REGENERATION OF SUPRASPINAL PROJECTIONS AFTER SPINAL CORD INJURY IN THE NEONATAL OPOSSUM, *MONODELPHIS DOMESTICA*

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by

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A thesis submitted in fulfilment of the requirements for the Degree of Doctor of Philosophy
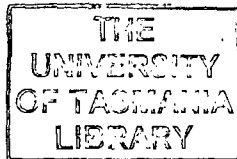
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ABSTRACT

The studies presented in this thesis define the developmental sequence of descending supraspinal projections in both control and spinally transected neonatal opossums using an axonal tracer (dextran amine) injected into the lumbar spinal cord at post-natal day (P) 7, P14, P21, P28, P35 and adulthood. All experiments were conducted in accordance with NHMRC guidelines and with the approval of the University of Tasmania Ethics (Animal) Committee. The numbers of retrogradely labelled neuronal cell bodies (indicating projections traced by the dye from the site of injection) were counted in brain sections from animals removed 4 days after injections. The time course of supraspinal projections reappearing across a complete mid thoracic spinal transection made at P7 was then compared to the normal developmental sequence after identical spinal injections in control animals. Supraspinal projections were found to traverse the injury site within one week of the operation and contributed in increasing numbers to lumbar projections across the lesion for up to 4 weeks post-lesion. Numbers of labelled neurons in adults were found to be much lower both for transected and uninjured, control animals. The distribution of neurons in different brain nuclei was similar to that of unlesioned control animals, thus projections appeared to emulate the normal sequence of development after transection. However, there were fewer projections to the lumbar spinal cord after a transection, as compared to control animals, indicating that while many axons were able to grow through an injury site, not all may persist until adulthood.

A double-labelled paradigm was employed to determine whether any fibres growing across the injury site were regenerated from axons severed by the transection. Lumbar spinal injections of dextran amine - Oregon green, made at P4, identified a population of supraspinal projections that were severed at P7 by a thoracic spinal transection. A different dextran amine – rhodamine was injected caudal to the lesion site at P7, P14, P21, P28 and P35 to label fibres growing across the lesion site. Neuronal cell bodies found containing both dyes indicated pre-lesion labelled axons that were able to regenerate their severed processes back across the injury site to encounter the second dye. Regenerating fibres increased from approximately 2 % of axons labelled with the first dye at P14, up to approximately 30 % at P35. It appears that regeneration of severed axons does occur following neonatal spinal injury at least in the first 4 weeks post injury.

ACKNOWLEDGEMENTS

ABBREVIATIONS

Al	Alar nucleus
Am	Nucleus ambiguus
CNS	Central nervous system
Coe	Locus coeruleus
CS	Superior colliculus
CSF	Cerebrospinal fluid
Dk	Nucleus Darkschewitsch
DRG	Dorsal root ganglion
EW	Edinger-Westphal nucleus
GAP	Growth associated protein
Hyl	Lateral hypothalamic nucleus
IFLM	Interstitial nucleus of the medial longitudinal fasciculus
L	Lumbar spinal segment
OS	Superior olive
P	Post-natal day
PHD	Paraventricular hypothalamic nucleus and dorsal hypothalamic area
PBS	Phosphate buffered solution
Rb	Red nucleus
RD	Dorsal medullary reticular nucleus
RGc	Gigantocellular reticular nucleus
RGcv	Ventral gigantocellular reticular nucleus
RaM	Raphe magnus nucleus
RP	Reticular pontine nucleus
RaO	Raphe obscurus nucleus
SEM	Standard error of the mean
T	Thoracic spinal segment
TrSi	Interpolar spinal trigeminal nucleus
TrSc	Caudal spinal trigeminal nucleus
WGA-HRP	Wheatgerm agglutinin- horse radish peroxidase
VstL	Lateral vestibular nucleus
VstM	Medial vestibular nucleus

STATEMENT
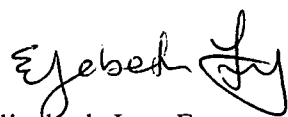
The work in this thesis has been undertaken exclusively for the use of a PhD in the area of Anatomy and Physiology, and has not been used for any other higher degree or graduate diploma in any University. All written and experimental work is my own, except where due reference is given.

Elizabeth Jane Fry

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CONTENTS

Chapter 1

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INTRODUCTION

1.1: THE FAILURE OF THE ADULT MAMMALIAN CNS TO REGENERATE

Nerve fibres within the adult central nervous system (CNS) do not regenerate following injury or insult, and this results in an irreversible loss of function. This has been a long observed clinical problem dating back to about 2500 BC from a description as "a disease one cannot treat" in the Edwin Smith Surgical Papyrus (Brested, 1930). Ramon y Cajal at the turn of the last century also agreed that an injury to the CNS was unchangeable and would be a challenging problem to solve:

" In adult centres, the nerve paths are something fixed, ended, immutable. Everything may die, nothing may be regenerated. It is for the science of the future to change, if possible, this harsh decree. " (Ramon y Cajal, 1928)

However, during his immaculate observations on nerve pathways and their response to injury, including the successful regeneration of severed peripheral axons, he noted that the initial response of a central nerve fibre to axotomy was encouraging. "Clubs of growth" sprouted at the proximal end of the severed nerve within 6 hours of the injury, but were aborted after extending only 1 mm towards the injury site and gradually resorbed and retracted (Ramon y Cajal, 1928).

From these and even earlier observations, a whole field of study has been devoted to what causes the sprouting to abort and how this may be reversed, with the eventual hope that the future outlook for victims of brain or spinal injury may be radically changed. Presently clinicians cannot offer much hope of recovery to spinal injury patients. They will still suffer permanent loss of function if the crucial communication pathways mediated by the spinal cord between the brain and periphery are severed. Sensation, movement, bladder control, heart and lung function, thermoregulation and sexual activity are all adversely affected and cause devastating disabilities accompanied by a great emotional and financial burden on patients, relatives, care-givers and society in general.

Continued research in recent decades has shaken the long established dogma of a non–regenerating CNS. It is increasingly apparent that nerve fibres in the CNS may indeed be responsive to experimental manipulation that can induce regenerative

growth following injury. These manipulations have included grafting tissue bridges of growth permissive substrate (such as peripheral nerve tissue or fetal spinal tissue) into injury sites, introduction of growth promoting substances, and the neutralising of negative influences within the CNS (see Section 1.1.2 and Table 1.3 for specific examples).

1.1.1 Why the CNS does not regenerate

Essentially, there are two possible explanations why the CNS is unable to regenerate following injury, both of which probably contribute to the answer.

1. The CNS microenvironment is inhibitory to growth (Ramon y Cajal, 1928; David and Aguayo, 1981) and/or;

2. the central neurons themselves lack the intrinsic capacity to regenerate (Guth, 1956).

It is notable that during development neurons are well able to regrow and the environment of the CNS is growth-supportive, but at a certain point the adult non-regenerative conditions appear to prevail (Ramon y Cajal, 1928). The idea that central axons do not have an intrinsic ability to regenerate was subscribed to for many years (Guth, 1956). Le gros Clarke (1943) interpreted the failure of CNS neurons to regenerate into peripheral nerve grafts as an incapacity for the process. However, other observations by Ramon y Cajal (1928) and Clemente (1964) describe sprouting and elongation of CNS neurons after injury before aborting the attempt at regeneration, rather than a complete absence of proliferation.

A convincing experiment by Aguayo and colleagues (David and Aguayo, 1981) illustrated that CNS fibres do indeed have the capacity to regrow after injury if given an appropriate substrate. When severed axons were presented with a peripheral nerve bridge between the adult rat medulla and spinal cord, they elongated into the transplanted bridge but growth was halted at the peripheral nervous system (PNS)/CNS interface. This also indicated the inhibitory nature of the CNS microenvironment, in comparison to other more permissive substrates such as PNS tissue. The Aguayo studies were in fact a repeat of much earlier experiments by one

of Ramon y Cajal's students (Tello, 1911). The modern studies had the advantage of good axonal tracing methods, which meant that the origin of axons growing into the graft could be clearly determined.

Elements of the CNS thought to contribute to the growth inhibitory nature of its microenvironment include myelin and/or associated molecules (Caroni and Schwab, 1988; Schnell and Schwab, 1990), and cells such as astrocytes, oligodendrocytes, oligodendrocyte precursors and microglia (see Fawcett, 1998 for review) that migrate into the lesion site. The absence of Schwann cells from the CNS is believed to be a crucial factor as in the regenerating PNS these act as a guide for regenerating axons and synthesise vital growth promoting molecules (Li and Raisman, 1994; also see Table 1.1 for information). It is now more widely accepted that spinal neurons do possess the potential to achieve regenerative growth but that this is somehow repressed by the local environment (Schwab and Bartholdi, 1996).

There are reports that the intrinsic cell body response of a central neuron appears unconducive to regeneration, meaning that CNS axons have a compounding disadvantage after injury compared to peripheral fibres (Grill and Tuszynski, 1999). For example, successfully regenerating peripheral axons upregulate the production of cytoskeletal proteins such as tubulin, microtubule-associated proteins and the growth-associated-protein GAP-43 after injury, while severed central axons exhibit attenuated changes in the production of these proteins (Bisby and Tetzlaff, 1992; Fournier and McKerracher, 1995). This suggests that the molecular machinery of central neurons imparts a less "vigorous" intrinsic capacity for achieving regeneration, in addition to the negative extrinsic influences exerted by the CNS microenvironment on central axons attempting to regrow (see Table 1.2).

A further complication when examining the cause of abortive CNS regeneration is that there are a variety of responses to axotomy by different populations of neurons. For example, motor axons survive axotomy and regenerate (if lesioned in the peripheral portion), Purkinje cells survive but do not regenerate, and retinal ganglion cells die (see Sofroniew, 1999 for review). Similarities in an injury response by specific populations are likely to be determined by shared intrinsic properties such as topography, function, projection, and developmental origin (Sofroniew, 1999). The

Table 1.1: Factors **extrinsic** to neurons that influence CNS regeneration

Problem	Cause	Key References
Inefficient phagocytosis and removal of debris after the injury	1. Delayed appearance of blood-borne macrophages at the site of injury. 2. CNS oligodendrocytes lack a phagocytic ability. 3. Few resident phagocytic cells in the CNS (microglia) and are slow to activate.	1. George and Griffin, 1994 2. Bignami and Ralston, 1969 3. George and Griffin, 1994
CNS inflammatory response may be non-conducive to regeneration.	1. Presence of microglia that may produce neurotoxic molecules. 2. Increased cytokine expression (eg interleukin -1, tumour necrosis factor-α) after CNS injury modulate migration and adhesion of inflammatory cells (eg microglia/ macrophages). These may have both growth promoting or inhibitory influences.	1. Giulin *et al.*, 1993; Perry *et al.*, 1993; Fitch and Silver, 1997 2. Schwab and Bartholdi, 1996
Axotomy induced neuronal cell death may occur after injury in the CNS but is dependant on neuronal cell type, collateral projections, distance of axotomy from cell body and age of the neuron.	1. Neurons may be deprived of target derived trophic support. 2. Some immature neurons are highly susceptible to axotomy induced cell death. 3. Collateral braches may provide support to axotomised neurons. 4. Axotomy proximal to neuronal cell body is associated with greater cell death.	1. Hamburger and Levi-Montalcini, 1949 2. Snider *et al.*, 1992 3. Fry and Cowan, 1972 4. Villegas-Perez *et al.*, 1993
Progressive necrosis of the damaged tissue for weeks after the injury prevents support of fibre outgrowth	Survival of axotomised neurons may be compromised by dying tissue.	Guth *et al.*, 1983; Oorschott and Jones, 1990
No intrinsic structural support or guidance for axons attempting to regenerate.	Schwann cells are absent from CNS, (present in PNS which can regenerate). Equivalent oligodendrocytes do not respond to injury by providing scaffolding for axonal outgrowth.	Lampert and Cressman, 1966; Bignami and Ralston, 1969
Absence of appropriate trophic molecules and growth promoting factors eg NT-3 after developmental period	Schwann cells in PNS are thought to produce trophic molecules but are absent from CNS.	Tello, 1911; Ramon y Cajal, 1928; Schnell *et al.*, 1994
Presence of **inhibitory myelin** associated molecules (Nogo, myelin-associated-glycoprotein) in adult CNS	Suppression of sprouting attempts by severed axons by inhibitory molecules.	Caroni and Schwab, 1988; Schnell and Schwab, 1990; McKerracher *et al.*, 1994; Chen *et al.*, 2000; GrandPre *et al.*, 2000;
Formation of a **glial scar** at the site of injury	The scar may act as a physical or chemical barrier to growing fibres	Windle, 1956; Cavanagh, 1970; Davies *et al.*, 1997
Upregulation of **growth inhibitory molecules** at the site of injury such as chondroitin sulfate proteoglycans, possibly associated with inflammatory cell recruitment to injury site or gliosis	Suppression of any sprouting that may occur after the injury.	Snow *et al.*, 1990; McKeon *et al.*, 1995; Davies *et al.*, 1997; Fitch and Silver, 1997

Table 1.2: Factors **intrinsic** to neurons that influence CNS regeneration

Problem	Cause	Key References
Expression of regeneration associated genes (eg GAP-43, c-jun, α-Tubulin, L-1, Tenascin- C) is compromised with inconsistent upregulation and "unvigourous" after CNS injury	1. Retrograde transport of injury signals (eg upregulated mRNA) to cell body is inefficient in CNS 2. Molecular machinery of CNS may not respond to injury in regenerative manner	1. Fernandez *et al.*, 1981; Kalil and Skene, 1986 2. Strittmatter *et al.*, 1995; Benowitz and Routttenberg, 1997
Resynthesis of cytoskeletal components eg neurofilaments, actin, tubulin, after injury is inefficient	Due to unvigourous response of growth associated genes eg Tα1-tubulin	Tetzlaff *et al.*, 1988; Bisby and Tetzlaff, 1992; Caroni *et al.*, 1997; Doster *et al.*, 1991
Failure to physically reconstruct severed fibres	Anterograde axonal transport of cytoskeletal components to aid fibre outgrowth is inefficient	McKerracher *et al.*, 1990; Fournier and McKerracher, 1995
Cell adhesion molecules (N-CAM, L1) and axonal guidance molecules (Netrins, Semaphorins, Ephrins) not correctly coordinated after axotomy in CNS	Suppressed or not upregulated once development finished.	Kleitman *et al.*, 1988; Zhang *et al.*, 1995;

ability of a cell body to mount a regenerative response after injury is also greatly affected by the distance between the site of axotomy and the cell body. However, there is no uniform principle in that different populations respond best to axotomy at different distances from the cell body due to the multifactorial influences upon the severed neuron (Lieberman, 1971).

The effect of myelin on the critical period

The presence of myelin has long been implicated as a major inhibitor to regrowth after injury in the CNS (Caroni and Schwab, 1988; Schnell and Schwab, 1990). A prime indicator of this association is that prior to the developmental onset of myelination, the immature CNS responds to injury with outgrowth of fibres and a degree of regeneration (Shimizu *et al.*, 1990; Bates and Stelzner, 1993; Hasan *et al.*, 1993; Wang *et al.*, 1998). Steeves and colleagues found that by delaying the onset of myelination in the chick embryo by immunologically-induced demyelination, the permissive period for functional repair following spinal transection was extended from E13 to E15 (Keirstead *et al.*, 1992). Subsequent experiments in the mature avian CNS showed that this technique facilitated axonal regeneration of approximately 6-19% of severed supraspinal neurons projecting to the spinal cord (Keirstead *et al.*, 1995). While the amount of regeneration observed after demyelination in the mature CNS was less than that produced following injury in the immature CNS, it confirmed that the appearance of myelin in the avian CNS has a greatly inhibitory influence on regenerative capacity.

It appears that a number of associated molecules are responsible; the two major ones were identified by Schwab and colleagues, initially named NI-250 and NI –35. The identity of the proteins composed of 250 kDa and 35 kDa components respectively, responsible for the powerful inhibition remained unknown for many years (Schwab and Bartholdi, 1996). This was despite the fact that a monoclonal antibody (IN-1) capable of neutralising both proteins had been successfully raised more than 10 years ago (Caroni and Schwab, 1988). However, recently the *Nogo* gene was identified (Chen *et al.*, 2000; Prinjha *et al.*, 2000). The gene was found to encode at least 3 major protein products: Nogo-A, Nogo-B and Nogo-C. Most importantly, Nogo-A

was recognised by the IN-35/250 antibody and probably corresponds to the rat protein NI-250, known to inhibit neurite outgrowth (Chen *et al.*, 2000).

In vitro studies with the isolated neonatal *Monodelphis* CNS at various stages of development have shown that there appears to be a certain age beyond which regenerative growth exhibited after a spinal crush will not occur. This time of development is associated with the differentiation of the glial cell population, the onset of myelination of axons, and expression of inhibitory proteins and neurotrophic factors (Varga *et al.*, 1995a). At post-natal day (P) 8-9 oligodendrocytes, astrocytes and myelin sheaths begin to develop in the *Monodelphis,* increasing to abundant levels by P14 (Varga *et al.*, 1995a). There is also a marked increase in the levels of nerve-growth inhibitory proteins at P10-P14 (Varga *et al.*, 1995b). This evidence supports the theory that these developmental events are highly related to the onset of non-permissive nature of the cord for axonal growth at P11-P14 in the neonatal *Monodelphis* (although this is also dependant on the spinal level at which the injury is made).

Myelin-associated glycoprotein is another well characterised myelin component that has been shown to have a potent inhibitory influence on the regrowth of axons in culture (McKerracher *et al.*, 1994; Mukhopadhyay *et al.*, 1994). This inhibitory influence may be blocked by firstly exposing neurons to neurotrophins prior to encountering the inhibitor molecule in culture (Cai *et al.*, 1999). The mechanism of this inhibitory suppression is mediated through a cAMP-dependant pathway, which activates protein kinase A or other, as yet unidentified down-stream proteins able to block or neutralise the inhibitory signals released by the myelin-associated glycoprotein (Cai *et al.*, 1999).

1.1.2 What would be required for a severed CNS axon to regenerate?

Should a central nerve fibre be injured and assuming the associated neuronal cell body survived, the regenerative process is more than a simple matter of damaged tissue rebuilding itself. Rather it is a highly complex series of events that requires the axon to sequentially sprout, enter, and grow through and beyond the injury site in a direction that allows recognition and re-entry into its specific correct target region.

There the axon must undergo synaptogenesis at the correct location, if necessary forming a correct topographic distribution to restore functional neural transmission and behaviour. Strategies to promote regeneration must address all these events with the final outcome being the return of lost function. No one therapy is likely to overcome such a multifactorial problem and thus a combination of therapies will be necessary. Given that much remains unclear or that studies show conflicting reports, a clinical cure is still some time away. Table 1.3 outlines strategies that attempt to fulfil the requirements for regeneration. Essentially, for successful regeneration, severed axons must recapitulate many of the events completed during development such as sprouting, outgrowth and extension of fibres, then form correct synaptic connections with appropriate targets such that signal transmission may mediate normal function – in spite of the fact that the adult CNS environment has now become non-permissive to axonal outgrowth.

1.2 THE IMMATURE CNS RESPONSE TO INJURY

As the immature CNS provides an environment that allows developing axons to grow through and reach their intended targets, it would be reasonable to assume that the immature CNS may also be permissive for severed axons to regrow after axotomy. This belief has been held since the turn of last century, although few scientists explored the idea in detail. Descriptions of fibre sprouting and recovery of function after injury in fetuses and newborn animals may be found among the early literature on regeneration, although this is more likely to have been considered evidence for plasticity, rather than a regenerative ability. Early last century, Ramon y Cajal noted in his comprehensive study of brain and spinal injury in cats and dogs that sprouting of injured axons occurs in one week old animals and that injury early in development produces far less irremediable damage than in the comparable adult or even late postnatal CNS (Ramon y Cajal, 1928).

Migliavacca (1928) also described the outgrowth of neurites across lesion sites in rat fetuses and some recovery of function. These early experiments on newborn animals or fetuses did not always have positive outcomes after injuries made in the CNS,

Table 1.3: Strategies to promote CNS regeneration

Objective	Method	References
Prevention of **secondary damage** such as tissue necrosis and cell death by suppression of the acute injury response or modulation of inflammatory response	Application of Anti-inflammatory agents 1. Methylprednisolone 2. LPS 3. Chloroquine 4. Others anti-inflammatory agents	1. Bartholdi and Schwab, 1995; Chen et al., 1996; Oudega et al., 1999 2. Guth et al., 1994 3. Giulian et al., 1989 4. Zhang et al., 1997, Chau et al., 1998
Prevention of **axotomy induced cell death** by neuroprotection	1. application of growth factors 2. blocking of cell death pathways (apoptosis or necrosis) with glutamate receptors of antagonists and ion channel blockers 3. protection against free radicals	1. Gimenez y Ribotta, 1997; Teng et al., 1998 2. Choi et al., 1987; Wrathall et al., 1997; Watanabe et al., 1998 3. Iadecola et al., 1997
Promotion of **outgrowth**	1. Application of neurotrophic factors, combined with grafts secreting factors 2. X-ray therapy 3. Electrotherapy	1. Xu et al., 1995a; Oudega and Hagg, 1996; Ye and Houle, 1997 2. Kalderon et al., 1990 3. Fehlings et al., 1988; Borgens et al., 1990
Prevention of **glial scar formation**	Suppression of glial reaction	Windle et al., 1952; Clemente, 1955; Gimenez y Ribotta et al., 1995; Vanek et al., 1998
Suppression/ neutralisation of **inhibitory influences** of CNS eg myelin associated products	1. Vaccination against myelin proteins 2. Neutralisation of inhibitory proteins (eg Nogo) with antibodies	1. Huang et al., 1999 2. Schell and Schwab, 1990; Bregman et al., 1995; Thallmair et al., 1998
Provide a **permissive environment** for sprouting central axons	Transplants and grafts of growth permissive tissue 1. Peripheral nerve 2. Fetal tissue grafts 3. Stem cell grafts 4. Schwann cell grafts 5. Olfactory ensheathing glia 6. Acellular matrix conduits 7. Fibroblasts	1. Tello, 1911; David and Aguayo, 1981; Cheng et al., 1997 2. Reier et al., 1983; Bregman and Reier, 1986; Iwashita et al., 1994 3. Gage et al., 1995; McKay, 1999 4. Bunge et al., 1994; Xu et al., 1995b; Guest et al., 1997 5. Li et al., 1997; Ramon-Cueto et al., 1998 6. Silver and Ogawa, 1983; Xu et al., 1995a; Oudega et al., 1997 7. Grill et al., 1997
Increase vigour of **cell body response.**	1. Replace neurons with stem cells 2. Application of molecules that increase intrinsic growth potential by upregulation of RAGs 3. Activate growth cone signal transduction cascade	1. McDonald et al., 1999 2. Benowitz et al., 1999, Lehmann et al., 1999 3. Song and Poo, 1999, Cai et al., 1999
Ensure correct outgrowth direction, target recognition and **correct reinnervation**	Provide specific guidance cues	Holt and Harris, 1998; Pasterkamp et al., 1999; Miranda et al., 1999; Reza et al., 1999
Functional **rehabilitation**	Functional Electrical stimulation and Body weight support	Barbeau et al., 1998

such as Hess (1955), causing some doubt as to the capacity of the immature CNS to produce true recovery. However, these negative results were not held in high regard by Chambers (1955) and Clemente (1955) when discussed alongside more positive outcomes from immature injury.

More recent work over the last decade has unequivocally established the immature CNS as possessing a much greater capacity than the adult CNS for outgrowth of fibres after an injury, plus recovery of function, and in a few cases some examples of regeneration have been produced. These are described in detail below.

This greater potential for repair in the immature CNS may be explained by the reduced form or absence of supposed inhibitory factors during development (Oorschott and Jones, 1990; see Table 1.1 and 1.2 for more information on inhibitory factors). For instance, in reference to the neonatal rat, nerve fibres are all as yet unmyelinated (Matthews and Duncan, 1971), astrocytes and other glial cells form only a minimal scar after injury as they are at very early developmental stages, if present at all (Prendergast and Stelzner, 1976). The phagocytic process after injury is completed very quickly, removing inhibitory products of degeneration by three days after the injury (Gilbert and Stelzner, 1979). More importantly, some axonal pathways, such as the corticospinal tract in rat (Schreyer and Jones, 1982), develop postnatally and therefore the spinal cord remains an environment that allows the growth of new fibres and synaptogenesis. Guidance cues and growth factors that are present to ensure the correct and appropriate innervation of growing fibres could well be available to regenerating axons in the immature CNS (Schwab and Bartholdi, 1996).

An example showing that the immature CNS has a greater plasticity for reorganisation and a more growth-permissive environment was illustrated when fetal grafts were implanted into a neonatal environment, and found to provide a bridge for host axons to grow through and provide a source of interneurons (Bregman and Kunkel-Bagden, 1988; Bregman and Bernstein-Goral, 1991). However, it was noted that fetal grafts were not as successful if implanted into the mature CNS unless growth factors were added (Bregman *et al.*, 1987) and tended to only act as a

neuronal relay for the transmission of impulses between the separated parts of the nervous system (Reier *et al.*, 1986).

CNS injury during the immature period also appears to produce fewer traumatic outcomes than an identical adult injury. Weber and Stelzner (1977) reported that spinal transection before P14 in rats resulted in little spinal shock as compared to an identical weanling lesion. Additionally, the lesion site in adult rats that were injured as neonates showed less scar forming gliosis as compared to adult animals lesioned as weanlings (Stelzner *et al.*, 1975).

When using the immature CNS as a model with which to examine axonal regrowth after injury, it must be recognised that often the period of time that is more permissive to regeneration will also be supporting much developmental fibre outgrowth in parallel. Therefore, should regrowth be seen following neonatal or embryonic CNS injury, it would be important to distinguish the relative contribution made by fibre growth from regenerating injured axons, and from subsequent development of undamaged axons. It is also of importance to determine whether regrowing fibres are able to complete the full regenerative process by growing through the injury and innervating the appropriate target regions to produce normal function. Consideration must be given to the fact that any functional recovery following immature CNS injury may be, at least in part, due to reorganisation of undamaged tissue and normal developmental growth.

1.2.1 Spinal injury in the immature marsupial *Monodelphis domestica*

The marsupial mammal has proven an ideal model with which to examine spinal injury at early stages of development due to the extreme immaturity of the young at birth (Fadem *et al.*, 1982). Much development, which in eutherian mammals would take place in the uterus, occurs *post partum* in the marsupial. In particular, the South American opossum *Monodelphis domestica* has notable advantages for studying a developing nervous system. This small, rat-like marsupial is a highly fecund, non-seasonal breeder producing litters of up to 12 pups. The species has a clean, hardy, docile nature in captivity and is a manageable laboratory animal. The *Monodelphis* is

pouchless, facilitating access to the young attaching to the teats immediately after birth. The *Monodelphis* has a short gestation period of 14 days (Fadem *et al.*, 1982). At birth (the first day of life is known as P0) the CNS is developmentally equivalent to an E13 rat or a 6-week-old human fetus (Saunders *et al.*, 1989) consisting of an embryonic forebrain without a cerebellum, and the pup is capable of little more than respiration, sucking and forelimb movement (Nicholls *et al.*, 1990). Nerve fibres are unmyelinated, few glial cells have differentiated and much developmental growth of fibres towards target regions in the spinal cord is still to occur (Møllgård *et al.*, 1994).

One great advantage of using the immature *Monodelphis* to study spinal injury is that the entire CNS may be dissected out from a P3 animal and maintained for up to 10 days in culture (Nicholls *et al.*, 1990). The *Monodelphis* spinal cord may therefore be examined for its response to injury either as an *in vitro* or *in vivo* system, each of which has different advantages (see Nicholls and Saunders, 1996 for review). While the *in vivo* situation better reflects the true processes, the complexity of biology can often confuse or mask the significance of contributing elements. The *in vitro* preparation, while only being useful for a period of days, allows the microenvironment to be more closely controlled. This is particularly useful when examining the influence of particular molecules or growth factors applied to the spinal cord.

Axon outgrowth in *Monodelphis*

One of the first preoccupations of any regeneration study is to determine whether there has been any axonal outgrowth after nerve injury. This has been observed after spinal lesions in neonatal *Monodelphis* in both *in vitro* preparations (Treherne *et al.*, 1992, Woodward *et al.*, 1993) and *in vivo* experiments (Fry, 1997). Most importantly, this has occurred in the absence of any external manipulations to promote outgrowth such as transplants or addition of growth factors.

In vitro preparations of entire isolated CNS from *Monodelphis* aged P3-7 were subjected to crush lesions at the thoracic level that completely separated the spinal cord beneath the pia mater. This immediately abolished all impulse conductivity along the cord from rostrocaudal portions. By 4 days after the injury, axons labelled

with a carbocyanine dye, DiI, or horseradish peroxidase, showed profuse outgrowth into the lesion site, whereupon they branched extensively and several extended up to 4 mm beyond the lesion. Nerve impulses were also restored across the lesion (Treherne *et al.*, 1992, Woodward *et al.*, 1993). Similar remarkable structural and also functional recovery was seen when the *Monodelphis* spinal cord was subjected to an identical thoracic crush lesion at P4-P8. Histological examination of the spinal cord immediately after the *in vivo* operation showed that all axons were severed, but 10 days later numerous fibres were detected growing across the crush site (Saunders *et al.*, 1995). The spinal cords of *Monodelphis* that received crush lesions at P4-P8 showed primarily normal gross spinal cord structure by 3 months of age with numerous fibres including myelinated axons present (Saunders *et al.*, 1995, 1998). These lesioned animals also showed significant functional recovery (Saunders *et al.*, 1998). One point to consider here is that while a crush lesion severs axons, it leaves the dura intact which provides a conduit along which axonal outgrowth prefers to extend (as seen in Varga *et al.*, 1996), and this makes it difficult to assess whether fibres would be capable of bridging a lesion in the absence of connection between severed ends. It also does not give complete assurance that all fibres were severed by the lesion. To completely remove all ambiguity about the validity of a transection, it would be necessary to cut the spinal cord in two. This would be very difficult in an *in vitro* situation due to the problem of keeping the opposed ends together within the culture system and therefore any such experiment is best suited to the *in vivo* situation, where the spinal cord is supported by surrounding tissue.

A cut lesion using spring scissors that completely transected the thoracic spinal cord (including the pia mater), was performed *in vivo* on *Monodelphis* aged P4-P7 (Fry, 1997; Saunders *et al.*, 1998) to compare the fibre outgrowth with the crushed *in vitro* preparations (Treherne *et al.*, 1992; Varga *et al.*, 1995, 1996). As a wide gap was created between severed ends in the spinal cord by this lesion, one might suppose that structural repair would be less likely to occur given the lack of connection, or at least that the process would take longer. This was indeed found to be the case in an *in vivo* study examining the reappearance of dorsal root ganglion (DRG) fibres across complete spinal transection in P7 *Monodelphis* (Fry and Saunders, 2000). In this study, fibres originating in DRG just caudal to a complete thoracic transection

were labelled with the retrograde tracer DiI (a carbocyanine dye) and were not seen to enter a tissue bridge across the lesion site until 14 days post-lesion (Fry and Saunders, 2000).

Spinal lesions performed in *in vivo* experiments greatly extended the time frame over which to observe repair process. *Monodelphis* at 2 and 6 months of age, which had received cut lesions at P6-8, showed a continuous and intact bridge of nerve fibres across the still obvious lesion site. The gross structure of the spinal cord had distinct deficits and the bridge across the lesion site was sometimes much thinner than the rostral and caudal cord (Saunders *et al.*, 1998).

1.2.2 Fibre outgrowth after injury in immature CNS in other species

The ability of the immature CNS to produce fibre outgrowth after injury is not confined to the *Monodelphis* and has been reported in other embryonic and neonatal mammals, such as the North American opossum, *Didelphis virginiana* (Xu and Martin, 1991), kitten (Howland *et al.*, 1995), rat (Bernstein and Stelzner, 1983; Saunders *et al.*, 1992) and hamster (Kalil and Reh, 1982). However, a distinction should be made between the ability of fibres to grow *around* partial lesions via undamaged tissue and actual reconnections being established *through* severed tissue. Another consideration is whether the axons are regenerating or originate from collateral sprouts from undamaged axons. The developmental status of lesioned tissue may influence whether the CNS is able to regenerate severed axons, or instead reorganise intact regions leading to the appearance of both structural and functional recovery. As development is not a uniform process across species, it can be difficult to compare fibre outgrowth during immature stages. The type of lesion is also an important consideration when examining the repair after injury as many studies employ only partial lesions, rather than complete transection. For example, an overhemisection of the spinal cord is a common lesion, by which one whole side of the cord is severed in addition to the dorsal columns of the contralateral side (see eg. Bernstein and Stelzner, 1983; Bates and Stelzner, 1993; Bernstein-Goral and Bregman, 1993).

Partial lesions will often be variable in extent and the undamaged cord may provide an easier route of outgrowth for fibres that may contribute to the observed recovery. However, as Saunders and Dziegielewska (2000) point out, partial lesion experiments have shown that the ability to grow through a lesion site, as opposed to around it, is mostly determined by a developmentally regulated growth-permissive period.

In many eutherian species, the window of time in which axons will regrow through a lesion site has ended before birth. Unilateral transection of the pyramidal tract in the medulla oblongata of infant hamsters (ie. within two weeks of birth in a species, which for a eutherian species, is born relatively immaturely) was followed by extensive growth of corticospinal fibres *around* the lesion site and down the spinal cord via an aberrant route in the unlesioned tissue as seen by ^{3}H proline labelling (Kalil and Reh, 1979). No fibres were able to grow directly through the lesion site, although the terminal pattern of innervation in the target region of the contralateral dorsal column nuclei and cervical spinal cord appeared normal. Subsequent studies with this model showed that synaptic connections had been made (Kalil and Reh, 1982) and that behaviour mediated by the lesioned pyramidal tract, such as fine manipulation during feeding, was preserved (Reh and Kalil, 1982). This behaviour disappeared if adult animals were given the lesion or were relesioned after neonatal injury. While it was initially suggested by the authors that regeneration of cut fibres may have contributed to the spared function along with collateral sprouting from other pathways, a subsequent study (Merline and Kalil, 1990) showed that the aberrant corticospinal pathways after early pyramidal tract lesions had arisen solely from undamaged or later developing cortical axons sprouting into the injury site.

While the hamster is less mature at birth than the rat (Kalil and Reh, 1979), its eutherian nature means that the CNS is relatively well developed at this time and may not be growth-permissive in all regions. This highlights the difficulty in establishing a CNS injury model in which outgrowth is produced directly through the lesion site with regeneration a further elusive outcome. When the mammalian CNS has been injured at even more immature stages such as in the *in utero* experiments by Migliavacca (1928) on E15 rats showing outgrowth of fibres post–injury, or in the cultured fetal rat CNS, axons labelled with DiI have been detected growing through a

complete crush lesion (Saunders *et al.*, 1992) as in similar experiments with the neonatal opossum *Monodelphis* (Varga *et al.*, 1996).

However, much of the fibre outgrowth seen may be explained by the large amount of subsequent developmental growth that is occurring at the time of injury and this must be distinguished from the regenerative outgrowth of cut axons. Fibre outgrowth, either through or around an injury site is likely to contain a high proportion of fibres attempting to manoeuvre towards their original intended target. It remains significant that any fibres may grow across an injury site with the proposed inhibitory influences (see Table 1.1), and this alone would be a notable achievement. An important question, which is central to the work presented in this thesis is whether any severed axons in the immature system have been able to regenerate following axotomy by the lesion, as distinct from continued developmental outgrowth of new axons.

1.2.3 Target reinnervation after immature injury

Aside from the important question of whether fibres growing through a lesion site are regenerative or have arisen from subsequent development, it is also of interest to see if they are able to make synaptic connections with appropriate targets. The *in vitro* opossum CNS preparation has been useful in examining this problem and evidence suggests that the immature spinal cord is able to achieve these requirements for functional regeneration.

Nerve fibres must also recover conduction after injury for any functional recovery to occur. Failure to detect propagation of an action potential across a crush immediately following the injury in *in vitro* opossum spinal cord preparations indicated that all conduction had been abolished by the lesion (Treherne *et al.*, 1992; Nicholls *et al.*, 1990). By the fourth or fifth day following a spinal cord crush, propagation of action potentials across the lesion site was observed in 80% of preparations and the amplitude approached normal (although not fully restored), despite only a small proportion of fibres crossing the injury site. Only the preparations with evidence of morphological repair also showed recovery of action potential conduction (Treherne *et al.*, 1992). Close inspection of the action potential showed 2 distinct peaks. The second peak could be blocked by a raised Mg^{2+} concentration (known to block

chemical synapses), both when added to the culture bath and when applied only to the distal half of the cord. This suggested that the second peak corresponded to activity mediated through synaptic connections made by the fibres growing through the lesion (Woodward *et al.*, 1993; Varga *et al.*, 1995a). Electrophysiological testing for action potential conduction across *in vivo* spinal crush lesions on P4-P8 *Monodelphis* revealed that conduction had returned to a substantial proportion of the normal level in lesioned cords by adulthood after the neonatal crush (Saunders *et al.*, 1998). Thus, both *in vitro* and *in vivo* systems are capable of restoring conduction across an injury.

Varga *et al.* (1996) examined under the electron microscope spinal cords from *Monodelphis* crush lesioned at 3 days of age at the cervical level and then cultured for a further 5 days. "Synaptic" structures formed by axons were observed growing next to the pial basal lamina. In this short period of post–injury repair active zones and vesicles were clustered within a presumptive presynaptic terminal indicating that axons growing across the lesion site were in the process of forming synaptic connections. As part of the same study, electrophysiological recordings were made from ventral spinal roots rostral to the lesion after stimulating dorsal roots caudal to the lesion. By 5 days post-lesion in culture, small but unequivocal discharges were recorded across the lesion site, further indicating that appropriate synaptic connections were forming (Varga *et al.*, 1996).

1.2.4 Functional recovery after spinal injury

The final test of repair is whether correct function returns to an animal with a completely transected spinal cord. It has been known for some time that often functional deficits are not apparent until more than 90% of axons are lost in CNS lesions (Fawcett, 1998) and that incomplete spinal lesions in adult rats can be followed by a substantial recovery of postural control (Windle *et al.*, 1958). Equally the regeneration of a few axons has been suggested to return a great degree of function (Fawcett, 1998) although there is little or no evidence for this. It may even be possible for an animal with a CNS injury to show a degree of behavioural recovery without restoration of the original correct topographic map, particularly in the case of brain injury. Rather than axonal regeneration, it is likely that an adaptive

process has taken place, reflecting a plasticity of the CNS only recently appreciated (Neumann and Woolf, 1999).

Functional recovery in *Monodelphis*

The development of locomotor behaviour in *Monodelphis* that had received either a complete crush or cut lesion at P7-P8 was remarkable and included the ability to perform complex tasks in adulthood (Saunders *et al.*, 1998). When generally observed for their walking ability, the adult animals that were spinally injured at P7-P8 did so normally and without apparent difficulty, using all four limbs, although minor abnormalities in gait were detected in some (Saunders *et al.*, 1998). Animals were observed climbing a beam, crossing two grids of different sizes, swimming, and swimming with climbing. These activities require interlimb coordination and intact vestibular pathways for balance along with other supraspinal tracts (Kandel *et al.*, 2000). The ability to swim using hindlimbs proves that locomotor action was not produced by local reflex circuits of the spinal cord upon contact with a hard surface. Overwhelmingly, the adult animals that received a spinal injury shortly after birth did not show differences from the control normal animals in their locomotor ability for all tasks using all four limbs. This suggested that at least some supraspinal pathways were mediating these functions from the lower spinal cord to the brain. Animals that received spinal cut lesions showed greater locomotor impairments than those that received spinal crush lesions and took significantly longer times than the controls to climb the beam and cross the grids (Saunders *et al.*, 1998). However, most importantly, they were still able to perform all tasks adequately, indicating a high degree of recovery and normal behavioural development. It has not yet been determined whether the capacity for locomotion was the result of correct target reinnervation or whether the animals have adapted to abnormal growth, as it appears possible for an animal to produce relatively normal behaviour with less than the full amount of fibre connection (Saunders *et al.*, 1998). Although it is not know if the axon growth producing the recovery was regenerative.

Functional recovery in other species

Functional recovery after injury in immature CNS injury has been reported on numerous occasions in other species. However, if the injury was made after birth in

eutherian mammals it would appear that rather than a regeneration of severed fibres mediating this function, an adaptive process occurs.

A study by McKenna and Whishaw (1999) examined paw-reaching capabilities in the rat before and after dorsal column lesion. The dorsal columns carry vital sensorimotor information that if damaged in humans, will compromise fine motor skills (Nathan *et al.*, 1986). Before the dorsal column lesion, the rats were highly proficient at retrieving a small pellet of food through a small aperture with their paw. After the lesion, the rats quickly recovered their ability to reach the food pellet with their affected paw, however they did not recover the normal range of movements. Thus the rats were able to effect adaptive movements, including abnormal rotation of the head, neck and shoulders, to achieve their locomotor intention. It is possible that other intact axonal pathways, such as the spinothalamic or spinoreticular tract (Giesler *et al.*, 1981; Tracey, 1995), carry the afferent information from the forelimb to the brain. However, there seem to be a remarkable ability to compensate for damage to major sensory fibres. Therefore, it is possible for a task to be performed by CNS injury-affected limbs by compensatory movements when the injury does not sever all axonal pathways. This makes the interpretation of experiments involving partial lesions extremely difficult.

1.2.5 Development of supraspinal pathways in the *Monodelphis*

When commenting on the response to injury in the immature CNS, it is relevant to know the developmental sequence of outgrowing fibres for that species, as this will be superimposed on any regenerative activity seen. In the present study, outgrowth of supraspinal projections after spinal transection in the *Monodelphis domestica* is examined. The normal developmental timetable of the axons outgrowth from different CNS nuclei in *Monodelphis* is shortly described below.

Martin and colleagues made injections of retrograde axonal tracers, Fast blue or wheat germ agglutinin- horse radish peroxidase (WGA-HRP), into cervical and lumbar spinal cord of neonatal *Monodelphis* pups aged from P0 to P26 (Wang *et al.*, 1992) and adults (Holst *et al.*, 1991). It was noted that Martin and colleagues define the first day of birth as P1, rather than conventional P0. Therefore for the purpose of

interpreting the results of their studies, reference to post-natal ages in the present thesis is consistently one day less than described by Martin and colleagues. Cell bodies backlabelled by the dyes were located in different brain nuclei to show the origin of fibre projections to these spinal regions. The cytoarchitecture of the *Monodelphis* brain is similar to the related North American opossum *Didelphis virginiana*, on which many neuroanatomical studies have been made (eg Martin *et al.*, 1975; Cabana and Martin, 1981; 1984 and Martin *et al.*, 1993) on which a brain atlas has been prepared (Oswaldo-Cruz and Rocha-Miranda, 1965). The terminology established for the *Didelphis* has been extended to cover the *Monodelphis*, which has not been studied as extensively. Some nomenclature from the rat brain (Paxinos and Watson, 1998) has been used for the *Monodelphis* where the *Didelphis* atlas does not describe the cytoarchitecture.

Holst *et al.* (1991) report some differences in the origins of projections between the *Didelphis* and *Monodelphis* opossums, particularly in the amount of isocortex containing corticospinal neurons, the number of corticospinal neurons, the presence of spinal projections from the amygdala and preoptic area, the degree of rubrospinal somatotopy and the origin of certain monoaminergic projections. However, in general it was found that the origins of descending supraspinal pathways were similar in the *Monodelphis* and *Didelphis*.

The developmental sequence of supraspinal outgrowth in the *Monodelphis* descending to the lumbar and cervical spinal cord was defined by Wang *et al.* (1992) after injection of retrograde axonal tracers into the spinal cord at different ages. The study showed that reticular neurons are among the first from the brain stem to reach the lumbar spinal cord, with the reticular formation being labelled from birth. Within the formation, neurons from the pontine reticular nucleus and gigantocellular reticular nucleus were consistently detected before the ventral regions of these nuclei. Labelled neurons in the lateral vestibular nucleus were present at P0, however they were more lightly labelled than those in the reticular formation suggesting that the vestibular neurons grow into the spinal cord later. Innervation from the lateral vestibular nucleus was seen to clearly precede the medial vestibular nucleus and inferior vestibular nucleus. Backlabelled cell bodies in the red nucleus were not observed until P4 from both lumbar and cervical spinal cord injections and were

thought to develop several days after the reticular and vestibular formations. Neurons labelled much later in development were detected in the deep cerebellar nuclei (P14), superior colliculus (P16) and cerebral cortex (P16) and projections only innervated the cervical levels of the cord (see Appendix A1).

Most fibre tracts known to project to lumbar regions in the adult *Monodelphis* as determined by Holst *et al.* (1991) were seen to have some projections in place by P6. There was evidence of transient corticospinal connections as some cortical labelling from cervical regions was detected in neonates outside areas labelled in the adult, but none was observed in brain stem regions.

The most rostral projections that extend from the brain to the lumbosacral regions of the spinal cord in the *Monodelphis* appear to originate from the hypothalamus in the adult (Holst *et al.*, 1991) with retrogradely labelled neurons appearing in the paraventricular hypothalamic nucleus, dorsal hypothalamic area, and lateral hypothalamic nucleus. Some brain stem nuclei such as the cuneate nucleus only project to cervical regions of the cord and cortical regions were only labelled by rostral cervical injections. The number of corticospinal neurons found and the areas of cortex containing them were smaller than that of the *Didelphis* (Nudo and Masterton, 1990a & b; Holst *et al.*, 1991). It is likely that cortical control of hindlimb movement in *Monodelphis* involves at least one relay in the spinal cord or brain stem (Martin *et al.*, 1975) given that direct cortical connections only reach the cervical region of the spinal cord. Other species that share this feature include the *Didelphis* and other marsupials, in addition to some placental mammals such as goats, elephants, tree shrews, sloths and armadillos (Armand, 1982). Amygdalospinal connections were found to be unique in the *Monodelphis* (Nudo and Masterton, 1990 a & b) and some degree of rubrospinal somatotopy appears to exist, which may correlate with the ability to use distal parts of the limbs independantly (Huisman *et al.*, 1982).

Lumbar spinal injections in the adult *Monodelphis* consistently produced greater numbers of labelled neurons in some nuclei (Holst *et al.*, 1991). Thus, these nuclei can be described as having more "robust" supraspinal projections (see Appendix A2).

The reticular formation, the lateral vestibular and rubral pathways were found to be particularly robust (Holst *et al.*, 1991).

There is not necessarily a simple caudal to rostral developmental origin of descending spinal projections as some more rostral regions (eg hypothalamus) innervate the spinal cord before other more caudal regions (eg red nucleus and medial vestibular nucleus). One definite trend is that an approximate ventrodorsal sequence of development is present and that those areas of the brain that mature earliest (eg. reticular formation), innervate the cord well before regions which mature later, such as the cerebral cortex (Wang *et al.*, 1992).

One issue to consider when determining the developmental timetable of supraspinal innervation is that the techniques used to trace the axonal projections back to their cell body origins in the brain may be several days off the correct time scale. It is possible that projections innervating the injected region of the spinal cord may fail to retrogradely transport the dye back to the cell body immediately or at all, and that newly developing axons may reach the injection site during the 1-4 days survival time allowed for the retrograde transport to occur. The former would indicate a false absence of projections at a certain age and the latter would indicate innervation earlier than the true time scale (Wang *et al.*, 1992). Thus, the time that the injected tracer remains in the cord is critical for accurate determination of a developmental timetable.

Following unilateral injections into the spinal cord by Holst *et al.* (1991) most of the labelled regions of the brain showed bilateral representation in nuclei with an ipsilateral predominance, with the exception of the isocortex, superior colliculus, red nucleus, paralemniscal nucleus and inferior vestibular nucleus which were predominantly contralateral. When lumbar injections of WGA-HRP were combined with contralateral hemisection of the thoracic cord, effectively preventing any transport of the retrograde marker along the contralateral tracts (in case of leakage across the midline at the time of injection) the laterality was determined with greater certainty. Few of the projections were found to be exclusively ipsilateral or contralateral (Holst *et al.*, 1991), with most found to be represented bilaterally with an ipsilateral predominance (see Appendix A3).

The level at which most contralateral projections cross the midline in the opossum was consistently observed to be above the cervical spinal level. Degeneration experiments by Martin *et al.* (1975) in the *Didelphis* found that lesions to the red nucleus of adult *Didelphis* produced degenerating axons located contralateral to the lesion at the cervical and lumbar spinal levels. The majority of rubral axons cross at the ventral tegmental decussation in the midbrain early in development (Xu and Martin, 1991). Reticular pontine fibres were found to be predominantly ipsilateral and the few that do cross the midline were found to do so also in the midbrain.

Vestibulospinal projections were seen to project mainly ipsilateral in the ventral funiculus. Some crossed to the contralateral side from the lesion at the cervical and rostral thoracic level and even in the lumbosacral level (Martin *et al.*, 1975). It is important to understand the laterality of normal projections when examining innervation of axons after an injury so that abnormal regrowth, if it occurs, may be observed.

The critical period for plasticity

The critical period for individual fibre tracts may vary depending on their developmental timetable (see Martin and Wang, 1997). Given that the developmental innervation of different projections from the brain to the spinal cord is highly asynchronous, it is proposed that an injury sustained at a certain stage of development will result in a differential response from individual fibre populations. It is thought that the developmental stage of an axon may greatly influence its ability to respond to injury in a positive manner (Wang *et al.*, 1994) either by growing *around* a lesion should there be an alternative path or even *through* the lesion site if it is still permissive. The time in development during which fibre populations were observed to grow again following injury (whether the growth be due to regeneration of cut axons or continued subsequent development of the tract) is known as the critical period. The end of the critical period appears to be closely tied to certain developmental events, most notably the onset of nerve fibre myelination and the maturation of associated cells (Keirstead *et al.*, 1992).

Therefore, it is of interest to know the identity of axons able to grow through a lesion and compare this to their developmental timetable. If there were no regeneration of

cut axons, it might be expected that the majority of fibres able to grow back across an injury were those that have not yet finished developing and were yet to reach their final adult innervation pattern. Indeed, it has been long observed that the corticospinal tract, which is a comparatively late developing tract in most species (eg, rat; Donatelle, 1977; hamster; Reh and Kalil, 1981) was able to grow around spinal lesions, performed in neonatal rats (Schreyer and Jones, 1982; Bernstein and Stelzner, 1983; Bates and Stelzner, 1993), while other brain stem projections such as the rubrospinal tract, also injured by spinal lesions appeared not to have a comparable plasticity with little evidence of outgrowth (Prendergast and Stelzner, 1976; Bregman and Bernstein-Goral, 1991).

There is a lack of comprehensive examination of the critical period of individual supraspinal projections in the *Monodelphis*, rather the whole spinal cord response to injury was examined in *in vitro* studies (Treherne *et al.*, 1992; Woodward *et al.*, 1993; Varga *et al.*, 1995a). Growth of axons and electrical conduction across and through a crush lesion were not seen in cultured spinal cords of *Monodelphis* aged P13- P14 over five days after a cervical lesion (Varga *et al.*, 1995a), as compared to cords from animals aged P3-P6 (Treherne *et al.*, 1992; Woodward *et al.*, 1993; Varga *et al.*, 1995a). Cords crushed at P11-P12 in the cervical region revealed only minimal axon growth and this is thought to be the beginning of a transition period after which axonal sprouting and growth through a lesion will not occur (Varga *et al.*, 1995a). There is a rostrocaudal developmental gradient in response to injury as cords also from P11-12 animals crushed in the lumbar region showed outgrowth of fibres across the lesion site (Varga *et al.*, 1995a). Studies by Martin and colleagues on the related *Didelphis* may be drawn upon to provide developmental comparisons with the *Monodelphis*, in regard to the critical period for individual tracts.

1.2.6 The development and response to injury in the *Didelphis* opossum.

While the *Monodelphis* and *Didelphis* share a close heritage as members of the family Didelphidae and both give birth to very immature offspring as characteristic of all marsupials (Archer, 1987), evidence suggests that perhaps they cannot be regarded as entirely comparable developmental models. The *Monodelphis,* with a 14 day gestation period is found to be more developed at birth than the *Didelphis*, born

12 days after conception (Qin *et al.*, 1993) and many developmental processes occur 2 to 4 weeks earlier in the *Monodelphis* (Smith and van Nievelt, 1997). In particular, the development of the brain is found to occur more rapidly in the *Monodelphis,* as indicated, for example, by cortical axons not projecting into the cervical cord until about P29 in the *Didelphis* compared to P16 in the *Monodelphis* (Wang *et al.*, 1992). However, these specificities aside, the *Didelphis* and *Monodelphis* do both exhibit a general pattern of growth, common to all mammals and information about processes in one species has relevance to the other (Rowe, 1997).

Comprehensive work of Martin and colleagues has defined many anatomical features of the *Didelphis*, including many studies defining the developmental timetable of axonal projections in the spinal cord and their response to injury. These are described below and were useful to draw upon when considering similar problems for the first time in the related *Monodelphis* in the present study, while bearing in mind that each species is unique.

The rubrospinal tract has been shown to develop postnatally in the *Didelphis* (Cabana and Martin, 1986) as opposed to prenatally in the cat (Bregman and Goldberger, 1982) and in the rat (Sheih *et al.*, 1985). In *Didelphis*, the tract retains plasticity after injury enabling growth around a thoracic hemisection lesion given during early development (P11–P14). A hemisection lesion refers to a complete transection of one half of the spinal cord in the transverse plane, while the other remains intact, unless otherwise stated. Outgrowth is primarily derived from late arriving axons, identified by double labelling with retrograde axonal tracers, Fast blue or WGA-HRP (Xu and Martin, 1991). The critical period of development after which this plasticity was not retained was identified as between P14 and P32 by both orthograde and retrograde axon tracing of rubrospinal axons (Martin and Xu, 1988).

Didelphis aged P19 that received hemisections of the thoracic spinal cord, followed by lumbar injection of Fast blue, did not show retrogradely labelled neurons in the reticular or vestibular nuclei, indicating that no projection from these nuclei had grown around or through the lesion. This was in contrast to animals hemisected at P4 or P11. The critical period for reticulospinal and vestibulospinal axons that innervate

the spinal cord earlier than rubral axons is therefore between P11 and P19, much earlier than that of rubral axons (Wang *et al.*, 1994).

The corticospinal tract has a prolonged period of postnatal development, not only in the opossums, but also in neonatal rats (Bernstein and Stelzner, 1981); cats (Bregman and Goldberger, 1982) and hamsters (Kalil and Reh, 1979) and when a hemisection is made interrupting this tract, axons are seen to grow around the injury site. The observed growth was thought to be due to both new growth of late arriving axons and some regeneration of severed axons (Bernstein and Stelzner, 1983). This suggestion was later confirmed by a double label experiment by Bates and Stelzner (1993) and is commented on later in Section 1.2.7.

The sequence of the development and loss of plasticity of different propriospinal and supraspinal pathways appear to be correlated to the maturation of the originating structures of the *Didelphis* brain. It appeared that in most cases, axons grew *around* the edges of an incomplete lesion and never *through* the severed tissue (Martin and Wang, 1997). However, it was noted that when a hemisection was made at P4, reticulospinal and vestibulospinal axons seemed to grow through the hemisection site as opposed to around it (Martin *et al.*, 1994). When this feature was examined more closely by Wang *et al.* (1996), it was found that after a complete transection of the thoracic spinal cord at P4, supraspinal axons had grown through the transection site, detected by retrograde tracing with Fast blue one month later. The axonal populations were identified by the presence of associated cell bodies in different brain stem nuclei. Fewer axons grew through the injury made at P11, and if transected at P19, even less axons grew around the injury, in addition to being more restricted in nuclei from which the axon originated. By P32, no axons growing through the lesion from caudal regions to the brain were detected. Neurons labelled in the medial pontine reticular nuclei (with reticular projections) and the dorsal part of the lateral vestibular nuclei (vestibular projections) were not detected after P11 while neurons in the red nucleus (rubral axons) and medullary raphe nuclei (raphealar axons) were detected at P25. This indicates a temporally different loss in the ability to grow through a lesion among the different axon populations of the brain stem (Wang *et al.*, 1996) as described earlier as asynchronous critical periods.

More recent studies have also documented the developmental sequence of ascending
axonal pathways such as the fasciculus gracilis (Wang *et al.*, 1997) and the dorsal
spinocerebellar tract (Terman *et al.*, 1996). It was shown by tracing Fast blue
injections from the lumbar cord and cholera toxin-horse radish peroxidase from
dorsal roots, that axons of the fasciculus gracilis pathway originating in dorsal root
ganglions reached the nucleus gracilis in the caudal medulla, by at least P4. These
fibres were able to grow through a complete thoracic transection if made at P5 and
the critical period for this recovery appeared to end between P7 and P11. The dorsal
spinocerebellar tract was also shown to grow through a thoracic lesion made between
P4 and P8, and the critical period ended at P12. There appear to be some differences
in the ability of ascending and descending axons to grow through a lesion. The
critical period of plasticity seemed to end earlier for ascending axons than
comparable plasticity of descending spinal axons in the *Didelphis* (Terman *et al.*,
1996).

1.2.7 The contribution of regenerating fibres to outgrowth across a lesion

Not all fibre projections had reached their final adult innervation patterns by birth in
the *Monodelphis* and therefore any growth detected through a lesion performed early
in life may have been due to subsequent axonal growth as part of normal
development after the injury. However, it is possible that axons severed by the injury
were able to regenerate their severed projections during development as reported by
several experiments examining fibre growth post injury. Kalil and Reh (1979) first
proposed in their study of corticospinal outgrowth after pyramidal lesions in neonatal
hamsters that neonatal CNS injury was followed by a combination of both
regenerative growth of cut axons and subsequent re-routing or outgrowth by later
developing fibres. Following this, regenerating axons were considered a feasible
source of axon outgrowth after immature CNS injury, by a number of research
groups (eg. Xu and Martin, 1991; Hasan *et al.*, 1991; Bates and Stelzner, 1993) using
a variety of species and this has been the focus of many subsequent studies. Some of
these are described below.

The developmental timetable of particular pathways is significant when determining
the contribution of regenerating fibres versus subsequent developmental growth after

injury. If a tract is seen to send fibres through a lesion site after it has finished developing (such as in the adult animal) then one may assume that the fibre outgrowth was most likely due to the regeneration of severed axons. As the critical period of outgrowth following injury appears to coincide with the end of the developmental growth for many tracts it becomes necessary to distinguish between the two types of fibre growth when examining the response to injury of the immature CNS.

Double label experiments to establish regeneration of severed axons

The experimental paradigm used to determine whether regenerative growth contributes to fibre outgrowth following injury to the spinal cord is a double label retrograde axonal tracing technique (eg Bates and Stelzner, 1993; Hasan *et al.*, 1993; Wang *et al.*, 1998; Zhang and McClellan, 1999; Becker and Becker, 2001). Neuronal cell bodies are labelled prior to an injury with one retrogradely transported dye injected into a caudal location to indicate axons that have already grown past the proposed site of injury. These would therefore be severed by a lesion performed after the first injection. Following a period of time for regeneration or repair to take place after the injury, a second different axonal tracer is injected caudal to the level of the injury to show all axons that have grown across the injury site. Those neuronal cell bodies that contain both dyes, may be interpreted as having had projections present prior to the lesion, that were severed and then regenerated back through the injury site to pick up the second dye. Those containing only the second dye may be subsequent developing axons, unless they failed to pick up the first dye prior to the injury. While this technique and certain variations have been the most widely utilised anatomical method of determining regeneration, it does present several methodological problems that must be considered when evaluating results.

The problems associated with double label experiments

Hasan *et al.* (1993) gave comprehensive descriptions of the potentially confounding factors of using the double label technique to determine the contribution by regenerating or subsequently developing axons to fibre growth across a lesion.

Firstly, the lesion must be a complete transection so that the only fibres able to pick up the second dye were those that grew across the lesion, as uninjured fibres would give the false impression of projections through the lesion site. Secondly, it is important that the dye injected first has not remained available for uptake in the spinal cord at the time of the second injection, allowing fibres to retrogradely transport both dyes back to the cell bodies simultaneously. Other considerations include whether the second dye is able to diffuse directly across the transection site, if any transynaptic labelling of supraspinal projections can occur, or if there is any leakage into the circulation causing direct uptake into the brain. There also may be considerable variation between the brightness and quality of labelling, in addition to the numbers of retrogradely labelled neurons, despite all attempts to make uniform injections.

However, when these factors have been accounted for as best as possible with a battery of control experiments accompanying the lesion experiments (see Discussion: Section 4.6: Critique of Methods), there have been several significant studies in the rat (Bates and Stelzner, 1993; Bernstein-Goral and Bregman, 1993), chick (Hasan *et al.*, 1993), opossum (Wang *et al.*, 1998) and hamster (Kalil and Reh, 1979; Merline and Kalil, 1990) which have all employed this technique to ascertain whether regeneration of cut axons contributes to the outgrowth following immature CNS injury.

Bates and Stelzner (1993) looked at the regeneration of the cortical projections after a right hemisection in the cervical spinal cord plus the left dorsal funiculus (ie. overhemisection) to sever the corticospinal tract at P4, P6 and P12 in neonatal rats. While both sides of the spinal cord were not completely transected, the regions containing the corticospinal tracts were bilaterally transected, thus meeting the criteria for a valid experiment looking only at the cortical fibres. The animals had received an initial injection of Fast blue dye two days prior to the lesion, which was removed by aspiration at the time of lesion to prevent false double labelling. Between two and seven months later the animals received a second injection of Diamidino yellow dye caudal to the lesion to show the corticospinal axons that had successfully grown through the lesion. The number of double-labelled neurons detected in the cortex was very small and of the thirteen cases examined, only 4 had

double-labelled cell bodies. Three of these animals were lesioned at the youngest age (P4), the 4th at P6, suggesting that immaturity was a determining element of the regenerative ability of cut axons. The number of axons that projected through the injury site (labelled with the second dye) was always a smaller number than those labelled with the initial injection, indicating that the corticospinal tract had many projections already at the time of injury and thus it was of a certain maturity when lesioned. The actual numbers of double labelled axons were small (2, 10, 15 and 2), and when presented as a proportion of the number of cell bodies labelled with the second dye were 4%, 15%, 17%, 9% respectively. This should be considered in light of the fact that there were many more axons labelled by the first injection than the second and these were potential regenerative candidates (being present through the corticospinal tract at the time of injury), as opposed to the axons showing the second label which arrived at the level of injury after the lesion or were regenerating. Were the data presented as a proportion of the potentially regenerative axons, which could be a more relevant observation, the percentages would be even lower.

It is most likely that the results were an underestimate of numbers of regenerating fibres, given that not all fibres in the corticospinal projection may have been labelled by both injections, and that only every third section (30μm) was collected and only cell bodies with a visible nucleus were counted. These experiments indicated that where a fibre tract is still developing and retains an associated plasticity, then it may regenerate a portion of its axons after injury. In the case of the neonatal rat, the corticospinal tract is the last population to develop and it would not be possible to examine the regeneration of other populations such as brain stem- spinal cord projection eg. rubrospinal tracts after injury before the critical period of these earlier developing tracts. It would necessitate doing lesions on embryonic pups *in utero* – technically difficult in terms of the survival of the pups post-operation.

Studies examining the regeneration of other early developing projections utilised the embryonic chick (Hasan *et al.*, 1993), and marsupial species such as the South American opossum *Monodelphis* (Varga *et al.*, 1996) or the North American opossum *Didelphis* (Xu and Martin, 1991; Wang *et al.*, 1998). Both *in ovo* chicks and early neonatal opossums have many supraspinal projections yet to develop and thus these fibres may have a plastic nature after injury. These studies have also

employed similar double labelling techniques with retrograde tracers to determine whether regeneration of brain stem projections contributed to fibre outgrowth post-injury.

Hasan *et al.* (1993) gave embryonic chicks an initial injection of fluorescent dextran amine (labelled with either Rhodamine, Fluorescein or Cascade blue) into the lumbar spinal cord 2 days prior to a complete thoracic lesion performed at E10-E15. The chicks were then injected with a second different dextran amine dye one week later, just caudal to the injury to show supraspinal projections that had grown back through the lesion site. Examination of the brain stem nuclei showed an increase in the mean number, range and distribution of double labelled cell bodies from E10-E12 transected chicks in each of the examined brain stem nuclei such as the gigantocellular reticular nucleus, the lateral vestibular nucleus and interstitial nucleus of the medial longitudinal fasciculus. The data were presented as percentages of neurons labelled with the second tracer that were double labelled and this increased from approximately 5% for chicks transected at E10 to nearly 22% for chicks transected at E12. The percentage of regenerated axons decreased between E13 to E15 in transected chicks and was interpreted as indicating the decline of the critical period. E13 is the age at which myelination begins in the chick and this process is implicated in the onset of the non-permissive state of the adult CNS. The suppression of the developmental onset of myelination in a separate study by spinal injection of a monoclonal antibody against galactocerebroside, a sugar associated with myelin (Keirstead *et al.*, 1992), extended the permissive period of outgrowth across the lesion for injuries up to E17.

