Abstract:

Title: The Effects of Dopamine and Estrogen upon Cortical Parvalbumin Expression

Name: Nicole R. Ross Degree: Ph.D. Year: 2001

Thesis directed by: Dr. Linda L. Porter, Associate Professor, Department of Anatomy, Physiology and Genetics, and the Neuroscience Program

Estrogen plays a protective role in several neurologic disorders known to involve alterations in both the dopamine system and (gamma amino butyric acid containing) GABAergic neurons that contain parvalbumin. Two studies were conducted to identify the effects of estrogen and dopamine upon cortical parvalbumin expression. In the first study, organotypic slice cultures of the frontal cortex were prepared from male rats (postnatal age 2/3) and maintained for 14 days in serum-enriched medium or media containing either: dopamine, estrogen, or a combination of the two. In control slices, parvalbumin immunoreactive neurons were primarily clustered near cortical layer V. All treatment conditions increased the distribution of parvalbumin labeled neurons in layer VI and enhanced the maturation of dendritic measures of labeled neurons in the deep layers (V-VI). Both estrogen and the combination treatment induced similar alterations in the superficial cortical layers, as well. In the second study, gonadectomy (male rats, postnatal age 2) induced long lasting decreased in the density of parvalbumin immunoreactive neurons throughout all cortical layers. Estrogen replacement restored parvalbumin expression to control levels in the superficial cortical layers, only. Gonadectomy also

increased dopaminergic neuron density in the substantia nigra, compacta and tyrosine hydroxylase fiber length in the cortex at 33 days postnatal. Estrogen replacement returned dopaminergic neuron density, but not cortical fiber length, to control levels. Neonatal 6-hydroxy dopamine (6-OHDA) lesions produced results in the cortex that were similar to those induced by gonadectomy, regardless of the loss in dopaminergic neurons. Taken together, these findings indicate that estrogen alters cortical parvalbumin expression and cortical and subcortical dopaminergic systems.

The Effects of Dopamine and Estrogen upon Cortical Parvalbumin Expression.

by

Nicole R. Ross

Dissertation submitted to the Faculty of the Neuroscience Program of the Uniformed Services University of the Health Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy 2001

Dedication:

This thesis work is dedicated to my husband Kemp and my daughter Sarah Phoebe. They have lovingly stood by me through my mood swings. Their support helped me complete this work. Kemp, thanks for standing by me. Sarah, thanks for being so understanding.

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Table of Contents:

Introduction:

Alterations in specific subclasses of cortical neurons are linked to a variety of brain disorders. For example, changes in neurons containing the calcium binding protein, parvalbumin, occur in disease states such as Alzheimer's disease (Aria et al, 1987; Solodkin et al, 1996; Brady and Mufson, 1997; Mikkonen et al, 1999), Creutsfeldt-Jakob disease (Ferrer et al, 1993; Guentchev et al, 1997) and schizophrenia (Beasley and Reynolds 1997; Kalus et al, 1997; Pierri et al, 1999). Such alterations may have drastic effects on cortical function and may contribute to the cognitive and behavioral disturbances exhibited in these diseases.

Parvalbumin is expressed in morphologically distinct subsets of gamma amino butryric acid containing (GABAergic) neurons (Conde et al, 1994). In the cerebral cortex, this protein is expressed primarily by chandelier and basket cells (Kawaguchi et al, 1987; Kawaguchi and Kubota, 1993, 1997), GABAergic neurons that exert potent inhibitory influences over pyramidal neurons (DeFelipe et al, 1985). The exact function of parvalbumin has yet to be elucidated. However, the protein is thought to protect developing and adult neurons from the effects of high levels of metabolic activity by buffering intracellular calcium levels (Chard et al, 1993). Accordingly, areas of high levels of metabolic activity contain large numbers of parvalbumin immunoreactive neurons (Cohen et al, 1993; Celio et al, 1986). Moreover, levels of expression of this protein vary with activity and therefore, decline in response to sensory deprivation (Gutierrez and Cusick, 1994

Decreases in parvalbumin expression are observed in the frontal lobe of schizophrenics (Beasley and Reynolds, 1997, Kalus et al, 1997). This particular cortical region is notably dysfunctional in schizophrenics (Andreasen et al, 1997). The frontal lobe is subdivided into distinct cytoarchitectural and functional regions which are connected to numerous cortical and subcortical regions including the hippocampus, thalamus, limbic areas and mesencephalic dopaminergic nuclei (see Fuster, 2001, for review). Specifically, the prefrontal region exerts control over cognitive, emotional and temporal processes (see Fuster, 2001, for review). This region does not mature functionally or morphologically until adolescence (Chunagani et al, 1987; Paus et al, 1999).

The complex series of events which occur during cortical maturation require a delicate balance of timing and appropriate circuit formation. Perturbations during this process may lead to cortical abnormalities thought to contribute to a developmental predisposition to schizophrenia. The dopaminergic system provides a rich innervation to the prefrontal cortex and is implicated in schizophrenia also continues to develop into adulthood. The density of dopaminergic fibers in the rodent frontal cortex gradually increases during postnatal development and peaks in adulthood (Verney et al, 1982; Kalsbeek et al, 1988). Cortical concentrations of tyrosine hydroxylase (TH), the rate limiting enzyme for dopamine production, peak earlier around 15 days postnatal (Chen et al, 1996), coinciding with peaks in dopamine receptor expression (Leslie et al, 1991, Shambra et al, 1994).

Cortical parvalbumin expression in GABAergic neurons also develops postnatally.

Somata immunoreactive for parvalbumin typically appear first in the frontal cortex around postnatal day 9 in the rat. Parvalbumin expression in somata first appear in layer V, progresses to neurons in layer VI, then to neurons in layer IV and finally to neurons in layers III-II. Somal labeling in each layer is succeeded by dendritic and axonal labeling two days later (Alcantara et al, 1993). At postnatal day 14, parvalbumin immunoreactive somata are normally observed in both the deep and superficial layers in the frontal cortex. By postnatal day 21, the adult level of parvalbumin expression is achieved in the normal rat: somal and fiber staining extend throughout layers II-VI, with particularly dense bands of labeled neurons in layers II-III and layer V (Alcantara et al., 1993).

The overlapping time-course of developing cortical dopamine and parvalbumin systems suggests that dopamine modulates parvalbumin expression. In fact, the absence of dopamine slows the maturation of neurons containing this protein *in vitro* (Porter et al, 1999). Conversely, neonatal increases in cortical catecholamine levels cause persistent increases in dendrite lengths and density of GABAergic neurons (Wang et al, 1995, 1996). Perturbations to the normal dopaminergic modulation of parvalbumin expression therefore, may be linked to the alterations in parvalbumin expressing neurons reported in schizophrenics.

Gonadal hormones also affect cortical development. Estrogen receptors are expressed in the neonatal (Miranda et al, 1992; Sandhu et al, 1986; MacLusky et al, 1979) and adult (Shugrue et al, 1997) rodent cortex. Whereas, the neonatal rodent brain is isolated from circulating estrogens through the action of the plasma estrogen binding protein, alpha feto protein, localized conversion of testosterone to estrogen occurs in the

cortex (MacKlusky et al, 1979). Therefore, both estrogen and its receptors are present in the cortex during development and may account for gender dimorphisms in the brain such as differences in the thickness of cortical hemispheres (see Sandhu et al, 1986, for review). Gonadal hormone manipulation can reverse this particular dimorphisms in both female and male rats (see Sandhu et al, 1986, for review).

Steroidal hormones also affect the development of the dopaminergic system (Adler et al, 1999; Kritzer et al, 1998; Stewart et al, 1994; Mack et al, 1991; Paden et al, 1982). For example, neonatal ablation of gonadal hormones in male rats increases dopamine levels in the cingulate cortex (Stewart et al, 1994). Furthermore, estrogen treatment blocks dopamine D2 receptors (Paden et al, 1982) and altered dopamine mediated behaviors, such as locomotor activity (Hafner et al, 1991). In light of these findings, alterations in either dopamine or estrogen levels could contribute to the cortical dysfunction seen in schizophrenia.

In fact, gender differences in the symptomology of schizophrenia suggest hormonal influences early in the disease process. For example, the age of onset of schizophrenic symptoms is significantly later in females than in males (Hartmann & Meyer, 1969; Lewine et al, 1981; H fner et al, 1989). Gender differences in structural brain abnormalities (Nopolous et al, 1997; Andreasen et al, 1986) which develop prior to symptom onset (see Harrison, 1995 for review), and neuroleptic response (Seeman, 1986) also exist. Furthermore, symptom severity in female schizophrenics is correlated with fluctuating estrogen levels (Hallonquist et al, 1993). Exploring the effects of estrogen on cortical development therefore, may aid in the understanding of the disease process.

It was established previously that dopamine alters parvalbumin expression in the cortex (Porter et al, 1999). The role of estrogen in modulating parvalbumin expression, however, was not addressed. Because estrogen affects the dopamine system, and dopamine enhances parvalbumin expression in the cortex, examining the effects of estrogen upon parvalbumin containing neurons may add to the understanding of how the expression of this protein is modulated. Because alterations in parvalbumin expressing neurons are reported in many neurologic disorders, identifying the mediators of parvalbumin expression may aid in the understanding of the pathophysiology of various diseases. Therefore, the experiments contained in this thesis were designed to elucidate the effects of dopamine and estrogen upon parvalbumin expression. To determine the roles of these factors in parvalbumin expression during cortical development, both an *in vitro* and *in vivo* approach were used.

The thesis is organized following the alternate thesis format outlined by the USUHS Neuroscience program and Graduate Education Committee. Therefore, the thesis contains a general introduction followed by two first-authored papers previously submitted to peer-reviewed journals, and then followed by a section linking the two papers and discussing the importance of the findings from the thesis experiments.

The Effects of Dopamine and Estrogen upon Cortical Neurons that Express Parvalbumin *In Vitro*

Nicole R. Ross, Ph.D[1]. and Linda L. Porter, Ph.D[1, 2]

[1]Neuroscience Program, and [2]Department of Anatomy, Physiology and Genetics,

Uniformed Services University of the Health Sciences, Bethesda, Maryland, 20814

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Corresponding Author: Linda L. Porter, Ph.D.

Department of Anatomy, Physiology, and Genetics

Uniformed Services University of the Health Sciences

4301 Jones Bridge Road, Bethesda, Maryland 20814.

Phone: (301) 295-3217

Facsimile: (301) 295-1715

Email: lporter@usuhs.mil

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Abstract:

The purpose of these experiments was to study the effects of dopamine (DA) and estrogen (EST) upon parvalbumin expression in rodent frontal cortex during development. Organotypic slice cultures of the frontal cortex were prepared from neonatal rats (postnatal day 2/3) and maintained for 14 days *in vitro* in serum-enriched medium and medium treated with either DA, EST (17 ß-estradiol), or DA+EST. Cultured slices were then fixed and immunostained for parvalbumin immunoreactivity. Under control conditions, parvalbumin immunoreactive somata and fibers were primarily found in the deep laminae. In comparison, slices in all treatment groups exhibited a pattern of parvalbumin expression that was significantly different than controls. Specifically, DA treatment increased the percentage of parvalbumin immunoreactive somata, dendritic length and density in the deep cortical layers, but not in the superficial cortical layers. Both EST and DA+EST treatments induced similar changes in both the deep and the superficial cortical layers. These treatment induced changes represent more mature patterns of parvalbumin expression when compared to controls, indicating that both DA and EST enhance cortical expression of the protein.

1. Introduction:

Many cognitive and behavioral disorders are thought to arise from structural abnormalities induced by altered cortical development. In particular, a predisposition to schizophrenia, a disease marked by a combination of cognitive and behavioral dysfunctions, is likely to arise from neurodevelopmental disturbances. This notion is supported by evidence that preschizophrenic children exhibit subtle characteristics of the disease. Furthermore, as reviewed by Harrison (1995), structural abnormalities exist at or before the onset of symptoms, indicating a neurodevelopmental component.

The neurotransmitter dopamine (DA) is not only implicated in the pathophysiology of schizophrenia (Horn et al, 1971;Weinberger, 1993), but alterations in its levels affect cortical development. In fact, similarities exist between cortical abnormalities caused by changes in DA levels during development and those observed in the brains of schizophrenics. For example, increases in prenatal levels of cortical DA induce changes in the dendritic morphology of pyramidal neurons *in vitro* (Reinoso et al, 1996) and *in vivo* (Jones et al, 1996). Furthermore, increases in catecholaminergic levels cause persistent increases in the density and the dendritic lengths of GABAergic neurons (Wang et al, 1995, 1996). Both pyramidal (Lewis et al, 1995) and GABAergic neurons (van Kammen et al, 1998; Ohnuma et al, 1999; Volk et al, 2000) are similarly altered in the brains of schizophrenics, whose normal DA function is compromised.

One particular subset of GABAergic neurons, those containing the calcium binding protein, parvalbumin, is altered in the disease. Decreases in the density of parvalbumin immunoreactive neurons are observed in the frontal (Beasley and Reynolds, 1997) and

anterior cingulate (Kalus et al, 1997) cortices of schizophrenics. Moreover, alterations in the axon cartridges of chandelier cells, GABAergic neurons that express parvalbumin, are observed in postmortem studies (Pierri et al, 1999) , indicating changes in the transmitter systems of these neurons.

Parvalbumin expression follows a distinct and sequential pattern of development in the intact rodent cortex. Immunoreactive somata appear earlier in some cortical areas than others, but typically appear first in layer V of each region. Parvalbumin expression progresses next to cortical layer VI, and finally to layers III-II. Labeled somata appear first in each layer, succeeded by dendritic and axonal labeling two days later (Alcantara et al, 1993). At postnatal day 14, parvalbumin immunoreactive somata are normally expressed in both the deep and superficial layers in the frontal cortex. By postnatal day 21, the adult level of parvalbumin expression is achieved in the normal rat: somal and fiber staining extends throughout layers II-VI, with particularly dense bands of immunoreactivity in layers II-III and layer V (Alcantara et al, 1993).

Expression of parvalbumin develops at a late postnatal stage in the cortex. Interestingly, DA receptor expression also peaks late, around 14-21 days postnatal (Leslie et al, 1991; Schambra et al, 1994). The concentrations of tyrosine hydroxylase (TH), the rate limiting enzyme for DA production, fluctuates developmentally in the rodent frontal cortex but, its highest level occurs at approximately 15 days postnatal (Chen et al, 1997). Qualitative studies show that the density of DA fibers in the rodent cortex gradually increases throughout postnatal development and peaks at adulthood (Verney et al, 1982; Kalsbeek et al, 1988). The overlapping time-course for expression of these cortical

systems allows for a role of DA in the development of parvalbumin immunoreactivity. In fact, DA appears to regulate the time-course of parvalbumin expression during postnatal development. The absence of DA in the developing frontoparietal cortex slows the maturation of neurons that contain this protein (Porter et al, 1999). Conversely, neonatal increases in DA levels in the anterior cingulate cortex augment the length of parvalbumin immunoreactive dendrites *in vivo* (Wang et al, 1995).

Gender differences in the expression of schizophrenic symptoms suggest a role for hormones in the disease process. The age of onset of schizophrenic symptoms is significantly later in females than in males (Hartmann et al, 1969; Lewine and Anderson, 1981; H fner et al, 1989). Differences in structural brain abnormalities, such as ventricular volume, also are apparent between male and female schizophrenics (Andreasen et al, 1986; Nopoulos et al, 1997).These structural differences may be both hormone-induced and developmental in origin. Moreover, males have a poorer response to neuroleptic treatment than females (Seeman, 1986). Further implicating hormones in the disease process, symptom severity in female schizophrenics is correlated with fluctuating estrogen (EST) levels (Hallonquist et al, 1993) and schizophrenic women sometimes exhibit low EST levels in comparison to normal women (Oades and Schepker, 1994). Therefore, exploring the effects of EST on cortical development seems integral to the understanding of the disease process.

Estrogen receptors are expressed in the neonatal (MacLusky et al, 1979; Sandhu et al, 1986; Miranda and Toran-Allerand) and adult (Shughrue et al, 1997) rat cortex, suggesting an intrinsically mediated role for this hormone in cortical development.

Additionally, steroidal hormones affect maturing cortical and subcortical DA systems (Paden et al, 1982; H fner et al, 1991; Mack et al, 1991; Stewart and Rajabi, 1994; Guivarc h et al, 1995; Kritzer et al, 1998; Adler et al, 1999). For example, blockade of localized conversion of testosterone to EST, followed by gonadectomy in neonatal male rats increases DA levels in the cingulate cortex as compared to controls (Stewart and Rajabi, 1994). Furthermore, DA D2 receptor antagonism (Paden et al, 1982) and altered DA mediated behavior (Håfner et al, 1991) occur with early EST treatment. Considering that gender differences exist in schizophrenia, and that EST alters the cortical DA system and DA mediated behavior, it is possible that an interaction between EST and DA plays a role in the developmental alterations seen in the disease.

It has been established that DA alters developmental parvalbumin expression in the cortex. The possibility of a role for EST in modulating parvalbumin expression has not been addressed. Therefore, our lab set out to determine whether EST alters cortical parvalbumin expression through its effects upon DA or through independent mechanisms.

