Research Paper

D2 dopamine receptors interact directly with N-type calcium channels and regulate channel surface expression levels

Alexandra E. Kisilevsky and Gerald W. Zamponi*

Department of Physiology and Biophysics; Hotchkiss Brain Institute; University of Calgary; Calgary, Alberta Canada

Key words: dopamine, N-type, G protein, signaling complex

N-type channels are located on dendrites and at pre-synaptic nerve terminals where they play a fundamental role in neurotransmitter release. They are potently regulated by the activation of a number of different types of pertussis toxin (PTX)-sensitive G\(\alpha_{i/o}\) coupled receptors, which results in voltage-dependent inhibition of channel activity via G\(\beta\gamma\) subunits. Using heterologous expression in HEK 293T cells, we show via whole cell clamp recordings that D2 receptors mediate both G\(\beta\gamma\) (i.e., voltage-dependent) and voltage-independent inhibition of channel activity. Furthermore, using co-immunoprecipitation and pull down assays involving the intracellular regions of each protein, we show that D2 receptors and N-type channels form physical signaling complexes. Finally, we use confocal microscopy to demonstrate that D2 receptors regulate N-type channel trafficking to affect the number of calcium channels available at the plasma membrane. Taken together, these data provide evidence for multiple voltage-dependent and voltage-independent mechanisms by which D2 receptor subtypes influence N-type channel activity.

Introduction

The central and peripheral nervous systems express multiple types of voltage gated Ca\(^{2+}\) channels (VGCCs) as defined by physiological and pharmacological criteria.\(^1\) Calcium entry through VGCCs serves as an important second messenger, initiating events such as hormone secretion, synaptic neurotransmission and gene expression. Each channel type is defined by its pore-forming \(\alpha_{1}\) subunit and is associated with unique properties and physiological roles. N-type channels (or Ca\(_{V}\)2.2 channels) contain the \(\alpha_{1B}\) subunit and are widely distributed across the nervous system. At presynaptic nerve terminals, calcium influx through these channels is responsible for evoked neurotransmitter release\(^2\,4\) thus directly regulating neurotransmission and synaptic output.

It is well documented that agonist activation of GPCRs coupled to pertussis-toxin (PTX) sensitive G\(\alpha_{i/o}\) subunits inhibit Ca\(_{V}\)2.2 channels in a membrane-delimited and voltage-dependent (VD) manner.\(^5\,13\) This VD inhibition is caused by binding of G\(\beta\gamma\) molecules to the calcium channel \(\alpha_{1}\), subunit and results in slowing of channel kinetics as well as a reduction in peak current amplitude.\(^14\,18\) A key hallmark of VD inhibition is its reversal upon strong membrane depolarizations which result in dissociation of the G\(\beta\gamma\) subunits from the channel molecule.\(^19\) Activation of GPCRs can also result in modulation of Ca\(_{V}\)2.2 channel activity in a voltage-independent (VI) manner that occurs over a slower time course than VD G\(\beta\gamma\)-mediated inhibition, and likely involves soluble second messengers.\(^20\,21\)

Dopamine (DA) receptors are one of the key GPCR families expressed in the mammalian brain, with far reaching physiological roles that include the regulation of locomotion, cognition and emotion.\(^22\,25\) To date, five different types of DA receptors (D1R-D5R) belonging to two subfamilies have been identified and pharmacologically characterized.\(^24\) In mammals, the D2R exists as both long and short splice variants (D2L and D2S, respectively), differing by a 29-amino acid insertion in the third intracellular loop.\(^26\) Activation of this receptor decreases cAMP production by negatively coupling to adenylyl cyclase via G\(\alpha_{i}\).\(^27\) and is known to mediate VD inhibition of N-type channel activity.\(^28\,32\)

We have recently described the regulation of N-type calcium channels by D1Rs.\(^35\) Here, we examine coupling of the D2R to the Ca\(_{V}\)2.2 calcium channel. We demonstrate that activation of this receptor subtype results in robust VD and VI inhibition of channel activity. Furthermore, we show that D2Rs physically associate with N-type calcium channels via interactions among the intracellular regions of both receptor and channel. Finally, we demonstrate a D2R-mediated modulation of Ca\(_{V}\)2.2 surface expression levels.

