**SPECIFICITY AND DIVERSITY IN THE VERTEBRATE NERVOUS
SYSTEM : AN ANALYSIS OF TWO GENES**

MAHENDRA D. WAGLE
(M.Sc., University of Mumbai-India)

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Abstract

This thesis describes two genes that may establish different identities in neurons and thus mediate the formation of synaptic connections. The first gene, *ephrinB2a*, is expressed strongly in posterior zebrafish tectal neurons that are contacted by retinal axons. Ectopic expression of *ephrinB2a* in the anterior midbrain, with the aid of baculovirus, causes stalling of retinal axons. EphrinB2a may thus signal some retinal axons that they have reached their target neurons. The second gene, *Rag1* (recombination activation gene-1), which mediates diversity in the immune system, is surprisingly also expressed in the vertebrate nervous system. Here, RAG1 protein is shown to be nuclear localized in a subset of differentiated mouse neurons. Chromatin immunoprecipitation, coupled with macroarray screening, identified a 5' repeat region in a LINE-1 retrotransposon, as a potential target of RAG1 in neurons. This raises the possibility that Rag1 may have a function in neurons by regulating a mobile element.

Keywords: vertebrate, zebrafish, ephrinb2, baculovirus, Rag1, chromatin immunoprecipitation, L1 retrotransposon.

Summary

Neuronal networks are built up through the connections of neuronal processes – axons and dendrites. Cues from surrounding tissues guide axons towards their targets during development of the nervous system. Once an axon reaches its target it needs to find a partner to make synaptic connections. Signals from the target itself could help the axon to make necessary modifications for synapse formation. To make precise connections it is also important that each neuron exhibit a unique identity.

This thesis describes the study of two molecules that are expressed in the nervous system. EphrinB2 a signal from target cells that could induce presynaptic modification and RAG1, a molecule that generates diversity in immune system, which is also present in specific subsets of neurons.

In this study, the role of EphrinB2 in the zebrafish visual system is examined. EphrinB2 belongs to a family of ligands for Eph receptor tyrosine kinases. It is B-type Ephrins which are transmembrane molecules. Ephrins are known for their role in topographic mapping of retinal ganglion cell axons on the optic tectum (O'Leary and Wilkinson, 1999; Wilkinson, 2000). EphrinB2 is known as a repellant cue for axon guidance and also has been found in a retinorecipient layer of chick tectum where RGC axons make synapses (Braisted et al., 1997).

With RNA-*in-situ* hybridization I found that zebrafish EphrinB2 is expressed in tectal neurons in the posterior part of the tectum when RGC axons enter the neuropil. Receptors for EphrinB2 on zebrafish RGC axons were detected by *in-vitro* receptor-ligand binding assays. As reported earlier in other systems, zebrafish RGC axons showed repulsive response to EphrinB2 in stripe assays. Studies with the

retinotectal projection mutant *"gnarled"* pointed out that the expression of ephrinB2 in ectopic cells in the anterior tectum of mutants could cause a premature stopping of RGC axons (Wagle et al., 2004). To verify this observation, a baculovirus-based gene expression system was developed which allowed temporal-spatial control over gene misexpression in zebrafish (Wagle and Jesuthasan, 2003). Ectopic expression of ephrinB2a in the anterior midbrain of wildtype embryos, with the aid of baculovirus, was found to inhibit RGC axon entry into the tectum. It is thus proposed that ephrinB2 may signal a subpopulation of RGC axons that they have reached their target neurons in the tectum.

The Recombination activating gene-1 (RAG1) is expressed in the vertebrate immune system and in the nervous system, including the zebrafish visual system (Chun et al., 1991; Frippiat et al., 2001; Jessen et al., 2001). RAG1 is well characterized for its role in generating diversity in immune system by V(D)J recombination (Schatz et al., 1989). Rag1 plays a key role in the initiation of this process of genomic rearrangement by recognizing and cutting recombination signal sequences (RSS) (Schatz et al., 1992). Detection of Rag1 transcripts in the mouse nervous system led to the idea that the genome may rearranged in neurons, but there has been no conclusive experimental evidence. In spite of the studies done over the last decade, the presence of RAG1 protein in neurons has not been demonstrated and its functions are questionable.

RAG1 protein was detected in specific neurons from the mouse brain at P10-14 and in neuronally differentiated P19 embryonic carcinoma cells. To identify potential RAG1 binding sites in neurons, chromatin immunoprecipitation (ChIP)

coupled with macroarray screening of a genomic YAC library was carried out. As a positive control, ChIP- DNA pulled down from thymocytes with anti-RAG1 antibody was used to generate probe. Signals obtained in this experiment partially overlapped those obtained from a T-cell receptor locus probe, showing the feasibility of this approach. ChIP-DNA from brain and neuronally differentiated P19 cells were then used to generate probes. A YAC clone that showed signal with both probes was analyzed further. Fine mapping by Southern analysis of BAC clones covering the YAC locus narrowed the potential target to a region which harbors a retrotransposon element. Binding of RAG1 to this region was further confirmed by analyzing ChIP-DNA from brain with the specific PCR. Analysis of the target sequence indicated the presence of a conserved heptamer found in the RSS. Although the YAC clone mapped to chromosome-9, PCR analysis and BAC macroarray screening with brain ChIP-DNA showed that the repeat region identified here as a potential target may not be specific to chromosome-9.

Identifying a retrotransposon as a potential target of RAG1 in neurons does not immediately answer the question of whether RAG1 could generate diversity in neurons as it does in the immune system. Nevertheless this finding indicates that RAG1 has distinct binding activity in neurons and puts us one step further in understanding the role of RAG1 in neurons.

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Abbreviation

A-P – anterior – posterior
BAC –Bacterial artificial chromosome
BDNF - Brain derived trophic factor
BMP – Bone morphogenic protein
ChIP – Chromatin Immunoprecipitation
CNS – Central nervous system
DAB - Diaminobenzidine
DAPI – 4,6-Diamidino-2-phenyindole, dilactate
DOPA - Dihydroxyphenylalanine
DSCAM - Down syndrome cell adhesion molecule
DRG – Dorsal root ganglion
D-V – Dorso-Ventral
FAK – Focal adhesion kinase
FGF – Fibroblast growth factor
GABA -gamma-aminobutyric acid.
LGN – Lateral geniculate nucleus
LINE – Long interspersed element
LMPCR – Ligation mediated PCR (polymerase chain reaction).
LTR – Long terminal repeat
MAPK – Mitogen activated kinase
NGF – Nerve growth factor
NMDA – N-methyl D-aspartate
PAK – p21 associated kinase
pcdh – Protocadherin
PCR – Polymerase chain reaction.
PSF - Pre m-RNA splicing factor
RAG –Recombination activating gene
R-C –Rostro-Caudal
RGC – Retinal Ganglion Cell
RPE – Retinal pigmented epithelium
RSS –Recombination signal sequence
SC- Superior colliculus
TGF – Transforming growth factor
YAC –Yeast artificial chromosome

Publications

1. **Wagle M**, Jesuthasan S. 2003. Baculovirus-Mediated Gene Expression in Zebrafish. Marine Biotechnology 5:58-63.

2. **Wagle M**, Grunewald B, Subburaju S, Barzaghi C, Le Guyader S, Chan J, Jesuthasan S. 2004. EphrinB2a in the zebrafish retinotectal system. J Neurobiol 59:57-65.

3. **Wagle M**, Jesuthasan S. 2005 A Specific Target For The Recombination Activation Gene-1 (*Rag1*) In Mouse Neurons. (Manuscript in preparation).

Chapter-I

Introduction

During embryonic development, the nervous system develops from a mass of neuroblasts (neuronal precursor cells). These cells divide and build a network of interconnected neurons. This is a crucial step in embryonic development, as a precise neuronal network is eventually responsible for most of the activities of an organism.

1.1 Central Nervous System (CNS) development

Various model organisms ranging from worms and insects to mammals have been used to understand neural development. With the help of mutants and other tools of genetic manipulation, the mechanisms of neural differentiation and nervous system patterning have been elucidated. As proposed by Goodman and Doe, the whole of neurogenesis can be viewed in eight steps (1) Induction and patterning of neuron forming regions, (2) birth and migration of neurons and glia, (3) generation of specific cell fates, (4) guidance of axonal growth cones to specific targets, (5) formation of specific synaptic connection, (6) binding of specific trophic factors for survival and differentiation, (7) competitive rearrangement of functional synapses and (8) continued synaptic plasticity during the life of an organism (Goodman and Doe, 1993). The first three steps are part of neural development and differentiation whereas the last three steps are activity-dependent. In this chapter, I will briefly describe the first steps of neural differentiation with a focus on axon guidance.

1.1.1. Neural differentiation

Soon after the embryo starts developing into a mulitcellular mass of cells from a single cell stage, it begins gastrulation. During this stage cells proliferate and

migrate. Involution of these cells converts the embryo into a multi-layered structure. Three germ layers – ectoderm, mesoderm and endoderm are formed. Specialized cell movements known as convergent-extension transform the embryo into a primitive body plan. Maternally deposited factors along with zygotically expressed genes pattern the embryo along the dorso-ventral (D-V) and anterior-posterior (A-P) axes. During gastrulation, a group of cells from the dorsal ectoderm is assigned a neuronal fate. These neuronal precursor cells migrate to their appropriate position to form the preliminary central nervous system in the form of a neural tube.

1.1.2. Neuralation and patterning of neural tube

In most vertebrate species, the anterior neural tube is formed by primary neuralation involving cell proliferation, invagination and pinching off from the rest of the cells, whereas the posterior part of the neural tube is formed by secondary neuralation in which the neural tube arises from a solid chord of cells which subsequently hollows out (Figure: 1.1A). The neural tube is patterned in A-P and D-V axis by the action of several genes. The anterior neural tube folds into forebrain (prosencephelon), midbrain (mesencephalon) and hindbrain (rhombencephalon) (Figure 1.1B). During the formation of preliminary brain structure from the anterior neural tube, optic vesicles are derived from the forebrain. Other sensory organs develop while the neural tube is transforming into the CNS. The posterior neural tube forms the spinal cord. Within the neural tube, neurons are specified to carry out different roles.

A

Neural plate

Neural Crest

Shaping

Epidermis

Folding

MHP   Notochord

Elevation

Convergence

Closure

Neural crest

Epidermis

Neural crest cells

Neural tube

B

Forebrain (Prosencephalon)

Telencephalon

Diencephalon

Midbrain (Mesencephalon) → Mesencephalon

Hindbrain (Rhombencephalon)

Metencephalon

Myelencephalon

spinal cord

C

Roof plate

Interneurons

Motor Neurons

Floor plate

Figure 1.1

Figure legends on pg. 4

(Adapted from: Developmental Biology-5th Edition: Scott F. Gilbert)

Figure 1.1: Schematic – Primary neuralation and patterning of neural tube

(Adapted & modified from Developmental Biology – 5th Edition: Scott F. Gilbert)

(A) The ectodermal plate consists of the neural tube in the middle and presumptive epidermis on either side separated by neural crest cells. The presumptive epidermis moves towards the center pushing the neural tube below it. This results in formation of an outer epidermis and neural tube contacted by neural crest cells that eventually migrate away from the neural tube to form peripheral neurons, glia and skin pigment cells.

(B) In the anterior region, the neural tube folds into three major structures

Prosencephalon (Fore brain): → Telencephalon and Diencephalon

Mesencephalon (Mid brain)

Rhombencephalon (Hind brain) → Metencephalon and Myelencephalon

Structures in the adult brain such as the olfactory lobe, hippocampus and thalamus are derived from these structures.

(C) the neural tube is patterned along the dorsal-ventral axis by signals from the ventral floor-pate and dorsal epidermis that specify different types of motor neurons and interneurons by activating transcription of specific genes.

1.2 Neuronal diversity

The nervous system of vertebrates comprises many types of neurons. In the human brain, there are approximately 10^{12} neurons of various types, for example there are about two dozen types of inhibitory neurons in the hippocampus alone (Parra et al., 1998). There is diversity in anatomy, gene expression and physiological properties. Morphologically, there are four different types of neurons, i.e. axonal, monopolar, bipolar, and multipolar. Based on their function in the nervous system, neurons are classified as sensory neurons, interneurons and motor neurons. Different types of sensory neurons are found within each sensory organ, depending on the stimulus they respond to. In the retina there are at least one dozen different types of ganglion cells (Devries and Baylor, 1997). Similarly in the olfactory epithelium each neuron has its own identity based on odorant receptor expression (Mombaerts et al., 1996). Motor neurons have distinct anatomical connectivities and gene expression properties. Neurons within the CNS have differences in neurotransmitter identities; they may be DOPAergic or GABAergic for example. They also differ by expression of surface molecules such as protocadherins.

This diversity is created by the action of several signaling molecules that act during the development of the nervous system. Two mechanisms have been described for neuronal fate specification: lineage dependency and extrinsic signal/morphogen dependency. Proneural genes belonging to bHLH family initiate neural fate and generate progenitor cells that are committed to differentiate (Bertrand et al., 2002). Studies in Drosophila have shown that lateral inhibition involving Notch-Delta signaling plays a crucial role in specification of neuronal fate in neuroblasts. A

similar mechanism exists in vertebrates as well (Lewis, 1998). Asymmetric cell division of neuronal progenitors allows the inheritance of cell fate determining factors to one daughter cell, thus resulting cells may be specified as neuronal or glial (Chia and Yang, 2002). Neuronal specification and diversity has been well studied in the CNS with respect to patterning of hindbrain along rostro-caudal axis and D-V axis in the neural tube. During development, FGF and several Hox genes pattern different regions of the brain to specify neurons within these structures (Dasen et al., 2003; Salie et al., 2005). The neural tube is patterned along the D-V axis by the action of TGF-β from dorsal and Sonic hedgehog from the ventral floorplate or notochord (Echelard et al., 1993; Roelink et al., 1994; Liem et al., 1995; Liem et al., 2000; Nguyen et al., 2000). Motor neurons and interneurons are specified along the D-V axis within the neural tube by the combinatorial effect of these factors (Figure 1.1C). These factors induce expression of transcription factors and genes which govern various properties of the neuron such as expression cell surface molecule/receptors, production and response to neurotransmitters. Thus various neurons are specified during the development of the nervous system. This allows neurons to carry out their specialized functions as well as to connect with their synaptic partner.

Sperry's chemoaffinity theory postulates a cytochemical specificity to individual neurons (Sperry, 1963). Cell surface molecules are the best candidate to satisfy this assumption. Indeed, in Drosophila, Down syndrome cell adhesion molecule (DSCAM) could generate diversity in neurons (Schmucker and Flanagan, 2004). The Dscam locus contains three arrays of alternative exons that are combined with 20 constant exons and two alternative transmembrane domain by alternative

RNA-splicing (Wojtowicz et al., 2004). This generates a huge repertoire of DSCAM molecules containing different extracellular domains. These molecules show homophilic interaction and are involved in axon guidance (Schmucker et al., 2000; Wojtowicz et al., 2004). The diversity in neuronally expressed DSCAM provides a mechanism for selective axon fasciculation and recognition of synaptic targets. Although vertebrate orthologs of Dscam do not show this diversity, other cell surface molecules such as protocadherins (*Pcdh*) exist and these are good candidates for generating diversity in the vertebrate nervous system (Serafini, 1999). The *Pcdh* genes are clustered in the genome and show similar organization as that of immunoglobulins or T-cell receptors (Wu and Maniatis, 1999; Wu et al., 2001). Like *Dscam*, individual *Pcdh* mRNA are generated by splicing of variable exons to the constant 3'end (Wu and Maniatis, 1999). Pcdh are localized in synapses, and have been proposed to offer synaptic specificity along with other cadherins (Kohmura et al., 1998; Serafini, 1999). Other molecules such as cochlear potassium channels and synaptic neurexins also show various isoforms through alternative RNA splicing mechanism and may further contribute to neuronal diversity (Black, 1998; Missler and Sudhof, 1998).

Thus neuronal diversity is achieved by the expression of various genes. In spite of this diversity and large number of neurons in the vertebrate nervous system, neurons are connected precisely to their targets. In fact this diversity is an essential criteria for building a complex neuronal network and its functionality.

1.3 Axon guidance –mechanism

Apart from differentiation and migration of neurons to their appropriate position in the embryo, it is also important that these neurons are connected to each other in a specific manner to build a functional neuronal network. Once neurons are specified, they send out processes called axons and dendrites to connect with each other. An axon can extend many cell diameters to connect to other neurons. Axons grow in a stepwise manner and surrounding tissue along the axon path may act as guide posts. Examples of such cells are those at the midline for peripheral axons (reviewed in Tessier-Lavigne and Goodman, 1996). The tip of the axon is called the growth cone. It has microtubules at the base and dynamic actin filaments that form finger like protrusions (filopodia) and web-like lamellipodia (Figure 1.2A). It also bears receptors at the surface that sense cues from surrounding tissues (reviewed in Tessier-Lavigne and Goodman, 1996)..

During embryonic development, axon guidance is mainly independent of neuronal activity and relies on surrounding cues. These cues could be in the form of secreted molecules or cell surface molecules that either attract or repel the growth cone (Figure 1.2). In the case of secreted signaling molecules, axon behavior is termed as chemoattraction or chemorepulsion, whereas in the case of guidance molecules bound to cell surface the phenomenon is known as contact mediated attraction or repulsion (Figure 1.2 B). Receptor ligand interactions at the growth cone lead to changes in the axon cytoskeleton. Signaling molecules may trigger different types of signaling pathways that eventually result in cytoskeletal rearrangements.

A

actin fillament

filopodia

lamellipodium

microtubules

B

Chemoattraction

Chemorepulsion

contact-dependent attraction

contact-dependent repulsion
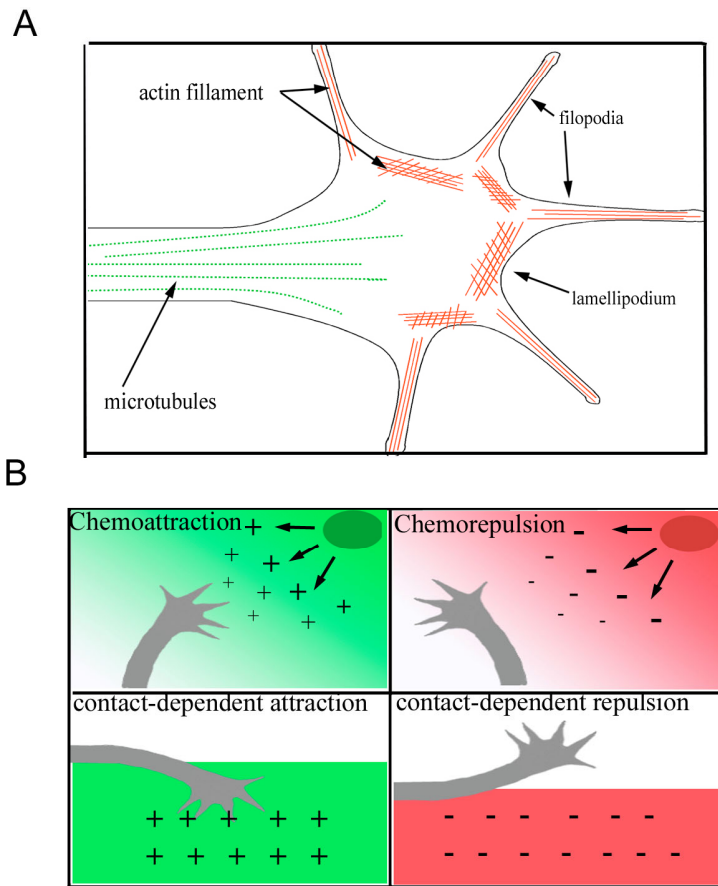
Figure 1.2

Figure 1.2: Schematic of growth cone structure and mechanism of growth cone
guidance

    (A) Diagrammatic representation of a growth cone showing actin
filaments at the tip forming lamellipodia and filapodia, whereas
microtubules are at the base of the growth cone and in the axon shaft.

    (B) Schematic describing attraction and repulsion by a diffusible gradient
of molecules – chemoattraction and chemorepulsion
or by physical contact with the source – contact dependant attraction
and repulsion.

(Adapted and modified from : Mueller, B. K. (1999). Annu Rev Neurosci 22: 351-88.)

1.4 Model systems and methods to study axon guidance

Several model organisms have been used over the last few decades to study axon guidance. Studies in invertebrates, mainly in *C. elegans* and *Drosophila,* have been successful in identifying many axon guidance molecules. The simple nervous system architecture, for example 302 total neurons in *C. elegans* and segmental arrangement in *Drosophila* allowed connection of individual neurons with their targets to be studied during development. Moreover these systems are easily amenable to genetic manipulation. Through the study of such simple systems, well-conserved mechanisms were elucidated. One example is the crossing of axons at the midline (Figure 1.3). Axons from peripheral neurons are attracted towards the midline and once they cross the midline they are kept away from the midline. The change in axon response to the same guidepost has been studied in depth in *Drosophila*. Molecules such as Roundabout, Commissureless, Netrins were identified by genetics and characterized extensively (Kaprielian et al., 2001). Thus genetics in invertebrates has been a powerful tool to identify guidance cues. Many of these genes have orthologs in vertebrates where they also function at midline crossing.

Biochemical approaches have also identified several cell adhesion and signaling molecules. A key requirement is an assay system to test effects of these molecules. Conventionally neuronal explants and co-cultures were used for axon guidance studies. Neuronal extensions (neurite growth) can be studied in response to secreted molecules by placing neurons in proximity to cells expressing those molecules (Kennedy et al., 1994; Serafini et al., 1994). Two assays that are widely used studying effects of various molecules on growth cone behavior are "stripe assays" for analyzing membrane

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associated molecules, and the "pipette assay" or "growth cone turning assay" for soluble molecules. Initial stripes assays developed by Bonhoeffer's group used stripes of membrane preparations from tectal cells to study the growth of retinal axons from anterior or posterior retina (Walter et al., 1987). In a modification of this assay, stripes of purified proteins have been used to examine the response of retinal axons (Drescher et al., 1995). Chemo- attraction or repulsion could be better studied in pipette assays. A glass capillary pipette holding a solution of the molecule to be tested is positioned close to growth cone. Pulses of these molecules create a concentration gradient between the pipette tip and growth cone. Growth cone response to this gradient could be studied by time-lapse microscopy (Lohof et al., 1992).
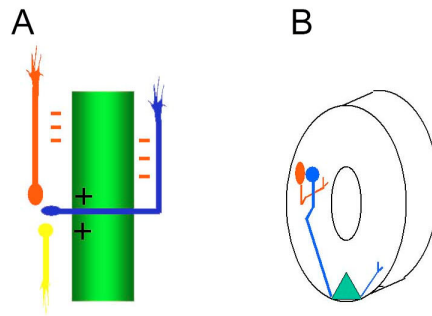
Figure 1.3

Figure 1.3: Axon guidance at the midline in *Drosophila* (A) and vertebrate (B) neural tube

Schematic representation of *Drosophila* ventral midline showing two types of axons – some axons are repelled away from the midline and grow parallel to midline on the ipsilateral side (red and yellow), whereas commissural axons are attracted towards the midline and cross it once, but after crossing the midline they are kept away from the midline and continue to grow on the contralateral side (Blue).Similar axons are shown in the diagrammatic representation of a cross-section of a vertebrate neural tube, some axons are attracted to the ventral floorplate, cross the midline and continue to grow on the contralateral side (blue), whereas some axons are kept away from the midline (Red)

(Adapted and modified from : Developmental Biology – 5th Edition: Scott F. Gilebert)

1.5 *Principles of axon guidance*

In 1892 Ramón y Cajal proposed that chemotactic cues guide axons in the nervous system (Ramón, 1892). About hundred years later the molecular nature of these cues became clear when several axon guidance molecules were identified based on approaches described above. Below is the brief summary of these molecules which describe the principles of axon guidance.

1.5.1   Netrins:

The idea of chemoattractant-mediated axon guidance was supported when Netrins were isolated from chick brain, based on their ability to promote outgrowth and reorient comissural axons in an in vitro assay system. (Kennedy et al., 1994; Serafini et al., 1994). Netrins form a small family of secreted proteins similar to laminin, an extracellular matrix protein. In mice, loss of Netrin-1 leads to abnormal commissural axons projection (Serafini et al., 1996). Similarly C.elegans mutant for UNC-5, a homolog of Netrin-1, show a defect in circumferential axon guidance. Also Netrin-A and Netrin-B are expressed in the Drosophila ventral nerve cord during commissure formation, and deletion of both these genes leads to formation of thinner than normal commissures (Harris et al., 1996; Mitchell et al., 1996). Thus the role of Netrin as a chemoattractant at the midline remains evolutionarily conserved.

Netrins act through their receptors known as Deleted in Colorectal Cancer (DCC) and UNC5H in mammals. Mice mutant for DCC shows similar defects in commissural axon projection as that of the Netrin-1 mutant (Fazeli et al., 1997). Surprisingly in C. elegans, the Netrin ortholog UNC-6 acts as an attractant for some axons and a repellant

for others. UNC-6 genetically interacts with the DCC ortholog UNC-40 for ventral guidance (Hedgecock et al., 1990; Chan et al., 1996), and UNC-5, a novel Ig superfamily member, is required for dorsal directed axon guidance by repulsive interaction (Culotti and Merz, 1998). Similarly Netrin-1 was also found to be bifunctional and repelled Xenopus spinal neurons under in vitro conditions (Ming et al., 1997). UNC-5 homologs in vertebrate have been identified and found to be expressed in neurons that are repelled by Netrin-1 in vitro (Leonardo et al., 1997). These studies showed that an axon guidance molecule could act as attractive or repulsive in different contexts by interacting with different receptors.

1.5.2   Semaphorins:

The molecular aspect of repulsive interactions in axon guidance was revealed by identification and characterization of Semaphorin. The first Semaphorin was identified in the grasshopper CNS (Kolodkin et al., 1992) and the subsequent one in vertebrate growth cone collapse study (Luo et al., 1993).

The semaphorins are a large family of cell surface and secreted guidance molecules. They show a characteristic ~ 420 amino acid "sema" domain at their –NH2 termini and are divided into eight classes (reviewed in (Raper, 2000). Semaphorins signal through a multimeric receptor complex which involves plexin, a family of transmembrane molecules, and neuropillins, along with other molecules such as L1 (a neuronal cell adhesion molecule), the Met receptor tyrosine kinase, and OTK (an inactive receptor tyrosine kinase in Drosophila) (Raper, 2000). Semaphorin III, also known as collapsin-1 in chicken, is the best studied semaphorin in vertebrates. It induces growth cone collapse in DRG (dorsal root ganglion) axons (Luo et al., 1993). Semphorin mainly functions as a

repulsive cue but has also been shown to act as an attractant in some cases (Wong et al., 1999; Raper, 2000). Sensory axons and spinal motor axons but not RGC axons are repelled from immobilized source of Sema III (Messersmith et al., 1995; Puschel et al., 1996; Varela-Echavarria et al., 1997).

1.5.3   Slit-Robo

Signals from the midline may attract or repel axons. After reaching the midline, some axons grow parallel to it on the ipsilateral (same) side while some axons cross the midline forming commissures and then grow parallel to the midline on the contralateral (other) side. The change in axon behavior at the midline was puzzling, as commissural axons are first attracted towards the midline but are kept away after crossing it.

In the Drosophila mutant roundabout (robo), axons that do not cross the midline usually do cross over, and those which cross it once are attracted towards the midline after crossing, resulting in formation of thick commissures (Seeger et al., 1993). Another mutant commissureless (comm) showed exactly the opposite phenotype to that of robo, displaying absence of nearly all commissures. Molecular characterization of these mutants and identification of Slit, a ligand for Robo, solved the midline crossing puzzle. Interaction of Slit with Robo leads to repulsion of axons, whereas Commissureless regulates the Robo-Slit interaction by controlling the localization of Robo at the growth cone during the midline crossing (Kidd et al., 1998; Keleman et al., 2002; Myat et al., 2002).

A C. elegans homolog of Robo, Sax-3 serves a similar function in axon guidance near the midline. Like in Drosophila, vertebrate Slit proteins are present at the ventral midline cells and commissural axons are repelled by Slit after crossing the midline (Brose

et al., 1999; Zou et al., 2000). Mice deficient for Slit1 and Slit2 do not show an obvious defect in midline guidance because of the presence of Slit3 at the midline in these mice. Nevertheless these mice show a defect in formation of the optic chiasm (Plump et al., 2002). Similar defects are seen in the zebrafish mutant astray/robo2 in which RGC axons make multiple errors before, during and after crossing the midline (Fricke et al., 2001). Thus, like Netrins the function of Robo-Slit at the midline seems to be conserved during evolution. These studies also revealed how the axon response to a guidepost or a guidance molecule could be reversed by receptor localization at the growth cone.

1.5.4   Eph-Ephrins

In visual system, most RGCs (retinal ganglion cells) send their axons to the contralateral optic tectum. RGC axons project to specific areas on the tectum with respect to their point of origin. For example, axons from anterior part of the eye terminate in the posterior region of the tectum and posterior axons in the anterior tectum. This is known as the topographic mapping of axons and has been an interesting question in the study of axon guidance. Sperry invoked the chemoaffinity theory, proposing that gradients of chemical tags may provide positional information to neurons in the eye as well as in the tectum (Sperry, 1963). Bonhoeffer's stripe assays provided experimental evidence to this theory (Walter et al., 1987). The molecular nature of this phenomenon began to become clear after the identification of Ephrins as guidance cues in the tectum (Drescher et al., 1995). There are two types of ephrin ligands viz. EphrinAs that are GPI anchored and EphrinBs which are transmembrane molecules. Accordingly there are EphA and EphB receptors. These molecules play important roles in retinotectal mapping i.e. mapping of RGC axons onto their target - the optic tectum in the brain (O'Leary and Wilkinson,

1999). The role of Eph-Ephrin signaling is well studied in the visual system (discussed in first section), but they are also known to be involved in axon guidance elsewhere in the nervous system.

The role of EphA4 and EphrinB3 during the formation of the corticospinal tract (CST) was elucidated in a genetic study, (Kullander et al., 2001; Kullander et al., 2001; Yokoyama et al., 2001). CST axons originate in the motor cortex and cross the midline once at the brain – spinal cord junction. In mice carrying a non-catalytic allele of EphA4 or deletion of EphrinB3, CST axons cross the midline a second time. It was suggested that EphrinB3 expressed by spinal cord midline cells repels EphA4 positive CST axons (Kullander et al., 2001). Midline Ephrins do not repel spinal cord commissural axons as EphB1 and EphA2 receptors are absent on ipsilateral axon segments but are up-regulated on distal axons segments after crossing the midline by localized protein translation and cell surface expression (Imondi et al., 2000; Brittis et al., 2002). Formation of the anterior commissure (AC) involves reverse signaling between EphrinBs expressed on AC, and EphB2 and EphA4 expressed in territories through which the AC migrates during midline crossing (Henkemeyer et al., 1996; Kullander et al., 2001).

Apart from axon guidance at the midline, Eph-Ephrins are also involved in creating patterned neuronal connections in the CNS and the peripheral nervous system. Similar to the topographic mapping in the visual system, Eph signaling acts in topographic projection involving the septum and hippocampus (Gao et al., 1996; Zhang et al., 1996). These studies show that EphrinA2 is expressed in a gradient on the septum whereas EphA5 is in the complementary gradient on the hippocampus. EphrinAs are also involved in patterning axon projections in other parts of the CNS, such as the thalamocortical

connection and cerebellum (Nishida et al., 2002; Dufour et al., 2003), reviewed in (Palmer and Klein, 2003). Axon projection of motor neurons within the lateral column of the spinal cord and their target muscles also show topographic mapping influenced by EphA4 (Helmbacher et al., 2000; Eberhart et al., 2002). Topographic mapping of axons from the vomeronasal organ (VNO) to the accessory olfactory bulb (AOB) involves EphrinA5 and EphA6 (Knoll et al., 2001). EphrinAs along with odorant receptors are also involved in axon projection of olfactory neurons (Cutforth et al., 2003).

1.5.5   Secreted molecules: Shh, BMP and Wnt

Secreted molecules such as Shh, BMP and Wnt, which play important roles in various developmental processes such as embryonic axis determination, neuronal differentiation and specification, also participate in axon guidance.

The first clue implicating the Shh pathway in axon guidance came from an observation that commissural axons do reach the ventral midline in mutant mice lacking Netrin or DCC (which are involved in attraction of commissure axons to the ventral midline). However, in double mutants lacking *Gli-2* (a component of Shh signaling pathway) and *Netrin*-1, commissural axons do not reach to the ventral midline (Charron et al., 2003). Thus Shh signaling may serve as an additional attractive cue from the midline to commissural axons. These observations were further supported by experiments in which the spinal cord was cultured with Shh expressing CHO (Chinese hamster ovary) cells and by *in vitro* growth cone turning assays. Shh is also known to affect growth cone behavior of RGC axons *in vitro* (Trousse et al., 2001).

In an in vitro assay, commisural axons were reoriented away from the roof plate (Augsburger et al., 1999). The behavior of commissural axons in co-culture assays with

roof-plate from wildtype and BMP-7$^{-/-}$ or GDF-7$^{-/-}$ mutant mice revealed that these molecules serve as repulsive cues (Butler and Dodd, 2003). Further analysis subsequently showed that BMP7 and GDF7 heterodimers cause growth cone collapse in commisural axons.

A family of secreted molecules, the Wnts, which function in nervous system development, are known to be involved in presynaptic axon remodeling in vertebrate synaptogenesis and maturation of Drosophila neuromuscular junctions (Hall et al., 2000; Krylova et al., 2002; Packard et al., 2002). Recently one Wnt family member, the Drl ligand Wnt-5 was found to function in axon guidance of anterior commissure axons in Drosophila (Yoshikawa et al., 2003).

1.5.6   Other signaling molecules

Apart from the major axon guidance molecules discussed above there are several neurotrophic growth factors, cell adhesion molecules and small molecules such as calcium and cyclic nucleotides that play important roles in axonal pathfinding.

