Abteilung Anatomie und Zellbiologie
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Estrogen synthesis and novel mechanisms of estrogen action in the developing brain

Dissertation
zur Erlangung des Doktorgrades der Humanbiologie
an der Fakultät für Medizin
der Universität Ulm

vorgelegt von
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aus Torun, Polen
2000

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Tag der Promotion: 04.05.2001

Moim Rodzicom i Bratu

For my Parents and my Brother

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Abbreviations

ABC	avidin-biotin peroxidase complex
AC	adenylate cyclase
APS	ammonium persulfate
ARN	arcuate nucleus
BB	bromophenol blue
BDNF	brain-derived neurotrophic factor
bFGF	basic fibroblast growth factor
BNST	bed nucleus of the stria terminalis
bp	base pair
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CRE	cAMP/calcium response element
CREB	cAMP response element binding protein
CSPD	disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2´-(5´-chloro)tricyclo[3.3.1.1.^{3,7}]decan}-4-yl) phenyl phosphate
Cyp	cyproterone acetate
DIG	digoxigenin
DIV	days in vitro
DTT	dithiothreitol
ED	embryonic day
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGTA	ethyleneglycol—bis(β-aminoethyl ether)N,N,N',N'-tetraacetic acid
EMSA	electrophoretic mobility shift assay
ER-α	estrogen receptor α
ER-β	estrogen receptor β
ERE	estrogen response element
EtBr	ethidium bromide
FCS	fetal calf serum
FF	xylencyanol blue
Forskolin	adenylate cyclase activator

GAP-43	growth-associated protein 43
GFAP	glial fibrillary acidic protein
H89	protein kinase A inhibitor H89
Hepes	N-(2-hydroxyethyl)-piperazine-N'-2-ethane
Hyp	hypothalamus
IBMX	3-isobutyl-I-methylxanthine
IGF	insulin-like growth factor
IP_3	phosphatidylinositol-3-phosphate
kDa	kilodalton
MAP-2	microtubule-associated protein 2
MAPK	mitogen-activated protein kinase
MEK	MAPK kinase
mPOA	medial preoptic area
MEM	minimum essential medium
MOPS	3 morpholinopropane sulfonic acid
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NBM	neurobasal medium
NGF	nerve growth factor
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pCREB	phosphorylated form of cAMP response element binding protein
PKA	protein kinase A
PKC	protein kinase C
PMSF	phenylmethylsulfonyl fluoride
POA	preoptic area
r^2	correlation coefficient
S	sesame oil
SDN-POA	sexually dimorphic nucleus of POA
SDS	sodium dodecyl sulfate
SON	supraoptic nucleus
SQ	adenylate cyclase inhibitor, SQ 22,536
TGF	transforming growth factor
TRIS	Tris(hydroxymethyl)-aminomethane
VMN	ventromedial hypothalamic nucleus

WB	western blotting

1. Introduction

1.1. Estrogen and the developing brain

Developmental processes in the mammalian brain are the result of complex interactions between epigenetic and genetic factors. Notwithstanding the importance of signals from the extracellular matrix, growth factors, and the cell-intrinsic developmental program, there is accumulating evidence that estrogen also interferes with neuronal differentiation. In the 1970s, it was shown by Toran-Allerand (1976) that estrogen enhances neurite outgrowth and arborization in organotypic hypothalamic cultures. Growth promoting effects were also described in neuronal cultures from other brain regions such as the cortex (Garcia-Segura *et al.*, 1989b), midbrain (Reisert *et al*., 1987), hippocampus (Gould *et al*., 1990), spinal cord (VanderHorst and Holstege, 1997), and pituitary (Chun *et al*., 1998). Moreover, synaptic density in the amygdala (Nishizuka and Arai, 1981a) and synaptic connectivity in the arcuate nucleus (ARN) (Matsumoto and Arai, 1980) appear to be highly dependent on estrogen. Estrogen action on neuronal plasticity is partly mediated by the regulation of the expression of cytoskeletal and other genes such as tau, class II β-tubulin, GAP-43, and neurofilament proteins (Ferreira and Caceres, 1991; Lustig *et al*., 1991; Rogers *et al*., 1991; Scovile *et al*., 1997). In addition, estrogen acts as a trophic factor by affecting neuronal survival (Chowen *et al*., 1992) and plays a protective role during neuronal apoptosis (Arai *et al.*, 1996). Importantly, many of the above mentioned perinatal effects of estrogen on neuronal survival and death, cell migration, synapse formation and cell differentiation, reveal sex-specific characteristics. In general, it is now safe to conclude that sex differences in brain structure and function are the result of an exposure of the developing brain to estrogen (Arnold and Gorski, 1984; Arnold and Breedlove, 1985). It is noteworthy that the developing brain itself is the source of estrogen. Thus, neuronal cells express the enzyme aromatase which converts circulating testosterone into estrogen (Naftolin *et al.*, 1975). During development, higher levels of testosterone found in males lead to higher estrogen production rates in the male brain (Weisz and Ward, 1980; Beyer and Hutchison, 1997).

Structural and functional sex differences in the vertebrate CNS are generally limited to particular brain regions and are measured in terms of the volume of nuclei, the number of neurons and synaptic connections. The sexual dimorphic nucleus of the medial preoptic area (SDN-POA) is 2.4 to 5.0 times larger in males than in females and results from three times higher numbers of neurons in this region (Gorski *et al.*, 1980). The volume of the

medial amygdala is also sexually dimorphic and larger in males compared to females (Nishizuka and Arai 1981b; Mizukami *et al*., 1983; Vochteloo and Koolhaas, 1987; Rajendren *et al*., 1993). In the hypothalamus, a region mainly involved in the control of reproductive behavior and integration of hormonal and neural responses, sexual differences in the structure of the ventromedial nucleus (VMN) and arcuate nucleus (ARN) are observed. In the VMN, differences between genders are seen in the size of neurons (larger in female) and in the overall size of the nucleus (larger in male) (Doerner and Staud, 1969). In the ARN, the structure of the synaptic connections differs between genders. The female ARN displays higher numbers of axospinous connections compared to males, whereas in males, there are more axosomatic connections than in females (Matsumoto and Arai, 1980). Other brain regions, not directly involved in reproductive behavior, also reveal sex differences and seem to be targets of estrogen during development and in adulthood. The hippocampus which is involved in learning, cognition, memory and stress responses, is influenced by steroids. Estrogen shows neuritotropic effects on axon growth of hippocampal neurons *in vitro* (Blanco *et al*., 1990), as well as dendrite spine formation (Lewis *et al*., 1995, Segal and Murphy, 1997). This organizational influence of estrogen on synapse formation is believed to affect sex differences in learning pattern between genders (Williams and Meck, 1991) In the striatum, estrogen effect and estrogen-induced sex differences are reported for dopamine release and dopamine-mediated behaviors (Becker, 1990; Di Paolo, 1994).

Currently, it is presumed that estrogen influences neuronal development in co-operation with other developmental signals such as growth factors and neurotrophins. Estrogen regulates also the expression of trkA and p75NTR receptors in differentiating PC12 cells (Miranda *et al.*, 1994; Sohrabji *et al.*, 1994 and 1995; Miranda *et al.*, 1994), and cooperates with the IGF-I signaling pathway in synaptic plasticity in the ARN and in the hippocampus (Azkoita *et al.*, 1999b; Cardona-Gomez *et al.*, 2000).

Estrogen not only influences neuronal morphology and function but can also influence glial cells in the CNS. Garcia-Segura *et al.* (1989a) and Toran-Allerand (1990) showed that estrogen promotes the differentiation of astroglia in both primary and explant cultures of the fetal hypothalamus. Moreover, estrogen affects astroglial morphology and the expression of the glial fibrillary acidic protein (GFAP) in the hypothalamus of young and adult animals (Garcia-Segura *et al*., 1989a and 1994). This effect is not restricted to the hypothalamus, since estrogen also affects GFAP expression in the hippocampus, striatum, and cerebellum (Garcia-Segura *et al.*, 1988; Suarez *et al*., 1992; Del Cerro *et al.*, 1995).

Microglia and astroglia take part in synaptic membrane remodeling by expressing ApoE protein, an important lipid carrier molecule. *In vitro* studies revealed that estrogen can increase the level of ApoE in microglia (Poirier 1999; Stone *et al*., 1997).

1.2. Estrogen synthesis in the brain

1.2.1. Aromatase

Androgens are converted to estrogens by aromatase, a member of the superfamily of enzymes collectively termed cytochrome P450 (Nelson *et al*., 1993). Aromatase is composed of a specific form of microsomal cytochrome P450 (aromatase cytochrome P450), and ubiquitous flavoprotein NADPH-cytochrome P450 reductase (Nebert and Gonzalez, 1987, Nebert *et al.*, 1989). The aromatization reaction takes place in the endoplasmic reticulum. Aromatase possesses a heme domain and a l-helix structure that together form the pocket for the substrate and catalyze the insertion of an oxygen into the substrate. NADPH-cytochrome P450 reductase provides reducing equivalents from NADPH. For the formation of one mole of estrogen, 3 moles oxygen and 3 moles NADPH are required. The substrates for aromatization are androgens that have an unsaturated A ring (for example testosterone) which can be converted to a phenolic ring structure characteristic for estrogens (Simpson *et al*., 1994).

1.2.2. Aromatase gene structure

Aromatase is found in the ovary (Hickey *et al.*, 1990), testis (Rommerts *et. al*., 1982), liver (Smuk and Schwers, 1977), placenta, intestine, adipose tissue (Longcope *et al.*, 1978; Mendelson *et al.*, 1982), skin fibroblasts (Berkovitz *et al.*, 1989) and brain (Naftolin *et al*., 1972). Rodent aromatase is coded by one single gene (cyp 19) containing 10 exons (Nebert and Gonzales, 1987; Hickey *et al.*, 1990; Terashima *et al.*, 1991). The open reading frame includes 9 exons encoding the active enzyme (Simpson *et al*., 1994). Exon 1 consists of at least 6 alternative forms which are flanked by particular promoters and differentially spliced. This alternative splicing results in a tissue specific regulation of aromatase expression at the transcription level. The splicing occurs at a common intron/exon boundary site upstream of the translation start site. Consequently, the enzyme protein expressed in different tissues is identical with respect to its structure (Simpson *et al.*, 1994). In human as well in mouse tissue, shorter and longer transcripts which are the result of two different polyadenylation sites have been found. The shorter transcript, lacking the

heme domain, codes for an inactive form of the enzyme. The functions of this transcript are not clear, however, it has been postulated that it may be involved in the regulation of aromatase activity (Simpson *et al.*, 1994; Terashima *et al*., 1991).

1.2.3. Aromatase in the brain

Aromatase has been detected in several brain regions such as the POA, hypothalamus, pituitary (Wagner and Morrell, 1996; Roselli and Resko, 1987), hippocampus (Roselli *et al*., 1984b; Mac Lusky *et al.*, 1987), striatum (Küppers and Beyer, 1999), and cerebellum (Roselli *et al.*, 1997). Studies carried out with different neural cell populations showed that aromatase expression and activity is limited to neurons and neither oligodendrocytes nor astrocytes show significant aromatase activity (Negri-Cesi *et al*., 1992; Beyer *et al.*, 1994). Light and electron microscopy studies further revealed that aromatase is present in the neuronal perikarya, dendrites, and axons (Naftolin *et al*., 1996; Beyer and Hutchison, 1997).

1.2.4. Regulation of aromatase activity

Aromatase activity is regulated predominantly at the transcription level by androgens, however, this effect is heterogeneous in distinct brain areas. Treatment with testosterone or dihydrotestosterone (DHT) has been found to increase aromatase activity in the Hyp/POA but not in the amygdala or cortex (Roselli *et al*., 1997). The region-specific mRNA expression/activity of aromatase in the brain can be explained, at least in part, by the action of the three different promoters: hypothalamic, ovarian and cortical, that are found in the brain (Kato *et al.*, 1997). Estrogen alone does not influence aromatase activity, but nevertheless in synergy with DHT, can stimulate aromatase activity in the Hyp/POA to the same level as testosterone (Roselli, 1991). In contrast, estrogen seems to exert a regulatory effect on the posttranscriptional level of aromatase (Abdelgadir, *et al*., 1994). Steroid hormones are not the only factors affecting aromatase activity. It has been shown that in gonadal and extragonadal tissues, aromatase activity is stimulated by cAMP (Evans *et al.*, 1987 ; Fitzpatrick *et al*., 1994). In contrast, cAMP inhibits aromatase expression in neuronal tissue (Lephart *et al*., 1992). Growth factors (bFGF, EGF, NGF, TGF), interleukin-6 as well activators of protein kinase A or protein kinase C (PKA, PKC respectively) have also been shown to influence aromatase activity in a brain region-specific manner (Lephart *et al.*, 1991; Abe-Dohmae *et al*., 1996).

1.2.5. Sex differences in aromatase activity

Sex differences in brain structure and functions are mainly the result of different estrogen levels during development. This implies the existence of sex differences in aromatase activity/expression in the brain of males and females. The level of aromatase activity in the SDN-POA, bed nucleus of the stria terminalis (BNST) and VMN is higher in males than in females (Roselli *et al.*, 1985). These sex differences are due to higher number of neurons that express aromatase (Beyer *et al.*, 1994). Consequently, no differences in enzyme kinetics between males and females are found in the brain (Beyer *et al.*, 1993; Roselli *et al.*, 1996). Since aromatase is predominantly regulated at the transcription level by androgens, as pointed out in chapter 1.2.4., higher levels of circulating androgens that have been reported during the perinatal period in males, might be responsible for higher aromatase levels in the male brain (Weisz and Ward, 1980; Roselli *et al.*, 1985). This idea is supported by findings which demonstrated that prepubertal gonadectomy abolishes sex differences in aromatase activity in the adult brain (Roselli *et al.*, 1998).

1.3. Estrogen signaling

1.3.1. Classical estrogen signaling

1.3.1.1. Structure and function of nuclear estrogen receptor-α and -β

To date, two distinct types of estrogen receptors (ER) have been identified: ER-α and -β. ER-α was first cloned from human breast carcinoma cells, and ER-β from rat prostate (Green *et al.*, 1986; Kuiper *et al.*, 1996). Both ER-α and -β genes consist of 8 exons coding 595 and 485 amino acid proteins, respectively. ERs are structurally composed of six distinct domains: A/B, C, D, E, and F (Figure 1).

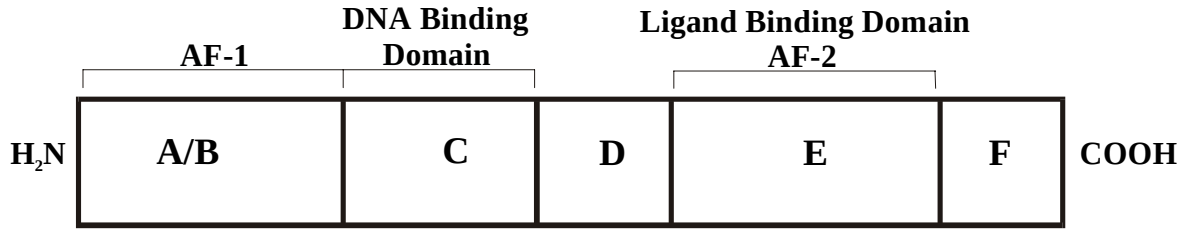

Figure 1. General structure of nuclear estrogen receptors.

Region A/B is located in the N-terminal region of each receptor and is highly variable between the members of the hormone-activated transcription factor family. It codes for the activation function 1 (AF-1) region responsible for constitutive transactivation of the

receptor. The action of this domain is cell-type specific. Domain C contains the DNA binding domain (DBD) and is the most conserved domain (95,5 % similarity) between ER-α and -β (Figure 2). The DBD consists of two zinc fingers responsible for receptor binding in the major groove of the DNA helix and stabilization of the transcriptional complex (Umesono and Evans, 1989; Elliston *et al.*, 1990; Schwabe *et al.*, 1993). Additionally, the C-region may bind heat shock protein 90 (Hsp90) (Chambraud *et al.*, 1990) and be responsible for nuclear localization of the receptor (Ylikomi *et al.*, 1992).

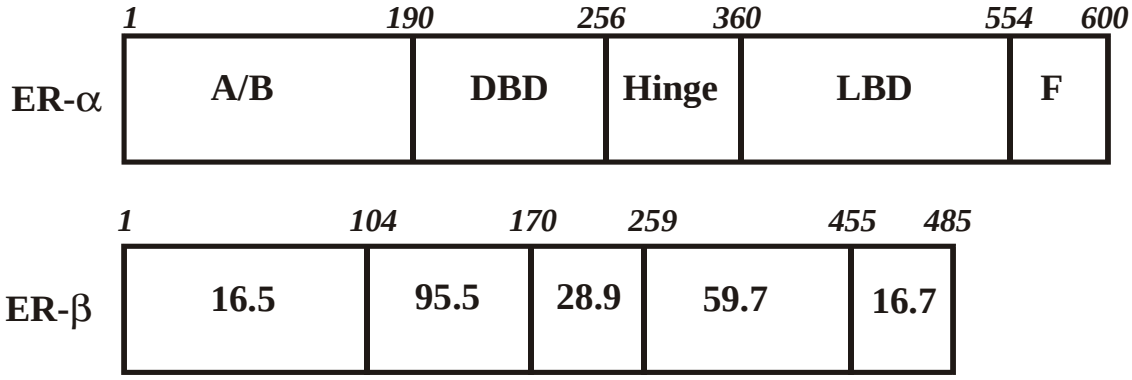

Figure 2. Comparison of the rodent estrogen receptor (ER) -α and -β structure. The bold numbers give the percentage of amino-acid homology between ER-α and ER-β. The numbers in italic represent the amino acid positions in the receptor protein.

Little is known about the D domain hinge region, which does not seem to be important for ER activation and transcriptional activity. The C-terminal E domain (ligand binding domain, LBD) contains the ligand-dependent transactivation AF-2 domain, Hsp90 binding region, a nuclear localization signal and dimerization domain (reviewed by Gronemeyer, 1992). The LBD is highly similar between ER-α and -β. The F domain is not required for hormone binding but takes part in the modulation of transcriptional activity of the receptor (Wooge *et al.*, 1992; Montano *et al.*, 1995).

1.3.1.2. Transcriptional properties of nuclear estrogen receptors

ERs are hormone-dependent transcription factors. Estrogen diffuses through the cellular plasma membrane and binds to ERs in the nucleus. Binding of estrogen causes conformational changes in the ER structure, the diffusion of Hsp90 proteins, and homo- or heterodimerization of ERs (Pettersson *et al.*, 1997). The ER-dimer then binds to an enhancer element in the promoter region of target genes, thereby stabilizing the

transcriptional activation complex (Shibata *et al.*, 1997). Transactivational properties of ERs are generally transmitted by binding of ER to estrogen-response elements (ERE), however, ER binding to AP-1 enhancer elements may also promote estrogen-dependent transcription (Paech *et al.*, 1997). Transcriptional activation by ER can be attributed to two regions: the N-terminal AF-1 and the ligand dependent AF-2 localized in the C-terminal hormone/ligand binding domain (LBD). AF-1 and AF-2 can activate transcription independently, synergistically, and act in a promoter and cell-specific manner (Tora *et al.*, 1989; Tzukerman *et al.*, 1994).

Activation of ERs is usually associated with receptor phosphorylation. Five phosphorylation sites are found in ERs. Four of them are localized in the AF-1 domain (Ser ^{104,106,118,167}). The phosphorylation of these sites is hormone-inducible, and it has been proposed that Ser^{118} is the major estrogen-inducible phosphorylation site in ERs. Point-directed amino acid mutations of these sites significantly decrease the transactivation properties of the receptor (Ali *et al.*, 1993; Le Goff *et al.*, 1994). The phosphorylation sites in the AF-1 domain are also target sites for EGF and IGF-I. Mutants lacking the N-terminal A/B domain are not activated by these factors (Bunone, 1996). The fifth phosphorylation site is located in the AF-2 domain (Tyr^{537}) and a mutation in this domain abolishes the activation of ER by estrogen or elevated cAMP levels (El-Tanani and Green, 1997). Data obtained from point mutations show that Tyr^{537} is necessary to form a three dimensional structure of ER, crucial for receptor interaction with co-activator proteins. Although many estrogenic substances or estrogen antagonists display similar binding affinities for both ERs (Kuiper *et al.*, 1997), studies of Paech and colleagues (1997) revealed that transactivation properties differ between ER-α and -β. These differences depend on the type of enhancer element in the promoter region of the target gene, i.e. ERE or AP-1. Antiestrogens such as tamoxifen or raloxifen activate AP-1 dependent transcription after binding to ER-β. ERs can be activated either directly by cognate estrogenic ligands or indirectly by a variety of other extracellular signals. Ocadaic acid, cAMP, EGF, IGF-I, and dopamine are able to activate ERs. However, these factors only induce a maximum 50% of the transcriptional activity measured in a reporter gene assay as compared with estrogen. This effect seems to be mediated through the activation of the Ras-Raf-MAPK cascade and involves Ser^{118} phosphorylation (Kato *et al.*, 1995; Bunone, 1996).

