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Rapid Signaling Mechanisms of Estrogens in the Developing Cerebellum

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Abstract

The steroid hormone 17 β -Estradiol regulates the normal function and development of the mammalian nervous system. Many of estradiol's effects are mediated via the nuclear hormone estrogen receptors ER α and ER β . In addition to regulating estrogen-responsive gene expression, estradiol also acts in an immediate and cell-specific fashion to regulate various intracellular signal transduction pathways. The goal of this review is to develop a contextual framework to understand the generalized function of estrogen during development of brain regions not known to be sexually specialized. However, it is first important to build this generalized framework on the more well-developed foundation of estrogen's gonad-driven sex-specific actions. As a result, a discussion of known and proposed mechanisms of estrogen actions in reproductive and other tissues will be presented. Building upon this information, a review of our research group's recent *in vitro* and *in vivo* studies that have focused on elucidating the mechanisms of estrogen actions in neurons of the non-sexually specialized cerebellum will be presented. While the full-spectrum of estrogen action during normal cerebellar development remains unresolved, results of recent studies have revealed a pathologic role for estrogen and estrogen receptors in medulloblastoma, common pediatric brain tumors that arise from cerebellar granule cell-like precursors. The potential use of anti-estrogen signaling agents as adjuvant therapy for medulloblastoma is proposed based on those finding.

Keywords

cerebellum; development; ERK; estrogen; non-genomic; signal transduction

Estrogens, often referred to as female sex hormones, regulate gene expression in target tissues and dramatically influence diverse physiological processes throughout the life of both males and females. The steroid hormone 17 β -estradiol (estradiol) is the most potent physiological estrogen. Estradiol exerts many of its influences through cognate nuclear hormone estrogen receptors ER α and ER β . When the receptors are activated by ligand binding, the ERs form dimers and associate with specific elements in the promoters of estrogen responsive genes, or interact with other transcription factors, to modify transcription through additional protein-protein interactions with various co-regulatory proteins (Edwards, 2000; Evans, 1988; McDonnell and Norris, 2002). The basic features of the classical mechanism of nuclear hormone receptor action are diagrammatically summarized in Figure 1.

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While many of estrogen's actions are mediated through the intracellular nuclear receptor mediated mechanism, it is now established that estradiol, steroidal and non-steroidal compounds with estrogen-like properties, also act in a cell-specific capacity through mechanisms that rapidly influence intracellular signaling in concert with nuclear ER mediated transactivation (Belcher and Zsarnovszky, 2001; Falkenstein et al., 2000; Farach-Carson and Davis, 2003; Kelly and Levin, 2001; Vasudevan et al., 2001; Vasudevan et al., 2005). These rapid signaling effects occur within minutes, are not blocked by transcriptional and translational inhibitors, and can be activated in many different types of cells by membrane-impermeable ligands; properties that indicate rapid signaling is initiated at the cells surface through a mechanism different from those mediated by the intracellular nuclear hormone receptor's ability to recruit co-regulators.

Although interest in the molecular mechanisms of rapid membrane-initiated estrogen action has surged during the last decade, the mechanistic details of these rapid signaling events remains poorly understood. It has become increasingly apparent that estradiol can influence intracellular signaling via rapid mechanisms which vary greatly from cell type to cell type, and that numerous types of binding sites and receptors are involved with mediating these rapid signaling actions. Understanding how these diverse molecular mechanisms are integrated into a coherent physiological outcome is a major challenge. However, it is reasonable to suggest that the total physiological effects of estradiol arise from the combination of rapid signaling mechanisms and longer-duration changes in estrogen responsive gene expression.

Due to the changeable cellular complexity of the brain, especially during developmental periods, understanding integrated and cell specific actions of estrogens in the brain are major challenges. As discussed below, we have used pharmacological, molecular and physiological approaches to understand mechanisms through which rapid estradiol effects are mediated in developing rhombencephalic neurons of the cerebellum and to determine whether and how environmental estrogens impact those actions. In this regard, we have been able to separate the physiological actions of rapid estradiol-induced ERK-signaling from longer duration estradiol-effects and found that rapid ERK-signaling and slower acting ER-mediated actions differentially regulate neuronal cell death, mitosis, and neuroprotection (Wong et al., 2003). Using in vitro and in vivo models our studies have also characterized new estrogen-initiated signaling pathways, and found that endocrine disrupting chemicals such as bisphenol A (BPA) can paradoxically act as extremely potent estrogen mimetics and as inhibitors of 17 β -estradiol activity (Belcher et al., 2005; Zsarnovszky et al., 2005).

For the purposes of this brief review, I have not attempted to identify every important research report related to the topic of rapid estrogen signaling, and the actions of endogenous or environmental estrogens in the developing brain. Rather than treat this as an opportunity to comprehensively review the literature, I have referenced a limited subset of studies and reviews, that are in some cases considered seminal exemplars, or which have greatly influenced the direction of the presented research. As a result, I have not cited many important and significant studies; it is hoped that the chosen references will lead the interested reader to discover the important work and findings of others in this dynamically changing area of research.

