THESIS

BEHAVIORAL EFFECTS OF ESTROGEN RECEPTOR BETA ACTING LOCALLY TO REGULATE THE EXPRESSION OF TRYPTOPHAN HYDROXYLASE 2 (TPH2) IN SEROTONERGIC NEURONS OF THE DORSAL RAPHE NUCLEI

submitted by

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In partial fulfillment of the requirements

for the Degree of Master of Science

Colorado State University

Fort Collins, CO

Fall 2008

COLORADO STATE UNIVERSITY

November 7, 2008

WE HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER OUR SUPERVISION BY NINA CAROLINE DONNER ENTITELDED: "BEHAVIORAL EFFECTS OF ESTROGEN RECEPTOR BETA ACTING LOCALLY TO REGULATE THE EXPRESSION OF TRYPTOPHAN HYDROXYLASE 2 (TPH2) IN SEROTONERGIC NEURONS OF THE DORSAL RAPHE NUCLEI" BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE.

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ABSTRACT OF THESIS

BEHAVIORAL EFFECTS OF ESTROGEN RECEPTOR BETA ACTING LOCALLY TO REGULATE THE EXPRESSION OF TRYPTOPHAN HYDROXYLASE 2 (TPH2) IN SEROTONERGIC NEURONS OF THE DORSAL RAPHE NUCLEI

Affective disorders often involve serotonin (5-HT)-related dysfunctions and are twice as common in women than men. Interactions between estrogen and the brain 5-HT system have long been proposed to contribute to sex differences in mood and anxiety disorders, but the mechanisms underlying this phenomenon have yet to be revealed. Estrogen signaling is mediated by two different receptors termed estrogen receptor alpha and estrogen receptor beta. While estrogen receptor alpha (ERalpha) has mainly reproductive responsibilities, in brain, estrogen receptor beta (ERbeta) has been shown to attenuate anxiety- and despair-like behaviors in rodent models. However, little is known about ERbeta regulation of function in the brainstem raphe nuclei. The raphe nuclei are the main 5-HT system of the brain, and projections from the dorsal raphe nuclei (DRN) innervate many important forebrain and limbic areas. The work presented in this thesis addressed the possibility that ERbeta may be involved in the

regulation of 5-HT gene expression specifically in DRN neurons. My studies examined the effects of systemic versus local, intracerebral application of the selective ERbeta agonist diarylpropionitrile (DPN) and the nonselective ER-ligand estradiol (E) on tryptophan hydroxylase 2 (TPH2) mRNA expression within the DRN of female rats. TPH2 is the brain-specific, rate-limiting enzyme catalyzing 5-HT synthesis, and is expressed in every 5-HT neuron. Thus, it provides an excellent tool to assess the capacity for 5-HT production with the DRN. In these studies, TPH2 mRNA expression was assessed via *in situ* hybridization. In addition, relevant behavioral parameters were tested in all animals to evaluate each compound's effect on two closely related, but yet different mental states, anxiety-like and despair-like behavior.

Both, chronic systemic and chronic local DPN administration to ovariectomized (OVX) female rats significantly enhanced TPH2 mRNA expression in mid- and caudal subregions of the DRN after 8 days of treatment. Respective controls received systemic vehicle (27% hydroxypropyl-beta-cyclodextrin) or blank control pellets. Local application of DPN caused a stronger effect than systemic drug delivery. Chronic local delivery of E (0.5 µM) increased TPH2 mRNA expression in the same subregions of the DRN as did DPN, but its overall effect was weaker compared to the selective ERbeta agonist. Interestingly, while systemic DPN-administration confirmed the anxiolytic nature of ERbeta in two separate anxiety tests (elevated plus maze and open field test),

the effect was lost when DPN was delivered locally. However, local DPN- as well as E-treatment both resulted in attenuated despair-like behavior, as measured in the forced-swim test. Chapter 3 describes the experimental design, results and interpretation of these studies in depth.

Taken together, my data indicate that local actions of ERbeta agonist onto DRN neurons are sufficient to decrease despair-like behavior, whereas ERbeta stimulation of other brain regions is necessary to alter anxiety-like behaviors. Correspondingly, ERbeta acts locally to control TPH2 mRNA expression and presumably 5-HT synthesis in the certain subregions of the rat DRN. These results suggest an important role of ERbeta for regulating cellular events in the female DRN, and offer new opportunities for therapeutic treatments of depressive disorders.

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ACKOWLEDGEMENTS

I thank my advisor Dr. Robert Handa for scientific advice and financial support, and my committee members Drs. Stuart Tobet, Colin Clay and Ronald Tjalkens for helpful critique. I especially thank Dr. Stuart Tobet for his availability and mental support in difficult times. In addition, the work presented would not have been possible without the other Handa Lab members, the social network they provided and their outstanding sense of humor. It was a pleasure to work with all of them, I am very grateful for that. To Michael Weiser I am thankful for *de novo* synthesis and provision of the ERbeta-selective compound diarylpropionitrile, and to Andrea Kudwa for help with ovariectomies. Most grateful, I am still for my early mentors and role models Dr. Gottfried Scholl, who sparked my fascination with biology, and Dr. Inga Neumann who never loses her excitement about science, and will always remain a great mentor and friend to me in scientific and non-scientific situations.

I also sincerely thank my parents who did not have the chance to enjoy a high school or university education themselves, but constantly provided an intellectually stimulating atmosphere during childhood. It is them who Anja, my sister, and I have to thank most for our deep interest in nature and the biology of living organisms and for encouraging us to pursue our dreams.

My boyfriend John deserves big thanks for being so supporting and understanding throughout the challenging last year, and for barbecuing so many awesome steaks to keep my metabolism going. Thanks!

To Amelia, Julia, John and all of my family

Chopping wood is so popular because it is

the one and only activity that makes

success instantly visible.

Albert Einstein

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CHAPTER 1

GENERAL INTRODUCTION

About one of five Americans suffers from at least one episode of major depressive disorder (MDD) during their life (Kessler et al., 1994; Varghese and Brown, 2001; Bloom, 2004). Furthermore, the prevalence for the incidence, duration, gravity and reoccurrence of depression is twice as high in women as in men (Earls, 1987; Nolen-Hoeksema, 1987). An alteration in serotonin (5-HT) neurotransmission is the leading hypothesis regarding the pathophysiology underlying MDD (Arango et al., 2002; Mann, 2003; Lesch, 2004). Other neuropsychiatric disorders, such as schizophrenia (Veenstra-VanderWeele et al., 2000), autism (Veenstra-VanderWeele and Cook, 2004), aggression and suicidal behavior (Arango et al., 2003), and attention deficit disorder (Gainetdinov et al., 1999; Quist and Kennedy, 2001), are also related to dysfunctions of the brain 5-HT system. Current antidepressants target the brain 5-HT system indirectly by inhibiting either the 5-HT transporter (SERT) or the monoamine oxidase (MAO), or by binding to 5-HT receptors on target neurons. However, the fact that tryptophan hydroxylase 2 (TPH2) itself – the brain-specific enzyme that catalyzes the rate-limiting step in 5-HT synthesis (Walther et al., 2003; Zhang et al., 2004) – is greatly associated with neuropsychotic disorders (Zill et al., 2004; Zhang et al., 2005; Bach-Mizrachi et al., 2006; Harvey et al., 2007; Maron et al., 2007) has not been paid proper regard yet. TPH2 could, in fact, provide the most direct target of the brain 5-HT system. Prior to the discovery of TPH2 (Walther et al.,

2003), researchers either measured TPH1, the enzyme that mainly catalyzes the hydroxylation of tryptophan in the periphery and the pineal gland (Patel et al., 2004), or they did not discriminate between the two isoforms (Singh et al., 1990; Boularand et al., 1995; Pecins-Thompson et al., 1996; Chamas et al., 1999; Lu et al., 1999; Rotondo et al., 1999). While these earlier studies are still of great value, they need to be interpreted with regard to the fact that there have always been two different genes each coding for one of the two isoforms. TPH1 is the non-brain-specific tryptophan hydroxylase, and its expression in the brain, including the raphe nuclei as the main site of 5-HT synthesis in the brain with projections to numerous forebrain areas (Abrams et al., 2004), is limited compared to TPH2 (Malek et al., 2005). Although a recent study drew attention to the potential involvement of TPH1 in the stress-reactivity of the dorsal raphe 5-HT system (Abumaria et al., 2008), TPH2 may be the preferable isoform to target in order to upregulate 5-HT synthesis due to its predominant expression in 5-HT neurons.

Ovarian hormones have often been implicated in modulation of 5-HT function (Joffe & Cohen 1998; McEwen & Alves 1999; Bethea et al. 1998), and among ovarian steroids, changing estrogen levels are thought to have the greatest effect on mood. Postpartum depression, as well as premenstrual syndrome (PMS), premenstrual dysphoric disorder (PMDD) and menopause depression are all associated with a sudden drop in circulating estrogen (Rubinow 1992; Halbreich et al. 1995; Buckwalter et al. 2001). The genomic actions of estrogen are accomplished by two distinct receptor systems, estrogen

receptor alpha (ERalpha) and estrogen receptor beta (ERbeta) (Green et al., 1986). Relevant literature has reported significant presence of ERbeta specifically in the dorsal raphe nuclei (DRN) of mammals (Shughrue et al., 1997a; Alves et al., 1998; Gundlah et al., 2001; Mitra et al., 2003; Sheng et al., 2004; Nomura et al., 2005; Vanderhorst et al., 2005), suggesting a potential role for ERbeta in the regulation of TPH2 gene expression (Pecins-Thompson et al., 1996; Lu et al., 1999; Hiroi et al., 2006). Furthermore, various rodent models have been used to show that ERbeta agonists attenuate anxiety- and despair-like behaviors (Krezel et al., 2001; Imwalle et al., 2005; Lund et al., 2005; Rocha et al., 2005). Considering the importance of 5-HT systems for emotional stability, I therefore proposed to investigate the hypothesis that ERbeta is involved in the regulation of 5-HT-neuronal gene expression and consequently in the modulation of emotionality.

The overall goal of my thesis research was to determine the function of ERs in the brainstem DRN, specifically to elucidate the neuronal and behavioral role of ERbeta in the regulation of serotonergic neurons in the DRN. The specific aim was to determine the effects of chronic systemic versus chronic local administration of estrogen or ERbeta selective agonist on anxiety and despair-like behavior and on TPH2 mRNA expression within the DRN.

In one experiment, female ovariectomized (OVX) Sprague-Dawley rats were either subcutaneously (s.c.) injected with selective ERbeta agonist diarylpropionitrile (DPN) or vehicle. In a second experiment, female OVX Sprague-Dawley rats were stereotaxically implanted, bilaterally, with small wax-

pellets to deliver DPN or E site-specifically to the DRN. Unoperated and animals receiving blank pellets served as controls. All animals were tested for anxiety-like behavior on the elevated plus maze (EPM) and in the open field (OF). The animals' active versus passive stress-coping strategies were analyzed in the forced-swim test (FST), an established test model for antidepressants and despair behavior. TPH2 mRNA levels were measured using *in situ* hybridization. My hypothesis was that local, 5-HT-neuronal ERbeta activation directly regulates the synthesis of TPH2, and that estradiol (E) may have a gradually different effect. I also expected the systemic and the local DPN-treatment to attenuate both anxiety- and despair-like behavior.

CHAPTER 2

LITERATURE REVIEW

5-HT AND ITS ROLE IN DEPRESSIVE DISORDERS

A. THE METABOLISM OF TRYPTOPHAN

The monoamine 5-HT is a key neurotransmitter of the brain (Cooper et al., 2003). It is derived from the amino acid tryptophan, and because tryptophan itself cannot cross the blood brain barrier it is taken up into the brain via a non-selective large amino acid transporter, LAT1 (Duelli et al., 2000; Killian and Chikhale, 2001), operating at the surface of the brain capillary endothelial cells. Only about 1% of the circulating tryptophan enters the brain, and on its way in it has to compete with all other large neutral amino acids (Baumann, 1979; Filippini et al., 1996; Allegri, 2003). Tryptophan is one of the eight essential amino acids that the human body cannot synthesize on its own, but must be absorbed from our food (Sidransky, 1985, 2002; Sarubin-Fragakis and American Dietetic Association., 2003; Davis, 2006). In fact, the so called “5-HT depletion studies” (Salomon et al., 1993; Delgado et al., 1994) - in which patients that were subject to a tryptophan-free diet showed severe downregulation of the peripheral and central 5-HT system - were the first to indicate a causal connection of 5-HT and the pathophysiology of many psychiatric disorders, most importantly depression.

Once in the extracellular fluid of the brain, tryptophan is transported into 5-HT neurons via a high-affinity neuronal tryptophan transporter. In the cytosol of the serotonergic neuron, tryptophan is hydroxylized at the 5-position by the rate-

limiting enzyme of serotonin biosynthesis, neuronal tryptophan-hydroxylase, also called TPH2 (Walther et al., 2003; Zhang et al., 2004). For biochemical details see Fig. 1. In peripheral tissue, including the pineal gland, this step is performed by TPH1, the peripheral isoform of the enzyme. In both cases, the product is 5-hydroxy-tryptophan which is then almost immediately converted into the neurotransmitter 5-HT via decarboxylation by a common enzyme, the aromatic amino acid decarboxylase (AADC). The AADC step is much faster than the TPH step, and therefore not rate-limiting. For an overview of tryptophan metabolism within the brain see Cooper et al. (2003).

In the brain, 5-HT is utilized in many ways, not all yet fully understood. It is known that it plays an important role in the regulation of basic homeostasis including body temperature and sleep (Sallanon et al., 1982; Goodrich et al., 1989; Rausch et al., 2003), emotions such as anger, aggression or mood in general (Van Praag, 1994; Schwartz et al., 1999; Giegling et al., 2006), and nutritional and reproductive functions like appetite, sexuality and arousal (Feist and Galster, 1974; Curzon, 1990; Menani et al., 2000; Popova and Amstislavskaya, 2002). Its postsynaptic effects can be inhibitory or excitatory, depending on the cell type and the receptor type that it interacts with. To date, about 15 genes encoding functional 5-HT receptors have been identified in the mammalian brain. With the exception of 5-HT3 receptors - which are ionotropic receptors - all others are metabotropic G-protein-coupled receptors (GPCRs) (Peroutka, 1992; Millan et al., 2008). Post-genomic modifications, such as alternative splicing or mRNA editing further broaden the range of 5-HT receptor

types. There are at least seven sub-classes of 5-HT receptors. 5-HT1 and 5-HT2 were the first neuronal 5-HT receptors identified, with 5-HT1 being mainly inhibitory, whereas 5-HT2 rather exerts excitatory effects (Murphy et al., 1998; Barnes and Sharp, 1999). 5-HT1_A receptors are autoreceptors that play a very important role in the immediate feedback / auto-regulation of 5-HT neurons, however, they are also found in postsynaptic membranes (Hjorth et al., 1996; Dos Santos et al., 2008). In contrast to classic neurotransmitter receptors, 5-HT receptors are rather localized around synapses (pre- or post-synaptically), but rarely within the synaptic cleft. The exact functions and intracellular signaling pathways of 5-HT-GPCR-interacting proteins are not yet fully understood, but may include fine-tuning of signaling, trafficking to or from the membrane, and desensitization.

Reuptake of 5-HT into neurons (and probably into glia cells) occurs by means of the serotonin transporter (SERT), a high-affinity monoamine transporter. This also serves as a major termination mechanism for the actions of 5-HT. SERT mRNA is almost exclusively expressed in cell bodies of the DRN and the median raphe nuclei, but SERT protein can be transported to distant 5-HT nerve terminals where it serves as a bi-directional plasma membrane carrier, depending on the extra-intracellular concentration gradient of 5-HT. Selective 5-HT-reuptake inhibitors (SSRIs) such as Prozac® are a major group of antidepressants (Apparsundaram et al., 2008; Narboux-Neme et al., 2008).

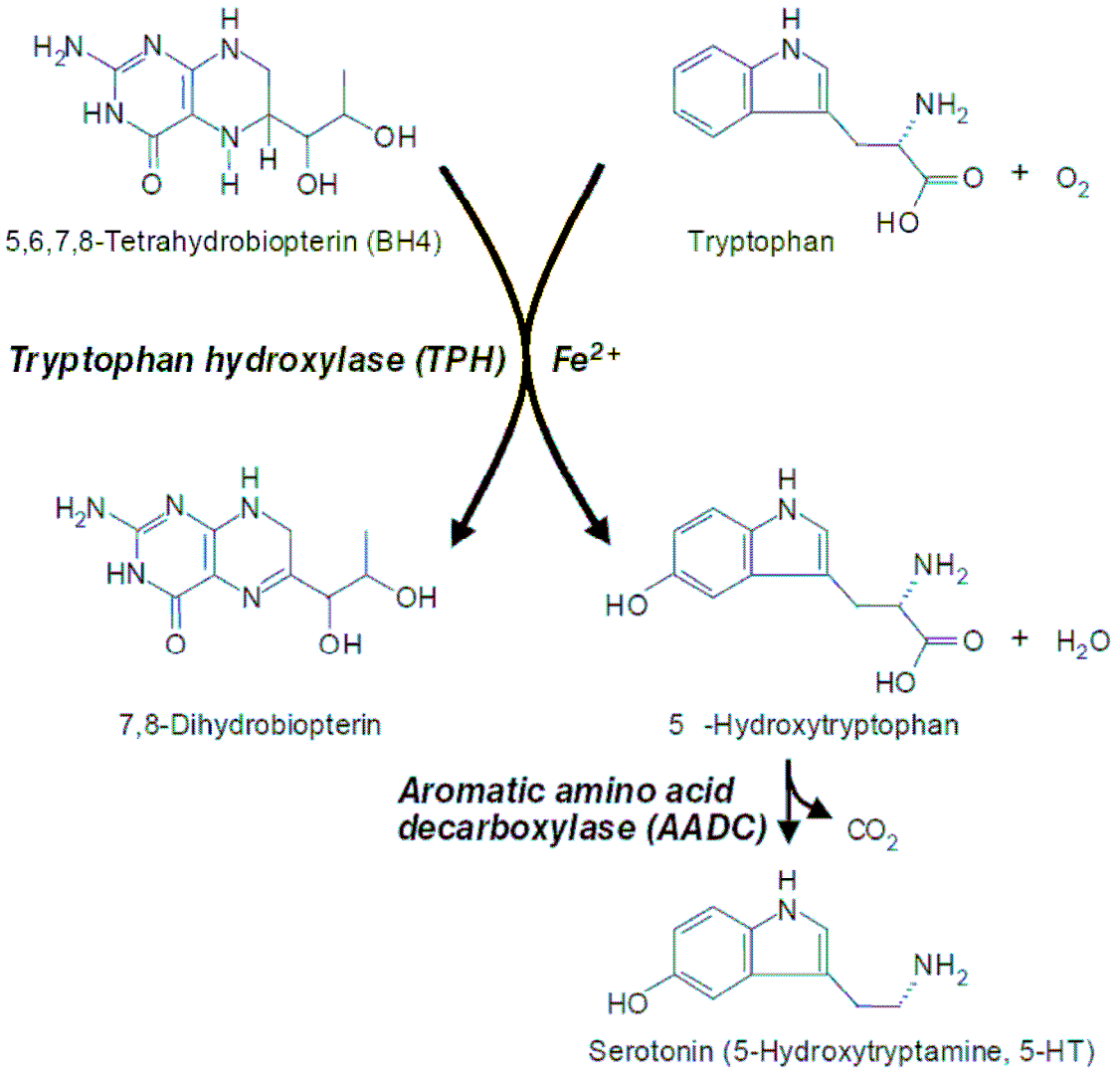


Figure 1. Schematic illustration of serotonin (5-HT) synthesis. Tryptophan-hydroxylase (TPH) is the rate-limiting (slow) enzyme during 5-HT production, catalyzing the hydroxylation of tryptophan with the help of the cofactor Fe^{2+} and the co-substrates O_2 and tetrahydrobiopterin (BH4). Aromatic amino acid decarboxylase (AADC) then rapidly decarboxylates the resulting 5-hydroxytryptophan to yield 5-HT. Figure from Walther and Bader (2003).

The cytosolic breakdown of 5-HT happens via deamination by monoamine oxidase (MAO). MAO is a target for another powerful class of antidepressants, the monoamine oxidase inhibitors (MAOIs). In the pineal gland, 5-HT is metabolized to melatonin by 5-hydroxyindole-O-methyltransferase. For the development of antidepressant treatments (such as tryptophan supplementation), it is important to recognize, however, that only about 1% of the body's tryptophan gets metabolized along the 5-HT pathway. The other 99% are metabolized differently, along the kynurenine pathway. The strength of the kynurenin pathway in one individual compared to another might therefore be an important factor influencing the availablity of the serotonin precursor tryptophan (Schmitz et al., 1974).