The neurotrophin family comprises nerve growth factors (NGF), brain derived neurotrophic factor (BDNF), NT3 and NT4/5. These factors are necessary for the development, maintenance and plasticity of the nervous system. Neurotrophins act through two types of receptors viz. a low affinity receptor p75 and a ligand specific receptor tyrosine kinase of the trk family (Segal and Greenberg, 1996). When injected into ventricles of neonatal rats, NGF evoked aberrant axon growth (Menesini Chen et al., 1978); it also promoted axonal growth of embryonic sensory neurons in vitro (Letourneau, 1978; Gundersen and Barrett, 1979). The role of neurotrophins in axon guidance has also been studied using DRG neurons. Gradients of NGF and BDNF in

culture conditions evoked either an attractive or inhibitory response in growth cones depending upon the neuronal type e.g. BDNF, NT3 and NT4/5 caused a chemotropic turning response in certain populations of embryonic DRG neurons whereas they served as inhibitory factors for growth cones of NGF dependent neurons (Paves and Saarma, 1997). BDNF also induced branching in RGC axons (Alsina et al., 2001).

Components of the extracellular matrix (ECM) have also been demonstrated to modulate neurite outgrowth. The myelin associated growth factor (MAG) in its soluble form was found to be a repulsive cue for cultured spinal neurons (Song et al., 1998). Soluble forms of cell adhesion molecules (CAM) such as neuronal CAM, L1 and neuronal cadherin (calcium dependent adherent proteins) can affect nerve growth by modulating cascades of secondary messenger systems. Many CAMs predominantly show homophilic interaction and could potentially influence growth cone guidance, axon fasciculation, and target recognition.

1.5.7 Interpretation of guidance cues (effect of Calcium and cyclic nucleotides)

Axon guidance molecules were initially described either as attractive, repulsive or in some cases bifunctional. Studies by Mu Ming Poo and colleagues provided an alternate viewpoint by showing that a guidance cue can be interpreted by the growth cone as attractive or repulsive depending on intrinsic factors such as calcium and cyclic nucleotide concentration (Terman and Kolodkin, 1999). For example, the attraction of cultured retinal neuron growth cones to netrin-1 and BDNF is converted to repulsion when these neurons are grown on a laminin substrate, which alters the level of cAMP in neurons (Song et al., 1997). Cultured spinal neurons show an attractive response to a gradient of a membrane permeable analogue of cAMP (Lohof et al., 1992). Mutant mice

lacking type-I adenylate cyclase activity shows disrupted patterning of the somatosenssory cortex (Abdel-Majid et al., 1998). The effect of cAMP may be mediated by protein kinase-A (PKA) through its substrate IP3 receptor and cytoskeletal proteins. Another cyclic nucleotide, cGMP has also been shown to have role in establishing connections of retinal and olfactory axons (Wu et al., 1994; Gibbs and Truman, 1998).

Extracellular and cytosolic levels of Ca2+ play a crucial role in regulating a wide range of growth cone behaviors. An inverse correlation of neurite extension rate with the frequency of Ca2+ transients in growth cones has been observed (Gu and Spitzer, 1995; Gomez and Spitzer, 1999). Growth cone collapse is sometimes associated with an increase in cytosolic Ca2+ whereas a lowering of extracellular Ca2+ concentration may increase neurite extension (Song et al., 1997; Gomez and Spitzer, 1999). The turning response of cultured Xenopus spinal neuron growth cones induced by Netrin-1 and BDNF can be abolished by removing extracellular Ca2+ (Ming et al., 1997; Song et al., 1997). Neural cell adhesion molecules such as L1 and NCAM can increase intracellular Ca2+ by opening Ca2+ channels. Calcium signaling can be transduced by calmodulin (CaM) and CaM dependent kinases (Zheng et al., 1994). Selective disruption Ca2+/CaM function in Drosophila embryos resulted in deviating axon growth, fasciculation, and pathfinding (VanBerkum and Goodman, 1995). The potential downstream target of Ca2+ is adenylate cyclase, which in turn regulates the level of cAMP.

1.6 Aim of the thesis

1.6.1   Study of EphrinB2a in zebrafish visual system

Differentiation of neurons and axon guidance have been studied for the past several decades. As described above, the molecular mechanism of neuronal

differentiation, axon pathfinding and topographic mapping has been elucidated to a great extent using various model organisms. The first part of this thesis examines a question that has received less attention, which is how axons from peripheral neurons find their synaptic partners within their target zone.

Axons are guided towards their target zone by different cues, but once axons reach their target, they need to change their growth behavior in order to make connection with their partners. At least three changes need to happen to form synapses in the target zone: axon should stop their growth so as not to overshoot the target, they should sort out into individual axons i.e. defasciculate and branch locally (arborize) and they should sequester proteins that are necessary for forming synaptic connections (presynaptic modification).

It is easy to conceptualize that the molecules expressed on the target, either in neurons or surrounding glial cells could induce these changes in the axon and thereby facilitate synapse formation. It is likely that a combination of signaling molecules is involved in this process. In the first part of the thesis (second chapter) I describe the study of one such signaling molecule, Ephrin-B2 - that is known to be involved in axon guidance as well as other cell migration phenomena (e.g. vasculogenesis and somitogenesis) and also recently suggested to be involved in target recognition (reviewed in (Palmer and Klein, 2003; Davy and Soriano, 2005).

1.6.2 Study of Rag1(Recombination activating gene-1) in neurons

Guidance cues help axons to reach their destination and may further help in target recognition but how does each neuron connect precisely to its right synaptic partner? To achieve this, each neuron should carry a specific address. At the molecular level this

would mean that each neuron carries specific molecule(s) that would be recognized by its synaptic partner. Even in a simplest vertebrate there would always be more synapses than the number of genes. So the question arises as to how such diversity is achieved. In Drosophila a family of cell adhesion molecules, DSCAM (Down syndrome cell adhesion molecule), plays an important role in this process. Large number of diverse DSCAM molecules are synthesized from a single transcript from the Dscam locus by alternate RNA splicing (Schmucker et al., 2000; Celotto and Graveley, 2001). But this phenomenon is specific to invertebrates, as the vertebrate DSCAM ortholog does not show this property. Cell adhesion molecules such as protocadherin may be involved in synapse formation. However, the question of how diversity is generated and maintained remains open

One intriguing possibility is that the vertebrate nervous system could generate diversity in neurons by genomic rearrangement analogous to the immune system. In the immune system, a large repertoire of antibodies is generated from a relatively small number of genes by genomic rearrangement. Such a process offers a large diversity and unique identity to each cell. Does a similar mechanism exist in neurons? Even before the mechanism of V(D)J recombination in immune system was known, Dreyer et al proposed the hypothesis of genetic reprogramming in the immune system and nervous system with reference to gold fish retinotectal projection (Dreyer et al., 1967). At least at the molecular level there seems to be a link between the immune system and the nervous system. Rag1 plays a key role in the initiation of this process of genomic rearrangement by recognizing and cutting recombination signal sequences (RSS). This molecule is also known to be expressed in the nervous system. Several groups have reported the presence

of Rag1 transcripts in the nervous system of various vertebrate model organisms and transgenic animals with reporter genes driven by the Rag1 promoter have shown expression in the nervous system (Chun et al., 1991; Frippiat et al., 2001; Jessen et al., 2001). Although mice lacking Rag1 do not show any obvious morphological defect in the brain, behavioral studies do show subtle defects in these mice (Mombaerts et al., 1992; Cushman et al., 2003). In spite of studies done over the last decade, the presence of RAG1 protein and its function in neurons remains to be established. In the second part of the thesis (chapter-3), I describe a study of Rag1 in neurons.

Chapter II

Development of a baculovirus mediated misexpression system and its application to the study of EphrinB2a function in Zebrafish visual system

2.1 Introduction

Though axon guidance molecules and cues that are necessary for topographic mapping have been extensively studied, molecules involved in target recognition within the CNS have not been well characterized. Once axons reach their target, they should stop growing further and arborize within the target. It is likely that the signals for target recognition are provided by the target itself. These signals should meet at least three conditions: firstly, they should be expressed postsynaptically, secondly the receptor for these signals should be present on presynaptic cells and lastly, these molecules should have a growth inhibitory effect on the axons. In case of motor neuron connections with their target muscles, a specific form of muscle derived laminin may act as "stop signal" for axonal growth (Martin et al., 1995; Noakes et al., 1995). Such stop signals have not been characterized for peripheral neurons. This study addresses the question by candidate gene approach using the visual system as a model.

2.1.1 Vertebrate visual system:

The vertebrate visual system has been a model for studying axon guidance of peripheral neurons. The vertebrate eye consists of a lens that projects the image of the surrounding environment onto the retina which includes photoreceptors. The information from photoreceptors is transmitted to the brain by retinal ganglion cells (RGCs) which send their axons to the visual centers in the midbrain. RGC axons from the entire retina first come together at the optic disc, form a fascicle and exit the eye as an optic nerve.

During the development of the visual system in vertebrates such as fish and amphibians, RGCs innervate a number of targets in the midbrain, with the most prominent being the optic tectum. In mammals, RGCs are connected to the lateral geniculate nuclei (LGN) and superior colliculus (SC) in the midbrain. These centers are connected to the primary visual cortex. A majority of RGCs send their axons to the contralateral tectum, but a population of RGC axons are connected to the ipsilateral centers.

Within the optic tectum, axons are sorted out depending on where they originate from. Axons from the anterior eye branch primarily in the posterior tectum, while those from the posterior eye branch in the anterior tectum. Similarly, axons from the dorsal eye branch in the ventral tectum, while those from the ventral eye arborize in the dorsal tectum. As they branch, the axons form synapses with tectal neurons. A family of receptor tyrosine kinases – Eph and their ligands Ephrins - are the main players in the topographic mapping of RGC axons.

2.1.2   Eph-Ephrins

Eph receptors constitute the largest subfamily of receptor tyrosine kinases (Tuzi and Gullick, 1994; Orioli and Klein, 1997). Its 13 members (in mammals) are subdivided based on their sequence similarity and ligand binding properties into subclass-A: *EphA1-EphA8* and subclass-B: *EphB1-EphB4, EphB6*. Their ligands are also subdivided into subclass-A: *EphrinA1-EphrinA5* which are associated with the cell membrane by a glycosyl phosphatidylinositol anchor and subclass-B: *EphrinB1-EphrinB3* which are transmembrane proteins. Ephrin orthologs have also been identified in invertebrates (George et al., 1998; Scully et al., 1999; Wang et al., 1999; Bossing and Brand, 2002). All Eph-Ephrins mentioned in this section are either referred to by the original

publication or as suggested by the Eph nomenclature committee (Eph_Nomenclature_Committee, 1997) and described by Nigel Holder & Rüdiger Klein (Holder and Klein, 1999). Binding of Ephrins to Eph is promiscuous (Figure 2.1). It is known that EphA4 can bind to EphrinB2 and EphrinB3 but not EphrinB1 (O'Leary and Wilkinson, 1999). This promiscuity could cause functional redundancy *in vivo* (Orioli et al., 1996; Feldheim et al., 2000).

The first Eph receptor was cloned in a screen for human homologs of viral oncogene (Hirai et al., 1987). Later Eph and Ephrin were extensively studied for their role in axon guidance and retinotectal topographic mapping (reviewed in (O'Leary and Wilkinson, 1999). But research over the last several years shows that Eph-Ephrin signaling is involved in several biological process during embryonic development, in the adult and in some pathological conditions (reviewed in (Palmer and Klein, 2003). Apart from their neuronal role, ephrins have been studied for their involvement in cell migration, segmentation and vasculogenesis (Holder and Klein, 1999; Palmer and Klein, 2003)

Eph-Ephrin signaling is unique among the RTK family because of its bidirectionality. Ephrins not only induce signaling downstream of the Eph receptor (known as forward signaling) but also signal into the cell that expresses them (referred as reverse signaling (reviewed in (Palmer and Klein, 2003; Davy and Soriano, 2005). Oligomerization and clustering of Eph receptors and Ephrins at the cell surface is essential for their signaling (Stein et al., 1998). Structural analysis showed that Eph and Ephrin interaction domains associate to form hetero tetramers (Himanen et al., 2001)

The bi-directional mode of signaling and knowledge about molecular architecture of Eph and Ephrin has facilitated the study of their roles in various context by perturbing

either one of the signaling. In addition to a complete null mutation (knockout) of either Eph or Ephrin, specific deletions or point mutations in the cytoplasmic domain of either receptor or ligand allows a dissection of the signaling pathway and an analysis of its effect in a particular process. Soluble or non-clustered ligands show different effects and enable their usage in a dominant negative approach (Durbin et al., 1998; Lackmann et al., 1998).

2.1.3   Neuronal roles of Ephrin

Eph-Ephrin signaling has been studied in various aspects of neurobiology. It was found to be involved in neurogenesis, axon guidance, synaptic plasticity and neuroregeneration (reviewed in (Palmer and Klein, 2003). Eph and Ephrins are localized in the ventricular zone (VZ) of the embryonic cortex (Stuckmann et al., 2001). Disruption of Eph-Ephrin signaling by the intraventricular infusion of soluble receptor or ligand in the subventricular zone (SVZ) results in disorganized migration pattern and increased proliferation (Conover et al., 2000)implying an involvement in stem cell differentiation. The involvement of Ephrins in axon guidance within the CNS has been discussed in the previous section. It is believed that molecules involved in axon guidance during embryonic development could be reused in regeneration. An up-regulation of Eph expression after injury was found in the adult spinal cord, hippocampus and cochlear nucleus (Pickles and van Heumen, 1997; Miranda et al., 1999; Moreno-Flores and Wandosell, 1999). An expression of EphrinA was observed in regenerating tectum of adult goldfish and zebrafish (Becker et al., 2000; Rodger et al., 2000).

EphB1
Elk, Cek6, Net, Hek6
Xelk

EphB2
Cek5, Nuk, Erk, Qek5
Tyro5, Sek3, Hek5, Drt
rtk3

EphB3
Cek10, Hek2, Mdk5
Tyro6, Sek4

EphB4
Htk, Myk, Tyro11
Mdk2
rtk5/rtk8

EphB6
Mep

EphB5
Cek9, Hek9

EphrinB1
LERK2, Elk-L, EFL3
Cek5-L, STRA1

EphrinB2
Htk-L, ELF2, LERK5
NLERK1
L5

EphrinB3
NLERK2, Elk-L3,
ELF6, ELF3,LERK8

EphA1
Eph, Esk

EphA2
Eck, Myk2, Sek2
G42/G50
rtk6

EphA3
Cek4, Mek4, Hek,
Tyro4, Hek4
rtk2

EphA4
Sek, Sek1, Cek8
Hek8, Tyro1,Pag
rtk1

EphA5
Ehk1, Bsk, Cek7
Hek7, Rek7
rtk7

EphA6
Ehk2, Hek12

EphA7
Mdk1, Hek11, Ehk3
Ebk, Cek11

EphA8
Eek, Hek3
rtk4/ZDK

EphrinA1
B61, LERK1
EFL1
L1

EphrinA2
ELF1, Cek7-L
LERK6
L3

EphrinA3
Ehk1-L, ELF2
LERK3

EphrinA4
LERK4 ELF4

EphrinA5
AL1, RAGS
LERK7, EFL5
L2/L4

Figure 2.1

Figure 2.1 : Eph-Ephrin classification and receptor ligand binding. Orthologs of Eph and ephrin in mammals, birds and Xenopus are shown in blue color, whereas zebrafish orthologs are shown in red color

(Reference: Mueller, B. K. (1999). Annu Rev Neurosci 22: 351-88
Holder, N. and R. Klein (1999). Development 126(10): 2033-44)

2.1.4   Ephrins in Retinotectal projection and topographic mapping

Friendrich Bonhoeffer and colleagues showed that cues in the posterior tectum had a repellant activity on temporal axons retinal *in vitro* (Walter et al., 1987; Walter et al., 1987). Subsequent molecular characterization revealed that positional information, which is critical for topographic mapping, is provided by gradients of ligands and receptors of the Eph family (O'Leary and Wilkinson, 1999; Wilkinson, 2000), which are present on endfeet of radial glial cells (the substrata for migration) and retinal ganglion cell axons. GPI-anchored ligands such as EphrinA5 (Drescher et al., 1995) and EphrinA2 (Cheng et al., 1995) are present in a high-posterior to low-anterior gradient in the optic tectum, and have been proposed to inhibit growth (Drescher et al., 1995) and branching (Yates et al., 2001) of retinal ganglion axons. Misexpression of EphrinA2 in chick or mutating EphrinA5 in mice caused abnormal axonal projection in the tectum (Nakamoto et al., 1996; Frisen et al., 1998). The double knockout of ephrinA2$^{-/-}$, ephrinaA5$^{-/-}$ shows a severe defect in anterior-posterior projection (Feldheim et al., 2000). Receptors for these ligands are present in the axons, either uniformly, as in the case of EphA4 (Holash and Pasquale, 1995), or in anterior-posterior gradients (e.g. EphA3 and Ephrin-A5) (Cheng et al., 1995; Connor et al., 1998). Though expression data from various organisms is sufficient to support the role of Eph receptors in topographic mapping, knockout of these receptor have not provided much information. But misexpression of EphA3 in a subset of RGCs and disturbing the gradient of EphA in the retina resulted in projection errors of both EphA3 overexpressing axons as well as wild-type axons. This lead to the theory that it's not the absolute levels of EphA in the axon but relative levels that are responsible for their termination zone in the tectum (Goodhill and Richards, 1999; Brown et al., 2000). These studies imply a more complex mechanism of retinoptic mapping than

the proposed model of repulsive interaction and complementary gradients of Eph and Ephrin in the retina and the tectum respectively. Moreover graded expression of EphrinAs in retina and EphA in tectum were found (Marcus et al., 1996; Connor et al., 1998; Hornberger et al., 1999; Marin et al., 2001). This raises a possibility that interaction between EphA on retinal axons and EphrinAs in the tectal cells may be regulated by ligands and receptors expressed within the same or neighboring axon and tectal cells (Knoll and Drescher, 2002).

Positional information along the dorso-ventral axis, appears to be provided by transmembrane ligands and receptors such as EphrinB1 and EphB2. The first evidence for the involvement of these genes was their dorsoventral gradient of expression in the radial glial cells of the tectum (Braisted et al., 1997) and retinal ganglion cell layer (Holash and Pasquale, 1995). In the mouse, forward signaling of EphB receptors by EphrinB1 is necessary for correct D-V patterning as indicated by the errors in *ephB2* and *ephB3* mutants (Hindges et al., 2002). In these mutant mice ventral axons termination zone was shifted to the lateral regions of superior colliculus with lower levels of EphrinB1. This suggests that EphrinB1 attract ventral axons. In the *Xenopus* embryo, EphrinB2 is present in a high-dorsal to low-ventral gradient in the retina, while EphB1 is distributed in a high-ventral to low-dorsal gradient in the tectum (Mann et al., 2002). Blocking EphrinB/EphB interaction by exogenous EphB or dominant negative EphrinB2 eliminated retinal projections to the ventral tectum. *In vitro* assays showed that dorsal EphrinB expressing axons preferred to grow on clustered EphB stripes. Together, this suggests that RGC axons from the dorsal eye appear to be attracted to the ventral tectum because of reverse signaling via EphrinB2. Whereas ectopic expression of EphrinB1 in

the developing chick tectum however, suggests a bifuctional role of EphrinB1 as the repellent and attractant in DV retinoptic mapping (McLaughlin et al., 2003)

Thus Ephrins have been studied in various model organisms with an aim to understanding their role in the topographic mapping of RGC axons. But cues that help axons to find their synaptic partner on the tectum (i.e. tectal neurons) remained unclear. It is possible that Ephrins along with other molecules may be involved in this process. This question could be addressed by studying the effect of candidate molecule on axons *in vivo* and *in vitro*. Hence, a model system that allows manipulation in live organism and neuronal explants would be ideal.

2.1.5   The zebrafish visual system

Over the last decade, the zebrafish has become an established vertebrate model organism to study early embryonic development. There are several advantages of using zebrafish. First of all, the early embryonic development in the zebrafish embryo is similar to that of amphibians and mammals. The external development allows study of early events by various manipulations in a live embryo. The almost transparent body of the larvae facilitates light and fluorescence microscopic studies. The comparatively short life cycle, ease of maintenance and breeding under laboratory conditions, high efficiency of spawning (typically 100-200 eggs from a single pair) makes it a good system for genetic studies. Indeed zebrafish has been the first vertebrate organism to be used for a large scale genetic mutagenesis screen (Nusslein-Volhard, 1994). The large-scale screens carried out at Tübingen and Boston generated a large number of mutants defective in various developmental processes (Development −1996 volume 123). Although genetic knockout techniques have not been implemented in Zebrafish, antisense morpholinos

(oligonucleotides with a modified sugar backbone) are widely used to block translation and perturb gene function during early embryonic development (Nasevicius and Ekker, 2000).

The advantages of zebrafish as a model organism and the bank of mutants defective in various aspects of visual system development have been useful in studying eye development and axon guidance (Karlstrom et al., 1996). The anatomy of the zebrafish visual system has been well documented. Analogous to other vertebrate species, the zebrafish eye consist of a lens derived from the ectoderm and a retina from the neural plate. The eye is covered with a melanin rich retinal pigmented epithelium (RPE). The RGCs in the eye project their axons to the optic tectum in the midbrain. Apart from the major target, which is the tectal neuropill, some RGC axons terminate at various pretectal targets known as AF1 − AF9 (Burrill and Easter, 1994) (Figure 2.2 B), within the optic tectum (Figure 2.2 A). Using lipophilic dyes such as DiI and DiO, the path of RGC axons can be traced in live larvae with fluorescence microscopy (Figure 2.2 C and D). This approach has been used to identify mutants defective in the retinotectal projection. Many of these mutants have been characterized and the mutant genes identified. For example characterization and cloning of *astray* mutant provided the information that Robo-Slit signaling (necessary for the commissural axon crossing the midline) is required for RGC axon guidance (Fricke et al., 2001). Along with molecular characterization of these mutants, behavioral tests have also been developed to study the zebrafish visual system (Rick et al., 2000). Tissue ablation studies have provided further information about development and functioning of the zebrafish visual system (Roeser and Baier, 2003).

Although zebrafish has been established as useful system to study early embryonic development there are several technical limitations in studying late developmental events such as axon pathfinding in the visual system, bone and gonad development. The effect of morpholino based knock down could be observed only up to 3dpf. Genetic knockout and gene replacement methods have not been established in zebrafish. Similarly the effects of overexpression with RNA injection at single-cell stage is limited to early stages of development and use of tissue specific or inducible promoters require the establishment of transgenic lines.

2.2   Aim of the project

The project involved the development of an easier and reliable technique for gene misexpression that would help in the study of the role of EphrinB2 *in vivo*. EphrinB2 expression has been detected in the visual system in other organisms. The aim of this project was to study the expression pattern of EphrinB2 in the visual system of zebrafish and its effect on RGC axons *in vivo* and *in vitro*.
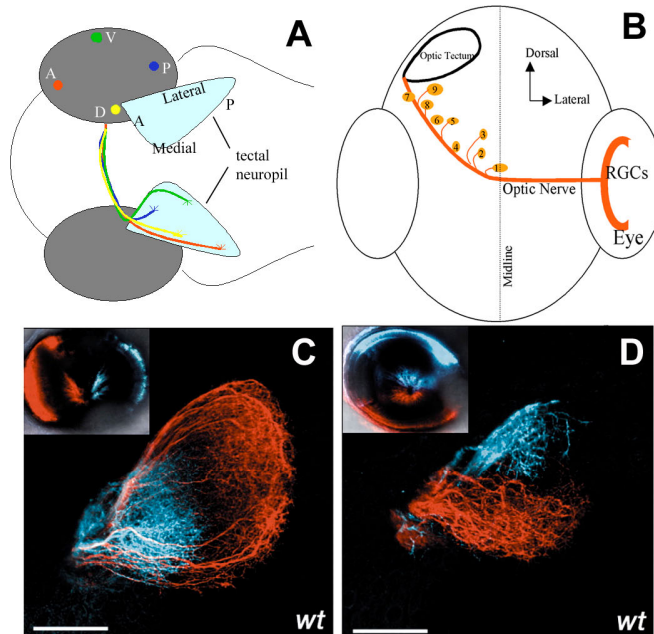
Figure 2.2

Figure 2.2: Zebrafish visual system.
(A) Schematic showing a dorsal view of zebrafish retinotectal projection
(B) Schematic showing the RGC axonal projection onto pretectal targets and tectum (Modified from: Gahtan, E 2004, J. Neurobiol 59:147-61)
(C & D) Optic tectum of 4-day-old zebrafish, with retinal axons labeled using DiI (red) or DiD (blue). Insets show a lateral view of the injected eye. (C) Axons from the anterior region of the eye(red) project to the posterior tectum. Axons from the posterior (blue) arborize in the anterior tectum. Similarly in (D) axons from the ventral retina (red) project to the medial portion of the tectum and axons from dorsal region (blue) arborize in the lateral regions.
(copied from: D'Souza. et al. (2005). Development 132(2): 247-56. with permission from The Company of Biologist Ltd)

2.3   *Methods*

2.3.1   Chemicals and general protocols

All chemicals were purchased from Sigma and BDH. Restriction endonucleases, and other DNA modifying enzymes were purchased from NEB, Promega and Roche. Ologonucleotide primers were purchased from GENSET. Fluorescent dyes were purchased from Molecular Probes. EphrinB2-Fc was purchased from R&D Systems.

Digestion of DNA with restriction endonuclease, ligation, agarose gel electrophoresis and other standard molecular biology experiments were carried out as described (Fred et al., 1987; Sambrook et al., 1989)

2.3.2   Zebrafish Adults and Embryos

Gnarled mutant embryos were obtained by crossing heterozygote carriers of gna^{tc36z}, which were identified by injection of DiI into the eyes of fixed 3 days-old fish. The *gnarled* mutant can be obtained as sperm samples from the Max Planck Institute for Developmental Biology in Tübingen, Germany. A live line no longer exists.

2.3.3   Constructs

A transfer plasmid (pGTV) containing GFP under the *Xenopus* EF1α promoter (Johnson and Krieg, 1994) was constructed by cloning a blunted DraIII/NotI fragment of pESG (Chien Chi-Bin, personal communication) into pAcSG2 (Pharmingen). To create a second expression cassette, the hsp70 promoter (Halloran et al., 2000) was cloned into the NotI/KpnI site of pGTV. The HSV-TK polyA signal was PCR amplified from pCEP4 (Invitrogen) and cloned into the ApaI/KpnI sites to generate the pGHT transfer plasmid. A blunted HindIII/StuI fragment of pCS2+nßgal (Fire et al., 1990) was cloned into the blunted ApaI site of pGHT. The transfer plasmid pGHT-Pp-nβGal was generated from

pGHT by removing polyhedrin promoter. A transfer plasmid pFB-HuC-GFP was constructed by cloning XhoI/NotI fragment of pBS-ΔEcoHuCP-EGFP (Park et al., 2000) into the SalI/NotI sites of pFastBac Dual (Life Technologies). A transfer plasmid pFBD-Unc76-GFP was constructed by cloning the XhoI /NotI fragment of XEX-76/eGFP (Dynes and Ngai, 1998) into the SalI/NotI site of pFastBac Dual. Full length EphrinB2 (PCR amplified from a c-DNA library clone) was cloned into pCS2+3'MT at the BamHI/ClaI sites. A SalI/SnaBI fragment of this was cloned into the XhoI (blunted)/NsiI sites of pFBD-Unc76-GFP to generate a transfer plasmid pFBD-U76G-CEB2m.

2.3.4   Virus production and injection

Viruses were prepared either by the conventional method i.e. by cotransfecting the viral DNA and targeting vector into SF9 cells with subsequent plaque purification and amplification or by the Bac to Bac method (Life Technologies). Viruses were amplified and purified as described by King and Possee (King and Possee, 1992). Briefly, medium from SF9 cells infected with virus was collected; centrifuged at 2500 rpm for 10 minutes at 4^0C to remove cell debris. The clear supernatant was filtered through a 0.2 μm filter. Viruses were concentrated by centrifugation at 24000 rpm at 4^0C for 1 hour using a 50.2Ti rotor. The glassy pellet was dissolved in a minimum volume of TE buffer. Viruses were further purified by layering on a 50%-10% sucrose step gradient and centrifuging at 24000 rpm at 4^0C for 1 hour in a SW41.2Ti rotor. Viruses forming a band at the interface of the 50% and 10% sucrose layers were collected and diluted in TE. These purified viruses were concentrated once again as above. Viruses were finally suspended in 20 μl of 0.1x TE.

For injection at the sphere stage, embryos with chorions were kept on a glass slide with minimum egg water (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl$_2$. 2H$_2$O, 0.33 mM MgSO$_4$.7H$_2$O) in a cluster of 10-15 embryos. For injections at the 6-somite and later stages, individual dechorionated embryos were embedded in 1.2% low melting temperature agarose (Bio-Rad). Concentrated viruses (1x10^{11} particles/ml) were injected in the intercellular spaces of zebrafish embryos using a gas pressure microinjector (PLI-100; Medical Systems Corp.). Virus particles were aspirated into the tip of injection needles, which were pulled from thin wall borosilicate capillaries (Clarks GC100TF).

2.3.5 X-gal staining

Embryos injected with viruses carrying the nβgal construct were stained as described previously (Muller et al., 1993). Embryos were washed in sodium phosphate buffer (100mM, pH 8.0), then fixed for 2 hours at 37^{0}C in fixing solution (100 mM phosphate buffer, pH 8.0; 5 mM EGTA; 2 mM MgCl$_2$; 1% glutaraldehyde). Fixed embryos were then rinsed two times in washing solution (100mM phosphate buffer, pH 8.0; 2 mM MgCl$_2$; 0.03% IGEPAL CA-630; 0.01% Na-deoxycholate). Fixed embryos were incubated in staining mix containing 1 mg/ml X-gal, 25 mM K$_4$Fe(CN)$_6$, 25 mM K$_3$Fe(CN)$_6$ dissolved in washing solution for 2 hours at 37^{0}C. Stained embryos were washed with washing solution.

2.3.6 DiI labeling

RGC axons in live embryos were labeled using DiI (Molecular Probes) dissolved in ethanol (2 mg/ml), which was injected into the eye using a gas pressure microinjector. For transneuronal labeling of tectal cells, embryos were fixed in 4% para-formaldehyde-PBS and DiI dissolved in chloroform (2 mg/ml) was injected into the eye. Embryos were

incubated at 28^0C for at least 36 hours. To photo-convert DiI, labeled embryos were incubated in DAB solution (0.7 mg/ml di-amino benzidine, 5 mg/ml heparin, 0.03% NiCl$_2$) for 15 min, and then exposed to green light using a conventional fluorescent microscope (Liu et al., 1999).

2.3.7 In-situ hybridization

Antisense RNA probes were prepared by in vitro transcription using T3, T7 or Sp6 RNA polymerase and Digoxigenin-UTP RNA labeling mix (Roche). The hybridization and staining was carried out using standard protocols (Westerfield, 1995).

2.3.8 Microscopy

For imaging, live embryos were embedded in 1.2% low melting temperature agarose. Embryos expressing GFP were imaged on a Zeiss Axioskop using a Bio-Rad MRC1024 laser scanning confocal microscope. For brightfield imaging, embryos were mounted in 70% glycerol in PBS and imaged with a compound microscope using a Princeton Instruments MicroMax camera.

2.3.9 Stripe assay

Stripes of zebrafish EphrinB2a (R&D Systems) were made with a silicon stamp on glass-bottom dishes (MatTek), which had been coated with poly-L-lysine. To cluster the proteins, 5 µg/ml of Ephrin-fc was incubated with 50 µg/ml Alexa 488-coupled goat anti-human antibody (Molecular Probes) for 30 minutes at room temperature. The clustered proteins were then placed on the silicon stamp for 10 minutes. The mixture was removed with a pipet and the stamp left to dry for a few minutes. The dish was then lowered onto an inverted stamp, enabling the transfer to protein to be seen. The dishes were subsequently coated with 50 µg/ml laminin for 1 hour. To prepare retinal axon

cultures, eyes of 2 day-old embryos were dissected using tungsten needles. These were then cleaned individually by passages through a few drops of sterile medium, broken into clumps and transferred to the culture dish containing L15/1%BSA/1%N1 (Sigma) buffered at pH 7.4 with Hepes and freshly supplemented with L-glutamine (2 mM).

2.3.10   Ligand binding assay

RGC cultures were washed with Hank's Buffered Salt Solution (HBHA; Hanks saline with 0.5 mg/ml BSA, 0.1% NaN_3, 20mM HEPES pH 7.3) and then incubated with 3 nM EphrinB2-Fc or HBHA buffer for 2 hours at room temperature. Cultures were washed in HBHA and fixed with 60% acetone, 3.7% formaldehyde, 20 mM HEPES pH 7.3 at room temperature for exactly one minute. After washing with 20 mM HEPES, 150 mM NaCl, protein bound to axons was detected using the anti-Fc antibody (Alexa 488 conjugated at 1:300 dilution). Images were captured with Leica DMIRBE microscope with a 100X oil immersion objective and Hamamatsu Orca II camera.

2.4   Results

2.4.1   Baculovirus can drive gene expression in zebrafish

Several methods have been established in other organisms for ectopic gene expression. In the chick, for example, viruses have been widely used to study cell lineage, brain patterning, axon guidance and vasculogenesis (Flamme et al., 1995; Friedman and O'Leary, 1996). A non-replicating virus that can infect zebrafish embryos, which is simple to use and can carry a large load, would be useful for experiments requiring localised gene misexpression. Thus the ability of *Autographa californica multiple nuclear polyhedrosis virus* (AcMNPV) to express genes in zebrafish was tested. This virus is known to infect a wide range of cells, including those from human, mouse,

Xenopus, and Drosophila, but can replicate only in insect cells (Sandig et al., 1996; Barsoum et al., 1997; Oppenheimer et al., 1999). It can also infect terminally differentiated cells such as neurons (Sarkis et al., 2000), and can carry inserts of 15 kb or even larger (Fraser, 1986; Boyce and Bucher, 1996).

To determine if baculovirus can infect zebrafish cells, viruses carrying Hsp70-LacZ or EF1α-GFP were made, concentrated to a high titre and injected into zebrafish embryos at different stages. When virus were injected into blastula-stage embryos, widespread expression was seen (Figure. 2.3 A, B), possibly due to the scattering of clonally related cells (Kimmel and Warga, 1987). When injections were carried out at later stages, e.g. at the 18-somite stage, expression was restricted to the site of injection (Figure. 2.3 C). GFP was detected within 6 hours of virus injection, and persisted for at least 5 days. Expression of the reporter gene was dependent on the promoter used. When virus containing EF1α-GFP was injected in the eye, retinal ganglion cells expressed GFP in only 3 out of 24 embryos, whereas when the zebrafish neural-specific HuC promoter (Park et al., 2000) was used, GFP expression was seen in retinal ganglion cells of all injected embryos (Figure. 2.3 D; n=20). Baculovirus were able to infect zebrafish at late stages of development. When virus carrying the HuC-GFP cassette were injected into the brain of fish at 19 dpf, GFP was detected in neurons; fluorescence persisted for at least 2 weeks after injection (Figure. 2.3 E).

