Calcium signaling cascade links dopamine D1–D2 receptor heteromer to striatal BDNF production and neuronal growth

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Although the perturbation of either the dopaminergic system or brain-derived neurotrophic factor (BDNF) levels has been linked to important neurological and neuropsychiatric disorders, there is no known signaling pathway linking these two major players. We found that the exclusive stimulation of the dopamine D1–D2 receptor heteromer, which we identified in striatal neurons and adult rat brain by using confocal FRET, led to the activation of a signaling cascade that links dopamine signaling to BDNF production and neuronal growth through a cascade of four steps: (i) mobilization of intracellular calcium through Gq, phospholipase C, and inositol trisphosphate, (ii) rapid activation of cytosolic and nuclear calcium/calmodulin-dependent kinase IIα, (iii) increased BDNF expression, and (iv) accelerated morphological maturation and differentiation of striatal neurons, marked by increased microtubule-associated protein 2 production. These effects, although robust in striatal neurons from D5−/− mice, were absent in neurons from D1−/− mice. We also demonstrated that this signaling cascade was activated in adult rat brain, although with regional specificity, being largely limited to the nucleus accumbens. This dopaminergic pathway regulating neuronal growth and maturation through BDNF may have considerable significance in disorders such as drug addiction, schizophrenia, and depression.

brain-derived neurotrophic factor activation | calcium signaling pathway | calcium/calmodulin-dependent kinase II | neuronal maturation | GPCR oligomerization

Dopamine promotes neuronal differentiation, maintenance, and survival (1–4) by modulating the transcription of different genes. Little however, is known regarding the molecular events that govern these dopamine-mediated effects. Evidence has emerged indicating a positive relationship between functions mediated by dopamine and brain-derived neurotrophic factor (BDNF) and its receptor TrkB (2–7). However, a direct mechanism bridging dopamine signaling to BDNF has not yet been described. Classically, dopamine exerts its actions through D1-like (D1, D5) and D2-like (D2, D3, D4) receptors, which regulate activation or inhibition of cAMP accumulation, through Gs/olf or Gi/o proteins, respectively (8). Other signaling cascades have also been reported (9, 10), including phosphatidylinositol turnover in brain through D1-like receptor activation (11, 12), but no such activation was observed when the cloned D1 receptor was expressed (13–15). These observations led us to the discovery of the dopamine D1–D2 receptor heteromer, which is able to mobilize intracellular calcium (15–18). However, the signaling cascade and the physiological functions of the dopamine D1–D2 receptor heteromer in brain are unknown.

Because calcium is involved in the activation of BDNF signaling (19), we hypothesized that this pathway may be central to dopamine activation of BDNF and subsequent neuronal maturation and differentiation.

In this context, we describe a signaling pathway that links dopamine action through the D1–D2 receptor heterooligomer to the expression of BDNF in postnatal striatal neurons and in adult rat brain by a mechanism involving activation of Gq, phospholipase C (PLC), the mobilization of intracellular calcium, activation of cytoplasmic and nuclear calcium/calmodulin (CaM)-dependent kinase IIα (CaMKIIα) and subsequently an increase in BDNF expression. Furthermore, we also highlight the physiological consequences resulting from the activation of this signaling pathway on neuronal maturation and growth during development and its existence in nucleus accumbens of adult rat brain. Finally, using confocal FRET, we demonstrate the presence of the D1–D2 receptor heterooligomer as a physical entity in striatal neurons and rat brain.

Results

Dopamine D1 and D2 Receptors Form Heterooligomers in Striatal Neurons. Immunocytochemistry revealed the majority of postnatal striatal neurons in culture for 7–21 days expressed D1 and D2 receptors mainly at the cell surface and on neurites with a high degree of colocalization in >90% of the neurons (Fig. L4 and Fig. S1a). Confocal FRET analysis showed that D1 and D2 receptors were in close proximity with a relative distance of 5–7 nm (50–70 Å) localized in microdomains, where FRET efficiency (E) ranged from 0.1 to 0.5, higher in the soma and proximal dendrites and lower in distal processes (Fig. 1B and Fig. S1b). Coinnunoprecipitation of D1–D2 receptor complexes from the striatum is shown (Fig. S1c). Data showed D2 receptor as a broad band of 55–70 kDa protein and an oligomeric form >170 kDa, whereas D1 receptor existed as bands at ∼55, ∼70, and ∼75–80 kDa, probably caused by different degrees of glycosylation. The data clearly indicate that D1 and D2 receptors can be coinmunoprecipitated from the striatum.