2. Materials and Methods

Culture Preparation:

Methods were followed as described previously (Porter et al, 1999). Briefly, organotypic slice cultures were prepared from male Sprague Dawley rat pups (Taconic), ages 2-3 postnatal days. All procedures were done in accordance with the NIH guide for the care and use of animals as outlined in a protocol approved by the USUHS IACUC. Appropriate measures were taken to alleviate pain and suffering. The pups were anesthetized by hypothermia until areflexive and quickly decapitated. Under sterile conditions, brains were rapidly dissected, removed, and placed in cold sterile MEM with 200 mM Tris buffer (dissecting medium). The frontal cortex was blocked and removed by making a coronal cut at the extreme frontal pole and a second cut approximately 2 mm caudal to the first. The pia matter was removed gently and the tissue was placed on a McIlwain tissue chopper. The right and left hemispheres were cut simultaneously into 250 μm coronal sections. Slices were washed into cold dissecting medium and separated by gentle agitation using a fire polished wide aperture pipette. Selected slices were placed upon Millicell-CM (Millipore, Bedford, MA) inserts immersed in cold dissecting medium. Four such inserts were placed in a single petri dish to ensure identical treatment conditions for all slices in a group. Slices were allocated randomly into control and experimental groups. Petri dishes contained at least 12 slices (3 slices per individual Millicell-CM insert). Each petri dish was subjected to one treatment condition. Control and all experimental groups were collected from a single litter (trial), prepared for culturing on the same day, and cultivated for the same duration. Seven trials were

repeated, each with a control and all treatment groups. For cultivation, dissecting medium was replaced with a sufficient amount of culture medium (MEM supplemented with 25% normal horse serum, NHS), to maintain an air to surface interface (Stoppini et al, 1991). The NHS was carbon filtered three times to remove steroidal agents (Hindawi et al, 1980). Phenol-red free MEM was used throughout the cultivation period, because of the potential steroidal action of this pH marker (Berthois et al, 1986). Serum levels were reduced from 25% NHS to 15% NHS after the first week of cultivation. Every 2-3 days, the medium was refreshed by removing half of the existing medium and replacing it with fresh medium. Control slices were maintained in culture medium supplemented with NHS alone. The experimental groups were supplemented with 10 μM DA (Porter et al, 1999), 10^{-7} M 17 ß-estradiol (EST) (Levy et al, 1996), or both 10 μM DA and 10^{-7} M EST for the entire cultivation period. The final DA concentration was obtained from stock solutions of 10 mM DA in 0.1 % ascorbic acid in sterile distilled water, which was also made weekly. The final EST concentration was prepared from a 1 mM stock of cyclodextrin-coated EST dissolved in water, which was made weekly. All control and experimental media contained comparable amounts of ascorbic acid to account for potential vehicle effects. Slices were maintained in a humid atmosphere at 36 ˚C (CO_2: 5.0%, 0_2: 20%) for 14 days.

Immunohistochemistry:

After 14 days *in vitro*, slices were harvested and immersion-fixed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer ($NaP0_4$) for one hour at room temperature (RT). Slices were washed twice in 0.1 M $NaP0_4$ and once in 0.05 M Tris

buffer with 0.6 NaCl (TBS) and 0.2% Triton X. The slices were then incubated at 4 ˚C for 48 hours in 0.05 M TBS and 0.2 % Triton X containing monoclonal antibodies raised in mouse against parvalbumin (1:10,000, Sigma, St. Louis, Missouri). Slices then were washed three times in TBS and incubated for 45 minutes in biotinylated 2˚ antibody (Vector) raised in horse against mouse and further processed according to the Vector ABC kit protocol. Parvalbumin immunoreactivity was visualized using diaminobenzadine (DAB) as a chromagen. Slices were separated from the Millicell-CM membrane, mounted on glass slides, dried, dehydrated and covered.

Data Collection and Analysis:

The laminar distribution and dendritic arborization patterns of parvalbumin immunoreactive cells within slices were determined using automated computer imaging systems. For the laminar distribution analysis, digitized images of the tissue were obtained at low magnification through a charge coupled device (CCD) camera attached to a Nikon Microscope. Labeled somata were identified and manually selected in slices, and their locations plotted using Image Pro (Media Cybernetics) software. The distribution of parvalbumin immunoreactive neurons relative to the depth of the cortex in each treatment group was determined for each 10% interval of the distance from the pia to the white matter.

For the analysis of dendritic arbors, images of neurons were obtained at high magnification through the CCD camera and digitized. Two to four labeled neurons from both the deep and superficial cortical layers were randomly selected from slices obtained from all trials from each group. The dendritic arbors of these neurons were reconstructed

using Neurolucida Image Analysis (Microbrightfield) software. Measures of density, length and branching of dendrites from neurons in the control and each treatment group were determined by Scholl analysis in which intersections between dendrites and concentric radii relative to the soma were detected and recorded. In addition, the somal size from measures made with the Neurolucida program (maximal cross-sectional area) was determined for each reconstructed neuron.

Statistical Analysis:

Data from the laminar distributions were analyzed statistically using a Chi Square test, to determine differences between the distribution of neurons in slices from control and all treatment groups. The [alpha] level was set at $p < 0.05$.

Data from the Scholl assays were analyzed statistically using a 2 way analysis of variance, with treatment (control, DA, EST, DA+EST), and litter as variables, between subject. The level [alpha] was set at $p < 0.05$. For post hoc comparisons, a Tukey's HSD test was used.

3. Results:

Laminar Distribution:

Cortical slices that contained clearly labeled parvalbumin immunoreactive neurons were analyzed quantitatively. The depth of the cortex from the pial surface to the border between the white matter and layer VI was divided into bins of equivalent 10% increments which were equated with specific laminae according to a previous analysis of Nissl stained slice cultures: cortical layer I corresponds approximately to 0-9% of cortical depth, layer II-III corresponds to 10-49% of cortical depth, layer V corresponds to 50-79% of cortical depth and VI corresponds to 80 -100% of cortical depth (personal communication; J-P Hornung). The number of parvalbumin immunoreactive neurons in each bin was averaged for slices (n = 57) among trials for the control and treatment groups.

For all treatment and control groups, labeled neurons in the slices exhibited a laminar organization typical to some stage in the normal sequence of parvalbumin expression during postnatal development. For each group however, the laminar distribution appeared to reach different levels of maturation. The laminar organization of immunoreactive neurons in representative slices from treatment and control groups is shown in Figure 1. Parvalbumin immunoreactive neurons are visible in cortical layer V of all experimental and control groups, but progress to layers VI and to layers II-III in treated slices. The distribution of parvalbumin immunoreactive neurons across the 10% increments varied for each group. In slices maintained in control medium, most parvalbumin immunoreactive neurons were observed in layer V (62% in bins 50-79%

cortical depth), but some were also evident in layer VI (23% in bins 80-100%) (Figure 2A). A few neurons were observed in the superficial layers (16% in bins 10-49%). For the most part, these latter profiles were labeled somata with lightly labeled dendrites, compared to the densely stained cell bodies and dendrites seen in the deeper layers.

In DA treated slices, parvalbumin immunoreactive neurons were distributed more evenly throughout both layers V and VI relative to those in the control slices (Figure 1B). Layer V contained 56% of all labeled neurons (compared to 62% in control slices), whereas layer VI contained a higher percentage, 30%, than in control slices (23%) (Figure 2A). The percentage of labeled neurons in the superficial layers of DA treated slices was nearly the same as that in controls (14% vs. 16%, respectively).

The EST treated slices exhibited a greater shift in neuronal distribution toward both layer VI and the superficial layers as compared to controls (Figure 1C). Layer V contained 49% of labeled neurons, compared to 62% in control slices. Layer VI contained 28% (compared to 23% in control slices), whereas layers II-III contained 23% of labeled neurons (compared to 16% in control slices) (Figure 2B).

Slice cultures treated with DA+EST also exhibited a more mature laminar pattern of parvalbumin expression relative to controls (Figure 1D). Fewer neurons were found in layer V of combined treated slices (50%), compared to that of controls (62%). The combined treatment resulted in a higher percentage of parvalbumin immunoreactive neurons (27%) in layer VI, when compared to control slices (23%). A higher percentage of immunoreactive cells was found in the superficial layers of the combined treatment slices (23%) than in untreated slices (16%) (Figure 2C). All treatment groups were

significantly different from the control group (Chi square; $p < 0.05$).

Dendritic Morphology:

The dendritic morphology of parvalbumin immunoreactive neurons was examined quantitatively. A Scholl analysis was used to determine the total dendritic length and branching density, and automated measures determined somal area of parvalbumin immunoreactive neurons in the deep (V-VI) (n = 217 neurons) and superficial (II-III) cortical layers (n = 137 neurons) for all of the treatment and control groups. An ANOVA determined that there were no statistically significant differences among trials for deep and superficial cortical layer dendritic length and density measures ($p > 0.800$, 0.100, respectively). Therefore, these data were pooled across trials.

Dendritic staining of neurons in the deep cortical layers of DA treated slices appeared more extensive than that of neurons in the control slices (Figure 3). The quantitative analysis confirmed that the dendrites of parvalbumin immunoreactive neurons in the deep cortical layers of slices treated with DA were significantly greater in total length when compared to those in controls ($p < 0.001$) (Figure 4A). Dendritic branching density of neurons (number of nodes) in the deep cortical layers also increased in slices treated with DA ($p < 0.01$) (Figure 4B).

Dendritic staining of neurons in layers II-III of control and DA treated slices appeared similar (Figure 5). In the superficial cortical layers, dendritic length and branching density of parvalbumin immunoreactive neurons in DA treated slices did not differ significantly from those in controls ($p > 0.070$, 0.200, respectively) (Figure 6), although DA treatment caused a trend towards an increase in total dendritic length of

parvalbumin immunoreactive neurons in the superficial cortical layers.

Immunoreactive neurons in slices treated with EST appeared to have more extensive dendritic labeling than those in controls (Figures 3 and 5). The EST treatment produced a significant increase in the total dendritic length and branching density of parvalbumin immunoreactive neurons in the deep cortical layers compared to controls ($p < 0.001$, 0.05, respectively) (Figure 4). In the superficial cortical layers, EST treatment significantly increased the dendritic length of parvalbumin labeled neurons, relative to controls ($p< 0.05$) (Figure 6A). However, dendritic branching measures in the superficial cortical layers did not differ significantly from those of controls ($p > 0.100$) (Figure 6B).

The combined treatment of DA+EST appeared to increase dendritic labeling of immunoreactive neurons (Figures 3 and 5). The total dendritic length of labeled neurons in the deep cortical layers of DA+EST treated slices was significantly greater than that of neurons of control slices ($p < 0.001$) (Figure 4A). Dendritic branching in the deep cortical layers was not significantly altered by DA+EST treatment ($p > 0.070$) (Figure 5A). A significant increase in the total dendritic length and branching density of neurons in the superficial cortical layers occurred with the DA+EST treatment, when compared to controls ($p < 0.001$, 0.05, respectively) (Figure 6).

Somal areas were measured in the deep and superficial cortical layers of cultured slices maintained in control and treatment conditions for 14 days *in vitro*. No significant differences between control and treatment conditions were found (ANOVA, $p > 0.600$, 0.200, respectively).

4. Discussion:

All the treatment conditions significantly altered the expression of parvalbumin in the rodent frontal cortex at 14 days *in vitro*. Specifically, the distribution of parvalbumin immunoreactive neurons was spread increasingly into cortical layer VI following treatment with DA, EST or DA+EST. The addition of EST, with or without DA, was necessary to induce a significant increase of parvalbumin immunoreactive neurons in the superficial cortical layers. Similar treatment effects were observed on total dendritic length. In the deep cortical layers, all treatment conditions caused an increase in dendritic length of parvalbumin immunoreactive neurons when compared to those in control slice cultures. Once again however, EST, with or without DA, was needed to increase the total dendritic length of neurons in the superficial cortical layers. The effects upon branching density of parvalbumin immunoreactive neurons varied with treatment. Whereas DA or EST alone caused an increase in the branching density of parvalbumin immunoreactive neurons in the deep cortical layers, the two together had no significant effect. In the superficial cortical layers however, only the combined effect of DA+EST was sufficient to cause a significant change in dendritic branching.

Technical considerations:

Neuronal morphology and cortical cytoarchitecture are maintained in organotypic slice cultures (Stoppini et al, 1991; Gåhwiler et al, 1997). This technique enables the study of the effects of controlled environmental conditions on the laminar pattern and dendritic morphology of neurons in a variety of cortical and subcortical regions. Relevant to this study, the density and distribution of parvalbumin immunoreactive neurons in the

culture system is similar to that of intact animals (Vogt Weisenhorn, 1998). Furthermore, the developmental pattern of parvalbumin expression is comparable. Adult patterns of parvalbumin expression emerge in organotypic slice cultures of the cortex under different conditions. For example, a mature pattern of parvalbumin expression develops in slices of parietal cortex co-cultured with striatal slices from postnatal day 0-2 rats and in frontoparietal cortex slices treated with DA for two weeks (Plenz and Aertsen, 1996; Porter et al, 1999). Vogt Weisenhorn and colleagues (1998) also report adult patterns of parvalbumin expression in parietal cortex, but only in explants taken from older animals (postnatal day 7).

Certain culture conditions may preclude optimal neuronal maturation. The charcoal-stripping process used to remove steroids from the serum in our experiments could affect the overall development of the cortical slices. Nutrients or other non-steroidal factors could be removed during the process. However, when deprived entirely of serum, cortical slices explanted at postnatal day 7 are not only viable, but they also develop normal patterns of parvalbumin expression (Vogt Weisenhorn, 1998). Although our slices are explanted at an earlier and potentially more vulnerable postnatal age, parvalbumin expression occurs and follows the predicted sequence of events.

In our slices, parvalbumin immunoreactivity is observed in neuronal phenotypes that express the protein *in vivo* and in laminar patterns consistent with normal cortical development. If the age at explant (2 days postnatal) is added to the amount of time in culture (14 days), then the slices in this study can be considered as comparable in age to postnatal day 16 in the intact rat. At postnatal day 16 *in vivo*, parvalbumin

immunoreactivity is observed in somata of both the deep and superficial cortical laminae and dendritic labeling is developing in the superficial, but more evident in the deep cortical layers (Alcantara et al, 1993).

Throughout this study, dendritic lengths were obtained by measuring visible portions of immunoreactive profiles. Although it is likely that parvalbumin is distributed throughout the entire extent of the dendrites, our measures cannot be considered with certainty to reflect total dendritic length, but only length in which parvalbumin immunoreactivity is visible.

Dopamine enhances parvalbumin expression

Considering that parvalbumin immunoreactivity normally occurs first in cortical layer V, and later extends into layer VI, and that immunoreactivity in fibers follows that in somata by approximately 2 days Alcantara et al, 1993), the increase in percentage of immunoreactive neurons in layer VI and the increase in dendritic length and branching of neurons in the deep cortical layers induced by DA treatment indicates an accelerated maturation in parvalbumin expression when compared to control slices.

The concomitant development of cortical DA and parvalbumin systems suggests several possible mechanisms for these findings. The frontal cortex in the rodent receives input from dopaminergic nuclei throughout postnatal development (Berger et al, 1983, 1991). Dopaminergic innervation density (Verney et al, 1982; Kalsbeek et al, 1988), concentrations (Chen et al, 1997) and receptor mRNA expression (Leslie et al, 1991; Schambra et al, 1994) peak postnatally, when parvalbumin expression is nearly mature. In fact, the entire population of GABAergic neurons continues to undergo dendritic

differentiation through postnatal weeks two to three (Vincent et al, 1995). Interestingly, appositions between GABAergic neurons and dopaminergic axons increase during this same period of development (Benes et al, 1996). The targets of these putative synapses are likely to be parvalbumin positive interneurons because studies indicate that the parvalbumin containing subclass of GABAergic neurons are contacted by mesocortical dopamine fibers, whereas the calbindin and calretinin containing subclasses are not (Porter et al, 1995; Sesack et al, 1998). Therefore, if direct synaptic input has the potential to increase parvalbumin expression, then the parvalbumin immunoreactive neurons are the ones that are likely to be affected. It does not seem however, that synapse formation is the cause of the DA induced increases in the parvalbumin immunoreactive dendrites because the mesocortical axons are severed during the slice preparation. Activation of the DA D2 receptor appears to be responsible, at least in part. Pharmacological blockade of the D2 receptor attenuates the DA induced changes in laminar pattern and dendritic morphology (Porter et al, 1999).

Dopamine s effects on superficial neurons which differentiate over a later time period are more subtle than on deep neurons. The modulation causes a slight, but not significant increase in dendritic measures. An earlier report (Porter et al, 1999) showed that DA alters dendritic morphology of parvalbumin immunoreactive neurons throughout all cortical layers. Those experiments did not necessitate serum filtration for the removal of steroids with the potential of loss of other nutrients. In the current study, this process may have adverse affects upon the maturation of the slices. Considering our findings that EST enhances parvalbumin expression, the inclusion of endogenous steroids may have

caused the differences between the two studies.

Estrogen enhances parvalbumin expression

The shift in distribution of parvalbumin expressing neurons from cortical layer V to layer VI, as well as to layers II-III is even greater in EST treated slices than in those treated with DA. Likewise, EST has a greater effect than DA on dendritic differentiation in that neurons in both deep and superficial cortical layers are affected. At postnatal day 14-16 neurons are still developing in the frontal cortex. The increase in the dendritic length and branching densities of parvalbumin immunoreactive neurons suggests an enhancement of maturation.

This report is the first to note an estrogenic influence upon the expression of parvalbumin in the cortex. Intrinsic and extrinsic mechanisms should be considered. As reviewed by Sawada and Shimohama (2000), EST has neuroprotective effects, perhaps through its antioxidant properties, upon DA neurons in the mesencephalon. Because all afferents, including mesocortical axons, are severed during the process of culturing, the observed effect of EST in these slices could not be caused by its actions on subcortical dopaminergic systems. Therefore, our culture system model clearly shows that EST acts directly on factors intrinsic to the cortex to enhance parvalbumin expression.

The presence of EST receptors (MacLusky et al, 1979; Miranda and Toran-Allerand, 1992; Shughrue et al, 1997; Butler et al, 1999) and the aromatase necessary for the localized conversion of testosterone to EST (Horvath et al, 1997) in the rodent cortex indicate a role for direct EST effects within the cortex. Neurons in organotypic slice cultures are capable of EST uptake (Toran-Allerand et al, 1983). In fact, EST uptake in

neurons in perinatal explants of mouse cingulate cortex enhances neurite growth (Toran-Allerand et al, 1983). In light of these findings, the EST induced effects observed in the current study may be mediated at least in part through cortical EST receptors.

