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No evidence for cross-talk between the adenosine A1 and the serotonin-1A receptor system after chronic A1 receptor stimulation

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ABSTRACT

Several studies show that different neurotransmitter receptor systems may interact with each other, for example, by means of a shared pool of G-proteins coupled to these receptors. Along these lines, there are data suggesting cross-talk between the adenosine and serotonin receptor systems. Such an interaction between these two receptor systems may also emerge under conditions of sleep loss which is characterized by increased adenosine turnover. In the present study, we tested whether chronic application of the adenosine A1 receptor agonist N6-cyclopentyladenosine (CPA) not only desensitizes the adenosine receptors but also affects serotonin-1A signalling. The present study shows that chronic injections with CPA caused a decrease in adenosine A1 receptor numbers and an increase in the amount of adenosine A1 receptor-activated G-proteins in a region-specific manner. On the level of physiological output, that is, CPA-induced and A1 receptor-mediated hypothermia, we found a reduced temperature response suggesting a desensitization of the receptor system. Nevertheless, the hypothermic response to the serotonin-1A receptor agonist 8-OH-DPAT was not altered in these animals, indicating that there had not been cross-desensitization of the response. In conclusion, the present study shows that chronic application of the A1 agonist CPA desensitizes the adenosine A1 receptor system but it does not alter serotonin-1A receptor-associated signalling as measured by the functional hypothermic response.
INTRODUCTION

A number of studies have shown that different neurotransmitter receptor systems, for instance, the serotonin-1A and adenosine A1 receptors may interact with each other. Particularly, it is known that adenosine A1 and serotonin-1A receptors activate a common pool of G-proteins and G-protein-coupled ion channels (Zgombick et al., 1989; Luscher et al., 1997). Therefore, elements of intracellular signal transduction pathways shared by different transmitter receptor populations might represent the means of cross-talk between receptors (Neubig, 1994; Bakker et al., 2004). Also, it has been reported that selective blockade of adenosine receptors by caffeine and theophylline in mice not only causes a significant increase in adenosine receptors but also other receptors, including serotonin receptors (Fastbom and Fredholm, 1990; Shi et al., 1993; Ralevic and Burnstock, 1998; Shi and Daly, 1999).

A real life situation that might involve cross-talk between serotonin-1A and adenosine receptors is sleep loss. Our earlier study in rats showed that chronic loss of sleep decreased the sensitivity of the serotonergic 1A receptor population (Roman et al., 2005). In that study, we hypothesized that this desensitization of the serotonin-1A receptor population after chronic sleep restriction might be a direct result of the presence of excess serotonin (Li et al., 1999), but also an indirect consequence of cross-talk between this receptor system and the adenosine receptor system; a phenomenon known as heterologous desensitization (Ferguson and Caron, 1998).

Adenosine, a metabolite of ATP, is produced at an increasing rate as a function of wakefulness and possibly signals decreasing availability of energy and, in turn, neuronal exhaustion in the brain. Along these lines, adenosine is thought to induce sleep and thereby promote neuronal recovery (Radulovacki, 1993; Porkka-Heiskanen et al., 1997; for review see Porkka-Heiskanen et al., 2002 and Basheer et al., 2004). It might be that increased adenosine turnover and stimulation of adenosine receptors under conditions of chronic prolonged wakefulness ultimately affects intracellular signalling pathways associated with the serotonin-1A receptor.

In the present study we examined whether chronic application of the adenosine A1 receptor agonist CPA would not only desensitize the adenosine receptors but might also result in reduced serotonin-1A receptor responses that we reported for chronically sleep restricted animals. In other words, we investigated whether pharmacological stimulation of adenosine receptors would mimic the effects of sleep loss. To study changes in adenosine A1 receptors and their coupling to G-proteins, we applied receptor and receptor-activated G-protein autoradiography. In order to study the functional sensitivity and output of the adenosine A1 receptor system and the serotonin-1A receptor system, we used radio telemetry to measure body temperature responses to adenosine and serotonin receptor agonists.
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MATERIALS AND METHODS

Animals and housing
In the present study, we used 18 young male Wistar rats (Harlan, Horst, The Netherlands) weighing approximately 250 g at the beginning of the experiment. Animals were housed under a 12 h light/12 h dark cycle, with lights on from 09.00 h to 21.00 h. The temperature of the room was kept at 21 ± 1°C. Rats were provided with food and water ad libitum. The experiment was approved by the Animal Experimentation Committee of the University of Groningen.

