

Impaired Cognition after Stimulation of P2Y₁ Receptors in the Rat Medial Prefrontal Cortex

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We hypothesize that cortical ATP and ADP accumulating in the extracellular space, eg during prolonged network activity, contribute to a decline in cognitive performance in particular via stimulation of the G protein-coupled P2Y₁ receptor (P2Y₁R) subtype. Here, we report first evidence on P2Y₁R-mediated control of cognitive functioning in rats using bilateral microinfusions of the selective agonist MRS2365 into medial prefrontal cortex (mPFC). MRS2365 attenuated prepulse inhibition of the acoustic startle reflex while having no impact on startle amplitude. Stimulation of P2Y₁Rs deteriorated performance accuracy in the delayed non-matching to position task in a delay dependent manner and increased the rate of magazine entries consistent with both working memory disturbances and impaired impulse control. Further, MRS2365 significantly impaired performance in the reversal learning task. These effects might be related to MRS2365-evoked increase of dopamine observed by microdialysis to be short-lasting in mPFC and long-lasting in the nucleus accumbens. P2Y₁Rs were identified on pyramidal cells and parvalbumin-positive interneurons, but not on tyrosine hydroxylase-positive fibers, which argues for an indirect activation of dopaminergic afferents in the cortex by MRS2365. Collectively, these results suggest that activation of P2Y₁Rs in the mPFC impairs inhibitory control and behavioral flexibility mediated by increased mesocorticolimbic activity and local disinhibition. *Neuropsychopharmacology* (2015) **40**, 305–314; doi:10.1038/npp.2014.173; published online 20 August 2014

INTRODUCTION

In the central nervous system adenosine 5'-triphosphate (ATP) is released in an activity dependent manner from neurons and glia cells into the extracellular space (Bodin and Burnstock, 2001; Pankratov *et al*, 2006; Heinrich *et al*, 2012). Its concentration increases notably under specific physiological states such as neural network oscillations (Khakh *et al*, 2003) but also after psychostimulant drug administration (Pintor *et al*, 1995) or during a variety of pathological conditions such as oxygen-glucose deprivation, epilepsy, neuroinflammation or neuronal injury (Burnstock *et al*, 2011) known to have impact on cognitive functions.

Once released into the extracellular space, ATP together with some of its breakdown products (eg adenosine 5'-diphosphate; ADP) acts at purine receptors (P2Rs) classified into several subtypes of ligand-gated cationic channels (P2X) and G protein-coupled receptors (P2Y) on the basis of their function, pharmacology and molecular cloning

(Abbracchio *et al*, 2009; Fischer and Krügel, 2007). Certain effects of ATP and ADP analogues, locally applied to the ventral striatum or ventral tegmentum, on behaviors in rats are attributed to stimulation of P2YR in mesolimbic circuits, in particular to the P2Y₁R subtype which is expressed, eg on dopamine neurons, and preferentially recognizes ADP over ATP (Waldo and Harden, 2004; Kittner *et al*, 2004; Krügel *et al*, 2001a).

With prolonged network activity (eg associated with performance of a certain cognitive task), levels of ATP and its breakdown products may accumulate in the extracellular space and be responsible for a decline in performance (ie fatigue). Given that local stimulation of P2YR facilitates dopaminergic neurotransmission and P2Y₁R antagonists suppress dopamine in basal ganglia as previously shown, we expected that P2Y₁R will affect dopamine release in cortical areas as well (Chandaka *et al*, 2011; Kittner *et al*, 2004; Krügel *et al*, 2001a). Knowing the importance of cortical dopaminergic signaling for performance of various cognitive tasks, we hypothesized that stimulation of P2Y₁Rs will affect cognitive behaviors, which depend on the prefrontal cortical function (Floresco *et al*, 2009; Arnsten, 2009).

The medial prefrontal cortex (mPFC) has attracted increasing attention because it is responsible for highly specialized complex cognitive functions such as planning of goal-directed actions, problem-solving and predicting forthcoming events, and because of its relevance for clinical cognitive impairment associated with schizophrenia,

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depression, attention deficit hyperactivity disorder or drug addiction (Arnsten and Rubia, 2012; Goldstein and Volkow, 2011; Lüscher and Malenka, 2011). The generation of appropriate goal-directed behavior relies, among others, on the intact interaction of the dorsolateral PFC and anterior cingulate cortex in primates, analogous to the rodent mPFC, with subcortical structures including the ventral tegmental area (VTA), striatum including nucleus accumbens (NAc) and other parts of the mesocorticolimbic circuit (Chudasama, 2011; Seamans *et al*, 2008).

In *ex vivo* and *in vitro* investigations ATP/ADP are important contributors to the regulation of prefrontal cortical activity. Functional postsynaptic P2Y₁Rs were found on layer V pyramidal neurons of rat mPFC (Luthardt *et al*, 2003). In these neurons, activation of P2Y₁R by ATP/ADP inhibits long-term depression (LTD), a synaptic plasticity phenomenon implicated in long-term memory, *via* reduction of Ca²⁺-influx through voltage-sensitive Ca²⁺-channels (Guzman *et al*, 2010). Therefore, in line with our hypothesis, one can expect modulatory effects of P2Y₁Rs on aspects of cognitive and executive functions involving attention, working memory, decision making, and the executive control of behavior, eg cognitive flexibility and adaptation.

To explore whether stimulation of P2Y₁Rs affects such prefrontocortical functions, we investigated behavioral effects of local administration of (*N*)-methanocarba-2Me-SADP (MRS2365), a highly potent selective non-nucleotide P2Y₁R-agonist with an EC₅₀ = 1.2 nM. At concentrations up to 1 and 10 μM, MRS2365 did not activate or inhibited either P2Y₁₂ or P2Y₁₃ receptors, respectively (Chhatiwala *et al*, 2004), nor hints for pharmacological relevant affinity at other targets exist.

