MOLECULAR STUDIES OF POSTNATAL STRIATAL DEVELOPMENT

By

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A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

1995

This manuscript has been read and accepted for the Graduate faculty in Biomedical Sciences in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy

6/12/95
Date

Executive Officer

6/9/95
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Abstract

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Transforming growth factor alpha (TGF–α) is expressed in the brain and affects cells by binding to the epidermal growth factor receptor (EGF-R). Using a ribonuclease protection assay, we found that TGF–α steady state mRNA levels in the mouse striatum peak during the first week of postnatal life. Temporally this peak correlates with the height of gliogenesis in the subependymal layer (SEL), which lies along the striatal border of the lateral ventricle. *In vitro* studies demonstrate that TGF–α can stimulate the proliferation of astrocytes, so glial fibrillary acidic protein (GFAP) mRNA levels were measured as well and it was observed that the peak of GFAP expression followed that of TGF–α by one week. Furthermore, in a TGF–α deficient mouse, *waved-1 (wa-1)*, a significant reduction of GFAP mRNA levels and immunostaining for GFAP was found in the striatum. Bromodeoxyuridine (BrdU) labeling combined with immunohistochemistry of normal postnatal day 6 brain showed that the proliferating cells in the SEL are EGF-R immunoreactive. In the *waved-1* SEL, there were fewer BrdU positive cells and there was a reduced level of ^{3}H-thymidine incorporation. EGF-R immunoreactive cells were found in the SEL of the

adult mouse brain. Taken together, our data suggest that the TGF–α/EGF-R signaling pathway is involved in postnatal mitogenic events in the brain.

Thyroid hormone is necessary for normal postnatal brain and body maturation. We noticed that the phenotype of the *weaver* mutant mouse, for example, a small body size and a delay in eyelid opening, was similar to the characteristics of hypothyroid mice. We show that postnatally the *weaver* mouse has significantly reduced serum thyroid hormone levels. In order to determine if the reduced thyroid hormone could affect the brain, we measured Nerve Growth Factor (NGF) mRNA levels in the hippocampus, striatum and cerebellum of *weaver* mice during the time the mutants are hypothyroid. We found significant reductions in striatal NGF mRNA in the striatum specifically at P18, which was reversible when postnatal thyroid hormone injections were administered to the *weavers*. Striatal Choline Acetyl Transferase (ChAT) activity, which is normally affected by hypothyroidism, is not affected in the *weaver* mouse; we did not find reductions in the striatum of *weaver* mice. We conclude that the phenotype of the *weaver* mice is not a direct result of reduced thyroid hormone, but rather, reduced thyroid hormone may contribute to the *weaver* phenotype.

TGF-α is a putative dopaminergic trophic factor. Therefore, we investigated whether changes in TGF–α mRNA levels accompanied nigrostriatal degeneration in the *weaver* mutant mouse. Using a ribonuclease protection assay, we detected a significant decline in TGF–α mRNA in the *weaver* dorsal striatum which correlated temporally with

known regional reductions in dopamine concentrations. We also found significant decreases in TGF–α mRNA in the *weaver* hippocampus, but not in the *weaver* olfactory tubercle. Since the *weaver* mice have reduced serum thyroid hormone levels, we measured brain TGF–α mRNA levels in normal mice compared to hypothyroid mice at P22. We found significantly reduced TGF–α steady state mRNA levels under hypothyroid conditions in the dorsal striatum and olfactory tubercle, but not in the hippocampus. Because TGF-α is made in glia during the third postnatal week, we determined if glial fibrillary acidic protein (GFAP) mRNA in the *weaver* mutant striatum was altered. We found an induction of GFAP mRNA in the dorsal striatum at P26. We gave thyroxine injections to the *weaver* mutant mice and were unable to reverse the deficit in TGF–α mRNA in the dorsal striatum. We conclude that TGF–α mRNA is reduced in target-regions of midbrain dopamine neurons in the *weaver* mutant mouse brain and that this reduction is probably not the direct result of reduced serum thyroxine levels.

Acknowledgments

I would like to acknowledge the time and talent that my thesis advisor, Dr. Mariann Blum contributed to this project. She not only provided all the materials for this project and actually performed some of the experiments in this body of work, but she also spent countless hours of time discussing Neurobiology with me. Her dedication to and enthusiasm for science served as a constant source of inspiration for me. I would also to thank my husband, Thomas Wesley Weickert for his love and support.

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CHAPTER 1

General Background

The neurotrophic hypothesis states that neuronal survival is dependent on polypeptide growth factors which are produced in limiting amounts in target fields (Davies 1988). This hypothesis is based on *in vitro* and *in vivo* studies which showed sympathetic and some sensory neurons to be dependent on nerve growth factor (NGF) for development and survival (Levi-Montalcini and Angeletti 1968). The preliminary goal of this thesis project was to detect mRNA for potential dopaminergic trophic molecules *in vivo.* Many growth factors, including basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), transforming growth factor alpha (TGF–α), brain-derived neurotrophic factor (BDNF), and glial-cell-line derived neurotrophic factor (GDNF) have been shown have a trophic effect on dopamine (DA) neurons in primary culture (Knusel et al., 1990; Casper et al., 1991; Hyman et al., 1991; Lin et al., 1993). TGF-α has been shown to be synthesized in the target regions of dopamine neurons *in vivo*, and it will be the factor focused on in this thesis.

We used the *weaver* mutant mouse model of nigrostriatal degeneration to determine if reduced levels of TGF-α mRNA could account for the degeneration of these mutant neurons. The dopamine neurons die during early postnatal development in the *weaver*; therefore, we first characterized the normal developmental expression profile of TGF-α mRNA in a major target region of midbrain dopamine neurons, the dorsal striatum. The trophic effects of EGF/TGF-α are thought to be mediated through glial cells and TGF-α is a potent mitogen for cultured glioblasts. We thought that TGF-α may serve more than one developmental role in the brain and we looked for evidence that TGF-α was a glial mitogen *in vivo.* During the course of our studies, we observed that the *weaver*

mutant mice suffered from hypothyroidism. Thus, we determined whether thyroid hormone itself could regulate the expression of TGF-α mRNA in the brain and potentially confound our measurements of TGF-α mRNA levels in the *weaver*. The molecular events regulating the production and the subsequent effects of trophic molecules on brain cells are incompletely understood. The goal of this thesis project is to gain a better understanding of the role that TGF-α plays in postnatal basal ganglia development. The rest of Chapter 1 reviews the neuroanatomy of the basal ganglia, the development of the basal ganglia, the localization of TGF-α and thyroid hormone receptors in the basal ganglia, and the phenotype of the *weaver* mutant mouse.

Basal Ganglia Neuroanatomy

The basal ganglia consist of several interconnected neuronal groups that lie underneath the cortical mantle. The basal ganglia receive inputs from every area of the cortex, and after neurochemical integration of this input, information is sent back to the cortex by way of the thalamus (Graybiel 1990). One of the main targets for midbrain dopamine neurons is the striatum, and therefore the striatum would serve as one of the major sources of target derived neurotrophic support for the dopamine neurons.

Striatum

The striatum, named for a striped appearance in gross neuroanatomical dissections, is the largest and most central component of the basal ganglia; it is divided by the internal capsule in the primate and

thus can be broken down into the rostral-dorsal caudate and more ventral-medial putamen (Heimer et al., 1985). The rodent caudate-putamen is a semicontinuos mass only interrupted sporadically by myelinated axon cables of cortical projection fibers. Therefore, a more accurate way to view these two cytoarchitecturally identical brain areas, especially in the rodent, is as representing one nuclear mass, called the striatum or caudate-putamen (Heimer et al. 1985). The terms striatum and caudate-putamen will be used interchangeably in this thesis. The dorsal aspect of the striatum lies medial to the cortex just under the corpus callosum and the lateral ventricle defines the rostral-medial boundary of the striatum. The nucleus accumbens and the olfactory tubercle comprise the ventral striatum and are often considered separately from the more dorsal caudate-putamen (Heimer et al. 1985).

The striatum is a mosaic made up of clusters of chemically similar neurons on a field of chemically dissimilar neurons (Graybiel 1990). The clusters can be delineated by a variety of neurochemical markers and are referred to as patches or striosomes. The background or matrix also is characterized by positive staining for many chemical markers. The most commonly used markers are Acetylcholinesterase for the matrix and μ-opiate receptors for the patch (Graybiel 1990). The most abundant neuronal cell type residing in the striatum (96% of striatal cells) is the medium spiny neuron, which are GABA-ergic projection neurons. The striatal Cholinergic neurons have very large cell somas and axons which remain entirely within the caudate-putamen; therefore, they are intrinsic interneurons. To summarize, there are different levels of organization: the striatum is part of a network of nuclei, termed together, the basal ganglia; the striatum can be grossly broken down into the dorsal and

ventral striatum; the striatum consists of macrocompartments, the patch and the matrix, and finally there are different neuronal subtypes found within each compartment.

Substantia Nigra

Three separate groups of midbrain dopamine neurons are among the most heavily labeled with monoamine histofluorescence (Dahlstrom and Fuxe 1965). These neurons are all located ventral to the cerebral aqueduct of the midbrain, the area referred to as the ventral tegmentum. The most caudally as well as most dorsally placed dopaminergic cell group is the retrorubral field or A8. Situated just dorsal to the substantia nigra pars reticulata is the A9 dopaminergic neuronal group; it is synonymous with the more common term substantia nigra pars compacta. The substantia nigra pars compacta has been described "capping" the pars reticulata as a two layered sheet with a dorsal and ventral tier. The medially located A10 dopaminergic neuronal group is also known as the ventral tegmental area (VTA).

Originating from cell bodies in the substantia nigra pars compacta (A9) are axons that form the bulk of the nigrostriatal tract. A dorso-ventral inversion of innervation has been recognized (Fallon and Loughlin 1985), where the ventral tier dopamine neurons project to the dorsal striatum and the dorsal tier dopamine neurons project to the ventral striatum. The dopamine neurons of the A10 can also project to the ventromedial caudate-putamen and to the nucleus accumbens (Bjorklund and Lindvall 1984). The mesocortical dopamine projection, which includes a heavy innervation of frontal regions and a sparse innervation of hippocampus, originates from both A9 and A10 (Bjorklund and Lindvall 1984).

Development

Neurons

As evidenced by birth dating studies with tritiated thymidine, the dopamine neurons of the rodent midbrain are generated on embryonic day 11 (E11) through E15 with peak production on approximately E13 (Hanaway et al., 1971; Lauder and Bloom 1974). Striatal neurons are also generated embryonically. GABA-ergic neurons born between E13 to E15 have been localized preferentially to the patch compartment in the adult, and those generated between E18 to E20 are found to reside in the matrix (van der Kooy and Fishell 1987). As early as E14.5, the dopaminergic neurites reach the ventro-lateral region of the developing striatum and innervation proceeds in a dorso-medial direction while zones of striatal neurogenesis are avoided (Tennyson et al., 1975; Specht et al., 1981). At E19 until well after birth, the dopamine innervation throughout the striatum is found in concentrated clumps, the dopamine islands (Olson et al., 1972; Tennyson et al. 1975; Voorn et al., 1988). No dramatic changes in dopaminergic innervation of the striatum happen during the first two weeks of postnatal life. However, during the third and fourth week of postnatal life, the concentration of dopamine increases by 440% to reach adult levels (Roffler-Tarlov and Graybiel 1987). During this time, morphological maturation of dopamine innervation is also achieved, the dopamine islands disappear and the dopamine innervation becomes homogeneous.

Glia

There is a specialized zone, which is termed the subependymal layer (SEL), in the neonatal caudate-putamen that has a particularly high rate of mitotic activity (Smart 1961; Smart and Leblond 1961; Sturrock and Smart 1980). It lies just underneath the ependyma that line the lateral ventricle in postnatal life. The SEL is thickest at the rostral medial edge of dorsal caudate-putamen. Prenatally, at E14, glioblasts begin to concentrate in the SEL zone of the lateral ventricles of the mouse forebrain (Sturrock et al., 1983). During early postnatal development, astroglial cells and oligodendroglia undergo final cell divisions in the SEL and then migrate tangentially along the corpus callosum in streams to populate the brain as mature glia (Smart 1961; Smart and Leblond 1961; Mc Dermott and Lantos 1990). Even though glia can be generated throughout life, the first two postnatal weeks mark a time when glial production is at a maximum in the rodent brain.

Recently, two different laboratories reported the successful disruption of the TGF–α gene by gene targeting techniques in embryonic stem cells. These mice were viable and exhibited waviness of coat and whiskers (Lu and Brown 1977; Luetteke et al., 1993; Mann et al., 1993). This mild phenotype was recognized to be similar to the phenotype of the mutant mouse *waved-1* (*wa-1*) which had already been described (Crew 1933). Both groups found that the wa-1 gene behaved as an allele to their disrupted TGF–α; consequently, they concluded that the mutant *waved-1* has a mutation in the TGF–α gene (Luetteke et al. 1993; Mann et al. 1993). There is some expression of TGF–α mRNA in the *waved* mutant mouse, but the levels are dramatically reduced (Luetteke et al. 1993; Mann et al. 1993).

Weaver Mutant Mouse

Neurons in the cerebellum and substantia nigra of the mutant mouse *weaver* undergo genetically triggered neurodegeneration shortly after birth (Rezai and Yoon 1972; Rakic and Sidman 1973). The weight of these mutants is considerably less than their normal siblings, and the behavioral phenotype is obvious at approximately P14 (Sidman et al., 1965) . The mutant mice exhibit constant tremor when at rest and show an inability to coordinate limb movements; they are often observed toppling over when attempting to walk. All the mice carrying the homozygous wv/wv condition lose 98% of their granule cells in the cerebellum (Rakic and Sidman 1973). When the behavioral phenotype is considered as the criteria to judge the "affected" mice, the *weaver* gene appears to be inherited in an autosomal recessive pattern; however, when, the cerebellum is examined histologically, it is clear that there is an intermediate phenotype expressed in the heterozygotes.

The *weaver* mutant mouse was recognized to have decreased levels of dopamine in the dorsal striatum compared to normal littermate controls (Lane et al., 1977). Subsequently, it was documented that this reduction in neurotransmitter levels was due to reduced TH + cell numbers in the midbrain (Triarhou et al., 1988). It is plausible that the cell death occurring in these two distinct populations, the *weaver* granule neuron and the *weaver* dopamine neuron share a common molecular mechanism, as no direct neuroanatomical relationship is known. The *weaver* disorder, as it affects the nigral neurons, can be viewed as failed development. At birth, "dopamine islands" are present in the *weaver* caudate-putamen and

thus it appears normal morphologically (Roffler-Tarlov and Graybiel 1987; Graybiel et al., 1990; Roffler-Tarlov et al., 1990). During the following three weeks, the mutant dopamine neurons fail to make the normal transition from the primarily patchy dopamine innervation to the more diffuse DA innervation. In *weaver* mutants, the early forming dopamine islands persist until P20, and dopamine content in the mutant striatum fails to undergo the rapid increase after P17. The adult *weaver* has a 69% decrease in cell number in the substantia nigra pars compacta (A9), a 56% decrease in A8 and a 26% decrease in A10 (Roffler-Tarlov and Graybiel 1987; Graybiel et al. 1990; Roffler-Tarlov et al. 1990). There is a 71% reduction in dopamine concentration in the adult *weaver* dorsal striatum compared to wild-type controls. The mutant ventral striatum is less affected than the dorsal striatum; there is a 36% reduction of dopamine in the *weaver* olfactory tubercle while no decrease in dopamine is detected in the *weaver* nucleus accumbens (Roffler-Tarlov and Graybiel 1987; Graybiel et al. 1990; Roffler-Tarlov et al. 1990).

Trophic Molecules

Neuron-Glia Relationships

Studies of cultured mibrain neurons indicate that a glial derived factor can support their survival and maturation of dopamine neurons (O'Malley et al., 1991). High affinity dopamine uptake activity is increased, dopamine neuron survival times are extended and dopaminergic neurites are longer and more branched when dopamine neurons are grown on a layer of glia as opposed to a poly-ornithine substrate (Prochiantz et al., 1981). The trophic effects of exogenously applied EGF/TGF–α and bFGF

have been shown to be mediated by glia (Casper et al. 1991; Engele and Bohn 1991; Alexi and Hefti 1993). Therefore, it has been proposed that glial cells synthesize and secrete factors which can influence the development and survival of neurons both *in vitro* and *in vivo*.

TGF–α & EGF-R

Transforming growth factor alpha protein (TGF–α), has a common secondary structure as EGF and is considered to be member of the epidermal growth factor family (Gill et al., 1987) . The secreted form of the TGF–α protein is about 50 amino acids in length and is processed from a larger membrane spanning precursor protein (Carpenter and Cohen 1990). TGF–α binds to the EGF receptor (EGFR) with high affinity and evokes a mitogenic response in a variety of cultured cell types (Massague 1983; Marquardt et al., 1984; Ma et al., 1992). The epidermal growth factor receptor (EGFR) is a cell-surface glycoprotein which uses the tyrosine kinase signaling pathway intracellularly. The viral *erb-B* oncogene encodes a version of the EGFR missing the ligand binding domain; and the intracellular tyrosine kinase domain of the mutant EGFR is constitutively active. Cells expressing *v-erb-B* respond to this artificially amplified intracellular phosphorylation event by continuously dividing. Many brain tumors of glial origin express high levels of EGFR when compared to non-transformed glia cells (Libermann et al., 1984). Injury to the central nervous system evokes the appearance of reactive astrocytes, which are capable of division and migration; these astrocytes increase in size, stain more intensely for GFAP, and show very prominent EGF receptor and TGF–α immunoreactivity (Nieto-Sampedro et al., 1988; Junier et al., 1991; Junier et al., 1993).

During embryonic development, rodent brain tissue binds only low levels of EGF until late gestation when there is a large increase in binding capabilities (Quirion et al., 1988). Binding of ^{125}I-EGF is highest perinatally and is concentrated in the forebrain, including cortical areas and the caudate-putamen (Quirion et al. 1988). Binding of EGF to brain or brain membrane extracts is greatly reduced in the adult rodent as compared to the neonate (Hiramatsu et al., 1988; Quirion et al. 1988). These studies imply that EGF receptor should be abundant in the perinatal period. Recently, EGF-R mRNA has been localized to the SEL by *in situ* hybridization (Seroogy et al., 1995).

An EGF-responsive population of precursors in the adult murine striatum has been identified using reaggregate culturing techniques. Adult striatal stem cells cultured on non–adhesive substrate proliferate in response to EGF and can be induced to differentiate into both neurons and glia by subsequent plating on poly-ornithine coated coverslips (Reynolds et al., 1992; Reynolds and Weiss 1992). These multipotent stem cells observed *in vitro* may reside in the SEL of the adult. The cells within the SEL of the adult brain can differentiate into both neurons and glia (Altman 1969; Lois and Alvarez-Buylla 1993; Luskin 1993; Lois and Alvarez-Buylla 1994). Cell tracing techniques have shown that neurons can be generated in the adult mammalian striatal SEL, can differentiate into GABA-ergic interneurons and can be incorporated into neuroanatomical circuitry of the adult olfactory bulb after a rostral migration (Lois and Alvarez-Buylla 1994). However, molecular factors that regulate the endogenous proliferation of these cells have not yet been identified.

EGF/TGF–α has been shown to support the survival and process outgrowth of cortical and subcortical neurons in primary culture (Kornblum et al., 1990). In addition, EGF/TGF–α can increase neuronal dopamine uptake and dopamine neuron survival in primary cultures from the mesencephalon (Knusel et al. 1990; Casper et al. 1991; Ferrari et al., 1991; Alexi and Hefti 1993). EGF has been shown to also exert neurotrophic effects on dopamine neurons *in vivo*. Intraventricular infusion of EGF resulted in a two-fold increase in the number of TH positive substantia nigra neurons after transection of the nigrostriatal pathway when lesioned animals with EGF infusions were compared to controls (Pezzoli et al., 1991). The region specific dopaminergic cell death in the *weaver* mutant could be caused by alteration in trophic support available from the targets. It seems that the dopamine neurons undergo a developmental failure during the third postnatal week of life so alterations in TGF–α mRNA concentration in the *weaver* mouse may be temporally specific.

Thyroid Hormone

Thyroid Hormone Effects

Thyroid hormone is made in the thyroid gland and enters the circulation as the inactive T4 bound in the blood by transthyretin, (Gubits et al., 1986). It can be brought into the brain cells by selective membrane transporters and it is converted to the active T3 by tissue deiodinases (Nikodem et al., 1990; Leonard 1992). The deiodinating enzymes are found in both neurons and glia in the brain (Leonard 1992). Serum levels of thyroid hormone must be kept within a normal range if brain

development is to proceed normally. Serum thyroid hormone levels peak at P15 in the mouse (Walker et al., 1979). Hypothyroidism induced postnatally initially results in a delay in maturation of the brain, such that both neurons and glia are kept in an immature state (Hamburgh 1968; Hamburgh et al., 1971).

Hypothyroidism affects the morphology of the medium spiny neurons in the striatum; they are smaller and have a reduction in the number of spines when they develop under hypothyroid conditions (Lu and Brown 1977). Biochemically, hypothyroidism has been shown to decrease striatal Choline Acetyl Transferase activity (ChAT), the synthetic enzyme for acetyl choline biosynthesis. One report shows that when rat pups age P0 are injected with ^{131} I, which destroys the thyroid gland, dopamine concentration in the caudate-putamen decreases by 42% (Rastogi and Singhal 1979). Also, a decrease in the levels of dopamine precursor tyrosine and a decrease in the dopamine metabolites DOPAC and HVA in the striatum was found (Rastogi and Singhal 1979). In the hypothyroid animals, the reduction in dopamine, as well as its precursor, suggest that there may be a reduction in the nigrostriatal innervation of the caudate-putamen, but this has yet to be examined.

Growth factor mRNA levels outside the brain have been shown to be affected by thyroid hormone manipulations. EGF is abundantly synthesized and stored in the granular convoluted tubules of the mouse submandibular glands (Gubits et al. 1986). Thyroid hormone affects the differentiation of the cells that synthesize EGF as well as affecting EGF synthesis itself (Gresik and Maruyama 1987). When compared to controls, levels of EGF protein and mRNA in the submandibular glands of hypothyroid mice were reduced by about 90% after four months of

propylthiouracil (PTU) treatment, a chemical that blocks the production of thyroid hormone (Gubits et al. 1986; Gresik and Maruyama 1987). Alterations in NGF protein produced by hypothyroidism provide an example of how hormonal changes can cause changes in brain development by acting through growth factor molecules. Cerebellar NGF protein levels rise in parallel with the rise in serum thyroid hormone levels in postnatal development (Walker et al., 1982). NGF immunoreactivity is first detectable on postnatal day 10 in the cerebellum and is totally abolished when animals are made hypothyroid (Legrand and Clos 1991). Injections of anti-NGF antibodies are able to reverse some of the beneficial effects of T4 treatment of hypothyroid rats (Legrand and Clos 1991). These observations suggest that the effects of thyroid hormone are partially mediated through NGF.

