ADENOSINE A2A RECEPTORS CONTROL NEURO-INFLAMMATION AND 
CONSEQUENT NEURONAL DYSFUNCTION TRIGGERED BY 
LIPOPOLYSACCHARIDE IN THE RAT HIPPOCAMPUS

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Abbreviations: DMSO, dimethylsulfoxide; EPSP, excitatory post-synaptic potential; IL-1β, 
interleukin-1β; JNK, c-jun N-terminal kinase; LPS, lipopolysaccharide; LTP, long-term 
potentiation; SCH 58261, 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4- 
triazolo[1,5-c]pyrimidine.
ABSTRACT

Recent studies have shown that the blockade of adenosine A_{2A} receptor affords a robust neuroprotection in different noxious brain conditions by mechanisms still to be resolved. Since A_{2A} receptors efficiently control peripheral inflammation, one possible mechanism could be the control of neuro-inflammation, which is associated with brain damage. Thus, we tested if the intracerebroventricular injection of a selective A_{2A} receptor antagonist (SCH58261) would attenuate the changes in the hippocampus triggered by intraperitoneal administration of lipopolysaccharide (LPS), which activates microglia and induces neuro-inflammation. LPS administration triggers an increase in inflammatory mediators like interleukin-1β that causes biochemical changes in neurons (p38 and JNK phosphorylation and caspase 3 activation) contributing to neuronal dysfunction typified by decreased long term potentiation (LTP), a form of synaptic plasticity. LTP, measured 30 min after the tetanus, was significantly lower in LPS-treated rats compared with control-treated rats, while SCH58261 prevented the LPS-induced change. The LPS-induced increases in phosphorylation of c-jun N-terminal kinase (JNK) and p38 and activation of caspase 3 were also prevented by SCH58261, which had no effect alone. Significantly, SCH58261 also prevented the LPS-induced recruitment of activated microglial cells and the increase in interleukin-1β concentration in the hippocampus, suggesting that A_{2A} receptor activation is a pivotal step in mediating the neuro-inflammation triggered by LPS. These results raise the possibility of using A_{2A} receptor antagonists as novel drugs aimed at controlling neurodegenerative diseases known to involve neuro-inflammation.
Neurological diseases account for approximately 30% of the total disease burden in Europe and neurodegenerative diseases, which prevail in the aging population, account for a significant proportion of these (1). A common event in neurodegenerative diseases is neuro-inflammation, characterized by the appearance of activated inflammatory cells in the brain with the consequent increase in the levels of inflammatory mediators (2). It is considered that neuro-inflammation begins with the activation of microglial cells, the inflammatory cells resident in the brain, in conjunction with other lymphoid cells infiltrating the brain (e.g. 3,4). Accordingly, the activation of microglial cells is a faithful sensor of pathologic events in the brain (5) and a decrease in the extent of neuro-inflammation is associated with a better prognosis in the progression of neurodegenerative diseases (e.g. 6).

Adenosine is a substance of great interest in the potential control of neuro-inflammation. Adenosine is an endogenous homeostatic modulator (7) and acts as neuroprotective substance in the central nervous system (8). In parallel, adenosine also acts as a potent modulator of inflammatory reactions in the periphery (9). Most importantly, the control by adenosine of the tissue damage caused by ischemic/reperfusion injuries in peripheral organs has been shown to depend on its ability to control tissue inflammation, mainly through A\textsubscript{2A} receptors (10). Adenosine A\textsubscript{2A} receptors are expressed in microglial cells, as well as A\textsubscript{1} and A\textsubscript{3}, but not A\textsubscript{2B} receptors (11). It has been suggested that A\textsubscript{2A} receptors could control microglial reactivity (e.g. 12), although the characterization and role of the adenosine receptors present in microglial cells is only starting to emerge (7). But, by analogy with the crucial control of inflammation in the adenosine-mediated control of peripheral tissue damage, the possibility that the neuroprotective role of adenosine in brain tissue could also involve a control of neuro-inflammation should be entertained. In fact, the blockade of adenosine A\textsubscript{2A} receptors, which have a low abundance in the neo- and limbic cortex (13), confer a robust neuroprotection in animal models of neurodegenerative diseases where neuro-inflammation is present, such as Parkinson’s and Alzheimer’s diseases, epilepsy or neurodegeneration caused by excitotoxicity or free radicals (14). Thus, it is conceivable that
adenosine, acting through $A_{2A}$ receptors, might control the extent of neurodegeneration through the control of neuro-inflammation.