Further, using dual-probe microdialysis, we provide evidence that the stimulation of prefrontal P2Y₁Rs by local infusion of MRS2365 influences mesocorticolimbic dopamine as a possible mechanism contributing to its behavioral effects. Immunohistochemical studies provide evidence, that the behavioral and neurochemical effects of MRS2365 may partly be mediated by P2Y₁Rs expressed on GABAergic interneurons.

METHODS AND MATERIALS

Animals

44 male Wistar rats for behavioral experiments and 10 for immunohistochemistry (Janvier Labs, Le Genest Saint Isle, France; 200–300 g body weight) as well 18 male Sprague-Dawley rats for microdialysis (Charles River, Bad Sulzfeld, Germany; 280–380 g body weight) were housed under standard conditions with free access to food and water and a 12 h light–dark cycle with lights on from 7:00 a.m. After implantation of the guide cannulae, animals were housed individually. Two days before training for delayed non-matching to position (DNMTP) and reversal learning tasks, rats were housed individually and restricted to 15 g food per day. Experiments were approved by the local governmental bodies according to the European Communities Council Directive (86/609/EEC) and Animal Welfare Offices (AbbVie Deutschland GmbH & Co KG and Landesdirektion Sachsen, Leipzig).

Bilateral Drug Infusion

MRS2365 [(1*R*,2*R*,3*S*,4*R*,5*S*)-4-[6-Amino-2-(methylthio)-9*H*-purin-9-yl]-2,3-dihydroxybicyclo[3.1.0]hex-1-yl]methyl] diphosphoric acid mono ester trisodium salt; Tocris, Ellisville, U.S.A.) was dissolved in artificial cerebral spinal fluid (ACSF, in mM: 126 NaCl; 2.5 KCl; 1.2 NaH₂PO₄; 1.3 MgCl₂ and 2.4 CaCl₂; pH 7.4). Bilateral guide cannulae implanted into the mPFC (AP = 3.2 mm, L = ± 1.5 mm, V = 3.1 mm relative to bregma at an angle of 15°) and fixed with dental cement (see Supplementary Material) were used to apply a bolus of 0.5 μl MRS2365 (0.05 pmol), or ACSF was infused bilaterally into the mPFC (0.2 μl/min). This dose of MRS2365 was selected by pilot dose-response and antagonist studies in the open field novelty as a primary test for biological activity and confirming the P2Y₁R selectivity (Supplementary Material, Supplementary Figure S2). Latin Square design was applied for pseudo-random order of ACSF and MRS2365 application in PPI (*n* = 14) and DNMTTP (*n* = 10) experiments. At least two training sessions without treatment were performed between drug tests in DNMTTP experiments. In PPI experiments, the second treatment session followed after two days. In the reversal learning task, animals received drug applications immediately prior to the sessions of retention and reversal phase.

Prepulse Inhibition (PPI) of the Acoustic Startle Response (ASR)

PPI was measured in a startle chamber (TSE, Bad Homburg, Germany) equipped with a movement-sensitive piezoelectric platform and two loudspeakers (Schneider *et al*, 2005). After 6 min habituation at continuous background sound pressure level (SPL) of 65 dB, the animals received 15 initial startling pulses of 110 dB for 24 ms followed by 20 pseudo-randomly ordered trials of 10 different types: pulse alone; control (without stimulus); prepulse alone (68 dB, 70 dB, 78 dB or 84 dB); pulse with preceding prepulse (prepulse 68 dB, 70 dB, 78 dB or 84 dB each 100 ms before the pulse) with an inter-stimulus interval between 4000 and 14 000 ms followed by 5 startling pulses to analyze habituation. PPI was calculated as % reduction of the ASR amplitude at a given prepulse intensity (100 × (mean startle response without prepulse – mean startle response with prepulse)/mean startle response without prepulse). Habituation was calculated as percentage of ASR from 15 initial pulses to 5 final startle pulses. ACSF or MRS2365 were administered immediately before the test.

Delayed Non-Matching to Position (DNMTP) Task

Experiments were conducted in operant chambers (MED Associates, East Fairfield, U.S.A.) (Sukhotina *et al*, 2008). Rats were trained to lever-press for food and to learn the DNMTTP rule. At start of each trial, randomly one of two levers was presented ('sample') and retracted after it was pressed. Following a variable delay (0, 8 or 16 s) the rat had to nose-poke for presentation of both levers. If the animal pressed the non-matching lever (opposite to 'sample'), it was a correct response, both levers were retracted, the food magazine was illuminated and the animal was rewarded. If the 'sample' lever (matching) was pressed again, a non-rewarded

incorrect response was recorded. Each of the 96 trials was followed by an inter-trial interval of 5 s with house light on. After 20 s given to respond, the lever was retracted and the house light extinguished for 20 s. Failure to respond was recorded as omission. This DNMTTP training took 4 weeks to reach criteria of: (1) $\geq 90\%$ correct without delay, (2) $\geq 50\%$ correct at 16 s delay, (3) between 40–60% left-hand side performance and (4) no or few omissions. Total choice accuracy, latency to press ‘sample’ lever (latency to sample), latency to take reward from food magazine (magazine latency), total number of nose-pokes, magazine entries, omissions, rewards as well as session length, left-hand side performance and failures to collect reward were analyzed. Choice accuracy was calculated for ‘same trials’ (expected correct response was the same as during the preceding trial) and ‘different trials’ (expected correct response was different from the one in the preceding trial). Once training criteria were met, tests were conducted with MRS2365 or vehicle applied immediately before the session.

Reversal Learning Task

The procedure was adapted as previously described (Boulougouris and Robbins, 2010). Rats ($n=20$) were shaped to nose-poke into the food magazine to initiate presentation of two levers and to respond on the individually defined correct lever, first under a fixed ratio (FR) 1 (one lever press for reward), later under FR3. Completion of FR3 requirements at correct lever, but not at incorrect lever, resulted in delivery of a single reward pellet. Responding on either lever was followed by retraction of both levers and by deactivation of house light for 5 s. One session consisted of a maximum of five blocks with 10 trials each. The rat had to nose-poke into the food magazine within 20 s to trigger presentation of the levers. There was a 10-s response window after the presentation of levers. Failure to respond resulted in termination of the trial recorded as omission.