Baculovirus carrying two expression cassettes were then made, to test whether two genes could be expressed independently. The zebrafish hsp70 promoter was used to regulate LacZ, while EF1α promoter was used to drive GFP. Twelve hours after virus injection into the eye primordia at the 18-somite stage, expression of GFP was recorded

by confocal microscopy. Embryos were then heat shocked and processed for LacZ activity six hours later. Strong X-gal staining was seen only in heat-shocked embryos (n=16) while untreated siblings (n=16) expressed only GFP (Figure. 2.4). Low level of X-gal staining in untreated siblings may be caused by leaky expression of β-gal through the Hsp70 promoter.

2.4.2 Baculovirus-mediated *EphrinB2a* misexpression affects segmentation

To test whether baculovirus-mediated misexpression can be used to manipulate a developmental process, virus carrying myc-tagged *ephrinB2a* under the CMV (cytomegalovirus) promoter, as well as the axonal tracer unc76-GFP driven by the EF1α promoter, were made. Virus were injected into the presomitic mesoderm, as ectopic *ephrinB2a* expression has been shown to affect segmentation (Durbin et al., 1998). Control virus, expressing only the fluorescent reporter (unc76-GFP), did not affect segmentation (Figure. 2.5 A,B), whereas the *ephrinB2a* virus caused defects in somite boundary formation (Figure. 2.5 C,D).
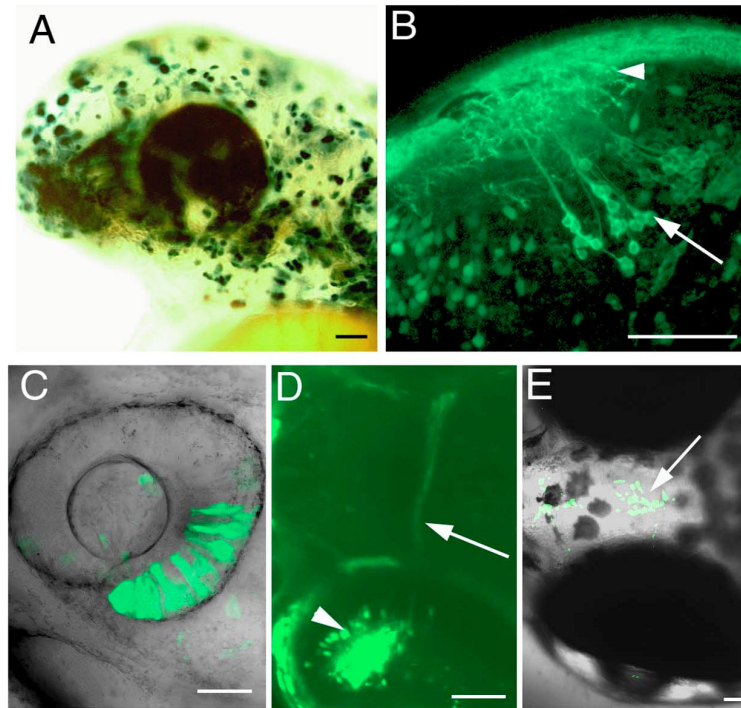
Figure 2.3

Figure. 2.3. Baculovirus-mediated gene expression in zebrafish. A: Lateral view of a 30-hour embryo with scattered expression of LacZ. The embryo had been injected at the sphere stage with virus carrying LacZ under the hsp70 promoter, then heat-shocked by a one-hour incubation at 37ºC, at 24 hours post-fertilization. B: Dorsal view of the midbrain of a 3 days-old fish, after injection in the animal pole at 30% epiboly with virus carrying unc76-GFP under the EF1α promoter. The arrow indicates a tectal neuron, while the arrowhead indicates retinal ganglion cell axons. C: Lateral view of the eye of an embryo 24 hours after virus carrying GFP under the EF1α promoter had been injected into the posterior region of eye primordia. D: Dorsal view of the head of a 3 days-old fish which had been injected at the 18-somite stage, in the anterior eye, with virus carrying eGFP under the HuC promoter. GFP expression is visible in RGCs (arrowhead) and axons (arrow). E: Dorsal view of the head of a 21 days-old fish which had been injected at 19 dpf, in the brain, with virus carrying eGFP under the HuC promoter. Fluorescent neurons are visible (arrow). Bar = 50 µm
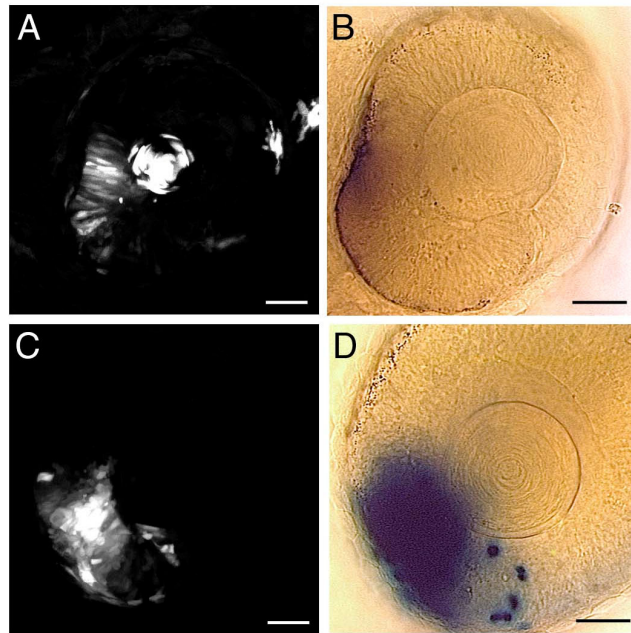
Figure 2.4

Figure. 2.4: Independent expression of two reporter genes. Embryos were injected in the eye primordia at the 18-somite stage with virus carrying two expression cassettes: EF1α GFP and hsp70- nβGAL. Panels A and C show the localized GFP expression 12 hours after injection, prior to X-gal staining. The embryo in panel C was heat-shocked and LacZ expression was detected 6 hours later (D). No LacZ expression was detected in the control embryo (B). Bar = 50 µm.
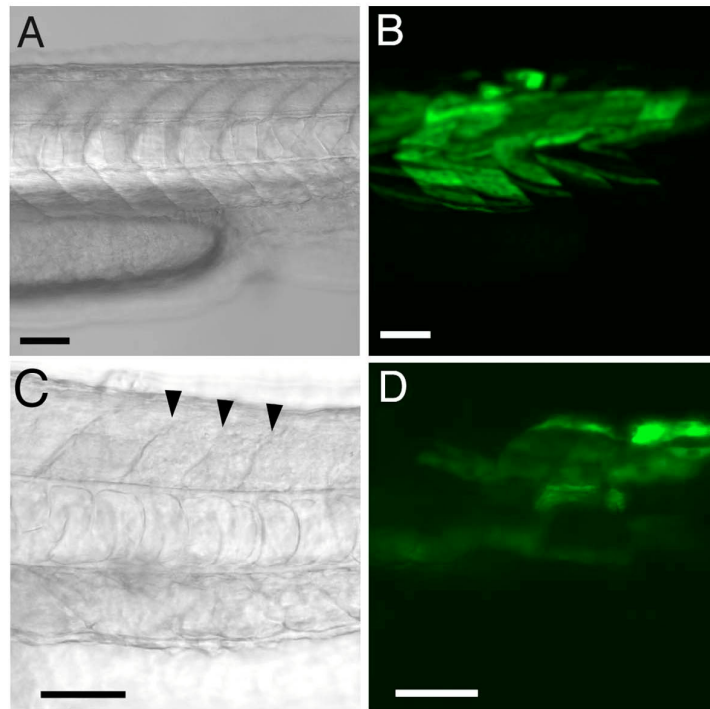
Figure 2.5

Figure. 2.5 The effect of baculovirus-mediated misexpression of EphrinB2a.
A-D: Lateral view of the trunk region of embryos 24 hours after the injection of baculovirus into the presomitic mesoderm (PSM) at the 6-somites stage. When virus carrying only EF1α̃ unc76-GFP was injected in the PSM, GFP was seen in somites (B) but no defects were detectable (A). When virus carrying CMV-ephrinB2a and EF1α̃ unc76-GFP was injected into the PSM, defects in somite boundary formation were seen (C, arrowheads) where cells expressed GFP (D). Bar = 50μm.

2.4.3   *EphrinB2a* expression in the optic tectum

The zebrafish has two orthologs of *ephrinB2*, *ephrinB2a* and *ephrinB2b* (Chan et al., 2001). In 3-days old zebrafish larvae, *ephrinB2a* is expressed by a subset of cells in the optic tectum (Figure. 2.6 A). Although there is no obvious gradation in the level of expression in the anterior-posterior or dorso-ventral axes, there appear to be many more *ephrinB2a* positive cells in the posterior tectum (see Figure. 2.9 A). These *ephrinB2a*-expressing cells may be tectal neurons, as their expression correlates with that of *deltaB*, which is expressed in post-mitotic neurons (Haddon et al., 1998) (Figure. 2.6 B). In particular, cells at the posterior boundary of the tectum appear to express both *ephrinB2a* and *deltaB*; cells closer to the neuropil only appear to express *deltaB*. Some of the *ephrinB2a*-expressing cells are directly contacted by RGC axons, as indicated by transneural labeling. DiI was used to label RGCs in fixed embryos, and these were incubated overnight at 28^0C to allow the dye to transfer to cells in physical contact (Bruce et al., 1997). Label was seen in scattered cells in the tectum, including those in the posterior tectum (Figure. 2.6 C). A combination of transneural DiI labeling and in-situ hybridization suggests that at least some of the *ephrinB2a* expressing cells are directly contacted by incoming RGC axons (Figure. 2.6 D). These cell bodies have the rounded morphology of tectal neurons, and not the elongated morphology of radial glial cells.
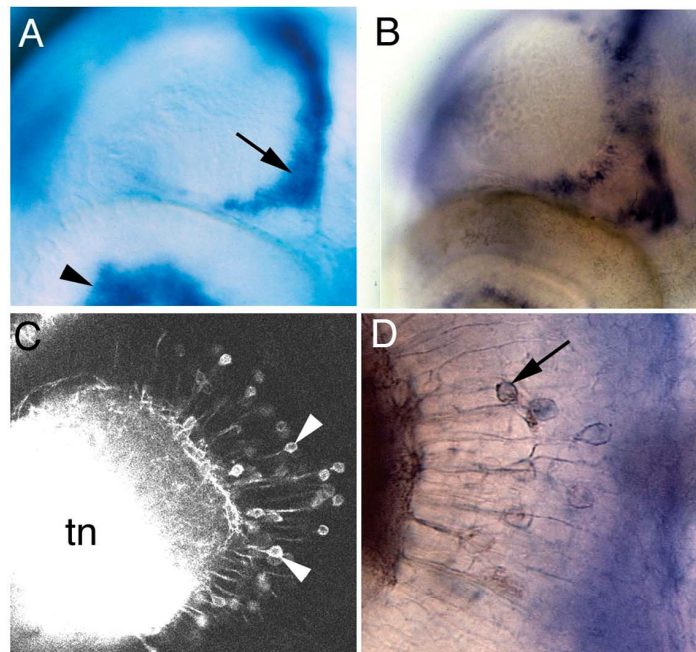
Figure 2.6

Figure 2.6. *EphrinB2a* expression in the zebrafish midbrain at 3 days
        post-fertilization (dpf), when the first synapses are being formed.

        (A) *EphrinB2a* is strongly expressed in a population of cells in the posterior
tectum (arrow). The ciliary margin of the eye is also labeled (arrowhead).

        (B) *DeltaB* expression in the optic tectum.

        (C) Transneural labeling with DiI. Arrowheads indicate tectal neurons,
which have been labeled by dye transfer from RGC axons which fill the
tectal neuropil (tn).

        (D) Combination of transneuronal DiI labeling and in-situ hybridization
with the ephrinB2a probe. The plasma membrane of some tectal cells has
been labeled by DiI transferred from incoming RGC axons. DiI was
photoconverted with DAB, and appears brownish black in this image.
Some ephrinB2a-expressing cells are labeled with DiI (arrow). Embryos
are shown in dorsolateral view, with anterior to the left.

2.4.4 Retinal ganglion cell axon behaviour in a mutant with ectopic tectal neurons

In 3-day old wild-type fish, the tectum is filled with RGC axons (Figure. 2.7 A), whereas in *gnarled* mutants the 3-day tectum is devoid of axons (Figure. 2.7 B). At 5-days post fertilization, RGC axons are found in the tectum of mutants, but they branch aberrantly (Trowe et al., 1996). Whole eye transplantation indicated that the defect lies in the brain of *gnarled* mutants, and not in the eye (Wagle et al., 2004). The brain of *gnarled* mutant fish is morphologically abnormal, as seen in optical sections of Syto-11 labeled fish (Figure. 2.8 A,B), and in transverse sections (Figure. 2.8 C,D). Specifically, there is a change in the shape of the neuropil and an increase in the number of cell bodies in the anterior tectum, in the region where RGC axons normally enter the tectal neuropil. It appears that ectopic tectal neurons have an inhibitory effect on RGCs – either delaying RGC axon entry into the tectum or causing them to misroute.

The increase in the number of cell bodies in the anterior tectum of *gnarled* mutant are likely to be caused by ectopic tectal neurons, as can be seen by the expression pattern of proneural genes such as *zash1b* and, and neural genes such as *deltaB* (Figure. 2.9 E,F). These ectopic tectal neurons in mutants express *ephrinB2a* (Figure. 2.9 D). Molecules thought to provide positional information in the optic tectum, such as *ephrinA2*, retain the anterior-low to posterior-high gradient seen in wildtype (Ober, 1996).
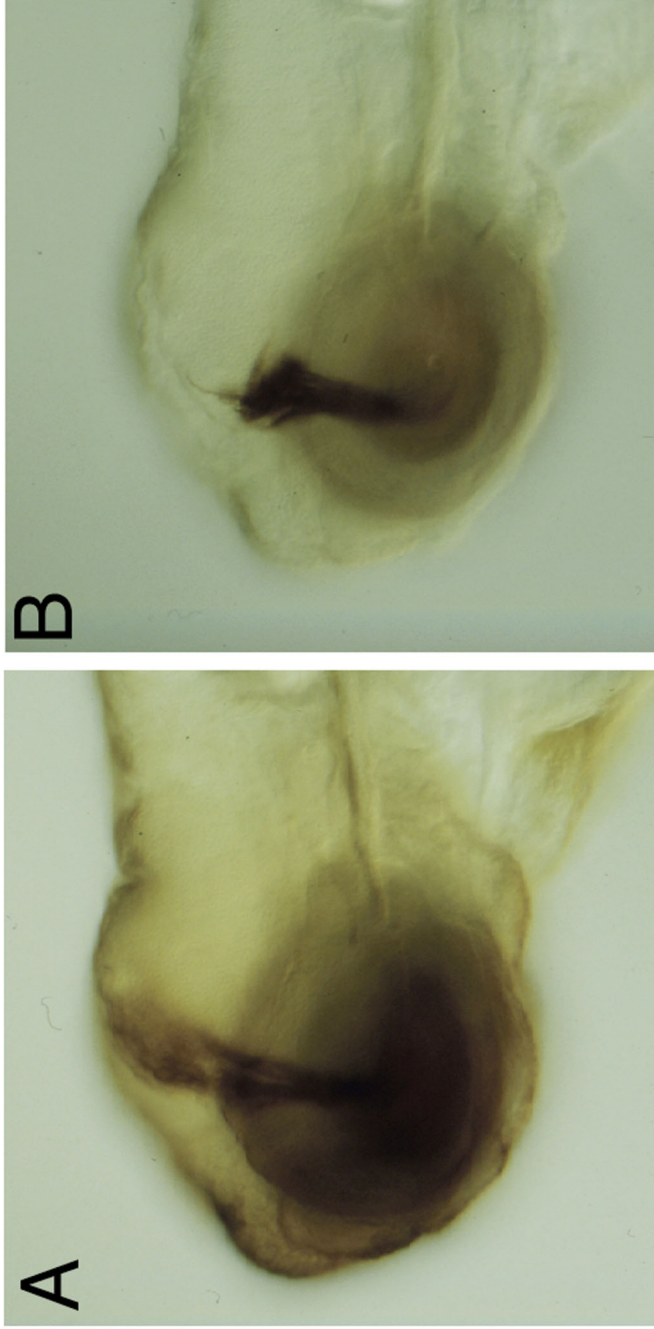
Figure 2.7

Figure. 2.7. Retinotectal projection defects in the gnarled mutant, as shown by labeling all RGCs in one eye with DiI. (A) Dorso-lateral view of a 3 dpf wild-type embryo. Retinal ganglion cell axon terminals fill the tectum. (B) A mutant sibling, with the tectum devoid of axons. The DiI-labeled RGC axons in the embryos in panels A and B have been photo-converted with DAB

(copied from: Wagle, et al. (2004). J Neurobiol 59(1): 57-65.)
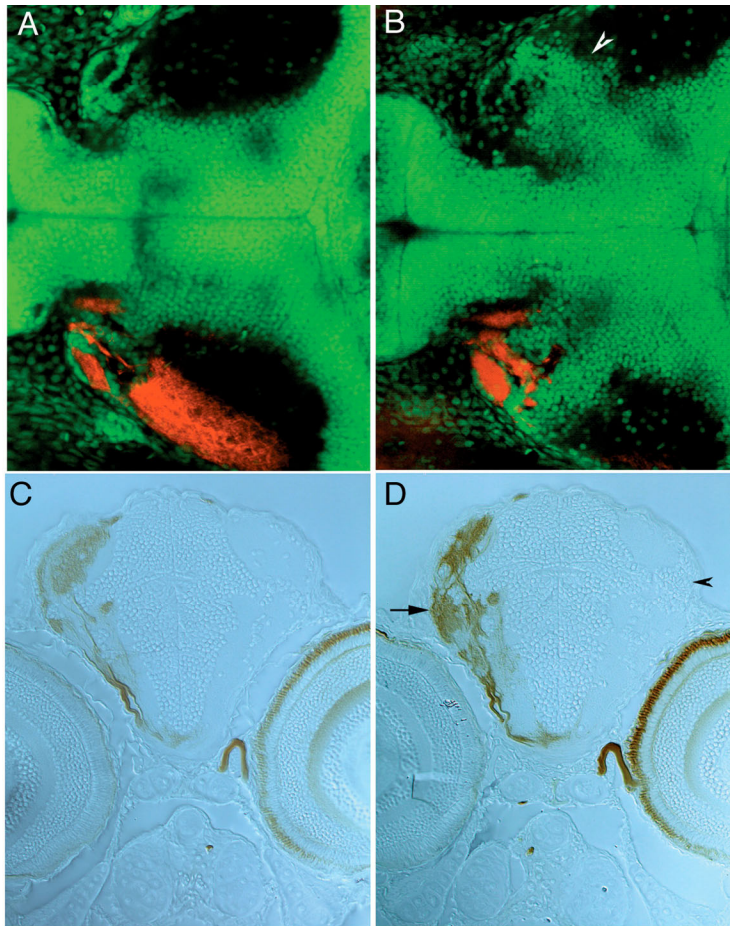
Figure 2.8

Figure. 2.8. Midbrain morphology in the *gnarled* mutant.
Horizontal sections, obtained by confocal microscopy of
wild-type (A) and mutant (B) embryos at 5 dpf. Nuclei are labeled
with Syto-11, while RGC axons from the right eye are labeled with
DiI. Note the increased number of cells in the anterior tectum
(arrowhead) in the mutant embryo. Transverse sections of
wild-type (C) and mutant (D) embryos at 5 dpf. All RGCs in the left
eye have been labeled with DiI, which was then photo-converted
with DAB. The tectal neuropil of the mutant embryo (D) is smaller
than that of the wild-type sibling (C). There is an increase in the
number of cells at the ventro-lateral margin of the tectum in mutants
(arrowhead), in the region where RGC axons enter the neuropil.
There are also more RGC axons in the pretectal area (arrow) of the
mutant.

(copied from Wagle. et al. (2004). J Neurobiol 59(1): 57-65.)
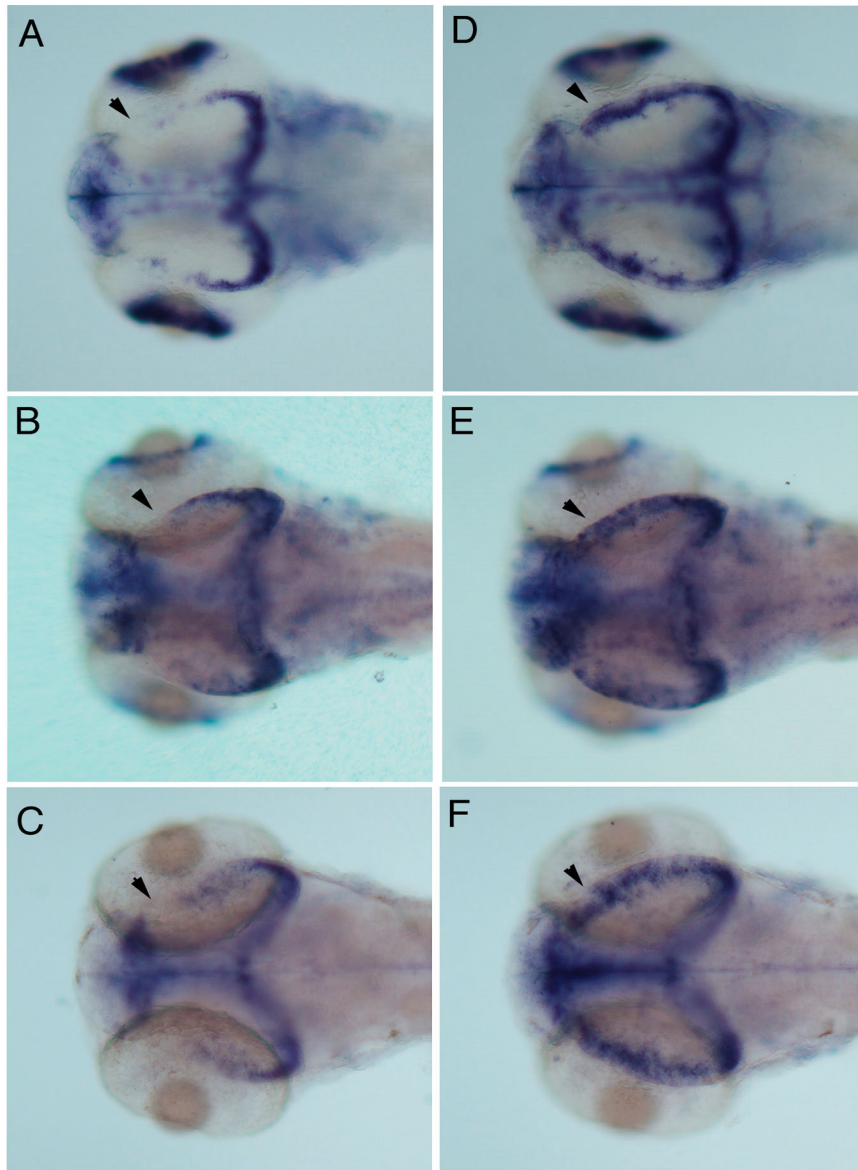
Figure 2.9

Figure. 2.9. Neurogenesis and gene expression in the midbrain of wild-type and *gnarled* mutant embryos. Expression of *ephrinB2a* (A and D), *deltaB* (B and E) and *zash1b* (C and F) at 3 dpf, in the midbrain of wild-type (panels A-C) and *gnarled* mutant (panels D-F) embryos. The expression domain of all three genes is expanded in mutant embryos. The anterior tectum (arrowhead) is devoid of expression in wild-type embryos, but expresses all three genes in mutant embryos. Embryos are shown in dorsal view, with anterior to the left.

(copied from: Wagle. et al. (2004). J Neurobiol 59(1): 57-65.)

2.4.5 Baculovirus-mediated *ephrinB2a* misexpression affects RGC axon migration

To test whether ectopic *ephrinB2a* alone can affect RGC axon entry into the tectum, baculovirus was used for misexpression in the anterior tectum. When virus carrying myc-tagged *ephrinB2a* under the CMV (cytomegalovirus) promoter, as well as the axonal tracer unc76-GFP driven by the EF1α promoter, were injected into the tectum at 40 to 48 hours post fertilization, defects in RGC axon migration occurred, as indicated by DiI labeling at 4 days post-fertilization. At this stage, axons from the anterior eye would normally have reached the posterior tectum, and this is what was seen in all embryos injected with virus carrying the reporter gene only (Figure. 2.10 A). In embryos injected with the *ephrinB2a*-carrying virus, axons from the anterior eye failed to reach the posterior tectum in 12 out of 21 embryos when reporter expression was seen in the anterior tectum (Figure. 2.10 B). Embryos with reporter expression in other parts of the brain did not have any defect in the migration of RGC axons. Hence, *ephrinB2a* is able to inhibit RGC axon entry into the tectum.
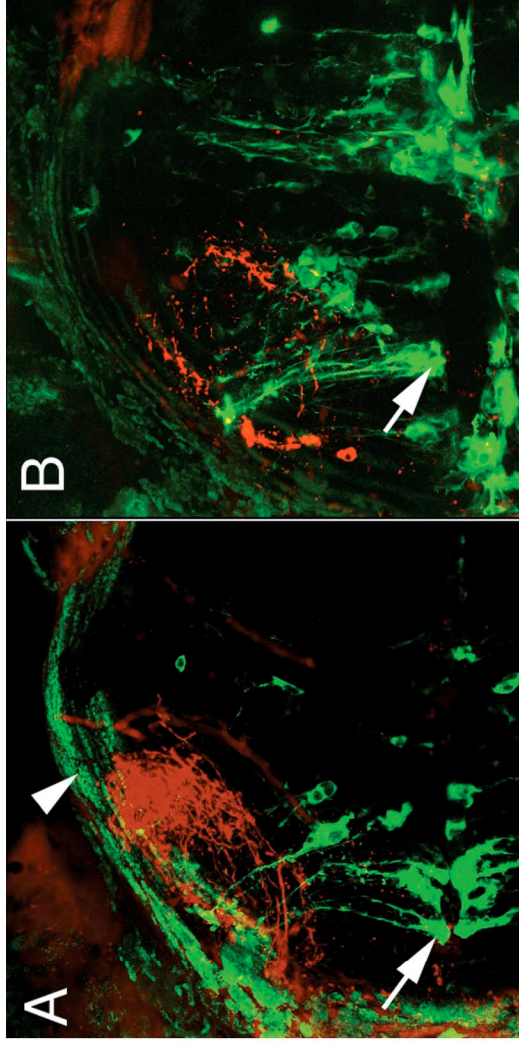
Figure 2.10

Figure. 2.10. :The effect of baculovirus-mediated misexpression of ephrinB2a. Dorsal view of the midbrain of embryos at 4 dpf, after injection of virus carrying either EF1α unc76-GFP (A) or CMV-ephrinB2a and EF1α unc76GFP (B) into the tectum at 42 to 48 hours post-fertilization. DiI was injected into the anterior contralateral eye 2 hours prior to imaging. Each image here is a projection of a series of optical sections, with GFP-expressing cells in green and DiI labeled axons in red. The green fluorescence at the outer periphery of the tectum (arrowhead) is due to the autofluorescence of xanthophores in the skin. In the control virus-injected embryo (A), RGC axons reach the posterior tectum, whereas in the ephrinB2a virus-injected embryo (B), RGC axons failed to reach to the posterior tectum. The arrows indicate glial cells expressing GFP.

53

2.4.6　　Effect of EphrinB2a on RGC axons *in vitro*

The ability of EphrinB2 to bind zebrafish RGC axons was tested *in vitro*, by incubating cultured neurons with purified EphrinB2-fc, and then lightly fixing the culture and labeling with a fluorescently labeled anti-Fc antibody. Punctate fluorescence was seen on axons (Figure. 2.11 B), indicating that the receptors for EphrinB2 are present on the growth cone. In cultures incubated with the buffer lacking EphrinB2-fc or heat inactivated EphrinB2-fc, no label was detected. To further examine the behavior of axons in response to EphrinB2a the stripe assay was used: stripes were printed on a glass-cover slip using a silicon stamp, and isolated retinal explants were placed over the stripes. Some degree of repulsion was seen in this assay (Figure. 2.11 C). Approximately 40% (n=200) of the axons from the explants grew exclusively in lanes between the EphrinB2a stripes, while the remaining 60% crossed at least one stripe. No avoidance was seen in the absence of EphrinB2a in the stripes.
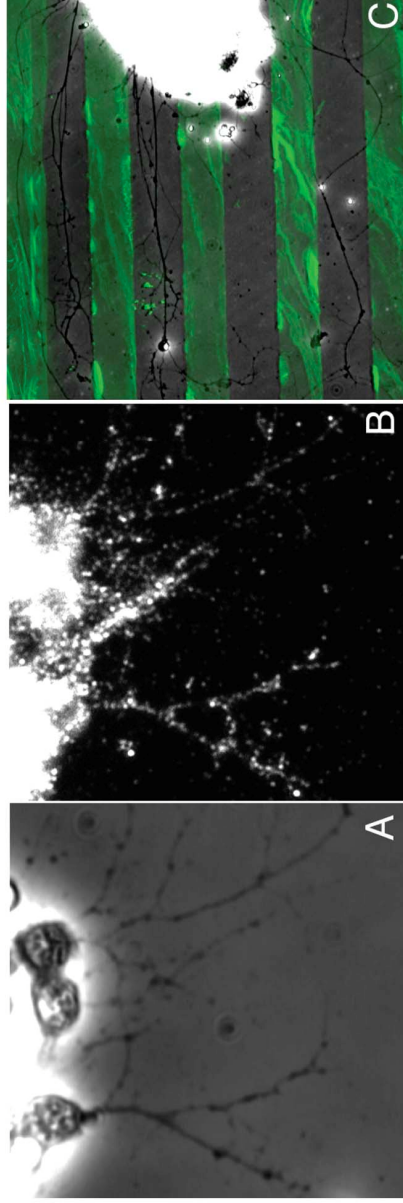
Figure 2.11

Figure. 2.11 : *In vitro* assays with zebrafish RGC axons. (A, B) Receptor-binding assay. Cultures were incubated with EphrinB2a-Fc and stained with anti-Fc antibodies (Alexa 488 conjugated). Specific staining was seen in cultures incubated with EphrinB2a-Fc (B). (C) The stripe assay with zebrafish retinal ganglion cell axons. Axons from the eye grow preferentially in between EphrinB2a stripes, which fluoresce in green because of the Alexa-488 antibody used to cluster the protein.

2.5 Discussion

To study the function of ephrinB2 *in vivo, a* baculovirus-based gene expression method in zebrafish was developed. The location of expression can be controlled by injection, and the timing can be controlled by using promoters such as the hsp70 promoter, as shown here, or a hormone inducible promoter. The most widely used method of misexpression at present, which is mRNA injection at the 1-cell stage, leads to ubiquitous expression of the gene of interest, whereas DNA injection leads to mosaic expression. Even with tissue-specific promoters, DNA injection does not allow restriction of expression to a cluster of cells, for example in restricted regions of the eye. Lipofection is less efficient as very few cells show expression. Baculovirus is thus useful when localized misexpression is required late in embryonic or larval development.

Baculoviruses are known to be taken up by cells via endocytosis (Hofmann et al., 1995), and they should thus be able to infect any cell type in the embryo, larvae or adult. Observations with injections of virus carrying GFP into the eye suggest that a critical parameter for obtaining expression is the choice of promoter. Very few RGCs expressed GFP when the EF1α promoter was used, while GFP expression was seen in all cases where the HuC promoter was used. More reproducible expression can thus probably be obtained using zebrafish promoters, rather than heterologous ones. The baculovirus has several advantages, in comparison to retroviruses (Burgess and Hopkins, 2000): they are easy to prepare, can carry large inserts and most importantly, can infect post-mitotic cells. Hence, baculoviruses can be used to manipulate differentiated neurons in the zebrafish brain. Even though the baculovirus genome remains episomal, and expression is transient, it should be sufficient for testing gene function.

At the time when this study was initiated, at least two other systems for ectopic expression of a gene of interest in the zebrafish were available. One utilises the Gal4-UAS system first developed in Drosophila (Brand and Perrimon, 1993) to drive gene expression reproducibly in selected cell types (Scheer and Camnos-Ortega, 1999). The other uses the hsp70 promoter, which can be activated locally by laser induction (Halloran et al., 2000). Both systems are effective, but require the establishment of stable transgenic lines. Recently expression of GFP reporter by localized delivery of DNA using electroporation in zebrafish has been demonstrated (Lyons et al., 2003; Teh et al., 2003). With the baculovirus, misexpression can be done without establishing transgenic fish, thus saving several months. Also, injection of the virus does not appear to have any deleterious effects on cells, so the method is only minimally invasive. For experiments requiring misexpression late in development, for example in studies of bone formation or gonad differentiation, baculovirus-mediated misexpression may be a useful method for testing gene function. It can be used to ectopically express genes, or to deliver antisense RNA or even double-stranded RNA, with the promoter determining what cell type is targeted.

Topography specific arborization, as mediated by EphrinA2 and EphrinA5 in the anterior-posterior axis or EphB1 in the dorso-ventral axis, is an important step in the establishment of the retinotectal projection. An additional part of this process is the recognition of synaptic partners. In the chick embryo, retinal ganglion cell axons form connections in three different layers within the tectum. Glycoproteins and cadherins have been implicated in the recognition of specific laminae, on the basis of their expression and the effect of an N-cadherin antibody. EphrinA5 may be present on some tectal

neurons in the chick (Rosentreter et al., 1998), and could thus be involved in mediating recognition. Chromophore-assisted laser ablation of ephrinA5 does not prevent arborization, however, but causes posterior shift (Sakurai et al., 2002). EphrinB2 has also been found in a retinorecepient layer (Braisted et al., 1997), but there has been no experimental test of its function in this system reported.

In the zebrafish larval tectum, two lines of evidence suggest that EphrinB2a could function as a target recognition signal. Firstly, *ephrinB2a* is expressed by tectal neurons that are contacted by RGC axons, as indicated by the transfer of DiI from RGC axons to these cells. Secondly, ectopic *ephrinB2a* is able to affect RGC axon migration.

