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A transgenic mouse model of neuroepithelial cell specific inducible overexpression of dopamine D1-receptor

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Abstract

Dopamine and its receptors appear in the brain during early embryonic period suggesting a role for dopamine in brain development. In fact, dopamine receptor imbalance resulting from impaired physiological balance between D1- and D2-receptor activities can perturb brain development and lead to persisting changes in brain structure and function. Dopamine receptor imbalance can be produced experimentally using pharmacological or genetic methods. Pharmacological methods tend to activate or antagonize the receptors in all cell types. In the traditional gene knockout models the receptor imbalance occurs during development and also at maturity. Therefore, assaying the effects of dopamine imbalance on specific cell types (e.g. precursor versus postmitotic cells) or at specific periods of brain development (e.g. pre- or postnatal periods) is not feasible in these models. We describe a novel transgenic mouse model based on the tetracycline dependent inducible gene expression system in which dopamine D1-receptor transgene expression is induced selectively in neuroepithelial cells of the embryonic brain at experimenter-chosen intervals of brain development. In this model, doxycycline-induced expression of the transgene causes significant overexpression of the D1-receptor and significant reductions in the incorporation of the S-phase marker bromodeoxyuridine into neuroepithelial cells of the basal and dorsal telencephalon indicating marked effects on telencephalic neurogenesis. The D1-receptor overexpression occurs at higher levels in the medial ganglionic eminence than the lateral ganglionic eminence or cerebral wall. Moreover, although the transgene is induced selectively in the neuroepithelium, D1-receptor protein overexpression appears to persist in postmitotic cells. The mouse model can be modified for neuroepithelial cell-specific inducible expression of other transgenes or induction of the D1-receptor transgene in other cells in specific brain regions by crossbreeding the mice with transgenic mouse lines available already.

Keywords

Ganglionic eminence; striatum; cerebral cortex; D1-receptor; neurogenesis; nestin

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Dopamine and its receptors appear in the developing brain early in the embryonic period before mature synaptic contacts emerge (Jung and Bennett, 1996a, b, Ohtani et al., 2003, Araki et al., 2007). This raises the possibility that dopamine plays a developmental role in the embryonic brain that is distinct from its role as a neurotransmitter at the synapse in the mature brain. Indeed, dopamine receptor activation can influence neurogenesis, neuronal migration and differentiation in the embryonic brain (Zhang and Lidow, 2002, Ohtani et al., 2003, Popolo et al., 2004, Stanwood et al., 2005, Zhang et al., 2005, Crandall et al., 2007) and neurogenesis in the mature brain (Hoglinger et al., 2004, Van Kampen et al., 2004, Van Kampen and Robertson, 2005). Dopamine imbalance or genetic deletion of dopamine receptors can perturb normal brain development and produce lasting changes in brain structure and function (Drago et al., 1998a, Stanwood et al., 2005, Bhidé, 2009, Frederick and Stanwood, 2009).

Dopamine imbalance can occur in the developing brain under a variety of circumstances, including exposure of the fetus to dopaminergic drugs such as cocaine, which impair dopamine receptor signaling mechanisms (Wang et al., 1995, Friedman et al., 1996, Simansky and Kachelries, 1996, Unterwald et al., 2003, Kubrusly et al., 2007). Fetal exposure to cocaine impairs neuronal migration (Crandall et al., 2004) and differentiation (Zachor et al., 1994, Zachor et al., 2000, Stanwood et al., 2001a, Stanwood et al., 2001b, Stanwood and Levitt, 2007, Lee et al., 2010) further underscoring dopamine's role in brain development. Other conditions associated with dopamine imbalance during the period of brain development include developmental disorders such as autism spectrum disorder, attention deficit hyperactivity disorder and schizophrenia (Biederman, 2005; Bloom, 1993; Weinberger, 1995; Benes, 2000; Schwartz et al., 2000).

Dopamine imbalance can be induced experimentally in the developing brain using a variety of techniques. Traditional experimental approaches include pharmacological methods of activating or antagonizing dopamine receptors or dopamine transporter in the developing brain (Crandall et al., 2004, Popolo et al., 2004, McCarthy et al., 2007) or toxin-induced selective depletion or inactivation of dopamine neurons (Drago et al., 1998b). Other approaches involve the use of mice lacking genes for dopamine receptor, transporter or monoamine synthetic enzymes (Cases et al., 1996, Drago et al., 1998a, Rios et al., 1999, Stanwood et al., 2005, Crandall et al., 2007). In these approaches, changes in dopamine function or gene expression occur globally, in all cell types of the brain throughout the period of brain development and at maturity. These methods cannot discriminate between dopamine's selective effects on proliferating precursor cells and postmitotic neurons. Moreover, in conditions such as fetal drug exposure, dopamine imbalance can occur transiently over short periods of gestation. The cell type specific and transient nature of dopamine receptor imbalance is difficult to model in the traditional pharmacological or gene knockout mouse models.