Thyroid Hormone Receptors

The effects of thyroid hormones are mediated through four different types of nuclear receptors that act to increase or decrease transcriptional rates of target genes by binding to thyroid response elements (TREs) in the DNA (Williams 1994). The receptor proteins are transcribed from two chromosomally separated genes, the α and ß. Each of their respective mRNAs can be alternatively spliced yielding two different forms; they are designated α1, α2, ß1 and ß2. The α2 receptor is distinct from the other three forms, in that it has no T3 binding ability; the others all bind T3 with high affinity (Nikodem et al. 1990; Williams 1994) . The α gene most closely resembles the oncogene *v-erb A* whose non-T3 binding product can lead to cellular transformation by the blockage of differentiation.

Thyroid hormone receptor mRNA has been localized to virtually every area of the nervous system by *in situ* hybridization on developing rat brain sections (Bradley et al., 1992). Hybridization signals for α1 and α2 transcripts can be detected as early as E13.5 in the neural tube and persist until adulthood in many brain areas, including the cerebellum, olfactory bulb, cortex, basal ganglia, hypothalamus and pituitary (Bradley et al. 1992). The ß1 and ß2 expression is both temporally and spatially restricted. The ß1 transcript is more prominent in the telencephalon and pituitary, and the ß2, once thought to be pituitary specific, was found at high levels in the pituitary and at low levels in the striatum and hippocampus (Bradley et al. 1992) . In the adult rodent caudate-putamen the thyroid hormone receptor mRNAs exist in distinct neuroanatomical patterns. The α2 specific signal is homogeneously distributed throughout the entire dorsal striatum. The α1 hybridization signal is more intense medially than laterally, whereas for both ß1 and ß2 the reverse is true. The ß1 hybridization signal exists in a gradient moving from no signal in the ventromedial regions of the caudate to a very intense band along the dorsal lateral boarder. A weak ß2 signal trims the lateral edge of the caudate-putamen (Bradley et al. 1992).

CHAPTER 2

Striatal TGF-α: Postnatal Developmental Expression and Evidence for a Role in the Proliferation of Subependymal Cells

Introduction

Polypeptide growth factors provide important information to brain cells during nervous system development, including signals to divide, migrate, and survive. The mRNAs encoding two of these factors, epidermal growth factor (EGF) and transforming growth factor alpha (TGF–α), exist in the murine central nervous system (Rall et al., 1985; Wilcox and Derynck 1988; Lazar and Blum 1992). EGF and TGF–α both bind to a signal transducing transmembrane protein known as the epidermal growth factor receptor (EGF-R) with equal affinities and exert comparable effects (Massague 1983; Marquardt et al. 1984; Gill et al. 1987; Carpenter and Cohen 1990). However, in both the embryonic and adult mouse brain TGF–α mRNA levels far exceed the levels of EGF mRNA. This relationship is especially evident in the striatum where the molar abundance of TGF–α mRNA is 100-fold higher than that of the EGF mRNA (Lazar and Blum 1992). Additionally, the regional levels of TGF–α mRNA are higher in the adult striatum than in any other brain region (Lazar and Blum 1992). In the rodent striatum, TGF–α mRNA has been shown to be present in both neurons and glia (Seroogy et al., 1993), whereas the TGF–α protein has been found only in astroglial cells (Fallon et al., 1990). Therefore, although TGF–α and EGF are both capable of activating the EGF-R, the available data regarding the abundance and localization of these two growth factors support the conclusion that TGF–α is a major physiological ligand for the EGF-R in the striatum.

Although EGF/TGF–α has been observed to have a number of effects on both neuronal and glial populations in cell culture, most studies report stimulation of glial proliferation as a major action of these factors (Leutz

and Schachner 1981; Simpson et al., 1982; Casper et al. 1991). To date, no studies have found EGF-R protein on proliferating cells in the normal brain, but it has been shown that EGF-R appears on reactive astrocytes, which can divide in response to brain injury (Gomez-Pinilla et al., 1988; Nieto-Sampedro et al. 1988; Topp et al., 1989; Junier et al. 1993). Further supporting the role of EGF/TGF–α in glial proliferation, EGF-R and TGF–α gene amplifications have been demonstrated in several malignant adult human gliomas (Libermann et al. 1984; Filmus et al., 1985; Yung et al., 1990).

Interestingly, many human gliomas can arise from the subependymal layer (SEL), a specialized zone in the rostrodorsal extent of the striatum (Globus and Kuhlenbeck 1942). In the rodent, the majority of glial cells are born in the SEL and move out of this zone to populate the telencephalon during the first two weeks of postnatal life (Smart 1961; Smart and Leblond 1961; Paterson et al., 1973; Korr 1980; Sturrock and Smart 1980; Levison and Goldman 1993). Cells in the SEL have also been shown to proliferate in the adult animal, and the daughter cells are believed to either migrate away or undergo cell death (Smart and Leblond 1961; Dalton et al., 1969; Korr et al., 1973; Morshead and van der Kooy 1992). In reaggregate cultures of the adult striatum, those cells which proliferate in response to EGF have been proposed to be derived from the SEL (Reynolds et al. 1992; Reynolds and Weiss 1992). Upon replating, these cells are able to differentiate into both neurons and glia; this finding shows the multipotency of these cells and identifies them as a population of dividing progenitors. The molecular signals regulating the proliferation of these cells *in vivo* remains to be determined.

In order to investigate the role of TGF–α in the developing striatum and adjacent SEL, we characterized the normal postnatal developmental profile of TGF–α mRNA in this region using a quantitative nuclease protection assay. One way a cell can regulate how much of a given protein is made is by changing steadystate mRNA levels, and thus, differences in mRNA levels across time reflect differences in the synthetic potential for that protein. We hypothesized that TGF–α may be a signal which is able to induce the division of astroblasts, so we also measured the levels of glial fibrillary acidic protein (GFAP) mRNA. The GFAP protein is restricted to astrocytes and is often used to monitor the development and maturation of astrocytes *in situ* and *in vitro* (Bignami et al., 1972; Trimmer et al., 1982; Morrison et al., 1985).

We use bromodeoxyuridine labeling/immunohistochemistry in conjunction with EGF-R immunohistochemistry, to provide *in vivo* evidence that dividing cells in the SEL have the potential to respond to TGF–α. The *waved-1* mouse has recently been recognized to have profound reductions in TGF–α mRNA and protein (Luetteke et al. 1993; Mann et al. 1993). We address whether the cells in the *waved-1* SEL are able to proliferate at a normal rate. We examine GFAP mRNA levels and GFAP immunostaining in the *waved-1* mutant to determine if there are fewer differentiated glial cells, as would be predicted if the TGF–α protein stimulates the proliferation of glial progenitors.

Materials and Methods

Tissue Collection for protection assay and proliferation assay

Adult C57Bl6, CBA, and *waved-1* mice were obtained from The Jackson Laboratory and maintained in the animal care facility of The Mount Sinai School of Medicine and cared for within institutional guidelines. Inbred pairs as well as mixed C57Bl6 female x CBA male crosses were monitored at least three times a week for births. F1 litters were collected at fourteen postnatal time points ranging from postnatal day 2 (P2) to P143. Animals were sacrificed and tissue was collected as previously described (Lazar and Blum 1992). For animals younger than P10, the brain dissection was done using iris spatulas with the aid of a dissecting microscope. Immediately after removal, the right and left striata were snap-frozen on dry ice and stored at -80°C in individual microfuge tubes. Acetylcholinesterase staining was used to verify that the dissected tissue was primarily the caudate-putamen. From the acetylcholinesterase staining it was determined that the dissection contained the adjacent lateral pallidum with little cortical contamination.

Cytoplasmic RNA isolation

Striatal tissue from five male and five female mice was randomly chosen from a total of approximately 40 animals collected at each age. After storage, the tissue to be assayed was removed from the freezer and the right and left striatum were weighed together as a pair (Table 2.1). The RNA isolation procedure was carried out as previously described (Jakubowski and Roberts 1992; Lazar and Blum 1992). A spectrophotometer was used to quantitate the µg of RNA in samples

(Table 2.1), and agarose electrophoresis was used to check the quality of the RNA in every sample. Appropriate amounts of RNA, 7 μg for the TGF–α mRNA protection assay and 2 μg for the GFAP mRNA protection assay were aliquoted, dried and stored in 30 μl hybridization buffer as previously described (Lazar and Blum 1992).

Preparation of Probes

The mouse TGF–α clone used for these studies was provided by Dr. R. Derynck (Wilcox and Derynck 1988) and subcloned as described (Lazar and Blum 1992). The mouse GFAP clone (Lewis et al., 1984), which was kindly provided by Dr. N. Cowan, was subcloned into the HindIII/PstI site of Bluescript II/KS vector. The 299 bp clone corresponds to bases 951-1250 of the GFAP coding domain. The clones were subsequently linearized by restriction digestion either 5' or 3' to the insert and used as DNA templates for *in vitro* transcription to generate sense and antisense RNA., as recommended by the manufacturer (Promega). The ^{32}P UTP labeled riboprobes (1-2 x 10^9 cpm/μg) were then purified by ethanol precipitation.

Ribonuclease Protection Assay

The solution hybridization RNase protection assay has been described in detail previously (Lazar and Blum 1992). Samples used to generate the standard curves contained different amounts of *in vitro* synthesized sense RNA ranging from 4 to 400 amoles for TGF–α and from 2 amoles to 60 amoles for GFAP. The tubes were prepared with increasing amounts of sense RNA (the standards), and brought up to 7 μg or 2 μg with yeast total RNA for the TGF–α and GFAP assays, respectively. The standards were

increased to a volume of 30 µl with hybridization buffer and stored at -70°C. The addition of radioactive probe, hybridization procedures, electrophoresis, and band quantitation were carried out as previously described (Lazar and Blum 1992). The amoles in each sample was predicted using linear regression.

Tissue Collection for Histology

C57Bl6 x CBA (F1) mice born in our colonies were scheduled for injections with 5-bromo-2' deoxyuridine-5' monophosphate (BrdU) on postnatal day 6. A 1 cc tuberculin syringe was used to subcutaneously inject the BrdU (50 µg/g body weight) or saline. Injected animals (as well as non-injected adults for EGF-R immunohistochemistry) were killed by decapitation; the brains were removed and cut coronally into four slabs approximately 2 mm to 3 mm thick. The tissue was positioned in a plastic cryomold filled with OCT embedding medium (Miles Inc.), frozen on dry ice, and stored frozen. A cryostat was used to cut the frozen blocks into 10 µm thick sections, which were then briefly thaw mounted onto Superfrost Plus slides (Fisher), and stored at -20°C. On the day of processing, the cut sections were fixed by immersion in 100% methanol for 10 minutes in the cold (for EGF-R and BrdU) or by immersion in 5% Acrolein in 0.1 M phosphate buffered saline (PBS, 0.1 M $NaPO_4$, pH 7.4) for 10 minutes (for GFAP). After the Acrolein fix, the slides were washed in PBS, 2 x 10 minutes.

Immunohistochemistry

Either following methanol fixation or at the time of thawing, the sections were circled with rubber cement. After the rubber cement hardened, the

sections were rehydrated in PBS for 20 min, changing the PBS after 10 min. Fifty µl of ICC buffer [10% rabbit serum (EGF-R) or 3% goat serum (GFAP), 0.3% Triton X-100 in PBS] was applied to each section, then incubated in a room-temperature humidified chamber for 30 min. After this blocking incubation, the slides were rinsed in PBS, and incubated overnight with 50 µl of primary antibody diluted 1:50 in ICC buffer. The primary anti-EGF-R sheep polyclonal antibody was synthesized against a recombinant protein corresponding to a portion of the cytoplasmic domain of human EGF-R [Upstate Biotechnology Institute (UBI)] . Western immunoblots carried out by UBI show one major immunopositive band, but four lighter bands can be revealed in longer exposures. A no primary anti-sera control, which was carried out for both the primary and secondary antibodies, show no positive immunostaining in the murine forebrain. The primary antibody for GFAP was made in rabbit (Biomeda). To monitor non-specific staining, control sections were incubated overnight with 50 µl of ICC buffer alone. The next day, sections were washed in PBS, and 50 µl of biotinylated anti IgG secondary antibody (Vector Laboratories) diluted 1:100 (anti-sheep) or 1:250 (anti-rabbit) in ICC buffer was applied to each section. After a 2 hour incubation, the slides were washed in PBS.

For the slides that were being processed for double labeling, fifty µl streptavidin-tetramethylrhodamine (Molecular Probes) diluted 1:1000 in ICC buffer was added to each section. After incubation, the sections were washed and post-fixed in 100% methanol at 4°C for 10 min. The following steps were done for the BrdU staining. The slides were incubated in 2N HCl at 37°C for 30 min or 1 hour to denature the DNA. To neutralize the acid, the slides were immersed in 250 ml of 0.1M sodium borate buffer (pH

8.5) for 15 min with three changes of the buffer. The slides were briefly rinsed with PBS and incubated for an hour with fluorescein-conjugated anti-BrdU primary antibody (Boehringer-Mannheim), at a dilution of 1:1 in ICC buffer. During the final washes, the slides were counterstained with the nuclear stain diamidinophenylindole (DAPI) (Sigma) diluted to 2 μg/ml in PBS. The rubber cement was removed and the slides were mounted using Perma-Fluor (Immunocon). The EGF-R and GFAP staining in the adult was carried out with the addition of peroxidase-conjugated ExtrAvidin (diluted 1:200 in PBS:Sigma) after incubation with secondary antibody. This incubation was followed with PBS washes and staining was revealed by reacting with diaminobenzidine (Vector). Staining was visualized and photographed using a Leitz or Zeiss microscope equipped with bright field and fluorescent illumination, appropriate filters and a camera for photomicroscopy.

Proliferation Assay

The frozen striata were weighed and then homogenized in 700 μl ice cold saline. The ^{3}H-thymidine was extracted following the protocol of Hellman and Ullberg (Hellman and Ullberg 1986). Briefly, 700 μl of ice-cold 10% tricholoric acid (TCA) was added to the each homogenate. The tubes were put on ice and placed on a shaking tray for 30 minutes. The samples were centrifuged for 5 minutes at 15,000 x rpm at 4°C. After a 70% EtOH wash, the pellets were dried in a speed vac. The pellet and 1 ml of SOLUENE 350 (Packard) were incubated in a 45°C water bath for three hours in 20 ml glass scintillation vials. The tubes were transferred to the bench top and incubated overnight. Next, 10 ml of HIONIC FLUOR

(Packard) was added to each vial and the total radioactivity in each sample was determined with a scintillation counter.

Results

TGF–α mRNA levels

TGF–α mRNA, expressed in attomoles per μg of the total RNA, changes during postnatal development (Figure 2.1A). Cytoplasmic TGF–α levels were approximately 3 fold higher at the end of the first week of postnatal of life (16.14 ± 1.35 amoles/μg) as compared to the levels after 8 weeks (5.59 ± .26 amoles/μg). Whereas significant differences in TGF–α mRNA concentrations between time points examined were easily detected by factorial ANOVA (F=9.169, p<.0001), we were unable to detect any sex differences across all the time points (F=.448, p=.505), nor was there any interaction effect (F=1.252, p=.253).

GFAP mRNA levels

The attomoles of GFAP mRNA per μg total RNA were found to increase steadily during the first two postnatal weeks of life (Figure 2.1B). The amount of GFAP mRNA/μg RNA at P2 is significantly lower than at all other time points examined (p< .01), with the exception of P6 where the levels are beginning to increase, but have not yet reached adult levels. The levels of GFAP mRNA increased by 3.5 fold from P2 (1.05 ± .16 amoles/μg) to P14 (3.72 ± .24 amoles/μg), and adult levels fall intermediate to these two values (2.51 ± .16 amoles/μg). Although analysis of variance demonstrated a significant difference in GFAP mRNA across postnatal development (F=3.738, p<.0001), we did not detect any significant differences between males and females overall or at any particular time point (F=2.228, p=.138; F=1.045, p=.415 respectively).

EGF-R and BrdU Immunohistochemistry -P6

After immunohistochemistry and counter staining of coronal mouse brain sections at P6, the SEL was easily identified with the DAPI nuclear counter stain by its characteristic shape, high cell density and juxtaposition to the lateral ventricle. As shown in figure 2.2B, many of the cells in the SEL had incorporated BrdU during the three hour pulse and thus were identified imunocytochemically as dividing cells. There were many cells in this zone that were positive for EGF-R immunoreactivity (Figure 2.2A). There were also a few cells outside this zone, especially in the developing corpus callosum, that were labeled by the EGF-R antibody. Many cells that were EGF-R positive were also double labeled with BrdU antibody. The EGF-R staining had a ring-like appearance characteristic of the staining of membrane–associated epitopes. Figure 2.3 shows a high power photomicrograph of cells along the callosal border of the SEL. Figure 2.3B is a phase-contrast photomicrograph of the border between the corpus callosum and SEL, which can be identified as a zone of dense cells running diagonally across the bottom of the photomicrograph. All of the BrdU positive nuclei (Figure 2.3A) are also EGF-R positive (Figure 2.3C), although the intensity of the EGF-R staining varies from one cell to the next.

EGF-R Immunohistochemistry –adult

In the adult mouse, the SEL of the forebrain is also strongly EGF-R immunoreactive. Although at maturity this zone has thinned out substantially, the staining appears to span the entire striatal border of the lateral ventricle extending laterally and ventrally (Figure 2.4A). In the adult, the intense staining of cells along the striatal border of the lateral

ventricle stands in marked contrast to cells along the septal border, which are only occasionally immunoreactive. There appears to be intense clusters of EGF-R immunoreactivity at the border between the dorsal striatum and corpus callosum, as shown in higher power in figure 2.4B. The staining intensity in this zone varies greatly from one cell to the next, but all staining appears to have a ring-like quality. In addition, some EGF-R staining is evident on cell bodies in the corpus callosum (especially near the SEL) and in the striatal parenchyma.

The waved-1 mutant mouse mRNA -developing

Using a nuclease protection assay, we compared the striatal levels of TGF–α and GFAP mRNA in normal Bl6 x CBA F1 mice and inbred *waved-1* mutant mice. We found striatal TGF–α mRNA levels to be significantly reduced (50%) in the *waved-1* mouse at P6 (Figure 2.5A; $t=7.496$, $p<.001$). The concentration of GFAP mRNA at P14 was also significantly reduced by 50% in the striatum of the *waved-1* mouse when compared to the normal strain (Figure 2.5B; $t=5.236$, $p<.01$).

The waved-1 mutant mouse mRNA –adult

Using a nuclease protection assay, we compared the adult (5 months of age) striatal levels of TGF–α and GFAP mRNA in normal Bl6 x CBA F1 mice and inbred *waved-1* mutant mice. The mutant animals showed a significant decrease in TGF–α mRNA steady-state level in adulthood, where mutant levels were only 10% of the normal strain (Figure 2.6A; $t=5.059$, $p< .01$). The levels of striatal GFAP mRNA in the adult mutant mouse were significantly reduced by approximately 50% (Figure 2.6B; $t=3.49$, $p<.01$).

The waved-1 mutant mouse -^{3}H-thymidine at P6

A quantitative determination of total ^{3}H-thymidine incorporation into the whole striatum of *waved-1* and normal P6 mice was made (N=5 in each group). The TCA perceptible radioactivity in counts per minute expressed as a ratio of tissue weight was graphed for *waved-1* and normal mice (Figure 2.7). Using a directional hypothesis and a non-paired t-test, we found that the *waved-1* mice incorporated significantly less ^{3}H-thymidine into striatal DNA than did the normal Bl6CBA mice ($t=-5.128$, $p<.001$).

The waved-1 mutant mouse-BrdU staining

In coronal sections, the SEL was identified by DAPI staining as a dense cellular zone lying next to the lateral ventricle. Although the SEL can be identified in both the normal and *waved-1* mouse, the thickness of this zone along the ventro-medial striatum is reduced in the mutant animal (see zone between arrows Figure 2.8A & Figure 2.8B). The normal P6 forebrain shows many BrdU labeled cells (Figure 2.8C) in the SEL, whereas in the SEL of the *waved-1* mouse fewer nuclei are BrdU positive (Figure 2.8D).

The waved-1 mutant mouse- GFAP immunostaining

The photomicrographs in Figure 2.9A and 2.9B show GFAP staining in 14 μm coronal sections of an adult normal mouse and an adult *waved-1* mouse neostriatum, respectively. Fasiculated bundles of the internal capsule can be seen in the upper left hand corner of both sections showing that they are derived from a similar rostro-caudal and dorso-ventral level.

There are more GFAP positive profiles detected in the normal striatum than in the mutant striatum.

Discussion

We have shown that steady state TGF–α mRNA levels are developmentally regulated in the postnatal murine striatum, reaching a maximum at the end of the first week of postnatal life (Figure 2.1A). Therefore, TGF–α is likely to play an important role in events occurring in the striatum at that time. One major occurrence during the first week of life is the division of glial precursors, which later differentiate into both astrocytes and oligodendrocytes (Smart 1961; Smart and Leblond 1961; Paterson et al. 1973; Korr 1980; Sturrock and Smart 1980; Levison and Goldman 1993). Levels of the mRNA encoding GFAP, a cytoskeletal protein considered to be a marker of differentiated astrocytes, peak one week following the peak of TGF–α expression (Figure 2.1B). There is a substantial lag between the height of glial cell proliferation (and TGF–α expression) and the height of glial maturation, as represented by GFAP mRNA levels, and the highest TGF–α mRNA levels correlate with the division rather than the differentiation of these cells. In order to further establish whether TGF–α could be acting as a mitogen for these glial progenitors, we went on to determine whether proliferating cells in the early postnatal SEL have EGF-R-like immunoreactivity.

We report that there is intense EGF-R immunostaining on proliferating cells in the SEL at postnatal day 6, as evidenced by double-label immunocytochemistry for EGF-R and BrdU (Figure 2.2 & 2.3). Although many of the EGF-R positive cells were not BrdU-positive, they still may have been in an active cell cycle but at a phase other than S during the three hour BrdU pulse. It appears, however, that most of the BrdU-positive cells in the SEL at postnatal day 6 were EGF-R-positive

(Figure 2.3), implying that the TGF–α/EGF-R pathway could be important in regulating the proliferation of this cell population. In contrast, the previously observed timing and localization of EGF-R immunoreactivity led authors to conclude that the EGF-R signaling pathway was not involved in mitogenic events in the developing brain (Gomez-Pinilla et al. 1988; Tucker et al., 1993). This apparent discrepancy could be due to differences in antibody specificity and/or fixation conditions used. Our findings agree with autoradiographic binding studies that indicate that EGF-R binding is prominent in neonatal forebrain (Adamson and Meek 1984; Quirion et al. 1988).

Division of precursor cells continues throughout postnatal life in the striatum (Smart 1961; Smart and Leblond 1961; Dalton et al. 1969; Korr et al. 1973; Kraus-Ruppert et al., 1973). When the adult striatum is cultured and maintained with EGF, a small fraction of cells survive and proliferate (Reynolds et al. 1992; Reynolds and Weiss 1992). Our results support the suggestion, as proposed by Reynolds and Weiss, that the EGF-responsive cells are derived from the adult SEL, because we have shown that this dividing cell zone which lies along the striatal border of the lateral ventricle is EGF-R immunoreactive in adult life (Figure 2.4). The SEL cells have the EGF-R protein on their cell surface and could be responding to either TGF–α or EGF, but we hypothesize that the *in vivo* mitogenic signal is actually TGF–α. Previous findings from our lab have shown that striatal TGF–α mRNA is much more abundant than EGF at 2 months of age (Lazar and Blum 1992), and our present results confirm that high levels of striatal TGF–α mRNA are sustained until at least 5 months of age (Figure 2.1A). Another group has localized TGF–α immunoreactive product in the striatum, and they reported intense

staining along the dorso-lateral border of the rodent striatum, and the staining appears especially strong along the lateral ventricle (Fallon et al. 1990). We speculate that one reason TGF–α gene expression is maintained at a high level in the adult striatal tissue (which includes the SEL) is that striatal TGF–α provides a mitogenic signal for cells in the SEL.

