THE EFFECT OF PERIPHERAL DEAFFERENTATION ON SPECIFIC AXONAL SYSTEMS AND GLIAL ELEMENTS IN THE MAIN OLFACTORY BULB OF THE RAT

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SHELLEY R. KING

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BY

© SHELLEY R. KING, B.Sc.

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ABSTRACT

Heterotypic collateral sprouting of neurotransmitter systems has been observed in many regions of the CNS following injury. The olfactory bulb (OB) receives direct input from the olfactory epithelium and its neuroanatomy and neurotransmitter content have been well characterized. Thus, the OB may provide a useful cortical model of lesion-induced plasticity. The present study has tested the hypothesis that heterotypic collateral sprouting of specific axonal systems, especially the serotonergic axons from the raphe, will occur following peripheral deafferentation of the OB. In addition, we hypothesized that an increase in the number of astrocytes would occur following deafferentation of the OB. In this study, serotonin, dopamine beta-hydroxylase, glial fibrillary acidic protein (GFAP) and S-100 (serotonergic growth factor) immunocytochemistry was performed to elucidate the response of different axonal systems and glial elements to peripheral deafferentation of the OB.

Deafferentation of the right OB was achieved by application of $ZnSO_4$ to the olfactory epithelium (OE) of adult male rats and postnatal day (PND) 10 rat pups. The effectiveness of the lesion was determined by the degree of the loss of tyrosine hydroxylase (TH) immunoreactivity in the periglomerular cells which require the presence of the OE to express the TH phenotype. The deafferentation resulted in a decrease in the density of serotonergic fibres in the deeper regions of the lesioned OB in adult animals which is in contrast to the increase in density evident in the PND 31 animals. The density of astrocytes expressing S-100, a proposed serotonergic

growth factor, did not correlate with the density of serotonergic innervation. In contrast to the serotonergic system, the density of noradrenergic fibres in the lesioned OB was increased in the adult animals, but remained unchanged in PND 31 rat pups. The densities of GFAP positive astrocytes in the lesioned OB of adult and PND 31 animals were similar to those of control animals. These results suggest that deafferentation of the OB of adult animals did not induce heterotypic sprouting of intact serotonergic fibres in the three week time period. On the contrary, the serotonergic fibres were selectively decreased, perhaps due to the potential neurotoxicity of zinc to these fibres. It appears, however, that the serotonergic fibres in the younger animals are capable of sprouting in response to OB deafferentation and this response may be related to the indirect neuromodulatory effects of zinc.

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INTRODUCTION

This thesis examines the hypothesis that heterotypic collateral sprouting of intact fibre systems, especially the serotonergic system, would occur following removal of the afferent input to the olfactory bulb (OB). In addition, we hypothesized that astrocytes might increase in number, thus displaying an increase in density in response to deafferentation of the OB. A brief history of research on plasticity within the central nervous system (CNS) will be presented first, followed by a review of the phenomenon of sprouting exhibited by neurons and the responses of glia after injury. In addition, the hypotheses addressed in this thesis and the rationale behind each hypothesis are presented in conjunction with similar studies of lesion-induced plasticity in other regions of the CNS.

1.1 Plasticity of the Central Nervous System

The ability of the nervous system to regenerate and form new synapses after injury or damage to the CNS has been the focus of much research since the early 1950. The study of lesion-induced plasticity is important in order to determine the mechanisms underlying recovery of function after injury and to compare how different regions of the CNS are reorganized. One means by which the brain reacts to injury is by growth or sprouting of axons. Historically, collateral sprouting has been defined as the growth of axon collaterals over some distance before they contact an area that has been denervated by a lesion. Homotypic collateral sprouting occurs

when undamaged fibres, containing the same transmitter as the damaged neuronal system, increase their projections. In contrast, heterotypic collateral sprouting occurs when the fibre system that responds to the damage is different from the system that had originally been injured.

The phenomenon of collateral sprouting of intact fibres was first demonstrated in the peripheral nervous system (Edds, 1953), however, much scepticism existed as to the ability of the neurons in the central nervous system to sprout in response to injury or deafferentation. The first evidence of collateral sprouting of intact axons was observed in the peripheral nervous system following partial denervation of the sympathetic ganglion (Murray and Thompson, 1957), while the first evidence of sprouting in the CNS was observed after partial denervation of the spinal cord of the cat (Liu and Chambers, 1958). Since the late 1950's the collateral sprouting response of the CNS after injury has been well documented by several investigators, using a variety of techniques, in several areas of the CNS including the septum (Raisman, 1969; Raisman and Field, 1973), dentate gyrus (Lynch et al.1973; Matthews et al.1976), hippocampus (Azmitia et al.1978; Zhou and Azmitia, 1986), striatum (Stachowiak et al.1984; Synder et al.1985), superior colliculus (Rhoades et al.1990), lateral geniculate nucleus (Rhoades et al.1990) and spinal cord (Polistina et al.1990; Wang et al.1991; Marlier et al.1992).

The ability of various fibre systems to sprout in many regions of the CNS has led to the belief that the sprouting phenomenon underlies recovery of function following CNS lesions. Three different systems, the serotonergic, noradrenergic and cholinergic systems, each with widely distributed cortical and subcortical projections, have been especially examined in relation to sprouting. Indeed, collateral sprouting of transplanted serotonergic neurons has been implicated in recovery of sexual function following spinal cord injury (Privat et al.1989). Similarly, partial recovery of motor behavior is attributed to homotypic collateral sprouting of supraspinal and/or propriospinal projections in response to deafferentation of the spinal cord (Goldberger and Murray, 1974). However, the exact mechanisms underlying the appearance of sprouting and functional recovery have not been clearly elucidated.

Although axonal sprouting is a phenomenon generally recognized after injury to the CNS, not all fibres systems exhibit sprouting following injury. Controversy also exists as to the occurrence of sprouting in certain areas. In addition to the reports of sprouting in the superior colliculus and lateral geniculate nucleus (Rhoades et al.1990), lack of sprouting has also been reported in these structures (Hickey, 1975; Stelzner and Keating, 1977; Chow et al.1981) following unilateral enucleation. Similarly, although sprouting has been extensively reported in the spinal cord following injury, absence of sprouting has also been reported (Rustioni and Molenaar, 1975; Beckerman and Kerr, 1976; Goldberger and Murray, 1982; Pubols and Bowen, 1988). The discrepancies in reports of sprouting in the same regions of the CNS may be due to the different methods of producing the injury . For instance, chemical

deafferentation of the spinal cord produced by ricin injection does not induce sprouting of fibres in the dorsal horn (Pubols and Bowen, 1988), while destruction of the primary afferents to the same region using another chemical, capsaicin, induces of sprouting of serotonergic fibres (Polistina et al.1990).

In addition to the conflicting reports of the occurrence of sprouting, the response of different neuronal systems to the same injury may not be identical. Thus, there appears to be some degree of selectivity in terms of which fibres sprout following injury, indicating that some neuronal systems may exhibit a greater degree of plasticity in response to a particular lesion. For instance, serotonergic fibres in the rat spinal cord have been reported to sprout after unilateral dorsal rhizotomy, while the noradrenergic innervation appears to be unaffected by the lesion (Wang et al.1991). The developmental stage of the animal at the time of injury may also be an important factor in determining the response to the lesion. In some areas of the CNS, the capacity of fibres to sprout is only evident following lesions in young or neonatal animals. In the striatum, for example,sprouting of the serotonergic system occurs after dopamine-depleting lesions in 3 day old rats, but not when comparable lesions are performed in adult animals (Stachowiak et al.1984; Synder et al.1985).

Several methods have been used to detect spouting of fibres following CNS lesions. Fibre density measurements, obtained using immunocytochemical staining, have been used by various investigators to detect lesion-induced sprouting (Luthman et al.1987; Rhoades et al.1990; Wang et al.1991; Zhou et al.1991; Marlier et al.1992). Additional methods of detecting sprouting, which are often used alone or

in combination with immunocytochemical techniques, include measuring biochemical levels of neurotransmitters and/or their metabolites (Stachowiak et al.1984; Luthman et al.1987; Zhou et al.1991; Baker et al.1993) and horseradish peroxidase retrograde tracing of the fibre projections to the denervated structure (Synder et al.1985). In the present study, immunocytochemical analysis is the method used to detect the response of fibre systems to deafferentation since this technique allows us to study changes in the density of axons as well as their distribution within the OB.

1.2 Plasticity of the Serotonergic System

The serotonergic system exhibits an exceptional ability to sprout in response to injury of the CNS. Homotypic collateral sprouting of serotonergic fibres has been demonstrated in the hippocampus following selective chemical lesion of the serotonergic afferents in the cingulum bundle (Azmitia et al.1978; Zhou and Azmitia, 1984; Azmitia and Zhou, 1986). Heterotypic collateral sprouting of the serotonergic axons has been established in the spinal cord following deafferentation (Polistina et al.1990; Wang et al.1991; Marlier et al.1992). In addition, specific depletion of dopamine, using the neurotoxin 6-hydroxydopamine, in the striatum (Stachowiak et al.1984; Synder et al.1985) and substantia nigra (Zhou et al.1991) results in heterotypic collateral sprouting of serotonergic fibres.

The plasticity observed in the serotonergic system following various types of injury to the CNS may be due to release of trophic factors. Of the many growth factors thought to be involved in regeneration or sprouting of axons in general, the S-100ß protein is believed to be specific for the serotonergic system (Azmitia et al.1990; Whitaker-Azmitia et al.1990).

The S100ß protein is a member of the S100 family of proteins which are in turn a part of the superfamily of calcium-modulated proteins (Van Eldik et al.1982). The S100ß protein is distributed throughout the nervous system with the majority of S-100ß believed to reside in glia (Bock, 1978). In the rat brain S100ß immunoreactivity reveals that the protein is strictly localized to astrocytes (Boyes et al.1986). S100ß is thought to be produced (Bock, 1978) and secreted (Suzuki et

al.1987; Van Eldik and Zimmer, 1987) by astrocytes in the brain. While the specific function of S100 has not been determined, potential neurotrophic activity of the protein has been demonstrated in cell culture experiments (Klingman and Marshak, 1985; Klingman and Hsieh, 1987; Van Eldik et al.1988). Recent studies have proposed that S100ß is a serotonergic growth factor (Azmitia et al.1990; Whitaker-Azmitia et al.1990) that is released by stimulation of 5-$HT1_A$ receptors on astroglial cells (Whitaker-Azmitia et al.1990).

The present study investigated the hypothesis that heterotypic collateral sprouting of intact serotonergic fibres would occur following peripheral deafferentation of the olfactory bulb and that potential changes in the serotonergic innervation may, in part, reflect an accompanying change in the number of astrocytes expressing S-100.

1.3 Plasticity of the Noradrenergic System

Like the serotonergic system, noradrenergic fibres also display the ability to sprout in response to injury. Homotypic collateral sprouting of intact noradrenergic axons has been reported following chemical induced axotomy of central noradrenergic neurons (Bendeich et al.1978; Kostrzewa et al.1978; Björklund and Lindvall, 1979; Nakai et al.1987; Fritschy and Grazanna, 1992) and partial denervation of the hippocampus (Gage et al.1983; Madison and Davis, 1983). Heterotypic collateral growth of noradrenergic axons in the lateral geniculate body (Stenevi et al.1972) and septum (Moore et al.1971) has been reported following unilateral ablation of the visual cortex and hippocampus, respectively.

The serotonergic and noradrenergic systems exhibit the ability to sprout after various lesions of the CNS. The response of these systems, however, has not been compared following peripheral deafferentation of the olfactory bulb. Comparing the response of the two systems to the same lesion may be important in determining the extent of reorganization following injury. The present study examined the hypothesis that a similar sprouting response of the serotonergic and noradrenergic systems would occur following peripheral deafferentation of the olfactory bulb.

1.4 Plasticity of the Cholinergic System

Homotypic sprouting of the cholinergic system has been observed following partial deafferentation of the hippocampus (Lynch et al.1973; Nadler et al.1977; Nadler et al.1977; Gage et al.1983) and destruction of cholinergic neurons following excitotoxic lesions of the nucleus basalis (Wenk and Oltom, 1984). In contrast to the plasticity of cholinergic axons observed in the cerebral cortex following injection of ibotenic acid, an excitotoxin, into the nucleus basalis, no evidence of sprouting of cholinergic axons was observed after injection of another excitotoxin, quisqualate, into the same structure (Henderson, 1991). Despite these interesting and potentially important observations of cholinergic axonal sprouting in the CNS, the response of the cholinergic system to peripheral deafferentation of the OB could not be determined in the present study due to the lack of a reliable marker for cholinergic axons.

1.5 Glial Response to Injury of the CNS

Gliosis is a ubiquitous process that occurs in the CNS following injury. Reactive astrocytes are often apparent in the local environment of the injury following damage to the CNS and may be closely associated with the regrowth of nerve fibres.

Reactive astrocytes have been reported following various types of injury to the CNS including cerebral stab wounds (Bignami and Dahl, 1976; Latov et al.1979; Mathewson and Berry, 1985; Takamiya et al.1988), cryogenic lesions (Amaducci et al.1981), laser injury (Schiffer et al.1986), optic nerve enucleation (Trimmer and Wunderlich, 1990) and transection of the spinal cord (Barrett et al.1984). Following injury, reactive astrocytes may increase in number around an injured area, either due to proliferation (Bignami and Dahl, 1976; Barrett et al.1984; Mathewson and Berry, 1985; Takamiya et al.1988) or migration (Rose et al.1976).

Glial fibrillary acidic protein (GFAP) is an immunocytochemical marker specific for the intermediate filaments of fibrous astrocytes in the CNS (Eng et al.1971; Bignami et al.1972). Identification of astrocytes using GFAP is a reliable and frequently used method of detecting astrocytes in the OB (Bailey and Shipley, 1993), in other regions of the CNS (Bignami and Dahl, 1973; Schiffer et al.1986; Takamiya et al.1988) and after injury to various sites in the CNS (Bignami and Dahl, 1976; Amaducci et al.1981; Takamiya et al.1988).

The present study investigated the hypothesis that an increase in the number of astrocytes would occur in the OB following peripheral deafferentation, thereby resulting in an increase in the density of astrocytes expressing the immunocytochemical marker for GFAP.

1.6 The Olfactory Bulb as a Cortical Structure to Investigate Lesion-Induced Plasticity.

The vertebrate olfactory epithelium (OE) contains a population of immature stem cells in the basal neuroepithelium which retain the ability to divide and differentiate into mature receptor neurons throughout the life of the animal (Graziadei and Monti-Graziadei, 1978b). Thus, the olfactory receptor neurons that have degenerated in response to noxious environmental stimuli or experimentally induced lesions are able to be replaced by new receptor neurons originating from the basal cells in the neuroepithelium (Graziadei and Monti-Graziadei, 1978a; Graziadei and Monti-Graziadei, 1980). In addition, the neuroanatomy and neurotransmitter content of the OB have been well characterized. These properties, coupled with the relative ease of accessibility of the OB for experimental manipulation, makes the OB a useful cortical structure for examining the plasticity of neurotransmitter systems after denervation.

The following sections briefly outline the structure of the olfactory neuroepithelium and main olfactory bulb since familiarity with these structures is necessary to understand the method of denervation used and the effects of peripheral denervation reported in this study.

1.6.1 The Olfactory Epithelium

The vertebrate olfactory epithelium (OE) is composed of three cellular elements: (1) sensory or primary olfactory receptor cells, (2) sustentacular or supporting cells and (3) basal cells (Moulton and Beidler, 1967; Graziadei and Monti-Graziadei, 1978a). The receptor cells are bipolar neurons with processes of varying length. The axons of the primary olfactory neurons (PON) are unmyelinated but are surrounded by glial cells, called ensheathing cells (Raisman, 1985; Doucette, 1989; Doucette, 1993) until they reach the olfactory bulb (OB). The axons of the PON leave the OE in discrete bundles, the *fila olfactoria*, and travel through the *lamina cribrosa* of the ethmoid bone to become the olfactory nerve layer (ONL) and terminate in the glomerular layer (GL), the second layer of the OB.

The supporting or sustentacular cells are columnar epithelial cells that provide mechanical support to the olfactory receptor cells by ensheathing the receptors. In addition to their role in support, these cells also serve a secretory function and have been implicated in maintaining proper nutrition for the receptor cells. The function of the basal cells has not been clearly elucidated. It appears, however, that these cells are replacement elements which are responsible for the regeneration of the olfactory receptor cells after injury ((Moulton and Beidler,1967; McLean and Shipley, 1992).

1.6.2 Neuroanatomy of the Olfactory Bulb

The neuroanatomy of the main olfactory bulb (MOB) has been well documented (Shepherd, 1972; Switzer et al.1985; McLean and Shipley, 1992). The MOB is a highly organized laminar structure consisting of a total of seven layers with clearly identifiable boundaries. The olfactory nerve layer (ONL) constitutes the first layer of the MOB and consists of the axons of PON and glia cells. The individual axons of PON penetrate the cribriform plate and enter the ventromedial aspect of the OB to terminate in several glomeruli (Pinching and Powell, 1971) within the glomerular layer (GL), the second layer of the OB. In the rat, the GL consists of approximately 3000 ovoid glomeruli (Meisami and Safari, 1981) and is a cell poor region with each glomerulus surrounded by a relatively thin layer of juxtaglomerular or periglomerular cells and glia. The dendrites and axons of the juxtaglomerular cells may ramify within the glomeruli (Cajal, 1911), surround the glomeruli within the periglomerular region (Cajal, 1911; Macrides and Davis, 1983; McLean et al.1989) or project to other areas of the ipsilateral bulb (Schoenfeld et al.1985). The dendrites of the mitral/tufted cells also project to the GL and make synaptic contacts with the axons of the PON. Subjacent to the GL is an another relatively cell poor region, the external plexiform layer (EPL). The EPL contains mainly superficial, middle and deep tufted cells as well as the dendrites of mitral/tufted and granule cells. The mitral cell layer (MCL) is immediately subjacent to the EPL and is a thin layer containing the somata of the mitral cells. The mitral cells have a single apical dendrite that projects mainly to the GL where it makes connections with the axons of the PON and to

other centrifugal inputs to the OB (Shepherd, 1972). The mitral cells, along with the middle and deep tufted cells (Schoenfeld and Macrides, 1984) from the EPL, constitute the output cells of the OB. The internal plexiform layer (IPL) lies immediately subjacent to the MCL and contains the axons and dendrites of mitral and granule cells, respectively. The next neuronal layer of the bulb, the granule cell layer (GCL), lies subjacent to the IPL. The GCL consists mainly of the somata of granule cells arranged in parallel rows. The granule cells do not have axons but have apical and basal dendrites that ramify in the EPL and GCL, respectively (Mori et al.1983; Orona et al.1983; Scott, 1986). The subependymal zone is the deepest layer in the MOB. Although a relatively cell poor region in the adult, the subependymal zone is a region of cell proliferation during development.

The accessory olfactory bulb (AOB) is located at the caudal-dorsal region of the olfactory bulb. Although the AOB is significantly smaller that the MOB, the cytoarchitecture of the two structures is similar. The AOB is a laminated structure, composed of relatively the same layers as the MOB, with the exception of the nerve layer and subependymal zone. In the AOB, it is the vomeronasal nerve that transmits information from the vomeronasal organ in the nasal septum to the glomeruli in the accessory GL. The accessory GL is less distinct than the GL in the MOB since the glomeruli are smaller and fewer in number. The periglomerular cells in the accessory GL do not surround the glomeruli, as in the MOB, but remain on either side of the glomeruli and do not contain dopamine. Subjacent to the accessory GL is the accessory EPL, which is less prominent than its counterpart in the MOB since the

cells of the next layer, the accessory MCL, are mixed in with the AEPL. The accessory IPL lies between the accessory MCL and lateral olfactory tract. The final layer of the AOB is the accessory GCL which is situated deep to the lateral olfactory tract and contains the same type of cells as the GCL of the MOB (McLean and Shipley, 1992).

Although both the MOB and AOB are important subdivisions of the olfactory system in macrosmatic animals such as rodents, only the effects of peripheral denervation of the MOB are discussed in the present study.

1.7 Neurotransmitter Content of the Olfactory Bulb

The olfactory bulb has numerous intrinsic neurons expressing various neurotransmitters/peptides and receives centrifugal afferent inputs from olfactory related cortices and subcortical regions. The olfactory related projections are linked to olfactory sensory and association functions. The afferent inputs to the bulb from the subcortical regions often have widespread projections to other areas of the CNS and are believed to serve a modulatory function. The olfactory related centrifugal afferents arise from the anterior olfactory nucleus, piriform cortex, periamygdaloid cortex, entorhinal cortex, nucleus of the lateral olfactory tract and amygdala (McLean and Shipley, 1992), while the dorsal and median raphe nuclei, locus coeruleus and nucleus of the diagonal band provide the other major "non-olfactory" subcortical sources of afferent input into the OB.

Although the OB receives many sources of afferent input, only the serotonergic, noradrenergic and cholinergic innervation will be described in the following sections. The present study focuses on the response of the serotonergic and noradrenergic systems to peripheral deafferentation of the OB. Descriptions of the other sources of afferent innervation have been recently reviewed (McLean and Shipley, 1992).

1.7.1 Neurotransmitters and Neuropeptides Intrinsic to the Olfactory Bulb

The specific neurotransmitter(s) of the PON has not, as yet, been conclusively determined although recent findings suggest that glutamate or aspartate are involved in neurotransmission (Trombley and Shepherd, 1994). Carnosine (Sakai et al.1988) and olfactory marker protein (Margolis, 1980) are two soluble proteins that are fairly specific to the PON but probably are not involved in neurotransmission. Calcitonin gene related peptide (Rosenfeld et al.1983) and substance P (Baker, 1986) have been observed in some fibres in the first layer of the MOB, the ONL. The specific origin of the latter two peptides has not been clearly defined but is believed to be associated with the trigeminal axons. In the GL, the PG cells have been reported to contain numerous neurotransmitters and neuropeptides including dopamine (Serby et al.1991; Davis and Macrides, 1983), GABA (Ribak et al.1977), substance P (Kosaka et al.1988), met-enkephalin (Davis et al.1982), vasoactive intestinal peptide (Gall et al.1986; Sanides Kohlrausch and Wahle, 1990), somatostatin (Scott et al.1987), cholecystokinin (Seroogy et al.1985; Matsutani et al.1988) and aspartic acid (Halasz, 1987). Although the PG cells contain a wide variety of transmitters and neuropeptides, the exact role of these substances in the processing of olfactory information has not been clearly elucidated. The tufted cells of the EPL have been reported to contain GABA (Kosaka et al.1987), cholecystokinin (Shepherd, 1972) and vasoactive intestinal peptide (Gall et al.1986; Sanides Kohlrausch and Wahle, 1990). The mitral cells in the next layer of the OB, the MCL, have been reported to contain N-acetyl-aspartyl-glutamate (Blakely et al.1987; ffrench-Mullen et al.1985) and

corticotropin releasing factor (Imaki et al.1989). The middle tufted cells of the MCL reportedly contain vasoactive intestinal peptide (Gall et al.1986) while the superficial to deep tufted cells have been reported to contain cholecystokinin (Seroogy et al.1985). Although the IPL contains relatively few cells, some of the cells have been reported to contain vasoactive intestinal peptide (Sanides Kohlrausch and Wahle, 1990). Finally, the cells in the GCL contain GABA (Ribak et al.1977), vasoactive intestinal peptide (Sanides Kohlrausch and Wahle, 1990), cholecystokinin (Shepherd, 1972) and met-enkephalin (Davis et al.1982).

