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# THEMED ISSUE: CANNABINOIDS

## REVIEW

# Adenosine–cannabinoid receptor interactions. Implications for striatal function

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Adenosine and endocannabinoids are very ubiquitous non-classical neurotransmitters that exert a modulatory role on the transmission of other more 'classical' neurotransmitters. In this review we will focus on their common role as modulators of dopamine and glutamate neurotransmission in the striatum, the main input structure of the basal ganglia. We will pay particular attention to the role of adenosine $A_{2A}$ receptors and cannabinoid $CB_1$ receptors. Experimental results suggest that presynaptic $CB_1$ receptors interacting with $A_{2A}$ receptors in cortico-striatal glutamatergic terminals that make synaptic contact with dynorphinergic medium-sized spiny neurons (MSNs) are involved in the motor-depressant and addictive effects of cannabinoids. On the other hand, postsynaptic $CB_1$ receptors interacting with $A_{2A}$ and $D_2$ receptors in the dendritic spines of enkephalinergic MSNs and postsynaptic $CB_1$ receptors in the dendritic spines of dynorphinergic MSN are probably involved in the cataleptogenic effects of cannabinoids. These receptor interactions most probably depend on the existence of a variety of heteromers of $A_{2A}$, $CB_1$ and $D_2$ receptors in different elements of striatal spine modules. Drugs selective for the different striatal $A_{2A}$ and $CB_1$ receptor heteromers could be used for the treatment of neuropsychiatric disorders and drug addiction and they could provide effective drugs with fewer side effects than currently used drugs.
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**Keywords:** adenosine $A_{2A}$ receptor; cannabinoid $CB_1$ receptor; dopamine $D_2$ receptor; receptor heteromers; striatum; basal ganglia disorders; drug addiction

**Abbreviations:** 2-AG, 2-arachidonylglycerol; BRET, bioluminescence resonance energy transfer; CNS, central nervous system; CREB, cAMP-responsive elements binding; DARPP-32, 32 kDa dopamine- and adenosine 3′,5′-monophosphate-regulated phosphoprotein; DGL, diacylglycerol lipase; FRET, fluorescence resonance energy transfer; GABA, γ-aminobutyric acid; GPCR, G protein-coupled receptor; MSN, medium-sized spiny neuron; NAPE-PLD, N-acyltransferase and N-acylphosphatidylethanolamine-phospholipase D; PKA, protein kinase A; Rluc, *Renilla luciferase*; THC, delta9-tetrahydrocannabinol; VTA, ventral tegmental area; YFP, yellow fluorescent protein

## The striatal spine module

The concept of 'local module' facilitates the understanding of the integrated role that a neurotransmitter has in a particular brain area. Also it facilitates understanding the role of interactions between different neurotransmitters. Local module is defined as the minimal portion of one or more neurons and/or one or more glial cells that operates as an independent integrative unit (Ferré *et al.*, 2007a). In a recent review we analysed the different types of local modules centred in the dendritic spines of striatal γ-aminobutyric acidergic (GABAergic) efferent neurons, also called medium-sized spiny neurons (MSNs), which constitute more than 95% of the striatal neuronal population (Ferré *et al.*, 2009a). MSNs receive two main extrinsic inputs: glutamatergic afferents from cortical, limbic and thalamic areas and dopaminergic afferents from the mesencephalon [substantia nigra pars compacta and ventral tegmental area (VTA)] (Gerfen, 2004). The glutamatergic and

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dopaminergic terminals make synaptic contact with the head and neck of the dendritic spines of the MSN (Gerfen, 2004). GABAergic intrinsic inputs from GABAergic interneurons and from MSN collaterals also contact the neck of the dendrite or the dendritic shaft close to the base of the dendritic spine (Ferré *et al*., 2009a). Because the dopaminergic and GABAergic inputs preferentially target the distal and proximal portions of the MSN dendritic tree, at least two different types of local modules can be distinguished. Each 'striatal spine module' includes the dendritic spine of the MSN, the glutamatergic terminal wrapped by glial processes and dopaminergic and/or GABAergic nerve terminals (Ferré *et al*., 2009a). But there are different phenotypes of MSNs that give rise to several additional types of striatal spine modules.

Two classes of MSNs, which are homogeneously distributed in the striatum, can be differentiated by their output connectivity and their expression of dopamine and adenosine receptors and neuropeptides. In the dorsal striatum (mostly represented by the nucleus caudate-putamen), enkephalinergic MSNs connect the striatum with the globus pallidus (lateral globus pallidus) and express the peptide enkephalin and a high density of dopamine $D_2$ and adenosine $A_{2A}$ receptors (they also express adenosine $A_1$ receptors), while dynorphinergic MSNs connect the striatum with the substantia nigra (pars compacta and reticulata) and the entopeduncular nucleus (medial globus pallidus) and express the peptides dynorphin and substance P and dopamine $D_1$ and adenosine $A_1$ but not $A_{2A}$ receptors (Ferré *et al*., 1997; Gerfen, 2004; Quiroz *et al*., 2009). These two different phenotypes of MSN are also present in the ventral striatum (mostly represented by the nucleus accumbens and the olfactory tubercle). However, although they are phenotypically equal to their dorsal counterparts, they have some differences in terms of connectivity. First, not only enkephalinergic but also dynorphinergic MSNs project to the ventral counterpart of the lateral globus pallidus, the ventral pallidum, which, in fact, has characteristics of both the lateral and medial globus pallidus in its afferent and efferent connectivity. In addition to the ventral pallidum, the medial globus pallidus and the substantia nigra-VTA, the ventral striatum sends projections to the extended amygdala, the lateral hypothalamus and the pedunculopontine tegmental nucleus. Finally, unlike the dorsal striatum, the substantia nigra pars reticulata is not a main target area for the ventral striatum, which preferentially directs its midbrain output to the substantia nigra pars compacta and the VTA (Heimer *et al*., 1995; Robertson and Jian, 1995; Ferré, 1997). It is also important to mention that a small percentage of MSNs have a mixed phenotype and express both $D_1$ and $D_2$ receptors (Surmeier *et al*., 1996).

