HOMOLOGUES OF THE HOMEOTIC AND SEGMENT POLARITY GENES ARE INVOLVED IN THE POSTNATAL DEVELOPMENT OF THE CAT VISUAL SYSTEM

by

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ABSTRACT

This thesis investigates the possible involvement of the homologues of the drosophila developmental genes in the postnatal development of the cat visual system. Initially, the cDNA's of homeotic genes *PBX1, PBX2* and the homologues of the segment polarity genes *BMP4, BMP6, BMP type II receptor, Wnt-1*, and *FrzB* were partially cloned in the cat. We report that the mRNA expression of these genes is developmentally regulated in the postnatal cat visual cortex. To further substantiate our hypothesis that the homologues of the drosophila developmental genes contribute to the postnatal development of the cat visual cortex, the expression of the beta-catenin protein was characterized in the visual system of normally developing and deprived kittens. The beta-catenin protein, which is a downstream effector of the Wnt-1 signalling pathway, is capable of functioning both as a transcription factor and a cell adhesion molecule. Consistent with its characterized role as a transcription factor, the beta-catenin protein becomes nuclearized in LGN neurons at the end of the period for thalamocortical plasticity. Hence, one of the putative functions of the beta-catenin protein in postnatal visual development is proposed to be ending thalamocortical plasticity. The role of the beta-catenin protein in cellular adhesion is to anchor the cadherin cell adhesion molecules to the actin cytoskeleton. Interestingly, the beta-catenin/cadherin cell adhesion system in neurons is located at synapses. Fittingly, both the cadherin and the beta-catenin proteins are expressed in the neuropil of the geniculate and the visual cortex and this expression is prominent in layer IV of the visual cortex. In addition, the temporal expression of these proteins correlates with the critical period. Furthermore, neuropil expression of beta-catenin and cadherin proteins is altered in the LGN in response to monocular deprivation. This finding suggest a role for these molecules in the competition occurring between X- and Y- cell arbours. In summary, beta-catenin appears to act as a multi-functional protein and contribute to different facets of postnatal visual development in the cat. These

findings endorse our original hypothesis that the homologues of the drosophila developmental genes are involved in the development of the cat visual system.

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INTRODUCTION

1.1 GENERAL INTRODUCTION

The visual areas of the brain from various species ranging from goldfish to primates have been extensively studied (Levine et al. 1993, Gaze et al. 1972, Shatz et al. 1978, Hubel et al. 1969, Kennedy et al. 1980, Van Sluyters et al. 1974, Maffei et al. 1992, Gordon et al. 1996, Meister et al. 1991). These studies which were initially undertaken to find remedies for a variety of different visual ailments have coincidentally contributed greatly to our understanding of overall brain development and behavior. For example, the activity dependent nature of the developing visual system (Wiesel et al. 1963, Hubel et al. 1977) has emphasized the importance of environmental factors and sensory experience during normal brain development. The activity dependent synaptic rearrangements identified in the visual system are a general characteristic of the developing brain and parallels are seen in the development of numerous brain areas (Webster et al. 1983, Meisami et al. 1981, Westrum et al. 1986, Wooseley et al. 1976, Garraghty et al. 1992, Pons et al. 1991). The examination of the mechanisms responsible for these synaptic rearrangements has provided significant insight into the manner in which neural circuits are formed and refined. Additionally, processes similar to synaptic rearrangements occurring during the development of the visual system are postulated to be the biological basis of learning and memory (Bienenstock et al. 1982, Bear et al. 1987). Clearly, the knowledge obtained from these studies is not merely limited to the visual system but instead, the mechanisms involved in visual development are applicable to general brain development and function.

Despite the wealth of knowledge available about the physiology and the anatomy of the developing kitten visual cortex, very little is known regarding the molecular mechanisms responsible for this event.

HYPOTHESIS: The homologues of the drosophila developmental genes are involved in the postnatal development of the kitten visual cortex.

The developmental genes initially characterized in the drosophila are highly conserved in all metazoa and play a prominent role in a large number of developmental events. In order to prove our hypothesis, the expression of some of the drosophila developmental gene homologues were examined by RT-PCR and Northern blot analysis during the critical period in kittens. To further verify our hypothesis the properties of one these developmental genes, *beta-catenin*, was extensively characterized in the developing visual system. The findings reported in this thesis suggest beta-catenin, which is the mammalian homologue of the drosophila *armadillo* gene, and the cell adhesion system it gives rise to with the cadherin molecules contributes to multiple facets of visual system development in the cat.

1.2 THE CHOICE OF THE CAT AS AN ANIMAL MODEL

There are both benefits and problems associated with using the cat as an experimental animal model in visual neuroscience. Historically, the cat has been a popular model animal in neuroscience research. Accordingly, the physiology and the anatomy of the cat brain is well characterized. Compared to mice and rats the organization of the cat visual system closely resembles that of the primate and therefore, the cat is a more relevant animal model to study than rodents (LeVay et al. 1978, Shatz et al. 1978, Gordon et al. 1996, Fagiolini et al. 1994). Additionally, compared to the primates, the cat visual system

is fairly immature at birth and hence, the cat provides a much more convenient system to examine early events in visual development (LeVay et al. 1978, Shatz et al. 1978). Also, cats are relatively inexpensive to purchase and to house compared to primates. Finally, since the visual areas of the cat brain develop more rapidly than the primate brain, time dependent studies are much easier to perform on these animals (Hubel et al. 1970, Hubel et al. 1969, Hubel et al. 1977, Blakemore et al. 1974, LeVay et al. 1978, Shatz et al. 1978). Unfortunately, the cat is not a suitable animal model for molecular biology. Gene replacement or gene knockout experiments are difficult to perform in cats because homologous recombination occurs very rarely in cells of highly complex animals (Alberts et al. 1994, Cappechi 1989). In addition, the cost associated with establishing transgenic cat strains is unreasonably high. Hence, gene expression in the cat can not be manipulated by using standard molecular biological techniques. Since the cat is not a suitable genetic animal model, very little information is available regarding the sequence of cat genes. The above statements outline the current dilemma in visual neuroscience. The cat possesses a sophisticated visual system, resembling that of the primate, which is well characterized both at the neuroanatomical and neurophysiological levels. However, this system can not be manipulated at the molecular level. Therefore, when choosing an animal model to study molecular events associated with visual development, one must sacrifice either genetic modifiability or work with a simpler visual system lacking many of the attributes found in humans.

This thesis has chosen the cat as its animal model because the focus of this research is to identify molecular mechanisms involved in the visual development of highly complex animals. The fact that cat cDNA sequence information is lacking is not a critical problem because many of the important neural molecules are highly conserved and these genes can be easily cloned in the cat by exploiting the extensive sequence homologies present amongst species. Similarly, many antibodies produced for use in other animals will cross react with cat proteins due to the high level of evolutionary conservation. The difficulties

associated with modifying gene expression in complex animals is the main problem with using the cat as an experimental model. Because of this reason, experiments aimed at determining gene function during development can not be performed on these animals. On the other hand, cats can be subjected to visual deprivation studies which will provide pertinent information regarding the behaviour of a particular molecule during visual development. This information can be used to form hypothesis about the function of the molecule involved.

1.3 ANATOMY AND PHYSIOLOGY OF THE DEVELOPING CAT VISUAL SYSTEM

1.3.1 Normal Development

The eyes of the cat are located in front of the head and face forward which results in the overlap of the two visual fields required for binocular vision. The cat visual system exhibits "contralateral bias" because the majority of the retinal ganglion cells (about 60%) from each eye cross the midline at the optic chiasm and make connections with the contralateral lateral geniculate nucleus (LGN) (Wiesel et al. 1963) (See figure 1.1). The remaining retinal neurons make connections with the ipsilateral LGN. Although each LGN receives input from both eyes, the connections from each eye are kept segregated in eye specific layers. Layer A of the LGN mainly receives input from the contralateral eye whereas layer A1 receives input from the ipsilateral eye and layer C receives connections from both eyes (Guillery 1970a). The LGN neurons connect to layer IV and to layer VI of the visual cortex. The segregation of inputs found in the lateral geniculate nucleus is retained in the cortex where the thalamocortical connections from the LGN form an alternating interdigitated series of eye specific columns referred to as "ocular dominance columns" (Hubel et al. 1969, Hubel et al. 1972, LeVay et al. 1978). Ocular dominance

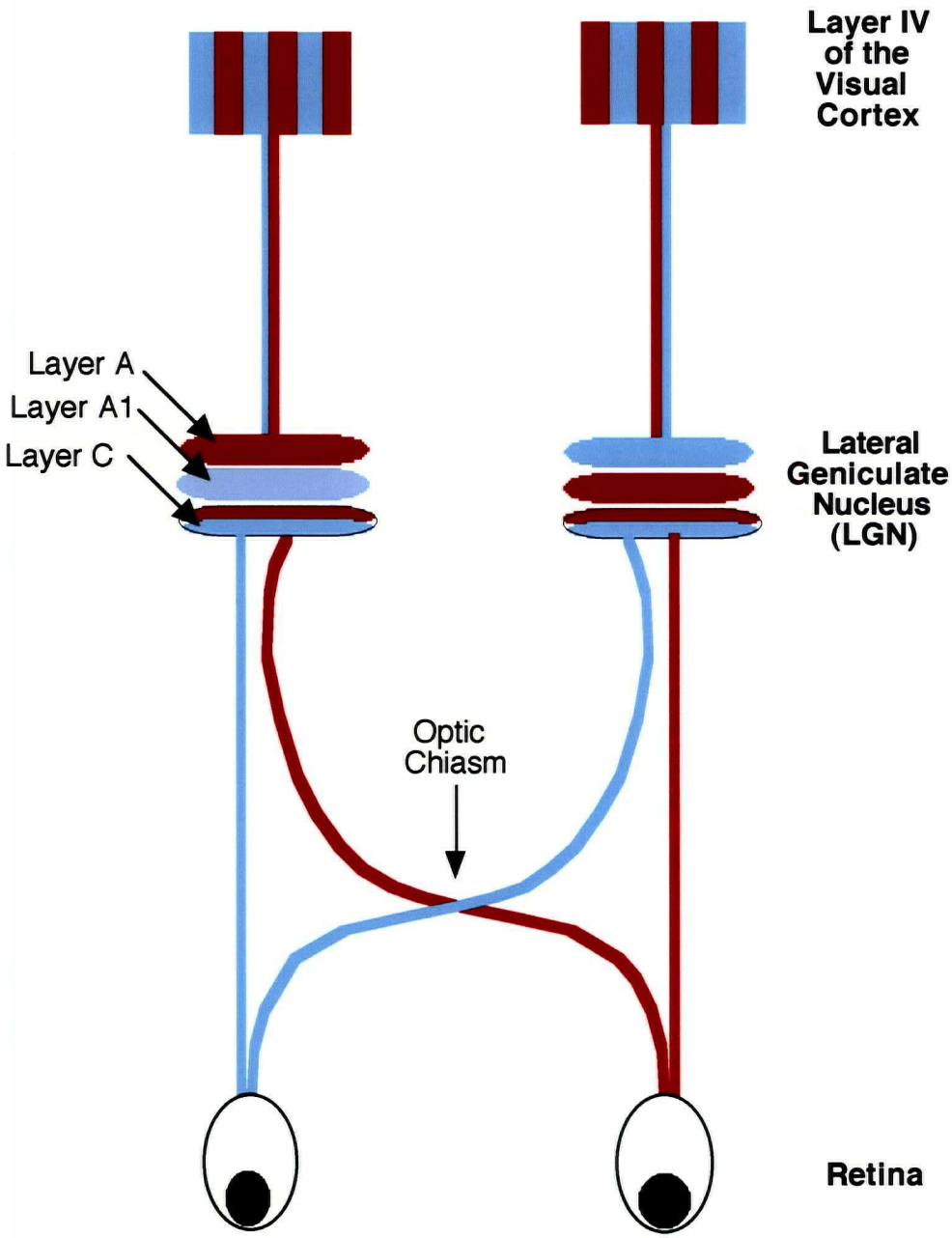

Figure 1.1 Anatomy of the Cat Visual System. The red and the blue colored lines represent the neural inputs originating from the two eyes. The inputs from the two eyes are segregated into eye specific layers in the LGN and ocular dominance columns in the visual cortex.

columns are roughly 400 - 600 microns wide and respond mainly to stimuli from only one of the eyes (Hubel et al. 1977, LeVay et al. 1985, Lowel et al. 1987, Swindale 1988). As determined by tracer injection experiments, ocular dominance columns are anatomically most prominent in layer IV and VI of the cortex. However, physiological experiments have established the presence of functional columns in layer II and III of the cortex (Weisel et al. 1963). Physiological experiments have determined that cells located in the centers of ocular dominance columns are largely monocular and respond only to stimuli from one eye whereas cells bordering two adjacent ocular dominance columns are mainly binocular and respond equally well to stimuli from either eye (Shatz et al. 1978, Shatz et al. 1984). Interestingly, neither eye specific layers in the LGN (Shatz et al. 1983, Shatz et al. 1984) nor ocular dominance columns (Shatz et al. 1978) are initially present in these structures. Synapses from either eye are intermixed at first and they become segregated by simultaneous strengthening and elimination. Fortunately, ocular dominance formation in the cat visual cortex occurs postnatally and the cat provides a convenient model system for the study and manipulation of synaptic reorganization (LeVay et al. 1978, Shatz et al. 1978).

A newborn cat's visual cortex is immature to the point that migratory events associated with the formation of cortical layers are not completed (Shatz et al. 1986). The ventricular zone is present at birth and cells responsible for the formation of layers 2/3 are in the process of migrating to their proper locations in the cortical plate. By this time, the connections from the LGN have grown into the subplate and await the completion of layer formation (Shatz et al. 1986, Friauf et al. 1990). A role for the subplate in ocular dominance column development is suggested by experiments which report that the ablation of this structure in early development prevents the formation of ocular dominance columns (Ghosh et al. 1990, Ghosh et al. 1992a, Ghosh et al. 1992b, Herrmann et al. 1994). Around 2 to 3 weeks of age, cortical layers become fully formed and the thalamic afferents from the LGN innervate layer IV of the cortex. Synaptic rearrangements do not occur prior

to the completion of cortical innervation by thalamic inputs from the subplate (Wiesel et al. 1963, Hubel et al. 1970). Thalamo-cortical connections in the cortex are initially non-segregated with regards to the two eyes. Instead, these connections are arranged in a retinotopic manner in the visual cortex of young kittens (LeVay et al. 1978, Tusa et al. 1978). The cues governing the formation of these initial LGN connections in the cortex are believed to be mainly chemo-attractant and chemo-repellent molecules (such as netrins and semaphorins) because these connections strictly avoid layers other than IV and VI in the cortex and thalamocortical connections are made in a retinotopic manner. Furthermore, the initial thalamo-cortical connections form independent of activity (Stryker et al. 1986, Reiter et al. 1986). By 3 to 4 weeks of age inputs from the two eyes become segregated and ocular dominance columns are established (Levay et al. 1978).

1.3.2 Activity Dependent Nature of the Developing Visual system

The most striking aspect of ocular dominance column development is its dependence on normal visual activity. If visual activity to only one eye is disrupted in a young kitten (induced by eyelid suture or patching and referred to as monocular deprivation), the normal eye expands the cortical territory of its ocular dominance columns by invading the cortical area normally occupied by the occluded eye (Shatz et al. 1978, LeVay et al. 1978, Wiesel et al. 1963, Hubel et al. 1970). This situation gives rise to a set of wide and narrow columns in the cortex which correspond to the open and deprived eye respectively. From a cellular point of view, during monocular deprivation thalamic neurons corresponding to the open eye expand their synaptic connections with cortical neurons at the expense of thalamocortical synapses belonging to the deprived eye (LeVay et al. 1978). Hence, in a monocularly deprived animal most cells in the cortex are responsive to stimuli from the open eye (Shatz et al. 1978). The severity of this effect is dependent on the length of the deprivation and the age of the animal (Hubel et al. 1970, Shatz et al. 1978). The period of time in which cats are susceptible to visual deprivation, called the "critical period", is considered to begin roughly 3 weeks after birth and end around 3

months of age (Wiesel et al. 1963, Hubel et al. 1970, Olson et al. 1980). However, the 3 week to 3 month time period is only a rough estimate of overall plasticity in the cat visual cortex and the length of the critical period varies significantly between cortical layers (Daw et al. 1992). Thalamocortical connections found in layer IV lose their ability to reorganize at around 50-60 days of age (Mower et al. 1985). The remaining layers II, III, V and VI exhibit a gradual reduction in plasticity beginning at 6 weeks of age until the end of the critical period which is approximately at 15 months of age (Daw et al. 1992). Thirty days of age is designated as the height of the critical period based on single unit recording experiments performed in the cortex of monocularly deprived animals (Hubel et al. 1970, Movshon et al. 1977, Olson et al. 1975). These experiments show that the most drastic and rapid synaptic shifts in response to monocular deprivation occur in 30 day old kittens. Ocular dominance shifts following monocular deprivation are more prominent in the cortical hemisphere contralateral to the deprived eye because in the cat the majority of the ganglion cells cross the optic chiasm and connect to the contralateral hemisphere (contralateral bias) (Hubel et al. 1963). After the critical period, the disruption of visual activity does not induce significant shifts in ocular dominance (Daw et al. 1992). However, rearing kittens in the dark can prolong the critical period and maintain plasticity in older animals (Cynader et al. 1976, Cynader et al. 1980, Cynader 1982). The absence of light seems to slow down the critical period but does not completely stop the age dependent loss of visual plasticity. Although dark reared adult cats undergo ocular dominance shifts in response to monocular deprivation, the extent of these shifts in dark reared cats is significantly less then those induced by monocular deprivation in normally developing young kittens (Cynader 1982, Mower et al. 1985, Mower 1991). In addition most of the cells exhibiting ocular dominance shifts reside in layer 2/3 of the cortex as opposed to layer 4. Interestingly, ocular dominance columns do not form in dark reared cats as long as they remain in the dark (Swindale 1981, Swindale 1988, Mower et al. 1985) although this issue is a point of contention (Stryker et al. 1986). Additionally, the

visual system of a dark reared cat matures and loses its plasticity quite rapidly once it is exposed to light (Swindale 1988).

1.3.3 Competitive Nature of the Developing Visual System

Based on the widening and shrinking of cortical ocular dominance columns in response to monocular deprivation, the two eyes are proposed to compete for cortical space during normal development (Wiesel et al. 1965, Guillery et al. 1970b, Guillery 1972, Sherman et al. 1974, Sherman et al. 1976). Support for this idea comes from reverse suture studies in which one eye is initially occluded and then opened at a later date while the originally non-occluded eye is then deprived of input (Blakemore et al. 1974, Blakemore et al. 1978). If the originally deprived eye is opened and allowed to receive normal visual stimulation while the originally open eye is sutured shut, then the originally deprived eye begins to recover some of the cortical territory it has lost. If this procedure is performed early enough during the critical period, the ocular dominance columns that receive input from the initially deprived eye not only recover to their normal size but expand into the area of the originally non-occluded eye. If this procedure is performed later on in the critical period, the ocular dominance columns only recover to their normal width. Reverse suturing following the critical period does not yield a detectable change from the normal pattern. The presence of activity dependent competition for cortical space during visual system development is further verified by findings in the xenopus nervous system. The retinal ganglion cells in the frog completely cross the midline and innervate the contralateral tectum (which is the primary visual area in the frog) in a retinotopic manner (Gaze et al. 1972, Scalia et al. 1974). Since a tectum normally receives input from only one eye there is no competition between the two eyes for tectal space. If a third eye is grafted onto a tadpole then the axons originating from the retinal ganglion cells of that eye grow and innervate one of the tecta (Constantine-Paton et al. 1978). Alternatively, one of the tecta can be ablated, forcing axons from both eyes of the frog to grow into the non-lesioned tectum (Law et al. 1980). In tecta that are innervated by two eyes, the connections from

each eye segregate into eye specific columns which are reminiscent of the ocular dominance columns found in cats and primates (Constantine-Paton et al. 1978, Law et al. 1980). These tectal columns exhibit the same activity dependent characteristics as the ocular dominance columns (Reh et al. 1985). In addition, the blocking of NMDA receptors in the tectum of these frogs prevents the formation of eye specific columns (Cline et al. 1987, Cline et al. 1990). These findings support the notion that in the developing visual system activity dependent competition exists between two sets of connections. Additionally, these findings argue against intrinsic mechanisms being involved in the formation of ocular dominance columns because segregation does not occur in the absence of activity. Furthermore, a normal frog is unlikely to have evolved specialized molecular machinery for the formation of eye specific columns because each tectum of the frog normally receives input from only one eye.

The model put forth to explain the formation of tectal columns in the three eyed frog is best summarized by the phrase "neurons that fire together wire together". In other words, since neurons from the same eye are more likely to fire at the same time, they will innervate the same target cells (Hebb 1949). The question that arises from this explanation is why form interdigitated columns as opposed to dividing the tectum into two continuous equal halves in which each half is driven by one of the eyes? The proposed explanation to this question is that columns are a trade-off between retinotopic organization where the innervation from the two eyes are interspersed and activity dependent synapse formation in which the innervation from the two eyes are completely segregated due to differential firing (Constantine-Paton 1981). Conceivably, the actions of chemoattractants, which are responsible for the formation of the retinotopic map, in conjunction with activity dependent Hebbian mechanisms of synapse formation may give rise to the observed columnar organization.

1.3.4 Importance of Concurrent visual activity

Another key aspect of visual system development in the cat is the concurrent firing of neurons. The importance of concurrent activity during visual development is best demonstrated by deficiencies observed in strabismic animals (Hubel et al. 1965, Shatz et al. 1977). Binocular cells, which are found at the borders of ocular dominance columns in the cat, arise presumably because the input from the two eyes do not have any sort of a competitive advantage over each other (Shatz et al. 1978). Since the inputs from both eyes activate the binocular cell with equal efficacy, the two inputs are able to innervate the same cortical cell. This situation can only arise if the inputs from the two eyes fire at the same time (Hebb 1949). In order for these two inputs to fire simultaneously the two eyes must be looking at the same point in the visual field. Accordingly, if an animal is made strabismic at an early age so that the two eyes look in different directions and do not share the same field of vision, then the cells in layer 4 of the visual cortex become almost entirely monocular (Hubel et al. 1965, Van Sluyters et al. 1980). In other words, since the inputs from the two eyes rarely fire concurrently in a strabismic animal, the cortical cells become innervated only by one of the eyes. This finding validates the notion of "cells that fire together wire together" and stresses the necessity of normal visual activity for the proper development of the visual system. A peculiar aspect of strabismic animals is that they form much wider ocular dominance columns than normal animals (Lowel 1994) and furthermore, the borders of ocular dominance columns in strabismic animals are sharply defined (Shatz et al. 1977, Shatz et al. 1978, Hubel et al. 1965). The number of monocular cells can also be increased in the visual cortex by doing reverse suture experiments (Blakemore et al. 1974). In reverse suture experiments, one eye is initially occluded and then opened at a later date while the originally non-occluded eye is then deprived of input. Because the two eyes are active during different times, the inputs become extensively segregated and most of the cortical cells become monocular.

1.3.5 Contribution of Spontaneous activity to visual development

By the time a primate is born, its visual cortex contains ocular dominance columns (Hubel et al. 1977) and similarly, a newborn cat has its retinal connections segregated into eye specific layers in the LGN (Shatz 1983). Since these animals do not receive visual activity prenatally, how can segregated synaptic arrangements such as ocular dominance columns or eye specific LGN layers be present in newborn primates and felines? Insight into this question was found by studying the prenatal formation of eye specific layers in the LGN (Shatz 1983). The development of eye specific layers in the LGN is restricted to the prenatal period of life and monocular deprivation can not induce synaptic shifts in the LGN of postnatal kittens (Shatz 1983) (although, LGN neurons corresponding to the deprived eye can shrink considerably following monocular deprivation (Hickey et al. 1977)). Similar to the segregation of thalamocortical connections in the postnatal cat visual cortex, competitive interactions between the two eyes appear to give rise to the formation of eye specific LGN layers in the prenatal cat (Shatz 1990). Since a prenatal kitten is unable to see, normal visual stimulation does not play a role in this process. However, injection of TTX into eyes of prenatal kittens prevents the formation of LGN layering revealing the necessity for some sort of activity for the prenatal segregation of retinogeniculate synapses (Shatz et al. 1988, Sretavan et al. 1988). Segregation of retino-geniculate inputs into eye specific layers in the LGN is believed to be facilitated by spontaneous activity. During early development, spontaneous, random waves of activity traverse the retina (Meister et al. 1991, Wong et al. 1993). Cells which are physically close to each other on the retina are likely to be included in the same wave of activity and fire together. Since "cells that fire together wire together", cells which are located close to each other on the retina will innervate proximal regions in the LGN. Because these waves of activity occur independently of each other in the two retinae, cells from the same eye are more likely to fire together. Hence, inputs from the two eyes segregate into eye specific layers in the LGN. Spontaneous activity is also believed to give rise to the formation of ocular

dominance columns in prenatal primates (Reiter et al. 1986, Stryker et al. 1986). However, the absence of ocular dominance column formation in dark reared cats is inconsistent with this notion. If spontaneous activity is capable of causing ocular dominance column formation in the absence of visual activity, then how does depriving an animal of visual activity prolong the critical period and prevent the formation of ocular dominance columns? The answer to this question is unknown but species differences between felines and primates may explain this outcome.

1.4 CELLULAR AND MOLECULAR MECHANISMS INVOLVED IN VISUAL PLASTICITY

1.4.1 Hebbian Mechanisms

As outlined by Hebb (1949), synapses that are active become stronger whereas synapses which are not used are eliminated. The experimental paradigms of long term potentiation (LTP) and long term depression (LTD) are proposed to be the cellular correlates of synapse formation. Long term potentiation is induced by applying a tetanic stimulus to the presynaptic cell (Andersen et al. 1977). This paradigm greatly increases the response of the postsynaptic cell to stimuli for a prolonged period of time lasting from hours to days. Conversely during LTD, presentation of a low frequency stimulus greatly decreases synaptic efficacy for a prolonged period of time (Lynch et al. 1977, Staton et al. 1989). LTP and LTD like processes are believed to occur during the normal development of the visual system and account for synaptic strengthening and elimination (Bienenstock et al. 1982, Bear et al. 1987, Kirkwood et al. 1994). Specifically, LTP like events are proposed to take place in active synapses resulting in the strengthening of these synapses whereas LTD like events are thought to be responsible for the weakening and eventual elimination of inactive synapses. Fittingly, LTP can only be induced experimentally in the visual cortex of young kittens and not in older animals (Komatsu et al. 1988, Kirkwood et

al. 1995). Unfortunately, the existence or absence of LTP and LTD like processes in normal visual development can not be demonstrated experimentally due to the shortcomings of present day electrophysiological techniques. Hence, the involvement of these experimental paradigms in the development of the visual system remains largely speculative.

1.4.2 The role of NMDA receptors in visual development

Despite the large amount of information available regarding the physiological and anatomical development of the cat visual system, the molecular mechanisms responsible for this event have remained elusive. As an obvious starting point for identifying molecules associated with visual plasticity, the role of different neurotransmitter systems in the postnatal development of the visual system has been studied. The NMDA receptors have received much attention during visual development because glutamate is the major excitatory neurotransmitter in the visual system of all vertebrates (Kleinschmidt et al. 1987, Bear et al. 1990, Rauschecker et al. 1990). In addition, the ability of NMDA channels to detect concurrent activity between pre and postsynaptic cells is consistent with Hebbian models of synaptic development (Nowak et al. 1984, Mayer et al. 1985) and the importance of NMDA channels in long term potentiation is well established (Collinridge et al. 1983). NMDA receptors are present throughout the visual system during the feline critical period. As would be expected of a molecule associated with visual plasticity, NMDA receptors are more abundantly expressed in young kittens (Bode-Greuel et al. 1989, Tsumoto et al. 1987, Fox et al. 1989). Whereas the NMDA receptors are involved in eliciting visual responses in layers II/III of the cortex throughout life, they are functional in the deeper layers of the cortex (IV, V and VI) only in young kittens (Fox et al. 1989). This laminar difference in NMDA activity is believed to be partially responsible for the plastic differences amongst cortical laminae (i.e. layer II/III plastic longer than layer IV) (Daw et al. 1992). Recent studies have also shown the subunit composition of the NMDA receptors to change with age (Nase et al. 1999). The NMDA channel EPSC's shorten with

age in a manner paralleling the critical period (Carmignoto et al. 1992). The expression of the NR2A NMDA subunit in older animals is responsible for this change (Flint et al. 1997, Nase et al. 1999). Fittingly, NR2A expression can be delayed by the plasticity-prolonging paradigm of dark rearing (Nase et al. 1999). Direct support for NMDA receptor involvement in ocular dominance column formation comes from three eyed frog studies in which the application of NMDA receptor antagonists cause the ocular dominance stripes to desegregate (Cline et al. 1987, Cline et al. 1990). Similarly, the infusion of NMDA antagonist into the kitten visual cortex interferes with plasticity (Bear et al. 1990, Bear et al. 1997). Unfortunately, the field of NMDA research is not free of controversy. One of the reasons for investigating NMDA receptor activity in the developing visual cortex is the putative role NMDA receptors are proposed to play in LTP and LTD. However, in kitten visual cortex slices LTP is found to rely on low threshold calcium channels and not on NMDA receptors (Komatsu et al. 1992, Komatsu 1994). The link between NMDA receptors and visual plasticity established by the use of pharmacological antagonists is also questionable. Since pharmacologically blocking NMDA function in the cortex reduces visual responses (Fox et al. 1989), the action of NMDA antagonists may simply block neural activity (i.e. action of these agents maybe analogous to TTX). Hence, NMDA receptors may not have a special role in plasticity. In support of this possibility is the finding that monocularly deprived kittens which have had their cortical NMDA receptors blocked exhibit an unexpected shift in ocular dominance towards the deprived eye (Bear et al. 1990). The same result is obtained in monocularly deprived kittens by suppressing general cortical activity by muscimol, a GABA agonist (Reiter et al. 1988). The similarity between these two experiments suggests that the pharmacological NMDA blockers are essentially blocking neural activity to the cortex and the NMDA receptors are merely involved in eliciting visual responses. These contentious issues will hopefully be resolved by examining knockout animals lacking the expression of the NMDA NR2A subunit. The

retention of visual plasticity past the normal critical period in NR2A knockout animals would clearly establish a role for the NMDA receptors in this process.

1.4.3 The role of metabotropic glutamate receptors in visual development

The metabotropic glutamate receptors have also been characterized during the critical period. The level of phosphoinositide turnover induced in the cortex by glutamate stimulation correlates with the critical period and during the height of the critical period, animals are reported to respond more potently to glutamate (Dudek et al. 1989, Bear et al. 1991). Since this effect is not observed with AMPA or NMDA, the increase in phosphoinositide turnover is likely due to the activity of PI-linked metabotropic glutamate receptors. The expression of the phosphoinosityl-linked metabotropic glutamate receptor mGluR5 in the visual cortex decreases with age (Reid et al. 1997, Daw et al. 1996). More importantly, following four weeks of age mGluR5 receptor's laminar distribution ceases to be ubiquitous in all layers of the cortex and becomes concentrated in layer IV. An overall decline in immunostaining for mGluR5 is observed with age and adult-like distribution in the visual cortex is established around 3 months of age. Clearly, the expression and the distribution of the mGluR5 receptor closely correlate with the critical period. However, blockade of metabotropic glutamate receptors by the pharmacological agent MCPG fails to prevent ocular dominance plasticity (Hensch et al. 1996). Although this result refutes the involvement of PI-linked metabotropic glutamate receptors in visual plasticity, the effectiveness of MCPG at blocking PI turnover induced by metabotropic glutamate receptor activity is questionable (Huber et al. 1998). Increase in cAMP levels by activation of metabotropic glutamate receptors is also reported to correlate with the critical period (Reid et al. 1996). However, since appropriate pharmacological agents are not available for selectively blocking cAMP levels induced by metabotropic glutamate receptors, this finding has also remained largely correlative. The conclusion derived from these studies is that glutamate is able to induce secondary signaling pathways more effectively during the critical period. However, it is not clear if this excessive secondary signaling is associated

with ocular dominance column formation or if it plays a completely different role in development.

1.4.4 The role of neuromodulatory neurotransmitters in visual development

In addition to glutamate, neuromodulatory neurotransmitters are implicated to be involved in the postnatal development of the cat visual system. Neuromodulatory neurotransmitters can have either inhibitory or excitatory effects depending on the postsynaptic cell type and receptor composition (Krnjevic et al. 1963). The neuromodulatory neurotransmitters characterized in the visual cortex include acetylcholine, noradrenaline, serotonin and dopamine. As in other sensory areas of the cortex, the visual cortex is innervated by neuromodulatory neurotransmitter producing cells from subcortical regions (Irle et al. 1984, Mizuno et al. 1969, Troiano et al. 1978, Watabe et al. 1982, Mulligan et al. 1988, Chazal et al. 1987, Tork et al. 1981). Although the visual cortex does not have any neurons that synthesize neuromdoulatory neurotransmitters, cortical neurons express receptors for neuromodulatory neurotransmitters and can readily respond to these neurotransmitters. The neuromodulatory neurotransmitter receptors have age specific laminar distributions in the visual cortex and the expression and the distribution of these receptors correlates with the critical period (Stichel et al. 1987, Prusky et al. 1988, Prusky et al. 1990, Liu et al. 1994, Aoki et al. 1986, Jia et al. 1994, Gu et al. 1990, Dyck et al. 1993a, Lewis et al. 1987). Similar to the metabotropic glutamate receptors, the neuromodulatory receptors are linked to cAMP or inositol phosphotidyl turnover secondary signaling pathways. More importantly, the selective blockade of these receptors by pharmacological agents disrupts visual plasticity and provides evidence for the involvement of neurotransmitters such as acetylcholine (Bear et al. 1986, Gu et al. 1993a), noradrenaline (Bear et al. 1986, Kasamatsu et al. 1979, Kasamatsu et al. 1981, Kasamatsu et al. 1985a, Kasamatsu et al. 1985b, Shirokawa et al. 1987), and serotonin (Gu et al. 1995, Wang et al. 1997) in visual plasticity. These neurotransmitters are believed to

control the excitability of cortical neurons by modulating the activity of NMDA receptors (Kojic et al. 1997, Kirkwood et al. 1999). The contribution of neuromodulatory neurotransmitters to visual plasticity is likely to determine the threshold activation levels for the NMDA receptors.

1.4.5 The role of immediate early genes in visual development

Immediate early gene (IEG) activity may also contribute to visual plasticity. The postnatal expression of immediate early genes in the kitten visual cortex is regulated in an age dependent manner (McCormack et al. 1992, Kaplan et al. 1995) and high levels of IEG expression can be maintained in older animals by dark rearing (Beaver et al. 1993). More importantly, immediate early gene expression is altered by monocular deprivation (Rosen et al. 1992). Although immediate early gene expression is modified by monocular deprivation in adult animals, visual activity is more effective at inducing immediate early gene expression in younger kittens and dark reared animals (Kaplan et al. 1996, Mower 1994). Since the expression of these transcription factors are upregulated in the presence of visual activity and decreased by deprivation paradigms (Mitchell et al. 1995), the immediate early genes are proposed to be involved in the process of synaptic rearrangements. According to this hypothesis, high immediate early gene expression levels are required to induce genetic processes which facilitate active neurons to expand their arbourization. Alternatively, immediate early gene expression may be necessary for maintaining synapses. Since in monocularly deprived animals immediate early gene expression is high in areas of the cortex corresponding to the open eye, synapses are maintained in these areas. Conversely, synapses are eliminated in areas of cortex corresponding to the closed eye because they express low levels of immediate early genes. Unfortunately, very little is known about the downstream genes that are directly regulated by the immediate early genes. Ergo, the actual molecular mechanisms activated by these genes during synaptic rearrangements are unknown. Furthermore, the involvement of immediate early genes in visual plasticity is based on various correlations between the

expression of these genes and the critical period. Experiments aimed at verifying the exact function of the IEG's during the critical period have not been performed due to the difficulties associated with modifying gene expression in the cat. Additionally, the regulation of immediate early gene expression by visual activity following the critical period argues against a role for these proteins in visual plastcitiy.

1.4.6 The role of neurotrophins in visual development

One of the best characterized family of molecules in the developing visual system is the neurotrophins. Neurotrophins have a diverse number of effects and are involved in various stages of development. The neurotrophins bind and act through specific tyrosine kinase receptors. NGF binds to TrkA, BDNF and NT4-5 bind to TrkB and NT3 binds to TrkC (Lamballe et al. 1991, Berkemeier et al. 1991, Ip et al. 1992, Klein et al. 1991, Cordon-Cardo et al. 1991). Initially, visual cortex neurons were proposed to release neurotrophins in limiting amounts (in an activity dependent manner) and the afferents originating from the LGN were suggested to compete for these factors (Maffei et al. 1992, Harris et al. 1997). According to this model, afferents which successfully receive neurotrophins are able to form synapses whereas afferents which do not take up neurotrophins are eliminated. The finding that cortical infusion of NT4 in the ferret is capable of preventing LGN cell shrinkage associated with monocular deprivation supports this hypothesis (Riddle et al. 1995). The case for the involvement of neurotrophins in visual plasticity is also bolstered by the activity dependent regulation of neurotrophin expression in the visual cortex (Castren et al. 1992, Schoups et al. 1995, Torasdotter et al. 1996). However, recent findings have caused a substantial amount of confusion regarding the role of neurotrophins in visual development. Since the ontogeny of TrkB receptor expression is consistent with the critical period, BDNF is believed to play a prominent role in visual plasticity (Cabelli et al. 1994, Allendoerfer et al. 1994). Infusion of BDNF or blocking of the BDNF receptor (trkB) in normal kittens during the critical period interferes with ocular dominance column formation and synaptic rearrangements induced by

monocular deprivation (Cabelli et al. 1995, Cabelli et al. 1997, Gaulske et al. 1996). The initial interpretation of this finding was that BDNF prolongs the critical period by keeping the cortex more plastic. However, recent work on transgenic mice models overexpressing BDNF has found that an excess of BDNF actually results in a shorter critical period and the visual cortex appears to mature faster with more BDNF (Huang et al. 1999, et al. Hanover 1999). These two paradoxical findings have challenged the validity of the original neurotrophin hypothesis. Additionally, cortical BDNF infusion in monocularly deprived animals during the critical period results in an unexpected shift in ocular dominance towards the closed eye (Gaulske et al. 1996). Furthermore, a factor which is involved in visual plasticity should be located in layer 4 of the cortex since this is the input layer for the LGN. Unfortunately, BDNF expression in the rat visual cortex is determined to be low in layer 4 while being high in layer 2/3, 5 and 6 (Cabelli et al. 1994). NGF infusion also prevents ocular dominance column formation and causes a shift in ocular dominance towards the closed eye of monocularly deprived animals (Carmignoto et al. 1993, Dommenici et al. 1993, Dommenici et al. 1994, Gaulske et al. 1996, Maffei et al. 1992). In addition, unlike BDNF, NGF infusion is capable of inducing plasticity in the adult visual cortex (Gu et al. 1994). However, since TrkA receptor expression in the visual cortex is restricted to cholinergic axons emanating from the basal forebrain (Springer et al. 1987) and the level of TrkA expression in the cortex is low compared to TrkB (Allendorfer et al. 1994, Cabelli et al. 1994), the importance of NGF in the developing visual system is questionable. The fact that relatively high levels of NGF infusion is required to cause an effect on visual plasticity is also concerning. Such high levels of NGF may result in promiscuous binding of this neurotrophin with Trk receptors other than TrkA. Another problem with determining the role of neurotrophins in visual development is contradictory findings reported by different laboratories. For example, NGF is reported to be ineffective at preventing LGN neuron shrinkage in monocularly deprived ferrets (Riddle et al. 1995) whereas this neurotrophin appears to be capable of preventing LGN cell shrinkage in

monocularly deprived rats (Domenici et al. 1993). Discrepancies such as these may be a result of species differences or alternatively, methodological issues (such as the concentration of the neurotrophin or differences in the application method) may have given rise to these paradoxical findings. The mechanism of action of neurotrophins is also unclear. Initially, neurotrophins were proposed to be activity induced signals involved in the strengthening and/or the maintenance of synapses (Maffei et al. 1992). This proposed role of neurotrophins is presumably accomplished by signaling through the Trk receptors and results in the maturation of synapses. However, this role for the neurotrophins is disputed by the finding that neurotrophins are able to modulate neural activity (Kang et al. 1995, Akaneya et al. 1997, Carmignoto et al. 1997). Hence, the neurotrophins may merely accentuate neural activity and they may not be involved in any downstream changes associated with the physical strengthening and maintenance of synapses.

1.4.7 Summary

In summary, a large amount of work has been performed identifying the physiological and anatomical characteristics of the developing visual cortex. Although several molecular factors which may play a role in visual development have been identified, the involvement of these factors in ocular dominance column formation and visual plasticity has not been definitively established. In addition, the mechanisms by which these molecules contribute to visual plasticity remains unclear. For example, neurotransmitters, immediate early genes and the neurotrophins are believed to cause the activation of gene cascades via various secondary signaling pathways. However, very little is known about the actual identity of genes which are associated with these signaling pathways. Clearly, a substantial amount of work has to be performed in order to unravel the molecular mechanisms responsible for the postnatal development of the cat visual system.

1.5 EXPERIMENTAL STRATEGIES AIMED AT IDENTIFYING MOLECULAR MECHANISMS INVOLVED IN VISUAL PLASTICITY

Based on the current status of visual neuroscience research, it is likely that many of the proteins involved in visual plasticity have not been identified. The identification of genes which are directly involved in the synaptic rewiring of the visual system is currently the primary area of research interest in the visual neuroscience field. A popular approach to solving this problem is to investigate differential gene expression in the visual system following various developmental and deprivation paradigms. For example, subtractive hybridization has been performed to determine the gene differences between the adult visual cortex and a highly plastic kitten visual cortex (Prasad et al. 1994). The logic behind this experiment is that genes involved in visual plasticity should be expressed at higher levels during the critical period. Unfortunately, many genetic processes which are unrelated to visual plasticity also occur in the developing kitten brain. Hence, the data obtained from these experiments is difficult to interpret. A recent study has compared gene expression differences between dark reared and normal adult visual cortecies (Yang et al. 1999). This experiment does not need to contend with the issue of age dependent developmental differences since both dark reared animals and normally reared animals are of the same age. Therefore, the only genetic differences between these animals should theoretically be those related to visual plasticity. However, dark reared cats are not as plastic as young kittens and there are also pathological conditions associated with long term confinement of kittens in darkness. Finally, genes regulated by neural activity have been identified by comparing normal visual cortecies with visual cortecies that have been silenced by TTX injections (Corriveau et al. 1998). Although the approach of studying differential gene expression is valid, it does suffer from certain serious limitations. The experimental methods available to study differential gene expression are not exhaustive and these methods will not yield all the molecular differences between two different conditions. Furthermore, the sensitivity of

these methods to detect differences in gene expression is an issue. Since the visual cortex is comprised of various cell types, gene expression differences occurring only in a particular cell type may go undetected when gene expression is compared between two tissue samples. If other cell types in the tissue express the same gene(s) at unaltered levels, the change in gene expression exhibited by the single cell type will not be sufficient to alter the overall gene expression levels in the tissue. This problem is of concern anytime gene expression is compared between different tissues. The standard way of overcoming this problem is to use a homogeneous cell population in a tissue culture system. Unfortunately, this option in not applicable because visual development is studied at the system level and can not be recreated in a tissue culture paradigm. Nonetheless, advances in methodology may soon allow gene expression to be compared between single cell types isolated from visual cortex tissue via fluorescence activated cell sorting. The comparison of gene expression from single cell types in normal and manipulated animals should overcome the above mentioned problems. (FACS isolation of cells from tissue is routinely performed at present. However, the amount of cells that are required for carrying out gene expression studies exceeds the number of cells that can be generated by FACSing. Although this experiment is potentially plausible at present using PCR based techniques, it is fraught with technical difficulties. Hence, it has not been successfully performed in the visual system.) Additionally, gene expression studies carried out on the visual cortex have focussed on the mRNA level and hence, translational and post-translational changes taking place during the critical period have not been characterized. This situation is likely to change in the recent future due to the advent of highly reproducible and easy to use two-dimensional protein systems. In summary, although examining the differences in gene expression between different paradigms is a valid approach to identifying the genes involved in visual plasticity, there are many technical issues to be considered when performing these sorts of experiments. Clearly, this approach is unlikely to identify every gene involved in visual plasticity and it may not be the most practical approach to solving this problem.