Dopamine and estrogen enhance parvalbumin expression

The DA+EST induced changes in parvalbumin expression are similar to that caused by EST treatment, alone. The increased percentage of parvalbumin expressing neurons in cortical layer VI, as well as in the superficial layers of the DA+EST treated slices again indicates a more mature pattern of parvalbumin expression than in either the control slices or the DA only treated slices. Furthermore, the DA+EST treatment induces dendritic differentiation of neurons in both the deep and superficial cortical layers. Enhanced parvalbumin expression in superficial neurons is indicative of a more mature stage of cortical development. In general, a continuum of increasing maturity is seen from control, to DA only, to EST or DA+EST treated slices. Whereas DA's effect upon parvalbumin expression seems confined to the deep cortical layers, EST's effect is noted throughout the cortex. However, differences in treatment effects of EST and DA+EST upon parvalbumin immunoreactive dendrites indicates and interaction between DA and EST suggest that the mechanisms by which each modulator functions may not be fully independent. It is possible that EST's influence on parvalbumin expression in the deep cortical layers occurs through DA receptor modulation.

Estrogen receptors are functional, but the hormone also acts on intrinsic aspects of the cortical dopamine system. After gonadectomy to deplete either estrogen or testosterone that is normally converted locally into estrogen in the brain, D1 and D2

receptor expression is reduced in the rodent frontal cortex (Boss and Di Paolo, 1996). Estrogen competes with DA (Paden et al, 1982), or alters the binding affinity of the dopamine D2 receptor (Håfner et al, 1991), subsequently blocking its activation. In light of estrogenic influences upon the dopamine system, the effect of EST treatment on parvalbumin expression in our system may be mediated in part through the DA D2 receptor. However, if EST acts by antagonizing the dopamine D2 receptors in the cortex, then DA s effect upon parvalbumin immunoreactivity should be attenuated upon additional treatment with EST. This attenuation does not occur. The possibility remains that estrogenic effects are modulated partly through the DA D2 receptor, but differences in effects between EST treatment and DA treatment suggest that separate mechanisms are also involved. Future studies are planned in which DA D2 receptor antagonists and/or EST receptor antagonists are co-applied with EST treatment to organotypic slice cultures.

Parvalbumin, one of several calcium binding proteins, regulates intracellular calcium levels. During development it is thought to protect neurons from excessive calcium accumulation associated with high neuronal activity. Alterations in its expression by manipulation of endogenous neuromodulators such as DA or EST at critical periods of development could cause subtle changes in cortical connectivity and subsequent processing. The changes in parvalbumin containing neurons observed in schizophrenia can be mimicked in our culture system. The increasing evidence that schizophrenia is linked to neurodevelopmental abnormalities highlights the importance of identifying factors that may contribute to anomalous development and subsequent pathology. Dopamine has long been implicated in schizophrenia, and gender differences in the

expression of the disease suggest a role for gonadal hormones. The interaction of these two systems and their effects on parvalbumin expression during development may prove useful towards understanding the origins of cortical malformations and subsequent cognitive dysfunction.

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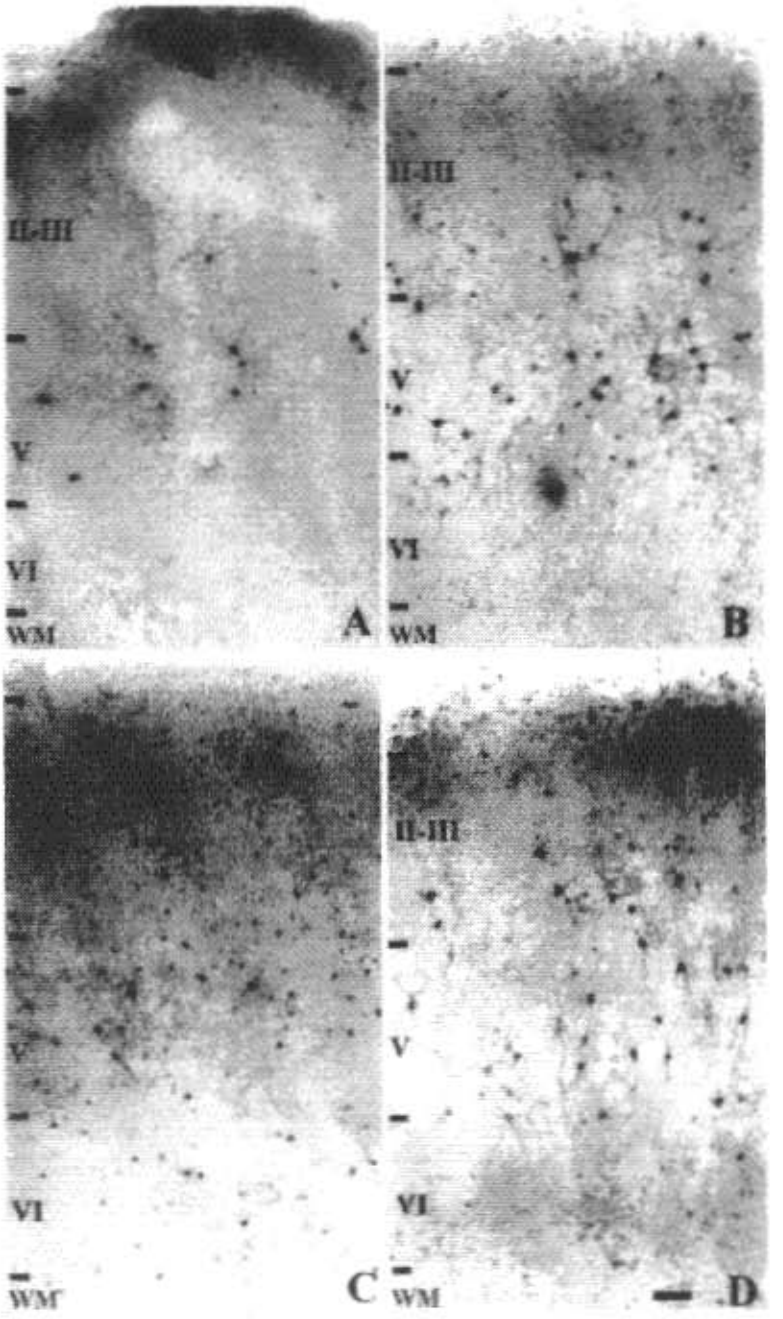

Figure 1. Laminar Distribution of Parvalbumin Immunoreactive Neurons: Micrographs of cortical slice cultures immunostained for parvalbumin at 14 days *in vitro*. *A*, Control slice: a band of labeled neurons is seen in layer V. *B*, DA treated slice: numerous labeled neurons are visible in layers V and VI. *C*, EST treated slice: a dense band of labeled neurons is apparent in layer V, but labeled neurons are also observed in layers II-III and layer VI. *D*, DA+EST treated slice: a dense band of labeled neurons exists in layer V. Labeling also occurs in layers II-III and layer VI. Scale bar = 150 μm (same for A-D).

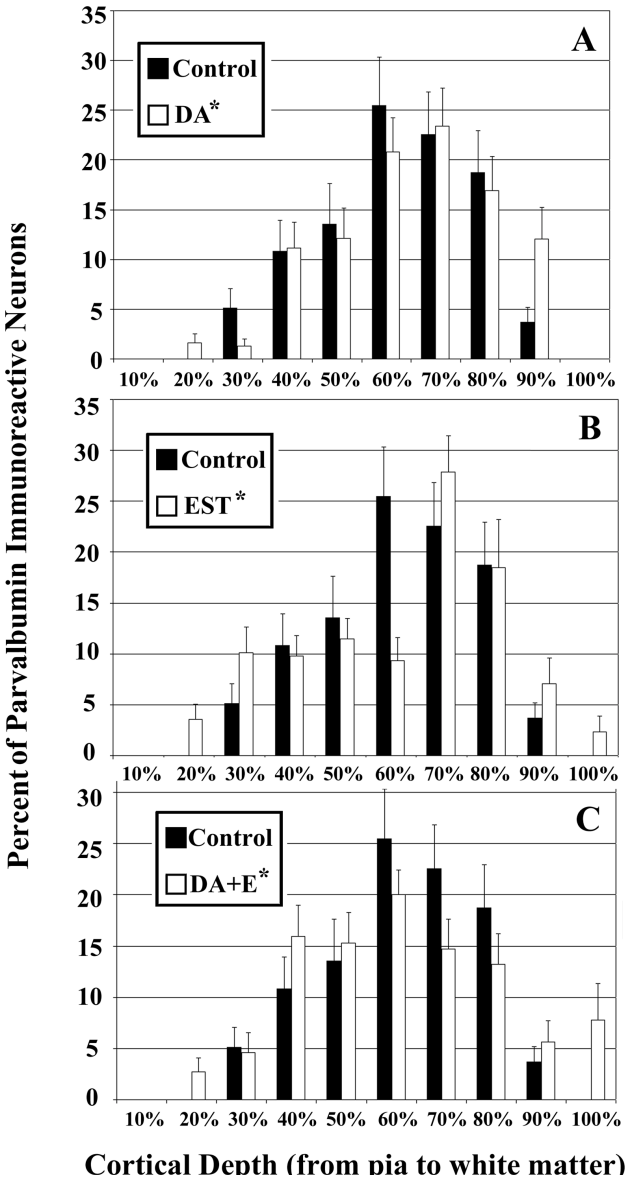

Figure 2. Distribution of Parvalbumin-IR Neurons: Histograms of the laminar distribution of parvalbumin immunoreactive (Parvalbumin-IR) neurons throughout the cortical depth, in slices maintained for 14 days *in vitro* (n = 57 slices). ***A***, Control vs. DA treatment: a greater percent of labeled neurons are found deeper in cortex (80-89%, cortical depth) of DA treated slices, compared to controls (Chi square, $p < 0.05$). ***B***, Control vs. EST treatment: a greater percentage of labeled neurons occurs in both deeper (70-100%, cortical depth) and superficial increments (10-29%, cortical depth) of EST treated slices, compared to controls ($p < 0.05$). ***C***, Control vs. DA+EST (DA+E) treatment: a greater percentage of labeled neurons occurs in both the deep (80-100%, cortical death) and superficial increments (10-39%, cortical depth) of DA+EST treated slices, compared to controls ($p<0.05$).

Parvalbumin Immunoreactive Neurons, Deep Cortical Layers

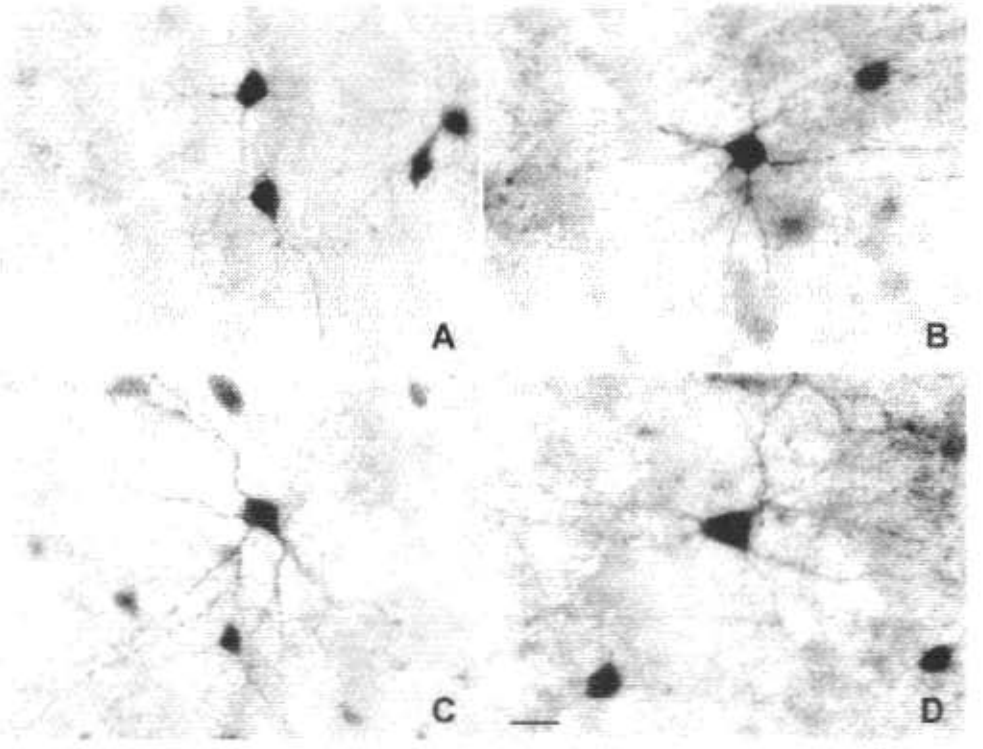

Figure 3. Morphology of Deep Cortical Layer Parvalbumin Immunoreactive Neurons: Micrographs of cortical slice culture, fixed and immunostained for parvalbumin at 14 days *in vitro*. ***A***, Control slice: labeled neurons exhibit moderately branching, lightly stained dendritic processes. ***B***, DA treated slice: labeled portions of dendrites appear to be longer than those of labeled neurons in control slices. Staining occurs in numerous secondary dendritic processes. ***C***, EST treated slice: dendritic labeling is intense and extends to numerous secondary processes. ***D***, DA+EST treated slice: labeled neurons exhibit extensively labeled dendritic processes, with immunoreactivity visible in secondary processes. Scale bar = 30 μm (same for A-D).

Dendritic Arborization of Parvalbumin-IR Neurons, Deep Cortical Layers

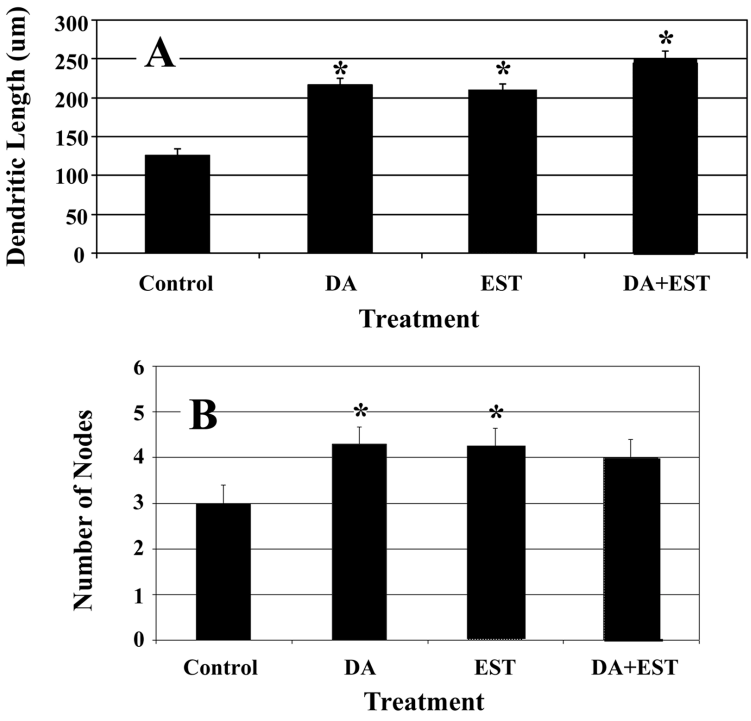

Figure 4. Dendritic Arborization of Parvalbumin-IR Neurons, Deep Cortical Layers: Histograms of dendritic arborization patterns of parvalbumin immunoreactive neurons in the deep cortical layers (n = 217 neurons). ***A***, Histogram of dendritic length: statistically significant increases (noted by *) in length occur with DA, EST, and DA+EST treatments (ANOVA) ($p < 0.001$, for all treatments). ***B***, Histogram of dendritic branching (nodes): statistically significant increases in nodes occur with DA and EST treatments ($p < 0.01$, for both treatments), but not with DA+EST treatment ($p > 0.05$). Bars indicate standard error.

Parvalbumin Immunoreactive Neurons, Superficial Cortical Layers

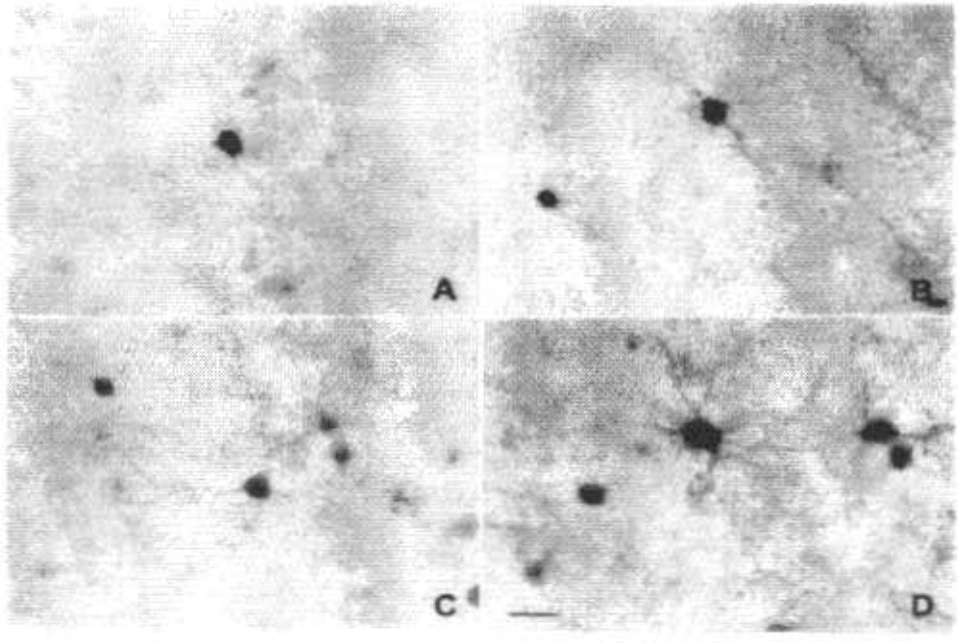

Figure 5. Morphology of Superficial Cortical Layer Parvalbumin Immunoreactive Neurons: Micrographs of cortical slice cultures, fixed and immunostained for parvalbumin at 14 days *in vitro*. ***A***, Control slice: labeled neurons exhibit lightly stained dendritic processes, with label extending only to proximal dendrites. ***B***, DA treated slice: labeled neurons resemble those seen in control slices, containing lightly stained dendritic processes, with labeling extending only to proximal dendrites. ***C***, EST treated slices: labeled neurons with more extensive labeling of dendritic processes are evident. ***D***, DA+EST treated slice: labeled neurons with more extensive labeling of dendritic processes are apparent, with labeling extending to secondary processes. Scale bar = 30 μm (same for A-D).

