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Thyroid hormone and adrenergic signaling interact to control pineal expression of the dopamine receptor D4 gene (*Drd4*)

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Abstract

Dopamine plays diverse and important roles in vertebrate biology, impacting behavior and physiology through actions mediated by specific G protein-coupled receptors, one of which is the dopamine receptor D4 (*Drd4*). Here we present studies on the >100-fold daily rhythm in rat pineal *Drd4* expression. Our studies indicate that *Drd4* is the dominant dopamine receptor gene expressed in the pineal gland. The gene is expressed in pinealocytes at levels which are ~100-fold greater than in other tissues, except the retina, in which transcript levels are similar. Pineal *Drd4* expression is circadian in nature and under photoneural control. Whereas most rhythmically expressed genes in the pineal are controlled by a norepinephrine-cAMP signaling, *Drd4* expression also requires thyroid hormone. This advance raises the questions of whether *Drd4* expression is regulated by this mechanism in other systems and whether thyroid hormone controls expression of other genes in the pineal gland.

Keywords

Drd4; thyroid hormone; (pineal gland); cAMP; norepinephrine; circadian

Introduction

Dopamine (DA) plays critical roles in neural processing (Missale et al., 1998, Tarazi and Baldessarini, 1999, Oak et al., 2000, Emilien et al., 1999, Neve et al., 2004, Volkow and Wise, 2005, Wise, 2004). In the central nervous system, DA controls a variety of physiological processes including motor function, cognition, mood, and endocrine secretion. It is also

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implicated in learning, motivation, mental disorders, obesity and addiction. In the peripheral nervous system, DA acts as an important regulator of catecholamine release, hormone secretion, renal function, and cardiovascular function (Amenta et al., 2002, Lokhandwala and Amenta, 1991, Emilien et al., 1999). In addition, DA plays a role in the visual system (Pozdeyev et al., 2008, Klitten et al., 2008, Pflug et al., 2008, Ruan et al., 2008).

The diverse physiological effects of DA are mediated by two classes of G-protein linked DA receptors (Drd) (Missale et al., 1998, Neve et al., 2004), referred to as D1-like and D2-like receptors. Members of the D1-like receptor family, the Drd1 and Drd5 receptor subtypes, are known to stimulate adenylate cyclase activity *via* a G_s mechanism. Members of the D2-like receptor family, the Drd2, Drd3 and Drd4 receptors, are known to inhibit adenylate cyclase activity *via* a G_i mechanism (Missale et al., 1998) and also to act independently of cAMP (Scarselli et al., 2001, Gu et al., 2006, Mei et al., 1995, Tang et al., 1994). In the retina, *Drd4* appears to modulate phototransduction through a mechanism that requires cAMP (Klitten et al., 2008, Ivanova et al., 2008, Nir et al., 2002, Nir et al., 2001).

Drd4 expression in the nervous system (Oak et al., 2000, Waddington et al., 2005, Tarazi and Baldessarini, 1999, Emilien et al., 1999) has become a focus of clinical attention because a polymorphism of this receptor has been associated with attention deficit hyperactivity disorder (Aguirre-Samudio and Nicolini, 2005, Tarazi and Baldessarini, 1999). This genetic evidence provides strong reason to suspect that expression of the Drd4 receptors impacts behavior. In addition, there is strong pharmacological evidence linking *Drd4* to behavior (Tarazi and Baldessarini, 1999).

Little is known about the regulation of *Drd4* expression in any tissue and knowledge about this would be of therapeutic and experimental importance. *Drd4* expression changes dynamically in the rodent pineal gland on a night/day schedule (Bai et al., 2008, Bailey et al., 2008, Humphries et al., 2002, Bailey et al., 2009). The current investigation extends this by determining that marked changes in *Drd4* expression require both neural and hormonal input. This is of special interest because the expression of many genes in the pineal gland is thought to be controlled by the neural input alone and there are no previous reports of effects of thyroid hormone on gene expression in this tissue. The new findings regarding *Drd4* expression are of special significance to investigators interested in the regulation of *Drd4* expression in genetic disorders and disease states involving DA signaling.