Rats were trained once a day until the criterion of at least nine correct trials in one block was achieved. If the criterion was not achieved, this step was repeated the next day. After rats achieved the criterion, this step was repeated next day under drug application (retention). When they fulfilled the criterion under drug application, an initial retention phase until criterion was repeated next day followed by a shift of position of the reinforced lever (within-session reversal) and the same criterion was applied. Reversal learning continued, if necessary, during the subsequent days. Percentage of correct trials, sessions to reach criterion and omissions were analyzed.

Microdialysis

The effect of prefrontocortical perfused MRS2365 on extracellular dopamine in this area and in the NAc was investigated by microdialysis (Krügel *et al*, 2003; Nikiforuk *et al*, 2010). Briefly, microdialysis probes (CMA/12, 3 mm length, 20 kDa cut-off, guide cannulae mPFC: AP = 3.2 mm, L = 0.6 mm, V = 0.2 mm; NAc: AP = 1.7 mm, L = 1.3 mm, V = 3.7 mm) were perfused with ACSF simultaneously in both areas (1.5 μ l/min) or MRS2365 in the mPFC as indicated in Figure 4. Dialysates for baseline dopamine were

taken beginning one hour after perfusion of probes and analyzed for dopamine after separation by HPLC by electrochemical detection (for more details see Supplementary Material).

Multiple Immunofluorescence

Double immunofluorescence labeling studies were performed as previously described (Franke *et al*, 2006). Slices were incubated with an mixture of mouse anti-microtubule associated protein-2 (MAP2, 1:1000, Chemicon), mouse anti-gial fibrillary acidic protein (GFAP, 1:1000, Sigma), mouse anti- γ -aminobutyric acid (GABA, 1:1000, Sigma), mouse anti-tyrosine hydroxylase (TH, 1:1000, Chemicon), mouse anti-synaptophysin (1:200, Sigma) or mouse anti-parvalbumin (1:1000, Sigma) and rabbit anti-P2Y₁R (1:400, Alomone Labs). Visualization was performed with a mixture of carbocyanine (Cy) 2-conjugated goat anti-mouse IgG and Cy3-conjugated goat anti-rabbit IgG. In experiments with rabbit anti-calretinin (1:1000, swant) a goat anti-P2Y₁R antibody (1:1000, Santa Cruz Biotechnology) was used followed by visualization with Cy2-conjugated donkey anti-rabbit and horse anti-goat IgG (20 μ g/ml in TBS containing 2% BSA each).

For triple immunofluorescence a mixture of rabbit anti-P2Y₁R (1:400), guinea pig anti-subtype 3 vesicular glutamate transporter (vGLUT3; 1:300 in 5% normal donkey serum in TBS, containing 0.3% Triton X-100) and mouse anti-MAP2 (1:1000) was applied overnight. After washing the sections were incubated with a mixture of Cy2-anti-guinea pig, Cy3-anti-rabbit and Cy5-anti-mouse IgG (all 20 μ g/ml) for 1 h and washed again. Immunofluorescence was investigated with a confocal laser scanning microscope (LSM 510 Meta, Zeiss, Oberkochen, Germany). No immunofluorescence was observed without the primary P2Y₁R antibody or by pre-adsorption of the antibody with the peptides used for their generation.

Statistical Analysis

Data are presented as mean \pm SEM. Effects of MRS2365 on the reversal task performance and ASR were evaluated using one-way analysis of variance (ANOVA). Effects of MRS2365 on the DNMTTP task performance, PPI and dopamine microdialysis were analyzed using a set of one- and two-way ANOVAs with repeated measures and *post hoc Tukey's test*. Rank transformation preceded DNMTTP data analysis by ANOVA.

RESULTS

Prepulse Inhibition (PPI) of the Acoustic Startle Response (ASR)

Treatment with MRS2365 (0.05 pmol) did not affect ASR amplitude during ‘pulse only’ trials and had no impact on habituation to repeated presentation of startle pulses (Figure 1a and b). MRS2365 had an overall attenuating effect on PPI (treatment: $F_{1,24} = 7.66$, $P = 0.01$, Figure 1c). This effect was seen only at low-to-intermediate prepulse intensities (treatment \times PPI interaction: $F_{3,72} = 2.84$, $P = 0.04$; Figure 1c). MRS2365 had no effects on startle

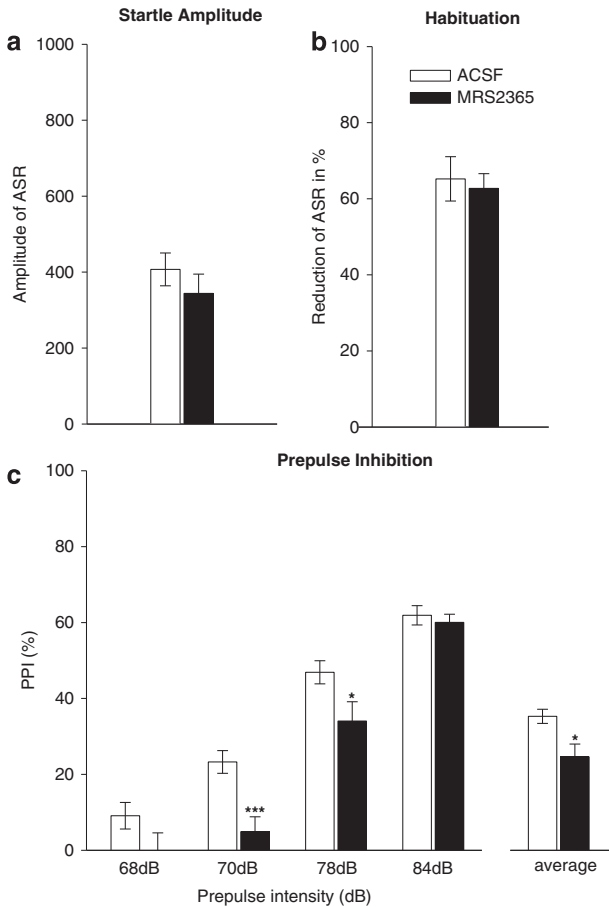


Figure 1 Effects of MRS2365 on the startle amplitude (a), startle habituation (b) and PPI of the ASR dependent on prepulse intensity (c) in comparison to ACSF-microinjected animals. MRS2365 was applied bilaterally into the mPFC at a dose of 0.05 pmol/site, ACSF was given in the equivalent volume of 0.5 μ l. Data are presented as mean \pm SEM ($n = 13$ each). * $P < 0.05$, *** $P < 0.001$ versus ACSF.

