Original Paper

Neuro-endocrinology

Neuroendocrinology 2011;94:113–123
DOI: 10.1159/000327138

Received: October 22, 2010
Accepted after revision: March 5, 2011
Published online: April 7, 2011

Phosphodiesterase 10A in the Rat Pineal Gland: Localization, Daily and Seasonal Regulation of Expression and Influence on Signal Transduction

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Key Words
Phosphodiesterase 10A · Circadian system · Pineal gland · Norepinephrine · cAMP · cGMP · Arylalkylamine N-acetyltransferase

Abstract
The cyclic nucleotide phosphodiesterase 10A (PDE10A) is highly expressed in striatal spiny projection neurons and represents a therapeutic target for the treatment of psychotic symptoms. As reported previously [J Biol Chem 2009; 284:7606–7622], in this study PDE10A was seen to be additionally expressed in the pineal gland where the levels of PDE10A transcript display daily changes. As with the transcript, the amount of PDE10A protein was found to be under daily and seasonal regulation. The observed cyclicity in the amount of PDE10A mRNA persists under constant darkness, is blocked by constant light and is modulated by the lighting regime. It therefore appears to be driven by the master clock in the suprachiasmatic nucleus (SCN). Since adrenergic agonists and dibutyryl-cAMP induce PDE10A mRNA, the in vitro clock-dependent control of *Pde10a* appears to be mediated via a norepinephrine → β-adrenoceptor → cAMP/protein kinase A signaling pathway. With regard to the physiological role of PDE10A in the pineal gland, the specific PDE10A inhibitor papaverine was seen to enhance the adrenergic stimulation of the second messenger cAMP and cGMP. This indicates that PDE10A downregulates adrenergic cAMP and cGMP signaling by decreasing the half-life of both nucleotides. Consistent with its effect on cAMP, PDE10A inhibition also amplifies adrenergic induction of the cAMP-inducible gene arylalkylamine N-acetyltransferase *(Aanat)* which codes the rate-limiting enzyme in pineal melatonin formation. The findings of this study suggest that *Pde10a* expression is under circadian and seasonal regulation and plays a modulatory role in pineal signal transduction and gene expression.

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Introduction

In most mammals, daily and seasonal changes in lighting conditions may be responsible for alterations in behavior and body physiology (for reviews, see [2–4]). These effects are mediated through a master clock located within the hypothalamic suprachiasmatic nucleus (SCN), which itself is entrained by the light-dark cycle through neural inputs from the retina (for a review, see [5]). This clock controls multiple circadian outputs, including the daily rhythm observed in the formation of the

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0028–3835/11/0942–0113$38.00/0

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hormone melatonin (for a review, see [6]) in the pineal gland. The transmission of signals from the SCN to the pineal gland takes place via a neural pathway that terminates in postganglionic superior cervical ganglia fibers which pervade the organ (for a review, see [7]). Under the master clock's influence, norepinephrine (NE) is released from the intrapineal nerve terminals during the night. By binding to α_{1B}- and β_1-adrenoceptors in pinealocytes, NE activates the enzymes adenylate cyclase and guanylate cyclase and hence results in formation of the cyclic nucleotides cAMP and cGMP (for reviews, see [8, 9]). Cyclic AMP – via protein kinase A and phosphoCREB – subsequently induces transcription of various genes including the arylalkylamine N-acetyltransferase *(Aanat)* gene that codes the penultimate enzyme (EC 2.3.1.87) in the formation of the hormone melatonin (for reviews, see [10, 11]). Cyclic GMP influences pineal function through posttranslational modification of proteins involved in pineal signal transduction [12–14].

Due to the central roles of cyclic nucleotides in the circadian/adrenergic regulation of the pinealocytes, phosphodiesterases (PDEs) – by promoting the inactivating of cyclic nucleotides – are of the highest putative significance for pineal function. In the rat pineal gland, PDE activities for cAMP and cGMP have been shown to display daily rhythms with peak values at night [15, 16]. Circadian regulation of the cAMP-specific PDE4B partly or fully accounts for the daily rhythm in the pineal PDE activity for hydrolysis of cAMP [17], but does not explain the daily alterations in pineal PDE activity for degradation of cGMP. Accordingly, further types of PDE other than PDE4B presumably form part of the molecular basis producing diurnal changes in PDE activity. Further clues concerning this gap in PDE activity are provided by evidence showing that the transcript amount of the dual-substrate-specific PDE10A displays daily changes with elevated values at night [1].

PDE10A is the single member of the PDE10 family, 1 of the 11 known PDE families, which are each characterized by differential sequence homology within the catalytic domain and affinity for cAMP and/or cGMP [18–20]. PDE10A exhibits properties of both a cAMP PDE and a cAMP-inhibited cGMP PDE. It hydrolyzes cAMP and cGMP with apparent K_m values of 0.05 and 3.0 μM, respectively. Of the 11 PDE families, PDE10A has the most restricted distribution. In the brain, the PDE10A expression pattern is highly conserved throughout the mammals [21]. Its most pronounced expression is found in the ventral and dorsal striatum, with a less extensive expression being evident in other areas of the brain including the hippocampus [22, 23]. Within the striatum, PDE10A is localized in the medium-sized spiny neurons [23–25] at the confluence of the midbrain dopaminergic and corticostriatal glutaminergic pathways. Consistent with the functional roles of these neurons and the targeting projections, *Pde10a*$^{-/-}$ mice are characterized by their altered locomotor activity [26, 27] and PDE10A inhibitors such as papaverine, TP-10 and MP-10 provide putative benefit in the treatment of psychotic symptoms ([28, 29]; for a review, see [30]).

In the present study, the *Pde10a* gene is shown to represent a target through which the SCN may partly influence signal transduction and *Aanat* expression in the pineal gland. This finding is of broader importance because it raises the possibility that PDE10A plays a role not only in motor function and the psychomotor domain, but also in the circadian system.

Materials and Methods

Animals

Animal experimentation was carried out in accordance with the European Communities Council Directive (86/609/EEC). Adult male and female Sprague-Dawley rats (body weights 150–180 g) were kept under standard laboratory conditions (illumination with fluorescent strip lights, 200 lx at cage level during the day and dim red light during the night; 20 ± 1°C; water and food ad libitum) under various light/dark (LD) cycles (LD 8:16, LD 12:12 and LD 16:8) for 3 weeks. Thereafter, the animals were killed in LD cycles or kept for one cycle either under constant dark (DD) or constant light (LL) and killed during the next cycle. Animals were euthanized (carbon dioxide) at the indicated time points. The pineal glands were carefully removed and either processed for immunohistochemistry or immediately frozen in liquid N_2 for Western blotting and RNA extraction.