Materials and Methods

Materials

[³²P] α-dCTP was purchased from GE Healthcare (Piscataway, NJ). [³⁵S] α-dATP was purchased from Perkin Elmer (Boston, MA). L-(−)-Norepinephrine, dibutyryl cAMP, (−)-isoproterenol, actinomycin D, puromycin, 3, 3', 5-triiodo-L-thyronine (T3), L-thyroxine (T4), insulin-like growth factor-1, nerve growth factor-β, and dexamethasone were obtained from Sigma (St. Louis, MO). N-2 supplement was purchased from Invitrogen (Calsbad, CA)

Animals, surgical procedures and tissue preparations

Rats (Male, Sprague Dawley, 150 to 200 grams) used for *in vivo* and organ culture experiments were obtained from Taconic Farms Inc. (Germantown, NY), with the exception of those used for radioactive *in situ* hybridization, which were obtained from Charles River (Sulzfeld, Germany). Animals were housed for two weeks in light:dark (LD) 14:10 lighting cycles in all cases except for those animals used for *in situ* hybridization, which were housed in LD 12:12.

For surgery, rats were anaesthetized with tribromethanol (500 mg/kg). A midline incision was made through the skin in the midline on the ventral side of the neck and the superficial neck

fascia was penetrated in the midline. After removal of the omohyoid muscle, the common carotid artery was visualized in the carotid trigone and the superior cervical ganglion was identified medial to the carotid bifurcation. For superior cervical ganglionectomy (SCGX), the ganglion was removed bilaterally together with a part of the sympathetic trunk. For decentralization of the superior cervical ganglion (DCN), a part of the sympathetic trunk was removed bilaterally just caudal to the ganglion; thus, the afferent input to the superior cervical ganglion was blocked while leaving the ganglion itself intact. The skin wound was closed with staples. Animals were sacrificed ten days after surgery.

Animals were sacrificed by CO₂ asphyxiation and decapitated. Tissues were prepared as described previously (Kim et al., 2005, Kim et al., 2007). Animal use and care protocols were in accordance with NIH guidelines, with Health Sciences Animal Policy and the guidelines of EU Directive 86/609/EEC (approved by the Danish Council for Animal Experiments). To treat animals with isoproterenol, the compound was first dissolved in 0.85% NaCl to a final concentration of 10 mg/ml and the appropriate volume (20 mg/kg rat) was injected subcutaneously at Zeitgeber time (ZT) 4; rats were killed at ZT 7, and their pineal glands were removed and immediately placed on solid CO₂ and stored at -80°C until use.

Organ culture

Rat pineal glands were cultured in defined culture medium (BGJ^b, Invitrogen, Carlsbad, CA) containing 1mg/ml BSA as described previously (Kim et al., 2007, Kim et al., 2005); BGJ^b medium does not contain added T3 or T4. In experiments where mRNA levels are reported, each analysis was performed on separate sets of pineal glands treated in culture on the same day.

Assays

mRNA

Northern blot: A published procedure was used (Kim et al., 2007, Kim et al., 2005). The hybridization probes used were based on the *Drd4* transcript (*Drd4* nucleotides 184-948, GenBank NM_012944). *Aanat* mRNA (arylalkylamine *N*-acetyltransferase) and 18S rRNA were detected as described previously (Kim et al., 2005). For each figure at least three blots were run, using total RNA extracts from different tissue pools for each lane. The image for each blot was captured using X-ray film and quantitated using ImageQuant TL software (GE Healthcare). The signal intensity of the positive band in each lane was normalized to 18S signal to correct for variations in loading.

Quantitative real time polymerase chain reaction (qRT-PCR)(Kim et al., 2007): Three pools of glands were prepared, each of which was composed of three rat pineal glands. Total RNA (5 µg/gland) was isolated from each pool using the RiboPure RNA isolation kit (Ambion, TX). Total RNA was then subjected to DNase treatment using TURBO DNA-free (Ambion) to remove contaminating genomic DNA. The amount and quality of RNA were assessed using a ND-1000 spectrophotometer (Nanodrop, Wilmington, DE) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). cDNA production was performed following the Superscript II protocol (Invitrogen) using 1 µg of DNase treated total RNA as starting material.

