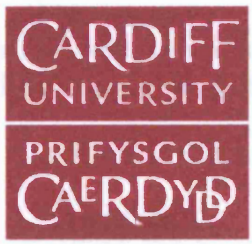

Temporal gene expression changes in the developing striatum

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A thesis presented for the degree of Doctor of Philosophy at the University of Wales, Cardiff.
October 2006

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Acknowledgements

Firstly I would like to thank my supervisors, Professor Anne Rosser and Doctor Mike Taylor for the opportunity to register for this degree and for their continued help and guidance during the course of my studies.

I would also like to thank Doctor Leslie Jones, Doctor Angela Hodges and Mister Gareth Hughes of the University Of Wales College Of Medicine, Dept of Genetics for their continued technical advice during the course of this work.

May I also thank the staff of the Brain Repair Group, who have been a pleasure to work alongside during my studies, particularly Doctor Claire Kelly, and Miss Ngoc Nga Vinh, without whose expertise and patience I would never have been able to get this far. Miss Ngoc Nga Vinh also helped immensely by carrying out a large proportion of the immuno-histochemistry and cell culture work that contributes towards Chapters Six and Seven. I would also like to thank the Huntington's Disease Association and their contributors, for supporting this project.

On a personal note, the carrying out of this project and writing of this thesis were also made possible by the continued encouragement, support and understanding of my partner Dorthe, my close friends and my family.

For

V.M.J.

Abstract

Huntington's disease (HD) is a neurodegenerative condition in which the predominant loss of neurons occurs in the striatum. At present there is no treatment for this condition, although neural transplantation may prove to be a viable therapeutic strategy if an appropriate source of donor cells can be identified. A major requirement of these donor cells is that they are able to differentiate into the cells lost to the disease process; that is, largely medium spiny projection neurons (MSNs). Currently, suitable donor cells (i.e. those already committed to developing into MSNs) can be extracted from foetal brain and early clinical trials have provided some evidence of efficacy when human foetal-derived striatum is transplanted into the brain of patients with HD. However, there is a major problem of supply and demand with respect to human foetal tissue and so alternative source of donor cells must be identified.

However, only a small proportion of animal studies in the literature report differentiation of MSNs from either animal or human stem cell sources and the percentage of mature MSNs is generally low, most cells becoming glia or taking on a 'default' GABA-ergic neuronal phenotype. Thus it is likely that stem cells will need to be 'directed' towards a MSN phenotype.

Knowledge of the molecular signals that cause striatal progenitors to differentiate into a MSN phenotype *in vivo* would help us to understand how to direct the fate of stem cell populations towards this phenotype *in vitro*.

In this thesis I have studied the genetic changes that occur during normal striatal development in the mouse with the aims being (i) to identify genetic markers of stages of differentiation for these cells and (ii) to identify genes important for striatal development with the ultimate aim of using this information to design protocols to direct the differentiation of stem cells towards a MSN phenotype.

I have studied the gene expression of the population of cells that make up the whole ganglionic eminence during its period of peak neurogenesis using Affymetrix micro array. I then validated the results of a subset of genes that were found to be significantly up-regulated using *in situ* hybridisation and then used these genes to characterise either primary cells that were differentiated *in vitro*, or cells that have been proliferated and then differentiated *in vitro*.

This study has not only provided a gene expression signature of a developing population of striatal precursors, enabling future experiments to compare and contrast expression patterns seen in different *in vitro* studies, but it has also highlighted *Foxp1* and *Foxp2* that have been shown to have a high degree of association with this period of development. This has encouraged future work in this laboratory in which the developmental functions of these genes in relation to MSN differentiation and development will be studied.

List of Abbreviations

BCIP	5-bromo-4-chloro-3-indolydl-phosphate
BLAST	Basic local alignment search tool
BME	β-mercapto-ethanol
CGE	Caudal ganglionic eminence
CNS	Central nervous system
CRABP	Cellular retinoic acid binding protein
CRL	Crown-rump length
DARPP32	Dopamine receptor precursor protein 32 Kda
DEPe	Diethylene pyrocarbonate
DMEM	Dulbecco's modified eagle's medium
DNA	Deoxyribonucleic acid
EC	Embryonic carcinoma
EG	Embryonic germ
EGF	Endothelial growth factor
EST	Extended sequence tag
FCS	Foetal calf serum
FGF	Fibroblast growth factor
FNPS	Foetal neural progenitor
GABA	Gamma-amino buteric acid
Gp *i/e*	Globus pallidus *internal/external*
HBSS	Hanks medium buffered saline solution
HD	Huntington's disease
HDAC	Histone deacetylase
hES	Human embryonic stem
IDV	Integrated density value
IVF	*in vitro* fertilisation
IVT	*in vitro* transcription
LGE	Lateral ganglionic eminence
mES	Mouse embryonic stem
MGE	Medial ganglion eminence
MM	Mismatch
MSN	Medium spiny neuron
NBT	nitro blue tetrazolium chloride
OD	Optical density
PCR	Polymerase chain reaction
PET	Positron emmision tomography
PFA	Paraformaldehyde
PGC	Primordial germ cells

PM	Perfect match
RALDH 1/2/3	Retinaldehyde 1/2/3
RNA	Ribonucleic acid
RNAi	Interferance RNA
SCID	Severe combined immuno deficient
SET-HD	Systematic evaluation of treatments for HD
SGZ	Sub-granular zone
SH	Subtractive hybridization
SICM	Inner cell mass
SNc/r	Substantia nigra *pars compacta/reticula*
STN	Sub thalamic nuclei
SVZ	Sub-ventricular zone
Ta	Annealing temperature
Tm	Melting temperature
VZ	Ventricular zone
WGE	Whole ganglionic eminence

Chapter One

1 Introduction.

1.1 Huntington's Disease.

Huntington's disease (HD) is one of the most prevalent monogenetic autosomally dominant neurodegenerative disorders in the developed world. The mutated gene that lies at the heart of the pathology is found on chromosome four (Hummerich *et al.*, 1994; Mangiarini *et al.*, 1996) and codes for a protein called Huntingtin. Although this gene is ubiquitously expressed, the main pathological changes that take place during the course of the disease do so in a single cell type; the medium spiny neuron (MSN) found in one distinct area of the brain called the striatum. As the disease progresses there is more widespread loss in other brain regions although the extent to which this is a primary process or secondary to degeneration of the striatum is not clear. The precise roles of this gene and its product are not known, although studies looking at Huntingtin knockout mice have found that this type of mutation leads to embryonic lethality (Duyao *et al.*, 1995; Zeitlin *et al.*, 1995; Nasir *et al.*, 1995).

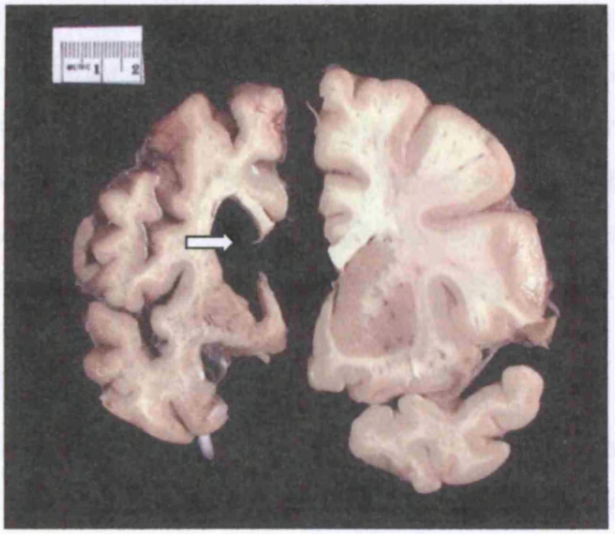


Fig.1. 1; A macroscopic comparison of an HD brain slice (left) and a normal control brain slice (right). Note the enlarged lateral ventricle (central white arrow) due to striatal atrophy and evidence of major neocortical atrophy in the advanced HD brain.

Although it is not fully understood how the mutated gene product causes neuronal degeneration, it is thought to be by some kind of 'gain of function' caused by the

abnormal poly-glutamine repeats (coded for by the codon CAG), whose numbers are amplified in the mutated form and somehow confers the molecule's toxicity. The normal upper limit of CAG repeats for the human Huntingtin gene is said to be 36. More recently it has become apparent that disease onset approaches 100% with repeat numbers of 39 and over, and that 36 to 39 repeats represents an intermediate range where risk of developing the disease has not been fully determined. There is also a reciprocal relationship between CAG repeat number and age of onset, so that the higher the number of CAG repeats on the mutant gene, the younger the onset (Rubinsztein, 2002), although this relationship is not sufficiently strong to allow a prediction of disease onset for individual subjects. When the repeat sequences are very long, a form of young onset HD results, in which the signs and symptoms are different; taking the form of bradykinesia, distonia and rigidity with chorea frequently being absent, and the affected children often developing epileptic seizures (Vonsattel and DiFiglia, 1998).

The disease affects between 3 and 7 people per 100,000 in most populations of western European descent (Harper and Newcombe, 1992), although in some instances this may be as high as 15 people per 100,000, explained by the distribution of predisposing alleles in the normal population of the country in question (Hayden et al., 1981). This is in contrast to other ethnic populations, where the prevalence may be much less; i.e. Japanese and African populations (<1 in 10,000,000 (Rubinsztein, 2002)).

HD has an average age of onset of between 30 and 50 years with mean disease duration being approximately 15-20 years from onset of symptoms to death. This timing means the sufferer may well have parented children before being diagnosed and each child then stands a 50/50 chance of inheriting the dominant mutation.

1.2 Symptoms of the disease.

The course of the disease tends to be slow, particularly in the early stages. Onset may be recorded with the start of slight, uncontrollable muscular movements, stumbling and clumsiness, lack of concentration, short-term memory lapses, depression and mood swings (sometimes including aggressive or antisocial behaviour). The cognitive and behavioural symptoms may precede the motor onset by up to ten years (Lawrence *et al.*, 1998; Kremer *et al.*, 2002). Great strain is often put on personal relationships due to

unexpected temper outbursts caused by the above symptoms and directed towards the partner or loved ones.

As the disease progresses sufferers begin to experience a wide range of symptoms which may include increased clumsiness, problems with balance, involuntary movements, tremor, dysarthria and dysphagia. These involuntary movements include chorea, dystonia and tics. The chorea (from the Latin verb meaning to dance) can be described as purposeless movements that affect the face, trunk and limbs that may appear in a series of groups that result in movements that appear to be complex. The movements can be made more complex by the patient's own (possibly subconscious) attempts to mask them by purposeful movements.

Cognitive changes can also result in a loss of motivation, initiative and/or organisational skills, some or all of which may result in the person appearing to be lazy. There also may be difficulty in concentrating on more than one activity at a time.

Emotional changes can also occur, resulting in stubbornness, frustration, mood swings and depression. However, unlike the motor and cognitive symptoms, psychiatric symptoms do not typically follow a progressive course, and can be greatly influenced by psychosocial aspects (Craufurd et al., 2001).

Unfortunately, the end stage of this disease leaves a patient who is mute, wheelchair bound and tube fed before an inevitable death usually occurring as a result of a secondary complication due to the immobility of the patient; the average age of death being 54-55 years old (Kremer et al., 2002).

HD is not just a personal tragedy in terms of the victims and their families but also expensive in economic terms. This is due to the fact that HD strikes sufferers at a point in life when they would normally be at peak earning potential. The progression of the disease is also long and drawn out, the sufferer needing full nursing care towards the latter stages, before an inevitable death; as such the costs to the community are enormous.

1.3 Therapeutic strategies for the treatment of HD.

At present, there is no cure for HD. Therefore, if a patient reports symptoms and is found to test positive for the mutation, the prognosis is bleak. Once symptoms start to appear, doctors can try to control the emotional problems by the means of medications used in standard psychiatric practice; however, many of these drugs have side effects such as fatigue, restlessness or hyper-excitability. There are also a number of drugs that may help with the choreiform symptoms, although these are of limited efficacy. Unfortunately at this time there are no treatments available that can alter the underlying course of this disease.

1.3.1 Developing novel therapeutic strategies.

A variety of approaches are being used world-wide to try to develop disease-modifying treatments for HD. These include strategies to target the pathogenic mechanisms of HD as well as more empirical approaches. In summary, general approaches include:

- Gene therapy
- Understanding the underlying cellular pathology of HD with the view of targeting specific pathways.
- Screening all known drugs for activity in cellular and model animal assays of HD.
- Neuronal protection.
- Cell replacement therapies.

A detailed discussion of these strategies is beyond the scope of this thesis and can be found in (Bonelli and Wenning, 2006; Handley *et al.*, 2006). However, a few examples are outlined below.

Gene therapy may include the introduction of either genes that code for proteins that will aid survival of the affected cells (Tuszynski, 2002; Bloch *et al.*, 2004) or molecules such as RNAi that interfere with the expression of the mutant Huntingtin protein itself (Rodriguez-Lebron *et al.*, 2005; Huang and Kochanek, 2005).

An example of a potential treatment that will utilise information on the pathogenic mechanisms of HD is based on recent evidence that gene transcription dysregulation may

be a contributing factor to the cell death seen in HD; one possible mechanism for this is the binding of mutant Huntingtin to components of histone acetylases preventing histone acetylation (Hockly *et al.*, 2003). Thus, a rational approach that is being considered is to administer histone deacetylase (HDAC) inhibitors (Ferrante et al., 2003). These have been shown to rescue lethality and degeneration in a *Drosophila* model of polyglutamine disease (Steffan et al., 2001), and dramatically improve motor impairments in the R6/2 transgenic HD mouse model (Hockly *et al.*, 2003).

Other groups are following up the hypothesis that HD is mediated through defects in mitochondrial pathways. Support for this comes from many areas including the recent finding that tauroursodeoxycholic acid, a bile acid normally produced by humans in very low levels, exhibits neuro-protective effects in the R6/2 transgenic HD model (Keene et al., 2002).

A number of groups have set up drug screen projects to screen candidate drugs using PC12 cells in culture. These projects provide candidate drugs for further investigation (Aiken *et al.*, 2004; Wang *et al.*, 2005b). A related approach is the SET-HD project which provides an objective and systematic evaluation of available pre-clinical and clinic data so that researchers can select the most promising agents for further study (http://www.huntingtonproject.org/).

Another possibility is cell therapy that will be discussed further below. It should be emphasised that it is likely than more than one of these approaches will be utilised in any one patient as it is likely that no one approach will completely stop the progression of this disease in its tracks.

1.4 Cell replacement therapies as a possible treatment for Huntington's Disease.

Cell replacement differs from most approaches outlined above in that it attempts to be restorative, based on the use of cell implantation to replace the striatal medium spiny neurons that have been lost to the disease process.

HD lends itself well to a cell replacement therapy due to the relatively focal loss of MSNs from the caudate nucleus and putamen. These neurons receive their major inputs from the cerebral cortex, thalamus and substantia nigra (pars compacta), and have

their primary outputs via GABA-ergic projections to the globus pallidus and the substantia nigra pars reticulata. Studies in animal models of HD have shown that cells dissected from the developing striatum of a developing foetus can show functional integration when transplanted into the lesioned striatum of experimental animal models of HD (Dobrossy and Dunnett, 1998; Nakao and Itakura, 2000; Dobrossy and Dunnett, 2005a; Dobrossy and Dunnett, 2005b).

Evidence from animal studies has demonstrated the efficacy of foetal striatal transplants in animal models of HD (Dunnett, 2000). Furthermore clinical studies have provided preliminary, but convincing evidence that human foetal striatal implants can produce clinical benefit (Bachoud-Levi *et al.*, 2006).

To date, the donor cells used in clinical neural transplantation trials have been derived directly from primary foetal striatum (Kopyov *et al.*, 1998; Bachoud-Levi *et al.*, 2002; Hauser *et al.*, 2002; Rosser *et al.*, 2002). However, although primary foetal tissue transplants are crucial to demonstrate the effectiveness, or otherwise, of transplantation strategies in HD, due to the ethical and practical constraints involved with the harvesting and use of these cells for use in research and medicine, there are severe limitations of supply and thus the chances of mainstream therapies adopting their use is negligible. For cell implantation to be widely adopted, therefore, it is imperative that alternative renewable sources of donor cells are identified.

A number of possible sources of donor cells can be considered. Some not discussed further here include primary foetal xenographs, where developing striatal cells are obtained from other species (most commonly pig) and are used to replace those that degenerate. So far xenografts have been performed in animal models (Isacson *et al.*, 1989; Armstrong *et al.*, 2002; Armstrong *et al.*, 2003) and in the clinical setting as pilot studies for a replacement therapy for Parkinson's disease patients. However, although the animal model studies have shown promising results, the clinical results showed little evidence of graft survival or functional improvement, either on Positron Emission Tomography (PET) scanning or on post mortem analysis, probably due to graft rejection.

Genetically modified cells lines are also a current research target. Using neurally committed cell lines functional benefit has been reported in animal models (Lundberg *et al.*, 1996) and some cells with characteristics of MSNs were seen (Saporta *et al.*, 2001).

However, other studies have reported good survival but still no expression of striatal-specific markers and no functional improvement (Fricker-Gates *et al.*, 2004b).

Stem cells have received considerable attention recently as a potential renewable source of cells for neural implantation and indeed there are already examples of stem cells being applied medically in other systems (skin (Wu *et al.*, 2006), bone marrow repopulation in cancer (Wang *et al.*, 2005a)). Stem cells are discussed in more detail below.

1.4.1 Stem cells

The definition of a stem cell is one that can divide by both symmetric division to produce two identical daughter cells and can also divide asymmetrically to give rise to another stem cell and a more differentiated progeny.

Depending on its developmental stage stem cells may have the potential to differentiate into all cells including placenta (totipotent), all cells of the developing organism (pluripotent) (e.g. ES cells), many (multipotent) (e.g. bone marrow stem cells), or even just one sub-type of specialised cell.

From this one cell the first four or so cleavages give rise to more totipotent cells (that may split to form twins, triplets etc), after which the generation of pluripotent stem cells confers the potential to form the different germinal layers of the embryo and all the cells in the adult organism. These latter cells are not, however, capable of forming cells of the extra-embryonic membranes (which are derived from the trophoblast).

There are at least three sources of pluripotent cells;

1.4.2 Pluripotential Stem cells.

1.4.2.1 Embryonic Stem (ES) Cells.

These can be isolated from the inner cell mass (ICM) of the blastocyst; at the stage of embryonic development when implantation occurs (Evans, 1989; Notarianni *et al.*, 1991; Thomson *et al.*, 1998).

ES cells are pluripotential and can be tested for this by injection into immuno-compromised nude (SCID) mice to produce teratomas that contain differentiated derivatives from all three germ layers (ectoderm, mesoderm and endoderm). These cells

have a wide therapeutic potential, but need to be directed towards the cell type of interest. However, there is an ongoing ethical debate as to whether they should be used due to the fact that they are presently harvested from eggs that are collected for *in vitro* fertilisation (IVF) but are surplus to requirements.

Those who oppose the use of ES cells do so because;

1. these cells have the potential to be implanted and grown to produce viable embryos and thus an argument is made that to use them would be destroying a human life, or
2. the potential for these cells being used to clone a human baby would be vastly increased (Sandel, 2004).

Human ES cells (hES cells) and mouse ES cells (mES cells) are thought to differ, in the fact that they show several morphological and behavioural differences (Odorico et al., 2001). Some signalling pathways may also differ, although many of the known pluri-potentiality genes expressed are also common to both species (Stojkovic et al., 2004). These differences make observations seen in one species harder to replicate/infer in other species and they also emphasise the need to validate results in the human cells that are under consideration for clinical use.

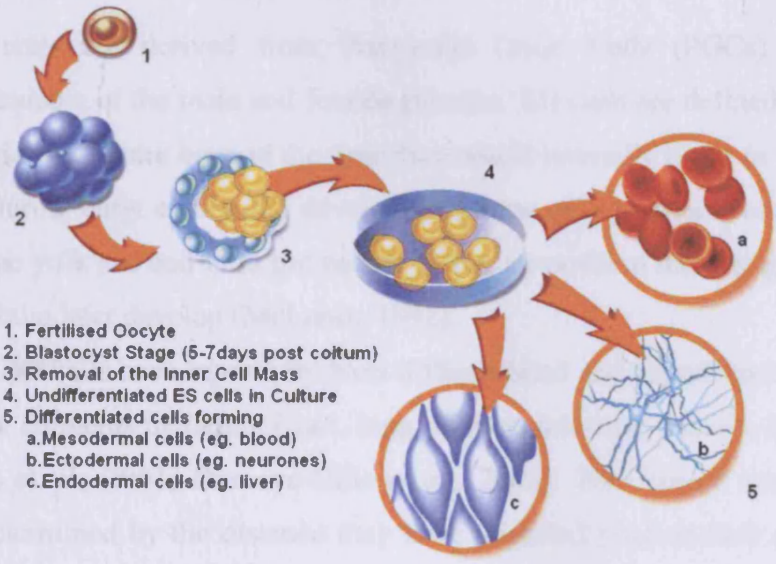


Fig. 1.2; Derivation, culture and differentiation of ES cells. Adapted from (C S and T Centre Museum of Science Boston M.A., 2005).

Despite the enormous therapeutic potential of ES cells, there are still many challenges in the use of these cells. For example, we currently know very little of how to control their expansion and differentiation to produce exclusive cells types tailored for a specific purpose. This said, research using ES cells is still very much in its infancy and these problems are recognised and may be overcome given time.

1.4.2.2 Embryonic Carcinoma (EC) Cells.

Pluripotent EC cells are derived from, and are the stem cells of, tumours known as teratomas (also known as teratocarcinomas). These growths arise from embryonic germ cells that have continued to divide and EC cells are considered to be the malignant counterparts to ES cells (Przyborski et al., 2004).

EC cells such as the mouse P19 embryonic carcinoma line have been used in research as alternatives to, and models of ES cell development and culture (although caution is required as they are clearly different cells). Although the actual differentiation capacity of EC cells is more limited than ES cells this can be used to advantage in providing a simpler and more robust experimental system for studying cell differentiation.

1.4.2.3 Embryonic Germ (EG) Cells.

These cells are derived from Primordial Germ Cells (PGCs) and are the embryonic precursors of the male and female gametes. EG cells are defined as PGCs that continue to divide in culture beyond the time they would normally do so *in vivo* (Labosky et al., 1994). During early embryonic development these cells migrate from the posterior endoderm of the yolk sac and hind gut before taking up position into the gonadal ridges where the genitalia later develop (McLaren, 1992).

Mouse EG cells have reportedly been differentiated and contributed to a range of tissues in foetal chimeras including heart, lung, kidney, intestine, muscle, brain and skin (Durcova-Hills *et al.*, 2001; Durcova-Hills *et al.*, 2003). The fate of mouse EG cells seems to be determined by the distance they have migrated prior to their removal from the embryo; the earlier the removal, the more likely they are to divide for longer, while if removed later, their ability to proliferate prior to differentiation seems to be progressively more restricted (McLaren, 1992).

Human EG (hEG) are less well understood with various studies in the literature reporting that human PGCs are prone to apoptosis or spontaneous differentiation when put into culture (Pesce *et al.*, 1993; Felici *et al.*, 1999). Some reports suggest that hEG cells are not as easy to manipulate in culture as mouse EG cells (Turnpenny et al., 2003), although one group do claim to have proliferated a 'possible' hEG population on a mouse STO feeder layer for around a year (Park *et al.*, 2004) while still remaining karyotypically normal (expressing high levels of alkaline phosphotase, OCT4 and certain surface markers). These cells were also reported to form embryoid bodies and differentiate into cell types from ectodermal, endodermal and mesodermal tissues (Park *et al.*, 2004).

Currently hEG cells need to be fully characterised, but may eventually prove useful for therapy as well as providing a model system to elucidate the mechanisms of differentiation of other types of stem cell.

1.4.3 Tissue Specific Stem cells.

1.4.3.1 Foetal Neural Precursors (FNPs).

Instead of obtaining ES, EC or EG cells and having to elucidate the signals needed to drive differentiation firstly towards firstly a neural lineage and then a specific cell type *in vitro*, an alternative approach is to isolate proliferative stem cells that are already destined to give rise to the required cell type.

Foetal Neural Precursors (FNPs) are cells that have been isolated from the neuro-epithelium of the embryonic CNS and can be defined as single cells that are clonal precursors of identical daughter cells as well as a defined set of differentiated progeny (Weissman *et al.*, 2001). These cells can be isolated from most parts of the foetal brain.

Striatal FNPs are derived from the Whole Ganglionic Eminence (WGE; the area in which striatal cells are born). FNPs can be encouraged to proliferate in culture with growth factors such as FGF2 and EGF (Tropepe *et al.*, 1999; Kelly *et al.*, 2003) to form spheres of cells also known as "neurospheres" or Embryonic Neuronal Precursors (ENPs). FNPs can be caused to differentiate into neurones by removal of EGF and FGF2 and the addition of serum or other growth factors (Arsenijevic *et al.*, 2001b; McKay, 2004) and exposure to a substrate.

FNPs are a potential source of cells that can be isolated from striatum for cell replacement therapies due to the fact that they are already restricted to a CNS lineage and can be more restricted to a striatal lineage. When FNPs were first explored as a potential donor cell source for HD it was hoped that cells derived from the developing striatum could be expanded in number *in vitro* whilst retaining their capacity to differentiate into MSNs.

However, the reality has proven more complex; A substantial proportion of neurons differentiating *in vitro* from FNPs appear to be MSNs, provided that the FNPs are derived from the developing striatum and providing that they are allowed to proliferate for a limited time only *in vitro*. The capacity to produce MSNs (identified by DARPP32-see below) declines rapidly with proliferation *in vitro* so that MSNs are seen after 10 days to 2 weeks of proliferation for mouse cultures and approximately 2-4 weeks for human. After longer periods of proliferation the cells can still differentiate into neurons, but have lost the capacity to differentiate into MSNs, even following transplantation into the CNS (Armstrong *et al.*, 2003; Zietlow *et al.*, 2005). PCR analysis of expression of a limited battery of genes suggests that the striatal FNPs have lost positional information as they proliferate in culture. That is, they appear to retain commitment to an MSN phenotype after a few divisions under these conditions, but in the absence of developmental signals *in vitro,* this capacity is lost.

These problems suggest that there are a number of changes happening over time within these cultures: The cells that are produced after multiple divisions are not the same cell phenotype as those that are produced from the cells originally dissected from the donor. Characterising the changes in gene expression that occur with proliferation *in vitro* may aid understanding of the changes that are taking place and help to define the intracellular and extra cellular signaling cascades that control their expansion and differentiation in order to provide the correct environment to obtain MSNs to use in a cell replacement therapy.

1.4.3.2 Adult neural stem cells.

Adult neural stem cells are stem cells found in discrete regions of the brain that have a differentiation capacity limited to the production of neural cells. To date there

have been reports of finding such cells in the sub granular zone (SGZ) of the dentate gyrus, within the hippocampus (Gage *et al.*, 1995; Gage *et al.*, 1998; Bottai *et al.*, 2003; Alvarez-Buylla and Lim, 2004; Emsley *et al.*, 2005; Becq *et al.*, 2005) and the sub ventricular zone (SVZ) where newly formed neurons migrate towards the olfactory bulb (Merkle *et al.*, 2004; Soares and Sotelo, 2004; Alvarez-Buylla and Lim, 2004); this has been reported to occur in other areas of the ventricular axis such as in the fourth ventricle and the spinal cord (Weiss et al., 1996).

Recent reports have also mentioned adult stem cells in other areas such as the cortex and amygdala (Arsenijevic *et al.*, 2001a) although opposing reports say these are actually satellite glial cells positioned near to neurons (Kornack and Rakic, 2001), and in smaller numbers still in the substantia nigra (Zhao et al., 2003) although again other reports argue against this (Frielingsdorf et al., 2004).

These cells present an attractive possibility for neural repair due to the fact that they are endogenous and present the possibility of autologous transplants or endogenous repair, thus reducing the problems of immuno-rejection. However, these cells remain difficult to isolate and culture *in vitro* and little is known about the signals they require to proliferate and differentiate so their potential as a possible donor cell line for use in a cell replacement therapy remains uncertain.

1.4.4 Transdifferentiation of other stem cell types.

Recent reports have claimed success in enabling other cell types such as haematopoietic and bone marrow stromal cells to transdifferentiate to become neuronal cell lines (Jang *et al.*, 2004; Saji *et al.*, 2004; Bossolasco *et al.*, 2005; Jori *et al.*, 2005). These reports have caused much heated debate in the literature due to the infrequency and difficulties in reproducing some of the experimental data, and the rarity of the transdifferentiation events (Filip *et al.*, 2004; Corti *et al.*, 2004) , (however, Cogle et al describe a clinical study where this is reported to have happened (Cogle *et al.*, 2004)

One suggestion supported by both historic (Ephrussi and Weiss, 1965) and recent studies (Terada *et al.*, 2002; Ying *et al.*, 2002; Vassilopoulos *et al.*, 2003; Wang *et al.*, 2003b), is that transplanted cells may not have trans-differentiated, as previously supposed, but rather the host tissue cells start to express genes from the donor cells due to

a process of cells fusion and then 'reduction division' (Medvinsky and Smith, 2003) to produce a cell that can express genes from both host and donor cells.

The general consensus is that more investigation and debate needs to occur before this strategy is likely to supply a possible donor cell type for transplantation therapies.

1.5 What is currently known about Striatal development: A Literature Review.

The striatum is a key component of the mammalian central nervous system (CNS), affecting motor function, cognition and behaviour in the individual. An understanding of the molecular and anatomical aspects of its development is key to developing new treatments to treat striatal diseases such as HD, and especially relevant to the cell replacement strategies discussed above.

However, due to its complex structure, it is only in the very recent past with the help of research into homologous molecules in organisms such as *Drosophila melanogaster*, *Rattus norvegicus* and *Mus musculus* that clues pertaining to the molecular mechanisms causing this region of the basal ganglia to grow, differentiate and develop, have begun to surface.

Ultimately, I would like to relate genetic changes precisely to embryological developmental stages. This has been attempted to some extent in Chapter Four. Previous studies have tended not to make this association in a direct way and so in the following section I have separately reviewed the anatomical development of the striatum and what is know about the associated gene expression changes.

1.5.1 The striatum.

The basal ganglia are comprised of a number of interconnected nuclei linking the basal ganglia to the cortex, the thalamus and the brainstem through several independent pathways. These nuclei are the striatum, the sub thalamic nuclei (STN), the internal and external segments of the globus pallidus (GP*i/e*) and the substantia nigra (SN) *pars compacta* and *pars reticulata* (SN*c* and SN*r*).

The human striatum consists of the caudate nucleus (from the Latin adjective *Caudatus* describing a tail-like appendage; referring to its physical shape), and the

putamen (from the word naming the hard stone of fruits such as the peach, plum or cherry). Both of these areas develop from an area of the embryonic telencephalon called the ganglionic eminence that, as the name suggests, goes on to form the whole of the basal ganglia later in development.

One of the principal cellular components of the striatum is the medium spiny neuron, which accounts for 90-95% of the striatal neuron population in the rodent and approx 85% in the human. These neurons have a medium-sized cell body approximately 20-25 μm in diameter, with dendritic filaments that are covered in spines (that are themselves covered in synaptic boutons) that begin about 20μm from the soma of the MSN and continue to the end of the dendrite which extends approximately 150-250μm (Kemp and Powell, 1971; Wilson and Groves, 1980). The most common way of identifying this mature phenotype currently is by immuno-histochemical detection of the DARPP32 protein.

The majority of afferents that project onto MSNs come from cortical and thalamic inputs that provide excitatory input (Hattori *et al.*, 1979; Bouyer *et al.*, 1984) and dopamine-ergic fibres from the SNc (Bouyer *et al.*, 1984; Freund *et al.*, 1984; Freund *et al.*, 1985). Other afferents come from neighbouring MSNs and also striatal interneurons (Wilson and Groves, 1980).

The major efferents of these GABA-ergic MSNs, (containing endogenous opioids, and substance P) project to the pallidum and substantia nigra in a topographically organised manner (Semba et al., 1987). Interneurons whose axons do not leave the striatum make up around 10% of the total striatal neuronal population. These include large cholinergic aspiny neurons (Bolam et al., 1984), and several types of medium sized aspiny neurons containing somatostatin and neuropeptide Y (DiFiglia and Aronin, 1982; Gerfen, 1992; Marin *et al.*, 2000), and those that contain the calcium-binding protein parvalbumin (Gerfen, 1992).

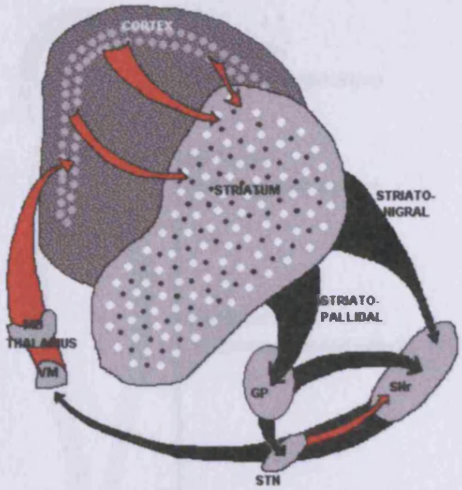


Fig.1.3; Schematic representation of the Striato-Pallidal and Striato-Nigral pathways.
(Red denotes excitatory, Black denotes inhibitory)

1.5.2 The Embryonic Origins of the Striatum.

Development of the vertebrate nervous system begins with neural induction when a dorsal region of the embryonic ectoderm becomes specified as neural plate. Neurulation then occurs as the neural plate folds in on itself forming the neural tube. This is lined by a pseudostratified columnar epithelium consisting of uncommitted progenitors from which the future CNS will arise (Kandel *et al.*, 2001; Jain *et al.*, 2001).

In the mouse the neural tube is first distinguishable at around E7 with regionalisation of the neural tube taking place from around E8. This leads to the rostral neural tube forming three primary vesicles; the forebrain (prosencephalon), midbrain (mesencephalon), hindbrain (rhombencephalon) and the caudal neural tube forms the spinal cord (Cecchi et al., 1999).

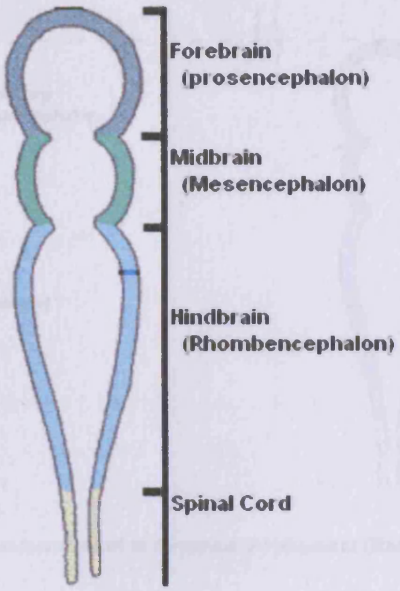


Fig. 1.4; A Schematic diagram of the mouse brain at E8

The neural tube can be further divided up using another model proposed by Peulles and Rubenstein (Rubenstein *et al.*, 1994; Rubenstein *et al.*, 1998; Puelles *et al.*, 2000). This model divides the prosencephalon into different prosomeres according to gene expression and fate mapping of the cells. It consists of three prosomeres numbered caudally to rostrally P1-P3 that are formed from the area situated around the notochord known as the epichordal region and give rise to the caudal diencephalon and the secondary prosencephalon which is formed from the precordal region of the neural tube that later gives rise to the rostral diencephalon and the telencephalon.

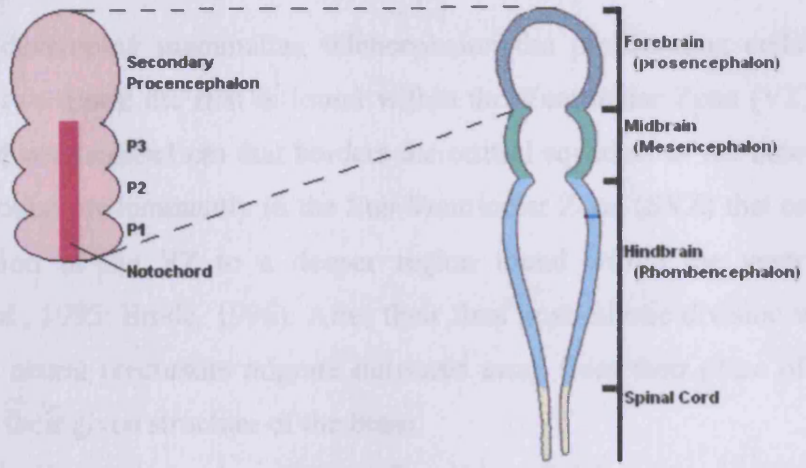


Fig. 1.5; The prosomere model of forebrain development (Rubenstein *et al.*, 1994).

The mesencephalon later gives rise to the midbrain and the rhomobencephalon splitting into two distinct parts; the metencephalon (which goes on to form the cerebellum) and the myelencephalon (which forms the medulla).

The origins of the striatum lie in the ventral telencephalic vesicle of the embryonic brain, derived from an area between the medial septum, and the border of the dorsal telencephalon that later develops into cortex.

Fig. 1.6; Schematic diagram showing formation of the telencephalon in coronal section showing the enlargement of the ventral telencephalon to form the striatum (MGE and LGE).

In mammalian development, the pallidum and striatum are derived from the transient swellings in the ventral region of the embryonic telencephalon known as the Medial Ganglionic Eminence (MGE), Lateral Ganglionic Eminence (LGE) and the caudal area where they both join, the Caudal Ganglionic Eminence (CGE). The LGE appears first, followed later by the MGE. These areas are collectively known as the Whole Ganglionic Eminence (WGE).

1.5.2.1 Timings of Striatal Development.

In the developing mammalian telencephalon the proliferating cells are found mainly within two areas; the first is found within the Ventricular Zone (VZ) forms the pseudostratified neuroepithelium that borders the central aqueduct of the neural tube and the second is found predominantly in the Sub-Ventricular Zone (SVZ) that extends from the basal portion of the VZ to a deeper region found within the ventricular wall (Takahashi *et al.*, 1995; Bhide, 1996). After their final post-mitotic division within these germinal areas neural precursors migrate outwards away from their place of origin and differentiate in their given structure of the brain.

The Ganglionic Eminence starts to form immediately after anterior neuropore closure (Kohtz *et al.*, 1998; Cecchi *et al.*, 2000) at around embryonic days 9.5 (LGE) to 10.5 (MGE) in the mouse (E9.5-E10.5 corresponds to Carnegie Stages 12-13 or 26-32 days post ovulation in the human embryo (Davidson et al., 2002) with the expression of ganglionic transcription factors such as GSH2, DLX1/2 and NKX2.1 (Rubenstein *et al.*, 1998; Corbin *et al.*, 2000). In humans the striatum begins to appear comparatively later, during the 6th week in development around Carnegie Stage 18 (O'Rahilly and Muller, 1999). It has been reported that there are sexual differences in the timing of peak neurogenesis within the murine striatum, with this point occurring earlier in females (E13 as opposed to E15 in males) suggesting that this process may be under the influence of certain sex hormones (Floerke-Nasher et al., 2000).

At E10.5 the murine CNS is still no more than a neural tube, formed from the cells of the neuro-ectoderm. These are progenitor cells that multiply to form yet more progenitors and also daughter cells (either immature neurons or glial cells) that will migrate outwards toward the periphery of the neural tube to take up their final positions before maturing and finally sending out axons and dendrites to form connections with their targets. As progenitor cells within the ganglionic eminence multiply, the neural tube also begins to bulge inwards as the cell populations within them enlarge.

The striatum develops in the ventral telencephalon just lateral to the peri-ventricular germinal zone of the whole ganglionic eminence. Early in CNS development, cells born within the germinal zone become post mitotic and begin their migration outward to take up the specific positions that they are destined to occupy in the adult

striatum. Recent evidence has shown the LGE to be the origin of most of the MSNs (Gerfen, 1992; Deacon *et al.*, 1994; Campbell *et al.*, 1995; Corbin *et al.*, 2000; Toresson *et al.*, 2000b; Polleux *et al.*, 2002), whilst MGE contributes fewer MSNs and most of the interneurons.

1.5.3 Patch and matrix organisation

These two areas contain cells that are born at different times, expressing different signals and transmitters, receiving innervation from different areas of the cortex and also projecting to different regions (Gerfen, 1992; Redies *et al.*, 2002).

The patches receive innervation from cortical neurons, originating in the deep parts of layer V and layer VI (Gerfen, 1992). The first identification of a patch/matrix type organisation was made when it was found that cells within these patches were enriched for μ-type opiate receptors (Pert et al., 1976). Later it was also noted that acetylcholine-esterase (AChE) staining in these areas (Graybiel and Ragsdale, 1978) was found to be weaker. MSNs within the matrix are innervated by projections from the superficial areas of layer V of the cortex (Gerfen, 1992). GABA-ergic striatal projection neurons from within the patches have also been found to have somewhat different projections than do the matrix neurons; e.g. they provide innervation to dopaminergic neurons within the substantia nigra *pars compacta* (SN*c*), whereas the matrix projects largely to the *pars reticulata* (SN*r*).

1.5.3.1 Patch/Matrix organisation during development.

The peak of patch formation occurs earlier than that of the matrices (Garel et al., 1999) with patch/matrix organisation starting to be evident from around E18.5 when Encephalin (Enk), DARPP32 and Dopamine Receptor-2 (DRD2) markers will stain early born striatal matrix neurons (Corbin et al., 2000) These neurons will later make up most of the striato-pallidal (indirect) pathway medium spiny projection neurons (Gerfen, 1992), Ebf-1 (a striatal homeobox gene (Garel et al., 1999) described below, Calbindin (Seto-Ohshima et al., 1988) and Somatostatin will mark later born matrix neurons (Corbin et al., 2000), which make up the majority of the striato-nigral (direct) pathway medium spiny projection neurons (Gerfen, 1992).

1.5.3.2 Cortical interneuron migration

From around E11, differentiated striatal neurons start to migrate outwards to eventually populate the striatum (Shimazaki *et al.*, 1999; Wichterle *et al.*, 2001); Peak striatal neurogenesis occurs at E14 in the mouse (Shimazaki et al., 1999) (although minor differences have been seen that are due to gender, see (Floerke-Nasher et al., 2000)) and around E15-16 in the rat (Fricker-Gates *et al.*, 2004a).

In vitro studies have shown that neurons migrating from within the ganglionic eminence not only populate the developing striatum, but some also populate areas of the cortex and hippocampus; a dramatically reduced number of GABA-ergic interneurons are found in these areas if DLX1 and DLX2 transcription factors (expressed in both the MGE and LGE, see below) are knocked out (Wichterle *et al.*, 1999; Wichterle *et al.*, 2001; Polleux *et al.*, 2002) suggesting that MGE and LGE also contribute. Previous studies have also indicated that the tangentially migrating neurons from the ventral to dorsal telencephalon gave rise to cortical GABA-ergic interneurons (Parnavelas *et al.*, 1991; Parnavelas, 2000). The use of 1,1'-dihexadecyl-3,3,3'-tetramethylindocarbocyanine perchlorate (Di I) tracer injected into the LGE as early as E11.5 resulted in the presence of a few of these same cells in the dorsal intermediate zone and cortical pre-plate 36 hours later. This number increased in slices taken one day later in development at E12.5 suggesting that E11.5 is the very beginning of this migration.

The actual source of these migrating neurones, though once thought to be just the LGE, has now been shown to also be the MGE; a recent study has reported that the number of cells migrating out of the MGE into cortex is two to three times greater than those originating in the LGE, indicating that most of the ganglionic eminence cells that migrate into the cortex are from the MGE (Polleux *et al.*, 2002). Interestingly, the same study also shows that a number of these cells don't stop in the neo-cortex, but go on to populate the hippocampus CA-1 to CA-3 areas, although they don't invade the Dentate Gyrus (Polleux *et al.*, 2002). Further evidence of this migration of MGE neurons into the hippocampus comes from a study of Nkx-2.1$^{-/-}$ mice (a gene that is not expressed in the LGE this early in development); in these mice the MGE is respecified to LGE and cell populations of the striatum (Marin et al., 2000), cortex (Sussel et al., 1999a) and hippocampus (Pleasure *et al.*, 2000) are all depleted. However, these populations are even

more depleted in the Dlx-1/2 knockout mouse, suggesting that the LGE is still the birthplace for some of these migratory neurons.

Recent advances in our understanding of neuronal migration have suggested that there are at least three types of neuronal migration involved in the development of the striatum, these being;

- Radial (or outward) migration from the LGE that go on to form the projection neurons of the adult striatum.
- Tangential (or lateral) migration from the MGE that develop to form the interneurons of the adult striatum, and
- Inward migration from the piriform pre-plate; this last group of cells are thought only to exist transiently during development as 'early generated neurons'. These cells mature much quicker than others and are thought to "provide the tract direction for growing striatal axons and/or accompany them to ensure their proceeding to the proper destination" (Hamasaki *et al.*, 2003).

1.6 Molecular regulation of the development of the striatum.

There is a growing literature relating to identification of the signals involved in striatal differentiation. As yet, however, this information is incomplete and the relationship between certain genes has not been fully elucidated and not all pathways have been defined. However, this information is important for placing any new genetic information from this work in context.

The MGE and LGE are defined not only by anatomical position but also by the expression of the many different genes throughout development. A crucial property of any progenitor is its ability to integrate extra-cellular signals with its intrinsically defined intra-cellular state to decide, or at least partially direct, the fates of their progeny (Livesey et al., 2004). It has been estimated through initial transcriptional profile studies of neural progenitors, that although there are around 2500 (Ramalho-Santos *et al.*, 2002; Ivanova *et al.*, 2002) genes up-regulated in a stem cell at any one time, only a small subset was found to be common to different stem cell types (although there were substantial differences in this subset between these studies, posing the question as to whether the genes found were actually indicative of "stemness" at all, see (Vogel, 2003) and

(Fortunel et al., 2003)). The expression level of genes may increase or decrease during the development and maturation of the cell itself and during changes in its environment. These changes are dependent on a host of temporal, environmental and/or spatial factors that may in turn give rise to other changes in the expression of "downstream" genes that cause other developmental processes to occur within the cell or its environment. The elucidation of these processes, even with the masses of data available from recently acquired profiling techniques, will take years of painstaking analysis as each part of the final combination is found, explained and then fitted precisely into the final puzzle.

1.6.1 What is known to date about the signals regulating striatal differentiation?

The following passage attempts to give an up-to-date description of the development of the striatum. This is a complex process and, although the adult rodent striatum is 90-95% medium spiny neurons, other cell types are also developing or migrating through this region. However, until very recently the available technologies have not allowed for the analysis of single cell types.

A host of molecular changes occur throughout development in response to fluctuations in the cells internal and external environment. Some of these changes regulate the expression of genes that code for many types of small molecules, some of which will act on the cell to influence its development and differentiation. These molecules range from signalling proteins such as Delta and Notch (Grandbarbe et al., 2003) that inhibit neuronal fate and promote glial cell fate and later promoting astrocytic differentiation, to the newly discovered potential of the post transcriptional control exerted by other elements such as gene silencing using RNA interference molecules (Fire et al., 1998). All of these changes are controlled through changes in the transcription of genes, and the way the gene products interact with each other.

1.6.2 Gene products linked with striatal development.

Transcription Factors are gene products that recognise specific target sites in the cis-regulatory elements of target genes and function to positively, negatively, competitively or differentially regulate target gene expression; either on their own, or in

coalition/competition with other molecules. Activity of these transcription factors may rise, fall, or disappear during cellular processes such as development or during normal activity. This regulatory complexity is especially evident in areas such as the brain.

A proportion of the genes that code for these transcription factors are known as Homeobox genes. These code for DNA-binding proteins that interact with other gene regulatory proteins to exert control over the antero-posterior patterning of the organism (Alberts et al., 2002). Expression control is achieved by the protein product of a homeobox gene binding to specific areas of DNA and regulating protein expression via a highly conserved DNA binding domain, coded for by a 180-base-pair segment (the "homeobox").

Another class of gene product that can also exert effects on development are morphogens. A cell's response to these molecules is finely graded, depending on the signal's concentration; high concentrations received by cells close to the source may direct cells onto one developmental pathway while lower concentrations received by cells further away from the source will have other effects. Therefore, depending on where cells are positioned in relation to the source, they may be driven in a variety of different ways, according to the concentration of morphogen they experience.

1.6.2.1 Sonic Hedgehog

Sonic hedgehog (SHH) (a homologue of the *Drosophila* signal protein *hedgehog*) is a prime example of a morphogen that has a variety of effects in the developing embryo (Fuccillo *et al.*, 2006a).

Within the telencephalon, cell fate is determined not just by concentration of SHH, but also by the actual timing of cell exposure to SHH (Kohtz *et al.*, 1998; Fuccillo *et al.*, 2004). Deletion of this gene in an *shh* mutant shows that it is vital for the generation of the globus pallidus and the striatum (Kohtz *et al.*, 1998; Gunhaga *et al.*, 2000), resulting in the telencephalon being greatly dysmorphic and reduced in size (Machold *et al.*, 2003) as well as changes in many other ventral structures along the rostra-caudal axis of the CNS. These changes lead to a dorsalisation (Kohtz *et al.*, 1998; Gunhaga *et al.*, 2000; Machold *et al.*, 2003) of the telencephalon, with the only part expressing ventral markers being a small region in the ventral midline. Further work

carried out on sonic hedgehog (Fuccillo *et al.*, 2006a) mutants with deletions of the dorsalising *Pax6 (Shh*$^{-/-}$ *Pax6*$^{-/-}$*)* do show a partial rescuing, resulting in the dorsal LGE reappearing (Fuccillo *et al.*, 2006b). Further analysis of ventral markers suggest that oligodendrocytes are also entirely absent from the shh mutant (Rallu *et al.*, 2002; Machold *et al.*, 2003). SHH is first expressed in the mesodermal notochord tissue and later within ventral regions of the neural tube known as the floor plate (Alberts et al., 2002). It induces ventral identity in a concentration-dependant manner (Shimamura and Rubenstein, 1997) and has been shown to induce the expression of NKX2.1 (also known as *Thyroid Transcription Factor-1* or TITF1) a ventral forebrain marker in the MGE (Gulacsi and Anderson, 2006). Although NKX2.1 and SHH are both expressed in the MGE, neither has been detected in the LGE, begging the question as to whether SHH actually has a role in LGE induction.

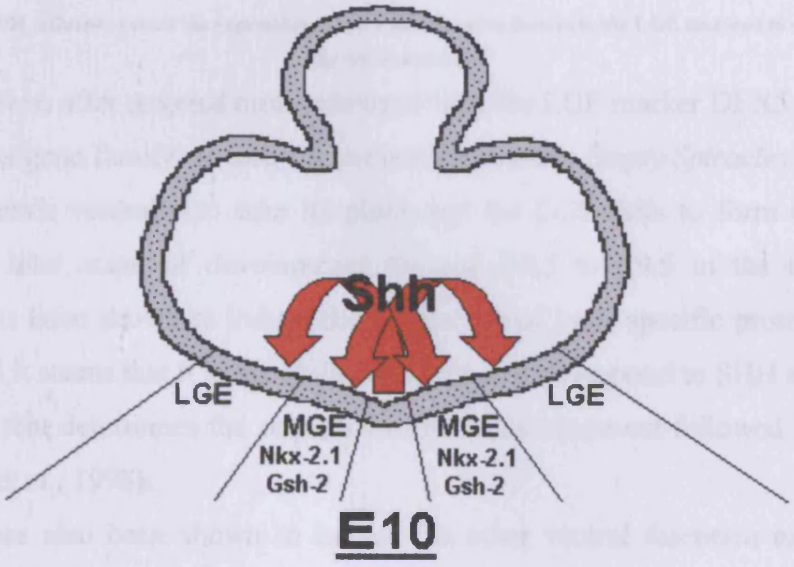


Fig. 1.7; At E10 SHH influences the expression of transcription factors that leads to ventralisation of the neural tube.

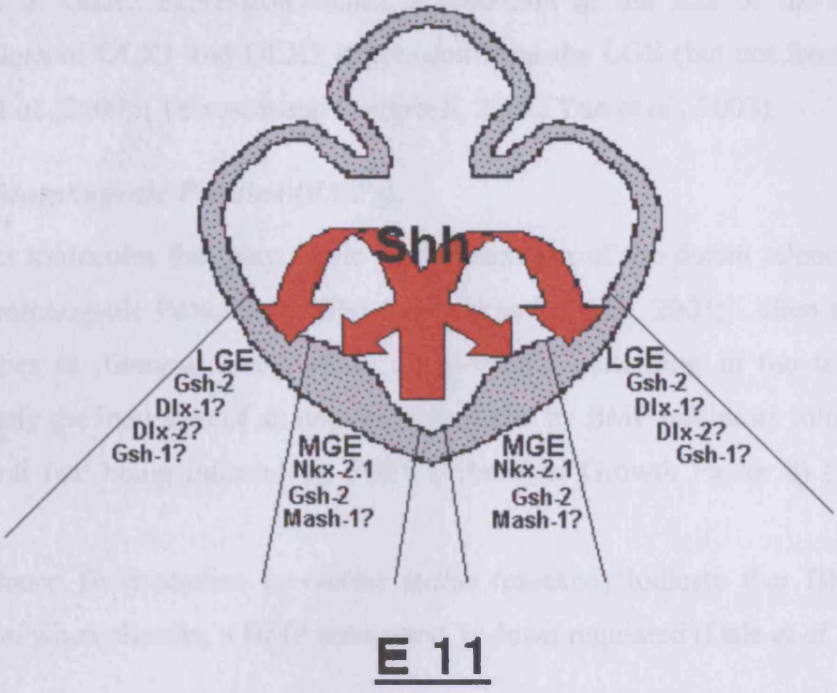


Fig. 1.8; At E11 SHH influence causes the expression of more transcription factors in the LGE that lead to ventral fate in both the MGE and LGE.

However, after targeted mutagenesis of Shh, the LGE marker DLX5 (a member of the *Distal-less* gene family) is lost, the neocortical marker *Empty Spiracles Homologue* 1 (EMX1) expands ventrally to take its place and the LGE fails to form (Kohtz et al., 1998). At a later stage of development (around E8.5 to E9.5 in the mouse), SHH expression has been shown to induce the expression of LGE specific proteins (Kohtz et al., 1998) and it seems that it is the ability of these cells to respond to SHH at this stage in development that determines the sequence of MGE development followed by that of the LGE (Kohtz et al., 1998).

Shh has also been shown to induce the other ventral forebrain markers DLX2 (possibly through the actions of *Genomic Screened Homeobox-2* (Gsh-2)) and the LIM homeodomain protein Islet1 (ISL1) (Kohtz *et al.*, 1998; Corbin *et al.*, 2000), whilst if SHH is expressed ectopically in the cerebral cortex at this time it results in the expression of NKX2.1, DLX2 (both normally ventral markers) and the LGE-specific marker CRBP (Cellular Retinoic Acid Binding Protein) (Shimamura and Rubenstein, 1997; Kohtz *et al.*, 1998; Gaiano *et al.*, 1999; Corbin *et al.*, 2000; Rallu *et al.*, 2002).

Loss of GSH2 expression causes a reduction in the size of the LGE and a subsequent loss of DLX1 and DLX2 expression from the LGE (but not from the MGE) (Toresson *et al.*, 2000b; Toresson and Campbell, 2001; Yun *et al.*, 2003).

1.6.2.2 Bone Morphogenic Proteins (BMPs).

Other molecules that play a role in dorsalisation of the dorsal telencephalon are the Bone Morphogenic Proteins (BMPs) (Gulacsi and Lillien, 2003; Lillien and Gulacsi, 2006). Studies in *Xenopus levis* show dorso-ventral patterning in the telencephalon involves firstly the induction of an anterior neural fate by BMP inhibitors followed by the ventral neural fate being induced by FGF8 (Fibroblast Growth Factor 8) (Lupo et al., 2002).

Evidence from studies in *Gallus gallus* (chicken) indicate that BMP7 causes ventralisation when chordin, a BMP antagonist, is down regulated (Dale *et al.*, 1999).

1.6.2.3 The Distal-less gene family

There are six murine DLX homologues (related to the *Drosophila Distal-less* homeobox gene family (Panganban and Rubenstein, 2002)) that are expressed in several tissues including the craniofacial primordia, limbs and central nervous system (Eisenstat *et al.*, 1999; Panganban and Rubenstein, 2002). Four of the *Dlx* genes (*Dlx 1/2/5* and *6* (Anderson *et al.*, 1997b; Eisenstat *et al.*, 1999)) are expressed in the developing LGE and have been described as being critical for striatal development.

The expression of DLX1 and DLX2 was originally thought to be indistinguishable, although work has indicated that DLX2 expression proceeds that of DLX1, which itself proceeds expression of DLX5, although early born striatal cells still express DLX5 and DLX6, indicating that these earlier born cells express DLX5/6 independently of DLX1/2 (Eisenstat et al., 1999). Single mutants for DLX1, DLX2 and DLX5 have been made, and have shown no discernable forebrain defects, suggesting there is some degree of genetic redundancy; i.e. the only defect shown to date in the DLX2 mutant is a lack of tyrosine-hydroxylase expressing olfactory bulb interneurons (Qiu *et al.*, 1995; Eisenstat *et al.*, 1999). In SHH mutants ($Shh^{-/-}$) it has also been found that DLX2 is lost, while in the $Shh^{-/-}/Pax6^{-/-}$ a broad domain of this gene is seen (Fuccillo *et al.*, 2006b).

However, the DLX1/2 double mutant shows an absence of the later-born (after E12.5 (Eisenstat et al., 1999)) GABA-ergic interneurons containing somatostatin, neuropeptide-Y and nitric oxide (Jain *et al.*, 2001). These later born striatal matrix neurons are believed to be derived from the sub ventricular zone (SVZ) of the developing striatum and are thought to remain in the SVZ apparently due to an arrest in their migration (Anderson *et al.*, 1997b; Eisenstat *et al.*, 1999). Tangential migration from the MGE to the cortex and pallidum of cells that are due to become the majority of neocortical interneurons in the neonate, is also decreased or absent (Anderson *et al.*, 1997a; Anderson *et al.*, 1997b). It seems that *Dlx* genes in general have a role to play in the development of GABA-ergic neurons, as the expression of the *Dlx* genes coincides with the location of virtually all neurons that use GABA as their transmitter. Further proof of this is shown by studies in which ectopic expression of *Dlx* genes has induced a GABA-ergic phenotype (Panganiban and Rubenstein, 2002).

1.6.2.4 Gsh-2

Gsh2 (the mammalian homologue of the Drosophila *Intermediate neuroblasts defective* (*Ind*) gene), also leads to the expression of *Mash-1* (*Mammalian achaete-scute-like homologue-1,* (also known as *AScl-1*) a gene that is closely related to the *Drosophila Achaete-Scute* gene and expressed in similar regions to *Dlx2* and implicated in striatal early-born cholinergic interneuron development (Jain *et al.*, 2001)) in the MGE; a loss of Gsh-2 will cause a subsequent loss of MASH1, affecting the generation of neural precursors in the MGE by E12.5 (Casarosa *et al.*, 1999; Kriks *et al.*, 2005) and altering the timing of production of SVZ precursors in the LGE. This leads to a perturbed generation of neuronal populations in the basal ganglia (Yun *et al.*, 2003) and cortex (as a proportion of cells born in the ganglionic eminence are destined to migrate tangentially towards dorsal regions to become cortex later in development (Casarosa *et al.*, 1999; Nery *et al.*, 2002)). As with the *Achaete-Scute* gene in *Drosophila*, MASH1 is confined to a subpopulation of neural precursor cells. Expression also precedes and ends upon neural differentiation in a similar fashion to its *Drosophila* homologue (Lo *et al.*, 1991).

Pax-6 (the murine *Pax-6*$^{-/-}$ mutant is known as *small eye* or *sey* and the mammalian homologue of the *Drosophila intermediate neuroblasts defective,* (*ind*) gene)

is a homeobox gene expressed in the dorsal telencephalon, a region that later develops into cortex, but not in the ventral telencephalon which later forms the striatum (Mastick *et al.*, 1997; Toresson *et al.*, 2000b). Conversely, GSH2, is expressed in the MGE (this is thought to be a result of the presence of Shh along the ventral antero-posterior axis) firstly at around E10.5 (Corbin et al., 2000), then spreading to the whole WGE later on, but not in the dorsal telencephalon as it is repressed by PAX6 (Quinn *et al.*, 2006). However, see (Yun *et al.*, 2001a; Yun *et al.*, 2003) who propose there is a small population of cells that double label for both GSH2 and PAX6 along the dorso-ventral border that may give rise to Tyrosine Hydroxylase^{+} (TH^{+}) cells that populate the telencephalon (Yun *et al.*, 2003). These two DNA regulating genes seem to cross repress one another and thereby regulate the expression of a further subset of genes that will cause the areas in question to develop certain fates, i.e. of cortex or striatum (Toresson et al., 2000b). The repressive interaction between these two genes appears to mark out this pallidal (dorsal) and sub-pallial boundary. As in the spinal cord, they have overlapping expression domains (Rallu et al., 2002). However, the repressive actions of GSH2 are not limited to the telencephalon; in the spinal cord class A progenitors GSH2 and subsequent MASH1 expression lead to the repression of *Ngn1*-expressing type dI2 neural progenitors cells in favour of dI3 neuronal progenitors (Kriks *et al.*, 2005). It has been shown *in vitro* that the loss of GSH2 in cultured LGE neurospheres may be the reason why these cells are increasingly restricted in their differentiation potential, the longer they are cultured, as the cells can no longer respond to contact-mediated region-specific developmental cues from primary cells that would otherwise cause them to differentiate into LGE neurons (Jensen *et al.*, 2004).

Loss of the ventralising *Gsh-2* gene (as in the *Gsh-2*$^{-/-}$ mutant) also allows the expression of *Pax-6* to spread into ventral areas (Chapouton *et al.*, 1999; Chapouton *et al.*, 2002; Suslov *et al.*, 2002) causing the expression of dorsal markers such as NGN1 and NGN2 (Corbin *et al.*, 2000; Fode *et al.*, 2000) (both basic-Helix-Loop-Helix (HLH) transcription factors) that are required for the production of cranial sensory neurons in the cortex (Casarosa *et al.*, 1999; Corbin *et al.*, 2000; Fode *et al.*, 2000) in areas that would otherwise have been ventral. However, later on in development it seems the molecular identity of these areas recovers; this is reported to be due to another member of this gene

family; *Gsh-1*, compensating for the loss of *Gsh-2* (Jain *et al.*, 2001). Furthermore, a loss of GSH2 at around E15.5 causes major path finding defects in corticofugal, corticothalamic, and thalamocortical fibres that would otherwise path through the striatum at this time before heading for their destinations (Yun *et al.*, 2003). It can be seen, therefore, that both *Pax-6*, *Gsh-2* and a number of other genes that they control are implicated in dorsal/ventral identity (Mastick *et al.*, 1997; Corbin *et al.*, 2000; Toresson *et al.*, 2000b; Yun *et al.*, 2001b). It seems they take a part in laying a 'molecular border line' down between the ventral ganglionic eminence and the dorsal cortex, as well as many other roles that they may play in cellular differentiation, development and normal activity.

1.6.2.5 Nkx-2.1

Another gene that is expressed in the foetus as early in development as the 3rd and 4th somite stages and in the MGE at around E9-E9.5 (Yun et al., 2003) is *Nkx-2.1*. As stated above, this gene is thought to be induced by the presence of Shh (Rubenstein et al., 1998) along the rostro-ventral axis of the neural tube at around E8.5 and is maintained in the presence of Shh (Gulacsi and Anderson, 2006); *Nkx-2.1* is expressed in cells that originate in the MGE but later migrate dorsally into the LGE (although not as far as the cortex (Chapouton et al., 1999)). At E14.5 and later stages, *Nkx2.1* expression is prominent in the developing globus pallidus and as development continues its expression can be detected in several other ventral telencephalic structures including the Bed Nucleus of the Stria Terminalis (BNST), parts of the septum, the ventral pallidum and parts of the amygdala (Sussel *et al.*, 1999a).

Mice lacking the *Nkx-2.1* gene have a deficiency in striatal cholinergic, calretinin, somatostatin, neuropeptide-Y and nitric oxide containing interneurons, indicating that this gene is implicated in striatal interneuron development (Jain *et al.*, 2001). *Nkx-2.1* deficient mice have also been found to not form pallidal structures; they lack basal forebrain Trk-A positive cells (probably the cholinergic neurons mentioned above (Jain *et al.*, 2001)) and also have reduced numbers of cortical cells expressing GABA, DLX2 and Calbindin; cells that would normally have been born in the MGE and migrated through

the LGE into the cortex (Sussel *et al.*, 1999b). Hippocampal cell numbers are also reduced (Pleasure *et al.*, 2000).

1.6.2.6 Foxg-1

Foxg-1, a gene formerly known as *Bf-1* (*Brain factor-1*, related to the *Drosophila Sloppy paired* and a member of the large *forked head* gene family) is a winged helix transcriptional factor first expressed at around E8.5-9 (Xuan *et al.*, 1995; Jain *et al.*, 2001), that acts as a negative regulator in the telencephalon and is expressed downstream of FGF8 (Storm *et al.*, 2006). A lack of expression of *Foxg-1*, as seen in the mutant *Foxg-1*$^{-/-}$, results in cortical hypoplasia and still birth (these mutants are only viable up to the age of E18.5 (Hanashima *et al.*, 2004; Martynoga *et al.*, 2005; Storm *et al.*, 2006)). This is thought to be due to progenitors differentiating early leading to reduction of the progenitor population and therefore a reduction in the final numbers of cells within the telencephalon (Xuan *et al.*, 1995). FOXG1 interacts with transcriptional co-repressors from the GROUCHO/TLE (*transducin-like enhancer of Split*) family and also associates with histone deacetylase to repress transcription of certain genes (Yao *et al.*, 2001) that would otherwise enhance neuronal differentiation.

In a recent study FOXG1 was found to suppress the earliest born cortical neurons (the Cajal-Retzius neurons of layer 1 of the cortex) in normal development, while in the *Foxg-1*$^{-/-}$ mouse there is an excess of these cells within the cortex (Hanashima *et al.*, 2004).

1.6.2.7 Ebf-1

Differentiation of striatal cells may also be associated with the expression of the transcription factor EBF1 (Early B-cell factor-1 also known as Olf-1, or Coe) which is expressed in both the LGE and MGE between E11 and E17.5 (Garel *et al.*, 1997). This gene has been found to be highly up-regulated in striato-nigral projection neurons, a subset of MSNs (Lobo *et al.*, 2006). In the normal brain, antibodies raised against EBF1 will highlight later born matrix neurons (Corbin *et al.*, 2000). The protein product of this gene contains a zinc-finger domain and a non-basic Helix-Loop-Helix dimerisation domain. Although the knockout model shows no defects within the striatum until E17.5, inactivation of this protein does affect cells within the SVZ/mantle transition in the LGE,

affecting the differentiation processes coincident with the migration of cells to the mantle zone, therefore leading to abnormal gene expression within the mantle later in development. Due to this, cell death later on in embryological development is increased (Garel *et al.*, 1999).

1.6.2.8 Meis-1/2

The *Meis* (*Myeloid Ecotropic Viral Integration Site*) family of homeobox genes belong to a super-group of transcription factors that have a Three-Amino acid Loop Extension (TALE). *Meis* genes are related to the *Homo-thorax* gene found in *Drosophila* and the *Knotted* (Kn) family of genes found in plant embryogenesis (Cecconi *et al.*, 1997). Three *Meis* genes have been found in vertebrates; *Meis-1/2/3*. However, only MEIS1 and MEIS2 have been detected in significant levels within the telencephalon; at around E10.5 MEIS1 is detected in low levels in the ventricular zone (VZ) of the ventro-lateral telencephalon while MEIS2 is present in high levels in the areas destined to become the LGE. Twenty four hours later at around E11.5, when the MGE and LGE become morphologically distinct MEIS1 is highly expressed in the CGE and developing amygdala and at lower levels in the LGE and MGE. This protein continues to be expressed in ventro-lateral regions of the striatum, cortex, ventral pallidum and medial septum (Toresson et al., 2000a). LGE precursors that have been passaged six times and then differentiated *in vitro* have also been seen to express Meis2, PBX and Dlx, all characteristics of LGE derived neurons (Parmar *et al.*, 2002). In drosophila, the *homo-thorax* protein is known to co-locate with another protein known as *extra-denticle* while in vertebrates MEIS1 and 2 are also known to act as co-factors with the *extra-denticle* TALE protein homologues PBX3 (Toresson et al., 2000a) and PBX1 (Knoepfler *et al.*, 1997; Swift *et al.*, 1998). Toresson and colleagues have also shown that DLX expression overlaps that of MEIS and PBX proteins within the telencephalon. MEIS2 has also been shown to interact with other TALE proteins to regulate gene transcription, for example, by competing with TGIF (Transforming Growth Interacting Factor) to differentially regulate (Meis-2 activates, TGIF represses) transcription of the D_{1A} dopamine receptor in striatal cells (Yang et al., 2000).

1.6.2.9 Retinoic Acid

The active metabolite of vitamin A, Retinoic Acid, is another morphogenic molecule that has been implicated in the differentiation of striatal neurons. This molecule works as another gene regulator, via ligand activated transcription factors known as retinoic acid α receptors (RARs) and retinoid acid χ receptors (RXRs), which are both nuclear receptors belonging to the steroid and thyroid hormone receptor family (Zetterstrom *et al.*, 1999).

In the spinal cord, Retinoic acid acts in opposition to FGF8, as a requirement for neuronal differentiation (Diez del Corral et al., 2003) and to specify neuronal development in the early neural tube.

Retinoic acid begins to appear in the developing CNS around E7, and Cellular Retinoic Acid Binding Protein (CRABP) assays have been detected in high amounts in the striatum in newborn rats (when compared to cerebellum, hippocampus and control samples). This expression can also be related to the areas of dopamine D2 receptor innervation within the striatum suggesting a functional relationship between these two molecules. Expression falls back to just above background levels by the time the rat pup reaches 5 weeks old (Zetterstrom *et al.*, 1999).

The appearance of retinoic acid is further confirmed by the presence of retinaldehyde dehydrogenase-3 (RALDH3) (an enzyme that oxidises retinol (vitamin A) to retinoic acid) within the LGE (Li et al., 2000) and RALHD2, (also known as retinaldehyde dehydrogenase-1) which has been found within dopaminergic nerve fibre terminals within the striatum, suggesting that retinoic acid may be involved in gene regulation within this area (Zetterstrom *et al.*, 1999). However, the expression of this gene is reduced in the LGE of Gsh2 mutants concomitant with a reduction of DARPP-32 (a phosphoprotein that marks striatal projection neurons but not striatal interneurons (Anderson and Reiner, 1991)). This deficit can be partially rescued by ectopic exposure to retinoic acid (Waclaw *et al.*, 2004). MSNs that express GABA, dynorphin, substance P and enkephalin have also been shown to express RARs and RXRs (Ferre et al., 1997). More support for this has been shown in a study in chick embryos by (Marklund *et al.*, 2004) which shows that retinoic acid acts to specify the intermediate zone neurons that later become the striatum early on in the formation of the neural tube. Marklund

describes this early influence of retinoic acid as coming from the ectoderm adjacent to the ventral and intermediate areas of the neural tube, and it is this that influences the intermediate cells to express markers such as MEIS2 and PAX6 (in the dorsal-most areas of the intermediate zone). Retinoic acid has limited or no effect on more ventral cells due to the opposing action of FGF, hence ventral cells remain under the influence of ventral transcription factors such as NKX2.1 (Marklund *et al.*, 2004).

During embryonic development of the striatum, glial cells (possibly radial glia) within the LGE are a localised source of retinoids, allowing cells that are migrating through the LGE into the developing striatum to come under the morphogenic influence of retinoids that could affect their differentiation. Findings that treatment of LGE cells with either RAR or RXR agonists also enhances the striatal neuronal characteristics of these cells further support this hypothesis (Toresson et al., 1999).

From the information provided by these earlier studies it can be seen that the differentiation, growth and maturation of the striatum involves a complex spatial and temporal interchange of many different molecular signals (the above list is by no means exhaustive). These signals act over the whole time period of striatal development from its initiation and neurogenesis at around E9.5 (Corbin et al., 2000), throughout its cellular proliferation until its final maturation. Some of these signals even change in their actions depending on the stage of development.

1.7 Aims of this project.

Small clinical trials of foetal striatal tissue implantation in HD have demonstrated the validity of a restorative approach. However, in order to become more widely available therapy a renewable supply of MSN precursors is required. Any stem cells grown in culture will require some direction towards an MSN phenotype. Thus, understanding the signals important for the development of this specific phenotype is important irrespective of the proposed cell source.

In this project the aim was to identify genes likely to be important in striatal development. This was done by characterising gene expression in striatal neural progenitors that later give rise to the MSNs of the adult striatum, over the peak time

period for MSN specification in this group of the neural progenitors, i.e. between the ages of E12 and E16.

Identification of such genes is important for a number of reasons;

- Identifying gene expression changes that are functionally relevant for striatal development will be important in the process of producing protocols to direct the differentiation of stem cells towards a functional MSN phenotype for transplantation. However, although primary associations may be made in this thesis, validation of a functional association would most likely take longer than could be accommodated in the time frame of this project and will not form a part of my work, but will be carried out by other researchers.
- To identify genes that are associated with striatal differentiation for use as markers of stages of striatal development. Genes used for this purpose do not necessarily have to be proven to be part of the differentiation machinery so long as they can be demonstrated to be strongly associated with the process. As described above, very few markers of striatal differentiation have been defined to date.

A panel of striatal markers is required for the following reasons;

- As part of the process of learning to direct the differentiation of cells toward a striatal phenotype a panel of markers is required to identify potentially useful protocols. To date reliable identification of striatal cells has relied on DARPP-32 labelling of transplanted cells (DARPP-32 staining of cells differentiated in culture is unreliable). A panel of markers to identify developing cells that have the potential to become MSNs would be extremely valuable for this work.
- Primary foetal tissue studies have demonstrated that the developmental stage at which cells are transplanted is crucial for their survival, integration and function. Defining and recognising the optimal stage at which to transplant directed stem cell populations would be greatly aided by a panel of developmentally associated striatal genetic markers.
- FNPs are a potential source of transplantable cells for HD but, as described above, they lose the capacity to differentiate into MSNs possibly due to loss of position

information. A panel of developmentally associated striatal genetic markers would be important in understanding this process and developing method of manipulating it.

Chapter Two

2 Methods

2.1 Dissection

All animals were treated within the constraints of the Animals (Scientific Procedures) Act 1986, the laboratory project licence and the author's own personal licence.

2.1.1 Manual dissection under microscope

This technique is described by Dunnett and Bjorkland (Dunnett and Björkland, 1992) for foetal rat dissection but a similar technique is suitable for foetal mice between E12 and E16. However, due to the differences in gestation length, (that of the rat being 21-22 days and that of the mouse being 19-20 days), the foetal mouse is bigger and more developmentally advanced than the foetal rat at a specific gestational stage, therefore the following chart shows the Crown-Rump length (CRL) for foetal mice during the age range dissected. The Carnegie Staging system, (devised by Franklin Mall of the Carnegie Institute in Washington, USA in the early 1900s), is an internationally recognised protocol for staging human embryos, based on the morphological characteristics of the embryo. Accurate staging in this case is important as the aim is to define genetic expression at discrete points in the development of the foetus.

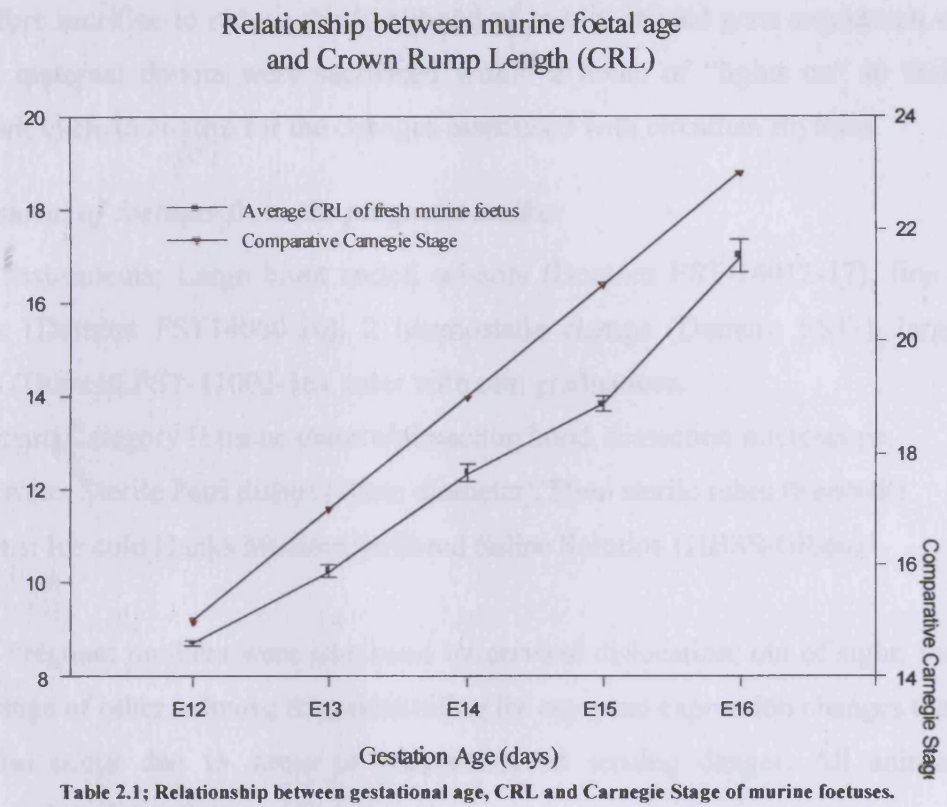


Table 2.1; Relationship between gestational age, CRL and Carnegie Stage of murine foetuses.

2.1.1.1 CRL measurement

The procedure adopted was simply to measure the foetuses straight after removal from the uterine horn and amniotic sac. A millimetre ruler was viewed alongside each foetus under low magnification and the CRL for each foetus recorded. This was used to produce a range of CRLs for the whole batch that was then taken and recorded before each dissection.

The CRL was used as a more accurate indication of age of the batch than estimating gestation from the time of mating (usually recorded using the day of vaginal plug as day 0): depending on environmental conditions murine females may also delay implantation (diapause) by a number of days (Lanman, 1970; Nieder and Weitlauf, 1984) which is particularly relevant when using mice for this study. Furthermore, animals could have mated any time during a 12 hour period, which is the standard period of pairing of a male and female for the purpose of mating.

Conditions were standardised to minimise variability between foetal tissue collections. In particular, pregnant mothers were food deprived from the afternoon of the

day before sacrifice to reduce the likelihood of insulin-related gene expression changes, and all maternal donors were sacrificed within 2 hours of "lights on" in their 12:12 light/dark cycle to control for the changes associated with circadian rhythms.

2.1.1.2 Removal of foetuses from the pregnant mother

Instruments; Large blunt ended scissors (Dumont FST-14012-17), fine pointed scissors (Dumont FST14060-10), 2 haemostatic clamps (Dumont FST-), large tissue forceps (Dumont FST-11002-16), ruler with mm graduations.
Equipment; Category II tissue culture/dissection hood, dissection microscope.
Plastic ware; Sterile Petri dishes (10cm diameter), 50ml sterile tubes (Sarstedt).
Solutions; Ice cold Hanks Medium buffered Saline Solution (HBSS-Gibco).

Pregnant mothers were sacrificed by cervical dislocation, out of sight, sound and smell range of other animals; thus controlling for any gene expression changes that would otherwise occur due to stress of anaesthesia or sensing danger. All animals were decapitated straight after sacrifice.

Dead animals were then laid ventral side upwards and a midline incision made first through the skin and then through the abdominal fascia from just above the genitalia to just below the xiphous sternum with blunt ended scissors. The severed abdominal layers were then retracted with forceps or clamps. The uterine horns were then removed and placed in a sterile 50ml tube containing 15-30ml ice cold HBSS.

Once both uterine horns from all animals had been dissected, they were transferred to a Category II dissection hood and placed into a 10cm sterile Petri dish containing ice cold HBSS. The amniotic sacs were removed with small pointed scissors from each uterine horn and then each foetus was cut from its amniotic sac and placenta and placed into a separate 10cm Petri dish containing ice cold HBSS for measurement of CRL.

2.1.1.3 Removal of the brain from the foetus.

Once the foetuses had been removed from the mother, their brains were removed; In addition to above the following equipment was used;

Instruments; Fine forceps (Dumont FST-number 5), Micro dissection/iridectomy scissors (Dumont FST-15000-08), Fine scalpel (Dumont FST-100035-00) with fine blade (Dumont FST-10035-15).

The foetuses were sacrificed by cervical severance or decapitation before further dissection was carried out.

The cranial bones do not start to calcify in the murine foetus until around E16.5 (unpublished observations), therefore any dissections before this time could be carried out using the lateral approach (Dunnett and Björkland, 1992) as shown below;

With the foetus illuminated from below, the outline of the brain could be made out within the developing cranium. A single cut was made with a fine scalpel blade, below the base of the brain and just above the eye, extending from the front of the head back to just in front of the mesencephalic flexure of the brain. Care was taken not to damage either the ventral mesencephalon of the ventral forebrain. The tips of fine forceps or the blunt side of the scalpel blade were then inserted under the skin at the apex of the cut and the overlying skin and meninges of the brain pulled away in a single motion, leaving the brain naked. Figures 1-4 show this carried out on an E14 murine embryo, however, much the same method was adapted for all ages between E12 and E16. A cut was then made across the mesencephalic flexure, or as low as possible on the brain and the brain removed and placed in a fresh Petri dish containing ice cold HBSS.

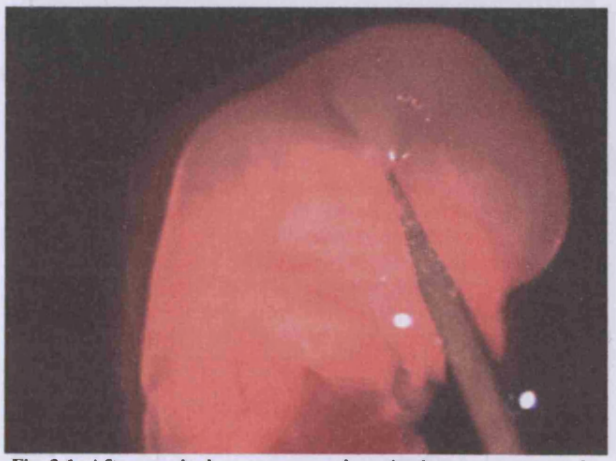


Fig. 2.1; After cervical severance or decapitation, a cut was made, beginning in the area of the muzzle, below the eye, and cutting towards the mesencephalic flexure.

2.1.1.4 Removal of the Whole Ganglionic Eminence.

The whole ganglionic eminences develop as 'heart shaped' structures on the rostro-ventral floors of the two lateral ventricles and can be made out in embryos of E12 and above, although by E16 and beyond the 'heart shape' is lost as the striatum grows to fill the lower part of the lateral ventricle. Dissection was carried out by laying the dissected brain ventral side facing down in a 10cm Petri dish containing ice cold HBBS. A longitudinal incision was made using iridectomy scissors along the upper surface of each of the cortical hemispheres, and the hemispheres then retracted outwards to reveal the inside of the lateral ventricle with the whole ganglionic eminence lying along the floor. Approaching the brain caudally with the iridectomy scissors lying parallel to the bottom of the Petri dish, a superficial incision was made under the 'heart shaped' bulge to separate it from the floor of the ventricle. This was repeated for the other hemisphere.

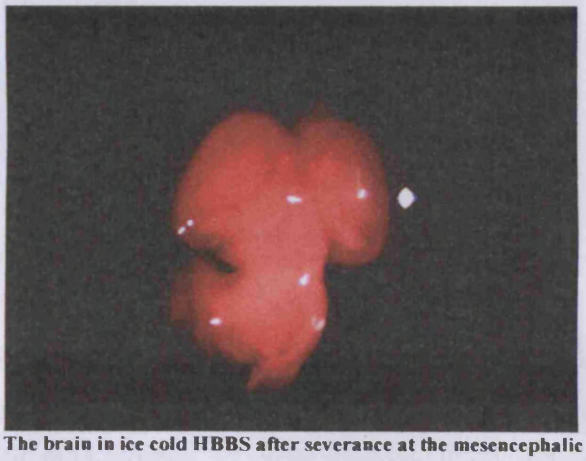


Fig. 2.2; The brain in ice cold HBBS after severance at the mesencephalic flexure.

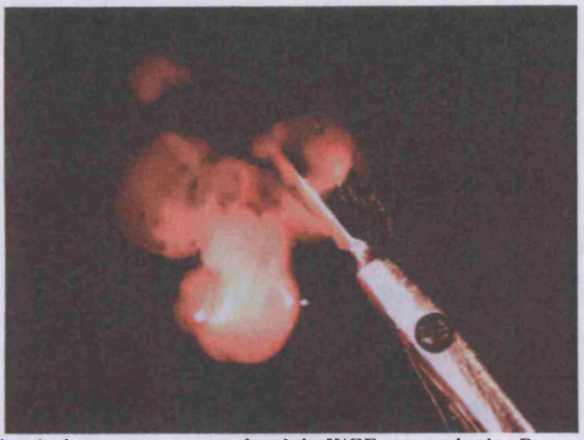


Fig. 2.3; The cortical hemispheres were retracted and the WGE removed using Dumont iridectomy scissors.

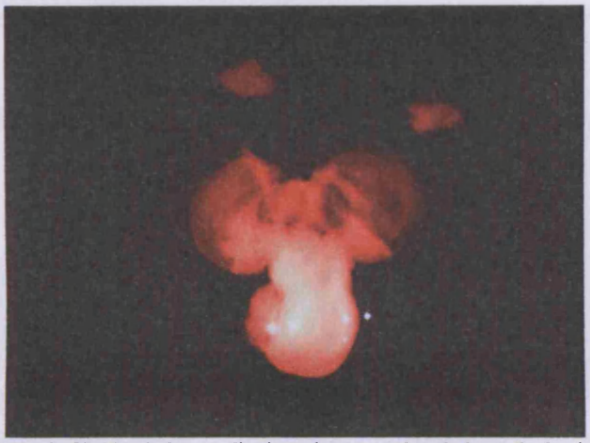


Fig. 2.4; The brain after removal of both whole ganglionic eminences (placed above the brain for illustration purposes).

The tissue was then placed in a suitable solution for fixing and either immuno-histochemistry or in situ hybridisation (4% Paraformaldehyde (PFA) for 1-2 hours then 25% Sucrose overnight then storage in -80oC) or for RNA extraction; (placed in to a Matrix D® lysing tube (Q Biogene) and 'snap frozen' in liquid nitrogen and stored at -80°C).

2.1.2 Immuno-cytochemistry and *in situ* Hybridisation

Whole foetal brains at E12, E14, and E16 were placed inside 1.5ml microfuge tubes filled with "Tissue-Tek" OCT compound (Miles Inc.) Care was taken to orient the brain rostro-caudally and the tube 'snap-frozen' and placed at -80°C until needed.

Brains were cut into 16μm sections on a Bright cryostat and mounted onto Superfrost Plus slides. These were stored at -80°C until further processing for either *in*

situ hybridisation or immuno. Sections were quenched (solution 1 below) for 5 minutes then washed in TBS (solution 2 below) three times for ten minutes each time. They were then blocked using 3% 'normal' serum (see antibody list) in TXTBS (solution 3 below) for 1 hour.

After blocking, sections were transferred (without washing) straight into primary antibody for varying amounts of time (see antibody list) in TXTBS with 1% 'normal' serum for at 4°C, after which they were washed three times in TBS, for ten minutes each time. Next, the sections were immersed in the secondary antibody (see antibody list for timings and conditions for each antibody) in TBS with 1% 'normal' serum.

2.1.2.1 Solution 1 Quench:

10ml	Methanol
10ml	Hydrogen peroxide 30%
80ml	Distilled water

2.1.2.2 Solution 2 TBS:

12g	Tris-Base
9g	Sodium Chloride
1000ml	Distilled water
	Adjust pH to 7.4 with Conc. HCl

2.1.2.3 Solution 3 TXTBS:

250ml	TBS
500µl	Triton X-100
	Adjust pH to 7.4 with Conc. HCl

2.1.3 RNA Extraction

This extraction method was advised by Dr Angela Hodges of the Dept of Medical Genetics at the Heath Hospital Cardiff, and has been shown to substantially increase final yields of labelled cRNA (personal communication).

10ml tubes were labelled for each sample of RNA and 2ml of ice-cold TRIzol for every sample of RNA being extracted was pipetted into a waiting 50ml tube. 1ml of ice-cold TRIzol was added to each sample whilst it was still in the matrix D Lysing tube and these were placed into a Fast prep agitator (Q Biogene) and homogenised on speed 4.0 for 30 seconds. The TRIzol and sample were then transferred into a 10ml polypropylene tube and another 1ml of ice-cold TRIzol was used to wash the Lysing Matrix D tube by and pipetting up and down a few times to ensure all the sample had been removed to the

10 ml tube. For small samples, spinning the Lysing Matrix D tube for a few seconds in a microfuge before pipetting with a yellow tip to obtain the last few µl's of TRIzol.

Samples were then incubated at room temperature for 5 minutes. 400µl of chloroform (reserved for RNA use only) was then added to each sample, before shaking vigorously for 15 seconds and incubating at room temperature for 5 minutes from finishing the last sample. Samples were then centrifuged at 12,000g for 15 minutes at 4°C. While samples were spinning, 1ml of isopropanol was added to the second set of 10ml tubes and an Eppendorf rack with one RNeasy (Qiagen) column, two 2ml collection tubes, one 1.5ml Eppendorf and 1 small eppendorf for (Genequant) sample, per sample of RNA was made ready; all tubes were labelled.

After centrifugation, the upper aqueous phase was transferred to a fresh 10ml tube. If extracting from small samples a 200µl yellow tip was used for the last few µl's to get in to the meniscus at the side of the tube, in order to take off all the aqueous phase. 1ml of isopropanol (reserved for RNA use only) was then added to each 10ml tube; this was mixed gently and incubated at room temperature for 10 minutes from finishing the last sample to allow precipitation.

Samples were then centrifuged 12,000g for 10 minutes at 4°C. After centrifugation, the supernatant was removed and the pellet washed carefully in 2ml of RNase-free 75% ethanol. Samples were then centrifuged again at 7,500g for 5 minutes at 4°C.

While samples were in the centrifuge the required amount of RLT buffer (RNeasy lysis buffer) with ß-Mercapto-ethanol (BME) was made up (350µl of RLT buffer with 1% BME) in a separate 50ml tube. After centrifugation the supernatant was removed and the pellet washed again in 2 ml of 75% RNase-free ethanol.

Samples were centrifuged at 7,500g for 5 minutes at 4°C before the supernatant was removed and the tube spun again to force the last of the ethanol to the bottom.

The pellet was dried for 5 minutes in a 45°C incubator/oven then resuspended in 100µl RNase-free water by vortexing followed by incubation at 65°C for 5 minutes and further vortexing.

2.1.3.1 RNeasy column cleanup

The RNA was purified further using the Qiagen RNeasy Mini kit as per the manufacturer's instructions:

Samples were mixed in the holding tube before adding 350µl of Buffer RLT (with 1% β–mercapto-ethanol) and mixing thoroughly. 250µl of RNase-free ethanol (96-100%) was then added to the diluted RNA and again mixed thoroughly (by pipetting, not vortexing). The sample (700µl) was then immediately added to an RNeasy mini column that had been placed in a 2ml collection tube. This was then centrifuged for 1 minute at 10,000g. (The eluate was then reapplied back on to the column and re-centrifuge for 1 minute at 10,000g).

The RNeasy column was then transferred into a new 2ml collection tube and 500µl of Buffer RPE (a phosphate washing buffer) pipetted onto the RNeasy column. This was centrifuged for 1 minute at 10,000g. The samples were then incubated with Qiagen RNase-free DNase for 15 minutes (to rid the sample of genomic DNA contamination) before another 500µl Buffer RPE pipetted onto the RNeasy column. This was again centrifuged for 1 minute at 10,000g before discarding the flow-through and centrifuging for another 2 minutes at 10,000g. The RNeasy columns and tube were then turned 180° in the centrifuge and spun again for a further 1 minute at 10,000g.

The RNeasy columns were then eluted in to freshly labelled 1.5ml eppendorf/collection tubes. 30µl of RNase-free water was pipetted directly onto the RNeasy silica-gel membrane before waiting 5 minutes and centrifuging again for 1 minute at 10,000g.

The sample left in the collection tube was then passed through the column again to Re-elute, or, if there was more than 150µg of RNA expected from the sample, a further 30µl of RNase-free water was pipetted directly onto the RNeasy silica-gel membrane and turning the column and collection tube in the centrifuge 180° and centrifuging for another 1 minute at 10,000g.

RNA was then quantified on a Genequant spectrophotometer to calculate the total yield of RNA, concentration, and 260/280nm ratio to check the quality of the RNA.

An aliquot (<=500ng/μl) was also sent for analysis on an Agilent chip to check quality, both from the visual 'gel picture' and the ribosomal RNA ratio. Total RNA was stored at -80°C.

2.2 Micro array methodology

The Affymetrix micro array process was carried out by the staff of the Micro array facility, The Department of Pathology, University of Wales College of Medicine (UWCM). Therefore the following is a summary of the protocol used by this facility; I observed this process in its entirety on one occasion.

2.2.1 Micro array protocol

Synthesis of double-stranded cDNA was performed using separate components from the standard Superscript Choice II cDNA synthesis kit. First-strand cDNA synthesis a T7-(dT)24 primer containing a T7 promoter site was used. After second-strand synthesis (using DNA polymerase 1 and T4 DNA polymerase) the double-stranded cDNA was "cleaned-up" sing an ethanol precipitation.

The IVT (In Vitro Transcription) was carried out using a BioArray HighYield RNA Transcript Labelling Kit from Enzo Life Sciences to make biotinylated cRNA, which was purified using RNeasy columns (Qiagen). Following quantification on a spectrometer at A260nm, the labelled cRNA is fragmented to 35-200 base fragments. Fifteen μg of fragmented cRNA are hybridized for sixteen hours at 45°C with the Mouse430A GeneChip Array using the GeneChip Hybridization Oven. Spiked hybridization controls include labelled transcripts from E. coli bioB, bioC and bioD, and cre from bacteriophage P1.

Following hybridization, the arrays are washed and stained in an Affymetrix GeneChip Fluidics Station 400. Staining was done in a three-step procedure starting with a streptavidin-phycoerythrin staining solution, followed by incubation with biotinylated anti-streptavidin and finally a second staining with streptavidin-phycoerythrin. Stained arrays were scanned with an Agilent GeneArray Scanner. Analysis of data was performed using the Affymetrix software Micro array Suite 5.0 (MAS 5.0) and Robust Multi

Averaging (RMA), GeneSpring from Silicon Genetics and the NIA (National institute of Ageing) Array Analysis ANOVA Tool.

2.3 Post Array Analysis

2.3.1 Primer design

Once a gene of interest had been identified the accession number was obtained via a search using the NCBI cross database search website (Geer et al., 2004) at http://www.ncbi.nlm.nih.gov/gquery/gquery.fcgi

The above website gave access to the gene accession number which could then be used to search for the sequence. Once the sequence was found this could be cut and pasted into primer analysis design software such as Primer 3 (Rozen and Skaletsky, 2000) or Oligo.

Factors taken into account when designing primers;

- Primer length was designed to be long enough for the sequence to be both unique, and not to be found at non-target sites. This length was usually between 18 and 30 bases; lengths of more than 24 base pairs do not confer greater specificity as they can hybridise with mismatching base pairs and decrease specificity.
- Primers were designed to have near 40-60% GC content.
- Complimentary areas of the sequence at the 3' end of primer pairs were also avoided where possible; this prevented amplification of the primers themselves to form primer-dimers. Thymidines should be avoided at the 3' end, as this base is more prone to mispairing than other nucleotides.
- Mismatches at the 3' end were also avoided as the last three nucleotides have to anneal to the template for the polymerase to catalyse extension.
- Sequences with the potential to form a secondary structure (such as hairpins) were avoided as this would destabilise primer annealing.
- Primers were designed to have a PCR product of between 300-400 base pairs if possible; if shorter than this they were likely to be less specific, longer and they were likely to diffuse slowly into the tissue to the target and would need to be hydrolysed before RNA probe hybridisation.

- If designing primers for genes that were known to be closely related (i.e. haemoglobin genes or Foxp1/Foxp2/Foxg1 etc), the primers were designed for areas of these specific gene sequences that were not common with close homologues.
- If there was a risk of genomic DNA contamination in the cDNA sample that was able to bind either the primer or a product of the primer such as an RNA probe, then primers were designed to include an intron (a piece of DNA that is not transcribed into mRNA). If these types of primer bind to genomic DNA, the product would be shown to be longer (therefore above) the mRNA product on a gel, so both could easily be differentiated from each other.
- Where primers were used that did not contain introns, an RT- (without the reverse transcriptase) stage was used during PCR to check for DNA contamination causing spurious results.

2.3.1.1 Primer melting temperature (T_m)

This is found by taking the base content of the primer; a primer with a high GC content will have a higher melting temperature due to the primer duplex having a higher number of hydrogen bonds holding it together than a primer with more AT base pairs.

2.3.1.2 Primer annealing temperature (T_a)

This is the temperature when 50% of the primer and its complementary sequence are present in a duplex state. Knowing this is necessary to establish the annealing temperature for PCR. Ideally the annealing temperature was designed to be low enough to guarantee efficient annealing of primer to target, but also high enough to minimise non-specific binding. Reasonable temperatures range from 55-70°C, and are generally set to around 5°C below the Tm of the primers (which were, where possible, designed to have a similar Tm). Higher annealing temperatures can reduce the formation of primer-dimers and non-specific products. Too low an annealing temperature will lead to non-specific annealing and therefore amplification.

2.3.1.3 PCR Buffer effects on amplification

In PCR, annealing occurs between the primers and their complimentary target sequences on the cDNA, this leads to amplification of the PCR product. However, due to the high concentration of primers needed to get efficient hybridisation during the short annealing time, primers may also anneal to non-complimentary sequences resulting in the non specific amplification of products in competition with, and reducing the yield of the specific PCR product.

2.3.1.4 Primer concentrations

For the optimisation steps this was set at 0.4 μM (see PCR Reaction Mix, below). Lower concentrations may result in a reduced yield while higher concentrations may result in the amplification of non-specific products.

2.3.1.5 Optimisation of Primers

PCR success depends upon the high ratio of specific to non-specific annealing of primer molecules which is influenced by a number of factors; including the PCR buffer and the concentration of the cations within the buffer which neutralises the negatively charged phosphate groups on the DNA backbone weakening the electro-repulsive forces between the DNA strands, facilitating the annealing between primer and template. In the absence of adequate free magnesium, Taq DNA polymerase (Promega) is inactive. Conversely, excess free magnesium reduces enzyme fidelity and may increase the level of non-specific amplification. Thus, it was important to empirically determine the optimal $MgCl_2$ concentration for each reaction. Primers were optimised using 1.0, 1.5, 2.0 or 3.0μM $MgCl_2$ concentration (see Buffer Mix, below).

The number of cycles through which each primer was hybridised was dependent upon the primer, and its ability to hybridise with its target. This can also be changed by changing the abundance of target cDNA; for the majority of primers used the cycle number was between 25 and 30.

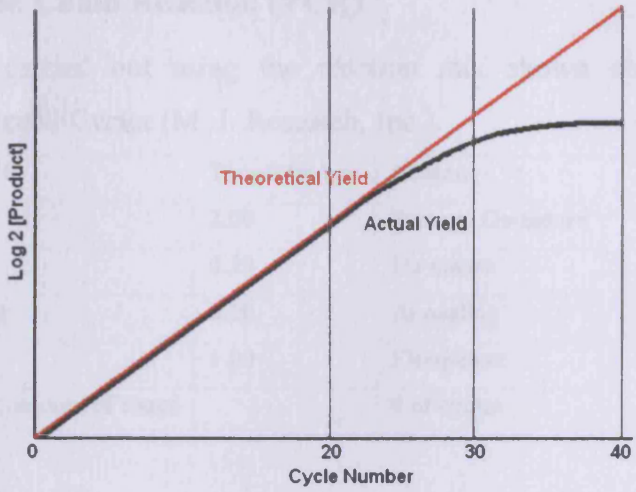


Fig. 2.5; Each PCR has a yield limit dependent on starting concentration of target cDNA.