“Classical” nuclear estrogen receptors

The classical estrogen receptors (ERs) are members of the steroid/thyroid superfamily of transcription-factor receptors, and share common modular domains with conserved structures and functions (Evans, 1988). The major protein isoforms of ER α and ER β are expressed from different genes and predicted to be about 47% identical (Enmark et al., 1997; Enmark and Gustafsson, 1999). The amino-terminal A/B region of these receptors is the most variable

domain and contains the N-terminal transactivation domain (AF-1). The DNA binding domain (region C) and the ligand binding domains of these receptors (located in the multifunctional C-terminal E/F region) are extremely well conserved. The C-terminal E/F region also contains structures that function in receptor dimerization and in ligand-dependent transactivation (AF-2) via protein/protein interactions with co-regulatory proteins (Gustafsson, 1999; Muramatsu and Inoue, 2000).

There are important differences in the gene regulatory functions of ER α and ER β . Both receptors are physiologically relevant ERs that bind estradiol with high affinity ($\sim K_d = 0.1$ nM) and can activate transcription of estrogen-responsive reporter gene constructs expressed in mammalian cell lines (Kuiper et al., 1996; Kuiper et al., 1997; Petersen et al., 1998). Although ER β binds estradiol and stimulates transactivation of estrogen responsive reporter constructs, levels of ER β mediated reporter activation were initially reported as lower than those activated by ER α in the same reporter system (Barkhem et al., 1998; Kuiper et al., 1996; Kuiper et al., 1997). However, it was subsequently found that the “long form” of human ER β 1 (hER β 1) and ER α have similar activity at a consensus ERE, activities greater than those mediated by the short-form of hER β 1 or the rat ER β 1. Those differences in receptor subtype activity correlated with ligand binding affinity, indicating that differential expression of ER β isoforms impact ERE-regulated gene expression in a ligand dependent fashion (Ramsey et al., 2004).

Both ERs normally undergo alternative splicing that results in expression of numerous receptor variants that modify the structure and function of these receptors. Studies comparing properties and activities of different alternative splice variants of ERs indicate that the different isoforms play a major role in determining the specific cellular response evoked by endogenous or environmental estrogens (Belcher, 1999; Bollig and Miksicek, 2000; Fasco et al., 2000; Fuqua et al., 1999; Omoto et al., 2003; Petersen et al., 1998; Shupnik, 2002). For example, the two major splice variants of ER β , ER β 1 and ER β 2, differ from one another by the insertion of a 54 base pair alternatively spliced exon (Kuiper et al., 1998). The inclusion of the alternative exon in the ER β mRNA results in an 18 amino acid insertion into the ligand-binding domain of the ER β 2 protein. The rat ER β 2 binds estradiol with about 35-fold lower affinity ($K_d = 5.1$ nM) than ER β 1 ($K_d = 0.14$ nM), and requires 1000-fold higher estradiol concentrations to activate comparable levels of transcription. Additionally, some endocrine disrupting chemicals (EDCs) that act as weak estrogens, preferentially bind ER β with ER β 1 binding most EDCs (e.g. BPA, coumesterol, genistein) with about 100-fold higher affinity than ER β 2 (Kuiper et al., 1998). The physiological significance of differential ER β isoform expression remains unknown; although it is speculated that expression of differing ratios of ER β 1 and ER β 2 might regulate cellular responsiveness to endogenous estrogens providing a cellular mechanism that allows a cell to respond in a dynamic fashion to concentrations of estrogens that would otherwise result in full activation if only ER α or ER β 1 were expressed (Belcher, 1999).

Estrogen and its receptors in the nervous system

In the brain, estradiol is well known as a fundamental regulator of the physiology and behaviors required for reproduction. Along with its role in regulating endocrine functions and sexual behaviors, estradiol also plays a significant role in normal development and sexual specialization of the mammalian CNS, with additional important neurotrophic and neuroprotective functions in the developing and adult brain (Beyer, 1999; Lee and McEwen, 2001; McEwen, 2002; Toran-Allerand, 1996; Wise et al., 2001). In humans, changes in estradiol concentrations, especially during the menstrual cycle and in postmenopausal women, can influence mental state and affect cognitive functions. Clinically, changes in the plasma estradiol concentration are associated with certain forms of depression and affective disorders, increased epileptic seizure frequency, Tourette's syndrome, schizophrenia, and Alzheimer's

disease (Fink et al., 1996; Klein and Herzog, 1998; Osterlund and Hurd, 2001; Woolley and Schwartzkroin, 1998). Estrogen has also been identified as a major factor responsible for increased sensitivity of females to the rewarding effects of drugs of abuse (Carroll et al., 2004). While estradiol is linked to these affects and disorders, a significant understanding of how it influences their etiology does not presently exist. Additionally, the findings presented below showing that ERs are expressed in primary human medulloblastoma, further supports previous findings suggesting a link between estrogen signaling and developmentally-related malignancies of the brain. Those results have shaped ongoing studies to develop new treatment strategies for medulloblastoma and related primitive neuroectodermal tumors by disrupting estrogen signaling mechanisms (Kirby et al., 2004).

