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Expression and function of dopamine receptors in the developing medial frontal cortex and striatum of the rat

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Abstract

The timeline of dopamine (DA) system maturation and the signaling properties of dopamine receptors (DRs) during rat brain development are not fully characterized. We used *in situ* hybridization and quantitative PCR to map DR mRNA transcripts in the medial frontal cortex (mFC) and striatum (STR) of the rat from embryonic day (E) 15 to E21. The developmental trajectory of DR mRNAs revealed distinct patterns of DA receptors 1 and 2 (DRD1, DRD2) in these brain regions. Whereas the mFC had a steeper increase in DRD1 mRNA, the STR had a steeper increase in DRD2 mRNA. Both DR mRNAs were expressed at a higher level in the STR compared to the mFC. To identify the functional properties of DRs during embryonic development, the phosphorylation states of cyclic AMP response element binding protein (CREB), extracellular signal-regulated kinase 1/2 (ERK1/2), and glycogen synthase kinase 3 beta (GSK3 β) were examined after DR stimulation in primary neuronal cultures obtained from E15 and E18 embryos and cultured for 3 days to ensure a stable baseline level. DR-mediated signaling cascades were functional in E15 cultures in both brain regions. Because DA fibers do not reach the mFC by E15, and DA was not present in cultures, these data indicate that DRs can become functional in the absence of DA innervation. Since activation of DR signal transduction pathways can affect network organization of the developing brain, maternal exposure to drugs that affect DR activity may be liable to interfere with fetal brain development.

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Keywords

dopamine receptors; prenatal brain development; medial frontal cortex; striatum; QPCR; primary neuronal culture

Introduction

The monoamine dopamine (DA) modulates neurotransmission and neuronal excitability via activation of second messenger cascades coupled to DA receptors (DRs). DRs are G-protein coupled receptors (GPCRs), characterized by the G-protein that they couple to: DRD1 “like” couple to $G_{\alpha s}$ and DRD2 “like” couple to $G_{\alpha i}$ (Girault and Greengard, 2004; Neve et al., 2004; Seamans and Yang, 2004; Bronson and Konradi, 2010). Abnormal function of the DA system has been associated with neuro-psychiatric disorders such as Parkinson’s disease, attention deficit hyperactivity disorder (ADHD), and schizophrenia (Barzilai and Melamed, 2003; Goto and Grace, 2007; Genro et al.). The DA system is furthermore involved in reward pathways and addiction to drugs such as cocaine and amphetamine (Kauer and Malenka, 2007).

While DA pathways have been studied in great detail in the adult brain, the role and functional state of the DA system during development is not well established in rats. In the rodent brain, DRs have been detected in mid to late embryonic development in the medial frontal cortex (mFC), a heavily interconnected brain area involved in attention, cognition, and working memory (Schambra et al., 1994). At around the same time, DA receptors appear in the striatum (STR), a brain area implicated in motor behavior, motivation, and reward (Sales et al., 1989; Jung and Bennett, 1996; Arnsten and Li, 2005; Araki et al., 2007; Van den Heuvel and Pasterkamp, 2008). During early brain development, events such as cell proliferation, differentiation, neuronal migration, and axon guidance are creating neuronal patterns and connections that determine brain function throughout life. Neuronal progenitor cells from the ventricular zone proliferate and begin to populate cortical layers V and VI around embryonic day (E) E15 in the rat brain (Kriegstein et al., 2006). Simultaneously, interneurons from the ganglionic eminences migrate tangentially into the cortex (Marin and Rubenstein, 2001). Midbrain ventral tegmental area (VTA) and substantia nigra (SN) neurons project via the medial forebrain bundle (MFB), arriving in the ventral and lateral regions of the rat STR at E14, and in the remaining areas of the STR by E18. MFB projections from the VTA continue past the STR and reach the subplate and intermediate zone of the mFC at E18 (Verney et al., 1982; Berger et al., 1983; Kalsbeek et al., 1988). DA positive fibers remain in this region for two days before entering the cortical plate at E20 (Van den Heuvel and Pasterkamp, 2008).

Because the rodent brain undergoes rapid changes during embryogenesis, a detailed characterization of the spatial and temporal expression patterns of DRD1 and DRD2 in the prenatal rat brain is essential. It is currently not known at what age DRs become functional and whether their signaling cascades in the embryonic brain reflect the known properties of DRs in adult animals.

In adult neurons, DRD1 activates adenylate cyclase, increases levels of cyclic nucleotides, activates protein kinase A (PKA) and mediates the phosphorylation of substrate molecules such as cyclic AMP response element binding protein (CREB), (Dudman et al., 2003) and extracellular signal-regulated kinase 1/2 (ERK1/2), (Valjent et al., 2000). DRD2 stimulation inhibits adenylate cyclase (Enjalbert and Bockaert, 1983) and activates beta arrestins and protein phosphatase 2A (PP2A) which inhibit protein kinase B (Akt), leading to the

dephosphorylation and activation of glycogen synthase kinase 3 beta (GSK3 β), a kinase involved in Wnt signaling (Cross et al., 1995; Beaulieu et al., 2009).