The availability of the *waved-1* mouse which expresses TGF–α at a dramatically reduced level allowed us to further test the concept that TGF–α stimulates proliferation of precursor cells. In contrast to the 90% reduction of TGF–α mRNA levels found in the adult mutant brain (Luetteke et al. 1993; Mann et al. 1993), we found only a 50% reduction in TGF–α mRNA expression in the developing striatum at postnatal day 6 (Figure 2.5A). Also at postnatal day 6, we showed that the mutant striatum has a lower proliferation index, as evidenced by significantly reduced ^{3}H-thymidine incorporation (Figure 2.6) and by the detection of fewer BrdU positive nuclei (Figure 2.7). Since it has been shown that these proliferating cells can differentiate into astrocytes, we expected to see a decrease in the GFAP mRNA in the mutant striatum at postnatal day 14, and indeed, there was a 50% reduction in the mRNA for this astrocytic cell marker when compared to the normal strain (Figure 2.5B). In the adult striatum, the mutant GFAP mRNA levels were also reduced by 50% (Figure 2.8B) and fewer GFAP immunoreactive astrocytes were detected in the neostriatum (Figure 2.9). We found that the adult levels of striatal TGF–α mRNA are reduced by 90% (Figure 2.8A) as was reported for the adult whole brain (Luetteke et al. 1993). Taking all of our findings together, we conclude that fewer glial cells are produced during gliogenesis in the mutant mouse, and that this is reflected by decreased

GFAP mRNA levels and decreased GFAP positive astrocytes in the *waved-1* striatum.

It is probable that TGF–α is not the only molecular signal regulating postnatal gliogenesis in the SEL. Many other polypeptide growth factors, including EGF, bFGF (basic fibroblast growth factor) and PDGF (platelet derived growth factor) have been shown to signal glial cells to divide in culture (Simpson et al. 1982; Kniss and Burry 1988; Richardson et al., 1988; Engele and Bohn 1991). One might speculate that if TGF–α mRNA is decreased, another functionally similar peptide could be upregulated to compensate for the lower levels of TGF–α. However, mRNA encoding EGF, a growth factor that is a glial mitogen structurally related to TGF–α and one that binds to the same receptor as TGF–α, was not found to increase in the whole brain of the adult *waved-1* mouse (Luetteke et al. 1993). We investigated whether there was an induction of EGF mRNA specifically in the striatum, but were unable to detect a change in striatal EGF mRNA levels (data not shown). The possibility that another mitogenic growth factor is induced in the mutant striatum still remains to be examined.

Our results show that at least two phases of TGF–α mRNA expression levels are distinguishable in the *waved-1* mutant mouse; there is a differential effect of the genetic mutation in young versus the adult striatum (Figure 5A & 6A). Interestingly, the cell type expressing TGF–α mRNA undergoes a developmental switch in the rodent striatum (Seroogy et al. 1993). At the time of gliogenesis, neurons are producing TGF–α. Later, in the mature striatum, TGF–α gene expression declines in neurons and becomes prominent in glial cells (Seroogy et al. 1993). Therefore, as the striatum matures, glial cells contribute more to the total TGF–α

mRNA pool. Since the *waved-1* mouse appears to have fewer astrocytes, there would be a decrease in the glial contribution to the total amount of TGF–α mRNA, and this could explain the greater reduction (90%) in the level of growth factor mRNA in the adult mutant animals as compared to the neonate (50%). Alternatively, one could propose that different cis- or trans–acting factors are used in the young brain as compared to the mature brain and that some later-used element is defective in the *waved-1* mouse. Future studies in our laboratory are aimed at understanding factors that regulate the postnatal gene expression patterns of TGF–α.

Novel receptors in the EGF-R family and cross-binding ligands are rapidly being reported (Lai and Lemke 1991; Orr-Urtreger et al., 1993); therefore, it becomes imperative to create a functional *in vivo* link between a particular ligand and a particular receptor. Both the TGF–α ligand and EGF-R have recently been shown to be critical for the development of normal hair follicles in the skin epidermis, and the authors conclude that this complimentary pair of proteins is a physiologically relevant one (Luetteke et al., 1994). In our system of study, the developing mouse striatum, we show : 1) that TGF–α mRNA is abundantly expressed, 2) that TGF–α mRNA levels increase at the peak of gliogenesis, 3) that dividing cells are equipped with the EGF-R, and 4) that in a mutant striatum deficient in TGF–α mRNA, glial production is reduced. Taken together, our data support the conclusion that TGF–α is the physiological ligand for the EGF-R in the striatum.

TGF–α /EGF may exert different effects on different populations of cells through different modes of action. Whereas, the mitogenic function of TGF–α in the SEL could be exerted through paracrine or autocrine mechanisms; the TGF–α synthesized in the developing striatum could

serve as a neurotrophic factor in the classical sense, affecting dopaminergic or cortical afferents in a retrograde manner. TGF–α /EGF has been shown to increase the survival of cortical and midbrain neurons *in vitro* (Knusel et al. 1990; Kornblum et al. 1990; Casper et al. 1991; Ferrari et al. 1991; Alexi and Hefti 1993), and EGF-R immunoreactivity has been demonstrated on neurons in the cortex (Gomez-Pinilla et al. 1988; Werner et al., 1988). Even though the presence of the EGF-R has not been demonstrated on dopaminergic neurons, the trophic effect of EGF/TGF–α could be mediated by another cell (Knusel et al. 1990; Casper et al. 1991; Alexi and Hefti 1993). Additionally, TGF–α could be affecting differentiated neurons or glia within the striatum. In support of this notion, we find inside the boundaries of the rodent caudate-putamen EGF-R positive cells (Figure 2.2 & 2.3), which preliminary observations suggest may be oligodendroglia. There is in fact previous evidence that EGF-R exists on oligodendroglia cultured from rat brain (Simpson et al. 1982). We also noticed that the cross sectional area of the myelinated cortico-spinal fiber bundles which pass through the rodent caudate-putamen appears to be smaller in the *waved-1* striatum as compared to the normal striatum (Figure 2.9). Since oligodendroglia are known to be generated in the SEL during the first two weeks of postnatal life in the rodent (Levison and Goldman 1993), it is possible that the generation and/or maturation of oligodendroglia is also adversely affected by the reduction in TGF–α in the *waved-1* mouse. It is likely that TGF–α has more than one function in the basal ganglia, and that these functions can vary throughout development.

Our findings identify TGF–α as a factor which regulates not only the division of glial precursors, but also the division of neuronal precursors. A

population of EGF responsive neuronal and glia progenitors have been proposed to exist in the adult mouse striatum (Reynolds and Weiss 1992). We find EGF-R positive cells residing primarily in the SEL of the adult mouse forebrain and thus, we have localized the likely endogenous source of the striatally derived EGF responsive stem cells. These progenitor cells in the SEL have been shown to differentiate into both neurons and astrocytes *in vivo* (Altman 1969, Alvarez-Buylla, et. al. 1990). If cells of the primate SEL (Lewis 1968; Mc Dermott and Lantos 1990) are also found to be EGF-R immunoreactive, it may be possible to exogenously stimulate primate striatal cells to divide and differentiate into new neurons. The possibility of generating replacement neurons could have profound clinical implications for patients with neurodegenerative disease.

Acknowledgments

Thank-you to Dr. Diana Casper and Suzie Snyder for intellectual input, to Dr. Patrick Hof, Dr. Victor Friedrich and Bob Wooley for photographical assistance, and to Jamie Rodriguez and Nancy Goodman for technical support. This work was supported by an Irma T. Hirschl Career Scientist Award and NIH grant AG08538 (M.B.).

Figures

Figure 2.1. Panel A and Panel B show the normal temporal profile for striatal TGF–α mRNA and GFAP mRNA respectively. The mRNA detected is expressed in am/μg total striatal RNA. The points represent the means ± s.e.m. for N=10 mice.

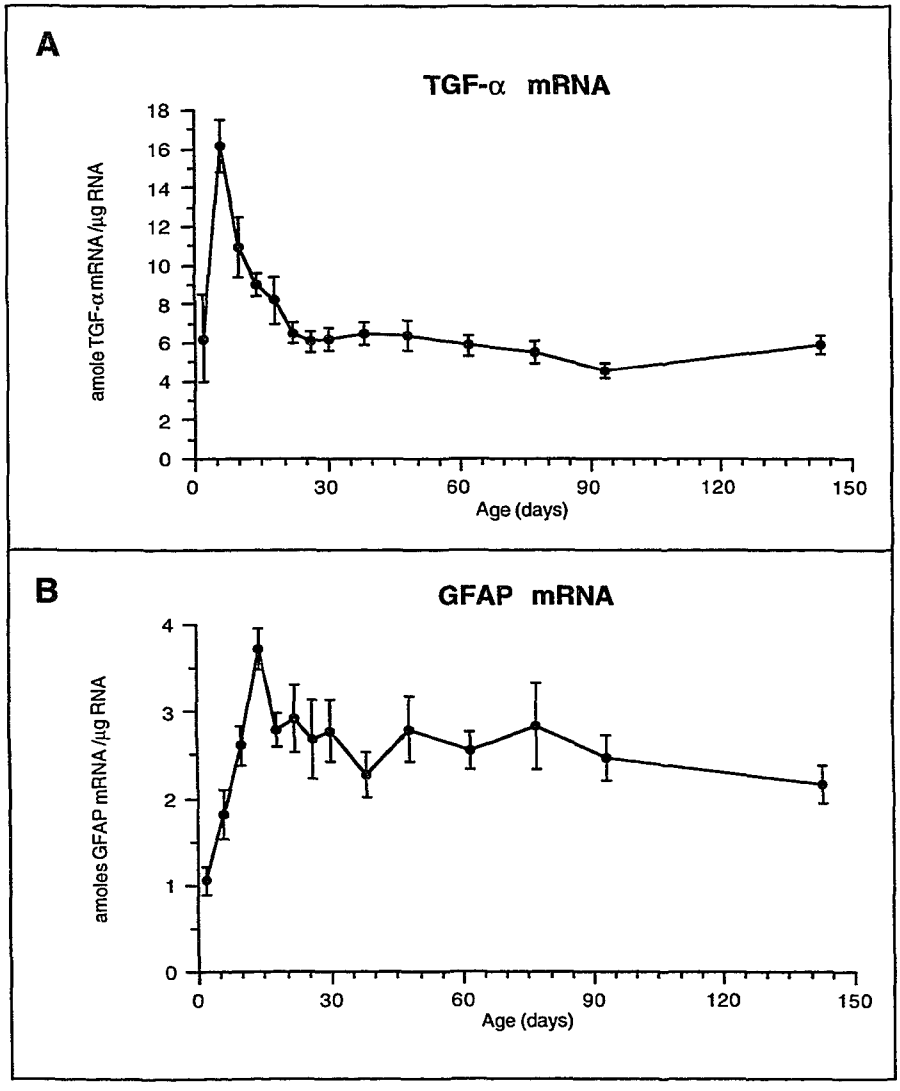

Figure 2.2. The boxed area in the sketch (top left hand corner in panel A) demarcates the area of the forebrain from which the photomicrographs were both taken. Panel A shows EGF-R immunoreactivety in P6 coronal section and panel B shows BrdU positive cells in the same field. The scale bar represents 10 μm.

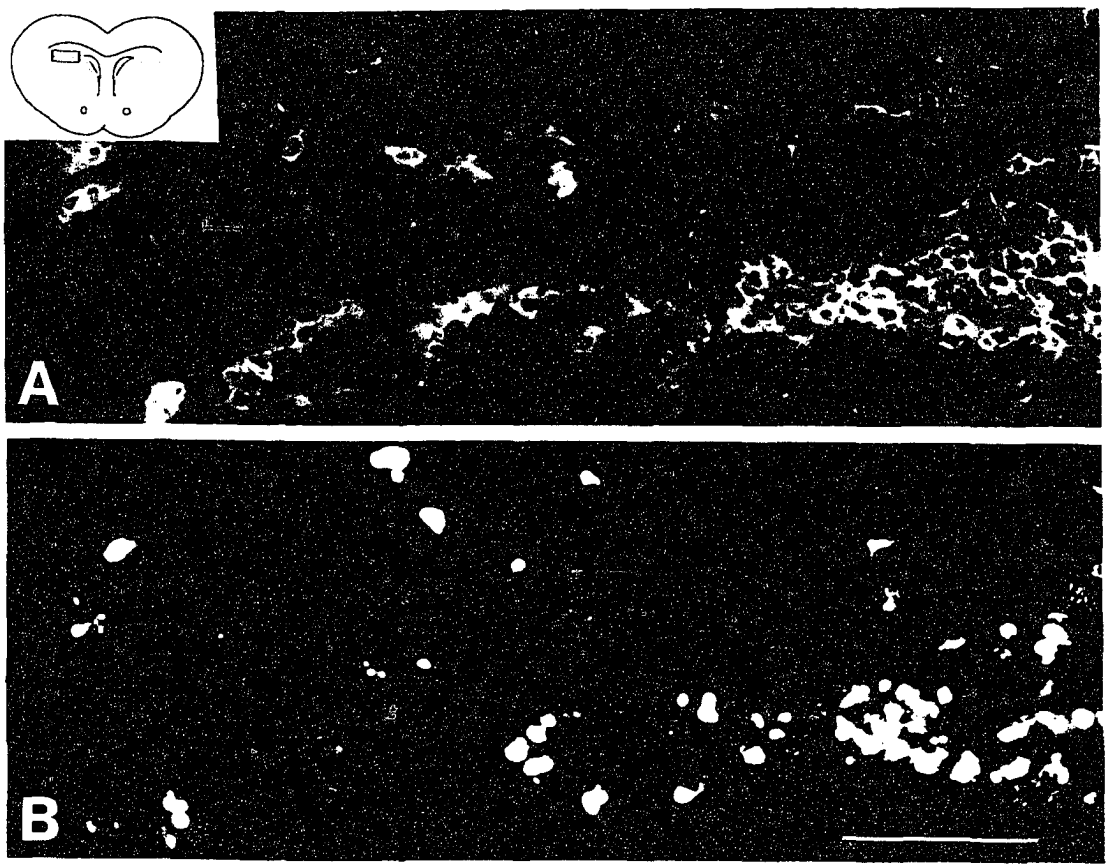

Figure 2.3. Panel A , B and C are all photomicrographs of the same field, where A shows BrdU immuno-positive nuclei, panel B shows all the cells in the field by phase contrast microscopy, and panel C shows EGF-R immuno-positive cells. The arrow in panel A, B and C points to one cell lying just outside the dorsal boundary of the SEL which is both BrdU positive and EGF-R positive. The scale bar represents 2 μm.

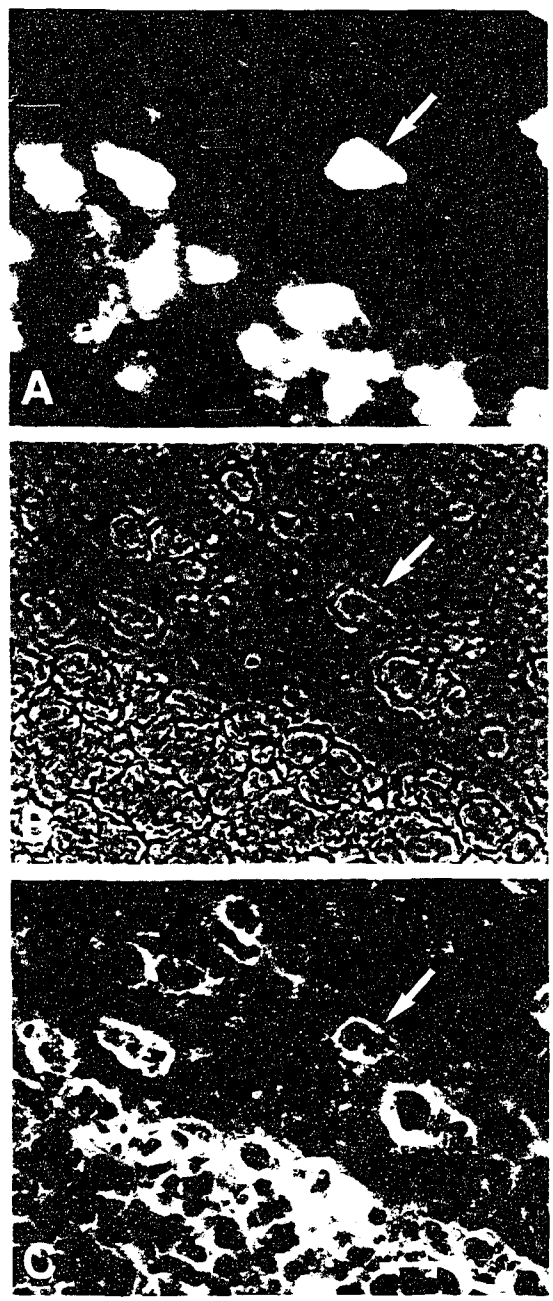

Figure 2.4. EGF-R immunoreactivety in a coronal section of the adult mouse forebrain is shown in the photomicrograph in panel A and B. Panel A shows a field encompassing the striatum (str), corpus callosum, (cc) and all margins of the lateral ventricle (lv); panel B is higher magnification of the boxed area in panel A. This highlights the EGF- R staining in the border between the striatum and corpus callosum. Panel C shows background staining in the non- primary control section. The scale bar equals 10 μm in panel A and 2 μm in panel B and 5 μm in panel C.

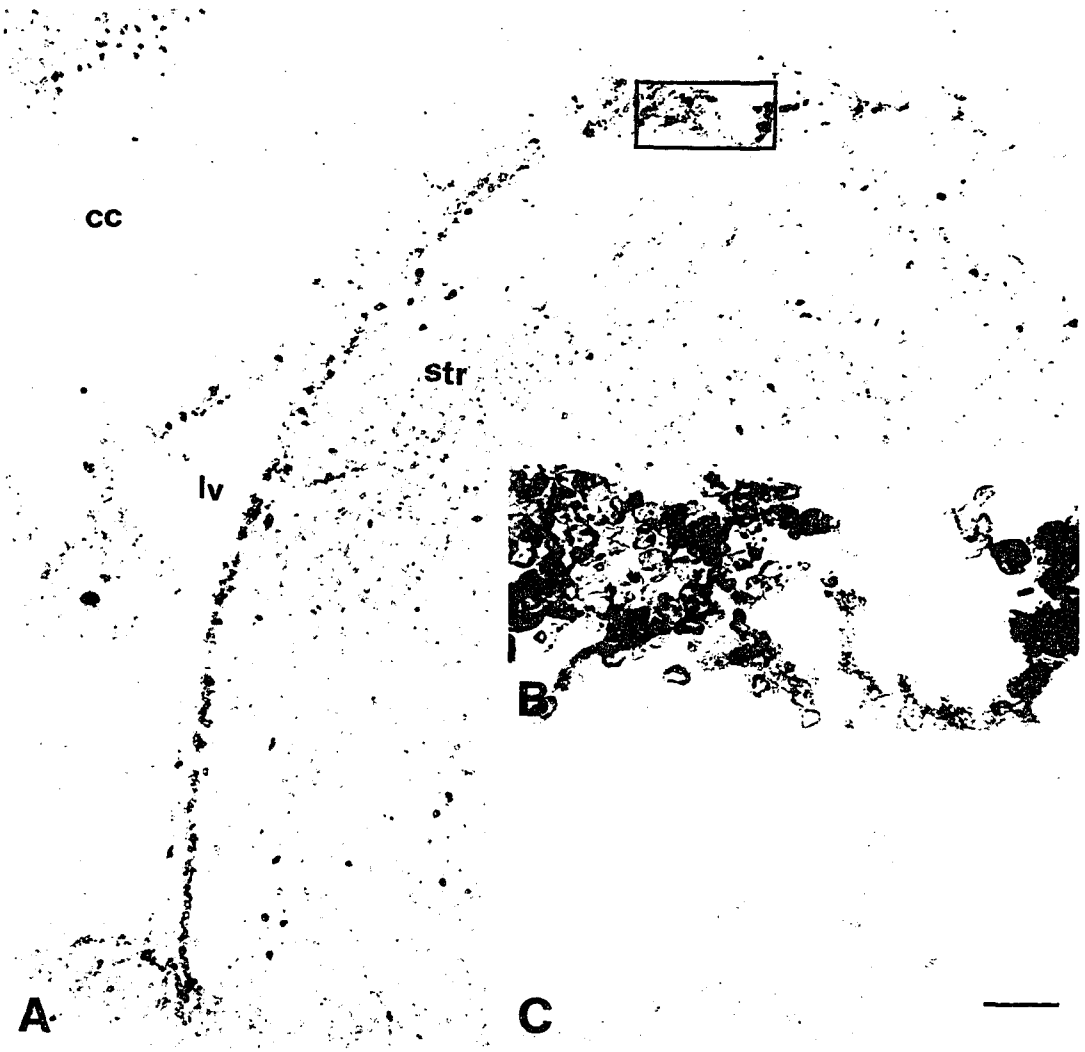

Figure 2.5. The TGF–α mRNA in the developing normal and *wa-1* brain is expressed in am/μg total RNA at postnatal day 6 (N=5). The GFAP in normal and *wa-1* is expressed as before, but RNA was extracted from tissue collected at postnatal day 14 (panel B, N=5).

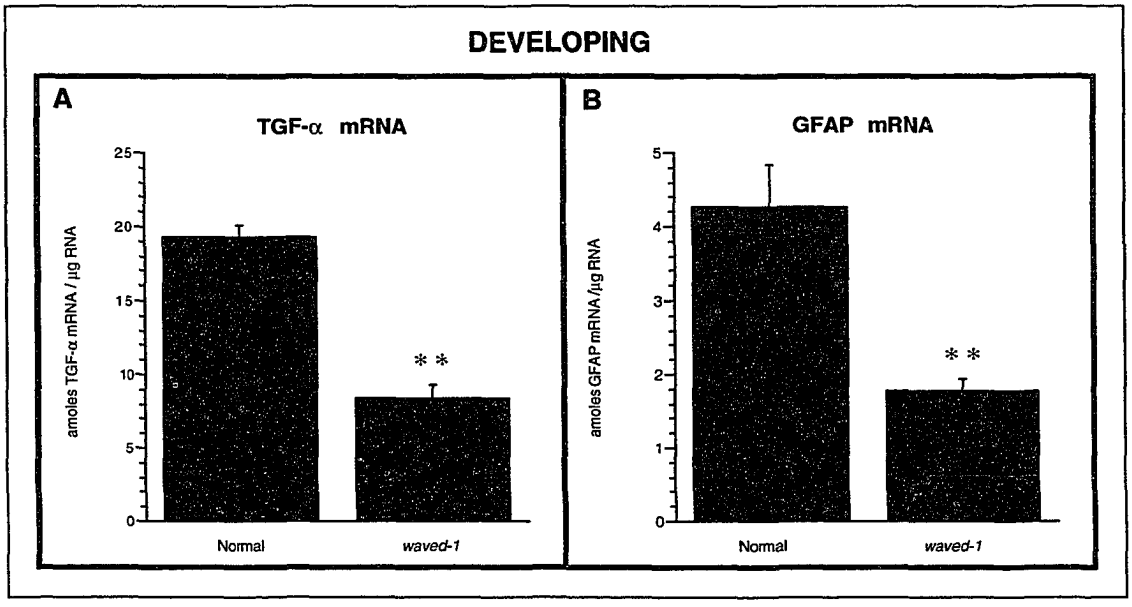

Figure 2.6. In panel A the means ± s.e.m. for striatal TGF–α mRNA expressed in am/μg total RNA are plotted for the adult normal mouse and *wa-1* mouse (N=5 and N=3 respectively). In panel B, a similar plot for GFAP mRNA is shown (N=5 for normal, and N=5 for *wa-1)*.