Coexpression, together with the coinmunoprecipitation, and the proximity of the receptors within same neurons indicated a physical interaction and heteromer complex formation between the natively expressed dopamine D1 and D2 receptors.

Intracellular Calcium Mobilization Through the Dopamine D1–D2 Receptor Heteromer. The calcium signaling pathway was evaluated by using camelone (20). In the absence of extracellular calcium, agonist treatments showed rapid increases in camelone FRET, reflecting an immediate rise in intracellular calcium (Fig. 1B Insets). Both dopamine and SKF 89395, a D1-D2 heteromer agonist (17, 18), dose-dependently mobilized intracellular calcium (Fig. 1B), with a higher maximal peak effect for dopamine (Emax 0.5 versus


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Ca\(^{2+}\) mobilization leads to rapid (insets) and dose-dependent mobilization of intracellular Ca\(^{2+}\) by dopamine or SKF 83959. (C) D1 antagonist SCH (10 \(\mu\)M) or D2 antagonist raclopride (10 \(\mu\)M) abolished intracellular Ca\(^{2+}\) mobilization triggered by D1–D2 receptor heteromer activation by 100 nM dopamine or 100 nM SKF 83959. (D) 2-APB (100 \(\mu\)M), inhibitor of IP3 receptors, abolished the intracellular Ca\(^{2+}\) increase elicited by 100 nM SKF 83959. (E) SQ 22536 (SQ, 10 \(\mu\)M), an adenylyl cyclase inhibitor, had no effect on dopamine-induced Ca\(^{2+}\) mobilization. Inhibitor of Gi/o proteins had no effect on dopamine-induced Ca\(^{2+}\) mobilization. *, significant difference from basal (P < 0.01). Data represent mean ± SEM of values from at least three different experiments.

Characteristics of the D1–D2 Receptor Heteromer-Mediated Calcium Release. Calcium mobilization studies performed in the absence of extracellular calcium excluded calcium entry via calcium channels. The exclusive involvement of intracellular calcium was confirmed by preincubation with 10 \(\mu\)M thapsigargin (TPG), which attenuated calcium release caused by SKF 83959 by 80% and that caused by dopamine by \(\approx 75\%\) (Fig. S2e). The source of intracellular calcium was assessed with 100 \(\mu\)M 2-aminoethoxydiphenyl borate (2-APB), which inhibits inositol trisphosphate (IP\(_3\)) receptor-dependent calcium stores and abolished calcium mobilization elicited by SKF 83959 (Fig. 1D).

Lack of involvement of the G\(_{s}\)-AC pathway in calcium mobilization by the D1–D2 receptor heteromer was shown by SQ22536 (SO; 10 \(\mu\)M), an inhibitor of AC, which had no effect on dopamine-dependent mobilization of intracellular calcium (Fig. 1E). Pertussis toxin (PTX), an inhibitor of G\(_{i/o}\) proteins, had no effect on the calcium release induced by SKF 83959 or dopamine (Fig. 1F), suggesting noninvolvement of G\(_{i/o}\) proteins in this process. G\(_{q}\) involvement was assessed by preincubation with YM 254890 (YM; 100 nM), a G\(_{q}\)-specific inhibitor (22). No significant effect on basal calcium levels was noted, but SKF 83959- and dopamine-triggered calcium mobilization was inhibited by 90% (Fig. 2A), implicating a role for G\(_{q/11}\). Immunocytochemistry of G\(_{q/11}\) (Fig. 2B) revealed it localized largely in the cytosol under basal conditions.
neurons were treated with 100 nM SKF 83959 or dopamine for 2–5 min, Gq was more concentrated at the cell surface and less present in the cytosol. Quantification of Gq fluorescence (Fig. S3) revealed agonists increased Gq at the cell surface by 28 ± 2.7% after 2 min and ∼65 ± 4% after 5 min (Fig. S3) of treatment, whereas cytosolic labeling decreased by 21% and 50%, respectively (Fig. S3). Together, these results indicated the involvement of Gq and the exclusion of Gs and Gi/o proteins, in calcium mobilization through the D1–D2 receptor heteromer.

The involvement of Gq and sensitivity of the calcium stores to IP₃ suggested a role for PLC. Treatment with U73122, a PLC inhibitor, attenuated calcium elevations seen with SKF 83959 and dopamine (Fig. 2C), indicating that PLC was involved in the calcium mobilization triggered by activation of the D1–D2 receptor heteromer. Thus identified is a signaling pathway in striatal neurons, whereby activation of the dopamine D1–D2 receptor heteromer led to the subsequent rapid activation and translocation of Gq, activation of PLC and mobilization of intracellular calcium from stores sensitive to IP₃.