qRT-PCR determinations were made using a LightCycler 2.0 (Roche Diagnostics, IN). Reactions (25µl volume) contained 0.5 µM primers, RT Real-Time SYBR Green mastermix (SuperArray Bioscience, MD) and cDNA according to the manufacturer's instructions. All incubations included an initial denaturation step at 95°C for 10 minutes, typically followed by 40 cycles of a 95°C denaturation for 15s, 30s annealing at 63°C then extension at 72°C for 30s. Primers used for qRT-PCR are described in Table 1; all primer pairs generated a product of the predicted size using mRNA prepared from pineal gland or striatum, as indicated by agarose

gel electrophoresis. Thereafter, specificity was demonstrated during every qRT-PCR run by melting curve analysis (T_m). Typically ~25 cycles were necessary to detect amplification. All qRT-PCR assays were linear ($r^2 > 0.99$) from 10^1 to 10^7 transcript copies.

Transcript copy number was determined using internal standards, which were either purified target PCR products quantified by absorbance at 260 nm or prepared by cloning target PCR products into pGEMT Easy vectors (Promega, WI). The identity of clones was determined by direct sequence analysis and by plasmid DNA digestion followed by agarose gel electrophoresis (2.0%, v/v). The products were visualized with ethidium bromide (EtBr, 0.5 μ g/ml). For each experiment, a set of 100-fold serial dilutions of each internal standard (10^1 – 10^7 transcript copies/1 μ l) was prepared and used to generate standard curves. Transcript number was determined using a 2 μ l sample of a 10-fold dilution of rat pineal cDNA prepared as mentioned above; values were normalized to the number of Gapdh transcript copies.

Radioactive *in situ* hybridization: A published method was used (Kim et al., 2007, Moller et al., 1997). Sagittal cryostat sections (12 μ m) were thawed and fixed for 5 min in 4% paraformaldehyde in PBS, washed 2×1 min in PBS, and acetylated (0.25% acetic anhydride in 0.9 % NaCl containing 0.1 M triethanolamine; 10 min). The sections were then dehydrated in a graded series of ethanols and delipidated in 100% chloroform (5 min). They were partially rehydrated in 100% and 95% ethanol (1 min each) and allowed to dry.

For hybridization of the cryostat sections, 35 S-labeled oligonucleotide probes were prepared based on the rat Drd sequences (Table 2). For detection of Drd1 and Drd5 transcripts, a mixture of two probes was used. The labeled probe solution was diluted in the hybridization buffer (10 μ l/ml) consisting of 50% (v/v) formamide, $4 \times$ SSC, $1 \times$ Denhardt solution, 10% (w/v) dextran sulfate, 10 mM DTT, 0.5 mg/ml salmon sperm DNA and 0.5 mg/ml yeast tRNA. A 200 μ l aliquot of hybridization buffer containing the labeled probe was placed on each section. The sections were then covered with Parafilm and incubated in a humid chamber overnight at 37 C. After hybridization, the slides were washed in $1 \times$ SSC for 4×15 min at 55 C, 2×30 min at room temperature, and rinsed twice in distilled water. The sections were dried and exposed to X-ray film for 1 to 2 weeks at 4 C. The pineal hybridization signals on X-ray films were quantified using “Image 1.42” (Wayne Rasband, NIH). Optical density was converted to dpm/mg tissue using simultaneously exposed 14 C-standards calibrated by comparison with 35 S brain-paste standards. Results are based on the analysis of pineal glands from four animals killed at night and four during the day.

Statistical analysis

All data are expressed as means $\pm$ S.E. values for the number of determinations indicated. Statistical analyses were performed using two-tailed Student's *t*-test for two groups and one-way analysis of variance (ANOVA) for multiple groups (*, $P < 0.01$).

Results

Day/night rhythm in Drd4 transcripts only seen in pineal gland and retina

Daily rhythms in Drd4 transcripts in the pineal gland and retina were evident from the results of northern blot, qRT-PCR analysis and radiochemical *in situ* hybridization histology (Fig. 1), extending previous reports (Humphries et al., 2002, Bai et al., 2008). Two transcripts (1.5 and 2.9 kb) were detected by Northern blot (Fig. 1A); the sequence of the major 1.5 kb band was determined to be identical to GenBank NM_012944. The minor 2.9 kb Drd4 band appears to be a splice variant or immature mRNA.

