CHAPTER 2
GENERAL METHODS FOR INJECTING FLUORESCENT TRACERS

2.1 INTRODUCTION

This chapter describes the incubation and housing conditions used in most experiments for this thesis and the neuroanatomical procedures used in Chapters 3-7. Any variations in the procedures, such as changing the survival period after injection and the injection of different volumes of tracer, will be detailed in the relevant chapter.

2.2 SUBJECTS AND HOUSING

Fertilised eggs (Black Australorp x White Leghorn) were obtained from Barter and Sons, Sydney, NSW. During the first 16 days of incubation, the eggs were incubated in an automatically turning, forced-draught incubator (Multiplo, Australia). The incubator turned the eggs at every second hour and maintained the temperature at 37-38 ^{0}C and 80% relative humidity. On every second day, water was added to the incubator and the temperature was checked.

On day 8 of incubation, the eggs were candled and all non-fertile eggs were removed from the incubator. On day E16/17 of incubation, the eggs were placed in wire-grid trays (30 x 30 x 10 cm) and moved to either a dark incubator or an illuminated incubator. The dark incubator was located in a dark room. The light incubator was illuminated with a 40 W light bulb suspended from the ceiling of the incubator so that the eggs were exposed to light continuously from day E17 of incubation to hatching (200-300 lux measured at the level of the eggs). The hatching incubators were maintained at 37-38 ^{0}C and approximately 95% humidity.

After hatching the chicks were housed in groups of 2 or 3 in grey metal cages (23 x 20 x 30 cm) with the front wall panel of transparent plastic. Food (chick starter crumbles, Fielders, Tamworth) and water were available ad libitum.

2.3 PROCEDURES USED FOR TRACER INJECTION

2.3.1. Fluorescent tracers

The five retrograde fluorescent tracers used were True Blue (TB, Sigma, St. Louis, USA), Fluorogold (FG, Fluorochrome Inc., England), rhodamine B isothiocyanate (RITC, Sigma, St. Louis, USA, No. R1755) and rhodamine-conjugated latex microspheres (red beads, Luma Fluor, New York), and fluroscein-conjugated latex microspheres (green beads, Luma Fluor, New York). Both red beads and green beads were prepared for use by Luma Fluor. However, TB, FG and RITC were prepared immediately prior to injection. RITC (2%) was dissolved in sterile, pyrogen-free water with an addition of 1% dimethylsulfoxide (DMSO). The FG (4%) was dissolved in sterile, pyrogen-free water only. A suspension of TB (5%) was prepared in sterile, pyrogen-free water. The solutions of RITC, FG and TB were homogenized for 20 minutes in an ultrasonicator. Since TB has low solubility in water, some of it tends to settle in the syringe at the time of injection. This property makes it difficult to control the dose of TB injected. To overcome this problem, the suspension of TB was homogenized 10 minutes again in an ultrasonicator prior to each injection.

2.3.2. Injection of the fluorescent tracers

On day 2 posthatching, the chicks were anaesthetized with equithesin (0.4 g magnesium sulphate, 0.8 g chloral hydrate, 17 ml pyrogen-free water, 3 ml Nembutal) given by intramuscular injection (3ml/kg body weight). The anaesthetized chick was mounted in a stereotaxic apparatus. The mouth bar of the stereotaxic apparatus was placed 45^0 below the horizontal axis of the stereotaxic apparatus (see Fig.2.1) according to the procedures outlined in Kuenzel and Masson (1988). The scalp was incised along the

midline to expose the skull. A small piece of skull over the injection target was removed. Because the skull of 2-day-old chicks is very thin and soft, a dental drill is difficult to manipulate and it is difficult not to make a lesion on the surface of the brain. Therefore, a sterile surgical blade (No.15) was used to remove a small piece of the skull. Then the chick received a pressure injection of fluorescent tracers into the target areas, the left and right visual Wulst in the forebrain or the left and right nucleus rotundus (Rt) in the thalamus, using a 1 µl Hamilton glass microsyringe attached to a 26 gauge needle. The microsyringes were sterilised overnight using Zephiran and then they were rinsed using sterile, pyrogen-free water to clean and remove Zephiran prior to injection.

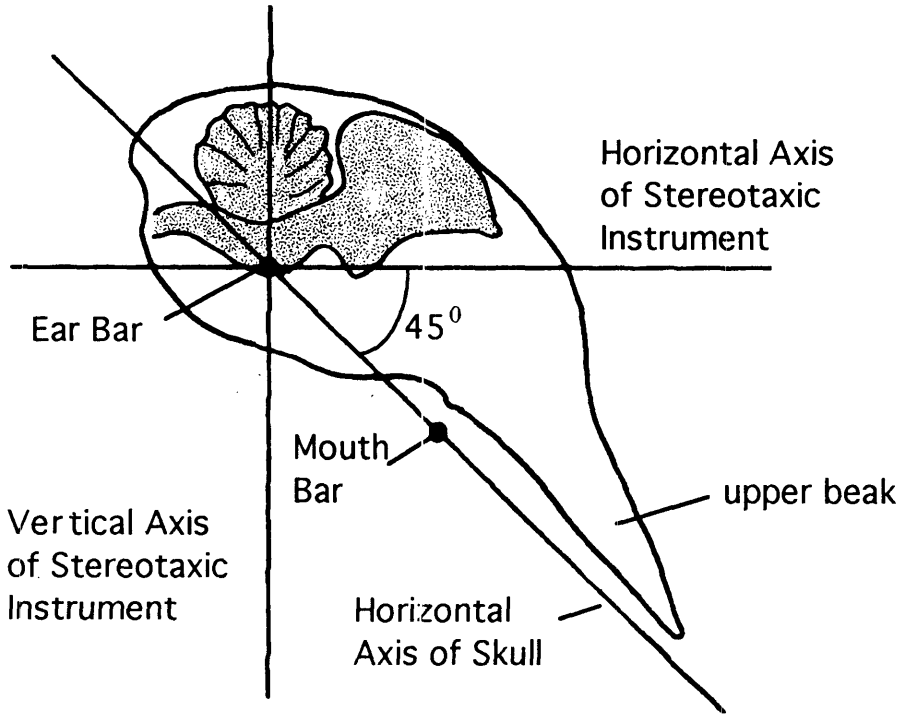

Figure 2.1 Orientation of the brain and skull of the chick mounted in the stereotaxic instrument

Reference was made to the atlas of Kuenzel and Masson (1988) for the 2-week-old chick brain, but the coordinates had to be adjusted for the 2-day-old chicks used and for the strain used in this study. The coordinates for placement of injections into the visual Wulst were A8.5-9.5 L (or R) 1.5 H1.5-2.0 (the centre of ear bar was A0.0, the midline of the brain was L0.0 (left) or R0.0 (right), and the dorsal surface of brain was H0.0). The coordinates for injections of Rt were A4.3-4.6 L (or R) 2.5 H7.1. The injections were made using a 1 µl Hamilton glass microsyringe attached with a 26 gauge needle. One minute after inserting the needle into the target area, 0.05 or 0.1 µl of tracer was injected slowly over a 2 minute period. To avoid leakage from the needle tip during advancement of needle through the brain, the plunger was raised to withdraw the column of fluid by 0.1µl before inserting the needle into the brain (Alheid et al., 1981). Before withdrawing, the needle was kept in place for 10 minutes to minimise the diffusion of the tracer into the needle track. Different tracers were injected into the left and the right hemispheres. After completing the injection of tracer and withdrawing the needle, the incision of skin on the head was sutured.

After injection of the tracers, the animals were allowed to survive for 4 days. Then, each chick was injected with a lethal dose (0.5 ml) of sodium pentobarbitone (300 mg/ml; Valabarb) and perfused transcardially. Prior to perfusion, 0.1 ml heparin (2500 I.U./ml) was injected into the left ventricle and allowed to circulate for approximately 1 minute; then the perfusion needle was inserted into the chick's left ventricle and the right auricle was cut. The chicks were perfused first with 30 ml of 0.1 M phosphate buffer (pH 7.4), for animals that had been injected with red beads or greed beads according to procedures of Katz et al. (1984) and Katz and Iarovici (1990), or physiological saline, for animals injected with TB, FG and RITC at 37 ^{0}C. This was followed by perfusion of 60ml of fixative, 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), at room temperature. After perfusion was finished, the brain was kept in the skull for half an hour. Then it was removed from the skull and post-fixed in the same fixative at 4 ^{0}C for

up to 4 weeks before sectioning.

2.4 HISTOLOGICAL PROCEDURES

2.4.1. Brain Sectioning

Before sectioning, the brains were placed in 10% sucrose dissolved in 0.1 M phosphate buffer and stored at 4 ^{0}C for 24 hours. The brains were sectioned into 40 µm coronal sections using a freezing microtome and the sections were collected in 0.1 M phosphate buffer. The sections were mounted alternately on chrom-alum gelatin coated slides and air-dried, thus giving two series of sections. One series was immersed in xylene for less than 1 minute and coverslipped with Fluoromount (BDH, England; for chicks injected with red and green beads) or DPX mountant (BDH, England; for those injected with only TB, FG and RITC), and the other series was stained with 0.1% cresyl violet and coverslipped (Disbrey and Rack, 1970).