Enkephalinergic and dynorphinergic MSNs can also be differentiated into two phenotypically different groups of neurons, which are heterogeneously distributed in the patch (striosomes) or the matrix compartments. One main characteristic of the patch compartment is that it has a much higher expression of μ-opioid receptors than the matrix compartment (Mansour *et al*., 1987; Gerfen, 2004). But there are also connectivity differences: patch-MSNs receive cortical input predominantly from periallocortical areas (such as the infralimbic and prelimbic cortices) while matrix-MSNs receive input from neocortical areas (Gerfen, 2004). Furthermore patch-dynorphinergic-MSNs project predominantly to the substantia nigra pars compacta and matrix-dynorphinergic-MSNs project predominately to the substantia nigra pars reticulata (Gerfen, 2004). In summary, there are different types of striatal spine modules, based on variations in localization on the dendritic tree and in enkephalinergic versus dynorphinergic and in patch versus matrix phenotypes (Ferré *et al*., 2009a).

## Adenosine and endocannabinoids in the striatal spine modules

The main sources of extracellular adenosine in the striatum still need to be established, but there is evidence suggesting that an important source is ATP co-released with glutamate from glia and glutamatergic terminals (Pascual *et al*., 2005; Schiffmann *et al*., 2007; Ferré *et al*., 2007a). ATP is then converted to adenosine by means of ectonucleotidases. In fact, most extracellular adenosine, ATP and glutamate seem to be glial in origin, but to depend on neuronal glutamate release. When glutamate and ATP receptors localized in astrocytic membranes closely apposed to glutamatergic synapses are activated, astrocytes release further glutamate and ATP (Hertz and Zielke, 2004). Therefore, an increased extracellular concentration of adenosine, coming from ATP released from nerve terminals and amplified by the adjacent glial processes, represents a signal of increased glutamatergic neurotransmission (Schiffmann *et al*., 2007; Ferré *et al*., 2007b). Recent studies also suggest that ATP could also be co-released with dopamine in the striatum (Cechova and Venton, 2008).

Most of the effects of adenosine in the central nervous system (CNS) are mediated by $A_1$ and $A_{2A}$ receptors, which are usually coupled to $G_{i/o}$ and $G_{s/olf}$ proteins respectively (Fredholm *et al*., 2001). $A_{2A}$ receptors are more concentrated in the striatum than anywhere else in the brain, while $A_1$ receptors are more widespread (Fredholm *et al*., 2001; Rosin *et al*., 2003; Schiffmann *et al*., 2007; Quiroz *et al*., 2009). In the striatal spine module, $A_{2A}$ receptors are localized predominantly postsynaptically in the dendritic spine of enkephalinergic but not dynorphinergic MSNs, co-localized with $D_2$ receptors (Ferré *et al*., 2007a,b; Quiroz *et al*., 2009). Striatal $A_{2A}$ receptors are also found presynaptically in glutamatergic terminals, where they form receptor heteromers with $A_1$ receptors (Ciruela *et al*., 2006). Recent studies have shown that these presynaptic $A_{2A}$ receptors are also segregated and that they are predominantly localized in glutamatergic terminals that make synaptic contact with dynorphinergic MSNs (Quiroz *et al*., 2009). In fact, *in vitro* and *in vivo* experiments indicate the existence of a selective presynaptic $A_{2A}$ receptor-mediated modulation of glutamatergic neurotransmission to dynorphinergic MSNs (Quiroz *et al*., 2009). Therefore, the recently proposed relative expression of $A_{2A}$ receptors in the different subtypes of striatal spine modules (Ferré *et al*., 2009a) already needs to be amended. With respect to the other elements of the striatal spine module, $A_{2A}$ receptors show little expression in the dopaminergic and GABAergic terminals (Rosin *et al*., 2003; Gomes *et al*., 2009). Nevertheless, recent studies suggest the existence of functional $A_{2A}$ receptors in the striatal collaterals of enkephalinergic MSNs, which activation facilitates

GABA release (Shindou *et al*., 2008). Less clear is the significance of results from some studies suggesting the existence of striatal $A_{2A}$ receptors localized in GABA terminals whose activation inhibits GABA release (Mori and Shindou, 2003). Although, contrary to $A_{2A}$ receptors, no segregation seems to apply to $A_1$ receptors, segregation of other receptors allows them to form different receptor heteromers in different striatal spine modules. As mentioned before, $A_1$ receptors heteromerize with $A_{2A}$ receptors in the glutamatergic terminals that contact dynorphinergic MSNs and also in these neurons they seem to form heteromers with $D_1$ receptors (Ferré *et al*., 1997; 2007a).

Endocannabinoids are membrane-derived signalling lipids that stimulate G protein-coupled receptors (GPCRs) that are targeted by delta9-tetrahydrocannabinol (THC), the main addictive ingredient of marijuana. Two major endocannabinoids, anandamide and 2-arachidonylglycerol (2-AG), have been discovered. Like classical neurotransmitters they are released from neurons following neuronal depolarization and $Ca^{2+}$ influx into the cell. Unlike classical neurotransmitters, they are not stored in vesicles, but are produced 'on demand' from endocannabinoid precursors by the action of enzymes localized in the plasma membrane (Di marzo *et al*., 1998; Freund *et al*., 2003; Piomelli, 2003). The enzymes necessary for the biosynthesis of anandamide are the $Ca^{2+}$-dependent N-acyltransferase and N-acylphosphatidylethanolamine-phospholipase D (NAPE-PLD). For the biosynthesis of 2-AG, the main enzymes involved are the $Ca^{2+}$-dependent and $G_{q\text{-}11}$-coupled receptor-activated phospholipase C and diacylglycerol lipase (DGL). The action of endocannabinoids is terminated by uptake or diffusion into cells followed by intracellular metabolism. Fatty acid amide hydrolase and monoacylglycerol lipase are the two primary enzymes involved in intracellular metabolism of anandamide and 2-AG respectively (Di Marzo *et al*., 1998; Freund *et al*., 2003; Piomelli, 2003). Finally, the existence of an endocannabinoid membrane equilibrative transporter has been postulated to explain cellular uptake of both anandamide and 2-AG (Piomelli, 2003). Some studies suggest that this transporter, which has not yet been identified, plays a role in endocannabinoid release (Ronesi *et al*., 2004; Adermark and Lovinger, 2007). However, the existence of a transporter has been questioned and, instead, fatty acid hydrolases maybe important for uptake of anandamide and 2-AG (Kaczocha *et al*., 2006).