We describe a transgenic mouse model in which dopamine receptor transgenes can be expressed in the neuroepithelial cells at experimenter-chosen intervals of brain development.

Experimental Procedures

Transgene construction, transgenic mice, and genotyping

B6-nestin-rtTA-IRES- β geo (nestin-rtTA)—The construction of this transgene is described in detail previously (Aoki et al., 2000, Mitsuhashi et al., 2001). Briefly, IRES- β geo was removed from pPGT.1.8IRES- β geo (gift from Dr. Austin Smith, University of Edinburg, Scotland) and inserted into the *Sma*I-*Sal*I site of pBSII KS(+) (Stratagene, La Jolla, CA) to generate the P_{nestin}^{rtTA} transgene. rtTA was removed from pUHD17-1 (gift from Dr. Hermann Bujard, University of Heidelberg, Germany) and inserted into a blunt-

ended *SpeI* site pBSII KS(+)/IRES- β -geo. A *NotI* linker was inserted in the *BglII* site of plasmid gITK (gift from Dr. Richard Josephson, NIH) to facilitate removal of the rat nestin intron II and TK minimal promoter. The *NotI* fragment was then inserted into a *NotI* site of pBSII KS(+)/rtTA-IRES- β geo.

B6-TRE-D1R-EGFP (D1R-EGFP)—PCR was performed to amplify the full-length mouse D1-receptor (D1R) gene, which was flanked by *MluI* and *NheI* restriction endonuclease sites. The amplicon was digested with *MluI* and *NheI* and cloned into pBi-EGFP (Clontech, Palo Alto, CA). pBi-TRE-EGFP/D1R plasmids were digested by the endonucleases *DraI* and *PacI* (New England BioLabs, Beverly, MA), respectively.

The nestin-rtTA and D1R-EGFP fragments were each injected separately into C57B6 mouse zygotes. In each case, the injected embryos were implanted in the oviducts of day 1 pseudopregnant foster females. Transgenic mice were identified in the resulting litter by Southern blot analysis and PCR using tail genomic DNA. An 829 bp fragment from the rtTA transgene was amplified using primers F-AAAAATAAGCGGGCTTTGCT and R-TGCTCCATTGCGATGACTTA. A 305 bp fragment from the D1R-EGFP transgene was amplified using EGFP primers, F-AAGCAGAAGAACGGCATCAAG and R-GCGGCGGTCACGAACCTC.

Nestin-rtTA - D1R-EGFP double transgenic (DT) mice were obtained by cross breeding hemizygous nestin-rtTA and hemizygous D1R-EGFP mice. Conception was ascertained by the presence of a vaginal plug. All the mice were housed in the institutional animal care facility with a 12 h light-dark schedule. Animal housing, breeding and experimental procedures described in this study conformed to institutional and NIH guidelines for the care and use of laboratory animals.

5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal) histochemistry to verify rtTA expression

Expression of rtTA was evaluated by histological analysis of pattern of expression of the IRES-linked β geo reporter gene using X-gal histochemistry. Hemizygous nestin-rtTA and wild-type mice were crossed and the timed pregnant dams carrying E13 embryos were anesthetized (Ketamine, 50 mg/kg body weight and Xylazine, 10 mg/kg body weight, i.p.). The embryos were removed from the anesthetized dams and immersion fixed in 2 % paraformaldehyde, 0.2 % glutaraldehyde, 0.1 M NaHPO₄, pH 7.3 for 1 h at 4 °C. After three washes in 0.01 % sodium deoxycholate, 0.02 % Nonidet P-40, 2 mM MgCl₂, 0.1 M NaHPO₄, pH 7.3, X-gal staining was performed for 12 to 16 h at 37 °C in X-gal buffer (2 mM MgCl₂, 5 mM K₃Fe(CN)₆, 5 mM K₄Fe(CN)₆, 0.1 M NaHPO₄, pH 7.3, 0.01 % sodium deoxycholate, 0.02 % Nonidet P-40) containing 1 mg per ml of 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal; Fisher Scientific, Pittsburgh, PA).

In additional experiments, fixed brains from E13 mice were embedded in 5 % agarose (Type VII; Sigma, St. Louis, MO) and sectioned on a Vibratome (TPI) at 200 μ m thickness in the coronal plane. Sections were immersed in X-gal buffer (X-gal; 8 mg per ml) and washed in PBS at 4 °C.

