Published in final edited form as:

Neuroscience. 2010 January 20; 165(2): 535. doi:10.1016/j.neuroscience.2009.10.017.

Activation of calcium/calmodulin-dependent protein kinase II α in the striatum by the heteromeric D1–D2 dopamine receptor complex

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Abstract

Synaptic plasticity in the striatum is a key mechanism that underlies processes such as reward related incentive learning and behavioural habit formation resulting from drugs of abuse. Key aspects of these functions are dependent on dopamine transmission as well as activation of calcium/calmodulin-dependent protein kinase II α (CaMKII α). In this study, we examined the ability of a recently identified heteromeric complex composed of D1 and D2 dopamine receptors coupled to Gq/11 to activate striatal CaMKII α . Using the dopaminergic agonist SKF83959, which selectively activates the D1–D2 complex, we demonstrated phosphorylation of CaMKII α at threonine 286 both in HEK 293 cells and in the murine striatum *in vivo*. Phosphorylation of CaMKII α by activation of the receptor complex required concurrent agonism of both D1 and D2 receptors and was independent of receptor pathways that modulated adenylyl cyclase. The identification of this novel mechanism by which dopamine may modulate synaptic plasticity has implications for our understanding of striatal-mediated reward and motor function, as well as neuronal disorders in which striatal dopaminergic neurotransmission is involved.

Keywords

dopamine receptor; SKF 83959; CaMKII α ; striatum; SKF 83822

Introduction

Calcium/calmodulin-dependent protein kinase II (CaMKII) is a calcium-sensitive serine/threonine protein kinase that plays a fundamental role in both long-term potentiation and depression of synaptic transmission (Lisman et al., 2002), functions which are generally accepted to be the primary, if not exclusive, cellular mechanisms that underlie learning and memory. An increase in intracellular Ca²⁺ typically leads to the binding of Ca²⁺ or calmodulin to CaMKII α , resulting in autophosphorylation of the enzyme at threonine 286 and its

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constitutive activation. Constitutively active CaMKII α has been shown to phosphorylate, and thus regulate, a number of proteins involved in synaptic transmission, most notably postsynaptic NMDA and AMPA receptors (Barria et al., 1997; Mammen et al., 1997; Derkach et al., 1999; Merrill et al., 2005). Phosphorylation of these receptors alters both their conductance properties as well as their levels at the plasma membrane, both of which are critical determinants in the development of long-term synaptic potentiation (LTP).

Transmission through the G protein-coupled receptors for dopamine also plays a key role in many forms of learning and memory. For instance, D1 and D2 receptors have been shown to be involved in the establishment of forms of memory that are mediated by the cortex (Rinaldi et al., 2007; Vijayraghavan et al., 2007). D2 receptors are critical for formation (Guarraci et al., 2000) and D1 receptors for extinction (El-Ghundi et al., 2001) of fear memory in the amygdala, and D1 and D5 receptors are required for motor-skill learning in the striatum (Willuhn and Steiner, 2008). At the cellular level, D1 receptors can enhance NMDA receptor-dependent LTP in the prefrontal cortex through activation of protein kinase A (PKA) (Gurden et al., 2000) and the D1 family of receptors plays a critical role in both LTP and LTD at corticostriatal synapses (Calabresi et al., 2007). Notably, it has also been shown that D1-like dopamine receptors can trigger activation of CaMKII by activating L-type calcium channels in neurons of the nucleus accumbens (Anderson et al., 2008).