As in other tissues, many of estradiol's effects on the brain are mediated through the nuclear hormone receptor activities of intracellular ER α and ER β . In addition to influencing the hypothalamus and other brain regions involved with reproduction, estradiol impacts many brain regions that are involved with non-sexually related motor and cognitive functions, such as the cerebellum (Beyer, 1999; Jakab et al., 2001; McEwen, 2002). Thus, it is not surprising that both ERs are present in the brains of adult mammals and are co-expressed in some brain regions with important region-specific differences in distribution (Laflamme et al., 1998; Mitra et al., 2003; Perez et al., 2003; Shughure et al., 1998). There is also much evidence for transient ER expression throughout much of the human and rodent brain during development (Brandenberger et al., 1997; Jakab et al., 2001; Lemmen et al., 1999; Raab et al., 1999; Weiland et al., 1997; Zsarnovszky and Belcher, 2001). For example, studies from our laboratory, and many others, indicate that ER α and ER β are expressed in neurons and glia of the developing rat prefrontal cortex and ER β is expressed in human and rodent cerebellar neurons and glia during development. Receptor expression in both brain regions is dynamically regulated in the developing cell populations (Belcher, 1999; Jakab et al., 2001; Zsarnovszky and Belcher, 2001). In contrast to other neuronal cell systems where ER α is expressed and appears to be the operative membrane ER, estrogen mediated effects in the cerebellar neurons are primarily driven by ER β . The generalized importance of ER β in the brain was highlighted from ER β knockout mouse model studies which demonstrated that ER β contributes to non-reproductive brain functions such as radial glia guided neuronal migration and spatial learning in females (Rissman et al., 2002; Wang et al., 2003).

Rapid Estrogen Signaling

Numerous studies have shown that estradiol can rapidly stimulate the activity of specific signaling cascades. In fact, it is difficult to identify an intracellular signaling cascade or a tissue that is not influenced by estradiol. In various cell types, estradiol can rapidly activate adenylate cyclase, increase intracellular [Ca²⁺], activate phospholipase C to generate inositol 1,4,5-trisphosphate and diacylglycerol, stimulate the phosphatidylinositol 3 kinase pathway, stimulate nitric oxide synthase to liberate nitric oxide, increase intracellular cGMP to activate protein kinase G, and activate mitogen activate protein (MAP) kinase pathways (Belcher and Zsarnovszky, 2001; Falkenstein et al., 2000; Ivanova et al., 2002; Nadal et al., 2001). The influence of estradiol on ERK1/2 signaling has been reported in many different cell types including various tumor cell lines (Di Domenico et al., 1996; Migliaccio et al., 1996), vascular endothelium (Chen et al., 2004; Chen et al., 1999), osteocytes (Endoh et al., 1997; Kousteni et al., 2001), central neurons (Singer et al., 1999; Watters et al., 1997; Wong et al., 2003) and glia (Beyer et al., 2002).

The ability of membrane impermeable estradiol to rapidly activate ERK-signaling in several different cell types indicates that rapid estrogenic effects are initiated at the plasma membrane (Kelly and Levin, 2001). However, a consensus identity of the membrane associated ER (mER) and the molecular mechanism through which the mER couples to the ERK-signaling pathway

does not exist. In fact, review of the literature leads one to conclude that many estradiol-binding sites may serve to initiate or modify rapid intracellular signaling in different cell types. Numerous membrane-associated estradiol-binding sites with an array of pharmacological properties, as well as a variety of different proteins, have been identified as putative membrane ERs (Baldi et al., 2000; Powell et al., 2001; Ramirez et al., 2001; Sak and Everaus, 2004). In some cells, including neurons, a small fraction of ER α or ER β can be associated with the plasma membrane (Levin, 1999; Razandi et al., 1999; Watson et al., 1999). Studies in endothelial cells and tumor cell lines support the ability of a variant of ER α to function as a membrane associated ER (Chen et al., 2004; Chen et al., 1999; Razandi et al., 1999; Razandi et al., 2003). The most clear cut example of a rapid ER α -centered mechanism of action is for the well-established rapid estrogen signaling mechanism that activates eNOS in vascular endothelium (Moriarty et al., 2006). Rapid estrogen signaling in human vascular endothelium increases the generation of nitric oxide (NO) by kinase mediated activation of eNOS. Here, a transcriptional variant of ER α (ER α 46) acts as a membrane associated receptor in a caveolin-1 associated multiprotein signaling complex located in specialized subdomains of the plasma membrane. Estrogen binding at ER α 46 stimulates c-Src activity leading to PI3K activity and AKT activation. Serine phosphorylation of eNOS by AKT stimulates nitric oxide synthase enzymatic activity resulting in increased NO generation. The vasoregulatory actions of E2-mediated NO generation may play an important role in homeostasis of vascular function (Moriarty et al., 2006).