**Induction of CaMKIIα Activation and Nuclear Translocation.** Because intracellular calcium changes affect CaM, we investigated whether CaMKIIα was modulated through the D1–D2 receptor heteromer-dependent calcium signal. In the absence of extracellular calcium, striatal neurons were treated with vehicle, SKF 83959, or dopamine for 2 or 5 min (Fig. 2D). In the basal state, a weak signal for phosphoCaMKIIα (pCaMKIIα) was observed mainly in the cytosol. Upon treatment, total pCaMKIIα fluorescence increased ∼2- and 4-fold after 2 and 5 min, respectively (Fig. 2E). Not only was there an increase in cellular pCaMKIIα levels, but there was a robust increase of pCaMKIIα in the nucleus (Fig. 2D). At basal state, no pCaMKIIα was observed in the nucleus, suggesting that it either translocated from the cytosol to the nucleus or preexisting nuclear CaMKIIα was activated. Quantification of nuclear pCaMKIIα levels showed that the effect was rapid, occurring within 2 min of treatment, and further dramatically increased after 5 min of treatment, when nuclear pCaMKIIα levels rose over basal by >12-fold (Fig. 2F). The SKF 83959-induced activation of CaMKIIα was blocked by raclopride (Fig. S4b), indicating the involvement of both dopamine D1 and D2 receptors. CaMKIIα was similarly activated in striatal neurons from D5−/− mice (Fig. S4a) treated (5 min) with dopamine or SKF 83959. Here again, the increase in pCaMKIIα in cytosol was accompanied by an increase in the nucleus (Fig. S4). No CaMKIIα activation by dopamine or SKF 83959 was observed in neurons from D1−/− mice. Taken together, these results indicated that calcium signaling, elicited by specific activation of the dopamine D1–D2 receptor heteromer, activated CaMKIIα in the cytosol and in the nuclei of striatal neurons with no involvement of the D5 receptor.

**Activation of BDNF in Striatal Neurons.** Because BDNF gene expression is modulated by nuclear isoforms of CaMKII (23), we assessed whether BDNF was activated by the D1–D2 heteromer signaling pathway. In the absence of extracellular calcium in the basal state, BDNF immunolabeling showed a weak expression level, mainly localized to cytosol, with little or no BDNF expression in neurites and axons (Fig. 3A, control). Treatment with dopamine or SKF 83959 showed a time-dependent increase in BDNF levels, evident after 1 h and significantly higher after 2 h at three and seven times higher than basal levels (Fig. 3A and B). It was noted that a
Consequences of Activating the D1–D2 Signaling Pathway: Neuronal Growth and Differentiation. Because the D1–D2 signaling cascade reached the nucleus through CaMKIIα and activated local synthesis of BDNF, we surmised a link may exist between the activation of the calcium signaling pathway and neuronal developmental and/or survival signaling by BDNF. Striatal neurons were treated with 10–100 nM SKF 83959 or dopamine from postnatal days 4–10 and compared with vehicle treatment. Treatments were performed overnight (6–14 h) in the absence of calcium, followed by removal of treatment media and replacement by fresh culture media. A significant proportion of neurons treated by the dopaminergic agonists differentiated earlier (Fig. 3D and Fig. S5), with a phenotype equivalent to that of neurons in culture for at least 15 days (Fig. 3D), when they usually reach maturation (3, 4). After agonist treatment, the neurons were no longer isolated as usually seen up to day 10, but instead had enhanced growth of neurites, forming connections with others at a distance, indicative of a more differentiated and mature stage (Fig. 3D and Fig. S5). We confirmed these observations by microtubule-associated protein 2 (MAP2) labeling (Fig. 3E and Fig. S5A), which is the major protein that regulates the structure and stability of microtubules, neuronal morphogenesis, cytoskeleton dynamics, and organelle trafficking in axons and dendrites (1). In newborn rat brain, MAP2a appears between postnatal days 10 and 20, during the dendritic growth phase when neurons have reached their mature morphology (1). In untreated cells, MAP2 labeling was restricted to the cell body and certain small processes, probably axons (Fig. 3E and Fig. S5A). In contrast, striatal neurons treated by SKF 83959 or dopamine showed dense MAP2 expression, within long processes extending between neurons making contact. This finding indicated that more MAP2, probably MAP2a, was produced because of dopamine agonist treatments and led to accelerated neuronal maturation and growth. The SKF 83959 effect was inhibited in the presence of raclopride, indicating the involvement of both D1 and D2 receptors (Fig. S5B). Moreover, this effect, while present in neurons derived from D5/–/– mice (Fig. S5C), was absent in neurons derived from D1/–/– mice (Fig. S5D), underscoring the necessary involvement of the D1 receptor. These data, as a whole, suggested that activation of the D1–D2 receptor heteromer signaling pathway was a key cascade transducing dopaminergic signals involved in the development, maturation, and differentiation of striatal neurons, through activation of BDNF signaling.