1.3.1.3. Localization of nuclear estrogen receptors in the brain

Comparative studies show that both ERs are widely distributed in the brain, although their individual expression pattern varies between different regions. In the adult rat brain, ER-α but not ER-β transcripts were detected in the VMN, whereas in the olfactory bulb, SON, cerebellum, and cerebral cortex ER-β is exclusively expressed. Both ER types are coexpressed in the BNST, POA, ARN, medial amygdala and hippocampus (Shughrue *et al*., 1997 and 1998). During development, the first signal for ER-α was obtained on ED14 in the Hyp, POA and amygdala using radioactive labeled diethylstilbestrol (synthetic estrogen) which bound to the receptor protein (Keefer and Holdergger, 1985). Ontogenetic studies in the rodent brain revealed the expression of both ER transcripts in the mouse striatum and hippocampus from ED15 until postnatal day (P) 15 as well as in adult animals (Küppers and Beyer, 1999; Ivanova and Beyer, 2000). In the rat cerebellum, ER-α and -β are present throughout the first two postnatal weeks (Belcher, 1999). The expression of ERα/β is not only restricted to neurons, since *in vitro* studies indicate that ER-α is expressed in astrocytes as well as in oligodendrocytes (Santagati, 1994). In addition, ER-β was detected in astroglia and microglia by immunocytochemistry (Azkoita *et al.*, 1999a; Mor *et al.*, 1999).

1.3.2. Non-classical estrogen signaling

Estrogen is generally thought to act as an activator of transcription via nuclear receptors but there is increasing evidence that estrogen can also influence cell physiology through other mechanisms. It is now generally accepted that estrogen stimulates distinct intracellular signaling cascades through interactions with membrane estrogen receptor (Fig. 3). Estrogen has been found to induce the release of calcium from intracellular stores in human oocytes (Tesaric and Mendosa, 1995) and to stimulate adenylate cyclase in breast cancer and uterine cells (Aronica *et al.*, 1994). Moreover, estrogen activates the phospholipase C (PLC), leading to an increase of IP_3 formation and a subsequent release of calcium from intracellular stores in rat osteoblasts (Le Mellay *et al.*, 1997). It is also established that estrogen can modulate the production of nitric oxide (NO) in endothelial cells by increasing NO synthase (NOS) mRNA expression as well as by NOS activation through the MAPK pathway (Hayashi *et al.*, 1995; Chen *et al.*, 1999). Together with the rapid time course, usually within seconds to minutes, and the pharmacological profile of these effects, i.e. kinetics and insensitivity to transcriptional and translational inhibitors,

the data suggest that the genomic mechanism of action is not involved (Aronica *et al.*, 1994; Chen *et al.*, 1999). Non-classical estrogen effects have also been reported for the CNS. In midbrain neurons, treatment with estrogens provokes a rapid and transient calcium release from intracellular stores via IP_3 signaling. This effect is stereo- and steroid-specific, and mediated through membrane receptors (Beyer and Raab, 1998). Estrogen also prevents glutamate-induced cell death by activation of the MAPK signaling pathway (Singer *et al.*, 1999) and acts as a neuroprotectant after experimental seizure in the rat hippocampus by inducing cAMP response element–binding protein CREB expression (Panickar *et al.*, 1998).

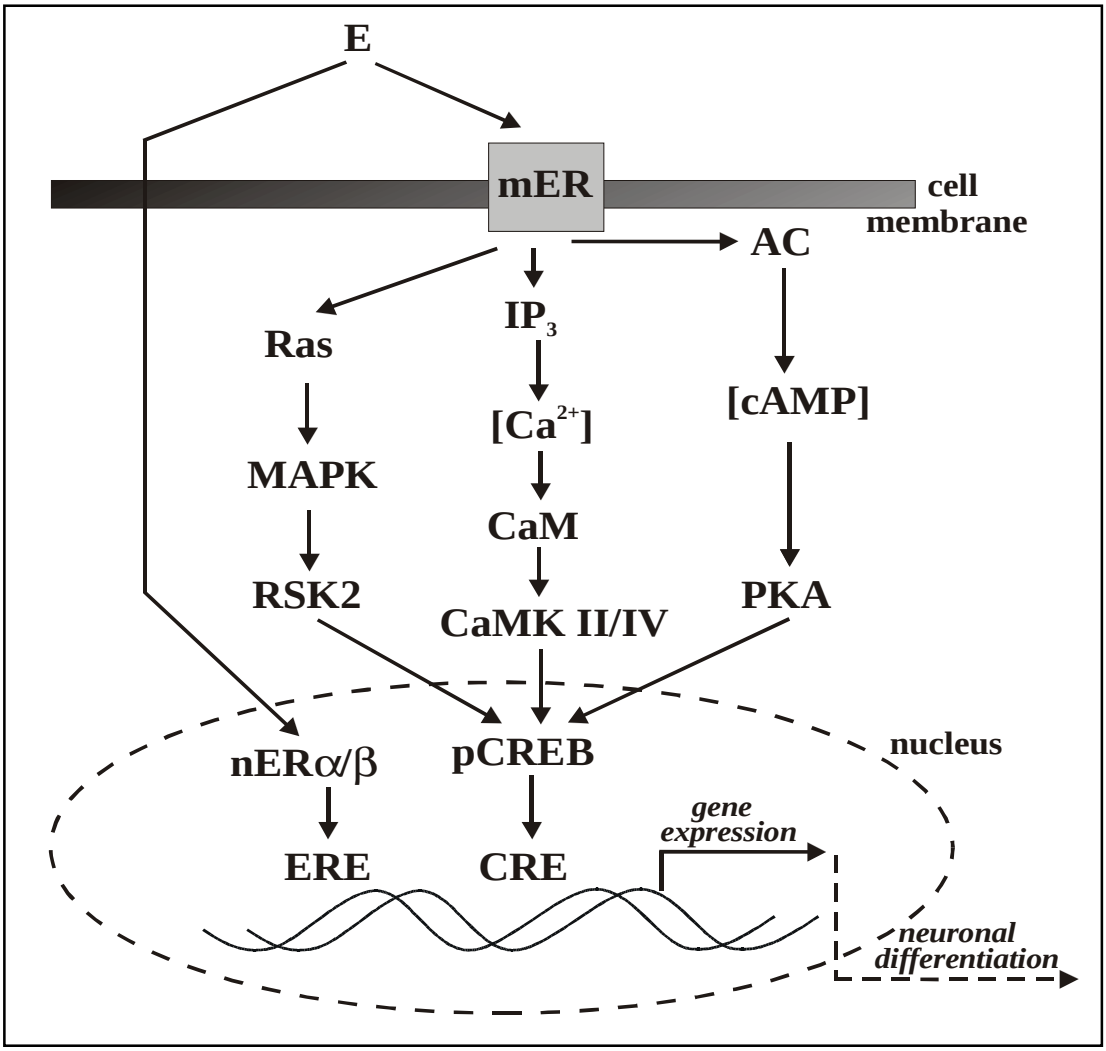

Figure 3. Schematic illustration of possible intracellular mechanisms of estrogen signaling in CNS. Classical signaling through nuclear estrogen receptors (nER) binding to the estrogen response element (ERE). Non-classical pathways via membrane ER (mER) involving: (1) mitogen activated protein kinases (MAPK), (2) IP^3, calmodulin (CaM) and CaM-dependent kinases II and IV (CaMK II/IV), and (3) adenylate cyclase/protein kinase A (AC/PKA) pathways through cAMP/calcium response element binding protein (CREB). CRE, cAMP/calcium response element; RSK ribosomal kinase

Despite numerous non-classical estrogen effects, knowledge about the structure and function of the putative membrane ER is still scant. Transfection of chinese hamster ovary cells (CHO) with nuclear ER-α or -β resulted in the integration of these receptors into the cell membrane (Razandi, 1999). It has been suggested that both, nuclear and membrane receptors, originate from one transcript, since the kinetic properties of these receptors appear to be identical. Studies of Pappas *et al.* (1995) showed that antibodies directed against different epitopes of nuclear ER-α recognize putative membrane ER on the cell membrane of GH_3/B6 rat pituitary tumor cells. Additionally, neuroprotective effects of estrogen in cortical neurons after glutamate toxicity is completely abolished by ICI 182,780, an extremly potent antagonist of nuclear ERs, suggesting that the membrane receptor may have similar properties compared to the nuclear ERs (Singer *et al*., 1999). In contrast, experiments with cerebral cortex explants showed that estrogen-induced activation of the MAPK pathway occurred in the wild type as well as in an ER-α knockout mouse. Neither specific ER-α or -β ligands nor ICI 182,780 were able to induce MAPK pathway or to block the estrogen action, respectively (Singh *et al.,* 2000). In neuroblastoma cells, ICI 182,780 as well as tamoxifen, another ER antagonist, did not affect estrogen induced MAPK phosphorylation and the transcription of a reporter gene driven by the *c-fos* immediate early gene promoter (Watters *et al.,* 1997). These findings altogether support the idea of a novel membrane ER in the brain distinct from classical nuclear ERs.

1.4. Aims and outline of this thesis

The present study is concerned with (I) the developmental regulation of estrogen synthesis in the brain, and with (II) the cellular and the molecular mechanisms that are involved in transmitting estrogen effects on differentiating midbrain dopaminergic neurons.

In the first part, we have analyzed the developmental expression and regulation of aromatase and nuclear ER-β in the mouse Hyp/POA and midbrain. The ontogenetic pattern and sex differences of aromatase and ERα/β mRNA expression were studied by semi-quantitative RT-PCR using tissues from embryonic and postnatal animals. Additionally, since androgens are thought to play an important role for the regulation of aromatase activity on the transcriptional level, we have tested this hypothesis by treating mouse embryos *in utero* from ED15 to ED20 with androgen receptor antagonist cyproterone acetate (Cyp).

The focus of the second part of this study was to characterize estrogen signaling mechanisms in midbrain dopaminergic neurons on the cellular and molecular level. Previous studies from our laboratory indicate that estrogen promotes the differentiation of dopaminergic neurons *in vitro*. It has been shown that these effects depend on the rapid activation of intracellular Ca^{2+}-dependent signaling cascades and CREB phosphorylation suggesting non-classical action. This view is supported by the presence of membrane ER on dopaminergic neurons (Beyer and Raab, 1998). Since the phosphorylation of CREB appears to be a key regulatory step in the transduction of estrogen effects on dopaminergic neurons, we have performed electrophoretic mobility shift assay (EMSA) to determine whether the increase in CREB phosphorylation results in increased binding of pCREB to a calcium/cAMP response element (CRE). In addition, we have investigated by western blot analysis whether estrogen is capable to stimulate the MAPK signaling pathway which has been shown to be an important intermediate step in transmitting estrogen effects in the brain. These experiments were carried out with neuronal and glial cultures separately in order to pinpoint the cell specificity of the observed estrogen effects. On the molecular level, we have studied the influence of estrogen on gene expression in midbrain neuronal cultures either by classical (through nuclear ER) or non-classical steroid signaling (through membrane ER). This was done by applying differential display PCR techniques in combination with the sequencing of the yielded PCR products.

2. Materials and Methods

2.1. Substances

Aprotinin	Sigma
Agarose	PeqLab
APS	Sigma
Anti-digoxigenin-AP Fab fragments	Boehringer Mannheim
BCA kit	Pierce Chem. Company
Blocking reagent	Boehringer Mannheim
CSPD	Boehringer Mannheim
Chloral hydrate	Sigma
Cyproterone acetate	Sigma
DTT	Sigma
β-Mercaptoethanol	Fluka
Ethidium bromide	Sigma
Formamide	ROTH
Forskolin	Biotrend
H89	Calbiochem
H.A.-Yellow	Hanse Analytik
Hepes	Serva
MEM	Instamed-Biochrom
MOPS	Merck
SDS	Sigma
SQ	RBI
FCS	Gibco
Poly-DL-ornithine	Sigma
PMSF	Sigma
TRIS	Merck
Triton X-100	Sigma

2.2. Enzymes

DNase (RNase free)	Boehringer Mannheim
M-MLV reverse transcriptase	Promega

Reverse Transcriptase	Promega
Taq-DNA polymerase	Gibco

2.3. Nucleotides

Hexanucleotide Mix, 10x conc	Boehringer Mannheim
PCR primers	Interactiva
DNA polymerization mix	Amersham Pharmacia Biotech
CREB consensus oligo	Promega
AP2 consensus oligo	Promega

2.4. Standards

100bp DNA ladder	Gibco
Mark 12 MW Standard-protein ladder	Novex

2.5. Antibodies

P44/42 MAP Kinase-antibody for total MAPK	New England Biolabs, Inc
PMAPK- antibody for phosphorylation form of MAPK	New England Biolabs, Inc
CREB-1 (24H4B) for Gel shift assay	Santa Cruz Biotechnology
Peroxidase conjugated affini-pure goat anti-rabbit Ig G (H+L)	Jackson ImmunoResearch
Peroxidase conjugated affini-pure goat anti-mouse Ig G (H+L)	Jackson ImmunoResearch

2.6. Specials

Micro Spin G-50 Columns	Amersham Pharmacia Biotech
Nick Spin Columns	Amersham Pharmacia Biotech
RNAimage mRNA differential display system	GenHunter Corporation
Gel Shift Assay Core System	Promega
Coomassie Protein Assay Reagent	Pierce
BCA Protein Assay Reagent A	Pierce
ECL Western blotting detection reagents	Amersham
Hybond-N: positively charged nylon membrane	Amersham
Glogos II Autorad Markers	Stratagene
Petri dishes 9.6 cm^2	Greiner
ABI Prism BigDye Ready Reaction Kit.	Perkin Elmer

2.7. Equipment

UV linker	Stratalinker 2400 Stratagene
PCR equipment	PCR DNA thermal cycler 480, Perkin Elmer
	Eppendorf Master Gradient, Eppendorf
Electrophoresis	Power Pack 300, BioRad
	Sub-CellGT Agarose gel electrophoresis System, BioRad
Vacuum Blotter	Vacuum Blotter Model 785, BioRad
Documentation system	Image Master VDS Software, Pharmacia
Centrifuges	Biofuge fresco, Heraeus
	Biofuge pico, Heraeus
Sequencing equipment	ABI Prism 310 Genetic Analyzer, Perkin Elmer

2.8. Animals

All experiments were carried out with BALB/c mice. Animals were kept in a 12 h dark/light cycle and fed a standard pellet diet with water provided *ad libitum.* Animals were mated during a 12 h period, and day 0 of pregnancy was defined as the day after copulation. For tissue preparation, animals were anesthesized with 1 ml/100g bodyweight of 25% chloridium hydrate in 0.9% NaCl, and embryos were delivered. Postnatal and adult animals were sacrificed with an overdose of ether and then decapitated. To investigate the effect of androgens on the regulation of aromatase expression, the androgen receptor inhibitor cyproterone acetate (Cyp) was used. Embryos were exposed *in utero* to 0.1 ml (100 mg/ml) Cyp dissolved in sesame oil (S) by a daily subcutaneous application to pregnant mice from ED15 until ED20. Control animals were treated with S only. The treatment was performed by HD Dr. Cordian Beyer. Control and treated embryos were delivered by caesarian section on ED20. Sex determination of fetuses and postnatal/adult animals was performed by the inspection of gonadal anlage/gonads and the anogenital distance. Prenatally, the testicular artery and sex cords allow to distinguish between sexes, since these features are absent in females. For our experiments, brain tissues from ED15 and ED17 embryos and individuals on P0, P10, P15 and P30 were used.

2.9. Tissue dissection

After removing meninges, tissue fragments containing the Hyp/POA were obtained by making a coronal cut anterior to the optic chiasm and a second cut at the caudal edge of the mammilary bodies. The excised region extended dorsally to the superior border of the third

ventricle and laterally to the edge of the tuber cinereum. In all cases, the optic chiasm and optic nerves were removed before collecting the hypothalamic samples. The midbrain was dissected by two cuts through the mesencephalic flexure at right angles to the neuraxis, immediately rostral and caudal to the developing tyrosine hydroxylase–immunoreactive cell groups. These tissue blocks comprise the anlage of the midbrain tegmentum. Ovaries were removed from adult female mice and used as positive control. All collected tissues were stored at -70°C until further use.

2.10. Preparation of neural cell cultures

Cultures were prepared from mouse hypothalamus or midbrain on ED15 and kept for 6 DIV. Tissue pieces were dissected and collected in a preparation buffer. After dissection, tissues were incubated for 15 min in 10 ml Versene containing 0.1% trypsin at RT. The enzymatic digestion was stopped by adding 10 ml dispersion buffer. Cells were mechanically dissociated by triturating through a plastic pipette. The obtained suspension was filtered through a 125 µm nylon mesh. Cells were centrifuged for 5 min at 1400 rpm at RT. The pellet was dissolved in 4 ml of MEM containing 5% FCS. The number of viable cells was determined by their ability to stain with trypan blue. Cells were plated at a density of 250 000 cells/cm^2 in 9.6 cm^2 petri-dishes coated with poly-DL-ornithine and were incubated in a water-saturated atmosphere of 5% CO_2 and 95% air at 37°C. On DIV2, the medium was completely changed to NBM without FCS and was renewed every second day.

2.11. Preparation of astroglial cell cultures

To establish astroglial cultures, the midbrain of newborn mice was dissected and incubated in Ca^{2+}- and Mg^{2+}- free Dulbecco's PBS containing 0.1% trypsin and 0.02% EDTA. After 20 min, trypsin action was terminated by transferring tissue pieces to a Ca^{2+}- and Mg^{2+}-free Hank’s balanced salt solution containing 10% FCS. Subsequently, the tissue was dissociated by trituration, filtered through a 50 µm nylon mesh, centrifuged at 1400 rpm for 5 min, and resuspended in NBM plus 10% FCS. Cells were seeded at a density of 4 x 10^4 cells/cm^2. Upon reaching confluency, cells were trypsinized and replated at lower densities. Plating was repeated two times before cultures were used for experiments.

Versene, pH 7.2

0.9 % NaCl, pyrogen-free

200 mg EGTA

Preparation buffer, pH 7.2

145 mM NaCl

4 mM KCl

15 mM Hepes

MEM- Minimum essential medium, Earle's culture medium, pH 7.2-7.4

9.6 g MEM

26 mM $NaHCO_3$

30 mM glucose

50 000 U/l penicillin

50 mg/l streptomycin

Dispersion solution, pH 7.2

MEM-Earle's culture medium with

10% FCS

30 mM glucose

15 mM HEPES

Petri dishes coated with poly-DL-ornithine

One ml of poly-DL-ornithine (0.5 mg/ml) was added per dish and left over night. After that, dishes were washed 3x with ddH_2O and left to dry.

2.12. Treatment of cell cultures

Cultures used for differential display PCR (ddPCR) were treated with 17β-estradiol (E, 10^{-8} M) and 17β-estradiol bound to BSA (E-BSA; 10^{-8} M of 17β-estradiol molecules) daily from DIV2 to DIV6. Neuronal cultures used for EMSA and WB were stimulated on DIV6 for 15 min with E and E-BSA at the same concentration as given above, or BSA (10^{-9} M). To inhibit E effects cultures were treated with E or E-BSA in the presence of the nuclear ER antagonist ICI 182,780 (10^{-6} M). As a positive control for WB, neuronal cultures were stimulated with BDNF (50 ng/ml). Glial cultures used for WB were treated for 7.5 min

with E, E-BSA, E/ICI 182,780, and FGF (positive control, 25 ng/ml). For EMSA, positive control cultures were exposed to forskolin plus IBMX (200 μM) which maximally stimulates CREB phosphorylation and inhibits PP1 phosphatase, respectively.

2.13. RNA isolation

Total RNA was isolated using TRI Reagent according to the manufacturers instructions. Briefly, tissue or cultured cells (pooled from up to three 9.6 cm^2 dishes) were lysed with 1 ml TRI Reagent. Then, phenol/chloroform extraction was performed by adding 0.2 ml chloroform, vortexing and centrifugation at 13000 rpm for 15 min at 4°C. The obtained aqueous phase was transferred to a new tube, precipitated in 50% isopropanol for 10 min at RT, and centrifuged at 13000 rpm for 15 min at 4°C. The pellet was washed with 200 μl 75% ice cold ethanol and centrifuged at 13000 rpm for 10 min at 4°C. The pellet was vacuum dried and dissolved in 20 to 40 μl DEPC-treated water. The concentration of isolated RNA was measured by spectrophotometry at 260 nm (an optical density (OD) of 1 corresponds to approximately 40 μg/ml RNA). RNA was stored at -70 °C.