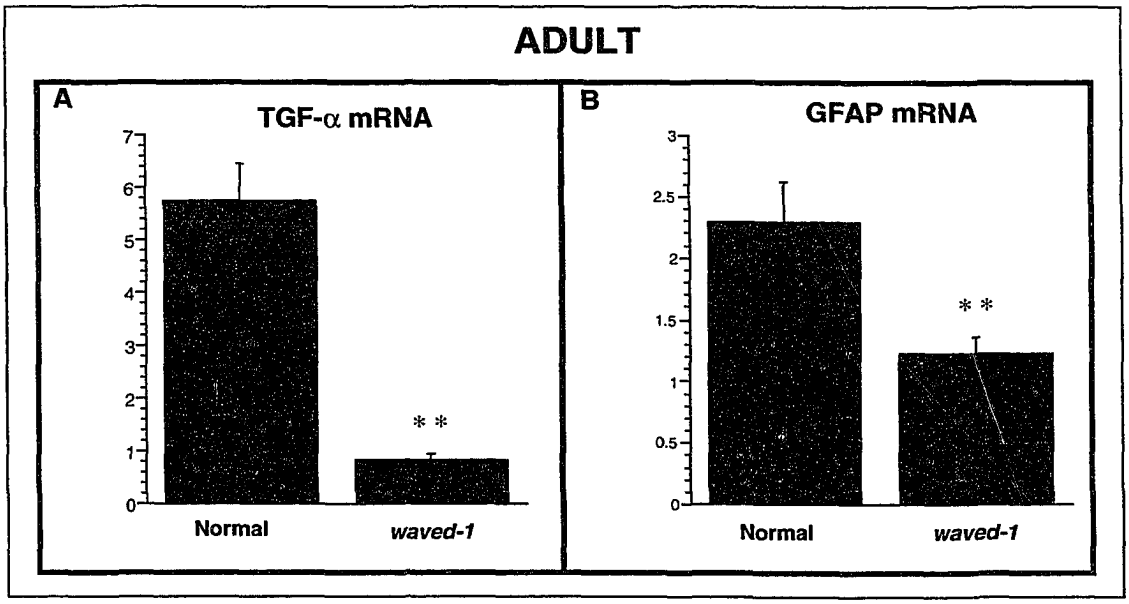

Figure 2.7. The amount of ^{3}H-thymidine incorporated into the normal and *wa-1* striatum at postnatal day six is expressed as a ratio of frozen tissue weight (N=5). The error bars represent ± s.e.m.

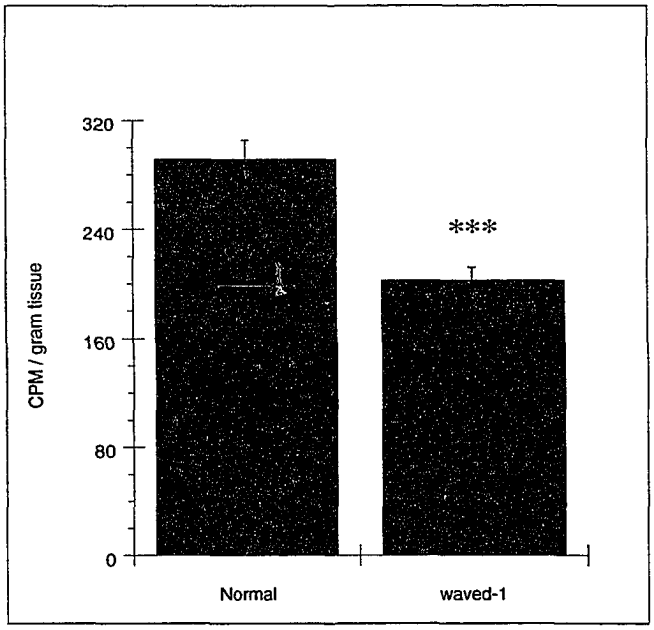

Figure 2.8. These photomicrographs are of 10 μm coronal sections of normal or *wa-1* forebrain. Panel A and C are from the normal mouse forebrain, whereas panel B and D are from the *wa-1* forebrain. Panel A and B show all the nuclei in the section stained with DAPI and panel C and D show the identical fields with the subset of dividing nuclei that labeled with BrdU. The scale bar equal 15 μm.

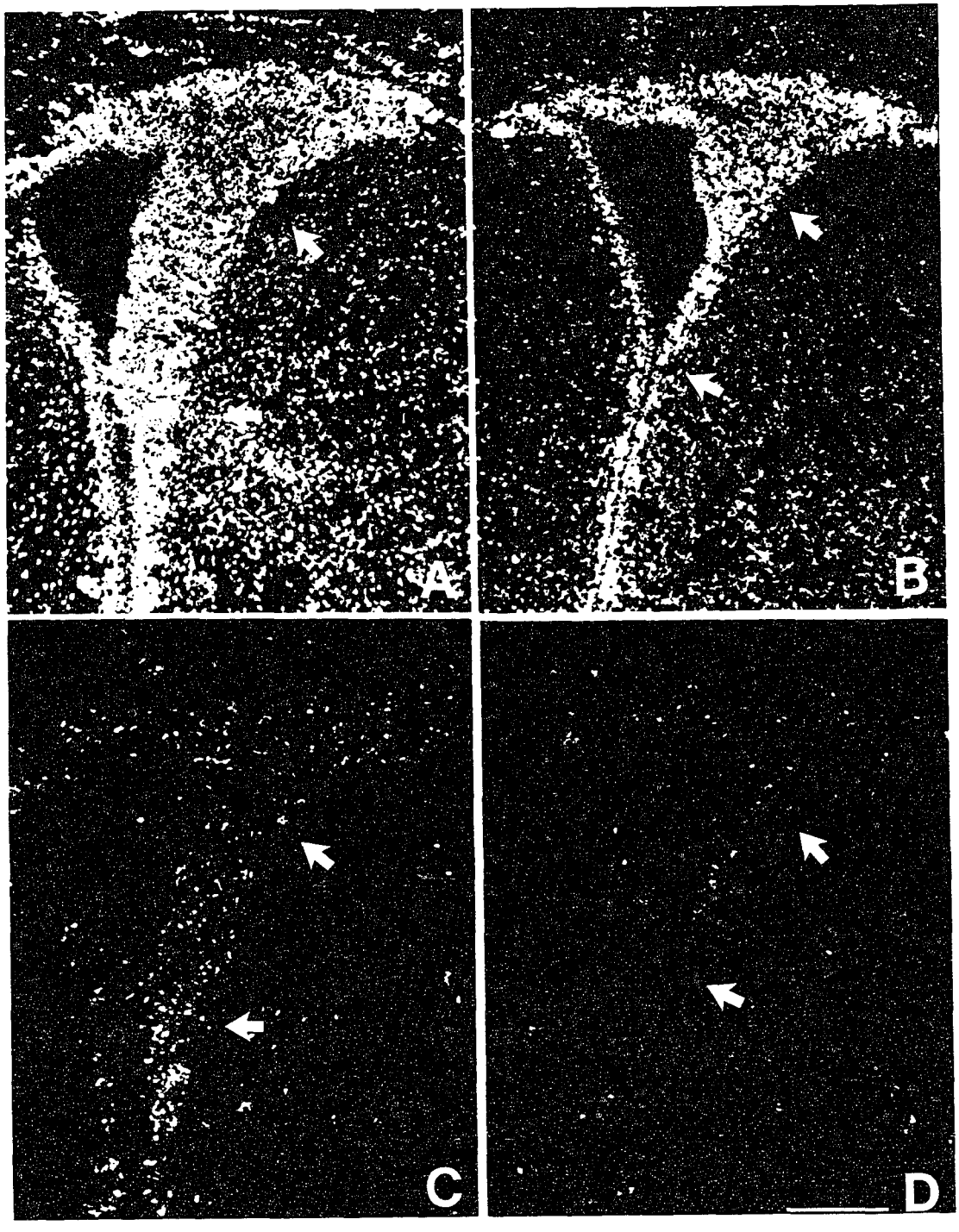

Figure 2.9. Panel A and B are photomicrographs taken from a 14 μm coronal section through the adult murine striatum. Panel A shows GFAP immunostaining in the normal mouse, while panel B shows GFAP immunostaining in the *wa-1* mouse. The scale bar equals 50 μm.

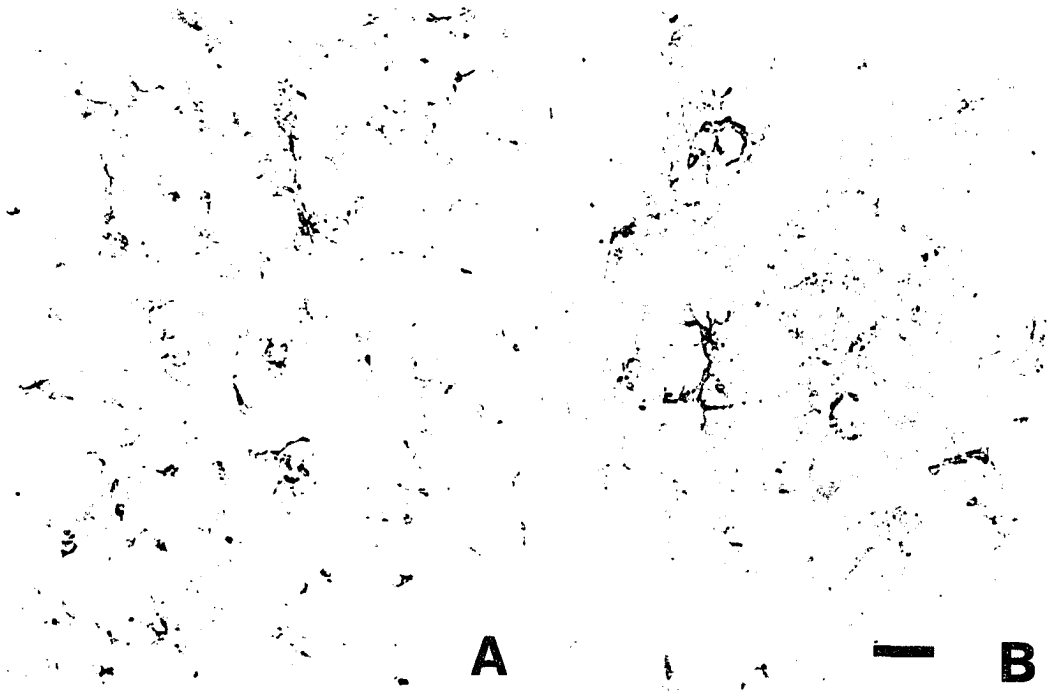

Table 2.1. The mean and s.e.m. for striatal weight in milligrams and total RNA isolated in micrograms are given for each of the postnatal time points under investigation (N≥12).

	Tissue Parameters	
Age (days)	Striatal Weight (mg)	Striatal Total RNA (μg)
2	6.34 (.69)	12.66 (1.30)
6	10.68 (.80)	18.86 (1.27)
10	12.68 (.68)	23.79 (1.74)
14	15.97 (1.02)	28.74 (2.24)
18	17.58 (1.15)	22.44 (1.73)
22	17.34 (.98)	22.34 (1.19)
26	19.08 (1.39)	22.84 (1.62)
30	21.03 (1.85)	23.52 (2.42)
38	15.76 (.93)	15.26 (1.02)
48	23.43 (.95)	19.96 (1.13)
62	21.95 (.94)	18.75 (1.10)
77	25.77 (1.63)	19.39 (2.26)
93	27.40 (1.83)	19.20 (1.63)
143	23.25 (1.05)	16.43 (1.36)

Values represent the mean ± (SEM) for N≥12

CHAPTER 3

Reduced Serum Thyroxine Levels in *Weaver* Mutant Mice, More than Just Another Phenotype

INTRODUCTION

To date, the genetic mutation which produces the mutant mouse *weaver* has not been identified. The *weaver* gene locus resides on murine chromosome 16 and follows an autosomal recessive pattern of inheritance (Lane et al. 1977; Mjaatvedt et al., 1993). By the end of the second postnatal week of life, the *weaver* mouse exhibits a constant tremor when at rest and walks with an uncoordinated gait (Sidman et al. 1965). Beginning around postnatal day 6 (P6), the cerebellum is visibly smaller upon gross examination; this led to the initial identification of the cerebellar granule cell as a site of neuropathology (Sidman et al. 1965). Subsequently, midbrain dopamine neurons and cerebellar Purkinje neurons in the *weaver* mutant mouse brain have also been shown to undergo abnormal cell death postnatally (Schmidt et al., 1982; Roffler-Tarlov and Graybiel 1987; Triarhou et al. 1988; Smeyne and Goldowitz 1990). Not only are *weaver* mutants distinguished from normal mice behaviorally and neuropathologically, but also, *weaver* mutant mice are 50% smaller than their normal litter mates (Sidman et al. 1965; Coscia and Fentress 1993). Recently, abnormal sperm cell development and degeneration was found to account for sterility in homozygous *weaver* males (Vogelweid et al., 1993; Harrison and Roffler-Tarlov 1994). Thus, the *weaver* mutation has deleterious effects on multiple systems of the mouse.

In the brain of *weaver* mice, granule neurons in the cerebellum and dopamine neurons in the midbrain, first, begin to differentiate, but then they fail to complete the last events of differentiation postnatally and subsequently undergo degeneration (Schmidt et al. 1982; Willinger and

Margolis 1985; Roffler-Tarlov and Graybiel 1987; Triarhou et al. 1988). The *weaver* granule neurons divide normally, exit the cell cycle and begin neurite outgrowth, but they fail to elongate processes, fail to express the molecular markers of granule neuron differentiation, TAG-1 and astrotactin, and fail to migrate out of the external granule cell layer (EGL) (Rezai and Yoon 1972; Rakic and Sidman 1973; Willinger and Margolis 1985; Gao et al., 1992). Additionally, the Purkinje neurons in the mutant cerebellum are found in ectopic positions and have reduced branching of their dendritic arbors (Hirano and Dembitzer 1973; Bradley and Berry 1978; Smeyne and Goldowitz 1990). The *weaver* dopamine neurons send axonal projections to innervate the neonatal striatum in a near normal pattern; but the terminals do not experience the normal outgrowth seen during the third and forth postnatal week, and the dopamine concentrations in the dorsal striatum fail to undergo the rapid increase seen after P14 (Schmidt et al. 1982; Roffler-Tarlov and Graybiel 1987). During the first few weeks of postnatal life, the vast majority of the granule neurons and about half of the *weaver* dopamine neurons in the pars compacta degenerate (Rezai and Yoon 1972; Rakic and Sidman 1973; Triarhou et al. 1988). Possibly, molecules which affect postnatal neuronal differentiation, maturation, and or survival could be altered in the *weaver* mutant mouse.

Thyroid hormone is known to be essential for normal postnatal brain cell maturation and survival. In normal mice, serum thyroid hormone levels increase from barely detectable on the day of birth and peak on P15 (Walker et al. 1982; Carassco and Blum unpublished observations). Circulating thyroxine contributes to postnatal body growth and determines timing of eyelid opening in the rodent (Hamburgh et al. 1971;

Schwartz 1983). The somatic abnormalities described for the *weaver* are remarkably similar to those described for hypothyroid rodents. As has been reported for the *weaver* mutant mice, mice suffering from hypothyroidism are usually about half the size of age-matched normal mice starting after P10 (Hamburgh et al. 1971; Nicholson and Altman 1972). When hypothyroidism is induced in new born rats and the gonads examined at puberty, hypothyroidism is associated with a decrease in the diameter of seminiferous tubules, arrested maturation of germ cells and increased degeneration of germ cells (Francavilla 1991). Recent descriptions of the *weaver* testes examined at puberty encompass a reduced diameter of seminiferous tubules, a delay in the maturation of sperm and an increase in the number of degenerating spermatids (Vogelweid et al. 1993; Harrison and Roffler-Tarlov 1994). Morphological abnormalities of the developing cerebellum of hypothyroid rodents include: a reduction in cerebellar size; a retardation of cell differentiation in the EGL; a decrease in Purkinje cells size; a multi-leveled layering of Purkinje cells; and an increase in the index of pyknotic granule cells (Nicholson and Altman 1972; Nicholson and Altman 1972; Rabie et al., 1979; Legrand and Clos 1991). These appear strikingly similar to the characteristics of the abnormal development in the *weaver* mutant cerebellum. The effects of hypothyroidism on the developing dopamine neurons has received much less attention. However, there are reports that chemically-induced hypothyroidism during postnatal development can significantly decrease dopamine content and tyrosine hydroxylase activity in the striatum especially when expressed on a per striatum basis (Rastogi et al., 1976; Kalaria and Prince 1985). Also, neonatal hypothyroidism can interfere with lesion-induced dopamine sprouting events in the olfactory

tubercle (Gottesfeld et al., 1987). Because similarities exist in the phenotype of hypothyroid and *weaver* mice, we hypothesized that the *weaver* mutants may be suffering from hypothyroidism. We were interested to see if this proposed reduction in circulating thyroid hormone levels could affect the brain and could potentially be a factor which contributes to the neurological deficits reported in the *weaver* brain.

It has been proposed that one way thyroid hormone may effect brain development is by altering the local synthesis of nerve growth factor (NGF) (Walker et al., 1981; Legrand and Clos 1991). NGF is a polypeptide trophic factor necessary for the differentiation and maintenance of specific neurons in the developing nervous system (for review see Thoenen and Barde 1980). In cultures of presumptive granule neurons from the cerebellum, NGF stimulates DNA synthesis (Confort, et al.,1991). In addition, intraventricular administration of antibodies to NGF can cause a delay in the disappearance of the EGL (Confort et al., 1991; Legrand and Clos 1991). Midbrain dopamine neurons are not responsive to NGF (Konkol et al., 1978; Schwab et al., 1979; Dreyfus et al., 1980), but NGF can stimulate striatal cholinergic neuron development (Gnahn et al., 1983; Patel et al., 1988; Altar et al., 1992). Thus, it may be that NGF affects dopamine neurons indirectly via cholinergic neurons. NGF protein and mRNA have been detected in high levels in the hippocampus, in moderate levels in the striatum and in low levels in the cerebellum of adult rats (Korsching et al., 1985; Shelton and Reichardt 1986). Coincident with the postnatal rise in serum thyroxine, NGF mRNA expression in the hippocampus and striatum increase from birth to peak at approximately P20, whereas cerebellar NGF mRNA peaks at P10 and returns back to baseline by P20 (Large et al., 1986; Auburger et al., 1987). NGF mRNA

and protein levels in the adult and neonatal brain have been shown to be regulated by thyroid hormone (Walker et al. 1979; Walker et al. 1981; Giordano et al., 1992; Figueiredo et al., 1993; Hashimoto et al., 1994). NGF protein levels in two-month-old *weaver* cerebellum and cerebrum were significantly reduced compared to normal litter mates (Matsui et al., 1990). Therefore, we asked if NGF mRNA levels were altered in the cerebellum, striatum or hippocampus of the developing *weaver* brain.

Neonatal thyroid deficiency leads to a significant reduction in brain, and more specifically striatal, choline acetyl transferase (ChAT) activity, the enzyme responsible for acetyl choline biosynthesis which is used as a marker for cholinergic neurons (Rastogi et al. 1976; Kalaria and Prince 1985). Additionally, neonatal injections of thyroxine can increase ChAT activity in the brains of thyroid hormone deficient and normal rats (Patel et al. 1988). While no reduction in ChAT activity was found in either the 9 month or 12 month old *weaver* striatum (Simon and Ghetti 1992), we hypothesized that there may be a developmental reduction of striatal ChAT activity in the *weaver*.

Materials and Methods

Animals / Tissue & Blood collection

Tested heterozygote *weaver* breeding pairs on a C57Bl6/6J Le–A^{wj} x CBA/CaGnLeF background, ordered from the Jackson Laboratory (Bar Harbor, ME), were maintained in the animal care facility of The Mount Sinai School of Medicine and cared for within institutional guidelines. Breeding pairs were monitored at least three times a week for births and litters were sacrificed by cervical dislocation or decapitation. The body weight of each individual mouse was taken and recorded at time of sacrifice. Striatal tissue was collected as previously described (Lazar and Blum 1992; Weickert and Blum 1995). Cerebellar halves of non–ataxic litter mates were sectioned and stained with hematoxylin and eosin (H & E) and were evaluated by at least two different individuals in order to distinguish +/wv from +/+.

Experimentally Induced Hypothyroidism

Newborn pups from a Bl6XCBA (BA) cross were rendered hypothyroid by adding Propylthiouracil (PTU) to the mothers diet beginning on the day of birth (P0). The standard laboratory chow was ground and mixed with 0.1% PTU and 0.001% PTU was added to the drinking water. Normal BA litters fed a non-altered diet served as controls. At P22, at least three different PTU-treated and control litters were sacrificed and brain tissue was collected as previously described (Lazar and Blum, 1992, Weickert and Blum 1995, Blum and Weickert 1995). The total T4 serum levels were measured using a commercially available RIA kit (see below).

Radioimmunoassay

Cardiac blood was drawn from the thoracic cavity by Pasteur pipettes. Blood was allowed to clot at 4°C, and then centrifuged at 10,000 X g for 20 minutes at 4°C. Serum was stored at -20°C until assayed. Total T4 was measured using a commercially available radioimmunoassay kit according to manufacture's recommendations (Coat–a-Count, Diagnostics Products Corporation, Los Angeles, CA).

Cytoplasmic RNA isolation / RNase protection assay

Tissue samples for the *weaver* and wild-type mice were matched according to postnatal age and sex; litter mate controls were used whenever possible. The total RNA was extracted from frozen brain regions and the S1 protection assay was run as previously described (Blum 1989). Synthetic sense RNA (standards) and antisense RNA (~ 2 x 10^9 CPM/μg) were transcribed using DNA from a bluescript vector with a cloned insert corresponding to bp 610-1000 of the NGF gene. The standards used to derive the regression line for prediction of amoles in samples ranged from 0.3 attomoles (am) to 5 am.

