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MEF-2 regulates activity-dependent spine loss in striatopallidal medium spiny neurons

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Abstract

Striatal dopamine depletion profoundly reduces the density of spines and corticostriatal glutamatergic synapses formed on D₂ dopamine receptor expressing striatopallidal medium spiny neurons, leaving D₁ receptor expressing striatonigral medium spiny neurons relatively intact. Because D₂ dopamine receptors diminish the excitability of striatopallidal MSNs, the pruning of synapses could be a form of homeostatic plasticity aimed at restoring activity into a preferred range. To characterize the homeostatic mechanisms controlling synapse density in striatal medium spiny neurons, striatum from transgenic mice expressing a D₂ receptor reporter construct was co-cultured with wild-type cerebral cortex. Sustained depolarization of these co-cultures induced a profound pruning of glutamatergic synapses and spines in striatopallidal medium spiny neurons. This pruning was dependent upon Ca²⁺ entry through Cav1.2 L-type Ca²⁺ channels, activation of the Ca²⁺-dependent protein phosphatase calcineurin and up-regulation of myocyte enhancer factor 2 (MEF2) transcriptional activity. Depolarization and MEF2 up-regulation increased the expression of two genes linked to synaptic remodeling – Nur77 and Arc. Taken together, these studies establish a translational framework within which striatal adaptations linked to the symptoms of Parkinson's disease can be explored.

Keywords

Plasticity; striatum; GABA; Dendritic spine; Patch Clamp; Parkinson's disease

Introduction

The principal medium spiny neurons (MSNs) of the striatum are richly innervated by pyramidal neurons residing in the cerebral cortex. The glutamatergic synapses they form are almost exclusively formed on spines that stud the dendrites of MSNs (Bolam et al., 2000). This cortical input is thought to carry information about sensory, motor and motivational state that guides striatal control of thought and movement (Graybiel et al., 1994).

One of the key modulators of this synaptic connection is dopamine (Albin et al., 1989). Dopamine has long been known to regulate the induction of long-term changes in the strength

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of corticostriatal synapses (Schultz, 2006); these changes are thought to underlie associative learning (Graybiel et al., 1994; Morris et al., 2004; Schultz, 2006). More recently, it has been shown that sustained perturbations in striatal dopamine levels alter the density of spines and synapses. For example, chronic elevation of striatal dopamine levels with psychostimulants increases MSN spine density (Kim et al., 2009), whereas dopamine-depleting lesions, mimicking Parkinson's disease (PD), trigger a rapid loss of MSN spines and asymmetric glutamatergic synapses (Day et al., 2006; Deutch et al., 2007). At least initially, the loss of spines in PD models is cell-type specific, occurring in striatopallidal MSNs that express D₂ dopamine receptors, but not striatonigral MSNs that express D₁ dopamine receptors.

In principle, the alterations in spine and synapse density triggered by psychostimulants or dopamine depletion could be the endstage of conventional forms of synaptic plasticity. The induction of long-term potentiation (LTP) has been reported to increase spine size, whereas the induction of long-term depression (LTD) has the opposite effect (Harvey and Svoboda, 2007; Matsuzaki et al., 2004; Tanaka et al., 2008; Yang et al., 2008; Zhang et al., 2008; Zhou et al., 2004). However, in the case of the striatum, dopamine depletion and the elimination of D₂ receptor signaling should promote LTP induction in striatopallidal MSNs (Shen et al., 2008). This should increase the size and apparent density of spines, not decrease them.

Synaptic scaling is another mechanism by which activity controls synaptic strength (Turrigiano, 2008). Synaptic scaling refers to a form of homeostatic plasticity aimed at maintaining cellular and network activity within an optimal range. For example, reducing somatic spiking for a prolonged period leads to a global up-regulation in synaptic glutamate receptors. This form of homeostatic plasticity appears to rely upon somatic Ca²⁺ entry through L-type Ca²⁺ channels opened during spiking. Lower than desired Ca²⁺ entry leads to a relative down-regulation in CaMKIV activity and diminished Arc transcription, resulting in increased trafficking of glutamate receptors into synapses (Shepherd et al., 2006). Although not studied nearly as thoroughly, sustained elevation in spiking could trigger a complementary form of synaptic scaling, leading to a global down-regulation in glutamate receptors at excitatory glutamatergic synapses. Synapse elimination could sit at one end of the spectrum of adaptations triggered by synaptic scaling mechanisms. Indeed, recent work has shown that increased Ca²⁺ entry through L-type Ca²⁺ channels can activate the transcription factor myocyte enhancer factor 2 (MEF2), leading to up-regulation of Arc and spine elimination (Flavell et al., 2006).

The adaptations seen in MSNs following dopamine depletion seem to fit neatly within this schema. Following depletion, the loss of D₂ receptor signaling elevates the intrinsic excitability of striatopallidal MSNs and promotes LTP induction at corticostriatal synapses (Surmeier et al., 2007). This combination of effects explains in large measure the overall increase spiking rates seen in this subset of MSNs in PD models (Mallet et al., 2006). This deviation from their activity set point should trigger synaptic scaling mechanisms to produce a compensatory down-regulation of excitatory synapses. To test this hypothesis, a corticostriatal culture model was used in which spines develop normally in striatal MSNs (Segal et al., 2003). To differentiate cortical and striatal neurons, cultures were generated with striata from mice expressing green fluorescent protein (GFP) under control of either the D₁ or D₂ receptor promoter. These studies revealed that prolonged depolarization of striatopallidal MSNs induces a profound decrease in the density of spines and glutamatergic synapses. This pruning depended upon Ca²⁺ entry through L-type Ca²⁺ channels with a Cav1.2 pore-forming subunit, activation of the Ca²⁺-dependent protein phosphatase calcineurin and elevation of MEF2 transcriptional activity, leading to increased expression of two genes linked to synaptic remodeling – *Nur77* and *Arc*.

Results

MSNs in corticostriatal co-cultures have spines and synapses

Primary cultures of striatal neurons have been widely used for a variety of purposes (Dudman et al., 2003; Falk et al., 2006; Surmeier et al., 1988). Because principal MSNs are GABAergic, these cultures are essentially devoid of glutamatergic neurons if done properly. In the absence of the normal glutamatergic input from cortical or thalamic neurons, MSNs do not develop mature spines (Fig. 1C). This situation can be corrected by co-culturing cortical pyramidal neurons with striatal MSNs (Segal et al., 2003). However, it is difficult to distinguish between cortical and striatal neurons solely on the basis of morphology. To make distinguishing cell-types possible, striata from mice expressing a D₂-GFP transgene were co-cultured with wild-type cortical neurons (Fig. 1D and 1E). The detailed dendritic morphology of striatal MSNs then could be readily analyzed following immunostaining with anti-GFP antibody. After 3 weeks in co-culture, most GFP-labeled cells met the morphological criteria for MSNs: small size soma (10-18 μ m), dense dendritic tree, and highly spiny dendrites (Fig. 1D and 1E). Occasionally, weakly expressing GFP immunoreactive cells with smooth, sparsely branching dendrites were observed and were most likely interneurons. All GFP-labeled cells expressed D₂ dopamine receptor protein (162 cells from 3 experiments Fig. 1F), but very few of them had immunoreactivity for D₁ receptor protein (2.7 ± 0.71 %, 311 cells from 3 experiments, Fig. 1G).