Evidence of Activation of the D1–D2 Receptor Heteromer Signaling Pathway in Adult Rodent Brain. Colocalization of D1 and D2 receptors in some neurons within the striatum (24–27), although representing an important finding, does not indicate whether such receptors form heteromers. Immunohistochemistry in rat striatum showed that certain neurons coexpressed D1 and D2 receptors, whereas others expressed only one of the receptors. In caudate putamen, fewer neurons coexpressed D1 and D2 receptors. Within the nucleus accumbens, there were more neurons coexpressing D1 and D2 receptors, mainly at the cell surface (Fig. 4 and Fig. S6). Confocal FRET analysis using fluorophore-labeled antibodies measured the interaction between the colocalized receptors, as illustrated for nucleus accumbens shell (Fig. 4) and core (Fig. S6). FRET analysis showed that in most neurons where D1 and D2 receptors were colocolated the receptors were physically close enough to generate a FRET signal, whereas, noncolocalized receptors were unable to generate any FRET (Fig. 4B and Fig. S6). The distance between the receptors showed D1 and D2 were in close proximity with an average distance of 4–7 nm (40–70 Å), and high FRET efficiency was detected in nucleus accumbens. These data clearly indicated in adult rat nucleus accumbens the presence of neurons coexpressing D1 and D2 receptors, which existed close enough to permit energy transfer and therefore may be considered to be physically interacting, forming heteromer receptors.

We then assessed whether BDNF expression in the adult rat striatum could be stimulated through activation of the D1–D2 heteromor. Rats were treated once daily for a total of three injections with saline (control), 0.4 mg/kg of SKF 83959, or 0.4 mg/kg of SKF 83822. Changes in BDNF expression were evident in nucleus accumbens (Fig. 4C) and not in caudate nucleus, which was in keeping with the distribution of the D1–D2 heteromers observed. Compared with saline-treated rats, SKF 83959-treated animals showed a 49 ± 9% increase in the number of BDNF-positive cells in nucleus accumbens core, whereas no increase was observed in SKF 83822-treated rats (Fig. 4D). Quantification of expression in individual neurons by densitometry showed that, compared with saline-treated animals, the level of BDNF increased in cells from animals treated with SKF 83959 (Fig. 4D Upper Right), whereas no increase was observed in SKF 83822-treated animals, confirming that the activation of the calcium pathway, and not the cAMP pathway, increased both the number of cells expressing BDNF and the concentration of BDNF within cells in the nucleus accumbens core. The number of BDNF-positive neurons of control rats was higher in the nucleus accumbens shell region than core region (Fig. 4D Left). This number was not significantly different from saline-treated rats in the shell region of SKF 83959- or SKF 83822-treated rats (Fig. 4D Lower Left), although a small increase was observed in SKF 83959-treated animals. However, when BDNF expression was quantified by densitometry in individual shell neurons the level of BDNF in cells from animals treated with SKF 83959 increased about 49 ± 9% (Fig. 4D Lower Right).
by a significant 26.8% compared with cells from saline-treated animals (Fig. 4D Lower Right), whereas no increase was observed in cells from animals treated with SKF 83823.

This finding suggests that the activation of the calcium pathway, and not the cAMP pathway, increased the production of BDNF within the nucleus accumbens core and shell neurons of adult rat brain after D1–D2 receptor heteromer activation.

Discussion
We describe a dopaminergic signaling pathway occurring in neonatal striatal neurons and adult rat brain, involving activation of the dopamine D1–D2 receptor hetero-oligomer-, Gq-, PLC-, and IP3 receptor-mediated intracellular calcium mobilization, resulting in CaMKII activation in both cytosolic and nuclear compartments, leading to enhanced BDNF production. This process resulted in enhanced neuronal maturation, differentiation, and growth. We also showed through confocal FRET the occurrence of heteromers of endogenously expressed native dopamine D1 and D2 receptors in striatal neurons in culture and in adult rat brain in situ.