Laser capture microdissection (LCM)

We examined whether expression of rtTA mRNA in the embryonic brain was limited to neuroepithelial domains (presumptive nestin expressing cells), as predicted. We collected samples of telencephalic proliferative and postmitotic domains from E12 and E13 nestin-rtTA hemizygous mice by using LCM (Araki et al., 2007). Embryos were removed from deeply anesthetized dams and the embryos were decapitated. The embryonic heads were

frozen in liquid nitrogen vapor and stored at -80°C . The frozen heads were sectioned on a cryostat at $70\mu\text{m}$ thickness in the coronal plane. The sections were mounted on RNase-free, MembraneSlides® (Molecular Machines and Industries, Glatbrugg, Switzerland), dried at room temperature and stored at -80°C . The sections were viewed under a Nikon TE2000 inverted microscope connected to CellCut® LCM equipment (Molecular Machines and Industries). The lateral ganglionic eminence (LGE), medial ganglionic eminence (MGE), dorsal cerebral wall and striatal differentiating field were identified and dissected by laser (UVCut software package; Molecular Machines and Industries). Immediately upon dissection, each sample was picked up on adhesive lids of $500\mu\text{l}$ mini IsolationCaps® (Molecular Machines and Industries) (Fig. 1 A, B). Each region of interest was collected from both hemispheres and pooled. We collected samples from 3–6 brains for each of the two embryonic stages.

Doxycycline administration to induce the D1R-EGFP transgene

Doxycycline (20 mg/ml dissolved in PBS, pH 7.0) was administered by oral gavage using a feeding needle to timed-pregnant dams resulting from crosses between nestin-rtTA and D1R-EGFP lines. The pregnant dams carried embryos of all 4 genotypes: wild type, double transgenic (DT), nestin-rtTA and D1R-EGFP. Thus, within the same litter the wild type embryos could serve as age-matched controls for the DT embryos. The doxycycline was administered twice daily (7 AM and 7 PM) on each of E12 and E13. The total daily dose of doxycycline was 25, 50 or 100 mg/kg body weight (each of the two daily administrations was 12.5, 25 or 50 mg/kg body weight). In the control group dams received saline instead of doxycycline.

Analysis of transgene mRNA expression

Following the doxycycline or saline administration on E12 and E13, pregnant dams carrying E14 embryos were anesthetized and the embryos were removed by hysterectomy. Upon verification of the age of each embryo forebrain was dissected and frozen in liquid nitrogen vapors. Tail samples were collected from each embryo for genotyping.

RNA isolation

RNA from the LCM samples or frozen forebrain samples was extracted with PicoPure RNA isolation kit (Arcturus-Molecular Devices, Mountain View, CA), and additional DNase digestion (Qiagen, Valencia, CA), according to manufacture's instructions. RNA, for validation of endogenous controls, collected by microdissection from cerebral cortex and striatum from E15 and P60 mouse brains, was isolated with RNAqueous-4PCR kit (Applied Biosystems, Foster City, CA) with DNase treatment according to manufacture's instructions.

Reverse transcription and quantitative real-time PCR

After validation of RNA sample quality and quantity with a Bioanalyzer 2100 (Agilent Technologies, Foster City, CA), $2\mu\text{g}$ of RNA were converted to cDNA by RT reaction using SuperScript II and random hexamer primers (Invitrogen, Carlsbad, CA). We used $0.5\mu\text{l}$ of cDNA per well in a $20\mu\text{l}$ reaction volume for PCR. Proprietary primers and fluorescent probes sequences were designed by Applied Biosystems TaqMan Gene expression assays. PCR for D1R-EGFP (F-AAGCAGAAGAACGGCATCAAG, R-GCGGCGGTCACGAAGTC), rtTA (F-AAAAATAAGCGGGCTTTGCT, R-TGCTCCATTGCGATGACTTA) and the reference gene 18s rRNA (TaqMan Probe, Hs99999901_s1, Applied Biosystems) was performed as follows.

TaqMan Fast Universal master PCR mix or Power SYBR Green master PCR mix was used for all PCR reactions (Applied Biosystems). Real-time PCR was performed in a Prism 7500

(Applied Biosystems) using the following thermal settings: 1 cycle of 2 min at 50°C, 1 cycle of 10 min at 95°C and 40 cycle of 15 sec at 95°C and 1min at 60°C. Relative mRNA expression for endogenous control genes was calculated by using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001, Araki et al., 2007). Cycle thresholds for all genes were selected 2 cycles past baseline amplification within the linear range on log scaling. All target gene expressions were normalized to the endogenous control 18s and the mean normalized expression was analyzed using Q-gene software (Simon, 2003, Araki et al., 2007). The data were analyzed by a Student's t-test or ANOVA using Prism 4 for Macintosh analysis package (GraphPad Software Inc., San Diego, CA).

Immunohistochemistry for EGFP, D1-receptor and nestin

Following the doxycycline or saline administration on E12 and E13, E14 embryos were collected as described above. The embryonic brains were removed and fixed by immersion in buffered 4% paraformaldehyde overnight at 4°C. Tail samples were collected for genotyping. The fixed brains were embedded in agarose and sectioned in the coronal plane on a Vibratome at 200 μ m thickness. The Vibratome sections were processed for EGFP immunohistochemistry (polyclonal anti-GFP antibody, 1:500, Abcam) using fluorescently tagged secondary antibody (anti-rabbit AlexaFluor 488, 1:500). In other cases, embryonic heads were snap frozen in liquid nitrogen vapors and sectioned in the coronal plane at 20 μ m thickness on a cryostat. The cryostat sections were fixed in buffered 4% paraformaldehyde for 10 min at room temperature and processed for D1-receptor (rat monoclonal, 1:1000; Sigma Chemical Co., St. Louis, MO) and nestin (mouse monoclonal, 1:100; Chemicon/Millipore, Billerica, MA) double-immunohistochemistry using fluorescently tagged secondary antibodies. The sections were examined using a Zeiss PASCAL laser scanning confocal microscope.