response latencies, duration of responses, time to maximal response or on startle responses induced by prepulses (not shown).

Delayed Non-Matching to Position (DNMTP) Task

Under treatment with ACSF, performance accuracy was above 90% at low delays, but gradually reduced to near-chance levels as delays increased to 16 s. MRS2365 (0.05 pmol) reduced overall accuracy at 8 and 16 s delay (delay by treatment interaction: $F_{2,59} = 5.1$, $P < 0.05$; effect of treatment: $F_{1,59} = 40.3$, $P < 0.001$; effect of delay: $F_{2,59} = 52.5$, $P < 0.001$; Figure 2a). This impairment was also evident during the trials where the correct lever was different from that in the preceding trial (delay by treatment interaction: $F_{1,9} = 3.8$, $P = 0.04$; effect of treatment: $F_{1,59} = 35.2$, $P < 0.001$; effect of delay: $F_{2,59} = 41.2$, $P < 0.001$; Figure 2c). For the 'same' lever trials, there were significant main effects of treatment ($F_{1,59} = 8.25$, $P < 0.05$) and delay ($F_{2,59} = 25.3$, $P < 0.001$) but no delay by treatment interaction (Figure 2b).

MRS2365 also shortened the session length ($F_{1,9} = 5.54$, $P = 0.04$), increased the frequency of magazine entries ($F_{1,9} = 8.56$, $P = 0.02$). MRS2365 had no effect on total omissions, latency to sample, magazine latency, reward, left-hand side performance, failure to collect food and the correct response latency (Supplementary Table S1).

Reversal Learning Task

Prior to MRS2365 treatment, the groups did not differ in task acquisition (Figure 3a). Treatment with MRS2365 did not influence the performance in the retention phase (Figure 3b) but resulted in a reduced percentage of correct trials during the reversal phase ($F_{1,17} = 9.43$, $P < 0.01$; Figure 3c). ACSF-treated rats needed less sessions to the

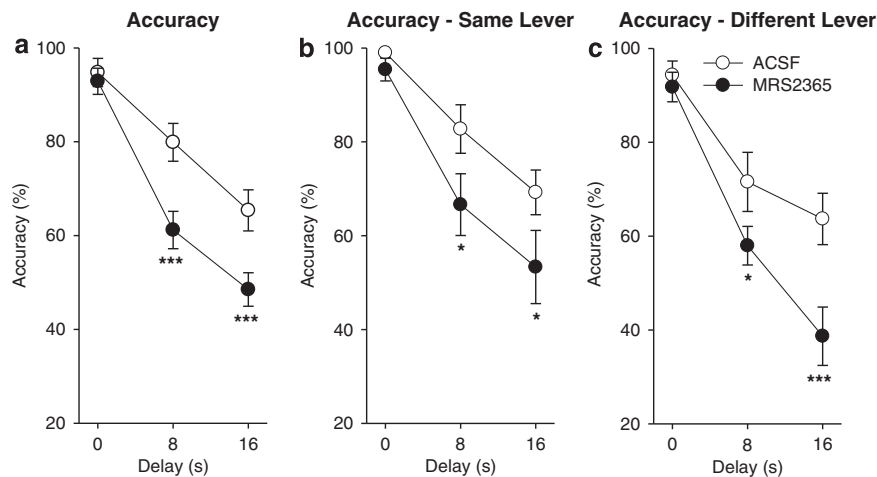


Figure 2 Effects of MRS2365 on the performance in the DNMTTP task in comparison to ACSF-microinjected animals. MRS2365 was applied bilaterally into the mPFC at a dose of 0.05 pmol/site, ACSF was given in the equivalent volume of 0.5 μ l. Data (mean \pm SEM; $n = 10$ for each group) are presented for delays of 0, 8 and 16 s as percent correct responses (choice accuracy) (a), as percent correct responses if the same lever as in the previous trial was presented ('same trial') (b) and if the opposite lever as in the previous trial was presented ('different trial') (c). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus ACSF.

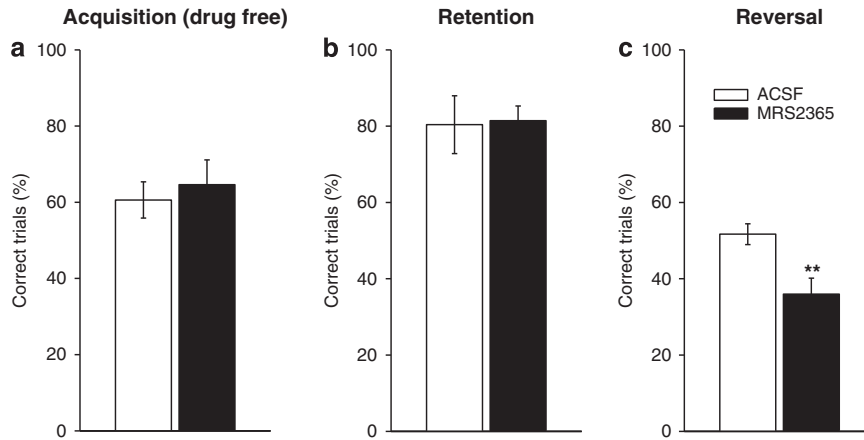


Figure 3 Effects MRS2365 on the performance in the reversal task. Percentage of correct trials is shown for the last acquisition session as well for retention and reversal sessions. Prior to retention and reversal sessions, MRS2365 was applied bilaterally into the mPFC at a dose of 0.05 pmol/side, ACSF was given in the equivalent volume of 0.5 μ l. Data are presented as the mean \pm SEM (ACSF: $n = 9$, MRS2365: $n = 10$). ** $P < 0.01$ versus ACSF.