2.14. Reverse transcription and polymerase chain reaction (RT-PCR)

Random-primed reverse transcription was performed in a total volume of 25 μl containing 2-10 μg of total RNA, 1.5 μl 10x hexanucleotide mixture, 200U M-MLV reverse transcriptase, 1x M-MLV reaction buffer, 0.2 mM dNTP for 1 h at 37°C. The enzyme was inactivated by heating samples for 5 minutes at 95°C. Amplification was performed in a total volume of 50 μl consisting 3 μl cDNA, 0.4 μM sense and antisense primers (see table 1), 0.2 mM dNTP, 1.5 mM $MgCl_2$, 1x reaction buffer and 1.2 U Taq Polymerase. 33 PCR cycles were performed. Amplification conditions were as follows: the first amplification cycle with 3 min denaturation at 95 °C, 1 min annealing at specific temperature (T_m) for each set of primers (see Table 1), and 2 min elongation at 72°C. The remaining 32 cycles were performed with 1 min denaturation.

Table 1. Primers applied in RT-PCR.

Name of gene, Genbank accession number	**Position of primers (nt)**	**Primer sequence**	**Product length (bp)**	**T_m**
β-actin AF195094	366-368 616-636	5'-CAG GTC CAG ACG CAG GAT GGC-3' 5'-CTA CAA TGA GCT GCG TGT GGC-3'	271	61°C
HPRT J00423	576-594 805-824	5'-GCT GGT GAA AAG GAC CTC T-3' 5'-CAC AGG ACT AGA ACA CCT GC-3'	249	61°C
Aromatase D00659	968-988 1256-1276	5'-CCT GAC ACC ATG TCG GTC ACT-3' 5'-GGG CTT AGG GAA GTA CTC GAG-3'	309	61°C
ER-β_{HYP} AJ000220	10-31 262-282	5'-TAC AGT CCT GCT GTG ATG AAC T-3' 5'-ACT AGT AAC AGG GCT GGC ACA-3'	273	59°C
ER-α_{MID} M38651	1682-1702 1691-1981	5'-GGC CTG ACT CTG CAG CAG CAG-3' 5'-GTT GGG GAA GCC CTC TGC TCC-3'	300	61°C
ER-β_{MID} AJ000220	557-575 751-771	5'-AAG ACA GAG AAG TGC CAG CGA-3' 5'-AGC CAG CTT CGT AAG GGA CAT-3'	217	59°C
MAP-2 M21041.1	253-273 796-816	5'-TGG ACA TCA GCC TCA CTC ACA-3' 5'-TGT GTC AGG AAC TAA GGC AGC-3'	563	56°C
GFAP X02801.1	1596-1616 2068-2088	5'-AGA GAA CAA CCT GGC TGC GTA-3' 5'-GGA GGG CTG CTA ACA TTT TCA-3'	493	64°C

T_m, specific annealing temperature; nt, nucleotide position in the respective gene sequence; bp, base pairs.

2.14.1. Visualization of PCR products

2.14.1.1. DNA agarose gel electrophoresis

PCR products were separated in a 1.5% agarose gel. Agarose gels were prepared by heating 1.5 g agarose in 100 ml of 1x TAE buffer until agarose was dissolved. 3 µl EtBr (10 mg/µl) were added to the solution. After that, the solution was poured into a gel tray. The gel was allowed to sit for 1 h. 10 µl PCR samples were loaded on the gel with an additional 1 µl loading buffer (50% glycerol, 0.05% BB). A 100 bp DNA ladder was loaded along with the PCR samples and used to estimate the size of PCR products. The

electrophoresis was carried out at 100 V for 45 to 60 min. The gel was examined under UV light (254 nm) and pictures of gels were taken using a fluorescent gel scanner. The specificity of the PCR products was tested by Southern blot analysis (see chapter 2.16.) or by sequencing.

1x TAE, pH 7.5
44.5 mM TRIS
45.5 mM boric acid
1 mM EDTA

2.14.1.2. Quantification of PCR products and linearity

Absolute ODs were calculated for each PCR product (aromatase, ERs). Pictures of gels were taken using the Image Master VDS and then ODs were calculated by using the Image Master VDS software (Pharmacia). For each individual sample, the OD of the corresponding β-actin or HPRT band was used as a reference and set to 100%. The relative OD of aromatase and ERs in each sample was expressed as percentage of the corresponding OD for β-actin or HPRT. In order to minimize inter-assay variations (≤ 5%), RNA isolation, RT-PCR, and quantification of samples from each gestational stage were performed simultaneously. In order to determine optimal PCR conditions for each set of primers, cDNA amplification was carried out for 28, 30, 32, 34, and 36/38 cycles. Since a linear relationship between the amount of PCR product and the number of cycles was shown (see Figure 4 and 5), 33 amplification cycles were chosen for all further experiments.

2.15. Southern blot analysis

The transfer of PCR products from agarose gels to the Hybond-N+ nylon membrane was carried out in the presence of 10x SSC buffer for 1 h in a Vacuum Blotter with 50 mbar pressure. The DNA was cross-linked by exposure to UV light at 1200 jouls/cm^2 for 1 min. 100 pmol of the 30-mer antisense hybridization oligonucleotides (see Table 2) for aromatase and ERβ were labeled using a digoxigenin (DIG) oligonucleotide tailing kit in the presence of 50 U terminal transferase and 0.05 mM DIG-UTP for 15 min at 37°C. The reaction was stopped with 20 mM EDTA, pH 8.0. The labeled nucleotides were separated from non-incorporated DIG-dUTP by ethanol precipitation using glycogen as carrier. The

membrane was prehybridized for 1h at RT in prehybridization buffer. 5 pmol/ml of the digoxigenin labeled oligonucleotide probe was added to the prehybridization buffer, and hybridization was carried out overnight at 42°C. After that, the membrane was washed twice in 2x SSC/0.1% SDS for 5 min at RT, twice in 0.1x SSC/1% SDS for 15 min at 60°C, and once in washing buffer for 5 min at RT. Subsequently, the membrane was transferred to blocking buffer for 30 min at RT. The anti-DIG Fab fragment was added to the blocking buffer (1:10000) and incubated for 30 min at RT. The membrane was then washed 2x in washing buffer and equilibrated in the detection buffer for 5 min. CSPD was diluted in the detection buffer (1:100) and used for chemiluminescence detection. The membrane was sealed in a plastic bag and exposed to an X-ray film for 5 to 30 min depending on the signal intensity.

Table 2. Oligonucleotides used for southern blot analysis.

Gene	Position of primers (nt)	Sequence
Aromatase	1177-1206	5'-CGGGTAGCCGTCAATTACGTCATCCTCCAG-3'
Estrogen receptor β	64-93	5'-TGGGCTTGCAGTCTGGCGAACAGGCCCACC-3'

nt, number of nucleotides and their position in the respective gene sequence.

20x SSC, pH 7.0

3 M NaCl

0.3 M sodium citrate

Prehybridisation buffer, pH 7.0

5x SSC

1% blocking reagent

0.1% N-lauryl sarcosine

0.02% SDS

Maleic acid buffer, pH 7.5

0.1 M maleic acid

0.15 M NaCl,

Washing buffer, pH 7.5

maleic acid buffer

0.03% (v/v) Tween 20

Blocking buffer

1% blocking reagent in maleic acid buffer

Detection buffer pH 9.5

0.1 M TRIS-HCl

0.1 M NaCl

50 mM $MgCl_2$

2.16. Differential display PCR

Differential display PCR (ddPCR) was performed using a commercially available RNAimage Kit.

2.16.1. Reverse transcription and PCR reaction

To divide total mRNA into three subpopulations, RNA was reverse transcribed using the 3'one-base-anchored oligo-dT primers included in the RNAimage Kit (see table 3). 0.2 µg of total RNA was denatured for 5 min at 65°C in a total volume of 19 µl containing 1x reverse transcription buffer, 20 µM dNTP and 4 pmol anchored primer. This mixture was preincubated for 10 min at 37°C. The reverse transcription was initiated by adding 100U M-MLV and incubated for 60 min. To stop the reaction, the mixture was incubated for 5 min at 75°C. This condition was chosen in order to prevent denaturation of cDNA/RNA complexes and possible mispriming by the arbitrary primer in the PCR reaction. PCR was performed in a total volume of 20 µl containing 1x PCR buffer, 2 µM dNTP, 2 µl cDNA, 4 pmol of one of the 5' arbitrary primers included in the RNAimage Kit (see

table 3), 4 pmol 3'one-base-anchored oligo-dT primer (used in reverse transcription), 10 µCi α-[^{35}S]dATP (1000 Ci/mmol), and 1.2 U Taq Polymerase. PCR conditions were: 1 min denaturation at 94°C, 2 min annealing at 40°C, 2 min elongation at 72°C, for 40 cycles followed by 1 cycle at 72°C for 5 min.

Table 3. Primers applied in different display PCR analysis

Name	**Sequence**
Anchoring primers	
H-T_{11}G	5'-AAGCTTTTTTTTTTG-3'
H-T_{11}A	5'-AAGCTTTTTTTTTTA-3'
H-T_{11}C	5'-AAGCTTTTTTTTTTC-3'
Arbitrary primers	
H-AP1	5'-AAGCTTGATTGCC-3'
H-AP2	5'-AAGCTTCGACTGT-3'
H-AP3	5'-AAGCTTTGGTCAG-3'
H-AP4	5'-AAGCTTCTCAACG-3'
H-AP5	5'-AAGCTTAGTAGGC-3'
H-AP6	5'-AAGCTTGCACCAT-3'
H-AP7	5'-AAGCTTAACGAGG-3'
H-AP8	5'-AAGCTTTTACCGC-3'

2.16.2. Separation and detection of PCR products on polyacrylamide gels

A 6% acrylamide gel stock solution (acrylamide/bisacrylamide 39:1) containing 8 M urea and 1x TBE was prepared. Polymerization was initiated by the addition of 25 µl TEMED and 250 µl 10% APS per 100 ml solution. The gel (600x300x0.3mm) was poured and allowed to polymerize for 1 h. The gel was initially run for 30 min at 2500 V. A 3.5 µl sample was mixed with 2 µl denaturing loading buffer and was denatured for 2 min at 80°C, immediately chilled on ice, and loaded onto the gel. Electrophoresis was then carried out at 2500 V until the FF reached the bottom of the gel. After electrophoresis, the gel was put on filter paper, covered with plastic wrap, and dried for 1 h at 80°C. The dried gel was then exposed to an X-ray film for 24-48 h at RT together with the fluorescent marker.

1x TBE

89 mM TRIS

89 mM boric acid

2 mM EDTA, pH 8.0

Loading buffer

95% formamide

10 mM EDTA, pH 8.0

0.09% FF

0.09% BB

2.16.3. Elution of differentially expressed PCR products from polyacrylamide gel and reamplification

The autoradiogram and dried gels were adjusted using fluorescent marker. Bands of interest were located by marking them with a needle at four corners. Bands were cut with a clean razor blade, transferred to 1.5 ml tubes, and soaked with filter paper in 100 µl ddH_2O for 10 min. The tubes were boiled for 15 min and then centrifuged for 2 min at 13000 rpm to pellet the gel and paper pieces. The supernatants were transferred to new tubes and DNA was precipitated with 0.05 M sodium acetate, 5 µg of glycogen and 75% ethanol at -80°C for 1 h. DNA was pelleted by centrifugation for 10 min at 4°C, washed with 200 µl 85% ice-cold ethanol, and vacuum dried. The DNA pellet was dissolved in 10 µl ddH_2O and 4 µl were used for the reamplification reaction. Reamplification was carried out in a total volume of 40 µl containing the same primer set and ingredients as in the PCR reaction, except dNTP (changed to 2 µM) and no isotopes. PCR conditions were as described above (see chapter 2.17.1.).

2.16.4. Separation of reamplified products and purification of cDNA probes

Reamplified products were separated on 3% low melting agarose gels containing 1 OD/ml H.A.-Yellow, in 0.5x TAE buffer. H.A.-Yellow was used to distinguish between different reamplification products with the same size but different content of adenine and thymidine. H.A.-Yellow binds to adenine and thymidine resulting in a slower migration of DNA fragments during electrophoresis proportional to increasing number of these nucleotides in the sequence. DNA fragments were excised from low melting agarose gels and were purified using QIAquick Gel Extraction Kit according to the manufacturer's instruction. Briefly, gel slices containing PCR products were dissolved in high salt buffer and loaded into columns. cDNA was adsorbed on silica-membranes and salts were washed out with buffer PE. Any residual PE buffer was removed by an additional centrifugation step. DNA was eluted with 30 µl of ddH_2O and used for cycle sequencing analysis.

2.17. Sequencing

Sequencing was performed using the ABI Prism BigDye Ready Reaction Kit.

2.17.1. Cycle sequencing and purification

Sequencing was performed with 2-4 µl purified reamplification product, 5 pmol adequate arbitrary or anchoring primer, and 2 µl BigDye Ready Reaction Mix (containing: dNTP, AmpliTaq, FS, rTtH DNA polymerases, pyrophosphatase, $MgCl_2$, fluorescent labeled ddNTP terminators) in a total volume of 10 µl. PCR conditions were as follows: denaturation 3 min at 96°C, followed by 36 cycles 96°C for 15 sec, 55°C for 2 min. pGEM-32f(+) and -21M13 control primer (included in kit) were used as a positive control in all sequencing procedures. Sephadex MicroSpin purification columns were used to remove not incorporated nucleotides. Briefly, columns were shortly vortexed to resuspend sephadex G-50 resins, placed in 1.5 ml eppendorf tubes, and centrifuged at 5000 rpm for 1 min. 10µl ddH_2O was added to each sample, and then samples were loaded into columns and centrifuged as detailed above. The obtained solution was used for sequence analysis.

2.17.2. Sequence analysis with capillary electrophoresis

5 µl purified sequence reaction was added to 20 µl TSR reagent, denatured at 90°C for 2 min, and chilled on ice. Sequence analysis was performed using ABI Prism 310 Genetic Analyzer. Samples were loaded into POP6 polymer containing 310GA capillary (47 cm length, 50µm∅) for 60 sec at 2.0 kV, and electrophoretic separation was performed for 35 min at 15 kV, at 50°C. The data were analyzed using the ABI PRISM sequencing analysis software.

2.18. Western blotting

2.18.1. Protein preparation

Cultured cells from mesencephalon were harvested in ice-cold 1x PBS and centrifuged for 5 min at 5000 rpm. The obtained pellet was sonicated in 100 µl probe buffer and denatured at 95°C for 5 min. The protein amount was determined using the ABC Protein Assay Reagent with BSA to establish a standard curve. Proteins were stored at -70°C.

Probe buffer, pH 6.8

62.5 mM TRIS-HCl

2% SDS

10% sucrose

5 µg/ml aprotinin

500 mM PMSF

2.18.2. Polyacrylamide gel electrophoresis and protein transfer

10 µg protein was denatured in 1x denaturing loading buffer, at 95°C for 5 min, chilled on ice, and loaded on 10% SDS polyacrylamide gel (acrylamide/bisacrylamide 29:1). Electrophoresis was performed with 1x electrophoresis buffer at constant 400 mA for 1 h. A standard-protein ladder (Mark 12 MW) was loaded along with the protein samples and used to estimate the size of proteins. Protein transfer was performed in 1x transferring buffer at 100V for 1 h to nitrocellulose membrane. The quality of transfer was checked by staining the membrane with 1x ponceau.

Stacking -upper gel

0.25 M Tris-HCl, pH 6.8

5% Acrylamide/bisacrylamide 29:1

0.1% SDS

0.05% TEMED

0.1% APS

Resolving –down gel

0.375 M TRIS-HCl, pH 8.8

10% Acrylamide/bisacrylamide 29:1

0.1% SDS

0.05% TEMED

0.1% APS

1x Electrophoresis buffer, pH 8.3

5 mM TRIS-HCl

50 mM glycine

0.1% SDS

1x Loading buffer

125 mM TRIS, pH 6.8

2% SDS

5% glycerol

0.006% BB

1x Transfer buffer, pH 8,3

39 mM glycine

48 mM TRIS

0.037% SDS

20% methanol

1x Ponceau

0.02% ponceau

0.3% trichloracetic acid

0.3% sulfosalicilic acid

2.18.3. Detection

The membrane was blocked for 30 min in blocking buffer and then incubated overnight at 4°C with the first antibody (1:500) in 1x incubation buffer. The membrane was washed 3x 10 min in washing buffer and incubated with the secondary antibody conjugated to horseradish peroxidase (1:3000) in incubation buffer for 2.5 h at RT. The membrane was then washed 3x for 10 min in washing buffer. The chemifluorescence reaction was performed using ECL western blotting detection kit. Briefly, equal volumes of detection solutions 1 and 2 were mixed and poured onto the membrane in an amount sufficient to completely cover the membrane surface. The membrane was incubated exactly 1 min. Then, the excess of the solution was drained off and the membrane was covered with saran wrap and exposed to X-ray film from 10 sec to 5 min depending on the signal intensity. For second labeling, the membrane was washed 3x in washing buffer after the first detection and the procedure was repeated with the next antibodies. In each experiment, the nylon membrane was first incubated with antibody directed against phosphorylated form of MAPK 42/44, and subsequently with antibody directed against total MAPK 42/44. The second reaction was performed in order to verify the level of proteins loaded onto the gel. The secondary antibodies, peroxidase conjugated affini-pure goat anti-mouse Ig G (H+L)

and peroxidase conjugated affini-pure goat anti-rabbit Ig G (H+L) for phosphorylated and total MAPK respectively, enable chemiluminescent detection with ECL Western blotting detection reagents.

1x TBS, pH 7.6
150 mM NaCl
50 mM TRIS

Washing buffer, pH 7.6
1x TBS
0.05% Tween 20

Blocking buffer, pH 7.6
1x TBS
5% non-fat dried milk
0.05% Tween 20

Incubation buffer, pH 7.6
1x TBS
0.05% Tween
0.5% non-fat dried milk

2.18.4. Quantification of MAPK phosphorylation

Four independent experiments were carried out for glial and neuronal cultures. Absolute ODs were calculated for each band (see chapter 2.15.1.2.) corresponding to the phosphorylated and non-phosphorylated MAPK 42 and MAPK 44. In each experiment, control values representing unstimulated cells were set to 100, and the values of the bands obtained from treated cultures were expressed as percentage of controls.

2.19. Electrophoretic mobility gel shift assay (EMSA)

EMSA was performed using the Gel Shift Assay Core System.

2.19.1. Nuclear protein isolation

After a 15 min stimulation, cultured cells from one 9.6 cm^2 petri dish were collected in ice-cold 1x PBS and centrifuged at 7000 rpm at 4°C for 10 min. The pellet was resuspended in 100 µl extraction buffer and shaken at 4°C for 15 min. Cell membranes were discarded by centrifugation at 13 000 rpm at 4°C for 15 min. The resulting supernatant was used for the gel shift assay analysis. The protein amount was determined using the Coomassie Protein Assay Reagent. BSA was used for the standard curve. Proteins were stored at -80°C.

1x PBS, pH 7.4

10 mM $Na_2HPO_4x2H_2O$

10 mM $NaH_2PO_4xH_2O$

Extraction buffer, pH 7.9

10 mM HEPES

400 mM NaCl

100 µM EGTA

500 µM DTT

500 µM PMSF

5% glycerol

2.19.2. Labeling of CRE consensus oligos

Labeling was performed in a total volume of 10 µl containing 3.5 pmol CRE consensus oligo (included in kit), 5-10 U T_4 kinase, 1x T_4 kinase buffer and 10µCi γ-^{32}P-ATP for 40 min at 37°C. The reaction was stopped by adding 1µl 0.5 M EDTA. 89 µl TE_{10} buffer was added to the reaction, and column chromatography with Nick Spin purification columns (containing G-50 Sephadex) was performed to remove not incorporated nucleotides. Briefly, the columns were shortly vortexed to resuspend sephadex resins, placed in 10 ml centrifuge tubes, and centrifuged at 1500 rpm for 5 min. The columns were equilibrated with TE_{10} buffer and samples were loaded into columns and centrifuged under the same conditions as described above. The activity of radiolabelled nucleotides was measured using a β-counter giving approximate values of 1-3 x 10^6 cpm/pmol.

TE_{10} buffer, pH 7.4- 8.0

10 mM TRIS-HCl

1 mM EDTA

2.19.3. Binding reaction and gel electrophoresis

An equal amount of protein from each sample was preincubated with 1x binding buffer in a total volume of 19 µl for 15 min at RT. The binding reaction was initiated by the addition of 0.03 pmol γ-^{32}P-ATP labeled CRE Consensus Oligo and incubated for 15 min at RT.

1x Binding buffer

20% glycerol

5 mM $MgCl_2$

2.5 mM EDTA

2.5 mM DTT

250 mM NaCl

50 mM TRIS-Cl, pH 7.5

0.25 mg/ml poly(dI-dC)•(dI-dC))

2.19.4. Competition reaction and supershift assay

Competition experiments were performed by the addition of 1.75 or 3.5 pmol unlabelled CRE Consensus Oligo as specific competitors or 3.5 pmol AP2 Consensus Oligo (included in the kit) as non-specific competitor to the preincubation mix. The binding reaction was performed as described in chapter 2.19.3. After 15 min binding reaction supershift assays were performed. 2µg CREB-1(24H4B) antibody for the shift assay was added to the binding mixture and incubated for 45 min at RT.