Chronic adenosine A1 receptor stimulation
The 18 rats were divided into two groups. Nine rats received daily subcutaneous (s.c.) injections of the potent and specific A1 receptor agonist N6-cyclopentyladenosine (CPA; Sigma, St. Louis, MO, USA) dissolved in physiological saline (0.25 mg/kg body weight) for five days. The functional sensitivity of adenosine receptors to CPA was assessed by measurements of the CPA-induced acute drop in body temperature (Dunwiddie and Worth, 1981; Ticho and Radulovacki, 1991). The concentration of the CPA was known to induce an intermediate or submaximal hypothermic response, based on a dose-response curve established previously (Roman and Meerlo, unpublished results). In addition to the rats receiving CPA, we used nine other rats that were injected with physiological saline over a period of five days. CPA and saline injections were given between 11.00 h and 12.00 h.

Serotonergic challenge
To examine the functional sensitivity of the serotonin-1A receptor system, rats were s.c. injected with the serotonin 1A agonist (±)-8-hydroxy-2-(di-n-propyl-amino) tetralin hydrobromide (8-OH-DPAT; 0.25 mg/kg body weight; Sigma, St. Louis, MO, USA) on the day that followed the chronic CPA treatment. The serotonergic agonist 8-OH-DPAT causes an acute hypothermic response that can be used as an indicator of central serotonin-1A neurotransmission, as has been shown in rats (Hjorth, 1985). The pharmacological challenges were performed between 11.00 h and 12.00 h. The sensitivity to the drug was determined by measuring the acute hypothermic response by means of radio telemetry.

Radio telemetry of body temperature
In order to record the adenosine A1 and serotonin-1A receptor-mediated drop in body temperature, we applied radio telemetry with chronically implanted transmitters (model TA10TA-F40; Data Sciences, St. Paul, MN, USA). Implantation of the transmitters in the abdominal cavity was performed under full anaesthesia (inhalation anaesthesia with a mixture of N2O, O2, and isoflurane). After surgery, the animals were allowed at least 10 days of recovery. The transmitters measured core body temperature and transformed temperature values into frequency coded radio signals. These radio signals were relayed to a PC by receivers placed under cages (model RPC-1; Data Sciences, St. Paul, MN, USA). Body temperature was sampled for 5 sec every 5 min and processed with Dataquest Labpro™ system (Data Sciences).

Collection and cutting of brain material
On the day after the 8-OH-DPAT challenge, rats were anesthetized on dry ice and decapitated. Brains were removed from the skull as quick as possible, frozen with liquid nitrogen and stored at -80°C until further processing. In a cryostatic microtome, 20-μm coronal sections were cut containing the prefrontal cortex (bregma 3.70 – 3.46 mm), the medial septum (bregma 1.20 – 0.96 mm), the hypothalamus (bregma -0.26 – 0.50 mm), the hippocampus/somatosensory cortex (bregma -2.12 – 2.36 mm) (Paxinos and Watson, 1986). Sections were mounted on SuperFrost®Plus glass slides (Menzel-Gläser, Menzel GmbH & Co KG, Braunschweig, Germany) and stored at -80°C.

Adenosine A1 receptor autoradiography
Adenosine A1 receptor autoradiography was performed according to published methods (Snowhill and Williams, 1986; Georgiev et al., 1993; Meerlo et al., 2004). Brain sections were preincubated in Tris/HCl buffer (0.17 M, pH7.6, Merck, Darmstadt, Germany) containing 0.5 IU/ml adenosine deaminase enzyme (EC 3.5.4.4, type VI from calf intestinal mucosa, Sigma) for 30 min. Preincubation was followed by a drying step in cool air stream. The incubation buffer was made by adding 0.5 IU/ml adenosine deaminase (Sigma) and 3 nM [3H]-cyclohexyladenosine (specific activity: 29 Ci/mmol; NEN Life Science Products, Zaventem, Belgium) to the Tris/HCl buffer. Sections were incubated in the incubation solution for 90 min. The incubation was followed by rinsing 3 times for 2 min in ice cold Tris/HCl buffer and once for 5 sec in ice cold destilled water. Rinsing was followed by a drying step in cool air stream. Then slides were desiccated with silica gel (Merck) overnight. On the next day, sections were placed in cassettes with standard autoradiographic [3H] micro-scales (Amersham, Roosendaal, The Netherlands) and Hyperfilm-3H film (Amersham) was placed on them. After an exposition time of 10 weeks, films were developed according to standard photographical procedures (Kodak D-19 developer containing paramethylaminophenol sulphate, sodium sulphite, hydroquinone, sodium carbonate, citric acid and potassium metasulphate in destilled water, and fixative containing 30% w/v sodium thiosulphate in destilled water).
Adenosine 1A receptor-activated G-protein autoradiography