DRs activated in the embryonic brain modify neuronal migration, cell cycle activity, and cell morphology (Sales et al., 1989; De Vries et al., 1992; Todd, 1992; Schmidt et al., 1996; Stanwood et al., 2001; Song et al., 2002; Zhang and Lidow, 2002; Ohtani et al., 2003; Popolo et al., 2004; Zhang et al., 2005; Crandall et al., 2007). Recent studies indicate that monoamines and their signaling pathways can modulate axon guidance events in the embryonic brain by altering levels of cyclic nucleotides in the growth cone (Ming et al., 1997; Halladay et al., 2000; Nishiyama et al., 2003; Bouchard et al., 2004; Bonnin et al., 2007). Moreover, stimulation of the DA system in the adult brain, via external factors such as drugs of abuse, has also been shown to regulate axon guidance molecules in various brain regions (Bahi and Dreyer, 2005; Jassen et al., 2006; Yetnikoff et al., 2007; Sullivan, 2011).

Knowledge of the expression and functional state of DRD1 and DRD2 during early embryonic development is vital for our understanding of how the DA system contributes to cortical and subcortical organization and thus might be involved in the developmental aspects of neuro-psychiatric disorders such as schizophrenia. In the present study, we address this question in the mFC and STR of the developing rat brain.

Experimental procedures

Animals

All animals were housed and maintained in accordance with the policies of Vanderbilt University, which is accredited by the Association for the Assessment of Accreditation of Laboratory Animal Care. Timed-pregnant female Sprague-Dawley rats (Charles River, Wilmington, MA) were anesthetized with pentobarbital (65mg/kg, Sigma, St. Louis, MO) and embryos were removed and washed in sterile phosphate buffered saline (PBS). Studies were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, and formal approval to conduct the experiments described has been obtained from the animal subjects review board at Vanderbilt University. All efforts were made to minimize the number of animals used and their suffering.

Primary neuronal cultures

mFC or STR tissue from E15 and E18 embryos were dissected under a stereo microscope (see figure 1), dissociated in media, and plated onto 6 well plates at a density of approximately 500,000 cells per well, as previously described (Rajadhyaksha et al., 1999). For stimulation of DRs, cells were grown for 72 hours in vitro and treated for 15 minutes with 50 μ M of the DR agonists (+)-SKF 82958 hydrobromide, or ($\pm$)-PPHT hydrochloride (N-0434) (Sigma). Experiments were carried out at least in duplicates and in at least two independent dissections.

Generation of nested RNA probes

RNA extraction and cDNA synthesis were performed as previously described (Sullivan, 2011). PCR products were used for vitro transcription with modifications of a published protocol (Kuppenbender et al., 2000). Two sets of nested primers were designed for each target sequence within DRD1 and DRD2 with the help of Primerblast (<http://www.ncbi.nlm.nih.gov/tools/primer-blast>), whereby the internal primer pair included sequences that encoded either Sp6 or T7 RNA polymerase recognition sites (table 1A). One μ g of the nested PCR product was used to synthesize digoxigenin-labeled RNA probes using Sp6 (sense) or T7 (antisense) polymerase with the Dig RNA Labeling Kit (Roche Applied Science, Porterville, CA).

In situ hybridization

Embryonic brains were fixed overnight in 4% paraformaldehyde (PFA), freeze-protected in a series of graded sucrose solutions (10-30%), and cut to a thickness of 20 μ m on a cryostat. Hybridization was carried out as described (Bonnin et al., 2007) with modifications. A cocktail of three digoxigenin-labeled probes, covering three separate mRNA stretches for each gene of interest was hybridized at a concentration of 0.25-ng/ μ l per probe to the sections for 18 hours at 60°C. Adjacent sections were incubated in parallel with antisense and sense probes to control for nonspecific binding, and each slide contained brain slices from an entire developmental set (E15, E17, E19, E21). Following hybridization, sections were blocked with 3% blocking reagent (Roche) and incubated overnight in alkaline phosphatase-conjugated anti-digoxigenin antibody at 1:2,000 dilution (Roche). The phosphatase reaction was carried out in a solution containing 0.2mM 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and 0.2mM nitroblue tetrazolium (NBT). Sections were dehydrated and mounted with Permount (Fisher). Images were captured using Stereo Investigator software (MBF BioScience, Williston, VT). Densitometric analyses were done with Kodak Imaging software using 2 animals per time point. Measurements were taken from the cortical plate of the mFC and the STR (see figures 2, 3). To control for nonspecific hybridization and endogenous phosphatase activity, the intensity of the antisense signal was normalized to the sense signal from each adjacent section.

Northern blots

Three μ g of whole rat brain RNA was loaded per well of a denaturing formaldehyde gel (1X 4-Morpholinepropanesulfonic acid (MOPS) with 10% formaldehyde). Following size-separation, RNA was electrophoretically transferred to a charged nylon membrane in 1X tris-acetate-ethylenediaminetetraacetic acid (EDTA) (TAE) buffer. The membrane was UV-crosslinked and dried overnight before hybridization. Prehybridization was carried out in NorthernMax Hybridization Buffer (Ambion, Austin, TX) followed by hybridization of probe at 0.25 ng/ μ l. The membrane was washed twice at room temperature in 2X saline-sodium-citrate (SSC) buffer and twice at 65°C in 0.2 $\times$ SSC for 30 minutes. The membrane was incubated in blocking solution (100mM Tris-HCl, 150mM NaCl with 3% blocking reagent; Roche) followed by alkaline phosphatase-conjugated anti-digoxigenin antibody at a 1:50,000 dilution (Roche). After washing in 0.1M maleic acid buffer (pH 7.5), the membrane was equilibrated with diluted (1:250) alkaline-phosphatase luminescent substrate 2-chloro-5-(4-methoxyspiro(1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1^{3,7}]decan)-4-yl)-phenylphosphate (CDP-Star) (Roche) and imaged using a Kodak I440 CS Imaging system (Kodak, Rochester, NY).