An alternate strategy to identifying the molecular events associated with visual plasticity is to apply the knowledge acquired from other developmental systems to the field of visual neuroscience. The members of a genetic cascade which was initially identified in the fruit fly drosophila melanogaster appears to be of prominent importance during development. These genes are highly conserved amongst species and appear to be ubiquitous to almost every type of developmental event. We hypothesized that these genes contribute to the postnatal development of the cat visual system and hence, the expression patterns of the cat homologues of the drosophila developmental genes were examined during the critical period and in various deprivation paradigms.

1.6 DROSOPHILA DEVELOPMENTAL GENES

1.6.1 Properties of the Drosophila Developmental genes

Epigenesis in drosophila is controlled by a gene hierarchy which forms a regulatory cascade that leads to the successive, step by step subdivision of the embryo into smaller units. This genetic hierarchy is comprised of three general categories of transcription factors known as the maternal genes, the segmentation genes and the homeotic genes. Homologues of these genes are present and highly conserved in all metazoa (Holland et al. 1996). The members of this gene hierarchy take part in a broad range of developmental events in various animals and are involved in the formation of a number of structures including the CNS.

The necessity for homeotic and segmentation gene function in the development of the vertebrae brain is well established. During embryonic development, the vertebrae hindbrain is subdivided along the rostrocaudal axis into compartments known as rhombomeres (Orr 1887, Vaage 1969). Rhombomeres restrict cell migration and prevent the intermixing of cells (Fraser et al. 1990). Since cells are subjected to only local cues and local cell to cell interactions, each rhombomere develops a unique phenotype (Fraser 1990).

The differential expression of homeotic genes amongst rhombomeres contributes to this phenotypic divergence (Wilkinson et al. 1989a, Murphy et al. 1989, Studer et al. 1996, Bell et al. 1999). This function of the homeotic genes is analogous to their role in drosophila development where they specify the identity of each body segment (Lewis 1978). In addition, the temporal and rostrocaudal expression of a particular homeotic gene in the both hindbrain and developing drosophila body is determined by its location on the chromosome (Wilkinson et al. 1989, Murphy et al. 1989, Lewis 1978, Graham et al. 1989, Izpisua-Belmonte et al. 1991). As in the hindbrain, the differential expression of the *Otx* and *Emx* homeotic gene families in the developing forebrain contributes to the anterior posterior patterning of this structure (Simeone et al. 1992, Boncinelli et al. 1993, Yoshida et al. 1997, Acampora et al. 1995, Acampora et al. 1996). Similarly, the segment polarity genes *Wnt* and *Engrailed* are necessary for the development of the midbrain (McMahon et al. 1990, McMahon et al. 1992, Wurst et al. 1994). In addition to the genes mentioned above, the activity of many other drosophila developmental genes are known to be required for the development of the CNS (Ericson et al. 1995, Wilkinson et al. 1989b, Sassai et al. 1995, Lindsell et al. 1995, De la Poma et al. 1997). Clearly, the homologues of the homeotic and other segmentation genes play an important role in the development of the vertebrae brain.

The mammalian homologues of these drosophila developmental genes contribute to various facets of CNS development such as cell induction (Ericson et al. 1995), cell type specification (Bell et al. 1999), proliferation and differentiation (McMahon et al. 1990, McMahon et al. 1992). However, the expression of many homeotic and segmentation genes persists in the postnatal brain (Levine et al. 1993, Frantz et al. 1994, Zhang et al. 1998). Although the function of these genes in postnatal developmental events has not been thoroughly examined, homeotic and other segmentation genes are implicated to be involved in plasticity (Levine et al. 1993), apoptosis (Mehler et al. 1997, Patapoutian et al. 2000) regulation of Trk receptor expression (Zhang et al. 1998), and synaptogenesis (Hall

et al. 2000). Based on the reported functions of these genes in brain development and their postnatal expression, we propose that the homologues of the drosophila developmental genes are likely to be involved in the postnatal development of the cat visual system.

1.6.2 Possible roles for segmentation genes in visual development

Since the homologues of the drosophila developmental genes are thoroughly characterized in other developmental paradigms, speculations can be made regarding the function of these genes in the visual system. Segregation and segmentation is a well examined characteristic of the visual system. As mentioned, retinal afferents in the LGN segregate into eye specific layers and eye specific ocular dominance columns form in the cortex. In addition, histochemical markers such as zinc and cytochromeoxidase give rise to distinct staining patterns in the cortex (Dyck et al. 1993b). Interestingly, neural function is segregated along these lines. In the primate visual system, color and motion are subdivided into the magno- and parvo-cellular pathways which can be distinguished by their differing CO staining patterns (Livingstone et al. 1984, Dow 1974). In the cat, which lacks color vision, CO blobs are involved in spatial frequency (Shoham et al. 1997). Although there are no histochemical markers for orientation selectivity, this function is also arranged in a columnar manner where cells of the same orientation are in the same column (Rose et al. 1974). Additionally, receptors such as NMDAR1 and serotonin (Trepel et al. 1998, Dyck et al. 1993c) are organized into columns in the cat visual cortex. Clearly, functional and physical compartmentalization of the visual system is evident. Since homeotic and segment polarity gene distribution is columnar (Akam 1987), a possible function of these genes may be to give rise to the observed organization of the visual system. This notion is supported by the finding that the drosophila serotonin receptor is expressed in columns and its expression is at least partly regulated by the pair rule gene *fushi tarazu* (Colas et al. 1995). Also, the transversely expressed stripe pattern of the pair rule genes in the drosophila embryo (Lawrence 1987) is reminiscent of the columnar arrangement of the interdigitated

ocular dominance columns. Furthermore, the transcription factor zif268 has functional domains in common with the drosophila *Kruppel* gene.

The homologues of the drosophila developmental genes clearly have properties which are capable of giving rise to the arrangement observed in the visual system. In particular, the segment polarity genes have characteristics which may be relevant to visual cortex development. These genes are usually components of cell-to-cell signaling pathways (i.e. they are usually ligand, receptor or a messenger) (for a review see Ingham et al. 1991). They are, for the most part, expressed in domains which can sometimes be stripe-like or columnar. As determined by experiments involving temperature sensitive mutants in drosophila, segment polarity genes need to act during a defined window of time similar to a critical period to have an effect. However, the most notable property of segment polarity genes is their ability to either enhance each other's expression or to exert mutual repression on each other. For example, in the case of mutual repression, a reduction in the expression level of a segment polarity gene results in its domain of expression being invaded by the opposing segment polarity gene. These properties of the segment polarity genes can potentially account for the observed narrowing and widening of the ocular dominance columns occurring following monocular deprivation in young animals. A role for segment polarity genes in visual development is supported by the finding that the segment polarity genes *wingless* and *decapentepeligic* are expressed in adjoining domains in the developing drosophila visual system and they are necessary for proper retinotopic organization (Kaphingst et al. 1994). Additionally, in the developing chick tectum the segment polarity gene *engrailed* is expressed in a concentration gradient and is also required for proper retinotopic arrangement (Friedman et al. 1996). Clearly, these genes possess characteristics that are capable of facilitating synaptic competition and ocular dominance column formation. In addition these genes are involved in visual development in less complex animals. Hence, these developmental genes, which were

initially characterized in the drosophila, have to be considered as ideal candidates for being involved in visual development and plasticity.

1.7 PROPERTIES OF BETA-CATENIN

Evidence for the involvement of *beta-catenin*, a mammalian homologue of the drosophila *armadillo* gene, in the postnatal development of the cat visual system is provided in this thesis. The beta-catenin protein functions both as a member of the cadherin-catenin cell adhesion system and as a transcription factor (Peifer et al. 1992, Peifer et al. 1994). The role of the beta-catenin protein during cellular adhesion is to anchor the intracellular domains of the cadherin molecules to the actin cytoskeleton. Ordinarily, the beta-catenin protein is rapidly degraded to prevent its accumulation in the cytosol (Aberle et al. 1997). Hence, beta-catenin protein expression is normally restricted to the periphery of the cell where it acts as a cell adhesion molecule (discussed further in the next section). However, beta-catenin protein expression is under tight post-translational regulation (Peifer et al. 1994). This post-translational regulation is influenced by factors such as Wnt-1 signaling, which is the homologue of the drosophila *wingless* gene, and the activity of the integrin linked kinase (Peifer et al. 1994, Novak et al. 1998). The activity of these genes prevents the rapid degradation of the beta-catenin protein and causes beta-catenin to accumulate in the cytosol (discussed in further detail in chapter 3). The accumulated beta-catenin protein is then translocated into the nucleus where it acts as a transcription factor (Schnieder et al. 1996). In addition to its function as a cell adhesion molecule, we report that the beta-catenin protein also functions both as a transcription factor in the postnatally developing cat visual system.

1.8 PROPERTIES OF CADHERINS

Due to the extensive interactions between the beta-catenin and the cadherin proteins, the expression of the cadherin proteins was also examined in the developing visual system of the cat. Beta-catenin and the cadherins constitute a prominent cell adhesion system in the body. The importance of cell adhesion molecules in neuro-development and neural connectivity is becoming apparent. Cell adhesion molecules such as N-Cam, the integrins and the cadherins are located at synapses (Persohn et al. 1989, Einheber et al. 1996, Martin et al. 1996, Uchida et al. 1996). These molecules contribute to processes such as synaptogenesis, target recognition, axon mobility and neuronal plasticity in the developing nervous system. Interestingly, many cell adhesion molecules are regulated by the activity of drosophila developmental genes.

Cadherins are a family of calcium dependent cell adhesion molecules that have homophilic binding properties (Leckband et al. 2000). Although heterophilic binding of cadherins does occur, normally a particular type of cadherin molecule only forms adhesive links with cadherin molecules which are of the same type. Since they are cell adhesion molecules, the cadherins are exclusively localized to the periphery of cells. Electron microscopy studies have localized the beta-catenin and cadherin proteins to synapses in neuronal tissue where the beta-catenin cadherin cell adhesion system borders the active zone (Uchida et al. 1996). Since, cadherins bind in a homophilic manner, they are present both pre and postsynaptically. The diversity in the types of cadherin and cadherin like molecules in the central nervous system is enormous (Redies 2000). In addition to the classic cadherins, numerous protocadherins, which are larger versions of the cadherin molecules (Sano et al. 1993), and numerous cadherin like neural receptors, which essentially are modified cadherin molecules, have been discovered (Kohmura et al. 1998). The homophilic binding properties of the cadherins and the diversity of cadherin types expressed in the brain has given rise to the theory that the differential expression of these

proteins contributes to the precise wiring of the nervous system (Obst-Pernberg et al. 1999). The specific and restricted expression of certain cadherin types to particular neural tracts gives merit to this notion (Arndt et al. 1996). This hypothesis is also consistent with the proposed functions of the cadherins which include axon motility, target recognition and synaptogenesis (Matsunaga et al. 1988).

These properties of the cadherin-catenin cell adhesion system are quite relevant to the postnatal development of the visual system. Clearly, pathfinding and synaptogenesis occurs during the critical period. Furthermore, the compartmental characteristics of the visual system such as eye specific LGN layers, Cytochrome oxidase blobs and interblobs, and the magno-/parvo- tracts may arise due to differential synaptic adhesion. In addition, the disruption of cadherin activity is reported to prevent the induction of LTP which suggests a direct link between these molecules and neural plasticity (Tang et al. 1998).

The remainder of this thesis will describe in detail our findings regarding the involvement of the drosophila developmental gene homologues in the postnatal development of the cat visual system.

Chapter 2

HOMEOTIC AND SEGMENT POLARITY GENE EXPRESSION IN THE POSTNATALLY DEVELOPING CAT VISUAL CORTEX

2.0 SUMMARY

In this chapter, the expression of various homologues of the homeotic and segment polarity genes were examined in the postnatal cat visual cortex by RT-PCR. The cDNA's of the *PBX1, PBX2, BMP4, BMP6, BMP-typeII-receptor, Wnt-1* and *FrZB* genes were partially cloned in the cat. Northern blot analysis revealed that the mRNA expression pattern of the *PBX1, PBX2, BMP4*, and *Wnt-1* genes are developmentally regulated in the postnatal cat visual cortex. The expression and developmental regulation of these genes during the critical period gives merit to the hypothesis that the homologues of the drosophila developmental genes are involved in the postnatal development of the cat visual system.

2.1 INTRODUCTION

The developmental genes initially identified in the drosophila appear to represent a general blueprint for development (Roelink 1996, Gellon et al. 1998, Christian 2000). These genes are highly conserved amongst species (Gellon et al. 1998, Holland et al. 1996a, Holland et al. 1996b) and are involved in the development of various structures including the nervous system (Reichert et al. 1999, Williams et al. 1998). In fact, the homologs of these genes play a role in almost every stage of neural development. The cellular decision for a neuronal or non-neuronal lineage is determined by the activity of the Notch gene (De la Pomma et al. 1997). Homologs of the homeotic and segment polarity genes are involved in cellular migration, proliferation, survival, apoptosis and target

McMahon et al. 1990, McMahon et al. 1992, Hall et al. 2000, Song et al. 1998, Friedman et al. 1996). The activity of homeotic genes contributes to the creation of different types of neurons (Valarche et al. 1993, Tissier-Seta et al. 1993, Pattyn et al. 2000). The expression of these genes persist into adulthood and their function is not limited to early neural developmental events. For example, the expression of certain homeotic genes is restricted to dopamenergic neurons in the adult brain (Valarache et al. 1993) and *Otx* genes are distributed in a lamina specific manner in the adult cortex (Frantz et al. 1994). Since the members of the genetic hierarchy identified in drosophila development have such a broad role in CNS development, it is not unreasonable to assume that the homologs of these genes may play a role in the postnatal development of the visual cortex. Accordingly, the expression of several homeotic and segment polarity genes in the developing visual cortex were examined, and the expression of the PBX1, PBX2, BMP4, BMP6, BMP type II receptor, Wnt-1 and the Frz B genes was detected in this structure.

2.1.1 Possible Role of Homeotic Genes in Visual Development

Columnar or segmental organization is a well characterized property of the cat visual cortex. The zinc ion, cytochromeoxidase protein, serotonin receptor subtypes and NMDAR1 protein distribution in the cat visual cortex is columnar (Dyck et al. 1993a, Dyck et al. 1993b, Dyck et al. 1993c, Treppel et al. 1998). In addition, ocular dominance columns and orientation columns are present in the visual cortex (Shatz et al. 1978, Wiesel et al. 1963). Furthermore, the cortex has a laminar arrangement with each lamina containing distinct types of neurons (Gilbert et al. 1981). Since one of the preeminent molecular factors responsible for the organization and development of compartments (i.e. rhombomeres or drosophila body segments) is the homeotic genes (Lewis 1978, Akam 1987, Lawrence 1987, Murphy et al. 1989), we propose that these genes contribute to the laminar and the columnar arrangement of the cat visual cortex.

The members of the homeotic gene family expressed in the postnatal visual cortex during the critical period are unknown. As a starting point, the expression of the *PBX*

homeotic genes was examined in the visual cortex because the *PBX* genes act solely as co-factors and facilitate the function of other homeobox genes (van Dijk et al. 1995, Chang et al. 1995, Neuteboom et al. 1995). Since *PBX* expression is required by several other homeobox genes, *PBX* homeotic genes are more widely expressed than other homeotic genes. Hence, they are more likely to be expressed in the visual cortex than most other homeotic genes. The main function of the PBX transcription factors is to facilitate cooperative binding. Cooperative binding refers to the process in which transcription factors form either homodimers with themselves or heterodimers with other transcription factors (Wilson et al. 1995). The newly formed complex alters the conformation of the transcription factors and increases the binding affinity of these proteins for particular promoter and enhancer sequences. Transcription factors that form complexes with co-factors such as the *PBX* genes are not able to function efficiently in their absence (Wilson et al. 1995, Chang et al. 1995). Hence, the expression of the *PBX* genes in the developing visual cortex entails the presence of other homeobox genes in this structure.

We have successfully cloned the *PBX1* and *PBX2* homeotic genes in the developing visual cortex. The *PBX* homeobox gene family has evolved from the drosophila *extradenticle* homeotic gene (Rauskolb et al. 1993) and has three identified members in mammals - *PBX1*, *PBX2* and *PBX3* (Kamps et al. 1990, Monica et al. 1991). These genes, which are largely identical to each other, exhibit significant divergence in their amino and carboxyl termini (Monica et al. 1991). The expression of these genes has been confirmed in the brain and the involvement of the *PBX1* gene in olfactory system development is reported (Redmond et al. 1996).

2.1.2 Possible role of Segment Polarity Genes in Visual Development

Segment polarity genes have properties which may also be relevant to the development of the visual system. Firstly, segment polarity genes are involved in cellular signaling (Ingham et al. 1994, Roelink 1996, Moon et al. 1997, Nakayama et al. 2000). They are either secreted proteins used for signaling, receptors for the signaling protein or

downstream components of the signaling pathway. Conceivably, the segment polarity genes may facilitate cellular communication amongst neurons during visual development. Secondly, segment polarity genes are capable of repressing each other's expression and forming adjacent non-overlapping domains of expression (Roelink 1996, Jiang et al. 1995, Gilbert et al. 1994). It is possible that the mutually exclusive expression domains set up by the segment polarity genes may be the basis for the formation of ocular dominance columns in the postnatal visual cortex. Appropriately, segment polarity genes are expressed in adjacent domains and demarcate particular structures in the developing nervous system (Rubenstein et al. 1999, Joyner 1996, Mastick et al. 1996).

Segment polarity genes give rise to the *wingless* (*Wnt-1* in vertebrates), *decapentapelgic* (*BMP2* and *BMP4* in vertebrates) and the *hedgehog* signaling pathways. The presence of any two of these pathways in the visual cortex would give merit to the hypothesis that segment polarity genes account for the competitive interactions occurring during the critical period. Accordingly, the expression of segment polarity genes belonging to the above mentioned signaling pathways was investigated in the postnatally developing visual cortex.

2.1.2.1 Members of the BMP Signaling Pathway Present in the Developing Visual Cortex.

Bone Morphogenetic Proteins or the BMP's are a group of proteins that induce bone formation in mesenchymal tissue (Wozney et al. 1988). Despite their name, the BMP's function is not restricted to bone development and their expression is readily detected in various different organs (Scheld et al. 2000, McElwee et al. 2000, Cheifetz et al. 1999) including neurons (Tomizawa et al. 1995, Harland 2000). The BMP's are secreted proteins that belong to the TGF-β superfamily of growth factors (Massague 1990). The BMP's have been found to perform a variety of functions in the developing nervous system including neural differentiation, proliferation, and synaptogenesis (Withers

et al. 2000, Zhu et al. 1999, Hattori et al. 1999). BMP's can also modulate neurotrophin activity by controlling the expression of the Trk receptors (Zhang et al. 1998).

The secreted protein *BMP4* and its corresponding receptor *BMP type II receptor* were cloned in the developing cat visual cortex. *BMP4* is the vertebrae homologue of the drosophila *decapenatpelgic* gene (Padgett et al. 1993) and the presence of *BMP4* along with its corresponding receptor, *BMP type II receptor* (Liu et al. 1995), verifies the presence of a segment polarity gene signaling pathway in the visual cortex during the critical period. The expression of *BMP6* (another secreted protein belonging to the TGF-β growth factor family) in the postnatal visual cortex was serendipitously discovered while attempting to clone BMP7 which is a protein involved in synaptogenesis (Withers et al. 2000).

2.1.2.2 Members of the Wnt Signaling Pathway Present in the Developing Visual Cortex.

The *wingless* gene and its effectors constitute another important segment polarity gene signaling pathway (Ramakrishna et al. 1993). The vertebrae homologue of the drosophila *wingless* gene is the *Wnt* genes. The *Wnt* genes are secreted glycoproteins involved in cellular communication and act through the G-coupled *Frizzled* receptors (Wodarz et al. 1998, Bhanot et al. 1996). Roughly 11 different members of the *Wnt* family have been identified in mammals(Dale et al. 1998). The *Wnt* family can be divided into two distinct categories based on their function (Torres et al. 1996); the *Wnt-1* class and *Wnt-5a* class. Interestingly, the members of the *Wnt-5a* class proteins antagonize the effects of the *Wnt-1* class proteins (Torres et al. 1996). Similar to the BMP's the Wnt proteins in the developing nervous system are reported to perform a variety of different functions including differentiation, migration, apoptosis, proliferation and axis formation (Patapoutian et al. 2000, McMahon et al. 1990, McMahon et al. 1992, Rubenstein et al. 1999, Smolich et al. 1994, Maloof et al. 1999, Shum et al. 1999, Wolda et al. 1993).

The expression of the Wnt-1 secreted protein was investigated in the cat visual cortex because *Wnt-1* is the homologue of the *wingless* gene and a possible inhibitor of

BMP4 signaling (Ramakrishna et al. 1993). The expression of the *Wnt-1* (or *wingless*) signaling pathway in the visual cortex verifies the presence of a molecular mechanism which can potentially antagonize the *BMP4* (*decapentapelgic*) signal. The expression of *the Wnt-1* and *BMP-4* signaling pathways during visual development gives merit to the notion that the interactions amongst segment polarity genes may be the molecular basis for the synaptic segregation occurring during the critical period. In addition, the expression of Frz-B, which is a secreted protein that prevents the Wnt proteins from binding to their receptor (Leyns et al. 1997), was detected. Hence, the Frz-B gene is hypothesized to be another candidate molecule that may be involved in synaptic segregation.

HYPOTHESIS: The PBX homeotic genes, the Wnt-1, Frz-B signaling genes and the BMP-4, BMP type II receptor genes are involved in the postnatal development of the cat visual cortex.

OBJECTIVES: 1) To identify homologues of the homeotic and the segment polarity gene families expressed in the developing cat visual cortex by performing RT-PCR.

2) To get sequence information for the homeotic and segment polarity genes expressed in the cat and to develop specific cDNA probes for these genes in the cat.

3) To illustrate that the expression of homeobox and segment polarity gene homolouges in the cat visual cortex are developmentally regulated.

2.2 METHODS

Animal Preparation

A total of 17 postnatal cats were used for the experiments described in this chapter (N=3 for 15 day old kittens, N=5 for 30 day old kittens, N=4 for 120 day old kittens and N=5 for adult cats). The animals were given an overdose of euthanol and then they were briefly perfused with ice cold RNAse free PBS solution. The brains were then removed, the visual cortecies were dissected, put into Falcon tubes and frozen in isoamyl alcohol. The tissue was kept frozen at - 80°C until processing.

Total RNA isolation

Trizol solution was warmed to room temperature before use (storage is at 4°C). Ten to fourteen volumes of Trizol reagent was added to per unit weight of frozen brain tissue. Adding more than ten volumes of Trizol reagent was found to significantly decrease DNA contamination and slightly improve the 260/280 ratio of the purified total mRNA product. The tissue was homogenized in trizol using a motorized homogenizer. Efforts were made to keep bubbles in the homogenate to a minimum. The homogenized samples were incubated for 5 minutes at room temperature to allow the complete dissociation of nucleoprotein complexes. Zero point two volumes of chloroform was then added to the Trizol reagent. The tubes were securely capped and shaken vigorously for 15 seconds and then incubated at room temperature for 3 minutes. The samples were then centrifuged at 12000 x g for 15 minutes at 4°C. After centrifugation the upper, colourless aqueous phase was carefully pipetted out and placed into a fresh tube without disturbing the interphase (which contains DNA) or the lower, red phenol-chloroform layer containing organic phase. In order to avoid disturbing the interphase while collecting all the aqueous phase, the organic phase was back extracted by adding an equal volume of DEPC treated distilled water. The solution was then mixed and centrifuged at 12000 x g for 5 minutes at 4°C. The second extract was then pooled with the original RNA extraction. An equal volume of

isopropanol was added to the RNA solution, mixed and incubated at room temperature for 10 minutes. After the incubation, the sample was centrifuged at 12000 x g for 10 minutes at 4°C. The supernate was carefully removed without disturbing the RNA pellet which had formed at the bottom of the tube. RNA pellet was washed twice by adding ice cold 75% ethanol (the tube containing the pellet was completely filled with 75% ethanol), mixed by finger vortexing and spun at 7500 x g for 5 minutes at 4°C. After the second wash, the supernatant was carefully removed and the sample was briefly pulse centrifuged to bring down any ethanol which had not been removed. The residual ethanol was removed and the tube was pulse centrifuged once again to ensure that no ethanol had remained. Vacuum or air drying was found to severely decrease the solubility of the RNA pellet. After all the ethanol was removed, the RNA pellet was dissolved in DEPC treated distilled water. The total RNA was then subjected to DNAse treatment in order to remove any residual DNA. The DNAse enzyme was removed by phenol chloroform extraction followed by reprecipitation of the RNA in ethanol and reconstitution in DEPC treated distilled water.

mRNA Isolation

Zero point two ml's of Dynabeads Oligo $(dT)_{25}$ was removed from the stock solution and was put in a fresh 1.5ml RNAse free Eppendorf tube. The tube was then placed in the Dynal MPC (the magnetic stand which collects the dynabeads) for 30 seconds and the clear solution was removed. The Dynabeads were then resuspend in 0.1ml of 2X Binding Buffer (20mM Tris-HCl (pH 7.5), 1.0M LiCl, 2mM EDTA). The volume of the total RNA was adjusted to 75µg's in 100µl's of DEPC treated water. This solution was mixed and heated at 65°C for 2 minutes in order to remove any secondary structures present in the RNA. During this step, the 2X Binding buffer was removed from the dynabeads and replaced with 0.1ml's of fresh binding buffer. The 0.1ml solution of total RNA was then added to the Dynabeads and the mixture was thoroughly mixed. This step was followed by a 5 minute hybridization of the Dyanbeads and the RNA on a rotating

roller at room temperature. The beads were then put on the Dynal MPC and the supernatant was removed. The beads containing the mRNA was then washed twice with 0.2ml's of washing buffer (10mM Tris-HCl (pH 8.0), 0.15mM LiCl, 1mM EDTA). During each wash the beads were thoroughly mixed and during the supernatant removal step, care was taken to remove all available solution. The mRNA was then eluted off the beads by applying 20µl's of Elution solution (2mM EDTA pH 8.0) directly to the beads and heating at 65°C for 2 minutes. The beads were then placed on the Dynal MPC and the remaining mRNA solution was placed into a new RNAse free Eppendorf tube and stored at - 80°C till use.

Reverse Transcription Reaction

One microgram of poly $(A)^+$ RNA was put into a fresh RNAse free tube and the total volume of the solution was adjusted to 14µl's with DEPC-treated distilled water. 1µl of 85µM oligo (dT) primer stock was added and the tubes were placed in 70°C for ten minutes in order to denature any RNA secondary structures. The tubes were then chilled on ice for ten minutes and the contents of the tubes were collected by brief centrifugation at room temperature. 2µl of 10X first strand buffer (0.5mM Tris-HCl (pH 8.3), 0.75M KCl, 0.03M $MgCl_2$), 1µl of RNAse Block Ribonuclease Inhibitor (40U/µl), 1µl of dNTP mix (25mM of each dNTP) and 1µl of MMLV-RT (20U/µl) was added to the tube. The contents of the tubes were gently mixed, and then the tubes were briefly centrifuged. The reaction was incubated at room temperature for 10 minutes followed by a 60 minute incubation at 37°C. After the reaction, the tubes were heated to 90°C for five minutes to inactivate the reverse transcriptase. Five hundred microliters of distilled water was added to the reaction and this diluted RT product was used as template for the PCR reactions.

PCR Primer design

Twenty-three base pairs long primers were designed using the primer program on the PC platform. The human homologue of the gene of interest was used as the template for the PCR primers. The selected primers had a GC content of between 50% to 60% and did not have more than 2 of the same nucleotide base adjacent to each other in their sequence. It was also ensured that the melting temperature of the 5 prime and the 3 prime primers did not differ more than 3°C. Once candidate primers were designed, the sequence of each primer was subjected to a Genbank searched. In order to improve the specificity of the PCR reaction, primers that had significant homology with animal genes other than the ones of interest were not used. Additionally, primers that had mismatches near the 3 prime end or significant mismatches with either the rat or the mouse sequence of the gene of interest were not used.

PCR

Prior to PCR all plasticware (i.e. tips and tubes) and non-enzymatic solutions were UV treated in order to remove any contaminating DNA. A typical PCR reaction consisted of 40mM Tricine-KOH (pH 9.2 at 25°C), 15mM KOAc, 3.5mM Mg $(OAc)_2$, BSA 75μg/ml, 0.2mM of each dNTP, 1μM of each primer, 1μl of the diluted reverse transcription reaction and 1μl of the 50X Advantage cDNA polymerase Mix from Clonetech in a 50μl final volume. The cycling conditions consisted of an initial cycle at 94°C for 1 minute to remove the antibody from the Taq enzyme followed by 30-40 cycles of 94°C for 30 seconds for denaturing, 47 - 62°C for 45 seconds for annealing and 68°C for 1 minute 30 seconds for extension in a Perkin Elmer 9600 thermal cycler.

Three prime end labeled Oligonucleotide Probes

Three prime ends of fifty-mer oligonucleotide were labeled in a 25µl reaction containing 5µl of 5x Co reaction buffer (0.7mM potassium cacodylate, 150mM Tris base, 5mM $CoCl_2$, 0.5mM DTT, pH7.2), 80ng of the primer, 10µl of [α-^{33}P] dATP and 2µl of terminal transferase enzyme. The reaction was incubated at 37°C for 90 minutes. Unincorporated nucleotides were removed by NucTrap Columns.

NucTrap Columns

NucTrap columns manufactured by Stratagene were used to remove unincorporated radioactive nucleotides from both double stranded DNA and oligo probes. Hence, they can be used for purifying both large DNA and small oligo probes. Seventy five microliters of 1x STE (0.1M NaCl, 20mM Tris-HCl (pH7.5), 10mM EDTA) was loaded onto the column and an empty 10ml syringe was attached. The STE was passed through the column by pushing down on the syringe. After the column was rehydrated, the probe was loaded onto the column in a volume of 75µl (STE or water was used to dilute the probe) and passed through using a 10ml syringe. The eluate from the column was collected into an Eppendorf tube. Another 75µl of 1x STE was loaded and passed through the column. Again the eluate was collected. Since the probe was radioactive, appropriate precautions such as shielding were in place during the purification procedure. Also, the syringe was never pulled back while it was attached to the column because this action would have disrupted the column.

Southern Blotting

The PCR products were electrophoresed, blotted onto nylon memberanes and the membranes were UV fixed. These blots were then put into hybridiztion buffer containing 6X SSC, 5X Denhardt's reagent, 0.5% SDS and 100µg/ml salmon sperm DNA (which

was boiled for 5 minutes before being added to the mixture) and pre hybridized for at least one hour at 42°C in a shaking water bath. Then the prehybridization buffer was removed and replaced with hybridization buffer containing 1million counts of the oligo probe/ml and the hybridization was allowed to proceed overnight at 42°C. The following day, the blot was washed twice in 2XSSC, 0.1% SDS for five minutes at room temperature followed by a 42°C wash for 30 minutes at room temperature with the same wash buffer. The temperature was then increased to 55°C and the membrane was washed once with 0.5XSSC, 0.1%SDS for thirty minutes followed by at least one wash with 0.2XSSC, 0.1%SDS. The membranes were then wrapped in saran wrap and exposed to X-ray film overnight.

Cloning

In order to polish the ends of the PCR product, 2.5U of Pfu DNA polymerase was added to 10µl's of the PCR product in a 0.2ml Eppendorf tube. The polishing reaction was incubated at 72°C for 30 minutes. After the polishing reaction the PCR product was electrophoresed and the band of interest was gel purified. Alternatively, the polished PCR products were stored at -20°C. The polished PCR products were ligated into the pCR-Script SK (+) vector by adding 1µl of pCR-Script SK (+) vector (10ng/µl), 1µl of 10x pCR-Script Direct Reaction Buffer, 0.5µl of 10mM rATP, 5.5µl of polished PCR product, 1µl of Srf I restriction enzyme (5U/µl) and 1µl (4U/µl) of T4 DNA ligase. An estimated insert to vector ratio of 40:1 to 100:1 was used. The tubes were mixed and incubated at room temperature for 1 hour (an overnight incubation at 16°C following the room temperature incubation substantially improves cloning efficiency). After the ligation

reaction, the samples were heated at 65°C for 10 minutes to inactivate the ligase enzyme and the samples were stored on ice until the transformation reaction.

Sequencing

Sequencing reactions were performed using the Amersham ThermoSequnase radiolabeled terminator cycle sequencing kit. Briefly, four tubes containing 2µl's of dGTP termination master mix containing 7.5µM dATP, dCTP, dGTP, dTTP and 0.5µl's of either [α-^{33}P] ddGTP, ddATP, ddTTP or ddCTP (each at 1500Ci/mmol, 450µCi/ml) were prepared for each clone which was to be sequenced. A mix containing the dGTP master termination mix and the appropriate [α-^{33}P] ddNTP was prepared and 2.5µl's of this mixture was added to the appropriate. The tubes were capped and kept on ice while the reaction mixture was made. A reaction mixture containing 2µl's of reaction buffer (260mM Tris-HCl, pH9.5, 65 mM $MgCl_2$), 50-200ng of DNA, 2.5pmol of primer, and 8 units of Thermo Sequenase polymerase (4U/µl in 0.0006U/µl *Thermoplasma acidophilum* inorganic pyrophospahatase; 50mM Tris-HCl, pH8.0, 1mM dithiothreitol (DTT), 0.1mM EDTA, 0.5% Tween-20, 0.5% Nonidet P-40, 50% glycerol), was prepared in a volume of 20µl's for each clone to be sequenced. Four point five microliters of the reaction mix was added to the tubes containing ddGTP, ddATP, ddTTP and ddCTP termination mixes. Once the mixture was aliquoted into the appropriate tubes, the tubes were mixed, spun down and placed into a thermal cycler machine which was preheated to 90°C. Thirty cycles at 95°C for 30 sec, 60°C for 30 sec and 72°C for 2minutes were performed. After the PCR reaction 4µl's of stop solution (95% formamide, 20mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF) was added to each sample. Samples were placed in -20°C until electrophoresis.

Labeling of Double stranded DNA

The plasmid DNA was cut with appropriate enzymes and the resulting insert was electrophoresed on a 1% low temperature agarose gel and gel purified. The amount of purified insert DNA was estimated by running a portion of the purified insert on another gel. The DNA was labeled using the Prime-It II Random Primer Labeling Kit distributed by Stratagene. Roughly 25ng's of DNA and 10µl's of random 9mer oligonucleotide primers (27 OD units/ml) was boiled together in a volume of 34µl's for 5 minutes. The tubes were then briefly centrifuged at room temperature to bring down the condensation. 10µl's of 5x primer buffer provided by kit (containing 0.1mM each of dATP, dGTP, dTTP) and 5µl's of [α-^{32}P] dCTP (3000Ci/mmol) was added to the tubes. 1µl of Exo(-)Klenow enzyme (5U/µl) was added and the reaction was incubated at 37°C for 30 minutes. Unincorporated nucleotides were removed by NucTrap Columns.

Northern blotting

3µg's of mRNA was loaded onto each RNA gel. The RNA gels were electrophoresed and then the RNA was transferred onto nylon memberanes. The blot was then prehybridized in a shaking water bath for 1 hour at 42°C in prehybridization solution. After the prehybridization, the prehybridization buffer was removed and hybridization buffer containing 1million count per ml of probe was added to the blot. The blot was incubated overnight at 42°C. The following day, the blot was washed twice for 5 minutes at room temperature with 2XSSC, 0.1% SDS solution. These washes were followed with 1 wash at 42°C with 2XSSC, 0.1%SDS for 30 minutes, 1 wash at 50°C with 0.5XSSC, 0.1%SDS for 30 minutes, 2 washes at 55°C with 0.2XSSC, 0.1%SDS. If longer DNA probes were used, the final washing temperature had to be increased to 60°C. After the

washes, the blots were wrapped in saran wrap and exposed to X-ray film for 3 to 10 days in a light tight cassette which was stored in -80°C.

The PBX1, PBX2, BMP4 and Wnt-1 probes were used on four different mRNA blots. After the northern blot analysis, the blots were stripped and reprobed with another marker. A blot was never reprobed with the same probe twice. The BMP6 northern blot analysis was repeated twice whereas the PBX1, PBX2, Wnt1 and BMP4 northern blot analysis was repeated three times (i.e. repeated on three different blots).

2.3 RESULTS

2.3.1 Technical Considerations

One of the reasons the drosophila melanogaster is a popular animal model for developmental genetic studies is its relative simplicity at the molecular level. The vast majority of the genes characterized in the drosophila are cloned in more complex animals where they perform similar functions (Gellon et al. 1998). This retention of function adds to the value of drosophila as a genetic animal model. However, hundreds of millions of years of evolution separate the drosophila from the mammals. During this period of time, new and more elaborate body types have arisen. In order to achieve this complexity an extensive amount of duplication and divergence has taken place at the genetic level (Holland et al. 1996a, Holland et al. 1996b, Williams et al. 1998). Accordingly, multiple variants or homologues of a single gene found in drosophila are present in mammals (Monica et al. 1991). Some of the recently evolved homologues of the drosophila developmental genes have distinct functions in mammals whereas other homologues are functionally redundant and safeguard against errors made during development (Depew et al. 1999, Bertuzzi et al. 1999). From an experimental point of view, the presence of multiple gene variants adds an increased level of complexity to identifying homologs of drosophila developmental genes expressed in the cat visual cortex during development.

Since the cat is not a popular genetic model, very little sequence information is available for this animal. Accordingly, before the expression of the homolougs of the drosophila developmental genes can be examined in the developing visual cortex, specific probes for these genes need to be obtained. Hence, attempts were made at partially cloning the cDNA's of various homeotic and segment polarity genes. PCR primers specific for individual genes were designed based on sequence information available from other species (mostly human). RT-PCR was then performed on cat cDNA using low annealing temperatures. The presence of multiple variants in mammals for drosophila developmental genes was addressed by trying to clone the mammalian homologue which most closely resembles its counterpart in drosophila both at the structural and functional levels.

2.3.2 Summary of the cloning strategy

Primers were designed to human sequences of the gene which was to be cloned in the cat. PCR was performed on cat cDNA using annealing temperatures ranging from 45°C - 62°C. The PCR products were electrophoresed and blotted as described in the methods section. Since low stringency PCR yields a lot of non-specific products, gene specific 50mer oligo probes were designed and these probes were used to screen the cDNA fragments generated by PCR via southern blot analysis. The PCR products which were identified by southern blot analysis were cloned and sequenced to confirm that the PCR fragment was actually from the gene of interest.

2.3.3 PBX1

PBX1 is a mammalian homologue of the *extradenticle* gene (Kamps et al. 1990). Figure 2.1 shows the partial cDNA sequence of the cat *PBX1* gene in the cat. The sequenced portions of the cat PBX1 cDNA have 93% and 98% identity with the mouse and human PBX1 cDNA's. This PBX1 cDNA fragment was obtained inadvertently while attempting to partially clone the cat PBX2 cDNA. This outcome is not surprising given the extensive homology between the different members of the PBX gene family. The PCR primers used to generate the cat PBX 1 cDNA fragment are

```
Cat    :    1 atcatccatcggaagttcagctccatccagatgcaacttaagcagagcacatgcgaggcc   60
             ||||||||  ||  ||||||||||||||||||||||||  ||  ||  ||||||||||||||||||
Mouse:1181 atcatccaccgcaagttcagctccatccagatgcagctgaaacagagcacatgcgaggcc 1240

Cat    :   61 gtcatgatcctgcgctcccggtttctggacgcgcggcggaagagacggaatttcaacaag  120
             ||||||||||||||||||||||||  |||||  |||  ||||||||||||||||||||||||||
Mouse:1241 gtcatgatcctgcgctcccggttcctggatgcgaggcggaagagacggaatttcaacaag 1300

Cat    :  121 caagcaacggaaatcctgaatgaatatttctattcccatctcagcaa  167
             |||||  ||  ||||||  ||||||||||||||||||||||||||||||||
Mouse:1301 caagccacagaaattctgaatgaatatttctattcccatctcagcaa 1347

                Blast score =  244 bits (123), Expect = 8e-63
                        Identities = 156/167 (93%)
                           Strand = Plus / Plus


Cat    :    4 ccaggttggagccaacgtgcagtcacaggtggatacctttcgccatgttatcagccagac   63
             ||||||||||||||||||||||  ||||||||||||||  ||||||||||||||||||||||
Human:1206 ccaggttggagccaacgtgcaatcacaggtggataccttcgccatgttatcagccagac 1265

Cat    :   64 aggaggatacagtgacggactcgcagccagtcagatgtacagtccgcagggcatcagtgc  123
             ||||||||||||||||  ||||||||||||||||||||||||||||||||||||||||||||
Human:1266 aggaggatacagtgatggactcgcagccagtcagatgtacagtccgcagggcatcagtgc 1325

Cat    :  124 taatggaggttggcaggatgccactacccccttcatcagtgacctcccctacagaaggccc  183
             ||||||||||||||||||||  ||||||||||||||||||||||||||||||||||||||||||
Human:1326 taatggaggttggcaggatgctactacccccttcatcagtgacctcccctacagaaggccc 1385

Cat    :  184 tggcagtgttcactctgatacctccaactg  213
             ||||||||||||||||||||||||||||||
Human:1386 tggcagtgttcactctgatacctccaactg 1415

                Blast Score =  385 bits (194), Expect = e-105
                        Identities = 206/210 (98%)
                           Strand = Plus / Plus
```

Figure 2.1 Partial cDNA sequence of the cat PBX1 gene.

CCATCGAACACTCGGACTATCGC and ACCAGTTGGAGACCTGAGACACG and these primers are specific to the human PBX2 cDNA.