2.3.1.6 Buffer Mix

	End Concentration	1.0µM	1.5µM	1.5µM	1.5µM	2.0µM	2.5µM	3.0µM
10 x PCR Buffer (µl)	1 x	29	29	58	116	29	29	29
MgCl2 [25µM] (µl)	Variable	11.6	17.4	35	70	23.2	29	35
DMSO (µl)	5%	14.5	14.5	29	58	14.5	14.5	14.5
dNTP [100µM] (µl)	0.8µM	2.3	2.3	4.6	9.2	2.3	2.3	2.3
dH2O (µl)	Variable	192.6	186.8	374	747	181	175.2	169.2
	End Volume (µl)	**250**	**250**	**500**	**1000**	**250**	**250**	**250**

2.3.2 Polymerase Chain Reaction (PCR)

PCR was carried out using the reaction mix shown above in a PTC-100 Programmable Thermal Cycler (M .J. Research, Inc.).

Step	Temperature/°C	Time/Min/sec.	Reason
#1	94	2.00	Primary De-nature
#2	94	0.15	De-nature
#3	T_m(Annealing)	0.20	Annealing
#4	72	1.00	Elongation
#5	Go to Step 2 x amount of times		# of cycles

2.3.2.1 Reaction Mix

PCR Reaction Volume:	20µl	End Concentration
Buffer Mix	17.2	
cDNA	1µl	
Primer 1 [10µM]	0.8µl	0.4µM
Primer 2 [10µM]	0.8µl	0.4µM
Taq Polymerase (Promega) [5 Units/µl]	0.2µl	1 Unit per reaction
Total (µl)	20µl	

2.3.2.2 Reverse transcription of RNA to obtain cDNA

This was done using Thermo-script Reverse Transcriptase (Invitrogen) and RNase H (Invitrogen).

2.3.2.3 For a 20 µl Reaction volume

Before starting a bucket of ice was filled (for all enzymes) and the thermal cycler set to 65°C hold. The following constituents were then added in order, to a thin walled/flat topped 0.2 ml PCR tube;

1 µg	RNA (total RNA)
1 µl of 50 µM	Random hexamers
0.5 µl	dNTP (Amersham) [100mM]
ad 13 µl	dH_2O

This was Incubated at 65°C for 5 min and then immediately placed on ice. It was then briefly centrifuged and the remaining constituents added;

4 µl	5x cDNA Synthesis Buffer
1 µl	RNasin
1 µl	0.1 M DTT
1 µl	ThermoScript RT (Invitrogen)[15 U/µl]

The following program was then entered into the thermal cycler;

Step	Temperature/°C	Time/Min.	Reason
#1	25	10.00	Mixing/Equalisation
#2	50	50.00	Annealing
#3	65	15.00	Extension
#4	85	5.00	Denaturing of RNA/cDNA

Any remaining RNA was digested by adding 1µl of RNase H [2 U/µl] and incubating at 37°C for 20 min.

A control reaction containing all constituents except the Thermoscript RT was also run to indicate if there were any genomic DNA 'carry over' that may interfere with upstream reactions. Gels were then run to check the amounts of cDNA in each sample before running an RT+ (and RT-) G3PDH PCR reaction to equimolarise (and check for genomic DNA carry over). The amounts of cDNA used in subsequent PCR reactions altered accordingly.

2.3.3 Cloning, transformation of primer products and subsequent purification of plasmids

Using the TOPO TA Cloning Kit (Invitrogen®) as per manufacturer's instructions:

2.3.3.1 Overview

Once the primers had been optimised and primer product made these were then cloned into the pCR®II-TOPO® plasmid vectors and then transformed into competent *E.coli.*

2.3.3.2 Cloning

The following ingredients were added to a 0.2ml PCR tube;

0.5 - 4.0µl	Fresh PCR product
1µl	Salt Solution (from kit)
Ad 5µl	RNase-free Water
1µl	TOPO® vector
6µl	Final Volume

The above ingredients were mixed gently and incubated at room temperature for 5 minutes before being placed on ice.

2.3.3.3 Transforming E.coli

The following were needed for each cloning reaction;

- 1 vial of competent *E.coli* cells thawed on ice.
- S.O.C. medium (included with kit) warmed to room temperature.
- 2 LB plates containing 50µg/ml ampicillin pre-warmed to 37°C for 30 minutes.
- Water bath equilibrated to 42°C.

2µl of the TOPO cloning reaction was pipetted into a vial of TOP10 *E.coli* and mixed gently by stirring with the tip of the pipette (not pipetted up and down).

This reaction mix was incubated for 10 minutes on ice.

The cells were then "heat shocked" for 30 seconds at 42°C then immediately transferred back to ice for 2 min.

250µl of room temperature S.O.C. was then added to the vial and allowed to mix with the cloning reaction.

The whole reaction mix was then transferred immediately to a 15ml Falcon® shaking tube (with the lid tight) and shaken at 37°C for 1 hour.

100µl was then taken and spread on a plate of agar (150µg/ml ampicillin), the remaining centrifuged at 1000 rpm for 3 minutes then after taking off a further ~750µl, a further 100µl was spread over another plate of agar (150µg/ml ampicillin) in case the transformation had not been efficient.

Plates were left upside down at 37°C overnight.

2.3.3.4 Plasmid Mini-prep.

Once the plasmids had been transferred into *E.coli* and grown into colonies on agar plates the plasmids containing the insert of the gene of interest and an SP6 and T7 promoter (one on either side) then had to be purified using a plasmid DNA mini-prep protocol which used an alkaline lysis to remove the bacterial DNA by utilising the fact that the *E.coli* genome is much longer than the plasmid DNA and therefore takes longer to re-nature.

The following equipment was needed for each plasmid mini-prep sample;

4 inoculation loops

4 separate 15ml Falcon® shaking tubes with ~15ml of LB (150µg/ml ampicillin)

1 agar plate (150µg/ml ampicillin)

GET solution (to make 200 ml) (Store at 4 °C)

Quantity	End concentration	Reagent	Stock Soln.
5ml	25mM	Tris-Cl pH 8.0	1M
1.8g	50mM	Glucose	
4ml	10mM	EDTA	0.5M
20mg	100µg/ml	Rnase A	

Alkaline-SDS (to make 200 ml) (Stable at RT)

1.6g	0.2M	NaOH	Mw=40.0
2g	1%	SDS	

KAc [K+ 3 M, Ac- 5 M] (to make 200 ml) (Store at 4 °C)

58.8g	KAc Mw = 98.14
23ml	Acetic acid-glacial
Ad 200ml	Distilled H2O

4 separate colonies of transformed *E.coli* were taken from the original agar selection plate for each sample.

Each was used to inoculate one 15ml Falcon® shaker tube.

Tubes were shaken at max speed (250rpm) overnight at 37°C.

The following morning a fresh agar (150μg/ml ampicillin) plate was taken and a grid drawn on the underside of it. A sample of each Falcon tube was then smeared into each square on the grid. These *E.coli* were used at a later stage to grow new colonies if they were proven to be good samples. This plate was put at 37°C overnight to grow and then stored in the fridge until needed.

Each Falcon tube was then centrifuged for 10 min at 300rpm, before the supernatant was tipped off and discarded and the lip of the tube wiped with a tissue.

The pellet was then resuspended in 100μl of GET by vortexing on maximum speed.

200μl of alkaline SDS was then added and mixed by gentle shaking until the solution became clearer (no more than 3 min.).

150μl of KAc solution was then added, and the reaction mixture shaken and transferred to 4°C for 20 min.

The reaction mixture was then centrifuged at 13500 rpm for 10 min.

A further purification step (an ethanol precipitation) was then carried out:

2.3.3.5 Chloroform purification of DNA

The supernatant was transferred into a 1.5ml eppendorf tube and 500μl of $CHCL_3$:IAA added. The reaction mixture was then vortexed for 3 min.

The reaction mixture was then centrifuged for 7 min. at 13500 rpm.

The supernatant was then transferred into a new eppendorf tube containing 1ml of ice cold EtOH before incubating at -20°C for 20 min.

The reaction mixture was then centrifuged at 13,500 rpm for 7 min and the supernatant poured off; the pellet was allowed to air dry.

Once totally dry (any EtOH would interfere with downstream reactions) the pellet was dissolved in 60μl of TE.

A 1% agarose gel was run using 2μl of the above to check for presence of the plasmid DNA (length of PCRII-TOPO was 4Kb plus the gene of interest PCR product).

2.3.3.6 EcoRI Restriction of Plasmids.

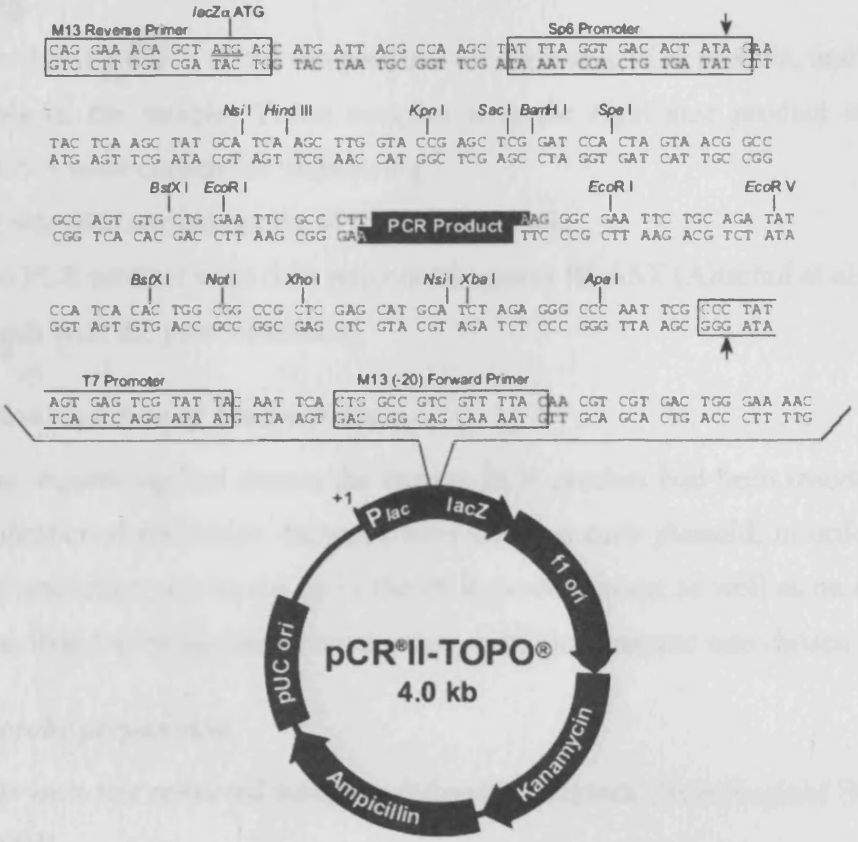


Fig. 2.6; PCRII-TOPO (Invitrogen®) map, showing the PCR product insertion site and the ECO-RI restriction sites either side of the PCR product insertion site. (Invitrogen life technologies, 2003)

Once the DNA had been visualised using a 1% agarose gel, it was restricted using the Eco-RI restriction enzyme, that cut the PCR product out of the plasmid in order for it to be sequenced;

The following constituents were made up per sample;

2µl	Plasmid
0.3µl (3 units per restriction)	Eco-RI
1.5µl	Eco-RI buffer
0.15µl	BSA
11.06	dH_2O
15µl	Total volume

This reaction mixture was incubated overnight at 37°C and a 1% agarose gel run the next morning.

The gels showed which of the samples had the right size PCR product, and how much was available in the sample. Those samples with the right size product and a large amount of DNA were chosen for sequencing.

Sequencing was then carried out by MWG-Biotech AG.

The plasmid/PCR product were then sequenced against BLAST (Altschul et al., 1997) for an exact match with the gene of interest.

2.3.3.7 Restriction digestion of Plasmid/insert

Once the sequencing had shown the correct PCR product had been inserted into the plasmid a number of restriction enzymes were tried on each plasmid, in order to check whether the restriction site would be in the PCR product insert as well as on one side of it; if this was found to be the case, then another restriction enzyme was chosen.

2.3.3.8 In situ probe preparation

All plasmids were test restricted using the following enzymes (New England Biolabs):

- BAMH1
- HINDIII
- XHOI
- XBAI

Using the following;

2µl	Plasmid
0.3µl (3 units per restriction)	Restriction Enzyme
1.5µl	Enzyme buffer
0.15µl	BSA
11.06	dH2O
15µl	Total volume

These were incubated for 3 hours and a 1% agarose gel run of the resulting DNA alongside an unrestricted sample of each was completed. If the resulting restricted plasmid only showed one band indicating one length of DNA (only one restriction site for

that particular enzyme in the plasmid) then the plasmid was midi-prepped to amplify plasmid number.

2.3.3.9 Midi-prep:

Plasmids numbers were amplified using the HiSpeed plasmid Midi Kit from Qiagen as per the manufacturer's instructions:

Colonies were picked from the refrigerated agar plates containing samples of colonies selected for test restriction. These colonies were seeded into 100ml of LB (containing ampicillin at 150μg/ml) and incubated overnight at 37°C.

The next morning the Elution buffer QF was pre-warmed to 50-60°C and buffer P3 pre-chilled to 4°C prior to starting.

The LB/bacteria were split into 50 ml aliquots and centrifuged at 5000rpm for 15 minutes before being resuspended in 6ml of buffer P1 lysis buffer and vortexed to get rid of clumps of cells. 6ml of buffer P2 was then added and the solution mixed gently by inverting then left to incubate for 5 minutes.

After incubation 6ml of chilled buffer P3 was added and the solution gently mixed shaking. The solution was then gently poured into a QIAfilter cartridge and incubated for 10 minutes before removing the cap using the plunger to push the solution into a pre-equilibrated HiSpeed Midi tip. The tip was allowed to clear by gravity as the DNA bound to the resin filter before being washed with buffer QC. The tip was finally eluted with 5ml of pre-warmed buffer QF. The DNA was precipitated by adding 3.5ml of isopropanol and incubated for 5 minutes before being centrifuged at 10,000rpm for 5 minutes and resuspended in 70% ethanol. This was transferred to an eppendorf microfuge tube and the ethanol removed and the pellet dried before resuspending in 300μl of H_2O.

2.3.3.10 Plasmid linearisation

This was done using the selected restriction enzymes (as above) using the following amounts:

7.5µl	Plasmid
1.0µl (20 units per restriction)	Restriction Enzyme
15µl	Enzyme buffer
1.5µl	BSA
125µl	dH2O
150µl	Total volume

2.3.3.11 Ribo-probe generation by purification and IVT

The plasmid linearisation was verified as complete by running a 1% agarose gel. The DNA was further purified using a chloroform purification step (outlined under Mini-prep subheading) and precipitated with 2.5 volumes of 100 % ethanol. The pellet was then washed with 70 % ethanol, dried and dissolved in DEPC-treated Water.

1µl	Linearised Plasmid DNA
2µl	DIG RNA Labelling mix 10x (Roche)
2µl	Transcription buffer 10x (Roche)
2µl	Polymerase (Roche) for T7 and SP6 sites
2µl	RNasin (Promega) 30 units/µl
20µl	Add dH_2O to total volume (11µl)

This solution was incubated at 37°C for 2 hours before adding 2µl of RNase-free DNase (Invitrogen) and being further incubated at room temperature for 15 minutes. Concentration and hybridisation conditions of each probe were then tested for each individual probe.

2.3.4 In situ hybridisation

All solutions that could be were treated with 0.5% DEPC (diethyl-pyrocarbonate) and then autoclaved to inactivate the DEPC. Glass boxes were baked, and hybridisation chambers washed in active DEPC water overnight. Powder free gloves were worn at all times.

2.3.4.1 Solutions for in situ:

DEPC = diethyl pyrocarbonate

X-phosphate = BCIP

BCIP = 5-bromo-4-chloro-3-indolyl-phosphate [M_r = 326.44; 4-toluidine salt = 433.6]

NBT = nitro blue tetrazolium chloride [M_r = 817.7]

2.3.5 Concentrated stock solutions

2.3.5.1 20x PBS pH 7.4 (1 litre)

160g/l	NaCl
4g/l	KCl
28.84g/l	Na_2PO_4 x $2H_2O$
4.14g/l	KH_2PO_4 x H_2O

+ 1 ml DEPC (diethyl pyrocarbonate);

over night on stirrer; autoclave for 45 min at 120 °C

2.3.5.2 20x SSC (1 litre)

175g/l	NaCl
88.3g/l	Tri-sodium citrate dihydarate

pH 7.0

+ 1 ml DEPC; over night on stirrer at RT; autoclave for 45 min at 120 °C

2.3.5.3 50x Denhardt solution (100 ml in DEPC treated H2O)

1g	Ficoll
1g	BSA (bovine serum albumin, non-acetylated)
1%	PVP (polyvinylpyrrolidone)

Heated to 50° on stirrer, filtered through 0,45 µm filter, aliquots 10ml stored at –20 °C. Dissolve on stirrer at 60°

2.3.5.4 TBS-T 10x (Detection buffer)

0.1M	Tris-HCl (1M in 100 ml)
87.7g	NaCl (1.5M)
10ml	Tween 20 (1%)

2.3.5.5 10x AP buffer (without $MgCl_2$ and Triton X-100)

121.1g	Tris-Base
58.4g	NaCl (1M)

Dissolved in 800ml water, pH adjusted to 9.5 with a few drops of conc. HCl,

Volume adjusted with water to 1 litre, autoclave

2.3.5.6 1M $MgCl_2$ (1 litre)

203.3g	$MgCl_2$ x $6H_2O$

2.3.5.7 10x TE (1 litre)

12.1g	Tris-Base (100mM)
3.72g	EDTA (pH8-10mM)

2.3.5.8 10% PFA (200ml)

20g	PFA
375µl	1M NaOH

2.3.6 1x solutions:

2.3.6.1 4% Para-formaldehyde (PFA) (fixative)

Diluted from 10% stock solution in PBS

2.3.6.2 Probe washing buffer

50% formamide (FA), 0.2 x SSC (To

increase stringency you can change the SSC into 0.1 x or increase the FA concentration).

2.3.6.3 1x PBS (1 litre)

Diluted from 20 x stock solution.

0.1% DEPC treated.

2.3.6.4 TBS-T, pH 7.5 (Detection buffer + Triton X-100)

Diluted from 10x stock solution

10mM	Tris-HCl
150mM	NaCl
0.1%	Tween 20

2.3.6.5 1 x AP buffer, pH 9.5 (1 litre)

Diluted from 10x stock solution and 1M

$MgCl_2$

100mM	Tris-Base
100mM	NaCl
50mM	$MgCl_2$ (1M in 50ml)

2.3.6.6 NTMT: (300 ml) = 1 x AP buffer + Triton X-100

100mM	Tris-HCl (pH 9.5-1M in 30ml)
50mM	$MgCl_2$ (1M in 15ml)
100mM	NaCl (5M in 6ml)
30 µl	Triton-X-100 (0.1%)

2.3.7 Staining solutions:

2.3.7.1 Pre-hybridisation/hybridisation buffer (10 ml)

5ml (100%)	Formamide (50% in 10ml)
2.5ml (20x)	SSC (5x in 10ml)
1ml (50x)	Denhardts (5x in 10ml)
100mg	250µg/ml yeast total RNA (10mg/ml)
100mg	500µg/ml herring sperm DNA (10mg/ml)

2.3.7.2 Ab blocking buffer:

For section staining:

3% Milk powder (Marvel) in 1x detection buffer = 30g/l detection buffer

2.3.7.3 Antibody working solution

Alkaline phosphatase antibody (1:2000 in 1 x blocking buffer (in 3% milk powder)) (Prepare immediately before use)

2.3.7.4 Development solution (10 ml)

45µl	75mg/ml NBT (337.5µg/ml)
35µl	50mg/ml X-Phosphate (175µg/ml)

Adjusted to 10 ml with NTMT buffer or 1x AP buffer (prepare before use).

2.3.7.5 Enhanced development solution (50 ml)

5g	Poly Vinyl Alcohol (PVA) (10%)
37.5ml	H_2O
5ml	10 x AP buffer (1 x in 50ml)
2.5mM	1 M $MgCl_2$ (50mM in 50 ml)

Mix levamisole, BCIP and NBT with NTMT or 1x AP buffer containing PVA.

[PVA to be dissolved in 1x AP buffer without Mg^{2+} at 90°C (do not allow to boil), then add $MgCl_2$ and other components]

2.3.7.6 NBT solution

Either bought in (Melford) or make up (below). Store at -20°C

75 mg/ml Nitroblue tetrazolium salt in 70% dimethylformamide

2.3.7.7 X-Phosphate solution (BCIP, (Melford))

Buy in (Melford) or make up. Store at -20°C

50 mg/ml 5-bromo-4-chloro-3-indolylphoshate (= BCIP) in 100% dimethylformamide

2.3.7.8 Levamisole stock solution

Store at -20°

24 mg/ml levamisole hydrochloride salt in 1x AP buffer

2.3.8 Hybridisation Protocol

2.3.8.1 Day 1

Sections were post fixed with 4% PFA in DEPC-treated 1 x PBS for 7 min. then washed twice for 5 min in PBS then once for 10 min in 0.1% DEPC/1 x PBS.

Sections were then equilibrated in DEPC-treated 5xSSC for 15 min before 2 further 5 min washes in 1 x PBS followed by a 5 min wash in 2 x SSC.

Each slide was then pre-hybridised in 50% formamide, 5x SSC containing 40 μg/ml ssDNA (denatured for 10 min at 100°C) for 3 hrs or more at 58°C.

They were hybridised in the hybridisation buffer with 0.4-0.8 μg/ml DIG labelled probe overnight at 58°C.

2.3.8.2 Day 2

Slides were hybridised by putting 250 μl of the hybridisation buffer onto each slide and covering them with parafilm in a sealed hybridisation chamber to prevent them drying out before being placed in a hybridisation oven.

Post hybridisation slides were washed in 5 x SSC for 2 min at 56°C in a water bath, then washed in 5 x SSC for 5 min followed by 2 x SSC for 5 min at 56°C and then in 0.2 x SSC for 5 min at 56°C before being washed in 50% formamide/0.2 x SSC for 20 min at 56°C in a water bath. This was followed by a further wash in 0.2 x SSC for 5 min at RT, and 2 x 5 min washes in 1 x TBS-T at RT for 5 min each.

Slides were then blocked using 3% milk powder in TBT-T at RT for 60 min Following block, the slides were transferred into hybridisation chambers and each was covered in 250μl of antibody solution (anti-DIG-AP in 3% milk powder/blocking solution) before being covered in parafilm and left at RT for 60 min.

After antibody binding slides were washed three times in 1 x TBS-T (for 2, 15 and 15 min periods) before being equilibrated in 1 x AP buffer/$MgCl_2$ for 5 min at RT.

The slides were then developed over-night in a hybridisation chamber, using 500μl of development solution, overlaid with a glass cover slip to prevent dehydration.

2.3.8.3 Day 3

Finally the reaction was stopped in TE buffer or water for 15 min, and slides were rinsed in ddH_2O and dehydrated and mounted in PVA/glycerol.

2.3.9 Cell Culture

2.3.9.1 Cell derivation and preparation

Batches of embryos (E12, E14 and E16) were collected from separate dams and placed into ice cold HBSS. Foetuses were dissected and the dissected WGE and cortical tissue was collected using a Pasteur pipette and left to settle in a 15ml tube containing HBSS solution on ice.

The medium was removed and 200µl of trypsin/DNAse was added to the tissue and incubated for 20 minutes at 37°C. Trypsin inhibitor and DNAse were then added, mixed and incubated for a further 5 minutes at 37°C. The tissue was then washed twice with Dulbecco's Modified Eagle's Medium (DMEM F-12) supplemented with 1%PSF, and then collected by centrifugation at 1000rpm for 3 minutes. The medium was poured off and the tissue was re-suspended in proliferation medium (DMEM F-12 + FGF2, 20ng/ml and EGF, 20ng/ml). The tissue was resuspended in 200µl normal medium and then triturated using a fire-polished pipette or no more than 10 strokes of a 200µl Gilson pipette to produce a single cell suspension.

Cells were counted under a haemocytometer using trypan blue exclusion to assess the viability of the cells:

To do this 10µl of the cell suspension was diluted in 40µl of differentiation medium (DMEM/F12-1%PSF, 2%B27 and 1% Foetal Calf Serum (FCS)), 10µl of this was mixed with an equal volume of trypan blue and then 10µl was transferred to the haemocytometer for counting. Cell viability was calculated according to the formula:

cells/square x dilution factor x 10 = cells/µl cell suspension

2.3.9.2 Differentiation of primary foetal cells in vitro

100,000 cells, in 30µl of the cell suspension was also plated onto poly-lysine coated cover slips and allowed to differentiate. Cells usually began to adhere within a few hours, but in general cells were allowed to adhere over night, after which the wells were flooded with 500µl of differentiation medium per well. Cultures were maintained in the same conditions as described above for 7 days. These were then stained using immuno-cytochemistry.

2.3.9.3 Derivation and passaging of FNPs

The cells were seeded at a concentration of 2,000,000 cells/well in 6 well plates with 5mls of proliferation medium (DMEM F-12 + FGF2, 20ng/ml and EGF, 20ng/ml). Cultures were maintained at 37°C in humidified 5% CO_2, 95% atmospheric air.

When proliferating in culture FNPs form free-floating spheres of cells (termed 'neurospheres') which continue to grow in size.

Passaging was performed by mechanical dissociation using a fire-polished pipette using 10 strokes or less to provide a single cell suspension. An aliquot of these cells (1/10) was trypsinised and a trypan blue count carried out in order to assess cell numbers for re-seeding at the correct density.

Dissociated cells were re-suspended at the same concentration as mentioned above in proliferation medium, except that B27 was replaced with 1%N2 after the first passage. Passaging was repeated every 7 days. Cells were fed every 2-3 days by replacing half the medium with fresh medium that was made up of twice the concentration of growth factors, thus maintaining the growth factor concentrations throughout.

2.3.9.4 Differentiation of FNPs

When cells were abundant an aliquot from each condition was removed for differentiation as per primary cells.

Chapter Three

3 Dissection Accuracy Study

3.1 Introduction

3.1.1 Why it was necessary to show that accurate dissection had been achieved?

This thesis aimed to record gene expression patterns within the developing whole ganglionic eminence, (WGE - the area known to contain the progenitor populations that gives rise to the cells of the adult striatum) over the time when the major part of its development takes place. Thus, it was important to restrict the dissection to this region and as far as possible to exclude non-striatal brain regions such as neighbouring cortical and septal regions. This required learning, practicing and perfecting a suitable dissection method.

However, it should also be emphasised, as noted elsewhere (Parmar *et al.*, 2002) the WGE is in developmental flux during the period of striatal development, so however anatomically accurate the dissection it will contain a small proportion of cells migrating to other brain regions; specifically cortex (see Chapter One, Introduction), although as far as can be estimated to date, these should be present in relatively small numbers at any particular gestational age.

Other sources of error include production of mRNAs that are not usually expressed in this area due to non-translated RNAs that are reported to function at the RNA level as regulators of gene expression (Fischer et al., 2002). There may also be underreporting of genes expressed in small discrete areas with the ganglionic eminences where the cellular content would be too small to allow any rare mRNA species to be recorded above background noise.

3.1.2 Consideration of alternative dissection methods.

Dissection is usually carried out using anatomical landmarks under a dissection microscope. However, consideration was also given to using more accurate ways of dissecting tissue.

Laser Capture Micro Dissection was evaluated for this purpose. This technique is capable of dissecting single cells, or larger regions containing hundreds of cells, and 'catapulting' these from a tissue slice mounted on a microscope slide into a microfuge tube, ready for RNA extraction to take place. However, the small amounts of cells collected mean that the resulting RNA would have to be amplified in order to achieve the amounts needed to run micro array analysis. Amplification itself would introduce another source of inaccuracy into the experiment, due to the differences in efficacy of available polymerases to recognise some mRNAs with shorter length poly A tails, and therefore introducing variability in amplification instead of amplifying all mRNAs in a linear fashion.

The need to amplify could be avoided by dissecting larger amounts of tissue. However, because the process of laser capture is slow, the amount of tissue that can be dissected from a given foetal brain is limited by the instability of the RNA prior to it being placed in a medium that inhibits the action of RNases. Thus, it was decided that the technique of hand-microscope dissection without the need for post-amplification of RNA, would be used.

3.1.3 Study rationale

Anatomical identification of WGE is straight forward and was confirmed on several occasions by a second dissector. The main source of error would be inclusion of substantial amounts of adjacent developing cortex. Thus, the presence of large amounts of a known dorsally expressed gene (Pax-6), which is known not to be expressed in the WGE at this time in development (Corbin *et al.*, 2000), apart from in a small number of cells on the pallidal/sub-pallial border (Yun *et al.*, 2001a), was used as a marker. As a control, areas of the cortex dissected under the same conditions should show high expression of Pax-6. The ventral marker (Gsh-2) was used to indicate the presence of WGE, while cortical dissections should show little or no expression of this gene (Corbin *et al.*, 2000).

Thus, an accurate dissection would be conferred by a presence of the WGE/ventral marker Gsh2 and low levels of expression of the cortical/dorsal telencephalon marker Pax6.

3.2 Methods

Foetal WGE and adjacent cortex were collected at E12, E14 and E16 using a dissecting microscope as outlined in Chapter Two. Tissue was snap frozen in liquid nitrogen to preserve the RNA and restrict the action of RNAses. Tissue was then stored at -80°C until analysis. RNA extraction was effected using the methods outlined in Chapter Two. The WGE samples were the same samples that were analysed by Micro Array.

3.2.1 Primer Design

Primers used by Corbin *et al* (Corbin *et al.*, 2000) were reproduced and used for this experiment. Pax-6, (forward primer; ctgtaccaacgataacatacc, reverse primer; cccttcgattagaaaacc; product size 454bp) and Gsh-2, (forward primer; gaggaacaatcacacaagc, reverse primer; ttcagaaaacaagacatacg, product size 580bp).

3.2.2 PCR

PCRs were carried out using the methods outlined in Chapter Two.

3.2.3 Equimolarisation calculations;

Each cDNA sample was used in a PCR to amplify a control gene (G3PDH)(Thellin *et al.*, 1999) primer product (forward primer; tccaccaccctgttgctgta, reverse primer; accacagtccatgccatcac; product size; 451bp) to ascertain its concentration.

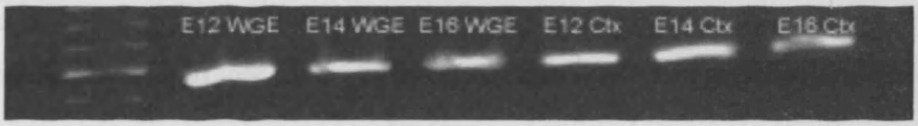


Fig. 3.1; 1% Agarose Gel showing the amplified PCR products for the G3PDH control gene primer, using 1µl of each sample. (Left:100bp DNA ladder)

From the above gel the following Integrated Density Values (IDV) for each lane/cDNA primer product sample were calculated by AlphaEase FC;

WGE	E12	E14	E16
	65736	36864	35340
Ctx	E12	E14	E16
	36704	47025	34485

Table 3.1; Saturation/IDV figures given by AlphaEaseFC® for 1µl of each of the cDNA samples.

The IDV value of 36500 was in the midrange of these figures and so was deemed to be the right amount of cDNA to use in all cases so each figure in Table 3.1 was divided against this figure to produce the amount to be loaded into PCR reactions in the future. Therefore the following amounts of each cDNA sample were used in all PCRs following:

WGE	E12	E14	E16
	0.56µl	0.99µl	1.03µl
Ctx	E12	E14	E16
	1.01µl	0.78µl	1.06µl

Table 3.2; Adjusted cDNA amounts after Equimolarisation.

Following equimolarisation the following PCR was ran to check the amounts of cDNA were correct.

Fig. 3.2; 1% Agarose Gel showing the amplified PCR products for G3PDH control gene primer, using the equimolarised/adjusted amount of each cDNA sample.

3.2.4 Data analysis

The gel was photographed in an ultra violet hood and the image of the PCR product amplification was carried out using AlphaImagerFC software. The spot density was then measured and the following calculation made to account for background intensity.

The Integrated Density Value (IDV) =∑ (each pixel value - background)

Because eqimolarisation cannot be totally accurate, the IDVs obtained in the experiment were then compared to the IDVs of the relevant G3PDH sample so that a comparable figure, between samples was obtained. This figure was then used for statistical analysis.

The IDV of each WGE PCR product was compared to the Ctx PCR product of the same age condition using a one-tailed, paired t-test (Microsoft Excel) to find whether the differences were significant.

3.3 Results

The equimolarised cDNA samples showed the following saturations when analysed using AlphaEaseFC;

WGE	E12	E14	E16
#1	40920	39936	41292
#2	30660	34560	30225
#3	60390	60900	56544
Ctx	E12	E14	E16
#1	44928	48825	43680
#2	28644	26640	26412
#3	55488	53312	48015

Table 3.3; Integrated Density Values (IDV) given by AlphaEaseFC for each of the cDNA PCR products samples after equimolarisation.

Equimolarised quantities of cDNA were then used in further experiments to amplify the two primer products Pax6 and Gsh2.

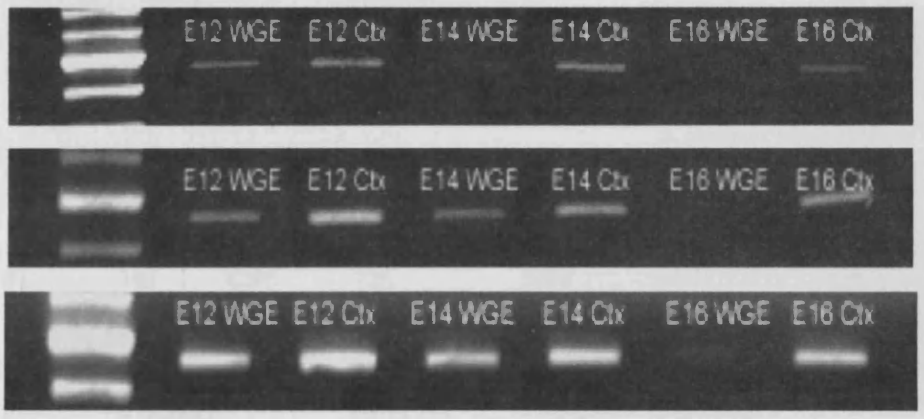


Fig. 3.3; 1% Agarose Gels showing the amplified PCR products of the Pax-6 primer on all cDNA samples

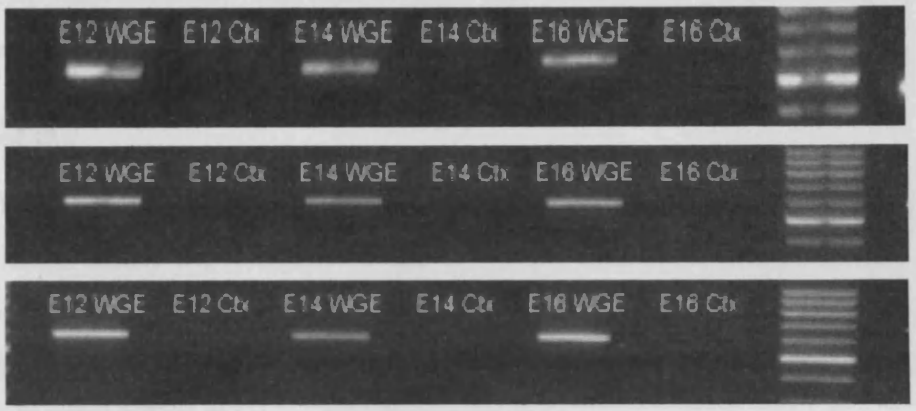


Fig. 3.4; 1% Agarose Gels showing the amplified PCR products of the Gsh-2 primer on all cDNA samples

3.3.1 Statistics; t test

	Gsh-2 E12 WGE	**E12 Ctx**	**E14 WGE**	**E14 Ctx**	**E16 WGE**	**E16 Ctx**
#1	42966	2904	35088	5390	36960	7098
#2	23430	0	16500	2376	23040	3072
#3	26180	4080	15180	1750	23584	0
p=	0.019329		0.034735		0.006833	
t Stat=	4.93744		3.59345		8.46640	
	Pax-6 E12 WGE	**E12 Ctx**	**E14 WGE**	**E14 Ctx**	**E16 WGE**	**E16 Ctx**
#1	363	4290	726	5456	1320	4433
#2	9207	20460	8096	13299	1116	14322
#3	10846	26845	9000	17578	2940	13440
p=	0.048852		0.018229		0.048739	
t Stat=	-2.95985		-5.09292		-2.96384	

Table 3.4; Integrated Density Values for cDNA PCR primer products when amplified using Pax-6 (cortical marker) and Gsh-2 (ventral marker) primers.

3.3.2 Description of results

These results show that there was significantly more Gsh2 expressed in the WGE samples of tissue, than there was Pax6. They also show the reverse; that is, that there was significantly more pax6 expressed in the cortical samples than there was Gsh2.

The amounts of Pax6 within the WGE samples was less, the more developed the foetuses were at the time of dissection, showing that as foetal age increased, so did the

accuracy of dissection and the less cortical and pallidal/sub-pallial border tissue was present within the WGE samples.

3.4 Discussion

These results indicate that the WGE dissections did contain the telencephalic marker Gsh-2, which was virtually absent in cortex, thus confirming that they included tissue from the Ganglionic Eminences.

These results also show that the WGE dissections contain significantly less of the dorsal marker Pax6 than did the cortical samples.

However, Pax6 was present in the WGE samples, and that this declined proportionally with increase in age. This may be explained by two factors;

- It may indicate that some surrounding or pallidal/sub-pallial border tissue was included in the dissection. The fact that Pax6 levels in the dissections decline as the embryonic age increases, illustrates the greater degree of difficulty in accurately dissecting the WGE from the rest of the brain in these early periods of development. At E12 the WGE is very small and it was easy to cut too deep, therefore taking pieces of the underlying cortex and from areas dorsal to the WGE. However, as the age increases, the likelihood of this happening decreases as the WGE becomes a more distinct structure in its own right.
- Cortical precursors may express Pax6 as they migrate through this region during development to populate the cortex and one would expect this at this early stage. Immuno-fluorescence has shown a small amount of cells that are double labelled for Pax6 and Gsh2 along the ventro-dorsal border (Yun *et al.*, 2003), although this contradicts the results found by Corbin *et al* (Corbin *et al.*, 2000).

Having obtained this result, ideally I would want to characterise the anatomical distribution of Pax6 particularly at the early developmental ages to distinguish between the two possibilities above. Indeed, this was attempted using immuno-histochemistry on histological slices of dissected WGE, using Pax6 antibodies but failed for technical reasons.

Chapter Four

4 Affymetrix Micro Array Analysis

4.1 Introduction

Although there is a literature on gene expression within the developing striatum (see Chapter One; Introduction), to date no laboratory has published a systematic search for gene expression changes over the period of maximum proliferation and development of this brain region. To achieve the aims of this study (which are outlined at the end of Chapter One) a method by which all of the significant gene expression changes within a defined period, was required.

4.1.1 Choice of method.

A number of techniques were considered for this purpose, including;

4.1.1.1 Subtractive hybridisation

This method uses subtraction cloning to select genes that are switched on or up-regulated in the experimental condition when compared with the control condition. Two samples, the normal "test" (control) mRNA sample and a "driver" (experimental) cDNA sample are mixed, denatured and re-annealed so that matching mRNA and cDNA samples are allowed to bind to each other. The resulting double stranded cDNA/mRNA hybrids are then isolated and discarded, as they are the genes that are found in both samples. The remaining, unpaired single stranded cDNA molecules ("subtracted" cDNA, derived from the genes expressed either more, or only in the "driver/experimental" sample) are then isolated and cloned for further analysis.

The main advantage of SH is that it can be used in a laboratory equipped to carry out basic recombinant DNA techniques. No large items of equipment are required since the method is based on PCR and microfuge tube reactions. Another advantage is that it allows the detection of low-abundance differentially expressed transcripts, such as those likely to be involved in signalling and signal transduction, and thus, may identify essential regulatory components in a number of biological processes. However, this technique only works for genes that are controlled at the transcriptional (DNA to RNA)

level. The expression of some gene products (proteins) are controlled at the translational (RNA to protein) level and hence would not be picked up by this method.

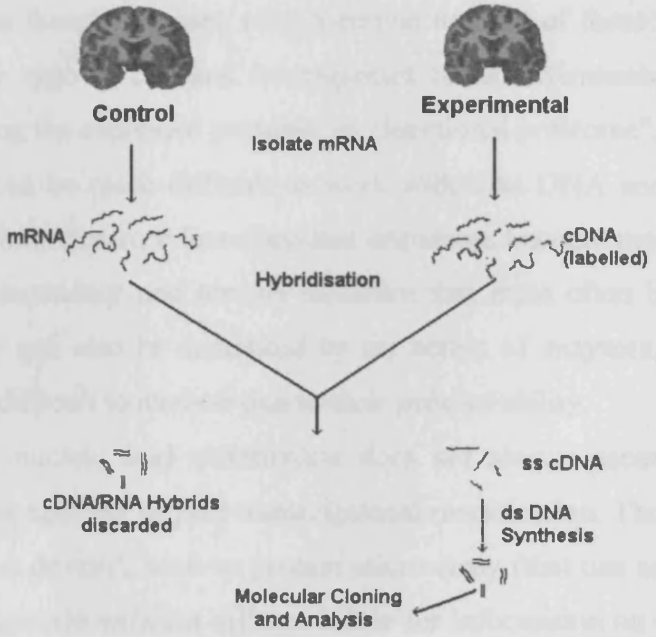


Fig. 4.1; Subtractive hybridisation can pick out genes that are either up-regulated, or expressed only in the experimental sample.

4.1.1.2 Proteomics.

Although all somatic (nucleated) cells within an organism contain the same chromosomes and therefore genes, only a certain number of those genes are expressed, depending on the type of cell and its responses to its environment. Proteomics is the science of studying the expressed proteins, or "functional proteome", of a cell.

Proteins can be more difficult to work with than DNA and RNA because they cannot be amplified; therefore less abundant sequences become more difficult to detect. They also have secondary and tertiary structure that must often be maintained during analysis and they can also be denatured by the action of enzymes, heat or light. Some proteins are also difficult to analyse due to their poor solubility.

However, nucleic acid quantitation does not always accurately reflect protein levels, due to gene splicing or post transcriptional modification. Therefore, new methods have recently been devised, such as protein micro array (that can accurately reveal what proteins are being made within a cell-see below for information on cDNA micro arrays), x-ray crystallography and nuclear magnetic resonance (NMR), which can define a protein's secondary and tertiary structure, in order to enhance our understanding of how it works. Proteomics is in its infancy, however, and the techniques involved are expensive and specialised.

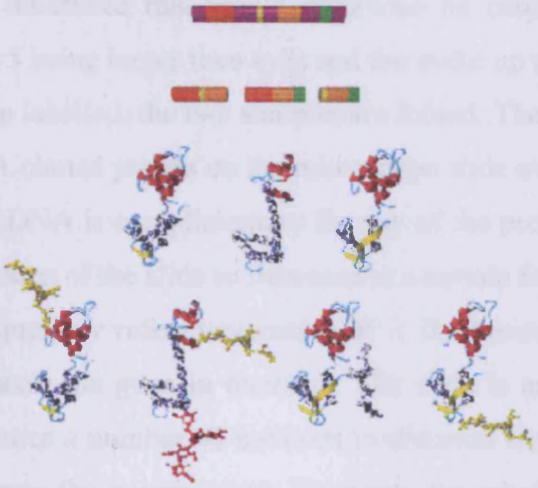


Fig. 4.2; A single gene can yield a number of splice variants, each of which produces a different protein which can undergo post-translational modification.

4.1.1.3 Nucleic acid Micro Arrays.

Micro array is a method of assaying large amounts of genetic information quickly and automatically. This is done by using a robot to make up a glass slide (commonly referred to as a chip), onto which thousands of separate microdots of genetic material in the form of DNA clones are printed. Each microdot contains one or more probes for an individual gene found in the organism being studied, i.e. yeast, fruit fly, mouse or human.

Samples are then taken from two tissues, a reference and a query (or experimental sample) or a series of query samples that can be compared against one another. The total RNA is extracted and transformed into cDNA with the aid of reverse transcriptases. This cDNA has been transcribed from mRNA extracted from the tissues in question and therefore contains information as to which genes are being expressed in the cell at the time of the RNA extraction.

In cDNA micro arrays the cDNA is then labelled with radioactive isotopes (macro arrays) or more commonly one of two fluorescent colours, cy5 (red) or cy3 (green); one for the query cDNA and one for the reference/control cDNA. This is commonly done in one of two ways, either with the 'first strand'/direct method, which links the marker molecule directly to the ribose molecule of the bases, or using the indirect method, attaching the marker via a bridging molecule. The latter method will label more efficiently, due to there being less steric hindrance that would otherwise be caused by the size of the fluorescent molecule (cy5 being larger than cy3) and the make up of the RNA itself.

Once the cDNA has been labelled, the two samples are mixed. Then the resulting solution is hybridised to the DNA cloned probes on the microscope slide over a period of between 16 and 48 hours. If the cDNA is complimentary for any of the probes on the slide, it will hybridise and cause that area of the slide to fluoresce at a certain frequency; depending on whether it is from the query or reference/control. If it fluoresces in both colours, then both tissue samples express the gene in question. The slide is analysed by a computer imaging system, which uses a number of methods to discount background labelling and mismatches in fluorescence (by normalising). However, the whole process relies on the quality of RNA extracted from the tissue sample. Without good quality RNA, cDNA cannot be made and labelled.

Because micro array technology is still evolving there are still problems with some of the methods; i.e. cDNA micro array has been quoted as being as low as 11% reproducible between experiments, (i.e. a coefficient of variation of 89%) (Hinman *et al.*, 2002)

although other studies show coefficients of variation to be as low as 12% (Yue *et al.*, 2001; Shi *et al.*, 2006; Frueh, 2006). The differences between each hybridisation are due to a host of factors including spatial heterogeneity, non-linear array-to-array variation and problems arising when correcting for background (Geschwind and Gregg, 2003). These are problems that can only be dealt with by using increasing numbers of replicates, thereby increasing the overall cost.

4.1.1.4 Affymetrix Micro array.

Affymetrix micro array differs mainly from normal two colour cDNA micro array in that it uses one-colour technology. The RNA is extracted from the cells, and transcribed into cDNA as usual. However, this cDNA is then labelled in an *in vitro* transcription reaction (IVT) with biotin, before being fractionated into small cDNA fragments and then hybridised to a micro array chip or Genechip (Affymetrix use these instead of glass microscope slides on which to print their arrays). The chip is then scanned, resulting in an image that is processed to give a numerical representation of each of the features (of which there are millions on each chip). This data can then be normalised and analysed in a number of computer programs. (For a more comprehensive insight into the RNA extraction and labelling please refer to Appendix A, for an overview refer to Chapter Two; Methods. The normalisation and data analysis will be described below). The Affymetrix technique is highly reproducible (Woo *et al.*, 2004) and can be used in a wide range of applications on a whole-genome scale, including gene and exon level expression analysis, novel transcript discovery, genotyping, and resequencing.

Although each Affymetrix chip was substantially more expensive than its cDNA counterpart, the fact that they were highly reproducible meant fewer chips would be needed to run an experiment. For these reasons it was Affymetrix micro arrays that were used as the primary method of investigation in this study above other possible methods.

However, it must be remembered that although micro arrays are a powerful tool in assaying gene expression in tissue, the data that is collected from such an experiment is only as good as the analysis that is done on it, and validation using other 'tried and tested' methods, of the most significant findings, must then be completed in order to justify any results they infer.

4.1.1.5 The Affymetrix Mouse 430A 2.0 GeneChip.

The most recent Affymetrix Genechip to be designed for *Mus musculus* at the start of this project was the Mouse 430A 2.0 array which contained 22,626 probe sets of 21 base pair

oligonucleotides, each interrogating one or more of 14,484 known genes, and over 8,000 ESTs (Extended Sequence Tags).

The MOE430A array chip has been compared to other techniques including cDNA and has consistently detected the largest number of significant genes and had the lowest false discovery rate. At the critical *p*-value of 0.01, 3% of the significant genes were expected to be false-positives, which was less than a half compared with other arrays (Woo *et al.*, 2004).

The probes were designed using sequences from GenBank (the NIH genetic sequence database, which is an annotated collection of all publicly available DNA sequences) (Benson *et al.*, 2004), dbEST, a division of GenBank; containing sequence data and other information on "single-pass" cDNA sequences or Expressed Sequence Tags (Boguski *et al.*, 1993), and the RefSeq collection (which includes a comprehensive, integrated and non-redundant set of sequences, including genomic DNA, transcript (RNA), and protein products).

The sequence clusters were created from the above databases in June 2002 and then refined by analysis and comparison with the publicly available draft assembly of the mouse genome from the Whitehead Institute for Genome Research in the same year.

4.2 Aims;

As discussed fully at the end of Chapter One, the aim of this chapter is a systematic search for gene expression changes during the period of peak striatal neurogenesis in the mouse.

The specific aims of this set of experiments were;

- To characterise the gene expression within the area of the developing mammalian brain that gives rise to the mature striatum.
- To identify a subset of expressed candidate marker genes that have the potential to be used as markers of striatal commitment in human embryonic striatal precursors.
- To identify a subset of candidate marker genes for future analysis, with the long term goal being to direct the differentiation of human FNPs towards a striatal phenotype.

4.3 Methods

Tissue was dissected and stored as stated in Chapter Two. RNA extraction was also carried out as per Chapter Two.

4.3.1 RNA Quantitation and quality checks

Before allowing the expensive and time consuming use and analysis of micro arrays all RNA samples were checked using the following methods that were commonly used by the majority of the micro array community in UK;

4.3.1.1 UV Spectrometry and Agilent 2100 Bioanalyser analysis.

These methods were used together to as each method had its strengths in defining different areas where RNA contamination or unreliable probe generation may have arisen.

4.3.1.2 Genequant Pro Spectrometry

Spectrometry is regarded generally as an accurate and reliable way to quantitate RNA at this time, however it does not distinguish between certain contaminants, as outlined below:

Genequant *Pro* (Amersham Biosciences) spectrometer readings were taken straight after RNA extraction (outlined in Chapter Two; Methods: RNA Extraction). The reading at 260nm allows the calculation of the concentration of nucleic acids (either DNA or RNA) in the sample. The Beer-Lambert law (Sambrook and Russell, 2000) was then used to compute the Optical Density (OD) of the sample and compare it to an OD of 1, which corresponds to 44.19µg/ml (Farrell, 1993) for single stranded RNA.

4.3.1.3 Agilent 2100 Bioanalyser readings

This equipment was used alongside the spectrometry results to gauge RNA quality before use on the Affymetrix genechip. One of the downfalls of spectrometry is that both DNA and RNA absorb UV light at 260nm; therefore it is hard to distinguish the difference between samples with high RNA content and those with low/high molecular weight genomic DNA (gDNA) or phenol contamination. By visualising the RNA using an electropherogram any contamination could be easily seen as peaks either side of the 18 and 28S ribosomal peaks normally seen in a clean sample.

4.3.1.4 RNA Degradation Plot (using R-Bioconductor (Bates et al., 2004))

This simple (shifted and scaled) plot enables the user to see the condition of the mRNA rather than presuming its condition by looking at either the total RNA or the ribosomal RNA. The plot considers the comparison between probes located at the 5' end of the genes/ESTs in question and the 3' end. This measured both the amount of degradation (mostly due to RNases) that occurred during the extraction of the RNA and the ability of the DNA Polymerase I to complete second strand synthesis during the preparation of the samples (see Chapter Two and Appendix A for protocols). A significant slope that did not follow the 'trend' of slopes of the majority of samples gave a hindsight indication as to a samples quality. However, this test was only used as confirmation of which Genechips to use in analysis as it was only possible to complete after the samples had been hybridised to a GeneChip.

4.3.1.5 Comparison Analysis and Normalisation using MAS (Micro Array Suite) 5.0®, and RMA (Robust Multi-array Averaging).

Normalisation was carried out utilising two well known and used methods. The separate results were then compared in parallel throughout later analysis and the final significant gene lists of the two methods then merged and the common genes from both accepted as being significant. This was done to reduce the false discovery of expression changes through relying on one method of normalisation.

4.3.1.6 MAS 5.0 Analysis.

Adapted from (Affymetrix, 2001a; Affymetrix, 2001b; Affymetrix, 2002).

After hybridisation of RNA from the three conditions (each condition having three repetitions) the resulting Mouse430A Genechips were scanned using an Affymetrix 2500 GeneArray scanner. Once the Genechip had been scanned, a .CEL file was generated by the Affymetrix MAS 5.0 software; this contained an image of the scanned Genechip and also calculations of the raw intensities for the probe sets on it.

The resulting image files were then normalised and analysed in MAS 5.0 (see "comparison analysis" below) to ascertain the intensity of each of the probes in order to calculate whether any of these transcripts could be assigned a call of Absent, Present or Marginal.

The Affymetrix system uses oligonucleotides with a length of 25 base pairs that are designed to probe selected known genes and EST's. Typically, each of these is

represented by between 16 and 20 pairs of oligonucleotides (each interrogating a different part of the gene or EST in question) referred to as probe sets. The first component of these probe pairs are referred to as perfect match (PM) probes. Each one of these is paired with a mismatch (MM) that is created by changing the 13th (middle) base with the intention of measuring non-specific binding. The PM and MM are referred to as a probe pair.

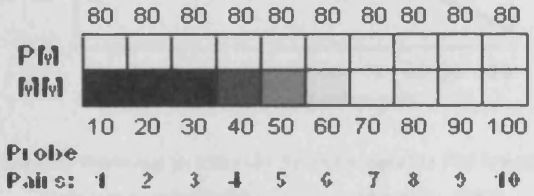


Fig. 4.3; In the probe set above, each probe pair is numbered from 1 to 10. All the PM intensities are 80 while the MM intensities increase from 10 to 100.

4.3.1.7 Detection p-value.

The detection *p*-value was determined using two steps;

- Calculation of the discrimination Score (R).
- Test of the Discrimination Scores against a used definable threshold (Tau).

The signal intensity of each probe cell within each pair was considered when determining whether the transcript was either detected (Present), or not (Absent). e.g. if there are a total of 10 probe pairs for a given gene, and in seven probe pairs the PM is of much higher intensity than the MM cell, then those probe pairs would each have an R score closer to 1.0, while the three probe pairs with the same or higher MM intensities would have R scores closer to 0.0 (see below). If all the PM cells within a probe set are fully saturated, the probe set would be given a Present call. Conversely, if a MM probe cell was saturated then that probe pair was rejected from further analysis.

Therefore;

$$\mathbf{R = (PM-MM)/(PM+MM)}$$

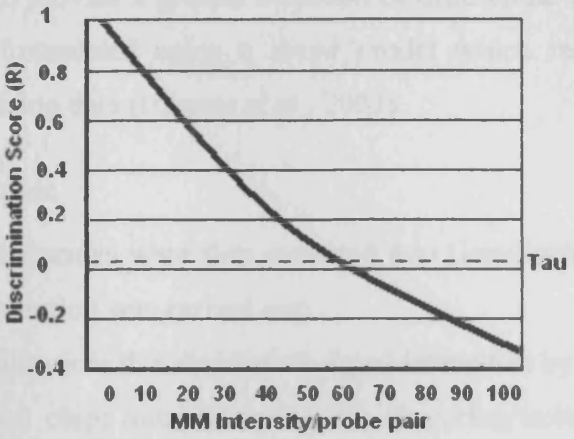


Fig. 4.4; As the MM probe cell intensity increases (x-axis) and becomes equal to PM intensity, the Discrimination (R) score decreases (y-axis) (adapter from (Affymetrix, 2002).

4.3.1.8 MAS 5.0 Normalisation and Scaling;

The MAS 5.0 software used Global scaling (i.e. all intensities scaled so that each array has the same average value; set at 100). A robust normalisation, (which was non-user modifiable), was then used to take account of unique probe set characteristics that were due to sequence dependant factors, such as the affinity for targets to their probes and the linearity of hybridisation of each probe pair in the probe set. If these factors were not taken into account, false positives in expression level changes could have resulted.

4.3.1.9 RMA Normalisation and scaling.

Although MAS 5.0 has been the benchmark method of normalising and scaling Affymetrix arrays since its introduction in 2001, it has been postulated that this method does not deal well with cases where there are non-linear relationships between arrays, as a baseline has to be chosen and the other chips normalised to this.

Therefore, to validate the normalisation/scaling processes used in MAS 5.0, another method known as RMA (Robust Multi-array Averaging (Irizarry *et al.*, 2003; Bolstad *et al.*, 2003)) was carried out with the kind help of Gareth Hughes, formerly of the Dept of Patholgy, UWCM).

RMA does not use a baseline array instead normalising both the PM and MM values instead of just the results of the votes of the probe pairs. The output from RMA was then transformed from Log^2 to decimal and treated in parallel with the MAS 5.0 data (see below).

RMA analysis uses a quantile normalisation (dividing all of the values in each condition into equal groups, each containing the same fraction of the total population).

This has been shown to provide a greater detection of differential expression, allowing a standard error to be formulated using a linear model which removed probe-specific affinities when normalising data (Irizarry *et al.*, 2003).

4.3.1.10 GeneSpring Analysis.

Data from all the arrays were then exported into GeneSpring® (Silicon Genetics inc.) and further normalisation was carried out;

Per chip Normalisation; this divided all signal intensities by the median value (the 50th percentile) for each chip; thus correcting for obscuring/technical variation due to scanner/hybridisation differences between chips and centering all values around the value of one.

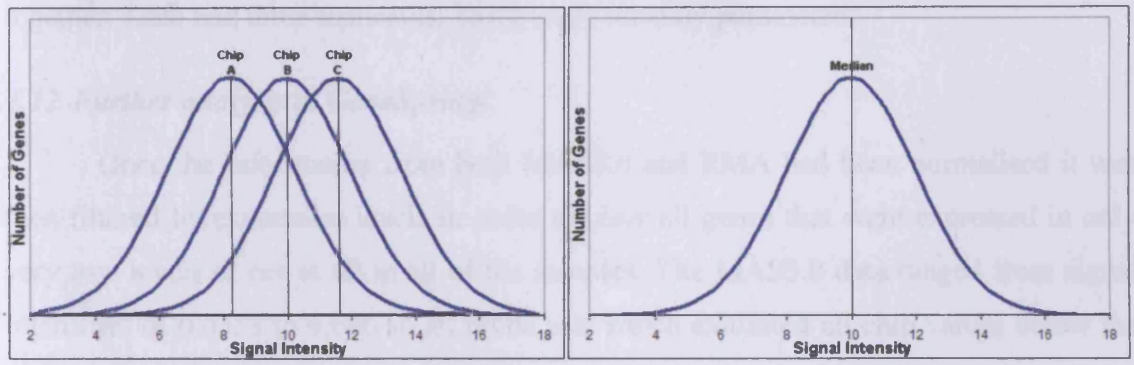


Fig. 4.5; Per Gene and Per Chip normalisations normalised each probe to the 50th percentile of that chip and then adjusted each chip until all the chips had similar medians.

Per Gene Normalisation; this calculated the median value of all genes in the array and divided them by the median value. Thus highlighting genes with large fold-changes in expression even when they are at low signal intensities; This meant that even at low signal intensity levels a large fold-change that would be otherwise be overlooked due to the size of the transcripts signal intensity were be recognised in the same light as significant changes at higher signal intensities that would be easier to find but were not as high a fold-change.

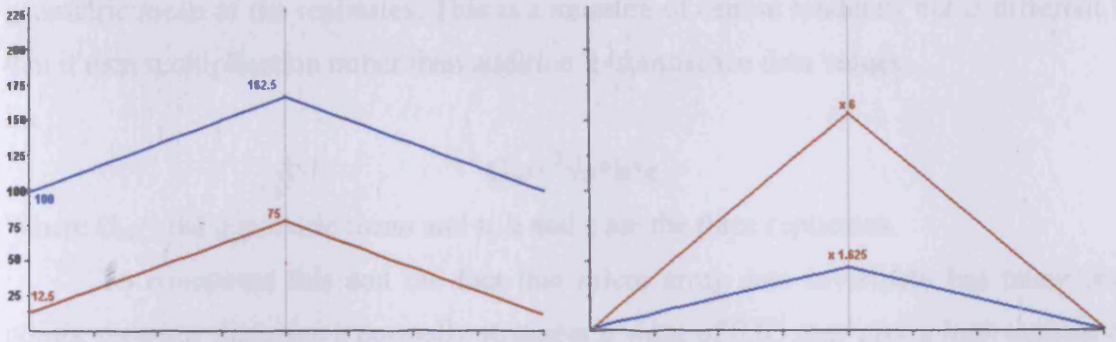


Fig. 4.6; The graph on the left shows the actual signal intensities (y-axis on the left graph) of two example genes which appear to have the same increase in expression. However, once the fold-change is measured (y-axis on the right graph) it can be seen the gene denoted by the red trace has a much higher fold-change.

4.3.1.11 Experimental interpretation.

In this experiment each condition (different embryological ages) were grouped together. Each had three replicates. These were the only parameters.

4.3.1.12 Further analysis in GeneSpring.

Once the information from both MAS5.0 and RMA had been normalised it was then filtered by expression levels in order to clear all genes that were expressed in only very low levels or not at all in all of the samples. The MAS5.0 data ranged from signal intensities of 0.0333 to 4,606 so all probe sets which exhibited all chip values below the cut off of 50 were withdrawn from further analysis (this is the number recommended by Genespring). The RMA data ranged from 19.87 to 25,785 after transformation from Log_2 to decimal so any probe sets with all chip values below that of 265 were withdrawn (I used this number as it discounted exactly the same number of genes as using the number 50 in the MAS5.0 data).

To ensure good separation of data and remove non-changing genes both data sets were then filtered on expression level changes. All expression changes over a 1.8-fold change were deemed, for the purposes of this study, to be of interest.

4.3.1.13 Statistical Analysis of Genes of Interest.

Because sample replicates representing small signal changes (low gene expression levels) usually have smaller variances than sample replicates showing much higher signal intensities (expression levels) micro array data is much harder to statistically analyse using the standard error of the mean of the replicates. The high cost of increasing the amount of replicates would also restrict this. Therefore micro array software uses the

geometric mean of the replicates. This is a measure of central tendency but is different in that it uses multiplication rather than addition to summarize data values.
i.e.

$$G_m = \sqrt[3]{a*b*c}$$

Where G_m = the geometric mean and a, b and c are the three replicates.

To counteract this and the fact that micro array data invariably has many data points, meaning that even a normally stringent *p* value of 0.05 may give a high number of false positives (5% of the resulting genes that pass the test) the ANOVA test used the Benjamini and Hochberg multiple testing correction (Hochberg and Benjamini, 1990; Benjamini and Bitterman, 1990; Reiner *et al.*, 2002). This ranked the genes found to be significant (p=0.05) in the ANOVA in order of *p* values, giving the least significant gene a value of 1 and the most significant gene the highest value. The *p* value (0.05) was then multiplied by the number of genes entering the test divided by each genes rank position and if the resulting number was less than the *p* value (p=0.05) then that gene passed the test. Instead of 5% of the original gene list being false positives this test reduced this to 5% of those genes passing the multiple testing correction being possible false positives.

A *post hoc* test was not done as expression between only two ages was tested (E12 and E16) for this experiment.

4.4 Results:

The following results show firstly, the quality assessment of the RNA samples according to the methods described above.

4.4.1 Genequant Pro Spectrometry results

RNA samples were initially checked immediately after extraction using a Genequant Pro (Amersham Biosciences) spectrometer;

Sample / rep	Mass (mg)	Dilution. factor	230	260	280	260/230	260/280	[conc] (ng/ml)	Yield (µg)	Yield (µg/mg)
E12a	~70	1:40	1.82	2.81	1.60	1.54	1.75	4498	134.9	1.97
E12b	59	1:40	1.39	2.27	1.26	1.63	1.8	3629	108.9	1.84
E12c	110	1:80	1.14	2.5	1.38	2.2	1.81	8012	240	2.18
E14a	152	1:40	1.882	2.865	1.520	1.522	1.885	4584	137.5	0.9
E14b	131	1:40	2.086	2.977	1.779	1.427	1.673	4763	142.9	1.1
E14c	118	1:80	1.024	1.932	1.032	1.887	1.872	6182	185	1.57
E16a	93	1:40	1.620	2.953	1.645	1.823	1.795	4725	141.7	1.5
E16b	138	1:40	2.476	2.939	1.557	1.187	1.888	4702	141	1
E16c	99	1:40	1.303	2.205	1.152	1.698	1.914	3528	105.8	1

Table 4.1; RNA samples were checked by spectrometry immediately after extraction.

4.4.2 Agilent 2100 Bioanalyser readings

The following results were obtained by the staff of UWCM Dept of Pathology at the micro array facility in the heath Hospital.

4.4.2.1 E12a RNA sample

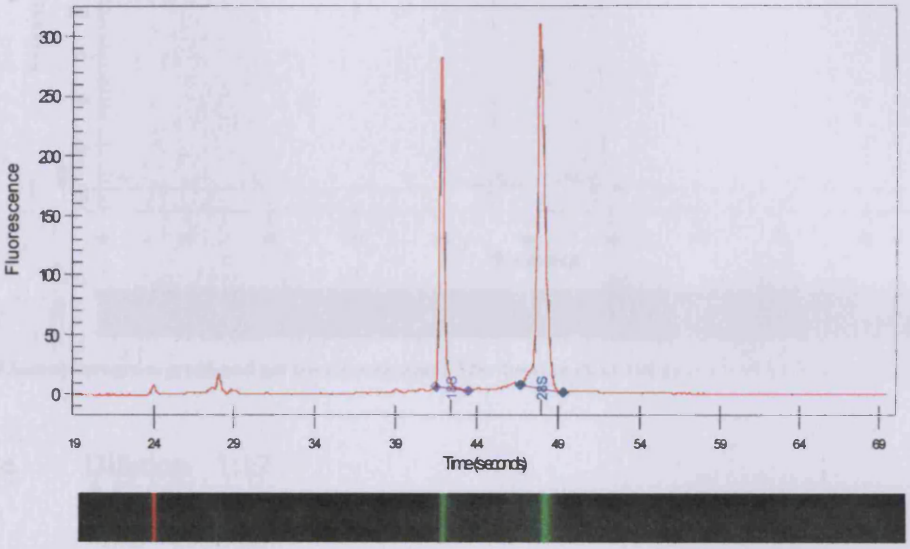


Fig. 4.7; Electropherogram graph and gel trace for sample E12a showing clear 18S and 28S rRNA bands and a flat base line

Sample Factor	Dilution 1:21				
Fragment	Name	Start time (secs)	End Time (secs)	Area	% of total Area
1	18S	41.45	43.45	225.62	27.96
2	28S	46.45	49.25	351.35	43.54

RNA Area	806.90
RNA Concentration (pg/µl)	6,527.41
rRNA Ratio [28S/18S]	1.56

4.4.2.2 E12b RNA sample

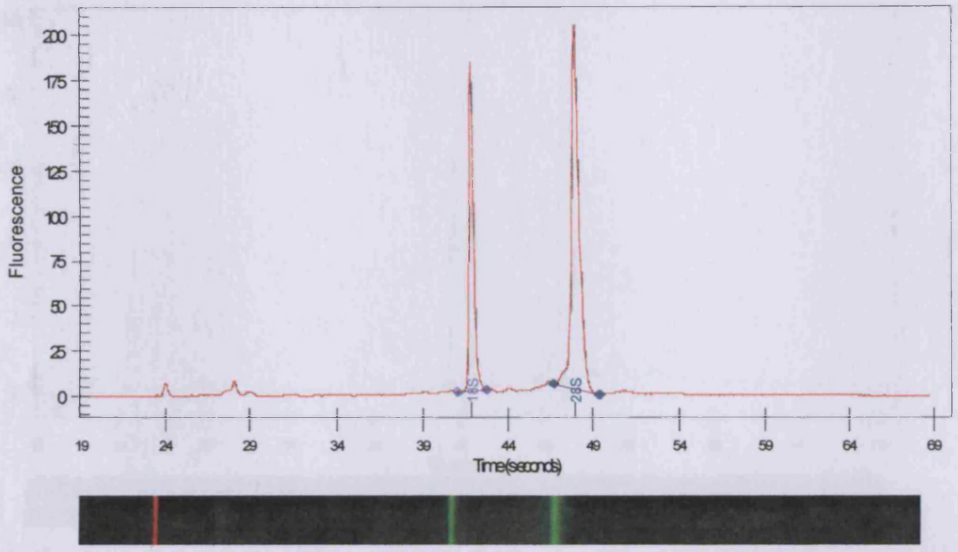


Fig. 4.8; Electropherogram graph and gel trace for sample E12b showing clear 18S and 28S rRNA bands and a flat base line

Sample Factor Dilution 1:17

Fragment	Name	Start time (secs)	End Time (secs)	Area	% of total Area
1	18S	41.00	42.70	143.96	27.23
2	28S	46.65	49.35	224.59	42.49

RNA Area	528.61
RNA Concentration (pg/µl)	4,276.19
rRNA Ratio [28S/18S]	1.56

4.4.2.3 E12c RNA sample

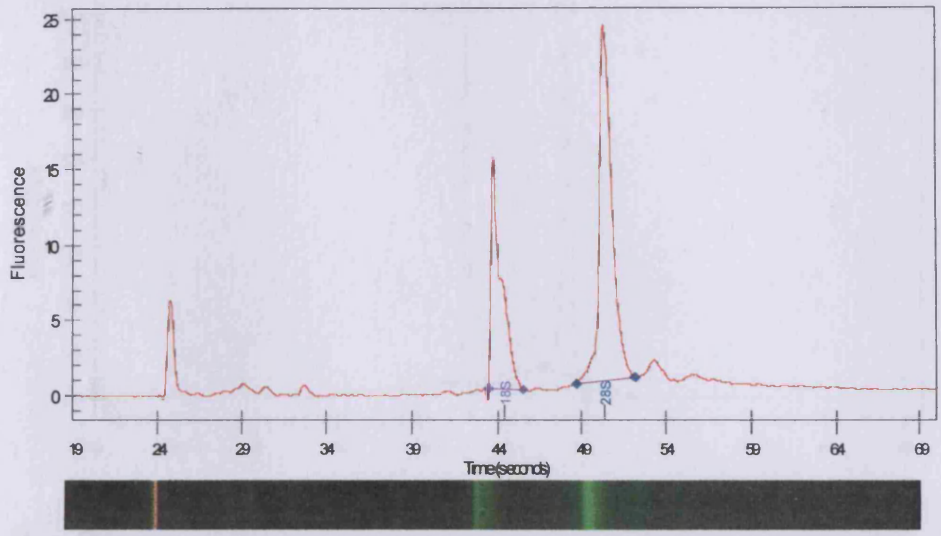


Fig. 4.9; Electropherogram graph and gel trace for sample E12c showing clear 18S and 28S rRNA bands and a reasonably flat base line

Sample Factor	Dilution	1:16				
Fragment	Name	Start time (secs)	End Time (secs)	Area	% of total Area	
1	18S	43.45	45.50	24.49	26.38	
2	28S	452.15	52.15	41.43	44.64	

RNA Area	92.82
RNA Concentration (pg/µl)	95.76
rRNA Ratio [28S/18S]	1.69

4.4.2.4 E14a RNA sample

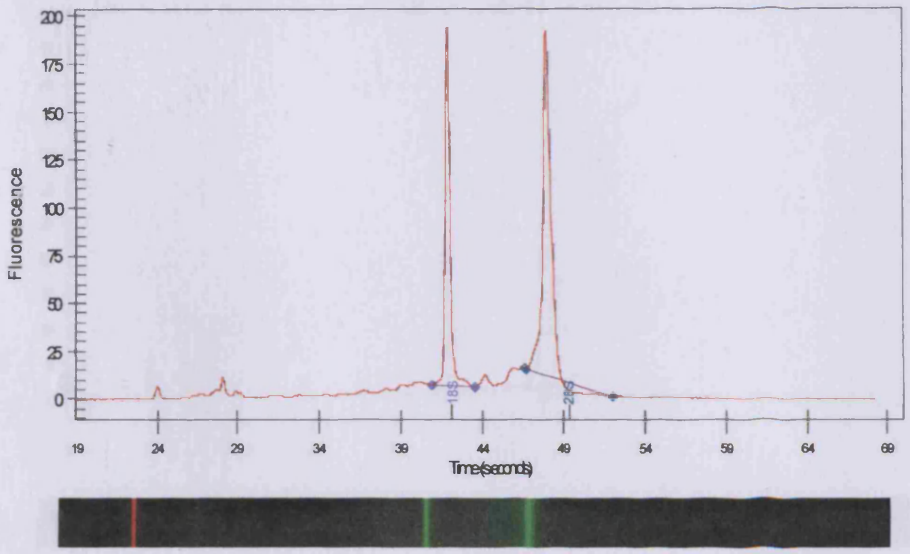


Fig. 4.10; Electropherogram graph and gel trace for sample E14a showing clear 18S and 28S rRNA bands and a flat base line

Sample Factor Dilution 1:22

Fragment	Name	Start time (secs)	End Time (secs)	Area	% of total Area
1	18S	40.90	43.50	165.04	22.09
2	28S	46.65	52.05	215.34	28.82

RNA Area	747.18
RNA Concentration (pg/µl)	6,044.351
rRNA Ratio [28S/18S]	1.30

4.4.2.5 E14b RNA sample

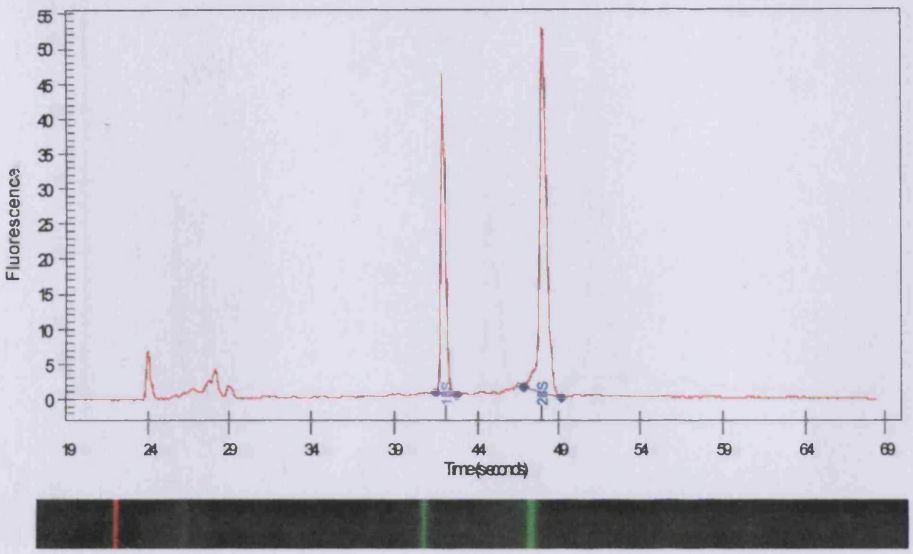


Fig. 4.11; Electropherogram graph and gel trace for sample E14b showing clear 18S and 28S rRNA bands and a flat base line.

Sample Factor	Dilution	1:22					
Fragment		Name	Start time (secs)		End Time (secs)	Area	% of total Area
1		18S	41.45		42.75	33.84	23.25
2		28S	46.85		49.15	53.49	36.74

RNA Area	145.56
RNA Concentration (pg/µl)	1,177.52
rRNA Ratio [28S/18S]	1.58

4.4.2.6 E14c RNA sample

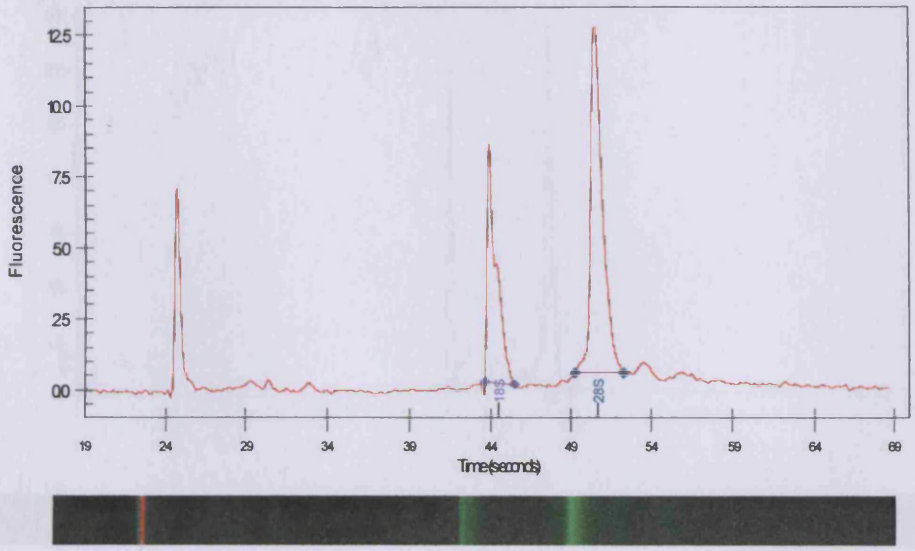


Fig. 4.12; Electropherogram graph and gel trace for sample E14c showing clear 18S and 28S rRNA bands and a reasonably flat base line.

Sample Factor	Dilution	1:13					
Fragment		Name	Start time (secs)	End Time (secs)	Area	% of total Area	
1		18S	43.60	45.45	12.64	25.67	
2		28S	449.25	52.25	20.51	41.65	

RNA Area	49.24
RNA Concentration (pg/µl)	50.80
rRNA Ratio [28S/18S]	1.62

4.4.2.7 E16a RNA sample

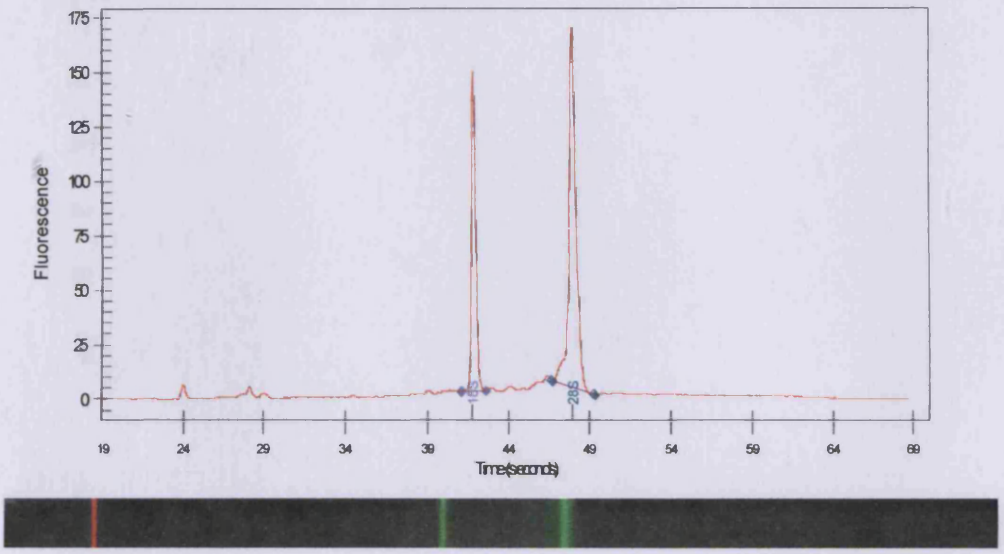


Fig. 4.13; Electropherogram graph and gel trace for sample E16a showing clear 18S and 28S rRNA bands and a flat base line.

Sample Factor Dilution 1:22

Fragment	Name	Start time (secs)	End Time (secs)	Area	% of total Area
1	18S	41.05	42.55	110.43	21.86
2	28S	46.65	49.25	179.12	35.45

RNA Area	505.45
RNA Concentration (pg/µl)	4,087.12
rRNA Ratio [28S/18S]	1.62

4.4.2.8 E16b RNA sample

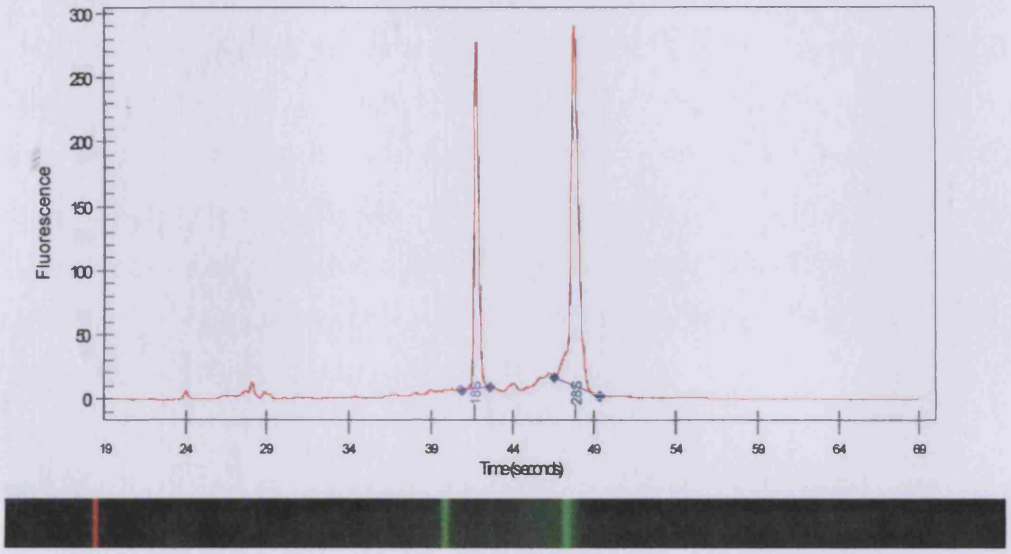


Fig. 4.14; Electropherogram graph and gel trace for sample E16b showing clear 18S and 28S rRNA bands and a flat base line.

Sample Factor	Dilution	1:22				
Fragment	Name	Start time (secs)	End Time (secs)	Area	% of total Area	
1	18S	40.80	42.55	213.13	23.68	
2	28S	46.50	49.30	316.81	35.20	

RNA Area	899.91
RNA Concentration (pg/µl)	7,279.81
rRNA Ratio [28S/18S]	1.49

4.4.2.9 E16c RNA sample

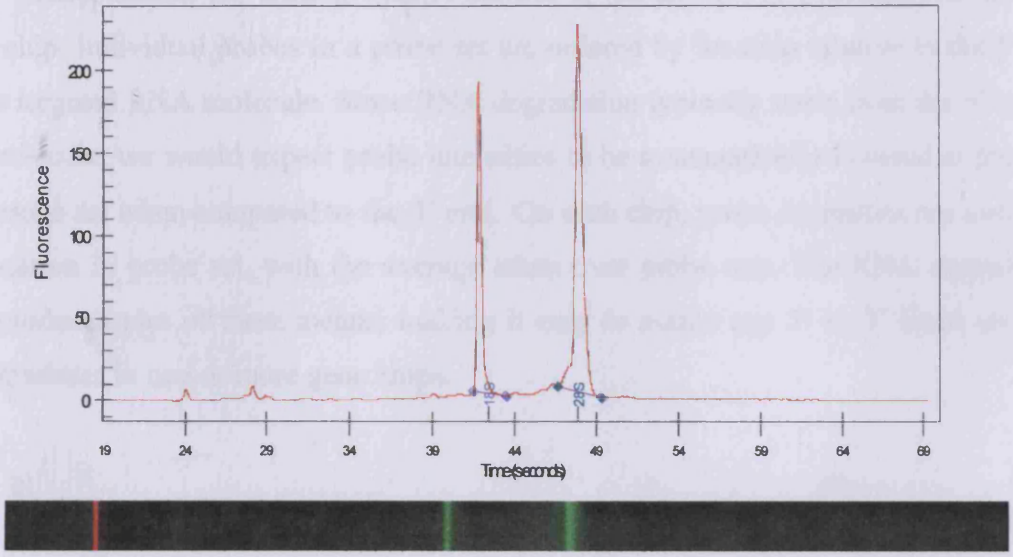


Fig. 4.15; Electropherogram graph and gel trace for sample E16b showing clear 18S and 28S rRNA bands and a flat base line.

Sample Factor	Dilution	1:16				
Fragment	Name	Start time (secs)	End Time (secs)	Area	% of total Area	
1	18S	41.45	43.45	150.82	25.72	
2	28S	46.60	49.25	253.81	43.28	

RNA Area	586.38
RNA Concentration (pg/µl)	4,743.55
rRNA Ratio [28S/18S]	1.68

4.4.2.10 RMA Degradation Plot

These results are used as quality control of the amount of hybridisation on each gene chip. Individual probes in a probe set are ordered by location relative to the 5' end of the targeted RNA molecule. Since RNA degradation typically starts from the 5' end of the molecule, we would expect probe intensities to be systematically lowered at that end of a probe set when compared to the 3' end. On each chip, probe intensities are averaged by location in probe set, with the average taken over probe sets. The RNA degradation plot produces plot of these means, making it easy to notice any 5' to 3' trend and any abnormalities in one or more gene chips.

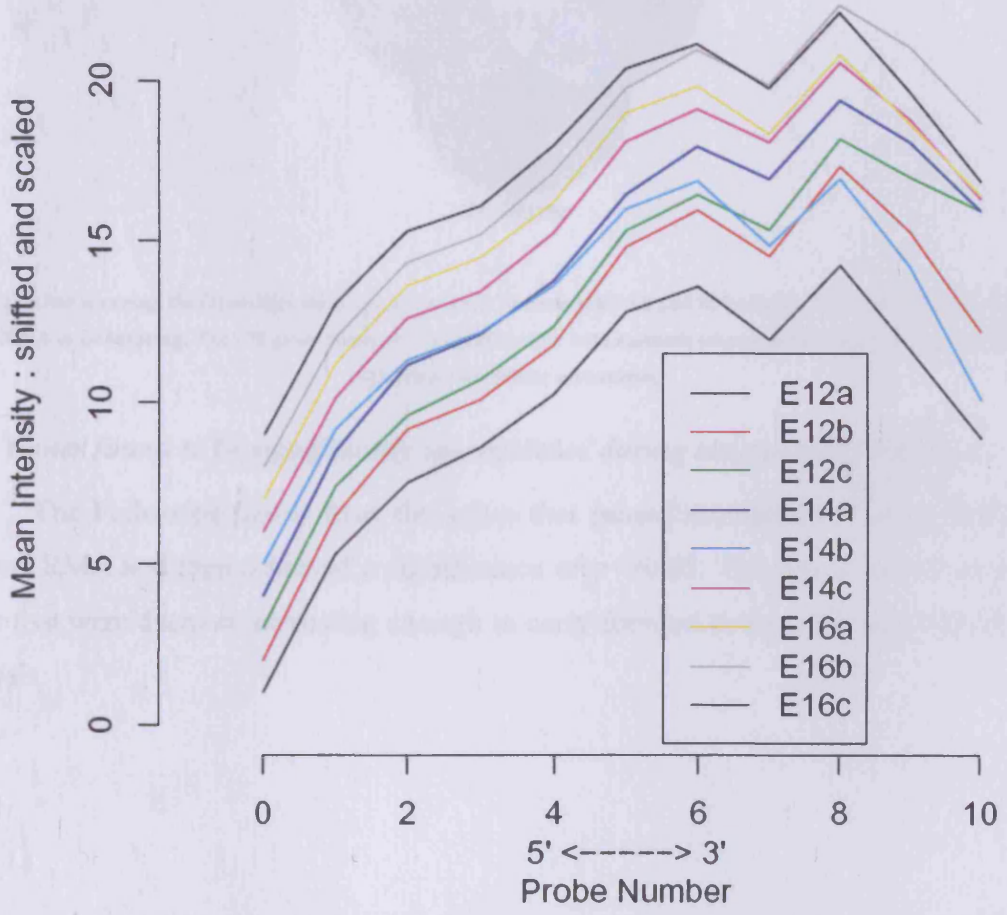


Fig. 4.16; RNA Degradation Plot showing all samples followed a common trend. This indicated, when viewed alongside the UV spectrometry and Agilent data that the RNA samples were of good quality (produced by RMA express (Irizarry *et al.*, 2003; Bolstad *et al.*, 2003)).

4.4.2.11 MAS 5.0 and RMA Normalisation

After normalisation using two methods (the Affymetrix MAS 5.0(Affymetrix, 2001a) and the RMA (Irizarry *et al.*, 2003; Bolstad *et al.*, 2003)) of normalisation, to reduce the chance of false positives from occurring, only the genes that passed both normalisations and a further ANOVA in Genespring with a significance value ($p<=0.05$) were carried through to follow up analysis/candidate marker gene analysis.

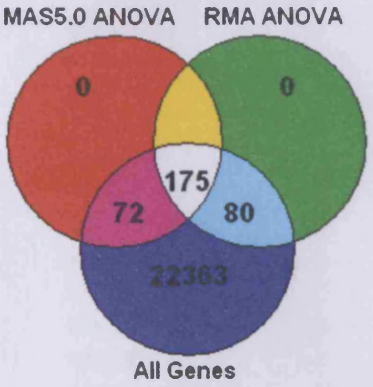


Fig. 4.17 After scanning the Genechips, data was normalised by both MAS 5.0 and RMA before both data sets were run using an ANOVA in Genespring. The 175 genes shown to be significant by both methods (shown in the white area) were selected as candidates for follow up analysis.

4.4.2.12 Genes found to be significantly up-regulated during striatal differentiation

The Following tables show the genes that passed normalisation using both MAS 5.0 and RMA and then achieved a significance of $p<=0.05$. The genes marked in red are those that were deemed interesting enough to carry forward to the next stage of follow up analysis.

Table 4.1; 175 Genes were found to be significantly up and down regulated between the ages of E12 and E16 ($p<0.05$) after RMA and MAS5.0 normalisation and ANOVA, ranked by order of significance for up and down regulated genes (Red genes denote those chosen for follow up investigation, Blue denotes the one unknown gene to be chosen, that was later discarded for technical reasons).

Affymetrix annotation	Common Name	Genbank ID	up/down regulated	RMA *p* value	MAS 5 *p* value
1435222_at	Foxp1	BM220880	up	0.000654	0.00767
1435221_at	Foxp1	BM220880	up	0.000837	0.00392
1451191_at	Crabp2	BC018397	up	0.000837	0.00392
1421142_s_at	Foxp1	BG962849	up	0.00113	0.00392
1451506_at	Mef2c	BB280300	up	0.00173	0.0306
1456283_at	Neto1	AV346211	up	0.00197	0.00672
1421141_a_at	Foxp1	BG962849	up	0.00197	0.00677
1423343_at	Slco1c1	BB667135	up	0.00197	0.00677
1424718_at	Mapt	M18775	up	0.00197	0.00677
1436363_a_at	Nfix	AW049660	up	0.00197	0.00677
1420534_at	Gucy1a3	AK004815	up	0.00197	0.00909
1449563_at	Cntn1	NM_007727	up	0.00197	0.0112
1423802_at	BC017634	BC017634	up	0.00197	0.0151
1448978_at	Ngef	NM_019867	up	0.00197	0.0152
1448154_at	Ndrg2	NM_013864	up	0.00208	0.00677
1450339_a_at	Bcl11b	NM_021399	up	0.00208	0.0119
1448194_a_at	H19	NM_023123	up	0.00208	0.013
1432466_a_at	Apoe	AK019319	up	0.00208	0.0177
1422052_at	Cdh8	NM_007667	up	0.00208	0.0306
1448330_at	Gstm1	NM_010358	up	0.00208	0.0405
1418782_at	Rxrg	NM_009107	up	0.00223	0.00863
1420533_at	Gucy1a3	AK004815	up	0.00234	0.0112
1452141_a_at	Sepp1	BC001991	up	0.00234	0.0128
1426106_a_at	Syt6	AB026810	up	0.00234	0.0189
1454906_at	Rarb	BB266455	up	0.00241	0.00909
1417466_at	Rgs5	NM_133736	up	0.00241	0.0228
1460214_at	Pcp4	NM_008791	up	0.00241	0.0229
1424400_a_at	Fthfd	AK007822	up	0.00249	0.0112
1421140_a_at	Foxp1	BG962849	up	0.00265	0.00466
1422014_at	Foxp2	AY079003	up	0.00265	0.0112
1416114_at	Sparcl1	NM_010097	up	0.00265	0.0128
1416149_at	Olig1	AB038696	up	0.00271	0.0175
1418594_a_at	Ncoa1	NM_010881	up	0.00277	0.00533
1425846_a_at	Caln1	AF282251	up	0.00277	0.0191
1455242_at	Foxp1	BM220880	up	0.00284	0.00338
1416342_at	Tnc	NM_011607	up	0.00284	0.0041
1416562_at	Gad1	AF326547	up	0.00284	0.0119
1448326_a_at	Crabp1	NM_013496	up	0.00284	0.0148
1416666_at	Serpine2	NM_009255	up	0.00299	0.0256
1436364_x_at	Nfix	AW049660	up	0.00311	0.0041
1420911_a_at	Mfge8	NM_008594	up	0.00329	0.0197
1421840_at	Abca1	BB144704	up	0.0035	0.0278
1416286_at	Rgs4	NM_009062	up	0.00367	0.00677
1434515_at	Ncoa1	BE996469	up	0.00386	0.0101

Table 4.1 (continued); 175 Genes were found to be significantly up and down regulated between the ages of E12 and E16 ($p<0.05$) after RMA and MAS5.0 normalisation and ANOVA, ranked by order of significance for up and down regulated genes (Red genes denote those chosen for follow up investigation, Blue denotes the one unknown gene to be chosen, that was later discarded for technical reasons)

Affymetrix annotation	Common Name	Genbank ID	up/down regulated	RMA *p* value	MAS 5 *p* value
1422541_at	Ptprm	NM_008984	up	0.00402	0.0113
1451516_at	Rhebl1	BC016521	up	0.00402	0.0241
1426517_at	Gnaz	AI326356	up	0.00406	0.0311
1428197_at	9430079M16Rik	AK020159	up	0.0042	0.0214
1417839_at	Cldn5	NM_013805	up	0.00444	0.02
1423551_at	Cdh13	BB776961	up	0.00448	0.0116
1421101_a_at	Ldb2	NM_010698	up	0.00452	0.0105
1451461_a_at	Aldo3	BC008184	up	0.0049	0.0153
1423186_at	Tiam2	BM228957	up	0.00522	0.0107
1434005_at	Rbms1	AW541585	up	0.00522	0.0128
1418062_at	Eef1a2	NM_007906	up	0.00522	0.0137
1423493_a_at	Nfix	BB315728	up	0.00542	0.00908
1419225_at	Cacna2d3	NM_009785	up	0.00542	0.0101
1448972_at	Gria1	NM_008165	up	0.00595	0.0137
1420871_at	Gucy1b3	BF472806	up	0.00694	0.00677
1417848_at	Gig1	AW413620	up	0.00694	0.00747
1451264_at	4930488L10Rik	BC019939	up	0.00694	0.00923
1450055_at	Vsnl1	NM_012038	up	0.00694	0.0112
1428443_a_at	Rap1ga1	AK005063	up	0.00694	0.0189
1449158_at	Kcnk2	NM_010607	up	0.00701	0.0197
1451021_a_at	Klf5	BI465857	up	0.00718	0.0112
1438802_at	Foxp1	BB021390	up	0.00774	0.0179
1454849_x_at	Clu	BB433678	up	0.00774	0.0307
1415812_at	Gsn	NM_010354	up	0.00783	0.0256
1416302_at	Ebf1	BB125261	up	0.00794	0.0151
1449468_at	Siat7e	NM_012028	up	0.00794	0.0175
1435239_at	Gria1	BQ175316	up	0.00794	0.0287
1448673_at	Pvrl3	NM_021495	up	0.00807	0.0151
1451475_at	Plxnd1	BC019530	up	0.00835	0.0474
1451804_a_at	Lrrc16	BC012229	up	0.00837	0.00579
1416718_at	Bcan	NM_007529	up	0.00839	0.0236
1419420_at	Siat7e	NM_012028	up	0.00862	0.01
1420872_at	Gucy1b3	BF472806	up	0.00864	0.0128
1416069_at	Pfkp	NM_019703	up	0.0092	0.0112
1451450_at	2010011I20Rik	AK008190	up	0.00971	0.0312
1427005_at	Plk2	BM234765	up	0.0102	0.0189
1416301_a_at	Ebf1	BB125261	up	0.0109	0.0351
1452981_at	Cntn1	AK004399	up	0.0109	0.0415
1415845_at	Syt4	AV336547	up	0.0111	0.0231
1435026_at	Spock2	BM117672	up	0.0112	0.00726
1424248_at	0710001E13Rik	BB159263	up	0.0118	0.00338
1422835_at	Kcnd2	BB051684	up	0.012	0.0342
1418950_at	Drd2	NM_010077	up	0.0126	0.0311
1428074_at	2310037P21Rik	BE981853	up	0.0126	0.0371

Table 4.1 (continued); 175 Genes were found to be significantly up and down regulated between the ages of E12 and E16 ($p<0.05$) after RMA and MAS5.0 normalisation and ANOVA, ranked by order of significance for up and down regulated genes (Red genes denote those chosen for follow up investigation, Blue denotes the one unknown gene to be chosen, that was later discarded for technical reasons).

Affymetrix annotation	Common Name	Genbank ID	up/down regulated	RMA *p* value	MAS 5 *p* value
1450930_at	Hpca	AK002992	up	0.0133	0.0189
1424852_at	5430401D19Rik	BB280300	up	0.0138	0.0163
1422064_a_at	Zbtb20	AY028963	up	0.0138	0.0228
1418453_a_at	Atp1b1	NM_009721	up	0.0158	0.0119
1417600_at	Slc15a2	NM_021301	up	0.0159	0.0311
1416783_at	Tac1	NM_009311	up	0.0166	0.028
1424341_s_at	Pcdha4	AW146252	up	0.017	0.035
1421027_a_at	Mef2c	AI595932	up	0.0174	0.026
1420981_a_at	Lmo4	NM_010723	up	0.0197	0.0361
1425452_s_at	AW125753	BC002154	up	0.0209	0.0202
1422474_at	Pde4b	BM246564	up	0.0214	0.0276
1420718_at	Odz2	NM_011856	up	0.0228	0.0114
1448710_at	Cxcr4	D87747	up	0.0228	0.0243
1416983_s_at	Foxo1	AI462296	up	0.026	0.0125
1433888_at	Atp2b2	AV343478	up	0.0288	0.0369
1439036_a_at	Atp1b1	AV152334	up	0.03	0.0311
1423836_at	Zfp503	BB447914	up	0.0304	0.0205
1424719_a_at	Mapt	M18775	up	0.0366	0.0398
1437689_x_at	Clu	AV152288	up	0.0368	0.0147
1425132_at	Neto1	AF448840	up	0.0432	0.0121
1415844_at	Syt4	AV336547	up	0.0454	0.0308
1450227_at	Ankrd6	BM199504	up	0.0461	0.0221
1434325_x_at	Prkar1b	BB274009	up	0.0468	0.0276
1437458_x_at	Clu	AV075715	up	0.0468	0.028
1437874_s_at	Hexb	AV225808	up	0.0468	0.0311
1418835_at	Phlda1	NM_009344	up	0.0468	0.0351
1437810_a_at	Hbb-bh1	AV311770	down	0.000654	0.015
1450736_a_at	Hbb-bh1	NM_008219	down	0.000837	0.00338
1437990_x_at	Hbb-bh1	AV147727	down	0.000837	0.00747
1422851_at	Hmga2	X58380	down	0.000837	0.00782
1448716_at	Hba-x	M26898	down	0.00173	0.00646
1450621_a_at	Hbb-bh1	NM_008221	down	0.00197	0.00677
1450781_at	Hmga2	X58380	down	0.00197	0.00765
1418026_at	Exo1	BE986864	down	0.00197	0.0342
1423348_at	Fzd8	AV345166	down	0.00208	0.00466
1450780_s_at	Hmga2	X58380	down	0.00208	0.00677
1448698_at	Ccnd1	NM_007631	down	0.00208	0.00767
1449939_s_at	Dlk1	NM_010052	down	0.00208	0.00767
1417419_at	Ccnd1	NM_007631	down	0.00208	0.0149
1436823_x_at	Hbb-y	AV148191	down	0.00223	0.00579
1418401_a_at	Dusp16	NM_130447	down	0.00241	0.0143
1430542_a_at	Slc25a5	AA823938	down	0.00265	0.0112
1422520_at	Nef3	NM_008691	down	0.00265	0.018
1416159_at	Nr2f2	AI463873	down	0.00277	0.00726

Table 4.1 (continued); 175 Genes were found to be significantly up and down regulated between the ages of E12 and E16 ($p<0.05$) after RMA and MAS5.0 normalisation and ANOVA, ranked by order of significance for up and down regulated genes (Red genes denote those chosen for follow up investigation, Blue denotes the one unknown gene to be chosen, that was later discarded for technical reasons).