Results

D2 receptors mediate VD and VI inhibition of Ca\(_{V}\)2.2 channels. Individual HEK 293T cells co-expressing the Ca\(_{V}\)2.2 \(\alpha_{1B}\) subunit (\(\alpha_{1B}\) and \(\alpha_{2-\delta}\) subunits), an EGFP marker, and the D2R were assessed for Ca\(_{V}\)2.2 channel activity following receptor activation with dopamine. All cells expressing exogenous D2Rs showed robust Ca\(_{V}\)2.2 current inhibition (Fig. 1A). Following stabilization of the inhibitory response, peak current amplitudes were reversibly reduced by 74.07 ± 2.18%, with 96.07 ± 6.31% recovery upon washout (Fig. 1A). In contrast, DA had no effect on channel activity in those cells in which exogenous D2Rs were omitted (data not shown). Activation
Dopamine modulation of N-type channels

Figure 1. Agonist activation of the D2 receptor results in robust voltage-dependent and voltage-independent inhibition of CaV2.2 channel activity. (A) Summary of peak current versus time during DA agonist application from individual HEK 293T cells co-expressing the CaV2.2 channel and the D2 receptor. Agonist application results in approximately 75% current inhibition. Inset: Representative current recording from an individual cell expressing the CaV2.2 channel and the D2 receptor, before [control] and after [DA] stimulation with DA. The relative proportion of inhibition resulting from VD and VI pathways was determined as described previously in detail.33 (B) Summary of the contribution of voltage-dependent (VD) and voltage-independent (VI) pathways to the overall magnitude of CaV2.2 current inhibition, following D2 receptor activation with DA. The relative proportion of inhibition resulting from VD and VI pathways was determined as described previously in detail.33 (C) Peak current amplitude as a function of time during DA application from individual HEK 293T cells co-expressing the CaV2.2 channel and the D2 receptor, following pretreatment with PTX. Inset: Representative current recordings from PTX-treated cells co-expressing the CaV2.2 channel and the D2 receptor, before [control] and after [DA] DA application. The vertical bar indicates 250 pA and the horizontal bar indicates 15 ms.

of the D2R produced both voltage-dependent (VD) and voltage-independent (VI) inhibition of current activity (Fig. 1B). Indeed, the hallmark characteristics of VD Gβγ-mediated inhibition, including kinetic slowing and relief of inhibition by a strong depolarizing pre-pulse, were observed (Fig. 1B). VD and VI mechanisms contributed equally to overall current inhibition, with a ratio of approximately 50:50 (VD: 43.10 ± 4.31%; VI: 35.70 ± 4.13%) (Fig. 1B). D2Rs signal to downstream effectors via the Gαi/o subunit and, hence, the functional effects on channel activity should be sensitive to inhibitors of Gαi/o signaling. Indeed, pre-treatment of D2R expressing cells with pertussis toxin (PTX) virtually abolished DA mediated inhibition of channel activity (Fig. 1C).

Overall, these data indicate that D2Rs effectively inhibit N-type calcium channels via a combination of Gαi/o-sensitive, voltage-dependent (i.e. Gβγ-mediated) and voltage-independent pathways.

D2 receptors physically associate with CaV2.2 channels. A number of recent studies have suggested the existence of physical signaling complexes between GPCRs and N-type calcium channels.34,35 To determine if a similar association could occur with the D2R, we performed co-immunoprecipitations of D2Rs and N-type calcium channels using rat striatal homogenate. D2Rs and N-type channels could be effectively co-immunoprecipitated from this tissue, indicating the existence of a receptor-channel signaling complex (Fig. 2A). Western blot analysis using an α-D2R antibody revealed two distinct bands in the homogenate (approximately 60 and 80 kDa, input lane), the heavier of which was also detected in the co-immunoprecipitation (middle lane). Multiple bands have been reported previously for the D2R and may indicate glycosylated receptors or receptor dimers.36 Overall, the data shown in Figure 2A suggest the existence of N-type channel-D2R complexes.