Figure 2.2 is a northern blot autoradiograph showing the mRNA expression levels of the *PBX1* gene in the postnatally developing cat visual cortex. The cat PBX1 mRNA specific 45'mer oligo probe GGAGGTCACTGACGAAGGGGTAGTGGCATCCTGCCAACCTCCATT was designed based on the cat nucleotide sequence obtained from the above mentioned experiments. This 45'mer oligo was used as the probe in the Northern Blot autoradiograph shown in Figure 2.2. As in humans (Kamps et al. 1990), 2 splice variants of the *PBX1* gene named *PBX1a* and *PBX1b* were detected in the developing cat visual cortex. The PBX1a mRNA expression in the visual cortex, which is high in 15 day old animals, is greatly reduced in 60 day and adult animals. A similar expression pattern is observed for PBX1b mRNA. Also, the PBX1b transcript is expressed at much lower levels than the PBX1a transcript.

2.3.4 PBX2

PBX2 is another mammalian homologue of the *extradenticle* gene (Monica et al. 1991). Figure 2.3 shows the partial cDNA sequence of the cat *PBX2* gene. The cat PBX2 cDNA has 90% identity with the human PBX2 cDNA. The cat PBX2 cDNA was generated by PCR using the primers CCATCGAACACTCGGACTATCGC and ACCAGTTGGAGACCTGAGACACG. These primers were designed based on sequence information available for the human PBX2 gene.

Figure 2.4 is a northern blot autoradiograph showing the mRNA expression levels of the *PBX2* gene in the postnatally developing cat visual cortex. The cat PBX2 mRNA specific 45'mer oligo probe GCAGAATAGGAATCTCCGTTGAGCCCGGGCATCCCCATAAACATGTCTCC was designed based on the cat nucleotide sequence obtained from the above mentioned cloning experiments. This 45'mer oligo was used as the probe in the Northern Blot autoradiograph shown in Figure 2.4. Similar to PBX1, PBX2 mRNA expression in the visual cortex is

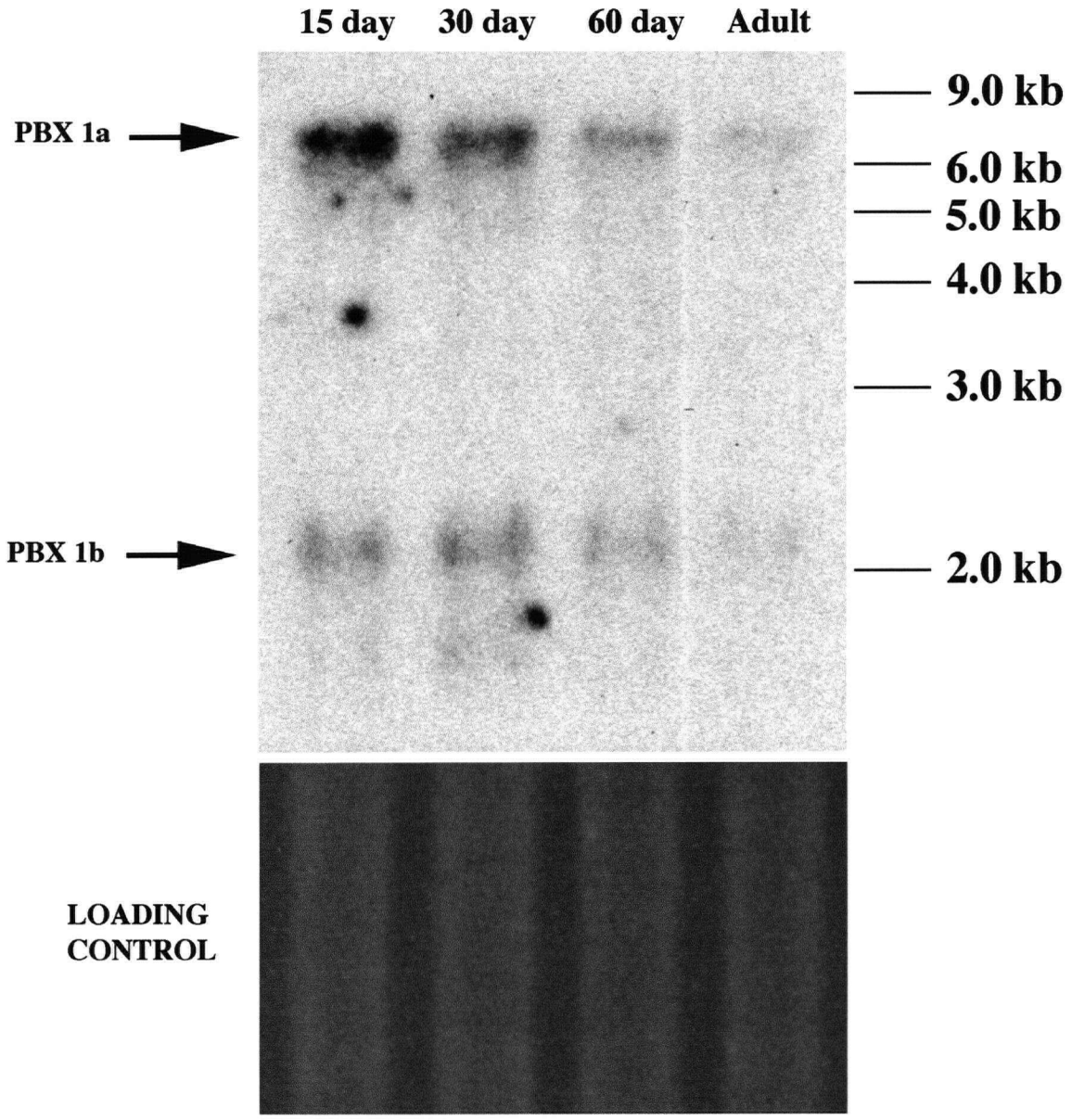

Figure 2.2: PBX-1 Gene mRNA expression in the postnatally developing cat visual cortex. The PBX-1 gene expresses 2 splice variants in the developing cat visual cortex. The 7.5 kb transcript is referred to as Pbx-1a and the 2.2 kb transcripts is named Pbx 1b. The expression of both splice variants is downregulated in the adult. The bottom panel shows the mRNA gel preparation (stained with ethidium bromide) for this Northern blot

```
Cat  :  1  agcaaacttgctcagatccgccacatctaccactcggagctggagaaatatgagcaggcg   60
           ||||||||||| |||||||| ||||| ||||||||||||||||||||| ||||||||||
Human:783  agcaaacttgcccagatccgtcacatataccactcggagctggagaagtatgagcaggca  842

Cat  : 61  tgtaacgagttcacaacccacgtcatgaacctgctgagggaacagagccgcacacggcct  120
           ||||| |||||||| ||||| ||||||||||||||||||| ||||||||||  ||||
Human:843  tgtaatgagttcacgacccatgtcatgaacctgctgagggagcagagccgcaccaggccc  902

Cat  :121  gtggcacccaaggagatggagcgcatggtgagcatcatccatcggaagttcagtgccatc  180
           ||||| ||||| |||||||| |||||||||||||||||||||| |||||||| ||||||
Human:903  gtggcccccaaagagatggaacgcatggtgagcatcatccatcgaaagttcagcgccatc  962

Cat  :181  cagatacagctcaagcagagcacctgtgaggctatcatgattctgcgttcccgcttcttg  240
           ||||| ||||| |||||||||||||| |||||| | ||||| ||||| ||||| ||| ||
Human:963  cagatgcagctgaagcagagcacctgcgaggctgtgatgatcctgcgctcccgtttcctg 1022

Cat  : 241 gatgccagacgaaaacgccgcaacttcagcaaacaggccactgaggtcctcaatgagtat  300
           |||||||||||||| ||||| |||||||||||||||||||||||||||||| |||||||||
Human:1023 gatgccagacgaaagcgccgtaacttcagcaaacaggccactgaggtcctaaatgagtat 1082

Cat  : 301 ttctactcacatctgagtaacccatatcctagtgaagaggctaaggaggagcttgcaa  358
           |||||||| || |||||||||||||||||||||| ||||| |||||||||||||| |
Human:1083 ttctactcccacctgagtaacccatatcctagtgaggaggccaaggaggagcttgcca 1140
```

Figure 2.3 Partial cDNA sequence of the cat PBX2 gene.
Blast Score = 444 bits (224), Expect = e-122
Identities = 324/358 (90%)
Strand = Plus / Plus

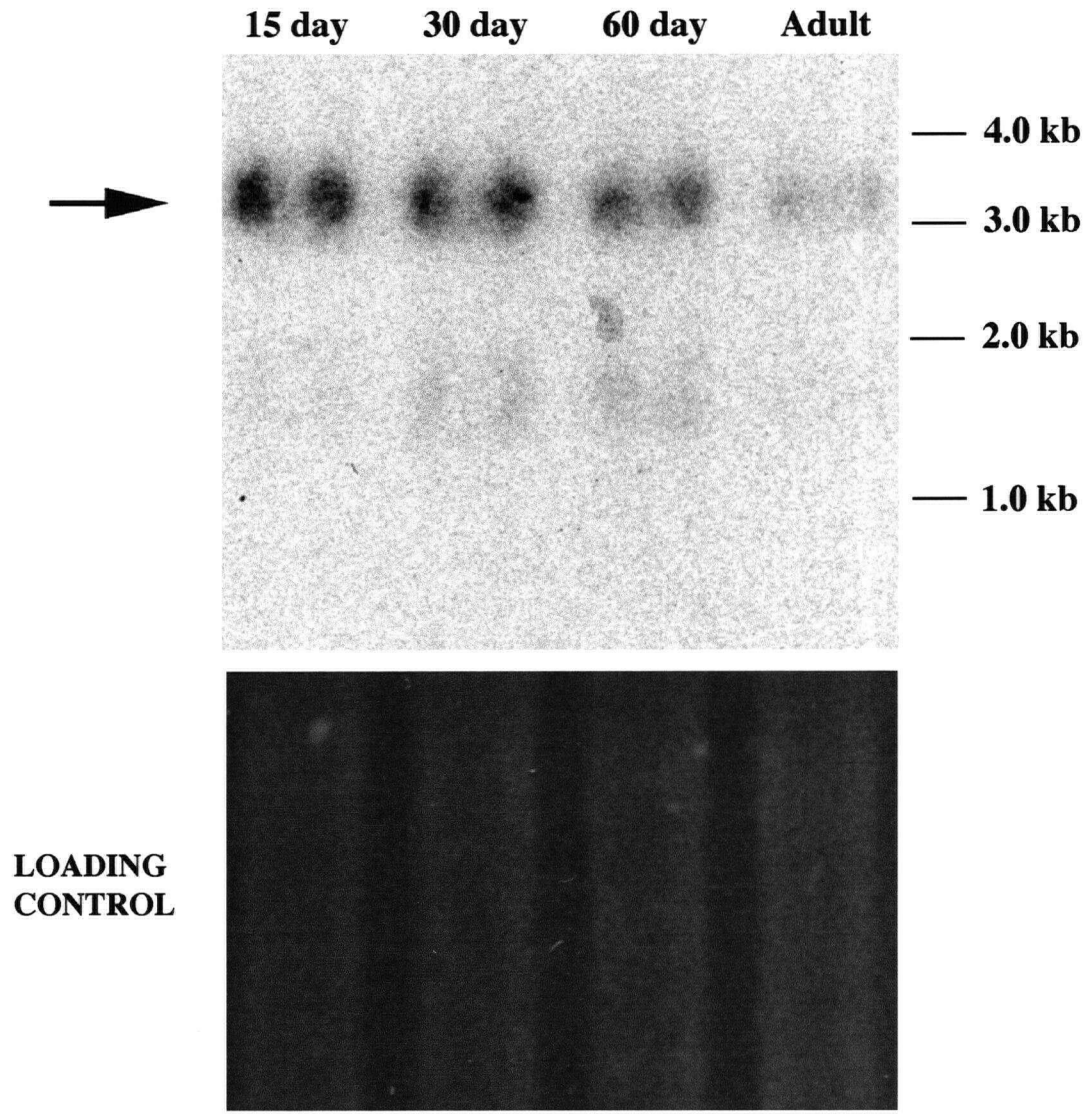

Figure 2.4 PBX-2 Gene mRNA expression in the postnatally developing cat visual cortex. The PBX-2 gene mRNA is expressed at relatively high levels in the visual cortex of young kittens but the expression of this mRNA decreases in the adult visual cortex. The developmentally regulated expression of this transcript in the postnatal visual cortex is consistent with the notion that the PBX-2 gene is involved in visual development. The bottom panel shows the mRNA gel preparation (stained with ethidium bromide) for this Northern blot analysis.

high in young kittens but the expression of this mRNA decreases in the adult. A splice variant is not observed for PBX2 mRNA.

2.3.5 BMP 4

BMP 4 is the mammalian homologue of the drosophila *decapentaplegic* gene (Padgett et al. 1993). Figure 2.5 shows the partial cDNA sequence of the *BMP 4* gene in the cat. The sequenced portions of the cat BMP4 cDNA have 89% and 93% identity with the human BMP4 cDNA. The BMP 4 cDNA was generated by PCR using the primers GACTTCGAGGCGACACTTCTGCA and GCCACAATCCAGTCATTCCAGCC. These primers were designed based on sequence information available for the human *BMP 4* gene.

Figure 2.6 is a northern blot autoradiograph showing the mRNA expression levels of the *BMP 4* gene in the postnatally developing cat visual cortex. The expression levels of the BMP 4 mRNA are fairly low (the autoradiograph shown in figure 2.6 was exposed for 10 days). The small smear observed in this autoradiograph suggests BMP4 splice variants are expressed in the developing visual cortex. This expression pattern was observed in three different northern blots and is unlikely to be an artifact. The possibility that the mRNA on these northern blots were degraded can be ruled out because when these blot were stripped and reprobed with the PBX1 probe sharp autoradiographic bands were obtained (as seen on figure 2.2). In the adult visual cortex, the higher part of the smear (or the larger splice variant) is missing whereas the lower part remains unaffected. Hence, the BMP 4 splice variants appear to be differentially regulated during the postnatal development of the cat visual cortex.

2.3.6 BMP 6

The partial BMP 6 cDNA sequence in the cat was obtained while trying to clone the cat BMP 7 cDNA, which is known to be involved in synaptogenic events (Withers et al. 2000). Figure 2.7 shows the partial cDNA sequence of this gene in the cat. The sequenced portions of the cat BMP6 cDNA have 92% and 94% identity with the human

```
Cat   :   5 ctggtcttgagtatcctgagcgccccgccagtagggccaacaccgtgagaagcttccacc  64
          ||||||||||||||||||||||||||| |||||  ||||||||||||||| ||||||||||
Human:388 ctggtcttgagtatcctgagcgcccggccagccgggccaacaccgtgaggagcttccacc 447


Cat   :65  acgaagaacatttggagaacatcccagggaccagcgaaaactctgattttcgtttcctct 124
          |||||||||||| ||||||||||||||||||||| |||||||||| ||||||||||||||
Human:448 acgaagaacatctggagaacatcccagggaccagtgaaaactctgcttttcgtttcctct 507


Cat   :125 ttaacctcagcagcatcccagagaacgaggtggtctcttccgcagagcttcgactcttcc 184
          |||||||||||||||||| |||||||||||| |||| || |||||||||| |||||||
Human:508 ttaacctcagcagcatccctgagaacgaggtgatctcctctgcagagcttcggctcttcc 567


Cat   :185 gggagcag 192
          ||||||||
Human:568 gggagcag 575
```

Blast Score = 278 bits (140), Expect = 7e-73
Identities = 176/188 (93%)
Strand = Plus / Plus
The 1st 4 bases in the cat sequence are agcc

```
Cat   :   1 cttgacccgacgccagagggccaaacgcagccccaagcatcacccacagcgggcccggaa   60
          |||||||||||||| ||||||||| || ||||| ||||||||| |||||||||| ||||
Human:1284 cttgacccgacgccggagggccaagcgtagccctaagcatcactcacagcgggccaggaa 1343


Cat   :  61 gaagaataagaatcgtcgtcgccactcgctctacgtggacttcagcga  108
          ||||||||||||  | || |||||||||||||| ||||||||||||||
Human:1344 gaagaataagaactgccggcgccactcgctctatgtggacttcagcga 1391
```

Blast Score = 127 bits (64), Expect = 1e-27
Identities = 97/108 (89%)
Strand = Plus / Plus

Figure 2.5 Partial cDNA sequence of the cat BMP4 gene.

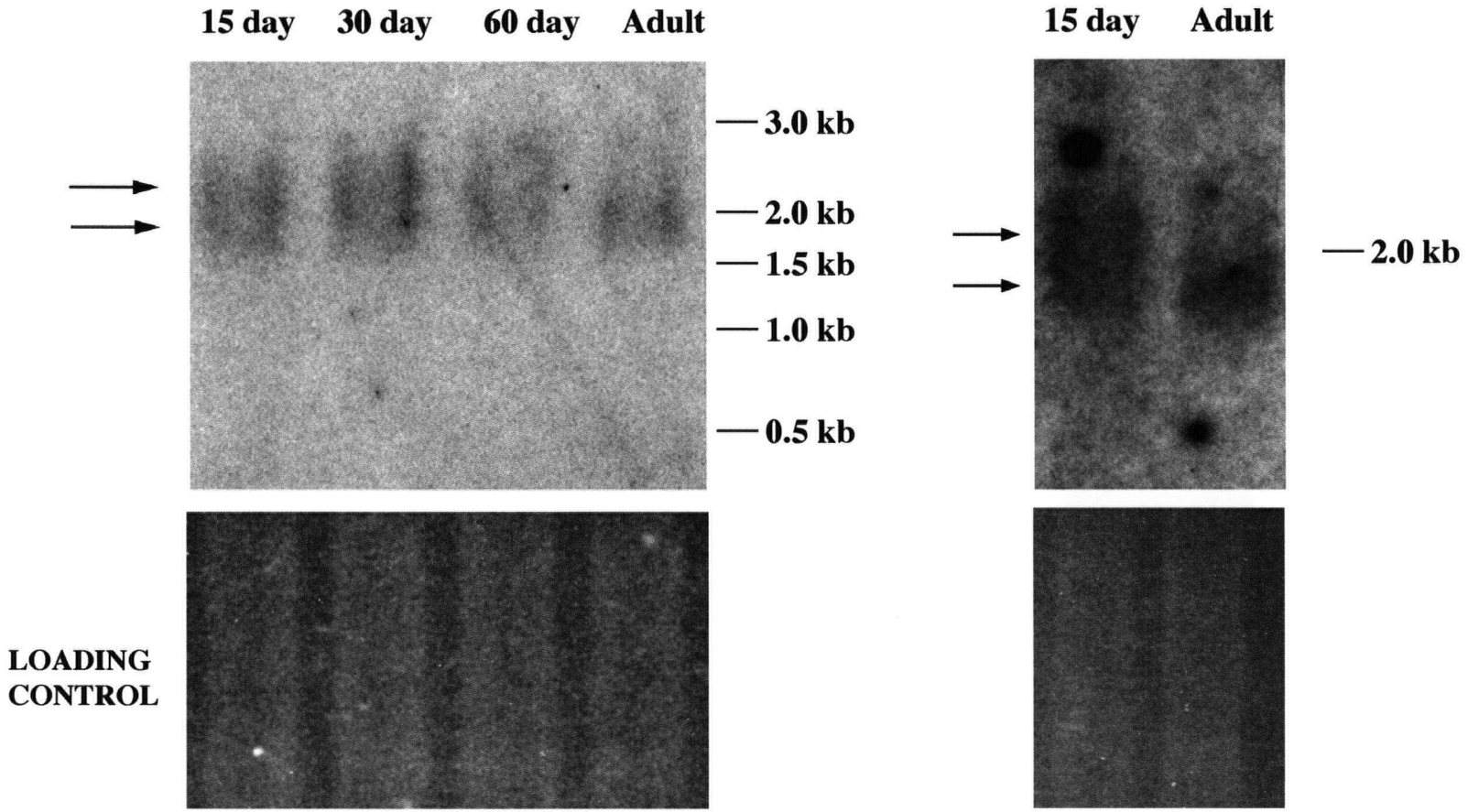

Figure 2.6 BMP4 Gene mRNA expression in the postnatally developing cat visual cortex. During visual cortex development in the cat, two similar sized splice variants (or doublets) of the BMP4 gene are expressed (pointed out by the arrows). The panel on the left is a northern blot autoradiograph containing visual cortex mRNA from 4 different ages whereas the panel on the right compares mRNA expression between 15 day and adult visual cortex. In the northern blot shown in the right panel, a higher percentage mRNA gel (2%) was electrophoresed for a longer period of time to better separate the two splice variants. An appreciable change in the expression level of the smaller BMP4 transcript is not detected in the visual cortex during the critical period. However, the larger BMP4 splice variant is not expressed in the visual cortex of adult cats. This differential regulation of the BMP4 splice variant expression may be relevant to the postnatal development of the cat visual cortex. The bottom panels show the mRNA gel preparations (stained with ethidium bromide) for these Northern blot autoradiographs.

```
Cat   :    1 caggacgtgtcgcgggtctccagcgcttcagattataacagcagtgagttgaaaacagcc   60
           ||||||||| |||||||||||| |||||||||| |||||||||| ||||||||||||
Human: 1353 caggacgtggcgcgggtctccagtgcttcagattacaacagcagtgaattgaaaacagcc 1412

Cat   :   61 tgcaggaaacatgagctttacgtgagcttccaagacctgggatggcaggactggatcatc   120
           |||||||| |||||||| || ||||| ||||||||||||||||||||||||||||||||
Human: 1413 tgcaggaagcatgagctgtatgtgagtttccaagacctgggatggcaggactggatcatt 1472

Cat   :  121 gcacccaagggctatgctgccaattactgtgatggaggatgctccttcccactcaatgca   180
           |||||||||||||||||||||||||||||||||||||||| |||||||||||||||| |||
Human: 1473 gcacccaagggctatgctgccaattactgtgatggagaatgctccttcccactcaacgca 1532

Cat   :  181 cacatgaacgccacaaaccacgcgatcgtgc  211
           |||||||| || || |||||||||| ||||
Human: 1533 cacatgaatgcaaccaaccacgcgattgtgc 1563
```

Blast Score = 299 bits (151), Expect = 2e-79
Identities = 196/211 (92%)
Strand = Plus / Plus

```
Cat   :    1 ctgtgggtcgtgaccccacagcacaacatggggcttcagctgagcgtggtgactcgggat   60
           |||||||| ||||| || |||||||||||||||| |||||||| ||||||||||||||||
Mouse: 1153 ctgtgggtggtgacaccgcagcacaacatggggctccagctgagtgtggtgactcgggat 1212

Cat   :   61 ggactcaacatcaacccccgagccgcaggcctggtgggcagagacggcccttacgacaag   120
           |||||| || |||||||||| || || ||||||||||||||||||||||||||||||||
Mouse: 1213 ggactccacgtcaacccccgtgcggcgggcctggtgggcagagacggcccttacgacaag 1272

Cat   :  121 cagcccttcatggtggccttcttcaaagtgagcgaggtccacgtgcgcaccaccaggtca   180
           |||||||||||||||||||||||| ||||||||||||||||||||||||||||||||
Mouse: 1273 cagcccttcatggtggccttcttcaaggtgagcgaggtccacgtgcgcaccaccaggtca 1332

Cat   :  181 gcctccagtcggcggcggc  199
           ||||||||||||||||||
Mouse: 1333 gcctccagtcggcggcggc 1351
```

Blast Score = 307 bits (155), Expect = 8e-82
Identities = 188/199 (94%)
Strand = Plus / Plus

Figure 2.7 Partial cDNA sequence of the cat BMP6 gene.

and mouse BMP6 cDNA's. This partial cat BMP 6 cDNA was generated by PCR using the primers GTACGGCTGTCGAGCAGGAAGAG and TAGAGGACGGAGATGGCATTGAG which are specific to the human BMP 7 gene. During southern blot analysis, the 50mer BMP 7 probe used to detect BMP 7 cDNA fragments detected this BMP 6 cDNA fragment. Interestingly, the BMP 6 fragment is of the same size as the expected BMP 7 fragment. This outcome is not surprising given the extensive homology between different members of the BMP family. Interestingly, the cat BMP 7 cDNA could not be cloned using this PCR strategy.

Figure 2.8 is a northern blot autoradiograph showing the mRNA expression levels of the BMP 6 gene in the postnatally developing cat visual cortex. The BMP 6 gene expresses two similar sized splice variants (more commonly known as doublets) in the visual cortex. The expression of the BMP 6 transcripts in the postnatal cat visual cortex appears to remain fairly constant throughout development.

2.3.7 BMP type II receptor

Figure 2.9 shows the partial cDNA sequence of the cat *BMP type II receptor* gene. This cat cDNA has 94% identity with the human BMP type II receptor cDNA. The BMP type II receptor was generated by PCR using the primers TTCCACCTCCTGACACAACACCA and CAGAATGAGCAAGACGGCAAGAG. These primers were designed based on sequence information available for the human *BMP type II receptor* gene.

Unfortunately, the BMP type II receptor mRNA could not be detected in the visual cortex by northern blot analysis. The failure to detect this mRNA by northern blot analysis suggests that the BMP type II receptor mRNA is expressed in very low levels in the developing visual cortex.

2.3.8 Wnt-1

Wnt-1 is a mammalian homologue of the drosophila *wingless* gene (Ramakrishna, et al. 1993). Figure 2.10 shows the partial cDNA sequence of the cat Wnt-1 gene. The cat

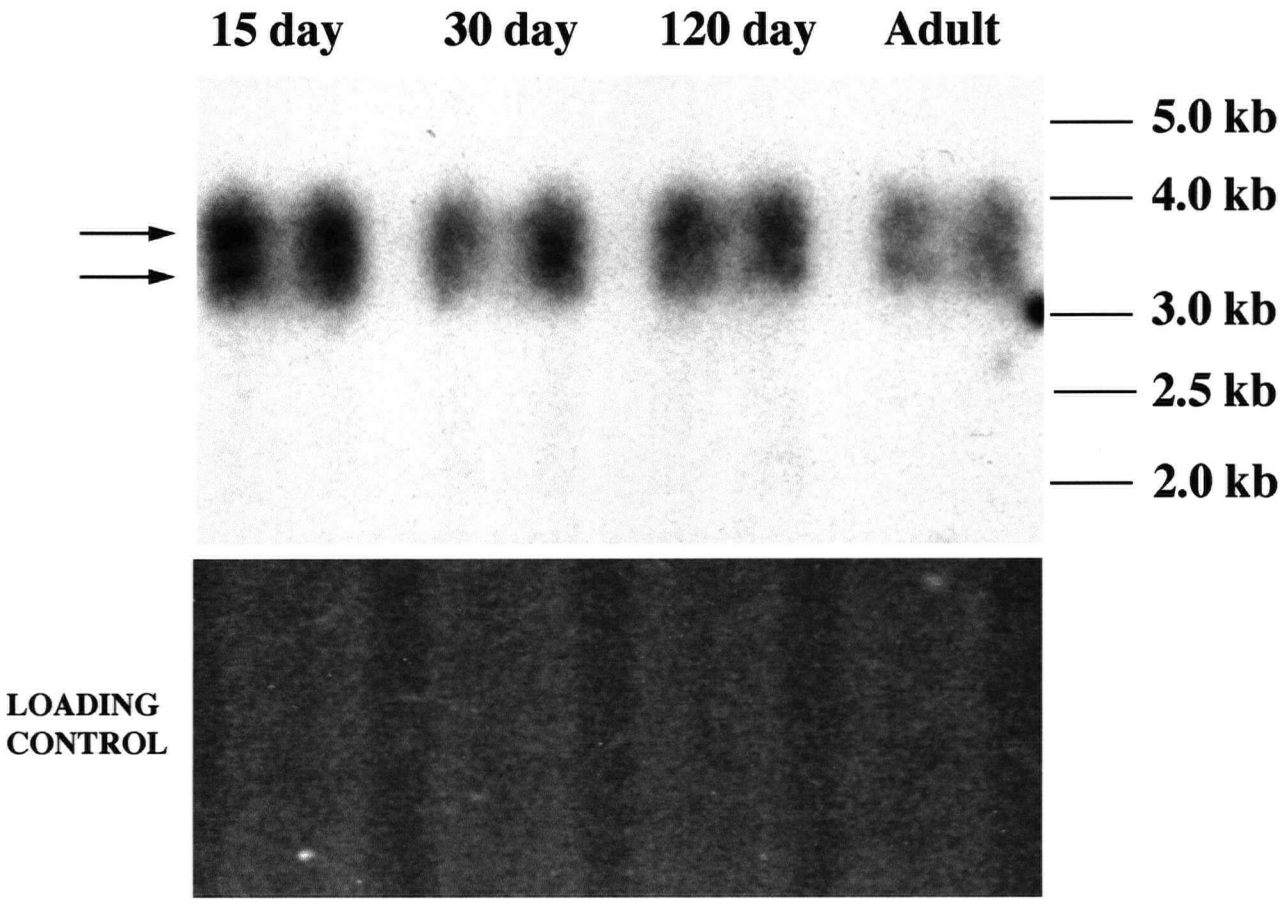

Figure 2.8 BMP6 Gene mRNA expression in the postnatally developing cat visual cortex. Two similar sized splice variants (or doublets) are expressed by the BMP6 gene (pointed out by the arrows) in the developing cat visual cortex. The BMP6 mRNA is expressed throughout the critical period and in the adult visual cortex. The bottom panel shows the mRNA gel preparation (stained with ethidium bromide) for this Northern blot autoradiograph.

```
Cat   :    1 atggaacatgacaacattgtccgctttatagttggagatgagagagttactgcagatgga   60
           ||||||||||||||||||| |||||||||||||||||||||||||| ||||||||||||
Human: 1162 atggaacatgacaacattgcccgctttatagttggagatgagagagtcactgcagatgga 1221

Cat   :   61 cgcatggaatatttgcttgtgatggagtattatcccaatggatctctctgcaagtatttg  120
           |||||||||||||||||||||||||||||| |||||||||||||| | ||||||||||||
Human: 1222 cgcatggaatatttgcttgtgatggagtactatcccaatggatctttatgcaagtattta 1281

Cat   :  121 agtctccatacaagtgattgggt  143
           |||||||| |||||||| |||||
Human: 1282 agtctccacacaagtgactgggt 1304
```

Figure 2.9 Partial cDNA sequence of the cat BMP type II receptor gene.

Blast Score = 220 bits (111), Expect = 1e-55
Identities = 135/143 (94%)
Strand = Plus / Plus

```
Cat   :    1 ggctgtcgggaaacggcgtttattttcgctatcacctccgccggggttacccattcggtg   60
           |||||||| |||||||||||||| |||||||||||||||||||||| ||||||||||||
Human: 2237 ggctgtcgagaaacggcgtttatcttcgctatcacctccgccggggtcacccattcggtg 2296

Cat   :   61 gcgcgctcctgctcagagggctccatcgagtcttgcacgtgcgactatcggcggcgcggc  120
           |||||||||||||||| || |||||||| || |||||||| ||||| ||||||||||||
Human: 2297 gcgcgctcctgctcagaaggttccatcgaatcctgcacgtgtgactaccggcggcgcggc 2356

Cat   :  121 cctgggggccccgattggcactnnnnnnnctgcagcgacaacatcgacttcggccgcctc  180
           || |||||||||| |||||||       |||||||||||||| ||||||||||||||
Human: 2357 cccgggggccccgactggcactgggggggctgcagcgacaacattgacttcggccgcctc 2416

Cat   :  181 ttcggcagggagtttgtggactccggggagaaggggcggg  220
           |||||| ||||||| |||||||||||||||||||||||||
Human: 2417 ttcggccgggagttcgtggactccggggagaaggggcggg 2456
```

Figure 2.10 Partial cDNA sequence of the cat WNT 1 gene.
Blast Score = 325 bits (164), Expect = 4e-87
Identities = 206/220 (93%)
Strand = Plus / Plus

Wnt-1 cDNA has 93% identity with the human Wnt-1 cDNA. The cat Wnt-1 cDNA was generated by PCR using the primers CCTCTTCGGCAAGATCGTCAACC and GGCCACTGTACGTGCAGAAGTTG. These primers were designed based on sequence information available for the human *Wnt-1* gene.

Figure 2.11 is a northern blot autoradiograph showing the mRNA expression levels of the *Wnt-1* gene during postnatal visual cortex development. Wnt-1 mRNA expression is low at 15 days of age, significantly increased at 30 days and 120 days of age and is reduced again in the adult visual cortex. The possibility that the 15 day mRNA is degraded on this blot was ruled out by reprobing the same blot with the PBX2 probe. As expected a strong signal was obtained with the PBX2 probe for the lane containing the15 day old visual cortex mRNA. This finding confirms that the observed autoradiographic pattern for the Wnt-1 northern blot analysis (which was repeated 3 times) is due to differential mRNA regulation and not to mRNA degredation. This mRNA expression pattern correlates with the critical period and suggests that the Wnt-1 protein is involved in the postnatal development of the visual cortex.

2.3.9 Frz-B

The Frz-B protein is a secreted form of the frizzled receptor (Wnt-1 binds and acts through the frizzled receptor)(Leyns et al. 1997). Similar to the truncated forms of the neurotrophin Trk receptors, the secreted form of the frizzled receptor has the ligand binding domain but lacks the intracellular protein domains. The Frz-B protein antagonizes Wnt signaling by binding to the secreted Wnt glycoproteins and preventing them from binding to the frizzled receptors. Figure 2.12 shows the partial cDNA sequence of the Frz-B gene in the cat. The cat Frz-B cDNA has 93% identity with the Bull Wnt-1 cDNA. The cat Frz-B cDNA was generated by PCR using the primers CTGCACCATTGACTTCCAGCACG and TCGAGTTCCTGCCAGACTTCTGA. Unfortunately, the expression of the Frz-B mRNA could not be detected using northern blot analysis.

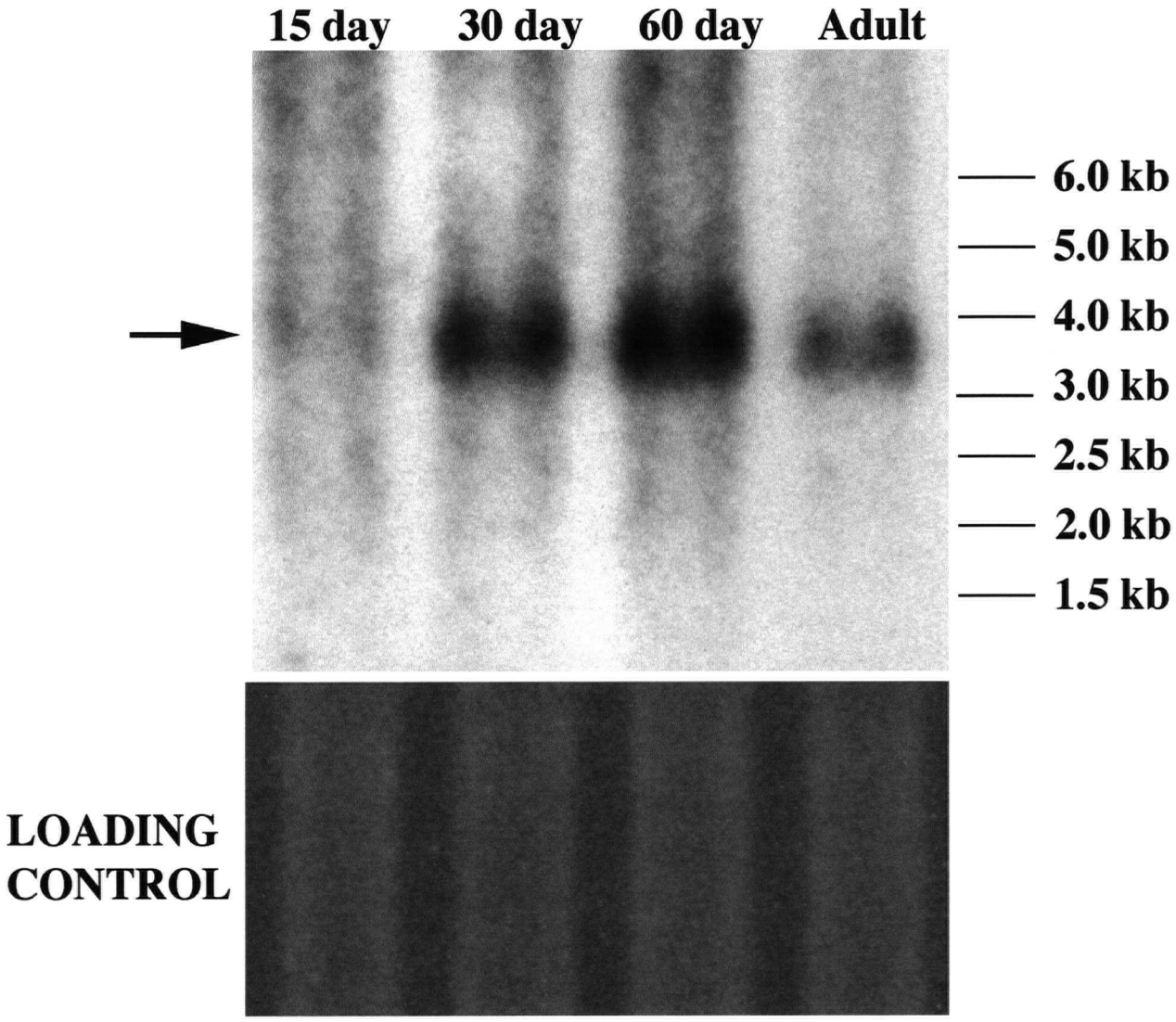

Figure 2.11 WNT-1 Gene mRNA expression in the postnatally developing cat visual cortex. At 15 days of age WNT-1 mRNA expression is not detected in the visual cortex. WNT-1 mRNA expression is upregulated in the 30 day and the 120 day old kitten visual cortex. In the adult visual cortex the expression of the WNT-1 mRNA is reduced. This developmental mRNA expression profile of the WNT-1 gene suggests a role for this molecule during the critical period. The bottom panel shows the mRNA gel preparation (stained with ethidium bromide) for this Northern blot analysis.

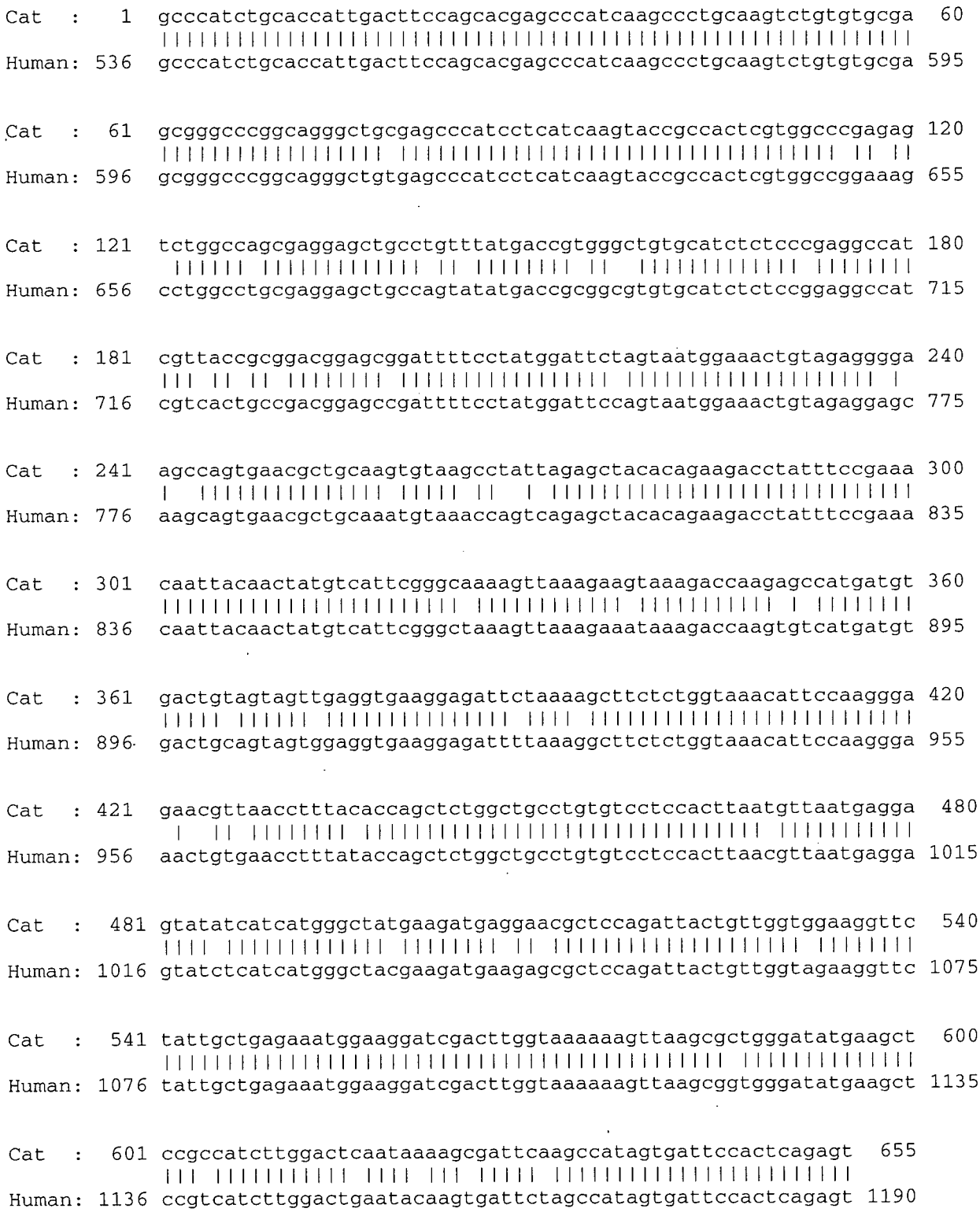

Figure 2.12 Partial cDNA sequence of the cat FrzB gene.
Blast Score = 902 bits (455), Expect = 0.0
Identities = 605/655 (92%)
Strand = Plus / Plus

2.3.10 Genes which could not be cloned using the RT-PCR approach

Unsuccessful efforts were made at cloning *engrailed-1, engrailed-2, sonic hedgehog, desert hedgehog, Indian hedgehog, chordin* and *noggin* genes. The *engrailed* and *hedgehog* genes are extensively characterized segment polarity genes that are known to be involved in neural development (Ericson et al. 1995, Zhu et al. 1999, Friedman et al. 1996). Chordin and noggin are secreted proteins (similar to Frz-B) that inhibit BMP activity (Bachiller et al. 2000). As mentioned before, we were also unable to clone the BMP 7 (a protein involved in synaptogenesis) cDNA in the cat.

The failure to partially clone these genes does not imply that they are not expressed in the cat visual cortex during the critical period. Technical problems associated with RT-PCR may have prevented us from cloning these transcripts. The presence of only a single nucleotide mismatch between the PCR primer and the cDNA template can significantly hinder the ability of the PCR primer to prime DNA elongation. Unfortunately, the PCR primers used in these experiments had to be designed based on sequence information available from other species and hence, nucleotide mismatches between the targeted cat cDNA templates and the PCR primers may have existed. In addition, numerous factors such as annealing temperature, the length of the elongation, the number of cycles, the concentration of the primers, nucleotides, template, or magnesium can effect the efficiency of a PCR reaction. Although the annealing temperature, the magnesium concentration and the number of PCR cycles were varied while attempting to clone the above mentioned cDNA's, it is possible that the PCR conditions used for the amplification of these transcripts may not have been optimal.