2.19.5. Gel electrophoresis

After incubation, 2 µl loading buffer was added to each sample. Samples were electrophoretically separated on 5% acrylamide gel (acrylamide/bisacrylamide 29:1) in 1x TBE buffer at 200V for 2-3 h. Gels were put on filter paper, covered with plastic wrap, dried at 80°C, and exposed to the X-ray film with an intensifying screen at -70°C for 1-2 days.

Loading buffer

250 mM TRIS-HCl, pH 7.5

0.02% BB

40% glycerol

1x TBE

89 mM TRIS-borate

89 mM boric acid

1 mM EDTA, pH 8.0

2.19.6. Quantification of EMSA bands

Absolute ODs were calculated for each EMSA band (see chapter 2.15.1.2.) In each experiment, control values were set to 100, and the values of the bands obtained from treated cultures were normalized to controls.

2.20. Statistical analysis

2.20.1. PCR

All values concerning aromatase and ERs mRNA levels given in the text and figures represent the means ± SEM. The number of independent observations (individual embryonic and postnatal animals) are given in parentheses in the figures or as (n) in the figure legends. At each embryonic and postnatal stage, fetuses and postnatal animals included in the analysis were derived from at least five different litters. Usually, only 1-2 male and female animals were used from one litter. For determining the effect of cyproterone acetate on aromatase mRNA expression, five pregnant mice were injected with an androgen receptor antagonist. As a control, we used newborns from five mothers that were treated with sesame oil only. Comparisons between different experimental groups and sexes were made by analyzing data by one-way analysis of variance (ANOVA) followed by „t" test with $p \leq 0.05$ as the level of statistical significance.

2.20.2. Electrophoretic mobility shift assay and Western blotting

Optical densities for EMSA and Western blotting are given in arbitrary units and expressed as percent of controls (set to 100, untreated cultures). All values given in the text and

graphs represent the means ± SEM. Differences between experimental groups were analyzed by one-way analysis of variance (ANOVA) followed by the posthoc Newman-Keuls multiple-range test with $p \leq 0.05$ as the criterion for statistical significance.

3. Results

3.1. Optimizing of RT-PCR conditions

The developmental expression of aromatase and ER mRNA was investigated using semi-quantitative RT-PCR analysis. PCR primers were designed using "oligo" DNA/RNA primer selection software (for sequences see Table 1). To establish optimal PCR conditions, temperature gradient PCR with temperatures in the range ± 5°C to the calculated annealing temperatures (T_m) were carried out. Table 4 shows the annealing temperatures that yielded one single band after PCR amplification and were consequently used for all experiments.

Table 4. Annealing temperatures and length of yielded RT-PCR products

Gene	**T_m**	**Length**
Aromatase	61°C	309 bp
Estrogen receptor β_{HYP}	59°C	273 bp
Estrogen receptor β_{MID}	59°C	217 bp
Estrogen receptor α_{MID}	59°C	300bp
β-actin	61°C	249 bp
HPRT	61°C	249 bp
MAP-2	56°C	563 bp
GFAP	64°C	493 bp

Additionally, regression analysis as a function of the intensity of the PCR signal and the applied PCR cycles was performed for each analyzed gene. For that purpose, PCR was run from 28 to 38 cycles. Figure 4 shows the cycle-dependency for aromatase, ER-β_{HYP}, ER-α_{MID}, and ER-β_{MID}. Since linear relationships between the amount of PCR products and the number of cycles were found (Figure 5) for each RNA between 28 and 36 cycles, 33 amplification cycles were chosen for all other experiments.

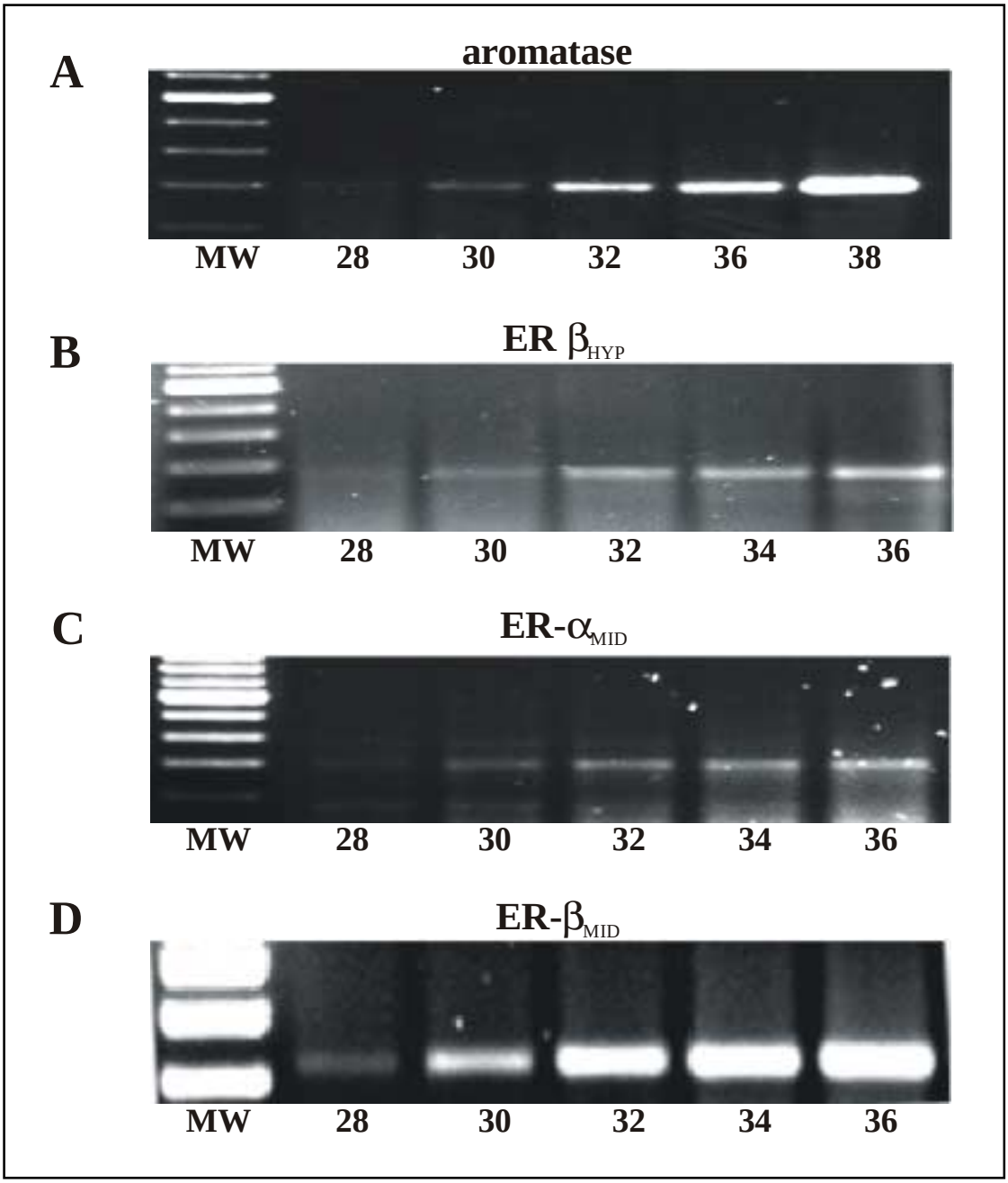

Figure 4. Cycle-dependency of aromatase **(A)**, estrogen receptor (ER)-β_{HYP}, **(B)**, ER-α_{MID} **(C)**, and ER-β_{MID} **(D)** mRNA expression obtained by RT-PCR of total RNA extracted from male newborn hypothalamic or midbrain tissue.

The specificity of RT-PCR products was confirmed for aromatase (data not shown) and ER-β_{HYP} (Figure 8) by Southern blot analysis with internal, DIG-labeled oligonucleotides (for sequences see Table 2). These experiments revealed specific hybridization signals.

Sequencing analysis using ABI Prism 310 Genetic Analyzer was performed for ER-α_{MID} and ER-β_{MID}, GFAP, and MAP-2 RT-PCR products. All four analyzed RT-PCR products revealed sequence similarities from 98 to 100% to the known sequences obtained from GenBank (accession numbers are given in Table 1).

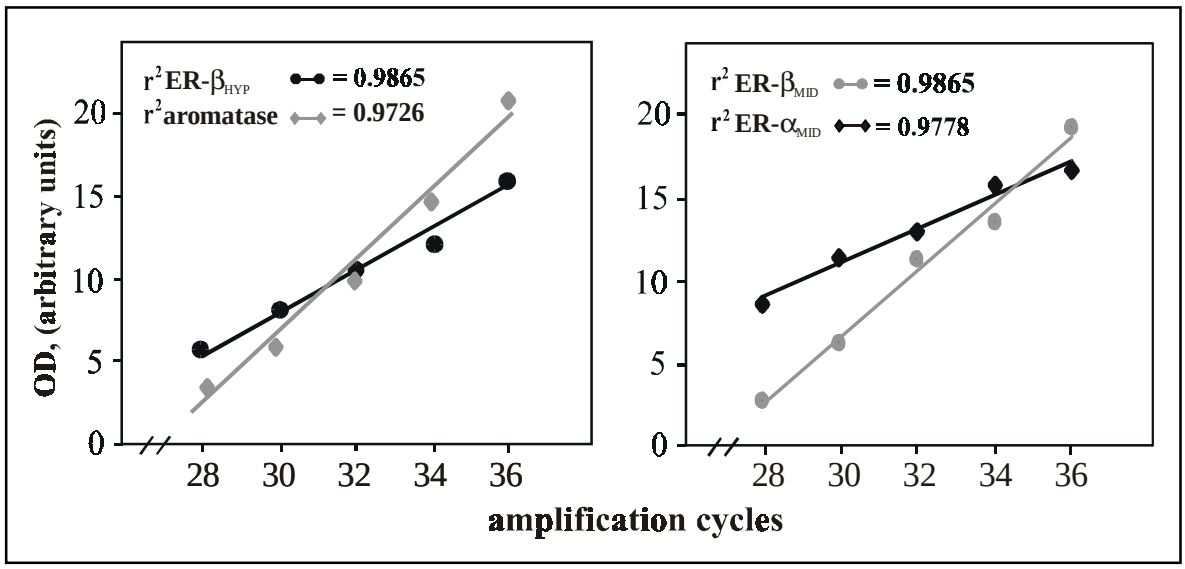

Figure 5. Cycle dependency of RT-PCR products for aromatase, estrogen receptor (ER)-$\beta_{HYP,}$ ER-$\alpha_{MID,}$ ER-β_{MID} obtained by semiquantitative fluorescent gel scanning; r^2- correlation coefficient.

3.2. Developmental expression of aromatase in the developing hypothalamus/ preoptic area

The qualitative and quantitative analysis of the developmental expression of aromatase mRNA in the hypothalamus at different pre- (ED15 and ED17) and postnatal stages (P0, P10, P15) as well as in young adults (P30) is shown in Figure 6. For each postnatal stage, 5 to 10 individuals were used. At ED15 and ED17, usually three individuals were pooled. Aromatase transcripts were detectable as early as at ED15 in both sexes. No significant difference in the expression pattern between sexes was observed prenatally. In males, aromatase expression increased stepwise until birth. Thereafter, expression levels decreased dramatically until P10, then increased again and remained at high levels until early adulthood (P30). In contrast to males, females exhibited lower levels of aromatase mRNA expression and no clear-cut developmental expression pattern from ED15 until P30. At P0 and P15, significant higher aromatase expression levels were found in males (P0, $p \leq 0.01$; P15 $p \leq 0.005$).

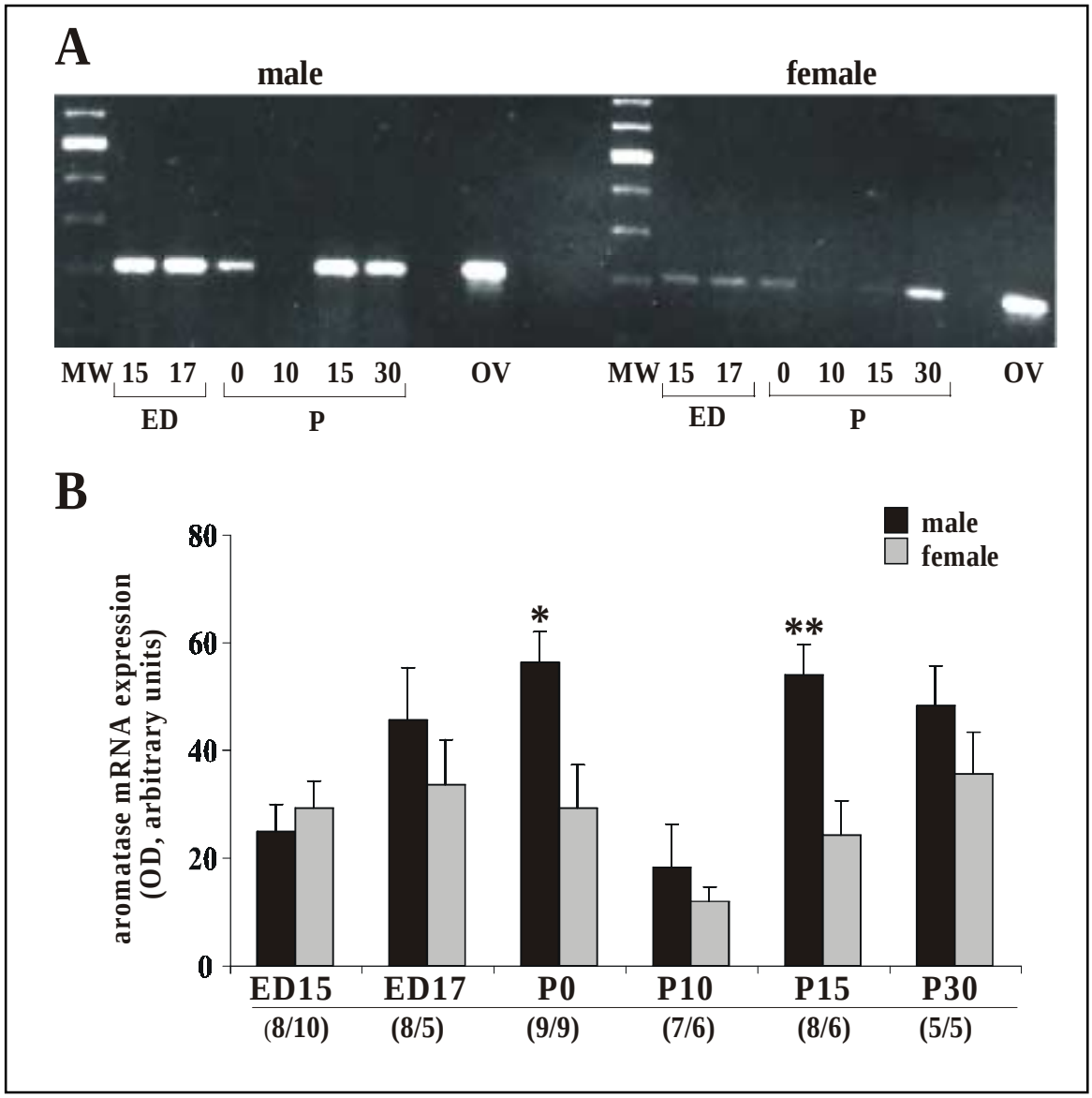

Figure 6. Developmental profile of aromatase mRNA expression in the hypothalamus/preoptic area (Hyp/POA) of male and female mice. **(A)** A representative agarose gels of aromatase RT-PCR products at different embryonic (ED) and postnatal (P) stages; mouse ovary (ov, positive control). **(B)** Semi-quantitative evaluation of RT-PCR analysis. The numbers of analyzed individuals are given in brackets.
*$p < 0.001$ P0 male vs. female
**$p < 0.005$ P15 male vs. female

3.3. Influence of androgens on developmental aromatase expression in the hypothalamus/preoptic area

In order to determine factors that are involved in the developmental regulation of aromatase mRNA expression, we have investigated the role of androgens. Embryos were exposed *in utero* from ED15 until ED20 to the androgen receptor antagonist, cyproterone acetate (Cyp), or to the vehicle (sesame oil, S). At ED20, embryos were delivered by cesarean section, and aromatase expression was investigated in the Hyp/POA. The data are given in Figure 7. Treatment with Cyp resulted in a significant (60 %, $p \leq 0.01$) reduction of aromatase mRNA levels in both sexes compared to untreated animals.

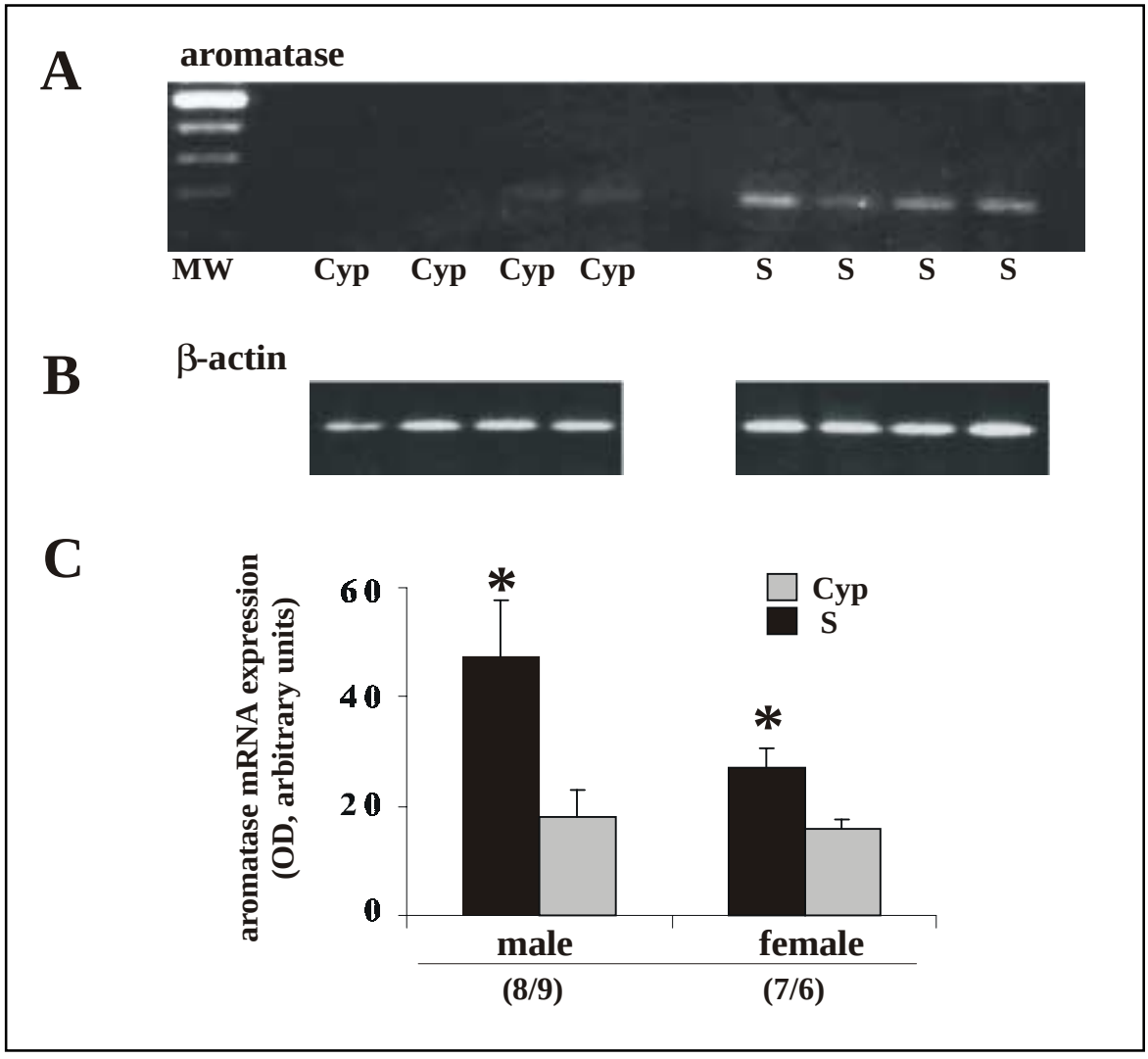

Figure 7. RT-PCR analysis of aromatase mRNA expression in the hypothalamus/preoptic area Hyp/POA of newborn mice treated *in utero* with cyproterone acetate (Cyp) or sesame oil (S). **(A)** Representative agarose gels of aromatase RT-PCR products. **(B)** β-actin mRNA expression. **(C)** Semi-quantitative evaluation of the above RT-PCR experiments. The numbers of analyzed individuals are given in brackets.
*p<0.01, Cyp-treated vs. S-treated

3.4. Estrogen receptor-β_{HYP} mRNA expression in the developing hypothalamus/ preoptic area

The qualitative and quantitative analysis of the ontogenetic expression of ER-β_{HYP} in the developing Hyp/POA is presented in Figure 8. As early as ED15, ER-β_{HYP} mRNA was detectable in both sexes. In the male Hyp/POA, expression of ER-β_{HYP} gradually increased during prenatal development until P0 and reached its highest level around birth. ER-β_{HYP} mRNA levels remained unchanged until P15 and declined thereafter in young adults. In females, ER-β_{HYP} mRNA expression did not fluctuate throughout pre- and postnatal development. Statistically significant sex differences in ER-β_{HYP} mRNA expression levels were measured from ED17 until P15 with higher values in males ($p \leq 0.05$).

