Phosphodiesterase 2 Inhibitors Promote Axonal Outgrowth in Organotypic Slice Co-Cultures

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Key Words

Axonal outgrowth • Biocytin • Development • Dopaminergic system • Organotypic slice co-culture • Phosphodiesterase • Regeneration • Repair • Tracing • Tyrosine hydroxylase

Abstract

The development of appropriate models assessing the potential of substances for regeneration of neuronal circuits is of great importance. Here, we present procedures to analyze effects of substances on fiber outgrowth based on organotypic slice co-cultures of the nigrostriatal dopaminergic system in combination with biocytin tracing and tyrosine hydroxylase labeling and subsequent automated image quantification. Selected phosphodiesterase inhibitors (PDE-Is) were studied to identify their potential growth-promoting capacities. Immunohistochemical methods were used to visualize developing fibers in the border region between ventral tegmental area/substantia nigra co-cultivated with the striatum as well as the cellular expression of PDE2A and PDE10. The quantification shows a significant increase of fiber density in the border region induced by PDE2-Is (BAY60-7550; ND7001), comparable with the potential of the nerve growth factor and in contrast to PDE10-I (MP-10). Analysis of

tyrosine hydroxylase-positive fibers indicated a significant increase after treatment with BAY60-7550 and nerve growth factor in relation to dimethyl sulfoxide. Additionally, a dose-dependent increase of intracellular cGMP levels in response to the applied PDE2-Is in PDE2-transfected HEK293 cells was found. In summary, our findings show that PDE2-Is are able to significantly promote axonal outgrowth in organotypic slice co-cultures, which are a suitable model to assess growth-related effects in neuro(re)generation.

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Introduction

Axonal projections and the basic organization of mid-brain dopaminergic (DAergic) neurons are the subject of interest when studying human neurological disorders, e.g. Parkinson's disease [1], schizophrenia [2], and drug abuse or addiction [3, 4]. The mesencephalon as a region of complex anatomical organization, and projection patterns of the DAergic neurons are divided into the retrorubral area

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(A8 cell group), substantia nigra (SN; A9 cell group), and the ventral tegmental area (VTA; A10 cell group) [5–7]. Tract-tracing techniques have revealed three projections to forebrain targets, namely the mesolimbic, the mesocortical, and the nigrostriatal pathways (for references, see [8]). The trajectories from the SN and VTA target all intrinsic nuclei of the basal ganglia while exhibiting preferential concentrations of terminals in the dorsal and ventral striatum (STR) [1, 8]. The balance between these projections is thought to be regulated by afferent DAergic signals from the VTA and SN pars compacta, acting on differentially distributed D1 and D2 dopamine (DA) receptors, e.g. on striatal medium spiny neurons. The activation of D1 receptors leads to an intracellular accumulation of cyclic adenosine 3',5'-monophosphate (cAMP) via the adenylyl cyclase and following activation of cAMP-dependent protein kinase. Otherwise, DA inhibits the adenylyl cyclase via D2 receptors (for references, see [9]).

It is well known that disruption of DAergic cell activity and the loss of ascending projections to the basal ganglia results in the emergence of fundamental pathological features. Due to the necessity for therapeutic strategies promoting neuro(re)generation, there is a clear need to develop appropriate test systems to assess the potential of different substances regarding regeneration and repair of neuronal circuits. Over the last years, we established *ex vivo* models of organotypic slice co-cultures of the DAergic system [10–12]. Parts of this system were reconstructed using tissue slices from the VTA/SN and STR or the prefrontal cortex, respectively, to analyze the cytoarchitectural organization of the VTA/SN and the innervations of the target regions by DAergic fibers. Our results demonstrated that tyrosine hydroxylase (TH)-immunopositive neurons are also able to develop their characteristic innervation patterns in organotypic slice co-cultures [10]. A number of intracellular and extracellular molecules are involved in the regulation of neuronal differentiation. Using our model system, we were able to identify a trophic support in axonal outgrowth after purinergic stimulation [11, 12]. Moreover, second messengers cAMP and cyclic guanosine 3',5'-monophosphate (cGMP), formed from the triphosphates ATP and GTP, appear to play prominent roles in regulating neuronal differentiation and neuroplasticity [13, 14]. Elevated intracellular levels of the cyclic nucleotides are important for axon regeneration, even beyond the required increase in cAMP levels needed to respond to most factors that support cell survival [15]. Cyclic nucleotide phosphodiesterases (PDEs) comprise a superfamily of metallophosphohydrolases that specifically cleave the 3',5'-cyclic

phosphate moiety of cAMP and/or cGMP and terminate the action of cyclic nucleotide signaling [16]. Eleven individual PDE subfamilies (PDE1–PDE11) with varying selectivity for cAMP or cGMP have been identified in mammalian tissues based on sequence similarities, inhibitor sensitivity, and biochemical properties. They are involved in mediating a range of different functions, including cytoskeletal rearrangement, gene transcription, and regulation of ion channel function [17, 18]. Recent data have shown that PDE2 and PDE10 are localized in the nigrostriatal system and hydrolyze both cAMP and cGMP. The inhibition of PDE2 selectively increases cyclic nucleotide levels, influencing synaptic plasticity and memory formation [19, 20]. Inhibition of PDE10 modulates neuronal activity within the striatopallidal and nigrostriatal pathway and leads to efficacy in behavioral models predicting an antipsychotic effect [21–24].

The aim of the present study was to characterize possible trophic/regenerative properties of selected PDE2 inhibitors (PDE2-Is; BAY60-7550 and ND7001) and PDE10-I (MP-10), as compared to the effect of the neurotrophin nerve growth factor (NGF) by using an organotypic slice co-culture model (VTA/SN+STR). To quantify the potential of stimulating fiber growth, density of neuronal fibers in the border region of slice co-cultures was determined using both biocytin-tracing technique and TH immunolabeling, and subsequent automated image quantification.