2.4.2. Episcopic-fluorescence microscope

The sections were examined using a Nikon (HB-10101AF) episcopic-fluorescence microscope with a 10 x (for TB, FG and RITC labelling) or 20 x (for the red beads and green beads labelling) UVFL objective and 10 x eyepiece. Filters were an UV-2A filter block for the TB and FG (including a 330-380 nm excitation filter, a dichroic mirror DM400 and a 420 nm barrier filter), a G-2A filter block for RITC and the red beads (including a 510-560 nm excitation filter, a dichroic mirror DM580 and a 590 nm barrier filter) and a B-2A filter block for the green beads (including a 450-490 nm excitation filter, a dichroic mirror DM510 and a 520 nm barrier filter). The first series of sections was used to examine the retrograde labelling pattern following injection of tracers into different regions of the brain, to count the number of labelled neurones and to measure various parameters of the injection site. The alternative sections stained with cresyl violet were used to determine the location of the labelled neurones and the injection sites of the tracers using standard light microscopy.

2.4.3 Counting of the labelled neurones

In the GLd: Following injection of different tracers into the left and right visual Wulst, the tracers were taken up and transported retrogradely into the cell bodies in the left and right nucleus geniculatus lateralis pars dorsalis (GLd) of the thalamus (Figure 2.2). By means of this double-injection procedure, the GLd neurones with uncrossed projections to the ipsilateral Wulst were labelled by the tracer injected to the ipsilateral visual Wulst (Fig. 2.2 a) and those with crossed projections to the Wulst were labelled by

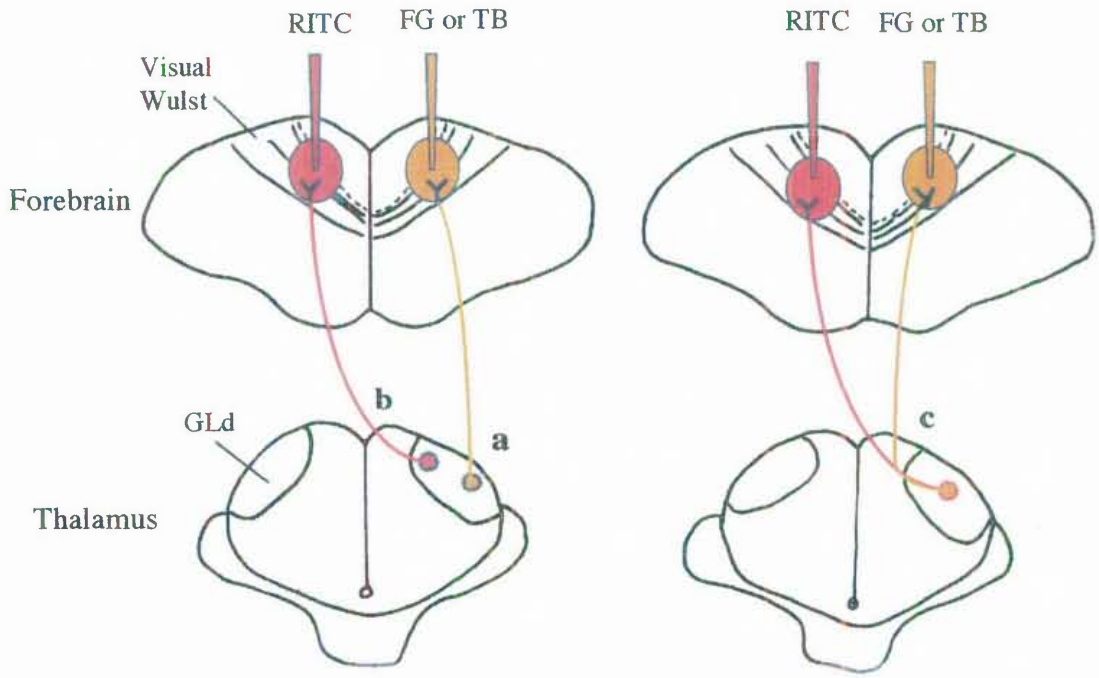

Figure 2.2 The double-labelling retrograde tracing technique used in this study to trace the thalamo-Wulst projections.
After the tracers (RITC, FG or TB) were injected into the visual Wulst, they were taken up by the nerve endings and transported retrogradely to cell bodies in the thalamus. a, an ipsilaterally projecting neurone which was labelled by tracer injected into the ipsilateral Wulst. b, contralateral projecting neurone which was labelled by the tracer injected into the contralateral Wulst. c, bilaterally projecting neurone which has an axon collateral so that it projects to both ipsilateral and contralateral Wulst. This neurone was double-labelled by tracers injected into both ipsilateral and contralateral visual Wulst.

the tracer injected into the contralateral visual Wulst (Fig. 2.2 b). If the neurones had collateral axons so that one branch projected to the left visual Wulst and the other projected to the right visual Wulst, they were double-labelled by the tracers (Fig. 2.2 c).

All labelled neurones in the GLd on each side of the thalamus were counted by using the method developed by Rogers and her colleagues (Rogers and Sink, 1988; Adret and Rogers, 1989; Rogers et al., 1993). An ocular micrometer grid (squares of 80 μm x80 μm at magnification of 100 x) was placed over the image of the labelled neurones to improve the accuracy of counting. Then, for each thalamic section, the number of labelled neurones counted was allotted into one of the following categories of cell numbers: 10 or less, 11-25, 51-100, 101-250, 251-500, 501-1000 and over 1000. For each brain the counts from all thalamic sections, using the middle value from each category, were added to give an estimation of the total number of the ipsilateral and contralateral cells (relevant to the injection side) labelled in the thalamus for each tracer. The double labelled neurones in the GLd were also counted (see Chapter 4).

In the optic tectum: Following injection of tracers into the nucleus rotundus (Rt), the tracers were taken up and transported retrogradely into the cell bodies in the layer stratum griseum centrale (SGC; layer 13 of the Cajal numerical system of nomenclature, p.20) of the optic tectum (Fig. 2.3). As discussed above, the ipsilateral projecting neurones (Fig. 2.3 a) and contralateral projecting neurones (Fig. 2.3 b) in SGC were labelled separately by the tracers injected into Rt on the same or the contralateral side. The bilaterally projecting neurones with collateral axons were double labelled by the two tracers injected, one of which was injected into the left Rt and the other of which was injected into the right Rt (Fig. 2.3 c). All labelled neurones in the entire SGC of each section were counted. The counts for each section were added to obtain the total number of the ipsilateral and contralateral labelled neurones (ipsilateral or contralateral with respect to the injection side) for each tracer. The double labelled cells in the optic tectum were also counted as reported in Chapter 5.

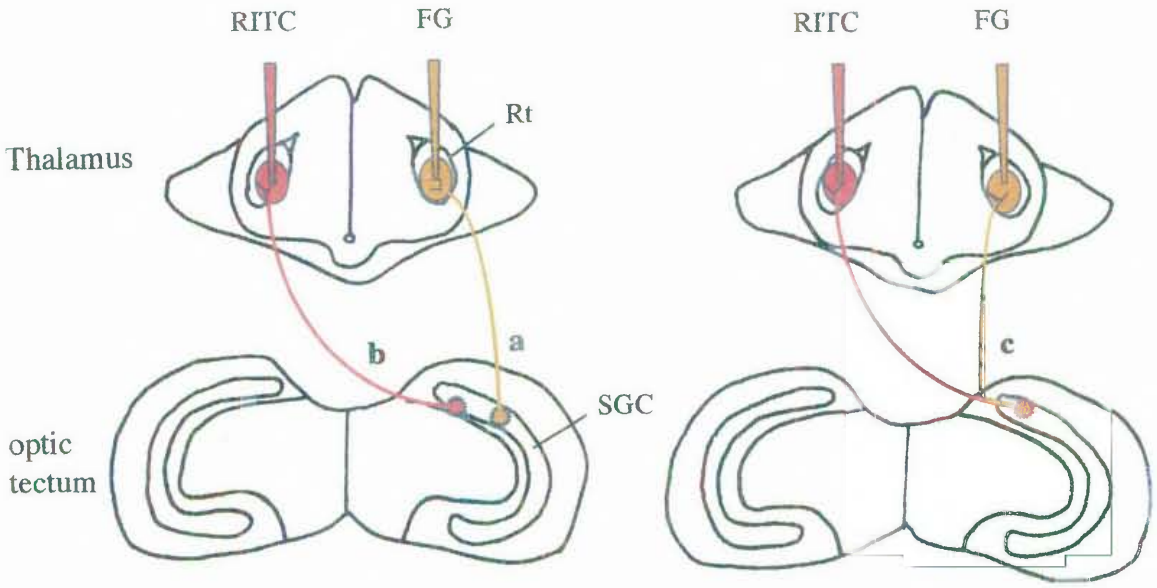

Figure 2.3 The double-labelling retrograde tracing technique used in this study to trace the tecto-rotundal projections.
After the tracers (RITC, FG or TB) were injected into the nucleus rotundus (Rt), they were taken up by the nerve endings and transported retrogradely to the cell bodies in the optic tectum. a, ipsilaterally projecting neurone which was labelled by tracer injected into the ipsilateral Rt. b, contralateral projecting neurone which was labelled by the tracer injected into the contralateral Rt. c. bilaterally projecting neurone which projects to both the ipsilateral and the contralateral Rt. This neurone was double-labelled by tracers injected into both the ipsilateral and contralateral Rt.

