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Stimulation of $P2Y_1$ Receptors Causes Anxiolytic-like Effects in the Rat Elevated Plus-maze: Implications for the Involvement of $P2Y_1$ Receptor-Mediated Nitric Oxide Production

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The widespread and abundant distribution of P2Y receptors in the mammalian brain suggests important functions for these receptors in the CNS. To study a possible involvement of the P2Y receptors in the regulation of fear and anxiety, the influences of the $P2Y_{1,11,12}$ receptor-specific agonist adenosine 5′-*O*-(2-thiodiphosphate) (ADPβS), the $P2X_{1,3}$ receptor agonist α,β-methylene ATP (α,βmeATP), the unspecific P2 receptor antagonist pyridoxalphosphate-6-azopheny l-2′,4′-disulfonic acid (PPADS), and the specific $P2Y_1$ receptor antagonist N^6-methyl-2′-deoxyadenosine-3′,5′-bisphosphate (MRS 2179) on the elevated plus-maze behavior of the rat were investigated. All tested compounds were given intracerebroventricularly (0.5 μl). ADPβS (50 and 500 fmol) produced an anxiolytic-like behavioral profile reflected by an increase of the open arm exploration. The anxiolytic-like effects were antagonized by pretreatment with PPADS (5 pmol) or MRS 2179 (5 pmol). Both compounds caused anxiogenic-like effects when given alone. Furthermore, the anxiolytic-like effects of ADPβS could be antagonized by pretreatment with the nitric oxide synthase (NOS) inhibitor N^w-nitro-L-arginine methyl ester (L-NAME). In addition, the anxiogenic-like effects of PPADS were reversed by the pretreatment with L-arginine (500 pmol), which is the natural substrate for NOS, but not by D-arginine (500 pmol), which is not. Immunofluorescence staining revealed the presence of $P2Y_1$ receptors on neurons in different brain regions such as hypothalamus, amygdala, hippocampus and the periaqueductal gray. Furthermore, the colocalization of $P2Y_1$ receptors and neuronal NOS (nNOS) on some neurons in these regions could be demonstrated. The highest density of $P2Y_1$- and nNOS-immunoreactivity was detected in the dorsomedial hypothalamic nucleus. Taken together, the present results suggest that $P2Y_1$ receptors are involved in the modulation of anxiety in the rat. The anxiolytic-like effects after stimulation of $P2Y_1$ receptors seem to be in close connection with the related nitric oxide production.
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Keywords: P2 purinoceptors; anxiety; PPADS; MRS 2179; plus-maze; nitric oxide

INTRODUCTION

In previous studies, it has been demonstrated that stimulation of P2 receptors, which belong either to the P2X ligand-gated ion channel family ($P2X_{1-7}$ subtypes) or to the P2Y G protein-coupled receptor family ($P2Y_{1,2,4,6,11,12}$ subtypes) (Ralevic and Burnstock, 1998; Bürnstock, 2001), is involved in the regulation of locomotion as well as in the expression of sensitization and reward (Kittner *et al,* 2001; Krügel *et al,* 2001a). It has been shown that the open field behavior after intra-accumbal injection of the adenosine 5′-triphosphate (ATP) analog 2-methylthio ATP (2-MeSATP) is characterized by an extended period of novelty-induced locomotion and additionally, after a latency time, by an increased exploration of the inner open field areas indicating anxiolytic-like properties of the P2 receptor agonist (Kittner *et al,* 1997). The aim of the present study was to clarify whether P2 receptors are involved in the regulation of fear and anxiety. For this purpose, the influence of the $P2Y_{1,11,12}$ receptor agonist adenosine 5′-*O*-(2-thiodiphosphate) (ADPβS), the $P2X_{1,3}$ receptor agonist α,β-methylene ATP (α,βmeATP), the nonspecific P2 receptor antagonist pyridoxalphosphate-6-azopheny L-2′,4′-disulfonic acid (PPADS), the $P2Y_1$ receptor-specific antagonist MRS 2179, and the combination of the respective agonists and antagonists on the elevated plus-maze behavior of rats was investigated after intracerebroventricular (i.c.v.) injection.

After the stimulation of $P2Y_1$ receptors by the physiological agonist adenosine 5′-diphosphate (ADP) (You *et al,* 1997) as well as by its analog ADPβS (Malmsjö *et al,* 1999;

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Rump *et al,* 1998), an enhanced formation of the free radical gas nitric oxide (NO) was observed. NO is known to be synthesized from L-arginine and oxygen by NO synthase (NOS) in the presence of NADPH, and plays a role in various signal transduction processes in the CNS. Three distinct isoforms of NOS have been identified: neuronal NOS (nNOS)—the isoform predominating in neuronal tissue, inducible NOS (iNOS)—the inducible isoform found in many cells and tissues, and the endothelial NOS (eNOS)—the isoform located in vascular endothelial cells (Alderton *et al,* 2001). It has been demonstrated that extracellular ATP induces a rise in cyclic GMP level, which is caused by Ca^{2+}-activated formation of NOS (Reiser, 1995). Therefore, any interaction between the P2 receptor-mediated signaling pathway and the NO system is of particular interest. There is growing evidence that NO is involved in the regulation of anxiety, although some controversial results have been reported. On the one hand, the NOS inhibitor N^g-nitro-L-arginine (L-NOARG) abolished the anxiolytic-like effects of NO (Caton *et al,* 1994) as well as of chlordiazepoxide (Quock and Nguyen, 1992), and the NOS inhibitor N^w-nitro-L-arginine methyl ester (L-NAME) produced anxiogenic-like effects in the rat elevated plus-maze (Vale *et al,* 1998). On the other hand, an anxiolytic-like action of L-NAME has also been reported in the same model (Volke *et al,* 1995).

The present study aimed to clarify whether the P2Y$_1$ receptor-mediated effects on anxiety are correlated with an enhanced availability of NO. Therefore, the influence of L-NAME and L-arginine on the P2Y$_1$ receptor-mediated effects on anxiety was studied. To clarify the possibility of a colocalization of P2Y$_1$ receptors and nNOS at the same neurons as a condition for a direct relation between P2Y$_1$ receptor stimulation and NO release, immunohistochemical studies were carried out in relevant brain regions, for example, the hypothalamus, amygdala, hippocampus, and periaqueductal gray, which are involved in the regulation of fear and anxiety.