Recently, a novel D1-D2 dopamine receptor complex has been identified in brain that activates Gq/11 and phospholipase C, resulting in intracellular calcium release and rapid phosphorylation of CaMKII α in the nucleus accumbens (Rashid et al., 2007). This complex could be specifically activated by the benzazepine SKF 83959, in contrast to SKF 83822, which was shown to activate only D1 dopamine receptors coupled to Gs and adenylyl cyclase (AC). In the present study we take advantage of the pharmacological specificity of these compounds targeting the Gq/11-coupled D1-D2 complex, as well as Gs-coupled D1 receptors, to examine the effects of activating these receptor systems on the phosphorylation state of CaMKII α in the striatum. The striatum is a region of the brain which displays high expression of both D1 and D2 dopamine receptor subtypes and which display co-localization of the two receptors in a significant proportion of neurons, particularly in the ventral striatum (Lee et al., 2004; Deng et al., 2006; Bertran-Gonzales et al., 2008). The characterization of the effects of D1 and D1-D2 receptor activation on CaMKII α in brain would have important implications for further understanding the role of dopamine receptor signaling in the mechanisms of synaptic plasticity that underlie striatal-mediated function.

Experimental Procedures

Animals

Mice were caged under standard conditions in accordance with the guidelines set up by the Canadian Council on Animal Care. They were given free access to food and water, and were kept on a 12-hour daylight schedule. All mice used in the experiments (10–15 week old males) were handled regularly and transferred to the testing room and allowed to acclimate to the surroundings for a few hours over several days prior to the experiment. While in the testing room, mice were also given saline injections 3–4 times prior to the day of the experiment in order to acclimatize them to testing conditions. All testing took place during the light phase of the light-dark cycle.

In all experiments, wild-type C57BL/6 (C57) mice were used, and in experiments where dopamine D1 knockout (D1 $^{-/-}$) mice were treated, the C57 mice acted as a separate control group. D1 $^{-/-}$ mutant mice lacking functional D1 receptors were initially generated through homologous recombination (Drago et al., 1994) and then backcrossed ten times to the C57BL/6 strain of mice. The resulting N11 generation were genotyped using a protocol described

previously (El-Ghundi et al., 1998), and were originally housed separately from the regular C57 mice, where they were kept on a reverse 12-hour day-light schedule. Approximately two weeks prior to each experiment, the 10–18 week old D1^{−/−} mice were transferred to the same room as the C57 mice and were acclimatized to the regular lighting schedule for 1 week before the experiment.

Drug preparation

Drugs were dissolved in physiological saline, and were injected intra-peritoneally at a volume of 0.1 ml/10g body weight. The doses of the individual drugs were determined based on pilot studies to obtain an optimal effect. SKF 83959 (Sigma-Aldrich, St. Louis, MO, USA) was tested at 2 mg/kg and 1 mg/kg, however all results presented are from studies where drug was applied at the dose of 1 mg/kg, unless otherwise indicated. SKF 83822 (Tocris, Elleville, MI, USA) was administered at a dose of 1 mg/kg, SCH 23390 (Tocris) at 0.5 mg/kg, raclopride (Sigma) at 1 mg/kg, quinpirole (Sigma) at 0.1 mg/kg and MK-801 (Tocris) at 0.2 mg/kg. SKF 83959 and SKF 83822 were first dissolved in dimethyl sulfoxide (DMSO), at <1% of total volume prior to being mixed with saline, as these compounds have low aqueous solubility. All other drugs were dissolved directly in physiological saline. In cases where drugs were dissolved in DMSO, an equivalent amount of DMSO was added to the saline and served as the vehicle control. In all other cases physiological saline was used.

Drug Treatment

On the day of each treatment experiment, mice were weighed and injected with a corresponding amount of agonist or vehicle (0.1 ml/10g). The animals were then killed by cervical dislocation 10, 30, 60 or 90 minutes after drug treatment. For antagonist studies, SCH23390 or raclopride was injected 10 minutes prior to agonist treatment. Raclopride was re-administered every 30 minutes when the treatment periods were longer than 30 minutes. Following drug treatments, the brain of each animal was immediately removed and placed on ice and striata were dissected out. Tissues were frozen immediately following dissection and stored at -70°C , or processed for analysis.

Tissue processing

Isolated striatal tissue was suspended in cell lysis buffer made up of 5 mM Tris, 0.2 mM EDTA and proteolysis inhibitors (5 $\mu\text{g/ml}$ leupeptin, 10 $\mu\text{g/ml}$ soybean trypsin, 10 $\mu\text{g/ml}$ benzamidine) and then sonicated for 15 seconds. Protein concentrations of the sample homogenates were determined and samples were then resuspended in sample buffer (125 mM Tris. Cl, pH 6.5, 4% SDS, 20% glycerol, 0.005% bromophenol blue and 5% β -mercaptoethanol) to the desired concentration, placed in boiling water for 3 minutes, removed and kept frozen at -70°C until required for Western blot analysis.

