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Rapid Synthesis and Synaptic Insertion of GluR2 for mGluR-LTD in the Ventral Tegmental Area

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The activation of metabotropic glutamate receptors (mGluRs) leads to long-term depression (mGluR-LTD) at many synapses of the brain. The induction of mGluR-LTD is well characterized, whereas the mechanisms underlying its expression remain largely elusive. mGluR-LTD in the ventral tegmental area (VTA) efficiently reverses cocaine-induced strengthening of excitatory inputs onto dopamine neurons. We show that mGluR-LTD is expressed by an exchange of GluR2-lacking AMPA receptors for GluR2-containing receptors with a lower single-channel conductance. The synaptic insertion of GluR2 depends on de novo protein synthesis via rapid messenger RNA translation of GluR2. Regulated synthesis of GluR2 in the VTA is therefore required to reverse cocaine-induced synaptic plasticity.

In the VTA, synaptic induction of mGluR-LTD requires burst firing [e.g., several repetitions of five stimuli at 66 Hz (*1*)]. mGluR-LTD can also be chemically induced by bath application of the selective mGluR group I agonist 3,4-dihydroxyphenylglycol (DHPG), which occludes the mGluR-LTD induced by synaptic activity (*2*). mGluR-LTD may therefore be mediated by mGluR1 or mGluR5, which in other parts of the brain induce LTD through heterotrimeric GTP-binding proteins (G proteins) of the pertussis toxin–insensitive G_q family (*3*).

We examined the expression of mGluR-LTD at excitatory synapses onto dopamine (DA) neurons of the VTA by monitoring excitatory postsynaptic currents (EPSCs) mediated by α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors (AMPAR) (fig. S1) and evoked by extracellular stimulation (*4*). Cocaine and other addictive drugs fundamentally change the efficacy (*5*–*7*) and the quality (*1*) of transmission

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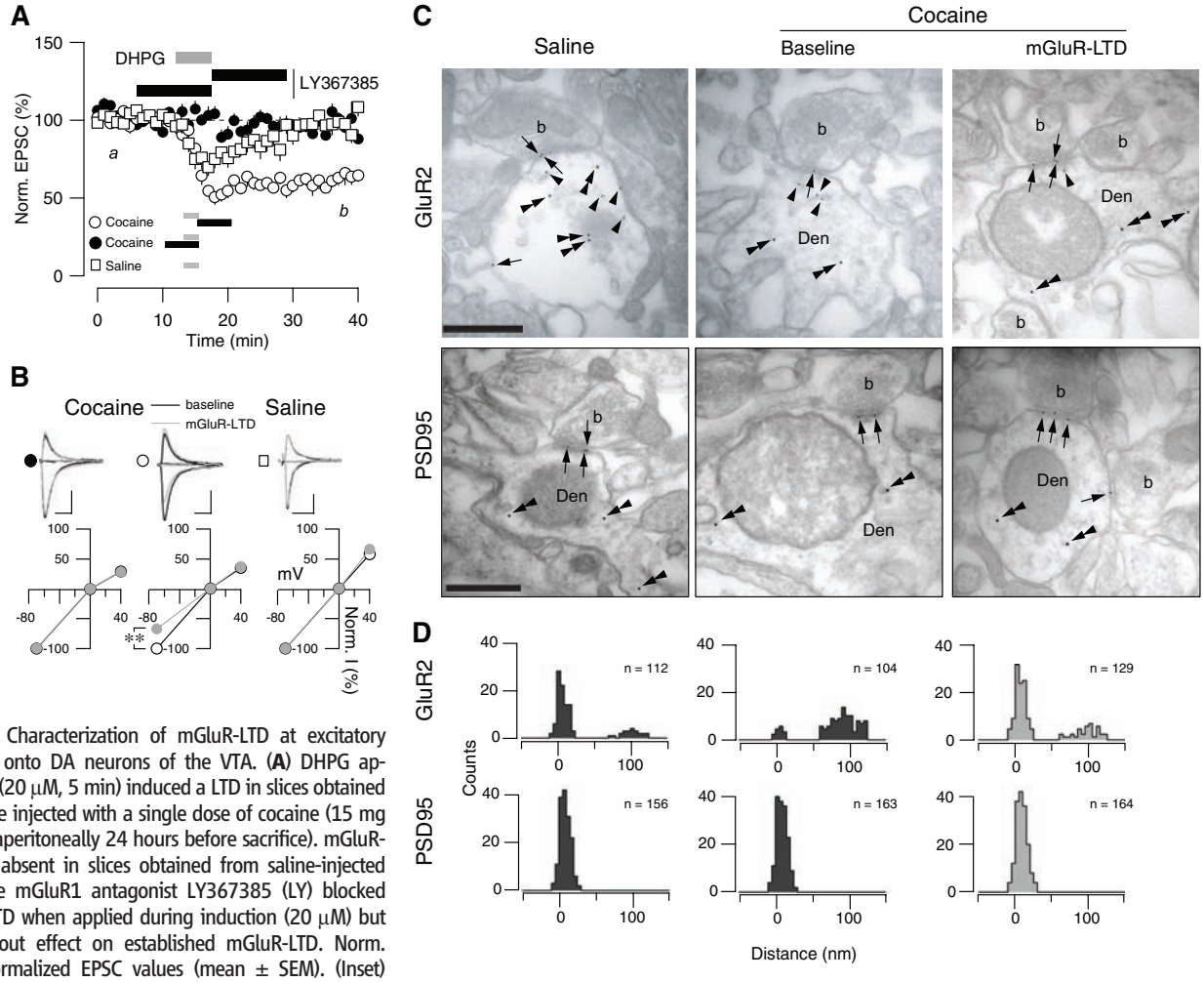

