[Frontiers in Bioscience 13, 2391-2399, January 1, 2008]

Adenosine A_1-A_{2A} receptor heteromers: new targets for caffeine in the brain

Sergi Ferre[1], Francisco Ciruela[2], Janusz Borycz[3], Marcello Solinas[4], Davide Quarta[5], Katerina Antoniou[6], Cesar Quiroz[1], Zuzana Justinova[1], Carme Lluis[2], Rafael Franco[2], Steven R. Goldberg[1]

[1]National Institute on Drug Abuse, Intramural Research Program, National Institutes of Health, Department of Health and Human Services, Baltimore, MD 21224, U.S.A., [2]Department of Biochemistry and Molecular Biology, Faculty of Biology, University of Barcelona, 08028 Barcelona, Spain, [3]Department of Psychology, Dalhousie University, Halifax, NS B3H 4J1, Canada, [4]Institut de Biologie et Physiologie Cellulaires, Centre National de la Reserche Scientifique-6187, University of Poitiers, 86022 Poitiers, France, [5]Drug Dependence and Behavioral Neurochemistry Department, Psychiatry-CEDD, GlaxoSmithKline S.p.A. Medicine Research Center, 37135 Verona, Italy, [6]Department of Pharmacology, Medical School, University of Ioannina, 45110 Ioannina, Greece

TABLE OF CONTENTS

1. Abstract
2. Targeting adenosine receptors
3. Targeting the striatal spine module
4. The striatal adenosine A1-A2A receptor heteromer
5. Conclusions
6. Acknowledgements
7. References

1. ABSTRACT

The contribution of blockade of adenosine A_1 and A_{2A} receptor to the psychostimulant effects of caffeine is still a matter of debate. When analyzing motor activity in rats, acutely administered caffeine shows a profile of a non-selective adenosine receptor antagonist, although with preferential A_1 receptor antagonism. On the other hand, tolerance to the effects of A_1 receptor blockade seems to be mostly responsible for the tolerance to the motor-activating effects of caffeine, while the residual motor-activating effects of caffeine in tolerant individuals seem to involve A_{2A} receptor blockade. These behavioral studies correlate with *in vivo* microdialysis experiments that suggest that A_1 receptor-mediated modulation of striatal glutamate release is involved in the psychostimulant effects of caffeine. Experiments in transfected cells demonstrate the ability of A_1 receptors to heteromerize with A_{2A} receptors and the A_1-A_{2A} receptor heteromer can be biochemically identified in the striatum, in striatal glutamatergic terminals. The striatal A_1-A_{2A} receptor heteromer provides a "concentration-dependent switch" mechanism by which low and high concentrations of synaptic adenosine produce the opposite effects on glutamate release. The analysis of the function of A_1-A_{2A} receptor heteromers during chronic treatment with caffeine gives new clues about the well-known phenomenon of tolerance to the psychostimulant effects of caffeine.

2. TARGETING ADENOSINE RECEPTORS

Caffeine is the most consumed psychoactive drug in the world (1). As a psychostimulant, it produces reinforcing effects, motor-activating effects and arousal (1). It was already proposed more than twenty years ago that the psychostimulant effects of caffeine and related methyl-xanthines, as well as their multiple non-central effects (including lipolysis in adipose tissue, increased renal blood flow, release of catecholamines and increased heart rate) were mainly due to antagonism of endogenous adenosine (2-4). Among the four cloned adenosine receptors (A_1, A_{2A}, A_{2B} and A_3 receptors), A_1 and A_{2A} receptors are the ones predominantly expressed in the brain. Caffeine is a non-selective adenosine receptor antagonist, with reported similar *in vitro* affinities for A_1, A_{2A} and A_{2B} receptors and with lower affinity for A_3 receptors (5,6). Physiological extracellular levels of adenosine can be sufficient to occupy and, therefore, stimulate A_1 and A_{2A} receptors. On the other hand, A_{2B} receptors have a lower affinity for adenosine and they are only activated by high pathological extracellular levels of adenosine (5). Thus, A_1 and A_{2A} receptors seem to be the preferential targets for caffeine in the brain, although their involvement in the psychostimulant effects of caffeine still remains controversial. A_1 receptors are widely expressed in the brain, while A_{2A} receptors are highly concentrated in the striatum (5, 7). The striatal localization of both receptors can underlie the reinforcing and motor

activating effects of caffeine, while A_1 receptors localized in the brainstem and basal forebrain and A_{2A} receptors localized in the hypothalamus have been suggested to be involved in caffeine-induced arousal (see below).

Based upon a correlation between potencies of a series of methylxanthines in stimulating locomotor activity of mice and in competing at adenosine receptors labeled with a tritiated A_1 receptor agonist, Snyder *et al.* (8) initially suggested that the motor stimulant effects of methylxanthines involve the blockade of A_1 receptors. At that time little was known about the role of A_{2A} receptors, especially because of the lack of specific A_{2A} receptor ligands. Nevertheless, later on, studies in mice and monkeys suggested that there was a better correlation between the locomotor depressant effects of several adenosine receptor agonists and their ability to bind to A_{2A} receptors (9,10). Also, there seemed to be a better correlation between the ability of several methylxanthines to produce motor stimulation in monkeys (increase in response rate in schedule-controlled behavior) and their ability to bind A_{2A} receptors (11). It was then suggested that blockade of A_{2A} receptors was mainly responsible for the motor stimulating effects of caffeine (11). This hypothesis was strengthened by more recent findings demonstrating the ability of A_{2A} receptor antagonists and the inability of some A_1 receptor antagonists to reproduce some biochemical and behavioral effects of caffeine (12,13). Furthermore, caffeine was found to be ineffective at producing motor activation in A_{2A} receptor knockout mice (14). Also, some results obtained in A_{2A} receptor knockout and wild-type mice even suggested that A_1 receptor antagonism induces motor depression, which could be responsible for the motor depressant effect observed with high doses of caffeine (13). However, these results were at odds with the results of other research groups which strongly suggested that A_1 receptor antagonists induce motor activation and that they potentiate the motor activating effects of A_{2A} receptor antagonists (15,16).