This study was designed to test if the blockade of $A_{2A}$ receptors could prevent the biochemical, morphological and functional consequences of an experimentally-induced neuro-inflammation triggered by administration of lipopolysaccharide (LPS). This toxin from Gram negative bacteria is a potent activator of inflammatory cells, including microglial cells (15-17), and considerable work has allowed us to understand the relation between the genesis of neuro-inflammation caused by LPS and neuronal dysfunction, using the hippocampus as an example (18-20).

**MATERIALS AND METHODS**

**Reagents**

Lipopolysaccharide from Escherichia coli (serotype 055:B5) was from Sigma, the kit for ELISA quantification of interleukin 1β (Duoset) was acquired from Genzyme Diagnostics, SCH58261 (5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine) was a generous gift from Scott Weiss (Vernalis, UK), the peptide Ac-DEVD-AFC was from Alexis, the anti-phospho-JNK antibody was from Santa Cruz Biotechnology, the mouse anti-CD11b was from Serotec, the mouse anti-α-tubulin antibody was from Zymed and the anti-phospho-p38 antibody was from Cell Signalling.

SCH58261 was prepared as a 5 mM stock solution in dimethylsulfoxide (DMSO) and aliquots were stored at $-20^\circ$C.

**Animals and experimental groups**

Groups of male Wistar rats (300-350 g) were used throughout this study and were handled in accordance with the EU guidelines for use of experimental animals. The rats were anesthetised by intraperitoneal injection of urethane (1.5 g/kg) and placed in a head holder in a stereotaxic frame to allow the intracerebroventricular (icv) injection of drugs into the third ventricle (2.5 mm posterior from Bregma and 0.5 lateral to the midline). The rats were then divided into 4 experimental groups: 1) control rats injected with 5 µl saline (with 0.0001%
DMSO) icv and 200 µl saline ip after 30 min; 2) LPS-treated rats injected with 5 µl saline (with 0.0001% DMSO) icv and 200 µl LPS (200 µg, dissolved in 200 µl saline) ip after 30 min; 3) SCH 58261-treated rats injected with 5 µl SCH 58261 (50 nM in 0.0001% DMSO) icv and 200 µl saline ip after 30 min; 4) LPS + SCH 58261-treated rats injected with 5 µl SCH 58261 (50 nM in 0.0001% DMSO) icv and 200 µl LPS (200 µg) ip after 30 min.

The dose of LPS administered was the same as that previously used in the studies exploring the effect of peripheral administration of LPS on the viability and functionality of hippocampal neurons (18-20). SCH 58261 was chosen since it is the most potent and selective A2A receptor antagonist available, as assessed by its sub-nanomolar affinity for A2A receptors (e.g. 13) and selectivity towards other adenosine receptors, which is best exemplified by the disappearance of the selective binding of SCH 58261 in A2A receptor knockout mice (13). We selected a concentration of SCH 58261 of 50 nM based on the equivalent effect of this concentration of SCH 58261 (applied through reverse-microdialysis) and ip injection of neuroprotective doses of 0.01-0.1 mg/kg (reviewed in 14) on the evoked release of glutamate (cf. 21,22).

**Electrophysiological recording of synaptic plasticity in the hippocampus in vivo**

Three hours after the injection of LPS (or saline) ip, a bipolar concentric stimulation electrode was placed in the perforant pathway (angular bundle, 4.4 mm lateral to lambda) and the recording electrode was positioned in the *stratum moleculare* of the dentate gyrus (2.5 mm lateral and 3.9 mm posterior to bregma). The depth of the electrodes was adjusted to obtain potentials with maximal amplitude and the intensity of stimulation was selected to trigger only a single post-synaptic potential. After obtaining a baseline for 15 min, the experimental protocol to induce long term potentiation (LTP) was initiated. This consisted of delivery of a tetanic pulse (3 trains of stimuli at 250 Hz during 200 ms with an inter-burst interval of 30 s) while the basal stimulation was delivered once every 30 seconds.