Based on observations in other species, such as in the crossing of the midline by RGC axons as well as neural crest migration in *Xenopus* (Smith et al., 1997), it is not surprising that EphrinB2a on its own acts as a repellent on some zebrafish ganglion cells *in vitro*. Nevertheless, it is intriguing that ectopic expression of *ephrinB2a* in the tectum, by baculovirus or in the context of the *gnarled* mutant, causes a stalling of RGC axons. Could EphrinB2a be a part of a group of signals that causes axons to stop at their target in the tectum? In principle, if Ephrins are cleaved from the membrane by proteases upon encountering a receptor on another cell, repulsion will occur. If, however, both receptor and ligand remain attached to their respective cells, it is possible that axons will stop (Holash et al., 1997). It is unlikely that EphrinB2 on its own can elicit all responses required for synapse formation as the stopping of axons was not reproduced *in vitro*. It may be that EphrinB2a acts in concert with other molecules, such as EphrinA2 and EphrinA5, as well as BDNF, to induce branch and synapse formation in the appropriate position, and to prevent further axonal outgrowth. The culture technique described here

should be useful for further studies addressing this question. For example, it will be interesting to see what combination of proteins can trigger an accumulation of pre-synaptic specialization on EphrinB2a stripes. Indeed recent research from several groups indicate that Eph-Ephrin play critical role in modulating multiple aspects of synaptic structure and physiology (Murai and Pasquale, 2004)

It would be informative to know the effect of eliminating *ephrinB2a* in tectal neurons. However, there is currently no simple way of doing this selectively. *EphrinB2a* has early functions in development (Durbin et al., 1998), and thus its function in RGC axon guidance cannot be studied using morpholinos knocked-down. Injection of soluble EphrinB2a will non-specifically inhibit signaling of ephrin-As, since EphrinB2 binds to EphA4, and would also interfere with reverse signaling that might be required for dorsoventral mapping. Overexpression of antisense RNA using baculovirus may be allow eliminating *ephrinB2a* in tectal neurons. Nevertheless this study shows that EphrinB2a fulfills the criteria for part of a stop signal in the tectum and implicates its involvement in target recognition by RGC axons.

Chapter III

Studying the Role of Rag1 (recombination activating gene-1) in Neurons

3.1 Introduction:

3.1.1 Similarities between the vertebrate adaptive immune system and the CNS: Molecular link

The nervous system and immune system both allow an organism to perceive its environment and to deal with it to survival. In vertebrates these systems are more complex with a highly evolved brain and adaptive immune system. Although these systems are derived from different lineages during embryonic development, similarities between the two have been investigated with great interest. Both these systems carry a common property: "memory". In case of the nervous system, memory involves the recording of a particular event and recalling it over time, whereas for the immune system it entails a quicker and robust response to the antigen that was experienced previously. It is tempting to speculate that these functions might share a similar underlying mechanism but there has been no experimental evidence supporting this idea.

Another feature that is common to these two systems is diversity. The immune system generates a huge repertoire of immunoglobulins and T-cell receptors that allows antigen recognition. Similarly, neurons have distinct anatomical connectivities, physiological properties, neurotransmitter identities and differences in surface molecules such as protocadherins (Wu and Maniatis, 1999). Considering this, CNS complexity seems to be comparable to the immune system. In 1967, even before the mechanism of adaptive immune system development was elucidated, Dreyer proposed a

hypothesis with reference to goldfish retinotectal projection suggesting that the mechanism of "genome reprogramming" could be involved in generating diversity in the adaptive immune system to recognize antigens and in neurons to recognize their synaptic partner (Dreyer et al., 1967). Genomic rearrangement at the immunoglobulin and T-cell receptor loci has been demonstrated in the immune system. Similar rearrangement of a reporter construct has been shown in the CNS, but these studies remain controversial and rearrangement of endogenous gene loci has not been reported in neurons (Matsuoka et al., 1991; Abeliovich et al., 1992).

For a functional network to develop, axons must be guided to their target zone and once there, they need to make precise synaptic connections. The first criterion is met by various axon guidance cues (discussed in Chapter 1). The neuronal-activity based model supports the second criteria of precise synaptic connection (Shatz, 1990; Penn and Shatz, 1999). But initial connections during embryonic development are mostly activity independent. One possibility is the involvement of cell surface molecule in cell to cell recognition. Indeed in *Drosophila,* a large number of cell surface molecules DSCAM synthesized from a single locus by an alternate splice mechanism have been shown to be involved in synaptogenesis (Schmucker et al., 2000; Celotto and Graveley, 2001). In vertebrates this function could partly be attributed to a large family of cell adhesion molecules: protocadherins (Wu and Maniatis, 1999). The recognition of synaptic partners by means of cell surface molecules parallels antigen recognition by immunoglobulins and T-cell receptors.

Similarities between the immune system and the nervous system have been found at the molecular level (Chun, 2001). Specific molecules such as Thy-1 (Stohl and Gonatas,

1977; Lancki et al., 1995), cadherins (Takeichi, 1991; Muller et al., 1997), major histocompatibility complex (MHC) (Germain, 1994; Corriveau et al., 1998) and immunoglobulins (Weiner and Chun, 1997) are present in both the immune and the nervous system. Genes encoding protocadherins and odorant receptors (OR) are arranged in clusters at genomic loci and ORs are expressed in a monoallelic fashion similar to immunoglobulins (Buck and Axel, 1991; Wu and Maniatis, 1999). Besides this, components essential for immune system development were found to be expressed in the nervous system. Moreover, deletion of some of these such as XRCC4 and Lig4 resulted in increased neuronal apoptosis (Sekiguchi et al., 1999). These studies once again raised the hypothesis of genome rearrangement in neurons analogous to V(D)J recombination in the immune system.

3.1.2 Development of the adaptive immune system

The major components of the adaptive immune system are T-cells and B-cells. Both develop from a common precursor known as pluripotent hematopoetic stem cells, which give rise to all blood cells. In the developing fetus, these cells are found in the liver but in the adult they are present in the bone marrow. The adult immune system consists of bone marrow and thymus as the primary lymphoid organs, where the initial development of T-cells and B-cells take place. It also involves secondary lymphoid organs such as the spleen, lymph nodes and appendix where these cells interact with antigens and later steps of development and activation can take place.

3.1.2.1   B-cell and T-cell development : Immunoglobulin and T-cell receptor structure

B-cells complete their development in the bone marrow (hence the name B-cells), whereas immature T-cells migrate from the bone marrow and mature in the thymus

(hence the name T-cells). Various cell surface molecules known as "cluster designation" (CD) have been characterized which serves as markers for developmental stages of both B-cells and T-cells. B-cells are destined to produce antigen receptor molecules known as "immunoglobulins" (Ig) whereas T-cell produce T-cell receptors (TCR) which interact with antigens when presented along with major histocompatibility complex (MHC) molecules by antigen presenting cells (APC).

Monomeric immunoglobulin is a "Y" shaped molecule consisting of two light and two heavy chains. Each monomeric molecule consists of two antigen binding sites. The C-terminal part of each chain is constant except for isotopic variation and is called CL (constant light) whereas the N-terminal region shows a high degree of sequence variability and is known as VL (variable light). The variable region of each chain is synthesized from a rearranged genomic locus (discussed below). There are five classes of immunoglobulins based on heavy chain composition. The classes and heavy chains are IgM- μ chain, IgD- δ chain, IgG- γ chain, IgA- α chain, and IgE- ε chain. Also there are two types of light chains, κ and λ. All classes exist in membrane bound as well as in soluble forms. B-cells can change the class of Ig they synthesize during their development and activation. This process is called "class switching". Precursor B-cells initially make the μ chain and continue further as immature naïve B-cells synthesizing membrane associated IgM. Thus IgM is the first class of antibody to appear on the surface of a B-cell. In its secreted form an IgM exists as a pentamer. After leaving the bone marrow, B-cells starts to produce surface IgD with the same antigen binding site as the IgM. At this stage they are called mature naïve B-cells and can respond to foreign antigen in peripheral lymphoid organs. The major class of Igs during the secondary

response is a monomeric IgG. The tail part of IgG called Fc can interact with macrophages and neutrophils through the Fc receptors. This helps in destroying the antigen recognized and coated by IgG. IgA is a dimeric molecule present in secretion such as saliva, tears, milk, respiratory and intestinal secretions. IgE binds to another class of Fc receptors present on mast cells and basophils.

Immature T-cells migrate to the thymus where they undergo various steps of maturation characterized by expression of cell surface molecules such as CD4, CD8 and others. Mature T-cells synthesize T-cell receptors containing α and β chains. T-cells entering the thymus do not express CD4 or CD8 and are called "double negative" . First the β-chain locus undergoes rearrangement and in the next step they become double positive expressing both CD4 and CD8. At this stage the TCR-α chain undergoes rearrangement. This stage accounts for 85% of lymphoid cells in the thymus at any given time. At the mature stage, T-cells express TCRs with either α and β or γ and δ chains and are positive for either CD4 or CD8 (single positive). During development in the thymus, T-cells also go through positive and negative selections. Those T-cells which recognize self MHC are positively selected and allowed to develop but those which recognize self components other than MHC undergo apoptosis (negative selection).

A

Light chain   Heavy chain
V_H
V_H
V_L
(Variable)
C_H1 C_H1
V_L
C_L
(Constant)
C_L
C_H2 C_H2
C_H3 C_H3

B

C

NTD   Core region

NLS ZF-A INT-NBD   ZF-B
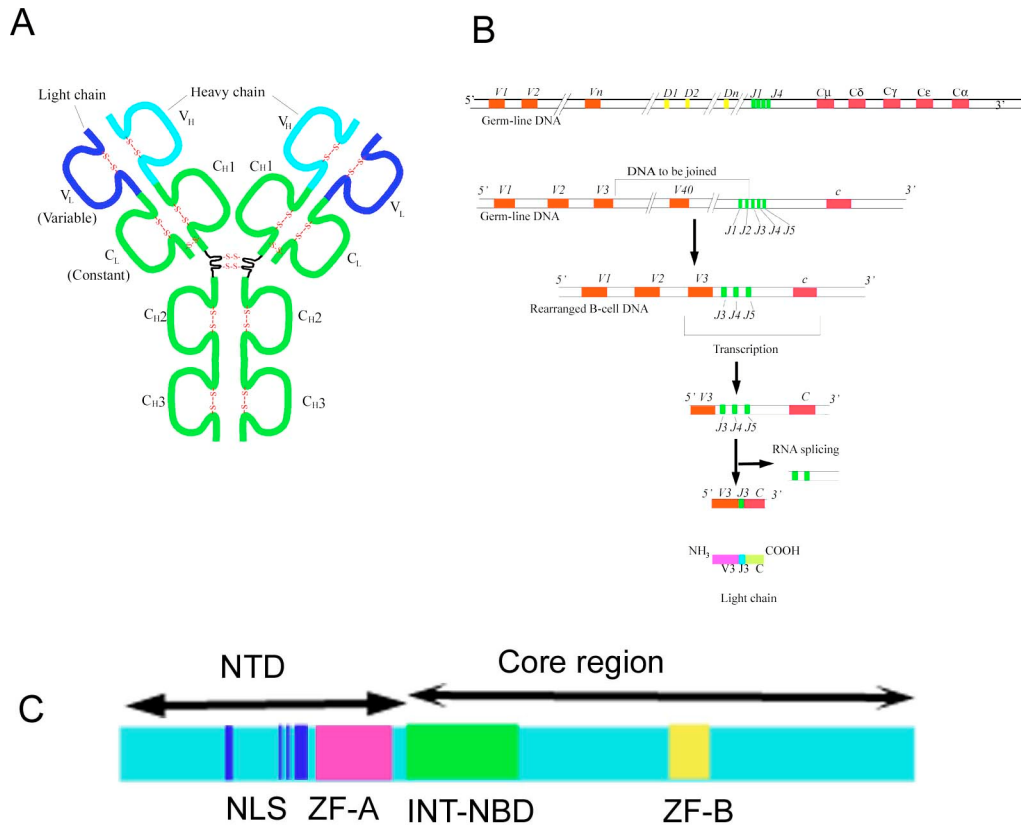
Figure 3.1

Figure 3.1:
(A): Schematic of a typical IgG molecule structure, showing variable and constant regions of the light chain and heavy chain .
(B) Schematic of Ig locus in germ line configuration and through the V(D)J rearrangement
(C) Domain structure of RAG1 molecules. NTD: N-terminal domain NLS: nuclear localization signal, ZF-A: RING zinc finger domain, ZF-B: zinc finger domain, INT-NBD: integrase homology, nonamer binding domain
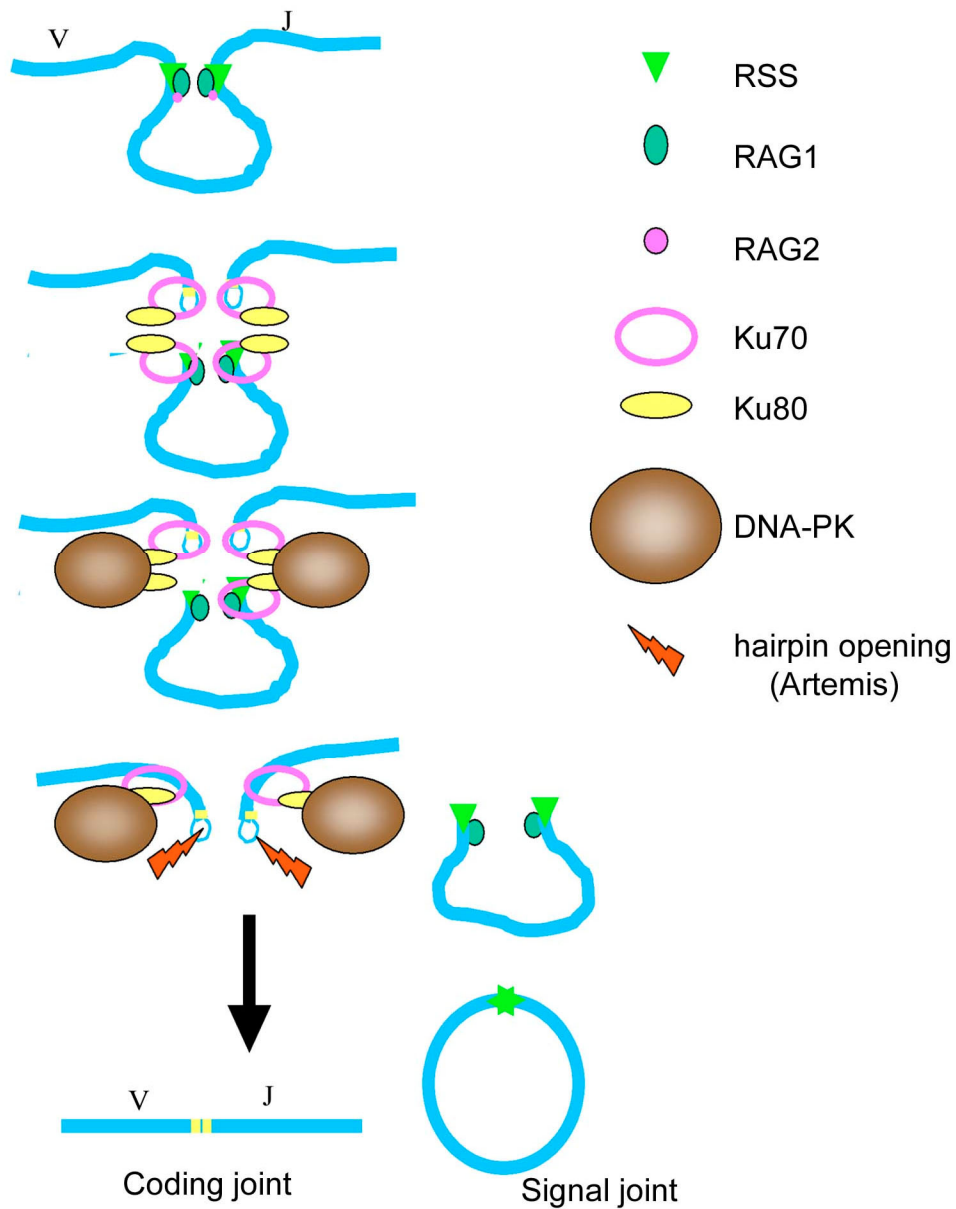
Figure 3.2

Figure 3.2: Schematic of V(D)J recombination pathway and molecules involved.
Two RSS undergoing recombination are recognized by RAG1 & RAG2,
and brought closer in a synaptic complex. After claveage the ends are held
together in a complex which includes Ku70/80, and DNA-PK. Hairpins
formed at coding ends are opened and ligated, signal ends remain
associated with RAG, and are eventually ligated.

(Adapted and modified from : Roth et al. 1995. Current Biology 5:496-99)

3.1.2.2 Genomic locus of immunoglobulins, TCR and V(D)J rearrangement : Role of Rag1

Both heavy and light chains of Igs and TCRs are synthesized from a cluster of genes spanning a genomic locus of several hundred kilobases. As mentioned above, both chains consist of a variable region which is synthesized from a combination of V and J segments. For example, in the human κ light chain locus, there are about forty V genes and five J genes in a cluster. Similarly the heavy chain is synthesized from a combination of V, J and D segments. Thus, in theory combinations of V and J genes in light chains and V, D, and J genes in heavy chains could potentially generate more than 10^6 different antigen binding sites. As shown in figure 3.1, these genes are arranged in a cluster and during the development of lymphocytes one of the V gene is combined with the J region and the intervening DNA is eliminated by a process which changes the original germline configuration and is known as V(D)J recombination (Tonegawa, 1983).

V(D)J recombination occurs between elements known as recombination signal sequences (RSS) that lie adjacent to each gene segment. The RSS contains a conserved heptamer and nonamer sequence separated by 12 or 23 bases. The process of V(D)J recombination requires recombination activating gene-1 (RAG1) and RAG2 proteins (Schatz et al., 1989; Oettinger et al., 1990) along with high mobility group protein-1 (HMG1) and HMG2 (Sawchuk et al., 1997; van Gent et al., 1997). Two RSS undergoing recombination are brought together in a specific complex by RAG proteins and cleaved subsequently such that two hairpin "coding ends" and two blunt "signal ends" are generated (McBlane et al., 1995). Thus RAG plays a key role at the very first step of V(D)J recombination. After the cleavage, a transient complex is formed in which both

signal and coding ends are held by RAG proteins (Hiom and Gellert, 1998; Tsai et al., 2002). The coding ends are subsequently released but signal ends remain bound to RAG proteins (Agrawal and Schatz, 1997). Coding ends are processed by DNA-dependent protein kinase (DNA-PK), Ku70, Ku80, XRCC4, DNA ligase, Artemis and terminal deoxy nucleotidyl transferase (TdT) (Figure 3.2 and reviewed in (Fugmann et al., 2000; Gellert, 2002). Hairpins at coding ends are opened, nucleotides are added or deleted and ends are subsequently ligated (Ramsden and Gellert, 1995; Roth et al., 1995). Depending on the original configuration of RSS in the germline arrangement, signal ends may be retained or deleted from the chromosome and eventually lost. Coding ends are retained in the chromosome and contribute to the expression of an antigen receptor peptide (light or heavy chains of Ig or TCR).

3.1.2.3   Rag1 structure, function and regulation

Owing to its identification as a key molecule in immune system development, RAG1 has been studied intensively for the past several years. It is a 119 Kd protein with 1043 amino acids. Residues 387 to 1008 (in human RAG1) constitute the core domain of the protein which is essential and sufficient for its activity (van Gent et al., 1995). The N-terminal region may have important functions *in vivo* and may be required for nuclear localization and enhancement of RAG1 activity (Rodgers et al., 1996; McMahan et al., 1997). Recently, the non-essential N-terminal domain of RAG1 was shown to have ubiquitin ligase activity *in vitro* (Yurchenko et al., 2003). The zinc RING finger domain in the N-terminal region and C2H2 zinc finger domain within the core region are thought to be involved in RAG dimerization (Rodgers et al., 1996; Bellon et al., 1997; McMahan et al., 1997). Five basic regions have been identified for interaction of RAG1 with

nuclear transport protein SRP-1 (Cortes et al., 1994; Cuomo and Oettinger, 1994; Spanopoulou et al., 1995). Two domains have been identified within the core region of RAG1; the first domain encompassing amino acids 392-448, is required for binding of RAG1-RAG2 to the nonamer of RSS, and the second domain, encompassing amino acids 504-526 is related to catalytic domain of prokaryotic integrase – a site-specific recombinase (Bernstein et al., 1996; Difilippantonio et al., 1996; Spanopoulou et al., 1996). The RAG2 interacting domain is located within the core region encompassing amino acids 504-1008 (McMahan et al., 1997).

RAG1, showing homology with prokaryotic integrase, is thought to have entered the genome of a vertebrate ancestor 450 million years ago, as part of a mobile element (Bernstein et al., 1996; Spanopoulou et al., 1996; Agrawal et al., 1998; Kapitonov and Jurka, 2005). Several similarities have been identified between the mechanism of V(D)J recombination and transposition (Thompson, 1995; Fugmann et al., 2000; Chatterji et al., 2004; Kapitonov and Jurka, 2005). Indeed RAG1 shows transposase activity *in vitro* and chromosomal translocations in some carcinomas have been attributed to the misregulation of V(D)J recombination (McGuire et al., 1989; Hiom et al., 1998; Melek and Gellert, 2000; Lee et al., 2002). Also a recent report shows that the intervening sequence with signal end can integrate elsewhere in the genome (Messier et al., 2003). Therefore the activity of RAG1 is controlled by its regulation at the expression level as well by restricting access to the genome.

The promoter region of RAG1 from mouse and humans has been characterized and binding sites for Ekaros, Ikaros and NF-Y were identified (Kurioka et al., 1996; Fuller and Storb, 1997). The expression of RAG1 and RAG2 is regulated by the region 5' to the

Rag2 gene (Yu et al., 1999). During the development of T-cells and B-cells, RAG1 is present at specific stages, for example RAG1 is expressed in pro-B cells during rearrangement of heavy chain but downregulated during expansion stage and expressed again in non-proliferating pre-B cells in which the light chain is rearranged (Nagaoka et al., 2000).

Rag proteins when expressed in non-lymphoid cells such as fibroblast do not cause rearrangement of endogenous Ig or TCR locus but could recombine artificial recombination substrate (Schatz et al., 1992). This implies that the process of V(D)J recombination is also regulated by controlling the access of RAG to RSS sites. One way to achieve this is by modulating the chromatin. Histone acetylation was found to play a role in active rearrangement (McMurry and Krangel, 2000). Other means of nucleosome modification may also control the accessibility of antigen receptor loci (Oettinger, 2004).

Besides regulating expression and access to the substrate, RAG1 activity is controlled by other factors to avoid transposition events. Though *in vitro* studies using core domains of RAG demonstrate the capability of transposition by V(D)J recombinase, full length proteins seems to be less efficient in generating hybrid joints (Sekiguchi et al., 2001). Recent report shows that the C-terminal region of RAG2 and GTP prevents transposase activity of RAG1 (Tsai and Schatz, 2003).

3.1.3 Rag-1: role in neurons – facts and hypothesis.

More than ten years ago it was shown that *Rag1* is expressed in specific regions of the brain such as the hippocampal formation, cerebellum and olfactory bulb (Chun et al., 1991). *Rag1* mRNA has been detected in the brain of vertebrates as diverse as mouse, salamander and zebrafish (Chun et al., 1991; Frippiat et al., 2001; Jessen et al., 2001).

This has led to speculation that neurons undergo some form of genomic rearrangement that is RAG1-mediated. An experiment to address this possibility, using a transgenic mouse carrying an inverted LacZ gene flanked by recombination signal sequences, lent some support to this idea, as X-gal stained cells were found only in the thymus and brain (Matsuoka et al., 1991). However, this experiment could not be reproduced, and remains controversial (Abeliovich et al., 1992). Another observation that has been suggested to support the notion that RAG1 has a role in the brain is that mice lacking *Rag1* have behavioral defects (Cushman et al., 2003). However, as these mice are also immunodeficient, the possibility that abnormal behavior is due to a defective immune system cannot be excluded. Moreover, the brain of mice lacking *Rag1* appears morphologically normal at the level of light microscopy (Mombaerts et al., 1992). Excessive cell death in the brain of double strand break repair mutants (Gao et al., 1998) indicates that the genome of neurons contains many breaks. However, there appears to be no exclusive link to *Rag1*, because the *xrcc4* and *Rag1* double mutant displays a similar phenotype as the *xrcc4* single mutant (Sekiguchi et al., 1999). Hence, in spite of some hints, there is no compelling evidence yet that *Rag1* has a function in the vertebrate nervous system.

3.2   *Aim of the project*

This project aims to study the role of RAG1 in neurons. Literature evidence from more than the last ten years have conclusively shown that RAG1 transcript is present in specific neurons. Exploring whether RAG1 aids genetic rearrangement in neurons and determining whether its role is related to neuronal development or function would be an eventual goal. But before addressing this, a few fundamental questions need to be

addressed. This study aims to check the presence and sub-cellular localization of RAG1 protein in neurons. If the RAG protein is localized in the neuronal nuclei, then being a DNA binding protein it would predictably have specific targets in neurons. Thus the second goal of this study was to test DNA binding of RAG1 in neurons and identify target(s).

3.3 Materials and Methods:

3.3.1 Antibody, enzymes, chemicals and general protocols:

The antibodies used in this study (SC363, SC5599) and the blocking peptide SC363P were purchased from Santa Cruz. These antibodies have been used for detecting RAG1 by western and immunoflurescence. (Kumaki et al., 2001; Bas et al., 2003; Vaitaitis et al., 2003; Hillion et al., 2005). ProteinA speharose beads were purchased from Amersham. Mouse YAC and BAC macroarrays were purchased from RZPD. All other chemicals were purchased from Sigma and BDH. Restriction endonucleases, and other DNA modifying enzymes were purchased from NEB, Promega and Roche. Oligonucleotide primers were purchased from GENSET.

Digestion of DNA with restriction endonuclease, ligation, agarose gel electrophoresis and standard molecular biology experiments were carried out as described (Fred et al., 1987; Sambrook et al., 1989)

3.3.2 Oligonucleotide primers :

DAR192 : 5_-AGCAAGGAAGTCCTGAAGAAGATCT-3

DAR216 : 5_-GATATCGGCAAGAGGGACAATAGCT-3_

DAR6 : 5-GAGCAGTGGGTAGGCGAAAGCTTAACCCC-3

DAR11: 5-AGTGCCACTAACTGCTGAGCCACCT-3

DAR321- 5-GGTCCACGTCCAGATGCCAACT-3

DAR322:5'- TCTCAGGGAAGATGGGCCTCTC-3'

DAR323: 5'-CCCTGTCAGCTTGGTTCAAAGGC-3

FM11: 5-CACTTCAGATC-3

FM25: 5-GCGGTGACTCGGGAGATCTGAAGTG-3

1F: 5'-TATCATAAACTGGGTGCAGC-3'

1R: 5'-GCTCGAGTCTGCTTTCACTA-3

2F: 5'- CGGGCTACCTTGCCAGCAGAGTCTT -3'

2R: 5'-AGTCTCGAGTCGAGCGGAAGGGACT -3'

HYAC-C: 5'-GCTACTTGGAGCCACTATCGACTACGCGAT-3'

LS2: 5'-TCTCGGTAGCCAAGTTGGTTTAAGG –3'

RSS-12-TOP:

5'-ATGCTTGACGTCCACAGTGATTCACATCATGACAAAAACCCCATGGATGCTT-3'

RSS-12-BOTTOM

5'-AAGCATCCATGGGGTTTTTGTCATGATGTGAATCACTGTGGACGTCAAGCAT-3'

RSS-23-TOP

5'ATGCTCTAGCGGCCGCGGTTTTTGTACGACTGAACATATCAAATCTTTCACTGTGGG

GCCCATCGTCA-3'

RSS-23-BOTTOM

5'TGACGATGGGCCCCACAGTGAAAGATTTGATATGTTCAGTCGTACAAAAACCGCGG

CCGCTAGAGCAT-3'

3.3.3 Buffers and solutions:

NPBS: 0.1% IGEPAL CA-630 (sigma- I-8896) in PBS

NPBS+: NPBS, 2% milk powder (fat free), 5% FBS, 0.02% Na-Azide.

TBS: 150mM NaCl, 20mM Tris-Cl pH 7.6

ChIP Lysis buffer: 0.1% (w/v) deoxycholic acid (Na-salt), 1mM EDTA, 50mM HEPES pH 7.5 (adjusted with KOH), 140mM NaCl, 1% (v/v) TritonX-100, add protease inhibitor (Roche) before use

ChIP Lysis Buffer 500: 0.1% (w/v) deoxycholic acid (Na-salt), 1mM EDTA, 50mM HEPES pH 7.5 (adjusted with KOH), 500mM NaCl, 1% (v/v) TritonX-100, protease inhibitor (Roche) added before use

TE/1%detergent washing solution: 10mM Tris-Cl pH 7.6, 1mM EDTA, 1% SDS

TE/0.67%detergent washing solution: 10mM Tris-Cl pH 7.6, 1mM EDTA, 0.67% SDS

LiCl/ detergent washing solution: 0.1% (w/v) deoxycholic acid (Na-salt), 1mM EDTA, 50mM, 0.5% (v/v) IPGEAL (sigma-), 10mM Tris-Cl pH 8.0, 250mM LiCl

Elution Buffer: 10mM EDTA, 1% SDS, 50mM Tris-Cl pH 8.0

Proteinase-K solution: 0.5μl of 20mg/ml glycogen (Roche), 5.0μl of 20mg/ml proteinaseK, 244.5 μl of TE pH 7.6

Complete Medium: DMEM (Dulbecco's Modified Eagle Medium) with 10% Fetal bovine serum and antibiotic (penicillin/streptomycin)

Retinoic Acid stock solution: Retinoic Acid was dissolved in ethanol to prepare 0.5mM stock

Ara-C: 5 mg/ml in water

3.3.4 Mice and tissue collection :

Mice pups at age 7 to 14 days were obtained from Laboratory Animal Center, Singapore. Mice were sacrificed by cervical dislocation or decapitation. Tissue was

collected by quick dissection. Brain and thymus dissected from pups were kept in ice-cold PBS till the next step.

3.3.5 P19 cells and differentiation into neurons:

Undifferentiated P19 embryonic carcinoma cells were obtained from American Type Culture Collection and maintained as described (Rudnicki and McBurney, 1987). Frozen stock of P19 cells was thawed at 37^0C and diluted in 10 ml of DMEM. Cell suspension was centrifuged at 200x g for 5 min. Cell pellet was washed with DMEM and diluted to 10ml centrifuged again at 200xg for 5min. Cells were suspended in 15 ml of complete medium (DMEM+ 10%FBS+ antibiotics) and grown in 75 cm^3 tissue culture flask at 37^0C with 5% CO_2 in a humidified incubator.

For routine splitting, high density cultures (up to 90% saturation) were split 1:15. Medium was discarded from the flask and cells were washed twice with PBS. To detach cells from the surface, 1 ml of 0.25% Trypsin, 2 mM EDTA mixture diluted in 4 ml of PBS was added and flasks were kept at 37^0C for not more than 5 min. 10 ml of complete medium was added to stop trypsinization and cells were monodispersed. 1 ml of this suspension with 14 ml of fresh complete medium was placed in a fresh tissue culture flask and incubated as above.

To induce the differentiation of P19 cells, cultures were treated with retinoic acid (RA). 0.5 µM of retinoic acid was added to monodispersed suspension of undifferentiated P19 cells at $5x10^6$ /ml density in 5 ml complete medium and placed in a 35 mm diameter non-tissue culture dish. Cultures were incubated at 37^0C with 5% CO_2 in a humidified incubator for 2 days. Small cell clusters were observed. The culture was transferred to a 15 ml falcon tube and cells were allowed to settle down. Medium was replaced with fresh

medium with 0.5µM RA and placed in the same dish and incubated for 2 more days. After 4 days of RA treatment, small spheres of cell clusters were formed. The culture was transferred to a falcon tube and clumps were allowed to settle down. Cell clumps were trypsinized and monodispersed as described above. Monodispersed cells were inoculated in 35 mm diameter tissue culture dishes at $1x10^6$ cells/dish and incubated at 37^0C with 5% CO_2 in a humidified incubator. Neuronally differentiated cells were visible after two days. Medium was replaced with fresh medium containing Ara-C at 5 µM. Incubation was continued under same condition and medium was replaced every 2 days.

3.3.6 Antibody staining

Thymocytes were dissociated from the thymus by triturating in ice cold PBS using tungsten needles and suspended at 5×10^6 cells/ml concentration. Cells were fixed with 1.0% formaldehyde for 15 mins at room temperature and then washed with PBS. Approximately 100 µl of cells were spread on charged glass slides (BDH superforst[+]). Cells were blocked with NPBS[+] (200-300 µl/slide) for at least one hour at room temperature. Anti-RAG1 antibodies diluted in NPBS[+] (1:250) were added (250 µl/slide) and incubated overnight at room temperature. Slides were washed with NPBS 5-6 times for 15 min each. Secondary antibodies (anti-rabbit – Alexa 488 conjugate; Molecular probes) diluted at 1: 300 in NPBS+ were added to each slide (300 µl/slide) and incubated in the dark at room temperature for 2 hours. Slides were washed with NPBS 3-4 times for 15 min each. Cells were counter stained with the DNA labeling dye Hochest (1:1000 dilution in PBS of stock 100 µg/ml) for 5 min at room temperature and rinsed with PBS. Slides were mounted in 70% glycerol in PBS

Pups were sacrificed by cervical dislocation. Viscera was dissected by standard procedure. Pups were perfused with 4% PFA in PBS by injecting into the heart. Perfusion was carried out only when tissues were to be used for immunohistochemistry. The brain was dissected out from the cranium and kept in 4%PFA for overnight fixation at 4^0C. the cerebellum was cut out from the rest of the brain. Both parts were embedded in cryo medium in a mould and frozen on dry ice. 20 μm section were cut using a cryostat and collected on charged slides. Sections were treated with 0.2% Triton-X100 in PBS for 30min to 1hour at room temperature and then washed with PBS three times for 5 min each. Antibody staining was carried out on sections as described above.

Neuronally differentiated P19 cells were stained with anti-RAG1 antibodies in a similar manner as described above for thymocytes with few changes. Anti-rabbit Alexa 568 conjugated secondary antibodies (1:300 dilution) were used to detect RAG1. Cells were also stained with anti-acetylated tubulin monoclonal antibodies (1: 200) and anti-mouse Alexa 468 conjugated secondary antibodies (1:300 dilution).