ChAT Assay

The procedure for the ChAT assay was adapted from that of Fonnum (Fonnum 1975). Frozen age-matched and sex matched striata were thawed and homogenized in 450 μl of 10 mM EDTA pH 7.4 on ice. Protein concentration in each sample was determined by the Bradford method. Five μl of 25% Triton X-100 was added to 20 μl (200 μg-600 μg protein) of the homogenized sample. Twenty-five μl of ChAT buffer [0.3 M NaCl, 0.10M $NaPO_4$, 40 mM EDTA, 1% BSA, 4 mM Choline Cloride, 2 mM

eserine salicylate and 0.8 mM ^{14}C-Acetyl CoA (2.50 mCi/mmol)] was added to each tube. The samples were incubated for 10' at 37°C, immediately transferred to ice and diluted with 450 µl of dH_2O. To each tube, 900 µl of extraction liquid (0.5% sodium tetraphenylboron, 85% toluene, and 15% acetonitrile) was added. The tubes were vortexed and centrifuged at 20,000 x g for 5'. The top aqueous layer was transferred into 10 ml of HIONIC FLUOR (Packard) and counted in a scintillation counter. The assay was first determined to be linear with respect to time and protein concentration. All incubations were set up within the linear range of the assay. Also, in order to monitor the specificity of the assay, all experimental runs were carried out with control samples incubated in the presence of a specific inhibitor of ChAT, napthyl vinyl peridnium (a generous gift from Dr. Peter Davies, Albert Einstein College of Medicine).

Thyroid hormone replacements

The L-thyroxine (Sigma) was initially dissolved in 0.1 N NaOH and stored frozen in concentrated aliquots (10 mg/ml). Mutant *weaver* mice were injected subcutaneously with a dose of 0.25 µg L-thyroxine/g body weight diluted in physiologic saline or with saline alone as a control. Injections were begun on P15 and continued until P18 on which day the mice were injected exactly 2 hours before sacrifice.

Results

Body weights

The average body weight in grams during postnatal development of the *weaver* mutants and litter mate controls is presented in Figure 3.1 (N=403 mice from our breeding colony). Using a two way ANOVA, the *weaver* mutant mice (n=119) were found to weigh ~50% less than their normal siblings, during the second and third postnatal week. [(for genotype, $F=55.669$, $p<.001$; for age x genotype, $F=4.722$, $p<.001$), with significant differences at P14, P18, P22, P26, P30 and P145 (all $p<.001$)]. The body weights of the wild type mice (n=121) and heterozygote mice (n=163) were not found to be statistically different from one another ($p>.05$).

Eyelid opening

Weaver mutants were checked for eyelid opening relative to the eyelid opening of normal litter mates from P13 to P14. As predicted by the laws of Mendelian genetics for an autosomal recessive trait, 25% (n=18) of all animals were judged phenotypically to be *weaver* mutants (Table 3.1). Only 1 of the 18 mutants had their eyes open on P13-14 (6%) whereas almost all of their normal siblings, (91%) had their eyes open.

Serum thyroxine levels

The serum thyroid hormone measurements were evaluated at three postnatal ages for *weaver* mutants versus heterozygote and wild type litter mates. A two way ANOVA followed by a Fishers Post-Hoc analysis revealed no detectable difference between total T4 levels in the *weaver* mice and normal litter mates at P14 ($p>.05$) but did show a detectable

difference between mutants and controls at P18 and P22 ($F=9.241$, $p<.01$). The serum thyroxine levels for the mutants was 63% control at P18 ($p<.01$) and 46% control at P22 ($p<.001$).

NGF mRNA levels in hippocampus, striatum and cerebellum of weaver mutants

During development, the hippocampal NGF mRNA levels were 10 fold higher than striatal NGF mRNA levels, and 100 fold higher than cerebellar NGF levels (Figure 3.3 & 3.4). At P18, the NGF mRNA expressed in attomoles per microgram total RNA (am/μg) was significantly reduced by 45% in the *weaver* striatum when compared to the wild-type striatum ($p<.01$, Figure 3.3B), but striatal NGF mRNA concentration was not significantly different between the *weaver* and the normal at P22 ($p>.05$, Figure 3.4B). No significant differences were detected between the hippocampal or cerebellar NGF mRNA levels in the *weaver* versus the wild-type ($p>.05$, Figure 3A & 3C, Figure 4A).

ChAT activity in striatum

The striatal ChAT activity, for *weaver* mice and age and sex matched wild-type controls, when expressed as nmoles acetyl choline synthesized per hour per milligram protein basis did not differ between the two genotypes at P18 (Figure 3.5A; $t=-.902$, $p=.197$) or at P22 (Figure 3.5B; $t=.252$, $p=.806$). The striatal ChAT activity for the PTU treated mice was significantly lower than the non-treated mice ($t=-2.936$, $p<.05$).

NGF mRNA levels and ChAT activity in striatum of weaver treated with thyroid hormone replacements

After four daily injections of L-thyroxine prior to sacrifice, both striatal levels of NGF mRNA (Figure 3.6A) and ChAT activity (Figure 3.6B) were induced in the *weaver* striatum ($p<.05$, and $p<.05$ respectively). The levels of NGF mRNA and ChAT activity in the striatum of the control *weaver* mice, which were injected with saline, were comparable to the levels of NGF mRNA and ChAT activity found in the non-injected *weaver* mice at P18 (compare figure 3.6A to Figure 3.3B and Figure 3.6B to Figure 3.5A).

Discussion

The *weaver* mutant mouse has reduced serum thyroid hormone levels after two week of age (Figure 3.2). This hypothyroidism appears to be of postnatal onset as the serum thyroid hormone levels are near normal at P14. The reduced thyroid hormone correlates with the lack of somatic growth of the mutants during this time (Sidman et al. 1965; Coscia and Fentress 1993) (Figure 3.1). Thyroid hormone works together with growth hormone to stimulate somatic growth during the third and fourth postnatal weeks of rodent life. It appears that there is a phase of "catch-up" growth for the mutants starting after the first month of postnatal life around the time of puberty (Figure 3.1, (Sidman et al. 1965). During the peripubertal period, gonadal hormones augment the ability of growth hormone to stimulate somatic growth and effects of thyroid hormone on peripubertal somatic growth are diminished (Hamburgh et al. 1971; Daughaday et al., 1975; Schwartz 1983). Perhaps the mutant mice have normal circulating gonadal hormones levels during the prepubertal period; this could explain the "catch-up" phase of growth from P30-P78 which we observed in the *weaver* mice.

The time of eyelid opening was delayed in *weaver* mice (Table 3.1) and usually lagged behind that of normal litter mates by one day. Unexpectedly, the *weaver* mutant mice were not found to have significantly reduced circulating thyroxine levels compared to normal litter mates at P14, the time of normal eyelid opening. We infer from this result that the timing of developmental events mediated by thyroid hormone is delayed possibly because *weaver* mutants may not be equipped with the normal molecular response mechanism to thyroid hormone.

A 40% reduction in serum thyroid hormone levels at P18 correlates with a 45% reduction in striatal NGF mRNA levels in the *weaver* (Figure 3.2 and Figure 3.3 B). This reduction is reversible when thyroid hormone replacement therapy is given to the *weaver* mouse (Figure 3.6 A). Therefore, decreased brain NGF mRNA levels in the *weaver* may due to decreased thyroid hormone levels at this time. The further reduction of serum thyroid hormone found at P22, does not correlate with further reductions of striatal NGF mRNA. Also, we did not find reduced NGF mRNA in the hippocampus of the *weaver* mice (Figure 3.3 A&B). Overall, these findings agree with previous reports that thyroid hormone effects on gene expression can be temporally and spatially restricted (Giordano et al. 1992; Mellstrom et al., 1994). Another interpretation of these data, would be that *weaver* NGF mRNA levels fail to reach their postnatal peak in the striatum at P18 as do their normal litter mates, and thus the ontogenetic rise in NGF mRNA is retarded in the mutant striatum. In support of this concept, we found a developmental delay in the down-regulation of striatal GDNF (Glial-Cell-Line-Derived Neurotrophic Factor) mRNA in *weaver* striatum at P18 (Blum and Weickert 1995). Both NGF and GDNF can be synthesized by forebrain astrocytes in culture (Houlgatte et al., 1989; Schaar et al., 1993; Ho et al., 1995) and it may be that glial development in general is delayed in the *weaver* striatum.

In *weaver* mice, cerebellar NGF mRNA levels were not found to be altered at P18. This suggests that NGF is not synthesized in granule neurons during the third postnatal week of life. Since, developmentally, NGF mRNA peaks 10 days earlier in the cerebellum as opposed to forebrain regions, this implies that cerebellar NGF mRNA may be regulated differently from telencephalic NGF mRNA (Auburger et al.

1987; Lu et al., 1989; Shannon et al., 1993). This idea is supported by a recent paper which reported an increase in forebrain NGF protein concentrations, and a decrease in cerebellar NGF protein concentrations, when thyroid hormone was administered to neonatal rodents (Hashimoto et al. 1994). Alternatively, we may be examining the cerebellar NGF mRNA levels of the *weaver* mutants after the time window of thyroid hormone responsiveness, as others found that the cerebellar NGF protein was altered by thyroid hormone injections only on P2 and not on P15 or P30 (Fifueriredo 1993).

NGF and thyroid hormone have been shown to be directly trophic for cholinergic neurons (Rastogi and Singhal 1979; Gnahn et al. 1983; Kalaria and Prince 1985; Hayashi and Patel 1987; Patel et al. 1988; Altar et al., 1991). Both striatal NGF mRNA and serum thyroxine are reduced in the *weaver* at P18 (Figure 3.2 & 3.3B), but we found no evidence for a developmental reduction in striatal ChAT activity (Figure 3.5). When *weaver* mice were injected with thyroxine they exhibited a normal response, that is ChAT was induced (Figure 6B, (Hashimoto et al. 1994). The fact that ChAT activity was normal in the *weaver* brain suggests that some of the systems normally affected by thyroid hormone reductions are intact in the mutant, therefore, only certain thyroid hormone response pathways seem to be affected in the *weaver*. Thyroid hormone exerts its effects on cells by binding to high affinity nuclear receptors and altering transcription of target genes (Evans 1988; Nikodem et al. 1990). Thyroid hormone receptors are abundant in the developing rodent brain including the developing caudate-putamen, hippocampus and cerebellum (Strait et al., 1990; Bradley et al. 1992; Mellstrom et al. 1994). Cholinergic neurons are more concentrated in the lateral quadrant of the striatum in a

gradient that is in register with the medial-lateral gradient of thyroid hormone β receptor subtype mRNA localization (Bradley et al. 1992). Neither of the neuronal regions thought to be primary targets of *weaver* gene action, the inner EGL layer or the substantia nigra pars compacta, contain this subtype of receptor mRNA, whereas they both do contain the α1 and α2 receptor subtype mRNA (Bradley et al., 1989; Mellstrom et al., 1991; Bradley et al. 1992) Therefore, it may be that thyroid hormone effects on striatal ChAT activity are mediated through the β form of thyroid hormone receptors, and that this response pathway is not compromised in the *weaver*. We suggest that the effect of the *weaver* gene may be down stream of the α1 and/or α2 thyroid hormone receptor subtype.

Functional disruption at some level of the hypothalamic-pituitary-thyroid axis is likely to be contributing to the reduced circulating thyroid hormone level found in the *weaver*. Since the pro-thyroid hormone releasing hormone (pro-TRH) protein levels in the adult *weaver* hypothalamus are normal it is not likely that the TRH neurons nor their TRH synthetic potential is the primary problem of the *weaver* mutant (Mitsuma et al., 1990). Also, *weaver* mice have been reported to respond to the ataxia–ameliorating effects of TRH, so TRH receptor responses are functional at least when analyzed for their effect on behavior (Mano et al., 1986; Mitsuma et al. 1990). One may propose that it is the release of TRH into the median eminence which is altered in the *weaver*. Dopamine can stimulate hypothalamic neurons to release TRH (Grimm and Reichlin 1973; Lewis et al., 1987). A decrease in hypothalamic dopamine concentrations has been reported in the *weaver*, therefore TRH release may be decreased in the mutants (Roffler-Tarlov and Graybiel 1986).

However, dopamine can also inhibit the synthesis and release of thyroid stimulating hormone (TSH) from the thyrotrophs, and in this case the decrease of dopamine may actually increase TSH release. Dopamine can have antagonistic effects on the hypothalamus and pituitary, therefore, the deficit in dopamine alone does not readily explain the reduction in thyroid hormone production (Shupnik et al., 1986; Ridgway et al., 1987) Thyroid hormone itself contributes to the overall regulation of thyroid hormone production by exerting direct negative feed-back control on TRH and TSH synthesis and secretion (see Williams 1994 for review). Since we suggest that some of the responses to thyroid hormone are abnormal in the mutant mice, it may be that thyroid hormone's ability to exert feedback control over its own production is compromised in the *weaver* mouse, rendering it hypothyroid during development.

The effects of reduced thyroid hormone on the developing brain are not identical to the neurological effects of the *weaver* gene; the granule cell loss is more severe in the *weaver* mouse than in hypothyroid rodent (Sidman et al. 1965; Nicholson and Altman 1972; Nicholson and Altman 1972; Rezai and Yoon 1972). Deprivation of thyroid hormone blocks granule neuron differentiation in the normal cerebellum, and can actually reduce the amount of dying granule neurons in the neonatal *weaver* cerebellum (Smeyne and Goldowitz 1990). This suggests that the *weaver* gene product may be induced by thyroid hormone. Cerebellar granule neurons cultured from normal brains, send out processes in response to tri-iodo-thyronine (T3), whereas cultured *weaver* granule neurons appear non-responsive to addition of T3 and they degenerate in culture with or with out T3 (Fischer et al., 1986). Thus, the *weaver* granule neurons are not rescued by the addition of thyroid hormone. Also, we noticed the

weavers injected with thyroid hormone were not significantly better upon gross examination. They still exhibited ataxia, had reduced body weights and had deflated cerebella compared to controls (unpublished observations). We infer from previously reported data combined with our own, that the abnormalities experienced as a consequence of carrying the *weaver* gene mutation are not likely to be a direct result of reduced thyroid hormone, but to be a consequence of an altered down stream event of thyroid hormone action that is cell-type specific.

To summarize, we have found that *weaver* mutant mice have reduced serum thyroxine levels at P18 and P22. We show that this reduced thyroid hormone relates to decreased NGF mRNA levels in the *weaver* brain in a region and age specific manner. We showed that the reductions of NGF mRNA levels in the *weaver* dorsal striatum may be due to decreased thyroid hormone levels as the deficit is reversed by thyroid hormone replacements. Thus, it appears that one of the *weaver* phenotypes is rescued by supraphysiologic thyroid hormone replacement. We show that striatal ChAT activity, which is reduced under hypothyroid conditions, is not similarly effected in the *weaver*; we suggest that certain thyroid hormone mediated developmental events are normal in the *weaver*. The hypothyroidism experienced by the *weaver* may contribute to some, but not all of the morphological and biochemical alterations found in the *weaver* brain and body.

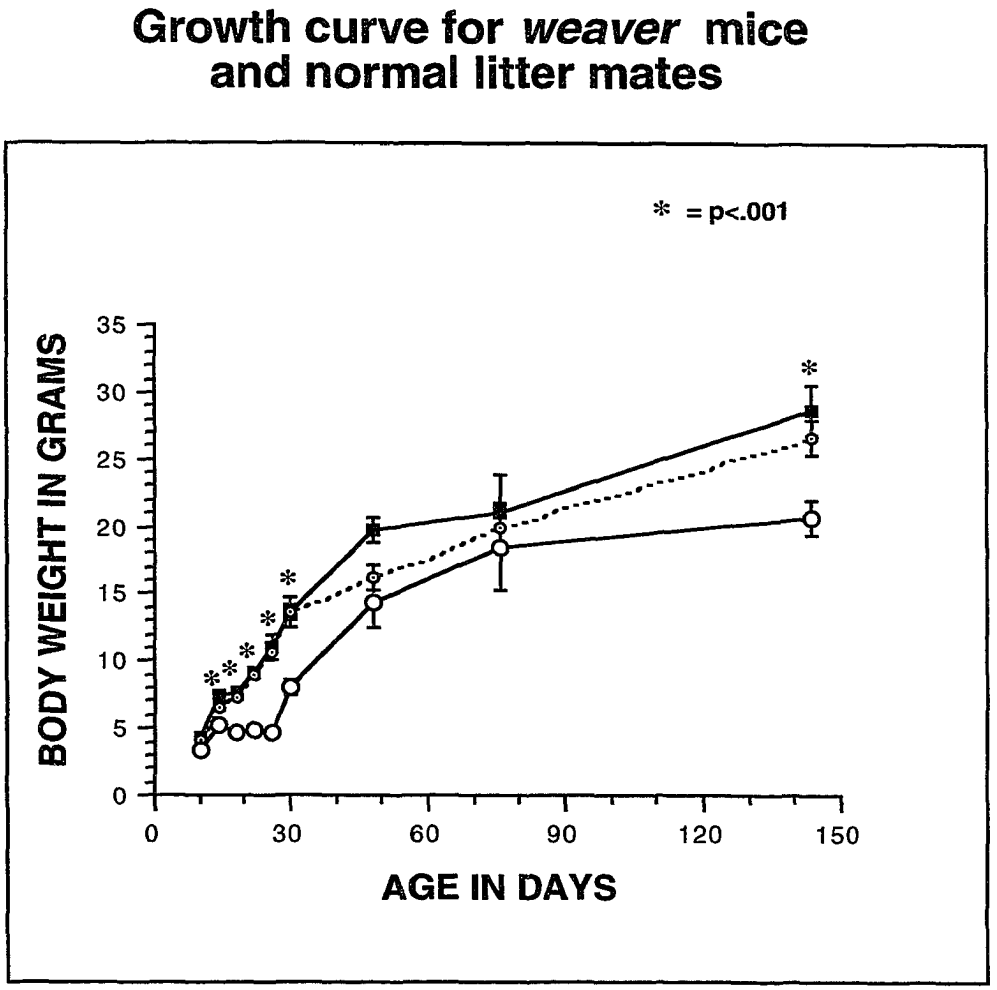

Figure 3.1. The mean (+/- s.e.m.) body weight per age in grams is shown for the wild-type (solid squares), heterozygote (open circles with dot) and homozygote *weaver* (open circles) mice during postnatal development. When the s.e.m. is smaller than symbol used to designate mean, no error bar can be seen on graph.

Litter	total # of Pups	# mutants with eyes open/ total # of mutants	# normal with eyes closed/ total #normals
1	8	0/2	6/6
2	6	0/1	5/5
3	9	0/3	6/6
4	7	0/2	4/5
5	5	1/2	2/3
6	5	0/1	4/4
7	9	0/1	8/8
8	7	0/1	4/6
9	8	0/3	4/5
10	7	0/2	5/5
totals	**71**	**1/18 (6%)**	**48/53 (91%)**

Table 3.1. This table shows the number of mutants and the number of normals with their eyes open at P13-P14. Mutants represent 25% of all animals observed.

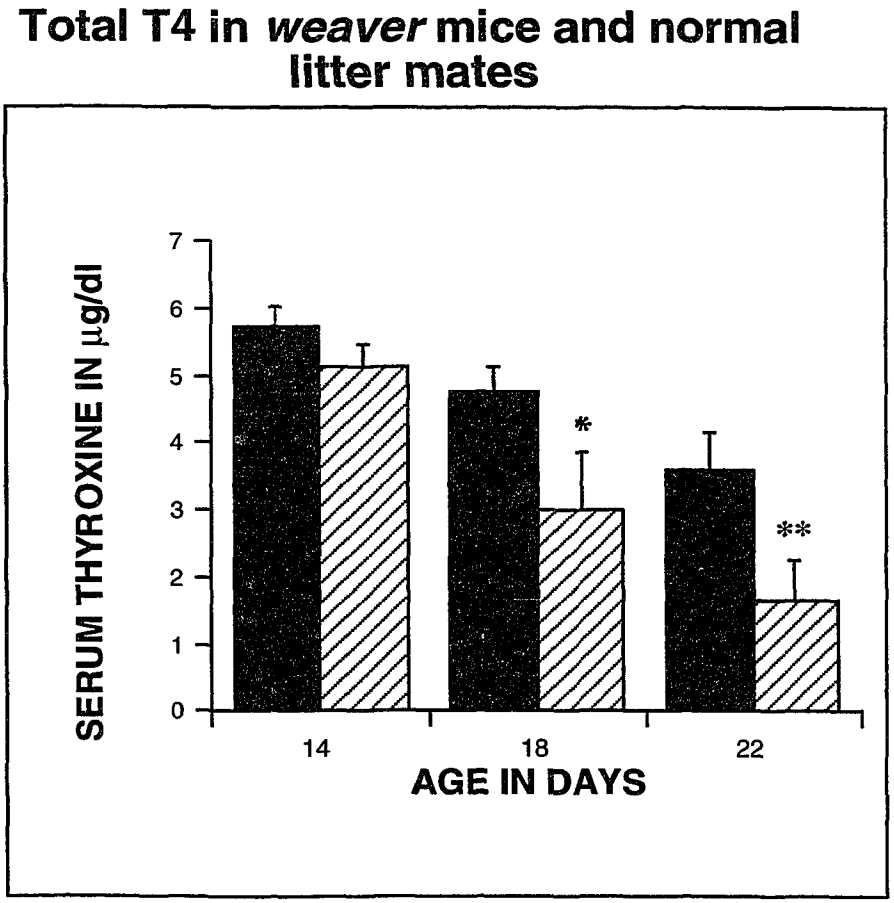

Figure 3.2. Serum total T4 levels expressed in µg/dl for *weavers* (striped bars) and normal litter mates (both wild-type and heterozygotes, solid bars) expressed at three time points (this work was done in collaboration with Emilce Carassco).