At the end of the electrophysiological recording, the rats were sacrificed by decapitation. The hippocampus was rapidly dissected at 4ºC, cut in 350 x 350 µm cubes, aliquotted and stored at -20ºC as previously described (e.g. 19) for subsequent biochemical assays.
Quantification of interleukin-1β concentration

The concentration of interleukin-1β (IL-1β) in hippocampal homogenates was assessed by enzyme-linked immunosorbent assay, as previously described (e.g. 18). Antibody-coated (2.0 µg/ml final concentration, diluted in 0.1 M sodium carbonate buffer, pH 9.5; monoclonal hamster anti-mouse IL-1β antibody) 96-well plates were incubated overnight at 4ºC, washed four times with PBS containing 0.05% Tween 20, blocked for 2 h at 37ºC with 250 µl of blocking buffer (PBS, pH 7.3, 0.1 M with 4% bovine serum albumin), and incubated with IL-1β standards (100 µl; 0-1000 pg/ml) or samples (supernatants of hippocampal samples homogenized in Krebs solution containing 2 mM CaCl₂) for 1 h at 37ºC. Samples were incubated with secondary antibody (100 µl; final concentration 0.8 µg/ml in PBS containing 0.05% Tween 20 and 1% bovine serum albumin; biotinylated polyclonal rabbit anti-mouse antibody) for 1 h at 37ºC, washed, and incubated in detection agent (100 µl; horseradish peroxidase-conjugated streptavidin; 1:1000 dilution in PBS containing 0.05% Tween 20 and 1% bovine serum albumin) and incubated for 15 min at 37ºC. 3,3',5,5'-Tetramethylbenzidine (100 µl; Sigma) was added, samples were incubated at room temperature and absorbance was read at 450 nm within 30 min. Values are expressed as pg IL-1β/mg of protein, quantified as described (23).

Analysis of p38 and JNK phosphorylation

Activation of JNK and p38 were quantified by assessing the density of the phosphorylated forms of the kinases using Western blot analysis, as previously described (e.g. 18,20). Samples of hippocampal homogenate were solubilized in 5% SDS to obtain a protein concentration of 1 mg/ml. Aliquot of homogenate (10 µl) were added to 10 µl of sample buffer (0.5 mM Tris-HCl pH 6.8, 10% glycerol, 10% SDS, 5% β-mercaptoethanol and 0.05% bromophenol blue) and samples were boiled for 5 min. These samples and the pre-stained molecular weight markers (Amersham) were separated on SDS gels (10%) and electro-transferred to polyvinylidene difluoride (PVDF) membranes (0.45 µm, from Amersham). After blocking for 2 h at room temperature with 5% milk in Tris-buffered saline (pH 7.6 containing
0.1% Tween 20 (TBS-T)), the membranes were incubated overnight with a mouse antibody against the phosphorylated form of JNK (1:2000 dilution) or with a mouse antibody against the phosphorylated form of p38 (1:300 dilution). After four washing periods for 10 min with TBS-T containing 0.5% milk, the membranes were incubated with the alkaline phosphatase-conjugated anti-mouse secondary antibody (1:2000 dilution, from Calbiochem) in TBS-T containing 1% milk during 90 min at room temperature. After five 10 min washes in TBS-T with 0.5% milk, the membranes were incubated with Enhanced Chemi-Fluorescence for 5 min and then analysed with a VersaDoc 3000 (Biorad).

The membranes were then re-probed and tested for tubulin immunoreactivity to confirm that similar amounts of protein were applied to the gels. Briefly, the membranes were incubated for 1 hour at room temperature with a 0.1 M glycine (pH 7.2) solution and blocked as previously described before incubation with the anti-tubulin antibody (1:1000). The membranes were then washed and incubated with alkaline phosphatases-conjugated secondary antibody as previously described.

**Analysis of caspase-3 activity**

Cleavage of the caspase-3 substrate (Ac-DEVD-AFC peptide) to its fluorescent product was used as a measure of caspase-3 activity, as previously described (20). Briefly, slices of hippocampal tissue were washed, homogenized in ice-cold lysis buffer (25 mM HEPES, 5 mM MgCl$_2$, 5 mM EDTA, 5 mM dithiothreitol, 2 mM phenylmethylsulfonfyl fluoride, 6.25 µg/ml pepstatin A, 6.25 µg/ml aprotinin, pH 7.4) and lysed by cycles of freezing and thawing. Aliquots of these samples (50 µl) were mixed with 50 µl of reaction buffer (50 mM HEPES, 2 mM EDTA, 20% glycerol, 10 mM dithiothreitol, pH 7.4) and 4 µl of caspase-3 substrate (final concentration 10 µM from a stock solution of 250 µM in reaction buffer) and added to 96-well plates. Samples were incubated at 37°C for 60 min in the dark, fluorescence was assessed (excitation 400 nm; emission 505 nm) and enzyme activity was calculated with reference to a standard curve of 7-amino-4-trifluoromethylcoumarin (AFC; 0-10 µM) versus absorbance. The protein concentration of each sample was determined using bovine serum albumin as a standard (23) and values expressed as nmol AFC/mg protein/min.
**Immunohistochemical analysis of microglial reactivity in the hippocampus**