Spines with a mushroom-like appearance richly invested the dendrites of co-cultured D₂ MSNs (Fig. 1D and 1E), in contrast to the situation in pure striatal cultures where D₂ MSNs had only sparse, filopodial-like dendritic protrusions (Fig. 1B,C). These mature looking spines were immunoreactive for PSD-95 and opposed by presynaptic profiles that were immunoreactive for the vesicular glutamate transporter 1 (vGlut1). The strong resemblance between D₂ MSNs in this co-culture model and those found *in situ* (Day et al., 2006; Wilson et al., 1983) argues it is a reasonable model for studying the mechanisms controlling spine stability.

Membrane depolarization and Ca²⁺ entry eliminates D₂ MSN spines

The loss of ambient, inhibitory D₂ receptor signaling is widely thought to elevate the excitability and spiking of striatopallidal MSNs following dopamine-depletion in PD models (Albin et al., 1989). One commonly used strategy for elevating neuronal activity is to block inhibitory GABAergic synaptic transmission (Turrigiano et al., 1998). However, GABA_A receptor antagonists have only modest effects on striatal activity in brain slices (unpublished observations), suggesting that in our cultures this would not be an effective means of mimicking the sustained elevation in activity thought to accompany dopamine depletion. Another commonly employed strategy to produce a sustained elevation in activity is to increase the extracellular K⁺ concentration (Franklin et al., 1992; Leslie et al., 2001; Moulder et al., 2003). Although this produces a sustained depolarization, as opposed to patterned, synaptically driven activity, it has the advantage of reproducibility.

To better understand the impact of elevating external K⁺ concentration, whole cell patch clamp recording was used to measure the response of cultured MSNs in the presence of ionotropic receptor antagonists. Changing the external K⁺ concentration from 4 mM to 12, 24 and then 35 mM produced a progressive depolarization as predicted by the Nernst equation (Fig. 2A). At 35 mM external K⁺, the membrane potential of MSNs appeared to be reasonably stable. The average membrane potential immediately after moving to the high K⁺ (35 mM) was around -31 mV (n=5); 24 hrs later, the average membrane potential of MSNs was -24 mV (n=4). Surprisingly, prolonged exposure to 35 mM K⁺ did not produce significant cell loss or signs of pathology (Fig. S1). Moreover, as described below, the physiology of MSNs was ostensibly intact after this treatment.

Membrane depolarization in this range opens voltage-dependent Ca^{2+} channels. To measure the time course and extent of Ca^{2+} entry, neurons were loaded with the Ca^{2+} dye Fura-2 AM and D_2 PLSM used to monitor changes in dye fluorescence following exposure to high K^+ concentrations. Striatopallidal MSNs were identified by their GFP expression (Fig. 2B). In the first few minutes following elevation of the external K^+ concentration to 35 mM (from 5.4 mM) in the presence of ionotropic receptor antagonists, the cytoplasmic Ca^{2+} concentration rose and then fell back to a level that was roughly 100 nM above baseline values (<20 nM, Fig. 2C-E). The amplitude of the initial rise in Ca^{2+} concentration varied between cells, but the steady-state level was very consistent (Fig. 2E). The elevation in cytosolic Ca^{2+} following exposure to 35 mM K^+ was entirely blocked by antagonizing L-type Ca^{2+} channels with nimodipine (10 μM , $P < 0.01$, Mann-Whitney Test, $n = 6$).

To determine how sustained elevation in cytosolic Ca^{2+} concentration would affect cellular morphology, co-cultures were incubated in media containing 35 mM K^+ for progressively longer periods of time and then the cultures fixed and analyzed. To eliminate the effects of ionotropic glutamate receptors, the experiments were conducted in the presence of both glutamate and GABA receptor antagonists (50 μM D-APV, 20 μM NBQX and 10 μM bicuculline). Membrane depolarization led to progressive loss of dendritic spines in striatopallidal MSNs (Fig. 2F,G). The pruning was progressive, as 8 hr treatment resulted in minimal spine loss (about 11%), while 24 hr treatment resulted in about 50% spine loss. Immunostaining for vGlut1 revealed a parallel loss of presynaptic terminals (Fig. 2F), indicating that both spines and synapses were lost (see below). Antagonizing both type 1 metabotropic glutamate receptors with AIDA (30 μM) and the ionotropic glutamate and GABA receptors did not alter the spine loss (Fig. S2). However, chelating extracellular Ca^{2+} with ethylene glycol tetra-acetic acid (EGTA, 2 mM) blocked depolarization-induced spine loss (Fig. S2), pointing to the importance of Ca^{2+} entry. Interestingly, 24 hr treatment with 35 mM K^+ had much less of an effect on the density of spines in D_1 MSNs identified post hoc by immunocytochemical staining of D_1 receptors (Fig. 2H; Fig. S3).

L-type Ca^{2+} channels are necessary for spine and synapse elimination induced by membrane depolarization

To determine if there was a causal linkage between depolarization and Ca^{2+} entry, co-cultures were exposed to high K^+ (35 mM) media in the presence of nimodipine, which attenuated the rise in cytosolic Ca^{2+} concentration with depolarization. Nimodipine prevented spine loss produced by 24 hr exposure to 35 mM KCl (Fig. 3A-B). Membrane depolarization has been shown to rapidly affect spine shape (Fischer et al., 2000; Okamura et al., 2004). In accord with these previous studies, depolarization significantly reduced the average spine head diameter ($P < 0.001$, t-test; $n = 410$); this could be seen most clearly in cumulative probability plots of spine head diameter in treated and control cultures (Fig. 3C). Nimodipine (10 μM) prevented spine heads from shrinking in the presence of high K^+ (Fig. 3C).

Our initial staining for vGlut1 suggested that spine retraction was accompanied by elimination of the presynaptic terminal (Trachtenberg et al., 2002). To provide a functional test of this inference, miniature excitatory postsynaptic currents (mEPSCs) were measured in striatopallidal MSNs. Depolarization (35 mM KCl for 24 hrs) significantly reduced mEPSC frequency (Fig. 3D,E), consistent with a global decrease in number of synapses. Co-exposure to nimodipine not only prevented the loss of spines, but also prevented the drop in mEPSC frequency (Fig. 3D,E). Thus, membrane depolarization that led to opening of L-type Ca^{2+} channels eliminated both dendritic spines and synapses in striatopallidal MSNs, as seen following dopamine depletion *in vivo* (Day et al., 2006).