QPCR

For mRNA analysis of DRs in tissue, samples were collected from E15, E17, E19, and E21 embryos and frozen at -80°C until RNA extraction. RNA extraction, cDNA synthesis, and QPCR were performed as previously described (Sullivan, 2011). Prior to cDNA synthesis, RNA was treated with DNase I, amplification grade (Invitrogen), for 15 minutes at room temperature to prevent DNA contamination. DNase activity was stopped with 25mM EDTA and incubation at 65°C. Primer sequences are listed in table 1B. All samples were examined in duplicate and values were normalized to the internal controls β -actin and 18S ribosomal RNA (18S rRNA), two genes that are among the more evenly expressed during development (McCurley and Callard, 2008). To ensure that the normalization control genes were not introducing false results, all data were analyzed without normalization, with each individual normalization control gene and with both normalization control genes combined. Although β -actin had a tendency to be regulated in the same direction as the dopamine receptor mRNAs, comparable statistical differences were seen in each analysis. Each QPCR plate had a standard curve of which efficiency and coefficient of determination values were

examined to verify the quality of the experiment. Expression levels were calculated using the formula $(1/2^{\Delta Ct})$ and all data were collected at the same fluorescence threshold. Five samples were analyzed for each time point with each sample generated from multiple animals. Each individual QPCR sample was composed of tissue from 4 animals for E15, 3 animals for E17, and 2 animals for E19 and E21.

Western Blotting

Primary neuronal cultures were harvested in 1X Laemmli buffer and sonicated. Samples were heated to 80°C for 10 minutes and separated on 10-20% Tris-Glycine gradient gels (Invitrogen). Proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Perkin Elmer, Waltham, MA) and membranes blocked with animal-free blocking solution (Vector Laboratories, Burlingame, CA). Primary antibodies were diluted in blocking solution and incubated with membranes overnight at 4°C. The following antibodies were used: anti-phospho CREB (Serine 133) 1:4000, anti-phospho ERK1/2 (P44/42 MAPK-Threonine 202/Tyrosine 204) 1:2000, and anti-phospho GSK3 β (Serine 9) 1:2000 (Cell Signaling, Danvers, MA). Membranes were washed 6 times in 50mM tris-buffered-saline with 0.05% tween-20 (TBS-T) and incubated for 30 min at room temperature with horseradish peroxidase (HRP)-conjugated secondary antibodies (Vector Laboratories) prepared in blocking solution. Blots were immersed in chemiluminescent reagents (Pierce, Rockford, IL) and exposed using a Kodak Imaging Station. MemCode Reversible Protein Stain (Thermo Scientific, Rockford, IL) was used prior to immunodetection to measure total protein per well. Proteins of interest were normalized to total protein (Aldridge et al., 2008) or beta actin (1:20,000; Sigma), detailed in the figure legends.

Statistics

Repeated measures analyses of variance (ANOVAs) were applied to developmental timecourses of each receptor, and multivariate ANOVAs were applied to compare differences in the developmental trajectories between DRD1 and DRD2 mRNA expression levels, as well as between mFC and STR. Post-hoc tests included paired t-tests to compare the expression of DRD1 to DRD2 within each sample at each time point and unpaired t-test for comparisons between the mFC and STR. For western blots and in situ hybridization, unpaired t-tests were used for 2-group comparisons and ANOVAs in cases of 3 or more groups. The JMP computer program (Cary, NC) was used for all analyses. Multiple comparison corrections were carried out for Western blots using the correction method developed by Benjamini and Hochberg (Benjamini and Hochberg, 1995), correcting for the three different phosphorylation antibodies within each time point and brain area. However, since Western data are only semi-quantitative and were obtained from independent dissections with independent control samples, multiple-comparison corrections might not be appropriate and may introduce false-negative findings. This is supported by the observation that some of the significant data that did not survive multiple-comparison corrections should be significant according to the literature (see 'Results' for details). We therefore present simple t-tests and note whenever data did not survive multiple comparison corrections.

Results

The individual probes for DRD1 and DRD2 detected a single band of mRNA transcript of the expected size (figure 1A). mRNA expression of DRD1 and DRD2 was assessed spatially and temporally by *in situ* hybridization in coronal brain slices of embryonic rats from E15 to E21, at two different levels for mFC and STR (figure 1B).