Four hours after the administration of LPS (200 µg in 200 µl, ip) under urethane anaesthesia, the heart was exposed and after clamping the descending aorta, a catheter was inserted in the ascending aorta. The animal was then perfused with saline with 4% sucrose (200 ml) while opening the right atria to allow the outflow of the perfusate. Rats were then perfused with 200 ml of 4% paraformaldehyde in saline with 4% sucrose. After its fixation, the brain was removed, maintained for 12 h in the same paraformaldehyde solution and subsequently for 48 h in a PBS solution with 30% sucrose. The brain was then frozen in dry ice and 20 µm coronal sections were prepared using a cryostat. The sections were stored in PBS with 0.01% sodium azide until mounting in slides coated with 2% gelatine with chromium and potassium sulphate. After drying at room temperature, the mounted sections were stored at -20°C.

The detection of microglia-like profiles was then carried out using an immunohistochemical detection of OX-42/CD-11b, an epitope which is up-regulated in activated microglia and to a lesser extent in macrophages (3,16). The sections were first rinsed for 5 min in PBS and then three times for 5 min with TBS (0.05 M Trizma base buffer containing 150 mM of NaCl, pH 7.2) at room temperature. The slides were blocked with TBS containing 0.2% Triton X-100 and 10% goat serum during 45 min. Sections were incubated in the presence of the mouse anti-CD11b antibody (1:200 dilution in TBS containing 0.2% Triton X-100 and 10% normal goat serum) for 72 hours at 4°C, rinsed three times for 10 min in TBS and subsequently incubated with goat anti-mouse secondary antibody conjugated with a fluorophore (Alexa Fluor 488) (1:50 dilution in 0.1 M phosphate buffer containing 0.2% Triton X-100 and 10% normal serum) for 2 hours at room temperature, then rinsed twice for 10 min in TBS and once for 10 min in distilled water. The sections were dehydrated and passed through xylol before mounting on slides, using Vectashield mounting medium (Vector Laboratories) and examined under a fluorescence microscope or a confocal microscope.
**Statistical analysis**

Values are presented as mean ± SEM of \( n \) experiments. Either a Student’s \( t \) test for independent means or a one-way analysis of variance (ANOVA) followed by *post hoc* Newmann-Keuls test was used to define statistical differences between values, which were considered at \( P<0.05 \).

**RESULTS**

**A\(_{2A}\) receptor blockade prevents the LPS-induced depression of synaptic plasticity**

We report that ip administration of LPS (200 µg in 200 µl) depressed LTP in perforant path-granule cell synapses *in vivo* confirming results of previous studies (18,19,24). In control rats, which received vehicle alone (5 µl 0.0001% DMSO icv and 200 µl ip), the mean percentage change in the slope of the population excitatory post-synaptic potentials (EPSP), measured 30 min after the high frequency train (compared with the EPSP slope in the 5 min immediately prior to tetanic stimulation) was 111.5±0.7% (\( n=5; \ P<0.05 \)). However, in rats injected with LPS, the corresponding mean percentage change in population EPSP slope was 102.5±0.8% (\( n=5 \)); thus LTP was not sustained in these rats (Figure 1).

The icv administration of the selective antagonist of adenosine A\(_{2A}\) receptors, SCH 58261 (5 µl of a 50 nM solution), did not significantly modify (\( P>0.05 \)) LTP in the dentate gyrus, when compared with control rats (Figure 1); the mean percentage change in EPSP slope 30 min following tetanic stimulation was 115.2±0.9% (\( n=5, \ P<0.05 \)). However, icv administration of SCH 58261 completely prevented the LPS-induced depression of synaptic plasticity in the dentate gyrus (Figure 1); the mean percentage change in mean EPSP slope 30 min after tetanic stimulation was 113.4±0.4% (\( n=5 \)), a value which is not significantly different (\( P>0.05 \)) from that found in control rats but significantly larger (\( P<0.05 \)) than that observed in rats injected only with LPS.