Affymetrix annotation	Common Name	Genbank ID	up/down regulated	RMA *p* value	MAS 5 *p* value
1421841_at	Fgfr3	NM_008010	down	0.00277	0.0152
1421180_at	Lix1	NM_025681	down	0.00277	0.0161
1418317_at	Lhx2	NM_010710	down	0.00288	0.00677
1452114_s_at	Igfbp5	BF225802	down	0.00289	0.0112
1437103_at	C330012H03Rik	AV293532	down	0.00311	0.0256
1418478_at	Lmo1	NM_057173	down	0.00356	0.00726
1418761_at	Igf2bp1	BB499476	down	0.00371	0.00767
1436717_x_at	Hbb-y	AV156860	down	0.00495	0.00747
1448363_at	Yap1	NM_009534	down	0.00637	0.0105
1427633_a_at	Pappa	AF439513	down	0.00694	0.0112
1417649_at	Cdkn1c	NM_009876	down	0.00694	0.0217
1417420_at	Ccnd1	NM_007631	down	0.00781	0.01
1417457_at	Cks2	NM_025415	down	0.00791	0.00747
1416802_a_at	Cdca5	NM_026410	down	0.00794	0.0112
1422670_at	Rohn	NM_009708	down	0.00794	0.0156
1428402_at	2810406K24Rik	AV140894	down	0.009	0.0471
1419700_a_at	Prom1	NM_008935	down	0.0114	0.0325
1425458_a_at	Grb10	AF022072	down	0.0126	0.0147
1420028_s_at	Mcm3	C80350	down	0.0128	0.02
1423146_at	Hes5	AV337579	down	0.0131	0.00909
1424882_a_at	2510015F01Rik	BC011230	down	0.0145	0.0168
1456010_x_at	Hes5	BB561515	down	0.0149	0.013
1423500_a_at	Sox5	AI528773	down	0.0168	0.0175
1428379_at	Slc17a6	BQ180367	down	0.017	0.013
1451346_at	Mtap	BG075139	down	0.0183	0.0278
1452654_at	Zdhhc2	BB224658	down	0.0186	0.00392
1419271_at	Pax6	BC011272	down	0.0187	0.0175
1439483_at	AI506816	BI438039	down	0.0221	0.011
1433924_at	Peg3	BM200248	down	0.0221	0.0431
1427300_at	Lhx8	D49658	down	0.0226	0.0247
1422839_at	Neurog2	NM_009718	down	0.0229	0.0221
1455972_x_at	Hadhsc	AV018774	down	0.0229	0.0402
1416558_at	Melk	NM_010790	down	0.0229	0.0466
1437807_x_at	Catna1	BB066232	down	0.0243	0.0191
1419380_at	Zfp423	NM_033327	down	0.0261	0.0189
1437033_a_at	Skp2	BB784099	down	0.0275	0.0362
1416076_at	Ccnb1	NM_007629	down	0.0288	0.0293
1448519_at	Tead2	D50563	down	0.0319	0.0197
1448777_at	Mcm2	NM_008564	down	0.0355	0.0181
1417541_at	Hells	NM_008234	down	0.0374	0.0147
1425457_a_at	Grb10	AF022072	down	0.0401	0.0272
1426001_at	Eomes	AB031037	down	0.0421	0.0471
1452241_at	Topbp1	BC007170	down	0.0495	0.0402

4.4.3 Reasons for selecting candidate genes to follow up;

Once the initial RMA and MAS5.0 normalisation and analysis had been completed and the results of both then compared it was found that there were still a very high number of genes that showed significant changes in expression levels over the time period assayed. Thus, additional criteria were set for significantly changing genes to select those deemed suitable for validation and further analysis. It was also decided to look at genes that only went up in expression between the ages of E12 and E16 for this project, as this reduced the number of genes and the functions and effects of these genes would be easier to study in future experiments that could be carried out using the facilities that were readily available to this laboratory group. Down regulation of genes was recognised as being equally important, but for reasons of time and cost this set of genes will be part of a separate study

The criteria were;

Genes significantly up regulated between the age of E12 and E16 that were also;

- Genes coding for transcription factors (as these are likely to important in differentiation and knowledge of these may lead directly to methods for directing differentiation of stem cell populations) or
- Genes previously known to be implicated in development/migration/axonal path finding or
- Genes coding for cell signalling proteins/receptors (as these genes are likely to be implicated in the signals that progenitor cells need in an *in vivo* environment to proliferate and/or differentiate normally) or
- Genes for which a function has not yet been identified (for obvious reasons novel genes that change in amounts during this period could be implicated in the development, proliferation or differentiation of striatal neurons, and as such are of interest).

Genes that were known to be involved in normal cell cycle and normal cell processes (housekeeping genes) were discounted at this point in the analysis.

A much smaller number of genes satisfied the above criteria, and are discussed below:

4.4.3.1 Foxp1 and Foxp2

The up regulation of these genes between the ages of E12 and E16 is in accordance with a small number of papers (Ferland *et al.*, 2003) and this lends confidence to the results reported here and also validate findings seen by other laboratories using other methods. However, new information from this screen is the high degree to which this gene is up-regulated during striatal development (*Foxp1* being the most significantly up-regulated in this particular screen).

The Fox gene family all contain a domain homologous to the drosophila 'forked head' domains and are transcription factors that promote terminal, rather than segmental development. The forked head motif (also known as 'winged helix') is a 110-amino acid DNA binding domain which has a common involvement in early developmental decisions of cell fates during embryogenesis and may function as a co-regulator of nuclear receptors, and in particular the oestrogen receptor. The Foxp sub family is a distant relation of the main Fox family; the only homology being an 80-amino acid region within the DNA binding domain. The Foxp genes also contain domains unique to this subfamily, such as a leucine zipper resembling the N-myc zip dimerisation domain, a zinc finger and a polyglutamine tract.

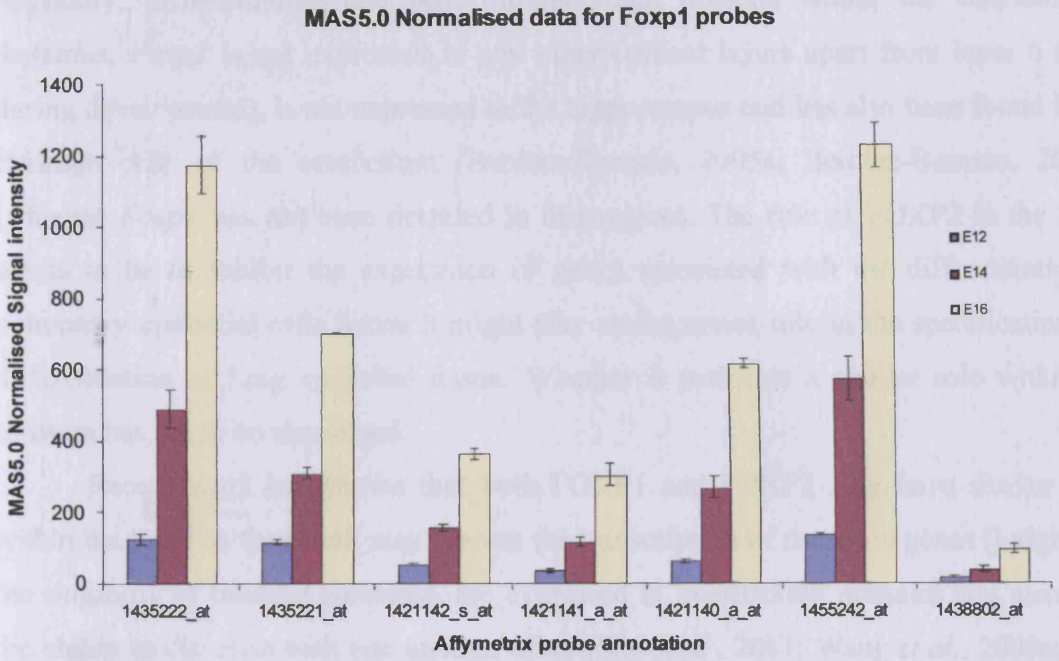
4.4.3.2 Foxp1

This gene was first described in neurons projecting from the striatum (Tamura *et al.*, 2000; Tamura *et al.*, 2003) having also been found to be expressed in lung and gut tissue (Shu *et al.*, 2001) .The FOXP1 protein has four known isoforms; FOXP1A, B, C and D which have been implicated in the development of a range of tissues including the CNS, heart lungs and liver and spleen, all of the isoforms apart from the B isoform have been found in the brain. All of the isoforms other than the C isoform repress gene transcription when bound to a protein binding site occurring within the SV40 and Interleukin-2 (IL-2) promoters (Wang *et al.*, 2003a).

Foxp1 is also thought to have tumour suppressing activity as it has been found to be varying in expression levels in a number of different types of tumours (Banham *et al.*, 2001; Banham *et al.*, 2005). The subfamily also has an HNF3 binding domain that indicates these transcription factors bind to DNA as monomers. However, recent work has suggested that the leucine zipper in combination with the zinc finger domain promotes self association amongst different isoforms and sub family members of the Foxp transcription factors (Wang *et al.*, 2003a). The 37-40 residue polyglutamine stretch

(seen in FOXP1A and B isoforms) may also infer some sort of modulatory effect on repression as these domains are known to mediate protein to protein interactions by either forming multimers (Perutz *et al.*, 2002) or interacting with polar amino acid-rich sequences (Imafuku *et al.*, 1998).

Foxp1 has been observed within the developing striatum, hippocampal CA1 fields and cortex (mostly within layers 3-5 but also in scattered amounts in layers 2-6) from E12.5 (Tamura *et al.*, 2000; Tamura *et al.*, 2003; Ferland *et al.*, 2003). The Striatal expression was not observed within the progenitor cells that reside near the ventricular zone, but in differentiating cells migrating away from this area and taking up positions in the newly formed striatum itself (Tamura *et al.*, 2000; Tamura *et al.*, 2003). This suggests FOXP1 may have a role in post migratory neuronal development. Later in development and throughout adult life expression of *Foxp1* remains high in the striatum although after development its expression in the cortex is more restricted from layers 3-5 to just layer 6 neurons within the cortex.



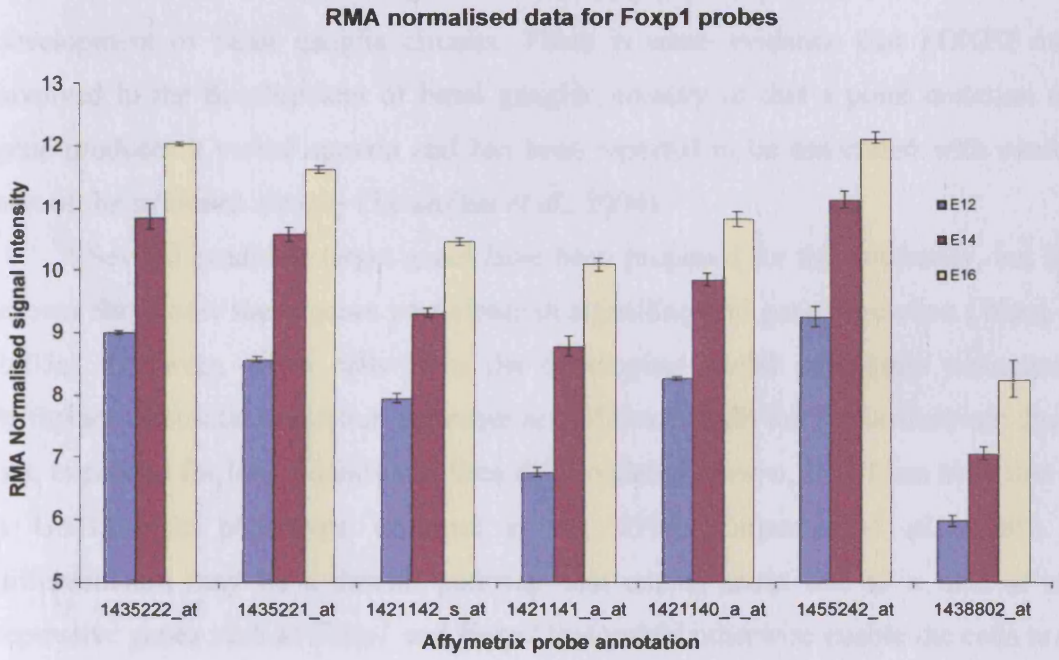


Fig. 4.18; MAS5.0 and RMA Normalised data for Foxp1 probes.

4.4.3.3 Foxp2

Foxp2 expression has also been previously reported in the developing striatum (Ferland *et al.*, 2003) from around E12.5 as well as other areas including the lung and gut epithelia (Shu *et al.*, 2001). In the brain, as with *Foxp1*, expression is seen within differentiated neurons. However, it seems that these two genes may be expressed in

different neuronal sub-types as although they are both expressed in high amount in post migratory, differentiating and post differentiating neurons within the striatum and thalamus; *Foxp2* is not expressed in any other cortical layers apart from layer 6 (even during development), is not expressed in the hippocampus and has also been found in the Purkinje cells of the cerebellum (Benitez-Burraco, 2005a; Benitez-Burraco, 2005b) (whereas *Foxp1* has not been detected in this region). The role of FOXP2 in the lungs seems to be to inhibit the expression of genes associated with the differentiation of pulmonary epithelial cells hence it might play an important role in the specification and differentiation of lung epithelial tissue. Whether it performs a similar role within the striatum has yet to be elucidated.

Recent work has shown that both FOXP1 and FOXP2 may have similar roles within the brain as they both may repress the transcription of the same genes (judging by the similarity of binding domains), are expressed in interlocking domains and also have the ability to dimerise with one another other (Shu *et al.*, 2001; Wang *et al.*, 2003a; Li *et al.*, 2004b). It may be that these genes have similar or parallel roles in the cells within the development of basal ganglia circuits. There is some evidence that FOXP2 may be involved in the development of basal ganglia circuitry in that a point mutation of this gene produces a verbal apraxia and has been reported to be associated with caudate (a part of the striatum) atrophy (Teramitsu *et al.*, 2004).

Several candidate target genes have been proposed for this subfamily, but little is known about how these genes participate in signalling and gene regulation (Wang *et al.*, 2003a). However, when cells from the developing lateral ganglionic eminence (the birthplace of striatal projection neurones and olfactory bulb inter-neurones) are dissected out, expanded for long periods and then differentiated *in vitro*, they form cells that adopt a GABA-ergic phenotype (Ahmed *et al.*, 1995; Carpenter *et al.*, 1999). This differentiation may be a default pathway that comes about due to a lack of certain repressive genes such as *Foxp1* and *Foxp2* that would otherwise enable the cells to adopt a separate differentiation pathway and possibly become striatal projection neurons. Therefore the expression of either one or both of these Foxp genes that are found to be up regulated genes during the development of the striatum, may be crucial to the continued differentiation and development of projection neurons within the developing striatum.

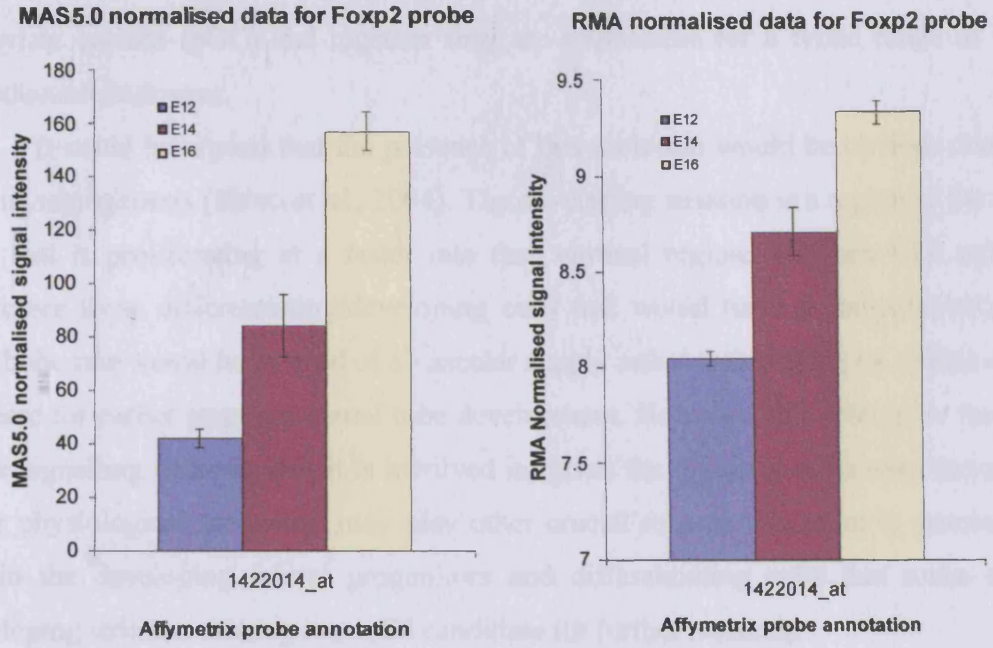


Fig. 4.19; MAS5.0 and RMA Normalised data for Foxp2 probe.

4.4.3.4 Guanylate Cyclase1β3

Guanylate Cyclase1β3 (human homologue GUCY1β1) is a subunit of soluble guanylate cyclase (sGC). This is a hetero-dymeric enzyme composed of α and β subunits that also contains a haem unit that enables Nitric Oxide (·NO) to bind to and activate the enzyme (Arnold *et al.*, 1977). sGC is the main and only conclusively proven receptor for the signaling agent ·NO and is also one of only two enzymes (the other being a membrane-bound form) that catalyses the conversion of guanosine 5'-triphosphate (GTP) to cyclic guanosine 3',5'-monophosphate (cGMP). Therefore this peptide is probably involved in many signal-transduction pathways including angiogenesis (Saino *et al.*, 2004), vascular and non-vascular smooth muscle relaxation, peripheral and central neural transmission, platelet reactivity (Hobbs, 1997), photo transduction (Semple-Rowland *et al.*, 1998) and possibly long term potentiation and depression (Jaffrey and Snyder, 1995). The activation of sGC and the resulting increase of cGMP allows sGC to transmit an ·NO signal to the numerous downstream elements of the cGMP signaling cascade which include cGMP-dependant protein kinases, cGMP-gated cation channels and cGMP-regulated phospho-diesterase.

sGC is a member of larger group of evolutionary related enzymes known as the nucleotide cyclases. These enzymes convert guanosine tri-phosphate (GTP) and adenosine tri-phosphate (ATP) into cyclic nucleotide monophosphates (cGMP and

cAMP). Other members of the group include adenylate cyclase (AC) and particulate guanylate cyclase (pGC) and together they are responsible for a broad range of signal transduction pathways.

It could be argued that the presence of this molecule would be obvious due to its role in angiogenesis (Saino *et al.*, 2004). The developing striatum is a region of the neural tube that is proliferating at a faster rate than cortical regions between E12 and E16. Therefore these differentiating/developing cells that would have a comparatively high metabolic rate would be in need of a vascular supply rather than relying on diffusion as is the case for earlier stages in neural tube development. However, this protein, or the nitric oxide signalling pathway that it is involved in, given the diversity of its roles throughout other physiological processes, may play other crucial roles at this point in development within the developing neural progenitors and differentiating cells that make up the developing striatum making it a valid candidate for further research.

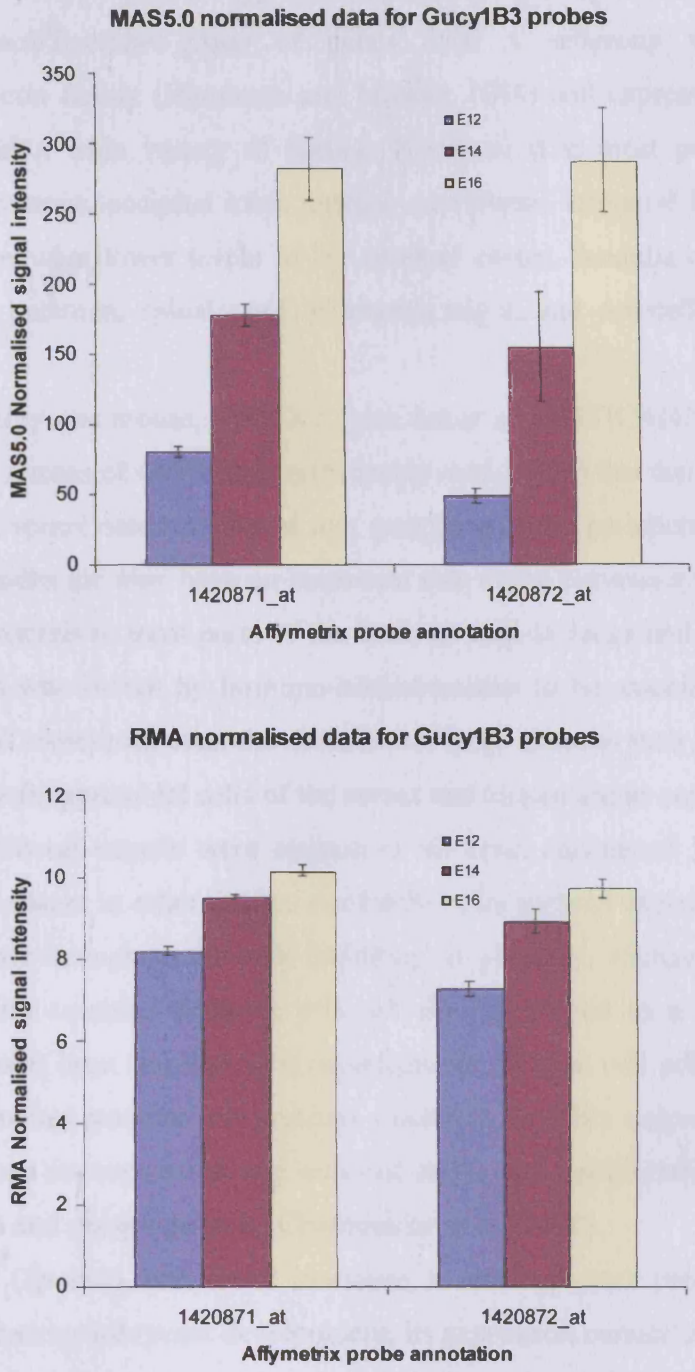


Fig. 4.20; MAS5.0 and RMA Normalised data for Gucy1B3 probes.

4.4.3.5 Spock 2 (Testican 2)

SPOCK2 is a putative extracellular heparin/chondroitin sulphate proteoglycan (Kohfeldt *et al.*, 1997; Vannahme *et al.*, 1999). The protein was first identified from a human cDNA library as being a protein of 424 amino acids that contain a signal peptide, a follistatin-like domain, a calcium binding domain, a thyroglobulin-like domain and a C-

terminal region with two putative glycoaminoglycan attachment sites (Vannahme *et al.*, 1999). The *Spock/Testican* group of genes form a subgroup within the BM-40/Sparc/osteonectin family (Hartmann and Maurer, 2001) and expression of *Spock2* in humans occurs in a wide variety of tissues. However, it is most pronounced in the thalamus, hippocampus, occipital lobe, nucleus accumbens, temporal lobe, and caudate nucleus with somewhat lower levels in the cerebral cortex, medulla oblongata, frontal lobe, amygdala, putamen, spinal cord, substantia nigra, and cerebellum (Marr *et al.*, 2000).

In the embryonic mouse, SPOCK2 (also know as TESTICAN2), is a 401 amino acid protein with a mass of 44678 dalton (Schnepp *et al.*, 2005) that has been found in the embryonic brain, spinal cord and dorsal root ganglions of the peripheral nervous system so it seems this molecule may have an important role in the nervous system. In the adult this expression extends to most parts of the brain as well as lungs and testis. In a recent study the protein was shown by immuno-histochemistry to be associated with the cell bodies, axons and sometimes even the dendrites of large neurons such as the mitral cells of the olfactory bulb, pyramidal cells of the cortex and hippocampus and Purkinje cells of the cerebellum. Blood vessels were stained in all brain regions as well as a diffuse staining that was absent in other tissues. Antibodies also showed expression in a number of endocrine glands throughout the body including the pituitary, (Schnepp *et al.*, 2005).

The protein contains domains that are closely related to a number of other molecules that have been implicated in neurogenesis such as cell adhesion molecules, growth factor binding proteins and protease inhibitors and this supports evidence of a correlation between its expression and areas of active cell proliferation and migration, axonal outgrowth and synaptogenesis (Charbonnier *et al.*, 2000).

Testican1 (*Spock1*), also found in mouse, is also expressed predominately in the nervous system during embryonic development; its expression correlating with periods of neuronal migration and axonal growth and path finding (Charbonnier *et al.*, 2000). In the adult mouse its expression is restricted to the brain where it is located in posts synaptic densities (Bonnet *et al.*, 1996). In the cerebellum transcripts of *Spock2* have been observed as early as E10.5 in the germinal trigone of the cerebellum (formed when granular layer precursors reach the surface of the cerebellar cortex and start to make connections) (Bonnet *et al.*, 1996; Charbonnier *et al.*, 2000). In the olfactory apparatus transcripts are again detected at E10.5 when the neuroepithelium begins to differentiate (Saito *et al.*, 1998) and expression increases through E12.5 as olfactory nerve axons

migrate up towards the olfactory cortex (Cuschieri and Bannister, 1975). At E14 in the mouse, *Spock* (this study was completed when only one gene of the Spock group had been found) was also detected in the olfactory cortex when synaptogenesis begins between the axons of the incoming olfactory axons and dendrites of the mitral cells of the olfactory bulb (Hinds and Hinds, 1976). In the spinal cord *Spock* is expressed by E10 around the time when neurogenesis begins and continues until E12 as migration of cells within the mantle layer increases. At E14 transcripts and protein are at their highest levels in the grey matter during the peak time of neuronal connection and then between E16 and E18 transcription is restricted to the dorsal and ventral horns, where synaptogenesis is most active at this time (Charbonnier *et al.*, 2000).

The expression of *Spock* in the peripheral nervous system also seems to suggest its role in the migration and differentiation of neurons (Lawson and Waddell, 1991; Ozaki and Snider, 1997).

In general, *Spock* expression correlates with the development of the mouse nervous system at times when cell adhesion, axon path finding and synaptogenesis are taking place. This supports its presumed involvement in neural development and gives a good reason to study its expression and functions within the developing striatum.

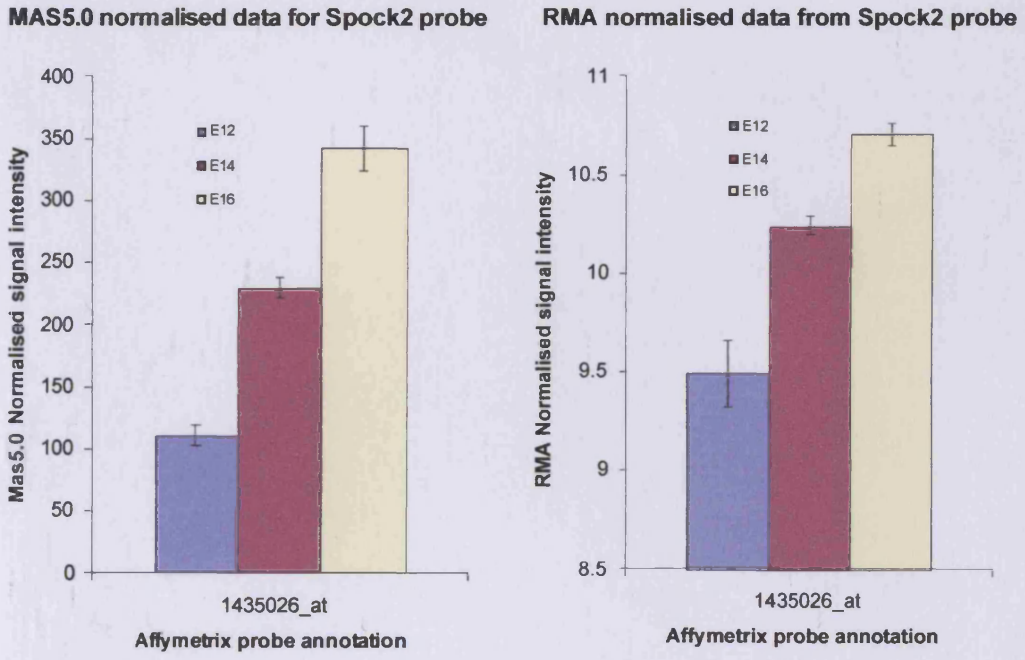
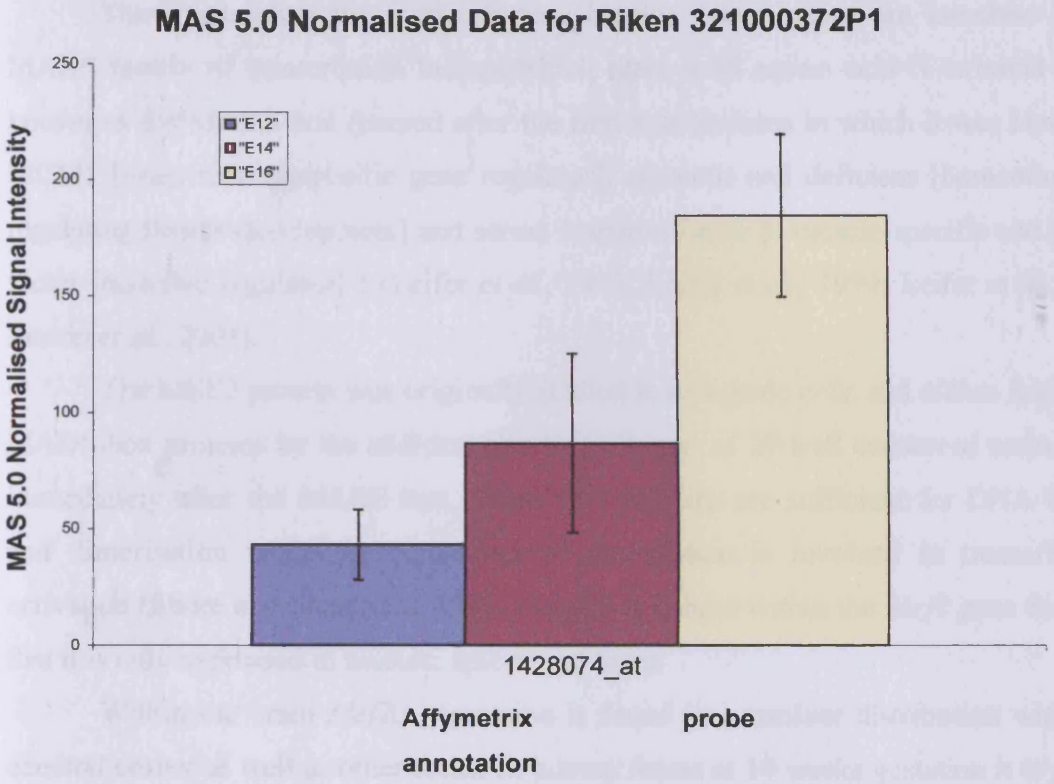


Fig. 4.21; MAS5.0 and RMA Normalised data for *Spock2* probe.

4.4.3.6 321000372P1 Riken (Brain Specific Binding protein)

This Riken clone had unknown function when found to be significantly up regulated between E12 and E16 in the WGE. However, due to the size of the known transcript of this clone, technical problems and the lack of information on it at the time, a suitable set of primers could not be made for follow on analysis (see Chapters Five onwards). Therefore this gene was dropped from the set of candidate marker genes.



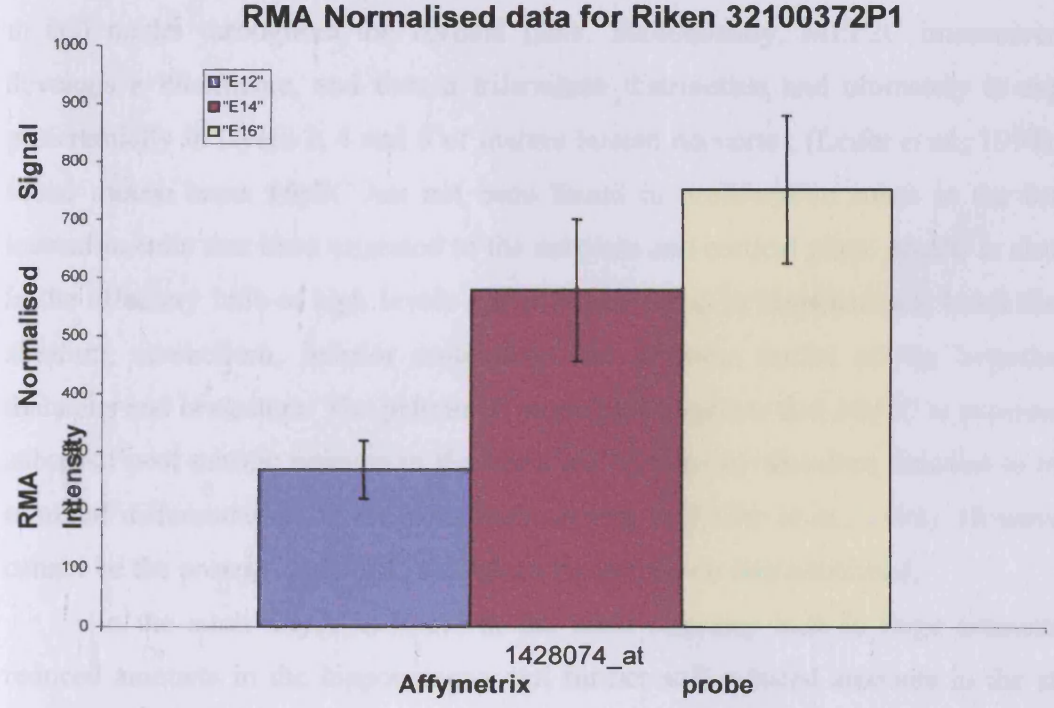


Fig. 4.22; MAS5.0 and RMA Normalised data for *Riken 3210037P1* probe.

4.4.3.7 Mef2c

The mammalian myocyte enhancer binding factor genes are members of the MADS family of transcription factors which share a 56 amino acid N-terminal region known as the MADS-box (named after the first four proteins in which it was identified: MCM1 [yeast mating-specific gene regulator], agamous and deficiens [homeotic genes regulating flower development] and serum response factor [a muscle-specific and growth factor inducible regulator]) (Leifer *et al.*, 1993; Leifer *et al.*, 1994; Leifer *et al.*, 1997; Janson *et al.*, 2001).

The MEF2 protein was originally studied in myogenic cells and differs from other MADS-box proteins by the addition of a 'MEF2-box' of 29 well conserved amino acids immediately after the MADS-box. These two domains are sufficient for DNA binding and dimerisation while the remainder of the protein is involved in transcriptional activation (Shore and Sharrocks, 1995). *Mef2C* is unique within the *Mef2* gene family in that it is only expressed in muscle, spleen and brain.

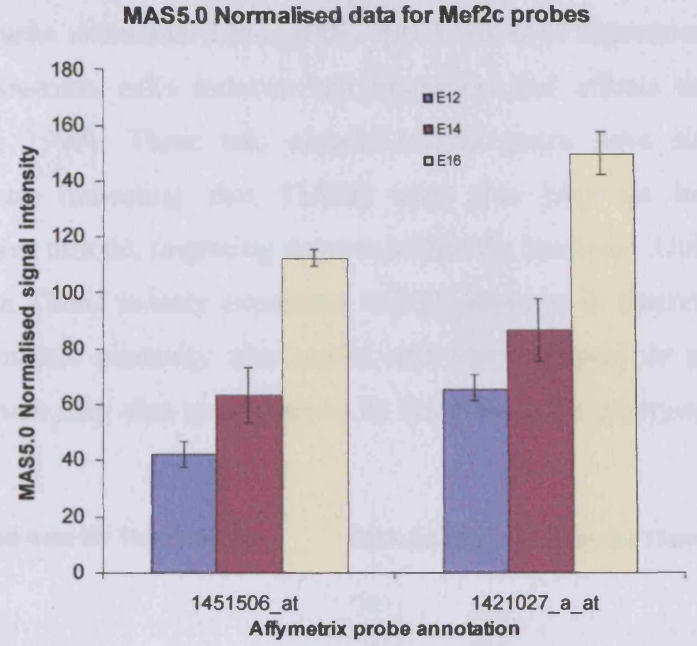
Within the brain *Mef2c* expression is found in a laminar distribution within the cerebral cortex as well as other areas. In human foetus at 14 weeks gestation it is present in cell nuclei throughout the cortical plate. Subsequently, MEF2C immunoreactivity develops a bilaminate, and then a trilaminate distribution and ultimately is expressed preferentially in layers 2, 4 and 6 of mature human neocortex (Leifer *et al.*, 1994). In the foetal mouse brain *Mef2C* has not been found in proliferative zones in the brain but instead in cells that have migrated to the subplate and cortical plate. *Mef2C* is also found in the olfactory bulb at high levels and at lower levels in hippocampus, basal forebrain, striatum, cerebellum, inferior colliculus, and in some nuclei of the hypothalamus, thalamus and brainstem. The pattern of expression suggests that *Mef2C* is expressed in a subset of post mitotic neurons in the brain and that it may therefore function to promote terminal differentiation of the cells that express it (Leifer *et al.*, 1994). However, this cannot be the proteins only task, as expression carries on into adulthood.

In the adult *Mef2c* is found in the adult olfactory bulb in large amounts, with reduced amounts in the hippocampus and further still reduced amounts in the striatum and trace expression in the thalamus (Lin *et al.*, 1996). Recent studies of *Ngn1* and *Ngn2* mutants suggest that Mef2c is a downstream transcription factor of NGN2 (Mattar *et al.*, 2004) and that although it seems that *Mash1* is actually down regulated in dorsal domains where *Ngn1* and/or *Ngn2* are expressed (Mattar *et al.*, 2004), when both *Mef2c* and *Mash1* are expressed in P19 cells the result is that the two transcription factors act

together to promote the expression of neuro-specific genes (Skerjanc and Wilton, 2000). This suggests that MEF2C, although expressed in both the cortex and more dorsal areas, is regulated by different genes in different areas and will act as a transcriptional regulator of different neuronal-specific genes depending on where it is expressed and what other transcription factors are available, hence it may play a pro-neuronal role in the developing striatum as well. Recent evidence has shown that MEF2C is regulated by Islet 1 (also a ventral forebrain marker) (Caubit *et al.*, 2005), within the anterior heart field during embryonic cardiac development (Dodou *et al.*, 2004), so it is possible that this gene is regulating *Mef2C* within the ventral telencephalon.

Human *Mef2C* has at least three different splice sites and at least 5 known splice variants. One of which includes the 'b' exon is found only in brain and not other tissues (while other variants that don't contain this sequence have been found both in the brain and in muscle) (McDermott *et al.*, 1993). MEF2C is also known to bind with other transcription factors (including b-HLH transcription factors) to form hetero and homo-dimers; these include myogenic factors and associated E-proteins. Interestingly, these interactions occur at the protein-DNA and the protein-protein level, so allowing for some potentially complex interactions to take place (Janson *et al.*, 2001).

Putative MEF2 binding sites have been found in a range of neuronal genes, including the somatostatin receptor, the NMDA-R1 receptor (NR-1), the tau microtubule associated protein and brain fatty acid binding protein. Neuro D, a transcription factor that contains an interposed MEF2 consensus sequence at nucleotides 779-788, is a bHLH transcription factor responsible for differentiation of hippocampal and cerebellar granule cells and also causes ectopic neuronal expression in *xenopus* when over expressed. MASH1 has also been shown to interact cooperatively with MEF2 proteins and E-proteins in neuronal cells (Black and Olson, 1998) and with MEF2C in P19 carcinoma cells (Skerjanc and Wilton, 2000) and it seems likely that MEF2C possibly acts with many other bHLH transcription factors as well.



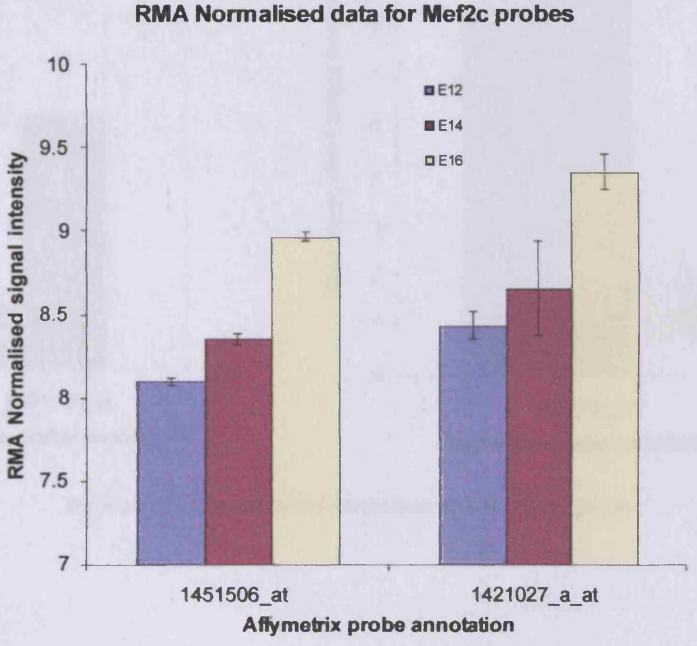


Fig. 4.23; MAS5.0 and RMA Normalised data for *Mef2c* probes.

4.4.3.8 Tiam2

Tiam2 (T-cell Lymphoma Invasion and Metastasis 2), also known as Stef (SIF and TIAM1-like exchange factor) has previously been found in the telencephalon of E13.5 mouse and also in the cerebral cortex, hippocampus and ependyma of adult mouse brains (Chiu *et al.*, 1999) . This gene is closely related to *Tiam1*, a transcript encoding a

guanine exchange factor that was first identified in a screen for genes that increased the invasiveness of T cell lymphoma lines (Habets *et al.*, 1994), is expressed during neuronal migration and neurite extension (Ehler *et al.*, 1997) and over expression of this gene in N1E-115 neuroblastoma cells induces cell migration and affects neurite outgrowth (Leeuwen *et al.*, 1997). These two closely related genes have similar embryonic expression patterns indicating that TIAM2 may also play an important role in development of post mitotic, migrating neurons within the forebrain. Unlike *Tiam1*, in the adult mouse brain *Tiam2* is only expressed in high amounts in discrete areas that also display either synaptic plasticity (the cortex and hippocampus) or neurogenesis (the dentate gyrus) which may also give clues to its function in the embryonic brain (Chiu *et al.*, 1999).

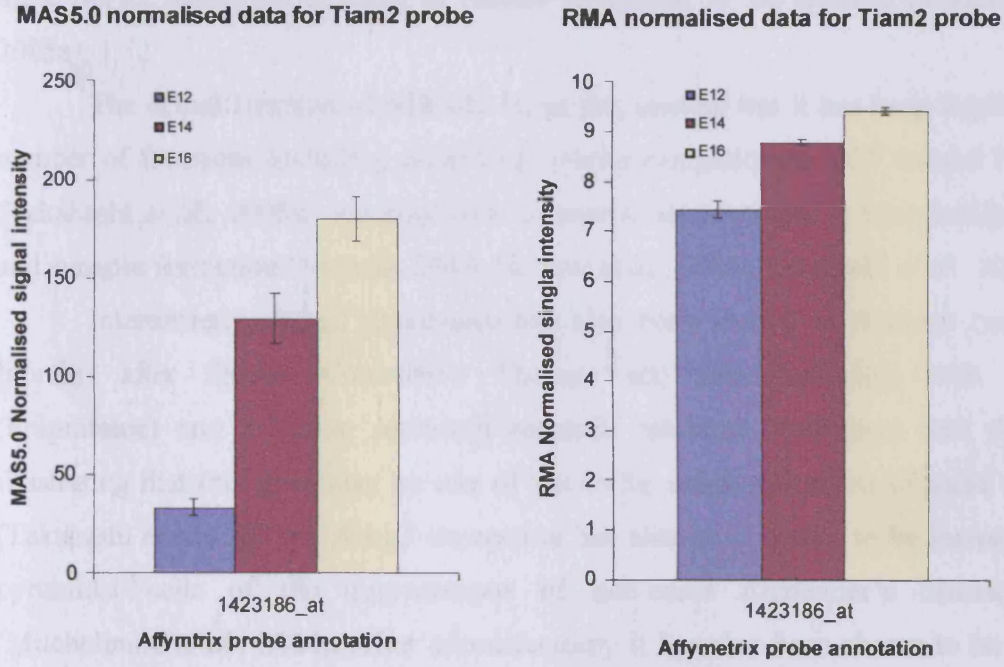


Fig. 4.24; MAS5.0 and RMA Normalised data for *Tiam2* probe.

4.4.3.9 Ndrg2

This gene is the second member of the four strong N-myc down regulated gene family that contains an α/β-hydrolase domain and shares a high degree of sequence homology.

NDRG2 itself has four isoforms (found in rat) of which none are actually repressed by the N-myc promoter (Okuda and Kondoh, 1999). NDGR2 is highly related

to N-Myc downstream-regulated protein 1, which, as well as being the best characterised protein in this family has putative links with a number of functions including stress responses, cell proliferation and differentiation as well as being reported to cause the genetic disorder called Hereditary Motor and Sensory Neuropathy-Lom (HMSN-L) (Chandler *et al.*, 2000; Kalaydjieva *et al.*, 2000).

Recent studies have shown that this gene is found throughout the brain but is predominately expressed within neurogenic regions of the brain such as the hippocampus and is localised to GFAP-positive astrocytes or radial glia (Nichols, 2003). Takahashi *et al* found that when P19 cells were treated with NGF, they began to sprout neurites. This neurite sprouting coincided with an increase in Ndrg2 mRNA and the resulting NDRG2 protein was found to localise to neurite growth cones. Furthermore, over expression of these *Ndrg2* transcripts resulted in further elongation of the neurites (Takahashi *et al.*, 2005a).

The actual function of NDGR2 is, as yet, unclear but it has been implicated in a number of functions including promoting neurite elongation in NGF treated PC12 cells (Takahashi *et al.*, 2005a) and may have a putative involvement in neural differentiation and synapse formation (Nichols, 2003; Nichols *et al.*, 2005; Takahashi *et al.*, 2005a).

Interestingly, *Ndrg2* expression has also been shown to decrease (mRNA and protein) after Electro Convulsive Therapy and after treatment with Tri-cyclic (imipramine) and selective serotonin reuptake inhibitor (sertraline) anti depressants illustrating that this gene may be one of the so far unknown targets of these treatments (Takahashi *et al.*, 2005b). *Ndrg2* expression has also been shown to be increased in the pyramidal cells of the hippocampus of late-onset Alzheimer's disease patients (Mitchelmore *et al.*, 2004). After adrenalectomy it has also been shown to be under the positive influence of corticosteroids (Nichols *et al.*, 2005). Its function in diseases such as Alzheimer's could be as part of a regulatory process in a response to an accumulation of mis-folded proteins within the cells in question; perhaps to chaperone these proteins away from the nucleus or take part in their degradation, or the increases in expression of this gene may be as a result of an epigenetic deregulation as part of the disease process (Mitchelmore *et al.*, 2004).

Expression of *Ndrg2* has been reported to be developmentally dynamic, being generally lower in the early stages of development and markedly increasing during later stages of embryonic development (Hu *et al.*, 2006).

The fact that it has been implicated in neural differentiation and synapse formation and is up regulated at the time of maximum proliferation of striatal cells suggests that this gene has important functions within the striatum during this period, also making it a worthwhile candidate for further investigation.

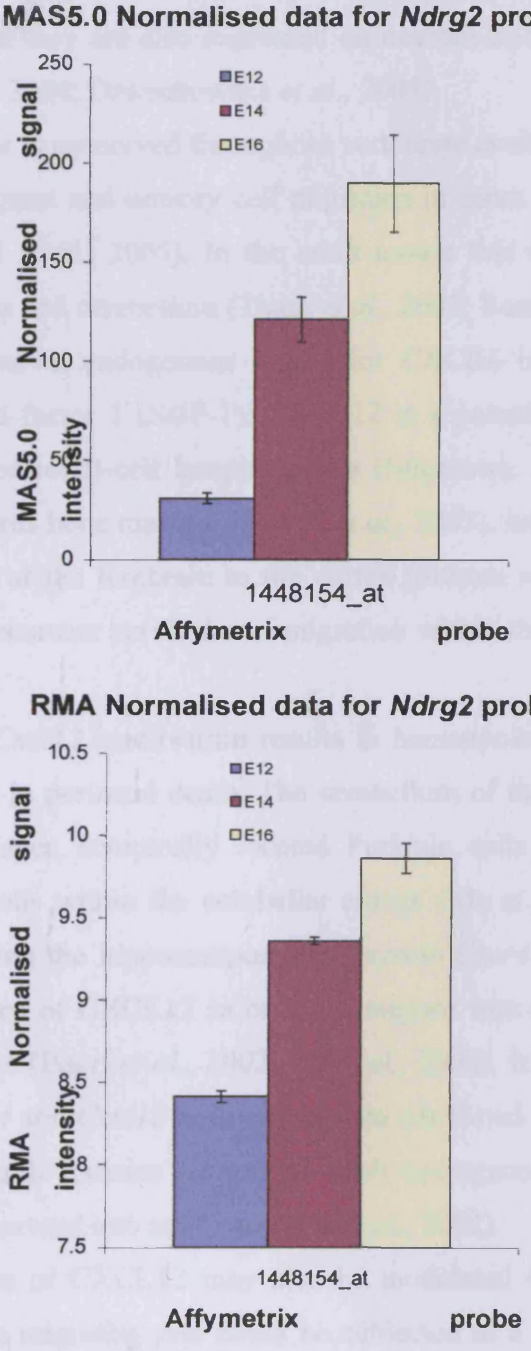


Fig. 4.25; MAS5.0 and RMA Normalised data for *Ndrg2* probe.

4.4.3.10 Cxcr4

This gene codes for The CXCR4 receptor; a seven trans-membrane domain chemokine receptor belonging to the G protein coupled receptor family that is also the co-receptor for the HIV glycoprotein gp120 (Lin *et al.*, 2003). It was first thought that this receptor was only expressed by glial cells (Banisadr *et al.*, 2002) but it has now been widely accepted that they are also expressed on neurons and oligodendrocytes and their precursors (Ji *et al.*, 2004; Dziembowska *et al.*, 2005).

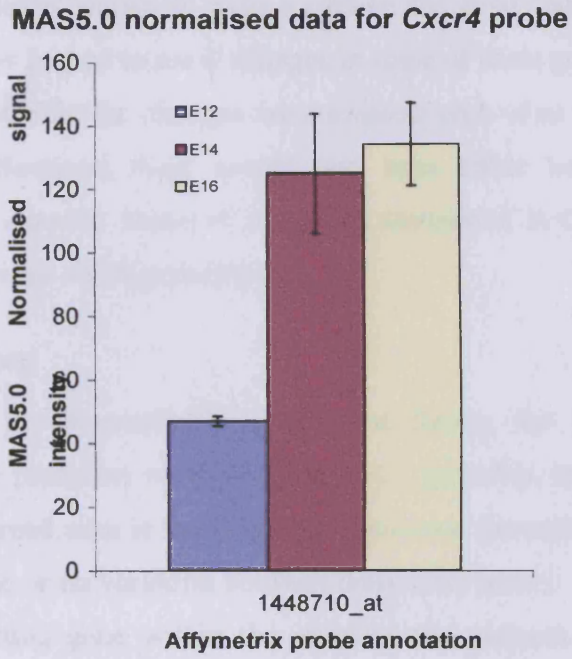
This receptor is conserved throughout vertebrate evolution as it has been found to control primordial germ and sensory cell migration in zebra fish (Doitsidou *et al.*, 2002; Li *et al.*, 2004a; Li *et al.*, 2005). In the adult mouse this receptor is expressed in the cortex, hippocampus and cerebellum (Tham *et al.*, 2001; Banisadr *et al.*, 2002).

The only known endogenous ligand for CXCR4 is CXCL12 (formally called stromal cell-derived factor 1 (SDF-1). CXCL12 is a potent leukocyte chemo-attractant that is known to control B-cell lymphopoiesis (Nagasawa, 2002), heamatopoietic stem cell migration towards bone marrow (Egawa *et al.*, 2001), interneurons that migrate from the ventral regions of the forebrain to the cortex (Stumm *et al.*, 2003a) and neural and oligodendrocyte precursors survival and migration within the striatum (Dziembowska *et al.*, 2005).

Cxcr4 and *Cxcl12* inactivation results in haemopoietic and CNS defects (Ma *et al.*, 1998) resulting in perinatal death. The cerebellum of these mutants has an irregular external granular layer, ectopically located Purkinje cells and clumps of abnormally migrated granule cells within the cerebellar anlage (Ma *et al.*, 1998). Dentate granular cell precursors within the hippocampus also express *Cxcr4* and require, amongst other factors, the influence of CXCL12 in order to migrate into a proper position within the dentate gyrus anlage (Bagri *et al.*, 2002; Lu *et al.*, 2002). It has been postulated that, as expression of *Cxcr4* and *Cxcl12* both extend into adulthood within the hippocampus and the hippocampus itself remains an area of adult neurogenesis, the role of CXCR4 and CXCL12 may also extend into adulthood (Lu *et al.*, 2002).

The function of CXCL12 may also be modulated by other long range chemo-attractants so that a migrating cell could be subjected to a number of different signals, each containing separate pieces of information about the pathway it is migrating along (Lazarini *et al.*, 2003). The response of glia and neurons to CXCL12 may also be different, as although some cortical neuronal precursors expressing *Cxcr4* respond to the chemo-attractant, they do not respond to the HIV glycoprotein GP120, whereas glial cells

responded to either signal in the same way. However, in differentiated cortical neurons a response was seen from both CXCL12 and GP120 to influence neuronal function (Lazarini *et al.*, 2000).



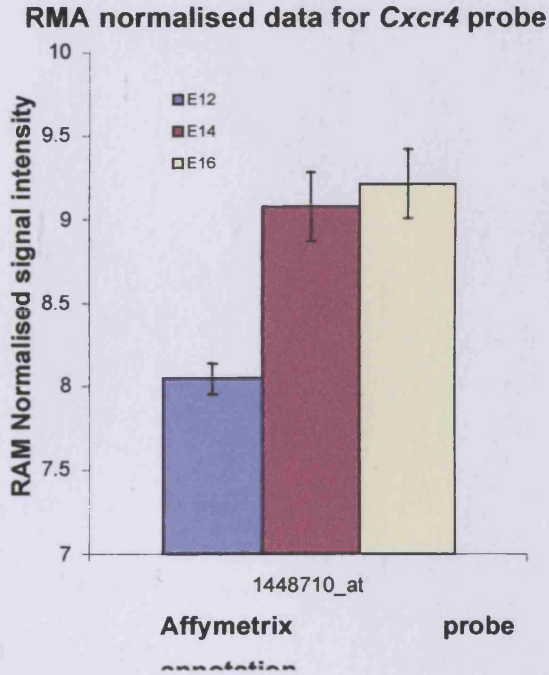


Fig. 4.26; MAS5.0 and RMA Normalised data for *Cxcr4* probe.

4.4.4 Known markers of striatal development.

As discussed in Chapter One, there are a number of genes that are have already been shown in other studies to be expressed during striatal development. Some of these genes have been clearly shown to have a role on the developmental process, but some haven't. Here I have looked to see if changes in some of these genes have been picked up in my screen and whether the changes are consistent with what would be predicted from previous studies. However, these results may have either less fold change or more variances between samples. Some of the genes mentioned in Chapter One are also not contained on the Mouse 430A genechip:

4.4.4.1 Sonic Hedgehog

This gene is comparatively unchanged during the period assayed. *Shh* is represented by one probe set on the Mouse 430A genechip, and although the data has wide variance the trend seen is for very low expression throughout the three time points tested. There is little or no variation between these time points. This is to be expected, as *Shh* is an early acting gene within the striatum that induces the expression of other important transcription factor that induce development of the MGE and LGE (see Chapter One).

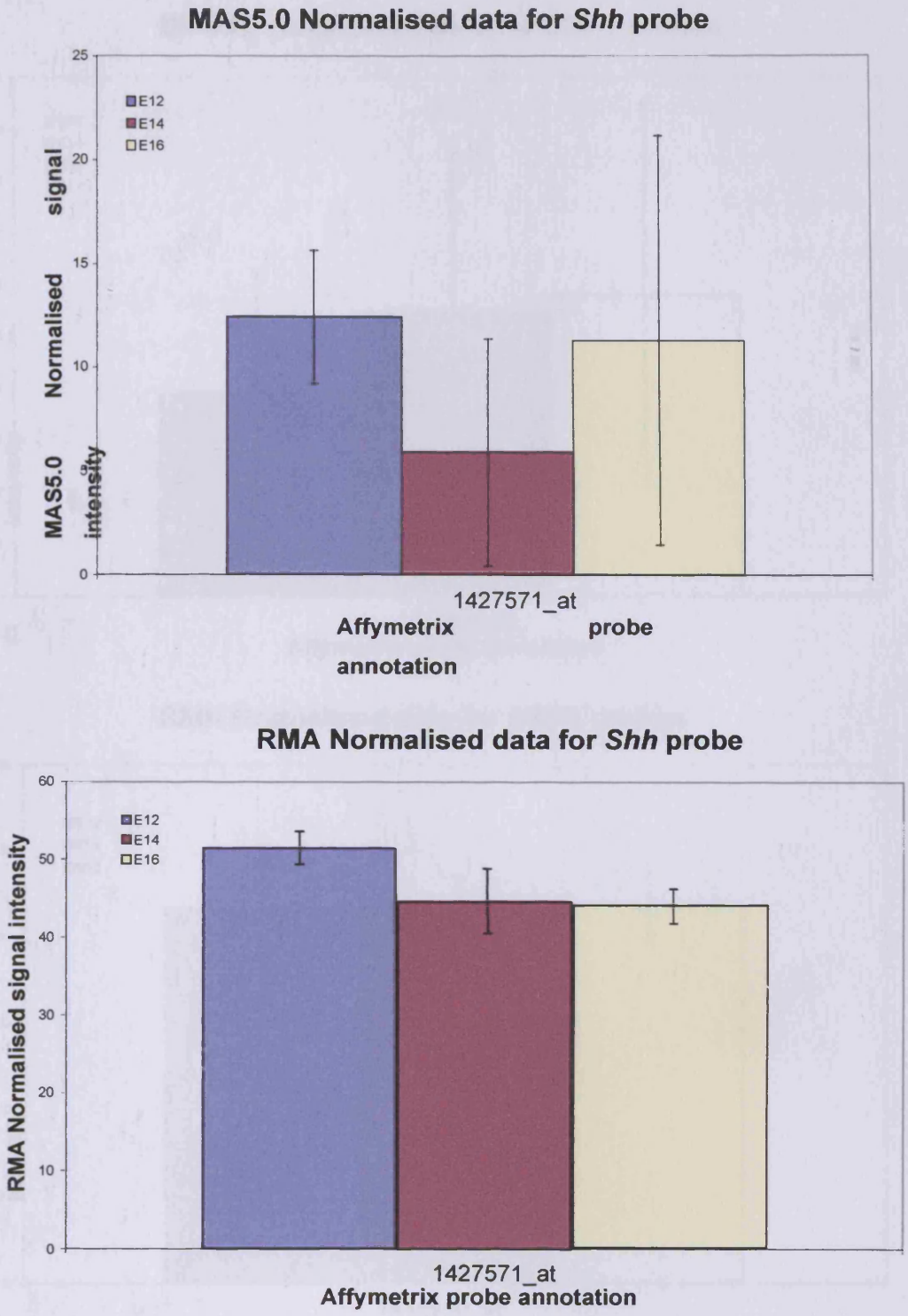
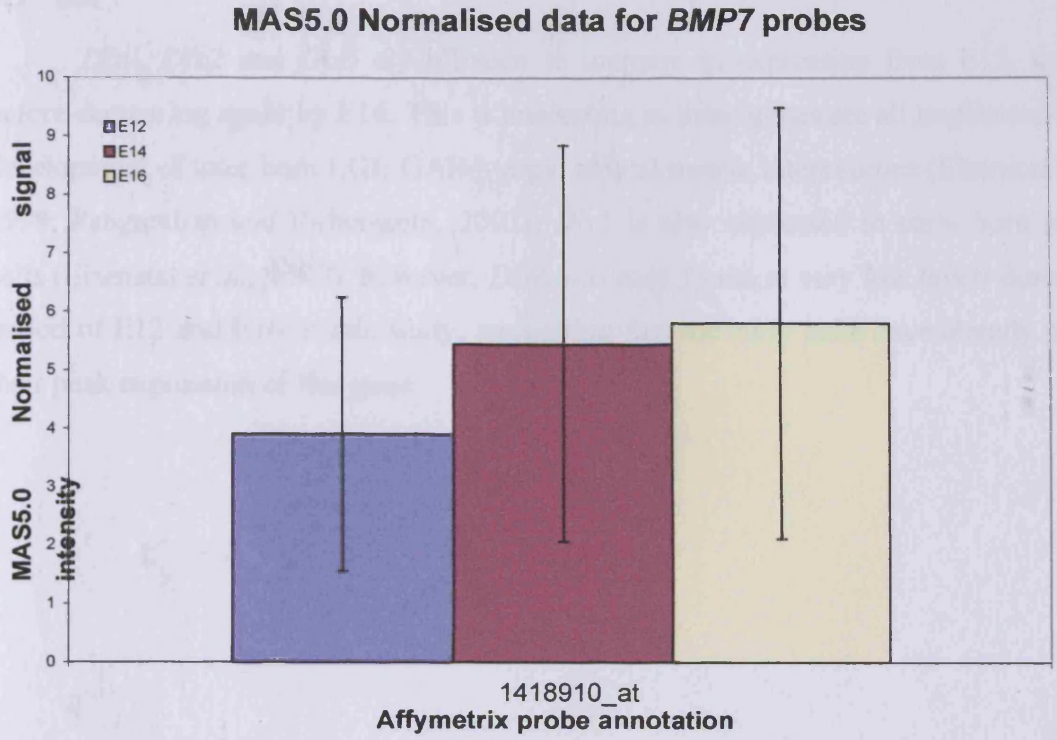


Fig. 4.27; Bar charts showing MAS5.0 and RMA normalised data for *Shh*

4.4.4.2 BMPs

Bmp7 is found to be expressed at very low concentrations throughout the period of study; again there are no surprises in this as this gene is active at much earlier stages of development (Dale *et al.*, 1999).



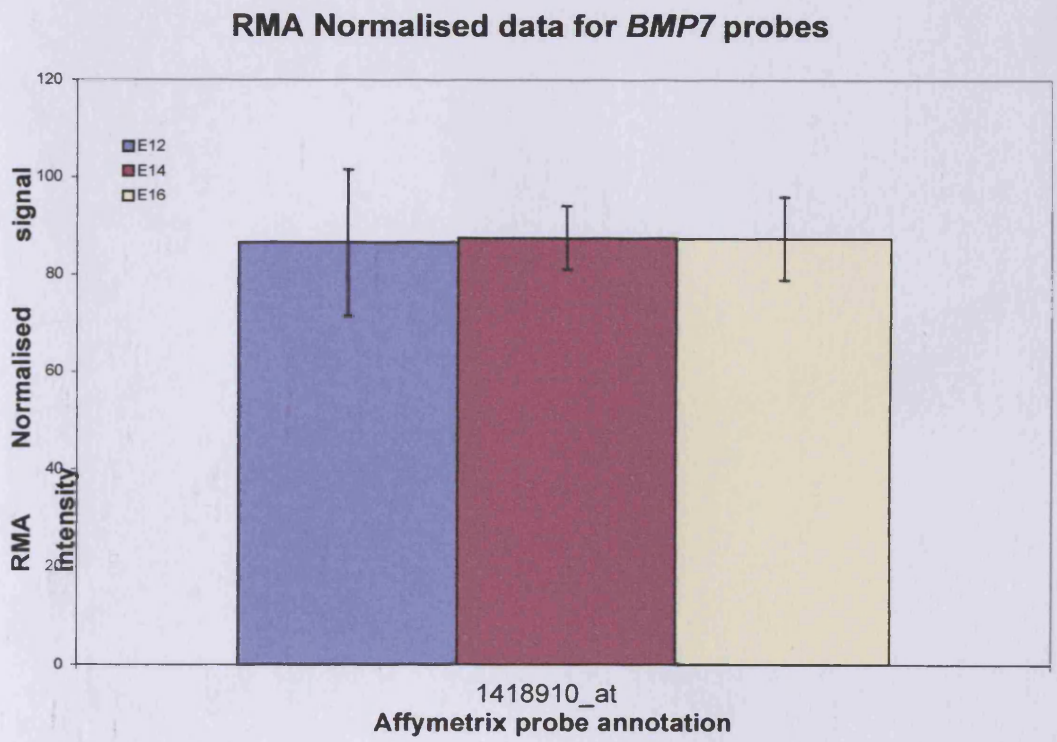
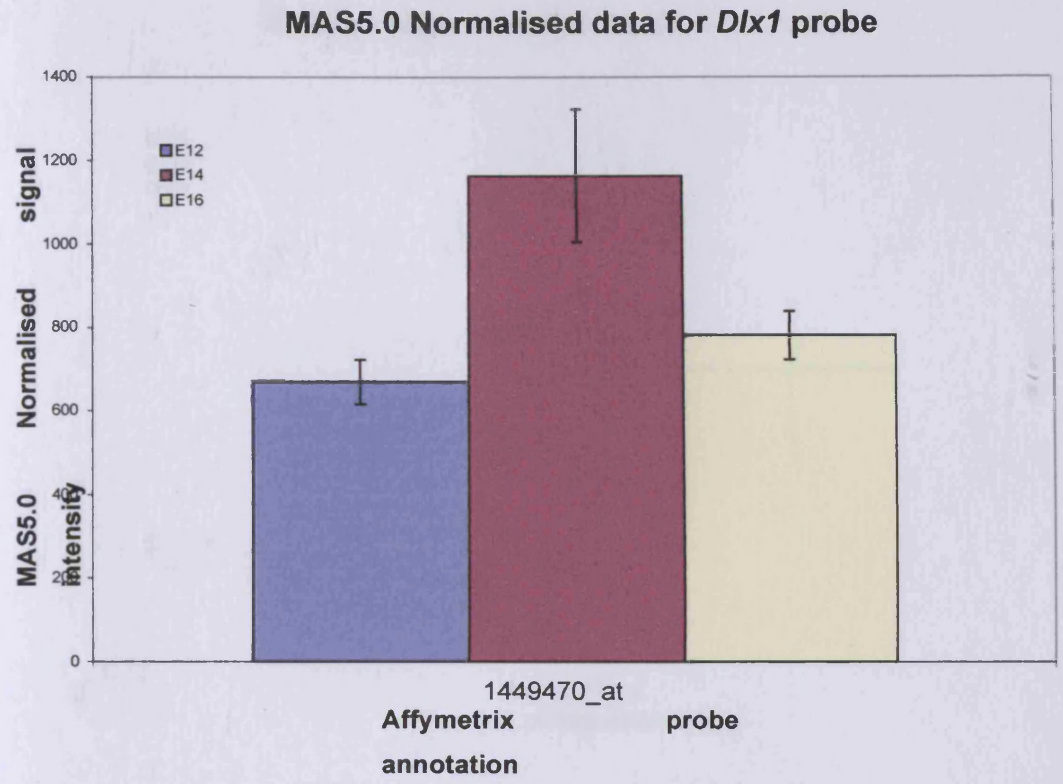


Fig. 4.28; Bar charts showing MAS5.0 and RMA Normalised data for *Bmp7*

4.4.4.3 Dlx

Dlx1, Dlx2 and *Dlx5* are all seen to increase in expression from E12, to E14, before decreasing again by E16. This is interesting as these genes are all implicated in the development of later born LGE GABA-ergic striatal matrix interneurons (Eisenstat *et al.*, 1999; Panganiban and Rubenstein, 2002). *Dlx5* is also expressed in early born striatal cells (Eisenstat *et al.*, 1999), however, *Dlx6* was only found at very low levels during the period of E12 and E16 in this study, suggesting that the early cells have already passed their peak expression of this gene.



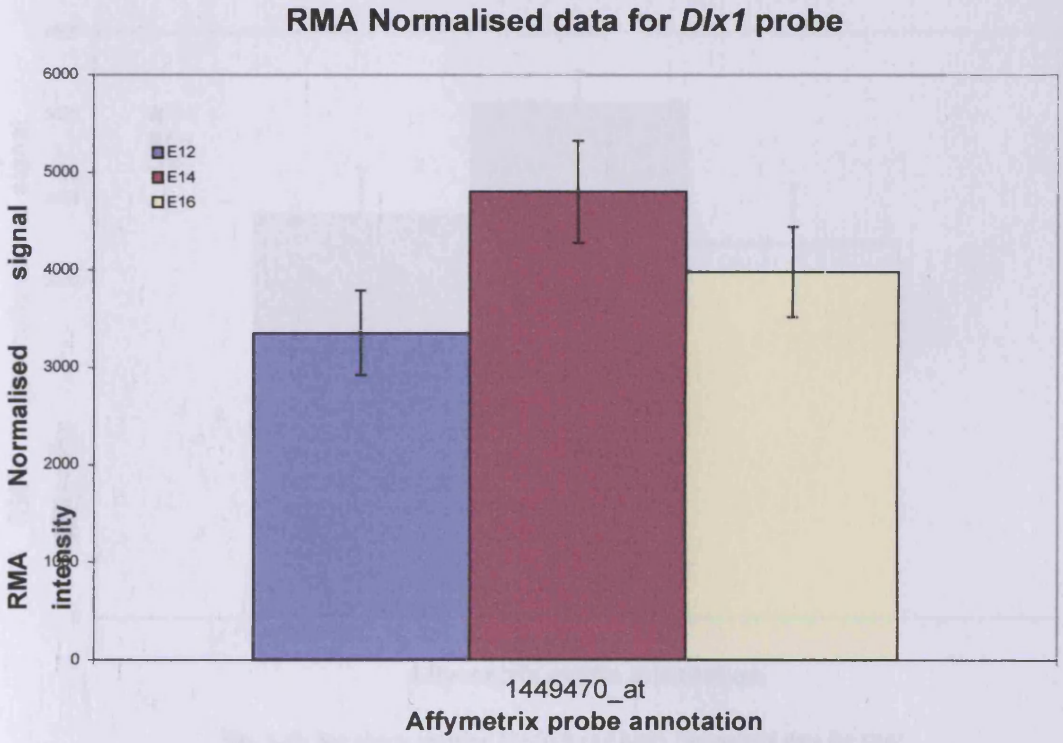
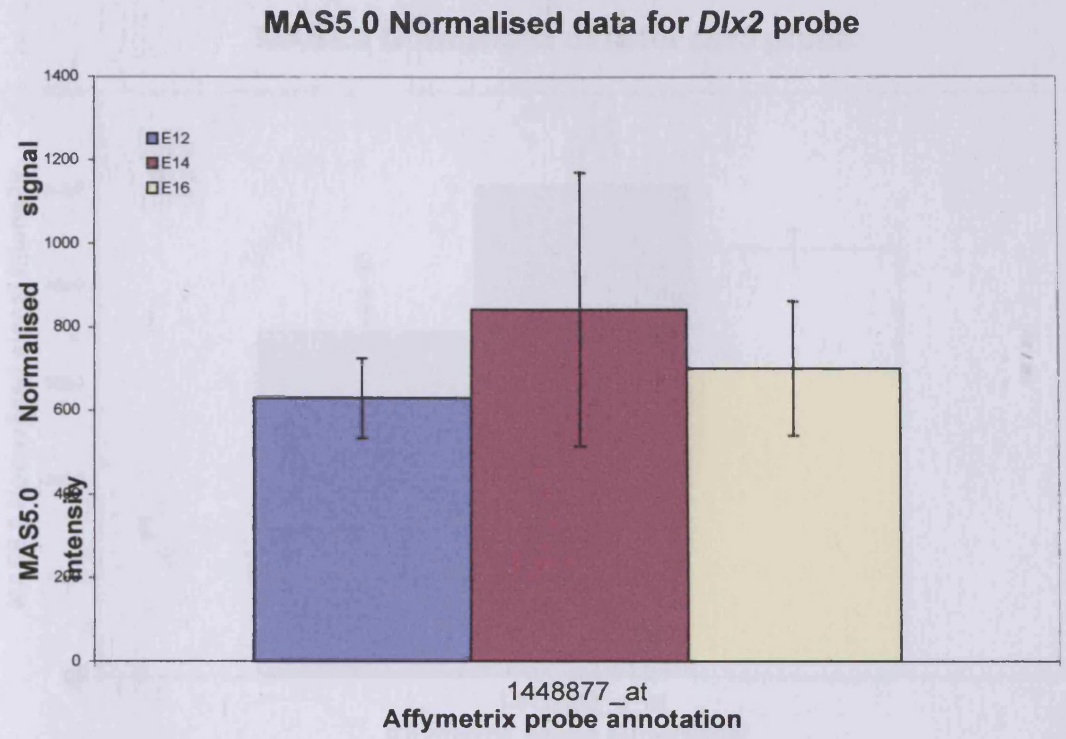


Fig. 4.29; Bar charts showing MAS5.0 and RMA Normalised data for *Dlx1*



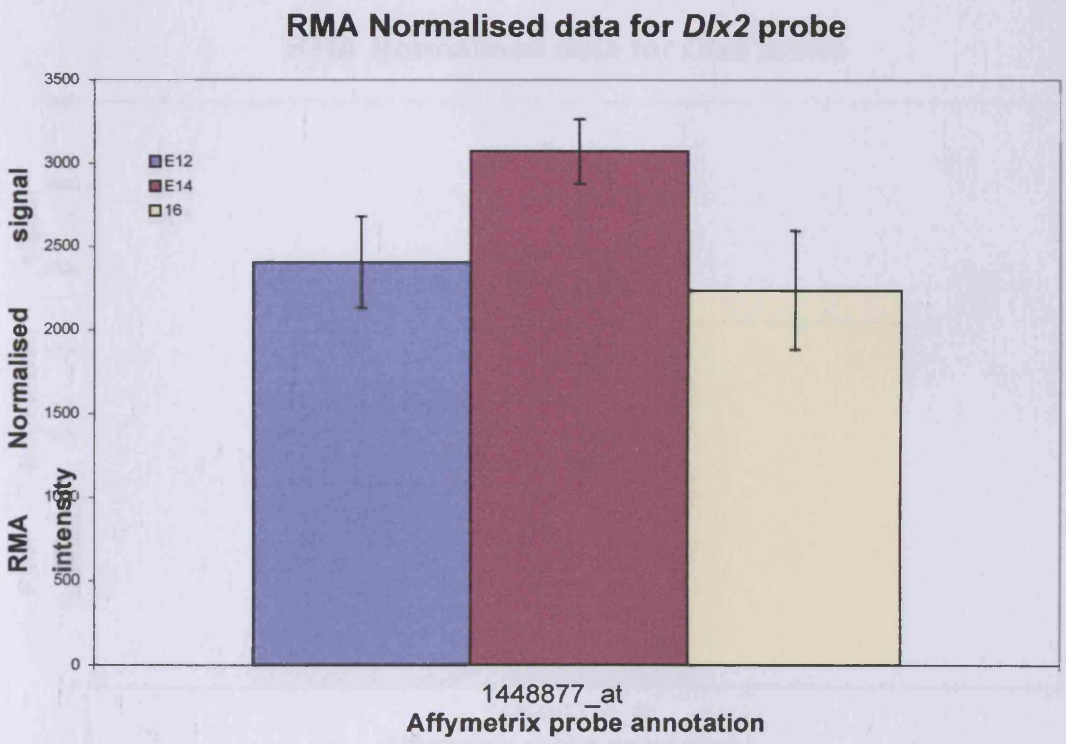
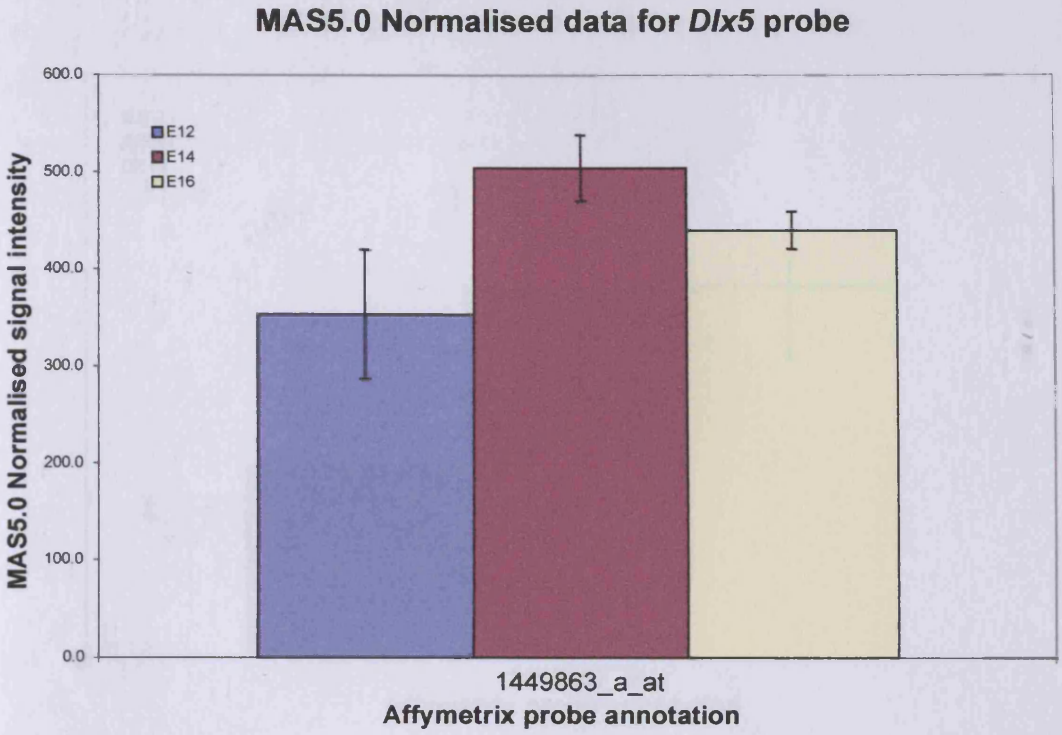


Fig. 4.30; Bar charts showing MAS5.0 and RMA Normalised data for *Dlx2*



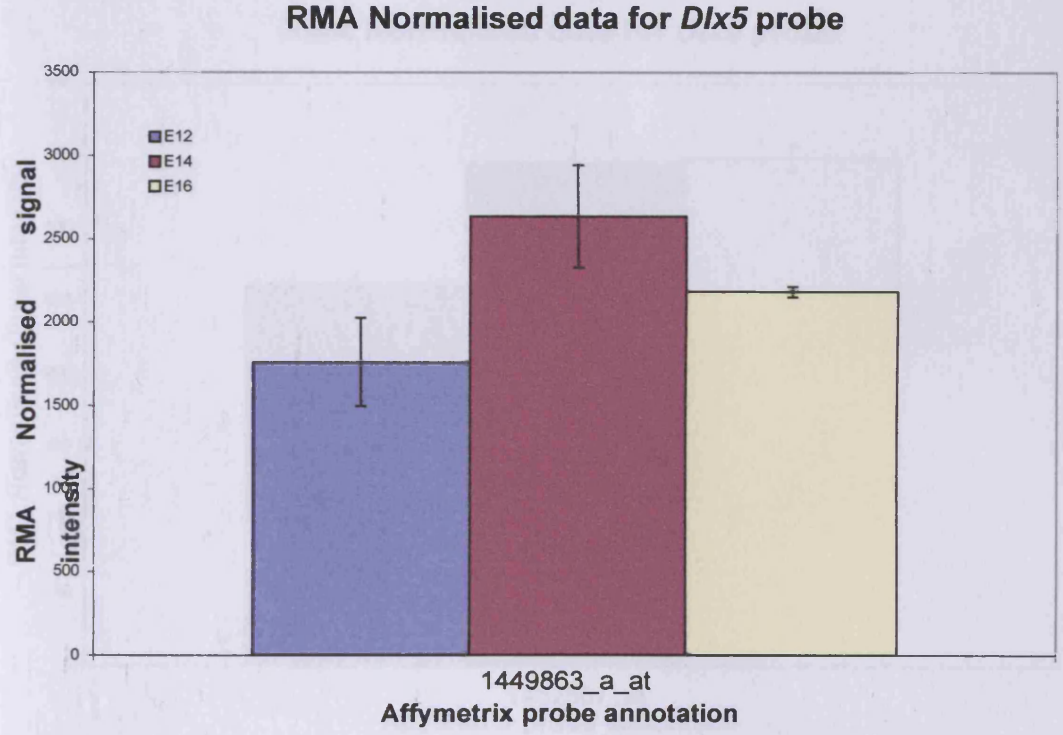


Fig. 4.31; Bar charts showing MAS5.0 and RMA Normalised data for *Dlx5*

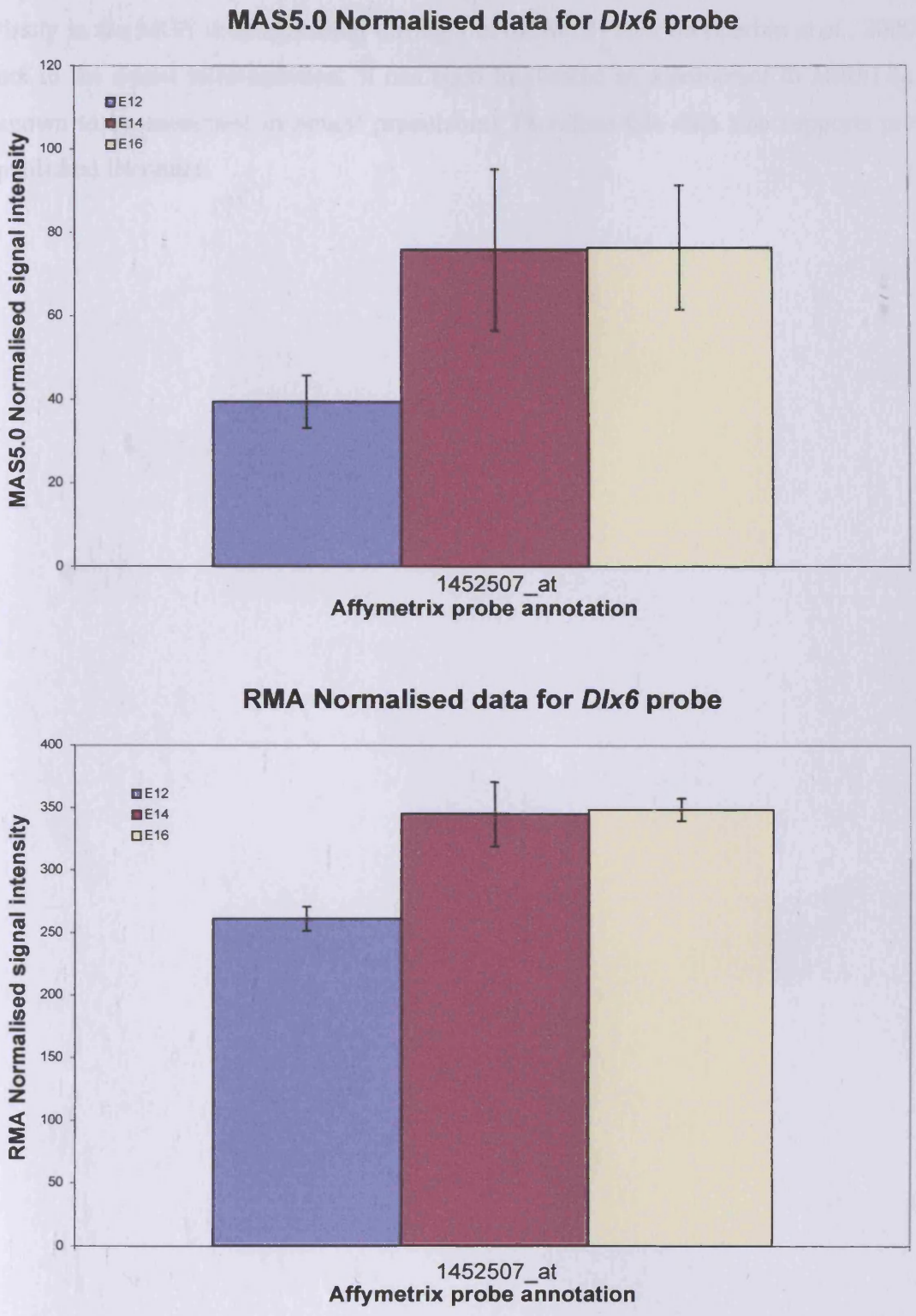


Fig. 4.32; Bar charts showing MAS5.0 and RMA Normalised data for *Dlx6*

4.4.4.4 Gsh2

This gene, heavily implicated in the development of neuronal precursors in the MGE and the timing of production of precursors in the LGE (Casarosa *et al.*, 1999; Kriks

et al., 2005) is highly active earlier on between the ages of around E10.5 and E12.5, firstly in the MGE then spreading throughout the WGE later on (Corbin *et al.*, 2000), but not in the dorsal telencephalon. It has been implicated as a precursor to *Mash1* (a gene known to be expressed in neural precursors) Therefore this data also supports previous published literature.

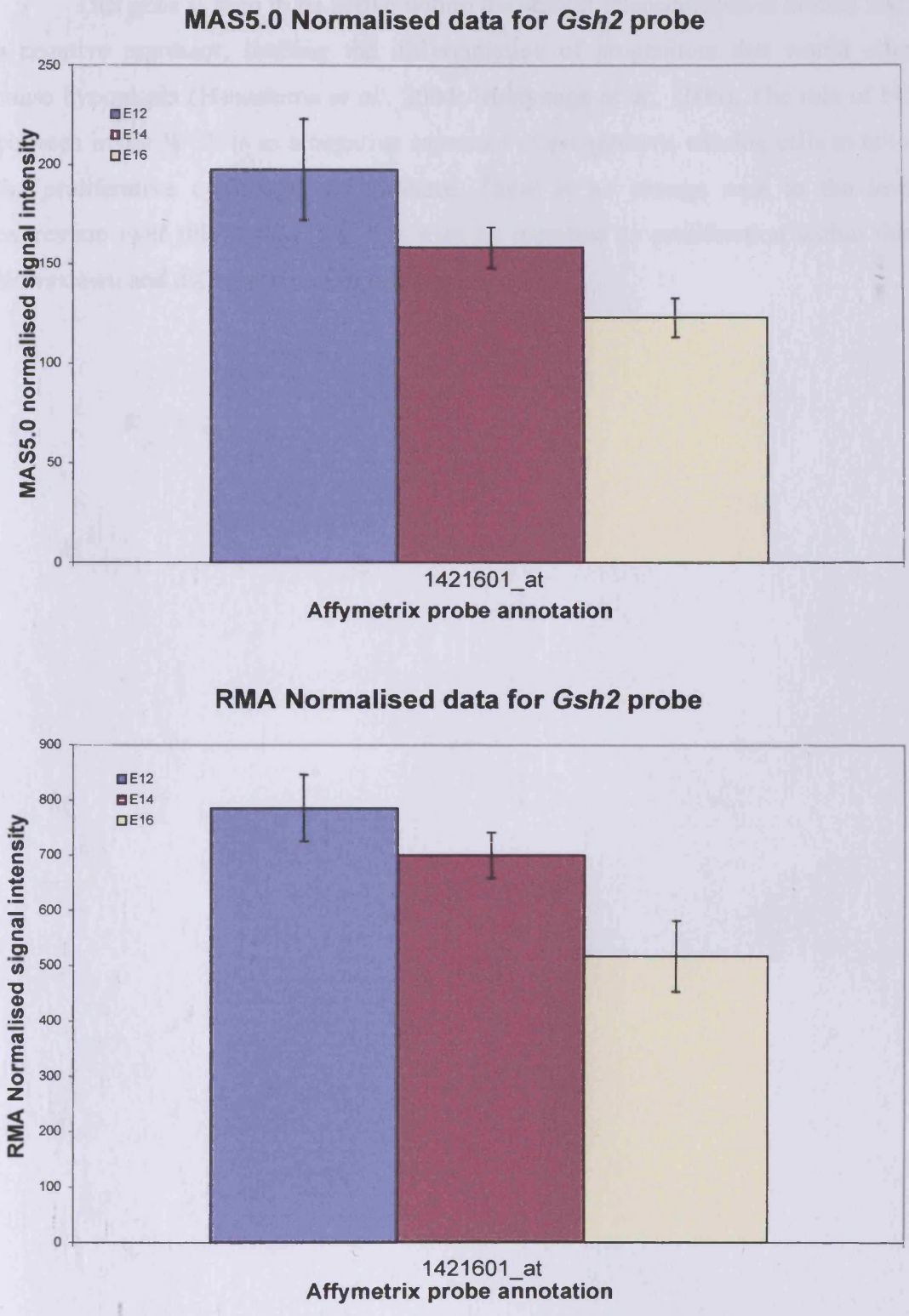
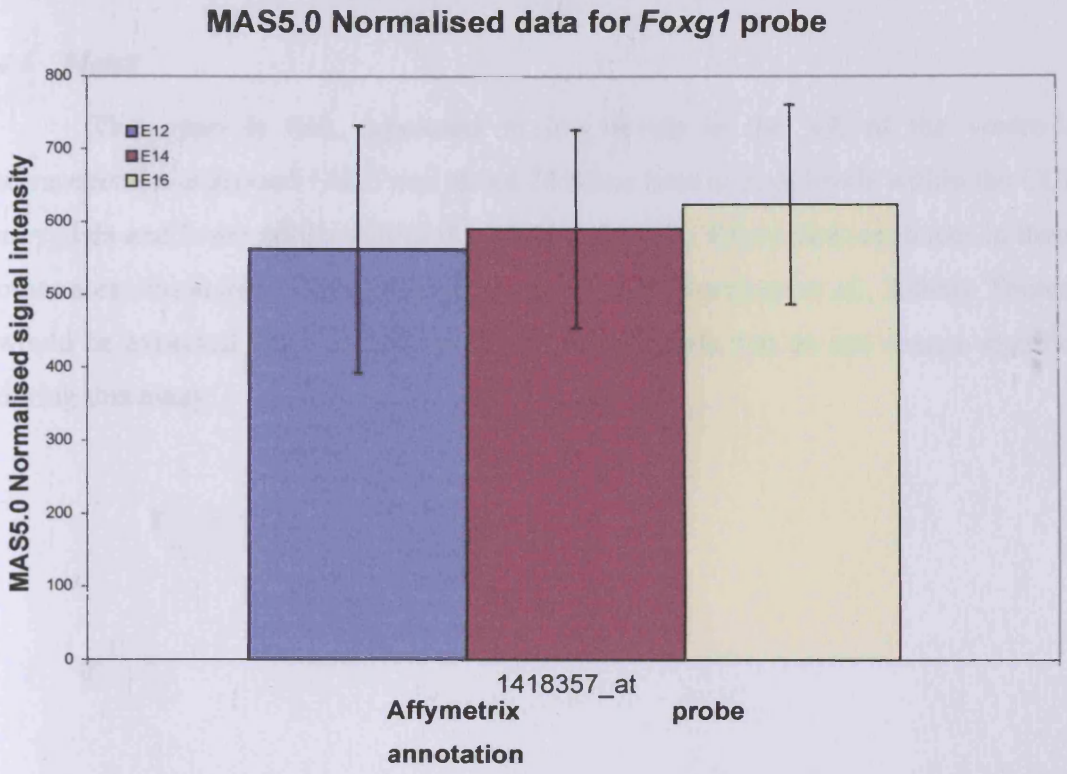


Fig. 4.33; Bar charts showing MAS5.0 and RMA Normalised data for *Gsh2*

4.4.4.5 Foxg1

This gene is seen to be active within the ventral telencephalon at around E8.5-9 as a negative repressor, limiting the differentiation of progenitors that would otherwise cause hypoplasia (Hanashima *et al.*, 2004; Martynoga *et al.*, 2005). The role of FOXG1 between in the WGE is as a negative repressor of progenitors, causing cells to fall out of the proliferative cycle and differentiate. There is no change seen in the levels of expression over this period, and this may be expected as proliferation within this area slows down and differentiation of these cells occurs.



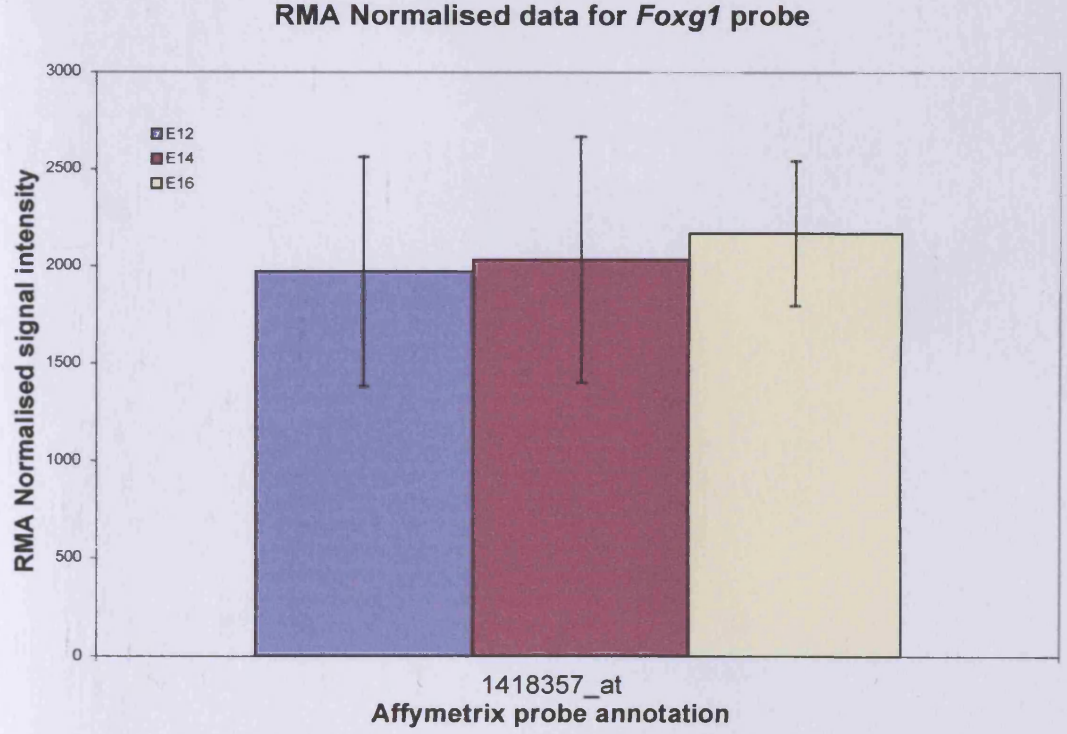
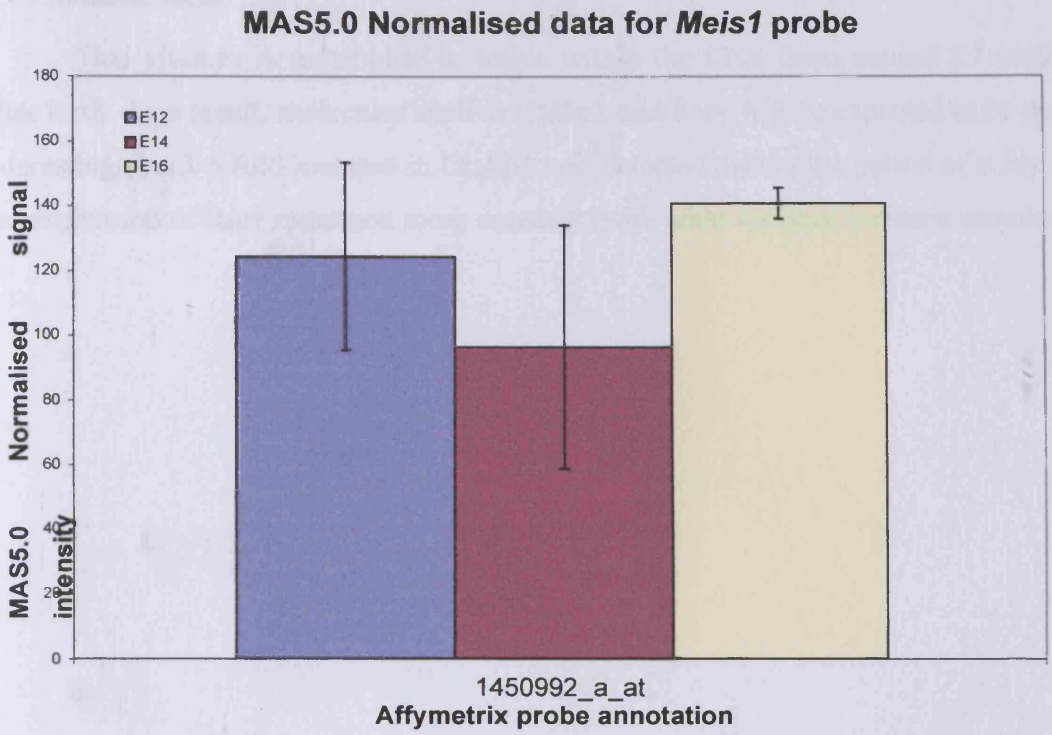


Fig. 4.34; Bar charts showing MAS5.0 and RMA Normalised data for *Foxg1*

4.4.4.6 Meis1

This gene is first expressed in low levels in the VZ of the ventro-lateral telencephalon at around E10.5 and about 24 hours later in high levels within the CGE and amygdala and lower levels within the MGE and LGE. Expression continues in these and other areas throughout development into adulthood (Toresson *et al.*, 2000a). Therefore it would be expected that this gene is found at low levels that do not change significantly during this assay.



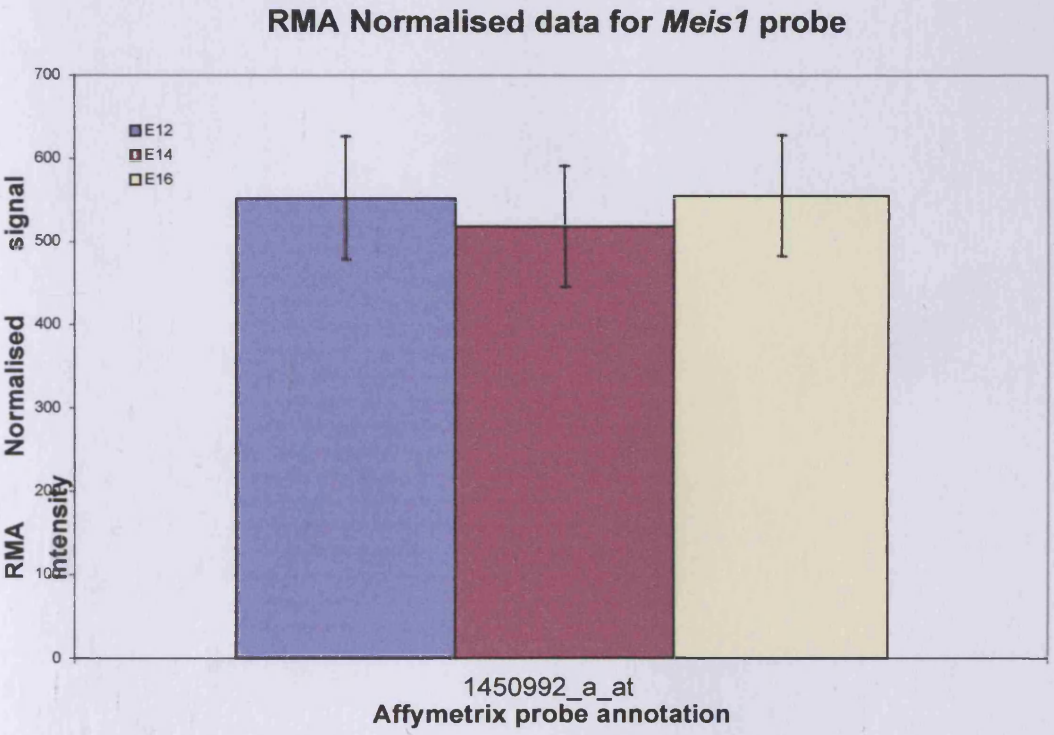


Fig. 4.35; Bar charts showing MAS5.0 and RMA Normalised data for *Meis1*

4.4.4.7 Retinoic Acid

This vitamin A metabolite is active within the CNS from around E7 until well after birth. As a result, molecules such as Crabp1 and Rarγ will be expected to be present. Interestingly, a 3-5 fold increase in Crabp1 was detected during the period of study while the expression of Rarγ remained more constant (with wide variance between samples).