criterion compared to the MRS2365-group (2.4 ± 0.6 versus 4.5 ± 0.6 ; $F_{1,17} = 5.60$, $P = 0.03$). MRS2365-treatment during the reversal phase was associated with an increase in the number of uncompleted trials (omissions; ACSF group: 24.7 ± 10.0 ; MRS2365 group: 84.9 ± 18.9 , $F_{1,17} = 7.41$, $P = 0.014$).

Microdialysis

Basal concentrations of dopamine in the mPFC and NAc were found to be 0.22 ± 0.05 fmol/ μ l and 0.41 ± 0.16 fmol/ μ l, respectively. MRS2365 caused a short-lasting increase in dopamine in the mPFC (Figure 4a; treatment: $F_{4,182} = 16.00$, $P < 0.001$; time: $F_{12,182} = 5.51$, $P < 0.001$; interaction: $F_{48,182} = 1.72$, $P < 0.01$). MRS2365 (1 and 10 μ M) increased dopamine at 40 min (1 μ M: $P < 0.001$, 10 μ M: $P < 0.01$; Figure 4a) and at 60 min (1 μ M: $P = 0.02$). Analysis of total dopamine (AUC_{0-180 min}) confirmed an inverted U-shaped dose-effect relationship ($F_{4,14} = 5.98$, $P < 0.01$; Figure 4b) significant at 1 μ M MRS2365 ($P = 0.01$).

Infusion of MRS2365 into the mPFC increased dopamine in the NAc (0.1 and 1 μ M; $P < 0.001$; Figure 4c). There were significant effects of treatment ($F_{4,182} = 12.82$, $P < 0.001$), time ($F_{12,182} = 4.47$, $P < 0.001$) but no interaction ($F_{48,182} = 0.84$, $P = 0.77$). MRS2365 (1 μ M) increased dopamine from 40 min onwards ($P = 0.02$; Figure 4c). Total dopamine in the NAc also displayed an inverted U-shaped concentration-response pattern ($F_{4,14} = 3.40$, $P = 0.04$; Figure 4d) significant at 1 μ M MRS2365 ($P = 0.03$).

Immunohistochemistry

Immunohistochemistry in the mPFC with MAP2 (Figure 5a and b) revealed that P2Y₁Rs are localized at neuronal structures, cell bodies and fibers. Double labeling with GFAP identified P2Y₁Rs also on astrocytic cell bodies and processes (Figure 5c and d). Pyramidal cells of typical triangular shaped soma are endowed with P2Y₁Rs (Figure 5e and f). The co-localization of P2Y₁Rs and synaptophysin (Figure 5g and h) strongly argues for presynaptic P2Y₁Rs, however not on TH-positive fibers (Figure 5i and j). P2Y₁Rs

are localised at cell bodies and processes of GABA-containing neurons (Figure 5k and l). These were parvalbumin-positive (Figure 5m and n) but not calretinin-positive interneurons (Figure 5o and p). P2Y₁Rs are also co-localized with vGLUT3, a marker for neurons releasing glutamate, but not primarily, in addition to acetylcholine, serotonin and even GABA (Figure 5q-s).

DISCUSSION

The present study provides experimental evidence suggesting for the first time that ATP/ADP-sensitive P2Rs in the mPFC may be involved in regulation of various behavioral functions. Stimulation of P2Y₁Rs by the selective agonist MRS2365 reduced PPI of the ASR, produced delay-dependent decreases in accuracy in the DNMT task, and impaired reversal learning. These effects may be mechanistically related to short- and long-lasting increases in dopamine in the mPFC and NAc, respectively, observed by microdialysis. P2Y₁Rs were found on pyramidal cells and parvalbumin-positive interneurons but not on TH-positive fiber tracts originating in the VTA, which argues for an indirect activation of dopaminergic afferents in the cortex by MRS2365. As the importance of the rodent mPFC in executive functions, is widely accepted (for review see: Chudasama, 2011), alterations in prefrontal extracellular ATP/ADP transferred *via* P2Y₁Rs might be responsible for behavioral abnormalities such as observed in this study.

The PPI of the acoustic startle reflex reflects a pre-attentive filtering process known as sensorimotor gating. While the acoustic startle reflex is mediated by brainstem circuits and the spinal cord, it can be extensively modulated by the activity of limbic and cortico-pallido-striato-thalamic circuitries. In our study, stimulation of P2Y₁Rs in the mPFC by MRS2365 affected neither the mean ASR nor the habituation of the ASR, but significantly reduced PPI. The impaired PPI evoked by P2Y₁R stimulation has a profile consistent with prefrontal dopamine D1R inhibition with the exception that prefrontal dopamine was increased by local application of the agonist (Ellenbroek *et al*, 1996).