2.4.4 Determining the c/i ratio

After counting the labelled neurones in GLd (for chicks with injection of tracer into the visual Wulst) or in the optic tectum (for chicks with injection of tracer into the Rt), the c/i ratio was calculated for each injection of tracer by using the following formula:

$$\text{c/i ratio} = \frac{\text{Number of labelled cells contralateral to the side of injection (c)}}{\text{Number of labelled cells ipsilateral to the side of injection (i)}}$$

Because different tracers have different extents of diffusion (see next paragraph

and Chapter 3, p. 75 and p. 80), it was hard to control the volume of the injection site even if the same amount of tracer was injected (Condé, 1987; Schmued and Fallon, 1986; Schmued et al., 1990; Adret and Rogers, 1989; Rogers and Rajendra, 1993). In addition, variations in the amount of tracer injected may occur when tracers such as TB are used (as discussed above, p. 55). This may make it difficult to compare directly the absolute number of neurones counted for the different tracers (see Chapter 3). Therefore the c/i ratio, which has been used to control the variations in the amount of tracer injected (Adret and Rogers, 1989; Rogers and Rajendra, 1993; Rogers et al., 1993), was used. Our data show that there were significant correlations between the volumes of tracers injected and numbers of ipsilateral and contralateral labelled neurones, but not between the volumes of tracers and the c/i ratios (see Chapter 4, p. 114 and Chapter 6, p. 177, p. 181). The c/i ratio could also be used to overcome the problem of variations between the different tracers following their injection into the visual Wulst (see p. 77 and p. 114).

2.4.5 Measurement of the volume of the injection site

For TB and FG, the injection site showed three concentric zones around the needle track (Fig. 2.4), as described by Schmued and Fallon (1986) and Güntürkün et al. (1993). Central zone 1 was the tip of the needle tract with a small region of necrosis, which contained the injected tracer. Peripheral zone 2 was the brightly fluorescent region surround the necrotic area. The most peripheral zone 3 exhibited labelled cells around the bright core (zone 1 and 2). The injection site for RITC showed three concentric zones also (Fig. 2.5). The zones 1 and 2 of RITC were similar to that of FG and TB, but zone 3 did not show any labelled cells. Zone 3 of the RITC injection was brighter than the outside surrounding areas. RITC diffused more widely to the surrounding area than did FG and TB. No studies have shown uptake of tracer from zone 3; therefore, following the methods of Adret and Rogers (1989) and Güntürkün et al.(1993), only the area of zone 1 plus zone 2 was measured. Hereafter, for FG, TB and RITC, 'area of injection site' will refer only to zones 1 and 2.

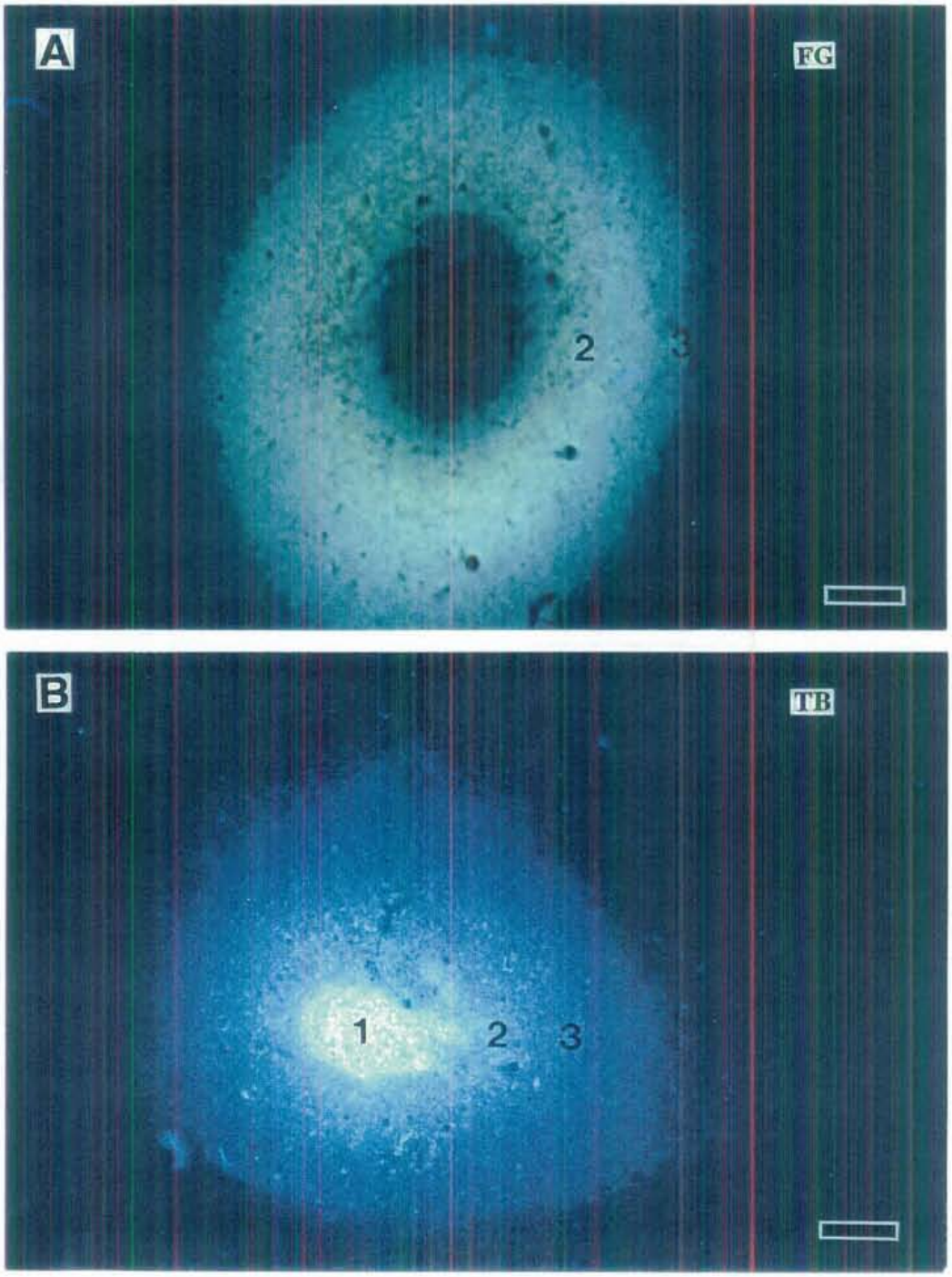

Figure 2.4 Photomicrographs of injection sites of FG (A) into the visual Wulst and TB (B) into Rt. The injection site contains three zones (1, 2 and 3; for detail see text). Scale bars: 200μm.

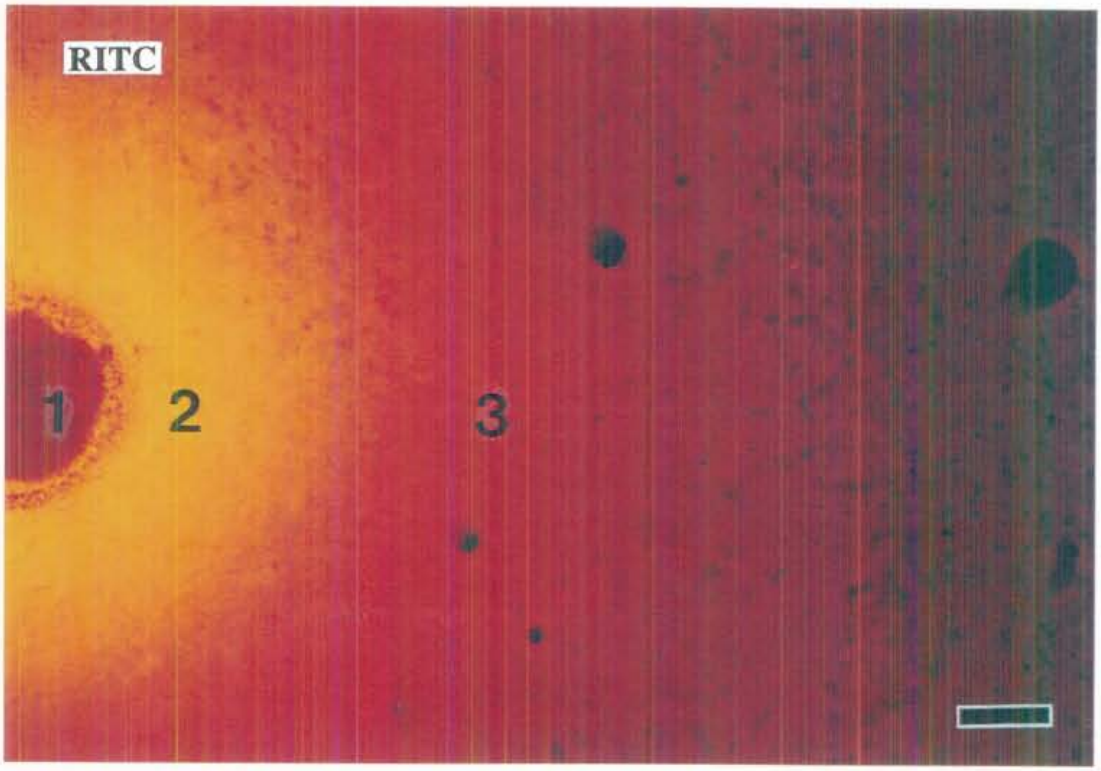

Figure 2.5 Photomicrograph of injection site of RITC into the visual Wulst.
Note that the injection site contains three zones (1, 2 and 3). Scale bar: 200μm.