Organ Culture

Pineal glands taken from rats kept under LD 12:12 were cultured as described previously [31]. Briefly, pineal glands taken from rats at zeitgeber time (ZT; hours after light onset) four were pre-incubated in BGJ_b medium (Gibco, Karlsruhe, Germany) at 37°C under an atmosphere of 95% O_2 and 5% CO_2. After 24 h of incubation, pineal glands were transferred into fresh medium containing the pharmacological substances of interest and incubated for 15 or 30 min for determination of cyclic nucleotide concentrations and for 3 h for measurement of RNA levels.

Light-Microscopic Immunocytochemistry

Immunostaining was investigated in pineal glands taken at ZT6 and ZT12 from rats kept under LD 12:12 for 3 animals from three independent experiments. Labeling was performed using an indirect fluorescence method (for details, see Spiwoks-Becker et al. [33]) with the mouse anti-PDE10A monoclonal antibody (1:500) (1:250; clone 24F3.F11 directed against rat PDE10A [26, 33]) and the rabbit anti-RIBEYE/CtBP2 (1:1,000; BD Biosciences,

Table 1. Primer sequences for the genes investigated

Gene	Accession number	Primer sequence 5′ to 3′		Length of PCR product, bp
Pde10a	NM_022236	forward: reverse:	GACCTCGCACTGTACTTTG CTGGCCATAGTTTCGTCAC	151
Aanat	*U40803*	forward: reverse:	GAAGGGAGACAGCAGTTC GTCCTGGTCTTGCCTTTG	431
Gapdh	NM_017008	forward: reverse:	ATGACTCTACCCACGGCAAG CTGGAAGATGGTGATGGGTT	89

Heidelberg, Germany) as primary antibodies. The binding sites of the primary antisera were revealed by goat anti-mouse (Alexa 488; 1:500) or goat anti-rabbit (Alexa 594; 1:500; both from Molecular Probes, Eugene, Oreg., USA) as secondary antisera. At least three randomly chosen labeled sections were examined per pineal gland and photographed using a confocal laser scanning microscope (LSM SP5, Leica Microsystems, Heidelberg, Germany). Multiple pairs of optical sections were recorded at different depths throughout the tissue sections and merged into a single plane (Image-Pro Plus 5.0, Media Cybernetics, Silver Spring, Md., USA). Photoshop software (Adobe Photoshop 7.0, San Jose, Calif., USA) was used to edit single pictures and to assemble figures.

Immunoprecipitation and Western Blot

For immunoprecipitation of PDE10A, tissue was homogenized in 1 ml phosphate-buffered saline containing protease inhibitors. Insoluble material was pelleted. For antibody immobilization, protein A-agarose beads (30 μl bead volume; Invitrogen, Carlsbad, Calif., USA) were washed four times with mouse anti-PDE10A monoclonal antibody (1:250) [23] at 4°C. Cell extracts corresponding to 500 μg (pineal gland) and 50 μg (striatum) protein amounts were applied overnight to the antibody-coupled beads at 4°C. Bound proteins were recovered after extensive washes in homogenization buffer. For Western blot analysis, samples were loaded on 4–12% NuPAGE Novex Bis-Tris gels (Invitrogen), separated and then blotted onto nitrocellulose transfer membranes (Protran, Schleicher & Schüll, Dassel, Germany). For immunodetection, membranes were blocked in 5% skimmed milk powder and the mouse anti-PDE10A monoclonal antibody (1:500) was applied overnight at 4°C. The horseradish-peroxidase-coupled secondary antibodies (goat anti-mouse-HRP 1:10,000; Dianova, Hamburg, Germany) were visualized using an ECL detection system (GE Healthcare Amersham, Freiburg, Germany).

RNA Isolation

The RNA of three samples (each obtained by pooling three pineal glands) was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. The amount of extracted RNA was determined by measuring the optical density at 260 and 280 nm.

Reverse Transcription and Quantitative PCR

Extracted RNA (1 μg) was reverse-transcribed using 4 U Omniscript reverse transcriptase (Qiagen, Hilden, Germany) in a total volume of 20 μl, which also contained 2.0 μl 10× buffer (supplied with the transcriptase), 0.5 mM of each deoxynucleotide triphosphate, 10 U ribonuclease inhibitor and 1 μM oligo d(T) primer. A sample without the addition of RNA was routinely included as a control. The reverse transcription (RT) mixture was incubated at 37°C for 60 min to promote cDNA synthesis. The reaction was terminated by heating the samples at 95°C for 5 min. The cDNA was then diluted 1:10 in RNase-free water and 5 μl aliquots were used for the polymerase chain reaction (PCR).

Real time PCR was carried out in a total volume of 25 μl containing 12.5 μl ABsolute™ QPCR SYBR® Green Fluorescein Mix (Abgene, Hamburg, Germany), 0.75 μl of each primer (10 mM) each, 6 μl RNase-free water and 5-μl sample. The primer sequences for the genes investigated *(Pde10a* and *Aanat)* are listed in table 1. PCR amplification and quantification were performed in an i-Cycler (BioRad, Munich, Germany) as follows: denaturation for 3 min at 95°C, followed by 40 cycles of 30 s at 95°C, 20 s at 60°C and 20 s at 72°C. All amplifications were carried out in duplicate. The amount of RNA was calculated from the measured threshold cycles (C_t) using a standard curve. The transcript amount of glyceraldehyde-3-phosphate dehydrogenase *(Gapdh)* was constitutively expressed over the 24-hour period. Values were then normalized with respect to the amount of glyceraldehyde-3-phosphate dehydrogenase GAPDH mRNA.