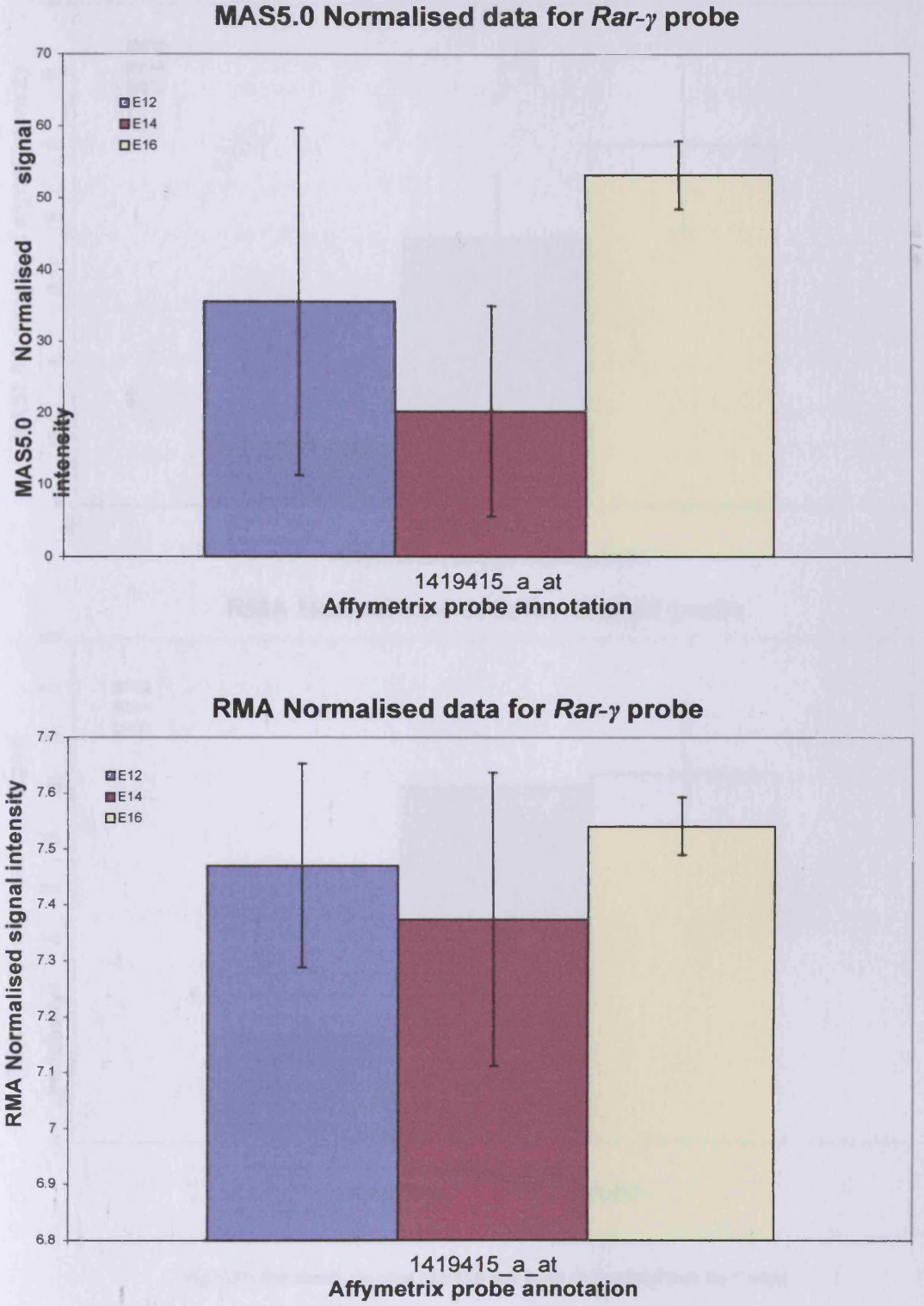


Fig. 4.36; Bar charts showing MAS5.0 and RMA Normalised data for *Rar-γ*

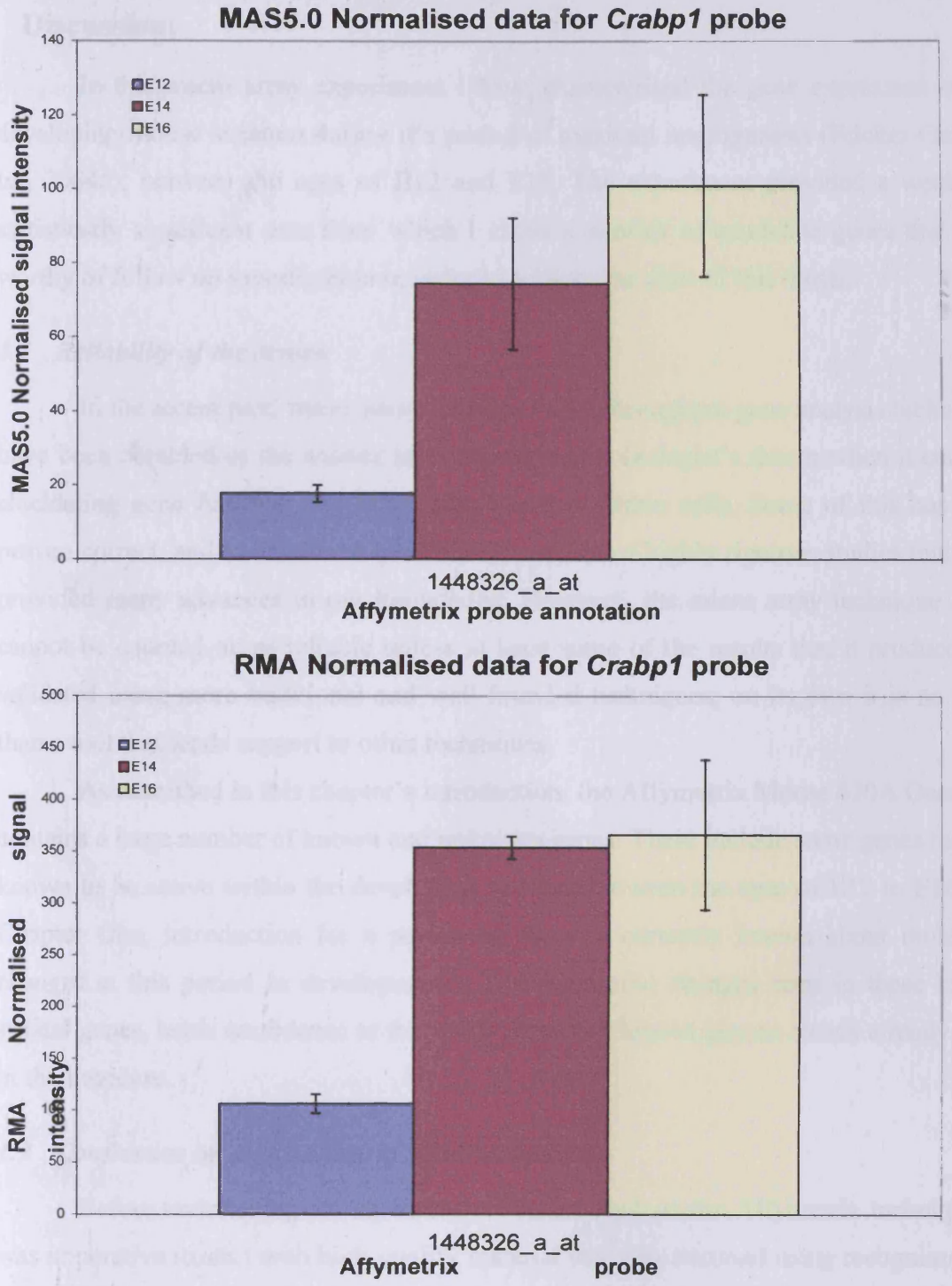


Fig. 4.37; Bar charts showing MAS5.0 and RMA Normalised data for *Crabp1*

4.5 Discussion;

In this micro array experiment I have characterised the gene expression of the developing mouse striatum during it's period of maximal neurogenesis (Fricker-Gates *et al.*, 2004a), between the ages of E12 and E16. The experiment provided a wealth of statistically significant data from which I chose a number of candidate genes that were worthy of follow up investigation in order to achieve the aims of this thesis.

4.5.1.1 Reliability of the screen

In the recent past, micro array and other high throughput gene analysis techniques have been heralded as the answer to every molecular biologist's dream when it came to elucidating gene function and expression changes within cells. Some of this has been proven correct, and to date there have been a number of highly rigorous studies that have provided many advances in our knowledge. However, the micro array technique alone cannot be counted on as reliable unless at least some of the results that it produces are validated using more traditional and well founded techniques; on its own it is no more than a tool that lends support to other techniques.

As described in this chapter's introduction, the Affymetrix Mouse 430A Genechip contains a large number of known and unknown genes. These include some genes that are known to be active within the developing striatum between the ages of E12 to E16 (see Chapter One, introduction for a review of what is currently known about molecular changes at this period in development). The expression changes seen in these known striatal genes, lends confidence to this study, showing known genetic trends already cited in the literature.

4.5.1.2 Confidence in good quality of RNA/techniques

Before undertaking an expensive procedure such as the Affymetrix technique, it was imperative to start with high quality material that was obtained using recognised and reproducible techniques. Therefore a lot of time went into choosing the right techniques of dissection (training and regular checking of my dissectional accuracy by an experienced observer), training in RNA extraction and the selection of the right micro array platform (Affymetrix) and Genechip (Mouse430A). The quality of RNA and the levels of quality control throughout the micro array process were therefore of a very high standard. Only one of the Genechips (E14b) failed to achieve the high levels of quality control (after it was found a small 'crop circle' irregularity had occurred on the actual

chip during the hybridisation process which would have affected the results) so was repeated. As such, I have confidence in the quality and reproducibility of the results achieved from this experiment.

4.5.1.3 Sources of error.

All the data was scaled and normalised (see methods section above for details) to correct for variations between the arrays. These variations could have arisen from two main sources;

- Biological (potentially interesting in this study) variation may arise from many sources including dissection time (minimised by limiting it to within a two hour window between 06:00 and 08:00 am), species variation (minimised by using only CD1 (ICR) mice from one supplier), variation between animals, ages of dams (these were all of similar/same age), sex of foetus (this was not controlled for due to difficulties in sexing at this age) or growth conditions (minimised here by housing all animals in the same conditions in adjacent cages).
- Technical (obscuring) variation (Hartemink *et al.*, 2001) could have arisen from experimental variables such as reagents, handling errors and quality of targets hybridised.

Of course, some sources of error cut across the boundaries, for example, the accuracy of dissection (discussed fully in Chapter Three).

The minimisation of variation was essential and was catered for in the initial design of the experiment; after this, scaling and normalisation provided another means to remove differences and facilitate comparison analysis. However, by only taking the genes that were found to be significant in both normalisation methods I may have missed some important genes. This is an intrinsic problem of the approach employed here. Of course, further validation was also performed using alternative experimental strategies and this is elaborated in the following chapter (Chapter Five).

4.5.1.4 The decision to analyse the E12 to E16 gestational window.

As with gene array experiments in general, this experiment produced a huge amount of data. In this thesis I have been able only to follow up a very small proportion of the information produced. Given the enormous volume of data, I selected the most prominent genes with the largest expression changes and the smallest variances between samples. The data that this experiment has provided is fully MIAME compliant, and as

such will be published on the web when any literature that arises from this work is published for acknowledgement. The data is already serving as a resource with a number of the up-regulated genes being actively assessed for functionality by other researchers. These studies will not be discussed further here.

The up-regulated genes selected from the screen are validated using PCR and in situ hybridisation and are discussed further in the following chapter.

Chapter Five

5 Validation of Micro Array using *In Situ* Hybridisation.

5.1 Introduction

In the previous chapter I described a series of micro array experiments in which a number of genes were found to be significantly up-regulated between the ages of E12 and E16 in the embryonic mouse striatum.

Due to the large numbers of genes assayed in each series of experiments, there is a chance that 'false positives' can appear in the data. To make sure the chances of this were minimal, the Affymetrix Mouse 430A chip was chosen, as it has been proven to show the least false positives (below 3% at a p value of 0.01) (Woo *et al.*, 2004). However, because this gene chip has over 22,000 genes on it, this still allows for an estimated 660 false positives to have been made in each experiment. This is controlled for by running gene chip replicates, which was done. However, due to the high cost of each micro array experiment the numbers of these replicates were limited to three replicates.

Using Affymetrix gene chips allowed me to obtain highly reproducible results, however, as with any technique its efficacy can only be validated when compared to similar results using tried and tested methods.

In order to validate the findings of the micro array experiments described in Chapter Four different methods of visualising gene expression changes were required. Furthermore, these alternative techniques also yield additional information about the candidate genes in question. It was decided to use semi-quantitive PCR and *in* situ hybridisation to gauge which of the genes were worthy of further interests and analysis.

PCR was used to confirm the gene array findings and also to assess whether the candidate genes in question were also expressed in neighbouring areas of brain, specifically, the neocortex.

In situ hybridisation was then used to analyse those genes that passed this initial assessment, to further confirm whether they were up regulated over the period in question and also to provide spatial information. Specifically, whether the candidate genes were expressed within the ventricular zone (VZ), or the sub-ventricular zone (SVZ) or within the maturing striatum itself (all will be included in the dissection of the striatum). The location of cells expressing the candidate gene may give clues as to whether these genes

are relevant to proliferation, migration, differentiation, maturation or normal activity seen within maturing cells within different parts of the developing brain (see Fig. 5.1).

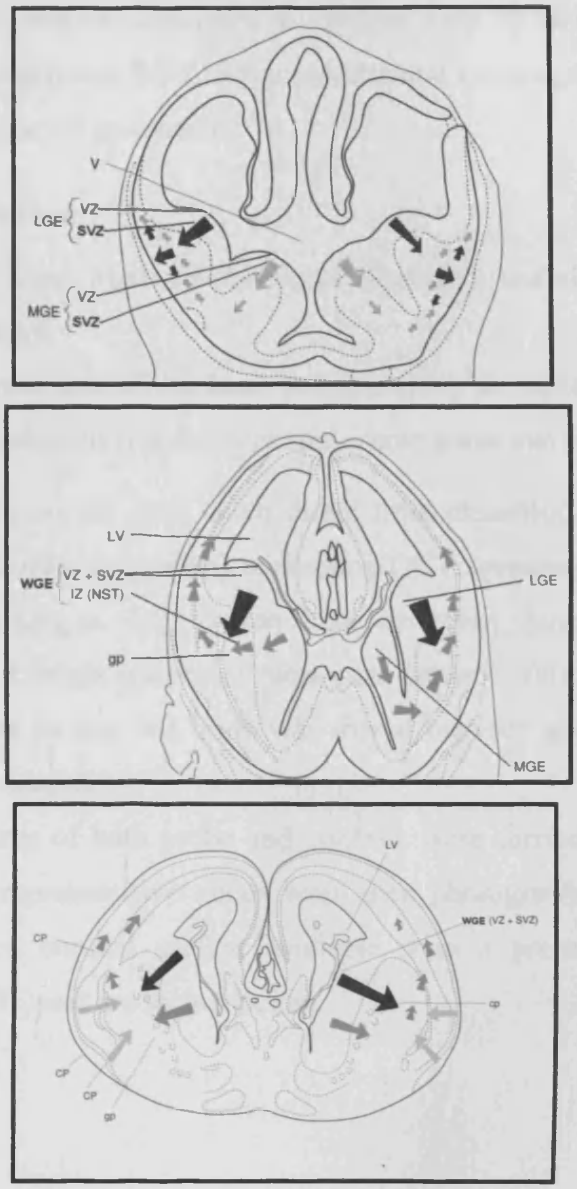


Fig. 5.1; Coronal sections around the level of the WGE at E12 (top), E14 (middle) and E16 (bottom). These diagrams depict the proliferation areas (**SVZ**=Sub Ventricular Zone, **VZ**=Ventricular Zone) and directions of migration of cells from the LGE (black arrows) that give rise to neurons that populate the neo-striatum (caudate putamen-**cp** and globus pallidus-**gp**), the MGE (dark grey arrows) that give rise to GABA-ergic interneurons that populate the striatum and dorsal areas such as the cortex and cortical pre-plate (**CP**) that transiently populate the neo-striatum (Hamasaki *et al.*, 2003). Figures adapted from (Schambra *et al.*, 1991)

5.2 Methods

PCR methods were as described in Chapter Two. PCR was carried out using a pooled sample of the original WGE RNA samples and cortical samples obtained from a later foetal dissection to act as controls.

5.2.1.1 In situ hybridisation.

See Chapter Two: Methods for foetal dissection techniques and *in situ* probe preparation and protocol.

Due to the very small size of the tissue being studied, the whole head was used in E12 and E14 samples, whereas just the dissected whole brain was used in E16.

These tissue samples were taken direct from dissection and placed in a plastic mould (made from a microfuge tube) containing OCT mounting gel and this was snap frozen using liquid nitrogen. Once frozen these were then stored at -80°C until cutting, which took place on a bright cryostat. Brains were cut into 16μm sections and placed on Superfrost plus slides, so that one brain was shared between several slides. Slides were stored at -80°C until needed.

Three replicates of both probe and controls were carried out on each candidate gene and the most representative slides were then photographed, cut and the picture mated to the nearest coronal section available from a pre-natal mouse brain atlas (Schambra *et al.*, 1991) and are shown below.

5.3 Results

5.3.1 PCR

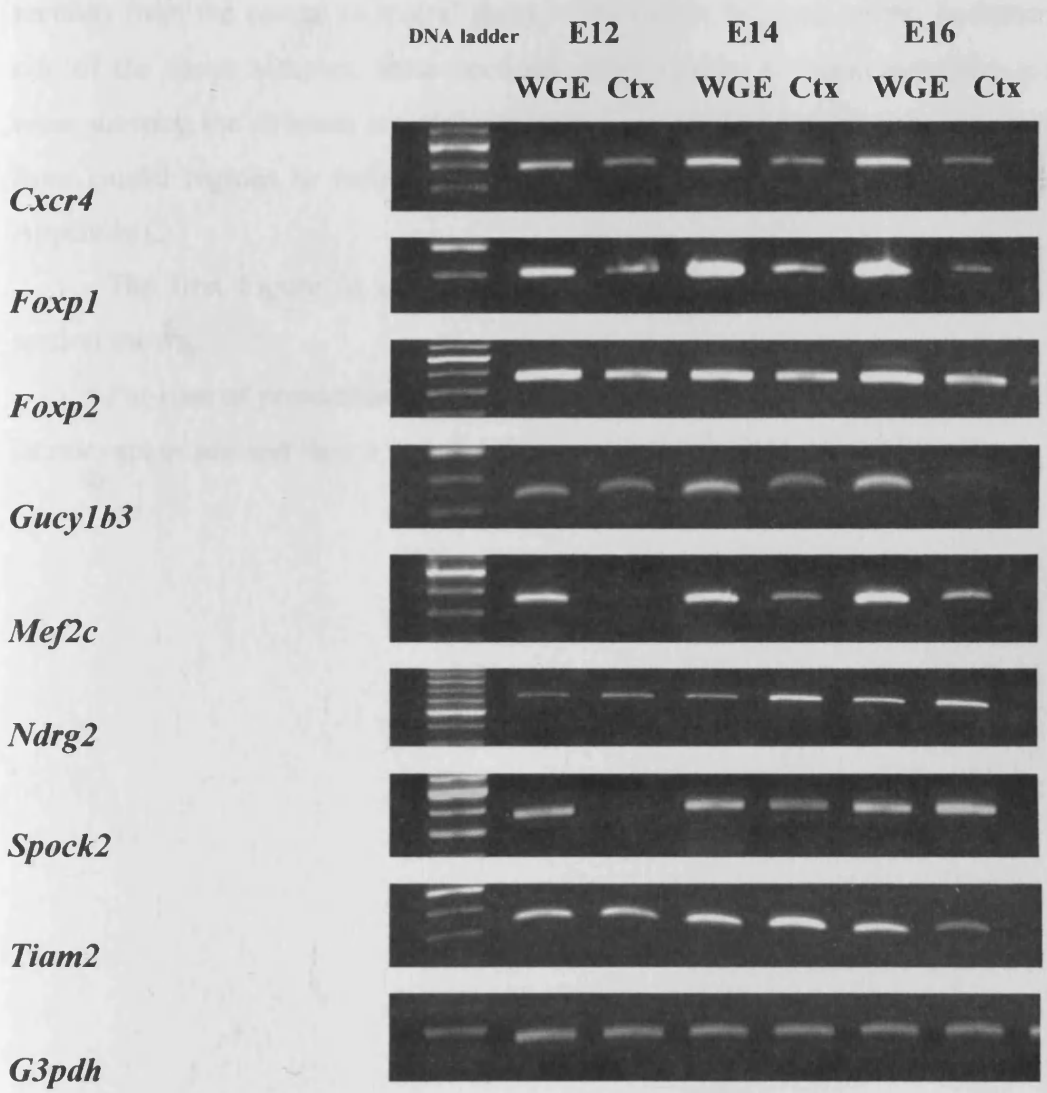


Fig. 5.2; Preliminary PCR results confirmed the micro array results, allowing progression to validation using *in situ* hybridisation.

The PCR results above provided a preliminary confirmation of the micro array experiment results as in all candidate genes a similar pattern of expression as that shown in the micro array, can be seen here. Having studied these preliminary results it was decided to design and make RNA probes for hybridisation for all the above candidate genes.

Expression of these candidate genes can also be viewed in the cortical samples. Many are expressed at lower levels in cortex, although up-regulation between E12 and E16 can be seen for some genes – for example, see *Mef2c*.

5.3.2 *In situ* hybridisations.

The following Figures show the results of the *in situ* hybridisation analysis carried out using the candidate genes selected in Chapter Four. The photographs show coronal sections from the caudal to rostral areas of the brains. Because of the limitations due to size of the tissue samples, these sections differ slightly between candidate genes. The areas showing the striatum are pictured below; the photographs showing the brain series, from caudal regions to rostral regions, for each embryonic age, are shown in full in Appendix C.

The first Figure in each set shows the approximate position of each coronal section shown.

For ease of presentation, there is some discussion specific to each gene following its relevant *in situ* and then a very brief summary at the end.

5.3.3 Foxp1

5.3.3.1 E12

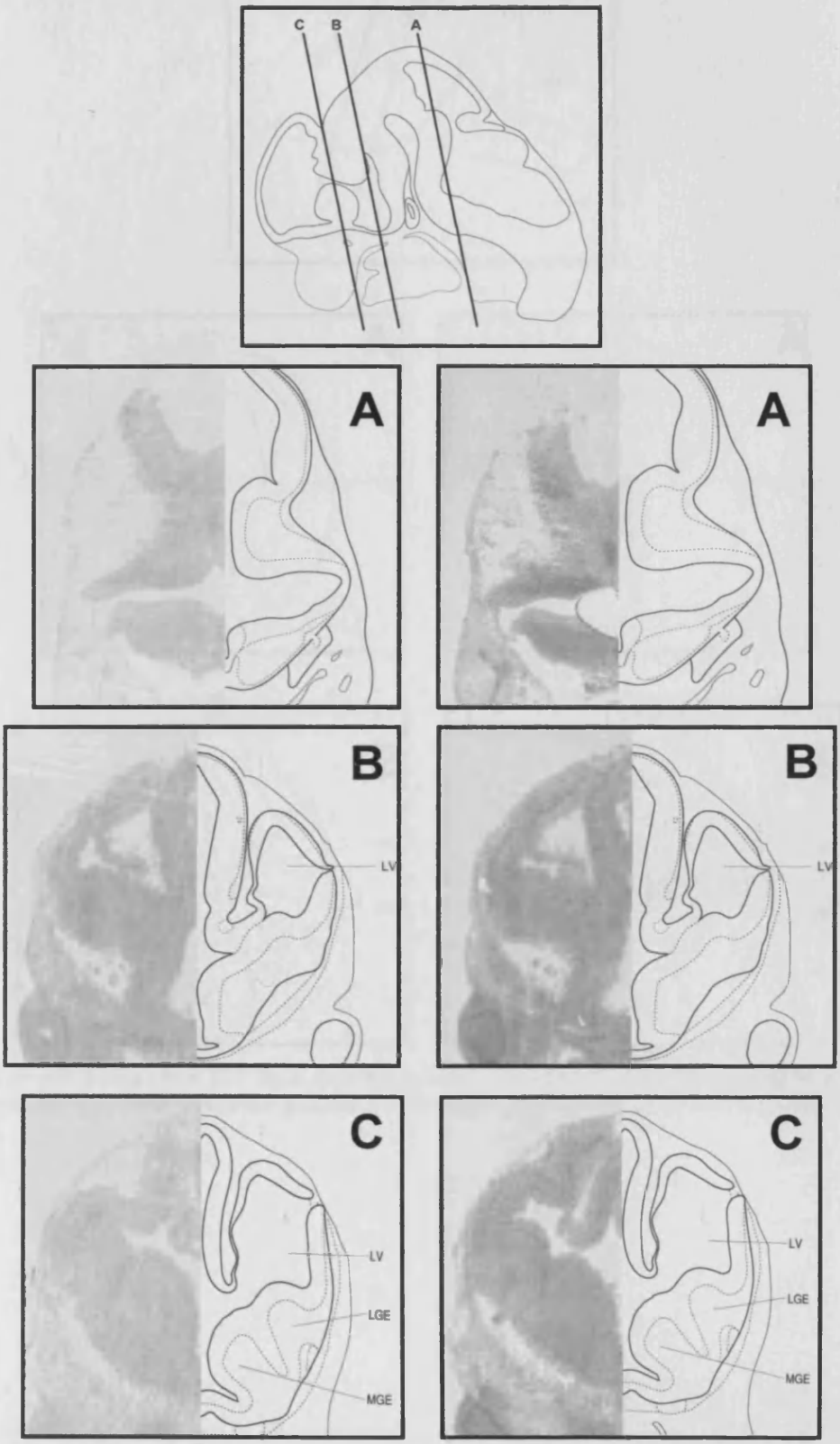


Fig. 5.3; Coronal sections through the E12 mouse brain showing control probe (left) and experimental probe (right) for Foxp1 (**V**=Ventricle, **LV**=Lateral Ventricle, **SVZ**= Sub Ventricular Zone, **VZ**=Ventricular Zone).

E14

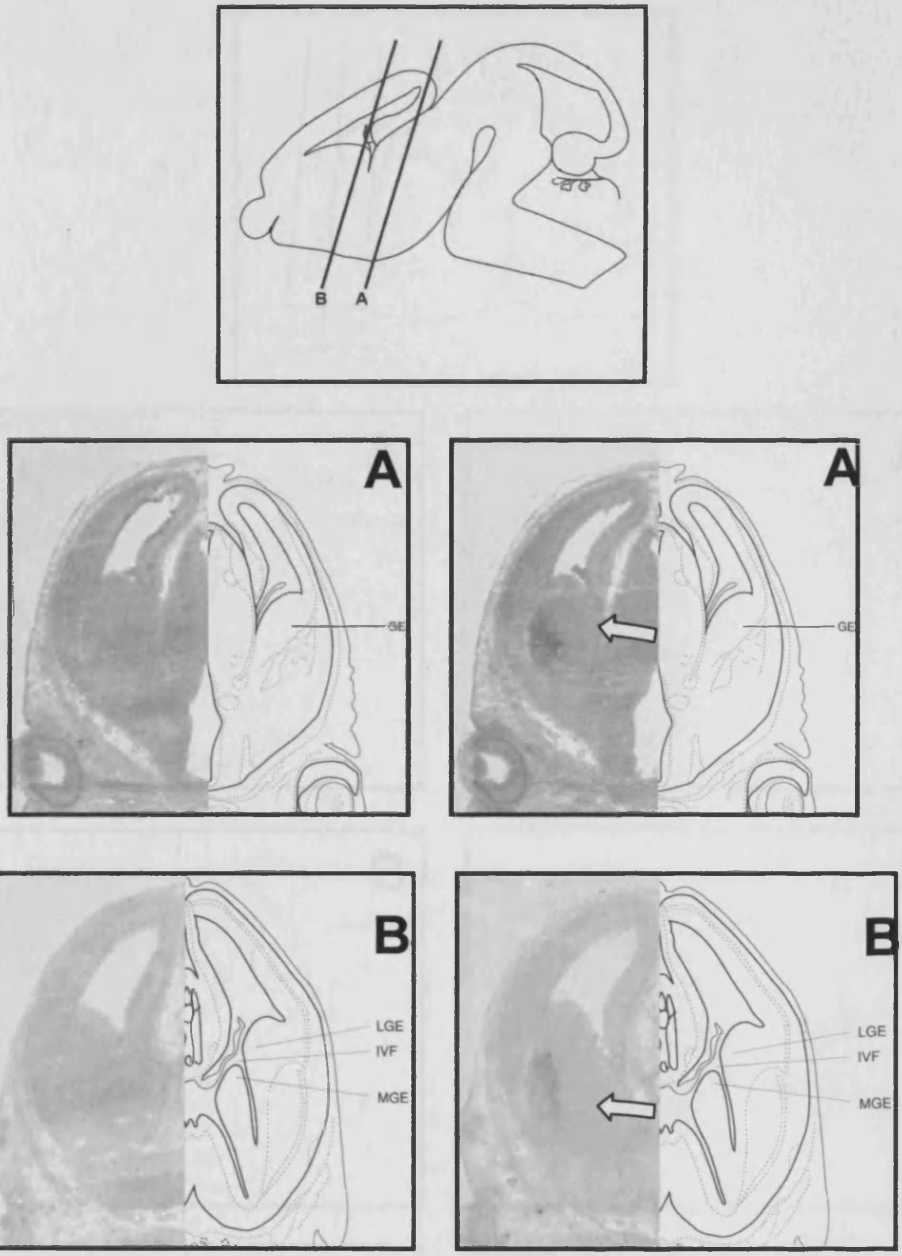


Fig. 5.4; Coronal sections from E14 Brain showing control probe (left) and experimental probe for *Foxp1*. **(SCbP=Superior cerebellar peduncle, IVF=Interventricular foramen of Munroe).**

5.3.3.2 E16

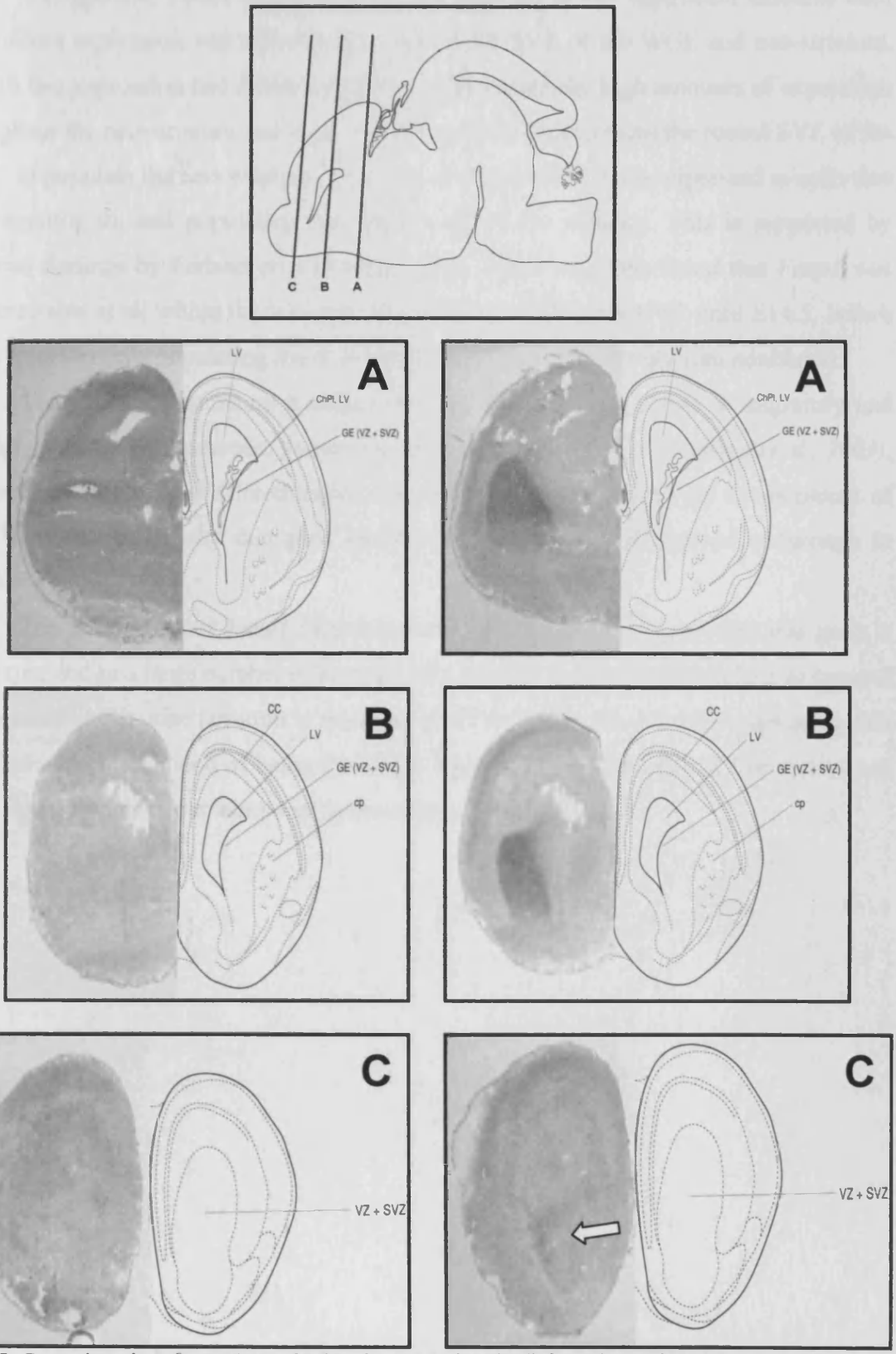


Fig. 5.5; Coronal sections from E16 Brain showing control probe (left) and experimental probe (right) for Foxp1. (**ChPL**=Choroid Plexus, **LV**= Lateral ventricle, **GE**=Ganglionic Eminence, **CC**=Corpus Collosum, **SVZ**=Sub Ventricular Zone, **VZ**=Ventricular Zone).

5.3.3.3 Foxp1

As expected, *Foxp1* expression was not detected in any significant amounts until E14, where expression was found within the caudal SVZ of the WGE and neo-striatum. At E16 this expression had increased to show comparatively high amounts of expression throughout the neo-striatum and in the cells that are migrating from the rostral SVZ of the WGE, to populate the neo-striatum. This indicates that this gene is expressed in cells that are migrating to, and populating the whole area of the striatum. This is supported by previous findings by Ferland *et al* (Ferland *et al.*, 2003) who also found that *Foxp1* was not detectable at all within the VZ, and did not appear within the SVZ until E14.5, before being found in cells populating the developing striatum at later stages into adulthood.

This evidence backs up a suggestion that this gene has a role in migratory and post migratory striatal neurons, as was postulated by Ferland *et al (Ferland et al., 2003)*, and perhaps in the final differentiation and then the ongoing life-long development of these cells (a reason why this gene remains at a high level of expression through to adulthood).

The sheer scale of *Foxp1* expression seen in this study suggests that this gene is also expressed in a large number of striatal cells: It seems a sensible hypothesis to suggest that because the murine striatum is made up of 95% MSNs, the cell type expressing this gene has a high likelihood of being the MSN. Future experiments that will be carried out in the Brain Repair Group aim to either confirm or discount this.

5.3.4 Foxp 2

5.3.4.1 E12

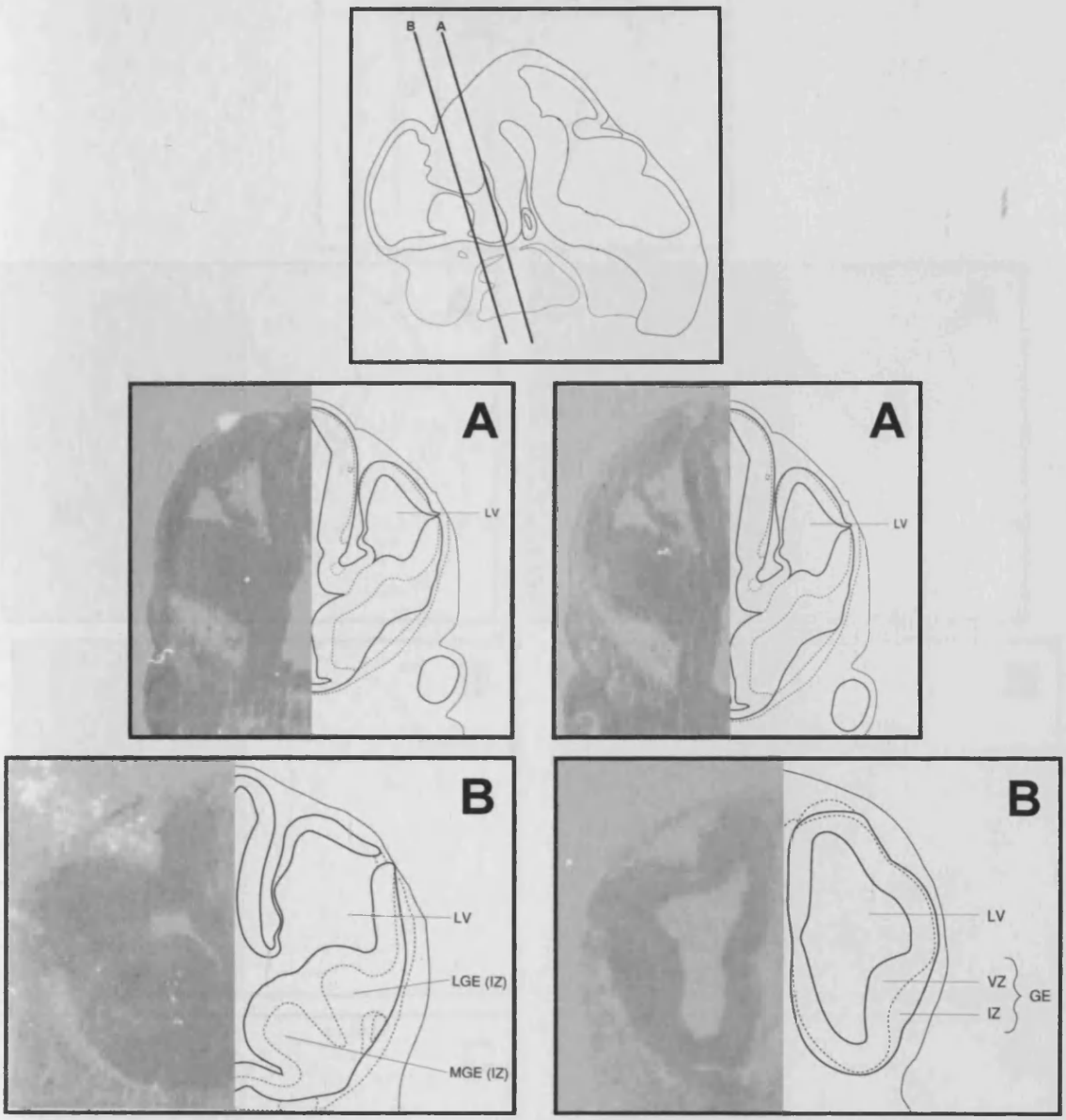


Fig. 5.6; Coronal sections from E12 Brain showing control probe (left) and experimental probe (right) for Foxp2. (**LV**= Lateral ventricle, **GE**=Ganglionic Eminence, **LGE**=Lateral Ganglionic Eminence, **MGE**=Medial Ganglionic Eminence, **IZ**=Intermediate Zone, **VZ**=Ventricular Zone).

5.3.4.2 E16

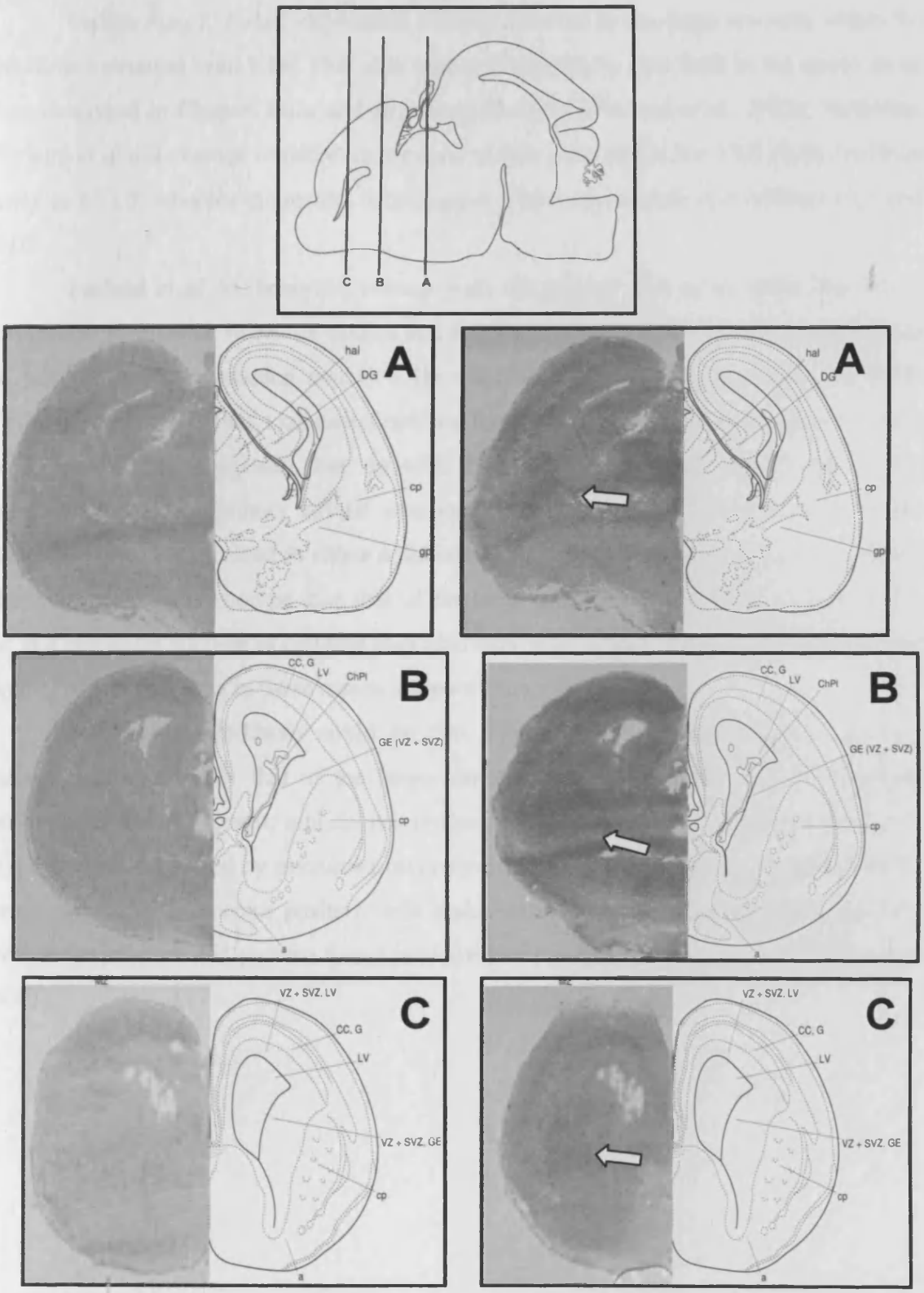


Fig. 5.7; Coronal sections from E16 Brain showing control probe (left) and experimental probe (right) for Foxp2. (**hal**=Lateral Habenula, **DG**=Dentate Gyrus, **cp**=Caudate Putamen, **gp**=Globus Pallidus, **CC, G**=Corpus Collosum, Genu, **ChPl**=Choroid Plexus, **a**=Nucleus Accumbens).

5.3.4.3 Foxp2

Unlike *Foxp1, Foxp2* expression was not detected in any large amounts within the WGE/neo-striatum until E16. This also mirrored the results seen both in the micro array data described in Chapter Four and in a previous study (Ferland *et al.*, 2003). However, Ferland *et al* did manage to detect expression of this gene within the SVZ of the WGE as early as E12.5, whereas the results here suggest a later up-regulation at between E14 and E16.

Ferland *et al* do, however, concur with the present data as to where the *Foxp2* expression is situated: Previous studies and this data suggest early expression is restricted to just the caudate putamen and to cells migrating towards this area from the SVZ. Ferland *et al* noted that expression within the mature adult mouse striatum is more widespread. They suggested that, as with *Foxp1*, this gene may have a role within migratory or post migratory striatal neurons (Ferland *et al.*, 2003). It may be that this candidate gene is expressed in either a different smaller subset of neurons and therefore a different neuronal phenotype than that of the large proportion of cells expressing *Foxp1*, or in a particular subtype of cell that may also expresses *Foxp1*. Evidence of the restricted spatial expression seen in these results supports this view.

A possible hypothesis could be that this gene is expressed within a separate neuronal type, possibly that of the larger aspiny neurones that also appear within the striatum to a lesser extent, and do not project outside the borders of the striatum; this hypothesis is supported by previous observations that show the majority of these GABA-ergic, substance P receptor positive cells make up around 13% of all GABE-ergic cells within the striatum and are also found predominantly in the caudate putamen (Chen *et al.*, 2003).

5.3.5 Cxcr4

5.3.5.1 E12

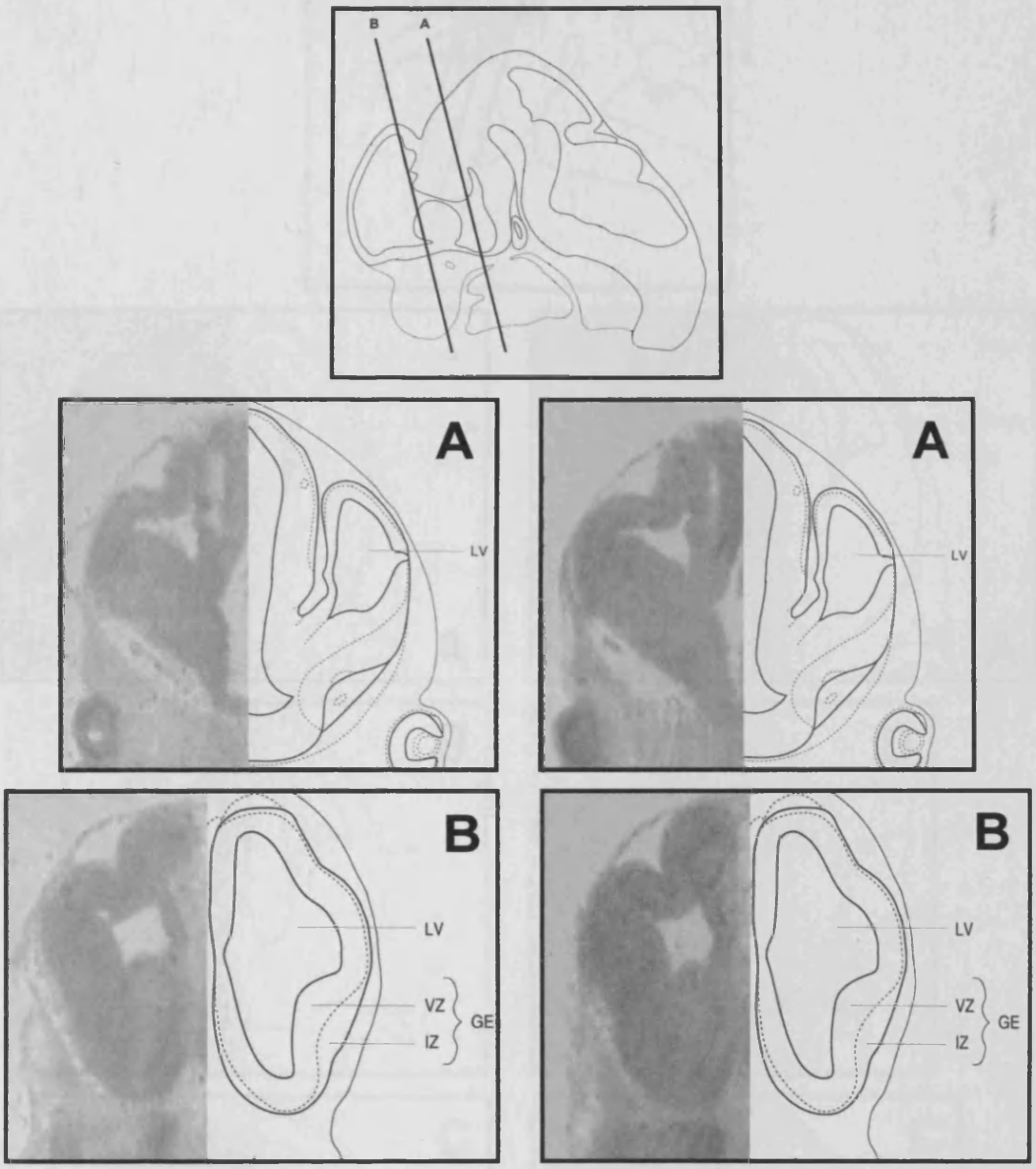


Fig. 5.8; Coronal sections from E12 Brain showing control probe (left) and experimental probe (right) for Cxcr4. (**LV**= Lateral ventricle, **GE**=Ganglionic Eminence, **IZ**=Intermediate Zone, **VZ**=Ventricular Zone).

5.3.5.2 E14

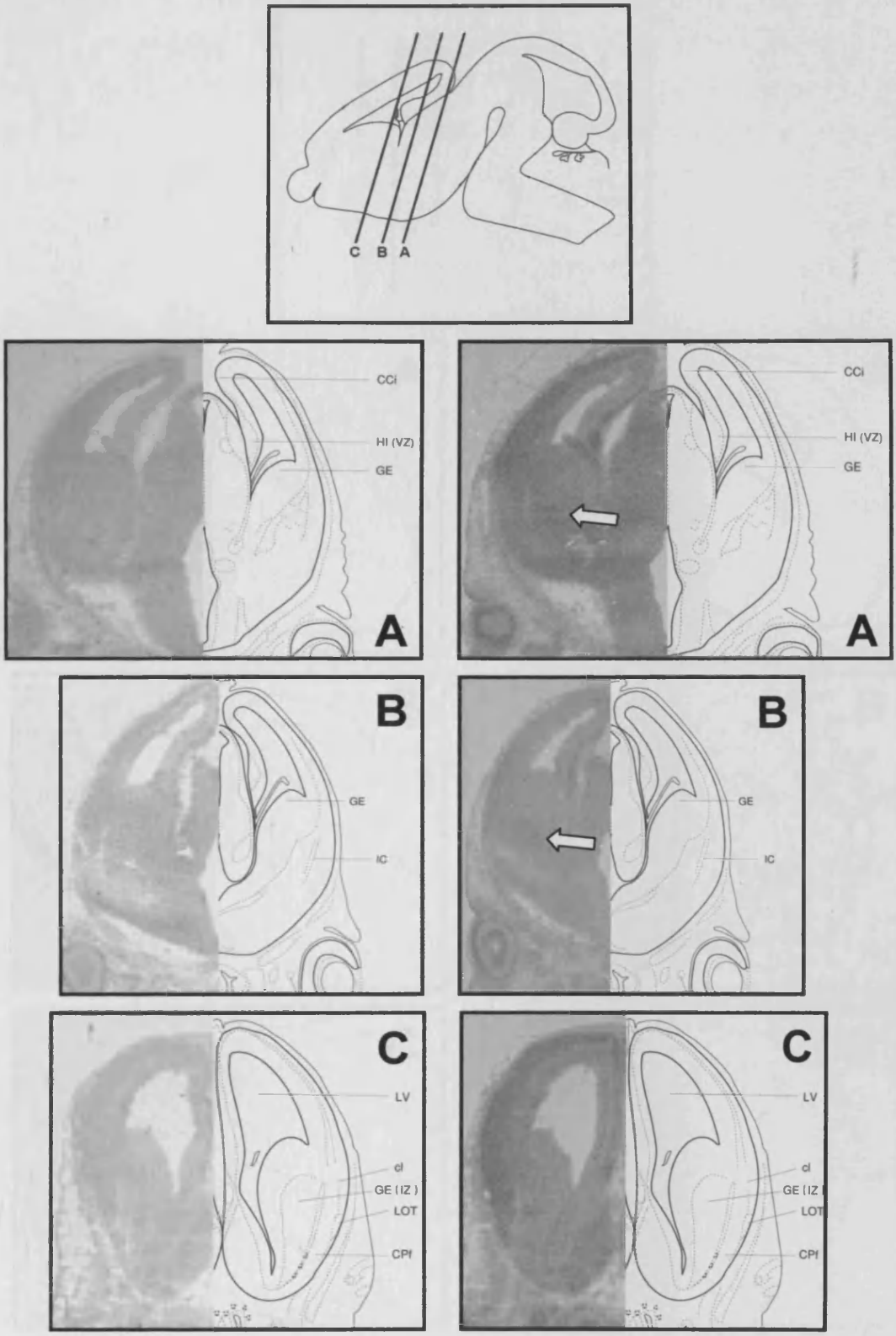


Fig. 5.9; Coronal sections from E14 Brain showing control probe (left) and experimental probe (right) for Cxcr4. (**HI**=Hippocampus, **VZ**=Ventricular Zone, **CCi**=Cingulate Cortex, **IC**=internal capsule, **LOT**=Lateral Olfactory Tract, **cl**=claustrum, **ic**=internal capsule).

5.3.5.3 *E16*

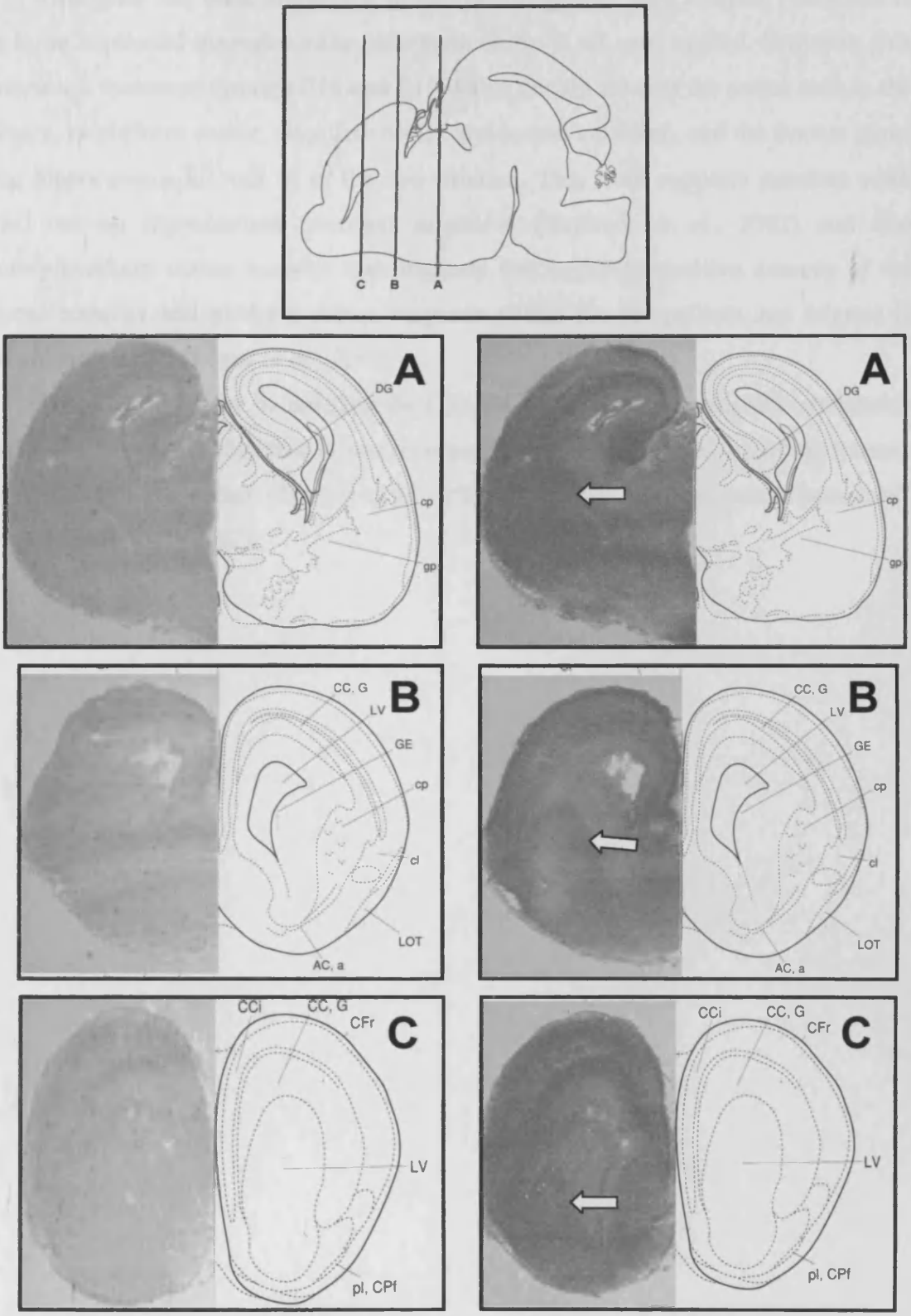


Fig. 5.10; Coronal sections from E16 Brain showing control probe (left) and experimental probe (right) for Cxcr4 (**pl,CPf**=Pyramidal layer of the pyriform cortex, **cp**=Caudate Putamen, **cl**=claustrum, **LOT**=Lateral Olfactory Tract, **AC,a**=Anterior Commisure (Anterior part), **GE**=Ganglionic Eminence, **LV**=Lateral Ventricle, **CC,G**=Corpus Collosum (Genu), **CFr**=Frontal Cortex, **DG**=Dentate Gyrus).

5.3.5.4 Crcx4

This gene has been implicated in neuronal migration (see Chapter Four) and is seen to be expressed throughout the embryonic brain at all ages studied. However, this expression is increased through E14 and E16 within certain areas of the cortex such as the olfactory, or piriform cortex, cingulate cortex and corpus collosum, and the dentate gyrus of the hippocampus as well as in the neo-striatum. This work supports previous work carried out on hippocampal neuronal migration (Banisadr *et al.*, 2002) and also olfactory/pyriform cortex neurons that suggests that calbindin-positive neurons of the claustral complex and piriform cortex originate within the sub-pallium and migrate to these areas by E16.5 (Legaz *et al.*, 2005).

These neurons may be utilizing the CXCR4/CXCL12 receptor/ligand relationship in order to achieve this objective as has been postulated for those cells migrating towards other regions of the cortex (Stumm *et al.*, 2003b) and the hippocampus (Tham *et al.*, 2001; Banisadr *et al.*, 2002).

5.3.6 Mef2c

5.3.6.1 E12

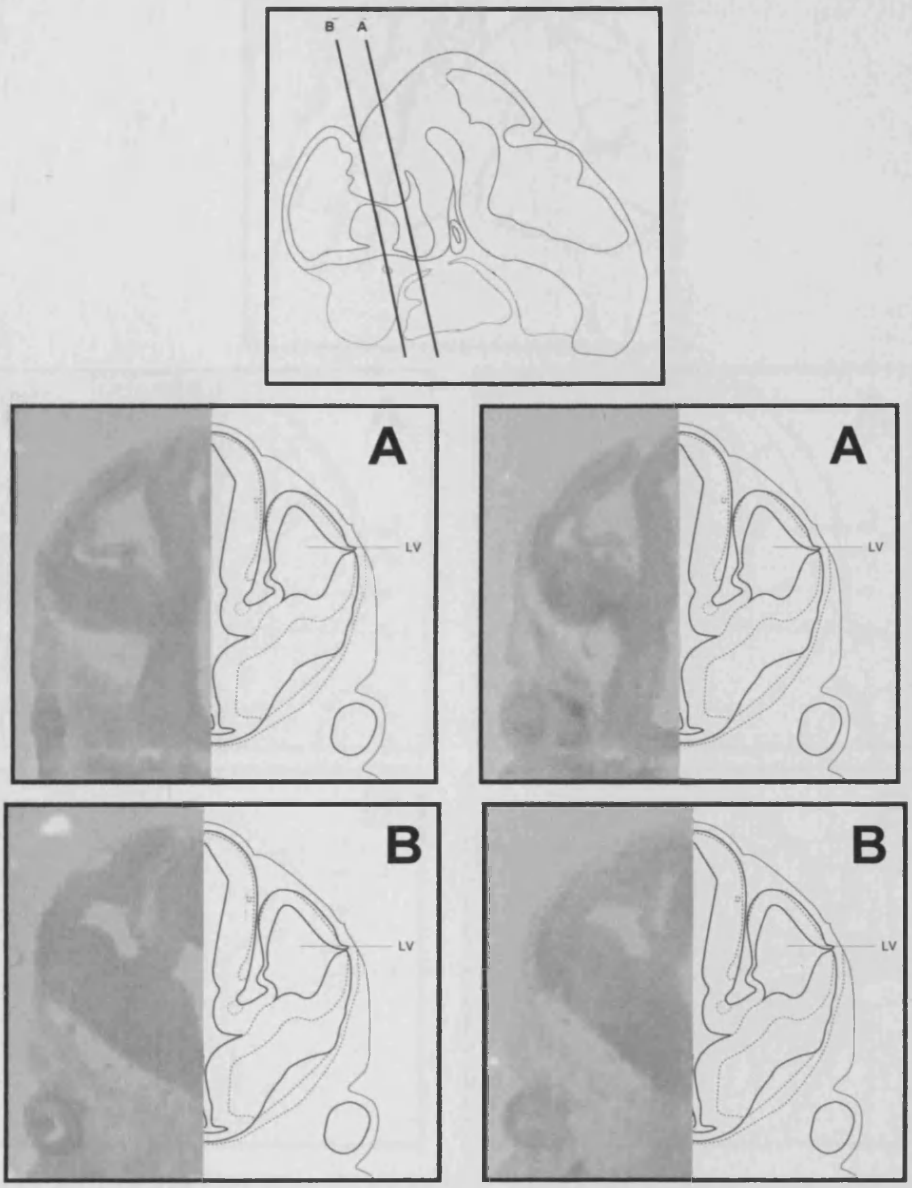


Fig. 5.11; Coronal sections from E12 Brain showing control probe (left) and experimental probe (right) for Mef2c. (**LV**= Lateral ventricle).

5.3.6.2 E14

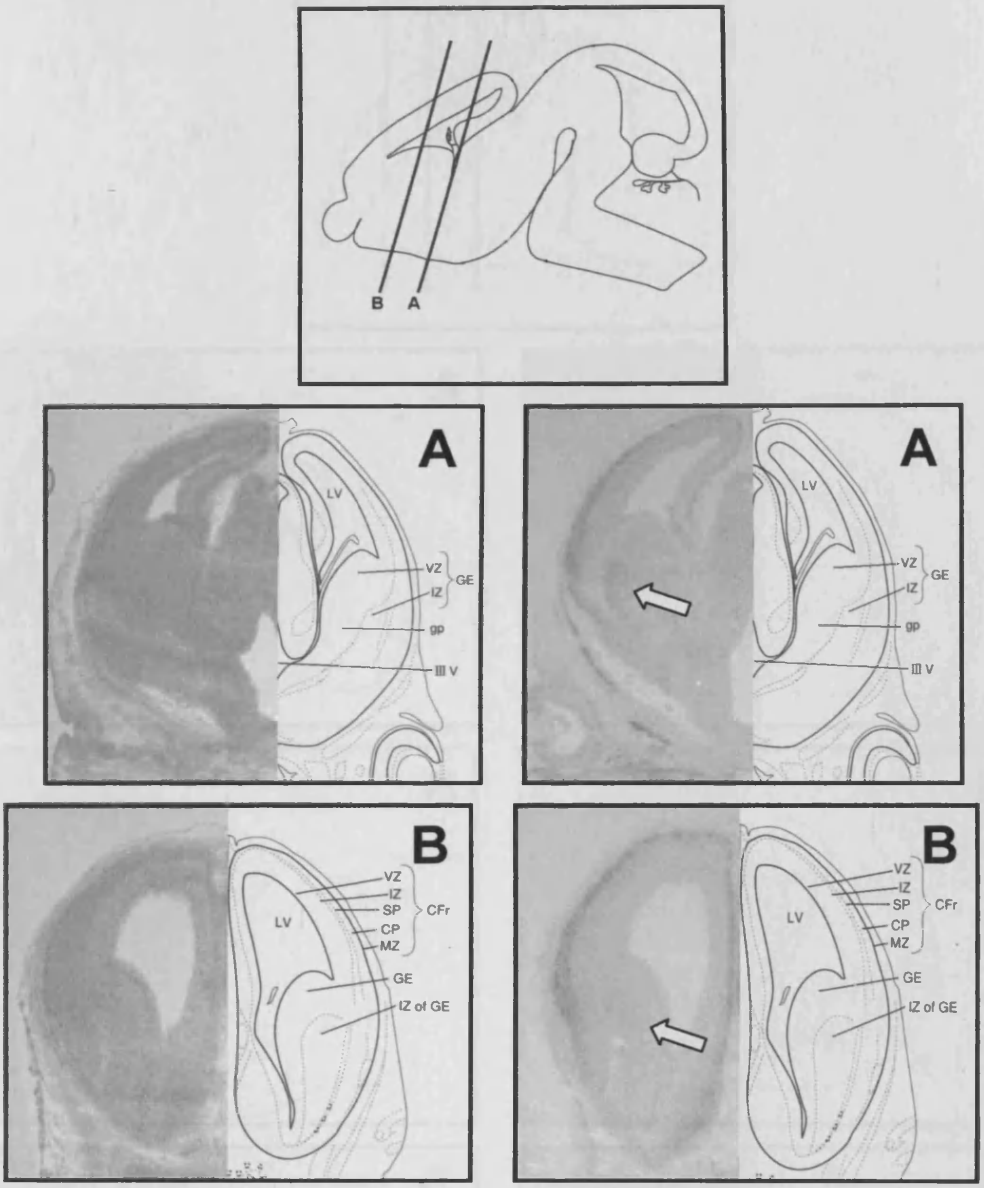


Fig. 5.12; Coronal sections from E14 Brain showing control probe (left) and experimental probe for *Mef2c* (**IIIV**=3rd ventricle, **CFr**= frontal cortex, **SP**=subplate, **MZ**=marginal zone, **CP**=Cortical Plate, **LV**=Lateral ventricle, **GE**=Ganglionic Eminence, **VZ**=Ventricular Zone, **IZ**= Intermediate Zone).

5.3.6.3 E16

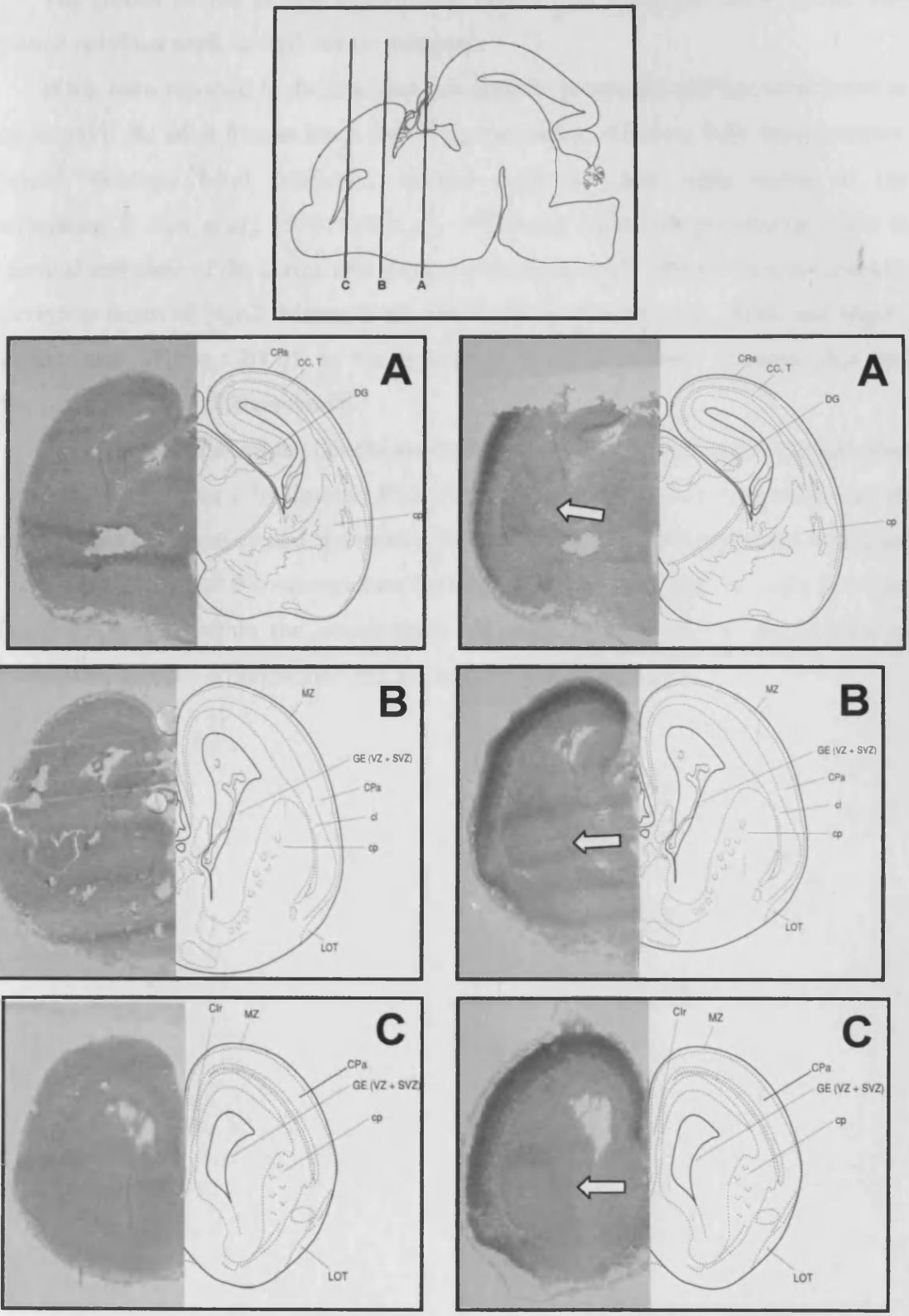


Fig. 5.13; Coronal sections from E16 Brain showing control probe (left) and experimental probe (right) for Mef2c. (**CRs**=Retro-spinal Cortex, **Clr**=Infra-limbic Cortex, **GE**=Ganglionic Eminence, **VZ**=Ventricular Zone, **LOT**=Lateral Olfactory Tract, **CC,T**=corpus collosum, trunk, **Cln**=insular/rhinal cortex).

5.3.6.4 Mef2c

The results of the *in situ* experiments carried out using the *Mef2c* probe also supported previous work carried out on this gene:

It has been reported in the past that this gene is pro-neural and has been found in many areas of the adult human brain including the cortex, olfactory bulb, hippocampus, thalamus, striatum, basal forebrain, inferior colliculus and some nuclei of the hypothalamus (Leifer *et al.*, 1994; Lin *et al.*, 1996) and within the developing brain; in the cortical sub-plate of the cortex and cortical plate (Lin *et al.*, 1996). It is a downstream transcription factor of Ngn2 (Mattar *et al.*, 2004), Islet1 (Caubit *et al.*, 2005) and Mash1 (Skerjanc and Wilton, 2000) so is expressed in post mitotic neurons that are differentiating or have differentiated.

This study confirmed the developmental expression of *Mef2c* within the sub plate and cortical plate, from E14 through E16. It also shows that some expression can be detected within the dorso-caudal and most of the rostral areas of the ganglionic eminence and caudate putamen of the neo-striatum by E16. This indicated that the cells in which mef2c is expressed within the neo-striatum are post mitotic, and in the process of differentiating or have differentiated and are starting to mature.

5.3.7 Gucy1β3

5.3.7.1 E12

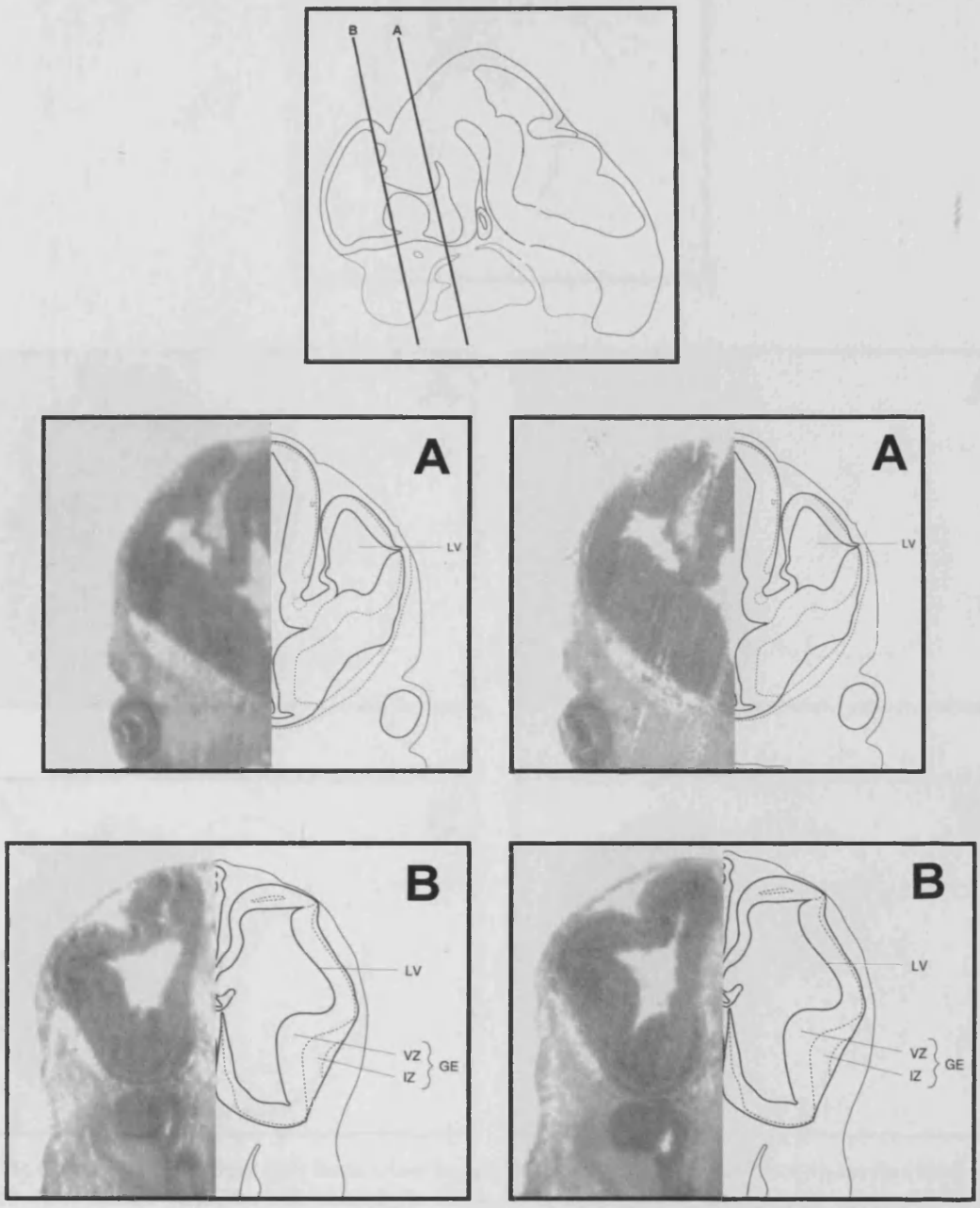


Fig. 5.14; Coronal sections from E12 Brain showing control probe (left) and experimental probe (right) for Gucy1β3. (**LV**= Lateral ventricle **GE**=Ganglionic Eminence, **IZ**=Intermediate Zone, **VZ**=Ventricular Zone).

5.3.7.2 E16

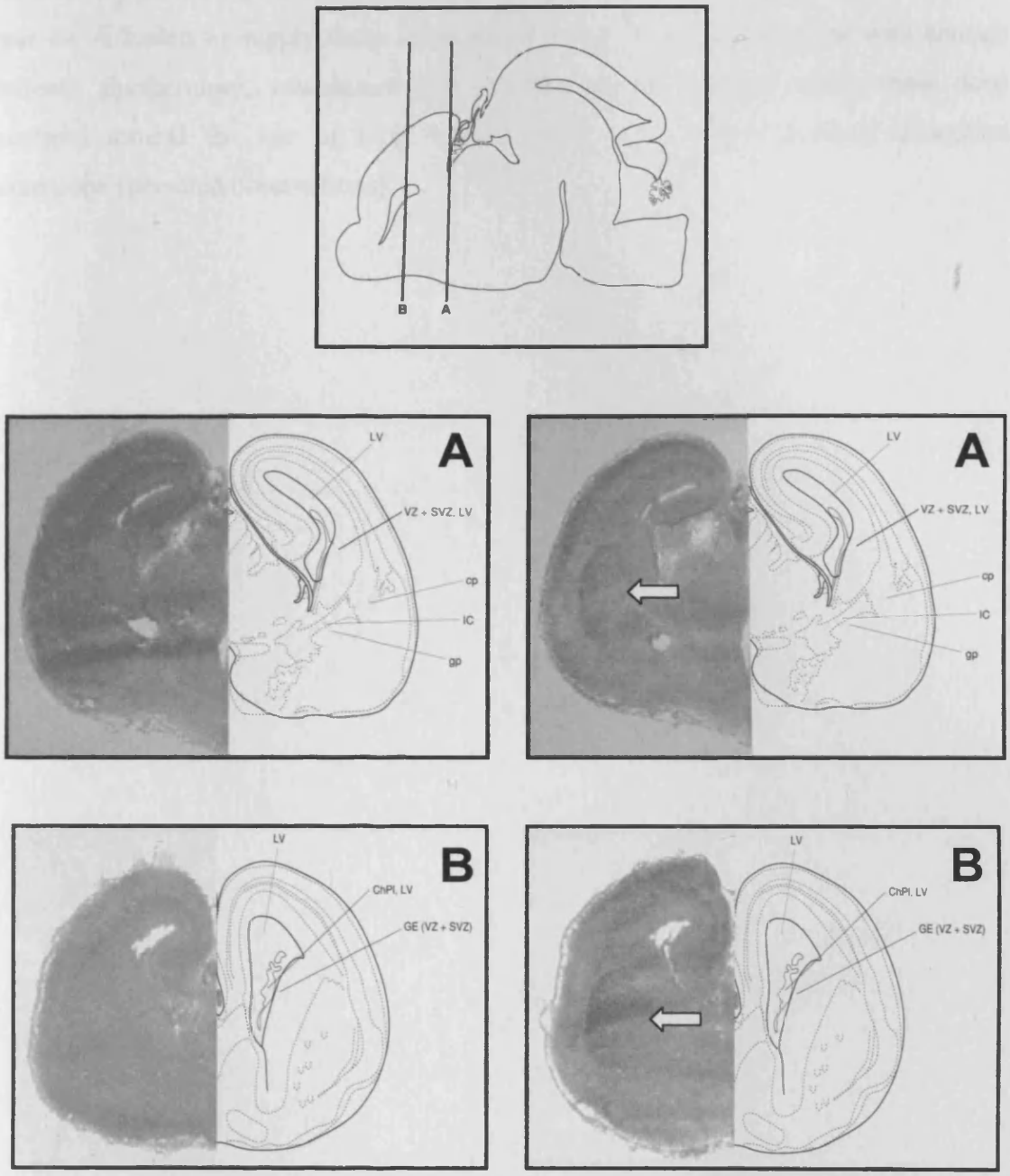


Fig. 5.15; Coronal sections from E16 Brain showing control probe (left) and experimental probe (right) for Gucy1β3. (**LV**=Lateral ventricle, **VZ**=Ventricular Zone, **SVZ**=Sub-Ventricular Zone, **cp**=caudate putamen, **IC**=Internal Capsule, **gp**=Globus Pallidus, **ChPl**=Choroid plexus, **GE**=Ganglionic Eminence)

5.3.7.3 Gucy1β3

The *in situ* data indicated very low expression of this gene before E16, mirroring the results described in Chapter Four. However, at E16, expression of this gene was found to be high within the caudate putamen of the neo-striatum and some areas of the cortex. This gene has been implicated in many signal-transduction pathways (see Chapter Four: Discussion) although the most probable function of this gene at this point in

development, in theses areas, is in the process of angiogenesis (Saino *et al.*, 2004), as the distances within the cortex and underlying structures of the developing brain become to great for diffusion to supply these increasingly metabolically active areas with enough nutrients. Furthermore, vasculature can be seen starting to form within these deep structures around the age of E16, when viewed under a low powered dissection microscope (personal observations).

5.3.8 Ndrg2

5.3.8.1 E12

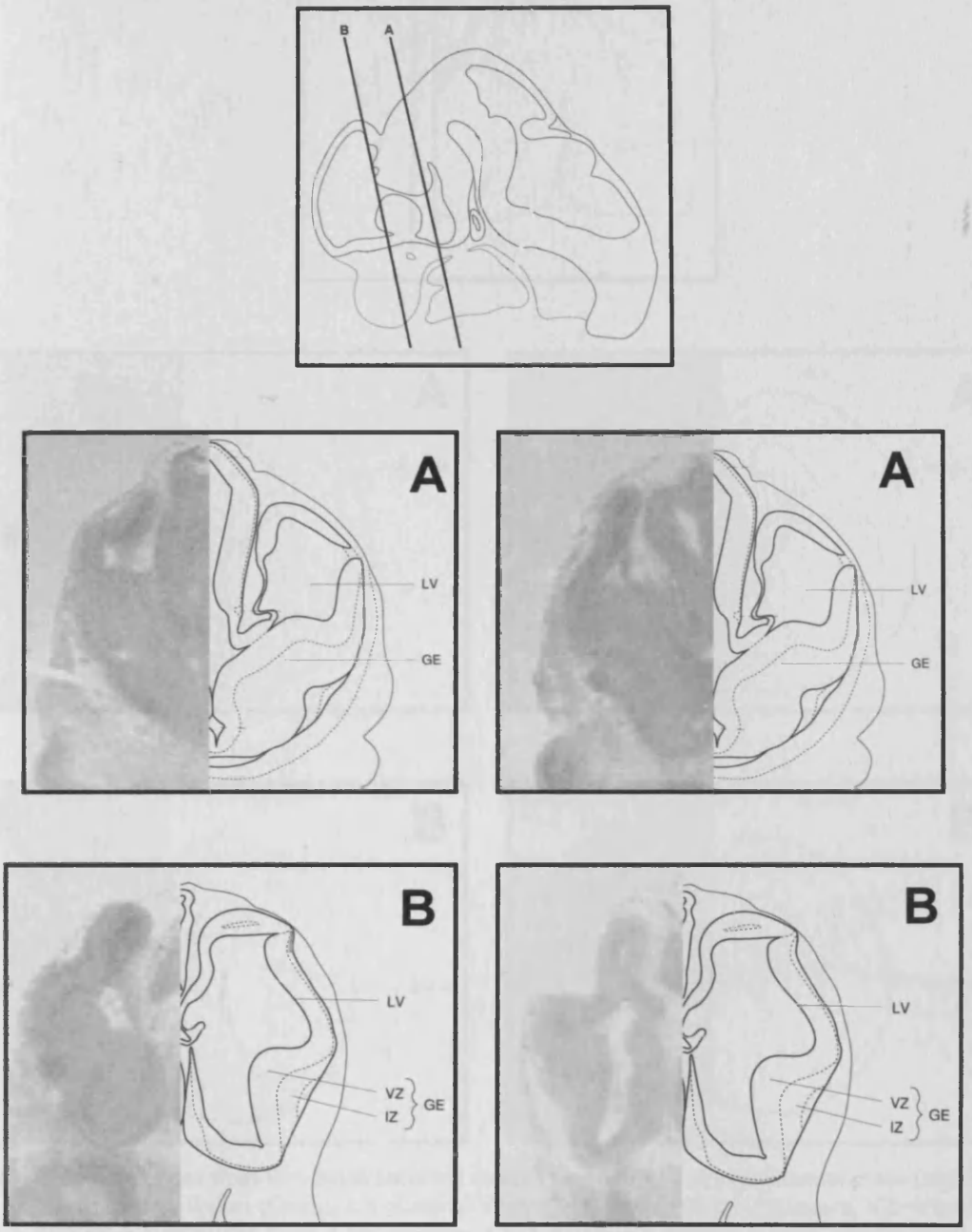
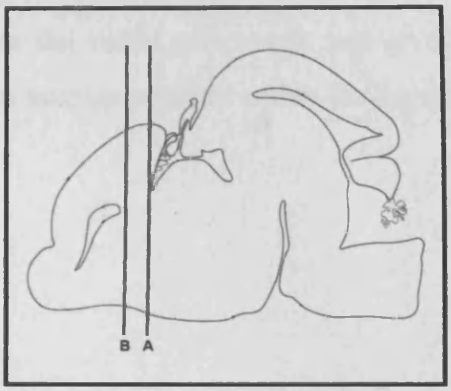


Fig. 5.16; Coronal sections from E12 Brain showing control probe (left) and experimental probe (right) for Ndrg2. (**LV**= Lateral ventricle **GE**=Ganglionic Eminence, **IZ**=Intermediate Zone, **VZ**=Ventricular Zone).

5.3.8.2 *E16*



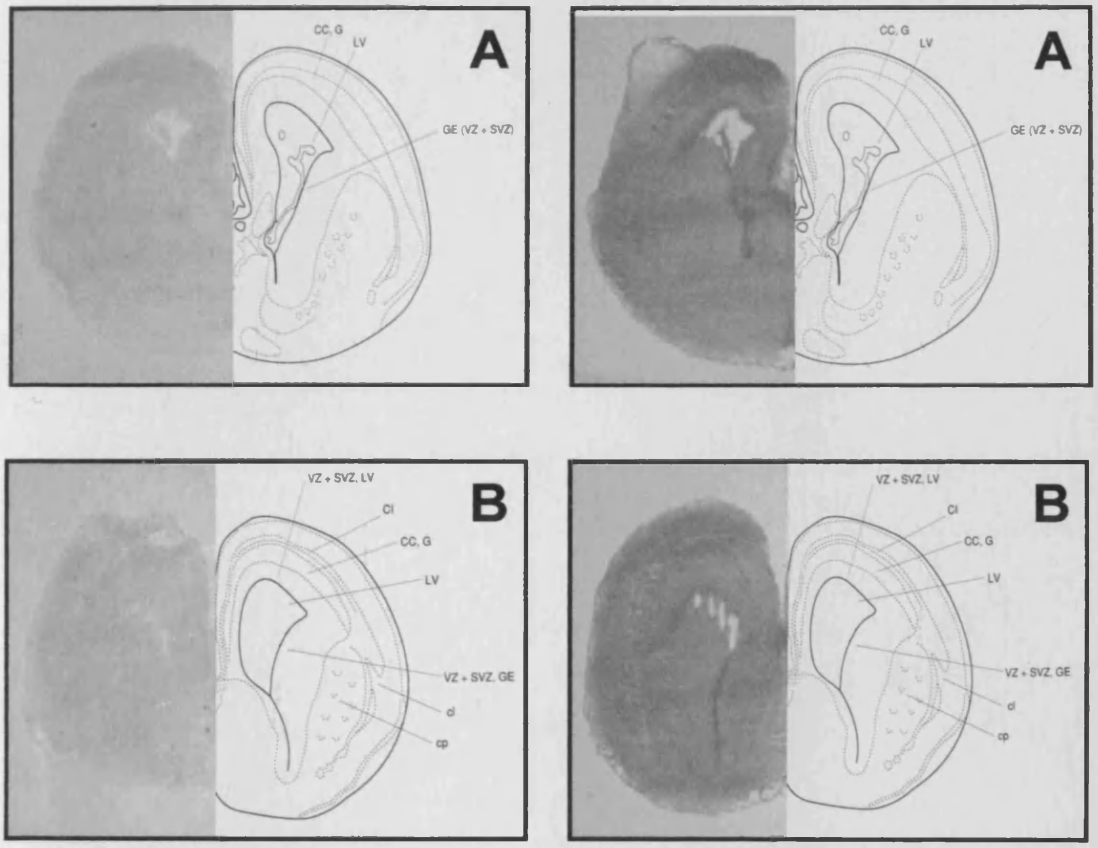


Fig. 5.17; Coronal sections from E16 Brain showing control probe (left) and experimental probe (right) for Ndrg2. (**CC,G**=Corpus Collosum (Genu), **LV**=Lateral Ventricle, **GE**=Ganglionic Eminence, **VZ**=Ventricular Zone, **SVZ**=Sub Ventricular Zone, **CI**=Cingulum, **CC,G**=Corpus Collosum (Genu), **cl**=Claustrum, **gp**=Globus Pallidus)

5.3.8.3 *Ndrg2*

The *in situ* experiment showed that *Ndrg2* was obviously up-regulated throughout the whole brain during the time window of study (E12 to E16). This is supported by previous studies (Nichols, 2003; Takahashi *et al.*, 2005a; Takahashi *et al.*, 2005b) that suggest that this gene is expressed by radial glia and GFAP-positive astrocytes, but could

also invoke growth cones in neurites, indicating it may be involved in neuronal migration away from the VZ. This is corroborated by the fact that expression density is undoubtedly highest within the VZ, where the radial glia reside and give rise to neurons that will migrate away from this area to take up position within the developing CNS.

5.3.9 Spock2

5.3.9.1 E12

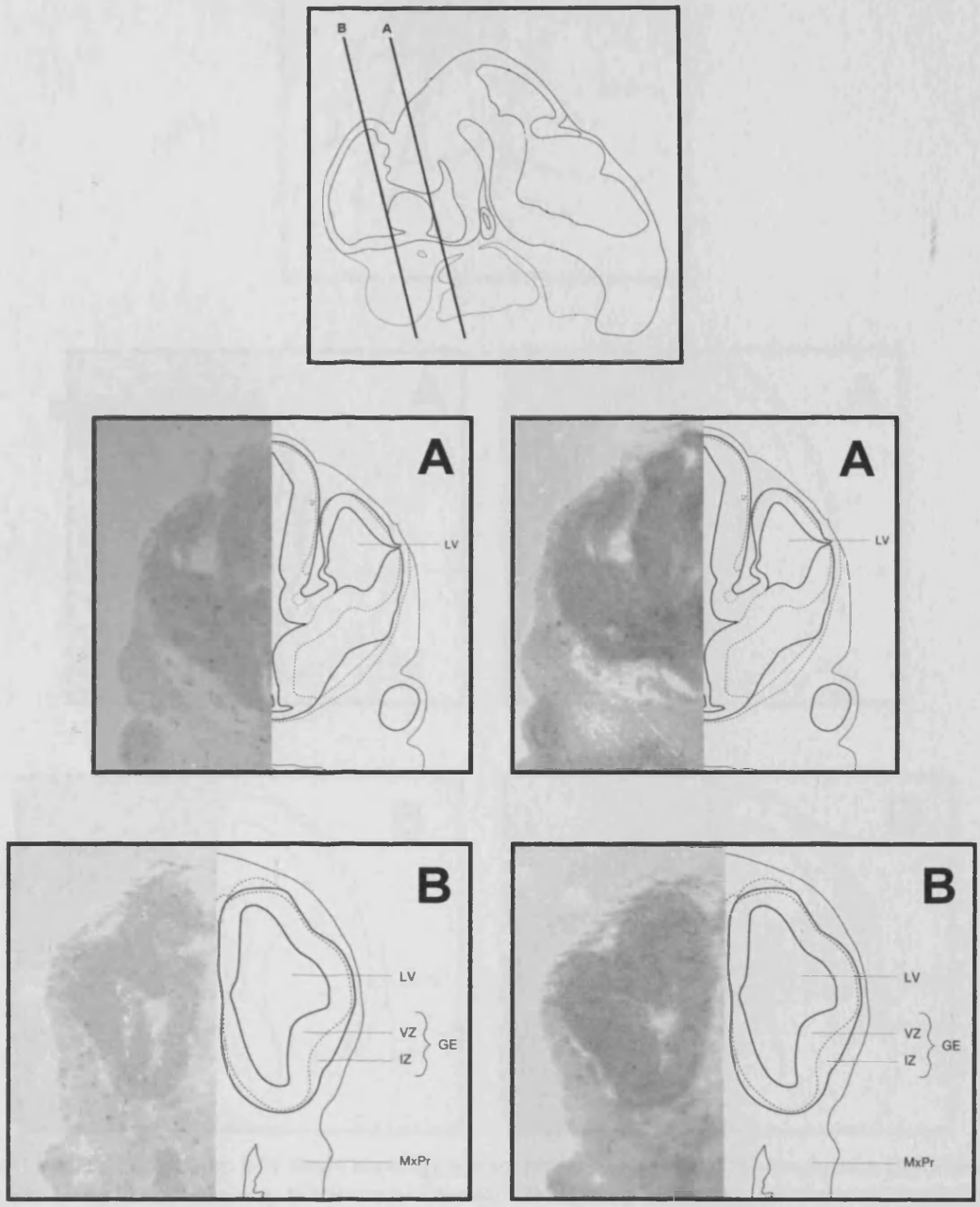


Fig. 5.18; Coronal sections from E12 Brain showing control probe (left) and experimental probe (right) for Spock2. (**LV**= Lateral ventricle **GE**=Ganglionic Eminence, **IZ**=Intermediate Zone, **VZ**=Ventricular Zone, **MxPr**=Maxillary Process).

5.3.9.2 E14

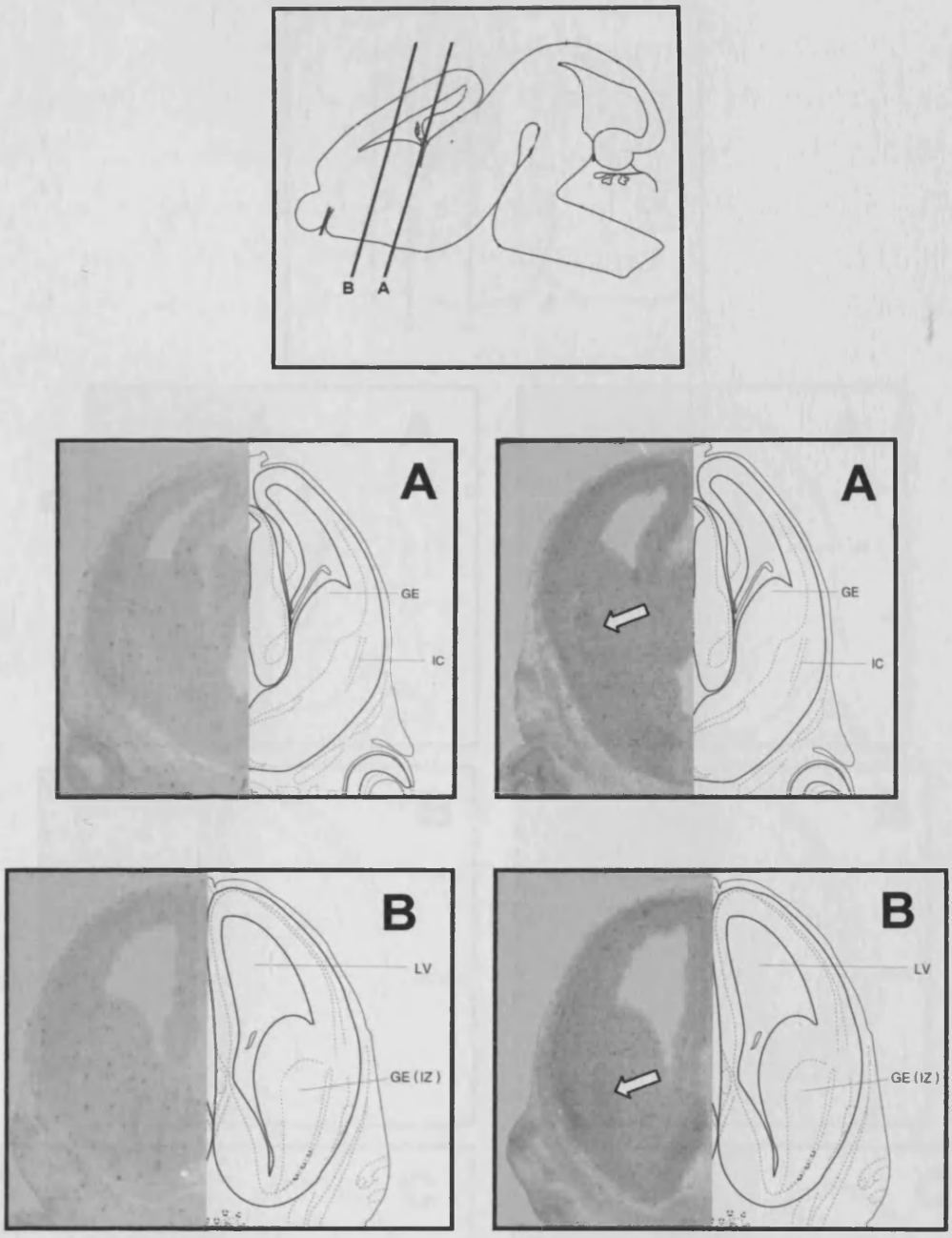


Fig. 5.19; Coronal sections from E14 Brain showing control probe (left) and experimental probe (right) for Spock2. (**GE**=Ganglionic Eminence, **IC**=Internal Capsule, **IZ**= Intermediate Zone, **LV**=Lateral Ventrical)

5.3.9.3 E16

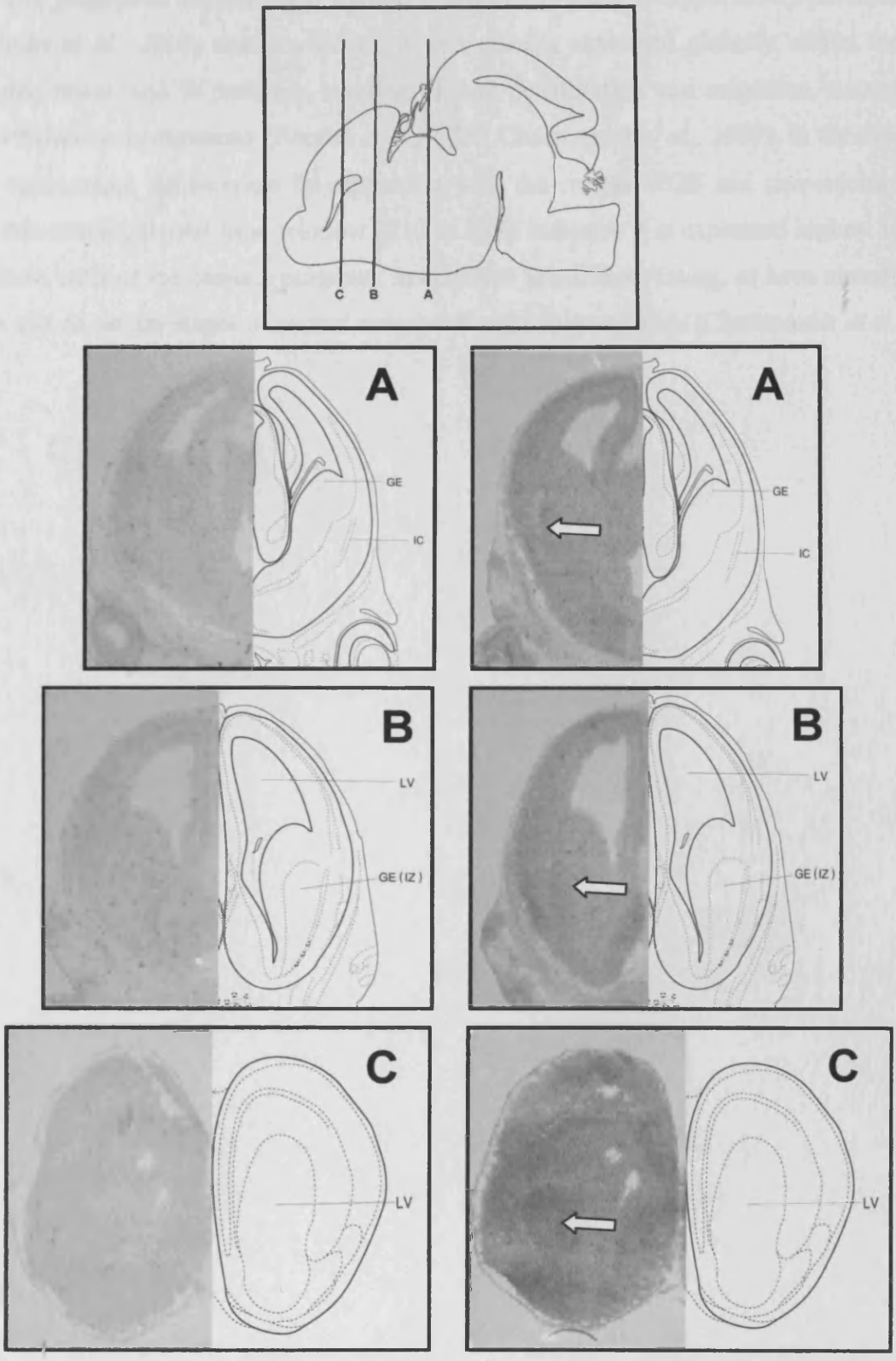


Fig. 5.20; Coronal sections from E16 Brain showing control probe (left) and experimental probe (right) for Spock2. (**LV**=Lateral Ventricle, **GE**=Ganglionic Eminence, **IC**=Internal Capsule, **IZ**=Intermediate Zone, **LV**=Lateral Ventricle)

5.3.9.4 Spock2

The patterns of expression of *Spock2* found in this study is supported by previous work (Marr *et al.*, 2000) that confirms that this gene is expressed globally within the developing brain, and is probably involved in cell proliferation and migration, axonal outgrowth and synaptogenesis (Bonnet *et al.*, 1996; Charbonnier *et al.*, 2000). In the case of this experiment, its increase in expression with the rostral WGE and neo-striatum during this developmental time window (E12 to E16) indicates it is expressed highest in post mitotic cells of the caudate putamen, in cells that are differentiating, or have already done so and are in the stages of axonal outgrowth and synaptogenesis (Charbonnier *et al.*, 2000).