Red beads and green beads remain confined to their injection site (Katz et al., 1984; Katz and Iarovici, 1990) as they have little diffusion. These tracers accumulated around the tip of the injection (zone 1) and no clear diffusion zone (zone 2) was observed (Fig. 2.6).

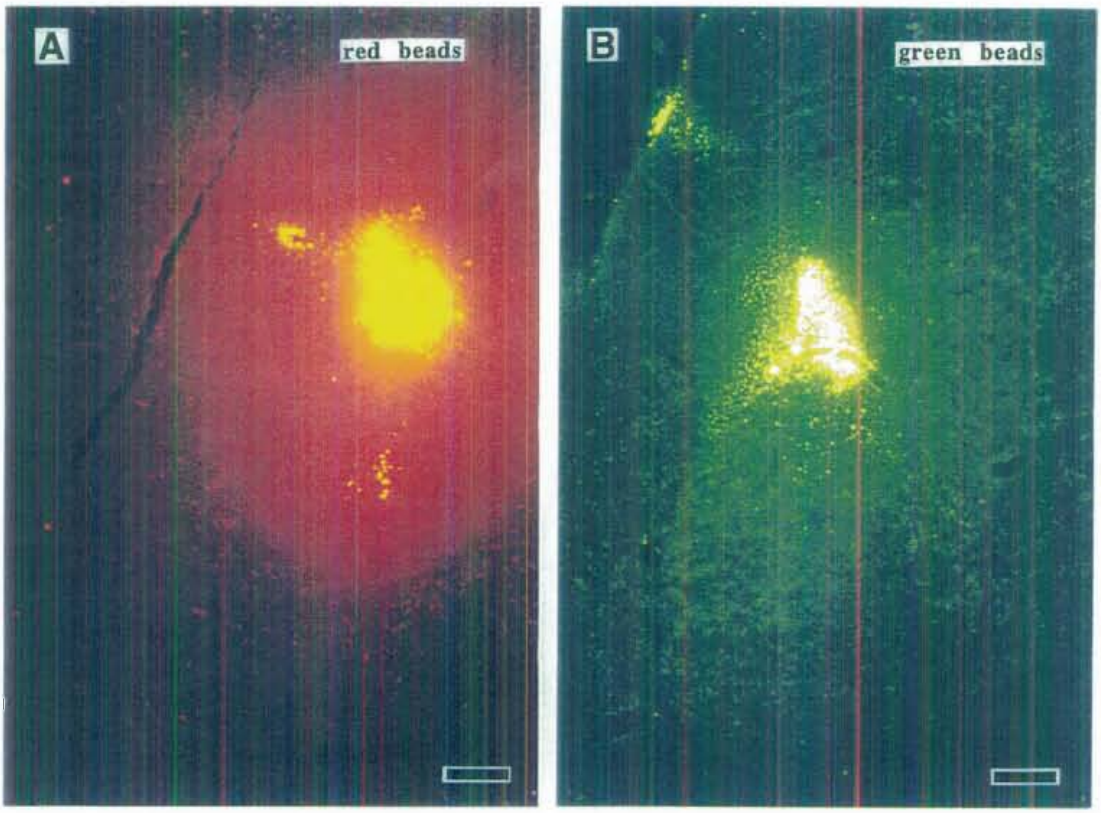

Figure 2.6 Photomicrographs of the injection sites of red beads and green beads. Note that the injection sites of red beads and green beads contain a clear zone 1 only. Scale bars: 200μm.

To calculate the volume of the injection site, the injection area of each section was measured using an ocular micrometer (squares of 200 x 200 μm) at low magnification (40x, Fig. 2.7). After all measurement of the series of sections for each chick, the values of the injection areas were multiplied by the thickness of the section and totalled to obtained the volume of the injection site for each tracer. This volume indicates the amount of dye injected.

2.4.6 Measurement of location of the injection site

In the visual Wulst: For each brain, the section showing the maximum area of the injection site of each tracer was selected to measure the placement of the injection site. For injections into the visual Wulst, the depth of injection from the surface of the brain

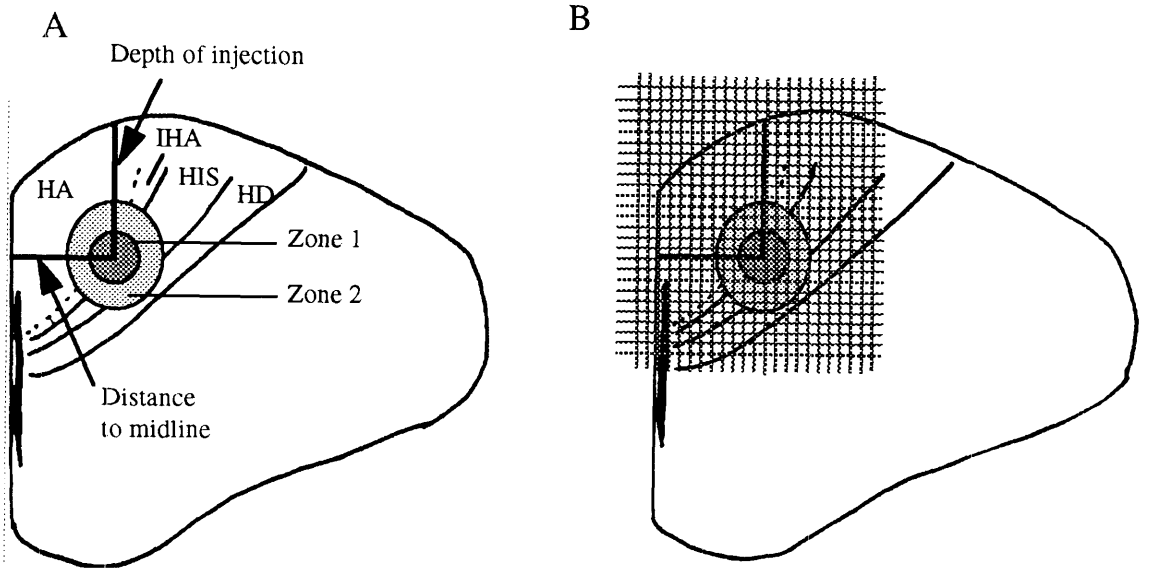

Figure 2.7 Measurement of parameters of the injection site in the Visual Wulst. A, an example of an injection site in the right visual Wulst. B, an ocular micrometer grid has been superimposed on the injection site of the section as in A. The injection area (for RITC, FG and TB, including zones 1 and 2; for red beads and green beads, only zone 1) was measured on each section containing the tracers. Depth of the injection site and distance to the midline were measured from the centre of the injection site on the sections with the maximum fluorescing area.

and the distance from the midline of the brain to the centre of the injection site were measured using an ocular micrometer grid (squares of 80 μm x80 μm at magnification of 100 x; Fig. 2.7). The rostral-caudal distance from the front end of the forebrain to the injection centre was calculated by counting the number of the sections and multiplying by the thickness of a section (40μm).

In the Rt: For each brain, the section showing the maximum injection site of tracer was selected to measure the depth of the centre of the injection from the dorsal boundary of Rt and its distance from the midline-boundary of the nucleus using an ocular micrometer grid (squares of 80 μm x80 μm, magnification 100 x; Fig. 2.8). The rostro-caudal distance from the first section on which Rt appeared to the centre of the injection

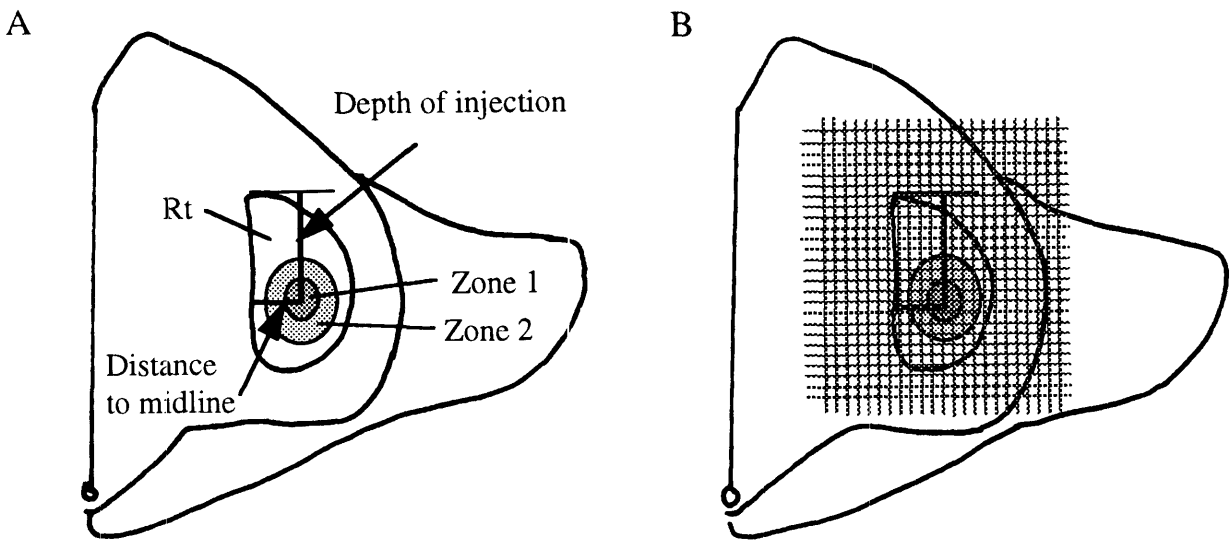

Figure 2.8 Measurement of parameters of the injection site in the Rt. A, an example of an injection site in the right Rt. B, an ocular micrometer grid has been superimposed of the injection site of the section as in A. The injection area (for RITC, FG and TB, including zones 1 and 2; for red beads and green beads, only zone 1) was measured on each section containing the tracer. Depth of the injection site from the dorsal boundary of Rt and distance to the midline-boundary of Rt were measured from the centre of the injection site on the sections with the maximum area of tracer.