HEK cell drug treatment and cell processing

HEK cell lines stably expressing either D1 receptors, D2 receptors or D1 and D2 receptors were generated as described (Lee et al., 2004). Drugs were dissolved in 100 μL DMSO, diluted in cell growth medium and then added to cells in 100mm dishes at a concentration of 1 μM (agonists) or 5 μM (antagonists). Cells were washed after five minutes of treatment and then harvested for lysis and Western blotting as described below. D1 cells were treated with either SKF 83959 alone or with control medium containing only DMSO. D1–D2-expressing cells were treated with saline or with SKF 83959 alone, SKF 83959 with SCH 23390 or SKF 83959 with eticlopride (Sigma-Aldrich).

Following treatment, cells were washed with phosphate-buffered saline (PBS) and resuspended in hypotonic cell lysis buffer containing protease and phosphatase inhibitors. Cells were

homogenized by sonicating for 15 seconds and sample buffer added to the lysate. Each lane for Western blotting contained 20 µg (HEK cells) or 50 µg (striatal tissue) protein. Where Western blot analysis was not immediately performed, the samples were kept frozen at -70°C.

Western Blot analysis

Tissue homogenates were separated by SDS-polyacrylamide gel electrophoresis and electroblotted onto PVDF transfer membranes for Western blotting. Membranes were blocked for 1 hour in 5% skimmed milk dissolved in TBS. and then incubated overnight at 4°C with primary antibody. Primary antibodies titers were first optimized using different concentrations and were diluted in 1% skimmed milk dissolved in Tris-buffered saline (TBS).. For analysis of animal tissue, mouse anti-pCaMKII (Affinity BioReagents, Golden, CO, USA) was used at a dilution of 1 in 1000, while the rabbit anti-pCaMKII (Cell Signaling Technology, Danvers, MA, USA) used in Western blot analysis of HEK cells was used at a dilution of 1:1000. Antibody directed against total CaMKII (anti-rabbit; Cell Signaling Technology) in both animal and cell tissue analysis was used at 1:1000 dilution. Antibody against glyceraldehyde-3-phosphate (GAPDH) (Abcam, Cambridge, MA, USA) was used as an internal loading control (dilution 1:10 000). The next day following overnight incubation, membranes were washed three times with TBS, 0.1% Tween-20 for 10 minutes each and then incubated for 1–2 hours at room temperature with HRP-conjugated species-specific secondary antibody.

Densitometry Measurement

Signal strength of the Western blot bands was quantified using Zeiss AxioVision 4 software, which measures the intensity of the signal based on pixel density. Optical density (O.D.) of the pCaMKII and CaMKII bands were measured relative to their respective GAPDH controls on the same blot, and total pCaMKII densitometry was then measured over total CaMKII band intensity, in order to rule out variances in band density between blots. The final calculations were performed as shown:

$$\frac{\text{O.D. of pCaMKII}}{\text{O.D. of GAPDH (pCaMKII)}} \div \frac{\text{O.D. of CaMKII}}{\text{O.D. of GAPDH (CaMKII)}} = \text{overall pCaMKII}$$

Statistical Analysis

Data were compared between control and drug-treated groups run on the same blot, and were analyzed using two-tailed, unpaired Student's *t* test. All data were expressed as mean ± S.E.M. Statistical significance was considered as *p* < 0.05.