MATERIALS AND METHODS

Animals

Adult male Wistar rats (WIST/Lei) weighing 300–320 g were used. The animals were housed under standardized humidity, temperature, and lighting conditions with a 12-h/12-h light/dark cycle (lights on at 7.00 am) and had free access to water and food. The animals were housed four or five per cage before and individually after surgery. The experiments were approved by the Committee on Animal Care and Use of the relevant local governmental body according to the German guidelines (BGBl.I p.1105) revised in 1998. All efforts were made to minimize the number of animals used and their suffering.

Drugs

All drugs were diluted and applied in artificial cerebrospinal fluid (aCSF; 126 mM NaCl, 2.5 mM KCl, 1.2 mM NaH_2PO_4, 1.3 mM $MgCl_2$, and 2.4 mM $CaCl_2$; pH 7.4). ADPβS and α,βmeATP were obtained from Sigma-Aldrich (Chemie GmbH, Deisenhofen, Germany). (L-NAME), L- and D-arginine were purchased from Tocris Cookson Ltd, (Bristol, UK) and N^6-methyl-2′-deoxyadenosine-3′,5′-bisphosphate (MRS 2179) from RBI (Natick, MA, USA).

Surgery and i.c.v. Injections

Rats were anesthetized with a combination of ketamine hydrochloride (100 mg/kg, i.p.; Ketanest®, Ratiopharm, Ulm, Germany) and xylazine hydrochloride (5 mg/kg, i.p.; Rompun®, Bayer, Leverkusen, Germany). Following placement in a stereotaxic frame, they were implanted with a 24-gauge guide cannula (bioflow catheters; Vygon, Ecouen, France). To avoid a permanent injury of the ventricle, the implanted guide cannula was placed immediately above it. The stereotaxic coordinates according to Paxinos and Watson (1986) were: AP = −1 mm rostral to bregma, ML = +1.5 mm lateral to the sagittal suture, DV = −2.5 mm mm below the surface of the hemisphere. The cannula was embedded in a socket mounted on the skull in dental acrylic cement (Technovit® 3040; Heraeus Kulzer, Wehrheim, Germany) and additionally fixed by two stainless-steel screws. After surgery, the animals were treated with benzyl penicillin (200.000 IE, i.m.; Retacillin compositum®, Jenapharm, Jena, Germany) for antibiotic prophylaxis and were allowed to recover for 6 days by individually housing before the maze exposure. The individually harbored animals showed only a slight, but no significant, tendency to higher anxiety-like behavior in comparison with the grouped, housed control animals.

The i.c.v. injections were made in freely moving rats, which were habituated to the injection procedure 2 or 3 days before the beginning of the experiments. Injection cannulae (26 gauge) connected to 10 μl syringes via PE-20 tubing were inserted into the guide cannula and protruded 1.5 mm beyond its end to reach the ventricle. All compounds were infused in a volume of 0.5 μl over 3 min using a microinfusion pump (TSE GmbH, Bad Homburg, Germany). The injection cannula was left in place for an additional minute after the application to allow diffusion of the solution. The purinergic agonists ADPβS and α,βmeATP, the P2Y$_1$ receptor antagonist MRS 2179 as well as L- and D-arginine were administered 5 min before the maze performance. The P2 receptor antagonist PPADS and L-NAME were injected 15 and 30 min, respectively, before the start of the maze exposure. Animals that received only antagonists or agonists got the vehicle as second injection. For histological evaluation, the animals received an i.c.v. injection of methylene blue and were killed by decapitation after completion of the experiments. Only rats showing the appropriate injection site were used for data analysis.

Elevated Plus-Maze

The elevated plus-maze is a widely used and extensively validated animal model of anxiety based on the natural aversion of rodents for open spaces and on the elevation of the maze (Handley and Mithani, 1984; Pellow *et al,* 1985).

The apparatus was made of wood with a black rubber floor. It consisted of two open arms (50 × 10 cm) and two enclosed arms (50 × 10 cm) with walls of 40 cm height, elevated 50 cm above the ground. The arms of the same type were opposite to each other, connected by an open central

area (10×10 cm). A camera was mounted vertically above the maze, and the behavior was scored from a monitor in an adjacent room. The investigation room was illuminated with a light intensity of 500 lx, resulting in a brightness of 650 lx at the open and 200 lx at the enclosed arms of the maze. At the beginning of the experiment, rats were placed in the center of the maze, facing the enclosed arms, and were observed for 10 min. Each animal was tested only once. Eight rats were tested at each dose for each compound. All tests were carried out between 0800 and 1300 h. The maze was thoroughly cleaned between each test. An increase in the percentage of time spent on the open arms (open $\times$ 100/open+enclosed) and in the percentage of open arms entries (open $\times$ 100/total entries) is interpreted as an anxiolytic-like response (Pellow *et al,* 1985), whereas the number of entries into enclosed arms provides a measure of general activity (File, 1991).

Double Immunofluorescence

The rats were transcardially perfused under thiopental sodium anesthesia with paraformaldehyde (2%) in sodium acetate buffer (pH 6.5) followed by paraformaldehyde (2%)/glutaraldehyde (0.1%) in sodium borate buffer (pH 8.5). Serial sections (50 μm thick) from the dorsomedial hypothalamic nucleus, the basomedial nucleus amygdala, the dorsal hippocampus (Figure 1) as well as from the periaqueductal gray (not shown) were obtained by using a vibratome (TSE, Bad Homburg, Germany) and collected as free-floating slices in 0.1 M Tris (pH 7.6).

