B protein levels in the whole striatum (**b**), nucleus accumbens (**c**), and caudate putamen (**d**) at a 14-day withdrawal period. **(e)** Densitometric quantification of Western blots in **b—d**. **(f and g)** Long-lasting reduction of striatal NR2B, but not NR1 and NR2A, proteins detected 1, 14, 28, or 60 days after repeated AMPH administration (*n* = 9, 7, 8, and 8 for saline group, *n* = 11, 8, 8, and 10 for AMPH group at respective 4 time points). The solid arrows indicate NR2B bands whereas the open arrows indicate weak cross-reactive bands detected by the anti-NR2B antibody, an internal reference for equal protein loadings. Data are expressed as means ± SEM. *p < 0.05 versus saline.

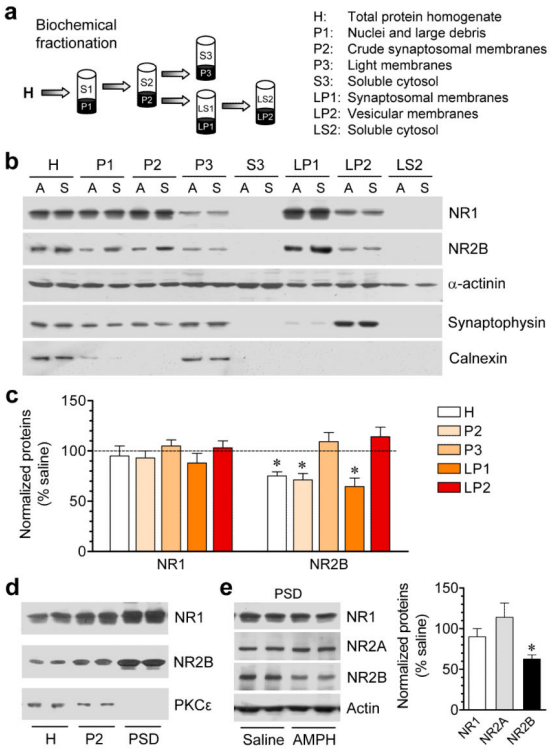

Figure 2. Repeated AMPH administration reduces NR2B proteins in specific subcellular compartments

(a) Schematic illustration of biochemical fractionation procedures. **(b and c)** Immunoblots (**b**) and the quantified data (**c**) illustrating effects of AMPH (A) and saline (S) on NR1, NR2B, α-actinin, synaptophysin, and calnexin protein levels in different subcellular fractions. Note that NR2B levels were reduced in the H, P1, P2 and LP1 fractions in AMPH-treated rats. **(d)** NR1, NR2B, and PKCε proteins in total protein homogenates (H), P2 pellets, and PSD fractions. Proteins were loaded at 10 μg/well for the H and P2, and 1.25 μg/well for the PSD. **(e)** AMPH reduced NR2B levels in the PSD. Rats were treated with saline or AMPH (4 mg/kg, i.p.) for 7 days, and the biochemical fractionation or PSD isolation of striatal tissue was performed 14 days after the last injection. Data are expressed as means ± SEM. *$p < 0.05$ versus saline.