1.7.2 Serotonergic Innervation of the Olfactory Bulb

Serotonergic fibres are widely distributed throughout the OB. However, the fibres that originate from the dorsal (DR) and median raphe (MR) nuclei innervate different layers (McLean and Shipley, 1987b). Thick fibres, presumably from the MR (Mamounas and Molliver, 1988), preferentially innervate the glomeruli, while thin fibers, presumably from the DR (Mamounas and Molliver, 1988), are found primarily in the inframitral regions of the bulb (McLean and Shipley, 1987b).

In the OB, the density of serotonin fibres is greatest in the GL. The density of fibres in the GL is estimated to be 2-3 times greater than the other bulb layers (McLean and Shipley, 1987a). Although the specific connections and the function of the serotonin fibres in the glomeruli have not been elucidated, the close proximity of these fibres and the PON terminals suggests that serotonin may have a direct or modulatory role in olfactory function. Given the constant turnover of synapses in the GL due to the continual regeneration of receptor cells and their axons in the

periphery and potential role of serotonin in the inhibition of synapse formation (Haydon et al.1984), serotonin may potentially be involved in regulation of synapse formation within the GL of the OB. Serotonin may also be associated with olfactory information processing since the fibres are highly concentrated in the glomeruli, where the first synaptic relay occurs from the OE. In this respect, we have recently shown that depletion of serotonin fibres in the neonate rat pup results in the inability of the pups to acquire or express a preference for a peppermint odor after a one trial conditioned odor training paradigm (McLean et al.1993).

1.7.3 Noradrenergic Innervation of the Olfactory Bulb

The locus coeruleus (LC), located in the rostral pons, provides the noradrenergic innervation to the bulb. In the rat, it is estimated that almost 40% of the neurons originating from the LC project to the bulb (Shipley et al.1985). The noradrenergic fibres innervate all layers of the OB with the exception of the ONL. The IPL and GCL receive the strongest noradrenergic innervation while the EPL and MCL have a moderate innervation. However, the GL receives only a few noradrenergic fibres (McLean and Shipley, 1987a; McLean and Shipley, 1991). The physiological role of noradrenaline has been extensively studied in the OB but its actual role is still debated. Noradrenaline has also been implicated in olfactory learning since noradrenergic antagonists have been shown to impede the ability of newborn rats to form learned odor preferences and noradrenergic agonists tend to enhance learning (Sullivan et al.1989).

1.7.4 Cholinergic Innervation of the Olfactory Bulb

The cholinergic projection to the bulb arises from the rostromedial portion of the horizontal limb of the nucleus of the diagonal band (NDB)(Macrides et al.1981; Carson, 1984; Zaborszky et al.1986). The cholinergic neurons are intermingled with GABAergic neurons located mainly in the lateral-caudal portion of the horizontal limb of the NDB (Zaborszky et al.1986). The cholinergic innervation of the bulb, as revealed by cholinesterase staining, is concentrated in the GL, EPL, IPL and GCL. The axonal distribution of the GABAergic innervation from the NDB is harder to determine since the periglomerular and granule cells provide intrinsic GABAergic innervation of the OB.

1.8 Peripheral Denervation of the Olfactory Bulb

A variety of methods have been used to deprive the olfactory bulb of sensory innervation. These methods include primary olfactory nerve axotomy, intranasal irrigation with zinc sulfate ($ZnSO_4$) or triton X-100 and cauterization of the external naris (Alberts, 1974). However, external naris closure, unlike the other methods of sensory deprivation, results in functional rather than anatomical deprivation (Brunjes and Frazier, 1986).

Denervation induced by intranasal irrigation with Triton X-100 or surgical manipulation results in degeneration of axons of the PON. However, since the stem cells remain undamaged following Triton X-100 irrigation or axotomy, new primary afferents to the olfactory bulb are able to be generated with complete regeneration reported to occur within 30-60 days post-treatment (Harding et al.1977; Graziadei and Monti-Graziadei, 1978a; Graziadei and Monti-Graziadei, 1980; Monti-Graziadei et al.1980; Baker et al.1983).

Intranasal irrigation or application of $ZnSO_4$ to the OE results in coagulation necrosis of the OE (Smith, 1938; Schultz, 1941; Schultz, 1960; Margolis et al.1974; Matulionis, 1975a; Harding et al.1978). Following necrosis, the OE separates from the underlying lamina propria and is sloughed off (Smith, 1938; Schultz, 1941). Since the stem cells lie in the basal olfactory epithelium they are believed to be cast off

along with the OE resulting in degeneration of the axons of the PON. The degenerative effects of $ZnSO_4$ have been reported to persist from 30 days to up 1 year following the lesion (Schultz, 1960; Mulvaney and Heist, 1900; Matulionis, 1975b). $ZnSO_4$ application was the method used to achieve deafferentation of the OB in this study since it results in longterm degeneration of the axons of the PON.

1.9 Effects of Peripheral Denervation on the Olfactory Bulb

Destruction of the primary sensory afferents to the olfactory bulb resulting from axotomy or intranasal irrigation of $ZnSO_4$ or Triton X-100 were the preferred methods of use in behavioral studies of olfactory impaired animals. These methods were thought to be specific to the peripheral nervous system while causing little, if any, direct damage to the CNS. However, degeneration of the primary sensory afferents induced by these methods produces profound morphological changes in the olfactory bulb. Denervation of the bulb is accompanied by (1) a 30-75% decrease in olfactory bulb weight (Margolis et al.1974; Harding et al.1978; Kawano and Margolis, 1982; Baker et al.1984); (2) atrophy or shrinkage of the glomerular layer and individual glomeruli (Margolis et al.1974; Harding et al.1978; Baker et al.1984); (3) shrinkage or complete absence of the olfactory nerve layer (Margolis, 1972; Baker et al.1984); (4) compacted periglomerular cells (Margolis et al.1974).

In addition to the morphological changes in the OB due to removal of the primary afferent input of the olfactory bulb several changes have been reported in neurotransmitter content. The periglomerular cells are by far the principal dopaminergic containing elements of the olfactory bulb (Halasz et al.1981; Halasz and Shepherd, 1983). Following afferent denervation the PG cells exhibit a drastic reduction in tyrosine hydroxylase (TH) immunoreactivity (Baker et al.1983; Baker et al.1984; Kream et al.1984; McLean and Shipley, 1988; Baker et al.1988; Biffo et al.1990). Accompanying the reduction of TH immunoreactivity is a drastic decrease in TH enzyme activity (Nadi et al.1981; Kawano and Margolis, 1982; Baker et

al.1988) and dopamine (DA) content of the denervated bulb (Nadi et al.1981; Kawano and Margolis, 1982; Baker et al.1983). However, reduction of TH immunoreactivity and decreased enzyme activity is not due to degeneration of the PG cells (Pinching and Powell, 1971; Graziadei and Monti-Graziadei, 1980) and the TH immunoreactivity returns to control levels upon regeneration of the afferent innervation following reversible lesions (Nadi et al.1981; Kawano and Margolis, 1982; Baker et al.1983; Baker et al.1984). Thus, the expression of the TH phenotype appears to be dependent upon the presence of the afferent input into the bulb (Nadi et al.1981; Baker et al.1983) and is believed to be mediated at the transcriptional level of TH mRNA (Ehrlich et al.1990). The association between TH and the olfactory nerve exists during development of the OB since the PG cells do not express TH immunoreactivity until contacted by the olfactory nerve (McLean and Shipley, 1988).

In comparison with the reduction in DA content, the biochemically determined levels of norepinephrine (NE) appear to remain unchanged from control levels with any slight increases attributed to the decrease in the size of the bulb after deafferentation (Nadi et al.1981; Kawano and Margolis, 1982; Baker et al.1983). It is not clear, however, if any sprouting of the NE axons into the denervated areas occurred in these studies.

1.10 Objectives of the Study

The main objectives of this study were to determine: (1) if peripheral denervation induced plasticity (or heterotypic collateral sprouting) of the serotonergic system in different layers of the OB; (2) if the potential increase in the density of serotonergic innervation, as a result of collateral sprouting, reflected an accompanying increase in astrocytic expression of S-100, a proposed serotonergic growth factor; (3) if increases in the number of $GFAP^+$ astrocytes occurred in the OB as a consequence of peripheral deafferentation; (4) if peripheral denervation affected the noradrenergic innervation of the OB; (5) if differences exist between adult and young animals in their axonal and astrocytic response to deafferentation of the OB.

METHODS

2.1 Animals

Adult male (250-275g) and postnatal day ten (PND 10), both male and female, Sprague Dawley rats (Charles River) were used in this study. The rats were housed in the animal care facility in the Health Sciences Centre at Memorial University. The animals were kept on a 12 hour light-dark schedule with food and water available *ad libitum*. Procedures performed on the animals were approved by the local animal care committee in accordance with the guidelines of the Canadian Association on Animal Care.

2.2 Anaesthesia

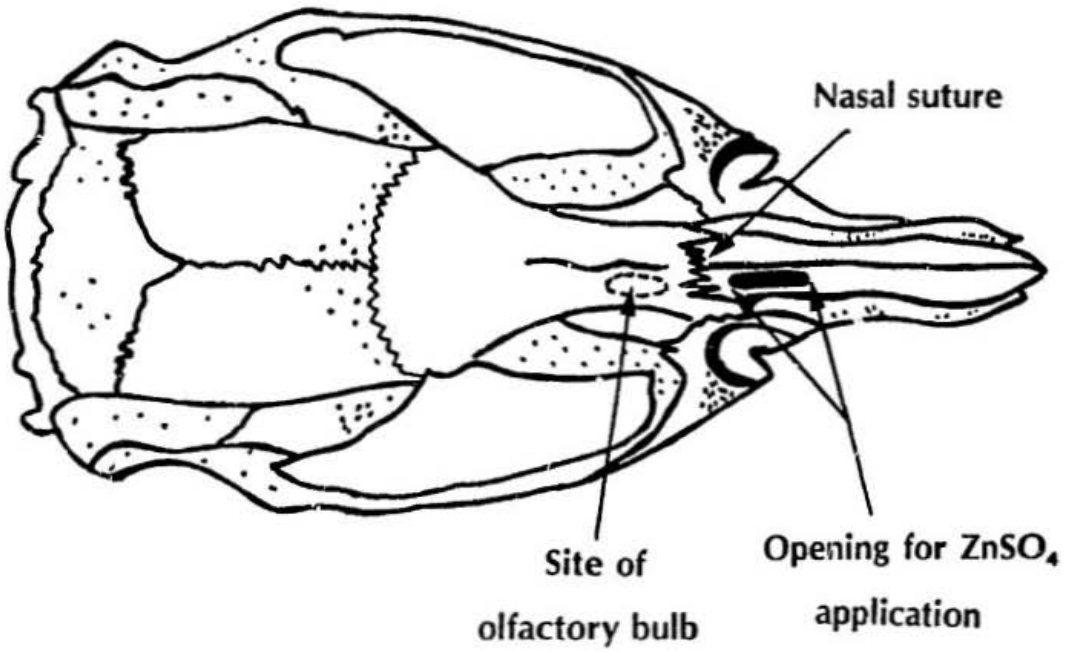
Adult rats were anaesthetized with sodium pentobarbital (40mg/kg, i.p.). The rats were given 0.5ml of atropine (1mg/ml, i.p.), 5-10 minutes prior to being anaesthetized, in order to reduce mucous secretions during surgery. Approximately equal numbers of PND 10 male and female rat pups from the same litter were anaesthetized by hypothermia. The pups were placed in an ice water bath for approximately three minutes or until they failed to respond to a paw pinch. The pups were kept on ice throughout the surgery.

2.3 Surgery

A longitudinal incision was made along the midline between the eyes of the anaesthetized animal. After retraction of the dermis a small hole was drilled immediately adjacent to the midline on the right side (Fig.1). The dorsum of the right nasal cavity was opened to expose the conchae and the respiratory and olfactory epithelium. Unilateral denervation of the OE was achieved by inserting Gelfoam (Upjohn), saturated with 20μl of 0.34M of $ZnSO_4$ (Sigma Chemical Co.) in 0.9% saline into the nasal cavity of PND 10 animals and 50μl of the same solution in adult animals. The cavity was sealed with a thin layer of sterile bone wax (Ethicon Ltd.) before closing the incision using stainless steel wound clips (Clay Adams) on the adult and 6.0 silk sutures (Ethicon Ltd.) on PND 10 animals.

Adult rats were kept under a heat lamp during and after surgery, in order to maintain body temperature, until they completely recovered from the anaesthetic. The rat pups were revived by gentle rubbing after removal from ice and placed under a heat lamp for approximately one half hour before being returned to the mother. The litters were then culled to eight pups to ensure standard nutrition.

Figure 1: Diagram of rat skull indicating site of $ZnSO_4$ application.



2.4 Perfusion

Twenty-one days after surgery the animals were deeply anaesthetized with sodium pentobarbital (80 mg/kg,i.p.) and perfused transcardially with ice cold 0.9% saline (two minutes) followed by 4% paraformaldehyde (Fisher Scientific) and 0.1% glutaraldehyde (Fisher Scientific) in 0.1M phosphate buffer (pH 7.4) for 30 minutes (600-1000 ml). The brains were removed from the skulls, placed in cold fixative for 1 hour and left overnight in cold 20% sucrose in 0.1M phosphate buffer.

In order to optimize the serotonin immunocytochemistry, the animals were given 50mg/kg of pargyline (Fisher Scientific), a monoamine oxidase inhibitor, and tryptophan (Fisher Scientific), a precursor to serotonin, in 0.9% saline i.p., 60 minutes and 30 minutes prior to perfusion, respectively.

2.5 Immunocytochemistry

The day after perfusion, frozen 30μm thick coronal sections of the OB were cut on a cryostat (-20°C) and placed immediately onto chrome alum subbed slides. The sections were then processed for immunocytochemistry (ICC), directly on the slides, with antibodies against tyrosine hydroxylase (TH), serotonin (5-HT), dopamine ß-hydroxylase (DBH), glial fibrillary acidic protein (GFAP) and S-100. The source and dilutions of the primary antibodies were as follows: (1) TH (Eugene Technical) 1:500, (2) 5-HT (INC Star) 1:3000, (3) DBH (Eugene Technical) 1:600, (4) GFAP (INC Star) 1:100 or 1:500, and (5) S-100 (INC Star) 1:10 or 1:100. The sections were (1) incubated in primary antibody in 0.2% Triton X-100 (Sigma Chemical Co.), 0.02% sodium azide (Fisher Scientific), 2% normal goat serum (NGS) in phosphate buffered saline (PBS) at 4°C for 36 hours in sealed plastic containers and then, (2) rinsed 3 times for 5 minutes in PBS on a shaker, (3) incubated in biotinylated goat anti-rabbit IgG (Vectastain Elite Kit, Vector labs) in a PBS solution containing 0.2% Triton X-100 for 1 hour at room temperature, (4) rinsed 3 times for 5 minutes each in PBS, (5) incubated in Vectastain avidin-biotin-peroxidase complex (Vector Labs) for 1 hour at room temperature, (6) rinsed 2 times for 5 minutes each in PBS. Visualization of the enzyme-labelled antibodies was achieved by incubating the sections in 0.05% diaminobenzidine dihydrochloride (DAB, Fisher Scientific) with 0.03% H_2O_2 in 0.1M phosphate buffer for 5-10 minutes. The slides were then rinsed 3 times for 5 minutes

each in phosphate buffer, submerged in deionized H_2O for 10-15 seconds, dehydrated and coverslipped using permount (Fisher Scientific) or prepared for silver intensification.

Silver intensification was performed in order to enhance sections processed for serotonin ICC when staining was considered to be less than optimal. Immediately after the DAB reaction the sections were (1) rinsed 3 times for 5 minutes each in 0.1M phosphate buffer and then, (2) rinsed 2 times for 15 minutes each in 2% sodium acetate (Fisher Scientific), (3) incubated overnight in 10% thioglycolic acid (Fisher Scientific) at 4°C, (4) rinsed 3 times for 15 minutes in sodium acetate and then (5) incubated in physical developer for 10-15 minutes. The physical developer consisted of 200 mg ammonium nitrate (Mallinckrodt), 200 mg silver nitrate (Fisher Scientific), 1 gm tungstosilicic acid (Polysciences,Inc.), 0.4 ml 37% formaldehyde (Fisher Scientific) in 100 ml distilled H_2O added slowly to an equal volume of 5% sodium carbonate (Mallinckrodt) in 100 ml distilled H_2O. Following immersion in the physical developer the slides were (1) rinsed in 1% acetic acid (Fisher Scientific) for 5 minutes, (2) rinsed 3 times for 5 minutes each in 2% sodium acetate (3) incubated in 0.05% gold chloride for 8-10 minutes at 4°C, (4) rinsed once for 5 minutes in 2% sodium acetate, (5) rinsed 2 times for 10 minutes each in 3% sodium thiosulphate (Mallinckrodt), (6) rinsed 2 times for 5 minutes each in 2% sodium acetate, and finally (7) rinsed 3 times for 5 minutes each in 0.1M phosphate buffer. The slides were then rinsed in deionized H_2O, dehydrated and coverslipped using permount.

2.6 Zinc Staining

A modified Timms sulphide silver method for detection of heavy metals (Haug, 1973; Danscher, 1981; Sloviter, 1982) was performed on five operated and three control adult animals to determine if increased amounts of zinc were present in the OB as a result of introduction of $ZnSO_4$ into the nasal cavity.

Three days following surgery, the animals were deeply anaesthetized with sodium pentobarbital (80 mg/kg, i.p.) and perfused transcardially with 11.7g Na_2S (Fischer Scientific) in 1000 ml of 0.1M phosphate buffer at room temperature for 30 minutes at a rate of 30 ml per minute (900-1000 ml) followed by neutral buffered formalin (90 ml 37% formaldehyde in 810 ml 0.1M phosphate buffer) for 25 minutes at a rate of 30 ml per minute (700-800 ml). The brains were removed from the skulls and placed in neutral buffered formalin for approximately 90 minutes. The brains were cut frozen at 30μm thickness in the coronal plane on a cryostat (-20°C) and the sections were placed immediately onto chrom alum subbed slides. Representative sections were taken from the OB and anterior olfactory nucleus (AON). The sections were then air dried for 15 minutes, fixed in 96% ethanol and hydrated prior to being submerged in developer. The developer consisted of the following solutions: (1) protecting colloid - 250g of powdered gum arabic (ICN Biochemicals) in 500 ml deionized H_2O made up at least 5 days prior to use and filtered through gauze, (2) citrate buffer - 25.5g citric acid (ICN Biochemicals) and 23.5g sodium citrate (ICN Biochemicals) brought to a final volume of 100 ml with deionized H_2O, (3) reducing agent - 5.67g hydroquinone (ICN Biochemicals) in 100 ml deionized H_2O prepared

just before use, (4) silver ion supply - 8.5g silver nitrate (Fisher Scientific) in 50 ml deionized H_2O which must be kept in the dark until used. The developer was prepared by carefully mixing 60 ml of (1), 10 ml of (2), 30 ml of (3) and adding 0.5 ml (4) immediately before use.

The slides were placed in glass Coplin jars, covered with developer and placed in a H_2O bath at 26°C. The slides were developed for 30-70 minutes with a complete set of slides for each animal removed from the developer every 5-10 minutes. The slides were rinsed thoroughly in H_2O to stop the staining, dehydrated, immersed briefly in xylene and coverslipped using permount.

2.7 Analysis

2.7.1 Quantification of the Deafferentation of the Olfactory Axons

Deafferentation of the olfactory axons was initially confirmed by visually examining the OB sections for loss of TH immunoreactivity in the PG cells which require the presence of the OE in order to express the TH phenotype (Nadi et al.1981; Baker et al.1983). This visual analysis has been the standard protocol by several laboratories (Harding et al.1978; Nadi et al.1981; Baker et al.1983; Baker et al.1984) and was the method that was initially adopted.

Following visual examination of OB sections, quantification of the number of PG cells exhibiting TH immunoreactivity was performed in the medial aspect of the circumference of the GL which consistently exhibited diminished TH immunoreactivity in deafferented animals. PG cells expressing TH immunoreactivity were counted (25X objective) with the aid of an image analysis system (Bioquant, R & M Biometrics). The number of TH immunoreactive cells that were present in three glomerular profiles were counted from a total of three sections per bulb per animal to obtain an average number of TH immunoreactive cells per glomerular profile for bulbs ipsilateral and contralateral to the lesion and in non-lesioned control animals. In the bulbs ipsilateral to the lesion, the PG cells expressing TH immunoreactivity were counted in the area of the GL where visual examination revealed the most obvious deafferentation, namely the medial aspect of the circumference of the GL regardless if diminished TH immunoreactivity extended throughout the entire circumference of the GL. Cells counted in bulbs contralateral to the lesion were taken

from the same area in the GL as cell counts for the ipsilateral bulb. In control animals the cell counts were taken from the medial aspect of the circumference of the GL, which often corresponded to the lesioned area in deafferented animals.

2.7.2 Density of Immunoreactive Fibres and Astrocytes

Following verification of peripheral deafferentation, adjacent sections from denervated and control animals were selected to determine the effect of the lesion on the density of innervation of serotonergic and noradrenergic immunoreactive axons in the OB. The effect of the lesion on density of GFAP and S-100 immunoreactive astrocytes was also examined.

Immunoreactive axons from control bulbs and bulbs contralateral and ipsilateral to the denervated OB were drawn using a camera lucida (40X objective) attachment to an Olympus microscope. Immunoreactive serotonergic fibres were drawn from the glomerular layer (GL), external plexiform layer (EPL), and infra EPL region, which included the mitral cell layer (MCL), internal plexiform layer (IPL) and granule cell layer (GCL), while noradrenergic immunoreactive axons were drawn from the EPL and infra EPL region. Similarly, the density of astrocytic cells expressing GFAP and S-100 immunoreactivity was determined in all OB regions examined using camera lucida drawings (20X objective) of cell distribution and image analysis.

The density of immunoreactive fibres ($\mu m/100\mu m^2$) and astrocytes ($cells/100\mu m^2$) in the different areas of the OB was quantitated using the camera lucida drawings transferred to an image analysis system (Bioquant, R & M Biometrics). A 200μm region of the camera lucida drawing, extending from the beginning to end of each region of the OB examined, was selected for analysis (Fig.2). The camera lucida drawings were then traced on a digitizing tablet

(SummaSketch, Summa Graphics) to obtain the density measurements. A total of three camera lucida drawings per animal per bulb region were used to obtain an average density of immunoreactive fibres and astrocytes in control and denervated animals.