Interestingly, depolarization also decreased the median mEPSC amplitude (Fig. 3F). This is consistent with models of synaptic scaling (Turrigiano, 2008) and could be part of an initial

attempt to restore activity to a set point. Nimodipine treatment prevented the re-scaling of mEPSC amplitude (Fig. 3D,F).

Enhanced L-type Ca^{2+} channel opening increases the effects of membrane depolarization

To see if membrane depolarization could be dissociated from L-type Ca^{2+} channel opening in the induction of spine loss, co-cultures were challenged with a lower concentration of K^+ (20 mM) for 24 hrs (in the presence of ionotropic receptor antagonists). Based upon the results in Fig. 1, this should depolarize cells to around -50 mV. This challenge did not induce a significant loss of spines (Fig. 4A,B). In fact, it significantly increased mEPSC frequency (not amplitude) (Fig. 4E,F), suggesting that modest depolarization elevated glutamate release probability, as there was no change in spine (synapse) number produced by this manipulation. However, adding the L-type Ca^{2+} channel agonist Bay K8644 (1 μM), which shifts the activation voltage dependence of L-type channels into the range produced by 20 mM K^+ (Grabner et al., 1996; Xu and Lipscombe, 2001), induced a robust loss of spines (Fig. 4A,B). As with stronger depolarization, the diameter of the residual spines was reduced in the presence of Bay K8644, but not with 20 mM K^+ treatment alone (Fig. 4C). The frequency of mEPSCs in striatopallidal MSNs also was lowered by co-treatment with Bay K8644 (Fig. 4D,E). However, mEPSC amplitude was not changed by treatment (Fig. 4F), a somewhat unexpected outcome given the change in spine dimensions.

Cav1.2 but not Cav1.3 L-type Ca^{2+} channels are required for membrane depolarization-induced spine loss

There are two variants of the L-type Ca^{2+} channel expressed by striatal MSNs (Olson et al., 2005). One possesses a Cav1.2 pore-forming subunit, the other a Cav1.3 subunit. Although both are sensitive to dihydropyridines, Cav1.2 channels have a higher affinity for nimodipine (Koschak et al., 2001; Xu and Lipscombe, 2001). Co-cultured D_2 MSNs robustly expressed Cav1.2 subunit protein that was distributed throughout the soma and dendritic shafts, but it was rarely found in spines (Fig. 5A). Localizing Cav1.3 protein was more problematic as the available antibodies cross-react with other proteins as judged by immunostaining in sections from Cav1.3 null mice (unpublished observations). Analysis of mRNA from co-cultures suggested that L-type channels were dominated by Cav1.2 subunits, suggesting that striatal expression of this subunit might be developmentally regulated and not prominent in cultures maintained for only a few weeks *in vitro*. Nevertheless, in an attempt to tease apart the contribution of these two channels to spine pruning, co-cultures were exposed to a relatively low concentration of nimodipine (1 μM) that should preferentially antagonize Cav1.2 channels and then depolarized with K^+ (35 mM). This lower concentration was very effective in reducing spine loss (Fig. 5B,D). To provide a more definitive test of the role of Cav1.3 channels, BAC D_2 mice were crossed with a line of mice lacking Cav1.3 L-type channels (Platzter et al., 2000) and co-cultures generated from the resultant line. Although deletion of Cav1.3 L-type channels attenuated spine loss following dopamine depletion (Day et al., 2006), deletion had no effect on depolarization-induced spine loss in the co-cultures (Fig. 5C,D). These results suggest that membrane depolarization-induced spine loss requires activation of Cav1.2 L-type Ca^{2+} channels, but not Cav1.3 Ca^{2+} channels.

Calcineurin activation is necessary for spine pruning

One of the potential signaling targets of Ca^{2+} entering through Cav1.2 L-type Ca^{2+} channels is the Ca^{2+} -dependent protein phosphatase calcineurin (or PP2B) (Nishi et al., 1999). Calcineurin is an important mediator of NMDA receptor -dependent spine loss in hippocampal neurons (Halpain et al., 1998) and L-type Ca^{2+} channel -dependent activation of MEF2 (Flavell et al., 2006). When calcineurin inhibitors (1 μM ascomycin and 4 μM cyclosporin A) were

applied to the co-cultures during high potassium treatment, depolarization-induced spine loss in striatopallidal MSN was significantly attenuated (Fig. 6A,B).

MEF2-dependent gene expression is necessary for spine pruning

Ca²⁺ entering through L-type (Cav1) Ca²⁺ channels regulates a variety of transcriptional programs (Calin-Jageman and Lee, 2008; Deisseroth et al., 1998; Dolmetsch et al., 2001). Some of these have been linked to alterations in spine and synapse density. To determine whether alterations in gene transcription were necessary for depolarization-induced spine loss in striatopallidal MSNs, two inhibitors were tested. First, the translation inhibitor cycloheximide (10 μ M) was added to the high K⁺ media at a concentration previously shown to inhibit protein synthesis (Park et al., 2008). Cycloheximide significantly attenuated depolarization-induced spine loss in D₂ MSNs (Fig. 6C,D). Next, the transcription inhibitor actinomycin D (10 μ g/mL) was tested. Actinomycin D also significantly attenuated depolarization-induced spine loss (Fig. S4). Neither inhibitor alone had any significant effect on spine density in the 24 hrs observation period (Papa and Segal, 1996).

One important target of calcineurin is MEF2 (Flavell et al., 2006). Dephosphorylation of MEF2 by calcineurin activates a transcriptional program that leads to down-regulation of synaptic density in hippocampal neurons. In cultured D₂ MSNs, MEF2s are highly expressed (Fig. S5). To determine the role of MEF2 in depolarization-induced spine loss here, short hairpin ribonucleic acid (shRNA) constructs were introduced into striatopallidal MSNs by single cell electroporation. Striatopallidal MSNs were examined two days (48 hrs) after transfection with either shRNA constructs targeting MEF2A and MEF2D or with a scrambled shRNA construct. The MEF2A/D constructs were clearly effective in reducing MEF2 expression (Fig. 7A), whereas the scrambled construct was without any obvious effect. Reducing MEF2 expression alone had had no effect on spine density 48 hrs after transfection. More importantly, reducing MEF2 expression significantly attenuated spine loss produced by depolarization (Fig. 7B,C), suggesting that calcineurin mediated dephosphorylation of MEF2 was a key step in the process underlying spine pruning.