site was calculated by the same method as used for determining the rostro-caudal position of injection in the Wulst (p.67).

2.5 Statistics

All of the data obtained in the histological studies, including c/i ratios, numbers of ipsilateral labelled neurones and contralateral labelled neurones and various parameters resulting from injection of different tracers, were analysed using ANOVA followed by 2-tailed unpaired t-tests for multiple comparisons. The Pearson correlation test was used to test for correlations between number of the contralateral and ipsilateral labelled cells, values of the c/i ratio and the volume of tracers injected. Correlations between the number of the ipsi- and contra-lateral labelled neurones, c/i ratio and other parameters of the injection site, such as depth, distance to the midline and distance to the rostral pole, were also analysed using Pearson correlation tests. For all statistical tests, the Statview statistical package (Haycock et al., 1992) was used and a probability value (P) of ≤ 0.05 was considered to indicate significant differences between groups.

CHAPTER 3
DIFFERENTIAL SENSITIVITIES OF THE TWO VISUAL PATHWAYS TO LABELLING BY FLUORESCENT TRACERS

3.1 INTRODUCTION

Since the initial discovery of fluorescent compounds as neuroanatomical tracers by Kuypers et al. (1977, 1979), numerous fluorescent retrograde tracers have been used to investigate the connections of neural pathways in various species (Bentivoglio et al., 1980; Thanos and Bonhoeffer, 1983; Katz et al., 1984; Kuypers and Huisman, 1984; Schmued and Fallon, 1986; Katz and Iarovici, 1990). Fluorescent tracing methods can be used as a simple and effective tool to perform double or even multiple labelling. In the latter case, two or more fluorescent tracers are injected into one or more brain areas of the same animal (Kuypers et al., 1980; Kuypers and Huisman, 1984). With use of multiple labelling, more detailed information has been obtained than by using single labelling.

The fluorescent tracers used contain various compounds with different structures and properties. In fact, some authors have reported that various fluorescent tracers are not always equally effective in retrogradely labelling certain neural pathways (Aschoff and Hollander, 1982; Kuypers and Huisman, 1984; Horikawa and Powell, 1986; Craig et al., 1989). Considerable variation exists between the different retrograde tracers.

Little is known about whether or not different neurones in different pathways have the same sensitivity to a particular tracer. Usually, studies comparing the labelling efficiency of retrograde tracers are performed in one particular neural pathway and rarely is the effectiveness of one tracer compared across different pathways because it is widely

assumed that the various tracers are equally effective in labelling different neural structures (for example, contralateral or ipsilateral projecting neurones) and different pathways, even though different numbers of neurones may be labelled by using different tracers. However, a recent study by Güntürkün et al. (1993) has shown that a fluorescent retrograde tracer, Fast Blue (FB), has structure-specific sensitivity: in the tecto-rotundal projections of pigeons, FB is much more effective in labelling the ipsilateral afferent neurones than the contralateral afferent neurones. By comparison another tracer, rhodamine B isothiocyanate (RITC), does not have this structure-specific sensitivity: RITC labels both the ipsilateral and contralateral afferent neurones (Güntürkün et al., 1993). Güntürkün et al. (1993) suggested that low-sensitivity tracers, like FB, might be unable to label contralaterally projecting neurones because the latter have small end-terminal arborisations.

It is still unclear whether or not this structure-specific sensitivity of retrograde tracers exists in the other neural pathways. As FB has been used successfully to label both the ipsilaterally and contralaterally projecting neurones from the thalamus to the visual Wulst (Miceli and Repérant, 1982), the different sensitivities of the contralaterally projecting neurones in the thalamus and the optic tectum may represent a difference between the two visual systems (i.e., in neuronal types). In the chick, another two retrograde tracers, Fluorogold (FG) and True Blue (TB), have been found to label both the ipsilaterally and contralaterally projecting visual neurones from the thalamus to the visual Wulst (Adret and Rogers, 1989), but it is not known whether FG and TB are also able to label both the ipsilaterally and contralaterally projecting neurones from the optic tectum to the rotundal nuclei.

Thus, it is important to investigate the effectiveness of labelling of the tracers used in this study (RITC, FG, TB) and also other tracers (red beads and green beads) in the two visual pathways. Based on the results of this chapter, suitable tracers will be chosen

for further experiments. As reviewed in Chapter 1, both the GLd-visual Wulst and tecto-Rt pathways have ipsilateral and contralateral projections. Therefore, the avian visual systems provide a convenient model in which to investigate whether there is a structural-specific (ipsilateral *vs* contralateral) or pathway-specific difference of retrograde labelling between various fluorescent tracers. A detailed study of the projections of both visual systems to the forebrain of the chick was undertaken to establish whether the visual neurones of the chick differ from those of the pigeon.

3.2 MATERIALS AND METHODS

Chicks were hatched from the eggs that had incubated with light exposure (200-300 lux at the eggs' surface) during days E17-21 of incubation. Both male and female chicks were used. The procedures used in this chapter are described in Chapter 2.

3.2.1 Experiment 1: Labelling by different tracers

The RITC was dissolved in 1% DMSO, and FG and TB were dissolved in water. (1) The thalamofugal pathway (n=10): on day 2 posthatching, 0.1 µl of RITC was injected into the visual Wulst of one hemisphere and 0.1 µl of FG or TB was injected into the visual Wulst of the other hemisphere (for details of the injection methods see Chapter 2, p. 55). The labelled neurones in the GLd were counted. (2) Tectofugal pathway (n=15): 0.05 µl RITC was injected into the Rt on the one side of the thalamus and 0.05 µl FG or TB was injected into Rt on the other side of the thalamus. All of the labelled neurones in the stratum griseum centrale (SGC) of the optic tectum were counted. After injection of the tracers, the chicks were allowed to survive for 4 days. Then the animals were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4, for details see Chapter 2, p. 57). After sectioning the brain, the sections were mounted alternately into two series and every second section was used to count the labelled neurones. The other series of sections was stained with cresyl violet (for details of the histological procedures see Chapter 2, p. 58).

3.2.2 Experiment 2: Effects of the interval between injection and sampling on FG and TB labelling of the tectofugal projections and the effect of DMSO

The results of the first experiment showed that the three tracers (RITC, FG and TB) labelled the ipsilateral and contralateral projecting neurones differently in the tectofugal projections, but this was not so in the thalamofugal projections. Therefore, the effects of different survival periods (2-10 days) and different vehicles (water or DMSO) on the effectiveness of labelling in the tecto-rotundal projections were tested. Because the purpose was to scan possible effects of the survival period, a small sample size was used (2 days, n=1; 4 days, n=2; 8 days, n=2; and 10 days, n=1). The tracers were injected into Rt of chicks on day 2 posthatching (n=3) or day 5 posthatching (n=3). RITC was dissolved in 1% DMSO, and FG and TB were dissolved in water (n=1) or 1% DMSO (n=5).

3.2.3 Experiment 3: Red beads and Green beads

In addition, in five 10-day-old chicks, 0.04 µl red beads and green beads were injected into Rt to investigate whether these two tracers may have differential labelling effectiveness in the tecto-rotundal projections. After injection of the tracers, the chicks were allowed to survive 4 days (n=3) or 8 days (n=2). After sectioning the brains, labelled neurones were counted in every fourth section.

3.2.4 Estimation of the lengths of thalamofugal and tectofugal projections

The different labelling effectiveness of FG, TB and green beads in the contralateral GLd-Wulst and the contralateral tecto-Rt projections (Experiments 1 to 3) raised the possibility that the length of these projections may be an explanation for the results. Ten chicks were used to estimate the distance between the injection site in the visual Wulst and labelled neurones in the contralateral GLd (n=5) compared to the distance between

the injection site in the Rt and the labelled neurones in the contralateral optic tectum (n=5). Since there is no established method for direct measurement of the length of neural pathways, the lengths of the two sets of projections were estimated using the following method.

