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Estradiol and isotype-selective estrogen receptor agonists modulate the mesocortical dopaminergic system in gonadectomized female rats

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ABSTRACT

The mesocortical dopaminergic pathway projecting from the ventral tegmental area (VTA) to the prefrontal cortex (PFC) contributes to the processing of reward signals. This pathway is regulated by gonadal steroids including estradiol. To address the putative role of estradiol and isotype-selective estrogen receptor (ER) agonists in the regulation of the rodent mesocortical system, we combined fMRI, HPLC-MS and qRT-PCR techniques. In fMRI experiments adult, chronically ovariectomized rats, treated with either vehicle, estradiol, ERα agonist 16α-lactone-estradiol (LE2) or ERβ agonist diarylpropionitrile (DPN), received a single dose of D-amphetamine-sulphate (10mg/kg, i.p.) and BOLD responses were monitored in the VTA and the PFC. Ovariectomized rats showed no significant response to amphetamine. In contrast, the VTA of ER agonist-substituted ovariectomized rats showed robust amphetamine-evoked BOLD increases. The PFC of estradiol-replaced animals was also responsive to amphetamine. Mass spectroscopic analysis of dopamine and its metabolites revealed a two-fold increase in both dopamine and 3,4-dihydroxyphenylacetic acid content of the PFC in estradiol-replaced animals compared to ovariectomized controls. qRT-PCR studies revealed upregulation of dopamine transporter and dopamine receptor in the VTA and PFC, respectively, of ER agonist-treated ovariectomized animals. Collectively, the results indicate that E2 and isotype-selective ER agonists can powerfully modulate the responsiveness of the mesocortical dopaminergic system, increase the expression of key genes related to dopaminergic neurotransmission and augment the dopamine content of the PFC. In a broader sense, the findings support the concept that the manifestation of reward signals in the PFC is dependent on the actual estrogen milieu of the brain.

1. Introduction

Accumulating evidence indicates that in rodents, 17β-estradiol (E2) enhances dopamine synthesis [1], release [2] and turnover [3] in areas associated with the mesolimbic and nigrostriatal pathways. We have revealed that E2 replacement increase D1 and D2 dopamine receptor expression in the frontal cortex of ovariectomized (OVX) rats [4]. This observation prompted us to explore the impact of E2 and isotype-selective ER agonists on the mesocortical system in female rats.

The ventral tegmental area (VTA) of the mesencephalon hosts the A10 dopaminergic cell group [5, 6] which gives rise to ascending mesolimbic and mesocortical projections [7-9]. The latter pathway heavily innervates the prefrontal cortex (PFC) in rodents [10], primates [11] and humans [12]. The information transfer between the VTA and the PFC is reciprocal [13, 14]. The VTA relays reward-related neuronal and metabolic signals to the PFC [15] predominantly via a dopaminergic neurotransmission [16, 17], while projecting pyramidal cells of the PFC utilize glutamate for communication with dopaminergic neurons of the mesocortical projection [18, 19]. The dopamine supply of the PFC is crucial for the maintenance of its basic, high level cognitive functions [20, 21]. Changes in the synaptic/extracellular content of dopamine [22], or alterations of the dopamine receptor-coupled signal transduction mechanisms [23, 24] evoke disturbances in mood [25], learning and memory [26] and processing of reward signals [16]. Dysfunction of the VTA-PFC unit has been implicated in the pathology of schizophrenia [27], bipolar disorder [28, 29] attention deficit hyperactivity disorder [30], and in the development of drug addiction [31]. It is noteworthy that effective medication of psychotic diseases partly relies on the selective targeting of dopaminergic neurotransmission [32].

In addition to the manifest psychotic diseases, an altered, deteriorating dopamine signaling characterizes the aging female brain [29, 33-35]. Obvious signs of decline in PFC-associated cognitive functions appear during perimenopause [18] . Many of the symptoms also reflect a

disturbed dopaminergic signaling [4, 36] which seems to correlate with the reduction in the levels of E2 [20]. The neuronal assembly of the VTA is also responsive to E2 [21] as resident dopaminergic neurons express ERα and ERβ [37-39]. Functional evidence has also been provided that the firing of VTA neurons is modulated by gonadal steroids [40]. Collectively, these data indicate that the gonadal hormone milieu may influence the function of dopaminergic neurons in both the VTA and the main neocortical target of these neurons, the neuronal assembly of the PFC.