In both brain areas, little expression of DR mRNAs was detected at early time points but expression increased for both receptors over time (figure 2, figure 3). In a preliminary

analysis, we used t-tests to compare the expression levels of DRD1 and DRD2 mRNA at each developmental time point after subtracting the sense intensity measure from the antisense intensity to correct for background levels (figure 4A, 4B). Whereas no significant differences were seen at early time points, levels of DRs were significantly different at E19 in the mFC and at E21 in the STR. This analysis suggested that the trajectories of DRs were divergent, with more DRD1 mRNA in the mFC, compared to DRD2, but more DRD2 than DRD1 in the STR.

The *in situ* method provided an anatomical overview over the brain areas of interest, but it was only semi-quantitative and for repeated measures relied on densitometry estimates across different slides with some variation in background intensities. For quantification purposes we employed QPCR analysis in samples from an independent cohort of embryonic rats, a method well suited for a comprehensive statistical analysis. Similar patterns to the *in situ* hybridization were found, with the exception that measurable amounts of mRNA could be detected at E15 (figure 4C, 4D). From E15 to E21 both receptors were significantly increasing in the mFC (DRD1: $F_{3,16}=47.0$, $p<0.0001$; DRD2: $F_{3,16}=191.7$, $p<0.0001$, (figure 4C). Levels of DRD1 increased more than levels of DRD2, and this difference was supported in a multivariate ANOVA which showed significant differences between DRD1 and DRD2 ($F_{3,16}=8.1$, $p<0.0017$; repeated measure [DRD1, DRD2] $\times$ embryonic day). In concordance, the ratio of DRD1/DRD2 in the mFC was different at different developmental stages ($F_{3,16}=24.5$, $p<0.0001$), (table 2A). Except for E15, DRD1 levels were significantly higher than DRD2 levels in the mFC at all time points (table 2A), supporting the preliminary findings in the *in situ* hybridization analysis.

In the STR, both receptors showed a steady increase from E15 to E21 as well (DRD1: $F_{3,16}=15.0$, $p<0.0001$; DRD2: $F_{3,16}=19.8$, $p<0.0001$), with significant differences between DRD1 and DRD2 ($F_{3,16}=17.1$, $p<0.0001$; repeated measure [DRD1, DRD2] $\times$ embryonic day). The ratio of DRD1/DRD2 in the STR was different at different developmental stages ($F_{3,16}=10.2$, $p<0.0005$), (table 2B). Levels of DRD2 were significantly higher than levels of DRD1 at all time points (figure 4D). The overall expression of both receptor mRNAs was higher in the STR than the mFC ($F_{3,16}=10.7$, $p<0.0004$ for DRD1; $F_{3,16}=16.3$, $p<0.0001$ for DRD2; repeated measure [mFC, STR] $\times$ embryonic day), (table 3; figures 2, 3, and 4).

To examine functional maturation of the receptors, the signaling properties of each receptor were evaluated with specific DR agonists in primary neuronal cultures. Neurons from both brain regions were isolated at two time points, E15 and E18, and grown for 72 hours to ensure a stable baseline following the disruption during dissociation. Dissociation causes the release of metabolites that lead to the activation of signal transduction pathways which could mimic DR-mediated signaling pathways. After 72 hours in culture neurons have re-grown their processes and established synaptic connections. The embryonic time points were chosen to examine the ability of DRs to activate second messenger pathways before and after DA fibers have reached the mFC and STR. DA fibers are not reaching to the mFC at E15, but will have arrived at the subplate and intermediate zone of the mFC at E18 (Verney et al., 1982; Berger et al., 1983; Kalsbeek et al., 1988). In the STR, DA fibers are starting to innervate at E15 with a high density observed at E18 (Verney et al., 1982; Berger et al., 1983; Kalsbeek et al., 1988).

Initially, we examined the expression of DR mRNAs during each day in culture. In mFC neurons plated at E15, we observed a significant induction of DRD1 mRNA over time in culture ($F_{5,11}=149.7$, $p<0.0001$), while the change in DRD2 mRNA was much smaller, though still significant over time ($F_{5,11}=8.4$, $p<0.0017$), (figure 5A). A multivariate ANOVA showed significant differences between DRD1 and DRD2 during time in vitro

($F_{5,11}=70.3$, $p\leq 0.0001$; repeated measure [DRD1, DRD2] $\times$ day in vitro). In STR cultures plated at E15, both DRD1 ($F_{5,12}=45.2$, $p\leq 0.0001$) and DRD2 ($F_{5,12}=60.3$, $p\leq 0.0001$) mRNAs were induced rapidly (figure 5B), and multivariate ANOVA showed significant differences between DRD1 and DRD2 during time in vitro ($F_{5,12}=10.4$, $p\leq 0.0005$; repeated measure [DRD1, DRD2] $\times$ day in vitro). mFC neurons plated at E18 had a much larger induction of DRD2 mRNA than on E15 ($F_{3,8}=18.8$, $p\leq 0.0006$) (figure 5C).