In some neuronal cells and some breast cancer cells, heterotrimeric G-protein coupled mechanisms appear involved in rapid estradiol-induced ERK-activation (Belcher et al., 2005; Filardo et al., 2000; Filardo, 2002; Kelly et al., 1999; Navarro et al., 2003; Razandi et al., 2003; Thomas et al., 2005). In MCF-7 breast cancer cells, conflicting results indicate that either ER α alone, or an orphan GPCR (GPR30), but not ER α or ER β , is required for estradiol to rapidly activate ERK1/2 via the same G-protein dependent mechanism of epidermal growth factor receptor (EGFR) transactivation (Fig. 2; pathway 1) (Filardo, 2002; Razandi et al., 2003). A role for ER β in rapid ERK-signaling is likely, but less well studied. Results from recombinant cell models indicate that ER β can be localized to the plasma membrane and can function to activate ERK1/2 signaling with unique temporal and pharmacological properties (Razandi et al., 1999; Wade et al., 2001).

How a membrane associated ER is coupled to ERK-signaling is at least, if not more confounding than the identity of the receptors. From the same cell types there is evidence supporting multiple mechanisms linking the ER to ERK-activation. Initially a mechanism was proposed where a membrane associated version of ER α directly activates the non-receptor tyrosine kinase c-Src to activate p21-Ras (Fig. 2; pathway 3) (Migliaccio et al., 1996). However, different studies demonstrated a direct interaction between ER α and the adaptor protein Shc (Fig. 2; pathway 3) (Song et al., 2002; Song et al., 2004). Another study has identified a different adaptor protein, initially characterized as an ER α co-activator (PELP1/MNAR), that mediates cross-talk between ER α and the ERK pathway (Fig. 2; pathway 3) (Barletta et al., 2004; Wong et al., 2002). Further, in cortical neurons, a complex containing Hsp90, B-Raf and ER α was identified by co-immunoprecipitation which was taken to indicate a direct interaction between neurotrophin and estradiol-mediated ERK signaling via B-Raf (Singh et al., 2000). The significance of the former observations is unknown since these results have not been confirmed by others, and as initially observed in MCF-7 cells, it was later suggested that c-Src activation may be an initiating event in ERK-activation in this cortical neuron model. Results in cerebellar granule cell neurons also indicate that c-Src kinase activation is required for estradiol to rapidly stimulate ERK-signaling (Belcher et al., 2005); results that further support a critical role for Src-kinase activity in the mechanism of rapid estradiol-induced ERK-activation (Fig. 2; pathway 4). Thus, the Src-kinase is most well-established as a central figure in many different rapid-steroid signaling mechanisms, a role supported by results from studies using both neuronal and non-neuronal models.

GPR30 as a rapidly acting mER

The orphan G-protein coupled receptor (GPCR), GPR30, was initially isolated from Burkitt's lymphoma cells and found to be widely expressed in peripheral tissues and the brain (Owman et al., 1996). The predicted 375 amino acid seven-transmembrane glycoprotein from humans has a calculated core molecular mass of ~46 kD, where as the rat ortholog (previously named GPR41) is predicted to encode a ~43 kDa core protein. Importantly, GPR30 is abundantly expressed in ER α -positive breast cancer cells, with little or no expression detected in ER α -negative cells (Carmeci et al., 1997). Northern blot and *in situ* hybridization analysis has revealed that GPR30 is expressed in most tissues and distributed throughout many different brain regions (O'Dowd et al., 1998). In the rat, we have also independently confirmed that GPR30 mRNA and protein is widely distributed and is detected in most tissues. The importance of GPR30's wide distribution pattern is unknown.

Similarly, the function of GPR30 is unclear. The correlation between ER α expression and increased GPR30 expression in breast cancer cells has lead to the suggestion that GPR30 might be involved with hormone responsiveness of breast cancer malignancies. In MCF-7 cells, GPR30 may be involved in progestin-induced growth inhibition through slow onset inactivation of ERK1/2 signaling (Ahola et al., 2002a; Ahola et al., 2002b; Ahola et al., 2002c). Results of over-expression studies in breast cancer cell lines differentially expressing GPR30, ER α , and ER β have lead to the proposal that GPR30 was the plasma membrane associated mediator of rapid ERK-signaling in breast cancer cells (Filardo et al., 2000). Those results also suggested that GPR30 mediates an estradiol-dependent activation of matrix metalloproteinases (MMPs) resulting in liberation of heparin-bound EGF (HB-EGF) which acts as a ligand to stimulate autophosphorylation of the EGFR resulting in ERK1/2 activation (see Fig. 2; pathway 1). The role of GPR30 as the mER was not supported by subsequent studies in breast cancer and endothelial cell lines where ER α was interpreted to act as a GPCR to induce transactivation of EGFR through Src-kinase dependent activation of MMPs which regulated liberation of HB-EGF to activate the EGFR (Prenzel et al., 1999; Razandi et al., 2003).