The in vitro autoradiography of adenosine A1 receptor-associated G-proteins was performed according to published methods with modifications (Sim et al., 1997; Laitinen, 1999; Moore et al., 2000; Hensler and Durgam, 2001). Brain sections were preincubated in the assay buffer containing 3 mM MgCl₂ (Merck), 100 mM NaCl (Sigma), and 0.2 mM EGTA (Merck) in Tris/HCl (50 mM, pH 7.4, Merck) for 15 min. Then, sections were incubated in the assay buffer containing 2 mM GDP (Sigma) and 9.5 mU/ml adenosine-deaminase (EC 3.5.4.4, type VI from calf intestinal mucosa, Sigma) for 30 min. After this, sections were incubated in the assay buffer, to which 2 mM GDP, 9.5 mU/ml adenosine-deaminase, 40 pM [³⁵S]GTPγS (specific activity: 1068 μCi/nM; Amersham), and 0.1 μM CPA (Sigma) was added for 90 min. In order to assess basal binding, CPA was omitted from the incubation buffer on some of the slides. After the 90-min incubation, slides were rinsed 3 times for 1.5 min in Tris/HCl buffer (50 mM, pH 7.4) and once for 5 sec in ice cold distilled water. Rinsing was followed by a drying step in cool air stream. Then, slides were desiccated with silica gel (Merck) overnight. On the next day, sections were placed in cassettes with standard autoradiographic [¹⁴C] micro-scales (Amersham) and Kodak BioMax films (Amersham) were placed on them. After an exposition time of 96 h at room temperature, films were developed according to standard photographic procedures (Kodak D-19 developer and fixative).

Data analysis and statistics

Autoradiograms were analysed with a computer-assisted image analysis system (Quantimet 500; Leica, Cambridge, England) by an experimenter who was unaware of the group assignment of animals. Binding was measured in forebrain areas including prefrontal cortex, septum, striatum, hypothalamus, CA1 and CA3 subregions of the hippocampus, dentate gyrus of the hippocampus and somatosensory cortex. After background correction, the optical density of [³H]-cyclohexyladenosine and [³⁵S]GTPγS binding was determined according to calibration curves that were generated with the aforementioned autoradiographic scales. Data were analysed with one-way ANOVA.

In the case of the serotonergic challenges, body temperature data between 30 min preceding the injection and 90 min after the injection were used. For the more prolonged CPA-induced hypothermic response data between 30 min preceding the injection and 180 min after the injection were processed. Body temperature data were subjected to analysis of variance (ANOVA) with repeated measures and the post hoc Tukey test. The level of significance was set to p=0.05. Data are expressed as group averages ± SEM.

RESULTS

Adenosine A1 receptor desensitization after chronic CPA injections

In response to the first injection of CPA, the body temperature of the rats decreased approximately 2.5°C within 50-60 min and only returned to baseline after about 180 min (Fig. 1). This hypothermic response was accompanied by an almost complete reduction of motor activity (data not shown). The consecutive daily injections with CPA resulted in a gradually diminished hypothermic response, suggesting a desensitization of the adenosine A1 receptor system (Fig. 1). ANOVA with repeated measures revealed a significant treatment effect (F(180,1224)=8.787; p<0.001). After all CPA injections, the body temperature response was different from the temperature after saline injection (Tukey, p<0.001 in each case). The temperature responses to CPA progressively diminished over the days, with the responses on day 3 to 5 being significantly smaller than the responses on the first two days.
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Figure 1. Chronic CPA injections desensitize the adenosine A1 receptor population. Rats were injected with CPA or saline on 5 consecutive days. The repeated daily injections with CPA resulted in a gradual desensitization of the adenosine A1 receptor population. All the CPA-induced hypothermic responses were significantly different from the saline-induced hyperthermia. Between temperature responses on day 1 and 2, no significant difference was found. The hypothermic response on day 2 significantly differed from that one on day 3. The temperature responses on day 3, 4, and 5 were not significantly different from each other.