B. THE DORSAL RAPHE NUCLEI – MAIN BRAIN SITE OF 5-HT SYNTHESIS

The main brain site for 5-HT synthesis are the neurons of the raphe nuclei, and within those the dorsal raphe nuclei (DRN). The DRN are grouped into rostro-caudally distinct subpopulations. 5-HT neurons of the rostral DRN innervate forebrain areas thought to be involved in motivational behaviors (Fig. 2), such as the frontal cortex, the caudate, putamen and substantia nigra, whereas 5-HT cells of the caudal DRN rather project towards limbic structures, such as the hippocampus, the entorhinal cortex and the lateral septum (Abrams et al., 2004). Collateral 5-HT projections from the mid DRN branch out to control functionally related targets and circuitries involved into autonomic control of anxiety and fear (Lowry, 2002; Lowry et al., 2005), such as the paraventricular

nucleus (PVN) of the hypothalamus and the central nucleus of the amygdala (CeA). This topography suggests a potentially regulatory role in emotion for all, and perhaps a unique functional property and behavioral implication for each of the 5-HT subpopulations in the DRN.

C. THE 5-HT HYPOTHESIS OF DEPRESSION

Dysfunction of 5-HT neurotransmission is the leading hypothesis regarding the pathophysiology of MDD (Arango et al., 2002; Mann, 2003; Lesch, 2004) and other neuropsychiatric disorders such as schizophrenia (Veenstra-VanderWeele et al. 2000), autism (Veenstra-VanderWeele & Cook 2004), aggression, suicidal behavior (Arango et al. 2003) and attention deficit disorder (Gainetdinov et al. 1999; Quist & Kennedy 2001). About one of five Americans suffers from an episode of major depressive disorder MDD during life (Kessler et al., 1994; Varghese and Brown, 2001; Bloom, 2004), and the prevalence for depression is twice as high in women than it is in men (Earls, 1987; Nolen-Hoeksema, 1987). Currently, the “receptor-theory of depression” has replaced the earlier “monoamine theory of depression”. Most current antidepressants target the brain 5-HT system indirectly by inhibiting either the reuptake-receptor SERT or the 5-HT metabolizing enzyme MAO (Feldstein et al., 1965; Schmauss et al., 1988; Baker et al., 1992), or by binding to 5-HT receptors on target neurons (Cryan and Leonard, 2000; Berrocoso and Mico, 2008; Navines et al., 2008). However, some antidepressants take several weeks to show the desired treatment effect (Frazer and Benmansour, 2002). One interpretation of this is that

more complicated post-synaptic changes, such as receptor expression levels in the target cells and of autoreceptors, are required before the drug is effective. Over the last years, more and more attention has been drawn toTPH2 itself (Walther et al., 2003; Zhang et al., 2004), which is greatly associated with neuropsychotic disorders (Zill et al., 2004; Zhang et al., 2005; Bach-Mizrachi et al., 2006; Harvey et al., 2007; Maron et al., 2007), and provides a direct target for control of brain 5-HT synthesis.

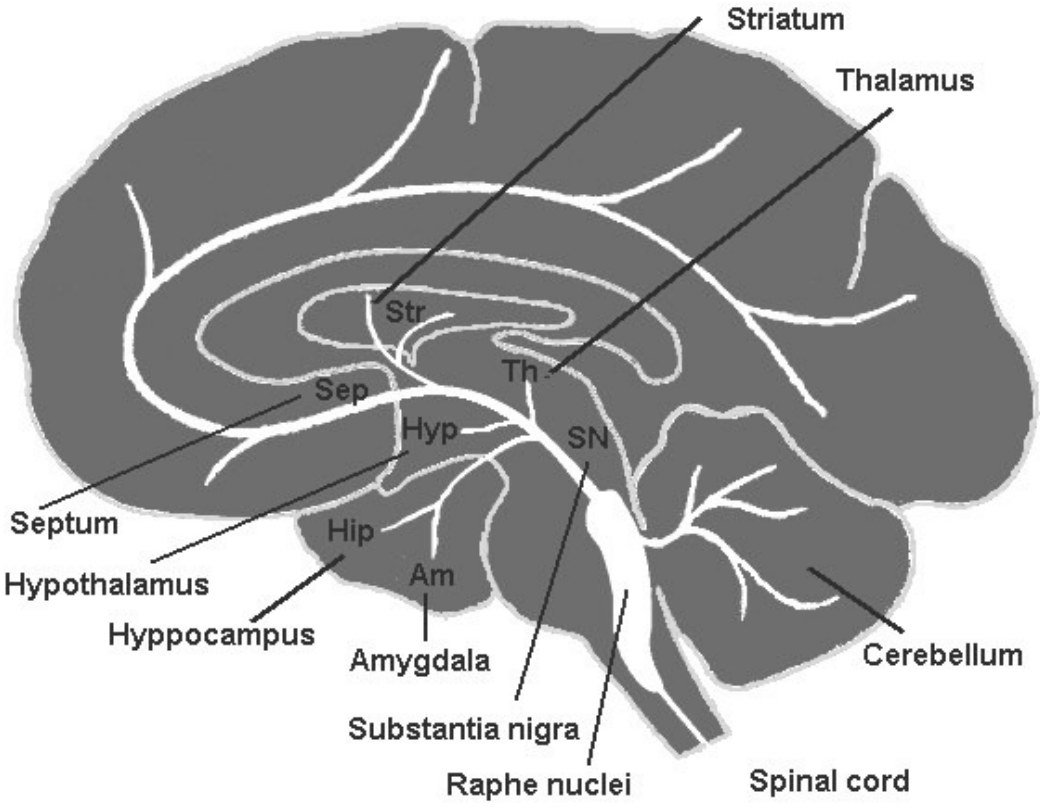


Figure 2. Schematic illustration of 5-HT projections from the dorsal raphe nuclei in the human brain. Important forebrain target areas include the thalamus (Th), striatum (str), the septum (Sep), the hypothalamus (Hyp), the hipocampus (Hip) and the amygdala (Am). Other axons innervate the mesencephalic substantia nigra and the cerebellum, or are located within the spinal cord, innervating targets of the pripheral nervous system. Figure modified from http://abdellab.sunderland.ac.uk.

THE NEURONAL TRYPTOPHAN-HYDROXYLASE: DISCOVERY & DISORDERS

TPH2 (Fig. 3) was recently discovered to be the neuronal-specific version of TPH (Walther and Bader, 2003; Zhang et al., 2004). Previous studies either did not discriminate between TPH1 and TPH2 or solely measured TPH1, the gene product that controls 5-HT synthesis in peripheral tissues and in the pineal gland, but shows a very weak expression in the DRN (Malek et al., 2005). TPH2 activity requires not only substrate, but also O_2 and BH4 (Fig. 1), for building the hydroxy-group. Soluble TPH2 is rather found in the pericaryal cytoplasm, whereas particulate TPH2 is more likely to be found in association with 5-HT synapses. Interestingly, TPH2 is only about 25-50% saturated with its substrate tryptophan under basal conditions (Hofto et al., 2008; Windahl et al., 2008). Tryptophan supplementation can thus hardly exhaust the enzyme's capacity. The activity of the enzyme can be increased by phosphorylation through protein kinase A, by Ca^{2+}-phospholipids and also by partial proteolysis (Winge et al., 2008). The drug *p*-chlorophenylalanine binds competitively and irreversibly to the enzyme, and can be used in experiments as a strong, long-term (weeks) inhibitor of 5-HTP synthesis (Jequier et al., 1967; Alexander et al., 1980; Petkov et al., 1995; Boot et al., 2002).

By now, various studies have linked defective TPH2 expression to emotional disorders, especially to MDD and suicide (Zill et al., 2004). For instance, Zhang et al. (2005) identified a single nucleotide polymorphism (SNP) in the TPH2 coding region in about 13% of a group of patients suffering from mild

anxiety to severe unipolar depression. When human TPH2 with the same SNP (replacement of the highly conserved Arg441) is expressed in cell culture, the same mutation causes an 80% reduction of 5-HT synthesis. Other studies revealed TPH2 SNPs in members of a French Canadian family with bipolar disease (Harvey et al., 2007), and among women with panic disorder (Maron et al., 2007). Paradoxically, TPH2 expression in the DRN of drug-free suicide victims was found to be 33% higher than in age-matched healthy controls (Bach-Mizrachi et al., 2006). Yet, earlier studies reported less 5-HT and less of its metabolite, 5-hydroxyindoleacetic acid, in the midbrain of suicide victims and in the CSF of suicide attempters (Mann et al., 1989; Placidi et al., 2001). This logical discrepancy could be explained by a compensatory elevation of TPH2 expression to regain 5-HT homeostasis in the brain, or possibly by a pathologic variation in the TPH2 gene as described above. All of the described genetic or functional studies (De Luca et al., 2005; Shink et al., 2005), however, suggest a link between chronically altered TPH2 expression and psychotic disorders.

SEX DIFFERENCES IN MOOD DISORDERS

Depressive, stress-related and anxiety disorders are twice as common in women as among men (Earls, 1987; Angold and Worthman, 1993; Weissman et al., 1993; Kornstein et al., 1995). Women also tend to respond differently to antidepressant medications than men (Kornstein, 1997; Gorman, 2006; Grigoriadis and Robinson, 2007), indicating that the cause and mechanism of mood disorders may be very different from males. In fact, various animal models

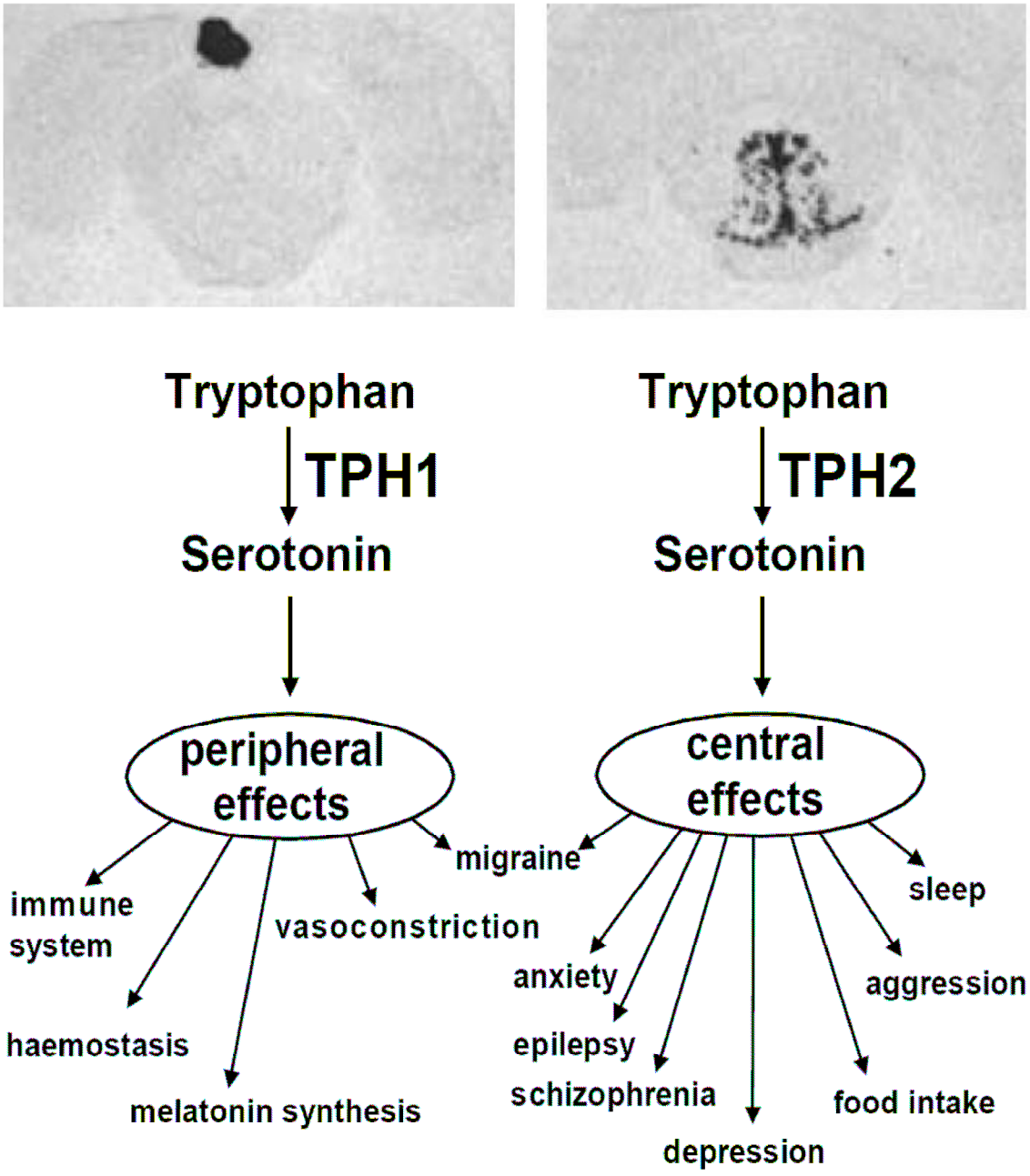


Figure 3. Overview of peripheral TPH1 versus brain-specific TPH2 functions. Although both enzymes catalyze the rate-limiting step for 5-HT synthesis, their role in physiology and various pathologies is very distinct. Top: *in situ* hybridization pictures of TPH1 mRNA expression in the pineal gland (left) and TPH2 mRNA expression in the DRN (right) of Sprague-Dawley rats. Bottom: Schematic representation of the peripheral versus central duality of the 5-HT system. Modified after Patel et al. (2004) and Walther and Bader (2003).

for affective disorders suggest profound sex differences in regulation of emotionality (Steenbergen et al., 1990; Caldarone et al., 2003; Toufexis, 2007) and in stress-induced performance deficits (Shors and Leuner, 2003; Shansky et al., 2006). In male rats, for instance, the exposure to an inescapable stressor greatly facilitates learning. In contrast, female rats respond to the same environmental event in the exact opposite way, and as a result are severely impaired in their ability to perform (Shors et al., 1998; Wood and Shors, 1998; Wood et al., 2001). Among all ovarian steroids, changing estrogen levels are thought to have the greatest effect on mood. Postpartum depression, as well as premenstrual syndrome (PMS), premenstrual dysphoric disorder (PMDD) and menopause depression are all associated with a sudden drop in circulating estrogen (Rubinow, 1992; Halbreich et al., 1995; Buckwalter et al., 2001). Regrettably, exactly these steroidal fluctuations and changing baselines in females, have led to a reluctance towards studying the etiology of female depression, and to the common misassumption that the female neurobiology of depression is simply an extension of that observed in males. Hence, the female prevalence for depressive disorders and their different response to antidepressant treatment indicate an essential role of ERs in the regulation of depression.

ESTROGEN RECEPTORS: STRUCTURE & FUNCTION

Estradiol has been reported to affect anxiety-related behaviors, yet a careful review of the literature shows that it can have both anxiogenic and anxiolytic effects. This initial contradiction may be explained by the functional difference between the two types of ER, ERalpha and ERbeta. ERalpha-selective agonists, such as propylpyrazoletriol (PPT, Stauffer et al. (2000)), are anxiogenic and increase the response of the hypothalamo-pituitary-adrenal (HPA) axis to a stressor, whereas ERbeta agonists (Meyers et al., 2001) like DPN (Fig. 4 and Table 1) attenuate anxiety- and despair-like behaviors and decrease the HPA stress response (Walf et al., 2004; Lund et al., 2005; Walf and Frye, 2005). In the forced swim test, DPN treatment could even dampen depressive-like behavior of flinders sensitive rats (Osterlund et al., 1999), a strain selectively bred for depressive-like behavior (Overstreet et al., 2006).

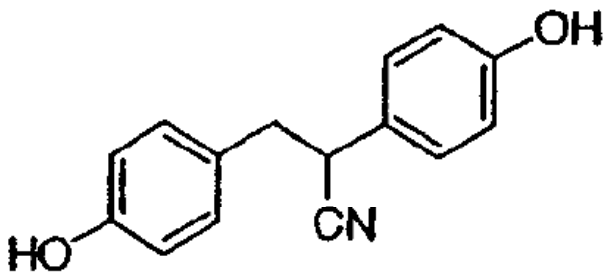


Figure 4. Chemical structure of the selective ERbeta agonist diarylpropionitrile (DPN). DPN binds to ERbeta with a 70- to 80-fold higher affinity than to ERalpha (Kuiper et al., 1998; Meyers et al., 2001).

Fig. 5 depicts the two different ER proteins, ERalpha and ERbeta, each a product of separate genes, and each having several isoforms that are created by posttranscriptional modifications such as alternative splicing (Chu and Fuller, 1997; Petersen et al., 1998; Price et al., 2000; Price et al., 2001; Chung et al., 2007). The exon structures of recently discovered splice variants of ERbeta are shown as well in Fig. 5. ERs belong to a family of steroid hormone receptors that are ligand-activated transcription factors, all members of the “nuclear receptor superfamily”. Steroid hormones such as estradiol are lipophilic allowing for free passage through membranes. Thus, estradiol and steroid compounds can cross the plasma membrane as well as the nuclear membrane. ERs can be cytosolic or nuclear. Ligand binding induces conformational changes in the ER that may first promote the transport of the complex through pores of the nuclear membrane, and then lead to receptor-dimerization, receptor–DNA interaction at an estrogen response element (ERE), recruitment of coregulators and other transcription factors, and finally – after notable remodeling of the chromatin at the DNA binding site - the formation of the pre-initiation complex that is necessary for exposing the promoter-sequence of the gene of interest and preparing the gene for transcription (Migliaccio and Marino, 2003; O'Lone et al., 2004; Marino et al., 2006). However, ERs not only regulate gene expression by binding to EREs, but also through protein–protein interactions with other transcription factors and coregulators (Tremblay and Giguere, 2001; Kang et al., 2002; Dutertre and Smith, 2003; Loven et al., 2004; Koide et al., 2007; Suzuki et al., 2007; Bovet et al., 2008; Ruegg et al., 2008). For instance, the nuclear receptors ERbeta1ð3

and ERbeta2δ3, two variants with a deletion of exon 3 coding for the DBD, colocalize with coactivator proteins of ER (cotransfected GFP-GRIP1 and endogenous CBP) in the presence of agonists (Price et al., 2001).

Besides classic genomic mechanisms ERs can also act via rapid, non-genomic (cytosolic) actions (Stirone et al., 2005; Mhyre and Dorsa, 2006). A prominent example is the membrane-anchored estrogen receptor GPR30 (Revankar et al., 2005). Another splice variant of ERbeta lacks the fourth exon (δ4), which codes for the nuclear translocation signal. ERbeta1δ4 resides mainly in the cytosol, and does not seem to bind estrogen (Price et al., 2000). While the cytosolic location of ERbeta1δ4 may suggest rapid, non-genomic actions of the splice variant, little is yet known about its actual purpose.

ERalpha and ERbeta share an almost identical (96%) DNA-binding domain (DBD) and bind to the same ERE (Kuiper et al., 1996). Although the ligand-binding domains (LBDs) are less homologous, the splice variant ERbeta1 (historically the first ERbeta to be identified) binds estradiol with almost the same affinity as does ERalpha (Kuiper and Gustafsson, 1997). The novel splice variant ERbeta2 (Chu and Fuller, 1997; Chung et al., 2007), carries an 18-amino acid insert between the fifth and the sixth exon within the LBD. This causes ERbeta2 to have a comparably low affinity for estradiol (Table 1). For both receptor types, ERalpha and ERbeta, synthetic ligands that can discriminate between the two types have been discovered, each displaying selective affinities for either ER, such as the ERalpha agonist PPT, or the ERbeta-specific agonist DPN (Table 1, Fig. 4). Natural discriminatory ligands, such as the ERbeta-favoring isoflavone,

Genistein (Table 1), had to be considered during the design of my experiments. Thus, all animals were fed a soy-free chow diet to avoid potential isoflavone effects.