Results

Activation of the D1–D2 receptor hetero-oligomer in HEK cells activates endogenous CaMKIIα

We previously characterized the generation of a G_q/PLC-mediated Ca²⁺ signal by co-activation of D1 and D2 receptors in HEK cells stably expressing both receptors (D1–D2_{HEK})(Lee et al., 2004; Rashid et al., 2007). A robust and rapid Ca²⁺ signal was observed after D1–D2_{HEK} cells were treated with SKF 83959, which acted as a full agonist for D1 receptor and a partial agonist for D2 receptor within a functional complex to activate Gq/11(Rashid et al., 2007). Notably, we demonstrated in both D1–D2_{HEK} cells and in the murine striatum that SKF83959 activates Gq/11 but not Gs/olf or Gi/o and this effect was absent in striatum of animals lacking D1 receptors or D2 receptors (Rashid et al., 2007). To examine if the observed increase in intracellular calcium resulted in activation of endogenous CaMKIIα, D1–D2_{HEK} cells were

treated with agonist for 5 minutes followed by cell lysis and Western blotting using antibodies for total CaMKII α and phosphorylated CaMKII α (Thr286). After treatment with 1 μ M SKF 83959, elevated levels of phosphorylated CaMKII α (pCaMKII α) were observed in comparison to D1–D2_{HEK} cells treated with saline ($179 \pm 16\%$) (Figure 1). When SKF 83959 was applied to HEK cells expressing D1 receptors alone or D2 receptors alone, there was no significant increase in pCaMKII α levels observed, indicating that both D1 and D2 receptors are required to generate the Ca²⁺ signal leading to CaMKII α activation. Similarly, the inclusion of SCH 23390 or eticlopride, selective antagonists for D1 and D2 receptors respectively, prevented any increases in pCaMKII α in response to SKF 83959. Therefore, activation of the Gq/11-coupled D1–D2 receptor complex resulted in specific activation/phosphorylation of CaMKII α .

Activation of the D1–D2 receptor complex activates striatal CaMKII α

To investigate whether activation of the D1–D2 receptor complex could specifically activate CaMKII α *in vivo*, intra-peritoneal injections of agonists and antagonists were given to C57/Bl6 mice followed by harvesting of striatal tissues at various times post-treatment. Protein solubilization was followed by Western blotting for total and phosphorylated CaMKII α .

Pilot experiments were performed beforehand to establish a time course and dose-dependence for the signaling pathways. CaMKII α activation was initially probed 10, 30 and 60 minutes following treatment with 1 mg/kg and 2 mg/kg SKF 83959. The two doses chosen were at the lower end of the spectrum of doses that have been previously used in other studies from the literature, in order to preserve specificity and minimize non-specific binding of the compound to other receptors. While SKF 83959 treatment at 1 mg/kg and 2 mg/kg both produced increases in CaMKII α activation when compared to control (data not shown), statistically significant results were obtained with 1 mg/kg of SKF 83959 and therefore this dose was used for all experiments subsequently.

When SKF 83959 was given to animals, no significant difference in striatal pCaMKII α levels was detected between the drug-treated and saline-treated mice following 10 minutes of treatment (Figure 2A). However, there was a significant increase in CaMKII α activation in the mice treated with SKF 83959 for 30 ($154 \pm 11.5\%$ of control), 60 ($161.5 \pm 24.69\%$) and 90 minutes ($199.5 \pm 21.32\%$). While the increase observed was not significantly different between time points, there was a trend towards increased CaMKII α phosphorylation with increasing time of drug treatment. Injection of SCH 23390 or raclopride, antagonists for D1 and D2 receptors respectively, just prior to SKF 83959 treatment prevented the agonist-mediated increase in pCaMKII α (Figure 2B,C), demonstrating the necessity for both D1 and D2 receptor activation in this pathway. The necessity for D1 dopamine receptors was further demonstrated with an absence of agonist mediated increases in pCaMKII α in animals lacking functional D1 receptors (Figure 2D). Similarly, treating animals with SKF 83822, which only activates adenylyl cyclase-coupled D1 receptors, did not result in any statistically significant increases in pCaMKII α at any of the time points tested post-injection (Figure 2E), indicating that activation of Gs/olf and subsequently AC had no direct role in striatal CaMKII α activation.