1. As the efferents from GLd cross over in the dorsal supraoptic decussation (SODd) to the contralateral side of the brain and then project through the fasciculus prosencephalic lateralis (FPL) to the visual Wulst (Hunt and Webster, 1972), the length of the contralateral thalamofugal projections was calculated using the following approximation (Fig. 3.1 A and B):

Length of the contralateral GLd to Wulst projections = ab+ bc + cd

ab= distance between the centre of the injection site in the visual Wulst and the section through both the central GLd and the supraoptic decussation (SOD)

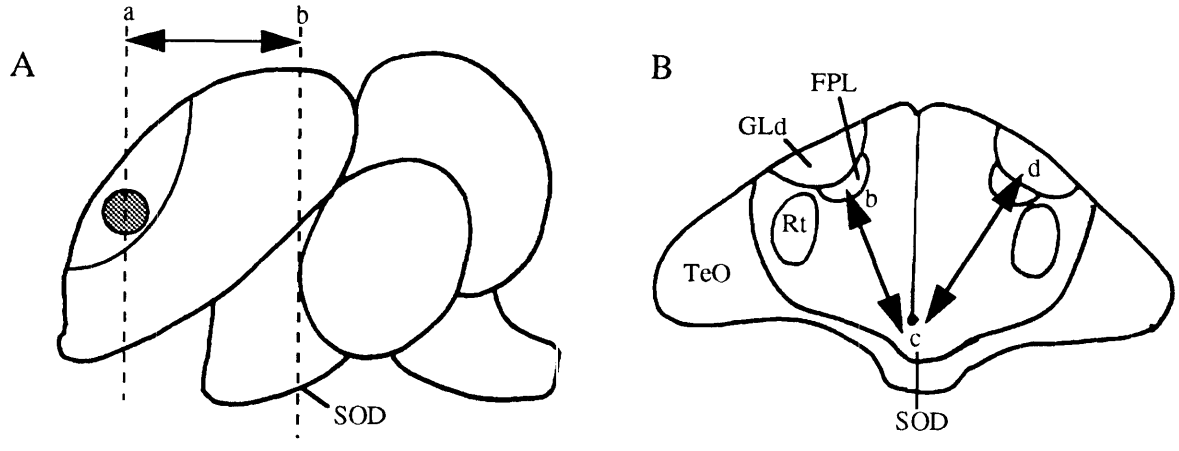

Figure 3.1 The method used to estimate the length of the contralateral GLd-Wulst projections (A and B). A. Outline drawing of the chick brain: dashed line a indicates the section through the centre of the injection site; dashed line b indicates the section through the supraoptic decussation (SOD). B. Drawing of transverse section through the central GLd and SOD as indicated by line b.

which was estimated by counting the number of sections between them and then multiplying the number of the sections by the thickness of each section (40μm; Fig. 3.1A);

bc= distances between FPL and SODd in the section through both the central GLd and SOD which was measured using an ocular micrometer (squares of 200 x 200 μm) at low magnification (40x; Fig.3.1B);

cd= distance between SODd and the contralateral GLd measured in the section through both the central GLd and SOD using an ocular micrometer (squares of 200 x 200 μm) at low magnification (40x; Fig. 3.1B).

As the tectal afferents project via the ventral supraoptic decussation (SODv) to Rt, the length of the contralateral tecto-Rt projections was estimated using the following formula (Fig. 3.2),

Length of the contralateral tecto-Rt projections = a'b' + b'c' + c'd'

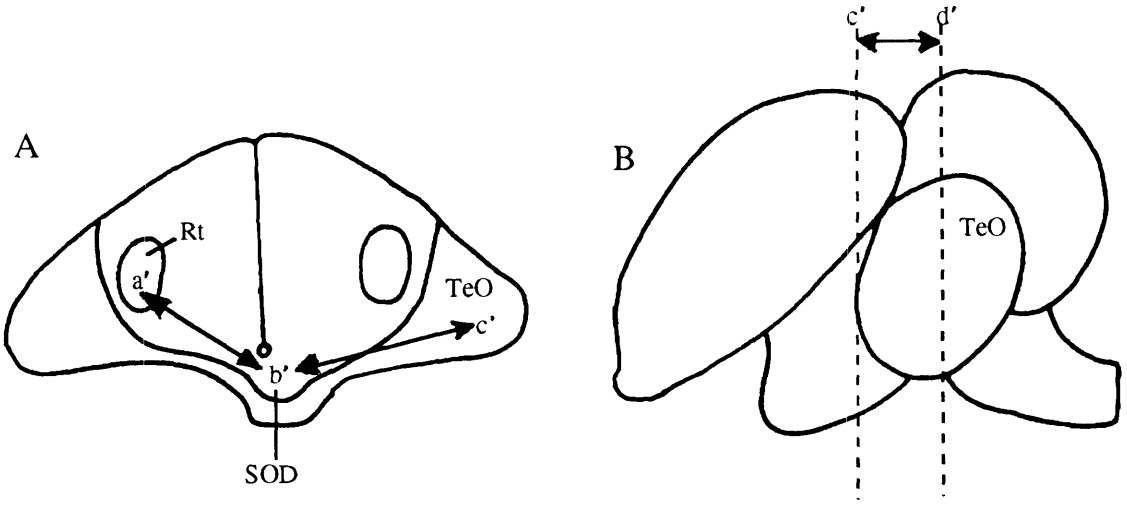

Figure 3.2 The method used to estimate the length of the contralateral tecto-Rt projections. A, Drawing of transverse section through SOD with first appearance of the tectum as indicated by line c in B. B, Outline drawing of the chick brain: dashed line c indicates the first section containing the tectum ; dashed line d indicates the section through the middle tectum. .

a'b'=distance between the centre of the Rt and SODv which was measured in the section through SOD that contained first appearance of the tectum, using an ocular micrometer (squares of 200 x 200 μm) at low magnification (40x; Fig. 3.2A);

b'c'=distance between SODv and the rostral tectum, measured in the section through SOD with the first appearance of the tectum using an ocular micrometer (squares of 200 x 200 μm) at low magnification (40x; Fig. 3.2A);

c'd'=distance between the first section containing the tectum and the middle of the optic tectum, calculated by counting the number of sections between these two regions and then multiplying the number of the sections by the thickness of each section (40μm; Fig. 3.2B).

3.3 RESULTS

3.3.1. Experiment 1: Labelling by RITC, FG and TB of neurones in the two visual pathways

3.3.1.1. Thalamofugal projections

In the 10 chicks injected with tracers in the visual Wulst, the centres of the injections were located in the region between IHA and HD and the injection sites contained IHA, HD and HIS. The fluorescent tracers diffused to HA also. The mean volume (± standard error, SE) of the injection site was 0.73±0.08 mm^3 for RITC, 0.61±0.11 mm^3 for FG and 0.31±0.04 mm^3 for TB. One way ANOVA showed that there was a significant difference in the volume of the injection sites for the three tracers ($F_{2,17}$=5.99, p=0.017). RITC had a significantly larger injection volume than TB (df=13, t=3.52, p=0.004, unpaired 2-tailed t-test). FG had also a larger injection site