2.4 DISCUSSION

To briefly summarize, the mRNA's of the *PBX1, PBX2, BMP4*, and *Wnt-1* genes are expressed in the visual cortex during the critical period in an age dependent manner. The mRNA expression of *BMP type II receptor* and the *Frz-B* genes are also detected in the developing visual cortex by RT-PCR. The developmentally regulated expression of these genes in the visual cortex gives merit to our original hypothesis that the homologues of the drosophila developmental genes are involved in the postnatal development of the cat visual system. Considering the number and the diversity of developmental events homeotic and segment polarity genes are associated with, the presence of these genes in the postnatal development of the visual cortex is not surprising. Furthermore, since the function of the homeotic and segment polarity genes are extensively characterized in other developmental models, the possible role of these genes in visual development can be speculated.

2.4.1 The cloning strategy

Since many of the genes postulated to be present in the visual cortex were successfully cloned, the cloning strategy described in this thesis appears to be an efficient way of identifying homologues of the drosophila developmental genes expressed in the cat. Gene specific PCR primers were used to partially clone these developmental genes in the cat instead of degenerate PCR primers because degenerate PCR primers have a tendency to be biased for and/or against certain sequences. Hence, it is naive to assume that all members of a particular gene family can be detected by using degenerate primers. More importantly, degenerate primers need to be designed to highly conserved regions of gene families so that common sequences amongst different members of the gene family are detected and amplified. Since only short, highly conserved cDNA sequences common to various members of the same gene family can be amplified using this approach, the sequences generated by degenerate primer PCR can not be used as gene specific probes for determining the expression pattern of an individual gene (i.e. they will cross react with

several members of the same gene family). Eventually, gene specific PCR primers need to be designed in order to get sequence information which is unique to the gene of interest. As a side note, due to the extensive homology present amongst the homologues of different drosophila developmental genes, low stringency PCR utilizing primers designed to a particular gene resulted in the amplification of other genes in the same family (i.e. PBX1 cDNA was obtained using PCR primers for PBX2). In other words, like degenerate primers, low stringency PCR can facilitate the cloning of multiple members of the same gene family. Furthermore, the aim of this chapter is not to characterize a single gene family but rather, to establish the expression of various different types of drosophila developmental gene homologues during the critical period. Clearly, the use of specific primers in combination with low stringency PCR is more suited for this purpose.

2.4.2 The expression of PBX Homeotic genes in the developing visual cortex

The expression of the *PBX* homeotic genes in the developing visual cortex strongly suggests the presence of other homeobox genes in this structure because the *PBX* homeotic genes act solely as cofactors and facilitate the activity of other homeobox genes (Rauskolb et al. 1993). Transcription factors containing particular amino acid motifs form complexes with the *PBX* genes (Neuteboom et al. 1995, Chang et al. 1995). Homeobox genes need to form these complexes in order to efficiently bind certain enhancer sequences and regulate transcription. Since the *PBX* genes do not induce gene expression by themselves, other homeobox genes which require the *PBX* genes as cofactors are likely to be expressed in the developing visual cortex.

The higher level of PBX1 and PBX2 mRNA expression in the visual cortex of young cats suggests the involvement of these genes in visual development. Two splice variants of PBX1 are found in the cat visual cortex. This finding is identical to the reported expression of the PBX1 transcript in humans (Kamps et al. 1990) but it is different from

the reported expression of a single PBX1 mRNA species in the developing olfactory bulb of the rat (Redmond et al. 1996). Species differences between rats and cats may be responsible for the observed splicing pattern difference. One purpose for having splice variants is to transport and localize transcripts to different cellular compartments. Since PBX1 strictly acts as a transcription factor, the splice variants are unlikely to be transported to any cellular compartment other than the nucleus. A more likely possibility is that the PBX1 splice variants encode proteins which have different affinities for binding different transcription factors (Monica et al. 1991). For example, a particular homeobox gene may only be able to form a heterodimer complex with the PBX1 protein encoded by the smaller splice variant. It is feasible that the expression pattern of the PBX1 mRNA splice variants in the visual cortex reflects the expression profile of the homeobox genes they interact with during development. Homeobox genes that form transcriptional complexes with PBX1 maybe highly expressed in the young visual cortex and the purpose for having high PBX1 expression levels during this period maybe to provide these homeobox genes with the necessary cofactor. Accordingly, the developmental regulation of PBX1 and its splice variants suggests that the homeobox genes which interact with PBX1 are likely to be developmentally regulated in the visual cortex during the critical period. Finally, it is also possible that each splice variant is expressed by a particular cell type.

PBX2 mRNA expression pattern in the developing visual cortex is almost identical to that of PBX1. Since *PBX1* and *PBX2* have an extensive amount of homology, the function of *PBX2* is speculated to be very similar to that of *PBX1*. The difference between the *PBX1* and *PBX2* genes may be limited to their ability to form complexes with different transcription factors. The possibility that the two *PBX* genes are performing the same function during the critical period also exists. In mammalian development redundancy of gene function is common amongst different members of a gene family. For example, knockout mice lacking a single member of the *Dlx* homeobox gene family (*Dlx* homeobox genes are expressed in the brain and contribute to forebrain development) do not appear to

be phenotypically different than normal mice (Depew et al. 1999). Mice begin to show noticeable phenotypic abnormalities following the knockout of at least two members of the *Dlx* gene family. These findings suggest that different members of the *Dlx* homeobox gene family are performing the same function during brain development. Given the extensive homology and similar mRNA expression profiles of the *PBX1* and *PBX2* genes, this functional redundancy may have extended into the *PBX* homeobox gene family.

The role of PBX as a cofactor for homeobox genes containing particular amino acid motifs is well established (Neuteboom et al. 1995, Chang et al. 1995). As mentioned, the presence of the *PBX1* and *PBX2* genes entails the expression of other homeobox genes in the developing visual cortex. The obvious question is how are the homeobox genes contributing to the development of the postnatal visual cortex? The developmental processes requiring segmentation have been completed well before birth, neurons have already attained their cell fates and cell division does not occur in the postnatal brain. Clearly, these well characterized functions of homeobox genes are unlikely to be taking place during the critical period. However, compartmentalization is an inherent characteristic of the visual cortex. Columnar organization of eye specific inputs, orientation, neurotransmitter receptors and blob, interblob arrangement of cytochrome oxidase in the visual cortex is well documented (Hubel et al. 1963, Shatz et al. 1977, Dyck et al. 1993c, Murphy et al. 1995). Given that the homeobox genes are involved in the development of various different compartmental structures (i.e. rhombomeres, drosophila body segments, spinal cord), the activity of these genes may contribute to the columnar organization of the cat visual cortex. Another function of the homeobox genes in the central nervous system is to regulate the expression of cell adhesion molecules (Cillo et al. 1996, Wang et al. 1996, Edelman et al. 1995, Valarche et al. 1993, Lincecum et al. 1998, Packer et al. 1997, Tissier-Seta et al. 1993). By differentially regulating the activity of cell adhesion molecules, homeobox genes are able to control the formation of synaptic connections in the spinal cord (Lumsden et al. 1995, Tosney et al. 1995). It is possible

that the homeobox genes are performing a similar function in the postnatal visual cortex. Support for this hypothesis is provided by the correlation that exists between the expression of cell adhesion molecules and the critical period which will be discussed later on in this thesis (Schoop et al. 1997, Corriveau et al. 1998)

2.4.3 Segment polarity gene expression in the developing visual cortex

Several members of the *BMP* gene family are also expressed and developmentally regulated in the visual cortex during the critical period. Splice variants which are detected for *BMP6* in the visual cortex have not been observed in previous studies examining brains from other species (Tomizawa et al. 1995). Hence, these splice variants may be novel to the cat or alternatively, the splice variants may be novel members of the *BMP* family which have not been characterized. The possible reasons for having *BMP* splice variants is identical to those discussed for the *PBX1* gene (i.e. produced by different cell types, transported to different cellular compartments or functionally different - bind different receptors). As mentioned, the BMP's are secreted proteins and the detection of the BMP type II receptor confirms the presence of the necessary machinery for BMP signaling in the visual cortex. The best characterized function of the BMP's is as signaling molecules involved in cell to cell communication (Nakayama et al. 2000). Signaling molecules are believed to contribute extensively to activity dependent synaptic rearrangements occurring in the developing nervous system (Goda 1994, Fitzsimonds et al. 1998, Haydon et al. 1994, Davis et al. 1998). However, this conjecture is largely hypothetical with regards to the critical period because the identity of these signaling molecules in the developing cat visual system (with the exception of the neurotrophins) is unknown. The developmentally regulated mRNA expression of the *BMP4* signaling molecules during the critical period suggests a role for this molecules in visual development.

Out of all the transcripts examined in this chapter, Wnt-1 mRNA expression parallels the critical period most accurately. Unlike the other transcripts examined where mRNA expression decreases with age, Wnt-1 expression is low in the visual cortex of

kittens which are 2 weeks of age. As discussed in the introduction, during the first 2 to 3 weeks after birth, neurons from the ventricular zone migrate into the cortical plate in order to complete the formation of the upper layers (Shatz et al. 1986). Following the completion of layer formation the thalamic connections in the subplate grow into layer IV of the cortex. Since segregation of thalamic inputs or synaptic modification does not occur before the LGN innervates the cortex, the critical period in the cat begins two to three weeks after birth. Hence, the absence of Wnt-1 expression in the visual cortex of 15 day old animals is consistent with the idea that Wnt-1 is involved in visual plasticity. The decrease in the expression of Wnt-1 mRNA in the adult visual cortex also correlates with the critical period. In addition to this correlation, the fact that Wnt-1 is a secreted signaling molecule capable of inhibiting the expression of the BMP4 gene (Baker et al. 1999) makes Wnt-1 an intriguing candidate molecule in visual plasticity research.

The interactions between the BMP4 and Wnt-1 signaling pathways have the potential to account for the formation of ocular dominance columns in the developing visual cortex. As mentioned, *BMP4* is the homologue of the drosophila *decapenteplegic* gene (Padgett et al. 1993) whereas *Wnt-1* is the homologue of the drosophila *wingless* gene (Ramakrishna, et al. 1993). The *DPP* and the *wingless* genes and their homologues (i.e. *BMP's* and *Wnt's*) are capable of forming adjacent non-overlapping domains by inhibiting each others expression (Jiang et al. 1995, Baker et al. 1999, Hirsinger et al. 1994). Often, these expression domains are in the form of interdigitated columns. If the expression domain of either one of the *Wnt-1* or *BMP4* genes is downregulated via experimental manipulation, its adjacent neighbour invades the expression domain of the downregulated gene (Penton et al. 1996, Jiang et al. 1995, Jiang et al. 1996). These characteristics of the *BMP4* and *Wnt-1* genes are somewhat reminiscent of the behaviour of ocular dominance columns following monocular deprivation. Furthermore members of the *Wnt* and *BMP* families are implicated to be associated with synaptic modifications and neural communication (Withers et al. 2000, Hall et al. 2000). Moreover, both of these molecules

are developmentally regulated in the cat visual cortex during the critical period. If the release of these secreted cell signaling molecules in the developing visual cortex is regulated by neural activity, the antagonistic nature of *Wnt-1* and *BMP4* can theoretically give rise to ocular dominance columns and account for the anatomical and physiological changes associated with monocular deprivation. Additionally, the expression of the *Frz-B* gene in the visual cortex, which is an inhibitor of *Wnt-1* activity, provides an alternative system by which ocular dominance columns can form during visual development. The expression of *Wnt-5a* type signaling genes, which antagonize *Wnt-1* function (Torres et al. 1996), was not examined in this thesis but these molecules may also contribute to the formation of ocular dominance columns.

2.4.4 Selection of the Beta-Catenin Protein For Further Characterization In The Postnatal Development Of The Cat Visual System.

Homologues of several homeotic and segment polarity genes were identified and shown to be developmentally regulated in the visual cortex during the critical period. Although the large body of information available about the function of these genes from other systems allows speculations to be made, the role of these genes during visual development can not be determined based solely on northern blot analysis. The young postnatal brain undergoes a plethora of processes which are associated with differentiation and maturation. Since these developmental processes occur at the same time as the critical period, it is difficult to assess if the homeotic and the segment polarity genes are involved in general brain development or if they contribute to visual plasticity. Further characterization is required in order to establish a role for these genes in postnatal visual development.

In order to establish the involvement of these genes in the development of the cat visual system more concretely, we decided to focus on and extensively characterize the behaviour of one of these genes in the postnatally developing cat visual cortex and lateral geniculate nucleus. The initial strategy for this thesis was to clone a group of

developmental genes, determine their expression profiles and then examine genes that have expression patterns paralleling the critical period. The northern blot analysis results reported in this chapter provide several lines of promising data and have led to some transcripts in the visual cortex (large amounts of mRNA is needed to detected these genes via northern blots) is the likely cause of this outcome. In addition, appropriate antibodies for the BMP4 and Wnt1 proteins are not available (especially antibodies against cat proteins). Hence, these genes could not be further characterized in the postnatally developing visual cortex. Accordingly, the role of these genes in visual development remains unverified.

Since the expression of the Wnt-1 mRNA correlates well with the developmental events occurring in the critical period, a reasonable approach seemed to be the investigation of downstream genes in the Wnt-1 signaling pathway. The best characterized downstream effector of Wnt-1 signaling is the *beta-catenin* gene, the homologue of the drosophila *armadillo* segment polarity gene (Moon et al. 1997). Like many other developmental genes identified in the drosophila, *beta-catenin* is reported to take part in a wide range of developmental events and has been intensively studied in mammals. Because of the attention this gene has received, molecular tools such as specific antibodies are available for the beta-catenin protein. As reported in the next chapter, the information gathered from the examination of this protein provides further support to the hypothesis that the homologues of the drosophila developmental genes are involved in the postnatal development of the cat visual system.

Chapter 3

BETA-CATENIN PROTEIN EXPRESSION IN THE POSTNATALLY DEVELOPING CAT VISUAL SYSTEM

3.0 SUMMARY

To further verify our hypothesis that the homologues of the drosophila developmental genes are involved in the postnatal development of the cat visual system, the expression pattern of the beta-catenin protein, which is the homologue of the drosophila *armadillo* gene, was examined in the cortex and LGN of kittens during the critical period. Beta-catenin protein appears to be expressed at fairly constant levels in the postnatally developing cat LGN and the visual cortex as determined by western blot analysis. Immunohistochemical analysis reveals that the beta-catenin protein is expressed in the neuropil of the visual cortex and the geniculate in young animals. Although this staining pattern is retained in the cortex throughout life, the beta-catenin protein becomes nuclearized in LGN cells between 50-60 days of age. Based on this finding nuclear beta-catenin protein is proposed to end the critical period for thalamocortical plasticity by altering gene expression in LGN neurons.

3.1 INTRODUCTION

If the homologues of the drosophila developmental genes are involved in the postnatal development of the cat visual system then their tissue distribution and temporal expression patterns should correlate with the critical period. Hence, we decided to extensively characterize the protein expression of one of the homologues of the drosophila

developmental genes in the visual system of normally developing and deprived kittens. In the last chapter we reported that the temporal expression of the Wnt-*1* mRNA in the visual cortex closely correlates with the critical period. Since the tissue distribution of *Wnt-1* could not be determined in the cat visual cortex by standard histological techniques, the temporal expression and the tissue distribution of the *beta-catenin* gene was examined in the postnatally developing cat visual system. *Beta-catenin* is a homologue of the drosophila *armadillo* segment polarity gene and a downstream component of the Wnt-1 signaling pathway (Peifer et al. 1992, Brown et al. 1998).

Unlike the *wingless* and *decapentapelgic* segment polarity genes, *armadillo* is not a secreted protein (Riggleman et al. 1989). It is a protein which can act both as a transcription factor and as a cell adhesion molecule (Peifer et al. 1992, van de Wetering et al. 1997). These functions of *armadillo* are conserved in the mammalian *beta-catenin* gene. The role of beta-catenin as a cell adhesion molecule is well established (McCrea et al. 1991, Bullions et al. 1998). Beta-catenin is found intracellularly and acts to anchor the calcium dependent cell adhesion molecules known as the cadherins to the actin cytoskeleton. Beta-catenin mediated attachment of cadherins to the actin cytoskeleton is required for the formation of stable adhesive links (Kitner et al. 1992, Nagafuchi et al. 1988, McCrea et al. 1991). Interestingly, electron microscopy performed on rat brain tissue sections has localized the beta-catenin and cadherin complexes to the synapse where they border the active zone (Uchida et al. 1996).

The regulation of the *beta-catenin* gene is mainly at the post-translational level (See figure 3.1). Cells have one pool of beta-catenin that is attached to the cytoskeleton via the cadherins and a free pool of beta-catenin (Papkoff 1997). Normally, cells produce a continuous amount of beta-catenin protein which is rapidly degraded (Pai et al. 1997, Aberle et al. 1997). The main protein responsible for beta-catenin degradation is the serine threonine kinase activity of glycogen synthase kinase 3 (GSK3) which is the mammalian homologue of the drosophila *shaggy/zest white 3* gene (Aberle et al. 1997). GSK-3 marks

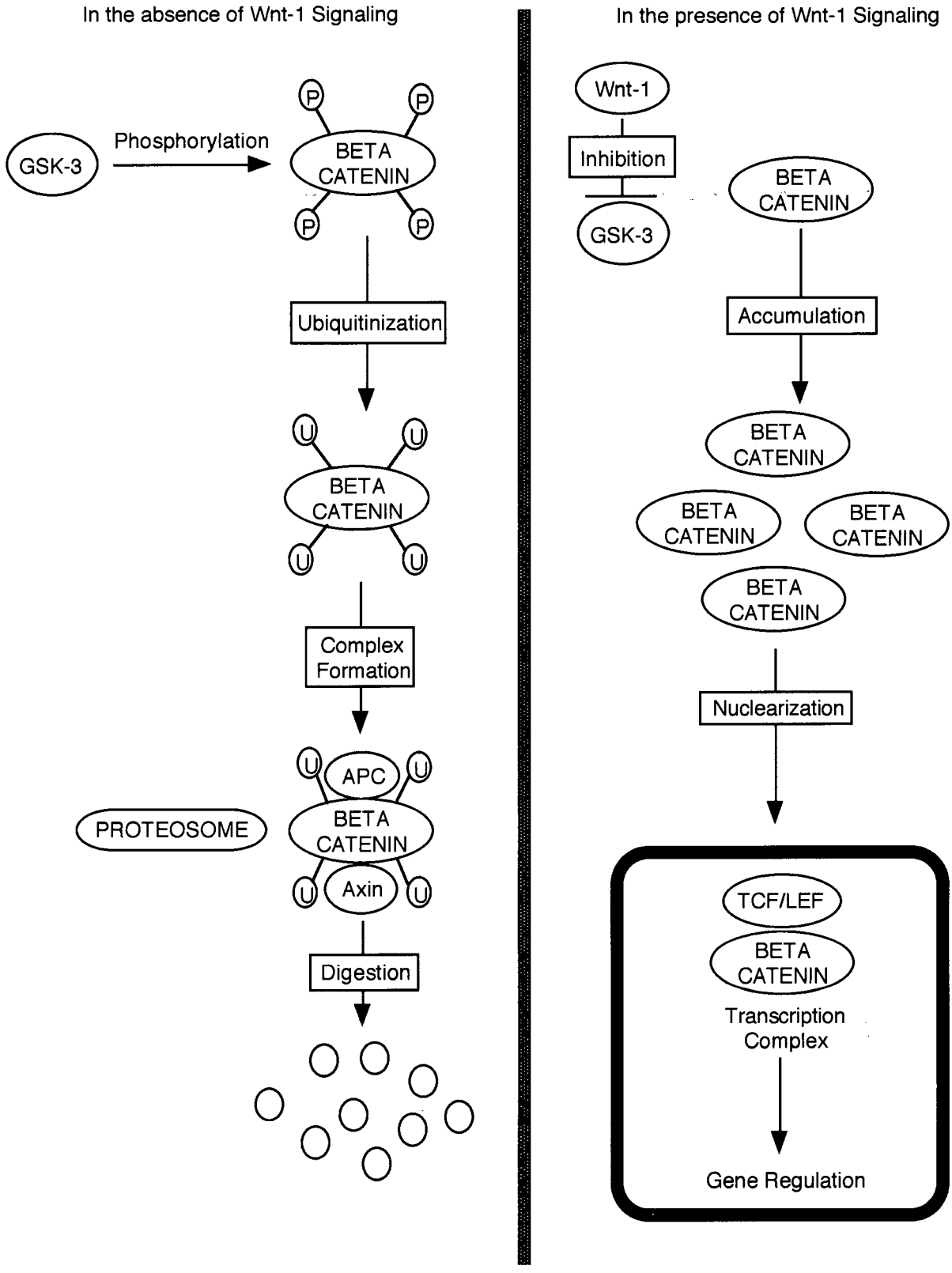

Figure 3.1 Post-translational Regulation of Beta-Catenin Protein Expression.

beta-catenin for ubiqutinization and eventual degradation by phosophorylating it (Aberle et al. 1997). Adenomatous polyposis coli (APC) and axin also play a role in beta-catenin degradation (Rubenfield et al. 1996, Hart et al. 1998). Factors such as Wnt-1 signaling, ILK (integrin linked kinase) and the activity of the *dishevelled* gene, which is regulated by the *Notch* signaling pathway, inhibit GSK-3 activity and prevent beta-catenin degradation (Cook et al. 1996, Yanagawa et al. 1995, Novak et al. 1998, Axelrod et al. 1996). Inhibition of GSK-3 leads to the increase in the free pool of beta-catenin in the cytoplasm which is then transported into the nucleus where it acts as a transcription factor (Cook et al. 1996, Novak et al. 1998).

In the nucleus, beta-catenin is unable to regulate transcription by itself and instead, it functions as a cofactor for the TCF (T-cell factor)/LEF transcription factors (Clevers et al. 1997, Huber et al. 1996, Molenaar et al. 1996, Behrens et al. 1996). TCF is an architectural protein which facilitates the formation of multi-protein transcriptional complexes (Oosterwegel et al. 1991). The activity of these complexes regulate the expression of various transcripts (Brannon et al. 1997, Riese et al. 1997) and cause cellular changes associated with beta-catenin nuclearization (Molenaar et al. 1996). Consistent with the behaviour of numerous other developmental genes, the inappropriate transcription factor activity of *beta-catenin* in adult tissue is involved in cancer pathogenesis (Bullions et al. 1998, Behrens 2000).

HYPOTHESIS: The tissue distribution and the expression profile of the beta-catenin protein in the postnatally developing cat visual system correlates with the critical period.

OBJECTIVES: 1.) To determine the temporal expression and the tissue distribution of the beta-catenin protein in the postnatally developing cat visual cortex and the LGN.

2.) To determine if the beta-catenin protein is present in the nuclei of either LGN or visual cortex cells in the postnatally developing cat.

3.2 METHODS

Partial cloning of beta-catenin cDNA:

See methods section in chapter 2.

Preparation of Animals:

For western blot analysis a total of 27 cats were sacrificed. Four animals were used for each age group with the exception of prenatal animals where N=2 and N=5 for 60 day old postnatal animals. 24 additional animals were used for immunohistochemical analysis N=4 for P(postnatal day)0, P20, P60 and adults and N=2 for P30, P45, P55, P120. Animals were euthanized with an overdose of euthanyl and then perfused briefly with cold PBS buffer (pH 7.4). The brains were then removed and frozen in isoamyl alcohol (LGN and visual cortex were dissected and pooled prior to freezing if the tissue was intended for western blot analysis). The frozen brains were stored at -80°C until use.

Antibody:

A monoclonal antibody specific against the mouse, rat and human beta-catenin proteins was purchased from Transduction Laboratories. This antibody was used in both immunohistochemistry and western blot analysis procedures.

Preparation of Protein Samples:

Boiling Method:

A piece of frozen brain tissue was put into suspension buffer containing 0.1M NaCl, 0.01M Tris-Cl (pH 7.6), 0.001M EDTA (pH 8.0), 1μg/ml aprotinin, 100μg/ml phenylmethylsulfonyl fluoride (PMSF). The suspension buffer was cooled on ice before use and the protease inhibitors aprotinin and PMSF were added immediately before use.

(Pipette tips and tubes which came into contact with PMSF were left in a NaOH solution overnight in the fume hood.) The brain tissue was teased apart using a pair of forceps. An equal volume of 2X SDS gel loading buffer containing 100mM Tris-Cl (pH 6.8), 200mM dithitothreitol, 4% SDS, 0.2% bromophenol blue was added to the sample and the solution was immediately boiled for 10 minutes. After boiling, the sample was briefly spun down and the high molecular weight chromosomal DNA was sheared by sonication for 1 minute at maximum speed. The purpose of this step is to decrease the viscosity of the protein sample. The sample was then centrifuged for 10 minutes at room temperature at 15000g and the resultant supernatant was transferred to a new tube. This sample was used for western blot analysis. It was usually aliquoted in order to avoid repeated freeze thaw cycles and placed into a -20°C freezer for short term storage or -80°C for long term storage.

Triple detergent Method:

Ten volumes of ice cold triple detergent containing 50mM Tris-Cl (pH 8.0), 150mM NaCl, 0.02% sodium azide, 0.1% SDS, 1%Nonidet P-40, 0.5% sodium deoxycholate, 100µg/ml PMSF, 1µg/ml aprotinin was added to one unit per weight of frozen brain tissue. As in the last method triple detergent was cooled on ice and the protease inhibitors aprotinin and PMSF were added immediately before use. The brain tissue was teased apart using a pair of forceps and the solution was incubated on ice for 30 minutes. Every ten minutes the solution was pipetted several times to improve solublization of the tissue. High molecular weight chromosomal DNA was sheared by sonication for 1 minute at maximum speed. Sonication was performed on ice. The solution was then centrifuged at 15000 x g for 5 minutes and the supernatant was aliquoted in order to avoid repeated freeze thaw cycles and placed into a -20°C freezer for short term storage or -80°C for long term storage.

Preparation of Nuclear Fraction:

LGN tissue was homogenized in PBS containing 2μg/ml each of Aprotinin and Leupeptin and 0.75mM PMSF until a homogeneous solution was obtained. All centrifugations were done on ice and the samples were kept on ice at all times. The homogenate was centrifuged and the supernatant removed. 200μl of CERI (PIERCE) solution was then added to the pellet containing the above listed protease inhibitors. The solution was vigorously vortexed to resuspend the cell pellet. The mixture was then incubated on ice for 10 minutes. 11μl of CERII solution (PIERCE) was added to the tube. The tube was vortexed at maximum setting for 5 seconds, incubated on ice for 1 minute and then vortexed again for another 5 seconds. The tubes were centrifuged at (16000 x g) for 5 minutes. The supernatant was removed and 100μl of NER buffer (PIERCE) was added to the insoluble pellet containing nuclei. The above listed protease inhibitors were added to the NER buffer before use. The solution was vortexed at maximum speed for 15 seconds and incubated on ice for 40 minutes. This sample was vortexed for 15 seconds at maximum speed every 10 minutes. After the 40 minute incubation, the tubes were centrifuged at (16000 x g) for 10 minutes. The supernatant containing the nuclear fraction was then transferred to a pre-chilled tube and stored at

-80°C.

Western Blot Analysis:

The dry Hybond-ECL membrane was soaked in 100% methanol for 5 seconds and then washed in distilled water for 5 minutes. The membrane was then placed into a heat sealable plastic bag containing 5% bovine serum albumin in PBS pH 7.5 (80mM disodium hydrogen orthophosphate anhydrous, 20mM sodium dihydrogen orthophosphate, 100mM sodium chloride) containing 0.1% Tween detergent (referred to as PBS-T) and incubated at 4°C overnight on a shaker table. All the washes and the rinses in this procedure were done

with PBS-T. The next day, the membrane was quickly rinsed twice, washed once at room temperature with shaking for 15 minutes and twice more for 5 minutes each at room temperature with shaking. After the washes, the membrane was incubated in a heat sealed bag with the diluted primary antibody (1:1000 dilution for cadherin, 1:2000 dilution for beta-catenin) in PBS-T for one hour at room temperature with shaking. Unbound primary antibody was washed off the membrane by two quick rinses, followed by a 15 minute wash at room temperature with shaking and then two more washes for 5 minutes each at room temperature with shaking. HRP conjugated secondary antibody diluted 1:1000 in PBS-T was then added to the membrane and the membrane was incubated in a heat sealed bag for 1 hour at room temperature with shaking. Unbound secondary antibody was washed off the membrane by two quick rinses, followed by a 15 minute wash once at room temperature with shaking and then four more washes for 5 minutes each at room temperature with shaking. During these washes, the detection solution (consisting of equal volumes of solution 1 and solution 2 from Amersham ECL western blotting analysis system) was prepared. The excess liquid from the washed membrane was drained onto a paper towel and the membrane was placed protein side up on a piece of Saran wrap. The remainder of the procedures described in this section were performed in the dark room. The membrane was then covered with the detection solution and incubated for exactly 1 minute. The membrane was removed from the detection solution and wrapped in a fresh piece of saran wrap. The membrane was put in a cassette with the protein side up and an autoradiography film (Kodak XAR) was placed on the membrane. The exposure times for the film varied from 30 seconds to 1 minute. The ECL signal was detected by developing the film.

Immunohistochemistry:

Glass slides containing 12 micron tissue sections were removed from the freezer and allowed to thaw at room temperature for at least 30 minutes. The slides were then fixed in an ice cold 50%methanol, 50%acetone solution for ten minutes at -20°C. The

fixation solution was made fresh each time and was cooled at -20°C for at least one hour. After fixation, the slides were dried for an additional 30 minutes. Wells were created by drawing circles around each tissue section on the slide with a PAP pen. The sections were not allowed to dry for the remainder of the immunohistochemistry procedure. The sections were then washed three times for five minutes each with PBS buffer at room temperature while shaking. Since fresh frozen sections contain a substantial amount of endogenous biotin/streptavidin sites, these sites were blocked using the ZYMED biotin/streptavidin blocking kit. Blocking of these sites was found to significantly decrease non-specific background staining with the vector ABC kit which utilizes a biotin/streptavidin based amplification system. Three drops of Reagent A, which contains streptavidin, from the biotin/streptavidin blocking kit was put onto each individual cat brain section (per section not per slide) and the slides were incubated in a humidified chamber for 10 minutes at room temperature with shaking. Tissue sections from one day old kittens only required one drop of this solution. After the ten minute incubation Reagent A solution was removed with a piptteman and the slides were quickly placed into a glass container containing fresh PBS. Once all the slides had Reagent A removed, they were washed three times with shaking for five minutes each in PBS buffer. After the final wash, excess fluid from each slide was removed with the aid of a pipetteman and three drops of reagent B, which contains biotin-m, from the biotin/streptavidin blocking kit was applied to each section and the slides were again incubated in a humidified chamber for ten minutes at room temperature with shaking. Following the incubation reagent B was removed and the slides were washed three times with shaking for five minutes each in PBS buffer. Excess solution from the slides was removed and 500 microliters of a 5% bovine serum albumin solution made in PBS was added to each slide and the slides were then incubated in a humidified chamber for one hour at room temperature with shaking. The purpose of this step was to block any non-specific sites. After the blocking step, the 5% BSA solution was removed and a 1%BSA solution made in PBS containing the primary antibody at a 1:100 dilution was added to the slides.

(The beta-catenin antibody was purchased from Transduction Laboratories). The slides were incubated with the primary antibody in a humidified chamber overnight at 4°C with shaking. Incubating for longer periods of time with the primary antibody solution (i.e. 48 or 72 hours) did not improve the final staining of the sections. The following day, the slides were washed three times with shaking for five minutes each in PBS buffer. A 1%BSA solution made in PBS containing the secondary antibody at a 1:1000 dilution was added to the slides and the slides were incubated in a humidified chamber for one hour at room temperature with shaking. While the slides were incubated with the secondary antibody, 20 micro liters of solution B and 20 micro liters of solution C from the ABC kit were added to 10mls of a 1%BSA solution made in PBS. The solution was mixed by inversion and was kept undisturbed at room temperature for at least 30 minutes. Once the secondary antibody incubation was completed, the secondary antibody solution was removed and the slides were washed three times with shaking for five minutes each in PBS buffer. The BC solution that was prepared earlier was then put onto each slide and the slides were incubated with this solution in a humidified chamber for one hour at room temperature with shaking. The slides were washed three times with shaking for five minutes each in PBS buffer after the incubation. The slides were then placed in a 500ml nickel DAB solution consisting of 5g of nickel ammonium sulphate, 1.7g of immidazole, 50mg of DAB and 5µl of 30% hydrogen peroxide in Tris buffer at pH7.4. The Nickel enhanced DAB reaction stains the slides a dark greyish blue color. Following the staining, the slides were washed three times for 5 minutes each in PBS and coversliped.

Double Labeling:

The slides were fixed and processed (i.e., had their biotin sites blocked) as described in the immunohistochemistry protocol. After the sections were incubated with the 5% BSA solution for 1 hour, one of the antibodies to be used in the double labeling procedure was added to the sections. The immunohistochemistry procedure was carried

out as described. Instead of coverslipping the sections at the end of the procedure, the sections were incubated with the ZYMED peroxidase blocking solution for ten minutes. After the completion of the immunohistochemistry procedure, the sections were not allowed to dry. The purpose of the peroxidase blocking solution is to deactivate the activity of horseradish peroxidase enzyme remaining from the initial immunohistochemistry step. The slides were washed three times for 5 minutes each in PBS with shaking. The slides were then blocked for 1 hour with 5% BSA in PBS and then the second antibody to be used in the double labeling procedure was added. The immunohistochemistry procedure was carried out as before except for the staining step. Instead of staining the slides in a Nickel DAB solution, which results in a greyish-blue color, the slides were incubated with DAB alone which results in a brownish red color. The 500 ml DAB solution contained 50mg of DAB and 1µl of 30% hydrogen peroxide. After the DAB reaction, the slides were washed three times for 5 minutes each in PBS and coversliped.

Coversliping:

The slides were put in distilled water for 1 minute for rehydration. The slides were then dehydrated by incubation for 1 minute in 70%, 5 minutes in 95%, and 10 minutes in 99% ethanol. The slides were then put in xylene for 20 minutes after which they were coverslipped with permount.

Nissel Staining:

Buffered Neutral Red Stain was made by adding 32ml's of acetate buffer, pH4.8, (stock solution made by adding 100ml of 0.1N acetic acid to 150ml of 0.1N sodium acetate) to 800ml's of 2% aqueous neutral red solution (16g Neutral Red, 185ml 1M acetic acid, 2.04g sodium acetate or 15ml of 1M sodium acetate solution, 200ml of distilled water, 400ml of 50% ethanol). This solution was stored at room temperature and filtered before each use.

3.3 RESULTS

3.3.1 Partial Cloning of the Cat Beta-Catenin cDNA

In order to confirm the expression of the *beta-catenin* gene in the visual cortex during the critical period, PCR primers were designed based on the nucleotide sequence of the human beta-catenin gene. Low stringency PCR was performed as described in chapter two and the resulting bands were cloned and sequenced. Figure 3.2 shows the partial cDNA sequence of the beta-catenin gene in the cat. The cat beta-catenin cDNA has 91% identity with the human beta-catenin cDNA. The beta-catenin cDNA fragment was generated by PCR using the primers GGTCACCTTGCAGCTGGAATTC and CCTGAGCAAGTTCACAGAGGACC.

Given that the beta-catenin gene is extensively regulated at the post-translational level (Aberle et al. 1997), the protein expression of this gene needs to be determined in the postnatally developing cat visual system. Also, since the beta-catenin protein is capable of entering the nucleus and acting as a transcription factor (Clevers et al. 1997), behaviour of this gene can not be assessed adequately in the visual system by simply using cDNA probes. Therefore, finding a suitable antibody against the cat beta-catenin protein is essential. Figure 3.3 shows the alignment of the cat and human beta-catenin amino acid sequences. The partially cloned beta-catenin cDNA in the cat is 100% identical with the human beta-catenin protein at the amino acid level. This finding is not surprising considering the human, mice and rat beta-catenin amino acid sequences are almost identical. Accordingly, beta-catenin antibodies generated for these species are likely to cross react with the cat beta-catenin protein.

3.3.2 Western Blot Analysis of the Beta-Catenin Protein in the Postnatally Developing Cat Visual Cortex

The Beta-Catenin antibody was purchased from Transduction Laboratories and this antibody is reported to cross react with the human, rat and mouse beta-catenin proteins. Figure 3.4 shows the quantitative western blot analysis performed in the developing cat

```
Cat  :    1 gtgaacagggtgccattccacgactagttcagttgctggttcgtgcccatcaagataccc   60
           |||| ||||||||||||||||||||||||||||||| |||||||| ||||| |||||||
Human:1797 gtgagcagggtgccattccacgactagttcagttgcttgttcgtgcacatcaggataccc 1856

Cat  :   61 agcgccgcacatctatgggcggaacgcagcagcagtttgtggagggagtccgtatggaag  120
           ||||||| || || ||||| || || |||||||| |||||||||||| ||||| |||||||
Human:1857 agcgccgtacgtccatgggtgggacacagcagcaatttgtggaggggtccgcatggaag 1916

Cat  :  121 aaattgttgaaggttgtaccggagcccttcatatcctagctcgggatgttcacaaccgaa  180
           |||| |||||||||||||||||||||||||| ||||||||||||||||||||||||||||
Human:1917 aaatagttgaaggttgtaccggagcccttcacatcctagctcgggatgttcacaaccgaa 1976

Cat  :  181 tcgtaatcagaggactaaataccattccattgtttgtgcagctgctttattctcccattg  240
           | || ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Human:1977 ttgttatcagaggactaaataccattccattgtttgtgcagctgctttattctcccattg 2036

Cat  :  241 aaaatatccaaagagtagccgcaggggtcctctgtgaacttgctcaggacaaggaggctg  300
           |||| |||||||||||||| ||||||||||||||||||||||||||||||||||| ||||
Human:2037 aaaacatccaaagagtagctgcaggggtcctctgtgaacttgctcaggacaaggaagctg 2096

Cat  :  301 cggaagccattgaagcggagggagccacggctcctctgacagagctccttcactccagaa  360
           | ||||| |||||||| |||||||||| |||||||||||||| | |||||||| || |
Human:2097 cagaagctattgaagctgagggagccacagctcctctgacagagttacttcactctagga 2156

Cat  :  361 acgaaggtgtggcaacatacgcagctgctgttttgttccgaatgtctgaggacaagcccc  420
           | |||||||||||| ||||| ||||||||||||||||||||||||||||||||||| |
Human:2157 atgaaggtgtggcgacatatgcagctgctgttttgttccgaatgtctgaggacaagccac 2216

Cat  :  421 aggactacaagaagcggctttcggtggagctgaccagttctctcttcagaac  472
           | || |||||||| |||||||| || |||||||||| ||||||||||||||
Human:2217 aagattacaagaaacggctttcagttgagctgaccagctctctcttcagaac 2268
```

Figure 3.2 Partial cDNA sequence of the cat Beta-catenin gene.

Blast Score = 634 bits (320), Expect = e-180
Identities = 434/472 (91%)
Strand = Plus / Plus

```
Cat   : 473 EQGAIPRLVQLLVRAHQDTQRRTSMGGTQQQFVEGVRMEEIVEGCTGALHILARDVHNRI 294
           EQGAIPRLVQLLVRAHQDTQRRTSMGGTQQQFVEGVRMEEIVEGCTGALHILARDVHNRI
Human: 529 EQGAIPRLVQLLVRAHQDTQRRTSMGGTQQQFVEGVRMEEIVEGCTGALHILARDVHNRI 588

Cat   : 293 VIRGLNTIPLFVQLLYSPIENIQRVAAGVLCELAQDKEAAEAIEAEGATAPLTELLHSRN 114
           VIRGLNTIPLFVQLLYSPIENIQRVAAGVLCELAQDKEAAEAIEAEGATAPLTELLHSRN
Human: 589 VIRGLNTIPLFVQLLYSPIENIQRVAAGVLCELAQDKEAAEAIEAEGATAPLTELLHSRN 648

Cat   : 113 EGVATYAAAVLFRMSEDKPQDYKKRLSVELTSSLFRT   3
           EGVATYAAAVLFRMSEDKPQDYKKRLSVELTSSLFRT
Human: 649 EGVATYAAAVLFRMSEDKPQDYKKRLSVELTSSLFRT 685
```

Figure 3.3 Alignment of the cat and the human beta-catenin amino acid sequences.

Blast Score = 300 bits (780), Expect = 5e-84
Identities = 157/157 (100%), Positives = 157/157 (100%)

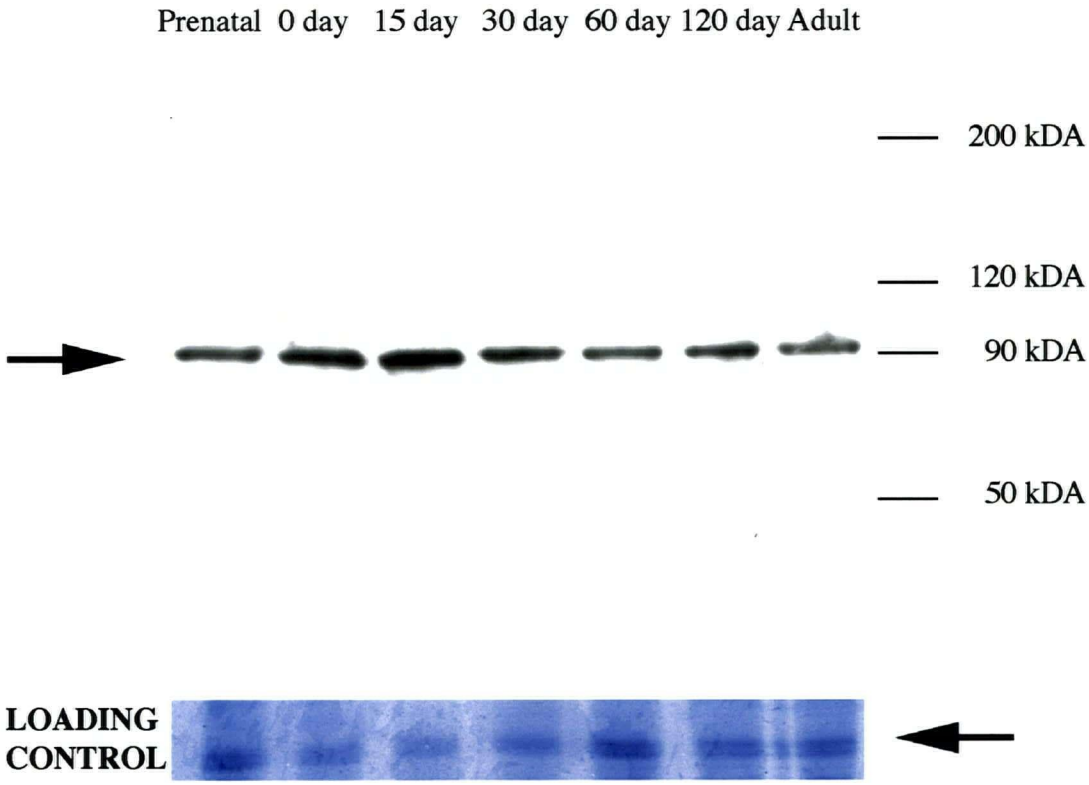

Figure 3.4 Beta-Catenin protein expression in the postnatally developing cat visual cortex. The top panel shows the western blot analysis performed on the developing cat visual cortex using the beta-catenin antibody purchased from Transduction Laboratories. This antibody specifically recognizes a single band of around 93 kDA in visual cortex protein preparations obtained from kittens of various different ages. Significant changes in the expression of the beta-catenin protein are not observed in the developing cat visual cortex. For the loading control (the bottom panel), the membrane used for the western blot analysis was stained with Coomasie blue.

visual cortex with the Transduction Laboratories beta-catenin antibody. The blots consist of prenatal, 0 day postnatal, 15 day postnatal, 30 day postnatal, 60 day postnatal, 120 day postnatal and adult visual cortex protein preparations. In each age a single band of roughly 93k Daltons is detected confirming the fact that the beta-catenin antibody produced by Transduction Laboratories cross reacts with the cat beta-catenin protein. Also, since only a single band is present in this western blot analysis, this antibody appears to specifically recognize the beta-catenin protein in the cat. Therefore, the Transduction Laboratories antibody is suitable for performing immunohistochemistry in the cat visual cortex to localize the distribution of the beta-catenin protein.