$P2Y_1$-immunofluorescence was performed as previously described (Franke *et al,* 2001). Briefly, after washing with Tris buffered saline (TBS, 0.05 M; pH 7.6) and blocking with 5% fetal calf serum (FCS) in TBS, the slices were incubated in an antibody mixture of rabbit anti-$P2Y_1$ receptor antibody (1 : 1500, SmithKline Beecham Pharmaceuticals, Hertord shire, UK) and mouse anti-NOS (NOS1, 1 : 1000, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) with 0.1% Triton X-100 in 5% FCS in TBS for 48 h at 4°C. Subsequently, the slices were incubated with the two secondary antibodies Cy3-conjugated goat anti-rabbit IgG (1 : 800; Jackson Immuno Research) and Cy2-conjugated goat anti-mouse IgG (1 : 400; Jackson Immuno Research, Baltimore, USA) in 5% FCS in TBS for 2 h. After intensive washing and mounting on slide glasses, all stained sections were dehydrated in a series of graded ethanol, processed through *n*-butylacetate, and covered with entellan (Merck, Darmstadt, Germany). Control experiments were carried out without primary antibodies.

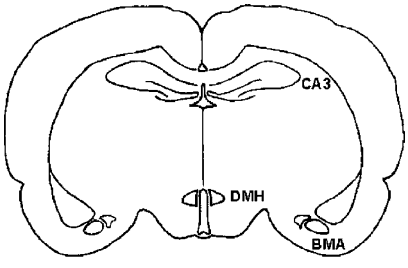

Figure 1 Schematic representation of the dorsomedial hypothalamus (DMH), the basomedial amygdala (BMA) and the dorsal hippocampus (for example the CA3 region) of the rat, characterizing the areas in which $P2Y_1$ receptor/ nNOS double-labelled cells were found.

Confocal Microscopy

The double immunofluorescence was investigated by a confocal laser scanning microscope (LSM 510, Zeiss, Oberkochen, Germany) equipped with an argon laser emitting at 488 nm and a helium/neon laser emitting at 543 nm. The two reaction products were distinguished by their different fluorescence: nNOS by the green Cy2 immunofluorescence and the $P2Y_1$ receptor subtype by the red Cy3 immunofluorescence.

Statistics

All results were expressed as means $\pm$ SEM ($n = 8$). In experiments using the elevated plus-maze, the percentage of time spent on the open arms (open $\times$ 100/open+enclosed) and the percentage of open arms entries (open $\times$ 100/total entries) were calculated for each animal. Additionally, the number of enclosed arm entries was recorded. To evaluate statistical differences in the elevated plus-maze experiments, one-way analysis of variance (ANOVA) was used followed by the Student–Newman–Keuls test for multiple comparisons. A probability level of $p < 0.05$ was considered to be statistically significant.

RESULTS

Influence of ADPβS on the Rat Elevated Plus-Maze Behavior

ADPβS had a significant dose-related anxiolytic-like effect, shown by an increase in the percentage of time spent on the open arms ($F(5,47) = 25.6$, $p < 0.001$) (Figure 2) and the

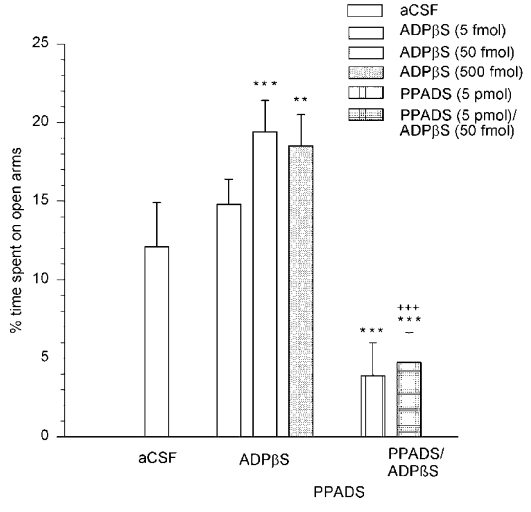

Figure 2 Means $\pm$ SEM percentage of time spent on the open arms in the elevated plus-maze of rats. The rats were tested 5 min after i.c.v. administration of ADPβ in various doses and 15 min after application of PPADS in comparison with vehicle (aCSF)-treated controls. $^{**}p < 0.02$, $^{***}p < 0.001$ compared with vehicle-treated controls, $^{+++}p < 0.001$ compared with the ADPβS (50 fmol) group, Student–Newman–Keuls test after one-way ANOVA.

Table 1 Mean ± SEM Percentage of Entries into Open Arms and Number of Enclosed Arm Entries made by Rats Tested in the Elevated Plus-Maze 5 min after i.c.v. Administration of ADPβS, α,βmeATP, or MRS 2179, respectively, and 10 min after PPADS

Treatment	% Open arm entries	Enclosed arm entries
aCSF	16.1 ± 1.3	16.7 ± 1.2
ADPβS		
5 fmol	16.9 ± 1.7	19.3 ± 1.1
50 fmol	21.6 ± 1.9*	16.6 ± 1.5
500 fmol	22.2 ± 1.5*	13.6 ± 1.1^{+}
PPADS 5 pmol	6.6 ± 1.6***	15.4 ± 1.2
PPADS/ADPβS 5 pmol/50 fmol	7.1 ± 1.8^{***+++}	14.5 ± 1.1
aCSF	13.9 ± 1.4	15.2 ± 1.8
ADPβS 50 fmol	18.2 ± 1.6*	15.9 ± 2.1
MRS 2179 5 pmol	7.4 ± 2.1*	17.2 ± 2.4
MRS 2179/ADPβS 5 pmol/50 fmol	8.6 ± 2.5^{*+}	16.5 ± 1.9
aCSF	21.8 ± 1.4	13.5 ± 2.4
α,βmeATP		
0.05 nmol	18.2 ± 2.2	11.6 ± 1.9
0.5 nmol	18.9 ± 1.3	12.4 ± 2.0
5 nmol	18.9 ± 1.2	14.6 ± 1.4

$^{*}p<0.05$, $^{***}p<0.001$ compared with the respective vehicle-treated control, $^{+}p<0.05$, $^{+++}p<0.01$ compared with 50 fmol ADPβS, Student–Newman–Keuls test after one-way ANOVA.

increased percentage number of open arm entries ($F(5,47)=18.5$, $p<0.05$) (Table 1). The anxiolytic-like effect of 50 fmol ADPβS was antagonized by 5 pmol PPADS ($p<0.001$). PPADS alone produced anxiogenic-like effects, indicated by a decreased percentage of time spent on open arms ($p<0.001$) and number of open arm entries ($p<0.01$) (Table 1). None of the drugs had a significant effect on the number of enclosed arms entries in comparison with the aCSF control group ($F(5,47)=2.5$). Significant differences were found only at 500 fmol ADPβS in comparison with the lower doses of 5 and 50 fmol of this compound ($p<0.05$) (Table 1).