The present rodent study was undertaken to reveal the putative role of E2 and isotype-selective ER agonists in the modulation of the VTA-PFC unit with the combined use of functional magnetic resonance imaging (fMRI), mass spectrometry and gene expression profiling. Amphetamine is known to increase synaptic dopamine levels through vesicular depletion [41] and reverse transport [42] in dopaminergic terminals. Using fMRI, responses to amphetamine [43] and its D and L stereoisomers [44] have been studied in rats by several laboratories [45-48]. Moreover, amphetamine is often used in rodent fMRI studies as a trigger to test the effect of a pharmacological manipulation such as D1 and D2 antagonism on regional brain activation [49]. Here, we used amphetamine in a modified acute treatment paradigm [43] to reveal the impact of the gonadal hormone milieu on the amphetamine-triggered blood-oxygenation-level-dependent (BOLD) response of the mesocortical pathway in OVX rats. Next, we applied mass spectrometry to investigate the levels of dopamine and its metabolites. Finally, real-time PCR was used to study the expression of a set of genes critical for dopaminergic neurotransmission. The findings of this study indicated that activation of the VTA-PFC axis by the psychostimulant D-amphetamine in OVX rats was subject to the availability of ER agonists. E2 and isotype-selective ER agonists altered dopaminergic neurotransmission via elevation of the dopamine content, and upregulation of dopamine receptors (D1A, D2, D3) and dopamine transporter (Slc6a3) in the PFC and the VTA, respectively.

2. Results

2.1. Functional MRI

To explore the effect of E2 on the mesocortical dopaminergic pathway, we followed the time course of the BOLD response to D-amphetamine [43, 50] in ovariectomized rats pretreated with either vehicle or E2 (Fig. 1). The selection of E2 regimen was based on our previous observation that E2 replacement with proestrous E2 levels enhanced mRNA expression of dopamine D1 and D2 receptors in the frontal cortex [4]. E2 replacement was carried out according to the protocol for Wistar rats reported by Cagampang *et al.* [51], to provide proestrous serum E2 levels (36pg/ml). In vehicle-treated ovariectomized rats, amphetamine had no significant effect on the BOLD responses neither in the PFC (Fig. 1A), nor the VTA (Fig. 1C). In ovariectomized rats treated with E2, amphetamine evoked positive BOLD response in the PFC (Fig. 1A) and the VTA (Fig. 1C), but not in the primary motor cortex (M1) (Fig. 1B). As indicated in Figure 1, we selected two time frames, between 1200 and 1300 sec (providing data for values referred as 4 min after amphetamine treatment), and between 2010 and 2110 sec (providing data for values referred as 17.5 min after amphetamine treatment) to quantitatively characterize the early and late phase responses to amphetamine, respectively, in the PFC, M1 and VTA.

Next, we compared the impact of pretreatments with E2 and isotype-selective ER agonists on the amphetamine response. 16α-lactone estradiol (LE2) and diarylpropionitrile (DPN) were synthesized and characterized by Schering AG [52] and the Katzenellenbogen laboratory [53], respectively. These compounds were less potent than E2 in many aspects (e.g. $RBA_{ER\alpha}$=15.4% for LE2, $RBA_{ER\beta}$=18% for DPN), therefore, they were used at a four-fold dose compared to that of E2. In the early sampling period at 4 min, we found significant BOLD increase in the PFC of E2-replaced rats (2.9%, p=0.005), but not in LE2- or DPN-treated animals (Fig. 2A). The M1 was not responsive to amphetamine in any treatment

group. In contrast, we detected increased BOLD in the VTA of E2-replaced (2.8%, p=0.009), LE2- (4.9%, p<0.001) and DPN-treated (3.5%, p=0.014) ovariectomized rats (Fig. 2A).

In the late sampling period at 17.5 min, we identified a blunted amphetamine-induced response in the PFC of E2-treated animals, which became non-significant (2.6%, p=0.058). A somewhat elevated but still not significant response was observed in the M1. On the other hand, the BOLD response in the VTA increased further in the E2-replaced (5.4%, p=0.008) and even more, in the LE2-treated animals (11.4%, p<0.001). In contrast, the response became non-significant in the VTA of DPN-treated animals (Fig.2B).