Since equal amounts of protein are loaded into each well (50μg) of the blot shown in figure 3.4, these blots are quantitative and can be used to determine beta-catenin protein expression during visual cortex development. The expression pattern shown on figure 3.4 was obtained on all of the western blots. Figure 3.4 shows that the beta-catenin protein expression is fairly constant in the visual cortex during development and does not appear to be regulated in an age dependent manner. In order to verify the validity of this expression pattern, western blot analysis using the beta-catenin antibody was repeated three times on three different blots. Since each beta-catenin antibody western blot analysis yielded a similar result, we are confident at claiming that the pattern shown in figure 3.4 is the actual profile of beta-catenin protein expression during development.

3.3.3 Western Blot Analysis of the Beta-Catenin Protein in the Postnatally Developing Cat LGN

Figure 3.5 shows the quantitative western blot analysis performed in the postnatally developing cat LGN with the beta-catenin antibody. The blot consists of LGN protein preparations from postnatal 0 day, 15 day, 30 day, 60 day, 120 day and adult cats. Each lane again shows a single band confirming the specificity of the beta-catenin antibody in the cat LGN. The expression of the beta-catenin protein does not appear to change with age in the postnatal LGN. As in the cortex, the expression pattern of the beta-catenin

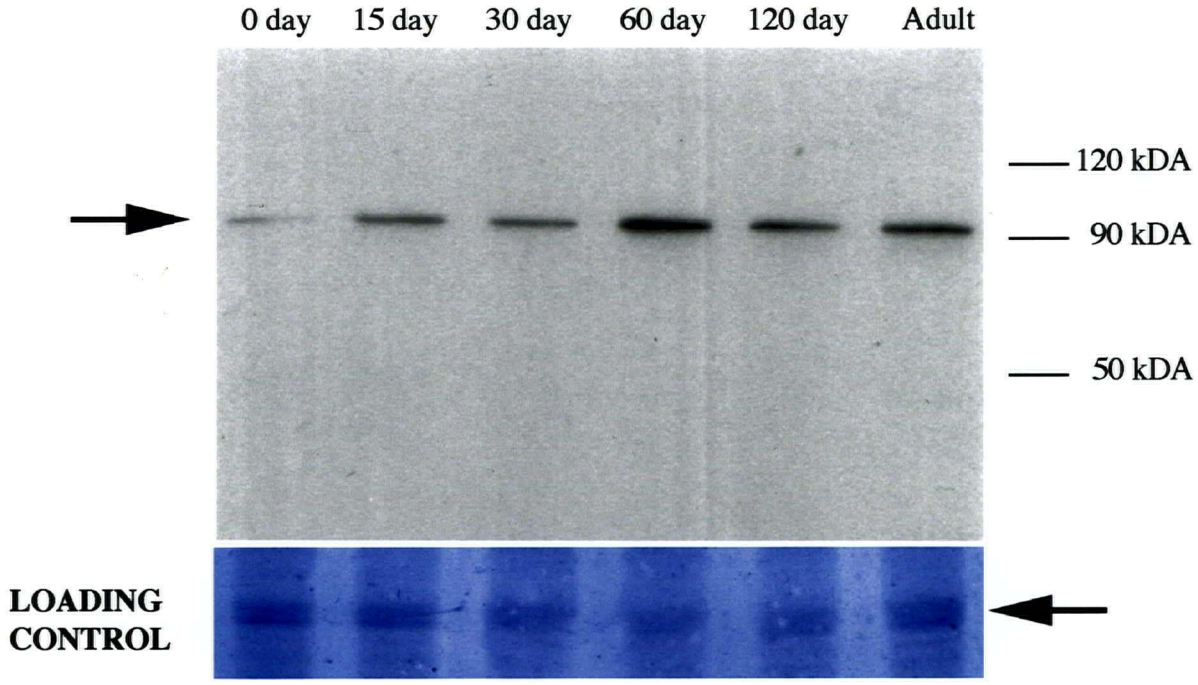

Figure 3.5 Beta-Catenin protein expression in the postnatally developing cat LGN. The top panel shows the western blot analysis performed on the developing cat LGN using the beta-catenin antibody purchased from Transduction Laboratories. This antibody specifically recognizes a single band of around 93 kDA in LGN protein preparations obtained from kittens of various different ages. The beta-catenin protein expression does not apper to vary in the developing LGN. For the loading control (the bottom panel), the membrane used for the western blot analysis was stained with Coomasie blue.

protein in the developing LGN was confirmed by repeating the western blot analysis thrice on three different protein blots. This expression profile of beta-catenin in the postnatal LGN does not correspond with the critical period.

3.3.4 Immunohistochemical localization of Beta-Catenin in the Postnatally Developing Cat Visual Cortex

The Transduction Laboratory beta-catenin antibody, whose specificity in the LGN and the cortex was established by western blot analysis, was used to localize beta-catenin protein expression in developing cat visual cortex tissue. Figure 3.6 shows a 60 day old cat visual cortex tissue section subjected to immunohistochemical analysis. Unlike the reported expression pattern of neurotransmitter receptors, the laminar distribution of the beta-catenin protein does not change with age. The postnatal ages examined include 0 day, 20 days, 30days, 45 days, 55 days, 60 days, 120 days and adults. Beta-catenin protein expression is high in the upper layers of the cortex with a slight overexpression in layer 4. The layer 4 expression of the beta-catenin protein is especially prevalent in the cingulate. Beta-catenin protein expression is not restricted to the visual cortex and this protein is present in other areas of the brain. This finding consistent with the reported function of beta-catenin as a synaptic protein expressed by various types of neurons.

Figure 3.7 shows the photograph of a visual cortex tissue section, which has been subjected to beta-catenin immunohistochemical analysis, taken at high magnification. This figure clearly shows that the beta-catenin protein expression is localized to the neuropil of the visual cortex. This distribution of beta-catenin is similar to the distribution of other synaptic proteins in the visual cortex such as synaptophysin. Neutral Red counter staining of the immunosection in Figure 3.7 reveals antibody staining of the beta-catenin protein is restricted to the neuropil and is absent from cell bodies of cortical neurons. Beta-catenin protein is not found in the nuclei of cortical neurons at any time during postnatal visual development. This finding is surprising considering the presence of Wnt-1 mRNA expression in the cortex during the critical period.

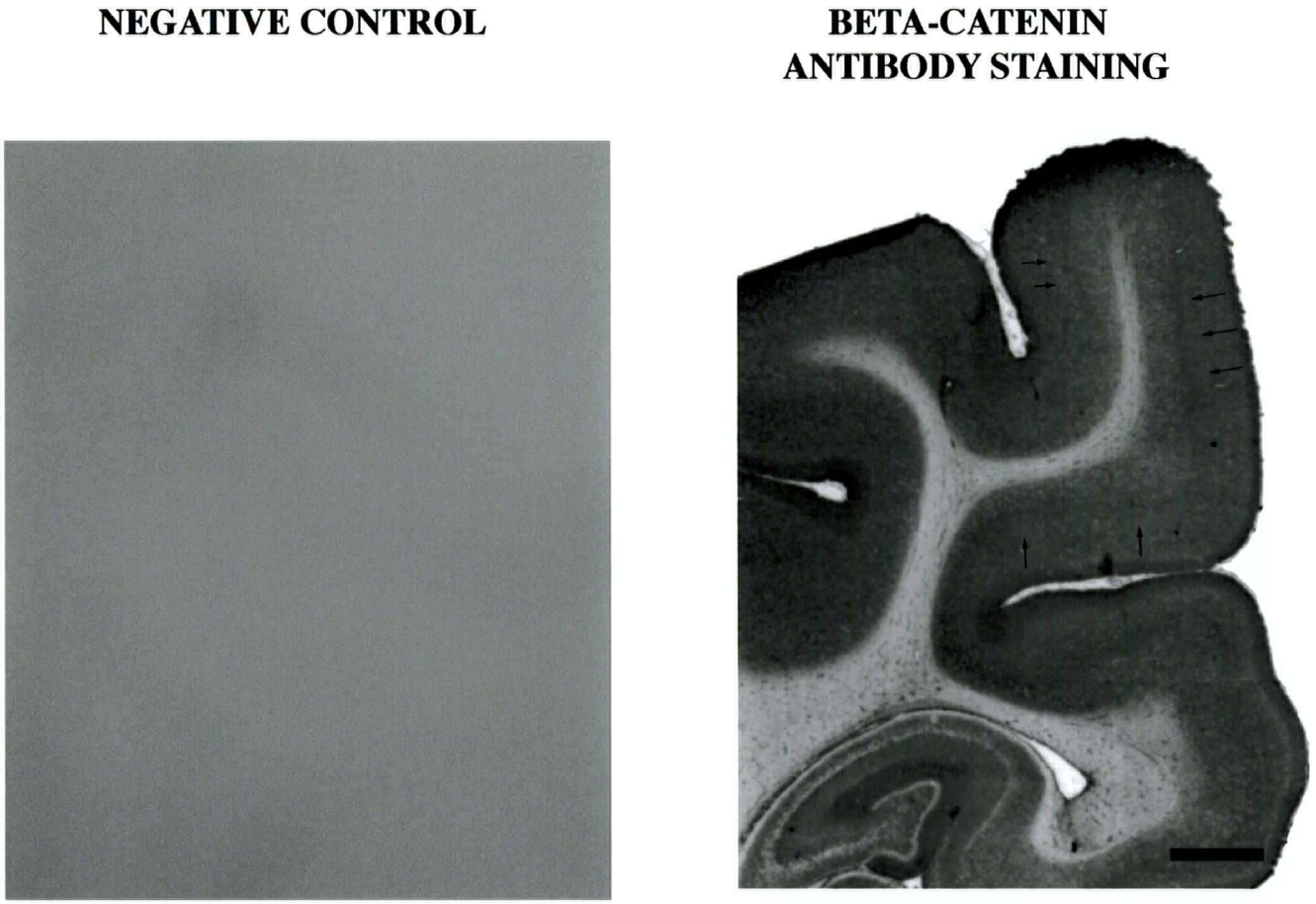

Figure 3.6 Beta-Catenin Antibody Staining in the Postnatal Cat Visual Cortex. As seen in the right panel, the beta-catenin antibody labels all layers of the visual cortex. However, a faint band of stronger beta-catenin antibody staining is observed in layer IV (arrows). This staining pattern of the beta-catenin antibody does not change with age and is not developmentally regulated. The negative control section shown in the left panel was not incubated with the primary antibody but it was subjected to all other steps of the immunohistochemistry procedure. (The contrast and brightness of the negative control figure was adjusted (enhanced) in order to show the absence of any artifactual staining. This is the reason for the darker background colour seen in the left panel.) The scale bar in this figure represents 2 mm's.

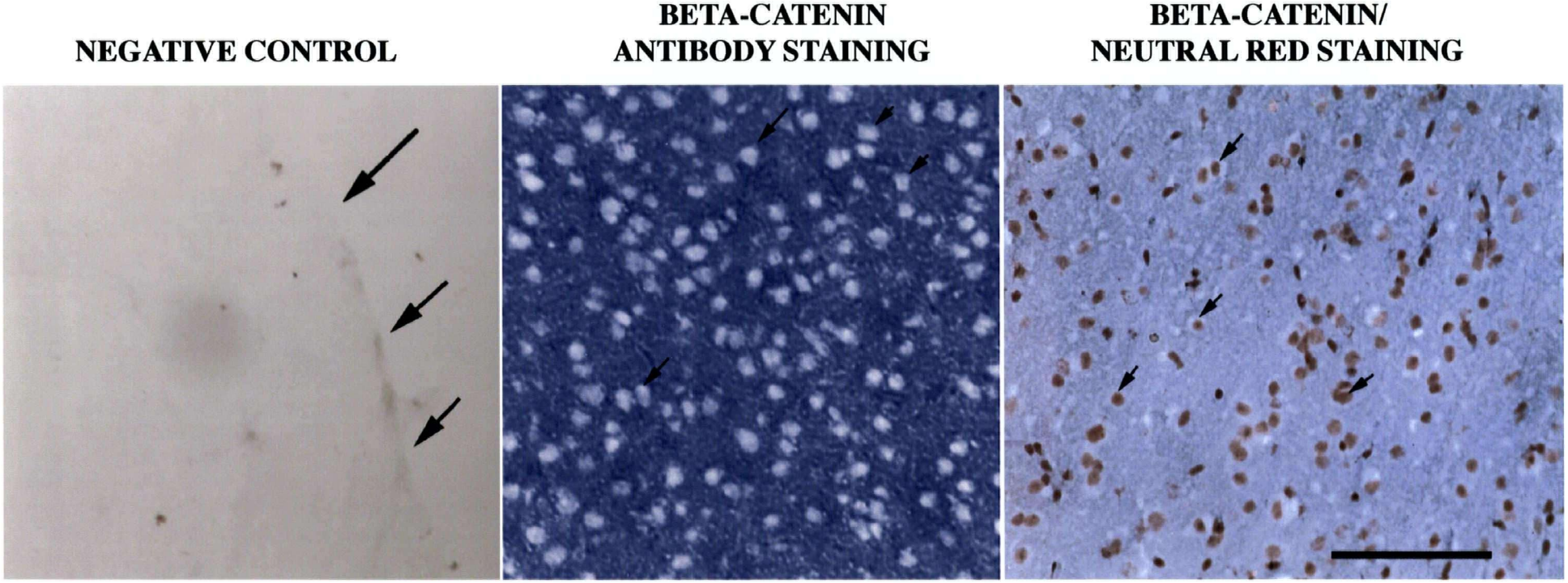

Figure 3.7 Cellular Location of the Beta-Catenin Protein in the Postnatal Cat Visual Cortex. The middle panel is a photograph of a visual cortex tissue section, which has been stained with the beta-catenin antibody, taken at high magnification. The arrows in this panel point to the white, unstained, circular cell bodies. This staining pattern of the beta-catenin antibody remains constant throughout development and the beta-catenin protein is restricted to the neuropil in the postnatal cat visual cortex. The right panel shows a beta-catenin antibody stained visual cortex tissue section which has been counterstained with neutral red. The arrows point to the neutral red stained nuclei and cell bodies and the blue staining is beta-catenin antibody labeling. (The beta-catenin antibody labeling in the counterstained tissue sections is much weaker because the neutral red stain has a tendency to wash out the antibody stain). The localization of the beta-catenin protein to the neuropil is consistent with its putative role in cell adhesion. The leftmost panel is the negative control. This tissue section was not incubated with the primary antibody but it was subjected to all other steps of the immunohistochemistry procedure (In order to get rid of background staining endogenous avidin/biotin sites were blocked - described in the methods section). The arrows in the negative control picture point to the artifactually labeled edge of the cortical tissue. The scale bar represents 0.08 mm's.

3.3.5 Immunohistochemical Localization of the Beta-Catenin Protein in the Postnatally Developing Cat LGN

Figure 3.8 shows immunohistochemical analysis performed in the postnatally developing cat LGN with the beta-catenin antibody. Beta-catenin protein expression in the lateral geniculate nucleus of a 0 day old kitten is restricted to the neuropil. This pattern is similar to the beta-catenin protein distribution observed in the postnatally developing visual cortex. The same distribution pattern is detected in the LGN of 20 day old animals. In kittens as old as 50 days of age, large unstained cell bodies are present in the LGN confirming the absence of the beta-catenin protein from the nuclei of geniculate cells. In kittens between 50 to 60 days of age, the beta-catenin protein is detected in the nuclei of LGN cells and unstained cell bodies are no longer observed. The nuclear localization of the beta-catenin protein is retained after 60 days of age and persists in the adult cat LGN.

The earliest expression of beta-catenin protein in the LGN was observed in a normal 47 day old kitten. Kittens as old as 45 days of age (n=2) lacked nuclear beta-catenin protein in the geniculate (30 day old animals also lacked nuclear beta-catenin). Additionally, no animal exhibited an intermediate state between having nuclear beta-catenin protein (the state seen in 60 day or adult animals) or lacking nuclear beta-catenin protein (the state seen in 0 or 20 day old animals). Nuclear beta-catenin protein was either strongly expressed in the LGN or completely absent.

To confirm that the beta-catenin antibody is labeling nuclei and to determine if the beta-catenin protein is becoming nuclear in neurons, double labeling experiments were carried out. The SMI-32 antibody, a well established neurofilament stain that specifically labels Y-cells (Campbell et al. 1989, Ang et al. 1991, Chaudhuri et al. 1996), was used in conjunction with the beta-catenin antibody to label cat lateral geniculate tissue sections. In figure 3.9, the brown SMI-32 labeling (the DAB stain) is clearly evident in neurons containing the blue beta-catenin nuclei (the nickel enhanced DAB stain). This result demonstrates that beta-catenin nuclearization does occur in neurons. However, although

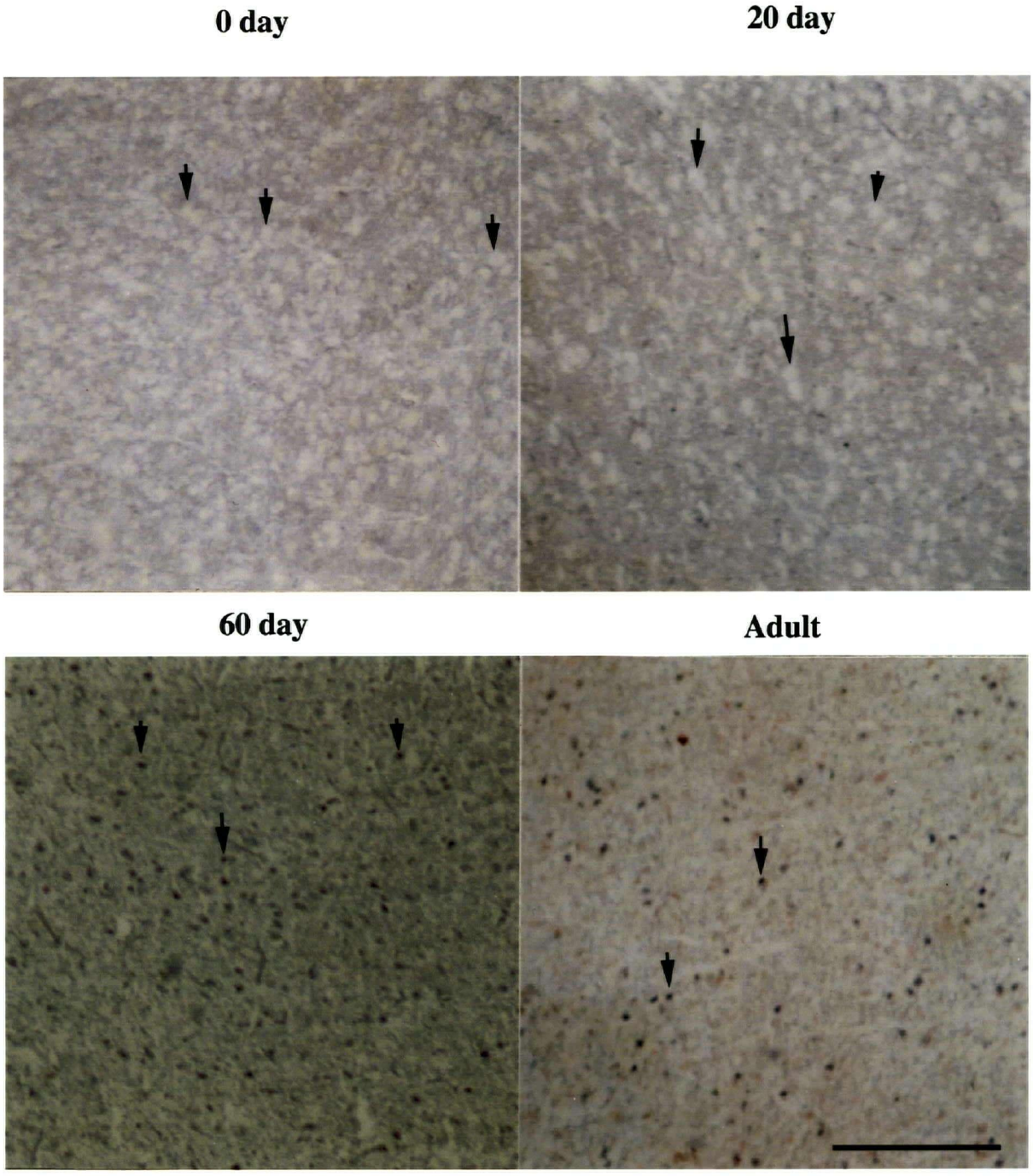

Figure 3.8 Expression of the beta-catenin protein in the postnataly developing cat LGN. In young postnatal animals (the top two panels) beta-catenin protein expression is restricted to the neuropil and the beta-catenin protein is not present in the nuclei of geniculate cells. Accordingly, white unstained cell bodies are observed in the LGN of young kittens (pointed to by arrows in 0 day and 20 day old animals). However, at 60 days of age nuclear beta-catenin staining is detected in the LGN (arrows) and the unstained white cell bodies are no longer observed. This staining pattern persists in the adult LGN (arrows point to nuclei). The scale bar represents 0.16 mm's.

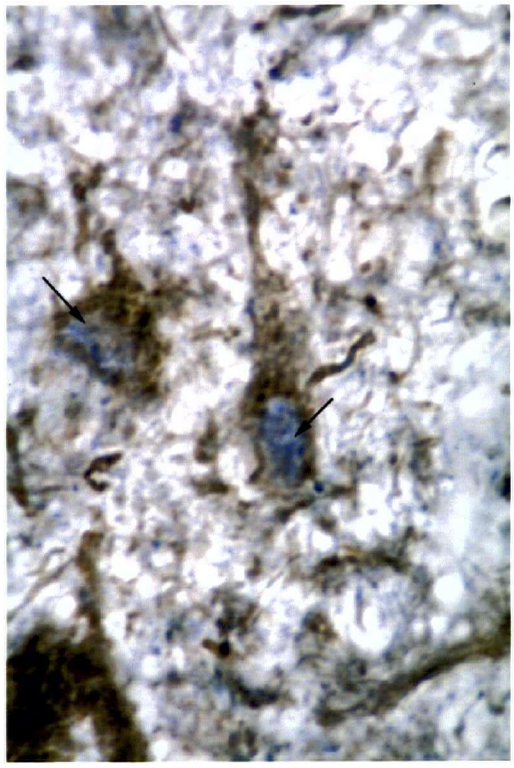

Figure 3.9 Beta-catenin and SMI-32 double staining in the LGN of older kittens. The blue nickel enhanced DAB beta-catenin protein staining (arrows) is observed in the nuclei of neurons which have been labeled with the brown SMI-32 neurofilament stain (regular DAB).

the co-localization between SMI-32 and beta-catenin staining is extensive, it is not complete and there are some cells that are only labeled with the SMI-32 antibody and some cells which are strictly labeled by the beta-catenin antibody. This finding indicates that the nuclear localization of the beta-catenin protein is not restricted to large neurons (i.e. not specific to Y cells) and nuclearization of beta-catenin is not determined by cell size.

3.3.6 Nuclear Beta-Catenin Protein Levels in the Postnatally Developing Cat LGN

In order to get a more accurate measurement of the amount of beta-catenin protein present in the nuclei of LGN neurons during the critical period, nuclear protein preparations were obtained from the lateral geniculates of postnatal 0 day, 15 day, 30 day, 60 day, 120 day and adult cats. Western blot analysis performed on these nuclear LGN preparations with the beta-catenin antibody is shown in figure 3.10. Beta-catenin protein levels in nuclei of geniculate cells become elevated in kittens 60 days of age and remain high throughout adulthood. These findings are consistent with the beta-catenin immunohistochemical analysis described in the postnatally developing LGN (discussed further in the last section).

3.4 DISCUSSION

These findings support a role for beta-catenin in the postnatal development of the visual system. The localization of the beta-catenin protein in the nuclei of geniculate neurons in the postnatally developing cat visual cortex correlate with the critical period. Since beta-catenin is a homologue of the *armadillo* segment polarity gene, the findings of this chapter solidify the hypothesis that the homologues of the drosophila developmental genes are involved in the postnatal development of the visual system.

3.4.1 Beta-Catenin Expression in the Postnatal LGN

Beta-catenin is likely to perform dual functions in the postnatally developing cat LGN. In the LGN of young kittens, the expression of beta-catenin is restricted to the

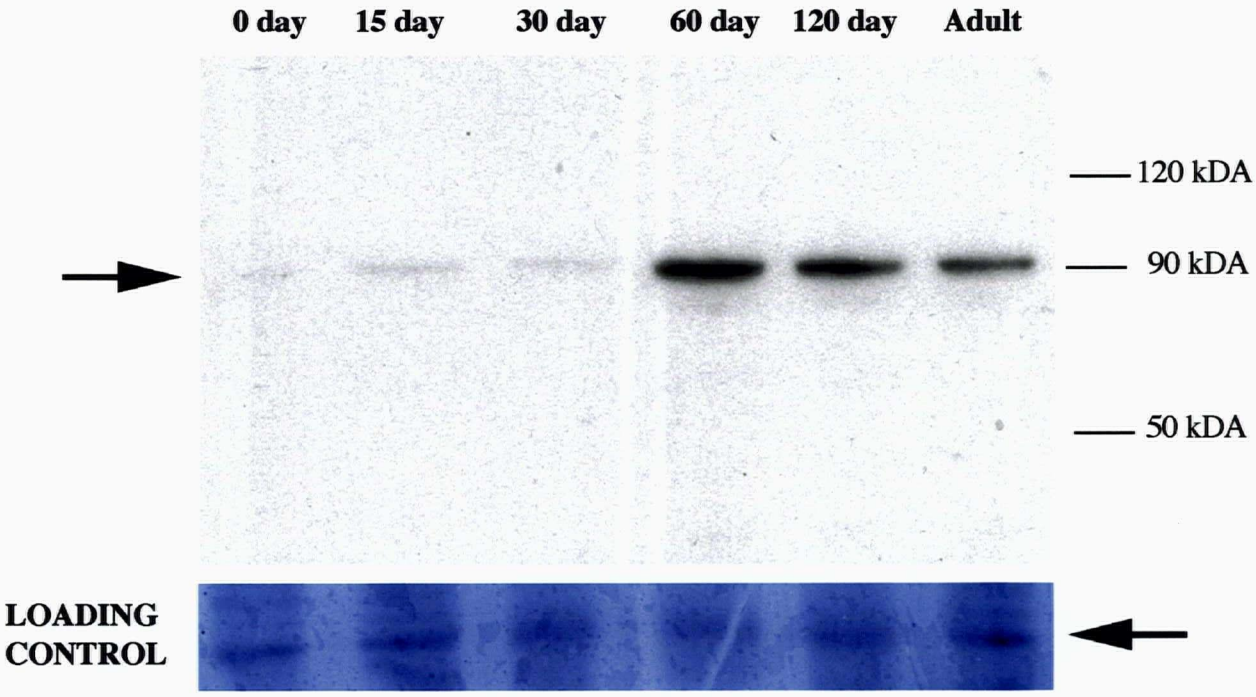

Figure 3.10 Beta-Catenin protein levels in the nuclei of geniculate cells during the postnatal development of the cat LGN. The top panel shows the western blot analysis performed on the nuclear protein preparations obtained from different aged postnatal cat LGN's. The beta-catenin protein is absent from the nuclear protein fraction of the LGN in cats younger than 60 days of age. This finding is consistent with the results of the immunohistochemical analysis described in Figure 3.8. For the loading control (the bottom panel), the membrane used for the western blot analysis was stained with Coomasie blue.

neuropil. This distribution pattern is consistent with the function of beta-catenin as a cell adhesion molecule. However, the beta-catenin protein levels are likely to be upregulated in a proportion of geniculate cells in postnatal kittens 60 days of age and older. The upregulation and the subsequent accumulation of the beta-catenin protein causes it to be transported into the nuclei of geniculate neurons where it is likely to function as a transcription factor. Interestingly, this nuclear localization of beta-catenin in LGN neurons is retained in the adult. These findings are quite surprising considering nuclear beta-catenin is usually associated with early events in development.

Beta-catenin protein becomes nuclear in the LGN at the end of the critical period for thalamocortical plasticity. Following 50-60 days of age, monocular deprivation fails to cause ocular dominance shifts in layer 4 of the visual cortex as determined by anatomical (tracer injections) and physiological (single unit recordings) techniques (LeVay et al. 1978, LeVay et al. 1980, Mower et al. 1985, Shatz et al. 1978). Since the beta-catenin protein becomes nuclear in LGN neurons around 50 days of age, we propose nuclear beta-catenin activity to be involved in ending thalamocortical plasticity. As mentioned in the introduction, beta-catenin acts as a transcription factor in the nucleus and regulates the expression of a variety of genes by forming complexes with the TCF and LEF proteins (Clevers et al. 1997, Huber et al. 1996, Molenaar et al. 1996, Behrens et al. 1996, Brannon et al. 1997, Riese et al. 1997, Molenaar et al. 1996). Conceivably, when the beta-catenin protein becomes nuclear in geniculate neurons, it may downregulate the expression of genes involved in facilitating synaptic plasticity or alternatively, beta-catenin may upregulate the expression of proteins which inhibit processes associated with synaptic rearrangements. This model implies that synapses in layer IV of the visual cortex are unable to undergo synaptic reorganization in kittens older than 60 days of age because thalamic processes have lost their plastic capabilities. Presumably, cortical neurons retain their plasticity past this age since beta-catenin nuclearization does not occur in the cortex. This notion is supported by the fact that plasticity is retained in the higher layers of the

cortex past 60 days of age and ocular dominance shifts in layer 2/3 of the cortex are detected in cats as old as 1 years of age (Daw et al. 1992). These findings argue for a presynaptic end to visual plasticity and stress the importance of presynaptic processes in synaptic rearrangements. However, the possible existence of processes involved in inhibiting plasticity specifically in layer IV of the visual cortex can not be ruled out.

Nuclear beta-catenin is unlikely to be the only factor responsible for ending visual plasticity. As mentioned, only a subset of geniculate neurons contain nuclear beta-catenin. Furthermore, nuclear beta-catenin is not observed in cortical neurons even during adulthood. Therefore, other processes associated with ending visual plasticity must be acting on cortical cells and geniculate neurons lacking nuclear beta-catenin activity. Beta-catenin is viewed as a factor that causes genetic changes in a subset of LGN neurons which prevents these neurons from undergoing synaptic rearrangements. Additionally, transcription factor activity of beta-catenin may only partially inhibit the plastic capabilities of neurons and other mechanisms may also be necessary for ending thalamocortical plasticity in geniculate neurons containing nuclear beta-catenin.

Two additional issues regarding beta-catenin nuclearization in the postnatal lateral geniculate need to be addressed. The beta-catenin protein starts going into the nuclei of LGN neurons in kittens that are around 50 days of age. Often the regulatory changes caused by transcription factors do not immediately alter the phenotypic behaviour of cells and this reason may explain why nuclear beta-catenin protein is detected in the geniculate slightly before the end of thalamocortical plasticity. For example, if beta-catenin activity leads to the activation of a transcription cascade, the proteins involved need to be synthesized and allowed to take effect. More importantly, genes downregulated by beta-catenin may exhibit phenotypic lag. Halting gene expression at the transcriptional level does not lead to immediate cessation of gene function (Rudd et al. 1990, Thilly et al. 1978, Penman et al. 1976). Previously synthesized proteins from the inhibited gene may remain undegraded and perform their function in the absence of newly synthesized protein. The

phenomenon of phenotypic lag is well documented for proteins which are highly stable and turn over slowly. The slight disparity between the nuclearization of beta-catenin in the LGN and the end of the period for thalamocortical plasticity can be accounted by these factors. Secondly, it is not clear why there are less nuclear beta-catenin containing cells in the LGN of adult animals. One possibility is that nuclear beta-catenin protein causes irreversible changes in some neurons which prevents them from being plastic whereas in other neurons beta-catenin protein may need to exert a constant level of inhibition in order to prevent plasticity.

Another important implication of having nuclear beta-catenin in adult neurons involves the role beta-catenin plays in cancer pathology. The nuclear localization of beta-catenin in non-neuronal tissue usually occurs during early development and the presence of nuclear beta-catenin in adult cells is an aberration associated with cancer metastasis (Oyama et al. 1994, Rubinfeld et al. 1997, Korinek et al. 1997, Porifi et al. 1997). Cancerous adult cells containing nuclear beta-catenin lose their adhesive properties, detach from their tissue of origin and spread the disease by migrating into other organs. In addition, beta-catenin activity is reported to contribute to the proliferation of cancerous cells in tumors (Tetsu et al. 1999). Clearly, these events do not occur in the adult geniculate neurons of cats. The previous statement does not imply that beta-catenin is performing a different function and effecting the expression of a completely different set of genes in neuronal and non-neuronal tissue. Instead, nuclear beta-catenin may exhibit differing effects in these tissues because certain pathways or cofactors which are present in non-neuronal cells may be absent in neurons and vice versa. If the reported effects of the nuclear beta-catenin protein in adult non-neuronal tissue requires the activity of certain cofactors which are lacking in neuronal cells then clearly, beta-catenin will not be able to induce the same changes in neurons. In other words, although beta-catenin may be necessary to cause the phenotypes observed during cancer, it may not be capable of carrying out this task by itself. As a side note, examination of factors which are lacking in geniculate neurons but

present in cells that become cancerous with beta-catenin nuclearization may provide important information regarding the processes involved in cancer.

3.4.2 Beta catenin expression in the postnatally developing cat visual cortex

The function of beta-catenin in the postnatally developing visual cortex appears to be as an adhesion molecule. Nuclear beta-catenin protein is not detected in the developing cat visual cortex at any age and its distribution is strictly restricted to the neuropil. A role for beta-catenin protein in cortical plasticity is suggested by the fact that its expression is slightly higher in layer IV of the visual cortex which is where thalamocortical connections reside. The known functions of the cell adhesion system beta-catenin constitutes with the cadherin proteins (such as synaptogenesis and target recognition (Huntley et al. 1999, Riehl et al. 1996, Matsunaga et al. 1988, Obst-Pernberg et al. 1999) - discussed in detail in the next chapter) further support a role for this protein in visual plasticity.

Despite the presence of Wnt-1 mRNA expression in the cortex, the beta-catenin protein does not become nuclear in cortical neurons at any age. This finding represents the complexity of the factors involved in Wnt-1 signaling and reflects the problems associated with determining the exact location of Wnt-1 signaling activity. In the previous chapter, the expression of Frz-B, a molecule that inhibits the function of Wnt-1 (Leyns et al. 1997), was confirmed in the developing cat visual cortex. Several other soluble factors similar to Frz-B, which prevent the Wnt-1 protein from binding to its receptor, are known to exist (Moon et al. 1997a, Brown et al. 1998) and are likely to be present in the visual cortex. Additionally, the Wnt-5A proteins, which are known to antagonize Wnt-1 signaling (Torres et al. 1996), may also be expressed during the critical period. Examination of all these molecules in the cortex is necessary in order to determine why beta-catenin does not become nuclearized in the cortex. It is important to point out that the Wnt-1 expression was detected at the mRNA level and the possibility exists that this transcript is not translated into protein. Although the beta-catenin protein does not become localized to the nuclei of

cortical neurons, it is possible that Wnt-1 protein may still play a role in regulating beta-catenin protein expression in the neuropil. Alternatively, the Wnt-1 protein may be a retrograde signal for the geniculate neurons. Support for this possibility is provided by the finding that Wnt molecules can act as retrograde messengers in the cerebellum (Hall et al. 2000). Conceivably, the Wnt-1 molecule secreted by cortical neurons may function as retrograde messengers and cause the nuclearization of beta-catenin in geniculate neurons thereby ending plasticity.

3.4.2 Function of beta-catenin in the developing visual system

Genes regulated by nuclear beta-catenin activity in LGN neurons need to be identified in order to verify the proposed role of beta-catenin in ending the critical period for thalamocortical plasticity and to determine the mechanisms involved in this process. Theories can be formulated about the transcriptional changes caused by nuclear beta-catenin in the LGN based on studies characterizing the transcription factor function of this protein in other systems. As noted, beta-catenin nuclearization in adult non-neuronal cells causes loss of adhesion allowing these cells to dislodge from their tissue of origin (Rubinfeld et al. 1997, Korinek et al. 1997, Porifi et al. 1997). This capability of beta-catenin is attributed to the direct inhibitory effect it exerts on cell adhesion molecules at the transcriptional level (Stewart et al. 2000, Nuruki et al. 1998). Beta-catenin is likely to control the expression of cell adhesion molecules in neurons since neurons express many of the same cell adhesion molecules as non-neuronal cells. The importance of cell adhesion molecules to the process of neuronal plasticity is beginning to emerge. The disruption of the cadherin and the integrin cell adhesion proteins are reported to interfere with the induction of LTP (Tang et al. 1998, Staubli et al. 1990). A correlation between cell adhesion molecule expression and visual plasticity is established by the work of Muller et al (Schoop et al. 1997) who report the adhesiveness of the visual cortex to decrease with age. Hence, we propose that nuclear beta-catenin is ending the critical period by downregulating the expression of cell adhesion molecules.

Since the role of beta-catenin in cell adhesion is to anchor the cadherin cell adhesion molecules to the actin cytoskeleton (Nagafuchi et al. 1988, Kitner et al. 1992, McCrea et al. 1991), the expression of the cadherin molecules in the postnatal visual system of the cat is investigated in the next chapter. Interestingly, certain kinds of cadherin cell adhesion molecules are downregulated by the transcription factor activity of beta-catenin (Stewart et al. 2000, Nuruki et al. 1998) and the ontogeny of the cadherin molecules in the developing visual cortex (reported in the next chapter) supports the hypothesis that nuclear beta-catenin activity in the LGN downregulates cell adhesion molecule expression.

Chapter 4

CADHERIN PROTEIN EXPRESSION IN THE POSTNATALLY DEVELOPING CAT VISUAL SYSTEM

4.0 SUMMARY

Since the role of the beta-catenin protein in cell adhesion is to anchor the cadherin proteins to the actin cytoskeleton, the distribution of the cadherin proteins in the postnatal cat visual system was examined using a pan-cadherin antibody. Western blot analysis shows the expression of the cadherin proteins is downregulated in the adult cat visual cortex and the lateral geniculate nucleus. Immunohistochemical analysis using the pan-cadherin antibody revealed that the cadherin proteins are largely expressed in layer IV of the cortex, which is the thalamic input layer. Based on the reported properties of the cadherin cell adhesion molecules in other systems, the cadherin proteins are likely to play an organizational role in the postnatal visual system and they are likely to contribute to the proper formation of thalamocortical connections.

4.1 INTRODUCTION

Beta-catenin and the cadherin proteins constitute a prominent cell adhesion system in the body. The role of the beta-catenin protein in this system is to anchor the cadherin molecules to the actin cytoskeleton (McCrea et al. 1991). Since the beta-catenin protein appears to act exclusively as a cell adhesion molecule in the visual cortex and in the lateral geniculate of young kittens, the ontogeny of the cadherin molecules was investigated in the developing cat visual system.

The cadherin family of calcium dependent cell adhesion molecules have received a great deal of attention in neurodevelopment (Volk et al. 1984, Volk et al. 1986, Redies et al. 1996, Martinek et al. 1997, Yagi et al. 2000, Redies 2000). The cadherin proteins are around 130kDa in size and consist of 5 extracellular domains which are repeats of each other (Tanihara et al. 1994). The first three extracellular domain repeats contain sites for calcium binding which are necessary for stabilizing the cadherin protein and preventing its proteolysis (Nose et al. 1988, Nose et al. 1990). The extracellular domain closest to the N terminal gives rise to the cell adhesion binding sites (Nose et al. 1990). The cadherin molecules are typically categorized based on their binding sites since cadherin molecules bind to each other in a strictly homophilic manner (Nose et al. 1988, Miyatani et al. 1989, Shapiro et al. 1995, Leckband et al. 2000). The cadherin proteins also contain a small transmembrane domain and a highly conserved intracellular domain that binds to the catenin proteins (Nagafuchi et al. 1988, Kitner 1992, McCrea et al. 1991). In addition, cadherin molecules need to form homo-dimers at the conserved HAV amino acid region in order to function properly (Blaschuk et al. 1990).

Numerous different types of cadherin and cadherin like molecules are expressed in the nervous system. The cadherin molecules are categorized into three general groups: the classic cadherins, protocadherins and the cadherin like neural receptors. The classic cadherin molecules can be subdivided further into two groups, named type I and type II, based on their amino acid sequences (Suzuki et al. 1991, Tanihara et al. 1994). Protocadherins are larger cadherin molecules containing six or seven cadherin like extracellular domains (Sano et al. 1993) and the intracellular binding region of the protocadherin proteins is significantly different than the classic cadherins (Sano et al. 1993). Finally, there are the cadherin like neural receptors (CNR's) which are very similar in structure to the classic cadherin cell adhesion molecules (Kohmura et al. 1998). Given the rapid rate in which new types of cadherin molecules are being discovered, additional classes of cadherin molecules are likely to be identified. As in other tissues, cadherins

are expressed in a temporally and spatially specific manner in the developing nervous system (Inoue et al. 1998, Suzuki et al. 1997, Matsunami et al. 1995, Rubenstein et al. 1999, Kimura et al. 1996, Arndt et al. 1996). Electron microscope studies in the brain have localized the cadherins to the synaptic terminals (Uchida et al. 1996) (figure 4.1). Involvement of cadherin proteins in synaptogenesis, neurite extension and target recognition is well established (Huntley et al. 1999, Riehl et al. 1996, Matsunaga et al. 1988, Obst-Pernberg et al. 1999). Cadherin molecules are also expressed in specific domains in both the adult and the developing brain (Rubenstein et al. 1999, Suzuki et al. 1997, Inoue et al. 1997). Based on these findings, the cadherin proteins are proposed to give rise to the complex and precise wiring pattern of neural connections found in the brain (Fannon et al. 1996, Martinek et al. 1997, Obst-Pernberg et al. 1999). According to this model, since cadherin proteins bind in a homophilic manner, neuronal processes expressing a particular type of cadherin molecule only make connections with other processes that express the same kind of cadherin molecule. The large number of cadherin protein subtypes expressed in the brain is postulated to facilitate the formation of various neural circuits. The existence of cadherin molecules that are specifically expressed by particular neural tracts gives merit to this hypothesis (Arndt et al. 1996, Suzuki et al. 1997, Obst-Pernberg et al. 1999, Inoue et al. 1998, Rubenstein et al. 1999).