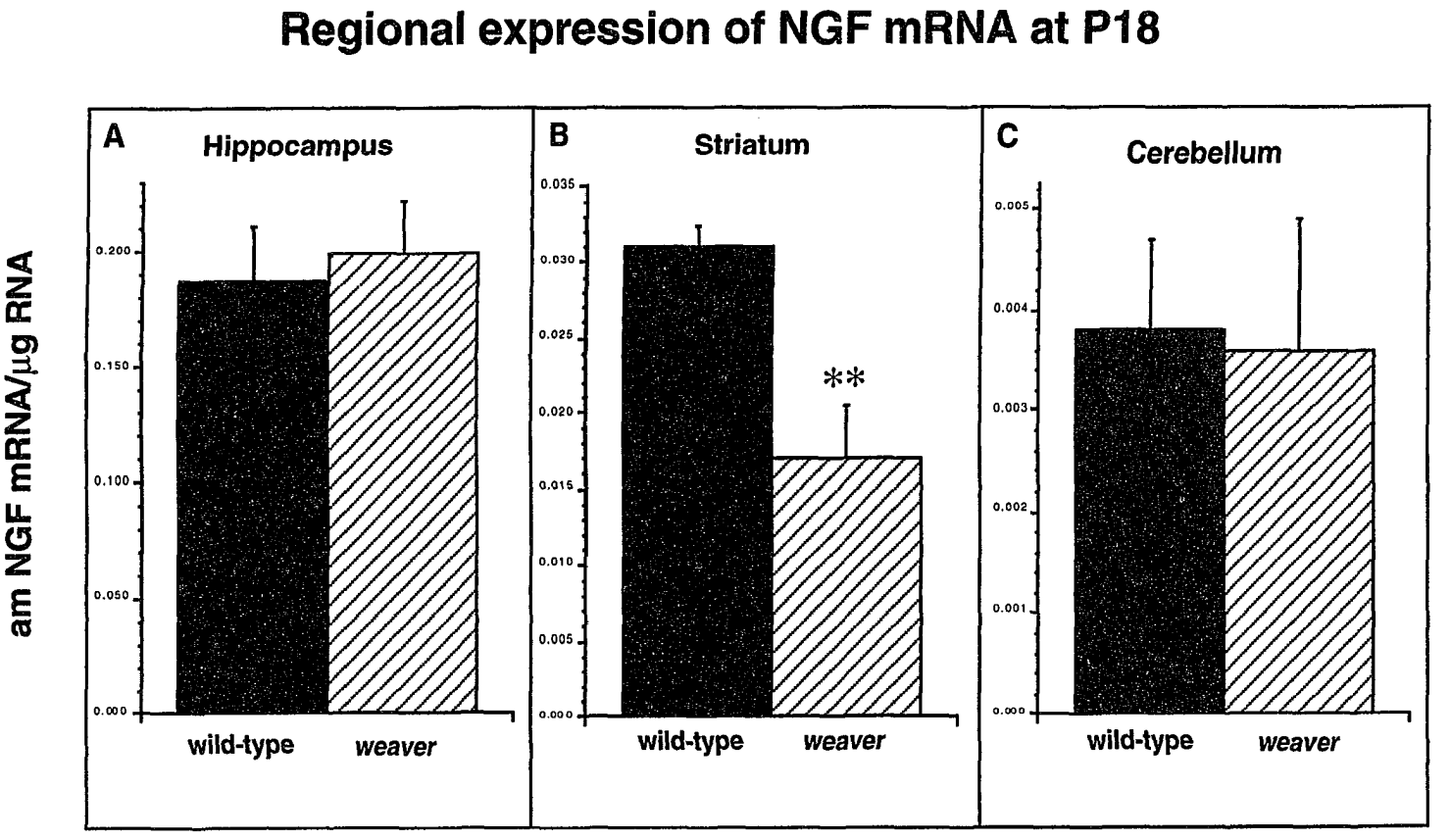

Figure 3.3. NGF mRNA expressed in attomoles/µg total RNA (+/- s.e.m.) are shown for the Hippocampus, Striatum and Cerebellum of wild-type (solid bars) and *weaver* mice (stripped bars) (n=5 per group). This work was done in collaboration with my Advisor, Dr. Blum.

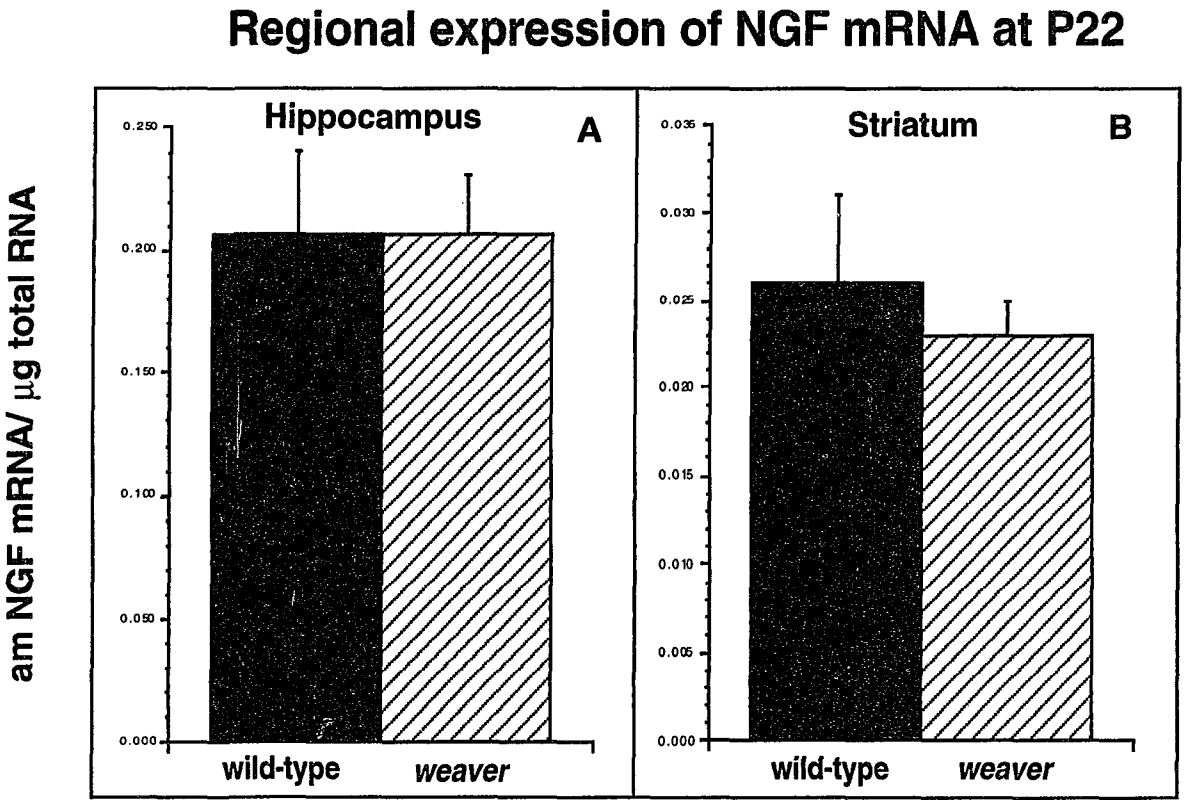

Figure 3.4. NGF mRNA expressed in attomoles/µg total RNA (+/- s.e.m.) are shown for the Hippocampus and Striatum of wild-type (solid bars) and *weaver* mice (stripped bars) (n=5 per group). This work was done in collaboration with my Advisor Dr. Blum.

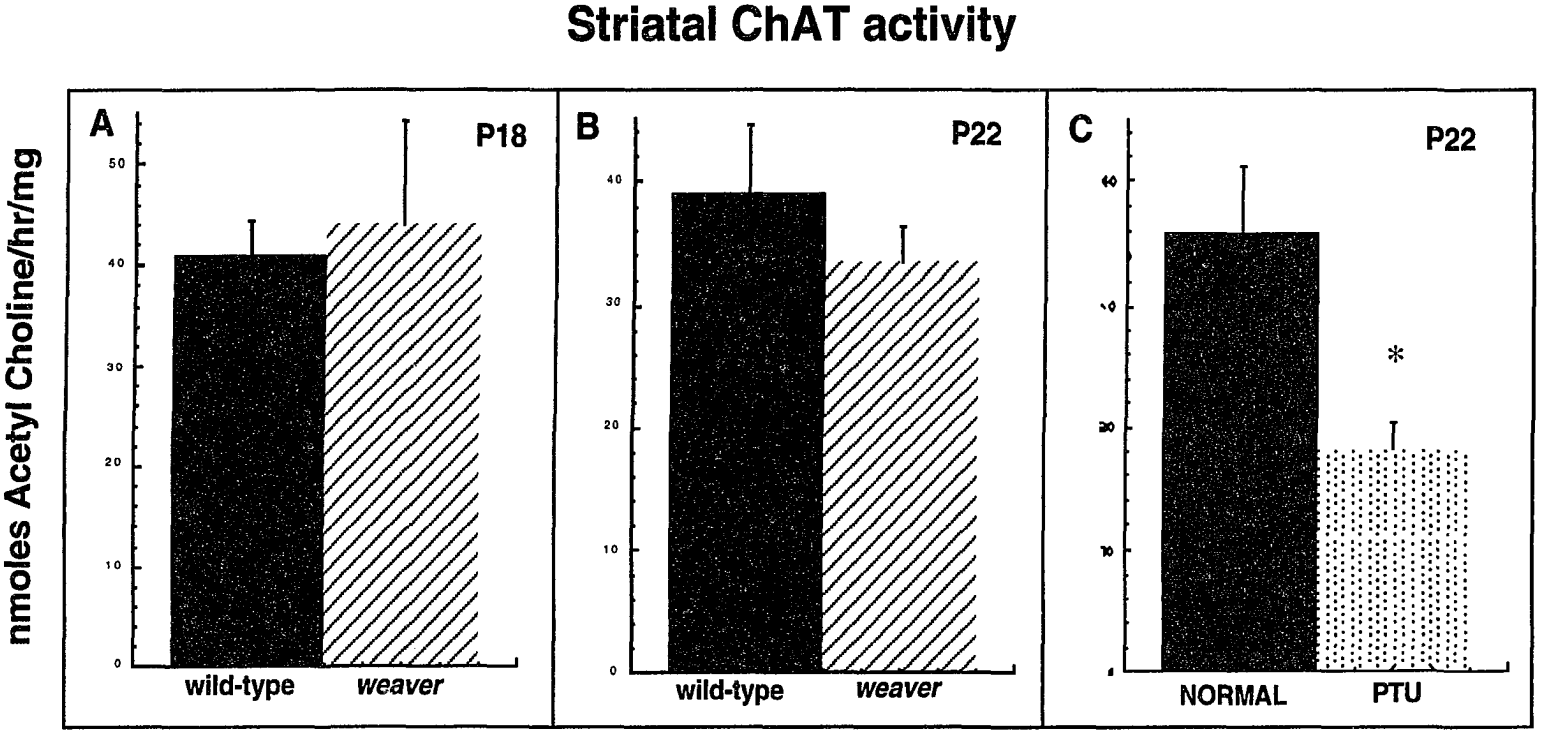

Figure 3.5. Panel A and B show the nmoles Acetyl Choline formed per hour per mg protein (nm/hr/mg) (+/- s.e.m.) for the striatum of the *weaver* (solid bars) and wild-type (stripped bars) mice at P18 and P22 (n=5 and n=6 per group respectively). The Acetyl Choline (nm/hr/mg) levels (+/- s.e.m.) for normal and experimentally induced hypothyroidism are shown in panel C (n=6 per group).

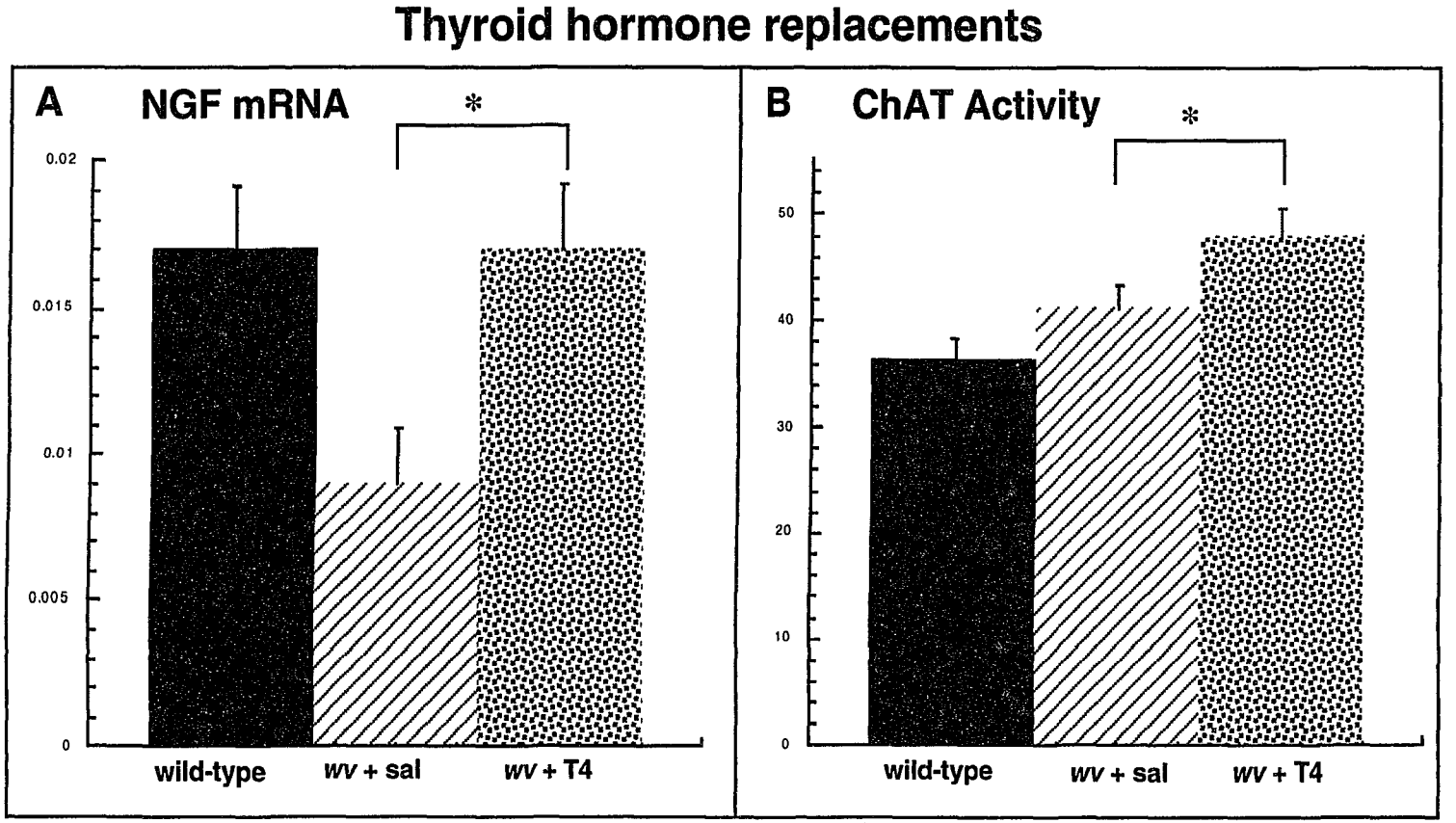

Figure 3.6. Biochemical measurements made in the striatum at P18 in wild-type mice (solid bars) and *weaver* mutant mice injected with saline (stripped bars) or thyroid hormone (patterned bars) (n=6, all groups except NGF *wv* + sal where n=3). Panel A shows NGF mRNA in attomoles/μg (+/- s.e.m.) and Panel B shows nmoles Acetyl Choline formed per hour per mg protein (+/- s.e.m.). Panel A was completed in collaboration with my Advisor, Dr. Blum.

CHAPTER 4

Decreased TGF–α Expression in Target Regions of Midbrain Dopamine Neurons in *Weaver* Mutant Mouse

Introduction

Two members of the epidermal growth factor family, transforming growth factor alpha (TGF–α) and epidermal growth factor (EGF), which both bind to and mediate comparable biological effects through the epidermal growth factor receptor (EGF-R) (Massague 1983; Marquardt et al. 1984; Gill et al. 1987; Carpenter and Cohen 1990), have been shown to be trophic for a variety of neuronal types including dopamine neurons (Knusel et al. 1990; Kornblum et al. 1990; Casper et al. 1991; Ferrari et al. 1991; Alexi and Hefti 1993). Recent evidence suggests that EGF-R mRNA is localized to nigrostriatal dopamine neurons in the rodent midbrain (Seroogy et al., 1994); this supports the concept that TGF–α/EGF could be directly trophic for midbrain dopaminergic neurons. TGF–α and EGF mRNA and protein have been shown to be present in target regions of midbrain dopamine neurons, specifically the dorsal striatum, olfactory tubercle, cerebral cortex and hippocampus (Wilcox and Derynck,1988; Fallon, et al.,1990; Lazar and Blum,1992; Seroogy, et al.,1993). In the murine striatum, TGF–α mRNA has been detected during postnatal development (Seroogy et al. 1993; Weickert and Blum 1995), and TGF–α's molar abundance throughout postnatal development is greater than that of other putative dopaminergic trophic factor mRNA's including: acidic fibroblast growth factor, basic fibroblast growth factor, brain derived neurotrophic factor, glial-cell-line-derived neurotrophic factor and epidermal growth factor (Lazar and Blum 1992; Blum et al., 1994; Blum and Weickert 1995). Midbrain neurons in primary cultures respond to EGF/TGF–α with increases in dopaminergic cell survival, increases in high affinity dopamine uptake and increases in number of tyrosine

hydroxylase (TH) positive neurites (Knusel et al. 1990; Casper et al. 1991; Ferrari et al. 1991; Alexi and Hefti 1993). Additionally, pretreatment of primary cultures with EGF can attenuate dopaminergic neuronal death after exposure to 1-methyl-4-phenlypyridinium (MPP+), a toxic metabolite of the dopaminergic neurotoxin 1-methyl-4-phenyl-1,2,3, tetrahydropyridine (MPTP) (Park and Mytilineou 1992). When MPTP-treated mice are supplemented with EGF, striatal TH levels are increased above those of non-growth factor MPTP-treated mice (Hadjiconstantinou et al., 1991). Thus, it has been demonstrated in many experimental systems that EGF/TGF–α can positively affect dopaminergic neuron viability, and therefore, can function as a potent dopaminergic trophic factor.

In the central nervous system of *weaver* mutant mice, midbrain dopamine neurons undergo abnormal cell death (see Ghetti and Triarhou 1992 for review). The nigral neurons projecting to the dorsal striatum appear to be more affected than those projecting to the ventral striatum (Roffler-Tarlov and Graybiel 1984). In the adult *weaver* brain, there is a 70% reduction in dopamine concentration of the dorsal striatum, a 34% reduction in the olfactory tubercle, and no change of dopamine concentration in the nucleus accumbens (Lane et al. 1977; Schmidt et al. 1982; Roffler-Tarlov and Graybiel 1984; Roffler-Tarlov and Graybiel 1986). Alterations in dopamine levels in the *weaver* hippocampus have received less attention; however, there is a significant decrease (66% reduction) in dopamine concentration in the adult *weaver* mutant hippocampus compared to normal litter mate controls (Chou et al., 1991). Thus, the amount of dopamine depletion found in the *weaver* brain varies from one target region of midbrain dopamine neurons to another.

The abnormal loss of dopamine neurons in the *weaver* mouse occurs during postnatal development. In the dorsal striatum, only slight differences in dopamine concentrations between *weaver* and wild type mice are seen at P14 (Roffler-Tarlov et al. 1990); this difference becomes more evident and reaches statistical significance by P21 (Schmidt et al. 1982; Roffler-Tarlov and Graybiel 1987; Roffler-Tarlov et al. 1990). Further reductions in dopamine concentrations in the striatum of *weaver* mice as compared to wild-type mice are seen at P30, when the dopamine depletion matches that found in the adult *weaver* (Roffler-Tarlov et al. 1990). The temporal course of dopamine loss in the *weaver* olfactory tubercle is similar to that in the dorsal striatum, although the overall change is not as great (Roffler-Tarlov and Graybiel 1987). The loss of dopamine in the *weaver* hippocampus during the course of development has not yet been investigated.

We hypothesized that decreased availability of a necessary dopaminergic trophic factor, specifically TGF–α, could result in dopaminergic cell death such as that seen in the *weaver* mutant. We tested this hypothesis by measuring target-derived TGF–α mRNA levels during the abnormal postnatal development and/or degeneration of midbrain dopamine neurons in the *weaver* brain. We predicted that the putative reduction of TGF–α mRNA in the mutant brain would vary regionally, such that target areas experiencing the largest dopamine depletion would have the greatest reduction in levels of TGF–α mRNA. Since we have observed that the *weaver* mutant animals become hypothyroid at a time coincident with dopamine neuron loss (Chapter 3), we explore the possibility that a decrease in circulating thyroid hormone level could influence TGF–α gene expression. We determined if TGF–α

levels in the mutant mouse can be affected by physiological thyroid hormone replacements.

It has been postulated that the developmental defect in the dopamine-containing systems in the *weaver* brain may result from abnormalities in neuron-glial interactions (Graybiel et al. 1990; Roffler-Tarlov et al. 1990), yet no studies have monitored striatal astroglial development in the *weaver* mutant basal ganglia. Many putative dopaminergic neurotrophic factors, including TGF–α, are synthesized in astroglial cells (Fallon et al. 1990; Seroogy et al. 1993). Reductions in thyroid hormone levels during development slow astroglial maturation, alter astroglial morphology and can decrease glial fibrillary acidic protein (GFAP) mRNA levels (Gould et al., 1990; Faivre-Sarrailh et al., 1991). We measured GFAP mRNA levels in the dorsal striatum of the *weaver* mouse during the third and fourth postnatal week, a time coincident with dopaminergic neuron loss and reduced serum thyroxine levels. We expected that striatal GFAP mRNA levels may be reduced in the mutant.

Materials and Methods

Animal breeding and tissue collection

Tested heterozygote *weaver* breeding pairs on a C57Bl6/6I Le–A^{wj} x CBA/CaGnLeF background (Jackson Laboratory, Bar Harbor, ME), were maintained in the animal care facility of The Mount Sinai School of Medicine and were cared for within institutional guidelines. The day of birth was designated as P0, and pups were sacrificed by litters at postnatal days 14 (P14), P22 and P26. Tissue dissections were performed as previously described (Lazar and Blum 1992; Weickert and Blum 1995). The olfactory tubercle examined in this study contained the ventro-medial portions of the nucleus accumbens, especially at more rostral levels since the lateral olfactory tract/rhinal fissure served as the dorsal landmark for the dissection, and a horizontal cut was made to the medial surface. The striatal dissection described previously (Lazar and Blum 1992; Weickert and Blum 1995) is analogous to what is termed here the dorsal striatum. Cerebellar halves of non–ataxic litter mates were sectioned and stained with hematoxylin and eosin (H & E) in order to distinguish +/*wv* from +/+ as previously described (Chapter 3). Animal collection continued over the course of four years, until at least five *weaver* mutant mice and age-matched, sex-matched wild-type controls could be collected at each postnatal time point examined.

Experimentally Induced Hypothyroidism

Newborn pups from a Bl6XCBA (BA) cross were rendered hypothyroid by adding Propylthiouracil (PTU) to the mothers diet beginning on the day of litter delivery (P0). The standard laboratory chow was ground and mixed

with 0.1% PTU and 0.001% PTU was added to the drinking water. Normal BA litters feed a non-altered diet served as controls. At P22, at least three different PTU-treated litters and control litters were sacrificed and blood was collected and total T4 levels were quantitated as previously described (Chapter 3).

Physiologic Serum Thyroxine Replacement

Starting at P15, five *weaver* mutant mice were subcutaneously injected with 0.01 ng/g body weight of L-thyroxine (T4, Sigma) daily for seven days. This dose of T4 was chosen because .025 ng/g body weight/day was shown to restore physiological levels of serum T4 to mice with non-detectable levels of T4 (Wilson 1985; Glasscock et al., 1991), and since *weaver* T4 levels are 60% control we replaced with 40% of the dose previously determined to be physiologic. Injections started at P15 and continued for 7 days until P21. As a control group, four *weaver* mutant mice were injected with saline in the same manner. Twenty-four hours after the last injection (P22), we sacrificed the mutants and collected the brains as previously described.

Cytoplasmic RNA isolation / RNase protection assay

The total RNA was extracted from frozen tissue as previously described (Jakubowski and Roberts 1992). Synthetic sense RNA and antisense RNA was transcribed using template DNA from a Bluescript vector with a cloned insert of either TGF–α (kindly provided by Dr. Rick Dyrenk) or (GFAP kindly provided by Dr. N. Cowan). Approximately 7 μg total striatal RNA, 10 μg total olfactory tubercle RNA, and 20 μg total hippocampal RNA was used for the TGF–α protection assay. From total

striatal RNA, a 2 μg sample was taken off for the GFAP protection assay. The protection assay was performed as previously described (Lazar and Blum 1992; Weickert and Blum 1995).

