Introduction

Dopamine is a catecholamine, which serve as a neurotrasmitter and a hormone. It is considered a monoamine because it contains one amino group that is connected to an aromatic ring by a two-carbon chain. Dopamine is also a precursor of noradrenaline and adrenaline. Dopamine is biosynthesized in the brain (in the substantia nigra and the ventral tegmental area) first by the hydroxilation of L-tyrosine to $_{L}$-DOPA via tyrosine hydroxilase. Then the carboxilation of $_{L}$-DOPA by DOPA decarboxilase produces dopamine. In some neurons, dopamine is further processed into noradrenaline by dopamine beta-hydroxylase and into adrenaline by phenylethanolamine *N*-methyltransferase. In neurons, dopamine is packaged after synthesis into vesicles, which are then released into the synapse in response to a pre-synaptic action potential (Elsworth *et al*., 1997).

There are three major divisions of dopaminergic pathways which innervate the forebrain and the basal ganglia. These correspond to the dopamine cells of the ventral tegmental area (VTA), the substantia nigra and the retrorubral area (Williams *et al.*, 1998). These dopaminergic neurons are functionally involved in higher motor activities and goal-directed behavior, which includes motivation, reward, learning and working memory. When impairment occurs, neurological and psychiatric disorders tend to occur (Schultz *et al.*, 1998). Dopamine acts in various regions of the brain, such as the pre-frontal cortex (PFC), the striatum (ST) and the hippocampus (HPC). In the pre-frontal cortex, a region known to control cognition and emotions, dopamine controls these two types of behaviors (Goldman-Rakic *et al.*, 1995). It is known that dopamine receptors play a key role in the modulation of the PFC working memory functions (Goldman-Rakic *et al.*, 1995). Pre-frontal cortex activation is reported to decrease dopamine release in the nucleus accumbens (NAcc) (Jackson *et al.*, 2001). One of the hypotheses postulated is that this decrease occurs via the pre-frontal cortex projections to the

ventral-tegmental area (VTA) GABAergic neurons. These neurons are thought to be able to suppress VTA-NAcc dopamine neuronal activity (Sesack and Carr *et al.*, 2002). On the one hand, low concentrations of D1 receptor antagonists are known to enhance memory-related neuronal responses (Goldman-Rakic *et al.*, 1995). Thus, administration of low doses of dopamine D1 receptor agonists improves spatial working memory performance (Arnten *et al.*, 1997). Results such as these lead to the conclusion that optimal levels of D1 receptors, hence dopamine, are required for the maintenance of PFC functions.

Dopamine has also an important role in the striatum, a sub-cortical region, which is known to be important in planning and modulating movements (Jog *et al.*, 1999). Thus, the modification of dopamine levels in the striatum in patients with Parkinson's disease provides an elucidate example. Parkinson's disease is a neurodegenerative disease that mainly impairs motor skills as well as speech and other functions (Jankovic *et al.*, 2008). Uptake of dopamine in the putamen, a region of the dorsal striatum, of Parkinson's disease patients is greatly reduced. In an early stage this was assessed by direct biochemical assays and more recently by uptake of the dopamine precursor ^{18}F-DOPA by positron emission tomography (PET) (Kandel, 1999). Patients with Parkinson's disease also have less synaptic activity both when active and when resting, but administration of dopamine agonists tend to attenuate this, increasing the blood flow to the supplementary motor and anterior cingulate areas (Kandel, 1999). This illustrates the important role subserved by dopamine in controlling movement-related areas in the brain.

When dopamine is released from synaptic or non-synaptic varicosities, the time course and local specificity of its action depends on the properties of the uptake/breakdown of dopamine. Dopamine is inactivated by reuptake via the dopamine

transporter (DAT) or by enzymatic breakdown by catechol-O-methyl transferase (COMT) and monoamine oxidase (MAO). Dopamine that is not broken down by enzymes can be repackaged into vesicles for re-use. Dopamine can also diffuse from the synapse and help regulate blood pressure (Kandel, 1999).

In the pre-frontal cortex (PFC), the dopamine clearance rates are slower than in the striatum (Garris *et al.*, 1994). Unlike in striatal regions, the PFC shows low levels of dopamine transporter expression, and the dopamine transporter accounts for only nearly 40% of the dopamine uptake in the PFC, compared with 95% in the striatum (Wayment *et al.*, 2001). A significant portion of the dopamine uptake in the PFC is mediated through the noradrenaline transporter and further catabolization by cathecol-O-methyltransferase (Morón *et al.*, 2002).

All the dopamine receptors known so far are G-protein coupled receptors (GPCRs), with 7 trans-membrane regions. Unlike the fast ionotropic receptors (glutamate receptors such as AMPA and NMDA), all GPCRs are essentially slower, since metabotropic receptors functionally inpact on cell metabolism and modulate other receptor systems and/or ion channels (Missale *et al.*, 1998). There are at least five dopamine receptors subtypes in the central nervous system, which are grouped into two major classes, depending on the G-protein coupling and the length of the third cytoplasmatic loop and the carboxyl tail. These classes are the D1 receptor family, which includes the D1 and D5 receptors (longer third cytoplasmatic loop and carboxyl tail) and the D2 receptor family, which includes D2, D3 and D4 receptors with shorter third cytoplasmatic loop and carboxyl tail (Jin *et al.*, 2001). Through their different G-protein coupling (the D1 receptor family couples to Gs proteins and the D2 receptor family couples to G_i proteins), the two classes have opposing effects on adenylyl cyclase activity and cAMP concentration.

Another neuromodulator controlling cAMP levels is adenosine. Adenosine is an endogenous purine nucleoside which exists in all cells and it is involved in several metabolic pathways, including nucleotide and nucleoside metabolism (Cunha *et al.*, 2005). Its basal intracellular levels range from 10 to 50 nM, but small changes in the energy charge of the cells, typified by a decrease in energy charge, can lead to an abnormal increase in the intracellular levels of adenosine (Cunha, 2001). The presence of bi-directional nucleoside transporters in cells indicates that the intracellular levels of adenosine should fluctuate in parallel to the extracellular levels. It is believed that adenosine can play a modulatory role in a wide variety of tissues (Fredholm *et al.*, 2005). Adenosine is not only released in a hormone-like way, but can also be formed through the breakdown of ATP released by cells, either in a regulated way or in response to noxious insults (Fredholm *et al.*, 2005).

There are four known types of adenosine receptors and all belong to the G-protein coupled receptors class. These receptors are classified as A_1, A_{2A}, A_{2B} and A_3 (Fredholm *et al.*, 2001). The A_1 receptor has been found to be ubiquitous throughout the entire body and it has an inhibitory function, since it is connected do $G_{\alpha i}$ proteins; this will lead to an inactivation of the enzyme adenylyl cyclase, resulting in a reduction of cAMP concentration (Fredholm *et al.*, 2001). This inhibitory action occurs in all the tissues where these receptors are present. More specifically, in the brain, A_1 receptors tend to slow metabolic activity by combination of two factors: presynaptically they can reduce the release of synaptic vesicles in glutamatergic terminals, while postsynaptically they stabilize the magnesium present in the NMDA receptors, preventing post-synaptic plastic changes in excitatory (Fredholm *et al.*, 2005). Several drugs are known to act as A_1 receptors agonists, such as 2-chloro-N^6-cyclopentyladenosine (CCPA). As antagonists, both caffeine (non-specific) and 8-

cyclopentyl-1,3-dipropylxanthine (DPCPX) antagonize A_1 receptors. In contrast, A_{2A} receptors stimulate adenylyl cyclase activity, increasing the concentration of cAMP because they are coupled to a $G_{\alpha s}$ protein (Fredholm *et al.*, 2001). Selective A_{2A} receptor antagonists include SCH58261, while selective agonists include CGS21680 (Fredholm *et al.*, 2005).

It is of major importance to know the distribution of receptors, because this allows knowing where the receptor-antagonist/agonist interaction occurs. Studies with radioligands or using antibodies provide information regarding the brain distribution of both A_1 and A_{2A} receptors. A_1 receptors are known to be widespread through the brain, being abundant in limbic and neocortical regions and basal ganglia (Fredholm *et al.*, 2005). These receptors are more abundant in neurons, but they can also be found in astrocytes, microglia and oligodendrocytes (Othman *et al.*, 2003), although at a lower density. In neurons, these receptors can be found mostly in synapses, mainly in nerve terminals (Rebola *et al.*, 2003). A_{2A} receptors are not as widespread as the A_1 receptors subtype, being largely confined to the basal ganglia, with a 20 fold higher density than in any other brain areas (Lopes *et al.*, 2004). A_{2A} receptors can be found in great amounts in the striatum, but this does not mean that they are exclusively found in the basal ganglia. Several studies, including binding (Lopes *et al.*, 2004), immunological (Rebola *et al.*, 2002) and functional assays showed that A_{2A} receptors are also found namely in limbic and neocortical brain regions. In these regions, A_{2A} receptors are mainly located in synapses, mostly in the pre-synaptic active zone (Rebola *et al.*, 2005).

The distribution of A_{2B} and A_3 receptors in the brain is not as well documented as that of the other two types. A_3 receptors have been found in neurons, as shown by binding, immunological and functional studies (Costenla *et al.*, 2001). Others have shown that A_3 receptor function is more evident in astrocytes and microglia rather than

in neurons (Abbracchio *et al.*, 1998). Concerning A_{2B} receptors, they are mainly located in astrocytes (Allaman *et al.*, 2003). Functional studies also suggested a possible neuronal location, yet further molecular studies are still needed to confirm this proposal (Mogul *et al.*, 1993).