To determine whether the D2R and the CaV2.2 channel interact directly, and to investigate which regions of each protein are responsible for their association, we conducted in vitro binding studies using GST- and 6xHis-fusion constructs of the intracellular regions of the receptor and the channel, respectively (Fig. 2B). These experiments revealed a biochemical interaction between the CaV2.2 II–III linker region and both the D2 loop-3 and the D2 C-terminus (Fig. 2C, top). Similarly, the distal portion of the CaV2.2 C-terminus was found to interact with the D2 loop-3 and the D2 C-terminus (Fig. 2C, bottom). These data are consistent with the co-IP data in Figure 2A, and indicate that the D2R interacts directly with the CaV2.2 calcium channel at multiple binding sites.
To verify the results from our protein biochemical analyses, we transfected HEK 293T cells with a single YFP-conjugated peptide corresponding to an intracellular region of the D2R, alone or in combination with the wild-type CaV2.2 channel. Using confocal microscopy, individual cells were subsequently examined for plasma membrane translocation of the YFP-peptide. We also carried out the converse experiment in which individual HEK 293T cells expressing a single YFP-conjugated peptide corresponding to an intracellular region of the channel, alone or in the presence of a 3xHA-tagged D2R, were examined for plasma membrane translocation.

Expression of the pEYFP-N1 vector produced diffuse cytoplasmic distribution of the YFP signal (data not shown). Similarly, individual peptides corresponding to the intracellular regions of the receptor or the channel alone were distributed throughout the cytoplasm (Fig. 3A and B). This was true even for the D2 C-terminal peptide, which contains a cysteine residue with the potential to be post-translationally modified and anchored to the plasma membrane. When co-expressed in the presence of the CaV2.2 channel or the 3xHA-tagged D2R, however, individual peptides exhibited marked plasma membrane translocation (D2 loop-3: 55%; D2 C-terminus: 16%; CaV2.2 II–III linker: 50%; CaV2.2 C-terminus: 29%) (Fig. 3A and B).

The results of our protein biochemical and membrane translocation analyses suggest that the D2R is capable of interacting directly with the CaV2.2 channel and that this interaction is mediated by distinct structural moieties on both the receptor and channel. Furthermore, our data indicate that minigenes encoding receptor/channel interaction sites find their appropriate targets in a mammalian cellular milieu.

Disruption of D2 receptor-CaV2.2 channel interaction alters receptor-mediated modulation of channel activity. To investigate whether or not disruption of specific receptor-channel interactions alters D2R mediated modulation of CaV2.2 channel activity, minigenes corresponding to the cytosolic regions of receptor-channel interaction were created. Individual HEK 293T cells expressing the CaV2.2 α1b subunit (+β1b and α2-δ1 subunits), the D2R, and a single minigene were assessed for channel modulation following receptor activation. Expression of each of the minigenes slowed the time course of channel inhibition (Fig. 4A). Furthermore, when compared to control, the degree of CaV2.2 current inhibition was significantly reduced in the presence of the D2 loop-3, the D2 C-terminus, or the distal region of the CaV2.2 channel (control: 76.20 ± 4.62%, D2 loop-3: 9.69 ± 7.09%, D2 C-terminus: 45.81 ± 9.54%, CaV2.2 C-terminus: 58.21 ± 5.70%) (Fig. 4B, left). This reduction in current inhibition was attributed to a loss of both VD
Dopamine modulation of N-type channels

To confirm the specificity of the inhibitory effects of individual peptides on CaV2.2 channel modulation, we first co-transfected HEK 293T cells with heterologous Kir 3.1/3.4 channels and exogenous Gβ1γ2, in the absence and presence of individual peptides, and examined the effect on GIRK channel conductance. GIRK channel conductance was unaffected by the presence of individual peptides corresponding to the D2 C-terminus or the distal portion of the CaV2.2 channel (Gβ1γ2: 50.37 ± 7.73 nS, D2 C-terminus: 54.18 ± 10.07 nS, CaV2.2 C-terminus: 52.49 ± 9.68 nS) (Fig. 4C, left). In contrast, the presence of the D2 loop-3 peptide significantly altered GIRK channel conductance in this assay (D2 loop-3: 30.86 ± 4.30 nS) (Fig. 4C, left). This result indicates that the D2 loop-3 peptide may exert non-specific effects on Gβγ signaling, perhaps through direct quenching of Gβγ subunits (note that certain types of GPCRs contain a physical Gβγ binding in their third intracellular loop37). We then co-transfected HEK 293T cells with heterologous Kir 3.1/3.4 channels and D2Rs, in the absence and presence of individual peptides, and examined the effect on GIRK channel conductance following stimulation with DA. The D2 loop-3 peptide was not examined due to the above noted non-specific effects (Fig. 4C, left). Following stimulation with DA, GIRK channel potentiation was unaltered by the presence of the D2 C-terminus or the distal portion of the CaV2.2 channel (control: 3.19 ± 0.35, D2 C-terminus: 3.34 ± 0.57, CaV2.2 C-terminus: 2.85 ± 0.35) (Fig. 4C, right). Hence, we conclude that the inhibitory effects of these individual peptides on D2-mediated CaV2.2 channel modulation (see Fig. 4A and B) do not occur as a result of a general interference with D2R mediated signaling. Instead, they are likely due to physical uncoupling of receptors from channels.