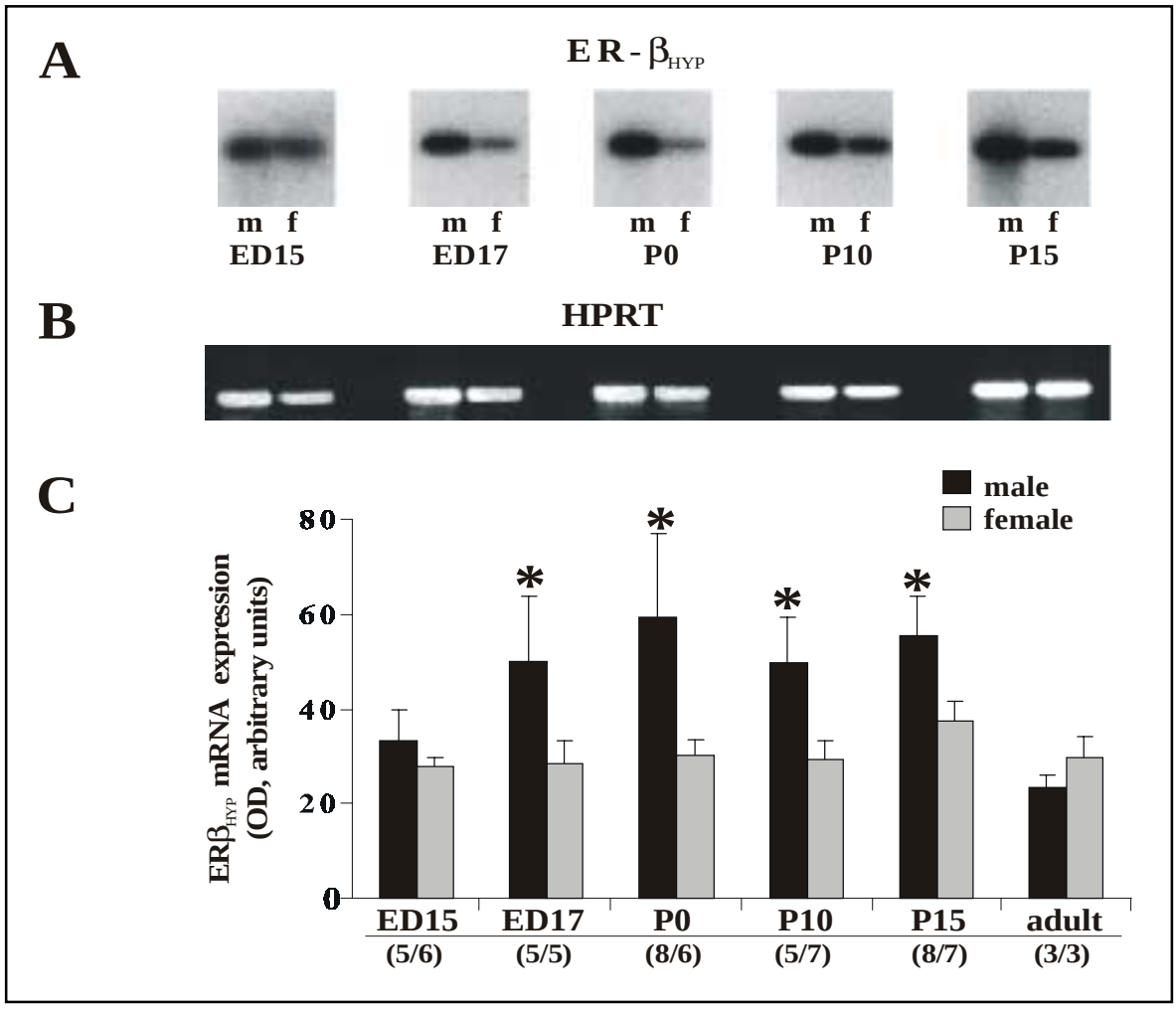

Figure 8. Developmental profile of estrogen receptor (ER)-β_{HYP} mRNA expression in the hypothalamus/preoptic area (Hyp/POA) of male and female mice. **(A)** Representative Southern blot of ER-β_{HYP} RT-PCR products from male (m) and female (f) mice at different embryonic (ED) and postnatal (P) stages. **(B)** Corresponding HPRT mRNA levels**. (C)** Semi-quantitative evaluation of RT-PCR experiments. The numbers of analyzed individuals are given in brackets.
*$p<0.05$ male vs. female

3.5. Estrogen receptor-α_{MID} mRNA expression in the developing midbrain

To investigate ER-α mRNA expression in the developing mouse midbrain, primers were designed targeting the ligand binding domain (LBD). The developmental pattern of ER-α_{MID} expression is shown in Figure 9. ER-α_{MID} mRNA transcripts were detected in both sexes at ED15, increased stepwise thereafter, and reached highest levels in adults. At each developmental stage, no significant differences in expression levels between males and females were observed.

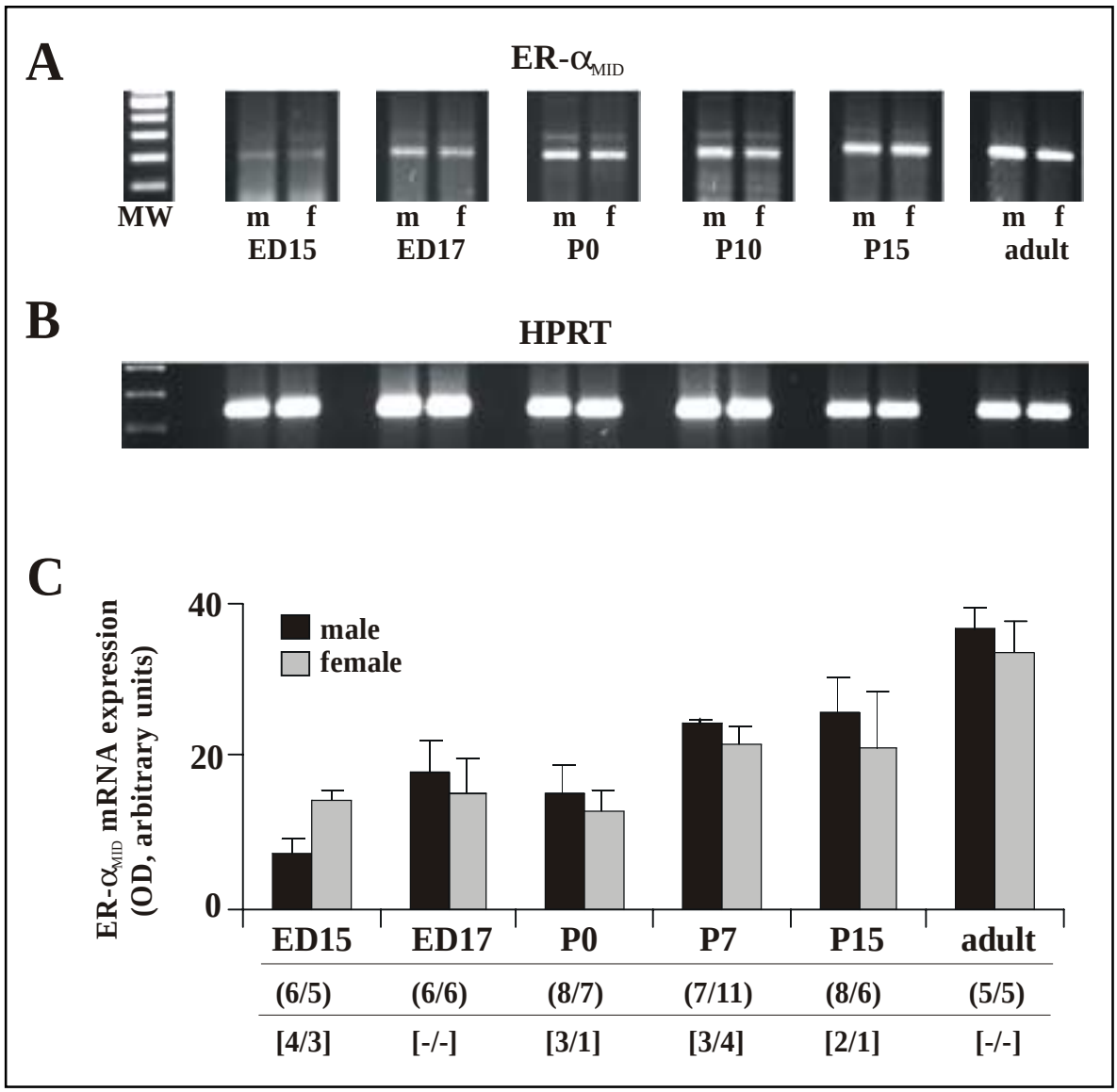

Figure 9. Developmental profile of estrogen receptor (ER)-α_{MID} mRNA expression in the midbrain of male and female mice. **(A)** Representative agarose gels of ER-α_{MID} RT-PCR products from male (m) and female (f) mice at different embryonic (ED) and postnatal (P) stages. **(B)** Corresponding HPRT mRNA levels**. (C)** Semi-quantitative evaluation of RT-PCR experiments. The numbers of analyzed individuals are given in round brackets. The number of samples below detectable level are given in square brackets.

3.6. Estrogen receptor-β_{MID} mRNA expression in the developing midbrain

ER-β_{MID} mRNA expression in the developing midbrain was studied by RT-PCR using primers located in the hinge region of the receptor. The developmental profile of ER-β_{MID} expression is shown in Figure 10. ER-β_{MID} transcripts were detectable in both sexes at ED15. Highest expression levels were observed at ED17 and P0 in both sexes. At P7, expression declined in both sexes and increased again until P15 and in adult animals. No significant differences in ER-β_{MID} mRNA expression between sexes were found at any developmental stage.

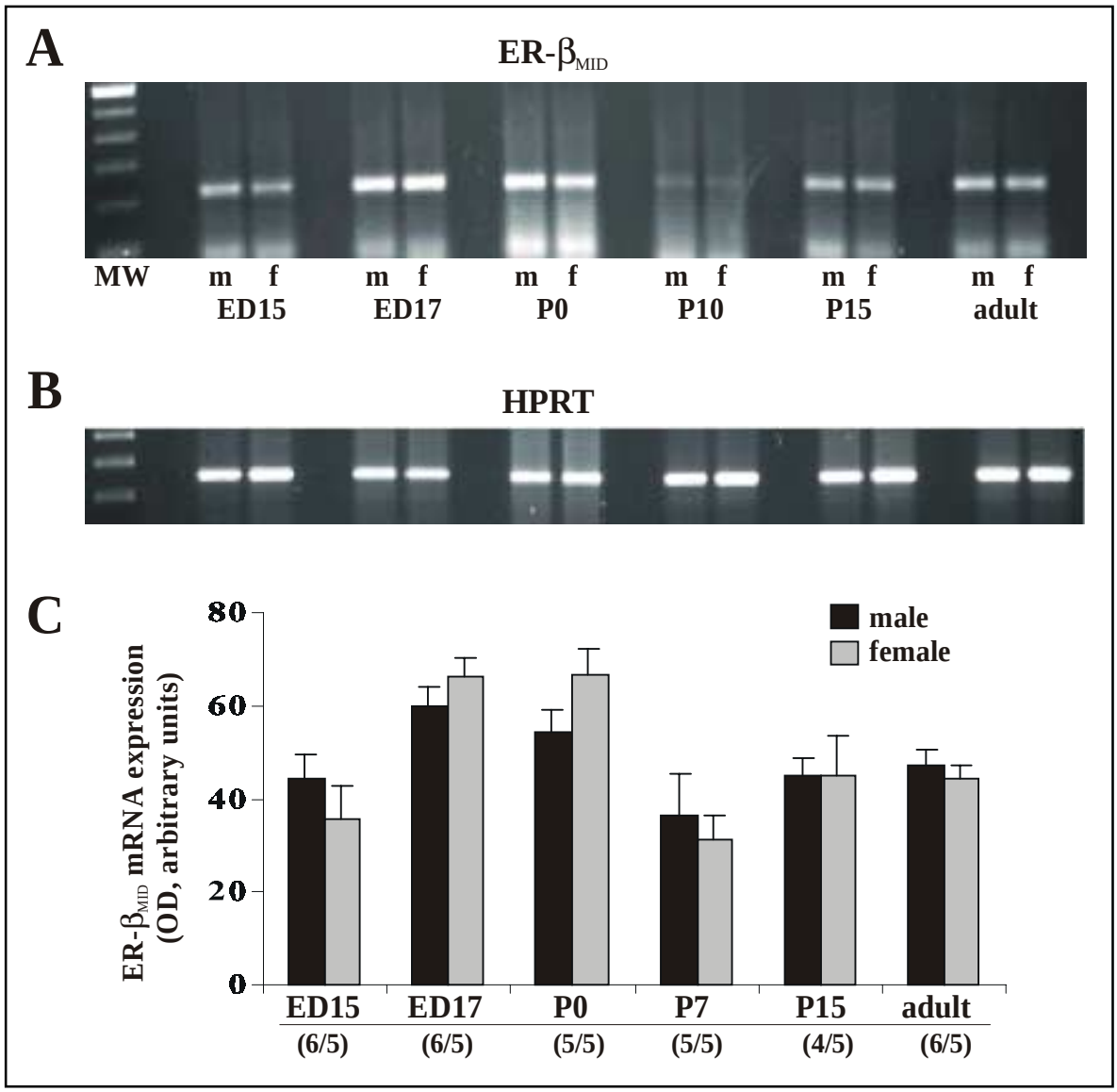

Figure 10. Developmental profile of estrogen receptor (ER)-β_{MID} mRNA expression in the midbrain of male and female mice. **(A)** Representative agarose gels of ER-β_{MID} RT-PCR products from male (m) and female (f) mice at different embryonic (ED) and postnatal (P) stages. **(B)** Corresponding HPRT mRNA levels**. (C)** Semi-quantitative evaluation of RT-PCR experiments. The numbers of analyzed individuals are given in brackets.

3.7. Estrogen effect on CREB binding to CRE

Estrogen effects on CREB phosphorylation/CRE-dependent transcription were analyzed by the electrophoretic mobility shift assay (EMSA). Primary midbrain cultures at DIV6 were treated with 17β-E (10^{-8} M) or E-BSA (10^{-9} M) for 30 min. Positive control cultures were treated with forskolin (adenylate cyclase activator, 10 μM) plus IBMX (inhibitor of intracellular phosphodiesterase, 200 μM). Short-term exposure of midbrain cultures to 17β-E or E-BSA raised the levels of CREB binding to CRE by 34% and 49%, respectively, compared to untreated controls. Stimulation with forskolin plus IBMX yielded a similar increase (44%) in CREB/CRE binding (Figure 11A and 11C). Super-shift and binding competition assays further proved the specificity of CREB binding. The super-shift experiments clearly demonstrated the specificity of CREB binding, since only the complex formed by CREB/CRE and the antibody directed against CREB and no other members of the CREB family resulted in a supershifted band with a slower mobility (Figure 11B, line a). The competition reaction showed that CREB binding (Figure 11B, line b) was not affected by the addition of an unlabeled, non-specific competitor AP2 consensus oligo (Figure 11B, line e). In contrast, an unlabeled CRE consensus oligo (specific competitor) reduced CRE labeled consensus oligo binding to CREB in a dose-dependent way (1.75 or 3.5 pmol) (Figure 11B, lines c and d).

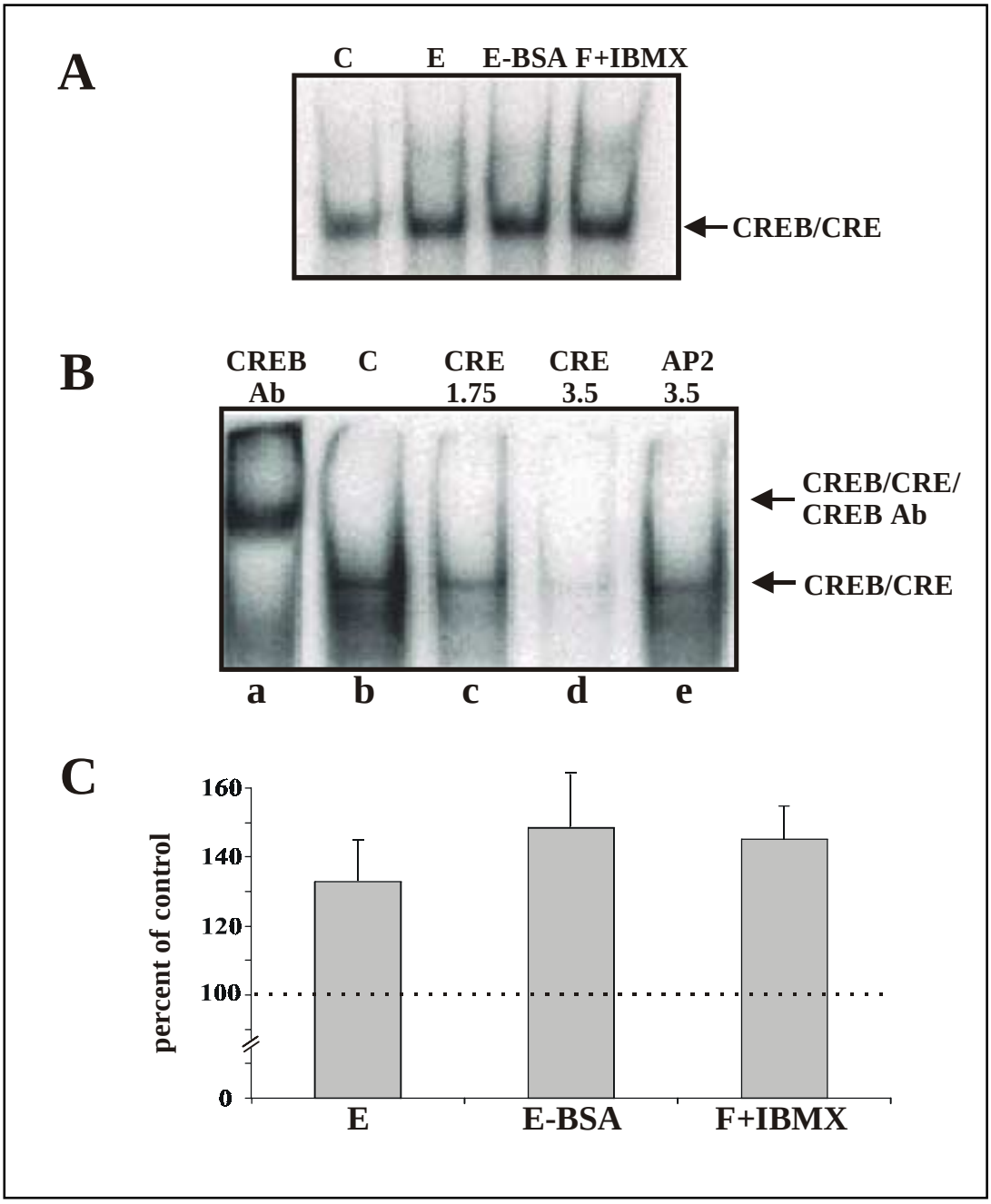

Figure 11. Effects of short-term treatment with 17β-estradiol (E) and E-BSA on CREB/DNA binding in cultured midbrain neurons**. (A)** Representative autoradiogram showing CREB/CRE binding after estrogen treatment. **(B)** Representative autoradiogram of supershift analysis (line a); dose dependent (1.75 and 3.5 mole) specific competition with non-phosphorylated CRE (lines c and d, respectively) and unspecific competition with non-phosphorylated AP-2 (line e). **(C)** Quantitative evaluation of E, E-BSA, and forskolin effect (F; F/IBMX treatment was used as a positive control). The dotted line in C shows levels from untreated controls.

3.8. Estrogen effects on MAPK signaling in midbrain cultures

3.8.1. Cell specificity and time-dependency of estrogen action

The effect of estrogen exposure on MAPK signaling in developing midbrain neurons and glia cells was analyzed by Western blotting using antibodies directed against the activated phosphorylated p44/42 MAPK proteins (ERK1 and ERK 2 respectively). Glial and neuronal cultures were studied separately to analyze the cell-specificity of estrogen effects. We first characterized different cell cultures with respect to their cellular composition. RT-PCR analysis revealed the absence of transcripts for the neuronal marker MAP-2 but strong signals for GFAP in glial cultures (Fig. 12). In neuronal cultures, MAP-2 mRNA levels significantly predominated over GFAP levels.