Both A_1 and A_{2A} receptors are expressed at somewhat high levels in brain areas in which dopaminergic innervations are present, which points to an interaction between these two neurotransmitter systems. Ferré *et al.* proposed a direct receptor-receptor interaction between A_{2A} and D_2 receptors, providing a possible mechanism to the many possible interactions between these two systems (Ferré *et al.*, 1991). The A_{2A}-D_2 intermembrane interaction was based on the finding that activation of A_{2A} receptors can reduce the binding affinity of D_2 receptor agonists to their receptor in the striatum (Ferré *et al.*, 1991). Further studies which include co-immunoprecipitation and confocal microscopy provided further support to the receptor-receptor interaction hypothesis (Fuxe *et al.*, 2007). In co-transfected fibroblast cells, A_{2A} and D_2 receptors aggregate in a heteromeric way (Ginés *et al.*, 2000), as revealed by co-immunoprecipitation and fluorescence resonance energy transfer (FRET). The A_{2A} and D_2 heteromeric receptor co-aggregates, co-internalizes and co-desensitizes, when treated with A_{2A} and D_2 agonists (Ferré *et al.*, 1997). Regarding A_1 receptors, studies with fibroblasts co-transfected with both receptor types showed a heteromeric immunoprecipitation with D_1 receptors; this heteromerization was prevented by pretreatment with D_1 agonists whereas activation of A_1 receptors promotes the aggregation of A_1 and D_1 receptors (Ginés *et al.*, 2000).

Adenosine can control nerve function in physiological condition, mainly by controlling neurotransmitter release (Cunha, 2001). The adenosine A_1 and A_{2A} receptors have a role in this control, however these two receptors affect this release in different

ways. A_1 receptors activation is known to inhibit neurotransmitter release (Cunha, 2001), however the mechanisms through which A_1 controls this phenomenon are yet unclear. A_{2A} receptors are known to have a facilitatory role in neurotransmitter release, and this facilitation is normally of small amplitude (Cunha, 2001). This facilitation can happen in two different ways. In some regions it can be seen only if A_1 receptors are present (Lopes *et al.*, 2002), but the A_{2A} receptor control of GABA release, which is A_1 receptor-insensitive, shows that this facilitation can also occur in the absence of A_1 receptors (Brooke *et al.*, 2004). The mechanism through which A_{2A} receptors facilitate neurotransmitter release is not yet clear, but evidence show that it may involve cAMP (Cunha, 2001) and protein kinase C (PKC) (Lopes *et al.*, 2002). Dopamine is no exception to this control, and work by Okada et al., showed an A_1 receptor inhibition and an A_{2A} receptor facilitation of dopamine release, mainly in the rat striatum (Okada *et al.*, 1996). However, this proposed ability of adenosine receptors to control the release of dopamine is not widespreadly accepted (Quarta et al., 2004).

Another important aspect that needs to be taken into account when exploring the adenosine-dopamine systems interaction is that endogenous adenosine acting on A_{2A} receptors has an excitatory role on striatal neurons via D_2 receptors-independent mechanisms. Several pharmacological studies using either dopamine depletion or D_2 receptors blockade showed that A_{2A} receptor-mediated effects do not depend necessarily on the integrity of the dopamine system.

Another supportive aspect of the adenosine-dopamine systems interaction is based on the activation of dopamine- and adenosine 3':5'-monophosphate-regulated phosphoprotein-32,000 (DARPP-32), which is considered the potential target for integrating adenosine and dopamine signaling at the cellular and network levels (Fredholm *et al.*, 2005). Midbrain dopaminergic neurons play a critical role in several

brain functions and abnormal signaling through dopaminergic pathways has been implicated in several neurological and pathological disorders, such as schizophrenia (Clinton *et al.*, 2005). DARPP-32 is a well-studied target for dopamine actions, as revealed by its location within the brain. In fact, the regional distribution of DARPP-32 in the rat brain follows the general pattern of dopaminergic innervations (Greengard *et al.*, 1984). DARPP-32 is mainly expressed in spiny neurons in the caudate-putamen. In these neurons, co-localization between the D1 receptors and DARPP-32 was found (Langley *et al.*, 1997). D_2 receptors are mainly found in the striatopallidal projections of these neurons (Langley *et al.*, 1997). The function of this protein seems to be controlled by receptor stimulation, and both dopaminergic and glutamatergic (NMDA) receptor stimulation seem to regulate the extent of DARPP-32 phosphorylation in opposite ways (Langley *et al.*, 1997). On the one hand, dopamine D_1 receptor stimulation enhances cAMP formation which will lead to the phosphorylation of DARPP-32 (Langley *et al.*, 1997). This phosphorylated form of DARPP-32 is known to be a potent inhibitor of protein phosphatase-1, which has a very important role in dopaminergic transmission (Svenningsson *et al.*, 2000). On the other hand, NMDA receptor stimulation will increase intracellular calcium levels, leading to activation of calcineurin and dephosphorylation of phospho-DARPP-32 (Greengard *et al.*, 1999). This will reduce the phosphatase-1 inhibitory activity of DARPP-32 (Greengard *et al.*, 1984). Not only D_1 receptors have influence in the phosphorylation state of DARPP-32. It is known that blockade of A_{2A} receptors or stimulation of D_2 receptors can also terminate A_{2A} agonist- or D_2 antagonist-induced phosphorylation of DARPP-32, but it also antagonizes the D_1 receptot agonist-induced DARPP-32 phosphorylation (Svenningsson *et al.*, 2000).

Another important aspect reinforcing the adenosine-dopamine interactions is the spatial localization of the adenosine receptors in comparison with the dopamine

receptors. A_{2A} receptors are localized in dopamine-innervated areas of the central nervous system, with a post-synaptic distribution similar to that of D_1 and D_2 dopamine receptors (Ferré *et al.*, 1991). This spatial overlap explains an important aspect of this interaction, showing that it occurs at a receptor level, at a membrane level. A_1 receptors, unlike A_{2A} receptors, are abundant in axons. This location of these receptors is probably associated with adenosine's homeostatic role (Cunha, 2001).

Adenosine receptors might also control dopamine metabolism by interacting with the two main enzymes responsible for the dopamine inactivation/degradation, monoamine oxidase (MAO) and cathecol-O-methyl transferase (COMT). A_{2A} receptors antagonists are thought to be therapeutic agents for the treatment of motor deficits associated with Parkinson's disease (Dall'Igna *et al.*, 2003). Several studies have reported that A_{2A} receptor antagonists can protect against the neurotoxicity caused by the neurotoxin MPTP (Chen *et al.*, 2001).

MAO-B is one of the two-subtypes of this enzyme, and its inhibitors are also known to be neuroprotective against MPTP neurotoxicity and enhance motor functions (Castagnoli *et al.*, 1997).

With this data present, some studies were made to examine the MAO-B inhibiting properties of several A_{2A} receptor antagonists, and to see if this inhibition of MAO-B can contribute to the known neuroprotection (Petzer *et al.*, 2003). Results showed that the studied A_{2A} receptor antagonists such as KW6002 have MAO-B inhibitory properties. This suggests that MAO-B inhibition can contribute to the neuroprotective potential of A_{2A} receptor antagonists, leading to the possibility of design of dual targeting drugs, which can lead to enhanced therapeutic potential in Parkinson's disease treatment (Petzer *et al.*, 2003).

These interactions between these two systems show that they do not interact only through their receptors but they can interact as a system, in a broader way.

Goals

The present study was designed to explore if A_{2A} receptors affect dopamine uptake, namely the transport of dopamine via the dopamine transporter DAT. This implied two parallel objectives, namely: i) to evaluate if A_{2A} receptors modified dopamine uptake; ii) to evaluate a possible co-localization between these two proteins at the synapse, more specifically in the pre-synaptic fraction.

The first objective was tackled using an agonist for the adenosine receptors, CGS21680, and an antagonist of the dopamine transporter, GBR12783, to see if A_{2A} receptors played a role in the dopamine uptake, in purified nerve terminals of either pre-frontal cortex or striatum. Also, an A_{2A} receptor antagonist, SCH58261, was used, in order to provide evidence if this facilitation of dopamine uptake was due to the activation of A_{2A} receptors.

The second objective was tackled using immunocytochemistry assays, which allowed assessing a possible co-localization between A_{2A} receptors and the dopamine transporter DAT in purified nerve terminals of rat striatum.

Matherials and Methods

Animals. Adult male Wistar rats were used, (2-3 months old) which were supplied by Charles River. Rats were sacrificed by decapitation after anesthesia under halothane atmosphere, following the European Union guidelines for handling experimental animals.

Preparation of purified nerve terminals (synaptosomes). Neural tissue is composed of neurons and their support cells, the glia. Neurons do not survive homogenization intact and the cell bodies are sheared from their processes, which break up into fragments. The plasma membrane of the cell fragments may reseal to form osmotically active particles and when such particles contain the organelles of the synapse they are known as synaptosomes. Subcellular fractions enriched in synaptosomes are sufficiently pure to permit the study of certain physiological and pharmacological aspects of synaptic function (Dunkley *et al.*, 2008). In the present study, the striata and pre-frontal cortices were isolated over an ice-cold platform and then homogenized separately in a sucrose solution [succrose 0.25M, HEPES 10 mM, albumin bovine serum (BSA) 1mg/ml, pH 7.4). This mixture was then centrifuged at 3000x*g* for 10 minutes at 4ºC and the supernatants were collected and centrifuged again at 14000x*g* for 12 minutes at 4ºC. The resulting pellet was re-suspended in a 45% Percoll solution made in succrose 0.32M, EDTA 1mM and DTT 0.25mM and then centrifuged at 14000x*g* for 2 minutes at 4ºC. The top layer was collected and re-suspended in a KHR solution of the following composition: NaCl 140mM, HEPES 10mM, KCl 5mM, glucose 5mM, pH 7.4, to wash any remaining Percoll. Another centrifugation at 14000x*g* was made for 2 minutes at 4ºC and the resulting pellets were kept at 4ºC for the assay.