While, GPR30 is increasingly considered an estrogen GPCR, based on a balanced assessment of the available evidence, it is not possible to state with any assurance that GPR30 plays any role in plasma membrane initiated rapid estradiol-induced ERK-signaling. In contrast to some binding studies, over expression studies suggest that GPR30 is not localized to the plasma membrane, but is instead an ER/Golgi-associated intracellular ER that stimulates PI3 kinase/AKT signaling in response to estradiol (Revankar et al., 2005). However, the results from another recently published over expression study suggests that GPR30 may yet have the capacity to function as an extracellular activated ER (Thomas et al., 2005). At least when over expressed *in vitro*, GPR30 remains a controversial candidate for an extracellular activated estrogen receptor that may in some cell types act to initiate ERK-signaling.

Rapid estrogen-induced signaling in the CNS

Based on results from other tissues and cell types, it is not surprising that the rapid actions of estradiol show a remarkable diversity in the CNS. These estradiol-mediated signaling events are of major importance during development and function of the brain (Kelly and Wagner, 1999; McEwen, 2002; Wong et al., 2003; Woolley, 1999; Zsarnovszky et al., 2005). Specific examples of rapid signaling actions include estradiol rapidly activating non-NMDA glutamate receptors in hippocampal neurons through a G-protein coupled and protein kinase A (PKA) dependent mechanism. In immortalized gonadotropin-releasing hormone (GnRH) cells, estradiol rapidly modulates cAMP levels via ER α -dependent activation of G $_{\alpha i3}$. (Navarro et al., 2003). In midbrain dopaminergic neurons, the IP3 kinase/AKT signaling pathway is rapidly estradiol-responsive (Ivanova et al., 2002). As we have found for cerebellar neurons (Wong et

al., 2003), in cultured cortical and hippocampal neurons and midbrain astrocytes, estradiol rapidly activates the ERK1/2 (Beyer et al., 2002; Singer et al., 1999). Thus, diverse rapid estrogen signaling mechanisms are active throughout many different regions of the brain.

ERK-mitogen activated protein kinase signaling in cerebellum

The MAPK pathways play critical roles in development of the nervous system by regulating growth, differentiation, and viability of both neurons and glia. In neurons, the ERK1/2 MAPK pathway is most well characterized as being activated by the binding of growth factors (e.g. EGF) or neurotrophins (e.g. BDNF) at their cognate receptor tyrosine kinases to initiate the sequential phosphorylation and activation of downstream effector kinases leading to phosphorylation of specific protein substrates (Fig. 2). As described above, GPCRs can also stimulate ERK1/2 signaling which facilitates crosstalk between growth factor and heterotrimeric GPCR-mediated signaling.

In immunohistochemical studies employing phospho-specific active-ERK1/2 (pERK) antiserum, the spatial and temporal patterns of activated-ERK1/2 in the developing and adult rat cerebellum have been characterized (Zsarnovszky and Belcher, 2004). In both males and females, the distribution and cell type-specificity of pERK-immunoreactivity (IR) followed identical age-related patterns. The initially higher levels of pERK-IR in immature Purkinje cells decreased during the second postnatal week and then increased at later times. By contrast, during the second postnatal week, immunopositive granule cell neurons increased, became decreased during much of later postnatal cerebellar development, and became absent in adults. In cerebellar glia pERK was present in the soma of maturing Bergmann glia beginning on postnatal day 4 and in astrocytes on postnatal day 10. Associated with weaning, pERK-IR was increased in all cell types on postnatal day 22. In adults, pERK-IR was confined to the Purkinje cell layer and scattered cells of the corpus medullare. Overall, the high degree of developmental plasticity in the spatiotemporal distribution of pERK-IR observed in the cerebellum revealed that the ERK-pathway had a dynamic role in regulating numerous developmental processes including glial migration, neuronal proliferation and differentiation, and postsynaptic information processing. The results of these studies also represented a foundation for studies aimed at determining whether estrogens influence ERK-signaling in the developing and mature cerebellum.

Estrogen receptor expression in the developing cerebellum

The idea that ERs might have a role in developing cerebellar cells arose from initial studies employing quantitative RT-PCR analysis of ER α and ER β transcript expression in purified populations of maturing cerebellar granule cells. Results of those studies demonstrated that ER mRNAs were present and that their expression and alternative splicing was modulated during early postnatal development in the rat cerebellum (Belcher, 1999).