There are two subtypes of cannabinoid receptors so far identified and characterized, $CB_1$ and $CB_2$ receptors. The $CB_1$ subtype is the one predominantly expressed in the adult CNS and it is considered the most abundant GPCR in the brain (Herkenham *et al*., 1990; 1991), although $CB_2$ receptors have also been found to be expressed in neurons and other brain cells (Ashton *et al*., 2006; Gong *et al*., 2006; Brusco *et al*., 2008a,b). $CB_1$ receptors are usually coupled to $G_i$ proteins (Demuth and Molleman, 2006) and are often localized presynaptically, where their stimulation usually inhibits neurotransmitter release (Di Marzo *et al*., 1998; Piomelli, 2003). In the striatal spine module $CB_1$ are localized both pre- and postsynaptically (reviewed in Ferré *et al*., 2009a). Presynaptically, $CB_1$ receptors are localized in GABAergic terminals of interneurons or collaterals from MSNs, and also in glutamatergic but not in dopaminergic terminals (Pickel *et al*., 2004; 2006; Köfalvi *et al*., 2005; Mátyás *et al*., 2006; Uchigashima *et al*., 2007). Postsynaptically, $CB_1$ receptors are localized in the somatodendritic area of MSN (Rodriguez *et al*., 2001; Pickel *et al*., 2004; 2006; Köfalvi *et al*., 2005) and both enkephalinergic and dynorphinergic MSNs express $CB_1$ receptors (Martín *et al*., 2008).

One of the best studied functions of endocannabinoids is retrograde signalling with stimulation of presynaptic $CB_1$ receptors and the consequent inhibition of neurotransmitter release. 2-AG, rather than anandamide, seems to be mainly responsible for endocannabinoid-mediated retrograde signalling in the striatum and, probably in most brain areas (Hashimotodani *et al*., 2007). A recent study has demonstrated that in the striatum DGL is mostly localized in the plasma membrane of the dendritic spines of MSNs (Uchigashima *et al*., 2007). The same study showed that, in MSNs, inhibition of DGL abolishes depolarization-induced suppression of inhibition and depolarization-induced suppression of excitation (Uchigashima *et al*., 2007), which depend on activation of postsynaptic $G_{q\text{-}11}$-coupled receptors and on activation of presynaptic $CB_1$ receptors with inhibition of GABA and glutamate release respectively (Hashimotodani *et al*., 2007). As mentioned before, $Ca^{2+}$-dependent and $G_{q\text{-}11}$-coupled receptor-activated phospholipase C is a main enzyme in 2-AG synthesis. The metabotropic glutamate receptor $mGlu_5$ is a $G_{q\text{-}11}$-coupled receptor that is co-expressed with DGL in the dendritic spines of MSNs and it has been found to be particularly involved in endocannabinoid-dependent retrograde signalling (Uchigashima *et al*., 2007). Studies performed in the hippocampus suggest that, unlike 2-AG, anandamide may be preferentially involved in anterograde signalling, as NAPE-PLD was found to be concentrated presynaptically, where it was associated with intracellular calcium stores in several types of excitatory axon terminals (Nyilas *et al*., 2008). Furthermore, fatty acid amide hydrolase is mostly localized postsynaptically (Freund *et al*., 2003).

## Adenosine $A_{2A}$ receptor–cannabinoid $CB_1$ receptor interactions

The very high expression of $A_{2A}$ and $CB_1$ receptors in the striatum (Herkenham *et al*., 1990; 1991; Fredholm *et al*., 2001; Rosin *et al*., 2003; Schiffmann *et al*., 2007; Quiroz *et al*., 2009) suggests that direct or indirect interactions between $A_{2A}$ and $CB_1$ receptors are involved in the modulation of motor activity and goal-directed behaviours. In fact, there is experimental evidence indicating that adenosine and adenosine ligands, by acting on $A_{2A}$ receptors, modulate some of the pharmacological effects of cannabinoids, particularly those dependent on striatal function (see below). If we also take into account that the density of $A_{2A}$ receptors is much higher in the striatum than anywhere else in the brain (Fredholm *et al*., 2001; Rosin *et al*., 2003; Schiffmann *et al*., 2007; Quiroz *et al*., 2009), we can infer that those pharmacological effects induced by cannabinoids that are strongly dependent on $A_{2A}$ receptor function probably involve $CB_1$ receptors localized in the striatum.

Cannabinoids produce a myriad of pharmacological (behavioural and autonomic) effects (Chaperon and Thiébot,

1999). Among those effects, hypolocomotion, hypothermia, antinociception and catalepsy constitute the so-called 'tetrad syndrome' of cannabinoid activity in experimental animals (Chaperon and Thiébot, 1999; Monory *et al*., 2007). These pharmacological effects of cannabinoids depend on $CB_1$ receptor activation, as they are counteracted by pharmacological blockade or genetic inactivation of $CB_1$ receptors (Ledent *et al*., 1999; Zimmer *et al*., 1999; Varvel *et al*., 2005; Monory *et al*., 2007). Surprisingly, recent studies using conditional knockout mouse lines lacking the expression of $CB_1$ receptors in different neuronal subpopulations indicate that the tetrad syndrome does not depend on functional expression of $CB_1$ receptors on GABAergic interneurons (Monory *et al*., 2007), as previously thought. Those studies strongly support the involvement of $CB_1$ receptors localized in corticostriatal glutamatergic neurons in the hypolocomotor effects of THC, while the cataleptic effects of THC seem to depend on the functional expression of CB1 receptors in MSNs, particularly $D_1$ receptor-containing neurons (Monory *et al*., 2007).

We also obtained indirect but compelling evidence for a main role of striatal $CB_1$ receptors in the motor-depressant effects of cannabinoids (Carriba *et al*., 2007). In our experiments, local infusion of the $CB_1$ receptor agonist WIN 55212-2 produced a decrease in exploratory locomotor activity in rats, which was completely counteracted by the previous systemic administration of two different $CB_1$ receptor antagonists, SR141716A and AM251. Furthermore, systemic administration of the selective $A_{2A}$ receptor antagonist MSX-3 completely counteracted the motor-depressant effects of WIN 55212-2 (Carriba *et al*., 2007). Previous studies had also shown that pharmacological blockade or genetic inactivation of $A_{2A}$ receptors reduced the cataleptic effects induced by systemic administration of the $CB_1$ receptor agonist CP55,940 in mice (Andersson *et al*., 2005) and that $A_{2A}$ receptor knockout mice show a decrease in the rewarding and aversive effects of low and high doses of THC respectively (Soria *et al*., 2006). Furthermore, we found that low doses of the $A_{2A}$ receptor antagonist MSX-3 reduce the reinforcing effects of THC and anandamide under a fixed-ratio schedule of intravenous drug injection in squirrel monkeys (Justinova *et al*., 2008). These studies suggest that not only the motor but also the addictive effects of THC are mediated by $CB_1$ receptors localized in the striatum and that $A_{2A}$ receptor stimulation is involved in those effects.