We showed that ≈90% of the cultured striatal neurons coexpress both D1 and D2 receptors. However, this number was lower in adult rat brain than in the cultured neurons and varied among the regions, with a high of ≈25% in the nucleus accumbens, and a lower degree of colocalization in the caudate putamen (CPu) (≈6%). Our results are consistent with the reported levels of D1 and D2 colocalization in cultured neurons and adult rat striatum. In fact, the D1 and D2 colocalization in the striatum has been a matter of long debate. It is believed that D1 and D2 receptors are localized in two anatomically segregated sets of neurons, forming the striatonigral D1-enriched direct pathway and the striatopallidal D2-enriched indirect pathway (28–30). Data from BAC transgenic mice, using EGFP driven by D1 or D2 receptor promoters, were consistent with this view (30–35). BAC data analysis also showed that at least 10–17% of medium spiny neurons (MSNs) coexpress both D1 and D2 receptors (30, 34, 35), with higher prevalence in ventral striatum (up to 17%) than in dorsal striatum (≈1–6%) (34, 35). Evidence for neuronal colocalization of D1 and D2 receptors has also been indicated by electrophysiological studies (36), immunohistochemistry (24, 25), electron microscopy (37), and retrograde labeling (26). In dissociated, cultured striatal neurons from fetal (38), neonatal (39), and 2- to 3-week-old (40) rats D1 and D2 were colocalized in a significantly higher number of neurons (60–100%) than in the adult striatum (15, 24, 27, 38, 39). The discrepancy between the results observed in adult striatum versus those observed in cultured striatal neurons may be caused in part by the lack in the cultures of afferents and glial cells. Another explanation may be a regulation of each receptor type during development, leading to different levels of receptor colocalization within brain regions.

Our study indicates that these colocalized receptors were in close physical proximity allowing the formation of receptor hetero-oligomers, both in striatal neurons in culture and in brain tissue. Furthermore, no known functional relevance has been attached to the neurons where D1 and D2 receptors colocalized, and our findings indicate that the receptor heteromers have a unique signaling pathway linking dopamine and BDNF through a rapid rise in calcium signaling and CaMKII activation. This finding may indicate a more significant role for the dopamine-activated calcium signaling cascade during striatal development, whereas it is relegated to a more specialized and circumscribed role in the adult striatum, largely confined to nucleus accumbens. These findings also suggest that in addition to the two separate direct and indirect pathways, another set of neurons form a third pathway, where both receptors interact to generate another specialized signal. This may help to explain, at least in part, the cooperativity between D1 and D2 receptors observed by many (40, 41).

Dopamine-induced control of gene expression, which is important in long-term synaptic plasticity (42, 43), has been shown to occur in striatum and other brain regions (43), but the molecular mechanisms of the information transfer from the cytoplasm to the nucleus of striatal neurons are still poorly understood (44). Our results showing activation of CaMKIIα both in the cytoplasm and nucleus, as an immediate consequence of the D1–D2 heteromer stimulation and calcium release, represents an expedient mechanism by which this information could lead to rapid gene regulation. Whether activated CaMKIIα was directly translocated to the nucleus or an intermediary component, such as the recently reported dopamine-controlled inhibition of nuclear protein phosphatase-1 (44), is responsible for the activation of preexisting nuclear CaMKIIα remains to be determined.

After the increase in CaMKIIα activity, we observed an increase in BDNF expression. We also observed, in rat brain, a significant increase in the production of BDNF by neurons from the nucleus accumbens, suggesting a regional specificity for the activation of this pathway in adult brain. It is notable that these results suggested that BDNF could be synthesized locally by striatal neurons. Thus, there exists a direct link between dopaminergic signaling and BDNF expression through activation of the D1–D2 receptor heteromer. Repeated activation of the D1–D2 heteromer pathway led to accelerated growth of the neurites and connections between striatal neurons, indicating enhanced maturation and differentiation of striatal neurons, with notably increased MAP2 expression. These results are consistent with the ability of dopamine and BDNF to promote neuronal maturation, differentiation, and survival and to stimulate lengthening and arborization of neuronal processes (1–4). Our results, modeled in Fig. 5, suggest that the effects of dopamine in this particular case are mediated through the D1–D2 receptor heteromer-, Gq-, PLC-, IP3-dependent signaling pathway, using calcium as a second messenger, which is responsible for CaMKIIα activation, notably in the nucleus, where it stimulates BDNF synthesis, which in turn activates protein synthesis responsible for neuronal maturation and differentiation. In adult brain, this signaling pathway is region-specific and highly circumscribed, likely confined to the neurons expressing the D1–D2 receptor heteromer.