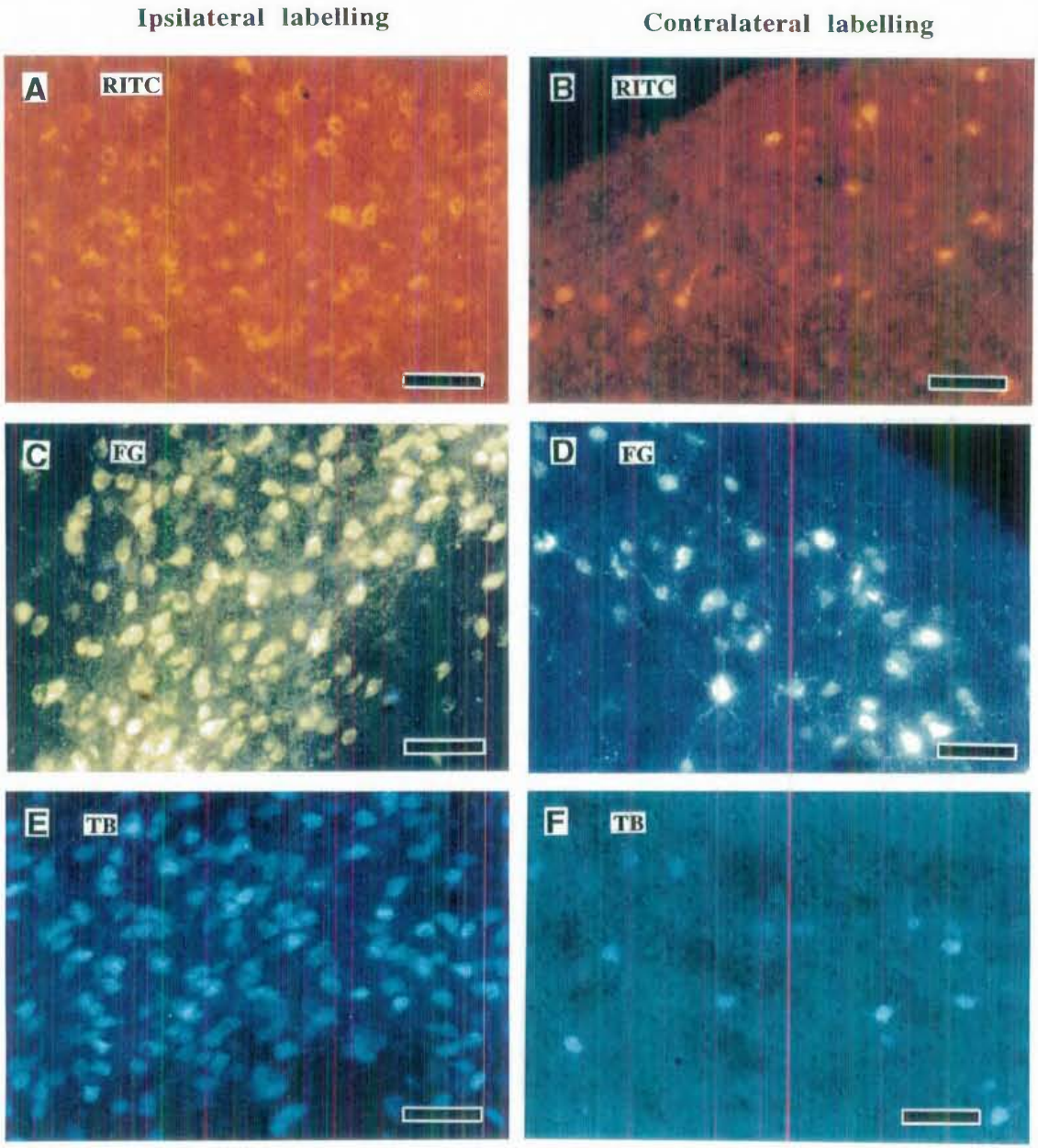

Figure 3.3 RITC, FG and TB labelling in the GLd following injection of these tracers into the visual Wulst.
A and B, RITC labelled neurones in the ipsilateral (A) and contralateral (B) GLd. C and D, FG labelled neurones in the ipsilateral (C) and contralateral (D) GLd. E and F, TB labelled neurones in the ipsilateral (E) and contralateral (F) GLd. Note that RITC, FG and TB all labelled both ipsilateral and contralateral GLd neurones. Scale bars: 50μm.

than TB (df=8, t=2.70, p=0.027, unpaired 2-tailed t-test). However, there was no significant difference between the volume of injection sites for FG and RITC (df=13, t=0.858, p=0.407, unpaired 2-tailed t-test).

Following injections of RITC, FG and TB into the visual Wulst, the labelled neurones were observed in both the ipsilateral and contralateral GLd (Fig. 3.3 and Table 3-1). There was a significant difference between tracers in the number of labelled neurones in both the ipsilateral ($F_{2,17}$=7.23, p=0.005, one-way ANOVA) and contralateral ($F_{2,17}$=5.45, p=0.015, one-way ANOVA) GLd. RITC labelled more GLd neurones than TB in both the ipsilateral (mean number ± SE for RITC 3741±299 *vs.* for TB 1791±262, df=13, t=4.2, p=0.001,unpaired 2-tailed t-test) and contralateral thalamus (mean number ± SE for RITC 1685±257 *vs.* for TB 553±46, df=13, t=3.1, p=0.009). FG also labelled more GLd neurones than TB in both the ipsilateral thalamus (FG 4030±684 *vs.* TB 1791±262, df=8, t=3.1, p=0.016) and the contralateral thalamus (FG 1786±318 *vs.* TB 553±46, df=8, t=3.8, p=0.005). There was no difference between RITC and FG in the number of labelled neurones in the GLd (ipsilateral df=13, t=-0.5, p=0.655; contralateral, df=13, t=-0.2, p=0.818). Although both RITC and FG labelled more GLd neurones than TB, there were no significant differences in the ratios of the contralateral to ipsilateral labelled neurones (c/i) between the three tracers ($F_{2,17}$=1.02, p=0.38, one-way ANOVA, Table 3-1). Thus, after injection of the three tracers into the visual Wulst, similar c/i ratios were obtained no matter which tracer was used.

SODd was also labelled by the tracers following placement in the Wulst (Fig. 3.4). As expected from the results that RITC, FG and TB all labell contralateral GLd neurones, SODd was labelled by all these tracers.

Table 3-1 The absolute counts and the ratio of the contralateral to ipsilateral labelled neurones (c/i) in the GLd after injection of RITC, FG and TB into the visual Wulst

	RITC				FG			
No.	Injection side	Ipsi-lateral	Contra-llateral	c/i ratio	Injection side	Ipsi-lateral	Contra-lateral	c/i ratio
W5	Left	4216	1024	0.243	Right	3034	896	0.295
W6	Right	5369	2909	0.542	Left	5700	1551	0.272
W7	Left	2767	959	0.345	Right	1929	1485	0.769
W8	Right	3229	2850	0.883	Left	5016	2319	0.462
W9	Left	2919	1273	0.437	Right	4473	2680	0.599
		⬇	⬇	⬇	Mean	4030	1786	0.479
					±SE	±684	±318	±0.094
					TB			
W1	Left	4073	939	0.231	Right	939	458	0.410
W2	Right	2889	2305	0.798	Left	2049	536	0.262
W3	Left	5191	1556	0.300	Right	2656	728	0.274
W4	Right	3565	2204	0.618	Left	1577	544	0.345
W10	Left	3197	839	0.262	Right	1558	499	0.320
	Mean	3741	1685	0.466	Mean	1791	553	0.322
	±SE	±299	±257	±0.075	±SE	±262	±46	±0.027

Note: 1.RITC was dissolved in 1% DMSO, and FG and TB were prepared with water only.

2. For each tracer, the injection of tracer into the left visual Wulst of some animals was matched by injection of the same tracer into the right Wulst of other animals. Thus, the data from the injections of tracers into both the left and right Wulst were used to calculate means and standard error.
3. "⬇": for RITC labelling, all of the data from 10 animals were used to calculate mean and standard error.
4. SE: standard error.

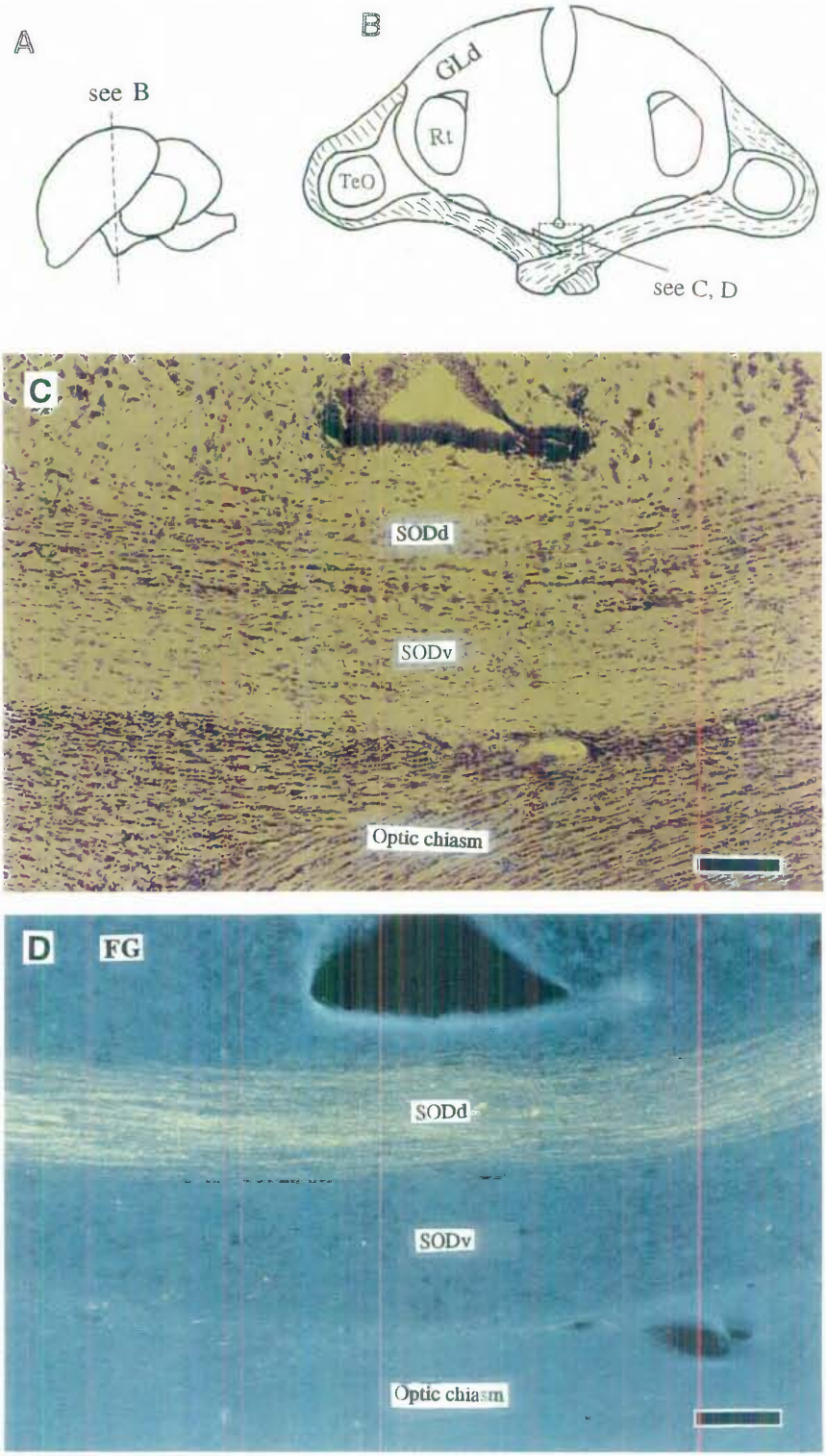

Figure 3.4 Dorsal supraoptic decussation labelled by FG injection into Wulst.