Materials and Methods

Materials/Substances

Selected PDE-Is with different subclass specificities were used (details, see table 1; structural formula, see fig. 1): BAY60-7550 (2-(3,4-dimethoxybenzyl)-7-((1R)-1-((1R)-1-hydroxyethyl)-4-phenyl-butyl)-5-methylimidazo[5,1-f][1,2,4]triazin-4(3H)-one; Alexis Biochemicals, San Diego, Calif., USA), ND7001 (3-(8-methoxy-1-methyl-2-oxo-7-phenyl-2,3-dihydro-1H-benzo[e][1,4]diazepin-5-yl)-benzamide), and MP-10 (2-[4-(1-methyl-4-pyridin-4-yl-1H-pyrazol-3-yl)-phenoxy]methyl]-quinoline 3) were synthesized at biocrea (Radebeul, Germany).

Furthermore, the following substances/factors were applied: NGF (Sigma-Aldrich, Taufkirchen, Germany), dimethyl sulfoxide (DMSO; AppliChem GmbH, Darmstadt, Germany), artificial cerebrospinal fluid (ACSF) of the composition (mM): 126 NaCl; 2.5 KCl; 1.2 NaH₂PO₄; 1.3 MgCl₂, and 2.4 CaCl₂ (pH 7.4; Hospital Pharmacy, University of Leipzig, Germany).

In vitro PDE Assay

PDE activity was measured by the conversion of [³H]-cAMP and [³H]-cGMP into [³H]-AMP and [³H]-GMP, respectively, as described previously [25]. PDEs 1B, 2A, 3A, 4A, 5A, 7B, 8A, 9A, 10A, and 11A were generated from full-length human recombi-

Fig. 1. Chemical structures of the PDE substrates cGMP and cAMP and of the PDE-Is BAY60-7550, ND7001, and MP-10.

nant clones. PDE6 was isolated from bovine retina as described previously [26]. PDE activity was measured with the preferred substrates, in detail, for PDE1B, 2A, 3A, 4A, 7B, 8A, 10A, and 11A cAMP was used and for PDE5A, 6, and 9A cGMP, at or below the K_m value (Michaelis-Menten constant). PDE1B and PDE2A were activated by Ca^{2+} /calmodulin and by cGMP, respectively. The other PDEs did not need any additional activation procedure. Inhibition of PDE activity was measured using a scintillation proximity assay at varied compound concentrations and optimized fixed enzyme concentrations for each enzymatic assay. IC_{50} (half maximal inhibitory concentration) values were calculated with the Hill2 parameter model from at least four independent experiments, done in duplicates.

Intracellular cGMP Assay

The selected PDE-Is BAY60-7550 and ND7001 were tested in a cellular test system to analyze the effects on intracellular cGMP levels. Human embryonic kidney (HEK) 293 cells, stably transfected with the full-length sequence of human PDE2A (NM002599), were cultured in collagen I-coated 96-well plates (70,000 cells/well) over night. On the next day, the test compounds were administered at a final DMSO concentration of 0.5% and incubated for 30 min at 37°C and 5% CO_2 . This was followed by incubation with the guanylyl cyclase (GC)-activating agent sodium nitroferricyanide(III) dihydrate (sodium nitroprusside, SNP, 100 μ M; Sigma-Aldrich) for 10 min. Wells incubated without test compound in the presence and absence of SNP were used as controls for statistical analysis. The level of intracellular cGMP was determined with the enzyme-linked immunosorbent assay system (cGMP EIA System; GE Healthcare UK Ltd., Little Chalfont, England).

Table 1. IC_{50} values (nM) of the PDE2-Is BAY60-7550 and ND7001, and the PDE10-I MP-10 against individual phosphodiesterases

PDE	BAY60-7550	ND7001	MP-10
hPDE2A	0.18	57	2,630
hPDE1B	426	>5,000	>5,000
hPDE3A	>5,000	>10,000	>5,000
hPDE4A	2,130	>10,000	2,230
hPDE5A	516	>10,000	>5,000
bPDE6	2,200	>10,000	>5,000
hPDE7B	1,740	>10,000	>5,000
hPDE8A	>5,000	>10,000	>5,000
hPDE9A	>5,000	>10,000	>5,000
hPDE10A	1,640	1,460	1.14
hPDE11A	>5,000	>10,000	>5,000

Overview of the IC_{50} values (nM) of the PDE2-Is BAY60-7550 and ND7001, and the PDE10-I MP-10 against individual human (h) PDEs as well as the bovine (b) PDE6.

Animals

Neonatal rat pups (WISTAR RjHan, own breed; animal house of the Rudolf Boehm Institute of Pharmacology and Toxicology, University of Leipzig) of postnatal day 1–5 (P1–5) were used for preparation of the organotypic slice co-cultures. The animals were housed under standard laboratory conditions, under a 12-

hour light/12-hour dark cycle and allowed access to lab food and water ad libitum.

All of the animal use procedures were approved by the Committee of Animal Care and Use of the relevant local governmental body in accordance with the law of experimental animal protection.

Preparation of Slice Cultures

Slice co-cultures were prepared from P1–5 old rats and cultivated according to the ‘static’ culture protocol described by Franke et al. [10] (for details, see also fig. 2). Briefly, rat pups were decapitated and the brains were removed from the skull under sterile conditions. The brains were apposed to an agar block and fixed onto the specimen stage of a slicer (Leica VT 1000S; Nussloch, Germany) with cyanoacrylate glue (Histoacryl®; B. Braun, Tuttlingen, Germany). Coronal sections (thickness 300 µm) were cut at mesencephalic (fig. 2: A) and forebrain levels (fig. 2: B). During the preparation of organotypic slice cultures of the ventral mesencephalon we did not attempt to separate the VTA and the SN. For further discussion, this area will be named VTA/SN. After preparation of the VTA/SN (fig. 2: A’, asterisk) and the STR (fig. 2: B’, asterisk), respectively, the slices were transferred into petri dishes filled with cold (4°C) preparation solution (MEM, Minimum Essential Medium, 2 mM glutamine; Invitrogen GmbH, Darmstadt, Germany) supplemented with the antibiotic gentamicin (50 µg/ml; Invitrogen GmbH). Selected sections were placed next to each other on moistened translucent membrane inserts (0.4 µm; Millicell-CM, Millipore, Bedford, Mass., USA) as co-cultures (VTA/SN+STR) and were put in 6-well plates each filled with 1 ml incubation medium (50% MEM, 25% Hank’s Balanced Salt Solution, 25% heat inactivated horse serum; glutamine to a final concentration of 2 mM; all from Invitrogen GmbH and with 0.044% sodium hydrogen carbonate, NaHCO₃; Sigma-Aldrich). The pH was adjusted to 7.2. The cultures were stored at 37°C in 5% CO₂ and the medium was changed 3 times a week.