**A\(_{2A}\) receptor blockade prevents the LPS-induced neuronal biochemical modifications**

Previous studies by our group have suggested that LPS-induced increase in hippocampal IL-1\( \beta \) and neuronal downstream signalling events (i.e. activation of kinases of the
stress pathway) mediate the depression of synaptic plasticity in the dentate gyrus (18-20). Here we asked whether the ability of SCH 58261 to reverse the LPS-induced impairment in LTP might be paralleled by a similar ability to attenuate the LPS-induced increases in phosphorylation of p38 and JNK and activation of caspase-3.

As shown in Figure 2, LPS increased the density of the phosphorylated forms of p38 and of JNK in hippocampal tissue to 131±6% and 129±9% of control values, respectively (n=5, P<0.05). Administration of SCH 58261 did not significantly change the density of the phosphorylated forms of p38 or JNK (103±8% and 98±9% of control, respectively; P>0.05 versus control, n=5), but prevented the LPS-induced increases in both p38 and JNK (100±5% and 98±9% of control; P<0.05 versus LPS, n=5).

In parallel with the effect of LPS on phosphorylation of JNK and p38, we report a significant increase in the activity of caspase-3 in hippocampal tissue prepared from LPS-treated, compared with control rats (n=5, P<0.05; Figure 3). Administration of SCH 58261 did not significantly change (P>0.05, n=3) the activity of caspase-3, but prevented the LPS-induced increase of caspase-3 activity so that there was a significant difference (P<0.05) in the enzyme activity in hippocampal tissue prepared from LPS-treated rats and in tissue prepared from rats which were treated with LPS and SCH 58261 (Figure 3).

A2A receptor blockade prevents the LPS-induced neuro-inflammation

To directly test if A2A receptors controlled microglia reactivity and the consequent generation of inflammatory mediators, we investigated if the blockade of A2A receptors could control activation of microglia and the previously-described LPS-induced increase in IL-1β (18).

The identification of microglia cells was carried out by immunohistochemical detection of anti-CD11b, a validated marker of activated microglia cells (3,16). As presented in Figure 4, we can conclude that 4 h after administration of LPS, there is an increase in the number of elements labelled with the anti-CD11b antibody (compare panels A and E from Figure 4). Furthermore, the labelled profiles displayed a more intense immunoreactivity and a morphology characteristic of early activated microglia in situ, i.e. enlarged cell body with short and thick processes (Figure 4F) (e.g. 3,16). Administration of SCH 58261 did not modify the
profile of CD11b immunoreactivity compared to control (n=4; Figures 4C and 4D), but prevented the LPS-induced changes in CD11b immunoreactivity (n=4; Figures 4G and 4H).

We report a significant increase in hippocampal concentration of IL-1β in rats treated with LPS (n=5, P<0.05; Figure 5). Administration of SCH 58261 alone did not significantly change (P>0.05, n=3) IL-1β concentration, but prevented the LPS-induced increase in IL-1β (n=3, P<0.05; Figure 5).

DISCUSSION

The main conclusion from this study is that the blockade of adenosine A2A receptors prevents the induction of a neuro-inflammatory situation triggered by lipopolysaccharide (LPS) and the consequent neuronal dysfunction and biochemical modifications in the hippocampus.