Fig. 1. Characterization of mGluR-LTD at excitatory synapses onto DA neurons of the VTA. (**A**) DHPG application (20 μM, 5 min) induced a LTD in slices obtained from mice injected with a single dose of cocaine (15 mg kg^{-1} intraperitoneally 24 hours before sacrifice). mGluR-LTD was absent in slices obtained from saline-injected mice. The mGluR1 antagonist LY367385 (LY) blocked mGluR-LTD when applied during induction (20 μM) but was without effect on established mGluR-LTD. Norm. EPSC, normalized EPSC values (mean ± SEM). (Inset) Gray bars, DHPG; black bars, LY. (**B**) Overlay of averaged traces of AMPAR-EPSCs recorded at −70, 0, and +40 mV before (black line) and after (gray line) DHPG application with corresponding *I-V* plots from representative cells in (A) at time points a and b. Scale bars indicate 5 ms (horizontal axis) and 50 pA (vertical axis). (**C**) Examples of postembedding immunoreactivity for tyrosine hydroxylase (TH) (double arrowheads), GluR2, and PSD95. Immunoparticles for GluR2 were found beneath (arrowheads) or within the postsynaptic densities (arrows) of asymmetrical synapses established by dendritic shafts (Den) of TH-positive neurons and axon terminals (b). As a control, PSD95 (arrows) always appeared in the postsynaptic densities. Scale bars, 0.5 μm. (**D**) Histograms compiling distances of GluR2 and PSD95 from the synapse (*n* particles in 60 identified synapses analyzed blindly from three hemislices of three mice for each condition).

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at this synapse. Twenty-four hours after a single injection of cocaine, the AMPA/*N*-methyl-D-aspartate (NMDA) ratio of evoked EPSCs (eEPSC) is increased, and the AMPA-EPSCs become rectifying. This suggests that new AMPARs are inserted into the synapse, of which a substantial fraction is devoid of the subunit GluR2. mGluR-LTD can reverse cocaine-induced synaptic plasticity (*1*), but the mechanisms underlying this process remain unclear.