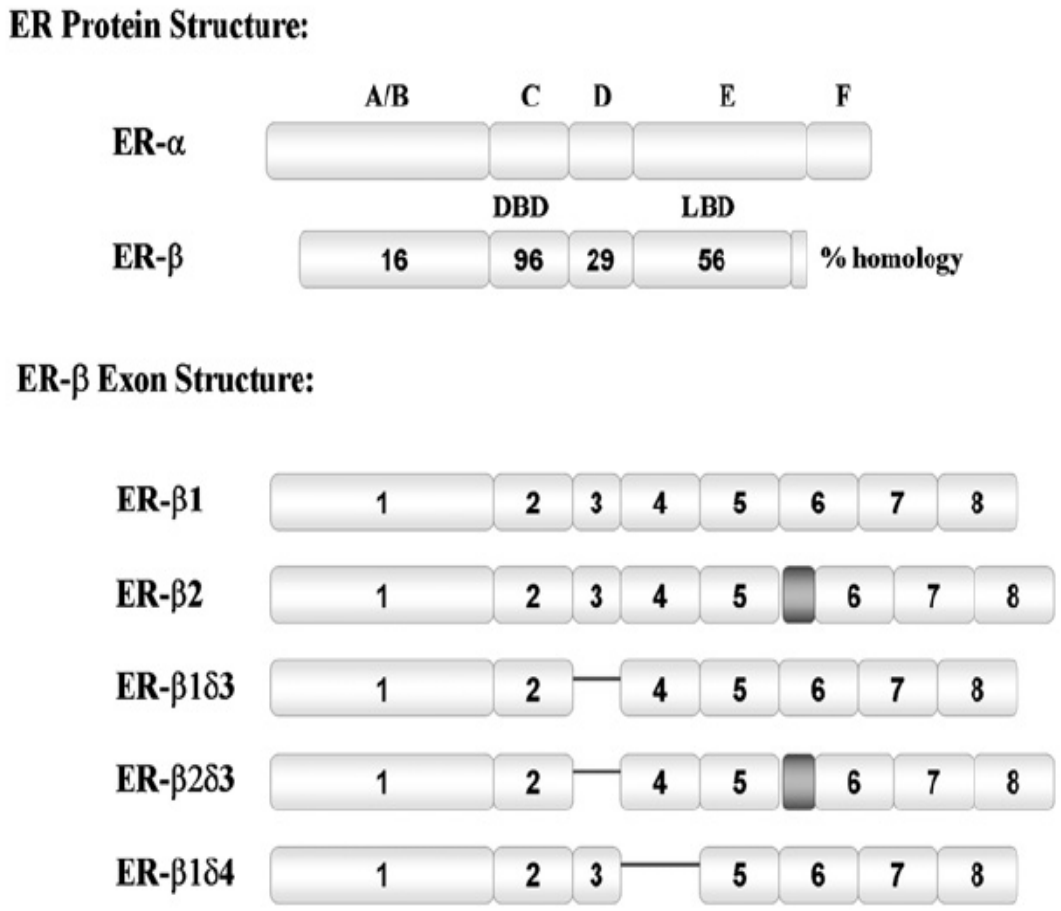


Figure 5. Schematic representation of the relative homology between ERalpha (ERα) and ERbeta (ERβ) protein (top panel) and ERβ splice variant exon structure (lower panel). Deletions are indicated by a single line, and insertions are indicated by a shaded box. DBD=DNA-binding domain, LBD=ligand-binding domain (Weiser et al., 2008).

Table 1

Binding affinities of estradiol and selected compounds for ERalpha and ERbeta, and for selected ERbeta isoforms

Compound	K_i (nM)	
	ERα	ERβ
Estradiol[a]	0.12	0.15
DPN[a]	195	2.5
PPT[a]	0.50	700
Diethylstilbestrol[b]	0.13	0.15
Moxestrol[b]	0.50	2.6
4-OH-Tamoxifen[b]	0.10	0.04
Genistein[b]	2.6	0.30

ER isoform	K_d (nM)	$T_{1/2}$ association (min)	$T_{1/2}$ dissociation (min)
ERα	0.13	354	12
ERβ1	0.15±0.02	<45	>60
ERβ2	1.84±0.19	165	8
ERβ1δ3	0.41±0.15	<45	ND
ERβ2δ3	1.44±0.82	165	ND

Top: Binding affinities (K_i) of estradiol (E) and selective natural (Genistein) or artificial (DPN, PPT, Diethylstilbestrol, Moxestrol, 4-OH-Tamoxifen) ligands for ERalpha and ERbeta (top). Bottom: Dissociation constant (K_d), association half-life and dissociation half-life ($T_{1/2}$) of [^{3}H]-estradiol for ERalpha and selected ERbeta isoforms. ND=not determined. Tables obtained from Weiser et al. (2008).

LOCALISATION & FUNCTION OF ESTROGEN RECEPTOR BETA IN THE BRAIN

Although ERalpha and ERbeta (also referred to as ERbeta1) both bind estradiol with about the same affinity, and bind to the same response element in DNA (Kuiper et al., 1996), they differ significantly in their neuroendocrine and behavioral function. ERalpha is vital for the control of reproduction in the brain and body (Ogawa et al., 1998; Hewitt and Korach, 2003), whereas studies of four different null mice mutants (βERKO) (either *neo* cassette insertions in the DNA-binding domain or stop codon inserts throughout the gene) indicate that ERbeta is not required for immediate reproductive functions or sexual behavior (Ogawa et al., 1999; Couse et al., 2000; Nomura et al., 2006). In contrast to that, both sexes of a new βERKO mouse strain without any transcription past exon 2 (Antal et al., 2008) are sterile. To date, studies agree that brain ERbeta functions as a regulator of emotion-related behavior (Walf et al., 2008b), anxiety and stress responses (Krezel et al., 2001; Imwalle et al., 2005; Lund et al., 2005; Rocha et al., 2005; Toufexis et al., 2007; Walf and Frye, 2007a), and possibly of the negative feedback control of anterior pituitary luteinizing hormone (Dorling et al., 2003). ERbeta has a similar binding affinity for estradiol as ERalpha (Kuiper et al., 1996), but posttranscriptional modifications of ERbeta, especially the novel splice variant ERbeta2 (Fig. 5), result in proteins that bind estradiol with a lower affinity. Such a dual receptor system could indicate adaptive changes in respective cells that would extend the range of sensitivity to higher concentrations of circulating estrogen (Petersen et al., 1998; Chung et al., 2007),

similar to the dual receptor system proposed for mineralocortocoid and glucocorticoid receptors (Reul and de Kloet, 1985).

Ovarian hormones, primarily estrogen, have been shown to modulate 5-HT function (Bethea et al., 1998; Joffe and Cohen, 1998; McEwen and Alves, 1999). However, the limited expression of ERalpha in the raphe puzzled researchers until ERbeta was discovered and localized in the DRN of ERalpha-knock-out (αERKO) mice (Shughrue et al., 1997a). Since then, ERbeta mRNA and / or protein have been repeatedly identified in the raphe nuclei of both sexes in mice (Mitra et al., 2003; Nomura et al., 2003; Vanderhorst et al., 2005) and primates (Gundlah et al., 2000; Gundlah et al., 2001), while ERbeta expression in the human DRN remains to be investigated. In guinea pigs, ERbeta, but not ERalpha is expressed in the raphe nuclei (Lu et al., 1999). The nature of ERs in the rat DRN is controversial as Lu et al. (2001) and Nomura et al. (2005) found that 5-HT neurons in the female rat DRN predominantly contain ERbeta, not ERalpha, but Sheng et al. (2004) did not find significant immunoreactivity for either ER in the rat DRN. Nonetheless, the qualitative variance between the employed anti-ERbeta antibody types and batches and the discovery of different splice variants raise the question whether the antibodies used may have failed to detect specific ERbeta variants. ERbeta1 is the original ERbeta; ERbeta2 is a splice variant containing an 18 amino acid insert in the ligand-binding domain; and deletion variants like ERbeta1δ3, ERbeta2δ3 and ERbeta1δ4 either lack the third or the fourth exon (Fig. 5). Since most ERbeta antibodies used in previous studies were created against the carboxy(C)-terminus of ERbeta, they should

automatically detect any splice variant, including ERbeta2. However, in my hands they only detected ERbeta in the far caudal DRN of female OVX rats, and in control regions like the cerebellum, but not in the rostral or mid DRN, where Chung et al. (2007) reported intense immunoreactivity of the novel splice variant ERbeta2. On one side of the argument, ERbeta was found to be expressed in the DRN of many other species, including mice, and is thus likely to be present in the raphe nuclei of rats as well. On the other side, a lot of studies show species differences in the expression pattern of ERs and other proteins (Young et al., 1995; Gundlah et al., 2000; Sheng et al., 2004; Warembourg and Leroy, 2004). Therefore, it remains to be determined if the failure to detect ERbeta in certain immunocytochemical (ICC) studies is due to true species-dependent variations, or to a regionally modified epitope in the C-terminus.

ESTROGEN RECEPTOR-MEDIATED GENE REGULATION IN 5-HT NEURONS

An ERbeta-selective mechanism is proposed for many of the numerous estrogen-serotonin interactions on mood and cognition (for review see Amin et al. (2005)). For example, ERalpha is found in non-5HT neurons of the rat DRN, where estrogen regulates expression of progestin receptors but not of TPH (Alves et al., 1998). However, the αERKO mouse also shows estrogen-induction of progestin receptors in the DRN, implying that other ERs, for example ERbeta, are involved (Alves et al., 1998). In the cynomolgus monkey, phytoestrogens from soy, which are mostly selective for ERbeta, improve mood and enhance 5-HT transmission in the DRN (Shively et al., 2003). Also, Nomura et al. (2005)

found that βERKO mice express significantly less TPH mRNA than wild type (WT) controls, whereas the synthesis of the enzyme was not altered in αERKO mice. A behavioral study reporting higher anxiety in βERKO mice compared to WT, additionally showed a reduced 5-HT content in the DRN of null mice (Imwalle et al., 2005). Most importantly, estradiol could be shown to selectively increase TPH2 expression in subregions of the DRN that are associated with attenuated anxiety (Hiroi et al., 2006).

One of my own pilot studies also indicated that ERbeta could regulate gene expression in TPH2 neurons. Chronic systemic treatment of female OVX rats revealed that ERalpha activation by PTT (1mg/kg, s.c.) increases, but selective ERbeta activation via DPN (2mg/kg, s.c.) decreases the number of c-*Fos*-immunoreactive TPH2 neurons within the dorsal mid DRN after exposure to forced swim stress (Fig. 6). The dorsal-mid DRN is of particular relevance for regulation of autonomic emotional responses, because collateral 5-HT projections from exactly this DRN subregion branch out to simultaneously innervate functionally related emotionality-regulating targets, such as the PVN and the CeA (Lowry, 2002; Lowry et al., 2005). The opposing effects of PPT versus DPN generally support the hypothesis that the two types of ERs (alpha and beta) hold functionally distinct roles that may include opposing regulation of 5-HT-neuronal stress-reactivity. Consistent with the indication of ERbeta2 in the DRN of female rats by Chung et al. (2007), my recent dual-ICC results furthermore suggest a predominant expression of ERbeta2 in TPH2 neurons and

in non-TPH2 cells of the female rat DRN. ERalpha does not colocalize with TPH2 (Fig. 7).

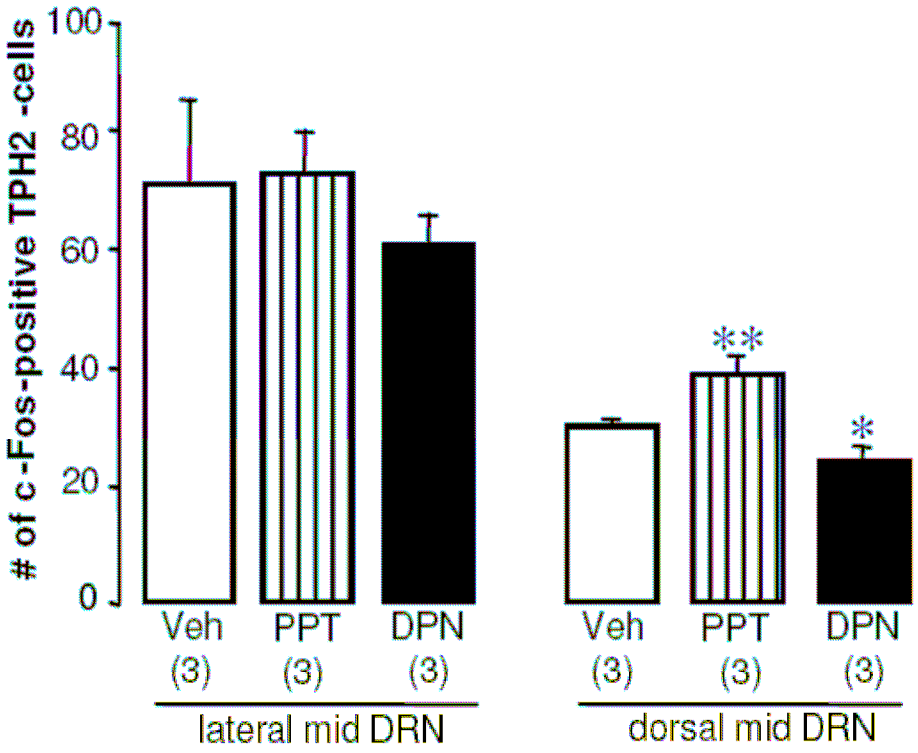


Figure 6. Seven days of daily subcutane treatment with DPN decreases acute c-Fos stress-reactivity of TPH2 neurons. PPT has the opposite effect. Female OVX rats were stressed for 5 min by forced swimming, and sacrificed via intracardial perfusion with 4% paraformaldehyde 1 h after termination of the stressor. Numbers in parenthesis indicate group size. $p^* < 0.05$, $p^{**} < 0.01$ vs. Veh (ANOVA, factor treatment, followed by Tukey's *post hoc* test)

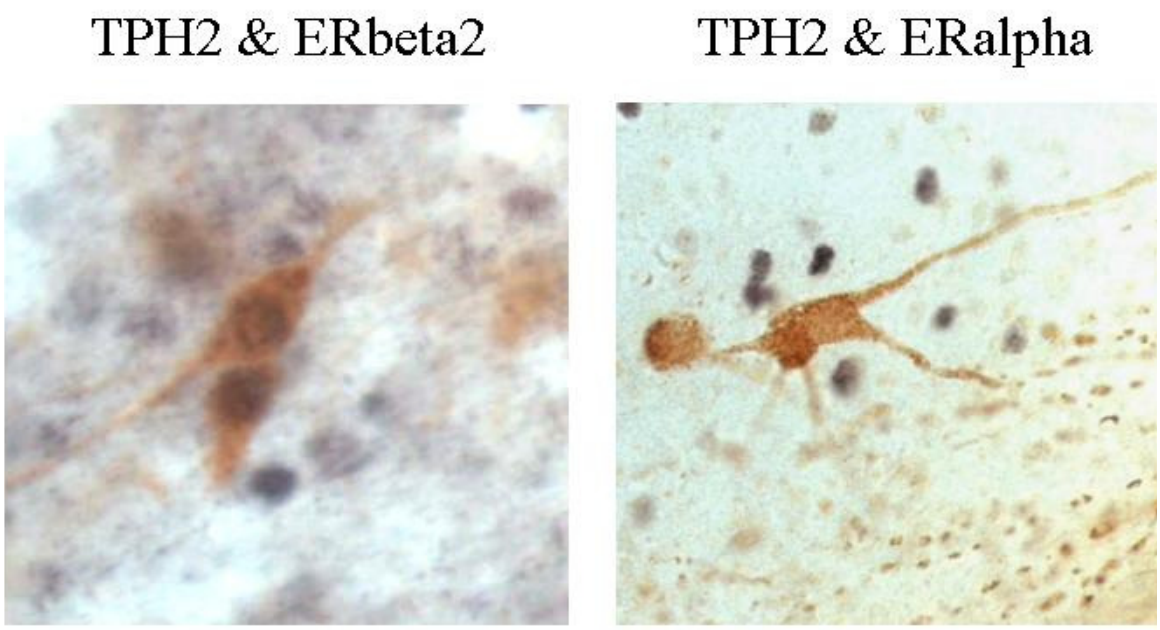


Figure 7. Photomicrographs showing dual-label ICC: ERbeta2-positive TPH2 neurons and ERalpha-negative TPH2 cells in the female rat DRN. The nuclear steroid receptors are labeled in black / purple, the cytoplasmatic TPH2 protein is visualized in brown. Note that nuclei other than within TPH2 neurons are also positive for ERbeta2, and that ERalpha-positive cells are located adjacent to the TPH2 neurons depicted. The TPH2 antibody was kindly provided by Drs. Kuhn and Sakowski. For methodological details see Sakowski et al. (2006). ERbeta2-ICC was performed after a protocol established by Chung et al. (2007).

SUMMARY & HYPOTHESIS

The evidence provided indicates that more detailed examination of ERbeta's involvement in the regulation of 5-HT-neuronal gene expression is required, especially with respect to the vast prevalence of depressive disorders and their impeding effect on the quality of life. The identification of disrupted TPH2 functionality or homeostasis in patients with mood disorders ranging from mild to major emotional disorders (Zill et al., 2004; Zhang et al., 2005; Harvey et al., 2007; Maron et al., 2007) and in suicide victims (Bach-Mizrachi et al., 2006) first proposed the involvement of this 5-HT-producing enzyme in the regulation of emotional health. It also bore the question of what may control the expression of TPH2. Estrogen's interactions with the serotonergic system (Amin et al., 2005; Hiroi et al., 2006), ERbeta's anxiolytic functions (for review see Weiser et al. (2008)), and the presence of the beta- but not the alpha type of ER in 5-HT DRN-neurons of various species (Shughrue et al., 1997a) then suggested a role for ERbeta in 5-HT-neuronal regulation. The phenotype of reduced TPH expression and lowered 5-HT content in the DRN of βERKO mice (Imwalle et al., 2005; Nomura et al., 2005) and the finding that ERbeta-selective agonists can alter 5-HT neurotransmission and gene expression in TPH2 neurons (Shively et al. 2003; our preliminary data) further strengthened my hypothesis that local ERbeta activation in 5-HT neurons of the DRN directly regulates TPH2 expression and decreases anxiety- and depressive-like behaviors. This hypothesis was to be tested via systemic delivery of ERbeta agonist in the first experiment, and local, site-specific delivery in the second experiment.

CHAPTER 3

Estrogen receptor beta acts locally to regulate the expression of tryptophan-hydroxylase 2 mRNA within serotonergic neurons of the rat dorsal raphe nuclei

ABSTRACT

Affective disorders are often associated with a disruption of the brain serotonin (5-HT) system and are twice as common in women compared to men. The median and dorsal raphe nuclei constitute the main source of 5-HT in the brain, and contain 5-HT cells that send projections to innervate important forebrain and limbic areas. Further, all 5-HT neurons of the raphe nuclei express tryptophan hydroxylase-2 (TPH2), the brain specific, rate-limiting enzyme for 5-HT synthesis. Previously, it was shown that ERbeta agonists attenuate anxiety- and despair-like behaviors in rodent models. Here, we tested the hypothesis that ERbeta is involved in the regulation of 5-HT gene expression in neurons of the dorsal raphe nuclei (DRN) by examining the effects of systemic versus local application of the selective ERbeta agonist diarylpropionitrile (DPN) on TPH2 expression within the DRN of female rats.

For the first experiment, young adult, ovariectomized (OVX) female rats were injected s.c. with DPN (2mg/kg) or vehicle (27% hydroxypropyl-beta-cyclodextrin) once daily for 8 days. Animals were tested for anxiety-like behavior in the open field (OF) and on the elevated plus maze (EPM) after 6 and 7 days of treatment, respectively. The results confirmed the anxiolytic nature of ERbeta, as

DPN-treated rats displayed more rears at the wall of the OF, and spent more time in the open arms of the EPM while entering open arms more frequently and with a shorter initial latency than controls. The following morning, all rats were killed 4 hours after the last injection under non-stress conditions. *In situ* hybridization revealed that systemic DPN-treatment significantly elevated basal TPH2 mRNA expression in the caudal and mid-dorsal DRN.

In a second experiment, young adult, OVX female rats were implanted bilaterally with wax pellets flanking the DRN. Pellets contained either 17-beta-estradiol (E, 0.5 μM), DPN (0.5 μM) or no hormone. Unoperated individuals served as additional controls. DPN- and E-treated rats displayed a more active stress-coping behavior in the forced-swim test (FST), as they struggled longer than controls. However, no significant differences in anxiety-like behaviors were found between any of the treatment groups in the OF or on the EPM. TPH2 mRNA in the DRN was measured using *in situ* hybridization. DPN significantly enhanced the TPH2 mRNA expression in the mid-dorsal and in the caudal DRN, compared to both control groups. Similarly, animals of the E-treated group also expressed more TPH2 mRNA than controls in the mid-dorsal DRN.

Taken together, these data indicate that local activation of ERbeta neurons in the DRN is sufficient to decrease despair-like behavior, whereas action of ERbeta in other brain regions is necessary to alter anxiety-like behaviors. These results suggest an important role of ERbeta for regulating cellular events in the DRN. ERbeta acts locally to control TPH2 mRNA levels and consequently 5-HT synthesis in the certain subregions of the rat DRN.