Membrane depolarization increases Nur77 and Arc expression in striatopallidal MSNs

MEF2 regulates the transcription of several genes linked to sculpting of synaptic connections. One of these genes is *Nur77*. Depolarization-induced activation of MEF2 increases the expression of *Nur77* in cerebellar granule cells, inhibiting differentiation of dendritic claws – a postsynaptic structure similar to dendritic spine (Shalizi et al., 2006). Depolarization also up-regulated *Nur77* expression in striatopallidal MSNs (Fig. 8A). *Nur77* was largely restricted to the nucleus, as judged by DAPI co-labeling (Fig. 8B). As in cerebellar granule neurons, the up-regulation in *Nur77* expression was significantly attenuated by antagonism of L-type Ca²⁺ channels or calcineurin (Fig. 8A,C).

Another MEF2 regulated gene implicated in synaptic sculpting is *Arc* (Flavell et al., 2006). Within 2 hrs, depolarization of co-cultures induced a significant up-regulation in the levels of *Arc* throughout the somatodendritic tree (Fig. 9A). With more sustained depolarization (6 hrs), *Arc* expression was still elevated (Fig. 9B). MEF2 activation was important to this response as knocking down MEF2 with shRNAs significantly attenuated the depolarization-induced up-regulation of *Arc* (Fig. 9C,D).

Discussion

Our studies define a novel form of striatal homeostatic plasticity. Sustained depolarization of co-cultures of cerebral cortex and transgenic striatum, mimicking elevated activity, induced a nearly 50% loss of spines and glutamatergic synapses in striatopallidal MSNs. This down-

regulation of synaptic connectivity was similar to that seen in animal models of PD (Day et al., 2006). The loss was dependent upon Ca^{2+} entry through L-type channels with a pore-forming Cav1.2 subunit, activation of the Ca^{2+} -dependent protein phosphatase calcineurin and up-regulation of MEF2. MEF2 up-regulation increased expression of two genes known to promote down-regulation of glutamatergic synapses – *Nur77* and *Arc* (Steward and Worley, 2001; Shepherd et al., 2006), providing the outline of a molecular mechanism for activity-dependent synaptic scaling.

A complementary, striatal form of homeostatic plasticity

As with many previous studies (Turrigiano, 2008), our work relied upon a culture model of the striatum. The advantage of this preparation is the ease with which neural activity can be reproducibly pushed up or down for hours or days. However, the model has potentially significant limitations. Certainly the cultures fail to recapitulate the cellular heterogeneity found *in situ*. MSNs receive inputs not only from cortical neurons but also from a variety of other brain structures, including the dopaminergic neurons of the mesencephalon. This might significantly alter the maturation of neurons and their response to perturbations. That said, the apparent normality of spine morphology and density in cultured striatopallidal MSNs demonstrates that the cortical input to MSNs, which is the predominant excitatory input, is sufficient for normal dendritic development.

In these co-cultures, sustained postsynaptic depolarization, produced by elevating extracellular K^+ concentration, induced a pruning of spines and synapses in striatopallidal MSNs. Patch clamp recordings showed that the magnitude of the depolarization was predicted by the Nernst equation, with 35 mM K^+ bringing the membrane potential to near -30 mV. Although this is suprathreshold for spike generation in cultured MSNs, sustained depolarization undoubtedly led to inactivation of voltage-dependent Na^+ channels and cessation of spiking. This inference is consistent with measurements of intracellular Ca^{2+} concentration that transiently rose and then fell back to near 100 nM with high K^+ treatment. This Ca^{2+} concentration is at the upper-end of what is generally considered to be the physiological range of basal intracellular Ca^{2+} concentration, suggesting that high K^+ treatment was an effective – if artificial – means of mimicking elevated postsynaptic activity. Other means of elevating activity, like blocking inhibitory GABAergic inputs, appeared to be a less reliable means of stimulating MSNs in our co-cultures; but more importantly, this means of stimulation requires engagement of ionotropic glutamate receptors, preventing a clean dissection of the routes of Ca^{2+} entry underlying spine pruning.

It is generally believed that cytosolic Ca^{2+} concentration is the controlled variable in homeostatic plasticity (Thiagarajan et al., 2005; Turrigiano, 2008). Two routes of Ca^{2+} entry appear to be particularly important in determining the activity signal for neurons: N-methyl-D-aspartate (NMDA) ionotropic glutamate receptors and L-type Ca^{2+} channels (Blackstone and Sheng, 1999). In hippocampal cultures, NMDA receptor opening leads to a loss or shrinkage of spines within minutes (Halpain et al., 1998). Because of its kinetics, this effect is likely to be locally mediated. Although a role for NMDA receptors in the striatal adaptations seen with dopamine depletion cannot be excluded, they were not necessary for the slower, global changes in spine density triggered by depolarization. While NMDA receptors were not necessary, L-type Ca^{2+} channels with a Cav1.2 pore-forming subunit were, based upon pharmacological and molecular tests. The sustained rise (~100 nM) in intracellular Ca^{2+} concentration produced by the modest depolarization used in our studies was almost entirely attributable to flux through Cav1.2 L-type Ca^{2+} channels. In the more commonly studied situation where neurons are subjected to a sustained reduction in activity (Desai et al., 1999), a drop in Ca^{2+} entry through L-type Ca^{2+} channels is thought to trigger transcriptional changes that globally scale-up synaptic AMPA receptors (Turrigiano, 2008). Thus, our work provides

a complementary example of where both the number of synaptic AMPA receptors fell in parallel with the number of detectable synapses, as judged by significant decreases in mEPSC amplitude and frequency with sustained depolarization. Elimination could be viewed as one end of a synaptic scaling spectrum, where global down-regulation of synaptic strength leads to the elimination of synapses that were relatively weak at the initiation of scaling.

In striatopallidal MSNs, sustained opening of L-type Ca^{2+} channels and Ca^{2+} entry led to the activation of the Ca^{2+} -dependent protein phosphatase calcineurin. This activation was necessary for the initiation of synaptic scaling as inhibitors of calcineurin effectively blunted the response to depolarization. Because interrupting either gene transcription or mRNA translation also prevented changes in scaling, calcineurin must be playing a role in nuclear signaling.

Two well-described transcriptional regulators targeted by calcineurin are MEF2 and nuclear factor of activated T-cells (NFAT). Both are robustly expressed in MSNs (Groth et al., 2008; Ruffle et al., 2006). Calcineurin dephosphorylates both MEF2 and NFAT proteins, increasing their transcriptional activity (McKinsey et al., 2002). Although a role for NFAT was not pursued, it was clear that MEF2 activation was necessary for depolarization-induced pruning, because of its sensitivity to MEF2 knockdown. MEF2 knockdown had no effect in unstimulated cultures. The inference that MEF2 activation can down-regulate glutamatergic synapses is consistent with recent work in hippocampal neurons (Flavell et al., 2006).