Measurement of cAMP and cGMP

To determine adrenergically induced cAMP and cGMP accumulation, pineal glands were immediately sonicated at the end of the incubation period in 40 μl 5% trichloroacetic acid (TCA) and centrifuged at 1,500 *g* for 10 min. The supernatant was then extracted three times with 200 μl water-saturated diethyl ether in order to remove the TCA. The residual ether was removed by heating the samples at 70°C for 5 min. Subsequently, the samples were reconstituted with 60 μl 0.05 M phosphate buffer (pH 7.4). The cyclic nucleotide content of the samples was determined using commercially available ELISA (enzyme-linked immunosorbent assay) kits (Alexis, Gruenberg, Germany). The samples were assayed at different dilutions, each dilution being assayed in duplicate. In brief, samples or standard solution were incubated together with cyclic nucleotide-specific rabbit antibodies and a cyclic nucleotide-acetylcholinesterase conjugate (tracer) at appropriate dilutions for 18 h at 4°C in microwell plates that had been precoated with mouse monoclonal anti-rabbit antibody. After a washing step, the plates were developed with Ellman's reagent for

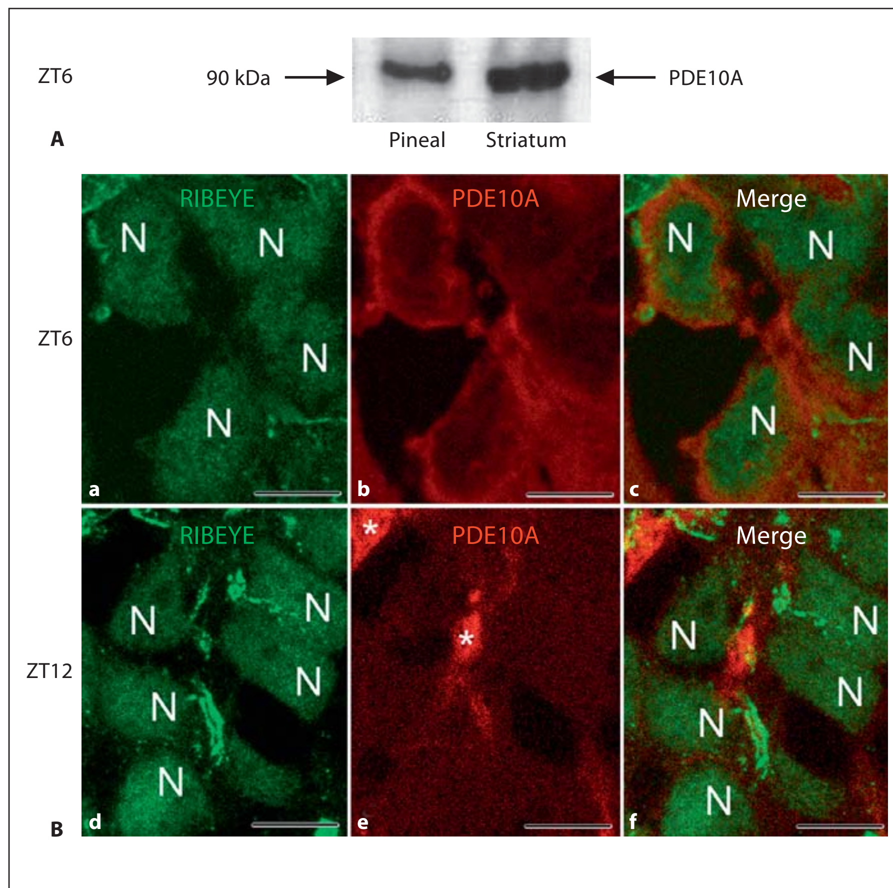

Fig. 1. Abundance and localization of PDE10A protein in the pineal gland of rats. **A** Western blot analysis of PDE10A protein in pineal gland at ZT6 (zeitgeber time 6). The monoclonal anti-PDE10A antibody recognizes a protein band of ~90 kDa characteristic for PDE10A [23] in the pineal gland (left lane) and striatum (right lane). The lanes were loaded with either PDA10A immunoprecipitated from 500 µg homogenate of pineal glands or 50 µg homogenate of striatum. **B** Confocal laser scanning micrographs of pineal gland at ZT6 (sections **a–c**) and ZT12 (sections **d–f**) labeled for RIBEYE/CtBP2 (sections **a**, **d**) and PDE10A (sections **b**, **e**). As seen in the merged image (sections **c**, **f**), RIBEYE/CtBP2-labeled cell nuclei (N, green) are surrounded by the PDE10A labeling of the cytoplasma (red). Note that PDE10A labeling of the cytoplasma is stronger at ZT6 (sections **b**, **c**) than at ZT12 (sections **e**, **f**). * Presumed glia cells. Bars = 5 µm.

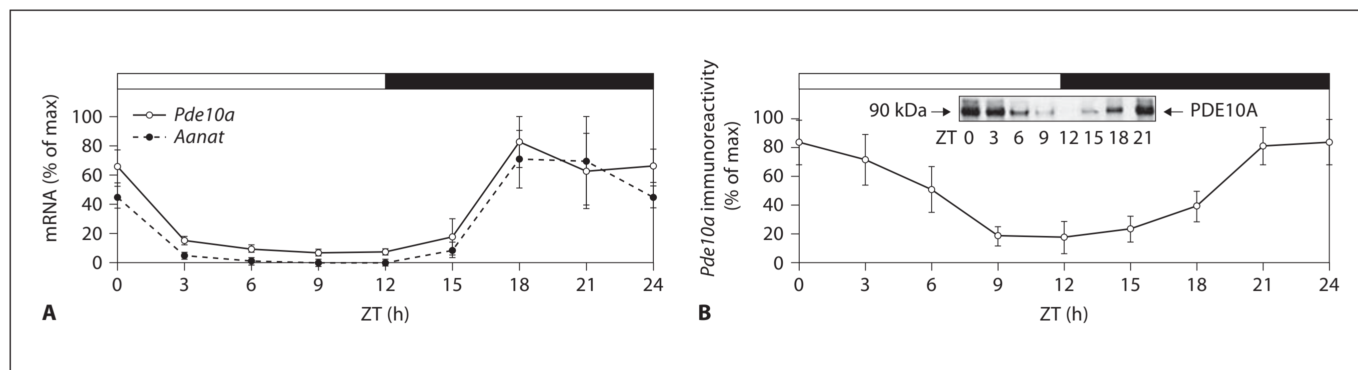

Fig. 2. Daily rhythm of *Pde10a* expression in the pineal gland of the rat. **A** Daily profiles in the PDE10A mRNA amount (open circles/solid lines) and AANAT mRNA amount (filled circles/dashed lines) under LD 12:12. ZT = zeitgeber time. The data were determined by real time PCR and normalized to the amount of GAPDH transcript. Maximum expression corresponds to 2.9 × 10^4 transcript copies of PDE10A mRNA and 2.2 × 10^6 transcript copies of AANAT mRNA, respectively. **B** Daily profile of PDE10A immunoreactivity in Western blot analysis. Data were obtained by densitometric measurement of the ~90 kDa band characteristic for PDE10A protein. The **inset** shows a representative Western blot analysis. In **A** and **B** solid bars indicate dark periods in LD 12:12. Data represent percentages of the overall maximal value. Each value is the mean ± SEM (n = 3; each derived from 3 (PCR) or 4 (Western blot analysis) pooled pineal glands).