Fixation of the Tissues

After 10 days in vitro (DIV), the cultures were fixed in a solution consisting of 4% paraformaldehyde, 0.1% glutaraldehyde, 15% picric acid in phosphate buffer (PB, 0.1 M; pH 7.4) for 2 h and subsequently washed with PB intensively. Following fixation, the co-cultures were cut into 50-µm thick slices by means of the vibratome.

Immunohistochemistry

(a) *Qualitative Characterization of PDEs.* Following pre-incubation in 1% H₂O₂ solution for 25 min as well as in blocking solution containing 10% normal horse serum in 0.1 M PB for 1 h, respectively, the slices were incubated with goat anti-PDE2A (1:100; Santa Cruz Biotechnology, Inc., Santa Cruz, Calif., USA) or rabbit anti-PDE10 (1:500; FabGennix Inc., Frisco, Tex., USA) for 48 h at 4°C.

(b) *Quantitative Characterization of TH-Positive Fibers.* After pre-treatment with H₂O₂ and incubation with blocking solution, the slices were incubated with mouse anti-TH (1:1,000; Chemicon, Temecula, Calif., USA) for 48 h at 4°C.

(a, b) After washing with PB, the slices were incubated with biotinylated anti-goat, biotinylated anti-rabbit or biotinylated anti-mouse immunoglobulin IgG (H+L) (1:65; Vector Laboratories, Inc., Burlingame, Calif., USA), respectively, for 2 h at room tem-

Fig. 2. Schematic illustration of the preparation procedure to obtain organotypic slice co-cultures of the DAergic system as described previously by Franke et al. [10]. Intact brains were removed from 1- to 5-day-old rats. Afterwards, transverse cuts were made at the level of A to isolate the mesencephalon and the striatal region (B). Additional horizontal cuts were made indicated by the dashed lines to separate the VTA/SN (A’ *) and the STR (B’ *). In the last step, selected coronal sections (thickness 300 µm) were placed next to each other on moistened membranes as co-cultures as shown in the pictures.

perature. Afterwards, the avidin-biotin-horseradish peroxidase complex (1:50; ABC-Elite Kit, Vector Laboratories, Inc.) was applied and visualized by 3,3'-diaminobenzidine hydrochloride (DAB; Sigma-Aldrich) as the chromogen. For the TH immunolabeling, nickel/cobalt-intensified DAB was used. After mounting on glass slides, the stained slices were dehydrated in a series of graded ethanol, processed through n-butylacetate and coverslipped with Entellan (Merck, Darmstadt, Germany).

Fig. 3. A detailed chronological scheme of the experimental procedure is illustrated. During the incubation period of the cultures, the treatment was executed 4 times (ST), starting on DIV 1 using the respective substances. At DIV 8, the biocytin tracing was performed as described and, finally, at DIV 10, the co-cultures were fixed and analyzed. ST = Substance treatment.

Immunofluorescence

Single Labeling. After washing with Tris-buffered saline (TBS, 0.05 M; pH 7.6) and blocking with TBS containing fetal calf serum (FCS, 5%) and Triton X-100 (0.3%), the slices were incubated with mouse anti-TH (1:1,000; Chemicon); mouse anti- β III-tubulin (1:400; Promega GmbH, Mannheim, Germany) or mouse anti-glial fibrillary acidic protein (GFAP, 1:1,000; Sigma-Aldrich), respectively, for 48 h at 4°C. The visualization of the respective primary antibodies was performed with carbocyanine (Cy)2-conjugated goat anti-mouse IgG (1:400; Jackson ImmunoResearch, West Grove, Pa., USA). In addition, slices were stained with the nucleic acid probe Hoechst 33342 (Hoe, final concentration 40 μ g/ml; Molecular Probes, Leiden, The Netherlands) to identify the cell nuclei.

Multiple Fluorescence Labeling. After the blocking step, performed as described above, the slices were incubated with an antibody mixture of rabbit anti-microtubule-associated protein-2 (MAP2, 1:500; Millipore, Temecula, Calif., USA), mouse anti-GFAP (1:1,000; Sigma-Aldrich) and goat anti-PDE2A (1:100; Santa Cruz Biotechnology, Inc.) or mouse anti-MAP2 (1:1,000; Millipore), goat anti-GFAP (1:300; Santa Cruz Biotechnology, Inc.) and rabbit anti-PDE10 (1:500; FabGennix Inc.), respectively, in TBS-containing 5% FCS and 0.3% Triton X-100 for 48 h at 4°C. The simultaneous visualization of the different primary antisera was performed with a mixture of secondary antibodies specific for the appropriate species IgG (rabbit, mouse, goat; all from Jackson ImmunoResearch). In detail, Cy2- (1:400), Cy3- (1:1000), and

Cy5- (1:100) conjugated IgGs were diluted in TBS supplemented with 5% FCS and 0.3% Triton X-100 and the mixture was applied for 2 h at room temperature.

TH-double immunofluorescence was performed using antibodies against mouse anti-TH (1:1,000; Chemicon) and goat anti-PDE2A (1:100; Santa Cruz Biotechnology, Inc.) or rabbit anti-PDE10 (1:500; FabGennix Inc.), respectively, in TBS-containing 5% FCS and 0.3% Triton X-100 for 48 h at 4°C. The visualization was performed with a mixture of DyLight™ 649-conjugated donkey anti-mouse IgG (1:100, color coded in green) and Cy3-conjugated donkey anti-rabbit or anti-goat (1:1,000; Jackson ImmunoResearch, each). Subsequently, all slices were stained with the nucleic acid probe Hoechst as described above.