Effects of full activation of the D1–D2 complex on CaMKII α phosphorylation

Although SKF 83959 is sufficient to specifically activate Gq/11 by acting as a full agonist for D1 receptors and a partial agonist for D2 receptors within a functional complex, we had previously demonstrated that SKF 83959 co-application with the D2/D3 agonist quinpirole, the latter acting as a full agonist for Gi/o-independent D2 receptors in the complex, resulted in more robust activation of Gq/11 and greater intracellular calcium release (Rashid et al., 2007). We therefore wanted to see if full activation of the complex by SKF 83959 and quinpirole resulted in robust phosphorylation of CaMKII α . We first examined the effects of quinpirole alone on CaMKII α phosphorylation. Treatment with 0.1 mg/kg quinpirole resulted

in a decrease in levels of phosphorylated CaMKII α when examined 60 and 90 minutes post-injection (Figure 3A), consistent with reports that D2/D3 dopamine receptor activation can decrease CaMKII α phosphorylation through activation of Gi/o signaling proteins (Gu and Yan, 2004). Based on this result, we used varying combinations of SKF 83959 and quinpirole in attempts to maximize the effects of activating the D1–D2 hetero-oligomer and minimize the effects of activating Gi/o-coupled D2/D3 receptors. The effects of quinpirole on decreasing pCaMKII α were found to be minimal when used at 0.1 mg/kg in combination with 1 mg/kg SKF 83959. When these concentrations of drug were used together, the CaMKII α phosphorylation in the striatum increased by $160.3 \pm 12.1\%$ at 60 minutes after injection and $186.7 \pm 16.2\%$ after 90 minutes (Figure 3B). Since these increases were not statistically different from those seen with SKF 83959 alone ($p=0.95$ at 60 min, $p=0.33$ at 90 min), the effects of quinpirole on Gi/o-coupled D2/D3 receptors to decrease pCaMKII α was most likely overcome by robust activation of the D1–D2-Gq/11 complex to an extent greater than that seen with SKF83959 alone.

Effects of NMDA receptor blockade on D1-D2 complex activation of CaMKII α

Calcium influx through NMDA receptors is a primary mechanism by which CaMKII α can be phosphorylated and activated. Since dopamine receptor activation has been shown to regulate NMDA receptor activity, we wanted to check if the effects of activation of the D1–D2 complex on CaMKII α phosphorylation were a direct consequence of dopamine agonist-mediated intracellular calcium release or occurred through dopamine receptor modulation of NMDA receptors. Animals were pre-treated with the NMDA receptor antagonist MK-801 10 minutes prior to the administration of SKF 83959 or saline (Figure 4). In response to MK-801 and SKF 83959, pCaMKII α levels were significantly higher than in MK-801 and saline-injected animals. When compared to earlier results obtained in animals treated with SKF 83959 in the absence of MK-801, the levels of pCaMKII α appeared to be slightly less 60 minutes after agonist treatment ($131.8 \pm 17.9\%$ for SKF 83959 and MK-801 vs. $154 \pm 11.5\%$ for SKF 83959 alone, $p=0.09$), and significantly lower at 90 minutes ($149.3 \pm 18.4\%$ for SKF 83959 and MK-801 vs. $199.5 \pm 21.3\%$ for SKF 83959 alone, $p=0.0047$). Overall, however, these results suggest that the increase in CaMKII α phosphorylation in response to D1–D2 complex activation was primarily due to activation of the Gq/11 signaling pathway.

Discussion

In this study we examined the cellular and *in vivo* effects of activating the recently defined Gq/11-coupled D1–D2 dopamine receptor complex that results in intracellular calcium release. We focused on the ability of this complex to regulate the phosphorylation, and presumably activation, of CaMKII α , a key protein kinase that has a critical function in the formation of long-term potentiation throughout the brain to provide a robust and time-dependent index of D1–D2 complex activation, since the direct measurement of the calcium signal is difficult to achieve in brain regions. Further, since CaMKII activation by rapid phosphorylation at Thr286 is very responsive to increases in intracellular calcium concentrations, we have been able to show excellent correlation with D1–D2-Gq/11 activation.