These results indicated that there was no detectable response to amphetamine in the PFC and the VTA in the absence of ovarian hormones (Fig. 2). However, chronic treatments with E2 or isotype-selective ER agonists turned the mesocortical system of ovariectomized rats responsive to amphetamine.

2.2. The levels of dopamine and its metabolites in the cerebral cortex of ovariectomized and

E2-replaced rats

The levels of dopamine and its major metabolites 3,4-dyhydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) and 3-methoxytyramine (3MT) were measured in the PFC, M1 and VTA by HPLC-MS. We compared neurotransmitter levels in ovariectomized rats treated with either vehicle or E2. The levels of dopamine and DOPAC in the PFC and M1 looked almost identical in ovariectomized rats. As a result of E2 treatment, ANOVA showed significant elevation in the levels of dopamine (358.5 *versus* 185.2 pmol/mg, p=0.002) and DOPAC (50.9 *versus* 23.4 pmol/mg, p<0.001) in the PFC, but not in M1 (Fig. 3). In the VTA, we found no alteration in the levels of dopamine and its metabolites (data not shown).

2.3. Targeted expression profiling

We explored the effect of treatments with E2 and isotype-selective ER agonists for 14 days on the expression of genes related to dopamine synthesis, metabolism, transport, binding and signal transduction (Table 1) in the PFC and the VTA in OVX female rats.

In the PFC, we observed upregulation of dopamine receptor genes including Drd1a, Drd2 and Drd3 (Fig. 4A-C). Drd1a expression was significantly increased by ERβ agonist DPN (RQ=1.471, p=0.042) and ERα agonist LE2 (RQ=1.706, p=0.003). Drd2 and Drd3 expression was upregulated by DPN (Fig. 4B,C). We measured mRNA expression of genes for regulator of G-protein signaling 2 (Rgs2), Rgs4, Rgs9 and Rgs14. While Rgs9 expression slightly increased in the LE2-treated group, this change did not reach statistical significance (RQ=1.822, p=0.082). The expression of other Rgs genes was unaffected.

In the VTA, we found no change in the expression of tyrosine hydroxylase (Th), GTP cyclohydrolase 1 (Gch1), dopa decarboxylase (Ddc), monoamine oxidase A (Maoa) and B (Maob), catechol-O-methyl-transferase (Comt), dopamine transporter (Slc6a3), norepinephrine transporter (Slc6a2) and vesicular monoamine transporter (Slc18a2). The only significant change was observed in Slc6a3 mRNA levels of the LE2-treated animals (RQ=1.451, p=0.017). Slc6a3 mRNA levels of E2-treated rats were slightly higher, but not different from levels of the ovariectomized controls (RQ=1.241, p>0.1) (Fig. 4D).

3. Discussion

3.1 Effects of E2 and isotype-selective ER agonists are region-specific and time-dependent

It has long been known that gonadal hormones modulate prefrontal functions via multiple mechanisms including actions on the mesocortical dopamine system [21, 47]. In this study, we addressed the putative role of E2 in the modulation of the rat mesocortical system. Strikingly, there is no BOLD response to high dose amphetamine challenge in the PFC and the VTA of vehicle-treated OVX animals. On the other hand, there is a characteristic BOLD response in E2-replaced OVX rats. In the PFC, early BOLD response to amphetamine is significantly higher in E2-treated animals compared to controls. However, the late BOLD response does not differ significantly from controls indicating desensitization of postsynaptic dopamine receptors. Isotype-selective ER agonists evoke some early BOLD response to amphetamine in the PFC, but the changes do not reach statistical significance. This result suggests that both ERα and ERβ are required for the enhanced early BOLD response in E2-replaced animals. In the VTA, E2 and isotype-selective ER agonists display overlapping, but not identical effects. The early BOLD responses are the most robust in LE2-treated OVX rats, and they are also significantly higher in E2- and DPN-treated animals. The late BOLD response increases further in the case of the three agonists and shows the highest increase in LE2-treated rats. These findings provide evidence that isotype-selective ER agonists can modulate the mesocortical system primarily through targeting dopaminergic cells of the VTA. The results underscore the importance of ERα in this action.