[^{3}H]Dopamine uptake assays. This assay was made using the synaptosomes prepared as described above. Each tube contained 50µl of buffer solution (134mM NaCl, 4.8mM

KCl, 1.3mM $CaCl_2$, 1.4mM $MgSO_4$, 3.3mM NaH_2PO_4, 12.7mM NaH_2PO_4, 10mM glucose, 1mM ascorbate, 1µM pargyline), or of a buffer solution with GBR12783 (2mM), an inhibitor of the dopamine transporter (DAT) to determine non-specific uptake of dopamine. Each tube also contained 3µl of either CGS21680 (30 nM), an A_{2A} receptor agonist or SCH58261 (50 nM), an A_{2A} receptor antagonist, or both. Finally 100µl of the synaptosomes preparation were added to each tube and the mixtures were incubated for 10 minutes at 37ºC, to equilibrate at this temperature. The reaction of dopamine uptake started when 50µl of [^{3}H]dopamine solution were added to each tube with a 15 seconds interval between tubes. The uptake reaction was for 3 minutes at 37ºC, after which 2.8ml of non-specific-uptake solution at 4ºC were added and the mixture was filtered through GF/B filters. After drying, 3ml of Universal scintillation liquid were added to each filter in a scintillation vial. The radioactivity content, disintegrations per minute (DPM), was determined using liquid scintillation counting using a TRI CARB® 2900TR liquid scintillation analyzer, for 4 minutes/tube. The specific activity was calculated by subtracting the average total DPM (disintegrations per minute) and the average non-specific DPM for each concentration of [^{3}H]dopamine. A standard curve was calculated using different concentrations of stock [^{3}H]dopamine ranging from 60 fmol/10µl to 600fmol/10µl of solution. The results of the standard curve were used to calculate a factor, to convert DPM into amount of fmol of [^{3}H]dopamine bound in the filters (*i.e.* taken up by nerve terminals). This was further normalized through the amount of protein quantificed as described below. We then used a formula ((specific binding/factor)/protein per 100µl) to obtain the amount of [^{3}H]DA (fmol) per mg of protein.

Bio-Rad assay for protein determination. The amount of protein in each sample was determined by the Bio-Rad assay method. This procedure, based on the method of Bradford (Bradford, 1976), is a dye-binding evaluation, exploiting the differential colour change of an acidic solution of Coomassie Brilliant Blue G-250 dye in response to different concentrations of protein. When dye-protein binding occurs, the maximum absorbance of the dye can range from 465 nm to 595 nm. Samples (2µl) were mixed with 78µl of deionised water in a 96-well plate, followed by the addition of 120 µl of 1:3 diluted Bio-Rad dye reagent to each well. The content of each well was mixed and the absorbance of each sample was read at 595 nm after 5 minutes of incubation, in a microplate reader spectrophotometer. The protein standard curve was prepared using lyophilized BSA 0.4% in deionized water, and the absorbance of BSA standard samples was used to construct the curve.

Immunocytochemistry. Sterilized, poli-L lysine covered coverslips with 1.2cm diameter were used in 12-well plates. Nerve terminals were prepared from the striatum or pre-frontal cortex of Wistar rats (n=3). Striata or pre-frontal cortices were homogenized in 10mL of succrose-HEPES medium (0.25mM succrose, 10mM HEPES, pH 7.4) and the homogenates were centrifuged at 2000x *g* for 3 minutes at 4ºC. The supernatants were again centrifuged at 9500x *g* for 13 minutes at 4ºC. The resultant pellet was resuspended in 2mL of succrose-HEPES medium. Nerve terminals (purified pre-synaptic fraction) were isolated using different Percoll gradients (3%, 10% and 23%) (Dunkley *et al.* 1988). Nerve terminals were fixed using 4% paraformaldehyde in phosphate buffer solution (PBS) (140mM NaCl, 3mM KCl, 20mM NaH_2PO_4, 1.5mM KH_2PO_4 pH 7.4) for 15 minutes at room temperature. Coverslip-mounted nerve terminals were permeabilized in 0.2% Triton-X 100 in PBS for 10 minutes at room

temperature and non-specific binding was subsequently blocked with 5% horse serum in PBS-3% BSA for 1 hour at room temperature. Double immunocytochemical labeling of nerve terminals was carried out by incubation for 1 hour at room temperature with mouse anti-SNAP 25 antibody (Sigma; 1:200 dilution) and rabbit anti-synaptophysin antibody (Chemicon; 1:200 dilution), with mouse anti-PSD-95 antibody (Chemicon; 1:200 dilution) and rabbit anti-synaptophysin antibody, with mouse anti-adenosine A_{2A} receptor antibody (Santa Cruz; 1:100 dilution) and rabbit anti-synaptophysin antibody, with rabbit anti-DAT antibody (Santa Cruz; 1:200 dilution) and mouse anti-synaptophysin antibody or with mouse anti-adenosine A_{2A} receptor antibody and rabbit anti-DAT antibody. After washing three times with 200µl PBS for 5 minutes, incubation was conducted for 1h at room temperature with the secondary antibodies: Alexa Fluor-594-(red) labeled goat anti-rabbit IgG antibody (1:200 dilution) and Fluor-594-(red) labeled goat anti-mouse IgG antibody (1:200 dilution), Alexa Fluor-488-(green) labeled goat anti-mouse IgG antibody (1:200 dilution), Alexa Fluor-488-(green) labeled goat anti-rabbit IgG antibody (1:200 dilution), all from Invitrogen. After this another series of washes was made with PBS.

Statistics. Data are mean ± S.E.M. or mean (95% confidence interval) of *n* experiments. Significance was considered at $P \leq 0.05$ using a Student's *t*-test.

Results

1. Control of [^{3}H] dopamine uptake by A_{2A} receptors

1.1. Effect of an A_{2A} receptor agonist in dopamine uptake

It was first investigated if an agonist of adenosine A_{2A} receptors, CGS21680 (30nM), modified the uptake of dopamine by either striatal or pre-frontal cortical synaptosomes. Results showed that CGS21680 (30nM) increased the dopamine uptake both in the striatum and the pre-frontal cortex (Fig. 1). However, this increase was more evident in the striatum compared to the pre-frontal cortex. This can be, possibly, due to a higher density of A_{2A} receptors present in the striatal nerve terminals (Rosin *et al.*, 1998).

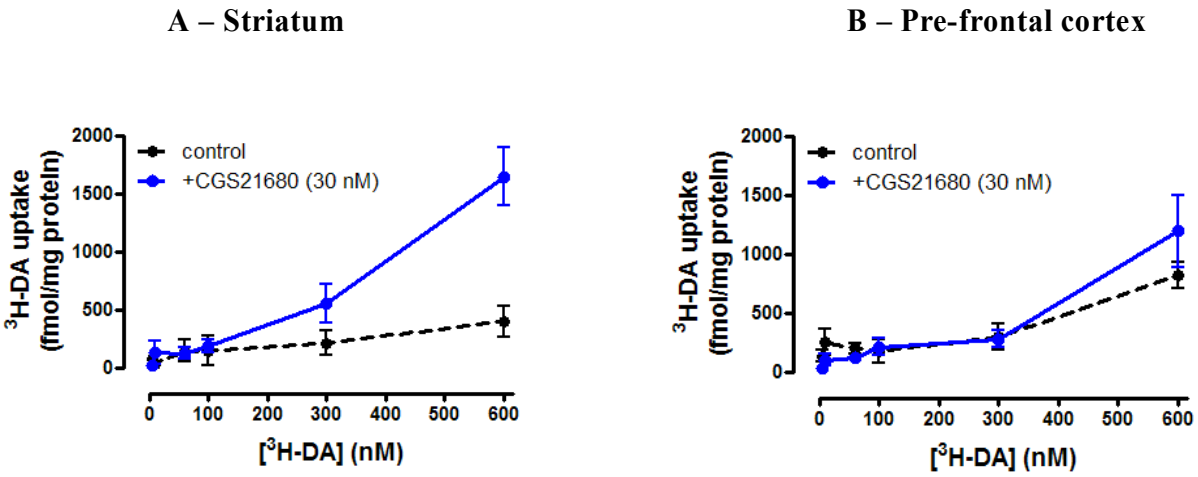

Fig. 1 – Effect of the A_{2A} receptor agonist CGS21680 (30nM) in dopamine uptake in the striatal (n=11) in A and pre-frontal cortical (n=10) in B synaptosomes. Results show that CGS21680 enhanced dopamine uptake in synaptosomes from both brain areas, although the amplitude of this facilitatory effect was larger in the striatum than the pre-frontal cortex.

1.2. Pharmacological characterization of CGS21680-induced facilitation of dopamine uptake

It was then attempted to pharmacologically demonstrate the involvement of A_{2A} adenosine receptors in the facilitation of [^{3}H] dopamine uptake in nerve terminals. This was investigated in striatal nerve terminals, where the effect of CGS21680 (30nM) was more evident. In this experiment an A_{2A} receptor antagonist, SCH58261 (50 nM) was used. Four conditions were tested – control, agonist alone, antagonist alone and both antagonist and agonist together. The results confirmed the increase in dopamine uptake by CGS21680 (30nM). Thus, CGS21680 (30nM) enhanced [^{3}H] dopamine uptake by 96.6% ± 65.5% (n=5). When A_{2A} receptors are antagonized by SCH58261 (50nM), CGS21680 (30nM) was now devoid of significant ($p>0.05$) effects on [^{3}H] dopamine uptake (-20.8% ± 20.5%, n=5), whereas SCH58261 (50nM) was also devoid of significant effects ($p>0.05$) when applied alone (-30.0% ± 26.6%, n=5) (Fig. 2).

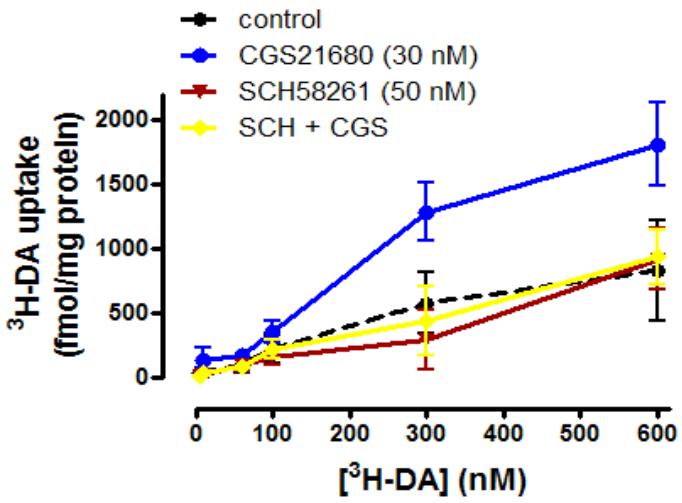

Fig. 2 – Effect of an A_{2A} receptor agonist alone (CGS21680 30nM), of an A_{2A} receptor antagonist alone (SCH58261 50nM) and of their simultaneous presence (SCH+CGS) in the dopamine uptake in striatal synaptosomes. CGS21680 applied alone significantly enhances dopamine uptake for concentrations of dopamine of 300nM and 600nM. Both SCH58261 administered alone and together with CGS21680 produce no statistically significant differences with the control group, showing that these conditions are devoid of effect on ^{3}H-dopamine uptake in the rat striatum.