After intensive washing and mounting on glass slides, all stained sections were dehydrated in a series of graded ethanol, processed through n-butylacetate and covered with Entellan.

Confocal Microscopy

Imaging of the fluorescence-labeled specimen was done using a confocal laser scanning microscope (LSM 510 Meta; Zeiss, Oberkochen, Germany). Excitation wave lengths were 633 nm (helium/neon2), 543 nm (helium/neon1), and 488 nm (argon). An ultraviolet laser (351/364 nm, Enterprise) was used to evoke the Hoechst fluorescence.

Treatment Procedure

According to the treatment procedure described in Heine et al. [12] (for a detailed time schedule, see fig. 3), the slice co-cultures were divided into different experimental groups and were treated with the following substances (final concentration): BAY60-7550 (10 μ M), ND7001 (10 μ M), MP-10 (10 μ M), and NGF (50 ng/ml). The experiments were performed in a blinded fashion, i.e. the compound identity was only provided after final data analysis.

The substances (except NGF) were dissolved in DMSO, sterilized by filters (0.2 μ m; Sarstedt, Nümbrecht, Germany) and finally added to 1 ml incubation medium. To exclude influences resulting from the solvent DMSO (final concentration 0.1%), an additional control group was used, treated only with ACSF (1%). The substances were applied at each medium change (4 times within the incubation period).

Tracing Procedure

At DIV 8, small biocytin crystals (Sigma-Aldrich) of similar size were placed on the VTA/SN part of the co-cultures (according to [10]) under binocular control (for detailed time schedule, see fig. 3). Cultures were left in contact with the crystals for 2 h to allow the uptake of biocytin, followed by careful rinsing with fresh incubation medium. Then, the cultures were reincubated with medium containing the substances to allow for anterograde transport of the tracer. After DIV 10, the cultures were fixed as described above and cut into 50- μ m thick slices by means of the vibratome.

The visualization of the traced axons was performed using a previously described protocol [10]. Briefly, the anterogradely transported tracer biocytin was labeled using the ABC-Elite Kit (1:50; Vector Laboratories, Inc.), in combination with nickel/cobalt-intensified DAB. After mounting on glass slides, all stained sections were handled as described above.

Fig. 4. **a** Overview of a DAergic nigrostriatal co-culture (VTA/SN+STR) using the biocytin-tracing technique in combination with DAB labeling. The small biocytin crystals had been placed onto the VTA/SN part of the co-culture; afterwards, the antero-grade tracer was transported from the cell bodies to the outgrowing fibers. The cell bodies (labeled black) of the VTA/SN and the respective outgrowing fibers, linking the border region and growing into the STR, are shown. **b–f** Examples of TH immunofluorescence labeling to characterize the expression of the DAergic marker in the co-cultures: TH-IR was observed on cell bodies and outgrowing fibers within the VTA/SN (**b**, **e**) as well as on fiber

processes in the border region (**c**, **d**) and the STR (**d**, **f**). In the border region, strong TH-IR was observed (e.g. arrowhead in **c**, **d**) on fibers, but also an increased number of ‘dot-like’ structures (e.g. arrows in **c**, **d**) was found. Finally, within the STR, a fine network of TH-positive fibers and ‘dot-like’ structures is characteristic (**f**). **g–i** The expression of β III-tubulin- (**g**), GFAP- (astroglial marker) (**h**), as well as MAP2- (neuronal marker) (**i**) positive fibers in the border region is demonstrated, together with the nuclear stain Hoechst (Hoe) 33342. Scale bars: **a** 10 μ m; **b–d**, **f** 20 μ m; **e** 50 μ m; **g–i** 10 μ m.

Automated Image Quantification

The quantification of outgrowing biocytin-traced fibers as well as of the TH-labeled fibers was performed within the border region of the co-cultures, i.e. the part where the two initially separated brain slices were grown together (fig. 4a). The following criteria were used for the selection of the biocytin-traced slices: (a) the tracer was correctly placed on the VTA/SN; (b) the major part of the VTA/SN was characterized by a dense network of biocytin-traced structures, and (c) no traced cell bodies had been observed in the target region STR (described in detail in [12]).

The raw images were obtained at 20× magnification from the whole border region by a usual transmitted light bright field microscope (Axioskop 50; Zeiss, Oberkochen, Germany) equipped with a CCD camera. Due to the usual problems with sensor noise and the large amount of light absorption and scattering inside the complex tissue structure of the stained co-culture slices, the axonal structures are typically blurred and image quality further suffers from inhomogeneous illumination. The border region is composed of both vesicular and fibrous structures (in the following only termed fibers) due to the dynamic transport properties of the tracer biocytin. Thus, a tailored image analysis pipeline had to be designed to quantify the fiber density in an automated manner. This procedure can be roughly split into two parts: image pre-processing and fiber detection.

Pre-Processing of the Images

At first, a deconvolution of the images was computed to reduce the blur and highlight the expected ‘true’ structures of the fibers. Since the real point-spread function of the microscope setup was lacking, the convolution kernel was estimated by a general Gaussian function (the width of the Gaussian is empirically set to 2 pixels), which is a reasonable guess in the general case of isotropic blur. The actual deconvolution was done with a damped least squares method (see [27]).

The deconvolution step inevitably leads to an increase in noise. Since noisy image structures would lead to a high number of false positive hits for subsequent detection of vesicular structures, a total variation-based smoothing step was applied to suppress single pixel noise in the images while preserving structures of interest [28].

Large-scale blurry structures in the background (due to structures from out-of-focus structures in the tissue) were subsequently removed by applying a top-hat transform, image structures larger than a given threshold were removed (a cutoff of 40 pixels was used here, see [29] for details).

Quantification of the Fiber Density

After pre-processing, the fiber structures appeared as well-defined bright regions that clearly stood out against the background. These regions were then extracted by Otsu thresholding [30].