At this point it is important to mention that there has been, and there is still, debate about the main localization of $CB_1$ receptors involved in the addictive effects of THC (Gardner, 2005; Lupica and Riegel, 2005). A common molecular mechanism contributing to the development of drug addiction that is shared by various drugs of abuse (including cannabinoids) is their ability to increase levels of extracellular dopamine in ventral striatum, especially in the shell of the nucleus accumbens (Koob, 1992; Robbins and Everitt, 1996; Di Chiara, 2002). The systemic administration of THC increases the firing rate of the VTA neurons and induces dopamine release in the nucleus accumbens (Chen *et al*., 1990; French *et al*., 1997; Tanda *et al*., 1997; Solinas *et al*., 2007). Although the involvement of $CB_1$ receptors from the VTA has been suggested, *in vivo* administration of THC directly into the VTA does not induce dopamine release in the nucleus accumbens, while direct infusion of THC into the nucleus accumbens does (Schlicker and Kathmann, 2001; Gardner, 2005; Lupica and Riegel, 2005). It has then been suggested that presynaptic $CB_1$ receptors that control striatal glutamate release (Robbe *et al*., 2001) are main targets for the dopamine-releasing effects of cannabinoids. Their stimulation would decrease the excitability of the striatal GABAergic dynorphinergic neurons that project to the mesencephalon and tonically inhibit dopaminergic cells in the VTA, therefore stimulating dopamine release in the nucleus accumbens (Schlicker and Kathmann, 2001). Relevant to the present review and adding more evidence for the striatal localization of $CB_1$ receptors involved in the addictive effects of THC, we found that in rats a behaviourally active dose of the $A_{2A}$ receptor antagonist MSX-3 significantly counteracted THC-induced, but not cocaine-induced, increases in extracellular dopamine levels in the shell of the nucleus accumbens (Justinova *et al*., 2008).

Going back to the striatal spine modules, and without discarding the possibility of indirect interactions between receptors localized in different neurons, there are two main localizations where close interactions between $A_{2A}$ and $CB_1$ receptors can take place: the dendritic spine of enkephalinergic MSNs and the glutamatergic terminals that make synaptic contact with dynorphinergic MSNs. From the experiments with conditional knockout mice and dopamine release mentioned above, it seems most probable that interactions between $A_{2A}$ and $CB_1$ receptors localized in glutamatergic terminals that contact dynorphinergic MSNs are primarily involved in the hypolocomotor and rewarding effects of THC. However, from results obtained with biochemical and electrophysiological experiments, it has been suggested that postsynaptic mechanisms are also involved in striatal $A_{2A}$ receptor-dependent $CB_1$ receptor function (Andersson *et al*., 2005; Tebano *et al*., 2009). Andersson *et al*. (2005) found that systemic administration of the $CB_1$ receptor agonist CP55,940 in mice produces catalepsy and striatal protein kinase A (PKA)-dependent phosphorylation at threonine 34 of 32 kDa dopamine- and adenosine 3′,5′-monophosphate-regulated phosphoprotein (DARPP-32). These effects were counteracted by a $CB_1$ receptor antagonist, by pharmacological blockade of $A_{2A}$ receptors or by genetic inactivation of $A_{2A}$ or $D_2$ receptors. Furthermore, $CB_1$ receptor agonist-induced catalepsy was significantly reduced in DARPP-32 knockout mice (Andersson *et al*., 2005). These results suggest that $CB_1$ receptors in enkephalinergic MSNs utilize $G_{s/olf}$ protein-dependent signalling, which is probably related to simultaneous molecular interactions between these $CB_1$ receptors with $A_{2A}$ and $D_2$ receptors (see below) and may be involved in the cataleptogenic effects of cannabinoids. In fact, in co-transfected cells or primary striatal neurons in culture, co-stimulation, but not individual receptor stimulation of $CB_1$ and $D_2$ receptors results in a $G_{s/olf}$ protein-dependent (pertussis toxin-insensitive) adenylyl cyclase activation (Glass and Felder, 1997; Kearn *et al*., 2005). Another study suggested that simply co-expressing $CB_1$ and $D_2$ receptors is sufficient to induce stimulation of adenylyl cyclase in response to $CB_1$ receptor activation (Jarrahian *et al*., 2004). Postsynaptic $CB_1$ receptors are also localized on dynorphinergic MSNs, where they probably have inhibitory effects, which are also probably involved in the cataleptogenic effects of cannabinoids (Monory *et al*., 2007). Those postsynaptic $CB_1$

receptors localized in dynorphinergic MSNs are probably coupled to $G_{i/o}$ proteins (Martín *et al*., 2008). As also pointed out by Andersson *et al*. (2005), their biochemical results could also involve a presynaptic mechanism, by a $CB_1$-mediated inhibition of striatal glutamate release in dynorphinergic MSNs (as glutamate receptor stimulation results in dephosphorylation of DARPP-32 at threonine 34; Svenningsson *et al*., 2004).

Both *in vitro* and *in vivo* electrophysiological experiments have shown that $CB_1$ receptor agonists produce a pronounced decrease in striatal synaptic transmission, mostly by decreasing excitatory neurotransmission and it is currently believed that this is predominantly related to inhibition of glutamate release (Robbe *et al*., 2001; Pistis *et al*., 2002; Kreitzer and Malenka, 2007). Tebano *et al*. (2009) recently showed that a $CB_1$ receptor-mediated decrease in striatal synaptic transmission (measuring extracellular field potentials in cortico-striatal slices) can also be partially, but significantly, reduced by pharmacological blockade or genetic inactivation of $A_{2A}$ receptors. The authors conclude that their results are more consistent with a postsynaptic mechanism, although a presynaptic component could not be ruled out (Tebano *et al*., 2009). According to Tebano *et al*. the postsynaptic mechanism in this case would depend on an interaction between $A_{2A}$ and $CB_1$ receptors in the enkephalinergic MSN. This interpretation would not fit with the above-postulated predominant $G_{s/olf}$-coupling-dependent stimulatory role of postsynaptic $CB_1$ receptors in enkephalinergic MSNs.