Several previous studies have already studied the cascade of events that lead to neuronal dysfunction after the administration of LPS and the consequent neuronal damage, particularly in the hippocampus (18-20, 24). Thus, LPS triggers an initial increase in the concentration of the pro-inflammatory cytokine IL-1β, which plays a key role in triggering neuronal dysfunction (18; reviewed in 25). The precise mechanism by which peripherally administered LPS triggers neuro-inflammation still remains to be defined (e.g. 26), but is thought to involve the activation of microglial cells (15,16). In response to a neuro-inflammatory situation, neurons react by up-regulating activation of the stress kinases p38 and JNK, which negatively impact on synaptic function leading to depression of LTP in dentate gyrus (19,20). In parallel, PARP cleavage and activation of caspase-3 are up-regulated leading to a delayed LPS-induced neuronal cell death (20,24). In the present study, we observed that the blockade with adenosine A2A receptors, using its selective antagonist SCH 58261 (7), prevented the principal modifications caused by LPS that are associated with the LPS-induced neuronal dysfunction. Thus, the administration of SCH 58261 prevented the ability of LPS to increase the phosphorylation of p38 and of JNK, as well the activation of caspase-3, and also prevented the LPS-induced depression of LTP. It is important to note that, in the absence of LPS, SCH
58261 was devoid of effects in any of these properties. It is therefore concluded that the blockade of adenosine A<sub>2A</sub> receptors conferred a robust neuroprotection in this model of neuroinflammation, as observed in other in vivo models of brain neurotoxicity. Thus A<sub>2A</sub> receptor blockade is also neuroprotective against either 6-hydroxydopamine- or MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine)-induced toxicity in the basal ganglia (two animal models of Parkinson’s disease, see e.g. 27), against malonate- or 3-nitropropionic acid-induced toxicity in the basal ganglia (two other animal models of Parkinson’s and/or Huntington’s diseases, see e.g. 28,29), against ischemia-induced cortical infarct (see e.g. 30) and against quinolinic acid-induced hippocampal damage (31). Since neurons are endowed with A<sub>2A</sub> receptors (e.g. 13), and A<sub>2A</sub> receptor blockade affords a direct protection in cultured neurons exposed to neurotoxic stimuli such as amyloidogenic peptides (32), it is likely that neuronal A<sub>2A</sub> receptors might directly protect neurons from the consequences of noxious brain stimuli, independently of any other brain cell type (discussed in 14). However, it is important to note that the blockade of A<sub>2A</sub> receptors is particularly effective when tested in vivo. In fact, blockade of A<sub>2A</sub> receptors affords a more robust neuroprotection in hippocampal regions upon in vivo ischemia (e.g. 30) when compared with the effect found upon chemical ischemia in hippocampal slices in vitro (33,34). This suggests that mechanisms other than a direct neuronal protection might be implicated in the neuroprotection afforded by A<sub>2A</sub> receptor blockade.

Here we report that blockade of A<sub>2A</sub> receptors decreased the extent of neuroinflammation caused by LPS, as evaluated by the levels of IL-1β, a master regulator of neuroinflammation that contributes to neurodegeneration (25). Thus, the present data indicate that the ability of A<sub>2A</sub> receptors to control the increase in brain levels of IL1β may play an important hitherto unrecognised role in the neuroprotective effect afforded by A<sub>2A</sub> receptor blockade. In striking parallel, we also observed that SCH 58261 decreased the number of activated microglial cells present in sections from the hippocampus of rats treated with LPS, which re-enforces the hypothesis that A<sub>2A</sub> receptors might control the genesis of neuro-inflammatory processes in pathological conditions. In fact, the activation of microglia is found in most brain noxious conditions such as ischemia, trauma, brain infection, Parkinson’s, Alzheimer’s or
Huntington’s diseases (reviewed in 6). The observations that microglia cells express A<sub>2A</sub> receptors (e.g. 11) and that exposure to LPS increased A<sub>2A</sub> receptor immunoreactivity in cultured microglial cells (unpublished observations by our group) re-enforce the hypothesis that A<sub>2A</sub> receptors might directly control the activation state of microglia (12).

It is particularly interesting that blockade of A<sub>2A</sub> receptors prevented the appearance of a neuro-inflammatory condition as well as the consequent neuronal dysfunction caused by neuro-inflammation, specifically the impairment in LTP. In contrast, in peripheral systems, the opposite was observed, i.e. it is the activation (rather than the blockade) of A<sub>2A</sub> receptors that attenuates the peripheral inflammatory system (9, reviewed in 10). In fact, the activation of A<sub>2A</sub> receptors triggers an ‘OFF signal’ in inflammatory responses in different types of peripheral lymphoid cells (reviewed in 10), in particular in macrophages and dendritic cells, that are cell types morphologically and functionally related to microglial cells (e.g. 35,36). Accordingly, the blockade of A<sub>2A</sub> receptors exacerbates the extent of damage of lesions involving inflammation in peripheral organs (e.g. 9), exactly the opposite of what we observed in the central nervous system. Although intriguing, these observations re-enforce the specific nature of neuro-inflammation and stress the care required in extrapolating from findings obtained in the study of peripheral inflammation to the brain. It remains to be clarified if the opposite findings regarding modulation by A<sub>2A</sub> receptors centrally and peripherally are due to different processes which trigger and/or control the release of cytokines from microglial cells and other lymphoid cells or if the differential effects of A<sub>2A</sub> receptors might be explained by the relationship between microglia and the neighbouring neurons and astrocytes which are known to modulate neuro-inflammatory processes (e.g. 37,38). The later hypothesis seems more likely since a recent study (Yu et al., 2004) showed that ischemic brain and liver injuries are regulated in opposite manners by A<sub>2A</sub> receptors located in bone marrow-derived cells (concluded based on the combined use of A<sub>2A</sub> receptor knockout mice and bone marrow transplants after bone marrow destruction by γ-irradiation). This study also supports an important role for A<sub>2A</sub> receptor blockade (through genetic inactivation of A<sub>2A</sub> receptors) in controlling the extent of cortical infarct after middle cerebral artery occlusion through the control of neuro-inflammation (39).
However, the authors concluded that the neuroprotection afforded by blockade of A\textsubscript{2A} receptors was mostly due to A\textsubscript{2A} receptors located in bone marrow-derived cells while blockade of the brain resident A\textsubscript{2A} receptors only contributed for 20% of neuroprotection in this ischemic brain injury model where extensive disruption of the blood-brain barrier and consequent massive invasion of peripheral lymphoid cells is expected (reviewed in 40). Interestingly, in another model of brain neurotoxicity where the disruption of the blood-brain barrier is not evident, it was concluded that non-neuronal brain-resident A\textsubscript{2A} receptors now played a prominent role in controlling the extent of neurodegeneration (J.F.Chen, personal communication).