3.3.7 Imaging:

Samples were imaged on a Zeiss LSM 510 laser scanning confocal microscope. A 100x 1.3 NA oil objective was used. Z-stacks were processed by deconvolution, using a blind deconvolution algorithm (Auto Deblur, AutoQuant Inc.)

3.3.8 Construction of artificial recombination substrate

A plasmid pEGRR carrying an artificial recombination substrate was prepared by modifying pEGFP-1 (clontech). RSS12 and RSS23 were prepared by annealing RSS-12-TOP+RSS-12-BOTTOM and RSS-23-TOP+RSS-23-BOTTOM respectively. RSS12 was inserted at the StuI site of pEGFP-1 to create pEGR. RSS23 was inserted at the SmaI site

of pEGR. The orientation of both RSS were confirmed by sequencing. The resultant pEGRR plasmid was introduced into undifferentiated P19 cells by lipofection using Lipofectamine (Invitrogen). P19 clones resistant to G418 were isolated and propagated separately. Retinoic acid treatment and differentiation was carried out as above. Cells were screened for GFP expression under fluorescent microscope with a 10x objective.

3.3.9 Chromatin immunoprecipitation

Single and double ChIP was performed as described by Aparicio and Farhnan (with some modifications - Figure 3.7) (Aparicio, 1999; Weinmann and Farnham, 2002)

3.3.9.1 Tissue preparation:

Thymocytes were dissociated from the thymus by triturating in ice cold PBS using tungsten needles. The brain was chopped into small pieces (~2 mm) with a surgical blade on a cold platform. These brain pieces were further homogenised in a 1.5ml centrifuge tube using a blue tip handheld homogenizer for 15-30sec pulse x 3 times. Tissue was transferred to a 15 ml falcon tube. Brain cells were suspended in 10ml ice-cold PBS by pipetting with a 5 ml surgical pipette. Cells were passed through 100 μm pore size mesh to eliminate clumps. Neuronally differentiated P19 cells were trypsinized and finally resuspended in ice-cold PBS.

3.3.9.2 Crosslinking:

In all cases cells were treated with 1% formaldehyde for 10 min at room temperature. Fixation was stopped by the addition of glycine to 0.125M and incubating at room temperature for 5 min. Cells were centrifuged at 500xg for 5 min at 4^0C. The cell pellet was washed 3 times with ice-cold TBS. Cells were suspended at approx. $1x\ 10^8$ cells/ml

in TBS and frozen at -80^{0}C till next step. Cells could be stored after fixation at -80^{0}C for several months.

3.3.9.3   Cell Lysis and preparation of soluble chromatin:

In all cases, approx. 10^8 cells were used in each experiment. Samples were processed in multiple tubes under identical condition. Frozen cells were thawed on ice, centrifuged at 1500x g for 5 min at 4 ^{0}C and resuspended in 500 µl of lysis buffer. Suspension was split into two 2.0 ml centrifuge tubes. Equal volume of glass beads (size 0.5 µm) were added to each tube and vortexed for 30 min at 4 ^{0}C. 250 µl of lysis buffer was added to each tube and vortexed briefly. Tubes were punctured with a heated 25 gauge needle and immediately placed on a 14 ml collection tube. Tubes were centrifuged at 1000x g for 3 min at 4^{0}C in a swinging bucket rotor. The sample expelled in the 14 ml tube contained soluble cell lysate and cell debris. The sample was mixed with a 1ml pipette tip (wide opening) and transferred to a fresh 1.5 ml centrifuge tube. To shear chromatin to approx. =<500bp fragments, samples were sonicated in 3 continuous pulses of 12 seconds duration each at the microtip setting 4 with Misonix incorporated XL2020 sonicator ultraprocessor. Tubes were held on ice for at least 2 mins. between two sonication pulses. After sonication, tubes were centrifuged at 14000rpm for 5 min. at 4 ^{0}C. Centrifugation was repeated for 15 min if necessary. Clear supernatant from two tubes was pooled into one 1.5 ml tube resulting in a pooled lysate from approx. 10^8 cells.

3.3.9.4   Incubation with antibodies and pull-down with beads:

Samples were incubated with either 4 µg of anti-RAG1 antibodies (SC363 or SC5599) or without antibodies at 4 ^{0}C overnight on a shaking platform (Nutator). ProteinG-sepharose beads were washed three times with ice-cold PBS and a 50% slurry

in PBS was prepared. 150 µl of 50% ProteinG-sepharose (Amersham) was added to each tube and incubated at 4 ^{0}C for 3 hours on the nutator. Tubes were either left on ice for 10min or centrifuged at 500x g for 5 min to pellet down beads. The supernatant was carefully removed without disturbing the beads. 1/10th of this supernatant was mixed with TE/1%detergent solution and kept aside as "total input". This was processed as described below to purify the DNA. Beads were washed twice with 1 ml of lysis buffer, followed by washes of 1 ml of lysis buffer-500, 1 ml of LiCl detergent solution, 1 ml of TE buffer for 5 min each at room temperature on a nutator. In each washing step, beads were separated from the washing solution by centrifuging tubes at 1000x g for 1 min at room temperature. DNA-protein complexes were eluted from beads by adding 200 µl of elution buffer and keeping tubes at 65 ^{0}C for 10 min.

3.3.9.5   Second round of antibody incubation and pull-down.

The eluate from the first round of immunoprecipitation was diluted with 1 ml of lysis buffer and corresponding samples were incubated with either 4 µg of anti-RAG1 antibodies (SC363 or SC5599) or without antibodies at 4 ^{0}C overnight on a shaking platform (Nutator). Incubation with beads and all washes were carried out as above. DNA-protein complexes were eluted from beads by adding 200 µl of elution buffer and keeping tubes at 65 ^{0}C for 10 min. Tubes were centrifuged for 5 seconds at full speed and the eluate was transferred to fresh tubes. Beads were again incubated with 300 µl of TE/0.67% detergent solution and the supernatant was mixed with the previous eluate.

3.3.9.6   Purifying double ChIP-DNA.:

All tubes containing the eluate (from with and without antibodies experiment) along with the "total input" were incubated at 65 ^{0}C overnight to reverse crosslink. 10 µl of

proteinase-K (20mg/ml) and 1 µl of glycogen (20mg/ml) was added to all tubes and incubated at 37 ^{0}C for 3 hours. 55 µl of 4M LiCl was added to each tube and mixed with 500 µl of phenol:chloroform: isoamyl alcohol (25:24:1) mixture by inverting tubes several times. Tubes were centrifuged at 14000 rpm for 10 min at room temperature. The aqueous phase was transferred to a fresh tube. DNA was precipitated by adding 1 ml of ethanol and mixing it by inverting tubes several times. Tubes were centrifuged at 14000 rpm for 15 min at room temperature. The supernatant was discarded and DNA pellet washed with 1ml of 75% ethanol. DNA pellets were air dried and dissolved in 25 µl of TE for all samples.

3.3.10 ChIP-DNA analysis by specific PCR:

All PCR assays were performed using 2µl of template DNA from each sample in a 20µl reaction with 1x Taq DNA polymerase buffer with 1.5 mM MgCl2, 200 µM dNTPs, and 1 µM primer. For amplification of Jα50 primer pairs DAR321 and DAR323 were used under PCR conditions: 95^{0}C-3 min, followed by 95^{0}C-1 min, 58^{0}C-1 min, 72^{0}C-1 min for 25 cycles and 72^{0}C-5 min. For amplification of Jκ, primer pairs DAR6 and DAR11 were used under PCR conditions: 95^{0}C-3 min, followed by 95^{0}C-1 min, 60^{0}C-1 min, 72^{0}C-1 min for 25 cycles and 72^{0}C-5 min. For amplification of brain target repeat region primers 2F and 2R (Figure 3.10 E) were used under PCR conditions: 95^{0}C-3 min, followed by 95^{0}C-1 min, 60^{0}C-1 min, 72^{0}C-1 min for 20 cycles and 72^{0}C-5 min. For unique region 1F and 1R (Figure 3.10 E) were used under PCR conditions: 95^{0}C-3 min, followed by 95^{0}C-1 min, 56^{0}C-1 min, 72^{0}C-1 min for 20 cycles and 72^{0}C-5 min. PCR amplification was checked on 2% TAE-agarose gel stained with Gel-Star

3.3.11 End-repair and adaptor ligation

ChIP-DNA was end repaired as described (Wilson and Mardis, 1997). Eluted ChIP-DNA was first incubated with T4 DNA polymerase with 100uM of dNTPs at 12^0C for 20 min followed by Klenow at 25^0C for 15 min. Enzymes were inactivated by heating the sample at 75^0C for 20 min. DNA was extracted with phenol:chloroform and ethanol precipitated with NaCl and glycogen. The DNA pellet was dissolved in water and treated with T4 polynucleotide kinase in T4 DNA ligase buffer containing ATP at 37^0C for 30 min. The enzyme was inactivated by incubating at 65^0C for 20 min.

3.3.12 LMPCR and DIG-labeled probe synthesis

Adapters were prepared by annealing oligos FM11 and FM25. These adapters were ligated to ChIP-DNA and was amplified by adding 1.24 µM of FM25, 320 µM dNTPs along with 1x PCR buffer and incubating for 3 min at 72^0C followed by the addition of Taq DNA polymerase and amplification under the following conditions: 94^0C-1 min, 60^0C-1 min, 72^0C-1 min for 30 cycles and 72^0C for 10 min.

LMPCR amplified DNA was used for preparing DIG labeled probe by PCR labeling method using PCR DNA-DIG probe synthesis kit (Roche 1636090) as per manufacturer's instructions. Incorporation of labeled nucleotide was checked by spotting various dilutions of probe and detecting by Alkaline Phosphatase conjugated anti-DIG antibody staining followed by developing with CDP-star (Roche - 1759051) substrate.

3.3.13 Screening YAC and BAC library macroarrays

An equal quantity of probe was used for a set of comparable experiments. A mouse genomic YAC library (Kusumi et al., 1993) spotted on a 22.2 x 22.2 cm nylon membrane (RZPD library no. 910), as well as a BAC library (RZPD library no. 710) was used for hybridization. The probe was hybridized to the macroarrays at 50^0C and washing

were carried out at 65^0C with high stringency wash buffer (twice with 2x SSC, 0.1%SDS 5 min. at room temperature followed by two washes of 0.1x SSC, 0.1%SDS for 15min at 65^0C). Hybridization signals were detected as above on Kodak-MS/ML X-ray film.

3.3.14 End sequencing of YACs

A positive YAC clone was received from RZPD (http://www.rzpd.de). The end sequence of YAC was obtained by plasmid-rescue method. Crude YAC DNA was prepared, digested with XhoI and self ligated. This was transformed into *E.coli* electrocompetent cells. Positive clones were selected on LB-Agar plates containing ampicillin. The plasmid insert was sequenced using HYAC-C and LS2 primers.

3.3.15 BACs southern hybridization

BAC clones covering the locus of mouse chromosome-9 to which YAC was mapped were received from RZPD. EcoRI and BamHI digests of these BAC DNA were resolved on a 0.8% TAE-agarose gel and transferred to nylon membrane. Southern hybridization with brain ChIP-DNA probe was carried out as described above.

3.3.16 Screening BAC subclone

A 5.7Kb EcoRI fragment of BAC-86O15 was gel purified and cloned into a pUC19 plasmid. These subclones were screened by probing with brain ChIP-DNA probe in a colony hybridisation assay under the hybridisation condition described above. Positive clones were sequenced using vector-specific primers. The insert was purified and digested with EcoRV. Southern analysis of this EcoRV digest with Brain-ChIP-DNA probe was performed as above.

3.4   Results :

3.4.1 Detection of RAG1 protein in thymocytes and neurons:

Previous *in situ* hybridization with radioactive probes has suggested that *Rag1* is expressed in the mouse brain, with higher levels of transcript being detected in the cerebellum and hippocampus (Chun et al., 1991). To determine if this gene is translated in neurons, immunofluorescent labeling with an antibody to the core domain of RAG1 was carried out. Antibodies were first tested on thymocytes, with pre-absorption being used as a control for specificity (Figure. 3.3 A-H). Strong nuclear labeling was detected (Figure. 3.3 I). The SC-363 antibody was then used to label sections made through the brains of p10 mice. Labeling was observed in the hippocampus (Figure. 3.4 A-F) and the cerebellum (Figure. 3.4 G-L). In the hippocampus (Figure. 3.4 A), nuclear-localized RAG1 was detected in cells forming the stratum pyramidale (Figure. 3.4 M). Some nuclear label was also seen in the granule neuron layer forming the stratum granulosum of the dentate gyrus, but these cells appeared to contain more cytoplasmically localized protein (Figure. 3.4 N). Cells in the purkinje cell layer of the cerebellum contained nuclear as well as cytoplasmic RAG1 (Figure. 3.4 O).
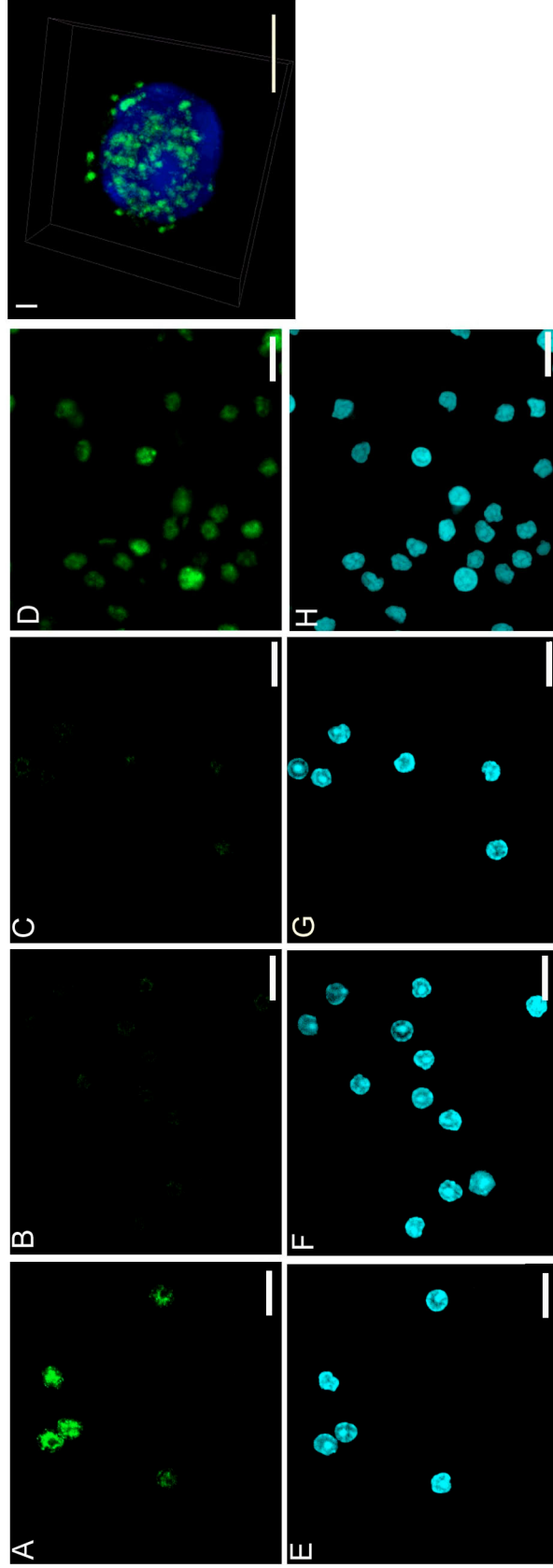
Figure 3.3

Figure 3.3: . Detection of RAG1 protein by indirect immunofluorescence. A-H: Thymocytes isolated from mice at P10, labeled with an anti-RAG1 polyclonal antibody SC363 (A), with no primary antibody (B), with a peptide-blocked anti-RAG1 polyclonal antibody SC-363 (C) or with anti-RAG1 polyclonal antibody SC-5599 (D). Corresponding images of DAPI-stained nuclei are shown below each panel (E-H). RAG1 appears to be present in nuclei as shown by 3D reconstitution of higher magnification image (I). Scale bar A-H :10 μ, I: 30 μ

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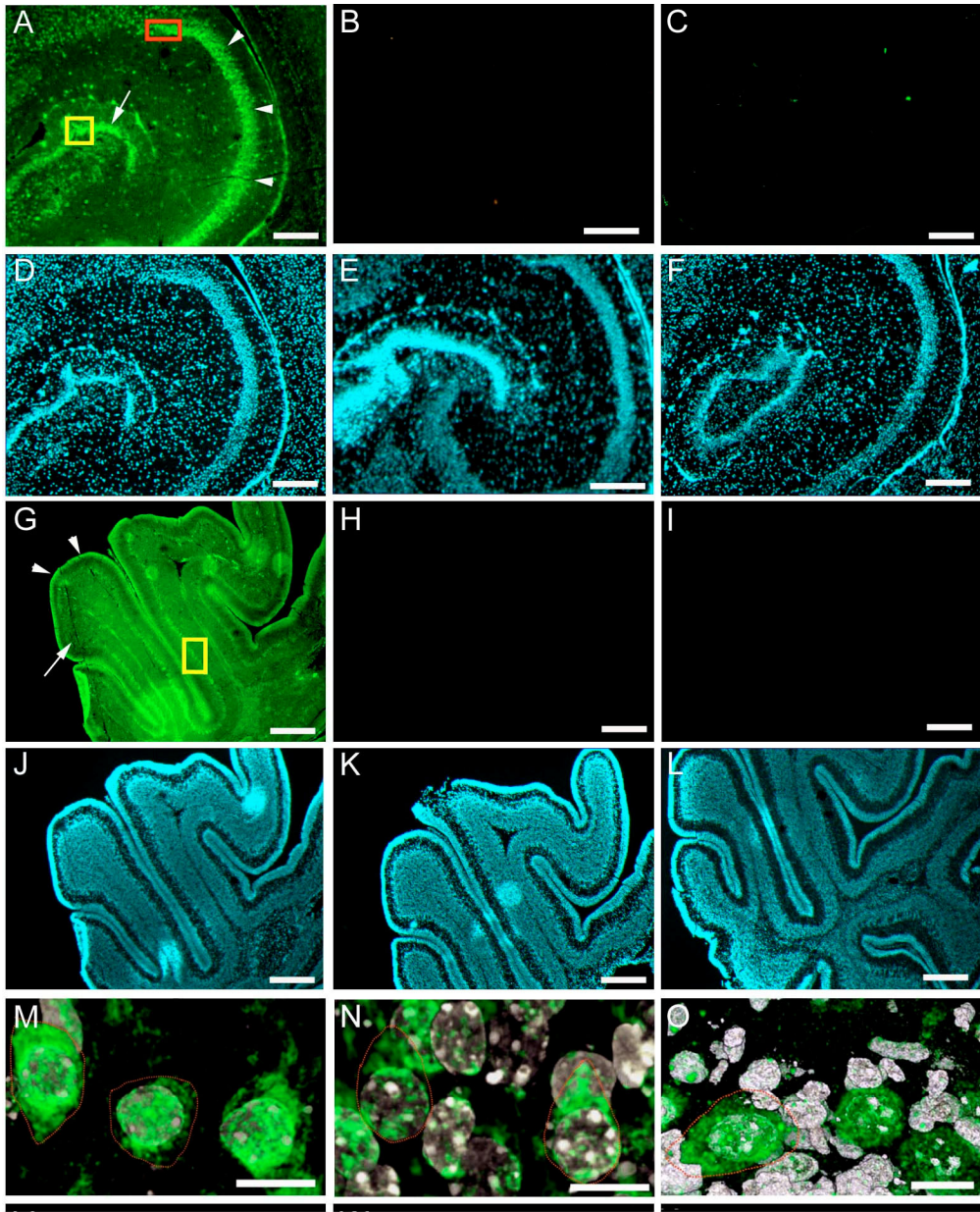
Figure 3.4

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Figure 3.4: **A-L:** Parasagittal sections through the hippocampus (A-F) and cerebellum (G-L) of neonatal mice, labeled with the SC-363 polyclonal antibody to RAG1 (A,G), without primary antibody (B,H) or with peptide-blocked antibody (C,I). Corresponding images of DAPI stained nuclei are shown below each panel (D-F, J-L). Strong labeling was detected in the stratum pyramidale (A; arrowhead) and stratum granulosum (A; arrow) of the hippocampus, as well as the granular layer (G; arrow) and external granular layer (G; arrowhead) of the cerebellum. **M-O**: Higher magnification images of the areas outlined with the red (M) and yellow (N) rectangles in panel **A**, and with the yellow rectangle (O) in panel **G**. Predominantly nuclear label was detected in cells of the stratum pyramidale (M), while more cytoplasmic labeling was seen in cells in the stratum granulosum (N). In the cerebellum (O), Purkinje neurons, which have large nuclei, contained nuclear and cytoplasmic RAG1. Scale bar A-L: 200μ and M-O: 10μ
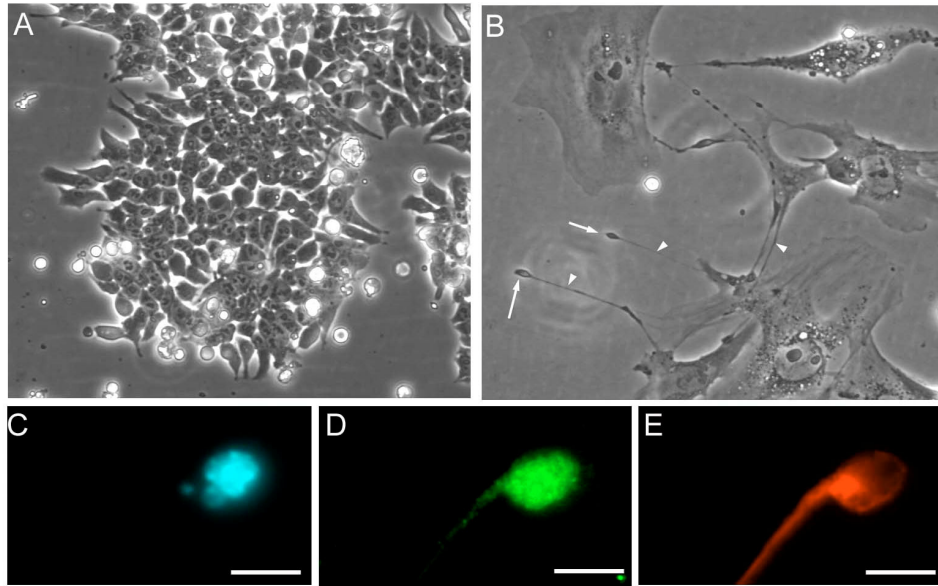
Figure 3.5

Figure 3.5: A-B: P19 embryonic carcinoma cell undifferentiated under normal growth conditions (A) and neuronally differentiated after retinoic acid treatment (B). Neuronal cell bodies are shown with arrow and neurite extension with arrowheads. Neuronally differentiated P19 embryonic carcinoma cell stained with DAPI (C), anti RAG1 polyclonal antibodies (D), and acetylated tubulin (E). Scale bar 10 μm

Mouse embryonic carcinoma cells from the P19 line show fibroblast morphology under normal growth conditions. With retinoic acid treatment, these cell can be differentiated into neuronal cells (Figure 3.5 A,B) (Jones-Villeneuve et al., 1982). The presence of RAG1 protein in these differentiated neuronal cells along with the neuronal marker acetylated tubulin was checked. RAG1 was detected in the cell bodies of differentiated neuronal cells whereas acetylated tubulin staining was mainly detected in neurites (Figure 3.5 C-E).

3.4.2 Checking the V(D)J like recombination in RAG1 expressing neuronally differentiated P19 embryonic carcinoma cells

Recombination of an artificial substrate containing RSSs in RAG1 positive neurons been reported earlier, but the conclusion of those studies remain controversial (Matsuoka et al., 1991; Abeliovich et al., 1992). After demonstrating the presence of RAG1 protein in the nucleus of neuronally differentiated P19 embryonic carcinoma cells, the possibility of V(D)J like recombination in these cells was checked. An artificial substrate containing RSS was prepared by modifying pEGFP-1 plasmid as shown in Figure 3.6. In the original configuration, the promoterless EGFP is not expressed. A recombination event between the 12 and 23 RSS flanking EGFP would result in loss of Kan^R/Neo^R, HSV-TK polyA, and pUC ori region bringing the SV40 promoter upstream of EGFP. Given that Rag1 can mediate transposition, it was postulated that the fragment containing Kan^R/Neo^R, HSV-TK polyA, and pUC ori might also be inserted into the genome. In a pilot experiment, EGFP expression under SV40 promoter was checked in undifferentiated and neuronally differentiated P19 EC. Linearized pEGRR plasmid (an artificial recombination substrate) was transformed into P19 cells and G418 resistant clones were picked. Cells from eight different clones were maintained in an undifferentiated state in medium containing G418.

During the differentiation of these cells, G418 selection was not applied. Differentiated cells were screened for EGFP expression. Cells from all clones (total no of cells – 1 x 10^8) were screened. No EGFP expression was detected. Presence of the recombination substrate in undifferentiated and differentiated cells was confirmed by PCR. A similar PCR analysis did not show recombined substrate in neuronally differentiated cells. A positive control in which the same pEGRR introduce into a B-cell line was lacking in this experiment due to the technical difficulties in maintaining B-cells.

Since this experiment did not show V(D)J like recombination in neuronal cells, alternative approach to check the biochemical function of RAG1 in neurons was implemented.
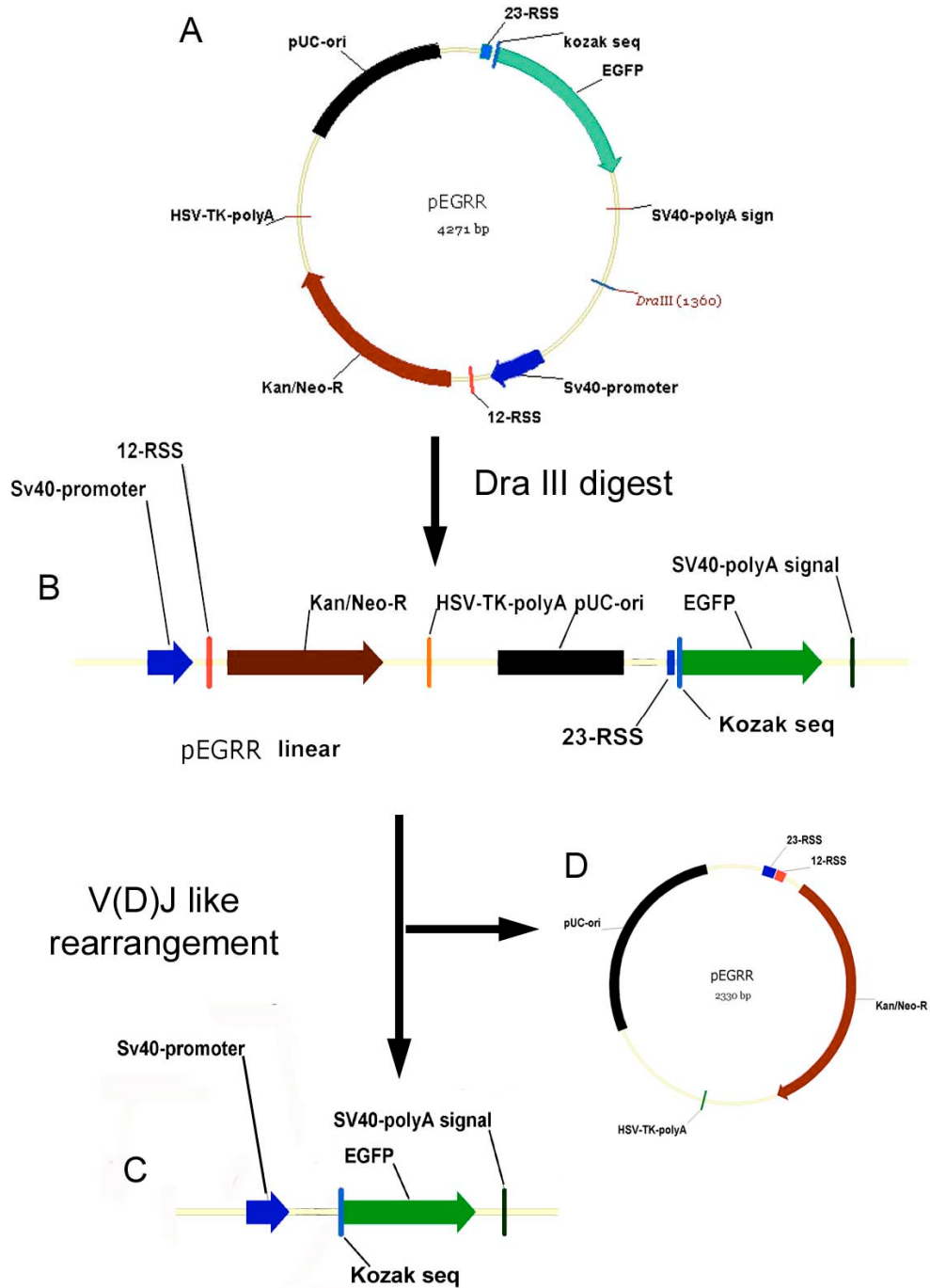
Figurre 3.6

Figure 3.6: Design of the artificial recombination substrate. A cicrular plasmid pEGRR (A). is linearized with Dra III resulting in the orginal configuration (B). V(D)J like rearrangement should result in EGFP immediately downstream of the SV40 promoter (C) and deletion/circularization of the Kan/Neo-R, pUC ori region (D)

3.4.3 Testing the possibility (standardization) of ChIP (chromatin immunoprecipitation) assay:

Chromatin immunoprecipitation has previously been used to confirm the binding of RAG1 to specific loci involved in the adaptive immune response. It is known that RAG1 remains associated with the intervening non-coding region during V(D)J recombination and that this locus can be pulled down in ChIP assays using anti-Rag1 antibodies (Perkins et al., 2002).

In a standard ChIP assay, formaldehyde fixed cells are lysed mechanically by vortexing with glass beads. The cell lysate is sonicated to obtain chromatin fragments of approx. 0.5-2 kb in size. Fragments associated with the DNA binding protein are pulled down using antibodies against the protein and proteinG-sepharose beads. This chromatin immunoprecipitated DNA (ChIP-DNA) is used to analyse the enrichment of the known targets of the DNA binding protein in a PCR based assay (Figure 3.7). To increase specificity of ChIP, samples were processed in two rounds of immunoprecipitation (Double ChIP assay Figure 3.7). The protocol was standardised using mouse thymocytes at P10-14. Two different polyclonal antibodies against RAG1 protein (SC363 and SC 5599) were used for ChIP. PCR assays for a specific locus (J-α50) showed the expected level of enrichment (~ 5-7%) compared to a non-specific locus (J-κ - undetectable) in the ChIP-DNA in the test experiment (with anti-RAG1 antibodies). Both loci (J-α50 and J-κ) were undetectable in the control experiments in which either preabsorbed antibodies or no primary antibodies were used for ChIP, further confirming the specificity of the immunoprecipitation (Figure 3.8 A-C).
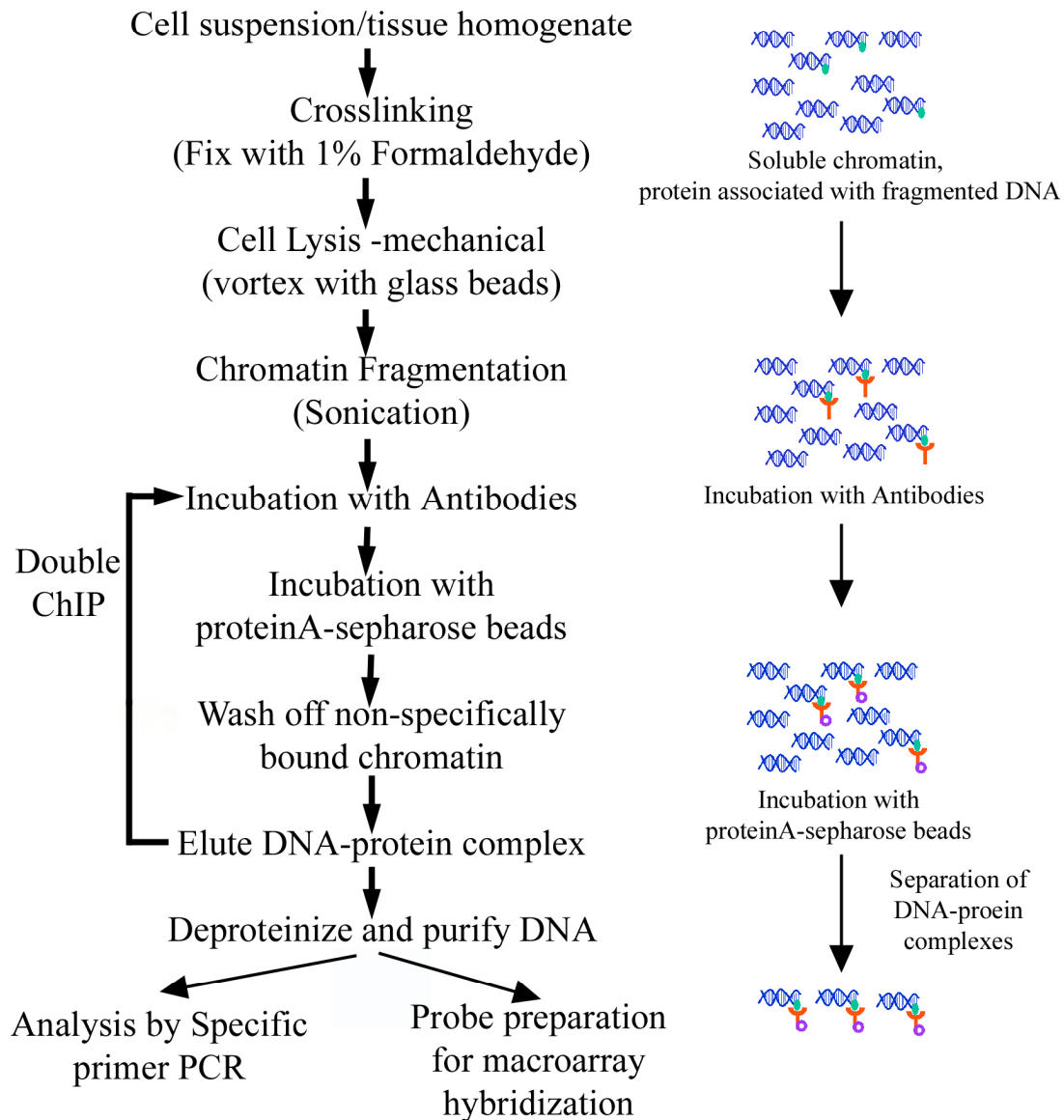
Figure 3.7

Figure 3.7: Flowchart of Double ChIP protocol and schematic
representation of principle of chromatin immunoprecipitation
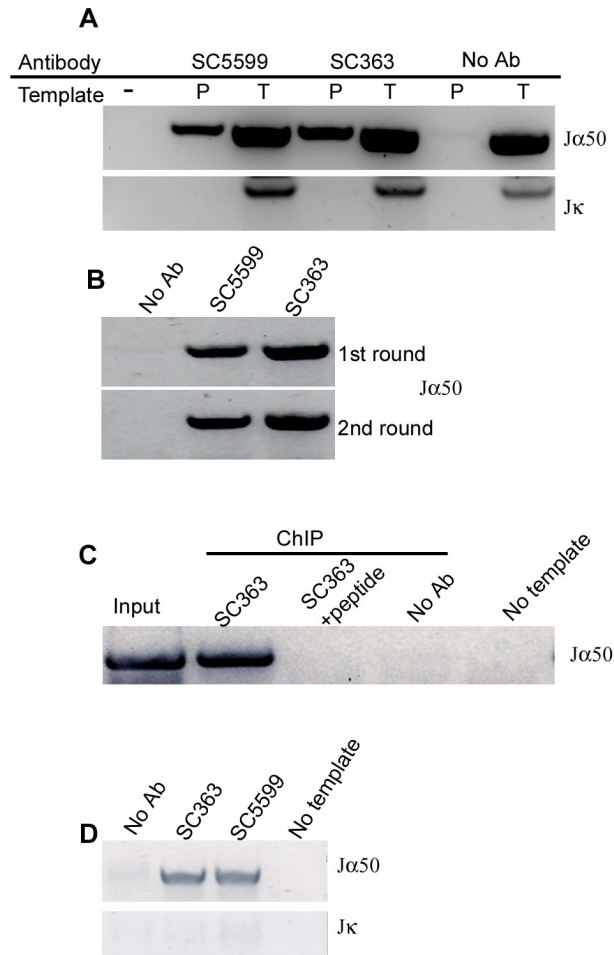
Figure 3.8

Figure legends on pg. 95

Figure 3.8: Analysis of chromatin immunoprecipitated DNA (ChIP-DNA) from thymocytes. **A.** PCR of specific target locus Jα50 – top panel and Jκ - bottom panel. Template DNA used for PCR are Lane1: no template DNA, Lane 2: ChIP DNA using anti-RAG1 polyclonal antibodies SC5599, Lane 4: ChIP DNA using anti RAG1 polyclonal antibodies SC363, Lane 6: ChIP DNA without using antibodies, Lane 3,5 and 7: 1/10th of total input DNA. **B.** PCR of specific target locus Jα50 using template DNA from single (top panel) and double ChIP (bottom panel). Lane 1: ChIP DNA without using antibodies, Lane: ChIP-DNA using anti-RAG1 polyclonal antibodies against the c-terminus of RAG1 (SC5599), Lane 3: ChIP-DNA using anti-RAG1 polyclonal antibodies against short peptide within the core domain of RAG1 (SC363). **C.** Specificity of anti-RAG1 polyclonal antibodies (SC363). Double ChIP-DNA analysis by PCR of specific target locus Jα50. Template DNA used for PCR are – Lane 1: 1/20th total input DNA. Lane 2: ChIP DNA using anti-RAG1 polyclonal antibodies (SC363). Lane 3: ChIP DNA using neutralized/peptide blocked anti RAG1 polyclonal antibodies (SC363). Lane 4: ChIP DNA without using antibodies. Lane 5: no template DNA control. **D.** PCR of specific target locus Jα50 – top panel and Jκ - bottom panel using LMPCR amplified ChIP DNA from thymocyte. Lane 1: ChIP DNA without using antibodies, Lane 2: ChIP-DNA using anti-RAG1 polyclonal antibodies –SC363, Lane 3: ChIP-DNA using anti-RAG1 polyclonal antibodies –SC5599, Lane 4: no template DNA control.