Subsequently, the image area occupied by the fibers was measured to obtain a reasonable estimate of the underlying axonal density of the analyzed specimen. Precisely, the ratio of the number of foreground pixels (fibers) against the total number of pixels in the images was taken, giving the percentage of area occupied by fibers in the focal plane.

Statistics

Statistical analysis of the intracellular cGMP assay was performed using Student’s t test. Statistically significant differences

were considered at a p level <0.05 (* p < 0.05, ** p < 0.01, *** p < 0.001; the error bars indicate the SEM).

Statistical analysis of the quantitative data, comparing the different treatment groups, was performed using a Wilcoxon Rank Sum test. Each individual group was compared to the control group DMSO. To correct for multiple pairwise testing between groups, a conservative Bonferroni p value correction was applied. Statistically significant differences were considered at a p level <0.05 (* p < 0.05, ** p < 0.01, *** p < 0.001). For each group, the treatment experiments were repeated for at least three individual preparations.

Results

IC₅₀ Values of Selected PDE-Is

The potency and selectivity of BAY60-7550, ND7001, and MP-10 were confirmed against individual human purified proteins of each PDE family (except for PDE6, which was isolated from bovine retina), and the results are shown in table 1.

The data of the IC₅₀ values against PDE2A indicated the following ranking: BAY60-7550 > ND7001 > MP-10. The PDE2-I BAY60-7550 inhibited the target enzyme with high potency with an IC₅₀ value of 0.18 nM, and ND7001 was characterized as a PDE2-I with moderate affinity (IC₅₀ = 57 nM). Both compounds are highly selective against all other PDE isoforms. MP-10 inhibited the activity of PDE10 with high potency (IC₅₀ = 1.14 nM) as well as high selectivity towards each individual isoform of the PDE family. In general, PDE proteins show a high degree of sequence homology across different species, especially within their catalytic domains (for review, see [17, 18]). Thus, it is very unlikely that PDE-Is show species-specific effects with regard to their inhibitory activity. We have tested, for example, the PDE2-I ND7001 in a reference experiment using rat and human PDE2 proteins and could not detect any significant differences in their inhibitory activity across species (data not shown).

Effects of PDE2-Is on Intracellular cGMP Level

The PDE2-Is BAY60-7550 and ND7001 had been selected for the cellular cGMP assay. Measurement of cGMP levels in HEK293 cells, stably transfected with the full-length sequence of human PDE2A, confirmed that PDE2-I BAY60-7550 (fig. 5a) and ND7001 (fig. 5b) increased cGMP levels in a dose-dependent manner. In the presence of SNP (100 μM), these compounds significantly increased cGMP levels as compared to the control group. These findings suggest that PDE2 may play a major role in the degradation of cGMP under conditions of

Fig. 5. Dose-dependent effects of PDE2-Is BAY60-7550 (**a**) and ND7001 (**b**) on intracellular cGMP levels in a PDE2-HEK293 cell line. BAY60-7550 and ND7001 (in the presence of the GC stimulator SNP) increased the intracellular cGMP level in a dose-dependent fashion. Values are shown in fmol cGMP per well. The

error bars indicate SEM. The numbers of independent experiments per compound were: BAY60-7550 ($n = 8$) and ND7001 ($n = 4$), performed in duplicates. Differences to SNP were considered to be statistically significant according to a p level < 0.05 (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

GC stimulations. The numbers of independent experiments per compound were: BAY60-7550, $n = 8$, and ND7001, $n = 4$, performed in duplicates.

Characterization of the Organotypic Co-Cultures (VTA/SN+STR)

In the present study, the VTA/SN and the STR, main parts of the DAergic nigrostriatal projection system, were prepared from P1–5 old rats, a period during which the rat exhibits an adult localization of the neurons, although the axonal network is still immature [31]. Following fixation of the cultures at DIV 10, the slices were characterized using different labeling techniques.

Biocytin Tracing

The morphology of the biocytin-traced cell bodies as well as the outgrowth of the fibers were analyzed in treated as well as untreated control co-cultures. An example of an untreated biocytin-traced co-culture is shown in figure 4a. In general, numerous biocytin-marked fibers (labeled in black) originating from the VTA/SN, ramified and crossed the border region between the two brain slices. Moreover, their fiber processes were observed to grow into the striatal part of the culture. The fiber pathways of the VTA/SN+STR co-cultures developed an innervation pattern similar to that found *in vivo* [10]. These findings are in accordance with other studies, showing that the projections of axons to their targets follow special stereo-

typical routes during the development of the central nervous system and that selected guidance molecules support this innervation [32–34].

Immunohistochemistry/Immunofluorescence

The expression of TH, a marker for DAergic neurons, was shown using immunofluorescence labeling in combination with confocal laser scanning microscopy. The results indicate the presence of TH-positive cell bodies and fibers in the VTA/SN (fig. 4b, e), whereas the expression of the marker was restricted to fibers within the border region (fig. 4c, d) and the STR (fig. 4d, f). Fluorescence images illustrating the differences in the expression of TH immunoreactivity (IR) in the respective anatomical parts of the DAergic system are shown in figure 4b–d. The pictures especially exemplify the expression of the ‘dot-like’ structures in the border region and the target area (also observed after biocytin tracing, see fig. 6). Furthermore, antibodies against neuronal markers, i.e. β III-tubulin and MAP2 and the astroglial marker GFAP, were used to prove the existence of neuronal processes and glial cells, especially within the border region of the co-cultures. A positive IR for all investigated markers was observed in VTA/SN and STR. Examples are shown in figure 4g–i, where the existence of β III-tubulin- (fig. 4g), MAP2- (fig. 4i), and GFAP- (fig. 4h) labeled fibers spanning the border region and, thus, linking the two slices could be verified. These results are consistent with the results

Fig. 6. Results of the biocytin tracing after substance treatments are demonstrated within the border region of the co-cultures. Compared to control conditions applying ACSF (**a**) and DMSO (**b**) an increase in biocytin-positive fibers in PDE2-Is-treated co-cultures using BAY60-7550 (**d**) and ND7001 (**e**) was observed.