We then performed a systematic study that compared the counteraction of the motor depressant effects of selective A_1 and A_{2A} receptor agonists by caffeine and selective A_1 and A_{2A} receptor antagonists in rats (17). The A_1 receptor antagonist CPT and the A_{2A} receptor antagonist MSX-3 produced motor activation at the same doses that selectively counteracted the motor depression induced by the A_1 receptor agonist CPA and the A_{2A} receptor agonist CGS 21680, respectively. Caffeine, at the doses that produced motor activation, was found to have a profile more similar to CPT than to MSX-3, since it more potently counteracted the motor depressant effects of CPA than of CGS 21680. Thus, caffeine showed a profile of a non-selective adenosine receptor antagonist, although with preferential A_1 receptor antagonism (17). A recent detailed behavioral analysis further supported the predominant role of A_1 receptors in the motor-activating effects of acutely administered caffeine. Thus, factor analysis (a statistical procedure that allows inter-correlated variables to be condensed into fewer dimensions, called factors) depicted a very similar three-dimensional profile for caffeine and the A_1 receptor antagonist CPT, which was different from a two-dimensional profile of the A_{2A} receptor antagonist MSX-3 (18). Recent experiments using genetic inactivation of A_1, A_{2A} or both receptors have revisited the previously hypothesized involvement of A_1 receptors in the depressant motor activation induced by high doses of caffeine (13). The results of those experiments support that A_1 receptor contributes to the stimulatory but not the inhibitory motor activating effects of caffeine (19).

Other results supporting the role of A_1 receptors in the psychostimulant effects of caffeine come from drug discrimination studies. Drug discrimination techniques have been used to classify and characterize the effects of abused drugs, including psychostimulants, since trained animals can be used to determine whether test substances are identified as being similar to the drug used for training (same pharmacological class) (20). Thus, in drug discrimination experiments, caffeine can substitute for other psychostimulants and the other way around (6,21,22). In rats trained to discriminate an injection of a motor-activating dose of caffeine from saline, CPT and the non-selective adenosine antagonist DMPX, but not MSX-3, produced significant caffeine-like discriminative effects. Furthermore, the A_1 receptor agonist CPA, but not the A_{2A} receptor agonist CGS 21680, dose-dependently reduced caffeine's discriminative effects (6). Therefore, these results strongly support a key involvement of A_1 receptors in the acute psychostimulant effects of caffeine (6).

Most radioligand-binding experiments show that A_1 and A_{2A} receptors have very similar affinities for caffeine (1,6). The mismatch between the *in vitro* and *in vivo* pharmacology of caffeine, with predominant *in vivo* A_1 receptor antagonism, could be related to a preferential occupancy of A_1 versus A_{2A} receptors by endogenous adenosine. In this context, adenosine has been found to be more potent at human adenosine A_1 than A_{2A} receptors transfected in mammalian cell line (23). Furthermore, we have recently reported that the affinity of the A_{2A} receptor for caffeine is selectively reduced in the A_1-A_{2A} receptor heteromer (see below and ref. 24).

Chronic exposure to caffeine differentially modifies its motor effects dependent on A_1 and A_{2A} receptor blockade. Thus, chronic exposure to caffeine in the drinking water results in partial tolerance to the motor effects of an additional acute administration of caffeine and total cross-tolerance to the motor effects of CPT, but not of MSX-3 (17). This suggests that tolerance to the effects of A_1 receptor blockade is mostly responsible for the tolerance to the motor-activating effects of caffeine and that the residual motor-activating effects of caffeine in tolerant individuals might be mostly because of A_{2A} receptor blockade. These results agree with other experimental findings showing a lack of tolerance of the motor activating effects of A_{2A} receptor antagonists with their repeated administration (25). Similarly, in unilateral 6-OH-dopamine-lesioned rats, chronic treatment with caffeine or repeated administration of an A_{2A} receptor antagonist was not associated with tolerance to the A_{2A} receptor

antagonist-induced potentiation of turning behavior produced by dopamine receptor agonists (26,27).

Although experiments with place- and taste-preference paradigms have demonstrated the reinforcing effects of caffeine (28-30), the lack of selectivity of the adenosine receptor ligands used in those experiments does not allow the establishment of a clear role of A_1 or A_{2A} receptors in the reinforcing effects of caffeine. In relation to the arousal-enhancing effects of caffeine, adenosine has been demonstrated to be a potent endogenous sleep-inducing substance, based on experimental evidence obtained from a variety of pharmacological and behavioral studies. For example, adenosine and its stable analogues are known to induce sleep when administered to rats, cats, and other experimental animals (31). The concentration of extracellular adenosine increases in the cortex and basal forebrain during sleep deprivation of cats and decreases during the recovery period after sleep deprivation (32). Because energy restoration is one of the functions of sleep, adenosine is proposed to be produced as a terminal product of energy metabolism and to act as a homeostatic regulator of energy in the brain during sleep. The well-known arousal-enhancing properties of caffeine depend on its ability to antagonize the sleep-promoting effects of adenosine (reviewed in refs. 33 and 34). Although previous results suggested that A_1 receptors are involved in sleep regulation by inhibiting ascending cholinergic neurons of the basal forebrain (31,32), more recent studies, which include experiments with A_{2A} and A_1 receptor knockout mice, indicate that A_{2A} receptors (most probably localized in the ventrolateral preoptic area of the hypothalamus) play a crucial role in the sleep-promoting effects of adenosine and the arousal-enhancing effects of caffeine (33,34).

3. TARGETING THE STRIATAL SPINE MODULE

As is the case for other psychostimulants, the motor and discriminative stimulus effects of caffeine are dopamine dependent. Caffeine's discriminative stimulus effects are counteracted by dopamine D_1 and D_2 receptor antagonists (at doses that do not decrease the rate of responding) (6, 35). Caffeine-induced motor activation can be counteracted by drugs that either block dopamine receptors or produce dopamine depletion (reviewed in refs. 36 and 37). Finally, although not directly demonstrated, it is most likely that the reinforcing effects of caffeine are also dopamine-dependent, since dopamine is crucial in most instances of positive reinforcement. Thus, rewarding stimuli fail to serve as effective reinforcers in dopamine-compromised animals (38).