90 min in the dark as described by the manufacturer. Plates were read at a wavelength of 405 nm. The concentrations of cyclic nucleotides were calculated by interpolation from a standard curve and expressed as pmol/pineal.

Statistical Analysis

All PCR data are given as the mean with SEM of three or more independent experiments. Statistical analyses were performed using Student's t test or Mann-Whitney U test for two groups and one-way ANOVA or nonparametric analysis of variance (Kruskall-Wallis test) for multiple groups. Based on the suggestion that ZT24 is equal to ZT0, cosinor analysis [32] was used to determine the acrophase (peak expression) and amplitude of oscillation (half the difference between the highest and lowest values). Statistical analysis was performed using SigmaStat (Version 3.10, Systat Software Inc., San Jose, Calif., USA), with the exception of Cosinor analysis which was performed using the statistic software 'R' (version 2.11.1, available free at www.r-project.org).

Results

Localization of PDE10A in the Pineal Gland

In the present study, the abundance and localization of the PDE10A protein in the pineal gland has been assessed (fig. 1). Western blot analysis (fig. 1A) revealed a protein band of ~90 kDa characteristic for PDE10A. A band of similar molecular weight was observed in extracts from rat striatum where the PDE10A protein has been demonstrated to be abundant [23]. Using confocal laser scanning microscopy (fig. 1B), the distribution of PDE10A in the pineal gland was also investigated. This was performed using double labeling immunohistochemical analysis for PDE10A and RIBEYE/CtBP2. Consistent with the presence of CtBP2 in the cell nuclei [33], the RIBEYE/CtBP2 antibody labeled the cell nuclei of pinealocytes (sections a and d in fig. 1B). Most of the CtBP2-labeled cell nuclei were seen to be surrounded by PDE10A immunoreactivity (section c in fig. 1B). This indicates that the PDE10A protein is primarily localized in the cytoplasma of pinealocytes. In addition to its presence in the cell somata, PDE10A immunoreactivity was occasionally also observed in structures which may represent glia cells (stars in section e of fig. 1B).

Daily Rhythms in PDE10A mRNA Levels

To investigate the daily regulation of the *Pde10a* gene, the levels of PDE10A transcript throughout the day were recorded (fig. 2A). Using quantitative PCR, the mRNA amount of PDE10A was found to undergo a daily regulation ($p = 0.01$) with an amplitude of 40.5% and acrophase (peak expression) at ZT 17.32 (i.e. 17.3 h after 'lights on'). The day/night ratio (ZT18 vs. ZT9) of the PDE10A mRNA amount was ~15-fold.

Aanat represents a prime example of a gene whose expression is driven by the master clock in the SCN. To normalize the experimental system used to previous studies and to compare the regulation of *Pde10a* with *Aanat* – in the same transcriptomes as those used for determination of PDE10A mRNA – the amount of AANAT transcript was measured. The daily course of mRNA amount was seen to be similar for *Pde10a* and *Aanat* ($p = 0.003$; amplitude: 36.5%; acrophase: ZT20.0), except that for *Aanat* the day/night ratio (ZT18 vs. ZT9) was higher (~160-fold).

Daily Rhythms in PDE10A Protein Levels

As with the transcript, the amount of PDE10A protein was seen to exhibit a daily rhythm ($p < 0.01$; amplitude: 47.6%) with a peak occurring at the early light phase (acrophase: ZT2.4) in Western blot analysis (fig. 2B). A comparison between the daily profiles in transcript amount (fig. 2A) and protein amount (fig. 2B) revealed that the latter is phase-delayed, with the temporal lag reflecting the time necessary to translate PDE10A mRNA into PDE10A protein. In agreement with the results obtained from Western blot analysis, PDE10A immunostaining in the cytoplasma of pinealocytes was more pronounced at ZT6 than at ZT12 in confocal laser scanning microscopy (fig. 1B).

Pineal PDE10A Is under Photoneural and Circadian Regulation

The increased PDE10A transcript amount at night suggests that *Pde10a* transcription may be dependent on light and dark phases. To test the hypothesis that *Pde10a* undergoes photoneural regulation, the influence of the lighting regime on the 24-hour profile in PDE10A mRNA was investigated (fig. 3A vs. 3B). This revealed that *Pde10a* expression is rhythmic under both lighting regimes (LD 8:16: $p = 0.02$, amplitude of 38.8%; LD 16:8: $p = 0.001$, amplitude of 51.4%) with nocturnal peak expression at an earlier ZT under LD 8:16 (acrophase: ZT17.2) (fig. 3A) than under LD 16:8 (acrophase: ZT19.2) (fig. 3B). Since the morning decrease occurred at the same time, irrespective of the lighting regime used, the duration of the PDE10A transcript elevation was increased under the lighting pattern with short photoperiods. The prolonged nocturnal transcription interval under short days may account for the increase in the morning level of PDE10A protein under short days (inset in fig. 3A) as compared to long days (inset in fig. 3B).