Wang *et al.* (1998) utilised the extreme immaturity of the North American opossum *Didelphis* at birth to also examine the growth of supraspinal connections through a spinal lesion made shortly after birth. Initial injections of Fast blue dye were made into the lumbar cord, 5 days before complete thoracic lesions at P9, P12, P19 and P24. A recovery period of 14 days was allowed and then a second injection of dextran amine rhodamine was given just caudal to the lesion to label all fibres that had grown through the lesion. Wang *et al.* (1998) described an asynchronous regenerative capacity of different populations of supraspinal fibres, dependant on the stage of development at the time of injury.

Many more double labelled axons were seen in the *Didelphis* pups that received lesions earlier in life (P9 and P12). After a P9 lesion, approximately 50% of the cell bodies labelled with the second dye in the reticular pontine nucleus, locus coeruleus, lateral vestibular nucleus, raphe nucleus and ventral medullary reticular nucleus were double labelled with both dyes, suggesting that in these nuclei a high proportion of fibres had regenerated from severed axons. The percentages of double labelled axons decreased synchronously for these populations at older lesion ages. No cell bodies in the reticular pontine nucleus or lateral vestibular nucleus were labelled with the second dye when the lesion was made after P12, while 25% and 20% respectively of the raphe nuclei and ventral medullary reticular neurons were double labelled. This indicated that the critical period in *Didelphis* for regenerative growth of the reticular pontine nucleus and lateral vestibular nucleus ended between P12 and P19, while ventral medullary reticular nucleus and raphe nuclei had not yet reached that point, highlighting the asynchronous regenerative ability of these different populations.

By comparison, the red nucleus did not show any regenerating projections after transections performed on P9 or P12 but did after transections at P19 and P24. Rubrospinal projections were not labelled by the first injection in the pups transected at P9 and P12 as presumably none had reached the lumbar cord by this time; however high numbers of neuronal projections were labelled only with the second dye, suggesting that these were all late arriving projections growing through the lesion. The rubrospinal tract is the last supraspinal projection to innervate the cord in the opossum and was the only population to show double labelled cell bodies after a P24 transection (Wang *et al.*, 1998). Thus, the latest developing tracts still showed a regenerative response to injury after axotomy at the oldest age examined.

The correlation between the developmental timetable of individual tracts and the end of a regenerative response following injury was supported by developmental studies in the opossum showing that the reticulospinal and vestibulospinal pathways innervate the lumbar cord before the rubrospinal in the *Didelphis* (Martin *et al.*, 1993). As is the case with the *Monodelphis*, corticospinal projections do not reach the lumbar or lower thoracic regions of the spinal cord (Cabana and Martin, 1984) and not are candidates for regenerative growth seen through the injury site. It did appear that all populations of supraspinal projections were able to regenerate some

proportion of their fibres following injury (in the absence of any external aids or influences), provided the injury occurred prior to the onset of the critical period for that pathway.

1.2.8 Regeneration in the *Monodelphis*

Regeneration of dorsal root ganglion (DRG) projections has been examined in the South American opossum in the cultured preparations of spinal cord (Varga *et al.*, 1995b). The dorsal columns of cultured P6 opossum spinal cords were injected with DiO, a carbocyanine tracer dye to prelabel dorsal tracts and cell bodies originating in associated proximal DRG. Following a complete crush lesion 1-2 days later, which effectively severed those pre-labelled fibres, a second carbocyanine dye DiI was injected near to the initial injection site. It was estimated from the number of cells counted that approximately 20% of DRG cell bodies had regenerating projections. It was noted that this was likely to be an underestimate as the labelling technique of inserting a glass electrode coated in crystals into the desired regions only labelled a very small number of fibres of those growing through the spinal level at which the lesion was made. Also this was just one population of fibres which pass through the cervical region and the response of other supraspinal projections to a crush injury *in vitro* was not known – though the number of fibres growing through the injury site was much greater than could be entirely attributable to the dorsal columns. Double labelled DRG projections were detected in the cultured spinal cords aged up to P16, which had been lesioned at P7–P8 (Varga *et al.*, 1996).

Luque *et al.* (1998) identified monoaminergic axons by staining whole mount preparations of cultured neonatal *Monodelphis* CNS with antibodies to tyrosine hydroxylase and serotonin. Most of these neurons were post-mitotic at birth when double labelled with BrdU. This technique has the advantage of labelling all cells of specific populations, as opposed to injection of dyes where not all fibres may have been in contact with the label so that some cell bodies may not have been labelled. Following a cervical crush lesion at P5 it was seen that approximately 8% of tyrosine positive fibres and 14% of the serotonin fibres had grown through the injury site by 5 days post-lesion. As these figures were based on counting bundles of axons through the lesion site rather than fine individual fibres (due to limitations of the resolution in

the whole mount preparations), it is likely that these are underestimates of the number of regrowing fibres. However, it cannot be determined whether cut axons of the monoaminergic population are truly regenerated from cut axons or the result of late arriving fibres reaching the cervical regions of the cord as it is not known whether the fibres were present at the time of injury. Luque *et al.* (1998) state that based on similar studies examining the regeneration of DRG projections (Varga *et al.*, 1995a), they would presume that a small portion of truly regenerated axons contribute to the outgrowth.

An elegant confirmation that regeneration does indeed contribute to a degree of outgrowth of *in vitro* fibres was provided by Varga *et al.* (1996), where severed DiI labelled DRG axons were directly visualised by time lapse video microscopy as regenerating following injury within two days. Evidence from the *in vitro* studies showing regenerating fibres in *Monodelphis* is highly significant and indicated a capacity of the developing nervous system to recover from injury, not only with profuse fibre outgrowth but that the nerve fibres in the immature CNS possess the molecular machinery and physical ability to regenerate after axotomy. The temporal limitations of the cultured spinal cord, however, do not lend themselves to examining the time course of repair beyond the 10 day culture period. It is of great interest to know whether regenerative outgrowth may be maintained, so that the fibres have the opportunity to elongate further through the injury site to correctly innervate the original intended target, as this would be required to mediate normal function. Of particular further interest would be the recovery of locomotor functions and whether the supraspinal projection responsible for mediating signals related to this function have the ability to regenerate after spinal injury, given that the loss of locomotor function is one of the more devastating aspects of spinal injury.

1.3 THE PRESENT STUDY

A relevant question has arisen – why do the highly coordinated events of axon extension, target innervation and establishment of functional synaptic connections occur with ease in the immature CNS as part of normal development, but when

injury occurs, recapitulating these events is only possible in the PNS and not in the adult CNS? Furthermore, might the immature CNS have a capacity to regenerate after injury, unlike the adult CNS? If so, this would provide a greater understanding of the failure to regenerate in the adult CNS.

Previous *in vitro* studies (Varga *et al.*, 1996; Luque *et al.*, 1998) looking at the regenerative capacity of the spinal cord have not commented on the ability of supraspinal projections to do so. Subsequent *in vivo* studies (Saunders *et al.*, 1998) examining the locomotor ability of adult *Monodelphis* given spinal crushes or complete cut transections showed that the supraspinal populations had been able to grow back through the injury site in animals that showed a relatively normal locomotor capacity. However, it was not known whether regenerative outgrowth by the supraspinal populations was responsible for this locomotion

Given that the immature CNS has a permissive nature for the outgrowth of developing nerve fibres, it is reasonable to suggest that they may also provide a permissive environment for the outgrowth and possible regeneration of severed axons after a central nervous system injury. In the present study, the immature CNS response to injury has been examined in the hope that by using a system in which axonal outgrowth is permitted to occur by either its virtue as a non-inhibitory microenvironment or with the intrinsic positive growth state of the developing neurons themselves, the keys to the inability of the adult CNS to regenerate may become apparent. It is necessary to establish a working model of regeneration to study the "normal" before one can hope to establish the complicated requirements for the full regenerative process resulting in the restoration of function after CNS injury.

The present study firstly defines the time course of when supraspinal projections are able to grow across a complete spinal transection made by cutting, rather than crushing as in Saunders *et al.* (1998), and secondly identifies which populations of brain neurons are able to do so. If only certain populations were able to grow across the lesion, knowing their identity might give clues as to what is necessary to overcome a growth inhibitory lesion site. Supraspinal projections are of particular interest as they mediate voluntary locomotor function, known to be restored after immature injury in the opossum. Lastly, the pivotal aim of the study was to

determine whether a portion of the fibre outgrowth across the lesion site was a product of regenerating axons severed by the lesion or was due solely to subsequent developmental outgrowth.

AIMS

The present study set out to establish whether the neonatal marsupial opossum *Monodelphis domestica* is able to restore supraspinal projections across a complete thoracic spinal transection performed at one week of age *in vivo*. It aimed to provide quantitative evidence of fibre outgrowth over a period of time, and to establish whether the development of supraspinal projections in transected animals differed greatly from that of control unlesioned animals. Specifically, the aims were:

1. To determine if supraspinal projections grow across a complete thoracic transection performed at one week of age in opossums.

2. To establish the time frame of fibre outgrowth through the lesion site and compare it to normal development.

3. To determine whether any particular supraspinal population shows a greater ability to traverse a lesion site.

4. Finally, to establish whether the regeneration of severed axons contributes to outgrowth seen across a transection site.

The overall aim was to establish an *in vivo* model of regeneration in the immature *Monodelphis* CNS as a platform from which to further investigate the capacity of the CNS to regenerate.

Chapter 2

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METHODS

2.1 ANIMAL MODEL: *MONODELPHIS DOMESTICA*

2.1.1 Husbandry

The animals used for this study were supplied from an established colony of *Monodelphis domestica* (grey short-tailed South American opossum) housed at the University of Tasmania Animal House, Hobart, Tasmania (see Figure 2.1a). Animals were kept in coloured polycarbonate boxes, lined with recycled paper pellets (see Figure 2.1b). Plastic nesting boxes were provided along with shredded paper for the animals to use as nesting material (see Figure 2.1b and c). Empty toilet rolls were placed in the boxes as toys. The colony is housed at an ambient temperature of 27° C in two separate rooms. Pregnant females and mothers with pups are kept in an inner room, where there is less staff activity and no adult males to reduce stress. Breeding pairs and males are housed in the other room. The mothers may cannibalise young under stressful conditions and all efforts were made to minimise stress. The daily light/dark cycle was 14/10 hours with the dark period beginning at 2 p.m. to prevent the nocturnal animals from being disturbed by staff during the whole of their sleep period.

Breeding program

Monodelphis may exhibit aggressive behaviour during the mating interactions, which on rare occasions have resulted in the death of one of the pair (usually the male). To discourage the initial aggressive interactions, the nesting boxes of intended mating pairs are swapped for two weeks prior to the mating to familiarise the animals with each other's smell. This had the effect of reducing the fighting between mating couples dramatically and increasing fertility rates. Pairs are mated all year round but there is a 3 month period of lower fertility during the middle of the year, usually June to August. Males are re-mated after 2 weeks, while females are rested for 4 weeks following either weaning of pups at 2 months or removal of pups for experiments.

Breeding pairs are housed together for 14 days after which the females are placed in the inner maternity room and the males are left in the outer room. Pregnant females, identified by weight gain, have a 14 day gestation period and give birth to litters of

Figure 2.1: The South American opossum, *Monodelphis domestica*

a) The adult is the size of a small rat and weighs approximately 100-120g.

b) The opossums are easily maintained in standard rat boxes lined with paper pellets with polycarbonate nesting containers and shredded paper.

c) The *Monodelphis* are housed in at 27°C in standard animal house facilities.

up to 12 pups. Pups remain continuously attached to the teats until around 21 days post-natal, after which they may move about independently in the nest (see Figure 2.2).

Diet

Water is provided from standard animal water bottles *ad libitum*. Animals are fed a daily diet of two teaspoons of tinned cat food, powdered milk, meat meal, vitamin supplement (Infant Pentavite), high protein cereal and peanut oil combined into a paste, plus several extra dry cat pellets. Banana slices are given 3 times a week and live mealworms are provided once a week or as a reward when disturbing the animal. Further details of maintenance and breeding management have been previously published (Fadem *et al.*, 1982; Saunders *et al.*, 1998).

2.2 SURGICAL PROCEDURES

2.2.1 Anaesthesia

All surgical procedures were conducted following NH&MRC guidelines and with the approval of the University of Tasmania Ethics Committee (Animal). For all manipulation and operations, the *Monodelphis* were anaesthetised to a surgical level (see Figure 2.3a). Prior to P14, as the pups remain permanently attached to the mother's teats, it was not possible to individually anaesthetise each pup. Pups aged P21 and older were individually anaesthetised. Details of anaesthesia are given below.

Adults

Adult *Monodelphis* were transported in their cages from the colony rooms to the operating theatre immediately prior to anaesthesia. Food was withheld on the day of the procedure. Inhaled Isoflurane (Isoflo, Abbott Australasia) was administered to the animals by a Tech – 3 anaesthetic machine, vaporised by 100% oxygen, delivered at 300 CC/min. Anaesthesia was induced in an enclosed belljar chamber filled with 5% Isoflurane, then transferred to a heated operating table to receive 2%

Figure 2.2: The South American opossum, *Monodelphis domestica*

a) The pouchless female *Monodelphis* allows simple access to the pups, born in litters of up to 14 young. Pups remain attached to the teats for up to 3 weeks after birth (seen here at P7).

b) A neonatal pup aged P7. The *Monodelphis* are born extremely immature after only 14-15 days gestation and are still in the very early stages of development at P7 when the thoracic spinal transection was made in the present study. At P7 the pups are approximately 1.5 cm long and weigh about 0.25 g.

c) A neonatal pup aged P35. The pups have grown rapidly since birth, now weighing approximately 5g, and although they spend time detached from the mother, they are is still highly dependant on her care. A fine fur covers the body, the eyes are still closed and the hindlimbs are able to partially support body weight. Rudimentary linear locomotion can be executed.

a

b

P7

P7

c

P35

Figure 2.3: Surgical procedures

a) Adult *Monodelphis* were maintained under anaesthesia by inhaled isoflurane administered by a Tech-3 anaesthetic machine, received through a gas mask while lying on a heated operating table under sterile operating conditions

b) Spinal injections of fluorescent dextran amines were made to the lumbar spinal cord of pups at different ages. The dye was injected with a glass pipette into the lumbar spinal cord, shown here in P7 animals attached to the anaesthetised mother.

c) Pups ages P7 attached to the anaesthetised mother were given a complete thoracic spinal transection, made with a microscalpel knife.

a

b
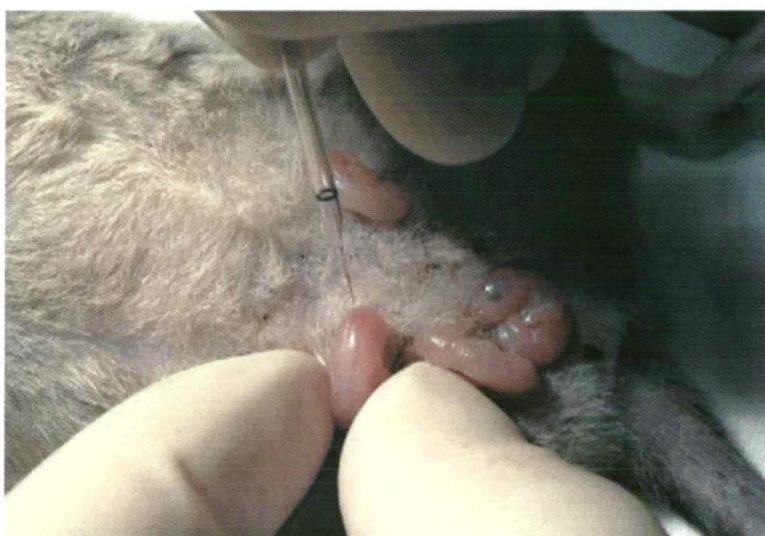
c
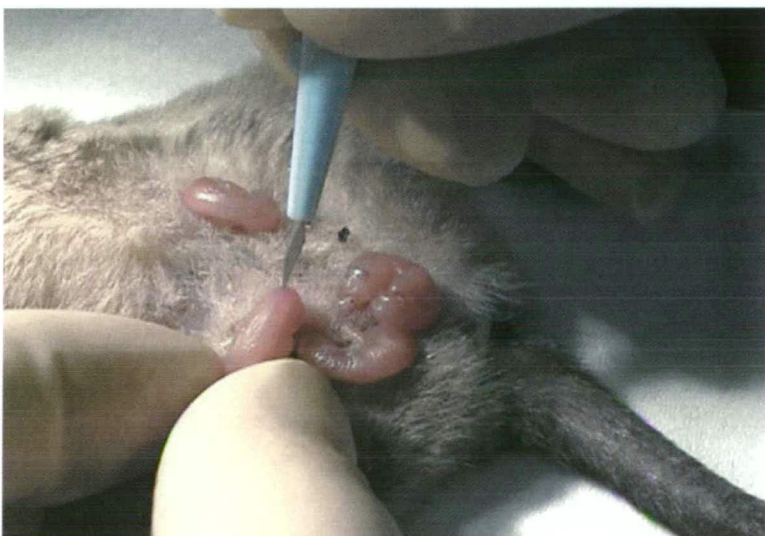
Isoflurane through a size 1 mask. The animals were maintained at surgical level of anaesthesia by this dosage for spinal injections, removal of pups, and for procedures performed on the neonatal pups. The level of anaesthesia was determined by the absence of withdrawal reflexes, relaxed musculature, and regular deep breathing. Only the nose of the animal was placed in the mask to ensure that the gas flow was dynamic and efflux respiration was blown away from the animal.

Where the animals were required to be anaesthetised for longer than 15 minutes, 1 ml of sterile saline was injected sub-cutaneuosly to reduce dehydration. For extended periods of anaesthesia, the dosage was reduced from 2% Isoflurane to 1.5% Isoflurane to prevent excess respiratory depression. Following the operation, the animal was removed from the operating table and placed in a recovery position on their side in their nesting box. Animals were observed closely until they regained consciousness, then were immediately returned to the colony holding room and given mealworms as a reward.

Neonatal pups aged P0 - P14

Pups do not begin to detach from the mothers teats until after P14 and must be manipulated while on the mother's abdomen. They receive traces of the inhaled Isoflurane through the mother's milk but were additionally exposed to inhaled Metafane (Methoxyflurane, Ophthalmic Labs) administered via a miniature face mask. They are easily immobilised by gently holding for spinal injections and spinal lesions.

Pups aged P21 - P35

By P21 the pups begin to move independently off the mother and may re-attach to different teats. At this age it was possible to individually anaesthetise the pups with inhaled Isoflurane delivered by the anaesthetic machine. Pups were anaesthetised in a small chamber filled with 5% Isoflurane, then placed on the heated operating table with their nose in a tiny modified mask to suit their small size. Pups were maintained at a surgical level of anaesthesia with 2.5% inhaled Isoflurane. The higher dosage, as compared to the adult dosage, was required due to the small lung volume of the pups,

and a deep surgical level of anaesthesia required at least 10 minutes inhalation of the anaesthetic.

Recovery from the anaesthetic was prolonged (up to 40 minutes) where the pup had been under for more than 10 minutes. The pups were placed under a heat lamp and closely monitored until full recovery from the anaesthesia and then returned to their mother in the colony holding room.

2.2.2 Spinal cord lesions

Under sterile conditions the anaesthetised female was placed in a supine position upon a heated operating table, displaying the suckling young (See Figure 2.3a). Due to the small size of the pups, an operating microscope (Zeiss) was employed to view the neonates during the operation. A small, medial incision was made through the dorsal skin with spring scissors, at a mid-thoracic level, to expose the vertebrae and spinal cord. A complete spinal transection at the mid-thoracic level (T4-6) was then performed with a fine scalpel blade (Morea instruments; see Figure 2.3c). It was possible to see the blade passing over the ventral surface of the vertebral column to ensure that the cord was completely lesioned in the dorsoventral plane. The small skin incision was gently pushed together with forceps and no adhesive was required for the wound to close. Any traces of blood or CSF around the wound were blotted away with cotton buds. The skin wound healed without scarring and in no cases did infection occur.

It was usual to remove at least one transected animal immediately following the operation on a litter. This served as a control for ensuring that the lesion was a complete transection of the spinal cord. After the operation, the mother with attached pups was returned to her nest and immediately taken to the colony room to recover from anaesthesia. Mealworms were promptly provided to the mother to reduce cannibalism of the pups. The spinal transected pups were maintained in the colony for different time periods from 1 day post-operation up to adulthood. They were weaned from the mother at 2 months of age.

Completeness of the spinal injury at P7

It was of importance that the spinal cord was completely severed by the transection as the present study assumed that no tissue connection remained between the rostral and caudal ends of the cord after the lesion was performed at P7. To establish that the lesion technique was a consistent and uniformly complete transection, whole litters of P7 animals were removed immediately following the injury. Sagittal sections of the whole body were stained with a histological stain (haematoxylin and eosin) to differentiate tissue types; the spinal injury site could be easily located and examined for completeness (see Figure 2.4). When all pups in a litter were shown to have an unequivocally complete transection of the thoracic spinal cord, the technique was considered reliable. After these preliminary experiments, only one animal was taken at random from larger transected litter at P7 (total of 25) as a check on the effectiveness of the operation. Removal of multiple young was found to predispose the mothers to cannibalise remaining pups.

2.2.3 Spinal injections

P3 – P14

Early neonatal pups do not have bony vertebral plates covering the spinal cord and a needle tip will easily pierce the developing dural membrane. The pups were gently held by hand while attached to the anaesthetised mother (see Section 2.2.1) and a small medial incision (0.25 cm in length) was made through the skin at desired injection location (either L1 or T8). At this age the skin is transparent and the exact spinal level may be determined by simply counting the ribs.

A 0.25-0.4 μl droplet of a fluorescent dextran amine was drawn into the narrow tip of a glass micropipette by capillary action. The pipette was attached to PVC tubing (Microline, diameter = 2mm) and the tip pushed gently into the spinal cord at a 45° angle. The dye was then injected by oral pressure into the cord (see Figure 2.3b). Any excess dye, blood or CSF was immediately blotted away with the cotton bud tip and the skin pushed together with forceps. The wound edges naturally sealed together within minutes.

Figure 2.4: Completeness of the thoracic spinal cord transection at P7

a) A sagittal section through the whole body of a P7 *Monodelphis* that has just received a complete spinal cord transection of the thoracic region stained with haematoxylin and eosin. Scale bar is 10mm.

b) The complete spinal transection in a P7 pup in a longitudinal section stained with haematoxylin and eosin. The surrounding vertebrae are visible either side of the spinal cord. It is possible to distinguish the white matter on the lateral edges of the cord from the grey matter in the centre with darkly stained cell nuclei. Scale bar = 100μm.

a
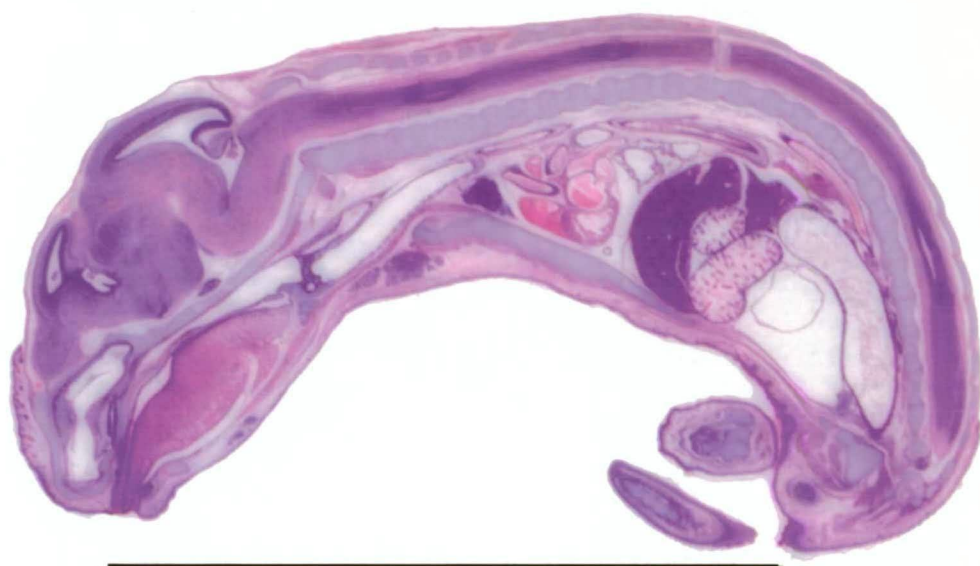
b
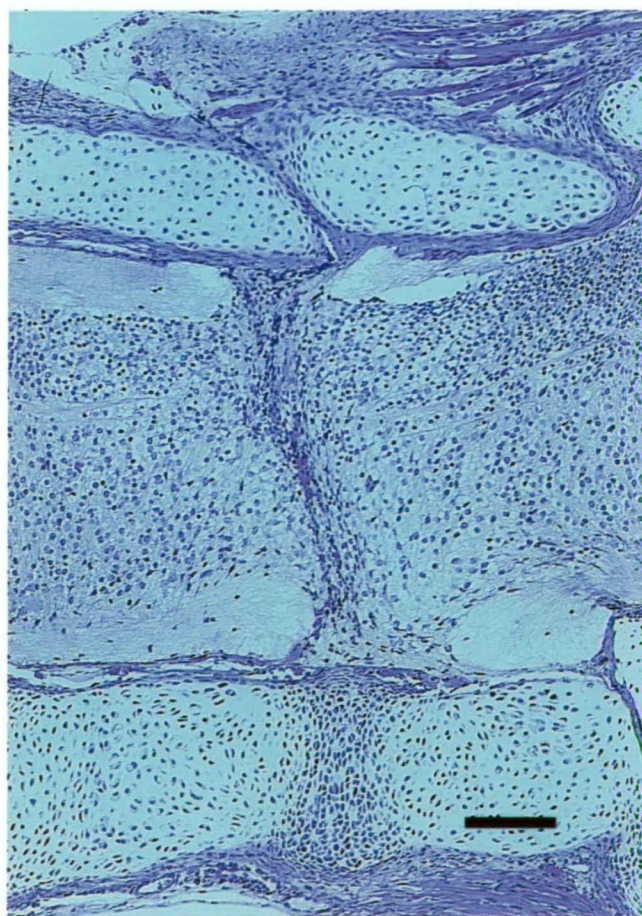
P21 – P35

Pups older than P20 were anaesthetised as described in Section 2.2.1. The vertebral plates have begun to develop by P14 and older and it was no longer possible to push the needle tip through the spinal covering. Following a medial incision through the dorsal skin at the desired spinal level (either L1 or T8) made with spring scissors, it was necessary to make a transverse cut through the developing vertebrae to expose the spinal cord. The incision was made between the plates, rather than through them, to reduce the impact of the surgery. The tip of a glass micropipette containing 0.4μl of a fluorescent dextran amine attached to PVC tubing was inserted through the transverse opening and the dye was injected by oral pressure into the cord. Any excess blood, dye or cerebrospinal fluid (CSF) was removed with a cotton bud and the skin pulled back together with forceps. A tiny amount of surgical glue (Histoacryl, Braun Surgical) was applied to the external skin to hold the wound together. It was found that the mothers would cannibalise the young if excess glue was applied to the skin.

Adult

Adult *Monodelphis* were anaesthetised (see Section 2.2.1) and placed in a prone position with their nose in the gas mask on a heated operation table covered by sterile drapes. The dorsal fur was shaven with a No. 1 razor and the area cleaned with alcohol. A lateral incision through the skin was made with a sterile scalpel blade (Swann Morton, size 15) of approximately 2 cm in length, exposing the 12th thoracic vertebrae. The wound was continuously moistened by sterile saline (0.9% w/v NaCl solution) throughout the duration of the operation (approximately 45 minutes). The operation was performed using a Zeiss operating microscope. The skin was held apart by loose artery forceps resting on either side of the incision. The dorsal spinous process of the vertebra was located and the attached muscle was pushed away from the bone with the scalpel to expose the dorsal surface of the T12 vertebra. Care was taken to avoid cutting the medial arteries visible through the muscle as they bleed profusely if cut.

The spinal column was immobilised by gripping the dorsal spinous process with shark's teeth forceps (FST). A small hole (1.5mm diameter) in the dorsal plate of the

vertebra was created by a high-speed dentist drill (Foreman) using a small sterilised drill (diameter = 1mm) exposing the T12 spinal cord over the right unilateral side. The pressure was applied to the drill in small pulsing movements so as not to disrupt the dura by the drilling process.

A 1.0 - 1.4µl droplet of fluorescent dextran amine was drawn into the narrow tip of a glass micropipette by capillary action. A short glass micropipette (3cm in length) was attached to the needle of a 5µl Hamilton syringe with parafilm to ensure an air tight seal. The depressor of the syringe was extended to create an air pocket, which pushed out the dye when depressed.

The syringe was mounted on a micromanipulator directly vertical above the drilled hole and lowered gently to pierce the dura cleanly with the attached glass capillary micropipette. The pipette needle was lowered until the ventral surface of the vertebra was reached to determine the depth of cord and then withdrawn in a dorsal direction to approximately half way through the depth. The syringe was then depressed slowly over one minute to allow the dye to diffuse gently out of the micropipette tip. The dye was delivered entirely into the spinal cord with little leakage into the subdural space. Any blood or dye was immediately blotted away by cotton buds. A small piece of compressed absorbable gelfoam (Upjohn) was placed over the hole in the vertebra to create an artificial "plug", and the skin and muscle realigned gently with forceps. The skin was sutured with a Sharpoint 4.0 suture needle with silk, and a powder antibiotic applied to the wound to prevent infection. Crushed aspirin was added to the drinking water as a post–operative analgesic.

2.2.4 Neuroanatomical axonal tracing

Single label retrograde tracing experiments

Axonal tracing experiments were used to determine which populations of long descending brain projections were able to grow through a complete spinal lesion. 0.4 - 1µl of 25% tetramethylrhodamine-labelled dextran amine (Fluororuby, Molecular Probes, MW 10000 in 2.5% Triton X-100 diluted in 0.1M Tris buffer, pH 8.0; see Appendix B1) was injected into the lumbar spinal cord at one week intervals following the lesion up to 5 weeks post-injury and at adulthood. The animals were

killed 4-7 days after the injection (procedure described in Section 2.2.3) to ensure that the dye has had sufficient time to back-label cell bodies in brain nuclei, should there be any projections across the injury site. Pups younger than 2 months of age were killed by an overdose of inhaled halothane (Fluothane, AstraZeneca), while adults received a lethal intraperitoneal injection of sodium pentobarbitone (0.2ml per 100gm of body weight; concentration = 60mg/ml).

Double label retrograde axonal tracing experiments

To determine whether a portion of the fibres able to grow through the lesion site are derived from truly regenerated cut axons, or entirely due to subsequent developmental growth, supraspinal projections were labelled with two different dyes before and after the spinal lesion (see Figure 2.5).

Animals were subject to three procedures.

1) First spinal injection of Oregon green at P4

P4 pups attached to the anaesthetised mother (see Section 2.2.1) were given a spinal injection of 0.25µl Oregon Green™ labelled dextran amine (Oregon green, diluted in 2.5% Triton X-100 in 0.1M TRIS buffer, pH 8.0) into the 1st lumbar segment (L1; see Section 2.2.3). At this age the pups are very small (approximately 10mm in length) and the injection was made into the middle of the cord. Ten pups were removed immediately following the injection at P4 to examine the injection site and ensure that the dye was contained within the spinal cord and had not extensively leaked into the CSF. The mother with attached pups was returned to the animal house. The purpose of the first spinal injection was to label the cell bodies of supraspinal projections which had grown past the mid–thoracic level at this early stage of development and would be severed by a complete spinal transection several days later.

2) Complete mid-thoracic spinal transection at P7

The mother was re-anaesthetised and the attached pups given a complete mid-thoracic spinal cord transection (see Section 2.2.2). Twenty five pups were removed at P7 immediately following the operation to check that the lesion was a complete

Figure 2.5: Double label axonal tracing protocol

a) A population of supraspinal neurons was retrogradely labelled by a lumbar injection of Oregon green made at P4.

b) Three days later, the thoracic spinal cord was completely transected and all brain neurons containing Oregon green would have had their lumbar projections severed.

c) Following a recovery period from 1 week to 4 weeks post-lesion, a second injection of Fluororuby was made caudal to the lesion site. Animals were killed 4 days after the second injection. Brain neurons that contained both dyes (yellow) indicated that these cells had a projections that was severed at P7 and regenerated back through the lesion site to contact the second dye. Neurons only containing Oregon green (green) indicated that the projection had been severed and had not grown back through the injury site. Neurons containing only Fluororuby (red) indicated that these cells had projections extending through the injury site as part of development subsequent to the injury

Double label axonal tracer protocol

A

brain

cervical

thoracic

lumbar

Oregon green injection

Post-natal day 4

B

spinal transection

Post-natal day 7

C

Fluororuby injection

One week intervals
P7 - P35
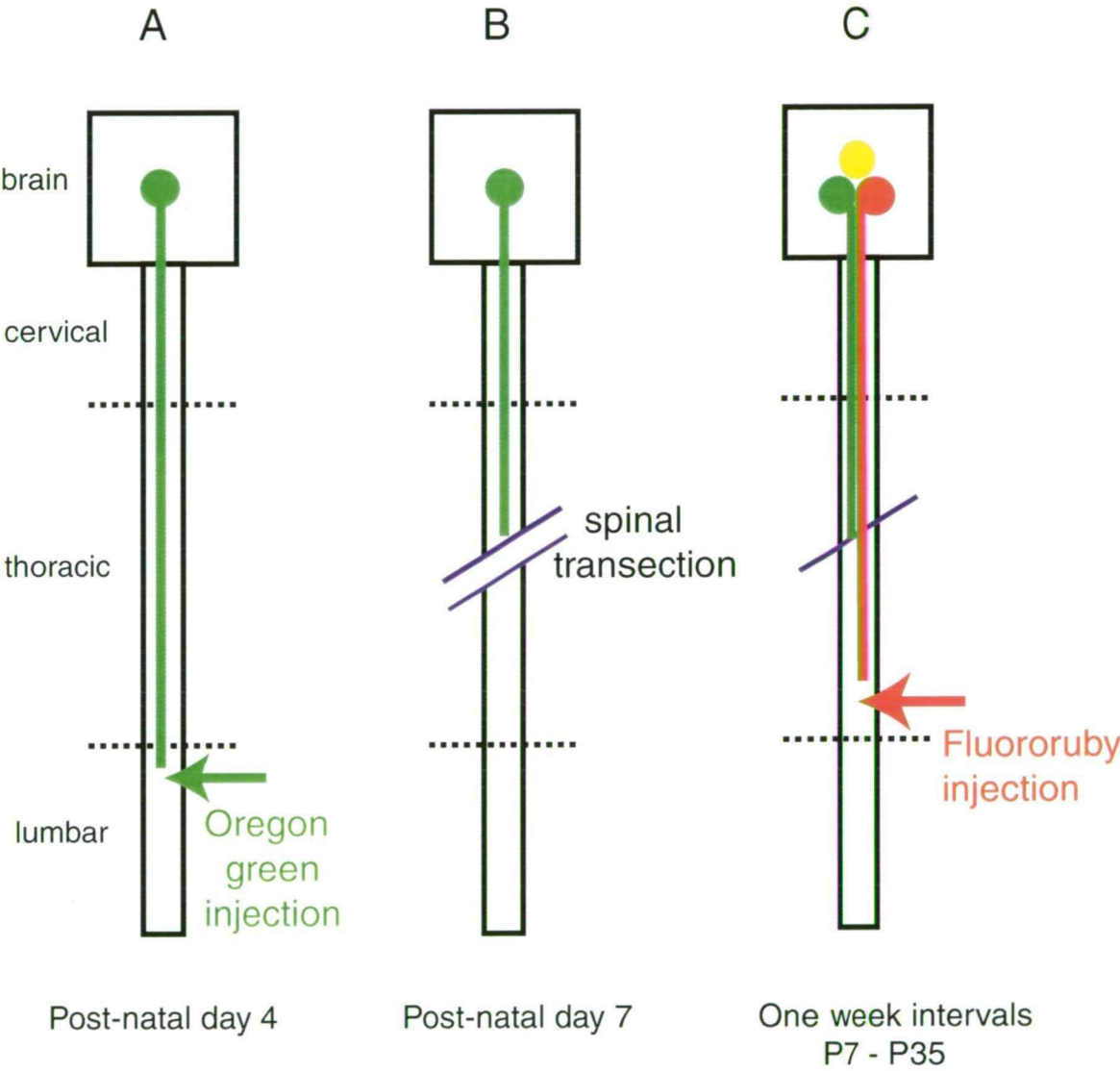
transection and that the initial injection of Oregon green had successfully back-labelled cell bodies in the brain. Some litters were injected but not lesioned, to provide age-matched controls for the axonal tracing experiments. The mother with pups was again returned to the animal house to recover from the operation.

3) Second spinal injection of Fluororuby at P7, P14, P21, P28, and P35

Pups were given a second injection into lumbar segment 1 of 0.25 µl of 25% tetramethylrhodamine-labelled dextran amine (Fluororuby, Molecular Probes, MW 10000) in 2.5% Triton X-100 in 0.1M TRIS buffer, pH 8.0. at different time points after the lesion. This injection would show all supraspinal projections that had grown across the complete transection, over what time course and what proportion had regenerated from severed axons (the latter indicated if neurons also contained Oregon green).

Control experiments for the double label protocol

a) Control animals were removed at P4 (n=10), immediately after Oregon green injection, to check uniformity of injection (see Table 2.1 and 2.2).

b) Following injection of Oregon green at P4, animals were removed at different times: P7 (n=7) P14 (n=3), P21 (n=3), P28 (n=3) and P35 (n=4), to check that the dye was localised in cell bodies by time of lesion, and to see if dye remained stable *in vivo* in the cell body (see Table 2.1 and 2.2).

c) P7 animals (n=25) were removed immediately following the lesion to check that the lesion was a complete transection (see Table 2.1).

d) Fluororuby injections were made at P7 (n=11), immediately after the lesion, to check that dye cannot leach across injury site, or through circulation to label brain neurons in the absence of a lesion (see Table 2.1 and 2.2).

e) Double injections were made in control uninjured animals at P7 (n=3), P14 (n=3), P21 (n=3), P28 (n=3) and P35 (n=3) to show the maximal amount of cell bodies that may be labelled in the brain stem by the double label protocol (see Table 2.1 and 2.2).

Table 2.1: The total number (n) of animals used in the present study

Age removed	Control	Spinally transected
P4	10	-
P7	7	25
P7 + 4	10	11
P14	3	7
P14 +4	6	12
P21	3	9
P21 + 4	7	10
P28	3	7
P28 +4	7	9
P35	4	6
P35 +4	5	15
Adult	6	8

Numbers include all animals used in spinal transection and spinal injections experiments. Adult animals were those older than 3 months; weaning is at approximately 2 months.

Table 2.2: Number of animals (n) used for the spinal injection experiments

Age removed	Control			Spinally transected		
	Oregon green	Fluororuby	Double labelled	Oregon green	Fluororuby	Double labelled
P7 + 4	3	3	3	11	4	4
P14 +4	3	3	3	10	3	3
P21 +4	3	4	3	8	6	6
P28 + 4	3	4	3	7	8	5
P35 +4	3	4	3	9	11	9
P42 + 4	3	4	3	4	4	4
Adult	-	6	-	-	8	-

Not all animals were found to have successful injections of both Oregon green and Fluororuby and therefore double label n values were not equal to that of the successful single label cases. Also some animals only received a single injection of either Oregon green or Fluororuby. The criteria for a successful injection included an appropriate unilateral injection in the lumbar spinal cord and below the lesion.

f) Injection sites of all uninjured and transected animals receiving injections (see Table 2.2) were examined to check that the injections were made in the correct location (unilaterally in lumbar spinal cord) and that Oregon green residue was not available for uptake beyond a few days post-injection at P4 to show misleading double labelled cell bodies.

g) The location of labelled cell bodies in control brains were checked to ensure that only neurons in regions known to have projections to the lumbar spinal cord in *Monodelphis* were labelled in case transynaptic transport of the dye was occurring.

h) The criteria for a successful injection included unilateral injection in the lumbar spinal cord, below the lesion, and evidence of some labelled neurons in the brain.

Removal and fixation of tissue

Four days following the last injection, pups younger than 2 months of age were killed by an overdose of inhaled halothane (Fluothane, AstraZeneca), while adults received a lethal injection of sodium pentobarbitone (0.2ml per 100gm of body weight; concentration = 60mg/ml). Tissue from pups aged P0-P14 was fixed by immersion while pups aged P21 and older were perfuse-fixed with 4% paraformaldehyde (see Appendix B1 for details) using a syringe pump infusing 2/3 of total blood volume circulating the body per minute. Blood volume was estimated to approximately equal 10% of total body weight and the perfusion rate was set at slightly lower (2/3) to prevent damage to blood vessels.

Perfusion rate = (Body weight x 0.1) x 0.66 ml per minute

As soon as the lethally anaesthetised animal had stopped breathing, the thoracic cavity was opened and the overlaying rib cage cut away with scissors. Care was taken not to sever associated blood vessels. A small slit was cut in the lower wall of the left ventricle. PVC tubing (diameter = 0.2mm) connected to a syringe pump was inserted into the slit and the right atrium also cut open to create an open circuit. Animals were initially perfused transcardially with 0.1M phosphate buffered saline (PBS) containing 2% sodium nitrate (to dilate the blood vessels) and heparin (15 units heparin in 1ml PBS) to prevent blood clots forming. Once the blood pushed out

of the right atrium had begun to run clear, the body was perfused with 4% paraformaldehyde fixing solution (in 0.1M PBS, pH 7.2, 4°C) of a volume approximately equal to the body weight of the animal.

Brains and the entire spinal cords (from cervical to sacral segments) were dissected out under a dissecting microscope and post-fixed for at least 24 hours in the same fixative. The injury site was photographed as a whole mount during the dissection with a Leica camera mounted to the microscope. Tissue was then stored in 4% paraformaldehyde at 4°C until further analysis.

Analysis of the tissue

Adult brains were cut in half across the coronal plane (in order to fit them under the vibratome stage) and both halves were coronally embedded in 5% agar blocks (High Gel Strength Agar; Sigma, USA in 0.1M PBS; see Appendix B1). Neonatal brains were embedded whole in the coronal plane. The agar block was glued to a stage immersed in 0.1M PBS and 100μm coronal sections were cut on a vibratome (Leica VT1000E).

All sections were collected with a fine brush, mounted on glass slides in an aqueous mounting medium (Ultramount, Dako) and coverslipped for viewing under a fluorescent microscope (Olympus BX50) through appropriate filter sets for each dye (see Table 2.3).

Table 2.3: Filter setting for fluorescent microscopy

	Fluororuby	Oregon green
Dichroic mirror	DM 570	DM500
Exciter Filter	BP530-550	BP470-490
Barrier Filter	BA590	BA515

Neurons in the brain retrogradely labelled with Fluororuby and Oregon green were visible at 10x magnification as brightly fluorescing red and green cell bodies respectively. For each 100µm brain section, the numbers of neurons labelled with either or both dyes found in different brain nuclei were counted and mapped onto camera lucida drawings of every 4th (adult) or 6th (neonates) serial section. The distribution and number of neurons retrogradely labelled from lumbar injections through a previously transected spinal cord was then compared to that of uninjured control animals.

At present there is no anatomical brain atlas for the *Monodelphis*. A detailed study by Holst *et al.* (1991) into the origins of supraspinal connections to the lumbar regions in *Monodelphis* has provided a source of reference from which brain nuclei have been identified and named in the present study. A brain atlas for the related species *Didelphis virginiana* by Oswaldo-Cruz and Rocha-Miranda (1968) was also used for additional information, as the two species have a similar cytoarchitecture of the brain (Holst *et al.*, 1991). For detailed listing of *Didelphis* connections see Appendix A.

Statistical analysis of counts of labelled neurons

The total numbers of neurons in the whole brain labelled with Oregon green or Fluororuby and those double labelled with both were counted for individuals in each age group. The mean ± standard error of the mean (SEM) was calculated for each age group. Where an individual did not contain double labelled neurons but did show both Oregon green and Fluororuby labelled neurons separately, the value of zero was included in the calculation of the mean as the two injections were considered to have been successful. It should be noted that the numbers of both Oregon green and Fluororuby labelled neurons included those that were double labelled.

The mean number of Fluororuby and double labelled neurons for the whole brain in control and spinally transected animals at each age was compared to the age with the highest value to see if there were significant changes over time. The means of Oregon green labelled neurons were compared against the P7 values as this should not have changed over time. The means of Oregon green, Fluororuby and double

labelled neurons in spinally transected animals for each age were then compared to the age –matched control means to see if a transection affected the number of labelled neurons. The comparisons of means were made by t-tests performed on SigmaStat ® statistical program.

For each nucleus in which Oregon green, Fluororuby and double labelled neurons were found, the mean ± SEM of the total number was calculated in both the transected and control groups. The total number found in particular nuclei in an individual was also represented as a proportion of the total number of labelled neurons for that individual. Where no labelled neuron were detected in a certain nuclei, but were present in other region of the brain, then the value in that nuclei for that individual was said to be zero and included in the calculation of the mean. The mean proportions of each nucleus were compared to see if significant changes occurred over time. The mean proportions for the youngest age at which labelling was obtained (P7 in controls and P14 in spinally transected) were compared to the means for the oldest age (P35 or adult) for different nuclei with each label.

Morphological examination of spinal cords: injury site & injection site.

The segments of the spinal cord containing the injury sites (approximately T4-T8) were dissected and were photographed on colour slide film as whole mounts under a dissecting microscope, illuminated by optic fibre lights at x1 magnification.

The segments of the spinal cord containing the injection sites were cut out of the spinal cord (T12-L2), embedded in 5% agar and sectioned transversely (single label experiments) or horizontally (double label experiments) at 100µm thickness on a vibratome. The sections were mounted on glass slides in aqueous mounting medium, examined under a fluorescent microscope with the appropriate filter set and photographed with colour slide film using a photomicrographic system (Olympus PM-30). The extent of the dye spread at the injection sites was recorded on camera lucida drawings of the injection site.

Some tissue was prepared for histological studies to examine the injury site at a cellular level. See Appendix B2 and B3 for details.

Chapter 3

RESULTS

Overview

The present study describes the distribution of neuronal cell bodies that grow axonal projections through a complete mid thoracic spinal lesion made at P7, 1, 2, 3, 4 weeks, and 6 months after injury in *Monodelphis* opossums. The main aim was to determine what proportion of severed axons have regenerated across the injury site. As the injury was made during the early developmental stage of life, the normal sequence of supraspinal innervation was initially determined for the time period examined.

Although supraspinal pathways that project to the lumbar cord in the adult already have some axonal projections that reach that level by P7 (Wang *et al.*, 1992), it would appear that much more developmental growth is still to occur before adult innervation is established (Holst *et al.*, 1991). Section 3.1 describes the developmental sequence of axonal projections from the brain to the lumbar spinal cord in control uninjured animals from P7 to adulthood.

Section 3.2 describes brain projections that reappear across a complete spinal transection in the first 4 weeks post-injury and the time course of innervation to the lumbar cord; a comparison is made with the development of projections in control animals.

Section 3.3 determines what proportion of the fibres that grow across a complete spinal transection have either regenerated from cut axons or were the result of subsequent development of new fibres.

3.1 THE DEVELOPMENT OF SUPRASPINAL PROJECTIONS TO THE LUMBAR SPINAL CORD IN *MONODELPHIS*

Lumbar injections of the retrograde axonal tracer Fluororuby were made at P7, P14, P21, P28, P35 and in adult control animals (see Methods: Section 2.2.4) to establish the origins of supraspinal projections in the lumbar cord over the developmental period studied (see Table 2.2 in Methods and Table 3.1.1).

Table 3.1.1: Number of control animals used for Fluororuby axonal tracing studies

Day of injection	P7	P14	P21	P28	P35	Adult
n	3	3	4	3	3	6

It was necessary to establish that spinal injections of Fluororuby in control animals labelled axonal projections at different ages in a consistent and reliable manner. Animals were removed 4 days after the injection in the case of pups aged P7–P35 and after one week in adults. The incubation times for the optimal labelling of neurons were established by preliminary studies. Shorter incubation times resulted in less optimal labelling of neurons with a high background in the tissue. The total amount of dye injected at each age was standardised in proportion to the cross sectional area of the lumbar spinal cord at that level (see Table 3.1.2).

The presence of long descending brain projections that extended to the lumbar regions of the spinal cord was confirmed by the presence of retrogradely labelled neurons in the brain nuclei. Labelled neurons were only found in the brain stem. Examination of 100μm vibratome sections of brains under a fluorescent microscope with appropriate optics (see Methods: Section 2.2.4) showed strongly fluorescent retrogradely labelled neuronal cell bodies, emitting light at rhodamine wavelength (580nm). The dye was accumulated in small granules in the cell soma and along processes in the plane of the section. Neurons were generally visible under x10 magnification (see Figure 3.1.1). At P7 and P14, the boundaries of fluorescent cell bodies were not clearly defined as the dye was not as heavily granulated in the cell

Table 3.1.2: Weight of animal, cross-sectional area of lumbar spinal cord and volume of dye injected in control animals at each age.

Age	n	Weight (g) mean ± SEM	Lumbar cross-section area (mm^2)	Volume range injected (μl)	Volume/area ratio $(\mu l/mm^2)$
P7	3	0.7 ± 0.2	0.1 ± 0.1	0.1 - 0.2	1.0 - 2.0
P14	3	1.8 ± 0.6	0.2 ± 0.1	0.3 - 0.4	1.6 - 2.1
P21	4	3.8 ± 1.1	0.3 ± 0.1	0.3 - 0.4	1.2 - 1.5
P28	4	6.9 ± 2.1	0.3 ± 0.0	0.5 - 0.6	1.6 - 1.9
P35	4	10.6 ± 3.2	0.4 ± 0.1	0.5 - 0.6	1.4 - 1.7
Adult	6	105.8 ± 31.9	0.6 ± 0.1	1.0 - 1.2	1.6 - 1.9

The volume of dye administered was calculated such that the ratio of the volume injected divided by the cross sectional area of the spinal cords remained between 1-2. It was apparent that the cross section was a closer indication of the developmental changes in size of the spinal cord, rather than the weight of the animal.

Figure 3.1.1: Retrogradely labelled neuronal cell bodies following lumbar spinal injection of dextran amine rhodamine (Fluororuby) at age P35 in *Monodelphis*.

a) Fluororuby labelled neurons in the lateral vestibular nucleus. Scale bar = 100μm.

b) Large multipolar neurons labelled with Fluororuby in the central region of the gigantocellular reticular nucleus. Scale bar = 100μm.

c) Small Fluororuby labelled neurons in the reticular pontine nucleus. Scale bar = 100μm.

a
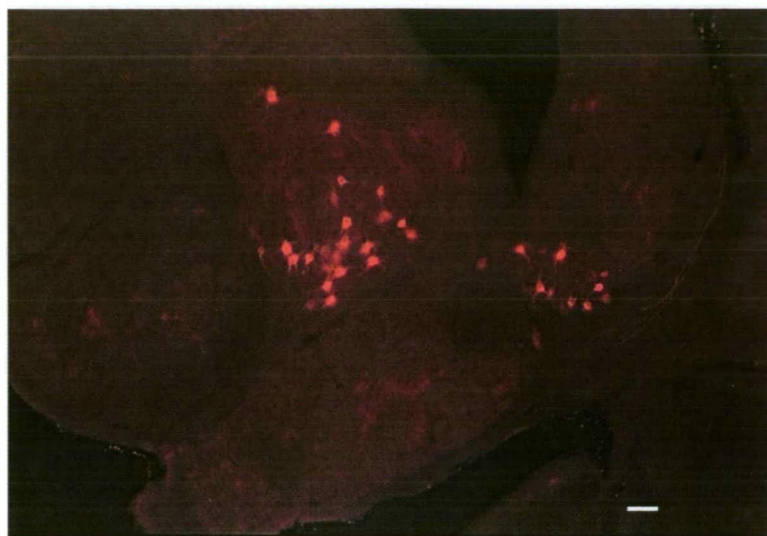
b
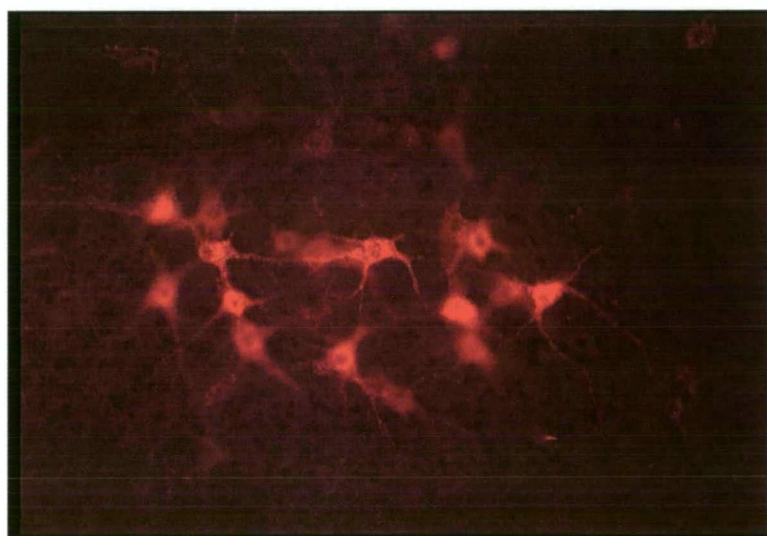
c
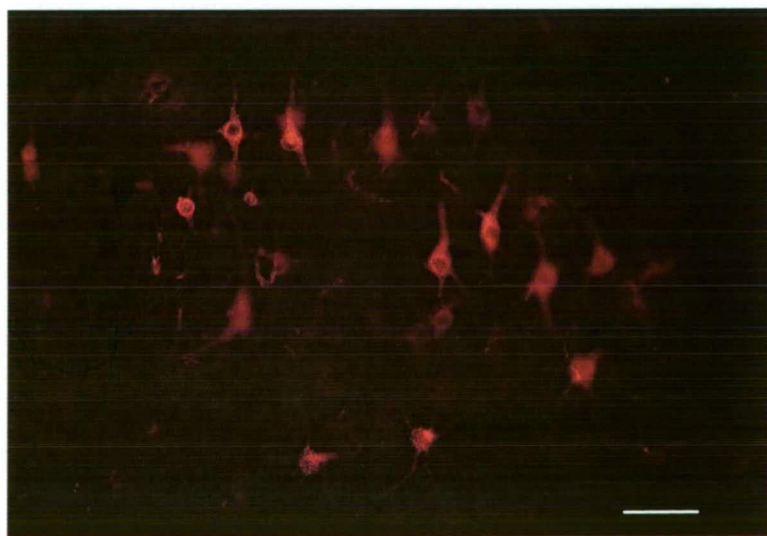
cytoplasm and background fluorescence of the brain section was often present (see Figure 3.1.2). In animals aged P21 and older, labelled neurons were much brighter.

The delineations of characteristic aggregations of neurons into nuclei in the brain were not easy to determine at the younger ages; and the distribution of neurons in brain sections often appeared indistinct under the fluorescent microscope. Illumination of sections under the light microscope was required to establish the exact location of labelled neurons in different nuclei, which were then mapped precisely onto camera lucida representations of the brains (refer to Figure 3.1.6 to Figure 3.1.11 for the location of the main nuclei that showed labelled neurons, and also their nomenclature). Due to the ambiguity of definite classification for some nuclei at younger ages, neurons found in several adjacent nuclei were grouped together to maintain comparability between the ages. These included:

Nuclei	Includes additional regions
Gigantocellular reticular nucleus	Ventral and lateral gigantocellular Reticular nucleus Paragigantocellular reticular nucleus
Raphe nuclei	Raphe palladis Raphe obscurus nucleus Raphe magnus nucleus
Dorsal medullary reticular nucleus	Intermediate medullary reticular nucleus Dorsal medullary reticular nucleus
Spinal trigeminal nucleus	Caudal spinal trigeminal nucleus Interpolar spinal trigeminal nucleus
Edinger-Westphal nucleus	Nucleus darkschewitsch
Hypothalamic nucleus	Paraventricular hypothalamic nucleus dorsal hypothalamic area lateral hypothalamic nucleus

The injection sites in the spinal cord were all sectioned and examined under the fluorescence microscope to ensure that the dye had entered the lumbar cord and that the injection was made in a uniform position on each occasion. The extent of the dye distribution and the exact spinal segmental level of the injection may influence the number of projections labelled. Examples from each age are shown as camera lucida representations in Figure 3.1.3a and micrographs of the injection sites are in Figure

Figure 3.1.2: Retrogradely labelled neuronal cell bodies in the brain from lumbar spinal injections of dextran amine rhodamine (Fluororuby) at age P7 in *Monodelphis*.

a) Fluororuby labelled neurons in the lateral vestibular nucleus. The granules are not as densely localised as in neurons seen at older ages (compare with Figure 3.1.1). Scale bar = 100μm.

b) Fluororuby labelled neurons in the reticular pontine nucleus. Scale bar as for a).

a
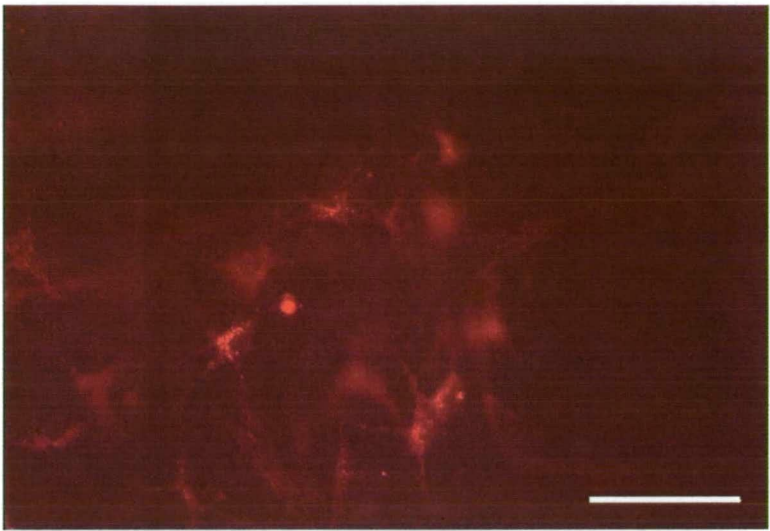
b
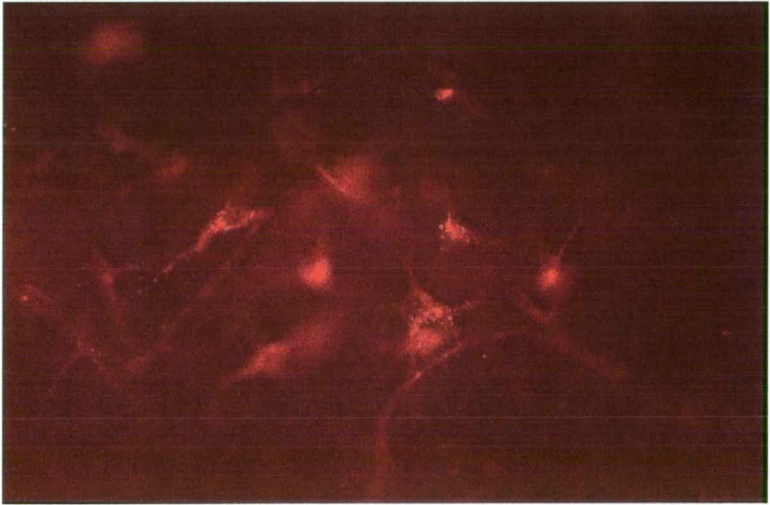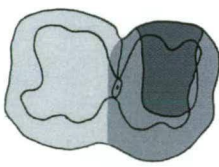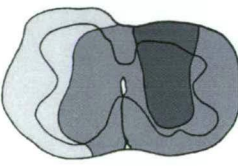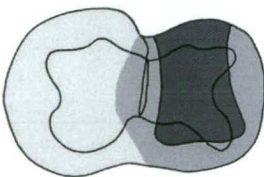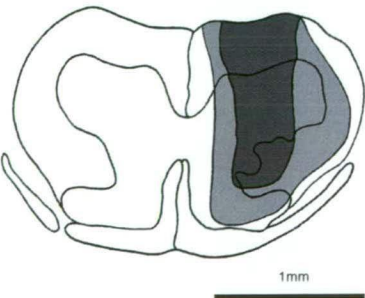
Figure 3.1.3: Injection sites of dextran amine rhodamine (Fluororuby) into the lumbar spinal cord of control *Monodelphis* at different ages.

a) Camera Lucida representations of the lumbar spinal cord sectioned in the transverse plane showing the typical appearance of the Fluororuby injection site from P7 to adult animals removed 4 days after the unilateral injection. The shaded grey areas represent the intensity of the fluorescence from the injected dye. Darkest grey indicates the site of injection and the lighter gray shows a diffuse halo of fluorescence. Scale bar = 1mm.

b) Transverse section of the adult lumbar spinal cord showing a discrete unilateral injection site of Fluororuby, with little diffusion to the contralateral side of the cord. The dye is accumulated into cell bodies and processes but remains visible in extracellular space in the immediate needle zone. Scale bar = 100μm.

c) Transverse section of the lumbar spinal cord from a P28 + 4 animal revealing the impact of the unilateral Fluororuby injection. The entire ipsilateral side of the spinal cord is brightly fluorescent and a halo of dye extends partially to the contralateral side. Scale bar = 100μm.

d) Sagittal section through the Fluororuby injection site in the lumbar spinal cord of a P21 + 4 animal showing that the impact of the injection reached right through the dorsoventral plane. The dye remained heavily localised at the level of injection and labelled cell bodies were detected in both rostral and caudal direction in the spinal cord indicating collateral projection. The dotted lines indicate the outline of the spinal cord tissue. Scale bar = 100μm.

Age	n	Injection site
P7	3	
P14	3	
P21	4	
P28	4	
P35	4	
adult	6	

1mm
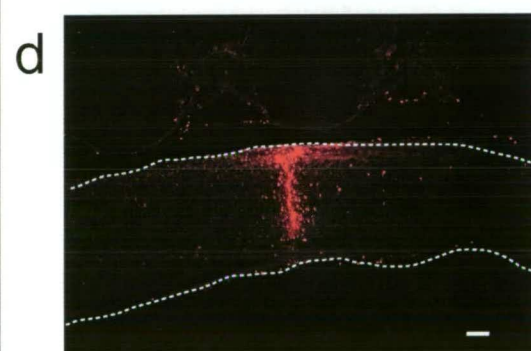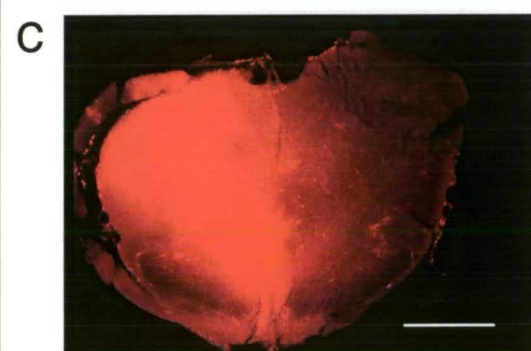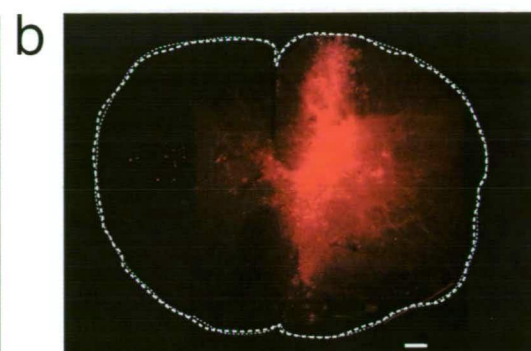
3.1.3b, c & d. Where the injection had been made at an incorrect level or where no dye was observed in the spinal cord, the animal was eliminated from the study.