To identify the mechanisms underlying mGluR-LTD in the VTA, we applied DHPG and recorded EPSCs in DA neurons. DHPG induced LTD in slices obtained from cocaine-injected mice but not in slices obtained from mice that were injected with saline (Fig. 1A) [46.9 ± 4.1% (mean ± SEM) compared with 3.76 ± 1.4%, n = 11 cells, $P < 0.01$]. This effect was blocked by the mGluR1 antagonist LY367385 (Fig. 1A, solid circles) (3.1 ± 2.7%, n = 7), but only when applied during the induction protocol; the same drug was ineffective when applied after mGluR-LTD was established (Fig. 1A, open circles). The current-voltage (*I-V*) relationship of the EPSCs was rectifying in slices from cocaine-treated mice and became linear after the induction of mGluR-LTD (*1*). In contrast, *I-V* curves plotted in slices from saline-treated mice were linear and did not change with DHPG treatment (Fig. 1B). Thus, activation of mGluR1 reverses cocaine-induced potentiation and leads to a reduction in the contribution of GluR2-lacking AMPARs to the EPSC, as expected (fig. S1) (*1*).

The redistribution of GluR2 was confirmed with postembedding immunogold labeling at the electron microscopy (EM) level. In slices from saline-treated mice, the majority of GluR2 labeling was observed at the synapse, along with a small cytoplasmic pool associated with intracellular membrane compartments. In slices from cocaine-exposed mice, the number of cytoplasmic GluR2 particles increased at the expense of synaptic labeling. Furthermore, DHPG treatment of slices from cocaine-exposed mice led to the reappearance of a predominantly synaptic pool. As a control, labeling of postsynaptic density (PSD95) was observed at synaptic locations in all three conditions (Fig. 1, C and D). Taken together, the electrophysiological and the EM observations demonstrate that, after cocaine exposure, GluR2 is redistributed toward intracellular compartments and that the induction of mGluR-LTD may restore basal conditions.

These results raise the possibility that LTD is expressed by the replacement of GluR2-lacking AMPARs with GluR2-containing AMPARs, which have a smaller single-channel conductance (γ) (*8*–*10*). We applied nonstationary fluctuation analysis (NSFA) to eEPSC and found that γ was significantly higher in slices from cocaine-treated mice compared with those from saline-treated controls (Fig. 2, A and B, and fig. S2). In slices from cocaine-treated mice, DHPG led to a significant relative decrease of γ (Fig. 2, C and D) (42.6 ± 6.9% versus 5 ± 10% in saline controls, n from 6 to 8, $P < 0.01$), yielding values similar to γ measured in saline controls where mGluR-LTD was absent (fig. S2). Importantly, the average number of receptors open at the peak (N) remained constant during mGluR-LTD (relative change for cocaine –4.4 ± 9.2%, for saline 1.2 ± 4.8%, n from 6 to 8). Thus, mGluR-LTD in the VTA is caused by a replacement of GluR2-lacking AMPARs with GluR2-containing AMPARs so that the total number of AMPARs remains constant.

Such an exchange could occur through lateral redistribution with an extrasynaptic pool or may involve the internalization of receptors. In the latter case, interfering with the endocytotic machinery should block mGluR-LTD. We therefore loaded the cells with a dominant negative peptide composed of 15 amino acids (D15) (*11*) that mimics the interaction site of dynamin with amphiphysin, two essential components for the internalization of clathrin-coated vesicles. D15 efficiently blocked the expression of mGluR-LTD without affecting baseline transmission in control conditions (Fig. 2C and fig. S2) (3.57 ± 2.1%, n = 7; for control, 48.6 ± 2.6%, n = 6; $P <$ 0.01). This is in contrast to previous reports in the hippocampus where a significant run-up was observed (*11*) and argues against a rapid constitutive recycling of AMPARs. To specifically interfere with the mobile pool of GluR2-containing receptors, we loaded cells with the active EVKI peptide that disrupts the interaction of GluR2 with PICK1 (*12*). This manipulation blocked the expression of mGluR-LTD (Fig. 2F) (13.1 ± 1.7%, n = 9, versus inactive pep2-SVKE control 47.1 ± 1.6%, n = 9, $P < 0.05$).