INTRODUCTION

Major depressive disorder (MDD) affects about 17% of Americans (Kessler et al., 1994; Williams et al., 2007), and is unquestionably a complex, heterogeneous disease (Winokur, 1997; Ellard, 2001; Weissman, 2002). A deficiency in serotonergic (5-HT) neurotransmission, however, is the leading hypothesis regarding the development and pathophysiology of this disease (Owens and Nemeroff, 1994; Arango et al., 2002; Perlis et al., 2002; Lesch, 2004). Furthermore, the incidence, duration, severity and rate of reoccurrence of depressive disorders are twice a high in women compared to men (Earls, 1987; Angold and Worthman, 1993; Weissman et al., 1993; Kornstein et al., 1995). Women also tend to respond differently than men to common antidepressant treatments, such as selective serotonin-reuptake inhibitors (SSRIs) (Kornstein, 1997; Gorman, 2006). This ratio together with numerous animal models reporting sex differences in the regulation of emotionality (Steenbergen et al., 1990; Caldarone et al., 2003; Shors and Leuner, 2003; Toufexis, 2007) and an interaction between estrogen and the serotonergic system (for review see (Amin et al., 2005)) suggest that estrogen receptor (ER)-mediated mechanisms may underlie the etiology of MDD.

In animal models, estradiol is capable to exert both, anxiolytic, but also anxiogenic effects, depending on the behavioral context (Koss et al., 2004; Hiroi and Neumaier, 2006). This ambiguity may be explained by the two different receptor systems, ERalpha and ERbeta. While ERalpha-selective agonists are anxiogenic, ERbeta-specific agonists, such as diarylpropionitrile (DPN), have

been shown to exert potent anti-anxiety effects (Walf et al., 2004; Lund et al., 2005; Maier and Watkins, 2005; Walf and Frye, 2005). The endogenous ligand estradiol binds to and activates both receptor types with about the same affinity (Kuiper et al., 1997). In flinders-sensitive rats, a rat strain selectively bred for depression, ERbeta agonists reduce the animals' passive floating and immobility behavior (Overstreet et al., 2006) during the forced swim test (FST), a test established to assess despair-like behavior in rodents by measuring their active versus passive stress-coping strategies (Porsolt et al., 1977). Rats of the same animal model also display abnormal levels of 5-HT receptor transcripts for 5-HT(2A) in the perirhinal cortex, piriform cortex, medial anterodorsal amygdala and in the hippocampus, a phenotype that is reversed by estrogen-treatment (Osterlund et al., 1999). Considering the importance of the 5-HT system for anxiety- and depressive disorders, we thus hypothized that local ERbeta activation regulates gene expression within 5-HT neurons, ultimately resulting in decreased anxiety- and despair-like behavior.

The brainstem dorsal raphe nuclei (DRN) are the primary 5-HT system of the brain. Distinct DRN subdivisions give rise to axons that innervate most forebrain areas, including areas crucial for the regulation of emotion and stress-coping behavior, such as the amygdala and the paraventricular nucleus of the hypothalamus (Imai et al., 1986; Petrov et al., 1992). Other subregions of the DRN send projections to motivational areas like the prefrontal cortex (Lowry, 2002; Abrams et al., 2004), while axons from the caudal DRN target limbic

structures, such as the hippocampus, the entorhinal cortex and the septum (Kohler and Steinbusch, 1982).

Within each 5-HT neuron, tryptophan-hydroxylase 2 (TPH2), the recently discovered, brain-specific version of the enzyme (Walther et al., 2003; Zhang et al., 2004), catalyses the rate-limiting step of 5-HT synthesis. Disruption or dysfunction of TPH2 itself is strongly correlated with affective disorders (Zill et al., 2004; Zhang et al., 2005; Haghighi et al., 2008), and abnormal TPH2 expression may be responsible for much of the pathology described.

The hypothesis that ERbeta-mediated actions may regulate the expression of TPH2 is supported by the robust expression of ERbeta within the DRN of mice (Shughrue et al., 1997a; Mitra et al., 2003; Nomura et al., 2005; Vanderhorst et al., 2005), primates (Gundlah et al., 2000; Gundlah et al., 2001) and guinea pigs (Lu et al., 1999), whereas ERalpha is only expressed to a miniscule extent in the DRN of most of these species. The nature of ERs in the rat DRN, however, remains controversial. In the female rat DRN, Lu et al. (2001) reported strong immunoreactivity for ERbeta1, the first ERbeta variant to be discovered (Green et al., 1986), and Chung et al. (2007) for ERbeta2 - a novel splice variant. In contrast, Sheng et al. (2004) did not detect any significant immunoreactivity for either ERbeta or ERalpha in female or male rat DRN.

Some studies already demonstrated that ERbeta activation can regulate gene expression in the brainstem. First, Alves et al (2000) discovered estrogen-mediated induction of progestin receptor expression in the DRN of ERalpha null mice (αERKO), suggesting a role for ERbeta. Later, ERbeta-selective

phytoestrogens were determined to improve mood and 5-HT neurotransmission in the cynomolgus monkey (Shively et al., 2003). Subsequently, Nomura et al. (Nomura et al., 2005) found significantly less TPH mRNA expressed in the DRN of βERKO mice than in wild type. βERKO mice also displayed increased anxiety-like behavior in conjunction with a lower 5-HT content in the DRN (Imwalle et al., 2005). Recently, estrogen-treatment was demonstrated to increase TPH2 expression especially in those DRN subregions that are associated with attenuated anxiety (Hiroi et al., 2006).

The described findings all support the hypothesis that ERbeta activation in the DRN may be sufficient to alter behavioral parameters and TPH2 gene expression in the DRN. Therefore, we examined the effects of chronic systemic versus local delivery of ERbeta agonist DPN in female, ovariectomized (OVX) rats on anxiety- and despair-like behavior as well as on TPH2 mRNA expression in all subdivisions of the DRN.

MATERIAL & METHODS

Animals

All animal surgeries, behavioral tests and experimental protocols followed NIH and AAALAC guidelines and were approved by the Animal Care and Use Committee at Colorado State University. Young adult female Sprague-Dawley rats (200-250 g body weight, Charles River Laboratories, Wilmington, MA) were kept under standard laboratory conditions (12:12 h light-dark cycle, lights on at 0600 h, 22 ℃, 60 % humidity, and *ad libitum* access to water and food). All rats were fed a phytoestrogen-free chow diet (Harlan Laboratories, San Diego, CA) for the entire duration of the experiment starting one week before ovariectomy (OVX) to avoid uncontrollable phytoestrogen effects. Surgical procedures were performed under isoflurane- (for OVX) or ketamine-anaesthesia (93% ketamine, 5% xylazine, 2% acepromazine; for stereotaxic wax pellet implantations). All animals were handled and their weight monitored every other day for the duration of both experiments.

Experimental design and surgical procedures

All rats underwent bilateral OVX through the dorsal approach one week after arrival, to remove circulating gonadal steroids, and to ensure a constant, high level of ERbeta expression within the brain (Suzuki and Handa, 2005). Chronic 8-day systemic or local, intracerebral treatment with ER ligands began one week after OVX. The animals' weight was measured every other day during

the treatment period, and all animals were double housed with a partner of equal treatment throughout each experiment.

Experiment 1: Systemic DPN treatment

Rats were injected s.c. with ERbeta agonist diarylpropionitrile (DPN, 2mk/kg, n=8) or vehicle (27% hydroxypropyl-beta-cyclodextrin in PBS, n=8) once per day at 0600 h. DPN was synthesized *de novo* following an established protocol (Lund et al., 2005). While estrogen binds to both ERalpha and ERbeta with almost the same affinity, the relative binding affinity of the selective agonist DPN is about 70 to 80-fold stronger for ERbeta than for ERalpha (Kuiper et al., 1998; Meyers et al., 2001). On days 6, rats were tested for anxiety-like behavior in the open field (OF) and on day 7 on the elevated plus maze (EPM). All animals were killed by decapitation on day 8 under basal, non-stress conditions between 1000 and 1200 h (4 h after the last DPN injection to avoid acute effects of steroid treatment). Their brains were removed, immediately fresh-frozen in dry-ice-cooled methylbutane (-40° C) and stored at -80° C until sectioning.

Experiment 2: Local DPN treatment

Three groups of rats were stereotaxically implanted bilaterally with wax pellets (each 2.0 mm long to target the ventro-dorsal extent of the DRN sufficiently) flanking the dorsal raphe. Stereotaxic coordinates were 8.0 mm posterior to bregma, ± 1.5 mm lateral of the skull's midline, 5.5 mm deep; and pellets were lowered into the brain at a 7° angle. Each pellet contained either 0.5 µM DPN (n=10), 0.5 µM 17-beta-estradiol (Sigma, St. Louis, MO; E, n=10) or

beeswax only (VWR International, Bristol, CT; vehicle control, n=10). To prepare the pellets for implantation, the tip of a sterile 22-gauge stainless steel outer cannula (Small Parts, Miami Lakes, FL) was packed with the respective compound, which was then lowered into the brain according to the stereotaxic coordinates mentioned above. A 28-gauge inner stylet was used to slowly expel the pellet from the outer cannula. After pellet implantation, the outer cannula was removed and the scalp sutured. At the time of the behavioral testing in the FST, the cranial incision site had healed completely on all individuals. Lund et al. (Lund et al., 2006) verified the diffusion of DPN and other steroids to be contained within a 0.5 mm radius around the pellet, ensuring that the compounds used in the present study successfully diffused into all rostro-caudal and medial-to-lateral subregions of the DRN without damaging any tissue within the target nuclei. Another control group of OVX animals remained unoperated for comparison with behavioral and cellular parameters of operated animals. All animals were tested in the OF and on the EPM on days 5 and 6 of treatment. On day 7, rats were subjected to the forced-swim test (FST) to assess despair-like behavior. As in the first experiment, all animals were killed by decapitation on day 8 between 1000 and 1200 h under basal, non-stress conditions, their brains removed, fresh-frozen and stored at -80° C until sectioning.

Behavioral testing & evaluation

All rats were tested in the open field (OF) and on the elevated plus maze (EPM) on two consecutive days for 5 min each, between 1000 and 1200 h. Both

the OF and the EPM are designed to assess anxiety-related behavior by creating a conflict situation between the rodent's natural explorative drive and its innate fear of open, exposed areas (Pellow et al., 1985).

The OF is an 80 x 80 cm open square box with 30 cm tall walls. Symmetrical lines drawn on the bottom of the box, divide the box floor into "protected" outer squares (adjacent to the walls) and "exposed" center squares. Light intensities were 65 lx in all squares facing the wall, and 80 lx in the center spare. At the beginning of the test, each animal was placed in the center of the OF. The following parameters were scored (Handa et al., 1993): locomotor activity (total of square line crossings), rears at walls, time spent in (exposed) center squares, time spent in (protected) outer squares, time spent grooming, number of fecal boli. Time spent in the center of the OF, and an increased number of rears, are both considered low-anxiety explorative behavior. More fecal boli and long grooming periods usually indicate a state of elevated anxiety or displacement-activity, respectively.

The EPM consists of a plus-shaped platform at about 80 cm elevation with two opposing closed arms (arms about 80 cm long with 30-cm tall walls) and two opposing open arms (no walls). All four arms are connected via a 10 x 10 cm neutral zone in the middle of the maze. Light intensities were 25 lx in the closed, 80 lx in the open, and about 60 lx in the neutral zone. Each rat was placed onto the mace facing one of the closed arms. The latency until first open arm entry, the time spent in the open and closed arms, the number of closed and open arm entries, the time spent grooming, and the number of fecal boli were recorded

(Handley and McBlane, 1993). The number of closed arm entries is generally used to describe overall locomotion and activity of the animal. The number of open arm entries and the time spent in the open (exposed) arms are evaluated as low-anxiety-like behavior, the time spent in the closed arms and an elevated number of fecal boli as high-anxiety-like behavior. Since grooming occurred in closed and for some animals in open arms, it was referred to as neutral (neither high- nor low-anxiety-like) behavior.

All rats of the second experiment were also exposed to the forced swim test (FST) for 5 min on day 7 after intracerebral pellet implantation. The FST is based on a rationale for testing and interpreting despair-like behavior in rodents (Porsolt et al., 2001). We intended to distinguish between mere explorative versus timid behavior (EPM and OF) on the one hand, and active versus passive, depressed behavior (FST) on the other hand. In accomplishment of the latter, the FST applies a strong physical challenge for the evaluation of active versus passive stress coping behaviors (Keay and Bandler, 2001). All animals swam for 5 min in tap water with a consistent temperature of 25 °C, and were removed and dried with a clean towel afterwards. The time paddling (normal stress-coping behavior: slow-pace front and hind leg movements to keep the nose over water), the time struggling (active stress-coping behavior: high-pace front leg paddling and strong hind leg strokes with the intension to escape the situation), the time spent floating (passive, despair-like stress-coping behavior: minimal leg movements, stiff, floating body posture) and the number of dives (active stress-

coping, exit-seeking behavior) was recorded. After each behavioral test, animals were returned to their home cages and housed with the same partner as before.

RNA isolation & RT-PCR

For the production of a riboprobe specific for tryptophan hydroxylase 2 (TPH2) mRNA, fresh-frozen brains from three separate female OVX Sprague-Dawley rats were cryocut (Leitz 1720 digital cryostat) at –12° C from bregma -6.5 mm to – 9.5 mm into 300-µm thick coronal brainstem sections. DRN tissue was collected from these sections via the micropunch sampling procedure (Handa et al., 1987; Price et al., 2000), using a blunted needle with a 1000-µm diameter and a dry-ice-cooled stage. The micropunched tissue samples were immediately transferred and pooled into nuclease-free microcentrifuge tubes with 250 µl GIT buffer (4 M guanidine isothiocyanate, 25 mM sodium citrate at pH 7.0, 0.5% sarcosyl, and 0.1 M beta-mercaptoethanol). Total RNA isolation from microdissected brain tissue was conducted on ice according to the protocol established by Chomczynski and Sacchi (1987). First, the tissue-buffer mixture was homogenized mechanically. Subsequently, 25 µl of 2 M sodium actetate (pH 4.0), 250 µl buffer-saturated phenol (pH 4.3) and 75 µl of chloroform–isoamyl alcohol at a 49:1 ratio were added. After vortexing, the reaction was allowed to sit on ice for 15 min, and was then spun at 14,000 *g* for 10 min to recover the aqueous phase. RNA was precipitated with ethanol, resuspended in GIT buffer, and precipitated a final time. RNA pellets were isolated in a last centrifugation step at 14,000 *g*, followed by two short wash steps with ice-cold 70% ethanol.

Total RNA was reconstituted in 20 µl RNAse-free water each and nucleic acid concentration determined at a spectrometer. 1 µg RNA was reverse transcribed with MMLV-RT (Invitrogen, Carlsbad, CA) using 1 µl oligo dT primers, dNTPs (100 mM each), 1st strand buffer (100 mM Tris–Cl–900 mM KCl–1 mM MgCl) and 2.5 mM DTT. The reaction was carried out at 37° C for 50 min, followed by heat-denaturation of the reverse transcriptase for 10 min at 95° C. The product, total cDNA from the DRN region, was stored at -20° C for later use.

Design of the TPH2 riboprobe

To generate a TPH2-specific plasmid DNA template for cRNA synthesis, a 583 bp fragment of the TPH2 cDNA was amplified by RT-PCR (forward primer: 5'-GGG GTG TTG TGT TTC GGG-3', reverse primer: 5'-GTG GTG ATT AGG CAT TCC-3'). Several online BLASTs, researching the 583 bp TPH2 cDNA sequence in an NIH-supported database (http://blast.ncbi.nlm.nih.gov), did not return any other matching sequences besides the gene of interest. PCR conditions were: 45 s denaturation at 95° C, 45 s annealing at 55° C, and 45 s elongation at 72° C. After 35 cycles, a final 7-min elongation step at 72° C was added. The 50 µl PCR reaction volume contained 1.5 mM Mg^{2+}, 0.2 mM dNTPs, 0.2 µM forward and reverse primer, 50 ng template cDNA, and 1.0 unit Taq DNA polymerase (Eppendorf, Westbury, NY). The PCR product was gel-purified (Qiagen, Valencia, CA) and TA-subcloned into the linearized 4.0 kb TOPO-vector pCR®II (Invitrogen, Carlsbad, CA) via the vector-attached topoisomerase I. The plasmid was then transformed into chemically competent TOP10 bacterial cells

(Invitrogen) for vector-amplification and selection of successful clones (ampicillin resistance and “white”-selection due to loss of lacZ gene expression). Successful clones were verified using restriction enzymes cutting within the desired insert and subsequent gel-electroporesis, and via sequencing (Retrogen, San Diego, CA). Sequencing also revealed the orientation of the cDNA insert within the vector. Once the sequence had been confirmed, a plasmid MAXI-prep (Qiagen) was performed to yield sufficient amounts of cDNA template. Antisense and sense (control) TPH2 cRNAs were transcribed from the plasmid in the presence of [^{35}S]-UTP, using either the restriction enzyme *Blp*I and T7 RNA-polymerase, or *Xba*I and Sp6 polymerase respectively. The specificity of the riboprobe was confirmed in two hybridisation test runs. The cRNA probe successfully detected TPH2 mRNA within the brainstem raphe nuclei, but did not hybridize within sections of the pineal gland where the peripheral isoform of the gene, TPH1, is expressed (Patel et al., 2004; Malek et al., 2005). The sense control probe did not hybridize with any specific areas in the brain.

Tissue preparation & In situ-hybridization (ISH)

A Leitz 1720 digital cryostat (-20° C) was used to cut and thaw-mount series of coronal 16-µm brainstem sections beginning at bregma –6.5 mm to –9.5 mm (Paxinos and Watson, 1998), covering the entire rostro-caudal extent of the DRN. Sections were thaw-mounted onto Superfrost plus slides (VWR Scientific, West Chester, PA), and stored at -80° C until assayed. For the ISH assay, tissue sections were thawed at room temperature, fixed within 10% paraformaldehyde,

acetylated with 0.25% acetic anhydride, dehydrated in a graded series of alcohols, and air-dried to prepare the tissue for the hybridization step. Sections were then incubated for 2 h in the pre-hybridization solution (50% formamide, 0.60 M NaCl, 0.02 M Tris, 0.01 M EDTA, 10% dextran sulfate, 2 M Denhart's solution, 50 mM dithiothreitol, 0.2% SDS, 100 mg/ml salmon testis DNA, 500 mg/ml total yeast RNA, and 50 mg/ml yeast transfer RNA) and again dehydrated. In the following step, sections were incubated with the actual hybridization solution, containing all components of the pre-hybridization solution plus the radiolabeled cRNA at a concentration of 2×10^7 cpm/ml, in moisturized chambers at 60° C overnight. After hybridization, slides were rinsed in 2 x SSC. Remaining non-hybridized RNA was digested in a 30 mg/ml RNase A solution for 30 min at 37° C. A final wash series in 0.1 x SSC preceded the final dehydration in graded alcohols.

Hybridization was first validated by opposing slides to a ^{35}S-sensitive Biomax MR film (Kodak, Rochester, NY) for 14 hours. Subsequently, hybridization was detected using photographic-emulsion-coated slide autoradiography. In a humidified dark room, all slides were slowly dipped twice into preheated (40° C) photographic emulsion (NTB-3; Kodak), air-dried, and stored at 4° C in the dark for 2 days. All slides were then developed using Kodak D-19 photographic developer, before counterstaining with cresyl violet.

Validation of pellet implantation

For single-cell identification and neuroanatomical validation of wax pellet placement, all sections were counterstained with 0.013% cresyl violet in 0.2 M

Walpole buffer (3 parts 0.2 M acetic acid and 2 parts 0.2 M sodium acetate) at pH 4.45. Sections were stained for 20 min, dehydrated in ascending alcohols, cleared in xylene, and mounted with Permount (Fisher Scientific, Pittsburg, PA). After evaluating each DRN series (12 sections per animal), using bright-field microscopy, the center location of each pellet was estimated and mapped in the corresponding coronal section in the rat brain atlas (Paxinos and Watson, 1998). Animals with pellets placed more than 0.5 mm away from the DRN in any direction were excluded from all data analysis. This criterion was defined empirically by Lund et al. (2006) who found the diffusion of [^{3}H]-labelled E to be confined within a 0.5 mm area surrounding the wax pellet. Based on this parameter for steroid diffusion, one of the individuals of the vehicle-group (now n=9) was excluded for analysis. Every other bilateral pellet placement passed the neuroanatomical criterion. See Fig. 13 for details.