To compensate for shrinkage of the olfactory bulb resulting from the deafferentation, density measurements obtained from bulbs ipsilateral and contralateral to the lesion were normalized with respect to control bulbs. The average area of each layer examined of the bulbs ipsilateral and contralateral to the lesion, for all operated animals, was divided by the average area of comparable layers of the control animals to obtain a normalization factor (NF). The average density measurements of each lesioned animal was then multiplied by the normalization factor to obtain the final density measurement for the ipsilateral and contralateral bulb (Rhoades et al.1990). For instance, if the area of the ipsilateral bulb decreased to 70% of the control, the fibre density in the ipsilateral bulb would be multiplied by 0.7 to obtain the final density measurement.

$$NF = A_1/A$$

Where: (1) A is the average area of the layer of the control bulb

(2) A_1 is the average area of the layer of the ipsilateral or contralateral bulb

Normalized Density = NF X D

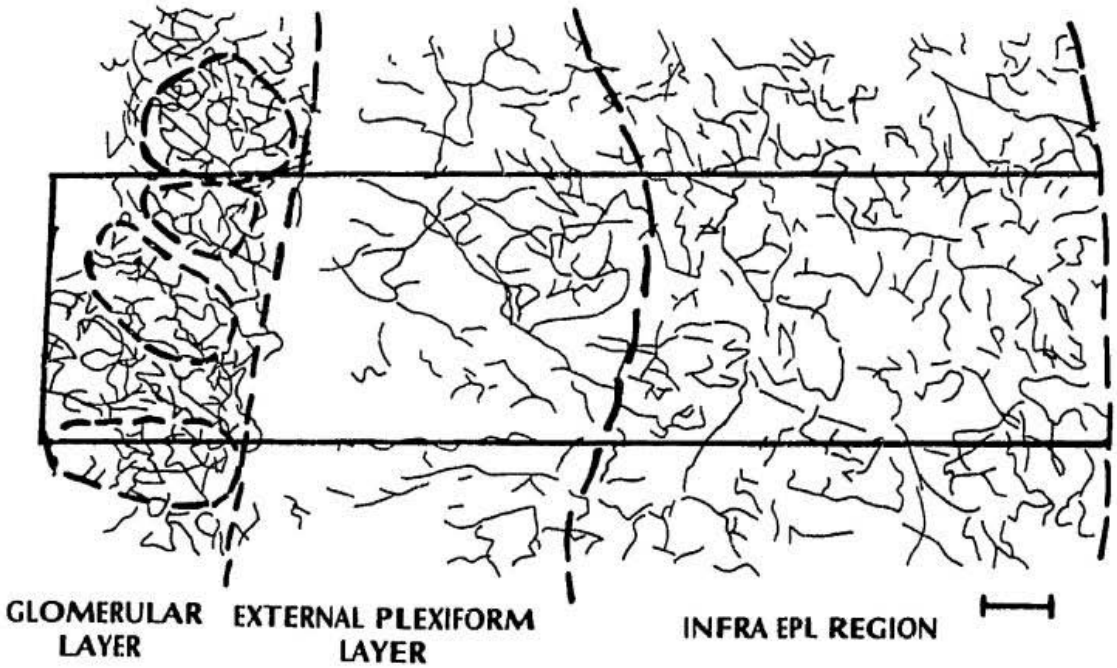
Where: (1) NF is the normalization factor

(2) D is the density of immunoreactive fibres or cells in the ipsilateral or contralateral bulb

A multivariate statistical analysis (MANOVA) is often utilized to test hypotheses involving more than three dependent variables. In this study the dependent variables were the density of immunoreactive fibres/cells in the control, contralateral and ipsilateral bulbs in each of the three regions of the OB examined while the independent variable was the $ZnSO_4$-induced lesion of the afferent input to the OB. The MANOVA tests if the means of the dependent variables are equal and indicates if the experimental treatment had a generalized effect which has a small probability of happening by chance (Kirk, 1968; Kirk, 1982). A MANOVA was performed on each separate area of the OB examined, namely the GL, EPL and infra EPL regions, to determine if significant differences existed among the normalized fibre/cell density measurements of the control, contralateral and ipsilateral bulbs. If the F-statistic generated by the MANOVA indicated that over all significant differences were apparent between the control, contralateral and ipsilateral bulbs in each region of the OB then further statistical analysis was performed to determine which comparisons were significantly different. A t-test may be used to compare

population means of a specific set of hypotheses (Kirk, 1968; Kirk, 1982). Therefore, in the present study an unpaired, two-tailed t-test was performed to determine if significant differences existed between the normalized fibre/cell density measurements of control animals and bulbs ipsilateral to the lesion. A post-hoc Tukey test may be used to determine if the experimental treatment results in significant differences between population means when the comparisons were unplanned or not originally defined by a specific set of hypothesis (Kirk, 1968; Kirk, 1982). Thus, in this study a post-hoc Tukey Multiple Comparisons test was performed to compare the normalized fibre/cell densities of control and contralateral bulbs and the density measurements obtained for bulbs ipsilateral and contralateral to the lesion. The data were plotted using SigmaPlot (Jandel Scientific).

Figure 2: Camera lucida drawing (40X objective) of immunoreactive serotonergic axons in a PND 31 control animal showing the 200 μm boxed region of a drawing selected for image analysis.



2.7.3 Relationship Between the Density of Serotonergic Fibres and Astrocytic Expression of S100

In order to determine if potential changes in the density of serotonergic fibres were related to a change in the astrocytic expression of S100, an analysis for linear correlation was performed between the density measurements obtained for serotonergic immunoreactive fibres and S100 immunoreactive astrocytes of lesioned and non-lesioned control animals in both adult and PND 31 animals. The analysis of linear correlation was performed by combining the density measurements obtained for serotonergic fibres and S100 astrocytes from all layers of the OB examined in control bulbs and bulbs ipsilateral and contralateral to the lesion with adults and PND 31 animals subjected to individual correlation analysis.

2.7.4 Relative Optical Density Measurements of Zinc Staining

An image analysis system (Bioquant, R & M Biometrics) was used to obtain relative optical density (R.O.D.) measurements in order to quantitate the intensity of zinc staining in control animals and in operated animals three days after unilateral application of $ZnSO_4$ to the OE. Relative values of optical density were used to control for variations in staining intensities or section thickness. The optical density (O.D.) measurements were determined for the GL, EPL and infra EPL region of three adjacent sections for the control animals and the lesioned animals in bulbs ipsilateral and contralateral to the lesion. For each section analyzed the O.D. of the middle of the ependymal zone was taken to represent the intensity of the background staining. The R.O.D. measurements of the GL, EPL and infra EPL were obtained by subtracting the O.D. of the background from the O.D. of the area of interest and dividing by the O.D. of the background. This calculation resulted in R.O.D. values between 0.0 (white or unreactive for zinc) and 1.0 (black or densely reactive for zinc).

RESULTS

3.1 Tyrosine Hydroxylase Immunocytochemistry

As mentioned previously, reduction of TH expression in the PG cells is indicative of a successful lesion of the olfactory epithelium and subsequent degeneration of the afferent olfactory neurons from the glomerular layer of the OB (Baker et al.1983; Baker et al.1984; Kream et al.1984; McLean and Shipley, 1988; Baker et al.1988; Biffo et al.1990). Reduction of TH immunoreactivity in the adult animals did not always occur throughout the entire circumference of the GL in the OB sections examined. However, reductions in TH immunoreactivity consistently occurred in the medial aspect of the GL in sections from the rostral and medial portions of the ipsilateral OB (Fig. 3a,b). Cell counts in this area of the GL indicated that in the ipsilateral bulb there was a significant decrease in the number of PG cells expressing the TH enzyme when compared to non-lesioned controls ($p<0.0001$) and when compared to bulbs contralateral ($p<0.001$) to the lesion (Fig.4). In addition, a significant decrease ($p<0.05$) in the number of PG cells expressing TH immunoreactivity in the medial aspect of the GL was observed in bulbs contralateral to the lesion in comparison to control animals (Fig.4).

The loss of TH staining in the younger animals was often more widespread than that observed in the adult animals, with almost complete loss of TH staining sometimes occurring throughout the entire circumference of the GL in PND 31 animals. In PND 31 animals (Fig.5) a significant decrease occurred in the number

of PG cells staining for TH in the medial aspect of the GL in bulbs ipsilateral to the lesion compared to non-lesioned controls ($p<0.0001$) and bulbs contralateral ($p<0.001$) to the lesion (Fig.6). A significant decrease ($p<0.05$) in the number of PG cells expressing TH was also apparent in the bulbs contralateral to the lesion in comparison to control animals (Fig.6).

In both the adult and PND 31 animals the loss of TH immunoreactivity consistently occurred in sections taken from the rostral and middle portions of the OB and more specifically in the medial region of the GL in each individual bulb section. Therefore, in order to optimize consistency, the density of fibres and astroglial cell bodies obtained from sections adjacent to those exhibiting consistent loss of TH staining were taken from a standardized location, namely the medial region of each area of the OB examined, in both adult and PND 31 animals.

Figure 3: Darkfield photomicrograph showing TH immunoreactivity in the glomerular layer (GL)in the contralateral (A) and ipsilateral (B) bulb of lesioned adult animal. The decline in the TH immunoreactivity in the GL of the ipsilateral bulb (B) is indicative of successful lesion of the primary olfactory neurons. Note the shrinkage of the GL and the absence of the olfactory nerve layer (ONL) in (B) which also indicates deafferentation of the OB. The TH immunoreactivity may appear more intense in (B) due to the shrinkage of the GL. Magnification is the same for (A) and (B) with the scale bar representing 50μm.

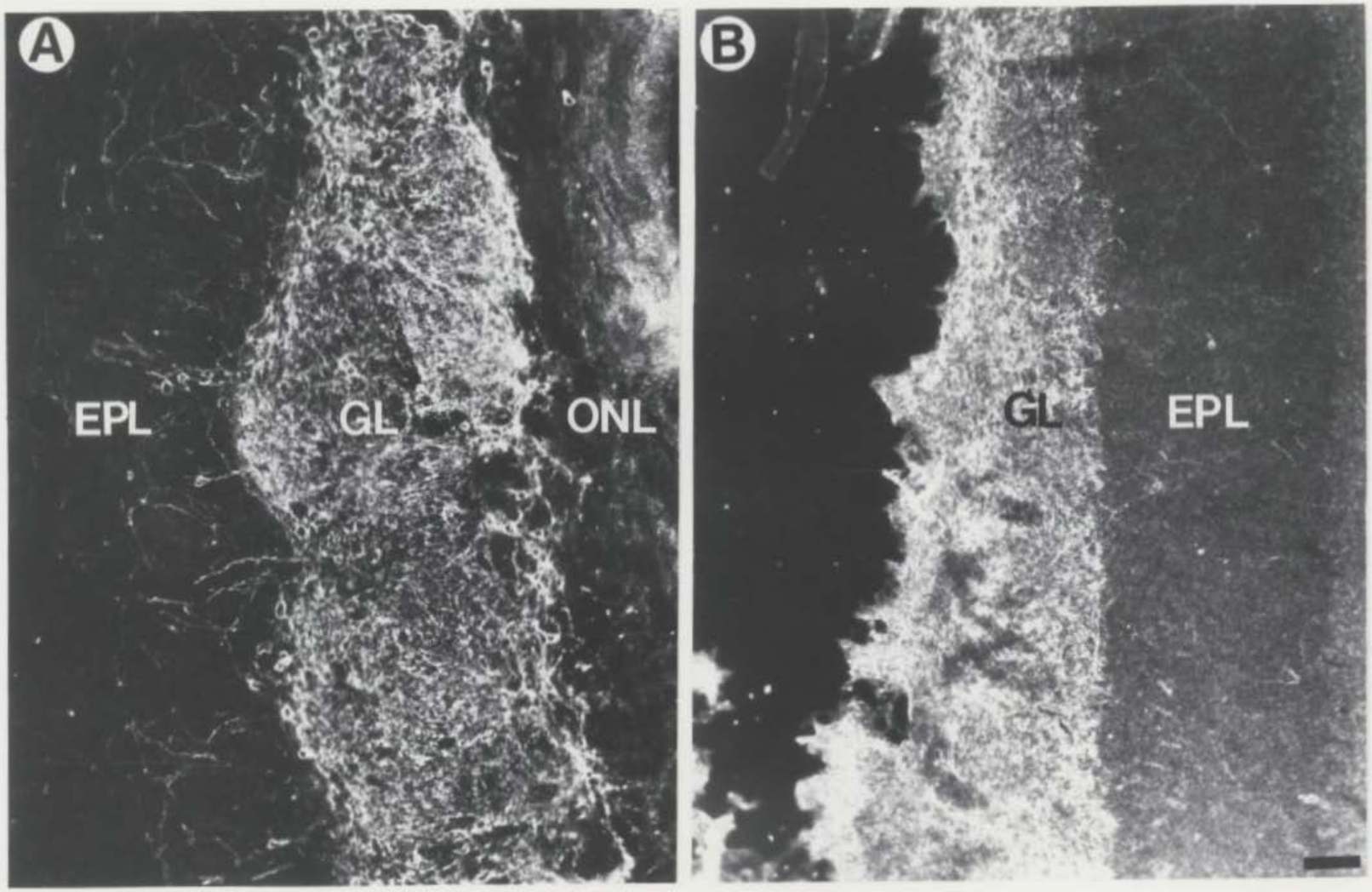

A
EPL
GL
ONL
B
GL
EPL

Figure 4: The mean number of PG cells per glomerular profile expressing TH immunoreactivity (±S.E.M.) in adult animals 21 days following peripheral deafferentation of the OB. The asterisks (***,$p<0.001$; **, $p<0.01$; *, $p<0.05$) represent significant differences in the number of PG cells expressing TH staining in lesioned and non-lesioned control animals.

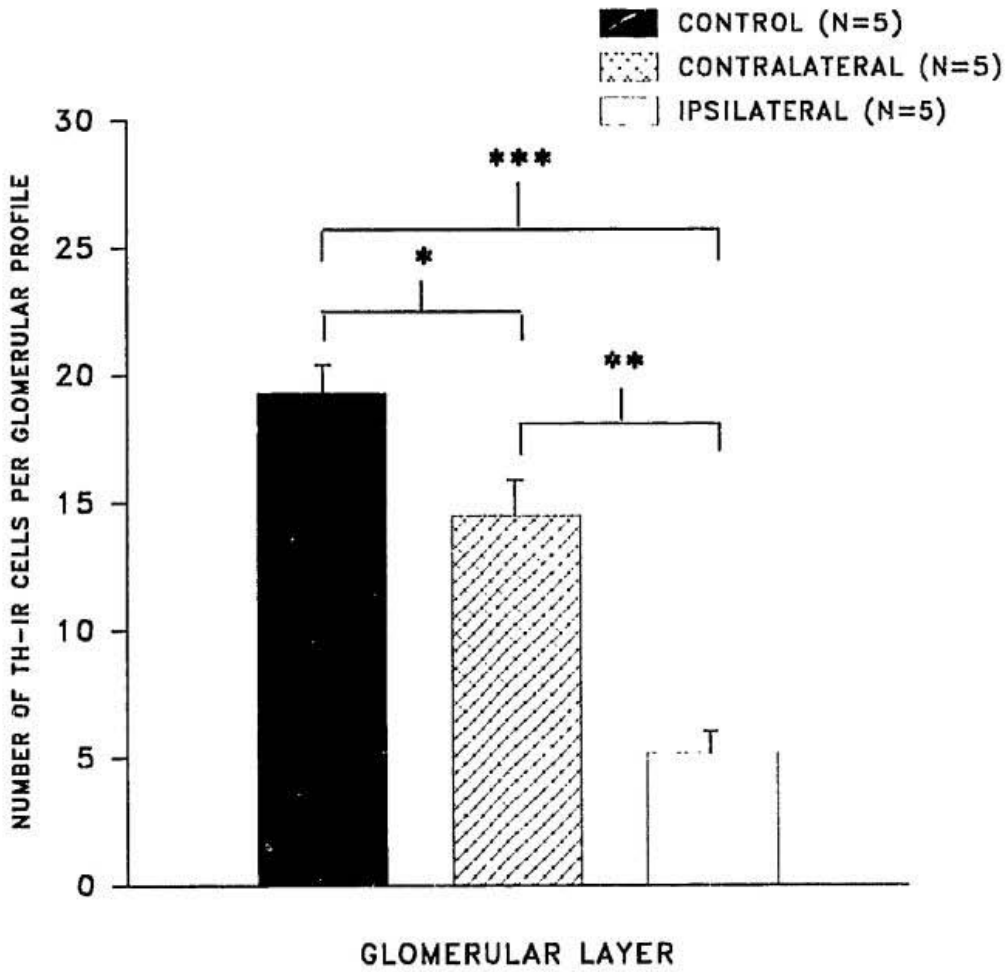


Figure 5: Darkfield photomicrograph showing TH immunoreactivity in the glomerular layer (GL) of a lesioned PND 31 animal. The decline in TH immunoreactivity in the bulb on the right in (A,B) is ipsilateral to the lesion site and is indicative of a successful lesion of the primary olfactory neurons. Magnification in (B) is higher than (A) with scale bars representing 50μm.

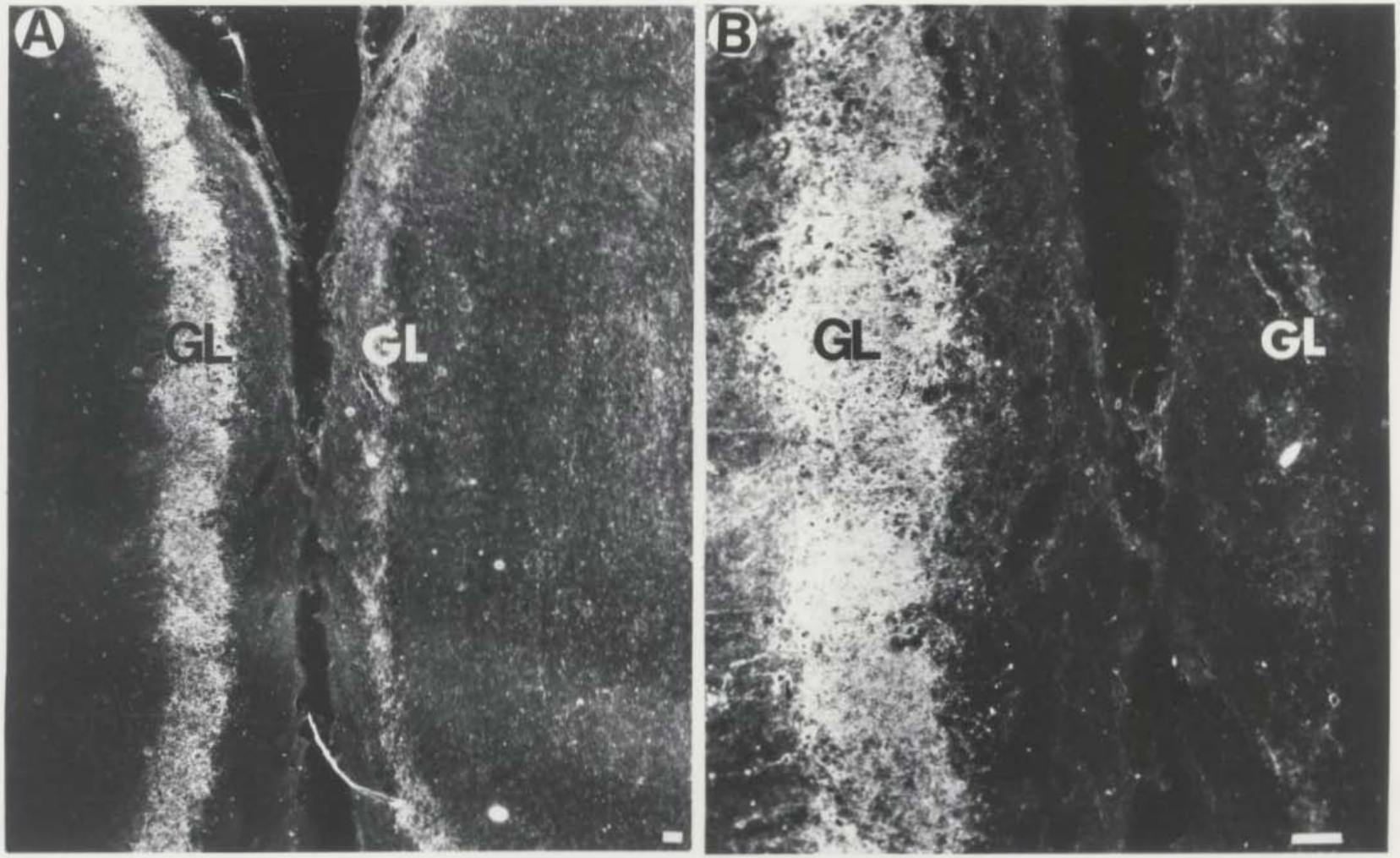
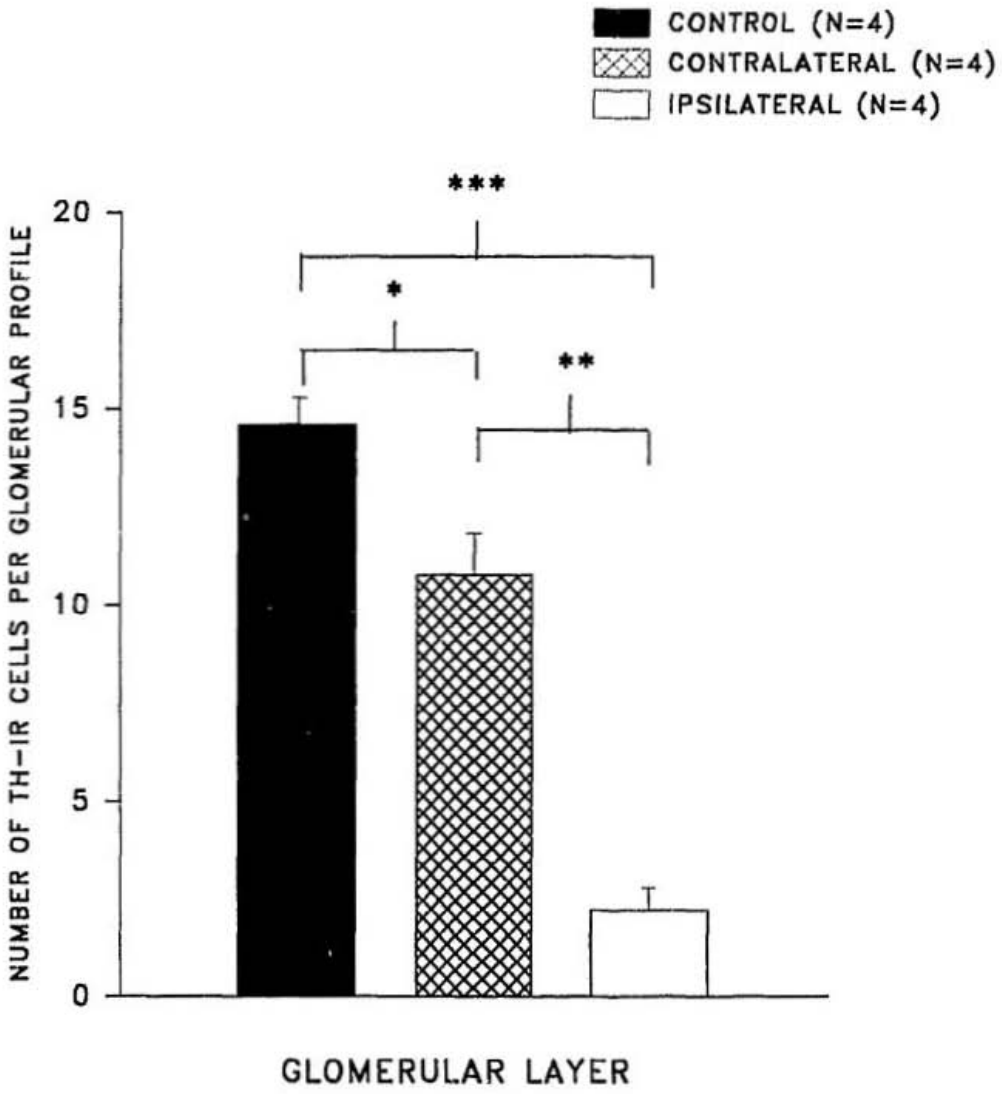

A
GL
GL
B
GL
GL

Figure 6: The mean number of PG cells per glomerular profile expressing TH immunoreactivity (±S.E.M.) in PND 31 animals 21 days following peripheral deafferentation of the OB. The asterisks (***, $p<0.001$; **, $p<0.01$; *, $p<0.05$) represent significant differences in the number of PG cells expressing TH staining in lesioned and non-lesioned control animals.