These effects were comparable with the effect evoked by the growth factor NGF (**c**). Co-cultures treated with MP-10 (**f**) were characterized by few biocytin-traced innervations within the border region. Scale bar: **a-f** 20 μ m.

found in our previous studies, demonstrating the expression of neuronal and glial fiber connections within VTA/SN + prefrontal cortex co-cultures [12].

Further light microscopic and triple immunofluorescence labeling studies with specific antibodies directed against PDE2A and PDE10 were performed to demonstrate the expression of these isoforms. Examples are shown in figure 7.

The light microscopic data indicated the expression of PDE2A on cell bodies and fibrous structures in the STR and VTA/SN (data not shown) and correlate with the immunofluorescence data. The immunofluorescence labeling was predominately found at the plasma membrane as well as in the cytoplasm (an example for the STR is given in fig. 7a–c, arrows). Moreover the co-localization of the PDE2A-IR on MAP2-immunopositive cells could be observed (example for VTA/SN; fig. 7f–h, arrows). We did not find PDE2-positive fibers in the border region (an example is shown in fig. 7d, e). Double immunofluorescence experiments using TH and PDE2A indicated no co-expression on cell bodies or fibers in all parts of the studied co-cultures, as shown in figure 7i–m.

Light microscopic images also confirmed the evidence of PDE10-marked cell bodies and fibers in the STR and the VTA/SN. Additional investigations using double immunofluorescence labeling with antibodies against TH and the PDE10 isoform indicated the expression of PDE10 on cell bodies and single fibers. On the other hand, no co-expression with TH-positive fibers (STR, VTA/SN) or the localization on TH-positive neurons (VTA/SN) could be found (fig. 7n–q).

The obtained results thus clearly underline the expression of the investigated isoforms in the VTA/SN+STR co-cultures during development.

Treatment of Organotypic Co-Cultures with PDE2- and PDE10-Is

Qualitative Results of the Fiber Growth (Biocytin Tracing)

Further co-cultures (VTA/SN+STR) had been used to study the influence of selected PDE-Is, with different subclass specificities, on fiber growth and sprouting. All experiments were performed in a blinded fashion, i.e. the compound identity was provided after final data analysis.

Fig. 7. Confocal images of multiple immunofluorescence labeling using antibodies against PDE2A (**a–m**) and PDE10 (**n–q**) in combination with MAP2 (neuronal marker), TH (DAergic marker), and Hoechst (Hoe) are shown. **a–h** PDE2A is expressed in the STR and VTA/SN especially on cell bodies (plasma membrane, cytoplasm; co-expression with MAP2 is indicated by arrows). No labeling was found on fibers in the border region (example given in

d, e). The double-labeling experiments with antibodies against TH indicated neither co-localization of PDEA2 and TH in the STR, VTA/SN, nor in the border region (**i–m**). PDE10 was found to be expressed on cell bodies and also on fibers (indicated by arrows in **n–q**), but no co-localization was found with TH-positive structures in the studied regions. Scale bars: **a–c** 5 μm ; **d–h** 10 μm ; **i** 20 μm ; **j, k** 20 μm ; **l–o** 10 μm ; **p, q** 20 μm .

Fig. 8. **a–d** TH-positive fibers after substance treatment (**a** ACSF; **b** NGF, **c** DMSO, **d** BAY60-7550 solved in DMSO) within the border region of the co-cultures are shown (scale bar: **a–d** 20 μ m). **e** Results of automated image quantification to measure the density of the TH-positive fibers in the border region of differently treated co-cultures in comparison to control conditions. After TH labeling, the fiber density was quantified, taking the ratio of the number of foreground pixels (fibers) over the total number of pixels in the images to reveal the size of the area occupied by fibers. The box-whisker plots represent the empirical distribution of the fiber densities in the different groups (the vertical axis corresponds to the percentage of image pixels belonging to labeled fibers). Compared with ACSF and DMSO, the growth factor NGF induced a significant increase in TH-positive fibers in the studied co-cultures. In contrast, the PDE2-I BAY60-7550 evoked only a small but significant increase of the number of TH-positive fibers, in comparison with the solvent DMSO. The numbers of analyzed images per group are: ACSF ($n = 8$), DMSO ($n = 20$), NGF ($n = 33$) and BAY60-7550 ($n = 94$). Differences were considered statistically significant at a p level < 0.05 (* $p < 0.05$, *** $p < 0.001$).

As described in the Methods section, the co-cultures were treated 4 times with the respective substances, over an incubation time of 10 days and, afterwards, the outgrowth of the biocytin-traced fibers in the border region was analyzed.

Differences in fiber outgrowth within the border region could be observed for the different treatments, and examples of these results are illustrated in figure 6. An increase in biocytin-positive fibers was apparent in PDE2-I- (e.g. BAY60-7750, fig. 6d) treated co-cultures in comparison to control conditions (ACSF, fig. 6a; DMSO, fig. 6b). The growth factor NGF evoked an effect comparable to the PDE2-Is under study (fig. 6c). Co-cultures treated with MP-10, an inhibitor of the PDE10, were characterized by fewer biocytin-traced innervations within the border region compared to the effects induced by the PDE2-Is (fig. 6f). Furthermore, the ‘dot-like’ structures

are of particular interest. They could be observed in the border region and the target region (STR) after substance treatment. This expression was also found after TH labeling of outgrowing fibers (see also fig. 4, 8). A significant increase in the number of puncta was noticeable after treatment with BAY60-7550.