Image analysis & Quantification of mRNA expression

All digital bright-field or dark-field images of cresyl-violet-counterstained, hybridized brain sections were captured by a Zeiss AxioCam HR camera on an Axioplan 2 microscope controlled by Axiovision, version 3.1, software. Three rostral, three mid and three caudal sections per animal were anatomically matched with the corresponding page in the rat brain atlas (Paxinos and Watson, 1998) and used for further image analysis with the help of ImageJ software (version 1.31, National Institute of Health, http://rsb.info.nih.gov). Matrices in shape of the brain regions of interest (see Fig. 11) were utilized to assess TPH2

mRNA expression within all subregions of the DRN via two different approaches. After subtraction of background activity (determined in an adjacent area devoid of silvergrain labelling), six (lateral mid DRN) or three values (dorso-rostral, ventro-rostral, dorso-mid, ventro-mid, dorso-caudal, ventro-caudal) per animal were averaged for each subregion. In the first approach (from here on called “area-analysis”), following inversion of the dark-field image, the area covered by black pixels was determined. Digital image inversion was background-corrected and standardized at the same inversion-threshold. The second approach (from here on called “intensity-analysis”) assessed single-cell intensity of mRNA expression by directly measuring the intensity of light reflected from the silvergrain-coverage of the cell of interest. Cells were picked randomly via a symmetrical grid overlaying the respective subregion. Only cells on grid crossings were analysed. Ultimately, the average intensity per cell and subregion was calculated.

Statistical Analysis

All data are expressed as the mean ± standard error of the mean (SEM). For studies with only two treatment groups, the non-parametric Mann-Whitney-*U* test was used for pair wise data comparison. Results of all studies with more than two groups were analyzed by one-way ANOVA (factor treatment) followed by Tukey’s post hoc test where appropriate, using SPSS 12.0 for Windows software. Results were considered significant at $p < 0.05$.

RESULTS

Weight gain

The overall weight gain (day 8 minus day 1) during the two experiments is shown in Table 2. Neither systemic delivery of DPN nor central, site-specific administration into the DRN caused significant differences in weight gain, compared to vehicle controls. Similarly, local delivery of E into the DRN did not impair normal weight gain. All E-treated rats gained the same amount of weight as vehicle controls.

Wax-pellet implantation *per se* did not affect the animals' weight gain either, as it becomes obvious in the comparison with unoperated control rats (Table 2).

Systemic delivery of DPN decreases anxiety-like behavior, but local DPN administration does not

The animals' anxiety-like behavior was tested in two different paradigms: the elevated plus maze (EPM), and the open field (OF). Both behavioral tests confirmed the anxiolytic effect of DPN when administered systemically (Fig. 8). On the EPM, DPN treatment caused the rats to enter the open arms sooner ($p < 0.01$), more often ($p < 0.05$), and stay on the open arms longer ($p < 0.05$) than vehicle controls (Fig. 8 A). The number of entries into the closed arms did not differ between the two treatment groups, indicating that DPN did not increase the rats' overall activity or locomotion behavior. None of the other parameters (time

grooming, time in closed arms, fecal boli) showed significant differences between any of the treatment groups.

In the OF (Fig. 8 B), s.c. DPN-treated animals displayed more rears at the walls than their vehicle-treated counterparts ($p < 0.01$). The total number of square line crossings did not differ between the two treatment groups, suggesting that there was no overall effect on activity. The time spent grooming and the numbers of fecal boli were not significantly different between the treatment groups.

Local, site-specific delivery of DPN or E did not alter any of the parameters measured in the EPM and the OF, compared to the vehicle group (Fig. 9, A and B). The animals from the unoperated control group did not differ from the vehicle control group in any of the behavioral tests (data not shown).

Local delivery of both DPN or E enhances active stress-coping behavior

Rats that were implanted with bilateral E- or DPN-pellets flanking the DRN, spent more time actively struggling in the water than did vehicle controls (Fig. 10; $F_{3,32}=4.628$; $p < 0.05$). Accordingly, DPN-treated rats tended to spend less time passively floating than vehicle-treated rats. Local E-treatment also caused the rats to float more than animals of the DPN group ($F_{3,32}=3.489$; $p < 0.05$). Animals of all treatment groups paddled (neutral stress-coping behavior) equal amounts of time. The number of dives did not differ between any of the groups.

Systemic DPN treatment increases TPH2 expression in the caudal and dorso-mid DRN

To test the hypothesis that local, site-specific ERbeta activation in the DRN is sufficient to upregulate TPH2 mRNA expression, ISH was performed. Two different types of image analysis, measuring either the area positive for TPH2 expression (Fig. 11 B), or single-cell intensity of TPH2 expression (Fig. 11 D), were applied.

Results from area-analysis: Compared to vehicle-treated animals, daily s.c. administration of DPN significantly enhanced TPH2 mRNA levels in the dorso-mid ($p < 0.05$), the dorso- caudal ($p < 0.05$) and the ventro-caudal ($p < 0.05$) DRN (Table 3 A). Accordingly, total TPH2 mRNA levels in the entire caudal DRN were significantly increased in the DPN group ($p < 0.01$; Fig. 11 A).

Results from single-cell intensity-analysis: The intensity-based analysis of TPH2 revealed significant differences in the same subdivisions of the DRN as the area-analysis. Compared to vehicle-treated animals, systemic DPN-treatment significantly enhanced TPH2 mRNA levels in the dorso-mid ($p < 0.01$), the dorso-caudal ($p < 0.01$) and the ventro-caudal ($p < 0.05$) DRN (Table 3 B). Accordingly, total TPH2 mRNA levels in the entire caudal DRN were significantly higher in the DPN group ($p < 0.05$; Fig. 11 C).

TPH2 mRNA expression in the rostral DRN was not elevated by DPN. In contrast, all rostral DRN-subregions were found to express slightly less TPH2 mRNA than vehicle controls (Table 3). This was also consistent throughout both types of image analysis. Fig. 12 displays representative dark-field

photomicrographs of TPH2 mRNA hybridization in the DRN of systemically DPN- or vehicle-treated rats.

Evaluation of ISH and placement of wax pellets flanking the DRN

To identify individual cells for the single-cell-analysis of TPH2 mRNA expression tissue was cresyl-violet-counterstained at an intensity sufficient to label the nucleus, but not dark enough to interfere with the counting and identification of silver grains. As shown in Fig. 13, cell nuclei could be easily identified (Fig. 13 C).

Fig. 13 also shows original bright-field (A) and dark-field (B) photomicrographs of the bilaterally implanted wax pellets placed to flank the DRN. A schematic of actual bilateral wax pellet localization in the brainstem of all animals in experiment 2 is depicted in Fig. 13 D. If the center of any of the two pellet was found to be more than 0.5 µm away from the midline of the DRN, the respective animal was excluded from all data analysis. This was true for one vehicle-treated rat (black dots, Fig. 13 D), which reduced the size of this group from 10 to 9 animals.

Local ERbeta activation further potentiates DPN's effect on TPH2 expression

When individual subregional area-values (Table 4 A) of TPH2 mRNA expression in the rostral, mid-, and caudal DRN were summarized (Fig. 14 A), it became apparent that local DPN-treatment had more than doubled the expression of TPH2 mRNA in the entire caudal DRN, compared to vehicle

($F_{3,32}$=6.040; $p < 0.01$) and unoperated controls ($p < 0.05$). In detail (Table 4 A), a more than 3-fold increase of the enzyme's expression in the dorso-caudal ($F_{3,32}$=13.720; $p < 0.01$) and a 2-fold increase in the ventro-caudal DRN ($F_{3,32}$=14.962; $p < 0.05$) had occurred, compared to vehicle-treated animals. In the dorso-mid DRN, local DPN-administration had also elevated TPH2 mRNA expression to about 2.5-fold of the amount expressed in vehicle controls (Table 4 A; $F_{3,32}$=18.197; $p < 0.01$), resulting in an about 1.7–fold overall increase of TPH2 mRNA expression in the entire mid-DRN, compared to vehicle- and unoperated controls (Fig.14 A; $F_{3,32}$=9.096; both $p < 0.01$).

Intensity-analysis (Fig. 14 B) revealed similar results as the area-analysis, regarding TPH2 mRNA expression in the rostral, mid- ($F_{3,32}$=5.830) and caudal ($F_{3,32}$=7.020) DRN after local DPN-treatment. In detail (Table 4 B), the average single-cell intensity within the dorso- ($F_{3,32}$=9.720) and ventro-mid DRN ($F_{3,32}$=5.502), as well as the dorso- ($F_{3,32}$=9.708) and ventro-caudal DRN ($F_{3,32}$=8.070) was significantly elevated, compared to vehicle-controls and - with exception of the ventro-mid DRN - versus E-treated animals ($p < 0.01$ each).

TPH2 mRNA expression in the rostral DRN was not elevated by DPN. Rather, all rostral DRN-subregions were found to express slightly less TPH2 mRNA than vehicle controls and E-treated animals. This was also consistent between the two types of analyses.

E-treated rats displayed similar, but weaker ($p < 0.05$ each) overall effects on TPH2 mRNA expression as the DPN group (Fig. 14 A: area-analysis of the mid-DRN $F_{3,32}$=7.902; Fig 14 B: intensity-analysis of the mid-DRN $F_{3,32}$=3.870,

and for the caudal DRN $F_{3,32}$=4.090). In specific, E significantly ($p < 0.05$ each) elevated the enzyme's expression in the dorso-mid (area-analysis: $F_{3,32}$=10.007; intensity-analysis: F=5.202) and in the dorso-caudal DRN (intensity-analysis: $F_{3,32}$=7.763), compared to the vehicle-treated group (Table 4 A and B).

Fig. 15 shows representative dark-field photomicrographs of TPH2 mRNA hybridization in the brainstem of locally DPN-, E-, or vehicle-treated rats.

FIGURES & TABLES:

Table 2

Weight gain of all animals during experiments 1 and 2

	Delta weight (day 8 minus day 1)			
	C (unoperated controls) (7)	Vehicle (8) (9)	E (10)	DPN (8) (10)
Study I systemic treatment	n.a.	25.16 ± 2.79	n.a.	31.17 ± 3.90
Study II local treatment	28.57 ± 1.87	25.00 ± 2.21	25.80 ± 3.30	30.50 ± 2.25

Weight gain between day 1 and day 8 of all animals used in study I (systemic, subcutane delivery of DPN versus vehicle) and study II (local, intracerebral application of DPN, estradiol (E) or vehicle. Numbers in parenthesis indicate group size of first (upper number) and second experiment (lower number). Values are shown as group means ± SEM. N.a. = not applicable.

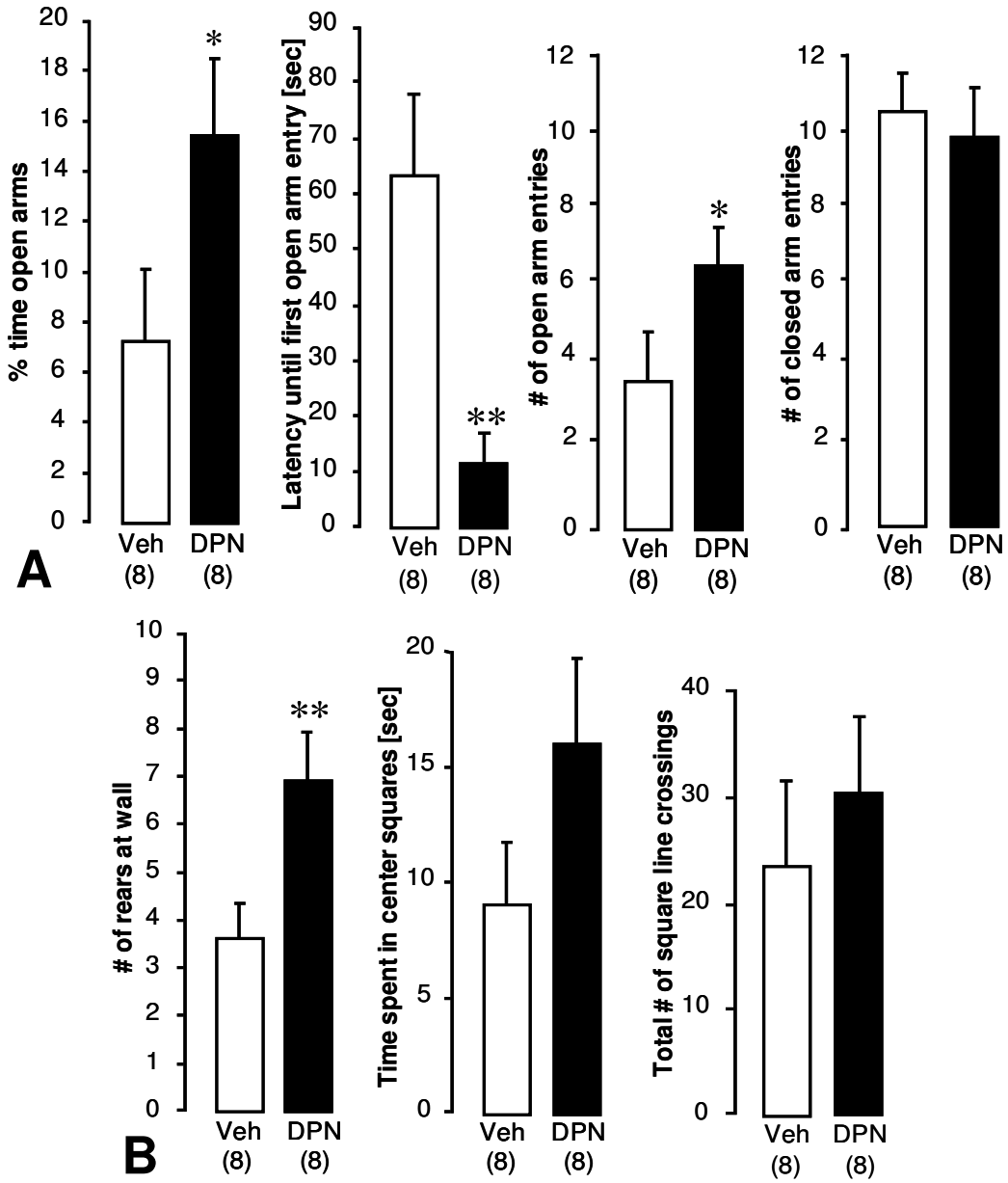


Figure 8. Systemic (subcutane) delivery of ERbeta agonist DPN was anxiolytic when animals were tested on the elevated plus maze (EPM, A) and in the open field (OF, B). Panel A displays the percent time the animals spent in the open arms of the EPM, the latency until the first open arm entry, the number of open arm entries and the number of closed arm entries (left to right). Panel B shows the number of rears at the walls of the OF, the time the animals spent in the center squares, and the total number of square line crossings. Each column represents the group mean ± SEM. Numbers in parentheses indicate group size. * ($p < 0.05$) and ** ($p < 0.01$) indicate significant differences versus vehicle controls (Mann-Whitney-U test).

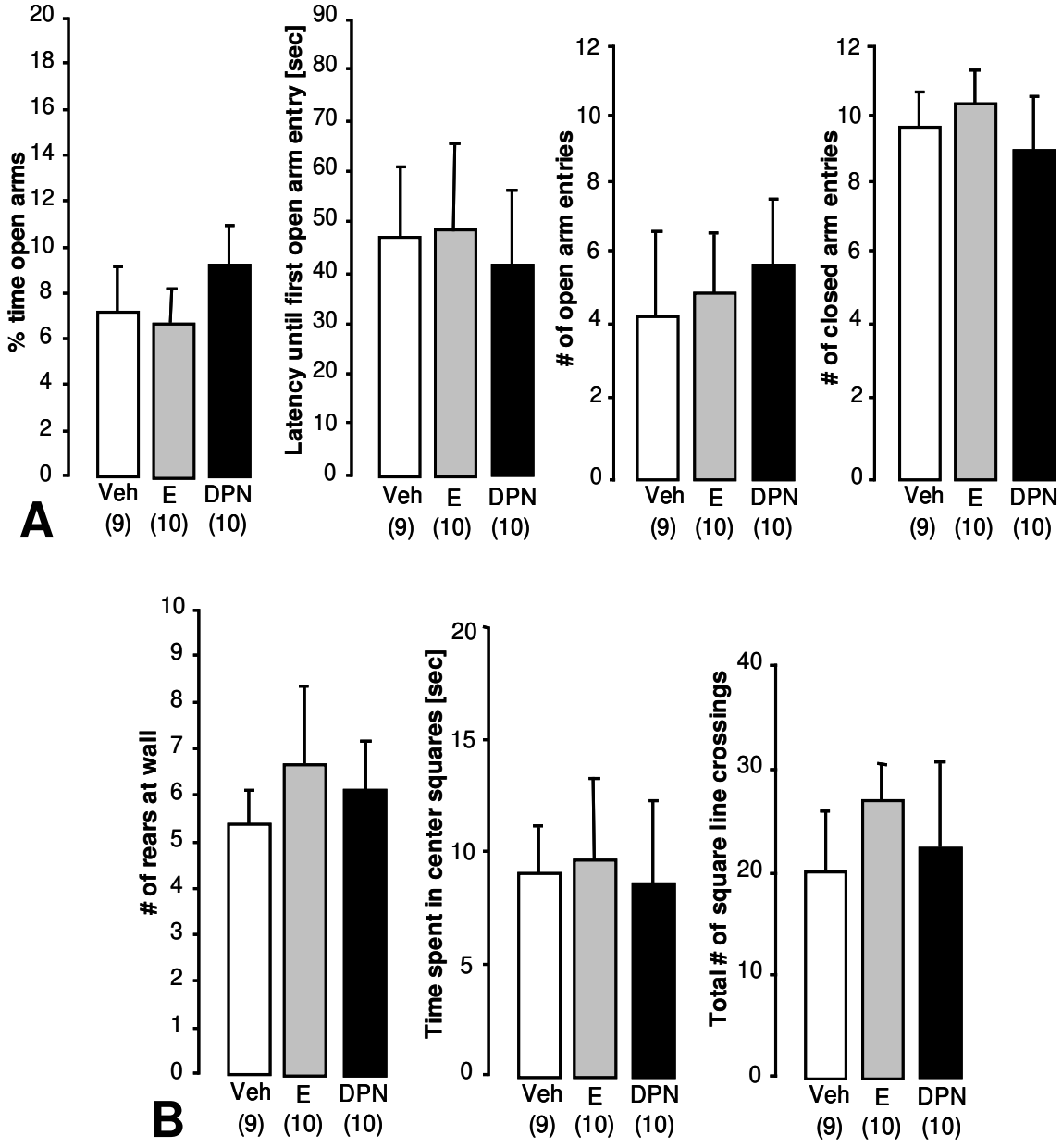


Figure 9. Effect of local (wax pellet-mediated) DPN- or E-treatment on behavior on the elevated plus maze (EPM, panel A) and the open field (OF, panel B), compared to vehicle control animals. Panel A displays (left to right) the percent time the animals spent in the open arms of the EPM, the latency until the first open arm entry, the number of open arm entries and the number of closed arm entries. Panel B shows the number of rears at the walls of the OF, the time the animals spent in the center squares, and the total number of square line crossings. Each column represents the mean ± SEM for 9-10 animals per group. Numbers in parentheses indicate group size. No significant differences were found between any of the treatment groups (ANOVA, factor treatment).

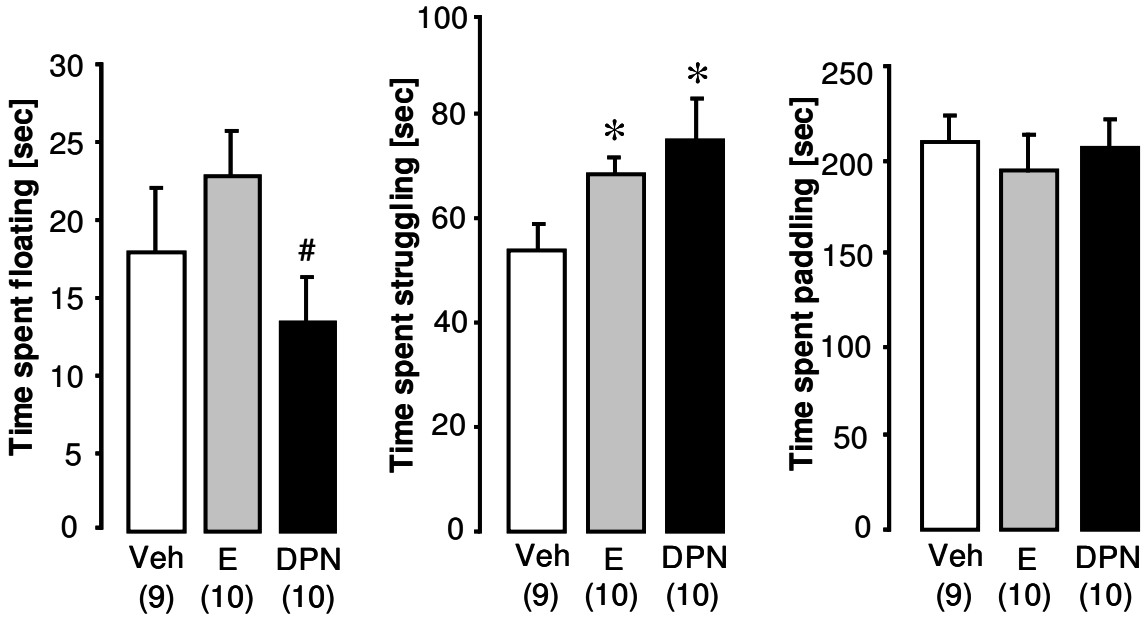


Figure 10. Effects of local (wax pellet-mediated) E- and DPN-treatment of female OVX rats in the forced swim test. Time spent floating (left), struggling (middle) and paddling (right) are shown. Each column represents the mean ± SEM for 9-10 animals per group. Numbers in parentheses indicate group size. * indicates significant difference ($p<0.05$) versus vehicle controls; # indicates significant difference ($p<0.05$) versus the E-treated group. ANOVA, factor treatment, was performed, followed by Tukey's *post hoc* test where appropriate.