3.2 Estrogen-dependent modulation of the mesocortical system involves tuning of prefrontal dopamine levels and dopamine receptor expression

In female rats, dopamine levels vary across the estrous cycle in a phase-dependent manner [54] due to E2-dependent increase of striatal dopamine synthesis, release and turnover [55]. In concert with these results, we showed a two-fold increase in the level of dopamine and

DOPAC in the PFC of E2-treated OVX rats compared to vehicle-treated controls. This finding suggests that in the absence of E2 dopamine levels fall sharply in the PFC of female rats. The relationship between dopamine levels and PFC function is not linear, but shows an inverted U-shaped curve [56, 57]. There is a narrow range of dopamine levels which is optimal for the normal function of the mesocortical pathway, and our results suggest that estrogens play a pivotal role to tune dopamine levels in the PFC. However, we found no alterations in the expression of genes encoding tyrosine hydroxylase, GTP cyclohydrolase 1, dopa decarboxylase, monoamine oxidase A and B, catechol-O-methyl-transferase. From these findings we conclude that the transmission of reward signals to the PFC, in particular its dopaminergic component, is largely dependent on the gonadal hormone milieu, and E2 regulates dopamine availability mainly through the modulation of intracellular signaling pathways [55, 58]. The tuning of dopamine levels has particular importance since dopamine effects follow a U-shaped dose-response curve in the PFC [57, 59, 60].

ER agonists alter the expression of several genes that are related to dopaminergic neurotransmission. Such ER agonist-dependent alterations of the mesocortical system include upregulation of D1A, D2 and D3 dopamine receptors in the PFC. Functional MRI has been used to investigate the effects of D1 and D2 antagonist pretreatments on the amphetamine response [49] in the rat brain. BOLD responses in the frontal cortex are attenuated by D2 antagonist sulpiride indicating that amphetamine effects are mediated, at least partly, by D2 dopamine receptors. Our finding that D2 expression increases in the PFC of E2 replaced animals compared to vehicle treated OVX rats may explain the enhanced BOLD response in the PFC of E2-replaced animals. Increased dopamine receptor expression in E2-treated rats are in agreement with earlier observations on the estrogen-induced upregulation of D1 and D2 expression in the frontal cortex of various rat models [4, 36]. It is important to note that D2 receptors are well-established molecular targets for the treatment of neuropsychiatric disorders [61], and drugs targeting D2 are the first choice of treatments for schizophrenia,

bipolar disorder and major depressive disorder. Of note, the incidence of these disorders increases in the absence of estrogen [62].

3.3 In the presence of ER agonists amphetamine evokes a lasting BOLD response in the VTA

In addition to influencing the PFC, estrogen hormones also target midbrain dopaminergic neurons. The effects of E2 are primarily mediated by ERα and ERβ, which are expressed in the VTA. ERα-immunoreactivity is sparse and found mostly in the paranigral nucleus of the VTA [39], while ERβ-immunoreactive nuclei are mostly present in dopaminergic and non-dopaminergic neurons of the dorsal VTA [37]. In addition to direct effects on midbrain dopaminergic neurons, estrogens may modulate dopaminergic cells through descending glutamatergic projections from the estrogen receptive PFC [19, 63, 64]. The amphetamine response in the VTA is modulated by both E2 and isotype-selective ER agonists. Of note, ERα agonist-treated animals produce the most robust response in the VTA. However, ERβ agonist DPN treatment also makes the VTA responsive to amphetamine indicating that both ER isotypes are involved in the mediation of E2 effects in the VTA. This is in concert with the finding of Shughrue *et al.* [63] reporting that the VTA expresses mRNA message and immunoreactivity specific for both ERα and ERβ.

A previous study has investigated estrogenic regulation of dopamine transporter mRNA expression in the VTA of OVX rats using *in situ* hybridization [58]. They have found hormonal regulation of dopamine transporter expression neither in OVX nor E2-replaced animals. In concert with this finding, we do not observe significant alteration in mRNA expression of dopamine transporter (Slc6a3) in E2- and ERβ agonist-treated animals, but find upregulation of Slc6a3 in ERα agonist-treated OVX rats using TaqMan-based real-time PCR. As amphetamine targets the dopamine transporter, estrogenic regulation of its expression may contribute to the estrogen-dependent amphetamine response of the VTA-PFC unit and may underlie gender differences in the vulnerability of the brain to drug abuse [65].