In summary, the experimental results mentioned so far suggest that presynaptic $CB_1$ receptors interacting with $A_{2A}$ receptors in cortico-striatal glutamatergic terminals that make synaptic contact with dynorphinergic MSNs are involved in the motor-depressant and addictive effects of cannabinoids. On the other hand, postsynaptic $CB_1$ receptors interacting with $A_{2A}$ and $D_2$ receptors in the dendritic spines of the enkephalinergic MSNs (and postsynaptic $CB_1$ receptors in the dendritic spines of dynorphinergic MSN, according to the conditional knockout mice experiments) are probably involved in the cataleptogenic effects of cannabinoids. In both cases, basal levels of $A_{2A}$ receptor activation would be necessary for $CB_1$ receptor function. These, so far hypothetical, mechanisms need to be reconciled with other more established functions of $A_{2A}$ receptors in striatal spine modules. For instance, how is it possible that $A_{2A}$ receptor stimulation in striatal glutamatergic terminals induces glutamate release (Ciruela *et al*., 2006; Quiroz *et al*., 2009) and yet $A_{2A}$ receptor stimulation is necessary for a $CB_1$ receptor-mediated inhibition of glutamate release?

The analysis of the differential effects of $A_{2A}$ receptor ligands in different animal models can already give some clues about these apparently contradictory findings. First it is important to remember that according to the most accepted model of basal ganglia function, stimulation of enkephalinergic or dynorphinergic MSNs leads to motor depression or motor activation, respectively, and that the final motor activation depends on the balanced activation of both striatal efferent neurons (Gerfen, 2004; DeLong and Wichmann, 2007). Results from different research groups have repeatedly shown that $A_{2A}$ receptor antagonists produce motor activation and $A_{2A}$ receptor agonists produce motor depression (see Karczkubicha *et al*., 2003, and references therein) and that $A_{2A}$ receptor agonists produce an increase in the striatal basal levels of glutamate, while $A_{2A}$ receptor antagonists do not significantly modify those basal levels (Quarta *et al*., 2004a,b), although they block striatal glutamate release induced by cortical stimulation (Quiroz *et al*., 2009). These pharmacological findings suggest that there is a tonic basal stimulation of postsynaptic striatal $A_{2A}$ receptors, and their blockade produces motor activation. These postsynaptic $A_{2A}$ receptors are probably only partially occupied by endogenous adenosine, explaining the ability of $A_{2A}$ receptor agonists to produce motor depression. On the other hand, there is little tonic activation of striatal presynaptic $A_{2A}$ receptors that produce glutamate release when stimulated, indicating that these receptors are only important during increases in input from cortical glutamatergic afferents, which increases striatal glutamate release and adenosine production (see above and Ferré *et al*., 2007a,b; Schiffmann *et al*., 2007). However, presynaptic $A_{2A}$ receptors that facilitate $CB_1$-mediated inhibition of glutamate release must be tonically activated, which would explain the blockade by $A_{2A}$ receptor antagonists of the putative effects of cannabinoids on dynorphinergic MSNs described above. Thus, there should be different pools of presynaptic and postsynaptic $A_{2A}$ receptors with different affinities for adenosine and possibly with different G protein-coupling or signalling (see above). This is where receptor heteromers come into play.

## Adenosine $A_{2A}$–cannabinoid $CB_1$ receptor heteromers

Receptor heteromer has been recently defined as a macromolecular complex composed of at least two (functional) receptor units or protomers with biochemical properties that are demonstrably different from those of its individual components (Ferré *et al*., 2009b). In a recent study, using a functional complementation assay, $D_2$ receptor homodimers with a single G protein were found to be a minimal signalling unit, which is maximally activated by agonist binding to one of the protomers, whereas additional agonist or inverse agonist binding to the second protomer blunts or enhances signalling respectively (Han *et al*., 2009). This allosteric modulation of signalling results from a direct interaction of the receptor homodimer (which should probably be called homodimeric $D_2$ receptor; see Ferré *et al*., 2009b, for nomenclature) with the single interacting G protein, rather than from a downstream effect (Han *et al*., 2009). A similar situation, two receptor units and one G protein, is most probably found in a receptor heterodimer, but in this case two different ligands interact within the heteromer, and several putative allosteric interactions like the one described for $D_2$ receptor homomers have also been described, for instance, in the $A_{2A}$–$D_2$ and $A_1$–$A_{2A}$ receptor heteromers (Ciruela *et al*., 2006; Ferré *et al*., 2008; 2009b). In these examples, stimulation of $A_{2A}$ receptors decreases the affinity of $D_2$ and $A_1$ receptors for their respective agonists (Ferré *et al*., 1991; Ciruela *et al*., 2006). It is currently believed that an allosteric interaction in a receptor heteromer (as well as in a receptor homomer) involves an

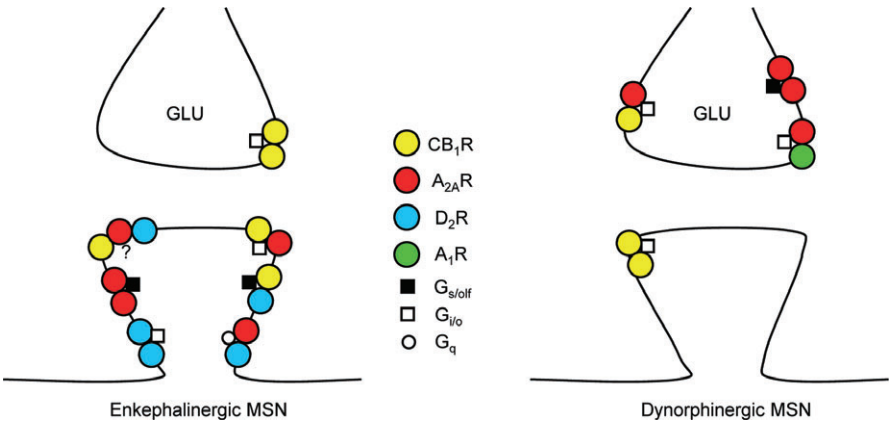


**Figure 1** Scheme showing putative localization and G protein coupling of adenosine $A_{2A}$ and $CB_1$ receptor oligomers in the striatal spines and glutamatergic terminals of enkephalinergic and dynorphinergic medium-sized spiny neurons (MSNs) discussed in the text. For simplicity, $A_1$ and $D_2$ receptors are only represented in the glutamatergic terminals that contact dynorphinergic MSNs and in the dendritic spines of the enkephalinergic MSNs respectively. However, $A_1$ receptors also seem to be located in glutamatergic terminals that contact enkephalinergic MSNs and also postsynaptically in both enkephalinergic and dynorphinergic MSNs. Similarly, $D_2$ receptors also seem to be localized presynaptically in striatal glutamatergic terminals.

intermolecular interaction by which binding of a ligand to one of the protomers induces structural changes on the second protomer that result in functional or pharmacological changes on this second protomer (Milligan and Smith, 2007; Ferré *et al*., 2007b; 2009b).