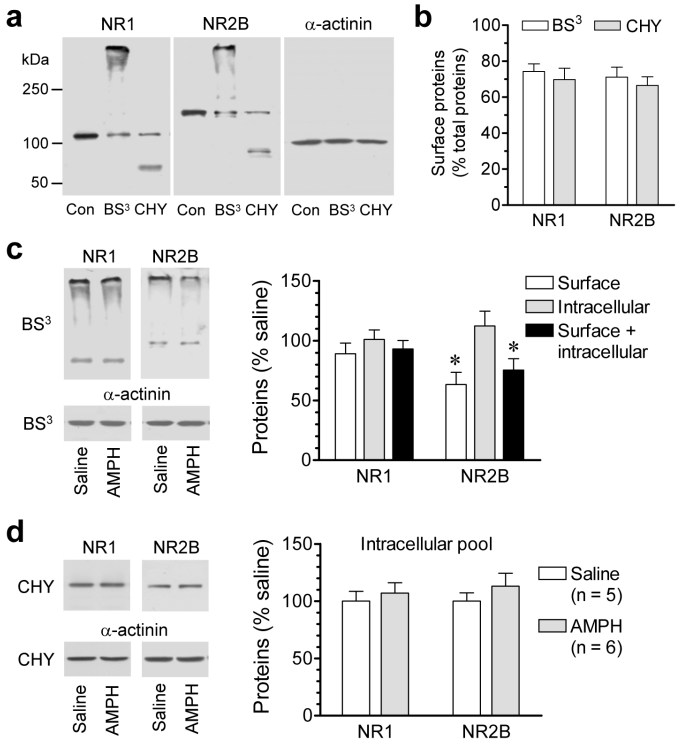

Figure 3. Repeated AMPH administration reduces NR2B proteins in the specific surface membrane pool

(a) Representative immunoblots from striatal tissue treated with BS^3 or chymotrypsin (CHY) or from control tissue (Con) without BS^3 or CHY treatment. **(b)** Quantification of basal surface expression of NR1 and NR2B from BS^3 cross-linking and protease experiments ($n = 4$). **(c)** AMPH reduced NR2B in the surface pool, but not in the intracellular pool, as revealed in BS^3 cross-linking experiments ($n = 8$ for saline group, $n = 11$ for AMPH group). **(d)** Effects of AMPH on the intracellular pool of NR2B as revealed in experiments with chymotrypsin. Rats were treated with saline or AMPH (4 mg/kg, i.p.) for 7 days, and cross-linking and protease assays with striatal tissue were performed 14 days after the last injection (**c** and **d**). Representative immunoblots are shown left to the quantified data (**c** and **d**). Data are expressed as means ± SEM. *$p < 0.05$ versus saline.

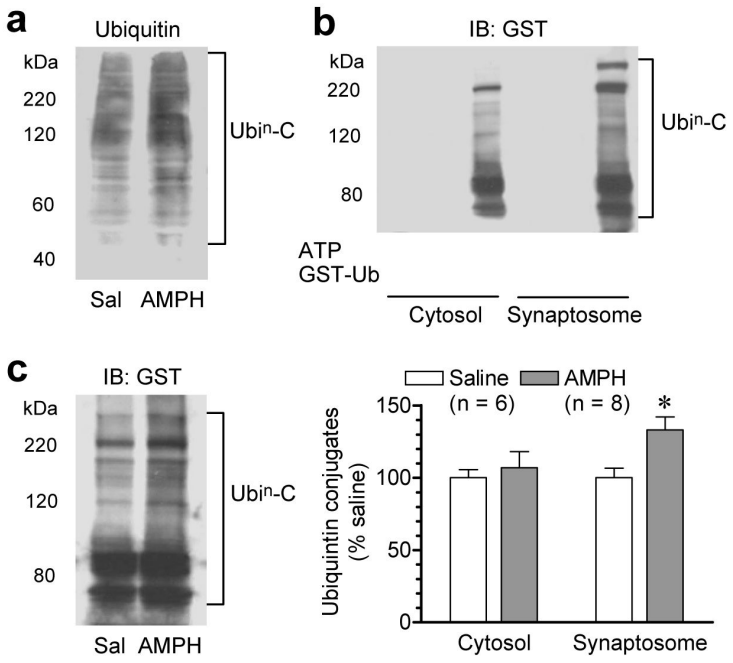

Figure 4. Repeated AMPH administration increases ubiquitin conjugation in striatal neurons

(a) AMPH increased the level of ubiquitin conjugates (Ubi^{n}-C) in synaptosomal fractions. **(b)** *De novo* ubiquitin conjugation of cytosolic and synaptosomal proteins. *In vitro* ubiquitin conjugation assays were conducted in the presence or absence of ATP and GST-ubiquitin (GST-Ub), followed by immunoblot (IB) analysis with an anti-GST antibody. **(c)** AMPH increased ubiquitin conjugation in synaptosomal, but not cytosolic, proteins. Representative immunoblots are shown left to the quantified data. Rats were treated with saline or AMPH (4 mg/kg, i.p.) for 7 days, and the biochemical assays with striatal tissue were performed 14 days after the last injection. Data are expressed as means ± SEM. *$p < 0.05$ versus saline.