These results indicate that A_{2A} receptors are indeed responsible for the facilitation of dopamine uptake in striatal synaptosomes.

2. Immunocytochemistry studies

The second major goal of this study was to confirm whether there was a co-localization of A_{2A} receptors and of the dopamine transporter (DAT) in striatal as well as pre-frontal cortical nerve terminals, which, if confirmed would strongly strengthen the hypothesis that A_{2A} receptors directly control the activity of DAT.

To carry out this goal, we used previously validated antibodies to evaluate their presynaptic relative localization, namely rabbit anti-DAT (1:200), mouse anti-A_{2A} receptor (1:100) and guinea pig anti-A_2A receptor (1:100). The first goal was to characterize the purity of the presynaptic nerve terminals prepared from the striatum and pre-frontal cortex. This was evaluated using antibodies against pre-synaptic and post-synaptic markers, namely mouse anti-SNAP-25 (1:200), rabbit anti-synaptophysin (1:200) and mouse anti-PSD-95 (1:200) antibodies. Synaptophysin, a protein located in synaptic vesicles and considered a pre-synaptic marker (Widenmann *et al.*, 1985), was used as reference. Thus, the marker of elements stained with synaptophysin was considered as the total number of nerve terminals.

2.1. Purity of the nerve terminals from the striatum and pre-frontal cortex

SNAP-25 (synaptosome-associated protein of 25 kDa) is a presynaptic protein (Oyler *et al.*, 1989). If the nerve terminals preparation was done correctly, one should expect to see most elements simultaneously labeled with synaptophysin as well as

SNAP-25. As illustrated in Figures 3 and 4, this scenario was indeed confirmed in both areas. Thus, the percentage of synaptophysin-positive labeled nerve terminals also labeled with SNAP-25 was 63.9% ± 2.2% (n=3) in the pre-frontal cortex and 57.2% ± 4.8% in the striatum (n=3).

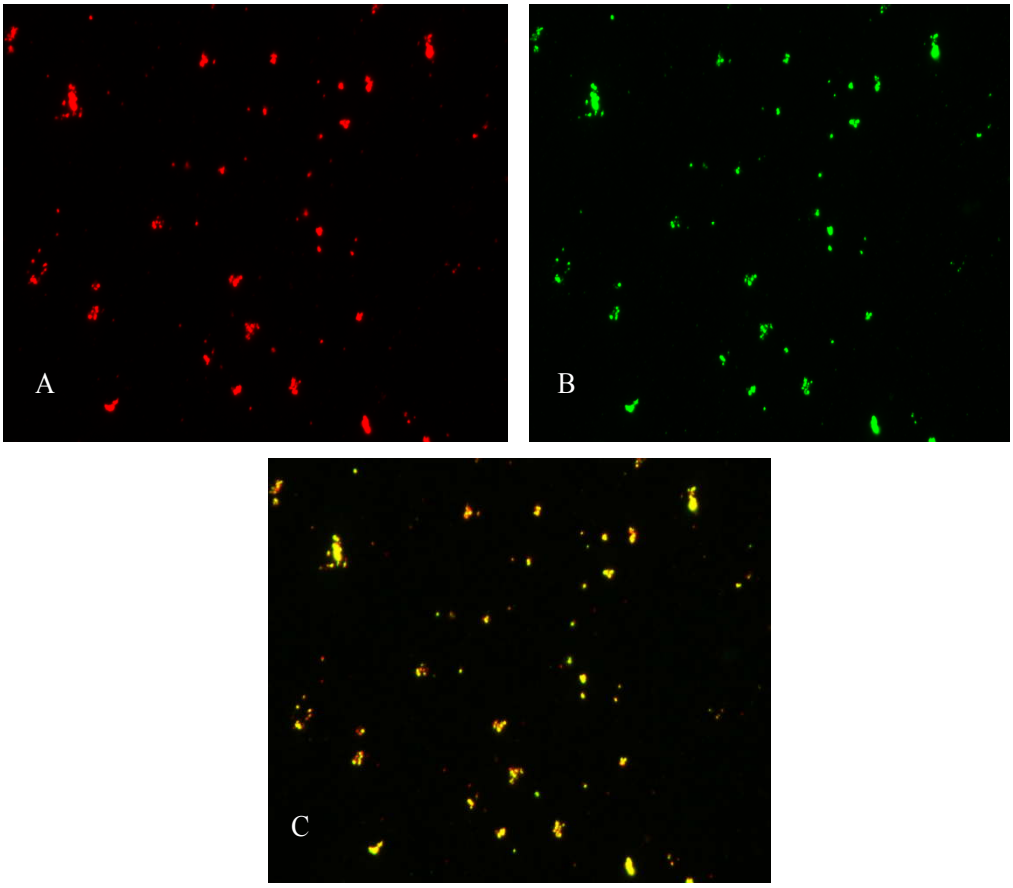

Fig. 3 – Co-localization of SNAP-25 immunoreactivity in pre-frontal cortical purified nerve terminals with synaptophysin immunoreactivity, a marker for nerve terminals. Immunoreactivity to reveal SNAP-25 was carried out using a mouse anti-SNAP-25 antibody (1:200 dilution) and an Alexa Fluor-594-(red) labeled goat anti-rabbit IgG antibody (1:200 dilution; A). Synaptophysin immunoreactivity was identified using a rabbit anti-synaptophysin antibody (1:200 dilution) and an Alexa Fluor-488-(green) labeled goat anti-mouse IgG antibody (1:200 dilution; B). The superimposition of images resulting from the double labeling experiments illustrates the predominant co-localization of SNAP-25 and synaptophysin (yellow spots; C) in pre-frontal cortical purified nerve terminals. These images are representative of 3 independent experiments, with similar results.

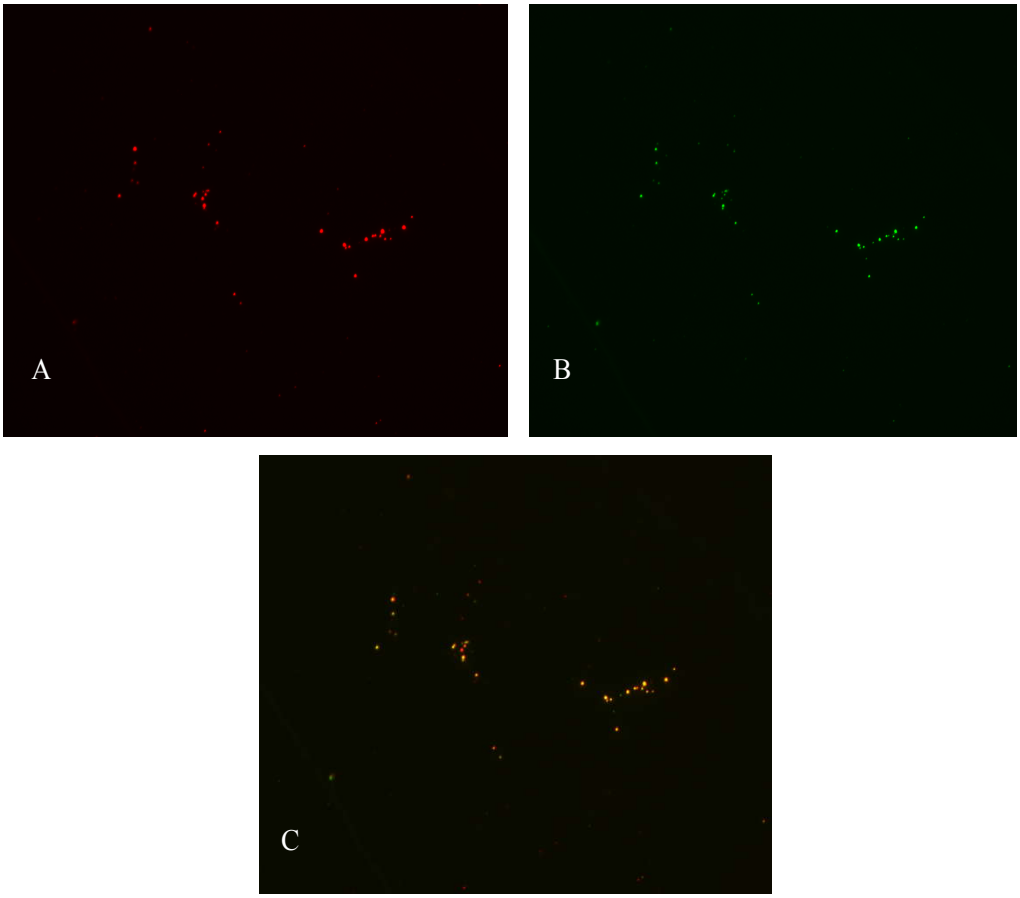

Fig. 4 – Co-localization of SNAP-25 immunoreactivity in striatal purified nerve terminals with synaptophysin immunoreactivity, a marker for nerve terminals. Immunoreactivity to reveal SNAP-25 was carried out using a mouse anti-SNAP-25 antibody (1:200 dilution) and an Alexa Fluor-594-(red) labeled goat anti-rabbit IgG antibody (1:200 dilution; A). Synaptophysin immunoreactivity was identified using a rabbit anti-synaptophysin antibody (1:200 dilution) and an Alexa Fluor-488-(green) labeled goat anti-mouse IgG antibody (1:200 dilution; B). The superimposition of images resulting from the double labeling experiments illustrates the predominant co-localization of SNAP-25 and synaptophysin (yellow spots; C) in striatal purified nerve terminals. These images are representative of 3 independent experiments, with similar results.

PSD-95 (postsynaptic density protein with 95 kDa) is a post-synaptic protein (Ziff, 1997). If the nerve terminals preparation was done correctly, one should expect to see only the labeling for synaptophysin and scarce localization of PSD-95. As illustrated in Figures 5 and 6, this scenario was indeed confirmed in both areas. Thus, the percentage of synaptophysin positive labeled nerve terminals also labeled with PSD-95

was 25.2% ± 11.5% (n=3) in the pre-frontal cortex and 14.6% ± 9.7% (n=3) in the striatum.