Cultures were treated for 15-minutes with either the DRD1 agonist SKF82958 or the DRD2 agonist PPHT, and the phosphorylation status of the second messenger proteins CREB, ERK1/2, and GSK3 β was assessed with western blots (figure 6 and 7). In E15 cultures, activation of DRD1 was observed in both brain regions (figure 6). SKF82958 increased ERK1/2 phosphorylation in the mFC at this early time point ($t_{(20)}=2.6$, $p\leq 0.016$), whereas in the STR CREB phosphorylation ($t_{(18)}=2.6$, $p\leq 0.02$), ERK1/2 phosphorylation ($t_{(15)}=3.6$, $p\leq 0.0027$) and GSK3 β phosphorylation ($t_{(18)}=3.8$, $p\leq 0.0015$) were increased. In E18 cultures, SKF82958 increased CREB phosphorylation in the mFC ($t_{(31)}=2.2$, $p\leq 0.033$), and STR ($t_{(22)}=2.9$, $p\leq 0.0089$), as well as ERK1/2 phosphorylation in the mFC ($t_{(30)}=2.8$, $p\leq 0.0082$), and STR ($t_{(24)}=2.2$, $p\leq 0.038$). Interestingly, the striatal data did not survive multiple-comparison corrections (Benjamini and Hochberg, 1995), though it is well-known that both CREB and ERK1/2 do get phosphorylated in STR cultures from E18 in response to DRD1 stimulation (Konradi et al., 1996; Rajadhyaksha et al., 1998; Brami-Cherrier et al., 2002; Dudman et al., 2003). GSK3 β phosphorylation status was unaffected in either brain region in E18 cultures.

DRD2 activation by the agonist PPHT decreased GSK3 β phosphorylation in E15 cultures in both the mFC ($t_{(16)}=2.4$, $p\leq 0.027$) and STR ($t_{(7)}=3.6$, $p\leq 0.0085$; mFC data do not survive multiple-comparison corrections), as well as in E18 cultures (mFC: $t_{(10)}=6.2$, $p\leq 0.0001$; STR: $t_{(7)}=2.4$, $p\leq 0.0494$; STR data do not survive multiple-comparison corrections), (figure 7). PPHT also decreased CREB phosphorylation in the mFC at E18 ($t_{(19)}=4.0$, $p\leq 0.0007$), and ERK1/2 phosphorylation in the STR at both time points (E15: $t_{(7)}=3.0$, $p\leq 0.020$; E18: $t_{(6)}=3.3$, $p\leq 0.017$; STR data at E18 do not survive multiple-comparison corrections).

The specificity of DR activation was assessed by co-treatment of E18 mFC neurons with the DRD1 antagonist SCH23390, and the DRD2 agonist PPHT. Antagonism of the DRD1 receptor in conjunction with DRD2 stimulation did not change PPHT-mediated dephosphorylation of GSK3 β ($F_{(3,8)}=7.5$, $p=0.0104$), (figure 8). Post hoc t-tests showed a significant difference between DMSO-control and PPHT ($t_{(4)}=4.6$, $p\leq 0.0103$) as well as DMSO-control and PPHT pretreated with SCH23390 ($t_{(4)}=3.6$, $p\leq 0.0234$) but not between DMSO-control and SCH23390 ($t_{(4)}=1.7$, $p\leq 0.1556$).

Discussion

The expression pattern and signaling properties of DRs in the rat brain change considerably during embryonic development, with unique developmental trajectories of mFC and STR. Whereas the mFC had higher levels of DRD1 than DRD2 mRNA, the opposite pattern was seen in the STR. At E15, expression levels of DRs were low in both brain regions, but increased steadily over the course of embryonic development, reaching higher levels in the STR than in the mFC. The DR expression data agree with trends reported in a previous study in the murine brain but are not fully comparable as the murine study had somewhat different time points (Araki et al., 2007). We furthermore extend the previous study by showing that DRD1 and DRD2 are functional by E15, and by providing a detailed description of DR expression at time points between E15 and birth.

DR expression rose sharply during mid gestation in both the mFC and STR. DRs in the mFC exhibited the greatest rate of change from E19 to E21, concurrent with the arrival of DA fibers at the subplate and intermediate zone of the mFC at E18 (Verney et al., 1982; Berger et al., 1983; Kalsbeek et al., 1988) and the innervation of the mFC at E20 (Van den Heuvel and Pasterkamp, 2008). The slope of DR mRNA expression in the STR was greatest from E17 to E19, as DA fibers innervate the STR. This pattern of expression suggests that DR mRNA transcripts are timed with the arrival of midbrain DA efferents.

To examine if DR mRNA expression in cortical and striatal neurons depends on DA axon innervation, we measured levels of DR mRNAs in cultured neurons isolated at either E15, before DA fibers innervate these areas or at E18, during innervation, and grown for 3 days in vitro. Similar to tissue, mRNA levels of both DRs increased steadily in the mFC and the STR, though the induction of DRD2 mRNA in the mFC was small when cultures were started at E15 and larger in E18 cultures. Since expression and functional activation of both DRs was detected at E15, DA axon innervation does not seem to be required for DR expression.

Causes for the moderate induction of DRD2 mRNA at E15 could include the need for external growth or guidance factors, absence of glial support in the cultures, missing environmental cues or an underrepresentation of DRD2 expressing neurons in the mFC at E15. At E15 the cortex consists of a thin layer of pre-plate cells, and only a subset of the DRD2-positive neurons may have matured (Kriegstein et al., 2006). Birth of the remaining DRD2 population may occur between E15 and E19, thus accounting for the larger induction of DRD2 mRNA in E18 cultured neurons.