The injection site became increasingly discrete and more delineated as the spinal cord developed (see Figures 3.1.3a). At P7, the dye dispersion encompassed the whole injected side of spinal cord and a halo of dye was present in the contralateral spinal cord. In animals from P14 to P35 (eg. see Figure 3.1.3c), while a diffuse halo of fluorescence extended across the whole cord, the needle zone was more discrete and localised with increasing age of the animals. In adult animals the dye did not appear to have diffused far from the site of injection. The unilateral injection showed a densely labelled central needle zone surrounded by a lighter halo of dye which was localised in the adjacent gray matter at the site of injection and in most cases did not spread to the contralateral side of the spinal cord (see Figure 3.1.3b). All control animals that showed an appropriate injection also had retrogradely labelled neurons in brain stem and midbrain nuclei. However, there was no evidence of cortical neurons being labelled. The location of labelled cell bodies in particular nuclei is described below.

3.1.1 Total number of labelled neurons in each brain

The total numbers of labelled neuronal cell bodies located in different brain nuclei were counted in 100μm sections through the entire brain. Table 3.1.3 shows the number of labelled neurons in control brains at each age. Figure 3.1.4 shows a scatter plot of the total number of labelled cell bodies counted in control brains following a lumbar injection. There was approximately a 4-fold increase in total number of labelled cell bodies from P7 (379.3 ± 122.4) to P35 (1645.3 ± 226.1, $P < 0.01$). A decline from P35 numbers was observed in adult animals (688.7 ± 207.6, $P < 0.01$). The labelling method used in the present study cannot be employed to estimate the actual total number of neurons in the brain that have lumbar projections as it is not possible to ensure that all descending fibres had taken up the dye. Therefore the term "total number of neurons" refers only to total number of labelled neurons found. Reasons why this technique does not label all neurons with lumbar projections are discussed in Critique of Methods (see Discussion: Section 4.6.2)

Table 3.1.3: Total number of Fluororuby labelled neurons in brains of control animals

Age of injection	n	Mean ± SEM	P t-test against P35 value
P7	3	379.3 ± 122.2	<0.01*
P14	3	758.3 ± 211.9	0.04*
P21	4	1458.0 ± 458.1	0.73
P28	4	1503.3 ± 228.8	0.67
P35	4	1645.3 ± 226.1	-
adult	6	688.7 ± 207.6	<0.01*

The mean ± SEM was calculated from counting neurons on both sides of the brain in 100µm coronal sections.

T-tests were made with Sigma Stat® comparing the mean values at each age against the highest numbers (P35) to determine whether there were significant differences.

*The P value of statistical significance was defined as $P \leq 0.05$.

Figure 3.1.4: The number of Fluororuby labelled neurons in control animals

Scatterplot of the number of labelled neurons counted in the brains of animals aged P7 to adult following lumbar injection of dextran amine rhodamine (Fluororuby). The open circles indicate the value recorded for individuals in different age groups and the closed red circles indicates the mean value for that age group. The P35 mean was found significantly different to the P7, P14 and adult mean values ($P < 0.05$).

Number of Fluororuby labelled neurons in control animals
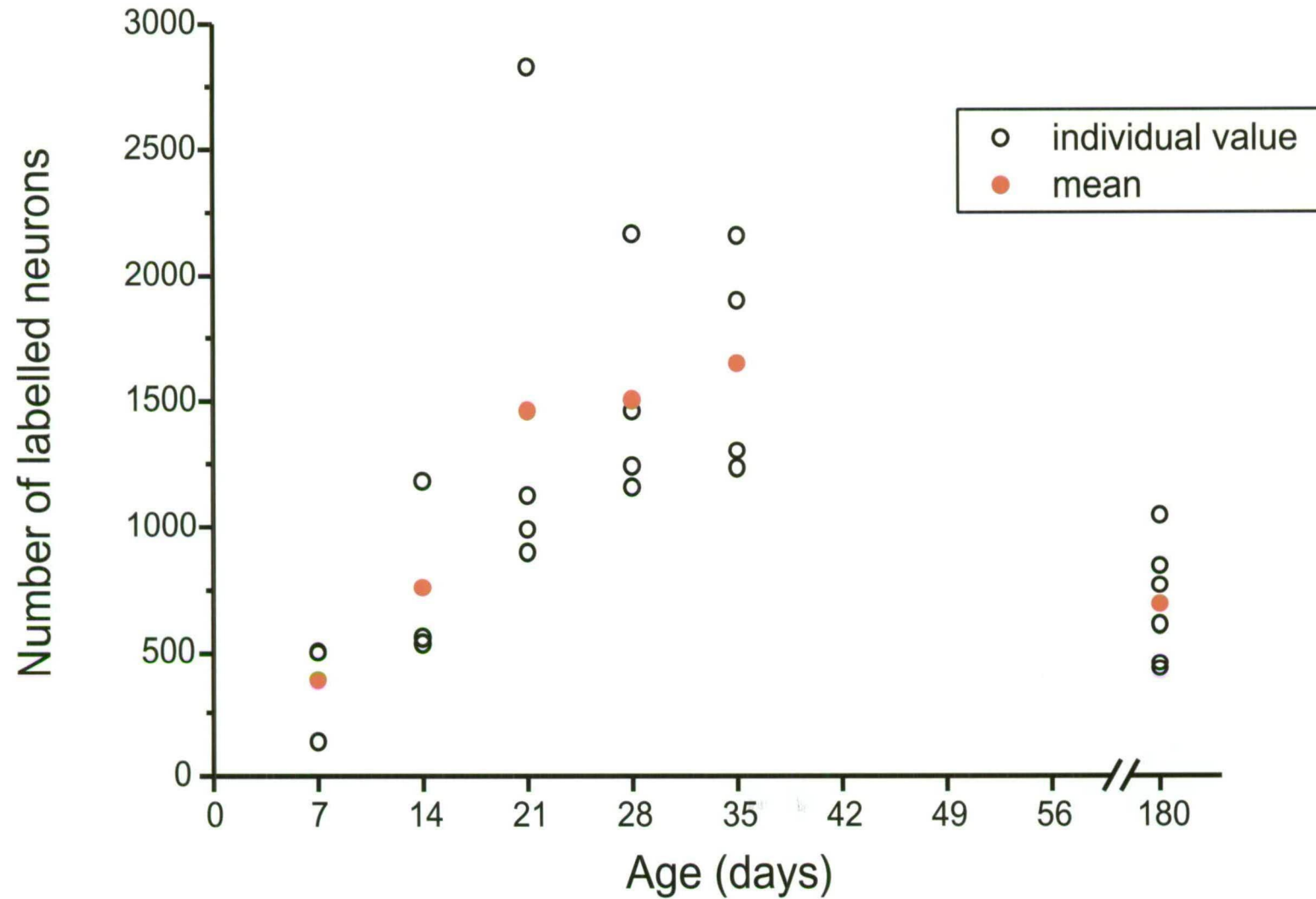
3.1.2 Distribution of cell bodies in different brain nuclei

In this section the origin of neurons with projections descending to the lumbar region was studied by mapping the location of labelled neurons into different nuclei. The numbers of labelled neurons in different individual nuclei were recorded at the time of counting total numbers of labelled neurons in each brain. These data for individual nuclei are presented in Table 3.1.4. A similar age-related trend to that seen for the total number of labelled neurons in the whole brain (see Table 3.1.3) was also observed for individual brain stem nuclei: a steady increase from P7 to P35, followed by a decline to adult numbers.

To directly compare the contribution that axonal projections from individual brain nuclei make to the lumbar spinal cord at different ages, the number of labelled neurons in different brain nuclei was expressed as a proportion of the total number of labelled cells in each brain. These data are presented in Table 3.1.5 and Figure 3.1.5. At P7 most of the projections originated from gigantocellular reticular nucleus (25.7 ± 1%), pontine reticular nuclei (27.7 ± 1.5%) and raphe nuclei (16.3 ± 1.2%) as indicated by the distribution of labelled neurons. Over the first 4 weeks after birth, the projections came from a much more diverse range of nuclei, with increasing contributions from the ventral medullary reticular nucleus, dorsal medullary reticular nucleus and lateral vestibular nucleus. In adult animals, the majority of projections to the lumbar cord originated from all the aforementioned nuclei with increasing numbers of labelled neurons appearing in the locus coeruleus, red nucleus and other smaller nuclei grouped together as "other nuclei". These included the nucleus ambiguus, caudal spinal trigeminal nucleus, medial vestibular nucleus, interstitial nucleus of the medial longitudinal fasciculus, Edinger-Westphal nucleus, nucleus Darkschewitsch, ventral part of central gray, paraventricular hypothalamic nucleus, dorsal hypothalamic area and the lateral hypothalamic nucleus.

To test whether changes in contributions made by different nuclei were of statistical significance, the proportion of total labelled cells found in each nucleus at P7 was compared (unpaired students t-test) with the proportion for each nucleus at P35 and in the adult (see Methods: Section 2.2.4). Due to the small n values in each group, the power of the t-test is low and thus the findings were interpreted with caution. It

Table 3.1.4: Number (mean ± standard error) of Fluororuby labelled neurons counted in each nucleus in control animals from P7 to adult.

Nuclei	P7		P14		P21		P28		P35		Adult		
n	3		3		4		4		4		6		
Ventral medullary reticular nucleus	19.7	± 5.8	52.3	± 24.0	69.3	± 19.3	102.8	± 20.0	147.5	± 35.7	113.5	±	38.1
Dorsal medullary reticular nucleus	26.3	± 8.4	79.0	± 41.2	243.0	± 165.9	165.0	± 34.1	236.0	± 19.8	105.2	±	29.1
Raphe nuclei	63.0	± 17.4	110.0	± 53.6	143.8	± 24.8	114.0	± 33.3	144.0	± 44.1	27.5	±	32.7
Gigantocellular reticular nucleus	100.7	± 26.0	169.0	± 17.5	380.0	± 58.2	326.3	± 43.8	282.5	± 30.1	132.8	±	40.0
Lateral vestibular nucleus	35.3	± 12.8	113.7	± 48.9	179.3	± 13.6	224.3	± 26.3	243.5	± 34.6	109.7	±	30.4
Reticular pontine nucleus	101.7	± 30.4	151.0	± 36.0	260.3	± 48.5	235.5	± 28.9	236.3	± 30.0	71.2	±	20.2
Locus coeruleus	13.0	± 2.1	20.0	± 4.0	78.0	± 51.6	81.0	± 14.5	110.3	± 45.2	17.5	±	3.9
Red nucleus	10.3	± 7.1	30.3	± 7.3	51.5	± 16.9	112.0	± 23.8	82.5	± 6.7	40.2	±	12.2
Other nuclei	9.3	± 0.7	33.0	± 3.4	53.0	± 2.9	142.5	± 4.9	146.3	± 3.6	70.2	±	2.9

Other nuclei = nucleus ambiguus, caudal spinal trigeminal nucleus, medial vestibular nucleus, interstitial nucleus of the medial longitudinal fasciculus, ventral part of central gray, paraventricular hypothalamic nucleus and dorsal hypothalamic area, Edinger-Westphal nucleus, and the nucleus Darkschewitsch.

Table 3.1.5: The proportion (% mean ± standard error) distribution of Fluororuby labelled neurons in different nuclei in control animals from P7 to adult.

Nuclei	P7	P14	P21	P28	P35	Adult
n	3	3	4	4	4	6
Ventral medullary reticular nucleus	5.4 ± 0.3	6.3 ± 1.3	4.9 ± 0.3	6.7 ± 0.5	9.6 ± 0.7	16.8 ± 5.2
Dorsal medullary reticular nucleus	7.1 ± 0.5	9.2 ± 2.3	12.3 ± 4.7	10.8 ± 0.7	13.3 ± 2.4	14.2 ± 2.0
Raphe nuclei	16.3 ± 1.2	15.3 ± 3.2	9.5 ± 0.9	7.4 ± 1.1	9.5 ± 0.7	4.8 ± 1.4
Gigantocellular reticular nucleus	25.7 ± 1.0	24.3 ± 2.3	27.9 ± 2.3	21.7 ± 1.4	17.8 ± 1.0	18.6 ± 2.8
Lateral vestibular nucleus	8.9 ± 0.8	13.9 ± 2.3	14.5 ± 2.4	15.1 ± 0.6	14.8 ± 0.2	16.8 ± 4.4
Reticular pontine nucleus	27.7 ± 1.5	20.3 ± 0.7	19.7 ± 2.7	15.9 ± 1.0	14.5 ± 0.6	9.9 ± 2.2
Locus coeruleus	4.4 ± 1.5	2.9 ± 0.7	4.0 ± 1.5	5.4 ± 0.2	6.0 ± 2.2	2.4 ± 0.4
Red nucleus	2.1 ± 1.4	4.2 ± 0.8	3.5 ± 0.4	7.3 ± 0.5	5.1 ± 0.3	6.2 ± 2.0
Other nuclei	2.6 ± 0.7	3.7 ± 0.3	3.7 ± 0.2	9.6 ± 0.3	9.4 ± 0.3	10.3 ± 0.4

Other nuclei = nucleus ambiguus, caudal spinal trigeminal nucleus, medial vestibular nucleus, interstitial nucleus of the medial longitudinal fasciculus, ventral part of central gray, paraventricular hypothalamic nucleus and dorsal hypothalamic area, Edinger-Westphal nucleus, and the nucleus Darkschewitsch.

Figure 3.1.5: The proportional contribution from different nuclei to the total profile of Fluororuby labelled neurons in the brain following lumbar spinal injection in control animals aged P7 to adult.

* denotes that t-tests made with Sigma Stat® between the proportion of the total at P7 with older ages for different nuclei showed statistical differences (P≤ 0.05).

<u>Abbreviations</u>

RV	Ventral medullary reticular nucleus
RD	Dorsal medullary reticular nucleus
Raphe	Raphe nuclei
RGc	Gigantocellular reticular nucleus
VstL	Lateral vestibular nucleus
RP	Reticular pontine nucleus
Coe	Locus coeruleus
Rb	Red nucleus
others	Other nuclei

Proportional distribution of Fluororuby labelled neurons in the brain stem of control animals
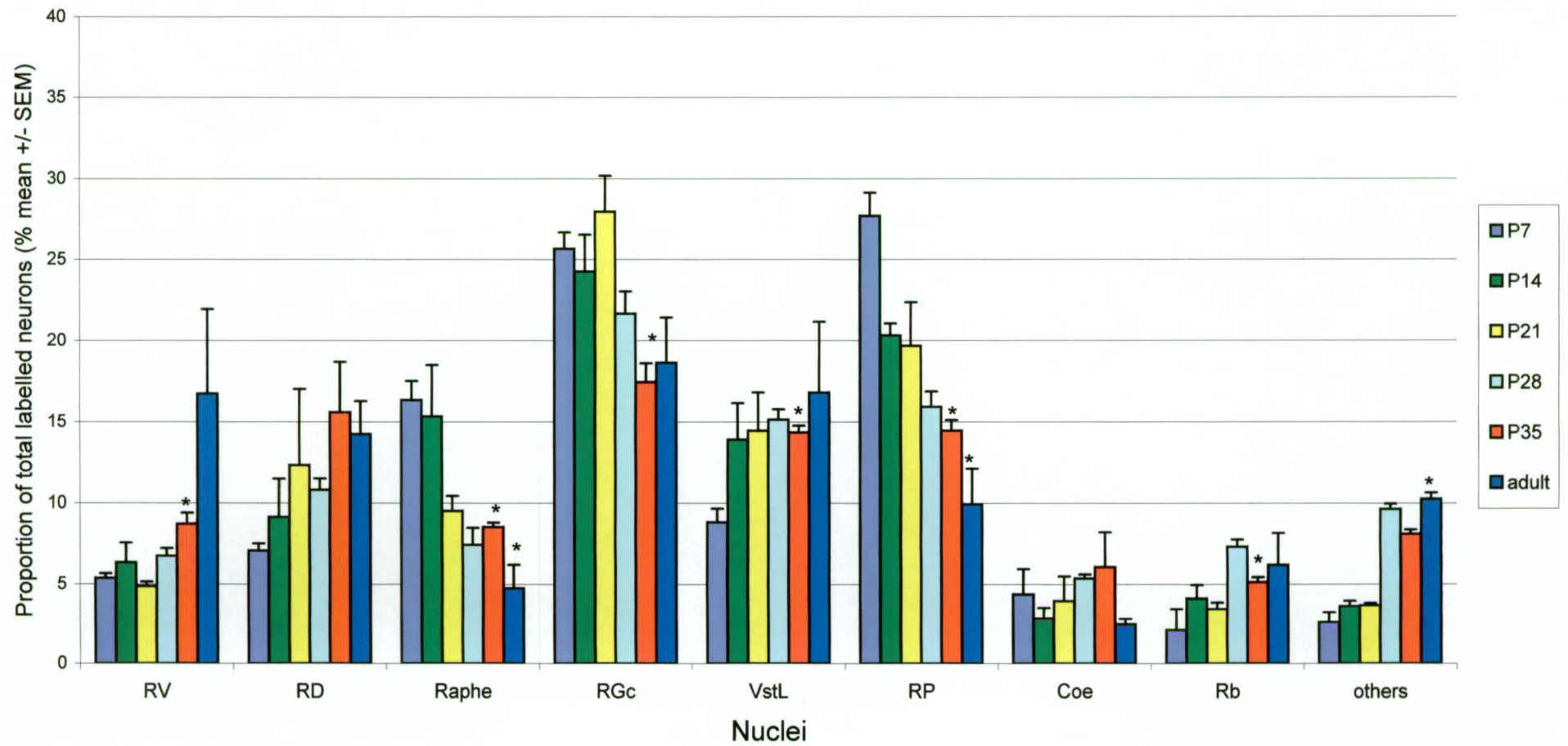
should be noted that all control data passed normality tests and had equal variance, thus validating the use of the student's t-test for comparisons. The P values for each test are presented in Appendix C1.

Between P7 and P35, there was a statistically significant increase observed in the proportion of labelled neurons found in the ventral medullary reticular nucleus (5.4 ± 0.3% and 9.6 ± 0.7%; P < 0.01), lateral vestibular nucleus (8.9 ± 0.8% and 14.8 ± 0.2%; P < 0.01) and red nucleus (2.1 ± 1.4% and 5.1 ± 0.3%; P = 0.05). The adult values for these nuclei appeared to reflect a similar increased proportion, but these did not reach statistical significance compared to P7 (P = 0.12, 0.25, and 0.18 respectively).

The dorsal medullary reticular nucleus, locus coeruleus and the combined "other nuclei" were also seen to make an increased proportional contribution to the total labelled neurons in the brain over time (see Figure 3.1.5). However, these did not reach statistical significance, with the exception of the "other nuclei" between P7 and adult animals (2.6 ± 0.7% and 10.3 ± 0.4%; P < 0.01). The locus coeruleus did not show any significant changes in its proportional contribution to the total number of labelled neurons between P7 and P35 (4.4 ± 1.5% and 6.0 ± 2.2%; P = 0.13) and the adult contribution (2.4 ± 0.4%; P = 0.18).

All the nuclei which contribute the majority of projections to the lumbar cord at P7 decreased their proportional contribution at later ages as projections from other nuclei increased. This was reflected by statistically significant decreases in the proportions contributed by the raphe nuclei between P7 and P35 (16.3 ± 1.2% and 9.5 ± 0.7%; P < 0.01) and between P7 and adult (4.8 ± 1.4%; P = 0.01). Significant decreases were also observed in the reticular pontine nucleus between P7 and P35 (27.7 ± 1.5% and 14.5 ± 0.6%; P < 0.01) and between P7 and adult (9.9 ± 2.2%; P < 0.01) and the gigantocellular reticular nucleus between P7 and P35 (25.7 ± 1.0% and 17.8 ± 1.0%; P = 0.03, see Table 3.1.5 and Appendix C1).

3.1.3 The origin of supraspinal projections

The location and number of labelled neurons in different nuclei showed significant differences over time reflecting the changes in the dominance of lumbar projections originating in the brain stem nuclei. The location of nuclei containing labelled neurons in control *Monodelphis* brain after a lumbar spinal injection showed a change in number and dominance of projections from certain nuclei through the developmental period examined. Camera lucida representations of brains are presented for each age showing the location and number of retrogradely labelled neurons counted in different nuclei (see Figures 3.1.6 to 3.1.11, refer to Table 3.1.5 for the appropriate SEM values). Further descriptions of the nuclear distribution of neurons at each age are provided below.

Post-natal day 7

Injections of Fluororuby into the lumbar cord of P7 animals produced labelled neurons (total = 379.3 ± 122.2) mainly in the reticular formation of the pons and medulla, and in the lateral vestibular nucleus (see Figure 3.1.6 and Figure 3.1.2). Reticular labelling was comprehensive throughout the formation, which included the dorsal medullary reticular nucleus, ventral medullary reticular nucleus, gigantocellular reticular nucleus, and reticular pontine nucleus and raphe nuclei. The majority of labelled neurons were from the gigantocellular reticular nucleus (25.7 ± 1%) and reticular pontine nucleus (27.7 ± 1.5%). The gigantocellular reticular nucleus encompasses a large region of the central brain stem at P7, and in the present study labelled neurons found in lateral, caudal and ventral regions were grouped together as in the developing animals the nuclear delineations were difficult to determine. However, most neurons labelled within the gigantocellular reticular nucleus were clearly located in the central region and were large, multipolar neurons.

Smaller contributions to the total number of labelled neurons were also detected from the locus coeruleus (13.0 ± 2.1), spinal trigeminal nucleus (2.3 ± 1.2), medial vestibular nucleus (7.0 ± 1.73) and red nucleus (10.3 ± 7.1). The red nucleus only showed labelled cell bodies in one animal out of three, suggesting that only a few

Figure 3.1.6: Distribution of labelled neurons in the brain stem after lumbar
Fluororuby injection in control P7 *Monodelphis*

Camera lucida representation of coronal brain sections from control
P7 + 4 brains showing the location and number of retrogradely
labelled neurons after an injection of Fluororuby into the lumbar
spinal cord at P7. Sections shown are from the caudal brain stem (a)
to the hypothalamus (h). One star represents up to 10 labelled
neurons.

P7 control *Monodelphis*

a

RD
RV
Am

b

RD
RV

c

TrSc
RGc
RGcv

d

Cb
VstM
TrSc
RGc
VstL
RaO
RGcv

e

Coe
RP
RaM

f

Iflm
Rb

g

Phd

h

Hyl

1mm
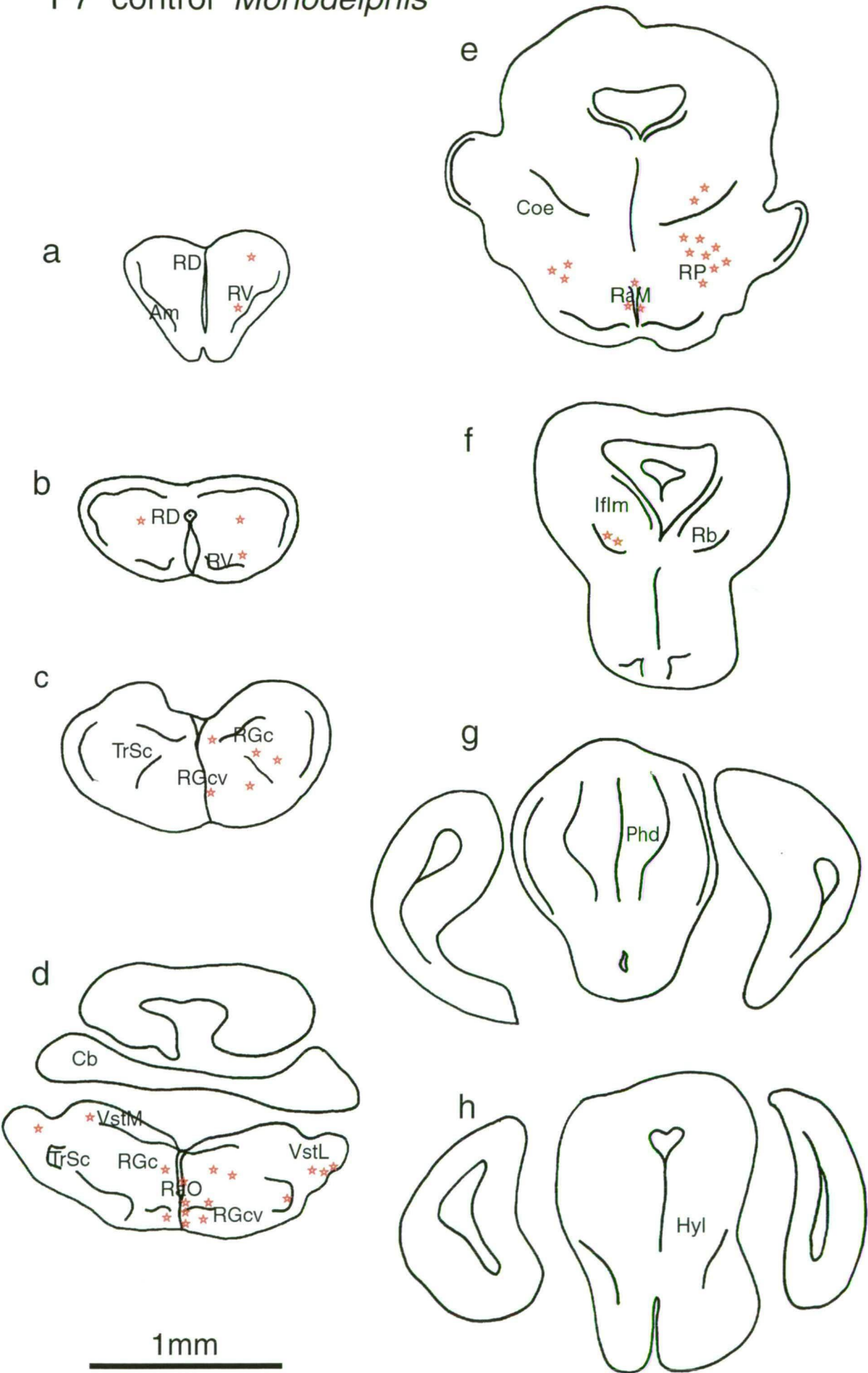
projections are present at this early developmental age. These were the most rostrally labelled neurons detected at P7. All other nuclei that contained cell bodies showed consistent labelling in the three animals examined. All three control brains examined at P7 showed labelling in the dominant nuclei (those with the highest number of labelled neurons) in remarkably consistent proportions of the total neurons (see Table 3.1.5).

Post-natal day 14

The total number of labelled neurons increased two–fold from P7 to P14 (from 379.3 ± 122.2 to 758.3 ± 211.9, P = 0.04), although the proportion contributed from each nuclei remained similar to the pattern observed at P7 (see Figure 3.1.7). The proportion from the lateral vestibular nucleus appeared to increase from 8.9 ± 0.8% at P7 to 13.9 ± 2.3%, but this was not statistically significant (P= 0.12). In contrast, the proportion contributed from the reticular pontine nucleus decreased significantly from 27.7 ± 1.5% at P7 to 20.3 ± 0.7% (P = 0.05). Small numbers of labelled neurons were also detected in the nucleus ambiguus (12.1 ± 7.2) where they had not been seen at P7 and one brain of the three examined showed 9 labelled neurons in the interstitial nucleus of the medial longitudinal fasciculus. No other rostral labelling was detected at this age. Labelling of small tightly clustered neurons in the red nucleus (30.3 ± 7.26) was also more consistent with all three brains showing fluorescent cell bodies. Both the number and proportional contribution of the nucleus ambiguus, interstitial nucleus of the medial longitudinal fasciculus, and red nucleus remained small.

Post-natal day 21

By P21, the total number of labelled cell bodies (1458.0 ± 458.1) had almost doubled from the P14 values and labelled neurons were now more consistently present in the smaller nuclei such as the nucleus ambiguus (24.3 ± 13.0), spinal trigeminal nucleus (8.8 ± 1.5) and medial vestibular nucleus (15.8 ± 3.1; see Figure 3.1.8). The interstitial nucleus of the medial longitudinal fasciculus (4.3 ± 2.5) showed the most rostral originating projections, although the labelling was inconsistent as only two of the 4 animals showed small numbers of labelled neurons. The red nucleus (51.5 ± 16.9) was consistently labelled in all animals in this age group.

Figure 3.1.7: Distribution of labelled neurons in the brain stem after lumbar
Fluororuby injection in control P14 *Monodelphis*

Camera lucida representation of coronal brain sections from control P14 + 4 brains showing the location and number of retrogradely labelled neurons after an injection of Fluororuby into the lumbar spinal cord at P14. Sections shown are from the caudal brain stem (a) to the hypothalamus (g). One star represents up to 10 labelled neurons.

P14 control *Monodelphis*

1mm

Figure 3.1.8: Distribution of labelled neurons in the brain stem after lumbar Fluororuby injection in control P21 *Monodelphis*

Camera lucida representation of coronal brain sections from control P21 + 4 brains showing the location and number of retrogradely labelled neurons after an injection of Fluororuby into the lumbar spinal cord at P21. Sections shown are from the caudal brain stem (a) to the hypothalamus (g). One star represents up to 10 labelled neurons.

P21 control *Monodelphis*

a RD, RV, Am, Cb

b TrSc, RD, RV

c VstL, VstM, RGc, TrSc, RaO, RGcv

d Coe, Cb, VstL, RP, RaM, RGc

e CS, GCv, aq, Coe, RP

f lflm, dk, Rb

g Hyl, Phd

1mm

The gigantocellular reticular nucleus (380.0 ± 58.2) made the largest contribution to the profile of labelled neurons at this age (27.9 ± 2.3%) and the proportions in other nuclei remained similar to that seen at P14 (compare with Figure 3.1.7). The contribution from the raphe nuclei appeared to decline from 15.3 ± 3.2% at P14 to 9.5 ± 0.9% at P21 (see Figure 3.1.5) but the difference was not significantly different.

Post-natal day 28

At P28 (see Figure 3.1.9) the total number of labelled neurons had increase to 1503.3 ± 228.8 and small numbers of labelled neurons were detected at further rostral locations in the brain than seen previously, in regions of the Edinger-Westphal nucleus and nucleus Darkschewitsch (16.0 ± 3.7), ventral part of central gray (14.0 ± 2.5), paraventricular hypothalamic nucleus, dorsal hypothalamic area and lateral hypothalamic nucleus (6.5 ± 3.8). The contributions from these nuclei to the total numbers of labelled neurons remained small but were similar in all 4 brains examined. Two of the 4 animals did not have labelled neurons in the paraventricular hypothalamic nucleus, dorsal hypothalamic area and lateral hypothalamic nucleus. However the presence of labelled neurons in these nuclei in two brains altered the proportional contribution by the reticular pontine nucleus (15.9 ± 1.0%) and gigantocellular reticular nucleus (21.7 ± 1.4%), although the gigantocellular reticular nucleus remained the highest proportion among the total labelled neurons (see Table 3.1.5). The lateral vestibular nucleus (224.3 ± 26.3) showed large numbers of labelled neurons at P28, particularly in the region of the nucleus that extended into the cerebellum in the more rostral regions of the brain. A small number of neurons was also found in the medial vestibular nucleus (26.8 ± 2.5). Faintly labelled neuronal cell bodies in the red nucleus (112.0 ± 23.8) appeared small and round and were tightly clustered in the now clearly delineated nucleus. Labelled neurons in the intermediate medullary reticular field were also counted as part of the dorsal medullary reticular nucleus as it was difficult to precisely determine where the nuclear boundaries lay. There were numerous labelled neurons in the intermediate medullary nucleus, which showed a decline in numbers by P28 although these neurons were included in the dorsal medullary reticular nucleus counts.

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Figure 3.1.9: Distribution of labelled neurons in the brain stem after lumbar Fluororuby injection in control P28 *Monodelphis*

Camera lucida representation of coronal brain sections from control P28 +4 brains showing the location and number of retrogradely labelled neurons after an injection of Fluororuby into the lumbar spinal cord at P28. Sections shown are from the caudal brain stem (a) to the hypothalamus (g). One star represents up to 10 labelled neurons.
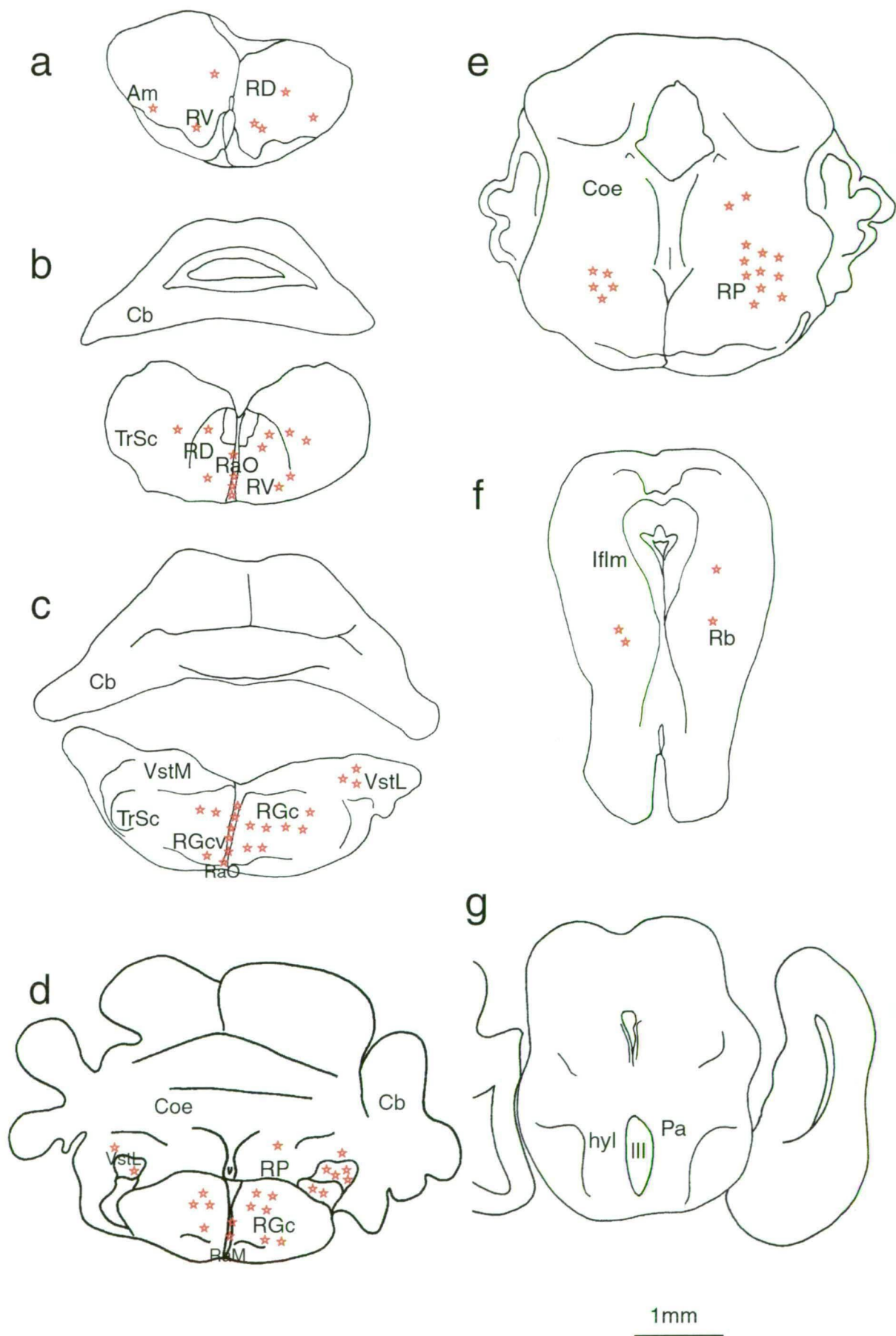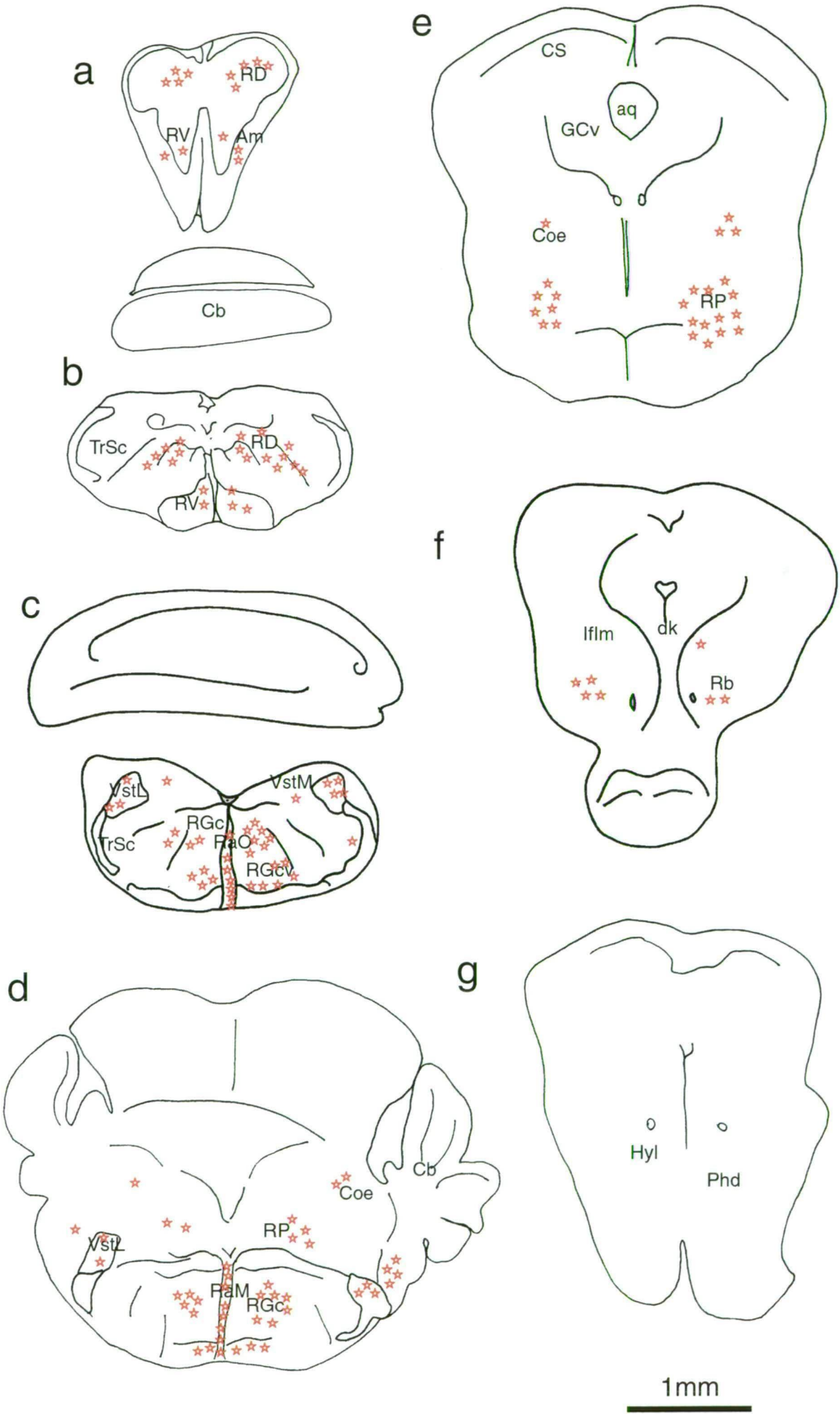
P28 control *Monodelphis*

a
RD
RV
Am

b
Cb
TrSc
RD
RV

c
Cb
VstL
VstM
RGc
TrSc
RGcv
RaO

d
Cb
IV
VstM
VstL
RGc
RGcv
RaM

e
Cl
aq
Coe
Cb
RP

f
CS
GCv
Dk
IFLm
ED
Rb

g
PHD
Hyl

1mm
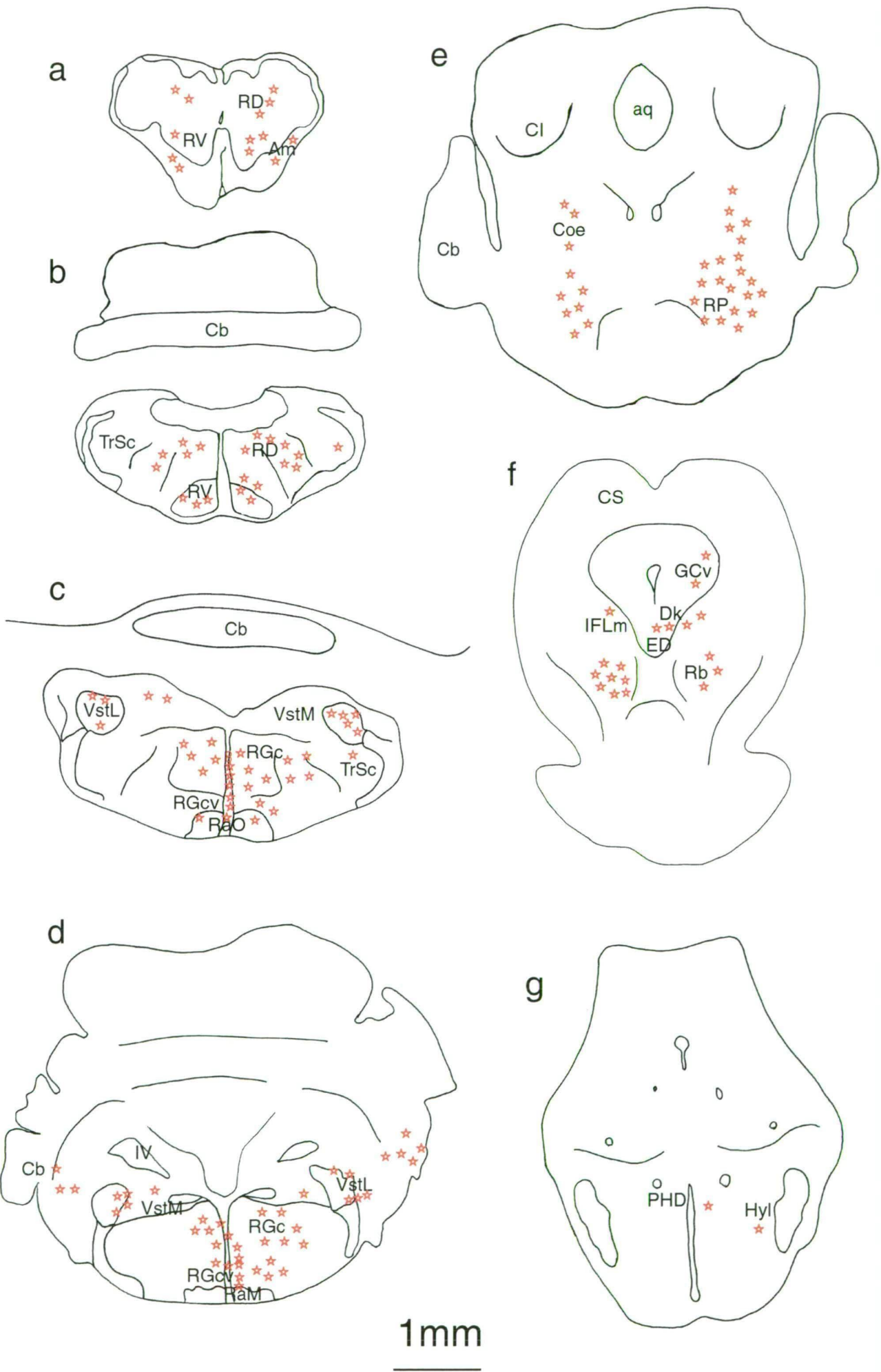
Post-natal day 35

At P35 (see Figure 3.1.10) the total number of labelled neurons (1645.3 ± 226.1) was the highest observed at any age and the proportions contributed by individual nuclei were more diverse than recorded at younger ages. Contributions from the lateral vestibular nucleus (14.8 ± 0.2%), gigantocellular reticular nucleus (17.8 ± 1.0%) and reticular pontine nucleus (14.5 ± 0.6%) still showed the majority of labelled neurons (see Table 3.1.5). The gigantocellular reticular nucleus and reticular pontine nucleus contributions were, however, proportionally less than in previous ages.

Adult

The total number of labelled neurons in the adult brain was significantly less (688.7 ± 207.6) than at P21, P28 and P35 (see Figure 3.1.11). Nuclear delineations were more clearly defined in the adult animal when viewed under the light microscope. Small numbers of labelled cell bodies were detected in several nuclei that were not seen at younger ages. These included the medial and parabranchial nuclei (3.5 ± 2.1) in the midbrain, the deep mesencephalic nucleus (2.7 ± 3.6), medial longitudinal fasciculus (6.4 ± 3.8), and superior colliculus (2.4 ± 2.2) but these were not consistently labelled and were not detected in 2 out of 6 brains.

The most rostrally labelled neurons in the adult brain were found in the paraventricular hypothalamic nucleus, dorsal hypothalamic area, and lateral hypothalamic nucleus. The counts for these hypothalamic regions (9.0 ± 2.7) were grouped together although the lateral hypothalamic nucleus was less consistently labelled and had very small numbers of labelled neurons in only 3 of the 6 brains examined.

Immediately caudal to these nuclei in the midbrain, the red nucleus (40.2 ± 12.2) showed the most numerous labelled neurons, although its contribution still remained proportionally less than that of the caudal reticular and vestibular nuclei. The Edinger-Westphal nucleus and nucleus Darkschewitsch (5.8 ± 2.5), ventral part of central gray (15.3 ± 6.0) and the interstitial nucleus of the medial longitudinal fasciculus (3.2 ± 1.0) showed small numbers of labelled neurons, while the reticular pontine nucleus presented numerous labelled neurons (71.2 ± 20.2). The spinal

Figure 3.1.10: Distribution of labelled neurons in the brain stem after lumbar Fluororuby injection in control P35 *Monodelphis*

Camera lucida representation of coronal brain sections from a control P35 + 4 brains showing the location and number of retrogradely labelled neurons after an injection of Fluororuby into the lumbar spinal cord at P35. Sections shown are from the caudal brain stem (a) to the hypothalamus (g). One star represents up to 10 labelled neurons.

P35 control *Monodelphis*

a

RD

RV

Am

b

TrSc

RD

RV

c

VstL

VstM

TrSc

RGc

RaO

RGcv

d

VstL

VstM

RGc

RaM

RGcv

e

GCv

aq

EW

Coe

Cb

RP

f

lFLM

dk

Rb

g

PHD

Hyl

1mm
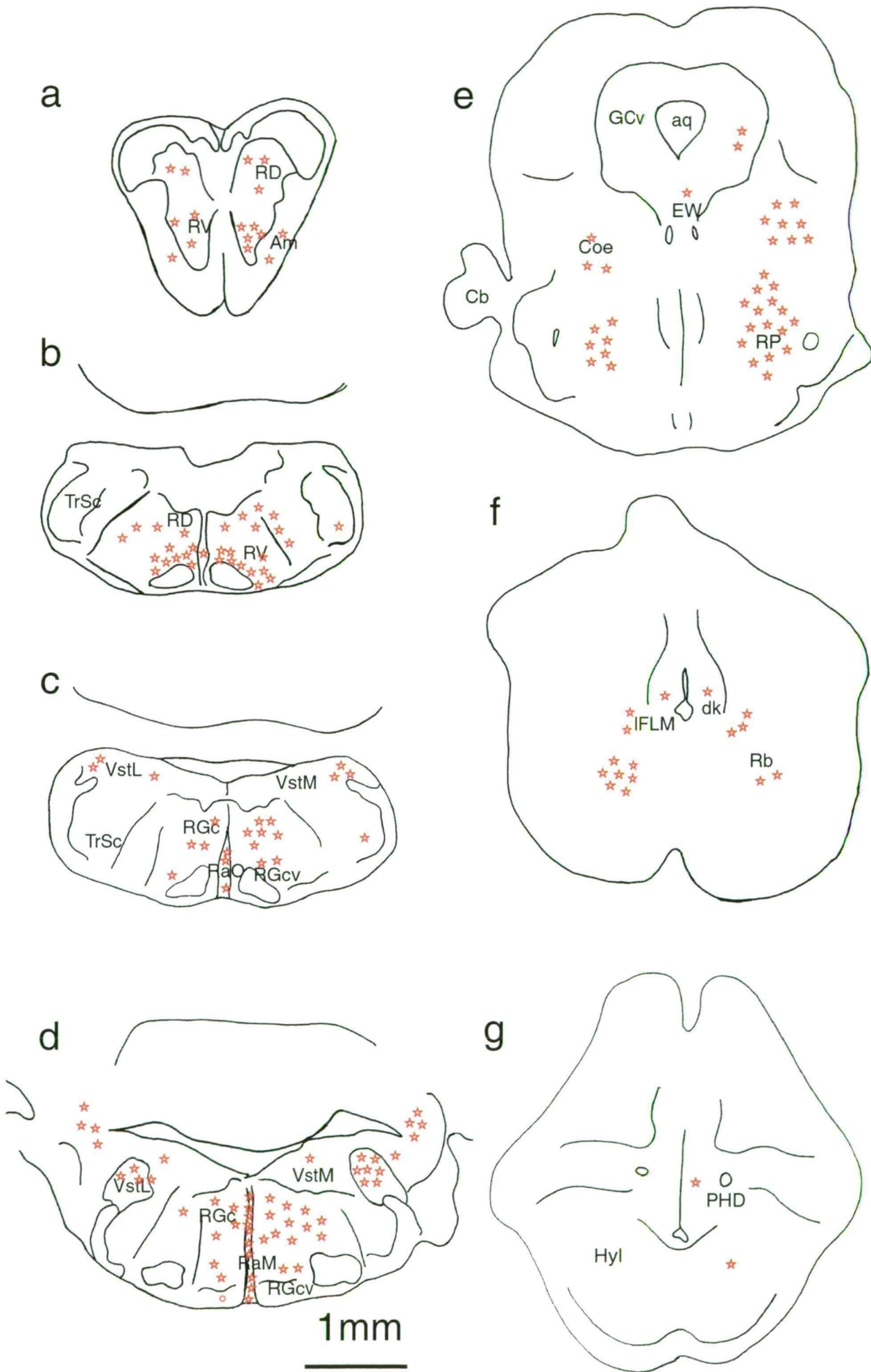
Figure 3.1.11: Distribution of labelled neurons in the brain stem after lumbar Fluororuby injection in control adult *Monodelphis*

Camera lucida representation of coronal brain sections from control adult brains showing the location and number of retrogradely labelled neurons after an injection of Fluororuby into the lumbar spinal cord one week earlier. Sections shown are from the caudal brain stem (a) to the hypothalamus (i). One star represents up to 10 labelled neurons.

Adult control *Monodelphis*

a
TrSe Gr
RD RV
Am

b
Gr
AI
TrSd RD
RV

c
VstM
TrSi RGc
RaO
RGcv

d
VstL VstM
TrSi RGc
OS RGcv
RaM

e
VstS VstL
TrSi RP
RGcv

f
GCv
IFLm
Coe
RP

g
CS
IFLm DK
Rb

h
CS
GCv
IFLm EW

i
PHD
Hyl

1mm
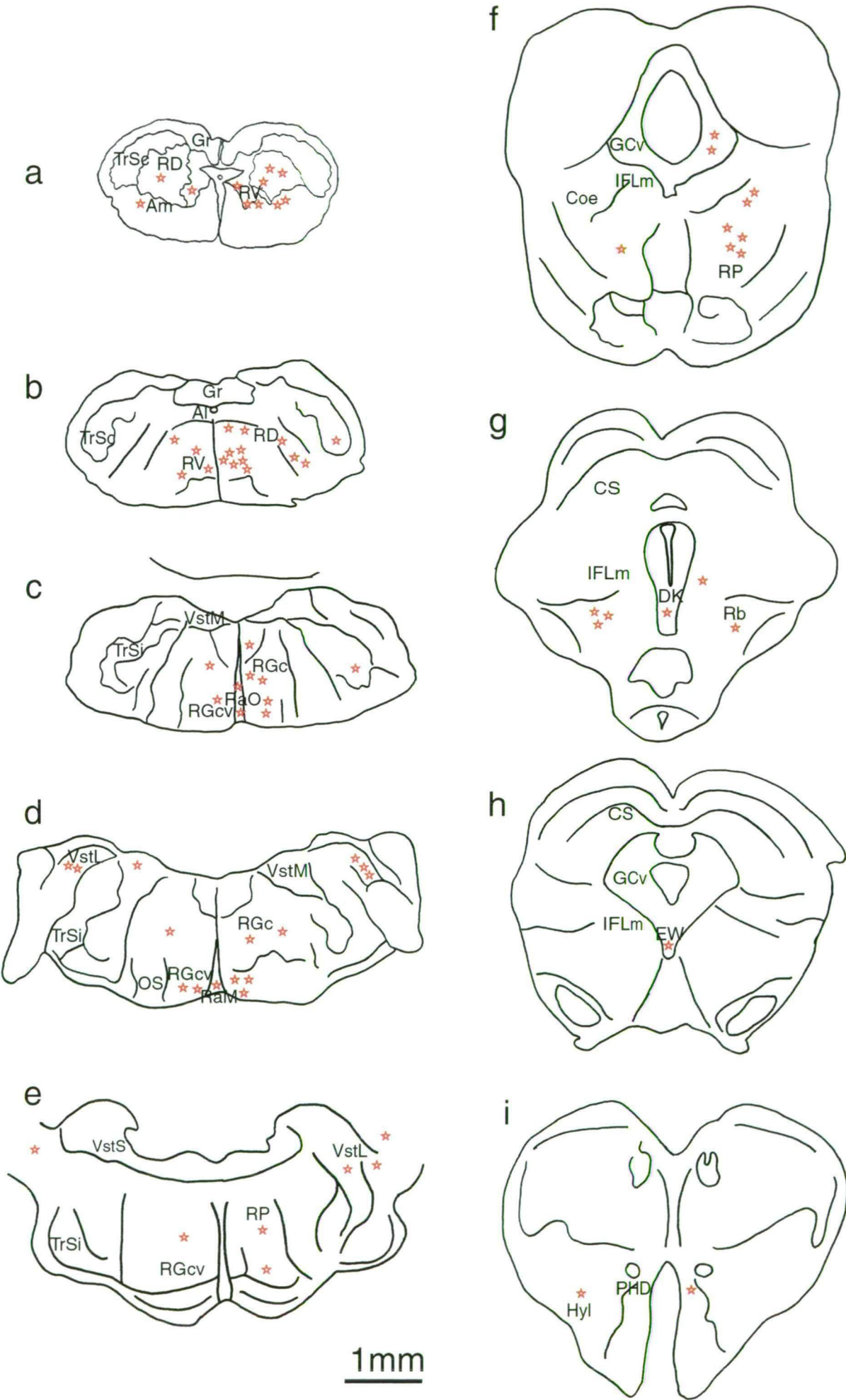
trigeminal tract (3.5 ± 1.6), medial and superior regions of the vestibular nucleus (18.2 ± 4.6) contributed only small numbers but this comprised a greater proportional contribution to the total than these nuclei made in the developing animals (see Figure 3.1.5 and Table 3.1.5).

The gigantocellular reticular nucleus (18.6 ± 2.8%) was still the highest contributor to the profile in adults and most labelled neurons appeared in the more central regions of this large nucleus through its rostocaudal axis. Giant cell bodies tended to be located in the central regions while more numerous large neurons were mainly clustered medially, in close proximity to the raphe obscurus nucleus and raphe magnus nucleus. In caudal regions of the gigantocellular reticular nucleus, a few labelled neurons were also detected in very dorsal positions close to the fibres of the hypoglossal nerve. The raphe nuclei showed a decline in the overall contribution over development and there was a further decline in the adult profile (4.8 ± 1.4%).

Towards the lateral aspects of the caudal midbrain, large numbers of labelled neurons were found in the lateral vestibular nucleus (109.7 ± 30.4) including the regions extending into the cerebellum which contained many labelled neurons (see Figure 3.1.1a). The proportion of the total number of labelled neurons made by the lateral vestibular nucleus increased significantly from P7 to P35 and this trend was also reflected by the large contribution made by these nuclei in adults (see Figure 3.1.5). The medial and superior vestibular regions showed much smaller numbers of labelled neurons with projections to the lumbar spinal cord (18.2 ± 4.6).

In the caudal medulla region, a few labelled neurons were seen in the interpolar spinal trigeminal nucleus and in the caudal spinal trigeminal nucleus (3.5 ± 1.6), while the nucleus ambiguus showed more (21.8 ± 4.5). Labelled neurons in this region were concentrated in the ventral medullary reticular nucleus (113.5 ± 38.1; 16.8 ± 5.2%) and dorsal medullary reticular nucleus (105.2 ± 29.1; 14.2 ± 2.0%) spanning a large extent of the medulla.

3.1.4 Laterality of projections

The lumbar spinal injections used in the present study were confirmed to be unilateral in most cases and this was reflected by the lateral asymmetry of the labelled neurons counted in the brain (see Table 3.1.6). All nuclei of the brain, in which labelled neurons were found, are represented bilaterally, but the labelling was predominantly found on the side ipsilateral to the injection. At P7, 32% of labelled neurons were on the contralateral side and at P35, 35% were contralateral.

Adult animals showed an even more pronounced ipsilateral labelling pattern following the unilateral lumbar injection with only 24% found on the contralateral side. It was also noted that the injected dye remained much more localised to the injection site in adult animals and did not appear to diffuse across to the contralateral side of the spinal cord after the injection. Labelled neurons in the reticular pontine nucleus and locus coeruleus remained mostly ipsilateral through all the ages examined while most other reticular neurons were more bilaterally presented. The red nucleus and medial vestibular nucleus both showed a contralateral predominance but were also bilaterally represented (see Figures 3.1.6 to 3.1.11).

Table 3.1.6: The number of labelled neurons counted on the ipsilateral and contralateral side of the brain after an injection of Fluororuby in control animals aged P7 to adult.

Age of injection	Total number of labelled cell (mean ± SEM)	Ipsilateral neurons (mean ± SEM)	Contralateral neurons (mean ± SEM)
P7	379.3 ± 114.4	226.4 ± 61.3	121.3 ± 34.2
P14	758.3 ± 228.6	522.7 ± 121.7	252.5 ± 70.4
P21	1186. 0 ± 357.6	817.4 ± 222.8	491.7 ± 154.7
P28	1503.3 ± 453.2	1022.6 ± 298.6	501.6 ± 143.9
P35	1645.3 ± 496.1	1111.7 ± 220.6	571.6 ± 165.6
Adult	688.7 ± 207.6	484.2 ± 97.3	168.4 ± 52.6

Note that approximately 70% of the total number of labelled neurons counted in brains were located ipsilateral and 30% contralateral to the side of injection.

3.2 RESTORATION OF SUPRASPINAL PROJECTIONS TO THE LUMBAR CORD FOLLOWING A SPINAL TRANSECTION IN NEONATAL *MONODELPHIS*

The restoration of axonal projections to the lumbar spinal cord in animals that had a complete spinal transection at P7 (see Methods) was investigated using injections of Fluororuby at different post-natal ages (see Table 3.2.1 for n). The study aimed to determine which populations of axons grow across the injury site, and whether injured projections that regrew were maintained to adulthood. Additionally the sequence of fibre outgrowth following injury was compared with the normal pattern of development as described for control animals (see Section 3.1).

It was observed from whole mount dissections of the spinal cord and subsequent histological examination that a structural bridge formed across the severed ends within days of the complete transection at P7. By P14, the tissue bridge showed fibres traversing the injury site and these were immunopositive for an antibody against the neuronal cytoskeletal protein, neurofilament, present in nerve fibres (see Figure 3.2.1a and Appendix B3).

By P21 and older, a substantial tissue bridge was observed across the lesion site in most spinally transected animals (see Figure 3.2.1b). It appeared that most transected animals showed some form of structural deficit (Figure 3.2.1c). There was a degree of variation in the amount of tissue in the bridge that grew across the injury, although it must be noted that a connection was always detected across the severed ends. Two examples of the variation of structural repair are shown in Figure 3.2.1b and Figure 3.2.1c of whole mount spinal cords, transected at P7. One cord (Figure 3.2.1b) has a substantial tissue reconnection across the lesion site at P35, while the other shows a thin thread like tissue bridge in adulthood (see Figure 3.2.1c).

Table 3.2.1: Number of spinal transected animals used for Fluororuby axonal tracing studies.

Day of injection	P7	P14	P21	P28	P35	Adult
n	3	3	6	8	11	8

Figure 3.2.1: Morphological repair was observed across the injury site within 7 days of complete spinal transection at P7 in *Monodelphis*

a) A longitudinal section through the injury site of P21 spinally transected animal stained with anti-neurofilament (SMI-312) shows that a bridge has formed across the injury site and is filled with fibres. Scale bar = 100μm.

b) A substantial tissue bridge across the thoracic transection site was established by P35 in most spinal cords, indicating that a large degree of repair occurs within the first few weeks post-injury. The lesion deficit was usually visible. Scale bar is 1mm.

c) The tissue bridge across the lesion site was variable and occasionally in adulthood was not substantial. Scale bar as for b).
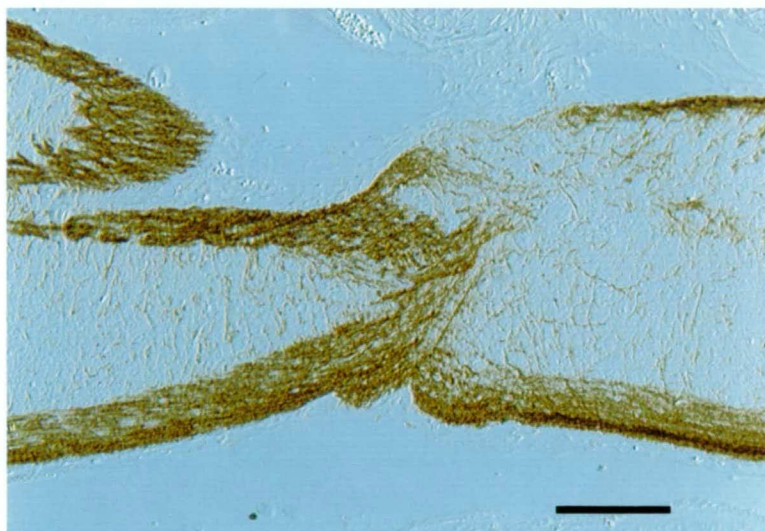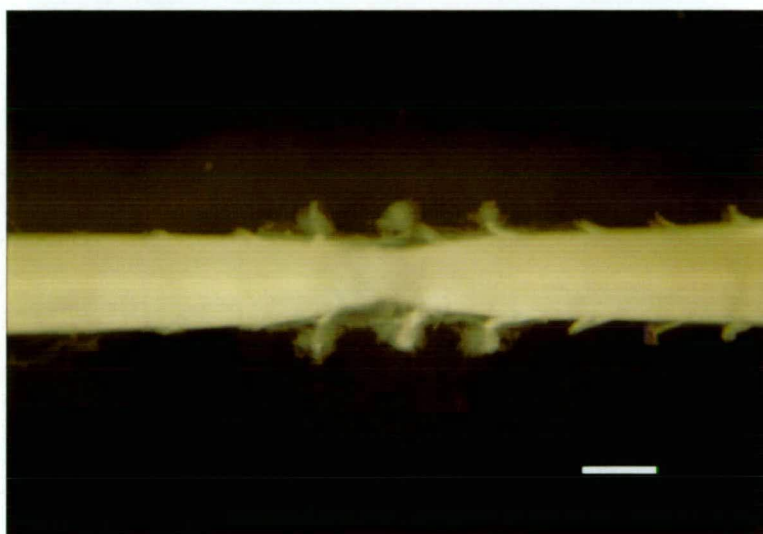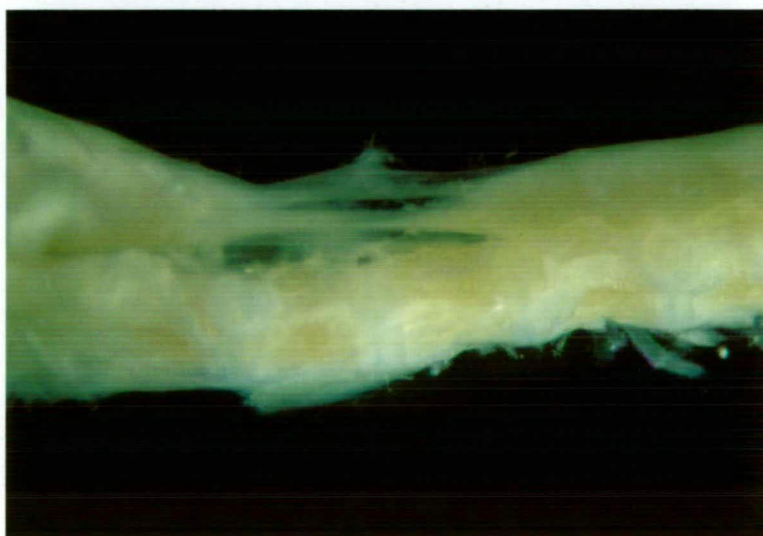
No significant differences were found between the weight of spinally transected and control animals or the cross sectional areas of the spinal cords at the lumbar level. Therefore identical amounts of dye were injected at each age in both control and spinally transected groups (see Table 3.1.2). The injection sites were later examined in all animals and found to be of similar appearance to the age-matched control injection sites as represented in Figure 3.1.3. In 9 animals the injection had not been made in the appropriate location and accordingly these animals were removed from the study.