Furthermore, the influence of the P2Y$_1$-receptor antagonist MRS 2179 (5 pmol) on the effects of 50 fmol ADPβS was studied. After pretreatment with MRS 2179, the anxiolytic-like effects of ADPβS (50 fmol) were abolished ($F(3,31)=38.2$, $p<0.001$). MRS 2179 (5 pmol) alone exerted anxiogenic-like properties ($p<0.001$) (Figure 3; Table 1).

Influence of α,βmeATP on the Rat Elevated Plus-Maze Behavior

α,βmeATP at all tested doses (0.05, 0.5, and 5 nmol) had no significant effect on the percentage of time spent on the open arms ($F(3,31)=0.65$, $p=0.59$) (Figure 4) as well as on the percentage of entries into the open arms ($F(3,31)=2.5$, $p=0.084$) (Table 1). Furthermore, the ANOVA for the number of enclosed arm entries revealed no significant effect of α,βmeATP on the general locomotor activity ($F(3,31)=0.27$) (Table 1).

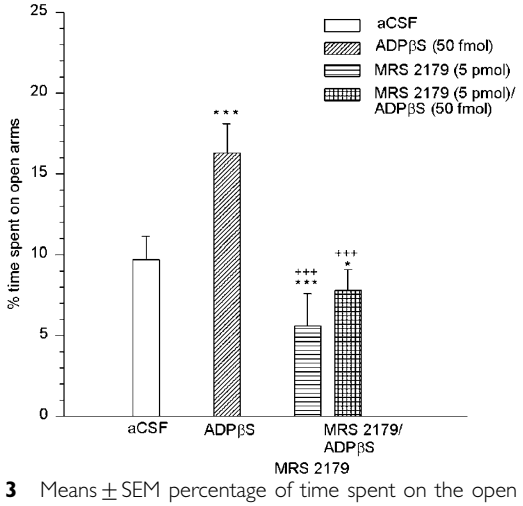

Figure 3 Means ± SEM percentage of time spent on the open arms in the elevated plus-maze of rats. The rats were tested 5 min after i.c.v. administration of ADPβS and 10 min after pretreatment with MRS 2179 in comparison with vehicle (aCSF)-treated controls. $^{*}p<0.05$, $^{***}p<0.001$ compared with vehicle-treated controls, $^{+++}p<0.001$ compared with the ADPβS group, Student–Newman–Keuls test after one-way ANOVA.

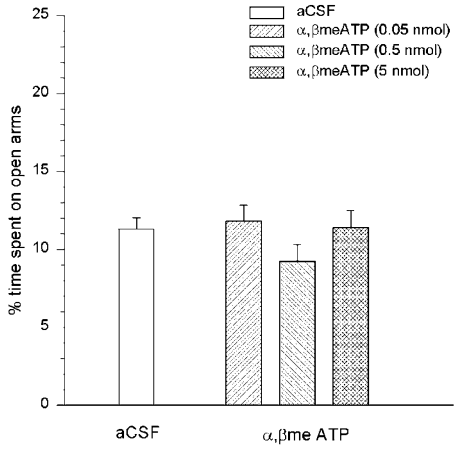

Figure 4 Means ± SEM percentage of time spent on the open arms in the elevated plus-maze of rats. The rats were tested 5 min after i.c.v. administration of α,βmeATP in various doses in comparison with vehicle (aCSF)-treated controls.

Influence of L-NAME on the Rat Elevated Plus-Maze Behavior

As shown in Figure 5, L-NAME (0.05–5 nmol) produced a dose-dependent decrease in the percentage of time spent on the open arms ($F(5,47)=19.2$, $p<0.001$). The decrease in the percentage of open arms entries reaches statistical significance at 5 nmol L-NAME ($F(5,47)=6.5$, $p=0.001$). The number of enclosed arm entries was unaltered at all tested doses ($F(5,47)=1.3$, $p=0.28$) (Table 2). The effects of L-NAME (0.5 nmol), were abolished by the pretreatment with L-arginine (0.5 nmol), indicating that the anxiogenic-like effect of L-NAME is caused by a decreased NO synthesis. L-Arginine alone (0.5 nmol) caused an increase in the percentage of time spent on the open arms ($p<0.05$) (Figure 5).

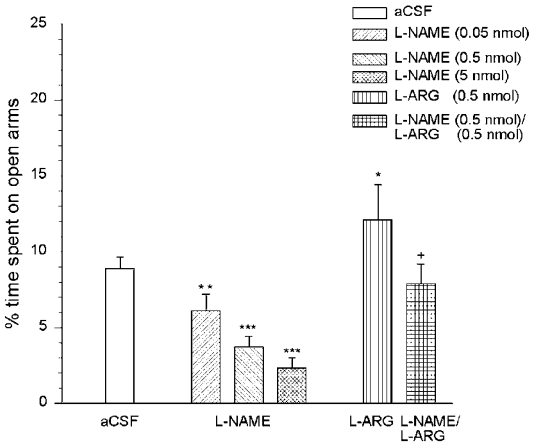

Figure 5 Means ± SEM percentage of time spent on the open arms in the elevated plus-maze of rats. The rats were tested 30 min after i.c.v. administration of L-NAME in various doses in comparison with vehicle (aCSF)-treated controls and 5 min after L-arginine (L-ARG) with and without pretreatment with L-NAME (0.5 nmol). $^{*}p<0.05$, $^{**}p<0.02$, $^{***}p<0.001$ compared with the vehicle-treated controls, $^{+}p<0.05$ compared with the L-NAME (0.5 nmol) group, Student–Newman–Keuls test after one-way ANOVA.