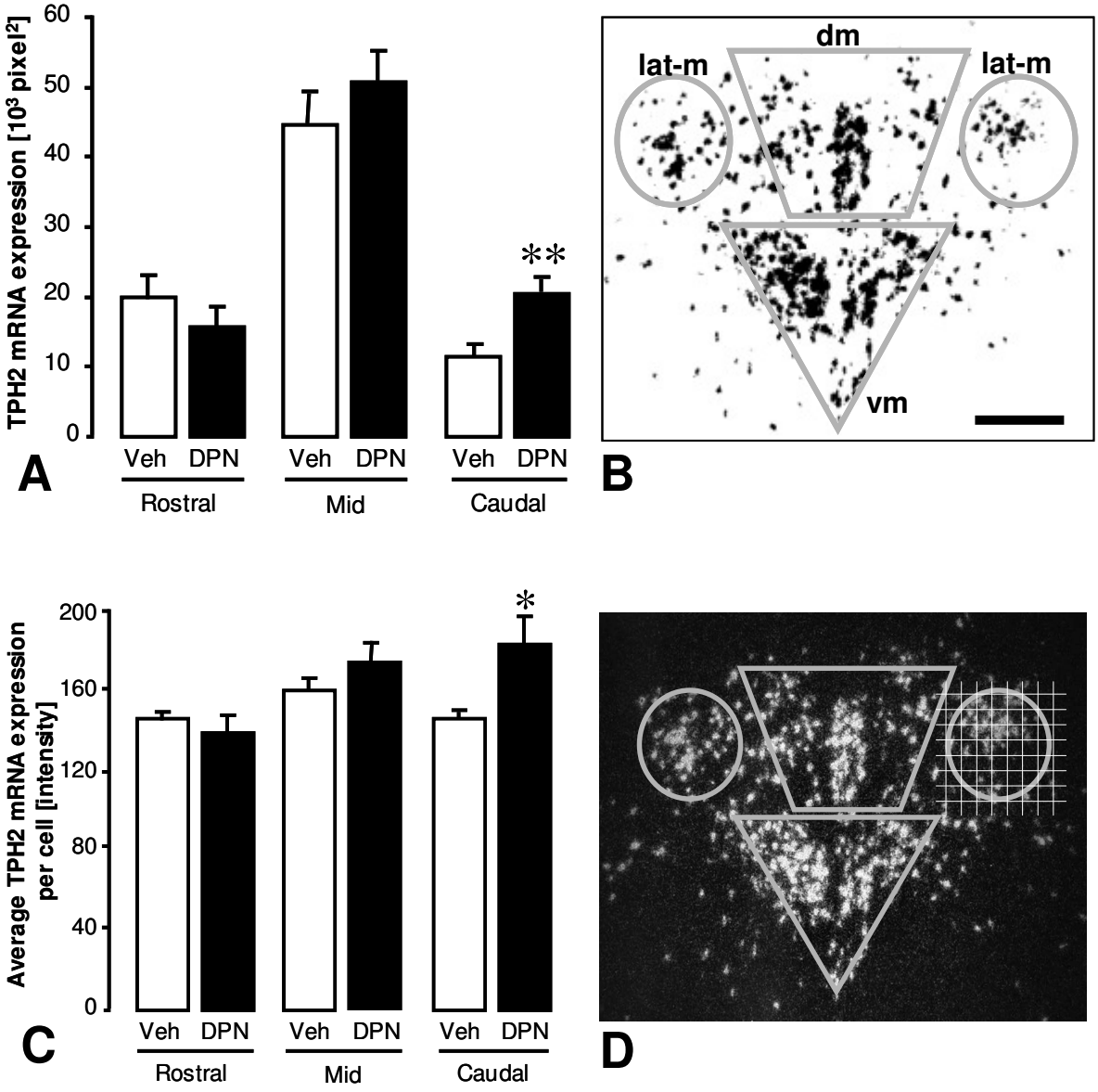


Figure 11. Systemic (subcutane) DPN-treatment of OVX females significantly enhanced the expression of TPH2 mRNA in the caudal, but not in the rostral DRN, compared to vehicle controls. All columns represent means ± SEM of n=8 per group. * ($p < 0.05$) and ** ($p < 0.01$) indicate significance versus respective vehicle controls (Mann-Whitney-U test). Two types of analyses were used to measure TPH2 mRNA expression. A: Results of the area-based analysis of TPH2 expression in the rostral, mid and caudal DRN of s.c. DPN- or vehicle-treated rats. B: Schematic representation of the area-analysis of TPH2 expression. The area of black pixels^2 in each of the subregions of the DRN (gray matrices) was measured in inverted, normalized dark-field pictures of the silvergrain-labeled brain regions, and subsequently summarized for total square-

pixels (pixels^2) in the rostral, mid and caudal DRN. dm = dorso-mid DRN, vm = ventro-mid DRN, lat-m = lateral mid-DRN. For individual subregional values see Table 3. Scale bar: 40 µm. C: Results of the intensity-based analysis of TPH2 mRNA expression in the rostral, mid and caudal DRN of s.c. DPN- or vehicle-treated rats. D: The intensity-analysis evaluated the average single-cell TPH2 mRNA expression per subregion (gray matrices) by measuring the intensity of the reflected light. All cells were selected randomly (only cells lying on cross lines of the white grid were analyzed). For individual subregional values see Table 3.

Table 3

Systemic delivery of DPN increases TPH2 expression in DRN subregions

A	TPH2 mRNA expression per area [pixel^2]	
DRN subregion	Vehicle (n=8)	DPN (n=8)
dorso-rostral	8100 ± 1260	6158 ± 1194
ventro-rostral	10214 ± 1382	9872 ±1394
dorso-mid	7808 ± 721	10710 ± 1105 *
ventro-mid	30810 ± 3515	34065 ± 2546
lateral mid	6574 ± 960	6123 ± 666
dorso-caudal	5120 ± 768	9965 ± 1931 *
ventro-caudal	6220 ± 831	10602 ± 1250 *

B	Average TPH2 mRNA expression per cell [pixel-intensity]	
DRN subregion	Vehicle (n=8)	DPN (n=8)
dorso-rostral	143.3 ± 10.4	135.9 ± 3.3
ventro-rostral	141.8 ± 21.3	126.0 ± 7.1
dorso-mid	207.0 ± 6.5	242.2 ± 4.2 **
ventro-mid	233.1 ± 15.8	239.4 ± 5.7
lateral mid	191.3 ± 3.3	198.6 ± 6.6
dorso-caudal	142.4 ± 6.7	170.6 ± 5.9 **
ventro-caudal	140.7 ± 18.0	182.0 ± 12.7 *

Part A lists individual subregion-specific values for TPH2 mRNA expression in the DRN, determined via area analysis [pixel^2]. Part B lists individual subregion-specific values for TPH2 mRNA expression, using single-cell analysis [pixel-intensity]. Animals were treated subcutaneously with DPN or vehicle. Each value listed represents the mean ± SEM. Numbers in parentheses indicate group size. * ($p < 0.05$) and ** ($p < 0.01$) indicate significance versus vehicle controls. Mann-Whitney-U test was performed between the two groups for all regions.

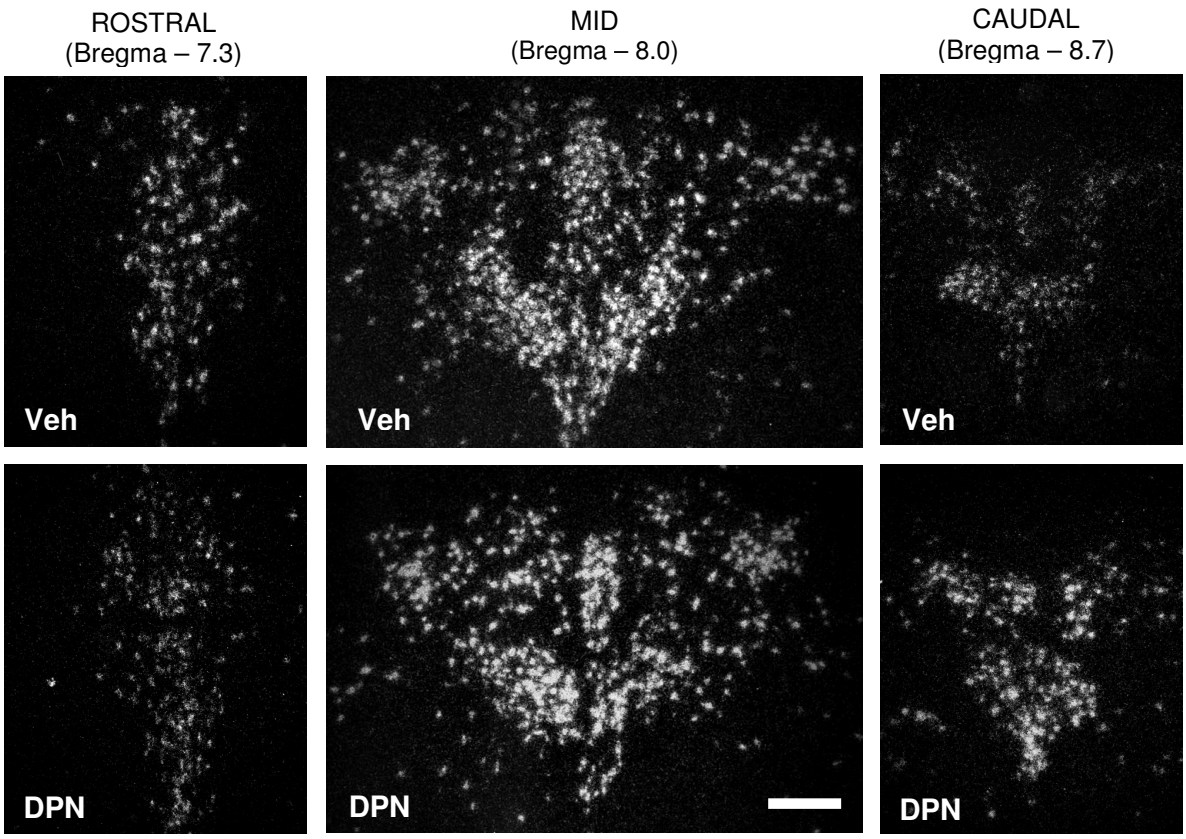
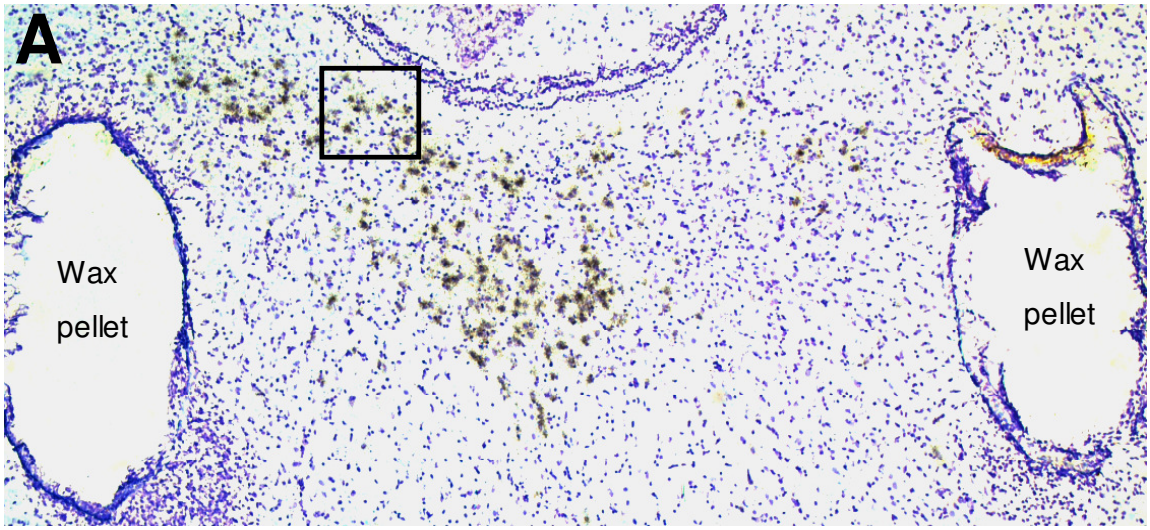


Figure 12. Representative dark-field pictures of TPH2 mRNA in the rostral (left side, bregma –7.3), mid- (middle, bregma –8.0), and caudal DRN (right side, bregma –8.7) of subcutaneously vehicle- or DPN-treated OVX animals. Vehicle-treated animals are shown in the top row of panels, DPN-treated rats in the lower row of panels. Scale bar: 40 µm.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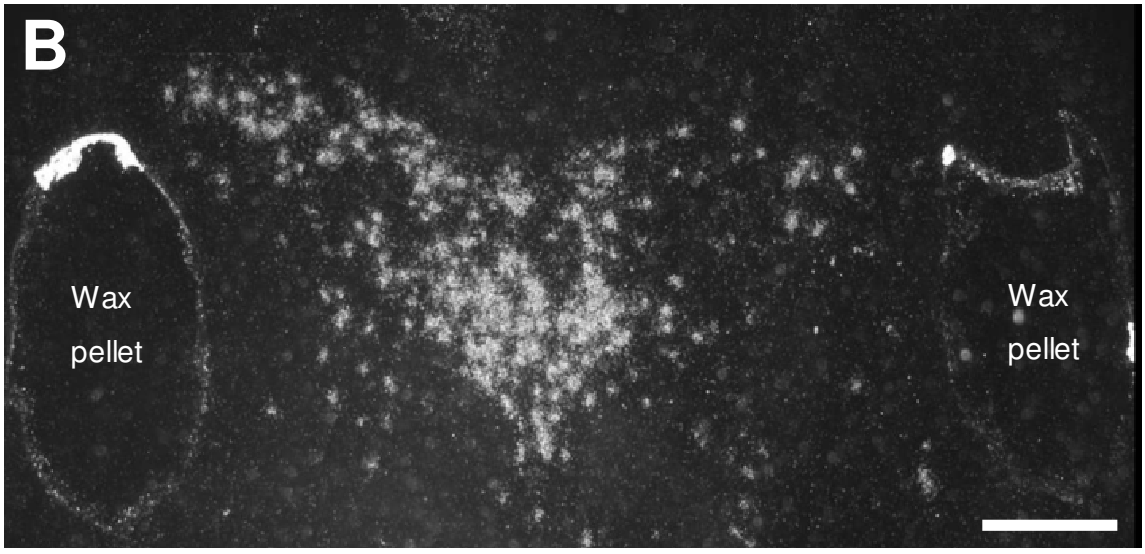
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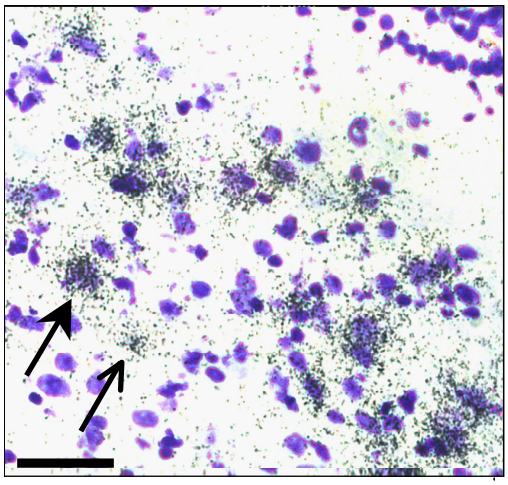

C

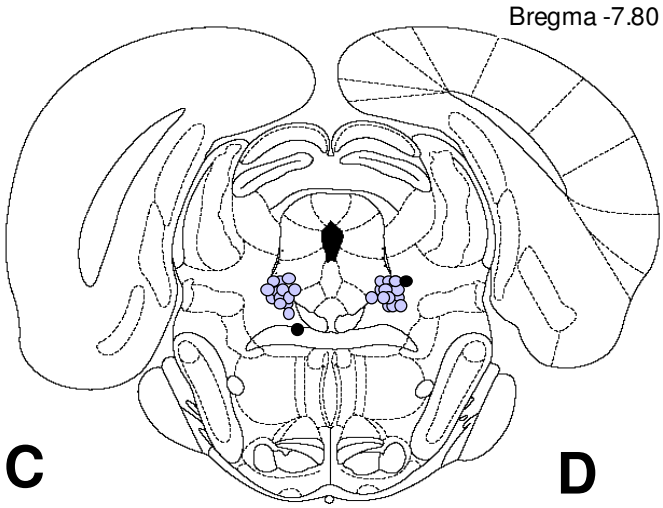
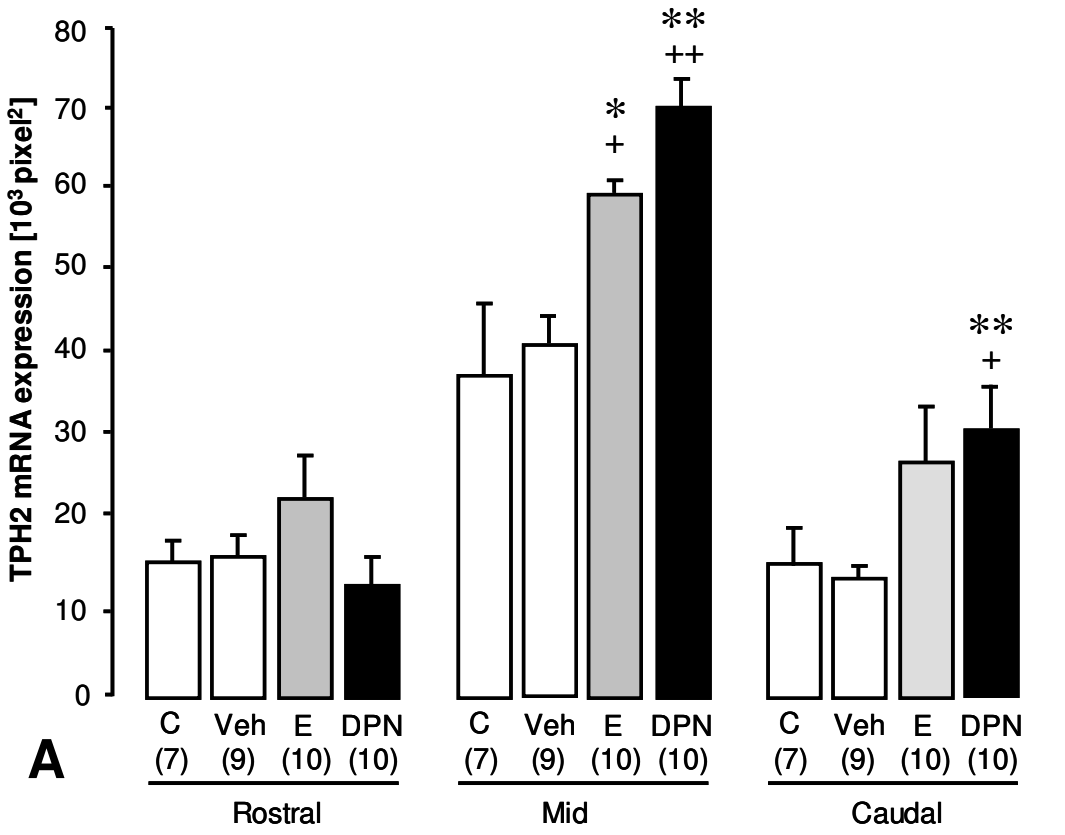

Bregma -7.80
D

Figure 13. Localization of wax pellets implanted in experiment 2. Wax pellets containing 0.5 µM DPN or 0.5 µM E were stereotaxically implanted left and right of the DRN. Bright-field (A) and dark-field (B, scale bar: 80 µm) images of silvergrain-labeled (small black grains) cells hybridized with a riboprobe detecting TPH2 mRNA, counterstained with cresyl-violet (purple), are shown. C: Magnification of the small square from picture A (scale bar: 40 µm). The filled arrow points to a silvergrain cluster above a cell nucleus (purple), the unfilled arrow points to a silvergrain cluster without an underlying nucleus. All single cell values for TPH2 expression were validated via positive nuclear (purple) counterstaining. The minimum spread of a compound diffusing from the pellet was estimated to be 0.5 mm (Lund et al. 2006). D: Schematic picture of the rat brainstem at Bregma -7.80 mm (Paxinos & Watson 1998). Each gray dot represents the center of an implanted wax pellet. The two black dots were considered outsiders, and stand for one individual that was excluded from data analysis.