Figure 2. Desensitization of the adenosine A1 receptor population does not change serotonin-1A receptor sensitivity. After the daily CPA injections for 5 days, rats were injected with the serotonin-1A receptor agonist 8-OH-DPAT (0.25 mg/kg). The injection of 8-OH-DPAT resulted in an immediate drop in body temperature. The 8-OH-DPAT-induced hypothermic response was not significantly different between rats that received 5 CPA injections and those that received saline injections.

Serotonin-1A receptor challenge
To test whether the desensitization of adenosine A1 receptors affects serotonin-1A receptor signalling, we measured the acute hypothermic response to the serotonin-1A agonist 8-OH-DPAT after desensitization of adenosine A1 receptors. The injection of 8-OH-DPAT caused an immediate hypothermia that reached its lowest value within 20-30 min, approximately 2°C below baseline temperature. Body temperature values returned to baseline within 90 min after the injection. The hypothermic response did not differ between rats that received CPA or saline for five consecutive days, suggesting that the desensitization of the adenosine A1 system had not affected the serotonin-1A signalling pathway (Fig. 2).

Adenosine A1 receptor autoradiography
Adenosine A1 receptor autoradiography was carried out in order to establish whether chronic treatment with CPA results in alterations in A1 receptor numbers in the brain (Fig. 3A). Binding was significantly reduced in the somatosensory cortex \(F(1,15)=7.39, p=0.016\), the CA1 \(F(1,15)=4.72, p=0.037\) and CA3 subfields \(F(1,15)=6.98, p=0.013\) as well as the dentate gyrus of the hippocampus \(F(1,15)=9.05, p=0.005\). Adenosine A1 receptor binding was somewhat lower in most other brain areas that we examined but these differences did not reach statistical significance (Fig. 4).
Cross-talk between serotonin and adenosine receptors

Adenosine A1 receptor-coupled G-protein autoradiography

To examine the amount of A1 receptor-associated inhibitory G-proteins, we carried out G-protein assays after CPA stimulations of brain sections (Fig. 3B). The results show that the binding of radioactively labelled GTP was slightly higher in most brain areas examined, but the increase reached the level of statistical significance only in the somatosensory cortex ($F_{(1,16)}=5.78$, $p=0.029$) (Fig. 5).

Figure 3. Representative autoradiograms of brain sections labelled for adenosine A1 receptors with $[^{3}H]$cyclohexyladenosine [A] and for the adenosine A1 receptor-activated G-proteins with $[^{35}S]$GTPyS [B]. Scale bar: 4 mm.

Adenosine A1 receptor auto-radiography

Chronic treatment with CPA resulted in a significant reduction of $[^{3}H]$cyclohexyladenosine binding to the adenosine A1 receptor (expressed as nCi/mg tissue) in the CA1 and CA3 regions and the dentate gyrus of the hippocampus, and in the somatosensory cortex. Alterations in binding were not significant in other brain areas examined. Abbreviations: PFX, prefrontal cortex; MS, medial septum; Hypo, hypothalamus; CA1 and CA3, cornu Ammonis 1 and 3; DG, dentate gyrus; SOMX, somatosensory cortex.