Bromodeoxyuridine (BrdU) administration and immunohistochemistry

Timed-pregnant dams resulting from crosses between nestin-rtTA and D1R-EGFP hemizygous mice were administered doxycycline or saline on E12 and E13 and a single injection of BrdU (Sigma Chemical Co., St. Louis, MO; 50 μ g/g body weight, i.p.) was administered to each dam on the 14th day of pregnancy. Two hours later the dams were anesthetized and the embryos were collected as described earlier. The embryonic brains were removed and fixed by immersion in 70% ethanol. Tail samples were collected for genotyping. The embryonic brains were processed for paraffin wax histology and sectioned in the coronal plane at 4 μ m thickness. BrdU immunohistochemistry was performed on the paraffin sections using a monoclonal anti-BrdU antibody (Becton Dickinson, Mountain View, CA; 1:75 dilution) and cobalt-nickel enhancement of diaminobenzidine reaction product according to methods described earlier (Bhide, 1996, Popolo et al., 2004, McCarthy et al., 2007). The sections were stained with 0.1% aqueous basic fuchsin. In these preparations, BrdU-labeled nuclei appear black and non-BrdU-labeled nuclei appear reddish pink (Ohtani et al., 2003, Popolo et al., 2004).

BrdU labeling index (BrdU LI; BrdU-labeled nuclei $\div$ all nuclei per defined area) was calculated by counting BrdU-labeled and unlabeled nuclei within a 120 $\times$ 240 μ m² sector of the lateral ganglionic eminence (LGE), a 120 $\times$ 120 μ m² sector of the medial ganglionic eminence and a 100 $\times$ 100 μ m² sector of the neuroepithelium of the frontal cortex (Popolo et al., 2004, McCarthy et al., 2007). The sector for the LGE was divided into twenty bins (each bin was 120 $\times$ 12 μ m²), the sector for the MGE into ten bins (each bin was 120 $\times$ 12 μ m²) and the sector for the cortical neuroepithelium also into ten bins (each bin was 100 $\times$ 10 μ m²). The sectors were aligned such that bin 1 was at the ventricular surface, with its long axis parallel to the ventricular border. The BrdU LI was calculated separately for each bin so that the data

could be analyzed separately for the ventricular and subventricular zones (VZ and SVZ, respectively).

The different sizes of the sectors and bins represent different sizes of the neuroepithelium in the different regions. The neuroepithelium of the LGE extends over a larger area compared to the other neuroepithelial domains, due to the large size of its subventricular zone (Bhide, 1996, Sheth and Bhide, 1997, Ohtani et al., 2003, Popolo et al., 2004). The different sizes also correspond to the sizes used in our previous work, facilitating comparisons across studies (Bhide, 1996, Sheth and Bhide, 1997, Ohtani et al., 2003, Crandall et al., 2004, Popolo et al., 2004).

Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL) analysis

Following the doxycycline or saline administration on E12 and E13, E14 embryos were collected as described above. The embryonic heads were snap-frozen in isopentane cooled to -40°C in liquid nitrogen. The frozen brains were sectioned on a cryostat in the coronal plane at a thickness of 20 μm and processed for TUNEL histochemistry according to manufacturer's instructions (Roche Diagnostics, Purchase, NY). The sections were stained with ToPro3 (Molecular Probes) to label DNA, coverslipped with Gelmount and examined under the confocal microscope to analyze TUNEL+ profiles in the neuroepithelial and postmitotic regions of the basal and dorsal telencephalon.

Results

rtTA expression in the embryonic brain

Expression of rtTA mRNA in forebrain samples of nestin-rtTA hemizygous mice was analyzed by X-gal histochemistry and PCR. We found that X-gal reaction product was restricted to the central nervous system. It was uniform and intense in the telencephalon, mesencephalon, rhombencephalon, spinal cord in E11 and E13 whole embryo preparations (Fig. 1 A, B). X-gal staining was not detectable in skeletal muscle or the viscera. To verify neuroepithelial cell specific expression, we performed the X-gal histochemistry in Vibratome sections of the E11 mouse brain. The X-gal reaction product was abundant in the MGE and relatively less robust expression was detected in the LGE and the dorsal cerebral wall (Fig. 1 C, D).

To further confirm neuroepithelial cell-specific expression of rtTA, we performed real-time quantitative PCR on samples obtained by LCM from the LGE, MGE, CGE, dorsal cerebral wall and striatal differentiating fields from E13 mice. We found that rtTA mRNA expression was the highest in the MGE, followed by LGE, CGE, dorsal cerebral wall and striatal fields (Fig. 1 E). The expression level in the MGE was nearly 8-fold higher than that in the LGE. These data support the X-gal labeling data, which showed highest X-gal labeling intensity in the MGE. ANOVA showed significant differences ($p = 0.02$) in rtTA mRNA expression level among the different brain regions analyzed. Tukey's multiple comparison test showed that MGE expression was significantly higher than that in all the other regions. Pair-wise comparisons between none of the other regions showed statistically significant differences.