Drd4 mRNA levels did not change on a day/night basis in other tissues (Fig.1). In addition, peak pineal and retinal levels at night were ~ 100-fold higher than in other tissues examined. The relatively high expression of *Drd4* in the pineal gland compared to other areas in the brain was also evident from the results of radiochemical *in situ* hybridization histology (Fig. 1C). High expression of *Drd4* in the pineal gland and retina is consistent with evidence that they share a common ancestral photodetector cell (Klein, 2004).

Analysis of the daily pattern of Drd4 mRNA in the pineal gland revealed that expression is highest during the last half of the night period and that a similar pattern of expression, characterized by a 1.5 kb band and a less prominent 2.9 kb band (Fig. 1A (Fig. 2A).

Drd4 is the dominant Drd gene expressed in the pineal gland and retina

The expression of additional *Drd* genes in the pineal gland and retina was examined by qRT-PCR and radiochemical *in situ* hybridization histology (Fig. 2B, C). qRT-PCR revealed that *Drd1* and *Drd3* were expressed at very low levels in both tissues; radio chemical *in situ* hybridization histology failed to detect Drd1 mRNA in the pineal gland; Drd2 and Drd5 mRNA were not detected by qRT-PCR; in addition, Drd5 mRNA was not detected by radiochemical *in situ* hybridization histology.

Physiological control of pineal Drd4 mRNA

We investigated the nature of the system that controls the rhythm in pineal Drd4 mRNA to determine whether it is under photoneural regulation. A large night/day-like difference in expression was found to persist in constant darkness (DD) (Fig. 3A), indicating that this rhythm is circadian in nature; this is also the case with many other genes in the pineal gland, including Aanat (Roseboom et al., 1996), which controls the large daily changes in melatonin production (Fig. 3A). The 24-h changes in expression of these genes is regulated by a neural system which includes a central circadian oscillator located in the suprachiasmatic nucleus (SCN); signals generated there at night stimulate the pineal gland (Klein, 1985a).

SCN stimulation of the pineal gland is prevented by light, which acts through a retinohypothalamic projection that terminates in the SCN (Klein, 1985a); light acts through these projections to block the neural output to the pineal gland. Here we found that exposure to light; either for five days (Fig. 3A) or for 5 h at night (Fig. 3B) prevents the nocturnal increase in *Drd4* expression, providing evidence of photic control, which is consistent with the interpretation that the SCN regulates the daily rhythm in *Drd4* expression in the pineal gland.

The SCN communicates with the pineal gland *via* a neural circuit that passes through the brain (paraventricular nucleus of the hypothalamus), the spinal cord and the superior cervical ganglia (SCG) of the sympathetic nervous system; the SCG innervate the pineal gland (Moore and Klein, 1974, Klein et al., 1971, Klein, 1985b, Klein and Moore, 1979, Klein et al., 1983, Yanovski et al., 1987, Ganguly et al., 2002, Roseboom et al., 1996). To determine if the increase in *Drd4* expression at night involves neural transmission via the SCG, these structures were surgically removed (SCGX) or decentralized (DCNT). Either procedure blocked the nocturnal increase in *Drd4* expression, as revealed by radiochemical *in situ* hybridization histology (Fig. 1C); northern blot and qRT-PCR confirmed that SCG decentralization blocked the nocturnal increase in Drd4 transcripts, whereas sham surgery did not (Fig. 3C, D).

Adrenergic/cAMP signaling and thyroid hormone control pineal Drd4 expression

Expression of many genes in the pinealocyte is mediated by the release of NE, which controls pineal function through β_1 - and α_{1b} -adrenergic receptors (Ganguly et al., 2002, Shyjan et al., 1990, Klein, 2007). Here we obtained *in vivo* evidence consistent with adrenergic control of

Drd4 expression in studies in which treatment with the β -adrenergic agonist isoproterenol increased pineal *Drd4* mRNA (Fig. 4).