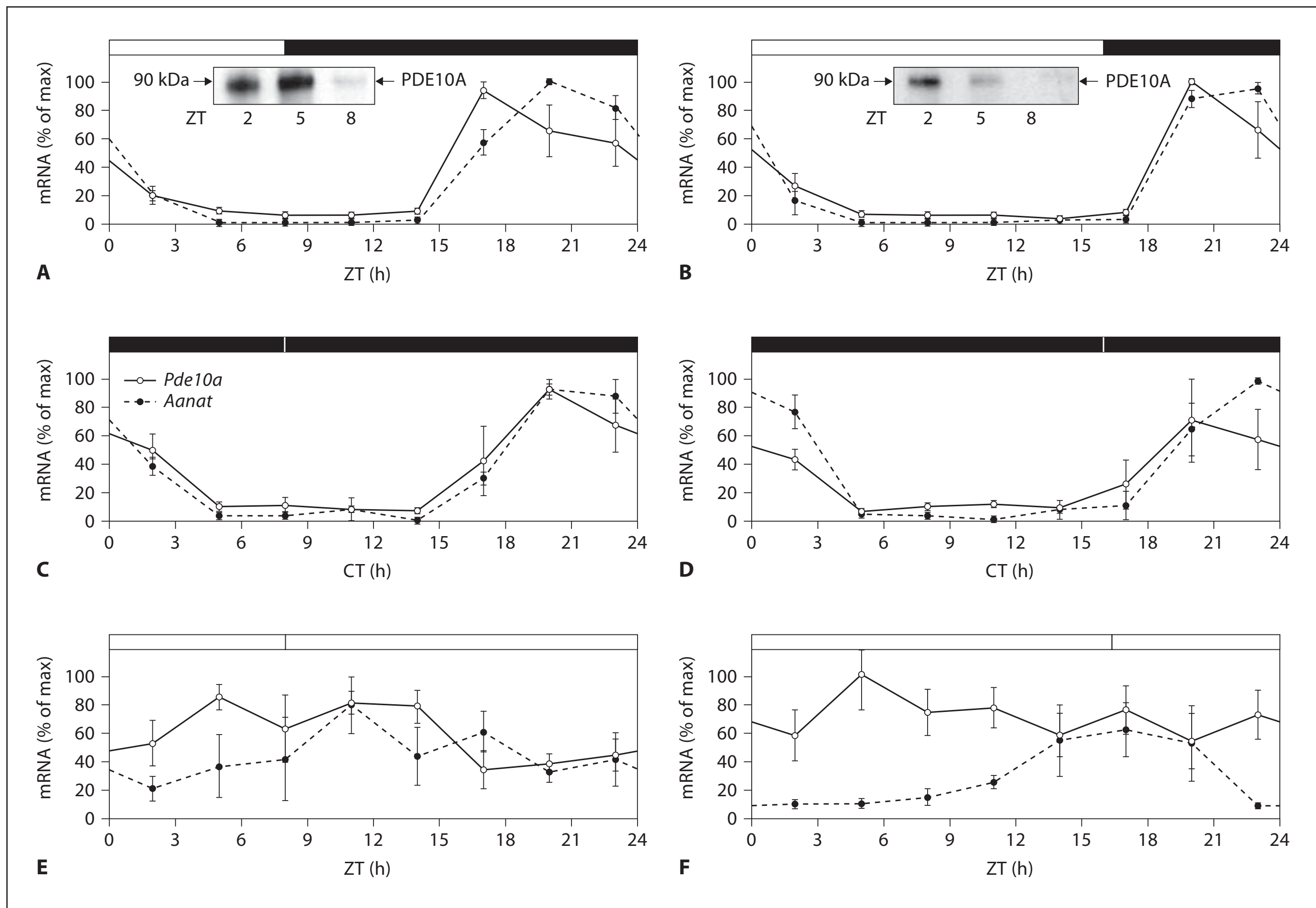

Fig. 3. Photoneural and circadian regulation of rat pineal *Pde10a* expression. Amounts of PDE10A mRNA (open circles, solid lines) and AANAT mRNA found (filled circles/dashed lines) under LD conditions (**A**, **B**), constant darkness (**C**, **D**) and under constant light (**E**, **F**). Before light treatment animals were adapted to either LD 8:16 (**A**, **C**, **E**) or LD 16:8 (**B**, **D**, **F**). ZT = zeitgeber time; CT = circadian time. The **insets** in **A** and **B** show representative Western blot analyses. The data were determined by real time PCR and normalized to the amount of GAPDH transcript. Data represent a percentage of the overall maximal value. Maximum PDE10A expression corresponds to 3.2×10^4 transcript copies under LD 8:16, 2.9×10^4 transcript copies under LD 16:8, 2.6×10^4 transcript copies under DD 8:16, 2.8×10^4 transcript copies under DD 16:8, 2.1×10^4 transcript copies under LL 8:16, and 1.9×10^4 transcript copies under LL 16:8. Maximum AANAT expression corresponds to 2.2×10^6 transcript copies under LD 8:16, 3.0×10^6 transcript copies under LD 16:8, 4.2×10^6 transcript copies under DD 8:16, 2.8×10^4 transcript copies under DD 16:8, 9.1×10^4 transcript copies under LL 8:16, and 1.8×10^5 transcript copies under LL 16:8. Each value is the mean ± SEM (n = 3; each derived from 3 (PCR) or 4 (Western blot analysis) pooled pineal glands).

The photoneural/photoseasonal regulation of the *Pde10a* gene may be mediated by the entrainment of the pacemaker in the SCN. To test this hypothesis, rats were kept in DD for one cycle (fig. 3C, D). Under these conditions the daily rhythm in the amount of PDE10A transcript was still seen, irrespective of whether the rats had previously been housed under LD 8:16 ($p < 0.05$; amplitude: 48.8%; acrophase: ZT19.3) (fig. 3C) or LD 16:8 ($p < 0.05$; amplitude: 39.0%; acrophase: ZT19.5) (fig. 3D). Thus, maintenance of the daily rhythm in the amount of the pineal PDE10A transcript did not require dark/light transitions but appears to be controlled by the SCN. The relevance of the SCN in the expressional control of the *Pde10a* gene was underlined by the finding that the clock-dependent stimulation of the pineal gland could be blocked by LL (fig. 3E, F). Under these conditions, the daily rhythm in the PDE10A mRNA amount was no longer evident, irrespective of whether the animals had pre-