Taken together, these data indicate that the formation of physical complexes between receptors and channels is a prerequisite for effective CaV2.2 channel modulation by D2Rs.

D2 receptors regulate CaV2.2 channel surface density. To determine if D2Rs can regulate CaV2.2 channel surface expression levels, we carried out receptor-channel colocalization experiments using confocal microscopy. Cultured HEK 293T cells were transiently transfected with the wild-type CaV2.2 channel (+β1b and α2-δ1 subunits), in the absence or the presence of a YFP-tagged D2R, and the ratio of channel/receptor surface expression to total cellular expression was quantified in the absence and the presence of DA.35 When expressed alone, the distribution of the CaV2.2 channel was unaffected by the application of DA (CaV2.2 control: 0.36 ± 0.02, CaV2.2 + DA: 0.36 ± 0.03) (Fig. 5A and C). In the absence of agonist, co-expression of the D2R with the CaV2.2 channel produced a significant increase in channel surface expression (CaV2.2 alone: 0.36 ± 0.02; CaV2.2 + D2: 0.63 ± 0.02) (Fig. 5B and C). In response to DA, a significant loss of surface expression of the D2R was observed (CaV2.2 + DA: 0.77 ± 0.01; CaV2.2 + D2 + DA: 0.67 ± 0.02, p < 0.001, t-test) (Fig. 5D). The internalization of the receptor was closely paralleled by a DA induced loss of CaV2.2 channel surface expression (CaV2.2 + D2 control: 0.63 ± 0.02; CaV2.2 + D2 + DA: 0.51 ± 0.02) (Fig. 5C), consistent with the idea of channel-receptor co-internalization.

To confirm that cytoplasmic CaV2.2 channels were internalized from the cell surface, we conducted similar experiments involving HA-CaV2.2 channels and YFP-D2Rs. Primary HA-antibody was applied to live cells prior to agonist stimulation, and cells were
Dopamine modulation of N-type channels

Figure 4. Disruption of D2-CaV2.2 channel interactions alters receptor-mediated channel modulation (A) Summary of peak current versus time during DA agonist application from individual HEK 293T cells co-expressing the CaV2.2 channel and the D2 receptor. Recordings were made in the absence and the presence of a single blocking peptide corresponding to a region of D2-CaV2.2 interaction (▼ D2 control, • D2 loop-3, △ D2 C-terminus, ○ CaV2.2 II–III linker, ■ CaV2.2 C-terminus (distal)). (B, left) Cells expressing peptides corresponding to the D2 loop-3, the D2 C-terminus, or the distal region of the CaV2.2 C-terminus show significant reductions in CaV2.2 current inhibition (*p < 0.05, one-way ANOVA). The degree of current inhibition was calculated 40 seconds following DA application (arrow in A). (B, right) In the case of the D2 loop-3, this reduction results from a loss of both VD and VI inhibition, while, in the case of the D2 C-terminus, this reduction results mainly from a loss of VD inhibition (*p < 0.05, t-test). (C, left) GIRK channel conductance is unaffected by the presence of minigenes corresponding to the D2 C-terminus or the distal portion of the CaV2.2 channel. Inset: Representative raw current trace from an individual HEK 293T cell co-expressing heterologous GIRK 3.1/3.4 channels, exogenous Gβ1γ2, and the D2 C-terminus. The recording, elicited by stepping from a holding potential of -35 mV to various test potentials between -130 mV and +60 mV, exhibits inward rectification at more depolarized potentials. The horizontal bar corresponds to 25 ms and vertical bar corresponds to 1500 pA. (C, right) D2 receptor activation in the presence of minigenes corresponding to the D2 C-terminus or the distal portion of the CaV2.2 channel does not interfere with potentiation of GIRK channel activity. Inset: Representative raw current trace from an individual HEK 293T cell expressing heterologous GIRK 3.1/3.4 channels and the D2 receptor before (control) and after [DA] agonist application.