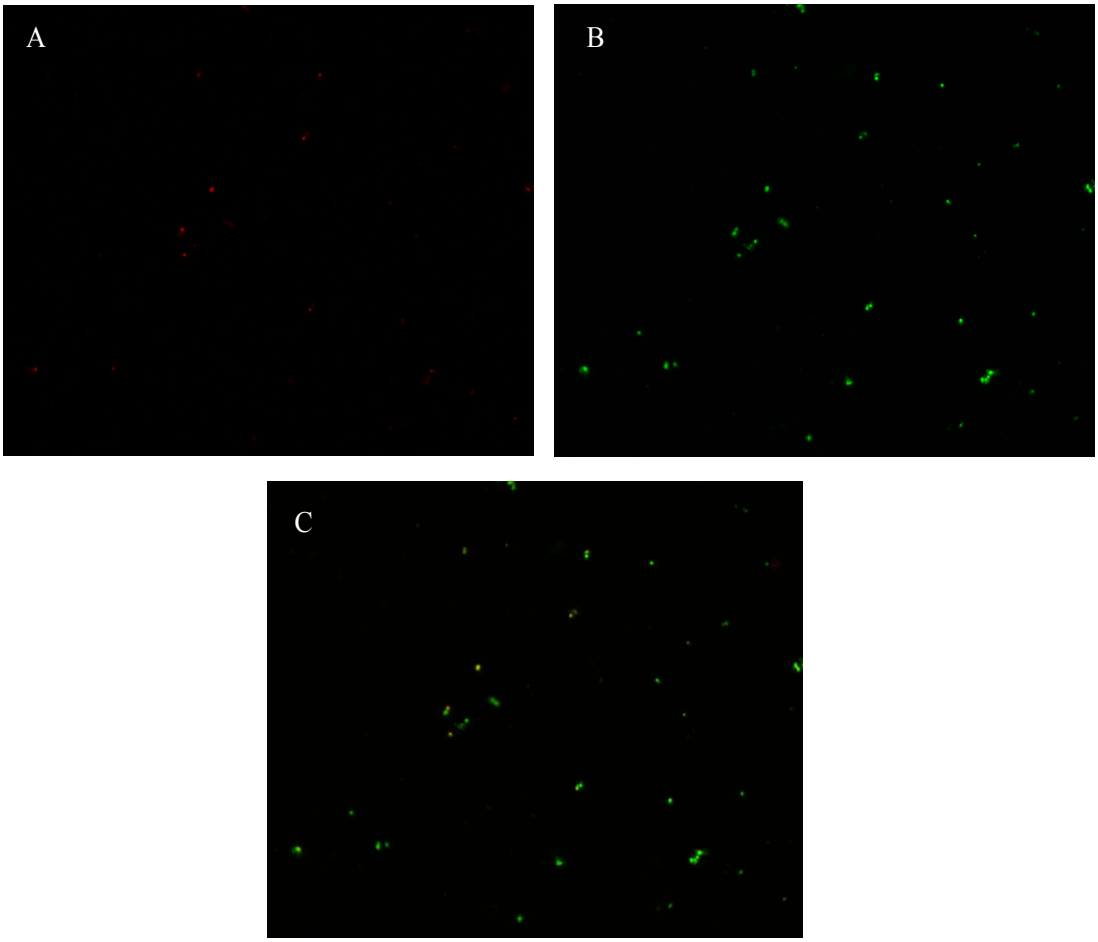

Fig. 5 – Co-localization of PSD-95 immunoreactivity in pre-frontal cortical purified nerve terminals with synaptophysin immunoreactivity, a marker for nerve terminals. Immunoreactivity to reveal PSD-95 was carried out using a mouse anti-PSD-95 antibody (1:200 dilution) and an Alexa Fluor-594-(red) labeled goat anti-rabbit IgG antibody (1:200 dilution; A). Synaptophysin immunoreactivity was identified using a rabbit anti-synaptophysin antibody (1:200 dilution) and an Alexa Fluor-488-(green) labeled goat anti-mouse IgG antibody (1:200 dilution; B). The superimposition of images resulting from the double labeling experiments illustrates the predominant co-localization of PSD-95 and synaptophysin (yellow spots; C) in pre-frontal cortical purified nerve terminals. These images are representative of 3 independent experiments, with similar results.

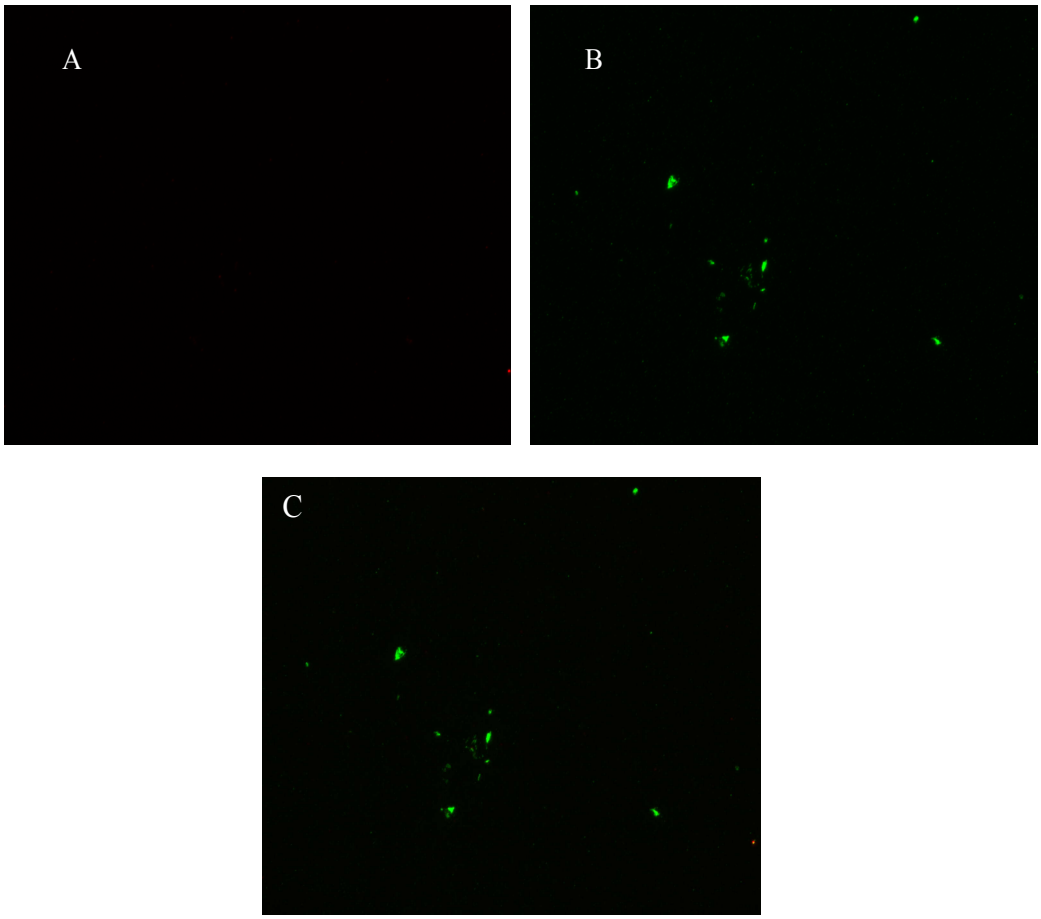

Fig. 6 – Co-localization of PSD-95 immunoreactivity in striatal purified nerve terminals with synaptophysin immunoreactivity, a marker for nerve terminals. Immunoreactivity to reveal PSD-95 was carried out using a mouse anti-PSD-95 antibody (1:200 dilution) and an Alexa Fluor-594-(red) labeled goat anti-rabbit IgG antibody (1:200 dilution; A). Synaptophysin immunoreactivity was identified using a rabbit anti-synaptophysin antibody (1:200 dilution) and an Alexa Fluor-488-(green) labeled goat anti-mouse IgG antibody (1:200 dilution; B). The superimposition of images resulting from the double labeling experiments illustrates the predominant co-localization of PSD-95 and synaptophysin (yellow spots; C) in striatal purified nerve terminals. These images are representative of 3 independent experiments, with similar results.

After conducting the immunocytochemistry studies, a statistical analysis was made, in order to further validate if the purified nerve terminal preparation was contaminated, or not, with the post-synaptic fraction.

A Student's *t*-test was made between two conditions: SNAP-25-synaptophysin and PSD-95-synaptophysin. This analysis was assessed in the pre-frontal cortical and striatal preparations. Results for the pre-fontal cortex show no significant differences

(p> 0.05), which means that the preparation doesn't contain only the pre-synaptic fraction and may possibly be contaminated with the post-synaptic fraction (Table I). On the other hand, results for the striatum show significant differences (p≤ 0.05) which means the striatal preparation was constituted by the pre-synaptic fraction (Table II).

	T-test for Independent Samples (testeT_PFC) Note: Variables were treated as independent samples										
Group 1 vs. Group 2	Mean Group 1	Mean Group 2	t-value	df	p	Valid N Group 1	Valid N Group 2	Std.Dev. Group 1	Std.Dev. Group 2	F-ratio Variances	p Variances
Snap25-Synapto vs. PSD95-Synapto	58,81333	59,48667	-0,018661	4	0,986006	3	3	51,16756	35,88729	2,032863	0,659443

Table I: Statistical analysis (*T*-test) results for the pre-frontal cortex. No statistically significant differences can be found between variables (p> 0.05).

	T-test for Independent Samples (testeT_ST) Note: Variables were treated as independent samples										
Group 1 vs. Group 2	**Mean Group 1**	Mean Group 2	t-value	df	p	Valid N Group 1	Valid N Group 2	Std.Dev. Group 1	Std.Dev. Group 2	F-ratio Variances	p Variances
Snap-Synapto vs. PSD-95 - Synapto	57,22267	14,55167	5,161831	4	0,006690	3	3	6,736111	12,63475	3,518152	0,442659

Table II: Statistical analysis (*T*-test) results for the striatum. Statistically significant\ differences were found (p≤ 0.05).

2.2 Localization of A_{2A} receptors in striatal and pre-frontal cortical nerve terminals

After confirming the purity of nerve terminals, we defined the percentage of nerve terminals equipped with A_{2A} receptors in the striatum and the pre-frontal cortex.