mGluR-LTD was blocked by an mGluR1 antagonist (Fig. 1, A and B). mGluR1 via Gq activates many pathways, including the extracellular signal–regulated kinase (ERK) (*13*) and phosphoinositide 3-kinase-Akt-mammalian tar-

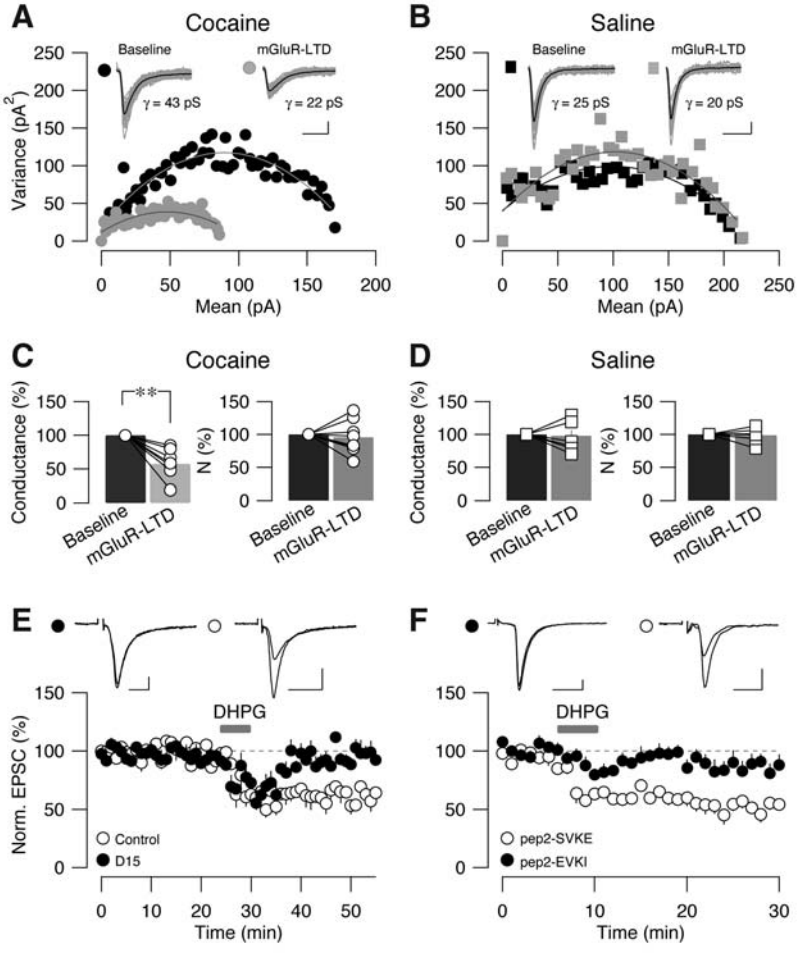

Fig. 2. mGluR-LTD expression involved the exchange of high-conductive AMPARs for low-conductive AMPARs. (**A** and **B**) Examples of *I-V* relationship before and after DHPG application in mice injected with cocaine (A) or saline (B). (Insets) Overlay of 20 consecutive traces. γ was estimated from the initial slope of the parabolic fit. (**C** and **D**) Relative changes in γ and number of channels (*N*) open at the peak of the response. (**E**). Disruption of the protein-protein interaction between dynamin-amphiphysin with a dominant negative peptide (D15, 1.5 mM) prevented mGluR-LTD. Averaged traces recorded before and after mGluR-LTD induction with and without D15 in the patch pipette. (**F**). Dialysis of DA neurons with the dominant negative peptide pep2-EVKI (100 μM) blocked mGluR-LTD, whereas its inactive form (pep2-SVKE, 100 μM) was without effect. Scale bars for (A), (B), (E), and (F) indicate 5 ms and 50 pA.

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get of rapamycin (mTOR) (*14*). Although the ERK inhibitor U0126 was inefficient in blocking mGluR-LTD (Fig. 3A) (36.4 ± 1.1%; for control, 41.1 ± 3.7%; *n* from 4 to 8; $P > 0.05$), we observed a block of the depression (6.45 ± 1.3%; for control, 46.5 ± 1.3%; *n* from 5 to 9; $P < 0.01$) with rapamycin (10- to 20-min preincubation), strongly implicating mTOR signaling in expression (Fig. 3B).