Discussion

Dopamine is the major catecholamine neurotransmitter in the mammalian brain and is vital for a number of neurophysiological functions. Indeed, aberrant dopaminergic signaling is associated with various neuropathological disorders.38-44 It is well established the D2Rs inhibit N-type calcium channel activity in neurons.45-48 Moreover, D2R-mediated VD inhibition of N-type channels has been described previously in expression systems49 and parallels that observed with many other types of Gαi/o linked receptors. Here, we show that D2Rs are also capable of robust VI modulation of CaV2.2 channels via direct effects on channel activity and, indirectly, via alteration of channel surface expression levels.

Our demonstration that D2Rs form physical signaling complexes with CaV2.2 channels in striatum is consistent with previous data showing that D2Rs selectively block N-type channels in this tissue, resulting in the functional inhibition of GABAergic and cholinergic neurotransmission.46,47 This result is in contrast to our recent demonstration that D1Rs selectively form signaling complexes with CaV2.2 channels in pre-frontal cortex but not in the striatum.43,44 As both D1Rs and D2Rs are highly expressed in both of these regions,24 subsequently fixed and stained with secondary antibody. Hence, only those cells initially located at the cell surface were visualized. Under these conditions, and similar to previous observations with the wild-type CaV2.2 channel, HA-tagged channel internalization was evident (HA-CaV2.2 + D2 control: 0.65 ± 0.01; HA-CaV2.2 + D2 + DA: 0.52 ± 0.02) (Fig. 5C). In keeping with results obtained using the wild-type channel, the extent of the reduction in HA-tagged channel surface expression was similar to the extent of the reduction in D2R surface expression (D2-YFP + HA-CaV2.2 control: 0.82 ± 0.01; D2-YFP + HA-CaV2.2 + DA: 0.65 ± 0.03) (Fig. 5D). These data indicate that the agonist-dependent increase in cytoplasmic CaV2.2 channels is a result of channel internalization. Moreover, they support the idea that CaV2.2 channels can be co-trafficked with D2Rs as part of stable, receptor-channel signaling complexes.
tissue-specific differences may underlie the unique ability of D2Rs to form complexes with CaV2.2 channels in the striatum. It is possible that a particular CaV2.2 channel splice variant that is capable of D2R (but not D1R) association is preferentially expressed in the striatum. Alternatively, D1Rs and CaV2.2 channels may be physically segregated in this tissue. It is noteworthy that, in forming physical signaling complexes with the CaV2.2 channel, D1Rs and D2Rs rely on distinct channel and receptor structural determinants, which may contribute to the observed tissue specificity of these interactions.

Our observation that disruption of specific D2-CaV2.2 channel interactions interferes with CaV2.2 channel activity indicates that the association between receptors and channels may serve to optimize acute receptor-mediated channel modulation, whereas disruption of this interaction weakens the coupling of the receptors to the channels. This finding is in contrast to our recent data illustrating that disruption of D1-CaV2.2 interactions does not affect D1R-mediated CaV2.2 channel modulation. On the other hand, both receptor subtypes were found to influence membrane expression levels of CaV2.2 channels, in both an agonist-independent and an agonist-dependent manner. Considering recent findings with ORL1/NOP receptors and GABAB receptors, such a receptor mediated effect on channel surface expression may indeed constitute a universal mechanism by which GPCRs modulate channel activity.