Attempts to confirm this using an *in vitro* organ culture preparation were initially unsuccessful: neither NE (Fig. 5) nor isoproterenol treatment alone increased *Drd4* expression (Bailey et al., 2009) (data not shown). This finding was especially interesting and surprising because adrenergic stimulation in organ culture has been found to increase expression of most genes that exhibit increased expression in the pineal gland at night (Humphries et al., 2004, Kim et al., 2007, Kim et al., 2005, Coon et al., 1997, Baler et al., 1996, Roseboom et al., 1996, Baler and Klein, 1995, Gaildrat et al., 2005, Bailey et al., 2009).

We examined the possibility that a factor critical for regulation of *Drd4* expression was missing in organ culture by testing effects of insulin-like growth factor-1, nerve growth factor- β , dexamethasone, and N-2 supplement, none of which altered the response to *Drd4* treatment (unpublished).

We also tested the effects of thyroxine (T4), and triiodothyronine (T3) because of the large daily rhythm in the activity in the pineal gland of type II deiodinase (Dio2), which converts T4 to T3 (Tanaka et al., 1987). In addition, the peak in *Drd4* mRNA occurs several hours after that of Dio2 mRNA (Bailey et al., 2008, Bai et al., 2008, Bailey et al., 2009). Suggesting the former could play a role in controlling the latter. Either T3 or T4 potentiated the effects of NE in a dose-dependent manner (Fig. 5C). Although maximal effects were seen at supraphysiological concentrations of T3 and T4, significant effects were obvious at physiological nanomolar concentrations. T4 was found to be less effective, which is consistent with the reported lower (~50-fold) affinity for thyroid hormone receptors (Gershengorn et al., 1979); these results also suggest that intracellular deiodination of T4 by induced levels of Dio2 does not appear to quantitatively reproduce the effects of T3 in this system (Rubio et al., 1991). The effect of T3 were not seen immediately, but increased during treatment; with maximal effects not seen until 9 to 12 hours following initiation of stimulation (Fig. 5D).

cAMP mediates effects of NE on *Drd4*

NE controls expression of many genes in the pineal gland through elevation of cAMP by a mechanism which involves simultaneous activation of β_1 - and α_{1b} -adrenergic receptors (Ganguly et al., 2002, Klein, 1985a, Simonneaux and Ribelayga, 2003, Maronde and Stehle, 2007). Such dual mechanisms, requiring two signals for full responses, have been described as “AND” gate or coincidence detection models.

We tested whether cAMP is involved in the control of *Drd4* expression by treating glands with the membrane permeant cAMP analog dibutyryl cAMP (Fig. 5A, B). By itself, it had a negligible effect; however, in combination with T3, dibutyryl cAMP increased *Drd4* expression to levels similar to those produced by cotreatment with NE and T3. These findings support the hypothesis that neural stimulation by NE of *Drd4* expression is mediated by cAMP signaling and that cAMP acts through an “AND” gate mechanism that requires thyroid hormone (Fig. 6).

Discussion

This study broadly highlights the issue of DA and T3 in pineal biology; the following discussion will first address the issues of DA signaling and then cover the subject of T3 control of *Drd4* expression.

DA signaling in the pineal gland

DA presence in the sympathetic nerves in the pineal gland is apparent from the fact that it is the immediate precursor of NE and from reports that DA is present in the pineal gland at levels which are ~10% those of NE (Kvetnansky et al., 1979). Moreover, because both transmitters are colocalized in the sympathetic nerve terminals, it is reasonable to predict that they are coreleased.

In contrast to NE, which is well recognized as the main regulator of melatonin synthesis and of the expression of many genes in the pineal gland (Ganguly et al., 2002), DA has received relatively little attention as regards control of pineal function. Limited evidence in the literature indicates that DA impacts melatonin production, Aanat activity or any other aspect of pineal biochemistry; some of this data is in conflict and problematical because of the relatively high concentrations of DA and DA agonists and antagonists that have been used (Klein and Weller, 1973, Santanavanich et al., 2005, Govitrapong et al., 1989). Such high concentrations raise the question of whether some of the effects claimed to be due to DA signaling are due to non-specific effects of DA on adrenergic receptors; this is predictable based on the structural similarity of DA and NE, which differ by a single hydroxyl group in the β position. In addition, most of reports in the literature are based on results obtained with the bovine pineal gland; accordingly, some differences may reflect species differences.