The involvement of mTOR in mGluR-LTD in the VTA suggests that this form of plasticity requires local translation. To directly test this possibility, we assessed the sensitivity of mGluR-LTD to two translational inhibitors. When cycloheximide (C-hex), which blocks translational elongation through an effect on the 60*S* ribosomal complex, was applied to the bath, we observed a significant reduction of the mGluR-LTD (Fig. 3C) (18.5 ± 2%, *n* = 10, $P < 0.05$). The residual depression during mGluR-LTD in C-hex was indistinguishable from that in interleaved experiments in which only C-hex was applied (13.2 ± 1.1%, *n* = 5, $P > 0.05$). To control for this nonspecific effect of C-hex, we obtained a within-cell control by applying DHPG first with and a second time without C-hex. We observed an almost-complete block of the depression in the presence of the translation inhibitor, whereas mGluR-LTD was restored after its washout (Fig. 3D, 13.1 ± 3.2% after first DHPG application; 45.3 ± 1.6% after washout of C-hex, *n* = 8, $P < 0.01$). Anisomycin, which inhibits translation via the 80*S* ribosomal complex, had no effect on baseline transmission but blocked mGluR-LTD, whether DHPG (Fig. 3E) (1.4 ± 2%, *n* = 14, $P < 0.01$) or brief trains of synaptic stimulation (Fig. 3F) (0.2 ± 2.1%, *n* = 7, $P < 0.01$) was used as induction protocol.

If mGluR-LTD depends on rapid translation, what is the protein that needs to be synthesized? One possibility is that GluR2-lacking AMPARs are removed from the synapse by a protein synthesized during mGluR-LTD. Alternatively, the synthesis of AMPARs that contain GluR2 may displace GluR2-lacking AMPARs, in which case GluR2 would be rapidly synthesized during LTD induction. The dendrites of many neurons contain mRNA for AMPAR subunits, and the machinery to integrate them into the membrane is also present (*15*, *16*). We therefore tested for synthesis and insertion of endogenous GluR2 by specifically interfering with the mRNA of GluR2. We chose two approaches, antisense oligonucleotide and small interfering RNA (siRNA), against a unique sequence in the N-terminal region of GluR2 (positions 1575 to 1595) (*17*). In both cases, the goal was not to deplete the endogenous protein but to rapidly and selectively prevent GluR2 mRNA from being translated. We filled the patch pipette with antisense oligonucleotide or siRNA and allowed free diffusion for 20 min while monitoring baseline synaptic transmission. With both interventions, baseline transmission was unaffected, whereas mGluR-LTD was abolished (Fig. 4, A to C) (for antisense oligonucleotide, 3.3 ± 1.2%; for siRNA-GluR2, 4.6 ± 1.6%; and for 66 Hz and siRNA-GluR2, 2 ± 3.1%; *n* from 8 to 18). As controls, we loaded the cells with scrambled versions of the oligonucleotide and siRNA, which did not block mGluR-LTD (for antisense oligonucleotide, 37.4 ± 1.7%; for siRNA-GluR2, 33.5 ± 5.1%; and for 66 Hz