The dopamine dependency of the psychostimulant effects of caffeine can be explained by the existence of important interactions between adenosine and dopamine in the brain and the main locus where these interactions seem to be particularly relevant is the "striatal spine module". The GABAergic striatal efferent neuron constitutes more than 90% of the striatal neuronal population (39). Also called medium sized spiny neuron, since it contains a high density of dendritic spines, the GABAergic striatal efferent neuron receives two main inputs, glutamatergic afferents from cortical, limbic and thalamic areas and dopaminergic afferents from the mesencephalon, either the substantia nigra pars reticulata or the ventral tegmental area (39). Both inputs converge in the dendritic spine. The glutamatergic terminal makes synaptic contact with the head of the dendritic spine, while the dopaminergic terminal makes synaptic contact preferentially with the neck of the dendritic spine (39). The dendritic spine, the dopaminergic and glutamatergic terminals and astroglial processes that wrap the glutamatergic synapse constitute the most common striatal local module, which we recently called striatal spine module (40). This arrangement allows dopamine neurotransmission to regulate glutamatergic neurotransmission. But glutamate is not only released synaptically to stimulate intrasynaptic glutamatergic receptors, mostly ionotropic receptors. There is also volume transmission of glutamate, which can spillover the synaptic cleft and by an amplificatory mechanism that involves the astroglia**,** stimulates extrasynaptic receptors localized both pre- and postsynaptically at the glutamatergic and dopaminergic synapses. These extrasynaptic glutamatergic receptors are metabotropic or ionotropic glutamate receptors which modulate glutamate and dopamine release. Similarly, dopamine is not only released synaptically but it can also spillover or be released by asynaptic varicosities and stimulate extrasynaptic receptors that are located both pre- and post-synaptically at both glutamatergic and dopaminergic synapses (reviewed in ref. 40).

In the striatal spine module adenosine is a key modulator of dopaminergic and glutamatergic neurotransmission. Until recently it was believed that the main source of extracellular adenosine was a paracrine-like formation. Extracellular adenosine would come mostly from intracellular adenosine, the concentration of which depends upon the breakdown and synthesis of ATP, which is metabolized to AMP and, then, by means of 5'nucleotidases it is converted to adenosine, which can be transported to the extracelular space by means of equilibrative transporters (41). However, recent studies suggest that astroglia play a fundamental role in the formation of extracellular adenosine which affects synaptic transmission. Astrocytes express glutamate receptors (mostly metabotropic) and ATP receptors which, when activated, induce astrocytes to release glutamate and ATP (42,43). Astroglial-released ATP can be converted to adenosine in the extracellular space by means of ectonucleotidases (44). Finally, there is an increasing amount of data suggesting the existence of a neurotransmitter-like formation of adenosine, a synaptic pool of adenosine. In this case, adenosine would come from ATP co-released with glutamate, which is metabolized to adenosine by means of ectonucleotidases (41).

What is the localization of adenosine receptors in the striatal spine module? There are two subtypes of GABAergic striatal efferent neurons, the striatopallidal neuron, also called enkephalinergic neuron, which expresses the peptide enkephalin and dopamine and

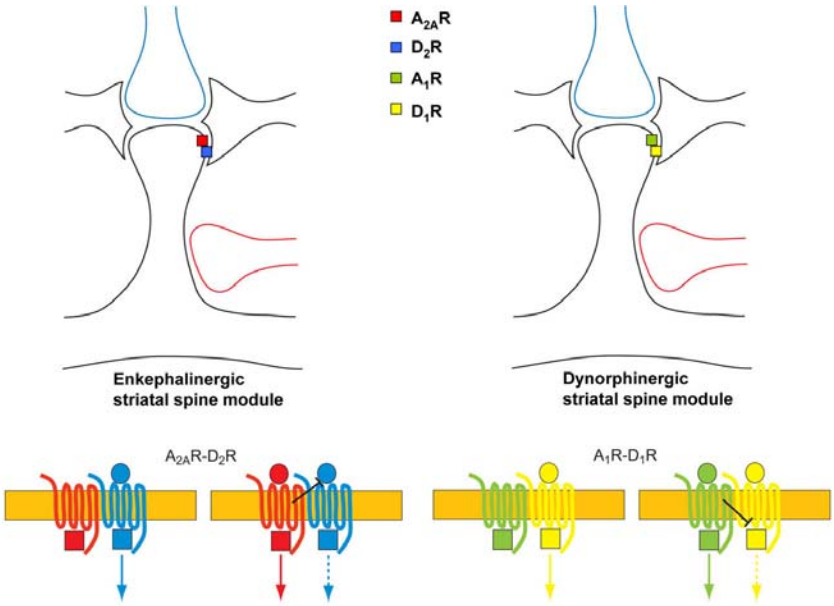

Figure 1. Adenosine-dopamine receptor heteromers in the striatal spine modules. Upper figures. Adenosine A_{2A}-dopamine D_2 receptor heteromers are localized in the dendritic spines of the GABAergic enkephalinergic neurons and adenosine A_1-dopamine D_1 receptor heteromers are localized in the dendritic spines of the GABAergic dynorphinergic neurons. Lower figures. Scheme of the A_{2A}-D_2 and A_1-D_1 receptor heteromers and their "intramembrane receptor-receptor interactions". Stimulation of A_{2A} receptor decreases the ability of dopamine to bind to the D_2 receptor in the A_{2A}-D_2 receptor heteromer. Stimulation of A_1 receptor decreases the G protein coupling to the D_1 receptor in the A_1-D_1 receptor heteromer.

adenosine receptors of the D_2 and A_{2A} subtypes and the striatonigral-striatoentopeduncular neuron, also called dynorphinergic neuron, which expresses dynorphin, dopamine and adenosine receptors of the D_1 and A_1 subtype (39-41,45,46). We demonstrated the existence of antagonistic interactions between A_{2A} and D_2 receptors that modulate the function of the enkephalinergic neuron and antagonistic interactions between A_1 and D_1 receptors that modulate the function of the dynorphinergic neuron (36,41,45,46). We and other groups demonstrated that A_{2A} receptors form heteromers with D_2 receptors and that A_1 receptors form heteromers with D_1 receptors in transfected cells (47-50). Importantly, we and other groups were able to consistently demonstrate some characteristic biochemical properties of the A_{2A}-D_2 and A_1-D_1 receptor heteromers, called "intramembrane receptor-receptor interactions", in different transfected cell lines which could be identified in striatal tissue, demonstrating the existence of both heteromers in the brain (51-58) (Figure 1).