In addition to allosteric modulations, GPCR oligomerization *per se* can produce changes in ligand recognition and G protein-coupling. With regard to changes in ligand recognition, for instance, the $A_{2A}$ receptor was found to have 13 times higher affinity for caffeine when co-transfected with $D_2$ than when co-transfected with $A_1$ receptors (Ciruela *et al*., 2006). With regard to changes in G protein-coupling, recent models indicate that only one G protein can bind to two receptor units (van Rijn and Whistler, 2009; Han *et al*., 2009). This means that a receptor heteromer will at least have to 'decide' to which G protein it should bind if its protomers are usually coupled to different G proteins. In some cases, the receptor heteromer can 'choose' a completely new G protein. For instance, dopamine receptors are classified as $D_1$-like, with $D_1$ and $D_5$ receptor subtypes, usually coupled to $G_{s/olf}$ proteins, or $D_2$-like, with $D_2$, $D_3$ and $D_4$ receptor subtypes, usually coupled to $G_{i/o}$ proteins. However, the $D_1$–$D_3$ receptor heteromer couples to $G_{s/olf}$ (Fiorentini *et al*., 2008) and the $D_1$–$D_2$ and $D_2$–$D_5$ receptor heteromers couple preferentially to $G_q$ proteins (Rashid *et al*., 2007; So *et al*., 2009). Although it still needs to be demonstrated, the $A_1$–$A_{2A}$ heteromer probably couples to $G_{i/o}$ and the $A_{2A}$–$D_2$ receptor heteromer couples to $G_q$ (Ferré *et al*., 2008) (Figure 1).

The functional interactions between adenosine $A_{2A}$, cannabinoid $CB_1$ and dopamine $D_2$ receptors mentioned above provide the framework for the possible molecular interactions between $A_{2A}$ and $CB_1$ receptors and for the existence of $A_{2A}$–$CB_1$ and $A_{2A}$–$CB_1$–$D_2$ receptor heteromers. $A_{2A}$–$CB_1$ receptor heteromerization has been demonstrated by means of bioluminescence resonance energy transfer (BRET) techniques in cells co-transfected with $A_{2A}$ receptors fused to *Renilla luciferase* (Rluc) and $CB_1$ receptors fused to a yellow fluorescence protein (YFP) (Carriba *et al*., 2007). It is important to point out that just the existence of BRET does not demonstrate the existence of actual physical contact between the fused proteins, but that there exists a very close proximity, which could depend on oligomerization. Nevertheless, when using BRET, physical contact can be shown by using several different experimental approaches. One possibility includes BRET saturation experiments, where a constant amount of the donor fusion protein (usually a receptor fused to RLuc) is co-expressed with increasing amounts of the acceptor fusion protein (usually a receptor fused to YFP). If there is oligomerization, saturation is reached when all receptor-Rluc molecules are specifically associated with their receptor-YFP counterparts. By contrast, if the BRET signal results from random collision promoted by high receptor density, a quasi-linear curve is obtained. A clear saturation BRET curve was obtained for the $A_{2A}$–Rluc–$CB_1$–YFP pair when constant amounts of the cDNA for the Rluc construct were co-transfected with increasing amounts of the plasmid cDNA for the YFP construct (Carriba *et al*., 2007). The existence of striatal $A_{2A}$–$CB_1$ receptor heteromers has also been inferred from confocal immunohistochemical analysis of rat brain sections and co-immunoprecipitation experiments from rat striatal membranes (Carriba *et al*., 2007). $A_{2A}$–$CB_1$ receptor co-localization was found predominantly in fibrilar structures, compatible with both dendritic processes and nerve terminals (Carriba *et al*., 2007).

The study of $A_{2A}$–$CB_1$ receptor interactions in co-transfected cells has shown that $CB_1$ receptor signalling through $G_{i/o}$ receptor-coupling (inhibition of forskolin-induced cAMP accumulation) depends on selective co-activation of $A_{2A}$ receptors (Carriba *et al*., 2007). Thus, $G_{i/o}$ receptor-coupling could constitute biochemical characteristics of $A_{2A}$–$CB_1$ receptor heteromers localized in striatal glutamatergic terminals that contact dynorphinergic MSNs and may be a main target for the motor-depressant and addictive effects of cannabinoids (Figure 1). These heteromers must coexist with $A_1$–$A_{2A}$ heteromers and $A_{2A}$ receptor homomers (Figure 1). It is our current working hypothesis that these receptor homomers and heteromers have different affinities for adenosine. Under basal conditions, adenosine seems to bind preferentially to