DRD1 agonists activate PKA and facilitate the phosphorylation of CREB and ERK1/2 (Valjent et al., 2000; Dudman et al., 2003). DRD1 and DRD2 have opposing effects on the Akt second messenger pathway and on GSK3 β phosphorylation: DRD1 agonists cause phosphorylation of GSK3 β , while DRD2 agonists cause dephosphorylation of GSK3 β (Iwakura et al., 2008; Beaulieu et al., 2009; Beaulieu and Gainetdinov; Souza et al., 2011). The phosphorylation patterns of CREB, ERK1/2 and GSK3 β were used to examine if DRs were functionally coupled to signal transduction pathways in embryonic neurons. In E15 cultures of the mFC, DRD1 was coupled to ERK1/2 phosphorylation. Coupling of DRD1 pathways was even stronger in mFC neurons cultured at E18, causing both CREB and ERK1/2 phosphorylation. In the STR, coupling of DRD1 to CREB and ERK1/2 signal transduction pathways was evident in neurons cultured at either E15 or E18. These findings are in agreement with studies that have shown cocaine-mediated phosphorylation of ERK1/2 exclusively in DRD1-expressing neurons, and inhibition of amphetamine-mediated CREB, ERK1/2, and Akt phosphorylation after pretreatment with the DRD1 antagonist SCH23390 (Bertran-Gonzalez et al., 2008; Shi and McGinty, 2011).

DRD2 activation with PPHT led to dephosphorylation of GSK3 β in both brain regions as early as in E15 cultures. PPHT mediated signaling was mediated by DRD2 and was not affected by a DRD1 antagonist. Inhibition of CREB phosphorylation by PPHT was observed in the mFC in E18 cultures, and of ERK1/2 phosphorylation in the STR in E15 and E18 cultures. This inhibition might have resulted from the inhibitory action of DRD2 on PKA pathways (Enjalbert and Bockaert, 1983) or an interaction of AKT with signal transduction pathways regulating CREB and ERK1/2 phosphorylation. The data indicate that DR mRNA expression as well as activation of DR signal transduction pathways can be induced in the absence of DA innervation, suggesting an internal timing mechanism of DR expressing neurons in mFC and STR.

Many of the previous studies that have examined DR-mediated modulation of Akt-GSK3 β activity were carried out in STR tissue or cultured neurons (Beaulieu et al., 2007; Iwakura et al., 2008). We present evidence that DRD2 activation increases GSK3 β activity in the mFC as well as the STR.

Conclusions

Since the mFC and STR are important for cognition, attention, movement, emotion, and learning, aberrant connectivity of these regions could impair behavior later in life, as seems to be the case in schizophrenia (Zalesky et al., 2011). DA could be a contributing factor in the organization of neuronal networks in DR-expressing neurons of the developing brain. Activation of DRs may influence developmental processes via molecules such as CREB, ERK1/2, and GSK3 β , all of which may signal to the nucleus to transcribe genes important for growth and development. Many intracellular signaling molecules affected by DR activity have the capability to modulate axon guidance events, including PKA, calcium in growth cones, and cyclic nucleotides (Ming et al., 1997; Halladay et al., 2000; Nishiyama et al., 2003; Bouchard et al., 2004; Bonnin et al., 2007). Any source of prenatal DA, including maternally supplied DA, could thus affect fetal brain development. The early expression and functionality of DRs and their capability to activate signaling cascades provides the DA system with a powerful position to influence the progression of brain development and neural network connectivity. Abnormalities in fetal DR function could thus lead to a miswiring of the brain with detrimental consequences for brain function later in life.

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- Development of dopamine receptors 1 and 2 (DRD1, DRD2) was examined in the rat
- mRNA transcripts were mapped in the medial frontal cortex (mFC) and striatum (STR) from E15 to E21
- The mFC had a steeper increase in DRD1 mRNA, while the STR had a steeper increase in DRD2 mRNA
- Both DR mRNAs were expressed at a higher level in the STR compared to the mFC
- DR-mediated signaling cascades were functional prior to dopamine innervation.

Figure 1. Generation of DR probes to measure mRNA transcripts in rat brain

Three non-overlapping RNA probes were generated for both DRD1 and DRD2 and combined for *in situ* hybridization studies. Each probe (labeled '1', '2', '3') was examined individually in Northern blots using RNA from whole rat brain (A). Arrows indicate the 28S and 18S rRNA bands. Expected size of DRD1 mRNA is approximately 4 kB and DRD2 is approximately 2.7kB (Bunzow et al., 1988; Zhou et al., 1990). The approximate size of the 28S band in rats is 4.8 kB and 18S is 1.9 kB. (B) Schematic of an embryonic rodent brain. For *in situ* analyses, probes were hybridized to coronal sections taken from the mFC (1) or STR (2). D=dorsal, C=caudal. (C-E) Dissection strategy for primary neuronal cultures, QPCR and Western blots. (C) Dorsal view with the first three cuts (1-3) that removed septum and midbrain. (D) Hemispheres were rotated to a sagittal view from the lateral ventricle onto the inside of the cortex. The part of the hippocampus that was not removed by cuts 2 or 3 was lifted up and FC was cut out as shown (cuts 4 and 5). (E) Coronal view to show how striatum was removed from RH and NC (cut 6). (F) Coronal brain slices from E15 to E21. The lighter colored regions indicate the approximate area of mFC and STR dissected. mFC was dissected rostral to this area, and STR caudal to this area. Abbreviations: FC: frontal cortex; HP, hippocampus; LV, lateral ventricle; NC, neocortex; OB, olfactory bulb; RH, rhinencephalon; SP, septum; ST, striatum. Scale bar 500μm.