The idea that D2Rs are coupled more efficiently (than D1Rs) to CaV2.2 channel activity is supported by the finding that, under identical experimental conditions, D2R activation produces a greater degree of overall CaV2.2 current inhibition when compared to activation of the D1R (75 versus 50% inhibition, respectively) (reviewed in Figure 5. The CaV2.2 channel traffics to and from the plasma membrane with the D2 receptor. (A) Representative confocal microscope images of HEK 293T cells expressing the CaV2.2 channel alone in the absence (control) or the presence (DA) of agonist. In both instances, the CaV2.2 channel is located throughout the cytoplasm and its sub cellular distribution is unaffected by DA. In each instance, the scale bar indicates 10 μm. (B) Representative confocal microscope images of HEK 293T cells co expressing the CaV2.2 channel and the D2 receptor, in the absence (control, top) or the presence (DA, bottom) of DA. Upon co expression with the D2 receptor, the channel is localized mainly to the plasma membrane (top). Conversely, following stimulation with DA, channel and receptor internalization is observed (bottom, arrows highlight internalized receptor/channels clusters). In each instance, the scale bar indicates 10 μm. For both panels A and B, the images reflect a single confocal plane. (C) Relative cell surface expression levels of wild type CaV2.2 channels or HA-CaV2.2 channels in the presence of the D2 receptor, assayed by confocal microscopy. (*p < 0.001, t-test). WT CaV2.2 channels were detected with a CaV2.2 antibody. In the case of the HA tagged CaV2.2 channels, live cells were treated with primary HA antibody prior to DA treatment, thus selectively labeling only those channels that were at the cell surface prior to DA application. Note that DA treatment leads to internalization of both WT CaV2.2 and HA-CaV2.2 channels from the cell surface. (D) Relative cell surface expression levels of YFP tagged D2 receptors (coexpressed with either WT CaV2.2 or HA tagged CaV2.2 channels), assayed by confocal microscopy. Note that the degree of receptor internalization closely parallels that observed for the CaV2.2 channel (C). (*p < 0.001, t-test.)
ref. 33). It is well established that signaling by different GPCRs may involve specific G-protein βγ subunit subtypes.50-52 Considering previous findings showing that N-type channels are differentially regulated by different G protein βγ subunit isoforms,53-56 this differential inhibition may be due to the coupling of these two receptor subtypes to different G protein βγ-dimers. It is also conceivable that the interaction of D1Rs and D2Rs with different regions of the channel alters the channel’s susceptibility to both VD and VI inhibition.

Overall, our data demonstrate that D2Rs modulate CaV2.2 channel activity via multiple VD and VI mechanisms, which may involve distinct receptor-channel interactions. Modulation of channel activity may occur indirectly, via DA receptor-mediated alteration of channel surface expression, or at the cell surface following receptor activation and G-protein mediated channel inhibition. This complex interplay between receptors and channels may provide for fine-tuning of intracellular calcium levels during dopaminergic signaling.

Materials and Methods

cDNAs. cDNAs encoding wild-type rat calcium channel subunits (α1B, β1b, and α2-δ1) were donated by Dr. Terry Snutch (University of British Columbia, Vancouver, BC). The construction of cDNAs encoding wild-type human GB1, and Gγt5, and 6His/Xpress-fusion proteins of the intracellular regions of the CaV2.2 channel have been described previously in detail.54,57 Constructs encoding the long isoform of the dopamine type 2 receptor (D2R) and wild-type G-protein inwardly rectifying potassium channels (GIRK), Kir 3.1 and Kir 3.4, were generously provided by Dr. Hubert van Tol, one of Canada's finest neuroscientists, and a wonderful individual. Hubert, we miss you.

GST-fusion proteins and pull down assay experiments. cDNAs encoding GST-fusion proteins of the intracellular regions of the D2R were generated using PCR or by the oligonucleotide synthesis core facility at the University of Calgary, as described previously for the intracellular regions of the D1R.33 Pull down experiments were carried out as described previously.33

YFP-tagged and pIRES minigenes. cDNAs encoding minigenes corresponding to the D2 loop-3, the D2 C-terminus, the CaV2.2 channel II–III linker, and the distal portions of the CaV2.2 channel C-terminus were generated using PCR or by the oligonucleotide synthesis core facility at the University of Calgary, as described previously in detail.33 In the case of the YFP-D2 C-terminal construct, the stop codon was removed.