Figure 4. Adenosine A1 receptor autoradiography. Chronic treatment with CPA resulted in a significant reduction of $[^{3}H]$cyclohexyladenosine binding to the adenosine A1 receptor (expressed as nCi/mg tissue) in the CA1 and CA3 regions and the dentate gyrus of the hippocampus, and in the somatosensory cortex. Alterations in binding were not significant in other brain areas examined. Abbreviations: PFX, prefrontal cortex; MS, medial septum; Hypo, hypothalamus; CA1 and CA3, cornu Ammonis 1 and 3; DG, dentate gyrus; SOMX, somatosensory cortex.
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The present study shows that chronic treatment with the adenosine A1 receptor agonist CPA leads to a significant reduction in the amount of adenosine receptors in various brain regions, particularly the dentate gyrus and CA1-3 subfields of the hippocampus as well as the somatosensory cortex. The reduction in A1 receptor numbers was paralleled by an increase in A1 receptor-linked G-protein numbers, which reached statistical significance for the somatosensory cortex. Overall, chronic stimulation of the adenosine A1 receptor system with CPA resulted in a gradually diminishing functional output of this signalling system, as measured by an attenuated hypothermic response to the CPA injections. However, despite of published reports on cross-talk between the adenosine A1 and serotonin-1A receptor system, we found no evidence for a change in the functional sensitivity of the serotonin-1A receptor system in the rats with a desensitized adenosine A1 system. Whereas, the hypothermic response to an adenosine A1 agonist was diminished in the CPA treated rats, the hypothermic response to a serotonin-1A agonist was unaltered.

The results of our study support earlier reports showing in vitro down-regulation of the A1 receptor system in cerebellar granule cells after chronic treatment with the receptor agonists CPA (Hettinger-Smith et al., 1996) and in vitro desensitization of the same receptors in smooth muscle cells by another adenosine receptor agonist, phenylisopropyladenosine (Ciruela et al., 1997). A number of studies have shown that prolonged stimulation of adenosine receptors leads to receptor desensitization by internalization and degradation of receptors or the uncoupling of receptors from their G-proteins (Bohm et al., 1997). Since the CPA-induced decrease of the hypothermic response and the reduced receptor numbers were not associated with significantly altered numbers of A1 receptor-coupled G-proteins in most brain areas, the desensitization perhaps most likely originated at the receptor level and not from beyond (Ferguson and Caron, 1998). Yet, chronic CPA treatment
resulted in increased G-protein numbers in the somatosensory cortex, which may reflect a compensatory mechanisms in response to decreased A1 receptor numbers. In line with this, a recent study has indicated that sleep deprivation in rats, known to increase extracellular adenosine levels, selectively increases the activity of adenosine A1 receptor-activated G-proteins in the frontal and cingulate cortex, while activities in basal forebrain areas remain unaltered (Alanko et al., 2004).

The second aim of the present study was to establish whether the sleep loss-induced functional desensitization of serotonin-1A system that we reported earlier (Roman et al., 2005; 2006), can be mimicked by chronic stimulation of the adenosine A1 receptors. The background for the study came from evidence showing that sleep loss leads to increased adenosine turn-over in the brain (Porkka-Heiskanen et al., 2002). This increased turn-over and accumulation of adenosine possibly results in higher receptor occupation, which affects receptors and further members of the signalling pathways (Yanik and Radulovacki, 1987; Ciruela et al., 1997; Basheer et al., 2001; Alanko et al., 2004). A number of studies have shown that the two receptor systems, adenosine A1 and serotonin-1A, share some elements of the signal transduction pathways and thereby may cross-talk (Zgombick et al., 1989; Neubig, 1994; Luscher et al., 1997; Bakker et al., 2004). Similarly, it has been demonstrated that the adenosine A1 receptor can interact with muscarinic acethylcholine M2, dopamine D1 and GABAergic receptors (Klinger et al., 2002; Fragata et al., 2006). A number of studies indicate that chronic treatment with the adenosine receptor antagonist caffeine can affect serotonin signalling (Shi et al., 1993; Jacobson et al., 1996; Shi and Daly, 1999). Although literature provides some evidence for cross-talk between the two receptor systems, our results do not show that chronic pharmacological adenosinergic stimulation would affect the functional sensitivity of the serotonin-1A receptor system in a way that we reported for sleep restriction. It still may be that CPA treatment has consequences for very specific serotonin-1A receptor-related brain functions that cannot be detected with the read-out used by this study.

In conclusion, our results show that chronic CPA treatment leads to the desensitization of the adenosine A1 receptor system in rats. This loss of functional sensitivity may be due to the loss of receptors on the neuronal cell surfaces rather than changes downstream to the receptors. On the other hand, our present study does not provide evidence for cross-talk between the adenosine and serotonin transmitter systems as measured on the level of functional output. Along these lines, it seems unlikely that the serotonin-1A receptor desensitization found in our earlier study could be due to sleep loss-induced adenosine accumulation.