3.2 Density of Serotonin Immunoreactive Fibres

In the adult animal, there was a significant decrease in the density of serotonergic fibres in the GL ($p<0.001$), EPL ($p<0.001$) and infra EPL ($p<0.05$) in the bulbs ipsilateral to the lesion compared to non-lesioned controls 21 days following deafferentation (Fig. 7,8). In the GL and EPL region, no significant differences in serotonergic fibre density were observed between bulbs contralateral and ipsilateral to the lesion or between contralateral bulbs and non-lesioned controls. However, the density measurements obtained for the contralateral bulbs in these regions exhibited a tendency (albeit non-significant) to lie between those values obtained for the non-lesioned control animals and bulbs ipsilateral to the lesion. In contrast to the GL and EPL comparisons, the infra EPL region exhibited a significant decrease ($p<0.05$) in fibre density between the bulbs contralateral and ipsilateral to the lesion (Fig.8).

In contrast to the adult animals, no significant differences occurred in the density of serotonergic fibres in the GL and EPL regions of lesioned and non-lesioned PND 31 animals (Fig. 9,10). In the infra EPL region, however, there was a significant increase ($p<0.05$) in the density of serotonergic fibres in the bulbs ipsilateral to the lesion compared to non-lesioned control animals (Fig.10). In the infra EPL region, the mean serotonergic fibre density measurements appeared to be the same in bulbs contralateral to the lesion and in control bulbs, yet a significant difference was observed between the control and the ipsilateral bulbs but not between the contralateral and ipsilateral bulbs (Fig.10). This difference in significance arises from

the differing, yet necessary, statistical methods performed to determine significance between the controls and the ipsilateral bulbs and between the ipsilateral and contralateral bulbs as explained in the methods section.

Figure 7: Darkfield photomicrograph of serotonergic immunoreactive fibres in the OB of control (A) adult animals and in the bulbs contralateral (B) and ipsilateral (C) to the lesion in denervated animals. Note the density of serotonin immunoreactive fibres appears greater in (C) than in (B) and (A) as consequence of shrinkage of the layers of the OB following deafferentation. Magnification is the same in (A), (B), and (C) with the scale bar representing 50μm. Abbreviations: glomerular layer (GL), external plexiform layer (EPL), mitral cell layer (MCL), granule cell layer (GCL).

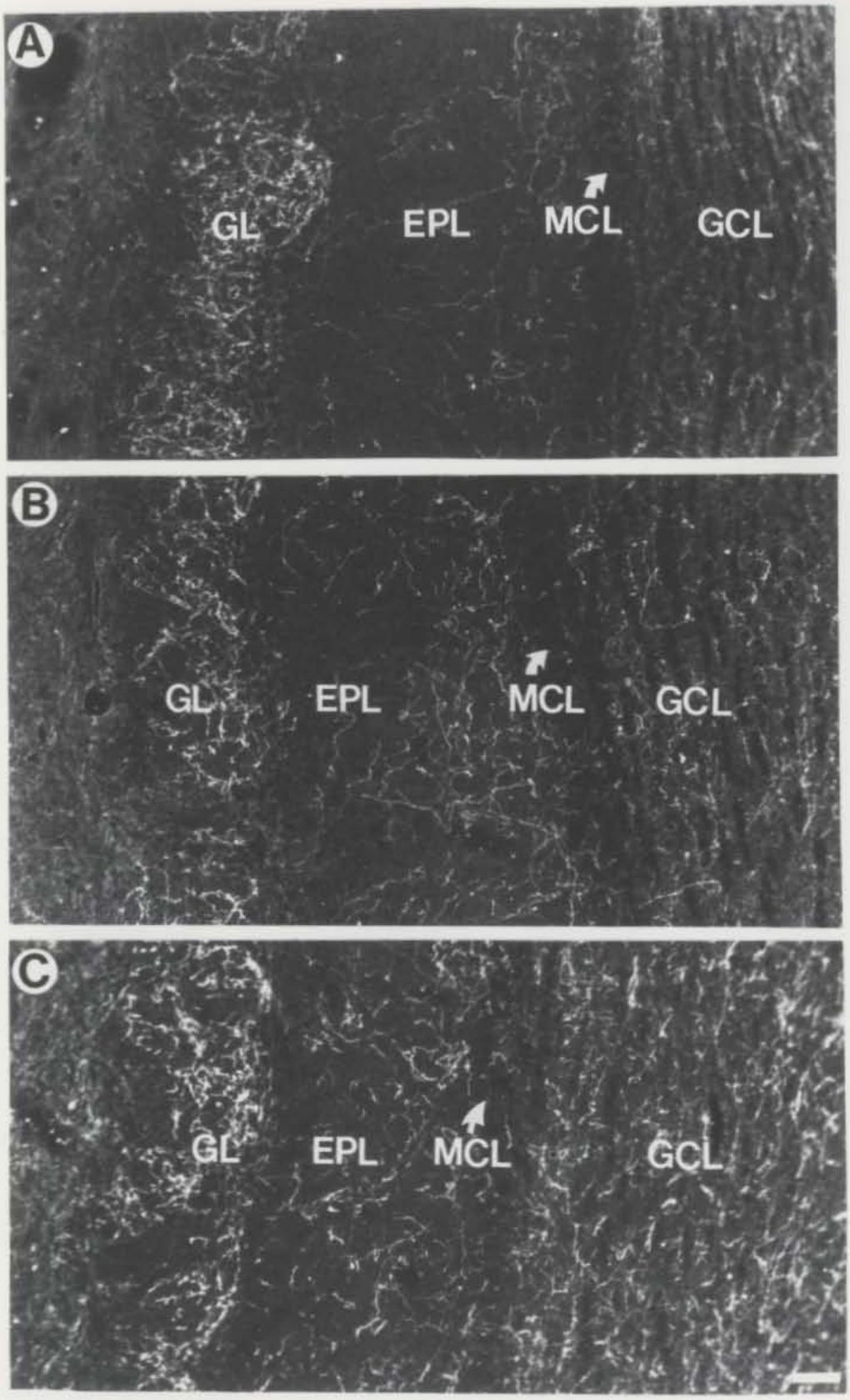

A
GL
EPL
MCL
GCL
B
GL
EPL
MCL
GCL
C
GL
EPL
MCL
GCL

Figure 8: Mean density of serotonergic immunoreactive fibres (± S.E.M.) in adult animals 21 days following peripheral deafferentation of the OB. The asterisks (**, $p<0.01$; *, $p<0.05$) represent a significant decrease in the density of serotonergic fibres in the bulbs ipsilateral to the lesion compared to control bulbs in the GL, EPL and infra EPL and between the contralateral and ipsilateral bulbs in the infra EPL region.

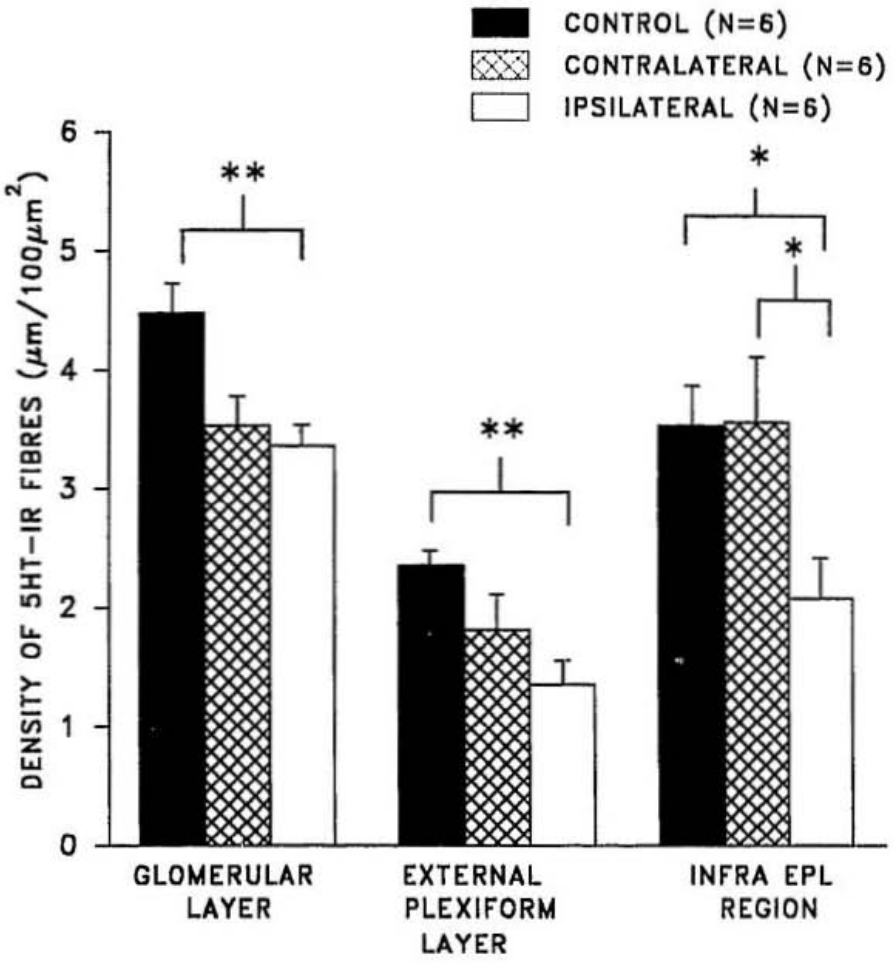


Figure 9: Darkfield photomicrograph of serotonergic immunoreactive fibres in the OB of control (A) PND 31 animals and in the bulbs contralateral (B) and ipsilateral (C) to the lesion in denervated animals. Note the density of serotonin immunoreactive fibres appears greater in (C) than in (B) and (A) as consequence of shrinkage of the layers of the OB following deafferentation. Magnification is the same in (A), (B), and (C) with the scale bar representing 50μm. Abbreviations: glomerular layer (GL), external plexiform layer (EPL), mitral cell layer (MCL), granule cell layer (GCL).

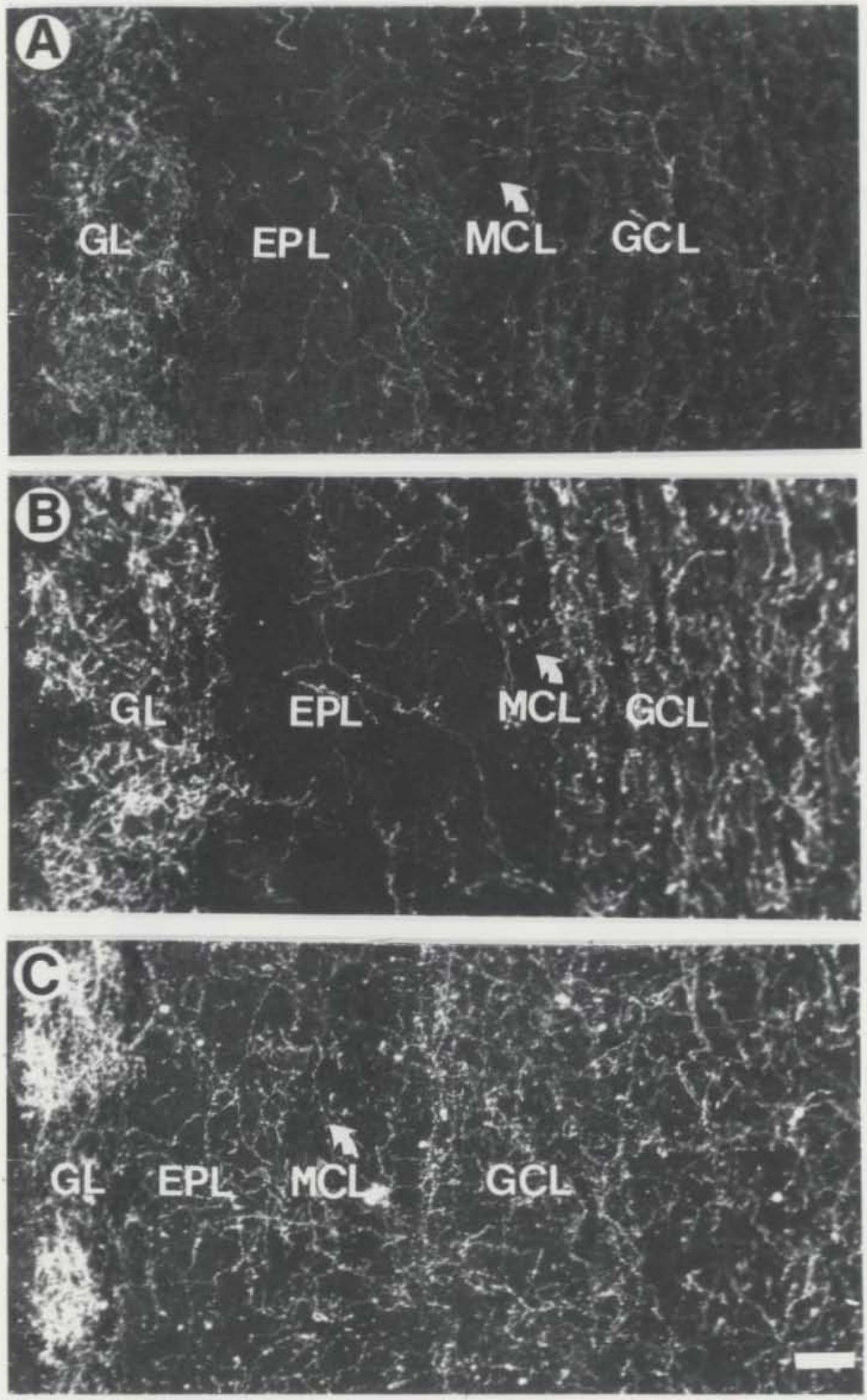
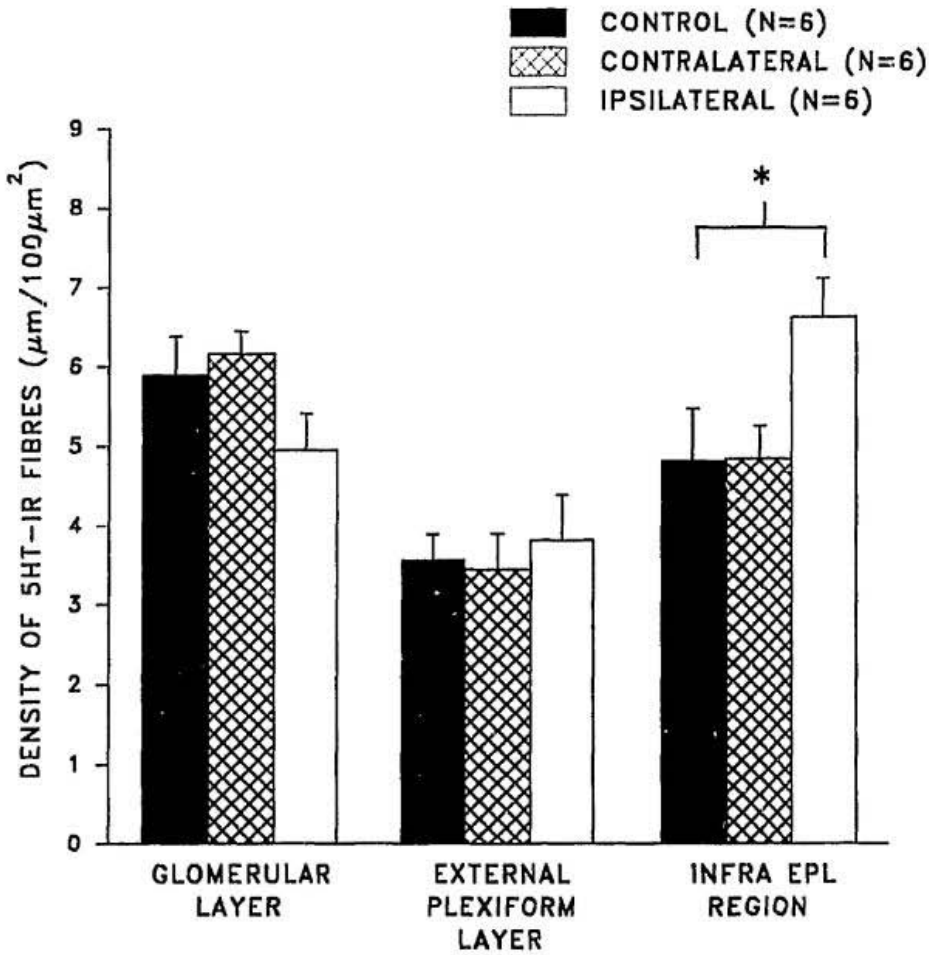

A
GL
EPL
MCL
GCL
B
GL
EPL
MCL
GCL
C
GL
EPL
MCL
GCL

Figure 10: Mean density of serotonergic immunoreactive fibres (±S.E.M.) in PND 31 animals 21 days following peripheral deafferentation of the OB. The asterisk represents a significant increase ($p<0.05$) in the density of serotonergic fibres in the infra EPL of bulbs ipsilateral to the lesion over the non-lesioned controls. The scale bar in the lower right of the figure represents 50μm.



3.3 Density of S-100 Immunoreactive Astrocytes

In the adult (Fig. 11,12) and PND 31 animals (Fig. 13,14), no significant differences in the density of S-100 immunoreactive astrocytes occurred between the lesioned and non-lesioned control animals in all regions of the OB examined.

Figure 11: Photomicrograph of S100 immunoreactive astrocytes in the OB of control (A) adult animals and in the bulbs contralateral (B) and ipsilateral (C) to the lesion in denervated animals. Magnification is the same in (A), (B), and (C) with the scale bar representing 50μm. Abbreviations: glomerular layer (GL), external plexiform layer (EPL), mitral cell layer (MCL), granule cell layer (GCL).

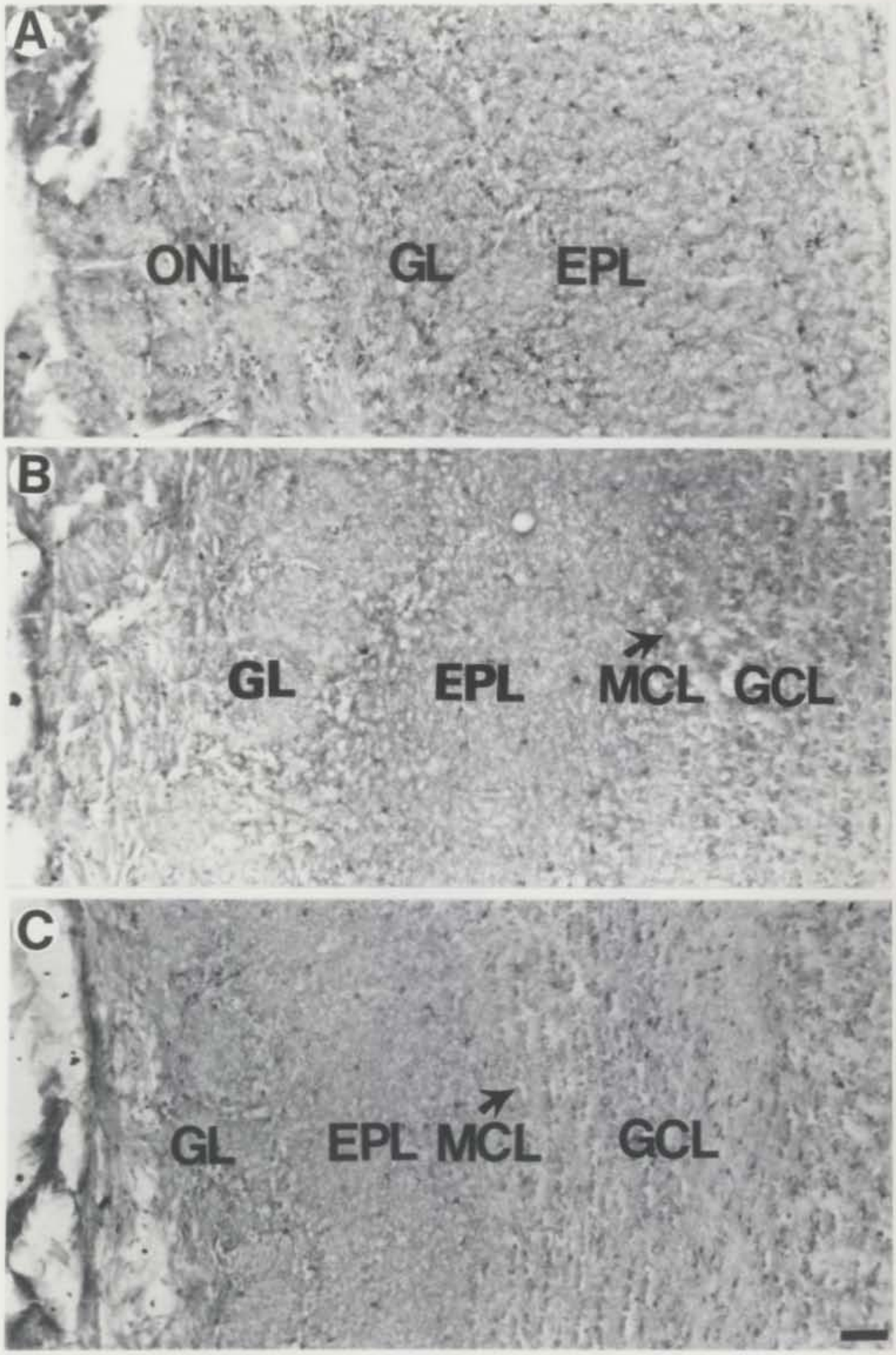

A
ONL
GL
EPL
B
GL
EPL
MCL
GCL
C
GL
EPL
MCL
GCL

Figure 12: Mean density of S100 immunoreactive astrocytes (±S.E.M.) in adult animals 21 days following peripheral deafferentation of the OB.