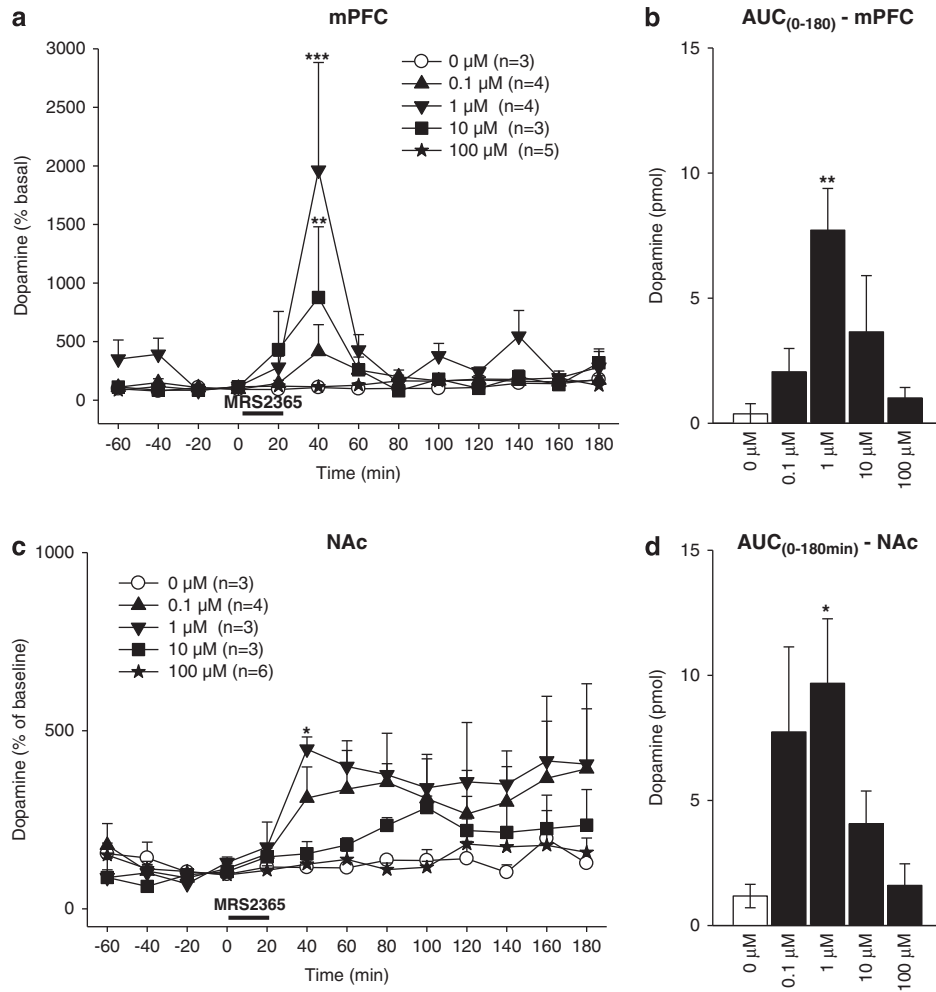


Figure 4 Effect of prefrontocortical infusion of MRS2365 on dopamine microdialysate concentrations in the mPFC (a and b) and in the ipsilateral NAc (c and d). MRS2365 was administered *via* retrodialysis in concentrations of 0.1, 1.0, 10, and 100 μM for 20 minutes each. Dopamine concentrations in microdialysates from the mPFC (a) and from NAc (c) after MRS2365-perfusion are presented as percent of basal concentration from the average of two pre-application levels. The total effect of MRS2365 on dopamine was calculated from the area under curve data collapsed over 0–180 min based on percent baseline change in the mPFC (a) and NAc (d). Data are given as means ± SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus ACSF.

Similar disruptions of PPI were observed in rats after inactivation of the mPFC leading to subcortical dopaminergic hyperactivity, eg entorhinal cortex lesion or N-methyl-D-aspartate (NMDA)-R blockade (Goto *et al*, 2002; Suzuki *et al*, 2002). In line, D2 dopamine receptor agonists disrupt PPI (Swerdlow *et al*, 2005). Mesolimbic hyperactivity and cortical hypoactivity associated with abnormal PPI are assumed to be related to various aspects of neuropsychiatric disorders (Swerdlow *et al*, 2005).

Further, stimulation of P2Y₁Rs by MRS2365 impaired accuracy of performance of the DNMTTP task. Effects of MRS2365 were delay-dependent suggesting specific effects on aspects of rodent working memory assessed in this task. This conclusion is supported by analysis of accuracy in trials where the sample lever was similar to or different from that in the previous trial. Effects of MRS2365 were largely independent of the trial type indicating that treatment did not induce side preference or increase probability of response perseveration. Interestingly, several other effects of MRS2365 were observed: decreased session length,

increased frequency of magazine entries and the tendency of increased nose-poke rate. These effects may point at impaired response inhibition or impulse control induced by P2Y₁R-stimulation in PFC.

Involvement of prefrontal P2Y₁Rs in the control of behavioral flexibility is verified by the reversal task results. Similar to what was observed in the DNMTTP experiments, MRS2365 did not interfere with the performance of an already learned task. However, during the reversal phase, MRS2365 seemed to impair the ability of animals to switch responding to another lever. This was evident as reduced percentage of correct trials, more sessions needed to achieve the criterion and more uncompleted trials.

The mPFC controls executive functions *via* several mechanisms including modulation of accumbal dopamine (Del Arco and Mora, 2008; Taber *et al*, 1995; Floresco *et al*, 2009). Previous investigations have shown that local administration of P2Y₁R agonists into NAc or VTA facilitates while antagonists decrease the release of dopamine under basal conditions (Krügel *et al*, 2001a; Krügel