In this case, and as A_{2A} receptors are known to be present in striatal and pre-frontal cortical synapses (Rebola *et al.*, 2005), if the nerve terminals preparation was done correctly, one should expect to see the labeling for synaptophysin as well as labeling for A_{2A}. As illustrated in Figures 7 and 8, this scenario was indeed confirmed in

both areas. Thus, the percentage of synaptophysin positive labeled nerve terminals also labeled with A_{2A} receptors was 35.2% ± 16.3% (n=3) in the pre-frontal cortex and 39.1% ± 10.9% (n=3) in the striatum.

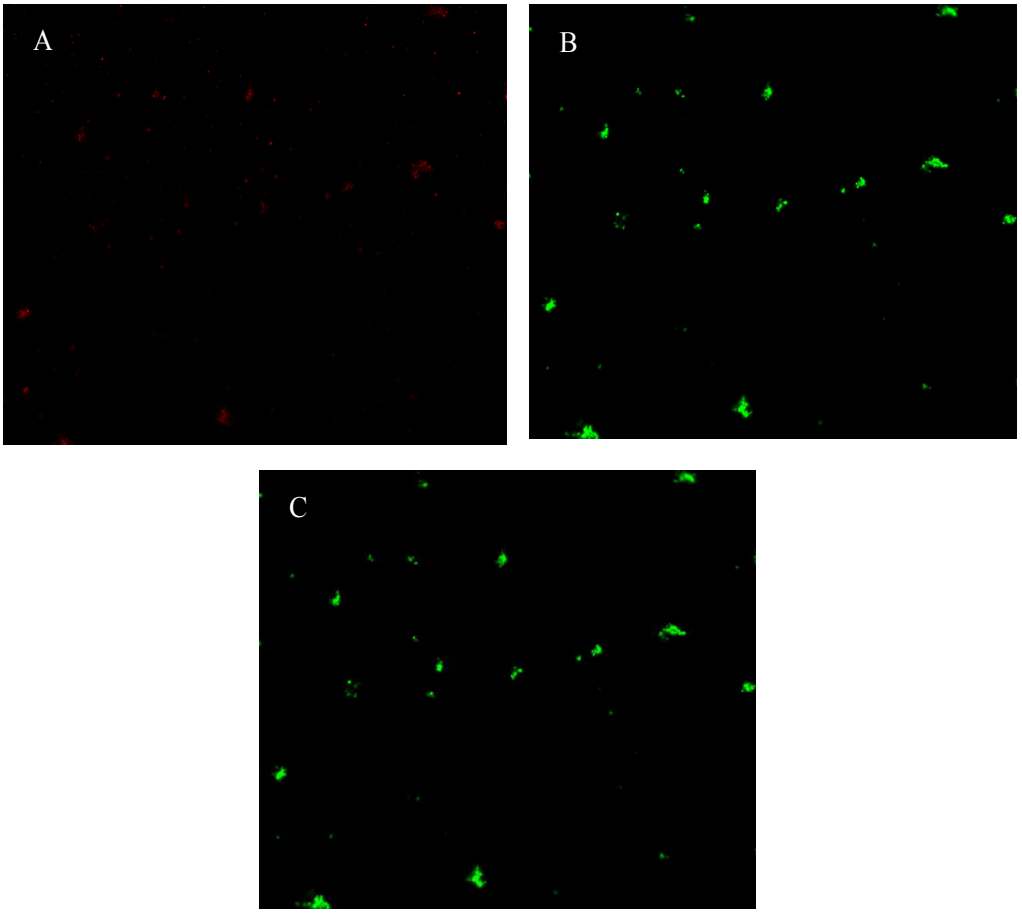

Fig. 7 – Co-localization of A_{2A} receptors immunoreactivity in pre-frontal cortical purified nerve terminals with synaptophysin immunoreactivity, a marker for nerve terminals. Immunoreactivity to reveal A_{2A} receptors was carried out using a mouse anti-A_{2A} receptor antibody (1:100 dilution) and an Alexa Fluor-594-(red) labeled goat anti-rabbit IgG antibody (1:200 dilution; A). Synaptophysin immunoreactivity was identified using a rabbit anti-synaptophysin antibody (1:200 dilution) and an Alexa Fluor-488-(green) labeled goat anti-mouse IgG antibody (1:200 dilution; B). The superimposition of images resulting from the double labeling experiments illustrates the predominant co-localization of A_{2A} receptors and synaptophysin (yellow spots; C) in pre-frontal cortical purified nerve terminals. These images are representative of 3 independent experiments, with similar results.

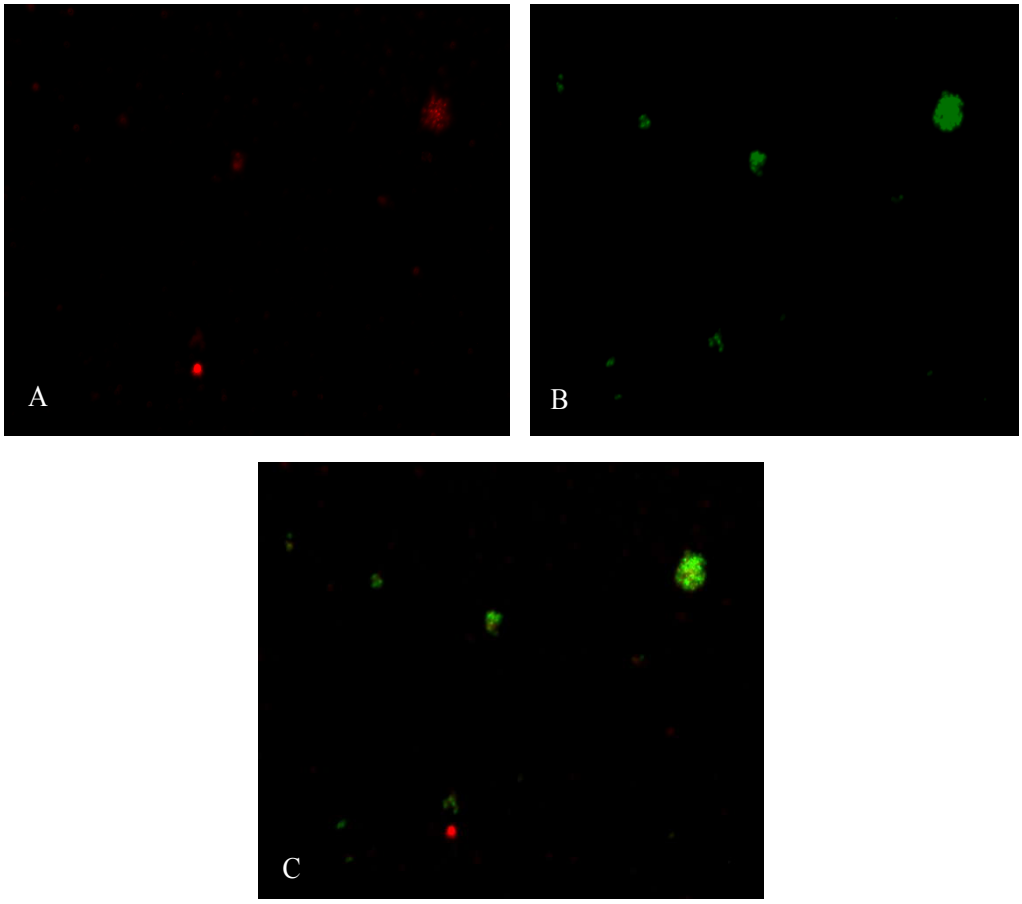

Fig. 8 – Co-localization of A_{2A} receptors immunoreactivity in striatal purified nerve terminals with synaptophysin immunoreactivity, a marker for nerve terminals. Immunoreactivity to reveal A_{2A} receptors was carried out using a mouse anti-A_{2A} receptor antibody (1:100 dilution) and an Alexa Fluor-594-(red) labeled goat anti-rabbit IgG antibody (1:200 dilution; A). Synaptophysin immunoreactivity was identified using a rabbit anti-synaptophysin antibody (1:200 dilution) and an Alexa Fluor-488-(green) labeled goat anti-mouse IgG antibody (1:200 dilution; B). The superimposition of images resulting from the double labeling experiments illustrates the predominant co-localization of A_{2A} receptors and synaptophysin (yellow spots; C) in striatal purified nerve terminals. These images are representative of 3 independent experiments, with similar results.

2.3 Localization of dopamine transporter DAT in striatal and pre-frontal cortical nerve terminals

Here, the percentage of nerve terminals equipped with the dopamine transporter DAT in the pre-frontal cortex and in the striatum was defined.

Since the dopamine transporter DAT can be found in the pre-synaptic fraction, and if the nerve terminals preparation was done correctly, one should expect to see the labeling for synaptophysin as well as the labeling for DAT. As illustrated in Figures 9 and 10, this scenario was indeed confirmed in both areas. Thus, the percentage of synaptophysin-positive labeled nerve terminals also labeled with DAT was 35.5% ± 22.1% (n=3) in the pre-fontal cortex and 18.8% ± 7.0% (n=3) in the striatum.