We first postulated that these postsynaptic A_1 and A_{2A} receptors, forming heteromers with D_1 and D_2 receptors, respectively, could provide a framework for adenosine-dopamine interactions in the brain and that they could be mostly responsible for the motor and reinforcing effects of caffeine (36,45). However, we found evidence for the existence of presynaptic effects of caffeine that could not be explained by its effects on the A_{2A}-D_2 and A_1-D_1 receptor heteromers. As shown by *in vivo* microdialysis, systemic administration of caffeine produced a significant increase in the extracellular concentrations of dopamine and glutamate in the ventral striatum, particularly in the most medial part, the medial shell of the nucleus accumbens (59,60). The same effect was observed with the systemic administration of CPT, while MSX-3 produced the opposite effect, a small but significant decrease in the extracellular concentration of dopamine and glutamate in the nucleus accumbens (61). Furthermore, chronic administration of caffeine in the drinking water completely counteracted the effects of caffeine or CPT on dopamine and glutamate, while the effect of MSX-3 was not modified (61). Thus, these biochemical changes correlated with our previous studies on motor activity. Dopamine release in the medial striatal compartments seems to be involved in invigoration of approach and in some aspects of incentive learning (for recent review, see ref (62)). In relation to psychostimulants, dopamine release in the very medial striatal compartments seems to be involved in both their locomotor-activating and reinforcing effects (62). Therefore, presynaptic mechanisms (striatal dopamine release) are probably involved in the motor, reinforcing and maybe discriminative stimulus effects of caffeine. In fact, the development of tolerance to the dopamine-releasing effects of caffeine in the nucleus accumbens could be one of the factors that explain its weaker reinforcing properties as compared with other psychostimulants.

4. THE STRIATAL ADENOSINE A_1-A_{2A} RECEPTOR HETEROMER

The effects of systemic administration of caffeine and CPT on striatal dopamine and glutamate were also replicated by local perfusion of these compounds in the nucleus accumbens (63). Furthermore, local perfusion of

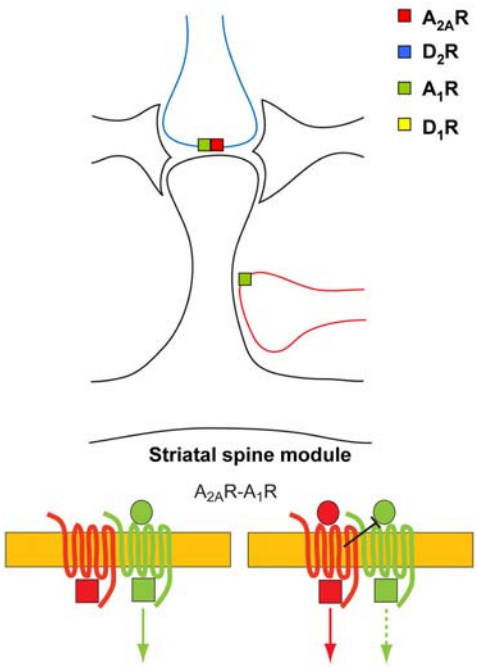

Figure 2. Adenosine A_1-A_{2A} receptor heteromers in the striatal spine modules. Upper figures. A_1-A_{2A} receptor heteromers are localized in the striatal glutamatergic terminals. A_1 receptors have also been found in a fraction of dopaminergic terminals. Lower figures. Scheme of the A_1-A_{2A} heteromer and its "intramembrane receptor-receptor interaction". Stimulation of A_{2A} receptor decreases the ability of adenosine to bind to the A_1 receptor in the A_1-A_{2A} receptor heteromer.

the A_{2A} receptor agonist CGS 21680 also produced an increase in the extracellular concentrations of dopamine and glutamate in the nucleus accumbens (63). The effects of A_1 receptor blockade or A_{2A} receptor stimulation were antagonized by blocking NMDA receptors, suggesting that the changes in dopamine release are secondary to glutamate release and stimulation of NMDA receptors localized in dopaminergic terminals (63). The results indicated that A_1 and A_{2A} receptors exert an important control of striatal glutamate release and, therefore, they would probably be co-localized in glutamatergic terminals. Nevertheless, it must be pointed out that there is also evidence for a glutamate-independent A_1 receptor-mediated modulation of striatal dopamine release, which depends on the existence of A_1 receptors in a fraction of striatal dopaminergic terminals (60) (Figure 2)

The A_1-A_{2A} receptor co-localization on striatal glutamatergic terminals was demonstrated by electron microscopy experiments, labeling A1 receptors with immunoperoxidase and A2A receptors with immunogold. Interestingly, presynaptic A1 and A2A receptors were mostly found inside the synapse (24) (Figure 2). Thus, they are in a position to modulate adenosine generated by synaptically released ATP. Furthermore, immunocytochemical experiments in striatal nerve terminal preparations showed that the majority of glutamatergic nerve terminals contain both A_1 and A_{2A} receptors (24). Functional studies were then performed in striatal nerve terminals in order to understand the significance of having two receptor subtypes (A_1 and A_{2A} receptors) of the same neurotransmitter (adenosine) with opposite functional effects (inhibition and stimulation of neurotransmitter release) co-localized in the same nerve terminals. In striatal nerve terminal preparations, stimulation of the A_1 receptor, with the A_1 receptor agonist CPA, decreases and stimulation of A_{2A} receptors with CGS 21680 potentiates potassium-induced glutamate release (24). Importantly, when both A_1 and A_{2A} receptors are stimulated, the response is not a counteractive effect, but the same than results from A_{2A} receptor stimulation, i.e., a potentiation of glutamate release (24). Furthermore, in the same kind of preparation, low concentrations of adenosine inhibit while high concentrations stimulate glutamate release (24). This would agree with a higher affinity for adenosine of the A_1 compared to the A_{2A} receptor (23) and would provide a mechanism for a fine-tuned modulation of glutamate release by adenosine, with low concentrations inhibiting and high concentrations stimulating glutamate release. But, what would be the mechanism by which A_{2A} receptor stimulation shuts down the effects of A1 receptor stimulation?