Labelled cell bodies in the brain nuclei of spinally transected animals were generally of identical appearance to those detected in the brains of control animals with brightly fluorescent granules accumulating in the cell soma and dendritic processes visible under x10 magnification in the plane of section (see Figure 3.2.2).

3.2.2 Total number of labelled neurons in spinally transected animals.

At each age investigated (injected at P7, P14, P21, P28, P35 and adult) the mean numbers of total labelled neurons counted in brains were less than those counted in age-matched control brains (compare Table 3.1.3 with Table 3.2.2). No labelled neurons were seen in the brains of spinally transected P7 animals that had received a Fluororuby injection within 10 minutes of the lesion. Small numbers of retrogradely labelled neurons (65.3 ± 16.9) were found in the brains of P14 animals injected one week after the lesion and this was significantly less than in the control P14 brains (758.3 ± 228.6, P = 0.03). The mean value for P21 spinally transected animals was 353.2 ± 176.1, a value nearly 5 times higher than that for P14. It was, however, significantly lower than the P21 control mean of 1186.0 ± 357.6 (P = 0.03) and also significantly lower than the P35 spinally transected value of 1234.1 ± 203.1 (P = 0.01). By comparison, the P21 and P35 values in control animals were not statistically different from each other (P = 0.73, see Table 3.1.3). The total number of labelled neurons in P28 spinally transected brains was higher than earlier ages at 1054.1 ± 336.5 and was not statistically different from its age-matched control value of 1503.3 ± 453.2 (P = 0.40), nor from the P35 spinally transected value (P = 0.63).

Figure 3.2.2: Retrogradely labelled neuronal cell bodies in spinally transected animals following lumbar spinal injection of dextran amine rhodamine (Fluororuby) at age P35

a) Numerous labelled neurons in the gigantocellular reticular nucleus in the brain stem of a spinally transected P35 animal included large multipolar cells, and smaller rounder neurons. Scale bar = 100 µm.

b) Labelled cell bodies were found in the lateral vestibular nucleus, including the dorsal region which extends into the cerebellum, although the cells were never as heavily clustered as in the pons region of the nuclei. Scale bar = 100µm.

c) Tightly clustered labelled cell bodies in the red nucleus in the midbrain region of a spinally transected animal. The neurons all have a similar small and round appearance with few dendritic processes. Scale bar = 100µm.

a
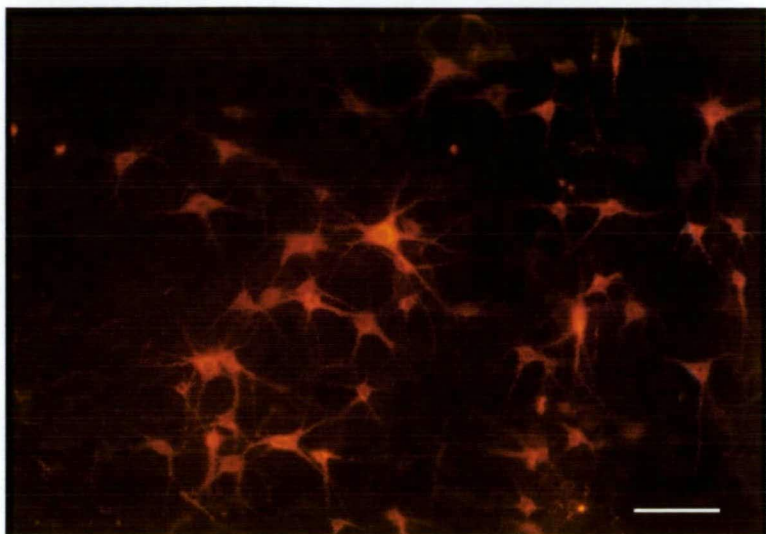
b
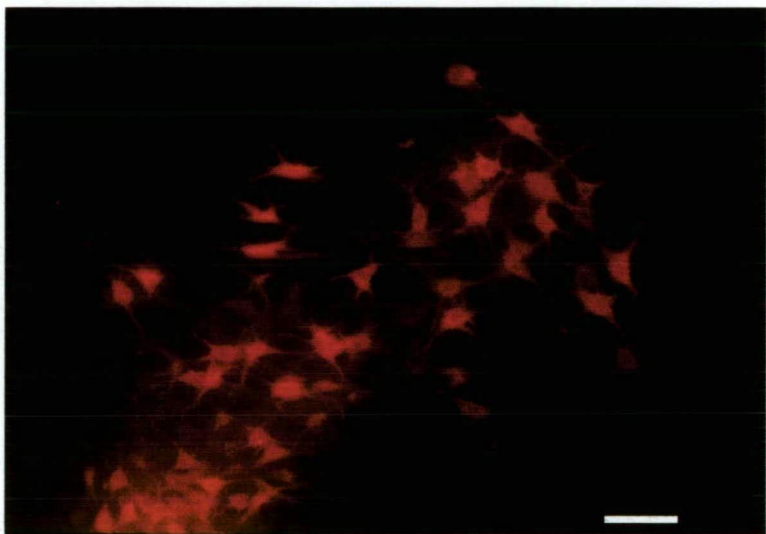
c
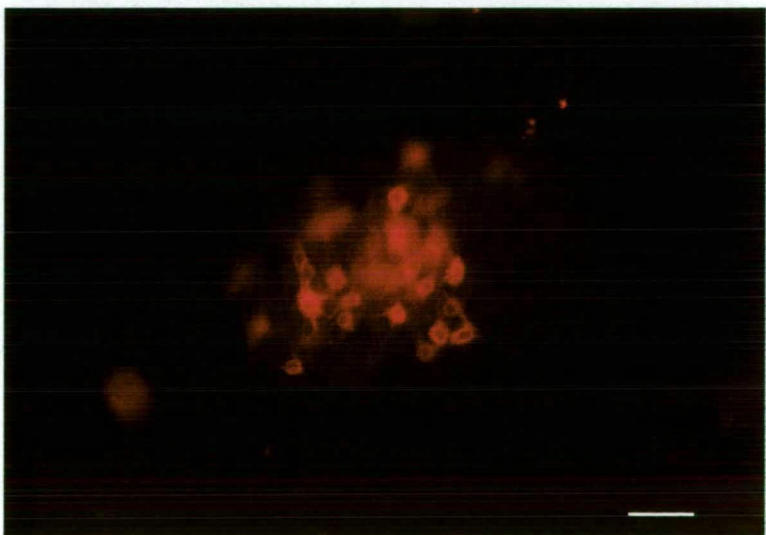
Table 3.2.2: Total number of Fluororuby labelled neurons after a lumbar injection in spinally transected animals

Age of injection	n	Mean ± SEM	P t-test against P35 value	P t-test against age-matched control value
P7	3	0.0 ± 0.0	-	-
P14	3	65.3 ± 16.9	0.01*	0.03*
P21	6	353.2 ± 176.1	0.01*	0.03*
P28	8	1054.1 ± 336.5	0.63	0.40
P35	11	1234.1 ± 203.1	-	0.28
Adult	8	186.8 ± 75.6	<0.01*	<0.01*

The mean ± SEM was calculated by counting all labelled neurons in brains cut at 100μm coronal sections. The t-tests was made using Sigma Stat ®. The P35 age group had the highest mean value and was chosen as the comparator to show whether other ages were different. The P7 values were nor included in the t-tests as the mean was zero.

*The P value of statistical significance was defined as $P \leq 0.05$.

The highest total number of labelled neurons in the brains of spinally transected animals was observed at P35 with 1234.1 ± 203.1 cells. This age also showed the highest value in control animals with 1645.3 ± 496.1 labelled neurons. There was no significant difference between the control and spinally transected mean values at P35 (P = 0.28).

There was a significant decline in total numbers from 1234.1 ± 203.1 at P35 to 186.8 ± 75.6 in adult animals (P < 0.01) as also observed between control P35 and control adult values (P < 0.01). The total numbers in the transected adult animals (186.8 ± 75.6) were significantly lower than the control adult values (688.7 ± 207.6, P < 0.01).

It would appear that using this technique of retrogradely labelling neurons, a plateau in total number of labelled cell bodies was reached at P21 in control animals and was maintained through the neonatal ages examined up to P35, after which numbers dropped in the adult. In spinally transected animals the plateau of labelled neurons was not established until a week later at P28 and a similar decline was also observed in adults (compare Figure 3.2.3 and Figure 3.1.4).

3.2.3 Distribution of labelled neurons in different nuclei after spinal transection

The number and distribution of all labelled neurons in different nuclei was recorded while total brain counts were being made and this was used to identify particular neuronal populations. The total numbers of labelled neurons in different nuclei at all ages were consistently lower in the spinally transected animals than for the same nuclei in age-matched controls (compare data in Table 3.1.4 and Table 3.2.3), although at P35 and P28 the difference was not significant. However, it was noticable that the nuclei that showed labelled neurons in control animals also showed neurons in some brains of transected animals. Some nuclei showed very small numbers after spinal transection and were not observed until one to two weeks later than the age at which they were first detected in control animals (see Table 3.2.4).

To enable direct comparisons of brain projections growing through the lesion site to the lumbar spinal cord both over the developmental period and against numbers from control animals, the distribution of labelled neurons in different brain nuclei was

Figure 3.2.3: The number of Fluororuby labelled neurons in spinally
transected animals

Scatterplot of the total number of Fluororuby labelled neurons counted in the brains of spinally transected animals aged P7 to adult. The open circles indicate the value for individual animals and the closed red circles indicate the mean values for each age. The P35 mean was found to be significantly different ($P \leq 0.05$) from the P14, P21 and adult mean values.

Number of Fluororuby labelled neurons in spinally transected animals
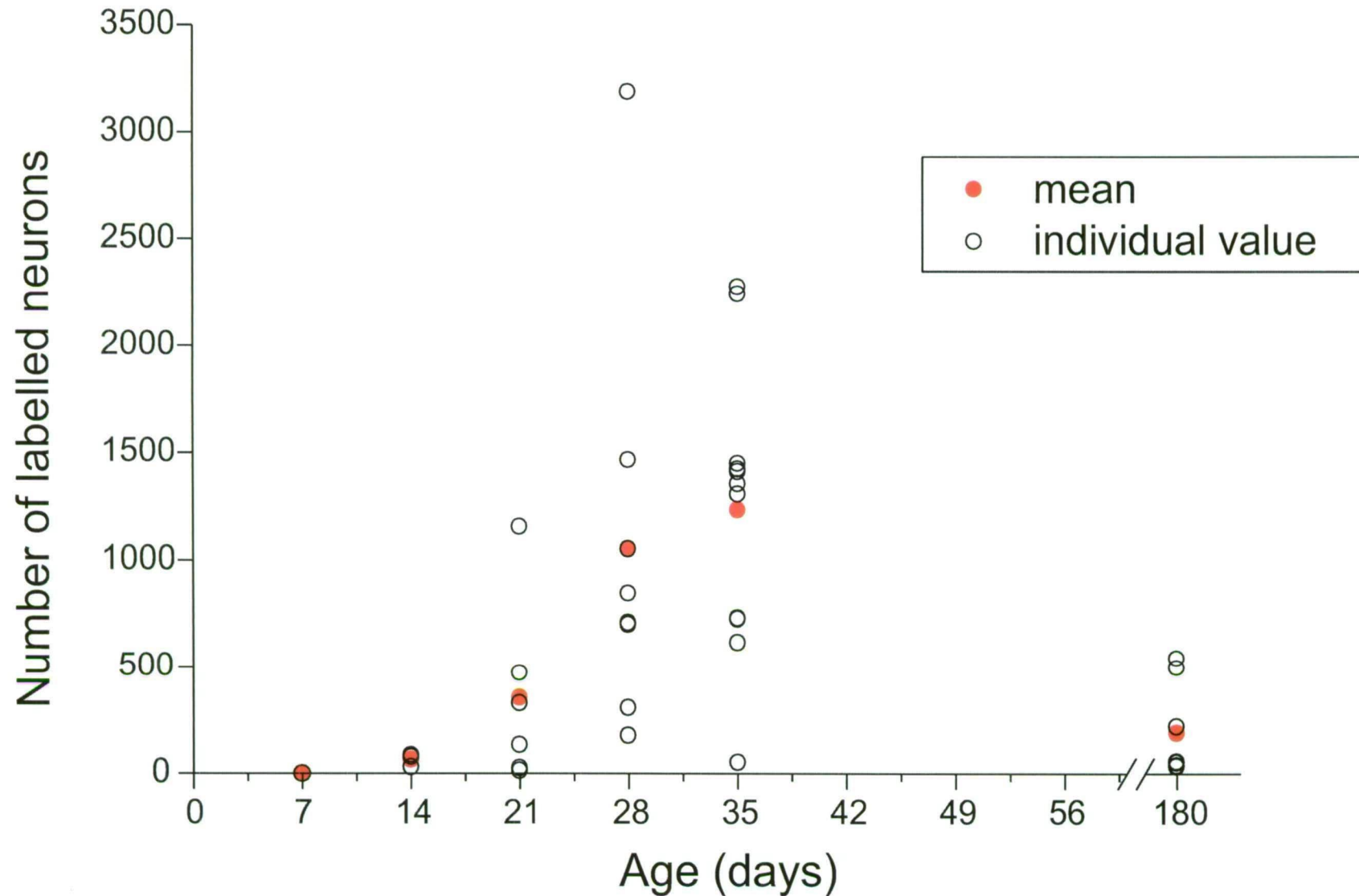
Table 3.2.3: The number (mean ± standard error) of Fluororuby labelled neurons counted in each nuclei in spinally transected animals from P7 to adult.

Nuclei	P7	P14	P21	P28	P35	Adult
n	3	3	4	4	4	6
Ventral medullary reticular nucleus	0.0 ± 0.0	3.3 ± 0.5	31.0 ± 24.5	103.5 ± 29.1	56.8 ± 12.4	29.8 ± 8.5
Dorsal medullary reticular nucleus	0.0 ± 0.0	3.7 ± 0.7	21.7 ± 12.9	156.8 ± 52.9	168.6 ± 27.1	23.8 ± 7.1
Raphe nuclei	0.0 ± 0.0	9.0 ± 1.7	12.5 ± 28.9	63.5 ± 70.5	56.5 ± 41.4	15.7 ± 7.0
Gigantocellular reticular nucleus	0.0 ± 0.0	17.3 ± 2.1	102.0 ± 45.3	328.8 ± 94.2	414.8 ± 77.0	57.3 ± 11.2
Lateral vestibular nucleus	0.0 ± 0.0	13.0 ± 1.1	62.0 ± 27.8	177.1 ± 54.8	129.5 ± 36.9	19.2 ± 6.4
Reticular pontine nucleus	0.0 ± 0.0	19.0 ± 2.4	77.0 ± 32.2	127.5 ± 50.2	144.4 ± 25.8	20.8 ± 7.4
Locus coeruleus	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	31.0 ± 17.3	57.5 ± 17.6	3.7 ± 1.7
Red nucleus	0.0 ± 0.0	0.0 ± 0.0	32.3 ± 25.8	41.6 ± 17.8	106.9 ± 26.8	24.3 ± 7.3
Other Nuclei	0.0 ± 0.0	0.0 ± 0.0	14.7 ± 1.6	24.4 ± 1.4	101.4 ± 7.2	38.2 ± 1.9

Other nuclei: nucleus ambiguus, caudal spinal trigeminal nucleus, medial vestibular nucleus, interstitial nucleus of the medial longitudinal fasciculus, ventral part of central gray, paraventricular hypothalamic nucleus and dorsal hypothalamic area, Edinger-Westphal nucleus, and the nucleus Darkschewitsch.

Table 3.2.4: The age at which Fluororuby labelled neurons first appear in different nuclei in control and transected animals

Nuclei	Control	Transected
Nucleus ambiguus	P14	P21
Ventral medullary reticular nucleus	P7	P14
Dorsal medullary reticular nucleus	P7	P14
Caudal spinal trigeminal nucleus	P7	P21
Raphe obscurus nucleus	P7	P14
Ventral gigantocellular reticular nucleus	P7	P14
Gigantocellular reticular nucleus	P7	P14
Lateral vestibular nucleus	P7	P14
Medial vestibular nucleus	P7	P21
Raphe magnus nucleus	P7	P14
Reticular pontine nucleus	P7	P14
Locus coeruleus	P7	P28
Red nucleus	P7	P21
Interstitial nucleus of the medial longitudinal fasciculus	P14	P28
Edinger-Westphal nucleus and nucleus Darkschewitsch	P28	P35
Ventral part of central gray	P28	P35
Paraventricular hypothalamic nucleus and dorsal hypothalamic area	P28	P35

standardised. This was achieved by expressing the number of labelled neurons in different brain nuclei as a proportion of the total number of labelled neurons in each brain. These data are presented in Table 3.2.5 and represented as a histogram in Figure 3.2.4.

In spinally transected animals, the majority of labelled neurons were found in only a few nuclei, while in control animals labelled neurons were more widely distributed through different regions of the brain stem (compare Table 3.1.5 with Table 3.2.5). At P14 the highest proportion of labelled neurons was found in the reticular pontine nucleus (30.1 ± 2.4%), whereas at all other ages the gigantocellular reticular nucleus contained the greatest proportion of labelled neurons (over 25% of the total number of labelled neurons in brains at P21, P28 and P35, see Figure 3.2.4).

At P14 the gigantocellular reticular nucleus, lateral vestibular nucleus and reticular pontine nucleus dominated the projection profile contributing 26.4 ± 5.4%, 21.6 ± 3.3% and 30.1 ± 2.4% respectively to the total labelled neurons. The dominance of the gigantocellular reticular nucleus remained high over the age range examined with no statistically significant change in its proportion from P14 to P35 and adult (P = 0.19 and P= 0.76 respectively; see Table 3.2.5 and Appendix C2).

By comparison, the lateral vestibular nucleus, which accounted for 21.6 ± 3.3% of projections at P14, declined significantly to 10.3 ± 2.5% at P35 (P=0.05) and 5.6 ± 1.2% in adults (P < 0.01). The reticular pontine nucleus showed a similar age-related decrease in numbers contributing 30.1 ± 2.4% of projections at P14, compared to 11.5 ± 1.6% at P35 (P < 0.01) and 6.6 ± 1.6% in adults (P< 0.01, see Table 3.2.5 and Appendix C2).

No labelled neurons were detected in the locus coeruleus, red nucleus or any of the nuclei grouped as "other nuclei", including the nucleus ambiguus, caudal spinal trigeminal nucleus, medial vestibular nucleus, interstitial nucleus of the medial longitudinal fasciculus and the Edinger-Westphal nucleus. As labelled neurons were detected in these nuclei at older ages, their proportional contribution correspondingly increased from P14 to adulthood. This resulted in a more diverse profile of projections in older animals (P35 and adult) compared to the younger animals (P14,

Table 3.2.5: The proportion (% mean ± standard error) of the total Fluororuby labelled neurons in different brain nuclei in spinally transected animals from P7 to adult.

Nuclei	P7		P14		P21		P28		P35		Adult	
n	3		3		4		4		4		6	
Ventral medullary reticular nucleus	0.0	± 0.0	5.3	± 0.7	5.4	± 1.8	12.9	± 3.0	6.1	± 1.8	11.2	± 2.0
Dorsal medullary reticular nucleus	0.0	± 0.0	5.7	± 0.9	7.8	± 1.7	14.9	± 2.9	14.3	± 1.8	11.5	± 1.3
Raphe	0.0	± 0.0	10.9	± 3.4	8.5	± 1.7	6.2	± 1.1	5.7	± 0.8	8.4	± 1.1
Gigantocellular reticular nucleus	0.0	± 0.0	26.4	± 5.4	26.7	± 1.6	26.0	± 2.8	31.5	± 2.4	27.4	± 1.3
Lateral vestibular nucleus	0.0	± 0.0	21.6	± 3.3	17.9	± 2.7	15.9	± 3.9	10.3	± 2.5	5.6	± 1.2
Reticular pontine nucleus	0.0	± 0.0	30.1	± 2.4	23.5	± 2.8	12.0	± 2.8	11.5	± 1.6	6.6	± 1.6
Locus coeruleus	0.0	± 0.0	0.0	± 0.0	0.0	± 0.0	2.0	± 0.8	3.7	± 1.0	2.1	± 0.9
Red nucleus	0.0	± 0.0	0.0	± 0.0	6.2	± 2.0	6.9	± 4.0	9.8	± 2.1	12.7	± 2.6
Other Nuclei	0.0	± 0.0	0.0	± 0.0	3.9	± 0.4	3.2	± 0.2	7.3	± 0.5	14.4	± 0.9

Other nuclei = nucleus ambiguus, caudal spinal trigeminal nucleus, medial vestibular nucleus, interstitial nucleus of the medial longitudinal fasciculus, ventral part of central gray, paraventricular hypothalamic nucleus and dorsal hypothalamic area, Edinger-Westphal nucleus, and the nucleus Darkschewitsch.

Figure 3.2.4: Proportional contributions from different nuclei to the total profile of Fluororuby labelled neurons in the brain following lumbar spinal injection in spinally transected animals aged P7 to adult.

* Denotes that t-test made with Sigma Stat comparing the mean of P14 proportions to that of older ages showed significant differences (P≤ 0.05).

Abbreviations

RV	Ventral medullary reticular nucleus
RD	Dorsal medullary reticular nucleus
Raphe	Raphe nuclei
RGc	Gigantocellular reticular nucleus
VstL	Lateral vestibular nucleus
RP	Reticular pontine nucleus
Coe	Locus coeruleus
Rb	Red nucleus
others	Other nuclei

Proportional distribution of Fluororuby labelled neurons in the brain stem of spinally transected animals

P21 and P28) where neurons were largely confined to three main nuclei, the reticular formation, raphe nuclei and vestibular nuclei (see Figure 3.2.4).

The proportion of labelled neurons seen in the raphe nuclei was not significantly different between P14 and P35 (P = 0.20) or adult animals (P = 0.59). In contrast, there was a significant increase in the proportional contribution from the red nucleus from P14 to adult values (P = 0.02) and in the "other nuclei" (P < 0.01) and dorsal medullary reticular nucleus (P = 0.03). The locus coeruleuar projections comprised only a small portion of the total profile at all ages (see Table 3.2.5) and were not detected at all before P28 (2.0 ± 0.8%). At P35, the proportion from this nuclei had increased significantly to 3.7 ± 1.0% and in adult animals (2.1 ± 0.9%) but were statistically not different from the earlier P14 value (P = 0.08 and P = 0.19 respectively; see Table 3.2.5 and Appendix C2).

3.2.4 The origin of supraspinal projections through a spinal transection site

Post-natal day 7

Labelled cell bodies were not detected in P7 animals that were given a lumbar spinal injection of Fluororuby dye immediately following the complete transection of mid thoracic spinal cord. This indicated that all projections were severed by the lesion and that the dye did not diffuse across the injury site to be taken up by severed axons. There was a possibility that neurons may take up the dye by blood-borne circulation through the tissue as all animals injected at ages P7 to P35 were allowed 4 days incubation time for retrograde axonal transport. The complete absence of labelling in the P7 animals suggested that this did not occur.

Post-natal day 14

The total number of labelled neurons detected in the brain at this age (65.3 ± 16.9) was small compared to later ages with only six nuclei showing labelled neurons (see Figure 3.2.5). Most cell bodies were localised in the gigantocellular reticular nucleus (17.3 ± 2.1), lateral vestibular nucleus (13.0 ± 1.1), and reticular pontine nucleus (19.0 ± 2.4; see Table 3.2.3). No labelled neurons were found in the midbrain, other than in the reticular pontine nucleus which was the most rostral labelling detected.

Figure 3.2.5: Distribution of labelled neurons in the brain stem after lumbar Fluororuby injection in spinally transected P14 *Monodelphis*

Camera lucida representation of coronal brain sections from spinally transected P14 + 4 animals showing the location and number of retrogradely labelled neurons after an injection of Fluororuby in the lumbar spinal cord. Sections shown are from the caudal brain stem (a) to the hypothalamus (g). One star represents up to 10 labelled neurons.

P14 spinally transected *Monodelphis*

1mm
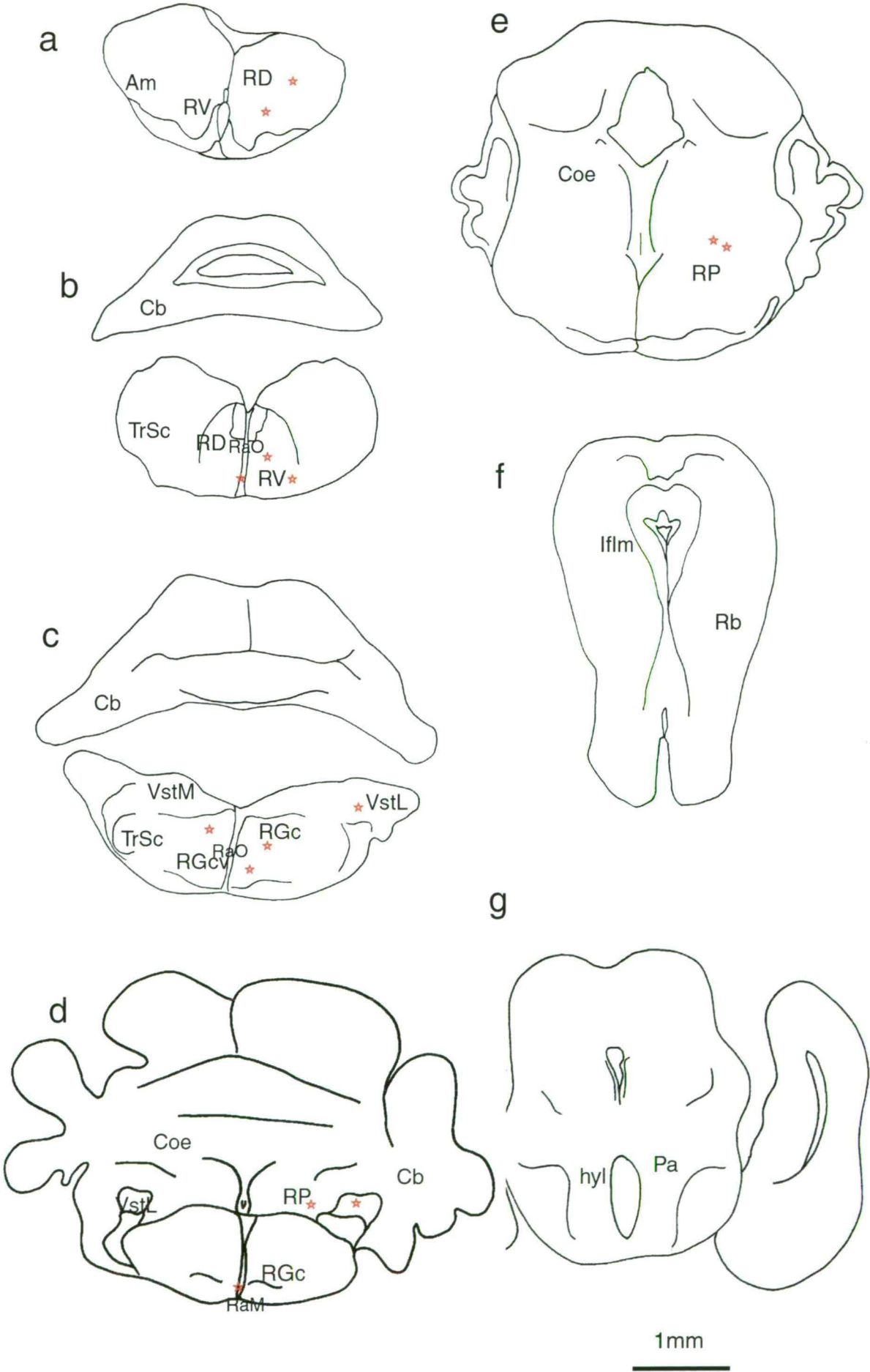
Post-natal day 21

By P21, the total number of labelled neurons detected after complete spinal transection at P7 increased (353.2 ± 176.1), but was only about a third of the number detected in control animals (compare with Table 3.1.3). Small numbers of labelled cell bodies appeared in the red nucleus (6.2 ± 2.0), nucleus ambiguus (0.4 ± 0.2), medial vestibular nucleus (3.0 ± 1.9, and caudal spinal trigeminal nucleus (0.4 ± 0.3) resulting in a more diversified projection profile (see Figure 3.2.6). These were not consistently found in all six brains examined at this age and only represented an equally small proportion of the total (see Table 3.2.5). The red nucleus (32.3 ± 25.8) showed the most rostral labelled neurons but again labelling was not consistent and was found in only three of the six brains examined. Despite the apparent decreasing dominance of the three main contributing nuclei, the lateral vestibular nucleus, gigantocellular reticular nucleus, and reticular pontine nucleus, they still remained the main contributors at P21 after a spinal transection (17.9 ± 2.7%, 26.7 ± 1.6%, and 23.5 ± 2.8 respectively; see Figure 3.2.4). The cell bodies of labelled neurons appeared more strongly fluorescent at this age compared to younger animals.

Post-natal day 28

At P28 the total number of labelled neurons following complete spinal transection at P7 (1054. 1 ± 336.5) was approximately two thirds of the control value (1503.3 ± 453.2). Labelled neurons were now found throughout the hindbrain and pontine nuclei (see Figure 3.2.7). The previously dominant reticular pontine nucleus showing 127.5 ± 50.2 neurons (see Table 3.2.3), decreased its proportional contribution to 12.0 ± 2.8% (see Figure 3.2.4) despite having more labelled neurons than younger animals. The lateral vestibular nucleus showed 177.1 ± 54.8 labelled neurons which was the highest number recorded at any age (see Table 3.2.3), although its contribution to the total seems to be a smaller proportion (15.9 ± 3.9%) than at P14 (21.6 ± 3.3%) or P21 (17.9 ± 2.7%), these differences did not reach statistical significance (see Figure 3.2.4). The gigantocellular reticular nucleus in spinally transected animals continued to contribute the greatest number of neurons 328.8 ± 94.2, as seen at all ages (except P14), representing 26 ± 2.8% of the total projections at P28.

Figure 3.2.6: Distribution of labelled neurons in the brain stem after lumbar Fluororuby injection in spinally transected P21 *Monodelphis*

Camera lucida representation of coronal brain sections from spinally transected P21+ 4 animals showing the location and number of retrogradely labelled neurons after an injection of Fluororuby in the lumbar spinal cord. Sections shown are from the caudal brain stem (a) to the hypothalamus (g). One star represents up to 10 labelled neurons.

P21 spinally transected *Monodelphis*

1mm

Figure 3.2.7: Distribution of labelled neurons in the brain stem after lumbar

Fluororuby injection in spinally transected P28 *Monodelphis*

Camera lucida representation of coronal brain sections from spinally

transected P28 + 4 animals showing the location and number of

retrogradely labelled neurons after an injection of Fluororuby in the

lumbar spinal cord. Sections shown are from the caudal brain stem

(a) to the hypothalamus (g). One star represents up to 10 labelled

neurons.

P28 spinally transected *Monodelphis*

1mm

No labelled neurons were found rostral to the pons, with the exception of one of five brains examined having a small number present in the interstitial nucleus of the medial longitudinal fasciculus (1.75 ± 1.75). The medial vestibular nucleus showed more consistent labelling of cell bodies than at earlier ages with 7.6 ± 2.7 neurons found in four of the five brains examined. The numbers of labelled neurons in these two nuclei were combined with those found in several others (caudal spinal trigeminal nucleus, nucleus ambiguus, ventral part of central gray, Edinger-Westphal nucleus, nucleus Darkschewitsch, and paraventricular hypothalamic nucleus and dorsal hypothalamic area) to obtain "other nuclei" values (see Table 3.2.3).

Post-natal day 35

The number of labelled neurons found in P35 brains after spinal transection was the highest of all age groups (1234.1 ± 203.1). This value was also not significantly different from the age-matched control group (1645.3 ± 496.1, P = 0.38). Again the majority of projections originated from the gigantocellular reticular nucleus with 414.8 ± 77.0 labelled neurons representing 31.5 ± 2.4% of the total (see Figure 3.2.8). The contributions from other nuclei were more evenly dispersed with each contributing between 3% and 15% (see Figure 3.2.4 and Table 3.2.5). The red nucleus was consistently labelled with substantial number of labelled neurons found in all 11 brains examined (106.9 ± 26.8). More rostral regions of the midbrain (most of which are included in the "other nuclei") also showed a moderate increase in the number of labelled neurons. Neurons in the interstitial nucleus of the medial longitudinal fasciculus (28.0 ± 12.8), and locus coeruleus (57.5 ± 17.7) were consistently labelled in all P35 brains examined. The ventral part of the central gray (7.2 ± 3.0), Edinger-Westphal nucleus (9.8 ± 3.0), paraventricular hypothalamic nucleus and dorsal hypothalamic areas combined (9.0 ± 7.0) showed labelled neurons in 8 brains, with none present in the remaining 3. One brain showed 74 labelled cell bodies in the dorsal and paraventricular hypothalamic nucleus but none in the ventral part of central gray. It was noted that the labelled neurons at this age appeared brightly fluorescent and of similar morphology to those seen in control P35 brains. More caudally the nucleus ambiguus also showed labelling with 8.7 ± 2.6 neurons detected.

Figure 3.2.8: Distribution of labelled neurons in the brain stem after lumbar
Fluororuby injection in spinally transected P35 *Monodelphis*

Camera lucida representation of coronal brain sections from spinally
transected P35 + 4 animals showing the location and number of
retrogradely labelled neurons after an injection of Fluororuby in the
lumbar spinal cord. Sections shown are from the caudal brain stem
(a) to the hypothalamus (g). One star represents up to 10 labelled
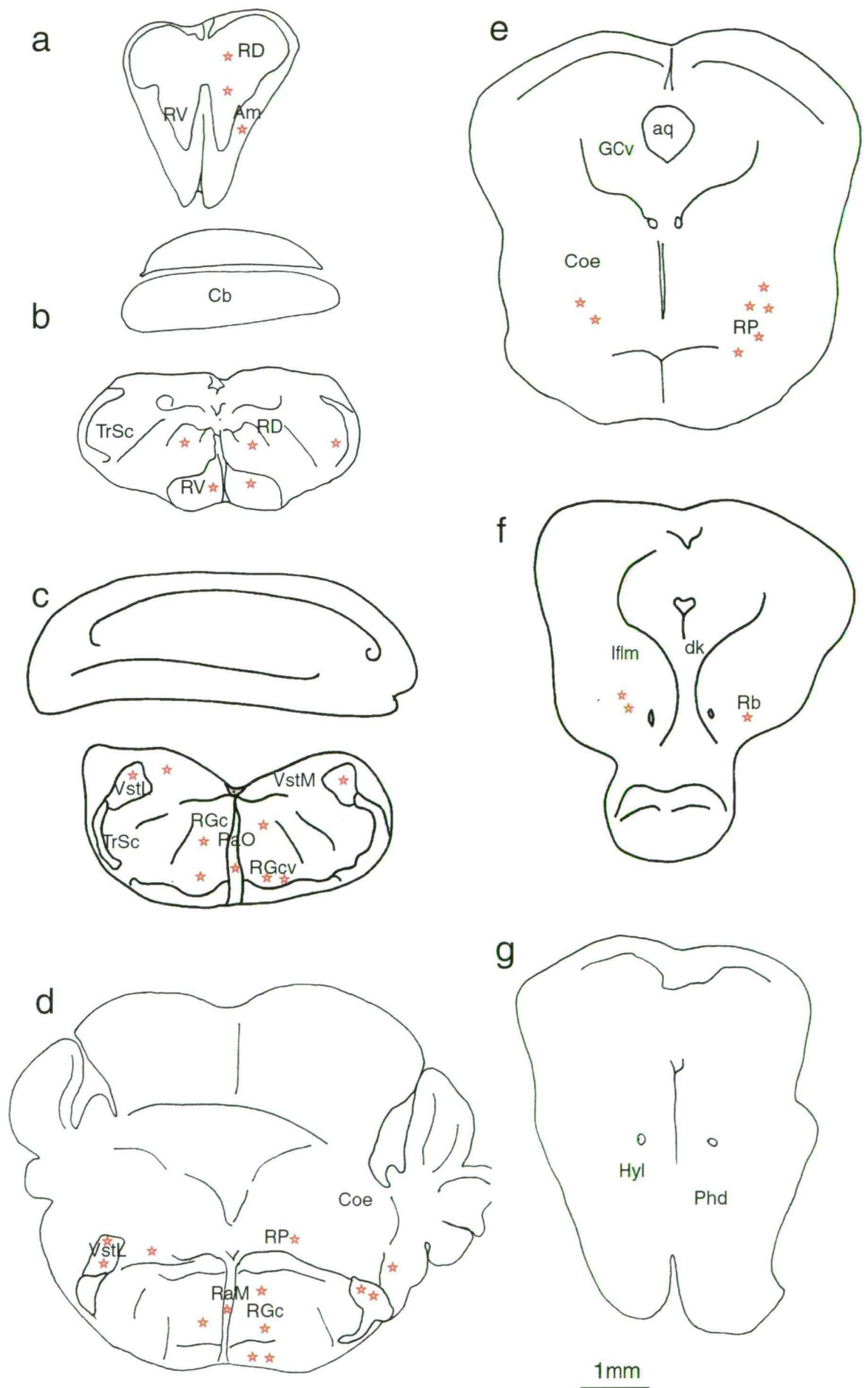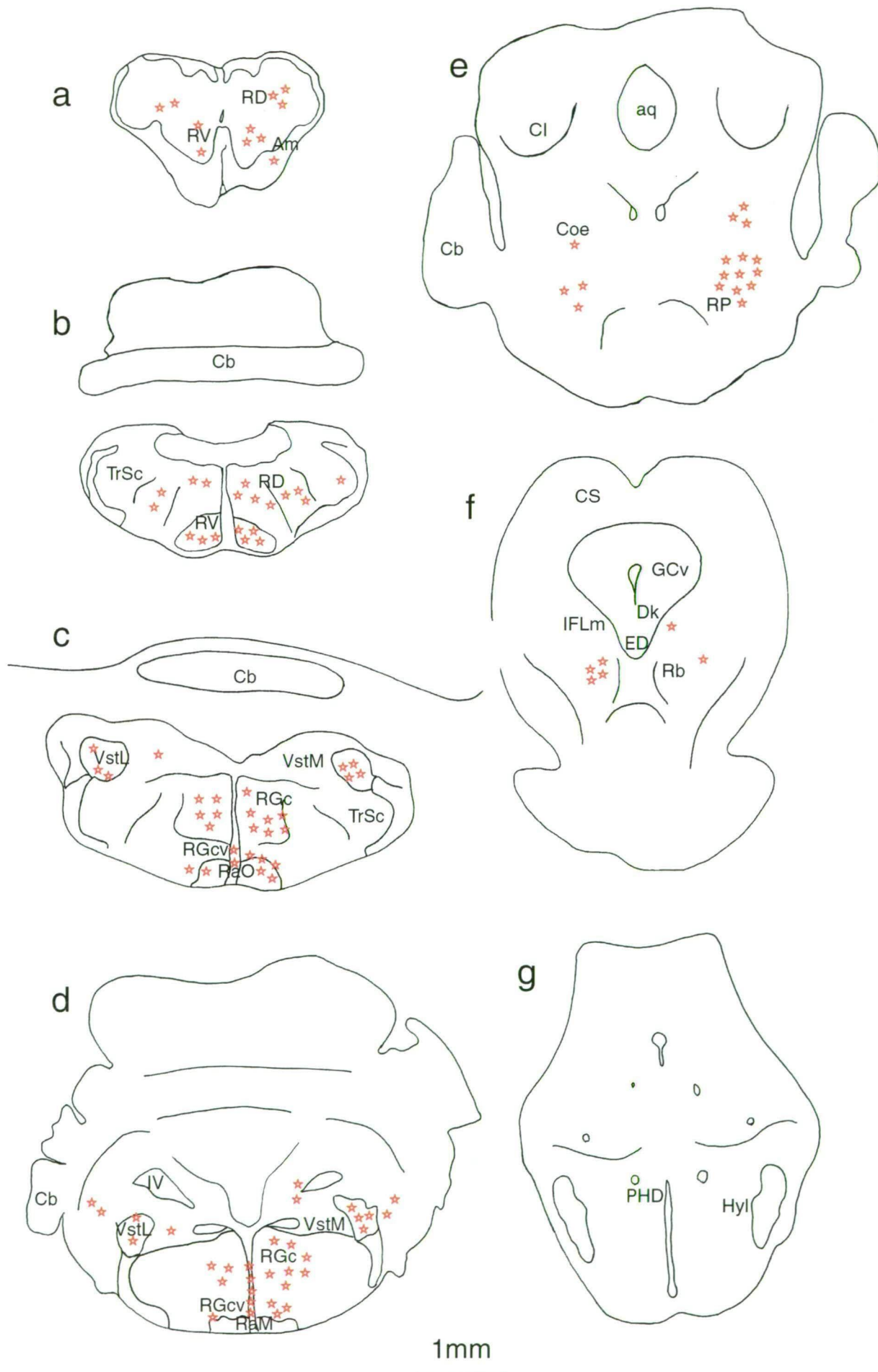neurons.

P35 spinally transected *Monodelphis*

1mm
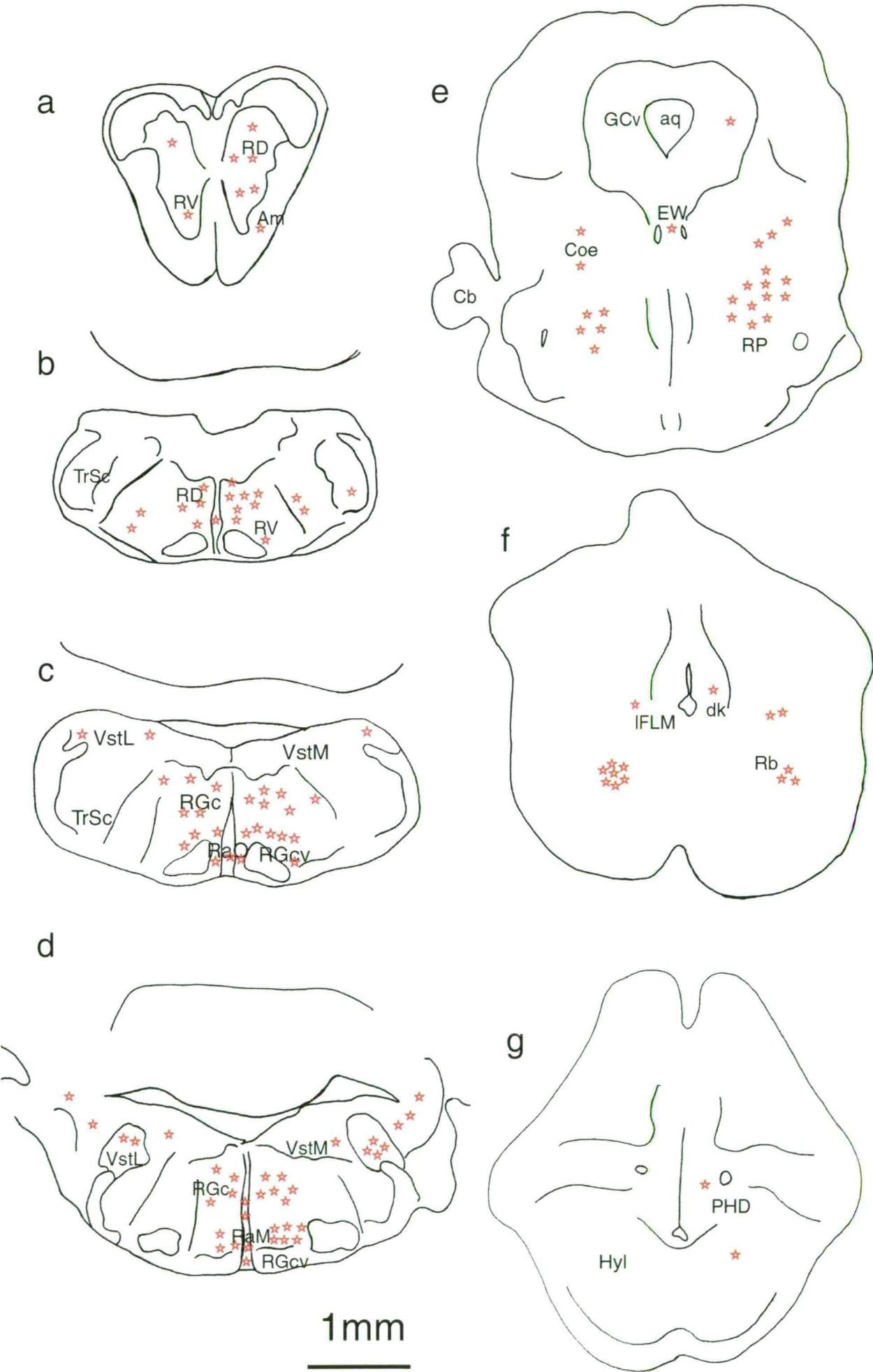
Adult

The total number of labelled neurons detected in adult animals following spinal transection at P7 was relatively small (186.8 ± 75.5), about one sixth of that recorded for P35 animals (1234.1 ± 203.1). However, the origin of the projections through the previous injury site to the lumbar cord was much more diverse, with contributions from all nuclei in the hind brain and midbrain which had shown labelled cell bodies at earlier ages (see Figure 3.2.9). The gigantocellular reticular nucleus again showed the highest number of labelled neurons (57.3 ± 11.2) of all nuclei contributing 27.4 ± 1.3% of all labelled neurons. Most labelled neurons appeared just lateral to the raphe nuclei with equal contributions from the caudal and central regions of these large nuclei. Labelled neurons in the gigantocellular reticular nucleus were either large multipolar cells containing dense brightly fluorescent granules in the soma or were small diameter cell bodies.

Regions of the midbrain were consistently labelled in the 8 adult animals examined with small numbers of fluorescent cell bodies in the paraventricular hypothalamic nucleus and dorsal hypothalamic area (9.0 ± 2.70), the ventral part of central gray (2.0 ± 0.6), the Edinger-Westphal nucleus and nucleus Darkschewitsch (3.2 ± 1.08), the interstitial nucleus of the medial longitudinal fasciculus (5.17 ± 2.2) and the red nucleus (24.3 ± 7.3; see Table 3.2.3). Labelled cell bodies in the reticular pontine nucleus and locus coeruleus were mainly found on the ipsilateral side in adult animals whilst the more caudal regions of the reticular formation had a more bilateral representation to both ipsilateral and contralateral sides of the spinal cord (see Figure 3.2.9). Small numbers of labelled neurons from the lateral vestibular nucleus (19.2 ± 6.4) were found extending into the dorsal cerebellar region.

3.2.5 Laterality of projections in spinally transected animals

The majority of labelled cell bodies appeared in nuclei on the ipsilateral side of the brain to the injected site (see Table 3.2.6). This was most pronounced for the reticular pontine nucleus and locus coeruleus. The gigantocellular reticular nucleus, ventral medullary reticular nucleus and dorsal medullary reticular nucleus showed a more bilateral representation. The red nucleus showed a more contralateral

Figure 3.2.9: Distribution of labelled neurons in the brain stem after lumbar Fluororuby injection in spinally transected adult *Monodelphis*

Camera lucida representation of coronal brain sections from spinally transected adult animals showing the location and number of retrogradely labelled neurons after an injection of Fluororuby in the lumbar spinal cord. Sections shown are from the caudal brain stem (a) to the hypothalamus (h). One star represents up to 10 labelled neurons.

Adult spinally transected
Monodelphis

1mm
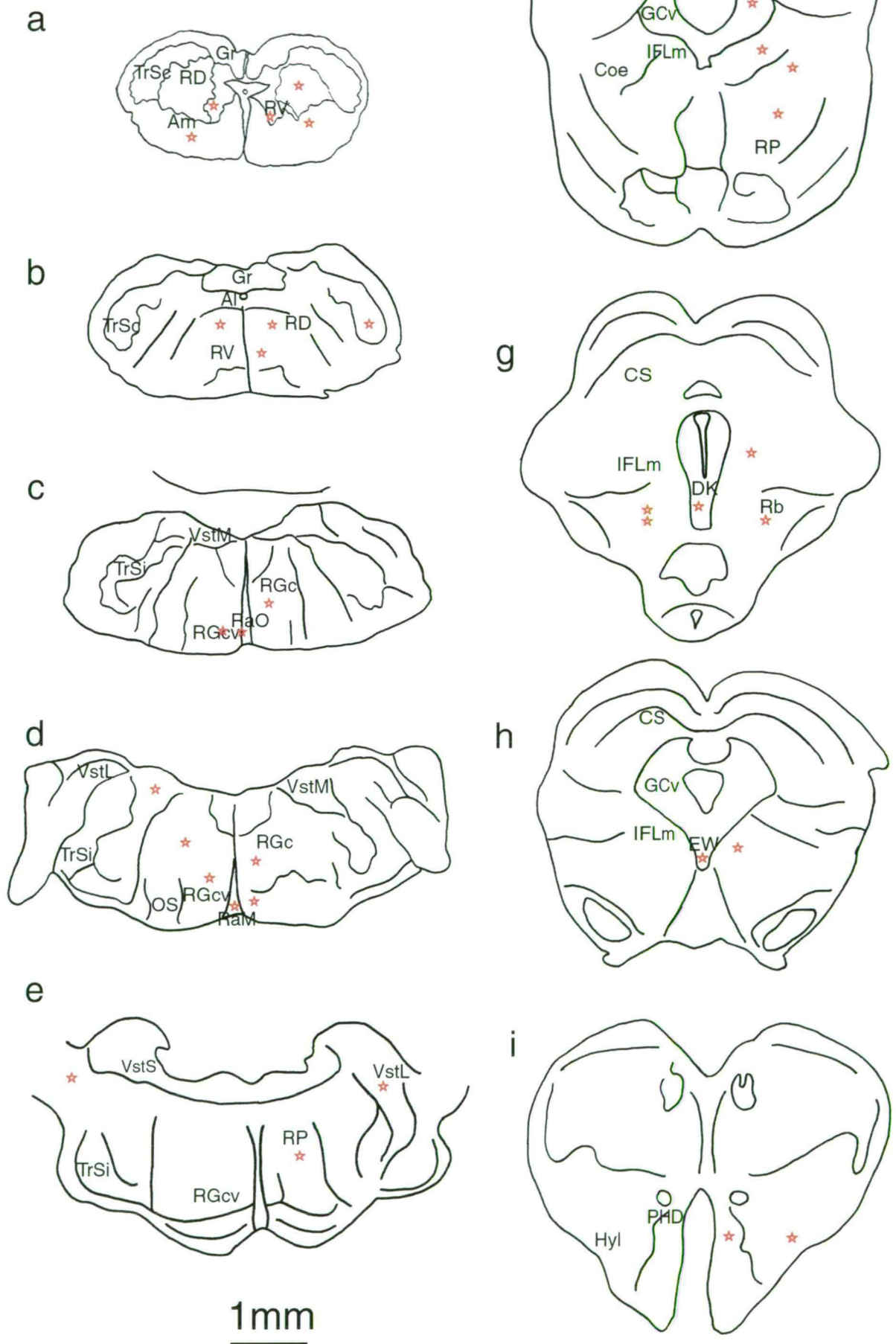
representation, as did the medial vestibular nucleus in small numbers. When considering the ipsilateral/contralateral balance of all labelled neurons in the brain, approximately 70% were found to be ipsilateral to the site of injection and 30% contralateral. This proportion was similar to that observed in control animals (see Table 3.1.6).

Table 3.2.6: The number of labelled neurons counted on the ipsilateral and contralateral side of the brain to an injection of Fluororuby in spinal transected animals aged P7 to adult

Age of injection	n	Total number of labelled neurons in brain (mean ± SEM)	Ipsilateral neurons (mean ± SEM)	Contralateral neurons (mean ± SEM)
P7	3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
P14	3	65.3 ± 16.9	48.7 ± 15.3	19.1 ± 5.2
P21	6	353.2 ± 176.1	245.6 ± 51.3	97.5 ± 25.3
P28	8	1054.1 ± 336.5	703.3± 187.7	344.9 ± 89.2
P35	11	1234.1 ± 203.1	781.6 ± 251.6	408.5 ± 101.2
P42	4	415.5 ± 159.7	321.4 ± 89.7	164.6 ± 54.1
Adult	8	186.8 ± 75.6	156.4 ± 32	49 .7 ±19.3

It was noted that approximately 70% of the total number of labelled neurons counted in brains were located ipsilateral to the side of injection, and 30% contralateral.

3.3 REGENERATION OF SUPRASPINAL PROJECTIONS IN THE NEONATAL OPOSSUM *MONODELPHIS DOMESTICA*

A double label experimental paradigm was used to determine whether descending projections from brain stem neurons were capable of regenerating back across a complete transection at the thoracic level in the spinal cord of developing opossums (see Figure 2.5 in Methods). The experiment distinguished axons regenerating across the injury site from fibre growth that was the result of subsequent development (see Table 3.3.1 for numbers of animals used).

The fluorescent dextran amine Oregon green was injected into the lumbar spinal cord in P4 animals to "pre-label" a population of brain neurons with lumbar projections that can be identified later as having been present at the time of injury. As these projections had already descended to lumbar levels by P4, they will be severed by the complete transection of the thoracic spinal cord at P7. A second, red coloured axonal tracer, Fluororuby, was then injected in the lower thoracic region at one week intervals after the transection (P14, P21, P28 and P35) to label those brain projections that had grown across the injury site. Brain neurons labelled with both dyes were assumed to have regenerated their axons; having initially projected to the lumbar cord to take up the green dye, been severed by the lesion and then regenerated across the lesion site to pick up the second red dye (see Figure 3.3.1). Neurons containing only the first dye would represent projections severed by the lesion that either did not regenerate or did not contact the second tracer. Neurons containing only the second dye would represent newly developing axons which have grown through the lesion site as part of the development subsequent to the lesion, although it is possible that the axons had not encountered the initial dye and were also regenerated from cut axons. It was thus likely that the number of double labelled cell bodies counted could represent an underestimate of the true amount of regeneration.

Oregon green was chosen as the first label as it provides a good optical contrast against Fluororuby. Fluororuby was chosen as the second dye as many more cells were likely to be labelled after the lesion as it was given at older ages. Individual

Table 3.3.1: Number of animals (n) used for the spinal injection experiments

Age	Control			Spinally transected		
	Oregon green	Fluororuby	Double labelled	Oregon green	Fluororuby	Double labelled
P7	3	3	3	11	4	4
P14	3	3	3	10	3	3
P21	3	4	3	8	6	6
P28	3	4	3	7	8	5
P35	3	4	3	9	11	9
Adult	-	6	-	-	8	-

Not all animals were found to have successful injections of both Oregon green and Fluororuby and therefore double label n values were not equal to that of the successful single label cases. Also some animals only received a single injection of either Oregon green or Fluororuby. The criteria for a successful injection included an appropriate unilateral injection in the lumbar spinal cord below the lesion.

Figure 3.3.1: Morphology of double labelled neurons.

a) A brain section cut at 100μm thickness showing Oregon green labelled neurons in the reticular pontine nucleus from a spinally transected P28 animal. Scale bar = 100 μm.

b) The same brain section as a) showing Fluororuby labelled neurons. Scale bar as for (a).

c) The two fluorescent micrographs were overlaid by digital manipulation to show several double-labelled neurons that appear as yellow. Those neurons were able to regenerate after spinal transection. Neurons labelled only with Oregon green indicate that they were not able to regenerate back across the injury site to pick up the second dye and neurons labelled only with Fluororuby indicate late developing fibres which were not present at the time of the first injection at P4. Scale bar as for a).

a
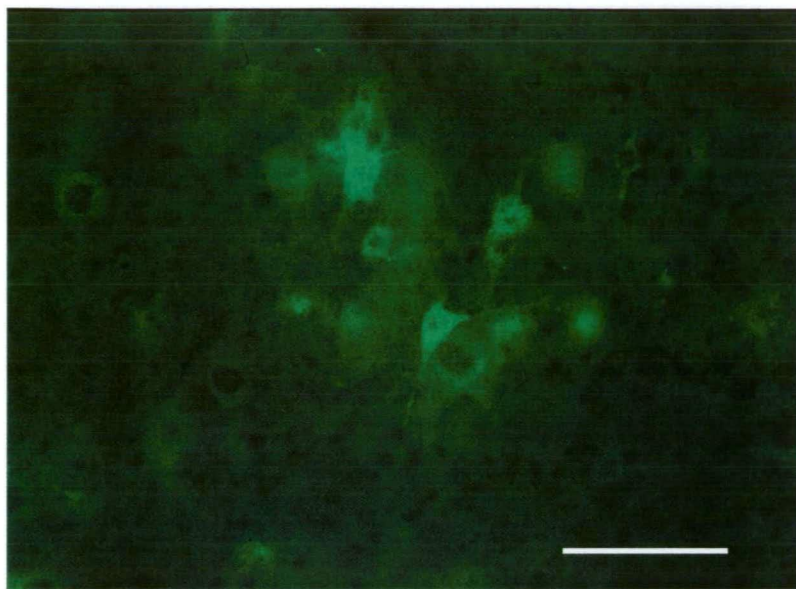
b
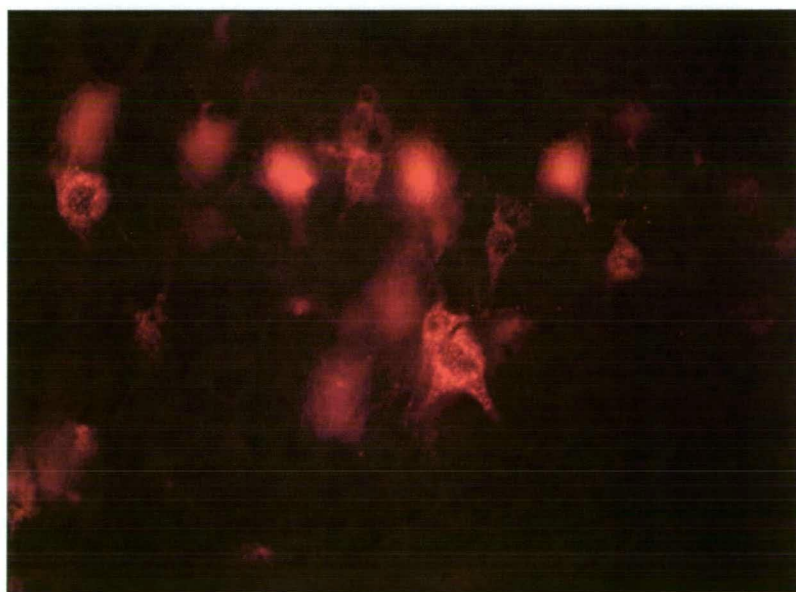
c
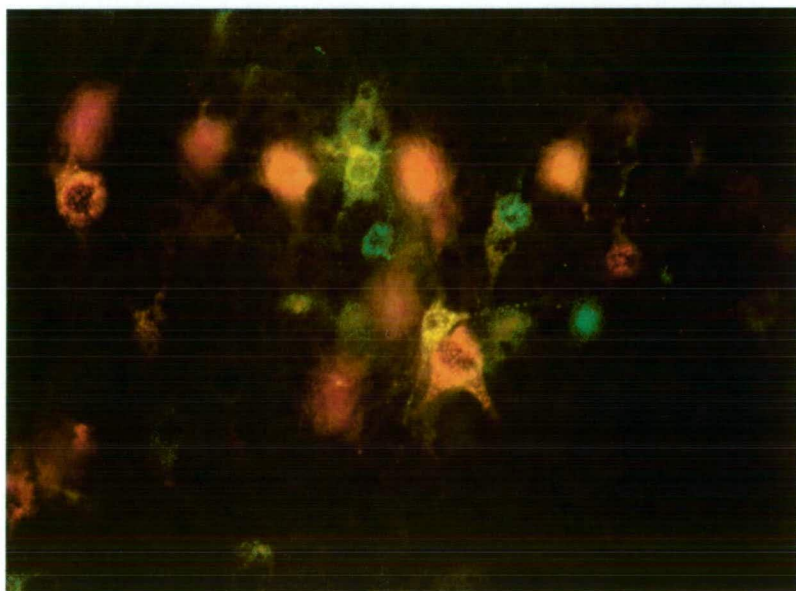
neurons were found to be more easily distinguished when fluorescing red rather then green.

3.3.1 Single label controls

The double label method relies on the assumption that neurons are able to take up both dyes equally and together into the same cell body. To establish that the presence of Oregon green in a neuronal cell body and associated process did not impact on the capacity for uptake of the second dye Fluororuby, single control injection experiments were conducted with both Oregon green and Fluororuby. These experiments determined the typical number, location and appearance of retrogradely labelled neurons that may be expected after injection of either dye alone. When compared to control double label experiments no differences in appearance or number of labelled neurons were found between animals that had received only one dye or both together.

The results for single label Oregon green injections are described initially, as while Fluororuby and Oregon green have almost identical chemical properties (with the exception of the colour of the attached fluorophore), it was critical to establish that Oregon green survives *in vivo* for at least 35 days after injection in comparison to the four days required for Fluororuby (see Table 3.3.1 for n). Additionally, it was important to know how long the first tracer persisted at the injection site, because it might remain available for uptake by any newly arriving "fibres en passage". This could introduce a methodological error in labelling where neuronal cell bodies may appear double labelled as a result of their projections contacting any residual Oregon green at the same time as the second injectate Fluororuby, given some time later.

Persistence of Oregon green at the injury site

The Oregon green injection sites in the lumbar spinal cord from P7 to P35 were examined to see if the injectate remained in the extracellular space. Immediately following the injection of Oregon green at P4, transverse sections revealed a predominantly unilateral injection site, although the dye had spread out across the whole diameter of the spinal cord and was fluorescing brightly in the extracellular space with indistinguishable fibres and cell bodies. This appearance was identical to

that of a Fluororuby injection site at P7 (see Figure 3.1.3 for comparison). By P14, the Oregon green injectate was no longer present in the extracellular space and was entirely localised in cell bodies at the site of injection or further distal in both the rostral and caudal directions (See Figure 3.3.7a & 3.3.7b). At P21 and older, the injection site could be identified by a concentration of Oregon green labelled cell bodies in the spinal cord, but was less visible than at P14.

Oregon green labelled cell bodies were of identical appearance to Fluororuby labelled neurons. The dye was accumulated in brightly fluorescing granules in the cell soma and along the processes (see Figure 3.3.2).

Number of Oregon green labelled neurons in control and spinal transected animals

The total number of Oregon green labelled neurons in control brains appeared consistent from P7 to P35 (Table 3.3.2 and Figure 3.3.3) with approximately 250 to 300 labelled neurons found in each brain. P14 to P35 total numbers were not found to be significantly different from the P7 numbers (see Table 3.3.2 for P values).

Spinal transected animals showed comparable values to those observed for control animals with similar total numbers of Oregon green labelled neurons with approximately 250 and 300 seen in animals aged P7 to P28 (see Table 3.3.3 and Figure 3.3.4). P14 to P28 numbers were not significantly different from P7 or from their age –matched controls (see Table 3.3.3 for P values). However P35 numbers (61.1 ± 21.4) were significantly less than the P7 value of 278.0 ± 57.1 $(P < 0.01)$. The total number of labelled neurons for P35 transected animals was significantly less than age –matched control values $(P < 0.01)$.

Distribution of Oregon green labelled neurons

Control animals

Oregon green labelled neurons were found in many nuclei through the brain stem in animals collected at one-week intervals following injection at P4. Both the number and proportional distribution of cell bodies in different nuclei appeared consistent in control animals from P7 to P35 (see Table 3.3.4 and 3.3.5, and Figure 3.3.5).