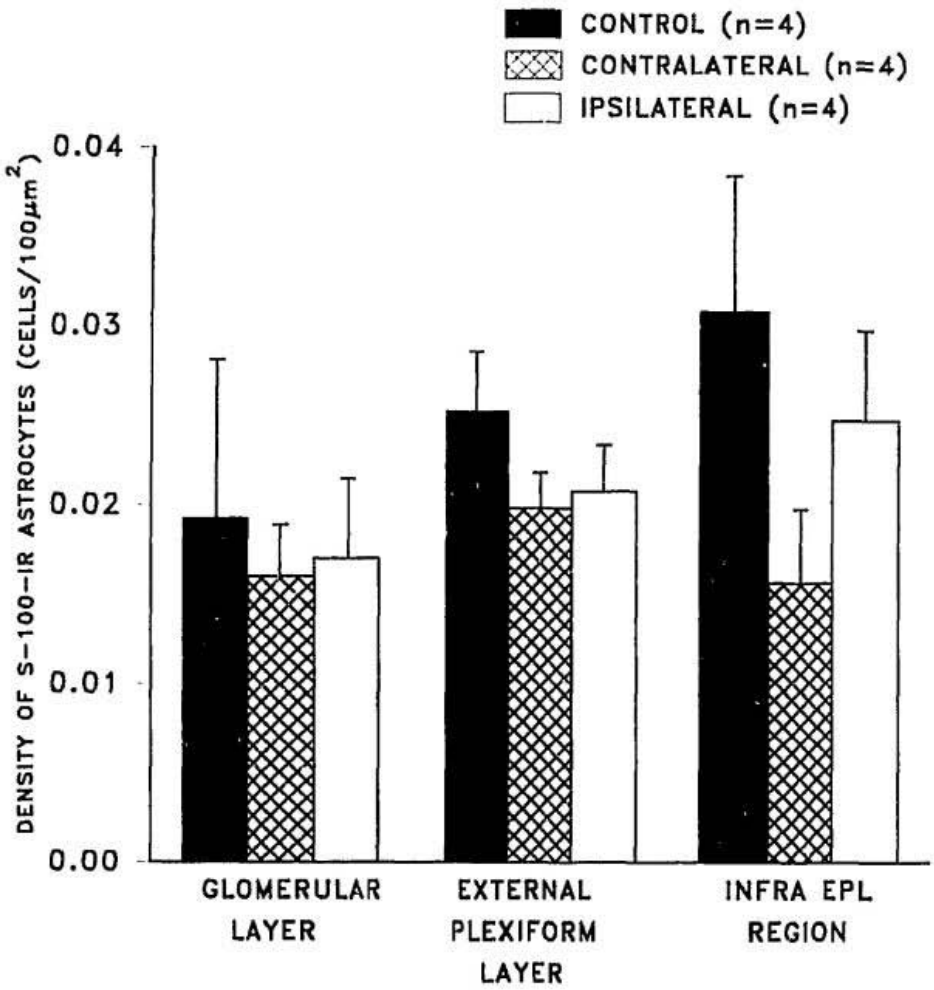


Figure 13: Photomicrograph of S100 immunoreactive astrocytes in the OB of control (A) PND 31 animals and in the bulbs contralateral (B) and ipsilateral (C) to the lesion in denervated animals. Magnification is the same in (A), (B), and (C) with the scale bar representing 50μm. Abbreviations: glomerular layer (GL), external plexiform layer (EPL), mitral cell layer (MCL), granule cell layer (GCL).

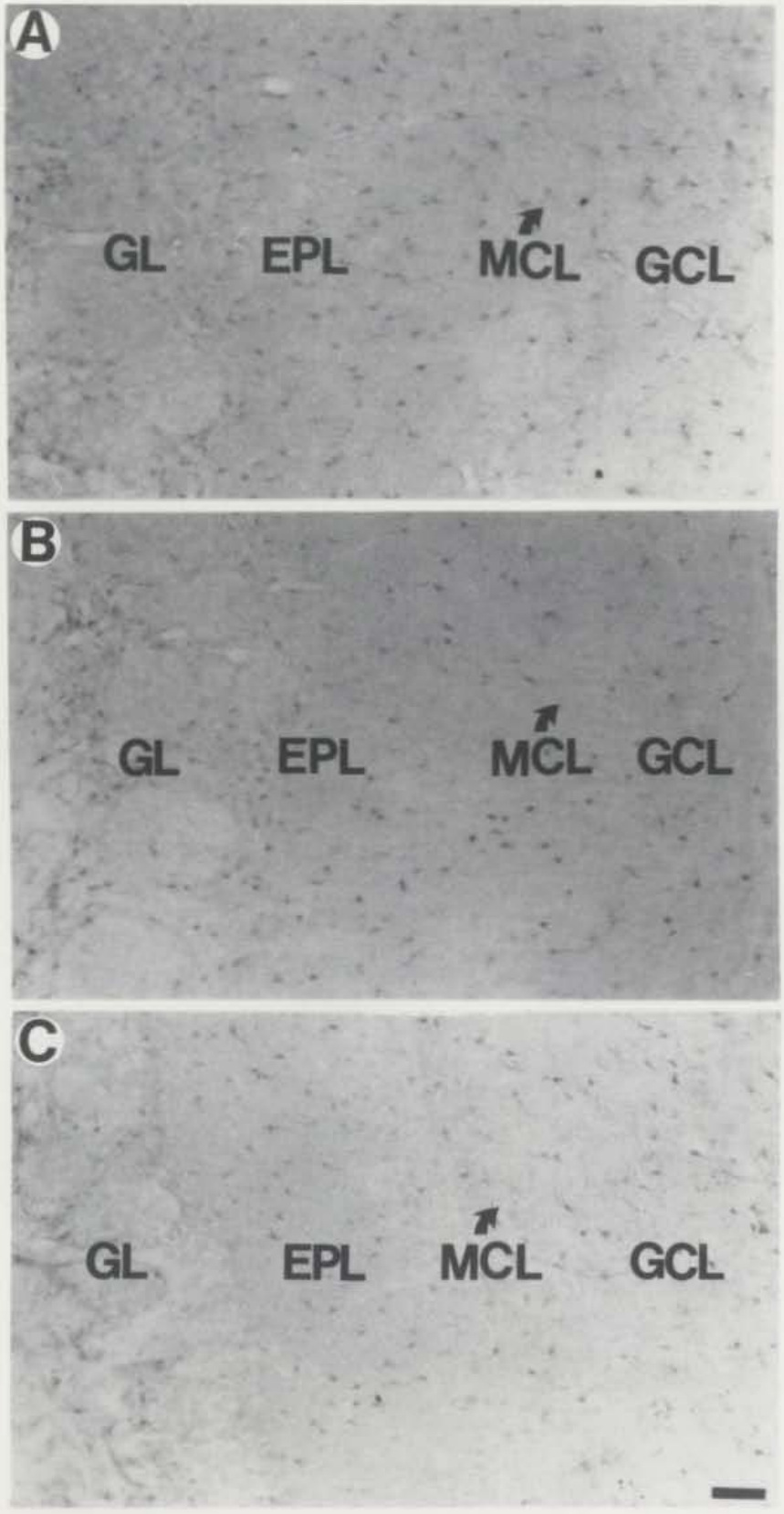
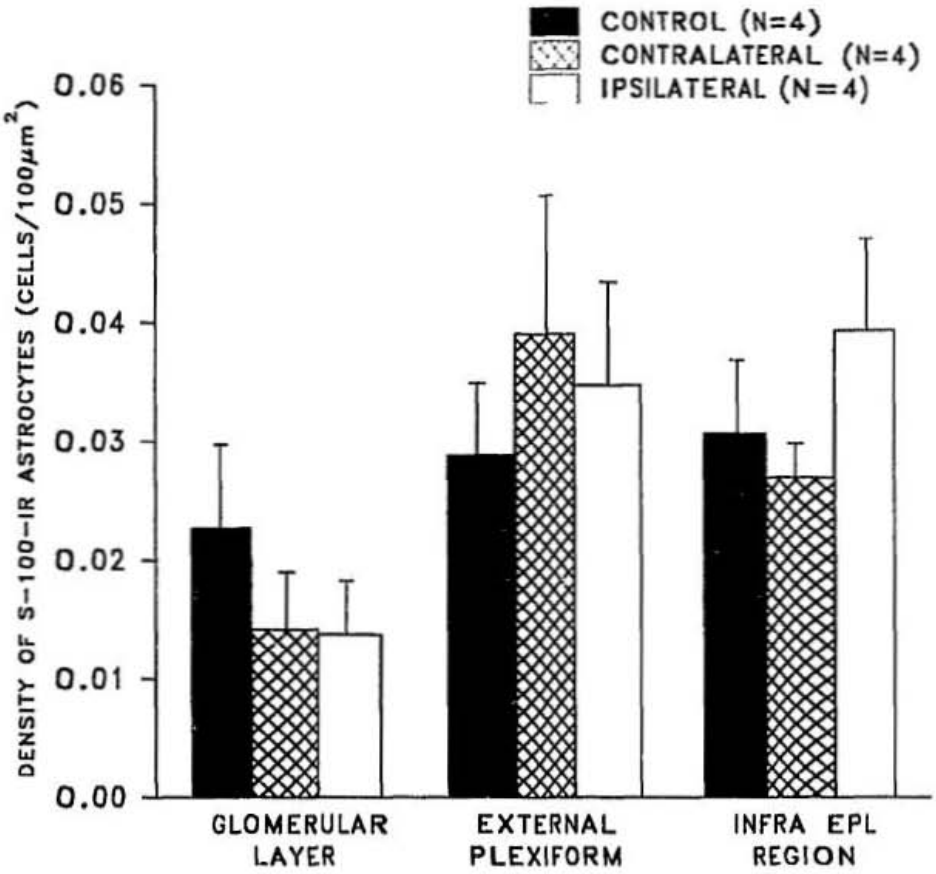

A
GL
EPL
MCL
GCL
B
GL
EPL
MCL
GCL
C
GL
EPL
MCL
GCL

Figure 14: Mean density of S100 immunoreactive astrocytes (±S.E.M.) in PND 31 animals 21 days following peripheral deafferentation of the OB.



3.4 Correlation of the Density of Serotonergic Fibres and Astrocytic Expression of S100.

In both the adult (Fig.15) and PND 31 (Fig.16) animals there was no correlation between the density of serotonergic innervation and the density of astrocytes expressing S-100 in lesioned and non-lesioned control animals 21 days following deafferentation of the OB.

Figure 15: Graph showing lack of correlation (R= -0.1844) between the mean density of serotonergic fibres and S100⁺ astrocytes in the OB of lesioned and non-lesioned adult animals 21 days following peripheral deafferentation.

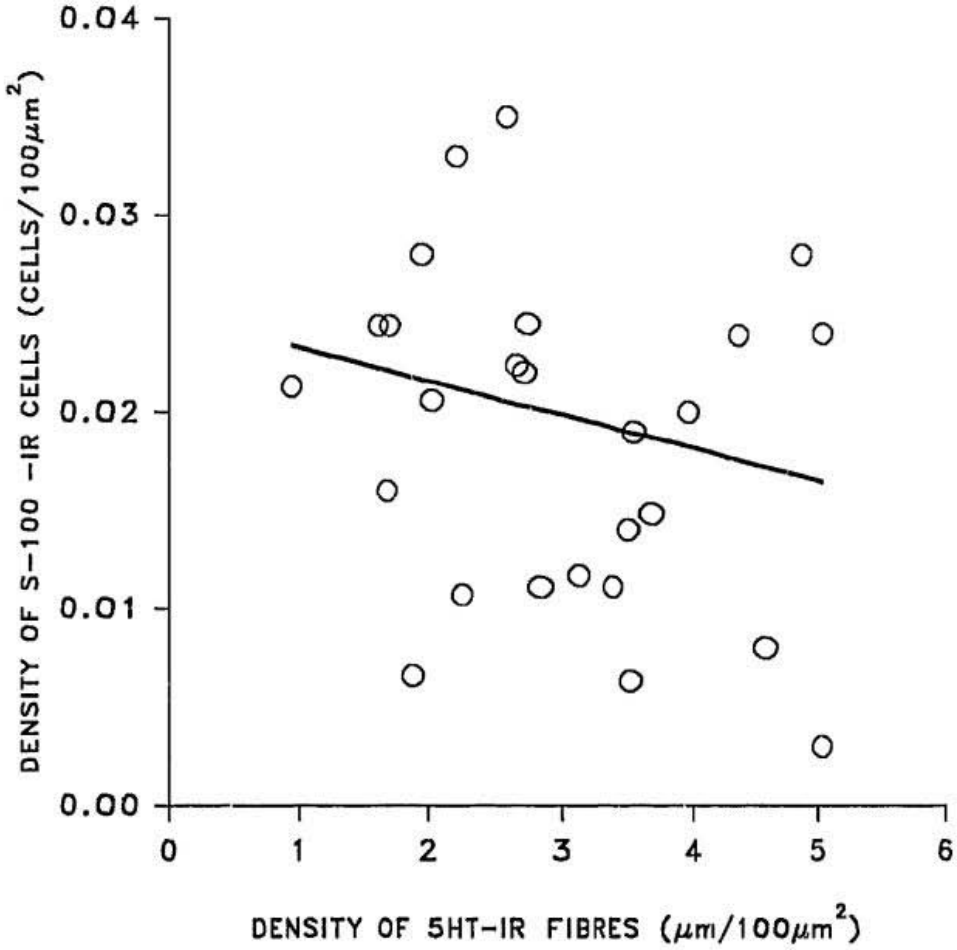
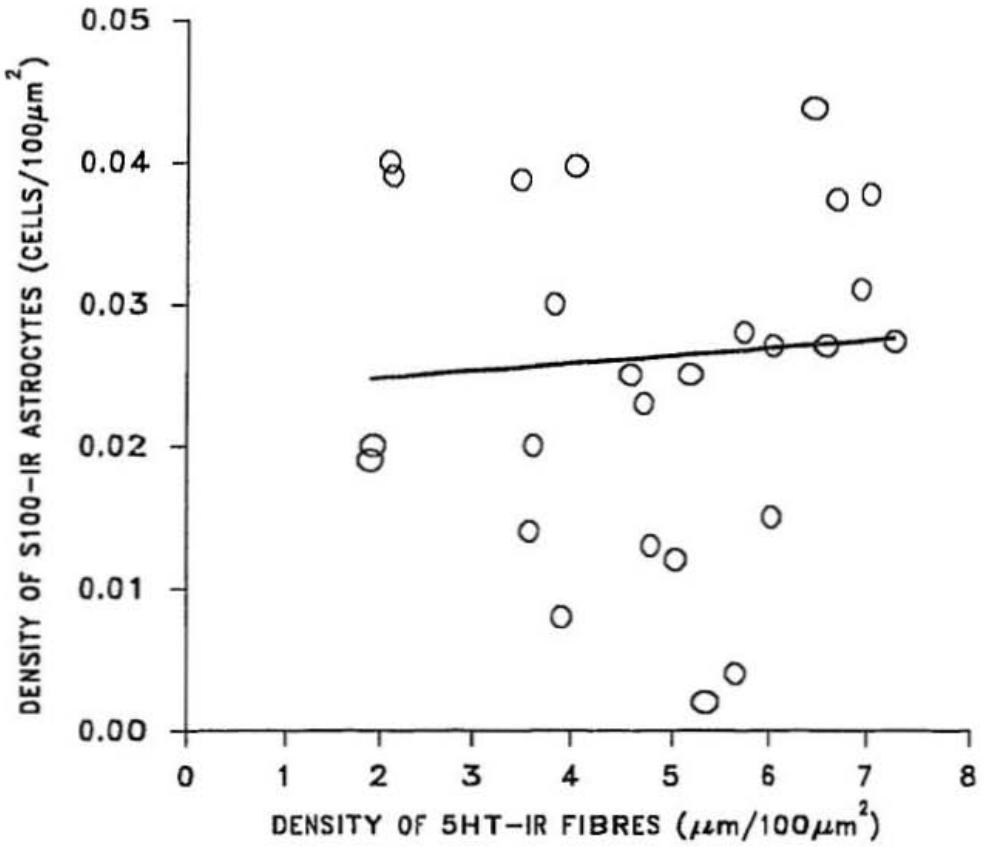


Figure 16: Graph showing lack of correlation (R = 0.0626) between the mean density of serotonergic fibres and S100⁺ astrocytes in the OB of lesioned and non-lesioned PND 31 animals 21 days following peripheral deafferentation.



3.5 Density of DBH Immunoreactive Fibres

Twenty-one days following deafferentation there was a significant increase ($p<0.05$) in the density of DBH immunoreactive fibres in the EPL in bulbs ipsilateral to the lesion compared to non-lesioned controls in the adult animals (Fig.17,18). In the EPL, the mean density of DBH immunoreactive fibres appeared to be the same in the ipsilateral and contralateral bulbs, yet a significant difference was observed between the ipsilateral and the control bulbs, but not between the contralateral and control bulbs (Fig.18). The discrepancy in significance arises from the different, yet necessary, statistical methods performed to determine significance between the ipsilateral and control bulbs and between the contralateral and control bulbs as explained in the methods section. In the infra EPL region, however, a significant increase in DBH immunoreactive fibre density was observed in bulbs contralateral to the lesion compared to the bulbs ipsilateral ($p<0.01$) to the lesion and control ($p<0.05$) animals (Fig.18).

In contrast to the adult animals, no significant differences were observed in the density of DBH immunoreactive fibres in the EPL and infra EPL regions of lesioned and non-lesioned PND 31 animals (Fig.19,20).

Figure 17: Darkfield photomicrograph of DBH immunoreactive fibres in the OB of control (A) adult animals and in the bulbs contralateral (B) and ipsilateral (C) to the lesion in denervated animals. Magnification is the same in (A), (B), and (C) with the scale bar representing 50μm. Abbreviations: glomerular layer (GL), external plexiform layer (EPL), mitral cell layer (MCL), granule cell layer (GCL).

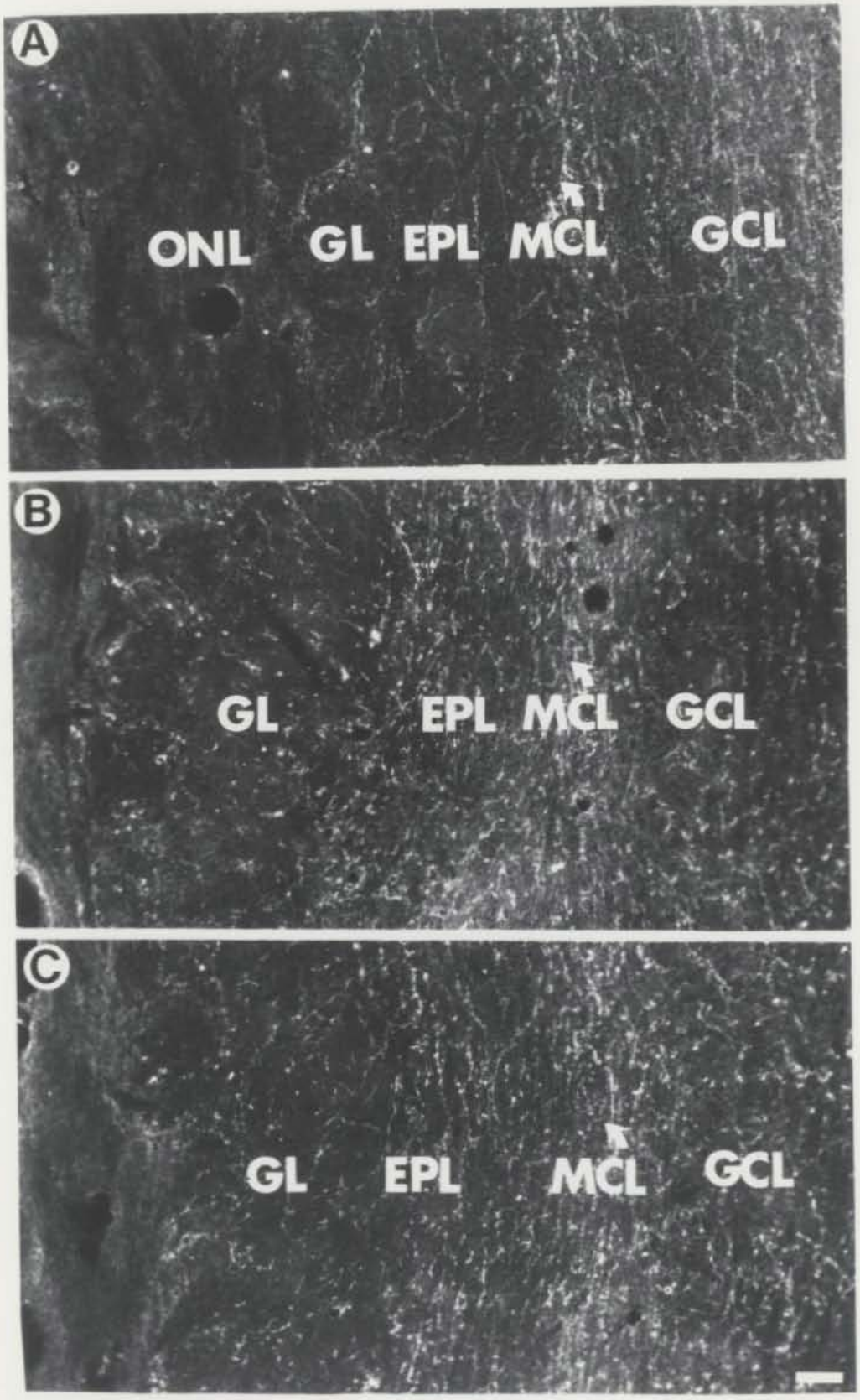

A
ONL
GL
EPL
MCL
GCL
B
GL
EPL
MCL
GCL
C
GL
EPL
MCL
GCL

Figure 18: Mean density of DBH immunoreactive fibres (±S.E.M.) in adult animals 21 days following peripheral deafferentation of the OB. The asterisk (*, $p<0.05$) represents a significant increase in the density of DBH immunoreactive fibres in the ipsilateral bulbs compared to control bulbs in the EPL. In the infra EPL the asterisks (*, $p<0.5$; **, $p<0.01$) represent a significant increase in fibre density in the contralateral bulbs compared to both the ipsilateral and control animals.

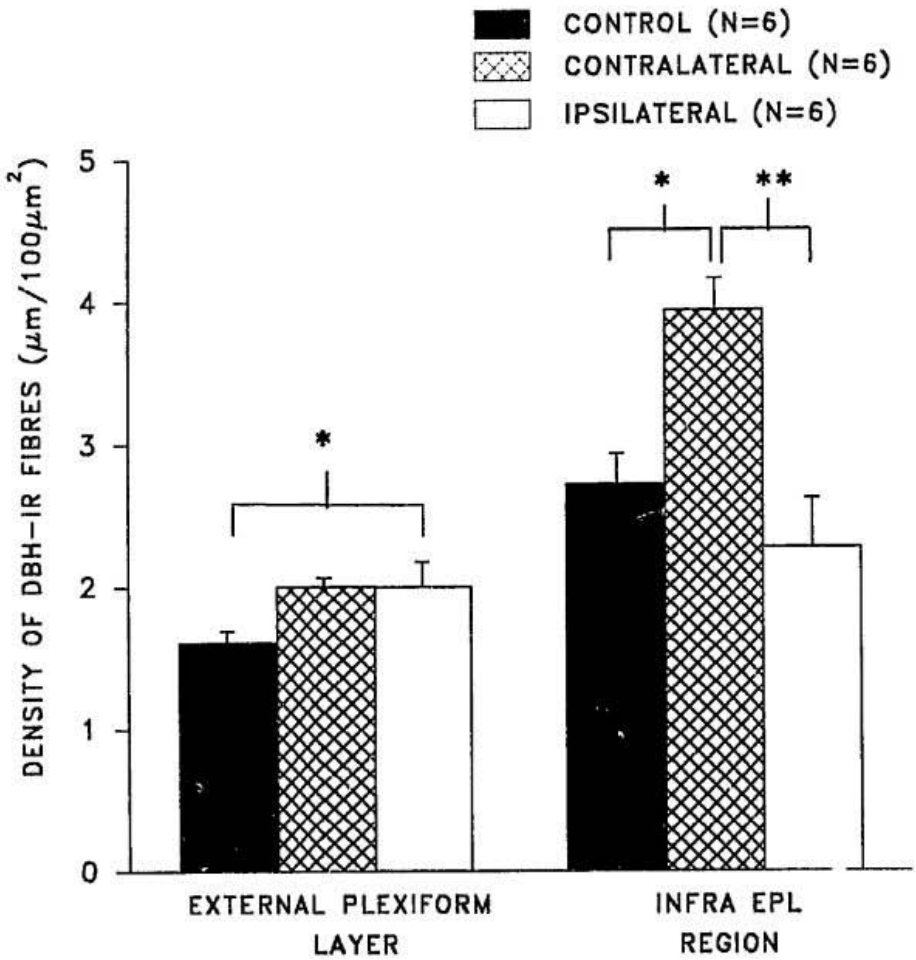


Figure 19: Darkfield photomicrograph of DBH immunoreactive fibres in the OB of control (A) PND 31 animals and in the bulbs contralateral (B) and ipsilateral (C) to the lesion in denervated animals. Magnification is the same in (A), (B), and (C) with the scale bar representing 50μm. Abbreviations: glomerular layer (GL), external plexiform layer (EPL), mitral cell layer (MCL), granule cell layer (GCL).