Summing up, we provide experimental evidence that in the absence of ovarian hormones the mesocortical dopamine system is not responsive to amphetamine challenge, but E2 replacement restores its responsiveness. Using isotype-selective ER agonists, we demonstrate that ERα agonist LE2 and ERβ agonist DPN are also able to modulate the dopaminergic transmission of reward signals from the VTA to the PFC. These new data may also explain the differential responsiveness of the mesocortical and mesolimbic dopaminergic systems to psychostimulants in the two sexes [66-69].

4. Experimental procedures

4.1. Reagents

Solvents (HPLC grade Acetonitrile, UHPLC grade formic acid) were obtained from Merck (Darmstadt, Germany) and Promochem (Queens, United Kingdom). Standards including DA (dopamine), DOPAC (3,4-dihydroxyphenylacetic acid), 3MT (3-methoxytyramine), HVA (homovanillic acid) were purchased from Sigma-Aldrich Hungary (Budapest, Hungary). E2 and DPN were obtained from Sigma (St.Louis, MO, USA) and Tocris (Ellisville, MO, USA), respectively. 3,17β-dihydroxy-19-nor-17α-pregna-1,3,5(10)-triene-21,16α-lactone (LE2) was synthesized and kindly provided for this study by Gedeon Richter Plc [70]. Alzet osmotic minipumps (model 2004) were purchased from Durect (Cupertino, CA, USA). Reverse transcription reagents, TaqMan assays and PCR master mix were ordered from Applied Biosystems (Foster City, CA, USA).

4.2. Treatments of Experimental Animals

4.2.1. Ethics statement

Protocols were reviewed and approved by the Animal Welfare Committee of IEM (No.: A5769-01, permission from the Municipal Agriculture Office, Budapest, Hungary). Experiments were carried out in accordance with the legal requirements of the European Community (Decree 86/609/EEC).

Female Harlan-Wistar rats were purchased from Toxicoop (Budapest, Hungary) and were housed under standard laboratory conditions with unrestricted access to phytoestrogen-free rodent diet (Harlan Teklad Global Diets, Madison, WI, USA).

4.2.2. Animals for fMRI studies

For fMRI experiments, young (2 month old) female rats (n=20) were deeply anesthetized intraperitoneally with a mixture of xylazine (12mg/kg) and ketamine (60mg/kg), and were ovariectomized (OVX) bilaterally as described earlier [36] to minimize the levels of endogenous estrogens. Rats were housed individually after surgery, and ten days later

received treatments with vehicle or one of the three estrogen receptor (ER) agonists E2, LE2, DPN. For chronic E2 treatments, we applied silicon capsules which were assembled according to the method published by Dubal and Wise [71]. The capsule (length: 50mm, inner/outer diameter: 1.5mm/3.0mm, Dow Corning, Buffalo Grove, IL, USA) contained a 40mm column of 20µg/ml E2 in sunflower oil. This capsule provided proestrous serum E2 levels on the second week of treatment according to the work of Cagampang *et al*. [51]. The authors implanted silicon capsules to young OVX Wistar rats and determined serum E2 levels using radioimmunoassay. Serum E2 levels corresponded to 35.8 ± 3 pg/ml nine days after treatment. For isotype-selective ER agonist treatments, we used the same capsules as for E2, but filled them with 80µg/ml of LE2 [52] or DPN [53] in sunflower oil. Capsules were implanted subcutaneously on the nape of the neck under anesthesia using xylazine and ketamine as described before. We made a small incision in the loose skin, dissected a pocket caudally, inserted the capsule and sewed the incision. Functional MRI was carried out on the second week of estrogen treatment.