3.4.4 Chromatin immunoprecipitation and Screening YAC library macroarray :

To identify binding sites for RAG1 in neurons, without *a priori* knowledge of what these sites might be, the method of chromatin immunoprecipitation followed by hybridization to a genomic array was adopted. Brain cells from P10 mice and neuronally differentiated P19 embryonic carcinoma cells were processed as above for ChIP.

A YAC library, with 6-fold coverage of the mouse genome and arrayed on nylon filters, was used. Each clone is spotted twice on the filter (within a 4 x 4 block, in one of the 8 patterns), so only clones that have both spots labeled are considered positive. Immunoprecipitated DNA was amplified by ligation-mediated PCR to generate sufficient probe. To establish conditions for hybridization, experiments were first carried out with thymocytes ChIP-DNA. To generate enough DNA for probe preparation, ChIP-DNA was amplified by LM-PCR. As shown in Figure. 3.8-D, LM-PCR did not lead to non-specific amplification of precipitated DNA. Amplified DNA was subsequently labeled with DIG and hybridized to the membrane. A number of YACs appeared positive (Figure. 3.9 A, B). To test whether any of these corresponded to T-cell loci, a probe was made from the Jα50 locus and the hybridization pattern compared. A degree of overlap was seen (Figure. 3.9 C, D), suggesting that the thymocyte probe was binding to YACs that contain RAG1 binding sites.

Probes were then generated from neurons. Two sources of neurons were used, the first being brains of p10 mice, and the second neuronally differentiated P19 cells. When probes from brain ChIP-DNA were hybridized at high stringency to the YAC array, a single YAC (WIBRy910H0698D) was positive (Figure. 3.10 A, B). At lower stringency, a second YAC (WIBRy910E0666D) was also positive for this probe (Figure. 3.10 D).

Both YACs, but no others, were positive with probes generated using either the SC363 or the SS595 antibody for ChIP. With probes made from P19 cells, using the SC363 antibody, the YAC - WIBRy910H0698D as well as others, were positive at high stringency (Figure. 3.11). The higher number of positives with the P19 probes may be either due to the higher number of nuclei that were RAG1-positive from this source, or because additional target sites are present.
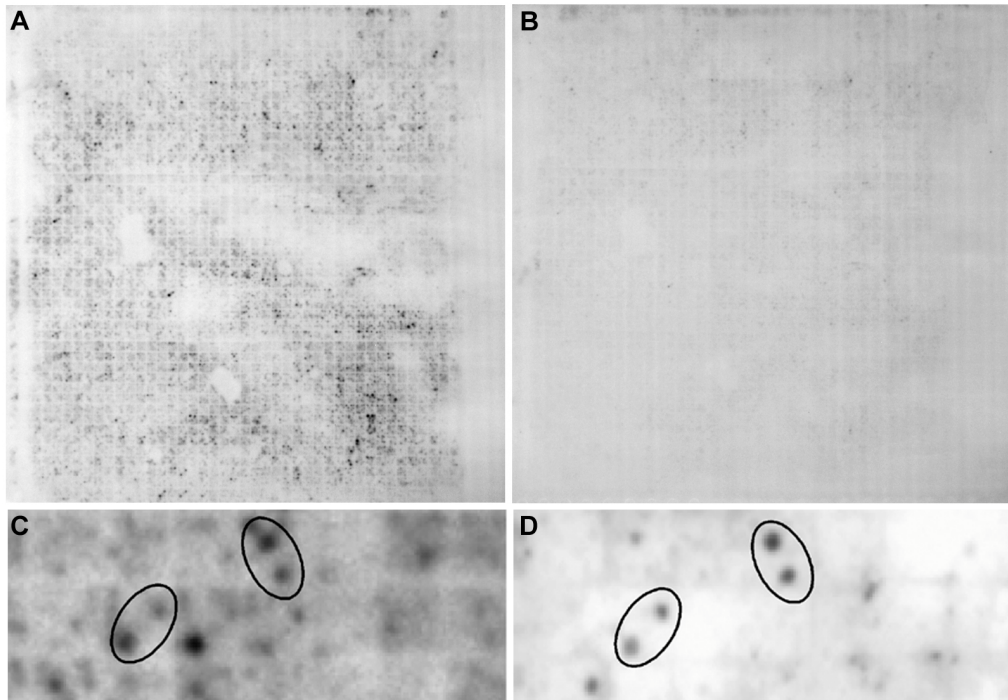
Figure 3.9

Figure3.9: Mouse genomic YAC library macrorarray hybridization with
thymocyte ChIP-DNA probe
ChIP-DNA probe from thymocyte with anti-RAG1 antibodies
(A) and without antibodies (B).
Comparison of hybridization of macroarray with ChIP-DNA
probe from thymocytes (C) and T-cell receptor specific probe
(D). YAC clones that were hybridized in both cases are circled.
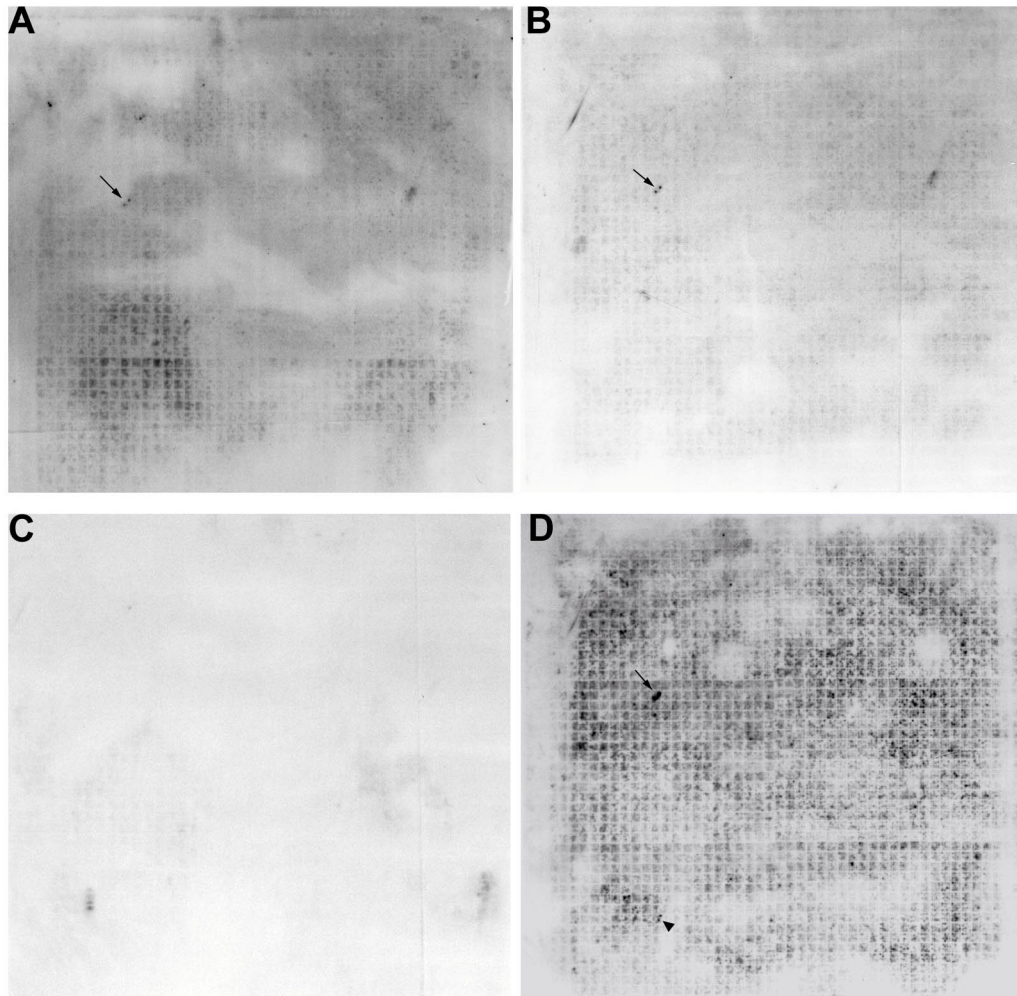
Figure 3.10

Figure 3.10: Mouse genomic YAC library macroarray hybridization
with Brain ChIP-DNA probe.
ChIp-DNA from mouse brain using SC-363 antibody
(A and D), SC-5599 antibody (B) and without antibody
(C) was hybridized at high stringency conditions (A-C)
and low stringency (D). Positive YACs clones are shown
with arrow and arrowhead.
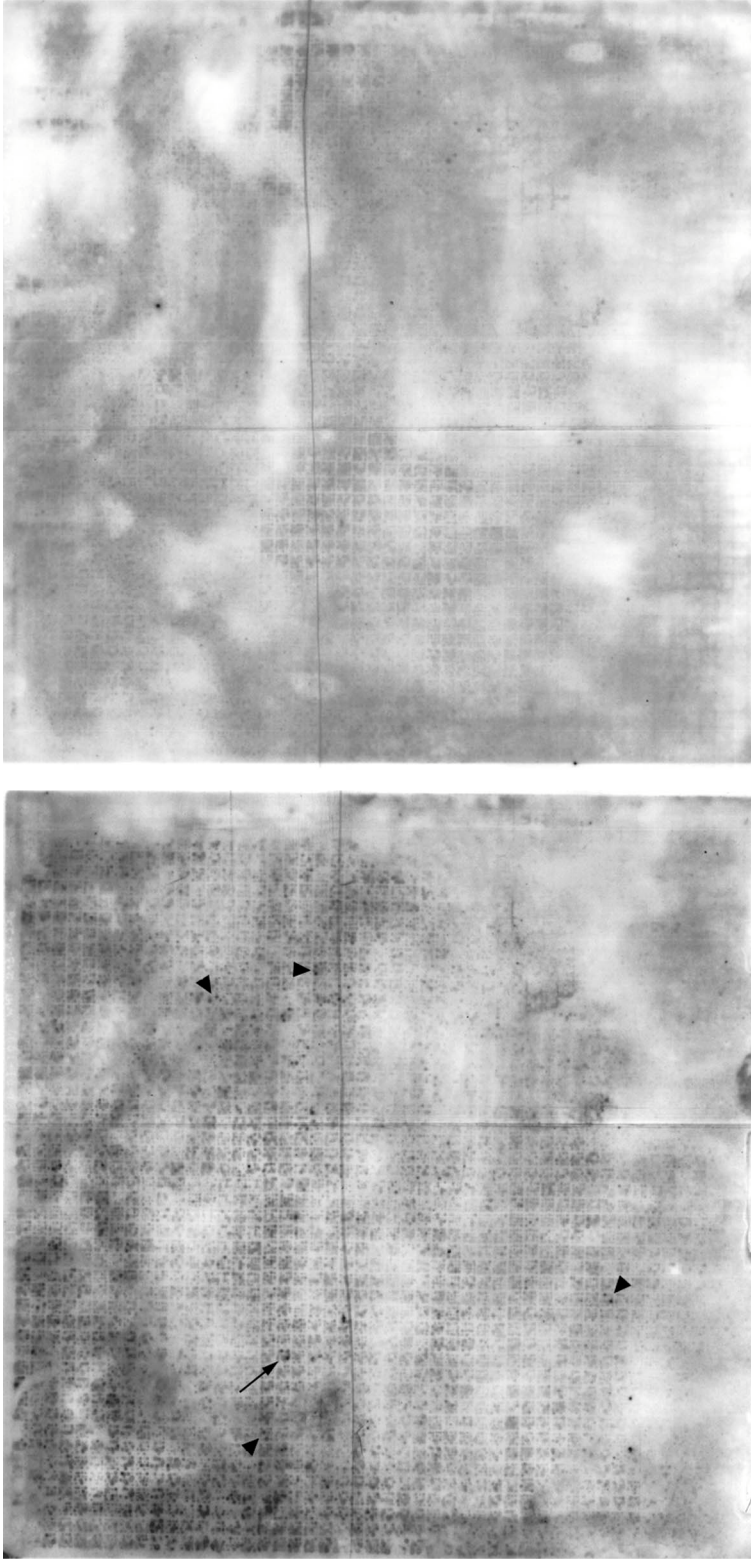
Figure 3.11

Figure 3.11: Mouse genomic YAC library macroarray hybridization with P19 ChIP-DNA probe ChIP-DNA from neuronally differentiated P19 embryonic carcinoma cells using SC-363 antibody (A) and without antibody (B) was hybridized at high stringency. The YAC clone that hybridized with brain and P19 ChIP-DNA is shown with the arrow. Additional YACs hybridized with P19 ChIP-DNA are shown with arrowheads

3.4.5 Mapping of YACs to their genomic locus

The sequence of the insert in both the YACs (WIBRy910H0698D and WIBRy910E0666D) identified using the brain probes was determined by plasmid rescue and end sequencing. Both YACs contained sequences that matched to the identical region of chromosome 9, namely band A5.2 (at approx. 32.9Mb). To further narrow down the region of putative RAG1 binding, BACs in the region of the YAC on Chromosome 9 were obtained (Figure. 3.12 A). Southern hybridization was used to narrow down the putative binding region to a 5.7 kb fragment in BAC 86O15 (Figure. 3.12 B, C). Following digestion with the EcoR-V restriction enzyme, this was further narrowed down to a 2.2 kb region.

3.4.6 Analysis of the putative RAG1 binding site

The region around the 2.2 kb site contains several features. Firstly, there are about 9.5 copies of a 208 nucleotide sequence (analyzed by Tandem Repeat Finder http://tandem.bu.edu/trf/trf.submit.options.html). Eight copies of the heptamer (CACAGTG) from the highly conserved RAG1 binding site, i.e. the recombination signal sequence (RSS), is also found within the repeat region, supporting the possibility of RAG1 binding to this region (Figure 3.13). Similar repeats occur elsewhere in the genome, but there is a 220 nucleotide unique sequence associated with the repeat that is found only on Chromosome 9. To test if binding occurs to the unique sequence or to the repeat region, PCR primers were designed to the repeat and unique region, and the immunoprecipitated DNA analyzed. Specific amplification was obtained with primers to the repeat region only (Figure. 3.12 D, E). PCR-ChIP was subsequently carried out on

thymocytes. No amplification was seen, suggesting that this region was not bound by RAG1 in thymocytes (Figure. 3.12 E). This is consistent with the results of the array hybridization using thymocyte probes, in which these YACs were not positive.

A second feature of the putative binding site is that it is located within a retrotransposon. The repeats are virtually identically to the monomers found in the 5'UTR of the A101-type LINE-1 element (Goodier et al., 2001). The sequence downstream to this repeat region shows almost 100% identity to ORF1 (mRNA U16672) and ORF2 of L1. A-type L1 retrotransposons are known to be transcriptionally active and to retrotranspose in cultured cells (DeBerardinis et al., 1998). ESTs of ORF1 and ORF2 have been found in several neonatal mouse cDNA libraries, including a cerebellar library (e.g. AU079979, BB251308), indicating that this element is transcribed *in vivo*. However, as the mouse genome contains approximately 730 copies of this element (ref: UCSC genome browser), it is not clear where the element originates from.

A

Chr. IX

33.0 33.1 33.2 33.3 33.4 33.5 33.6

1   2   3   4   5   6   7

B 1 2 3 4 5 6 7 8
B E B E B E B E B E B E B E B E

10.0—
8.0—
6.0—
5.0—
4.0—
3.0—
2.0—
1.5—

C 1 2 3 4 5 6 7 8
B E B E B E B E B E B E B E B E

—10.0
—8.0
—6.0
—5.0
—3.0
—2.0
—1.5
—1.0

D 28 56 84 112 140 168 196 222

- 5.7 Kb potential target
- 2.2 Kb potential target
- Transposase
- Reverse Transcriptase

E 1F 2F
1R 2R

F

ANTIBODY
+ -
1 2 3 4

				Template source	Region amplified
				brain	unique
				brain	repeat
				thymus	repeat
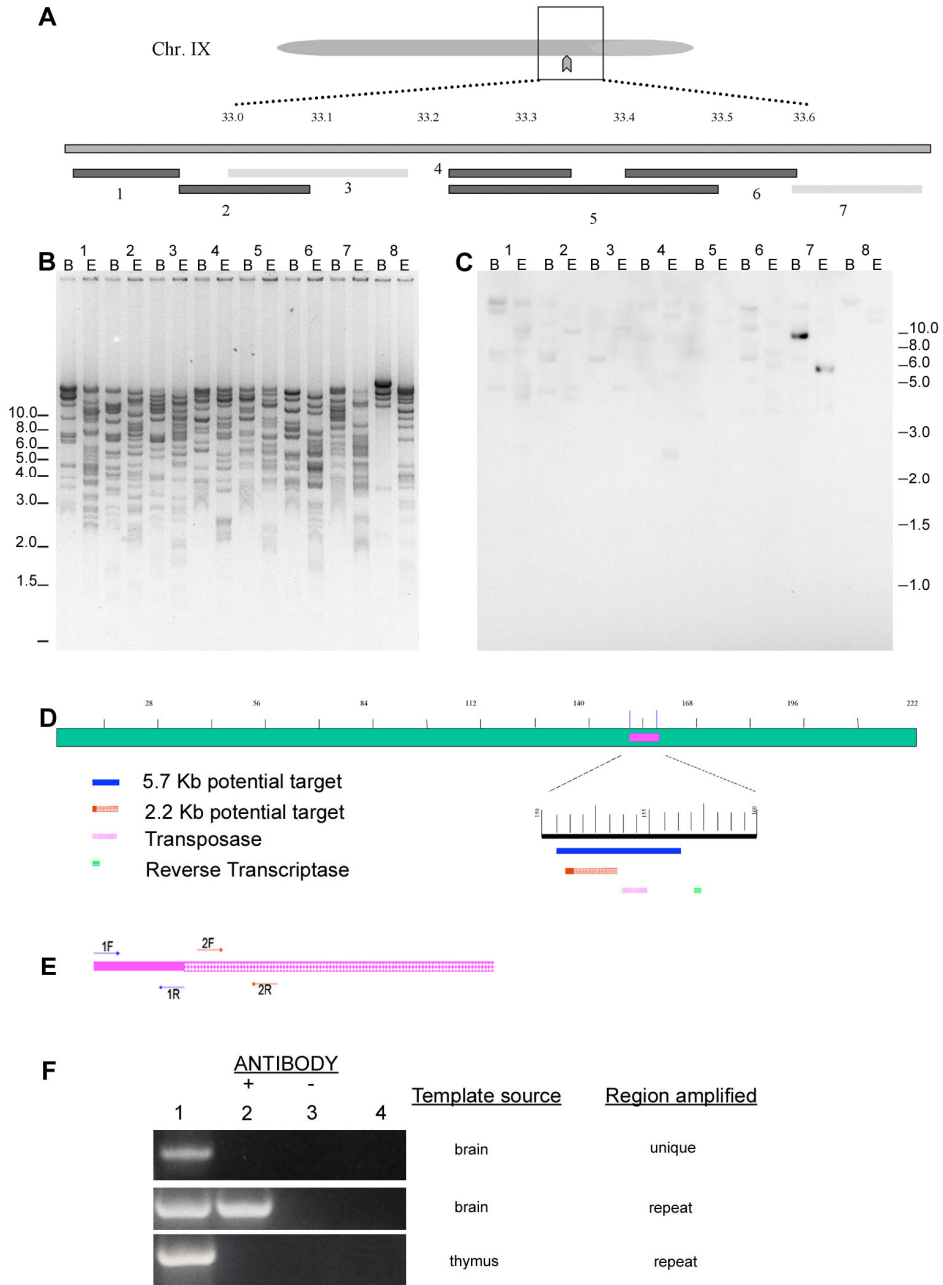
Figure 3.12
Figure legends on pg. 104

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Figure 3.12: Fine mapping of potential RAG1 target in brain **A.** BAC contig covering the region of mouse chromosome-9 to which the positive YAC was mapped. BAC clones denoted by numbers are 1: RP23-65J2, 2: RP24-11A6, 3: RP23-84E4, 4: RP86-B13, 5: RP23-93M1, 6: RP24-308I2, 7: RP24-86O15. **B.** BamH-I and EcoR-I digest of BAC clones, resolved on 0.8% agarose gel stained with Gel-star. **C.** Southern analysis of BAC digest with brain ChIP-DNA probe. Letters on the top of panel (B) and (C) denote B:BamH-I , E: EcoR-I and numbers 1-7 denotes BAC clones as in panel (A), BAC clone 8 is from a zebrafish library and serves as a negative control for Southern hybridization. **D.** Schematic of BAC 86O15. Blue bar showing the region corresponding to the 5.7Kb band that hybridised with brain ChIP-DNA probe shown in panel (C), Red bar shows further fine mapped 2.2 Kb potential target. Predicted tranposases and reverse transcriptase are shown with pink and green bars respectively. **E,F.** PCR analysis of the ChIP-DNA from thymocyte and LMPCR amplified ChIP-DNA from brain for the potential target region of RAG1 in brain. The schematic (E) shows the location of primers to the unique region (1F and 1R) and repeat region (2F and 2R). Top panel in (F) shows the PCR for unique region using ChIP-DNA from brain. PCR for the repeat region using brain ChIP-DNA (middle panel) and thymus ChIP-DNA (bottom panel). Template DNA used for PCR are Lane1 – total input, Lane2 - ChIP DNA using anti RAG1 polyclonal antibodies, Lane3 - ChIP DNA without using antibodies, Lane4 - no template DNA control.

```
   1    tatcataaac tgggtgcagc agatggccag tgttttgtgg gagaaagcgt
        tgctcagtag ctgctgtcat gaagaccttt aggttaattt aaagctaaga
 101    gcacactatt ggtccaatga tggaaaagta ggtattcatc atgttgccta
        tccagagtct gctagtgatt ttgcccaact ccaaacagta atgcaagaat
 201    agtgaaagca gactcgaGCC CCGGGCTACC TTGCCAGCAG AGTCTTGCCC
        AACACCTGCA AGGGCCCACA TGGGACTCCC CACGGGAACC TAAGACCTCT
 301    GGTGAGTGGA CCACAGTGCC TGCCCCAATC CAATCGCGCG GAACTCGAGA
        CTGCGGTACA TAGGGAAGCA GGCTACCCGG GCCTGATCTG GGGCACAAGT
 401    CCCTTCCGCT CGACTCGAGA CTCGAGCCCC GGGCTACCTT GCCAGCAGAG
        TCTTGCCCAA CACCCGCAAG GGTCCACACG GGACTCCCCA CGGGACCCTA
 501    AGACCTCTGG TGAGTGGATC ACAGTGCCTG CCCCAATCCA ATCGCGTGGA
        ACTTGAGACT GCGGTACATA GGGAAGCAGG CTACCCGGGC CTGATCTGGG
 601    GCACAAGTCC CTTCCGCTCG ACTCGTGACT CGAGCCCCGG GCTACCTTGC
        CAGCAGAGTC TTGCCCAACA CCCGCAAGGG TCCACACGGG ACTCCCCACG
 701    GGACCCTAAG ACCTCTGGTG AGTGGATCAC AGTGCCTGCC CCAATCCAAT
        CGCGCGGAAC TCGAGACTGC GGTACATAGG GAAGCAGGCT ACCCGGGCCT
 801    GATCTGGGGC ACAAGTCCCT TCCGCTCGAC TCGAGACTCG AGCCCCGGGC
        TACCTTGCCA GCAGAGTCTT GCCCAACACC CGCAAGGGTC CACACGGGAC
 901    TCCCCACGGG ACCCTAAGAC CTCTGGTGAG TGGATCACAG TGCCTGCCCC
        AATCCAATCG CGTGGAACTT GAGACTGCGG TACATAGGGA AGCAGGCTAC
1001    CCGGGCCTGA TCTGGGGCAC AAGTCCCTTC CGCTCGACTC GAGACTCGAG
        CCCCGGGCTA CCTTGCCAGC AGAGGCTTGC CCAACACCCG CAAGGGTCCA
1101    CACGGGACTC CCACGGGAC CCTAAGACCT CTGGTGAGTG GATCACAGTG
        CCTGCCCCAA TCCAATCGCG CGGAACTTGA GACTGCGGTA CATAGGGAAG
1201    CAGGCTACCC GGGCCTGATC TGGGGCACAA GTCCCTTCCG CTCGACTCGA
        GACTCGAGCC CCGGGCTACC TTGCCAGCAG AGTCTTGCCC AACACCCGCA
1301    AGGGTCCACA CGGGACTCCC CACGGGACCC TAAGACCTCT GGTGAGTGGA
        TCACAGTGCC TGCCCCAATC CAATCGCGTG GAACTTGAGA CTGCGGTACA
1401    TAGGGAAGCA GGCTACCCGG GCCTGATCTG GGGCACAAGT CCCTTCCGCT
        CGACTCGAGA CTCGAGCCCC GGGCTACCTT GCCAGCAGAG GCTTGCCCAA
1501    CACCCGCAAG GGTCCACACG GGACTCCCCA CGGGACCCTA AGACCTCTGG
        TGAGTGGATC ACAGTGCCTG CCCCAATCCA ATCGCGCGGA ACTTGAGACT
1601    GCGGTACATA GGGAAGCAGG CTACCCGGGC CTGATCTGGG GCACAAGTCC
        CTTCCGCTCG ACTCGAGACT CGAGCCCCGG GCTACCTTGC CAGCAGAGGC
1701    TTGCCCAACA CCCGCAAGGG TCCACACGGG ACTCCCCACG GGACCCTAAG
        ACCTCTGGTG AGTGGATCAC AGTGCCTGCC CCAATCCAAT CGCGCGGAAC
1801    TTGAGACTGC GGTACATAGG GAAGCAGGCT ACCCGGGCCT GATCTGGGGC
        ACAAGTCCCT TCCGCTCGAC TCGAGACTCG AGCCCCGGGC TACCTTGACA
1901    GCAGAGTCTT GCCCAACACC CGCAAGGGCC CACACGGGAC TCCCCACGGG
        ACCCTAAGAC CTCTGGTGAG TGGAACACAG CGCCTACCCC AATCCAATCG
2001    CGTGGAACTT GAGACTGCGG TACATAGGGA AGCAGGCTAC CCGGGCTTGA
        TCTGGGGCAC AAACCCCTTC CACTCCACTC GAGCCCCGGC TACCTTGCCA
2101    GCTGAGTCGC CTGACACCCG CAAGGGCCCA CACAGGATTC CACACGTGAT
        CCTAAGACCT CTAGTGAGTG GAACACAACT TCTGCCAGGA GTCTGGTTCG
2201    AACACCAGAT
```

Figure 3.13: Sequence of 2.2Kb target region. The unique sequence is indicated in red color text (lower case), monomer of repeat sequence is in blue color text (bold type). RSS heptamer sequence CACAGTG is shown in pink color bold text type (underlined).

3.4.7 BAC macroarray hybridization

The failure of primers to the unique region to amplify the ChIP-DNA raises the possibility that RAG1 binding actually occurs to another genomic locus that contains this motif and not chromosome-9. Indeed, an analysis of mouse genome indicates the existence of numerous repeats of this 208 nucleotide sequence. The fact that the only one YAC clone was positive may be due to the nature of the YAC library. Hence a BAC library with 11 times coverage of mouse genome, (RPCI-23 created by Kazutoyo Osoegawa, Dr. Pieter J. de Jong , and Minako Tateno) arrayed on 7 nylon membranes (RZPD library no. 710) was screened with the brain ChIP-DNA probe. Fifty six BACs appeared positive, out of these 44 clones were mapped to the mouse genome based on Ensemble mouse genome assembly -2005. Chromosome location and sequence information was not available for 12 clones. Mapping of these 49 BACs revealed 24 different loci on various chromosomes. Three of these loci which mapped to chromosome X, 2, and 14, showed the presence of a 5'UTR of the L1 element. Most of other contained L1 ORFs but lacked 5'UTR repeats.   In addition three loci were found that did not contain L1 elements (Table 3.1). None of the BACs mapped to TCR or immunoglobulin locus.