We had previously demonstrated that different D1 receptor agonists can activate different repertoires of G proteins, presumably through distinct D1 receptor-containing complexes. While the benzazepine SKF 83822 activates only Gs/olf through D1 receptors, SKF 83959 was shown to activate only Gq/11 in a manner that was dependent on the presence of D1 and D2 receptor subtypes, and SKF 81297 could activate both Gs/olf and Gq/11 through its ability to act on either population of D1-containing receptors (Rashid et al, 2007). Using the pharmacological specificity of selectively activating the D1–D2 complex using SKF 83959, we demonstrated phosphorylation of CaMKII α at Thr286 in striatum *in vivo*. This effect was

dependent on activation of both D1 and D2 receptor subtypes within the functional complex by agonist, and was not obtained in D1^{-/-} mice. The effect was attenuated by blockade of either the D1 or D2 receptor by selective antagonists. The CaMKII phosphorylation was independent of receptor pathways that modulated adenylyl cyclase as substantiated by the lack of effect of SKF 83822 treatment. In heterologous cells expressing D1 and D2 receptors, treatment with SKF 83959 induced rapid and robust CaMKII phosphorylation. Both receptors were shown to be required for this effect as shown by attenuation of effect by pretreatment with either the D1 or D2 selective antagonists. The generation of phosphorylated CaMKII in the striatal neurons of mice was demonstrated to be caused by signaling through the D1–D2 receptor heterooligomer in the most part, with a small contribution to the signal mediated by NMDA receptors. Therefore, we propose that the D1–D2 receptor complex represents a novel mechanism by which dopamine receptor signaling could activate CaMKII α directly and thus regulate synaptic plasticity in the striatum as well as other brain regions.

Other studies have also linked dopamine neurotransmission with modulation of CaMKII α . Activation of D4 receptors has been shown to activate CaMKII α in prefrontal cortical neurons through activation of PLC (Gu and Yan, 2004) and a recent study indicates that D1 receptor-mediated activation of L-type calcium channels can result in activation of CaMKII α in the nucleus accumbens (Anderson et al., 2008).

Dopaminergic transmission in the striatum plays a key role in both the short-term and long-term plasticity that underlie motor skill learning, the brain's perception of reward, and behavioural habit formation resulting from drugs of abuse (Self et al., 1996; Koob et al., 1998; Costa, 2007). The mechanisms by which dopamine may influence plasticity presumably depend on the dopamine receptor subtypes involved and the signaling pathways to which they are coupled. Some of the already characterized effects of dopamine receptor signaling on synaptic plasticity also include modulation of glutamate receptor function (Barria et al., 1997; Derkach et al., 1999), activation of transcription factors such as *fos* (Nagai et al., 2007) and regulation of ion channel activity (Rotaru et al., 2007; Anderson et al., 2008).

Neuronal calcium signaling can regulate many aspects of cellular function, such as excitability, transmitter release, gene transcription and synaptic plasticity. The finding that the D1–D2 heteromer mediated calcium signal has the ability to modulate CaMKII α activity is of particular significance, given the important role CaMKII α has in the induction of LTP and LTD (Malenka and Bear, 2004) and in the process of synaptic plasticity in striatum (Mayford et al., 1996) as well as other brain regions. Since dopamine has been shown to have a key role in synaptic plasticity in striatum (Centonze et al., 2001; Tang et al., 2001), our results provide one of possibly multiple mechanisms linking dopamine action directly to one of the most important mediators of this change. CaMKII α activation in nucleus accumbens has been shown to be necessary for the induction of behavioral sensitization to psychostimulants, so it is intriguing to suggest that dopamine involvement in such processes may be through a D1–D2 heteromer and Gq/PLC mediated calcium signal activation with subsequent involvement of CaMKII α .