Subsequent immunocytochemical studies analyzed ER β expression in the cerebellum at different times during the postnatal development period. Expression of ER β varied with age and cell-type, but not sex (Jakab et al., 2001). Highest levels of ER β -IR were detected in all major types of cerebellar neurons during periods of active neurite extension, and in some glial cells during migration. Throughout the first week after birth, ER β -IR was localized to scattered migrating glia and differentiating, but not mitotic, granule cell precursors, in the external germinal layer. Differentiating granule cells expressed detectable levels of ER β throughout development and mature granule cells retained low, but detectable levels of ER β into adulthood.

Peak intensities of ER β -immunostaining coincided with the initiation of axonal growth in granule cells, and in Purkinje cells the initiation of dendrite growth and significant extracellular

interactions with descending granule cells. Once induced, expression of ER β remained high during maturation of Purkinje cell dendrites, and was maintained in the adult. From the third postnatal week, ER β -IR was also detected in the later developing Golgi, stellate and basket neurons. Based on the observed temporal and cell-type specific changes in ER β expression and the correlation of these changes with known events during cerebellar development, the results were collectively interpreted to suggest that ER β was involved in post-mitotic developmental events related to migration, extension of cellular processes of cerebellar neurons and glia and the regulation of synaptic dynamics (Jakab et al., 2001).

Rapid Estrogen Signaling mechanism in cerebellar granule cells

Results published in Wong et al. confirmed that estrogen directly impacts the development and maturation of cerebellar neurons (Wong et al., 2003). In those *in vitro* studies primary neonatal rat cerebellar granule cell models were used to investigate the effects of estradiol on granule cell development and to characterize rapid estradiol-mediated signaling in these neurons. Low concentrations of 17 β -estradiol, 17 α -estradiol, and 17 β -estradiol-BSA were found to specifically stimulate phosphorylation of the ERK1/2 with unique pharmacological profiles. These pharmacological studies also clearly revealed that ICI182,780 is a highly efficacious agonist of rapid estrogen signaling, an observation that has since proven useful in studying the mechanism and physiological actions of rapid ERK-signaling in the absence of classical transactivation effects mediated via nuclear estrogen receptors (Belcher et al., 2005; Wong et al., 2003).

The physiological consequence of rapid estrogen signaling was analyzed in dissociated primary explant cultures of developing cerebellar neurons. Rapid estrogen signaling through the ERK-pathway was found to increase oncotic/necrotic, but not apoptotic, programmed-cell death in a subpopulation of sensitive granule cells. Rapid estrogen signaling also increased mitogenesis from the granule cell precursors that were refractory to estradiol-induced toxicity. In contrast, long-term estrogen exposures that induced activation of rapid signaling and nuclear ER transactivation were found to decrease granule cell neurogenesis, but protected the maturing neurons already present, an effect that resulted in a net increase in viable granule cells following exposures. As one would predict if these activities were generalize, rather than sexually specialized, no differences were detectable in the effects on granule cells isolated from males or females. Significantly, these studies showed that estrogen exposure during critical developmental periods could influence the final balance of neuronal numbers in the cerebellum by regulating genesis, viability and cell death of granule cells. The impact of endogenous and environmental estrogen action on granule cell precursors, and the long term consequences of inappropriate exposure to estrogenic compounds during epigenesis is an area of continued investigation.

In a follow-up study, the signaling mechanism of rapid estrogenic ERK-signaling by estradiol and ICI182,780 in primary cerebellar neurons was analyzed in more detail (Belcher et al., 2005). In this study ICI182,780 was again employed as a second rapid signaling agonist, with the results reinforcing the previous conclusion that, while less potent, ICI 182,780 is a highly efficacious agonist of rapid estrogenic ERK-signaling.

The temporal nature of rapid estrogen mediated ERK-signaling was rapid and transient, similar to the actions of EGF at the EGFR. However, rapid estrogen signaling in granule cells did not involve transactivation of the EGFR. Instead, it was observed that estrogen mediated its effects on ERK-signaling through a pathway coupled to G α_i and required protein kinase A (PKA) and c-Src kinase activity to link estradiol to the ERK-signaling module. Estradiol thus appears to activate ERK1/2 through a G-protein dependent mechanism that resembles a Gs/Gi switching mechanism first described for β_2 -adrenergic receptor (Fig 2; pathway 4). Additional

experiments also revealed that protein phosphatase 2A (PP2A) activity was rapidly stimulated by estradiol through a separate intracellular initiated rapid-signaling pathway that unlike rapid ERK-signaling, was independent of G α i and PKA, and was not stimulated by membrane impermeable estradiol. This first demonstration of rapid signaling effects of estradiol on protein phosphatase activity could account for the transient nature of estradiol-induced ERK activation in these neurons.