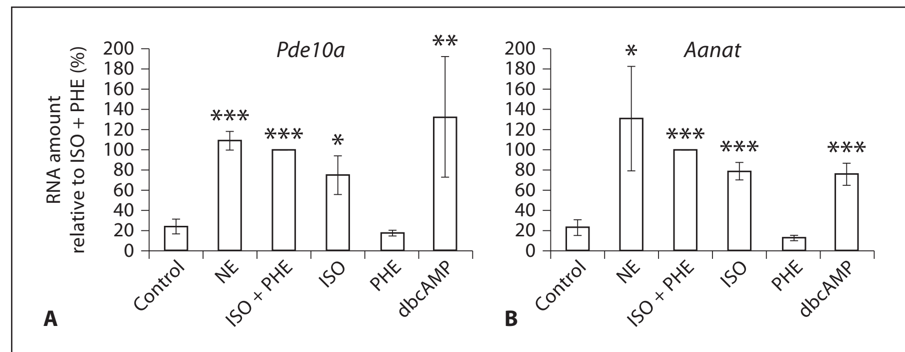

Fig. 4. In vitro effects of adrenergic agonists and the cAMP protagonist dbcAMP on the mRNA levels of PDE10A (**A**) and AANAT (**B**). Pineal glands were pre-incubated for 24 h and then treated with either NE (10^{-7} M), a combination of the β-adrenergic agonist ISO and the α-adrenergic agonist PHE (each 10^{-7} M), ISO (10^{-7} M), PHE (10^{-7} M) or dbcAMP (10^{-3} M) for 3 h. Data were determined by real-time PCR and normalized to the amount of GAPDH transcript. Data represent a percentage of the value obtained with ISO and PHE. Hundred percent expression corresponds to 1.4×10^4 transcript copies of PDE10A mRNA and 5.7×10^5 transcript copies of AANAT mRNA. Each value represents the mean ± SEM for three or more samples. * $p < 0.05$ compared with control, ** $p < 0.01$, *** $p < 0.001$ compared with control.

viously been housed under LD 8:16 (fig. 3E) or LD 16:8 (fig. 3F).

Consistent with the hypothesis that *Pde10a* expression is regulated by the clock in the SCN, the observed effects of the light conditions on the amount of PDE10A mRNA paralleled those seen for AANAT mRNA [34] (fig. 3).

Pineal Pde10a Expression Is Induced by Adrenergic Agonists and cAMP

The pineal gland is controlled by the endogenous clock and involves the action of the transmitter NE on α- and β-adrenergic receptors. To investigate the SCN-dependent/adrenergic regulation of the *Pde10a* gene, cultured pineal glands were treated with NE (10^{-7} M), a combination of the β-adrenergic agonist ISO and the α-adrenergic agonist PHE (each 10^{-7} M), ISO alone (10^{-7} M) or PHE alone (10^{-7} M) (fig. 4). The amount of PDE10A mRNA was seen to increase in response to treatment with NE ($p < 0.001$), ISO plus PHE ($p < 0.001$) or ISO alone ($p < 0.05$), but not with PHE alone. This is consistent with the conclusion that the endogenous clock in the SCN controls the expression of the *Pde10a* gene via the action of NE, primarily on β-adrenoceptors.

The question of whether an established second-messenger system of NE in the pineal gland, i.e. the cAMP → protein kinase A pathway, mediates the regulation of *Pde10a* transcription was further addressed by treating pineal glands with dibutyryl-cAMP (dbcAMP, 10^{-3} M), an agent acting as a cAMP mimic and activator of protein kinase A (fig. 4A). Dibutyryl-cAMP was seen to increase the accumulation of the PDE10A transcript ($p < 0.01$), and thus suggests that the SCN-dependent/adrenergic regulation of the *Pde10a* gene is mediated via the cAMP/protein kinase A pathway.

In agreement with this hypothesis the observed effects of pharmacological treatment on *Pde10a* expression were similar to those found for *Aanat* expression [35] (fig. 4).

PDE10A Inhibitor Papaverine Increases Adrenergically Stimulated cAMP and cGMP Accumulation

PDE10A is a dual-substrate PDE capable of hydrolyzing both cAMP and cGMP. To investigate a possible regulatory role of PDE10A in adrenergic cyclic nucleotide signaling in the pineal gland, the effect of the PDE10A-specific inhibitor papaverine (10^{-6} M, 10^{-7} M) [36] on the cyclic nucleotide responses to NE (10^{-7} M) was examined (fig. 5). The PDE10A inhibitor papaverine – even at a concentration of 10^{-7} M – enhanced the responses of both cyclic nucleotides to NE after 15 min of incubation ($p < 0.05$). This indicates that PDE10A, by decreasing the half-life of the cyclic nucleotides, dampens adrenergic cAMP and cGMP signaling in rat pinealocytes.

To obtain a measure of full PDE inhibition, the effect of the unspecific PDE inhibitor IBMX (3-isobutyl-1-methylxanthine; 10^{-4} M) was also determined. IBMX significantly increased NE-stimulated cAMP and cGMP formation after 15 min (cAMP: $p < 0.05$; cGMP: $p < 0.05$), with significantly higher values after 30 min of incubation (cAMP: $p < 0.05$; cGMP: $p < 0.05$) (fig. 5). Irrespective of the type of cyclic nucleotide and the time of incubation