The results presented here support the hypothesis that DA acts through Drd4 receptors to control rodent pineal function. This is indicated by the finding that *Drd4* as the dominant *Drd* gene expressed in the pineal gland and that expression of other dopamine receptor genes is less than 1% that of *Drd4*, according to the results of qRT-PCR, of radiochemical *in situ* hybridization histology and of microarray studies (Bailey et al., 2008, Bailey et al., 2009). Previous studies have indicated that Drd1 and Drd2 mRNA is present the bovine pineal gland; however, these were based on non-quantitative PCR methods, making it difficult to compare to those obtained here using qRT-PCR.

The precise role that DA and Drd4 receptors play in control of pineal physiology requires further study. From a physiological point of view, it is possible that the intensity of the pineal response to sympathetic stimulation reflects the ratio of NE to DA, which is controlled by dopamine β -hydroxylase. Accordingly, factors that decrease the activity of this enzyme would be expected to increase the amount of DA and as a result would suppress NE stimulation of Aanat.

The discovery that *Drd4* is highly expressed in the pineal gland relative to other tissues is of special interest for several reasons. First, the unusually high expression of *Drd4* in the pineal gland also makes this tissue an attractive model to use to study the biology and pharmacology of this receptor. Second, DA related drugs are used clinically in treating Parkinson's disease and schizophrenia (Dagher and Robbins, 2009, Scharfetter, 2004); such agents could modulate pineal function through Drd4 receptors and in this way modulate circadian physiology.

T3 regulation of Drd4 expression

The studies in this report have revealed a mechanism of regulation which has not been reported previously for *Drd4* in any tissue or for any gene in the pineal gland. Specifically, expression of *Drd4* is controlled by an "AND" gate mechanism which requires both cAMP and T3 or T4, as outlined in Fig. 6, in which cAMP has two parallel roles, one of which is to act on expression of *Drd4* and the second being to induce expression of Dio2 (Chik et al., 2007, Murakami et al., 1997), which converts the relatively inactive T4 to the highly active T3. A hypothetical explanation of this is that concomitant activation of a thyroid hormone receptor regulatory

element and a cAMP regulatory element in the *Drd4* promoter is required to induce *Drd4* expression. Another possibility is that T4 and T3 act to stabilize *Drd4* mRNA.

A previous report has indicated that *in vitro* treatment with supraphysiological concentrations (> 5 μ M) T3 or T4 enhances melatonin production by cultured pineal glands (Nir and Hirschmann, 1978). However, we have been unable to confirm this in unpublished results using a range of concentrations (1 nM to 1 μ M). Accordingly, the physiological relevance of the earlier observation remains in question, as do their relationship to the current study.

We have been unable to determine whether *Drd4* protein exhibits a daily rhythm similar to that of *Drd4* mRNA, because of technical limitations. The daily changes in *Drd4* mRNA may or may not be translated into daily changes in *Drd4* protein, dependent upon on the turnover rate of the protein, which might exist is a relatively stable pool. This could reflect membrane localization and receptor recycling. This situation exists in the pineal gland for α_{1b} -adrenergic receptor, in which the encoding mRNA exhibits a daily rhythm whereas the encoded protein does not (Coon et al., 1997). The relatively stable nature of the α_{1b} -adrenergic receptor indicates that abundance is a long-term integrated reflection of prior neural stimulation. Further work on the *Drd4* receptor is necessary to determine whether this is also the case for this receptor, or if it changes in parallel to *Drd4* mRNA.

These studies point to the necessity of determining whether thyroid hormone alters the expression of other genes in the pineal gland or the effects of NE on gene expression. It is known that NE treatment alone does not fully mimic the changes in gene expression seen to occur at night in the pineal gland (Bailey et al., 2009). In addition to *Drd4* a set of ___ other genes are either not expressed by NE treatment following organ culture; in addition, other genes, which don't exhibit a night/day difference, are expressed at very low levels following several days in organ culture. One explanation for these observations is that they reflect the absence of T3. T3 is known to act through T3 receptor dimers; it is also known to act through dimers formed by T3 receptors and retinoic acid receptors (Yen, 2001). Accordingly, it is reasonable to predict that addition of both T3 and retinoic acid will alter expression of some genes in the pineal gland; this possibility has been raised based on the results of gene expression analysis (Bailey et al., 2009), but remains to be investigated.