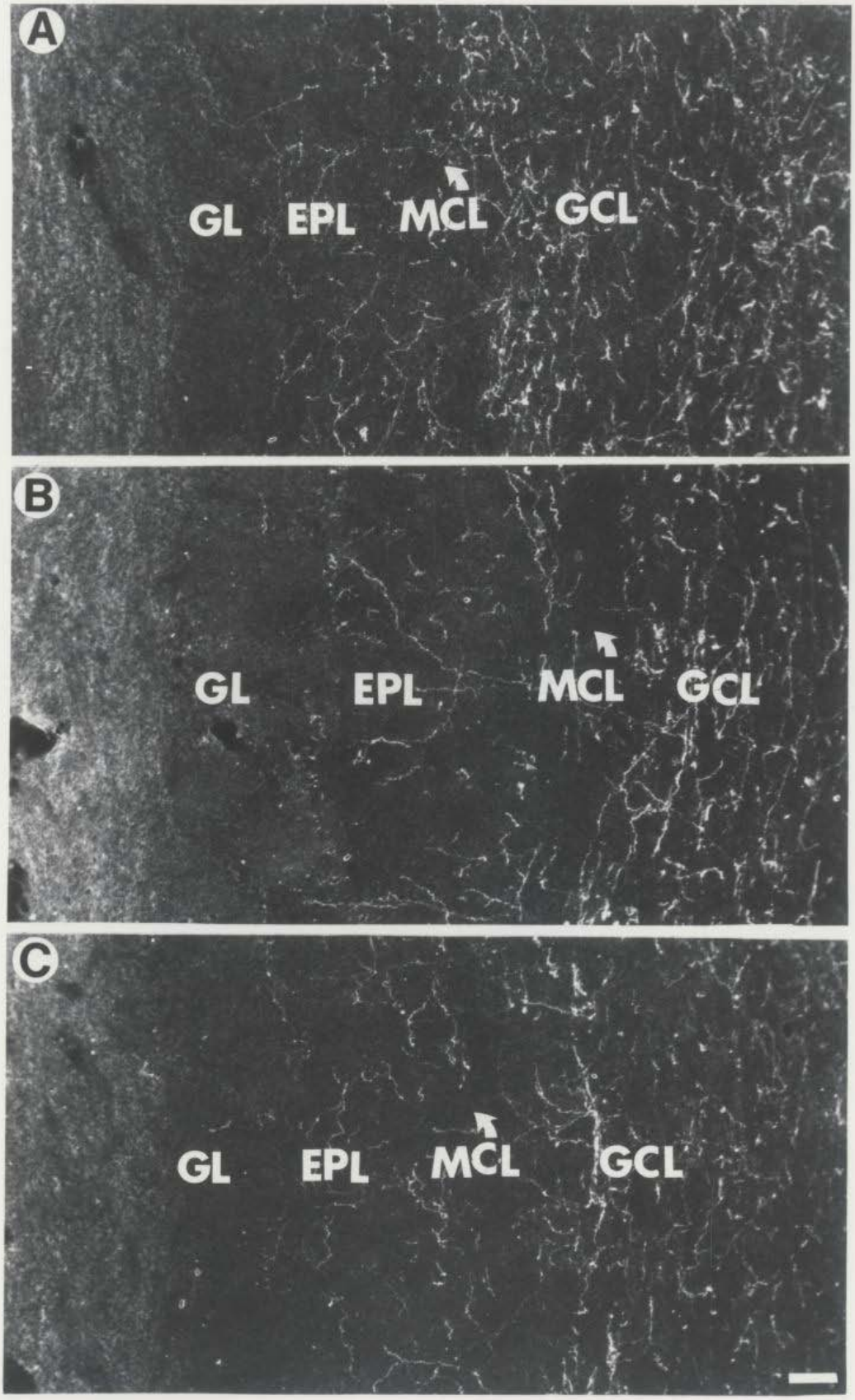
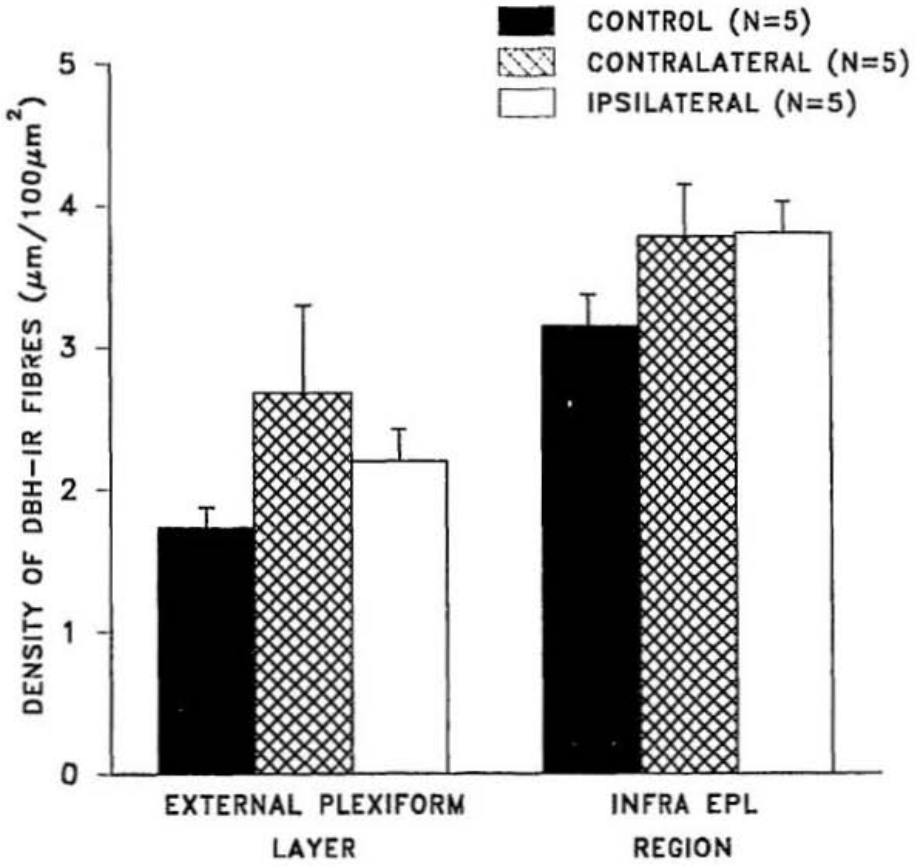

A
GL
EPL
MCL
GCL
B
GL
EPL
MCL
GCL
C
GL
EPL
MCL
GCL

Figure 20: Mean density of DBH immunoreactive fibres (±S.E.M.) in PND 31 animals 21 days following peripheral deafferentation of the OB.

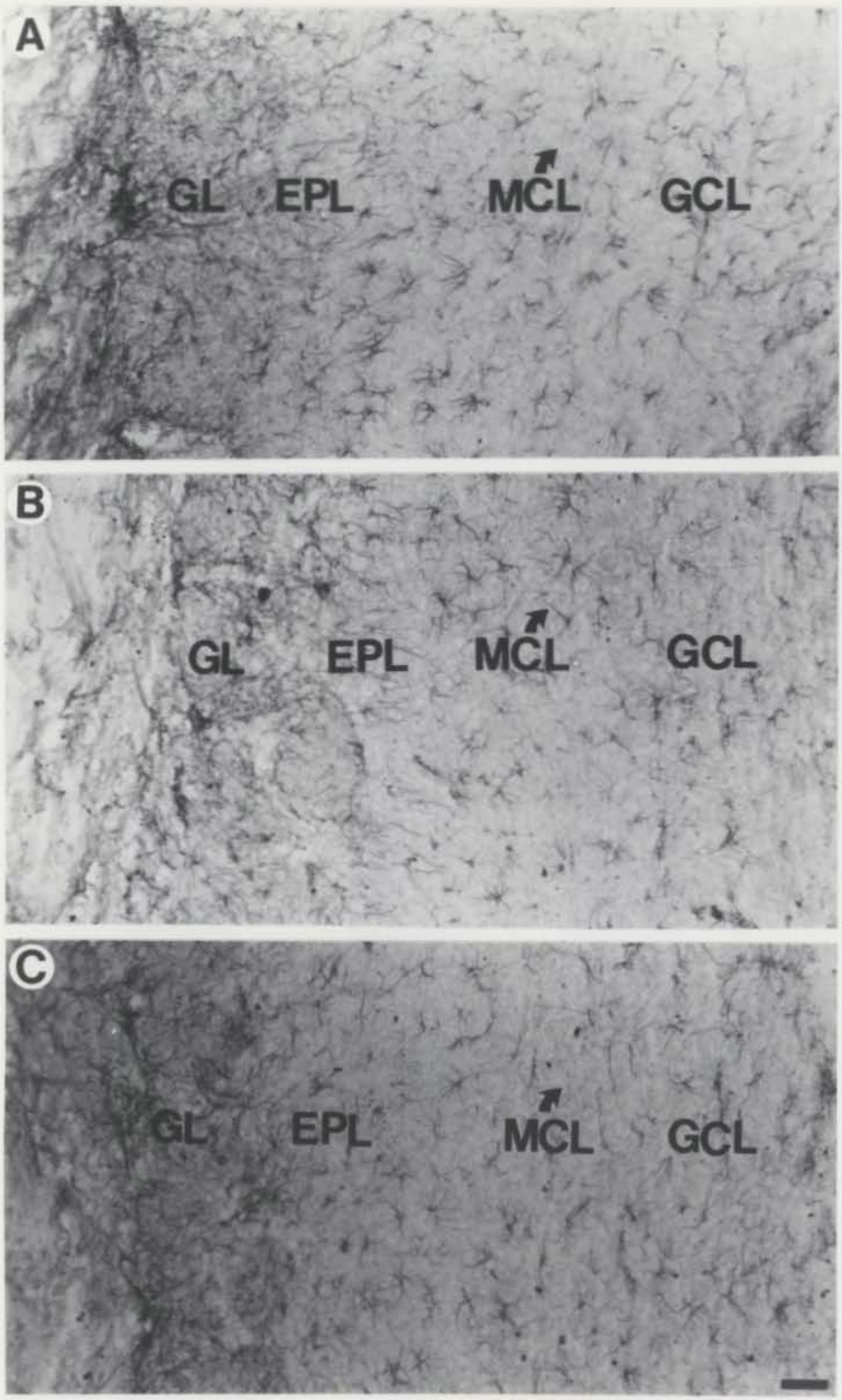


3.6 Density of GFAP Immunoreactive Astrocytes

Twenty-one days following peripheral deafferentation no significant differences in the density of GFAP immunoreactive astrocytes were apparent in any region of the OB examined in lesioned and non-lesioned adult (Fig.21,22) and PND 31 (Fig.23,24) animals.

In PND 31 animals, a consistent, generalized increase in $GFAP^+$ astrocytes was observed in bulbs ipsilateral and contralateral to the lesion compared to non-lesioned controls. Although this generalized increase in density occurred in all regions of the OB examined no significant differences in astrocytic density were apparent (Fig.24).

Figure 21: Photomicrograph of GFAP immunoreactive astrocytes in the OB of control (A) adult animals and in the bulbs contralateral (B) and ipsilateral (C) to the lesion in denervated animals. Magnification is the same in (A), (B), and (C) with the scale bar representing 50μm. Abbreviations: glomerular layer (GL), external plexiform layer (EPL), mitral cell layer (MCL), granule cell layer (GCL).


A
GL
EPL
MCL
GCL
B
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EPL
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GCL
C
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EPL
MCL
GCL

Figure 22: Mean density of GFAP immunoreactive astrocytes (±S.E.M.) in adult animals 21 days following peripheral deafferentation of the OB.

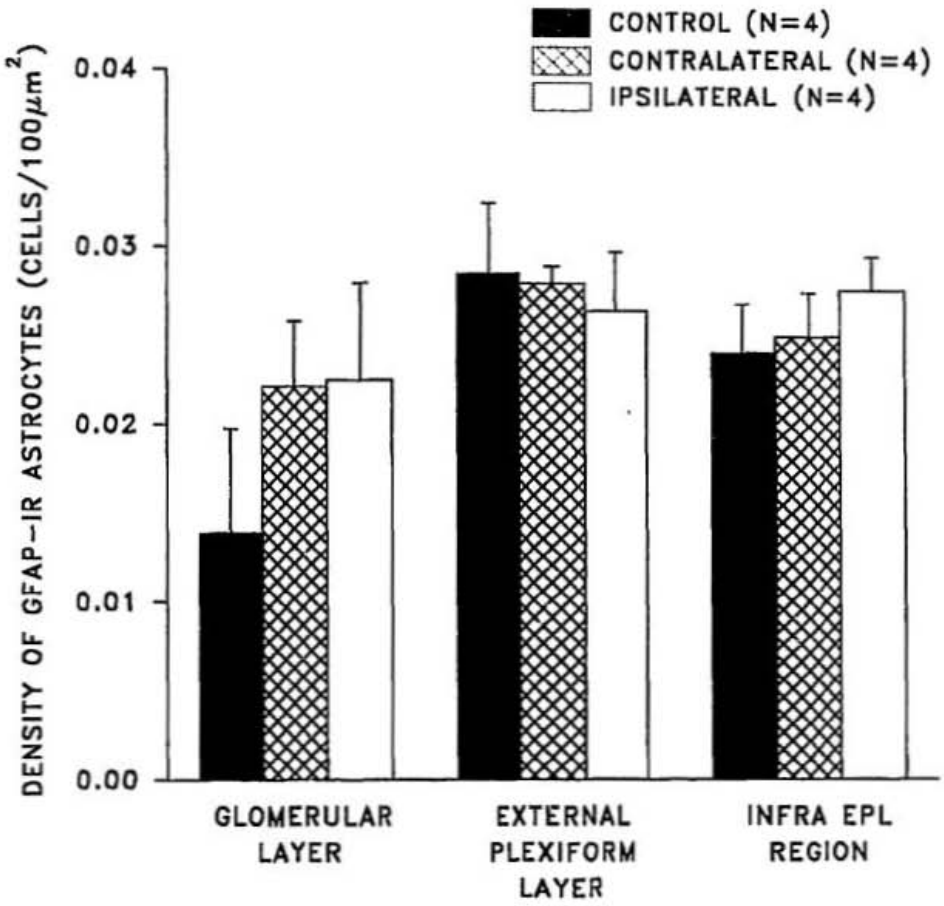
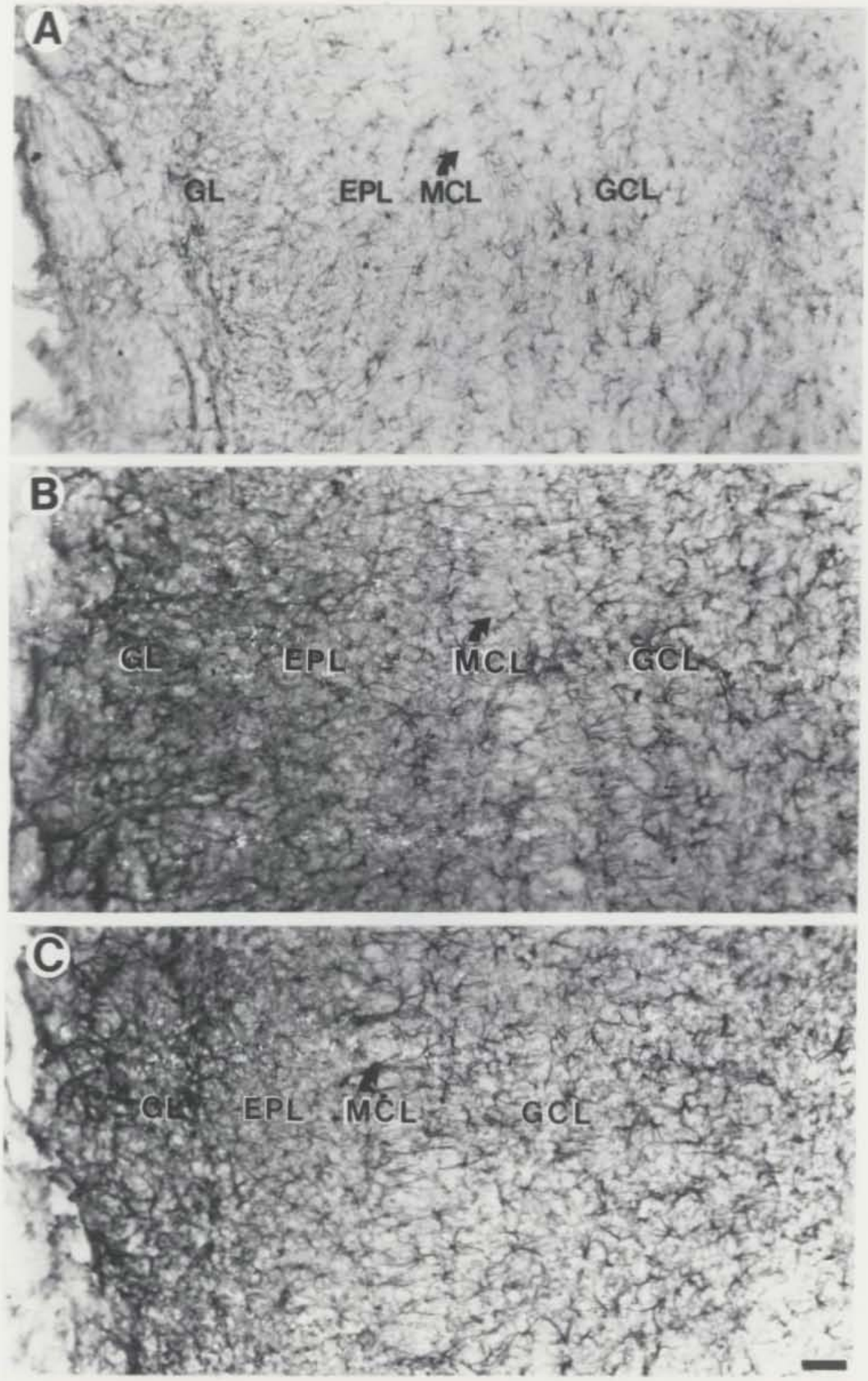


Figure 23: Photomicrograph of GFAP immunoreactive astrocytes in the OB of control (A) PND 31 animals and in the bulbs contralateral (B) and ipsilateral (C) to the lesion in denervated animals. Magnification is the same in (A), (B), and (C) with the scale bar representing 50μm. Abbreviations: glomerular layer (GL), external plexiform layer (EPL), mitral cell layer (MCL), granule cell layer (GCL).


A
GL
EPL
MCL
GCL
B
GL
EPL
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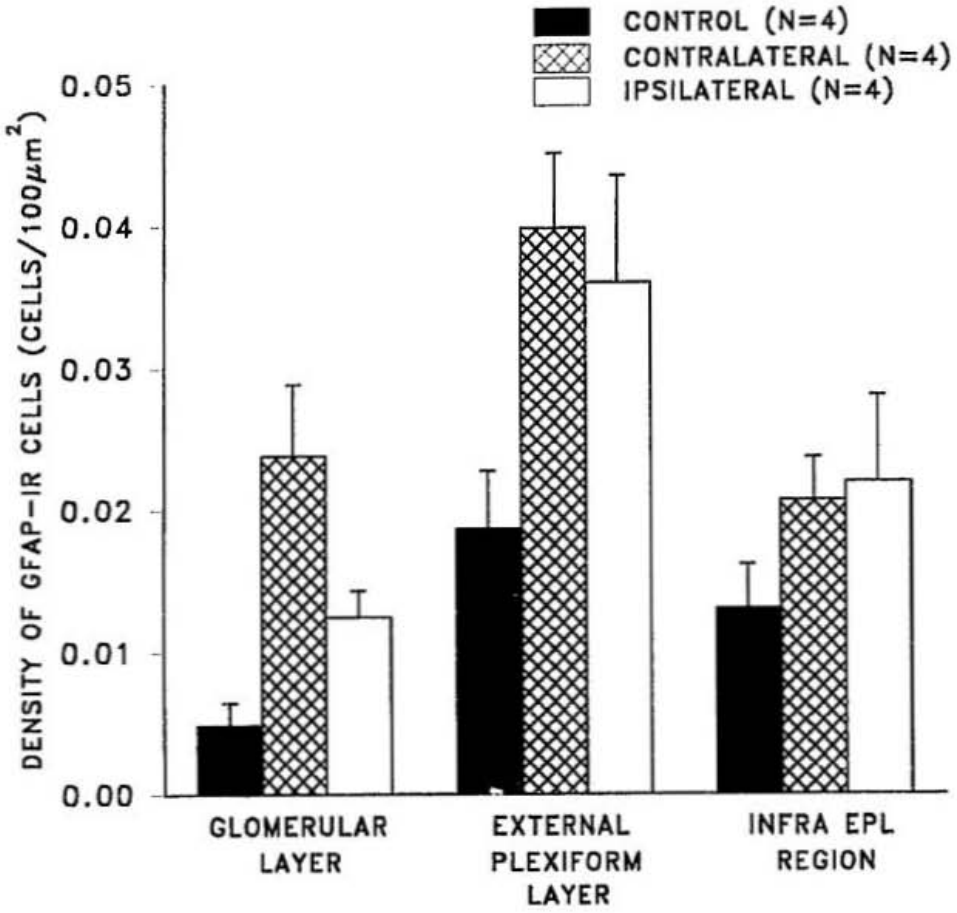
GCL

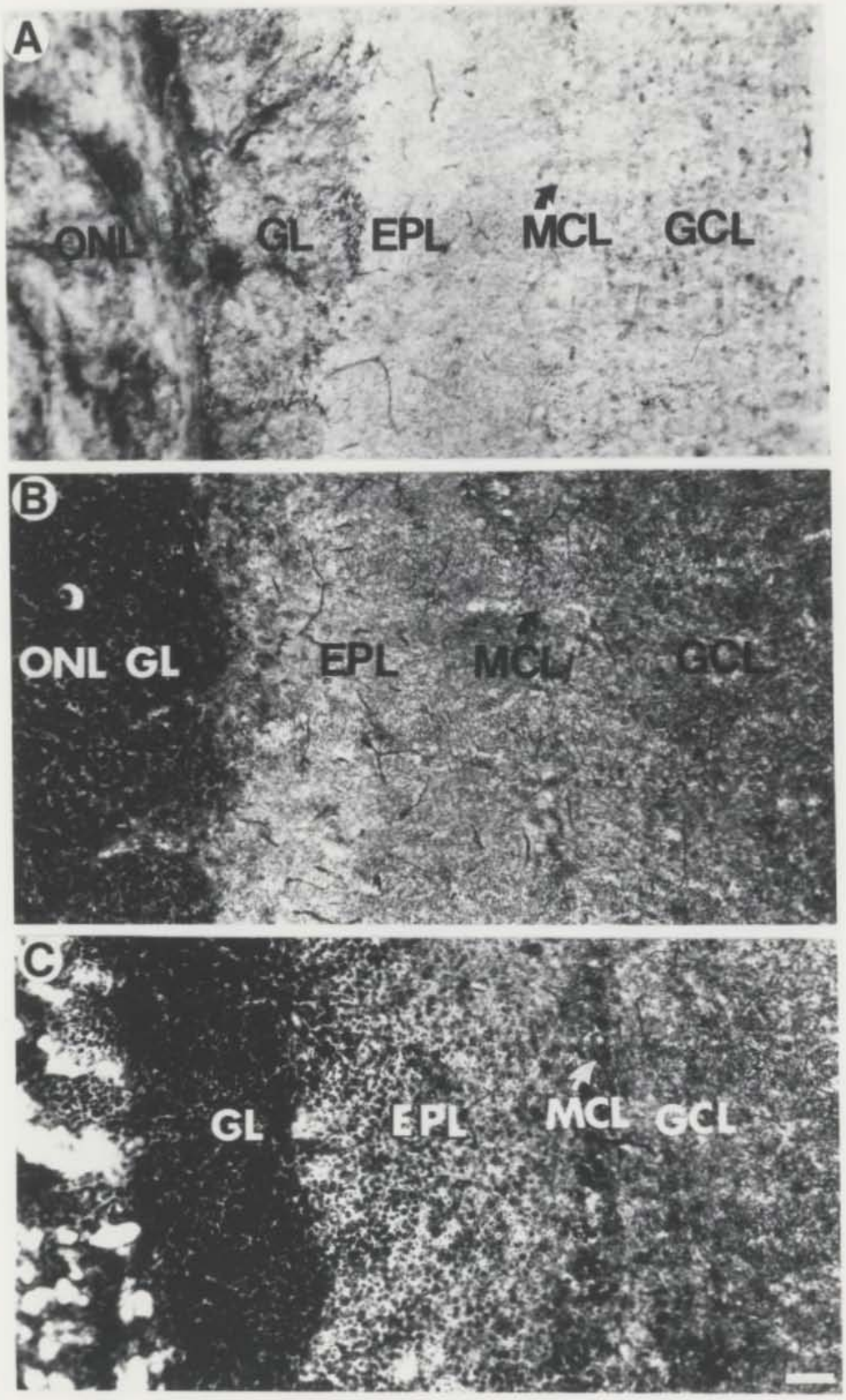
Figure 24: Mean density of GFAP immunoreactive astrocytes (±S.E.M.) in PND 31 animals 21 days following peripheral deafferentation of the OB.