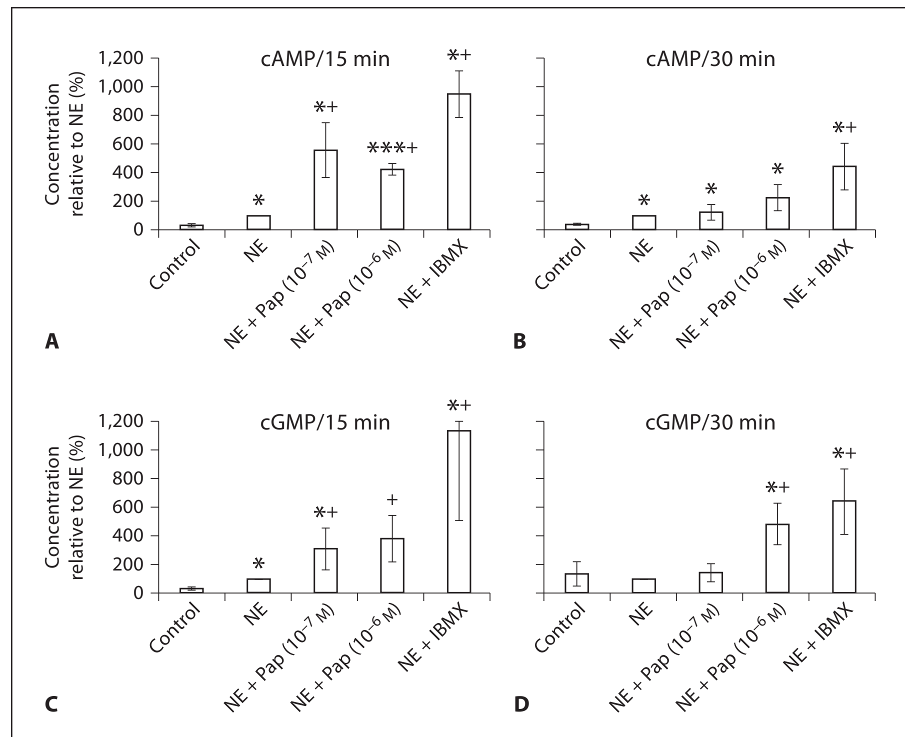

Fig. 5. In vitro effects of the PDE10A-specific inhibitor papaverine (10^{-6} M, 10^{-7} M) and the unspecific PDE inhibitor IBMX (10^{-4} M) on NE-elevated cAMP (**A**, **B**) and cGMP (**C**, **D**) levels. Pineal glands obtained from rats kept under LD 12:12 at ZT 4 were preincubated for 24 h and then treated with NE (10^{-7} M) in the presence or absence of PDE inhibitors for 15 min (**A**, **C**) or 30 min (**B**, **D**). The data shown represent relative values calculated as percentages of the value obtained with NE alone. Each value is the mean ± SEM for at least three samples. * $p < 0.05$, *** $p < 0.001$ compared with control; $^{+}$ $p < 0.05$ compared with NE-treated pineal glands.

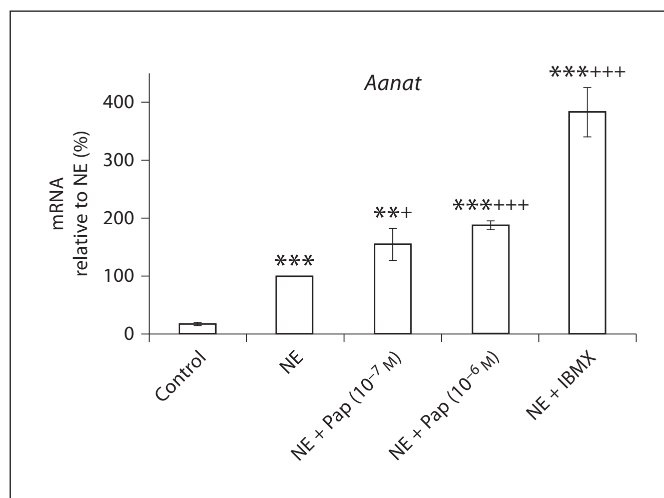

Fig. 6. In vitro effects of the PDE10A-specific inhibitor papaverine (10^{-6} M, 10^{-7} M) and the unspecific PDE inhibitor IBMX (10^{-4} M) on the amount of NE-elevated AANAT transcript. Pineal glands were preincubated for 24 h and then treated with NE (10^{-7} M) in the presence or absence of PDE inhibitors for 3 h. The data shown represent relative values calculated as percentages of the value obtained with NE alone. Each value is the mean ± SEM for at least three samples. ** $p < 0.01$, *** $p < 0.001$ compared with control; $^{+}$ $p < 0.05$, $^{+++}$ $p < 0.01$ compared with NE-treated pineal glands.

investigated, the effect of IBMX appeared to be stronger than that of papaverine. This suggests that further types of PDE are also relevant as far as decreasing the adrenergic cyclic nucleotide signaling in pinealocytes is concerned.

PDE10A Inhibitor Papaverine Increases the Amount of Adrenergically Elevated AANAT Transcript

Since cAMP mediates the adrenergic induction of *Aanat*, the observed cAMP hydrolyzing activity of PDE10A may downregulate the adrenergic induction of *Aanat* expression. To test this hypothesis, the effect of the PDE10A-specific inhibitor papaverine (10^{-6} M, 10^{-7} M) [36] on the AANAT mRNA response to NE (10^{-7} M) was determined (fig. 6). The PDE10A inhibitor papaverine was seen to enhance the adrenergically elevated AANAT mRNA levels (10^{-7} M: $p < 0.05$; 10^{-6} M: $p < 0.001$). Therefore, by decreasing the intracellular cAMP level, PDE10A dampens the adrenergic induction of pineal *Aanat* gene expression. To obtain a measure of full PDE inhibition with regard to *Aanat* expression, the effect of the unspecific PDE inhibitor IBMX was examined (fig. 6). The influence of IBMX on *Aanat* expression ($p < 0.001$) was found to be stronger than that of papaverine. This is consistent with the concept that PDE10A acts in concert with further types of PDEs to downregulate the adrenergic induction of *Aanat*.

Discussion

Within the brain, PDE10A is expressed at much higher levels in the striatum than in other areas across several mammalian species [21]. This suggests an evolutionarily conserved role for this enzyme in the regulation of information processing throughout the basal ganglia and, thus, in motor function [26, 27] and the psychomotor domain [28, 29]. Recent evidence suggests further roles of PDE10A in the mammalian brain. Thus, PDE10A appears to be involved in the visual system [37] and in the SCN-dependent control of pineal function [1; this study].

In this study, it was found that PDE10A protein and transcript is abundant in the pineal gland of rat where PDE10A protein is primarily expressed in the cytoplasma of pinealocytes. In accordance with a recent report [1], the amount of PDE10A transcript in the pineal gland of the rat was found to undergo a pronounced day/night rhythm with peak expression in the second half of the dark phase and to increase in response to treatment with NE and dbcAMP. The results presented demonstrate that this rhythm is translated into changes in the amount of PDE10A protein and is generated by the pacemaker in the SCN via β-adrenergic cAMP/protein kinase A signaling. These results show that the *Pde10a* gene should be considered as belonging to the list of 'circadian genes' of the pineal gland whose transcription is controlled by the SCN through a primarily β-adrenergic pathway [1; for a review, see 38]. Cyclic AMP/protein kinase A signaling also regulates the subcellular targeting of PDE10A in rat striatum [39]. Therefore, the significance of this pathway for the expressional control of PDE10A (as shown in this study) highlights the general importance of cAMP/protein kinase A signaling for the regulation of PDE10A-dependent cyclic nucleotide inactivation.