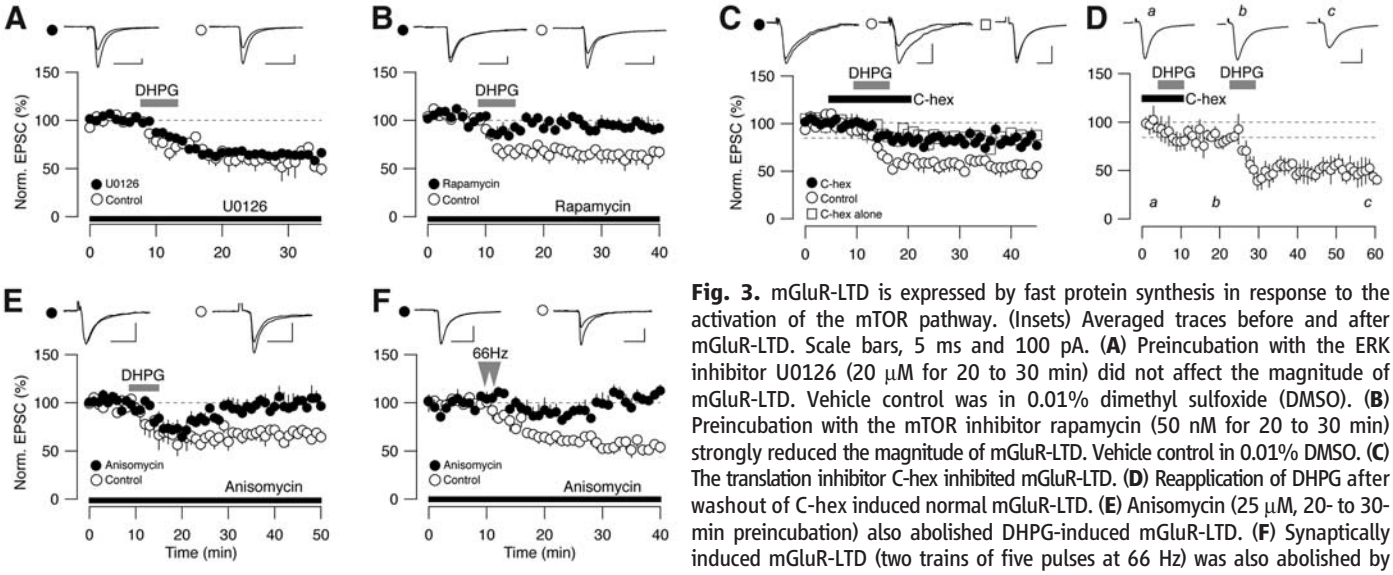

Fig. 3. mGluR-LTD is expressed by fast protein synthesis in response to the activation of the mTOR pathway. (Insets) Averaged traces before and after mGluR-LTD. Scale bars, 5 ms and 100 pA. (**A**) Preincubation with the ERK inhibitor U0126 (20 μM for 20 to 30 min) did not affect the magnitude of mGluR-LTD. Vehicle control was in 0.01% dimethyl sulfoxide (DMSO). (**B**) Preincubation with the mTOR inhibitor rapamycin (50 nM for 20 to 30 min) strongly reduced the magnitude of mGluR-LTD. Vehicle control in 0.01% DMSO. (**C**) The translation inhibitor C-hex inhibited mGluR-LTD. (**D**) Reapplication of DHPG after washout of C-hex induced normal mGluR-LTD. (**E**) Anisomycin (25 μM, 20- to 30-min preincubation) also abolished DHPG-induced mGluR-LTD. (**F**) Synaptically induced mGluR-LTD (two trains of five pulses at 66 Hz) was also abolished by anisomycin (25 μM, 20- to 30-min preincubation).