Quantification of Fiber Density (Biocytin Tracing)

Starting from light microscopic images, the density of the biocytin-positive fibers was measured for all treatment groups in the border region of the co-cultures using the automated image quantification described above. The respective results of the tested substances are shown as box-whisker plots in figure 9. A significant increase in neuronal fiber outgrowth after application of the PDE2-Is BAY60-7550 and ND7001 was observed, with BAY60-7550 exhibiting the strongest effect. These results were of

Fig. 9. Automated image quantification to measure the fiber density in the border region of different treated co-cultures in comparison to control conditions. After biocytin tracing, the fiber density was quantified, taking the ratio of the number of foreground pixels (fibers) over the total number of pixels in the images to reveal the size of the area occupied by fibers. The box-whisker plots represent the empirical distribution of the fiber densities in the different groups (the vertical axis corresponds to the percentage of image pixels belonging to traced fibers). The analysis revealed a significant increase in neuronal fiber outgrowth after application of PDE2-Is BAY60-7550 and ND7001, with effects comparable to the stimulation capacity of NGF. The inhibitor of PDE10, MP-10, caused a significantly lower fiber density in the border region, similar to the results of ACSF and DMSO. The numbers of analyzed images per group were: ACSF (n = 22), DMSO (n = 62), NGF (n = 41), BAY60-7550 (n = 101), ND7001 (n = 103), and MP-10 (n = 76). Statistically significant differences were considered at a p level <0.05 (***) p < 0.001).

comparable effect size as for the neurotrophic factor NGF. MP-10 (PDE10-I) caused a clearly lower fiber density in the border region, akin to the effects observed in the control groups. Moreover, no significant differences in fiber density could be observed between the ACSF and the DMSO control group, thus excluding adverse effects resulting from the solvent DMSO. The numbers of analyzed images per group are: ACSF (n = 22), DMSO (n = 62), NGF (n = 41), BAY60-7550 (n = 101), ND 7001 (n = 103), and MP-10 (n = 76).

Treatment of Organotypic Co-Cultures with PDE2-I and NGF – Characterization of DAergic Fibers Qualitative Results of the Fiber Growth (TH Immunolabeling)

Treatment of the co-cultures with ACSF, NGF, DMSO, and BAY60-7550 (solved in DMSO) and immunolabeling with antibodies against TH indicated differences in the

expression of TH-positive fibers in the border region (examples are shown in fig. 8a–d). Especially the effect of NGF as trophic factor was clearly visible.

Quantification of the Fiber Density (TH Immunolabeling)

Moreover, the density of the TH-positive fibers was analyzed and quantified for all treatment groups as described above. The results are shown in figure 8e. The growth factor NGF induced a significant increase in TH-positive fibers in the studied co-cultures as compared to ACSF and DMSO. In contrast, the PDE2-I BAY60-7550 evoked a smaller (in comparison to the biocytin tracing) but statistically significant increase in the density of TH-positive fibers under these conditions (as compared with the DMSO group). The observed difference in effect size of BAY60-7550 treatment between biocytin tracing and TH labeling suggests an involvement of additional neurotransmitter populations, other than the TH-positive (DAergic) subgroup. Although small differences between DMSO and ACSF exist, they were not found to be statistically significant.

The numbers of analyzed images per group are: ACSF (n = 8), DMSO (n = 20), NGF (n = 33), and BAY60-7550 (n = 94). Differences were considered statistically significant at a p level <0.05 (* p < 0.05, ** p < 0.01, *** p < 0.001).

Discussion

The characterization of determinants of innervation patterns and the activities of growth promoting factors are of special interest for the development of clinical strategies to promote fiber development and, thereby, the regeneration of neuronal circuits. Using the ex vivo model of organotypic slice co-cultures, consisting of the VTA/SN and the STR, in combination with biocytin tracing, TH immunolabeling, and subsequent automated image quantification, it was shown that substances with the potential to inhibit PDE2 (BAY60-7550, ND7001) were able to increase neuronal fiber outgrowth, in contrast to the studied PDE10-I (MP-10) and the control substances ACSF and DMSO.

The data suggest a role for PDE2-Is, possibly mediated via cGMP, in the induction of fiber outgrowth in the nigrostriatal projection system. In PDE2-transfected HEK293 cells, a dose-dependent increase of intracellular cGMP levels in response to PDE2-Is, the previously described reference compounds BAY60-7550 and ND7001,

could further be shown. These data indicate that inhibition of PDE2 results in a dose-dependent augmentation of intracellular cGMP levels.

In vivo studies on rats have shown that DAergic neurons emerged around embryonic day 10, with the first nerve fibers appearing in the STR around embryonic days 14–15. The innervations are completed at about P15 [35]. Thus, the situation in the established ex vivo model strongly correlates with the developmental processes of physiological neuronal circuits. The observed neurite outgrowth pattern is consistent with our own previous data obtained under in vivo conditions [10].

PDEs are involved in distinct tasks in the brain, both in the mature rat brain and at embryonic as well as post-natal stages when significant developmental and maturational events are in progress [19, 20, 36]. An increase in PDE activity was reported during development of the brain from fetus to adulthood [37, 38]. Our data indicate an expression of PDE2A and PDE10 on cells in the DAergic co-culture preparations, consequently underlining a role of these isoenzymes in early development.

Hydrolyzing PDE families have distinct expression patterns within the central nervous system. For instance, striatal medium spiny neurons express mRNA for the PDE1B, 2A, 4B, 7B, 9A, and 10A isoforms (for references, see [39, 40]). A prominent PDE2A-IR was found besides the STR in the isocortex, hippocampus, and basal ganglia. Moreover, a high expression was observed in the SN and VTA on cell bodies and terminals [20, 36], which is supported by the findings presented in this work. PDE10 mRNA and protein are highly enriched in medium spiny neurons in the STR, within the cell bodies and dendrites as well as on SN axons [22, 41, 42]. PDE10A is associated with post-synaptic membranes of these medium spiny neurons and their dendrites and spines [43].

Under physiological conditions, the dual substrate enzyme PDE2(A) plays a critical role in the feedback control of cellular cAMP and cGMP levels, resulting in acute and long-term changes of neuronal functions. The basal PDE2 activity in neurons is low, but it is stimulated by an acute increase in cGMP following GC activation during signal transduction. In detail, a characteristic feature of PDE2 is the positive cooperativity with the substrate cGMP due to an allosteric cGMP binding site at N-terminal domains [40, 44]. This cyclic nucleotide stimulates cAMP degradation such that higher concentrations of cGMP inhibit cAMP hydrolysis and low levels of cGMP enhance the rate of cAMP hydrolysis [39, 44]. Besides the described features of PDE2, also special GAF domains of PDEs 5, 6, 10, and 11 bind cGMP and may regulate the catalytic activity

or protein-protein interactions (for references, see [45]). As a consequence, inhibition of PDE2 selectively increases cGMP and cAMP in brain active synapses [19, 44]. Incubation of cultured neurons and hippocampal slices with the selective PDE2-I BAY60-7550 resulted in increased cGMP levels [44]. This is in accordance with our present data, indicating a dose-dependent increase of intracellular cGMP levels in response to the investigated PDE2-Is obtained in the HEK cell line. Inhibition of the PDE10A causes also increased levels of cAMP and cGMP [40], with the difference that the regulation of the two cyclic nucleotides by PDE10A is independent [22].