By means of co-immunoprecipitation and BRET techniques, we demonstrated the existence of A_1-A_{2A} receptor heteromers in co-transfected human embryonic kidney (HEK) cells (24). We then demonstrated the existence of an intermolecular cross-talk, an intramembrane receptor-receptor interaction in the A_1-A_{2A} receptor heteromer in co-transfected HEK cells, by means of radioligand-binding techniques (24). In cells only transfected with A_1 receptors, competitive-inhibition experiments with the radio-labeled A_1 receptor agonist [^{3}H]R-PIA and the A_{2A} receptor agonist CGS 21680 showed that CGS 21680 displaces A_1 receptor binding only at high concentrations, when it loses its selectivity for A_{2A} receptors. On the other hand, in cells co-transfected with A_1 and A_{2A} receptors (but not in mixtures of cells co-transfected with either A_1 or A_{2A} receptors) low concentrations of CGS 21680 also counteract A_1 receptor binding. This shows the existence of an intramembrane interaction in the A_1-A_{2A} heteromer, by which stimulation of A_{2A} receptors decreases the affinity of A_1 receptor for agonist binding. We could then use this biochemical characteristic of the heteromer as a "biochemical fingerprint" and identify the A_1-A_{2A} receptor heteromer in the brain. In fact, the same results were obtained when we performed the same kind of competitive inhibition experiments in membrane preparations from rat striatum (24). This demonstrates the existence of A_1-A_{2A} receptor heteromers in the striatum. It also shows that an important part of the A_1 receptors in the striatum are forming heteromers with A_{2A} receptors, otherwise the intramembrane A_1-A_{2A} receptor interaction would not be detected. Thus, the A_1-A_{2A} receptor heteromer provides a "concentration-dependent switch" mechanism by which low and high concentrations of synaptic adenosine produce the opposite effects on glutamate release.

The A_1-A_{2A} receptor heteromer gives a rationale for the existence of heteromers of isoreceptors (receptors

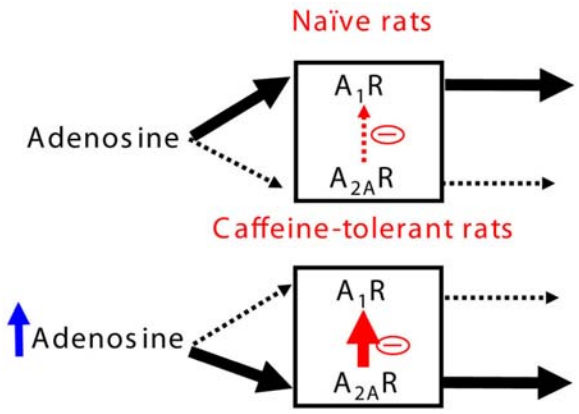

Figure 3. Scheme of the role of adenosine A_1-A_{2A} receptor heteromers in the effects of caffeine. Upper figure. In naïve rats, the low levels of endogenous adenosine and the higher affinity of A_1 receptor for the agonist gives a preferential A_1 receptor-mediated signaling in the A_1-A_{2A} receptor heteromer. Under these conditions, the administration of caffeine induces a preferential blockade of A_1 receptor function. Lower figure. In rats chronically treated with caffeine, the higher adenosine levels and decreased affinity of the A_{2A} receptor for caffeine allows endogenous adenosine to stimulate A_{2A} receptors even in the presence of caffeine. A_1 receptor signaling is turned down because of its blockade by caffeine and because of the increased A_{2A} receptor-mediated inhibition of A_1 receptor agonist binding. Under these conditions an additional administration of caffeine produces a blockade of A_{2A} receptor signaling.

for the same neurotransmitter) and demonstrates that neurotransmitter heteromers composed of isoreceptors with different affinities for their endogenous neurotransmitter and different signaling pathways can act as concentration-dependent processors that exert a fine-tuned modulation of neurotransmission. In this case we have a neurotransmitter released or formed in the synaptic space which acts on synaptically or perisynaptically located heteromers. A weak input results in the stimulation of the receptor with the highest affinity for the neurotransmitter, while a strong input results in the additional stimulation of the other receptor, with the establishment of the intermolecular cross-talk between both receptors an a different neuronal response.

Finally, we also analyzed if chronic treatment with caffeine alters the function of the striatal A_1-A_{2A} receptor heteromer. In fact, a remarkable finding was an increased intermolecular cross-talk in the A_1-A_{2A} receptor, with a significant increase in the potency of A_{2A} receptor agonist-mediated inhibition of A_1 receptor agonist binding (24). The affinity of the striatal A_{2A} receptor for caffeine was also reduced (24). One additional variable has to be taken into account when analyzing the effects of chronic caffeine administration, which is an increase in the plasma and extracellular levels of adenosine. Thus, Conlay *et al.* (64) demonstrated that the same treatment with caffeine that we used in our biochemical experiments (1 mg/ml in the drinking water for 14 days) leads to a 10-fold increase in plasma adenosine levels (64). The following model can explain the above mentioned experimental findings (Figure 3): In naïve rats, due to the relatively low levels of endogenous adenosine and the higher affinity of A_1 receptor for the agonist, there is a preferential A_1 receptor-mediated signaling in the A_1-A_{2A} receptor heteromer. Under these conditions, the administration of caffeine induces a preferential blockade of A_1 receptor function in the A_1-A_{2A} receptor heteromer. In rats chronically treated with caffeine, the higher adenosine levels and low affinity of the A_{2A} receptor for caffeine compared to the A_1 receptor allows endogenous adenosine to stimulate A_{2A} receptors even in the presence of caffeine, which does not reach enough concentration to compete with adenosine for binding A_{2A} receptors. The A_1 receptor signaling, on the other hand, is turned down because of its blockade by caffeine and because of the strong A_{2A} receptor-mediated inhibition of A_1 receptor agonist binding. Under these conditions an additional administration of caffeine produces a blockade of the residual A_{2A} receptor signaling (Figure 3).

5. CONCLUSIONS

In previous reports, A_{2A}-D_2 receptor heteromers localized in striatal enkephalinergic neurons and A_1-D_1 receptor heteromers localized in striatal dynorphinergic neurons were suggested to be main targets responsible for the pshychostimulant effects of caffeine (36,45,46). In fact, striatal enkephalinergic neurons contain the highest density of A_{2A} receptors in the brain (7) and their blockade seems to be mainly responsible for the antiparkinsonian effects of A_{2A} receptor antagonists (7,65,66). Nevertheless, the above reviewed experimental work strongly suggests that the A_1-A_{2A} receptor heteromer localized in striatal glutamatergic nerve terminals plays an important role in the motor-activating and, also possibly, in the reinforcing and discriminative-stimulus effects of caffeine. Furthermore, the analysis of the function of A_1-A_{2A} receptor heteromers during chronic treatment with caffeine allows a better understanding of the well-known phenomenon of tolerance to the psychostimulant effects of caffeine.

6. ACKNOWLEDGEMENTS

This work was supported by the NIDA IRP funds. F. Ciruela is currently holding a Ramón y Cajal research contract signed with the "Ministerio de Educación y Ciencia". This work was supported by grant SAF2006-05481 to R. F. and SAF2005-00903 to FC from "Ministerio de Educación y Ciencia".