Doxycycline-induced expression of D1R-EGFP mRNA

Expression of the D1R-EGFP transgene in embryonic brains was induced by oral administration of doxycycline to timed-pregnant dams resulting from crosses between nestin-rtTA and D1R-EGFP lines. The pregnant dams carried embryos of all 4 genotypes: wild type, double transgenic (DT), nestin-rtTA and D1R-EGFP. Thus, within the same litter the wild type embryos could serve as age-matched controls for the DT embryos.

Doxycycline was administered twice daily (at 7 AM and 7 PM) on E12 and E13. Embryonic forebrains were collected at 9 AM on E14 for analysis of the D1R-EGFP transgene expression by using quantitative PCR for EGFP mRNA.

EGFP mRNA induction occurred following doxycycline administration in the DT embryos and not in the WT littermates (Fig. 2 A). The D1R-EGFP transgene was expressed in the DT embryos with doxycycline doses of 50 or 100 mg/kg bodyweight/day but not at 0, or 25 mg/kg body weight/day (Fig. 2B). The wild type embryos from the same litter did not show transgene expression at any of the doxycycline doses (Fig. 2 A, B). In all the other experiments, we used the 50 mg/kg/day doxycycline dose.

Additional experiments using quantitative real-time PCR confirmed D1R-EGFP transgene expression in the DT embryos exposed to 50mg/kg/day doxycycline administration (Fig. 2 C; arrows). The D1R-EGFP transgene expression was also noted in DT embryos not exposed to doxycycline (Fig. 2 C arrowhead). However, quantitative analyses showed >1,500-fold induction of EGFP transgene expression in the doxycycline-exposed DT embryos (0.00106 ± 0.0009 copies/18s copy) compared to non-doxycycline-exposed DT embryos ($5.99 \times 10^{-7} \pm 5.7 \times 10^{-7}$ /18s copy). The extremely low expression in the non-doxycycline exposed DT embryo may be merely background.

EGFP immunofluorescence confirms D1R-EGFP transgene expression

We analyzed EGFP reporter expression in the embryonic brains by immunohistochemistry. EGFP labeling was present only in the E14 DT embryonic brains and not in the brains of wild type littermates upon doxycycline exposure on E12 and E13 (Fig. 3). The EGFP was detected in the ganglionic eminence and the cerebral wall (Fig. 3). At higher magnification, EGFP-labeled profiles resembled bipolar neuroepithelial cells with robust expression in the MGE compared to the LGE and cerebral wall (Fig. 3 H – J). The regional differences in rtTA mRNA expression (MGE > LGE/cerebral wall; Fig. 1) were not fully reflected in the intensity of immunohistochemical labeling for EGFP (Fig. 3).

Doxycycline-induced D1R protein overexpression in neuroepithelial cells

We analyzed D1R protein expression by immunohistochemistry in sections of the forebrain from E14 DT and WT embryos following exposure to 50 mg/kg/day doxycycline on E12 and E13 (Fig. 4). In both the genotypes, intensity of D1R fluorescence was the highest in the striatal differentiating fields, followed by the LGE (Fig. 4). The MGE and frontal cortical neuroepithelium showed the lowest intensity of D1R immunofluorescence. The intensity of the immunofluorescence was greater in the DT embryos compared to WT littermates in regions analyzed, with the striatal differentiating fields showing the largest difference (Fig. 4).

To confirm D1R expression in neuroepithelial cells we performed double immunofluorescence for nestin and D1R. We found that D1R expression occurred in nestin-positive cells in both the WT and DT E14 embryos exposed to doxycycline on E12 and E13. Examples of sections through the MGE of WT (Fig. 5 A – B) and DT (Fig. 5 C – F) embryos reveal labeling with D1R (A, C, E) and nestin (B, D, F). The DT embryos showed significantly higher intensity of D1R immunofluorescence compared to the WT littermates (Fig. 5 A–D). Merged image reveals D1R- and nestin co-localization in the MGE in the DT embryo (Fig. 5 F).

D1R-EGFP transgene induction does not cause cell death

We used TUNEL analysis to determine if cell death was increased in DT embryos exposed to 50 mg/kg/day doxycycline dose, which was sufficient for transgene induction without

causing embryo mortality. We examined LGE, MGE, neuroepithelium of the cerebral wall, striatal differentiating fields and cortical plate for TUNEL-positive profiles in E14 WT and DT embryos exposed to doxycycline on E12 and E13. We found only a very small number of TUNEL-positive profiles (less than one profile per 10 sections) in WT and DT embryos in any given brain region although positive control sections processed for TUNEL histochemistry at the same time as the embryonic sections showed clearly positive TUNEL profiles (Fig. 5 K). TUNEL histochemistry in the MGE and striatal differentiating fields, where the transgene expression and the D1R protein overexpression achieved the highest levels among all the brain regions examined are shown for WT and DT embryos in Figure 5 (G – J). The frequency of occurrence of the TUNEL profiles was not different between the genotypes, suggesting that doxycycline exposure and induction of the D1R-EGFP transgene did not contribute to cell death.