Because of the major roles played by both dopamine and BDNF in many aspects of neuronal maturation and survival, any disequilibrium in the presently described D1–D2 heteromer pathway linking dopamine to BDNF may have dramatic consequences that undermine neuronal morphology, adaptation, and survival, potentially leading to neuropsychiatric disorders. Alterations in BDNF levels caused by complications in the prenatal development period, early childhood events, or adult stress were associated with neuropsychiatric disorders such as schizophrenia (45), depression (46), or drug addiction (47). A neurotrophin hypothesis linking dysfunction of BDNF to the emergence of symptoms of schizophrenia has been postulated (48), and there are reports of associations between dysfunction of calcium signaling and its related proteins, including Gq, IP3, and CaMKII, with schizophrenia (49). A link between D1 and D2 receptors was shown to be missing in postmortem striata from patients with schizophrenia and Huntington disease (50).

The importance of the different receptor signaling complexes in mediating specific dopamine functions is being revealed. Ultimately, alterations of one dopamine receptor in early developmental stages, or even in the adult, may induce differences in the balance of the heteromeric/homomeric complexes and be at the origin of complicated diseases, such as schizophrenia, depression, or drug addiction.

In summary, we have demonstrated a signaling pathway triggered by the activation of the dopamine D1–D2 receptor heteromer that bridges the action of dopamine to BDNF expression, with an important physiological function of neuronal maturation and growth. A dopamine signaling pathway using calcium as a second messenger, targeting the nucleus through CaMKII activation and leading to enhanced BDNF production, may potentially be of considerable importance in the postnatal development of striatal neurons and in nucleus accumbens function in the adult.
signaling cascade described here may indicate the presence of a third pathway consisting of neurons containing D1 and D2 receptor heteromers, in addition to the already well-characterized direct and indirect pathways. Finally, our findings also represent an opportunity for drug discovery strategies to target particular signaling pathways within the dopaminergic system.

Materials and Methods
Confocal Microscopy FRET and Data Processing. Paraformaldehyde-fixed striatal neurons or floating sections (10 μm) from rat brain were incubated for 24 h at 4 °C with primary antibodies highly specific to D1 and D2 receptors (15) and the specific-specific secondary antibodies conjugated to Alexa Fluor 488 and Alexa Fluor 550 dyes, respectively. Anti-D2-Alexa Fluor 550 was used as a donor dye, while anti-D1-Alexa Fluor 488 was used as an acceptor dye. The immunofluorescence was acquired with a Leica TCS SP2 AOBS spectral confocal microscope with lasers emitting at 405 nm of the Ar laser and at 488 nm of the Krypton laser. Images were then generated based on the described algorithm (51), in accordance with an algorithm (51) (Tables S1 and Table S2). The processed FRET (pFRET) images were then generated based on the described algorithm (S1), in which: pFRET = UFRET – ASBT – DSBT, where UFRET is uncorrected FRET and ASBT and DSBT are the acceptor and the donor spectral bleed-through signals, respectively. The rate of energy transfer efficiency (E) and the distance (r) between the donor (D) and the acceptor (A) molecules were estimated by selecting small regions of interest (ROI) using the same images and software, based on the following equations: F = 1 – [(1/2)QD + pFRET × (1/QD + QA) × (A/D)], where QD is the donor image in the presence of acceptor, and QA and QD are collection efficiencies in the donor and acceptor channels, respectively, and QA and QD are the quantum yields. E is proportional to the sixth power of the distance (r) separating the FRET pair, r = R0/[(1/E) – 1]. R0 is the Förster’s distance.

Calcium Measurements Using Cameleon YCG-1. Calcium mobilization was measured by using cameleon YCG-1 (generous gift from M. Ikura, University of Toronto), an engineered calcium indicator based on the dependence of CaM conformation on elevations of calcium concentration (20). An increase in calcium binding to CaM leads to a decrease in the distance separating the two flanking proteins, CFP and YFP, and results in a measurable FRET change (20). Using a single excitation wavelength at 405 nm, which solely excites CFP, images and fluorescence values data for both CFP and YFP were collected. The experiments were performed in live neurons in the absence of extracellular calcium, and the data obtained from each individual cell were used to calculate the ratios, reflective of the energy transferred. The background signal was subtracted from the values obtained after drug injection.

Additional materials, methods, and related references are available in SI Text.

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