Dendritic Arborization of Parvalbumin-IR Neurons, Superficial Cortical Layers

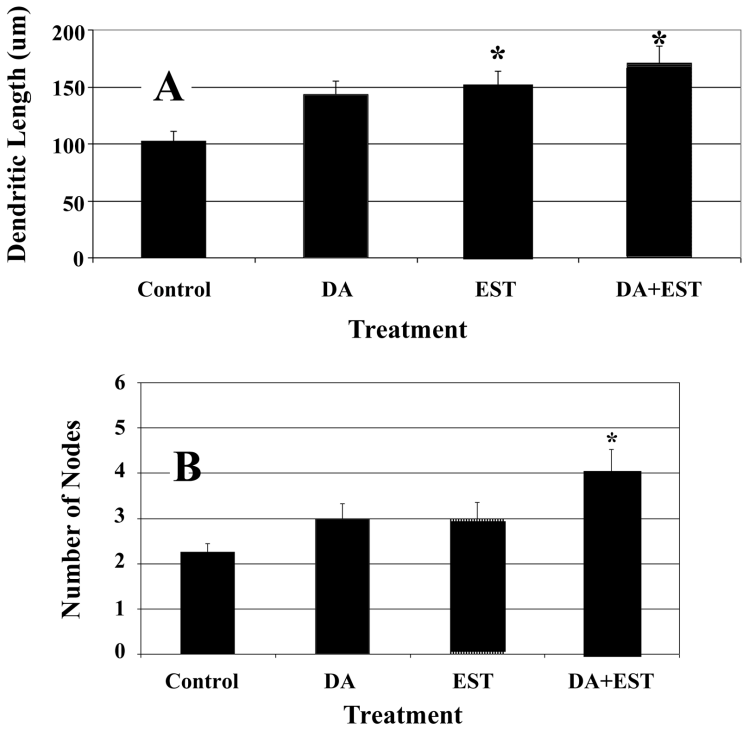

Figure 6. Dendritic Arborization of Parvalbumin-IR Neurons, Superficial Cortical Layers: Histograms of the dendritic arborization of parvalbumin immunoreactive neurons in the superficial cortical layers (n = 137 neurons). ***A***, Histogram of dendritic lengths: statistically significant increases (noted by *) in length occur with EST (ANOVA, $p < 0.05$) and DA+EST ($p< 0.001$) treatments, but not with DA treatment ($p > 0.05$). ***B***, Histogram of dendritic branching (nodes): a statistically significant increase in nodes , occurs with DA+EST treatment ($p < 0.05$) but not with DA or EST treatments ($p > 0.05$, for both treatments). Bars indicate standard error.

The Effects of Estrogen upon Developing Cortical Parvalbumin Expression and the Dopamine System *In Vivo*

Nicole R. Ross, Ph.D[1]. and Linda L. Porter, Ph.D[1, 2]

[1]Neuroscience Program, and [2]Department of Anatomy, Physiology and Genetics, Uniformed

Services University of the Health Sciences, Bethesda, Maryland, 20814

Submitted: Cerebral Cortex

Corresponding Author: Linda L. Porter, Ph.D.

Department of Anatomy, Physiology, and Genetics

Uniformed Services University of the Health Sciences

4301 Jones Bridge Road, Bethesda, Maryland 20814.

Phone: (301) 295-3217

Facsimile: (301) 295-1715

Email: lporter@usuhs.mil

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Key Words: Neonatal, Gonadectomy, Rodent, CNS, 6-hydroxydopamine

Abstract:

Estrogen is implicated in several neurologic disorders in which dopaminergic dysfunction and alterations in parvalbumin expression are noted. This study was designed to determine the effects of estrogen on cortical parvalbumin expression and the dopaminergic system during development. Neonatal male rats were either gonadectomized (GDX), gonadectomized and estrogen supplemented (GDX+E), or sham-operated (control). Decreases in the density of parvalbumin-immunoreactive somata occurred in the deep cortical layers of GDX and GDX+E rats at 12, 16 and 33 days postnatal. Decreases also occurred in the superficial layers of GDX rats at 16 and 33 days postnatal. At 33 days postnatal, tyrosine hydroxylase (TH) fiber length and Substantia nigra, pars compacta (SNc) dopaminergic neuron density were increased in GDX and GDX+E rats. The effects of neonatal 6-OHDA lesions were similar to those seen in GDX animals. Neonatal 6-OHDA lesions produced results that were similar to those of GDX animals, in that, parvalbumin expression was decreased and TH fiber length was increased in the deep layers at 33 days postnatal, regardless of the decrease in dopaminergic neurons. Our findings indicate that estrogen enhances parvalbumin expression and alters cortical and subcortical dopamine systems. These findings may aid in the elucidation of the pathophysiology of a number of neurologic disorders.

Introduction:

Estrogen is implicated in numerous neurologic disorders. For example, estrogen replacement therapy is associated with improved cognitive function in female Alzheimer s patients (Henderson et al, 1996; Baldereschi et al, 1998; Asthana et al, 1999). Estrogen may also lessen cognitive impairment (Fernandez and Lapane, 2000) and motor dysfunction (Tsang et al, 2000) in Parkinson's patients. In addition, differences in age of onset of symptomology (Hartmann and Meyer, 1969; Lewine et al, 1981; Håfner et al, 1989), structural brain abnormalities (Andreasen et al, 1986; Nopoulos et al, 1997) and response to neuroleptic treatment (Seeman, 1986) exist between male and female schizophrenic patients, suggesting hormonal modulation of the expression of the disease. The mechanisms through which estrogen acts on the cortex may help to elucidate its putative protective influence in various disorders.

Gonadal hormones have extensive and long term effects on the cortex. As reviewed by Sandhu et al (1986), manipulation of gonadal hormones alters the development of hemispheric dimorphisms normally seen in the cortex . Estrogen levels alter cerebral blood flow (Dietrich et al, 2001) which , in turn, may alter cortical function. Steroidal hormones also influence cortical and subcortical dopamine systems (Paden et al, 1982; Håfner, 1991; Mack et al, 1991; Stewart et al, 1994; Kritzer et al, 1998; Adler et al, 1999). For example, gonadal hormone depletion increases normal dopamine levels in the rodent cingulate cortex during postnatal development (Stewart et al, 1994). Furthermore, dopamine D2 receptor antagonism (Paden et al, 1982) and altered dopamine mediated behavior, like locomoter activity (Håfner et al, 1991) occur with estrogen treatment.

Whereas, many of these effects occur during development of the nervous system, they may manifest later as functional disorders.

Alterations in GABAergic neurons are reported in several of the neurologic disorders in which estrogenic modulation is implicated. Specifically, alterations in a particular subset of cortical GABAergic neurons, those containing the calcium binding protein parvalbumin, occur in Alzheimer's disease (Aria et al, 1987; Solodkin et al, 1996; Brady and Mufson, 1997; Mikkonen et al, 1999) and schizophrenia (Beasley and Reynolds, 1997; Kalus et al, 1997). Moreover, alterations in axon cartridges of chandelier cells, one subtype of GABAergic neuron that expresses parvalbumin, are observed in schizophrenics (Pierri et al, 1999), indicating changes in the transmitter systems of these neurons. Therefore, it is possible that cortical function may be altered through estrogenic modulation of parvalbumin levels in GABAergic neurons.

In fact, previous studies in our lab indicate that estrogen enhances the maturation of parvalbumin expressing neurons, *in vitro* (Ross and Porter, In Press). Exogenously applied dopamine however, alters parvalbumin expression in a similar manner (Porter et al, 1999; Ross and Porter, In Press). Furthermore, increased levels of dopamine augment the length of parvalbumin immunoreactive dendrites in the anterior cingulate cortex in the intact animal (Wang et al, 1995). Because estrogen affects the dopaminergic system, the estrogenic modulation of parvalbumin expression may be mediated through changes in dopaminergic function in the intact animal.

Parvalbumin is expressed in GABAergic neurons found in areas of high metabolic activity (Celio et al, 1986; Cohen et al, 1993) and may protect developing neurons from

hyperexcitability. Parvalbumin containing neurons provide inhibitory input onto pyramidal neurons (DeFelipe et al, 1985) and may play a vital role in regulating cortical activity. Consequently, subtle alterations in these neurons may alter cortical connectivity and function, factors that may contribute to neurologic dysfunction.

Recent research has targeted estrogen's influence on the cortical and subcortical dopamine systems, but little is known about the effects of estrogen upon cortical neurons. Our previous findings from *in vitro* studies suggest that both dopamine and estrogen are important modulators of the development of parvalbumin expressing neurons. Similarities between the influences of estrogen and dopamine on these neurons, and estrogen s potential to alter the dopaminergic system raise questions as to whether they act on parvalbumin expression through independent mechanisms. Our current study uses a model of neonatal gonadectomy and estrogen replacement to explore estrogen's effect upon the development of cortical parvalbumin expression in the intact rodent frontal cortex, as well as, its effect upon the developing mesocortical dopamine system. We also seek to determine the effects of neonatal midbrain dopamine lesions upon cortical parvalbumin expression and their correlation to the gonadectomy induced changes.

Materials and Methods:

A total of forty-three male and three female Sprague-Dawley rats were used for this study. Of the forty-three male rats, 12 were used as controls 24 were used in the experimental groups included in the hormone study, and six were used in the neonatal lesion study. An additional three male rats that were not included in the hormone study were used in the radioimmunoassay study due to differences in the dosage of 17-ß estradiol pellet. An additional three females were included in the radioimmunoassay study. Surgical procedures were conducted upon male rats at 2 days postnatal. All procedures were done in accordance with the NIH Guide for the Care and Use of Animals as outlined in a protocol approved by the USUHS IACUC.

The study was subdivided into two parallel experiments, both performed in an intact animal model. The first experiment examined the effects of hormone manipulation upon parvalbumin expression in the frontal cortex, and cortical and subcortical dopamine systems during development. The second experiment examined the effect of neurotoxic lesions of the dopamine system upon developing cortical parvalbumin expression.

Animals were randomly assigned to experimental or control groups. Two treatment groups and one control group were included in the hormone study. Gonadectomies were performed on male rats in both treatment groups (n = 24) to deplete gonadal hormones. Treatment animals were further subdivided into two groups; 1) animals receiving the gonadectomy only (GDX) and 2) animals receiving both the gonadectomy and estrogen replacement (GDX+E) (n = 12 male rats/group). Estrogen was supplied by implanting a slow release 17 ß-estradiol pellet in the GDX+E rats at the time of gonadectomy. The

procedure allowed for a steady and continuous dosing until time of sacrifice. The control group of animals underwent a sham operation in which all surgical procedures were similar to the treated rats, except that gonads were left intact after being identified (n = 12 male rats). Four animals from each treatment and the control group were sacrificed at one of the following time points: 12, 16, or 33 days postnatal. Brain tissue was harvested after sacrifice. The effects of treatments upon both cortical parvalbumin expression and the dopaminergic system were determined.

One treatment group and a control group were included in the neurotoxic lesion experiment (n = 4 male rats/group). The treatment rats were subjected to injections of 6-hydroxy dopamine (6-OHDA) at two days postnatal to disrupt dopaminergic input into the cortex. Because the hormone and lesion experiments were done in parallel, the 33 day postnatal control group used in the hormone experiment was also used for the lesion experiment. As a control measure for the administration of the neurotoxic lesions, all non-lesioned animals received sham injections with the vehicle for the 6-OHDA. Lesioned and control animals were sacrificed at 33 days postnatal. Brain tissue was harvested after sacrifice. The effects of treatment upon both cortical parvalbumin expression and the dopaminergic system were determined.

Gonadectomy:

Male rats, two days postnatal, were deeply anesthetized by hypothermia until areflexive. This procedure allows for a rapid recovery and high survival rate. A transverse incision was made in the skin, midway between the umbilicus and the anal opening. Another transverse cut was made in the same area, through the underlying muscle and

peritoneum. The testes were located, withdrawn from the abdomen and cut from the spermatic chord. The deep tissues and skin were quickly sutured.

17 ß-estradiol Administration:

Estrogen pellets which slowly released 17 ß-estradiol throughout the survival period were administered to the GDX+E group to maintain a continuous supply of estrogen. The dose of estrogen used in the GDX+E treatment group was determined prior to the experiment. Radioimmunoassay (RIA) of estrogen levels was performed on blood samples taken from animals implanted with different pellet sizes. The desired dose was that at which blood serum levels of 17 ß-estradiol in the 33 day postnatal male rats reached physiological levels comparable to that in unmodified female rats of the same age.

The implantation procedure was performed at the time of GDX under one episode of anesthesia. One pellet of 0.72 mg 17 ß-estradiol (Innovative Research of America, Sarasota, FL) was inserted into each animal in the GDX+E group. The pellets were placed subcutaneously into the dorsal-scapular region. A small skin incision was made at the back of the neck. The pellet was quickly inserted and the skin opening sutured. Pellets were left in place throughout the animals survival periods.

Radioimmunoassay Procedures:

Two groups of male GDX+E rats (3 rats/group), both with a different pellet size, and three normal female rats were bled from tail cuts at 12, 16, and 33 days postnatal. Blood samples were pooled, collected into uncoated tubes, and allowed to clot for 30 minutes at room temperature. The serum was centrifuged, pipetted from the plasma to

separate the two, and stored at -80 ^{o}C. The RIA was conducted as per directions from the ImmunoChem^{TM} Double Antibody 17 ß-estradiol ^{125}I RIA kit (ICN Biomedicals, Costa Mesa, CA). The 60 day release, 0.72 mg pellet of 17 ß- estradiol produced serum levels of estrogen that ranged from a concentration of 37 - 196 pg/ml in 33 postnatal day GDX+E rats. The levels in 33 day postnatal normal females fell within this range (49 - 53 pg/ml).

6-OHDA Administration:

The neurotoxin 6-OHDA was administered to four male rats at postnatal day two to disrupt the dopamine system. Animals were pretreated with the norepinephrine uptake inhibitor, desiprimine (25 mg/kg, subcutaneous), thirty minutes prior to injection of neurotoxin to protect the norepinephrine system from damage. Animals were then deeply anesthetized by hypothermia until areflexive. Rats were placed in a specifically designed holder and given a single intracisternal injection of either 6-OHDA (75 µg) in 10µl of vehicle (ascorbic acid/saline), or vehicle only (control group), into the cisterna magna (Luthman et al, 1995, 1997). The solution was injected with a Hamilton syringe adapted with a stop-marker to ensure appropriate depth of needle insertion.

Animal Recovery and Survival:

Animals were rapidly warmed after surgery and returned to the mother after recovery from anesthesia. They were kept with their littermates until time of sacrifice (or until weaning at 22 days postnatal). Animals were kept on a 12 hour light/dark cycle. Weaned animals were fed rat chow and given access to water *ad libitum*.

Animal Sacrifice:

Rats were deeply anesthetized with ketamine (60 mg/kg, IM) and xylazine (4 mg/kg, IM) at age 12, 16, or 33 days postnatal. They were intracardially perfused with a prewash of 0.9% NaCl in 0.1 M $NaPO_4$ (PBS), followed by 4% paraformaldehyde in PBS, and then with a solution of PBS and 20% sucrose. Brains were harvested and post-fixed in 4% paraformaldehyde for two hours, then placed in PBS and 30% sucrose and refrigerated for two days.

Immunohistochemical procedures:

Brains were cut in the coronal plane at 30μm in thickness. Three alternating series of sections through the frontal cortex were collected for immunostaining of either TH or parvalbumin, or Nissl staining. Midbrain sections containing the substantia nigra pars compacta (SNc) were collected in series. Alternating sections were stained for either TH or Nissl. For TH immunostaining, tissue was washed two times for ten minutes each in Tris Buffered Saline (TBS), and then incubated for 30 minutes in a blocking solution containing 0.5% bovine serum albumin (BSA) and 0.1% Triton-X in TBS. Sections were incubated overnight at room temperature in a solution containing 0.5% BSA, 0.5% Na Azide and monoclonal antibodies raised against TH in rabbit (1:1000, Protos Biotech Corp., New York, NY). Sections were then washed in TBS and incubated for one hour at room temperature in biotinylated 2^o antibody against rabbit IgG (Vector Laboratories Inc., Burlingame, CA). Sections were then processed according to the Vector ABC kit protocol. Immunoreactivity was visualized using diaminobenzidine (DAB) as a chromagen.

For parvalbumin immunostaining, sections were washed twice in TBS and once in

TBS containing 0.1% Triton-X, and then incubated overnight at room temperature in monoclonal antibodies raised in mouse against parvalbumin (1:10,000, Sigma, ST. Louis, Missouri) in TBS. Tissue was then washed in TBS and incubated for one hour at room temperature in the biotinylated 2^{o} antibody raised in horse against mouse IgG (Vector Laboratories Inc., Burlingame, CA) and processed according to the Vector ABC kit protocol. Parvalbumin immunoreactive neurons were visualized using DAB as a chromagen.

Data Collection and Analysis:

The density of parvalbumin immunoreactive neurons in the deep and superficial layers of the frontal cortex was determined stereologically using a non-biased three level sampling design (Systems Planning and Analysis Inc., Alexandria VA). A series of sections through the frontal cortex was analyzed for each animal in all treatment and control groups for all ages. Sections were visualized through a Nikon microscope and digitized. Nine alternating sections (every sixth section beginning from a random start point) were examined within consistent boundaries of the frontal cortex for each animal, beginning at the anterior commissure and ending at the decussation of the corpus callosum. Separate stereological analyses were performed in the superficial cortical layers (I-III) and deep cortical layers (V-VI). Boundaries between the layers were determined visually using cytoarchitectural landmarks and referring to Nissl stained sections. The density of labeled neurons in each section was determined using the optical disector probe to estimate immunoreactive neurons in randomly selected volumetric samples (2-5/section, frame area = 500 μm^2). Results from separate layers were analyzed

across sections and animals then, treatment and control groups were compared statistically.