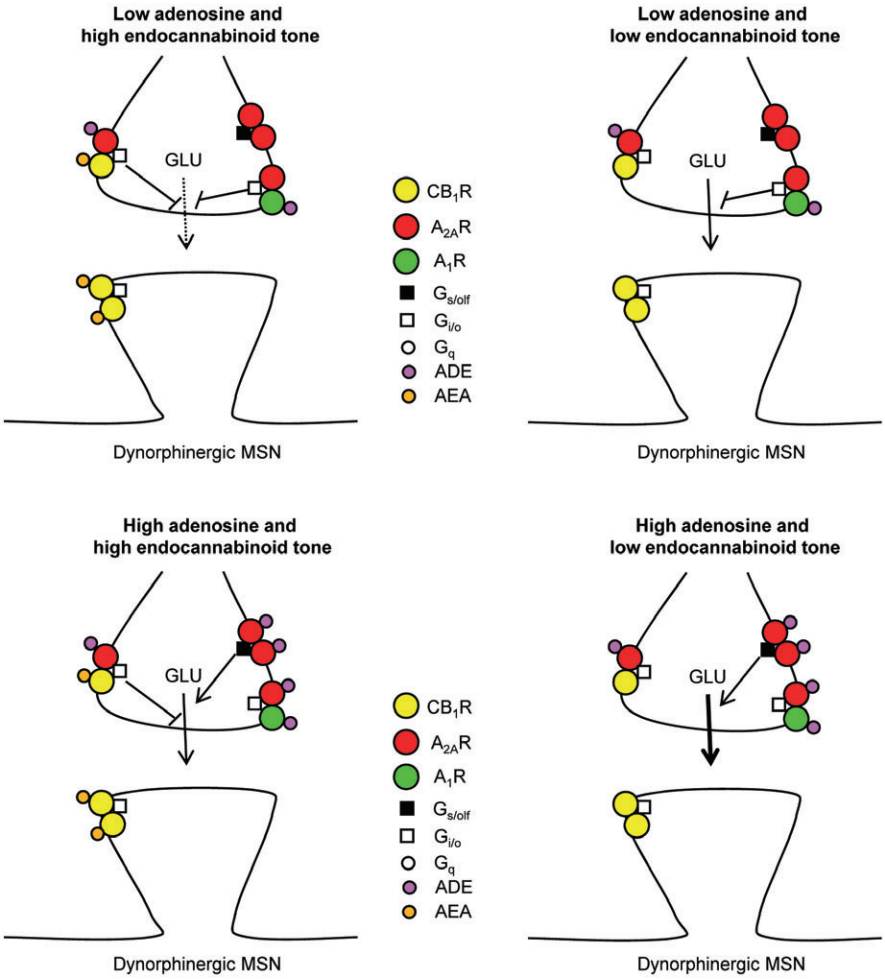


**Figure 2** Scheme showing the potential putative fine tuning for the modulation of glutamate release to the dynorphinergic medium-sized spiny neuron (MSN) that would depend on adenosine and endocannabinoid tone as well as to the different affinities of adenosine receptor homomers and heteromers for adenosine. With low adenosine tone, adenosine would bind preferentially to $A_1$ receptor in the $A_1$–$A_{2A}$ receptor heteromer, which would inhibit glutamate release, and to $A_{2A}$ receptor in the $A_{2A}$–$CB_1$ receptor heteromer. Co-stimulation of $A_{2A}$ and $CB_1$ receptors in the $A_{2A}$–$CB_1$ receptor heteromer is necessary for its signalling, which would inhibit glutamate release. Higher adenosine tone would be needed for adenosine to bind to the $A_{2A}$ receptor in the $A_{2A}$ receptor homomer, which stimulates glutamate release and in the $A_1$–$A_{2A}$ receptor heteromer, which blocks $A_1$ receptor-mediated inhibition of glutamate release. Therefore, low adenosine and high endocannabinoid tone would produce the weakest and high adenosine and low endocannabinoid tone would produce the strongest glutamate release. ADE, adenosine; AEA, anandamide.

the $A_1$ receptor in the $A_1$–$A_{2A}$ heteromers (as previously suggested in Ciruela *et al.*, 2006) and to the $A_{2A}$ receptor in the $A_{2A}$–$CB_1$ receptor heteromer. This is associated with a relatively low degree of glutamate release, which can be further inhibited by endocannabinoid release. However, under conditions of strong input, the increased adenosine formation might also activate $A_{2A}$ receptors in the $A_1$–$A_{2A}$ receptor heteromer (which inhibits its signalling due to the antagonistic allosteric interaction in the heteromer) and in the $A_{2A}$ receptor homomers, which stimulates glutamate release (Ciruela *et al.*, 2006). This can provide a very fine tuning for the modulation of glutamate release to the dynorphinergic MSN that depends on the levels of adenosine and endocannabinoids, with low adenosine and high endocannabinoid tone producing the weakest and high adenosine and low endocannabinoid tone producing the strongest glutamate release (Figure 2).

$A_{2A}$–$D_2$ receptor heteromerization has been demonstrated in mammalian transfected cells with co-immunoprecipitation, BRET and fluorescence resonance energy transfer (FRET) techniques (reviewed in Ferré *et al.*, 2008). By using computerized modelling, pull-down techniques and mass spectrometric analysis, it was shown that an electrostatic interaction between the arginine (Arg)-rich epitope located in the amino-terminal portion of the third intracellular loop of the $D_2$ receptor and a single phosphate group from a casein kinase phosphorylable serine localized in the distal portion of the carboxy-terminus of the $A_{2A}$ receptor is involved in $A_{2A}$–$D_2$ receptor heteromerization (Canals *et al.*, 2003; Ciruela *et al.*, 2004). The Arg–phosphate electrostatic interaction between epitopes located in intracellular domains is obviously not the only interaction responsible for $A_{2A}$–$D_2$ receptor heteromerization. Thus, a significant but not complete reduction of BRET is observed when transfected cells express mutated $D_2$ receptors that lack the key amino acids involved in the Arg–phosphate interaction (Ciruela *et al.*, 2004), indicating that other receptor domains are also involved. Most probably,

intramembrane domains play an important role in $A_{2A}$–$D_2$ receptor heteromerization, as has been demonstrated for other GPCR homomers and heteromers (Guo *et al*., 2008; González-Maeso *et al*., 2008; Han *et al*., 2009). Nevertheless, the significant modification of BRET with mutated receptors indicates that the Arg–phosphate interaction is necessary to provide the final quaternary structure of the heteromer, which in fact determines its function. Patch-clamp experiments in identified enkephalinergic MSNs demonstrated that disruption of the Arg–phosphate interaction in $A_{2A}$–$D_2$ receptor heteromers (by intracellular addition of small peptides with the same sequence than the receptor epitopes involved in the Arg–phosphate interaction) completely eliminates the ability of the $A_{2A}$ receptor to antagonistically modulate the $D_2$ receptor-mediated inhibition of neuronal excitability (Azdad *et al*., 2009).