3.7 Zinc Staining

Three days following introduction of $ZnSO_4$ into the nasal cavity of adult animals, significant increases were apparent in the R.O.D. measurements in the GL ($p<0.01$) and infra EPL region ($p<0.05$), indicating that increased amounts of zinc were present in the bulbs ipsilateral to the lesion over controls (Fig. 25,26). In addition, the R.O.D. measurements for the contralateral OB fall between those of the ipsilateral bulb and the non-lesioned controls (Fig. 26).

Figure 25: Brightfield photomicrograph of zinc staining in the the OB of control (A) adult animals and in the bulbs contralateral (B) and ipsilateral (C) to the lesion in denervated animals. Magnification is the same in (A), (B), and (C) with the scale bar representing 50μm. Abbreviations: glomerular layer (GL), external plexiform layer (EPL), mitral cell layer (MCL), granule cell layer (GCL).


A
ONL
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B
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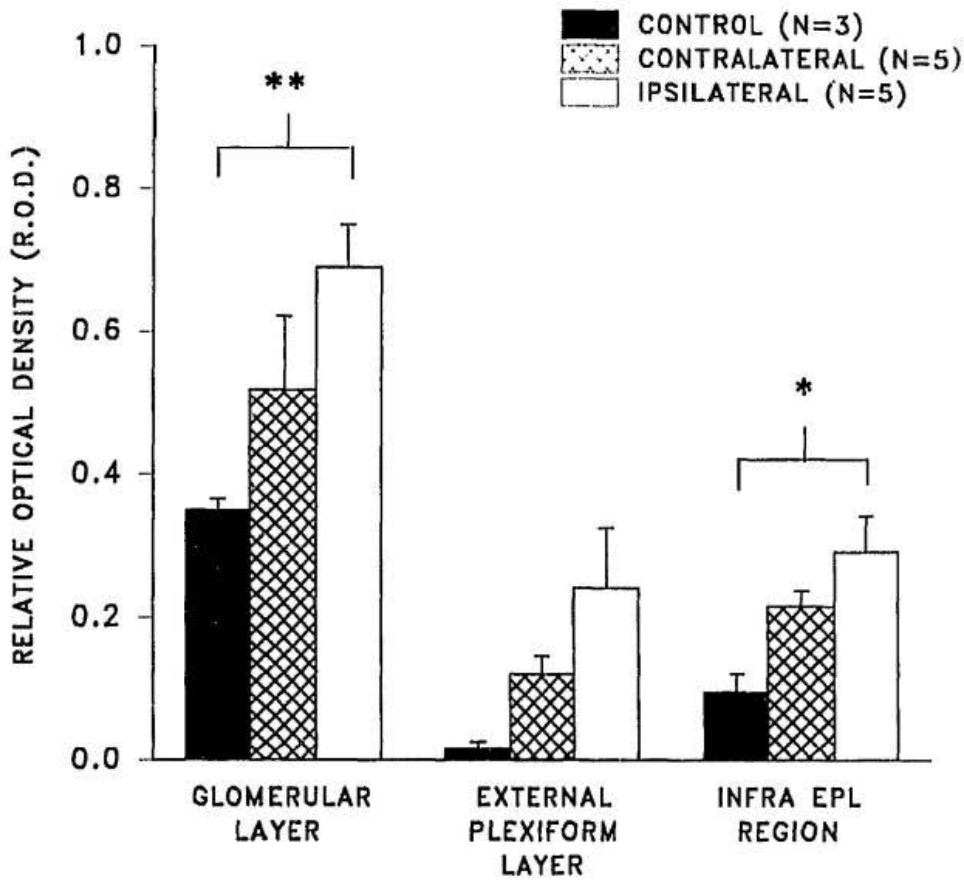
MCL
GCL
C
GL
EPL
MCL
GCL

Figure 26: Mean relative optical density (R.O.D.) measurements (±S.E.M) of the intensity of zinc staining in the OB of control adult animals and three days following peripheral deafferentation of the OB. The asterisks (**, $p \leq 0.01$; *, $p \leq 0.05$) represent a significant increase in R.O.D measurements in the bulbs ipsilateral to the lesion compared to controls.



DISCUSSION

This thesis examined the hypothesis that heterotypic collateral sprouting of intact fibre systems, especially the serotonergic system, would occur following removal of the PON input to the OB. In addition, this study examined the hypothesis that astrocytes might increase in number in response to deafferentation of the OB. These hypotheses were based on similar studies of lesion-induced plasticity in other regions of the CNS. The results of the present study suggest that in the OB of adult animals the serotonergic system does not respond to deafferentation by sprouting, a response that is observed in many other regions of the CNS following deafferentation in the adult. In contrast to the expected increase in serotonergic innervation, the density of serotonergic fibres was decreased significantly in the adult in all regions of the OB following peripheral deafferentation. Serotonergic fibres in the neonate appeared to respond differently to deafferentation: the density of serotonergic fibres in PND 31 animals was increased significantly in the deeper regions of the OB following deafferentation, indicating that at least some serotonergic fibres in the OB of younger animals sprout in response to deafferentation. The difference in the serotonergic responses of the adult and PND 31 animals following deafferentation concurs with reports of lesion induced sprouting in other regions of the CNS where young animals tend to be more plastic in their response to injury (Stachowiak et al.1984; Synder et al.1985). However, although the sprouting phenomenon is not apparent in adult animals in these reports, the density of fibres does not decrease

which is in contrast to the findings in the OB where the density of some fibres decreases. Interestingly, the astrocytic expression of the S100 protein, a proposed serotonergic growth factor, did not correlate with the density of serotonergic innervation in the adult or PND 31 animals. In contrast to the changes observed in the serotonergic innervation in the adult animals, the density of the noradrenergic fibres was significantly increased in the deeper regions of the OB following deafferentation. In PND 31 animals, however, no significant differences in the density of noradrenergic innervation were apparent in any region of the OB following deafferentation. Thus, in the OB, the differences in the serotonergic and noradrenergic response to deafferentation indicate that different transmitter systems respond differently to the same lesion. Finally, no significant changes in the density of astrocytes expressing GFAP were observed in any regions of the OB examined in the PND 31 and adult animals 21 days following deafferentation.

These results, especially those pertaining to the serotonergic system in the adult animals, lead us to suggest that the responses to deafferentation of the OB may be different to responses reported in other regions of the CNS due to several possibilities which will be elaborated upon in subsequent sections of the discussion. Since tissue shrinkage and the variability in the effectiveness of the lesion and the extent of damage induced in animals may be important variables relevant to the study of lesion-induced deafferentation, a few points for technical consideration will be addressed in the first two sections. The responses of the serotonergic, noradrenergic and glial systems to OB deafferentation will then be discussed. The unique features

of the OB, which may be an important factor contributing to the results observed in the present study, will also be elaborated upon in the discussion. In addition, the method of zinc sulfate deafferentation and the potential neurotoxicity and modulatory effect of the compound will be discussed in light of the present results observed in the serotonergic system.

4.1 Tissue Shrinkage Following Deafferentation

Shrinkage of brain tissue often occurs following injury and can lead to incorrect interpretation of the sprouting phenomenon. For instance, the density of fibres may be perceived as increased even though the actual number of fibres remained constant if shrinkage is not taken into account. Conversely, if shrinkage is not taken into account, degeneration of fibre systems may be masked by the decrease in the area of the tissue. For example, if the density of innervation decreases in conjunction with a decrease in the area of the tissue, the fibre density may appear similar to control values. Thus, the fibre system would be perceived as being unaffected by the lesion.

Shrinkage was relevant to the present study as shrinkage of the GL and the OB in general has been reported following OB deafferentation (Margolis et al.1974; Harding et al.1978; Baker et al.1984). We, therefore, accounted for any shrinkage in the OB by obtaining the density of fibres and glial elements using normalization factors as outlined in the methods section.

4.2 The Effectiveness of Deafferentation

In the present study, although a significant decrease was apparent in the number of TH immunoreactive cells in the adult animals, degeneration of the axons of the PON did not extend throughout the entire GL of the lesioned OB. This indicated that either the axons of the PON had regenerated within the 21 day post-lesion time period or that some of the axons of the PON were spared during the initial application of $ZnSO_4$. In contrast, PND 31 animals exhibited a more pronounced degeneration of the axons of the PON as indicated by an almost complete loss of TH immunoreactive PG cells throughout the entire GL in some animals. The possible explanations for the difference between olfactory deafferentation of the pups and adult rats are considered below.

Although the ability of $ZnSO_4$ to destroy the olfactory epithelium has been well documented in several species (Smith, 1938; Schultz, 1941; Mulvaney and Heist, 1900; Harding et al.1978) some controversy exists concerning the time course of regeneration of the olfactory sensory cells after application of $ZnSO_4$ to the OE. Regeneration of sensory cells has been reported to occur after 30 days in the rabbit (Mulvaney and Heist, 1900), 72 days in the mouse (Matulionis, 1975b) and 6 months to 1 year in the monkey (Schultz, 1960). In the present study, a 21 day post-lesion time period was employed in order to ensure degeneration of the axons of the PON. This time frame is in keeping with other $ZnSO_4$-depletion studies in the rat which showed that OE input into the glomeruli remains at very low levels even one month after deafferentation using TH immunoreactivity and biochemistry as an indication

of deafferentation (Baker et al.1983; Baker et al.1984). In addition, the time period chosen was before the time period reported for potential regeneration of the sensory cells after application of zinc in other species and provides sufficient time for serotonergic/noradrenergic fibre and glial cell reorganization.

The differences in the extent of deafferentation within the same age group or between the younger animals and the adults may be a consequence of the structure of the OE. The convoluted nature of the OE renders it difficult to administer equal amounts of $ZnSO_4$ to all areas of the epithelium with intranasal irrigation or dropwise application of $ZnSO_4$. Therefore, some of the stem cells may remain undamaged and able to generate new primary olfactory neurons. Indeed, it is difficult to obtain total deafferentation as indicated by Baker (1990), Baker et al. (1983,84). In the present study, Gelfoam was saturated with $ZnSO_4$ and packed into the nasal cavity in order to obtain prolonged, yet continuous, contact of the PON and the $ZnSO_4$ solution and to optimize the amount of solution reaching the stem cells in different areas of the OE. The presence of the Gelfoam within the nasal cavity may have provided a physical barrier to PON regeneration as well as a means of maintaining a long term source of $ZnSO_4$ to destroy any afferent axons which may have initially escaped injury. In addition, in PND 10 rat pups the Gelfoam may have provided a more extensive physical barrier to regeneration of the axons of the PON due to the smaller size of the nasal cavity compared to adult rats, thereby accounting for the more extensive deafferentation often observed in the younger animals. At any rate, for analysis purposes only the regions subjacent to obvious TH depletion were analyzed

in the present study. Thus, only regions with the most obvious deafferentation, and probable reactivity to deafferentation, were analyzed. Although the extent of the lesion varied between animals, the most consistent deafferentation occurred in the medial aspect of the circumference of the GL in sections taken from the rostral to medial extent of the OB. Therefore, this region was chosen for all subsequent analysis of fibre/cell densities.

Admittedly, the lack of total deafferentation is certainly a complicating factor in the present study. For instance, although only deafferented regions of the OB were selected for analysis it is possible that the OB requires total PON deafferentation in order to produce significant changes in the axonal and glial complement of the bulb. Compared to other deafferentation paradigms cited in this thesis, including the spinal cord (Polistina et al.1990; Wang et al.1991; Marlier et al.1992), visual system (Rhoades et al.1990) and noradrenergic deafferentation to the striatum (Stachowiak et al.1984; Synder et al. 1985), the deafferentation of the OB was less complete. Future studies may require more complete deafferentation of the OB in order to provide more confident comparisons between the responses observed in the OB and the responses observed in other systems. In preliminary studies at the onset of this thesis, mechanical deafferentation of the olfactory nerves was attempted by surgically cutting the nerves as they enter the OB. However, this procedure produced considerable direct trauma to the OB and more variable deafferentation than that observed by $ZnSO_4$ deafferentation. Therefore, chemical deafferentation using $ZnSO_4$ was determined to be most likely to provide deafferentation.

Another potential question is whether responses to deafferentation of the OB may have been observed if longer survival times had been employed. However, it should be noted that longer survival times (48 days post deafferentation) were attempted during the course of the study. In most of these animals, however, there was little, if any, loss of TH immunoreactivity, suggesting that undamaged OE neurons may have reinnervated the OB by 48 days post deafferentation. Therefore, these animals were not included in any analysis because of the questionable effectiveness of deafferentation.

In both adult and young animals, unilateral application of $ZnSO_4$ to the OE produced significant decreases in the number of PG cells staining for TH in bulbs ipsilateral and contralateral to the lesion when compared to controls. Thus, it appears that some of the $ZnSO_4$ must have entered the nasal cavity of the contralateral OB, possibly due to perforation of the nasal septum during surgery or the presence of a window in the nasal septum. In this respect, studies have shown that rats can detect (Slotnick and Pazos, 1990; Hunt and Slotnick, 1991) and discriminate odors (Hunt and Slotnick, 1991) through an intra-nasal communication channel which is perceived as a septal window. In addition to the loss of TH staining in the contralateral bulb, relative optical density measurements of zinc staining in the adult animals indicated that the amount of zinc in the contralateral bulb of adult animals

was generally higher than the controls but less than bulbs ipsilateral to the lesion. Although the contralateral bulb is often used as an internal control in many studies of zinc-induced peripheral denervation of the OB, it appears that unlesioned animals should be used to provide adequate controls.

4.3 Difference in the Serotonergic Response Following Peripheral Deafferentation of the OB in Adult and PND 10 Animals

Although the serotonergic system displays a tremendous capacity to sprout in many regions of the CNS following injury in adult animals, the results of the present study indicated that heterotypic collateral sprouting of serotonergic fibres does not occur within 21 days following peripheral deafferentation of the OB in adult animals.

An important consideration which may influence the interpretation of lack of sprouting of serotonergic fibres in the OB is whether or not the 21 day post-lesion time period is sufficient to observe sprouting of fibres. However, sprouting of serotonergic fibres has been reported to occur within one to two weeks in the spinal cord (Wang et al.1991; Marlier et al.1992) and hippocampus (Azmitia and Zhou, 1986) following injury. Thus, it appears that the 21 day post-lesion time period employed in this study should have provided ample time for the appearance of sprouting of serotonergic fibres in the OB.

In contrast to the widespread decrease observed in serotonergic fibre density in bulbs ipsilateral to the lesion in adult animals, an increased density of serotonergic fibres was observed in the ipsilateral infra EPL region in PND 31 animals, indicating that the serotonergic system may have the potential to sprout following OB deafferentation in the younger animal. In an effort to explain the interesting, yet perplexing, differences in the response of the serotonergic system following deafferentation of the OB of young and adult animals, several hypotheses are put forth in the following sections.

4.3.1 Correlation Between the Decrease in Serotonergic Input and Loss of PON Axonal Input: A Functional Loss

Lack of serotonergic sprouting following deafferentation of the OB in adult animals may potentially be related to an impoverished sensory environment resulting from the removal of the afferent innervation to the OB. Morphological changes are apparent in the brains of animals exposed to enriched or complex environments. For instance, the thickness of the occipital cortex is increased in animals reared in enriched environments (Bennett et al.1964). The change in the thickness of the occipital cortex is attributed to an increase in dendritic branching, paralleled with and increase in neuronal synapse number (Volkmar and Greenough, 1972; Greenough et al.1973; Turner and Greenough, 1985; Kilman et al.1991). Conversely, deafferentation of the OB, resulting in an impoverished sensory environment, may have potentially led to a decrease in dendritic and axonal complement of the OB, including the serotonergic innervation. In this respect, decreases in the number and size of synaptic terminals have been reported following deprivation of the visual system (Bliss Tieman, 1994), although changes in serotonergic axons were not specifically examined in that study.

Although negative changes in morphology of the visual system occur in response to deprivation, the serotonergic fibres in the superior colliculus and lateral geniculate nucleus sprout following enucleation (Rhoades et al.1990). In addition, deafferentation of portions of the spinal cord leads to sprouting of serotonergic fibres in the deafferented areas (Polistina et al.1990; Wang et al.1991). Thus, even though

the sensory input into these three regions (the visual system, spinal cord and olfactory bulb) can be deprived in similar ways, at least one component of these systems, the serotonergic component, reacts to deafferentation in different ways. It appears, therefore, that the response of the serotonergic system depends not only upon the loss of major sensory/functional inputs, but in the case of the OB depends upon some additional factor or factors.

An obvious, yet important, difference between the OB and the visual system and spinal cord is the ability of the primary sensory afferents to the OB to be regenerated throughout the life of the animal. Therefore, the differences in the ability of the serotonergic fibres to sprout in the OB following deafferentation as opposed to structures such as the visual system and spinal cord, may be related to fundamental differences in sensory inputs to these systems and the role of the serotonergic system to respond or influence the sensory inputs. Serotonin has been implicated in the inhibition of synapse formation (Haydon et al.1984). Thus, a potential role of serotonin in the OB may be regulation of synapse formation in the GL as a consequence of the continual turnover of synapses due to regeneration of olfactory neurons in the periphery. In this study, maintenance of the serotonergic fibres may potentially be dependent upon a normal sensory environment and the turnover of synapses in the GL. That is, without OE axon input to the GL, there may have been a decrease of a trophic influence of PON axons on the serotonergic fibres. Therefore, without a specific function, decreased sensory stimulation and/or trophic influence the serotonergic fibres may have retracted or degenerated. Supporting this hypothesis

is the fact that the density of serotonergic axons increases in the glomeruli during postnatal development (McLean and Shipley, 1987) which corresponds to increased postnatal input to the glomeruli. Whether the PON axons actually have a trophic influence upon serotonergic axons has not been conclusively determined. A confounding observation in the present study is that deafferented PND 31 animals displayed increased serotonergic axon density in the deeper layers of the OB relative to controls. Although interesting, this finding is somewhat perplexing since increased serotonergic fibre density was not observed in the deep layers of the deafferented OB in adult animals. A potential explanation for the differences between the responses of the serotonergic fibres in young and mature rats is offered in the following sections which deal with the neurotoxic effects of zinc, which was used to achieve deafferentation of the OB.

4.3.2 The Lack of Correlation of S100 Expression and the Density of Serotonergic Fibres

It was originally hypothesized that changes in serotonergic fibre density would correlate with changes in the number of astrocytes expressing S100. However, in the present study, the decrease in the density of serotonergic fibres in the OB of adult animals did not reflect a corresponding change in the number of astrocytes expressing S100 in the bulb. Similarly, in the infra EPL region of PND 31 animals, the number of cells expressing S100 did not correspond with the increase in serotonergic innervation.

The rationale for originally hypothesizing that there may be a positive correlation between the number of astrocytes expressing S100 and the density of serotonergic fibres in the OB was based on work originating from Azmitia's laboratory. Tissue culture studies have provided evidence that the S100 protein may be a growth factor that is specific for serotonergic neurons (Azmitia et al.1990; Whitaker-Azmitia et al.1990), since addition of S100 to immunoreactive serotonergic raphe neurons in culture increases neurite outgrowth and the uptake of [^{3}H] 5-HT. Release of S100 in culture has shown to be activated following stimulation of 5-HT_{1A} receptors on astroglial cells using ipsapirone, a 5-HT_{1A} agonist (Whitaker-Azmitia et al.1990). 5-HT_{1A} receptors are present in the OB (Albert et al.1990; Pompeiano et al.1992) and could, therefore, help mediate serotonergic neurite growth.

In addition to tissue culture studies, a recent study suggests that serotonin levels in the hippocampus influence the immunocytochemical expression of S100 (Haring et al.1993). Significant decreases were observed in the density of S100 positive structures 7 days following depletion of serotonin, using parachlorophenylalanine (PCPA) to prevent synthesis of serotonin. The reduction in S100 immunoreactivity was independent of changes in the actual number of glial cells expressing S100 since diminished immunoreactivity occurred in the astrocytic processes and not as a consequence of a decrease in the number of astrocytes. The reduction in S100 immunoreactivity is presumably due to diminution of 5-HT_{1A} receptor stimulation (Haring et al.1993) resulting in reduced expression of the protein. In the present study although the density of serotonergic fibres is decreased significantly in the adult animals, the level of serotonin remaining is much more than would be expected following serotonergic depletion by PCPA administration in the hippocampus. Thus, the amount of serotonin available in the OB following deafferentation may be sufficient to stimulate astrocytic 5-HT_{1A} receptors thereby maintaining the immunocytochemical expression of S100.