Evidence for the involvement of cadherin proteins in activity dependent plasticity is also present. In the developing somatosensory cortex N-cadherin expression is regulated in an activity dependent manner (Huntley et al. 1999). In hippocampal slices, the induction of LTP is blocked by preventing cadherin dimerization (Tang et al. 1998). Neural activity also appears to stabilize and strengthen cadherin mediated synaptic adhesion (Tanaka et al. 2000). Since events like synaptogenesis, formation of neural circuits and activity dependent plasticity are associated with visual development, the beta-catenin/cadherin cell adhesion system is an intriguing molecular candidate for being involved in postnatal visual system development.

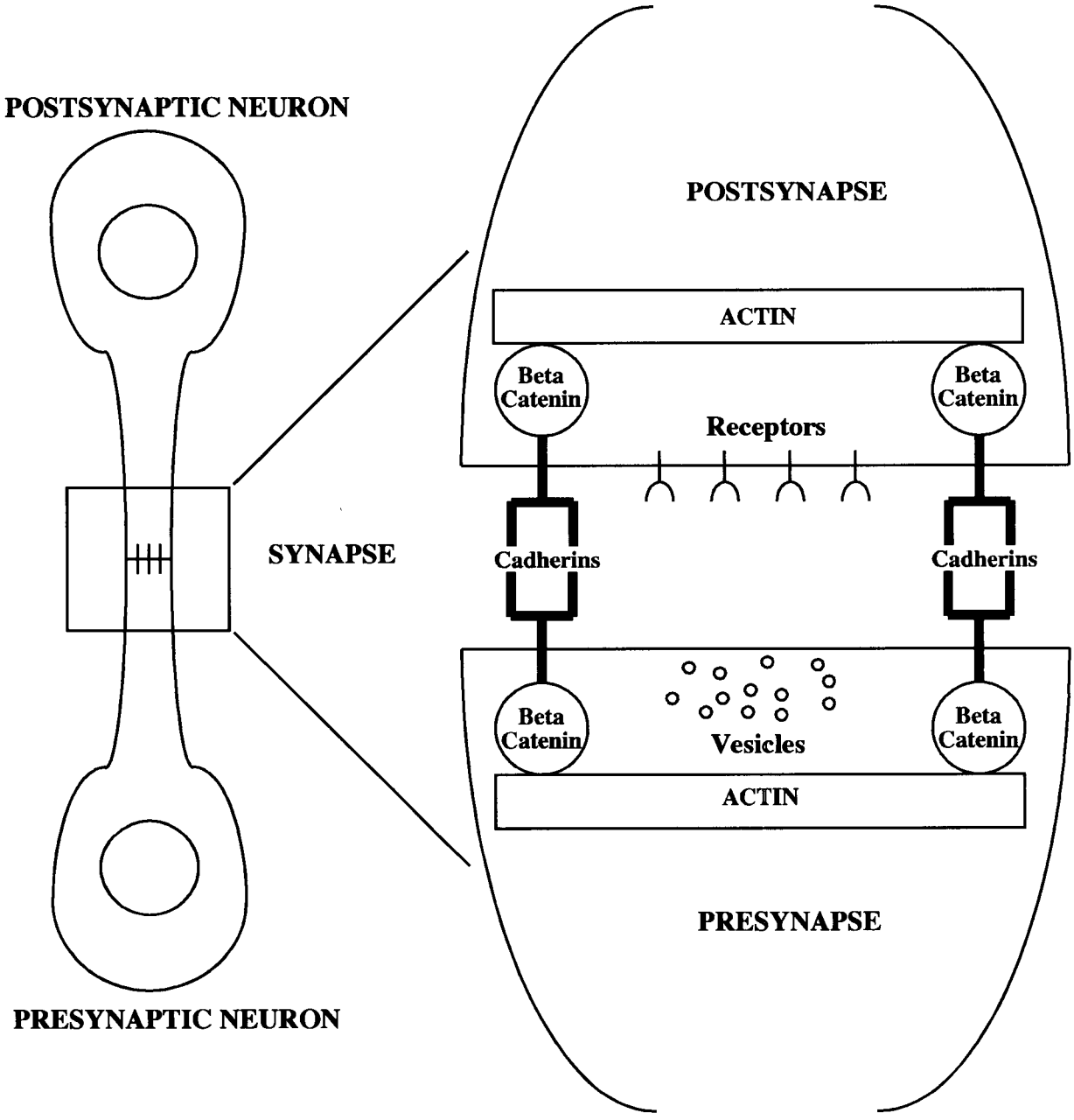

Figure 4.1 Cadherin Protein Distribution in Neurons.

HYPOTHESIS: The tissue distribution and the expression profile of the cadherin proteins in the postnatally developing cat visual system correlate with the critical period.

OBJECTIVES: 1.) To determine temporal expression and the tissue distribution of the cadherin proteins in the postnatally developing cat visual cortex and the LGN.

4.2 METHODS

Animal preparation, Immunohistochemistry and Western blots:

See Methods Section in Chapter three. 5 additional animals N=1 P0, N=2 P60 and N=2 Adult had to be sacrificed for western blot analysis in order to generate more tissue. The pan-cadherin antibody was purchased from SIGMA.

4.3 RESULTS

4.3.1 Partial Cloning of Cadherin Genes Expressed in the Postnatal Cat Visual Cortex

In order to determine the different kinds of cadherin molecules expressed in the developing visual cortex, the cDNA's of various cat cadherin genes were partially cloned using the RT-PCR strategy described in chapter 2. Initially, the expression of cadherin 6, 8 and 11 were examined in the developing visual cortex. These cadherins were examined because they demarcate neural circuits and their expression is retained in the adult brain (Suzuki et al. 1997). Figures 4.2, 4.3 and 4.4 show the partial cDNA sequences of the cat

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Cat   :    1 ttgaccgagaaacattgctgtggcacaacattacagtaatagcaacagagatcaataatc   60
           ||||||||||||||| |||| ||||||||||||||||| ||||||||||||||||||||||
Human:1457 ttgaccgagaaacactgctatggcacaacattacagtgatagcaacagagatcaataatc 1516

Cat   :   61 caaagcaaagcagccgagtccctctatatattaaagtcctagatgtcaatgacaatgccc  120
           |||||||||| || ||||| ||||||||||||||||| ||||||||||||||||| ||||
Human:1517 caaagcaaagtagtcgagtacctctatatattaaagttctagatgtcaatgacaacgccc 1576

Cat   :  121 cggaatttgctgaattctatgaaacctttgtctgtgaaaaggcaaaagcagatcagttga  180
           | |||||||||||| |||||||||| |||||||||||||| ||||| ||||||||||||
Human:1577 cagaatttgctgagttctatgaaacttttgtctgtgaaaaagcaaaggcagatcagttga 1636

Cat   :  181 ttcagaccctacgagccattgacaaagacgacccttatagtgggcaccaattctcattct  240
           |||||||||| |  ||  ||||||| || |||||||||||||| |||||||| || || |
Human:1637 ttcagaccctgcatgctgttgacaaggatgacccttatagtggacaccaattttcgtttt 1696

Cat   :  241 ctttggcccctgaagcaaccagtggctcaaactttaccattcaagacaacaaagataaca  300
           | |||||||||||||| ||||||||||||||||||||||||||||||||||||| ||||
Human:1697 ccttggcccctgaagcagccagtggctcaaactttaccattcaagacaacaaagacaaca 1756

Cat   :  301 cagcaggaatcttaactcggaaaaatggctataatagacacgagatgagcacctatctct  360
           | || ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Human:1757 cggcgggaatcttaactcggaaaaatggctataatagacacgagatgagcacctatctct 1816

Cat   :  361 tgcctgtggtca  372
           ||||||||||||
Human:1817 tgcctgtggtca 1828
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Figure 4.2 Partial cDNA sequence of the cat cadherin 6 gene.

Blast Score = 507 bits (256), Expect = e-142
Identities = 343/372 (92%)
Strand = Plus / Plus

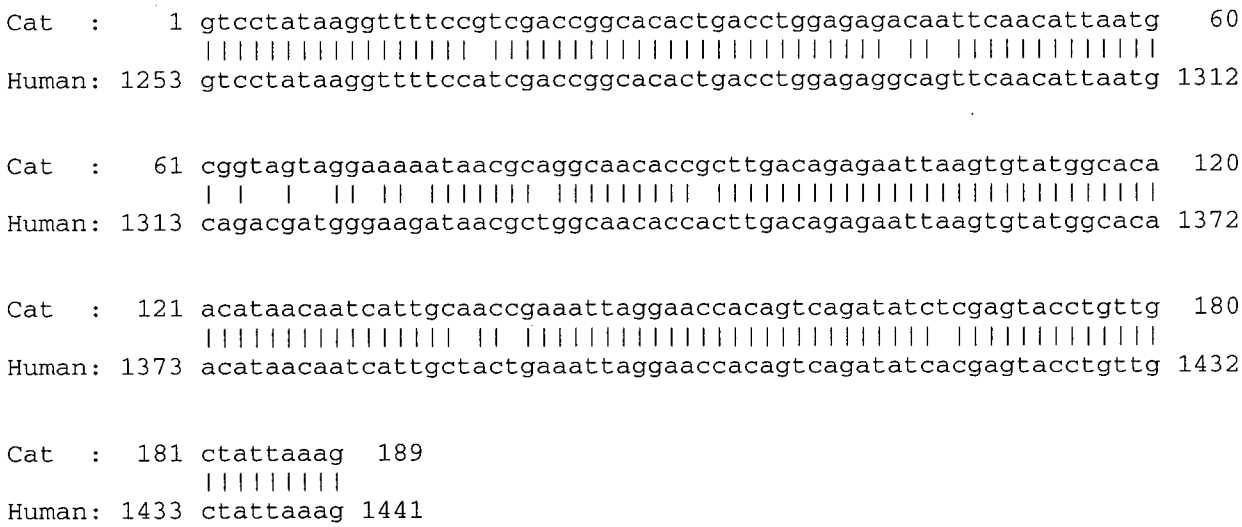


Figure 4.3 Partial cDNA sequence of the cat cadherin 8 gene.

Blast Score = 256 bits (129), Expect = 2e-66
Identities = 174/189 (92%)
Strand = Plus / Plus

Figure 4.4 Partial cDNA sequence of the cat cadherin 11 gene.

Blast Score = 440 bits (222), Expect = e-121
Identities = 272/286 (95%)
Strand = Plus / Plus

cadherin 6, 8 and 11 genes. The degree of identity between the human and the partially cloned cat cadherin cDNA's is 92% for cadherin 6 and cadherin 8, and 95% for cadherin 11. The cadherin 6 cDNA was generated by PCR using the primers ATGCTGCCAGGAATCCTGTCAAG and TGACCACAGGCAAGAGATAGGTG, cadherin 8 cDNA was generated using primers AACCTCTGGAGCCTCCTTCTGAA and GCGTTGTCATTGACATCCAGCAC, and cadherin 11 cDNA was generated using the primers CGATCGTCACACTGACCTCGACA and GTACAAGTCCTGCTTCTGCCGAC. The amino acid sequence of these cDNA's and their homology to the human amino acid sequence is shown in Figure 4.5. As expected, the homology between the cat and the human cadherin genes is more extensive at the protein level. Clearly, cadherin antibodies specific to the human versions of these proteins should cross-react with the cat cadherin proteins.

The presence of all three of the cadherin genes postulated to be expressed in the visual cortex led to the realization that a large number of cadherin types are likely to be expressed in this structure. The cloning and examination of each cadherin type expressed in the visual cortex is an enormous task and beyond the scope of this thesis. Additionally, it is unclear which cadherin molecule(s) is of importance in postnatal cat visual development. Hence, instead of examining the expression profile of one cadherin protein, we decided to monitor the expression of several cadherin molecules at once. The cumulative expression pattern of a group of cadherin molecules was determined in the developing cat visual system by using a pan-cadherin antibody capable of recognizing several different cadherin proteins.

4.3.2 Selection of the Pan-Cadherin Antibody

The pan-cadherin antibody produced by SIGMA was used for immunohistochemical and western blot analysis. This is a well characterized antibody which is known to cross react with several members of the classic cadherin gene family. The specificity of this antibody in other species is well established and it has been used in

CADHERIN 6 AMINO ACID SEQUENCE

```
Cat   : 374 DRETLLWHNITVIATEINNPKQSSRVPLYIKVLDVNDNAPEFAEFYETFVCEKAKADQLI 195
          DRETLLWHNITVIATEINNPKQSSRVPLYIKVLDVNDNAPEFAEFYETFVCEKAKADQLI
Human: 447 DRETLLWHNITVIATEINNPKQSSRVPLYIKVLDVNDNAPEFAEFYETFVCEKAKADQLI 506

Cat   : 194 QTLRAIDKDDPYSGHQFSFSLAPEATSGSNFTIQDNKDNTAGILTRKNGYNRHEMSTYLL  15
          QTL A+DKDDPYSGHQFSFSLAPEA SGSNFTIQDNKDNTAGILTRKNGYNRHEMSTYLL
Human: 507 QTLHAVDKDDPYSGHQFSFSLAPEAASGSNFTIQDNKDNTAGILTRKNGYNRHEMSTYLL 566

Cat   :  14 PVV   6
          PVV
Human: 567 PVV 569
```

Blast Score = 250 bits (632), Expect = 8e-67
Identities = 120/123 (97%), Positives = 121/123 (97%)

CADHERIN 8 AMINO ACID SEQUENCE

```
Cat   : 187 PIRFSVDRHTDLERQFNINAVVGKITQATPLDRELSVWHNITIIATEIRNHSQISRVPVA   8
          PIRFS+DRHTDLERQFNINA  GKIT ATPLDRELSVWHNITIIATEIRNHSQISRVPVA
Human: 417 PIRFSIDRHTDLERQFNINADDGKITLATPLDRELSVWHNITIIATEIRNHSQISRVPVA 476

Cat   :   7 IK   2
          IK
Human: 477 IK 478
```

Blast Score = 118 bits (293), Expect = 1e-27
Identities = 58/62 (93%), Positives = 59/62 (94%)

CADHERIN 11 AMINO ACID SEQUENCE

```
Cat   : 285 YSIDRHTDLDRFFTINPEDGFIKTTKPLDREETAWLNISVFAAEIHNRHQEAKVPVAIRV 106
          YSIDRHTDLDRFFTINPEDGFIKTTKPLDREETAWLNI+VFAAEIHNRHQEA+VPVAIRV
Human: 419 YSIDRHTDLDRFFTINPEDGFIKTTKPLDREETAWLNITVFAAEIHNRHQEAQVPVAIRV 478

Cat   : 105 LDVNDNAPKFAAP 67
          LDVNDNAPKFAAP
Human: 479 LDVNDNAPKFAAP 491
```

Blast Score = 151 bits (378), Expect(2) = 1e-46
Identities = 71/73 (97%), Positives = 73/73 (99%)

```
Cat   : 70  PYEGFICESDQTKPLSNQPIVTI 2
          PYEGFICESDQTKPLSNQPIVTI
Human: 491 PYEGFICESDQTKPLSNQPIVTI 513
```

Blast Score = 52.7 bits (124), Expect(2) = 1e-46
Identities = 23/23 (100%), Positives = 23/23 (100%)

Figure 4.5 Alignment of the cat and the human cadherin amino acid sequences.

the rat brain for immunohistochemical localization of cadherin proteins (Tang et al. 1998, Benson et al. 1998). These experiments have shown this antibody to colocalize with synaptophysin and other synaptic markers. Furthermore, this antibody can block the induction of LTP in rat hippocampal slices by interfering with cadherin function (Tang et al. 1998).

4.3.3. Western Blot Analysis Of Cadherin Protein Expression In The Postnatally Developing Cat Visual Cortex

Figure 4.6 shows a western blot analysis performed in the postnatally developing cat visual cortex using the SIGMA pan-cadherin antibody. This blot consists of visual cortex protein preparations obtained from prenatal, 0 day postnatal, 15 day postnatal, 30 day postnatal, 60 day postnatal, 120 day postnatal and adult cats. In each age a single band of roughly 130kDaltons is detected confirming the fact that this antibody is able to recognize the cat cadherin proteins. Despite the use of a pan-cadherin antibody capable of recognizing multiple cadherin types, only a single band is observed in the western blot analysis shown in figure 4.6. This outcome is not too surprising because many cadherin proteins (especially the classic cadherin molecules) are 130 kDA in size (Tanihara et al. 1994). Since only a single band of the appropriate size is observed in this western blot, the SIGMA pan-cadherin antibody appears to specifically recognize the cadherin proteins in the cat. Although the ability of the SIGMA pan-cadherin antibody to recognize several different cadherin types is well established, this antibody clearly does not recognize every possible kind of cadherin molecule expressed in the cortex. Protocadherins which are larger than 130 kDa and other cadherins of variant sizes are not detected by this antibody. Hence, the Sigma antibody is likely recognizing a subset of the cadherin molecules present in the visual cortex. However, because western blot analysis using this antibody yields only a single band, the possibility that this cadherin antibody is detecting only one cadherin protein can not be ruled out (Based on the reported expression of cadherin types in the brain and the PCR results mentioned in the cloning experiment it is unlikely that there is

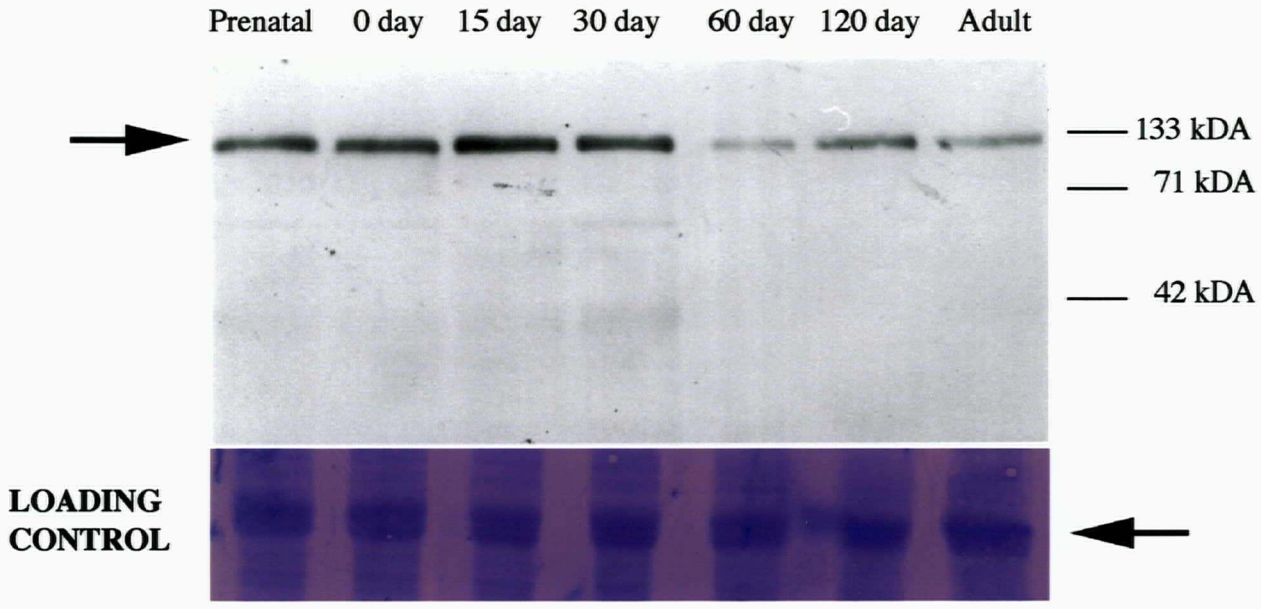


Figure 4.6 Cadherin protein expression in the postnatally developing cat visual cortex. The top panel shows the western blot analysis performed on the postnataly developing cat visual cortex using the SIGMA pan-cadherin antibody. This antibody specifically recognizes a single band of around 130 kDA in visual cortex protein preparations obtained from kittens of various different ages. The expression of these proteins is high in the young visual cortex but it declines sharply around 60 days of age. This expression pattern for the cadherin proteins is consistent with the timing of the critical period. For the loading control (the bottom panel), the membrane used for the western blot analysis was stained with Coomasie blue.

only one type of cadherin molecule expressed in the postnatal cat visual cortex). In addition, the pan-cadherin antibody may be biased in its ability to recognize certain types of cadherin proteins.

Since equal amounts of protein was loaded into each well (100 μg), the western blot analysis shown in figure 4.6 can be used to compare cadherin protein expression levels in the postnatally developing cat visual cortex. This expression pattern for the cadherin proteins in the developing visual cortex was confirmed by repeating the western blot analysis thrice on three different protein blots. Clearly, cadherin protein expression in the developing visual cortex parallels the critical period. A high level of cadherin protein expression is observed in the visual cortex of young kittens. This expression decreases around 60 days of age and cadherin protein levels remain reduced in the adult visual cortex.

4.3.4. Western Blot Analysis of the Cadherin Protein Expression in the Postnatally Developing Cat LGN

Figure 4.7 shows western blot analysis performed in the postnatally developing cat lateral geniculate nucleus using the SIGMA pan-cadherin antibody. This blot consists of LGN protein preparations obtained from postnatal 0 day, 15 day, 30 day, 60 day, 120 day and adult cats. As in the visual cortex, each lane in the western blot contains a single band confirming the specificity of the cadherin antibody in the cat LGN. Also, the western blot analysis of the cadherin proteins in the LGN was repeated three times to ensure that the observed expression pattern is not artifactual. The expression pattern of the cadherin proteins in the LGN is different than in the visual cortex but this expression profile is consistent with the critical period since a large decrease in cadherin protein expression occurs in the geniculate of adult cats.

4.3.5. Immunohistochemical Localization of Cadherin Proteins in the Postnatal Visual Cortex

The SIGMA pan-cadherin antibody characterized by western blot analysis in the last two sections was used to localize cadherin protein expression in the postnatally developing

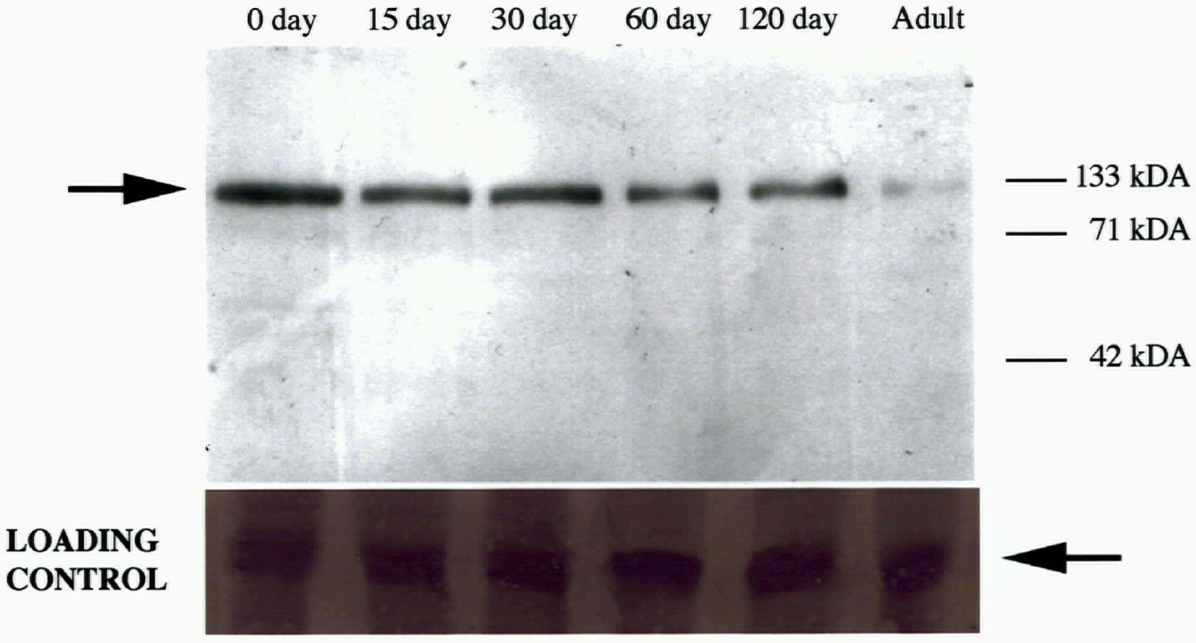


Figure 4.7 Cadherin protein expression in the postnatally developing cat LGN. The top panel shows the western blot analysis performed on the developing cat LGN using the SIGMA pan-cadherin antibody. This antibody specifically recognizes a single band of around 130 kDA in LGN protein preparations obtained from postnatal kittens of various different ages. The expression of this protein is high in the geniculate of young animals but it declines sharply in the adult LGN. For the loading control (the bottom panel), the membrane used for the western blot analysis was stained with Coomasie blue.

cat visual cortex. Figure 4.8 shows a 60 day old visual cortex tissue section from a cat subjected to immunohistochemical analysis with the pan-cadherin antibody. Since immunohistochemical analysis was performed using a pan-cadherin antibody, the observed staining pattern likely represents the cumulative expression pattern of several different types of cadherin molecules in the visual cortex. Similar to the observed expression of the beta-catenin protein discussed in the previous chapter, the laminar distribution of the cadherin protein in the developing visual cortex does not change with age. Postnatal ages examined include 0 day, 20 days, 30days, 45 days, 55 days, 60 days, 120 days and adults. Interestingly, this antibody strongly labels layer IV of the visual cortex, which is the thalamic input layer. Additionally, the pan-cadherin antibody staining is much more intense in visual areas of the cortex. Heavy pan-cadherin antibody staining is observed in layer IV of cortical area 17 and 18 (primary and secondary visual areas in the cat). This staining pattern in the visual cortex is consistent with the restricted expression patterns reported for the cadherin proteins in other brain areas. Cadherin staining is also visible in the higher layers of the cortex (2/3).

Similar to beta-catenin and synaptophysin, cadherin staining is observed in the neuropil of the lateral geniculate and visual cortex (shown in Figure 4.9). Neutral Red staining clearly illustrates cadherin labeling to be outside of cell bodies. Unlike beta-catenin, cadherin proteins do not have transcriptional activity and they do not translocate into the nuclei of cells. Accordingly, cadherin protein expression is restricted to the neuropil, as seen in Figure 4.8, throughout development in both the cortex and the LGN. This distribution of the cadherin proteins is consistent with their adhesive functions.

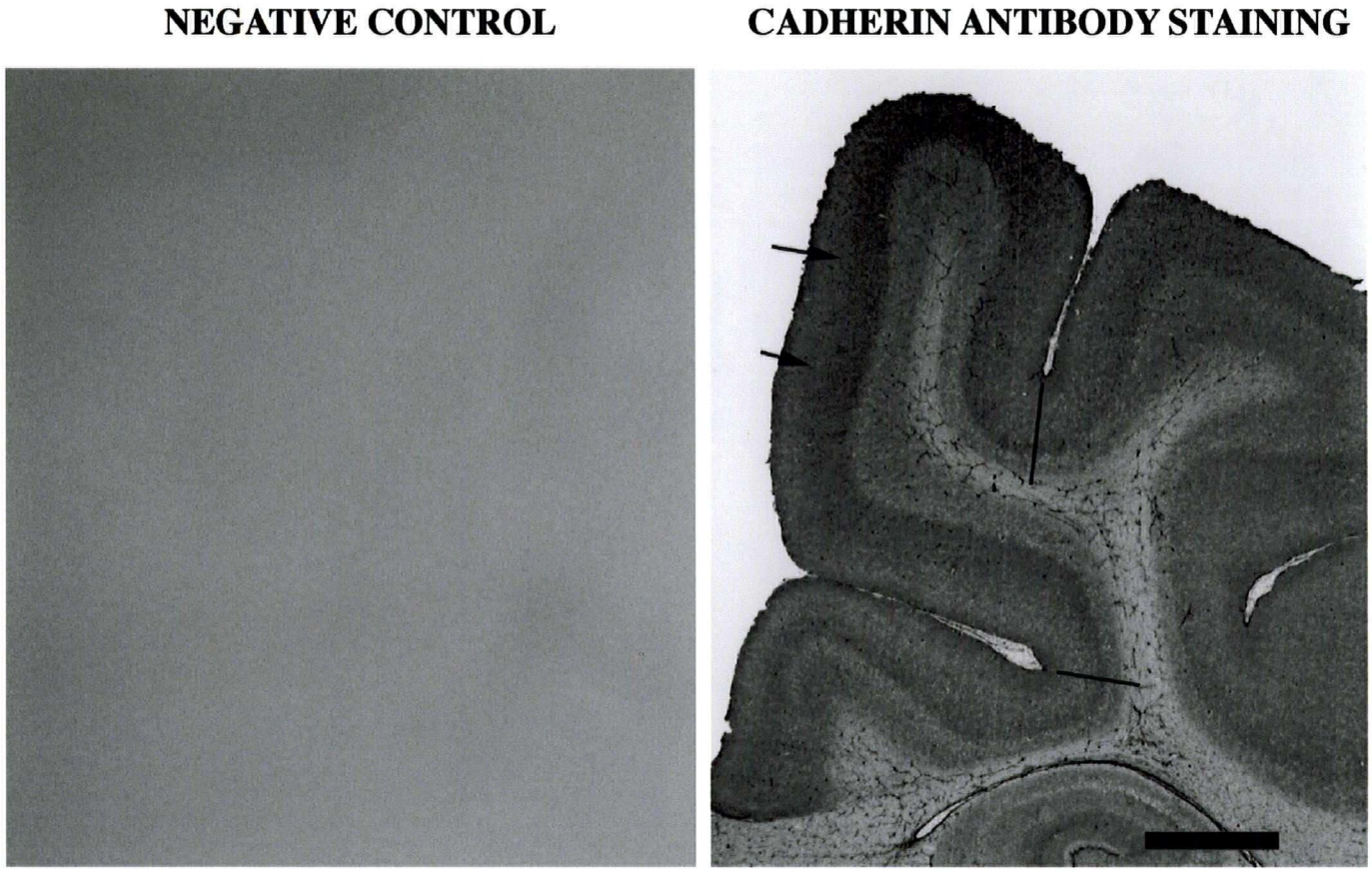


Figure 4.8 Pan-Cadherin Antibody Staining in the Postnatal Cat Visual Cortex. The pan-cadherin antibody strongly labels layer IV of the visual cortex (arrows). Weaker labeling is also seen in all other layers. In addition, the pan-cadherin antibody stains area 17 and 18 of the visual cortex more intensely than other cortical areas (the two black lines in the figure demarcate the combined borders of area 17 and 18 of the visual cortex). The laminar staining pattern of the pan-cadherin antibody does not change with age and is not developmentally regulated. The negative control tissue section shown in the left panel was not incubated with the primary antibody but it was subjected to all other steps of the immunohistochemistry procedure. (The contrast and brightness of the negative control figure was adjusted (enhanced) in order to show the absence of any artifactual staining. This is the reason for the darker background colour seen in the left panel.) The scale bar in this figure represents 2 mm's.

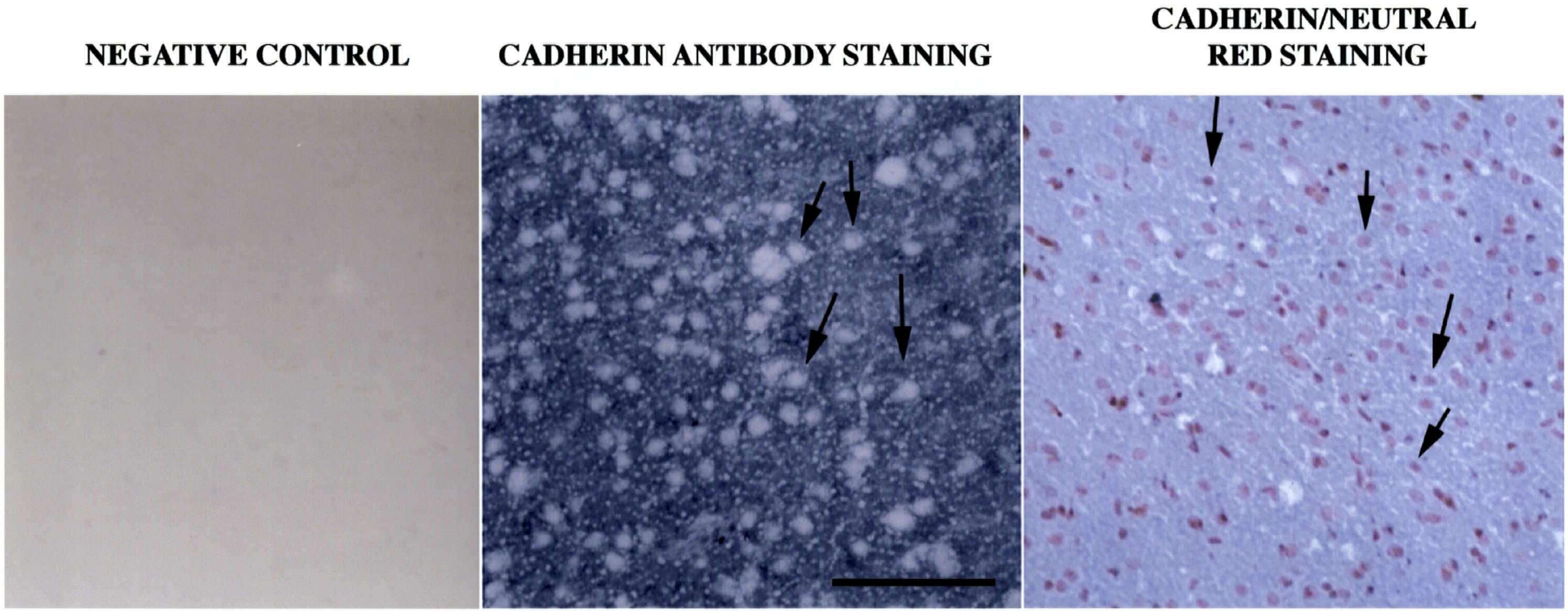


Figure 4.9 Cellular Location of the Cadherin Proteins in the Postnatal Cat Visual Cortex. The middle panel is a photograph of a visual cortex tissue section, which has been stained with the pan-cadherin antibody, taken at high magnification. The arrows in this panel point to the white, unstained, circular cell bodies. This staining pattern of the cadherin antibody remains constant throughout development and the cadherin proteins are restricted to the neuropil in the postnatal cat visual cortex. The right panel shows a pan-cadherin antibody stained visual cortex tissue section which has been counterstained with neutral red. The arrows point to the neutral red stained nuclei and cell bodies and the blue staining is pan-cadherin antibody labeling. (The pan-cadherin antibody labeling in the counterstained tissue sections is much weaker because the neutral red stain has a tendency to wash out the antibody stain). The neuropil localization of the cadherin protein is consistent with its putative role in cell adhesion. The leftmost panel is the negative control. This tissue section was not incubated with the primary antibody but it was subjected to all other steps of the immunohistochemistry procedure (In order to get rid of background staining endogenous avidin/biotin sites were blocked - described in the methods section). The scale bar represents 0.08 mm's.

4.4 DISCUSSION

The findings in this chapter support the involvement of the cadherin molecules in the postnatal development of the cat visual cortex. Firstly, expression of these proteins in the visual cortex closely correlates with the critical period. Cadherin molecule expression in the cortex decreases substantially at 60 days of age and remains low in adulthood. Secondly, cadherin proteins detected by the pan-cadherin antibody clearly label layer IV of the cortex. Layer IV is the input layer of the cortex and neurons in this layer form connections with the afferents from the geniculate. A molecule that is expressed in layer IV of the cortex and whose expression correlates with the critical period is likely to be involved in the reorganization of thalamocortical connections. The case for the involvement of cadherin molecules during visual development is further bolstered by the well characterized properties of these molecules in other areas of the brain. The synaptically localized cadherin proteins (Uchida et al. 1996) act as cell adhesion molecules and are believed to take part in such processes as synaptogenesis, target recognition, and axon migration (Arndt et al. 1996, Benson et al. 1998, Huntley et al. 1999, Riehl et al. 1996, Matsunaga et al. 1988, Obst-Pernberg et al. 1999, Redies et al. 1996, Rubenstein et al. 1999, Suzuki et al. 1997). Unfortunately, the results of the activity dependent studies carried out in Chapter 5 do not support the involvement of these molecules in activity dependent modifications occurring in the visual cortex.

The age dependent decrease in cadherin protein expression in the developing visual system supports the proposed role of nuclear beta-catenin in downregulating the expression of cell adhesion molecules during the critical period. The downregulation of cadherin protein expression detected in the LGN of older kittens may be partially mediated by the transcription factor activity of the nuclear beta-catenin protein in geniculate neurons. In addition to its downregulation in the LGN, cadherin protein expression in the visual cortex decreases with age. LGN processes projecting into the cortex may be partially responsible for this observed decrease since cadherin molecules are located on both presynaptic and

postsynaptic terminals (the cadherin proteins residing in the presynaptic portion of the thalamocortical connections are synthesized by geniculate neurons). Unfortunately, presynaptic thalamic terminals can not be separated from cortical tissue and the proteins present in these terminals had to be included in the visual cortex protein preparations used for Western Blot analysis. Since most of the cadherin protein expression is in layer IV of the visual cortex (the input layer of the LGN), the cadherins proteins located in thalamic terminals are likely to make a significant contribution to the overall cadherin protein levels in visual cortex protein preparations. In addition, the timing of beta-catenin nuclearization in the LGN and the decrease in cadherin expression in the visual cortex occur almost simultaneously. This finding further supports the proposed role of nuclear beta-catenin in downregulating the expression of cell adhesion molecules in LGN neurons. Additionally, the nuclearization of beta-catenin is reported to downregulate the expression of only certain types of cadherin molecules (Stewart et al. 2000, Nuruki et al. 1998). Since the pan-cadherin antibody used for monitoring cadherin levels may also detect cadherin proteins which are not downregulated by beta-catenin activity, the observed decrease in cadherin protein expression in the LGN and the visual cortex may not be reflective of the regulatory action imposed by nuclear beta catenin. In other words, the expression of specific types of cadherin proteins in the visual cortex and the LGN may be downregulated in a much more drastic manner during the critical period. As an aside, the regulatory effects of the beta-catenin protein in cancerous cells is not limited to the cadherins but it also encompasses other cell adhesion molecules. Hence, the expression of cell adhesion molecules other than the cadherins may be downregulated in LGN neurons by nuclear beta-catenin. However, beta-catenin is probably not the only factor responsible for the downregulation of cadherin protein expression in the LGN because beta-catenin nuclearization is only observed in a subset of neurons in the LGN. The downregulation of cadherin protein expression in a small number of cells is unlikely to cause a decrease in the overall levels of cadherin proteins in the cortex or the LGN. Regardless, there is a decrease in cell adhesion molecule

levels during the critical period in both the cortex and the LGN. One of the processes responsible for this downregulation is proposed to be the nuclear activity of beta-catenin in LGN neurons.

The presence of cadherin proteins which are expressed mainly in layer IV of the visual cortex suggests these molecules to function as target recognition cues for axons originating from geniculate neurons and other cortical areas. Since various cadherin proteins are expressed in specific domains and demarcate specific brain areas, the cadherin proteins are believed to facilitate the proper wiring of neurons (Suzuki et al. 1997, Inoue et al. 1997, Inoue et al. 1998, Kimura et al. 1996, Matsunami et al. 1995, Redies et al. 1996, Redies 2000). Because cadherin molecules bind in a homophilic manner (Nose et al. 1988, Nose et al. 1990, Miyatani et al. 1989, Leckband et al. 2000) the thalamocortical axons arising in the geniculate must express the same types of cadherin molecules as the cortical cells in layer IV. By restricting the expression of a subset of cadherin molecules to layer IV and LGN neurons, axons originating from the geniculate may be guided to make connections in layer IV of the visual cortex. According to this model, since other layers of the cortex do not express cadherin proteins which are identical to those located on thalamic axons, geniculate neurons have no choice but to make connections with layer IV cortical cells. Similarly, a sub population of neurons in the cortex expressing a particular type of cadherin protein may be restricted to forming synaptic connections with neurons in the visual cortex containing the same cadherin molecules. Hence, cadherin molecules may be carrying out an organizational function in the visual cortex and facilitating the formation of proper connections between visual areas of the cortex and the thalamus. The involvement of N-cadherin in the organization of thalamocortical synapses during barrel cortex development supports this conjecture (Huntley et al. 1999).

The restricted expression domains of cadherin cell adhesion molecules provide indirect support for the involvement of homeotic genes in postnatal visual development. Cadherin protein expression patterns characterized in non-visual areas of the brain, which

are both lamina and brain region specific, are very reminiscent of the reported distribution pattern of the homeobox genes. As discussed in the introduction, homeotic and segment polarity genes are expressed in specific domains and give rise to various patterns such as columns and stripes (Wilkinson et al. 1989a, Wilkinson et al. 1989b, Murphy et al. 1989, Bell et al. 1999). The similarity between cadherin molecule and homeotic gene expression is not coincidental. Homeotic and homeobox genes regulate the expression of various cell adhesion molecules (Valarache et al. 1993, Tissier-Seta et al. 1993, Edelman et al. 1995, Edelman et al. 1998, Cillo et al. 1996, Wang et al. 1996, Tosney et al. 1995) including the cadherins (Lincecum et al. 1998, Packer et al. 1997). By differentially regulating the activity of cell adhesion molecules, homeotic genes are able to subdivide and cause differentiation amongst a group of similar cells. For example, during early brain development, the neural tube gets divided into compartments known as rhombomeres. Each rhombomere expresses a distinct combination of homeobox genes that activates the expression of a unique set of cell adhesion molecules (Wilkinson et al. 1989a, Wilkinson et al. 1989b, Murphy et al. 1989, Bell et al. 1999). The restriction of cell to cell interactions and cellular migration to individual rhombomeres causes the rhombomeres to diverge from each other and leads to the formation of different nervous system structures (Fraser et al. 1990). Similarly, the differential expression of *LIM* homeobox genes in the spinal cord causes each level of the spinal cord to express a distinct set of cell adhesion molecules (Lumsden 1995, Tosney et al. 1995). The differential expression of adhesion molecules by spinal cord neurons enables the spinal cord to make connections with different peripheral neurons. In other words, differential adhesion allows the spinal cord to become properly wired with the appropriate peripheral neurons. In the last paragraph, this sort of an arrangement was proposed to exist between the visual cortex and lateral geniculate afferents. In addition, if the homeobox genes regulate the expression of cadherin molecules in the developing cat visual cortex then their distribution in this structure may be similar to the distribution of the cadherin proteins. The data in chapter two specifically

show the expression of various developmental genes to decrease, including the cofactor *PBX* family of homeobox genes, in the adult visual cortex. Hence, the expression of the *PBX* homeotic genes and the cadherin molecules parallel each other during visual development. Concievably, the downregulation of homeotic gene expression in older kittens may cause cell adhesion molecule expression to decrease (the data presented in this chapter and Schoop et al. 1997) in the adult visual cortex.