***In vivo* studies of rapid estradiol and BPA effects in the cerebellum**

Detailed *in vivo* pharmacological study of rapid estrogen signaling in neurons at differing stages of development presents numerous challenges (e.g. temporal considerations and the impacts of steroid binding proteins on bioactive concentrations of steroid). To decrease uncontrolled variables associated with systemic exposures, an *in vivo* model system where the cerebellum of anesthetized neonatal and adult rats was directly exposed to known concentrations of estrogens by intracerebellar infusion was developed (Zsarnovszky et al., 2005). Using that approach, the rapid impact of estradiol on active ERK-IR in the cerebellum was pharmacologically characterized. Initially, the dose-dependency of rapid estradiol the endocrine disrupting chemical BPA on estrogen-mediated ERK1/2-phosphorylation was investigated. Quantifiable immunostaining techniques using phosphorylation-state specific ERK antiserum showed that estradiol influenced ERK-signaling in an age, cell type and concentration dependent fashion that was independent of sex. In the developing cerebellum, the spatial and temporal profile of estradiol-induced ERK activation reflected a modulatory role in maturing excitatory synapses and/or an indication of excitatory activity in the immature synapses. In the mature cerebellum, estradiol was found to increase pERK in interneurons, but eliminated pERK from the axon hillock of Purkinje cells, suggesting that estradiol-induced activation of ERK-signaling in inhibitory interneurons may lead to disinhibition of Purkinje cells. In the different populations of sensitive cerebellar neurons, the ability of estradiol to mediate changes in ERK-activity correlated very well with the changing patterns of ER β expression during development (Jakab et al., 2001), suggesting that ER β may mediate the rapid estradiol-induced actions in the cerebellum.

Significantly, detailed dose-response analysis revealed that physiologically relevant concentrations of estradiol and BPA acted as equally efficacious and equally potent ligands. Specifically, estradiol and BPA, induced ERK-activation in a non-monotonic dose dependent fashion both in granule cell cultures and *in vivo* with a similar potency. Paradoxically, injection of a mixture of active concentrations of estradiol and BPA completely blocked the ERK-stimulatory actions of each compound alone. When a maximally active concentration of estradiol (10^{-10} M) was coinjected with as little as 1 pM BPA, >50% inhibition was observed. The blockade of effects observed upon co-administration of estradiol/BPA suggests that they act as mutual-disruptors of each others rapid-estrogenic actions. The incongruous ability of BPA to mimic the rapid effects of estradiol with comparable potency and efficacy, and to also act as very potent disruptor of rapid signaling, highlights the complexity associated with the effects of estrogenic endocrine disruptors.

Malignant childhood brain tumors as estrogen responsive tumors

Primitive neuroectodermal tumors (PNETs) are the most common form of pediatric brain tumor (Ries et al., 1999). Most often these malignant childhood brain tumors arise from neuroepithelial precursor cells in the cerebellum, and less frequently in the cerebral cortex (Wechsler-Reya and Scott, 2001). Because the normal PNET precursor cells from the cerebrum and cerebellum transiently express high levels of estrogen receptors (Jakab et al., 2001; Zsarnovszky and Belcher, 2001), we hypothesized that PNETS and medulloblastoma are estrogen responsive tumors.

Initial studies in the cerebrocortical-derived cell line PFSK1 revealed that PNET cells expressed variants of both ER α and ER β and that low physiological concentrations of estradiol rapidly stimulated ERK1/2 phosphorylation and nuclear translocation within 15 minutes of exposure (Kirby et al., 2004). However, exogenously added 17 β -estradiol (E2) could not stimulate PFSK1 growth. This lack of effect is believed due to maximally elevated levels of growth stimulation via autocrine/paracrine PDGF signaling (Kirby et al., 2004). However, estradiol was shown to increase PFSK1 cell migration via a nuclear receptor transactivation mediated pathway that also required ERK-signaling. Those results suggested that rapid signaling actions and ER-mediated transactivation collaborate to increase the invasive nature of PNETs. The findings of this study supported the possibility that PNETs which share common progenitors with estrogen-responsive cerebral and cerebellar neurons, were estrogen responsive tumors.

Because medulloblastoma are the most common malignant brain tumor in children and because they arise from cerebellar granule cell-like precursors (Ries et al., 1999; Wechsler-Reya and Scott, 2001), the impact of the rapid and nuclear hormone receptor mediated actions of estradiol on these neuroectodermal tumors was analyzed in detail. It was hypothesized that like normal granule cell precursors; malignant medulloblastoma cells express ER β and are estrogen-responsive. As in PFSK1 cells, preliminary western blot analysis indicated that ER β -like proteins are expressed in two medulloblastoma-derived cell lines (Daoy; D283Med) representing the differentiated “glial” and “neuronal” phenotypic profiles of medulloblastoma. Immunohistochemical studies found significant amounts of ER β in all 22 primary medulloblastoma tumors that were analyzed (e.g. Fig. 4). Preliminary xenograft studies have also confirmed that estradiol regulates metastatic medulloblastoma cell invasion and tumor growth *in vivo*, and that anti-estrogen chemotherapeutics effectively block estrogen sensitive tumor growth/invasion. These *in vitro* and *in vivo* studies suggest that novel estrogen-signaling therapeutic approaches could be useful for treating these pediatric brain tumors.