4.2.3. Animals for dopamine measurements

For neurotransmitter studies, young female rats (n=10) were OVX and treated either with vehicle or E2 as described above. After decapitation, the PFC, M1 and VTA were isolated using mold-assisted manual cut and frozen immediately. To collect PFC and M1, a 3-mm-thick coronal slice between bregma 5.2 and 2.2 was prepared from the forebrain with two razor blades positioned in a rat brain matrix. Then, the medial prefrontal cortex was dissected out manually from both hemispheres using bilateral sagittal cuts 1mm lateral from the midline and horizontal cuts through the ventral tip of the forceps minor. To collect VTA samples, a 3-mm-thick coronal brain slice between bregma -3.8 and -6.8 was prepared with two razor blade cuts aided by the brain matrix. A single VTA block was manually dissected from this to form

an isosceles trapezoid with two bases of 4mm (dorsally) and 2mm (ventrally), an altitude of 2mm and to legs medial to the substantia nigra. Samples were stored at -80°C.

4.2.4. Animals for quantitative real-time PCR studies

For gene expression analysis, young female rats (n=20) were OVX, treated either with vehicle or ER agonists and kept for two weeks. Animals were deeply anesthetized with pentobarbital (40mg/kg, ip.) and perfused transcardially with 100ml of cold fixative solution containing 10% RNA*later* (Qiagen, Heidelberg, Germany) in phosphate buffered saline. The relevant areas were dissected manually as described before. Samples were stored in RNA*later* at -80°C.

4.3. Functional MRI

Functional MRI experiments were performed on a 9.4T ASZ Varian MRI system (Varian, CA, USA) with a free bore of 210mm, which contains a gradient coil with inner size of 120mm (180µs rise time). For excitation an actively RF-decoupled 2 channel volume coil system with inner size 72mm was used and a fix tuned receive-only phase array rat brain coil located directly above the dorsal surface of the rat's head. Rats were anaesthetized with isoflurane (for inducement at a concentration of 5%, which was followed by a reduced 1-1.6% concentration for maintenance of anesthesia during scanning) administered in compressed air. The anesthetized rat was transferred into the magnet. Ventilation and body temperature of the animal were controlled; the latter was monitored by a rectal probe and maintained at 37 ± 1ºC via a thermostatically controlled air flowed around the rat.

Scout pictures were obtained in coronal and sagittal planes in order to set the anatomical and functional images. Anatomical scans were acquired using gradient echo multi slice, (GEMS) sequence with a field of view, FOV 35x35mm, slice thickness 1mm, gap 0.2mm. Nine slices were received in interleaved order; the scanner's default coronal orientation was slightly changed to get a standard anatomically horizontal plane according to the Paxinos rat atlas by

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setting the following parameters: echo time, TE=3.83msec, repetition time, TR=200msec, flip angle 45º, averages 3, dummy scans 4, data matrix 192x192, total scan time 2min.

An interleaved triple-shot gradient-echo echo planar imaging, EPI sequence with compressed segments was used for T2*-weighted MR images by applying the following parameters: TE=10msec, TR=3000msec, flip angle 90º, averages 1, dummy scans 4, data matrix 64x64, repetitions 1000. FOV and slice parameters were the same as in the anatomical setup.

After 1000sec control period, D-amphetamine was administered intraperitoneally at a dose of 10mg/kg in a volume of 1ml/kg. Fresh D-amphetamine hemisulphate (Sigma-Aldrich Co.) solution was prepared daily in sterile saline. Each animal was involved only in a single experiment and received only a single dose of the drug. The results of each measurement were stored in the scanner's own file format (fdf-files). These files were converted to the widely used nifti-format (Neuroimaging Informatics Technology Initiative) by a Matlab script. Data analysis and visualization were performed also by Matlab scripts.

For creating T-maps, paired t-test was performed on each voxel's two time intervals (pre-injection baseline and post-injection) to determine the significant differences between the baseline signal and post-injection signal (see Fig. 2). So that, every voxel had a T-value, and voxels with T value above the limit, highlighted.

Region of interest (ROI) analysis was also performed. ROIs were manually defined in the scans for the PFC, M1 and VTA according to the atlas of Paxinos and Watson (1986). The average normalized (to baseline) BOLD signal intensity changes across all pixels within these ROIs were determined between 200sec and 300sec, and between 1010sec and 1110sec after amphetamine administration. Factorial ANOVA followed by Fisher post-hoc test was used to calculate the statistical significance of difference among treatments in each ROI. Further details concerning fMRI measurements are given in a previous study [72].