Tissue culture and transient transfection. Human embryonic kidney tsA-201 (HEK 293T) cells were transfected as above but cDNA encoding a single minigene (in pIRE2-EGFP, Clontech) was transfected in place of the EGFP marker. Culture dishes were placed at 29°C, 6–9 h post-transfection, to prevent cell overgrowth.

Voltage clamp recordings. CaV2.2 channel current recordings were carried out as described previously in detail,33 with the exception that D2R stimulation was achieved via application of dopamine to a final concentration of 100 mM. In brief, currents were elicited by stepping from a holding potential of -100 mV to a test potential of +20 mV. D2R receptor activation resulted in inhibition of current activity that was comprised of both voltage dependent (VD) and voltage independent components. Voltage dependent (VD) modulation reflects the percentage of inhibited current that could be recovered upon application of a 50 ms prepulse to +150 mV prior to the test depolarization. Voltage independent (VI) modulation is defined as the percentage of inhibited current that is resistant to prepulse relief (see inset in Fig. 1B).

Voltage clamp recordings of individual HEK 293T cells expressing heterologous Kir 3.1/3.4 channels were performed as described for CaV2.2 channels. However, GIRK recordings were carried out using external recording solution comprised of 25 mM KCl, 10 mM HEPES, 10 mM glucose, and 116 mM NaCl (pH 7.4 adjusted with NaOH), and internal pipette solution comprised of 100 mM potassium gluconate, 40 mM KCl, 10 mM HEPES, 5 mM EGTA, 1 mM MgCl2 and 5 mM NaCl (pH 7.4 adjusted with KOH). For GIRK channel recordings following agonist activation of the D2R, internal recording solution was supplemented with 2.6 mM MgATP and 0.6 mM LiGTP. GIRK channel activity was tested by holding individual cells at -35 mV followed by application of a voltage ramp from -120 to +60 mV over 525 ms. Only cells displaying inward rectification were used for analysis and whole cell GIRK conductance was obtained by a linear fit to the inward current observed between the potentials of -100 mV and -60 mV during the voltage ramp. For measurement of modulation of GIRK channel activity following stimulation with DA, only cells with current amplitudes less than 2.0 nA were used for analysis.

Preparation of rat brain homogenate and co-immunoprecipitation experiments. Rat striatal homogenate was prepared and co-immunoprecipitation experiments were carried out as described previously in detail.33 D2Rs were visualized using a primary α-D2R antibody (Biotrend, 1:500) and a secondary HRP-conjugated donkey α-rabbit Ig antibody (Amersham, 1:5000).

Confocal microscopy. Membrane translocation and co-localization experiments were carried out as described previously in detail.33

Statistics. All sample means are reported ± SEM. All statistical analyses were carried out using SigmaStat 2.03 (SPSS Inc.). Statistically significant differences between means were assessed using the student’s t-test or a one-way ANOVA at the confidence level indicated.

Acknowledgements

This work was supported by an Operating Grant from the Canadian Institutes of Health Research to GWZ. GWZ is a Scientist of the Alberta Heritage Foundation for Medical Research (AHFMR) and a Canada Research Chair. AEK is supported by studentships from the AHFMR and the Heart & Stroke Foundation of Canada. We would like to dedicate this paper to the memory of Dr. Hubert van Tol, one of Canada’s finest neuroscientists, and a wonderful individual. Hubert, we miss you.
Dr. Terry Snutch (University of British Columbia, Vancouver, BC) graciously managed the peer review of this manuscript.

References

1. Carrell WA. Structure and regulation of voltage-gated Ca2+ channels. Annu Rev Cell Dev Biol 2000; 16:521-55.

2. Ishikawa T, Kaneko M, Shin HS, Takahashi T. Presynaptic N-type and P/Q-type Ca2+ channels mediating synaptic transmission at the calyx of Held of mice. J Physiol 2005; 568:199-209.

3. Westenbroek RE, Hell JW, Warner C, Dubel SJ, Snutch TP, Carrell WA. Biochemical properties and subcellular distribution of an N-type calcium channel alpha subunit. Neuron 1992; 9:1099-115.

4. Westenbroek RE, Sakura T, Elliott EM, Hell JW, Starr TV, Snutch TP, Carrell WA. Immunoochemical identification and subcellular distribution of the alpha1a subunits of brain calcium channels. J Neurosci 1995; 15:6403-18.