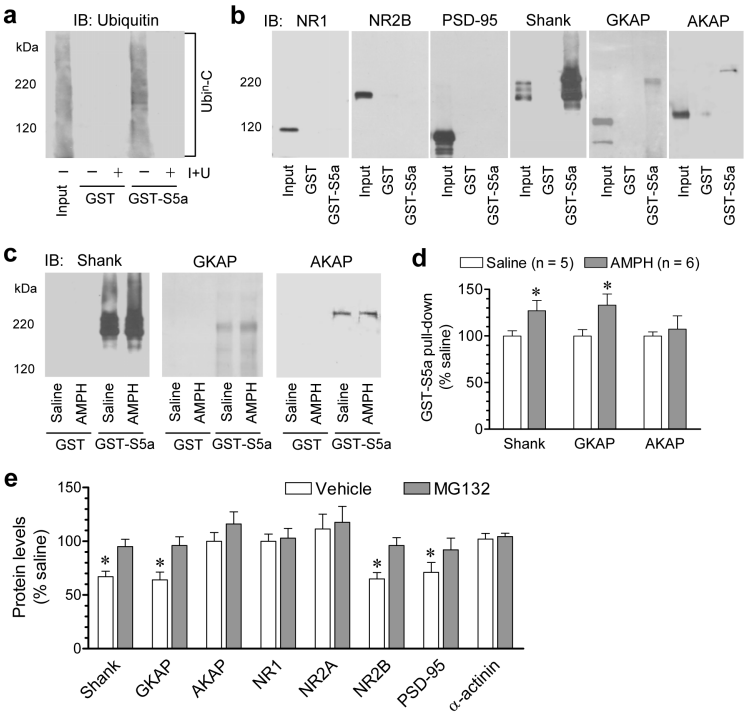

Figure 5. Repeated AMPH administration remodels excitatory synapses via the ubiquitin-proteasome system

(a) Polyubiquitinated conjugates (Ubi^n-C) in GST-S5a precipitates detected by immunoblot (IB) analysis with an anti-ubiquitin antibody. Denatured striatal synaptosomal proteins were used in pull-down assays in the presence or absence of the deubiquitinating enzymes isopeptidase-T (I) and UCH-L3 (U). **(b)** Identification of polyubiquitinated proteins in GST-S5a precipitates by immunoblots with specific antibodies. Note that Shank, GKAP, and AKAP were displayed as higher molecular mass species in the GST-S5a precipitates. **(c and d)** Polyubiquitination of Shank, GKAP, and AKAP in saline- or AMPH-treated rats. **(e)** Incubation of MG132 (5 μM, 3 h) reversed the reduction of NR2B and scaffolds in synaptosomes from AMPH-treated rats (n = 6 for saline/vehicle group, n = 7 for AMPH/vehicle group, n = 8 for AMPH/MG132 group). Rats were treated with saline or AMPH (4 mg/kg, i.p.) for 7 days, and the assays on striatal synaptosomal fractions (**c**) or slices (**e**) were performed 14 days after the last injection. Data are expressed as means ± SEM. *p < 0.05 versus saline.