D1R-EGFP transgene induction decreases BrdU labeling index in the E14 brain

The BrdU labeling index was calculated in the MGE, LGE and neuroepithelium of the dorsal cerebral wall (CW; presumptive frontal cortex) in E14 embryos following doxycycline exposure on E12 and E13. The labeling index in each brain region was analyzed separately for the ventricular and subventricular zones (VZ and SVZ) and also for the entire neuroepithelium (VZ + SVZ). These 3 regions were chosen to represent high (MGE), medium (LGE) and low (CW) transgene expression based on PCR and X-gal histochemistry data (Fig. 1). We found that the BrdU labeling index was significantly reduced in the VZ, SVZ as well as VZ + SVZ regions of the MGE and LGE in the DT embryo compared to the WT littermate (Fig. 3 6, B). In the CW, only the SVZ showed statistically significant reduction in the BrdU LI in the DT embryo (Fig. 6 C).

Discussion

We report development of a double-transgenic mouse model for inducible expression of the D1R transgene in neuroepithelial cells of the embryonic forebrain. Expression of rtTA mRNA, β -geo reporter and D1R protein indicate that the transgene induction is more robust in the MGE than in the other neuroepithelial regions. Moreover, even though the transgene is induced selectively in the neuroepithelium, the D1R protein overexpression persists in postmitotic cells of the striatum after the cells exit from the neuroepithelium.

The mouse model consists of two transgenic lines each carrying nestin-rtTA or TRE-D1R-EGFP transgenes that when bred together confer cell-type and temporal specificity to transgene expression. The nestin intron II enhancer/promoter targets the rtTA to neuroepithelial cells of the developing and mature brain (Zimmerman et al., 1994, Aoki et al., 2000, Mitsuhashi et al., 2001) whereas the TRE component of the TRE-D1R-EGFP transgene confers temporal specificity of transgene expression due to its responsiveness to exogenous doxycycline (Shockett and Schatz, 1996, Shin et al., 1999, Mitsuhashi et al., 2001).

Initially, we confirmed transgene expression by examining the expression of the IRES-linked β geo reporter in the nestin-rtTA mouse embryo by using X-gal histochemistry (Fig. 1A, B). Additional verification was obtained by analysis of rtTA mRNA expression by LCM and real-time quantitative PCR (Fig. 1C). These analyses collectively confirmed the highest expression levels in the MGE with relatively lower levels in the LGE and cerebral wall (Fig. 1).

We confirmed doxycycline-induced expression of the D1R-EGFP transgene by analysis of EGFP mRNA (Fig. 2), EGFP protein (Fig. 3) and D1R protein expression (Figs. 4 and 5). The mRNA expression was confirmed in the forebrain of the DT embryos.

Immunohistochemistry showed EGFP expression in the MGE, LGE and the cerebral wall. However, the regional differences in expression level of rtTA (Fig. 1; MGE>LGE) were not recapitulated by the intensity of EGFP immunohistochemical labeling (Fig. 3). Thus, the EGFP immunofluorescence may serve only as a qualitative measure of transgene expression. Double-immunohistochemistry for nestin and D1R confirmed D1R protein expression in nestin-positive neuroepithelial cells as well as in postmitotic cells of the striatal differentiating fields (Fig. 4). The over-expression of the D1R in striatal postmitotic cells illustrates persisting expression of the protein even after exit from the cell cycle.

The transgene induction was doxycycline dose dependent, as it occurred at doxycycline dose of 50 but not 25 mg/kg/day. Similar results were obtained in our earlier study in which expression of p27^{Kip1} transgene was induced with doxycycline using the same nestin-rtTA construct as that used here (Mitsuhashi et al., 2001). As in our earlier study, in the present study also a doxycycline dose of 100mg/kg/day resulted in selective resorption of DT embryos. The embryonic lethality is unlikely to be due to doxycycline toxicity because only the DT embryos and not embryos of the other genotypes (WT, nestin-rtTA or D1R-EGFP) were resorbed, even though the entire litter was exposed to the same high doxycycline dose. In our earlier study (Mitsuhashi et al., 2001) using the same nestin-rtTA construct that is used here, we showed that transgene mRNA induction occurred by 6 hr after doxycycline administration and reverted to basal levels by 48 hr. Thus, our transgenic mouse model is capable of regulatable gene expression that consists of rapid (within 6 hr) induction of the transgene.