7. REFERENCES

1. Fredholm, B. B., Battig, K., Holmen, J., Nehlig, A. & Zvartau, E. E. Actions of caffeine in the brain with special reference to factors that contribute to its widespread use. *Pharmacol Rev* 51, 83-133 (1999)
2. Fredholm, B. B.: Are methylxanthine effects due to antagonism of endogenous adenosine? *Trends Pharmacol Sci* 1, 129-132 (1980)

3. Rall, T. W.: Evolution of the mechanism of action of methylxanthines from calcium mobilizers to antagonists of adenosine receptors. *Pharmacologist* 24, 277-297 (1982)
4. Snyder, S. H.: Adenosine as a neuromodulator. *Annu Rev Neurosci* 8, 103-124 (1985)
5. Fredholm, B. B., Ijzerman, A. P., Jacobson, K. A., Klotz, K. N. & Linden J. International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. *Pharmacol Rev* 53, 527-52 (2001)
6. Solinas, M., Ferré, S., Antoniou, K., Quarta, D., Justinova, Z., Hockemeyer, J., Pappas, L. A., Segal, P. N., Wertheim, C., Muller, C. E. & Goldberg S. R. Involvement of adenosine A1 receptors in the discriminative-stimulus effects of caffeine in rats. *Psychopharmacology* 179, 576-586 (2005)
7. Schiffmann, S. N., Fisone, G., Moresco, R., Cunha, R. & Ferré, S. Adenosine A2A receptors and basal ganglia physiology. *Prog Neurobiol* (in press)
8. Snyder, S. H., Katims, J. J., Annau, Z., Bruns, R. F. & Daly, J. W. Adenosine receptors and behavioral actions of methylxanthines. *Proc Natl Acad Sci USA* 78, 3260-3264 (1981)
9. Durcan, M. J. & Morgan, P. F. Evidence for adenosine A2 receptor involvement in the hypomobility effects of adenosine analogues in mice. *Eur J Pharmacol* 168, 285-290 (1989)
10. Spealman, R. D. & Coffin, V. L. Behavioral effects of adenosine analogs in squirrel monkeys: relation to adenosine A2 receptors. *Psychopharmacology* 90, 419-421 (1986)
11. Spealman, R. D. Psychomotor stimulant effects of methylxanthines in squirrel monkeys: relation to adenosine antagonism. *Psychopharmacology* 95, 19-24 (1988)
12. Svenningsson, P., Nomikos, G. G., Ongini, E. & Fredholm, B. B. Antagonism of adenosine A2A receptors underlies the behavioural activating effect of caffeine and is associated with reduced expression of messenger RNA for NGFI-A and NGFI-B in caudate-putamen and nucleus accumbens. *Neuroscience* 79, 753-764 (1997)
13. El Yacoubi, M., Ledent, C., Menard, J. F., Parmentier, M., Costentin, J. & Vaugeois, J. M. The stimulant effects of caffeine on locomotor behaviour in mice are mediated through its blockade of adenosine A (2A) receptors. *Br J Pharmacol* 129, 1465-1473 (2000)
14. Ledent, C., Vaugeois, J. M., Schiffmann, S. N., Pedrazzini, T., El Yacoubi, M., Vanderhaeghen, J. J., Costentin, J., Heath, J. K., Vassart, G. & Parmentier, M. Aggressiveness, hypoalgesia and high blood pressure in mice lacking the adenosine A2a receptor. *Nature* 388, 674-678 (1997)
15. Jacobson, K. A., Nikodijevic, O., Padgett, W. L., Gallo-Rodriguez, C., Maillard, M. & Daly, J. W. 8- (3-Chlorostyryl)caffeine (CSC) is a selective A2-adenosine antagonist *in vitro* and *in vivo*. *FEBS Lett* 323, 141-144 (1993)
16. Popoli, P., Reggio, R., Pezzola, A., Fuxe, K. & Ferré S. Adenosine A1 and A2A receptor antagonists stimulate motor activity: evidence for an increased effectiveness in aged rats. *Neurosci Lett* 251, 201-204 (1998)
17. Karcz-Kubicha, M., Antoniou, K., Terasmaa, A., Quarta, D., Solinas, M., Justinova, Z., Pezzola, A., Reggio, R., Muller, C. E., Fuxe, K., Goldberg, S. R., Popoli, P. & Ferré, S. Involvement of adenosine A1 and A2A receptors in the motor effects of caffeine after its acute and chronic administration. *Neuropsychopharmacology* 28:1281-1291 (2003)
18. Antoniou, K., Papadopoulou-Daifoti, Z., Hyphantis, T., Papathanasiou, G., Bekris, E., Marselos, M., Panlilio, L., Muller, C. E., Goldberg, S. R. & Ferré, S. A detailed behavioral analysis of the acute motor effects of caffeine in the rat: involvement of adenosine A1 and A2A receptors. *Psychopharmacology* 183, 154-162 (2005)
19. Halldner, L., Aden, U., Dahlberg, V., Johansson, B., Ledent, C. & Fredholm, B. B. The adenosine A1 receptor contributes to the stimulatory, but not the inhibitory effect of caffeine on locomotion: a study in mice lacking adenosine A1 and/or A2A receptors. *Neuropharmacology* 46, 1008-10017 (2004)
20. Solinas, M., Panlilio, L. V., Justinova, Z., Yasar, S. & Goldberg, S. R. Using drug-discrimination techniques to study the abuse-related effects of psychoactive drugs in rats. *Nat Protoc* 1, 1194-1206 (2006)
21. Mumford, G. K. & Holtzman, S. G. Qualitative differences in the discriminative stimulus effects of low and high doses of caffeine in the rat. *J Pharmacol Exp Ther* 258, 857-865 (1991)
22. Munzar, P., Justinova, Z., Kutkat S, W., Ferré, S. & Goldberg, S. R. Adenosinergic modulation of the discriminative-stimulus effects of methamphetamine in rats. *Psychopharmacology* 161, 348-355 (2002)
23. Fredholm, B. B., Irenius, E., Kull, B. & Schulte, G. Comparison of the potency of adenosine as an agonist at human adenosine receptors expressed in Chinese hamster ovary cells. *Biochem Pharmacol* 61, 443-448 (2001)
24. Ciruela, F., Casado, V., Rodrigues, R.J., Lujan, R., Burgueno, J., Canals, M., Borycz, J., Rebola, N., Goldberg, S.R., Mallol, J., Cortes, A., Canela, E.I., Lopez-Gimenez, J.F., Milligan, G., Lluis, C., Cunha, R.A., Ferré S. & Franco, R. Presynaptic control of striatal glutamatergic neurotransmission by adenosine A1-A2A receptor heteromers. *J Neurosci* 26, 2080-2087 (2006)
25. Halldner, L., Lozza, G., Lindstrom, K. & Fredholm, B. B. Lack of tolerance to motor stimulant effects of a selective adenosine A (2A) receptor antagonist. *Eur J Pharmacol* 406, 345-354 (2000)
26. Popoli, P., Regio, R. & Pezzola, A. Effects of SCH 58261, an adenosine A (2A) receptor antagonist, on quinpirole-induced turning in 6-hydroxydopamine-lesioned rats. Lack of tolerance after chronic caffeine intake. *Neuropsychopharmacology* 22, 522-529 (2000)
27. Pinna, A., Fenu, S. & Morelli, M. Motor stimulant effects of the adenosine A2A receptor antagonist SCH 58261 do not develop tolerance after repeated treatments in 6-hydroxydopamine-lesioned rats. *Synapse* 39, 233-238 (2001)
28. Brockwell N. T. & Beninger R. J. The differential role of A1 and A2A adenosine receptor subtypes in locomotor activity and place conditioning in rats. *Behav Pharmacol* 7, 373-383 (1996)
29. Fedorchak P. M., Mesita J., Plater S. A. & Brougham K. Caffeine-reinforced conditioned flavor preferences in rats. *Behav Neurosci* 116, 334-346 (2002)
30. Myers K. P. & Izbicki E. V. Reinforcing and aversive effects of caffeine measured by flavor preference