The involvement of cAMP/cGMP during neuronal development in synaptic plasticity and memory formation has been described [19, 36, 44]. Recent data suggest a role of cAMP (via cAMP-dependent protein kinase) in neurogenesis and synaptogenesis during neuronal differentiation in NG108-15 cells [14]. The particular role of exchange proteins directly activated by cAMP (Epac) will be of specific interest for further studies as they are known to be involved in mediating cAMP responses. These are implicated in various cellular processes such as integrin-mediated cell adhesion and cell-cell junction formation or influencing, for example, cortical actin cytoskeleton (for review, see [46]).

cGMP can regulate directional guidance of growth cones [13, 47] and is involved in the outgrowth of cortical neurons during maturation [32]. The observed trophic effects clearly show that inhibitors of PDE2 are able to modulate neuronal fiber outgrowth in VTA/SN+STR co-cultures in contrast to the studied PDE10-I (MP-10), suggesting an involvement of PDE2, via cGMP signaling, in synaptic plasticity. Data from Boess et al. [44] confirm that inhibition of PDE2, e.g. by the selective PDE2-I BAY60-7550, increases cGMP/cAMP in cultured neurons and hippocampal slices, resulting in enhanced long-term potentiation of synaptic transmission, which is thought to be a key factor implicated in pro-cognitive activity without affecting basal synaptic transmission. A role of PDE2A activity in memory processes is further supported by the results of Song et al. [47] and Rutten et al. [19] showing that BAY60-7550 reportedly improves performance of rodents in recognition memory tasks.

Inhibition of PDE10 promotes effects modulating basal ganglia function in ways that suggest a particular therapeutic utility in the treatment of psychosis in schizophrenia [22, 23, 48]. It has also been shown that PDE10-I increases phosphorylation of key cAMP-dependent substrates, such as cAMP response element-binding protein, extracellular receptor kinase, or, more recently, the

AMPA-receptor GluR1 subunit [48–51]. Sotty et al. [51] further suggest a modulatory role of PDE10A on mesolimbic DAergic neurotransmission, via the D1-regulated feedback loop controlling midbrain DAergic neuronal activity. Therefore, investigating its modulatory role to affect the confluence of the corticostriatal glutamatergic and the midbrain DAergic pathways will be of particular interest.

The regulation of cAMP synthesis by DA and other neurotransmitter receptors has been extensively studied. But the importance of cAMP degradation by PDEs and the precise roles of each PDE isoform in DAergic signaling are not yet completely understood, owing to the diversity of PDE isoforms expressed in the STR and the complexity of their potential regulation (for review, see [24]). The authors conclude that the inhibition of PDEs upregulates cAMP/PKA signaling in three neuronal subtypes, resulting in (a) the stimulation of DA synthesis at DAergic terminals, (b) the inhibition of D2 receptor signaling in striatopallidal neurons, and (c) the stimulation of D1 receptor signaling in striatonigral neurons. However, it should be noted that the striatopallidal system as well as the corticostriatal glutamatergic projection are not constituents of the organotypic slice co-culture system used in the present study – possibly causing the different inhibitory effects of the investigated PDE-Is (especially for PDE10-I). However, the mechanism of inhibition responsible for the observed effects of the two kinds of PDE-Is needs further experimental investigations, especially the role of PDE10 to affect the corticostriatal glutamatergic and the midbrain DAergic pathways.

Finally, in the present study, the growth-promoting effect of the PDE-Is was compared with the stimulating capacity of NGF, which elicited a significant increase in fiber outgrowth intensity. Neurotrophins, like NGFs and their receptors, are the major regulators of neuronal survival during development, after injury or nervous system diseases [52]. NGF promotes neurite extension through a cAMP-independent signaling pathway involving Ras, PKC, and extracellular signal-regulated protein kinase [53]. It has been shown that cAMP also induces neuronal differentiation in PC12 cells and that the co-treatment of NGF and dibutyryl cAMP exhibits synergistic effects on neurite outgrowth [54]. Moreover, NGF increases cGMP level and activates PDEs in PC12 cells [55]. Also other trophic molecules have been shown to enhance survival and neurite outgrowth of mesencephalic DAergic neurons in single cell cultures or slice cultures [56–58]. Treatment of organotypic slice co-cultures with BDNF resulted in enhanced survival of TH-positive neurons and an increased growth of TH-positive fibers into the striatal

tissue [58]. GDNF has been shown to act as a powerful chemoattractant for outgrowing DAergic axons in organotypic co-cultures [33, 59] as well as under in vivo conditions [60]. Finally, Thompson et al. [60] showed that axonal regrowth in the nigrostriatal trajectory can be further enhanced by the addition of trophic support by overexpression of GDNF in the striatal target.

In summary, cAMP/cGMP are involved in survival, repair, and remodeling in the nervous system both during development and after injury, and PDE2 appears to act as an important regulator in these processes. In the present study, we were able to analyze the specific potential of PDE-Is in mediating the growth of defined fiber connections under controlled conditions, using the ex vivo model of organotypic slice co-cultures of the nigrostriatal system in combination with an automated image quantification. Hence, our results strongly support a trophic role of PDE2-Is in shaping interneuronal connections during the early phase of neuronal development. The characterization of the molecular and functional basis underlying the observed effects of PDE2-Is in regeneration and repair of disrupted neuronal circuits will be an important next step to foster the development of improved ways for the treatment of neurological disorders.

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