4.4. HPLC-ESI-MS

4.4.1. Sample preparation

200µl ultra-pure water was added to 50mg brain tissue. The tissue was homogenized and cells were explored 6x10sec with a high energy ultrasonicator, UIS250V (Hielsher Ultrasound Technology, Teltow, Germany) applying ice cooling between the cycles. Samples were vortex mixed (V-1 plus, Biosan Medical-Biological Research &Technologies, Warren, USA) and centrifuged (Thermo Fisher Scientific, Hudson, New Hampshire, USA) at 10,000rpm for 10min. After spinning, the supernatants were loaded in Centrifugal Filter Devices (Amicon Ultra with 10K cut off membrane, Merck-Millipore, Darmstadt, Germany) and centrifuged at 10,000 rpm for 30min. The filtrates were loaded into autosampler vials and HPLC-MS part of the experiment was carried out.

4.4.2. Measurements

Analyses were performed with a complex Ultimate 3000 (Dionex, Sunyvale, USA) micro HPLC system equipped with a quaternary pump, a degasser, and a Bruker Esquire HCT mass spectrometer (Bruker Daltonics, Bremen, Germany). Separations were performed on a Kinetex PFP (100mm x 2.1mm i.d., particle size 2.6µm) column (Phenomenex, Torrance, USA). The flow rate was 100µl/min; the injection volume was 5µl, the temperature was kept at 4°C in the autosampler and 40°C in the column compartment. Hystar 3.2 (Bruker Daltonics, Bremen, Germany) and Bruker Esquire Control 5.3 software were used for controlling the instrument. Data acquisition and spectrum evaluations were performed by means of Bruker Data analysis 4.0 software. An isocratic gradient consisting of mobile phases A and B (A: ultra pure water-0.1% formic acid; B: acetonitrile–0.1% formic acid) was applied for the chromatographic separation and the mixing ratio was 1:1. A Bruker iontrap mass spectrometer, equipped with an ESI source was used for mass detection. The ionization source was operated with an endplate potential of 4kV in the positive ion mode and 3kV in the

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negative ion mode. The following electrospray parameters were kept constant during the analysis: drying gas (N2) flow 6l/min, drying gas temperature 220°C, nebulizer pressure 9psi. The multiple reaction monitoring mode was used for selective and sensitive detection of dopamine and its metabolites. The most intense precursor-to-fragment transitions were used for analysis: DA 153.18 → 137.10, 3-MT 167.21 → 151.00, DOPAC 168.15 → 123.90, HVA 182.17 → 136.70.

4.5. Quantitative Real-Time PCR

Total RNA was isolated from the PFC, M1 and VTA samples using the RNeasy Lipid Tissue Mini Kit (Qiagen). RNA analytics included A260nm/A280nm readings using a Nanodrop Spectrophotometer and capillary electrophoresis using Nano RNA Chips on 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA). All RNA samples displayed RNA integrity numbers above 8.2.

We selected inventoried assays for twenty genes of our interest (Table 1). Each assay consisted of a FAM dye-labeled TaqMan MGB probe and two PCR primers. We followed mRNA expression of genes involved in dopamine synthesis (Th, Gch1, Ddc, Dbh), reuptake (Slc6a3, Slc6a2, Slc18a2), metabolism (Maoa, Maob, Comt), binding (Drd1a, Drd2, Drd3, Drd4, Drd5) and signaling (Rgs2, Rgs4, Rgs9, Rgs14, Adcy5). We used glyceraldehyde-3-phosphate dehydrogenase (Gapdh), hypoxanthine guanine phosphoribosyl-transferase (Hprt) as endogenous controls. Expression of these genes did not vary among treatment groups of the study. Reverse transcription and real-time PCR were run as described earlier [4]. The ViiA7 RUO (Applied Biosystems) software and relative quantification against calibrator samples (ΔΔCt) were used for analysis. Relative quantity (RQ=$2^{-\Delta\Delta Ct}$) was used to characterize gene expression in the various experimental groups. PCR experiments conformed to minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines [73].

In PCR data evaluation, group data were expressed as RQ(mean)±standard deviation (SD). Statistical significance of the changes in gene expression was analyzed using ANOVA followed by Newman-Keuls post-hoc test (STATISTICA software version 11.0, StatSoft Inc., Tulsa, OK).