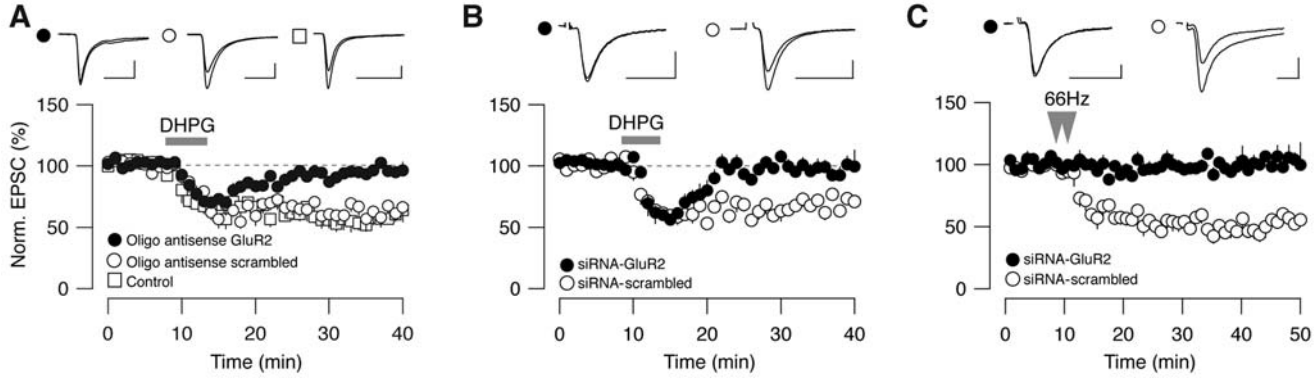

Fig. 4. Specific targeting of GluR2-mRNA abolished mGluR-LTD. (Insets) Averaged traces before and after mGluR-LTD. Scale bars, 5 ms and 50 pA. (**A**) Antisense oligonucleotide against the N-terminal sequence of GluR2-mRNA (250 μM, positions 1575 to 1595) dialyzed intracellularly blocked mGluR-LTD. (**B**) Double-stranded siRNA (30 nM) targeting GluR2-mRNA abolished mGluR-LTD. (**C**) Synaptically induced mGluR-LTD was also abolished by the GluR2-siRNA.

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and siRNA-GluR2, 49.3 ± 3.3%; *n* from 5 to 7; $P < 0.01$ against corresponding active siRNA).

Our results define two unexpected features of mGluR-LTD in the VTA. First, we found that mGluR-LTD in the VTA is not due to the simple removal of AMPARs from the synapse; rather, AMPARs that lack the GluR2 subunit are selectively endocytosed and immediately replaced by AMPARs that contain GluR2. Second, we found that de novo synthesis of GluR2 is required for LTD.

Protein synthesis is involved in many forms of synaptic plasticity, typically during the maintenance phase several hours after induction (*18*). However, forms of synaptic plasticity that rely on local protein synthesis on a much faster time scale have been described (*19*, *20*), and components of the translation machinery, such as polyribosomes and mRNA are present in dendrites (*15*, *16*). However in most cases, the identity of the proteins synthesized on demand remains unknown. Unbiased strategies to fill this gap, such as cDNA microarrays in dendritic fractions, have revealed mRNA of several genes, including ribosomal proteins, transcription factors, or components of the induction cascade for plasticity (*21*, *22*). mGluRs can initiate such synthesis in expression system. DHPG drives the translation of recombinant GluR2 mRNA into receptor subunits, which are then detected in the cell membrane (*23*, *24*).

In the VTA, GluR2 seems to be both (i) synthesized in response to the induction of plasticity and (ii) directly involved in the expression of plasticity. Thus, as in other systems, LTD does lead to AMPAR withdrawal, but in the VTA these receptors are replaced by newly synthesized AMPARs so that the total number of AMPARs at synapses remains constant. Thus, synapses express LTD because the conductance of the newly synthesized GluR2-containing AMPARs is smaller than that of GluR2-lacking AMPARs that were present before LTD.

Our data are reminiscent of a form of synaptic plasticity observed in stellate cells of the cerebellum that is associated with a loss of rectification (*25*, *26*); however, in that case, GluR2-containing receptors are present at extrasynaptic sites and incorporated through lateral movement (*25*). In contrast, in the VTA, GluR2 appears to be a protein that needs to be synthesized in order for synapses to express mGluR-LTD. This does not exclude the possibility that other proteins must also be synthesized, for example to form functional heteromeric AMPARs.

Our results may be of physiological and pharmacological relevance in the context of drug addiction. Several studies implicate synaptic plasticity in DA neurons of the VTA in core components of drug addiction (*27*). Analogous to pain sensitization or ischemia (*10*), the Ca^{2+} permeability of the AMPARs could play an important role in the pathological process. Activation of mGluR1 may restore Ca^{2+}-impermeable transmission, and regulated synthesis of GluR2 may be an important requirement to reverse cocaine-induced synaptic plasticity.

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Materials and Methods
Figs. S1 and S2

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