Results

Overall

Confirming the regional variation of TGF-α mRNA expression we have previously reported in the adult mouse (Lazar and Blum 1992), the steady state level of TGF–α mRNA expressed in attomoles/μg total RNA in the striatum was about 5-fold higher than that for either the olfactory tubercle or hippocampus during development (Figure 4.1, 4.3 & 4.4). For both *weaver* mutants and wild-type control mice, brain levels of TGF–α mRNA declined significantly from P14 to P22. This developmental decline was attenuated during the fourth postnatal week in wild-type mice. For the PTU treated mice, the levels of serum T4 were below detection in the RIA, whereas the T4 level of normal BA mice was 4.5 μg/dl at P22 (data not shown).

TGF–α mRNA levels in Dorsal Striatum

In both genotypes examined, striatal TGF–α mRNA levels expressed in attomoles (am)/μg total RNA decrease during the third and fourth postnatal week ($F=45.015$, $p<.001$; Figure 4.1A). The TGF-α mRNA levels are significantly different in the mutant animals compared to controls ($F=12.458$, $p<.002$). A significant reduction in TGF–α mRNA in the *weaver* striatum is evident at P22 when values are reduced by 25% ($p<.05$). This reduction becomes even more dramatic at P26 where values of TGF–α mRNA concentration are reduced by 61% compared to control levels ($p<.01$). In the striatum of PTU treated mice, the TGF- α mRNA levels were reduced by 40% ($t=-9.66$, $p<.001$) at P22 (Figure 4.1B).

GFAP mRNA levels in Dorsal Striatum

The striatal GFAP mRNA levels decline slightly, but not significantly, during the third postnatal week in both the wild-type and *weaver* mice (Figure 4.2A, F=2.885, p=.0732) No differences in the levels of expression between the two genotypes was detected at either P14 or P22 (main effect for genotype, F=1.269, p=.2698) However, there is a 239% induction of GFAP mRNA in the mutant striatum at P26 (Figure 4.2A, p<.005). We detected a significant decrease in GFAP mRNA levels in the PTU treated striatum at P22 (Figure 4.2B, t=2.74, p<.05).

TGF–α mRNA levels in Olfactory Tubercle

In the olfactory tubercle, the concentration of TGF–α decreases by 50% for both the wild-type and *weaver* mice during the third postnatal week (F=33.361, p<.001, Figure 4.3A). No statistically significant differences in TGF–α mRNA levels between *weaver* mutants and wild-type controls were detected in the olfactory tubercle at any of the time points examined (F=.258, p=.617). With experimentally induced hypothyroidism, TGF–α mRNA levels in the olfactory tubercle were 44% of controls (t=-5.792, p<.001, Figure 4.3B).

TGF–α mRNA levels in Hippocampus

Hippocampal TGF–α mRNA levels decline during the second postnatal week in both the *weaver* mice and wild-type mice (F=24.072, p<.0001; Figure 4.4A), but in the *weaver* mice the levels of TGF–α fall significantly below that of the wild-type controls (F=14.515. p=.001). At P22 and P26, the *weaver* hippocampal TGF–α mRNA levels are significantly reduced when compared to wild-type controls (both p<.05). During the fourth

postnatal week, the mutant TGF–α mRNA concentration in the hippocampus was approximately 31% less than that of normal control values. We were unable to detect a significant difference in hippocampal TGF–α mRNA levels in mice rendered hypothyroid starting from birth and examined at P22 (t=.996, p=.343; Figure 4.4B).

TGF–α mRNA levels in Striatum of T4 injected weavers

The total RNA of striata of *weavers* injected with physiological does of T4 or with saline as a control was evaluated in the TGF-α protection assay. No significant differences in striatal TGF–α mRNA levels could be detected between the two treatment groups at P22 (t=-.986, p=.3620).

Discussion

During the third and fourth postnatal week of life, TGF–α mRNA was detected in target areas of midbrain dopaminergic neurons in both wild-type and *weaver* mutant mice. TGF-α mRNA steady state levels varied regionally in accordance with our previously reported findings in adult Swiss Webster mice (Lazar and Blum 1992). As we have previously reported in a normal (C57Bl6/CBA) strain of mice, the striatal TGF–α levels decreased during the third postnatal week (Figure 4.1A, (Weickert and Blum 1995). Although the absolute levels of TGF–α mRNA differed somewhat between these two reports (possibly due to strain differences or interassay variability), the magnitude of the developmental change in TGF–α was similar for both the normal profile presented previously and the wild-type profile presented here. In the olfactory tubercle and the hippocampus (Figure 4.3A and 4.4A), we found that the temporal expression levels of TGF–α mRNA also decreased significantly from P14 to P22. The fact that TGF–α appears to be down-regulated coordinately in regionally distinct brain areas implies that some common underlying regulatory mechanism may operate to lower TGF–α mRNA levels during this time in development.

In support of our original hypothesis, we found significantly diminished TGF–α mRNA levels in the *weaver* dorsal striatum at a time coincident with both dopamine neuron loss in the *weaver* midbrain and the decrease in dopamine concentrations in the *weaver* striatum relative to the control striatum (Roffler-Tarlov and Graybiel 1986; Triarhou et al. 1988; Roffler-Tarlov et al. 1990). We were not able to detect a difference in TGF–α mRNA at P14 in the early phases of dopaminergic cell death in

the *weaver* (Gupta et al., 1987; Roffler-Tarlov and Graybiel 1987; Triarhou et al. 1988; Graybiel et al. 1990). Rather, we detected a diminution of striatal TGF–α mRNA which exactly parallels the temporal reduction in dopamine concentration in the striatum of the *weaver* relative to controls (Roffler-Tarlov et al. 1990). The reduction of dopamine and the decrease in TGF–α mRNA in the mutant are correlated across time; consequently, we do not know if the reduction in TGF–α mRNA is a cause or is a consequence of dopaminergic cell death in the *weaver* brains. Since TGF-α/EGF can stimulate dopamine neuron survival in culture (Knusel et al. 1990; Casper et al. 1991; Ferrari et al. 1991; Alexi and Hefti 1993), we speculate that it may be more causally related to dopaminergic neuron loss.

As predicted, we did find that the reduction in TGF–α mRNA can be region specific. In the olfactory tubercle, where the loss of dopaminergic innervation is not as profound as it is in the dorsal striatum (Graybiel et al. 1990), we were unable to detect a difference in the TGF–α mRNA levels. In the hippocampus, a target-region experiencing a reduction in dopamine concentrations similar to that of the dorsal striatum (Chou et al. 1991), we did detect significant reductions in TGF–α mRNA. We do not know how this reduction in TGF-α mRNA relates to the loss of dopamine concentration in this tissue because the time course of dopamine depletion in the *weaver* hippocampus remains to be determined.

We have previously reported that the *weaver* mutant mice have reduced serum thyroid hormone levels during the third and fourth postnatal weeks of life. Thyroid hormone profoundly affects brain development during the postnatal period (see Stein et al., 1989 for review). Thyroid hormone's major site of cellular action is believed to be genomic,

where thyroid hormone binds to nuclear receptors which in turn act as cis–acting transcriptional factors which can augment or dampen the transcriptional rates of target genes (Evans 1988; Nikodem et al. 1990). It has been postulated that one way thyroid hormone can exert control over the developing brain is to regulate local synthesis of neurotrophic factors (Walker et al. 1979; Legrand and Clos 1991). Our data showing that brain TGF–α mRNA steady state levels are reduced in hypothyroid mice lends further support to this concept. It has been suggested that the 5' region of the TGF-α gene confers thyroid hormone responsiveness (Raja et al., 1991); however, whether the transcriptional rate of the TGF–α gene in brain cells is directly modifiable by thyroid hormone remains to be investigated.

The data obtained from the dorsal striatum suggest that TGF-α mRNA may be reduced in the *weaver* due to the decreases in thyroid hormone. TGF–α mRNA levels are found to be reduced in both the *weaver* and in the hypothyroid dorsal striatum (Figure 1A &B). However, when we gave injections of T4 to replace physiologic levels of serum thyroxine to the *weaver* mice, we were not able to induce the expression of TGF–α in the dorsal striatum; therefore, we suspect that the lack of T4 itself is not directly responsible for the reduction in TGF-α mRNA found in the *weaver* striatum. We have previously proposed that the action of the *weaver* gene is down stream of thyroid hormone action (Chapter 3), and it may be that, in the *weaver* dorsal striatum, thyroid hormone may not be able to induce TGF-α mRNA levels. Alternatively, it may be that the level of thyroid hormone we gave in this study was not sufficient to return TGF-α mRNA levels to baseline; we plan to test this idea by giving

a supraphysiologic dose of thyroid hormone from P15 until P26 followed by TGF-α mRNA measurement in the dorsal striatum.

We were unable to find reductions in TGF–α mRNA in the *weaver* olfactory tubercle; however, we did find reductions in olfactory tubercle TGF–α mRNA under conditions of experimentally induced hypothyroidism. This would suggest that thyroid hormone's ability to stimulate TGF-α gene expression in the *weaver* olfactory tubercle is not altered in the *weaver*. It also suggests that the brain levels of thyroid hormone may not be that different in the *weaver* and wild-type despite significantly reduced serum levels. In the *weaver* hippocampus, we did find reductions in TGF–α mRNA, but were unable to detect alterations in TGF–α mRNA under hypothyroid conditions. This result suggests TGF-α is not regulated by thyroid hormone in the hippocampus, and it suggests that another factor contributes to the regulation of TGF-α mRNA. We speculate that dopamine itself may contribute to the regulation of steady state TGF–α mRNA levels, and that the region specific reduction of TGF–α mRNA in the *weaver* mutant brain may be related to the region specific reduction in dopamine content.

The decrease in striatal TGF–α gene expression observed in the *weaver* dorsal striatum occurs during a time in development when striatal glia are normally upregulating TGF–α mRNA (Seroogy et al. 1993). Therefore, one may propose that the glial expression of TGF–α is more likely to be affected in the mutant brain. Some reports show that the cellular site of action of *weaver* mutation includes glial cells (Rakic and Sidman 1973; Hatten et al., 1984), but no studies have actually looked specifically for evidence of glial cell abnormalities in the *weaver* striatum. We found no evidence for altered GFAP mRNA levels at P14 or P22, however we did

detect a large induction of GFAP mRNA in the mutant striatum at P26. We speculate that the increased GFAP mRNA levels found in the weaver brain represent a glial reaction to dopamine terminal degeneration in the *weaver* dorsal striatum. In support of this idea, an upregulation of GFAP protein in the dorsal striatum occurs in response to degenerating dopamine terminals of the MPTP treated mouse (Stromberg et al., 1986; Reinhard et al., 1988). We did find a reduction of GFAP mRNA at P22 in hypothyroid mice, but not in *weaver* mice. An overall reduction of GFAP mRNA in the *weaver* striatum may have been masked by an upregulation of GFAP mRNA in reactive astrocytes. In studies of reactive glial cells in the hypothalamus, TGF–α and GFAP are coordinately upregulated (Junier et al. 1991; Junier et al. 1993), but we did not find an upregulation of TGF–α mRNA in the *weaver* dorsal striatum when GFAP mRNA levels were increased. While it may be that the molecular reaction of astroglial cells to brain injury are brain region specific; it also may be that TGF-α mRNA is not inducible by injury in the mutant glia. The fact that TGF-α is not increased in the *weaver* striatum at P26 suggests that an upregulation of TGF-α may not be a necessary component of an astrocytic reaction. Examination of TGF-α and GFAP mRNA expression in a neuroanatomical context could help to elucidate the interrelationship of these molecular events in the mutant striatum.

The reduced levels of striatal TGF-α mRNA may explain not only the premature death of the dopamine neurons through an inadequate supply of neurotrophic support, but may also explain the failure of the dopamine neurons to undergo the normal postnatal transition from the patchy innervation to the diffuse innervation (Roffler-Tarlov and Graybiel 1987; Graybiel et al. 1990). In the striatum, immature astroglial cells surround

the dopamine islands early in postnatal life and these immature glia express tenascin which is inhibitory to neurite outgrowth (Grierson et al., 1990; O'Brien et al., 1992; Wang et al., 1994). Mature astroglial cells contain cues that are supportive of dopaminergic neurite outgrowth (Prochiantz et al. 1981; Denis-Donini et al., 1984). TGF–α pre-pro-protein has been shown to be expressed in mature astrocytes (Fallon et al. 1990) and has the ability to stimulate the synthesis of neurite promoting molecules such as laminin and cytotactin (Manthorpe et al., 1983; Pixley et al., 1987). One of the ways TGF–α has been proposed to trophically influence dopaminergic neurons is to increase the adhesive properties of astroglial cells and to encourage neuron to glia contact (Alexi and Hefti 1993). It may be that TGF–α can directly provide, or indirectly induce, an adhesive substrate for dopaminergic neurites to successfully innervate the matrix compartment of the striatum during the third and fourth postnatal weeks of murine life. If our hypothesis is correct, then in the *weaver* mutant striatum where TGF-α mRNA and presumably protein levels are reduced, this morphological transition would fail.

The studies described here show that the mRNA for the putative dopaminergic neurotrophic factor, TGF-α, is significantly reduced in the dorsal striatum and hippocampus of the *weaver* mutant mouse. Also, we demonstrate that thyroid hormone reductions can mimic the change in TGF-α mRNA in the dorsal striatum, but not in the olfactory tubercle or hippocampus. The decrease of TGF-α mRNA in the dorsal striatum may be due to a region-specific alteration in the ability of the mutant to respond to thyroid hormone, as we have previously suggested (Chapter 3). On the other hand, we have shown that TGF-α mRNA is reduced in the dorsal striatum and hippocampus, and both regions are known to

experience a reduction in dopamine concentration. It may be that dopamine is able to regulate TGF-α mRNA. We speculate that both an altered response to thyroid hormone and a decrease in dopamine could contribute to the reduced TGF-α mRNA levels found in the *weaver* dorsal striatum, but further experiments are necessary to distinguish between these possibilities. Overall, our studies support the hypothesis that reduced target-derived trophic support may be a factor contributing to dopamine neuron loss in the *weaver*. It would be interesting to see if TGF-α replacement therapy may increase *weaver* dopamine neuron survival.

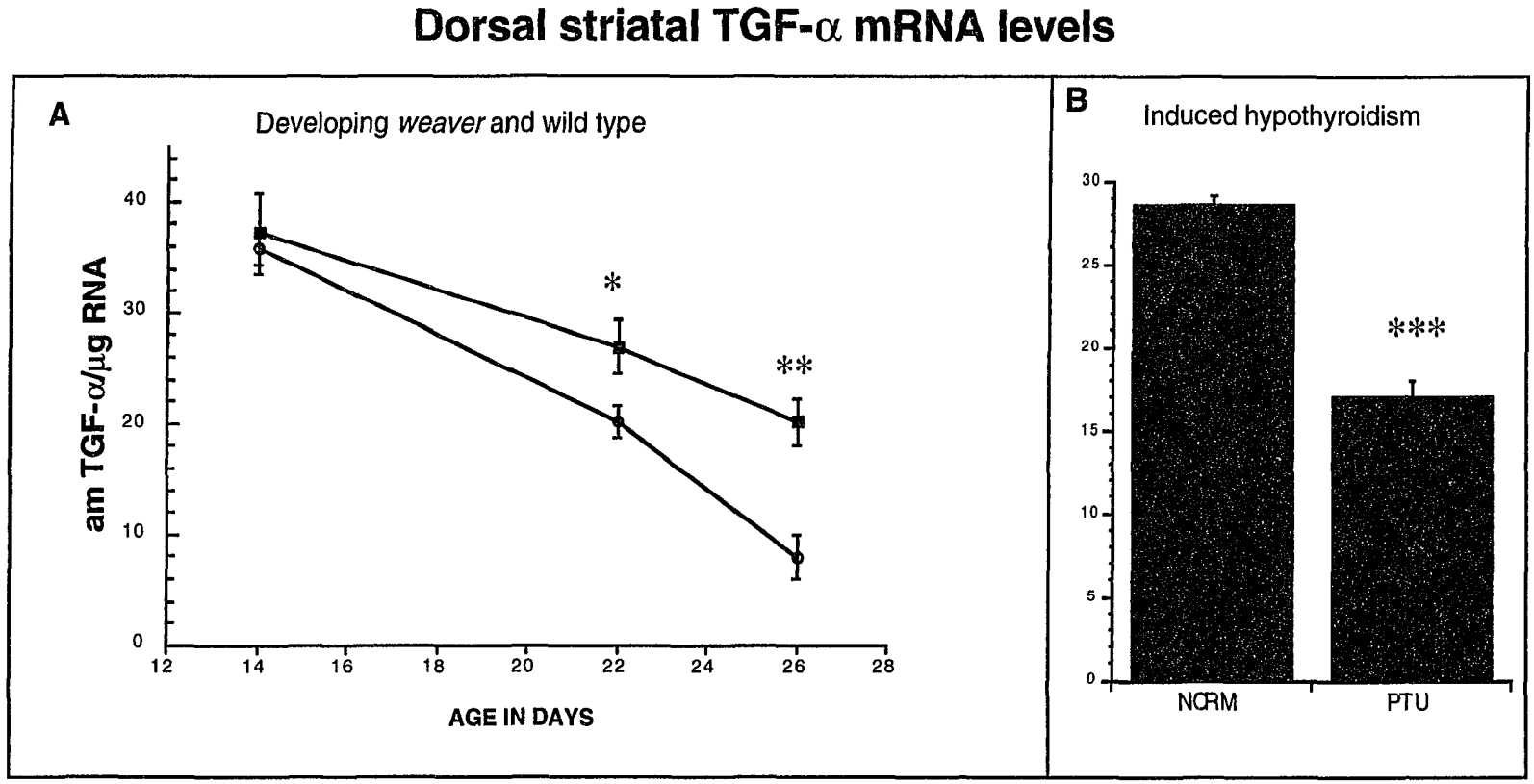

Figure 4.1. Panel A shows the mean (+/- s.e.m.) level of TGF-α mRNA for wild-type (solid squares) and *weaver* (open circles) mice at three different postnatal time points (n=4-5/group). Panel B shows the mean (+/- s.e.m.) level of TGF-α mRNA in normal mice and hypothyroid mice at P22 (n=6/group).

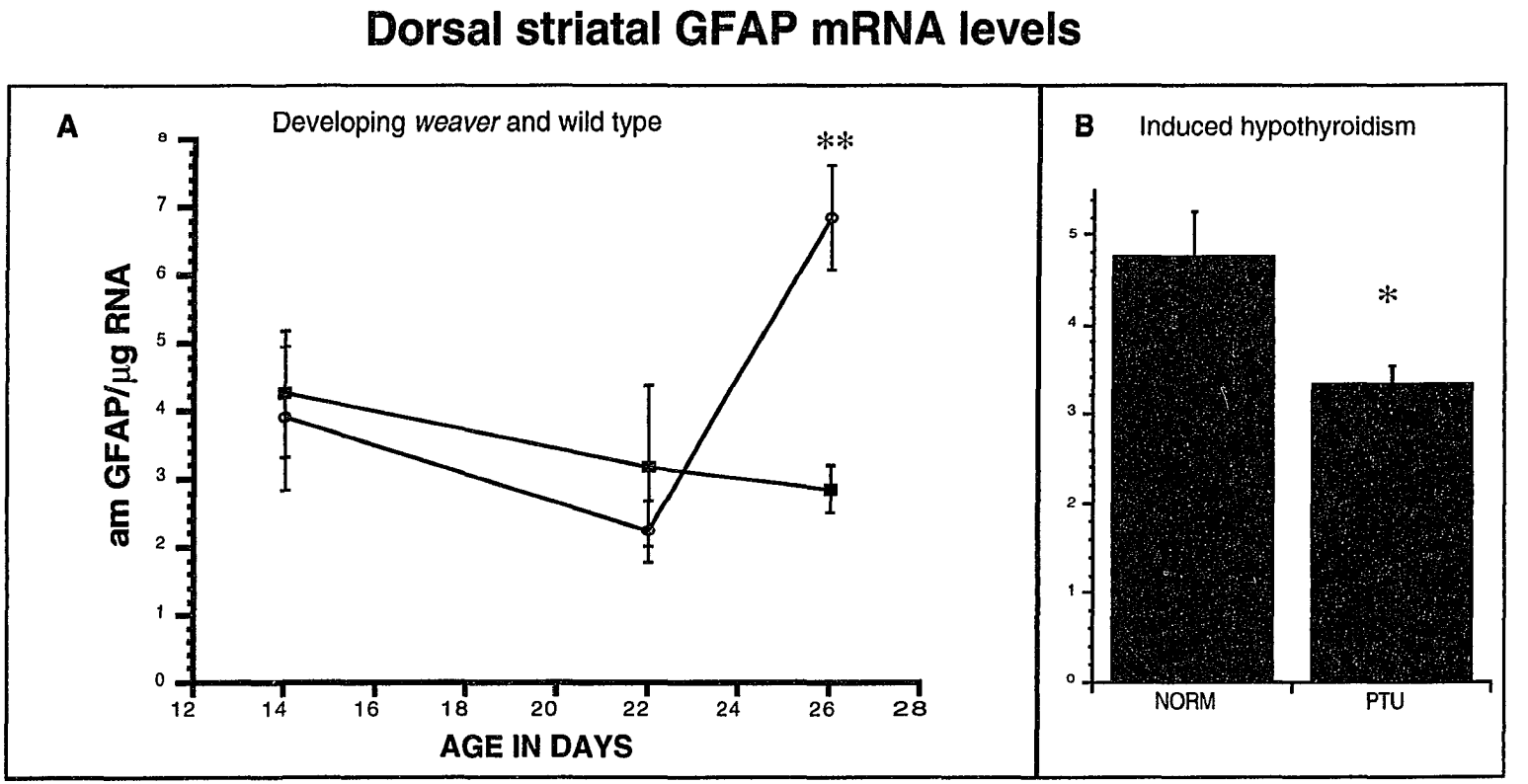

Figure 4.2. Panel A shows the mean (+/- s.e.m.) level of GFAP mRNA for wild-type (solid squares) and *weaver* (open circles) mice at three different postnatal time points (n=5-8/group). Panel B shows the mean (+/- s.e.m.) striatal GFAP mRNA level in normal mice and hypothyroid mice at P22 (n=6/group).