Chapter 5

Activity Dependent Expression Of Beta-Catenin And The Cadherin Proteins

5.0 SUMMARY

In order to determine the effect of visual activity on beta-catenin and cadherin protein expression, immunohistochemical analysis was performed on animals subjected to various visual deprivation paradigms (monocular eyelid suture, enuclation, strabismus followed by monocular eyelid suture or enucleation and optic tract lesions). Beta-catenin and cadherin protein expression is not modified in the cat visual cortex by these visual deprivation paradigms. Nuclear beta-catenin protein expression also appears unaltered in the LGN of animals subjected to either monocular deprivation or optic tract lesions. However, following monocular deprivation, the beta-catenin and the cadherin proteins are expressed in higher amounts in layers of the geniculate corresponding to the deprived eye. This finding implies a role for the beta-catenin and the cadherin cell adhesion system in the competition occurring between X- and Y- cells in the postnatal LGN.

5.1 INTRODUCTION

As discussed in the first chapter, synaptic rearrangements in the visual system are largely dictated by activity (Wiesel et al. 1963, Hubel et al. 1977). Activity dependent synaptic changes contribute not only to the development of the visual system but to the formation of various other neural structures including the spinal cord and the somatosensory cortex (Webster et al. 1983, Meisami et al. 1981, Westrum et al. 1986, Wooseley et al. 1976, Garraghty et al. 1992, Pons et al. 1991). The molecular mechanisms contributing to synaptic modifications in the developing visual cortex are also

believed to be involved in learning, memory and synaptogenesis (Bienenstock et al. 1982, Bear et al. 1987). Essentially, activity dependent synapse formation, which is a recurring theme in neurodevelopment, is of paramount importance in the proper formation of neural circuits. Although the contribution of neural activity to synapse formation and maintenance is well established both in the visual system and elsewhere, the molecular events that facilitate these processes remain elusive.

Any molecule involved in the synaptic reorganization of the cat visual system is likely to be regulated in an activity dependent manner. Obviously, molecules associated with synaptic modification need to be present and functioning during the actual modification process. Since interfering with normal visual activity can induce synaptic rearrangements in the postnatally developing visual system (Shatz et al. 1978, Antonini et al. 1993, Cynader et al. 1980, Cynader et al. 1982, Olson et al. 1975, Wiesel et al. 1963) the expression of molecules involved in synaptic rearrangements should be altered in response to these deprivation paradigms. Examples of molecules regulated by visual activity include the neurotrophins (Castren et al. 1992, Schoups et al. 1995, Torasdotter et al. 1996), immediate early genes (Kaplan et al. 1996, Mower 1994) and the cytochrome oxidase (Wong-Riley 1979) proteins. Since molecules involved in visual plasticity are likely to be regulated in an activity dependent manner, the expression of the beta-catenin and the cadherin proteins was examined in the visual systems of kittens subjected to various deprivation paradigms.

The function and the expression of both the cadherin and beta-catenin molecules are influenced by activity in various regions of the CNS. For example, N-cadherin is expressed in an activity dependent manner during the formation of "barrels" in the developing somatosensory cortex (Huntley et al. 1999). Given the similarity between barrel cortex and visual cortex development, it is not unreasonable to assume that cadherin expression will be regulated in an activity dependent manner in the visual cortex. Tissue culture experiments have also shown beta-catenin protein distribution to be altered by

neural activity (Murase et al. 1999). Additionally, the cadherins dimerize and become more resistant to protease digestion in response to neural activity (Tanaka et al. 2000). Furthermore, the ability to inhibit LTP induction by preventing cadherin molecule dimerization strongly suggests the cadherins to be an integral part of activity dependent plasticity (Tang et al. 1998).

HYPOTHESIS: Beta-catenin and the cadherin cell adhesion protein expression is regulated in an activity dependent manner in the postnatal cat visual system.

OBJECTIVES: 1.) To determine if beta-catenin or cadherin protein expression can be altered by the monocular deprivation paradigm.

2.) To determine if beta-catenin or cadherin protein expression can be maintained at high levels following the optic tract lesion paradigm which prolongs the critical period.

3.) To determine if the distribution of nuclear beta-catenin can be altered by monocular deprivation or optic tract lesion paradigms.

5.2 METHODS

Animals used

For monocular deprivation studies 26 kittens were used. 40 day old postnatal kittens were monocularly deprived for 3 days N=4, for 7 days N=3, for 2 weeks N=2 or 5 weeks N=2. Alternatively 4 kittens of the same age were monocularly enucleated for 2 weeks N=2 or 5 week N=2. 6 postnatal kittens were made strabismic around 20 days of age and then monocularly deprived for 1 week at 40 days of age N=2, for 2 weeks N=2 or 5 weeks N=2. 2 postnatal kittens which were also strabismic were enucleated at 40 days of age and 1 was allowed to survive for 2 weeks whereas the other was allowed to survive

for 5 weeks. 2 animals made strabismic at postnatal day 15 and monocularly deprived for 5 days at age 55 days were also used. 1 cat made strabismic at postnatal day 15 and monocularly deprived for 5 days at 155 days was also examined.

For optic tract lesion experiments 5 postnatal kittens were used. These animals had their optic tracts lesioned around 20 days of age and allowed to survive until 90 days of age N=2 or 120 days of age N=3.

Immunoprecipitation

300-400mg of brain tissue was placed into ice cold solubilization solution containing 50mM Tris-Cl (pH7.7), 0.5% NP-40, 100mM NaCl, 2.5mM EDTA, 10mM NaF, 0.2mM sodium orthovanadate, 1mM sodium molybdate, 40μg/ml PMSF, 1μM pepstatin, 0.5μg/ml leupeptin, 1μg/ml aprotinin. Note that all the solutions and the tubes used for immunoprecipitation were cooled on ice before use and all the microcentrifugation was performed at 4°C. Additionally, the protease inhibitors PMSF, pepstatin, leupeptin and aprotinin were added from stock solutions immediately prior to use and fresh solubilization buffer was made each time. The brain tissue in the solubilization buffer was teased apart with forceps and the solution was allowed to sit on ice for 30 minutes. Every ten minutes the solution was pipetted to improve solublization of the tissue. After the thirty minutes, the solution was centrifuged at 15000 x g for 1 minute and the supernatant was transferred to a new tube (pre-cooled) on ice. Note that at times this preparation was found to be quite cloudy after only 1 spin and if the immunoprecipitation procedure was carried out using a cloudy solution, precipitates would form during the antibody incubation step. Successful immunoprecipitation could not be attained (as determined by western blot analysis) when precipitates were present during the antibody incubation step. The cloudiness is believed to be caused by myelin since the supernatants had a whiteish color. Spinning an additional 2 to 3 times for 1 minute at 15000 x g and aspirating the supernatant into a new tube each time resolved this problem. The protein sample was quantitated and

the appropriate volume of the sample containing 1mg of total protein was placed into a fresh tube (300-400mg of brain tissue roughly yields 3-5mg of total protein which is enough to carry out several immunoprecipitation reactions concurrently). The total volume of the protein solution was increased to 500µl's with solubilization buffer. 60µl's of this solution was set aside and labeled as the Pre- fraction. 8µg's of antibody was added to the protein solution and the antibody incubation was allowed to proceed overnight at 4°C on a rotator. Fresh solubilization solution was prepared the following day because protease inhibitors do not remain active overnight. 50µl's of protein G beads were spun down in a new eppendorf tube at 10000 x g in 4°C for 1 minute. 25µl's of solubilization buffer was added to the beads and the beads were spun down as before. This wash step was repeated 3 times. After the final wash, 25µl's of solubilization buffer was added and the protein G bead slurry was added to the protein sample which had been incubating overnight. The antibody/protein complex was incubated with the protein G beads for 1 hour at 4°C on a rotator. After this incubation, the protein/antibody complex is attached to the protein G beads and not in solution. The beads were pelleted by centrifuging in 4°C for 1 minute at 10000 x g. 60µl's of the supernatant was set aside as the Post- fraction while the rest of the supernatant was discarded. The pelleted protein G beads were washed 5 times with 1ml of solubilization solution followed by 2 washes with a 100mM NaCl solution to remove any proteins non-specifically bound to the beads. After the washes the beads were resuspended in 25ul's of loading buffer, boiled for 5 minutes and electrophoresed.

5.3 RESULTS

5.3.1 Effects Of Monocular Deprivation on Beta-Catenin Protein Expression In The Postnatal Visual Cortex And The LGN

In this set of experiments, 5-6 week old postnatal kittens had one of their eyes sutured shut or enucleated. These kittens were allowed to survive for 3 days, 7 days, 2 weeks or 5 weeks before being sacrificed. Figure 5.1 is a picture of a visual cortex from a kitten deprived for 5 weeks stained with the beta-catenin antibody. Layer IV staining in the cortex is continuous and beta-catenin expression is not altered in response to visual deprivation. Hence, monocular deprivation does not appear to cause a detectable change in beta-catenin protein levels in the developing visual cortex. Changes in beta-catenin staining are also not observed in the cortex of animals deprived for shorter lengths of time.

However, a clear change in the distribution of the beta-catenin protein in the LGN is observed in response to monocular deprivation. Figure 5.2 shows beta-catenin staining performed in the lateral geniculate of a postnatal kitten subjected to 5 weeks of monocular deprivation beginning at 5 weeks of age. Interestingly and unexpectedly, the layers of the LGN exhibiting darker beta-catenin neuropil staining correspond to the closed eye. This staining pattern was observed consistently in all animals which were deprived of visual activity for five weeks (normal animals monocularly deprived (n=2), strabismic animals monocularly deprived (n=2) and enucleated animals (n=2)). Beta-catenin expression in the LGN can be altered with 2 weeks of deprivation but this effect is relatively weak compared to the effect obtained with longer deprivation periods (normal animals monocularly deprived (n=2), strabismic animals monocularly deprived (n=2)). Changes in beta-catenin protein expression in the LGN are not observed in animals subjected to monocular deprivation or enucliation periods shorter than two weeks.

Finally, Figure 5.3 shows nuclear beta-catenin distribution in the LGN of a postnatal kitten monocularly deprived for 5 weeks. Monocular deprivation or enculeation does not appear to effect the nuclearization of the beta-catenin protein since the distribution

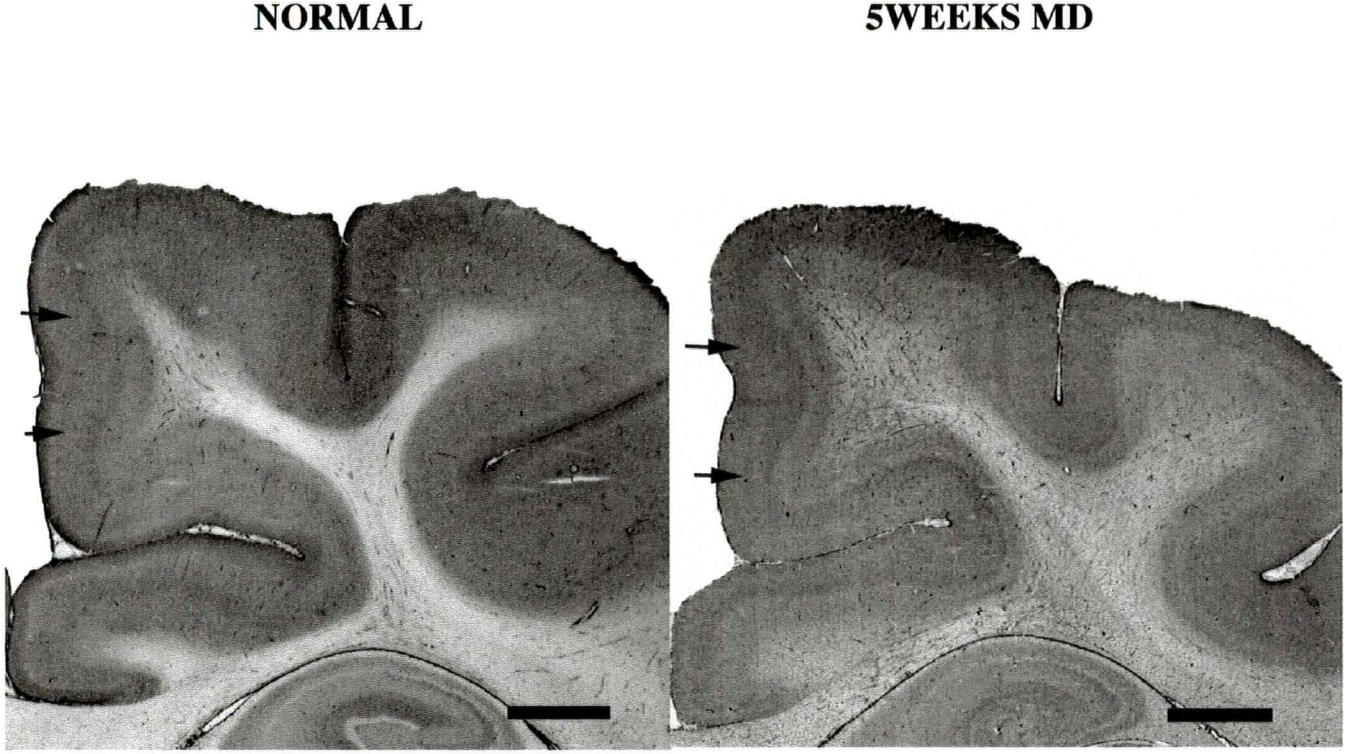


Figure 5.1: Beta-Catenin Protein Expression In the Visual Cortex of Normal and Monocularly Deprived Postnatal Kittens. The panel on the left shows a visual cortex from a normally developing postnatal kitten whereas the right panel shows a visual cortex from a kitten which has been monocularly deprived for 5 weeks. Both tissue sections have been subjected to immunohistochemical staining using the beta-catenin antibody obtained from Transduction Laboratories. The arrows in both figures point to layer IV which expresses the beta-catenin protein at a slightly higher level. The staining pattern for the beta-catenin protein does not appear to change following monocular deprivation. In both normal and deprived kittens the staining pattern for the beta-catenin protein is continuous in layer IV and the other layers. Alterations in beta-catenin protein expression associated with monocular deprivation (i.e. columnar staining patterns) are not seen in the deprived visual cortex. The scale bar in both figures represents 2mm's.

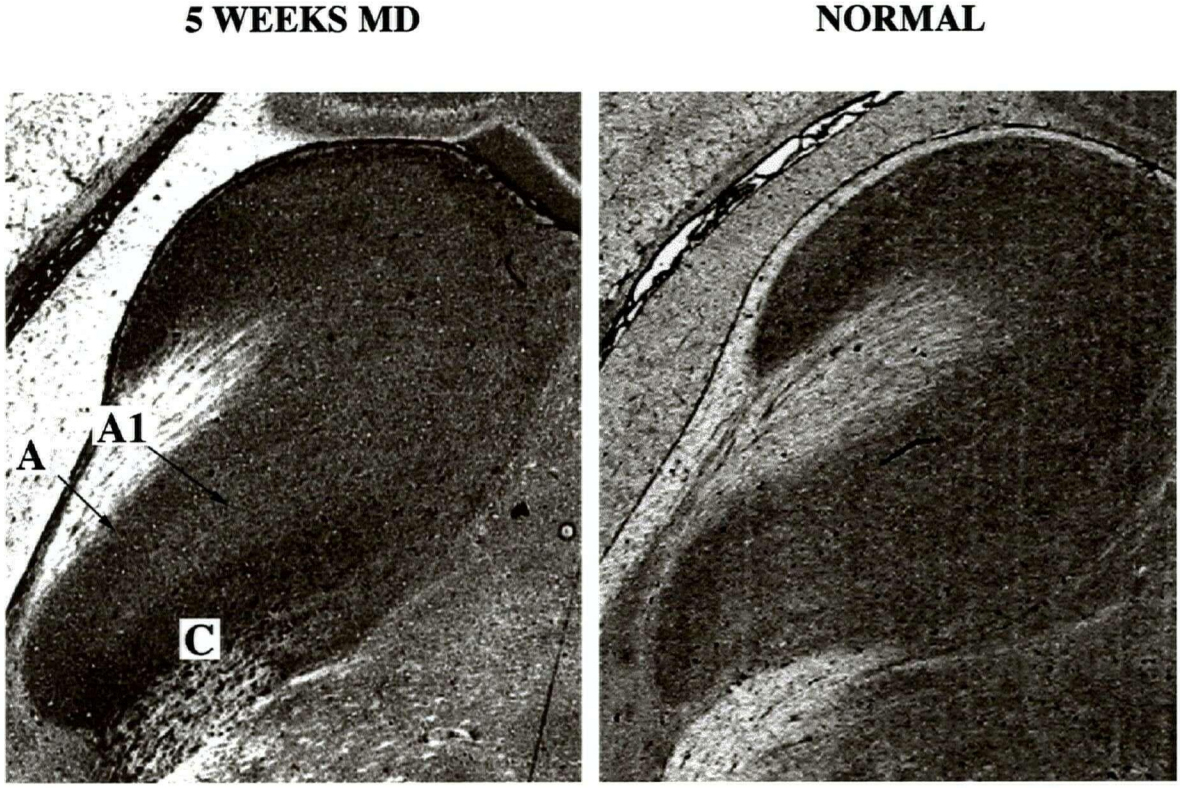


Figure 5.2 Beta-Catenin Antibody Staining in the LGN of Monocularly Deprived and Normal Postnatal Kittens. The left panel is a picture of an LGN from a kitten which has been monocularly deprived for 5 weeks whereas the right panel is an LGN from a normal kitten. The LGN shown in the monocularly deprived animal is the geniculate which is contralateral to the deprived eye. Layer A and Layer C in this LGN, which are innervated by the deprived eye, contain stronger beta-catenin antibody staining compared to the A1 layer of the LGN which is innervated by the non-deprived, ipsilateral eye. The scale bar for this figure represents 2mm's.

Figure 5.3 Nuclearization of the beta-catenin protein in the postnatal cat LGN following monocular deprivation. The picture shown in this figure is a cross section of a geniculate from a kitten which has been monocularly deprived for 5 weeks. Nuclear beta-catenin protein is found in all layers of the LGN and its widespread distribution is not distrupted by this deprivation paradigm. The deprived eye was contralateral to this LGN.

of nuclear beta-catenin in the LGN in normal and deprived animals is similar. Following monocular deprivation, cells containing nuclear beta-catenin are distributed through out the geniculate and they do not preferentially concentrate on either the deprived or non-deprived eye layers.

5.3.2 Effects of Monocular Deprivation on Cadherin Protein Expression In The Postnatal Visual Cortex And The LGN

Cadherin protein expression was also assessed by immunohistochemistry in postnatal kittens either monocularly deprived or enucleated at 5-6 weeks of age. These animals were sacrificed following 3 days, 7 days, 2 weeks or 5 weeks of deprivation. Similar to beta-catenin, monocular deprivation did not alter pan-cadherin antibody staining in the visual cortex of deprived animals (Figure 5.4). However, pan-cadherin protein expression is higher in layers of the LGN corresponding to the closed eye (figure 5.5). The similar expression patterns of the beta-catenin and the cadherin proteins following monocular deprivation is consistent with the proposal that these molecules attach together to function as a cell adhesion system.

5.3.3 Effect of Optic Tract Lesion on the Expression of Beta-Catenin and Cadherin Proteins

By inhibiting activity to the cortex, the optic tract lesion paradigm prolongs the critical period in the visual cortex (Singer 1982). An optic tract lesion is made after the optic chiasm and unlike an optic nerve lesion where the input from only one eye is silenced, the optic tract lesion paradigm disconnects all the inputs to one LGN and its corresponding cortical hemisphere (Figure 5.6). Since optic tract lesions prolong the critical period in the geniculate and the cortex, older kittens which have had their optic tracts lesioned at an early age are expected to express plasticity related proteins at higher levels. 17 Day old postnatal kittens were subjected to unilateral optic tract lesions and allowed to survive until 120 days of age. In this paradigm, the lesioned side of the brain retains a plastic LGN and cortex whereas the contralateral side of the visual system essentially develops normally and loses

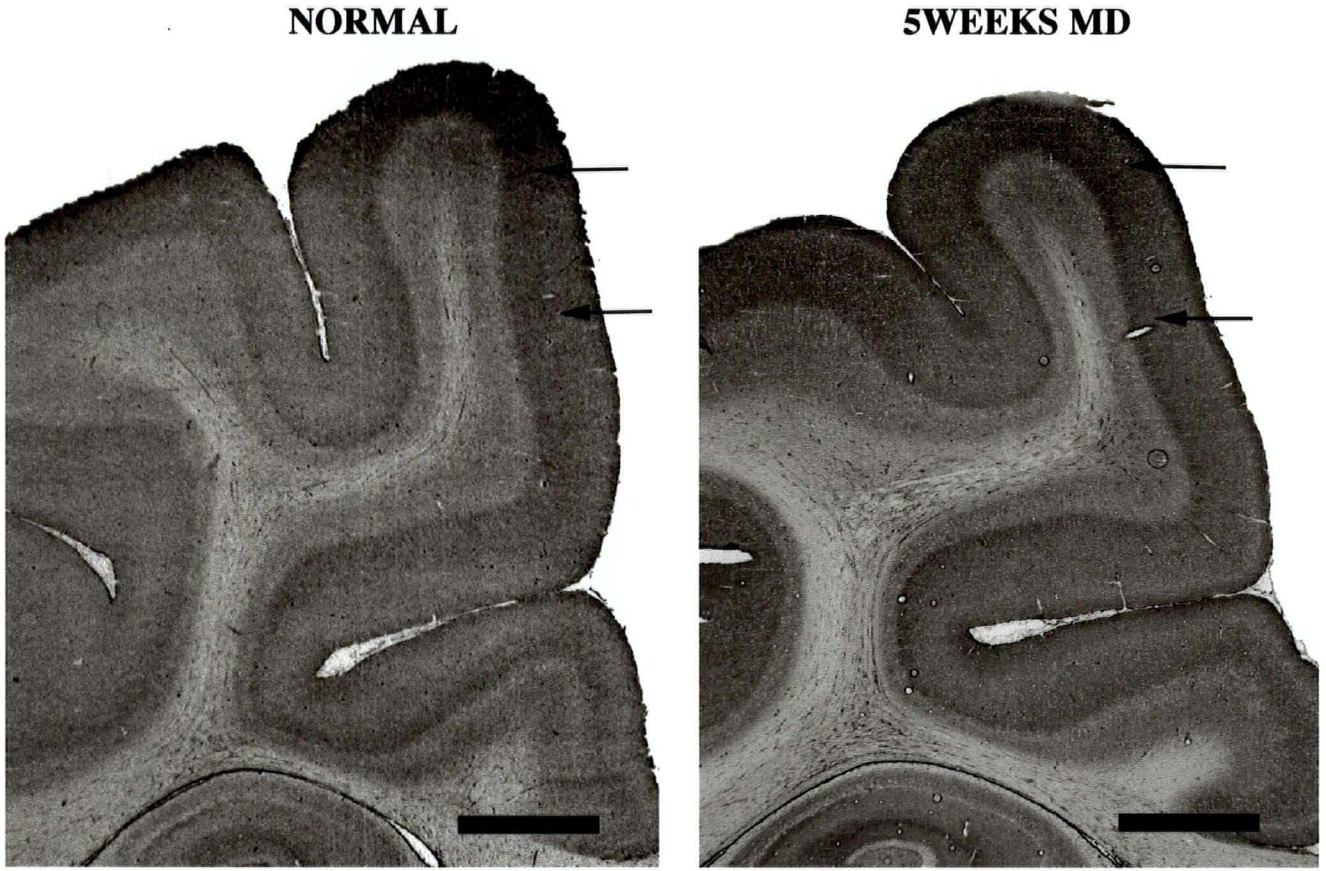


Figure 5.4 Pan-Cadherin Protein Expression In the Visual Cortex of Normal and Monocularly Deprived Postnatal Kittens. The panel on the left shows a visual cortex from a normally developing postnatal kitten whereas the panel on the right shows a visual cortex from a kitten which has been monocularly deprived for 5 weeks. Both tissue sections have been subjected to immunohistochemical staining using the pan-cadherin antibody obtained from SIGMA Laboratories. The arrows in both figures point to layer IV which expresses the pan-cadherin protein at a higher level. The staining pattern for the pan-cadherin protein does not appear to change following monocular deprivation. In both normal and deprived kittens the staining pattern for the pan-cadherin protein is continuous in layer IV and the other layers. Alterations in pan-cadherin protein expression associated with monocular deprivation (i.e. columnar staining patterns) are not seen in the deprived visual cortex. The scale bar in both figures represents 2mm's.

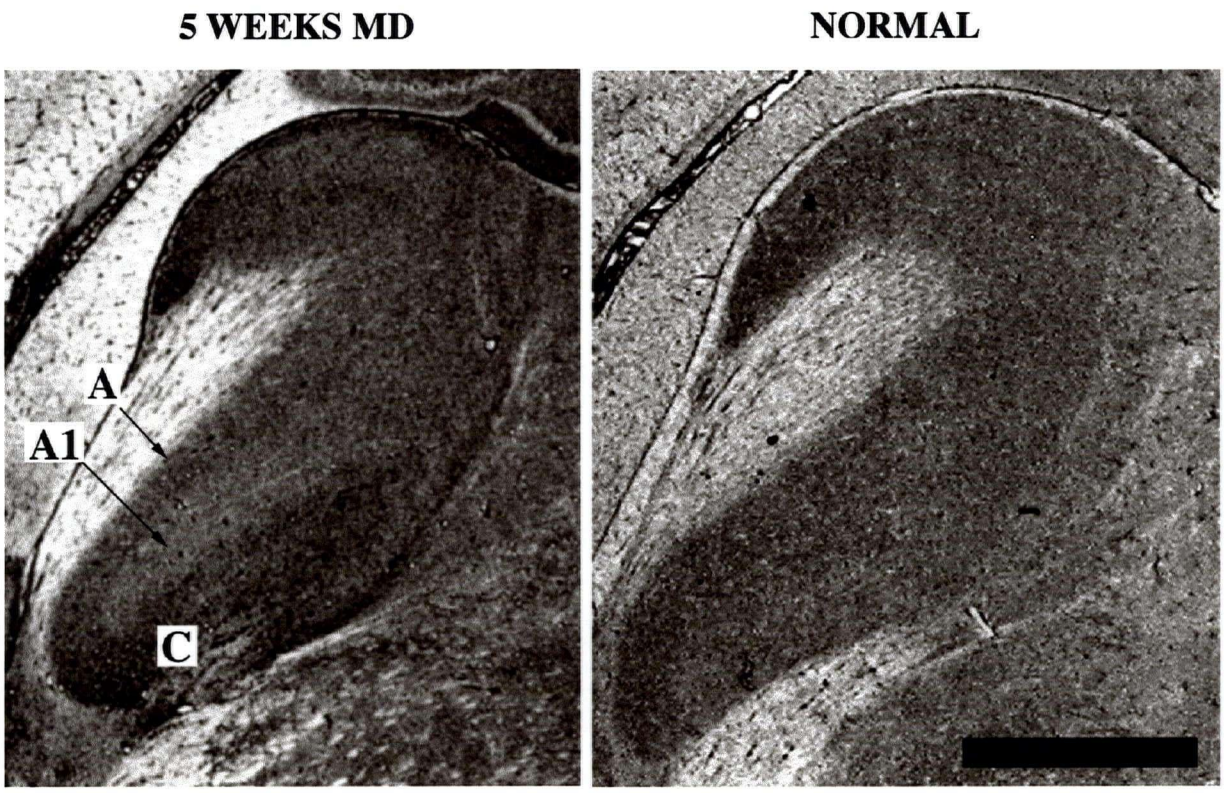


Figure 5.5 Pan-Cadherin Antibody Staining in the LGN of Monocularly Deprived and Normal Postnatal Kittens. The left panel is a picture of an LGN from a postnatal kitten which has been monocularly deprived for 5 weeks whereas the right panel is an LGN from a normal postnatal kitten. The LGN shown in the monocularly deprived animal is the geniculate which is contralateral to the deprived eye. Layer A and Layer C in this LGN, which are innervated by the deprived eye, contain stronger pan-cadherin antibody staining compared to the A1 layer of the LGN which is innervated by the non-deprived, ipsilateral eye. The scale bar for this figure represents 2mm's.

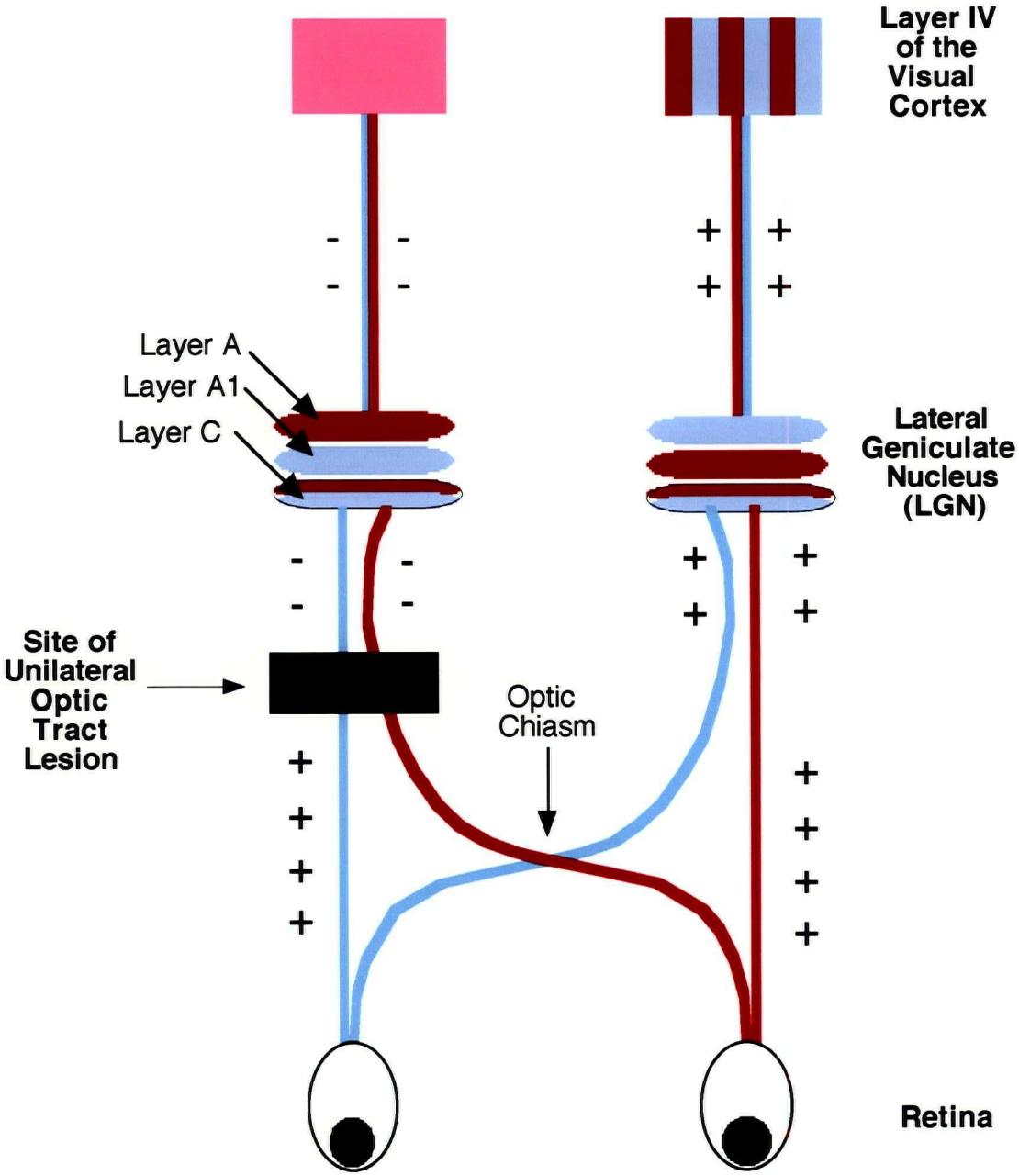


Figure 5.6 Unilateral Optic Tract Lesion. The red and the blue colored lines represent the neural inputs originating from the two eyes. The black rectangle represents the location of the lesion which is after the optic chiasm. This lesion prevents visual activity from reaching the LGN and the cortex. + and - signs represent the presence or the absence of visual activity.

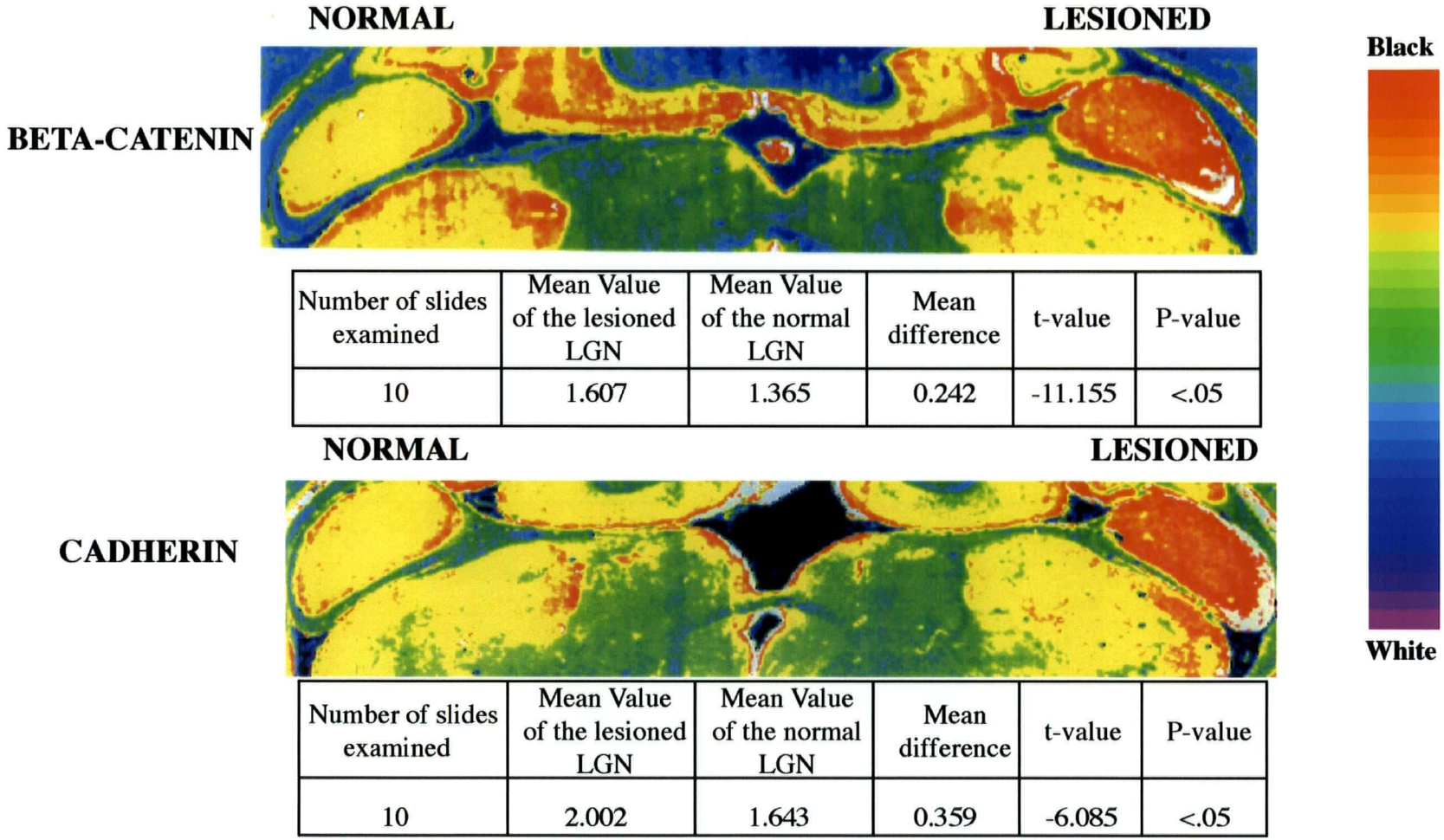
its plasticity. Hence, the unlesioned cortex and LGN can be used as a control to determine if immunohistochemical changes arise as a result of the lesions.

The expression of the beta-catenin and the cadherin proteins are unaltered in cortical structures following optic tract lesions. These findings are consistent with the reported failure of the monocular deprivation paradigm to alter the expression of the cadherin and beta-catenin proteins in the visual cortex. However, the LGN's ipsilateral to the optic tract lesions exhibit higher amounts of beta-catenin and cadherin protein expression compared to the unlesioned LGN's (figure 5.7). In this figure, brain tissue sections containing the LGN's were digitized and psudeocolor images were produced using the NIH Image computer program. The statistical analysis (shown in the tables) was performed by measuring the grayscale intensity of both the lesioned and the normal LGN on each tissue section (n=10 sections). The student t-test analysis on these sections verifies the presence of a staining difference between the normal and the lesioned LGN. These findings are also consistent with the monocular deprivation studies which show activity dependent changes in the expression of beta-catenin and cadherin proteins to occur only at the thalamic level.

In addition, nuclear beta-catenin distribution appears unaffected in animals subjected to optic tract lesions. As seen in figure 5.8 both the lesioned and the normal geniculates appear to exhibit the same number of beta-catenin positive cells. However, we can not conclude that the optic tract lesion or the monocular deprivation paradigms do not modify nuclearization of beta-catenin. The level of nuclear beta-catenin in individual cells or the cells containing nuclear beta-catenin may change in response to these paradigms.

5.3.4 Co-Immunoprecipitation of The Beta-Catenin And The Cadherin Proteins

The corresponding increase in both beta-catenin and cadherin protein expression in the LGN following the deprivation paradigms described above suggests beta-catenin and the cadherin proteins to extensively interact with each other in the developing LGN. The binding of the beta-catenin protein to the cadherin molecules is well established and is



Number of slides examined	Mean Value of the lesioned LGN	Mean Value of the normal LGN	Mean difference	t-value	P-value
10	1.607	1.365	0.242	-11.155	<.05

Number of slides examined	Mean Value of the lesioned LGN	Mean Value of the normal LGN	Mean difference	t-value	P-value
10	2.002	1.643	0.359	-6.085	<.05

Figure 5.7 Neuropil expression of the beta-catenin and the cadherin proteins in the postnatal cat LGN following unilateral optic tract lesions. The pictures in this figure show beta-catenin and pan-cadherin antibody staining in the LGN of 120 day old postnatal cats which have had their optic tracts unilateraly lesioned at an early age. Pictures of the stained tissue sections were taken with a digital camera and the false color images were generated by using the NIH Image program. The red color in the lesioned geniculates indicate that the beta-catenin and the cadherin proteins are more highly expressed in these LGN.

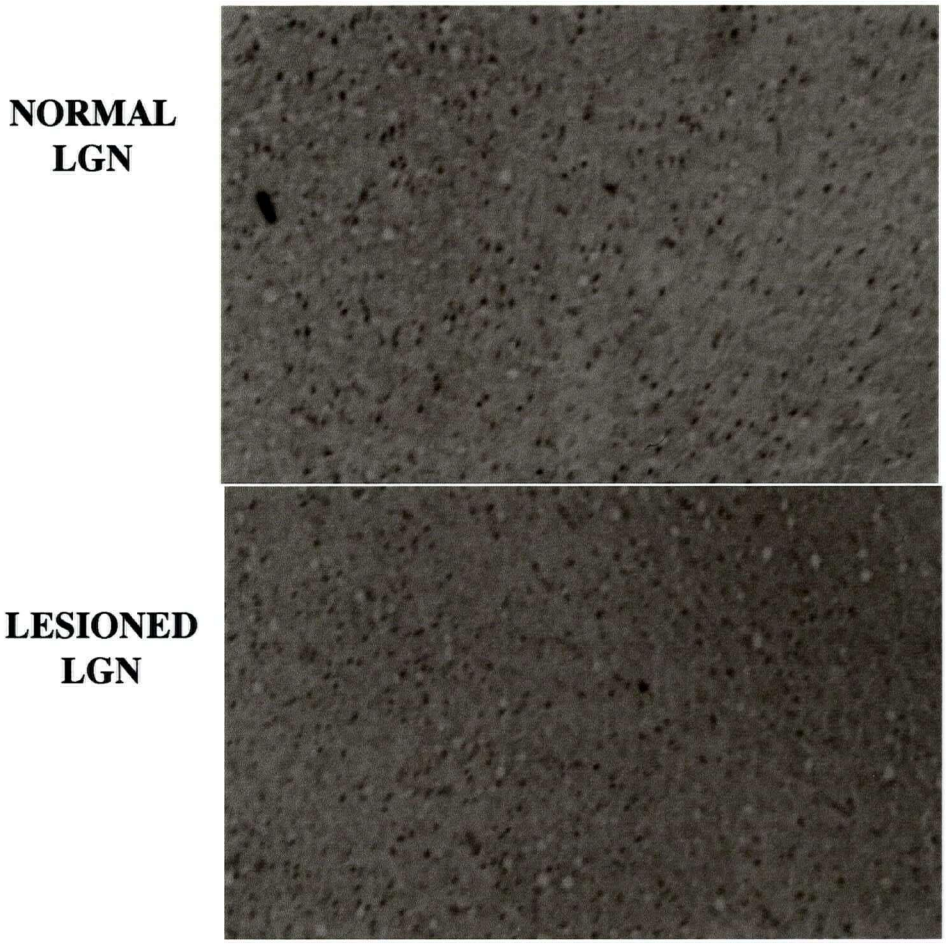


Figure 5.8 Nuclearization of the beta-catenin protein in the postnatal cat LGN following optic tract lesions. The two LGN's shown in this figure are from the same 120 day old postnatal animal. The LGN shown in the top panel was allowed to develop normally whereas the LGN in the bottom panel had its optic tract lesioned at an early age. Nuclear beta-catenin protein is found in both LGN's and the staining pattern in the two pictures seem to be similar. Hence, unilateral optic tract lesion paradigm doesn't appear to influence the nuclearization of the beta-catenin protein.

required for proper cell adhesion (Kitner 1992, Nagafuchi et al. 1988, McCrea et al. 1991). However, cadherin molecules can interact with catenins other than beta-catenin such as plakoglobin (McCrea et al. 1991). Since the exact type of cadherin proteins which are being detected by the pan-cadherin antibody in the LGN are unknown, the interaction between the beta-catenin protein and the cadherin molecules detected by the pan-cadherin antibody needs to be verified.

In order to demonstrate the interaction between these two molecules, beta-catenin and cadherin proteins were co-immunoprecipitated. In the top panel of figure 5.9, the beta-catenin protein was immunoprecipitated and the proteins obtained by this procedure were electrophoresed on a gel and blotted onto a membrane. This membrane containing the immunoprecipitated beta-catenin protein (and presumably, all the other proteins it interacts with) was subjected to western blot analysis using the pan-cadherin antibody. The fact that cadherin proteins are present in the immunoprecipitate obtained with the beta-cateninantibody proves that beta-catenin and cadherin proteins detected by the pan-cadherin antibody attach to each other in the developing visual system. The middle panel of the same figure further verifies this interaction. During this experiment the pan-cadherin antibody was used to immunoprecipitate the cadherin proteins and then this precipitate was screened with the beta-catenin antibody. The positive signal observed shows that the beta-catenin protein can be "pulled down" or co-immunoprecipitated with the cadherin proteins detected by the pan-cadherin antibody and hence, beta-catenin and the cadherin proteins are physically connected to each other in the cat visual system.