The length of TH labeled fibers was estimated using unbiased stereology to assess the effects of estrogen on the cortical dopamine system. Measures were taken from the same cortical region as that examined for parvalbumin immunoreactive neuron density. The series of sections adjacent to those used for the parvalbumin neuron counts were examined. Again, nine alternating sections with a sampling rate of every sixth section were examined within consistent boundaries for each animal, beginning at the anterior commissure and ending at the decussation of the corpus callosum in each series. Separate analyses were conducted for deep and superficial cortical layers. A three level fraction design was used. Measuring frames (2-5/section, frame area = 500 μm^2) with sine weighted lines were placed randomly over the cortical surface area. The number of intersections between the grid lines and TH fibers through the Z axis (8 μm probe depth, 4 μm guard height) was used to estimate total TH fiber length in the measuring frame. Data was averaged across the series of sections for all animals in each group.

The number of dopaminergic neurons in the SNc was also estimated stereologically in experimental and control animals. Nine alternating sections (every third section) through the SNc were analyzed using a two-level sampling design. As before, sections were digitized and a detailed stereologic analysis was conducted using an optical disector probe to estimate dopaminergic neurons in randomly selected volumetric samples (2-5/section, frame area = 500 μm^2) in each section. Estimates were averaged across the series of sections for all animals in each group.

Statistical Analysis:

For the hormone study, neuron density and fiber length measures in treatment and control groups were analyzed statistically using a 2 way analysis of variance. The experiment follows a 3 X 3 factorial design, with treatment condition (3: normal, GDX, GDX+E) and survival time (3: 12,16, and 33 postnatal days) as variables, between subject. The [alpha] level was set at $p < 0.05$. A Tukey s HSD test was used for *post hoc* comparisons. For the lesion study, neurons and fiber measures in the treatment and the control groups were analyzed Student's t-test. To determine whether the 6-OHDA injections were effective in depleting dopaminergic neuron density in the substantia nigra pars compacta, a one-tailed students T-test was used. The [alpha] level was set at $p < 0.05$. To determine whether dopamine depletion induced significant changes in parvalbumin neuron density and TH fiber length measures, a two-tailed students T-test was used. The [alpha] level was set at $p < 0.05$.

Results:

Density of Parvalbumin Immunoreactive Neurons in the Frontal Cortex:

Parvalbumin expression followed the normal sequence of laminar pattern formation during postnatal development in all treatment and control groups of both experiments (Alcantara et al, 1993). In the hormone experiment, labeled neurons were observed primarily in the deep cortical layers of all groups with only sparse labeling in the superficial cortical layers at 12 days postnatal. During the next four days, the density of immunoreactive neurons increased in both superficial and deep laminae. A substantial increase occurred in the superficial layers, but only a moderate increase was observed in the deep layers. From postnatal day 16 to 33, the density in layers II-III remained virtually unchanged. In the deep cortical layers however, the density of labeled neurons continued to increase between these time points. Whereas the general developmental progression of laminar distribution was similar across all groups, the density of parvalbumin immunoreactive neurons varied among treatment and control animals.

The relative distribution of parvalbumin immunoreactive neurons across all treatment conditions at 12 days postnatal is shown in figure 1. At this age parvalbumin immunoreactive neurons appeared primarily in the deep cortical laminae of the frontal cortex of all groups. Labeled neurons in the superficial layers were sparse. In general, labeling appeared to be reduced throughout the frontal cortices of both GDX and GDX+E groups, compared to controls.

In fact, analyses of the stereological data revealed treatment-induced reductions in neuronal density in both deep and superficial laminae as shown in Figure 2. At 12 days

postnatal, both GDX and GDX+E rats exhibited significant decreases in the density of parvalbumin expressing neurons in cortical layers V-VI, compared to control animals (ANOVA, $p = 0.014$, 0.001 respectively). The decreased density of labeled neurons in the superficial cortical layers did not meet our criteria for being statistically different from controls ($p = 0.100$), perhaps because of the paucity of labeled neurons in these layers at postnatal day 12.

At 16 days postnatal, the density of labeled neurons throughout the depth of the cortex increased over that at postnatal day 12 (Figure 3), particularly in the superficial cortical layers. The density of parvalbumin immunoreactive neurons in both the deep and superficial cortical layers of GDX treated animals was significantly lower than in control animals ($p = 0.001$, 0.006, respectively). Parvalbumin immunoreactive neuron density was also significantly lower in the deep cortical layers of GDX-E treated animals, compared to controls ($p = 0.009$). In the superficial cortical layers however, the density of labeled neurons in the cortices of GDX-E treated animals was restored to control levels ($p = 0.672$) (Figure 2).

At 33 days postnatal the density of labeled neurons in the deep cortical layers increased, but that in the superficial layers was nearly the same as at postnatal day 16 (Figure 4). Parvalbumin immunoreactive neuron density was significantly lower in the deep and superficial cortical layers of GDX treated rats ($p = 0.034$, 0.036, respectively), compared to control rats. Parvalbumin immunoreactive neuron density was significantly lower only in the deep cortical layers of GDX+E treated rats ($p = 0.04$), but restored to control levels in the superficial layers (Figure 2).

Cortical TH Immunoreactive Fibers in the Frontal Cortex:

The total estimated length of cortical TH immunoreactive fibers varied across the developmental time points studied. TH immunoreactive fiber length in the deep cortical layers of control rats developed in a distinct pattern over time. Immunoreactive fibers were present throughout the depth of the cortex by postnatal day 12. The total fiber length throughout the deep cortical layers increased between 12 and 16 days postnatal, then decreased between 16 and 33 days postnatal in control animals. Similarly, TH immunoreactive fiber length throughout the superficial laminae increased slightly between 12 and 16 days postnatal, then decreased between 16 and 33 days postnatal. A peak in total fiber length occurred in the deep cortical layers at postnatal day 16 (Figure 5).

An interesting trend in the pattern of TH immunoreactive fiber development was noted in GDX treated animals. In control rats TH fiber length in the deep cortical laminae increased between 12 and 16 postnatal days and decreased between 16 and 33 days postnatal. The opposite pattern occurred in GDX treated animals, in that there was a decrease in total length between 12 and 16 days postnatal and a subsequent increase between 16 and 33 postnatal days (Figure 5A). The GDX-E treated animals showed a pattern of development similar to control animals, with a peak in fiber length in the deep cortical layers at postnatal day 16.

Compared to the deep cortical layers, very little variation in the pattern of TH immunoreactive fiber length occurred in the superficial cortical layers across the postnatal ages studied in control and treatment animals (Figure 5B). The GDX and the GDX+E

treated animals exhibited the same pattern of development as control animals, with only a slight increase in fiber length noted between 12 and 16 days postnatal and a slight decrease between 16 and 33 days postnatal.

Treatment induced changes in TH immunoreactive fiber length were age-dependent. No significant differences were found among treatment and control conditions in the deep layers at both 12 and 16 days postnatal (ANOVA, $p = 0.062, 0.198$ respectively), or in the superficial cortical layers ($p = 0.287, 0.608$, respectively). At 33 days postnatal, however, significant differences between experimental and control animals were noted (Figure 6). The GDX animals exhibited significant increases in total TH immunoreactive fiber length in the deep cortical layers, compared to control animals ($p = 0.001$) (Figure 7). The GDX+E rats also exhibited a significant, albeit smaller, increase in TH fiber length compared to controls in the deep cortical layers ($p = 0.042$). No significant differences were found between treatment and control animals in the superficial cortical layers ($p = 0.489$).

Effects of Gonadectomy on the Density of Dopaminergic Neurons in the SNc:

Dopaminergic neurons were evident in the SNc at all postnatal ages in all treatment and control groups. In control animals, the dopaminergic neuron density remained relatively stable between 12 and 16 days postnatal, then slightly decreased between 16 and 33 days postnatal. In GDX treated animals however, the density of labeled neurons remained relatively stable between 12 and 16 days postnatal, then increased between 16 and 33 days postnatal, examples of TH immunoreactive neurons in the SNc of all groups at 33 days postnatal are shown in figure 8. The GDX+E treated animals were similar to

control animals at all ages.

No significant differences were observed in the SNc among the GDX, GDX+E and control groups at 12 and 16 days postnatal (p = 0.664, 0.333, respectively). At 33 days postnatal however, gonadectomy caused a significant increase in dopaminergic neuron density compared to control rats (p = 0.041). Conversely, the GDX+E treated animals did not differ significantly from the controls (p = 0.697) (Figure 9).

Effect of Dopamine Depletion:

Neonatal 6-OHDA lesions appeared to partially deplete dopaminergic neurons in the SNc of 33 day old animals (Figure 10). In fact, statistical analysis revealed a significant decrease (65%) in dopaminergic neuron density compared to controls, indicating that the lesions were effective (Student's T test, p = 0.047) (Figure 11A). Interestingly, an increase in cortical TH immunoreactive fiber length was noted in the frontal cortex of lesioned rats, particularly in the deep laminae (Figure 12 A and B). The increase was statistically different from cortical values for fibers in the deep cortical layers (p = 0.005), but the superficial layers were unaffected (p = 0.069) (Figure 11B).

The density of parvalbumin immunoreactive neurons was not correlated with the lesion induced changes in the TH fiber length. There appeared to be an overall decrease in neuronal density (Fig 12 C and D) which reached significant levels in the deep cortical layers of lesioned rats, compared to controls (p = 0.02). Parvalbumin immunoreactive neuron density in the superficial cortical laminae did not significantly differ from that in control rats (p = 0.225) (Figure 11C).

Discussion:

Neonatal gonadectomy significantly decreases the density of parvalbumin immunoreactive neurons throughout the layers of the rodent frontal cortex during postnatal development. Estrogen replacement restores this density to control levels in the superficial cortical layers, but not the deep. Neonatal gonadectomy also alters the development of the dopaminergic system. TH positive fibers in the frontal cortex are increased by gonadectomy in postnatal day 33 rats. Fiber length is increased in the deep, but not the superficial, cortical layers. Estrogen replacement does not restore this measure to control levels. Interestingly, gonadectomy increases dopaminergic neuron density in the SNc at 33 days postnatal. Estrogen replacement returns this density to control levels. Similar to the effects of gonadectomy, neonatal 6-OHDA lesions decrease the density of cortical parvalbumin immunoreactive neurons and increase TH immunoreactive fiber length in the deep cortical layers at 33 days postnatal, in spite of a decrease in SNc dopaminergic neuron density in 33 day-old rats.

Technical Considerations:

In this study we used anti-TH antibodies as a marker for cortical dopamine fibers. Tyrosine hydroxylase is common in the biosynthetic pathway for catecholaminergic fibers. However, studies indicate that TH is relatively selective for dopaminergic fibers in the rodent frontal cortex (Hokfelt, 1974; Emson, 1978; Berger, 1984). Therefore, it is likely that the alterations in cortical TH expression seen in this study are indicative of changes in the dopamine system.

Stereologic counts were taken from a restricted region of the frontal cortex, which

included frontal 2, cingulate 1, and cingulate 3 subdivisions (Paxinos and Watson, 1986).

One sixty-day time release 17 ß-estradiol pellet was administered to each GDX+E rat at two days postnatal. Radioimmunoassay studies revealed that variations in blood serum levels of 17 ß-estradiol occurred over time, with levels at the earlier ages greater than physiological levels in controls. However, blood serum levels in GDX+E male rats were comparable to physiological levels of normal female rats by puberty.

Hormone Manipulation Affects Cortical Parvalbumin Expression:

We reported previously that exogenously applied estrogen enhances parvalbumin expression in organotypic slice cultures of the frontal cortex. These developmental changes were particularly evident in neurons located in the superficial cortical layers (Ross and Porter, In Press). The current study shows that a similar phenomenon occurs in an intact animal model. Specifically, gonadectomy, with consequent hormone depletion, induces a decrease in the density of parvalbumin expressing neurons, but only in the superficial cortical layers. Estrogen replacement ameliorates the laminar specific reduction in labeled neurons. Similar mechanisms may be responsible for the *in vitro* and *in vivo* findings.

Estrogen's effect upon parvalbumin expression in our current and previous experiments may be mediated by direct hormonal actions on the cortex. In fact, in the slice culture preparation, the cortex is isolated from all afferent inputs. Therefore, only endogenous mechanisms are in place. Estrogen s ability to bind dopamine receptors (Paden et al, 1982), the presence of estrogen receptors (MacLusky et al., 1979; Yokosuka et al., 1995; Shugrue et. al., 1997; Butler et al., 1999), and the aromatase necessary for the

localized conversion of testosterone to estrogen (Horvath et al., 1997) in the developing cortex allows for localized regulation of cell function..

The *in vivo* model however, is susceptible to extracortical influences, which need to be considered as contributing factors to our observed changes. For example, dopamine independently and similarly affects cortical parvalbumin expression. Exogenously applied dopamine enhances cortical parvalbumin expression in cortical slice cultures (Porter et al, 1999). Steroidal hormones influence the dopaminergic system in many ways and, therefore may mediate changes in parvalbumin expression through this neurotransmitter system.

Alterations in TH positive fiber density in the cortex (Kritzer et al, 1998; Adler et al, 1999), as well as, dopaminergic neuron density in the SNc (Zsarnovszky et al, 2000) occur following gonadectomy in adult rats. For example, two weeks after gonadectomy, adult male rats show increases in the density of TH immunoreactive fibers in the deep cortical layers (Adler et al, 1999). Our findings show that neonatal gonadectomy has a similar effect on cortical TH immunoreactive fibers which persist into adulthood. Here again, the length of cortical TH immunoreactive fibers is increased in the deep cortical layers of 33 day-old GDX.

Gonadectomy induced changes in fiber length measures do not occur until 33 days postnatal, although slight shifts from control levels appear earlier. Fiber length in the deep cortical laminae of control rats increases between 12 and 16 days postnatal and decreases thereafter. The opposite pattern occurs in GDX animals, but estrogen replacement restores this developmental pattern to control levels. This effect of hormonal

manipulation appears to affect only the deep layers. This change in cortical TH fiber development could selectively alter synapse and circuit formation of cortical output neurons in layers V-VI.

Interestingly, the mesocortical dopaminergic system emerges in a laminar specific pattern. Dopaminergic afferents targeting the deep cortical layers primarily develop prenatally, whereas those to the superficial cortical laminae reach the cortex postnatally (Berger et al, 1991). Distinct origins and functions have been proposed for the two inputs, but supporting evidence is lacking.

Similar to the dopaminergic projections, the maturation of cortical GABAergic neurons also extends into postnatal ages (Vincent et al, 1995). Furthermore, appositions between GABAergic neurons and dopaminergic axons increase in number during postnatal development (Benes et al, 1996). These contacts are likely to target only the GABAergic neurons which express parvalbumin (Porter et al, 1995; Sesack et al, 1998). In fact, early dopaminergic fiber growth into the cortex enhances parvalbumin expression in midbrain-cortex co-cultures (Porter et al, 1999), possibly because of ongoing synapse formation between the two neuronal subtypes. Therefore, hormone induced changes in cortical TH fibers could contribute to the alterations in parvalbumin expression. This possibility seems unlikely, however, given their polarized responses to estrogen depletion. The failure of a greater TH fiber presence to maintain the density of cortical parvalbumin expressing neurons may result from inappropriate timing of fiber ingrowth or inappropriate targeting of postsynaptic neurons.

Hormones alter other aspects of mesencephalic dopamine function. Changes in

dopamine receptor expression (Bosse et al, 1996) and alterations in binding affinity (Hafner et al, 1991) occur with gonadal hormone manipulation. In addition, castration at three weeks postnatal increases dopamine release from the substantia nigra of adult male rats (Knoll et al, 2000), whereas, chronic estrogen treatment decreases dopamine release in adolescent male and female rats (Knoll et al, 2000). Any or all of these mechanisms may be involved in altering parvalbumin expression.

Hormone Manipulation Affects Substantia Nigra Dopaminergic neurons:

The gonadectomy increased neuron density in SNc and TH fiber length in the cortex, but restoration of these measures to normal levels by estrogen replacement occurred only in the SNc. Estrogen therefore, appears necessary for the normal development of mesencephalic dopaminergic neurons, but ineffective in restoring fiber outgrowth to appropriate cortical connections.

Dopamine depletion alters parvalbumin expression in vivo:

To determine whether estrogen s influence on cortical parvalbumin expression was linked to its influence on the developing dopamine system, we disrupted the dopaminergic system in neonatal rodents. Lesioned rats exhibited significant decreases in dopaminergic neuron density in the SNc which was correlated with decreases in the density of parvalbumin immunoreactive neurons in the deep layers of the frontal cortex. This result was expected as our past experiments indicated that dopamine levels affect the development of cortical parvalbumin expression *in vitro*. Our study is the first to note that manipulation of the postnatal dopaminergic system alters the development of a specific population of GABAergic neurons, those expressing parvalbumin, in the intact rat.

We did not anticipate the increase in cortical TH immunoreactive fiber length after

the neurotoxic lesion. A compensatory increase in axons of surviving cells may occur in response to the loss of SNc dopamine neurons. The increase in TH fibers following the 6-OHDA lesions, like that after gonadectomy, was not associated with a restoration of parvalbumin expression. The peak in TH fibers after gonadectomy at 33 days postnatal may occur after maturation of parvalbumin expression.

Estrogen and dopamine may influence parvalbumin expression through separate mechanisms. The hormone induced alterations in parvalbumin expression do not correspond with changes in midbrain and cortical dopamine systems. Whereas gonadectomy induces changes in TH immunoreactive fibers only in the deep cortical layers at 33 days postnatal, it affects parvalbumin expression at all postnatal ages and throughout all cortical layers. Moreover, estrogen replacement does not ameliorate gonadectomy induced changes in TH fibers. Estrogen is effective in restoring parvalbumin immunoreactive neuron density to control levels , at least in the superficial layers. Depletion of SNc dopaminergic neurons by neurotoxic lesioning causes somewhat different effects in that the density of parvalbumin immunoreactive neurons decreases only in the deep cortical layers. These layer specific hormone induced changes suggest that estrogen and dopamine have separate and unique effects upon parvalbumin expression. Estrogen may act primarily on neurons in the superficial layers, and dopamine on the deep cortical neurons. Laminar specific differences in the parvalbumin immunoreactive neuron density and dopamine receptor distribution could contribute to such a phenomenon.