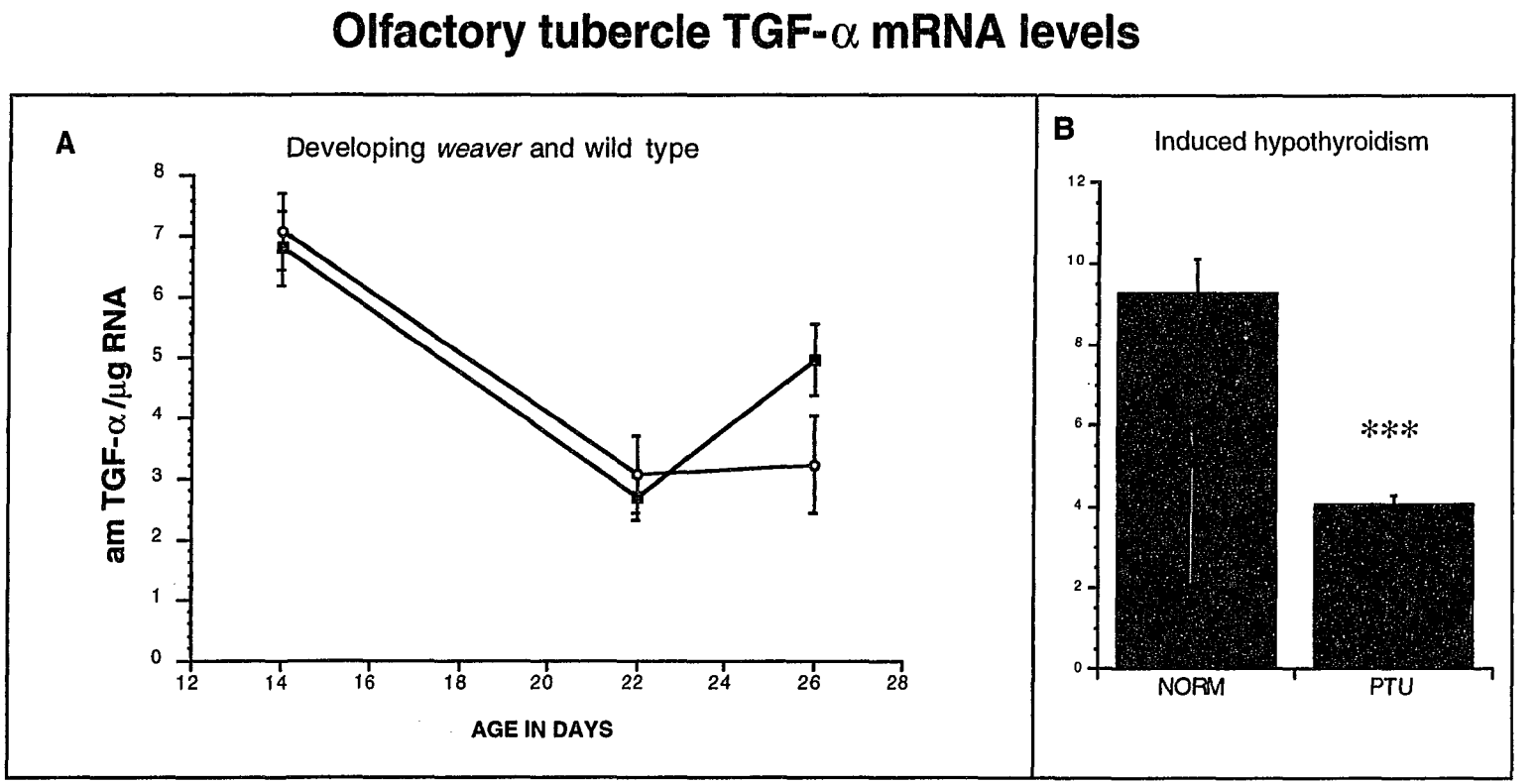

Figure 4.3. Panel A shows the mean (+/- s.e.m.) level of TGF-α mRNA for wild-type (solid squares) and *weaver* (open circles) mice at three different postnatal time points (n=4-5/group). Panel B shows the mean (+/- s.e.m.) level of TGF-α mRNA in normal mice and hypothyroid mice at P22 (n=6/group).

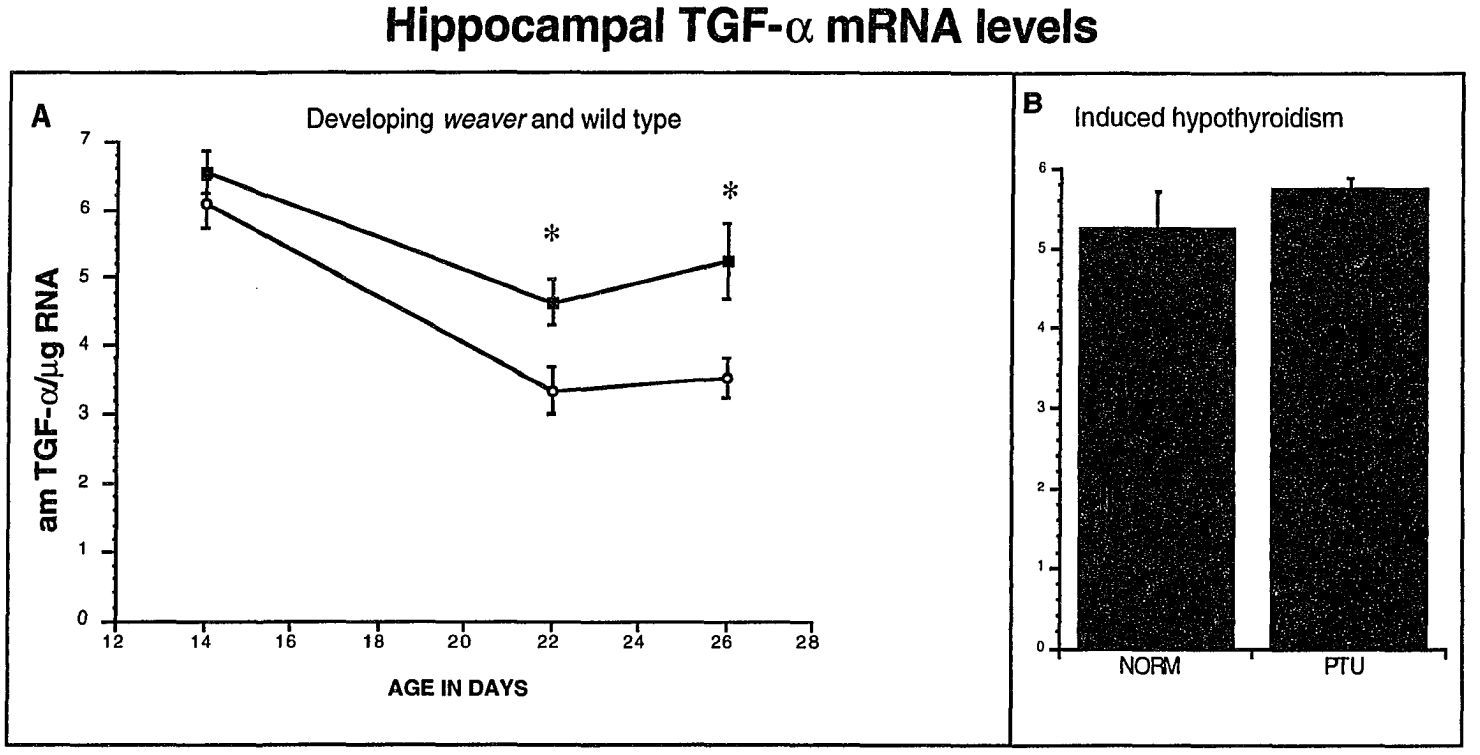

Figure 4.4. Panel A shows the mean (+/- s.e.m.) level of TGF-α mRNA for wild-type (solid squares) and *weaver* (open circles) mice at three different postnatal time points (n=4-5/group). Panel B shows the mean (+/- s.e.m.) level of TGF-α mRNA in normal mice and hypothyroid mice at P22 (n=6/group).

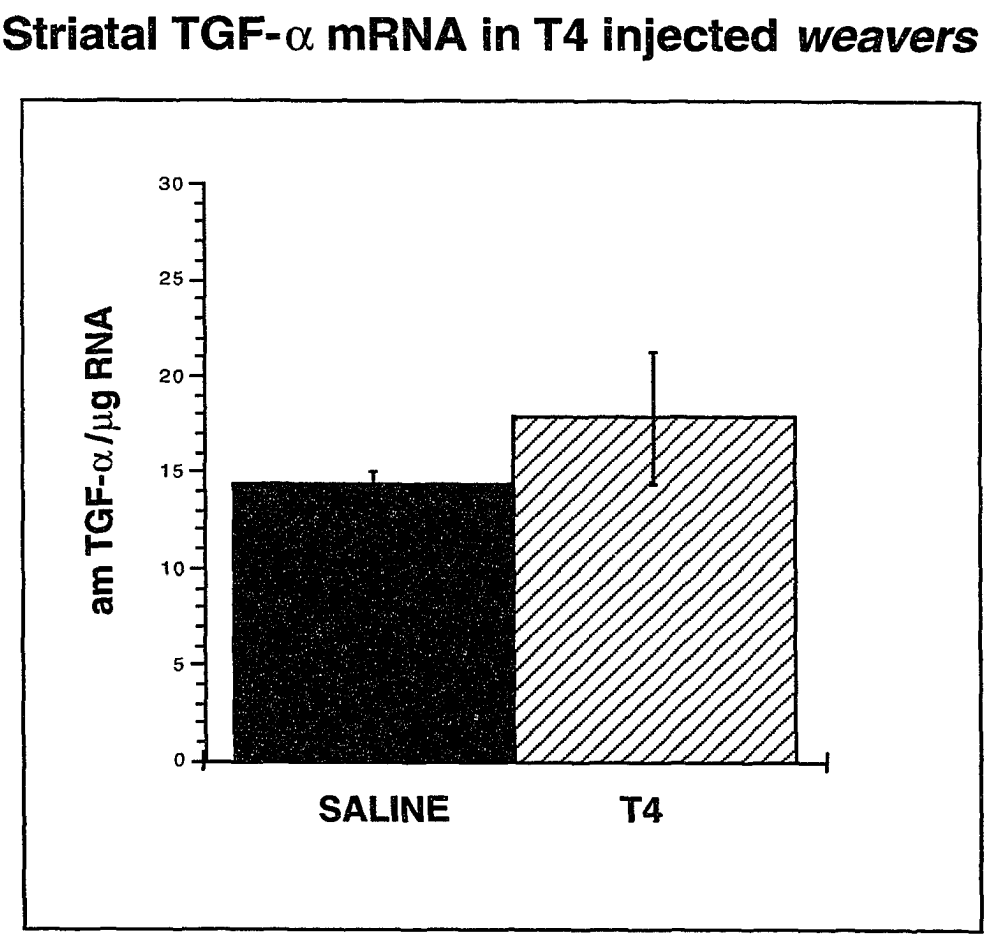

Figure 4.5. TGF-α mRNA levels expressed in attomoles/μg total RNA in *weaver* mice injected with saline (solid bar, n=4), or with thyroid hormone (stripped bar, n=4).

CHAPTER 5

General Discussion

Mammalian brain development proceeds by an exponential increase of multi-potent precursor cells followed by progressive restriction of cell fate. There is a multitude of hormonal signals, cell contact mediated signals, and local diffusable signals which interact to specify the complement of genes to be expressed in a given cell. The cellular pattern of gene expression ultimately determines cellular phenotype. Molecular factors not only provide signals to brain cells during development, but are likely to function to maintain cell and tissue integrity throughout life. Thus, any given factor be it endocrine, juxtacrine or paracrine may serve multiple roles during the life of an organism. The reports presented here just begin to define some aspects of the complex interplay between hormonal factors and growth factors during the postnatal development of the basal ganglia, and show that any one molecule, such as TGF-α, is likely to have more than one function depending on the developmental time period under study.

Simple organisms such as *Caenorhabditis elegans (C. elegans)* and *Drosophila melanogaster* have been used to identify molecules involved in cell proliferation and differentiation of multi-cellular organisms. Membrane bound tyrosine kinase receptors play a key role in mediating critical cell to cell interactions during the specification of cell fate (for review, see Pawson and Bernstein 1990). Multipotent precursors in the vulval cell lineage of *C. elegans* respond to inductive signals provided by the lin-3/let-23 proteins (Aroian et al., 1990; Hill and Sternberg 1992; Sommer et al., 1994). The mammalian homologue of the let-23 protein is most likely EGF-R (Aroian et al. 1990; Aroian et al., 1994) and the lin-3 gene product is transmembrane protein containing a single extracellular EGF-like repeat, and thus is similar to members of the mammalian EGF

family, which includes EGF and TGF-α (Hill and Sternberg 1992). The evidence provided in this thesis supports the concept that this signaling pathway is employed in the development of such complex structures as the central nervous system of the more recently evolved mammals. Thus, there appears to be a functional conservation of the molecular signals used to generate cells of multi-cellular organisms throughout evolution.

This thesis contributes information about molecular events involved in postnatal development of the murine striatum. These studies focused on two phases of postnatal development, a neonatal period and a juvenile period. The neonatal period encompassed the time of birth (P0) until the end of the second postnatal week of life (P14), and the juvenile period spanned the third and fourth postnatal week, from P14 until P26. Rodents are atricial at birth, and during the neonatal period their brain is relatively immature. At birth, the majority of neurons residing in the caudate-putamen have been generated and are situated within the parenchyma of the developing striatum, but a majority of astrocytes and oligodendrocytes are still being generated in the subependymal layer (SEL) during the neonatal period. One of the major striatal afferent systems, the dopamine projection reaches the caudate-putamen several days before birth, although the innervation pattern is not adult-like. The morphology of dopamine terminals matures during the juvenile period. This thesis presents *in vivo* evidence that TGF–α may stimulate mitogenesis and may also serve as a neurotrophic factor for dopamine neurons.

Comments on findings for the neonatal period

One of the major findings of this thesis is that striatal transforming growth factor alpha (TGF–α) provides a mitogenic signal to dividing glioblasts in the SEL (Chapter 1). Yet, nothing is currently known about what form of TGF–α protein mediates this events; TGF-α could be in the membrane bound form or the secreted form at P6. During the time of rapid glial cell generation, striatal neurons would be the likely source of TGF–α, since the mRNA for TGF–α has been localized to the striatal neurons at P6 (Seroogy et al. 1993). However, the localization of the mature TGF–α protein has not been reported in the neonate. It may be that neural-derived TGF–α diffuses across the striatal tissue to stimulate proliferation via the secreted form. Alternatively, TGF–α may signal cell proliferation via the membrane bound form. In this case only those SEL cells reaching the outskirts of the proliferative zone and physically abutting differentiated neurons would be stimulated to divide. This seems unlikely, as many cells well within the boundaries of the SEL are marked with BrdU (Chapter 2).

Model of TGF-α action in the neonatal period

EGF/TGF-α was first characterized as a mitogen when it was added to cultured cells in a processed, secreted form. It is well established that the secreted form of EGF/TGF-α is a mitogen, but little is known about the mitogenic potential of the membrane-bound form and it may be that in this form TGF-α actually functions as a differentiation factor as suggested by Seroogy et. al. Also, cell membranes of differentiated neurons have been shown to contain a factor(s) that can suppress astroglial proliferation (Hatten et al. 1984; Hatten 1987). In order to address both of the

proposed functions of TGF-α, we suggest the following model: at P6, TGF–α is made by differentiated neurons and can be found in both the secreted and membrane bound forms. The secreted form of TGF–α would diffuse from a source, the striatal parenchyma, to a sink, the lateral ventricle. Along this route, TGF–α would encounter the precursor cells in the SEL, which are equipped with the EGF-R (Chapter 2, (Seroogy et al. 1995), and these multipotent stem cells would respond to the secreted form of TGF–α with a proliferative response. Cell numbers would increase and cells lying along the edge of this proliferative zone would be "pushed up" against differentiated neurons of the striatum. Once the dividing precursor contacted the differentiated neurons, the EGF-R on the dividing cell surface could bind to the membrane bound form of TGF–α on the surface of the differentiated neurons. This binding could serve to stimulate differentiation by blocking the ability of the secreted form of TGF–α to stimulate proliferation. In this way, TGF–α could be involved in signaling both division and differentiation of glioblasts in the central nervous system. Often proliferation rates are linked to rates of differentiation, and further studies which investigate the presence of the membrane bound versus the secreted form of TGF–α along with studies in which the neuroanatomical location of these proteins could be mapped are necessary to fully interpret the precise molecular mechanism by which TGF–α stimulates stem cell proliferation in the postnatal murine brain.

Comments on findings for the juvenile period

During the juvenile period, intrinsic striatal neurons and astrocytes as well as striatal afferents are undergoing their final stages of maturation. The cholinergic interneurons of the striatum increase in soma size during

the second and third postnatal week of life (Woolf and Butcher 1981; Gould et al., 1991). Also, the activity of ChAT increases by some four-fold during this period (Hashimoto et al. 1994). At P14, the number of proliferating cells in the SEL has decreased considerably, and a majority of striatal astrocytes are maturing as marked by increases in GFAP expression (Smart 1961; Smart and Leblond 1961; Sturrock and Smart 1980); Chapter 2). During the third week of life, the dopamine concentration in the striatum increases by 440% and the dopaminergic innervation of the striatum undergoes a morphological shift (Roffler-Tarlov and Graybiel 1987; Graybiel et al. 1990). The island-like innervation, characteristic of the striatum at birth, begins to expand into the matrix compartment (Olson et al. 1972; Tennyson et al. 1975; Roffler-Tarlov and Graybiel 1987; Graybiel et al. 1990). The postnatal sprouting of dopaminergic axons, burst of cholinergic neuron development, and increase in glial maturation are coincident in time with the rise in serum thyroid hormone levels. There is abundant evidence that thyroid hormone is involved in the maturation of the cholinergic neurons (Rastogi and Singhal 1979; Kalaria and Prince 1985; Patel et al. 1988), and in the maturation of astroglial cells (Ruel and Dussault 1985; Aizenman and de Vellis 1987; Gould et al. 1990; Faivre-Sarrailh et al. 1991; Gavaret et al., 1991). Also, experimentally -induced hypothyroidism can cause a significant reduction in dopamine content and can interfere with lesion induced sprouting events in the striatum (Rastogi et al. 1976; Gottesfeld et al. 1987). It may be that thyroid hormone is an important underlying factor which contributes to the overall coordinate maturation of the basal ganglia system during the juvenile period.

Thyroid hormone, which affects brain cells by binding to nuclear receptors of the steroid and thyroid hormone superfamily, can direct transcriptional events and regulate programs of gene expression (Evans 1988). The mechanism of thyroid hormone action is complex because the activated thyroid hormone receptor does not exert effects in isolation, but rather is part of a complex machinery in which many different transcriptional elements can interact to exert combinatorial control over activation of gene expression (He et al., 1989; Lazar 1993; Williams 1994; Williams 1994). The precise transcriptional control depends on the complement of transcription factors present in any one cell; which is determined by the developmental history of the cell. Thyroid hormone acting during postnatal development is a relatively late event in cell fate restriction and many cells have already diverged down specified developmental pathways. Therefore, each cell type in the brain will possess a unique set of transcription factors and will exhibit a unique response to thyroid hormone. In this context, it is easily appreciated that the effects of thyroid hormone will vary with respect to brain region and time window under study. Our studies in chapter 3 and 4 support the concept that the effects of thyroid hormone on gene expression within the brain are region and time specific. The molecular effect of thyroid hormone on gene expression in the developing brain will depend on more detailed information concerning the combinatorial actions of the ever-expanding array of nuclear transcriptional factors. Identification of the *weaver* gene may ultimately give insight into events down-stream of thyroid hormone action that are critical to the development of both granule and midbrain neurons.

Models of trophic support have proposed that growth factors are made in the target fields of neurons and many growth factors have been shown to be synthesized in target regions of midbrain dopamine neurons. Further, many different growth factors have been shown to be trophic for dopamine neurons in culture (see Casper 1995 for review). This raises the following question: why are there so many different dopaminergic trophic factors which are all potentially available to the dopamine neurons *in vivo*? We provide evidence that a putative dopaminergic trophic factor can have more than one function *in vivo*, and we speculate that different growth factors may each act on dopamine neurons at distinct times in development. The distinct temporal expression profiles of aFGF, bFGF. BDNF, GDNF and TGF-α mRNA support this concept (Blum et al. 1994). We used the *weaver* mutant mouse model of nigrostriatal degeneration to demonstrate that TGF-α mRNA is made in limiting amounts in the targets of dopamine neurons during the nigrostriatal degeneration seen in the *weaver* (Chapter 4). We also have found that the synthesis of other putative dopaminergic neurotrophic factor mRNA's, BDNF and GDNF, are not reduced in the *weaver* striatum at this time ((Blum and Weickert 1995) and (unpublished observations). It may be that GDNF or BDNF are important for dopaminergic survival at earlier or later points in the life history of dopamine neurons.

Model of TGF-α action in the juvenile period

When dopamine neurons first contact the developing striatum at E14.5, they synapse on striatal neurons which have been shown to provide a stop signal for dopaminergic outgrowth (Olson et al. 1972; Tennyson et al. 1975; Denis-Donini et al., 1983). From the time the dopamine afferents

finish arriving in the striatum (around the time of birth) until the end of second postnatal week of life they are held in this clumped immature innervation pattern (Graybiel et al. 1990). During the first two weeks of murine life, development of the dopamine neurons is arrested and a majority of glial cells are being generated and are beginning the maturational process (Chapter 2). In the normal striatum, the glial expression of TGF–α is upregulated during the third postnatal week (Seroogy et al. 1993) This astroglial TGF-α upregulation not only marks the maturation of glial cells, but may also function to stimulate/support dopaminergic neurite outgrowth from the concentrated islandic innervation to the homogeneous innervation of the mature striatum. As many studies have identified glial cells as the source of dopaminergic trophic molecules (see Casper 1995, for review), this transition may enable the dopaminergic terminals to derive trophic support from a wider spectrum of cells including mature glial cells. We speculate that in the *weaver* striatum, when TGF–α mRNA levels are reduced, the *weaver* dopamine neurites would fail to make the developmental transition necessary to derive full glial trophic support and inevitably degenerate as a result.

Future Directions

Little is actually known about the molecular nature of glia-neuron trophic interaction *in vivo,* and further studies examining the expression of glial derived factors within a neuroanatomical and developmental context are necessary to begin to define the molecules mediating dopaminergic axonal growth/maturation *in vivo*. A logical follow-up study to the work presented here, would be to examine the TGF–α deficient

mice, *waved-1*. First, to determine if striatal TGF–α mRNA is altered during the juvenile period, and if so to ascertain if the developmental transition in the dopaminergic innervation pattern of the striatum is normal. Also, it would be interesting to map the cellular localization of the different forms of the TGF-α protein during the neonatal and juvenile period. A low-dose injection of MPTP in the mouse, where initial dopaminergic toxicity is followed by dopaminergic sprouting, could be used to further elucidate the role of TGF-α in dopaminergic neurite outgrowth. One could evaluate the time course and localization of TGF-α mRNA expression in relationship to reactive gliosis and regeneration. A better understanding of the molecular events surrounding the sprouting of dopaminergic neurons in both normal development and in lesion models would have important clinical implications.

Potential Clinical Relevance

Nigrostriatal dopamine neurons are known to degenerate in Parkinson's disease brains, and the progressive loss of dopamine neurons appears to underlie the deterioration in motor functioning of these patients. In order to develop treatments for this disease, research efforts are aimed at determining ways to prevent or delay the death of the endogenous dopaminergic neurons or to transplant exogenous dopaminergic neurons into dopamine depleted striatum. In either case, it is useful to identify trophic molecules that can encourage the survival of dopaminergic neurons and/or induce the sprouting of dopaminergic neurites. Our studies show that TGF–α is a factor deserving of further attention in this regard.

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