TPH2 mRNA expression [10³ pixel²]
80
70
60
50
40
30
20
10
0
**
++
*
+
**
+
C (7)
Veh (9)
E (10)
DPN (10)
Rostral
Mid
Caudal
A

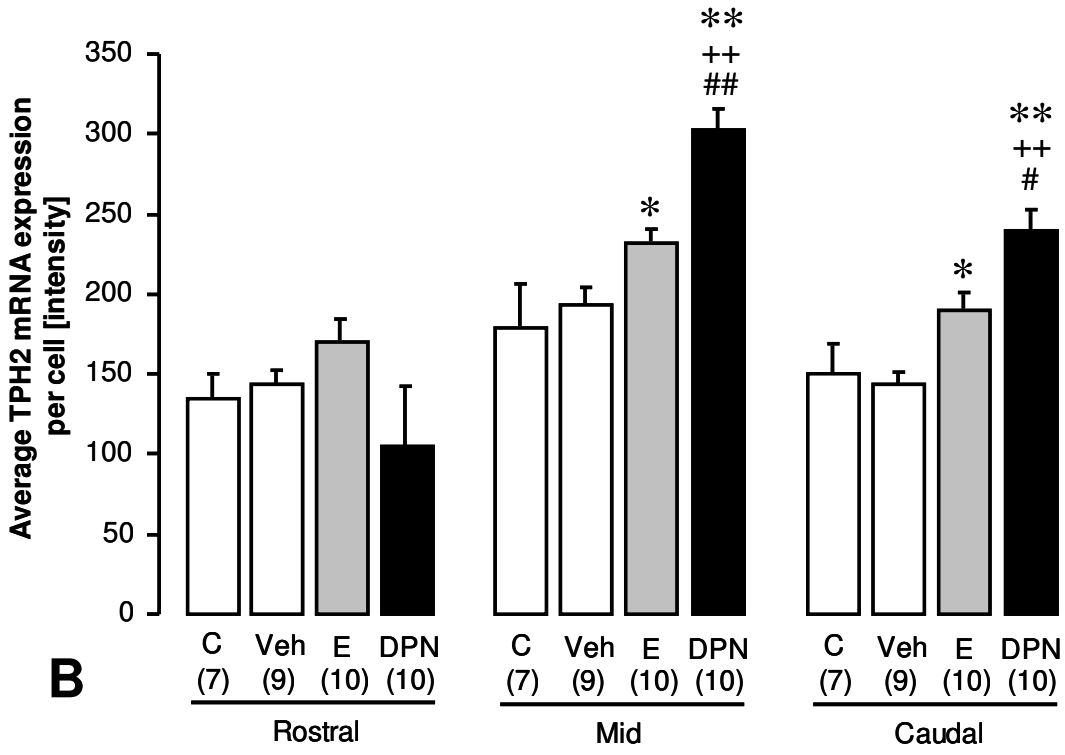

Average TPH2 mRNA expression per cell [intensity]
350
300
250
200
150
100
50
0
**
++
##
*
**
++
#
*
C (7)
Veh (9)
E (10)
DPN (10)
Rostral
Mid
Caudal
B

Figure 14. Local DPN- and E-pellets flanking the DRN in female OVX rats elevated the expression of TPH2 mRNA in the mid- and caudal DRN. Panel A shows data retrieved from area analysis. Panel B shows data retrieved from single-cell analysis. Each bar represents the mean ± SEM. Numbers in parentheses indicate group size. * ($p<0.05$) and ** ($p<0.01$) indicate significant differences versus vehicle controls; # ($p<0.05$) and ## ($p<0.01$) indicate significance versus the E-treated group; + ($p<0.05$) and ++ ($p<0.01$) versus unoperated control animals (C). ANOVA, factor treatment, was performed, followed by Tukey's *post hoc* test where appropriate. For individual subregional values see Table 4.

Table 4

DPN and E both act locally to enhance TPH2 expression in DRN subregions

A	TPH2 mRNA expression per area [pixel^2]		
DRN subregion	Vehicle (n=9)	E (n=10)	DPN (n=10)
dorso-rostral	7836 ± 1013	12545 ± 3987	5927 ± 1273
ventro-rostral	8921 ± 1650	10182 ± 732	7454 ± 2644
dorso-mid	9165 ± 1016	16324 ± 1231 *	24110 ± 2513 **, ##
ventro-mid	28029 ± 2797	34554 ± 3095	35116 ± 3535
lateral mid	5747 ± 1810	10352 ± 1948	12074 ± 3598
dorso-caudal	6162 ± 1055	16707 ± 4872	20138 ± 4831 **
ventro-caudal	8461 ± 577	11847 ± 2342	17061 ± 3283 *

B	Average TPH2 mRNA expression per cell [pixel-intensity]		
DRN subregion	Vehicle (n=9)	E (n=10)	DPN (n=10)
dorso-rostral	138 ± 21.0	158 ± 15.3	110 ± 32.0
ventro-rostral	150 ± 7.8	155 ± 11.4	121 ± 19.4
dorso-mid	132 ± 11.1	166 ± 13.8 *	213 ± 9.8 **, ##
ventro-mid	138 ± 11.9	154 ± 3.3	193 ± 29.2 *
lateral mid	129 ± 22.5	153 ± 10.7	201 ± 37.9
dorso-caudal	140 ± 16.6	197 ± 7,4 *	238 ± 17.0 **, ##
ventro-caudal	151 ± 9.9	180 ± 15.2	242 ± 11.5 **, ##

Part A lists individual subregion-specific values for TPH2 mRNA expression in the DRN, determined via area analysis [pixel^2]. Part B lists individual subregion-specific values for TPH2 mRNA expression, using single-cell analysis [pixel-intensity]. Each value listed represents the mean ± SEM. Numbers in parentheses indicate group size. * ($p < 0.05$) and ** ($p < 0.01$) indicate significance versus vehicle controls. ## ($p < 0.01$) indicates significance versus the E-treated group. TPH2 mRNA expression levels did not differ for any subregion between the vehicle- and the unoperated control group (data not shown). ANOVA, factor treatment, was performed, followed by Tukey's *post hoc* test where appropriate.

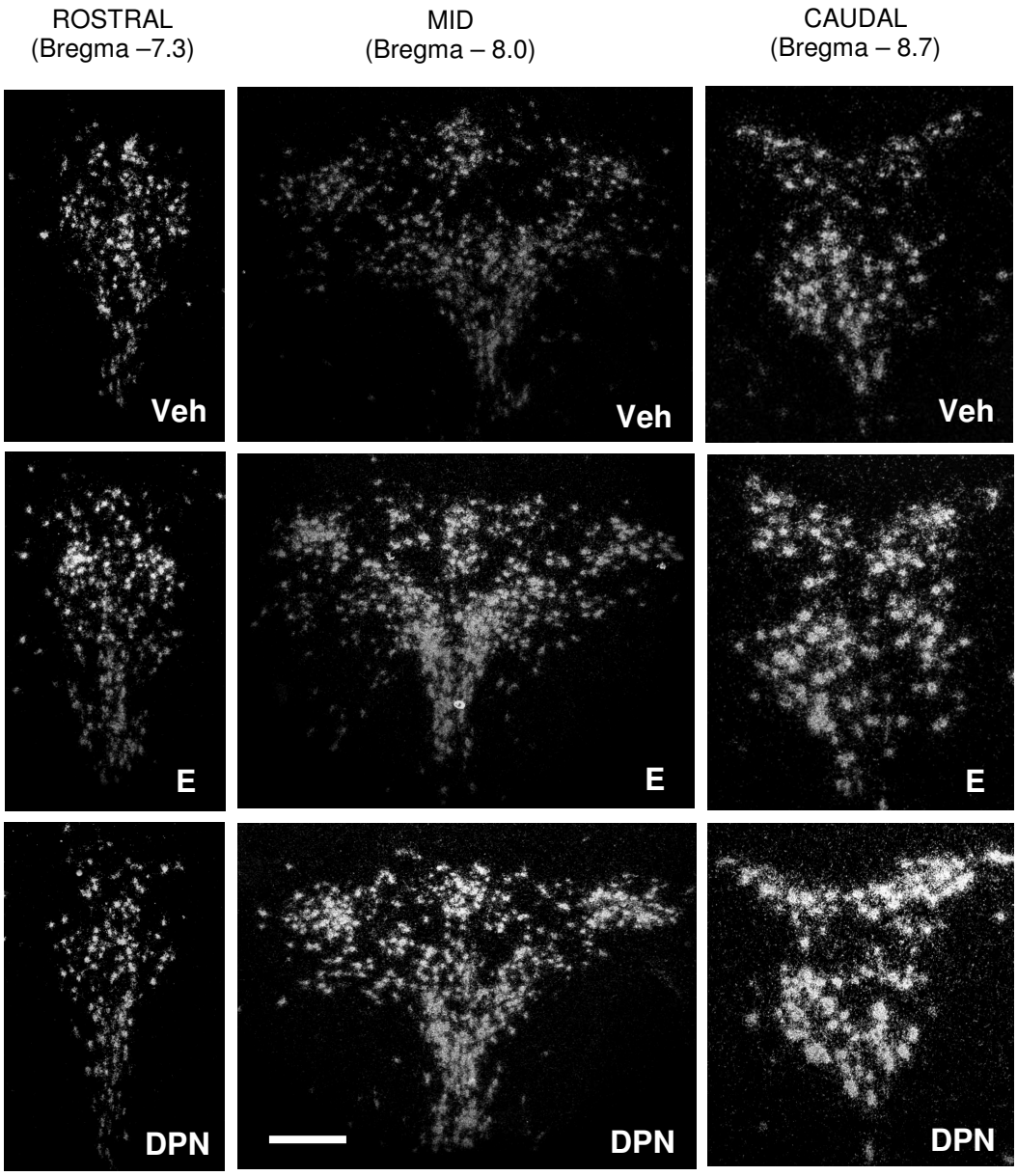


Figure 15. Representative dark-field pictures of silvergrain-labeled cells expressing TPH2 mRNA in the rostral (left side, bregma –7.3), mid- (middle, bregma –8.0) and caudal DRN (right side, bregma –8.7) of locally vehicle-, E- or DPN-treated female OVX animals. Vehicle-treated animals are shown in the top row of panels, E-treated rats in the middle row of panels, and DPN-treated rats in the lower row of panels. Scale bar: 40 µm.

DISCUSSION

Our results show that, compared to vehicle controls, both systemic and local activation of ERbeta in or around the DRN increased the expression of 5-HT-neuronal TPH2 mRNA in a subregion-dependent manner. Estrogen treatment resulted in similar overall effects, but to a lesser extent than DPN. Furthermore, animals treated locally with DPN or E showed decreased despair-like behavior. However, only systemic delivery of DPN decreased anxiety-like behavior, while local administration of the ERbeta-selective compound failed to have the same effect. This indicates that ERbeta actions within the DRN are sufficient to regulate despair-like behavior, but that other brain circuitries are necessary to control anxiety-like behavior.

The observed DPN- or estrogen-induced increase in TPH2 mRNA expression was mainly restricted to the dorso-mid and the caudal DRN. These findings are consistent with observations by Hiroi et al. (2006), who reported elevated TPH2 mRNA expression specifically in the dorso- and ventro-caudal DRN following systemic estrogen treatment in female OVX rats. Furthermore, recent studies in rats and mice indicate that depression-related paradigms (Keeney and Hogg, 1999; Becker et al., 2007) like social defeat (Gardner et al., 2005) or inescapable stress (Grahn et al., 1999; Amat et al., 2005) selectively activate the dorsal and caudal parts of the DRN, subregions that give rise to projections targeting forebrain areas involved in the control of emotional behavior (Lowry et al., 2005; Lowry et al., 2008). The mid-dorsal DRN, for instance, sends out collateral projections to emotion- and stress-related brain areas (Lowry,

2002). These branched projections could, for instance, simultaneously modulate the hypothalamic PVN and the central amygdala (CeA). 5-HT axons from the caudal DRN target limbic structures like the hippocampus, the entorhinal cortex and the septum (Kohler et al., 1982; Kohler and Steinbusch, 1982), indicating that an alteration in TPH2 expression and 5-HT neurotransmission by estrogens may improve memory and learning deficits that are associated with depression (Shors et al., 1998; Burriss et al., 2008; Liu et al., 2008). Within the entire caudal DRN it is again the dorso-caudal subdivision that has been suggested to play a crucial role in affective disorders (Commons et al., 2003). In clinical studies of drug-free, depressed suicide victims, exactly those dorso-caudal DRN subregions exhibited elevated TPH2 protein and mRNA expression (Bonkale et al., 2006; Bach-Mizrachi et al., 2008). However, while a pathological increase in TPH2 expression may reflect a compensatory feedback response to low overall 5-HT concentrations in the brain of depressed patients (Mann et al., 1989; Owens and Nemeroff, 1994; Placidi et al., 2001), therapeutic up-regulation of TPH2 may be beneficial, ultimately boosting 5-HT neurotransmission. More detailed studies in animal models, quantifying TPH2 protein and local 5-HT release and turnover within the DRN itself (autoregulation) and in target areas, are required to address this issue satisfactorily.

In our experiments, systemic delivery of DPN resulted in reduced anxiety-like behavior, confirming the anxiolytic nature of ERbeta (Krezel et al., 2001; Imwalle et al., 2005; Lund et al., 2005; Rocha et al., 2005; Walf and Frye, 2007a, b; Walf et al., 2008a). In contrast, local DPN- or estrogen-treatment at the raphe

nuclei failed to decrease anxiety-like behavior, indicating that anxiety-regulation may primarily involve other brain areas, such as the hypothalamic PVN (Herman et al., 2002a; Donner et al., 2007; Blume et al., 2008; Neumann, 2008), the lateral septum (Henry et al., 2006), the amygdala (Bosch et al., 2007), and the bed nucleus of the stria terminalis (Davis et al., 1997; Walker et al., 2003).

Our studies demonstrate, however, that local delivery of DPN or estrogen is sufficient to decrease despair-like behavior. While recent studies revealed an antidepressant function of ERbeta (Walf et al., 2004; Rocha et al., 2005; Hughes et al., 2008), our experiments suggest a concrete site of action for the observed effect. Thus, high anxiety and despair-like behavior may be closely related regarding the phenotype of depression (Chaby et al., 1993; Leibbrand et al., 1999; Farabaugh et al., 2005; Godart et al., 2006; Mittal et al., 2006), but they may be two distinct mental states in their neuroanatomical origin. The fact that estrogen-treated rats in our experiment spent more time immobile / floating in the FST than their DPN-treated counterparts contradicts previous studies in mice and rats, which found that DPN and estrogen generally decrease the time spent immobile during the FST (Walf et al., 2004; Rocha et al., 2005). However, our study delivered estrogen locally via wax pellets flanking the DRN, whereas the mentioned other reports administered estrogen and DPN systemically. It is unclear at this point, why the behavioral effects of DPN or estrogen differ into opposing directions in this particular parameter. In accordance with previous studies (Walf et al., 2004; Rocha et al., 2005), both estrogen and DPN did, indeed, increase the time rats spent actively struggling during the FST. Further

studies will have to confirm if, and explore how, ERbeta-mediated elevation of TPH2 mRNA expression in the mid-dorsal and caudal DRN, actually causes an attenuation of despair behavior.

Overall, local estrogen-treatment had similar, but less intense effects on TPH2 mRNA expression and on despair-like behavior than the selective ERbeta agonist. This gradual difference may be explained with a simultaneous action of estrogen on both ERalpha and ERbeta, and with the suggested opposing functions of the two ER types (Couse et al., 2000; Pike et al., 2000; Shapiro et al., 2000; Liu et al., 2002; Lindberg et al., 2003; Toufexis et al., 2007). While increased ERalpha mRNA and single nucleotide polymorphisms (SNPs) in the gene coding for ERalpha are associated with mental illness, specifically with depression (Perlman et al., 2005; Mill et al., 2008), ERbeta-mediated actions have been found to exert anxiolytic and antidepressant effects in various animal models (Krezel et al., 2001; Imwalle et al., 2005; Lund et al., 2005; Rocha et al., 2005; Walf and Frye, 2007a, b; Walf et al., 2008b). Estrogen itself binds to both receptor types with about the same affinity (Kuiper et al., 1997; Lund et al., 2005), and could, thus, activate two functionally opposing mechanisms, both ultimately balancing TPH2 mRNA expression and emotionality.

Immunohistochemical receptor localization studies have found both ERbeta1 (Lu et al., 2001) and ERbeta2 (Chung et al., 2007), a novel splice variant carrying an 18-amino acid insert between the fifth and the sixth exon in the ligand-binding domain, in the DRN of female rats. However, due to quality differences in antibodies used for immunohistochemical studies of ERbeta, the

exact expression pattern of ERbeta and its splice variants in the rat DRN is still controversial (Sheng et al., 2004). In mice, Nomura et al. (2005) revealed that ERbeta, but not ERalpha is located within 5-HT neurons. In rat, we also hypothize that ERbeta (Lu et al., 2001) and possibly splice variants of ERbeta (Chung et al., 2007) are expressed within 5-HT neurons of the DRN. Based on preliminary studies in our lab (data not shown), ERalpha may only be expressed in local non-5-HT-, but possibly GABAergic (Hart et al., 2001; Su et al., 2001) neurons, indicating that ERalpha could interfere with the negative feedback loop (Haddjeri et al., 2000; Liu et al., 2000) regulating 5-HT-neuronal function.

Differences in the expression of ERalpha versus ERbeta in the midbrain of rats (Shughrue et al., 1997a; Shughrue et al., 1997b; Lu et al., 2001), mice (Nomura et al., 2005; Vanderhorst et al., 2005), guinea pigs (Lu et al., 1999; Warembourg and Leroy, 2004), and cats (VanderHorst et al., 1998) generally evoke the notion that profound species differences may exist regarding the modulation of the 5-HT system by gonadal steroids. While ERbeta seems to be the predominant ER to be expressed in the 5-HT-neural DRN of rodents, ERbeta expression in the human DRN remains to be investigated, but is likely, since ERbeta mRNA levels also dominate over ERalpha in the DRN of primates (Gundlah et al., 2000; Gundlah et al., 2001). ERbeta may, thus, directly and positively regulate TPH2 gene transcription.

The molecular or neurotransmitter-mediated mechanisms by which ERbeta and ERalpha may directly or indirectly modulate TPH2 gene expression are still unknown. Most ER-induced changes in gene transcription are due to

classic effects of the steroid receptors acting as nuclear transcription factors. More and more studies, however, discover rapid, non-genomic mechanisms of ER action within the cell (Cato et al., 2002; Mhyre and Dorsa, 2006; Levin, 2008).

The observation that local estrogen-treatment significantly increased TPH2 in the mid-dorsal and in the caudal DRN, raises the question whether physiological changes of circulating estrogen concentrations in cycling women have similar effects, and if, for example, an abnormal ratio of ERalpha versus ERbeta expression or a disruption of normal ER-regulation of TPH2 expression in the midbrain contribute to severe menstrual mood disorders like the premenstrual syndrome (Rubinow, 1992; Arpels, 1996; Schmidt et al., 1998) or premenstrual dysphoric disorder (Gorman, 2006). Our results further elucidate the roles that ERbeta and ERalpha may play in the regulation of mood disorders in females. Considering the clinical relevance of an intact system for 5-HT production (Zill et al., 2004; Zhang et al., 2005; Bach-Mizrachi et al., 2008) and the importance to address the sex ratio in depressive disorders (Kornstein et al., 1995; Grigoriadis and Robinson, 2007), our studies may also help to develop sex-specific treatment of depression (Gorman, 2006).