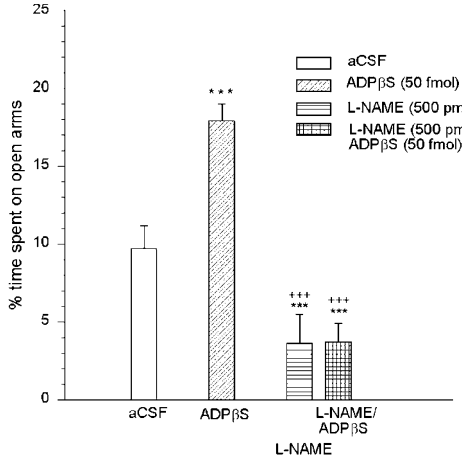

Figure 6 Means ± SEM percentage of time spent on the open arms in the elevated plus-maze of rats. The rats were tested 5 min after i.c.v. administration of ADPβS or 30 min after L-NAME in comparison with vehicle (aCSF)-treated controls. $^{***}p<0.001$ compared with vehicle-treated controls, $^{+++}p<0.001$ compared with the ADPβS group, Student–Newman–Keuls test after one-way ANOVA.

Influence of the Pretreatment with L-NAME on the Anxiolytic-Like Effect of ADPβS

The pretreatment with L-NAME (0.5 nmol) antagonized the anxiolytic-like effect of ADPβS (50 fmol) with respect to the percentage of time spent on the open arms ($F(3,31)=41.2$, $p<0.001$) (Figure 6). The percentage of open arm entries was also decreased by the pretreatment with L-NAME ($F(3,31)=3.48$, $p=0.029$) (Table 2) in comparison with the aCSF and the ADPβS group. The ANOVA revealed that the general locomotor activity was not changed in the different groups ($F(3,31)=0.89$, $p=0.45$) (Table 2).

Table 2 Mean ± SEM Percentage of Entries into Open Arms and Number of Closed Arm Entries Made by Rats Tested in the Elevated Plus-Maze 30 min after i.c.v. Administration of L-NAME, 10 min after PPADS, and 5 min after ADPβS or L- or D-Arginine

Treatment	% Open arm entries	Enclosed arm entries
aCSF	16.4 ± 1.3	14.6 ± 0.6
L-NAME		
0.05 nmol	13.0 ± 2.1	12.8 ± 1.4
0.5 nmol	11.4 ± 0.8	14.9 ± 1.1
5.0 nmol	6.7 ± 1.9*	13.3 ± 1.4
L-NAME/L-Arginine 0.5 nmol/500 pmol	14.1 ± 2.2	15.1 ± 1.9
L-Arginine 500 pmol	17.4 ± 1.7	17.0 ± 2.8
aCSF	17.5 ± 1.5	14.1 ± 1.8
ADPβS 50 fmol	20.6 ± 1.9	17.1 ± 2.2
L-NAME/ADPβS 0.5 nmol/50 fmol	15.7 ± 1.6^{+}	15.4 ± 1.3
L-NAME 0.5 nmol	14.6 ± 1.7^{+}	14.3 ± 1.7
aCSF	19.2 ± 1.4	14.9 ± 2.1
PPADS 5 pmol	8.8 ± 1.7**	14.5 ± 2.7
PPADS/L-Arginine 5 pmol/500 pmol	28.2 ± 1.9^{++}	17.2 ± 2.9
PPADS/D-Arginine 5 pmol/500 pmol	9.6 ± 0.9^{**++}	16.3 ± 2.0
L-Arginine 500 pmol	23.2 ± 2.7**	15.6 ± 1.7
D-Arginine 500 pmol	17.3 ± 1.7	12.8 ± 1.8

$^{*}p<0.05$, $^{**}p<0.02$ compared with the respective vehicle-treated control, $^{+}p<0.05$, $^{++}p<0.02$ compared with ADPβS (50 fmol) or PPADS (5 pmol), respectively, Student–Newman–Keuls test after one-way ANOVA.

Influence of the Pretreatment with L- or D-Arginine on the Anxiogenic-Like Effect of PPADS

The pretreatment with L-arginine (500 pmol) completely antagonized the anxiogenic-like effect of PPADS (5 pmol) with respect to the percentage of time spent on the open arms ($F(5,47)=10.5$, $p=0.006$) (Figure 7) as well as to the open arm entries ($F(5,47)=18.6$, $p<0.001$) (Table 2), whereas the pretreatment with D-arginine was without influence on the PPADS effect. L-Arginine alone showed anxiolytic-like properties indicated by an increase of the percentage of time spent on the open arms ($p=0.014$) and the open arm entries ($p=0.014$). D-Arginine was without effect on any of the measured parameters in the plus-maze ($p=0.99$). The general locomotor activity was not affected by either of the treatment regimens.

Immunohistochemistry

Immunohistochemical staining revealed the presence of P2Y$_1$ receptors on neurons in relevant brain regions such as hypothalamus, amygdala, hippocampus, and periaqueductal gray, which are known to be involved in the regulation of anxiety and fear. Especially the labeling of the dorsomedial nucleus of the hypothalamus shows a high density of P2Y$_1$-immunoreactivity (IR) (Figure 8b and e). A weakly expressed P2Y$_1$ IR was found in the basomedial nucleus of the amygdala (Figure 8h) and the dorsal hippocampus, for example in the CA3 region (Figure 8k). In the basolateral nucleus of the amygdala and the periaqueductal gray, only scarce P2Y$_1$-IR was detected (data not shown).