Figure 3.3.2: Retrogradely labelled neuronal cell bodies at P28 after spinal injection of Oregon green dextran amine at P4 in P7 spinally transected animals.

a) Fluorescently labelled neurons were numerous in the gigantocellular reticular nucleus. Scale bar = 100µm.

b) The reticular pontine nucleus also contained brightly labelled cells bodies for several weeks after injection. Scale bar = 100µm.

a
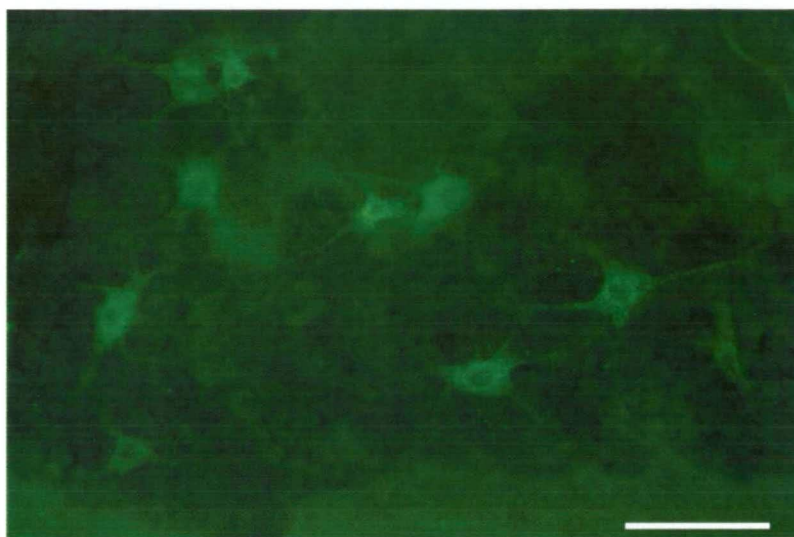
b
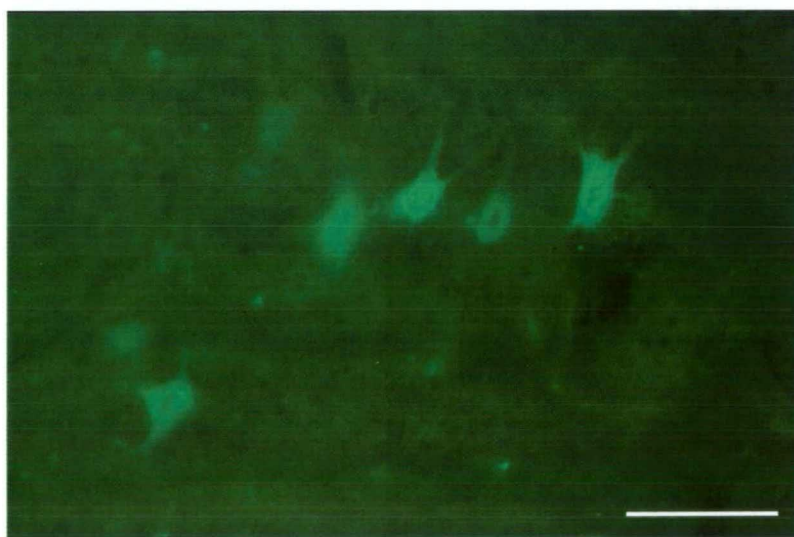
Table 3.3.2: Total number of Oregon green labelled cells in control animals after P4 spinal injection.

Age	n	Mean ± SEM	P t-test against P7 value
P7	3	243.3 ± 56.6	-
P14	3	336.7 ± 90.0	0.43
P21	3	263.0 ± 29.7	0.77
P28	3	270.7 ± 26.0	0.68
P35	5	255.0 ± 28.7	0.86

The mean and standard error were calculated from the total number of neurons counted on both sides of the brain in 100μm coronal sections from individual animals. T-tests were performed using Sigma Stat ®, comparing the mean of the P7 values with other ages to determine if there was a decrease over time.

Figure 3.3.3: The number of Oregon green labelled neurons in control animals

Numbers of labelled neurons in control animals aged P7 to P35, following a lumbar spinal injection of Oregon green at P4. The open circles represent the individual counts and closed circles indicate the mean value at each age. The mean values from P14 to P35 were found not to be significantly different (P<0.05) from the P7 value indicating that the number of Oregon green labelled cells remained constant over this period.

Number of Oregon green labelled neurons in control animals
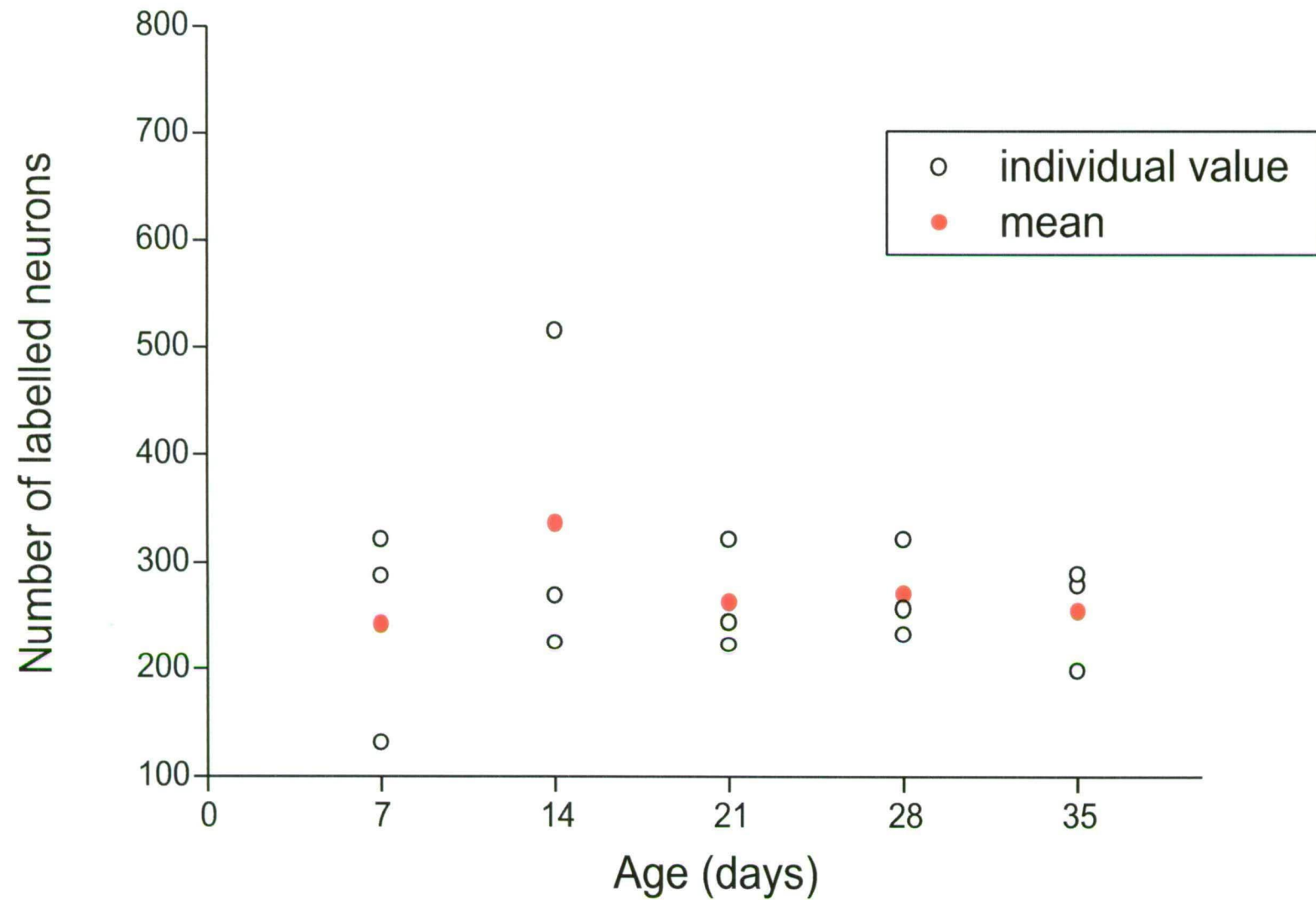
Table 3.3.3:Total number of Oregon green labelled neurons in spinally transected animals

Age	n	mean ± SEM	P t-test against P7 value	P t-test against control value
P7	11	278.0 ± 57.1	-	0.77
P14	10	267.8 ± 68.0	0.91	0.62
P21	8	252.3 ± 63.4	0.77	0.92
P28	7	244.0 ± 95.4	0.75	0.87
P35	10	68.0 ± 21.5	<0.01*	<0.01*

The mean and standard error were calculated from the total number of neurons counted on both sides of the brain in 100μm coronal sections from individual animals. T-tests were performed using Sigma Stat ®, comparing the mean of the P7 values with other ages to determine if there was a decrease over time. T-tests were also made comparing the mean value for each age against the age-matched control mean.

*The P value of statistical significance was defined as $P \leq 0.05$.

Figure 3.3.4: The number of Oregon green labelled neurons in spinally
transected animals

Numbers of labelled neurons in spinally transected animals aged P7 to P35,
following a lumbar spinal injection of Oregon green at P4. The open circles
represent the individual counts and closed circles indicate the mean value at
each age. The mean values at P14, P21, P28 and P35 were not found to be
significantly different from the P7 value indicating that the number of Oregon
green labelled cells remained constant over the period examined. None of
the values for spinally transected animals were significantly different from
age–matched control values, with the exception of P35 ($P \leq 0.05$).

Number of Oregon green labelled neurons in spinally transected animals
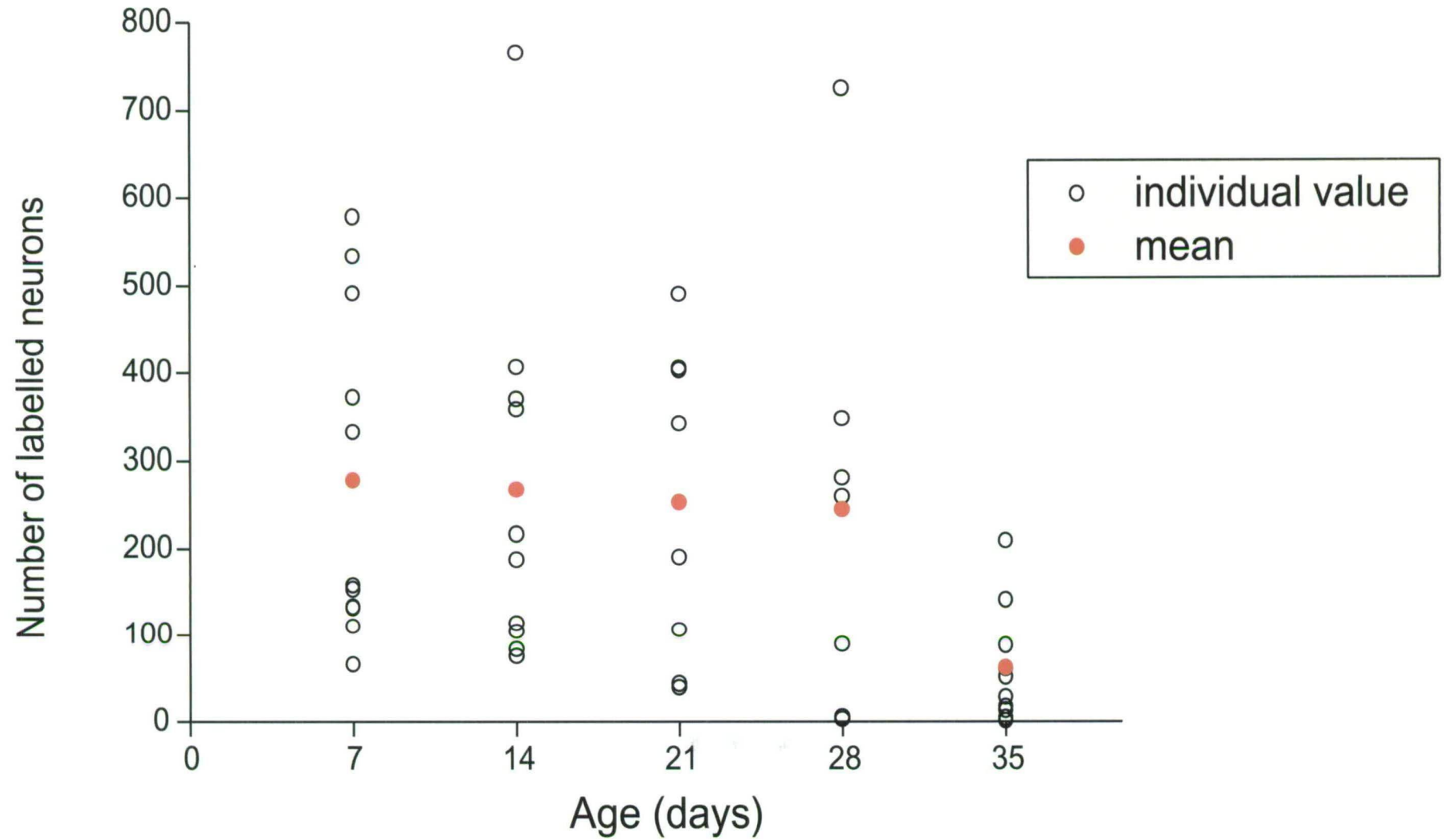
Table 3.3.4: Number (mean ± standard error) of Oregon green labelled neurons counted in each nucleus in control animals from P7 to P35

Nuclei	P7			P14			P21			P28			P35		
n	3			3			3			3			3		
Ventral medullary reticular nucleus	13.3	±	2.9	19.3	±	2.9	15.3	±	2.4	13.3	±	1.8	15.0	±	3.7
Dorsal medullary reticular nucleus	15.3	±	2.7	17.3	±	3.8	10.0	±	2.0	18.0	±	1.7	18.6	±	2.9
Raphe nuclei	24.0	±	14.6	28.0	±	17.2	20.0	±	5.6	23.0	±	7.0	16.3	±	12.6
Gigantocellular reticular nucleus	70.6	±	17.4	90.6	±	37.7	71.3	±	8.4	71.6	±	9.4	59.3	±	7.8
Lateral vestibular nucleus	42.6	±	13.6	68.0	±	16.6	48.6	±	4.4	44.3	±	6.3	54.6	±	11.7
Reticular pontine nucleus	61.3	±	10.8	82.6	±	20.2	66.0	±	11.0	73.0	±	1.0	61.0	±	10.0
Locus coeruleus	6.0	±	2.0	13.3	±	4.9	11.6	±	2.4	8.6	±	2.6	9.0	±	1.7
Red nucleus	5.0	±	5.0	13.0	±	2.8	12.3	±	3.8	11.6	±	3.3	14.3	±	0.6
others	5.6	±	0.6	4.3	±	1.0	7.6	±	0.4	7.0	±	0.4	6.6	±	0.6

Other nuclei = nucleus ambiguus, caudal spinal trigeminal nucleus, medial vestibular nucleus, interstitial nucleus of the medial longitudinal fasciculus, ventral part of central gray, paraventricular hypothalamic nucleus and dorsal hypothalamic area, Edinger-Westphal nucleus, and the nucleus Darkschewitsch.

Table 3.3.5: The proportion (% mean ± standard error) of the total number of Oregon green labelled neurons for different brain nuclei in control animals from P7 to P35.

Nuclei	P7	P14	P21	P28	P35
n	3	3	3	3	5
Ventral medullary reticular nucleus	5.6 ± 0.3	6.1 ± 0.6	5.8 ± 0.5	4.9 ± 0.3	5.7 ± 1.0
Dorsal medullary reticular nucleus	6.5 ± 0.5	5.3 ± 0.3	3.8 ± 0.7	6.7 ± 0.8	7.5 ± 1.2
Raphe nuclei	9.8 ± 0.6	8.0 ± 0.9	7.7 ± 0.7	8.6 ± 1.0	6.7 ± 0.8
Gigantocellular reticular nucleus	27.9 ± 2.5	26.1 ± 4.9	27.2 ± 2.2	26.2 ± 1.4	24.0 ± 2.4
Lateral vestibular nucleus	16.5 ± 2.3	20.4 ± 0.5	18.6 ± 1.1	16.3 ± 1.0	20.9 ± 2.5
Reticular pontine nucleus	26.3 ± 2.9	24.9 ± 0.8	24.8 ± 1.4	27.4 ± 2.2	23.6 ± 1.6
Locus coeruleus	3.4 ± 2.1	3.8 ± 0.6	4.4 ± 0.6	3.1 ± 0.6	3.5 ± 0.3
Red nucleus	1.6 ± 1.6	4.3 ± 1.3	4.5 ± 0.9	4.2 ± 1.2	5.7 ± 0.4
others	2.5 ± 0.3	1.1 ± 0.2	3.2 ± 0.2	2.6 ± 0.2	2.5 ± 0.2

Other nuclei = nucleus ambiguus, caudal spinal trigeminal nucleus, medial vestibular nucleus, interstitial nucleus of the medial longitudinal fasciculus, ventral part of central gray, paraventricular hypothalamic nucleus and dorsal hypothalamic area, Edinger-Westphal nucleus, and the nucleus Darkschewitsch.

Figure 3.3.5: The proportional contribution from different nuclei to the total profile of Oregon green labelled neurons in the brain following lumbar spinal injection in control animals. No statistical differences were found between the P7 numbers and other ages.

Abbreviations

RV	Ventral medullary reticular nucleus
RD	Dorsal medullary reticular nucleus
Raphe	Raphe nuclei
RGc	Gigantocellular reticular nucleus
VstL	Lateral vestibular nucleus
RP	Reticular pontine nucleus
Coe	Locus coeruleus
Rb	Red nucleus
others	Other nuclei

Proportional distribution of oregon green labelled neurons in control animals
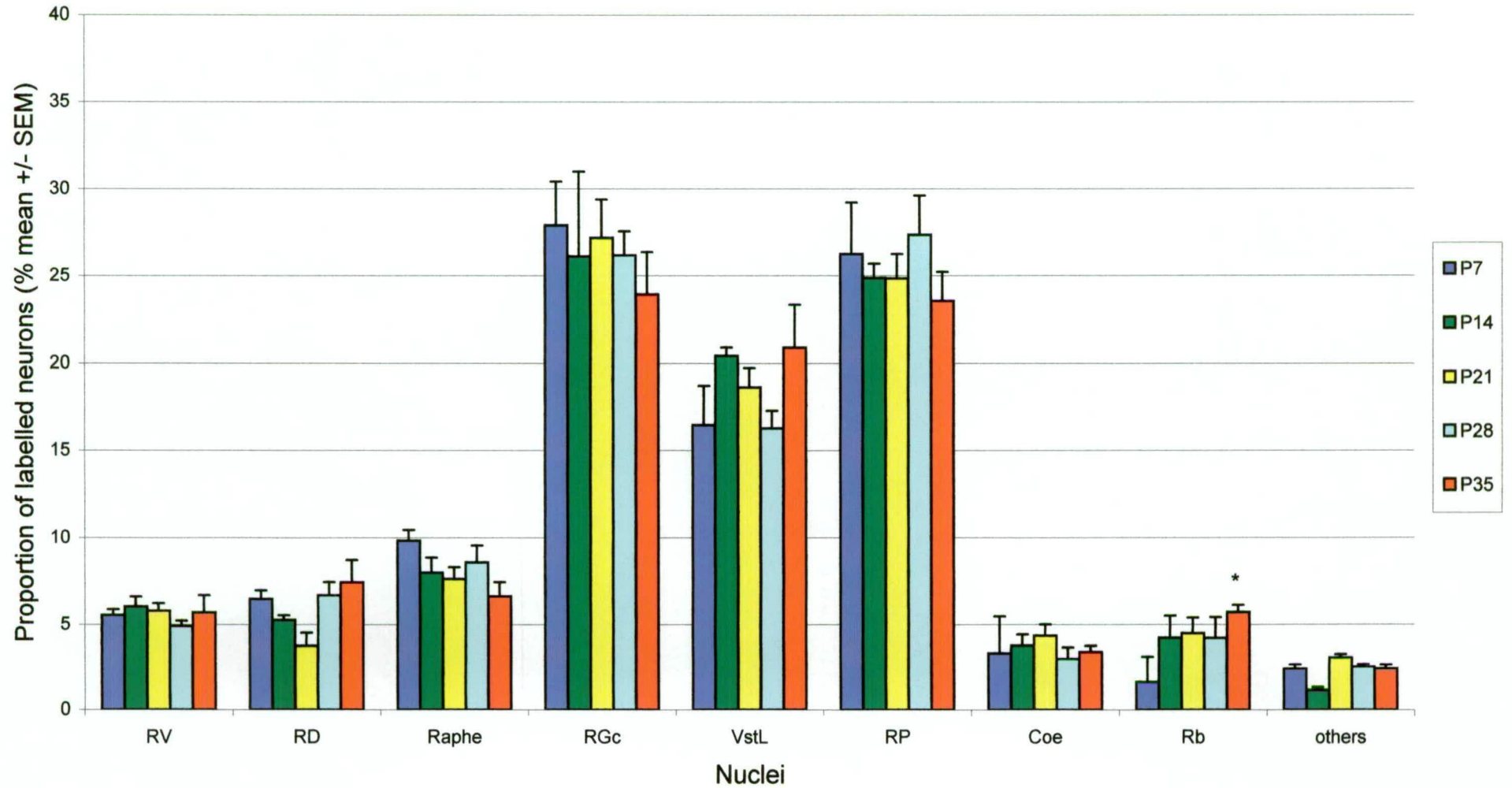
At all ages after the P4 lumbar injection of Oregon green, the gigantocellular reticular nucleus, lateral vestibular nucleus and reticular pontine nucleus contained the largest proportion of labelled neurons at all ages. At P7 these nuclei showed 27.9 ± 2.9%, 16.5 ± 2.3% and 26.3 ± 2.9% of all labelled cells respectively, and the proportions did not significantly differ from those at any of the subsequent ages examined (see Table 3.3.5 and Figure 3.3.10). Minor contributions to the total number of cells at P7 were also found from the ventral medullary reticular nucleus (5.6 ± 0.3%), dorsal medullary reticular nucleus (6.5 ± 0.5%), raphe nuclei (9.8 ± 0.6%), and locus coeruleus (3.4 ± 2.1%). The proportion of neurons seen in these nuclei (<10% of the total) appeared consistent at all ages (see Figure 3.3.5) and P7 proportions were not significantly different from P35 (see Appendix C3).

Small numbers of neurons were found in the red nucleus at all ages, which was the most rostral labelling observed for an Oregon green injection at P4. At P7 the red nucleus represented a small proportion of the total number (1.6 ± 1.6%) and was consistently observed at all other ages, and the proportion was never seen to be greater than about 6% (5.7% ± 0.4 in P35 animals). The nucleus ambiguus, caudal spinal trigeminal nucleus and medial vestibular nucleus also showed small numbers of labelled neurons from P7 to P35. These nuclei were grouped together collectively as "other nuclei " and at P7 only represented 2.5 ± 0.3% of the total number of labelled neurons. These nuclei contained a similarly small proportion of total neurons at all ages (see Table 3.3.5).

No Oregon green labelled neurons were found in the interstitial nucleus of the medial longitudinal fasciculus, ventral part of central gray, paraventricular hypothalamic nucleus and dorsal hypothalamic area, or lateral hypothalamic nucleus at any age following a lumbar injection at P4 in control animals (see Figures 3.3.10, 11, 12, 13 and 14). The distribution and numbers of Oregon green labelled neurons after injection at P4 in control animals were similar to that seen after Fluororuby injections in control P7 animals (see Section 3.1.3).

Spinally transected animals

The number and distribution of Oregon green labelled neurons in spinal transected animals did not appear to differ from that observed in the age-matched controls at P7, P14, P21, and P28 (compare Table 3.3.4 with Table 3.3.6 and Table 3.3.5 with Table 3.3.7). The decline in total numbers observed at P35 was reflected in a similar decline in numbers of labelled neurons from individual nuclei at this age (see Figure 3.3.4 and Table 3.3.6).

The proportional distribution of labelled cell bodies between different nuclei in the brain appeared consistent from P7 to P35 (see Table 3.3.7 and Figure 3.3.6). At P7 most of the labelled cell bodies were located in the gigantocellular reticular nucleus (28.0 ± 4.4%), the lateral vestibular nucleus (22.6 ± 5.4%) and the reticular pontine nucleus (31.1 ± 8.1%). The contribution from each of these nuclei did not appear different from other ages (P14, P21, P28 and P35, see Table 3.3.7). Despite an observed decline in total numbers at P35, the labelled cell bodies were still distributed between the different nuclei in similar proportion to younger ages (see Figure 3.3.6).

The ventral medullary reticular nucleus and dorsal medullary reticular nucleus consistently showed smaller numbers at P7 (12.5 ± 4.5 and 8.8 ± 3.2 respectively) and at this age contributed 5.3% ± 2.1 and 4.5% ± 2.5 respectively to the total. The number and proportional representation of labelled neurons seen at P7 did not change significantly for all ages up to P35 (see Appendix C4).

The locus coeruleus showed very small numbers at each age, consistently less than 2.5% of the total numbers, while cell numbers in the raphe nuclei appeared to increase in proportion from 4.6 ±1.7% at P7 to 11.0 ± 1.7% at P35 but this was not significantly different (P=0.14, see Table 3.3.7 and Appendix C4).

No Oregon green labelled neurons were detected at any age in transected animals in the interstitial nucleus of the medial longitudinal fasciculus, ventral part of central gray, Edinger-Westphal nucleus, nucleus Darkschewitsch, paraventricular hypothalamic nucleus, dorsal hypothalamic area or lateral hypothalamic nucleus. Small numbers (< 5) were seen at each age in the nucleus ambiguus, medial

Table 3.3.6: Number (mean ± standard error) of Oregon green labelled neurons counted in different nuclei in spinally transected animals from P7 to P35

| Nuclei	P7	P14	P21	P28	P35
n	11	10	8	7	9
Ventral medullary reticular nucleus	12.5 ± 4.5	16.5 ± 4.6	9.2 ± 1.7	10.6 ± 3.9	2.6 ± 1.2
Dorsal medullary reticular nucleus	8.8 ± 3.2	13.4 ± 4.1	15.5 ± 4.4	15.8 ± 5.2	3.2 ± 1.0
Raphe nuclei	7.5 ± 22.8	25.8 ± 12.0	15.0 ± 15.8	16.1 ± 22.6	7.3 ± 6.7
Gigantocellular reticular nucleus	79.5 ± 11.9	71.7 ± 16.2	72.0 ± 11.8	68.6 ± 20.8	19.4 ± 4.3
Lateral vestibular nucleus	76.6 ± 22.1	44.3 ± 11.1	54.1 ± 14.8	56.5 ± 20.5	14.2 ± 6.2
Reticular pontine nucleus	87.4 ± 21.4	75.0 ± 20.9	79.7 ± 19.4	86.8 ± 32.1	17.5 ± 5.8
Locus coeruleus	2.9 ± 1.1	10.0 ± 6.9	3.0 ± 1.1	6.5 ± 2.4	1.5 ± 0.7
Red nucleus	0.0 ± 0.0	1.1 ± 1.1	0.0 ± 0.0	0.3 ± 0.2	0.0 ± 0.0
others	6.5 ± 0.4	10.5 ± 0.8	3.5 ± 0.2	8.5 ± 0.5	2.0 ± 0.1

Other nuclei = nucleus ambiguus, caudal spinal trigeminal nucleus, medial vestibular nucleus, interstitial nucleus of the medial longitudinal fasciculus, ventral part of central gray, paraventricular hypothalamic nucleus and dorsal hypothalamic area, Edinger-Westphal nucleus, and the nucleus Darkschewitsch.

Table 3.3.7: The proportion (% mean ± standard error) of the total number of Oregon green labelled neurons for different brain nuclei in spinally transected animals.

Nuclei	P7	P14	P21	P28	P35
n	11	10	8	7	9
Ventral medullary reticular nucleus	5.3 ± 2.1	6.1 ± 1.5	4.7 ± 0.7	2.9 ± 0.8	3.1 ± 0.9
Dorsal medullary reticular nucleus	4.5 ± 2.5	5.4 ± 1.0	5.8 ± 0.9	4.5 ± 1.2	4.9 ± 1.0
Raphe nuclei	4.6 ± 1.7	7.1 ± 1.3	5.9 ± 0.8	10.9 ± 3.5	11.0 ± 1.7
Gigantocellular reticular nucleus	28.0 ± 4.4	24.2 ± 2.5	26.9 ± 2.8	24.9 ± 4.3	31.2 ± 1.8
Lateral vestibular nucleus	22.6 ± 5.4	20.1 ± 2.5	21.0 ± 1.0	22.8 ± 2.6	19.5 ± 3.0
Reticular pontine nucleus	31.1 ± 8.1	30.2 ± 3.5	31.9 ± 1.7	29.2 ± 2.0	26.6 ± 3.2
Locus coeruleus	2.0 ± 1.6	2.3 ± 0.9	1.6 ± 0.7	2.0 ± 0.9	1.3 ± 0.6
Red nucleus	0.0 ± 0.0	0.3 ± 0.3	0.0 ± 0.0	0.1 ± 0.1	0.0 ± 0.0
others	1.8 ± 0.3	4.3 ± 0.3	2.1 ± 0.1	2.6 ± 0.2	2.3 ± 0.2

Other nuclei = nucleus ambiguus, caudal spinal trigeminal nucleus, medial vestibular nucleus, interstitial nucleus of the medial longitudinal fasciculus, ventral part of central gray, paraventricular hypothalamic nucleus and dorsal hypothalamic area, Edinger-Westphal nucleus, and the nucleus Darkschewitsch.

Figure 3.3.6: The proportional contribution from different nuclei to the total profile of Oregon green labelled neurons in the brain following lumbar spinal injection in spinally transected animals. No statistical differences were found between the P7 proportions and other ages.

Abbreviations

RV	Ventral medullary reticular nucleus
RD	Dorsal medullary reticular nucleus
Raphe	Raphe nuclei
RGc	Gigantocellular reticular nucleus
VstL	Lateral vestibular nucleus
RP	Reticular pontine nucleus
Coe	Locus coeruleus
Rb	Red nucleus
others	Other nuclei

Proportional distribution of Oregon green labelled neurons in spinally transected animals
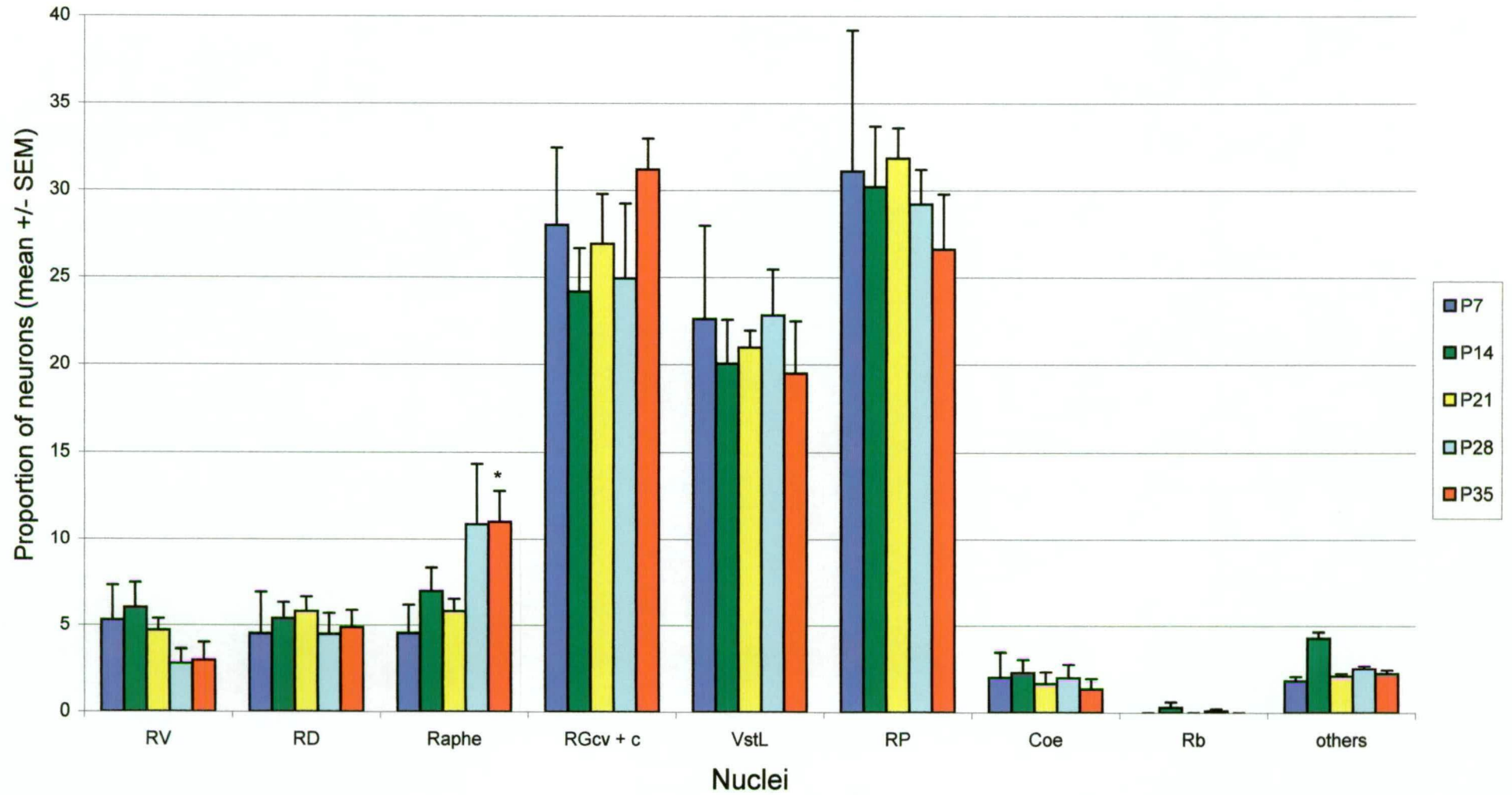
vestibular nucleus, and caudal spinal trigeminal nucleus comprising approximately 3% of the total number seen at each age (see Table 3.3.6 and 3.3.7). These results were reflected in the control Oregon green results (see Section 3.3.1) and also in control P7 animals injected with Fluororuby (see Section 3.1.3).

The red nucleus showed a very small number of labelled neurons at P14 (1.1 ± 1.1) and P28 (0.3 ± 0.2) which amounted to a negligible contribution to the total at these ages (0.3 ± 0.3% and 0.1 ± 0.1% respectively). No Oregon green labelled neurons were found in the red nucleus at P7, P21, or P35. The red nucleus contained Oregon green labelled neurons at all ages in control animals, although in small numbers (see Table 3.3.4).

3.3.2 Control double label studies

These studies were conducted to establish the maximum number of double-labelled cells that could be expected in the brain after spinal injections of the two dyes in the absence of a spinal lesion. Because both the first and second injection may not reach all fibres in the spinal cord, it is unlikely that all supraspinal projections would be exposed to both dyes. Additionally the injections were administered unilaterally, which should further restricted the number of labelled neurons to be detected in the brain to only those with projections reaching the side ipsilateral to the injection. Therefore it was important to have a comparative double label control group to compare the spinally transected animals to more accurately assess the effect of the lesion on the number of labelled neurons found.

It was also important to establish whether similar total numbers of neurons were labelled in animals that received only a single injection of either tracer (Oregon green or Fluororuby) to those animals in the double label studies that received injections of both dyes. The presence of one dye in a cell body or axon might impact on the capacity of the neurons to receive a second dye at a later time.

Injection sites

The injection sites from the double labelling studies were examined in all animals from each age group (see Figure 3.3.7). It was found that Oregon green, injected at

Figure 3.3.7: Double label injection sites

a) Sagittal section from a P7 Oregon green injected spinal cord. The dye was injected at P4 into the lumbar region and remained localised to the injection site. Scale bar = 100µm.

b) Sagittal section of a P28 spinal cord that had been injected with Oregon green at P4. Within days the dye had dissipated from the extracellular space and was not available for further uptake by *fibres en passage*. Some dye had localised in cell bodies along the spinal cord near the site of injection. Scale bar = 100µm.

c) Sagittal section of a P28 + 4 double labelled spinal cord examined under fluorescent optics for rhodamine showing the DAR injection site made at P28. Scale bar as for (b).

d) The same sagittal section as c) examined under fluorescent optics for fluorescein showing that no Oregon green was available at the site of the second injection. This may otherwise have given rise to inappropriate labelling of axons growing to the region where the dye was injected, and were not present at the time of the lesion. Scale bar as for b).
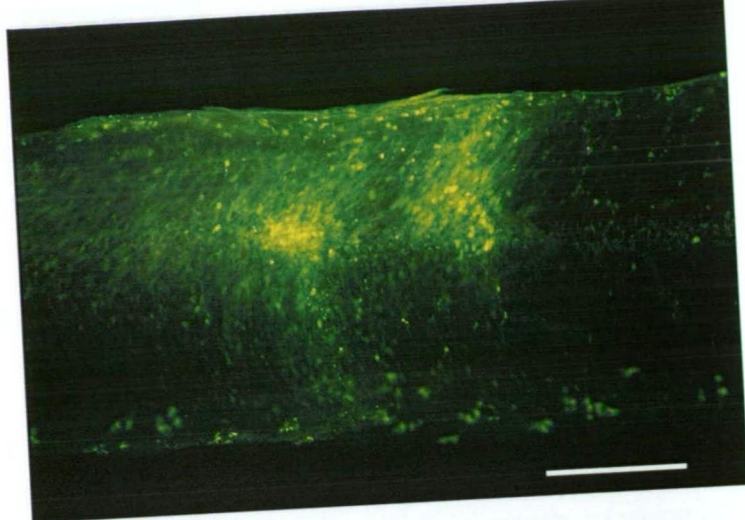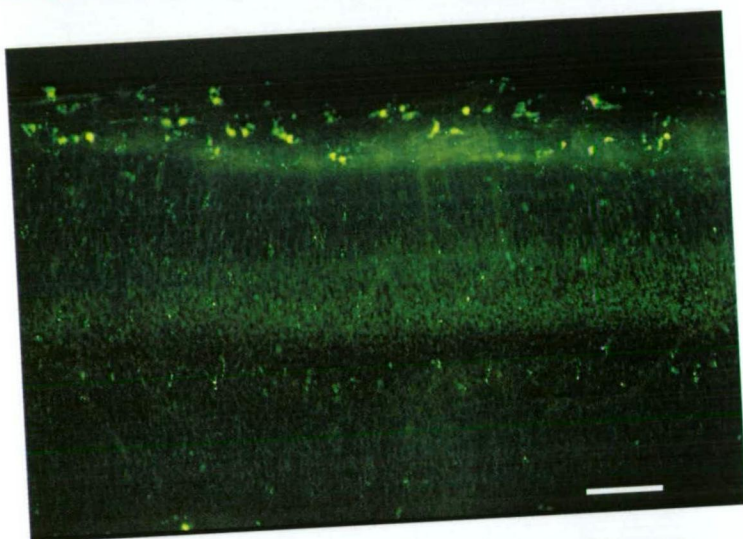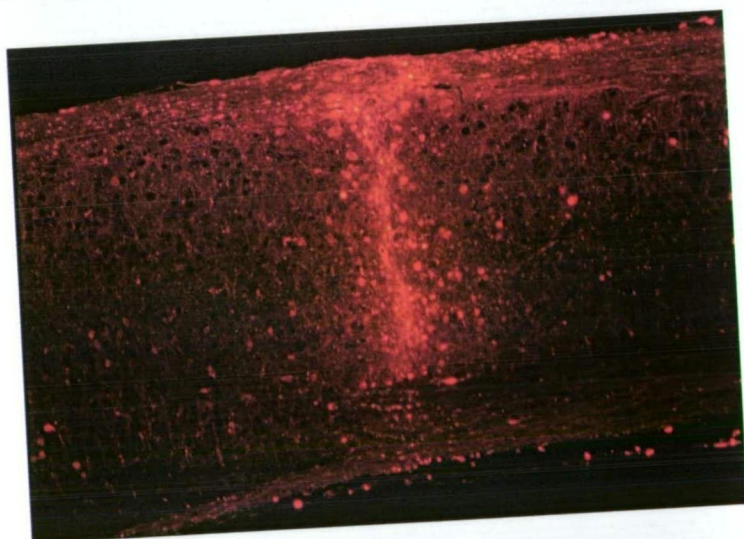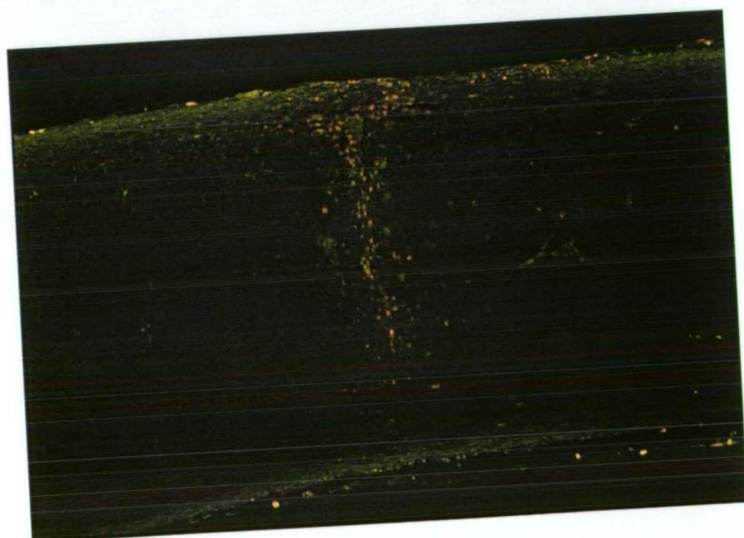
P4, dissipated from the extracellular space at lumbar segment 1 relatively quickly. Therefore it is unlikely that it was available for retrograde transport by projections arriving after transection and thus falsely double labelling neurons. The second dye was injected caudal to the injury site (T6) and rostral to the first injection site so that the two dyes had distinctly separate sites of impact. If these criteria were not met, the animals were rejected from the study.

Comparison of Oregon green numbers from single label and double label experiments

The number of Oregon green labelled neurons found in control animals, which had only received a single injection of Oregon green dye at P4, was compared with the number found in control animals which had also received the second Fluororuby injection at P7, P14, P21 and P35. There was no significant difference in Oregon green numbers between single labelled and double labelled groups. Similarly the total number of Fluororuby labelled neurons in the control single label studies (see Sections 3.1.1) was not significantly different from the number of Fluororuby neurons in control animals that had also received a pre- lesion injection of Oregon green. Therefore data from both double labelled and single labelled control animals have been pooled when calculating overall mean numbers for control P4 Oregon green injections and Fluororuby post-lesion injections.

It did not appear that the presence of one dye competed with or affected the capacity of the axons to take up and transport the other. Where a brain showed neurons labelled with both dyes but no double labelled cells, the number of double labelled cells was recorded as zero and included in the calculation of the mean value at each age. However, all control double label brains containing single labelled neurons with Oregon green and Fluororuby, showed some double labelled neurons (see Figure 3.3.1).

3.3.3 Numbers of double labelled neurons

Control animals

The total numbers of double labelled cells found in control brains were consistent from P7 (70.0 ± 19.6) to P35 (79.7 ± 2.2) with no difference in the numbers between ages (see Table 3.3.8 and Figure 3.3.8).

As all the Oregon green labelled neurons have the potential to regenerate their severed axonal processes, the number of double labelled neurons was also expressed as a proportion of total number of Oregon green labelled neurons. It was found that approximately 25 – 30% of neurons which were labelled by the first injection of Oregon green also contained the second label of Fluororuby from P7 to P35 in control animals (see Table 3.3.8 and Figure 3.3.9).

Spinally transected animals

In spinally transected animals, no double labelled neurons were found in the brains of P7 animals (see Table 3.3.9). A small number was detected at P14 with 5.0 ± 2.6 double labelled neurons representing 2.4 ± 2.2% of the total number of Oregon green labelled neurons for this age. A small increase in double labelled neurons was observed in P21 animals, showing 15.2 ± 5.9 cells, which represented 6.5 ± 3.4% of Oregon green labelled cells. This number was not significantly different from the P14 value (see Table 3.3.9, Figure 3.3.8 and Figure 3.3.9).

The highest number of double labelled neurons was found at P28 with 59.0 ± 26.7 cells, and this number was significantly higher than P21 double labelled numbers. P28 transected animals also showed an apparent increase in the proportion of single labelled Oregon green neurons to 17.3 ± 9.1%, this value was not significantly different from the highest proportion at P35. At P35 the mean number of double labelled neurons had declined to 19.6 ± 5.6 but this represented an increased proportion of the total number of Oregon green labelled cells at 29.9 ± 6.7%. This value was similar to the proportion of double labelled cells in control animals (32.0 ± 3.6%).

Table 3.3.8: Total number (mean ± SEM) of labelled neurons after injection of both Oregon green and Fluororuby in control animals

Age	First Dye (Oregon green)	Second dye (Fluororuby)	Double labelled	P t-test against P7 double label numbers	Proportion of Oregon green neurons with both labels	P t-test against P7 proportion
P7	243.3 ± 56.6	379.3 ± 122.2	70.0 ± 19.6	-	27.8 ±2.1%	-
P14	336.7 ± 90.0	758.3 ± 211.9	80.3 ± 13.3	0.38	25.7± 5.1%	0.71
P21	263.0 ± 27.7	1458.0 ± 458.0	74.7 ± 9.6	0.76	28.4 ± 2.2%	0.86
P28	270.7 ± 26.0	1503.3 ± 228.8	70.3 ± 8.8	0.86	25.8 ± 0.8%	0.43
P35	255.0 ± 28.7	1645.3 ± 226.1	79.7 ± 2.2	0.46	32.0 ± 3.6%	0.37

T-tests were made with P7 numbers and proportion of double labelled Oregon green neurons to see if the values declined over time. The single label values also include those neurons that were double labelled.

Figure 3.3.8: Numbers of double labelled neurons in spinally transected and age-matched control animals.

Numbers of double labelled neurons after spinal injection of Oregon green at P4 and Fluororuby at P7 to P35 in both spinally transected (black) and control animals (red). The open circles represent individual values and the closed circles represent the mean value at each age. The mean values from P7 to P35 were not significantly different.

In spinally transected animals, the highest numbers of double labelled neuron numbers were found at P28, which was significantly different from P21 values. Numbers of double labelled neurons were statistically different from control values at P21 and P35. P28 numbers of double labelled neurons were not different between spinally transected and control animals.

Number of double labelled neurons
in control and spinally transected animals
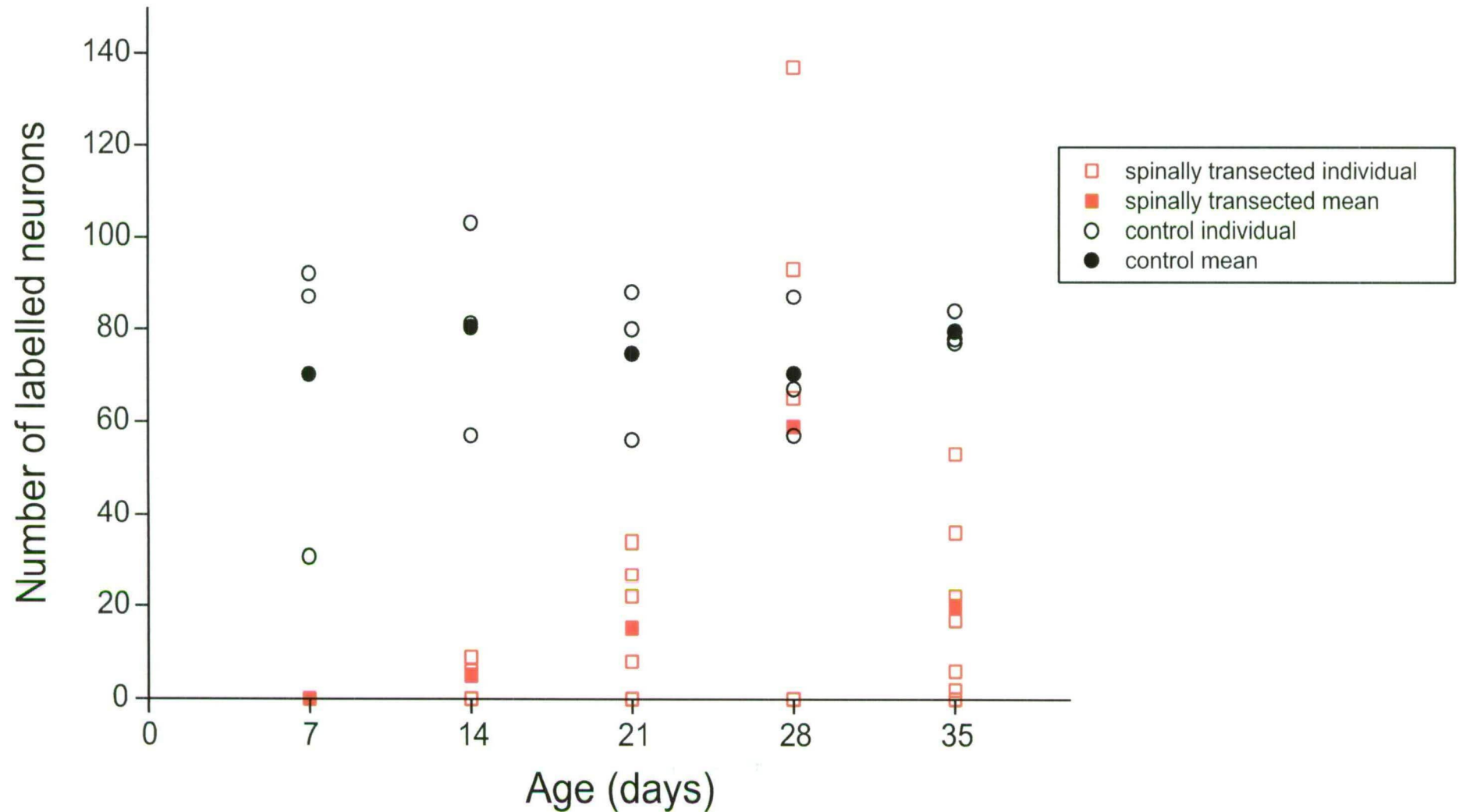
Figure 3.3.9: The proportion of Oregon green labelled neurons that were double labelled in control and spinally transected animals.

Approximately 30% of Oregon green labelled neurons were consistently double labelled in control animals at all ages (shown in black), while in spinally transected animals (shown in red) a significant increase was observed from P14 values to P35 values. P35 values were not statistically different between control and transected animals.

Proportion of Oregon green labelled neurons that were double labelled
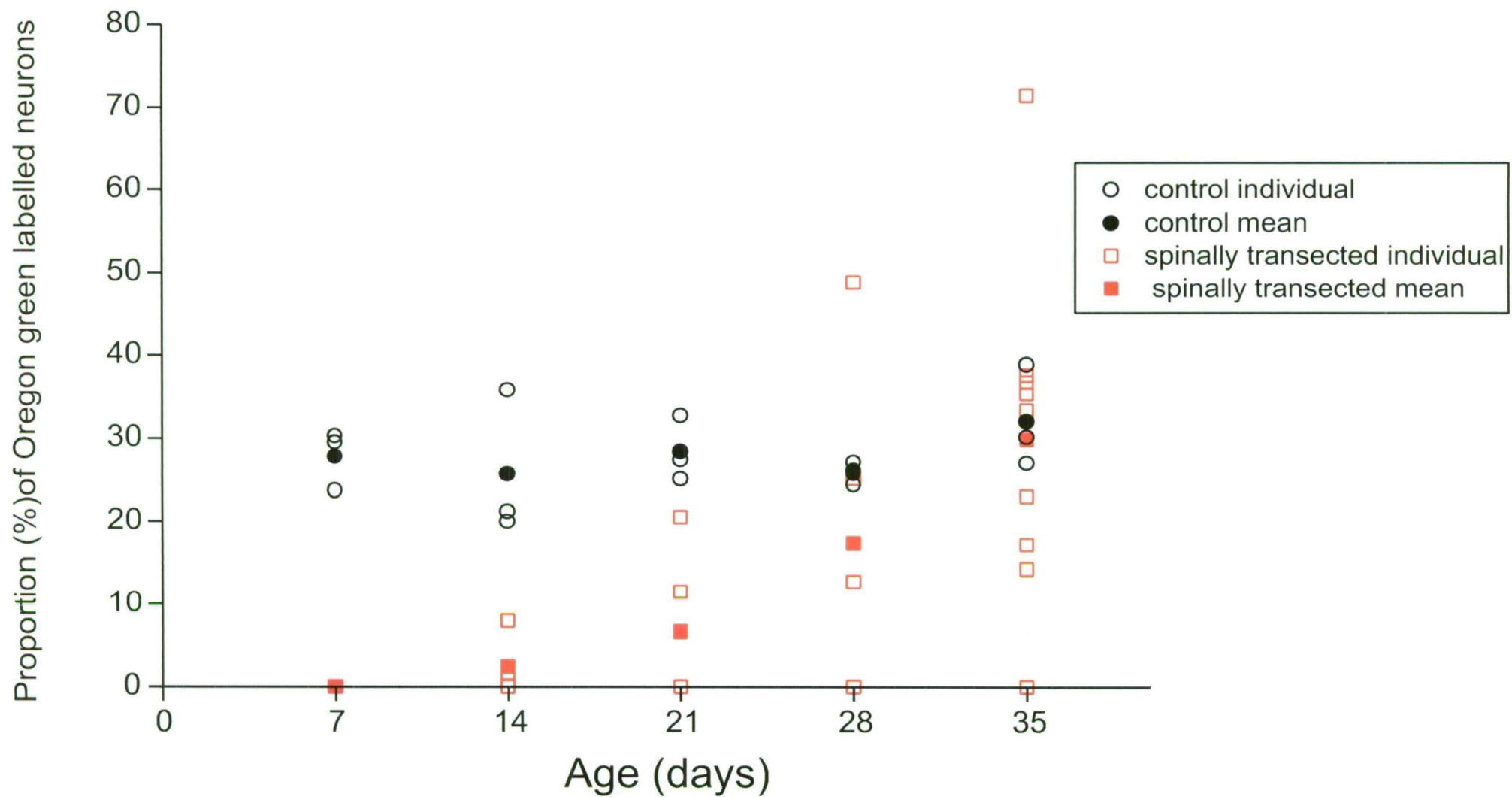
Table 3.3.9: Total number (mean ± SEM) of labelled neurons after injection of both Oregon green and Fluororuby in spinally transected animals

Age	Oregon green labelled neurons (first dye)	Fluororuby labelled neurons (second dye)	Double labelled neurons	P t-test against P28 double labelled numbers	Proportion of Oregon green labelled neurons with Fluororuby	P t-test against P35	P t-test control against transected double labelled numbers
P7	278.0 ± 57.1	0.0 ± 0.0	0.0 ± 0.0	-	0.0 ± 0.0 %	-	-
P14	267.8 ± 68.0	65.3 ± 16.9	5.0 ± 2.6	0.39	2.4 ± 2.2%	0.01 [#*]	0.01 [#]
P21	252.3 ± 63.4	353.2 ± 176.1	15.2 ± 5.9	0.05*	6.5 ± 3.4%	<0.01*	<0.01*
P28	244.0 ± 95.4	1054.1 ± 336.5	59.0± 26.7	-	17.3 ± 9.1%	0.16	0.33
P35	68.0 ± 21.5	1234.1 ± 203.1	19.6 ± 5.6	0.35	29.9 ± 6.7 %	-	<0.01*

#Mann- Whitney rank sum test performed as data failed equal variance test.
T-tests were made against the P28 number for double labelled numbers and the P35 Oregon green proportion of double labelled neurons, as these were the highest values. The single label values also include those neurons that were double labelled.

* Denotes that the t-test showed significant differences in compared means (P≤ 0.05).

3.3.4 Distribution of double labelled cells

Control animals

All control animals that had successful injection sites for both dyes showed double labelled neurons (see Methods: Section 2.2.4 for criteria of successful injection). With the exception of the caudal spinal trigeminal nucleus, all other nuclei that were found to contain Oregon green labelled neurons, also showed double labelled cell bodies (see Figures 3.3.10, 11, 12, 13 and 14). However, in some brains where the number of Oregon green labelled neurons in certain nuclei was very small, double labelled neurons were not always found there. For example, the nucleus ambiguus was only found to have double labelled neurons in one of three animals aged P28, despite all 3 animals at this age having Oregon green labelled neurons in this nuclei.

Double labelled cells in control animals consistently appeared in nuclei that showed well established projections by P7 (as determined by control single label injections of Fluororuby; see Section 3.1.3). At P7, most of the double labelled neurons were located in the gigantocellular reticular nucleus (20.0 ± 4.3), the lateral vestibular nucleus (14.6 ± 6.0) and the reticular pontine nucleus (17.0 ± 3.6; see Table 3.3.10 and Figure 3.3.10). Total numbers of double labelled cells found in these nuclei remained constant up to P35, with 20.0 ± 3.2, 18.3 ± 1.8 and 16.7 ± 1.7 neurons respectively. These numbers were not significantly different to P7 values (P = 1.0, P = 0.59 and P = 0.94 respectively, see Appendix C5).

The proportional contribution of neurons through the brain showed a consistent labelling pattern at all ages (see Table 3.3.11 and Figure 3.3.15). At P7, the gigantocellular reticular nucleus contributed 28.7 ±1.8% of the total labelled neurons, the lateral vestibular nucleus showed 18.5% ± 4.6 and the reticular pontine nucleus represented 26.1 ± 3.4%. The proportion present in these nuclei did not waiver much from these values from P7 to P35, where 25.2 ± 2.9%, 23.0 ± 2.2% and 20.8 ± 1.5% of double labelled cells were still seen in the three major nuclei. No statistical differences were detected between P7 and P35 values for these nuclei (see Appendix C6).

Figure 3.3.10: Distribution of labelled neurons in the brain stem of control P7 +4 *Monodelphis* after lumbar Oregon green injection at P4 and Fluororuby injection at P7.

Camera lucida representations of coronal brain sections from control P7 + 4 animals showing the location and number of retrogradely labelled neurons after a lumbar Oregon green injection at P4 and Fluororuby injection at P7. Sections shown are from the caudal brain stem (a) to the hypothalamus (h).

Green star = up to 10 Oregon green (single) labelled neurons

Red star = up to 10 Fluororuby (single) labelled neurons

Yellow star = up to 10 double labelled neurons

P7 Control *Monodelphis*

a

RD
RV
Am

b

RD
RV

c

TrSc
RGc
RaO
RGcv

d

e

Coe
RP
RaM

f

Iflm
Rb

g

Phd

h

Hyl

1mm
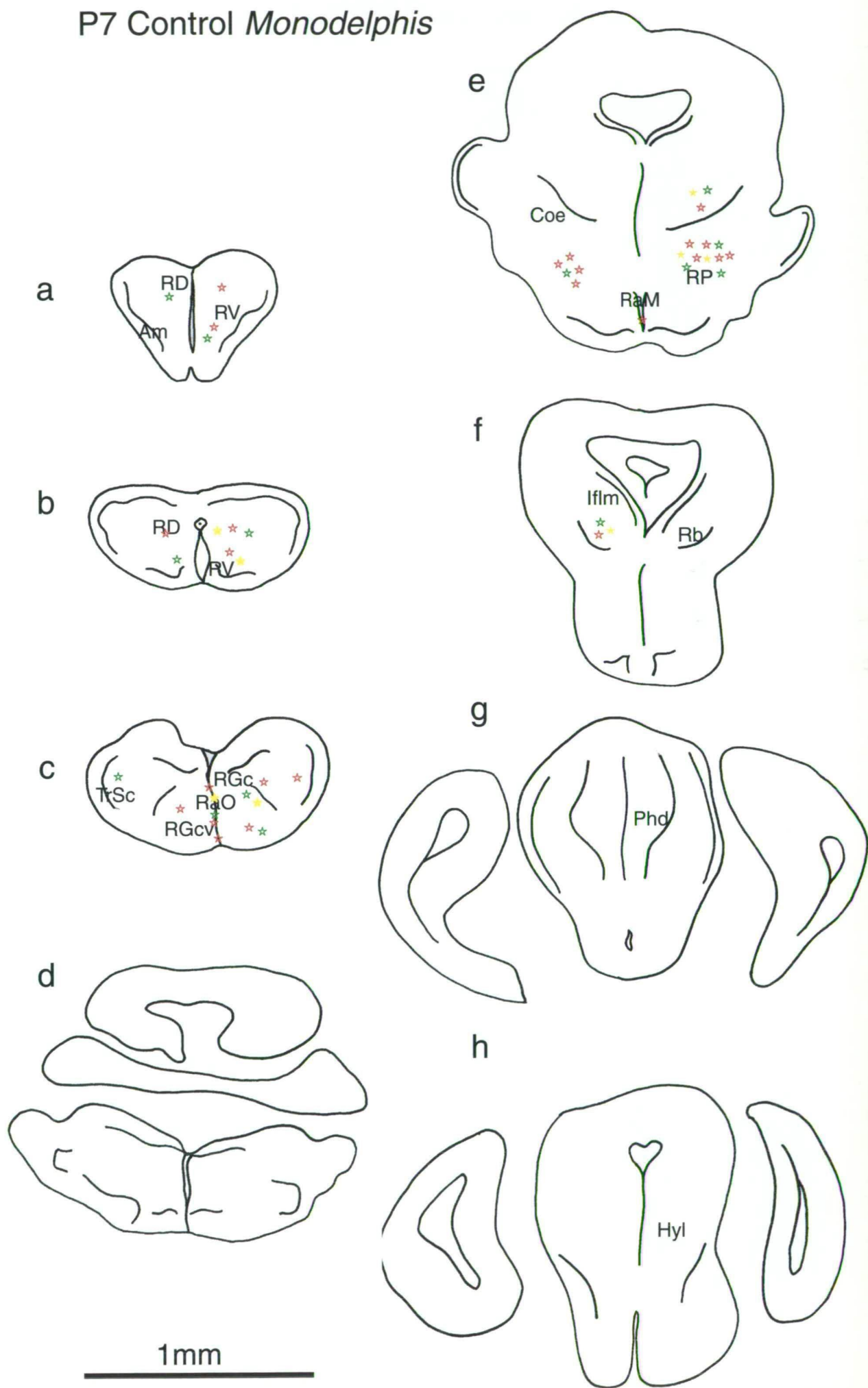
Figure 3.3.11: Distribution of labelled neurons in the brain stem of control

P14 + 4 *Monodelphis* after lumbar Oregon green injection at

P4 and Fluororuby injection at P14.

Camera lucida representation of coronal brain sections from control

P14 + 4 animals showing the location and number of retrogradely

labelled neurons after a lumbar Oregon green injection at P4 and

Fluororuby injection at P14. Sections shown are from the caudal

brain stem (a) to the hypothalamus (g).