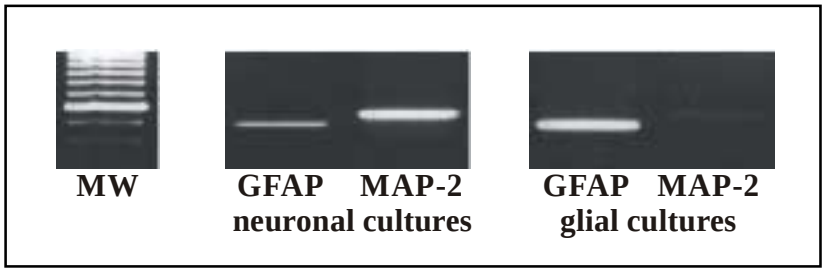

Figure 12. Representative agarose gels of RT-PCR analysis for GFAP and MAP-2 mRNA expression in neuronal and glial cultures.

In total cell lysates of control neuronal and glial cultures, only low levels of the phosphorylated isoforms p42 and p44 of MAPK were detected by Western blotting (Figures 14A and 15A). After E treatment (10^{-8}M), with time intervals ranging from 7.5 to 60 min, a rapid and transient increase of p44/42 MAPK phosphorylation was observed in midbrain glial cultures (Figure 13). Precisely, estrogen-mediated p44/42 MAPK induction occurred at a maximum after 7.5 min and decreased to control values after 15 min (Figure 13 A/B). In contrast, treatment of neuronal cultures with E for the same time intervals did not influence phosphorylation levels of MAPK (data not shown). To further characterize the signaling events that might participate in the observed estrogen effects, we have treated midbrain cultures with E or E-BSA together with either ICI 182,780 or BAPTA, to inhibit the respective intracellular signaling pathways. The application of E-BSA alone fully mimicked the effects of estrogen and significantly ($p \leq 0.01$) increased

the levels of phosphorylated p44/42 MAPK compared to controls. Again, this effect was seen in glial but not neuronal cultures (Figures 14A/B and 15A/B). Treatment with the estrogen receptor antagonist ICI 182,780 did not prevent the estrogen-dependent phosphorylation. In contrast, treatment with BAPTA (calcium chelator) to interrupt intracellular Ca^{2+} signaling abolished the observed estrogen effects on p44/42 MAPK phosphorylation in glial cultures ($p \leq 0.05$; Figures 15A/B). In addition, administration of BDNF in neuronal (Figure 14A) and FGF (Figure 15A) in glial cultures was performed to obtain a high degree of p44/42 MAPK phosphorylation (positive controls). Under these conditions, a 7-8 fold and 3-4 fold increase in p44/42 MAPK phosphorylation was measured in glial and neuronal cultures, respectively (Figures 14B and 15B).

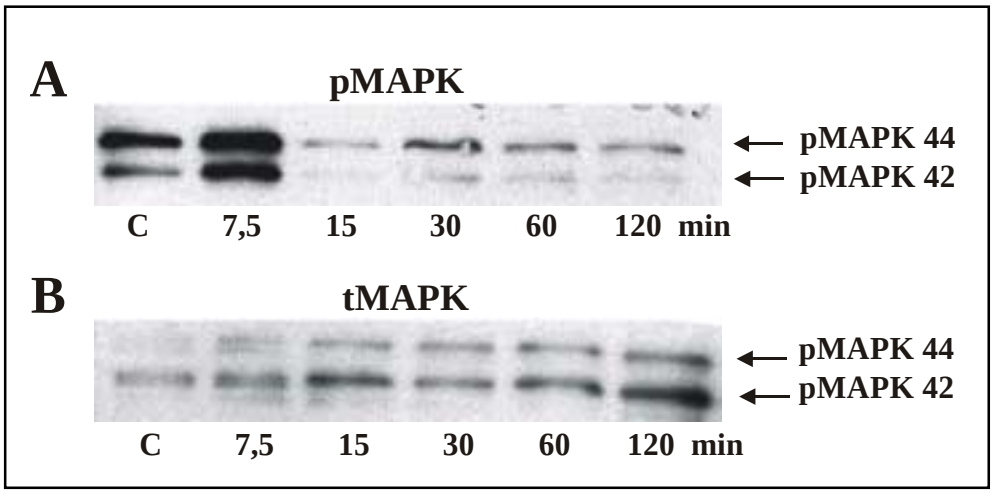

Figure 13. Time dependency, of p44/42 MAPK phosphorylation after estrogen treatment in glial cultures analyzed by Western blotting. **(A)** Representative blot for the phosphorylated form of p44/42 MAPK (pMAPK). **(B)** Corresponding levels of total p44/42 MAPK (tMAPK). C, control

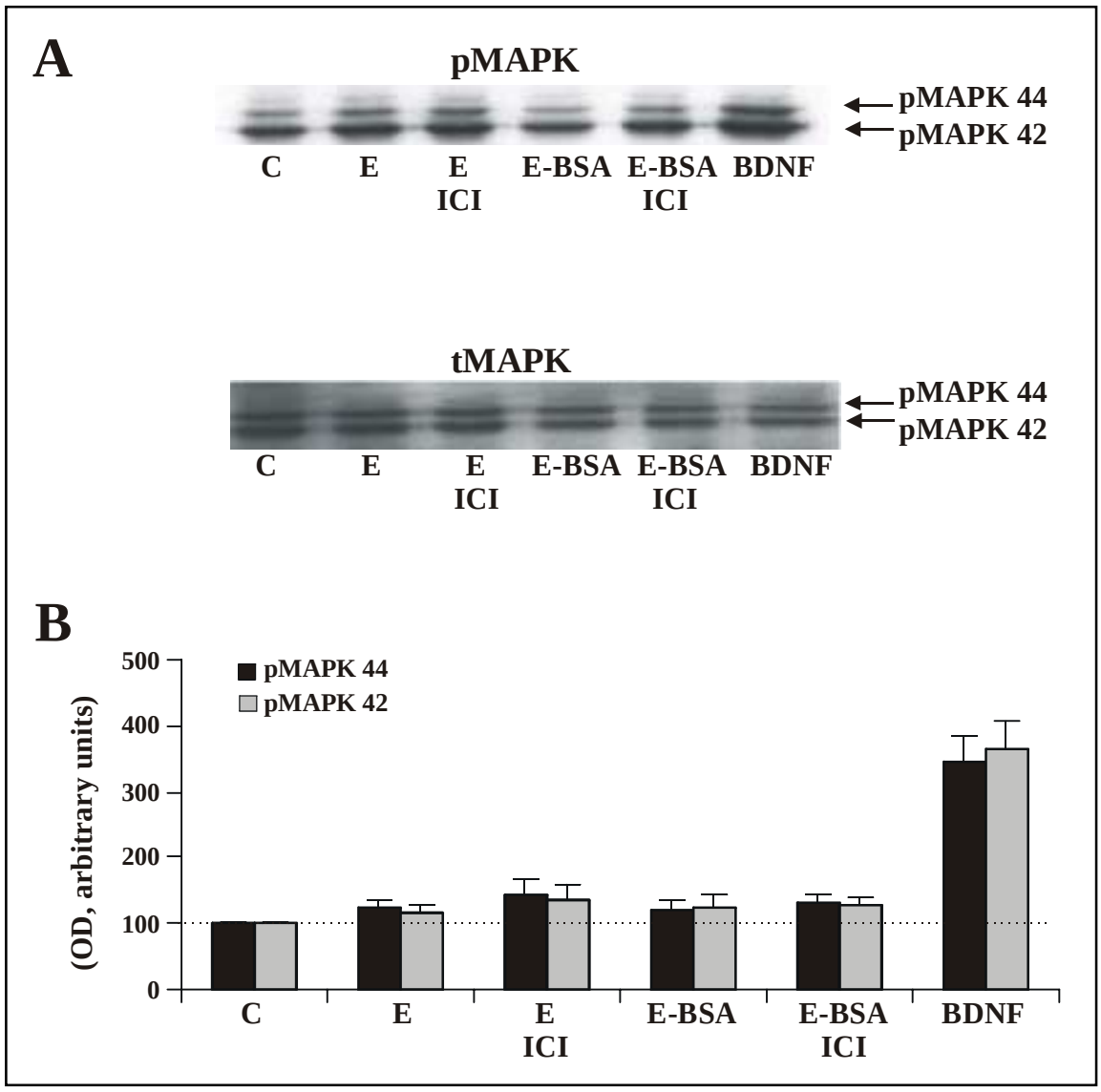

Figure 14. The influence of estradiol on p44/42 MAPK phosphorylation in midbrain neuronal cultures**. (A)** Representative Western blot for the phosphorylated forms (pMAPK) and the corresponding total amount of p44/42 MAPK (tMAPK). **(B)** Quantitative evaluation of estrogen effects.

C, control; E, 17β-estradiol, E-BSA, membrane impermeable form of E; ICI, ICI 182,780, inhibitor of estrogen receptors; BDNF, brain-derived neuronal factor (treatment used as positive control). The dotted line in (B) shows control levels.

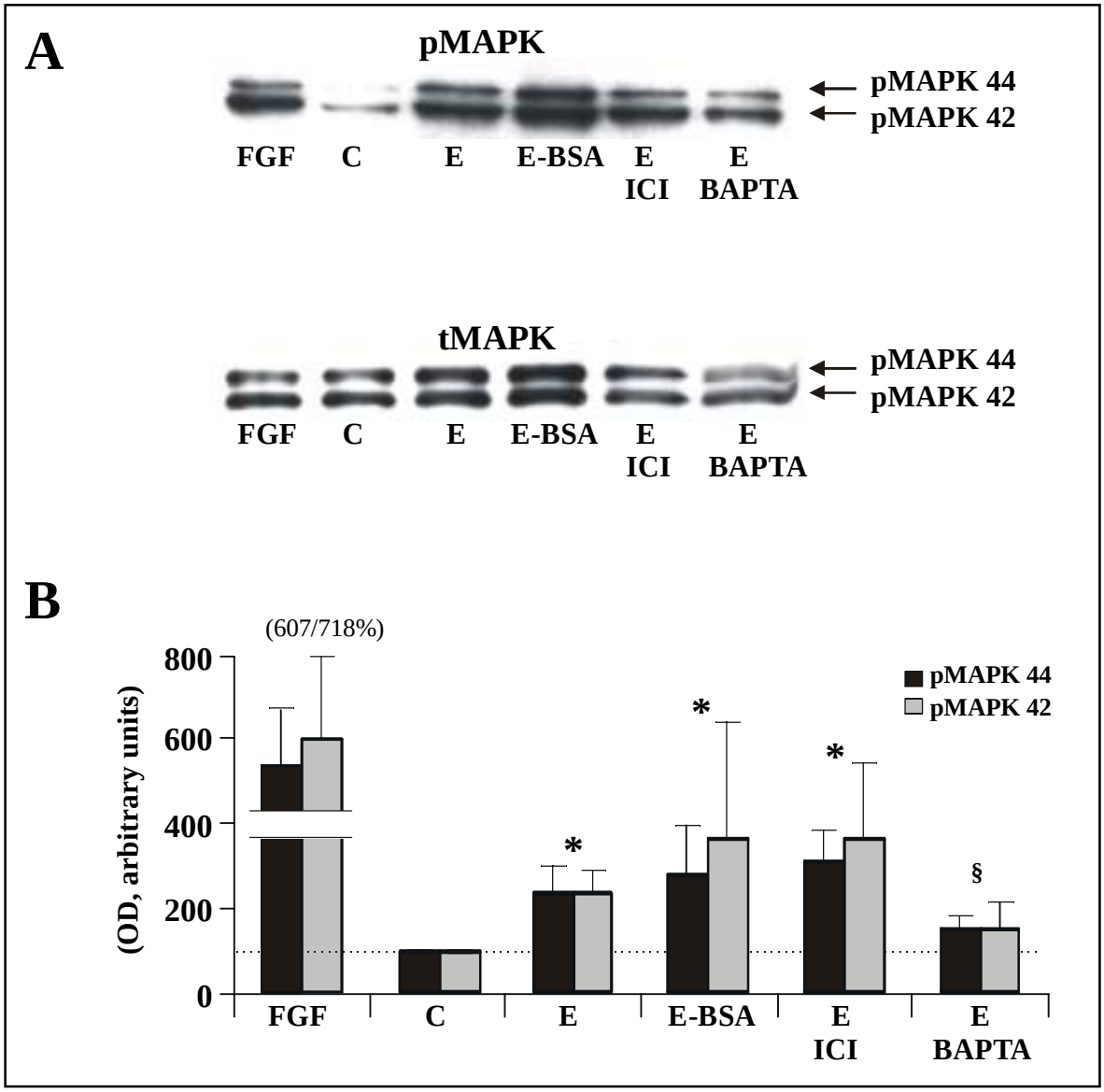

Figure 15. Influence of estradiol on p44/42 MAPK phosphorylation in midbrain glial cultures**. (A)** Representative Western blot for the phosphorylated forms (pMAPK) and the corresponding total amount of p44/42 MAPK (tMAPK). **(B)** Quantitative evaluation of estrogen effects.

C, control; E, 17β-estradiol; E-BSA, membrane impermeable form of E; ICI, ICI 182,780, inhibitor of estrogen receptors; FGF, fibroblast growth factor used as positive control. The dotted line in (B) shows control levels.

*$p < 0.01$ treatment vs. control

§$p < 0.05$ BAPTA treatment vs. E treatment

3.9. The effect of estrogen treatment on gene expression in midbrain neuronal cultures

Differential display RT-PCR (ddRT-PCR) analysis was used to investigate the influence of estrogen on gene expression in midbrain cultures. Reverse transcription with anchoring primers directed against the polyA-tail of mRNA allowed to divide the total mRNA into three subpopulations. Amplification of obtained cDNAs with anchoring and arbitrary primers under unspecific PCR conditions resulted in a number (approximately 50) of different products per each RT-PCR reaction. In total, approximately 3600 RT-PCR products from 72 RT-PCR reactions were obtained with sizes ranging from 100 to 600 bp. Separation of these products in denaturing polyacrylamide gel and autoradiographic detection allowed to discriminate differences in mRNA expression levels in midbrain cultures between controls and estrogen-treated cultures (Figure 16). As shown in Figure 17, treatment with estrogen can abolish, induce as well as up- or down-regulate gene expression. After estrogen treatment, in total, the levels of 48 transcripts were found to be different as compared to control. Twenty five of them were obtained by RTs prepared with $HT_{11}C$, twelve with $HT_{11}A$, and eleven with $HT_{11}G$ anchoring primers. These products were then isolated from polyacrylamide gels and sequenced. By comparing the homologies of these transcripts with sequences available from GenBank, we were able to identify 22 out of 48 RT-PCR products. The other 26 products revealed homologies of less than 30% or did not show any homology to known genes and were therefore not further detailed. The list of the analyzed genes is given in Table 4. From the 22 identified transcripts, E and E-BSA treatment abolished the expression of four (Figure 17A) and switched on the expression of one gene (Figure 17B). Additionally, induction of transcription of three genes (Figure 17C) was observed after E treatment only, and expression of one transcript was abolished after E-BSA treatment only (data not shown). A specific upregulation of another 5 genes by E and three genes by E-BSA, and two by both E and E-BSA treatment was seen (data not shown). Downregulation of expression occurred with three genes after E treatment only (data not shown).

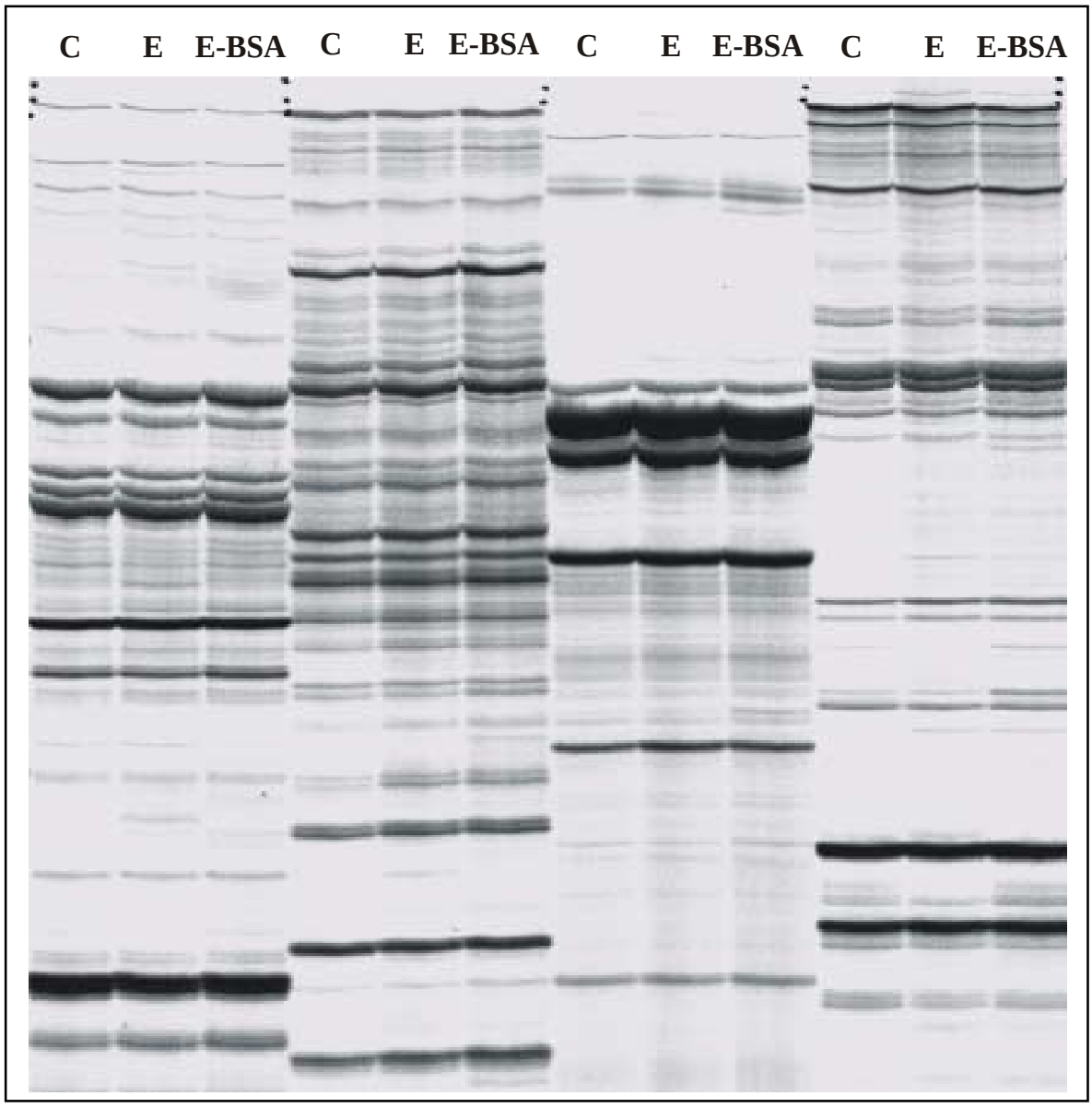

Figure 16. A representative autoradiogram of ddPCR analysis of neuronal midbrain cultures stimulated with E or E-BSA treatment.
E, (17-β estradiol); E-BSA, membrane impermeable construct of E; C, control.

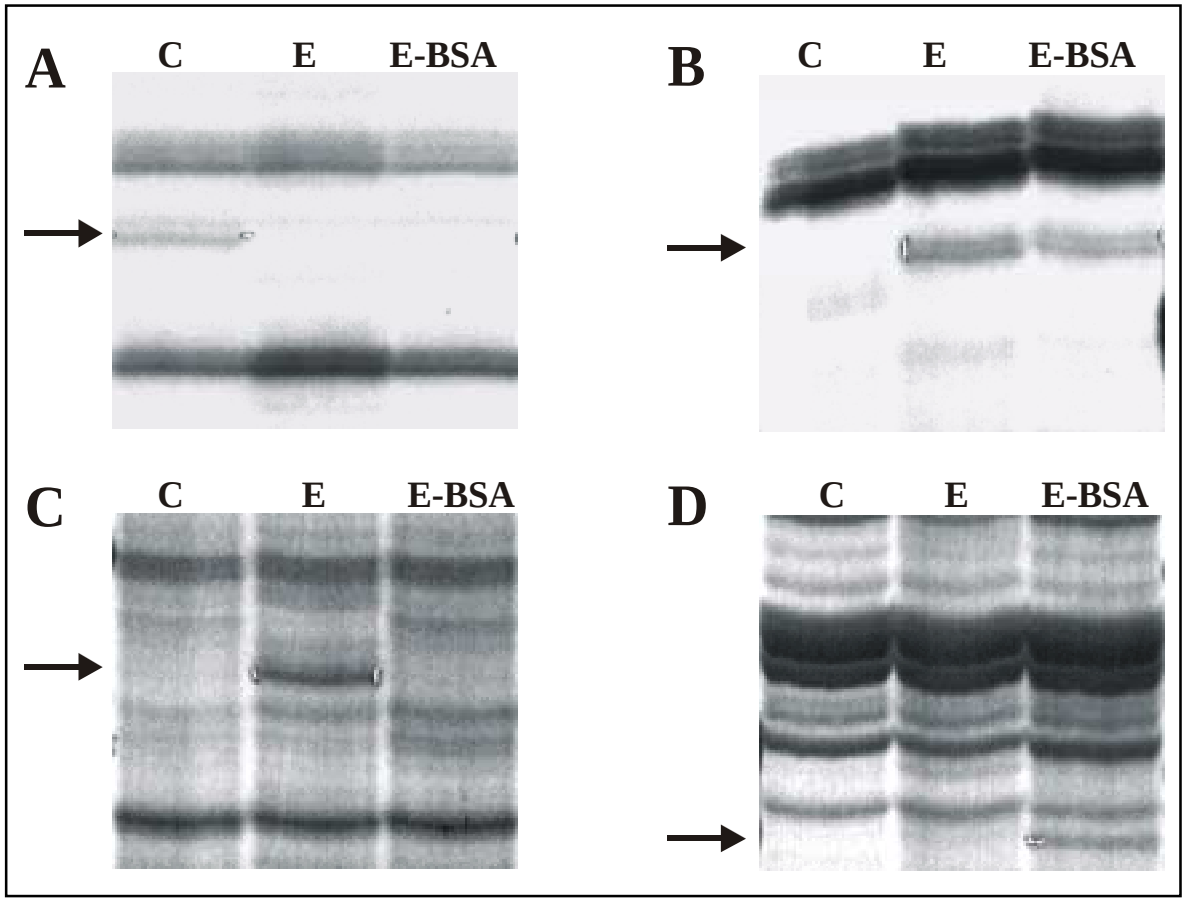

Figure 17. Effect of estrogen treatment on gene expression in neuronal midbrain cultures. Representative autoradiograms demonstrating that E and E-BSA treatment can abolish **(A)** or induce **(B)** mRNA expression of distinct genes. **(C)** and **(D)** examples of transcripts whose expression is induced by E or E-BSA treatment, respectively.
E, 17-β estradiol; E-BSA, membrane impermeable construct of E; C, control.