5.3.10 Tiam2

5.3.10.1 E12

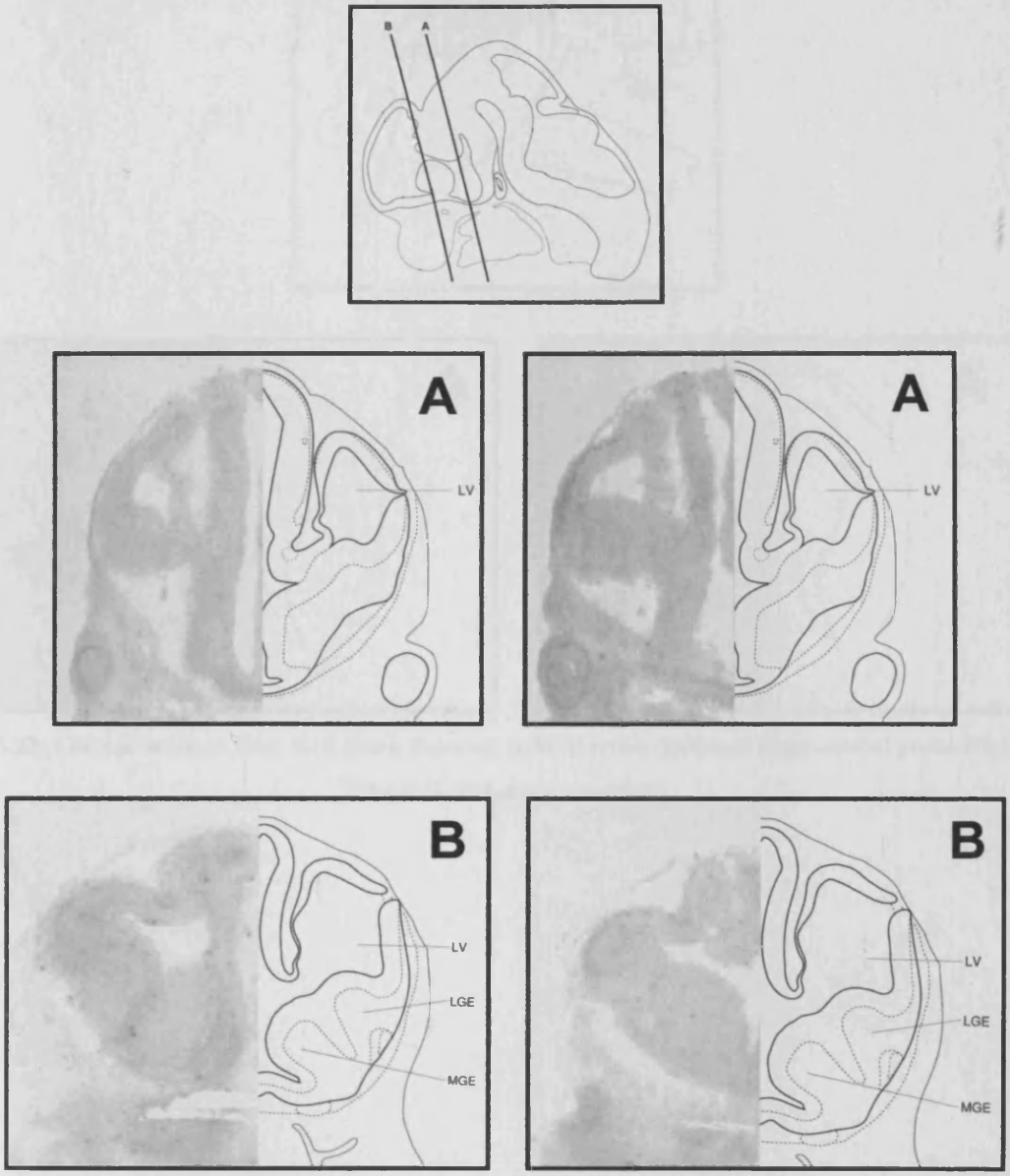


Fig. 5.21; Coronal sections from E12 Brain showing control probe (left) and experimental probe (right) for Tiam2. (**LV**= Lateral ventricle **LGE**=Lateral Ganglionic Eminence, **MGE**=Medial Ganglionic Eminence).

5.3.10.2 E14

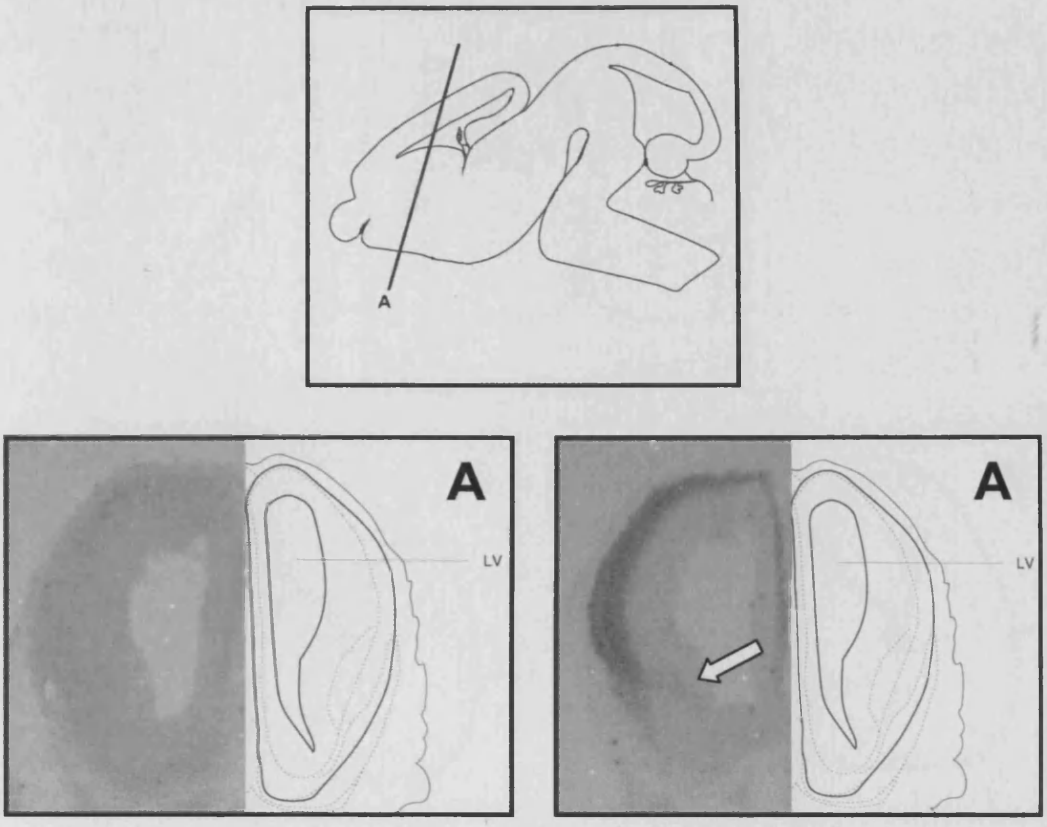


Fig. 5.22; Coronal sections from E16 Brain showing control probe (left) and experimental probe (right) for Tiam2. (**LV**=Lateral ventricle).

5.3.10.3 E16

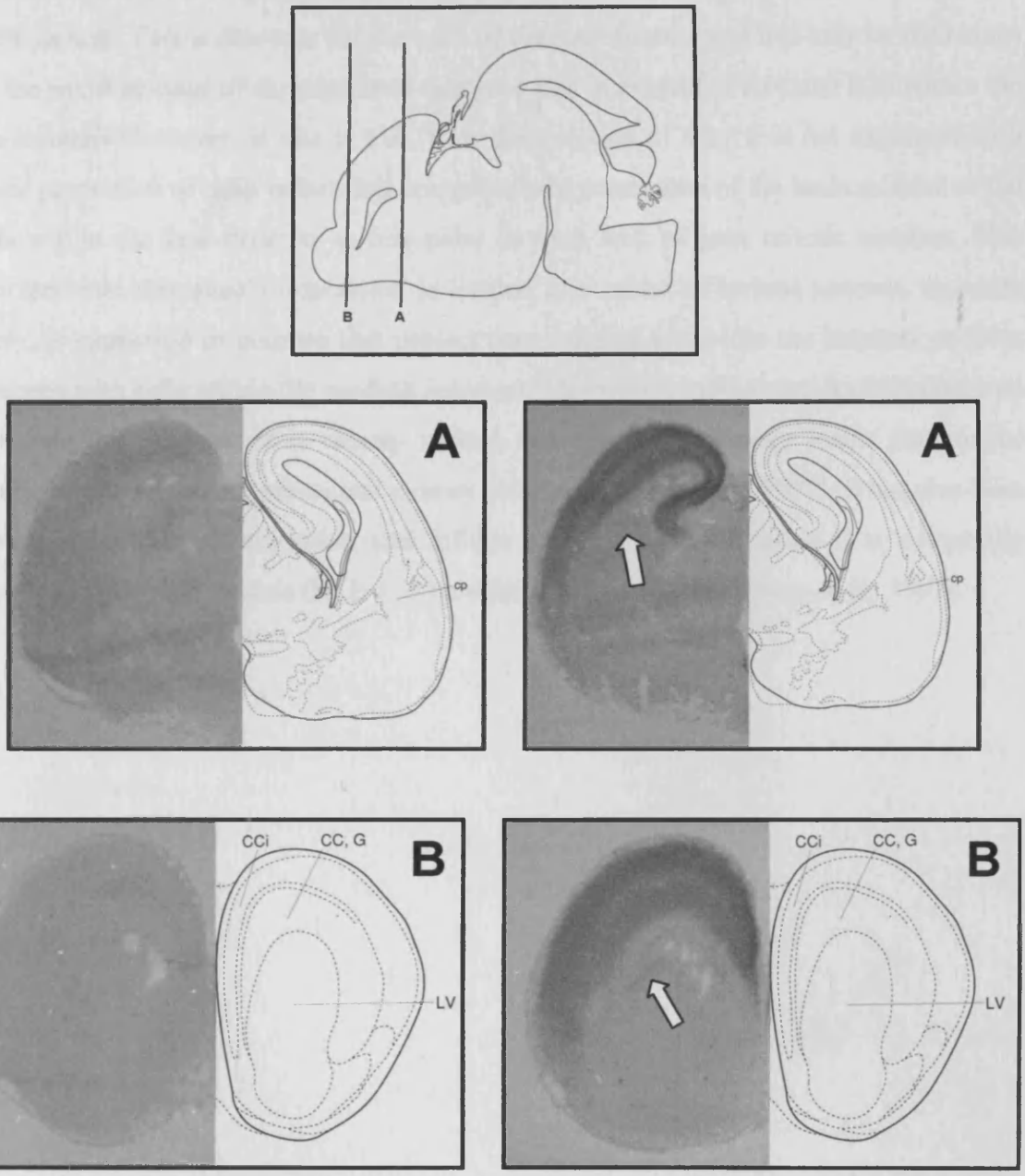


Fig. 5.23; Coronal sections from E16 Brain showing control probe (left) and experimental probe (right) for Tiam2. (**cp**=Caudate Putamen, **LV**=Lateral Ventricle, **CCi**=Cingulate Cortex, **CC,G**=Corpus Collosum, Genu).

5.3.10.4 Tiam2

Previous literature on this gene has stated that in the adult mouse this gene is only expressed in areas of the brain that display either synaptic plasticity (the cortex and hippocampus) or neurogenesis (the dentate gyrus) and it is this finding that may give a clue as to the function of this gene (Chiu *et al.*, 1999). Expression is confirmed within the

foetal brain, with a high expression within the hippocampus, cortex and corpus collosum; all areas that are developing and involved in plasticity and synaptogenesis at around E16 in the mouse. This is also true for the cells of the neo-striatum and this may be the reason for the small amount of expression of this gene that is evident at E14 and E16 within the neo-striatum However, if this is true, then the question of why it is not expressed in a larger proportion of cells within this comparatively young area of the brain as most of the cells within the neo-striatum at this point in time will be post mitotic neurons. This indicated that this gene's expression is limited to a subset of striatal neurons, or, more likely, is expressed in neurons that project from cortical areas into the striatum, to form synapses with cells within the caudate putamen. This would support previous literature on this gene that suggests it is closely related to a guanine exchange factor that maybe involved in neuronal migration and neurite extension (Ehler *et al.*, 1997). It has also been shown to induce cell migration and affects neurite outgrowth when it is ectopically expressed in *in vitro* models (N1E-115 neuroblastoma cells). (Leeuwen *et al.*, 1997).

5.4 Discussion

The experiments described in this chapter have validated the findings of the micro array experiment described in Chapter Four for all the genes selected. They have also allowed us to visualise the spatial expression patterns of the candidate genes selected from the work carried out in Chapter Four, as well as confirm/validate their temporal expression patterns found earlier.

The spatial expression patterns of these genes is important to know as we can tell from the approximate position of the cells in which the particular candidate gene is expressed, whether it is in a cell that is proliferating, migrating, differentiating or developing.

A further set of *in situ* experiments, giving the spatial information of these candidate genes further through development, and perhaps after maturation of the brain may well have given us a wider picture of which genes are, in fact, involved in synaptogenesis and migration, and which are perhaps genes expressed in most areas anyway. This may be useful to pursue in future experiments.

Many of the genes described here have been described previously as associated with the developing striatum. However, they have generally not been prominent in this respect, for example, Foxp1 and 2 which are highly up-regulated in this screen have been associated with striatum in a small number of studies but have not been widely associated with striatal development. One contribution of my study is to bring genes such as Foxp1 and 2 to prominence.

Although, as stated in Chapter Four, one previously unknown gene was chosen to follow up as a candidate marker gene, due to the technical difficulties in making suitable primers for this cDNA clone, it had to be dropped before the *in situ* hybridisation experiment.

Although a number of the genes that have been followed up in this chapter have also been seen to be expressed in cortex, this does not necessarily count them out as markers of striatal differentiation. The fact that these genes may have different expression patterns than other cells, in certain genes, may well be utilised to build up a phenotype's genetic signature over certain periods such as differentiation, which will set it apart from other cell that may express some of the same gene, just at different times, or in different amounts.

5.4.1 Conclusions

This *in situ* data has expanded on what was found during the micro array screen described in Chapter Four. It has also confirmed and been supported by previous reports on some of the candidate genes, and has raised some very interesting hypotheses about some of those genes about which only small amounts was known. Only future experiments will give answers as to whether these hypotheses are correct, but if they hold true then at least two of the candidate genes highlighted during the micro array screen may well be good markers for different sub-types of developing striatal neurons that are migrating out of the SVZ and into the neo-striatum to form the mature striatum.

Chapter Six:

6 Gene expression changes during differentiation of mouse primary foetal striatal cells *in vitro.*

6.1 Introduction

As set out in the introduction to this thesis, in order for most stem cell populations to be used for cell replacement on Huntington's disease, they will need to be directed towards a striatal phenotype and then transplanted into the adult CNS at the appropriate developmental stage as it is crucial to have cells of the same phenotype as the ones lost to the disease process. In addition, the cells must be transplanted at the appropriate developmental stage; if they are taken from foetuses that are too young, then the phenotype will not have been specified, and if from foetuses that are too mature, then they will be unable to survive and integrate into the host circuitry. This knowledge has been obtained from studies in which rat embryos of different gestational stages were used to derive striatal cells for implantation into quinolinic acid-lesioned rats (a lesion model of HD) (Fricker *et al.*, 1997a; Fricker *et al.*, 1997b). The optimum gestational window was assessed according to the gestational stage at which foetal striatum produced the optimum transplants in terms of both histological appearance and improvements on behavioural testing. Such transplant experiments are ultimately the only way in which cells can be tested fully for functionality. However, such experiments are labour-intensive, costly, and take a substantial period of time from transplantation of the cells to the read-out (typically 12-18 weeks minimum). Thus, it is not an appropriate approach for screening for cellular phenotype.

It is likely that screening of developing cells for their capacity to produce MSNs will become important when developing protocols to 'direct' cells towards striatal MSN phenotypes as this process is likely to be complex and to require multiple systematic manipulations of many factors. We know that development of the nervous system depends on the temporal and spatial gene expression changes in neural progenitor cells that allow them to give rise to distinctive cell types in the mature CNS (Pierani *et al.*, 1999; Briscoe *et al.*, 2000). These changes could involve many combinations of transcription factors and morphogens, some of which have different actions depending on concentration (see introduction: Chapter One), Commitment to a neuronal phenotype is

thought to be a progressive process during which time the cell will be firstly controlled by extrinsic signals that trigger certain events within the cells own transcription machinery that will lead it along its developmental pathway to its final phenotype (Edlund and Jessell, 1999). Thus it will be necessary to have a relatively simple screening procedure for a striatal-like phenotype following multiple manipulations of the culture conditions, as it will not be possible to take all cells right through a transplant paradigm for practical and economic reasons. Thus a 'read-out' or 'genetic signature' that can be used in developing cells that have not yet expressed mature phenotypic markers is required and developmentally regulated gene expression changes are an obvious tool for this.

I have already identified a number of gene changes associated with striatal differentiation and in this chapter I explored the extent to which these gene changes are associated with striatal differentiation *in vitro*. This was important to do, because it cannot be assumed that striatal cells developing *in vitro*, and thus separated from their normal cell contacts and exposure to morphogens, will behave in exactly the same way as they would have done *in vivo*. That this is the case, is suggested by a number of findings: For example, striatal cells that develop *in vitro* express much lower levels of DARPP-32 than would be seen in normal brain tissue, suggesting that the phenotype has not developed fully (Nakao *et al.*, 1994). A second reason for wishing to characterise the developmental gene expression patterns on this population of cells is to select cells at the optimum developmental stage for transplant purposes. Once protocols are available for directing cells towards an MSN phenotype, it will be necessary to have methods for recognising when cells are at the optimum developmental stage; such methods are not currently available. As discussed above, cells that are either too immature or too mature developmentally are not able to reconstruct brain circuitry in a functional way. A developmental 'gene signature' is a potential way of being able to achieve this.

A further use for 'developmental gene signatures' would be to analyse the characteristics of neural precursor cells. Although they do maintain certain aspects of their molecular specification even after significant expansion periods (Parmar *et al.*, 2002), there are time dependant changes in gene expression (Zietlow *et al.*, 2005), In order to interpret changes in expression of these genes in cells allowed to differentiate in vitro after spending time in culture in proliferating conditions (as done in Chapter Seven), it is key that the gene expression patterns have been characterised during the differentiation of primary (non-expanded) developing striatum,

It must also be remembered that some of the candidate marker genes in this study have never been studied within the context of foetal striatal development so nothing is known about their expression *in vitro*, whether it is markedly changed from that seen *in vivo*, and if it is, whether this has an effect on the cells phenotype and genotype. This knowledge is a necessary pre-requisite to developing methods for directing cells towards the phenotype we require in treating neurodegenerative diseases such as HD and PD.

An important starting point was to assess candidate marker gene expression patterns when the cells are just taken out of the foetus, and differentiated straight away *in vitro* using standard tissue culture techniques. E12, E14 and E16 primary cells, dissected from the developing WGE were to be used, as had been done in the micro array assay, except in this experiment, they would be differentiated *in vitro*. Cells of the same developmental stages were dissected from the developing cortices of the same foetuses in order to understand the specificity of these changes.

6.1.1 Analysing the problem.

Micro array or Q-PCR would have allowed us to fully quantitate the expression of the candidate genes in samples of cells taken at different stages of culture. However, the availability and expense of these techniques precluded their use in this experiment.

Instead semi-quantitive RT-PCR was used to give an indication of the levels of expression of certain maker genes within culture samples at different stages of expansion. RT-PCR was deemed to be within the project budget and the equipment needed was readily available to the laboratory. The primers were also available and had been optimised for use in the earlier *in situ* experiments.

To demonstrate that neuronal differentiation occurred in cultures derived from all ages of foetal striatum used in this study and that numbers of neurons differentiating from the culture did not vary widely between conditions, immuno-cytochemistry was carried out on differentiated cells in order to illustrate the presence of neurons within the culture using antibodies against GABA (the majority of neurons in this brain region would be expected to be GABA-ergic) and confirmed by βIII-tubulin. Additional histological analysis was performed to visualise the products of the two most highly up-regulated genes. Double-staining was performed to see whether FOXP1 and FOXP2 were co-localised in the same cells.

6.1.2 Aims.

As discussed in Chapter Four of this thesis, a reproducible panel of candidate marker genes, deemed to be most interesting over the period of maximal striatal neurogenesis, were selected for use.

These candidate marker genes were supplemented by four other genes known to be involved in either enhancing or repressing pro-neural genes; these were

- *Groucho/TLE* (Transducing-Like Enhancers of Split)/Grg (Groucho related gene). This gene family is known to bind to members of the Hairy Enhancer of Split (HES) family of transcription factors to cause inhibition of neuronal differentiation and extended proliferation of neuronal progenitors (Hatakeyama *et al.*, 2004; Kageyama *et al.*, 2005),
- *Mash-1* (a pro-neural bHLH transcription factor that causes neural progenitor cells to differentiate into neuronal lineages (Parras *et al.*, 2002)) and
- *Meis-2*, a transcription factor expressed in both the developing and adult murine striatum (Cecconi *et al.*, 1997; Toresson *et al.*, 2000a).
- *Islet-1*, a marker of striatal neurons is a LIM-homeodomain protein, and marks the overwhelming majority of neurons of the striatal complex (Toresson and Campbell, 2001) and has been implicated in neuronal differentiation which also lends support to the hypothesis that striatal progenitors are still present within the culture after prolonged expansion, although whether they are still specified to differentiate into mostly MSNs, as *in vivo* is not clear from this work.

The aims of this experiment were to test selected up-regulated candidate marker genes from the Affymetrix micro array study (Chapter Four) to;chart the differentiation of embryonic striatal cells *in vitro*. This would provide data on the validity of these genes for use as markers of MSNs in cultured cells and give an indication of how closely differentiation of striatal cells *in vitro* mirrors that *in vivo*.

6.2 Methods.

Dissection of tissue, tissue culture, RNA extraction and RT-PCR were all carried out as per methods laid out in Chapter Two; Methods.

6.2.1 Primer Optimisation

As RT-PCR is a semi-quantitative method of measuring the mRNA signal in tissue it was imperative that each primer was optimised so that the cycle number used would produce a product that was in the linear phase of amplification.

The following primers and conditions were used in this experiment:

Primer pairs	Forward	Reverse	$MgCl_2$ Buffer conditions	Annealing Temp. (°C)	Optimal Cycle #	Primer length (bp)
Cxcr4	AGG AAA CTG CTG GCT GAA AAG	GAT GAC TCC CAA AAG GAT GAA G	1.5	55	30	383
Foxp1	GCA GCA GCT CTG GAA AGA AG	GCA GAC TTG GAG AGG GTG AC	1.5	55	30	481
Foxp2	AAG TTT GGG CTA TGG AGC AG	GTC ACA AGT TCG TTC TCA TTC C	1.5	58	30	342
ucho/TLE/Grg	TAA GGT GTG GGA CAT CAG CC	TGT CAG CTC TGC CTT TAT GC	1.5	54	30	193
Gucy1B3	TGA GTG TGA GGA TGA ACT GAC TG	AGG AAG GAC AGA ATA CAG CAA TG	1.5	55	32	327
Islet1	CAG CAA GAA CGA CTT CGT GA	GGA CTG GCT ACC ATG CTG TT	1.0	54	30	779
Mash1	AAG TCA GCG GCC AAG CAG GAT AAG	CGC AGC GTC TCC ACC TTG CTC ATC T	1.5	58	30	245
Mef2c	CTG TGC GAC TGT GAG ATT GC	ACT GTT ATG GCT GGA CAC TGG	1.5	55	30	344
Meis2	GAC TCC GAG AGT TAT AAC TA	ACT AAA GGA CAC ATG GAC GG	1.5	58	34	351
Ndrg2	CCA GGG ACA GAC TCA CTC TG	GTC TCG GCG TTG TTG TAG C	1.5	55	30	649
Spock2	CCT TCT TCA ACT CCT GTG ATA CC	CCT CTT CTG TCT CCT TTT CTT CC	1.5	55	28	356
Tiam2	TCG GAG GAA GTG GAA ACA ATA C	CTG GTC TGG CTC TTT AGC AGT C	1.5	58	30	329
G3PDH	TCC ACC ACC CTG TTG CTG TA	ACC ACA GTC CAT GCC ATC AC	1.5	55	25	451

Table 6.1; PCR Primers used during this experiment, with optimised conditions

Primers were optimised with 0.5µl of control cDNA (a mixture of equal aliquots of E12, E14 and E16 Ctx and WGE primary cell cDNA samples). Once the primers were shown to have been optimised to a cycle number that was within the exponential amplification phase of the PCR each sample was run.

6.2.2 Differentiation experiment.

Three biological replicates of both WGE and cortical tissue were dissected from three separate batches of embryos (each biological replicate sample was derived from tissue pooled from all the pups of a separate time-mated CD1 (ICR) dam). The tissue was dissected as per the methods set out in Chapter Two and put into single cell suspension by trypsinisation. The cell samples were counted and put in to differentiation media in either six well plates (seeded at 2,000,000 cells/well) or in 24 well plates (plated at 100,000 cells/well) and differentiated at 37°C in a tissue culture incubator. The following time points were selected for analysis:

1. Day 0 (primary cells)
2. Day 4
3. Day 7

and

4. Day 10

At the above times, samples were taken from the six well plates (containing at least 2,000,000 cells) or, in the case of Day 0, straight after dissociation and counting; these were centrifuged at 3000 x g for 3 minutes, the media taken off and replaced with RNA Later stabilising solution. All samples were frozen to -80°C pending RNA extraction. Cells plated on cover slips were fixed with 4% PFA (for those cells being stained for β-Tubulin), 4% PFA/Gluteraldehyde (for those cells being stained for GABA) or ice cold Methanol (for those cells being stained for FOXP1 and FOXP2) and then washed three times with PBS before being stored at +4°C in PBS azide pending immuno cytochemistry.

RNA samples for both experiments were processed using Qiagen Qia-shredder tubes and RNeasy kits with RNAse free DNAse (as per manufacturer's instructions) followed by cDNA synthesis using Thermoscript RT protocol (outlined in Chapter Two; Methods).

6.2.2.1 Experimental Controls

This experiment compared the expression of all the candidate marker genes and three supplementary genes with the expression of a known housekeeping gene; glyceraldehyde-3-phosphate dehydrogenase (*G3pdh*).

The abundance of *G3pdh* in eukaryotic cells is known to be relatively unaffected by external factors (Thellin *et al.*, 1999). In molecular analysis the level of *G3pdh* is often used as a reference value for comparison between different cell lysates or gel loadings in order to compare different samples.

cDNA samples were then equimolarised using *G3pdh* as a reference to make sure all samples being run were of approximately the same concentration. Once each of the cDNA samples (n=3 for each condition/area of brain and Day) had been equimolarised the cDNA was probed for the supplementary and candidate marker genes using PCR.

Data used for genetic expression of primary cells (Day 0) was the same for both this study and the expansion study described in Chapter Six.

6.2.2.2 Analysis.

The results of PCR experiments were quantified by mixing 2µl of x10 DNA loading buffer into the 20µl PCR reaction and then running 10µl of this mixture on a 1% agarose gel at 117 volts for 24 minutes before visualisation using spot densitometry (AlphaEase2200 imaging system and AlphaEaseFC software). Each gel was automatically analysed by the software for gel background and each sample was normalised using these figures to account for changes in light conditions and exposure times. This method gave a cDNA concentration figure for each of the genes probed for in each sample. This figure was then normalised against the corresponding sample's *G3pdh* figure (obtained after equimolarisation) in order to account for any minor differences in cDNA concentration that could not be catered for during equimolarisation. Data were analysed using analysis of variance a (Statistica 5.0; Statsoft inc.).

6.3 Results.

For all experiments, values were normalised against *G3pdh* expression. The average (n=3) was taken from each for the description. Results from each gene will be described first. The graphs show the levels of expression compared to the control gene (*G3pdh*) expression seen at the same time point. Although all the genes were analysed using *G3pdh* as a control gene in order to equimolarise between samples, the reader should be aware that the aim intended is to be able to compare the expression of the candidate marker/supplementary gene, at different time points in this cell population's differentiation, compared with the expression levels of that particular gene at Day 0/primary cells that have just been dissected out of the foetus.

The results will then be summarised at the end of this results chapter.

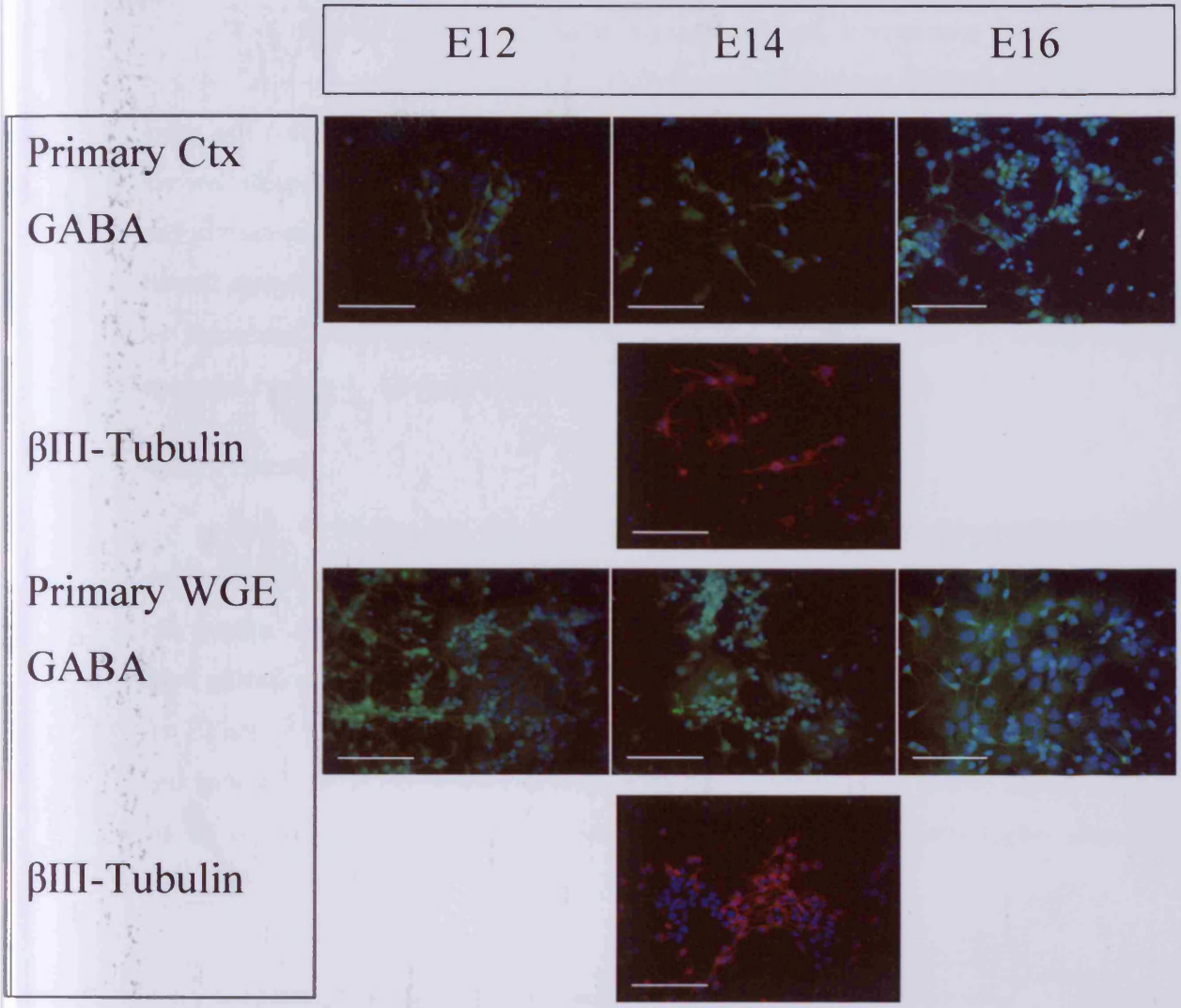


Fig. 6.1; GABA staining in primary cultures after 7 days differentiation *in vitro*. GABA was used to label neurons (most striatal neurons are known to be GABA-ergic). All cultures showed a similar number of neurons per field (although quantification was not performed for technical reasons). E16 cells showed a raised proportion of large Hoechst (blue) stained nuclei, which, from previous studies indicates an increasing number of astrocytes. This was fully expected as by E16 the gliogenic switch has occurred with increased astrocytes production. β-III Tubulin (Red) also shows neuronal presence in E14 primary differentiated cultures (Scale bar =200μm).

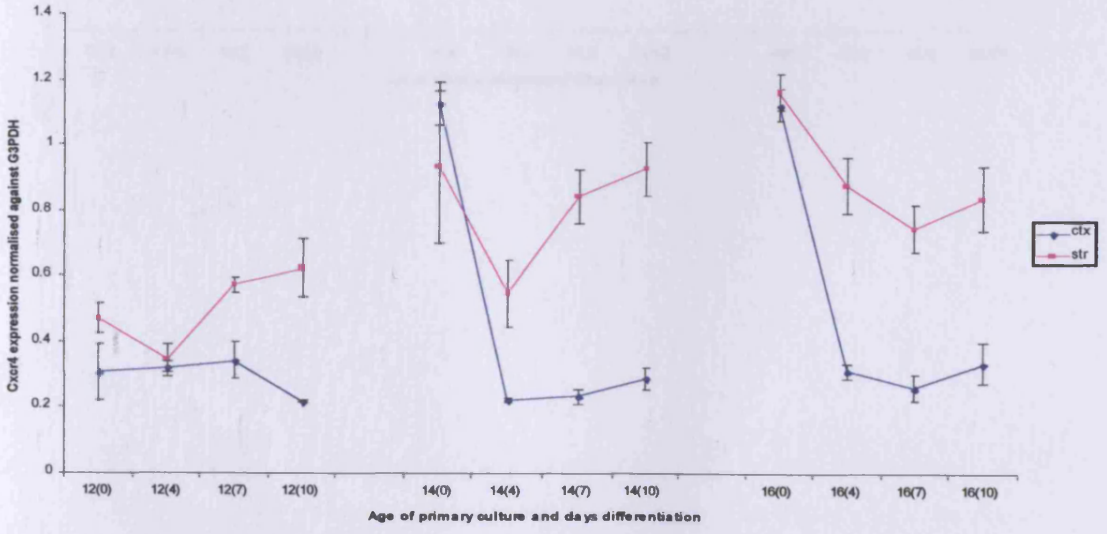
6.3.1 Gene changes in differentiating cells

For many of the genes, a dip in expression levels were seen at day 4 compared to day 0 with some recovery by day 7. This dip may have been masked in genes in which there was continued fall of expression levels. The reason for this decline is not presently known, although one explanation is that it is related to damage done during the process of cell extraction and preparation as a single cell suspension. During this process there may be cell membrane damage and cells tend to 'round up' and lose their processes. This may be associated with a fall in some levels of gene expression and recovery may have occurred by day 7. All gene levels are described relative to *G3pdh*.

6.3.2 Cxcr4

Day 0 levels can be seen to rise with increasing developmental age as demonstrated in Chapters Four and Five, thus confirming the micro array data and the *in situ* results. An ANOVA demonstrated levels of expression to be higher in striatal tissue at all gestational ages ($F_{(1,4)}=24.29, p<0.05$; $F_{(1,4)}=152.72, p<0.001$; $F_{(1,4)}=129.31, p<0.001$ for E12, E14, and E16 respectively). Indeed, cortical levels remain low in E12 cultures and appear to drop off steeply in E14 and E16 cultures, whilst striatal levels rise a little during the differentiation of E12 cells, remain relatively stable at a higher level in E14 cells and fall in E16 cells.

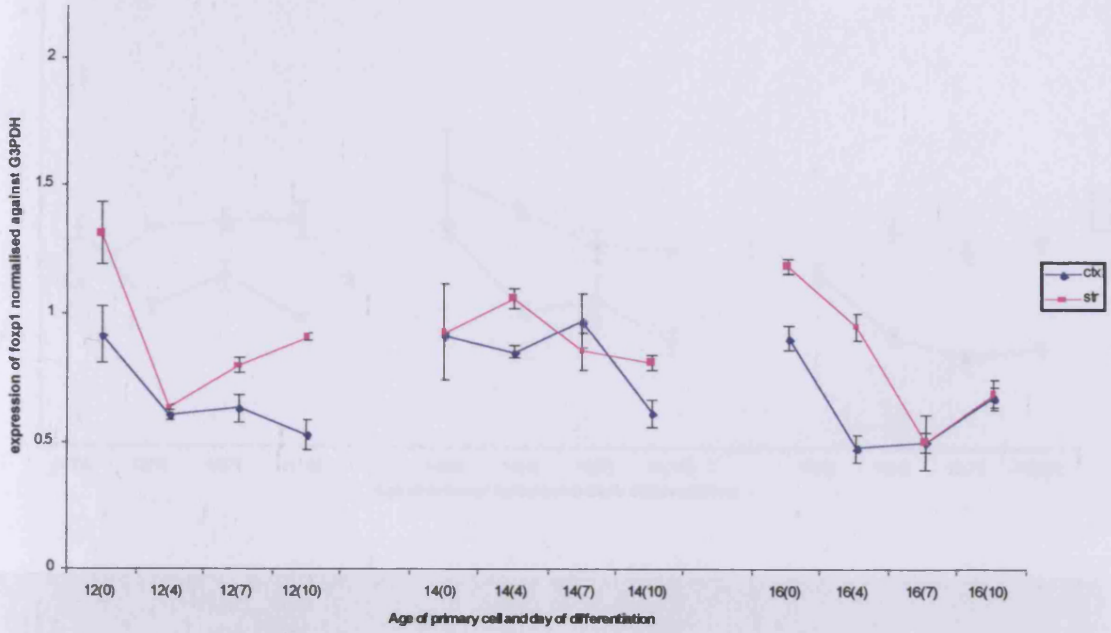
Fig 6.2; Expression of *Cxcr4* in varying ages of primary FNP culture at 0, 4, 7 and 10 days differentiation



6.3.3 *Foxp1*

Day 0 levels didn't change dramatically over the gestational window examined for either striatum or cortex. Levels of *FoxP1* were generally higher in the striatum at E12 ($F_{(1,4)}$=92.35,$p<0.001$), but this effect had disappeared by E14 and E16. During the differentiation of E12 cells levels fell significantly in striatum over the differentiation period (effect of day; $F_{(3,6)}$=22.58,$p<0.01$); there was also a fall in cortical tissue. There was little change in levels of both areas at E14 and levels declined overall in E16 cultures ($F_{(3,6)}$=29.94,$p<0.001$), although may have been rise by Day 10 although this was not significantly so.

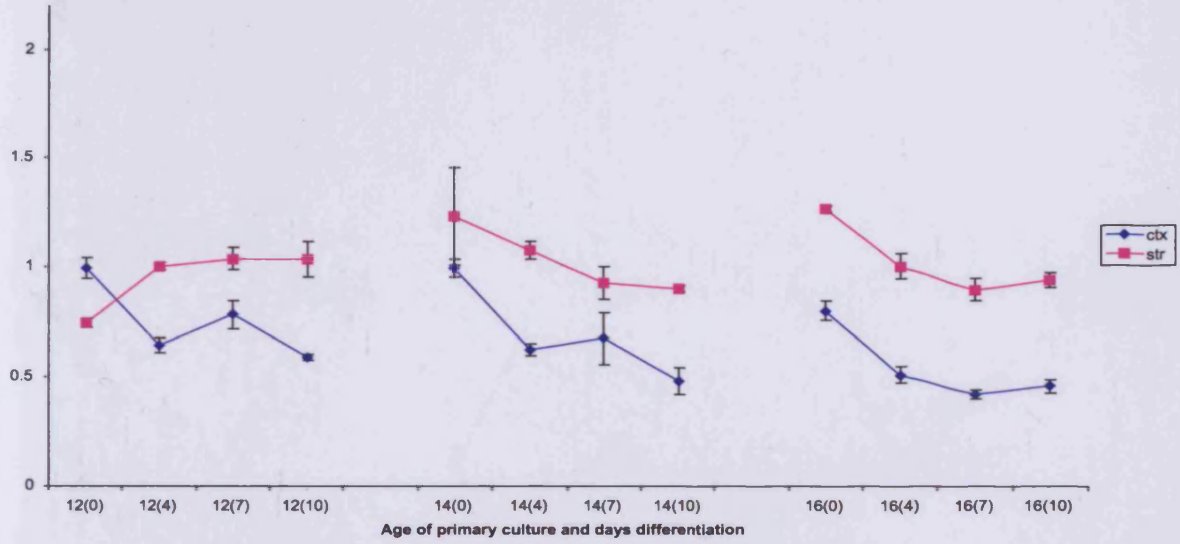
Fig 6.3; Expression of *Foxp1* in varying ages of primary FNP culture at 0, 4, 7 and 10 days differentiation



6.3.4 *Foxp2*

Primary striatal cells increased expression of *Foxp2* with increased gestational age which agreed with the micro array data. Across all conditions striatal expression levels were higher than cortex ($F_{(1,4)}=180.36, p<0.01$; $F_{(1,4)}=23.44, p<0.01$; $F_{(1,4)}=814.09, p<0.001$ for E12, E14 and E16 respectively). Striatally derived cell expression of *Foxp2* rose in E12 throughout the differentiation period ($F_{(3,6)}=8.42, p<0.05$), levels remained largely steady in E14 cells while a significant fall in expression was observed in E16 striatal cells ($F_{(3,6)}=11.72, p<0.01$).

Fig 6.4; Expression of *Foxp2* in varying ages of primary FNP culture at 0, 4, 7 and days differentiation



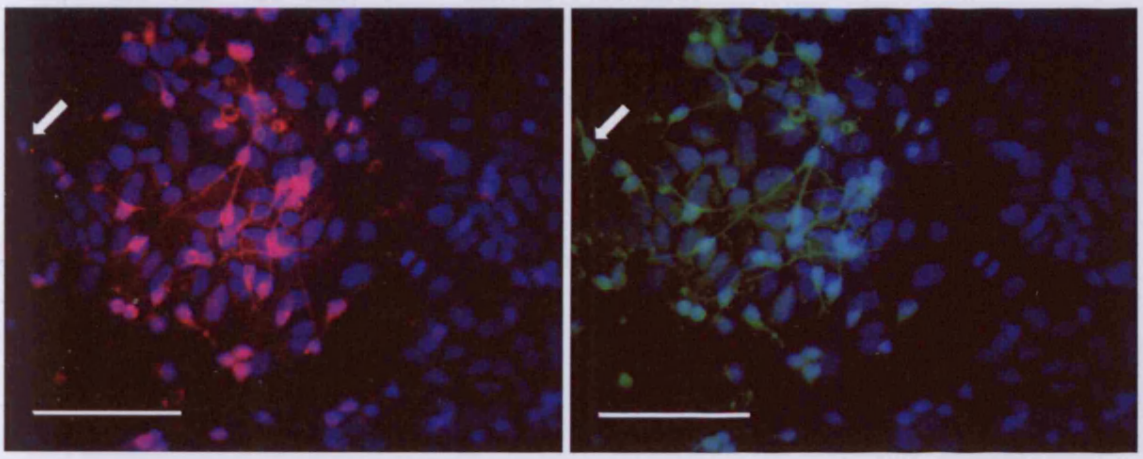
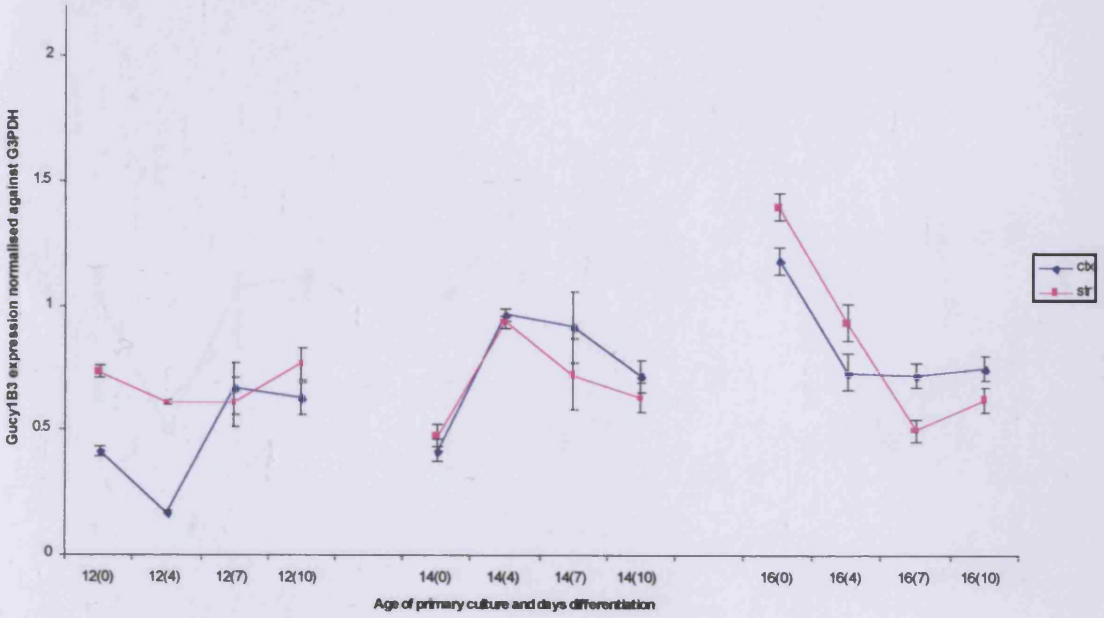


Fig. 6.5; This Figure shows the expression of FOXP1(Red) and FOXP2 (Green) in E16 of primary WGE after 7 days of differentiation. Arrows indicate cells single labelled for Foxp2 (no FOXP1) (Nuclei stained Blue with Hoechst) (Scale bar =200μm).

6.3.5 *Gucy1β3*

Primary expression levels of *Gucy1β3* showed an increase between E12 and E16 confirming the results seen in the micro array. E12 striatally derived cells showed little expression changes of this gene through differentiation, although cortically derived cells showed an overall increase over the period of differentiation. E14 striatal cells also showed no change over the differentiation period although E16 cells showed a significant fall in expression ($F_{(3,6)}$=169.22,p<0.001). There was no significant difference between striatal and cortical expression in any of the age groups.

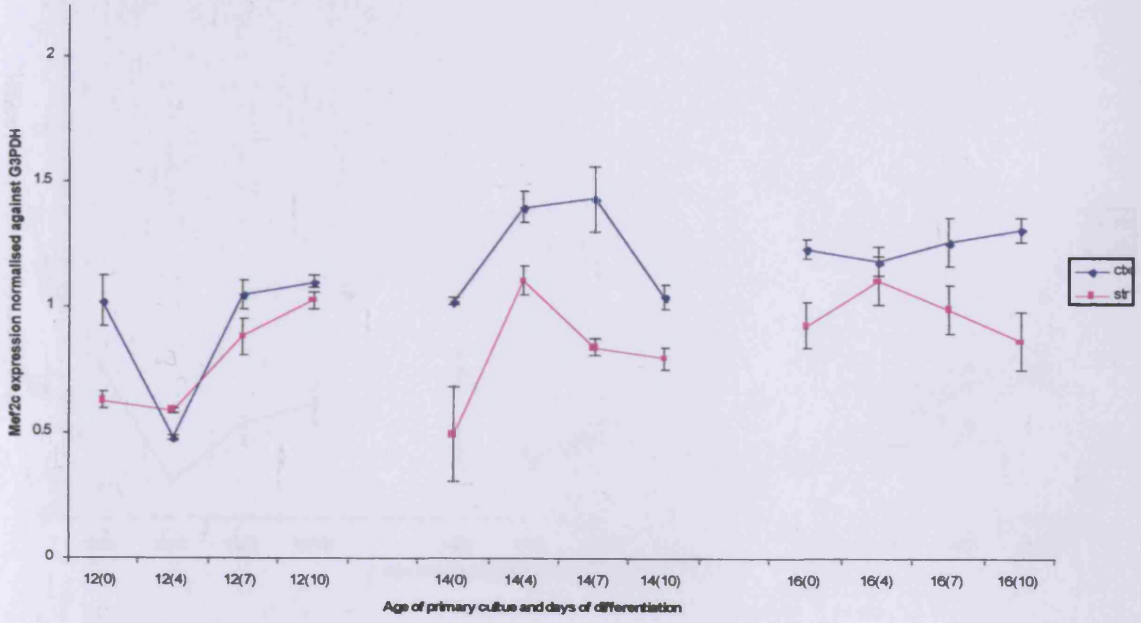
Fig 6.6; Expression of G *ucy1B3* in varying ages of primary FNP culture at 0, 4, 7 and 10 days differentiation



6.3.6 *Mef2c*

Mef2c levels were seen to rise between E12 and E16 in primary cells, mirroring the Affymetrix micro array results. E12 expression was seen to increase over the differentiation period ($F_{(3,6)}=18.10, p<0.01$), E14 expression was seen to rise slightly overall ($F_{(3,6)}=5.48, p<0.05$), while E16 levels stayed reasonably stable throughout the period of differentiation. There was no difference between cortical and striatal expression in E12 cells, although a difference between cortical and striatal cells was observed in E14 $F_{(1,4)}=48.38, p<0.01$) and E16 $F_{(1,4)}=59.69, p<0.01$).

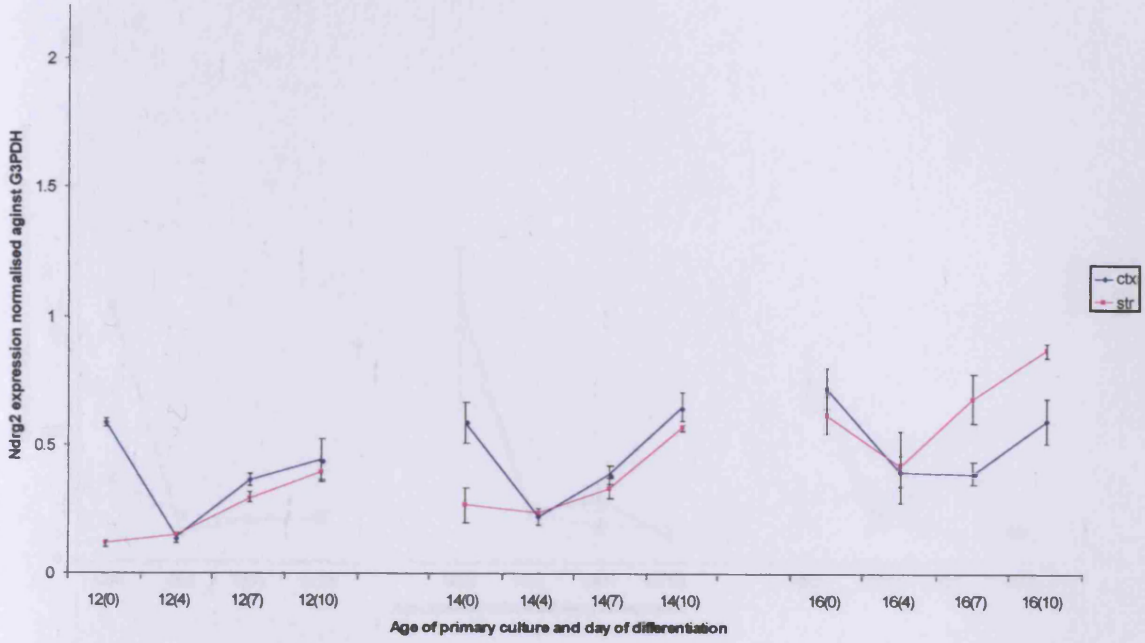
Fig 6.7; Expression of *Mef2c* in varying ages of preimary FNP culture at 0, 4, 7 and 10 days differentiation



6.3.7 *Ndrg2*

Ndrg2 primary cell expression was seen to rise between E12 and E14, again mirroring the micro array and *in situ* results. E12 cells showed a significant increase in expression of this gene over the differentiation period ($F_{(3,6)}=25.29, p<0.001$). E14 cells also showed an increase in expression ($F_{(3,6)}=12.63, p<0.01$), however, no significant increase was observed in E16 cells through the period of differentiation. There was no significant difference between striatal and cortical cells in any of the gestational stages.

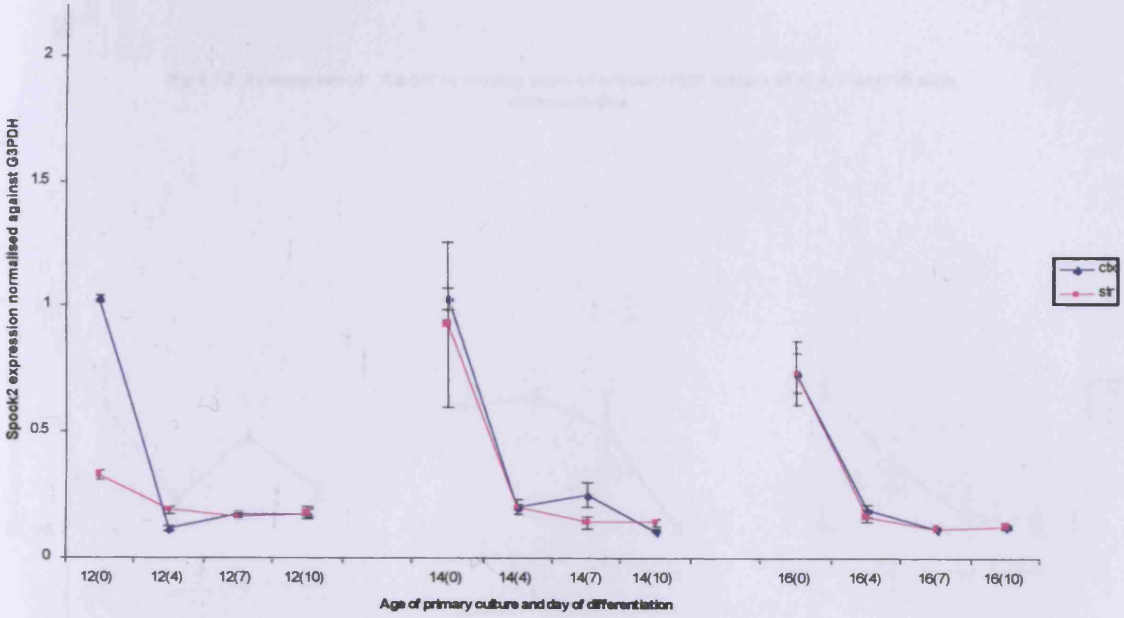
Fig 6.8; Expression of *Ndrg2* in varying ages of primary FNP culture at 0, 4, 7 and 10 days differentiation



6.3.8 *Spock2*

Levels of *Spock2* were seen to rise between E12 and E16 primary cells which support the results of the micro array and *in situ*. However, E12 Striatal cell expression levels decreased over the differentiation period ($F_{(3,6)}$=13.18,$p<0.01$). E14 expression also fell significantly through the differentiation period ($F_{(3,6)}$=5.54.$p<0.05$, as did E16 ($F_{(3,6)}$=22.69,$p<0.01$). No significant difference was observed between expression seen in striatal and cortical cells.

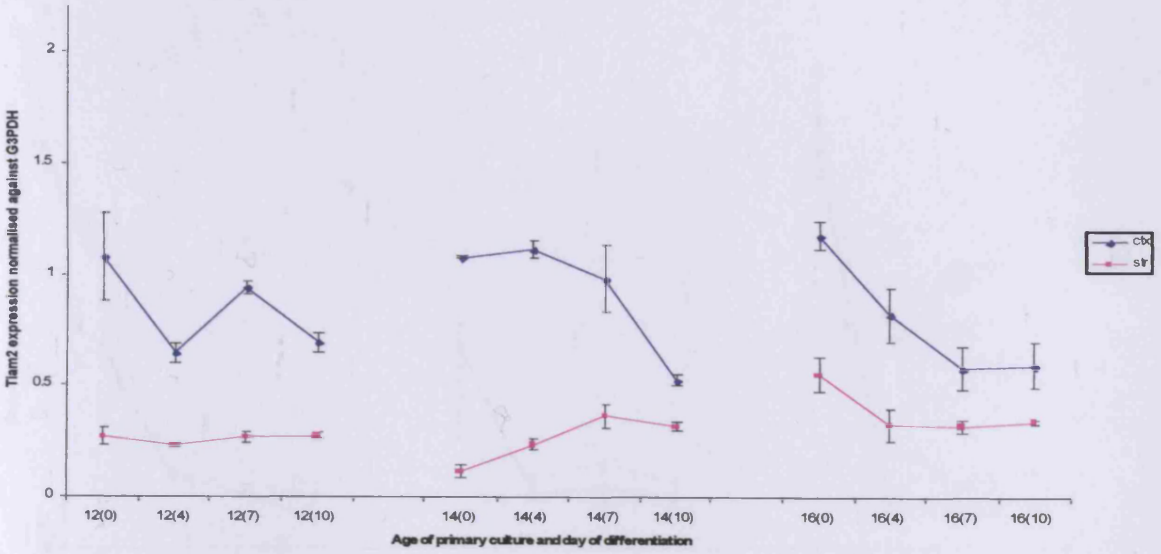
Fig 6.9; Expression of *Spock2* in varying ages of primary FNP culture at 0, 4, 7 and 10 days differentiation



6.3.9 *Tiam2*

Levels of *Tiam2* increased between the ages of E12 and E16 primary cells, supporting the results seen in the micro array. There was no change in the low expression levels of *Tiam2* seen during the differentiation of E12 cells, compared to the higher expression seen in E12 cortical cells ($F_{(1,4)}=46.45, p<0.01$). E14 cells showed an increase in *Tiam2* expression levels ($F_{(3,6)}=11.80, p<0.01$) showing a significant difference to E14 cortical expression, which fell over the same period ($F_{(1,4)}=298.48, p<0.001$). E16 striatal expression fell during differentiation, ($F_{(3,6)}=6.20, p<0.05$) which was also observed to be different to E16 cortical cells, which had higher expression levels throughout the same period ($F_{(1,4)}=30.62, p<0.01$).

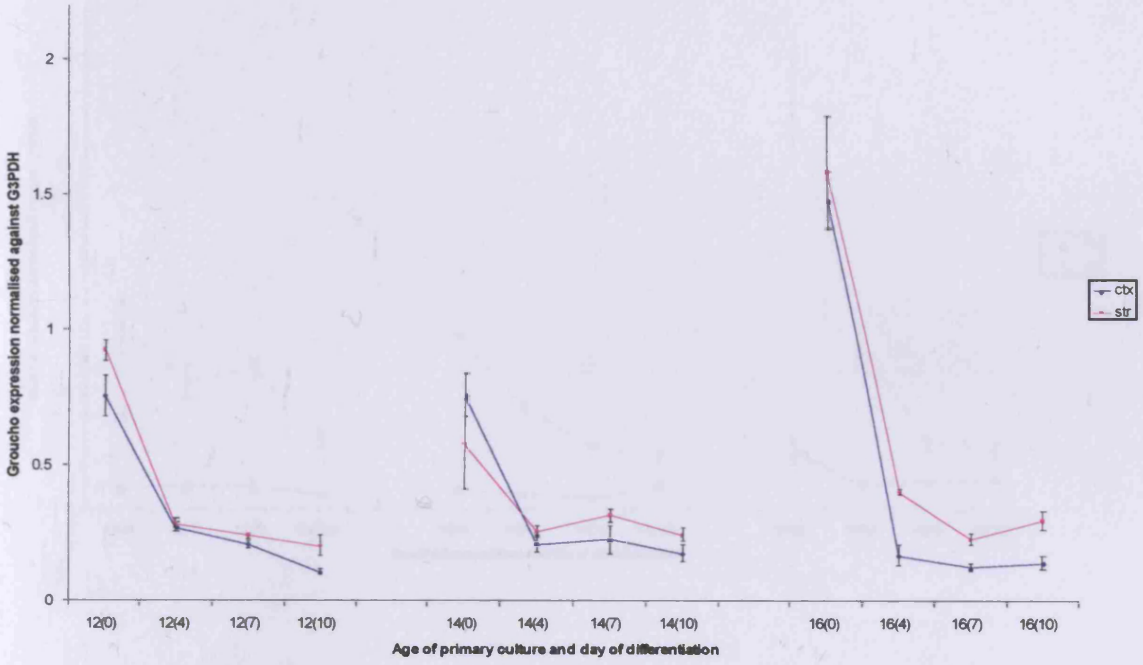
Fig 6.10; Expression of *Tiam2* in varying ages of primary FNP culture at 0, 4, 7 and 10 days differentiation



6.3.10 Groucho/TLE/Grg

Expression levels of *Groucho/TLE/Grg* increase between primary E12 and E16 cells, supporting the micro array results seen in Chapter Four. However, E12 and E16 expression levels in both striatally and cortically derived cells are reduced through the differentiation period ($F_{(3,6)}=112.24, p<0.001$) and ($F_{(3,6)}=38.43, p<0.001$) respectively (E14 cells also show a downward trend although this was not significant). Striatal cells expressed higher levels of *Groucho/TLE/Grg* in E12 cells ($F_{(1,4)}=65.66, p<0.01$), although there was no significant differences between E14 and E16 striatal and cortical expression of *Groucho/TLE/Grg*.

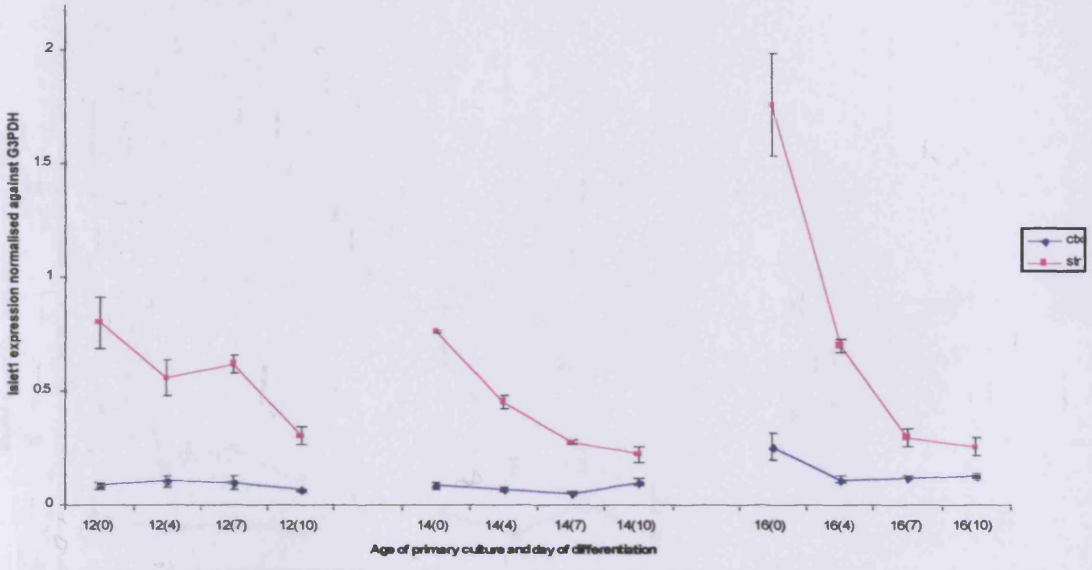
Fig 6.11; Expression of *Grouch/TLE/Grg* in varying ages of primary FNP culture at 0, 4, 7 and 10 days differentiation



6.3.11 Islet1

Levels of striatal expression of *Islet1* increased between E12 and E16 primary cells, supporting the results seen in the micro array study described in Chapter Four. Expression was reduced through the differentiation period in E12 ($F_{(3,6)}=6.91, p<0.05$), E14 ($F_{(3,6)}=97.46, p<0.001$) and E16 cells ($F_{(3,6)}=33.65, p<0.001$). This was significantly different from cortically derived cells, that showed very low levels of expression of *Islet1* throughout the study in all three gestational ages ($F_{(1,4)}=228.01, p<0.001$), ($F_{(1,4)}=830.88, p<0.001$) and ($F_{(1,4)}=115.15, p<0.001$) for E12, E14 and E16 cells respectively.

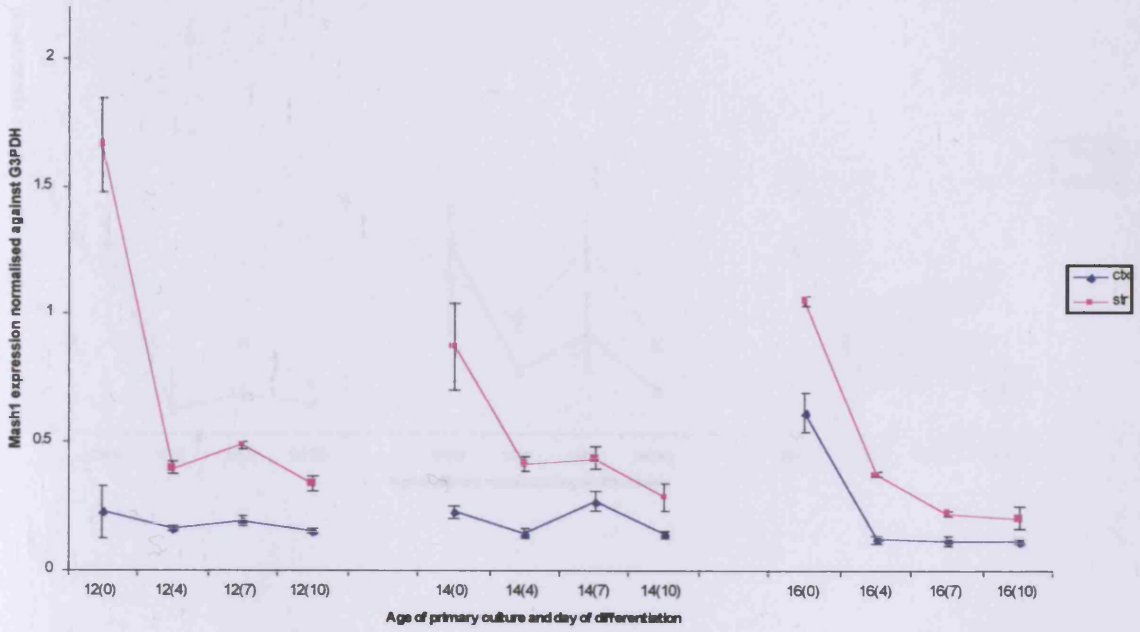
Fig 6.12; Expression of *islet1* in varying ages of primary FNP culture at 0, 4, 7 and 10 days differentiation



6.3.12 Mash1

Mash1 expression was seen to reduce between the gestational ages of E12 and E14 in striatal cells and increase slightly in cortical cells. In all gestational ages expression of *Mash1* reduced significantly by Day 4 and remained low throughout differentiation ($(F_{(3,6)}=44.55, p<0.001)$, $(F_{(3,6)}=6.39, p<0.001)$ and $(F_{(3,6)}=277.54, p<0.001)$ for E12, E14 and E16 respectively). This was significantly different to expression in cortical cells in all three gestational ages ($(F_{(1,4)}=87.64, p<0.001)$, $(F_{(1,4)}=107.24, p<0.001)$ and $(F_{(1,4)}=107.70, p<0.001)$ for E12, E14 and E16 respectively).

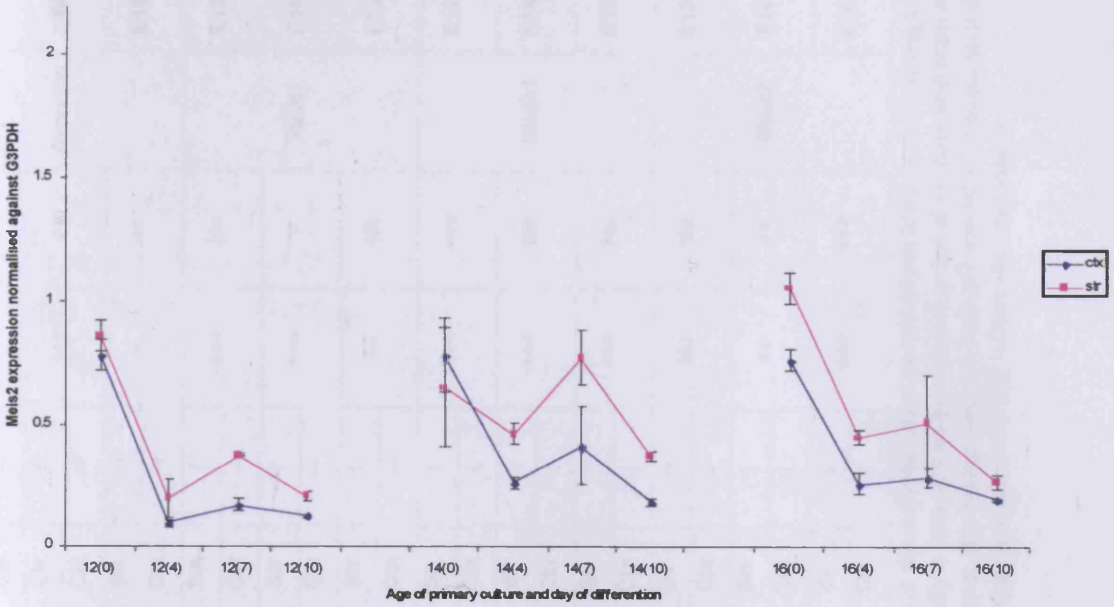
Fig 6.13; Expression of *Mash1* in varying ages of primary FNP culture at 0, 4, 7 and 10 days differentiation



6.3.13 Meis2

Primary levels of *Meis2* were similar in all three gestational ages in both striatum and cortex. Expression levels of *Meis2* reduced throughout the differentiation period in E12 striatal cells ($F_{(3,6)}$=47.06,$p<0.001$) and E16 cells ($F_{(3,6)}$=18.11,$p<0.01$) but not significantly so in E14 striatal cells. Cortical cells were also seen to reduce expression. Striatal levels of Meis2 were generally higher than cortical levels, although this was only seen to be significant at E12 ($F_{(1,4)}$=24.67,$p<0.01$).

Fig 6.14; Expression of *Meis2* in varying ages of primary FNP culture at 0, 4, 7 and 10 days differentiation



Gene	Age	Brain Area	change (Day 0 - Day 10)	incr or decr signif?	a signif. diff betw areas?	Gene	Age	Brain Area	change (Day 0 - Day 10)	incr or decr signif?	a signif. diff betw areas?	Gene	Age	Brain Area	change (Day 0 – Day 10)	incr or decr signif?	a signif. diff betw areas?
Cxcr4	E12	Str	→	No	+	*Mef2c*	E12	Str	↑	+++	No	*Groucho*	E12	Str	↓	+++	++
		Ctx	→					Ctx	→					CTx	↓		
	E14	Str	→	+	+++		E14	Str	↑	+++	++		E14	Str	↓	+++	No
		Ctx	↓					Ctx	→					Ctx	↓		
	E16	Str	↓	+++	+++		E16	Str	→	+	++		E16	Str	↓	+++	No
		Ctx	↓					Ctx	→					Ctx	↓		
Foxp1	E12	Str	↓	+	+++	*Ndrg2*	E12	Str	↑	+++	No	*Islet1*	E12	Str	↓	++	+++
		Ctx	↓					Ctx	→					Ctx	→		
	E14	Str	→	+	No		E14	Str	↑	+++	+		E14	Str	↓	+++	+++
		Ctx	↓					Ctx	→					Ctx	→		
	E16	Str	↓	+	No		E16	Str	↑	++	No		E16	Str	↓	+++	+++
		Ctx	↓					Ctx	→					Ctx	→		
Foxp2	E12	Str	↑	+++	++	*Spock 2*	E12	Str	↓	+++	+++	*Mash1*	E12	Str	↓	+++	+++
		Ctx	↓					Ctx	↓					Ctx	→		
	E14	Str	↓	++	++		E14	Str	↓	+++	No		E14	Str	↓	+++	+++
		Ct	↓					Ctx	↓					Ctx	→		
	E16	Str	↓	+++	++		E16	Str	↓	+++	No		E16	Str	↓	+++	+++
		Ctx	↓					Ctx	↓					Ctx	↓		
Gucy1 β3	E12	Str	→	+++	+	*Tiam2*	E12	Str	→	No	No	*Meis2*	E12	Str	↓	+++	++
		Ctx	↑					Ctx	↓					Ctx	↓		
	E14	Str	↑	++	No		E14	Str	↑	++	++		E14	Str	↓	+	No
		Ctx	↑					Ctx	↓					Ctx	↓		
	E16	Str	↓	+++	No		E16	Str	↓	+++	+++		E16	Str	↓	+++	No
		Ctx	↓					Ctx	↓					Ctx	↓		

Table 6.2; Summary of the gene expression changes seen in this study over the differentiation period (from primary cells through to Day 10 of differentiation). The arrow shows the general trend seen in the levels of expression of each gene. Ideally a comparison between all genes at all ages and brain regions would have been run on one PCR and one gel, however, due to practical reason this was not possible. However, the background on each gel was taken into account during each analysis. There were also no major differences between age groups in cell culture (see histology).

6.4 Discussion

In this study I looked at the expression patterns of my candidate genes in primary murine neural striatal cells as they differentiated *in vitro* over a ten day period. It was important to characterise the gene expression patterns occurring during *in vitro* differentiation of these cells in order to be able to interpret the differentiation of cells in other conditions; for example the differentiation of striatal precursors that have been allowed to proliferate in vitro (as in Chapter Seven) and the differentiation of ES cells after exposure to protocols to 'direct' their phenotype. Although it wasn't the primary aim of this study, it is also interesting to assess how closely striatal differentiation *in vitro* recapitulates that occurring *in vivo*.

In order to understand the changes seen in this experiment, it is important to put the timing of the *in vitro* differentiation experiments into context. The cells dissected out at E12 would still have carried on developing as I put them into culture so by Day 4 of differentiation *in vitro* these cells may be expected to be comparable in age to E16 primary cells. Likewise, Day 4 of differentiation for E14 cells may be expected to be the equivalent to E18 and thus is already beyond the time point looked at in the Affymetrix experiment described earlier. It is important to bear this in mind when analysing the above results. However, although there is a wealth of experimental data demonstrating that striatal cells between E12 and E16 are able to differentiate *in vitro* into cells with characteristics of MSNs (Fricker *et al.*, 1997a; Fricker *et al.*, 1997b), it cannot be assumed that the differentiation of such cells will necessarily occur precisely as it would *in vivo*, perhaps because the insult of cell preparation causes damage and perturbs certain cellular processes or because cells plated in a culture dish are not exposed to all the developmental signals that they would be exposed to *in vivo*.

It must also be remembered that not all cells in the developing foetal brain at E12, E14 or E16 are differentiating; a proportion are still proliferating or may have differentiated at an earlier stage. Furthermore, although the majority of cells within the developing striatum during this time are neuronal, there are also other populations of cells within the developing striatum, including glial progenitors. Such cells may have an effect on the overall gene expression within the cultures, although even by E16, the majority of striatal cells are neurons and neuroblasts and so this effect would be expected to be

relatively small. However, these results still allow us to estimate what is actually happening to the cell populations we dissect out of the developing foetus (at varying ages) after they have been put into culture and differentiated *in vitro*. As such, it is relevant to take the candidate marker genes picked from the earlier micro array and try to work out which of these genes, if any, is a good marker of striatal differentiation.

Changes in gene expression were analysed in both developing striatum and cortex. As shown in previous chapters, none of the genes were found to be expressed exclusively in striatum, although some were expressed in much higher levels in striatum; i.e. *Foxp1, Foxp2*. These genes may well be useful as striatal markers during differentiation. Clearly, the exclusive expression of a gene in the striatum would be significant as it would indicate that the gene had the potential to be used as a striatal-specific marker. To date, the only clearly striatal specific marker we have available is DARPP-32, which is found exclusively in terminally differentiated MSNs and not developing cells. A striatal specific developmental marker would be useful as it would provide the potential to be able to select and purify striatal cells for cell transplantation purposes. However, this was not the aim of the current project and changes that occur in both brain areas can still be useful for the purposes of developing a gene expression 'signature' of developing striatal cells.

Some of the changes seen during this study may not be meaningful in terms of cell development, for example, in many of the genes studied, there was a fall in expression at Day 4 with an increase in expression by Day 7. This initial drop could be interpreted as the reaction to being put into culture with a substantial proportion of the cells being stressed in the initial stages with subsequent recovery.

There were also some technical limitations that also need to be born in mind when interpreting these results:

- Due to limitations in the numbers of samples that could be run on the one PCR machine available and the space available on one agarose gel, it was not possible to run all genes and all ages on one gel. For any one gene at each of the three gestational ages, all differentiation time points for both striatum and cortex were run on the same gel and so were directly comparable. However, by comparing each gene's expression level with that of a housekeeping gene that remains

constant, and by taking into account the background of all the gels while analysing them I have attempted to make all the data comparable

- Gene expression levels in all cases relate to a population of cells and not individual cells, although at the developmental ages examined, the majority of cells are differentiated or developing MSNs. One method of analysing this further would be to use antibodies for proteins that have been coded for by these transcripts and this was done for the Foxp genes, although at the time of writing antibodies were not available for the majority of the candidate genes.
- Due to RT-PCR being a semi-quantitative method of analysis it can be said that the results of this screen can only be used as "indications" of the true gene expression changes that are occurring at the different stages of culture tested. However, this method can be argued as being as valid for detecting quantitative differences in gene expression as long as the following have been adhered to: (i) the PCR cycle number has to be proven to be within the linear phase of amplification; (ii) the use of replicates and statistical analysis must prove significance (Halford, 1999). (In this instance the cycle number had been optimised) and 3 biological replicates of each condition were used. This therefore reduced the chances of Type 1 errors being made (where the null hypothesis is rejected but in fact is true; i.e. that there was no difference between expression levels of a gene between the start of expansion/differentiation and the end point of the experiment).
- Other examples of errors that could have occurred have already been discussed on previous chapters and include inaccuracies in gauging gestational age, dissection inaccuracies, cell miscounting, pipetting errors during the RNA extraction, RT-PCR and quantification of cDNA product.

6.4.1.1 Interpreting gene changes in differentiating cells.

For many, although not all, of the genes examined, the changes in the primary tissue (Day 0) samples changes with gestational age in accordance with the results of the Affymetrix study; i.e. *Cxcr4, Foxp2*, etc. However, this was not the case with all the candidate marker genes studied; i.e.*Foxp1, Spock2* and *Tiam2*. These differences in results could be due to experimental error or due to the differences in cell populations

present between normal development and what is found *in vitro*, although at this stage it is hard to say. Further replicates would need to be carried out know for sure.

As cells differentiated over the 10 days in culture, clear and significant changes in expression levels could be seen for some of the genes – i.e. those changes seen in the levels of *Foxp1, Foxp2, Ndrg2 Spock2* and *Tiam2,* although in others there was more noise; i.e. *Cxcr4, Mef2c* and *Gucy1β3*. In order to be used as markers of striatal differentiation, genes would have to have clear changes that were reproducible. In order to see if the changes seen in this study were reproducible, more replicates would ideally have to be carried out.

This is, to our knowledge, the first systematic longitudinal study of gene expression changes carried out in striatal cells differentiated *in vitro*.

Some of the changes seen in this study were largely anticipated; It is known from previous studies (Tamura *et al.*, 2000; Shu *et al.*, 2001; Tamura *et al.*, 2003; Ferland *et al.*, 2003; Takahashi *et al.*, 2004) that *Foxp1* and *Foxp2* are both expressed in the developing and mature brain, so these results are in accordance with what we know. However, both of these genes were shown to drop in expression levels in E16 cells that were between 4 and 7 days into their differentiation. What is not known is whether the levels of these repressor genes fluctuate between these periods. These results could also be due to the cultures being diluted by the increasing numbers of glia that are differentiating at this stage of development, or by the fact that some of the cells that express these genes are dying off earlier on in the differentiation period due to the insult of being put into culture.

6.4.1.2 Specific gene changes; those that recapitulate developmental stages

The majority of genes shown in this study may well be recapitulating normal development as was seen in both the micro array study described in Chapter Four and the *in situ* study described in Chapter Five. For instance; *Groucho/TLE/Grg, Mash1, Meis2, Islet1, Mef2c* and *Foxp2* possibly including *Foxp1, Ndrg2* and *Cxcr4*, all look as if they increase after being put into culture, although some of them do have a period in the first four to seven days when expression seems to be reduced, although this could be down to the cells rounding up and trying to deal with the stress of trypsinisation and being put into culture as discussed earlier. *Spock2* is certainly reduced *in vitro*, and *Tiam2* does not increase in expression as indicated by the Affymetrix results. Why this is, is not known at

this point, but further studies looking into the function of these genes may well provide answers.

The study of additional genes also gave a lot of information as these genes did react as they might *in vivo*;

Groucho/TLE/Grg codes for a gene that inhibits neuronal differentiation so it could be expected for this gene to fall sharply once differentiation began. The primary (Day 0) levels also support previous literature that indicates that E16 is the peak of neurogenesis in the striatum (Fricker-Gates *et al.*, 2004a).

Islet1 is known to be expressed within the developing LGE, marking out a domain known to generate striatal projection neurons. Once these cells have been born, this gene is down regulated (Skogh *et al.*, 2003). Another hypothesis suggests that this gene continues to be expressed in just a small amount of cholinergic cells that reside within the striatum (Wang and Liu, 2001), however, if this were the case then it might be that the conditions of culture either do not favour the differentiation of these cells or the amounts of Islet1 expressed are too small to be picked up by this technique.

Mash1 is a pro neural gene that works in conjunction with other transcription factors to specify neuronal identity (Lo *et al.*, 1991; Guillemot and Joyner, 1993; Joyner and Guillemot, 1994; Hirsch *et al.*, 1998; Horton *et al.*, 1999). Expression of *Mash1* is found in the VZ at around E10.5-E12.5, and spreads into the SVZ in cells that continue to divide as they migrate (Porteus *et al.*, 1994; Casarosa *et al.*, 1999). As these cells differentiate this expression is reduced as is seen in this study.

Meis2 has been reported to be a major influencing component of striatal differentiation neurons born in the LGE at around E10.5 onwards (Toresson *et al.*, 1999; Toresson *et al.*, 2000a). By E16.5 this gene is reported to be down regulated within the striatum, only to be found in a small area of the cortical plate (Toresson *et al.*, 2000a). Thus, these results support the previous literature and the data suggests this gene follows a normal expression pattern.

In conclusion, this study has indicated that once the cells are put into culture, after an initial period the surviving cells adopt gene expression patterns that would loosely mimic what is seen in normal development, in most genes. However, as development continues, the amount of glia differentiating within the culture rises and this might have an effect on the overall gene expression seen within these populations of cells as the gene

pool is 'diluted' with genes specific to the rising numbers of glial cells being born. The more these patterns are reproduced, the more reliable we can assume these changes to be, and the more confident we can be in using some of them for markers of striatal differentiation.

Chapter Seven

7 Characterisation of gene expression changes in proliferating foetal neural precursors (FNPs).

7.1 Introduction

In previous chapters I identified a number of genes that are shown to be up-regulated in the WGE over the peak period of striatal neurogenesis and have validated this data for a small subset of genes using other methods of analysis. In Chapter Six I have used some of these genes to examine whether foetal striatal tissue allowed to differentiate in culture recapitulates some of the gene expression changes seen *in vivo* In this chapter I used the same techniques to characterise gene expression changes in foetal neural precursors (FNPs), derived from the foetal striatum that have been allowed to proliferate in culture.

Although trials in both animal models and in the clinical setting have provided 'proof of principle', future mainstream clinical use of primary tissue remains unlikely for reasons of supply and demand. However, FNP cell populations may provide a source of viable cells that may be suitable for this purpose and are also more amenable to quality control procedures (see discussion of FNPs in introduction: Chapter One). FNPs that have been derived from the developing striatum and have been allowed to proliferate for a relatively short period of time *in vitro* are able to differentiate into MSNs as indicated by DARPP-32 expression, but previous studies have shown that once these cells are expanded *in vitro* over a greater number of passages, their differentiated phenotype changes: The essential changes are that astrocytes become more abundant with increasing time *in vitro* (Quinn *et al.*, 1999) and although, neurons continue to develop from the expanded cells these neurons no longer express DARPP-32 and no longer display a striatal (MSN) phenotype. PCR of short-term and long-term expanded cells has demonstrated some gene expression changes in the late-expanded cells (Zietlow *et al.*, 2005) that suggest that loss of positional information may be a factor in these changes. It has also been reported that the post transplant survival of grafts using long-term (20 weeks) expanded cells is reduced when compared with shorter-term expanded cells (4 weeks) (Zietlow et al., 2005).

There are a number of possible reasons for these changes;

- There is positive selection for a non-striatal lineage as the period of culture continues, perhaps due to the culture conditions.
- The FNPs that are forced to proliferate *in vitro* in the presence of EGF and FGF2 become no longer capable of responding to signals that would otherwise drive their differentiation towards a striatal neuronal phenotype. As cells proliferate *in vitro*, cells that had been committed to a striatal phenotype eventually die and new cells are born that are not exposed to the normal sequence of developmental signals that would be present in the foetal brain at this time and so they are not properly committed to this phenotype.

Until now we have had very limited tools for exploring the mechanisms for change in these expanded cell populations. This work represents a potential advance as the future study of these issues would be greatly facilitated if markers of MSN differentiation were available.

In this experiment the expression of the same genes characterised for primary cell differentiation in Chapter Six (including the genes that were not identified as especially highly up-regulated in my screen, but have been identified in previous literature as being important for striatal development) are assessed for their expression in striatally derived FNPs that have been expanded in culture.

In this study PCR was used to assess gene expression patterns occurring in striatal-derived FNP cells during their period of *in vitro* expansion (Day 0, Day 2, Day 4, Day 7, Day 14 and Day 28). Cells allowed to proliferate for 28 days were then allowed to differentiate on a substrate for a further 7 days *in vitro*, for comparison with cells allowed to differentiate immediately after isolation form the foetal brain in Chapter Six.

FNPs can be derived from foetal striatum at various gestational ages and we have ourselves derived them from mouse and rat cells ranging from E11 to E20. At later times, the cultures produce mostly glia but up to E16 also produce neurons. The FNP cultures tend to have a greater potential to produce neurons, the younger they are in terms of their gestational age of derivation, however, we cannot assume that the cultures producing greater proportion of neurons are the ones that will most readily produce striatal neurons, given the right conditions. From experiments to date, all striatally

derived FNPs lose the capacity to produce MSNs with time in culture, and so at this stage of the study, it was felt important to perform these studies on FNPs derived from foetal striatum over a range of gestational ages.

A second related reason is that in the original micro array screen, many of the genes seen to be up-regulated were expressed at low levels at E12 and high levels at E16, and thus it was possible that we might also observe differences in the response of genes to the proliferating conditions, depending on whether they were 'switched on' or not at the start of the proliferation period.

7.2 Methods.

Dissection of tissue, tissue culture, RNA extraction and RT-PCR were all carried out as per methods laid out in Chapter Two; Methods.

All methods and materials were the same as those used in Chapter Six (apart from those changes mentioned below) using the same PCR primers.

7.2.1 Experimental design

7.2.2 *Expansion experiment*

Three biological replicates from both WGE and cortical tissue were dissected from murine embryos at each gestational age (E12, E14 and E16). Each biological replicate sample was derived from pooled tissue of pups from at least 1 separate time-mated dam. The tissue was then dissected as per the methods set out in Chapter Two and put into single cell suspension by trypsinisation. Equal amounts of cells (2,000,000/well) were then cultured in expansion medium (for constituents see Chapter Two; Methods) in six-well plates at 37°C in a tissue culture incubator. Aliquots of cells were taken out for analysis at the following stages;

1. Primary Tissue (Day 0)
2. Day 2
3. Day 4
4. Day 7
5. Day 14

and

6. Day28.

Each aliquot was divided into two. One sample was allowed to differentiate to assess the production of neurons and astrocytes and to ensure that the gene expression patterns were not due to artefacts of the culture conditions: for example, on occasions (for reasons that are poorly understood) FNP cultures may turn out to contain pure populations of astrocytes with an absence of neurons. Cells were plated onto a substrate on four cover-slips in 24-well plates (plated at 100,000 cells per cover-slip) and allowed to differentiate for 7 days (in differentiation media; for constituents see Chapter Two).

The rest of the cells were then centrifuged (at 3000 x g for 3 minutes) into a pellet, the media removed and replaced with RNA Later stabilisation solution before being frozen at -80°C pending RNA extraction. Where there was a shortage of cells due to poor expansion of cell numbers, only the latter was carried out. Cells plated on cover-slips were fixed with 4% PFA (for those cells being stained for βIII-Tubulin), 4% PFA/0.2% Gluteraldehyde (for those cells being stained for GABA antibody) or ice cold Methanol (for those stained for the Foxp1 or Foxp2 antibodies) and then washed three times with PBS before being stored at +4°C in PBS azide, pending immuno cytochemistry.

7.2.3 Differentiation of cells after 28 Days proliferation

In addition to the above, at 28 days expansion, samples of cells differentiated for 7 days on a substrate were also prepared for RNA extraction as above.

All RNA samples and PCRs were processed as outlined in Chapter Two and analysed in the same way as those in Chapter Six. However, unlike in Chapter Six, where there were less samples, due to number of samples in this experiment, not all were run at the same time, or on the same gel (i.e. although E12 Day 0 to Day 28 Foxp1 samples were all run in the same PCR and all were run on the same gel, different ages; i.e. E12, E14 and E16 could not be run together due to lack of space in the PCR machine or on the gel). However, background was taken into account on analysis of the PCR products, using the AlphaEase2200 imaging system, the PCR conditions were the same and the gels were made and run using exactly the same ingredients and conditions. Because of this, caution must be taken when comparing between different age primary cells, and also different genes.

Results.

The following results show firstly the primary data (pictures of the 1% agarose gels taken by the AlphaEase2200 imaging system) on which the spot-denso analysis was carried out (note *G3pdh* PCR products for each sample are shown at the bottom of Figures 6.1a-6.1c). These are followed by a series of graphs plotting those normalised expression levels with SEMs and descriptions of the gene expression changes compared with the *G3PDH* expression, including results of the statistical analysis if significant. Comparisons can be made between the results from the E12, E14 and E16 cultures as all can be related to *GAPDH* levels. However, some caution is required as each set of PCR product for each gene was run on separate gels (due to the sheer number of samples and space restrictions on the gels). However, it is worth noting that the primary (Day 0) results are the same samples as those used in Chapter Six and, as discussed in that chapter, are largely consistent with the micro array results described in Chapter Four.

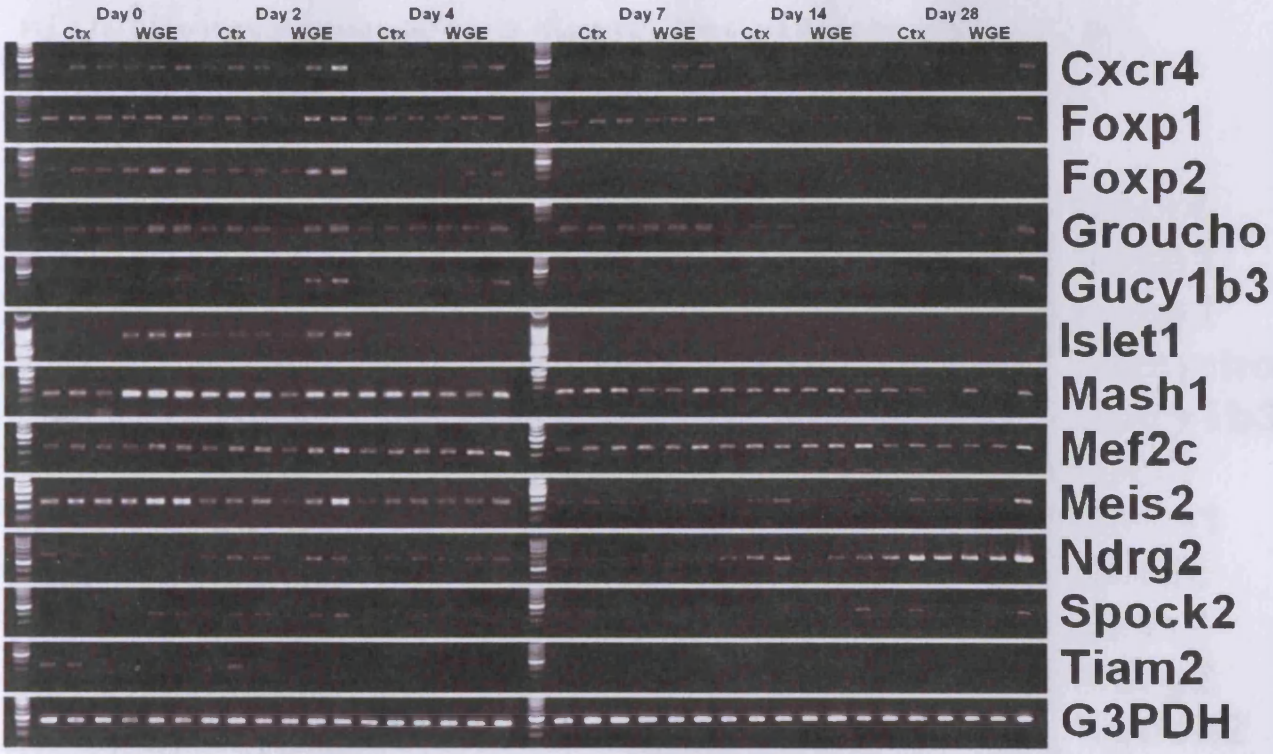


Fig. 6.1a; E12 primary tissue expanded over 28 days *in vitro*. The above shows three replicates for both Ctx and WGE at Day 0 (primary), Day 2, Day 4, Day 7, Day 14 and Day 28.

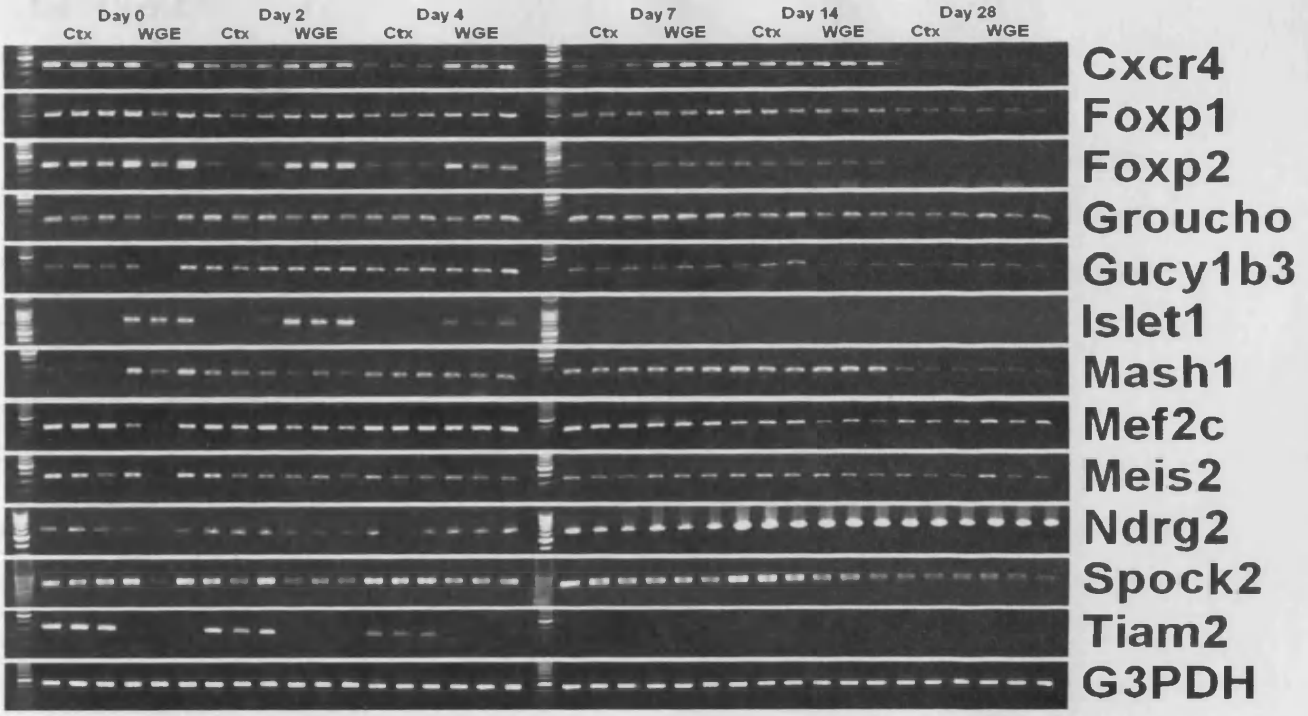


Fig. 6.1b; E14 primary tissue expanded over 28 days *in vitro*. The above shows three replicates for both Ctx and WGE at Day 0 (primary), Day 2, Day 4, Day 7, Day 14 and Day 28.

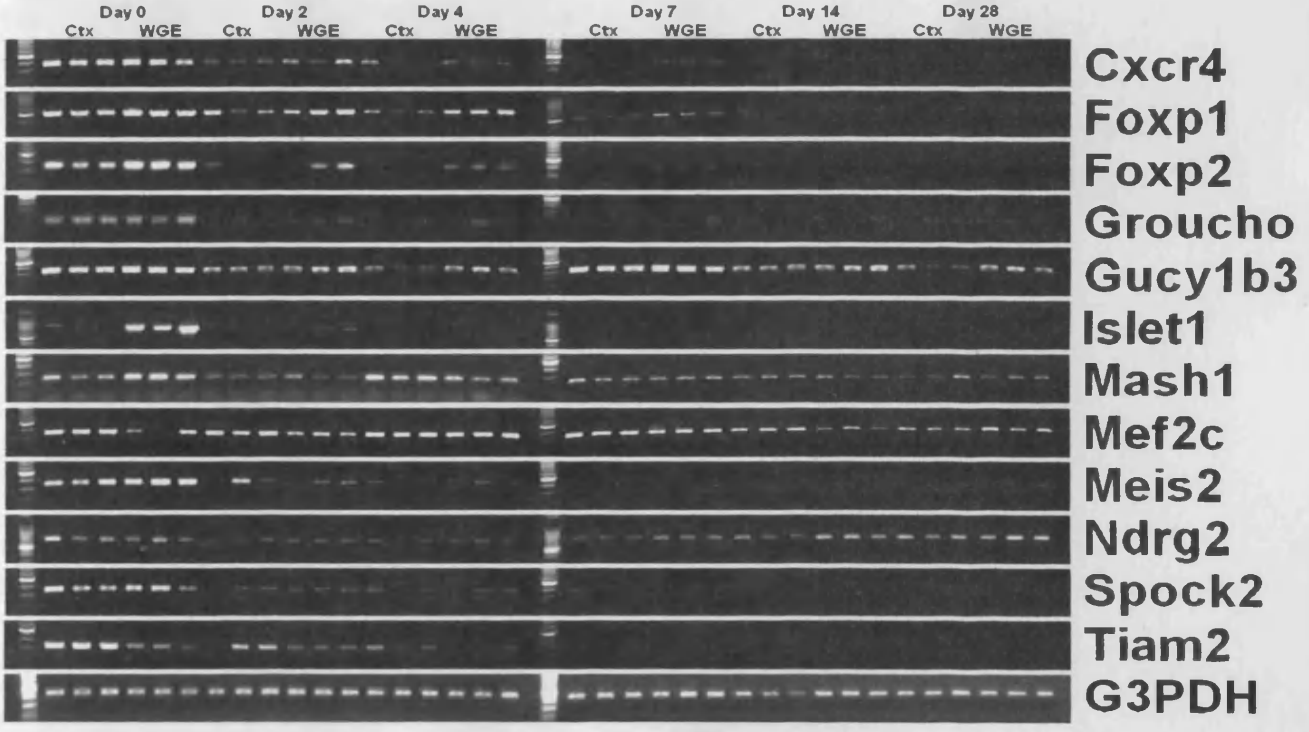


Fig. 6.1c; E16 primary tissue expanded over 28 days *in vitro*. The above shows three replicates for both Ctx and WGE at Day 0 (primary), Day 2, Day 4, Day 7, Day 14 and Day 28.