Green star = up to 10 Oregon green (single) labelled neurons

Red star = up to 10 Fluororuby (single) labelled neurons

Yellow star = up to 10 double labelled neurons

P14 control *Monodelphis*

a

Am
RD
RV

b

Cb
TrSc
RD
RaO
RV

c

Cb
VstM VstL
TrSc
RGc
RGcv
RaO

d

Coe Cb
VstL
RP
RGc

e

Coe
RP

f

Iflm
Rb

g

hyl Pa
III

1mm
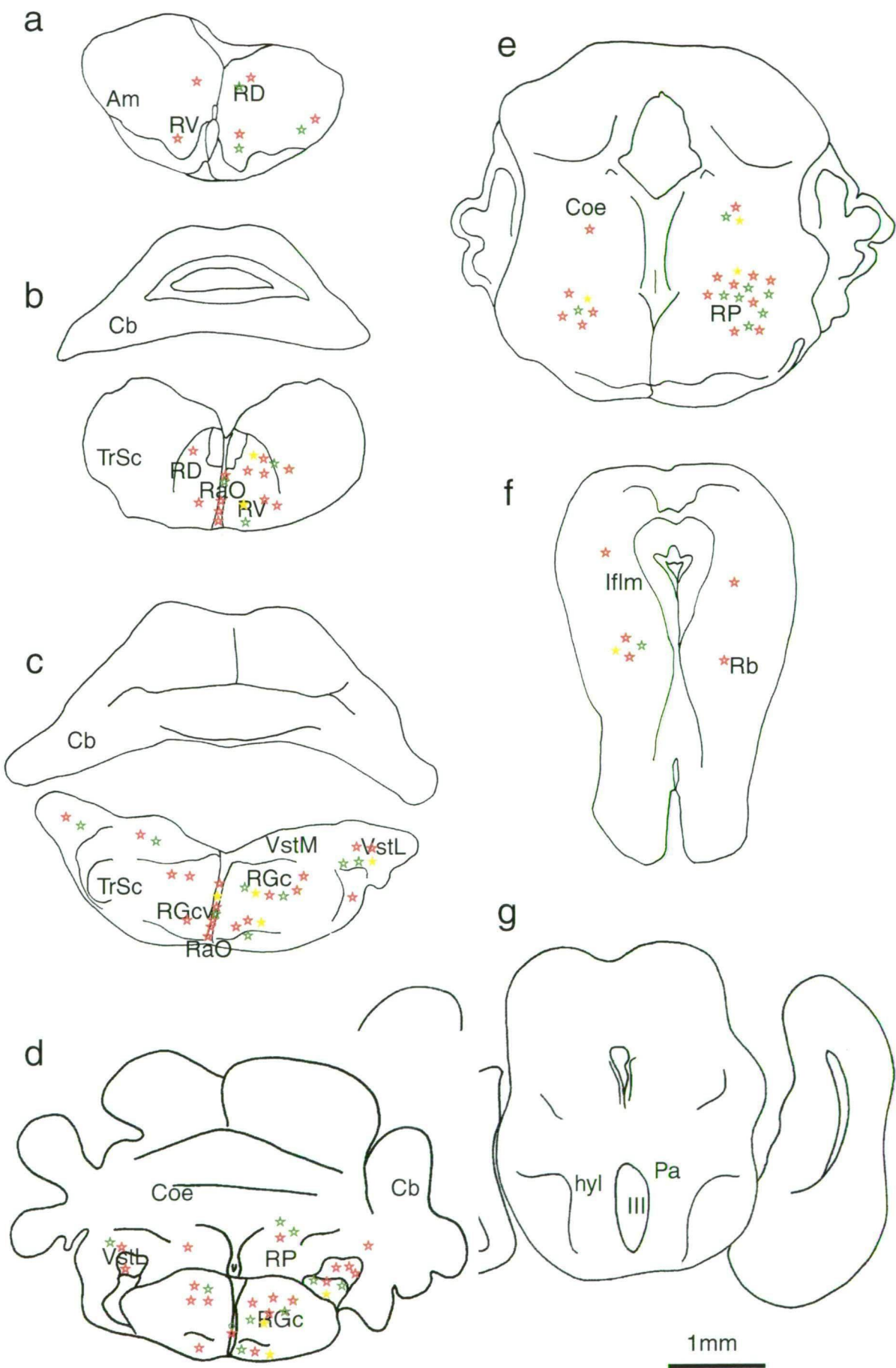
Figure 3.3.12: Distribution of labelled neurons in the brain stem of control

P21 + 4 *Monodelphis* after lumbar Oregon green injection at P4

and Fluororuby injection at P21.

Camera lucida representation of coronal brain sections from control

P21 + 4 animals showing the location and number of retrogradely

labelled neurons after a lumbar Oregon green injection at P4 and

Fluororuby injection at P21. Sections shown are from the caudal

brain stem (a) to the hypothalamus (g).

Green star = up to 10 Oregon green (single) labelled neurons

Red star = up to 10 Fluororuby (single) labelled neurons

Yellow star = up to 10 double labelled neurons

P21 control *Monodelphis*

1mm
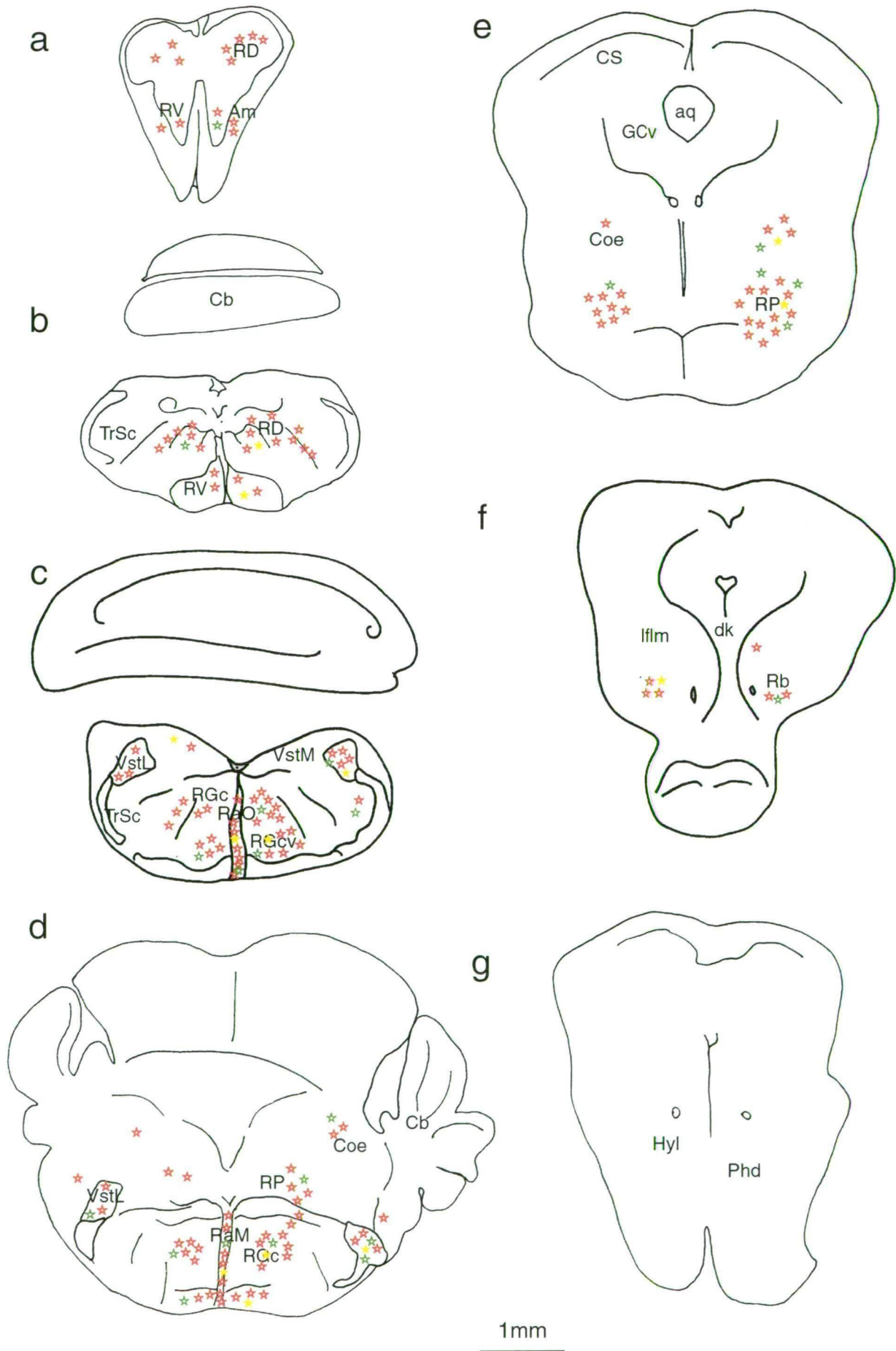
Figure 3.3.13: Distribution of labelled neurons in the brain stem of control
P28 + 4 *Monodelphis* after lumbar Oregon green injection at P4
and Fluororuby injection at P28.

Camera lucida representation of coronal brain sections from control
P28 + 4 animal showing the location and number of retrogradely
labelled neurons after a lumbar Oregon green injection at P4 and
Fluororuby injection at P28. Sections shown are from the caudal
brain stem (a) to the hypothalamus (g).

Green star = up to 10 Oregon green (single) labelled neurons

Red star = up to 10 Fluororuby (single) labelled neurons

Yellow star = up to 10 double labelled neurons

P28 control *Monodelphis*

1mm
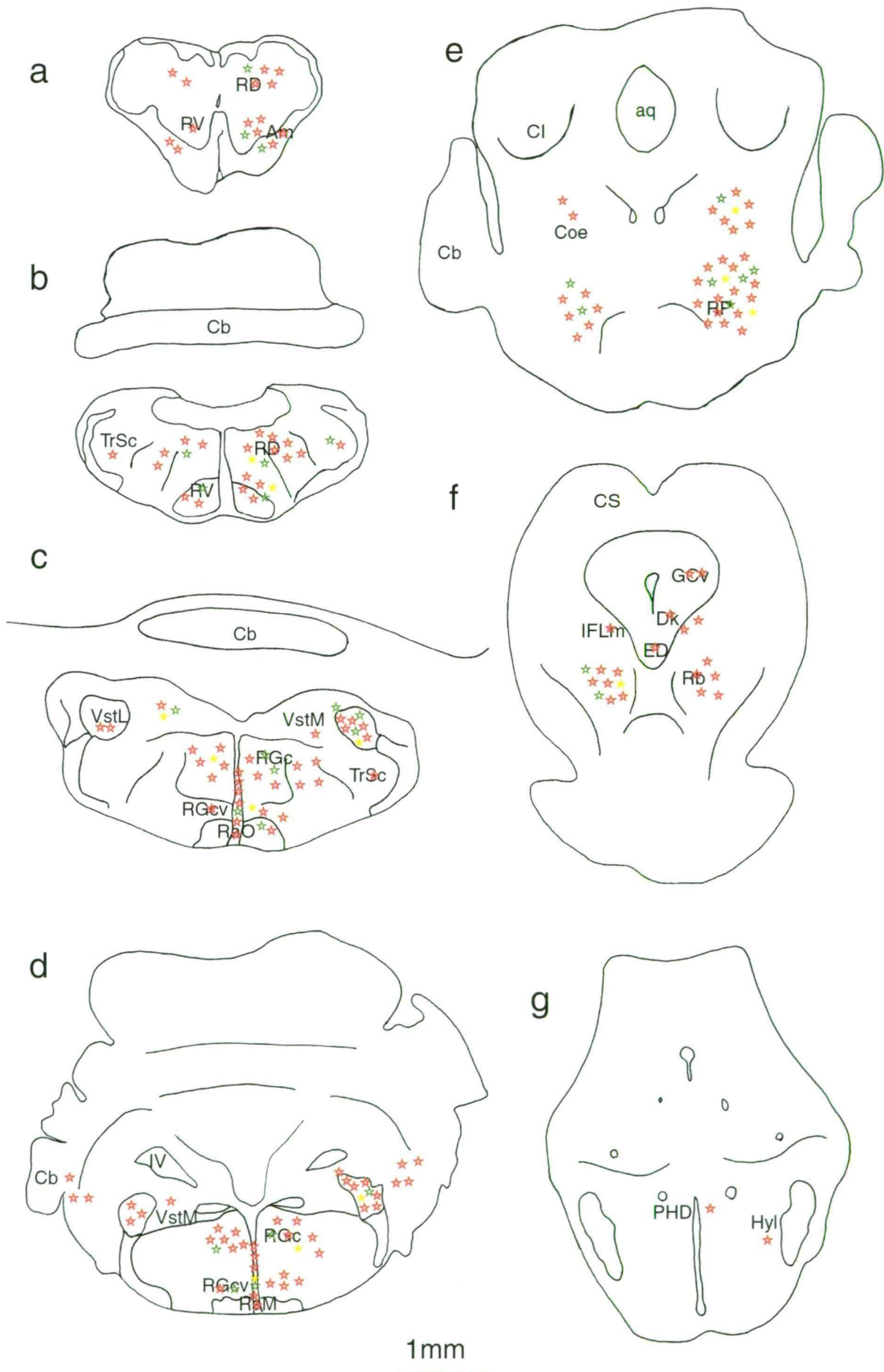
Figure 3.3.14: Distribution of labelled neurons in the brain stem of control

P35 + 4 *Monodelphis* after lumbar Oregon green injection at P4
and Fluororuby injection at P35.

Camera lucida representation of coronal brain sections from control
P35 + 4 animals showing the location and number of retrogradely
labelled neurons after a lumbar Oregon green injection at P4 and
Fluororuby injection at P35. Sections shown are from the caudal
brain stem (a) to the hypothalamus (g).

Green star = up to 10 Oregon green (single) labelled neurons

Red star = up to 10 Fluororuby (single) labelled neurons

Yellow star = up to 10 double labelled neurons

P35 control *Monodelphis*

a

RD
RV
Am

b

TrSc
RD
RV

c

VstL
VstM
TrSc
RGc
RaO RGcv

d

VstL
VstM
RGc
RaM
RGcv

e

GCv aq
EW
Coe
Cb
RP

f

IFLM dk
Rb

g

PHD
Hyl

1mm
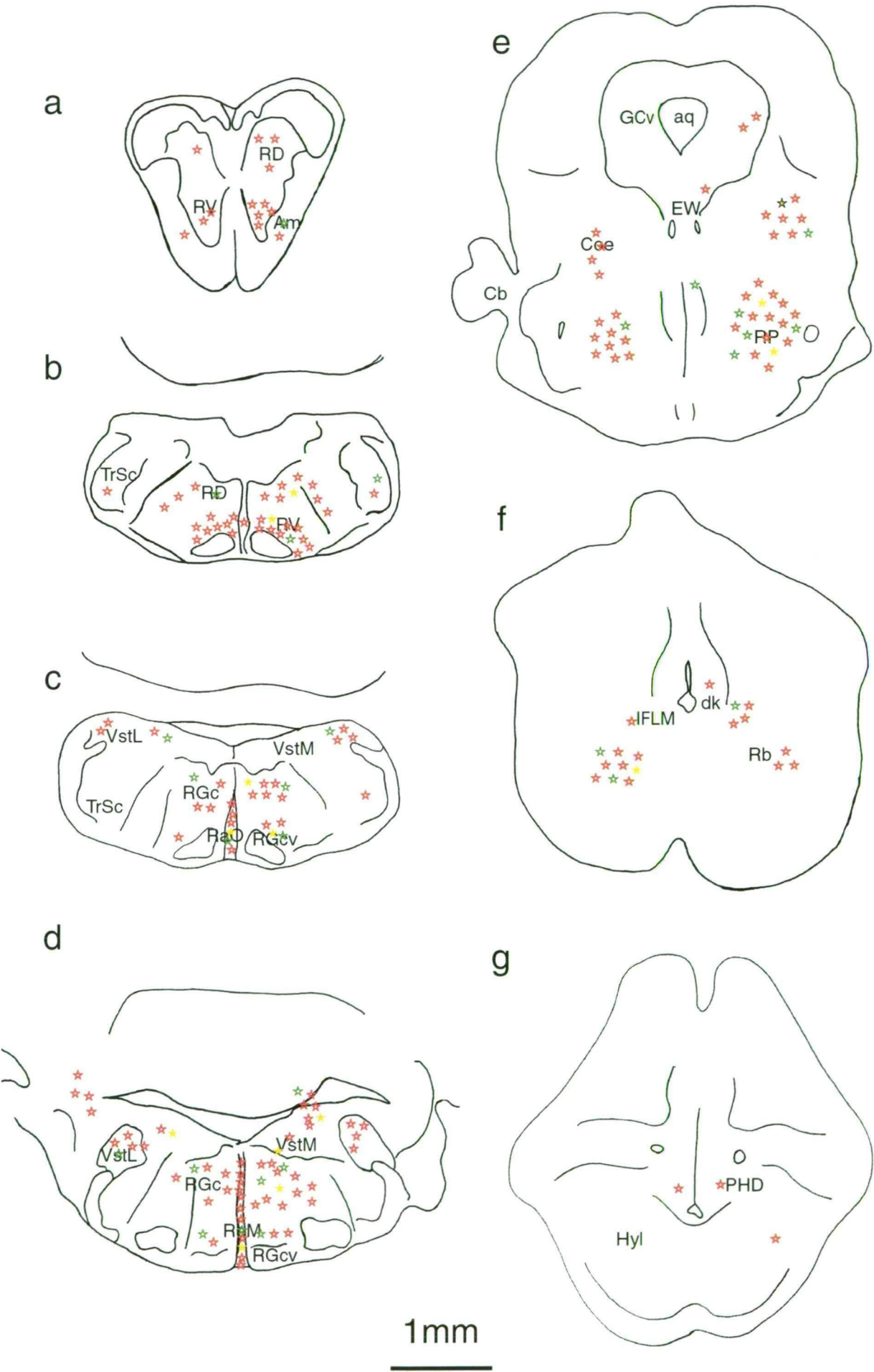
Table 3.3.10: Number (mean ± SEM) of double labelled neurons counted in each nucleus in control animals from P7 to P35

Nuclei	P7	P14	P21	P28	P35
n	3	3	3	3	3
Ventral medullary reticular nucleus	4.7 ± 1.8	5.0 ± 0.0	4.0 ± 0.0	4.0 ± 0.6	4.0 ± 1.0
Dorsal medullary reticular nucleus	4.7 ± 2.2	4.3 ± 0.7	3.0 ± 0.6	5.0 ± 0.6	5.7 ± 0.3
Raphe nuclei	8.0 ± 6.5	7.7 ± 2.5	6.7 ± 3.8	6.3 ± 4.9	5.0 ± 2.1
Gigantocellular reticular nucleus	20.0 ± 4.3	22.7 ± 9.0	20.0 ± 2.3	19.7 ± 1.7	20.0 ± 3.2
Lateral vestibular nucleus	14.7 ± 6.0	17.3 ± 2.0	15.3 ± 3.2	12.3 ± 4.4	18.3 ± 1.8
Reticular pontine nucleus	17.0 ± 3.6	19.3 ± 3.7	18.7 ± 2.0	17.0 ± 3.1	16.7 ± 1.7
Locus coeruleus	0.3 ± 0.3	1.3 ± 0.7	2.3 ± 0.3	1.7 ± 0.3	4.0 ± 1.0
Red nucleus	0.7 ± 0.7	2.7 ± 0.7	3.3 ± 1.3	2.7 ± 0.9	5.7 ± 0.9
Other nuclei	0.7 ± 0.7	2.7 ± 0.7	1.3 ± 0.0	2.7 ± 0.9	5.7 ± 0.9

Other nuclei = nucleus ambiguus, caudal spinal trigeminal nucleus, medial vestibular nucleus, interstitial nucleus of the medial longitudinal fasciculus, ventral part of central gray, paraventricular hypothalamic nucleus and dorsal hypothalamic area, Edinger-Westphal nucleus, and the nucleus Darkschewitsch.

Table 3.3.11: The proportional (% mean ± standard error) distribution of double labelled neurons in different brain nuclei for control animals

Nuclei	P7	P14	P21	P28	P35
n	3	3	3	3	3
Ventral medullary reticular nucleus	6.6 ± 1.2	6.6 ± 1.2	5.6 ± 0.8	5.7 ± 0.2	5.0 ± 1.1
Dorsal medullary reticular nucleus	6.7 ± 2.0	5.4 ± 0.4	4.1 ± 0.9	7.1 ± 0.2	7.1 ± 0.4
Raphe nuclei	11.8 ± 1.3	9.3 ± 1.8	8.7 ± 1.1	8.9 ± 1.1	6.3 ± 0.6
Gigantocellular reticular nucleus	28.7 ± 1.8	27.3 ± 5.6	26.6 ± 1.9	28.2 ± 1.7	25.2 ± 2.9
Lateral vestibular nucleus	18.5 ± 4.6	22.0 ± 1.3	20.4 ± 2.9	16.8 ± 4.0	23.0 ± 2.2
Reticular pontine nucleus	26.1 ± 3.4	23.8 ± 1.8	25.2 ± 0.9	24.8 ± 5.0	20.8 ± 1.5
Locus coeruleus	1.1 ± 1.1	1.8 ± 1.0	3.2 ± 0.3	2.5 ± 0.7	5.0 ± 1.2
Red nucleus	0.7 ± 0.7	3.8 ± 1.6	4.3 ± 1.3	3.6 ± 0.9	7.2 ± 1.3
Other nuclei	0.0 ± 0.0	0.0 ± 0.0	2.0 ± 0.1	2.4 ± 0.3	0.4 ± 0.1

Other nuclei = nucleus ambiguus, caudal spinal trigeminal nucleus, medial vestibular nucleus, interstitial nucleus of the medial longitudinal fasciculus, ventral part of central gray, paraventricular hypothalamic nucleus and dorsal hypothalamic area, Edinger-Westphal nucleus, and the nucleus Darkschewitsch.

Figure 3.3.15: The proportional contribution from different nuclei to the total profile of double labelled neurons following spinal injection in control animals. The proportion in the raphe nuclei at P7 was found to be statistically different from P35 as indicated by *.

Abbreviations

RV	Ventral medullary reticular nucleus
RD	Dorsal medullary reticular nucleus
Raphe	Raphe nuclei
RGc	Gigantocellular reticular nucleus
VstL	Lateral vestibular nucleus
RP	Reticular pontine nucleus
Coe	Locus coeruleus
Rb	Red nucleus
others	Other nuclei

Proportional distribution of double labelled neurons in control animals
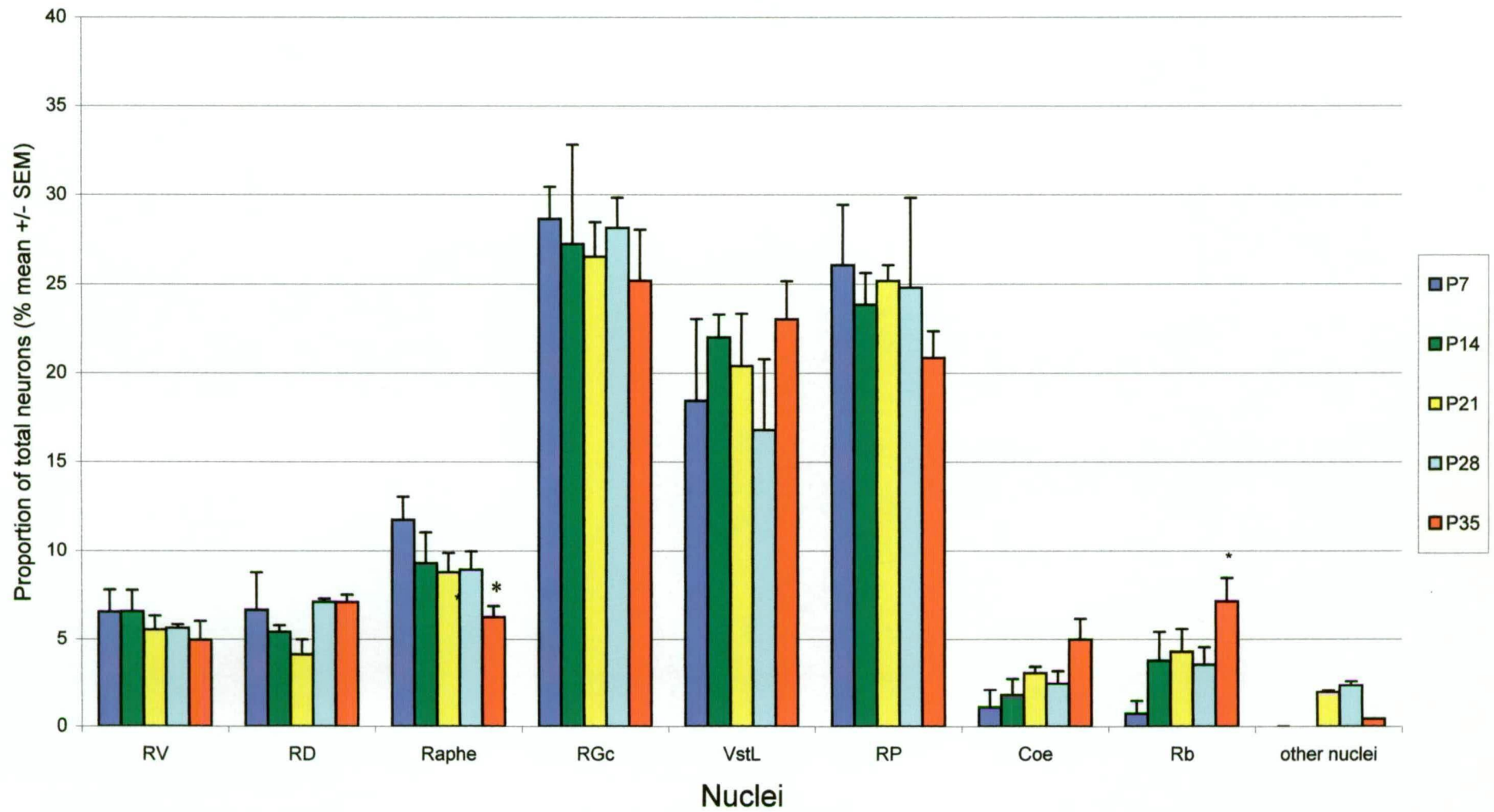
Other nuclei which show consistent labelling of neurons after single injections of either Fluororuby or Oregon green by P7 included the dorsal medullary reticular nucleus, ventral medullary reticular nucleus and the raphe nuclei. However, these represented only small proportions of the total double labelled neurons, although seen consistently from P7 to P35 (see Figure 3.3.15). At P7, the ventral medullary reticular nucleus, dorsal medullary reticular nucleus, and raphe nuclei contributed to the total number; 6.6 ± 1.2%, 6.7 ± 2.0% and 11.8 ± 1.3% respectively and these proportions at all other ages were within 2-4% of the P7 values (See Table 3.3.11).

The locus coeruleus, red nucleus, nucleus ambiguus, and medial vestibular nucleus, which had only small Oregon green labelled neurons at all ages, showed an increase in double labelled neurons from P7 reaching a peak at P35 (approximately 5 cells, see Table 3.3.10). The proportional contribution for the locus coeruleus appeared to increase from P7 to 5.0 ± 1.2% at P35, although not significantly (P = 0.08), while the red nucleus showed a significant increase from P7 to P35 with 7.2 ± 1.3% (P = 0.01). The medial vestibular nucleus showed small numbers of inconsistently double labelled cells at P21 (1.33 ± 0.33) and P28 (1.0 ± 0.6) in 2 of the 3 brains examined at each age as did nucleus ambiguus with double labelled cells at P28 (0.7 ± 0.7) in one of the three brains examined.

The proportion of Oregon green labelled neurons that were double labelled remained consistent in control animals aged P7 to P35 (about 30%) in the ventral medullary reticular nucleus, dorsal medullary reticular nucleus, Raphe nuclei, gigantocellular reticular nucleus, lateral vestibular nucleus, and reticular pontine nucleus (see Table 3.3.12), with no significant differences between these ages for statistical analysis (see Appendix C7). The locus coeruleus and red nucleus showed a gradual increase in proportion of Oregon green labelled neurons that were double labelled.

Spinal transected animals

Post-natal day 7

No double labelled neurons were found in any regions of the brain of P7 animals that had received a pre-injury injection of Oregon green at P4 and the second injection of

Table 3.3.12: The proportion (% mean ± standard error) of Oregon green labelled neurons that were double labelled in control animals

| Nuclei	P7	P14	P21	P28	P35
n	3	3	3	3	3
Ventral medullary reticular nucleus	32.7 ± 6.0	27.0 ± 3.9	27.3 ± 3.9	30.3 ± 3.3	28.3 ± 5.4
Dorsal medullary reticular nucleus	29.6 ± 11.7	26.6 ± 5.9	30.5 ± 1.8	27.9 ± 2.8	32.3 ± 6.4
Raphe nuclei	32.2 ± 7.2	24.9 ± 16.8	31.9 ± 6.9	28.9 ± 9.7	31.6 ± 6.3
Gigantocellular reticular nucleus	28.6 ± 3.1	26.9 ± 8.1	28.1 ± 4.0	26.4 ± 7.4	32.0 ± 5.0
Lateral vestibular nucleus	30.6 ± 6.0	27.2 ± 4.3	31.1 ± 5.1	26.3 ± 5.4	35.9 ± 5.5
Reticular pontine nucleus	27.3 ± 1.5	24.9 ± 5.9	28.9 ± 2.9	23.2 ± 4.0	28.6 ± 4.3
Locus coeruleus	3.3 ± 3.3	12.4 ± 8.5	21.3 ± 3.9	24.6 ± 8.7	43.5 ± 6.5
Red nucleus	4.4 ± 4.4	20.9 ± 2.9	25.7 ± 2.3	22.4 ± 3.1	39.8 ± 6.6
Other nuclei	0.0 ± 0.0	0.0 ± 0.0	23.3 ± 0.3	41.1 ± 4.8	16.7 ± 2.4

Other nuclei = nucleus ambiguus, caudal spinal trigeminal nucleus, medial vestibular nucleus, interstitial nucleus of the medial longitudinal fasciculus, ventral part of central gray, paraventricular hypothalamic nucleus and dorsal hypothalamic area, Edinger-Westphal nucleus, and the nucleus Darkschewitsch.

Fluororuby at P7 immediately following the lesion. Animals were removed 4 days after the second injection, as for all ages.

Post-natal day 14

By P14, very small numbers of double labelled cells (5.0 ± 2.6) were found in two of the three brains from spinally transected animals examined at this age and these represented only 2.4 ± 2.2% of the total number of Oregon green cells detected in P14 brains (see Table 3.3.9 and Figure 3.3.9). It should be noted that when individual Oregon green and Fluororuby labelled neurons were detected in a brain, that individual was included in the calculation of the double labelled means, even if the number of double labelled cells was zero. Thus at P14, three animals showed both dyes in brain stem nuclei, but only two animals showed double labelled cells. However, the mean was calculated including all three, as the presence of both dyes indicated that it would have been possible to label regenerating neurons should there have been any.

The gigantocellular reticular nucleus showed 1.3 ± 0.4 cells in central region of the hindbrain nuclei (labelling in only one animals) and the lateral vestibular nucleus showed 0.7 ± 0.4 in the rostral hindbrain (labelling in only one of three animals). The reticular pontine nucleus showed 3.0 ± 0.8 labelled neurons through the midbrain (labelling in 2 animals, see Table 3.3.13 and Figure 3.3.16). The proportional distribution of such small total numbers will magnify the contribution from each of the nuclei as observed in Table 3.3.14 where the reticular pontine nucleus was seen to contribute 40.7 ± 20.6% to the total number.

The proportion of Oregon green labelled neurons that was double labelled in the lateral vestibular nucleus was low (0.7± 0.4%), while a higher proportion were double labelled in the gigantocellular reticular nucleus, and reticular pontine nucleus (12.1 ± 3.3% and 6.5 ± 2.6% respectively, see Table 3.3.15).

Post-natal day 21

By P21, the number of double labelled cells in the brains of spinally transected animals had apparently increased to 15.2 ± 5.9, but this was not significantly

Table 3.3.13: Number (mean ± SEM) of double labelled neurons counted in different brain nuclei for spinal transected animals from P7 to P35

Nuclei	P7*	P14	P21	P28	P35
n	3	3	6	5	9
Ventral medullary reticular nucleus	- -	0.0 ± 0.0	1.3 ± 0.7	2.6 ± 1.1	1.1 ± 0.5
Dorsal medullary reticular nucleus	- -	0.0 ± 0.0	0.8 ± 0.5	3.6 ± 1.6	1.7 ± 0.6
Raphe	- -	0.0 ± 0.4	1.2 ± 1.7	1.8 ± 9.8	3.9 ± 1.4
Gigantocellular reticular nucleus	- -	1.3 ± 0.4	4.3 ± 1.5	6.2 ± 4.3	6.6 ± 1.8
Lateral vestibular nucleus	- -	0.7 ± 0.4	3.3 ± 1.4	14.4 ± 9.3	2.1 ± 0.8
Reticular pontine nucleus	- -	3.0 ± 0.8	4.2 ± 1.6	17.0 ± 9.5	3.8 ± 1.3
Locus coeruleus	- -	0.0 ± 0.0	0.0 ± 0.0	1.2 ± 1.2	0.4 ± 0.3
Red nucleus	- -	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
others	- -	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

Other nuclei = nucleus ambiguus, caudal spinal trigeminal nucleus, medial vestibular nucleus, interstitial nucleus of the medial longitudinal fasciculus, ventral part of central gray, paraventricular hypothalamic nucleus and dorsal hypothalamic area, Edinger-Westphal nucleus, and the nucleus Darkschewitsch.

*no labelled neurons detected

Figure 3.3.16: Distribution of labelled neurons in the brain stem of spinally transected P14 + 4 *Monodelphis* after lumbar Oregon green injection at P4 and Fluororuby injection at P14.

Camera lucida representation of coronal brain sections from spinally transected P14 + 4 animals showing the location and number of retrogradely labelled neurons after a lumbar Oregon green injection at P4 and Fluororuby injection at P14. Sections shown are from the caudal brain stem (a) to the hypothalamus (g).

Green star = up to 10 Oregon green (single) labelled neurons

Red star = up to 10 Fluororuby (single) labelled neurons

Yellow star = up to 10 double labelled neurons

P14 spinal transected *Monodelphis*

1mm
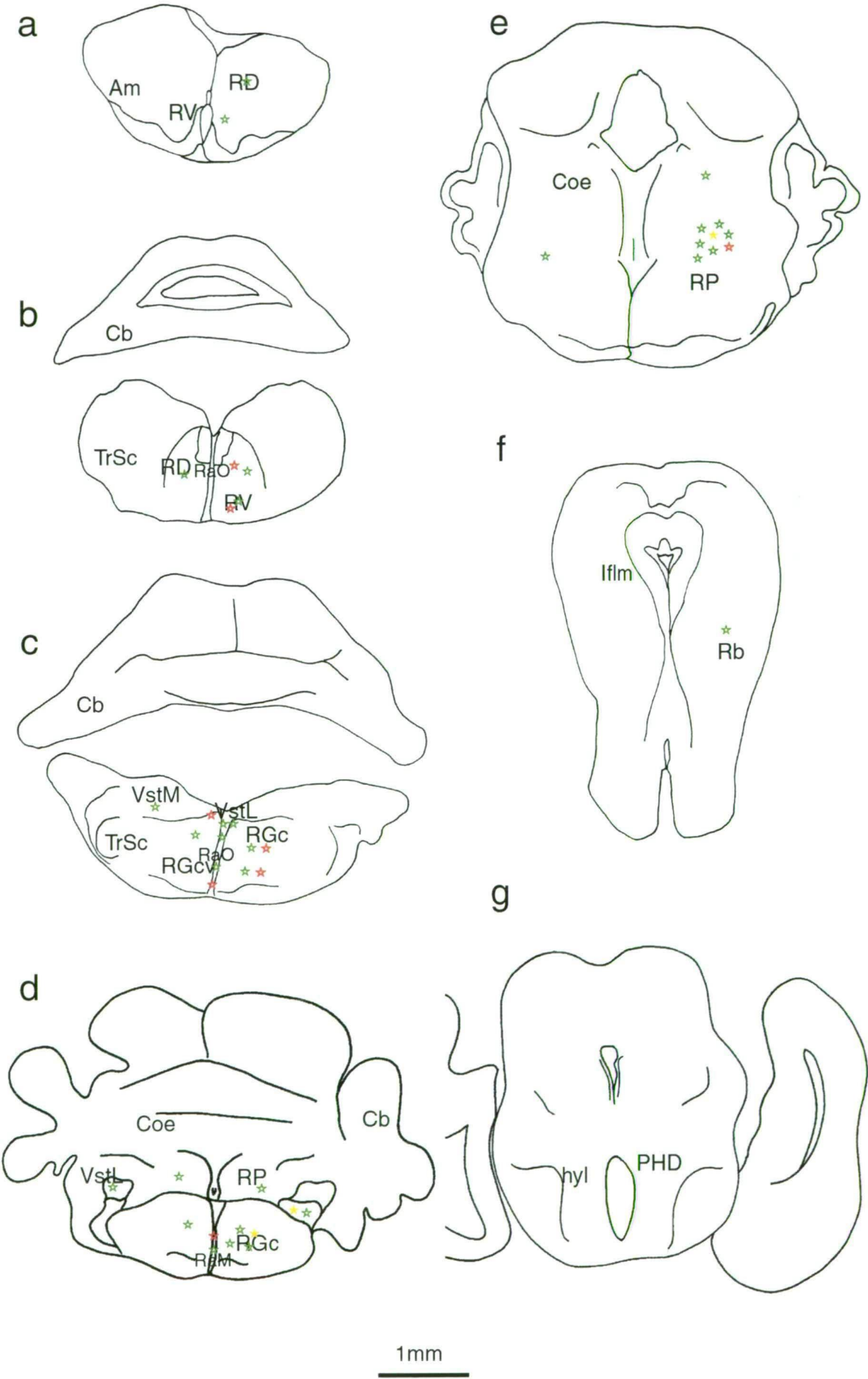
Table 3.3.14: The proportional (% mean ± standard error) of double labelled neurons in different brain nuclei for spinal transected animals

Nuclei	P7*	P14	P21	P28	P35
n	3	3	6	5	9
Ventral medullary reticular nucleus	- -	0.0 ± 0.0	5.2 ± 2.4	2.7 ± 1.1	4.2 ± 1.7
Dorsal medullary reticular nucleus	- -	0.0 ± 0.0	2.7 ± 1.4	3.9 ± 1.8	6.2 ± 2.4
Raphe nuclei	- -	0.0 ± 0.0	4.1 ± 1.4	4.0 ± 1.3	16.6 ± 4.3
Gigantocellular reticular nucleus	- -	14.8 ± 7.4	17.9 ± 3.1	14.1 ± 3.8	28.6 ± 3.8
Lateral vestibular nucleus	- -	11.1 ± 11.1	14.7 ± 4.0	12.9 ± 6.7	8.7 ± 3.7
Reticular pontine nucleus	- -	40.7 ± 20.6	22.1 ± 7.1	16.1 ± 7.3	18.4 ± 5.4
Locus coeruleus	- -	0.0 ± 0.0	0.0 ± 0.0	1.8 ± 1.8	6.2 ± 5.5
Red nucleus	- -	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Other nuclei	- -	0.0 ± 0.0	0.0 ± 0.0	4.5 ± 0.4	0.0 ± 0.0

Other nuclei = nucleus ambiguus, caudal spinal trigeminal nucleus, medial vestibular nucleus, interstitial nucleus of the medial longitudinal fasciculus, ventral part of central gray, paraventricular hypothalamic nucleus and dorsal hypothalamic area, Edinger-Westphal nucleus, and the nucleus Darkschewitsch.

*no labelled neurons detected

Table 3.3.15: The proportion (% mean ± standard error) of Oregon green labelled neurons that were double labelled in spinal transected animals

Nuclei	P7 *	P14	P21	P28	P35
n	0/3	2/3	4/6	3/5	7/8
Ventral medullary reticular nucleus	- -	0.0 ± 0.0	10.3 ± 4.8	21.3 ± 8.4	45.4 ± 15.9
Dorsal medullary reticular nucleus	- -	0.0 ± 0.0	3.0 ± 1.9	22.2 ± 9.8	47.9 ± 15.6
Raphe nuclei	- -	0.0 ± 0.4	7.6 ± 7.4	12.4 ± 14.1	34.9 ± 15.9
Gigantocellular reticular nucleus	- -	12.1 ± 3.3	8.1 ± 6.5	16.0 ± 12.5	34.5 ± 14.2
Lateral vestibular nucleus	- -	0.7 ± 0.4	8.5 ± 3.3	18.1 ± 10.8	17.2 ± 7.7
Reticular pontine nucleus	- -	6.5 ± 2.6	8.7 ± 4.7	18.3 ± 12.0	20.7 ± 7.1
Locus coeruleus	- -	0.0 ± 0.0	0.0 ± 0.0	6.7 ± 6.7	4.3 ± 4.5
Red nucleus	- -	0.0 ± 0.0	0.0 ± 0.0	0.8 ± 0.6	0.0 ± 0.0
Other nuclei	- -	0.0 ± 0.0	0.0 ± 0.0	51.7 ± 4.7	0.0 ± 0.0

Other nuclei = nucleus ambiguus, caudal spinal trigeminal nucleus, medial vestibular nucleus, interstitial nucleus of the medial longitudinal fasciculus, ventral part of central gray, paraventricular hypothalamic nucleus and dorsal hypothalamic area, Edinger-Westphal nucleus, and the nucleus Darkschewitsch.
*no labelled neurons detected

different from P14 (P = 0.39), and it remained a small proportion of the total number of Oregon green labelled neurons (6.5 ± 3.4%, see Table 3.3.9 and Figure 3.3.9). Only four of the six brains examined at this age showed double labelled cells, although all animals were included in the calculation of the mean value for this age as all six animals showed each dye in separate neurons.

Very small numbers of double labelled cells were found in the more caudal regions of the hindbrain in the ventral medullary reticular nucleus (1.3 ± 0.7) and dorsal medullary reticular nucleus (0.8 ± 0.5, see Figure 3.3.17). The raphe nuclei, including the raphe obscurus nucleus and raphe magnus nucleus through the reticular formation also showed small numbers of double labelled neurons at P21 (1.2 ± 1.7, see Table 3.3.13). These numbers represented minor proportions of the total (5.2 ± 2.4%, 2.7 ± 1.4%, and 4.1 ± 1.4% respectively, see Table 3.3.14). However, these nuclei were not consistently double labelled as in some animals, no double labelled neurons were found. Small numbers of double labelled cells were always found in the gigantocellular reticular nucleus (4.3 ± 1.5), the lateral vestibular nucleus (3.3 ± 1.4) and reticular pontine nucleus (4.2 ± 1.6) but due to the low mean number for the occasionally labelled nuclei, these represented large proportions of the total double labelled neurons with 17.9 ± 3.1%, 14.7 ± 4.0% and 22.1 ± 7.1% respectively. The proportional contribution of double labelled neurons from these nuclei were not significantly different to that in animals aged P28 and P35 (see Appendix C9). An increasing proportion of Oregon green labelled neurons was double labelled at this age in different nuclei (see Table 3.3.15).

Post-natal day P28

The total number of double labelled neurons at P28 appeared much higher than at any other age with 59.0 ± 26.7 cells found, but this was only significantly different from the P21 numbers (P = 0.05, see Appendix C8). This represented 17.3 ± 9.1% of the total number of Oregon green labelled neurons, however, only three of the five brains collected at this age contained double labelled cells (see Figure 3.3.9 and Figure 3.3.18).

Figure 3.3.17: Distribution of labelled neurons in the brain stem of spinally transected P21 + 4 *Monodelphis* after lumbar Oregon green injection at P4 and Fluororuby injection at P21.

Camera lucida representation of coronal brain sections from spinally transected P21 + 4 animals showing the location and number of retrogradely labelled neurons after a lumbar Oregon green injection at P4 and Fluororuby injection at P21. Sections shown are from the caudal brain stem (a) to the hypothalamus (g).

Green star = up to 10 Oregon green (single) labelled neurons

Red star = up to 10 Fluororuby (single) labelled neurons

Yellow star = up to 10 double labelled neurons

P21 spinally transected *Monodelphis*

1mm

Figure 3.3.18: Distribution of labelled neurons in the brain stem of spinally transected P28 + 4 *Monodelphis* after lumbar Oregon green injection at P4 and Fluororuby injection at P28.

Camera lucida representation of coronal brain sections from spinally transected P28 + 4 *Monodelphis* showing the location and number of retrogradely labelled neurons after a lumbar Oregon green injection at P4 and Fluororuby injection at P28. Sections shown are from the caudal brain stem (a) to the hypothalamus (g).

Green star = up to 10 Oregon green (single) labelled neurons

Red star = up to 10 Fluororuby (single) labelled neurons

Yellow star = up to 10 double labelled neurons

P28 spinal transected *Monodelphis*

a

RD
RV
Am

b

Cb
TrSc
RD
RV

c

Cb
VstL
VstM
RGc
TrSc
RGcv
RaC

d

Cb
IV
VstL
VstM
RGc
RGcv
RaM

e

Cl
aq
Coe
Cb
RP

f

CS
GCv
Dk
IFLm
ED
Rb

g

PHD
Hyl

1mm
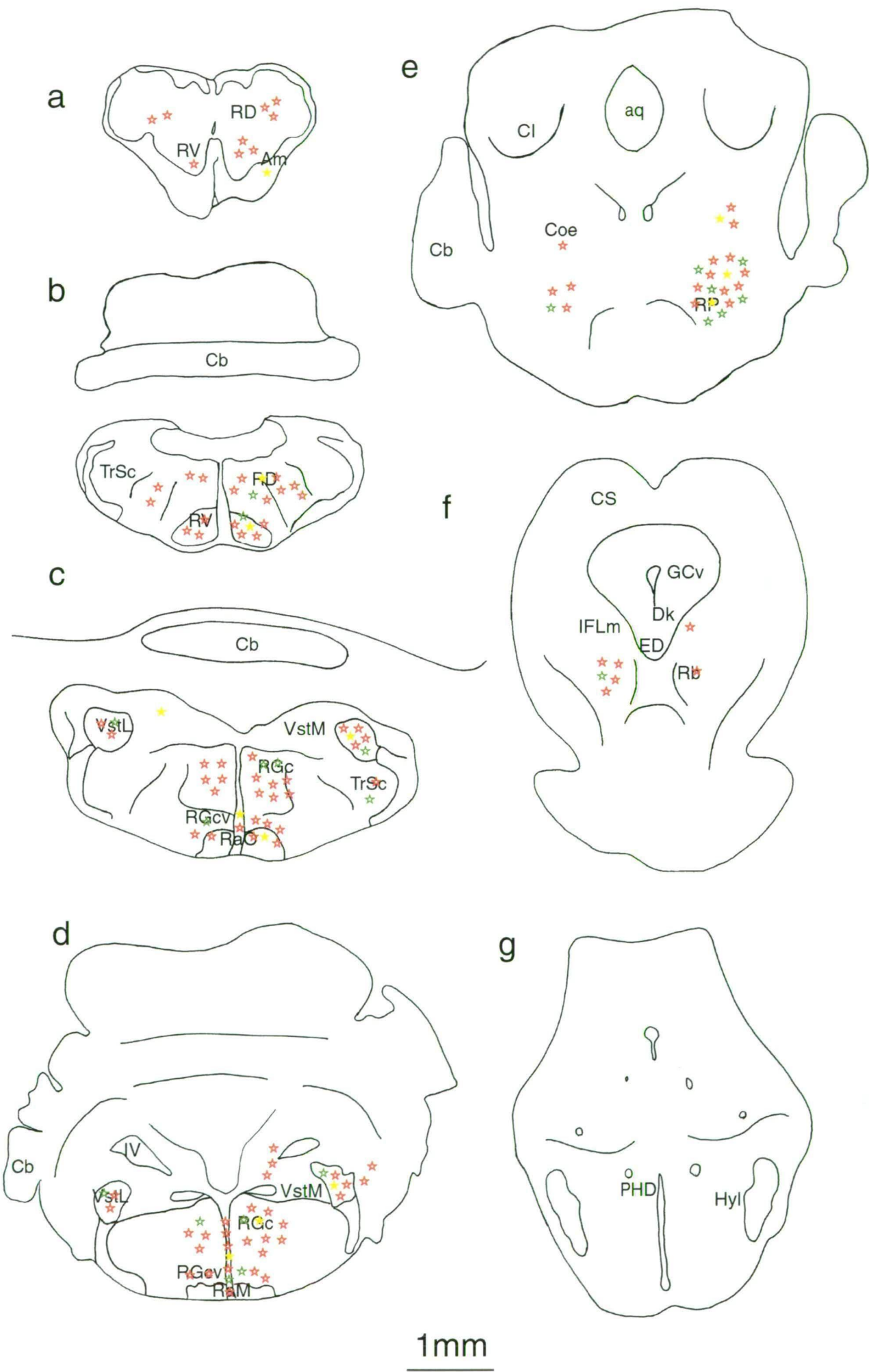
Again, the majority of double labelled neurons were found in the gigantocellular reticular nucleus (6.2 ± 4.3), lateral vestibular nucleus (14.4 ± 9.3) and reticular pontine nucleus (17.0 ± 9.5) representing 14.1 ± 3.8%, 12.9 ± 6.7% and 16.1 ± 7.3% respectively (see Table 3.3.13 and Table 3.3.14). One brain in particular showed high numbers with 50 double labelled neurons in the lateral vestibular nucleus. Double labelled cells were also found in the locus coeruleus in one brain but in very small numbers (1.8 ± 1.8). Minor contributions to the total were also made by the ventral medullary reticular nucleus (2.7 ± 1.1%), dorsal medullary reticular nucleus (3.9 ± 1.8%) and raphe nuclei (4.0 ± 1.3%). The proportion of Oregon green labelled neurons that were double labelled increased was approximately 9% in the different nuclei showing double labelled neurons (see Table 3.3.15). The exception to this was the dorsal medullary reticular nucleus with 3.0 ± 1.9% of Oregon green labelled neurons that were double labelled.

Post-natal day P35

The number of double labelled cells found at P35 after spinal transection were small at this age (19.6 ± 5.6) compared to earlier ages, but as the number of Oregon green labelled cells also dropped at this age, the proportion of Oregon green labelled cells which were double labelled was consistent with that seen at younger ages (29.9 ± 6.7%; see Table 3.3.9 and Figure 3.3.9). Double labelled cells were found in all nuclei that had been observed to contain double labelled cells at younger ages, including the locus coeruleus. Eight of the nine animals examined at this age showed double labelled cells (see Figure 3.3.19)

Once again, the gigantocellular reticular nucleus had the greatest number of labelled neurons with 6.6 ±1.8 which was 28.6 ± 3.8% of the total. Other nuclei in the reticular formation comprised a large portion of the remainder with the reticular pontine nucleus containing 18.4 ± 5.4% and the raphe nuclei contributing 16.6 ± 4.3%. The dorsal medullary reticular nucleus and ventral medullary reticular nucleus showed smaller proportions at 4.3 ± 1.7% and 6.2 ± 2.4% respectively. The lateral vestibular nucleus contributed 8.7 ± 3.7% of the total, but double labelled cells were restricted to the hindbrain region. Unlike control animals, double labelled neurons were never detected in the dorsal cerebellar region in P35 spinally transected

Figure 3.3.19: Distribution of labelled neurons in the brain stem of spinally transected P35 + 4 *Monodelphis* after lumbar Oregon green injection at P4 and Fluororuby injection at P35.

Camera lucida representations of coronal brain sections from spinally transected P35 + 4 *Monodelphis* showing the location and number of retrogradely labelled neurons after a lumbar Oregon green injection at P4 and Fluororuby injection at P35. Sections shown are from the caudal brain stem (a) to the hypothalamus (g).

Green star = up to 10 Oregon green (single) labelled neurons

Red star = up to 10 Fluororuby (single) labelled neurons

Yellow star = up to 10 double labelled neurons
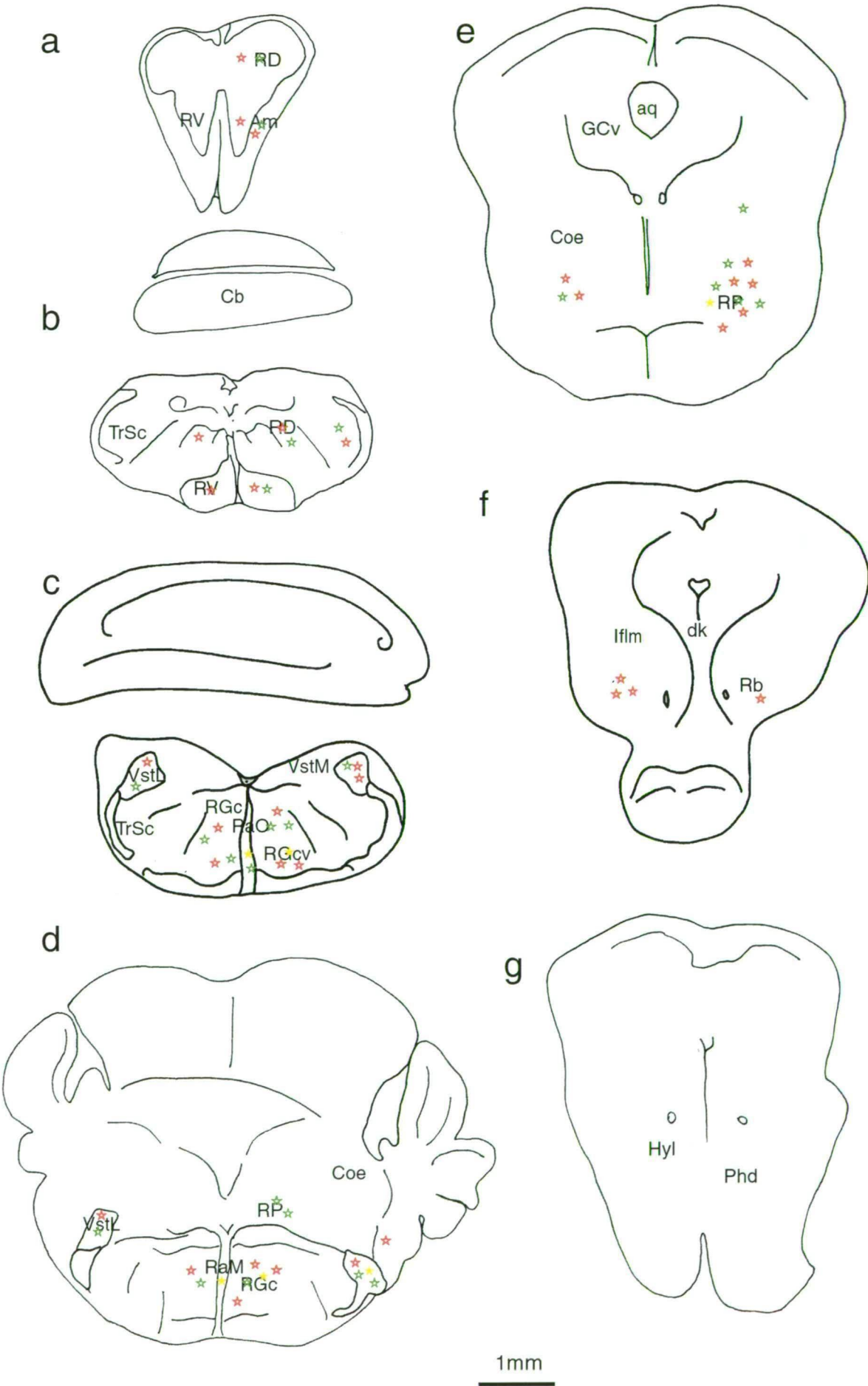
P35 spinal transected *Monodelphis*

a

RD
RV
Am

b

TrSc
RD
RV

c

VstL
VstM
TrSc
RGc
RaO RGcv

d

VstL
VstM
RGc
RaM
RGcv

e

GCv aq
EW
Coe
Cb
RP

f

IFLM dk
Rb

g

PHD
Hyl

1mm
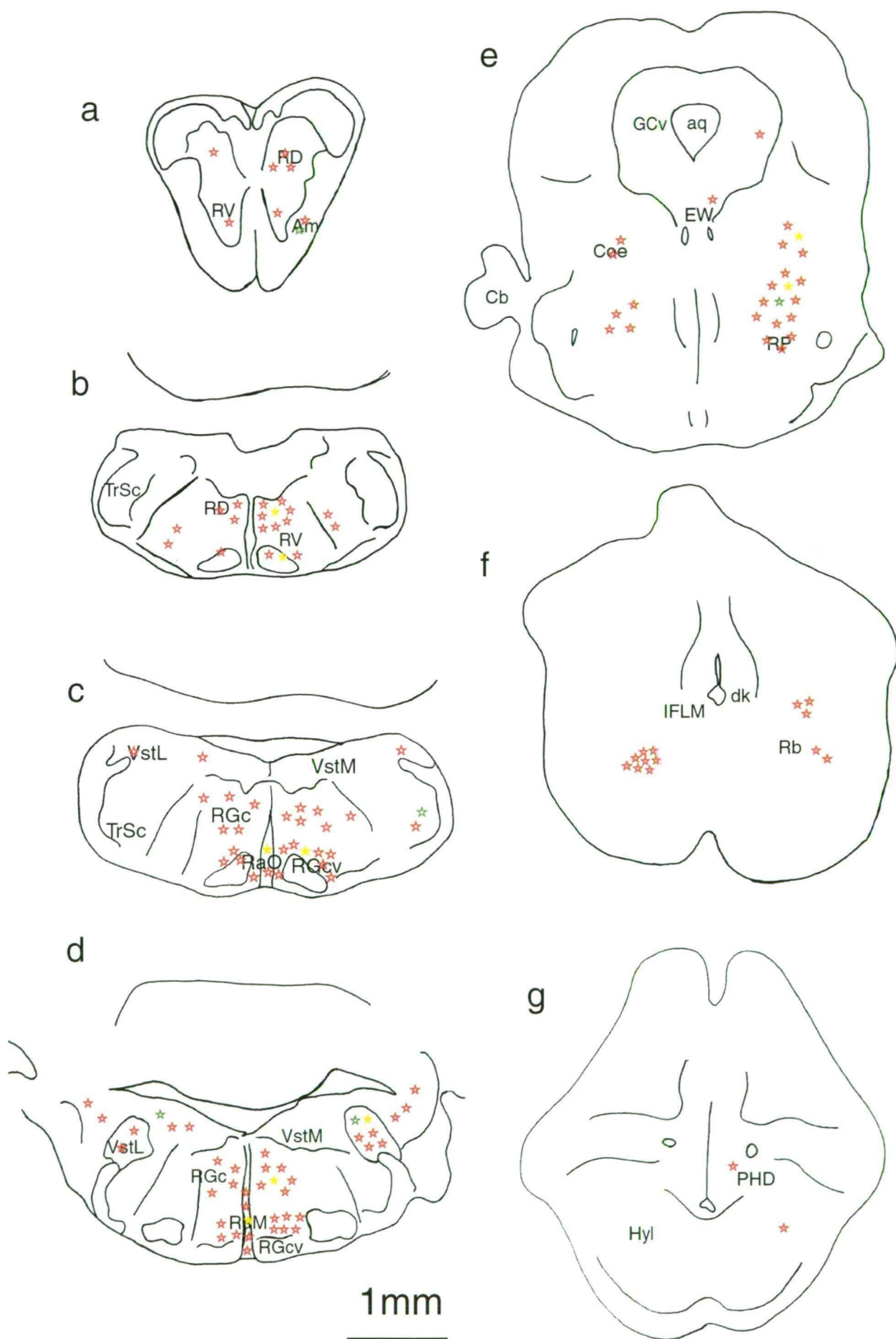
animals, although many individual Fluororuby and Oregon green labelled neurons were found here. The locus coeruleus also contributed a small proportion of the total with 6.2 ± 5.5% of cells.

Approximately 40% of Oregon green labelled neuron in the ventral medullary reticular nucleus, dorsal medullary reticular nucleus, raphe nuclei and gigantocellular reticular nucleus were double labelled, while only about 20% were double labelled in the lateral vestibular nucleus and reticular pontine nucleus (see Table 3.3.15). Despite the decline in the total number of Oregon green and double labelled neurons in different nuclei at P35 from P28 numbers in spinally transected animals (see Table 3.3.6 and Table 3.3.13 respectively), the proportion of Oregon green labelled cells that were double labelled at P35 was consistently higher than that at P14, P21 and P28. However, no statistical differences were found (see Appendix C10). Very few of the locus coeruleus and red nucleus neurons were double labelled and only a small proportion of Oregon green labelled neurons was also double labelled (see Table 3.3.15).

3.3.5 Laterality of Projections

Most of the double labelled neurons were found in the same (ipsilateral) side of the brains to the injection sites in transected animals (see Table 3.3.16). Only a small number of double labelled neurons were found in the contralateral red nucleus whereas all other nuclei showed a strong ipsilateral predominance. Oregon green labelled neurons showed very similar distribution to that of P7 Fluororuby injections but with a slightly more bilateral representation.

In control animals, a similar lateral distribution of double labelled neurons in the brain stem was observed to that after control Fluororuby injections of approximately 70% located ipsilateral to the injection, and 30% contralateral (see Table 3.1.6 and Table 3.3.16.)

Table 3.3.16: The number of double labelled neurons counted on the
ipsilateral and contralateral side of the brain after an injection of
Oregon green at P4, followed by Fluororuby injections in control
animals and spinally transected animals aged P7 to P35.

Age	Control			Spinally transected		
	double labelled neurons (mean ± SEM)	Ipsilateral neurons (mean ± SEM)	Contralateral neurons (mean ± SEM)	double labelled neurons (mean ± SEM)	Ipsilateral neurons (mean ± SEM)	Contralateral neurons (mean ± SEM)
P7	70.0 ± 19.6	50.2 ± 6.9	25.4 ± 4.5	0.0 ± 0.0	-	-
P14	80.3 ± 13.3	53.7 ± 4.7	32.7 ± 3.9	5.0 ± 2.6	5.0 ± 2.6	-
P21	74.7 ± 9.6	57.9 ± 6.7	27.8 ± 5.2	15.2 ± 5.9	12.2 ± 2.1	3.3 ± 2.3
P28	70.3 ± 8.8	47.4 ± 3.2	23.8 ± 4.1	59.0 ± 26.7	39.4 ± 2.1	23.8 ± 4.3
P35	79.7 ± 2.2	59.3 ± 5.3	26.8 ± 5.1	19.6 ± 5.6	18.1 ± 3.2	3.2 ± 2.1

Note that approximately 70% of the total number of double labelled neurons counted
in brains were located ipsilateral to the side of injection, and 30% were contralateral
in control animals. However, in spinally transected animals, most double labelled
neurons were located ipsilaterally.

SUMMARY OF RESULTS

3.1: THE DEVELOPMENT OF SUPRASPINAL PROJECTIONS TO THE LUMBAR SPINAL CORD IN *MONODELPHIS*

- The normal developmental innervation of supraspinal projections to the lumbar spinal cord in *Monodelphis* was established by Fluororuby injections made into the lumbar spinal cord of control animals at P7, P14, P21, P28, P35 and adulthood.

- The total number of retrogradely labelled neurons obtained after lumbar spinal injection increased 4-fold from P7 to P35 then declined in adult animals to approximately twice that of P7 animals. A plateau in the number of labelled neurons was reached at P21 to P35.

- All major supraspinal populations (dorsal medullary reticular nucleus, ventral medullary reticular nucleus, raphe nuclei, gigantocellular reticular nucleus, lateral vestibular nucleus, reticular pontine nucleus, locus coeruleus, red nucleus) showed retrogradely labelled neurons after lumbar spinal injection at P7.

- Other nuclei (nucleus ambiguus, Edinger-Westphal nucleus, nucleus Darkschewitsch, paraventricular hypothalamic nucleus, dorsal hypothalamic area, interstitial nucleus of the medial longitudinal fasciculus, and ventral part of central gray) did not appear until between P14 and P28.

- All supraspinal populations represented in adult profiles were detected by P28 and the contribution from different nuclei to the profile of labelled neurons the brain stem became increasing diverse over development.

3.2 RESTORATION OF SUPRASPINAL PROJECTIONS TO THE LUMBAR CORD FOLLOWING A SPINAL TRANSECTION IN NEONATAL *MONODELPHIS*

- The developmental innervation of supraspinal projections at P7, P14, P21, P28, P35 and adulthood to the lumbar spinal cord in *Monodelphis* across a complete spinal transection was established by Fluororuby injections made in to the lumbar spinal cord.

- Supraspinal projections grew across a complete thoracic spinal transection made at P7 within one week post-injury.

- The total number of labelled neurons obtained after lumbar spinal injection in spinally transected animals increased significantly from P14 to P35, with a plateau at P28 and P35, followed by a significant decline in adulthood.

- Numbers of labelled neurons were mostly lower in spinally transected animals than that in control animals, but at P28 and P35 spinally transected and age-matched control values were not significantly different.

- All supraspinal populations detected in the profile of control animals showed retrogradely labelled neurons after a lumbar injection of Fluororuby in spinally transected animals.

- The gigantocellular reticular nucleus, lateral vestibular nucleus, and reticular pontine nucleus showed the most consistent contribution to the profile of labelled neurons in the early post-injury ages. While the gigantocellular reticular nucleus remained the highest contributor, the profile became increasing diverse by P35 and adulthood, and did not appear different to that obtained from control animals with a similar lateral distribution.

- Labelled neurons in brain stem nuclei in transected animals appeared for the first time 1-2 weeks later than in age matched control animals, but in a similar temporal sequence.

3.3 REGENERATION OF SUPRASPINAL PROJECTIONS IN THE NEONATAL OPOSSUM *MONODELPHIS DOMESTICA*

- A double label experiment was used to determine whether the supraspinal projections growing across the spinal transection had regenerated from injured neurons or were subsequently developing fibres.

- Animals were spinally injected with Oregon green at P4 to label a population of neurons that would have projections severed by a spinal transection at P7. The numbers of these neurons remained stable in control animals, when removed at P7, P14, P21, P28 and P35. In spinally transected animals, Oregon green numbers were stable from P7 to P28 and then a decline was observed at P35.

- Oregon green labelled neurons showed an identical distribution to that seen for control Fluororuby injected P7 animals,

- Control animals that received a double injection of Oregon green at P4 and Fluororuby at P7, P14, P21, P28, or P35 showed consistent numbers and distribution of double labelled neurons through brain stem nuclei at all ages. Approximately 30% of neurons labelled with Oregon green were double labelled at all ages and in different nuclei.