The above-mentioned antagonistic interaction between $A_{2A}$ and $D_2$ receptors is probably related to the existence of an allosteric modulation in the $A_{2A}$–$D_2$ receptor heteromer. An antagonistic $A_{2A}$–$D_2$ receptor interaction has been demonstrated in many different membrane preparations from different transfected mammalian cells and from rat and human striatal tissues and implies the ability of $A_{2A}$ receptor stimulation to change the binding characteristics (decrease the affinity) of the $D_2$ receptor for agonists (Ferré *et al*., 1991; 2008). The antagonistic interaction in the $A_{2A}$–$D_2$ receptor heteromer provides a mechanism for the well-known ability of $A_{2A}$ receptor agonists and antagonists to selectively counteract or potentiate, respectively, the motor-activating effects of dopamine $D_2$ receptor agonists. This interaction also seems to be fundamental for the motor-activating effects of $A_{2A}$ receptor antagonists (and also non-selective adenosine receptor antagonists such as caffeine) and provides a rational for the use of $A_{2A}$ receptor antagonists in Parkinson's disease (Ferré *et al*., 1997; 2001; Muller and Ferré, 2007).

In addition to the allosteric modulation in the $A_{2A}$–$D_2$ receptor heteromer, $A_{2A}$ and $D_2$ receptors can also interact at the second messenger level when not forming heteromers, and this has been repeatedly demonstrated both in cell culture and in the brain (reviewed in Ferré *et al*., 2008). In this case, however, it is the stimulation of $D_2$ receptors that counteracts the effects of $A_{2A}$ receptor stimulation. $A_{2A}$ receptors, through their coupling to $G_{s/olf}$ proteins, can potentially stimulate adenyl-cyclase, with phosphorylation of several PKA substrates, such as DARPP-32, cAMP-responsive elements binding (CREB) and AMPA receptors and the consequent increase in the expression of different genes, such as *c-fos* or *preproenkephalin* in the enkephalinergic MSN (Ferré *et al*., 2007a,b; 2008; Schiffmann *et al*., 2007). $D_2$ receptors, on the other hand, can potentially couple to $G_{i/o}$ proteins and counteract the ability of $A_{2A}$ receptor stimulation to signal through the cAMP/PKA cascade (Ferré *et al*., 2007a,b; 2008; Schiffmann *et al*., 2007). Both types of antagonistic $A_{2A}$–$D_2$ receptor interactions (in the heteromer and between homomers) coexist in enkephalinergic MSNs (Figure 1). Co-stimulation of $A_{2A}$ and $D_2$ receptors implies a simultaneous $A_{2A}$ receptor-mediated inhibition of the $D_2$ receptor-mediated modulation of neuronal excitability and a $D_2$ receptor-mediated inhibition of the $A_{2A}$ receptor-mediated modulation of gene expression, which provides a clear example of a functional dissociation between neuronal excitability and gene expression. This apparently incompatible coexistence of reciprocal antagonistic $A_{2A}$–$D_2$ receptor interactions can be explained by the presence in the same cell of $A_{2A}$ and $D_2$ receptors homomers and heteromers.

In addition to the $A_{2A}$–$CB_1$ and $A_{2A}$–$D_2$ receptor heteromers, $CB_1$–$D_2$ and $A_{2A}$–$CB_1$–$D_2$ receptor heteromerization has been shown by biophysical techniques (BRET, FRET, sequential BRET-FRET, BRET-bimolecular complementation) in transfected cells (Carriba *et al*., 2008; Marcellino *et al*., 2008; Navarro *et al*., 2008) (Figure 1). As mentioned before, when $CB_1$ and $D_2$ receptors co-expressed in the same cells are co-stimulated they couple to $G_{s/olf}$, which results in stimulation of adenylyl cyclase (Glass and Felder, 1997; Kearn *et al*., 2005). It is most probable that $G_{s/olf}$-coupling is a biochemical property of $CB_1$–$D_2$ receptor heteromers (Kearn *et al*., 2005). One question that needs to be resolved is if $CB_1$–$D_2$ receptor heteromers couple constitutively to $G_{s/olf}$ (Jarrahian *et al*., 2004) or if they only switch upon co-agonist treatment (Kearn *et al*., 2005). That G protein-coupling might depend on which protomers are stimulated in the receptor heteromer constitutes an attractive possibility. If it were a common property of receptor heteromers, it might also be involved in the presynaptic control of glutamate release mentioned above, with the $A_1$–$A_{2A}$ receptor coupling to $G_i$ or $G_s$ proteins upon stimulation of $A_1$ stimulation or $A_1$–$A_{2A}$ receptor co-stimulation respectively. Also a similar co-agonist treatment-dependent G protein switch could take place in the $A_{2A}$–$CB_1$ receptor heteromer. We also need to determine whether the $A_{2A}$–$CB_1$–$D_2$ receptor heteromers exist in enkephalinergic MSNs and, if they do, we need to determine their properties and function.

## General conclusions

The adenosine–cannabinoid receptor interactions reviewed here provide a clear example of the importance of looking for the functional significance of receptor heteromers in the CNS. Local modules (such as the striatal spine module) provide the best framework for studying receptor heteromer function, which can lead to a better integrative view of the role of multiple neurotransmitters in different brain areas and circuits (such as the striatum and the basal ganglia). It is becoming evident that receptor heteromers can provide an explanation for the previously unsuspected existence of multiple functions of a single GPCR subtype, not only in different elements of a local module, but even in the same cell. These multiple functions depend on direct molecular interactions with other receptors, which results in significant changes in their ligand-binding and G protein-coupling properties. The selective localization and function, as well as the unique ligand-binding properties of receptor heteromers provide the obvious possibility of using receptor heteromers as targets for drug development. Drugs selective for different $A_{2A}$ and $CB_1$ receptor heteromers could be used for the treatment of neuropsychiatric disorders and drug addiction, and they could provide effective drugs with fewer side effects than currently used drugs. For instance, the $CB_1$ receptor antagonist rimonabant was marketed as an anti-obesity drug and was also proposed as a potential anti-smoking treatment. However, it

targets peripheral as well as CNS receptors, thus promoting side effects, such as anxiety and depression (Christensen *et al*., 2007; Mitchell and Morris, 2007). This led to FDA disapproval in USA and its removal from the market in Europe. We already mentioned that the $A_{2A}$–$D_2$ receptor heteromer can be a target for Parkinson's disease, while drugs selective for presynaptic $A_{2A}$ receptor heteromers ($A_1$–$A_{2A}$ and $A_{2A}$–$CB_1$) could be of use in diyskinetic disorders (Quiroz *et al*., 2009) and in THC addiction (see above).

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## Conflict of interest

The authors state no conflict of interest.

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