7.2.4 *Cxcr4.*

7.2.4.1 *E12* (Fig. 6.2a)

E12 striatal cells showed a significant reduction in expression of *Cxcr4* over the 28 day expansion period ($F_{(5,10)}=3.84, p<0.05$), with a decline towards Day 14 followed by a slight (insignificant) rise at Day 28. There was no significant difference between the *Cxcr4* expression patterns of striatally derived cells and cortically derived cells.

7.2.4.2 *E14* (Fig. 6.2b)

E14 striatally derived cells also showed a reduction in expression of *Cxcr4* ($F_{(5,10)}=5.67, p<0.01$). This overall reduction over 28 days was similar to that seen in cortically derived cells. These low levels of expression at 28 days were maintained after differentiation for 7 days, with no significant increases in expression seen.

There was also found to be a difference in *Cxcr4* expression patterns between striatally and cortically derived cells throughout the expansion period ($F_{(1,4)}=13.89, p<0.05$).

7.2.4.3 *E16* (Fig. 6.2c)

Cxcr4 expression in striatally derived cells was reduced in the 28 day expansion period ($F_{(5,10)}=49.52, p<0.001$). There was no significant difference seen between striatal and cortical expression patterns of this gene.

Fig. 7.2a *In vitro* expression of *Cxcr4* in expanded E14 Ctx & Str

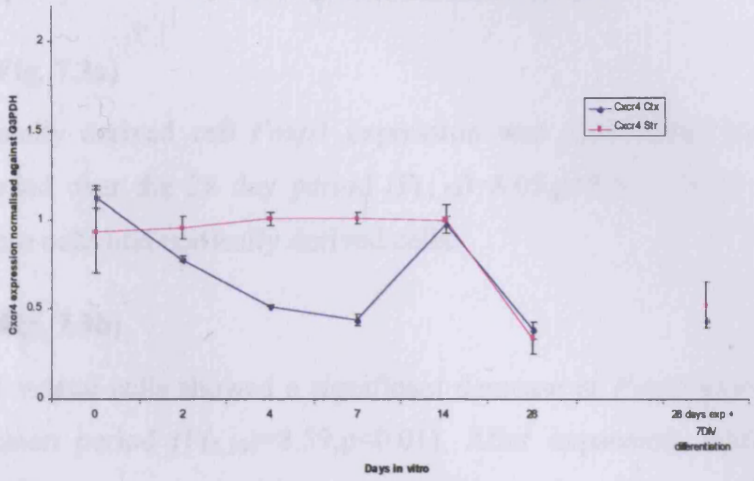


Fig. 7.2b; *In vitro* expression of *Cxcr4* in expanded E14 Ctx & Str

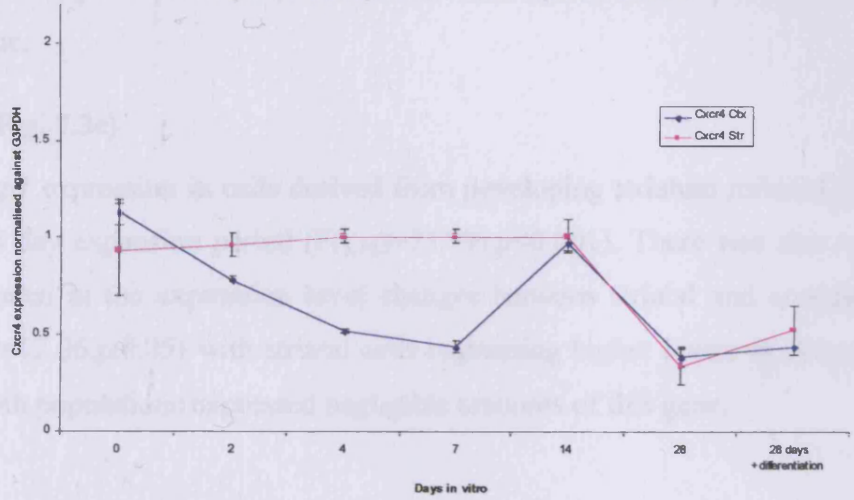
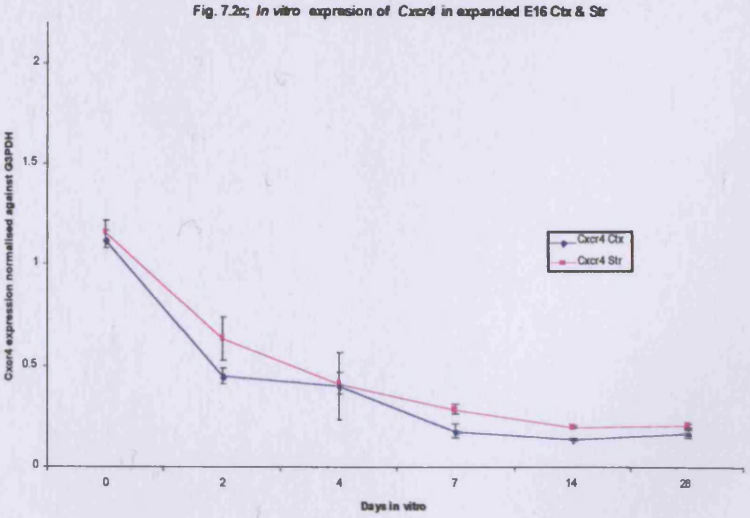


Fig. 7.2c; *In vitro* expression of *Cxcr4* in expanded E16 Ctx & Str



7.2.5 Foxp1

7.2.5.1 *E12* (Fig. 7.3a)

Striatally derived cell *Foxp1* expression was significantly reduced as the cells were expanded over the 28 day period ($F_{(5,10)}$=8.05,p<0.01). There was no difference between these cells and cortically derived cells.

7.2.5.2 *E14* (Fig. 7.3b)

E14 striatal cells showed a significant decrease in *Foxp1* expression over the 28 days expansion period ($F_{(5,10)}$=8.59,p<0.01). After expansion, these levels of *Foxp1* expression did not change significantly on differentiation. There was also no significant difference in *Foxp1* expression between cortically derived cells and those derived from striatal tissue.

7.2.5.3 *E16* (Fig. 7.3c)

Foxp1 expression in cells derived from developing striatum reduced significantly over the 28 day expansion period ($F_{(5,10)}$=71.79),p<0.001). There was also a significant difference seen in the expression level changes between striatal and cortically derived cells ($F_{(1,4)}$=12.06,p,0.05) with striatal cells expressing higher levels of *Foxp1* until Day 14 when both populations expressed negligible amounts of this gene.

Fig. 7.3a; *In vitro* expression of *Foxp1* in expanded E12 Ctx & Str

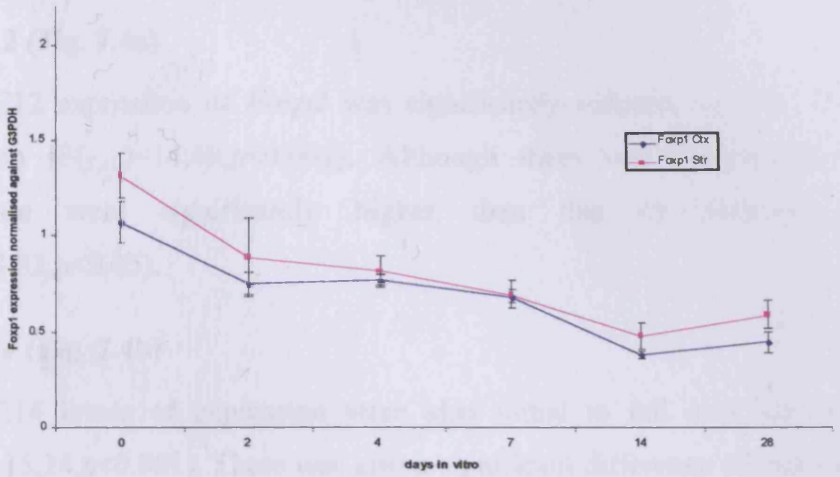


Fig. 7.3b; *In vitro* Expression of *Foxp1* in expanded E14 Ctx & Str

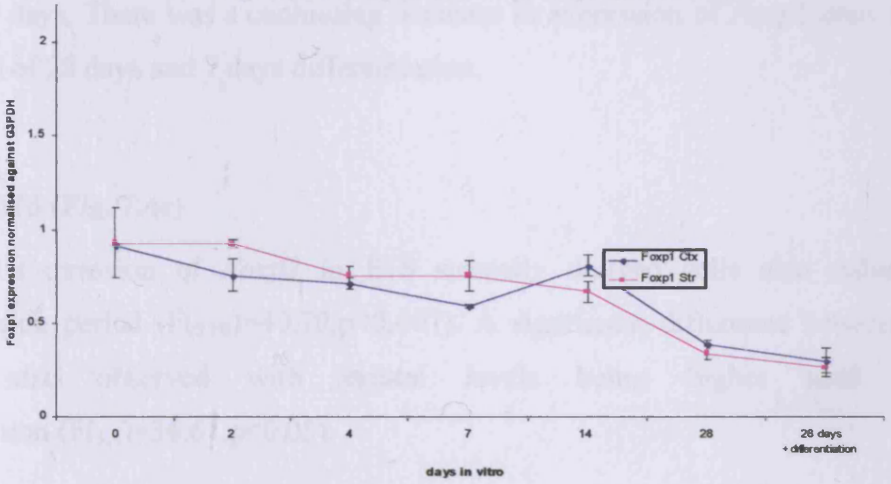
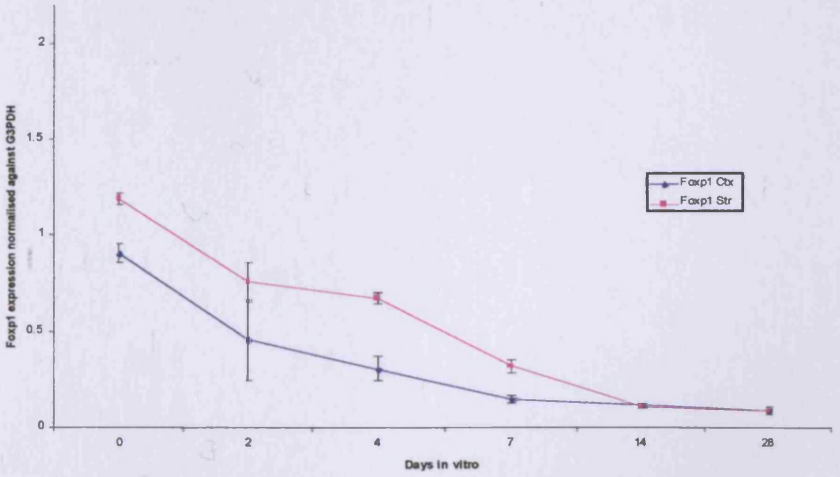


Fig. 7.3c; *In vitro* expression of *Foxp1* in expanded E16 Ctx & Str



7.2.6 Foxp2

7.2.6.1 *E12* (Fig. 7.4a)

E12 expression of *Foxp2* was significantly reduced over the 28 day period of expansion ($F_{(5,10)}=14.49, p<0.001$). Although there was a reduction, the levels of expression were significantly higher than that of cortically derived cells ($F_{(1,4)}=9.32, p<0.05$).

7.2.6.2 *E14* (Fig. 7.4b)

E14 levels of expression were also found to fall over the expansion period ($F_{(5,10)}=15.24, p<0.001$). There was also a significant difference of expression levels over time between brain areas ($F_{(1,4)}=52.30, p<0.05$) with striatal levels being higher for the first 7 days. There was a continuing decrease in expression of *Foxp2* after the expansion period of 28 days and 7 days differentiation.

7.2.6.3 E16 (Fig. 7.4c)

Expression of *Foxp2* in E16 striatally derived cells also reduced over the expansion period ($F_{(5,10)}=40.70, p<0.001$). A significant difference between brain areas was also observed with striatal levels being higher until day 7 of expansion.($F_{(1,4)}=36.61, p<0.05$).

Fig. 7.4a; *In vitro* expression of *Foxp2* in expanded E12 Ctx & Str

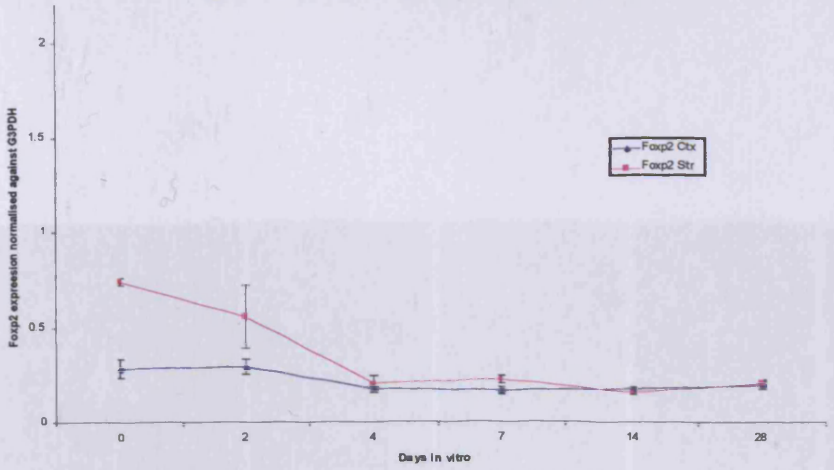


Fig. 7.4b; *In vitro* expression of *Foxp2* in expanded E14 Ctx & Str

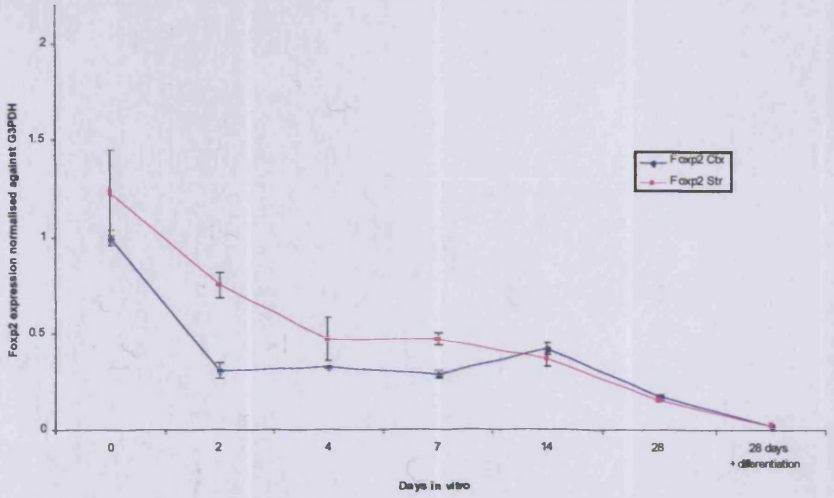
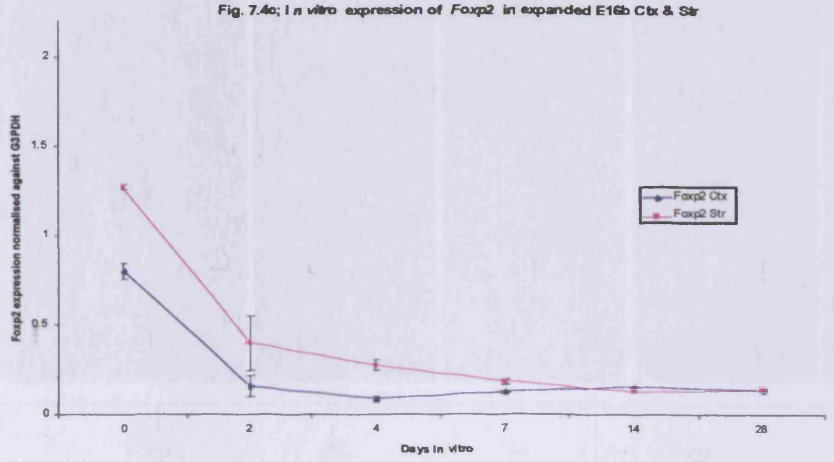


Fig. 7.4c; I*n vitro* expression of *Foxp2* in expanded E16b Ctx & Str



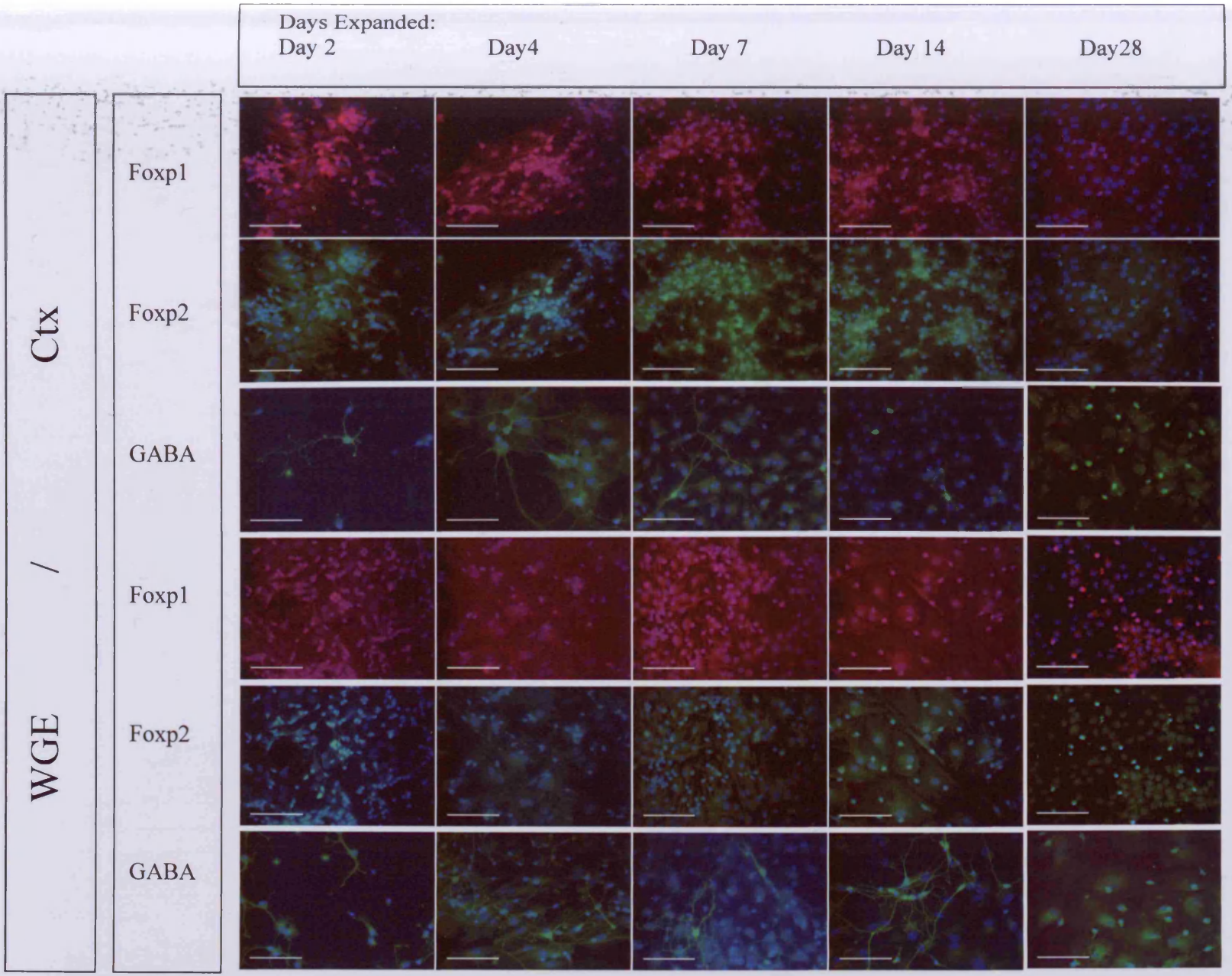


Fig. 7.5; High magnification fluorescent microscopy showing FOXP1 (red), FOXP2 (green) and GABA (green) protein expression in E14 cells expanded *in vitro* for the specified periods, and then differentiated over 7 days. A nuclear stain (Hoechst) has also been used (Scale bar = 200μm). Note different sized nuclei of neurons (small) and astrocytes (large).

Gucy1β3

7.2.7.1 *E12* (Fig. 7.6a)

No significant changes were seen in the expression of this gene in striatally derived cells during the 28 day expansion period. There was also no significant difference between the expression of this gene between brain areas.

7.2.7.2 *E14* (Fig. 7.6b)

E14 striatal cells showed a significant reduction in expression of *Gucy1β3* during the 28 day expansion. ($F_{(5,10)}=7.90$),$p<0.01$). There was no difference between the expression levels of this gene in striatally derived and cortically derived cells. On differentiation after expansion, this gene continued to fall in expression levels.

7.2.7.3 *E16* (Fig. 7.6c)

E16 striatally derived cells showed a significant reduction in expression of this gene over the expansion period ($F_{(5,10)}=58.62$),$p<0.001$). However, no difference was observed between cortically and striatally derived cell expression.

Fig. 7.6a; *In vitro* expression of *Gucy1B3* in expanded E12 Ctx & Str

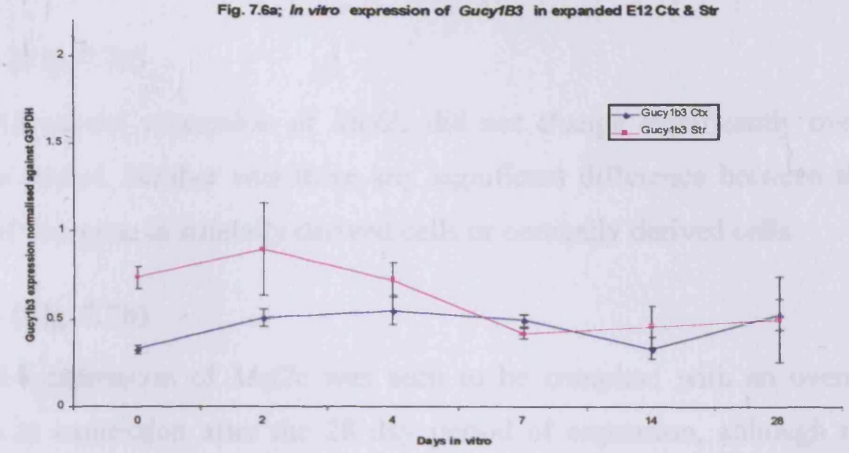


Fig. 7.6b; *In vitro* expression of *Gucy1B3* in expanded E14 Ctx & Str

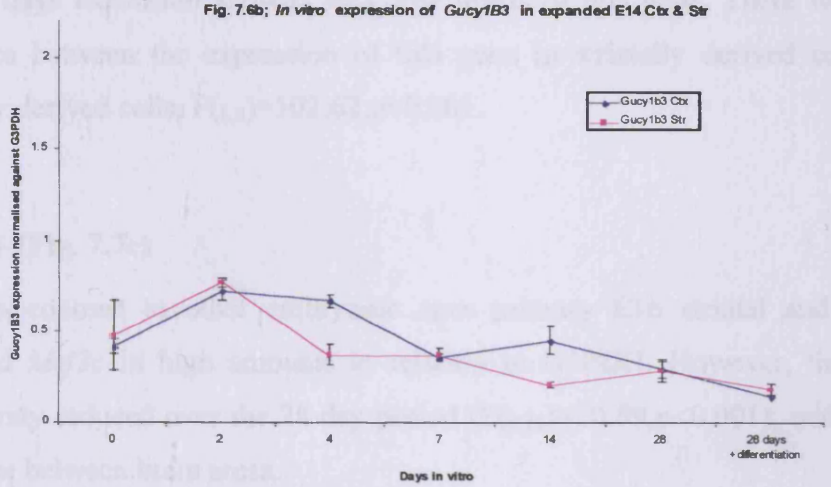
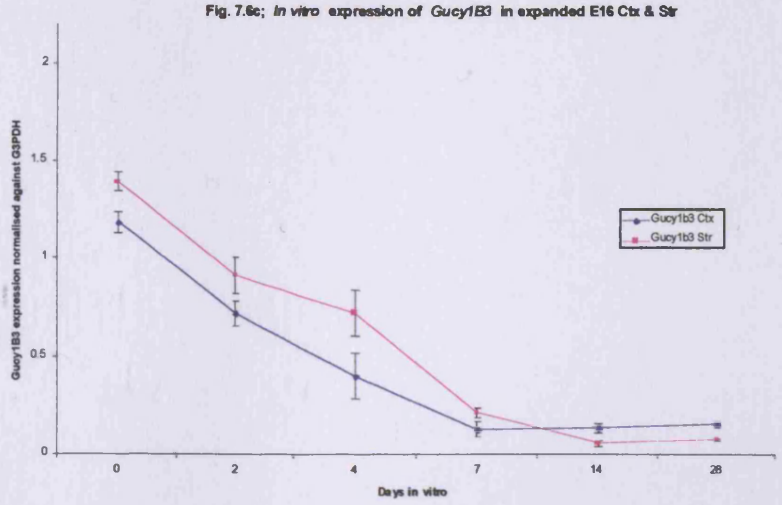


Fig. 7.6c; *In vitro* expression of *Gucy1B3* in expanded E16 Ctx & Str



7.2.8 Mef2c

7.2.8.1 *E12* (Fig. 7.7a)

E12 striatal expression of *Mef2c* did not change significantly over the 28 day expansion period. Neither was there any significant difference between the expression patterns of this gene in striatally derived cells or cortically derived cells.

7.2.8.2 *E14* (Fig. 7.7b)

E14 expression of *Mef2c* was seen to be complex; with an overall significant reduction in expression after the 28 day period of expansion, although rising initially between Day 2 and Day 7 ($F_{(5,10)}=6.32, p<0.01$). Striatal cells that were differentiated after 28 days expansion showed very low levels of this gene. There was a significant difference between the expression of this gene in striatally derived cells and that in cortically derived cells; $F_{(1,4)}=102.62, p<0.001$.

7.2.8.3 *E16* (Fig. 7.7c)

In contrast to other embryonic ages primary E16 striatal and cortical tissue expressed *Mef2c* in high amounts in relation to G3PDH. However, these high levels significantly reduced over the 28 day period ($F_{(5,10)}=20.89, p<0.001$), with no significant difference between brain areas.

Fig. 7.7a; *In vitro Mef2c* expression in E12 expanded Ctx & Str

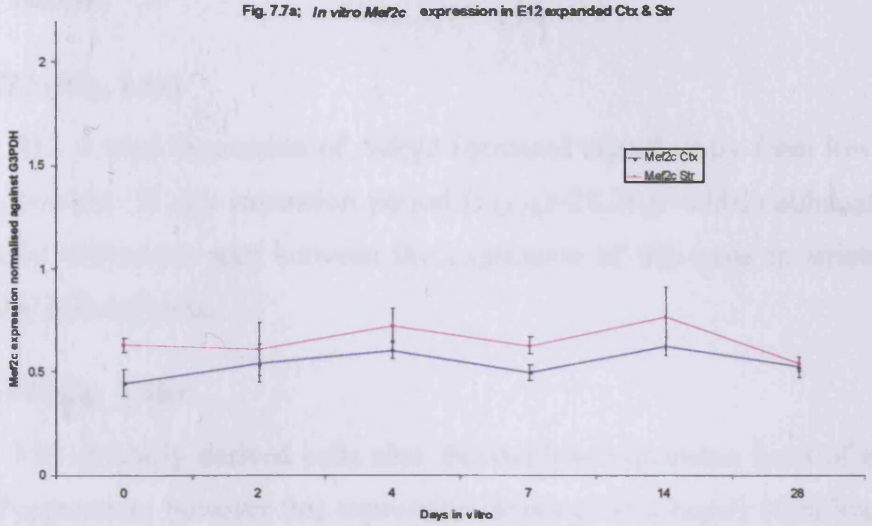


Fig. 7.7b; *In vitro* expression of *Mef2c* in expanded E14 Ctx & Str

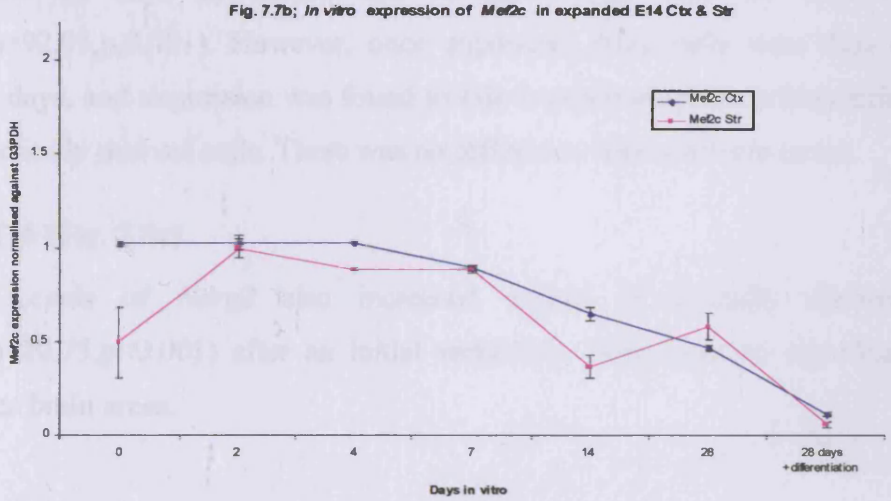
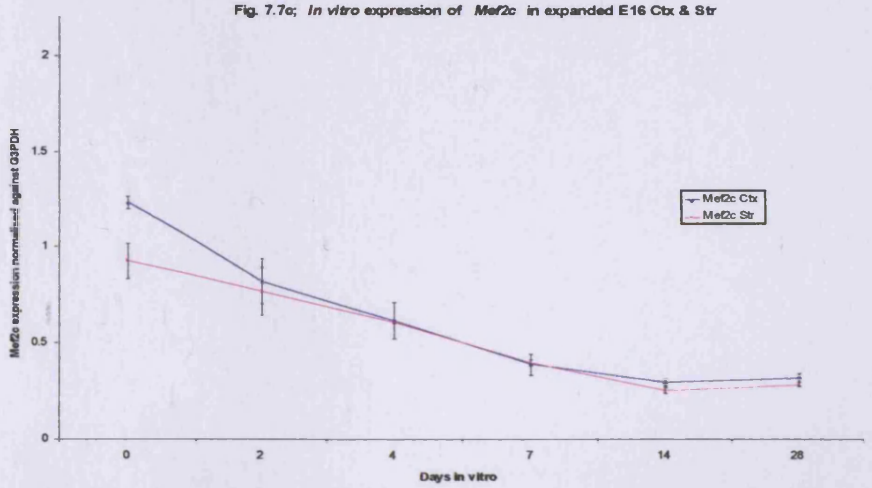


Fig. 7.7c; *In vitro* expression of *Mef2c* in expanded E16 Ctx & Str



7.2.9 Ndrg2

7.2.9.1 *E12* (Fig. 7.8a)

E12 striatal expression of *Ndrg2* increased significantly from low levels to high levels, over the 28 day expansion period ($F_{(5,10)}$=28.28,p<0.001) although there was no significant difference seen between the expression of this gene in striatally derived or cortically derived cells.

7.2.9.2 *E14* (Fig. 7.8b)

E14 striatally derived cells also showed low expression level of this gene at the start of expansion; however this expression increased in a highly significant manner over the first 14 days in culture and remained high until 28 days of expansion ($F_{(5,10)}$=92.93,p,0.001). However, once expanded, these cells were then differentiated over 7 days, and expression was found to fall to minimal levels in both striatally derived and cortically derived cells. There was no difference between brain areas.

7.2.9.3 *E16* (Fig. 7.8c)

Levels of *Ndrg2* also increased overall in striatally derived E16 cells ($F_{(5,10)}$=20.75,p<0.001) after an initial reduction. There was no significant difference between brain areas.

Fig. 7.8a; *In vitro* expression of *Ndrg2* in expanded E12 Ctx & Str

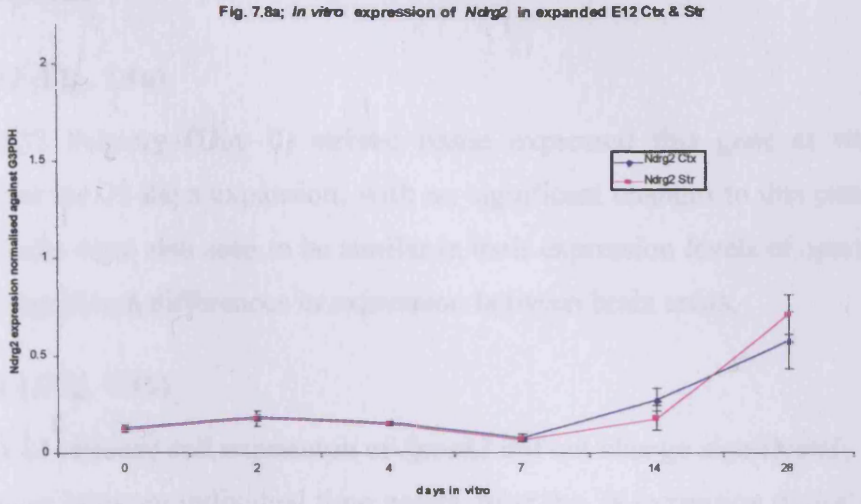


Fig. 7.8b; *In vitro* expression of *Ndrg2* in expanded E14 Ctx & Str

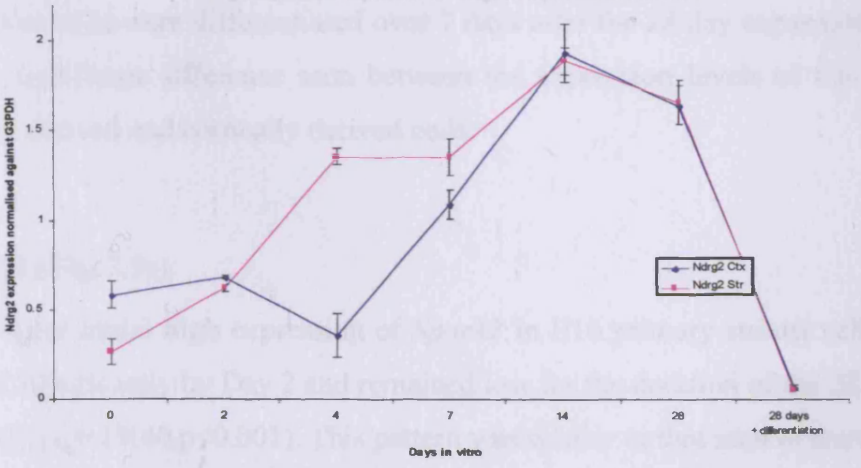
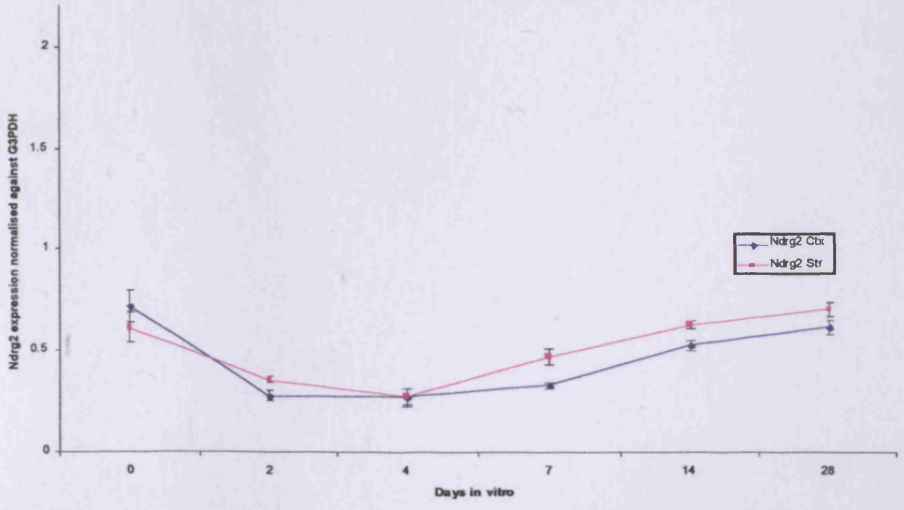


Fig. 7.8c; *In vitro* expression of *Ndrg2* in expanded E16 Ctx & Str



7.2.10 Spock2

7.2.10.1 *E12* (Fig. 7.9a)

E12 Primary (Day 0) striatal tissue expressed this gene at very low levels throughout the 28 days expansion, with no significant changes to this pattern. Cortically derived cells were also seen to be similar in their expression levels of *Spock2* throughout, with no significant differences in expression between brain areas.

7.2.10.2 *E14* (Fig. 7.9b)

E14 primary cell expression of *Spock2* did not change significantly either over the duration, or between individual time points, over the 28 expansion period (due to a wide variance between replicates). However, expression levels did reduce to negligible levels when these cells were differentiated over 7 days after the 28 day expansion period. There was no significant difference seen between the expression levels of this gene between striatally derived and cortically derived cells.

7.2.10.3 *E16* (Fig. 7.9c)

After initial high expression of *Spock2* in E16 primary striatal cells, these levels reduced significantly by Day 2 and remained low for the duration of the 28 day expansion period ($F_{(5,10)}=19.40, p<0.001$). This pattern was similar to that seen in cortical cells.

Fig. 7.9a; *In vitro* expression of *Spock2* in expanded E12 Ctx & Str

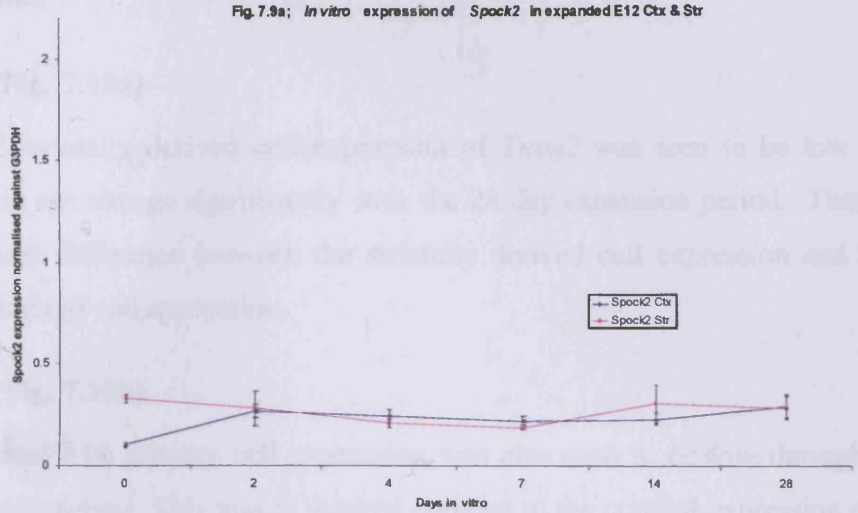


Fig. 7.9b; *In vitro* expression of *Spock2* in expanded E14 Ctx & Str

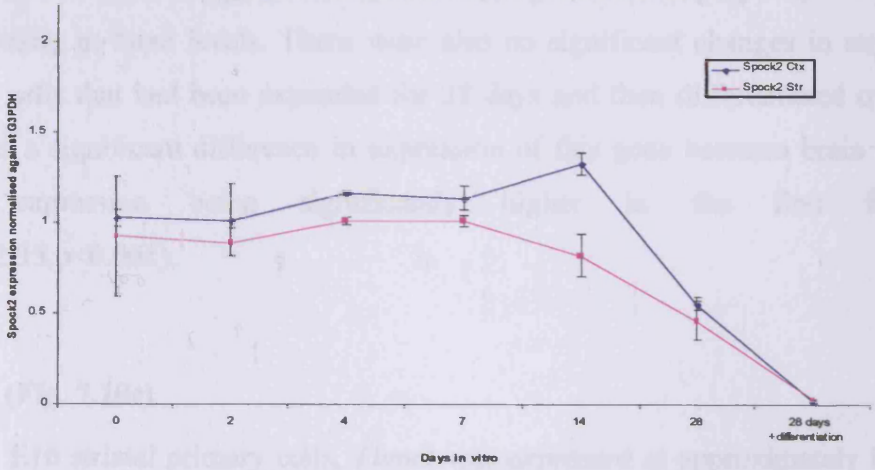
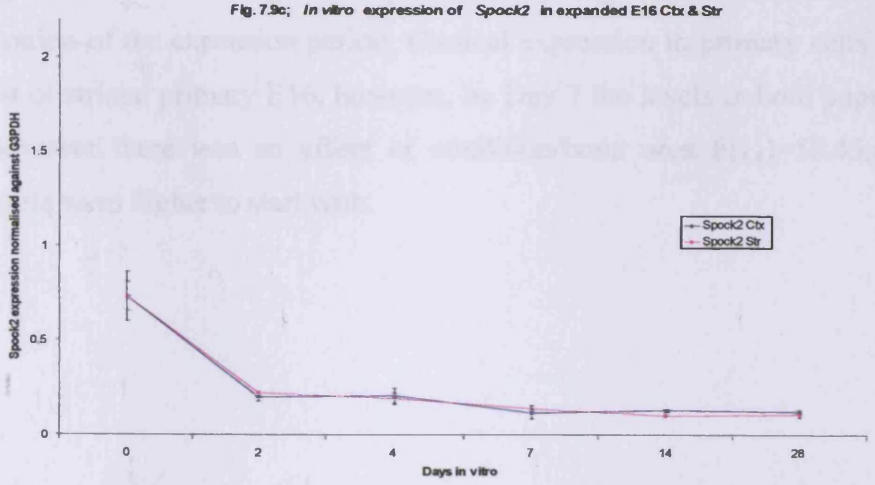


Fig. 7.9c; *In vitro* expression of *Spock2* in expanded E16 Ctx & Str



7.2.11 Tiam2

7.2.11.1 *E12* (Fig. 7.10a)

E12 striatally derived cell expression of *Tiam2* was seen to be low in primary cells and did not change significantly over the 28 day expansion period. There was also no significant difference between the striatally derived cell expression and that of the cortically derived cell expression.

7.2.11.2 *E14* (Fig. 7.10b)

Striatal E14 primary cell expression was also seen to be low throughout the 28 day expansion period. This was in marked contrast to the cortical expression of this gene that was seen to start at high levels before reducing dramatically by Day 7 of expansion and remaining at these levels. There were also no significant changes in expression of Tiam2 in cells that had been expanded for 28 days and then differentiated over 7 days. There was a significant difference in expression of this gene between brain areas, with cortical expression being significantly higher in the first four days $F_{(1,4)}=292.35, p<0.001$).

7.2.11.3 *E16* (Fig. 7.10c)

In E16 striatal primary cells, *Tiam2* was expressed at approximately half that of G3PDH. However, this was significantly reduced by Day 7 and remained at a low level for the duration of the expansion period. Cortical expression in primary cells was nearly double that of striatal primary E16, however, by Day 7 the levels in both population was similar, however there was an effect of condition/brain area $F_{(1,4)}=58.45, p<0.01$) as cortical levels were higher to start with.

Fig. 7.11a; *In vitro* expression of *Tiam2* in expanded Ctx & Str

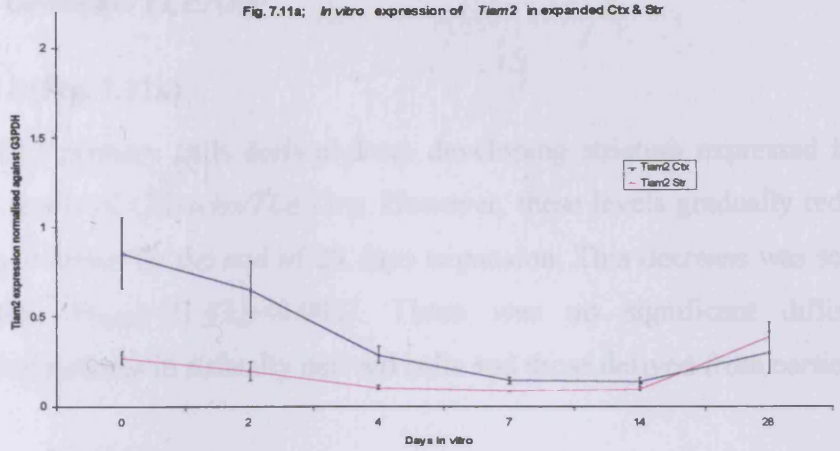


Fig. 7.10b; *In vitro* expression of *Tiam2* in expanded E14 Ctx &

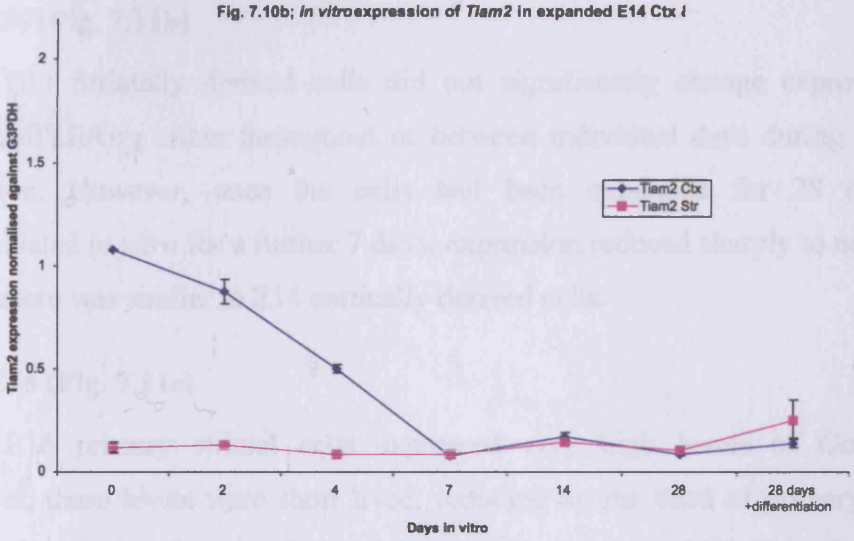
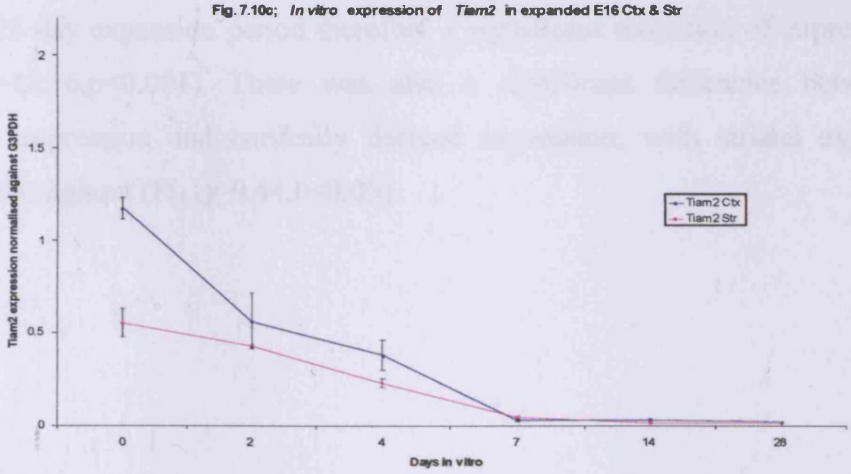


Fig. 7.10c; *In vitro* expression of *Tiam2* in expanded E16 Ctx & Str



7.2.12 Groucho/TLE/Grg

7.2.12.1 *E12* (Fig. 7.11a)

E12 primary cells derived from developing striatum expressed high (similar to *G3pdh*) levels of *Groucho/TLE/Grg*. However, these levels gradually reduced to around half Day 0 levels by the end of 28 days expansion. This decrease was seen to be highly significant ($F_{(5,10)}=21.43, p<0.001$). There was no significant difference between expression patterns in striatally derived cells and those derived from cortical areas.

7.2.12.2 *E14* (Fig. 7.11b)

E14 Striatally derived cells did not significantly change expression levels of Groucho/TLE/Grg either throughout or between individual days during the 28 days of expansion. However, once the cells had been expanded for 28 days and then differentiated *in vitro* for a further 7 days, expression reduced sharply to negligible levels. This pattern was similar in E14 cortically derived cells.

7.2.12.3 *E16* (Fig. 7.11c)

E16 primary striatal cells expressed very high levels of *Goucho/TLE/Grg*. However, these levels were short lived, reducing to one third of primary cell levels by Day 2 of expansion. Levels of expression remained at around this level for the remainder of the 28 day expansion period therefore a significant reduction of expression was seen ($F_{(5,10)}=12.16, p<0.001$). There was also a significant difference between Striatally derived expression and cortically derived expression, with striatal expression being higher throughout ($F_{(1,4)}=9.44,0<0.05$).

Fig. 7.11a; *In vitro* expression of *Groucho* in expanded E12 Ctx & Str

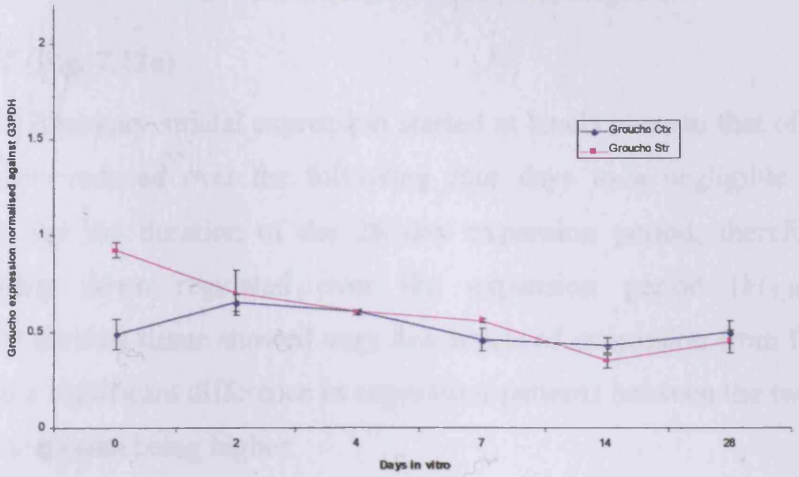


Fig. 7.11b; *In vitro* expression of *Groucho* in expanded E14 Ctx & Str

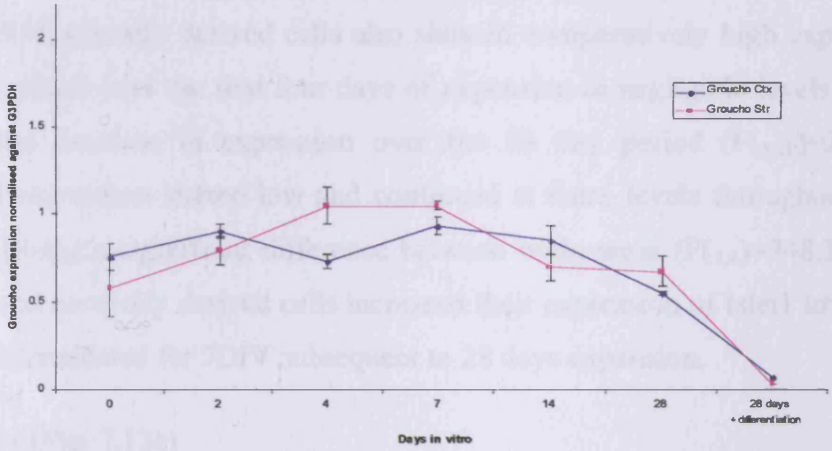
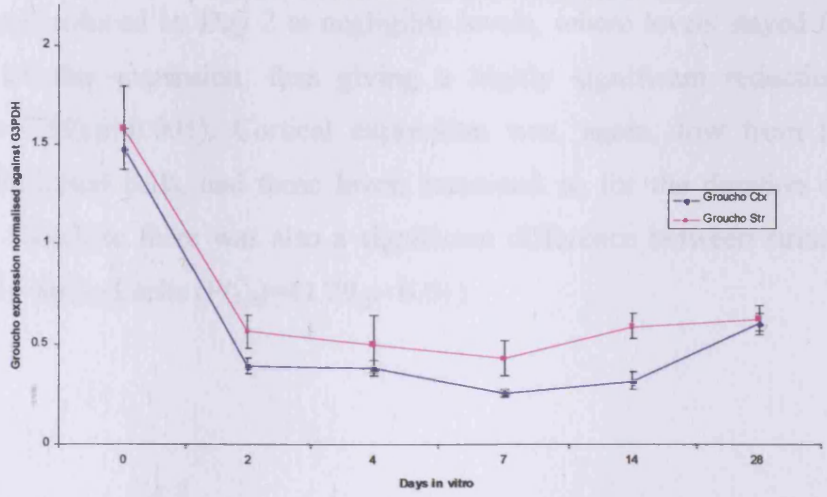


Fig. 7.11c; *In vitro* expression of *Groucho* in expanded E16 Ctx & Str



7.2.13 Islet1

7.2.13.1 *E12* (Fig. 7.12a)

E12 primary striatal expression started at levels close to that of *G3pdh*. However, these levels reduced over the following four days to a negligible level, where they remained for the duration of the 28 day expansion period, therefore this gene was significantly down regulated over the expansion period ($F_{(5,10)}=19.23, p<0.001$). Cortically derived tissue showed very low levels of expression from Day 0, and as such there was a significant difference in expression patterns between the two brain areas, with striatal expression being higher.

7.2.13.2 *E14* (Fig. 7.12b)

E14 striatally derived cells also showed comparatively high expression of *Islet1* which reduced over the first four days of expansion to negligible levels, thus showing a significant decrease in expression over the 28 day period ($F_{(5,10)}=215.46, p<0.001$). Cortical expression started low and continued at these levels throughout the expansion period, giving a significant difference between brain areas ($F_{(1,4)}=248.19, p<0.05$). Both striatal and cortically derived cells increased their expression of Islet1 to high levels after being differentiated for 7DIV subsequent to 28 days expansion.

7.2.13.3 *E16* (Fig. 7.12c)

Primary E16 striatal tissue expressed *Islet1* at very high levels although this was very much reduced by Day 2 to negligible levels, where levels stayed for the remainder of the 28 day expansion, thus giving a highly significant reduction in expression ($F_{(5,10)}=47.59), p<0.001$). Cortical expression was, again, low from the start in E16 primary cortical cells, and these levels remained so for the duration of the expansion period. Therefore there was also a significant difference between striatally derived and cortically derived cells ($F_{(1,4)}=41.29, p<0.01$).

Fig. 7.12a; *In vitro* expression of *Islet1* in expanded E12 Ctx & Str

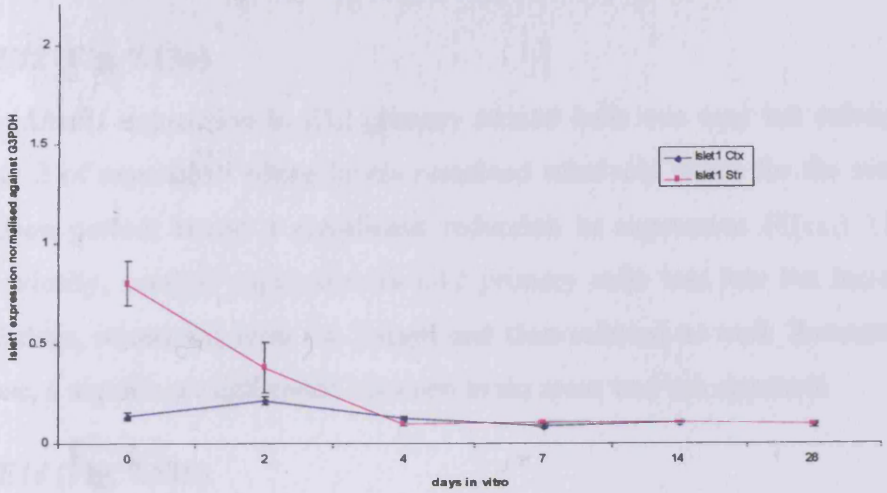


Fig. 7.12b; *In vitro* expression of *islet1* in expanded E14 Ctx & Str

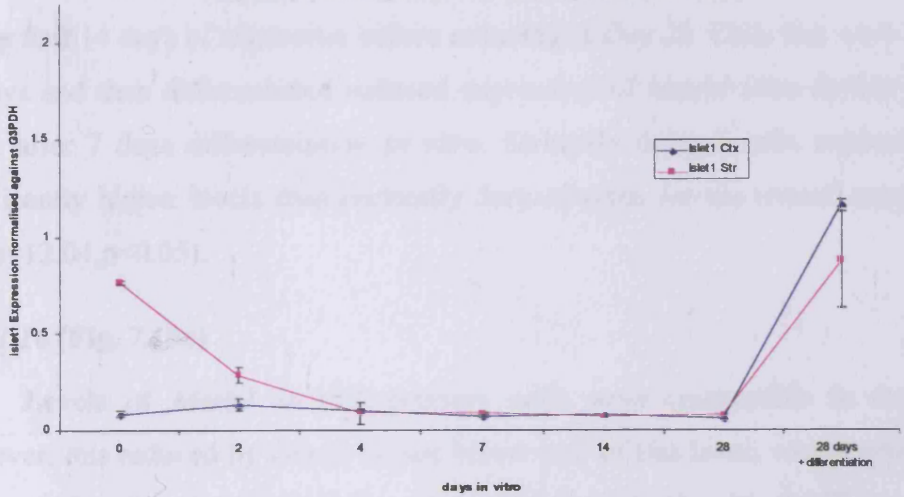
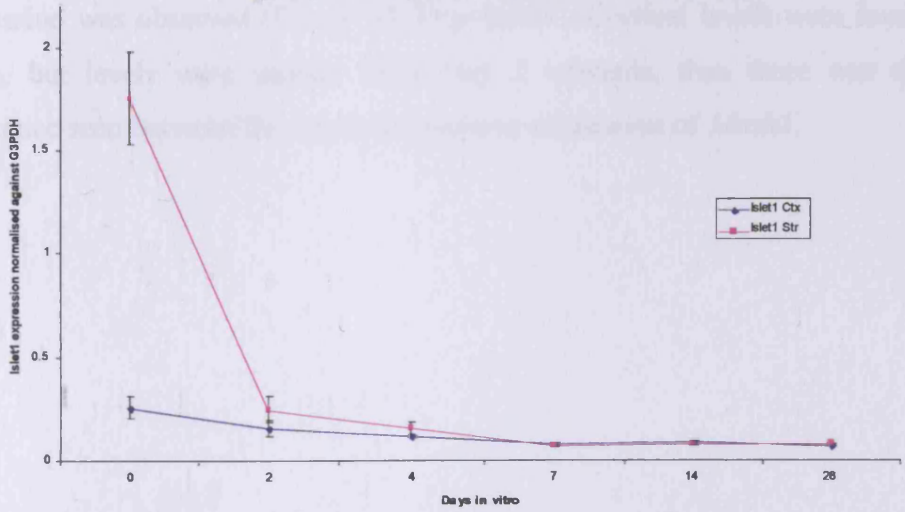


Fig. 7.12c; *In vitro* expression of *Islet1* in expanded E16 Ctx & Str



7.2.14 Mash1

7.2.14.1 *E12* (Fig. 7.13a)

Mash1 expression in E12 primary striatal cells was very but reduced half of this by Day 2 of expansion where levels remained relatively stable for the remainder of the expansion period, hence a significant reduction in expression ($F_{(5,10)}=13.51, p<0.001$). Paradoxically, cortical expression in E12 primary cells was low but increased over the next 2 days, remaining high for 2 days and then reduced as well. Because of this initial increase, a significant difference between brain areas was not apparent.

7.2.14.2 *E14* (Fig. 7.13b)

E14 primary striatal cells expressed *Mash1* at high levels, which remained as such for the first 14 days of expansion before reducing at Day 28. Cells that were expanded for 28 days and then differentiated reduced expression of *Mash1* even further to negligible levels after 7 days differentiation *in vitro*. Striatally derived cells expressed *Mash1* at significantly higher levels than cortically derived cells, for the overall expansion period ($F_{(1,4)}=12.04, p<0.05$).

7.2.14.3 *E16* (Fig. 7.13c)

Levels of *Mash1* in E16 primary cells were comparable to that of *G3pdh*. However, this reduced by Day 2 to just below half of this level, where expression levels remained for the duration of the expansion period, thus a significant decrease in expression was observed ($F_{(5,10)}=35.20, p<0.001$). Cortical levels were lower in primary tissue, but levels were similar from Day 2 onwards, thus there was no significant difference seen between the two brain areas in expression of *Mash1*.

Fig. 7.13a; *In vitro* expression of *Mash1* in expaneded E12 Ctx and Str

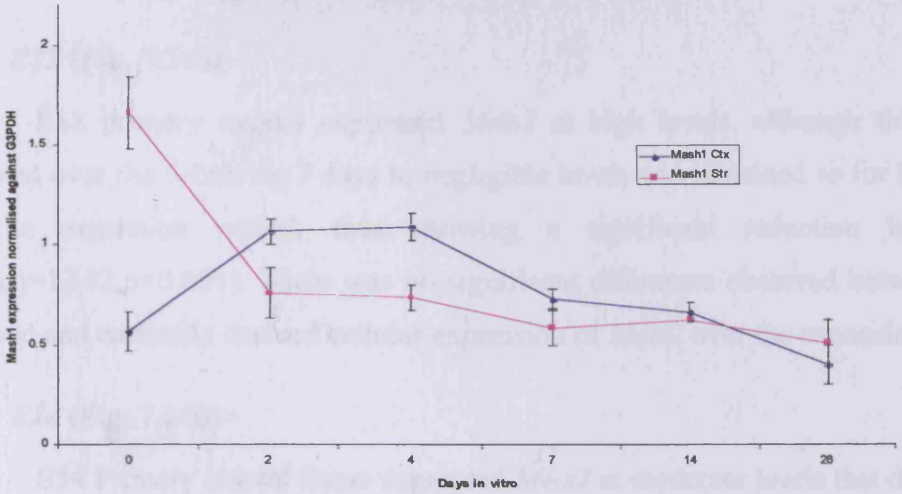


Fig. 7.13b; *In vitro* expression of *Mash1* in expanded E14 Ctx & Str

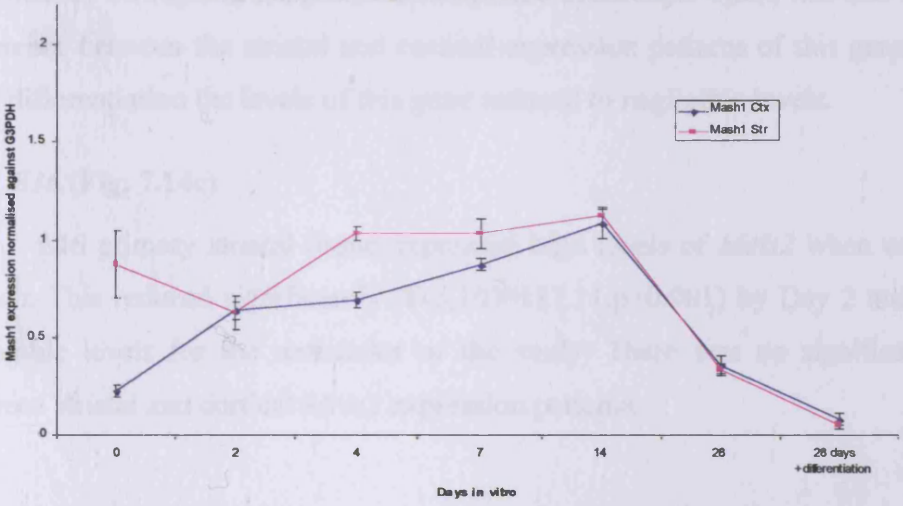
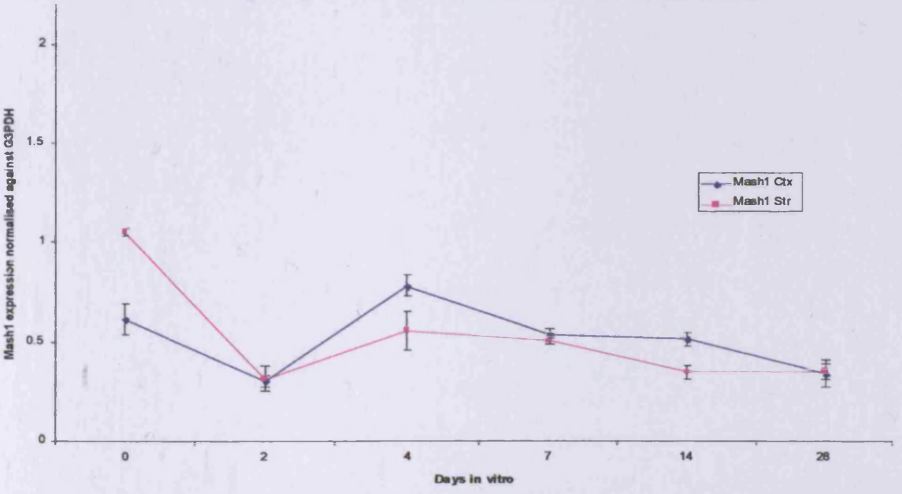


Fig. 7.13c; *In vitro* expression of *Mash1* in expanded E16 Ctx & Str



7.2.15 Meis2

7.2.15.1 *E12* (Fig. 7.14a)

E12 primary striatal expressed *Meis2* at high levels, although this expression reduced over the following 7 days to negligible levels and remained so for the remainder of the expansion period, thus showing a significant reduction in expression ($F_{(5,10)}=12.42, p<0.001$). There was no significant difference observed between striatally derived and cortically derived cellular expression of *Meis2* over the expansion period.

7.2.15.2 *E14* (Fig. 7.14b)

E14 Primary striatal tissue expressed *Meis2* at moderate levels that did not change significantly throughout the proliferation period of 28 days. There was also no significant difference between the striatal and cortical expression patterns of this gene. After seven days differentiation the levels of this gene reduced to negligible levels.

7.2.15.3 *E16* (Fig. 7.14c)

E16 primary striatal tissue expressed high levels of *Meis2* when compared with *G3pdh*. This reduced significantly ($F(5,10)=117.11, p>0.001$) by Day 2 and remained at negligible levels for the remainder of the study. There was no significant difference between striatal and cortical *Meis2* expression patterns.

Fig. 7.14a; *In vitro* expression of *Meis2* in expanded E12 Ctx & Str

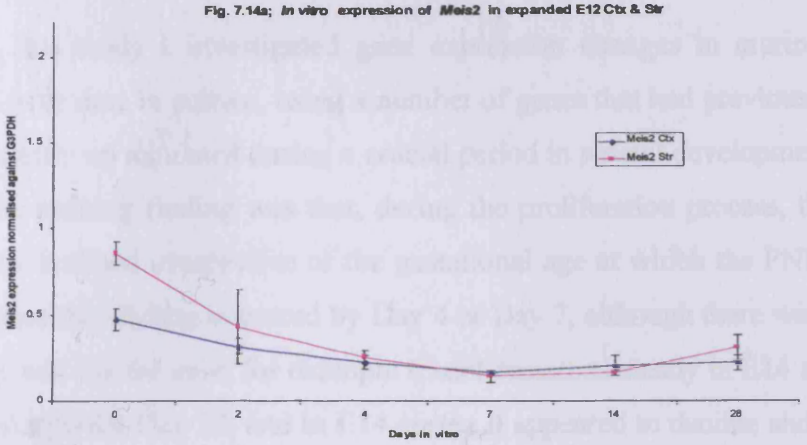


Fig. 7.14b; *In vitro* expression of *Meis2* in expanded E14 Ctx & Str

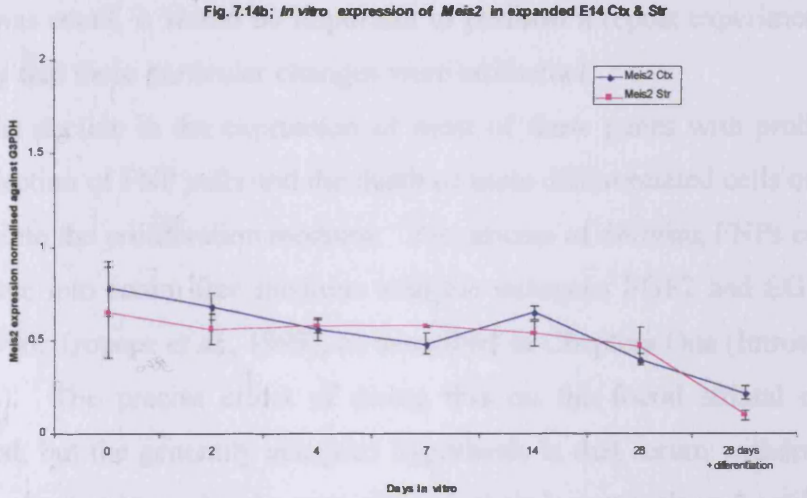
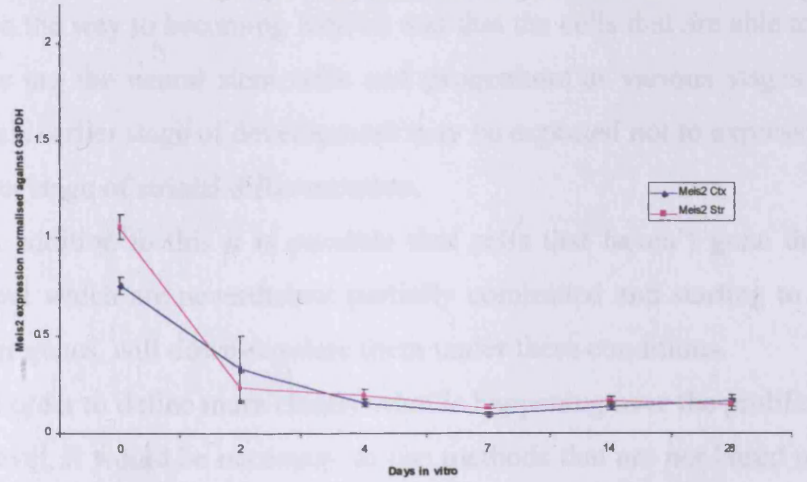


Fig. 7.14c; *In vitro* expression of *Meis2* in expanded E16 Ctx & Str



7.3 Discussion

In this study I investigated gene expression changes in murine striatal FNPs, expanded over time in culture, using a number of genes that had previously been found to be significantly up regulated during a crucial period in striatal development.

One striking finding was that, during the proliferation process, the expression of most genes declined irrespective of the gestational age at which the FNPs were isolated. In most cases this decline occurred by Day 4 or Day 7, although there were some genes in which this was not the case, for example Cxcr4 remained steady in E14 striatum and then declined sharply by Day 28, and in E14 cortex it appeared to decline and then increase at Day 14 before decreasing again at Day 28. However, although the variance of these samples was small, it would be important to perform a repeat experiment to exclude the possibility that these particular changes were artifactual.

The decline in the expression of most of these genes with proliferation may be due to selection of FNP cells and the death of more differentiated cells once striatal tissue is placed into the proliferation medium. The process of deriving FNPs entails placing the foetal tissue into serum free medium with the mitogens FGF2 and EGF (Reynolds and Weiss, 1996; Tropepe *et al.*, 1999), as described in Chapters One (Introduction) and Two (Methods). The precise effect of doing this on the foetal striatal cells is not well understood, but the generally accepted hypothesis is that serum withdrawal leads to the death of cells that have already gone through their last mitosis and are in the process of, or have completed, terminal differentiation (i.e. in our studies, this would include neurons that are on the way to becoming MSNs) and that the cells that are able to survive in these conditions are the neural stem cells and progenitors at various stages of commitment. Cells at this earlier stage of development may be expected not to express genes associated with a later stage of striatal differentiation.

In addition to this it is possible that cells that haven't gone through their final mitosis, but which are nevertheless partially committed and starting to express some of the marker genes, will down-regulate them under these conditions.

In order to define more clearly what is happening over the proliferation period at a cellular level, it would be necessary to use methods that are not based on analysis of the whole cell population. One possibility would be to analyse differentiating cells using antibodies to the protein products of the genes assessed in this study. This was not carried

out here, except for *Foxp 1* and *2*, due to specific antibodies not being available for many of the gene products. Antibodies are now available for a number of the proteins and others could be generated to produce a useful panel of histological markers.

In this study, *Foxp 1* and *2* were used to stain FNP cultures that had been expanded between Days 0 and 28 and then allowed to differentiate *in vitro* for 7 days and this is discussed further below. However, in brief, the most striking finding was that, although neuronal proportion has declined by later time points, in particular 28 days, *Foxp 1* and *2* staining is still clearly seen in cells with a neuronal phenotype at these times. For *Foxp 1*, this is in accordance with the PCR findings which demonstrate steady expression levels up to Day 14. For *Foxp 2*, however, expression levels on a population basis have declined substantially by Day 14 and 28, but the differentiated cultures demonstrate expression of *Foxp 2* in the majority of neurons. This suggests that at later time points, the FNPs are still able to up-regulate *Foxp 1* and *2* when they differentiate, which may suggest that they still have the capacity to become MSNs (and indeed, this is in agreement with the study of Zietlow et al (Zietlow *et al.*, 2005) showing that after 14 days of proliferation mouse MSNs are able to differentiate into DARPP-32 positive neurons when transplanted into the adult mouse striatum).

The immuno-fluorescence study also demonstrates the decline in the proportion of cells differentiating into neurons by this time point. Recent data suggests that the capacity of FNPs to produce neurons by 28 days remains constant, but that the capacity to produce astrocytes accelerates and thus the neuronal population is 'diluted' (C Kelly, personal communication). However, this data also illustrates the importance of going on to study this problem using antibodies to the gene products so that the changes in the neuronal population alone can be better understood – in the present study the contribution of astrocytes to the gene expression changes cannot be assessed. This will require proper quantification of neuronal, astrocytic and gene markers, which was not carried out here due to time limitations.

As mentioned above, not all of the genes decline in expression over the proliferation period. In particular, this can be seen for *Mash1*, which is maintained in the striatal-derived cells and increases in the cortically-derived cells up to Day 14 in E14-derived cells, and *Ndrg2*, which again increases with a similar pattern in E14-derived cells. *Mash1* has been shown to be associated with neuronal specification and may be

expected to be present in neuroblasts (Parras *et al.*, 2002), so this is consistent with the generation of neuronal progenitors. The decline in levels after E14 may be associated with the 'dilution' of neuroblasts with glioblasts as described above.

Groucho/TLE/Grg is also implicated in maintaining proliferative programs within neuronal progenitors (Hatakeyama *et al.*, 2004; Kageyama *et al.*, 2005), and the data from this study support this; showing that it is expressed at relatively high levels in striatal cells over the period of 28 days expansion in the E12 and E14 cultures with down-regulation in the E14 differentiated cells. However, the high levels observed in primary E16 with a fall in levels by day 2 are more difficult to explain.

Ndrg2 also increases with proliferation time *in vitro*, and this is most striking in the E14-derived cells. This gene is implicated in neurite extension and is particularly found in neurogenic regions of the brain such as the hippocampus, where it has been localised to radial glia and astrocytes (Nichols, 2003) and may also have a putative involvement in neuronal development (Nichols *et al.*, 2005). It may be that because neuronal differentiation is being reduced at this point in development, in favour of glial cell differentiation, (as seen when cells derived from earlier embryos switch from being predominately neurogenic to predominately gliogenic (Sun *et al.*, 2003) and alluded to earlier, in Fig. 6.1; Chapter Six) we see an increase in expression of this gene.

An interesting, but as yet unsubstantiated finding, is that for a number of the genes, expression levels and/or expression patterns were subtly different in the E14, as compared to the E12 and E16 cells: Examples of this were *Ndrg2*, where expression levels increased dramatically in the E14 cells, *Groucho/TLE/Grg* where levels increased a little in the E14 cells but fell in the E12 and E16 cells, and *Mash1* which followed a similar pattern. The precise reasons for this are not clear, however, it may be associated with the fact that striatal cells from E14 embryos show better graft integration when transplanted into the adult striatum (Fricker *et al.*, 1997a; Fricker *et al.*, 1997b; Watts *et al.*, 2000), and it may be that this is related in some way to the greater capacity of the E14 populations to retain levels of expression of genes important to their specification and identity. However, exactly what this relationship would be is not clear. It is notable that many of the genes that are expressed differently in the E14 FNPs are associated with neuroblast proliferation and thus it may be that E14 is a good age for isolation of FNPs. Indeed, studies of rat FNPs demonstrate that E12 and E14 are good ages for obtaining

FNP cultures with a high neuronal potential (Kelly *et al.*, 2005) although this has not been systematically determined for mouse.

There are very few studies in the literature that attempt to analyse the characteristics of FNPs in this way. However, a study by Parmar *et al* (Parmar *et al.*, 2002) in which mouse FNPs were analysed in a similar way; the medial (MGE) and lateral (LGE) parts of the developing striatum were assessed separately and changes were found to be greater in MGE derived than LGE derived cultures. However, this study also revealed changes over a period of expansion (6 passages) and showed that many of the markers for ventral cells were down regulated (i.e. *Dlx* genes). However, there were some differences between their study and this one; most notably that Parmar *et* al found that the gene Islet1 continued to be expressed in expanded cultures, whereas it appears to be dramatically reduced in expression here. Cells from all three areas (MGE, LGE and Cortex) also showed ectopic expression of genetic markers that were known to be not expressed within the areas of dissection, and although the researchers rightly point out that this may have been due to inaccurate dissection, it may also have been due to the populations of cells becoming a more multi potent form of progenitor (possibly capable of forming both, many or all types of neurons from the three areas), or could be due to aberrant gene expression associated with the proliferation conditions.

7.3.1 Differentiation of FNPs after 28 days expansion

Having characterised the gene changes in FNPs expanded in culture over a period of 28 days and having shown that many of the genes associated with striatal differentiation were down-regulated in proliferating populations of cells, it was important to find out whether these genes could be up regulated again once the cells were allowed to differentiate. For practical reasons this was restricted to E14 FNPs after 28 days of proliferation and the cells were allowed to differentiate *in vitro* for 7 days. The results were compared to primary E14 cells that were allowed to differentiate *in vitro* that have already been described in Chapter Six. In this part of the experiment, only Islet1 was seen to increase after the cells were allowed to differentiate. This gene is known to mark the majority of striatal neurons (Toresson and Campbell, 2001) and is also implicated in neuronal specification generally (Curtiss and Heilig, 1998). This suggests that at least a proportion the population of cells may still be capable of becoming striatal neurons,

although from this work it is not known whether these progenitors would differentiate into MSNs, as they do in the majority of primary E14 ENPs, and the fact that most of the other striatal associated genes remain expressed at low levels would suggest that this is not the case. However, as discussed above, it needs to be emphasized that the expression levels are on a population basis and that it will be necessary to follow up these findings with studies to look at the presence of the gene product using antibodies.

7.4 Conclusion

From this study it can be concluded that the expansion of FNPs in the presence of FGF2 and EGF does cause changes in the expression of genes found in previous chapters to be associated with striatal differentiation, and that most of these genes are not up-regulated with subsequent differentiation. The extent to which this is an indication that the cells have lost the capacity to express these gene after proliferation and the extent to which the down regulation is an artefact due to 'dilution' of neuronal precursors by astrocytic precursors has yet to be determined.

This work has taken forward our understanding of the changes that are occurring during FNP proliferation, but it is clear that, for the future of these cells as a potential alternative cell source for neural transplantation, further work is required to fully understand the effect that proliferation has on these cells.

Chapter Eight

8 Final Discussion.

Neural transplantation augers to be an effective strategy for treating neurodegenerative disorders such as HD by replacing the MSNs that are the predominant cells lost in this condition; however, its clinical application is limited by the absence of a suitable renewable donor source of cells capable of differentiating into MSNs. With this in mind, this thesis has attempted to find gene expression changes associated with MSN differentiation in order to;

- (i) identify potential markers of MSN differentiation to facilitate studies aimed at producing donor cells for HD transplantation (by increasing our understanding of striatal FNPs; allowing sorting of FNPs in order to purify a progenitor cell population for expansion and/or transplantation and contributing to work to direct precursors towards this phenotype), and
- (ii) to identify genes that are potentially part of the machinery of striatal differentiation.

Towards this aim, I have completed a screen of gene expression during the period of peak neurogenesis in the developing mouse striatum and I have validated these results for a small proportion of these genes. I have then used these results to characterise the *in vitro* differentiation of developing striatal cells and interrogate foetal striatally derived precursors when expanded in vitro.

8.1.1 The gene expression screen.

8.1.1.1 Would it have been beneficial to study gene expression at earlier/later embryonic ages?

The original micro array study was successful in so far as it produced highly significant results for a number of gene expression changes seen during the period studied. The screen looked at gene expression changes between the embryonic ages of E12 and E16, as previous studies have found that this period is when peak neurogenesis occurs in the mouse (Shimazaki *et al.*, 1999). However, peak neurogenesis defines the period during which most MSNs were born, but it is still possible that progenitor cells

undergoing differentiation to MSNs are specified to do so in the preceding hours or days before this time (i.e. before E12). Thus there may be important gene expression changes that occurred before E12 that may be important for understanding the pathways that regulate MSN differentiation and so it may be advantageous to look at striatal gene expression at an earlier time point; for example E10 or E11. This is technically difficult because the size of the developing murine brain at these stages in development is so small. In order to ensure accuracy, another method of dissection, for example laser capture micro dissection (LCMD), would most likely have to be employed. To do this would mean that the later time points would also have to be dissected using the new method (in order to ensure continuity in methods and to control for differences that may occur due to different techniques being used).

8.1.1.2 Was the right technique used?

LCMD has been developed to allow accurate dissection of very small areas of tissue and this may have provided a suitable technique. However, because LCMD is designed to dissect such a small area, it presented two problems for the work carried out in this thesis:

(i) As this was the first systematic screen of its kind for striatal development, it was necessary to obtain, as far as possible, the whole developing striatum as there may be intra-striatal regional differences in development and dissection of a subset of the developing neurons may have lead to bias: the developing ganglionic eminence contains many different cells types which later migrate into many different areas of the brain as well as the adult striatum. If LCMD had been used, some of these cell populations may have been missed while others may have been over represented;

(ii) The small size of the tissue pieces would have introduced the need for RNA amplification, which at the time the project was commenced, was not thought to be reliable due to the possibility of non-linear amplification. Having said this, non-linear amplification has turned out to be much less of a problem than was originally anticipated and does not appear to have inhibited other researchers from using this technique (see (Pietrzyk *et al.*, 2004; Fink and Bohle, 2005; Mizuarai *et al.*, 2005)).

8.1.1.3 Was the appropriate area dissected?

In this study, population gene expression within the WGE was observed. It was valid to look at gene expression within the WGE initially for two reasons:

- Firstly, it is not fully understood how the LGE and MGE interact to produce the normal medium spiny neurons that populate the adult striatum.
- Secondly, although there may be no interaction between these areas it may be that the developing cells within the MGE play a crucial role in guiding the differentiation of MSNs.

Previous studies have shown that WGE, when transplanted into the striatum, produces functional striatal-like tissue, and although transplanted LGE would also produce striatal-like tissue, the functionality of that tissue has not yet been determined experimentally (although see (Parmar *et al.*, 2002) for a molecular approach to this).

This is, however, an important issue and future experiments that compare the LGE and MGE using micro array and more refined dissection techniques would be very useful in determining the functionality of these areas.

8.1.1.4 Did the criteria for selection of the candidate marker genes achieve the aims set out?

From the original data set produced from this micro array I selected a number of candidate marker genes to follow up, and the expression changes seen in these genes using the micro array were successfully validated primarily using PCR and then by using *in situ* hybridisation.

The candidate marker genes that I chose for further investigation were all significantly up-regulated between E12 and E16, thus making them easier to study than other genes that were shown to be significantly down-regulated during the same period.

However, it cannot be stressed enough that down regulated genes may also be very important in directing the differentiation and development of the cell population within the whole ganglionic eminence at this point in development. This point is illustrated further when we see that two of the most significantly changed candidate markers chosen are both transcription factors that repress the expression of other genes, thus causing down regulation in other genes. The question still remains as to which genes

are being down-regulated because of these gene's influences and what would happen if this repression was reduced or enhanced.

Another point to remember is that although I selected genes that were highly up regulated between E12 and E16, this method of selection automatically dismisses those genes that were only up, or down regulated by a small amount, and it cannot be assumed that subtle changes in gene expression are unimportant. However, the use of micro array, the validation methods used and using *in situ* hybridisation meant that my methods would not have been sensitive enough to highlight these genes without also increasing the rate of false positives.

The way the micro array data was analysed was very conservative, in order to reduce the amount of false positives that could arise from the analysis. I chose the two most robust and commonly used normalisation methods (MAS 5.0 and RMA) and then merged the results, effectively discounting any genes that did not pass both normalisations at the required p-value level. I did this to discount any arguments that, at the time of doing these experiments, were being highlighted in the literature, about the efficacy of some normalisation techniques. However, in doing this I also discounted many genes that would otherwise have been highlighted as being significant and worthwhile candidate marker genes.

The above points highlight an area of caution which must be borne in mind when analysing datasets as big as those produced by micro array and other high throughput assays: That is, that because the researcher has so much data at hand from such an experiment, he/she also is at liberty to 'cherry pick' the most significantly changed, or easily validated results; but in doing so, they are also at risk of missing vital data that may be overlooked as being insignificant when in fact, biologically these small, or downward changes in gene regulation may be very important.

8.1.1.5 Were the selection criteria right?

The primary aim, once the results had been analysed from the micro array experiment, was to focus on those changes that were deemed to be 'interesting', and to leave to one side genes known to be involved in other functions apart from the differentiation and maturation of the striatum (such as so called 'house-keeping' genes). I especially wanted to look at transcription factors (i.e. the controlling genes) that may have been implicated in specifying certain changes to do with differentiation and

maturation within the cells that populate the developing striatum at the times chosen. *Foxp1, Foxp2* (Tamura *et al.*, 2000; Shu *et al.*, 2001; Tamura *et al.*, 2003; Ferland *et al.*, 2003; Wang *et al.*, 2003a) and *Mef2C* (Leifer *et al.*, 1993; Leifer *et al.*, 1994; Leifer *et al.*, 1997; Janson *et al.*, 2001) all proved to be interesting genes that are seen to be up regulated during this period of peak neurogenesis.

I also wanted to select genes implicated in axonal path finding and migration, as these may well be expressed in the developing MSNs as they move to, and take up residence within the neo-striatum. *Spock2* (Cuschieri and Bannister, 1975; Charbonnier *et al.*, 2000), *Cxcr4* (Doitsidou *et al.*, 2002; Stumm *et al.*, 2003b; Dziembowska *et al.*, 2005) and *Ndrg2* (Nichols, 2003; Nichols *et al.*, 2005; Takahashi *et al.*, 2005a; Takahashi *et al.*, 2005b) were all genes that fulfill these criteria and as such proved very interesting to study.

Thirdly, signaling receptors or proteins that may be implicated in progenitor differentiation were also interesting and *Gucy1β3* and the putative Brain Specific Binding Protein (2310037P21Rik) (about which very little was known at the start of this study) were both worthy candidates for study.

Novel genes found to be significantly changed in expression over the period studied were also of interest. One novel gene putatively named Brain Specific Binding Protein (2310037P21Rik) and now known to be homologue of the human gene Ras Induced Senescence1 (RIS1), was found to be highly up-regulated during the period of study. However, at the time of the experiment, the corresponding Affymetrix probe had been designed around a gene fragment that proved too small to design working primers for in order to carry this gene through to validation. Due to these difficulties, this gene had to be dropped from further investigation.

8.1.1.6 Have we found markers for striatal differentiation?

The genes studied here have proven worthwhile candidates for providing a cell gene expression signature that appears to be associated with striatal differentiation. We may have also highlighted gene markers that are largely striatal specific in *Foxp1* and *Foxp2,* although they occur in other regions during neural development. Indeed, it appears from our studies, and those of others, that many of the genes up-regulated during striatal differentiation are also up-regulated during cortical differentiation, and some are also known to be important for differentiation of other tissues (eg *Mef2C,* which is a key

gene involved in muscle differentiation). This does not distract from their value as markers of striatal differentiation in the context in which they are used here as the aim is to have a 'signature' of gene expression changes rather than to rely on one or two gene changes specific to the striatum.

However, it may be that some of the markers eventually turn out to be useful for the identification of striatal tissue. For example, *Foxp1* and *Foxp2* are expressed within the developing striatum and continue to be expressed in the adult striatum, and may be useful specific markers used in conjunction with DARRP-32 to mark cells grown ***in vitro*** in culture and grafting experiments. However, although there is a clear association of the expression of *Foxp1* and *2* with striatal development, no link or function has been proven as yet.

As such we now have a panel of genes that provide a gene expression signature of the population of progenitor cells that give rise to the mature striatum. Once fully validated this panel of genes can be used in future as a reference to how a cell population dissected from the WGE should behave (with respect to gene expression) over the period of peak neurogenesis.

8.1.1.7 Further Work

The micro array described in this thesis has now been validated and will be published on the World Wide Web so that other groups may also make use of the information it provides. The information has already become the subject of follow up studies within the laboratory to find the role of *Foxp1* and *2* within the developing striatum. A *Foxp1* deletion is known to be embryonically lethal due to its activity in development of other areas such as the lungs. *Foxp2* mutations have been implicated in a familial speech disorder. Further work will provide a more detailed history of these genes functions.

As well as the follow-up work to understand the functional roles of the genes from this screen in striatal development, it will also be important to further quantify the gene expression patterns of the developing WGE, using a wider set of embryonic ages. This may give clues as to which genes are being repressed by the *Foxp* genes, as well as giving more genetic information about the population as a whole. It will also be important to investigate the down-regulated genes as described above.

Further *in vitro* work should also be carried out to further validate the differentiation results seen after 28 days expansion, with differentiation at earlier time points such as Day 2, Day 4, Day 7 and Day 14, that may give clues as to when these cell populations become incapable of differentiating into striatal-like cells.

8.1.2 In conclusion

This thesis has studied the gene expression of the population of cells that make up the whole ganglionic eminence during its period of peak neurogenesis using Affymetrix micro array. It has then validated the results of a subset of genes found to be significantly up-regulated during this period using *in situ* hybridisation and then used these genes to characterise either primary cells that were differentiated, or cells that have been proliferated and then differentiated *in vitro*.

The genes highlighted from the micro array provide not only a gene expression signature of a developing population of striatal precursors, enabling future experiments to compare and contrast expression patterns seen in different *in vitro* studies, but it has also highlighted *Foxp1* and *Foxp2* that have been shown to have a high degree of association with this period of development. This has encouraged future work in this laboratory in which the developmental functions of these genes in relation to MSN differentiation and development will be studied.

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Appendices

9 Appendix A

9.1 Affymetrix array

The following protocol was carried out by technicians at the Department of Pathology, University of Wales College of Medicine (UWCM).

9.1.1.1 Synthesis of double stranded cDNA from total RNA

(NOTE: T7-(dT)24 primer – Must be HPLC purified).

Starting material: 10μg of high quality, total RNA (see above).

First strand synthesis was carried out by adding the following to an RNase-free tube;

	dH_2O (variable)
	RNA (10μg)
	T7-(dt) 24 primer
1μl	(100pmol/μl)

This was incubated at 70 °C for 10 minutes, then briefly centrifuged and placed on ice.

Once on ice the following was added (in order);

4μl	5x First Strand Buffer
	0.1M DTT (mixed well to resuspend
2μl	any precipitate)
1μl	10mM dNTP mix

This was incubated at 42°C for 2 minutes. After incubation, 2μl of Superscript II Reverse Transcriptase was added and the total volume made up to 20μl before incubating at 42°C for 1 hour.

Second strand synthesis was carried out by placing the first strand reaction on ice, then centrifuging briefly to bring down the condensation on sides of tube. The following reagents were then added;

91µl	DEPC treated water
30µl	5x Second Strand Reaction Buffer
3µl	10 mM dATP; dCTP; dGTP; dTTP
1µl	10 U/µl DNA Ligase
4µl	10 U/µl DNA Polymerase I
1µl	2 U/µl RNase H

This gave a final volume of 150µl.

The contents of the tube were mixed and then briefly centrifuged before being incubated at 16°C for 2 hours in a water bath. After this 2µl of T4 DNA Polymerase (10Units) was added and the reaction was incubated for 5 min at 16°C, after which, 10µl 0.5M EDTA was added.

9.1.1.2 Clean-up of double stranded cDNA

NOTE: Use 'Heavy' 0.5 ml or 1.5 ml Phase Lock Gel tubes (PLG).

Immediately prior to use, PLG tubes were centrifuged at max speed for 20-30 seconds then 162µl of the above sample was added to the pre-spun PLG tube. An equal amount of phenol:chloroform:isoamyl alcohol (25:24:1) was then added and thoroughly mixed (do not vortex). This was centrifuged at maximum speed for 5 minutes (to separate the phases).

Note: after centrifuging some PLG may remain at the bottom of the tube. If a second extraction is necessary and maximum tube volume is not exceeded, more organic solvent can be added to the tube, mixed and re-centrifuged.

Once centrifuged, the upper aqueous phase was removed and pipetted into a fresh tube, where the following was then added:

	0.5 volumes of 7.5 M NH4Ac
	2.5 volumes of absolute EToH (-20oC)
1µl	Glycogen (5mg/ml)

This was left to precipitate for at least 1 hour, or overnight.

This was mixed briefly then centrifuged at 12,000xg for 20 min at 4°C. The supernatant was removed and the pellet washed with 0.5ml of 80% ethanol (previously stored at –20°C). This was again centrifuged at 12,000xg for 5 min at 4°C and the

supernatant removed. This ethanol wash was repeated before air drying the pellet (taking care not to "over-dry" the pellet as this stops it from going back into suspension, but bearing in mind that any residue ethanol may interfere with the IVT reaction).

The dried pellet was resuspended in 12µl of DEPC-treated water, heated to 65°C for 5 minutes and then left on ice for between 30 minutes and 1 hour to rehydrate; 1µl of this sample was kept for Agilent® chip analysis.

The cDNA sample concentration was then analysed using a system that could differentiate between RNA, ssDNA and dsDNA (the Picogreen® system was used by the UCWM Affymetrix Micro array facility). The sample was then stored at –80°C until required.

9.1.1.3 In vitro transcription (IVT)

Synthesis of Biotin Labelled cRNA kit required the use of an Enzo Bio-Array High Yield RNA Transcript Labelling Kit (available from Affymetrix). Reactions were done in a thermo cycling PCR machine (requiring the reactions to be set up in PCR tubes).

The following reaction components were added to an RNase-free 1.5ml microfuge tube in the order indicated:

10µl	Template cDNA (from the above reaction- this equated to 1µg of cDNA)
4µl	10x HY Reaction Buffer
4µl	10x Biotin Labelled Ribo-nucleotides
4µl	10X DTT (mixed well to remove any precipitate)
4µl	RNAse Inhibitor mix
4µl	20X T7 RNA polymerase
12µl	DEPC-Treated Water to a final volume of 40µl

Contents were carefully mixed then briefly centrifuged (a pulse for 5 seconds) then incubated immediately in a 37°C water bath, for 6 hours. The contents were gently mixed every 30-45 minutes during this incubation. This reaction must not be left overnight.

9.1.1.4 IVT cleanup

Kit required: RNeasy mini kit (Qiagen).

Note: Buffer RLT should NOT contain β-mercaptoethanol as this contributes to fluorescence of the Affymetrix GeneChip arrays and leads to high background.

Sample volume was adjusted to 100µl with 60µl of RNase-free water and 350µl Buffer RLT added and mixed by pipetting. 250µl of absolute ethanol was then added and mixed by pipetting. The sample (700µl) was then loaded onto an RNeasy mini column which was then placed into a 2ml collection tube. This was centrifuged at 8000xg for 15 seconds; the sample was then passed through the column again and centrifuged before discarding the flow-through and collection tube. The column was then transferred to a new 2ml tube and 500µl Buffer RPE added. This was centrifuged at 8000xg for 15 seconds, the flow through discarded and the column replaced in the 2ml collection tube. Another 500µl Buffer RPE was added to the column which was then centrifuged at 8000xg for 2 minutes (to dry the membrane) before discarding the flow-through and collection tube. The column was placed into a fresh 1.5ml collection tube and centrifuged at 8000xg for 1 minute before discarding tube and flow-through. The column was then placed into a new 1.5ml tube and 30µl of RNase-free water pipetted directly onto the RNeasy silica-gel membrane. This was allowed to stand for 1 minute before centrifuging at 8000xg for 1 minute (to elute the DNA). A further 30µl of RNA-free water was then pipetted onto the membrane, the column and tube turned 180° then allowed to stand for 1 minute and centrifuged for a further minute at 8000xg.

9.1.1.5 Ethanol Precipitation of cRNA

0.5 volumes of RNAse-free 7.5 M NH4 Acetate, 2.5 volumes of RNAse-free 100% ETOH and 1µl of glycogen were added to the sample and mixed well. This was then left to precipitate overnight at -20°C before being centrifuged at 12,000xg at 4°C for 30 minutes. The pellet was then washed with 80% ETOH, centrifuged at 12,000xg at 4°C. This was repeated before air drying the pellet briefly and resuspending in 34µl of RNAse-free water. The sample was then heated to 65°C for 5 minutes then left on ice for between 30 minutes and 1 hour to rehydrate.

A 1µl aliquot of this purified unfragmented cRNA was taken for Agilent® analysis.

9.1.1.6 cRNA Quantification and Fragmentation

The adjusted cRNA yield was then worked out:

Adjusted yield = (Amount of cRNA measured after IVT, in μg) – (Amount of total RNA used in the cDNA synthesis reaction) (Fraction of cDNA synthesis reaction used in IVT)

UWCM Affymetrix micro array facility generally did not proceed to the cRNA fragmentation unless they had around 15μg of adjusted cRNA.

The fragmentation reaction was performed in microfuge tubes; these were then used to make the Hybridisation Cocktail.

The following reaction was made up;

1-32μl	cRNA to make 15μg
8μl	5x Fragmentation Buffer
	RNAse-free water to a final volume of 40μl

This reaction was incubated at 94°C for 35 minutes; then placed on ice. A 1μl aliquot was then taken for later Agilent chip analysis. The remaining sample was stored at -20oC until the hybridization.

9.1.1.7 Agilent Analysis

Agilent Lab-on-a-chip chips were used to examine cDNA, cRNA and fragmented RNA.

9.1.1.8 Test3 hybridisation

The Affymetrix GeneChip Test3 Array provides a convenient and accurate method to determine the quality of a labelled target prior to its analysis on the GeneChip expression arrays. It contains probe sets representing a subset of characterized genes from various organisms, including mammals, plants, and bacteria. Additionally, the GeneChip Test3 Array contains a subset of human and mouse housekeeping genes (shown to be expressed early in fetal development and throughout adulthood).

The above samples were routinely hybridised to Test3 chips. The protocol was ran in such a way so when making a hybridization cocktail for a full chip, there would automatically be enough to run on a Test3 chip without the need to process more sample.

2x and 1x hybridisation buffers were made up. The 20x Genechip Eukaryotic control and B2 Oligo tubes were heated to 60°C for 5 minutes to ensure that the cRNA was fully resuspended.

The chips were taken out of the bags and left to fully equilibrate at room temperature. The hybridisation rotisserie oven was switched on to warm up.

The hybridisation cocktail was made up using the following;

15µg	Fragmented cRNA (usually 40µl fragmentation reaction)
5µl	Control Oligo B2
15µl	20x Eukaryotic Hybridisation Controls
3µl	Herring Sperm DNA (10mg/ml)
3µl	Acetylated BSA (50mg/ml)
150µl	2x Hybridisation Buffer
	RNAse-free water to a final volume of 300µl (usually 84µl)

This hybridization cocktail was heated to 99°C for 5 minutes, followed by 45°C for 5 minutes in a PCR machine.