In conclusion, our results confirmed the hypothesis that chronic, local activation of ERbeta alters TPH2 mRNA expression in the DRN in a subregion-dependent manner, and, at the same time, changes emotion-related behavior. 5-HT neurons of the DRN may be the key regulators of despair-like behavior, whereas other brain circuitries seem to be necessary to alter anxiety-related behavior.

CHAPTER 4

DISCUSSION

The goal of these studies was to determine the role of estrogen receptors for the regulation of TPH2 within 5-HT neurons of the DRN. Four main findings were derived. First, local ERbeta activation within the midbrain is sufficient to decrease despair-like behavior in female OVX rats. Second, local ERbeta activation does not decrease anxiety-like behavior, whereas systemic activation does. Third, systemic, as well as local ERbeta activation elevates TPH2 mRNA expression in a subregion-dependent manner, with local treatment being more efficient than peripheral s.c. administration; and fourth, local administration of estradiol exerts similar effects on TPH2 mRNA expression and behavior as does DPN, but to a lesser extent. While each individual result is discussed in detail the last part of the previous chapter, I will broaden the discussion further and explore potential mechanisms of action, ways for signal transmission and mood regulation, estrogen receptor splice variants, involvement of other steroid receptors, and possible future research directions in this chapter.

A functional, well-balanced system for 5-HT production is essential for mental health, and at the core of this system acts TPH2, the rate-limiting enzyme for 5-HT synthesis in the brain (Walther et al., 2003). When this system gets out of balance, either due to a mutation in the TPH2 gene (Sun et al., 2004; Zill et al., 2004; De Luca et al., 2005; Shink et al., 2005; You et al., 2005; Zhang et al., 2005; Harvey et al., 2007; Lim et al., 2007; Lopez de Lara et al., 2007; Maron et al., 2007; Haghighi et al., 2008) or, often in correlation, due to an abnormal

mRNA and protein level of TPH2 (Boldrini et al., 2005; Bach-Mizrachi et al., 2006; Bonkale et al., 2006; Lim et al., 2007; Bach-Mizrachi et al., 2008), it may change 5-HT release in target areas, 5-HT turnover and feedback regulation, as well as 5-HT auto- and target cell receptor densities. Depending on the subdivision of the DRN, 5-HT axons from the DRN innervate many different forebrain areas (Lowry et al., 2008). Thus, it is not surprising that these subdivisions can react differently to the same stimulus, in my experiments an ERbeta-selective agonist. Other interesting questions are 1) what ER splice variants are involved and where are they located, 2) upon ERbeta activation, what are the neuronal circuits and changes within synapses on the way from the DRN to target neurons, and 3) what are the intracellular mechanisms in 5-HT neurons that ultimately lead to an ERbeta-mediated alteration in TPH2 transcription?

Based on results from our and other laboratories (Shughrue et al., 1997a; Lu et al., 2001), ERbeta seems to be the predominant ER that is expressed in 5-HT neurons of the rat DRN, and is likely to regulate TPH2 gene expression directly through classic genomic actions. So far, however, an ERE in the TPH2 promoter sequence has not been discovered. Nonetheless, ERbeta could interact with numerous other transcription factors or transcription-enhancing proteins to increase the expression of TPH2. The gradually attenuated effect of estrogen on TPH2 expression compared to DPN-treatment could suggest an opposing action of ERalpha, as seen in other tissues (Couse et al., 2000; Liu et al., 2002). However, in rat, none or only miniscule immunoreactivity for ERalpha

has been reported within the DRN (Shughrue et al., 1997a; Sheng et al., 2004), and dual-ICC studies from our lab confirm the lack of ERalpha in brainstem TPH2 neurons. Instead, a high density of intense ERalpha-immunoreactive cells is localized within the periaqueductal gray (PAG) dorsal of the DRN (Murphy et al., 1999; Loyd and Murphy, 2008), and some ERalpha-positive cells are found within the lateral DRN, often adjacent to TPH2 neurons, but never colocalized with TPH2 (unpublished results). ERalpha could, for example, be expressed in the nuclei of PAG-typical cholinergic neurons, which are especially active during social defeat, an animal paradigm for depression (Kroes et al., 2007). If those neurons actually project onto 5-HT neurons of the DRN, varying levels of circulating estrogen (and thus also estrogen-levels in the brain), could indirectly influence the activity of TPH2 expression. ERalpha could also be invoked in brain regions farther away, such as the hypothalamic PVN, which not only receives input from the DRN, but also sends out projections towards the DRN (Abrams et al., 2004; Lowry et al., 2008) In another dual-ICC study, I identified some ERalpha-positive neurons in and around the lateral DRN to be positive for glutamic acid decarboxylase, a marker for GABAergic neurons. Since GABA is the brain's most important inhibitory neurotransmitter, this might indicate that ERalpha can enhance the inhibitory input towards the DRN, possibly interacting with negative feedback loops that control the DRN (Liu et al., 2000; Tao and Auerbach, 2003). Identification of ERalpha in GABAergic interneurons of the hippocampus corroborates the concept that ERalpha may enhance GABAergic neurotransmission (Hart et al., 2001; Su et al., 2001). Fig. 16 displays a

schematic model for these predicted inhibitory afferents of the DRN, and for relative expression levels of ERalpha and ERbeta depending on the rostro-caudal and ventro-dorsal axes of the DRN.

Depending on the nature of the postsynaptic 5-HT receptors, 5-HT can be either inhibitory or excitatory towards the target neuron. Most efferents from the DRN to the forebrain are indirect. For instance, 5-HT projections from the dorso-mid DRN mainly target glutaminergic neurons surrounding the hypothalamic PVN. Increased 5-HT release onto those excitatory neurons could, thus, indirectly inhibit the stress-reactivity of the PVN via an inhibition-of-excitation principle (Herman et al., 2002b). This hypothesis is corroborated by the fact that, in humans, depression is commonly characterized by a hyperactive HPA axis and hypercortisolemia (Gillespie and Nemeroff, 2005).

Most current antidepressant-treatments target the 5-HT reuptake mechanism via SSRIs, and are based on the so-called “receptor theory” of depression, asserting that each drug must ultimately change postsynaptic receptor expression and signaling pathways before it is effective. The same reasoning is used for the typical one- to two-week delay before the patient recognizes an improved state of mind (Cowen and Sargent, 1997; Wilson, 2007). Nonetheless, boosting the 5-HT system directly by enhancing mRNA and presumably protein levels of TPH2, offers an alternative or additional therapeutic tool, especially if low expression of this enzyme is the cause and core of the pathology. A recent study showed that the SSRI-antidepressant fluoxetine ultimately also resulted in upregulated TPH2 expression (Shishkina et al., 2007).

A combinatory treatment of depression using common SSRIs together with TPH2-targeting drugs may be beneficial.

Projections from the caudal DRN, the subdivision where I noticed the most profound elevation of TPH2 expression after DPN- or estrogen-treatment, target limbic structures like the hippocampus, the septum and the entorhinal cortex (Abrams et al., 2004). With regard to depression-induced learning- and memory-deficits reaching all the way to severe debilitation of intellectual performance (Horan et al., 1997; Shors et al., 1998; Basso and Bornstein, 1999; Burt et al., 2000; Rose and Ebmeier, 2006; Burriss et al., 2008), this is of particular interest. Importantly, stress- and depression-induced performance deficits in women, but not in men, and in female animals, but not in males, are one of the most obvious and remarkable sex differences in depressive disorders (Shors and Leuner, 2003). In the cerebellum, ERbeta seems to enhance the induction of synaptic long-term potentiation, and to improve motor-related memory (Andreescu et al., 2007). Within the hippocampus, ERbeta has also been shown to improve memory and stimulate synaptic plasticity (Liu et al., 2008). Thus, it is quite possible that ERbeta-induced elevation of TPH2 mRNA expression in the caudal DRN may cause increased 5-HT neurotransmission towards the hippocampus, resulting in improved memory consolidation or storage. Future studies should address this possibility.

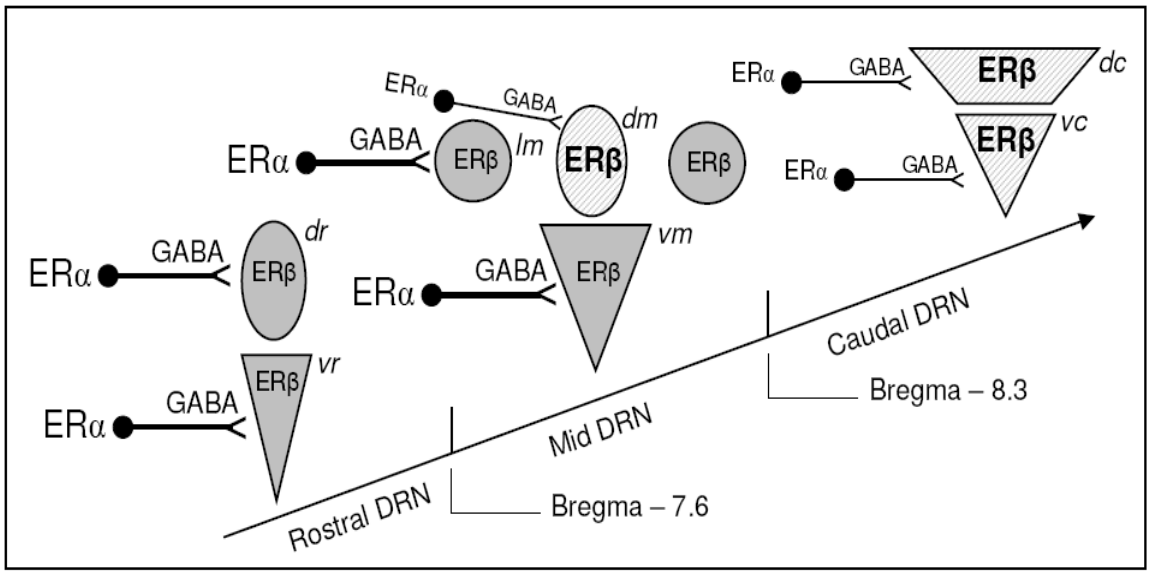


Figure 16. Schematic illustration of proposed neuroanatomical afferents to the rostro-caudally oriented subdivisions of the DRN, suggesting opposing actions of ERalpha and ERbeta on TPH2-neuronal function. Font size represents relative levels of receptor expression. GABA = gamma-aminobutyric acid, dr=dorso-rostral, vr=ventro-rostral, dm=dorso-mid, vm-ventro-mid, lm=lateral mid, dc=dorso-caudal, vc=ventro-caudal.

The intracellular and / or nuclear mechanism by which ERbeta regulates the expression of TPH2 mRNA remains to be revealed. Most likely, following ligand binding, ERbeta acts as a classic steroid hormone receptor, initiating transcription at a response element on the DNA, activating and collecting other transcription factors into an initiation (of transcription) complex, ultimately causing a signaling cascade that leads to DNA remodeling and TPH2 promoter activation.

In the dorsal raphe, the expression of TPH1, the non-brain-specific version of the enzyme which is only expressed at very low levels in the brain (Malek et al., 2005; Abumaria et al., 2008), is regulated by cAMP (Boularand et al., 1995), indicating a regulatory effect of CREB (cAMP response element binding protein) on TPH1 transcription. It needs to be determined, if the control of TPH2 gene expression works in a similar way. The many options of posttranscriptional or posttranslational modifications of the enzyme's activity must also be considered. Elevated TPH2 expression is likely but not guaranteed to result in elevated enzyme concentration in the neuron; and that, again, does not necessarily mean that the enzyme always possesses the same activity. In contrast to TPH1, TPH2 contains an additional 41 amino acids at the N-terminus. Murphy et al. (2008) revealed that amino acids 10-20 in this N-terminal region of TPH2 carry an important sequence that normally keeps the enzyme's expression at relatively low levels. When this region is removed, TPH2 mRNA expression increases. Another modification of the enzyme's activity is posttranslational phosphorylation. Phosphorylation of serine 19, a protein kinase A consensus site located in the same N-terminal domain, leads to increased stability of TPH2, and consequently

increases the 5-HT output of the enzyme (Murphy et al., 2008). Thus, it would be of interest to investigate whether non-genomic, rapid signaling actions of other membrane-bound or cytosolic ERs regulate TPH2 activity at the protein level.

In rat, it is still unclear exactly which DRN cell population contains ERalpha or ERbeta and what splice variants that concerns. However, there is reason to believe that the DRN contains novel splice variants of ERbeta in addition to the already detected ERbeta2 (Chung et al., 2007). My hypothesis is that the origin of the controversial results regarding the presence or absence of ERbeta in the rat DRN lies in a C-terminal truncation of the receptor in this particular brain region. The antibodies against ERbeta were all created to detect one or several epitopes along the C-terminal ligand-binding domain. Thus, the reason as to why I can identify high expression levels of ERbeta mRNA with RT-PCR (preliminary results), but some otherwise very reliable antibodies do not detect ERbeta protein (Sheng et al., 2004) in the rat DRN, may be explained with one or more novel splice variant mRNAs lacking some part of the C-terminus (Fig. 17). If such variants existed, the consequence on the binding affinity of various ligands, including estrogen and DPN, would need to be determined for each novel ERbeta variant. Possibly, certain subregions of the DRN may express only one or the other subset of ERbeta splice variants. A unique expression pattern like that could further explain the subregion-dependent regulation of TPH2 mRNA expression, which was observed in the work presented. At this point, however, these proposed truncated splice variants are purely hypothetical. Therefore, a future study could be to characterize via RT-PCR the expression

pattern of ERs in the DRN of OVX rats without treatment, and of OVX rats with chronic systemic estradiol-substitution, in order to assess potential effects of ligand availability on receptor expression.

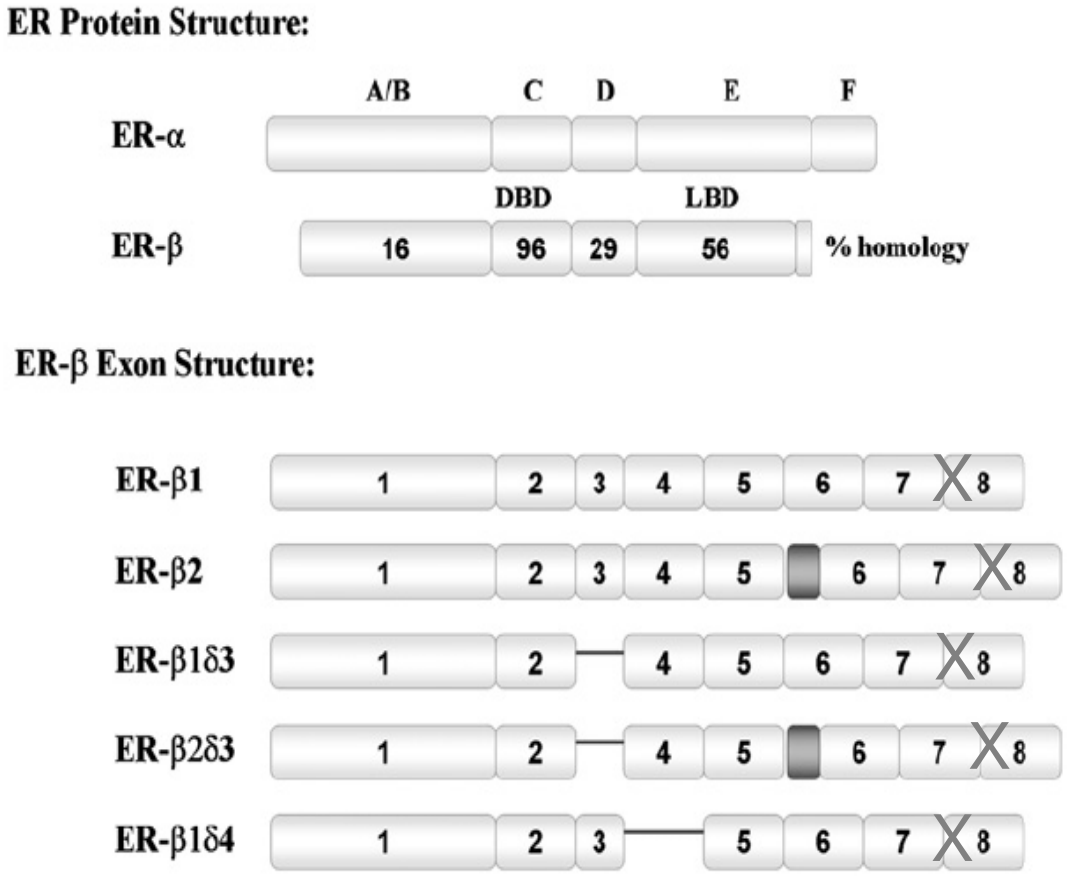


Figure 17. Proposed C-terminal truncation of ERbeta in the DRN. “X” represents the expected site of truncation. Modified after Weiser et al. (2008).

CONCLUSION

The overall conclusion of this thesis is that ERbeta regulates the expression of TPH2 mRNA within selective subdivisions of 5-HT neurons in the female rat DRN, and that this is correlated with decreased despair-, but not anxiety-like behavior. Future studies will have to address the molecular mechanism of transcriptional modulation through ERbeta, which splice variants are expressed and translated within each subdivision of the DRN, and if an increase in TPH2 mRNA expression actually results in elevated TPH2 protein, 5-HT synthesis and 5-HT release in target areas.

LIST OF ABBREVIATIONS

5-HT	5-hydroxytryptamine / serotonin
[^{35}S]-UTP	radioactively labeled uridine-triphosphate
α	alpha
αERKO	ERalpha knock-out (mouse), ERalpha null mutation
β	beta
βERKO	ERbeta knock-out (mouse), ERbeta null mutation
δ	delta; indicates a missing exon, created by alternative splicing
AAALAC	Association for Assessment and Accreditation of Laboratory Animal Care
AADC	aromatic amino acid decarboxylase
ANOVA	analysis of variance
BH4	tetrahydrobiopterin
BLAST	Basic Local Alignment Search Tool
C	unoperated control group; OR: C- = carboxy- (terminus)
Ca^{2+}	Calcium ion
cAMP	cyclic andosine-monophosphate
cDNA	complementary DNA
CeA	central amygdala
CREB	cAMP response element binding protein
cRNA	complementary ribonucleic acid (riboprobe)
DBD	DNA-binding domain
DTT	dithiothreitol

dc	dorso-caudal
dm	dorso-mid
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DPN	diarylproprionitrile; diarylproprionitrile-treated group
dr	dorso-rostral
DRN	dorsal raphe nuclei
E	(17-beta-)estradiol; estradiol-treated group
EDTA	ethylendiaminetetraacetic acid
EPM	elevated plus maze (anxiety behavior test)
ERalpha	estrogen receptor alpha
ERbeta	estrogen receptor beta
ERE	estrogen response element
Fe^{2+}	iron ion
FST	forced swim test (despair behavior test)
GABA	gamma(γ)-aminobutyric acid
GIT buffer	guanidine-isothiocyanate buffer
GPCR	G-protein-coupled receptor
^{3}H	Tritium (radioactive)
HPA	hypothalamo-pituitary-adrenal
ICC	immunocytochemistry
ISH	*in situ* hybridization
Kb	kilo-bases (10^3)

K_d	dissociation constant
KCl	potassium chloride
K_i	binding affinity
LacZ	beta-galactosidase
LAT	large amino acid transporter
LBD	ligand-binding domain
lm	lateral mid
MAO	monoamine oxidase
MAOI	monoamine oxidase inhibitor
MDD	major depressive disorder
Mg^{2+}	magnesium ion
MgCl	magnesium chloride
MMLV-RT	Moloney Murine Leukemia Virus - Reverse Transcriptase
mRNA	messenger ribonucleic acid
n.a.	not applicable
NaCl	sodium chloride
NIH	National Institutes of Health
O_2	molecular oxygen
OF	open field (anxiety behavior test)
OVX	ovariectomy / ovariectomized
PAG	periaqueductal gray
PCR	polymerase chain reaction
PMDD	premenstrual dysphoric disorder

PMS	premenstrual syndrome
PPT	propylpyrazoletriol
PVN	paraventricular nucleus (of the hypothalamus)
RT-PCR	reverse transcription-polymerase chain reaction
s.c.	subcutane
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
SERT	5-HT transporter
SNP	single nucleotide polymorphism
SSC	sodium chloride with sodium citrate (buffer)
SSRI	“selective” serotonin reuptake inhibitor
$T_{1/2}$	half-life
TA-	thymine 3’-overhang (used during so called “TA-cloning”)
Taq pol.	heat-resistant DNA polymerase from *thermus aquaticus*
TOPO	topoisomerase
TPH2	tryptophan hydroxylase 2 / neuronal tryptophan hydroxylase
Tris-Cl	tris base with hydrochloric acid
Veh	vehicle-treated group
vc	ventro-caudal
vm	ventro-mid
vr	ventro-rostral
WT	(genetic) wild type

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