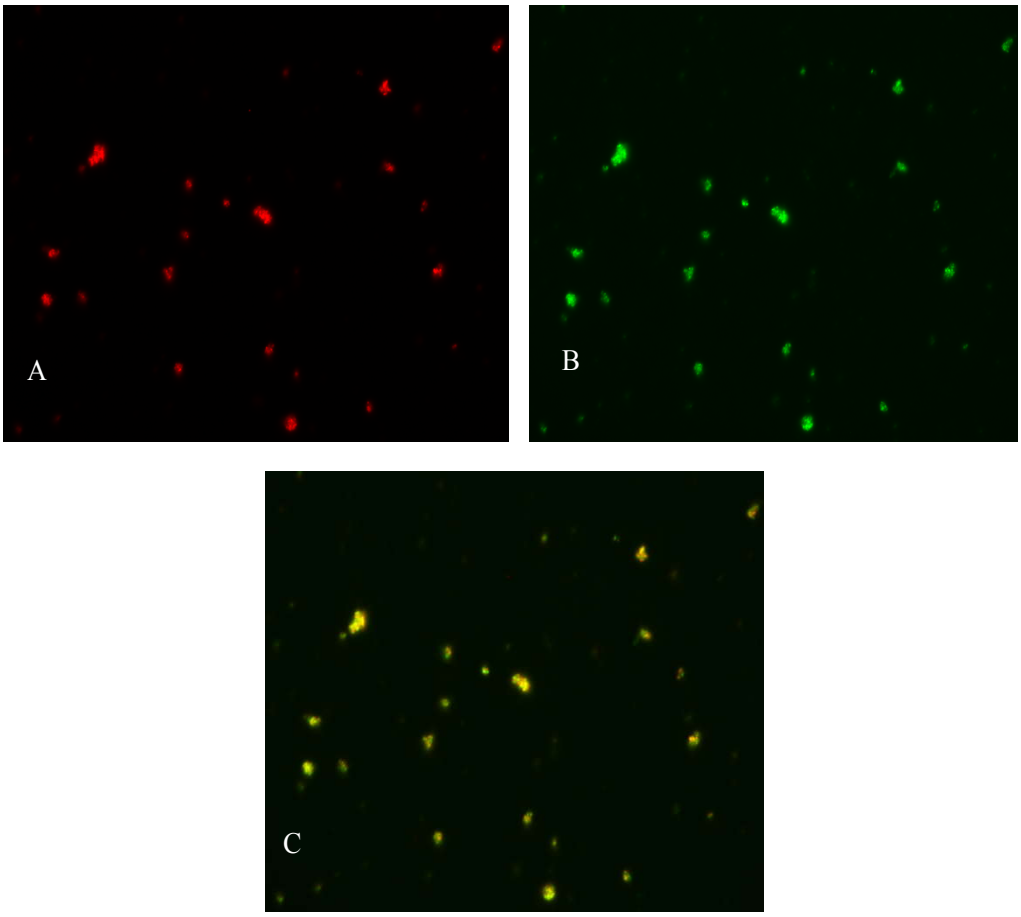

Fig. 9 – Co-localization of dopamine transporter (DAT) immunoreactivity in pre-frontal cortical purified nerve terminals with synaptophysin immunoreactivity, a marker for nerve terminals. Immunoreactivity to reveal DAT was carried out using a rabbit anti-DAT antibody (1:200 dilution) and an Alexa Fluor-488-(green) labeled goat anti-rabbit IgG antibody (1:200 dilution; B). Synaptophysin immunoreactivity was identified using a mouse anti-synaptophysin antibody (1:200 dilution) and an Alexa Fluor-594-(red) labeled goat anti-mouse IgG antibody (1:200 dilution; A). The superimposition of images resulting from the double labeling experiments illustrates the predominant co-localization of DAT and synaptophysin (yellow spots; C) in pre-frontal cortical purified nerve terminals. These images are representative of 3 independent experiments, with similar results.

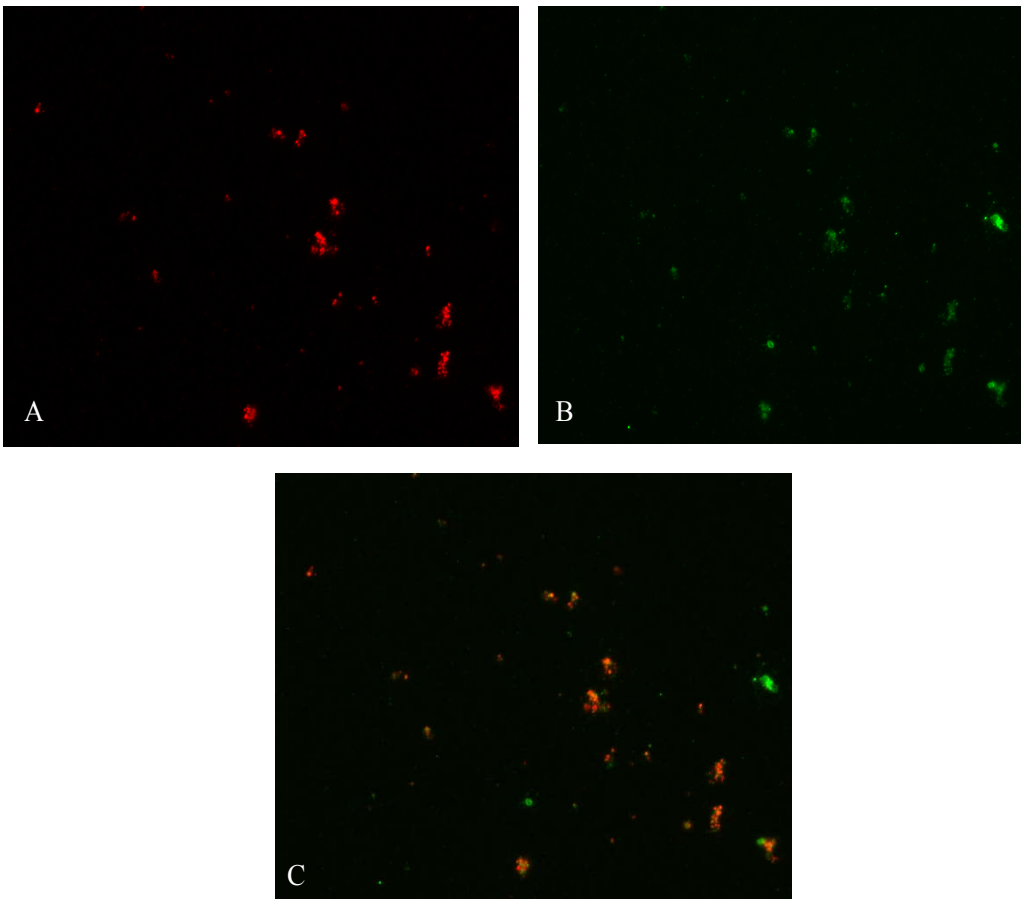

Fig. 10 – Co-localization of dopamine transporter (DAT) immunoreactivity in striatal purified nerve terminals with synaptophysin immunoreactivity, a marker for nerve terminals. Immunoreactivity to reveal DAT was carried out using a rabbit anti-DAT antibody (1:200 dilution) and an Alexa Fluor-488-(green) labeled goat anti-rabbit IgG antibody (1:200 dilution; B). Synaptophysin immunoreactivity was identified using a mouse anti-synaptophysin antibody (1:200 dilution) and an Alexa Fluor-594-(red) labeled goat anti-mouse IgG antibody (1:200 dilution; A). The superimposition of images resulting from the double labeling experiments illustrates the predominant co-localization of DAT and synaptophysin (yellow spots; C) in striatal purified nerve terminals. These images are representative of 3 independent experiments, with similar results.

2.4. Co-localization between DAT and A_{2A} receptors in pre-frontal cortical and striatal nerve terminals

These preparations were made to assess a possible co-localization between the adenosine A_{2A} receptors and the dopamine transporter DAT. In this case there are two

different co-localization percentages as displayed in Figures 11 and 12. These represent either the percentage of A_{2A} receptors positively labeled nerve terminals also labeled with DAT (A_{2A} receptors are considered as 100%) or the percentage of DAT positively labeled nerve terminals also labeled with A_{2A} receptors, respectively. In the first case, this percentage of co-localization was 21.3% ± 7.9% (n=3) in the pre-frontal cortex and 16.7% ± 5.1% (n=3) in the striatum.

As for the second case, the percentage of DAT-positive labeled nerve terminals also labeled with A_{2A} receptors was 22.3% ± 11.1% (n=3) for the pre-frontal cortex and 28,5% ± 8,4% (n=3) for the striatum.

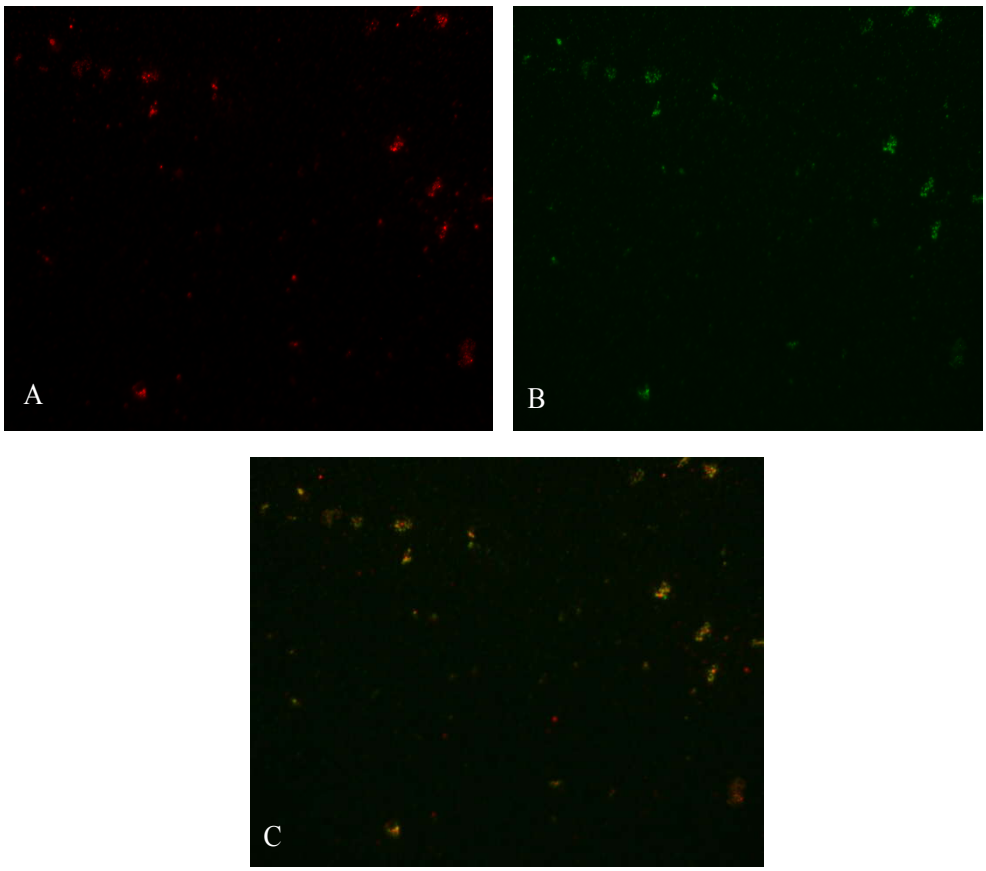

Fig. 11 – Co-localization of dopamine transporter (DAT) immunoreactivity in pre-frontal cortical purified nerve terminals with A_{2A} receptors immunoreactivity. Immunoreactivity to reveal DAT was carried out using a rabbit anti-DAT antibody (1:200 dilution) and an Alexa Fluor-488-(green) labeled donkey anti-goat IgG antibody (1:200 dilution; B). A_{2A} receptors immunoreactivity was identified using a mouse anti-A_{2A} receptors antibody (1:100 dilution) and an Alexa Fluor-594-(red) labeled goat anti-mouse IgG antibody (1:200 dilution; A). The superimposition of images resulting from the double labeling experiments illustrates the predominant co-localization of DAT and A_{2A} (yellow spots; C) in pre-frontal cortical purified nerve terminals. These images are representative of 3 independent experiments, with similar results.