The labeling of nNOS reveals a high density in the dorsomedial hypothalamus (Figure 8a and d). The expres-

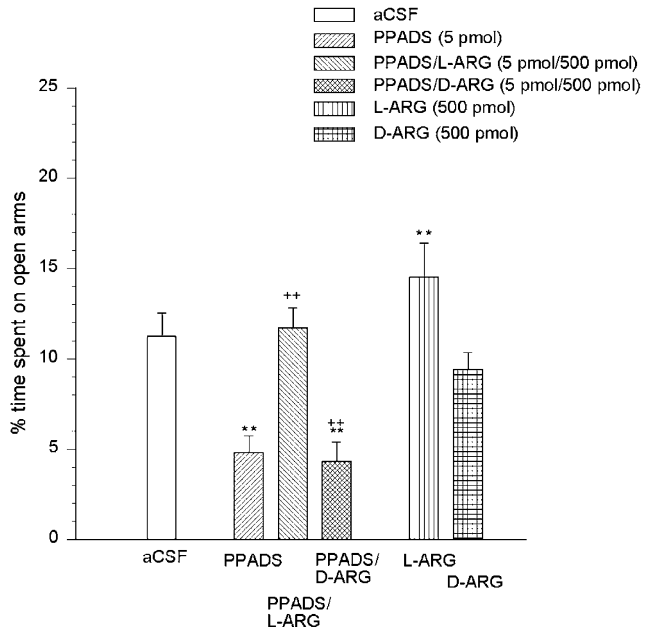

Figure 7 Means ± SEM percentage of time spent on the open arms in the elevated plus-maze of rats. The rats were tested 15 min after i.c.v. administration of PPADS or 5 min after L- or D-arginine (L-, D-ARG) in comparison with the vehicle (aCSF)-treated controls. $^{**}p < 0.02$ compared with vehicle-treated controls, $^{++}p < 0.02$ compared with the PPADS group, Student–Newman–Keuls test after one-way ANOVA.

sion of nNOS-IR in the basomedial nucleus amygdala and the hippocampal CA3 region (Figure 8g and j) was considerably lower. The colocalization of P2Y$_1$-IR and nNOS-IR at various neurons in the studied regions is documented in Figure 8(f, i, l).

DISCUSSION

The metabolically stable P2 receptor agonist ADPβS, preferential for adenine nucleotide-sensitive P2Y$_1$, P2Y$_{11}$, and P2Y$_{12}$ receptors, produced anxiolytic-like effects in the rat elevated plus-maze after i.c.v. administration at doses between 5 and 500 fmol. Both the percentage of time spent on the open arms and the percentage of open arm entries were increased by this ADP analog. The highest tested dose of ADPβS (500 fmol) significantly decreased the enclosed arm entries that were used as an indicator of drug-induced changes of general locomotor activity. It has been shown that the effects of compounds on the elevated plus-maze behavior such as amphetamine were confounded by an increase of general activity (Dawson *et al,* 1995). In the turned around sense, a decreased locomotion could be responsible for the tendency to a weaker anxiolytic-like effect after 500 fmol ADPβS in comparison with 50 fmol. An attenuated locomotor response could be a result of a predominant activation of presynaptic dopamine D2 autoreceptors in the nucleus accumbens or the striatum (Hu and Wang, 1988) after a slightly enhanced dopamine release induced by the ADP analog. Another explanation could be a P2 receptor-mediated glutamate release in the same area (Krügel *et al,* 2001b), which is known to reduce locomotor activity (Schmidt and Kretschmer, 1997). The high potency of ADPβS to produce anxiolytic-like effects agrees with various *in vitro* studies that documented the high affinity of ADPβS to bind to rat brain P2Y$_1$ receptors (Vöhringer *et al,* 2000). It seems unlikely that P2Y$_2$, P2Y$_4$, or P2Y$_6$ receptors are involved in the ADPβS response for the tested doses.

Pretreatment with the P2 receptor antagonist PPADS abolished the ADPβS-induced effects. The anxiogenic-like effects of PPADS alone suggest that an endogenous ATP acting at P2 receptors is involved in the regulation of anxiety and fear. PPADS is a nonselective P2 receptor antagonist. In addition to the blockade of various P2X receptors, PPADS also acts as an effective antagonist at the P2Y$_1$ receptor, whereas it is completely ineffective at the P2Y$_{11}$ receptor (Communi *et al,* 1999). PPADS does not recognize P2Y$_2$ receptors up to a concentration of 30 μM and is also a relatively ineffective antagonist at the uridine nucleotide-sensitive P2Y$_4$ and P2Y$_6$ receptors (Charlton *et al,* 1996; Bogdanov *et al,* 1998; Dol-Gleizes *et al,* 1999). PPADS also fails to block P2Y$_{12}$ receptor-mediated effects (Nicholas, 2001). It has been shown that only P2Y receptors coupled to phospholipase C, but not those negatively coupled to adenylate cyclase, were inhibited by PPADS (Boyer *et al,* 1994). In this view, the anxiolytic-like effect of ADPβS appears to be mediated by an activation of phospholipase C, leading to the production of inositol-1,4,5-trisphosphate (IP$_3$) and to the mobilization of intracellular Ca^{2+} resulting in the stimulation of a variety of signaling pathways such as protein kinase C, phospholipase A$_2$, Ca^{2+}-dependent K^+-channels as well as NOS and subsequent NO formation.

It is thought that neither cell death (Chan and Lin-Shiau, 2001) nor unspecific effects of PPADS such as the inhibition of ectonucleotidase activity (Windscheif *et al,* 1995) or the inhibition of IP$_3$-induced Ca^{2+} mobilization by a nonspecific mechanism (Vigne *et al,* 1996) contribute to the PPADS-mediated effects at the tested dose. Furthermore, it should be emphasized that in contrast to the P2 receptor antagonists suramin and reactive blue 2 no direct glutamate antagonistic properties were found for PPADS (Fröhlich *et al,* 1996; Motin and Bennett 1995; Gu *et al,* 1998). The anxiolytic-like effects of ADPβS were also abolished after pretreatment with MRS 2179, which acts as a specific P2Y$_1$ receptor antagonist (Boyer *et al,* 1998). MRS 2179 alone produced anxiogenic-like effects similar to those caused by PPADS, suggesting that the blockade of P2Y$_1$ receptors is mainly involved in mediating the PPADS-induced anxiety.

No effects were observed after i.c.v. administration of α,βmeATP up to doses of 5 nmol. This ATP analog, which is resistant to enzymatic degradation (Kennedy and Leff 1995), acts on homomeric P2X$_1$ and P2X$_3$ receptors as well as on heteromeric P2X$_4$/P2X$_6$ receptors, but it is known to be inactive at P2Y receptors (Ralevic and Burnstock, 1998; Nörenberg and Illes, 2000). In this view, an involvement of P2X receptors on the regulation of anxiety appears unlikely, although it cannot be completely excluded.