Table 3.1: BAC macroaary data, chromosome location of BAC clones

BAC #	loci #	Filter ID	X1	Y1	X2	Y2	Ensemble ID	Chromosome	From	To	Genes within or nearby the BAC location	Repeats within BACs
1		402.2.558	133	72	134	75	RP23-236B22	1	122883953	123111631	dipeptidylpeptidase 10 isoform 2	42bpx1.9-L1MD-TF2 retrotransposon, **L1Md ORFs**
2		402.2.558	93	147	94	50	RP23-235C6	1	122950980	123111631	dipeptidylpeptidase 10 isoform 2	42bpx1.9-L1MD-TF2 retrotransposon, **L1Md ORFs**
3		402.4.507	47	106	47	108	RP23-319K15	1	122963061	123138996	dipeptidylpeptidase 10	42bpx1.9-L1MD-TF2 retrotransposon, **L1Md ORFs**
4	1	402.4.507	41	198	43	200	RP23-341I16	1	123014336	123227225	dipeptidylpeptidase 10 isoform 2	42bpx1.9-L1MD-TF2 retrotransposon, **L1Md ORFs**
5		402.4.507	31	213	33	215	RP23-341F18	1	122923709	123110841	dipeptidylpeptidase 10 isoform 2	42bpx1.9-L1MD-TF2 retrotransposon, **L1Md ORFs**
6		402.4.507	195	9	192	10	RP23-314O10	1	122964197	123166521	dipeptidylpeptidase 10 isoform 2	42bpx1.9-L1MD-TF2 retrotransposon, **L1Md ORFs**
7		402.5.541	39	183	36	184	RP23-473L17	1	122963063	123153541	dipeptidylpeptidase 10	
8		402.4.556	63	67	64	70	RP23-382C12	4	150859383	151068494	------------------	SINE
9		402.4.556	23	72	24	75	RP23-382B20	4	150859408	151068497	------------------	
10	2	402.4.556	37	79	40	80	RP23-406A17	4	150888574	151131195	------------------	
11		402.4.556	62	104	65	105	RP23-403L12	4	150918542	151155359	------------------	
12		402.5.541	153	16	155	17	RP23-452M18	4	150859228	151068498	------------------	
13		402.5.541	153	17	154	20	RP23-452M18	4	150859228	151068498	------------------	
14		402.2.510	137	46	137	48	RP23-176G21	6	115021961	115249428	AGP-7, Autophagy-7-ubiquitin activating enzyme, Histamine H1 receptor	**Chr 6-1** 39bp-heptamer containing repepatx6.5copies
15	3	402.4.507	211	96	214	96	RP23-300M6	6	115021961	115133119	AGP-7, Autophagy-7-ubiquitin activating enzyme, Histamine H1 receptor	39bp-heptamer containing repepatx6.5copies
16		402.5.541	52	234	55	235	RP23-479B14	6	115023223	115101376		
17		402.2.558	93	197	94	200	RP23-239I6	6	4525301	4686532	protein phosphatase 1, regulatory (inhibitor) subunit 9A; neurabin; neural tissue-specific F-actin binding protein	**L1Md ORFs**
18		402.4.556	166	107	166	110	RP23-366K15	6	4496279	4655916	protein phosphatase 1, regulatory (inhibitor) subunit 9A; neurabin; neural tissue-specific F-actin binding protein	**L1Md ORFs**
19	4	402.5.541	147	159	150	160	RP23-480A19	6	4529412	4761785	protein phosphatase 1, regulatory (inhibitor) subunit 9A; neurabin; neural tissue-specific F-actin binding protein	**L1Md ORFs**
20		402.5.541	28	72	29	75	RP23-454B19	6	4451912	4637889		**L1Md ORFs**
21		402.5.541	28	71	30	72	RP23-454B19	6	4451912	4637889		**L1Md ORFs**
22	5	402.4.556	24	7	23	9	RP23-424O20	8	82883409	83100584	**1.**RGS19-interacting protein 1 (GAIP C-terminus interacting protein GIPC) (RGS-GAIP interacting protein) (Synectin) (SemaF cytoplasmic domain associated protein 1) (SEMCAP-1). **2.**Prostaglandin E2 receptor, EP1 subtype (Prostanoid EP1 receptor) (PGE receptor, EP1 subtype). **3.**DEAD (Asp-Glu-Ala-Asp) box polypeptide 39 **4.** Protein kinase C-like 1. **5.** CD97 antigen precursor	
23		402.4.556	7	46	7	48	RP23-394G23	8	82823910	83132029		
24	6	402.4.507	156	143	158	145	RP23-342D17	18	7753678	7954397	similar to palmitoylated membrane protein 7	
25		402.4.556	70	94	67	95	RP23-385N11	18	7751867	7978683	similar to palmitoylated membrane protein 7	partial **L1Md-A101** detected
26		402.5.541	147	114	150	115	RP23-480J19	14	61068330	61239347	NK2 transcription factor related,Homeobox protein Nkx-3.1. , mitochondrial solute carrier protein	**L1Md with 5'UTR repeat region 208bpx5.6**
27	7	402.5.541	160	19	157	20	RP23-458M17	14	61084637	61263849		
28	8	402.2.508	178	61	180	62	RP23-140D13	7	50569551	50845499	Cezanne 2, Zinc finger A20 domain containing protein ?	204bpx2.9-similar to RT, GAG protein, **L1Md-A101** partial
29	9	402.2.510	143	86	145	82	RP23-216P20	8	15804954	15979377	Csmd1, CUB and Sushi multiple domain protein	42bpx2.5-L1MD-TF2 retrotransposon, **L1Md-A101** ORF1 partial
30	10	402.2.558	192	49	195	50	RP23-260G10	13	107516844	1077688947		20bpx2.3-similar to tumor supressor gene, **L1Md-A101** ORF partial
31	11	402.1.505	27	81	27	83	RP23-31P19	X	114660407	114924706	------------------	**L1Md-A101 A-typeL1 208bpx5**

Table 3.1 continued

BAC #	loci #	Filter ID	X1	Y1	X2	Y2	Ensemble ID	Chromosome	From	To	Genes within or nearby the BAC location	Repeats
32	12	402.2.558	47	94	50	95	RP23-259N15	3	149136492	150136490	Latrophilin 2 (Calcium-independent alpha-latrotoxin receptor 2),MUS MUSCULUS 0 DAY NEONATE CEREBELLUM CDNA PRODUCT: FRAGMENT,MUS MUSCULUS ADULT MALE TESTIS CDNA PRODUCT	42bpx1,9-L1-TF repeat, 39bpx2 similar to GAG polyprotein, **L1Md-A101** partial ORF
33	13	402.2.558	66	237	66	240	RP23-221A11	6	88185413	88362182	Coatomer gamma-2 subunit (Gamma-2 coat protein) (Gamma-2 COP),MUS MUSCULUS 11 DAYS EMBRYO HEAD CDNA PRODUCT:UNKNOWN EST FRAGMENT,Cellular nucleic acid binding protein (CNBP),, gene model 461; H1 histone family, member X,Ras-related protein Rab-7	77bp and 49bp repeat matching to cDNA clone, 39bpx2 similar to GAG protein, **L1Md-A101** partial ORF
34	14	402.4.507	127	51	127	53	RP23-320F23	4	45904271	46092215	similar to TBC1 domain family member 2 (Prostate antigen recognized and indentified by SEREX) (PARIS-1),coronin, actin binding protein 2A; coronin 4,,novel gene similar to GABA-B receptor 2,	40bpx23.6
35	15	402.4.507	132	51	132	53	RP23-320F22	8	1277710827	127932654	Neuropilin-1 precursor (A5 protein).	37bpx2,3-SINE, **L1Md-A101**ORF1, pheromone receptor VR3R4
36	16	402.4.507	11	32	11	35	RP23-292J22	6	77221126	77670634	Alpha-2 catenin,leucine-rich repeat transmembrane neuronal 1	**L1Md-A101** partial ORF1
37	17	402.4.507	11	37	11	40	RP23-292I22	18	84484440	84777570	smillar to zinc binding alcohol dehydrogenase, novel zinc finger protein	L1Md-A102 partial
38	18	402.4.507	180	11	179	14	RP23-302N13	12	88052227	88236977		**L1Md-A101** partial ORF1, GAG protein
39	19	402.4.507	140	229	137	230	RP23-315C21	3	91404787	91574191	novel gene	**L1Md-A101** partial ORF1, GAG protein
40	20	402.4.556	171	17	171	20	RP23-362M14	X	88164493	88442959	G protein-coupled receptor PGR15L (CLOSE TO BAC)	**L1Md-A101** partial ORF1 212bpx2
41	21	402.4.556	51	23	51	25	RP23-412L14	4	150888574	151089072		-------
42	22	402.4.556	30	51	29	54	RP23-376F19	2	22027342	22203981	Close -myoll, G-protein receptor	**L1Md-A101 208bpx26.6copies**
43	23	402.4.556	93	56	95	57	RP23-430E6	13	1074426068	1077712352		**L1Md-A101** ORF1and2
44	24	402.5.541	104	183	101	184	RP23-473L4	5	88928041	89121776	Probable inner membrane protein OXA1L2, ankyrin repeat domain protein 17 isoform a	**L1Md-A101** L1Md-A102
45		402.1.505	117	75	120	74	RP23-28B1	N-M	------	------	------	------
46		402.2.508	147	49	150	50	RP23-116G19	N-M	------	------	------	------
47		402.2.508	186	107	186	110	RP23-78K11	N-M	------	------	------	------
48		402.2.508	113	67	114	70	RP23-94C2	N-M	------	------	------	------
49		402.2.508	188	117	189	120	RP23-96I11	N-M	------	------	------	------
50		402.2.510	134	52	133	54	RP23-206F22	N-M	------	------	------	------
51		402.2.510	137	47	133	49	RP23-206G22	N-M	------	------	------	------
52		402.4.556	177	39	180	40	RP23-404I3	N-M	------	------	------	------
53		402.4.556	17	46	17	48	RP23-394G21	N-M	------	------	------	------
54		402.4.556	159	128	156	129	RP23-402G17	N-M	------	------	------	------
55		402.4.556	205	231	204	234	RP23-375B8	N-M	------	------	------	------
56		402.5.541	62	204	65	205	RP23-479H12	N-M	------	------	------	------

Table 3.1: Mouse genomic BAC macroarrays screened with Brain ChIP-DNA probe resulted in 56 positive clones, 44 clones were mapped to 24 loci (column-2). Co-ordinate position of these positive BAC clones on 7 different filters and their Ensemble ID numbers are shown in column 3-8. Chromosomes to which these BACs were mapped and their location (in base pair numbers) shown in column 9-11. Genes within in or nearby the BAC location and repeats found within the BAC are shown in column 12 and 13. Pink boxes highlights BAC carrying L1Md-A101 5' repeats.

3.5   *Discussion :*

I have used immunofluorescence followed by chromatin immunoprecipitation to investigate whether RAG1 may have a function in the vertebrate nervous system. This study shows that the RAG1 protein is present in specific neurons in the mouse brain, which have previously been identified by *in situ* hybridization to express *Rag1*. This correlation, together with the absence of signal with peptide-blocked antibody, indicates that the label obtained provides an accurate description of the distribution of RAG1 in the brain. RAG1 localization does not appear to be identical in all neurons: some neurons have more nuclear protein whereas others have more cytoplasmic RAG1.

An artificial recombination substrate was introduced in P19 cells to check the possibility of V(D)J like recombination in neuronal cells. Recombination was not observed in neuronally differentiated P19 cells either phenotypically (EGFP expression) or at a molecular level by PCR assays. This is consistent with previous studies by Chun *et al.* reporting a lack of recombination in P19 cells using an artificial substrate (Chun et al., 1991) and the recent report suggesting that RSS are not used for recombination in neuronally differentiated P19 cells (Kawabata et al., 2004). This is likely because the RAG2 is not expressed in these cells.

But nuclear localization of RAG1 in P19 cells as well as in brain cells was sufficient grounds to investigate RAG1 interaction with DNA in these cells.

A relatively new approach of double chromatin immunoprecipitation coupled with whole genome macroarray screening was used in this study. Double ChIP approach was shown to enhance the specificity without losing the efficiency of pull down (Weinmann

and Farnham, 2002). Indeed, in this study I find that after two rounds of immunoprecipitation the specific TCR locus region Jα50 shows a similar level of amplification from ChIP-DNA as after the first round, whereas the non-specific locus does not show any amplification (Fig. 3.8). The ChIP & chip approach in which the pulled down DNA is used as a probe to screen microarray chips has been used (Ren et al., 2000). This approach is useful in identifying unknown targets of transcription factors with the use of specific microarrays. In this study I used whole genomic macroarray to scan the entire genome as there was no prior information about the location of potential RAG1 targets in neurons. In a YAC library screen, brain ChIP-DNA probe hybridized to a single clone, whereas the BAC library screen resulted in 56 positive clones.

Chromatin immunoprecipitation, using either whole brains or a cell line as source of nuclei, suggests that RAG1 binds to specific loci in the genome of neurons. Analysis of the positive YAC clone identified a putative binding site that contains eight copies of the conserved heptamer found in all vertebrate recombination signal sequences. The nonamer, which is less well conserved, especially near embedded heptamers (Fanning et al., 1998) and in cryptic recombination signals (Cowell et al., 2003), was not detected. This site turned out to be the L1 repeat located on chromosome-9. The unique sequence associated with this repeat region at chromosome-9 locus did not amplify from brain ChIP-DNA suggesting that the repeat bound by RAG1 is located elsewhere in the genome. Consistent with this, the 5 positive BAC clones containing L1 repeat regions map to different chromosomes.

It is clear from some cancers, such as Burkitt's lymphoma, that RAG1 can cut DNA and mediate transposition in cryptic sequences (Vaandrager et al., 2000; Raghavan et al.,

2004). Also recent studies showing similarity of RAG1 and its recognition sites to *transib* transposon supports that RAG1 alone may function as transposases (Kapitonov and Jurka, 2005). To prevent unwanted RAG1 induced breaks, access to the genome is normally tightly regulated. The fact that RAG1 binding occurs at this site in neurons, raises the possibility that binding has a useful function.

An unexpected finding is that the site identified here lies in the 5' region of a L1Md A-type retrotransposon. Although mobile elements such as retrotransposons are sometimes considered junk DNA, there is evidence that these elements can sometimes be specifically regulated and even have physiological roles (Britten, 1997). In the *Drosophila* visual system, for example, transcription of a retrotransposon is activated when axons contact their target (Mozer and Benzer, 1994), presumably because of a factor transported by the axons. In fission yeast, the LTR of retrotransposons are able to silence adjacent genes through an RNAi-dependent mechanism (Schramke and Allshire, 2003). A similar phenomenon occurs in mouse cells, where RNA of the VL30 retrotransposon binds to PSF (pre m-RNA splicing factor) protein and enables the induction of genes normally repressed by PSF (Song et al., 2004). Recent studies shows that L1 retrotansposition occurs in neural progenitor cells, and may have a role in generating neuronal diversity (Muotri et al., 2005; Ostertag and Kazazian, 2005).

It should be informative next to determine whether and how RAG1 affects L1 elements, and if this affects neurons. One possibility is that binding of RAG1 the retrotransposon may causes DNA breaks. I have attempted to use terminal transferase dependent PCR (TD-PCR), which has been used for detecting DNA adducts (Komura and Riggs, 1998) to detect endogenous DNA breaks at TCR locus and A-type L1. This

approach did not yield significant information probably due to high level background (unbroken) DNA in the sample. It is possible that RAG1 binding to 5' region of the L1 may have other effects such as controling the expression of L1 ORF or the neighboring genes. It would be worth checking expression of L1 ORF in wildtype and *rag1* mutant mice brains.

The A-type L1 repeats are found only in the mouse genome. This means that the potential site identified here is applicable only to the mouse genome. Other binding sites may exist, both in mouse and other vertebrates. A further analysis of BAC clones as well as YAC clones bound by ChIP-DNA from neuronally differentiated P19 might identify these other sites.

In summary, this study establishes that RAG1 protein is present in neurons, in regions of the brain where a LacZ reporter has suggested that recombination can occur (Matsuoka et al., 1991). Chromatin immunoprecipitation indicates that RAG1 can bind to the genome of mouse neurons specifically in a repeat associated with a retrotransposon. This indicates that RAG1 has DNA binding activity in neurons and binds loci that are distinct from those in thymocytes. Thus the data reported here represent initial steps in unraveling the question of whether RAG1, which revolutionized the vertebrate immune system, has had any effect on evolution of the vertebrate nervous system. Further molecular and biochemical analysis along with behavioral and physiological studies should solve the puzzle of *Rag1* expression in neurons.

Appendix

Studies presented in Chapter-2 on *gnarled* mutant (in figure 2.7, 2.8 and 2.9) were carried out by other members of Dr. Jesuthasan's lab.

References

Abdel-Majid RM, Leong WL, Schalkwyk LC, Smallman DS, Wong ST, Storm DR, Fine A, Dobson MJ, Guernsey DL, Neumann PE. 1998. Loss of adenylyl cyclase I activity disrupts patterning of mouse somatosensory cortex. Nat Genet 19:289-291.

Abeliovich A, Gerber D, Tanaka O, Katsuki M, Graybiel AM, Tonegawa S. 1992. On somatic recombination in the central nervous system of transgenic mice. Science 257:404-410.

Agrawal A, Eastman QM, Schatz DG. 1998. Transposition mediated by RAG1 and RAG2 and its implications for the evolution of the immune system. Nature 394:744-751.

Agrawal A, Schatz DG. 1997. RAG1 and RAG2 form a stable postcleavage synaptic complex with DNA containing signal ends in V(D)J recombination. Cell 89:43-53.

Alsina B, Vu T, Cohen-Cory S. 2001. Visualizing synapse formation in arborizing optic axons in vivo: dynamics and modulation by BDNF. Nat Neurosci 4:1093-1101.

Aparicio OM. 1999. Characterization of Proteins Bound to Chromatin by Immunoprecipitation from Whole Cell Extracts. In: Ausubel FM, Brent R, Moore DD, Seidman JG, Smith JA, Stuhl K, Ausubel FM, Brent R, Moore DD, Seidman JG, Smith JA, Stuhl Ks. Current Protocols in Molecular Biology. John Wiley & Sons, Inc. p 21.23.21-21.23.12.

Augsburger A, Schuchardt A, Hoskins S, Dodd J, Butler S. 1999. BMPs as mediators of roof plate repulsion of commissural neurons. Neuron 24:127-141.

Barsoum J, Brown R, McKee M, Boyce FM. 1997. Efficient transduction of mammalian cells by a recombinant baculovirus having the vesicular stomatitis virus G glycoprotein. Hum Gene Ther 8:2011-2018.

Bas A, Hammarstrom SG, Hammarstrom ML. 2003. Extrathymic TCR gene rearrangement in human small intestine: identification of new splice forms of recombination activating gene-1 mRNA with selective tissue expression. J Immunol 171:3359-3371.

Becker CG, Meyer RL, Becker T. 2000. Gradients of ephrin-A2 and ephrin-A5b mRNA during retinotopic regeneration of the optic projection in adult zebrafish. J Comp Neurol 427:469-483.

Bellon SF, Rodgers KK, Schatz DG, Coleman JE, Steitz TA. 1997. Crystal structure of the RAG1 dimerization domain reveals multiple zinc-binding motifs including a novel zinc binuclear cluster. Nat Struct Biol 4:586-591.

Bernstein RM, Schluter SF, Bernstein H, Marchalonis JJ. 1996. Primordial emergence of the recombination activating gene 1 (RAG1): sequence of the complete shark gene indicates homology to microbial integrases. Proc Natl Acad Sci U S A 93:9454-9459.

Bertrand N, Castro DS, Guillemot F. 2002. Proneural genes and the specification of neural cell types. Nat Rev Neurosci 3:517-530.

Black DL. 1998. Splicing in the inner ear: a familiar tune, but what are the instruments? Neuron 20:165-168.

Bossing T, Brand AH. 2002. Dephrin, a transmembrane ephrin with a unique structure, prevents interneuronal axons from exiting the Drosophila embryonic CNS. Development 129:4205-4218.

Boyce FM, Bucher NL. 1996. Baculovirus-mediated gene transfer into mammalian cells. Proc Natl Acad Sci U S A 93:2348-2352.

Braisted JE, McLaughlin T, Wang HU, Friedman GC, Anderson DJ, O'Leary D D. 1997. Graded and lamina-specific distributions of ligands of EphB receptor tyrosine kinases in the developing retinotectal system. Dev Biol 191:14-28.

Brand AH, Perrimon N. 1993. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development 118:401-415.

Britten RJ. 1997. Mobile elements inserted in the distant past have taken on important functions. Gene 205:177-182.

Brittis PA, Lu Q, Flanagan JG. 2002. Axonal protein synthesis provides a mechanism for localized regulation at an intermediate target. Cell 110:223-235.

Brose K, Bland KS, Wang KH, Arnott D, Henzel W, Goodman CS, Tessier-Lavigne M, Kidd T. 1999. Slit proteins bind Robo receptors and have an evolutionarily conserved role in repulsive axon guidance. Cell 96:795-806.

Brown A, Yates PA, Burrola P, Ortuno D, Vaidya A, Jessell TM, Pfaff SL, O'Leary DD, Lemke G. 2000. Topographic mapping from the retina to the midbrain is controlled by relative but not absolute levels of EphA receptor signaling. Cell 102:77-88.

Bruce LL, Christensen MA, Fritzsch B. 1997. Electron microscopic differentiation of directly and transneuronally transported DiI and applications for studies of synaptogenesis. J Neurosci Methods 73:107-112.

Buck L, Axel R. 1991. A novel multigene family may encode odorant receptors: a molecular basis for odor recognition. Cell 65:175-187.

Burgess S, Hopkins N. 2000. Use of pseudotyped retroviruses in zebrafish as genetic tags. Methods Enzymol 327:145-161.

Burrill JD, Easter SS, Jr. 1994. Development of the retinofugal projections in the embryonic and larval zebrafish (Brachydanio rerio). J Comp Neurol 346:583-600.

Butler SJ, Dodd J. 2003. A role for BMP heterodimers in roof plate-mediated repulsion of commissural axons. Neuron 38:389-401.

Celotto AM, Graveley BR. 2001. Alternative splicing of the Drosophila Dscam pre-mRNA is both temporally and spatially regulated. Genetics 159:599-608.

Chan J, Mably JD, Serluca FC, Chen JN, Goldstein NB, Thomas MC, Cleary JA, Brennan C, Fishman MC, Roberts TM. 2001. Morphogenesis of prechordal plate and notochord requires intact Eph/ephrin B signaling. Dev Biol 234:470-482.

Chan SS, Zheng H, Su MW, Wilk R, Killeen MT, Hedgecock EM, Culotti JG. 1996. UNC-40, a C. elegans homolog of DCC (Deleted in Colorectal Cancer), is required in motile cells responding to UNC-6 netrin cues. Cell 87:187-195.

Charron F, Stein E, Jeong J, McMahon AP, Tessier-Lavigne M. 2003. The morphogen sonic hedgehog is an axonal chemoattractant that collaborates with netrin-1 in midline axon guidance. Cell 113:11-23.

Chatterji M, Tsai CL, Schatz DG. 2004. New concepts in the regulation of an ancient reaction: transposition by RAG1/RAG2. Immunol Rev 200:261-271.

Cheng HJ, Nakamoto M, Bergemann AD, Flanagan JG. 1995. Complementary gradients in expression and binding of ELF-1 and Mek4 in development of the topographic retinotectal projection map. Cell 82:371-381.

Chia W, Yang X. 2002. Asymmetric division of Drosophila neural progenitors. Curr Opin Genet Dev 12:459-464.

Chun J. 2001. Selected comparison of immune and nervous system development. Adv Immunol 77:297-322.

Chun JJ, Schatz DG, Oettinger MA, Jaenisch R, Baltimore D. 1991. The recombination activating gene-1 (RAG-1) transcript is present in the murine central nervous system. Cell 64:189-200.

Connor RJ, Menzel P, Pasquale EB. 1998. Expression and tyrosine phosphorylation of Eph receptors suggest multiple mechanisms in patterning of the visual system. Dev Biol 193:21-35.

Conover JC, Doetsch F, Garcia-Verdugo JM, Gale NW, Yancopoulos GD, Alvarez-Buylla A. 2000. Disruption of Eph/ephrin signaling affects migration and proliferation in the adult subventricular zone. Nat Neurosci 3:1091-1097.

Corriveau RA, Huh GS, Shatz CJ. 1998. Regulation of class I MHC gene expression in the developing and mature CNS by neural activity. Neuron 21:505-520.

Cortes P, Ye ZS, Baltimore D. 1994. RAG-1 interacts with the repeated amino acid motif of the human homologue of the yeast protein SRP1. Proc Natl Acad Sci U S A 91:7633-7637.

Cowell LG, Davila M, Yang K, Kepler TB, Kelsoe G. 2003. Prospective estimation of recombination signal efficiency and identification of functional cryptic signals in the genome by statistical modeling. J Exp Med 197:207-220.

Culotti JG, Merz DC. 1998. DCC and netrins. Curr Opin Cell Biol 10:609-613.

Cuomo CA, Oettinger MA. 1994. Analysis of regions of RAG-2 important for V(D)J recombination. Nucleic Acids Res 22:1810-1814.

Cushman J, Lo J, Huang Z, Wasserfall C, Petitto JM. 2003. Neurobehavioral changes resulting from recombinase activation gene 1 deletion. Clin Diagn Lab Immunol 10:13-18.

Cutforth T, Moring L, Mendelsohn M, Nemes A, Shah NM, Kim MM, Frisen J, Axel R. 2003. Axonal ephrin-As and odorant receptors: coordinate determination of the olfactory sensory map. Cell 114:311-322.

Dasen JS, Liu JP, Jessell TM. 2003. Motor neuron columnar fate imposed by sequential phases of Hox-c activity. Nature 425:926-933.

Davy A, Soriano P. 2005. Ephrin signaling in vivo: Look both ways. Dev Dyn 232:1-10.

DeBerardinis RJ, Goodier JL, Ostertag EM, Kazazian HH, Jr. 1998. Rapid amplification of a retrotransposon subfamily is evolving the mouse genome. Nat Genet 20:288-290.

Devries SH, Baylor DA. 1997. Mosaic arrangement of ganglion cell receptive fields in rabbit retina. J Neurophysiol 78:2048-2060.

Difilippantonio MJ, McMahan CJ, Eastman QM, Spanopoulou E, Schatz DG. 1996. RAG1 mediates signal sequence recognition and recruitment of RAG2 in V(D)J recombination. Cell 87:253-262.

Drescher U, Kremoser C, Handwerker C, Loschinger J, Noda M, Bonhoeffer F. 1995. In vitro guidance of retinal ganglion cell axons by RAGS, a 25 kDa tectal protein related to ligands for Eph receptor tyrosine kinases. Cell 82:359-370.

Dreyer WJ, Gray WR, Hood L. 1967. The genetic, molecular and cellular basis of antibody formation:some facts and a unifying hypothesis. Cold Spring Harbor Symp Quant Biol 32:353-367.

Dufour A, Seibt J, Passante L, Depaepe V, Ciossek T, Frisen J, Kullander K, Flanagan JG, Polleux F, Vanderhaeghen P. 2003. Area specificity and topography of thalamocortical projections are controlled by ephrin/Eph genes. Neuron 39:453-465.

Durbin L, Brennan C, Shiomi K, Cooke J, Barrios A, Shanmugalingam S, Guthrie B, Lindberg R, Holder N. 1998. Eph signaling is required for segmentation and differentiation of the somites. Genes Dev 12:3096-3109.

Dynes JL, Ngai J. 1998. Pathfinding of olfactory neuron axons to stereotyped glomerular targets revealed by dynamic imaging in living zebrafish embryos. Neuron 20:1081-1091.

Eberhart J, Swartz ME, Koblar SA, Pasquale EB, Krull CE. 2002. EphA4 constitutes a population-specific guidance cue for motor neurons. Dev Biol 247:89-101.

Echelard Y, Epstein DJ, St-Jacques B, Shen L, Mohler J, McMahon JA, McMahon AP. 1993. Sonic hedgehog, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity. Cell 75:1417-1430.

Eph_Nomenclature_Committee. 1997. Unified nomenclature for Eph family receptors and their ligands, the ephrins. Eph Nomenclature Committee. Cell 90:403-404.

Fanning L, Bertrand FE, Steinberg C, Wu GE. 1998. Molecular mechanisms involved in receptor editing at the Ig heavy chain locus. Int Immunol 10:241-246.

Fazeli A, Dickinson SL, Hermiston ML, Tighe RV, Steen RG, Small CG, Stoeckli ET, Keino-Masu K, Masu M, Rayburn H, Simons J, Bronson RT, Gordon JI, Tessier-Lavigne M, Weinberg RA. 1997. Phenotype of mice lacking functional Deleted in colorectal cancer (Dcc) gene. Nature 386:796-804.

Feldheim DA, Kim YI, Bergemann AD, Frisen J, Barbacid M, Flanagan JG. 2000. Genetic analysis of ephrin-A2 and ephrin-A5 shows their requirement in multiple aspects of retinocollicular mapping. Neuron 25:563-574.

Fire A, Harrison SW, Dixon D. 1990. A modular set of lacZ fusion vectors for studying gene expression in Caenorhabditis elegans. Gene 93:189-198.

Flamme I, von Reutern M, Drexler HC, Syed-Ali S, Risau W. 1995. Overexpression of vascular endothelial growth factor in the avian embryo induces hypervascularization and increased vascular permeability without alterations of embryonic pattern formation. Dev Biol 171:399-414.

Fraser M. 1986. Ultrastructural Observation of Viron Maturation in Autographa californica Nuclear Polyhedrosis Virus Infected Spodoptera frugiperda Cultures. Journal of Ultrastructure and Molecular Structure Research 95:189-195.

Fred MA, Brent R, Kingston RE, Moore D, Seidman JG, Smith JA, K. S, Fred MA, Brent R, Kingston RE, Moore D, Seidman JG, Smith JA, K. Ss. 1987. Current Protocols in Molecular Biology. John Wiley & Sons.

Fricke C, Lee JS, Geiger-Rudolph S, Bonhoeffer F, Chien CB. 2001. astray, a zebrafish roundabout homolog required for retinal axon guidance. Science 292:507-510.

Friedman GC, O'Leary DD. 1996. Retroviral misexpression of engrailed genes in the chick optic tectum perturbs the topographic targeting of retinal axons. J Neurosci 16:5498-5509.

Frippiat C, Kremarik P, Ropars A, Dournon C, Frippiat JP. 2001. The recombination-activating gene 1 of Pleurodeles waltl (urodele amphibian) is transcribed in lymphoid tissues and in the central nervous system. Immunogenetics 52:264-275.

Frisen J, Yates PA, McLaughlin T, Friedman GC, O'Leary DD, Barbacid M. 1998. Ephrin-A5 (AL-1/RAGS) is essential for proper retinal axon guidance and topographic mapping in the mammalian visual system. Neuron 20:235-243.

Fugmann SD, Lee AI, Shockett PE, Villey IJ, Schatz DG. 2000. The RAG proteins and V(D)J recombination: complexes, ends, and transposition. Annu Rev Immunol 18:495-527.

Fuller K, Storb U. 1997. Identification and characterization of the murine Rag1 promoter. Mol Immunol 34:939-954.

Gao PP, Zhang JH, Yokoyama M, Racey B, Dreyfus CF, Black IB, Zhou R. 1996. Regulation of topographic projection in the brain: Elf-1 in the hippocamposeptal system. Proc Natl Acad Sci U S A 93:11161-11166.

Gao Y, Sun Y, Frank KM, Dikkes P, Fujiwara Y, Seidl KJ, Sekiguchi JM, Rathbun GA, Swat W, Wang J, Bronson RT, Malynn BA, Bryans M, Zhu C, Chaudhuri J, Davidson L, Ferrini R, Stamato T, Orkin SH, Greenberg ME, Alt FW. 1998. A critical role for DNA end-joining proteins in both lymphogenesis and neurogenesis. Cell 95:891-902.

Gellert M. 2002. V(D)J recombination: RAG proteins, repair factors, and regulation. Annu Rev Biochem 71:101-132.

George SE, Simokat K, Hardin J, Chisholm AD. 1998. The VAB-1 Eph receptor tyrosine kinase functions in neural and epithelial morphogenesis in C. elegans. Cell 92:633-643.

Germain RN. 1994. MHC-dependent antigen processing and peptide presentation: providing ligands for T lymphocyte activation. Cell 76:287-299.

Gibbs SM, Truman JW. 1998. Nitric oxide and cyclic GMP regulate retinal patterning in the optic lobe of Drosophila. Neuron 20:83-93.

Gomez TM, Spitzer NC. 1999. In vivo regulation of axon extension and pathfinding by growth-cone calcium transients. Nature 397:350-355.

Goodhill GJ, Richards LJ. 1999. Retinotectal maps: molecules, models and misplaced data. Trends Neurosci 22:529-534.

Goodier JL, Ostertag EM, Du K, Kazazian HH, Jr. 2001. A novel active L1 retrotransposon subfamily in the mouse. Genome Res 11:1677-1685.

Goodman CS, Doe CQ. 1993. Embryonic development of the *Drosophila* central nervous system. In: Bate M, Martinez Arias A, Bate M, Martinez Arias As. The Development of *Drosophila melanogaster*. NY: Cold Springs Harbor. p 1131-1206.

Gu X, Spitzer NC. 1995. Distinct aspects of neuronal differentiation encoded by frequency of spontaneous Ca2+ transients. Nature 375:784-787.

Gundersen RW, Barrett JN. 1979. Neuronal chemotaxis: chick dorsal-root axons turn toward high concentrations of nerve growth factor. Science 206:1079-1080.

Haddon C, Smithers L, Schneider-Maunoury S, Coche T, Henrique D, Lewis J. 1998. Multiple delta genes and lateral inhibition in zebrafish primary neurogenesis. Development 125:359-370.

Hall AC, Lucas FR, Salinas PC. 2000. Axonal remodeling and synaptic differentiation in the cerebellum is regulated by WNT-7a signaling. Cell 100:525-535.

Halloran MC, Sato-Maeda M, Warren JT, Su F, Lele Z, Krone PH, Kuwada JY, Shoji W. 2000. Laser-induced gene expression in specific cells of transgenic zebrafish. Development 127:1953-1960.

Harris R, Sabatelli LM, Seeger MA. 1996. Guidance cues at the Drosophila CNS midline: identification and characterization of two Drosophila Netrin/UNC-6 homologs. Neuron 17:217-228.

Hedgecock EM, Culotti JG, Hall DH. 1990. The unc-5, unc-6, and unc-40 genes guide circumferential migrations of pioneer axons and mesodermal cells on the epidermis in C. elegans. Neuron 4:61-85.

Helmbacher F, Schneider-Maunoury S, Topilko P, Tiret L, Charnay P. 2000. Targeting of the EphA4 tyrosine kinase receptor affects dorsal/ventral pathfinding of limb motor axons. Development 127:3313-3324.

Henkemeyer M, Orioli D, Henderson JT, Saxton TM, Roder J, Pawson T, Klein R. 1996. Nuk controls pathfinding of commissural axons in the mammalian central nervous system. Cell 86:35-46.

Hillion S, Saraux A, Youinou P, Jamin C. 2005. Expression of RAGs in peripheral B cells outside germinal centers is associated with the expression of CD5. J Immunol 174:5553-5561.

Himanen JP, Rajashankar KR, Lackmann M, Cowan CA, Henkemeyer M, Nikolov DB. 2001. Crystal structure of an Eph receptor-ephrin complex. Nature 414:933-938.

Hindges R, McLaughlin T, Genoud N, Henkemeyer M, O'Leary DD. 2002. EphB forward signaling controls directional branch extension and arborization required for dorsal-ventral retinotopic mapping. Neuron 35:475-487.

Hiom K, Gellert M. 1998. Assembly of a 12/23 paired signal complex: a critical control point in V(D)J recombination. Mol Cell 1:1011-1019.

Hiom K, Melek M, Gellert M. 1998. DNA transposition by the RAG1 and RAG2 proteins: a possible source of oncogenic translocations. Cell 94:463-470.

Hirai H, Maru Y, Hagiwara K, Nishida J, Takaku F. 1987. A novel putative tyrosine kinase receptor encoded by the eph gene. Science 238:1717-1720.

Hofmann C, Sandig V, Jennings G, Rudolph M, Schlag P, Strauss M. 1995. Efficient gene transfer into human hepatocytes by baculovirus vectors. Proc Natl Acad Sci U S A 92:10099-10103.

Holash JA, Pasquale EB. 1995. Polarized expression of the receptor protein tyrosine kinase Cek5 in the developing avian visual system. Dev Biol 172:683-693.

Holash JA, Soans C, Chong LD, Shao H, Dixit VM, Pasquale EB. 1997. Reciprocal expression of the Eph receptor Cek5 and its ligand(s) in the early retina. Dev Biol 182:256-269.

Holder N, Klein R. 1999. Eph receptors and ephrins: effectors of morphogenesis. Development 126:2033-2044.