- Spinally transected animals that were injected before and after the injury showed small numbers of double labelled neurons appearing at P14, one week post-injury. The number increased significantly up to P28, and then declined at P35. The number of double labelled neurons in P28 spinally transected animals was not statistically different to that of P28 control numbers, but at other ages, the spinally transected double labelled numbers were significantly lower.

- The proportion of Oregon green labelled neurons that were double labelled in spinally transected animals increased significantly from approximately 2.5% at P14 up to approximately 30% in P35 animals.

- Double labelled neurons were consistently found in the gigantocellular reticular nucleus, lateral vestibular nucleus, and reticular pontine nucleus, with smaller contributions from the dorsal medullary reticular nucleus, ventral medullary reticular nucleus, and the raphe nuclei.

Chapter 4

DISCUSSION

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4.1 NEONATAL *MONODELPHIS* AS A MODEL FOR REGENERATION

The present study demonstrated that in the neonatal *Monodelphis,* a large number of supraspinal projections were able to grow across a complete spinal cord transection made *in vivo* at one week of age. Following injury, fibres began to reappear in the injury site within 7 days and their numbers increased rapidly over the next 4 weeks. Regenerating axons contributed to this outgrowth and were detected within one week post-injury in initially small, but then increasing proportions for the first 4 weeks post injury. Additionally, it was established that supraspinal projections descended to the lumbar spinal cord across a complete thoracic transection in a similar developmental sequence to that of control animals, although in lesser numbers. It would appear that repair after neonatal spinal injury follows a relatively normal pattern of development. Fibre outgrowth across the lesion was maintained into adulthood, although the survival of regenerating axons following transection could not be determined beyond 4 weeks post-injury due to technical limitations (see Section 4.6). The ability to grow axons across the injury site was not confined to particular neuronal populations as all brain stem nuclei seen to project to lumbar levels in control animals, contributed to the axonal outgrowth across the transection site. Regenerating neurons were also distributed throughout all populations of brain stem nuclei that project to the lumbar regions at P7.

Quantitative axonal tracing studies were conducted defining the developmental descent of supraspinal projections to the lumbar spinal cord in both control and spinally transected animals. While previous studies showed that adult *Monodelphis* receiving a spinal transection at P7 had supraspinal innervation to the lumbar regions (Saunders *et al.,* 1998), the time course of the appearance of axons across an injury during the early stages of repair had not been examined. Additionally, the vital question of whether a proportion of transected axons contributing to the fibre outgrowth through a complete spinal transection was regenerating as opposed to newly developed axons, has been examined in the present study using a quantitative approach.

The proposal that a degree of regeneration may occur after axotomy in the CNS under certain conditions is a well established idea. Previous studies of "lower"

vertebrates have shown that supraspinal projections are capable of regenerating a small proportion of severed axons even in the absence of growth promoting aids in many different species. Lampreys (Yin and Selzer, 1983; Zhang and McClellan, 1999), salamanders (Davis *et al.*, 1990), zebrafish (Becker and Becker, 2001), teleost fish (Stuermer *et al.*, 1992) and lizards (Duffy *et al.*, 1992) have all been demonstrated to show spontaneous regeneration of supraspinal projections in adults which leads to considerable behavioural recovery. Other "higher" evolutionary groups showed regeneration only during development; these included frogs (Beattie *et al.*, 1990) and embryonic chicks (Hasan *et al.*, 1993). In mammalian species, it would also appear that during embryonic and early postnatal periods, the CNS is capable of a large degree of plasticity. This plasticity may not necessarily be the result of regeneration of severed axons directly back through the original lesion site. Rather fibre growth may navigate around an incomplete lesion, as seen in neonatal rats (Stelzner *et al.*, 1979), kittens (Bregman and Goldberger, 1982) and newborn hamsters (Kalil and Reh, 1979; Kalil and Reh, 1982). A high degree of functional recovery may be observed after incomplete lesions of the developing mammalian CNS, although it sometimes is mediated by aberrant connections (Reh and Kalil, 1982; Bregman and Goldberger, 1983- III). A limiting aspect of experiments examining fibre outgrowth in immature eutherian mammals is that after birth, only the very latest developing pathways remain to complete their growth. Thus, the permissive period for developmental growth and possible regeneration after injury does not extend for very long after birth even in eutherian species, it is born at a relatively early stage of development comparative to other species, such as the rat.

What has been less observed is the spontaneous regeneration of the earlier developing supraspinal connections after complete transection in mammals. The best evidence for regenerative axonal outgrowth through a spinal cord lesion so far has been seen in the developing *Didelphis* opossum, where a double label experiment, similar to the present study, showed that all groups of supraspinal projections were capable of regeneration after a complete thoracic spinal transection given at postnatal ages from P10 to P25 (Wang *et al.*, 1998). However, this study focused on determining the critical period for regeneration and fibre outgrowth after the lesion,

rather than the time course of the regenerative process as examined in the present study.

Other highly suggestive evidence that regeneration could occur after the transection of thoracic spinal cord in another marsupial, a one week-old *Monodelphis,* was described in experiments performed on *in vitro* preparations of the opossum spinal cord. Detailed *in vitro* experiments involving crush lesions to the spinal cord at one week of age showed significant outgrowth of fibres within 5 days of the lesion (Treherne *et al.*, 1992). Further extension of these experiments by labelling fibres with DiI and closely observing the response of individual axons to axotomy with time–lapse video microscopy revealed a regenerative response (Varga *et al.*, 1996). However, the *in vitro* system is limited due to the short term survival of the tissue in culture (up to about 10 days, Stewart *et al.*, 1991).

A double labelling experimental paradigm has previously been employed to determine the contribution of regenerating and/or subsequent developmental outgrowth to the profile of supraspinal projections seen to grow across the complete lesion site (Hasan *et al.*, 1993; Wang *et al.*, 1998). The present study used a similar technique, and is confined to examining supraspinal projections, which are of particular interest as they mediate locomotor function that may be tested in parallel behavioural studies (see Saunders *et al.*, 1998 for details). A detailed discussion of the methods chosen in this study is presented in the Critique of Methods (see Section 4.6).

Although a neonatal marsupial offers a unique model in which to study the process of regeneration, it also poses a complication due to its immaturity. Any growth seen post-injury could be a mixture of normal development and possible regeneration. The possibility of continued neurogenesis contributing to the observed repair is not thought to be a significant source of recovery (Hasan *et al.*,1993 and Terman *et al.*, 2000). This is discussed further in Section 4.5.

The first part of this thesis (Results: Section 3.1) was devoted to establishing the normal pattern of development of supraspinal projections down to the lumbar spinal cord. In the second part (Results: Section 3.2) the growth of fibres across a complete spinal transection was described and the third part (Results: Section 3.3) established

to what degree regenerative growth contributed to repair across the injury site, given that a large amount of development was yet to occur in the opossum after the time of transection. The results from all three sets of experiments are discussed together below.

4.2: THE DEVELOPMENT OF DESCENDING SUPRASPINAL PROJECTIONS TO THE LUMBAR REGION OF THE SPINAL CORD IN *MONODELPHIS*

It was important to firstly establish the normal developmental timetable of supraspinal innervation in order to determine whether outgrowth across injury site following a lesion at P7 followed a similar pattern of innervation. Injections of Fluororuby made at P7 in control, non-operated animals showed that by this age, all supraspinal populations of neurons that contribute to the majority of lumbar projections in adulthood, were represented although some in small numbers. These included most regions of the reticular formation: the gigantocellular reticular nucleus (including the lateral and ventral paragigantocellular regions), ambiguus nucleus, ventral and dorsal medullary nuclei, raphe nuclei and the pontine reticular nucleus. The lateral vestibular nucleus, red nucleus and locus coeruleus also showed projections from P7, although numbers were small (see Results: Table 3.1.4 and Figure 3.1.6). These projections would therefore be severed by the transection at P7.

A comprehensive, but non-quantitative study by Wang *et al*. (1992) described a similar developmental sequence of supraspinal innervation to the lumbar spinal cord of the *Monodelphis* from birth (P0) to P26 but showed a wider repertoire of supraspinal labelling by P7 than seen in the present study. As previously noted in the Chapter 1: Introduction, Martin and colleagues define the first day of birth as P1, rather than the conventional P0. Therefore, reference to post-natal ages in the present thesis is consistently one day less than described by Martin and colleagues (eg. Wang *et al*., 1992). Adult labelling patterns were observed by P7 while in the present study some nuclei present in adults were not detected until P28. For example, the presumptive paraventricular hypothalamic nucleus showed labelled cell bodies after a P1 lumbar injection of Fast blue (Varga *et al*., 1996), while neurons from the same presumptive nucleus in the present study did not appear until P28 (see Results:

Figure 3.2.7 and Table 3.2.4). It was noted by Wang *et al.* (1992) that neurons in the paraventricular hypothalamic nucleus and dorsal hypothalamic area were only seen when a large volume of dye was injected and in the present study these nuclei were only ever detected in small numbers even at older ages. On the other hand, Holst *et al.* (1991) also found numerous labelled neurons in these nuclei. This highlights the possibility that the present study could be less sensitive at labelling small populations of neurons using the chosen dyes and injection techniques. The rationale behind the methodology used is discussed further in the Critique of Methods (see Section 4.6). Other neuronal populations detected by Wang *et al.* (1992) at P7 that were not seen in the present study included the interstitial nucleus of the medial longitudinal fasciculus, Edinger-Westphal nucleus, nucleus Darkschewitsch, ventral part of central gray, nucleus ambiguus, lateral and ventromedial hypothalamic regions. All of these nuclei were seen at older ages in the present study but again only with small numbers of labelled neurons.

Therefore, it was possible that supraspinal populations other than those detected by the control Fluororuby injections in the present study (Results: Section 3.1) had projections present in the lumbar cord that would have also been severed at P7. Nonetheless as results obtained after Fluororuby injection into control P7 *Monodelphis* were consistent (as were the Oregon green results from P4 injections), the profile obtained for P7 was considered an accurate and reliable reflection of the projections present at the time of injury using methods applied in this study.

As the *Monodelphis* pups matured, the later developing pathways showed greater representation such that the projection profile become more diverse. While the nuclei that dominated the profile at P7 (gigantocellular reticular nucleus, reticular pontine nucleus, and raphe nuclei) remained the more consistently represented populations at all ages in the *Monodelphis*, their proportional contribution to the total numbers of neurons labelled decreased significantly over time (see Results: Figure 3.1.5) as other nuclei were represented by increasing proportions. These nuclei included the ventral medullary reticular nucleus, dorsal medullary reticular nucleus, lateral vestibular nucleus, locus coeruleus, red nucleus and the smaller nuclei grouped together as "other nuclei", including the nucleus ambiguus, interstitial nucleus of the medial longitudinal fasciculus, and the medial vestibular nucleus (see Results: Table

3.1.4). All nuclei observed in the present study to contribute to the lumbar supraspinal profile in the adult *Monodelphis* were already present in P28 control pups (compare Results: Figure 3.1.9 and Figure 3.1.12). Labelled neurons appeared more widely distributed throughout the brain stem as the Edinger-Westphal nucleus, nucleus Darkschewitsch, ventral part of central gray, paraventricular hypothalamic nucleus and dorsal hypothalamic area all showed lumbar projections for the first time at P28. This highlights the asynchronous development of different supraspinal pathways. Wang *et al.* (1992) make the statement that those regions of the brain that mature first show the first projections to the spinal cord.

Adult animals showed the greatest diversity of labelled neurons appearing in different nuclei with a more even distribution than at younger ages. Pups aged P7, P14 and P21 showed that the majority of labelled neurons were found in the gigantocellular reticular nucleus, reticular pontine nucleus and raphe nuclei, indicating that projections from these nuclei descend to the lumbar spinal cord earlier that the populations such as the red nucleus and locus coeruleus. This has implications for the ability of a particular population of neurons to respond to injury as early developing tracts may not retain the ability for outgrowth for very long, reducing the plasticity of the CNS in an asynchronous manner (Wang *et al.*, 1998).

It would seem that the rapid descent of many newly developing fibres occurs in *Monodelphis* up to P21 as shown by the increasing total number of labelled neurons (see Results: Figure 3.1.4). After this time, the outgrowth did not appear as prolific but the diversity of projections continued to increase, indicating a possible developmental organisation process to reach the adult formation (see Results: Table 3.1.5 and Figure 3.1.5).

The highest numbers of labelled neurons in control animals were recorded at P35 after a single injection of Fluororuby (1645.3 ± 226.1), although, statistically, the numbers were not significantly different from those of the control P21 and P28 (see Results: Table 3.1.3). Thus, the period between P21 and P35 is likely to represent the end of developmental growth of the descending fibres. In adult animals, however the total numbers of labelled neurons were significantly lower than the high plateau between P21 to P35 (688.7 ± 207.6) and may indicate a decline in projections to the

lumbar spinal cord resulting from a developmental refinement process whereby projections that do not make functional connections are eliminated (see O' Leary, 1992 for review). Alternative technical explanations for the decline in numbers that are likely to further explain this observation are discussed in the Critique of Methods (see Section 4.6).

An interesting parallel was seen between the number of labelled neurons counted in control animals over a developmental period in the present study and control *Didelphis* in a similar transection experiment described by Wang *et al.* (1996). While quantitative data was only provided for three nuclei (reticular pontine nucleus, lateral vestibular nucleus and red nucleus) following Fast blue injections in Wang *et al.* (1996), it was noted that injections in control animals age-matched to the P4, P11 and P19 transected animals (including 30 – 40 days survival time) showed decreasing numbers in older animals (see Figure 6 in Wang *et al.*, 1996) A similar observation was made in the present study between P35 and adult. For example in Wang *et al.* (1996), red nucleus numbers counted in control animals age –matched to the animals transected at P4 plus survival time (P35 – P45) showed approximately 600 neurons counted in both sides of the brain and in the age-matched controls to P20 animals (plus survival time, P50 – P60), the red nucleus showed about 400 neurons on either side of the brain. No comment was made in reference to this observation by Wang *et al.* (1996).

Fewer newly descending projections appeared to penetrate the lumbar cord after P21, given that the total number of projections did not increase after this time in control animals (see Results: Table 3.1.3). Cabana (2000) agrees that in *Monodelphis*, the bulk of descending pathways grow into the lumbosacral cord within the first 3 - 4 weeks after birth.

4.3: RECONSTRUCTION OF A TISSUE BRIDGE ACROSS A COMPLETE SPINAL CORD TRANSECTION

Within one week of complete transection in the present study, the spinal cord showed a tissue connection across the injury site when examined in whole mounts in nearly

all individuals although with varying degree of repair (see Results: Figure 3.2.1). This was an important aspect of the present study, for if no tissue reconnection was made then no supraspinal projections would be able to reach the lumbar cord. It was also important that a complete transection was made at P7 in order to comment on the repair time course assuming that all connections were severed. This has been established in the Chapter 2: Methods.

It was observed by the author in complementary morphological experiments (Fry, unpublished observations), not included in the present study, that a tissue bridge can form across the lesion site within 3-5 days post injury and that the bridge contained nerve fibres when stained with antibodies against neuronal elements. It is therefore likely that the fibres may have entered the injury site prior to one week post-injury (7 days is the first time point used in the present study). It should be noted that the tracing protocol requires axons to extend several segments past the thoracic injury site to the lumbar cord, but the histological examination of the injury site itself showed fibres that have only just entered the lesion at approximately 4 days post-injury. It is therefore likely that fibres can be detected in the injury site by histological markers before projections were backlabelled by axonal tracers. This is discussed further in the Critique of Methods (see Section 4.6).

Other studies have also shown that fibre outgrowth, not necessarily that derived from supraspinal origins, appears rapidly after an injury to the immature CNS (eg Shimizu *et al.*, 1990; Treherne *et al.*, 1992; Varga *et al.*, 1996; Terman *et al.*, 2000). Complete spinal transection experiments on P4 *Didelphis* pups showed anatomical continuity between the cut ends of the spinal cord within 48 hours of the thoracic injury; silver stained sections of the injury site indicated the presence of axons crossing the lesion site, although the origin of these fibres was not determined (Terman *et al.*, 2000). Similar rapid repair was also observed after a complete transection of the spinal cord in embryonic chicks aged E5, with large numbers of nerve fibres detected across the lesion site within 48 hours of the injury (Shimizu *et al.*, 1990).

Previous studies using the *Monodelphis* have shown that ascending fibres originating from the dorsal root ganglion reappeared across a complete thoracic transection made at P7, by 2 weeks post-injury (Fry, 1997). In that study, the lesion used

(complete cut with scissors) caused a wider separation between the severed ends and this may explain the greater delay. Saunders *et al.* (1998) performed similar lesions to Fry (1997), but did not examine the morphology of the injury site until 2 months post-lesion when a remarkable degree of repair was observed.

In vitro studies in *Monodelphis* showed that a crush injury in the cultured preparation of the spinal cord aged P4 – P10 resulted in profuse outgrowth of fibres across the lesion site by 5 days post-injury (Treherne *et al.*, 1992, Varga *et al.*, 1995). It must be noted that this injury did not result in a complete separation of the severed ends of the spinal cord, although it did sever all axonal projections as indicated both morphologically and by the absence of electrical recording from either side of the lesion.

When the injury site was examined as a whole mount dissection, the anatomical continuity of the bridge between the severed ends was observed to be established by one week post-injury in all transected pups (n = 7) although the lesion deficit was visible as a thinner area over one to two spinal segments (see Results: Figure 3.2.1). This degree of structural repair was also previously observed by Terman *et al.* (2000) when making histological examinations of *Didelphis* spinal cords completely transected at P6.

4.4: THE TIME COURSE OF DEVELOPMENTAL AND REGENERATIVE SUPRASPINAL OUTGROWTH FOLLOWING SPINAL INJURY

Only a few studies have provided quantitative data describing the capacity of the supraspinal projections to regenerate in higher vertebrates (Wang *et al.*, 1998; Bates and Stelzner, 1993; Hasan *et al.*, 1993). However, specific details of experimental protocols must be considered when making any comparisons as differences in species, the age and stage of development at which the lesion is made, the location of lesions, the time and amount of dye injected, which tracer was used and counting techniques employed will all have a significant impact on the amount of regeneration likely to be observed. The studies most directly comparable to the present work were made in the developing North American opossum *Didelphis* (Wang *et al.*, 1998),

neonatal rat (Bates and Stelzner, 1993) and embryonic chick (Hasan *et al.*, 1993), as all of these studies examined regeneration of brain projections after embryonic or neonatal CNS injury in the absence of any growth promoting aids. Another difficulty in making comparisons is that most other double label experiments have chosen to only look for regenerative outgrowth at one or two time points post-injury, rather than construct a time course, as in the present study. However, it is possible to look at different studies to obtain a comparative picture of the time course of regenerative outgrowth. Each stage of repair examined in this thesis is discussed in relation to other studies describing comparable stages of development.

4.4.1: One week post-injury: P14

A small number of brain neurons was retrogradely labelled after lumbar injection of Fluororuby given at P14 to animals transected at P7, indicating that the tissue bridge across the thoracic lesion supported the outgrowth of some descending supraspinal axons for several segments caudal to the lesion. The total number of axons projecting through the injury site at this age was an order of magnitude less than that of control P14 animals (65.3 ± 16.9 compared with 758.3 ± 211.9 respectively; see Results: Table 3.2.2 and Table 3.1.3) indicating that supraspinal innervation to the lumbar cord in transected animals is just beginning.

Not only was the number of retrogradely labelled neurons less in animals with injured cords, but also the number of nuclei that contained labelled neurons was restricted. By P14 in control animals, most of the supraspinal projections seen in adult *Monodelphis* were detected, while transected animals showed only six nuclei contributing to the small number of projecting neurons labelled after the injury (see Table 3.2.3). These nuclei were all of the reticular formation (dorsal and ventral medullary reticular nucleus, gigantocellular reticular nucleus, raphe nuclei and reticular pontine nucleus) and also the lateral vestibular nucleus. The reticular projections have been observed as the most robust populations among the supraspinal projections in the *Monodelphis* (Martin *et al.*, 1975) with consistent and numerous labelled cell bodies and it would appear that this extended to growth following a complete spinal transection.

The gigantocellular reticular nucleus and reticular pontine nucleus were two of the three major contributing regions to first show projections across the injury site at P14 suggesting that the axons of the reticulospinal pathways were able to respond relatively quickly after injury. As this pathway is one of the earliest developing supraspinal tracts in the opossum, along with the vestibular pathways (Wang *et al.*, 1994), they may be in the process of extending many projections to the lumbar spinal cord at the time of injury. The new growth may be poised to continue past the lesion site before later developing pathways, such as the rubrospinal fibres, have reached the thoracic level in greater numbers (Xu and Martin, 1992). This may also be the case for the lateral vestibular nucleus that equally presents a large proportion of the first fibres to cross the injury site, and is known to be early developing (Wang *et al.*, 1992).

Importantly, among the supraspinal projections growing across the injury site in the short repair period of one week, a small number of these (5.0 ± 2.6, n=3) were found to have regenerated from severed axons, as demonstrated by double labelled neuronal cell bodies found in the brain stem. While these only represented 2.4 ± 2.2% of the neurons labelled with Oregon green before the injury and thus available to regenerate, it should be noted that the maximum proportion of Oregon green labelled neurons that were double labelled in control animals (P14 to P35) was approximately 30%, and therefore the proportion of regenerating fibres recorded is probably an underestimate. This is discussed further in the Critique of Methods (see Section 4.6).

Of the few fibres that were regenerating by P14, all were from the reticular pontine nucleus, gigantocellular reticular nucleus and lateral vestibular nucleus (see Results: Table 3.3.13). These nuclei appear to have a vigorous response to axotomy and they also showed the highest proportional contribution to total outgrowth (regenerative as well as developmental) across the lesion at this age. Double labelled neurons appeared in two out of three P14 spinally transected animals, despite the presence of both dyes in separate neuronal populations in all three animals. By contrast, all control animals showed double labelled cells, indicating that if regenerated projections were present below the lesion in transected animals, at least a proportion of these (about 30%) was likely to be labelled. It would appear that the majority of

the supraspinal projections seen across the injury site at P14 was the result of developmental outgrowth of the descending pathways, given the regenerative contribution was relatively small at this stage (5.0 ± 2.6 double labelled neurons compared to 65.3 ± 16.9 labelled with Fluororuby).

Previous studies of repair within one week post-lesion

Retrograde tracing experiments in the embryonic chick aged P5 that involved injecting wheatgerm-agglutinin horseradish peroxidase (WGA-HRP) complex caudal to the lesion 3 days following the transection also reported labelled brain stem neurons (Shimizu *et al.*, 1990). Supraspinal projections contributed to this early repair across the injury site in a manner similar to that found in the present study. These projections were also labelled after the lesion was made at E10, but not if made at E15 (Shimizu *et al.*, 1990). The projection profile was only examined after 3-5 days following the lesion so no information may be gained about the changes to the composition of supraspinal projections to the lower regions of the spinal cord over an extended post-injury period. An extension of this work was made by Hasan *et al.* (1991) in a study primarily focused on functional repair following spinal transection in the embryonic chick. The study also described the appearance of tracer-labelled supraspinal projections through a thoracic transection made at E3 to E14 after 7 days of repair. It was found that chicks transected from E3 to E12 showed virtually no differences in number and distribution of labelled neurons in supraspinal nuclei, while chicks transected at E13 or E14 showed only a few labelled neurons. It was noted that they appeared primarily in reticular and vestibular nuclei, as also observed in the present study, including the gigantocellular reticular nucleus and raphe nuclei in the medulla, as well as the lateral vestibular nucleus and reticular pontine nucleus in the pons. Very small numbers of labelled neurons were observed in the red nucleus and locus coeruleus, which showed no outgrowth in the present study at one week post-injury (see Results: Table 3.2.3). The numbers obtained after control injections in the chick were higher than those in the present study which may be the result of using WGA – HRP, known to be more sensitive than Fluororuby (Köbbert *et al.*, 2000), although Hasan *et al.* (1991) alternated between the two axonal tracers. The numbers obtained in E13 and E14 transected chicks, while higher

overall, were more comparable with those obtained after 1 week post-injury in the present study (see Table 2, Hasan *et al.*, 1991).

It appears that supraspinal outgrowth following injury during early developmental periods occurs readily in several species, but that the degree of outgrowth is dependant on the time at which the injury occurs. Preliminary experiments in which lesions were made at P14 in the *Monodelphis*, followed by identical spinal injection (as described in Methods: Section 2.2.2 and 2.2.3) did show outgrowth of axons through the injury site but in lesser numbers than in the P7 injured animals (Fry, unpublished observations). The time beyond which no further growth would occur after lesion *in vivo* has not yet been established for the *Monodelphis*.

Further evidence that regeneration can occur within one week of injury in the immature CNS was observed in an *in vitro* study with cultured *Monodelphis* spinal cords by Varga *et al.* (1996) who used time lapse video microscopy to directly observe single regenerating sensory fibres after a crush injury in a 5-day-old spinal cord. Within 18 hours a sprouting fibre originating in a dorsal root ganglion immediately caudal to the crush was seen to grow past the injury site for approximately 100μm. This would appear to be a very vigorous regenerative response by the dorsal root ganglion sensory fibres. However, there may be significant differences in response to injury between axonal populations. DRG projections are known to possess a high potential for regeneration associated with their peripheral portion (Neumann and Woolf, 1999). Also the injury in Varga *et al.* (1996) study was a crush that did not completely sever all tissue connections either side of the lesion and thus regenerating fibres have a structural scaffold along which to extend. Additionally, the protocol of the present study required fibres to extend several spinal segments caudal to the lesion to encounter the post-lesion injected Fluororuby. As this would be a distance of at least 400μm and up to several mm in P14 pups (Fry, unpublished observations), there was likely to be a greater delay in detecting regenerative outgrowth across the lesion. The *in vitro* studies are however limited by tissue survival of only up to 10 days in culture and so no further time course of events could be followed. However, these experiments provided the best evidence so far for regeneration in the immature opossum spinal cord and together

with the present study, establish that true regeneration of severed axons takes place following injury to the spinal cord projection in an immature mammalian CNS.

Another study that dealt with the *in vivo* regenerative outgrowth of supraspinal projections at one week post–lesion was performed in embryonic chicks (Hasan *et al.*, 1993). Hasan *et al.* (1993) examined anatomical and functional repair of supraspinal projections in embryonic chicks before and after thoracic spinal lesions made at E10 to E15 using a double label injection protocol. This study showed that regenerating projections contributed to the outgrowth of fibres growing across embryonic spinal transection within one week of complete transection observed previously in Shimizu *et al.* (1990) and Hasan *et al.* (1991). Hasan *et al.* (1993) had a different approach to the present study, as the focus was to establish the critical period *in ovo* when regeneration was no longer possible. Brains were examined for double-labelled neurons at one week after the injury and no comments on the persistence of regenerating cells found in animals beyond this time point were made. Nevertheless the study of Hasan *et al.* (1993) showed that the numbers, range and distribution of regenerating double-labelled neurons increased in E10 to E12 transected chicks. Transections made at E13 to E15 produced much less outgrowth across the injury, reflected by lower numbers of second dye-labelled neurons and double labelled neurons, indicating the onset of non-permissive growth period in the chick CNS.

Hasan *et al.* (1993) studies presented the proportion of double labelled neurons by calculating the number of double labelled cells compared to the total number of cells containing the second dye. The numbers labelled with the second dye potentially indicate projections that have grown across the lesion site, either by regeneration or subsequent development, thus showing the contribution by regenerating fibres to total successful growth of axons after the injury. Increasing proportions of neurons were found to be double labelled from approximately 5% in E10 transected chicks to approximately 20% at E12. No regenerating neurons were detected in animals lesioned after this time.

The present study has defined the proportion of regenerating fibres as the percentage of those labelled by the first dye (Oregon green). This focused on what proportion of

severed axons was able to regenerate. In contrast to the present study, Hasan *et al.*
(1993) showed that similar numbers of neurons were labelled with both the first and
second dyes in animals transected E10 to E12, which suggested that little
developmental outgrowth occurred between the first and second injection. The
present study showed a large difference between the numbers of labelled neurons
obtained after the first injection at P4 and the second injection at P7, P14, P21, P28
and P35 indicating that subsequent development of fibres extend to the lumbar spinal
cord during this time. To make a direct comparison between the present study and
the study of Hasan *et al.* (1993), the proportions of regenerating neurons may be
recalculated as proportions of the total neurons labelled with the first dye (as in the
present study) from the data presented in Table 1 of Hasan *et al.* (1993; see p26 of
Chapter 1: Introduction). Data for the gigantocellular reticular nucleus, lateral
vestibular nucleus and interstitial nucleus of the medial longitudinal fasciculus (the
only nuclei reported) then shows that the proportions of double labelled neurons
were approximately 8% in animals transected on E10 and appeared to decrease to
4% in animals transected at E12. It would also appear that the reticular and vestibular
nuclei in the Hasan *et al.* (1993) study were also particularly vigorous in their
response to injury and were amongst the first to regenerate with a large contribution
of axons growing through the injury site. The numbers of neurons labelled with the
first dye after injection at E10 – E15 increased with age suggesting continued
developmental growth of fibres between these ages. Thus, between E10 and E15
there would be a greater number of axons available to regenerate. Most of the
developmental growth to the lumbar spinal cord in the chick has ended by E11
(Okada and Oppenheim, 1985; Hasan *et al.*, 1991) and therefore limited numbers of
fibres remain to contribute to subsequent developmental growth across the injury. It
was thought to be unlikely that subsequent development could be responsible for all
the fibres growing across the lesion site as numbers of neurons labelled with the
second dye in control and transected animals were similar.

In the present study the proportion of regenerating fibres at one-week post lesion was
approximately 2.5% of those labelled by the first injection. By comparison, the chick
study (Hasan *et al.*, 1993) suggests that following the transection at E10, a
regenerative response was mounted with greater initial vigour within the first week

than the P7 transected *Monodelphis* of the present study. This may be because the CNS of the chick at E10 is at earlier stage of development than the opossum at P7, although the period of developmental outgrowth of fibres to the lumbar cord is thought to end at E11 (Okada and Oppenheim, 1985). However, the present study has shown that developmental outgrowth continues to the lumbar spinal cord until at least P21 in the *Monodelphis* and thus it is unlikely that the E10 chick would be at a much earlier stage of development than the P7 *Monodelphis*. Additionally, a transection at E12 in the chick showed similar values to that of the P7 *Monodelphis* (approximately 4% and 2% respectively).

4.4.2: Two weeks post-injury: P21

By two weeks post injury, increasing numbers of supraspinal axons had extended across the transection site but the numbers were still lower than in control animals (approximately 350 labelled neurons compared to 1450 in control animals). Nevertheless similar developmental patterns were observed between the profile of supraspinal populations of transected and control *Monodelphis* pups. Brain stem nuclei were represented in similar proportions between the two groups and this indicates that relatively normal developmental innervation occurred in the early repair period, although with less total fibres growing through to the same lumbar level.

Monodelphis examined at P21 (n=6) following spinal transection at P7 showed that the majority of projections from the lumbar spinal cord originated in the gigantocellular reticular nucleus, lateral vestibular nucleus, and reticular pontine nucleus. This was also the case in the control animals (compare Results: Figure 3.1.8 and Figure 3.2.6) although some differences also existed. The nucleus ambiguus and red nucleus also showed small numbers of projections across the injury site for the first time at P21, which was one and two weeks respectively behind their detection in control animals. The locus coeruleus and interstitial nucleus of the medial longitudinal fasciculus did not show any labelling in transected P21 animals unlike the control P21 animals, suggesting that these nuclei have a lag in their outgrowth ability after a spinal lesion (see Results: Table 3.2.4).

The contribution of regenerating fibres to the growth across the injury site at P21 remained small with approximately 15 double labelled neurons found in transected P21 animals (see Results: Table 3.3.9). The source of these projections included the appearance for the first time of labelled neurons in the dorsal medullary reticular nucleus, ventral medullary reticular nucleus and raphe nuclei adding to the profile along with the dominant gigantocellular reticular nucleus, reticular pontine nucleus and lateral vestibular nucleus. The number of regenerating neurons represented approximately 7% of the neurons labelled with the first dye. It should be noted that the maximum amount of double labelled cells obtained in the control injection at P21 remained approximately 30% thus the true number of regenerating neurons is probably higher.

At two weeks post-injury it would appear that the majority of outgrowth was derived from developmental growth of fibres still in the process of descending to the lumbar spinal cord. Double labelled neurons (approximately 15) made a small contribution to the total (350). No double labelled neurons were observed in the red nucleus, locus coeruleus, medial vestibular nucleus, nucleus ambiguus, and caudal spinal trigeminal nucleus suggesting that none of these populations have regenerating fibres by P21 descending to the lumbar regions despite all these nuclei having a small number of projections growing through the injury site as indicated by neurons labelled with Fluororuby. Most outgrowth through the injury site at P21 from these nuclei was likely to be the result of subsequent development (see Results: Figure 3.3.17). This may illustrate the lag between the early appearance of subsequently developing fibres and slower regenerative outgrowth where axons have to reassemble cytoskeletal elements and recover from the effect of axotomy (Conti and Selzer, 2000).

Delayed reappearance of regenerating fibres through the injury site

The cause of why there is a lag between the reappearance of regenerating fibres as compared to subsequently developing fibres is likely to be a physical effect. During development, a growth cone is formed at the leading edge of the growing axons and it exerts a pulling force driven by a microtubule interaction, extending fibres in a

direction determined by other extrinsic forces such as guidance molecules (Guthrie, 1999).

A different mechanism for axonal extension has been proposed for regenerating neurons, based on studies of lower vertebrate such as goldfish (Lanners and Grafstein, 1980) optic nerve, the lamprey spinal cord (Lurie *et al.*, 1991), rat optic nerve (McKerracher *et al.*, 1993b) and parallel observations on the PNS.

Instead it is proposed that regenerative extension of severed axons in the lamprey CNS is due to an internal propulsive force exerted by the transport of neurofilaments along the microtubules into the growing tip of an axons (Jacobs *et al*, 1997). The proposed push mechanism of regenerative axonal extension is likely to be much slower than the more dynamic actin/myosin interaction which pulls microtubules into the growth cone because that is based on a molecular machine while microtubules transport along the axons provide the propulsive force for neurofilaments to invade the growth cone. The reason why the pull mechanism is not employed after axotomy by regenerating axons may be due to developmental changes in the CNS microenvironment. Distances to travel are much further in the adult and a greater calibre of fibre is required with a greater tensile strength. Additionally, the growth cone may no longer be able to establish the stability of actin with the extra cellular matrix due to developmental changes in the adhesion molecules (Conti and Selzer, 2000). It is entirely possible in the present study that, a different mechanism of axonal extension between regenerating and developmental outgrowth is present as suggested by the observed delay. A closer examination of the molecular basis for such processes would be required to answer this problem.

Preliminary immunohistochemical studies of the injury sites in the transected spinal cords in the present study showed that the severed ends fill almost immediately with neurofilaments and growth cones, in greater density than control non-transected spinal cords (Fry, unpublished observations). The high density of neurofilaments may suggest the presence of regenerating axons from injured neurons extending by the regenerative mechanism described above.

Previous studies related to 2 weeks post-injury

The present work can be most directly compared to that of Wang *et al.* (1998), which investigated whether regeneration of supraspinal projections contributed to outgrowth of fibres across complete thoracic lesions made at different stages of development in the North American opossum (*Didelphis*). Their study also employed a double label axonal tracing technique, although it only examined the fate of axons at one time-point post-injury (two weeks), and thus did not give an indication of further capability of axons to regenerate beyond this time. It also only examined fibres that had grown a short distance across the lesion without investigating their ability to grow for considerable distances.

Wang *et al.* (1998) study calculated the proportion of double-labelled cells as a proportion of those labelled with the second dye, in contrast to the present study (see above). Spinal transections were made at P9, P12, P19 and P24 in *Didelphis* pups and the P10 lesion data were most likely to be the best comparison to the P7 transection experiments of the present study. Wang *et al.* (1998) reported that approximately 50% of neurons labelled with the second dye were double labelled in the reticular pontine nucleus, locus coeruleus, lateral vestibular nucleus, raphe nuclei and ventral medullary reticular nucleus after complete spinal transection at P9 in *Didelphis*. This indicated that approximately half the fibres from these populations that had been able to grow through the injury site were derived from regenerating fibres. If the data from the present study at two weeks post-injury are calculated using the Wang *et al.* (1998) formula, then a much smaller proportion of fibres that grow through the injury site have regenerated (about 4.3%).

While no quantitative data were presented in Wang *et al.* (1998), it was apparent from a representative camera lucida plot of a P9 transected animal that many more neurons contained the first dye than the second dye. Should the proportion of double-labelled cells be represented as a proportion of all those labelled with the first dye, as in the present study, a much smaller percentage would be obtained. This was in contrast to the present study where many more neurons were labelled with the second dye. It is likely that the majority of outgrowth across the injury site in the present study was due to subsequent developmental of new projections, while in the

Didelphis, as there was less fibre growth through the lesion in total, fibres from regenerating neurons contributed a higher proportion.

The fact that the lesion was made at P7 in the present study as opposed to P9 in the Wang *et al.* (1998) study on *Didelphis* may explain these differences. It may have allowed more fibres growth to traverse the lesion. Additionally, as the number of projections growing across the lesion in the present study did not reach maximum numbers until 4 weeks post lesion, it is possible that more fibres would have been able to grow through the lesion at later time points in Wang *et al.* (1998). What is obvious, however, is that regenerative outgrowth may proceed even when the neonatal CNS is more mature and very little developmental outgrowth remains. It appears that the permissive nature of the immature CNS maybe retained until all projections have reached their final innervation pattern.

4.4.3: Three weeks post-injury: P28

By three weeks post-lesion, the injury site often appeared to have undergone considerable structural repair and in some cases it was difficult to determine the exact site of the injury (see Results: Figure 3.2.1). There was a significant increase in numbers of Fluororuby labelled neurons detected in the brain stem of transected animals at P28 as compared to the younger ages suggesting that many more fibres have grown across the injury site since P21. The maximum number of labelled neurons was observed at P35 but this value was not significantly different from P28 (see Results: Table 3.2.2 and Figure 3.2.3).

The spinally transected animals lag behind in the appearance of fibres from certain nuclei compared to control animals and it would seem that the spinal cord following injury has the capacity to increase the number of projections beyond P28. This is in contrast to control animals that showed no increase in the number of labelled neurons beyond P21 (see Results: Table 3.1.3 and Figure 3.1.4). This suggests that the spinal cord remains permissive to outgrowth of fibres at least up to P28 and that final adult innervation pattern is not yet established despite the fact that little continued developmental growth occurs afterwards. The asynchronous nature of different tracts development means that even if most have finished their outgrowth, the CNS

environment must remain permissive until the final organisation of the nervous system has been achieved. For example, the red nucleus and locus coeruleus continue to increase their proportional contribution to the total profile up to adulthood (see Results: Figure 3.1.5).

An important observation made at P28 was that the number of regenerating fibres was the highest at this age with nearly 60 double labelled neurons found in the brain stem (mean value from 6 animals; see Results: Table 3.3.9 and Figure 3.3.8). This was the closest value to the number of double labelled cells found in the control animals (about 70) suggesting that despite the delay, a transected spinal cord is capable of regrowing close to the original normal innervation over time. This still remains only a small proportion of the total projections crossing the lesion site at this age (approximately 1000), the majority of fibre outgrowth across the lesion site appeared to be the result of subsequent development. The design of the present study was such that it was not possible to determine the absolute contribution of regenerating neurons to total outgrowth across the lesion site, only a proportion.

The proportion of axotomised neurons that were double labelled increased from P21 to P28; one likely explanation is that it takes some time for the fibres to grow through the lesion site and reach the lumbar spinal cord where the second injection was made. Populations that contributed to this regenerative growth included all of the major reticular nuclei seen at P21 (dorsal medullary reticular nucleus, ventral medullary reticular nucleus, gigantocellular reticular nucleus, raphe nucelli , and reticular pontine nucleus) and also the vestibular nuclei (lateral and medial). The locus coeruleus and nucleus ambiguus also showed small numbers of double labelled neurons. A notable absence from the regenerative profile was that of the red nucleus (see Results: Figure 3.3.17).

However, while there was evidence of a few rubral projections appearing for the first time at P7 (see Results: Section 3.1), this tract was at very early stages of development. A similar observation was also made by Wang *et al.* (1992), who first detected retrogradely labelled rubral neurons at P3 in small numbers in *Monodelphis*. The Oregon green injections in the present study were made at P4 and thus very few rubral projections would have been present at the lumbar level at this age. As the

technique appeared only to double label approximately 30% of the total neurons containing the first dye (see Results: Figure 3.3.9), the chances of seeing neurons from nuclei with very few projections in a double label profile, were very small. Additionally, neurons at very early stages of development appear more susceptible to axotomy (Snider *et al.*, 1992). This has been observed previously by Xu and Martin (1992) in the red nucleus of *Didelphis* lesioned at P12 , P22, P 30, P40 and P50, where up to 75% of rubral neurons had degenerated by 30 days post-lesion. An element of axotomy-induced cell death may be an influence on the absence of rubral neurons in the regenerating profile. On the other hand cell numbers of these neuronal populations were so low at the age of first injection that it is not possible to make a definite statement regarding the response of rubral neurons to axotomy.

Evidence for repair at 3 week post-injury: related studies.

A double label study in the rat by Bernstein–Goral and Bregman (1993) further supported the concept that eutherian mammals are able to regenerate their severed axons should they either be lesioned early in development or if presented with a more conducive environment (in this case, fetal implants). They asked the additional question of whether axons might extend the regenerative outgrowth beyond the transplant, as this had important implications for a success to reach correct targets to support functional recovery.

The Bernstein–Goral and Bregman (1993) study also examined the regenerative capacity of different neuronal populations, including the corticospinal tract. However, this study made an over-hemisection lesion of the dorsal funiculus at the thoracic level, rostrocaudal gradient of development meant that the corticospinal tract was not actually severed by the lesion made at 1 or 2 days of birth as it had not yet descended to that level. Therefore, it was not possible to examine the regenerative response of this pathway, but only those of earlier developing populations. In this study, the raphe, coerelar and rubral projections were examined in addition to the corticospinal tract. The study demonstrated that by 3-6 weeks after the injury approximately 30% - 35% of neurons labelled with the first dye in the red nucleus, locus coeruleus and raphe nuclei regenerated their severed axons up to 5 mm past the lesion site. This figure is comparable to that found after double injection

in the present study, although the second injection made at T10 may be placed at distance greater than 5mm from the lesion (T4-T6), particularly in older animals. In the Bernstein – Goral and Bregman (1993) study the proportion of double labelled neurons fell to approximately 10% when the second dye was injected more than 5 mm past the injection site. In the absence of the transplant, no regenerating neurons appeared to be present in the red nucleus, locus coeruleus or raphe nuclei. The absence of double-labelled neurons without a transplant was interpreted as being due to axotomy-induced cell death without the trophic support of the transplants. Therefore, if a lesion is made even at very early stages of post-natal development in the rat, axotomy will cause cell death in most neuronal populations. The developing *Monodelphis* CNS environment, in contrast to the rat, seems to support the extension of fibres for a further distance beyond the lesion. It would seem that fetal transplants allow both regenerating axons and subsequently developing axons to grow through a spinal lesion and then extend for considerable distance beyond the lesion (Bernstein –Goral and Bregman, 1993).

As these experiments (Bernstein –Goral and Bregman, 1993) pooled the data from animals collected 3-6 weeks after the lesion, it was not possible to determine whether there was a difference between these 2 ages. No differences were found in the regenerative capacity of the red nucleus, locus coereolus and raphe nuclei (about 30%). In the present study at 3 weeks post-injury (P28), differences were seen in each of these nuclei: the red nucleus showed no double labelled neurons, the raphe nuclei showed approx 4% increasing to 16%, at 4 weeks post injury and the locus coeruleus showed approximately 2%, increasing to 6%. It is important to consider that the neonatal rat, even immediately after birth, has a more developed nervous system than the *Monodelphis* and most pathways in the rat will be reasonably mature at the time of injury, whereas in the *Monodelphis* the different nuclei will be at more asynchronous earlier stages of development.

4.4.4: Four weeks post-injury: P35

The present study showed that a substantial degree of repair occurred after complete transection at P7 and suggests that transected animals complete relatively normal development of supraspinal projections to the lumbar regions. At P35, the number of

Fluororuby labelled neurons found in spinally transected animals (1234.1 ± 203.1) was not significantly different from that of age-matched controls (1645.3 ± 226.1; see Results: Table 3.2.2 and Figure 3.2.2). Both transected and control animals appeared to have a similar developmental innervation of supraspinal projections to the lumbar spinal cord, which is an important indication that the transected spinal cord may achieve the correct reorganisation. Complementary behavioural studies also indicate that functional connections have been made (Saunders *et al.*, 1998), although the possibility of adaptive reinnervation cannot be discounted. More specific transynaptic tracing would be required to determine whether specific neurons innervate the correct targets.

In transected animals, the numbers of Fluororuby labelled neurons plateaued at P28 - P35, one to two weeks behind that of the control animals which occurred at P21 (see Results: Table 3.1.3 and 3.2.2). This delay between cut and control animals has been consistently observed and is most likely due to the delayed growth of new fibres traversing the gap created by the lesion. Otherwise the growth rate of supraspinal innervation compares well with the uninjured control animals. The fact that such a degree of structural recovery occurs with a substantial tissue bridge across the lesion site (see Results: Figure 3.2.1) and also with near-normal total number of projections, demonstrates the great capacity of the immature opossum spinal cord to recover from injury.

Spinally transected animals aged P35 showed the full diversity of supraspinal populations seen in control adult animals, including the prominent projections from the reticular and vestibular nuclei (see Results: Figure 3.2.3). This demonstrated that all supraspinal populations detected by the present study as having lumbar projections in *Monodelphis*, were capable of sending fibre outgrowth across the complete transection made at P7. A more important consideration is whether all these populations were able to regenerate after transections of their fibre projections. Unfortunately due to the experimental design, it was inevitable that some populations were not labelled by either the P4 Oregon green injection. These include the interstitial nucleus of the medial longitudinal fasciculus, paraventricular hypothalamic nucleus, dorsal hypothalamic area and ventral part of central gray. Wang *et al.* (1992) reported the presence of all these populations by P7 when

examining the origins of lumbar supraspinal projections over development using Fast blue injections. Reasons why the present study and Wang *et al.* (1992) reported different timetables for the development of some populations are discussed further in the Critique of Methods (see Section 4.6).

There were a few interesting differences observed between the distribution of populations in transected and control animals at P35. Firstly, gigantocellular reticular nucleus indicated that it had most lumbar projections with consistent numerous labelled neurons at all ages in spinally transected animals (see Results: Figure 3.2.4), while the control animals showed a decreasing contribution from this nuclei (see Results: Figure 3.1.5). This indicates that the gigantocellular reticular nucleus has a particularly vigorous response to outgrowth after injury by comparison with other nuclei. This is in contrast to the lateral vestibular nucleus, which in control animals increased its proportional contribution from P7 to adulthood, reflecting a continued outgrowth of the pathway over development such that it became one of the most dominant components of the adult profile (see Results: Figure 3.1.5). However, in transected animals the opposite trend was seen: the lateral vestibular nucleus was one of the major contributors immediately following injury but then over development (P7 to P35) its proportional contribution decreased to be one of the smaller components of the adult profile (see Results: Figure 3.2.4). This may reflect a less vigorous ability of the lateral vestibular nucleus to extend outgrowth (both regenerative and developmental) across the injury site.

All other populations appear to reflect similar processes to those seen in control animals: the ventral medullary reticular nucleus, dorsal medullary reticular nucleus, red nucleus, locus coeruleus, collective "other nuclei" all increased their proportional contribution over time as they developed. The reticular pontine nucleus and raphe nuclei were among the earliest projections to reach the lumbar regions and this was reflected by a decrease in proportional contribution for both. They were likely to have completed developmental extension of projections into the lumbar spinal cord prior to those nuclei that increased their proportional contributions over development.

Contribution of regenerating axons to outgrowth at P35

The total number of double labelled neurons at P35 in transected animals (19.6 ± 5.6) was less than that for P28 (59.0 ± 26.7), although the decline was not statistically significant due to large scatter (including one animal with none, and two with less than 10). This was mirrored by a similar decline in total Oregon green labelled neurons at this age from stable numbers at younger ages (see Results: Table 3.3.9). As the number of double labelled neurons is dependant on Oregon green cell numbers, this may reflect a degree of cell death among the axotomised neurons caused by an inability to reach their intended target and associated trophic support required for survival (see Snider *et al.*, 1992 for review on axotomy induced cell death). The fact that Oregon green cell numbers did not fall in control P35 animals suggests that axotomy had some effect on the long term survival of neurons. Interestingly, an increasing proportion of the total double labelled Oregon green cells remaining at P35 in transected animals was double labelled (approximately 30%), despite the decline in total numbers of these neurons (see Results: Table 3.3.9 and Results: Figure 3.3.9). This value was not significantly different from that seen for control animals at P35 (approximately 30%) and indicated that most of neurons initially severed by the transection that were labelled with Oregon green, regenerated their axons by P35. The proportion of double labelled Oregon green cells in some individual nuclei at P35 was found to be up to 45% in the ventral medullary reticular nucleus and dorsal medullary reticular nucleus, approximately 35% in the raphe nuclei and gigantocellular reticular nucleus, and approximately 20% in the reticular pontine nucleus and lateral vestibular nucleus (see Table 3.3.15). While it may appear that the ventral medullary reticular nucleus and dorsal medullary reticular nucleus have a greater ability to regenerate than other nuclei, the variation between the individual animals meant that it was difficult to make such distinctions. Given that control animals showed similar variation in individual nuclei (see Results: Table 3.3.7), it was concluded that by P35, all the six nuclei named above showed a similar ability to regenerate a large proportion of their neurons with lumbar projections surviving transection at P7. The locus coeruleus showed a much lower proportion of double labelled Oregon green cells in spinally transected animals (see Table 3.3.15) but as the numbers of Oregon green cells and double labelled cells were very

small (less than 2; see Results: Table 3.3.6 and Table 3.3.14), this nucleus was not a consistent presence in the regenerating profile.

The ability to regenerate following axotomy may confer great survival capacity on an axotomised neuron; this is likely to be related to the fact that those axotomised neurons (Oregon green labelled) that regenerate through the lesion site to reach a correct target have access to the appropriate trophic support (Snider *et al.*, 1992).

Other studies that have examined axotomy-induced cell death in the neonatal nervous system have also found that approximately 30 days after axotomy, large numbers of neurons die. For example, in the neonatal *Didelphis* red nucleus, 75% of neurons died 30 days after spinal transection (Xu and Martin, 1992) and in the facial nerve of neonatal rats 90% of neurons had died 30 days after transection (Soriede, 1981).

The neurons that remained at P35 in the double label experiment were therefore likely to consist mostly of those that regenerated and reached their target. The survival of neurons that were unable to regenerate across the lesion site or to make an appropriate connection with a target would have had a limited survival capacity in the absence of target derived trophic support (Oppenheim, 1985).

The gigantocellular reticular nucleus and reticular pontine nucleus were particularly consistent among the regenerating profiles at P35 (indicated by double labelled neurons), as also observed at P14 to P28, confirming their ability to extend not only large numbers of subsequently developing fibres back through the injury site, but also a small number of regenerating fibres (see Results: Figure 3.3.19). While the contribution of regenerating fibres from each nucleus remained much less than the total outgrowth across the injury site, it would appear that those populations that comprise the majority of projections in control animals would also do so after transection in operated animals.

The fact that most populations present at P7 have shown a capacity for some regenerative outgrowth by P35 (see Section 4.4.3) means that the ability to regenerate is not necessarily associated with particular supraspinal population. Similar conclusions were also drawn by Bernstein-Goral and Bregman (1993).

Rather it is more likely to be associated with the developmental stage at which the injury occurred. This has been proposed previously by Martin and colleagues, who showed that the time at which the injury occurs has the most influence over the populations that may regenerate or extend developmental outgrowth across a lesion site (Wang *et al.*, 1996; Wang *et al.*, 1998).

Other studies examining repair at 4 weeks post-injury.

A study by Wang *et al.* (1996) examined the growth of supraspinal axons across a transection lesion of thoracic spinal cord in *Didelphis*, made over a wide range of post-natal ages (P4, P11, P19, P25, and P32). The study found that after lesions made at P4, all supraspinal populations labelled in age-matched control animals were seen to grow back through the lesion by 30 –40 days later. This was a similar finding to the present study in the *Monodelphis* examining outgrowth in animals 4 weeks post lesion (compare Results: Figure 3.1.10 with Figure 3.2.8). In the Wang *et al.* (1996) study lesions made at older ages, even a few days later, produced outgrowth that became asynchronously represented with the medial reticular pontine nucleus and lateral vestibular nucleus no longer in the profile of projections through the thoracic injury site, along with lesser numbers than seen in control animals. Progressively less numbers of labelled neurons were detected in the brain stem in animals lesioned at P19 and P25, with only medullary raphealar and rubral projections seen after lesions made at P25. This highlighted the fact that these particular populations have longer critical periods for outgrowth after injury, as compared to other reticular and vestibular projections (Wang *et al.*, 1994). Animals lesioned at P32 showed no further outgrowth of supraspinal projections and this was considered to be the end of the critical period for all supraspinal projections in *Didelphis*.

A similar study also in *Didelphis* (Wang *et al.*, 1998) showed that regenerating axons contributed to the outgrowth in similarly decreasing numbers and originated from restricted locations, with increasing age at which the lesion was made. Lesions made at P24 showed that regenerating axons were entirely restricted to the red nucleus and the only other nucleus showing outgrowth (not regenerative) was the medullary raphe (Wang *et al.*, 1998).

4.4.5: Adult animals

Adult animals that had received a complete spinal transection at one week of age showed remarkable structural recovery (see Results: Figure 3.2.1) such that in some cases it was difficult to determine the exact location of the lesion. There was a degree of variation within this repair but all animals showed the maintenance of supraspinal projections that had grown across the injury since the P7 transection.

Spinally transected adult animals showed a relatively small number of Fluororuby labelled neurons (186.8 ± 75.6). In fact the total number was less than that counted for all other ages except at P14 (see Results: Table 3.2.2). It was a significant reduction from the maximal P35 values, and similar reduction was also observed between the control P35 and control adult values. This may partially be due to a developmental refinement process whereby extraneous neurons are eliminated by a naturally occurring cell death cycle along with the withdrawal of transient projections (see Vaux, 1993 for review). Stolp *et al.* (2002) examined the gigantocellular reticular nucleus in the brain stem of the developing *Monodelphis* opossum for the presence of apoptotic neurons. Haematoxylin and eosin stained sections showed that neuronal cell bodies displaying the morphological characteristics of apoptotic cells were most abundant at P14 and by P35, very few were visible. This suggests that developmental cell death occurs earlier in development and does not explain the decline in numbers in the adult. On the other hand, cells displaying characteristics of necrosis were seen to increase significantly by P35 in transected animals (Stolp *et al.*, 2002)

Alternatively, another explanation would be that technical aspects of the present study also may have influenced the number of labelled neurons; such as the fact that the larger adult spinal cord architecture is more densely packed and complex, thus the injected dye may not contact so many projections in the lumbar spinal cord despite the volume of dye administered pro ratio (see Results: Table 3.1.2). This point is discussed further in the Critique of Methods (see Section 4.6.2).

In the present study it was not possible to determine whether regenerating axons contributed to the fibre outgrowth seen in the adult animals as in the present study,

the dextran amine dyes were not found to be stable in neuronal cell bodies for periods of time longer than 5-6 weeks. However, the presence of some regenerating neurons at P35 meant that some were likely to remain until adulthood. This continued survival of regenerating neurons has important implications for the application of future remedies to promote regeneration and the window of time in which they do survive to mediate function in the nervous system.

Other studies examining repair in adult animals after neonatal CNS injury

It is well documented that immature animals can show a high degree of structural and functional recovery from CNS injury by adulthood in many different species, eg. cat (Bregman and Goldberger, 1983), rat (Bernstein and Stelzner, 1983), *Didelphis* (Wang *et al.*, 1998), and also the *Monodelphis* (Saunders *et al.*, 1998).

Adult *Monodelphis* which received either a thoracic cut or a crush lesion at P7 or P8 were shown in adulthood to have substantial numbers of supraspinal projections to the lumbar cord across the injury in many of the nuclei also shown in the present study (Saunders *et al.* 1998). The distribution of neurons was also very similar to that observed in the present study. These same animals were also shown to have developed relatively normal behavioural functions, including the ability to swim using all limbs and climb a pole. These activities require intact supraspinal projections suggesting that the projections identified by the tracing study as growing through the injury contribute to this behaviour. Similar behavioural tests were made on the adults used in the present study and their behaviour was observed not to be different from that of control animals or those tested in Saunders *et al.*, 1998 (also Fry, unpublished observations).

The question of whether regenerating neurons maintain their projections into adulthood was addressed by Bates and Stelzner (1993) in rats using a double label paradigm after overhemisection of the cervical spinal cord at P4, P6 and P12. When these animals were examined as adults, 2 - 7 months later, small numbers of corticospinal projections were double labelled in four out of 13 cases. This confirms that immature neurons which regenerate after axotomy are capable of long term survival but this ability appears to be confined to a small percentage of all those axotomised. The next step would be to establish if those surviving neurons have any

special characteristics and whether it is possible to rescue other neurons that at present seem to be incapable of regenerating.

4.5: OTHER DEVELOPMENTAL EVENTS IN THE SPINAL CORD

4.5.1:The endpoint of developmental outgrowth after spinal transection

The present study indicated that the fibre outgrowth across the injury site continued for longer in transected animals during development than seen in control animals (see Results: Figure 3.1.4 and Figure 3.2.3). However, it should be noted that the ability to extend outgrowth in normal, control animals could well be retained for longer than the time of cessation of new projections penetrating the lumbar region (indicated by no further increase in numbers of labelled neurons). This could be related to the target innervation process where once enough fibres have reached a certain level, the competition for limited targets and associated trophic support means that no further fibres are extended (Burek and Oppenheim, 1996). After an injury, the situation is different as fewer fibres have reached the targets at the comparative time and thus outgrowth of newly arriving fibres may be observed for a longer period. The present study demonstrated that the opossum spinal cord remains permissive to new outgrowth for at least up to 3 weeks after spinal transection.

The possibility that continued neurogenesis of newly descending brain stem or spinal neurons also contributed to repair seen was considered. Hasan *et al.* (1993) discounted this possibility in the chick as the supraspinal neurons became post-mitotic prior to the time at which the lesion was made. The chick and the opossum may have different developmental time tables. However, Terman *et al.* (2000) found many dividing cells in the thoracic cord at P5 in the *Didelphis* and at P20 small numbers around the central canal. Terman *et al.* (2000) examined the contribution of newly proliferated cells to the reconstruction of the spinal cord after complete transection in *Didelphis* at P5 or P20 with sub-cutaneous injections of BrdU over a time course post –injury (hours – days) . The results of this study were slightly ambiguous suggesting that dividing cells were detected in the vicinity of the lesion site but that it was unclear whether any newly divided neurons were contributing to

the structural repair. As no gray matter was observed through the repair site of the P20 lesioned spinal cord, it was unlikely that any cell division had created new tissue, but that in the P5 lesioned cases dividing neurons or glia were present through the injury site.

As no cell bodies (neuronal or glial) were detected by the histological examination of the repaired spinal cord in the present study (see Results: Figure 3.2.1), it was considered that neurogenesis does not play a large role in the reconstruction of the tissue bridge after spinal transection.

4.5.2: Myelination

The onset of myelination is also often associated with the end of the permissive period for outgrowth (Caroni and Schwab, 1988; Schnell and Schwab, 1990) due to the presence of inhibitory membrane-bound Nogo proteins (Chen *et al.*, 2000). Varga *et al.*, (1995) examined the cultured *Monodelphis* spinal cord for the development of myelin at P6 – P14 using electron microscopy and immunohistochemistry. At P8 a few thinly myelinated fibres appeared in the ventral region of the cervical spinal cord along with small numbers of oligodendrocytes. The myelin sheaths became well compacted over the examined period and the number of myelinated axons and oligodendrocytes increased significantly, spreading through to the dorsal region of the cervical spinal cord. The myelination process was described as proceeding from the brain stem in a caudal direction down the spinal cord, and from ventromedial to lateral and dorsal regions of the spinal cord reflecting the developmental gradient of the *Monodelphis* CNS. Astrocytes became morphologically distinguishable from radial glial cell at P9 in the ventral region of the cervical cord and numbers increased in the ventral horns up to P14. The radial glial scaffolding had given way to astrocytic cells by this time.

Preliminary investigations of the comparative development of myelin in the thoracic spinal cords of control and P7 spinally transected *Monodelphis* when aged P14 and P14, were made using electron microscopy, but were not a major part of this thesis project. Examination of epon embedded section of thoracic spinal cord from control P14 *Monodelphis* under the electron microscope showed some lightly myelinated

axon bundles at different stages of development mainly located in the ventrolateral regions (see Figure 4.1a and 4.1b). By contrast, sections through the centre of the similar thoracic region that encompassed the injury made one week earlier at P7 showed no sign of myelination in the ventrolateral region, despite the presence of many axons through the injury site (see Figure 4.1c). A notable difference in the cellular organisation between the control and transected spinal cords was the presence of many astrocytic processes throughout the injury site of transected spinal cords in close association to the nerve fibres (see Figure 4.1d). This may illustrate a glial accumulation at the site of injury forming a scaffolding of the tissue bridge across the lesion site, through which many fibres extend.

By P35 in the control animals, large diameter heavily myelinated axons bundles were seen in the same ventrolateral thoracic spinal region, densely packed among smaller myelinated axons (see Figure 4.2a). In transected animals aged P35 while many myelinated axons bundles were visible through the thoracic injury site, the large diameter, heavily myelinated bundles were absent (see Figure 4.2b).

These studies are only preliminary and would have to be extended over a longer period of time using more material. However, it would seem that the initial outgrowth across the injury site, whether regenerative sprouts or subsequent development, is unlikely to be myelinated and the normal development of this process may be delayed after injury. As myelination probably does not start, or is very preliminary, until an axon reaches its target and has begun to form a synaptic connections (Dr. Tessa Gordon, personal communication), the absence of myelination may indicate that while fibres have grown through the injury site at P14, neither regenerating or subsequent development have yet made synaptic connections with targets. By P35, however, this process may have been completed.