conditioning in caffeine-naive and caffeine-acclimated rats. *Physiol Behav* 88, 585-596 (2006)
31. Basheer, R., Strecker, R. E., Thakkar, M. M. & McCarley, R. W. Adenosine and sleep-wake regulation. *Prog Neurobiol* 73, 379-396 (2004)
32. Porkka-Heiskanen, T., Strecker, R. E. & McCarley, R. W. Brain site-specificity of extracellular adenosine concentration changes during sleep deprivation and spontaneous sleep: an *in vivo* microdialysis study. *Neuroscience* 99, 507-517 (2000)
33. Huang, Z. L., Urade, Y. & Hayaishi, O. Prostaglandins and adenosine in the regulation of sleep and wakefulness. *Curr Opin Pharmacol* 7, 33-38 (2007)
34. Ferré, S., Diamond, I., Goldberg, S. R., Yao, L., Hourani, S. M., Huang, Z. L., Urade, Y. & Kitchen, I. Adenosine A (2A) receptors in ventral striatum, hypothalamus and nociceptive circuitry Implications for drug addiction, sleep and pain. *Prog Neurobiol* (in press)
35. Powell K. R., Koppelman L. F. & Holtzman S. G. Differential involvement of dopamine in mediating the discriminative stimulus effects of low and high doses of caffeine in rats. *Behav Pharmacol* 10, 707-716 (1999)
36. Ferré, S., Fuxe, K., von Euler, G., Johansson, B. & Fredholm, B. B. Adenosine-dopamine interactions in the brain. *Neuroscience* 51, 501-512 (1992)
37. Garrett B. E. & Griffiths R. R. The role of dopamine in the behavioral effects of caffeine in animals and humans. *Pharmacol Biochem Behav* 57, 533-541 (1997)
38. Wise R. A. Dopamine, learning and motivation. *Nat Neurosci Rev* 5, 483-494 (2004)
39. Gerfen, C. R. Basal Ganglia. In: Paxinos, G. (Ed.), *The Rat Nervous System*. Elsevier Academic Press, Amsterdam, pp. 445-508 (2004)
40. Ferré, S., Agnati, L. F., Ciruela, F., Lluis, C., Woods, A. S., Fuxe, K. & Franco, R. (2007) Neurotransmitter receptor heteromers and their integrative role in 'local modules': The striatal spine module. *Brain Res Rev* 55, 55-67 (2007)
41. Ferré, S., Borycz, J., Goldberg, S. R., Hope, B. T., Morales, M., Lluis, C., Franco, R., Ciruela, F. & Cunha, R. Role of adenosine in the control of homosynaptic plasticity in striatal excitatory synapses. *J Integr Neurosci* 4, 445-464 (2005)
42. Newman E. A. New roles for astrocytes: Regulation of synaptic transmission. *Trends Neurosci* 26, 536-542 (2003)
43. Hertz L. & Zielke R. Astrocytic control of glutamatergic activity: astrocytes as stars of the show. *Trends Neurosci* 27, 735-743 (2004)
44. Pascual O., Casper K. B., Kubera C., Zhang J., Revilla-Sanchez R., Sul J. Y., Takano H., Moss S. J., McCarthy K. & Haydon P. G. Astrocytic purinergic signaling coordinates synaptic networks. *Science* 310, 113-116 (2005)
45. Ferré, S., Fredholm, B. B., Morelli, M., Popoli, P. & Fuxe, K. Adenosine-dopamine receptor-receptor interactions as an integrative mechanism in the basal ganglia. *Trends Neurosci* 20**,** 482-487 (1997)
46. Agnati, L. F., Ferré, S., Lluis, C., Franco, R. & Fuxe, K. Molecular mechanisms and therapeutical implications of intramembrane receptor/receptor interactions among heptahelical receptors with examples from the striatopallidal GABA neurons. *Pharmacol Rev* 55, 509-550 (2003)
47. Ginés, S., Hillion, J., Torvinen, M., Le Crom, S., Casado, V., Canela, E. I., Rondin, S., Lew, J. Y., Watson, S., Zoli, M., Agnati, L. F., Verniera, P., Lluis, C., Ferré, S., Fuxe, K. & Franco, R. Dopamine D1 and adenosine A1 receptors form functionally interacting heteromeric complexes, *Proc Natl Acad Sci USA* 97, 8606-8611 (2000)
48. Hillion, J., Canals, M., Torvinen, M., Casado, V., Scott, R., Terasmaa, A., Hansson, A., Watson, S., Olah, M. E., Mallol, J., Canela, E. I., Zoli, M., Agnati, L. F., Ibanez, C. F., Lluis, C., Franco, R., Ferré, S. & Fuxe K. Coaggregation, cointernalization, and codesensitization of adenosine A2A receptors and dopamine D2 receptors. *J Biol Chem* 277, 18091-18097 (2002)
49. Canals, M., Marcellino, D., Fanelli, F., Ciruela, F., de Benedetti, P., Goldberg, S. R., Neve, K., Fuxe, K., Agnati, L. F., Woods, A. S., Ferré, S., Lluis, C., Bouvier, M. & Franco, R. Adenosine A2A-dopamine D2 receptor-receptor heteromerization: qualitative and quantitative assessment by fluorescence and bioluminescence energy transfer. *J. Biol. Chem.* 278, 46741-46749 (2003)
50. Kamiya, T., Saitoh, O., Yoshioka, K. and Nakata, H. Oligomerization of adenosine A2A and dopamine D2 receptors in living cells. *Biochem Biophys Res Commun* 306, 544-549 (2003)
51. Ferré, S., von Euler, G., Johansson, B., Fredholm, B. B. & Fuxe, K. Stimulation of high-affinity adenosine A2 receptors decreases the affinity of dopamine D2 receptors in rat striatal membranes. *Proc Natl Acad Sci USA* 88, 7238-7241 (1991)
52. Dixon, A. K., Widdowson, L. & Richardson, P. J. Desensitisation of the adenosine A1 receptor by the A2A receptor in the rat striatum. *J Neurochem* 69, 315-321 (1997)
53. Dasgupta, S., Ferré, S., Kull, B., Hedlund, P. B., Finnman, U. B., Ahlberg, S., Arenas, E., Fredholm, B. B. & Fuxe, K. Adenosine A2A receptors modulate the binding characteristics of dopamine D2 receptors in stably cotransfected fibroblast cells. *Eur J Pharmacol* 316, 325-331 (1996)
54. Salim, H., Ferré, S., Dalal, A., Peterfreund, R. A., Fuxe, K., Vincent, J. D. & Lledo, P. M. Activation of adenosine A1 and A2A receptors modulates dopamine D2 receptor-induced responses in stably transfected human neuroblastoma cells. *J Neurochem* 74, 432-439 (2000)
55. Kudlacek, O., Just, H., Korkhov, V. M., Vartian, N., Klinger, M., Pankevych, H., Yang, Q., Nanoff, C., Freissmuth M. & Boehm, S. The human D2 dopamine receptor synergizes with the A2A adenosine receptor to stimulate adenylyl cyclase in PC12 cells. *Neuropsychopharmacology* 28, 1317-1327 (2003)
56. Ferré, S., Popoli, P., Gimenez-Llort, L., Finnman, U. B., Martinez, E., Scotti de Carolis, A. & Fuxe, K. Postsynaptic antagonistic interaction between adenosine A1 and dopamine D1 receptors. *Neuroreport* 6, 73-76 (1994)
57. Ferré, S., Torvinen, M., Antoniou, K., Irenius, E., Civelli, O., Arenas, E., Fredholm, B. B. & Fuxe, K. Adenosine A1 receptor-mediated modulation of dopamine D1 receptors in stably cotransfected fibroblast cells, *J Biol Chem* 273, 4718-4724 (1998)