MEF2 regulates the expression of several genes but two that have demonstrated roles in controlling synaptic strength are *Arc* and *Nur77* (Flavell et al., 2006; Shalizi et al., 2006). *Arc* expression is rapidly up-regulated by synaptic stimulation and membrane depolarization (Steward et al., 1998), and *Arc* protein subsequently moves to the site of dendritic synapses where it promotes endocytosis of AMPA receptors (Rial Verde et al., 2006). *Nur77* is a transcription factor belonging to a family of orphan nuclear receptors that is highly expressed in striatum and prefrontal cortex (Levesque and Rouillard, 2007; Pols et al., 2007). Recently, *Nur77* has been shown to inhibit postsynaptic dendritic differentiation and synapse formation (Shalizi et al., 2006). In line with these actions, both *Nur77* and *Arc* were up-regulated by MEF2-dependent signaling following depolarization of striatopallidal MSNs, suggesting an involvement in synaptic scaling.

Relevance of homeostatic plasticity to Parkinson's disease

The primary goal of our studies was to gain insight into the cellular mechanisms underlying the elimination of spines and synapses in striatopallidal MSNs in animal models of PD. It is widely thought that the loss of inhibitory D_2 receptor signaling in this model elevates the excitability of this subtype of MSN, inducing a network dysfunction underlying the motor symptoms of the disease (Albin et al., 1989). Although initially based upon indirect measures of activity, more recent work has largely supported this framework in suggesting that D_2 dopamine receptors decrease glutamate release and dendritic excitability, as well as elevate the amount of synaptic input necessary to achieve a given level of spiking (Surmeier et al., 2007). The loss of spines and synapses following dopamine depletion takes days to complete, putting it in the right time frame for homeostatic plasticity and synaptic scaling. Although the depolarization achieved by elevating extracellular K^+ concentration is an imperfect means of mimicking the effects of removing dopamine, the similarity in the effects is striking.

As mentioned above, one mechanistic difference between these two studies is the role of Cav1.3 L-type Ca^{2+} channels. Because they are activated at sub-threshold membrane potentials and positioned near synapses (Olson et al., 2005), they are important regulators of synaptic plasticity. For example, Ca^{2+} entry through these channels promotes LTD at corticostriatal synapses (Adermark and Lovinger, 2007). Genetic deletion of these channels increases the

density of MSN spines and synapses *in vivo* and attenuates the effects of dopamine depletion on spine density (Day et al., 2006). Thus, *in vivo*, Cav1.3 channels appear to participate local dendritic mechanisms controlling synaptic downsizing. Although we found no role for these channels in synaptic pruning induced by high K⁺ treatment, this could be because this manipulation essentially bypasses the normal synaptic mechanisms to directly depolarize the somatic membrane. Somatic depolarization directly activated high threshold Cav1.2 Ca²⁺ channels positioned in this region. These channels, because of their peri-somatic location, are perfectly suited to influence calcineurin signaling to the nucleus. In this scenario, the increased excitability of striatopallidal MSNs following dopamine depletion would produce spine and synapse elimination by a local and global processes: a local process involving synaptic Cav1.3 channels and a global process involving somatic Cav1.2 channels and the signaling cascade described here. The elevated engagement of somatic Cav1.2 channels following dopamine depletion would depend upon glutamatergic synaptic inputs being effectively transduced by the dendrites, a process that would be compromised by genetic deletion of Cav1.3 channels. This conjecture is consistent with the role of cortical excitatory input in producing spine loss (Neeley et al., 2007). It is also consistent with the up-regulation of *Nur77* in striatopallidal MSNs following 6-hydroxydopamine lesioning (St-Hilaire et al., 2003). To provide a definitive test, the impact of virally delivered MEF2 shRNA on synaptic scaling following dopamine depletion is currently being examined.

If MEF2-dependent transcriptional events underlie synaptic scaling in PD models does it point to a potential therapy? It is difficult to see how the loss of much of the cortical connectivity with the striatum would not be a major impediment to proper movement control, making its preservation a desirable goal. However, it isn't clear that a global elevation in spiking would come without serious consequences either (Bevan et al., 2002). Recent work by our group suggests that synaptic scaling is only the first step in the attempt to restore spiking to normal levels. The second step is a down-regulation of intrinsic excitability, as seen in other cell types following sustained perturbations in activity (Desai, 2003). From a network standpoint, it is possible that increasing the reliance upon this type of adaptation, rather than synaptic scaling, would be more desirable, increasing the therapeutic attractiveness of interrupting rapid synaptic adaptations.

Experimental methods

Cell culture

Corticostriatal co-cultures were prepared as described previously (Segal et al., 2003). Striatal cultures were prepared from one to two day old mouse pups harboring a bacterial artificial chromosome transgene containing the D₂ receptor promoter and a GFP reporter construct (Heintz, 2001). Cortices were dissected from E18-19 C57BL mouse embryos. Tissues were digested with papain (Worthington Biochemical Corporation) and dissociated with 1 mL pipet tips as described elsewhere (Brewer, 1997). The striatal cells and cortical cells were mixed at a ratio of 3:1 and plated on 12 mm coverslips coated with polyethylenimine (Sigma) at a density of 1×10⁵/cm². Coverslips were placed in 24-well plates with Neurobasal A medium (Invitrogen) supplemented with 0.5 mM glutamine (Invitrogen), 1×B27 (Invitrogen), 50 mg/L penicillin/streptomycin (Invitrogen), 50 ng/mL BDNF (Sigma) and 30 ng/mL GDNF (Sigma). After initial plating, one quarter of the medium was exchanged with fresh medium without BDNF and GDNF every 3-4 days.

Drug treatment

Drug treatments were carried out after 16-20 DIV. Cultures were depolarized by adding KCl to the medium in the presence of ionotropic glutamate and GABA receptors blockers: 50 μM D-APV (Tocris), 20 μM NBQX (Sigma), 10 μM bicuculline (Sigma). In control groups, NaCl

was substituted for KCl. The following reagents were used at the indicated concentration: 10 μ M nimodipine (Sigma), 1 μ M Bay K8644 (Tocris), 2 mM EGTA (Sigma), 4 μ M cyclosporin A (Sigma), 1 μ M ascomycin (Sigma), 10 μ g/mL Actinomycin D (Tocris), 10 μ M cycloheximide (Sigma).