The unspecific NOS inhibitor L-NAME produced dose-dependent anxiogenic-like effects. These results conform with studies of Vale *et al* (1998) after intraperitoneal (i.p.) injection of L-NAME. Anxiogenic-like effects after the inhibition of NOS were also reported by other authors. For example, the NOS inhibitor L-NOARG decreased the exploration of an elevated plus-maze system (De Oliveira *et*

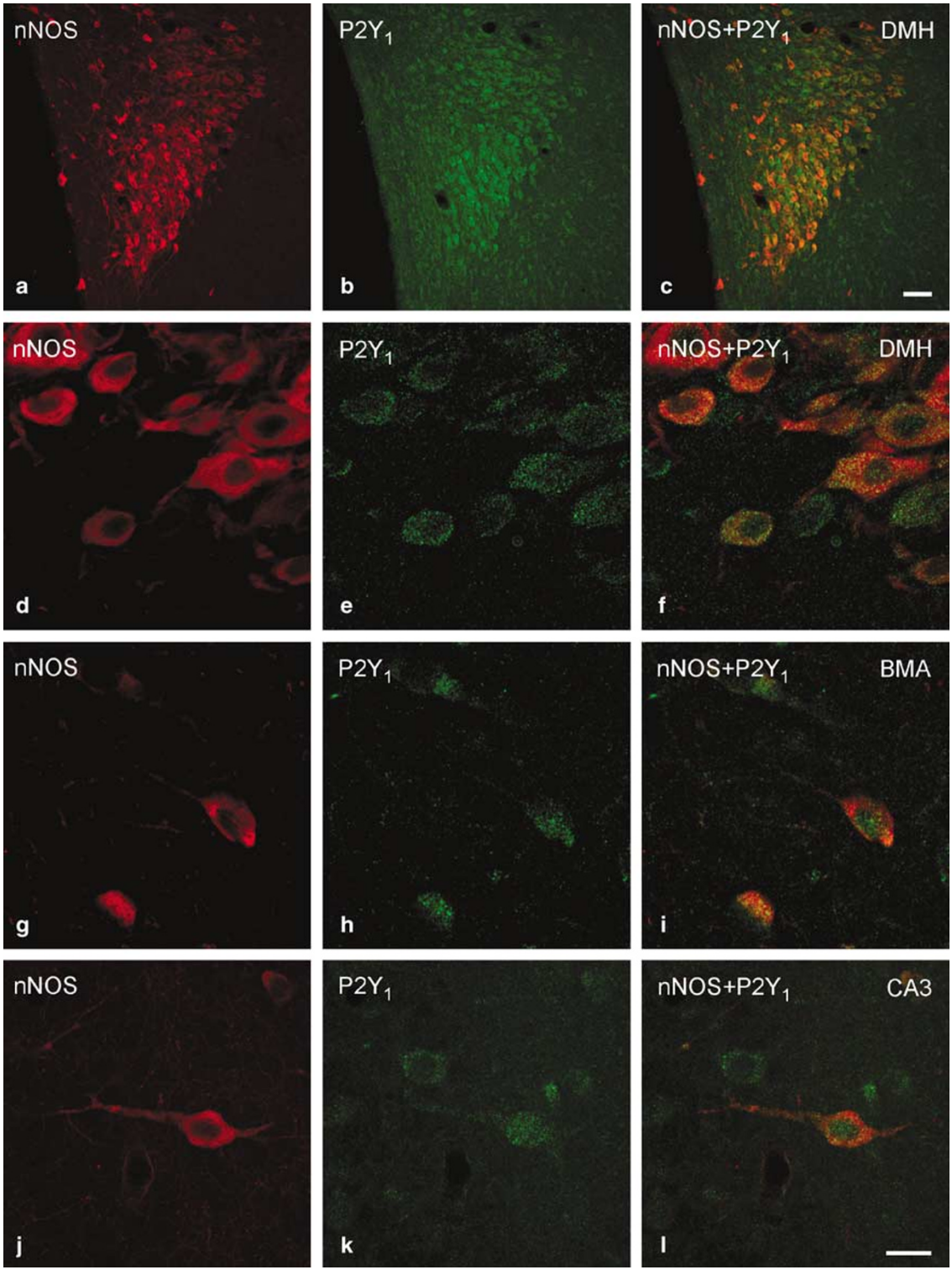

Figure 8 Confocal images of double immunofluorescence of the $P2Y_1$ receptor subtype and nNOS on cells in different brain regions of the rat under normal conditions. (a–c) Dorsomedial hypothalamus (DMH), (d–f) dorsomedial hypothalamus (greater magnification), (g–i) basomedial amygdala (BMA), (j–l) hippocampus (CA_3 region). By color coding Cy3 labelling appears as green ($P2Y_1$ receptor), whereas immunoreactivity for Cy2 is red (nNOS). Scale bar: (a–c): 50 μm; (d–l): 20 μm.

al, 1997) and was able to antagonize the anxiolytic-like effects of NO (Caton *et al,* 1994) as well as of chlordiazepoxide (Quock and Nguyen, 1992). Anxiogenic-like effects were also reported after intra-amygdala or intra-hippocampal injection of L-NOARG (Monzon *et al,* 2001). On the one hand, eNOS-deficient mice showed an increase in anxiety-related behavior in the plus-maze in comparison with the wild-type controls (Frisch *et al,* 2000). On the other hand,

anxiolytic-like effects of L-NAME have been reported in the rat elevated plus-maze after i.p. injections (Volke *et al,* 1995) as well as after administration into the periaqueductal gray area (Guimaraes *et al,* 1994). However, in both studies the anxiolytic-like response occurred only in a small dose range at lower doses, whereas at higher doses anxiogenic-like effects became apparent. In the present study, a weak but significant anxiolytic effect of L-arginine alone was observed, whereas other authors failed to show any anxiolytic effects of L-arginine on the elevated plus-maze (Yildiz *et al,* 2000, Volke *et al*, 1997). Possible reasons for these incompatible findings may consist in a different basal tonus of fear in the animals used and in different application routes.