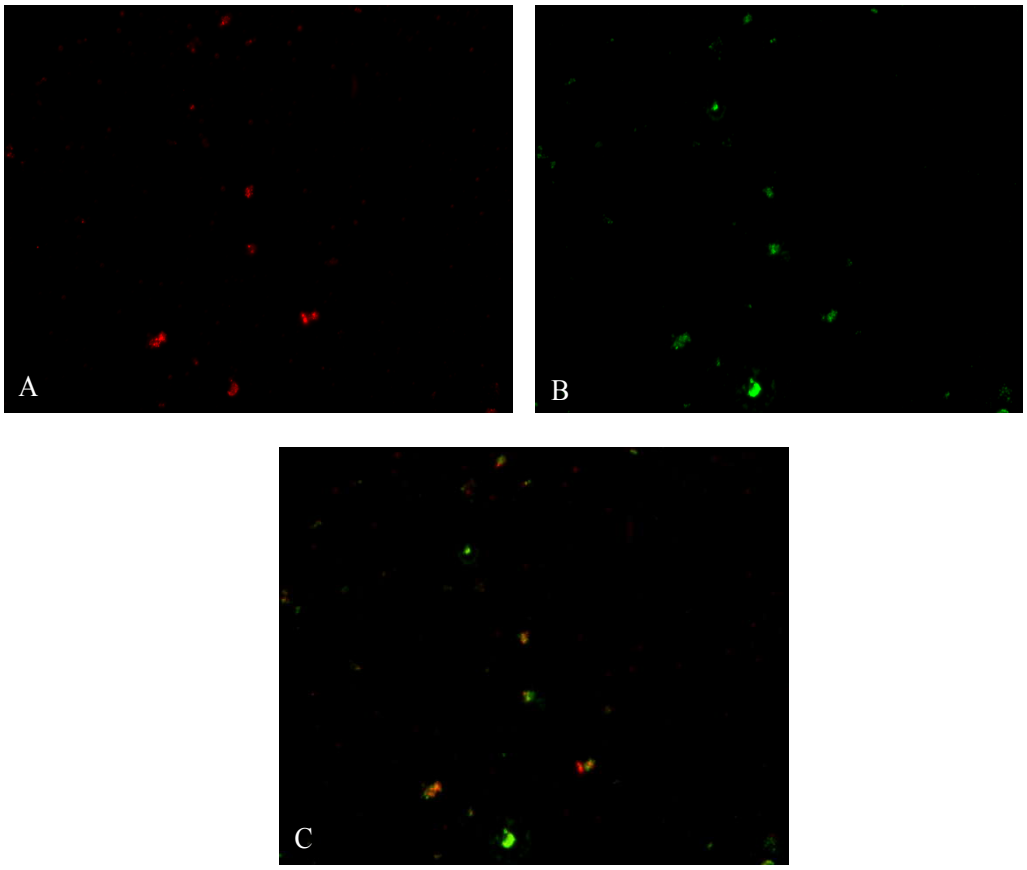

Fig. 12 – Co-localization of dopamine transporter (DAT) immunoreactivity in pre-frontal cortical purified nerve terminals with A_{2A} receptors immunoreactivity. Immunoreactivity to reveal DAT was carried out using a rabbit anti-DAT antibody (1:200 dilution) and an Alexa Fluor-488-(green) labeled donkey anti-goat IgG antibody (1:200 dilution; B). A_{2A} receptors immunoreactivity was identified using a mouse anti-A_{2A} receptors antibody (1:100 dilution) and an Alexa Fluor-594-(red) labeled goat anti-mouse IgG antibody (1:200 dilution; A). The superimposition of images resulting from the double labeling experiments illustrates the predominant co-localization of DAT and A_{2A} (yellow spots; C) in pre-frontal cortical purified nerve terminals. These images are representative of 3 independent experiments, with similar results.

Discussion

Radioactive dopamine uptake assays were conducted in the striatum and in the pre-frontal cortex. In a first stage, experiments were conducted to see if adenosine A_{2A} receptors intervened in a modulatory way in the dopamine uptake, conducted by the dopamine transporter DAT. Results showed that, when DAT was inhibited by GBR 12783, and A_{2A} receptors were activated with CGS21680, an increase in the dopamine uptake occurred, both in the striatum and in the pre-frontal cortex. With these results it is possible to implicate A_{2A} receptors in the dopamine uptake in both the studied structures. However, the increase in the striatum was greater than in the pre-frontal cortex. This difference in uptake can be due to the number of this type of receptors in these two structures (Rosin *et al.*, 1998). These results show that, by activating A_{2A} receptors, dopamine uptake is enhanced, which constitutes the first evidence that A_{2A} receptors might control dopamine metabolism. Other evidences, such as the A_{2A} receptor/MAO interaction, reinforce the A_{2A} receptor-mediated control of dopamine metabolism. MAO-B is one of the two types of the monoamine oxidase enzyme. A_{2A} receptors antagonists are known to inhibit MAO-B, controlling the dopamine metabolism.

In this work is reported for the first time that the adenosine A_{2A} receptors are found co-localized with the dopamine transporter DAT in rat striatal and cortical pre-synaptic membranes. This co-localization was seen using immunocytochemistry assays, in purified nerve terminals. A_{2A} receptors are known to control dopamine release in dopaminergic nerve terminals. Golembiowska *et al.* proved this control by inhibiting the A_{2A} receptors with a potent antagonist, ZM241385. Rats treated with the antagonist showed a higher increase in dopamine release, when comparing to control animals (Golembiowska *et al.*, 2004). This control can be due to the fact that they are co-localized in the pre-synaptic fraction, as reported in this work.

Results show a significative co-localization rate, however some limitations inherent to the technique may have altered the results. Steps such as homogenization of the material or even re-suspension of the pellets can lead to the contamination of the purified nerve terminals preparation with the post-synaptic fraction or to the formation of pre-synaptic fraction aggregates, which can lead to errors when analyzing co-localizations.

The discovery of a co-localization of these two proteins, through immunocytochemistry studies, gives a new insight to this interaction, providing evidence that adenosine A_{2A} receptors may act directly on the dopamine transporter DAT. If this interaction occurs, it can be of importance to further studies regarding, for example, Parkinson's disease. One particularly known characteristic of Parkinson's disease patients is a decrease in dopaminergic function (Jankovic et al. 2008). This decrease, specifically in the putamen and in the globus pallidus leads to bradikinesia, which refers to difficulty in planning, initiating and executing movements (Jankovic et al. 2008). A_{2A} receptors control of dopamine uptake may be relevant for Parkinson's disease, as it can provide new insights to new treatments for the disease. Parkinson's disease is characterized by loss of dopamine uptake sites (González *et al.*, 2000) resulting in an impairment of dopamine uptake. Ikeda *et al.* showed that an A_{2A} receptors agonist, CGS21680, decreased dopamine uptake, however an A_{2A} receptor, KW6002 reversed that effect, increasing the dopamine uptake in PC-12 cells (Ikeda *et al.*, 2002). Blockade of A_{2A} receptors may also lead to the activation of the vesicular monoamine transporter, which will lead to the clearance of MPP^+ and consequent neuronal protection (Ikeda *et al.*, 2002). This shows the relevance of the A_{2A} receptor

control of dopamine uptake for Parkinson's disease, as A_{2A} receptor antagonists may act as new drugs to the treatment of some parkinsonian symptoms.

The new data here presented gives an alternative way to control the dopaminergic system, through adenosine A_{2A} receptors in specific areas of the brain, as the pre-frontal cortex and the striatum. It also reinforces the "fine-tuning" role adenosine has throughout the brain, controlling and adjusting other neurotransmitter systems (Fredholm et al., 2005).

Conclusion

Several conclusions can be taken from this work, and also, some future questions can arise, when considering the data here presented.

Firstly, one can conclude that A_{2A} adenosine receptors are an indispensable factor in the correct functioning of the dopamine uptake in the rat striatum and pre-frontal cortex.

Another key conclusion to take is that they both are co-localized in the pre-synaptic region of the synapse, which was showed by the immunocytochemistry tests, which suggests a direct interaction between these two proteins.

It is know that the orphan receptor GPR 37 binds to the dopamine transporter and that it modulates this transporter's activity. *Gpr37*-null mutant mice showed an enhancement in dopamine uptake in the striatum (Marazziti *et al*., 2007). These two proteins were found co-localized in mouse purified nerve terminals and also in transfected cells (Marazziti *et al.*, 2007). With these results present, one can think of future work to be done on this field. Questions such as how A_{2A} receptors and the dopamine transporter connect, and if there are other proteins, such as GPR 37, that bind to the putative A_{2A}/DAT heterodimer in order to facilitate dopamine uptake in the rat striatum and pre-frontal cortex can be useful to fully understand the role of A_{2A} receptors in the facilitation of dopamine uptake.

It is important to know if indeed A_{2A} receptors and DAT interact physically, because this can provide new insights on how do A_{2A} receptors facilitate the dopamine uptake, either if it is through the way they bind or if there are other factors that influence this facilitation.

The question regarding other proteins, such as GRP37, that can couple to this heterodimer, is also of great importance. If, indeed, other proteins take part in this facilitation of dopamine uptake, it means that this process can be complex.

Finally, if there are other proteins binding to both A_{2A} receptors and DAT, biochemical studies need to be made in order to understand properly which signals lead them to bind to the other proteins.

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