Our results indicate that neonatal hormone manipulation has long term effects upon developing parvalbumin and dopamine systems in the intact animal. Estrogen s effects appear to be layer specific. Neonatal manipulation of the dopaminergic system also alters the developmental expression of parvalbumin *in vivo*. In the current study, the specificity of estrogen e effect upon parvalbumin immunoreactive neurons in the superficial cortical layers, as well as the selectivity of dopamine depletion to alter parvalbumin expression in the deep cortical layers suggests that estrogen s effects are not medicated by alterations of the dopamine system. However, it is possible that estrogen may alter the activity of cortical dopamine receptors, thereby altering deep cortical layer parvalbumin expressing neurons. It remains to be determined how estrogen s effect upon parvalbumin is mediated. The relevance of parvalbumin expressing neuron function to numerous CNS disorders is increasingly apparent. These findings therefore, may aid in understanding the pathophysiology of a multitude of brain disorders.

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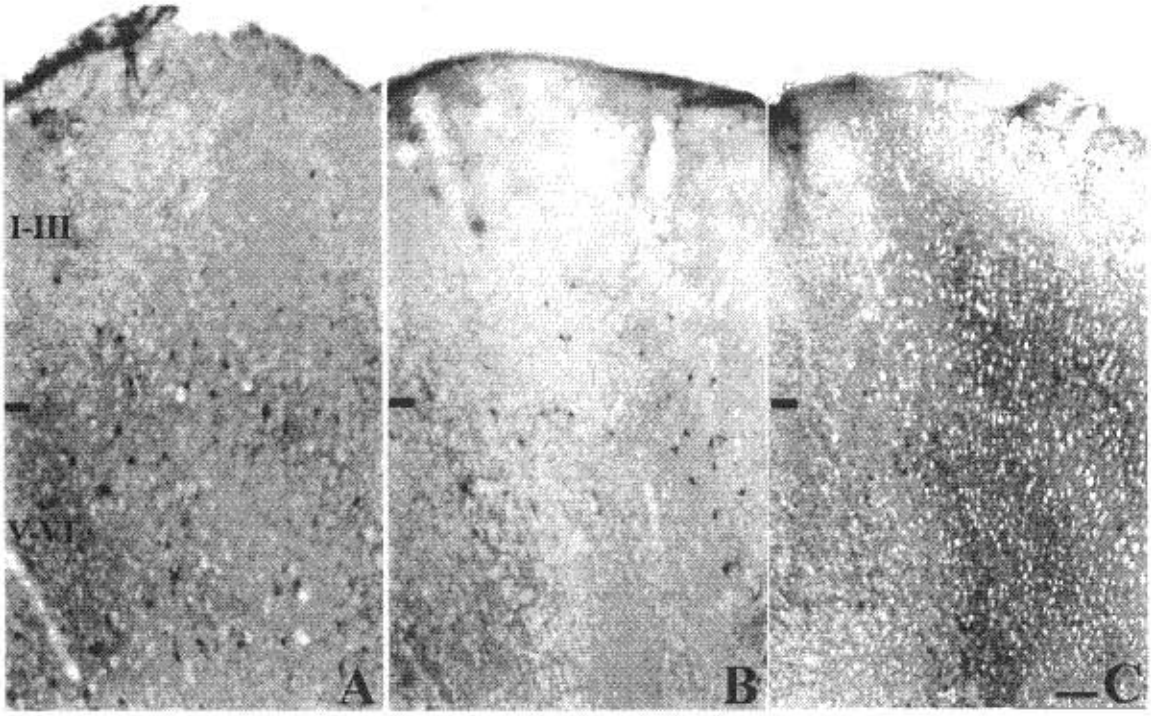

Figure 1. Photomicrographs of representative sections of the frontal cortex of 12 day old animals. Immunostaining is for parvalbumin. ***A***, section from a control animal: somal labeling is noted primarily in layers V-VI, with only a sparse number of labeled somata seen in the superficial cortical laminae. ***B***, section from a GDX treated animal: sparse somal labeling is noted in both the deep and superficial laminae. *C*, section from a GDX+E treated animal: very few somata are apparent in cortical layers V-VI. Scale bar for A-C = 150 μm.

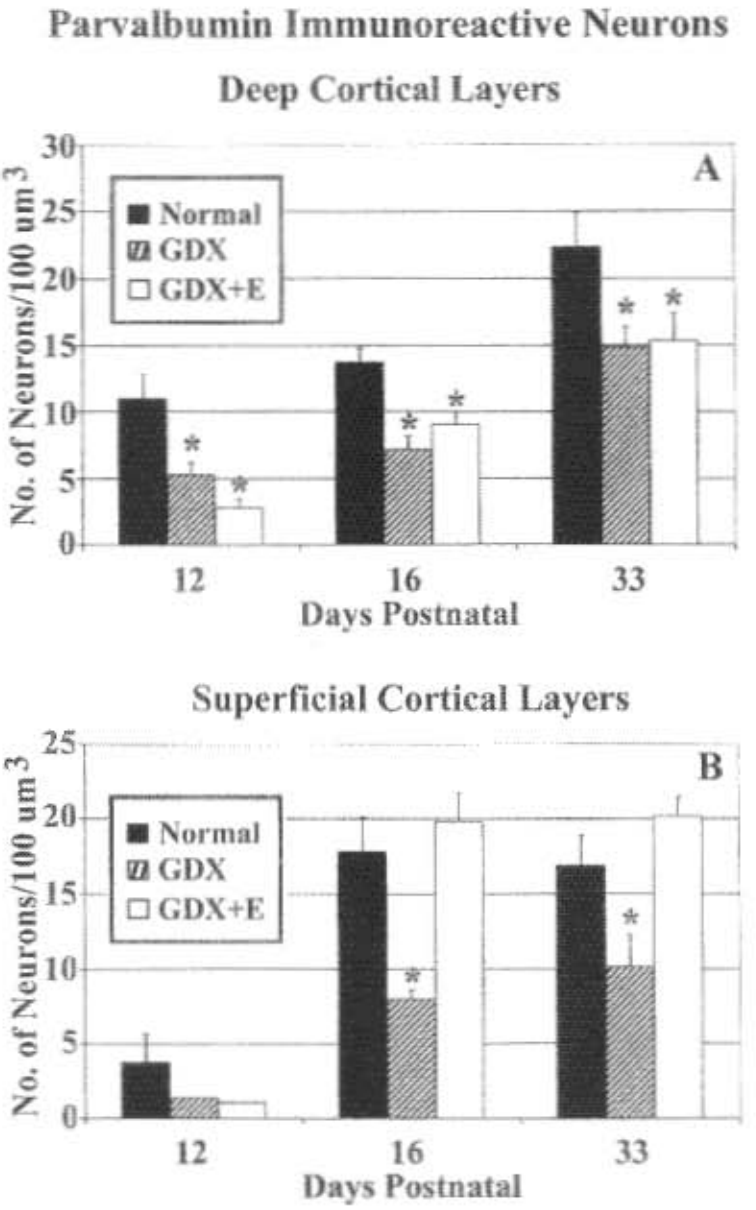

Figure 2. Histograms of the density of labeled neurons in the deep and superficial laminae at all postnatal ages. ***A***, Deep cortical layers: decreases in the density of labeled neurons are noted in the GDX and GDX+ E treated animals at all ages (ANOVA, GDX: $p = 0.014, 0.001, 0.034$, respectively; GDX+E: $p = 0.001, 0.009, 0.04$, at 12, 16, and 33 days respectively). ***B***, Superficial cortical layers: decreases in the density of labeled neurons are noted only in GDX treated animals at 16 and 33 days postnatal ($p = 0.006, 0.036$, respectively). * = statistically significant at $p < 0.05$.

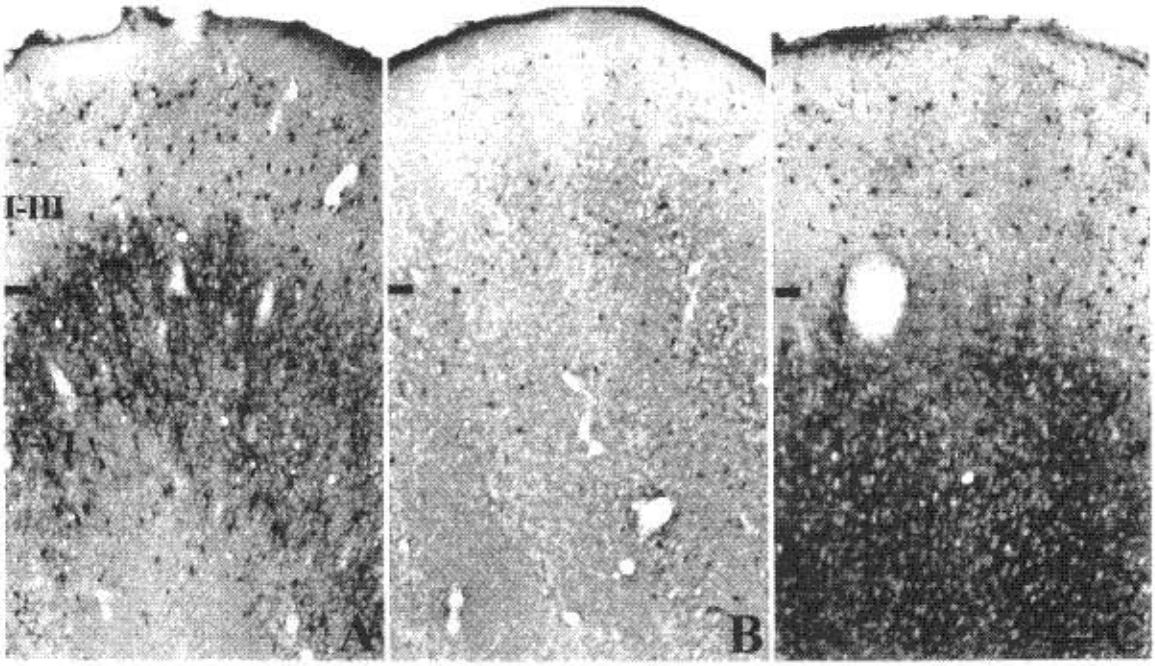

Figure 3. Photomicrographs of representative sections of the frontal cortex of 16 day old animals. Immunostaining is for parvalbumin. ***A***, section from a control animal: somal and fiber labeling are noted throughout all cortical laminae. ***B***, section from a GDX treated animal: somal labeling is noted primarily in the superficial cortical layers, with sparse labeling in the deep cortical laminae. ***C***, section from a GDX+E animal: somal and fiber labeling are noted throughout all cortical laminae. Scale bar for A-C = 150 μm.

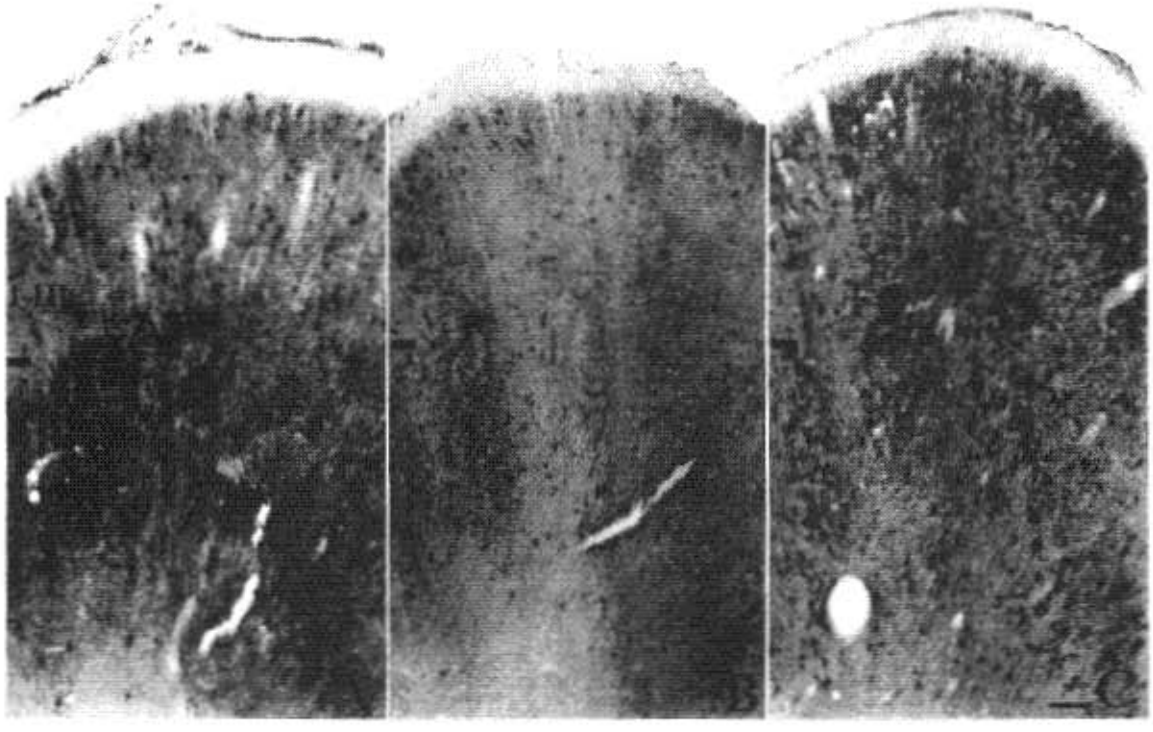

Figure 4. Photomicrographs of representative sections of the frontal cortex of 33 day old animals. Immunostaining is for parvalbumin. *A*, control: somal and fiber labeling are noted throughout all cortical laminae. *B*, section from a GDX animal: labeled neurons are apparent throughout all cortical layers, but are relatively sparse in the deep laminae. *C*, section from a GDX+E animal, somal and fiber labeling are noted throughout all cortical laminae. Scale bar for A-C = 150 μm.

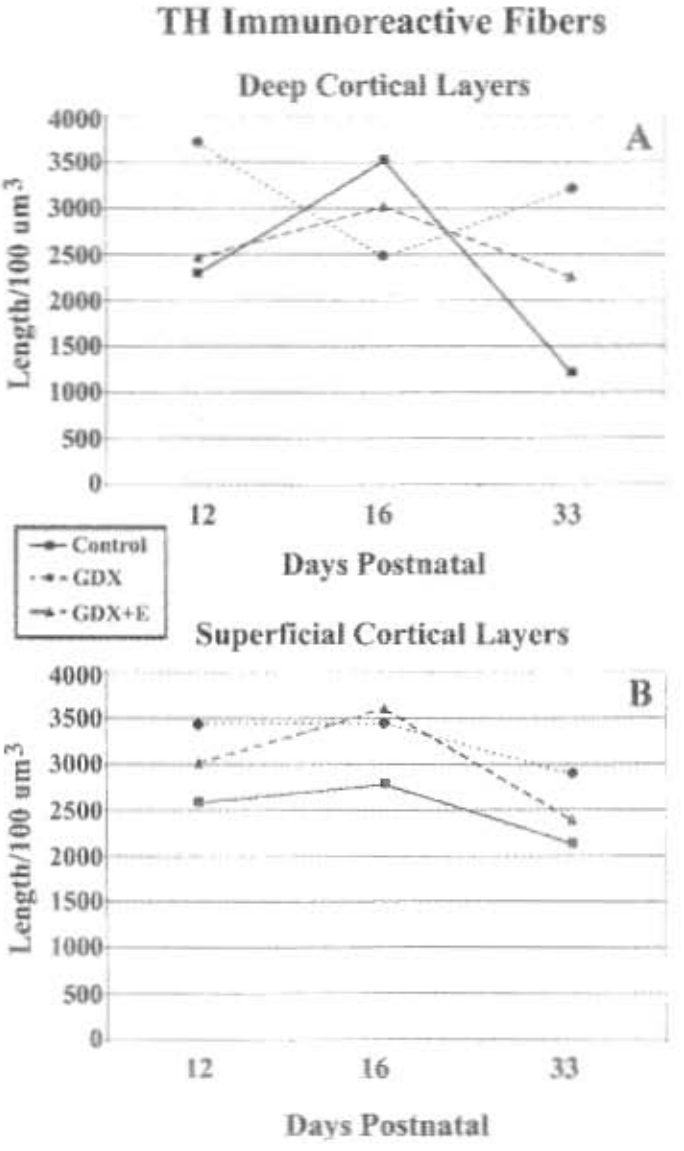

Figure 5. Graphs showing the of TH immunoreactive fiber measures at all postnatal ages. *A*, fiber length in the deep layers: fiber length increases between 12 and 16 days postnatal and decreases between 16 and 33 days postnatal in control animals. Fiber length decreases between 12 and 16 days postnatal, and increases between 16 and 33 days postnatal in GDX animals. A similar pattern to the control animals is noted in GDX+E treated animals. *B*, fibers in the superficial layers: similar measures are noted across all treatment and control conditions. Fiber length increases slightly between 12 and 16 days postnatal and decreases slightly between 16 and 33 days postnatal.