Transfection and constructs

pSuper-MEF2A, pSuper-MEF2D and pSuper-scramble expressing shRNAs targeting MEF2A and MEF2D mRNAs, and scrambled shRNA were described before (Flavell et al., 2006). For knockdown of MEF2, 1 μ g/ μ L EGFP, pSuper-MEF2A and pSuper-MEF2D constructs in Tris-EDTA buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0) were mixed at 2: 1: 1 (w/w). For control, 1 μ g/ μ L EGFP and pSuper-scramble were mixed at 1: 1 (w/w). Individual GFP labeled striatopallidal MSNs in 15 DIV corticostriatal co-culture were transfected by single cell electroporation (SCE), using Axoporation 800A (Axon Instruments, Union City, CA), according to manufactory protocols with some modification. Briefly, the culture on a coverslip was transferred to a 35 mm dish with hibernate A medium (Brainbits) supplemented with 0.5 mM glutamine (Invitrogen) and 1 $\times$ B27 (Invitrogen) on an invert microscope. Micropipette with a tip diameter of 0.5-0.7 μ m was filled with plasmid mixture. Individual GFP labeled striatopallidal MSNs were identified and micropipette tip was gently pressed against the cell membrane. Plasmid delivery was accomplished with 1 s train of 1 ms rectangular pulses (5-7 V) at 100 Hz. After transfection, the culture medium was replaced and the cultures were put back into the incubator. Twenty-four hours later, high potassium treatment was carried out.

Immunocytochemistry

Cultures were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4) for 20 min at room temperature. Fixed cells were incubated in blocking buffer containing 0.2% Triton X-100, 1% BSA, 5% normal goat or donkey serum (Jackson ImmunoResearch Laboratories) and 0.01% sodium azide in PBS for 1 hour at room temperature. The cultures were then exposed to primary antibody (dilution was dependent on antibody used) in blocking buffer overnight at 4°C. After brief wash in PBS, the cells were incubated with suitable secondary antibody for 1 hour at room temperature. After rinsing in PBS for 30 min, the coverslips were mounted with Prolong Gold anti-fade reagent (Invitrogen). The following primary antibodies were used: rabbit anti-GFP (1:10000, Abcam); FITC-conjugated goat anti-GFP (1:5000, Abcam); mouse anti-PSD-95 monoclonal (1:200, Affinity Bioreagents); rabbit anti-vGlut1 (1:500, Synaptic Systems); rabbit anti-D₂ dopamine receptor (1:400, Chemicon), rat anti-D₁R dopamine receptor monoclonal (1:500, Sigma), rabbit anti-Nur77 (1:500, Santa Cruz), mouse anti-MEF2 monoclonal (1:1000, Santa Cruz) and mouse anti-MEF2D monoclonal (1:200, BD Biosciences). The secondary antibodies from Invitrogen were diluted by 1:1000. Cy3 conjugated donkey anti-rat antibody (Jackson ImmunoResearch Laboratories) was diluted by 1:500. Image acquisition was performed using a NA1.4, 63X oil immerse objective in a LSM 510 META Laser Scanning Microscope (Zeiss).

Dendritic spine quantification

Images of neurons were analyzed using Metamorph image analysis software (Universal Imaging Corporation). Dendritic spines were defined as dendritic protrusions that were less than 4 μ m and were clearly connected to dendrites. For each cell, spines on 100 -150 μ m dendritic segments located at least 20 μ m away from soma were counted and spine density was calculated. To measure the spine head width, a line was drawn across the widest part of a spine (Fig. 3C). Threshold was set at half of the maximum fluorescence intensity of the line and threshold distance of the line was read as spine head width. 20-30 spines on one to two dendritic segments were analyzed in each cell, which was also used in spine density analysis. Each experimental condition was repeated at least once.

Nur77 and Arc quantification

For Nur77 quantification, cultures were stained with GFP antibody and Nur77 antibody. To visualize the nuclei, the cultures were also stained with 4,6 -diamidino-phenylindole (DAPI). A Z- stack of images for each fluorescence channel was taken with a LSM510 confocal microscope. The images were captured from randomly selected fields, but with the same microscope settings. For quantification, the images were collapsed into one plane using maximum projection. The threshold in DAPI channel was set in MetaMorph software to define the area of the nucleus. The integrated fluorescence intensity of Nur77 staining in the nucleus of a GFP labeled cell was calculated automatically.

Similar method was used to quantify Arc immunostaining. The soma area of a GFP labeled cell was defined manually in GFP channel using Metamorph software. The average somatic intensity of Arc immunostaining was measured with the software.

Fluorescence imaging of Ca^{2+}

Co-cultures containing GFP-expressing MSNs were imaged using a commercial 2P laser scanning system (Radiance 2100 MPD, Bio-Rad) with an upright microscope (BX51, Olympus) and 60X water immersion objective (0.9 NA, Olympus). The scanhead was optically coupled to a Ti:sapphire pulsed infrared laser (Chameleon Ultra, Coherent) whose output intensity was regulated by an electro-optical modulator (M350-80, Conoptics). Excitation of GFP was performed at 950 nm, and emission collected at 525 $\pm$ 25 nm by a multialkali photomultiplier tube. Single images were formed by integrating (accumulating) six scans of 512 pixels $\times$ 512 pixels $\times$ 8-bits, and z-stacks were formed using 0.7 μm step size. Projected images were formed from z-stacks in order to visualize simultaneously cell bodies and dendrites. We selected areas containing one to three EGFP-expressing cells for examining Ca^{2+} responses. Ca^{2+} imaging was performed on co-cultures loaded with 10 μM fura-2/AM (Invitrogen/Molecular Probes) in Hank's Buffered Salt Solution (HBSS) for 60-90 min at 37 $^{\circ}$ C, washed in HBSS, and imaged at room temperature in Hibernate A medium (BrainBits). Ratiometric 2P imaging of Ca was performed using sequential excitations at 700 and 780 nm (five images per wavelength collected at 1 Hz) providing a ratio image every 10 sec. Emission was collected in 8-bit photon-counting mode using custom software (VB script, Microsoft) and laser dwell time of 6 μsec per pixel. Laser power at the sample was controlled by custom software (PowerCal, Dr. John Dempster, Univ. of Strathclyde, Scotland) and maintained at 5-6 mW for each wavelength. Hibernate A medium with, and without, high potassium was delivered by a gravity-fed system, which allowed complete exchange of bath contents within 2 minutes. The ratiometric system was calibrated using known Ca^{2+} -EGTA standards (Invitrogen/Molecular Probes) added to fura-2 K-salt (Invitrogen/Molecular Probes) in PBS imaged in microwell chambers following established procedures (Grynkiewicz et al., 1985).