In the present study, although the density of glial cells expressing the immunocytochemical marker for S100 did not correlate with changes in density of serotonergic fibres it is possible that changes occurred in astrocytic production of S100 thereby resulting in differences in the biochemical levels of the protein. For instance, the astrocytic production of S100 may have been up or down regulated

with the differences in the levels of the protein affecting the density of serotonergic fibres in adult and PND 31 animals. Future studies measuring the biochemical levels of the S100 protein would be needed to confirm if changes in the amount of S100 occur following deafferentation.

4.3.4 The Potential Effect of Zinc on the Serotonergic Innervation of the OB

4.3.4.1 Potential Neurotoxic Effect of Zinc on Serotonergic Fibres in Adult Animals

Zinc sulfate has been routinely used to remove the afferent input to the OB since the early 1940's. However, recent studies have suggested that the presence of excess zinc in the CNS may be neurotoxic in its effects (Yokoyama et al.1986; Choi et al.1988; Lees et al.1990). Thus, the potential neurotoxicity of the compound, coupled with the ability of substances to be transported from the OE to the OB (Shipley, 1985; Baker and Spenser, 1986; McLean et al.1989; Lafay et al.1991) warranted examination of the amount of zinc present in the OB following introduction of the metal into the nasal cavity.

Zinc is normally found in many areas of the CNS (Donaldson et al.1973) with regional variations occurring in the actual zinc concentration. In the OB a heavy granular pattern of Timm staining, which indicates the presence of zinc, is normally found in the GL and EPL, while lighter staining occurs in the IPL and GCL (Friedman and Price, 1984). Zinc-containing neurons in the CNS are believed to accumulate zinc in synaptic vesicles of presynaptic terminals (Huang, 1967; Ibata and Otsuka, 1969; Assaf and Chung, 1984; Sloviter, 1984; Friedman and Price, 1984; Pérez-Clausell and Danscher, 1985; Aniksztejn et al.1987). Release of zinc into the synaptic cleft occurs following normal stimulation or electrical excitability (Howell et al.1984; Assaf and Chung, 1984; Charton et al.1985). However, the specific function of endogenously released zinc during neurotransmission has not been elucidated.

Although zinc is believed to participate in normal synaptic functioning, recent studies have suggested a neurotoxic effect of zinc. Direct application of zinc to cultured cortical cells (Yokoyama et al.1986; Choi et al.1988) and injection of zinc into the hippocampus (Lees et al.1990) have been shown to produce neurotoxicity. The specific factors responsible for neurotoxicity associated with increased concentrations of zinc have not been identified. Zinc reportedly disrupts normal tubulin assembly (Smart and Constanti, 1983; Kress et al.1981), produces changes in extracelluar pH (Chung and Assaf, 1984) and cell membranes (Bettger and O'Dell, 1981), inhibits electron transport (Nicholls and Malviya, 1967) and alters excitatory amino acid activity (Choi et al.1988). All of these factors could adversely modify neuronal function and result in cell death.

The present study investigated whether zinc could enter the brain of adult animals because of the potential of neuronal toxicity of compound as mentioned above. It was hypothesized that the lack of sprouting observed in the serotonergic system (which was originally expected to sprout) may, in part, be attributed to the toxic effects of zinc. In the present study, relative optical density measurements (R.O.D.) of the intensity of zinc staining indicated a significant increase in the amount of zinc in the bulbs ipsilateral to the lesion compared to controls in the GL and infra EPL regions of the OB of adult animals following introduction of the metal into the nasal cavity. In the EPL, the distribution of R.O.D. measurements for zinc staining was similar to the pattern obtained for the GL and infra EPL regions, yet no significant differences in staining intensity were apparent. The lack of significance may be

attributed to the small sample size since it is unlikely that transport of zinc from the GL to the infra EPL could occur without entering the EPL which is situated between the GL and infra EPL. At any rate, given the ability of other substances to be transported from the OE to the OB (Shipley, 1985; Baker and Spenser, 1986; McLean et al.1989; Lafay et al.1991), it is likely that zinc was transported transneuronally and/or transynaptically into the OB, thereby accounting for the significant increases in the quantity of the metal in GL and infra EPL regions of the OB compared to controls.

In the adult animal, the density of the serotonergic innervation in the ipsilateral OB was significantly decreased compared to controls in all areas examined which appeared to inversely correlate with the increase in intensity of zinc staining, especially in the GL and infra EPL regions. The decrease in the density of serotonergic fibres could have resulted from one, or possibly a combination, of the neurotoxic effects of zinc listed above.

In PND 31 animals the density of serotonergic innervation was not significantly decreased in any region of the OB following deafferentation which is in contrast to the widespread decrease in density observed in the adult animals. In fact, a significant increase in serotonergic fibre density was observed in the infra EPL region of the OB of PND 31 animals, indicating sprouting of serotonergic fibres. Although zinc staining was confined to the adult animals, we are confident that increased amounts of zinc would be present in the OB of the PND 31 animals following introduction of the metal into the nasal cavity through similar transport

mechanisms as mentioned previously. The difference in serotonergic response of adult and PND 31 animals, if indeed increased amounts of zinc are neurotoxic to these fibres, is difficult to determine. However, the different responses may be related to differences in the relative toxicity of zinc during development. For instance, the neurotoxic effects of zinc have been reported to be less prominent in immature neurons in culture when compared to mature neurons (Choi et al.1988). Thus, the difference in serotonergic response in adult and PND 31 animals may be partially due to a decreased neurotoxic effect of zinc in the younger animals. Future studies may be able to determine if serotonergic neurons/axons from the adult are more vunerable to zinc than those from neonate brains.

4.3.4.2 Potential Neuromodulatory Effect of Zinc and the Association with Increased Serotonergic Fibre Density in PND 31 Animals

Although excess zinc has been associated with neurotoxicity, at lower physiological concentrations zinc appears to serve a modulatory function. For instance, zinc reportedly enhances γ-Aminobutyric acid (GABA) transmission in cultured neocortical cells (Zhou and Hablitz, 1993). GABA has long been recognized as the major inhibitory transmitter in the CNS, but its neurotrophic function during the early developmental period has only recently been established.

Addition of GABA or 4,5,6,7-tetrahydro-isoxazolo[5,4-c]pyridine-3-ol (THIP), a GABA agonist, to cultured cerebellar granule cells has been shown to enhance morphological development of these cells with respect to the formation of cytoplasmic organelles and neurites (Hansen et al.1984; Meier et al.1985) and the

effect appears to be restricted to early postnatal development (Hansen et al.1988).

In the OB, high levels of GABA are present in the periglomerular regions, MCL, EPL and GCL (Austin et al.1979). In addition, GABA has been localized in granule cells and their processes and is believed to exert an inhibitory action on the dendrites of mitral cells (Halasz and Shepherd, 1983). Recent studies utilizing *in situ* hybridization techniques have indicated that mRNA for various $GABA_A$ receptor subunits are present on cells in the EPL, MCL and GCL (Zang et al.1990; Laurie et al.1992; Poulter et al.1993). Thus, the high levels of GABA and the expression of GABA receptor subunit mRNA in the same regions of the OB suggests that GABA receptors are present on cells in the EPL, MCL and GCL.

Interestingly, in the infra EPL layer of PND 31 animals there was a significant increase in the density of serotonergic fibres in the ipsilateral OB which may indicate collateral sprouting of serotonergic fibres. Collateral sprouting of serotonergic fibres in this region may potentially be related to the effect of zinc on GABA receptors. If zinc does indeed enhance the flow of chloride through GABA receptors, thus potentially releasing increased amount of GABA, then excess GABA could be present in the OB during the time when GABA may have neurotrophic influences.

Although excess GABA in PND 10 animals (day of zinc application to the OE) may exert neurotrophic effects on serotonergic fibres thereby accounting for the increased density of these fibres, several questions arise concerning this hypothesis. GABA is present in all layers of the OB whereas sprouting of serotonergic fibres is only evident in the infra EPL region. Thus, if increased GABA during the early

postnatal period is indeed involved in the serotonergic sprouting response in PND 31 animals then sprouting of these fibres would be expected to occur in other regions of the OB which may also experience increased GABA following exposure to zinc. A final difficulty with the hypothesis that GABA exerts trophic influences on the serotonergic fibres is whether serotonergic fibres in the OB have GABA receptors. The precise relationship between the cells containing GABA and/or GABA receptors and the serotonergic fibres in the OB has not been clearly elucidated making it difficult to conclusively determine the neurotrophic effect of GABA on serotonergic fibres. However, a recent study has shown that serotonergic neurons in the median and dorsal raphe nuclei can express the immunocytochemical marker for the alpha-1 and alpha-3 subunits of the $GABA_A$ receptor, suggesting that serotonergic neurons in raphe nuclei contain GABA receptors (Gao et al,1993). Thus, it is possible that the serotonergic fibres in the OB, which originate from the median and dorsal raphe nuclei, may contain GABA receptors and be influenced by the trophic effects of increased GABA following zinc application.

4.3.5 Consolidation of Factors Potentially Affecting Serotonergic Axon Sprouting

The degeneration of serotonergic fibres throughout the OB in adult animals is in contrast to reports of sprouting within serotonergic system in other regions such as the spinal cord (Polistina et al.1990; Wang et al.1991; Marlier et al.1992), hippocampus (Azmitia and Zhou, 1986) and visual system (Rhoades et al.1990) following deafferentation. We are confident, however, that the density measurements obtained in this study are accurate and reflect a true picture of post-lesion effects in

the OB. It appears that there may be two factors that differ between the present study and those involving the visual system and spinal cord. The continual renewal of the PON axons throughout the life of the animals is unique to the olfactory system. In addition the use of $ZnSO_4$ to remove the primary afferents to the OB differs from the visual system and spinal cord where the afferents to these areas were removed by enucleation or transection, respectively.

In the present study it is quite possible that degeneration of serotonergic fibres may have occurred as a consequence of an impoverished sensory environment. However, given the sprouting response of serotonergic fibres following surgical removal of the afferents to the visual system and spinal cord, it is more likely that loss of serotonin in the OB may be due to differing roles of the transmitter within these three systems. Continual renewal of the PON axons throughout the life of the animal may necessitate the prcsence of serotonergic fibres to inhibit synapse formation (Haydon et al.1984) and may be a function of serotonin that is unique to the OB. Thus, removal of the PON may decrease the need for the presence of serotonin and may lead to degeneration or retraction of the serotonergic fibres. Perhaps an even more important factor influencing the lack of serotonergic sprouting in the OB of adult animals following deafferentation was the use of zinc sulfate to lesion the afferent input. In most studies where heterotypic sprouting of serotonergic axons occurs, deafferentation is achieved by surgically lesioning the afferent input (Polistina et al.1990; Rhoades et al.1990; Wang et al.1991) or by specific chemical depletion of another transmitter system (Stachowiak et al.1984; Synder et al.1985; Zhou et

al.1991). Although chemical deafferentation using $ZnSO_4$ is the most common method used to remove PON inputs to the OB, the potential neurotoxic properties of zinc, coupled with the increased amounts of the metal present in the zinc-deafferented OB, lead us to suggest that the use of zinc was a major contributing factor to the observed decrease in serotonergic innervation in the adult animals. With respect to the increased density of serotonergic fibres in the deeper layers of the lesioned OB in PND 31 animals, it is possible that zinc may be less neurotoxic in the younger animals or may have modulated the GABA receptors in the OB during the early postnatal period, a time when GABA may exert neurotrophic effects upon the serotonergic fibres. These hypotheses, however, would have to be examined in future studies.

The use of non-chemical deafferentation of the OB, rather than $ZnSO_4$ deafferentation, may be an appropriate approach in future studies to examine the plastic response of cells and centifugal afferents in the OB. This would provide a more equivalent deafferentation of the OB to that produced by dorsal root deafferentation of the spinal cord or enucleation of the visual system. However, the problem with surgical deafferentation of the OB is that it is extremely difficult to perform successfully. In fact the approach was attempted without success in preliminary work leading to this thesis research. Therefore, the commonly used chemical deafferentation was the method of choice in this study to achieve deafferentation of the OB.

4.4 The Effect of OB Deafferentation on the Density of Noradrenergic Innervation

Studies on the response of the noradrenergic system to deafferentation of the OB report that the biochemical levels of noradrenaline remain unchanged from control values, with any slight increase in the noradrenaline level attributed to shrinkage of the OB following deafferentation (Nadi et al.1981; Kawano and Margolis, 1982; Baker et al.1983). However, biochemical studies could not determine any redistribution of fibres which was examined in the present study by dopamine-ß-hydroxylase immunocytochemistry. In normal rats at all postnatal ages, the noradrenergic innervation is densest in the granule cell layer and the internal plexiform layer with sparser input to the EPL and relatively little input to the GL (McLean and Shipley, 1991). In the present study, significant increases were observed in the density of noradrenergic innervation in the ipsilateral EPL region of the lesioned OB in adult animals implying that sprouting of the noradrenergic fibres occurred in that layer. The increase in the density of noradrenergic fibres in this study was not attributable to shrinkage of the OB as in previous reports (Nadi et al.1981; Kawano and Margolis, 1982; Baker et al.1983) since the decrease in area of the EPL and infra EPL regions of the lesioned OB were normalized with respect to control animals. Thus, it is possible that the slight biochemical increases in noradrenaline levels found in previous studies indeed reflected lesion-induced sprouting of the noradrenergic fibres. Similarly, heterotypic sprouting of noradrenergic axons has been reported in the lateral geniculate body (Stenevi et al.1972), superior colliculus (Rhoades and Hess, 1983; Nakamura et al.1984) and septum (Moore et al.1971)

following denervation or enucleation. In addition, sprouting of noradrenergic fibres has been reported in the atrophied neocortex (Coyle and Molliver, 1977).

Sprouting of noradrenergic fibres was also apparent in the contralateral infra EPL region following deafferentation of the OB of the adult, implying that anatomical changes may occur in sites removed from the area of injury. This increased density of noradrenergic innervation in the contralateral OB is somewhat perplexing. A possible explanation may be obtained by observations made in the atrophied neocortex where noradrenergic fibres increase their terminal arborizations in target areas regardless of changes in the cells in these regions. This phenomenon suggests that noradrenergic fibres are naturally programmed to maintain a certain density of terminals and possess high intrinsic growth regulation (Foote et al.1983). Thus, atrophy of the ipsilateral OB following deafferentation may have induced hyperinnervation of noradrenergic fibres in the contralateral bulb. This hypothesis could be tested more directly by removing one olfactory bulb and determining if noradrenergic fibres hyperinnervate the remaining OB.

In PND 31 animals no significant differences were observed in the density of noradrenergic fibres in any region of the lesioned OB examined when compared to control animals. However, generalized increases in fibre density were apparent in the EPL and infra EPL regions in bulbs contralateral to the lesion compared to control animals. Although it is possible that the noradrenergic fibres in the younger animals

are capable of responding to deafferentation in a similar way as the adult animals, future studies involving more PND 10 animals would be needed to determine if the generalized increase in noradrenergic fibre density is statistically significant before definite conclusions may be drawn.

Direct application of zinc to the OE did not appear to adversely affect the density of the noradrenergic innervation in the adult or PND 31 animal, unlike the speculated neurotoxic effect of compound on the serotonergic fibres in the adult animals. It is possible that the noradrenergic fibres may simply be resistant to the toxic effects of zinc due to some intrinsic property that renders them different from the serotonergic fibres or that zinc may have differential effects on the two fibre systems, however we are unaware of any reports corroborating these theories. The theories that serotonergic and noradrenergic fibres may be differentially affected by zinc would require further investigation possibly by application of zinc to cultured serotonergic or noradrenergic neurons.

4.5 Lack of Significant Change in the Density of GFAP$^+$ Astrocytes Following Deafferentation of the OB

Although astrocytes have been found to increase in the area of injury following damage to the CNS (Bignami and Dahl, 1976; Rose et al.1976; Barrett et al.1984; Mathewson and Berry, 1985; Takamiya et al.1988), the results of the present study suggest that no significant change in the density of GFAP$^+$ astrocytes is apparent 21 days following deafferentation of the OB of adult or PND 31 animals.

Proliferation of astrocytes is often observed after various types of injury to the CNS. The astrocytic response may be confined to the site of injury (Latov et al.1979) or occurs in areas distal to the lesion (Amaducci et al.1981; Schiffer et al.1986; Takamiya et al.1988). The specific factors that induce proliferation of astrocytes after injury are not known. Several stimuli, however, have been proposed as the cause of astrocyte proliferation including myelin breakdown (Osterberg and Wattenberg, 1962), influx of serum proteins (Roher et al.1991), increased extracelluar space (Cook and Wisniewski, 1973), nerve degeneration (Barrett et al.1981) and cytoarchitectural disruption (Mathewson and Berry, 1985).

Nerve degeneration and cytoarchitectural disruption, two factors that potentially induce astrocytic response to injury, are evident in the OB following deafferentation. Denervation of the axons of the PON is accompanied by degeneration of the terminals of the PON (Doucette et al.1983) and a subsequent shrinkage or atrophy of the GL or individual glomeruli (Margolis et al.1974; Harding et al.1978; Baker et al.1984). The lack of significant change in the density of

astrocytes indicates that the astrocytes in the OB may not increase in response to PON degeneration or disruption of the cytoarchitecture of the GL. However, given the magnitude of the astrocytic response in other areas of the CNS following injury it is unlikely that the astrocytes in the OB are unique in their response to deafferentation. A potential and probable reason fo the lack of apparent astrocytic response to deafferentation of the OB is the length of the post-lesion time period employed in this study. The astrocytic response appears to be optimum within 2-7 days following injury (Rose et al.1976; Mathewson and Berry, 1985; Takamiya et al.1988; Herrera and Cuello, 1992) and steadily declines, reaching almost control values by 2-3 weeks post-lesion (Mathewson and Berry, 1985; Takamiya et al.1988; Herrera and Cuello, 1992). Thus, if astrocytes did, indeed, increase in response to deafferentation of the OB the density measurements would most likely be similar to control values at the end of the three week time interval employed in this study. Although the post-lesion time period may have been too long for detection of astrocytic response to deafferentation it was necessary to ensure degeneration of the PON and to allow sufficient time to detect potential sprouting of fibre systems. Future studies monitoring the response of $GFAP^+$ astrocytes at different post-lesion times may provide additional information on the astroglial response to deafferentation of the OB. In addition, to determine if the potential changes in density of astrocytes is related to proliferative activity, double labelling of astroglia with GFAP and tritiated thymidine or bromodeoxyuridine (marker for cell proliferation) would facilitate investigation of cell division and mitotic activity.

In PND 31 animals, although no significant increase in the density of GFAP$^+$ cells occurred following deafferentation of the OB, a generalized tendancy for increased density is apparent in all regions of the lesioned OB. This may relate to a change in the expression of intermediate proteins of glial cells. During development radial glia are present in the CNS of mammals and are believed to transform into mature astrocytes (Wallace et al.1991). Unlike mature astrocytes, radial glia in rodents do not contain GFAP (Bignami and Dahl, 1974b). These immature glial cells, however, contain vimentin (VIM), another intermediate filament protein (Schnitzer et al.1981; (Bignami et al.1982). Transition from VIM to GFAP in rodents, occurs between the second or third postnatal week (Pixley and de Vellis, 1984). After injury, radial glial cells in the newborn appear to switch from VIM to GFAP production (Bignami and Dahl, 1973; Bignami and Dahl, 1974a; Bignami and Dahl, 1974b; Bignami and Dahl, 1976) and are considered to be early forms of mature astrocytes. Since deafferentation of the OB of PND 10 animals in the present study was performed during the period when maturation of glial cells was still occurring, we hypothesize that if there was a significant increase in the density of GFAP$^+$ astrocytes observed 21 days post-lesion, the increase may reflect greater numbers of radial glia producing GFAP earlier in development due to a forced switch from VIM to GFAP production. Thus, more astrocytes would express GFAP as a result of the injury thereby accounting for the generalized, yet nonsignificant, increase in density of GFAP immunoreactive astrocytes in PND 31 animals. Future studies comparing the density of VIM$^+$ and GFAP$^+$ glia in the PND 10 rat pup prior to injury and 21 days

post-lesion may provide insight on the magnitude of radial glia switching to GFAP production in response to injury.

4.5.1 Additional Responses of Astrocytes Following Injury

Although there was no significant increases in the density of GFAP$^+$ astrocytes in adult or PND 31 animals, it is possible that other changes, characteristic of the reactive astrocyte, could have occurred. For instance, increases in the intermediate filaments of astrocytes with a subsequent increase in the intensity of GFAP immunostaining (Bignami and Dahl, 1976; Amaducci et al.1981) have been reported after injury to the CNS. However, qualitative analysis of the intensity of GFAP immunostaining in the adults in the present study did not appear to differ between lesioned and control animals.

In addition, *in situ* studies have reported increases in GFAP mRNA in the hippocampus following unilateral lesions of the entorhinal cortex (Steward et al.1990) and in copper-deficient brindled mice that have genetically programmed damage of the CNS due to deficits in oxidative metabolism (Shafit-Zagardo et al.1988). However, the increase in GFAP mRNA in those studies did not reflect an increase in the number of astrocytes, but was attributed to increases in the cellular levels of the protein. Therefore, while the density of GFAP$^+$ astrocytes in the lesioned OB of adult and PND 31 was not significantly different from controls, the actual message for GFAP could be increased as a result of injury. Future studies could utilize *in situ* hybridization to localize and quantitate changes in GFAP mRNA in the OB after peripheral denervation.

CONCLUSION

This study has found that heterotypic sprouting of the serotonergic system did not occur in the OB of adult animals following $ZnSO_4$ lesioning of the OE, but instead decreased in its innervation density. In PND 31 animals treated similarly with $ZnSO_4$, however, the increased density of serotonergic fibres in the deeper regions of the OB may indicate sprouting of these fibres. The differences in the serotonergic response in adult and PND 31 animals may be due to differential effects of zinc with respect to the direct neurotoxicity and indirect neuromodulatory (via enhanced GABA release) properties of the compound. In addition, it appears that the astrocytic immunocytochemical expression of S100 in the OB does not correlate with changes in the density of serotonergic innervation of adult or PND 31 animals. Therefore, if S100 expression is correlated with serotonergic axon growth as it is in other systems, then it is not through more glial cells expressing S100 but perhaps through existing S100 positive cells expressing more of the protein.

In contrast to the serotonergic system, the noradrenergic system appeared to increase its axonal projections in the adult animals following deafferentation, yet no changes were apparent in the PND 31 animals. The differences in the response of the two fibre systems may be related to the differences in the reorganization of the OB following deafferentation. It is also conceivable that differential effects of the potential neurotoxic properties of zinc on the two systems could have accounted for the difference. Finally, this study has shown that the long-term density of astrocytes in the OB is not affected by removal of the afferent input in either PND 10 or adult animals.

Several of the rather unexpected results observed in this study may be attributable to direct neurotoxicity and/or indirect neuromodulatory effect of zinc application. Future studies using non-chemical deafferentation of the olfactory bulb may be helpful to further elucidate some of the questions raised in the present studies.

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