We detected very low levels of D1R-EGFP transgene expression in DT embryos that were not exposed to doxycycline (Fig. 2C). The expression in the doxycycline-exposed DT embryos was nearly 2000-fold higher than that in the non-doxycycline-exposed DT embryos suggesting that the expression in the latter may be either low level leakage of expression or minor DNA contamination.

To assess whether the overexpressed D1R was functional, measures such as D1R agonist induced cAMP synthesis (Araki et al., 2006, Kubrusly and Bhide, 2010) were not suitable here because they would not permit discrimination between overexpression in the neuroepithelium and postmitotic regions of the embryonic brain. Since a consistent effect of activation of the D1-like receptors (D1 and D5 receptors) on neuroepithelial cells of the embryonic brain is significant decreases in BrdU incorporation (Song et al., 2002, Zhang and Lidow, 2002, Ohtani et al., 2003, Popolo et al., 2004, Zhang et al., 2005, Araki et al., 2006, McCarthy et al., 2007), we used BrdU labeling index to assess D1R function in the neuroepithelium.

We found significant decreases in BrdU labeling index in the ventricular and subventricular zones of the MGE and LGE and the subventricular zone of the cerebral wall in the doxycycline-exposed DT embryos (Fig. 6), in agreement with earlier studies on the effects of dopamine receptor activation on cell proliferation in the embryonic forebrain (Song et al., 2002, Zhang and Lidow, 2002, Ohtani et al., 2003, Popolo et al., 2004, Zhang et al., 2005, Araki et al., 2006, McCarthy et al., 2007). The cerebral wall did not show statistically significant differences in the overall BrdU LI (i.e. VZ + SVZ), although the BrdU LI was decreased in the SVZ of the CW (Fig. 6). The relatively small effect in the cerebral wall may reflect lower levels of transgene expression in this region (Fig. 1). In addition to serving as a functional readout of D1R overexpression, the BrdU data also show that overexpression of the D1R can influence neurogenesis in the intact embryo. Previous studies on this subject had used D1-like or D2-like receptor agonists or antagonists [reviewed in (Bhide, 2009)], which can influence the activity of more than one dopamine receptor. Here, we overexpressed only the D1R and examined the effects within 48 hrs of initiation of the

overexpression, a period of time that minimizes the possibility of confounding effects produced by compensatory changes in the other dopamine receptors.

The doxycycline-inducible gene expression system has been used extensively in neuroscience research. Both rtTA and tTA systems (also known as tet-on and tet-off systems, respectively) are widely employed (Mayford et al., 1996, Mansuy et al., 1998, Mitsuhashi et al., 2001, Sakai et al., 2002, Hwang et al., 2006, Kellendonk et al., 2006, Hong et al., 2007, Millecamps et al., 2007, Liu et al., 2008). The transgenic mouse model reported here has a number of unique advantages for studying the role of dopamine D1 receptor activation in neuroepithelial and postmitotic cells. The nestin intron-II enhancer/promoter used in the present model targets the rtTA gene to precursor cells in the embryonic as well as mature CNS (Aoki et al., 2000). Therefore, our nestin-rtTA transgenic line can be used to induce D1-receptor expression in the adult brain subventricular zone, where balance between D1- and D2-receptor activity regulates neurogenesis (Hoglinger et al., 2004, Van Kampen et al., 2004, Kippin et al., 2005, Van Kampen and Robertson, 2005). The D1R-EGFP transgenic line used here can be crossed with mouse lines expressing rtTA or tTA under the control of neuron-specific promoters (Mayford et al., 1996, Sakai et al., 2002, Hong et al., 2007, Millecamps et al., 2007) to induce D1-receptor expression in postmitotic neurons of specific brain regions. Thus, the use of two separate lines of mice each carrying the rtTA or the D1R-EGFP transgene confers significant versatility to our model.

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List of abbreviations

BrdU	5-bromo-2-deoxyuridine
CGE	Caudal ganglionic eminence
CW	Cerebral wall
D1-R	Dopamine D1 receptor
DT	Double transgenic
E	Embryonic day
EGFP	Enhanced green fluorescent protein
LCM	Laser Capture Microdissection
LGE	Lateral ganglionic eminence
LI	Labeling index
MGE	Medial ganglionic eminence
rtTA	Reverse tetracycline trans activator
SVZ	Subventricular zone
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling
VZ	Ventricular zone
X-gal	5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside

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Figure 1.

Expression of rtTA in hemizygous nestin-rtTA mouse embryos analyzed by X-gal histochemistry (A – D) and quantitative real-time PCR (E). Expression of the IRES-linked β geo reporter gene was analyzed using X-gal histochemistry β in whole embryo (A, B) and in coronal sections of the rostral forebrain (C, D). The X-gal reaction product was restricted to the central nervous system in embryonic day 11 (E11) and E13 hemizygous nestin-rtTA embryos and was absent from wild type (WT) littermates. In the rtTA embryos, the reaction product was uniform and intense in the telencephalon (Te), mesencephalon (Me), rhombencephalon (Rh) and the spinal cord (SC). It was absent from skeletal muscle and viscera. Coronal Vibratome sections of the E11 brain (D, E) confirmed robust X-gal labeling

in the medial ganglionic eminence (MGE) of the basal forebrain and traces of reaction product in the lateral ganglionic eminence (LGE) and the dorsal cerebral wall (CW). D is a higher magnification view of the labeling. Quantitative real-time PCR of RNA isolated from samples of the MGE, LGE, caudal ganglionic eminence (CGE), CW and striatal differentiating fields (STR) collected from E13 brain sections by laser capture microdissection showed nearly 8-fold higher expression of rtTA mRNA in the MGE compared to the other brain regions confirming the intense X-gal labeling in the MGE.