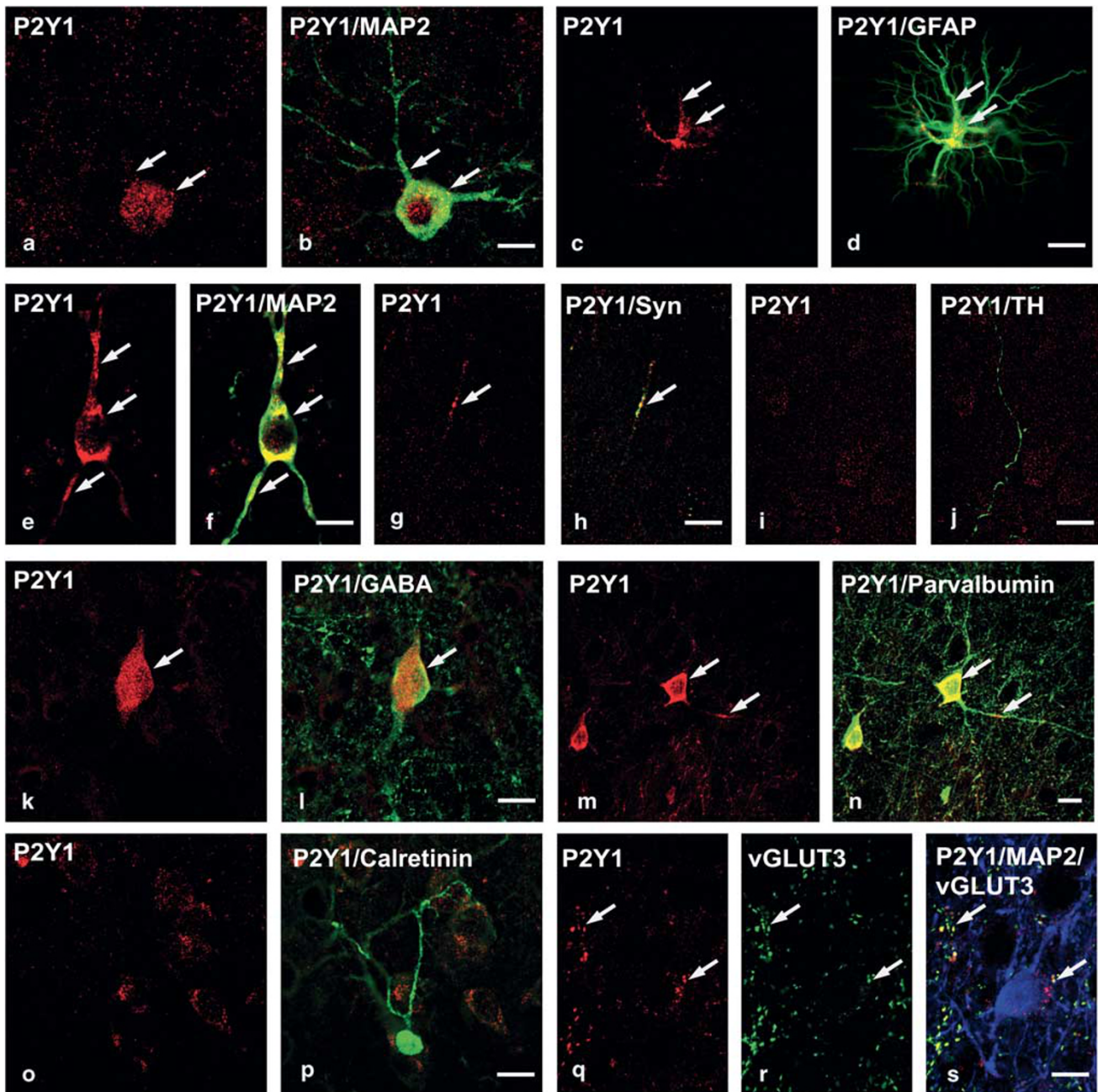


Figure 5 Immunocytochemical identification of P2Y₁R-localisation in prefrontocortical layer V. P2Y₁Rs (red) are localized on neuronal structures labeled with MAP2 (green; a and b) and on astrocytes identified by GFAP-labeling (green; c and d). Neurons with the typical triangular shape of pyramidal cells (e and f) bear P2Y₁Rs at the apical dendrite (upper arrow), at the cell body (middle arrow) and at basal dendrites (lower arrow). P2Y₁Rs are presynaptically located as indicated by co-labeling with synaptophysin, see arrow (green; g and h) but not on TH-positive fibers (i and j). Further, P2Y₁Rs were found on GABA-containing neurons (green; k and l) and parvalbumin-positive neurons (green; m and n) at cell bodies and origin of processes. Calretinin-positive neurons (o and p) were not labeled for P2Y₁Rs. Finally, P2Y₁Rs were co-localized with dot-like vGLUT3 structures (green; q, r and s) and in strong association with the neuronal marker MAP2 (blue) as indicated by arrows. Scale bars = 10 μm.

et al, 2001b; Kittner *et al*, 2004). Our microdialysis data demonstrate that stimulation of prefrontal P2Y₁Rs also increased subcortical dopamine in the NAC. An inverted U-shaped dose-dependency of purine-mediated effects on dopamine is similar to that reported in earlier studies on stimulation of phosphoinositide hydrolysis in NG108-15 cells by the P2Y_{1,12} agonists, ADPβS and 2MeSADP, and on exploratory activity by 2-methylthio-ATP (Kittner *et al*, 2000; Sak *et al*, 2000) or MRS2365 (present study).

Dopamine facilitating effects by MRS2365 were more pronounced and short-lasting in the mPFC compared to the NAC where dopamine was elevated for a much longer time similar to effects after NMDA-R antagonist microinjection (Del Arco and Mora, 2009).

It is known that working memory depends on dopamine in the PFC, however the relationship between dopamine and working memory performance is not linear and its inverted U-shape was suggested to indicate existence of

dopaminergic activity optimal for the function of PFC (van Schouwenburg *et al*, 2010; Arnsten, 2009; Floresco *et al*, 2009). The acute transient activation of prefrontal P2Y₁Rs seems to trigger the deviation from optimal dopamine in the mPFC and this might contribute to the observed behavioral effects, eg decreased behavioral flexibility (van Schouwenburg *et al*, 2010; Floresco *et al*, 2009). Thus, even though present behavioral and microdialysis studies were conducted in rats of two different strains for which differences in the sensitivity to PPI-disrupting effects are reported (Swerdlow *et al*, 2000), microdialysis results support the behavioral relevance of the used dose of MRS2365 in operant tasks as it matches the most effective dopamine-enhancing concentration of 1 μM (assuming a recovery of 10% for MRS2365 at the membrane similar to ATP; Krügel *et al*, 2001b). Similar relationships between dose (behavior) and concentration (microdialysis) were found for the less selective agonist 2-methylthio-ATP in the ventral tegmentum (Krügel *et al*, 2001a).