The discussion of inconsistent results from studies using the elevated plus-maze had also to consider that there are different stages of the elevated plus-maze behavior. At first, a stimulation of anxiety-like behavior may result in a facilitated passive avoidance response. The animals remain in the enclosed arms. A more potent stimulation of anxiety-like behavior may result in a situation in which the animal is motivated to search for an escape route from the maze (Kalynchuk *et al*, 1998). In the present study, the increased anxiety-like behavior after L-NAME as well as after PPADS and MRS 2179 administration resulted in a stimulation of passive avoidance (animals remained in the enclosed arms) without stimulation of the escape behavior.

To avoid the cardiovascular effects of L-NAME, the compound was administered into the lateral ventricle (Rees *et al,* 1990). It has been shown that the i.c.v. administration of L-NAME did not cause any alteration of blood pressure and heart rate (Hamada *et al,* 1995; Chikada *et al,* 2000). Considering the distribution properties of NOS inhibitors after application into the lateral ventricle (Greenberg *et al,* 1997; Salter *et al,* 1995), only regions in close proximity to the ventricle such as the thalamus, the hypothalamus or the striatum are possible targets for mediating the anxiogenic-like effects after L-NAME application.

Although the nNOS is the predominant isoenzyme of NOS in the neuronal tissue, a contribution of eNOS or iNOS to the L-NAME-evoked effects cannot be excluded. The selective nNOS inhibitor 7-nitroindazole is not an alternative to L-NAME in this respect, because of its monoamine oxidase (MAO)-B inhibitory effects (Castagnoli *et al,* 1997; Royland *et al,* 1999). MAO inhibitors were shown to have anxiolytic-like properties in the elevated plus-maze (Griebel *et al,* 1998).

Pretreatment with the NOS inhibitor L-NAME not only led to an expression of enhanced anxiety, but also completely abolished the anxiolytic-like effects of ADPβS, suggesting that these effects are in close relation to enhanced NO formation. Considering the assumption that NOS is stimulated by an increase of intracellular Ca^{2+}, L-NAME acts downstream to the P2 receptor-mediated effects. In fact, ADPβS did not attenuate the L-NAME-induced anxiety.

The influence of the pretreatment with L- and D-arginine on the PPADS-mediated effect was also investigated in this study. When PPADS pretreated rats obtained L-arginine, the anxiogenic-like influence of PPADS on the elevated plus-maze behavior was abolished, while by the treatment with D-arginine the PPADS-mediated effect remained unchanged. In summary, the results of these experiments demonstrate that NO is involved in mediating the behavioral effects of P2Y$_1$ receptor stimulation. Confirming this possibility, the blockade of the P2 receptors by PPADS may lead to a decrease of the NO release, an effect that is synergistic with the action of NOS inhibitors. Further evidence for a close relationship between P2 receptors and NO production is given by a study of Liu *et al* (2000), which shows that pretreatment of astrocytes with P2 receptor antagonists including PPADS results in a down regulation of interleukin-1β-stimulated NOS expression.

The present immunohistochemical data show a distribution of the P2Y$_1$ receptors in the rat brain, which agrees well with that observed in human brain (Moore *et al,* 2000). From CNS areas known to be involved in the regulation of anxiety and fear, the highest density of P2Y$_1$-IR was found in the area of the dorsomedial hypothalamus. Moreover, a considerable part of the neurons in this hypothalamic nucleus showed a coexpression of P2Y$_1$ receptor- and nNOS-IR. The dorsomedial hypothalamus is associated with various physiological functions such as the regulation of anxiety and the modulation of food intake (Vanhatalo and Soinila, 1998; Shekhar and Keim, 1997). For example, the blockade of GABA$_A$ receptors in the dorsomedial hypothalamus increases corticosterone and ACTH plasma levels (Keim and Shekhar, 1996), whereas lesion of this nucleus causes anxiolytic-like effects (Inglefield *et al,* 1994). In the present study, immunohistochemical staining has demonstrated that P2Y$_1$ receptors and nNOS are colocalized in a population of neurons in the dorsomedial hypothalamus. Therefore, it is conceivable that this area is at least one possible site of action participating in the P2Y$_1$ receptor-mediated effects on fear and anxiety. This assumption is supported by preliminary experiments with infusion of ADPβS (50 fmol) directly into the dorsomedial hypothalamus. The ADPβS-treated animals showed a significant decrease in anxiety, but no changes in locomotor activity (H Kittner, unpublished observation).

An inhibition of the dorsomedial hypothalamus after stimulation of P2Y$_1$ receptors may be mediated by an activation of the NO-cyclic GMP pathway, followed by an opening of ATP-sensitive K^+ channels. P2Y$_1$- and nNOS-IR was found to a lesser extent in the basomedial amygdala and the dorsal hippocampus.

Although only a small number of neurons showing P2Y$_1$- and nNOS-IR was found in the basolateral amygdala and the periaqueductal gray, we cannot reliably exclude an involvement of these structures in the elevated plus-maze behavior. It has been shown that NO release, for example, only from few non-noradrenergic neurons in the locus coeruleus may influence a large population of noradrenergic neurons, which in turn may control the neuronal activity in various brain regions (Xu *et al,* 1998). Nevertheless, it seems unlikely that the basomedial amygdala and the dorsal hippocampus considerably participate in the ADPβS-induced anxiolytic-like response because the basomedial amygdala is mainly involved in mediating feeding and social behavior as well as emotion-related learning (Petrovich *et al,* 1996). The dorsal hippocampus does not seem to play an important role in controlling the behavior during the first exposure to the plus-maze system (Treit and Menard 1997; File *et al,* 1998, 2000).

In conclusion, the results of the present study reveal that $P2Y_1$ receptors are involved in the modulation of anxiety. The anxiolytic-like effects after stimulation of the $P2Y_1$ receptor seem to be in close relationship with the ensuing formation of NO.

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