Figure 2.

D1R-EGFP transgene induction in E14 double transgenic (DT) embryos. (A) Transgene mRNA was detected by PCR in forebrain samples obtained from E14 DT mice but not wild type (WT) littermates following doxycycline administration for 2 days, on E12 and E13. (B) Transgene induction was doxycycline dose dependent and occurred at doxycycline doses of 50 or 100 mg/kg/day but not at 0 or 25 mg/kg/day. The transgene induction occurred in forebrain samples of DT embryos and not in WT embryos even though both genotypes were exposed to the same doxycycline dose in utero. (C) Approximately 1,796-fold higher induction of the D1R-EGFP transgene occurred in the DT embryos exposed to doxycycline (arrows) compared to DT embryos not exposed to doxycycline (arrowhead). The extremely

low level of expression of the transgene in the DT embryos in the absence of doxycycline (arrowheads) is likely background. DNA base-pair ladder is show in the left lane.

Figure 3.

Immunohistochemistry reveals EGFP reporter (surrogate for D1R-EGFP transgene) expression in the forebrain of E14 embryos exposed to doxycycline (50 mg/kg/day) on E12 and E13. EGFP expression (green) is detected in double transgenic (DT; A–C; H–J) embryos but not in wild type (WT; E–G) littermates. Nuclei are labeled with ToPro-3 (blue). EGFP expression occurs in the medial and lateral ganglionic eminences (MGE and LGE, respectively) and in the cerebral wall (CW). Expression in the MGE (H) is more intense than that in the LGE (I) or CW (J). Many EGFP expressing cells resemble neuroepithelial cells in that they have long processes directed away from the lateral ventricle (LV) and towards the pial surface.

Figure 4.

Immunohistochemistry reveals dopamine receptor (D1R) overexpression in the forebrain of E14 wild type (WT) and double transgenic (DT) littermate embryos exposed to doxycycline (50 mg/kg/day) on E12 and E13. A cartoon of a coronal section through an E14 forebrain shows the brain regions in which D1R expression was analyzed (A). D1R expression is significantly higher in the DT embryo compared to the WT embryo in the lateral ganglionic eminence (LGE; B, C), medial ganglionic eminence (MGE, D, E) and striatal differentiating fields (STR, F, G). In both the genotypes, the striatal differentiating fields show the highest D1R expression (F, G) among all the regions analyzed. Scale bar in G = 50μm and applies to all micrographs.

Figure 5.

Immunohistochemistry for D1-receptor (D1R) and nestin in the medial ganglionic eminence (MGE) of E14 wild type (WT) and double transgenic (DT) littermate embryos exposed to doxycycline (50 mg/kg/day) on E12 and E13. Lower magnification images show D1R and nestin expression in the MGE of WT (A, B) and DT (C, D) embryos. The D1R labeling in the MGE of the WT (A) is low, compared to that in the DT embryo (C). The higher D1R expression in the MGE of the DT embryo is due to doxycycline-induced overexpression of the D1R transgene. The higher magnification images of the MGE from the DT embryos show cell surface labeling with the D1R antibody (E) and its co-localization with nestin labeling (F). TUNEL histochemistry (G – J) did not reveal positive profiles in E14 wild type

(WT) or DT embryos exposed to doxycycline (50 mg/kg/day) on E12 and E13 in the MGE (G, H), where transgene expression was the highest or in the striatal the differentiating fields (STR; I, J), where the D1R overexpression was the highest. Inset (K) shows an image from a positive control section showing TUNEL+ profiles (green). The control section is from an adult mouse brain subjected to controlled cortical impact from our earlier study (Whalen et al., 2008). Sections processed for TUNEL (E – I) were counterstained with ToPro3 to label all nuclei (blue).

Figure 6. Bromodeoxyuridine (BrdU) labeling index (LI) in the lateral ganglionic eminence (LGE; A), medial ganglionic eminence (MGE; B) and neuroepithelium of the dorsal cerebral wall (CW; C) in embryonic day 14 (E14) double transgenic (DT) and wild type (WT) littermates following doxycycline exposure (50 mg/kg/day) in utero on E12 and E14. The BrdU exposure was for 2 hr. The BrdU LI is significantly decreased in the ventricular zone (VZ), subventricular zone (SVZ) and VZ + SVZ of the LGE and MGE (A, B) and in the SVZ of the CW of the DT embryo compared to the WT littermates.