While the hybridisation cocktail is denaturing the Test3 chip was filled with 100µl of 1x Hybridisation Buffer. They were then pre-hybridised in the rotisserie oven for 10 minutes at 45°C, rotating at 60 rpm.

The hybridisation cocktail was then centrifuged at maximum speed for 5 minutes in order to remove any insoluble material from the hybridisation mixture. After centrifuging and when the chips have finished pre-hybridising, the chips were taken out of the oven and the hybridisation solution removed from the Test3 chips and replaced with 80µl of hybridisation cocktail.

The chips were then hybridised in the rotisserie oven for at least 16 hours at 45°C, rotating at 60 rpm.

9.1.1.9 Washing, Staining and Scanning Test3 chips

All solutions used were filter sterilized before use and the fluidics station was primed with buffers A and B by running the Prime protocol.

The Test3 chips were removed from the rotisserie hybridisation oven and the hybridisation cocktail was removed and stored at -20°C. The chip was filled with 100µl of wash buffer and placed in the fluidics station.

1200µl of Stain Solution was made up in an eppendorf;

600µl	2x Stain Buffer
540µl	RNAse-free water
48µl	Acetylated BSA (50mg/ml)
12µl	SAPE (1mg/ml)

This was then split in to two 600µl aliquots in separate eppendorfs.

600µl of Antibody solution was then made up in an eppendorf using;

300µl	2x Stain Buffer
266.4µl	RNAse-free water
24µl	Acetylated BSA (50mg/ml)
6µl	Normal Goat IgG (10mg/ml)
3.6µl	Biotinylated Antibody (0.5mg/ml)

The washing and staining protocol was then run adding the staining tubes and antibody tubes when required. At the end of the protocol each chip was checked for bubbles (if found, replace the chip in the fluidics station and refill with Wash Buffer A). If no bubbles were found the chip was scanned in the scanner.

The following quality controls were then checked before the sample could proceed onto being hybridised onto a full chip;

The chip was seen to be well aligned (no more than 2 pixel rows out).

No scratches or fluff obliterated the chip features.

The scale factor was below around 0.75, and similar for all chips.

The raw Q was around 1.

All Bio spiked controls were called as present with the exception of BioB.

The Bio and Cre spiked controls were called as present and increasing in intensities.

The relevant housekeeping genes were all present and at reasonable levels.

None of the relevant housekeeping genes had a 3'/5' ratio of above 3.

9.1.1.10 Full Chip hybridisation

Full chips were unpackaged and left to equilibrate to room temperature while rotisserie oven was allowed to warm up.

The frozen hybridisation medium that was previously withdrawn from the Test3 chip was allowed to thaw and then heated to 99°C for 5 minutes followed by 45°C for 5 minutes in PCR machine.

While the hybridisation medium was denaturing the full chip (GeneChip Mouse430A array) was filled with 250µl of 1xHybridisation buffer. The full chips were then pre-hybridised in the rotisserie oven for 10 minutes at 45°C, rotating at 60rpm.

After denaturing, the Hybridisation cocktail was centrifuged at maximum speed for 5 minutes in order to remove any insoluble material. When both the hybridisation cocktail and the chips are ready the chips were taken from the rotisserie oven, the pre-hybridisation medium removed and replaced with 200µl of hybridisation cocktail.

The chip was allowed to hybridise in the rotisserie oven for 16 hours or more at 45°C, rotating at 60 rpm, with the chips secured in the oven racks.

9.1.1.11 Washing, Staining and Scanning full chips

All solutions such as Buffer A and Buffer B were filter sterilized before use. Antifoam was then added to Buffer A after filtration. The Fluidics station was primed and the chips removed from the rotisserie oven. The Hybridisation Cocktail was then removed from the chip and stored at -20°C and the chip filled with 250µl of Hybridisation buffer A and placed in the fluidics station.

1200µl of Stain Solution was made up in an eppendorf;

600µl	2x Stain Buffer
540µl	RNAse-free water
48µl	Acetylated BSA (50mg/ml)
12µl	SAPE (1mg/ml)

This was then split in to two 600µl aliquots in separate eppendorfs.

600µl of Antibody solution was then made up in an eppendorf using;

300µl	2x Stain Buffer
266.4µl	RNAse-free water
24µl	Acetylated BSA (50mg/ml)
6µl	Normal Goat IgG (10mg/ml)
3.6µl	Biotinylated Antibody (0.5mg/ml)

The stain protocol (EukGE-WS2) was run on each chip adding the Stain Solution when required.

When the fluidics station requested the antibody solution (after the second wash with Buffer A), the chip was instead taken out of the fluidics station and scanned under a different filename. This was in order to give a pre-amplification back up of the data, just in case the chip had reached saturation. Once this was done, the chip was replaced in the fluidics station and the antibody solution and SAPE added in turn.

Once the protocol had finished (approximately 2 hours) the chip was checked for bubbles and if free from bubbles, placed in the scanner.

10 Appendix B

10.1 MIAME (Minimal Information about A Micro array Experiment):

10.1.1 Experiment Design/Type of Experiment:

This experiment was designed as a time course experiment to measure the gene expression changes within the Whole Ganglionic Eminence (WGE) at the embryonic ages of E12, E14 and E16.

10.1.1.1 Experimental factors:

The only Experimental parameters tested in this experiment were the gene expression changes seen between the three embryonic ages of E12, E14 and E16.

Each experimental array was hybridised once.

Three replicates for each age group were tested. Each RNA sample replicate was extracted from a pooled sample of WGE tissue dissected from the embryos of three dams.

10.1.1.2 Quality control steps taken:

Dissectional accuracy was checked using semi-quantitive RT-PCR of WGE RNA to probe for the dorsally expressed Pax-6 gene.

RNA was quality was checked using both UV spectrometry and the Agilent 2100 Bioanalyser.

TEST3 chips were run before hybridising to the Affymetrix Mouse430a Genechips.

10.1.2 Samples used, extract preparation and labelling:

10.1.2.1 The origin of the biological sample

Samples were taken from the CD1(ICR) strain of *Mus musculus*; time mated dams were supplied by Harlan UK Ltd (Bicester). These were housed in 12:12 conditions and fed on an expanded supplementary diet (due to pregnancy).

Dams were food deprived for 12 hours prior to sacrifice (taking account of genetic changes due to short term effects caused by eating and insulin release).

Dams were sacrificed and the tissue dissected within 2 hours of lights on (between 06:00 and 08:00) to take account of genetic changes due to circadian rhythms.

Tissue was flash frozen in liquid nitrogen immediately after dissection and kept at -80°C until RNA extraction (as described in Chapter Two; Methods).

All samples were processed using the same methods (outlined in Chapter Two; Methods).

All samples were treated under blind conditions after RNA extraction.

All samples were labelled using the same techniques, in the same laboratory, by the same technician (outlined in Chapter Two; Methods).

All Mouse430a Genechips were from the same print batch and each contained 64 "spiked in" control genes.

10.1.3 Hybridization procedures and parameters:

10.1.3.1 The protocol and conditions used during hybridization, blocking and washing:

These are described in full in Chapter Two; Methods.

10.1.4 Measurement data and specifications:

10.1.4.1 Type of scanning hardware and software used:

An Agilent GeneArray Scanner was used to scan the GeneChip. This information was then saved in a data (*.dat) file.

10.1.5 Type of image analysis software used:

MAS (Microarray Suite) version 5.0.0.032 (2001;Affymetrix) was then used to generate a cell intensity file (*.cel) from the image data file (*.dat). The cell analysis algorithm analyzes the *.dat and computes a single intensity value for each probe cell on an array convert the (*.dat) files into cell intensity (*.cel) files.

10.1.5.1 A description of the measurements produced by the image-analysis software:

MAS 5.0 computed the signal intensities of all 22,690 probes; These figures were saved as a data (*.dat) file. MAS 5.0 was used the Expression Analysis algorithm to compute the cell intensities (which were stored in a cell (*.cel) file). These arbitrary figures ranged from 0 to 7671.2 and described the amount of biotin fluorescence picked up during the image analysis from each probe on the Genechip. These figures were used by RMA and MAS 5.0 to normalise the data by two different methods before looking at the data in GeneSpring and the NIH online Micro Array Analysis ANOVA tool.

10.1.5.2 The complete output of the image analysis before data selection and transformation (spot quantitation matrices).

The complete data set, before any normalisation (the (*cel) file for each chip) can be viewed on the Compact Disk in Appendix D.

10.1.5.3 Data selection and transformation procedures.

As described in Chapter Four; Methods

10.1.5.4 Final gene expression data table(s) used by the authors to make their conclusions after data selection and transformation (gene expression data matrices).

The complete expression data used to make conclusion on temporal expression changes of selected genes can be viewed in the results section of this chapter.

10.1.5.5 Array Design:

All samples were hybridised to Mouse430a Affymetrix GeneChips (from the same print batch). All technical specifications and probe locations/details can be found on the Affymetrix website at http://www.affymetrix.com/index.affx.

11 Appendix C

11.1 *In situ* hybridisations

11.1.1 Foxp1

11.1.1.1 E12

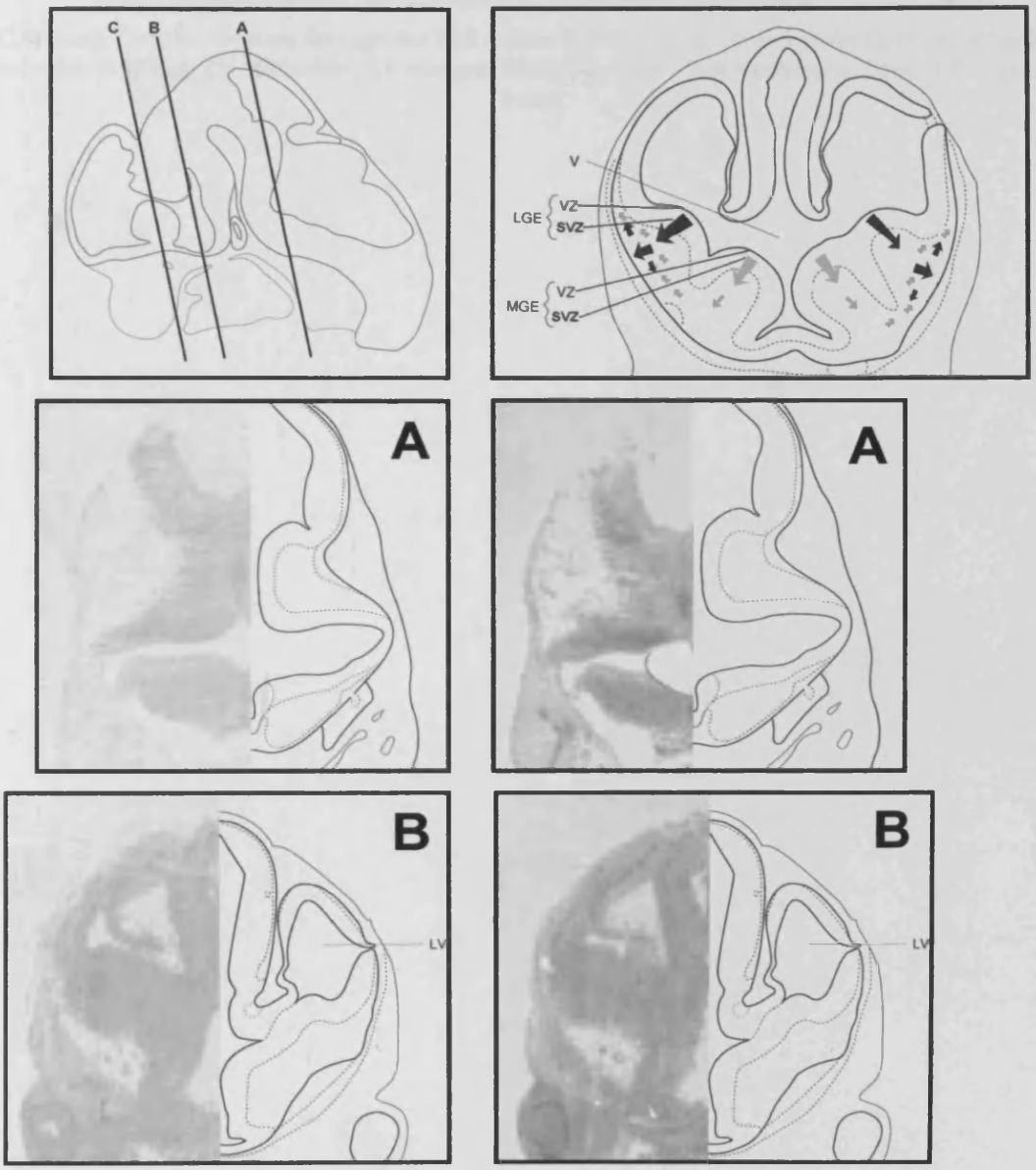

Fig. C.1; Coronal sections through the E12 mouse brain showing control probe (left) and experimental probe (right) for *Foxp1* (V=Ventricle, LV=Lateral Ventricle, **SVZ**= Sub Ventricular Zone, **VZ**=Ventricular Zone).

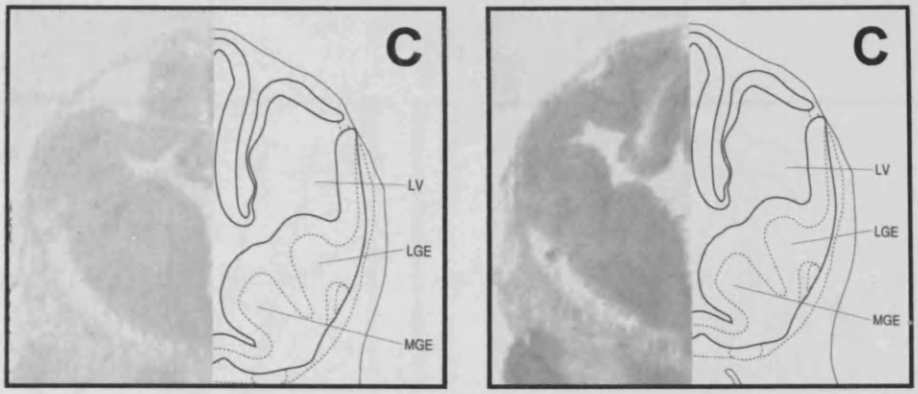

Fig. C.1(cont); Coronal sections through the E12 mouse brain showing control probe (left) and experimental probe (right) for *Foxp1* (V=Ventricle, LV=Lateral Ventricle, **SVZ**= Sub Ventricular Zone, **VZ**=Ventricular Zone).

11.1.1.2 E14

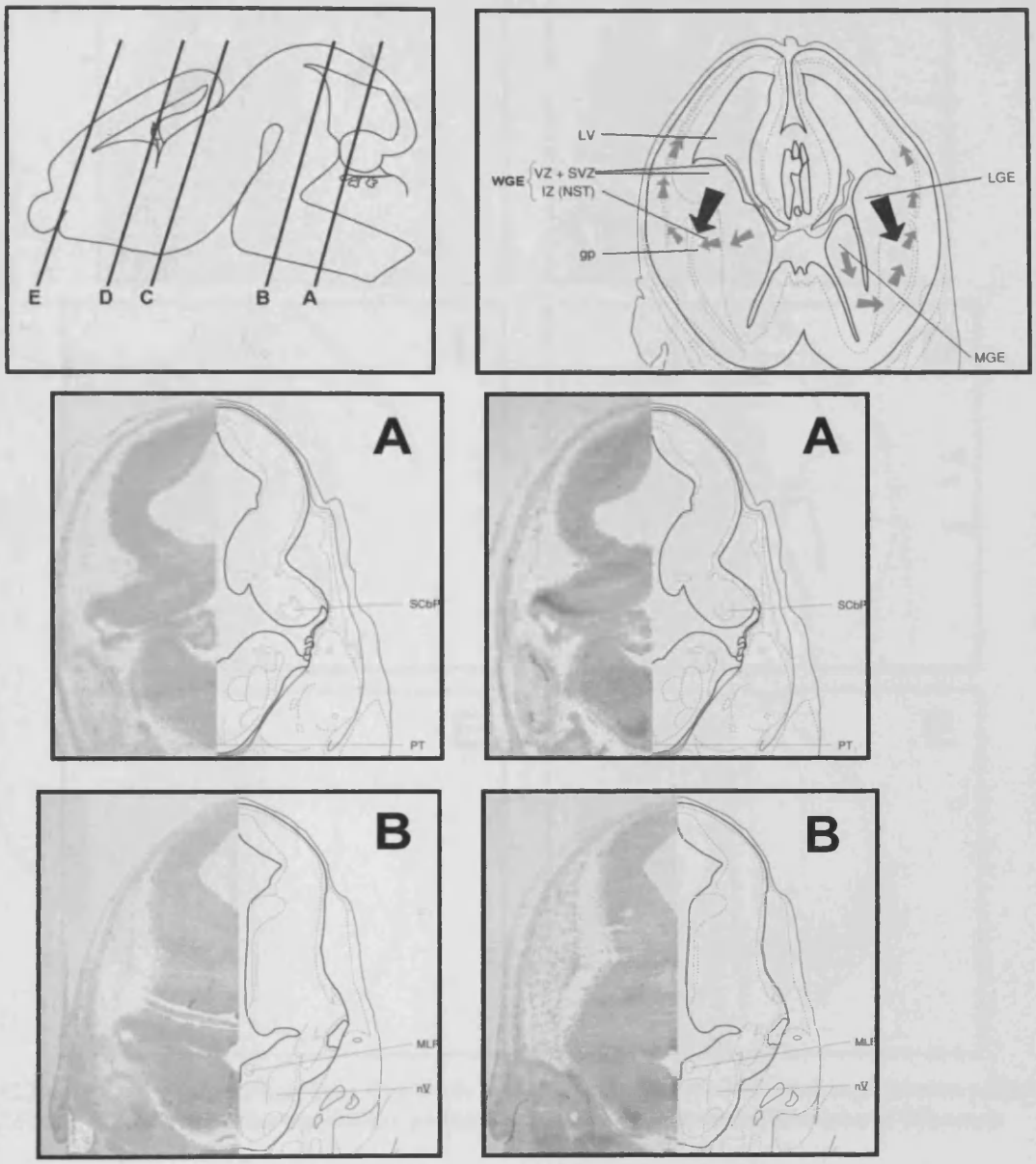

Fig. C.2; Coronal sections from E14 Brain showing control probe (left) and experimental probe for *Foxp1*. **(SCbP=Superior cerebellar peduncle, IVF=Interventricular foramen of Munroe).**

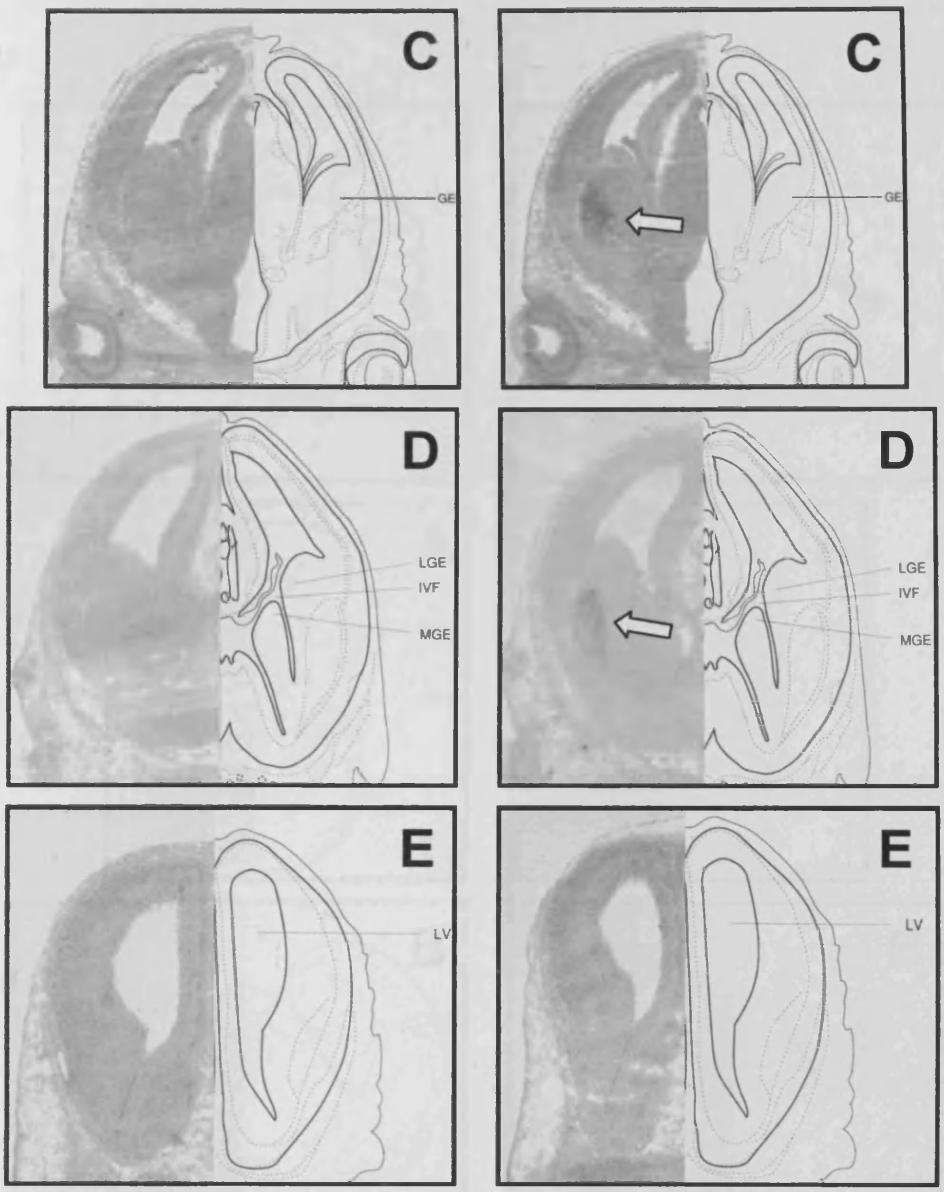

Fig. C.2(cont); Coronal sections from E14 Brain showing control probe (left) and experimental probe for *Foxp1*. **(SCbP=Superior cerebellar peduncle, IVF=Interventricular foramen of Munroe).**

11.1.1.3 E16

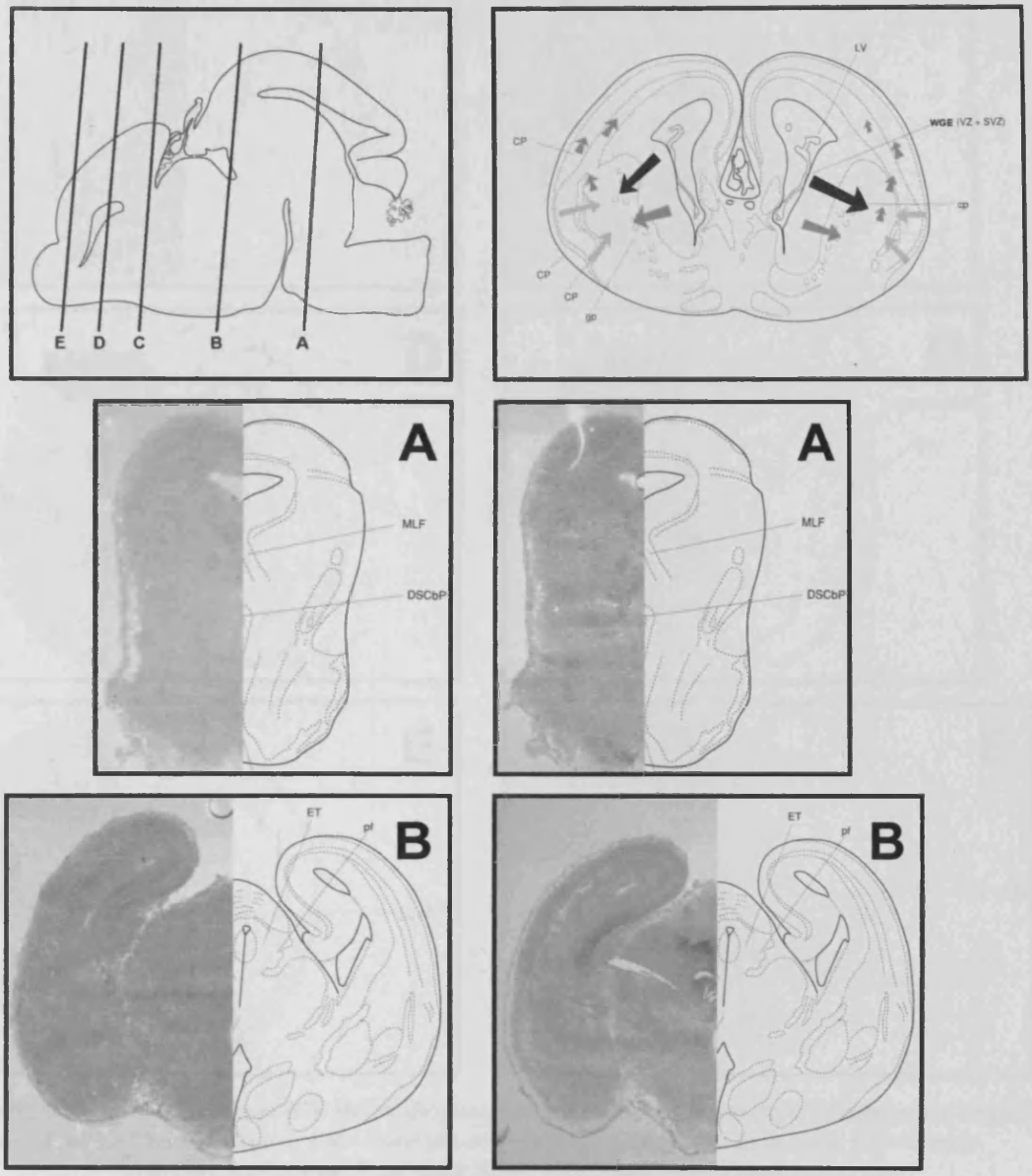

Fig. C.3; Coronal sections from E16 Brain showing control probe (left) and experimental probe (right) for *Foxp1*. (**ChPL**=Choroid Plexus, **LV**= Lateral ventricle, **GE**=Ganglionic Eminence, **CC**=Corpus Collosum, **SVZ**=Sub Ventricular Zone, **VZ**=Ventricular Zone).

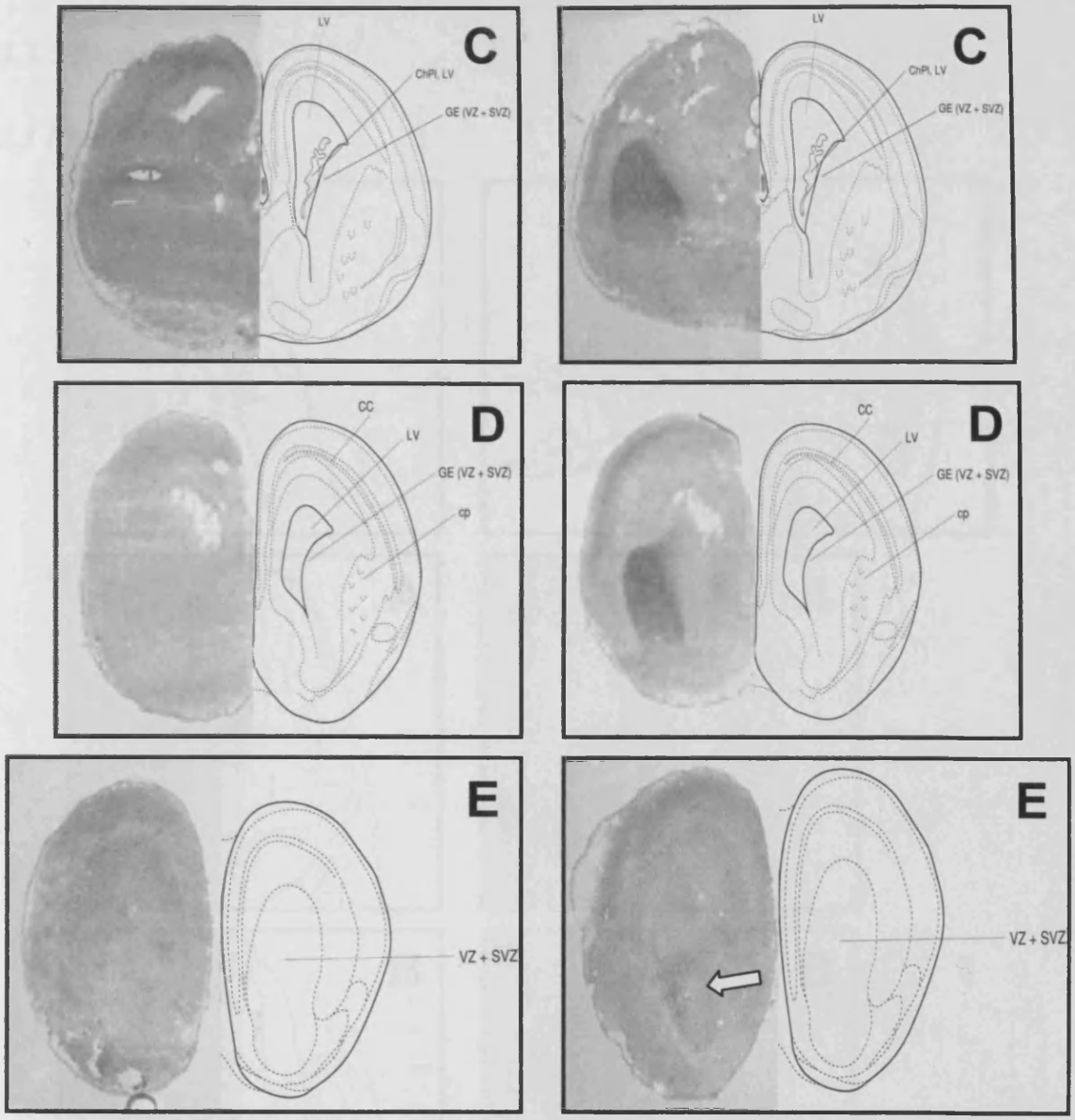

Fig. C.3(cont); Coronal sections from E16 Brain showing control probe (left) and experimental probe (right) for *Foxp1*. (**ChPL**=Choroid Plexus, **LV**= Lateral ventricle, **GE**=Ganglionic Eminence, **CC**=Corpus Collosum, **SVZ**=Sub Ventricular Zone, **VZ**=Ventricular Zone).

11.1.2 Foxp 2

11.1.2.1 E12

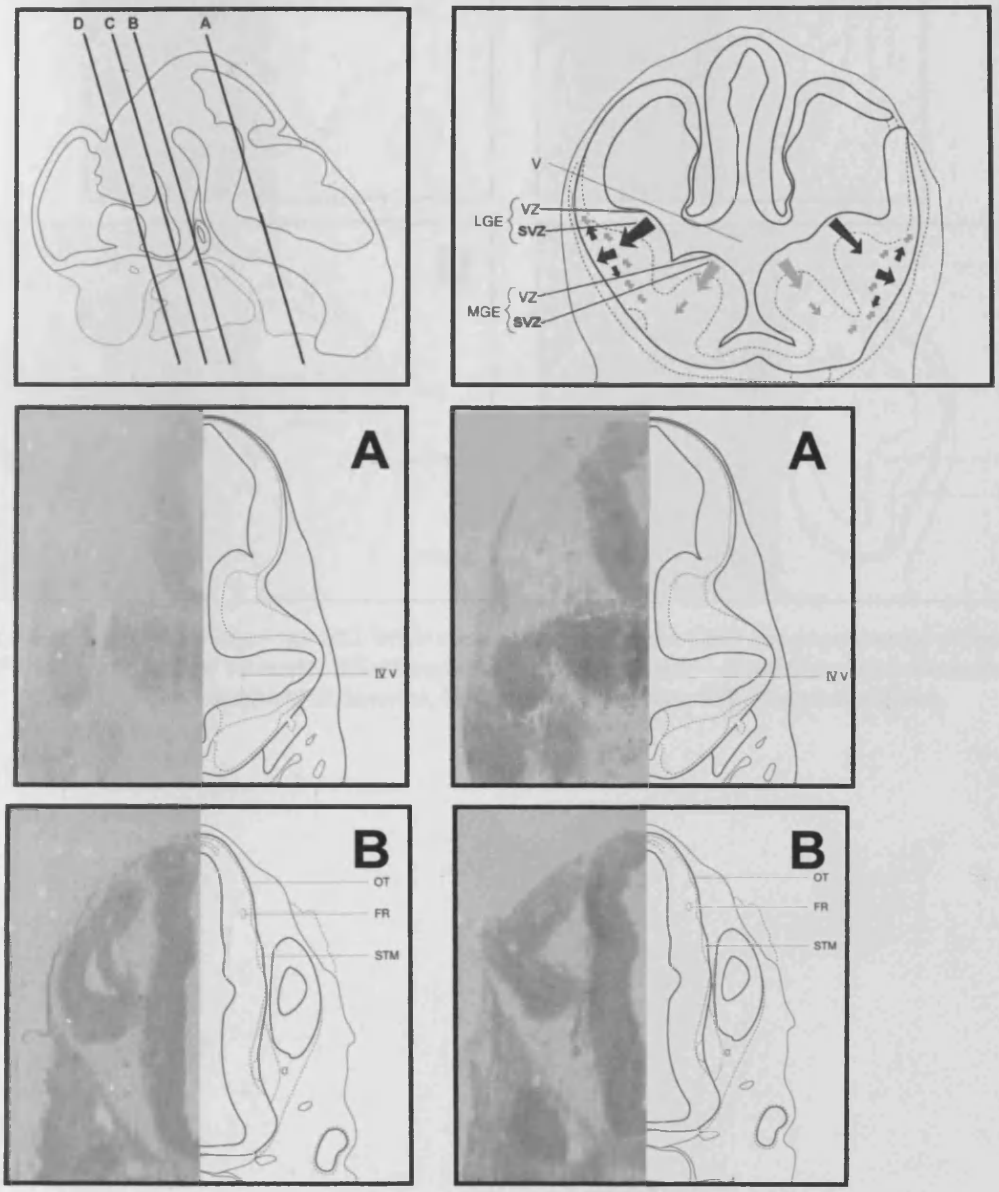

Fig. C.4; Coronal sections from E12 Brain showing control probe (left) and experimental probe (right) for *Foxp2*. (**LV**= Lateral ventricle, **GE**=Ganglionic Eminence, **LGE**=Lateral Ganglionic Eminence, **MGE**=Medial Ganglionic Eminence, **IZ**=Intermediate Zone, **VZ**=Ventricular Zone).

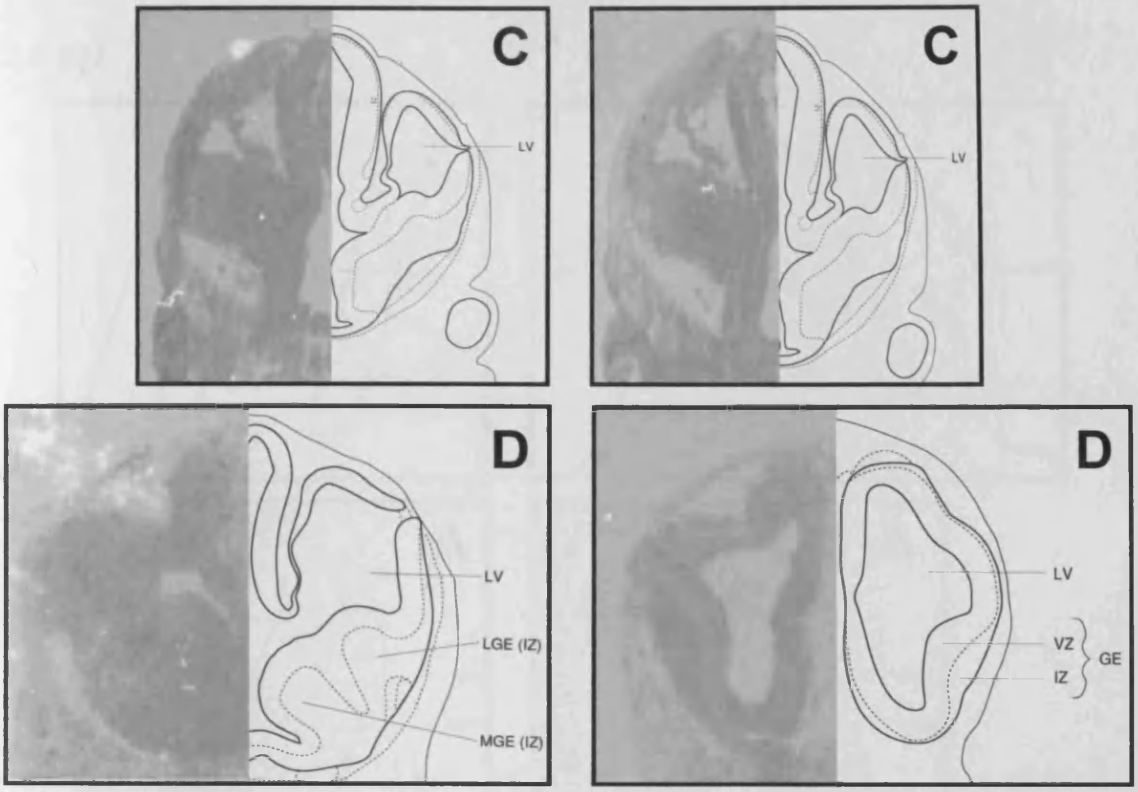

Fig. C.4(cont); Coronal sections from E12 Brain showing control probe (left) and experimental probe (right) for *Foxp2*. (**LV**= Lateral ventricle, **GE**=Ganglionic Eminence, **LGE**=Lateral Ganglionic Eminence, **MGE**=Medial Ganglionic Eminence, **IZ**=Intermediate Zone, **VZ**=Ventricular Zone).

11.1.2.2 E14

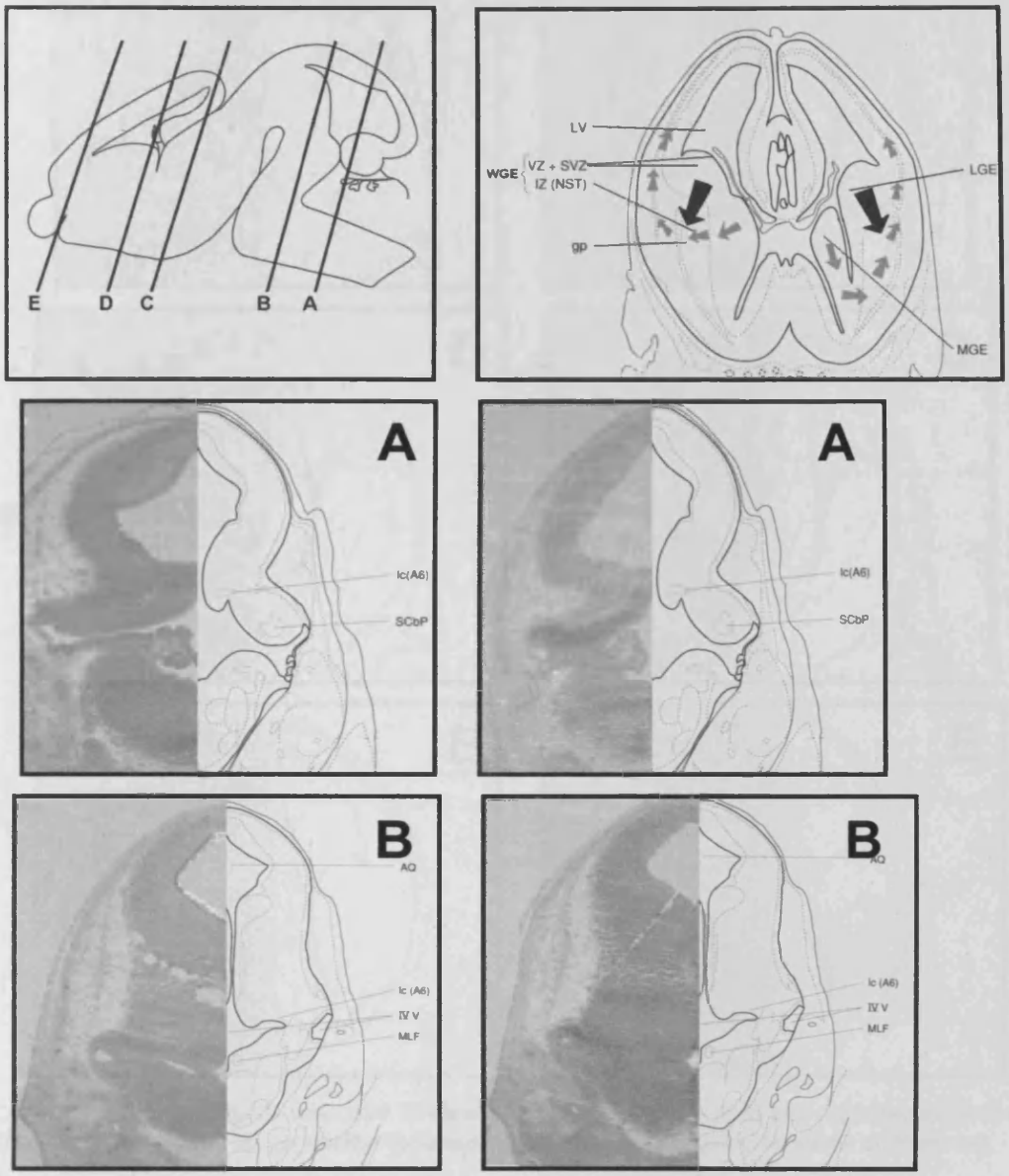

Fig. C.5; Coronal sections from E14 Brain showing control probe (left) and experimental probe for *Foxp2*. **(SCbP=Superior cerebellar peduncle, IVF=Interventricular foramen of Munroe).**

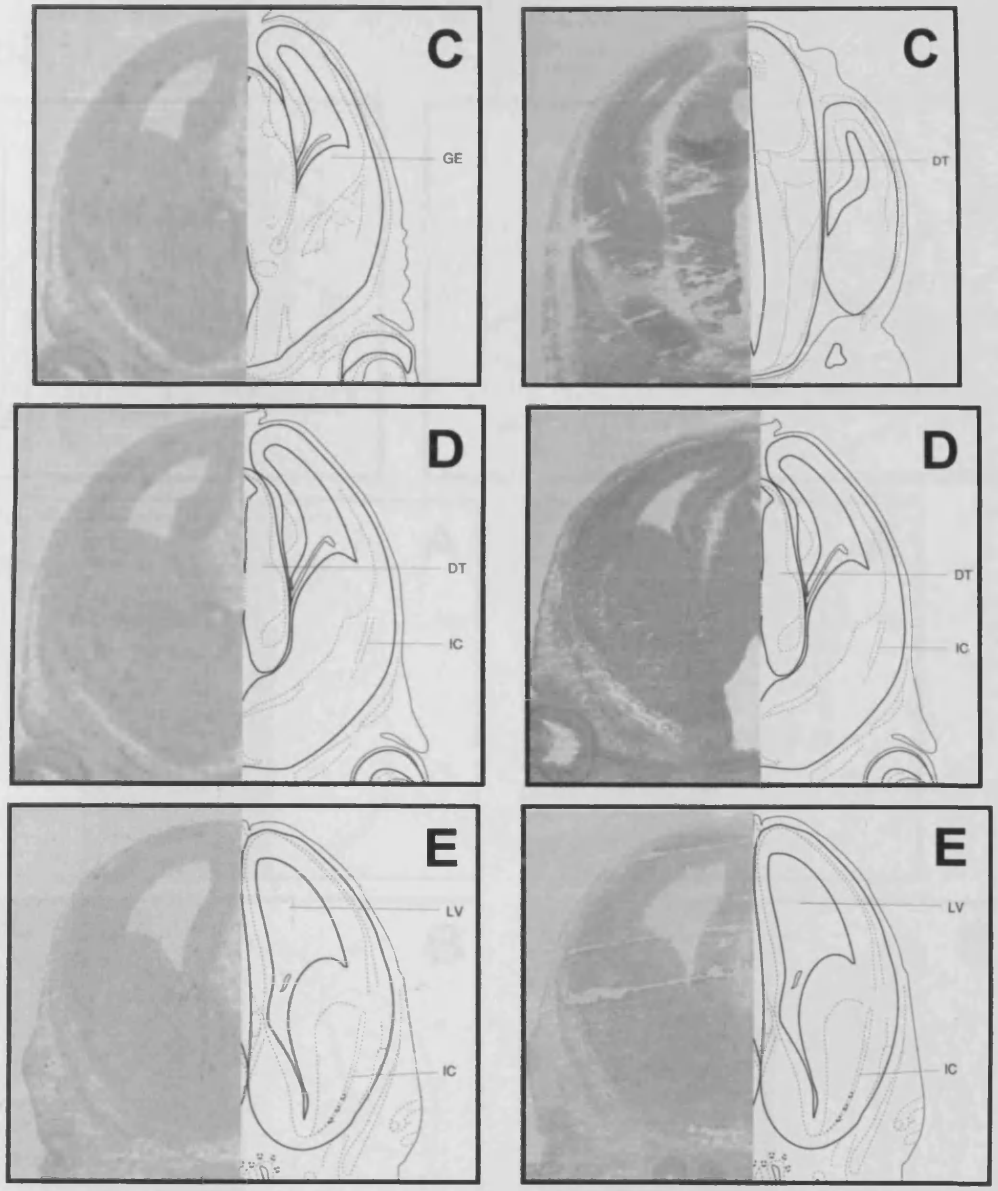

Fig. C.5(cont); Coronal sections from E14 Brain showing control probe (left) and experimental probe for *Foxp2*. **(SCbP=Superior cerebellar peduncle, IVF=Interventricular foramen of Munroe).**

11.1.2.3 E16

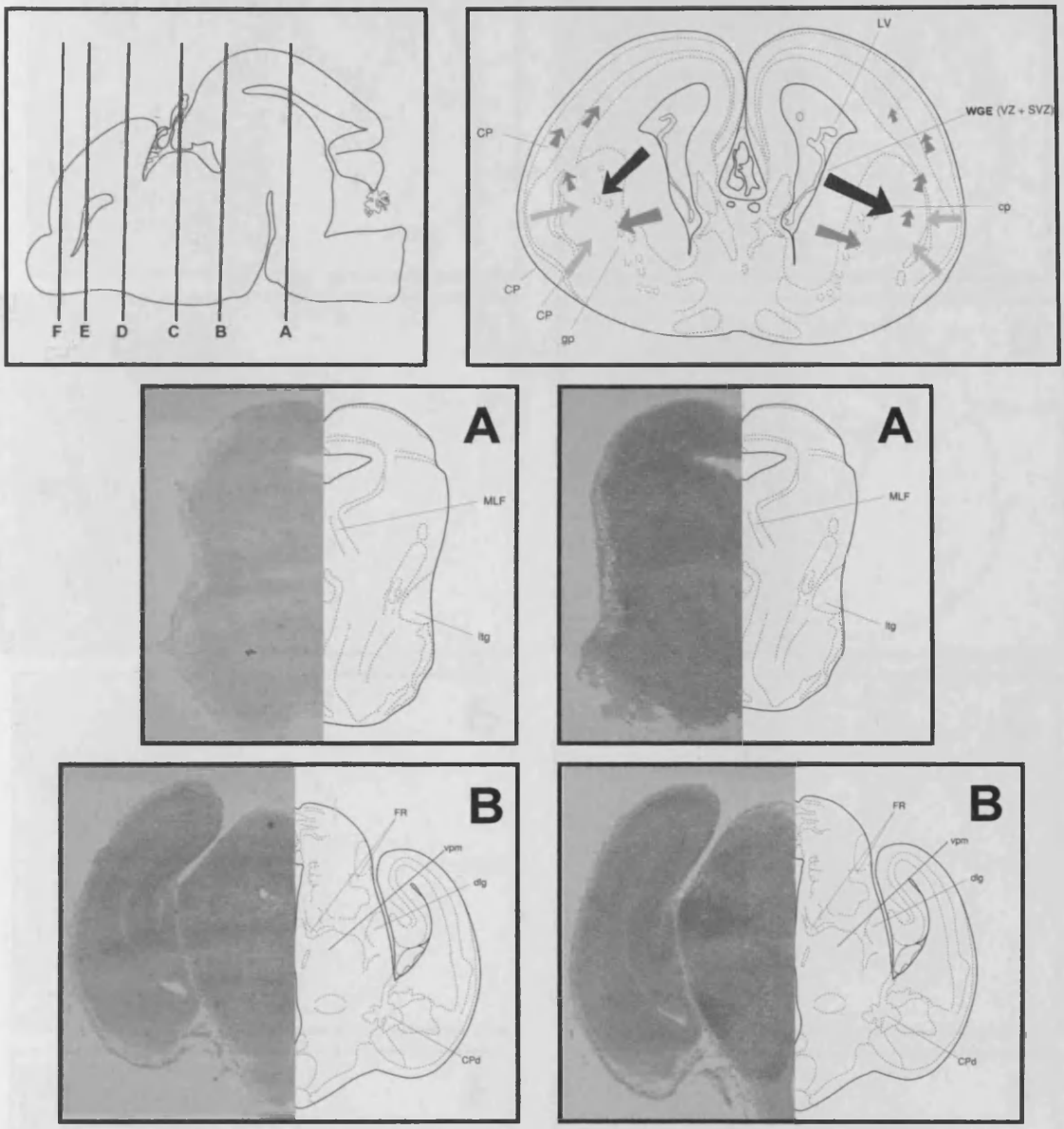

Fig. C.6; Coronal sections from E16 Brain showing control probe (left) and experimental probe (right) for *Foxp2*. (**hal**=Lateral Habenula, **DG**=Dentate Gyrus, **cp**=Caudate Putamen, **gp**=Globus Pallidus, **CC, G**=Corpus Collosum, Genu, **ChPl**=Choroid Plexus, **a**=Nucleus Accumbens).

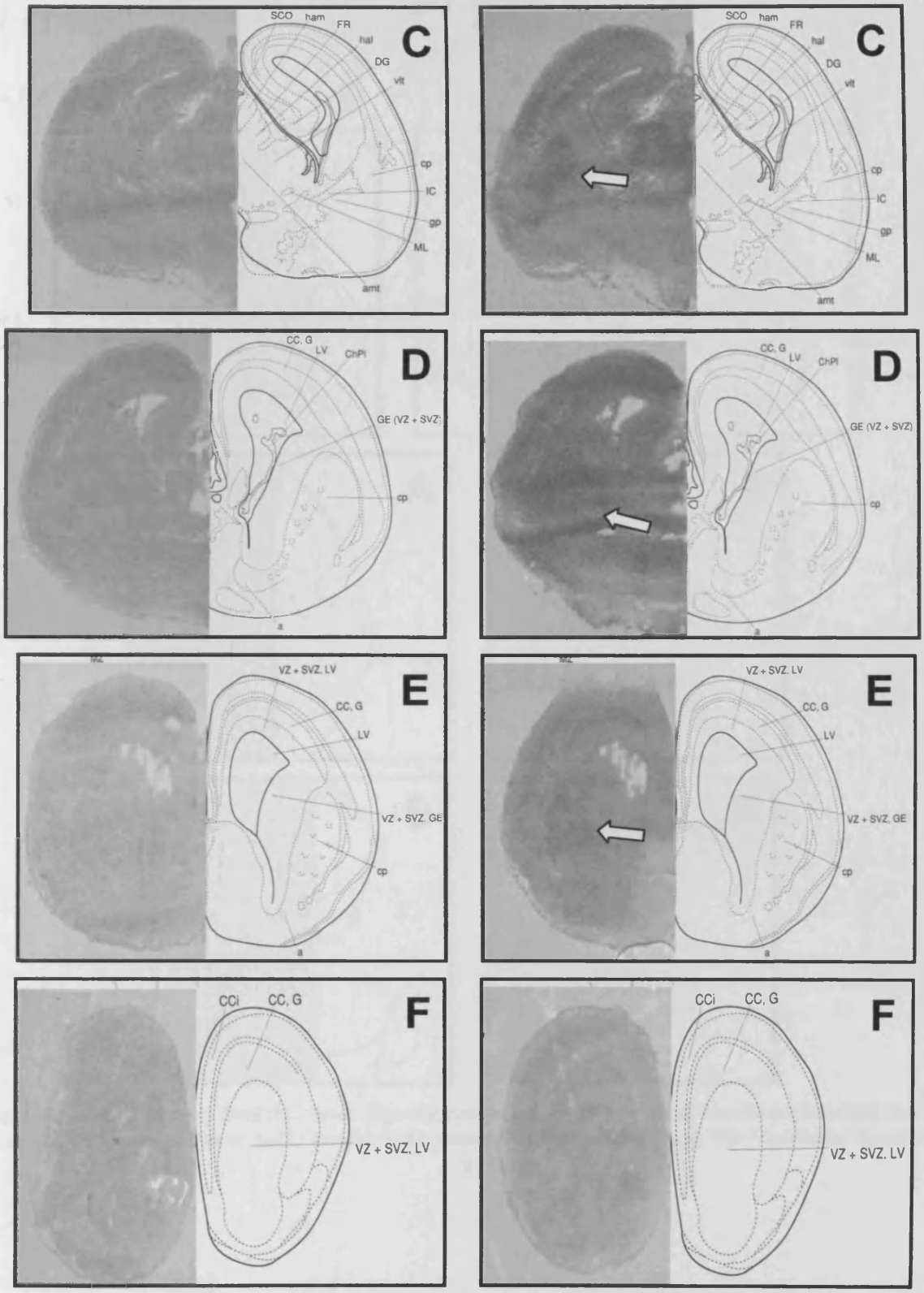

Fig. C.6(cont); Coronal sections from E16 Brain showing control probe (left) and experimental probe (right) for *Foxp2*. (**hal**=Lateral Habenula, **DG**=Dentate Gyrus, **cp**=Caudate Putamen, **gp**=Globus Pallidus, **CC, G**=Corpus Collosum, Genu, **ChPl**=Choroid Plexus, **a**=Nucleus Accumbens).

11.1.3 Cxcr4

11.1.3.1 E12

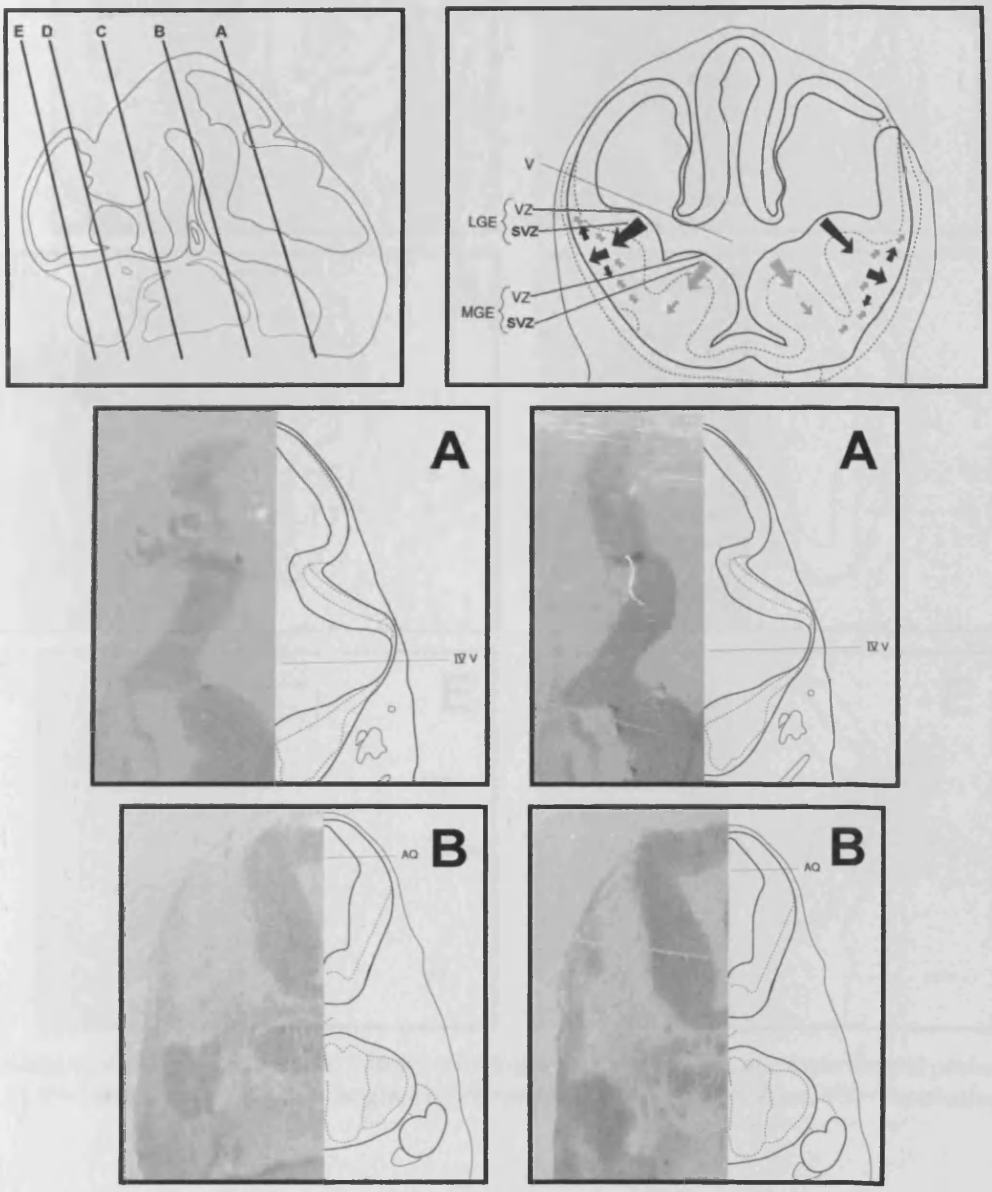

Fig. C.7; Coronal sections from E12 Brain showing control probe (left) and experimental probe (right) for *Cxcr4*. (**LV**= Lateral ventricle, **GE**=Ganglionic Eminence, **IZ**=Intermediate Zone, **VZ**=Ventricular Zone).

v

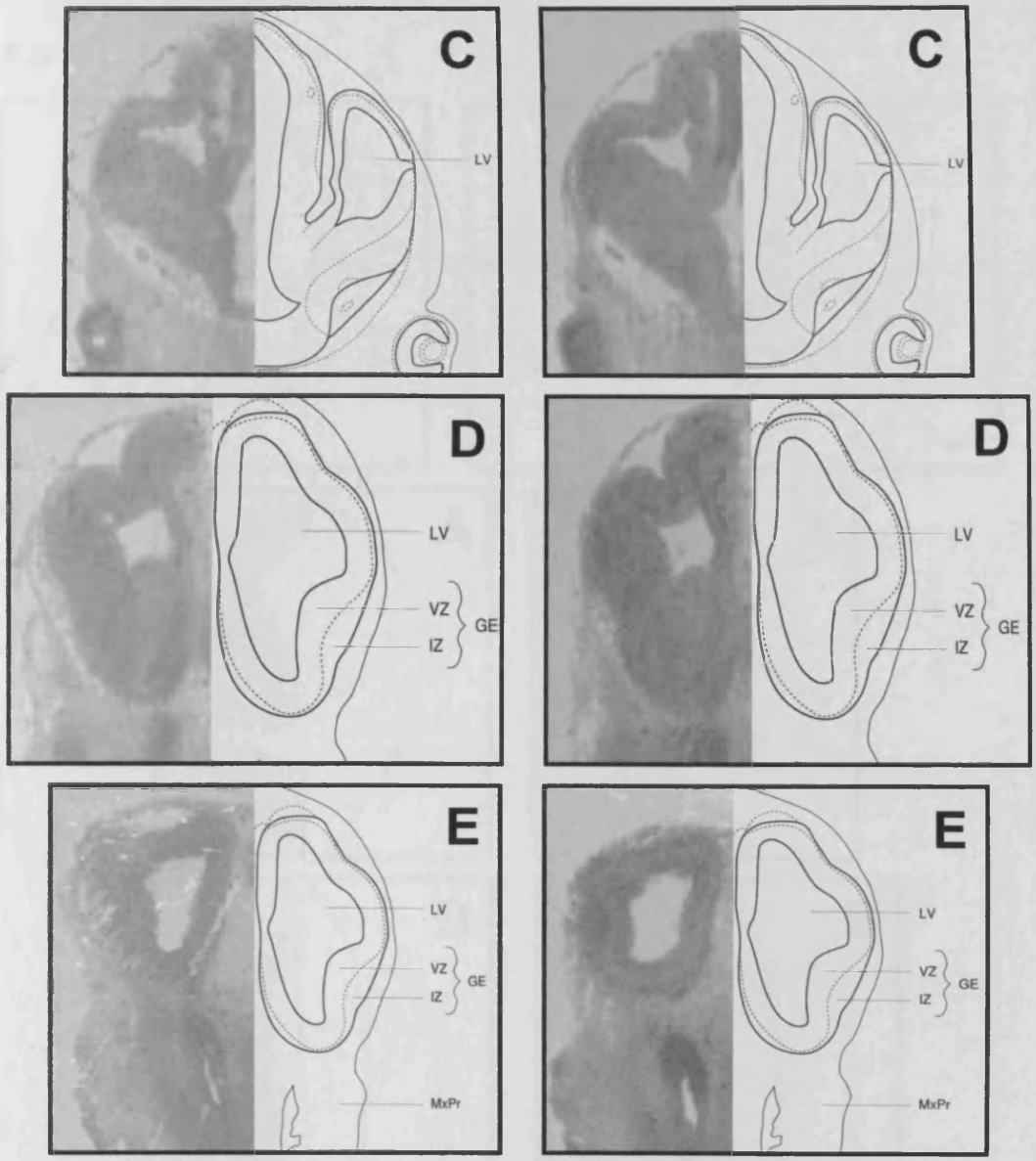

Fig. C.7(cont); Coronal sections from E12 Brain showing control probe (left) and experimental probe (right) for *Cxcr4*. (**LV**= Lateral ventricle, **GE**=Ganglionic Eminence, **IZ**=Intermediate Zone, **VZ**=Ventricular Zone).

11.1.3.2 E14

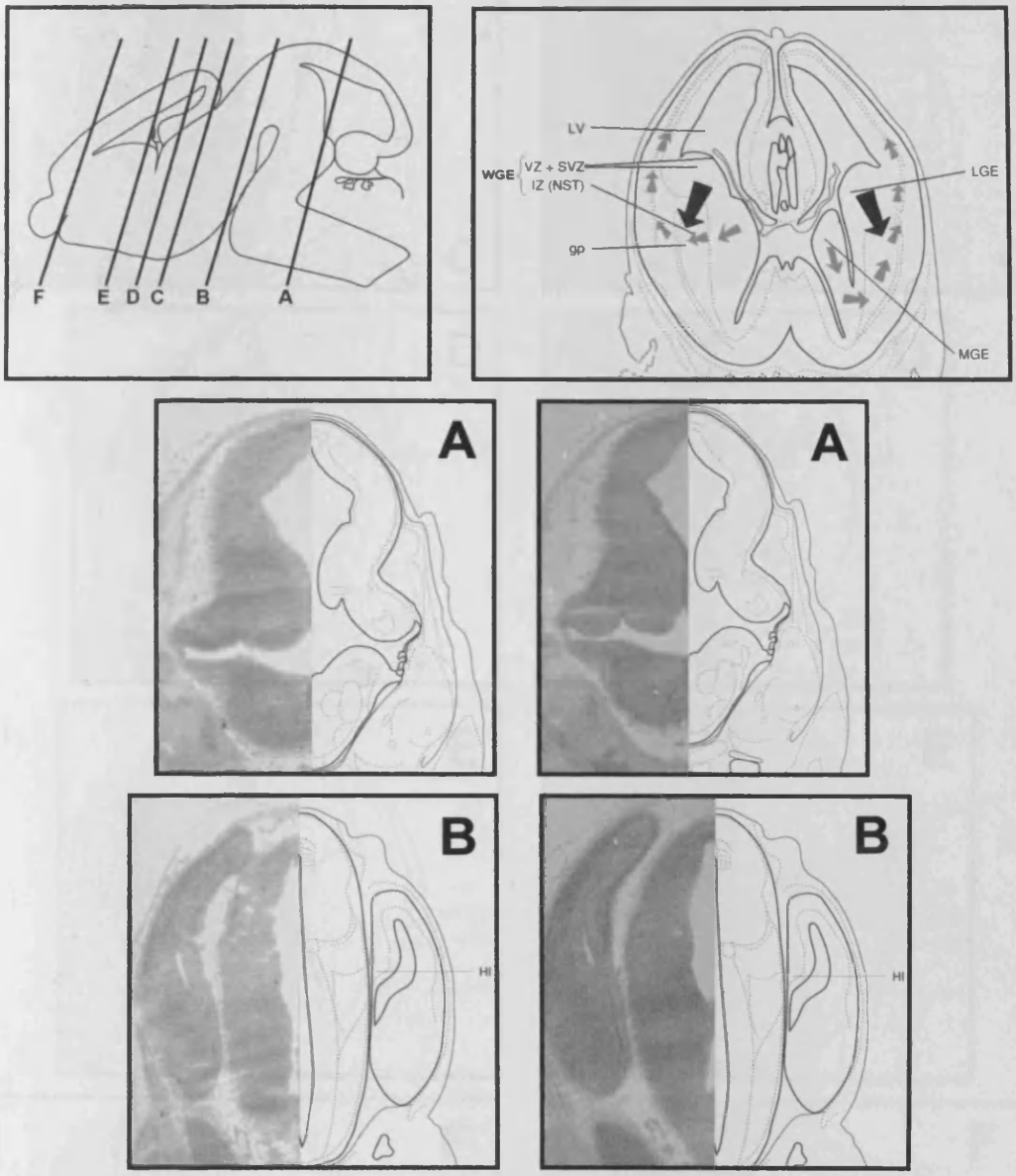

Fig. C.8; Coronal sections from E14 Brain showing control probe (left) and experimental probe (right) for *Cxcr4*. (**HI**=Hippocampus, **VZ**=Ventricular Zone, **CCi**=Cingulate Cortex, **IC**=internal capsule, **LOT**=Lateral Olfactory Tract, **cl**=claustrum, **ic**=internal capsule).

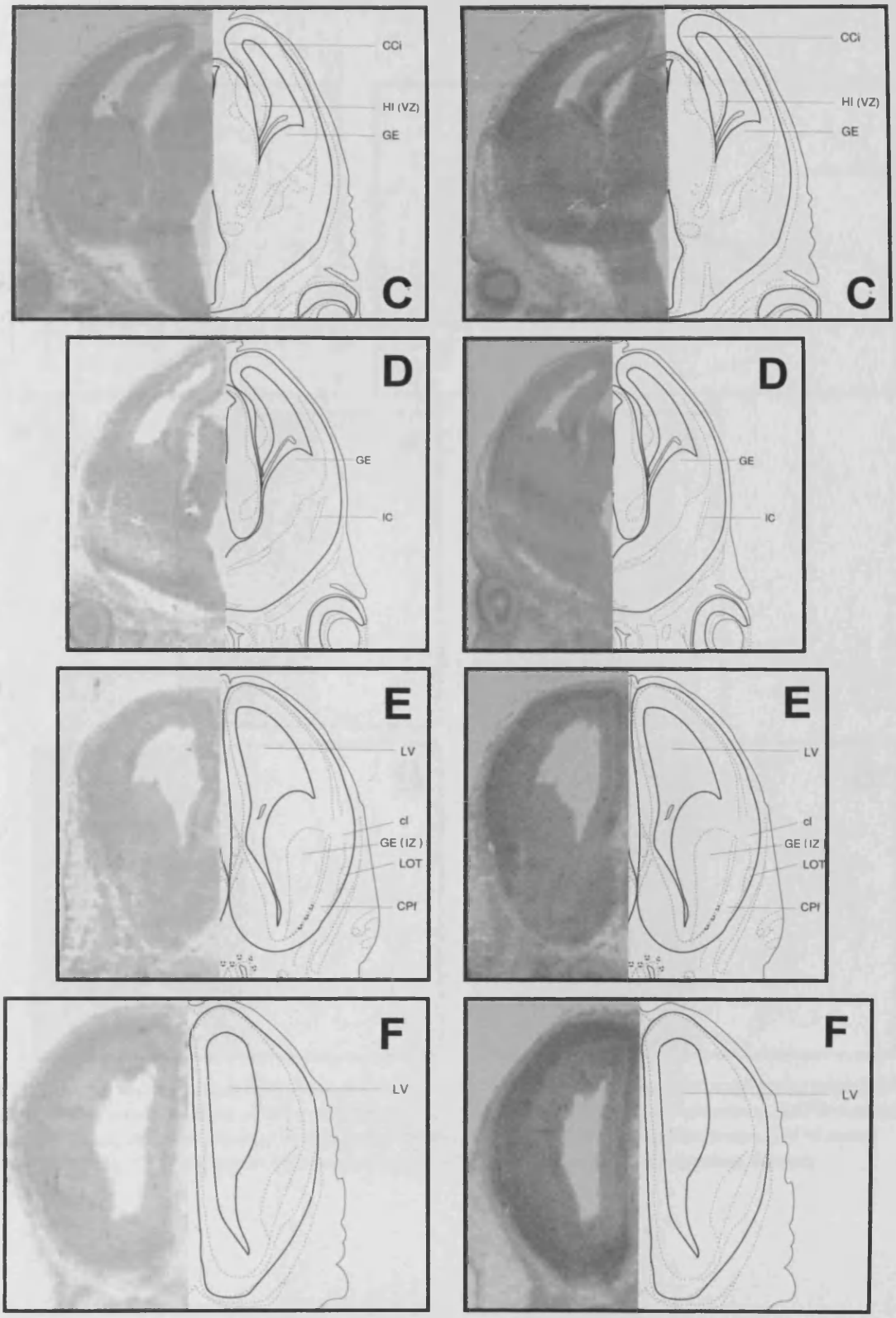

Fig. C.8(cont); Coronal sections from E14 Brain showing control probe (left) and experimental probe (right) for *Cxcr4*. (**HI**=Hippocampus, **VZ**=Ventricular Zone, **CCi**=Cingulate Cortex, **IC**=internal capsule, **LOT**=Lateral Olfactory Tract, **cl**=claustrum, **ic**=internal capsule).

11.1.3.3 E16

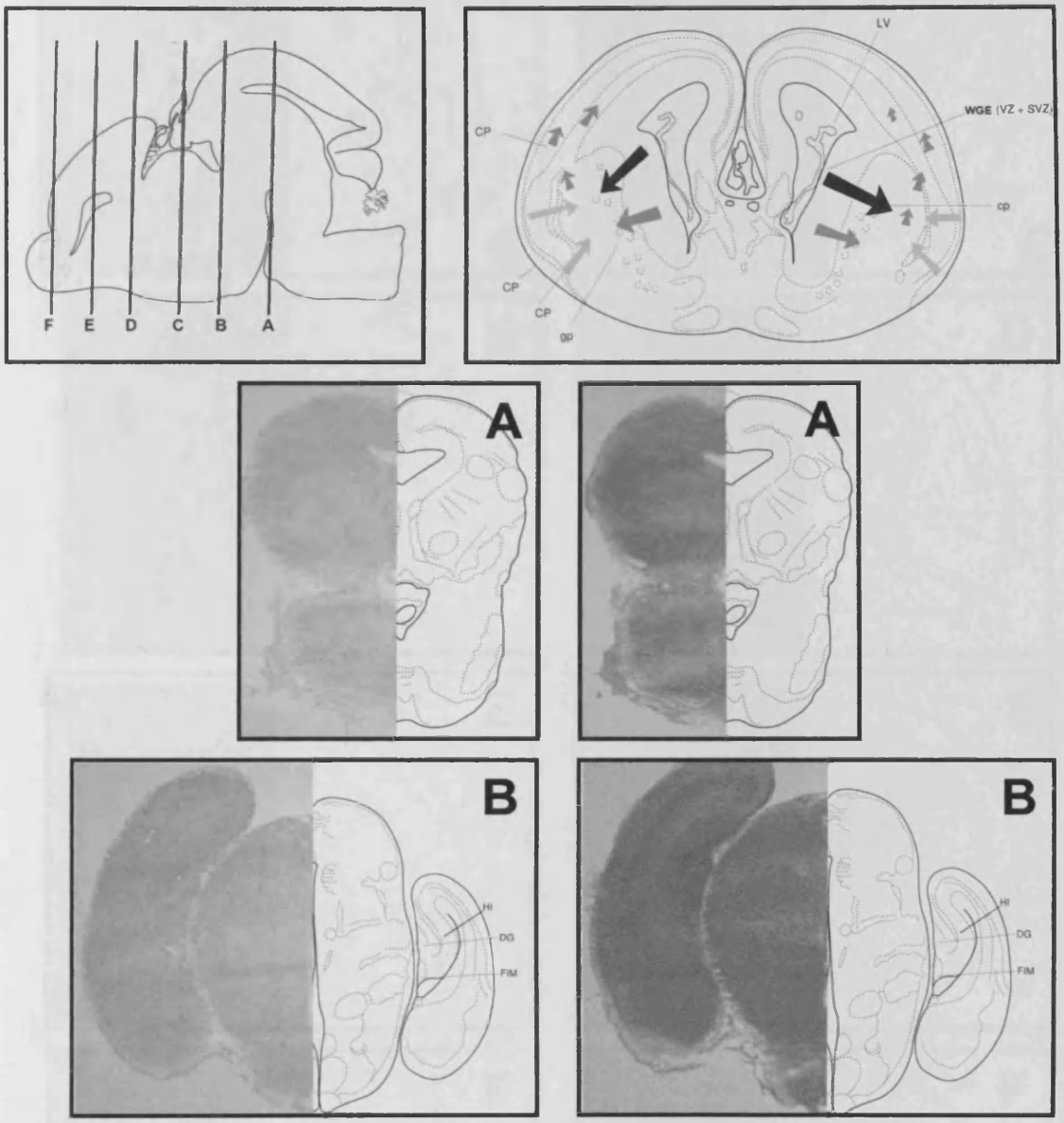

Fig. C.9; Coronal sections from E16 Brain showing control probe (left) and experimental probe (right) for *Cxcr4* (**pl,CPf**=Pyramidal layer of the pyriform cortex, **cp**=Caudate Putamen, **cl**=claustrum, **LOT**=Lateral Olfactory Tract, **AC,a**=Anterior Commisure (Anterior part), **GE**=Ganglionic Eminence, **LV**=Lateral Ventricle, **CC,G**=Corpus Collosum (Genu), **CFr**=Frontal Cortex, **DG**=Dentate Gyrus).

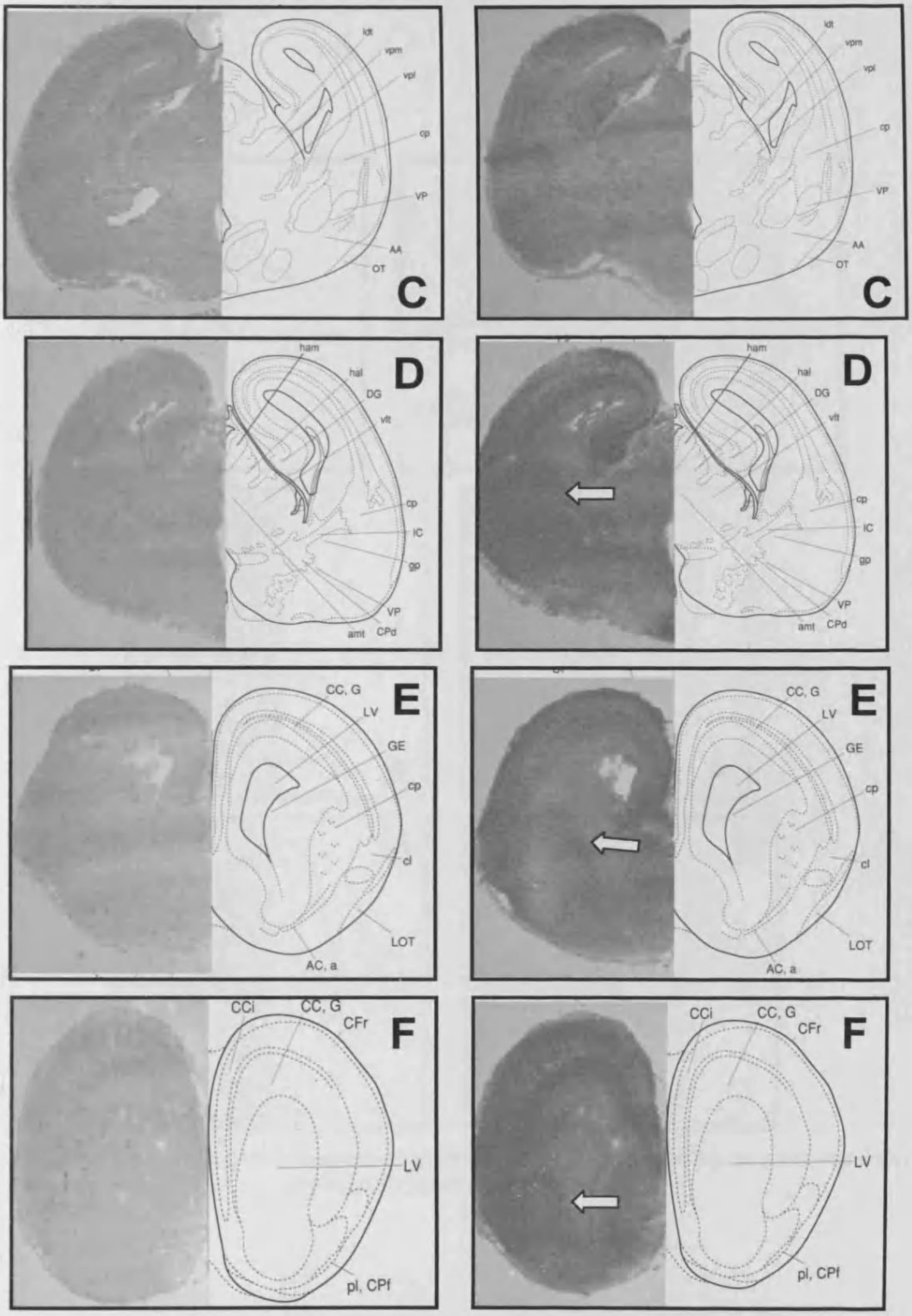

Fig. C.9 Coronal sections from E16 Brain showing control probe (left) and experimental probe (right) for *Cxcr4* (**pl,CPf**=Pyramidal layer of the pyriform cortex, **cp**=Caudate Putamen, **cl**=claustrum, **LOT**=Lateral Olfactory Tract, **AC,a**=Anterior Commisure (Anterior part), **GE**=Ganglionic Eminence, **LV**=Lateral Ventricle, **CC,G**=Corpus Collosum (Genu), **CFr**=Frontal Cortex, **DG**=Dentate Gyrus).

11.1.4 Mef2c

11.1.4.1 E12

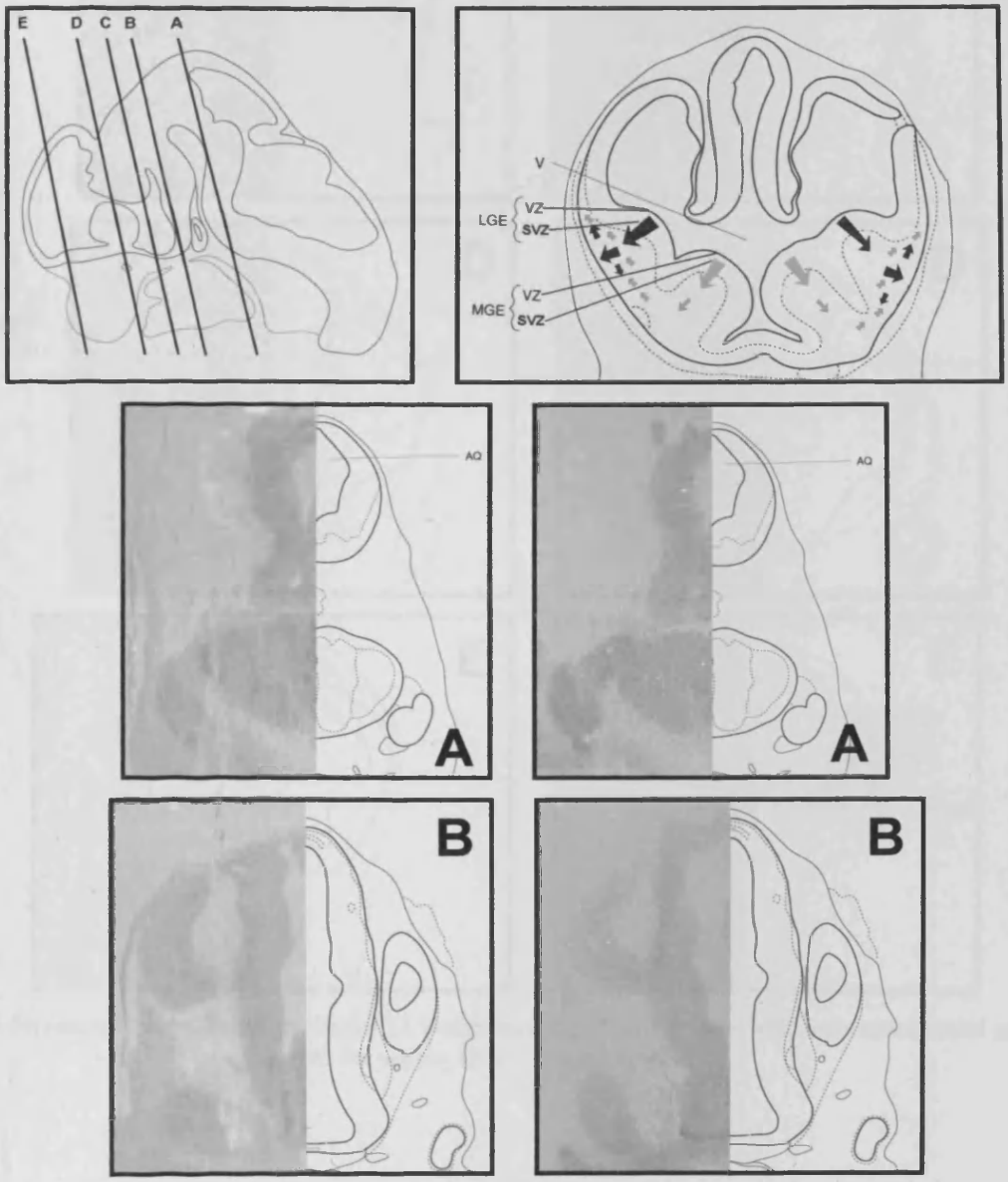

Fig. C.10; Coronal sections from E12 Brain showing control probe (left) and experimental probe (right) for *Mef2c*. (**LV**= Lateral ventricle).

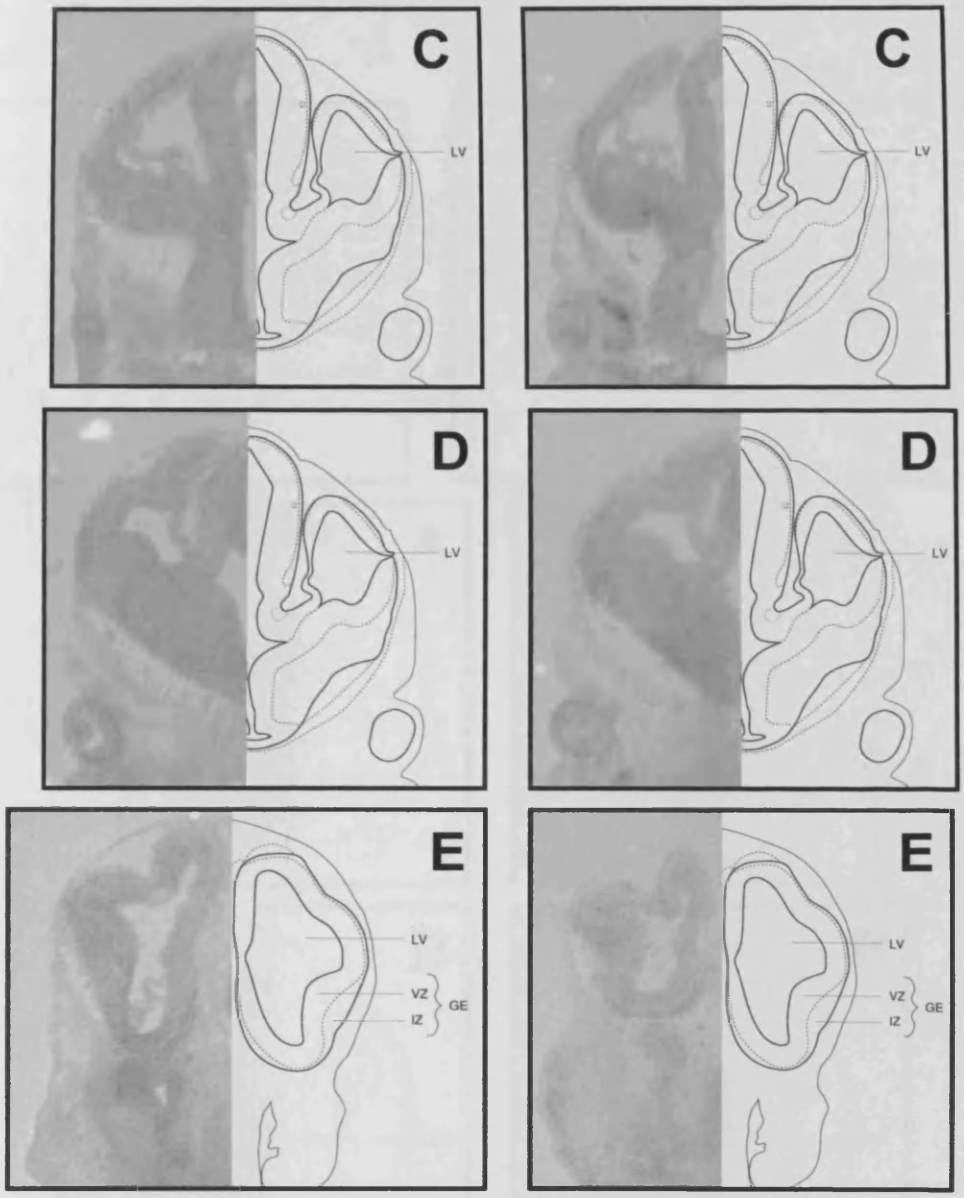

Fig. C.10(cont); Coronal sections from E12 Brain showing control probe (left) and experimental probe (right) for *Mef2c*. (**LV**= Lateral ventricle).

11.1.4.2 E14

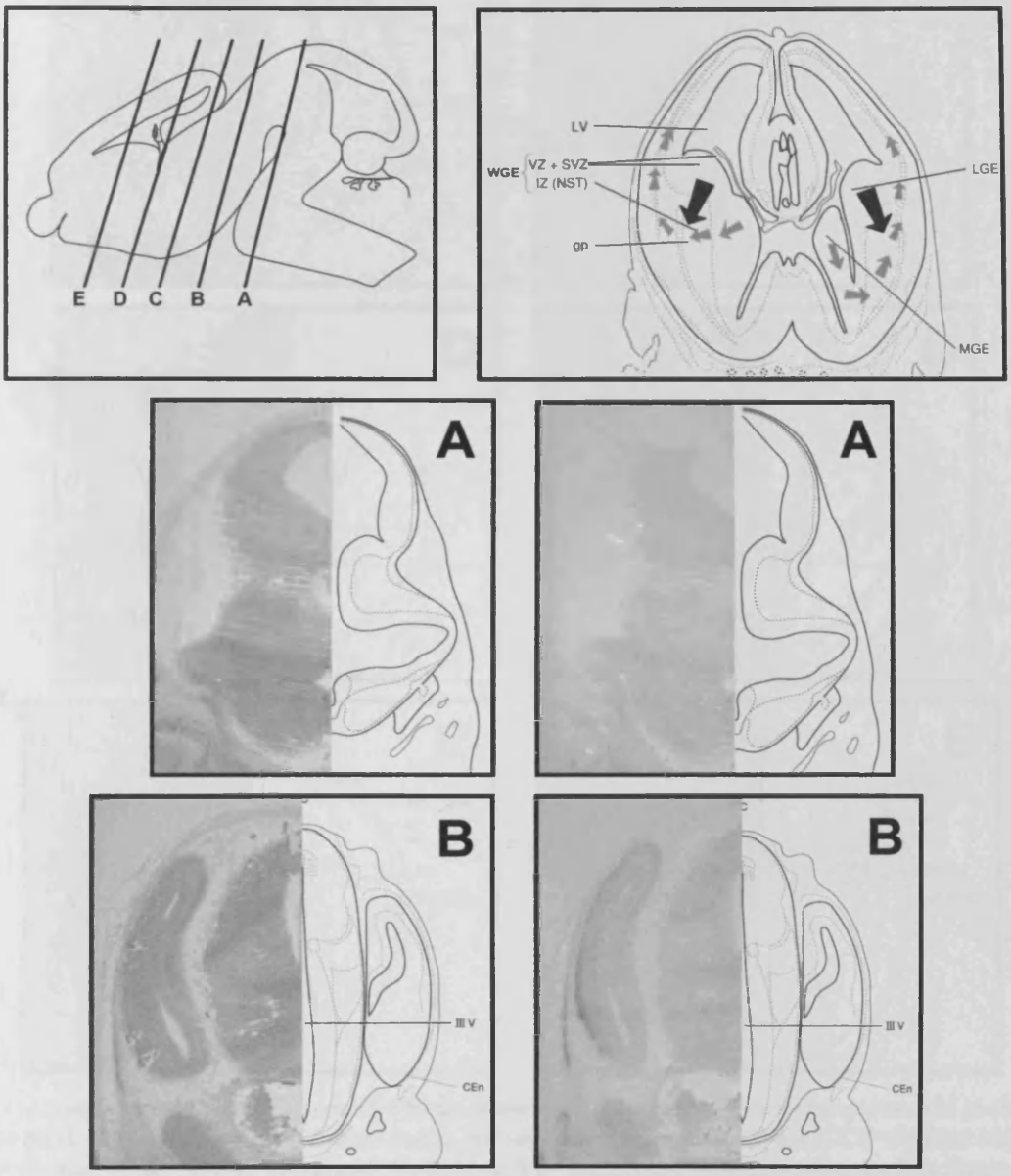

Fig. C.11; Coronal sections from E14 Brain showing control probe (left) and experimental probe for *Mef2c* (**IIIV**=3rd ventricle, **CFr**= frontal cortex, **SP**=subplate, **MZ**=marginal zone, **CP**=Cortical Plate, **LV**=Lateral ventricle, **GE**=Ganglionic Eminence, **VZ**=Ventricular Zone, **IZ**= Intermediate Zone).

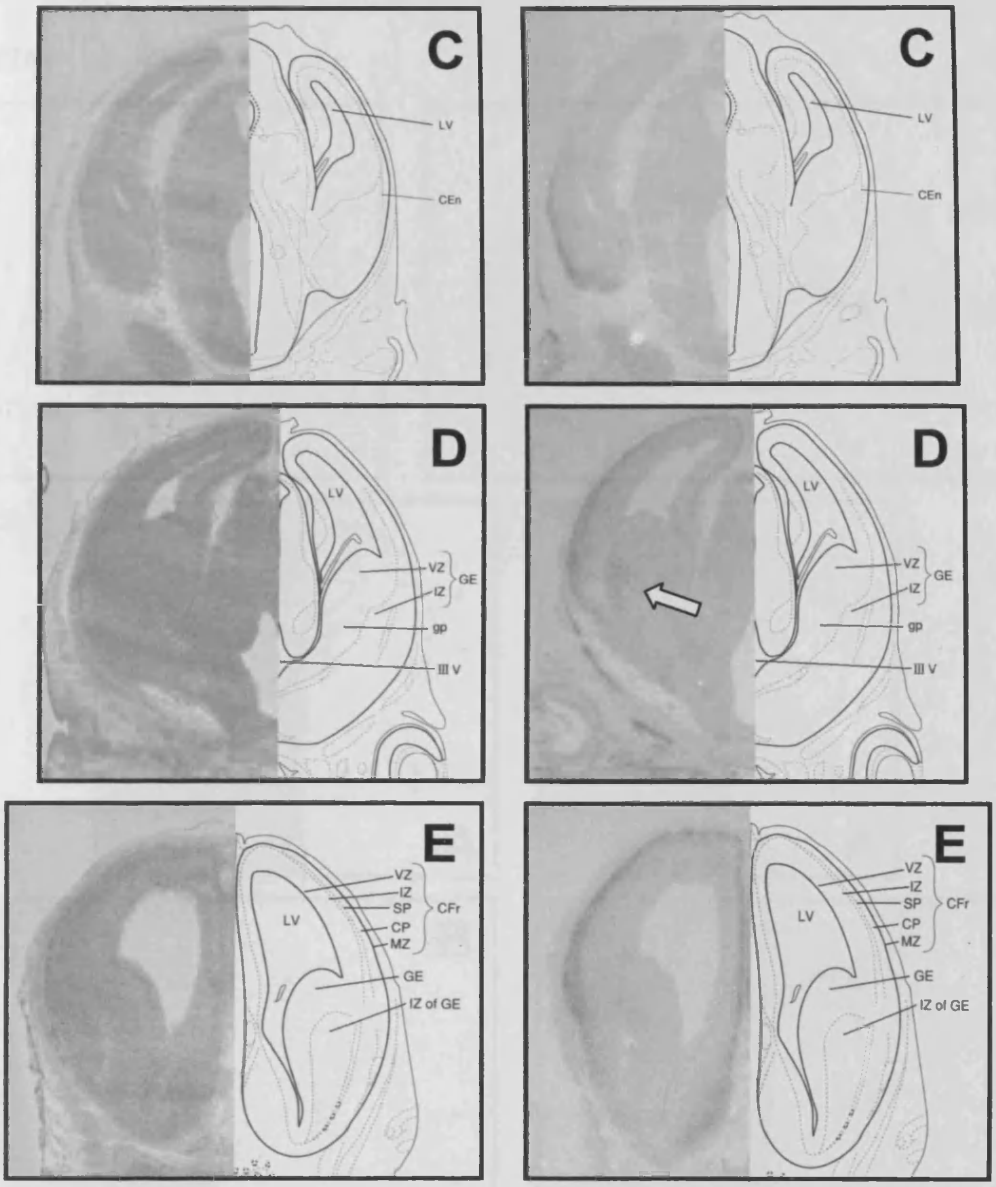

Fig. C.11(cont); Coronal sections from E14 Brain showing control probe (left) and experimental probe for *Mef2c* (**IIIV**=3rd ventricle, **CFr**= frontal cortex, **SP**=subplate, **MZ**=marginal zone, **CP**=Cortical Plate, **LV**=Lateral ventricle, **GE**=Ganglionic Eminence, **VZ**=Ventricular Zone, **IZ**= Intermediate Zone).

11.1.4.3 E16

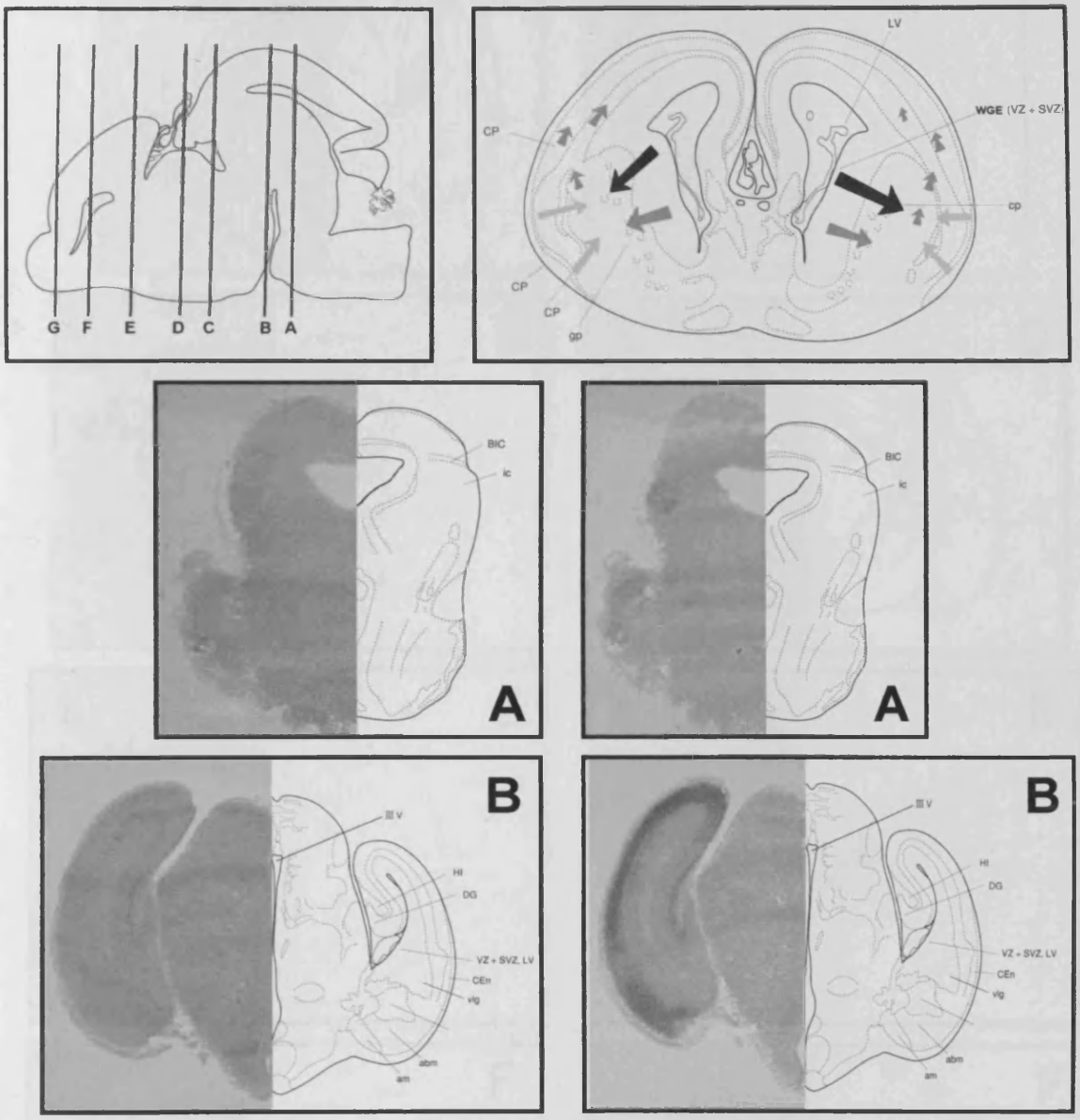

Fig. C.12; Coronal sections from E16 Brain showing control probe (left) and experimental probe (right) for *Mef2c*. (**CRs**=Retro-spinal Cortex, **Clr**=Infra-limbic Cortex, **GE**=Ganglionic Eminence, **VZ**=Ventricular Zone, **LOT**=Lateral Olfactory Tract, **CC,T**=corpus collosum, trunk, **Cln**=insular/rhinal cortex).