MRS2365-induced increase in prefrontal dopamine and related behavioral responses do not seem to be mediated by direct presynaptic P2Y₁R-stimulation on dopaminergic terminals as no P2Y₁R-immunoreactivity was found on prefrontal TH-positive fibers. Therefore, multisynaptic mechanisms are suggested to be involved. Our immunohistochemical data provide evidence for P2Y₁R-positive subpopulations of mPFC neurons, which had morphological features of pyramidal cells or were positive for GABA or parvalbumin.

Previous slice studies have already revealed the presence of functionally active prefrontal postsynaptic P2Y₁Rs on pyramidal cells (Luthardt *et al*, 2003). As these cells are known to synapse in the VTA onto dopamine neurons that project back to the mPFC, the stimulation of P2Y₁Rs might activate the mPFC-VTA-mPFC circuit (Carr and Sesack, 2000). In line with that, the firing rate of dopamine neurons can be regulated depending upon glutamatergic stimulation within the VTA (Wang *et al*, 1994). Dopamine neurons have synaptic contacts within the VTA with GABA interneurons and GABA neurons projecting to the NAc. Thereby, prefrontal P2Y₁Rs might modulate the activity of mesoaccumbal neurons and dopamine release in the NAc indirectly (Carr and Sesack, 2000). This hypothesis is supported by facilitating effects of the GABA_A-antagonist bicuculline infused into the VTA on dopamine in the NAc (Krügel *et al*, 2001a). Furthermore, mPFC neurons also may facilitate accumbal dopamine by an indirect pathway involving the pedunculo pontine tegmentum and the laterodorsal tegmentum, which in turn stimulate mesoaccumbal projection neurons (Del Arco and Mora, 2008).

Behavioral alterations induced by MRS2365 in the present study are similar to motor hyperactivity and cognitive impairment found after inhibition of prefrontal NMDA-Rs with phencyclidine (Del Arco *et al*, 2008). NMDA-R antagonists such as 3-[(R)-2-carboxypiperazin-4-yl]-propyl-1-phosphonic acid (CPP) applied to the PFC caused lasting elevations of accumbal dopamine providing evidence that hypofunction of prefrontal NMDA-Rs produces corticolimbic hyperactivity by activation of subcortical areas (Del Arco *et al*, 2008).

Interestingly, at PFC layer 5 pyramidal cells, the activation of P2Y₁Rs inhibited both, the current responses to

NMDA and the NMDA-component of excitatory postsynaptic potentials evoked by electrical stimulation (Luthardt *et al*, 2003). The nature of that interaction at pyramidal cells or GABA interneurons (see below) is unclear. Heteromers, similar to A_{2A}R-D2R, as well ion channel receptor-tyrosine kinase heteromers possibly might be involved (Agnati *et al*, 2010). At all, this interference is similar to the action of NMDA-R antagonists and might have impact on the activity of pyramidal cells. It is suggested that NMDA-R blockade increases the activity of pyramidal efferent projections by attenuation of feedback inhibition by GABA interneurons endowed with NMDA-Rs (Del Arco *et al*, 2011; Homayoun and Moghaddam, 2007).

Further, P2Y₁Rs on non-pyramidal cells strongly argue for their participation in prefrontal disinhibition. Among P2Y₁R-positive non-pyramidal cells, we identified GABA-positive and parvalbumin-, but not calretinin-positive neurons. Given that parvalbumin and calretinin expression characterizes different classes of interneurons, P2Y₁Rs might have specific functions in prefrontal inhibition (Kawaguchi and Kubota, 1998).

Calretinin-containing interneurons are suggested to mediate the overall network disinhibition in the PFC because they preferentially target dendrites of other GABA neurons rather than pyramidal cells (Hashimoto *et al*, 2003).

Parvalbumin interneurons primarily contact soma and axon initial segments of pyramidal cells and thereby deliver a unique inhibitory input to them (Marin, 2012). Fast-spiking parvalbumin interneurons are involved in oscillatory activity, which enables pyramidal cells to fire synchronously and is crucial for cognitive functions. Alterations in parvalbumin neuron-mediated inhibition are attributed to cognitive deficits in schizophrenia (Gonzalez-Burgos and Lewis, 2008). The observed behavioral changes by MRS2365 and the expression of P2Y₁Rs on parvalbumin interneurons fit in this concept.

Whereas Guzman *et al* (2010) identified postsynaptic P2Y₁Rs, the co-localization with synaptophysin, an integral membrane glycoprotein of small synaptic vesicles, also confirms a presynaptic function, probably in the modulation of transmitter release.

A co-expression of P2Y₁Rs and vGLUT3 in strong association with neuronal structures was also observed suggesting a contribution of P2Y₁Rs to the neuronal release of glutamate from cortical neurons not primarily glutamatergic, eg GABA interneurons or excitatory interneurons (Herzog *et al*, 2004; Franke *et al*, 2006). Even though the significance of P2Y₁Rs in this interaction is completely unknown, the vGLUT-dependent increased vesicular accumulation of primary neurotransmitters (vesicular synergy) proposed by El Mestikawy *et al* (2011) is a conceivable mechanism of ATP/ADP contribution to GABA/glutamate co-transmission and thereby to the modulation of prefrontal synaptic activity.

In conclusion, the results indicate the involvement of P2Y₁Rs in the prefrontal cortical control over certain cognitive behavioral responses. This control may become more significant under conditions of augmented ADP-signaling (eg after prolonged exhausting network activity, associated with neuroinflammation or neuronal injury).

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Author Contributions

AB and UK designed the study. HK performed the behavioral experiments and statistical analysis. KD conducted the microdialysis, HF managed the immunohistochemistry. HK, AB and UK prepared the main parts of the manuscript, which was approved by all authors.

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