Broad implications of the findings of this study

Data is not currently available to indicate or suggest that cAMP/T3 control of *Drd4* expression seen in the pineal gland occurs in other tissues. However, this is of some interest in light of the possibility that thyroid hormone and cAMP might interact to alter *Drd4* expression in humans. Thyroid hormone levels change as a function of thyroid status; and, cAMP levels change in response to a variety of ligands and drugs. Their interaction through the “AND” gate mechanisms controlling *Drd4* expression could modulate DA signaling in humans.

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Figure 1. Tissue survey of Drd4 mRNA

Total RNA was prepared from tissues obtained during the day (ZT7) and night (ZT19). **A.** Northern blot analysis. **B.** Quantitation of Drd4 transcripts; qRT-PCR was done as described in Materials and Methods. Primers used are described in Table 1. Abbreviations are HYPO, hypothalamus; CTX cerebral cortex; CB, cerebellum; AD, adrenal gland; PG, pineal gland; PIT, pituitary gland; Muscle, skeletal muscle. Results show mean $\pm$ SE of three samples. **C.** Radioactive *in situ* hybridization histological analysis of Drd4 mRNA. Brains, removed during the day (ZT6) and night (ZT18), were obtained from untreated animals (Sham), superior cervical ganglionectomized animals (SCGX) and from animals with decentralized SCG (DCNT). Tissue was removed two weeks after surgery and prepared for radioactive *in situ*

hybridization histological analysis using probe *Drd4* (Table 2). Scale bars = 1 mm. The Day and Night signals for Sham pineal glands are 43.4 ± 6.6 and 572.9 ± 88.5 dpm/mg tissue; the values for the other groups were not statistically different from the Day Sham value. For further details see the Materials and Methods.

A **B** **C**

Figure 2. Drd transcripts in the pineal gland and retina

A. *Drd4* transcripts were determined by Northern blot. Following housing for two weeks in LD (14:10) animals were killed and pineal glands were obtained at the times indicated. **B.** qRT-PCR was performed on gland and retina removed at the indicated times. *Drd1* and *Drd3* mRNAs were detectable at low levels in both tissues. *Drd2* and *Drd5* mRNAs were not detectable. The ZT1, 7, and 13 data points are double plotted. Data shown are the mean $\pm$ SE of three replicates. *, Statistically different from the ZT7 group, $P < 0.01$. **C.** Radiochemical *in situ* hybridization histological analysis of *Drd* transcripts. Scale bars = 1 mm. The probes used are given in Table 2. Note that a positive signal for *Drd1* transcripts was detected in the striatum. For further details see the Materials and Methods.

Figure 3. Physiological control of pineal Drd4 mRNA

Animals were housed as described in Materials and Methods for at least two weeks. **A.** Drd4 mRNA in constant lighting or constant darkness: Animals were maintained for five days in constant lighting (LL), constant darkness (DD) or in LD. Glands were obtained during the day and night; mRNA was extracted and detected by northern blot. Blots were stripped and Aanat mRNA was measured to confirm rhythmicity. Blots were then stripped again and 18S rRNA was determined for normalization purposes. Results show mean $\pm$ SE of three samples. *, Statistically different from the Day group, $P < 0.01$. **B.** Effect of a 5 h pulse of light at night on Drd4 mRNA: One group of animals was maintained in light for 5 h light at night (Night, PL), tissue was obtained as indicated and Drd4 mRNA was measured by qRT-PCR. Results show mean $\pm$ SE of three samples. *, Statistically different from the Night group, $P < 0.01$. **C** and **D.** Effect of decentralization of the SCG (DCNT) on Drd4 mRNA: Animals were maintained for two weeks following surgical removal of the SCG or sham surgery. Drd4 mRNA was determined by qRT-PCR (**C**) and by northern blot (**D**). For further details see the Materials and Methods.

Figure 4. In vivo treatment with isoproterenol elevates pineal Drd4 mRNA levels

Animals were injected subcutaneously with isoproterenol (20 mg/kg rat) at ZT 4 and sacrificed 3 hours later. Drd4 mRNA (~1.5 and 2.9kb) was detected by northern blot analysis. The blot was hybridized sequentially with probes for Drd4, Aanat and 18S rRNA. For further details see the Materials and Methods.