Table 4. List of genes which have been identified to be differentially regulated by estrogen.

No.	**Name of gene**	**Regulation**	**Seq similarity**
1	JNKa	E (+)	30%
2	ryanodine receptor type 2 (RyR2)	E (+)	30%
3	serine/threonine kinase	E (+)	30%
4	cyclin D3	E (↓)	30%
5	growth hormone gene and promoter	E (↓)	30%
6	mammary-derived growth inhibitor (MDGI)	E (↓)	30%

Table 4 continued….

7	regucalcin	E (↑)	30%
8	insulin receptor substrate-2 (IRS2)	E (↑)	78%
9	fibroblast growth factor-4 (FGF-4)	E (↑)	30%
10	presenilin-1 gene, alternatively spliced transcripts	E (↑)	30%
11	hexokinase II	E (↑)	30%
12	putative transmembrane GTPase	E, E-BSA (-)	70%
13	translation initiation factor IF2	E, E-BSA (-)	80%
14	FUN12 protein	E-BSA, E (-)	80%
15	Mus musculus castaneus haplotype cas-S2 mitochondrial D-loop	E-BSA, E (-)	80%
16	heat-shock protein (dnaJ)	E, E-BSA (+)	40%
17	potassium channel (KCNQ2)	E, E-BSA (↑)	30%
18	LIM-kinase1 (Limk1)	E, E-BSA (↑)	30%
19	mitochondrial genes coding for three transfer RNAs (specific for Phe, Val and Leu), 12S ribosomal RNA, and 16S ribosomal RNA	E-BSA (-)	80%
20	IGIF precursor polypeptide	E-BSA (↑)	80%
21	glycine receptor, alpha 1 (startle disease/hyperekplexia, stiff man syndrome) (GLRA1)	E-BSA (↑)	30%
22	tyrosine kinase receptor	E-BSA (↑)	70%

Transcripts induced (+), abolished (-), up-regulated (↑), down-regulated (↓) after distinct treatment.

4. Discussion

4.1. Methods

4.1.1. Cell culture

It is generally accepted that dissociated neuronal and glial cell cultures are suitable model systems to investigate the differentiation of brain cells. In particular, primary cultures can be used to study the influence of single factors on the differentiation of distinct cell types under defined conditions. Numerous reports have detailed the application of primary cultures to study cell differentiation. Earlier studies from our group have shown that cultured neuronal and glial cells from midbrain and diencephalon differentiate into morphologically and functionally mature cells (Engele *et al.*, 1989; Beyer *et al.*, 1990, 1992). Cultures used for our studies were initiated from ED15 midbrain tissue. At this developmental stage, a clear distinction between the different brain regions is already possible. Furthermore, estrogen is known to influence the brain development at this early time point.

Astroglial cultures were prepared from newborn mice and were kept in a medium containing 10% fetal calf serum. The high serum concentration is necessary to stimulate astroglia proliferation and differentiation. Nevertheless, this treatment results in the elimination of most of the neuronal cells. Glial cells were passaged several times before performing experiments to increase their purity. The cellular composition of neuronal and glial cultures was controlled by RT-PCR for the glial and neuronal markers, GFAP and MAP-2, respectively. As expected, neuronal cultures were composed of a large fraction of glial cells (the measured OD for GFAP was approximately 20% of those for MAP-2). Glial cultures showed no contamination with neuronal cells as revealed by PCR (signals for MAP-2 were not detectable).

4.1.2. RT-PCR

RT-PCR is a well-established molecular method that allows the specific amplification of distinct mRNA fragments from total RNA extracted from tissue or cell culture. In this study, RT-PCR analysis was not only used for qualitative, but also quantitative, analysis of aromatase, ER-α and -β mRNA expression in the hypothalamus and midbrain. The primers were designed using the oligo DNA/RNA primer selection software for each investigated gene. Highly specific reaction conditions (annealing temperature and salt concentration) allowed to obtain distinct PCR products of the size corresponding to the molecular size

calculated from the known gene sequences (see table 1). The efficiency of each RT-PCR reaction was controlled by including positive and negative controls. RTs from mouse ovary expressing aromatase and ER-α/β mRNAs were used as positive controls. PCR mix without RT was applied as a negative control. In RNA preparations, contaminating DNA may be present. In order to exclude samples containing DNA contamination, PCR for the house keeping gene HPRT was carried out for each sample. The forward and reverse primers for HPRT were localized in two different exons. Amplification with these primers can either result in a 273 bp product from mRNA or a 1100 bp product from DNA. In each case, only those samples with the 273 bp product were used for further studies. The RT-PCR for HPRT was also used for the relative quantification of the studied mRNAs (Mullis *et al.,* 1994). In order to perform relative comparisons with low interassay variations between samples at different developmental stages and from different sexes, RNA extraction, RT and PCR reactions were carried out under constant conditions at each step of the experiment. To verify the specificity of RT-PCR products, Southern blotting or sequencing analysis were performed. For Southern blot analysis, we used DIG-labeled oligonucleotides located within the amplified sequences. Stringent hybridization and washing conditions resulted in specific signals for both, aromatase and ER-β_{HYP} RT-PCR products. The sequencing analysis allowed not only to characterize the nucleotide sequence of the RT-PCR products for ER-α and -β in the midbrain, but also to determine the nucleotide sequences of differentially expressed genes in the ddPCR analysis.

4.1.3. Differential display PCR (ddPCR)

The ddPCR procedure was developed by Liang and Pardee (1992) and is a suitable method to identify and characterize differentially expressed genes. This method is based on the reverse transcription of mRNA and followed by PCR using anchoring 3'oligo-dT primers and arbitrary 13-nucleotides 5'primers. The RT-PCR reaction, using these primers at low annealing temperature, results in a number of PCR products which differ in size. In this study, a commercial ddPCR kit with one-base anchored primers was used. These primers have a significant advantage over two-base anchored primers, eliminating the high background smear and reducing the number of RT reactions from 12 to 3. Some of the PCR products, obtained after purification from the sequencing gel and reamplification may contain a significant proportion of co-migrating contaminating sequences which have a similar size but are different in their nucleotide composition and, thereby cause false-

positive results. In order to avoid this problem, reamplified products were run in agarose gels with H.A.-Yellow (polyethylene glycol (PEG) 6000 covalently coupled to bisbenzamide). This dye causes a retardation of the DNA migration in relation to the A+T content and allows to distinguish between sequences with the same size but different nucleotide composition (Wawer, 1995).

4.1.4. EMSA

The electrophoretic gel shift assay provides a simple and rapid method for the analysis of DNA-protein interactions (Revzin, 1989). This method is widely used to study sequence specific DNA-binding proteins such as transcription factors and is based on the observation that complexes of protein and DNA migrate through a non-denaturing polyacrylamide gel at slower rates compared to protein-free DNA fragments or double-stranded oligonucleotides. The gel shift experiment should reveal just one single DNA-protein complex, however, the appearance of a single retarded band does not necessarily mean that a specific complex is formed. To test the specificity of retarded complexes, competition reactions with specific competitors (unlabeled CRE oligonucleotides) and non-specific competitors (unlabeled AP-2 oligonucleotides) were performed. Since the binding reaction can result in more than one protein bound to a distinct consensus element, such as other members of a transcription factor family, the specificity of CREB was further proved by the use of antibodies directed specifically against CREB.

4.2. Developmental expression and regulation of aromatase in the hypothalamus

It is widely accepted that circulating androgens are aromatized to estrogen in the developing brain by aromatase and can masculinize brain structures and functions (Arnold and Gorski, 1984; Arnold and Breedlove, 1985). Estrogen formation is required for the sexual differentiation of CNS and may influence the number of neurons and neuronal connections as well as neuroendocrine function of the brain (Toran-Allerand, 1976; Matsumoto and Arai, 1980; Becker, 1990; Williams and Meck, 1991). The hypothalamus is involved in several reproductive behaviors and is the center of hormonal and neural responses of the organism. Therefore, the hypothalmus is one of the most comprehensively studied brain structures in relation to the influence of estrogen on brain differentiation. In the present study, the developmental profile of aromatase mRNA expression in the male and female mouse Hyp/POA was examined focusing on possible perinatal sex differences. Semi-quantitative RT-PCR analysis was used to examine animals from ED15 until P30

(young adults). Furthermore, we investigated the mechanisms that may be involved in controlling the transcription of the aromatase gene during ontogeny. Since androgens have been shown to regulate aromatase mRNA expression in a region-specific manner (Abdelgadir *et al.*, 1994; Wagner and Morrell, 1996; Roselli *et al.*, 1997) in the adult, the influence of testosterone on aromatase expression during development was investigated in tissues from fetuses treated *in utero* with the androgen receptor antagonist cyproterone acetate (Cyp). RT-PCR analysis showed that aromatase expression is sexually different during hypothalamic development with significantly higher mRNA transcript levels at P0 and P15 in males compared to females (see Figure 6). At these stages, aromatase levels were approximately two times higher in males. These data suggest that the observed sex differences in aromatase activity (MacLusky *et al.,* 1985; Lephart *et al.,* 1992; Beyer *et al.,* 1993) are the result of a higher expression rate of the aromatase gene in males. Additionally, higher levels of aromatase activity in males around birth accompanied by higher numbers of cells expressing aromatase protein but not the result of different kinetic properties of this enzyme between sexes (Beyer *et al*., 1993, 1994). In the past, several studies attempted to correlate the developmental profile of diencephalic aromatase activity with aromatase mRNA expression. Using RNA blot hybridization or fluorometric PCR techniques, these reports demonstrated highest aromatase mRNA expression around birth in the rat (Lephart *et al.*, 1992) and in the mouse (Harada and Yamada, 1992). However, these studies were unable to detect any sex differences at the expression level. In contrast, semiquantitative in situ hybridization showed significant differences of aromatase mRNA transcript levels between sexes in the Hyp/POA during the first postnatal week (Lauber *et al.*, 1997). The data presented here support the latter findings and previous studies demonstrating sex differences in aromatase activity in the newborn mouse hypothalamus (Selmanoff *et al*., 1977). We then focused on the role of androgens in regulating aromatase expression. Our studies showed that *in utero* treatment of embryos with the antiandrogen Cyp results in a significant decrease in aromatase expression in the male and female Hyp/POA. These data imply that aromatase expression, in general, and sex differences during development, are controlled by circulating androgens. The regulation of the activity and expression of aromatase was investigated by several groups. In the adult hypothalamus, testosterone increased aromatase activity in an androgen receptor-dependent manner (Roselli *et al.,* 1984a). Using *in situ* hybridization, Wagner and Morrell (1996) showed that castration decreases the level of aromatase mRNA in Hyp/POA. It has also been demonstrated that the perinatal secretion of gonadal steroids enhances aromatase

activity in the adult brain and determines sex differences in estrogen formation and androgen responsiveness of aromatase in adulthood (Roselli *et al.*, 1996; Roselli and Klosterman, 1998). *In vitro* experiments also revealed that testosterone increases aromatase activity in embryonic diencephalic cell cultures by increasing the number of cells expressing aromatase as well as by doubling aromatase activity per neuron (Beyer *et al.*, 1994; Hutchison *et al.*, 1994). In line with a direct androgenic regulation of hypothalamic aromatase expression is the finding that aromatase containing cells co-express androgen receptors (Beyer and Hutchison, 1997). As shown in adults, the data presented here show that androgen regulates the aromatase activity in the developing hypothalamus (see Figure 7). Sex differences in hypothalamic aromatase activity *in vivo* may be the result of higher circulating androgen levels in males (Weisz and Ward, 1980). The fluctuations in androgen production by the testis during development could also explain the developmental pattern of aromatase expression in the mouse Hyp/POA. Highest plasma levels of androgen are usually observed shortly before birth and coincide with high aromatase expression levels in the brain (Weisz and Ward, 1980). The decrease of aromatase expression on P10 is due to decline in testosterone production by the testis after birth in male pups (Weisz and Ward, 1980). In the second postnatal week, significantly higher aromatase mRNA levels on P15 appear to be again the result of higher testosterone production rates in the second/third postnatal week in mice. At this stage, the onset of puberty is accompanied by changes in the cellular composition of the testis which might be responsible for higher androgen production rates in males (Vergouwen *et al.*, 1991).

4.3. Estrogen receptor expression in the brain

4.3.1. Estrogen receptor-β in the developing hypothalamus/preoptic area

Until recently, target cells for estrogen were thought to express only one type of ER, namely ER-α. This nuclear receptor is widely distributed in the adult brain (VMN, BNST, POA, ARN, medial amygdala and hippocampus) and represents a hormone-inducible transcription factor that can regulate the transcription of target genes upon binding to estrogen response elements (ERE) (Klein-Hitpass, 1988*).* Although estrogen synthesis is several times higher in the male Hyp/POA compared to females during development (Beyer *et al.*, 1993), inverse sex differences, i.e. lower levels of ER-α mRNA expression in males compare to female, have been reported (Yokosuka *et al.*, 1997). These findings make it difficult to explain how sex-specific aspects of neuronal development are brought

about by estrogen during perinatal brain development. This discrepancy is further demonstrated by data showing that estrogen produced in the male brain can down-regulate the expression of ER-α mRNA (Yokosuka *et al.*, 1997). The discovery of ER-β may reconcile this conflicting data. The ER-β is also a member of the steroid receptor superfamily and was recently cloned from the rat prostate (Kuiper *et al.*, 1996). Molecular analysis of the ER-β sequence revealed a high homology with the ER-α sequence and the presence of all known distinct functional domains which are typical for the steroid receptor superfamily (Kuiper *et al.*, 1996). Several studies have demonstrated the presence of ER-β in several regions of the adult brain, such as the amygdala, hippocampus, striatum, cerebral cortex, and Hyp/POA by in situ hybridization and RT-PCR (Kuiper *et al.*, 1996; Shurgue *et al.*, 1997). In addition, both ERs appear to be expressed individually in a region-specific manner or are co-expressed in other brain areas (BNST, POA, amygdala, cerebral cortex, and hippocampus) (Shurgue *et al.*, 1997).

In our study, we have analyzed for the first time the developmental expression of ER-β in the Hyp/POA. We also described distinct sex differences in ER-β expression. Our semiquantitative RT-PCR analysis revealed perinatally significantly higher levels of ER-β mRNA transcripts in males compared to females (see Figure 8). The data presented here point to the possibility that estrogen signaling through ER-β might account for sex differences in the Hyp/POA, since the higher amounts of ER-β mRNA transcripts in the male Hyp/POA coincide with higher aromatase mRNA expression and activity levels around birth (see chapter 3.2; Beyer *et al.*, 1993). Although ER-α and -β show similar affinities for their ligands, variations in the expression of these receptors can lead to differences in the structure of distinct parts of the brain. Recent studies have reported that estrogen treatment induces two distinct morphological phenotypes in neuroblastoma cells depending on the type of ER to be activated. Estrogen action via ER-α results in an increase of the length and number of neurites, whereas ER-β activation modulates only neurite elongation (Patrone *et al*., 2000). These results reveal that the influence of estrogen on neuronal differentiation might be determined by the presence of distinct ER subtypes. The high expression levels of ER-β mRNA in the developing Hyp/POA compared to ER-α suggests a major role in transducing estrogen signals via this ER subtype. This does not necessarily exclude an additional role for ER-α signaling. Since ER-α and β are known to form heterodimers (Petterson *et al.*, 1997), we also have to take into consideration that estrogen effects might be transmitted via such heterodimers. The use of specific inhibitors

or ligands for either ER-α or ER-β might help in the future to characterize the precise contribution of each of the receptor subtypes during brain development.

4.3.2. Estrogen receptor-α and -β in the developing midbrain

Although not directly involved in reproduction, the midbrain is also one of the brain regions that is influenced by estrogen during development as well as in adulthood. Estrogen has been shown to stimulate neurite outgrowth and tyrosine hydroxylase mRNA expression in developing midbrain dopaminergic neurons (Reisert *et al.,* 1987; Raab *et al.,* 1995a). Apart from affecting dopaminergic neuron differentiation, estrogen is also capable of inducing the dopaminergic phenotype in undifferentiated human neuroblastoma cells (Agrati *et al.*, 1997). In the adult nigrostriatal dopaminergic system, estrogen influences the expression of dopamine transporters (Bosse *et al.*, 1997) and the presynaptic dopamine release at the striatal level (Becker, 1990). In the adult midbrain, ER-α mRNA and protein, as well as ER-β mRNA were detected within the substantia nigra and ventral tegmental area and were particularly localized in dopaminergic neurons (Shughrue *et al*., 1997; Kritzer, 1997). Our study describes the developmental expression of the ER-α and -β in the mouse ventral midbrain. Transcripts of both receptors are present in midbrain from ED15 until adulthood in both sexes. In contrast to the Hyp/POA no significant sex differences in mRNA expression of both receptors were observed. However, there are clear-cut developmental differences in the expression pattern of ER-α and -β mRNA. Whereas ER-β mRNA expression dominates pre- and early postnatally, ER-α expression observed as early as on ED15, increases later during development and reaches maximum levels in adults (see Figures 9 and 10). Expression of ER-α as shown in Figure 9 was not consistent within distinct experimental groups. The ER-α expression was not detected or was below detection levels in some individuals at E15, P0, P7, and P15. At present, it is difficult to explain this heterogeneity. One possibility could be that differential splicing of ER-α mRNA which we have found in the developing midbrain (Raab *et al.,* 1999) accounts for these individual differences.

The presence of nuclear ERs in the developing midbrain clearly indicates that estrogen is involved in the regulation of differentiation and for function of midbrain neurons through a classical genomic mechanism (Reisert *et al.,* 1987; Raab *et al.,* 1995a). This is also supported by the transient expression of the estrogen-forming enzyme aromatase in the rodent midbrain around birth and during the first two postnatal weeks (Raab *et al.,* 1995b).

Interestingly, no sex differences were detectable during ontogenesis in ER-α and -β expression levels. At first glance, this appears to be contradictory to the presence of sex differences with respect to midbrain dopaminergic neurons (Beyer *et al.,* 1991). However, already in previous studies, we have demonstrated that sex differences within the nigrostriatal dopaminergic system develop along a cell-intrisic program rather than being induced by estrogen (Reisert and Pilgrim, 1991).

4.4. The influence of estrogen on developing midbrain dopaminergic neurons by non-classical signaling

The expression of ER-α and -β in the developing midbrain clearly suggests that estrogen can influence the phenotype and physiology of developing dopaminergic neurons. Besides its genomic action, there is growing evidence that estrogen can also interact with putative membrane receptors and thereby influence the physiology of nerve cells within seconds to minutes (Pietras and Szego, 1977; Schumacher, 1990; Pappas *et al.,* 1995; Ramirez *et al.,* 1996). This mechanism is termed "non-classical" estrogen action and known to affect the neuronal physiology (Wong and Moss, 1991; Thompson and Moss, 1994; Watson *et al.,* 1999) and morphology (Garcia-Segura *et al.,* 1989b; Reisert *et al*., 1987; Gould *et al*., 1990). Estrogen, by binding to its membrane receptor (Beyer and Raab, 1998; Razandi, 1999), might stimulate intracellular signaling cascades such as PKA and PKC (Kelly *et al.,* 1999), MAPKs (Singh *et al.,* 1999), and IP_3/calcium release (Beyer and Raab, 1998). Additionally activation of those signaling pathways then might lead to a subsequent stimulation of nuclear transcription factors such as CREB, c-fos, and c-jun (Herdegen and Leah, 1998). Previous studies from our laboratory showed that estrogen treatment of midbrain neuronal cultures induced a rapid release of calcium from intracellular stores in dopaminergic neurons. These effects were completely mimicked by a membrane-impermeable construct of estrogen, E-BSA, and were not inhibited by the antiestrogen tamoxifen (Beyer and Raab, 1998). Moreover, 17β-estrogen and E-BSA increased the neurite length and branching of midbrain dopaminergic neurons. Again, these effects were not abolished by the simultaneous treatment with an ER inhibitor, ICI 182,780, but were inhibited by treating the cultures with the adenylate cyclase inhibitor SQ, the PKA inhibitor H89, or the calcium chelator BAPTA (Beyer and Karolczak, 2000). In this study, we have investigated non-classical estrogen effects on midbrain neurons and glia with

respect to the activation of the nuclear transcription factor CREB as well as MAPK activation and the subsequent events on the molecular level.