For a control, the immunoprecipitation reactions were probed with antibodies for proteins that do not associate with beta-catenin or the cadherins (such as c-fos and jun). The absence of these proteins (jun was used as a control in the top panel whereas c-fos was used as a control in the middle panel) in the immunoprecipitated protein fractions shows the immunoprecipitation reactions to specifically contain proteins which interact with either beta-catenin or the cadherins. Furthermore, the bottom panel shows beta-catenin and the

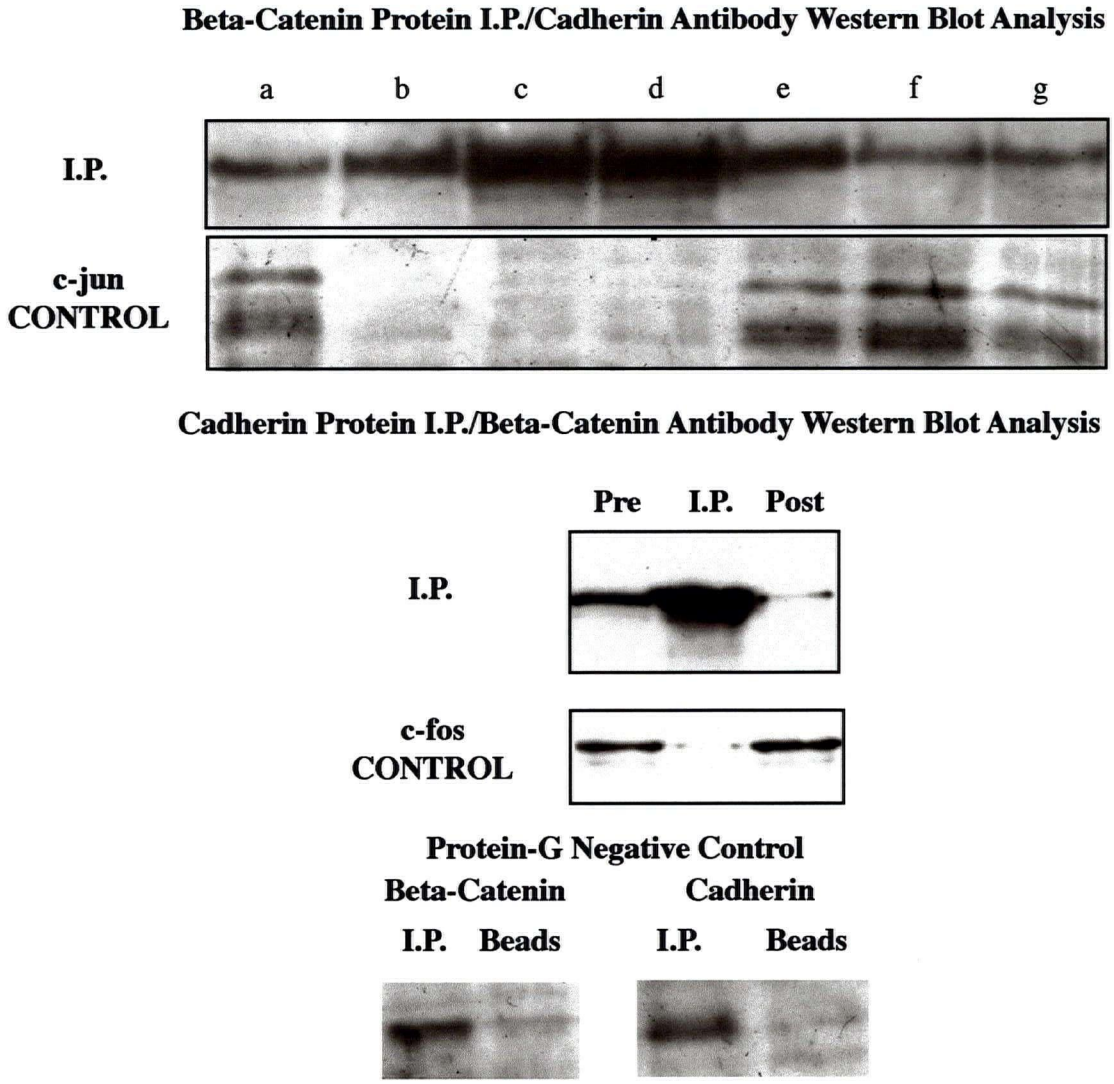


Figure 5.9 Coimmunoprecipitation of the Beta-Catenin and the Cadherin Proteins. In the top panel western blot analysis was performed on the immunoprecipitate obtained with the beta-catenin antibody using the pan-cadherin antibody. Lane a) shows the pre-fraction or the protein preparation before immunoprecipitation with the beta-catenin antibody. Lane b) is immunoprecipitation performed with 2μg of beta-catenin antibody incubated with the protein preparation for 2 hours. The immunoprecipitation reaction shown in lane c) also contained 2μg of beta-catenin antibody but was incubated overnight whereas the immunoprecipitation reaction in lane d) contained 8μg of beta-catenin antibody and was incubated overnight. Lane e), f) and g) are the post-fractions (i.e. the protein preparation after the beta-catenin antibody and the proteins it attaches to are removed) corresponding to the immunoprecipitation reactions performed in lanes b), c) and d) respectively. The bottom half of the top panel shows western blot analysis performed on the same blot with the c-jun antibody (the immunoprecipitation blot was stripped and reprobed with the c-jun antibody). The c-jun protein is absent in the immunoprecipitation fraction (lanes b, c and d). The middle panel shows western blot analysis performed with the beta-catenin antibody on proteins immunoprecipitated with the pan-cadherin antibody. The bottom half of the middle panel shows the reprobing of the same blot with the c-fos antibody which shows the c-fos protein not to be immunoprecipitated by the pan-cadherin antibody. The bottom panel shows a control where only beads were added to the protein preparation. Beta-catenin and the cadherin proteins are found not to be pulled down by beads alone.

cadherin proteins do not to simply attach to the Protein G beads. Finally, the fact that higher yields of co-immunoprecipitated protein could be obtained using either higher concentrations of antibody or longer incubation periods (lanes b,c and d of the top panel)illustrates the specificity of the immunoprecipitation reaction (short incubation periods with the cadherin antibody i.e. 2 hours repeatedly failed to yield a immunoprecipitation product).

5.4 DISCUSSION

The important finding of this chapter is that the cadherin and catenin cell adhesion molecules are upregulated in layers of the LGN corresponding to the visually deprived eye in monocularly deprived animals. Additionally, the optic tract lesion paradigm, which prolongs the critical period (Singer 1982), is capable of retaining high levels of beta-catenin and cadherin protein expression in the LGN. The regulation of the beta-catenin and the cadherin molecules by visual activity supports the involvement of these proteins in the postnatal development of the cat visual system. Unexpectedly, changes in the expression of these proteins in response to activity were not observed in the visual cortex. Also, nuclear localization of beta-catenin was unaltered by visual deprivation. Since visual activity effects the expression of the beta-catenin and cadherin cell adhesion molecules only in the LGN, these molecules may be performing a different set of function in the geniculate as compared to the cortex. This conjecture is not unreasonable based on the fact the cytoarchitecture and the neuro-chemical makeup of the cortex are considerably different than the geniculate and various molecules are differentially expressed between the cortex and the LGN.

5.4.1 Activity Independent Regulation of Beta-catenin and the Cadherin Proteins in the Postnatal Cat Visual Cortex

Whereas cadherins are clearly expressed in an activity dependent manner in the LGN, changes in the expression of these proteins in the visual cortex following monocular deprivation or optic tract lesions is not observed. This finding is unexpected given the expression profile of the cadherin and the beta-catenin proteins correlates well with the critical period for ocular dominance column formation. The involvement of these proteins in visual plasticity is also bolstered by the fact that the pan-cadherin antibody heavily stains the input layer of the thalamic terminals in layer IV and visual areas of the cortex. However, based on the anatomical and physiological properties of the developing visual cortex any molecular mechanism involved in synaptic rearrangements is likely to be regulated in an activity dependent manner. Hence, the involvement of these proteins in activity dependent synaptic modifications is somewhat doubtful in the visual cortex. Conceivably, the role of cadherin molecules in layer IV may be limited to facilitating the formation of proper neural circuits (discussed further in the last chapter).

The absence of activity dependent regulation in the cortex is contradictory to the reported behaviour of cadherin proteins examined in other regions of the nervous systems. For example, N-cadherin expression is reported to decrease in skeletal muscles in response to neural activity (Hahn et al. 1992). The expression of N-cadherin also decreased in dorsal root ganglia by neural stimulation (Itoh et al. 1997). In the developing barrel cortex N-cadherin is concentrated strictly at the thalamocortical synaptic junctional complex during the initial formation of thalamocortical connections (Huntley et al. 1999). Synaptic reorganizations induced by deprivation paradigms are accompanied by N-cadherin expression at newly forming synapses. The cellular redistribution of the beta-catenin protein also occurs in tissue culture neurons deprived of activity (Murase et al. 1999). Furthermore, the activity dependent regulation of these proteins in the geniculate is reported in this chapter. Since these findings contradict the reported activity independent regulation

of beta-catenin and the cadherin proteins in the visual cortex, the possibility that technical problems are responsible for this outcome needs to be considered. A possible technical problem may have arisen from the use of a pan cadherin antibody. As noted in the previous chapter, the differential expression of cadherin molecules allows the formation of various neural circuits. The pan-cadherin antibody heavily labels layer IV and the visual areas of the cortex. Hence, these cadherin proteins are likely to contribute to the organization of the visual cortex and facilitate appropriate connections to form in layer IV. However, the connections that are made onto layer IV are not strictly from the lateral geniculate nucleus. Layer IV receives extensive input from layer VI, the claustrum (LeVay et al. 1981) and neuromodulatory neurons from various subcortical areas (Irle et al. 1984, Mizuno et al. 1969, Troiano et al. 1978, Watabe et al. 1982, Mulligan et al. 1988, Chazal et al. 1987, Tork et al. 1981). In order for these connections to form appropriately, cadherin types other than those necessary for thalamocortical connections need to be expressed in layer IV of the visual cortex. Since some of the non-thalamic connections in layer IV form in an activity independent manner (Perkel et al. 1984), layer IV is likely to contain cadherin molecules that are not regulated by activity. Unfortunately, the pan-cadherin antibody does not recognize cadherin molecules based on their activity dependence. The pan-cadherin staining in the visual cortex following monocular deprivation represents the cumulative protein distribution pattern of both activity dependent and independent cadherin genes. Hence, the presence of cadherin molecules regulated in an activity independent manner in layer IV of the cortex may have prevented the staining pattern of the pan-cadherin antibody from changing following monocular deprivation. In other words, there may be cadherin molecules in layer IV of the visual cortex whose expression is regulated by visual activity. The expression of individual types of cadherin molecules needs to be examined in order to determine if activity dependent members of this cell adhesion family are expressed in the visual cortex. In addition, since beta-catenin interacts with various different cadherin proteins, its expression reflects the cumulative

expression pattern of the classical cadherins. Therefore, the constancy of beta-catenin expression in layer IV of the cortex following monocular deprivation may be attributable to the presence of cadherin molecules regulated in an activity independent manner.

5.4.2 Activity Independent Regulation of Nuclear Beta-Catenin Expression

Another important finding of this chapter is that beta-catenin nuclearization appears to occur in an activity independent manner. Neurons containing nuclear beta-catenin are present in animals subjected to optic tract lesions and the distribution of neurons containing nuclear beta-catenin do not correspond to specific layers of the LGN in monocularly deprived animals. Beta-catenin nuclearization is not proposed to be involved in causing synaptic modifications because nuclear beta-catenin in the LGN is not observed until around 7 weeks of age but monocular deprivation before this period causes significant shifts in ocular dominance. Instead, beta-catenin is believed to cause changes at the transcriptional level that inhibits the neuron from undergoing synaptic reorganization. Therefore, the absence of nuclear beta-catenin redistribution following monocular deprivation is not inconsistent with the suggested function for this protein. In addition, paradigms prolonging the critical period act on higher layers of the cortex and these paradigms are ineffective at prolonging the critical period for thalamocortical neurons (Mower et al. 1985). Hence, the findings of the optic tract lesion experiments can not be deemed as contradicting the proposed role of beta-catenin nuclearization in ending thalamocortical plasticity. Furthermore, the presence of age related factors involved in ending the critical period is well established. Experimental paradigms can only prolong the critical period and are unable to maintain a high level of visual plasticity in older cats (Cynader et al. 1980, 1982). An adult dark reared cat is not as plastic as a thirty day old kitten and accordingly, the synaptic reorganization resulting from monocular deprivation is not nearly as drastic in these animals. Hence, beta-catenin nuclearization may also be an age related, activity independent molecular mechanism contributing to the end of thalamocortical plasticity.

Although changes in beta-catenin nuclearization in response to visual deprivation are not observed in this chapter, the possibility that visual activity effects nuclearization of beta-catenin can not be disregraded. Feasibly, the level of beta-catenin protein in the nucleus may be altered in individual cells by monocular deprivation or optic tract lesions. Alternatively, cells containing nuclear beta-catenin may be different in monocularly deprived animals.

5.4.3 Activity Dependent Changes in Beta-Catenin and Cadherin staining in the LGN

Beta-catenin and cadherin protein expression is altered in the LGN of kittens following monocular deprivation. Also, the expression level of these proteins is greater in the geniculate of older cats subjected to optic tract lesions. This activity dependent regulation of beta-catenin and the cadherin proteins supports the involvement of these proteins in the postnatal development of the cat visual system. We are tempted to devise a presynaptic molecular model for ocular dominance column formation based on the activity dependent regulation of cadherin and beta-catenin protein expression in the LGN. Although this conjecture is supported by various observations (i.e. layer IV staining, expression correlating with the critical period), this hypothesis can not be justified because changes in cadherin or beta-catenin staining in layer IV thalamocortical connections is not observed in response to monocular deprivation. Therefore, the function of these molecules in the LGN is likely to be associated with postnatal developmental events occurring in this structure rather than the cortex. Unlike the visual cortex, eye specific layers form in the cat geniculate prior to birth (Shatz 1983, Shatz et al. 1984). Furthermore, reorganization of eye specific layers in the LGN does not take place postnatally and unlike the ocular dominance columns of the cortex, eye specific layers of the LGN in kittens do not expand and contract postnatally in response to activity dependent paradigms such as monocular deprivation (Shatz et al. 1988, Garraghty et al. 1988). However, cellular competition between different neuronal classes does exist in the postnatal kitten LGN and this cellular

competition is affected by visual activity (Guillery et al. 1970b, Garrey, et al. 1977, Sur et al. 1984, Sur 1988).

Increases in the neuropil staining of beta-catenin and pan-cadherin antibody is observed in the A and C lamina of the LGN following monocular deprivation. The A lamina is innervated by the X cells and the Y cell from the contralateral eye (Guillery1970a, Guillery et al. 1970b). The A1 lamina is innervated by the X and Y cells from the ipsilateral eye. The C lamina is innervated by Y cells from the contralateral eye and W cells of the ipsilateral eye (Guillery1970a, Guillery et al. 1970b). X cells are not found in layer C of the LGN. The retinogeniculocortical pathways constituted by X and Y cells give rise to morphologically and functionally different neural streams. The X pathway in the cat is somewhat analogous to the parvocellular pathway (the cat lacks extensive color vision) and is involved in detecting spatial frequency (Shoham et al. 1997). Similarly, the Y pathway in the cat is involved in motion processing and is equivalent to the magnocellular pathway in the monkey (Frishman et al. 1983). As the magno- and the parvo- names imply, the X cells are smaller in size as compared to Y cells. The development of these two cell types also differs in the postnatal lateral geniculate nucleus.

Compared to the adult LGN the X-cell terminal fields are wider in the A and the A1 layers of the kitten LGN whereas the Y-cell terminal fields in the same layers of the kitten geniculate are much narrower than in the adult LGN (Sur et al. 1984). The contraction of X-cell terminals and the expansion of Y-cell terminals in the LGN occurs gradually with age (Sur 1988). The adult terminal field patterns for X- and Y-cells in the LGN is achieved in kittens around 12 weeks of age (Sur 1988). Interestingly, the development of Y-cells is much more susceptible to the effects of monocular deprivation (Friedlander et al. 1982, Garraghty et al. 1986a). In a monocularly deprived animal, the Y-cells fail to expand their arbors in A layers of the LGN (i.e. A or A1 but not C) corresponding to the deprived eye (Garraghty et al. 1986a, Garraghty et al. 1986b). In these deprived A layers, the age related shrinkage of X-cell terminals does not occur and the X-cells retain large terminal

fields (Garraghty et al. 1986a, Garraghty et al. 1986b). These data imply that the absence of visual activity stunts the growth of Y-cell arbors. The growth of the Y-cell arbors is hypothesized to be accompanied by competitive pruning of X-cell connections and hence, giving rise to the observed shrinkage of X-cell arbors with age. However, the observed competition between X and Y cells is not simply based on activity dependent competition because Y cells grow normally and expand their arbors in the C laminae even in the absence of visual activity. The expansion of Y cell arbors occurs in C laminae not only in the absence of monocular deprivation but also during binocular deprivation where there is absolutely no visual input in this lamina. The ability of Y cells to mature normally in lamina C is attributed to the absence of X cell arbors in this layer of the LGN. The lack of activity in layer C is inconsequential to Y cells because they do not have to compete with X cells in this layer. Hence, both cellular and activity dependent competition appears to be involved in determining the final terminal fields of X- and Y-cells in the postnatally developing LGN.

The activity dependent regulation of beta-catenin and the cadherin molecules in the geniculate suggests the involvement of these molecules in the development of X and Y cell connections in the postnatal LGN. The higher expression levels of the beta-catenin and the cadherin proteins corresponding to the deprived A layers of the LGN may favour the retention of X cell inputs as opposed to the formation of Y cell connections. The overexpression of beta-catenin and the cadherins may either stabilize the X-cell connections or alternatively, these cell adhesion molecules may inhibit Y-cell arbours from expanding. The higher expression levels of these molecules in layer C of the LGN is irrelevant since X cells are not present in this laminae. Changes in the cytoskeleton associated with the development of X- and Y- cell arbours are reported and support the proposed role for beta-catenin and the cadherins in the competition between X and Y cells (Bickford et al. 1998). SMI-32 protein is a neurofilament protein that is selectively expressed by Y-cells (Chaudhuri et al. 1986). Electron microscope studies have revealed the presence of this

protein in synapses(Bickford et al. 1998). Following monocular deprivation, the expression of SMI-32 decreases in the neuropil of A layers corresponding to the deprived eye (Bickford et al. 1998). Neuropil staining of SMI-32 is retained in the C laminae since Y cells expand their arbors in this layer lacking X cells. Compared to SMI-32, beta-catenin and cadherin expression have the opposite response to visual deprivation. These findings support the notion that overexpression of beta-catenin and the cadherin proteins favours the retention of X-cell connections. (The beta-catenin and the cadherin protein expression in the LGN can not be cell type specific like SMI-32 (i.e. these proteins can not be expressed by X-cells alone) because these proteins are expressed in layer C of the LGN lacking X cells and containing Y cells.)

It is unclear if higher levels of beta-catenin and cadherin expression in the LGN following optic tract lesions are associated with retention of plasticity. Firstly, optic tract lesions can only maintain high levels of beta-catenin and cadherin protein expression in the LGN of older animals and not in the cortex (although the reasons for this finding may be identical to the problems associated with the use of the pan cadherin antibody discussed earlier). The distinct responses of these two visual structures to the same deprivation paradigm are hard to explain in terms of ocular dominance plasticity. Secondly, paradigms extending the critical period for ocular dominance formation fail to prolong the period during which X and Y cell competition occurs. Possibly, the higher expression levels of these proteins following optic tract lesions in the LGN may simply reflect the upregulation of beta-catenin and the cadherins in the absence of activity. The regulatory effect of activity on these molecules may be specific to the LGN and may not transpire in cortical structures. This possibility is in agreement with the results of the monocular deprivation studies.

The experiments discussed in this section support a role for beta-catenin/cadherin cell adhesion system in the postnatal development of X- and Y- cell terminals in the postnatal LGN. The alteration in beta-catenin and cadherin protein expression following monocular deprivation suggests these molecules to be involved in the cellular and activity

dependent competition taking place between X- and Y- cell arbours. Since beta-catenin is the homolgue of the *armadillo* segment polarity gene, this result supports the hypothesis that segment polarity genes are involved in the synaptic organization of the postnatal cat visual system (discussed in chapter 2). Clearly, these findings bolster the argument that the homologues of the drosophila developmental genes are involved in the development of the postnatal cat visual system.

Chapter 6

SUMMARY AND GENERAL DISCUSSION

6.1 INTRODUCTION

The aim of this thesis is to establish the hypothesis that the homologues of the drosophila developmental genes are involved in the postnatal development of the cat visual system. In order to achieve this goal, several homeotic and segment polarity genes were partially cloned in the cat and their developmental regulation during the critical period was demonstrated by Northern blot analysis. To provide further evidence for this hypothesis the expression of the beta-catenin protein, the mammalian homologue of the *armadillo* segment polarity gene, was characterized in the visual cortex and the LGN of normally developing and visually deprived kittens. The findings of this thesis indicate beta-catenin to be a multifunctional protein which is likely to contribute to various aspects of postnatal visual development in the cat brain. The evidence provided for the involvement of beta-catenin in visual development strongly endorses our original hypothesis that the mammalian genes originating from the drosophila developmental genes contribute to the postnatal development of the cat visual system.

6.2 THE INVOLVEMENT OF DEVELOPMENTAL GENES IN VISUAL PLASTICITY

Various classic developmental genes are expressed in the visual system during the critical period. Figure 6.1 briefly summarizes the findings of this thesis and illustrates the putative functions of these genes. The *PBX1* and *PBX2* homeotic genes, which are known cofactors of other homeobox genes, are expressed during the critical period (van

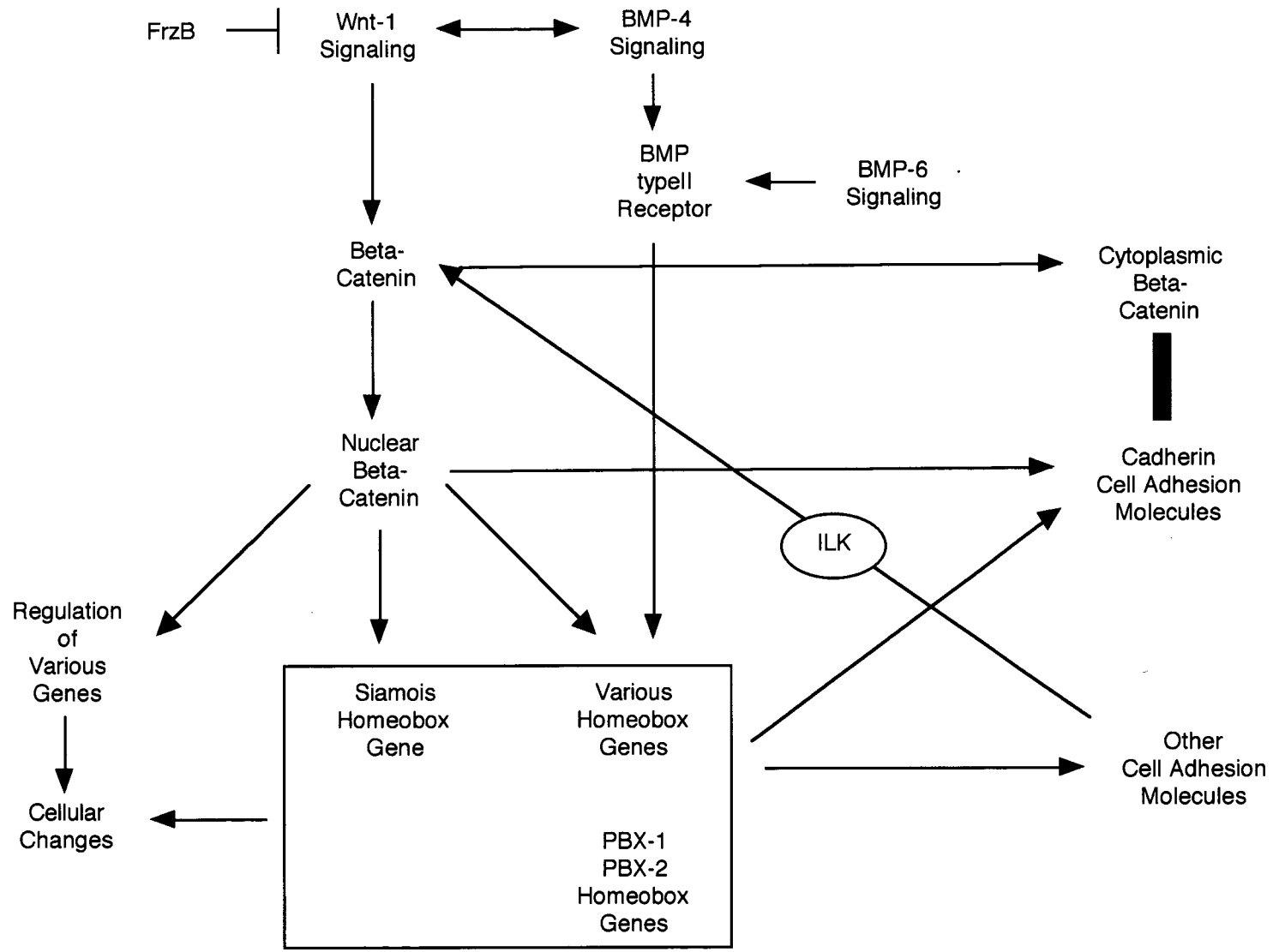


Figure 6.1 : Summary of Drosophila Developmental Gene Expression and Their Putative Functions in the Postnatal Cat Visual System.

Dijk et al. 1995, Chang et al. 1995, Neuteboom et al. 1995). Hence, additional homeobox genes that form transcriptional complexes with these *PBX* genes are quite likely to be expressed in the developing visual cortex. Also, the expression of the *siamois* homeobox gene is likely to be present in the LGN of kittens over the age of 60 days because the expression of this protein is upregulated by the nuclear activity of beta-catenin (Brannon et al. 1997). The possible relevance of these genes to the postnatal development of the visual system arises from the reported involvement of homeotic genes in segmentation and compartmentalization (Lewis 1978, Akam 1987, Lawrence 1987, Murphy et al. 1989). We believe that homeotic genes are likely to contribute to the organization of the visual cortex and these genes may be involved in the formation of structures such as ocular dominance columns, cytochrome-oxidase blobs, and interblobs.

In addition, the bone morphogenic and wnt signaling pathways, which have originated from the *decapentaplegic* (Padgett et al. 1993) and *wingless* (Ramakrishna et al. 1993) segment polarity genes, are expressed in the developing cat visual cortex. The importance of cellular communication and signaling in the development of the visual system is well established. Although the BMP and Wnt signaling pathways were not extensively characterized in the visual cortex, their presence and age dependent regulation is indicative of a role for these molecules in visual development. Since the Wnt and the BMP pathways can act in a competitive and antagonizing manner towards each other, the action of these genes may potentially be the molecular basis for the synaptic organization occurring in the developing visual cortex.

The presence and the developmentally regulated expression of the segment polarity and homeotic genes in the visual cortex gives merit to our initial hypothesis. However, the strongest evidence for the involvement of the homologues of the drosophila developmental genes in visual development arises from the characterization of the *beta-catenin* gene, the homologue of the *armadillo* gene (Peifer et al. 1992). (For detailed discussion of the subjects discussed below please refer to the appropriate chapter in the thesis)

6.2.1 Evidence for the involvement of the beta-catenin protein in postnatal visual development

The data obtained in this thesis indicate that the beta-catenin protein is performing two distinct functions in the postnataly developing cat visual system. Since the beta-catenin protein is present in the nuclei of LGN neurons and the neuropil of the cortex and the geniculate, this protein is likely to act both as a transcription factor and as a cell adhesion molecule in the developing visual system. Because the transcription factor activity of beta-catenin is quite distinct from its adhesive function, there is no reason to assume that these two separate functions of the beta-catenin molecule contribute to the same aspect of visual development. Hence, in addition to having multiple functions the beta-catenin protein may be contributing to different facets of postnatal development in the cat visual system.

6.2.1.1 Involvement of Nuclear beta-catenin in ending thalamocortical plasticity

Beta-catenin is translocated into the nuclei of LGN neurons at the end of the period for thalamocortical plasticity. The simultaneous occurrence of these two events implies a role for beta-catenin in ending thalamocortical plasticity. By acting as a transcription factor and altering the expression of various genes, beta-catenin is proposed to inhibit plasticity in LGN neurons. Since nuclear beta-catenin is only observed in geniculate neurons and not in cortical neurons, neurons in the LGN are postulated to lose their plasticity before cortical neurons. This possibility is supported by the fact that thalamocortical plasticity is lost prior to cortical plasticity (LeVay et al. 1980, Mower et al. 1985). Higher layers of the cortex (such as layer 2/3) retain plasticity until 1 years of age whereas thalamocortical connections of layer IV do not undergo any further synaptic rearrangements following roughly 50-60 days of age (Mower et al. 1985, Daw et al. 1992). The absence of plasticity in layer IV of the cortex following 50-60 days of age can be explained by the inability of the geniculate neurons to modify their synaptic connections. The importance or the involvement of cortical factors in determining ocular dominance plasticity is not being disputed by these

findings. The nuclear activity of beta-catenin is merely being presented as a presynaptic mechanism that contributes to ending thalamocortical plasticity.

Nuclear beta-catenin distribution in the LGN does not appear to be modified by activity dependent paradigms such as monocular deprivation or optic tract lesions. Beta-catenin nuclearization is not proposed to be involved in causing synaptic modifications because nuclear beta-catenin in the LGN is not observed until around 7 weeks of age but monocular deprivation before this period causes significant shifts in ocular dominance. Hence, the results of the monocular deprivation experiments do not contradict the proposed role of nuclear beta-catenin as a plasticity-inhibiting factor. In addition, the failure of the optic tract lesion paradigm to prevent or to delay beta-catenin nuclearization in the LGN does not contradict the proposed function of nuclear beta-catenin because plasticity-prolonging paradigms are ineffective at prolonging thalamocortical plasticity (Mower et al. 1985). As determined by single unit recordings, ocular dominance shifts in dark reared kittens (subjected to monocular deprivation) occur largely in cortical layers other than layer IV (Cynader et al. 1980, Cynader 1982). Accordingly, anatomical changes in ocular dominance columns can not be induced in these animals by monocular deprivation (Mower et al. 1985). These findings indicate the critical period for thalamocortical plasticity to be largely age dependent. Age related events involved in ending visual plasticity for non-thalamocortical connections also exist, since older cats subjected to plasticity-prolonging procedures are less plastic than normally reared young kittens (Cynader et al. 1980, Cynader 1982). In summary, our proposal of an age related end to plasticity in a subset of LGN neurons brought about by the transcriptional factor activity of beta-catenin is consistent with the reported characteristics of the developing visual system.

Concievably, beta-catenin may inhibit the plastic capabilities of geniculate neurons by acting as a transcription factor and downregulating the expression of cell adhesion molecules. The involvement of cell adhesion molecules in neuronal plasticity is well established and a role for these molecules in visual plasticity is supported. Nuclear beta-

catenin activity in non-neuronal adult tissue is associated with cancer and cancer metastasis (Tetsu et al. 1999, Porfiri et al. 1997, Bullions et al. 1998). Nuclear beta-catenin activity in cancerous cells downregulates the expression of cell adhesion molecules. Accordingly, these cancerous cells lose their adhesive properties and detach from their tissue of origin (Oyama et al. 1994). Although the nuclearization of beta-catenin in the LGN does not cause cancer, it may cause an overall decrease in synaptic cell adhesion molecule expression (in addition to the one observed for the cadherins). This decrease in cell adhesion molecule expression is proposed to be responsible for the cessation of plasticity in the geniculate. In support of this hypothesis is the findings of Schoop et. al. 1997 who report the adhesiveness of the visual cortex to decrease in an age related manner. Coincidentally, this decrease in cortical adhesiveness parallels the critical period. In addition, the expression of MHC like cell adhesion molecules in the developing visual cortex and the LGN correlate with the critical period and the expression of these cell adhesion molecules is regulated by visual activity (Corriveau et al. 1998). Also, cell adhesion molecules are required in processes such as synaptogenesis and target recognition (Edelman et al. 1998, Uryu et al. 1999, Lilienbaum et al. 1995, Matsunaga et al. 1988). Finally, the downregulation of cadherin protein expression in the visual system of older kittens (reported in this thesis) supports the involvement of cell adhesion molecules in visual plasticity. If high levels of cell adhesion molecules are required to facilitate synaptic rearrangements (i.e. cell adhesion molecules are expressed more abundantly in younger animals), then the downregulation of these proteins by nuclear beta-catenin activity may cause thalamocortical connections to lose their plasticity. The possibility that beta-catenin ends thalamocortical plasticity by alternate means can not be ruled out considering the activity of nuclear beta-catenin is likely to influence the expression of numerous genes. However, based on the data currently available regarding the function of this protein, the above proposal appears to be the most logical.

6.2.1.2 The Expression of the Beta-Catenin protein in the postnataly developing cat visual cortex

The expression of the beta-catenin protein in the postnatal visual cortex is restricted to the neuropil. This distribution of the beta-catenin protein is consistent with its role as a cell adhesion molecule (McCrea et al. 1991). Since the role of the beta-catenin protein in cell adhesion is to anchor the cadherin molecules to the actin cytoskeleton, the expression of the cadherin proteins was also examined in the developing visual cortex.

The role of the beta-catenin/cadherin cell adhesion system in the developing visual cortex appears to facilitate the formation and wiring of neural circuits. The expression of both the beta-catenin and the cadherin proteins is most prominent in layer IV of the cortex. In addition, the pan-cadherin antibody strongly labels the visual areas of the cortex. A well-characterized property of the cadherins is that they specifically label certain cortical regions and contribute to the wiring of neural circuits (Rubenstein et al. 1999, Suzuki et al. 1997, Inoue et al. 1997, Huntley et al. 1999, Riehl et al. 1996, Matsunaga et al. 1988, Obst-Pernberg et al. 1999). The model by which cadherin molecules are believed to give rise to specific neural circuits is summarized in figure 6.2. In this figure there are three hypothetical neurons that express A-cadherin, B-cadherin or A- and B- cadherin. Due to the homophilic binding properties of cadherin molecules, cells expressing A-cadherin can only make connections with other cells expressing A- cadherin. The same situation is true for cells expressing B-cadherin. However, cells expressing A- and B- cadherin can make connections with both. We propose that the cadherin proteins expressed in layer IV of the visual cortex may be providing a target for axons originating from various different areas of the brain. Axons originating from brain areas that make connections with layer IV such as the thalamus, clastrum and layer VI are likely to express at least some of the same cadherin molecules as layer IV. Based on the model presented in figure 6.2, these axons will be able to make connections with layer IV neurons if they express the same types of cadherins. Each neural pathway connecting to layer IV (for example the callastrum and

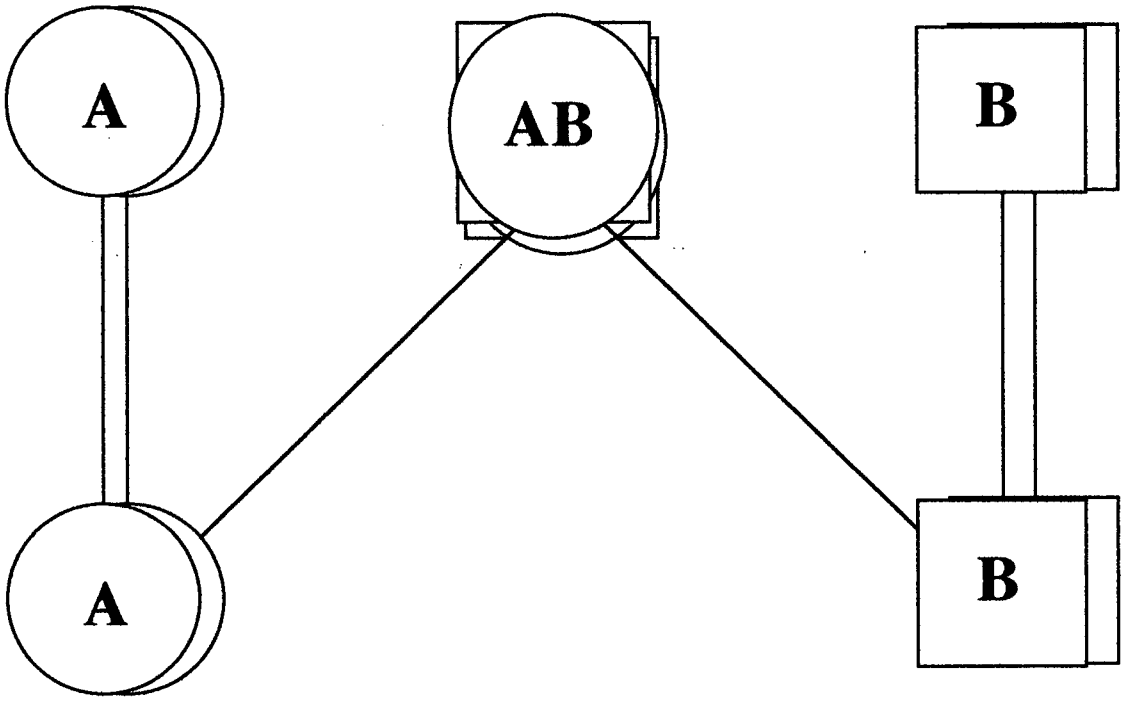


Figure 6.2 Synaptic organization mediated by the cadherin proteins.

thalamic connections) is likely to express different cadherin molecules so that each type of input can form synapses with appropriate neurons.

Although a correlation between cadherin protein expression and the critical period exists in the visual cortex, these proteins appear to be expressed in an activity independent manner in this structure. Feasibly, these proteins may contribute to the age related decrease in plasticity in the visual cortex and may not be involved in activity dependent synaptic modifications. However, activity dependent changes in cortical cadherin protein expression may have been missed due to the use of a pan-cadherin antibody. The fact that some connections in layer IV are made in an activity independent manner supports this notion (Perkel et al. 1984). Activity dependent regulation of cadherin and beta-catenin expression has been discovered in various other neural structures (Huntley et al. 1999, Murase et al. 1999, Tanaka et al. 2000, Tang et al. 1998). The activity dependent changes reported in the expression of the beta-catenin and the cadherin proteins in the LGN verifies the ability of visual activity to regulate the expression of these molecules. Hence, the examination of individual cadherin protein types is likely to identify specific species of cadherin molecules which are regulated by neural activity in the developing visual cortex. The discovery of such molecules would confirm our belief that the beta-catenin/cadherin cell adhesion system is involved in synaptic modification events taking place during the formation of ocular dominance columns.

As an aside, these data are advocating further examination of cell adhesion molecule expression in the developing visual cortex. It seems intuitive that cell adhesion molecules, which are the glue that form and hold synapses together, would be involved in the wiring of neural circuits. An obvious target for research is identifying and characterizing the cadherin molecules that are specific to various neural streams. Based on previous research (Suzuki et al. 1997, Arndt et al. 1996, Kimura et al. 1996), cell adhesion molecules that are specific to both anatomical and functional neural pathways in the visual system are likely to exist. Characterizing the expression patterns of these molecules will be an arduous task

given the number of different cadherin, protocadherin and cadherin like molecules expressed in the brain. In addition, the integrins constitute another cell adhesion system which may be involved in visual development. Certain members of the integrin family are localized to the synapse and they contribute to neural development and neural plasticity (Einhebner et al. 1996, Lilenbaum et al. 1995, Martin et al. 1996, Staubli et al. 1990). Efforts should also be made towards better understanding the role of CAM's, which do not require calcium for their function, in visual development.

6.2.1.3 Involvement of the Beta-catenin and the Cadherin proteins in X- and Y- cell competition

Beta-catenin and cadherin protein expression in the geniculate decreases with age. Following monocular deprivation, beta-catenin and cadherin protein levels increase in layers of the LGN which receive input from the deprived eye. Since changes in the expression of these proteins are not detected in the cortex in response to activity, activity dependent regulation of the beta-catenin and the cadherin proteins in the geniculate is unlikely to be involved in the development of thalamocortical connections. The only well established activity dependent event occurring in the postnatal LGN is the development of X and Y cell connections (Guillery et al. 1970b, Garrey, et al. 1977, Sur et al. 1984, Sur 1988). Hence, the beta-catenin and cadherin proteins expressed in the postnataly developing LGN are likely to contribute to this event. The altered levels of cadherin and beta-catenin protein expression in the neuropil of the LGN in response to monocular deprivation is proposed to provide the X-cell arbours with a competitive advantage and prevent the Y-cells from taking over X-cell terminals. This proposed role of the beta-catenin/cadherin cell adhesion system is consistent with the reported function of other cytoskeletal proteins, such as SMI-32, during the postnatal development of X- and Y-cell terminal fields in the geniculate (Bickford et al. 1998). The evidence for the involvement of

the beta-catenin protein in X- and Y- cell competition supports the hypothesis that segment polarity genes may be involved in synaptic organization occuring during the critical period.

6.2.1.4 Summary of the evidence implicating a role for the beta-catenin protein in postnatal visual development

The data discussed in this section provide several lines of evidence supporting the involvement of beta-catenin in the postnatal development of the cat visual system. First, the beta-catenin protein along with the cadherin proteins is expressed in layer IV of the visual cortex and is proposed to play an organizational role. Second, the beta-catenin protein becomes nuclear in a subset of LGN neurons at the end of the critical period for thalamocortical plasticity. Third, the expression of beta-catenin is regulated in an activity dependent manner in the developing LGN and it may be involved in the competition taking place between X- and Y- cells for synaptic terminals. Taken together, these data strongly endorse the involvement of beta-catenin in visual development and ergo, these data bolster the hypothesis that the mammalian counterparts of the drosophila developmental genes are involved in the postnatal development of the cat visual system.

6.2.2 Significance of the findings presented in this thesis

We have established the expression of the homeotic and segment polarity genes in the visual cortex during the critical period and provided evidence for their involvement in visual development. The segment polarity and homeotic genes comprise two large gene families. Unfortunately, the expression of only a few of these genes could be investigated in this thesis. Hence, the results of this thesis can be used as a basis for designing experiments to investigate the function of other homologues of the drosophila developmental genes in visual development. For example, based on the results presented in this thesis, it is not unreasonable to assume that the Notch signaling pathway or the pair rule genes are playing a role in the postnatal development of the cat visual system. In addition, hypothesis regarding the function of the developmental genes identified in Chapter 2 can be formed and the expression of these genes can be further characterized in

the visual system. Also, examining the activity of genes that are known to interact with the homeobox and segment polarity genes identified in chapter 2 (or beta-catenin) can expand upon the data presented in this thesis. By verifying the presence of the homeotic and segment polarity genes in the developing visual areas of the postnatal brain, this thesis will hopefully promote further studies examining the role of other homologues of the drosophila developmental genes in the postnatal development of the visual system.

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