In conclusion, the present study provides evidence to support the hypothesis that blockade of adenosine A\textsubscript{2A} receptors attenuates the LPS-induced neuro-inflammation and the consequent neuronal dysfunction in the hippocampus caused by LPS administration. These pioneering observations suggest that antagonists of A\textsubscript{2A} receptors might constitute a novel pharmacological strategy to control a diversity of brain pathological conditions which involve neuro-inflammation, such as cerebro-vascular disease, Alzheimer’s disease or brain trauma.

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Figure 1- Effect of the administration of lipopolysaccharide (LPS, 200 µg in 200 µl, ip) and/or the selective antagonist of adenosine A2A receptors, SCH 58261 (5 µl of a 50 nM solution, icv) on long-term potentiation (LTP) measured in the rat dentate gyrus in vivo. Rats were anesthetized with urethane (1.5 mg/kg) and separated in four experimental groups: 1) control (administration icv and ip of saline – open circles); 2) SCH 58261 (administration of SCH 58261 icv and saline ip – open squares); 3) LPS (administration of saline icv and LPS ip – filled circles); 4) LPS + SCH 58261 (administration icv of SCH 58261 and of LPS ip – filled squares). The ordinates represent the slope of the excitatory post-synaptic potentials (EPSPs) recorded in the cell body region of the dentate gyrus after stimulation of the perforant pathway at a frequency of 0.033 Hz. The arrow indicates the period of application of a high frequency stimulation period (3 trains of stimuli at a frequency of 250 Hz during 200 ms with an inter-burst interval of 30 s). Each point represents the mean±SEM of 5 experiments. Note that SCH 58261 did not modify LTP but prevented the LPS-induced depression of LTP.

Figure 2- Effect of the administration of lipopolysaccharide (LPS, 200 µg in 200 µl, ip) and/or the selective antagonist of adenosine A2A receptors, SCH 58261 (5 µl of a 50 nM solution, icv) on the density of the phosphorylated forms of two stress activated kinases, p38 (panels A and C) and JNK (panels B and D) in the rat hippocampus. The rats were separated into 4 groups: 1) control (administration icv and ip of saline); 2) SCH 58261 (SCH 58261 icv and saline ip); 3) LPS (saline icv and LPS ip); 4) LPS + SCH 58261 (SCH 58261 icv and LPS ip). Four hours and 45 min after LPS injection, the rats were sacrificed, the hippocampus dissected and homogenized for Western blot analysis. Panels A and B present a Western blot comparing the density of immunoreactivity of the phosphorylated form of p38 (panel A) and of JNK (panel B) in the hippocampus of each group of rats. The re-probing of the same gels against tubulin is displayed below. Panels C and D present the average results (mean±SEM), obtained in 3-5 experiments, comparing the density of immunoreactivity of the phosphorylated form of p38 (panel C) and of JNK (panel D) in the hippocampus of each
group of rats. *P<0.05 between the indicated bars. Note that SCH 58261 did not modify the density of phosphorylation of these two activated stress proteins but prevented the ability of LPS to increase their density.