The circadian/adrenergic regulation of the transcription of the *Pde10a* gene provides a basis for the photoperiod-dependent control of PDE10A expression. As is the case for other pineal genes [34], the nocturnal rise in the amount of PDE10A transcript occurred at an earlier ZT under the short-day regime (LD 8:16) than under the long-day regime (LD 16:8) (fig. 3A vs. B). Since the morning decrease is simultaneously 'locked' to light onset under both lighting regimes, the duration of the increase in PDE10A expression is also lengthened under the short-day regime. As a result, the per night rate amount of PDE10A transcription and protein de novo formation should be higher under short days than under long days. The results showing that the postnocturnal level of PDE10A protein amount is higher under the short-day cycle than under the long-day cycle (inset in fig. 3A vs. inset in fig. 3B) is in agreement with this suggestion. It therefore appears reasonable to add the PDE10A protein to the group of pineal proteins whose level of expression depends not only on daily but also on seasonal lighting conditions [13, 40–42].

With regard to the role of PDE10A in pinealocytes, the PDE10A inhibitor papaverine [36] was seen to enhance the cAMP and cGMP responses to adrenergic stimulation in the present study. This indicates that PDE10A dampens adrenergic cyclic nucleotide signaling by decreasing the half-life of the cyclic nucleotides. Accordingly, a cAMP-dependent elevation of the PDE10A protein should result in a decrease in the intracellular cAMP concentration, and thus represents a means of negative feedback in adrenergic cAMP signaling. In the pineal gland, cAMP and cGMP mediate the influence of adrenergic stimulation on gene expression (for a review, see [43]) and posttranslational protein modification [12–14]. Therefore, the regulatory role of PDE10A in adrenergic cyclic nucleotide signaling suggests an influence of PDE10A on pineal function. Consistent with this hypothesis is the finding in this study that PDE10A inhibition by papaverine was seen to enhance the adrenergic induction of the *Aanat* gene which codes the key enzyme in melatonin formation.

The significance of PDE10A for adrenergic cyclic nucleotide signaling and *Aanat* expression is also of interest in relation to the daily profile in the amount of PDE10A protein. Adrenoceptor-dependent activation of adenylate and guanylate cyclase causes a rapid increase in cAMP and cGMP levels in pinealocytes. As a result, formation of the cyclic nucleotides in pinealocytes is stimulated as long as NE is released from intrapineal nerve endings, i.e. during the entire dark phase. In contrast, the effect of adrenergic elevation of the PDE10A protein has a time course of hours and the PDE10A protein amount begins to gradually increase only after midnight. Accordingly, the negative influence of PDE10A on adrenergic cyclic nucleotide signaling and *Aanat* expression comes into effect around midnight and cumulates during the course of the second half of the night, with a peak being reached around the time of light onset (fig. 2B). Therefore, the contribution of PDE10A in the circadian/adrenergic regulation of the pineal gland may be to downregulate the adrenergic stimulus and *Aanat* expression late at night by gradually increasing the destruction of cAMP and cGMP. Noteworthy is the finding that the amount of PDE10A protein transiently remains at a high level after light onset

when the adrenergic stimulus is terminated. Therefore, an additional role of PDE10A might be to catabolize the remaining cyclic nucleotides after light onset and in that way to 'lock' the termination of the cyclic nucleotide signal and *Aanat* induction to light onset.

The role of PDE10A for adrenergic cyclic nucleotide transduction appears to also be important in relation to the photoperiod-dependent changes in the PDE10A protein amount. Thus, the higher morning PDE10A protein amount under the short-day regime may facilitate a more rapid destruction of remaining nucleotides and, thus, a sharper cessation of the adrenergic stimulus and *Aanat* induction at light onset. This might guarantee that the pineal gland, even under the short-day regimen, has enough time to recover from the adrenergic stimulus and to get ready for the next stimulus. The influence of the photoperiod on PDE10A expression suggests that the *Pde10a* gene belongs to the group of other genes of the pineal gland whose expressional control imprints the seasonal lighting conditions on the adrenergic signal transduction cascade [40–42].

The unspecific PDE inhibitor IBMX is more effective than papaverine in increasing the cyclic nucleotide and the AANAT mRNA responses to adrenergic stimulation. Additional types of PDEs therefore appear to regulate adrenergic cyclic nucleotide signaling, highlighting the decisive role that cyclic nucleotide degradation plays in adrenergic signal transduction in pinealocytes. Consistent with the involvement of different types of PDEs in adrenergic cyclic nucleotide signaling, PDE4B has also been reported to decrease the cAMP response to adrenergic stimulation in rat pinealocytes [17]. As is the case with PDE10A, PDE4B is also under circadian regulation, and PDE4B mRNA and protein [17] display daily changes parallel to those of PDE10A (as shown in this study). Accordingly, both types of PDE appear to play a similar role in the circadian/adrenergic control of pineal function, i.e. in the termination of the adrenergic stimulus. Due to their differential substrate specificity, both types of PDEs appear to influence different pathways of adrenergic signaling, whereas PDE10A affects both cAMP and cGMP signaling [this study], PDE4B has been seen to only target cAMP signaling [17]. It is also of interest to note that PDE10A and PDE4B have been demonstrated to be localized in different subcellular compartments in rat striatum [44]. If the situation is similar in the pineal gland, then circadian regulation of both types of PDE may enable the SCN to concurrently control distinct subcellular compartments of cAMP signaling.

In conclusion, the present study has examined the role of PDE10A in the context of the circadian system and adds *Pde10a* to the pineal genes that are controlled by the master clock in the SCN. A putative role of PDE10A is its contribution to the cessation of the adrenergic signal and *Aanat* transcription as part of the SCN-dependent control of pineal function. The findings presented provide a basis for further studies using PDE10A inhibitors and *Pde10a*$^{-/-}$ mice in which the impact of PDE10A on the output of the pineal gland, i.e. melatonin formation and circadian regulation of body function could be investigated. Within the scope of such investigations, the role of PDE10A inhibitors whose putative benefit in the treatment of psychotic symptoms has entered clinical testing (for a review, see [30]) may also be of pharmacological interest.

Acknowledgements

We thank Frank S. Menniti and Timothy M. Coskran for the anti-PDE10A antiserum; Ute Frederiksen, Kristina Schäfer, Ursula Göringer-Struwe, Ilse von Graevenitz and Annette DeCuvry for their excellent technical assistance; Bettina Wiechers-Schmied, Susanne Rometsch and Stephanie Becker for secretarial help, and Frank Menniti for his critical reading of the manuscript. The data contained in this study are included in the thesis presented by Oliver Rickes as a partial fulfillment of his medical doctorate degree at the University Medical Center of the Johannes Gutenberg University, Mainz.

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