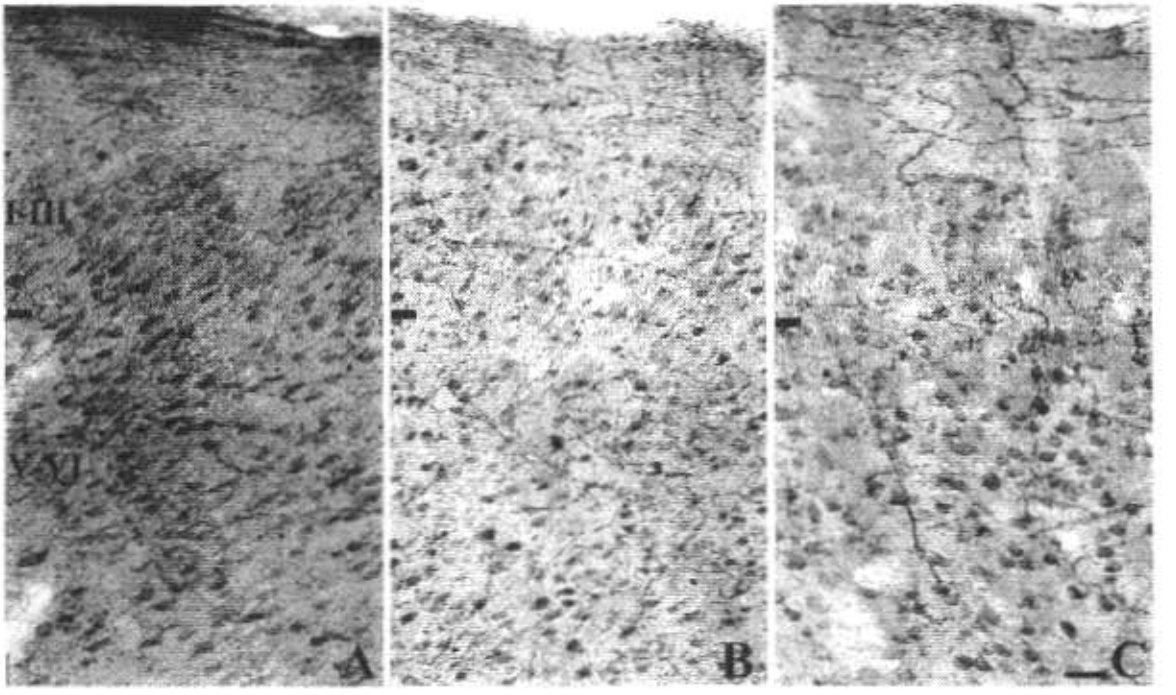

Figure 6. Photomicrographs of representative sections of the frontal cortex of 33 day old animals. Immunostaining is for TH. *A*, a section from a control animal: labeled fibers are seen coursing horizontally, occasionally vertically, throughout the superficial cortical laminae. Sparse labeling is noted in the deep cortical layers. *B*, a section from a GDX animal: extensive fiber labeling is noted in both the deep and superficial cortical laminae. *C*, a section from a GDX+E animal: extensive fiber labeling is mainly noted in the superficial cortical laminae, with occasional fibers apparent in layers V-VI. Scale bar for A-C = 150 μm.

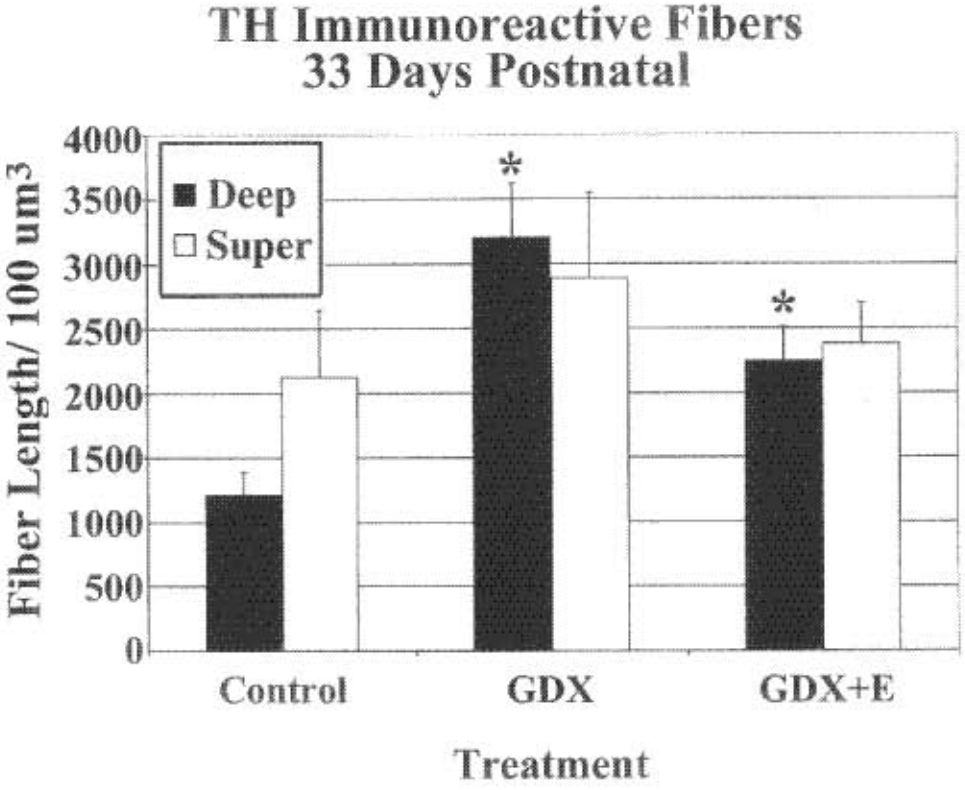

Figure 7. Histogram of fiber length in the deep and superficial cortical laminae from 33 days postnatal animals. Increases occur only in the deep cortical layers of GDX and GDX+E treated animals as compared to controls (ANOVA, $p = 0.001, 0.042$, respectively). * = statistically significant at $p < 0.05$.

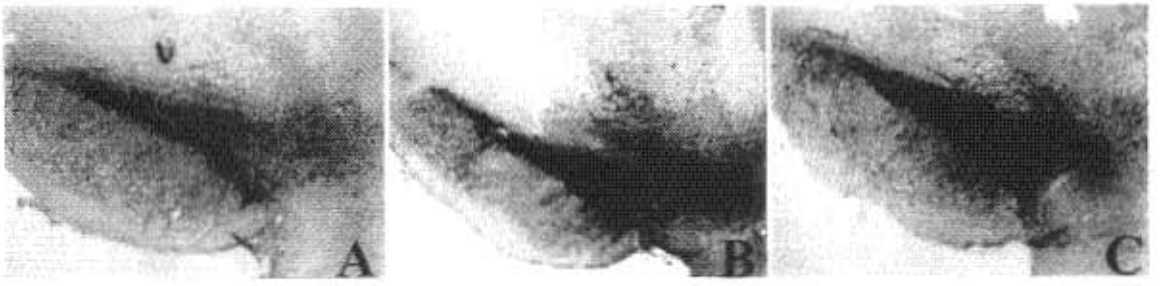

Figure 8. Photomicrographs of representative sections of the SNc of 33 day old rats. Immunostaining is for TH. ***A***, a section from a control animal: dense TH labeling is apparent in neurons of the SNc. ***B***, a section from a GDX animal: TH immunoreactive neuron density increased, compared to controls. ***C***, a section from a GDX+E animal: TH immunoreactive neuron density is similar to that in control animals. Scale bar for A-C = 200 μm.

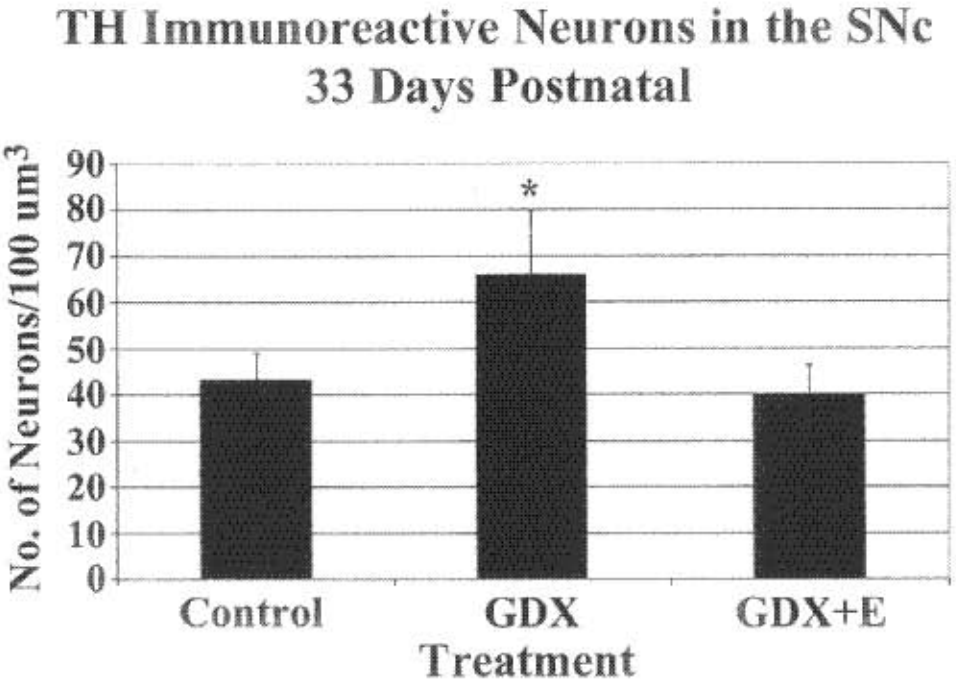

Figure 9. A histogram of labeled neuron density in the SNc at 33 days postnatal. An increase in neuron density is noted in GDX animals compared to controls. No change in density is apparent with GDX+E treatment, compared to controls (ANOVA, $p = 0.041$ for GDX, 0.697 for GDX+E). * = statistical significance at $p < 0.05$.

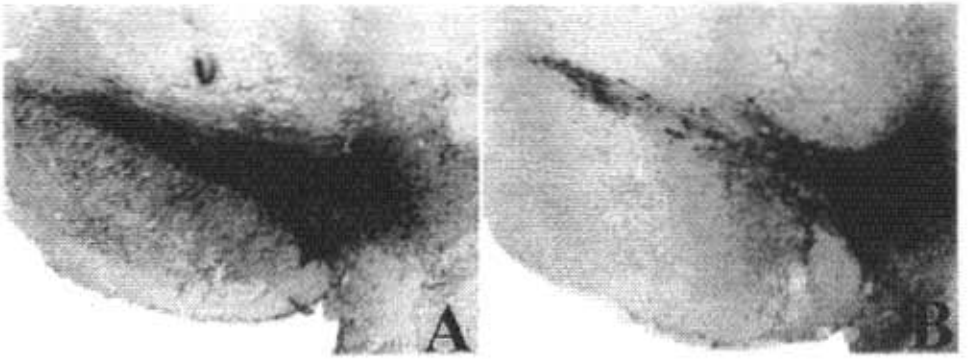

Figure 10. Photomicrographs of representative sections of the SNc of 33 day old rats. Immunostaining is for TH. ***A***, section from a control animal: TH labeled neurons in the SNc are dense. ***B***, section from a 6-OHDA treated animal: a decrease in the density of TH immunoreactive neurons is apparent compared to controls. Scale bar for A and B = 200 µm.

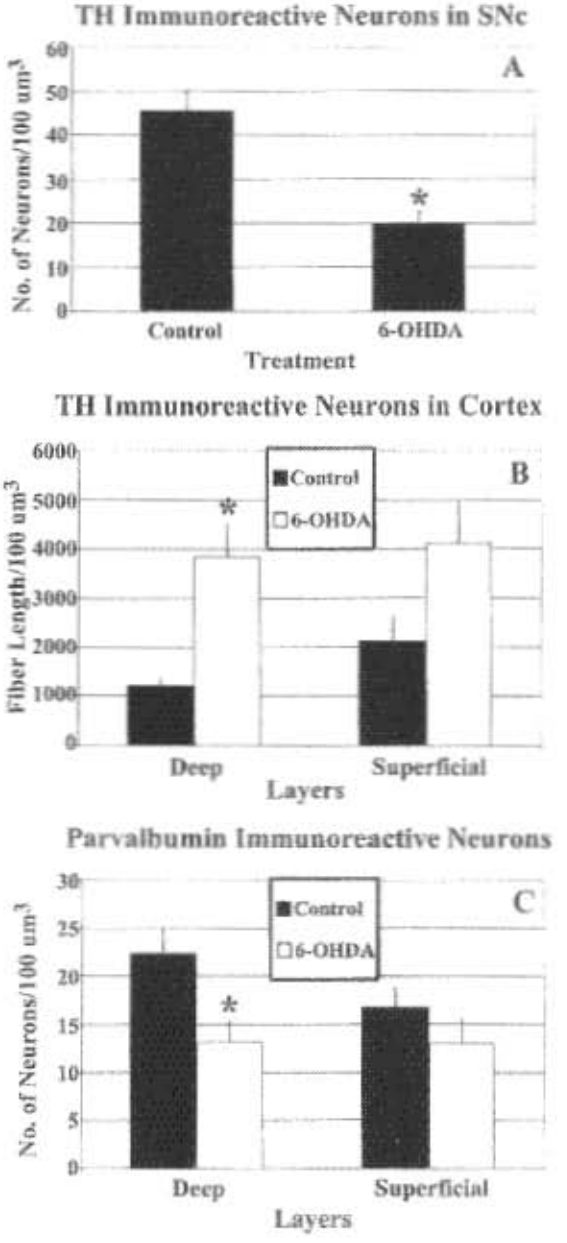

Figure 11. Histograms of dopaminergic neuron density in the SNc at 33 days postnatal. *A*, A decrease in labeled neuron density is noted in 6-OHDA lesioned animals, compared to controls (students' T Test, $p = 0.005$). *B*, A histogram of TH fiber length in the cortex at 33 days postnatal. An increase in estimated fiber length is noted only in the deep cortical layers of 6-OHDA lesioned animals ($p = 0.02$). *C*, A histogram of cortical parvalbumin immunoreactive neuron density at 33 days postnatal. A decrease in neuron density is noted only in the deep cortical layers of 6-OHDA lesioned animals ($p = 0.02$). * = statistical significant at $p < 0.05$.

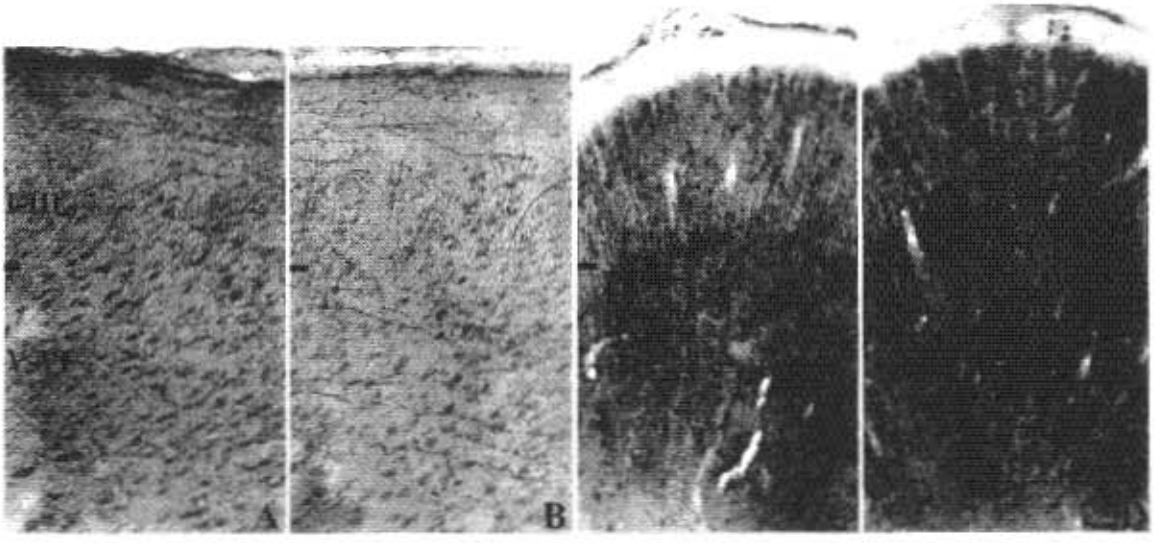

Figure 12. Photomicrographs of representative sections of the frontal cortex of 33 days postnatal animals. Immunostaining is for TH or Parvalbumin. ***A***, a section from a control animal: TH Immunoreactive fibers are seen throughout the superficial cortical laminae. Sparse labeling is noted in the deep cortical layers. ***B***, a section from a 6-OHDA animal: extensive fiber labeling is noted in both the deep and superficial cortical laminae. ***C***, a section from a control animal: parvalbumin labeled somata and fibers are noted throughout all cortical laminae, with particularly dense bands in layers II and V-VI. ***D***, a section from a 6-OHDA animal: a decrease in the density of parvalbumin immunoreactive somata is noted only in the deep cortical laminae, compared to that in controls. Scale bar for A-D = 150 μm.

Discussion:

The majority of hypotheses presented in these experiments were accepted. Both dopamine and estrogen enhance parvalbumin expression *in vitro*. In the *in vitro* study, the combined treatment enhanced parvalbumin expression, as well. However, the hypothesis that the comined treatment of dopamine and estrogen would produce alterations upon cortical parvalbumin expression could not be adequately answered in these experiments. In the *in vivo* study, the hypothesis that estrogen modulates parvalbumin expression is partially accepted. Estrogen modulates parvalbumin expression primarily in the superficial cortical layers. As predicted, estrogen also modulates the mesencephalic dopamine system. However, estrogen seems to have little effect upon cortical dopamine fiber alterations in the *in vivo* model. Therefore, the hypothesis that estrogen modulates the dopamine system is partially accepted.

The findings from these studies indicate that both dopamine and estrogen enhance the maturation of cortical interneurons that express the calcium binding protein, parvalbumin, in the developing cortex. They act at least in part through mechanisms intrinsic to the cortex. The *in vitro* studies revealed that both neuromodulators similarly affected cortical parvalbumin expression. The laminar pattern of parvalbumin expression was accelerated by dopamine and estrogen. Treatment of slice cultures with either estrogen, dopamine, or both, resulted in more mature patterns of laminar distribution of labeled neurons. Moreover, because the dendritic length and branching of labeled neurons increased in response to both treatments, the differentiation of individual neurons was

accelerated by both treatments. The influence of estrogen in promoting neuronal maturation was somewhat more potent than that of dopamine. Estrogen enhanced maturation of neurons in all laminae, whereas dopamine alone was affective only in the deep layers.