4.4.1. The influence of estrogen on CREB binding in midbrain neurons

One of the best characterized transcription factors which can be stimulated by exogenous signals is CREB. CREB activates the transcription of different target genes in response to a wide array of heterogenous stimuli. This includes the activation of protein kinases such as PKA, MAPKs, and CaMKs. All these kinases phosphorylate CREB at a particular residue, Ser^{133}, that is required for CREB-dependent transcription (Montminy, 1990). Phosphorylated Ser^{133} is also needed for CREB binding protein (CBP) that serves as a molecular bridge for transcription factors and polymerase II/transcription factor complexes (Kwok *et al.,* 1994; Arias *et al.*, 1994). The phosphorylation of Ser^{133} is not critical for CREB dimerization but seems to be essential for CREB binding to CRE (Yamamoto *et al.,* 1988; Alberts *et al.*, 1994; Bullock and Habener, 1998). It has been shown that calcium signaling is involved in the phosphorylation of CREB via the activation of CaMK IV and certain CaMK II isoforms which are present in the nucleus (Dash *et al.*, 1991; Heist and Schulman, 1998). Apart from the activation of CaMKs, changes in intracellular Ca^{2+} also influence other second messenger systems such as cAMP levels by the activation of calmodulin-sensitive adenylate cyclases (Mons *et al.*, 1998). The activation of adenylate cyclases results in an increase of cAMP levels, followed by an activation of PKA and the subsequent phosphorylation of CREB (Murphy and Segal, 1997). The present study was performed to investigate whether short-term estrogen treatment is able to increase CREB binding to CRE in midbrain neuronal cultures. We could show that short-term treatment with 17β-estradiol as well as E-BSA increased the amount of CREB binding to CRE. We further proved that this effect is limited to CREB and not to other members of the CREB family such as ATF-1, CREM by performing supershifts with an antibody directed specifically against CREB (see Figure 11). These data are supported by the finding that the number of TH/pCREB (phosphorylated form of CREB) cells is significantly increased after estrogen treatment (Beyer and Karolczak, 2000). These effects were abolished by the co-application of estrogen together with SQ, H89, or BAPTA but not by ICI 182,780. A calcium release from intracellular stores (Beyer and Raab, 1998) seems to be important for the observed CREB phosphorylation in midbrain dopaminergic neurons, since the number of double-labeled TH/pCREB cells was reduced to control levels after co-treatment of cultures with estrogen and the calcium chelator BAPTA. The fact that the application of

cAMP/PKA inhibitors also prevented CREB phosphorylation implies a possible cross-talk between the two different signaling pathways. Therefore, we conclude that the increase of intracellular Ca^{2+} levels represents the first step in a series of signaling events necessary for the estrogenic stimulation of CREB phosphorylation. It is, moreover, suggested that cAMP/PKA signaling is intertwined with calcium signaling through calmodulin-dependent adenylate cyclases (Dash *et al.,* 1991; Cooper *et al.,* 1995). Our study further indicates that estrogen promotes the development of dopaminergic neurons via a "non-classical" signaling mechanism including calcium signaling and CREB activation.

There is a large body of evidence that neurotrophins are important factors for the development of dopaminergic neurons (Hyman *et al.,* 1994; Horger *et al.,* 1998; Strudel *et al.,* 1995; Burvenich *et al.,* 1998). BDNF is one of the neurotrophins that is involved in the regulation of developmental processes of dopaminergic neurons. For instance, BDNF is involved in the survival and neuronal outgrowth of dopaminergic neurons (Engele and Bayatti, 1999; Ostergaard *et al.,* 1996). It has been shown that two CRE consensus sequences are present in the BDNF promoter (Shieh *et al.,* 1998). One of them, a "novel calcium response element", is required for the calcium-dependent regulation of BDNF expression in both adult and embryonic cortical neurons. The second element matches the consensus CRE and is required for transactivation of BDNF in postnatal but not embryonic neurons. Putative EREs have also been detected in the BDNF promoter region (Sohrabji *et al.,* 1995). The presence of CRE and ERE sequences implies that estrogen may regulate BDNF expression either by direct action through nuclear ERs or by a mechanism which requires membrane ERs and calcium/CREB signaling. In the hippocampus, it has already been shown that estrogen influences the spine density of dendrites by controlling BDNF transcription (Murphy *et al.,* 1998). Other groups have also demonstrated that estrogen is involved in the regulation of BDNF expression in different brain regions such as the hippocampus and cortex (Singh *et al.,* 1995; Sohrabji *et al.,* 1995; Miranda *et al.,* 1994; Gibbs, 1999). Together with unpublished data from our laboratory which show that estrogen increases BDNF expression in developing midbrain neurons, these findings support the idea that estrogen controls the spatial and temporal expression of BDNF in the different brain regions. Further studies using "antisense" technique are necessary to show that the estrogen/cAMP/pCREB signal cascade is involved in generating estrogen effects in midbrain neurons.

4.4.2. Estrogen-dependent stimulation of MAPK in neuronal and glial midbrain cultures

The MAPKs regulate a diverse array of functions, such as growth and proliferation, differentiation, and apoptosis of cells (Pages *et al.*, 1993; Schaefer and Weber, 1999; Meyer-Franke *et al.*, 1998; Anderson and Tolkovsky, 1999). MAPK activation is well characterized in the context of growth factor signaling through tyrosine kinase receptors (van der Geer *et al.*, 1994). Several studies have shown that estrogen can also activate MAPK in different tissues. In particular, estrogen was found to activate MAPK in bone cells (Endoh *et al.*, 1997), cardiomyocytes (Nuedling *et al.*, 1999), and smooth muscle cells in the uterus (Ruzycky, 1996). In the CNS, rapid estrogen-mediated activation of MAPKs has been reported for hippocampal neurons and in cortical organotypic explants (Singh *et al.*, 1999; Singer *et al.*, 1999) as well as in neuroblastoma cells (Watters *et al.*, 1997). The activation of MAPK in cortical explants by estrogen is induced via the activation of B-Raf and was not inhibited by ICI 182,780. Singh and colleagues (1999) suggested that the latter activation of MAPK may be a consequence of a multimeric complex formation consisting of at least the ER-α, hsp90, and B-Raf, and that phosphorylation of any of the members of this complex can propagate activation of MAPK. At present, it is difficult to explain how estrogen activates B-Raf. Although ICI 182,780 does not block the observed increase of MAPK phosphorylation, it cannot be excluded that estrogen acts via one of the nuclear ER, since the efficacy of ICI 182,780 and other ER antagonists has been characterized principally only on the basis of their ability to prevent the transcriptional activation via ERE. Estrogen also did not activate *trk*, which is an upstream activator of B-Raf. It has been suggested that estrogen can evoke the phosphorylation of some earlier identified ER/signaling proteins, associated within the complex and causes the conformational change or increase of the ER/B-Raf complex. Thus, the formation of this complex provides an alternative model to the “classical” way of ER action.

Estrogen influences calcium levels in dopaminergic midbrain neurons (Beyer and Raab, 1998). It is known that changes in calcium levels might affect neuronal functions via MAPK activation involving Ras signaling (Farnsworth *et al.*, 1995). Thus, it appears feasible that estrogen activates MAPK in midbrain dopaminergic neurons through increased calcium levels. However, this was not the case in the studies presented here. Short-term treatment with 17β-estradiol and E-BSA did not activate MAPK in midbrain neurons (see Figure 14). Instead, an increased phosphorylation of MAPK was observed

only in astrocytes (see Figure 15). Nevertheless, the effect of estrogen on MAPK activation also requires increased calcium levels and is not due to a direct action via nuclear ERs, since BAPTA abolished but ICI 182,780 did not affect this effect. There are only a few studies published so far that show a direct influence of estrogen on astroglial cells (Garcia-Segura *et al.*, 1989b; Toran-Allerand, 1990). At present, it is not known which effects are induced by estrogen-dependent MAPK activation in midbrain astroglia. Estrogen might influence astroglial morphology as it has been shown for diencephalic and cortical astrocytes (Mong and McCarthy, 1998 and 1999; Mong *et al.*, 1999; Abe and Saito, 2000). The latter effects were recently shown to require the activation of the PKC/MAPK pathway (Abe and Saito, 2000). It is known that testosterone influences (via its byproduct estrogen) the density of dendritic spines in the arcuate nucleus. These morphological changes are brought about by astroglial cells that exhibit rapid stellation and reduce the surface of neuronal connections (Mong *et al.*, 1999). Additionally, astrocytes which synthesize and secrete a considerable number of factors important for neuronal survival and differentiation (Vernadakis, 1988), might mediate specific estrogen effects in the developing brain. Since neurons and glia compose closely connected functional system estrogen might influence neuronal survival and differentiation through the regulation of astroglial functions.

4.4.3. The effect of estrogen on gene expression in midbrain neurons

The differentiation of midbrain neurons requires the realization of distinct cell-intrinsic developmental programs as well as the action of epigenetic signals (Reisert and Pilgrim, 1991; Engele and Bayatti, 1999). Short-term action of estrogen on calcium and MAPK signaling might induce the expression of immediate-early genes, such as *c-fos* and *c-jun*, which subsequently regulate waves of gene expression. In contrast to immediate-early genes, the delayed-response genes generally code for proteins that influence the functions and cell physiology directly. It is generally thought that the transcription of the delayed-response genes is regulated, at least in part, by immediate-early genes (Curran and Morgan, 1995). In our study, we have specifically investigated long-term effects of estrogen (4 days treatment) in midbrain neuronal cultures in order to detect the delayed-response genes. To discriminate between "classical" and "non-classical" signaling through nuclear ERs or membrane ER, respectively, we have used 17β-estradiol and E-BSA in our experiments.

By applying ddPCR and sequencing, we could detect a considerable number of genes (see Table 4) that are under estrogenic control in the midbrain. We found that estrogen effects

are not restricted to the classical signaling route via nuclear ERs, since some of the genes showed differences in their transcription levels also after E-BSA treatment. This suggests that estrogen can also affect gene expression through membrane ER.

Some of these genes have previously been shown to play a role during brain development and to be under estrogenic control. The function of the c-Jun NH_2-terminal kinase family (JNKs, also called stress activated protein kinases, SAPKs) in the brain is not well-understood. However, there is evidence that JNKs are potent effectors of apoptosis and neuronal degeneration (Mielke and Herdgen, 2000). It is also known that during early development JNKs are required for brain-region specific apoptosis (Kuan *et al.*, 1999). The induction of JNK expression in the midbrain points to the possibility that estrogen might, apart from its trophic function, be involved in the regulation of apoptotic processes during midbrain development. The insulin receptor substrate (IRS), also up-regulated by estrogen, mediates the signal from receptor-associated tyrosine kinases of insulin or insulin like growth factor (IGF) receptors to downstream effectors such as the phosphatidylinositol-3'-kinases (PI-3-Ks) (Yamada, 1997; Numan and Russell, 1999). Furthermore, estrogen and IGF-I are closely connected co-acting trophic factors which can increase the survival and differentiation of fetal hypothalamic neurons (Garcia-Segura *et al.*, 1996). Fibroblast growth factor-4 (FGF-4) was also up-regulated by estrogen in the midbrain. Members of the FGF family are involved in the regulation of a variety of developmental processes in brain, e.g. proliferation and migration of neuronal progenitor cells, neuronal and glial differentiation, neurite outgrowth and synapse formation (Ozawa *et al.*, 1996). The up-regulation of FGF-4 by estrogen in the midbrain indicates a possible influence of this factor for dopaminergic neurons during development. Indeed, FGF-4 has been shown to be important for the induction of the dopaminergic phenotype in the hindbrain (Ye *et al.*, 1998). Additionally, another member of the FGF family, basic FGF, in co-operation with IGF-I increase the dopaminergic cell survival after the transplantation of embryonic dopaminergic neurons in the striatum (Zawada *et al.* 1998). Studies presented here showed that estrogen treatment increased Ca^{2+}-binding protein regucalcin mRNA levels in the midbrain. Investigations in rat neuronal cells suggested a major role of this protein for the inhibition of protein phosphatases (Yamaguchi *et al.*, 2000). In non-neuronal tissues, estrogen has been shown to have a tissue-specific influence on the expression of regucalcin mRNA (Yamaguchi and Oishi, 1995; Kurota and Yamaguchi, 1996). The observed increase of regucalcin mRNA expression implies that estrogen might also regulate the intensity of intracellular signal via serine/threonine or tyrosine kinases in neuronal tissue.

Not only regulatory proteins but also proteins showing structural properties of kinases were upregulated by estrogen. The Limk-1 is involved in the assembly of actin filaments (Wang *et al.,* 2000). Up-regulation of this protein by estrogen demonstrates another aspect of estrogen effects on midbrain neurons. Estrogen action on midbrain neuronal differentiation might not only be confined to its regulatory role in the expression of factors involved in the morphological changes such as FGF or BDNF, but also estrogen in the expression of factors required during the cytoskeleton rearrangement.

The ddPCR techniques allowed us to identify a considerable number of genes that seem to be under estrogen-regulated transcriptional control. However, at present, the function of many of these genes in the brain/midbrain is not known. Further studies have to address this problem.

4.5. Estrogen and developing dopaminergic system

During the last ten years, several studies from this and other groups have demonstrated that estrogen interferes with developmental processes of dopaminergic neurons at several levels (reviewed by DiPaolo, 1994; Beyer, 1999). Thus, estrogen stimulates the expression of tyrosine hydroxylase (Raab et al., 1995a; Küppers *et al*., in press) and affects the dopamine transporter capacity (Engele, 1989). In addition, the morphological maturation of this cell population is also promoted by estrogen. Treatment with estrogen increases the synaptic efficacy indirectly by stimulating neurite outgrowth and plasticity (Beyer and Karolczak, 2000). These *in vitro* findings are supported by the demonstration that the estrogen-forming enzyme aromatase is strongly expressed in the perinatal midbrain at the time period when dopamine neurons develop (Raab, 1995b). Moreover, both nuclear ERs appear also to be present in the midbrain during this developmental stage (Raab *et al.*, 1999). Despite the presence of nuclear ERs, our most recent findings clearly suggest that estrogen-mediated effects on dopamine neuron differentiation depend not only on classical nuclear estrogen signaling but also on non-classical estrogen action which involves the activation of membrane ERs coupled to the calcium/cAMP/PKA/pCREB signaling cascades (Beyer and Karolczak, 2000). The data presented here, furthermore, demonstrate that the above mentioned estrogen-dependent signaling events influence the expression of different classes of genes in the midbrain. In addition, we found not only dopaminergic neurons but also astroglial cells in the developing midbrain to be direct targets for estrogen. Altogether, these observations suggest that the development of midbrain

dopaminergic neurons relies on a concerted action of cell-intrinsic properties and epigenetic signals, the latter arising from other neurons and surrounded astrocytes. From our data, we hypothesize that the contribution of estrogen to this process is founded in its ability to coordinate the spatial and temporal expression of other developmental signals in the midbrain.

5. Summary

Estrogen synthesized *in situ* in the CNS by the enzyme aromatase plays an important role during brain development. In particular, estradiol stimulates the morphological and functional differentiation of distinct neural systems but is also a critical factor for the determination of sex-specific properties of neural structures and functions. The present study focuses on three different aspects of estrogen action in the developing brain.

The first part is concerned with the developmental expression of the estrogen-synthesizing enzyme aromatase and the nuclear estrogen receptor (ER-β) in the Hyp/POA of male and female mice. Although estrogen is known to influence the sexual differentiation of hypothalamic neurons, i.e. the masculinization occurs in the presence of estrogen whereas low estrogen levels result in "female-like" brain structures, previous studies have failed to clearly demonstrate sex differences in estrogen production and ER expression in the Hyp/POA. The expression analysis was performed by applying a semi-quantitative RT-PCR method. Our studies revealed a stepwise increase in the expression of aromatase and ER-β, showing highest levels around birth and during the first postnatal week. Sex differences in aromatase expression were found perinatally and again at the beginning of the third postnatal week. In both cases, higher expression levels were observed in males. In addition, the ER-β expression pattern also revealed a clear-cut sex difference perinatally with higher levels in males compared to females. These data demonstrate that the onset of sexual differentiation of hypothalamic functions coincides with a sex-specific expression pattern of aromatase and ER-β. We might, therefore, assume that signaling through ER-β is necessary for transmitting developmental estrogen effects, i.e. in a sex-specific way, on hypothalamic cells.

The second part focuses on the developmental regulation of aromatase in the Hyp/POA. At both developmental stages, when aromatase expression peaks in males, high testosterone production rates are described for the testes. Thus, we followed the idea that circulating androgens might regulate the expression of hypothalamic aromatase. In order to study this, we have treated pregnant mice from embryonic day 15 until birth with the androgen receptor antagonist cyproterone acetate (Cyp) by daily subcutaneous injections. On the calculated day of birth, the fetuses were born by caesarian section, and the expression profile of aromatase was analyzed. In both sexes, Cyp treatment massively reduced

aromatase mRNA levels compared to oil controls. These data clearly show that circulating androgens not only are the substrate for the aromatase but also directly stimulate the expression of aromatase in the hypothalamus through an androgen receptor-dependent mechanism.

The third part of this study is devoted to the emerging new field of "non-classical" cellular steroid hormone signaling. There is growing evidence that estrogen (and also other steroids) transmits its cellular effects not only through classical nuclear receptors (ER-α/β), but can, in addition, bind to specific plasma membrane receptors. These receptors are coupled to distinct intracellular signaling cascades. In previous studies, we have shown that estrogen stimulates the release of calcium from intracellular stores upon binding to membrane ERs in the midbrain. Secondary to this effect, we describe here that the cAMP/PKA/pCREB signaling pathway appears to be activated in midbrain neurons, whereas in astroglial cells which also represent targets for estrogen, the MAP-kinase pathway is induced. Our data further suggest that in both cases, the estrogen-evoked primary signal appears to be rapid change of intracellular calcium homeostasis. It is of great interest that rapid non-classical estrogen effects in the midbrain contribute to the differentiation of midbrain dopaminergic neurons. However, our study also shows that classical and non-classical estrogen signaling are both involved in estrogen-mediated developmental effects on dopaminergic cells. This is specifically shown by the gene expression analysis data which revealed different genes to be regulated by classical or non-classical estrogen signaling.

Taken together, our findings demonstrate that estrogen contributes to different processes of neuronal differentiation. This includes sex-specific aspects of cellular development in the hypothalamus/POA which appear to result from sex differences in local estrogen synthesis and signaling through nuclear ER-β. On the other hand, estrogen affects, irrespective of the sex, the morphological and functional differentiation of midbrain dopaminergic neurons. The latter effect requires classical action through nuclear ERs but also non-classical action via not yet characterized membrane receptors. In particular, the newly established steroid signaling mechanisms will become more important in the future for understanding the complexity of cellular steroid effects.

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Acknowledgements

I would like to express my gratitude to all my friends and colleagues in the Department of Anatomy and Cell Biology who supported me during the scientific work for my thesis.

In particular, I wish to thank my supervisor, HD. Dr. C. Beyer, for his invaluable help and advice throughout these investigations and for his ideas and willingness to discuss any problems, whenever necessary. I would also like to thank him for offering me the possibility to spend some time in the laboratory of Prof. L.M. Garcia-Segura in Madrid.

I also would like to thank Prof. Dr. Dr. hc. Ch. Pilgrim for inviting me to join his department and for supporting me during my PhD and Prof. Dr. J.B. Warchol from The Medical Academy in Poznan for his understanding and giving me the opportunity to come to Germany.

Thanks also to my colleagues in the laboratory: Gita, Nadhim, Maciej, Eva and Tatiana for their practical help and advice during the planning of experiments. Additional thanks to Nadhim and Maciej for their help in solving problems with "crashing" computers. In addition, I would like to acknowledge Gabriele Noack’s fine technical assistance in cell culturing.

Special thanks go to Dr. Gita Pezeshki for her excellent help while writing this thesis.

Finally, I would like to dedicate this thesis to my parents Alina and Tadeusz Karolczak, as well as to my Brother Piotr. They encouraged me during my studies and during the time I spent in Germany.

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