Figure 2. *In situ* hybridization of DRD1 development in rat mFC and STR

Representative photomicrographs of *in situ* hybridization of coronal sections of embryonic rat brains at E15, E17, E19, and 21. A combination of three DRD1 probes was used to visualize the expression of receptors in mFC (left panel) and STR (right panel). For each time point, a representative antisense slice was placed next to a sense slice to show background levels of hybridization. Boxes indicate the regions in which *in situ* densitometry was quantitated. Scale bar is 500µm.

Figure 3. *In situ* hybridization of DRD2 development in rat mFC and STR

Representative photomicrographs of *in situ* hybridization of DRD2 receptors in coronal sections of embryonic rat brains from E15 to E21. A combination of three DRD2 probes was used to detect receptors in mFC (left panel) and STR (right panel). For each time point, a representative antisense slice was placed next to a sense slice to show background levels of hybridization. Boxes indicate the regions in which *in situ* densitometry was quantitated. Scale bar is 500µm.

Figure 4. DR mRNA expression measured by *in situ* hybridization and QPCR

Densitometric analyses of *in situ* hybridization results for DRD1 and DRD2 reveals significant increases in mFC (A) and STR (B) in a preliminary statistical analysis. Shown are the levels of intensity of antisense probes, normalized to the background signal generated by sense probes from an adjacent section. N=12-17 area measurements in 2 slices per time point. QPCR was used to measure mRNA transcript levels of DRD1 and DRD2 in samples from mFC (C) and STR (D). Values were normalized to the control genes beta actin (ACTB) and 18S rRNA. Inserts: Magnification of expression levels at E15. n=5 samples/time point. *p<0.05, **p<0.01, *** p<0.001 in the comparison of DRD1 to DRD2 at individual time points; mean $\pm$ SEM. Brackets around asterisks denote that these data are semiquantitative. Paired t-tests were used in (C) and (D).

Figure 5. DR mRNA expression in mFC and STR neuronal cultures increases over time

The developmental trajectory of mRNA transcript levels of DRD1 and DRD2 was examined in primary culture from mFC (A) and STR (B) plated at E15, and primary culture from mFC at E18 (C). DIV = day in vitro starting 24 hours after plating as DIV 1. Values were normalized to the control genes beta actin (ACTB) and 18S rRNA. Data mean $\pm$ SEM; paired t-tests: * $p < 0.05$, ** $p < 0.01$; $n = 3$ per time point. Expression levels for both DRs were significantly altered over time in all experiments (see 'Results').

Figure 6. DRD1-mediated activation of signal transduction pathways in embryonic neuronal cultures from the rat mFC and STR

Phosphorylation of CREB (A, B), ERK1/2 (C, D), and GSK3 β (E, F) was measured in embryonic neurons from mFC (A, C, E) or STR (B, D, F) at E15 and E18 in response to 15-minute treatments with the DRD1 agonist SKF82958. All neurons were cultured for 3 days. Bands were normalized to total protein on the membrane. Representative blots are shown beneath each histogram. Data mean $\pm$ SEM; t-tests: * = $p < 0.05$, ** = $p \leq 0.01$. N=10-14 per group.

Figure 7. DRD2-mediated activation of signal transduction pathways in embryonic neuronal cultures from the rat mFC and STR

Phosphorylation of CREB (A, B), ERK1/2 (C, D), and GSK3 β (E, F) was measured in embryonic neurons from mFC (A, C, E) or STR (B, D, F) at E15 and E18 in response to 15-minute treatments with the DRD2 agonist PPHT. All neurons were cultured for 3 days. Bands were normalized to total protein on the membrane. Representative blots are shown beneath each histogram. Data mean $\pm$ SEM; t-tests: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. N=4-12 per group.

Figure 8. PPHT mediated activation of GSK3 β is specific for DRD2

Co-treatment of E18 mFC neuronal cultures for 15 minutes with the potent DRD2 agonist PPHT and the DRD1 antagonist SCH23390. Antagonism of DRD1 in conjunction with PPHT did not affect PPHT-mediated activation of GSK3 β . Bands were normalized to β -actin. Representative blots are shown beneath histogram. Data mean $\pm$ SEM; t-tests: * = $p < 0.05$. N=3 per group.

Table 1

List of primer sequences used for in situ probe synthesis (A) and QPCR (B).