The spinal cord diameter of the lumbar region continued to increase through development until adulthood (see Results: Table 3.1.2) suggesting that at least some developmental events continue after the outgrowth of new projections to the lumbar regions has ended. The development of non–neuronal elements within the spinal cord, such as glial cells, would contribute to an increasing diameter of the spinal cord. Synaptogenesis and myelination take place after a fibre terminations

Figure 4.1: Electron micrographs of P14 control and spinally transected
Monodelphis spinal cord

a) A transverse section through the ventrolateral mid-thoracic region
    of a P14 control *Monodelphis* spinal cord. Most of the axons are
    as yet unmyelinated (indicated by white stars) but some are
    becoming myelinated (Ax) and can be seen at different stages of
    this process. Scale bar = 5 μm.

b) A high power micrograph of the box region in a) showing axon
    bundles at different stages of myelination. Ax 1 and Ax 2 are only
    lightly myelinated while Ax3 shows a thicker compact myelin
    sheath. Scale bar = 1 μm.

c) A section taken through the mid-thoracic injury site of a P14
    spinally transected *Monodelphis* showing that the same
    ventrolateral region as a) contains only small unmyelinated axon
    bundles. Scale bar = 5 μm.

d) The injury site also showed a proliferation of glial processes (GP)
    extending through the surrounding spinal tissue. This was not
    observed in control animals. Scale bar = 1μm.

a

b

Ax3

Ax2

Ax1

Ax

Ax

Ax

control P14

control P14

c

d

GP

GP
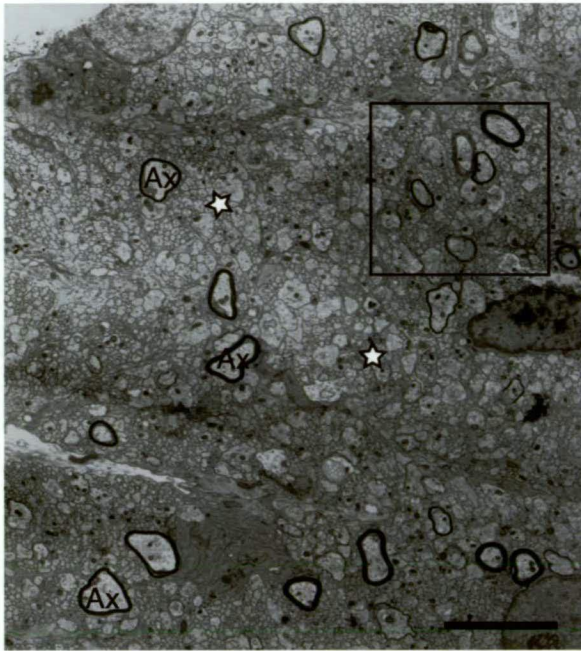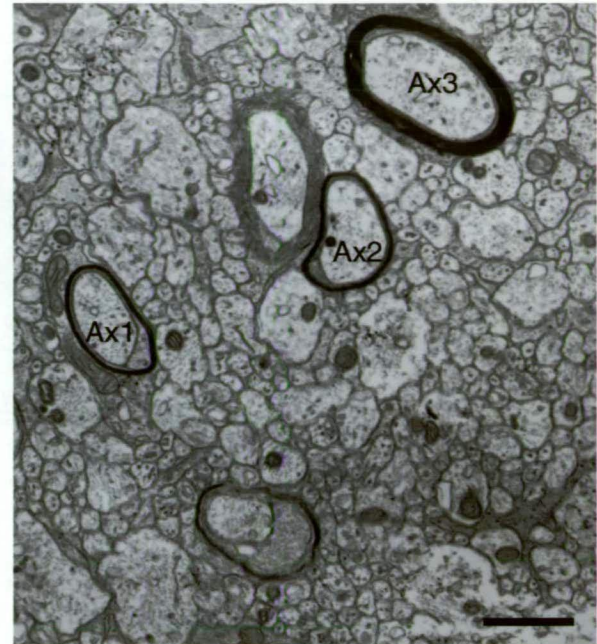
spinally transected P14
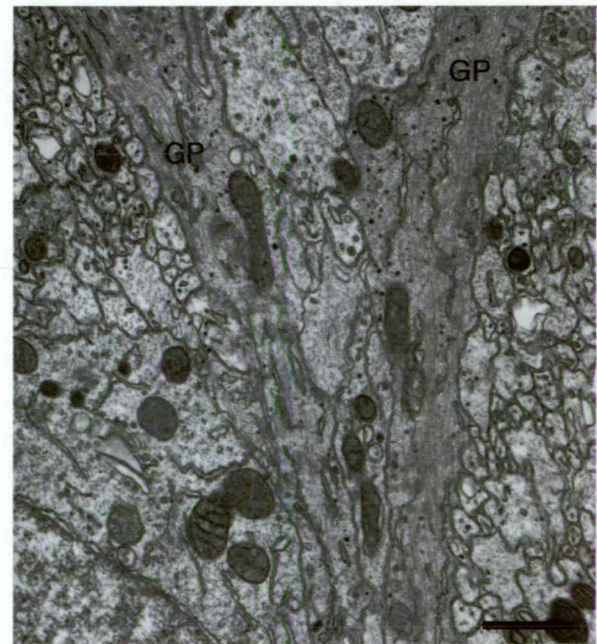
spinally transected P14

Figure 4.2: Electron micrographs of P35 control and spinally transected *Monodelphis* spinal cord

a) The ventrolateral mid-thoracic region of a P35 control *Monodelphis* spinal cord showing large diameter, heavily myelinated axon bundles (LDAx), along with smaller myelinated axon bundles (Ax). Scale bar = 5 µm.

b) The injury site of a P35 *Monodelphis*, spinally transected at P7, showing that many small, myelinated axon bundles (Ax) are present through the same spinal cord region as seen in a), along with unmyelinated bundles (indicated by the stars). Note the absence of large diameter myelinated bundles. Scale bar = 5µm.

a

Ax

LDAx

LDAx

LDAx

Ax

Ax

P35 control

b

Ax

Ax
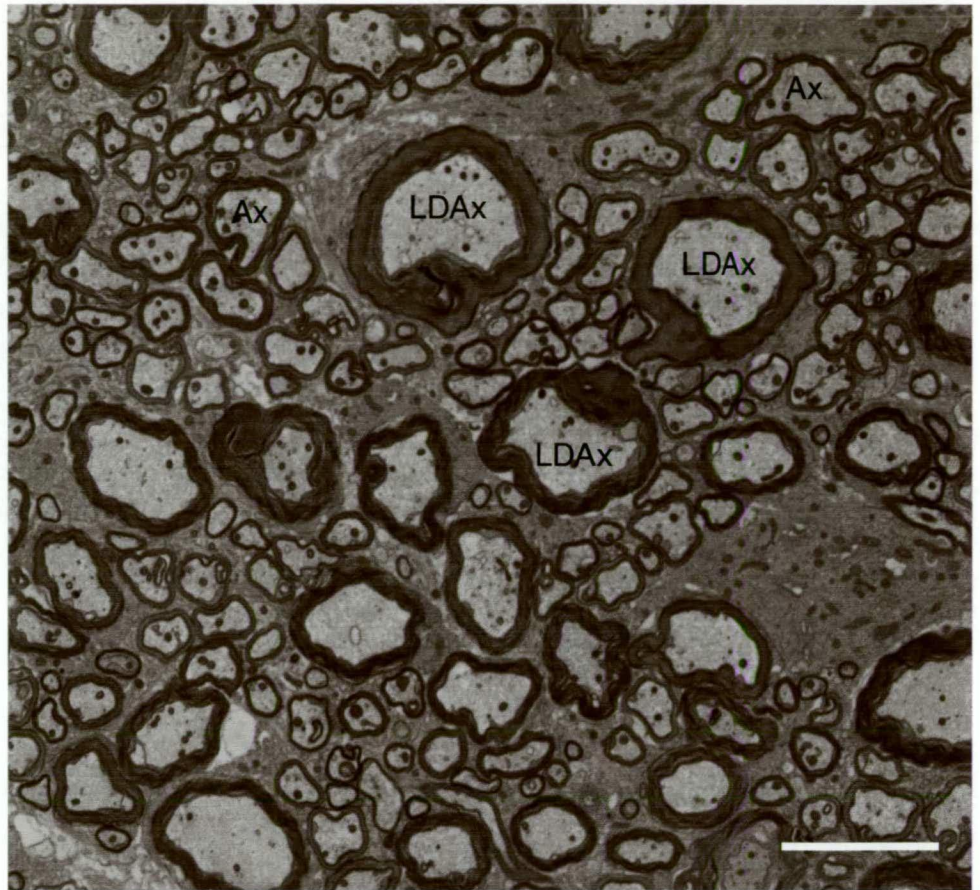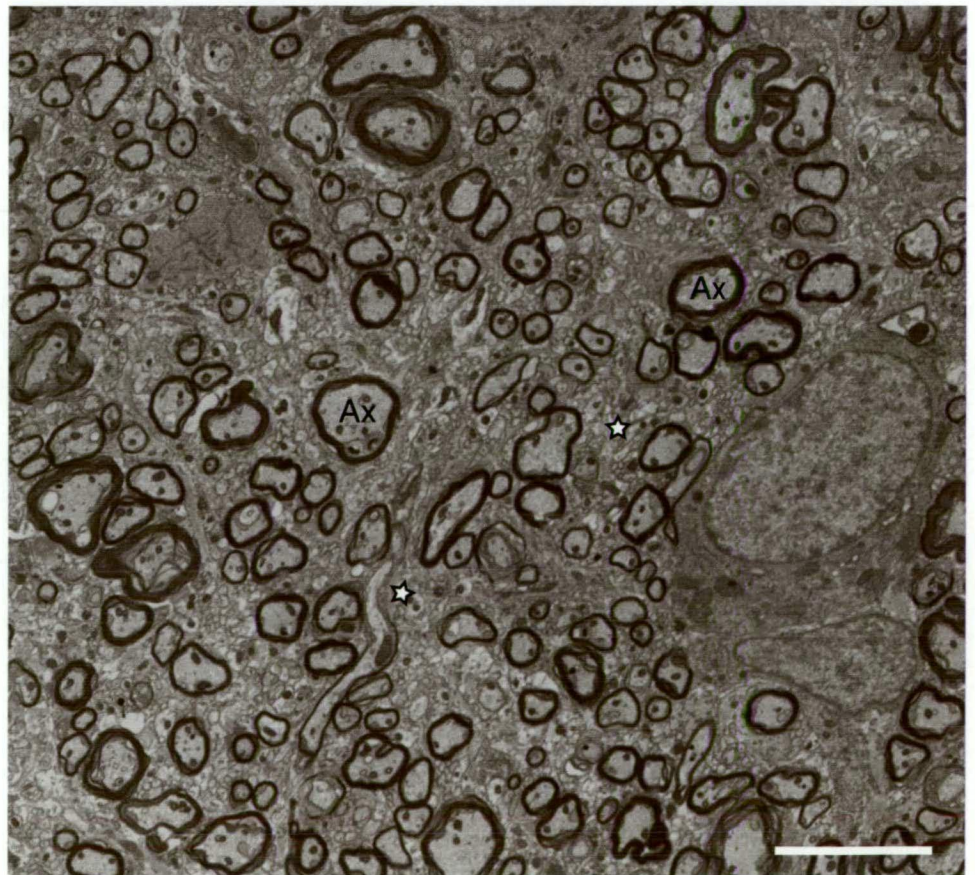
P35 spinally transected

outgrowth, followed by a maturation process of both in order to achieve a fibre calibre and conduction velocity capable of mediating a mature behavioural expression and sensory information (Cabana, 2000). While the present study has not examined the onset of these further processes required for the complete development of the nervous system, it is useful to draw on other studies in the opossum that have commented on these events.

A study by Gingras and Cabana (1999) examined the development of synaptic structures in the *Monodelphis* as it occurs over a similar period to that of the outgrowth of newly descending fibres and may give an indication of when development of the spinal cord is finished. Immunolabelling with antibodies against synaptic proteins in lumbosacral region of the spinal cord showed an increasing density of labelling initially only in the white matter at birth, spreading through the gray matter until 5-7 weeks post-natal (Gingras and Cabana, 1999). At this time, immunostaining was no longer observed in the white matter and resembled the adult pattern of protein distribution. After 8 weeks, the density of labelling in the gray matter decreased and this was suggested to be the result of the differentiation and growth of the tissue, along with the possible elimination of synapses during normal development (Cabana, 2000). This would support findings of the present study that the number of projections during the developmental period may be in fact greater than the adult due to a pruning process of refining synaptic connections.

However, it was noted that the process of myelination in *Monodelphis* appeared more closely correlated with the *maturation* of the observed motor behaviour, where greater speed and accuracy of movements is expressed. Gingras and Cabana, (1999) suggested a correlation between synaptogenesis and the *appearance* of more mature motor behaviour in *Monodelphis* such as quadrupedal locomotion (Pflieger *et al.*, 1996) and the expression of sensorimotor reflexes observed during the 4th to 7h weeks of age (Cassidy *et al.*, 1994). The intensity of immunolabelling with anti-myelin antibodies in the lumbar cord showed a similar pattern of development to that for synaptic proteins but lagged about 2-3 weeks behind indicating the myelination is one of the last tasks in the development of the CNS.

Myelin was not observed in *Monodelphis* spinal cord until the second post-natal week, beginning at P8 and developing asynchronously through the rostrocaudal, ventrodorsal, and lateromedial planes, reflecting the asynchronous development of the spinal cord and different pathways (Møllgård *et al.*, 1994; Cabana 2000); this was also observed in the present study. The full completion of development probably occurs in *Monodelphis* after P40 and thus was not part of the present study. However, in some preliminary studies of myelination in control and experimental animals, using EM, it appears that a degree of myelination has occurred by P14, but was not observed after by one week post-injury. By P35, fibres appeared heavily myelinated in transected spinal cords, but not necessarily of the same calibre as those in control animals (see Figure 4.2).

4.6: CRITIQUE OF METHODS

4.6.1 Appropriateness of the *Monodelphis* as an animal model

The greatest advantage of *Monodelphis* for the experiments such as these in the present study is their extreme immaturity at birth, which allows for all the operations to be performed on neonates before the plasticity of the developing spinal cord is lost (Varga *et al.*, 1995a; Nicholls and Saunders, 1996; Fry and Saunders, 2000). This is in contrast to the rat where fibre growth and repair are not seen following a crush injury after E15-16 *in vitro* (Saunders *et al.*, 1992) requiring that the crush operation be performed either on a CNS in culture or *in utero*.

Another advantage of the *Monodelphis* is the high fecundity of these animals. A particular problem that did emerge was that despite the high numbers of young born, a great many were cannibalised by the mother after the operation. The mother had a greater tendency to cannibalise her young in transected litters as compared to control pups, although cannibalism was intermittently observed in the breeding stock litters that were not interfered with. This appears to be a normal feature of maternal behaviour as noted before by Fadem *et al.* (1982). This cannibalism may have introduced an element of bias with regard to the animals studied, as it was not

possible to examine or determine the cause of death of cannibalised pups. Some pups may not have survived the trauma of the spinal injury, or the mother may have sensed the paralysis in some of her offspring and reacted by cannibalising them. This was an unavoidable consequence of the *in vivo* nature of the present study.

4.6.2 Experimental design

This study used several key techniques: in particular, injection of the axonal tracer dye into the spinal cord at different ages (P4, P7, P14, P28, P35 and adult) and transection of the spinal cord transection performed at P7 only. There was a battery of control experiments that were conducted in parallel to the main experiments to ensure that all serious sources of potential error were accounted for, or at least considered when evaluating the results.

The spinal cord transection

The operation technique of complete transection was chosen for the present study in order to establish with certainty that all spinal cord tissue was severed and that fibres which reappeared across the lesion site could only have grown *through* the transection. Other experiments that employ spinal crushes (Treherne *et al.*, 1992; Saunders *et al.*, 1998) or hemisections (Xu and Martin, 1991) leave some tissue connection around the circumference of the injury site that may cause fibres to grow preferentially along remaining structure rather than forge their own pathway through a discontinuous region when a complete transection is performed. Performing complete transections removed all ambiguity as to the source of axon growth detected across the injury site. It could not be the result of undamaged axons sending collateral sprouts into the site of injury, as might be the case if only a partial lesion is made. Rather all outgrowth must have crossed *through* the injury site.

All spinal cords from animals removed at P7 (n=25) that were examined either in whole mount, agar embedded 100μm sections, or 5 μm paraffin wax embedded section (stained with histological agents to show tissue differentiation) showed complete separation between rostral and caudal ends (see Methods: Figure 2.4). This was a good indication that requirement of complete transection for the study had been satisfied, although it was not possible to know with absolute certainty that this

was also the case in all animals removed at older ages. Another good indication that projections to the lumbar spinal cord were severed was that no neuronal cell bodies in the brain stem were labelled after injection of Fluororuby immediately following the lesion on P7. By contrast, P7 control animals showed substantial labelling. Examination of lesioned spinal cord in longitudinal sections under the fluorescent microscope also showed no evidence of fibres containing the dye in the spinal cord rostral to the lesion. As this was observed in all transected animals showing labelled cell bodies in the brain it indicated that the dye itself was unlikely to have crossed the lesion. Additionally no dye localised in cell bodies rostral to the lesion from collateral innervation to the lumbar levels was observed, further indicating a complete transection. This was also observed in control animals. Once the effectiveness of the spinal cord section method was established, one pup was removed at random following the operation on litters to check the continued consistency of the lesion procedure. All checks showed that the spinal cord had been completely severed.

Validity of a double label experiment

A further possible area of ambiguity encountered when examining fibre outgrowth after immature spinal cord injury relates to the time during which the spinal cord has a "plastic" response to injury. The age at which the lesion was made in the present study coincided with the time of prolific developmental outgrowth of many different ascending and descending pathways in *Monodelphis* (Wang *et al.*, 1992, Terman *et al.*, 1996; present study). Therefore, after a spinal lesion made during this early stage of development, fibres seen growing across the injury site could be derived either from regeneration of severed fibres present across the level of injury already by P7 or from subsequent developmental outgrowth. The principal aim of the present study was to distinguish between these two sources of fibre outgrowth following complete spinal transection.

To achieve this, it was necessary to physically identify a population of neurons both before and after the spinal lesion to see whether severed neuronal fibres do regenerate. The use of a double label paradigm to retrogradely label fibre projections back to their cell bodies has been a method of choice for a number of studies (Bates and Stelzner, 1993: Bernstein-Goral and Bregman, 1993; Hasan *et al.*, 1993; Wang *et*

al., 1998; Zhang and McClellan, 1999; Doran and LeDoux, 2000). While it is acknowledged by all these studies that the double label technique does have technical complications and generally underestimate the numbers of regenerating fibres, all agree that if rigorous controls are applied, then the protocol is an excellent method of distinguishing between regenerating and developmental growth across an injury site in the central nervous system. These complications and controls are elaborated on further below as addressed in the present study.

The choice of dextran amine dyes

In order to determine the proportion of fibres that are regenerating across an injury site it was first necessary to establish which supraspinal populations have innervated the lumbar spinal cord at the time of injury in control animals, and how this pattern changed over the time course of the study (P7 – P35 and adult). The results of these experiments were described in Results: Section 3.1. Secondly, it was necessary to determine the timetable of fibres growing back across the injury site (described in Results: Part 3.2) in order to compare it to the sequence of events in control animals. Finally, a double label tracing study was designed to distinguish between regenerating and subsequently developing fibres descending across thoracic injury site (described in Results: Part 3.3).

Axonal tracers are widely used for establishing the origins of fibre projections (Köbbert *et al.*, 2000; Novikova *et al.*, 1997), both in developmental studies (eg Wang *et al.*, 1992; Lakke, 1997) and injury studies (eg Kalil and Reh, 1979; Bregman and Goldberger, 1982; Bernstein and Stelzner, 1983; Treherne *et al.*, 1992; Wang *et al.*, 1996; Saunders *et al.*, 1998). As a quantitative approach was taken in the present study, the tracer used was required to be reliable and able to consistently label neuronal cell bodies. The amount of dye injected was adjusted so that it remained a uniform proportion of the cord diameter which increases over the developmental period studied (see Results: Table 3.1.2).

The present study employed two fluorescent conjugated dextran amine dyes (Fluororuby and Oregon green) to label fibre projections from the lumbar spinal cord to their neuronal origin in the brain stem. These particular dextran amines were found to be ideal tracers, whether injected as single labels or as part of the double

label study. They are non-toxic, clearly visible in retrogradely labelled cell bodies and importantly, the red and green florescence was easily distinguished from each other with little spectral overlap. The method of administering accurate amounts of dye into the spinal cord in a glass pipette by mouth pressure produced uniform, consistent labelling of neuronal cell bodies in terms of location and number of cells counted. The dye was heavily pigmented in solution and so easily visible entering the spinal cord. The dye was weighed out to ensure accurate amounts were injected.

Dextrans are hydrophilic polysaccharides that are very water soluble and resistant to cleavage by cellular enzymes (Köbbert *et al.*, 2000). These chemical characteristics give the fluorescent conjugate a long lasting presence in the neuronal cell body and reduce photobleaching when examining sections under excitatory fluorescent light. The stability of the dye in the cell allowed extended viewing of sections under the fluorescent microscope, thus it was possible to count neuronal cell bodies and take photographs without significant photobleaching. Fluorescently conjugated dextrans also have the advantage of being fixed by paraformaldehyde on the lysine residues. Vibratome sections of the brain and spinal cord required no further processing before viewing which was highly advantageous when examining and counting cells in large numbers of sections.

WGA-HRP is also a commonly employed neuroanatomical tracer (eg Martin and Xu, 1988; Shimizu *et al.*, 1990). It is a highly sensitive marker but requires substantial immunohistochemical processing for visualisation. The apparent distribution of WGA-HRP may be exaggerated by diffusion of the HRP reaction product (Köbbert *et al.*, 2000). The present study required a relatively discrete site of injection as only lumbar projections should be labelled (see Results: Figure 3.1.3). The use of a dextran with a simple processing protocol was the preferred tracer for the present study. Fast blue is another common alternative axonal tracer and is a sensitive, reliable dye in opossums (Wang *et al.*, 1992). However, it fades rapidly with exposure to light and over time, which is highly disadvantageous when making comprehensive counts of labelled cell bodies over a time course as in the present study. Fluororuby has been reported to label less neurons than the blue dyes (Fast blue, True blue and Granular blue; Vercelli *et al.*, 2000). This was also observed by

Wang *et al.* (1998) when comparing the number of neurons labelled by Fast blue and Rhodamine B dextran (which is very similar to Fluororuby).

Fluororuby is a widely used dextran amine and its properties are well described (Schmued *et al.*, 1990). Oregon green is a relatively newly released product (Molecular Probes) and is thus less described. However, it was observed to produce identical results to that of Fluororuby in the present study when both dyes were injected at P7 in control animals. The location and appearance of labelled neurons was identical. The two dextrans were chosen for their contrasting fluorophores, and all other properties appeared to be similar.

Dextrans have a fast axonal transport speed of up to 2 cm/day and the dye accumulates in small vesicles that are actively carried along the cytoskeleton of axons and processes (Köbbert *et al.*, 2000). The four day (or one week in adults) incubation period between injection of Fluororuby into the lumbar region of the cord and removal of the animals was more than ample time for the dye to reach the neuronal cell bodies of origin in the brain stem as even P35 animals had an average crown rump length of not more than 5 cm and adults not more than 15 cm.

Oregon green was required to have been retrogradely transported to the neuronal cell bodies of projections by the time the transection was made at P7. As the injection was made at P4 when the pups are not more than 10 mm in length, there would have been also ample time for this. No increase in total number of Oregon green labelled neurons after spinal injection at P4 was recorded in the control animals from P7 to P35 (see Results: Table 3.3.1). Rather the number remained relatively stable and then decreased in the adult. This indicated that the neurons were unlikely to have taken up the dye at later time points due to dye remaining available for continued uptake from the extracellular space after the initial injection at P4.

The vesicles containing the dye remained in the cell body for a long period of time and these gave a brightly fluorescent granular appearance to the neurons (see Results: Figure 3.1.1) so that they were easily visible for counting. The longevity of Oregon green in the cell soma was the main concern in the present study. It was important that Oregon green remained stable *in vivo* as animals were not removed for up to 5 weeks after the initial injection was given and labelled neurons should be

detectable at these time points. Injections of Oregon green in control animals showed that the number of labelled cell bodies in brain stem nuclei remained stable and consistent from P7 to P35 and this satisfied an essential requirement for a valid double label study (see Results: Table 3.3.1). Other studies also report that dextran amines remain relatively stable *in vivo* for up to 12 weeks, although it was acknowledged that some decline in numbers does occur during this period (Novikova *et al.*,1997). Thus, the smaller number of neurons labelled with Oregon green in P35 transected as opposed to control animals was likely to be related directly to the axotomy that occurred at P7 and not due to the instability of the dye.

It has been observed that the antero/retrograde direction of transport of tracers is rather species dependent. For example, Vercelli *et al.* (2000) observed that biocytin is an almost exclusive anterograde tracer in kittens, while has retrograde movement in mice. In the opossum, Fluororuby exhibited excellent retrograde transport as did Oregon green, although Fluororuby is often used as an anterograde tracer (Köbbert *et al.*, 2000).

One of the features of Fluororuby that was particularly suited to the present study was that its gives a "snapshot" of the projection profile at a certain time point. This is related to the mode of transport of the dye. Fluororuby is taken up primarily by neurons and fibres injured by the injection (Köbbert *et al.*, 2000, Glover *et al.*, 1986) and to aid this process Triton was added to the diluent as a permeabliliser of membranes. Fluororuby may also be incorporated in the axon terminals of *fibres en passage* but at a much lesser degree than, for example, Fast blue which is predominantly taken up by this route (Vercelli *et al.*, 2000). Thus, Fluororuby is much better suited to the present study, giving an indication of the fibres present at a certain day of development. It was less likely than other dyes (such as Fast Blue) to continue to enter more axons (resulting in more labelled neurons), during the 4 day incubation period. However, the possibility of some *fibres en passage* picking up the dye could not be discounted. The different ages of animals in the present study are thus referred to as the day of injection but include four days incubation eg P7 = P7+ 4 etc. This was to indicate that any labelled cell bodies counted in the brain of these animals may have projections either already reaching the lumbar spinal cord on the day of injection, or arriving during the next 4 days. It is likely that the majority of

cell bodies received the dye from the initial injection, given the properties of Fluororuby outlined above, although a minimal amount of newly developed projections may begin transporting the dye during the incubation time. The fact that Oregon green cell numbers did not increase from P7 values at any later ages, further supported the contention that these dextrans do not label many additional projections after the time of injection (see Results: Table 3.3.1).

Lack of transynaptic labelling of neurons

It was important to establish that the axonal tracers did not diffuse transynaptically via intrinsic spinal neurons that may have descended past the lesion site, which would falsely indicate brain stem-spinal projection. This was considered unlikely as fluorescent dextrans do not diffuse easily across cell membranes (Hasan *et al.*, 1993; Vercelli *et al.*, 2000) and no non-neuronal cells were observed to be labelled. There was no evidence that this occurred in the present study, as only regions observed to be labelled in control animals in both the present and other studies on the origins of supraspinal projections in *Monodelphis* (Wang *et al.*, 1992; Holst *et al.*, 1991) were in known regions with direct projections to the spinal cord. For instance, no labelled neurons were ever observed in the cortical regions. It is known that the corticospinal tract only reaches the cervical region of opossum spinal cord, well distal from the lumbar injections of the present study (Martin *et al.*, 1975).

The possibility that neurons may have been labelled from blood-borne transport of the dyes was also considered. While the chance that the dye entered the CSF circulation or blood circulation at the time of injury cannot be completely discounted, the fact that only known supraspinal connections were labelled indicates that there was a negligible element of non-specific labelling via blood or CSF, if any.

Uniformity of lesions

Any manipulation of the newborn opossum is fraught with technical difficulty due to the extremely small size of the pups (not more than 10 mm in length at birth, Nicholls and Saunders, 1996). It is an unavoidable consequence of injecting very small amounts of dye unilaterally into the spinal cord that some variation between individuals in the number of neurons backlabelled will occur. It is widely accepted that all tracing experiments and *in vivo* studies will show a degree of variation

(Hasan *et al.*, 1991; Hasan *et al.*, 1993; Bates and Stelzner, 1993; Zhang and McClellan, 1999; Doron and Le Doux, 2000; Becker and Becker, 2001). Therefore small discrepancies in the amount or location of the injection may have a magnified effect on number and location of labelled neurons. It was not always possible to guarantee that all injected dye was successfully placed into the spinal cord as occasionally some leaked back through the injection hole. The injection site of Fluororuby was observed to be a discrete region in adult animals (see Result: Figure 3.1.3), where the unilateral injection showed a densely labelled core needle zone surrounded by a fainter halo of dye localised in the immediate gray matter at the site of injection. The dye did not appear to have reached the contralateral side of the spinal cord which satisfied the aim of only exposing the spinal cord to the dye in a unilateral manner, allowing the laterality of projections to be observed.

There are also differences in the density of the spinal cord tissue during development and this may have influenced the depth of penetration of the dye contacting fibres, although as dextrans require some interruption to axonal integrity, this is probably not a significant concern. The injection sites became more discrete and localised with increasing age of the animals (see Results: Figure 3.1.3). At P7, a brightly fluorescent injection core was observed to cover almost the whole ipsilateral side of injection. The injection site became more discrete as the cord grew and a diffuse halo of fluorescent dye extended across the whole cord in all the developing animals from P7 to P35. A combination of the increase in size of the cord relative to the needle and the fact that the spinal cord elements become more densely packed over development may explain this observation. The maturation of glial cells and associated reduction of interstitial spaces, for instance, are likely to contribute to this. In developing animals, (P7 – P35) the injectate appeared to diffuse through the whole unilateral injected side of the cord to a similar degree, and therefore the dye should have hit a similar proportion of projections present at the spinal level injected (see Results: Figure 3.1.3). The differences in numbers of labelled cell bodies in the brain should thus be related to the number of projections innervating the lumbar spinal cord at the age examined. As the number of labelled cell bodies increased from P7 to P35 control animals injected with Fluororuby (see Results: Table 3.1.3), this can be reasonably assumed to be the case.

In the adults, however, the number of neurons labelled after control injections of
Fluororuby was found to be significantly less than that in younger animals. In adults,
the injection site was observed to be more discrete than younger animals and thus the
dye may not have hit as many projections in the cord. The amount of dye injected
was adjusted to allow for the increase in diameter (see Results : Table 3.1.2),
however, as the adult spinal cord is more densely packed, the dye may diffuse
through a lesser proportion of the cord to partially explain the difference in number
of neurons labelled at different ages.

The spread of injectate in developing animals has also been observed by other
authors (cited by Vercelli *et al.*, 2000). However, as Fluororuby mostly enters axons
from the membrane disruption caused by the needle and the presence of a detergent
Triton in the injectate, the effect of a localised injection site was reduced.
Additionally, the same size needle was used at all ages and would have impacted on
a smaller region of the spinal cord in older animals which may be a further
contributing factor to the observed decline in total numbers in adult brain.

The alternative possibility considered was that brain stem projections may undergo
developmental cell death and/or withdrawal of transient projections between P40 and
adulthood as part of a developmental refinement process in the CNS (see Vaux, 1993
for a review). While it was beyond the scope of the present study to examine further
developmental processes between P35 and adulthood, it has been noted previously
by Stolp *et al.* (2002) that indications of developmental cell death in the brain stem
(apoptotic cells detected by Haematoxylin and Eosin histology stains) were seen at
P14 and not at P35. It was concluded to be unlikely that a great deal of
developmental refinement would occur after P35 and the decline in adult cell
numbers was more likely to be due to technical aspects as outlined above.

Validity of the counting method

As the present study aimed to make a quantitative comparison between the number
of supraspinal projections to lumbar cord in control and spinally transected
Monodelphis, it was important that the method for counting labelled neurons was
reliable. Extensive work published by Martin and colleagues (Wang *et al.*, 1992 and
Holst *et al.*, 1991) studied the projections from the brain to the spinal cord using Fast

blue and True blue as opposed to Fluororuby in the present study. Only selected representative brains at different ages were shown in those studies without recording quantitative data regarding the number of neurons present. For this reason, in the present study, it was necessary to establish the expected number and identity of brain projections in control *Monodelphis* using the new protocol to make valid comparisons with spinally transected animals.

The total number of labelled neurons counted for each brain would be an underestimate of the total projections at the level of injection. Firstly, only one side of the cord was labelled to enable the contralateral/ipsilateral distribution of projections to be observed, secondly the injection would not contact all fibres (see Results: Figure 3.1.3). As it is known that Fluororuby primarily labels axons injured by the single injection, it would not be possible to label all fibres present in the lumbar spinal cord. The primary aim of the control injections was to establish a baseline of numbers seen by the technique used and compare other data gathered in the course of the present study.

Brain sections were cut at 100μm thickness and it is possible that some labelled neurons would not have been counted should they not fall into the plane of section despite counting all labelled neurons from all section through the brain. The thickness of 100μm was chosen as the largest neurons in the opossum were estimated to be about 30 – 40 μm (Wang *et al.*, 1998).

It must be noted that fibres must grow several spinal segments past the mid- thoracic injury site to reach the lumbar cord before they can to pick up the second dye and this would cause a time lag between fibres crossing through the injury site and being detectable by the second injection. This would result in an underestimate of the number of projections counted although the incubation period of 4 days should partially account for this problem.

The control double label injections consistently showed that approximately 30% of neurons labelled with Oregon green from a P4 injection would also show labelling from Fluororuby injected at ages from P7 to P35. This could be considered the maximum amount of cells available for double labelling and it is widely acknowledged by many groups undertaking tracing studies (Hasan *et al.*, 1993; Bates

and Stelzner, 1993, Wang *et al.*, 1998; Bernstein-Goral and Bregman, 1993) that it is difficult to obtain much more accurate results. For comparative studies between different ages and between control and transected animals, the fact that the number of neurons detected was likely to be an underestimate was not considered a great problem as it was consistent for all ages.

Validity of the statistics used

The statistics used in the present study were employed to highlight the likely differences in the means of comparative groups (either for different nuclei, different ages or between transected and control animals) using a basic t-test. It was recognised that in an *in vivo* study, n numbers are generally low and this can compromise the power of statistical tests. Most comparisons were made between either the highest or lowest mean and to the other means, to see if they differed from the extremities. It was neither practical, nor meaningful to do further statistical tests. When data from the same material are tested many times for a statistical significance this greatly increases the chance of incorrectly rejecting the null hypothesis (that there is not difference between the 2 means), when it is actually correct (Type 1 error). The chance of incorrectly rejecting the null hypothesis is magnified each time a set of dependant data are tested on multiple occasions. This was a concern in the present study. For example, the data describing the proportions of total number of neurons in the brain were dependant data, as the counting of cells in an incorrectly classified nucleus at anytime will impact on the values of all other categories. Statistical differences were interpreted with caution, but did indicate the dynamic changes in nuclei contribution to lumbar projections over development, with and without a lesion and in single or double label experiments.

Anatomical influence on the projection profile.

Some consideration must be given to the location of tracts in the spinal cords and whether this may give a greater chance of being contacted by the injection. It would appear from proportional comparisons made between different nuclei that the lumbar spinal cord of control *Monodelphis* aged P7 to P35 and also adults, have a large proportion of reticulospinal projections. The vestibulospinal tract appeared the other major contributor, followed by the rubrospinal tract (see Results: Figure 3.1.5).

Orthograde autoradiographic tracing techniques using ^{3}H leucine were employed by Martin *et al.* (1982a) to describe the innervation of raphe and reticular projections from injected nuclei to the spinal cord in the *Didelphis*. They emphasised that these projections are particularly robust in the opossum showing consistent and numerous labelled cell bodies, and thus were highly likely to encounter the dye in the present study. While the numbers of projections that reach the lumbar spinal level were less than to cervical regions, the dorsolateral region of the lumbar spinal cord and most regions of the laminae of Rexed were shown to be well innervated by reticular projections. For example, injections to the raphe obscurus nucleus and adjacent gigantocellular reticular nucleus showed extensive innervation to dorsolateral, lateral and ventrolateral funiculi, along with a robust presence through the laminae of rexed – with the notable exception of the laminae I and II. The ventral paragigantocellular reticular nucleus and adjacent raphe magnus nucleus were seen to innervate similar region with the additional presence in the laminae II and I (Martin *et al.*, 1982). It was therefore expected that the reticular formation would be highly represented in the lumbar projections established by the present study and that as they are among the earliest developing pathways, this will be reflected through all the ages examined from P7. This indicated that the methodology of the present study gave an accurate illustration of the projections from the brain innervating the lumbar spinal cord.

It was noted that some other regions of the reticular formation may only be traced to the thoracic levels such as the retrofacial nucleus and caudal regions of the ventral and dorsal medullary reticular levels (Martin *et al.*, 1981). On occasion a small number of labelled neurons was detected in the presumptive facial nucleus of an adult animal. Examination of the injection site in the spinal cord revealed that the dye had been injected in more rostral thoracic location and such animals were subsequently discarded from the study. However, this was an indication that the method was sensitive to small changes in the level of injection and for this reason all effort was made to standardise the injection site. The very caudal region of the dorsal medullary reticular nucleus and ventral medullary reticular nucleus in the hindbrain were not noted to contain labelled neurons and this was due the absence of lumbar innervation by projections.

A further indication that the tracing techniques was a true reflection of the origins of supraspinal projections to the lumbar spinal cord was that the laterality of different pathways seen in the control *Monodelphis* in the present study were similar to that observed in other tracing studies in both the *Monodelphis* (Holst *et al.*, 1991; Wang *et al.*, 1992), and also the related *Didelphis* opossum (Martin *et al.*, 1975; Martin *et al.*, 1982). In general, all populations were bilaterally represented but most had an ipsilateral predominance. Exceptions to this were the red nucleus, locus coeruleus and medial vestibular nucleus, which showed a contralateral predominance after lumbar injection.

The level at which the supraspinal projection cross to the contralateral side of the spinal cord in their descent from the brain stem might impact on the laterality represented if this occurred at the level of, or below the injury. While little information was found for the *Monodelphis*, comprehensive neuroanatomical studies in the *Didelphis* indicated that most appear to cross (if they do so at all) in the mid or hindbrain (Martin *et al.*, 1975). Therefore, fibres should arrive at the injury site in the normal formation. Any aberrant growth pattern after injury might be indicated by variation from the lateral distribution seen in control animals. As there appeared to be very little difference between the control and transected animals with regard to the laterality of projections to the lumbar spinal cord, it was suggested that fibres growing through the injury site (either from regenerative outgrowth or subsequent development) should be able to find their correct targets given that they have descended in the appropriate formation. Whether they make functional synaptic connection upon arrival into a correct target region is a further question beyond the scope of the study.

The opossum's CNS appears generally comparable to that of most eutherian mammals (Martin *et al.*, 1982) and the results of the present study also found the neuroanatomy of the *Monodelphis* to be similar to that of commonly examined mammals such as rat and human. Much of the neuroanatomical characterisation of the opossum brain has been examined in the North American opossum, *Didelphis virginiana* by Martin and colleagues over many years (eg Martin *et al.*, 1975; Martin *et al.*, 1982) and the response to injury during development has been compared to their studies. The *Monodelphis* has also been the subject of neuroanatomical

examination, but has been less comprehensively studied (Holst *et al.*, 1991; Wang *et al.*, 1992, Qin *et al.*, 1993; Cassidy and Cabana, 1993; Terman *et al.*, 1997; and Molnár *et al.*, 1998).

Some differences do exist between the *Didelphis* and *Monodelphis* as described in the Introduction (see Section 1.2.6), which emphasises the need to determine the developmental sequence of innervation for each species, particularly if comparing the outgrowth following a injury during the developmental period. However, the two species share many similarities and where aspects have not been examined in one species, it is logical to look for indications within the other closely related opossum. Previous studies with the *Didelphis* have been drawn upon where no information was found on the *Monodelphis*, but cannot be considered as the same (see Holst *et al.*, 1991 for further details).

4.6.3 Limitations of the present study

One of the main concerns was that spinal injections would have given an underestimate of the projections that innervate the lumbar spinal cord. This problem was reduced by comparing numbers of labelled neurons obtained between control and transected animals, between different ages or between different nuclei. However, it may have the effect of hiding some populations entirely from a projection profile if only a few fibres from those nuclei had grown through the injury site. The fact that only 30% of neuronal cell bodies in the brain of control animals containing the first dye (Oregon green) were double labelled with the second dye, highlighted the magnitude of the underestimation. It must be noted that fibres need to grow several spinal segments past the mid- thoracic injury site to reach the lumbar cord before they can pick up the dye and this would cause a time lag between fibres crossing through the injury site and to detected by the injection. This would result in an underestimate of the number of projections counted although the incubation period of 4 days should partially account for this problem. The most effective way to increase the chances of labelling more neurons and expose more fibres to the dye would be to make multiple bilateral injections, although this would increase variability in numbers of labelled neurons with each extra injection. It would also be

technically difficult to make more than one injection on either side of the spinal cord in very small animals.

Another limitation was that because of the experimental design, the study could only deal with the regenerative ability of supraspinal axon populations that had already descended to the lumbar spinal cord by the time of the first injection and were severed by the lesion made at P7. Lesions would need to be made in older animals (eg P14, P21 and P28) in order to determine the regenerative capacity of populations such as the interstitial nucleus of the medial longitudinal fasciculus, paraventricular hypothalamic nucleus, dorsal hypothalamic area, Edinger-Westphal nucleus, ventral part of central gray and nucleus Darkschewitsch. Also the regenerative capacity of neuronal populations that contributed only a small component of the lumbar profile at P7 such as red nucleus, nucleus ambiguus, and locus coeruleus would be more clearly established if lesions were made at a stage of development when they would have a greater number of fibres injured. Injuries in older animals would also define the end of the critical period when the spinal cord no longer supported the growth of fibres after transection. This would be an important point when comparing regenerating and non-regenerating systems to identify crucial differences between the two.

There are many other populations of fibres that may also contribute to growth through the injury site, such as ascending dorsal column fibres that were not studied in this thesis. These could be studied with brain stem injections with retrograde or anterograde tracers (such as carbocyanine dyes or other dextrans) which could be injected into the dorsal root ganglion or spinal cord. Another important biological point that was not part of this study is the determination whether the supraspinal projections that regenerated across the injury site were capable of reinnervating the correct targets and mediating normal behavioural function. These are important components of the regenerative process and while achieving regeneration of severed axons is no small feat, correct innervation of targets and completion of synaptogenesis are necessary for restoration of complete function to spinally injured animals (including humans). It has been previously established that *Monodelphis*, spinally transected at P7, develop near normal behavioural function in adulthood and

were observed to complete tasks such as swimming and beam climbing which require supraspinal input (Saunders *et al.*, 1998).

While the present study showed unequivocally that adult animals that had been spinally transected at one week of age supported supraspinal innervation to the lumbar cord, it was beyond the scope of the study to determine whether regenerating fibres were still contributing to the adult profile. The present study did not describe the fate of axotomised neurons beyond P35, as the survival of the first dye appeared to diminish over time. The number of Oregon green labelled neurons in transected animals was reduced at P35 compared to the younger ages, while it remained constant in control animals, indicting a likely effect of axotomy. A degree of axotomy-induced cell death may have contributed to this and 4 weeks post injury may be a significant time point for the survival of regenerating neurons. Limited survival of axotomised neurons would greatly influence the number of double labelled neurons counted.

4.7: IMPLICATIONS OF THIS STUDY

Confirming the presence of regenerating axons after spinal transection in neonatal *Monodelphis* in the absence of any promoting strategy has enormous significance for further development of this experimental approach to spinal injury research. While it has been established that the developing *Monodelphis* spinal cord was able to respond with fibre outgrowth after complete transection *in vivo*, both in previous studies (Saunders *et al.*, 1998; Fry, 1997) and the early part of the present work, this could have been entirely due to continued subsequent growth from uninjured axons. A regenerative capacity of at least some of the severed axons means that a working model of CNS regeneration has been established. While the subsequent outgrowth of fibres through an injury demonstrated certain plasticity, given the possible inhibitory nature of the lesion site, it has limited scope for studying regenerative processes. Regeneration is the desired outcome as in the future it would be important to discover the elements of the immature CNS which are permissive for regeneration, and at what point these features are lost and why. It may then be possible to reverse, delay or replace these crucial features of a regeneration-permissive mammalian CNS

in situations where injury results in irreversible damage to CNS neurons, such as in the adult mammal.

It was found that an increasing proportion of the axotomised neurons was able to regenerate over a 4-week post-injury time course examined, from approximately 3% at one week post-injury 30% by 4 weeks post injury. The control experiments showed that in the present experimental design, 30% was the maximum amount of neurons that can be double labelled (thus indicating regenerating neurons). The proportion was some times larger, up to approximately 45% for the ventral medullary reticular nucleus and dorsal medullary reticular nucleus although the standard error was high (see Results: Table 3.3.15). This shows that a large proportion of the axotomised supraspinal projections appear to regenerate. A second important point is that it is necessary to allow the regenerating axons sufficient time to complete outgrowth after injury as many regeneration studies only looked at short time points post-injury (eg. Hasan *et al.*, 1993; Wang *et al.*, 1998).

This model also allows examination of what local environmental conditions influence regeneration. If the molecular composition of a regenerating system can be determined, then it may be possible to observe differences with a non-regenerating system, with a view to altering negative or inhibitory elements that may be preventing this process. It would also be important to establish the fate of axotomised neurons: which ones survived axotomy, were they capable of regenerative outgrowth and whether regenerating fibres innervate the correct targets to mediate functional behaviour.

A further important observation from these studies is that all supraspinal populations consistently represented at P7 as having lumbar projections appeared to have the ability to regenerate after the injury made at one week of age and that it was not restricted to certain groups of axons. This suggest that if given the right conditions after injury (even in the adult) it may be possible for all supraspinal populations to show a regenerative response and that an injury made at P7 is well within the critical period for outgrowth of all axon populations present at this time.

The injured spinal cord supported a relatively normal development innervation time table, although it appeared to take approximately one–two weeks longer for the

appearance of the same tracts. This was probably due to having to overcome the gap created by the lesion. Whether this innervation mediates correct and appropriate functional connections is an important further question. It is possible that an adaptive process is taking place to complete the developmental outgrowth to the lumbar spinal cord, but little evidence was found to suggest this, given that the developmental sequence appeared similar between control and spinally transected animals.

5.CONCLUDING SUMMARY

The main aim of the present study was to establish whether the substantial repair observed previously after injury in the immature spinal cord of *Monodelphis* (Varga *et al.*, 1995; Saunders *et al.*, 1998) was due to regeneration or to subsequent developmental growth of axons. An additional aim was to determine the time frame of the process of repair.

The work described in this thesis has established the neonatal opossum *Monodelphis domestica* as a model for studying the contribution of axonal regeneration to the recovery from spinal injury. The prolific growth of axons across the lesion makes it possible to study the process of target innervation and functional synapse formation following spinal injury, as well as the developmental regulation of neuronal and local environmental factors that determine the extent to which regeneration and functional recovery can occur at different stages of spinal cord and brain development.

Firstly, the developmental innervation pattern of supraspinal projections descending to the lumbar spinal cord was established for control animals aged P7, P14, P21, P28, P35, and adult by a series of single spinal injections of Fluororuby into the first lumbar (L1) segment at each age. These experiments showed that all of the supraspinal populations were still growing new projections to the lumbar spinal cord by one week of age when spinal transections were made, although different populations were at different asynchronous stages of development. The control experiments established the normal sequence of innervation against which experimental animals could be quantitatively compared.

Experimental animals all received a complete thoracic spinal transection at P7. Single label spinal injections with Fluororuby were made in the lumbar spinal cord caudal to the lesion at P7, P14, P21, P28, P35 and adult to determine whether supraspinal populations of neurons extending axons the injury site were following the same developmental patterns as established for control animals. It was found that a structural tissue bridge across the transection site was achieved by one-week post injury and this contained fibres from supraspinal nuclei, mainly reticular and vestibular projections.

Transected animals showed fewer supraspinal projections to the lumbar region than control animals, but all populations of neurons seen in control animals were also represented through the injury site at least by P35. The sequence of their appearance was very similar to that of control animals, although time of first appearance was often delayed by 1-2 weeks in transected animals, probably due to the physical reconstruction of the spinal cord required. The number of labelled neurons with projections growing through the injury site increased by about 4 orders of magnitude between one week to 4 weeks post-injury.

The last part of the present study aimed to determine to what extent regeneration of severed axons had contributed to the fibre growth seen across the injury site, given that much subsequent developmental outgrowth of all supraspinal pathways was yet to occur after P7. The experimental design used was able to distinguish between the two sources of outgrowth: firstly populations of neurons that had axons extending to the spinal cord into the lumbar levels (at least to L1) at P4 were labelled with Oregon green. This population of labelled neurons showed consistent numbers and distribution from P7 to P35 (period of regeneration investigated) and all had their lumbar projections cut by a complete transection at P7. Subsequently a second tracer (Fluororuby) was introduced to label neuronal cell bodies that had their processes extending across the injury site to the lower thoracic region. Any neuronal cell body labelled with the first dye had their processes axotomised, any with the second dye were those that had processes extending through the injury site, any that were double labelled had regenerated. Control double label experiments were also made on uninjured animals to establish the maximum number of double labelled neurons that could be obtained with this approach. The proportion of double labelled (regenerating) axons was found to be on average 30% of those labelled with the first dye, but with some differences in different brain stem nuclei (20 - 40%).

It was confirmed by the presence of double labelled neurons in the brainstem nuclei that regenerating fibres were amongst the initial fibres to cross the lesion site at one week post-lesion, but at that time point these were only a small portion of total (about 2-3%). The proportion of regenerating axons increased up to about 30% at 4 weeks post-injury, which was the maximum proportion of double labelled neurons found in control animals. The delay in reaching this peak may be due to the intrinsic

capability of the some neurons to regenerate. Regenerating neurons remained a small proportion of the total fibres across the injury site (less than 1%), most of which arose from subsequent development.

The present study confirms the presence of a window of regeneration during the development of a mammalian CNS for up to 5 weeks of age (P35) in *Monodelphis* and that following injury during the first week of life, fibre outgrowth across the injury site is maintained to adulthood. The accessibility and reproducibility of regeneration studies with the developing *Monodelphis* CNS has great potential for determining the characteristics of a regenerating mammalian system in the absence of any promoting aids, and exploring the non-regenerative nature of the adult CNS. For similar studies in rodent, it would be necessary to operate and inject the spinal cord *in utero.*

Having successfully established that the immature *Monodelphis* spinal cord is capable of supporting the growth of regenerating fibres, a wide range of aspects of regeneration may be studied in this accessible and reproducible working model. There are many immediate questions arising from the findings of the present study arising that would need to be addressed before long term aims are explored. One of the first questions would be to establish the long term fate of regenerating axons, as there was some indications that severed neurons may have a compromised survival following transection. It would be important to confirm that they survive beyond the 5 week developmental period examined. The next obvious question would be to see whether regenerating neurons are innervating the correct target regions to make synaptic connection able to mediate normal functional behaviour. Determination of the regenerative capacity of those brainstem populations that were later developing would establish the critical period of the developing *Monodelphis* CNS, an important piece of information when considering the local environment of a regeneration-permissive CNS.

If the permissive elements for axonal growth in the immature CNS are identified and transposed into the non-permissive adult CNS, the eventual hope would be to "return" the adult CNS back to the immature state following a spinal or brain injury, so that repair and regeneration would occur.

Chapter 5

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APPENDIX A

Appendix A1: The age at which labelled neurons were first detected in supraspinal nuclei after lumbar spinal injection over development in *Monodelphis*, according to Wang *et al.* (1992).

Age (Post-natal day)	Nuclei
P0 (day of birth)	Raphe magnus nucleus
	Dorsal medullary reticular nucleus
	Ventral medullary reticular nucleus
	Gigantocellular reticular nucleus
	Lateral paragigantocellular reticular nucleus
	Reticular pontine nucleus
	Locus coeruleus
	Raphe obscurus nucleus
	Raphe palladis nuclei
	Interstitial nucleus of the medial longitudinal fasciculus
	Tegmentum
	Mesencephalic lateral germinal nucleus
	Paraventricular hypothalamic nucleus
	Dorsal hypothalamic area
	Ventromedial hypothalamic region
P1	Some with dorsal vagus nerve
	Vagus nerve
P2	Ventral gigantocellular reticular nucleus
	Ventral reticular pontine nucleus
	Principle sensory trigeminal nucleus
P3	Medial vestibular nucleus
	Inferior vestibular nucleus
	Red nucleus
	Mesencephalic trigeminal nucleus
	Edinger-Westphal nucleus
	Hypothalamus lateral
P6	similar to adult lumbar injection
P14	no longer in ventromedial hypothalamus

NB. Wang et al. (1992) described the first day after birth as P1, not P0 as in the present study.

Appendix A2: Labelling patterns in supraspinal nuclei after lumbar spinal injection in adult *Monodelphis*, according to Holst *et al.* (1991)

Numerous neuronal labelling	Less numerous neuronal labelling
Paraventricular hypothalamic nucleus	Periventricular hypothalamic nucleus
Red nucleus	Lateral hypothalamic area
Edinger-Westphal nucleus	Nucleus Darkschewitsch
Paralemniscal nucleus	zona incerta
Reticular pontine nucleus	fields of forel
Locus coeruleus	Postcommissural nucleus
Locus subcoeruleus , pars alpha	Mesencephalic trigeminal nucleus
Gigantocellular reticular nucleus	Interstitial nucleus of the medial longitudinal fasciculus
Ventral gigantocellular reticular nucleus	Central substantia grisea
Hypoglossal nucleus	Deep Mesencephalic nucleus
Raphe magnus nucleus	Lateral parabranchial nucleus
Raphe obscurus nucleus	Medial parabranchial nucleus
Dorsal hypothalamic area	Lateral paragigantocellular reticular nucleus
Raphe pallidus	Medial vestibular nucleus
Ventral medullary reticular nucleus	Superior vestibular nucleus
Dorsal medullary reticular nucleus	Nucleus X
Nucleus ambiguus	Caudal spinal trigeminal nucleus
Lateral vestibular nucleus	Interpolar spinal trigeminal nucleus
	Solitary tract

Appendix A3: The lateral predominance of some brainstem nuclei in adult *Monodelphis* according to Holst *et al.* (1991).

Predominantly Ipsilateral	Predominantly Contralateral
Interstitial nucleus of the medial longitudinal fasciculus	Red nucleus
Edinger-Westphal nucleus	Paralemniscal nucleus
lateroventral part of central gray	Medial vestibular nucleus
Locus coeruleus	Inferior vestibular nucleus
Locus subcoeruleus, pars alpha	Superior colliculus
Caudal locus coeruleus	
Ventral locus coeruleus	
Lateral vestibular nucleus	
Caudal reticular pontine nucleus	
Caudal part of central gray	

APPENDIX B

Appendix B1: Formulas

Paraformaldehyde Fixative Solution (4%)

8% Paraformaldehyde mixed with equal volume 0.2M Phosphate Buffer (pH = 7.3)

Phosphate buffer = 23ml of 0.2M NaH_2PO_4
                   77ml of 0.2M Na_2HPO_4

Fresh fixative is used on tissue each time, makes 200ml.

Bouins Fixative

Picric acid	750 ml
Formaldehyde (40%)	250 ml
Glacial acetic acid	50 ml

Phosphate Buffered Saline (PBS) Stock (x5)

pH = 7.2	
NaCl	0.73M
Na_2HPO_4	0.04M
NaH_2PO_4 $2H_2O$	0.01M

pH adjusted with NaOH and 1 x stock solution diluted with distilled H_2O.

Stock solution is 5 x working solution

TRIS HCl Buffer stock solution (x10)

0.5M, pH = 7.6
TRIS = hydroxymethyl aminomethyl

TRIS (Trizma base, Sigma)	60.57g
HCL	300 -350 ml
H_2O add water until 1 litre volume and pH = 7.6	

Appendix B2: Preparation of tissue for histology

Tissue that had been initially fixed in 4% Paraformaldehyde and dissected out was washed in tap water for 5 minutes then reimmersed in Bouin's Fixative overnight. The tissue was then subject to the following preparation for histological examination

1) washed briefly in tap water.
2) dehydration through graded alcohols:
   a) 70% ethanol, several washes over 24 hours.
   b) 95% alcohol, 3 washes over 3 hours.
   c) 100% alcohol, 3 washes over 3 hours.
3) cleared in 100% chloroform for 24 hours.
4) embedded in paraffin wax by slowly infiltrating tissue with 3 changes of melted wax at 60°C under increasing vacuum pressure.
   a) 0 PSI for 1.5 hours
   b) 15 PSI for 1.5 hours
   c) 25 PSI for 1.5 hours
5) blocked in wax and left to set overnight.

The tissue blocks were cut in a longitudinal plane by a Bright rotary microtome with a Feather disposable blade, at 5 for Haematoxylin and Eosin staining. The sections were floated in a 50°C water bath for mounting on slides coated with subbing solution and dried overnight in a 37°C oven. Immunohistochemistry was also performed on some sections.

Appendix B3: Immunohistochemistry protocol for Neurofilament staining

<u>Table 2.5.3</u> Antibodies used for Neurofilament labelling

Antibody	Name	Dilution	Source
Primary	SMI-312 (NF)	1:5000	Sternberger, Monoclonals Incorporated, USA
Secondary	Rabbit Anti-Mouse Immunoglobulins	1:25	Dakopatts, Denmark
Tertiary	monoclonal Mouse PAP (peroxidase-anti-peroxidase complex)	1:100	Dakopatts, Denmark
Stain substrate	3,3'-diaminobenzidine tetrahydrochloride (DAB) (chromogen and substrate)		Dako Liquid DAB+ for enhanced sensitivity kit

All dilution are made in standard blocker (2% gelatin in 0.1M PBS with 0.2% Tween)

Procedure of neurofilament marker staining
Slides were:
1) incubated at 60°C for 20 minutes to soften the paraffin.
2) dewaxed in standard method
3) incubated in 1:10 hydrogen peroxidase:methanol bath at 37°C for 20 minutes to remove endogenous peroxidase from tissue.
4) washed in 0.1M PBS with 0.2% Tween for 3 x 5 minutes.
5) incubated with 300µl of primary antibody SMI-312 (1:5000 dilution) overnight at 4°C. Antibody was applied to slides lying flat and coverslips placed on raised cotton threads to prevent damage to the sections.
6) washed in 0.1M PBS with 0.2% Tween for 3 x 5 minutes.
7) incubated with secondary antibody (rabbit antimouse immunoglobulins) for 2 hours at room temperature.
8) washed in 0.1M PBS with 0.2% Tween for 3 x 5 minutes.
9) incubated with tertiary antibody (mouse PAP) for 2 hours at room temperature. Coverslipped.
10) washed in 0.1M PBS with 0.2% Tween for 2 x 5 minutes.
11) washed in Tris HCl buffer solution (see Appendix for formula) for 1 x 5 minutes.
12) incubated with DAB for maximum of 15 minutes. Placed into distilled water when stain had optimally developed at individual rate.
13) washed thoroughly under running tap water for 10 minutes.
14) dehydrated through the following baths.
 a) 70% alcohol for 30 seconds while agitated
 b) 95% alcohol for 1 x 5 minutes
 c) 100% alcohol for 2 x 5 minutes
 d) xylene for 2 x 5 minutes
15) mounted in DPX and dried overnight in 37°C oven.

APPENDIX C

Appendix C1: P values from t-tests made with Sigma Stat® comparing the proportion of Fluororuby labelled neurons in different nuclei at P7 against P35 and adult data in control animals.

Nuclei	t-test P7 against P35	t-test P7 against Adult
Ventral reticular medullary nucleus	<0.01*	0.12
Dorsal reticular medullary nucleus	0.90	0.50
Raphe nucleus	<0.01*	0.01*
Gigantocellular reticular nucleus	0.03*	0.08
Lateral vestibular nucleus	0.04*	0.25
Reticular pontine nucleus	<0.01*	<0.01*
Locus coeruleus	0.13	0.18
Red nucleus	0.05*	0.18
Other nuclei	0.07	<0.01*

All data passed tests for normality and equal variance performed by Sigma Stat®.

* denotes that t-tests made with Sigma Stat® between the proportion of the total at P7 with older ages for different nuclei showed statistical differences ($P \leq 0.05$). These ages were tested to determine if changes were observed over time.

Appendix C2: P values from t-test made with Sigma Stat® comparing the proportion of rhodamine labelled neurons in different nuclei at P7 against P35 values and adult values in spinally transected animals.

Nuclei	t-test (P) P14 against P35	t-test (P) P14 against adult
Ventral medullary reticular nucleus	0.14	0.12
Dorsal medullary reticular nucleus	0.05*	0.03*
Raphe nucleus	0.20	0.59
Gigantocellular reticular nucleus	0.19	0.76
Lateral vestibular nucleus	0.05*	<0.01*
Reticular pontine nucleus	<0.01*	<0.01*
Locus coeruleus	0.08	0.19
Red nucleus	0.08#	0.02*
Other nuclei	0.08	<0.01*

Where data did not pass tests for normality by Sigma Stat® the non-parametric Mann -Whitney rank sum test was performed.

* Denotes that the T-test showed significant differences in compared means (P< 0.05). These ages were tested to determine if a change were observed over time.

Appendix C3: P values from t-test made with Sigma Stat® comparing the proportion of Oregon green labelled neurons in different nuclei at P7 against P35 values in control animals.

Nuclei	t-test (P) P7 against P35
Ventral medullary reticular nucleus	0.14
Dorsal medullary reticular nucleus	0.11
Raphe nucleus	0.06
Gigantocellular reticular nucleus	0.19
Lateral vestibular nucleus	0.08
Reticular pontine nucleus	0.12
Locus coeruleus	0.14
Red nucleus	0.05*
Other nuclei	0.24

* Denotes that the T-test showed significant differences in compared means ($p < 0.05$).

Appendix C4: P values from t-test made with Sigma Stat® comparing the
proportion of Oregon green labelled neurons in different nuclei at
P7 against P35 values in spinally transected animals.

Nuclei	t-test (P) P7 against P35
Ventral medullary reticular nucleus	0.14
Dorsal medullary reticular nucleus	0.24
Raphe nucleus	0.03*
Gigantocellular reticular nucleus	0.19
Lateral vestibular nucleus	0.24
Reticular pontine nucleus	0.12
Locus coeruleus	0.14
Red nucleus	0.15
Other nuclei	0.24

* Denotes that the T-test showed significant differences in compared means
(P< 0.05).

Appendix C5: P values from t-tests made with Sigma Stat® comparing the mean number of double labelled neurons in different nuclei at P7 against P35 values in control animals.

Nuclei	t-test P7 against P35
Ventral medullary reticular nucleus	0.76
Dorsal medullary reticular nucleus	0.67
Raphe nucleus	0.23
Gigantocellular reticular nucleus	1.00
Lateral vestibular nucleus	0.59
Reticular pontine nucleus	0.94
Locus coeruleus	0.25
Red nucleus	0.01*
Other nuclei	0.37

* Denotes that the T-test showed significant differences in compared means ($P \leq 0.05$).

Appendix C6: P values from t-tests made with Sigma Stat® comparing the proportions of double labelled neurons in different nuclei at P7 against P35 values in control animals.

Nuclei	t-test P7 against P35
Ventral medullary reticular nucleus	0.37
Dorsal medullary reticular nucleus	0.85
Raphe nucleus	<0.01*
Gigantocellular reticular nucleus	0.23
Lateral vestibular nucleus	0.42
Reticular pontine nucleus	0.23
Locus coeruleus	0.43
Red nucleus	0.01*
Other nuclei	-

* Denotes that the T-test showed significant differences in compared means (P≤ 0.05).

Appendix C7: P values from t-tests made with Sigma Stat® comparing the proportions of Oregon green labelled neurons that were double labelled in different nuclei at P7 against P35 values in control animals.

Nuclei	t-test P7 against P35
Ventral medullary reticular nucleus	0.37
Dorsal medullary reticular nucleus	0.85
Raphe nucleus	0.42
Gigantocellular reticular nucleus	0.12
Lateral vestibular nucleus	0.16
Reticular pontine nucleus	0.23
Locus coeruleus	<0.01*
Red nucleus	<0.01*
Other nuclei	-

* Denotes that the T-test showed significant differences in compared means ($P \leq 0.05$).

Appendix C8 : P values from t-tests made with Sigma Stat® comparing the mean of double labelled neurons in different nuclei at P21 against P28 values and P35 values in spinally transected animals.

Nuclei	t-test (P) P21 against P28	t-test (P) P21 against P35
Ventral medullary reticular nucleus	0.34	0.79
Dorsal medullary reticular nucleus	0.25	0.37
Raphe nucleus	0.14	0.17
Gigantocellular reticular nucleus	0.43	0.43
Lateral vestibular nucleus	0.23	0.19
Reticular pontine nucleus	0.23	0.19
Locus coeruleus	0.66	0.51
Red nucleus	-	-
Other nuclei	-	-

T-tests were made against P21 values as there were the age at which double labelled cells were first consistently found in different brain nuclei.

Appendix C9: P values from t-tests made with Sigma Stat® comparing the proportions of double labelled neurons in different nuclei at P21 against P28 values and P35 values in spinally transected animals.

Nuclei	t-test P21 against P28	t-test P against P35
Ventral medullary reticular nucleus	0.48	0.75
Dorsal medullary reticular nucleus	0.65	0.32
Raphe nucleus	0.33 [#]	0.28 [#]
Gigantocellular reticular nucleus	0.99	0.14
Lateral vestibular nucleus	0.83	0.34
Reticular pontine nucleus	0.62	0.71
Locus coeruleus	0.89 [#]	0.64 [#]
Red nucleus	-	-
Other nuclei	-	-

T-tests were made against P21 values, as there were the age at which double labelled cells were first consistently found in different brain nuclei.
[#] Mann- Whitney rank sum test performed as data failed equal variance test.

Appendix C10: P values from t-tests made with Sigma Stat® comparing the proportions of Oregon green labelled neurons that were double labelled in different nuclei at P21 against P35 values in spinally transected animals.

Nuclei	t-test P21 against P35
Ventral medullary reticular nucleus	0.12
Dorsal medullary reticular nucleus	0.06
Raphe nucleus	0.12
Gigantocellular reticular nucleus	0.12
Lateral vestibular nucleus	0.24
Reticular pontine nucleus	0.31
Locus coeruleus	-
Red nucleus	-
Other nuclei	-