Thus, the identification of the D1–D2 activated calcium signal provides a direct link to the modulation of CaMKII α in striatum and the prevalence of this complex in this region could play an important role in the functional regulation of synaptic plasticity by dopamine. Coupled with our results, it is interesting to speculate that multiple pathways can link dopamine with CaMKII α activation and that these pathways may differ by distinct temporal and spatial features to the activation profile of this important kinase. Further elucidation of the regulation of the D1–D2-Gq/11-mediated pathway leading to CaMKII α activation may allow greater insight into the putative links between dopamine dysregulation and alterations in learning and memory associated with neuropsychiatric disorders such as schizophrenia and drug addiction.

Acknowledgments

This work was supported by a grant from the NIH National Institute on Drug Abuse. SRG is the holder of a Canada Research Chair in Molecular Neuroscience.

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Fig.1. D1–D2 hetero-oligomer-mediated Ca^{2+} signaling activates CaMKII α in HEK cells
(A) Western blot of cell lysates with anti-pCaMKII α or anti-CaMKII α antibodies after treatment with dopamine receptor agonists and/or antagonists. SKF 83959 (1 μM) treatment of HEK cells stably co-expressing D1 and D2 receptors resulted in increased levels of phosphorylation of CaMKII α at threonine 286 (lane 2) when compared to saline treated cells (lane 1). This effect of SKF 83959 was absent in cells expressing the D1 receptor alone (lane 5), the D2 receptor alone (lane 6–8) or in cells expressing both receptors after treatment with D1 antagonist 5 μM SCH 23390 (lane 3) or D2 antagonist 5 μM eticlopride (lane 4). **(B)** Quantitative data from Western blots are expressed as percentage of CaMKII α activation over control ($n = 3$ experiments per individual treatment condition). *, $P < 0.05$ compared with control.

Fig.2. Activation of the D1–D2 receptor complex results in phosphorylation of striatal CaMKIIα (A) Mice treated with 1mg/kg SKF 83959 (SKF) i.p. expressed higher levels of striatal pCaMKIIα compared to saline controls (sal) at 30, 60 and 90 min after injection. (B)(C) SKF 83959-mediated increases in phosphorylated CaMKIIα were prevented by pretreating animals with 1mg/kg SCH 23390 (SCH) or 0.5mg/kg raclopride (rac). (D) In animals lacking functional D1 receptors (D1^{-/-}), SKF 83959 had no effect on levels of pCaMKIIα. (E) No significant increases in pCaMKIIα were observed at 30, 60 or 90 min after animals were injected with SKF 83822 (SKF). Quantitative data from Western blots are expressed as percentage of CaMKII activation over saline control ($n = 4$ animals per individual treatment condition). *,

$P < 0.05$ compared with saline; **, $P < 0.01$ compared with saline. [both SKF drugs are labelled the same- call them SKF 59 and SKF 22.]

Fig.3. D1–D2 receptor heterooligomer signaling activates striatal CaMKIIα

(A) Activation of $G_{i/o}$ coupled D2 receptors with 0.1mg/kg quinpirole (quin) decreased striatal pCaMKIIα by 35–40%, but not total CaMKIIα when compared to saline controls (sal) at both 60 and 90 min after injection. (B) Mice treated with 1mg/kg SKF 83959 and 0.1mg/kg quinpirole, however, showed increasing levels of striatal pCaMKIIα with time compared to saline controls, comparable to levels attained after treatments with SKF 83959 alone (Figure 2). For both A and B, quantitative data from Western blots are expressed as percentage of CaMKIIα activation over saline control ($n = 5$ animals per treatment condition). *, $P < 0.05$ compared with saline ; **, $P < 0.01$ compared with saline.

Fig.4. D1–D2 heterooligomer-mediated phosphorylation of CaMKII α is independent of NMDA receptor activity

Mice treated with 1mg/kg SKF 83959 and MK-801 had higher levels of striatal pCaMKII α , but not total CaMKII α , compared to saline controls at both 60 and 90 min after injection. The increase in pCaMKII α was not significantly different from that seen with SKF 83959 alone (Figure 2) at 60 min, and was only slightly less than after SKF 83959 alone at 90 min.

Quantitative data from Western blots are expressed as percentage of CaMKII α activation over saline control ($n = 10$). *, $P < 0.05$ compared with saline.