A. Primer pairs used to synthesize RNA probes						
Name	Genbank #	Exons covered	direction	bp From /To	Size	sequence
DRD1 outer pair 1	NM_012546.2	exon 1	forward reverse	585/959	375	5'-ACCTCCTGGAGGACACCGA-3' 5'-AGAGCCACAGAAGGGCACC-3'
DRD1 outer pair 2	NM_012546.2	exon 1	forward reverse	22/428	407	5'-TGAGGGGCAAGTCCCGGAA-3' 5'-AGCCCAAGTACCTGTCCACGCT-3'
DRD1 outer pair 3	NM_012546.2	exon 1	forward reverse	1423/1815	393	5'-GCCACGAGTTCCCTTGGGCTT-3' 5'-ATCCCACTCCTGCTGTAAAGGT-3'
DRD2 outer pair 1	NM_012547.1	exons 2-4	forward reverse	429/865	437	5'-AGGCAGACAGGCCCCACTACA-3' 5'-TCCGAAGAGAGCTGGGAGGA-3'
DRD2 outer pair 2	NM_012547.1	exons 7-8	forward reverse	1273/1701	429	5'-TCCATCCCAACCGGCTACA-3' 5'-TGTGCAGGCAGGGGCAGAC-3'
DRD2 outer pair 3	NM_012547.1	exons 5-7	forward reverse	898/1259	362	5'-TGCCAAACCTGCCCTTTGTGGTC-3' 5'-GCTGGTGTGACTGGGAGGGAT-3'
DRD1 nested pair 1	NM_012546.2	exon 1	forward reverse	665/901	284	5'-AAGCATTTAGGTGACACTATACTTTACATCCCGTAGCA-3' 5'-AAGCTCTAATACGACTCACTATAGGGAACACCCCATGATCAGAG-3'
DRD1 nested pair 2	NM_012546.2	exon 1	forward reverse	69/361	340	5'-AAGCATTTAGGTGACACTATACTGCCAGGGAGAGGATT-3' 5'-AAGCTCTAATACGACTCACTATAGGGTGACGCCGTAGGACATGA-3'
DRD1 nested pair 3	NM_012546.2	exon 1	forward reverse	1459/1733	322	5'-AAGCATTTAGGTGACACTATAACAGGAGATCCCTCTGCTGCTT-3' 5'-AAGCTCTAATACGACTCACTATAGGGTGCTCGGACAGTTTAGCACCTG-3'
DRD2 nested pair 1	NM_012547.1	exons 2-4	forward reverse	506/793	335	5'-AAGCATTTAGGTGACACTATACTGGTGTGCATGGCTGTATC-3' 5'-AAGCTCTAATACGACTCACTATAGGGCTTGGAGCTGTAGCGTGTGT-3'
DRD2 nested pair 2	NM_012547.1	exons 7-8	forward reverse	1394/1658	312	5'-AAGCATTTAGGTGACACTATAAGGCAAAACCCGGACCTCCCT-3' 5'-AAGCTCTAATACGACTCACTATAGGGAGGCCCTTGCAGAACTCGATG-3'
DRD2 nested pair 3	NM_012547.1	exons 5-7	forward reverse	943/1216	321	5'-AAGCATTTAGGTGACACTATACTGGTCCCTTCAATCGTCACTCTGC-3' 5'-AAGCTCTAATACGACTCACTATAGGGTGGGGGACTGTGCTTGACAG-3'

B. Primer pairs for QPCR					
Name	Genbank #	Exons covered	direction	bp From /To	sequence
DRD1	NM_012546.2	exon 1	forward reverse	400/570	171 5'-GCGTGATCAGCTGGACAGG-3' 5'-AGGGCCATGTGGCTTTGCC-3'
DRD2	NM_012547.1	exons 2-4	forward reverse	592/806	215 5'-GGTGGCCACACTGGTAATGCC-3' 5'-CAGTAACTCGCGCTTGGAGCT-3'

B. Primer pairs for QPCR						
Name	Genbank #	Exons covered	direction	bp From / To	Size	sequence
18s RNA	V01270.1	exon 1	forward reverse	1544/1720	177	5'-TGGCTCAGCGTGTGCCCTACC-3' 5'-TAGTAGCGACGGCGGTGTG-3'
Beta actin	NC_005111	exons 4-5	forward reverse	798/967	170	5'-CTATGAGCTGCCTGACCGT-3' 5'-TGGCATAAGAGGTCCTTACCGGA-3'

Table 2

Ratio of DRD1 over DRD2 mRNA in the developing rat brain, measured with QPCR.

A. mFC	Developmental timepoint	DRD1/DRD2	Paired t-test
	E15	0.90 ± 0.04	0.0470
	E17	1.97 ± 0.15	0.0046
	E19	2.35 ± 0.16	0.0002
	E21	1.37 ± 0.12	0.0398
B. STR			
	E15	0.45 ± 0.07	0.0325
	E17	0.82 ± 0.05	0.0488
	E19	0.82 ± 0.05	0.0149
	E21	0.67 ± 0.05	0.0066

Table 3

Ratio of DR mRNA in the mFC versus STR, measured with QPCR.

A. DRD1	Developmental timepoint	STR/mFC	t-test
	E15	0.59 ± 0.10	0.0609
	E17	3.42 ± 0.66	0.0007
	E19	6.90 ± 1.32	0.0011
	E21	3.72 ± 0.64	0.0073
B. DRD2			
	E15	1.27 ± 0.28	0.4745
	E17	7.74 ± 0.56	0.0002
	E19	18.72 ± 2.18	0.0001
	E21	7.67 ± 1.40	0.0015