5. Mathie A, Berenholtz L, Hille B. Inhibition of N- and L-type calcium channels by muscarinic receptor activation in rat sympathetic neurons. Neuron 1992; 8:907-14.

6. Golard A, Siegelbaum SA. Kinetic basis for the voltage-dependent inhibition of N-type calcium current by somatostatin and norpinephrine in chick sympathetic neurons. J Neurosci 1993; 13:3884-94.

7. Ikeda SR, Schofield GG. Somatostatin blocks a calcium current in rat sympathetic ganglion neurons. J Physiol 1989; 409:221-40.

8. Shapiro MS, Hille B. Substance P and somatostatin inhibit calcium channels in rat sympathetic neurons via different G protein pathways. Neuron 1993; 10:11-18.

9. Shapiro MS, Loose MD, Hamilton SE, Nathanson NM, Gomezza J, Wes J, Hille B. Assignment of muscarinic receptor subtypes mediating G-protein-modulation of Ca(Mv)2+ channels by using knockout mice. Proc Natl Acad Sci USA 1995; 92:10889-94.

10. Yan Z, Song WJ, Surmeier J. D2 dopamine receptors reduce N-type Ca2+ currents in rat neostriatal cholinergic interneurons through a membrane-delimited, protein-kinase-C-invasive pathway. J Neurophysiol 1997; 77:1003-15.

11. Liposits D, Königsmark S, Tien RW. Alpha-adrenergic inhibition of sympathetic neurotransmitter release mediated by modulation of N-type calcium channel gating. Nature 1989; 340:639-42.

12. Caulfield MF, Jones S, Vallis Y, Buckley NJ, Kim GD, Milligan G, Brown DA. Mucarmin M-current inhibition via G alpha q/11 and alpha-adrenergeic inhibition of Ca2+ current via G alpha s in rat sympathetic neurons. J Physiol 1994; 477:415-22.

13. Telford HW, Kisulevsky AE, Peloquin JB, Zamponi GW. Scanning mutagenesis reveals a role for serine 189 of the heterotritmeric G-protein beta1 alpha subunit in the inhibition of N-type calcium channels. J Neurophysiol 2006; 96:465-70.

14. Zamponi GW, Bourin R, Nelso N, Dargot J, Snutch TP. Crosstalk between G proteins and protein kinase C mediated by the calcium channel alpha1 subunit. Nature 1997; 385:442-6.

15. Zamponi GW, Snutch TP. Modulation of voltage-dependent calcium channels by G proteins. Curr Opin Neurobiol 1998; 8:351-6.

16. Zamponi GW, Snutch TP. Decay of prepulse facilitation of N-type calcium channels during dopamine denervation. Eur J Neurosci 2000; 15:388-96.

17. Beedle AM, McRory JE, Poirot O, Doering CJ, Altier C, Barrere C, Hamid J, Barrere AM. G protein-induced trafficking of voltage-dependent calcium channels. J Biol Chem 2006; 281:287-39.

18. Altier C, Khoosravani H, Evans RM, Hamed S, Peloquin JB, Vartiainen VA, Chen L, Beedle AM, Ferguson SS, Meighrani A, Dubel SJ, Bourne E, McRory JE, Zamponi GW, ORL1 receptor-mediated internalization of N-type calcium channels. Nat Neurosci 2006; 9:51-60.

19. Lee SF, O'Dowd BF, George SR. Homo- and hetero-oligomerization of G protein-coupled receptors. Life Sci 2003; 74:173-80.

20. Geourgouzis Z, Leonardis I, Mazarakou G, Merkouris M, Hyde K, Ham H. Selective interactions between G protein subunits and RG54 with the C-terminal domains of the mu- and delta-opioid receptors control opioid receptor signaling. Cell Signal 2006; 18:771-82.

21. Seabrook GR, Knowles M, Brown N, Myers J, Sinclair H, Patel S, Freedman SB, McAllister G. Pharmacology of high-threshold calcium currents in GH3B1 pituitary cells and their regulation by activation of human D2 and D4 dopamine receptors. Br J Pharmacol 1994; 112:728-34.