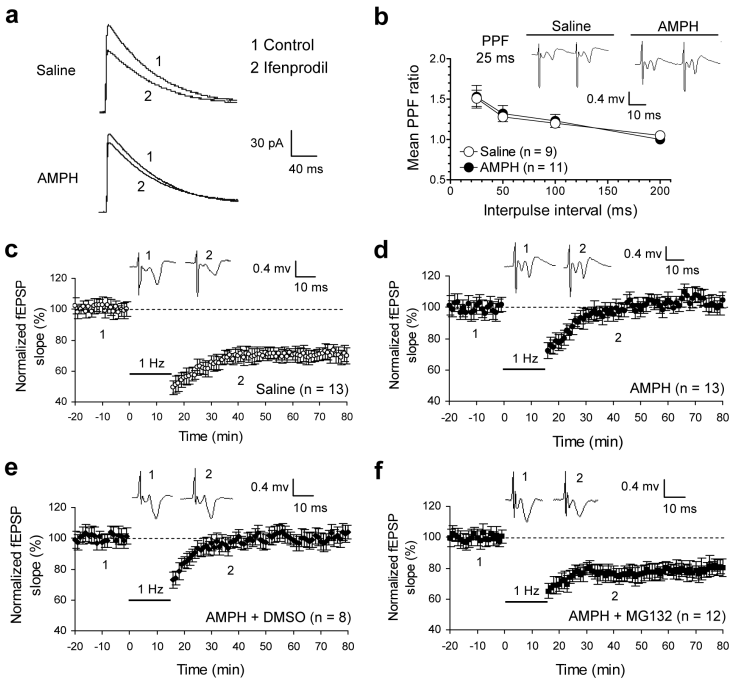

Figure 6. Repeated AMPH administration affects NMDAR-mediated ifenprodil-sensitive EPSCs and synaptic plasticity at cortico-accumbal glutamatergic synapses

(a) Representative traces illustrating effects of ifenprodil (10 μM) on NMDAR-mediated EPSCs measured at +40 mV/10 μM NBQX in chronic saline- or AMPH-treated rats (*n* = 7–8 cells, 7 animals per group). **(b)** PPF recorded in the NAc of chronic saline- and AMPH-treated rats. PPF was measured with pairs of presynaptic stimulation pulses at intervals of 20, 50, 100, and 200 ms. **(c)** LTD recorded in the NAc of chronic saline-treated rats. **(d)** LTD recorded in the NAc of chronic AMPH-treated rats. **(e and f)** LTD recorded in the NAc of chronic AMPH-treated rats in the presence of DMSO (**e**) and MG132 (**f**). NAc slices were incubated with DMSO or MG132 (5 μM) for 3 h prior to the recording. Rats were treated with saline or AMPH (4 mg/kg, i.p.) for 7 days. EPSCs, PPF, or LTD were recorded in NAc slices 14 days following the last injection. Data are expressed as means ± SEM.

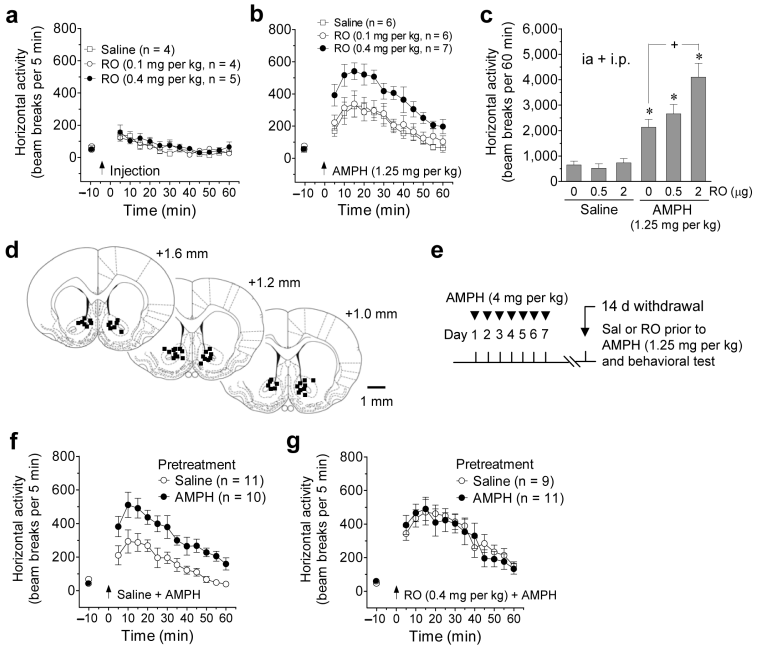

Figure 7. Effects of RO25–6981 (RO) on behavioral responses to acute or chronic AMPH administration