Similar findings were observed in our parallel studies performed on intact animals. Neonatal gonadal hormone depletion significantly decreased the density of parvalbumin expressing neurons in all layers of the rodent frontal cortex throughout development. Estrogen replacement returned the density of labeled neurons to control levels, but only in and not until 16 days postnatal. The possibility that estrogen-induced effects on parvalbumin expression might be mediated through its influence on the dopaminergic system was considered. Therefore, the effects of hormone manipulation on cortical and subcortical dopaminergic systems was examined. At 33 days postnatal, gonadectomy altered cortical and subcortical dopaminergic systems. Surprisingly gonadectomy increased the density of parvalbumin immunoreactive neurons in the SNc, and increased the length of TH immunoreactive fibers in the cortex, but not until 33 days postnatal when the cortex is fully matured. Estrogen was ineffective in restoring fiber length, but returned dopaminergic neuron density in the SNc to control levels. Direct manipulation of the dopaminergic system by administration of the neurotoxin, 6-OHDA, caused an expected decrease in dopaminergic neurons density in the SNc. However, the neonatal 6-OHDA lesions induced changes in the cortex similar to those of gonadectomy, in that parvalbumin expression was decreased and TH immunoreactive fibers were increased only in the deep cortical layers, and at 33 days postnatal.

The treatment induced shifts in distribution of parvalbumin-expressing neurons to both cortical layer Vi and the superficial layers were expressed in this thesis as changes in the percentage of parvalbumin labled neurons. These changes in percentage reflected changes in the relative distribution of parvalbumin-containing neurons in these slices, rather than increases or decreases in the amount of neurons in the various layers.

The estrogen-induced shift in distribution of labeled neurons and the increase in dendritic measures in isolated slice culture preparations suggest that estrogen enhances the maturation of parvalbumin expressing neurons through mechanisms intrinsic to the cortex. The presence of estrogen receptors in the cortex (MacLusky et al., 1979; Yokosuka et al., 1995; Shugrue et. al., 1997; Butler et al., 1999) and the aromatase necessary for the localized conversion of testosterone to estrogen (Horvath et al., 1997) presents a possible mechanism for a direct hormonal effect upon cortical parvalbumin expression. The findings from the *in vivo* experiments supported the results from the *in vitro* project. Estrogen treatment protected against the gonadectomy-induced decrease in density of parvalbumin expressing neurons in 16 and 33 day old rats. This protection, however, only occurred in the superficial cortical layers.

Cortical estrogen receptors are functional, but the hormone also acts on intrinsic aspects of the cortical dopamine system. Gonadal hormone depletion reduces dopamine D1 and D2 receptor expression in the rodent frontal cortex (Bosse et al., 1996). Estrogen alters the binding affinity of the dopamine D2 receptor (Hafner et al, 1991) and competes with dopamine (Paden et a l, 1982), subsequently blocking its activation. In light of estrogenic influences upon the dopamine system, the effect of estrogen treatment on

parvalbumin expression in these experiments could be mediated in part through the dopamine D2 receptor.

The presence of dopamine enhanced parvalbumin expression in the deep cortical layers in organotypic slice cultures. Conversely, dopamine depletion resulted in decreased parvalbumin expression in the deep cortical layers in the *in vivo* model. Dopamine D1 and D2 receptors are located on parvalbumin containing interneurons located primarily in the deep cortical layers (Le Moine and Gaspar, 1998). The dopamine D2 receptor mediates dopamine induced alterations in GABAergic neurons (Retaux et al, 1991) and parvalbumin expression (Porter et al, 1999). The preponderance of the dopamine receptors in the deep cortical layers may account for the selective effect of dopaminergic modulation on parvalbumin expression in neurons of the deep cortical layers.

In the studies described in this thesis, only one dose for each treatment was studied. A higher or lower dose of estrogen or dopamine could produce difference effects upon parvalbumin expression. The determination of a dose response curve for estrogen and dopamine treatment may be beneficial towards elucidating the effects of these treatments upon parvalbumin expression.

The normal pattern of the emergence of dopaminergic fibers in the cortex suggests that postnatal disruptions in the dopaminergic system may have layer specific effects upon cortical development. Mesocortical dopaminergic fibers develop in a layer specific manner. Fibers which target the deep layers enter the cortex prenatally. Fibers targeting the superficial cortical layers enter the cortex postnatally (see Berger et al, 1991, for review). The timing of their entry into the cortex coinciding with ongoing developmental events

may determine which neurons are susceptible to dopamine induced changes.

Estrogen induced alterations in neurons that express parvalbumin occurred throughout the cortical laminae in the *in vitro* experiment. However, in the *in vivo* experiment, estrogen s effect on parvalbumin expression was limited to the superficial cortical layers. Early gonadectomy may disrupt both the earlier-established dopamine input pathway and the ongoing formation of the late occurring input pathway. Therefore the gonadectomy-induced alterations in parvalbumin expression in the deep cortical layers may be mediated by alterations in the dopamine input. Because estrogen is known to alter cortical dopamine receptor function, it is also possible that estrogen-induced enhancement of parvalbumin expression noted in the *in vitro* experiments resulted from dopamine receptor alterations.

The findings from the *in vivo* study indicate that gonadal hormone manipulation affects cortical and subcortical dopamine systems. Gonadectomy increased the density of dopaminergic neurons in the SNc and TH fiber length in the cortex of 33 day old male rats. Chronic estrogen treatment only returned dopaminergic neuron density to control levels. Alterations in the cortical dopamine system therefore, are long lasting and are unaffected by estrogen.

From the findings of the organotypic slice culture experiment and from previous studies *in vivo* (Wang et al, 1995, 1996), we anticipated that increased dopamine levels would increase parvalbumin expression. In the intact animal however, the gonadectomy-induced increase in dopaminergic measures did not correlate with an increase in parvalbumin expression. To the contrary, the density of neurons that express parvalbumin

was decreased in the cortex. These findings suggest that the gonadectomy-induced alterations in parvalbumin expression were unrelated to changes in the dopamine system.

Parvalbumin is thought to protect neurons from excessive calcium accumulation associated with high levels of neuronal activity during development. Alterations in its expression by manipulation of endogenous neuromodulators such as dopamine or estrogen at critical periods of development could cause subtle changes in cortical connectivity and subsequent processing. The involvement of parvalbumin in numerous neurologic disorders highlights the importance of identifying the factors that may contribute to anomalous development of neurons that express this protein. Our findings that dopamine and estrogen enhance parvalbumin expression in the developing cortex provide clues towards understanding the origins of cortical malformations and subsequent cognitive dysfunction.

Limitations and Future Directions:

The parvalbumin containing subclass of GABAergic neurons was focused upon in this thesis because previous studies showed that altered dopamine concentrations do not affect other populations of neurons identified by calcium binding proteins they produced (Porter et al, 1999). Determining whether postnatal perturbations in the dopaminergic system induces changes in parvalbumin in the intact animal, however, had not been addressed. Because estrogen influences the dopamine system, the studies were also expanded to include estrogen induced effects and dopamine-estrogen interactions. These studies were focused on only one particular subclass of GABAergic neurons. Therefore, possible alterations induced by dopamine or estrogen upon other populations

of neurons were not elucidated.

The mechanisms by which dopamine and estrogen act on parvalbumin expression were not determined, but changes in cortical activity medicated by the glutamate system may be involved. in a previous study, Porter et al (1999) found that the dopamine-induced alterations in parvalbumin expression were blocked by co-application with the NMDA receptor antagonists MK801 or AP5. In fact, facilitatory effects of dopamine upon glutamatergic activity in layer V pyramidal neurons have been observed (Cepeda et al, 1992; Henze et al, 2000). Due to the extent of the research conducted in the thesis and the difficulties with assessing dopamine induced changes in cortical activity in the intact animal. alterations in cortical activity due to treatment were not addressed.

Estrogen also may act by altering glutamatergic activity. Estradiol-induced increases in fiber outgrowth of cultured cortical neurons is blocked by AP5 administration, whereas estrogen receptor antagonist ICI 184-780 administration does not block this estrogen-induced alteration (Briton et al, 1997). Furthermore, estradiol decreases cortical NMDA and AMPA receptor binding in the rat (Cyr et al, 2000a). Therefore, estrogen may alter neuronal function through modulation of glutamate receptors. The organotypic cortical slice culture preparation would be an excellent model to determine whether estrogen alters parvalbumin expression through glutamatergic mechanisms. The effects of co-application of estrogen and NMDA receptor antagonists on parvalbumin expression would address this issue.

Changes in cortical activity may also be elicited by alterations in the GABAergic system. Estrogen alters GABAergic function in the cortex. Ovariectomy, with subsequent

estrogen depletion, increases the amount of GABA-A receptor binding sites in the cortex, which is returned to normal levels by estrogen replacement (Juptner et al, 1991). Furthermore, estrogen may block stress-induced increases in benzodiazepine receptor (one type of GABA receptor) density (Bitran et al, 1998). These findings indicate possible mechanisms by which estrogen may alter GABAergic function. GABAergic neurons inhibit cortical output neurons. Therefore, these estrogen-induced alterations may alter cortical activity levels. Because increased parvalbumin expression coincides with high levels of cortical in parvalbumin expression.

Alterations in the neuronal phenotypes that express parvalbumin may account for the overall increases observed in GABAergic neuron density and GABA release in the cortex induced by dopamine (Wang et al, 1995; Grobin and Deutch, 1998). A stereological determination of the ration of total GABAergic neurons to parvalbumin expressing neurons would help to determine the validity of this notion. Such a study could be executed with our *in vitro* model.

It was previously demonstrated in the culture preparations that dopamine D2 receptor agonists alter the dendritic arborization of parvalbumin containing neurons (Porter et al, 1999). These findings suggest that receptor mediated mechanisms contributed to the dopamine-induced enhancement of parvalbumin expression in the current studies. because estrogen alters dopaminergic receptor function and binds directly to the D2 receptor, the co-application of estrogen and the dopamine D2 receptor antagonist, eticlopride are currently being conducted on slice cultures in our laboratory.

The effects of these manipulations on parvalbumin expression will be determined.

Estrogen receptor modulation may also be involved in the hormone induced alterations. To determine whether estrogen mediates cortical parvalbumin expression through estrogen receptors, an estrogen receptor antagonist, such as ICI 182-780, could be administered concurrently with estrogen, to cortical slice cultures. Furthermore, using ICI 182-780 to block estrogen receptor activation in the cortex may be a useful approach to discern the mechanisms by which estrogen affects parvalbumin expression *in vivo*. An estrogen receptor-beta knockout mice has been produced (Nilsson et al, 2000), and this model could be used to further elucidate the role of specific estrogen receptors upon cortical parvalbumin expression.

Hormonal manipulation of neurotransmitters other than dopamine may also play an important role in cortical development. Ovariectomy of adult rhesus monkeys increases norepinephrine fiber density in the cortex and estrogen replacement returns this measure to normal levels (Kritzer and Kohama, 1999). Gonadectomy in the male rat, however, does not affect norepinephrine fiber density in the cortex (Kritzer, 2000), highlighting the roles for gender specific hormones in development. Ovariectomy also increases serotonergic fiber density in the primate cortex (Kritzer and Kohama, 1999) and decreases 5-HT2A mRNA levels and receptor binding in the rodent frontal cortex. Estradiol restores these levels to normal (Cyr et al, 2000b). Therefore, hormone-induced alterations in the serotonergic system cannot be excluded as contributors to the changes in parvalbumin expression seen in the current studies. Koestner and Hornung (1999), however, were unable to detect changes in parvalbumin expression after manipulation of

the serotongeric system in slice cultures taken from neonatal animals. However, manipulations in older animals may reveal changes in the later developing parvalbumin-containing neurons. Hormone induced manipulations of both the noradrenergic and serotonergic neurotransmitter systems could help determine how both of these systems alter different subclasses of neurons during development *in vivo*.

Gender specific differences in parvalbumin expression have not yet been explored. Anatomical studies comparing the development of parvalbumin expression in the male and female rodent cortex should be conducted. The effects of other gonadal hormones, particularly testosterone, upon parvalbumin were beyond the scope of this thesis, but would help to understand differences in the cortical development and function between males and females. Furthermore, because the hormonal effects upon parvalbumin expression were noted in the frontal cortex, gender differences in cognition and emotion may be related to differences in the expression of this protein. However, the contribution of these neurons to cortical processing and behavior remains speculative. Behavioral studies examining locomotor activity or cognitive impairment could be examined using the in vivo model described in this thesis. However, because of the possible involvement of numerous neurotransmitter systems, only a correlation, and not causation, could be inferred between parvalbumin alterations and behavioral dysfunctions.

The functional consequences of the alterations in parvalbumin expression seen in these studies are difficult to assess. The neurons that express this protein have been identified morphologically and their physiologic activity has been studied in isolated slice

preparations. It is know that parvalbumin-containing neurons exert potent inhibitory influences over cortical output neurons and, therefore, influence over all cortical activity. Changes in the expression of parvalbumin may help to elucidate the pathophysiology of numerous neurologic disorders in which alterations in GABAergic neurons have been observed.

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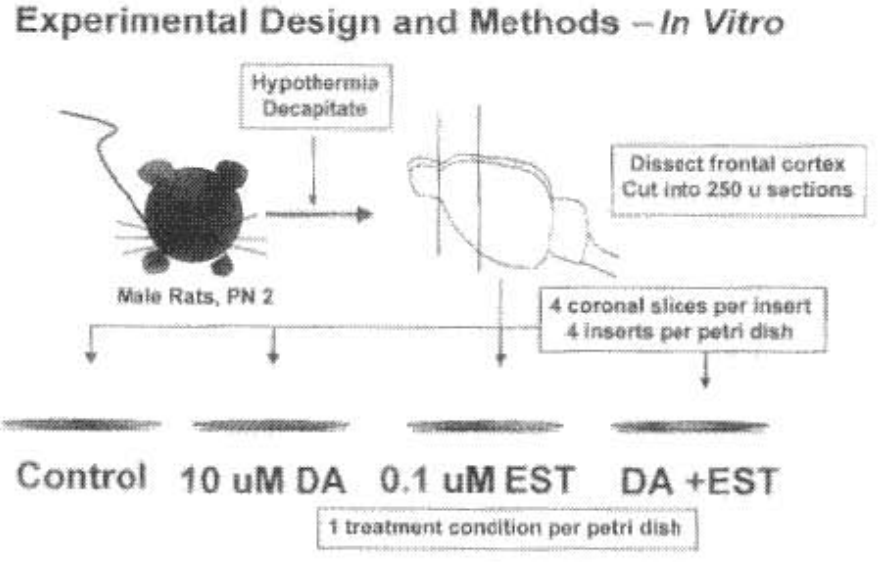
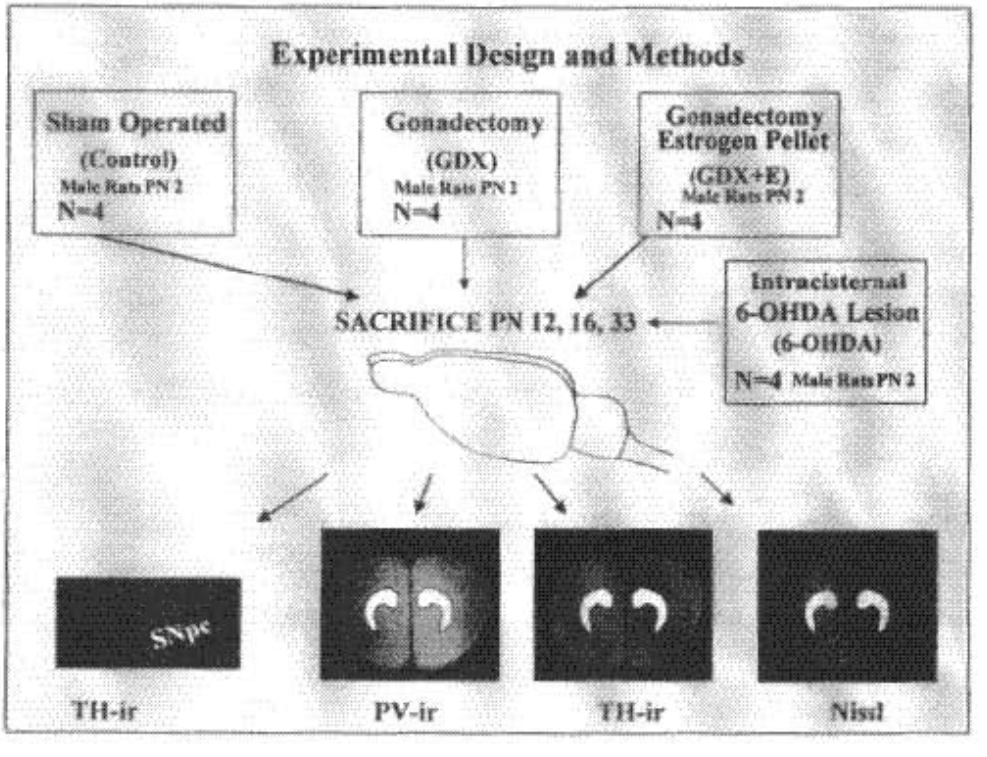

Experimental Design and Methods – *In Vitro*
Hypothermia
Decapitate
Dissect frontal cortex
Cut into 250 u sections
Male Rats, PN 2
4 coronal slices per insert
4 inserts per petri dish
Control
10 uM DA
0.1 uM EST
DA +EST
1 treatment condition per petri dish
* Maintain Slices for 14 days *in vitro*


Experimental Design and Methods
Sham Operated
(Control)
Male Rats PN 2
N=4
Gonadectomy
(GDX)
Male Rats PN 2
N=4
Gonadectomy
Estrogen Pellet
(GDX+E)
Male Rats PN 2
N=4
Intracisternal
6-OHDA Lesion
(6-OHDA)
N=4 Male Rats PN 2
SACRIFICE PN 12, 16, 33
SNpc
TH-ir
PV-ir
TH-ir
Nissl