58. Cao Y., Sun W. C., Jin L., Xie K. Q. & Zhu X. Z. Activation of adenosine A1 receptor modulates dopamine D1 receptor activity in stably cotransfected human embryonic kidney 293 cells. *Eur J Pharmacol* 548, 29-35 (2006)
59. Solinas, M., Ferré, S., You, Z. B., Karcz-Kubicha, M., Popoli, P. & Goldberg, S. R. Caffeine induces dopamine and glutamate release in the shell of the nucleus accumbens. *J Neurosci* 22, 6321-6324 (2002)
60. Borycz, J., Pereira, M. F., Melani, A., Rodrigues, R. J., Kofalvi, A., Panlilio, L., Pedata, F., Goldberg, S. R., Cunha, R. A. & Ferré, S. Differential glutamate-dependent and glutamate-independent adenosine A1 receptor-mediated modulation of dopamine release in different striatal compartments. *J Neurochem* 101, 355-363 (2007)
61. Quarta, D., Ferré, S., Solinas, M., You, Z. B., Hockemeyer, J., Popoli, P. & Goldberg, S. R. Opposite modulatory roles for adenosine A1 and A2A receptors on glutamate and dopamine release in the shell of the nucleus accumbens. Effects of chronic caffeine exposure. *J Neurochem* 88, 1151-1158 (2004)
62. Ikemoto, S. Dopamine reward circuitry: Two projection systems from the ventral midbrain to the nucleus accumbens-olfactory tubercle complex. *Brain Res* doi:10.1016/j.brainresrev.2007.05.004
63. Quarta, D., Borycz, J., Solinas, M., Patkar, K., Hockemeyer, J., Ciruela, F., Lluis, C., Franco, R., Woods, A. S., Goldberg, S. R. & Ferré, S. Adenosine receptor-mediated modulation of dopamine release in the nucleus accumbens depends on glutamate neurotransmission and N-methyl-D-aspartate receptor stimulation. *J Neurochem* 91, 873-880 (2004)
64. Conlay, L. A., Conant, J. A., deBros, F. & Wurtman, R. Caffeine alters plasma adenosine levels. *Nature* 389, 136 (1997)
65. Ferré, S., Popoli, P., Gimenez-Llort, L., Rimondini, R., Muller, C. E., Stromberg, I., Ogren, S. O. & Fuxe, K. Adenosine/dopamine interaction: implications for the treatment of Parkinson's disease. *Parkinsonism Relat Disord* 7, 235-241 (2001)
66. Morelli, M., Di Paolo, T., Wardas, J., Calon, F., Xiao, D. & Schwarzschild, M. A. Role of adenosine A2A receptors in parkinsonian motor impairment and L-DOPA-induced motor complications. *Prog Neurobiol* (in press)

Key Words: Caffeine, Psychostimulants, Adenosine Receptors, Receptor Heteromers, Striatum, Review

Send correspondence to: Dr Sergi Ferre, National Institute on Drug Abuse, I.R.P., N.I.H., D.H.H.S., 5500 Nathan Shock Dr., Baltimore, MS 21224, U.S.A, Tel: 410-550-1586, Fax: 410-550-1648, E-mail: sferre@intra.nida.nih.gov