(a) A systemic injection of RO25–6981 (0.1 or 0.4 mg/kg, i.p.) did not alter spontaneous motor activity. **(b)** Co-administration of RO25–6981 (0.1 or 0.4 mg/kg, i.p., 5 min prior to AMPH at 1.25 mg/kg, i.p.) dose-dependently augmented AMPH-stimulated horizontal activity. **(c)** Intra-accumbal (ia) injection of RO25–6981 augmented AMPH-stimulated horizontal activity (*n* = 5–7 per group). RO25–6981 (0.5 or 2 μg/0.4 μl/side) was injected 5 min prior to a systemic injection of AMPH (1.25 mg/kg, i.p.). **(d)** Locations of microinjection sites within the NAc from **c**. **(e)** An inset outlining a sensitization treatment regimen and pharmacological analysis protocol. **(f and g)** Behavioral responses to challenge injections of saline + AMPH (**f**) or RO25–6981 + AMPH (**g**). The standard 7-day sensitization regimen was given followed by challenge treatments at a 14-day withdrawal time. Saline or RO25–6981 (0.4 mg/kg, i.p.) was given 5 min prior to AMPH (1.25 mg/kg, i.p.). Data are expressed as means ± SEM. *p < 0.05 versus saline. +p < 0.05 versus AMPH alone.

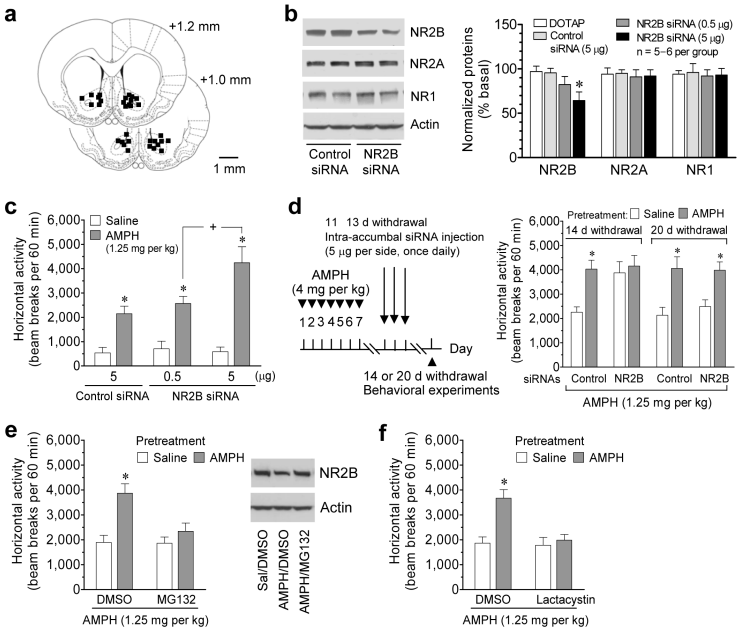

Figure 8. Effects of NR2B siRNAs on behavioral responses to AMPH

(a) Locations of bilateral microinjection sites within the NAc. **(b)** Intra-accumbal injection of NR2B siRNAs reduced total levels of NR2B proteins in the injected side. **(c)** Intra-accumbal injection of NR2B siRNAs augmented behavioral responses to acute AMPH injection in naive rats (*n* = 4, 5, 4, 6, 5, and 6 from left to right). **(d)** Effects of intra-accumbal injection of NR2B siRNAs on behavioral responses to a challenge AMPH injection in saline- and AMPH-treated rats (*n* = 8, 9, 14, 11, 8, 9, 12, and 10 from left to right). **(e and f)** MG132 and lactacystin reduced the behavioral sensitization (*n* = 8–12 per group). Bilateral intra-accumbal injections of DMSO, MG132 (0.2 μg/1 μl), or lactacystin (0.2 μg/1 μl) were made 3 h prior to a challenge saline or AMPH injection at 14 days of withdrawal. NR2B levels were tested in the injected sites (inset) 3 h after intra-accumbal injections of DMSO or MG132 in rats pretreated with chronic saline or AMPH at a 14-day withdrawal period. In naive rats, siRNAs were injected bilaterally into the NAc at 0.5 or 5 μg/0.5 μl (once daily for 3 days), and protein assays (**b**) or behavioral assessments (**c**) were conducted 1 day after the final siRNA injection. In chronic experiments, behavioral experiments (**d**) were conducted 1 (14 days withdrawal) or 7 (20 days withdrawal) days after the final siRNA injection. Data are expressed as means ± SEM. *p < 0.05 versus control siRNAs (**b**), acute saline (**c**), or chronic saline (**d-f**). +p < 0.05 versus 0.5 μg of NR2B siRNAs + AMPH (**c**).