Hornberger MR, Dutting D, Ciossek T, Yamada T, Handwerker C, Lang S, Weth F, Huf J, Wessel R, Logan C, Tanaka H, Drescher U. 1999. Modulation of EphA receptor function by coexpressed ephrinA ligands on retinal ganglion cell axons. Neuron 22:731-742.

Imondi R, Wideman C, Kaprielian Z. 2000. Complementary expression of transmembrane ephrins and their receptors in the mouse spinal cord: a possible role in constraining the orientation of longitudinally projecting axons. Development 127:1397-1410.

Jessen JR, Jessen TN, Vogel SS, Lin S. 2001. Concurrent expression of recombination activating genes 1 and 2 in zebrafish olfactory sensory neurons. Genesis 29:156-162.

Johnson AD, Krieg PA. 1994. pXeX, a vector for efficient expression of cloned sequences in Xenopus embryos. Gene 147:223-226.

Jones-Villeneuve EM, McBurney MW, Rogers KA, Kalnins VI. 1982. Retinoic acid induces embryonal carcinoma cells to differentiate into neurons and glial cells. J Cell Biol 94:253-262.

Kapitonov VV, Jurka J. 2005. RAG1 core and V(D)J recombination signal sequences were derived from Transib transposons. PLoS Biol 3:e181.

Kaprielian Z, Runko E, Imondi R. 2001. Axon guidance at the midline choice point. Dev Dyn 221:154-181.

Karlstrom RO, Trowe T, Klostermann S, Baier H, Brand M, Crawford AD, Grunewald B, Haffter P, Hoffmann H, Meyer SU, Muller BK, Richter S, van Eeden FJ, Nusslein-Volhard C, Bonhoeffer F. 1996. Zebrafish mutations affecting retinotectal axon pathfinding. Development 123:427-438.

Kawabata M, Kawabata T, Saeki K. 2004. DNA rearrangement activity during retinoic acid-induced neural differentiation of P19 mouse embryonal carcinoma cells. Acta Med Okayama 58:263-270.

Keleman K, Rajagopalan S, Cleppien D, Teis D, Paiha K, Huber LA, Technau GM, Dickson BJ. 2002. Comm sorts robo to control axon guidance at the Drosophila midline. Cell 110:415-427.

Kennedy TE, Serafini T, de la Torre JR, Tessier-Lavigne M. 1994. Netrins are diffusible chemotropic factors for commissural axons in the embryonic spinal cord. Cell 78:425-435.

Kidd T, Brose K, Mitchell KJ, Fetter RD, Tessier-Lavigne M, Goodman CS, Tear G. 1998. Roundabout controls axon crossing of the CNS midline and defines a novel subfamily of evolutionarily conserved guidance receptors. Cell 92:205-215.

Kimmel CB, Warga RM. 1987. Indeterminate cell lineage of the zebrafish embryo. Dev Biol 124:269-280.

King LA, Possee RD. 1992. The Baculovirus Expression System A laboratory Guide. London: Chapman and Hall. 107-126.

Knoll B, Drescher U. 2002. Ephrin-As as receptors in topographic projections. Trends Neurosci 25:145-149.

Knoll B, Zarbalis K, Wurst W, Drescher U. 2001. A role for the EphA family in the topographic targeting of vomeronasal axons. Development 128:895-906.

Kohmura N, Senzaki K, Hamada S, Kai N, Yasuda R, Watanabe M, Ishii H, Yasuda M, Mishina M, Yagi T. 1998. Diversity revealed by a novel family of cadherins expressed in neurons at a synaptic complex. Neuron 20:1137-1151.

Kolodkin AL, Matthes DJ, O'Connor TP, Patel NH, Admon A, Bentley D, Goodman CS. 1992. Fasciclin IV: sequence, expression, and function during growth cone guidance in the grasshopper embryo. Neuron 9:831-845.

Komura J, Riggs AD. 1998. Terminal transferase-dependent PCR: a versatile and sensitive method for in vivo footprinting and detection of DNA adducts. Nucleic Acids Res 26:1807-1811.

Krylova O, Herreros J, Cleverley KE, Ehler E, Henriquez JP, Hughes SM, Salinas PC. 2002. WNT-3, expressed by motoneurons, regulates terminal arborization of neurotrophin-3-responsive spinal sensory neurons. Neuron 35:1043-1056.

Kullander K, Croll SD, Zimmer M, Pan L, McClain J, Hughes V, Zabski S, DeChiara TM, Klein R, Yancopoulos GD, Gale NW. 2001. Ephrin-B3 is the midline barrier that prevents corticospinal tract axons from recrossing, allowing for unilateral motor control. Genes Dev 15:877-888.

Kullander K, Mather NK, Diella F, Dottori M, Boyd AW, Klein R. 2001. Kinase-dependent and kinase-independent functions of EphA4 receptors in major axon tract formation in vivo. Neuron 29:73-84.

Kumaki S, Villa A, Asada H, Kawai S, Ohashi Y, Takahashi M, Hakozaki I, Nitanai E, Minegishi M, Tsuchiya S. 2001. Identification of anti-herpes simplex virus antibody-producing B cells in a patient with an atypical RAG1 immunodeficiency. Blood 98:1464-1468.

Kurioka H, Kishi H, Isshiki H, Tagoh H, Mori K, Kitagawa T, Nagata T, Dohi K, Muraguchi A. 1996. Isolation and characterization of a TATA-less promoter for the human RAG-1 gene. Mol Immunol 33:1059-1066.

Kusumi K, Smith JS, Segre JA, Koos DS, Lander ES. 1993. Construction of a large-insert yeast artificial chromosome library of the mouse genome. Mamm Genome 4:391-392.

Lackmann M, Oates AC, Dottori M, Smith FM, Do C, Power M, Kravets L, Boyd AW. 1998. Distinct subdomains of the EphA3 receptor mediate ligand binding and receptor dimerization. J Biol Chem 273:20228-20237.

Lancki DW, Qian D, Fields P, Gajewski T, Fitch FW. 1995. Differential requirement for protein tyrosine kinase Fyn in the functional activation of antigen-specific T lymphocyte clones through the TCR or Thy-1. J Immunol 154:4363-4370.

Lee GS, Neiditch MB, Sinden RR, Roth DB. 2002. Targeted transposition by the V(D)J recombinase. Mol Cell Biol 22:2068-2077.

Leonardo ED, Hinck L, Masu M, Keino-Masu K, Ackerman SL, Tessier-Lavigne M. 1997. Vertebrate homologues of C. elegans UNC-5 are candidate netrin receptors. Nature 386:833-838.

Letourneau PC. 1978. Chemotactic response of nerve fiber elongation to nerve growth factor. Dev Biol 66:183-196.

Lewis J. 1998. Notch signalling and the control of cell fate choices in vertebrates. Semin Cell Dev Biol 9:583-589.

Liem KF, Jr., Jessell TM, Briscoe J. 2000. Regulation of the neural patterning activity of sonic hedgehog by secreted BMP inhibitors expressed by notochord and somites. Development 127:4855-4866.

Liem KF, Jr., Tremml G, Roelink H, Jessell TM. 1995. Dorsal differentiation of neural plate cells induced by BMP-mediated signals from epidermal ectoderm. Cell 82:969-979.

Liu Q, Sanborn KL, Cobb N, Raymond PA, Marrs JA. 1999. R-cadherin expression in the developing and adult zebrafish visual system. J Comp Neurol 410:303-319.

Lohof AM, Quillan M, Dan Y, Poo MM. 1992. Asymmetric modulation of cytosolic cAMP activity induces growth cone turning. J Neurosci 12:1253-1261.

Luo Y, Raible D, Raper JA. 1993. Collapsin: a protein in brain that induces the collapse and paralysis of neuronal growth cones. Cell 75:217-227.

Lyons DA, Guy AT, Clarke JD. 2003. Monitoring neural progenitor fate through multiple rounds of division in an intact vertebrate brain. Development 130:3427-3436.

Mann F, Ray S, Harris W, Holt C. 2002. Topographic mapping in dorsoventral axis of the Xenopus retinotectal system depends on signaling through ephrin-B ligands. Neuron 35:461-473.

Marcus RC, Gale NW, Morrison ME, Mason CA, Yancopoulos GD. 1996. Eph family receptors and their ligands distribute in opposing gradients in the developing mouse retina. Dev Biol 180:786-789.

Marin O, Blanco MJ, Nieto MA. 2001. Differential expression of Eph receptors and ephrins correlates with the formation of topographic projections in primary and secondary visual circuits of the embryonic chick forebrain. Dev Biol 234:289-303.

Martin PT, Ettinger AJ, Sanes JR. 1995. A synaptic localization domain in the synaptic cleft protein laminin beta 2 (s-laminin). Science 269:413-416.

Matsuoka M, Nagawa F, Okazaki K, Kingsbury L, Yoshida K, Muller U, Larue DT, Winer JA, Sakano H. 1991. Detection of somatic DNA recombination in the transgenic mouse brain. Science 254:81-86.

McBlane JF, van Gent DC, Ramsden DA, Romeo C, Cuomo CA, Gellert M, Oettinger MA. 1995. Cleavage at a V(D)J recombination signal requires only RAG1 and RAG2 proteins and occurs in two steps. Cell 83:387-395.

McGuire EA, Hockett RD, Pollock KM, Bartholdi MF, O'Brien SJ, Korsmeyer SJ. 1989. The t(11;14)(p15;q11) in a T-cell acute lymphoblastic leukemia cell line activates multiple transcripts, including Ttg-1, a gene encoding a potential zinc finger protein. Mol Cell Biol 9:2124-2132.

McLaughlin T, Hindges R, Yates PA, O'Leary DD. 2003. Bifunctional action of ephrin-B1 as a repellent and attractant to control bidirectional branch extension in dorsal-ventral retinotopic mapping. Development 130:2407-2418.

McMahan CJ, Sadofsky MJ, Schatz DG. 1997. Definition of a large region of RAG1 that is important for coimmunoprecipitation of RAG2. J Immunol 158:2202-2210.

McMurry MT, Krangel MS. 2000. A role for histone acetylation in the developmental regulation of VDJ recombination. Science 287:495-498.

Melek M, Gellert M. 2000. RAG1/2-mediated resolution of transposition intermediates: two pathways and possible consequences. Cell 101:625-633.

Menesini Chen MG, Chen JS, Levi-Montalcini R. 1978. Sympathetic nerve fibers ingrowth in the central nervous system of neonatal rodent upon intracerebral NGF injections. Arch Ital Biol 116:53-84.

Messersmith EK, Leonardo ED, Shatz CJ, Tessier-Lavigne M, Goodman CS, Kolodkin AL. 1995. Semaphorin III can function as a selective chemorepellent to pattern sensory projections in the spinal cord. Neuron 14:949-959.

Messier TL, O'Neill JP, Hou SM, Nicklas JA, Finette BA. 2003. In vivo transposition mediated by V(D)J recombinase in human T lymphocytes. Embo J 22:1381-1388.

Ming GL, Song HJ, Berninger B, Holt CE, Tessier-Lavigne M, Poo MM. 1997. cAMP-dependent growth cone guidance by netrin-1. Neuron 19:1225-1235.

Miranda JD, White LA, Marcillo AE, Willson CA, Jagid J, Whittemore SR. 1999. Induction of Eph B3 after spinal cord injury. Exp Neurol 156:218-222.

Missler M, Sudhof TC. 1998. Neurexins: three genes and 1001 products. Trends Genet 14:20-26.

Mitchell KJ, Doyle JL, Serafini T, Kennedy TE, Tessier-Lavigne M, Goodman CS, Dickson BJ. 1996. Genetic analysis of Netrin genes in Drosophila: Netrins guide CNS commissural axons and peripheral motor axons. Neuron 17:203-215.

Mombaerts P, Iacomini J, Johnson RS, Herrup K, Tonegawa S, Papaioannou VE. 1992. RAG-1-deficient mice have no mature B and T lymphocytes. Cell 68:869-877.

Mombaerts P, Wang F, Dulac C, Chao SK, Nemes A, Mendelsohn M, Edmondson J, Axel R. 1996. Visualizing an olfactory sensory map. Cell 87:675-686.

Moreno-Flores MT, Wandosell F. 1999. Up-regulation of Eph tyrosine kinase receptors after excitotoxic injury in adult hippocampus. Neuroscience 91:193-201.

Mozer BA, Benzer S. 1994. Ingrowth by photoreceptor axons induces transcription of a retrotransposon in the developing Drosophila brain. Development 120:1049-1058.

Muller F, Lele Z, Varadi L, Menczel L, Orban L. 1993. Efficient transient expression system based on square pulse electroporation and in vivo luciferase assay of fertilized fish eggs. FEBS Lett 324:27-32.

Muller KM, Luedecker CJ, Udey MC, Farr AG. 1997. Involvement of E-cadherin in thymus organogenesis and thymocyte maturation. Immunity 6:257-264.

Muotri AR, Chu VT, Marchetto MCN, Deng W, Moran JV, Gage FH. 2005. Somatic mosaicism in neuronal precursor

cells mediated by L1 retrotransposition. Nature 435:903-910.

Murai KK, Pasquale EB. 2004. Eph receptors, ephrins, and synaptic function. Neuroscientist 10:304-314.

Myat A, Henry P, McCabe V, Flintoft L, Rotin D, Tear G. 2002. Drosophila Nedd4, a ubiquitin ligase, is recruited by Commissureless to control cell surface levels of the roundabout receptor. Neuron 35:447-459.

Nagaoka H, Yu W, Nussenzweig MC. 2000. Regulation of RAG expression in developing lymphocytes. Curr Opin Immunol 12:187-190.

Nakamoto M, Cheng HJ, Friedman GC, McLaughlin T, Hansen MJ, Yoon CH, O'Leary DD, Flanagan JG. 1996. Topographically specific effects of ELF-1 on retinal axon guidance in vitro and retinal axon mapping in vivo. Cell 86:755-766.

Nasevicius A, Ekker SC. 2000. Effective targeted gene 'knockdown' in zebrafish. Nat Genet 26:216-220.

Nguyen VH, Trout J, Connors SA, Andermann P, Weinberg E, Mullins MC. 2000. Dorsal and intermediate neuronal cell types of the spinal cord are established by a BMP signaling pathway. Development 127:1209-1220.

Nishida K, Flanagan JG, Nakamoto M. 2002. Domain-specific olivocerebellar projection regulated by the EphA-ephrin-A interaction. Development 129:5647-5658.

Noakes PG, Gautam M, Mudd J, Sanes JR, Merlie JP. 1995. Aberrant differentiation of neuromuscular junctions in mice lacking s-laminin/laminin beta 2. Nature 374:258-262.

Nusslein-Volhard C. 1994. Of flies and fishes. Science 266:572-574.

Ober E. 1996. Charakterisierung von Mutanten mit Einfluß auf die

retinotectale Projection des Zebrabaerblings Danio rerio. In: Eberhard Karls Universitaet Tuebingen.

Oettinger MA. 2004. How to keep V(D)J recombination under control. Immunol Rev 200:165-181.

Oettinger MA, Schatz DG, Gorka C, Baltimore D. 1990. RAG-1 and RAG-2, adjacent genes that synergistically activate V(D)J recombination. Science 248:1517-1523.

O'Leary DD, Wilkinson DG. 1999. Eph receptors and ephrins in neural development. Curr Opin Neurobiol 9:65-73.

Oppenheimer DI, MacNicol AM, Patel NH. 1999. Functional conservation of the wingless-engrailed interaction as shown by a widely applicable baculovirus misexpression system. Curr Biol 9:1288-1296.

Orioli D, Henkemeyer M, Lemke G, Klein R, Pawson T. 1996. Sek4 and Nuk receptors cooperate in guidance of commissural axons and in palate formation. Embo J 15:6035-6049.

Orioli D, Klein R. 1997. The Eph receptor family: axonal guidance by contact repulsion. Trends Genet 13:354-359.

Ostertag EM, Kazazian HH, Jr. 2005. LINEs in mind. Nature 435:890-891.

Packard M, Koo ES, Gorczyca M, Sharpe J, Cumberledge S, Budnik V. 2002. The Drosophila Wnt, wingless, provides an essential signal for pre- and postsynaptic differentiation. Cell 111:319-330.

Palmer A, Klein R. 2003. Multiple roles of ephrins in morphogenesis, neuronal networking, and brain function. Genes Dev 17:1429-1450.

Park HC, Kim CH, Bae YK, Yeo SY, Kim SH, Hong SK, Shin J, Yoo KW, Hibi M, Hirano T, Miki N, Chitnis AB, Huh TL. 2000. Analysis of upstream elements in the HuC promoter leads to the establishment of transgenic zebrafish with fluorescent neurons. Dev Biol 227:279-293.

Parra P, Gulyas AI, Miles R. 1998. How many subtypes of inhibitory cells in the hippocampus? Neuron 20:983-993.

Paves H, Saarma M. 1997. Neurotrophins as in vitro growth cone guidance molecules for embryonic sensory neurons. Cell Tissue Res 290:285-297.

Penn AA, Shatz CJ. 1999. Brain waves and brain wiring: the role of endogenous and sensory-driven neural activity in development. Pediatr Res 45:447-458.

Perkins EJ, Nair A, Cowley DO, Van Dyke T, Chang Y, Ramsden DA. 2002. Sensing of intermediates in V(D)J recombination by ATM. Genes Dev 16:159-164.

Pickles JO, van Heumen WR. 1997. The expression of messenger RNAs coding for growth factors, their receptors, and eph-class receptor tyrosine kinases in normal and ototoxically damaged chick cochleae. Dev Neurosci 19:476-487.

Plump AS, Erskine L, Sabatier C, Brose K, Epstein CJ, Goodman CS, Mason CA, Tessier-Lavigne M. 2002. Slit1 and Slit2 cooperate to prevent premature midline crossing of retinal axons in the mouse visual system. Neuron 33:219-232.

Puschel AW, Adams RH, Betz H. 1996. The sensory innervation of the mouse spinal cord may be patterned by differential expression of and differential responsiveness to semaphorins. Mol Cell Neurosci 7:419-431.

Raghavan SC, Swanson PC, Wu X, Hsieh CL, Lieber MR. 2004. A non-B-DNA structure at the Bcl-2 major breakpoint region is cleaved by the RAG complex. Nature 428:88-93.

Ramón yCS. 1892. Le Rétine de vertébrés. La Cellule 9:119-258.

Ramsden DA, Gellert M. 1995. Formation and resolution of double-strand break intermediates in V(D)J rearrangement. Genes Dev 9:2409-2420.

Raper JA. 2000. Semaphorins and their receptors in vertebrates and invertebrates. Curr Opin Neurobiol 10:88-94.

Ren B, Robert F, Wyrick JJ, Aparicio O, Jennings EG, Simon I, Zeitlinger J, Schreiber J, Hannett N, Kanin E, Volkert TL, Wilson CJ, Bell SP, Young RA. 2000. Genome-wide location and function of DNA binding proteins. Science 290:2306-2309.

Rick JM, Horschke I, Neuhauss SC. 2000. Optokinetic behavior is reversed in achiasmatic mutant zebrafish larvae. Curr Biol 10:595-598.

Rodger J, Bartlett CA, Beazley LD, Dunlop SA. 2000. Transient up-regulation of the rostrocaudal gradient of ephrin A2 in the tectum coincides with reestablishment of orderly projections during optic nerve regeneration in goldfish. Exp Neurol 166:196-200.

Rodgers KK, Bu Z, Fleming KG, Schatz DG, Engelman DM, Coleman JE. 1996. A zinc-binding domain involved in the dimerization of RAG1. J Mol Biol 260:70-84.

Roelink H, Augsburger A, Heemskerk J, Korzh V, Norlin S, Ruiz i Altaba A, Tanabe Y, Placzek M, Edlund T, Jessell TM, et al. 1994. Floor plate and motor neuron induction by vhh-1, a vertebrate homolog of hedgehog expressed by the notochord. Cell 76:761-775.

Roeser T, Baier H. 2003. Visuomotor behaviors in larval zebrafish after GFP-guided laser ablation of the optic tectum. J Neurosci 23:3726-3734.

Rosentreter SM, Davenport RW, Loschinger J, Huf J, Jung J, Bonhoeffer F. 1998. Response of retinal ganglion cell axons to striped linear gradients of repellent guidance molecules. J Neurobiol 37:541-562.

Roth DB, Lindahl T, Gellert M. 1995. Repair and recombination. How to make ends meet. Curr Biol 5:496-499.

Rudnicki MA, McBurney MW. 1987. Cell culture methods and induction of differentitation of embryonic carcinoma cell lines. In: Robertson EJ, editor.Robertson EJs. Teratocarcinoma and Embryonic Stem Cells: A Practical Approach. New York: IRL Press. p 19-50.

Sakurai T, Wong E, Drescher U, Tanaka H, Jay DG. 2002. Ephrin-A5 restricts topographically specific arborization in the chick retinotectal projection in vivo. Proc Natl Acad Sci U S A 99:10795-10800.

Salie R, Niederkofler V, Arber S. 2005. Patterning molecules; multitasking in the nervous system. Neuron 45:189-192.

Sambrook J, Fritsch EF, Maniatis T. 1989. Molecular Cloning - A Laboratory Manual. New York: Cold Spring Harbour Laboratory Press.

Sandig V, Hofmann C, Steinert S, Jennings G, Schlag P, Strauss M. 1996. Gene transfer into hepatocytes and human liver tissue by baculovirus vectors. Hum Gene Ther 7:1937-1945.

Sarkis C, Serguera C, Petres S, Buchet D, Ridet JL, Edelman L, Mallet J. 2000. Efficient transduction of neural cells in vitro and in vivo by a baculovirus-derived vector. Proc Natl Acad Sci U S A 97:14638-14643.

Sawchuk DJ, Weis-Garcia F, Malik S, Besmer E, Bustin M, Nussenzweig MC, Cortes P. 1997. V(D)J recombination: modulation of RAG1 and RAG2 cleavage activity on 12/23 substrates by whole cell extract and DNA-bending proteins. J Exp Med 185:2025-2032.

Schatz DG, Oettinger MA, Baltimore D. 1989. The V(D)J recombination activating gene, RAG-1. Cell 59:1035-1048.

Schatz DG, Oettinger MA, Schlissel MS. 1992. V(D)J recombination: molecular biology and regulation. Annu Rev Immunol 10:359-383.

Scheer N, Camnos-Ortega JA. 1999. Use of the Gal4-UAS technique for targeted gene expression in the zebrafish. Mech Dev 80:153-158.

Schmucker D, Clemens JC, Shu H, Worby CA, Xiao J, Muda M, Dixon JE, Zipursky SL. 2000. Drosophila Dscam is an axon guidance receptor exhibiting extraordinary molecular diversity. Cell 101:671-684.

Schmucker D, Flanagan JG. 2004. Generation of recognition diversity in the nervous system. Neuron 44:219-222.

Schramke V, Allshire R. 2003. Hairpin RNAs and retrotransposon LTRs effect RNAi and chromatin-based gene silencing. Science 301:1069-1074.

Scully AL, McKeown M, Thomas JB. 1999. Isolation and characterization of Dek, a Drosophila eph receptor protein tyrosine kinase. Mol Cell Neurosci 13:337-347.

Seeger M, Tear G, Ferres-Marco D, Goodman CS. 1993. Mutations affecting growth cone guidance in Drosophila: genes necessary for guidance toward or away from the midline. Neuron 10:409-426.

Segal RA, Greenberg ME. 1996. Intracellular signaling pathways activated by neurotrophic factors. Annu Rev Neurosci 19:463-489.

Sekiguchi JA, Whitlow S, Alt FW. 2001. Increased accumulation of hybrid V(D)J joins in cells expressing truncated versus full-length RAGs. Mol Cell 8:1383-1390.

Sekiguchi JM, Gao Y, Gu Y, Frank K, Sun Y, Chaudhuri J, Zhu C, Cheng HL, Manis J, Ferguson D, Davidson L, Greenberg ME, Alt FW. 1999. Nonhomologous end-joining proteins are required for V(D)J recombination, normal growth, and neurogenesis. Cold Spring Harb Symp Quant Biol 64:169-181.

Serafini T. 1999. Finding a partner in a crowd: neuronal diversity and synaptogenesis. Cell 98:133-136.

Serafini T. 1999. Finding a partner in a crowd: neuronal diversity and synaptogenesis. Cell 98:133-136.

Serafini T, Colamarino SA, Leonardo ED, Wang H, Beddington R, Skarnes WC, Tessier-Lavigne M. 1996. Netrin-1 is required for commissural axon guidance in the developing vertebrate nervous system. Cell 87:1001-1014.

Serafini T, Kennedy TE, Galko MJ, Mirzayan C, Jessell TM, Tessier-Lavigne M. 1994. The netrins define a family of axon outgrowth-promoting proteins homologous to C. elegans UNC-6. Cell 78:409-424.

Shatz CJ. 1990. Impulse activity and the patterning of connections during CNS development. Neuron 5:745-756.

Smith A, Robinson V, Patel K, Wilkinson DG. 1997. The EphA4 and EphB1 receptor tyrosine kinases and ephrin-B2 ligand regulate targeted migration of branchial neural crest cells. Curr Biol 7:561-570.

Song H, Ming G, He Z, Lehmann M, McKerracher L, Tessier-Lavigne M, Poo M. 1998. Conversion of neuronal growth cone responses from repulsion to attraction by cyclic nucleotides. Science 281:1515-1518.

Song HJ, Ming GL, Poo MM. 1997. cAMP-induced switching in turning direction of nerve growth cones. Nature 388:275-279.

Song X, Sui A, Garen A. 2004. Binding of mouse VL30 retrotransposon RNA to PSF protein induces genes repressed by PSF: effects on steroidogenesis and oncogenesis. Proc Natl Acad Sci U S A 101:621-626.

Spanopoulou E, Cortes P, Shih C, Huang CM, Silver DP, Svec P, Baltimore D. 1995. Localization, interaction, and RNA binding properties of the V(D)J recombination-activating proteins RAG1 and RAG2. Immunity 3:715-726.

Spanopoulou E, Zaitseva F, Wang FH, Santagata S, Baltimore D, Panayotou G. 1996. The homeodomain region of Rag-1 reveals the parallel mechanisms of bacterial and V(D)J recombination. Cell 87:263-276.

Sperry RW. 1963. Chemoaffinity in the Orderly Growth of Nerve Fiber Patterns and Connections. Proc Natl Acad Sci U S A 50:703-710.

Stein E, Lane AA, Cerretti DP, Schoecklmann HO, Schroff AD, Van Etten RL, Daniel TO. 1998. Eph receptors discriminate specific ligand oligomers to determine alternative signaling complexes, attachment, and assembly responses. Genes Dev 12:667-678.

Stohl W, Gonatas NK. 1977. Distribution of the thy-1 antigen in cellular and subcellular fractions of adult mouse brain. J Immunol 119:422-427.

Stuckmann I, Weigmann A, Shevchenko A, Mann M, Huttner WB. 2001. Ephrin B1 is expressed on neuroepithelial cells in correlation with neocortical neurogenesis. J Neurosci 21:2726-2737.

Takeichi M. 1991. Cadherin cell adhesion receptors as a morphogenetic regulator. Science 251:1451-1455.

Teh C, Chong SW, Korzh V. 2003. DNA delivery into anterior neural tube of zebrafish embryos by electroporation. Biotechniques 35:950-954.

Terman JR, Kolodkin AL. 1999. Attracted or repelled? Look within. Neuron 23:193-195.

Tessier-Lavigne M, Goodman CS. 1996. The molecular biology of axon guidance. Science 274:1123-1133.

Thompson CB. 1995. New insights into V(D)J recombination and its role in the evolution of the immune system. Immunity 3:531-539.

Tonegawa S. 1983. Somatic generation of antibody diversity. Nature 302:575-581.

Trousse F, Marti E, Gruss P, Torres M, Bovolenta P. 2001. Control of retinal ganglion cell axon growth: a new role for Sonic hedgehog. Development 128:3927-3936.

Trowe T, Klostermann S, Baier H, Granato M, Crawford AD, Grunewald B, Hoffmann H, Karlstrom RO, Meyer SU, Muller B, Richter S, Nusslein-Volhard C, Bonhoeffer F. 1996. Mutations disrupting the ordering and topographic mapping of axons in the retinotectal projection of the zebrafish, Danio rerio. Development 123:439-450.

Tsai CL, Drejer AH, Schatz DG. 2002. Evidence of a critical architectural function for the RAG proteins in end processing, protection, and joining in V(D)J recombination. Genes Dev 16:1934-1949.

Tsai CL, Schatz DG. 2003. Regulation of RAG1/RAG2-mediated transposition by GTP and the C-terminal region of RAG2. Embo J 22:1922-1930.

Tuzi NL, Gullick WJ. 1994. eph, the largest known family of putative growth factor receptors. Br J Cancer 69:417-421.

Vaandrager JW, Schuuring E, Philippo K, Kluin PM. 2000. V(D)J recombinase-mediated transposition of the BCL2 gene to the IGH locus in follicular lymphoma. Blood 96:1947-1952.

Vaitaitis GM, Poulin M, Sanderson RJ, Haskins K, Wagner DH, Jr. 2003. Cutting edge: CD40-induced expression of recombination activating gene (RAG) 1 and RAG2: a mechanism for the generation of autoaggressive T cells in the periphery. J Immunol 170:3455-3459.

van Gent DC, Hiom K, Paull TT, Gellert M. 1997. Stimulation of V(D)J cleavage by high mobility group proteins. Embo J 16:2665-2670.

van Gent DC, McBlane JF, Ramsden DA, Sadofsky MJ, Hesse JE, Gellert M. 1995. Initiation of V(D)J recombination in a cell-free system. Cell 81:925-934.

VanBerkum MF, Goodman CS. 1995. Targeted disruption of Ca(2+)-calmodulin signaling in Drosophila growth cones leads to stalls in axon extension and errors in axon guidance. Neuron 14:43-56.

Varela-Echavarria A, Tucker A, Puschel AW, Guthrie S. 1997. Motor axon subpopulations respond differentially to the chemorepellents netrin-1 and semaphorin D. Neuron 18:193-207.

Wagle M, Grunewald B, Subburaju S, Barzaghi C, Le Guyader S, Chan J, Jesuthasan S. 2004. EphrinB2a in the zebrafish retinotectal system. J Neurobiol 59:57-65.

Wagle M, Jesuthasan S. 2003. Baculovirus-Mediated Gene Expression in Zebrafish. Marine Biotechnology 5:58-63.

Walter J, Henke-Fahle S, Bonhoeffer F. 1987. Avoidance of posterior tectal membranes by temporal retinal axons. Development 101:909-913.

Walter J, Kern-Veits B, Huf J, Stolze B, Bonhoeffer F. 1987. Recognition of position-specific properties of tectal cell membranes by retinal axons in vitro. Development 101:685-696.

Wang X, Roy PJ, Holland SJ, Zhang LW, Culotti JG, Pawson T. 1999. Multiple ephrins control cell organization in C. elegans using kinase-dependent and -independent functions of the VAB-1 Eph receptor. Mol Cell 4:903-913.

Weiner JA, Chun J. 1997. Maternally derived immunoglobulin light chain is present in the fetal mammalian CNS. J Neurosci 17:3148-3156.

Weinmann AS, Farnham PJ. 2002. Identification of unknown target genes of human transcription factors using chromatin immunoprecipitation. Methods 26:37-47.

Westerfield M, editor.Westerfield Ms. 1995. The Zebrafish Book. A guide for laboratory use of zebrafish (Danio rerio). Eugene: University of Oregon Press.

Wilkinson DG. 2000. Topographic mapping: organising by repulsion and competition? Curr Biol 10:R447-451.

Wilson RK, Mardis ER. 1997. Shotgun Sequencing. In: Birren B, Gree ED, Klapholz S, Myers RM, Roskams J, Birren B, Gree ED, Klapholz S, Myers RM, Roskams Js. Genome Analysis A Laboratory Manual. Cold Spring Harbor Laboratory Press. p 397-454.

Wojtowicz WM, Flanagan JJ, Millard SS, Zipursky SL, Clemens JC. 2004. Alternative splicing of Drosophila Dscam generates axon guidance receptors that exhibit isoform-specific homophilic binding. Cell 118:619-633.

Wong JT, Wong ST, O'Connor TP. 1999. Ectopic semaphorin-1a functions as an attractive guidance cue for developing peripheral neurons. Nat Neurosci 2:798-803.

Wu HH, Williams CV, McLoon SC. 1994. Involvement of nitric oxide in the elimination of a transient retinotectal projection in development. Science 265:1593-1596.

Wu Q, Maniatis T. 1999. A striking organization of a large family of human neural cadherin-like cell adhesion genes. Cell 97:779-790.

Wu Q, Zhang T, Cheng JF, Kim Y, Grimwood J, Schmutz J, Dickson M, Noonan JP, Zhang MQ, Myers RM, Maniatis T. 2001. Comparative DNA sequence analysis of mouse and human protocadherin gene clusters. Genome Res 11:389-404.

Yates PA, Roskies AL, McLaughlin T, O'Leary DD. 2001. Topographic-specific axon branching controlled by ephrin-As is the critical event in retinotectal map development. J Neurosci 21:8548-8563.

Yokoyama N, Romero MI, Cowan CA, Galvan P, Helmbacher F, Charnay P, Parada LF, Henkemeyer M. 2001. Forward signaling mediated by ephrin-B3 prevents contralateral corticospinal axons from recrossing the spinal cord midline. Neuron 29:85-97.

Yoshikawa S, McKinnon RD, Kokel M, Thomas JB. 2003. Wnt-mediated axon guidance via the Drosophila Derailed receptor. Nature 422:583-588.

Yu W, Misulovin Z, Suh H, Hardy RR, Jankovic M, Yannoutsos N, Nussenzweig MC. 1999. Coordinate regulation of RAG1 and RAG2 by cell type-specific DNA elements 5' of RAG2. Science 285:1080-1084.

Yurchenko V, Xue Z, Sadofsky M. 2003. The RAG1 N-terminal domain is an E3 ubiquitin ligase. Genes Dev 17:581-585.

Zhang JH, Cerretti DP, Yu T, Flanagan JG, Zhou R. 1996. Detection of ligands in regions anatomically connected to neurons expressing the Eph receptor Bsk: potential roles in neuron-target interaction. J Neurosci 16:7182-7192.

Zheng JQ, Felder M, Connor JA, Poo MM. 1994. Turning of nerve growth cones induced by neurotransmitters. Nature 368:140-144.

Zou Y, Stoeckli E, Chen H, Tessier-Lavigne M. 2000. Squeezing axons out of the gray matter: a role for slit and semaphorin proteins from midline and ventral spinal cord. Cell 102:363-375.