In summary, the actions of estrogens in the cerebellum, and other non-sexually associated regions of the brain, are not yet fully appreciated. While we and others have accumulated strong evidence that estrogens impact cerebellar neurogenesis, viability and migration during development, a clear picture of estrogenic mechanisms and their physiological actions is yet to be exposed. As a whole the study of rapid estrogen signaling in neurons of the developing cerebellum, stands as a clear and striking example of the diverse and dynamic nature of estrogen signaling in the different cell types of a single region of the brain. It is now apparent that the mechanisms of estrogenic signaling are dynamically cell-specific, and changeable during epigenesis in ways that are incompatible with “classical” nuclear hormone receptor theory. Additional investigation into the mechanisms of estrogen action, other steroid hormones and their metabolites, and the intrinsic and environmental factors that modify the physiological impact of steroid-action across the life-time, will undoubtedly result in additional discovery that afford unanticipated opportunity for improved treatments of many human diseases.

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Figure 1. Classical model of nuclear hormone receptor action

Shown is a cartoon describing the “classical” nuclear receptor mechanism of ligand-mediated estrogen receptor (ER) effects. Ligand binding induces a conformational change of the receptor leading to dissociation from a chaperone complex containing heat shock proteins. Activated receptors dimerize and bind at estrogen response elements (ERE) in the promoters of estrogen responsive genes. Ligand/receptor complex binding of EREs results in recruitment of co-regulatory complexes to the promoter which modifies target gene transcription through protein/protein interactions with the basal transcription complex.

Figure 2. Models for the mechanisms of cross-talk between receptor tyrosine kinase/growth factor and rapid estradiol-induced activation of ERK1/2

This schematic diagram illustrates proposed mER-mediated mechanisms that can mediate rapid estradiol-induced ERK1/2 activation. Pathway 1 represents G-protein-like transactivation of epidermal growth factor receptor (EGFR) proposed to occur via GPR30 or ERα induction of MMP-mediated heprin-bound EGF (HB-EGF) shedding; Pathway 2 shows the mechanism proposed to involve direct interaction of ERα with Shc which acts to couple the activated ER to the ras/ERK pathway; Pathway 3 is a cascade involving the interaction of the ER with the adaptor protein PELP/MNAR 1, or directly interacting with c-Src to stimulate c-Src kinase, which in turn activates Ras to link the activated ER to ERK-MAPK pathway; Pathway 4 shows the G-protein and Src-kinase dependent, EGF-receptor independent pathway operative in cerebellar granule cells. The site of inhibition for specific antagonists used to elucidate the pathway of rapid cerebellar signaling are indicated. The dotted arrow indicates the identified rapid intracellular estrogen signaling pathway that stimulates protein phosphatase 2A (PP2A) activity in granule cells. This phosphatase pathway is independent of Gαi-signaling and is also PKA independent. PP2A activation is required, along with rapid ERK-activation, for estrogen to induce calpain-mediated cell death in granule cell precursors. Key protein kinase activation phosphorylation sites are indicated with a red P. The PI3K pathway is included to illustrate that EGF-receptor activation in granule cells activates both the ERK & PI3K pathways, but estrogen only stimulates ERK-signaling.

Figure 3. Changing distribution of ERβ expression in the cerebellar cortex during postnatal development

The diagram presents the temporal and cellular profile of ERβ expression in the mid-line vermis of the cerebellar cortex. The changing spatial and morphological properties of maturing Purkinje cells and granule cells are represented. The approximate time after birth in postnatal days is indicated by a timeline at the bottom of the figure. Immunopositive cells are indicated in red. Abbreviations: EGLd –differentiating sublayer of the external germinal layer; EGLm – mitotic sublayer of the external germinal layer; IGL - internal granule cell layer; ML - molecular layer; PL -Purkinje cell layer.

Figure 4. Immunohistochemical analysis demonstrating ER β expression in primary medulloblastoma

(A) Immunohistochemical detection of ER α in an ER-positive breast carcinoma (positive control; ab: 6F11). (B) A representative medulloblastoma (6 yr old male) section DAB stained for ER α ; no immunopositive cells are detectable. (C) A serial section of the same tumor was stained following treatment with non-specific serum. (D) An additional serial section was stained with anti-ER β -specific monoclonal antibody 14C8 demonstrating strong ER β -specific immunostaining. Tissue samples were obtained by the Cooperative Human Tissue Network which is funded by NCI. Scale bar is 50 μ m.