Figure 5. Adrenergic stimulation of Drd4 mRNA requires T3

A and **B**. After 48 h of control incubation in organ culture, glands were treated as indicated for 12h; the concentrations of NE and T3 were 1 μ M and that of DB cAMP was 0.5 mM. **A**. Northern blot analysis: The blot was sequentially hybridized for Drd4 and Aanat mRNAs and 18S rRNA. **B**. qRT-PCR analysis: Drd4 mRNA was measured by qRT-PCR. Data are the mean $\pm$ SE of three samples. *, Statistically different from the CONT group, $P < 0.01$. **C**. Dose-response studies: Glands were treated for 12 h with the indicated doses of NE and T3 or NE and T4. Drd4 mRNA was measured by qRT-PCR. Mean $\pm$ SE of three samples. *, Statistically different from the untreated group, $P < 0.01$. **D**. Time course of the effect of T3: The indicated treatments were initiated 48 h after the start of culture. Drd4 mRNA was measured by qRT-PCR. Mean $\pm$ SE of three samples. *, Statistically different from the 0 h treatment group, $P < 0.01$. The insert is representative of the results of three experiments in which Drd4 mRNA (~1.5 and 2.9kb) was detected by Northern blot. For further details see Materials and Methods. The abbreviations used are CONT, control; T3, triiodothyronine; NE, norepinephrine; DB, dibutyryl cAMP.

Figure 6. “AND” gate regulation of *Drd4* expression

The model indicates that *Drd4* expression in the pineal gland is controlled by NE and T4. NE acts through a dual adrenergic mechanism involving α_{1b} -adrenergic receptors (α_{1b} -AR) and β_1 -adrenergic receptors (β_1 -AR) to elevate cAMP (Ganguly et al., 2002). cAMP induces expression of Type II deiodinase (*Dio2*) and accumulation of encoded (*Dio2*), which converts thyroxine (T4) to the more active triiodothyronine (T3). Expression of *Drd4* requires both T3 and cAMP for full induction. Although not indicated, any agonist or drug which elevates cAMP can substitute for NE; and, the downstream effects of elevation of cAMP on *Drd4* expression will be a function of thyroid status. Additional investigation is required to determine whether this mode of regulation is active in systems other than the pineal gland.

Table 1
List of primers used for qRT-PCR

In all cases the primers spanned an intron. For further details see Materials and Methods.

Target	Primer Identity	Primer Sequence	Product size (bp)
Drd1	Drd1-S	GCTGACTGGGCTGACTATGG	77
	Drd1-AS	CATCTCTCCAAATGCCCTCT	
Drd2	Drd2-S	AGCCTTGCTGTGGCTGAT	91
	Drd2-AS	TCCTGCTGAATTCCACTCA	
Drd3	Drd3-S	CAGAACAGCCAGTGCATCAG	66
	Drd3-AS	ATGCAGCCTCAGACAGGAAG	
Drd4	Drd4-S	TGGTGTGCTCTCTTTGTCT	76
	Drd4-AS	GCCATGAGGGTGTACAGA	
Drd5	Drd5-S	TTTGACATCATGTGCTCCACT	60
	Drd5-AS	ACGGTCCACGCTGATGATAC	
Gapdh	Gapdh-S	TGGTGAAGGTCGGTGTGAACGGAT	310
	Gapdh-AS	TCCATGGTGGTGAAGACGCCAGTA	

Table 2
List of probes used for radiochemical *in situ* hybridization histology

For further details see the Materials and Methods.

Probe Name	Probe Sequence	Genebank Number
Drd1	CGGCCTCATCCATGGTAGAAGTGTG	NM_012546
Drd1	CCCATGATCAGAGACAGCGTCTTTAGAACT	NM_012546
Drd4	GACAATCTGAGAAGAGGGGACCTGAGTAG	NM_012944
Drd5	CAAGGTCCAGACGATTAGGAGAGTCAGGAG	NM_012768
Drd5	AAAGGCATAGATGATGGGATTGAGAGAGGA	NM_012768