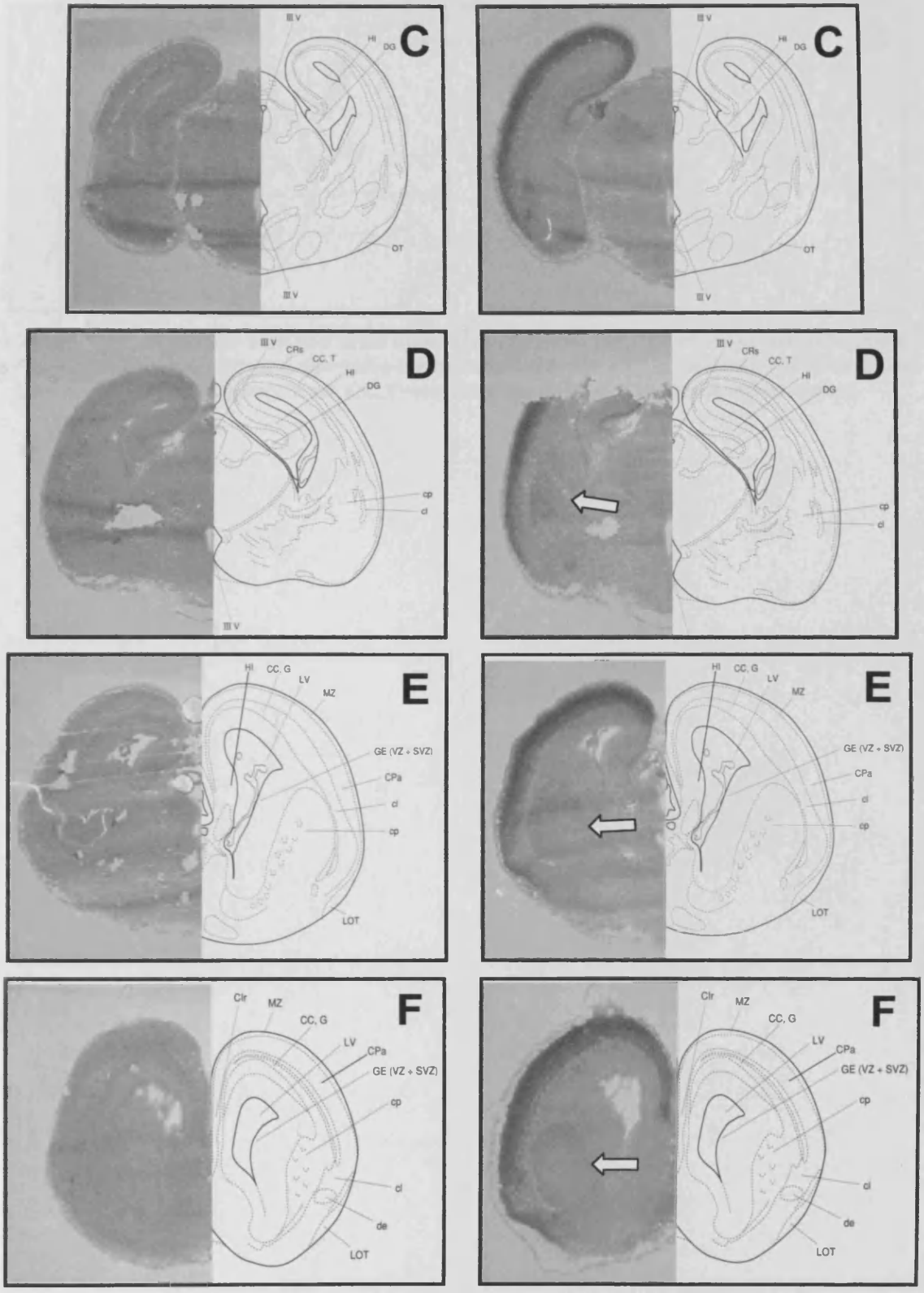

Fig. C.12(cont); Coronal sections from E16 Brain showing control probe (left) and experimental probe (right) for *Mef2c*. (**CRs**=Retro-spinal Cortex, **Clr**=Infra-limbic Cortex, **GE**=Ganglionic Eminence, **VZ**=Ventricular Zone, **LOT**=Lateral Olfactory Tract, **CC,T**=corpus collosum, trunk, **Cln**=insular/rhinal cortex).

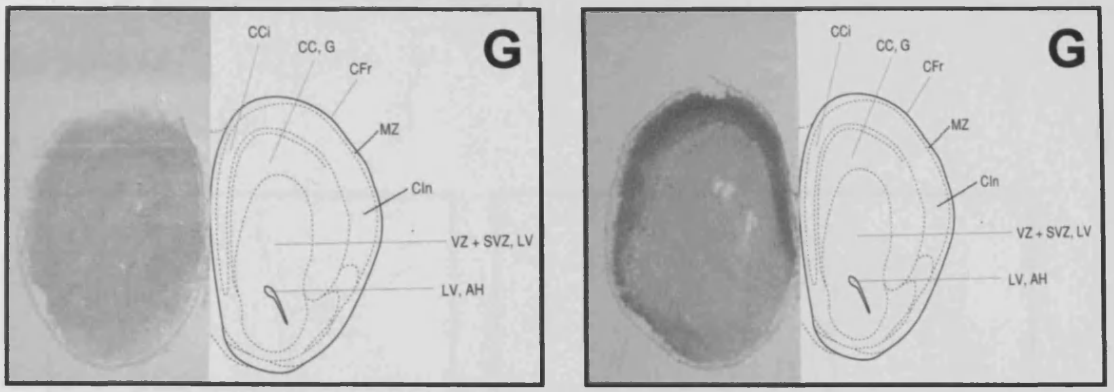

Fig. 12(cont); Coronal sections from E16 Brain showing control probe (left) and experimental probe (right) for *Mef2c*. (**CRs**=Retro-spinal Cortex, **Clr**=Infra-limbic Cortex, **GE**=Ganglionic Eminence, **VZ**=Ventricular Zone, **LOT**=Lateral Olfactory Tract, **CC,T**=corpus collosum, trunk, **Cln**=insular/rhinal cortex).

11.1.5 Gucy1β3

11.1.5.1 E12

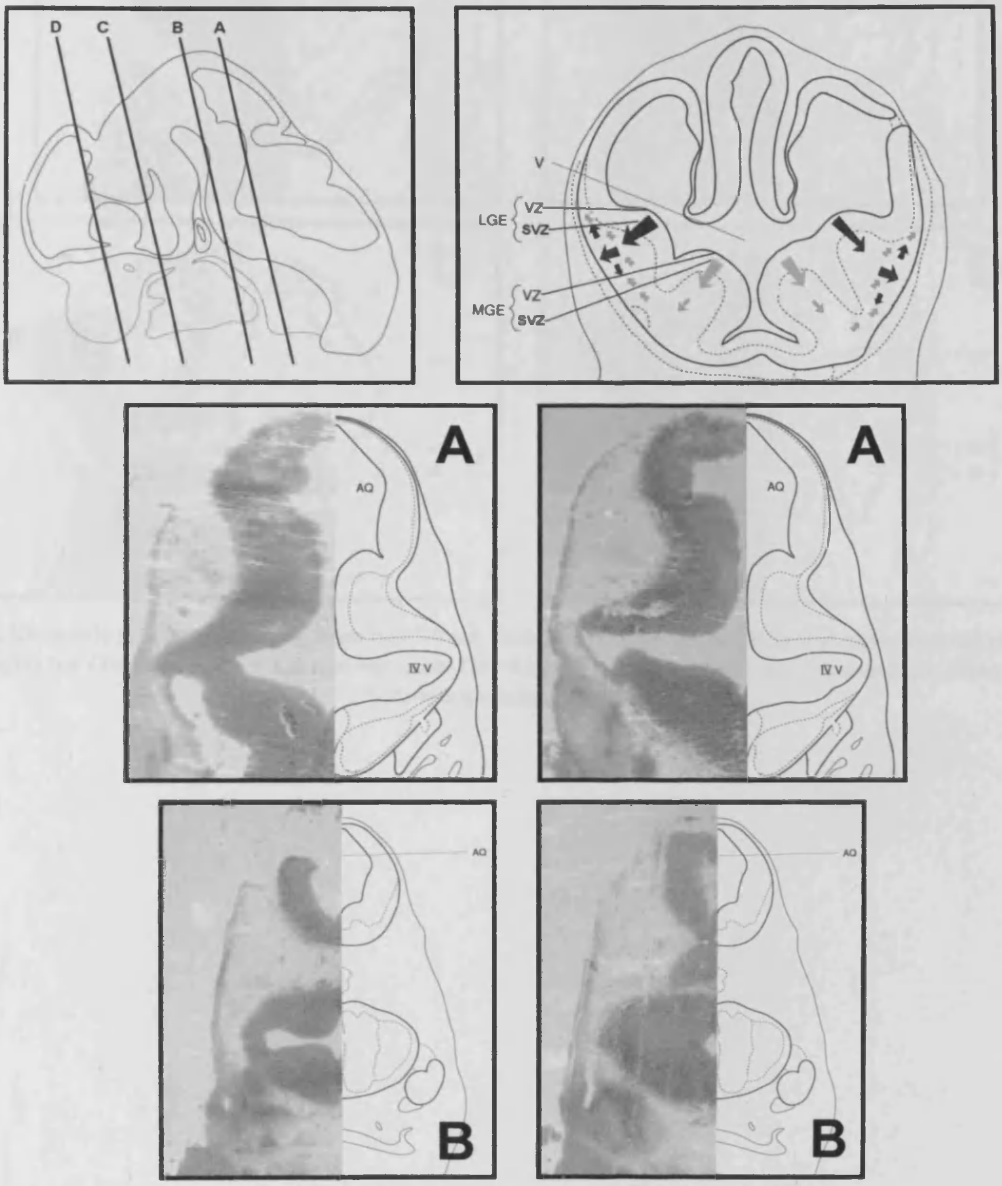

Fig. C.13; Coronal sections from E12 Brain showing control probe (left) and experimental probe (right) for *Gucy1β3*. (**LV**= Lateral ventricle **GE**=Ganglionic Eminence, **IZ**=Intermediate Zone, **VZ**=Ventricular Zone).

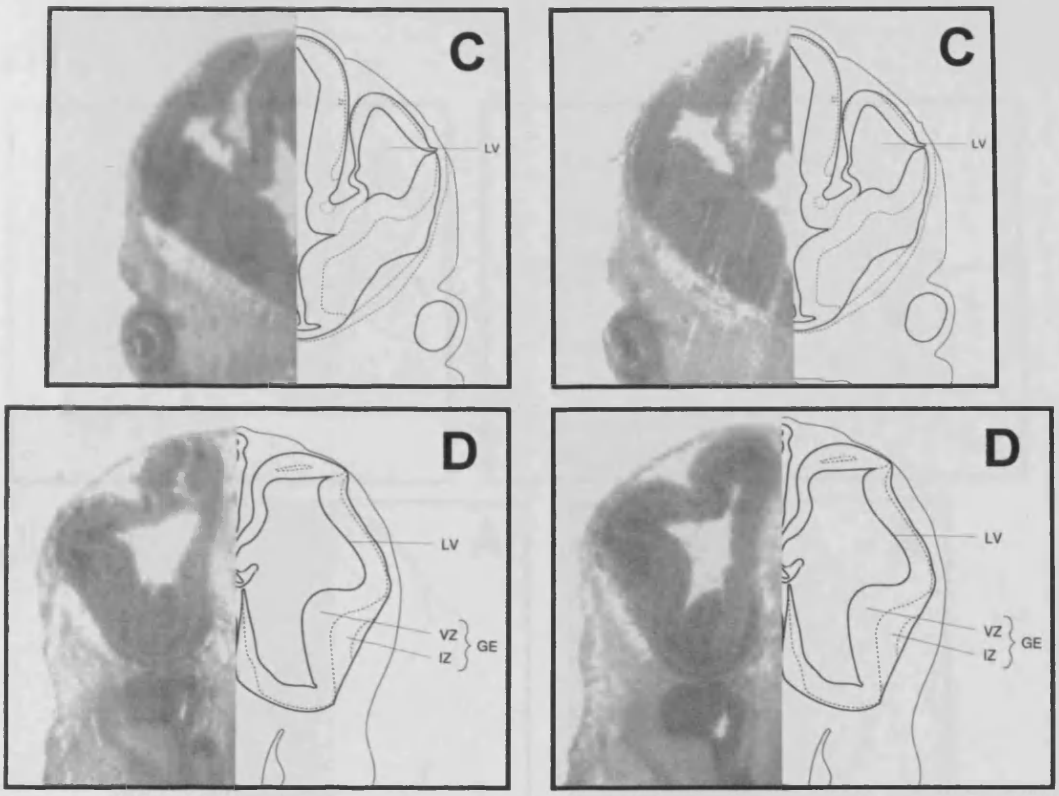

Fig. C.13(cont); Coronal sections from E12 Brain showing control probe (left) and experimental probe (right) for *Gucy1β3*. (**LV**= Lateral ventricle **GE**=Ganglionic Eminence, **IZ**=Intermediate Zone, **VZ**=Ventricular Zone).

11.1.5.2 E14

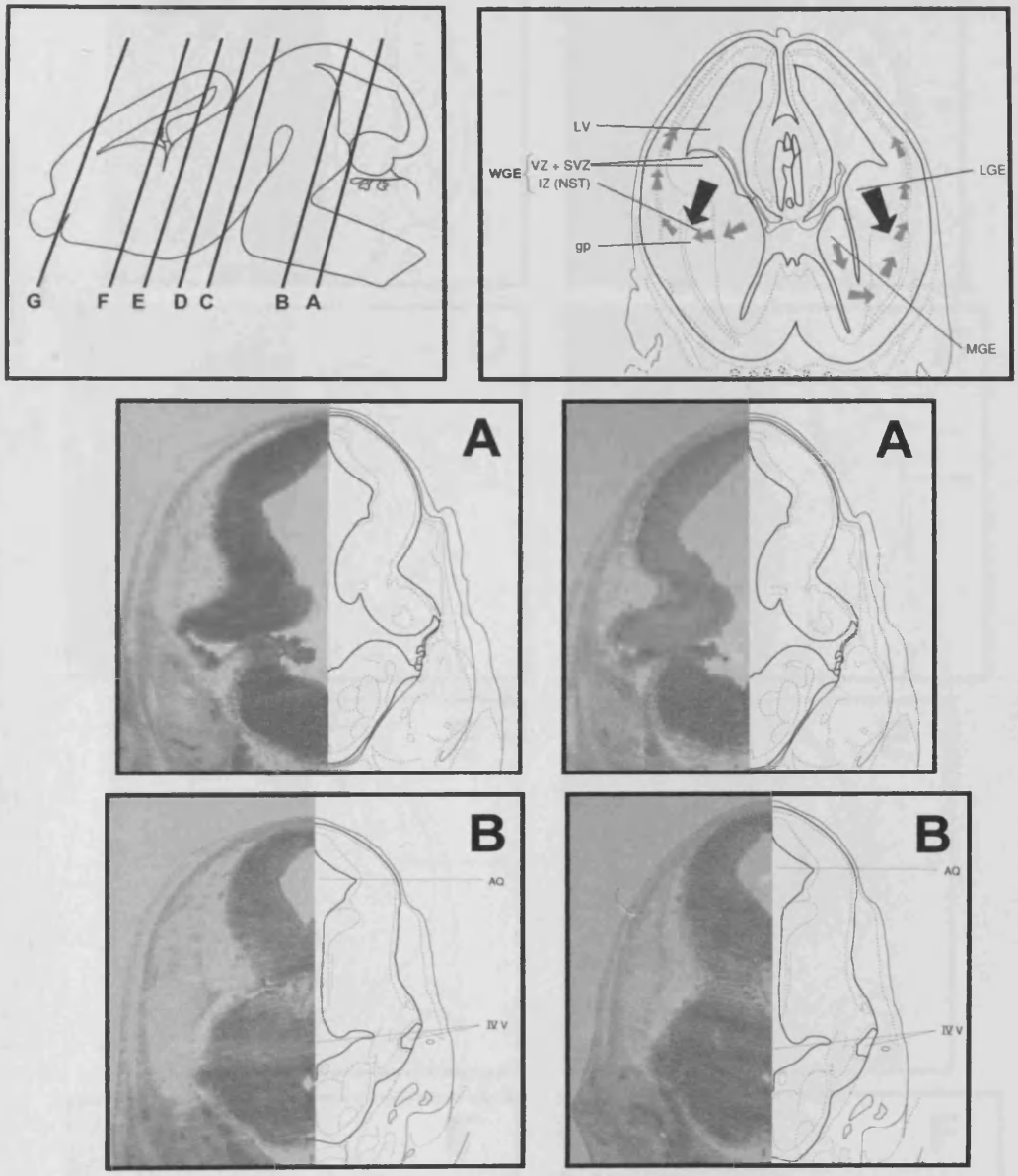

Fig. C.14; Coronal sections from E14 Brain showing control probe (left) and experimental probe for Gucy1β3 (**IIIV**=3rd ventricle, **CFr**= frontal cortex, **SP**=subplate, **MZ**=marginal zone, **CP**=Cortical Plate, **LV**=Lateral ventricle, **GE**=Ganglionic Eminence, **VZ**=Ventricular Zone, **IZ**= Intermediate Zone).

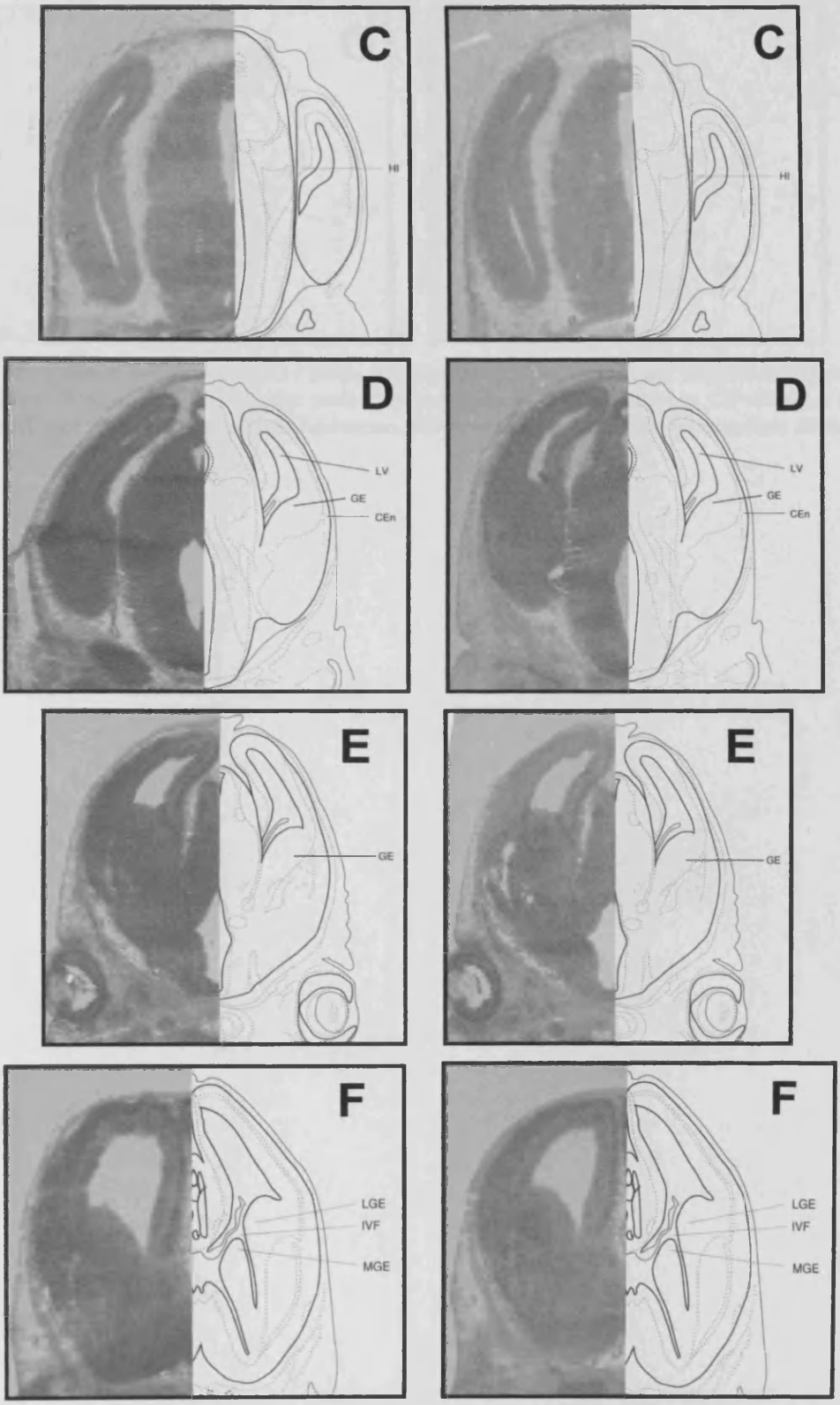

Fig. C.14(cont); Coronal sections from E14 Brain showing control probe (left) and experimental probe for Gucy1β3 (**IIIV**=3rd ventricle, **CFr**= frontal cortex, **SP**=subplate, **MZ**=marginal zone, **CP**=Cortical Plate, **LV**=Lateral ventricle, **GE**=Ganglionic Eminence, **VZ**=Ventricular Zone, **IZ**= Intermediate Zone).

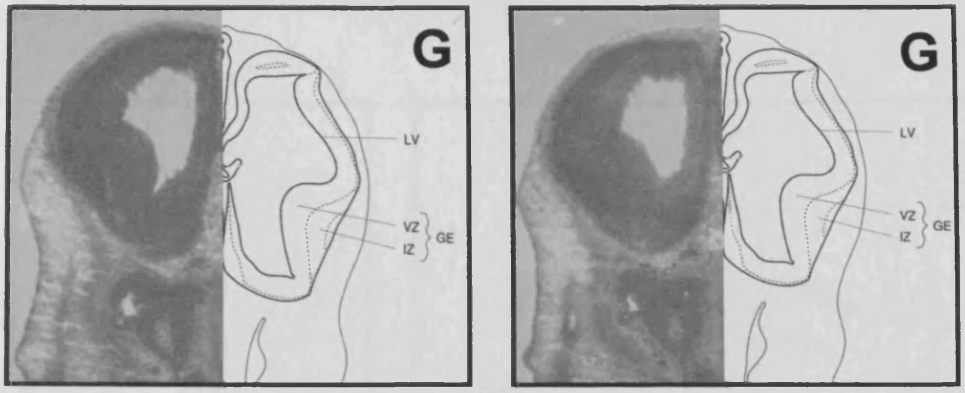

Fig. C.14(cont); Coronal sections from E14 Brain showing control probe (left) and experimental probe for Gucy1β3 (**IIIV**=3rd ventricle, **CFr**= frontal cortex, **SP**=subplate, **MZ**=marginal zone, **CP**=Cortical Plate, **LV**=Lateral ventricle, **GE**=Ganglionic Eminence, **VZ**=Ventricular Zone, **IZ**= Intermediate Zone).

11.1.5.3 E16

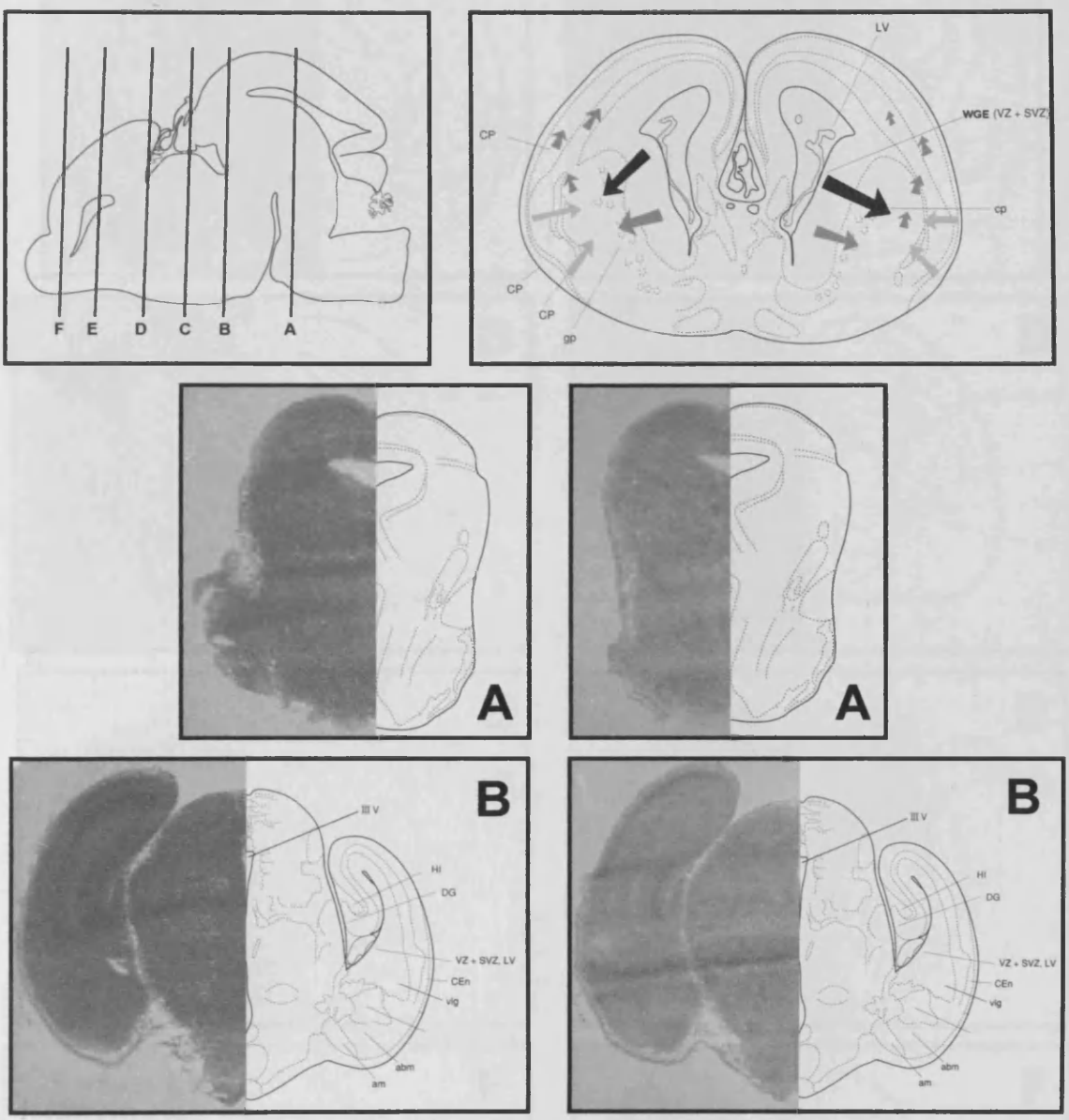

Fig. C.15; Coronal sections from E16 Brain showing control probe (left) and experimental probe (right) for Gucy1β3. (**LV**=Lateral ventricle, **VZ**=Ventricular Zone, **SVZ**=Sub-Ventricular Zone, **cp**=caudate putamen, **IC**=Internal Capsule, **gp**=Globus Pallidus, **ChPl**=Choroid plexus, **GE**=Ganglionic Eminence)

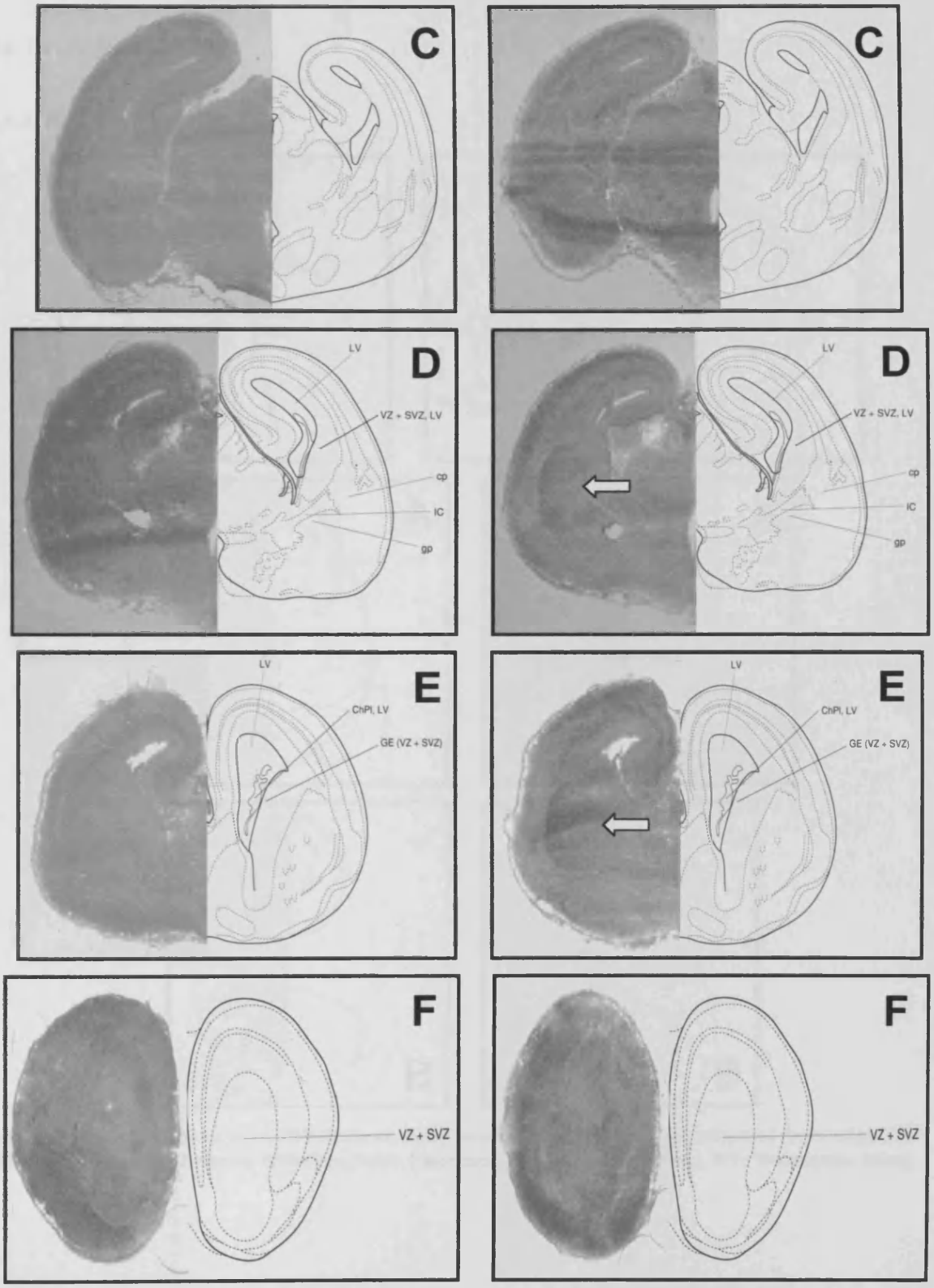

Fig. C.15(cont); Coronal sections from E16 Brain showing control probe (left) and experimental probe (right) for Gucy1β3. (**LV**=Lateral ventricle, **VZ**=Ventricular Zone, **SVZ**=Sub-Ventricular Zone, **cp**=caudate putamen, **IC**=Internal Capsule, **gp**=Globus Pallidus, **ChPl**=Choroid plexus, **GE**=Ganglionic Eminence)

11.1.6 Ndrg2

11.1.6.1 E12

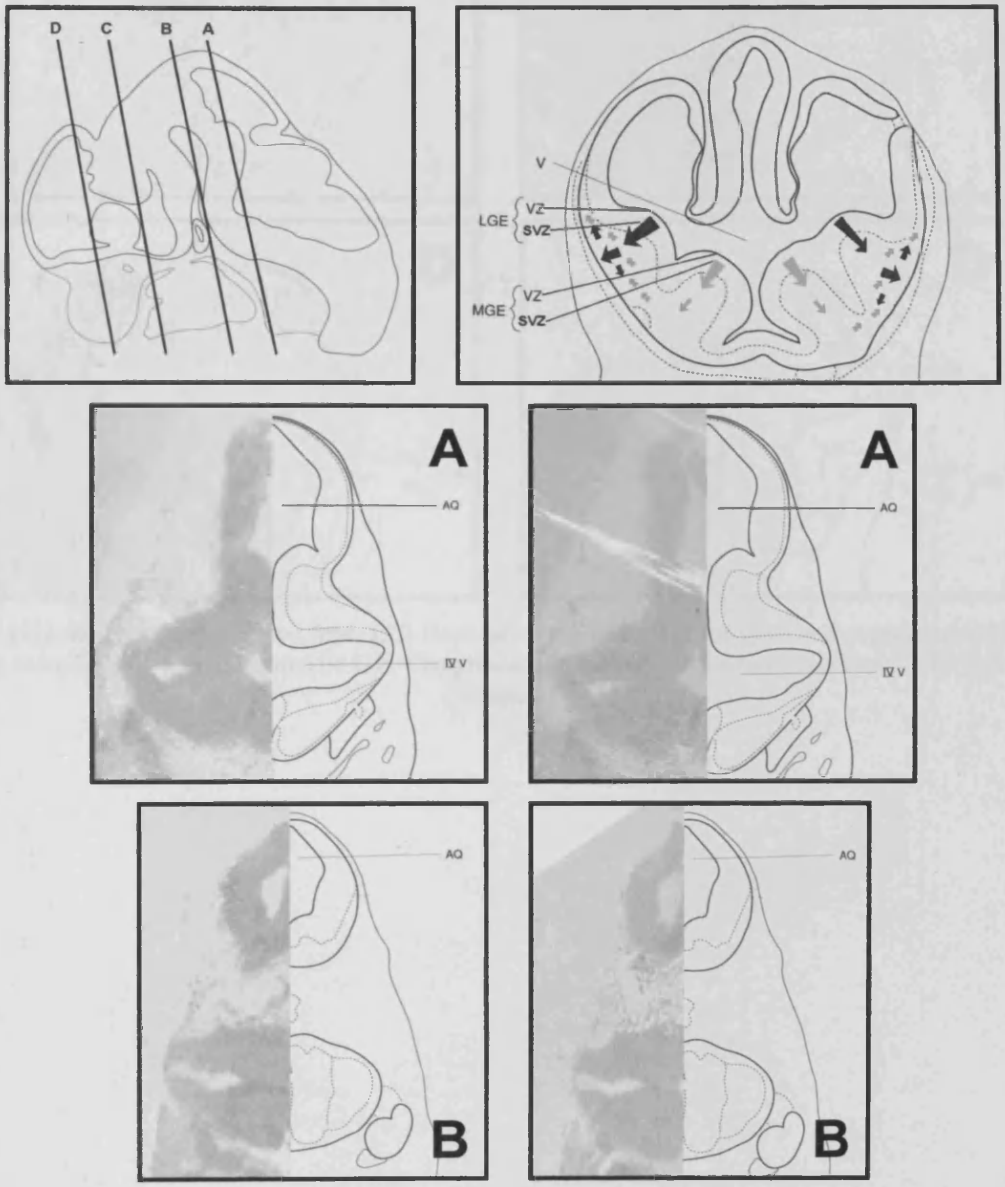

Fig. C.16; Coronal sections from E12 Brain showing control probe (left) and experimental probe (right) for Ndrg2. (**LV**= Lateral ventricle **GE**=Ganglionic Eminence, **IZ**=Intermediate Zone, **VZ**=Ventricular Zone).

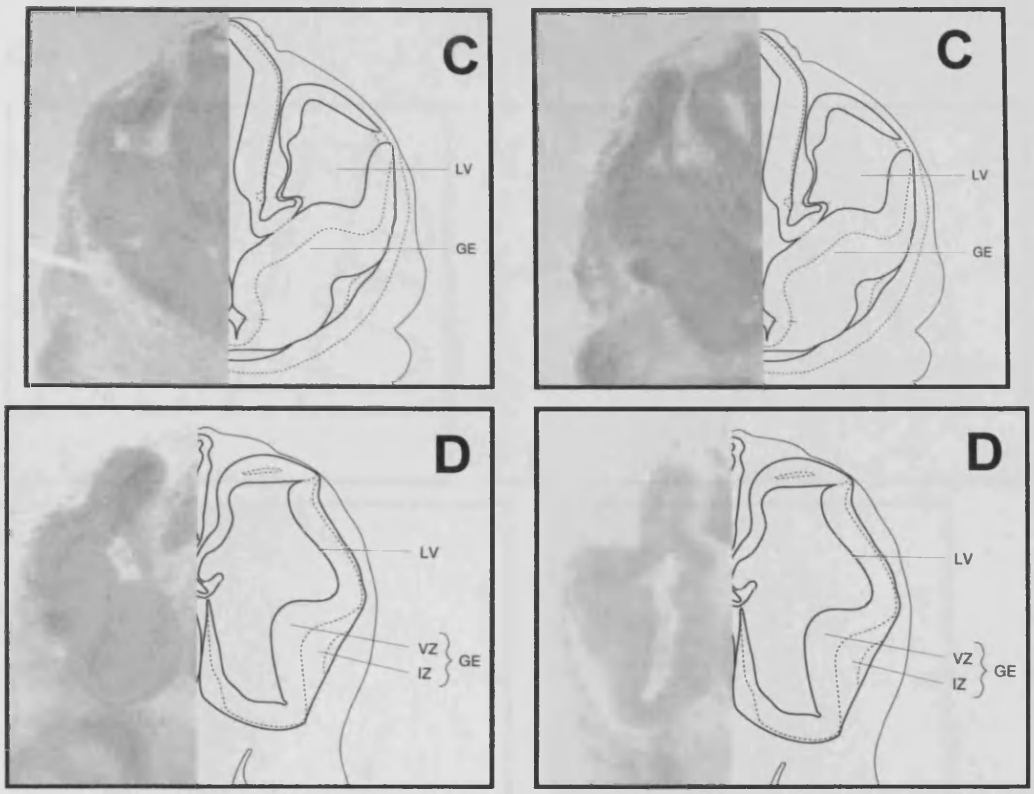

Fig. C.16(cont); Coronal sections from E12 Brain showing control probe (left) and experimental probe (right) for Ndrg2. (**LV**= Lateral ventricle **GE**=Ganglionic Eminence, **IZ**=Intermediate Zone, **VZ**=Ventricular Zone).

11.1.6.2 E14

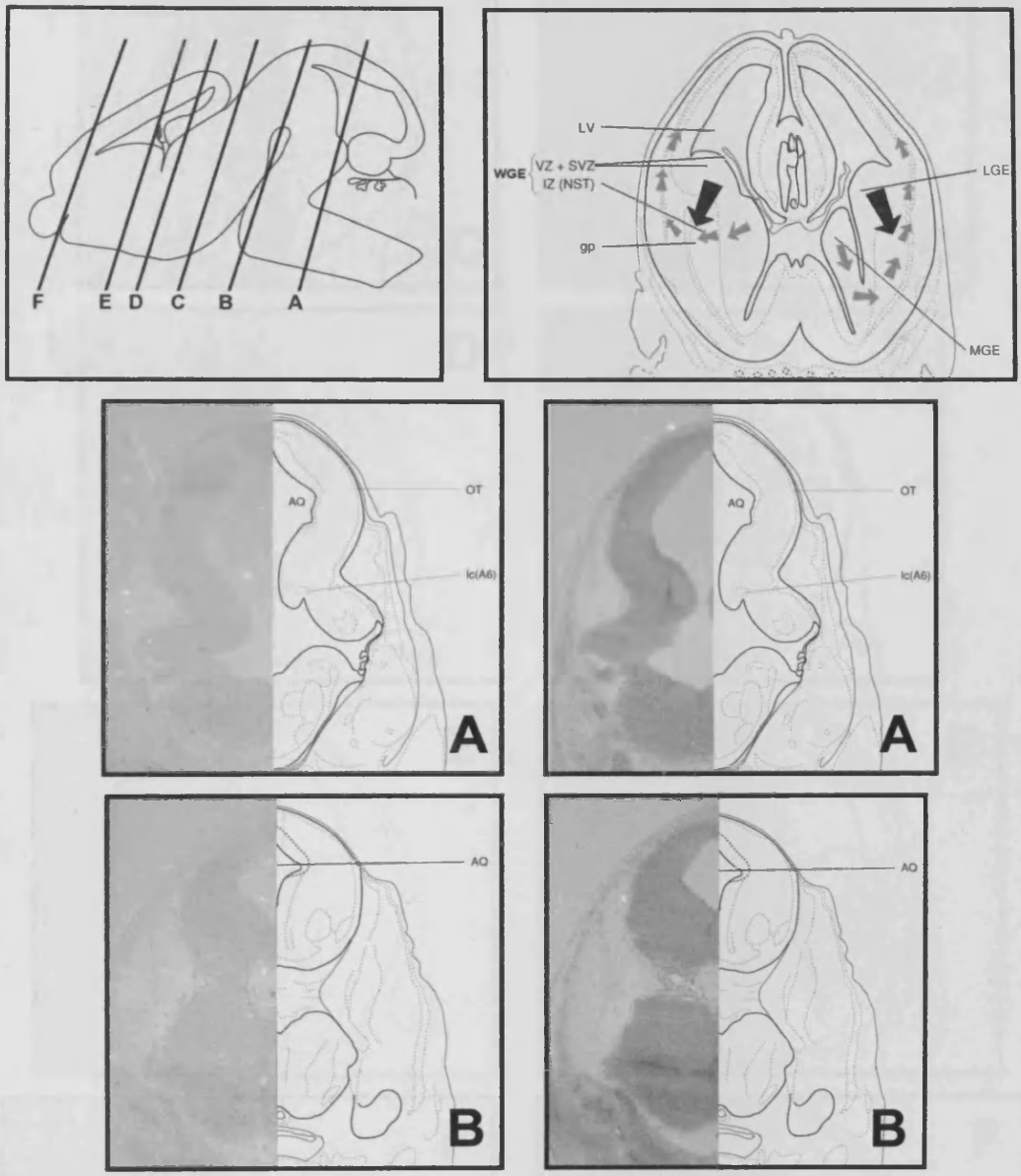

Fig. C.17; Coronal sections from E14 Brain showing control probe (left) and experimental probe for *Ndrg2* (**IIIV**=3rd ventricle, **CFr**= frontal cortex, **SP**=subplate, **MZ**=marginal zone, **CP**=Cortical Plate, **LV**=Lateral ventricle, **GE**=Ganglionic Eminence, **VZ**=Ventricular Zone, **IZ**= Intermediate Zone).

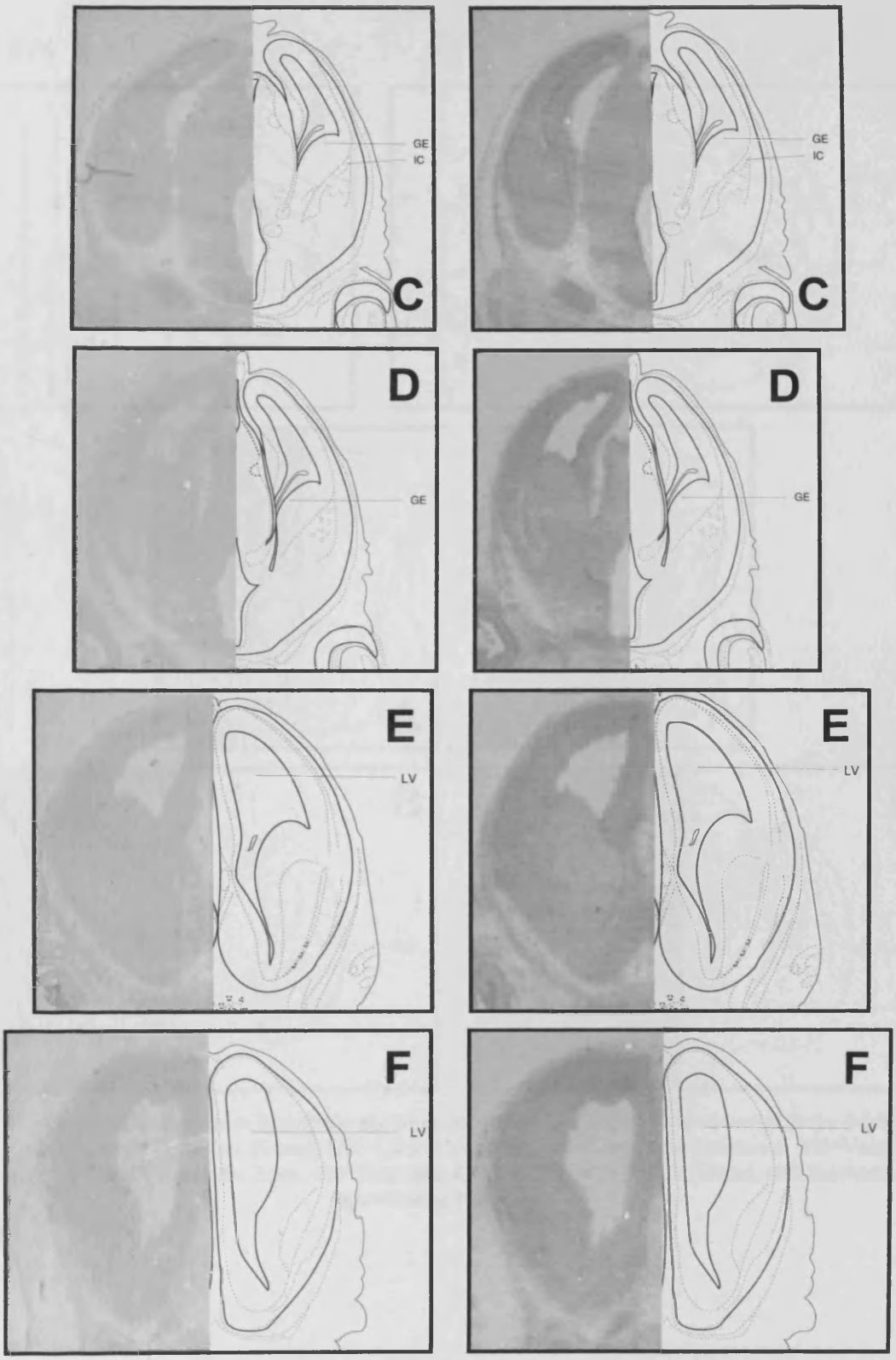

Fig. C.17(cont); Coronal sections from E14 Brain showing control probe (left) and experimental probe for Ndrg2 (**IIIV**=3rd ventricle, **CFr**= frontal cortex, **SP**=subplate, **MZ**=marginal zone, **CP**=Cortical Plate, **LV**=Lateral ventricle, **GE**=Ganglionic Eminence, **VZ**=Ventricular Zone, **IZ**= Intermediate Zone).

11.1.6.3 E16

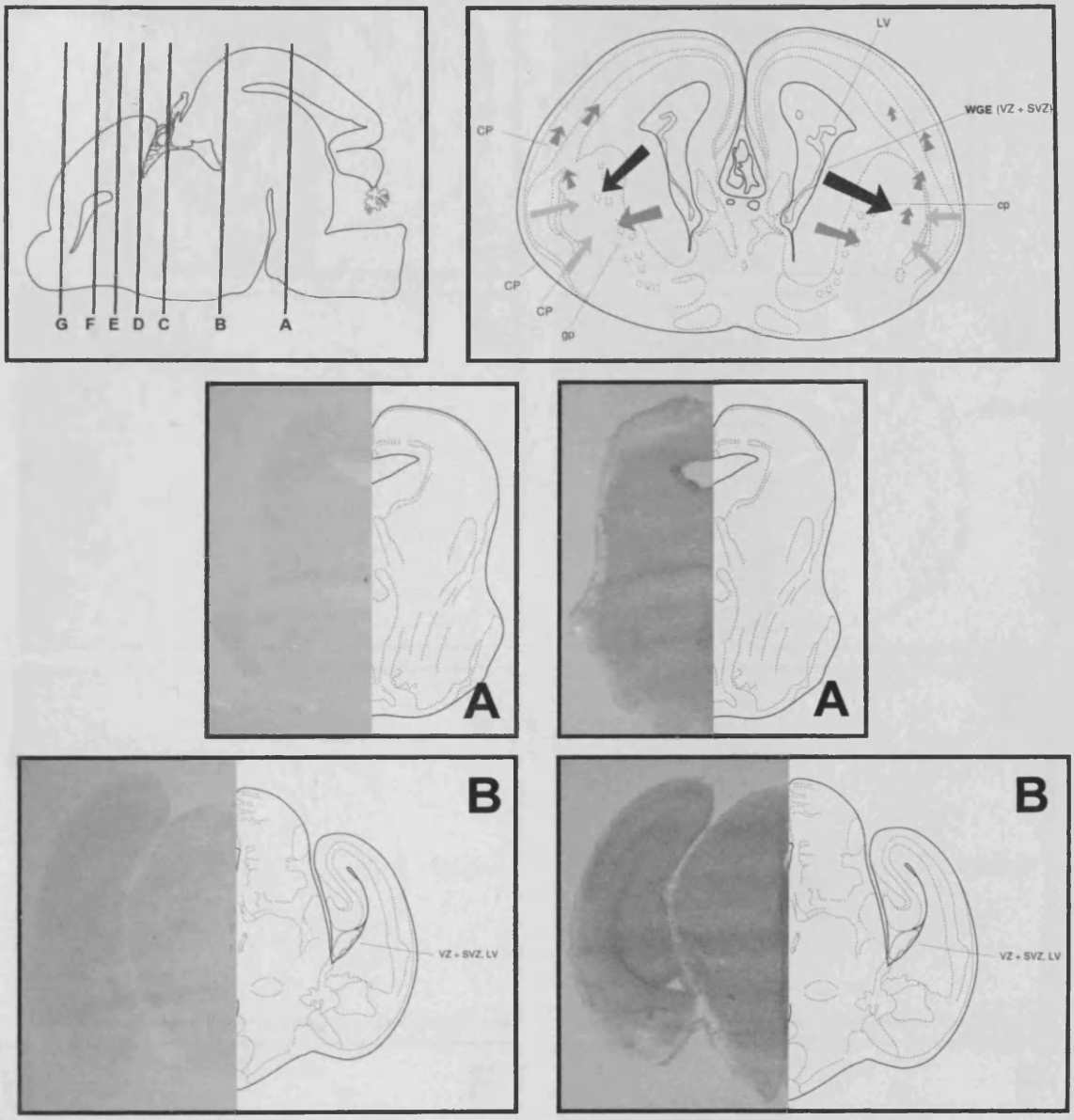

Fig. C.18; Coronal sections from E16 Brain showing control probe (left) and experimental probe (right) for Ndrg2. (**CC,G**=Corpus Collosum (Genu), **LV**=Lateral Ventricle, **GE**=Ganglionic Eminence, **VZ**=Ventricular Zone, **SVZ**=Sub Ventricular Zone, **Cl**=Cingulum, **CC,G**=Corpus Collosum (Genu), **cl**=Claustrum, **gp**=Globus Pallidus).

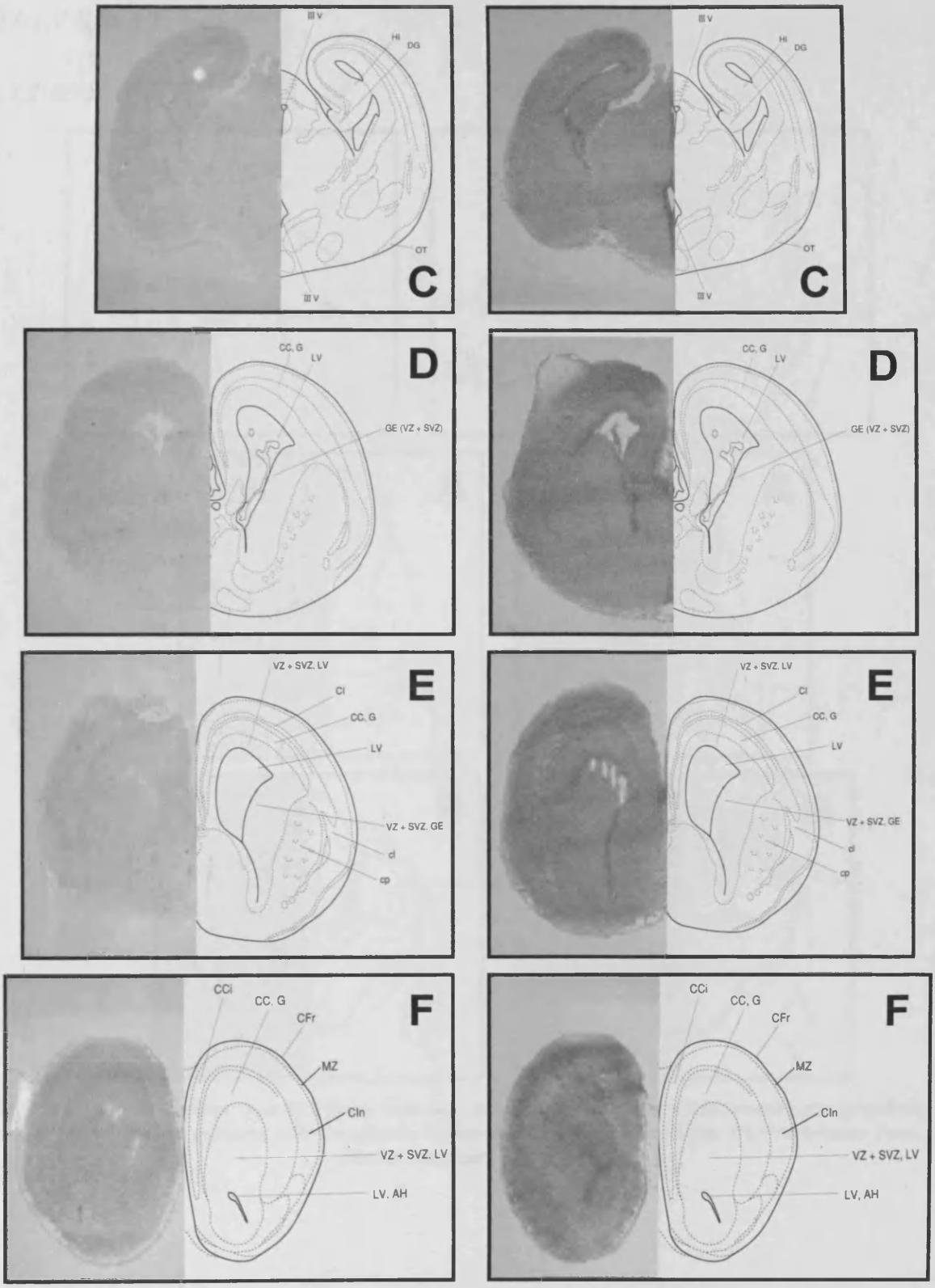

Fig. C.18; Coronal sections from E16 Brain showing control probe (left) and experimental probe (right) for Ndrg2. (**CC,G**=Corpus Collosum (Genu), **LV**=Lateral Ventricle, **GE**=Ganglionic Eminence, **VZ**=Ventricular Zone, **SVZ**=Sub Ventricular Zone, **CI**=Cingulum, **CC,G**=Corpus Collosum (Genu), **cl**=Claustrum, **gp**=Globus Pallidus).

11.1.7 Spock2

11.1.7.1 E12

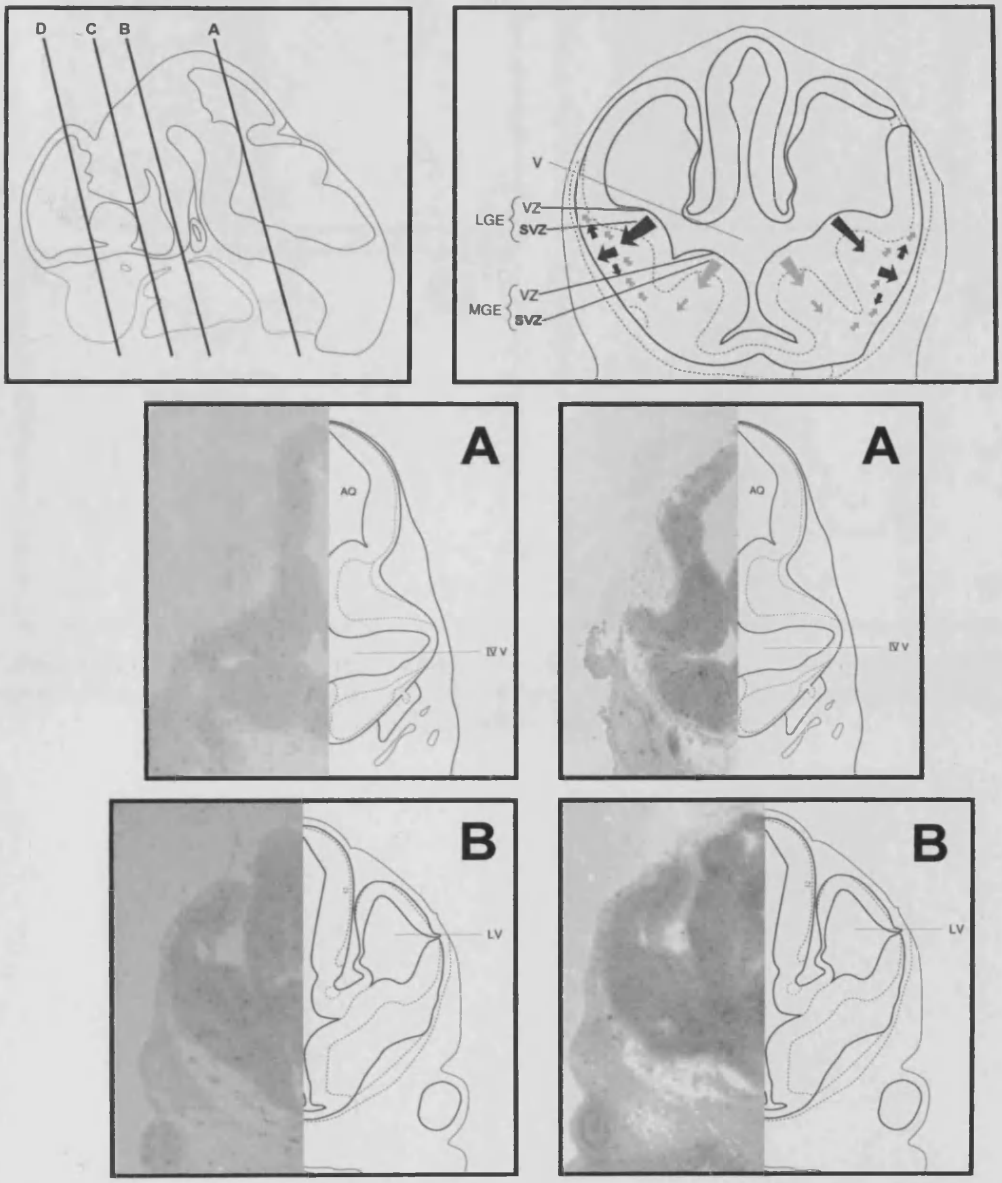

Fig. C.19; Coronal sections from E12 Brain showing control probe (left) and experimental probe (right) for Spock2. (**LV**= Lateral ventricle **GE**=Ganglionic Eminence, **IZ**=Intermediate Zone, **VZ**=Ventricular Zone, **MxPr**=Maxillary Process).

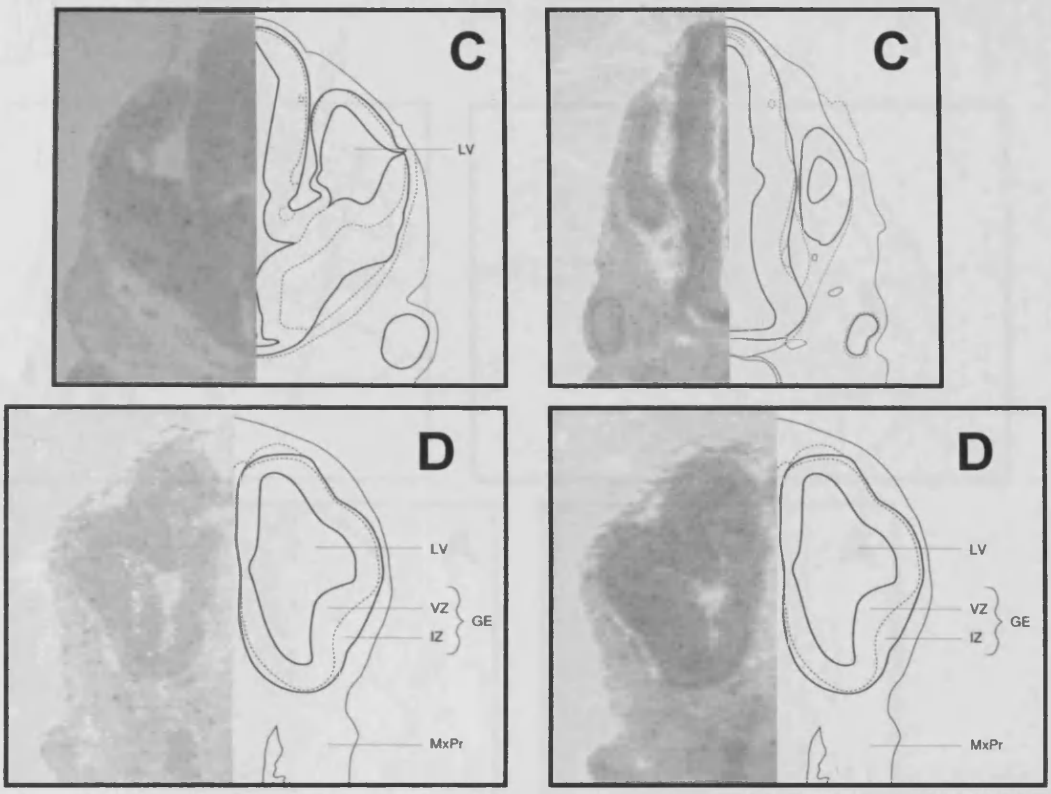

Fig. C.19(cont); Coronal sections from E12 Brain showing control probe (left) and experimental probe (right) for Spock2. (**LV**= Lateral ventricle **GE**=Ganglionic Eminence, **IZ**=Intermediate Zone, **VZ**=Ventricular Zone, **MxPr**=Maxillary Process).

11.1.7.2 E14

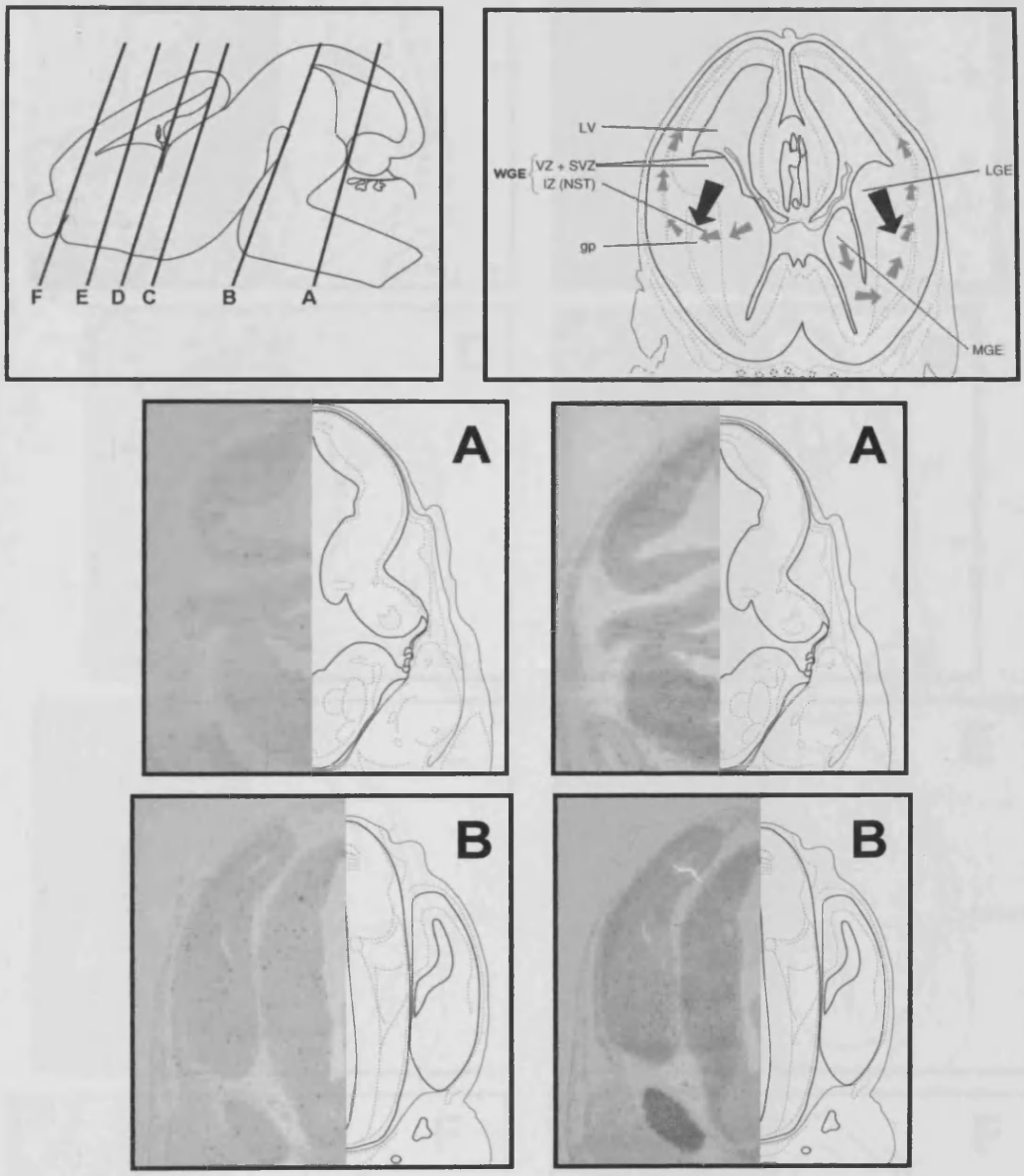

Fig. C.20; Coronal sections from E14 Brain showing control probe (left) and experimental probe (right) for Spock2. (**GE**=Ganglionic Eminence, **IC**=Internal Capsule, **IZ**= Intermediate Zone, **LV**=Lateral Ventrical).

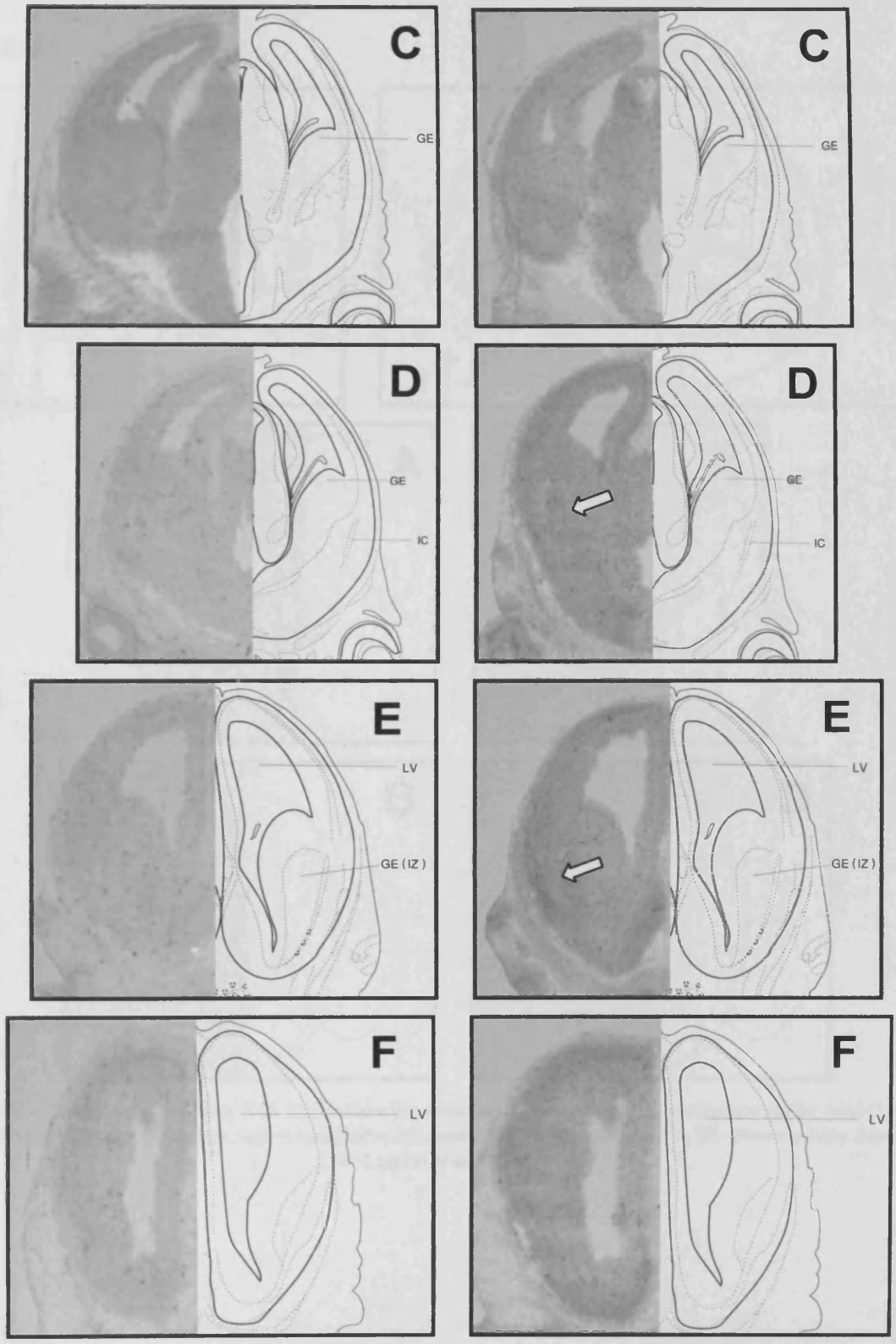

Fig. C.20(cont); Coronal sections from E14 Brain showing control probe (left) and experimental probe (right) for Spock2. (**GE**=Ganglionic Eminence, **IC**=Internal Capsule, **IZ**= Intermediate Zone, **LV**=Lateral Ventrical).

11.1.7.3 E16

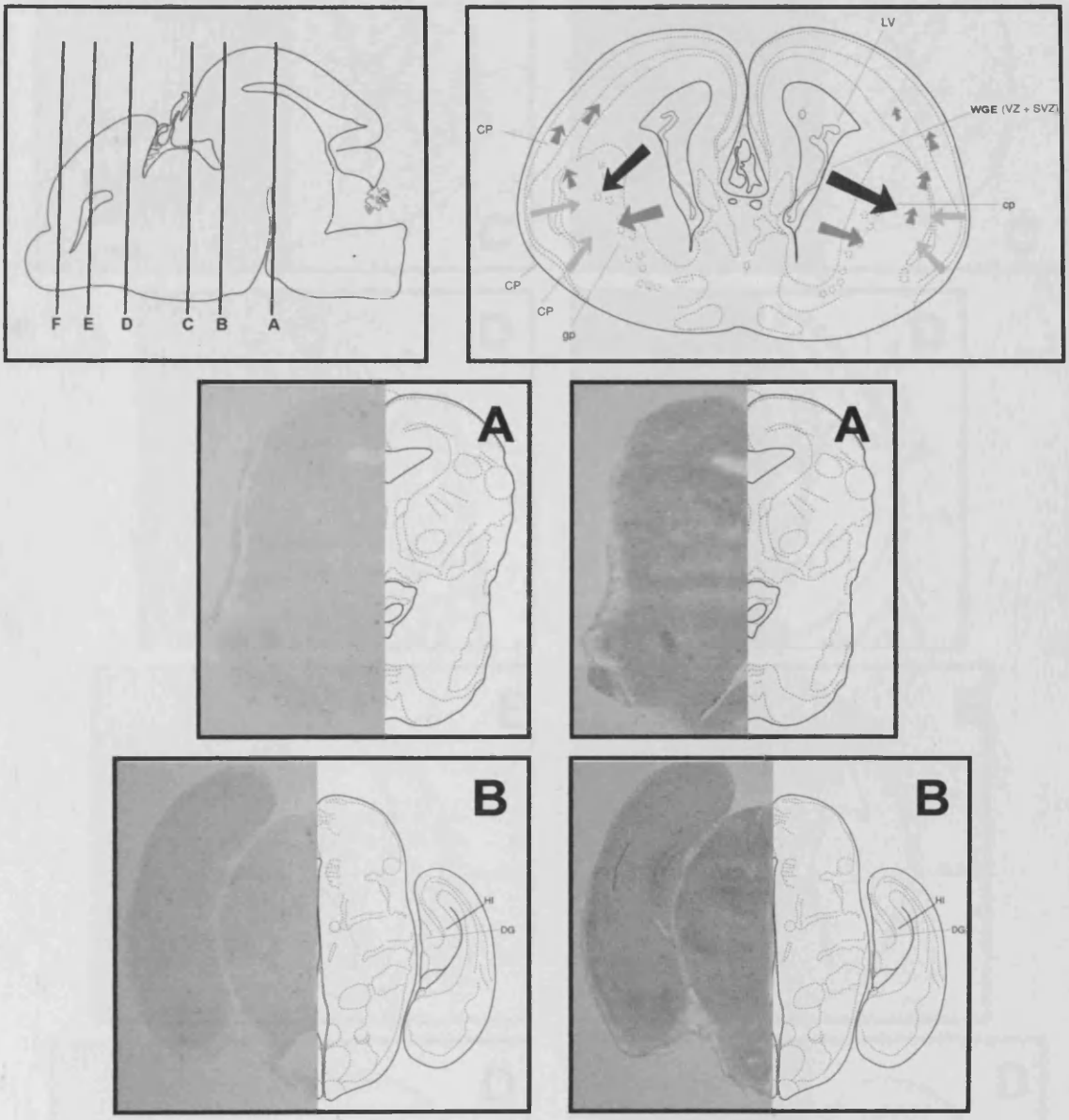

Fig. C.21; Coronal sections from E16 Brain showing control probe (left) and experimental probe (right) for Spock2. (**LV**=Lateral Ventricle, **GE**=Ganglionic Eminence, **IC**=Internal Capsule, **IZ**=Intermediate Zone, **LV**=Lateral Ventricle).

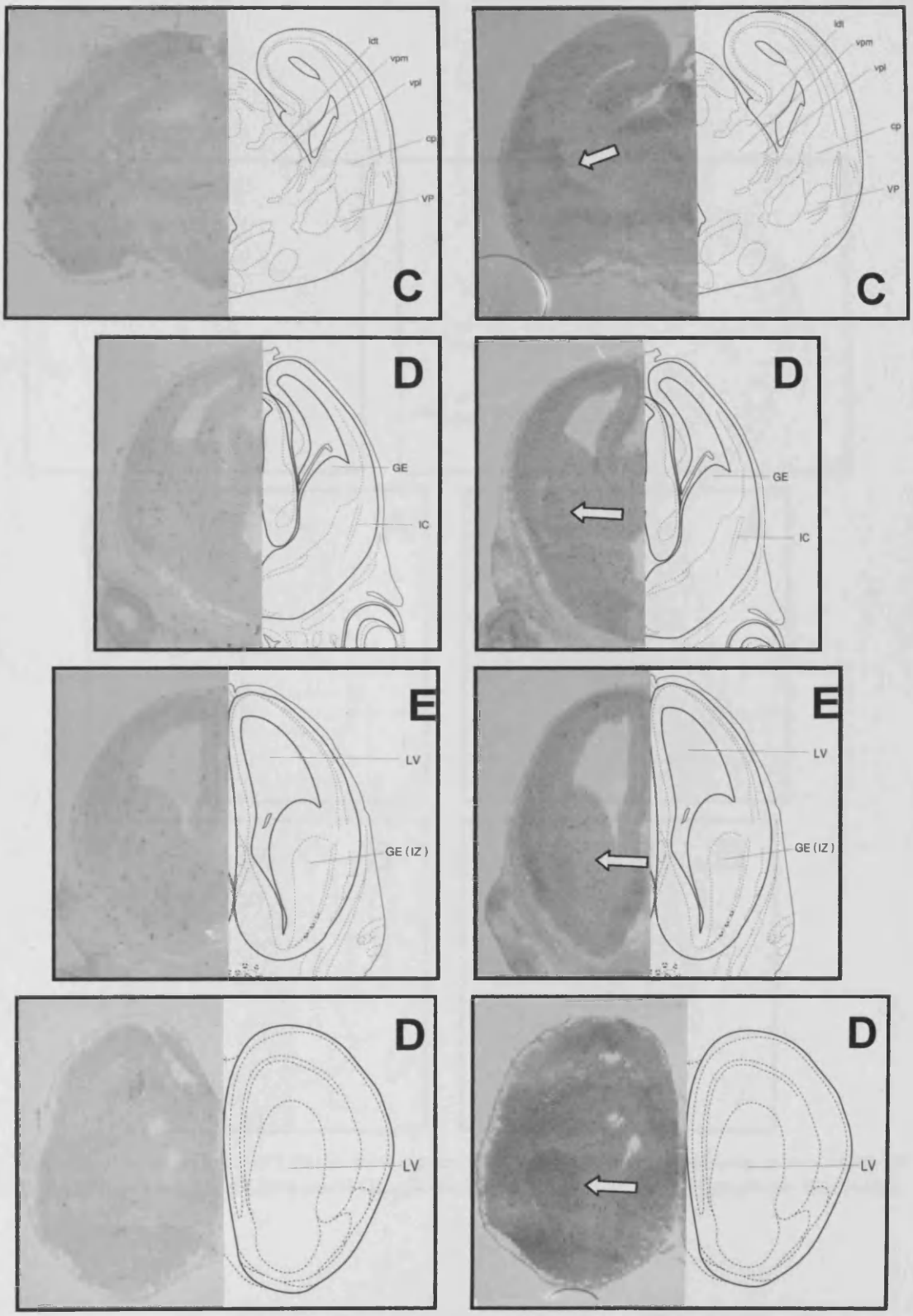

Fig. C.21(cont); Coronal sections from E16 Brain showing control probe (left) and experimental probe (right) for Spock2. (**LV**=Lateral Ventricle, **GE**=Ganglionic Eminence, **IC**=Internal Capsule, **IZ**=Intermediate Zone, **LV**=Lateral Ventricle).

11.1.8 Tiam2

11.1.8.1 E12

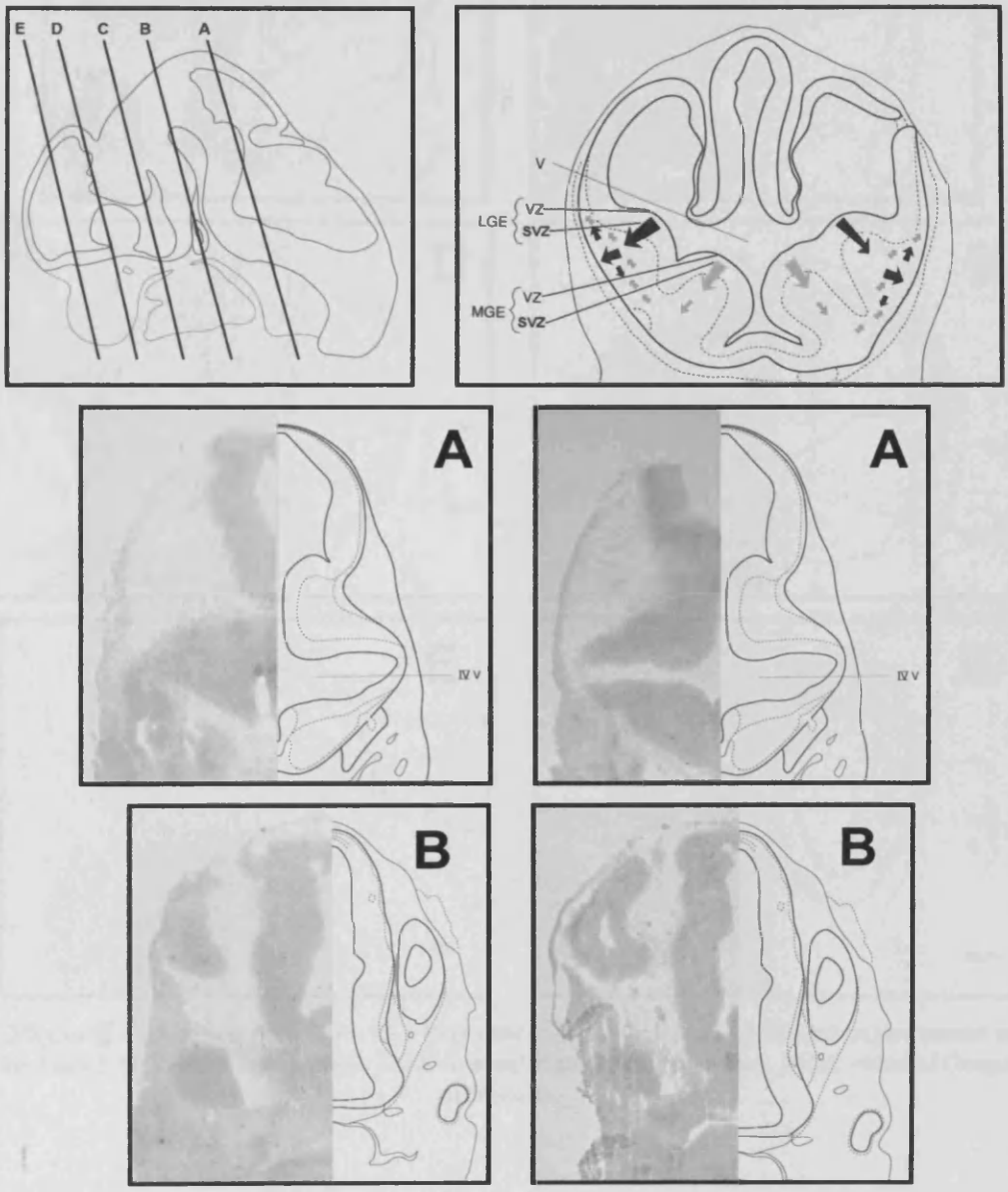

Fig. C.22; Coronal sections from E12 Brain showing control probe (left) and experimental probe (right) for Tiam2. (**LV**= Lateral ventricle **LGE**=Lateral Ganglionic Eminence, **MGE**=Medial Ganglionic Eminence).

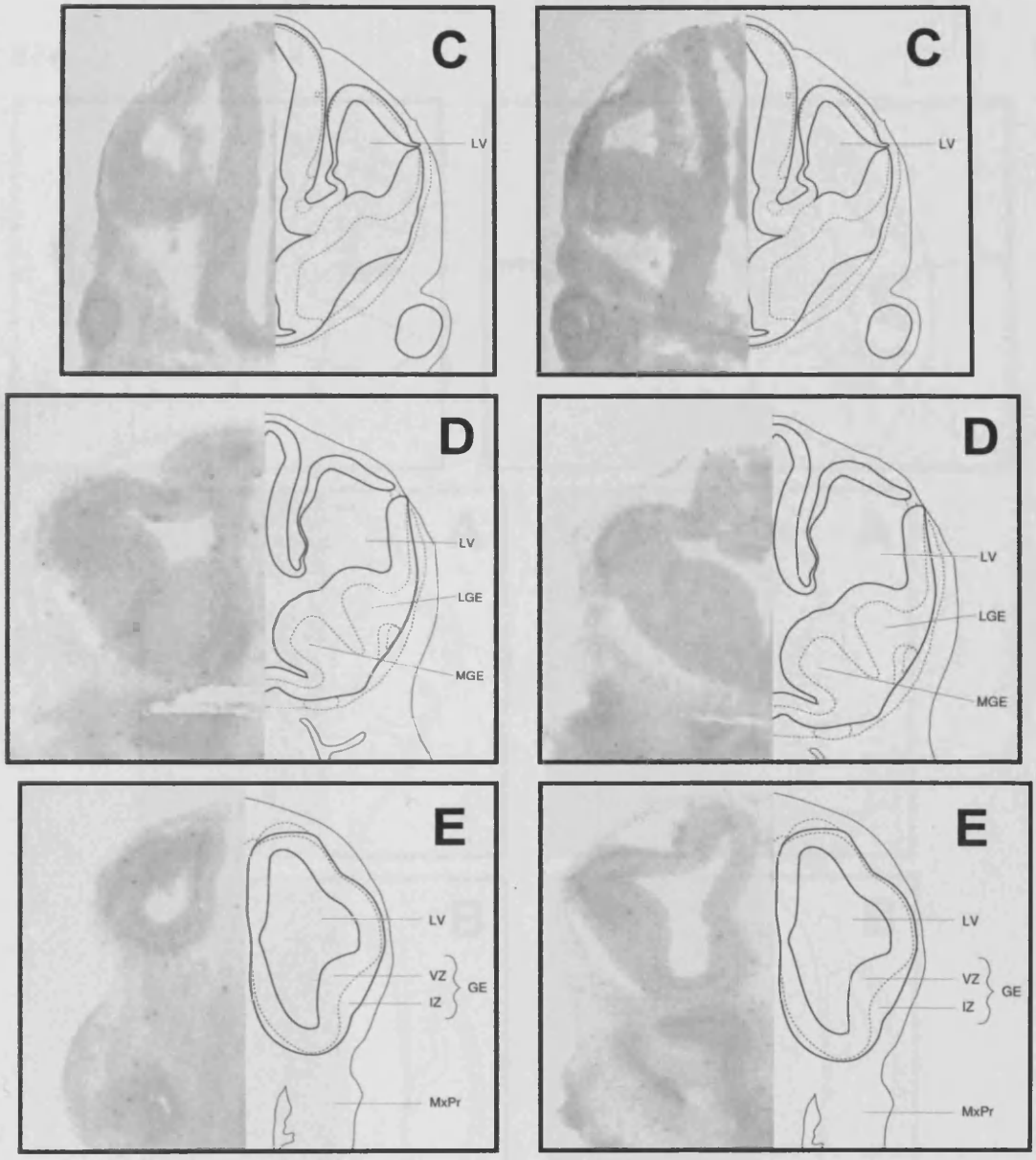

Fig. C.22(cont); Coronal sections from E12 Brain showing control probe (left) and experimental probe (right) for *Tiam2*. (**LV**= Lateral ventricle **LGE**=Lateral Ganglionic Eminence, **MGE**=Medial Ganglionic eminence).

11.1.8.2 E14

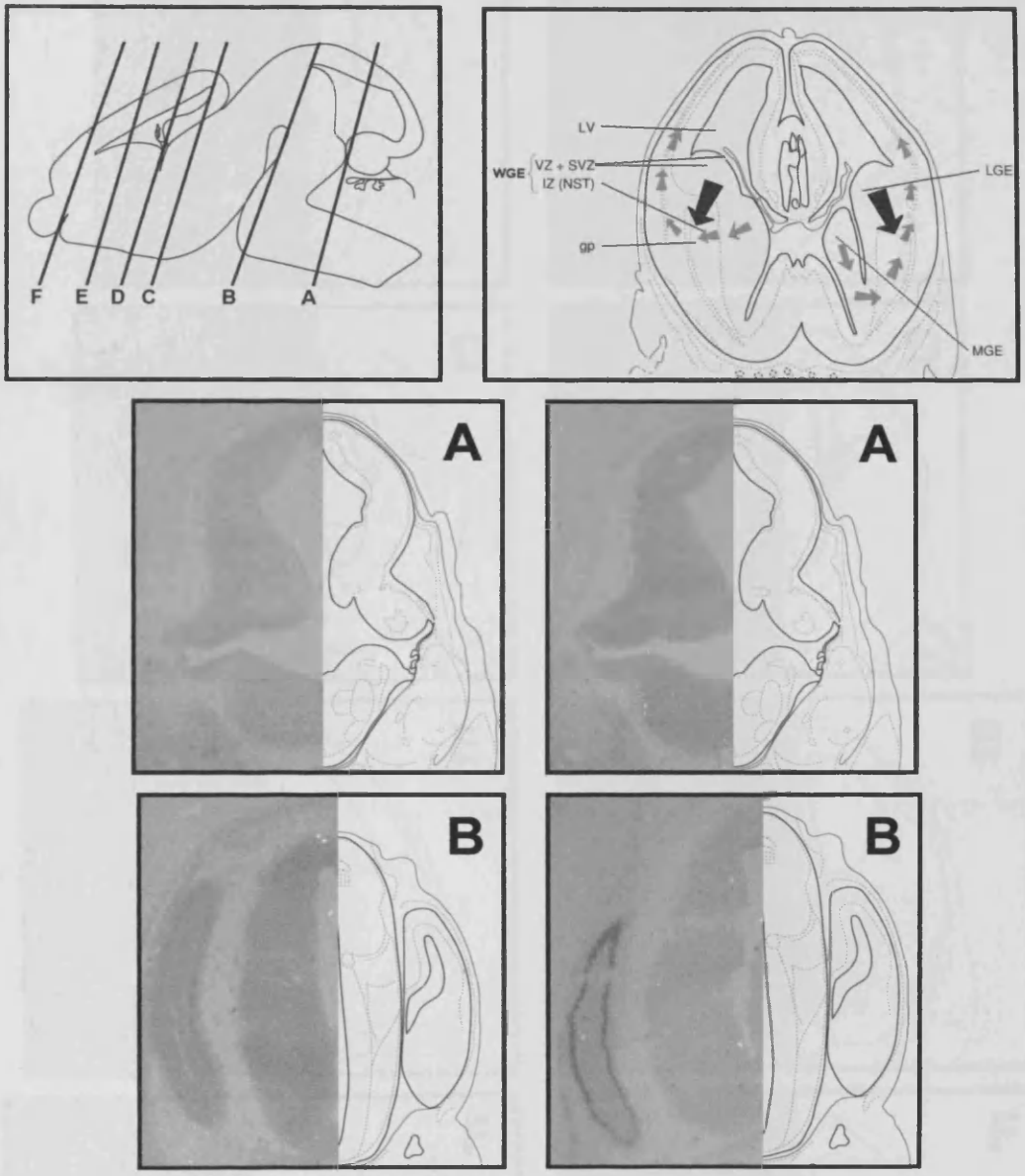

Fig. C.23; Coronal sections from E16 Brain showing control probe (left) and experimental probe (right) for Tiam2. (**LV**=Lateral ventricle).

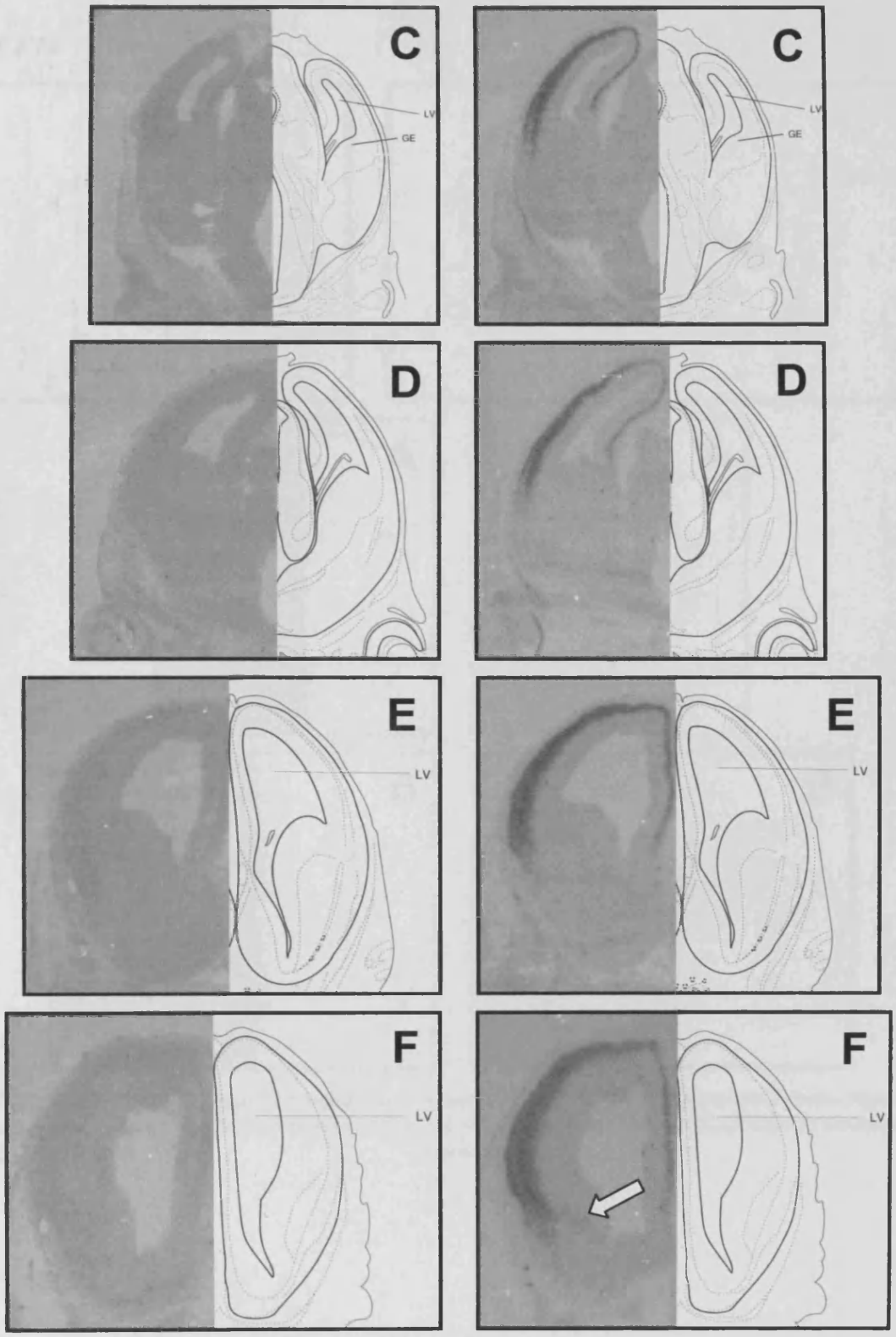

Fig. C.23(cont); Coronal sections from E16 Brain showing control probe (left) and experimental probe (right) for Tiam2. (**LV**=Lateral ventricle).

11.1.8.3 E16

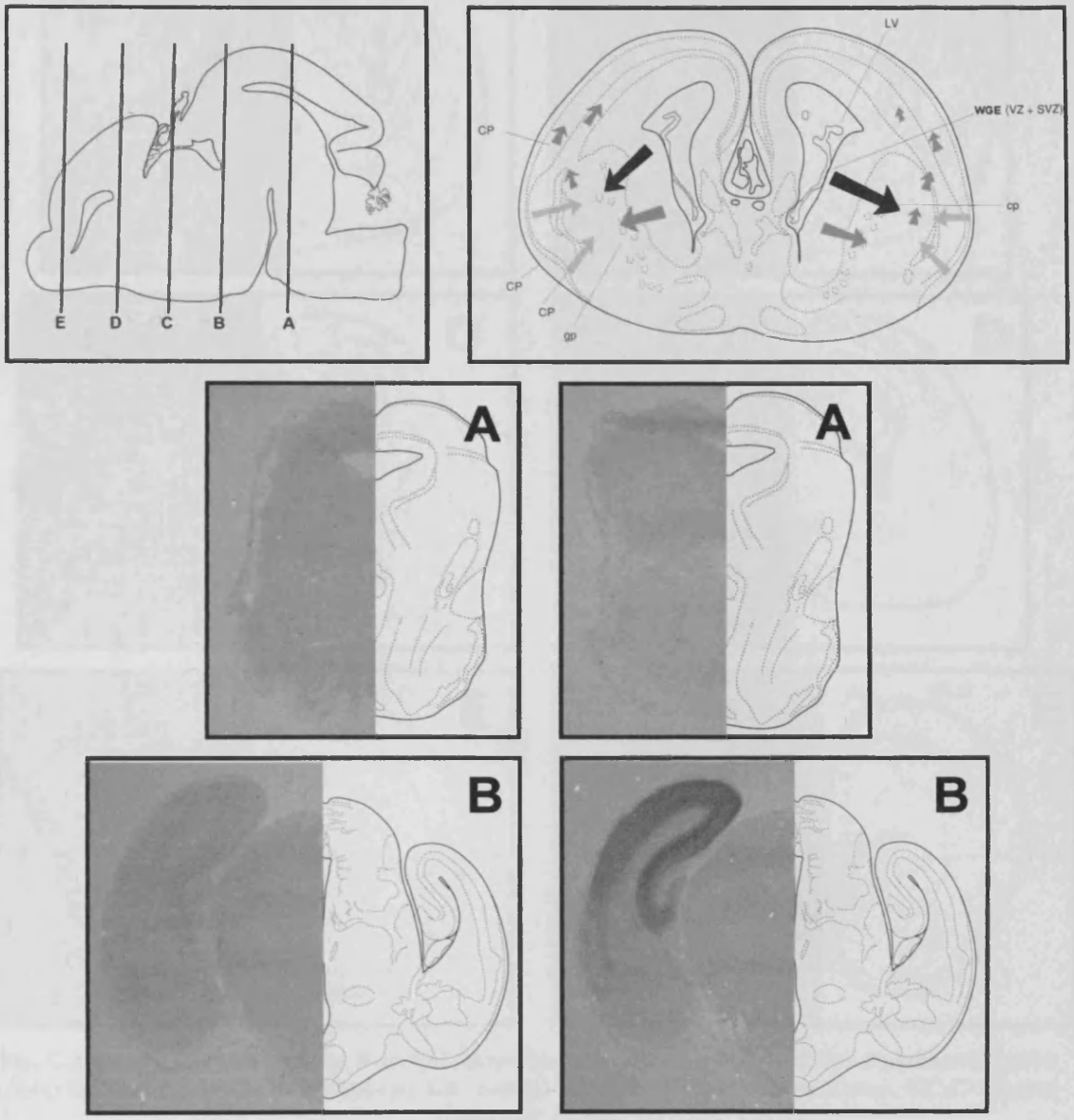

Fig. C.24; Coronal sections from E16 Brain showing control probe (left) and experimental probe (right) for Tiam2. (**cp**=Caudate Putamen, **LV**=Lateral Ventricle, **CCi**=Cingulate Cortex, **CC,G**=Corpus Collosum, Genu).

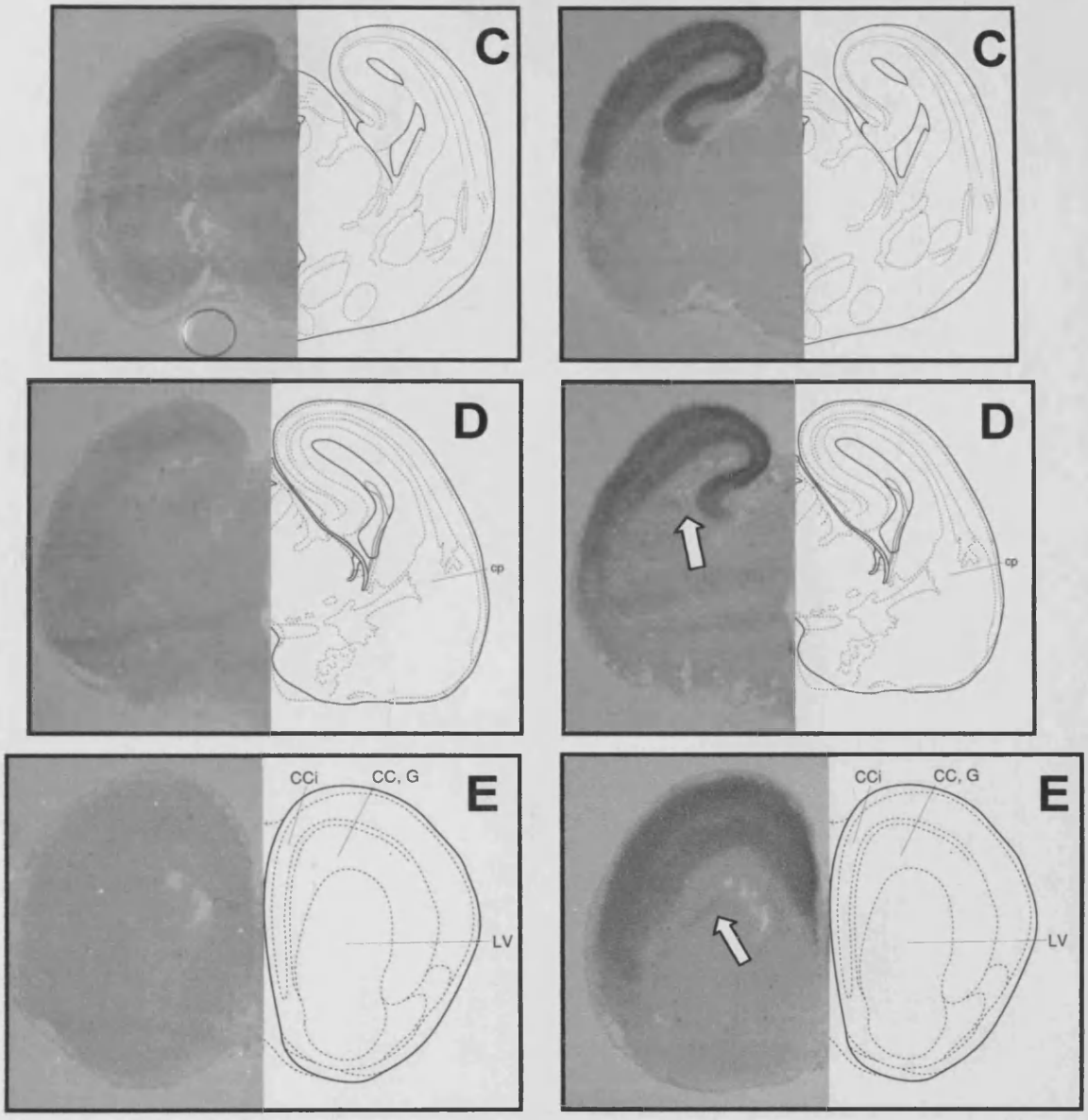

Fig. C.24(cont); Coronal sections from E16 Brain showing control probe (left) and experimental probe (right) for Tiam2. (**cp**=Caudate Putamen, **LV**=Lateral Ventricle, **CCi**=Cingulate Cortex, **CC,G**=Corpus Collosum, Genu).