Electrophysiology

Striatal cells and cortical cells were co-cultured for 21 days. GFP labeled striatopallidal MSNs were identified visually before recording. The external solution contained (in mM): NaCl 129; KCl 4; MgCl_2 1; CaCl_2 2; HEPES 10; glucose 10; bicuculline 0.010; TTX 0.0005. The pH of the solution was adjusted to 7.4 and osmolarity to 300 mOsm/L. The internal solutions was (in mM) potassium gluconate 136.4; KCl 17.5; NaCl 9; MgCl_2 1; HEPES 10; EGTA 0.2; pH 7.4; 290-300 mOsm/L. Miniature AMPA-mediated excitatory postsynaptic currents (mEPSCs) were measured from whole-cell voltage patch-clamp recordings with a gap-free recording using Pulse 8.4 software data acquire system (HEKA, Germany). Signals were low-pass filtered at 1 kHz, and digitized (sampled) at 10 kHz and were amplified with an Axopatch 200B patch-clamp amplifier (Axon Instruments). EPSCs were recorded at a holding potential of -70 mV at room temperature ($\sim 22^{\circ}\text{C}$). Patch pipettes were pulled from borosilicate glass and had a resistance of approximately 3-5 $\text{M}\Omega$. Internal pipette solution contained the following (in

mM): CsMeSO₃ 120; NaCl 5; TEA-Cl 10; HEPES 10; QX314 5; EGTA 1.1; ATP-Mg₂ 4; GTP-Na₂ 0.3; pH 7.2 adjusted with CsOH; 270–280 mOsm/L. Electrophysiological signals were analyzed using Clampfit 9.2 (Axon Instruments) and Mini Analysis Program 6.0.3 (Synaptosoft).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Medium spiny neurons in cortical co-cultures have mature dendritic architecture and synaptic connectivity. (A) Scheme of the preparation of corticostriatal co-culture. (B) Quantification of the spine density of EGFP labeled neurons in pure striatal cultures and corticostriatal co-cultures. Spine density was significantly high in co-cultures (Striatum, median=2.8, n=14; Co-culture, median=11.0, n=14; ***p<0.001, Mann-Whitney Rank Sum test). (C) A EGFP-labeled neuron in a pure striatal culture. (D) to (G), Images of EGFP-labeled neurons in corticostriatal co-cultures stained with antibodies against PSD95, vGlut1, D₂R or D₁R. Scale bar: low magnification images, 10 μ m; high magnification images 5 μ m.

Fig. 2.

Membrane depolarization induces Ca^{2+} influx and spine loss. (A) Membrane depolarization of D₂ MSNs in response to elevated extracellular potassium concentration (mM, n=5 for each concentration). The membrane potentials measured correlate to those predicted by Nernst equation. (B-E) Membrane depolarization induces L-type Ca^{2+} channel-dependent Ca^{2+} elevation in D₂ MSNs. Images of Fura-2 AM loaded D₂ MSNs in Corticostriatal co-culture were captured using two-photon microscopy. Images of two EGFP-labeled cells stimulated with 35 mM KCl in presence of ionotropic receptor blockers are shown at excitation wavelength 950 nm (B), and 700 nm (C) and 780 nm (D). Scale bar, 10 μ m. Ca^{2+} concentration in the somas of D₂ MSNs was determined by computing the ratio 700/780 images. (E) Changes of Ca^{2+} concentration relative to baselines are shown as a function of time for D₂ MSNs stimulated by membrane depolarization (n=4, black traces) or D₂ MSNs stimulated in the presence of 10 μ M nimodipine (n=6, red traces). (F) A D₂ MSN in corticostriatal co-cultures treated with 35 mM KCl for 24 hours in presence of ionotropic receptor blockers at 20 DIV. Bar: upper panel

10 μ m; lower panel, 5 μ m. (G) Time course of the change of spine density in D₂ MSNs after membrane depolarization. Spine density is shown in mean $\pm$ standard deviation ($p<0.001$, one way ANOVA; Ctrl, 11.69 ± 1.66 , $n=12$; 8 hrs, 10.41 ± 1.13 , $n=16$; 16 hrs, 8.42 ± 1.99 , $n=14$; 24 hrs, 5.79 ± 0.96 , $n=14$). (H) Spine losses in D₂ and D₁ MSNs after 24 hours of membrane depolarization. (35 mM KCl treated groups are shown in shadows. D₂ MSNs control, median=11.3, $n=21$; D₂ MSNs with 35 mM KCl, median=5.3, $n=23$; D₁ MSNs, median=10.8, $n=21$; D₁ MSNs with 35 mM KCl, median=9.5, $n=24$. * $p<0.05$, *** $p<0.001$, Mann-Whitney Rank Sum Test)

Fig. 3.

L-type Ca^{2+} channels are necessary for spine and synapse elimination. (A) Images of D₂ MSNs in corticostriatal co-cultures treated with 35 mM KCl and ionotropic receptor blockers for 24 hours, in absence or presence of 10 μM nimodipine. Bar, upper panels 10 μm ; lower panels, 5 μm . (B) Quantification of spine density showing that nimodipine blocked the membrane depolarization-induced spine loss (control, median=11.9, n=15; +K⁺, median=5.6, n=18; +K⁺+nimodipine, median=11.9, n=13). (C) Cumulative frequency plot of spine head width showing that nimodipine blocked the reduction of spine size induced by membrane depolarization (control, median=0.5, n=412; +K⁺, median=0.40, n=410; +K⁺+nimodipine, median=0.50, n=333; +K⁺ vs. control and +K⁺ vs. +K⁺+nimodipine, $p < 0.001$, Mann-Whitney

Rank Sum Test). Insert showing method of measuring the spine head width in Metamorph software. Scale bar, 2 μ m. (D) Examples of mEPSCs recording from the D₂ MSNs treated as in (A). (E) Box plot showing membrane depolarization resulted in reduction of mEPSC frequency (control, median=2.17, n=19; +K⁺, median=1.29, n=14), which was blocked by nimodipine (+K⁺+nimodipine, median=2.92, n=18). (F) Box plot showing membrane depolarization resulted in reduction of mEPSC amplitude (control, median=15.74, n=19; +K⁺, median=11.89, n=14), which was blocked by nimodipine, (+K⁺+nimodipine, median=18.15, n=18). *p<0.05, ***p<0.001, Mann-Whitney Rank Sum Test.

Fig. 4.