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Table 1   The list of genes and the applied TaqMan assay IDs.

symbol	name	TaqMan ID
Dopamine synthesis, reuptake and metabolism		
Th	tyrosine hydroxylase	Rn00562500_m1
Gch1	GTP cyclohydrolase I	Rn00577450_m1
Ddc	dopa decarboxylase	Rn00561113_m1
Dbh	dopamine b-hydroxylase	Rn00565819_m1
Slc6a3	dopamine transporter	Rn00562224_m1
Maoa	monoamine oxidase a	Rn01430950_m1
Maob	monoamine oxidase b	Rn00566203_m1
Slc6a2	norepinephrine transporter	Rn00580207_m1
Comt	catechol-O-methyltransferase	Rn99999091_m1
Slc18a2	vesicular monoamine transporter 2	Rn00564688_m1
Dopamine receptors and signaling molecules		
Drd1a	dopamine D1 receptor	Rn03062203_s1
Drd2	dopamine D2 receptor	Rn00561126_m1
Drd3	dopamine D3 receptor	Rn00567568_m1
Drd4	dopamine D4 receptor	Rn00564071_m1
Drd5	dopamine D5 receptor	Rn00562768_s1
Rgs2	regulator of G protein signaling 2	Rn00584932_m1
Rgs4	regulator of G protein signaling 4	Rn01490867_g1
Rgs9	regulator of G protein signaling 9	Rn00570117_m1
Rgs14	regulator of G protein signaling 14	Rn01442096_m1
Adcy5	adenylyl cyclase V	Rn00575059_m1

Expression of dopamine signaling-related genes was followed by real-time PCR using TaqMan gene expression assays. Details for each gene expression assay can be found on the manufacturer's website (www.lifetechnologies.com).

Figure captions

Figure 1   The time course of BOLD responses of the prefrontal cortex (PFC) (**A**), the primary motor cortex (M1) (**B**) and the ventral tegmental area (VTA) (**C**) to amphetamine in ovariectomized (OVX) rats pre-treated with either vehiculum (OVX+VEH) or 17β-estradiol (OVX+E2). BOLD responses represent the mean of five measurements. Time frames highlighted in blue indicate the sampling periods used for the statistical evaluation of amphetamine's effect.

Figure 2   BOLD responses in OVX rats pretreated with vehicle (OVX+VEH), E2 (OVX+E2), ERα agonist 16α-LE2 (OVX+LE2) and ERβ agonist DPN (OVX+DPN) after 4min (**A**) and 17.5min (**B**) of amphetamine administration. Color-coded columns show the average BOLD responses, which were normalized to the pre-drug BOLD responses, obtained from the first (1200-1300sec) and second (2010-2110sec) sampling periods. Asterisks indicate significant differences in BOLD responses after amphetamine treatment compared to the OVX+VEH group. Red asterisks mark significant differences of BOLD responses after amphetamine administration compared to the OVX+E2 group (factorial ANOVA followed by Fisher posthoc test, * indicates $p < 0.05$, **$p < 0.01$, ***$p<0.001$).

Figure 3   Concentration of dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) and 3-methoxytyramine (3MT) in the prefrontal cortex (**A**) and the primary motor cortex (**B**) of ovariectomized (OVX) rats treated with either vehicle (OVX+VEH) or estradiol (OVX+E2). Error bars show SD of eight samples for each group. ** indicates $0.001< p <0.01$.

Figure 4   Expression of dopamine receptors including Drd1a (**A**), Drd2 (**B**) and Drd3 (**C**) in the prefrontal cortex and dopamine transporter (Slc6a3) in the ventral tegmental area (**D**) of ovariectomized rats pretreated with vehicle (OVX+VEH), E2 (OVX+E2), LE2 (OVX+LE2) and DPN (OVX+DPN). The effects of ER agonists were compared to that of vehicle.

Statistical significance of the alterations was assessed by analysis of variance (ANOVA) with Newman-Keuls post hoc test. Asterisks indicate statistically significant changes: * corresponds to $p < 0.05$, ** to $0.001 < p < 0.01$.

A   PFC   **B**   M1