**Figure 3**- Effect of the administration of lipopolysaccharide (LPS, 200 µg in 200 µl, ip) and/or the selective antagonist of adenosine A<sub>2A</sub> receptors, SCH 58261 (5 µl of a 50 nM solution, icv) on the activity of caspase-3 in the rat hippocampus. The rats were separated into 4 groups: 1) control (administration icv and ip of saline); 2) SCH 58261 (SCH 58261 icv and saline ip); 3) LPS (saline icv and LPS ip); 4) LPS + SCH 58261 (SCH 58261 icv and LPS ip). Four hours and 45 min after LPS injection, the rats were sacrificed, the hippocampus dissected and homogenized for the fluorimetric analysis of caspase-3 activity, quantified by the hydrolysis of its selective substrate, Ac-DEVD-AFC peptide, which produces a fluorescent product (emission at 505 nm after excitation at 400 nm). The ordinates represent the activity of caspase-3 expressed in nmols of 7-amino-4-trifluoromethylcoumarine (AFC), normalized by the concentration of protein in each sample. Each bar represents the mean±SEM of 5 experiments. *P<0.05 between the indicated bars. Note that SCH 58261 did not modify the activity of caspase-3 but prevented the LPS-induced increase in caspase-3 activity.

**Figure 4**- Effect of the administration of lipopolysaccharide (LPS, 200 µg in 200 µl, ip) and/or the selective antagonist of adenosine A<sub>2A</sub> receptors, SCH 58261 (5 µl of a 50 nM solution, icv) on the appearance of reactive microglia in the rat dentate gyrus. The rats were separated into 4 groups: 1) control (administration icv and ip of saline; panel A); 2) SCH 58261 (SCH 58261 icv and saline ip; panel B); 3) LPS (saline icv and LPS ip; panel C); 4) LPS + SCH 58261 (SCH 58261 icv and LPS ip; panel D). Four hours and 45 min after the drug treatments, the rats were perfused with paraformaldehyde for fixation of the brain, which was sliced (20 µm). The coronal sections were labelled by immunohistochemistry using a mouse anti-CD11b antibody (a marker of activated microglia cells) and a goat anti-mouse secondary antibody labelled with Alexa Fluor 488 (emits green) (the insert figures in
each panels display the pattern of labelling and morphology of a single microglia cell, obtained from the presented picture, at higher magnification). The pictures presented are representative of 3-4 experiments with qualitatively similar results. Note that LPS increase the general density of CD11b positive elements (panel C) displaying a morphology characteristic of activated microglia cells (see inserted Figure in panel C) and these LPS-induced modifications were prevented by SCH 58261 (panel D), which by itself was devoid of effects (panel B).

**Figure 5**- Effect of the administration of lipopolysaccharide (LPS, 200 µg in 200 µl, ip) and/or the selective antagonist of adenosine A<sub>2A</sub> receptors, SCH 58261 (5 µl of a 50 nM solution, icv) on the activity of caspase-3 in the rat hippocampus. The rats were separated into 4 groups: 1) control (administration icv and ip of saline); 2) SCH 58261 (SCH 58261 icv and saline ip); 3) LPS (saline icv and LPS ip); 4) LPS + SCH 58261 (SCH 58261 icv and LPS ip). Four hours and 45 min after LPS injection, the rats were sacrificed, the hippocampus dissected and homogenized for analysis by ELISA of interleukin-1β (IL-1β) levels. Each bar represents the mean±SEM of 5 experiments. *P<0.05 between the indicated bars. Note that SCH 58261 did not modify the levels of IL-1β but prevented the LPS-induced increase in IL-1β levels.
Fig. 1

**in vivo LTP**

EPSP slope (%)

- ○ Control
- ● LPS
- □ SCH 58261
- ▣ LPS + SCH 58261

**TIME (min)**

0 10 20 30 40

HFS
Fig. 2

A

B

C

D

Ctr  LPS  SCH  SCH  LPS

P-p38

P-JNK

tubulin

0

50

100

150

*  *

density of P-JNK (% of control)

0

50

100

Control  LPS  SCH  SCH+LPS

*  *

density of P-p38 (% of control)

Control  LPS  SCH  SCH+LPS

*  *

Control  LPS  SCH  SCH+LPS
Fig. 3

CASPASE-3 ACTIVITY
nmol AFC/mg/min

control  LPS  SCH  LPS+SCH

0  10  20  30

*  *

23
Fig. 5

Control LPS SCH SCH+LPS

ng IL-1β / mg protein

0.0 0.2 0.4 0.6

* *