Enhanced L-type Ca^{2+} channel opening increases the effects of membrane depolarization. (A) Images of D₂ MSNs in corticostriatal co-cultures treated with 20 mM KCl and ionotropic receptor blockers for 24 hours, in the absence or presence of 1 μM Bay K8644. Bar: upper panels 10 μm ; lower panels, 5 μm . (B) Quantification of spine density showing that Bay K8644 treatment decreased spine density in the D₂ MSNs depolarized by 20 mM KCl (+K⁺, median = 10.1, n = 15; +K⁺+Bay K8644, median = 5.9, n = 14). (C) Quantification of spine head width showing Bay K8644 treatment decreased the spine size in the D₂ MSNs depolarized by 20 mM KCl (+K⁺, median = 0.50, n = 336; +K⁺+Bay K8644, median = 0.45, n = 335; $p < 0.001$, Mann-Whitney Rank Sum Test). (D) Examples of mEPSCs recording from the D₂ MSNs treated as

in (A). (E) Box plot showing that Bay K8644 treatment reduced mEPSC frequency in D₂ MSNs depolarized by 20 mM KCl (+K⁺, median = 3.46, n=16; +K⁺+Bay K8644, median=1.82, n=12). (F) Box plot showing that Bay K8644 treatment had no significant effect on mEPSC amplitude (+K⁺, median = 17.09, n=16; +K⁺+Bay K8644, median=16.82, n=12; p=0.981 Mann-Whitney Rank Sum Test). **p<0.005, ***p<0.001, Mann-Whitney Rank Sum Test.

Fig. 5. Cav1.2 but not Cav1.3 L-type Ca²⁺ channels are required for membrane depolarization-induced spine loss. (A) Expression of Cav1.2 L-type Ca²⁺ channel in a D₂ MSN. Lower panel shows dendritic expression of Cav1.2 L-type Ca²⁺ channel. (B) A D₂ MSN in corticostriatal co-culture treated with 35 mM KCl and ionotropic receptor blockers for 24 hours in the presence of 1 μM nimodipine. (C) A Cav1.3 deficient D₂ MSN in corticostriatal co-culture treated with 35 mM KCl and ionotropic receptor blockers. (D) Quantification of spine density shows that 1 μM Nimodipine treatment blocks the membrane depolarization-induced spine loss (+K⁺, median=6.2, n=15; +K⁺+1 μM nimodipine, median=13.2, n=15), and membrane depolarization induces spine loss in D₂ MSNs deficient of Cav1.3 Ca²⁺ channels (control, median=10.0, n=14;

+K⁺, median=3.9, n=17). ***p<0.001, Mann-Whitney Rank Sum Test. Scale bar: low magnification images, 10 μ m; high magnification images 5 μ m.

Fig. 6.

Calcineurin and protein synthesis are necessary for spine pruning. (A) A D₂ MSN in a corticostriatal co-culture treated with 35 mM KCl and ionotropic receptor blockers for 24 hours in the presence of calcineurin inhibitors ascomycin (1 μM) and cyclosporin (4 μM). (B) Quantification of spine density in D₂ MSNs treated as indicated. Calcineurin inhibitors attenuated the membrane depolarization-induced spine loss (+K⁺, median=5.2, n=15; +K⁺+Asc/CsA, median=7.9, n=15). (C) A D₂ MSN in a corticostriatal co-culture treated with 35 mM KCl and ionotropic receptor blockers for 24 hours in the presence of protein synthesis inhibitor cycloheximide (10 μM). (D) Quantification of spine density in D₂ MSNs treated as indicated. Cycloheximide attenuated the membrane depolarization-induced spine loss (+K⁺, median=5.8, n=16; +K⁺+CHX, median=10.8, n=15) ***p<0.001, Mann-Whitney Rank Sum Test. Scale bar: low magnification images, 10 μm; high magnification images 5 μm.

Fig. 7.

MEF2 activity is necessary for membrane depolarization-induced spine loss in D₂ MSNs. (A) D₂ MSNs transfected with indicated shRNA expressing constructs at 15DIV and stained with generic anti-MEF2 antibody or anti-MEF2D antibody 48 hours later. Transfected D₂ MSNs are shown in yellow squares, while untransfected ones are shown in blue squares. Scale bar, 20 μm. (B) A D₂ MSN in corticostriatal co-culture transfected with MEF2 shRNA and treated with 35 mM KCl and ionotropic receptor blockers for 24 hours. Scale bar: low magnification images, 10 μm; high magnification images 5 μm. (C) Quantification of spine density in D₂ MSNs treated as indicated. Knockdown of MEF2 blocks membrane depolarization-induced spine loss in D₂ MSNs (+K⁺+Scrambled shRNA, median=4.2, n=15; +K⁺+MEF2A/2D shRNA, median=7.9, n=15). *** p<0.001, Mann-Whitney Rank Sum Test.

Fig. 8. L-type Ca^{2+} channel- and calcineurin-dependent induction of Nur77 expression in D₂ MSNs in response to membrane depolarization. (A) Images of D₂ MSNs treated with 35 mM KCl and ionotropic receptor blockers for 24 hrs in absence or presence of nimodipine or Ascomycin/Cyclosporin A. Cultures were stained with anti-GFP antibody (Green), anti-Nur77 antibody (red) and 4,6-diamidino-phenylindole (DAPI, blue). (B) A representative image at a focal plane (1 micron thick) through the soma of a depolarized cell marked in (A) showing that most of Nur77 staining is localized in nucleus. (C) Quantitative analysis of Nur77 staining in the nuclei of D₂ MSNs showing that KCl treatment increases intensity of Nur77 staining (control, median=4318, n=99; +K⁺, median=9206.5, n=198), and nimodipine or Asc/CsA blocks the depolarization-induced Nur77 increase (+K⁺+nimodipine, median=3014.5, n=198; +K⁺+Asc/

CsA, median=1876.5, n=104). *** $p < 0.001$, Mann-Whitney Rank Sum Test. Scale bar: low magnification images, 10 μm ; high magnification images 5 μm .

Fig. 9.

Membrane depolarization induces MEF2-dependent Arc expression. (A) A D₂ MSN in a corticostriatal co-culture treated with 35 mM KCl and ionotropic receptor blockers for 2 hours and stained with anti-GFP and anti-Arc antibodies. High magnification images (right panels) show Arc expression in dendrites. (B) Quantification of average fluorescence intensity of Arc immunostaining in the soma area of D₂ MSNs depolarized for 2 hours and 6 hours. (control median=2.47, n=33; 2 hours with K⁺, median=27.28, n=21; 6 hours with K⁺, median=11.4, n=25). (C) Upper panel shows the image of a D₂ MSNs in non-transfected culture. Middle and lower panels show the images of D₂ MSNs in corticostriatal co-cultures transfected with indicated shRNAs and depolarized for 2 hours. Transfected cells are shown in yellow squares, an untransfected cells is shown in a blue square. Note that different microscope setups were used for experiments in (A) and (C). (D) Quantification showing MEF2 RNAi significantly reduces membrane depolarization-induced Arc expression (scrambled shRNA, median=23.13, n=13; MEF2A/2D RNAi, median=15.13, n=15). **p<0.005, *** p<0.001. Mann-Whitney Rank Sum Test. Scale bar: low magnification images, 10 μ m; high magnification images 5 μ m.