A, outline drawing of the chick brain. B, schematic drawing of transverse section from the cutting as indicated by the dashed line in A; the area enclosed in dashed lines indicates the area shown in the photomicrographs C and D. C, a photomicrograph showing Nissl staining of the supraoptic decussation. D, the dorsal supraoptic decussation (SODd) labelled by injecting FG into the visual Wulst. The SODd was also labelled by injecting TB and RITC into the visual Wulst, not shown here. Scale bars: 100μm.

3.3.1.2 Tectofugal projections

Out of 15 injection placements intended for Rt, seven were outside of Rt. These seven chicks were excluded from further analysis for labelling efficiency. Significant differences between tracers in the volume of tracer at injection site were found ($F_{2,13}$=4.18, p=0.04, one-way ANOVA). The mean volumes (± SE) of the injection site were 0.31± 0.04 mm^3 for RITC, 0.38± 0.09 mm^3 for FG and 0.13 ±0.02 mm^3 for TB. Although TB had a smaller injection site than RITC (RITC *vs.* TB, df=10, t=2.63, p=0.025, unpaired 2-tailed t-test) and FG (FG *vs.* TB, df=6, t=2.77, p=0.033), the RITC and FG injection sites were of a similar volume (df=10, t=-0.88, p=0.401).

As shown in Fig. 3.5 and Table 3-2, RITC, FG and TB labelled the ipsilateral tectal neurones after being injected into Rt. A one-way ANOVA analysis has shown that there is no significant effect of tracer on the number of the ipsilateral labelled neurones ($F_{2,13}$=2.51, p=0.12). However, both RITC and FG tended to label more ipsilateral tectal neurones than TB (mean number ± SE: RITC 13743±1912 *vs.* TB 6861±1077, df=10, t=2.4, p=0.037; FG 13875±3576 *vs.* TB 6861±1077, df=6, t=1.9, p=0.10, unpaired 2-tailed t-test). Differential labelling between tracers occurred in the contralateral tectal neurones ($F_{2,13}$=12.09, p=0.001, one-way ANOVA). Although RITC-labelled neurones were present in the contralateral tectum, cells labelled with FG or TB were found only occasionally in the tectum contralateral to the side of the injection site in the Rt (Fig. 3.5). There were no differences between RITC and FG labelling of the ipsilateral tectal neurones (df=10, t=0.01, p=0.97). However, RITC labelled about 9 times more contralateral tectal cells than FG (RITC 6774±1222 vs. 789±309, df=10, t=3.4, p=0.007, unpaired 2-tailed t-test). Although RITC labelled twice as many ipsilateral tectal neurones than did TB, it labelled 35 times more contralateral tectal cells than TB (RITC 6774±1222 vs. TB 220±113, df=10, t=3.7, p=0.004, unpaired 2-tailed t-test).

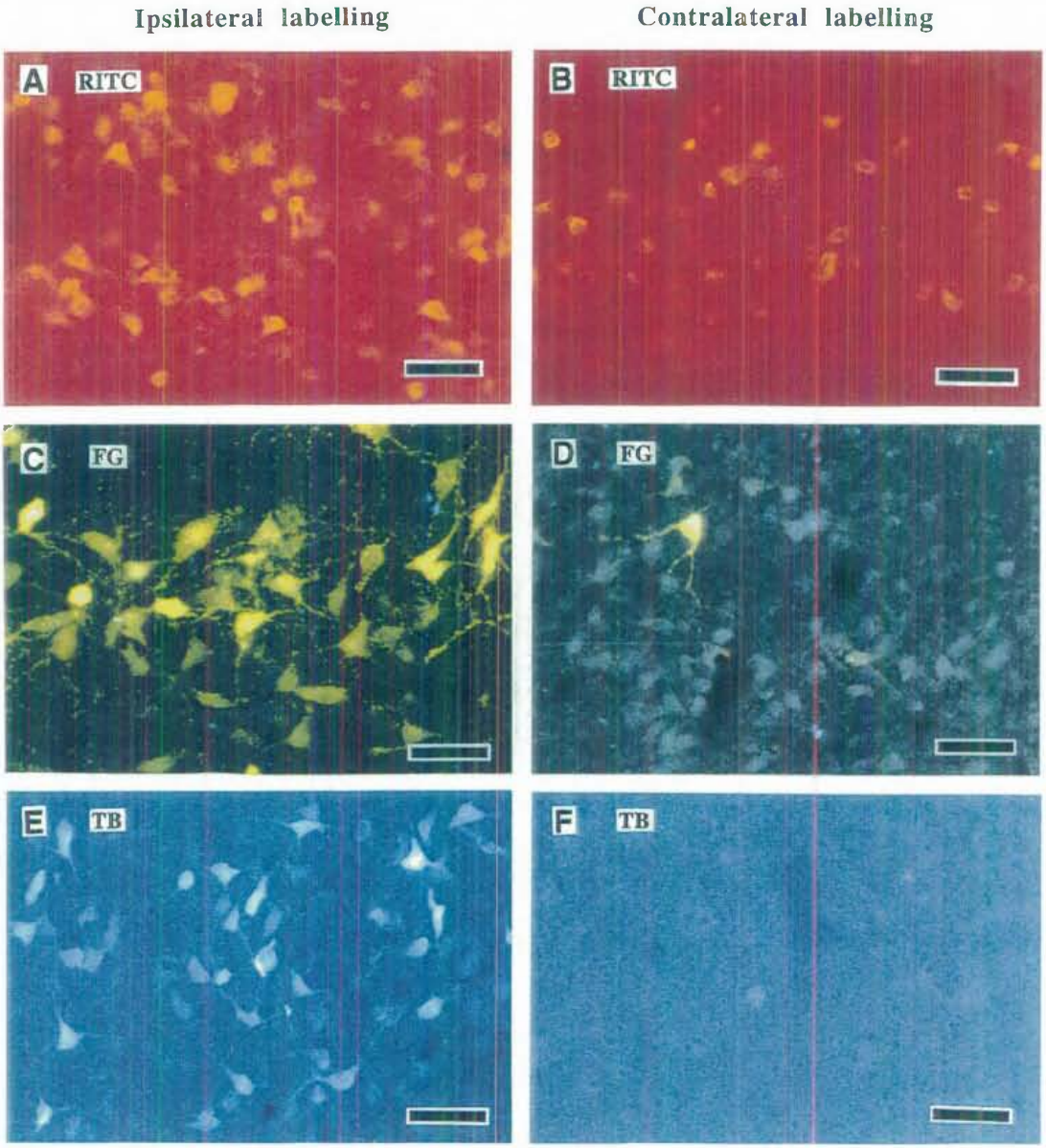

Figure 3.5 RITC, FG and TB labelling in the optic tectum following injection of these tracers into Rt. RITC labelled both ipsilateral (A) and contralateral (B) tectal neurones well. FG and TB labelled effectively only the ipsilateral tectal cells (C and E), but they occasionally labelled a few contralateral tectal cells (D and F). Scale bars: 50μm.

Table 3-2 Number of ipsilateral and contralateral labelled neurones in the optic tectum and the c/i ratio after injecting RITC, FG and TB into Rt

	RITC				FG			
No.	Injection side	Ipsi-lateral	Contra-lateral	c/i ratio	Injection side	Ipsi-lateral	Contra-lateral	c/i ratio
R9	Right	9353	4216	0.545	Left	14425	1287	0.089
R22	Right	8606	3422	0.398	Left	4899	102	0.021
R29	Left	11661	5919	0.508	Right	22392	1334	0.060
R43	Left	7402	4267	0.576	Right	13785	433	0.030
		↓	↓	↓	Mean	13875	789	0.050
					±SE	±3576	±309	±0.015
					TB			
R15	Right	14724	10313	0.700	Left	8787	218	0.025
R16	Right	14684	6818	0.464	Left	5031	98	0.019
R39	Left	24428	11849	0.485	Right	4963	27	0.005
R47	Left	11275	2252	0.200	Right	8666	536	0.06
	Mean	13743	6774	0.485	Mean	6862	220	0.027
	±SE	±1912	±1222	±0.051	±SE	±1077	±113	±0.012

Note: 1.RITC was dissolved in 1% DMSO, and FG and TB were prepared with water only.

2. For each tracer, injection of tracer into the left visual Rt of some animals was matched with injection of the same tracer into the right Rt of other animals. Thus, the data from the injections of tracers into both the left and right Rt were used to calculate the mean and standard error.
3. "↓": for RITC labelling, all of the data from 8 animals were used to calculate mean and standard error.
4. SE: standard error.

The differential labelling of contralateral versus ipsilateral neurones by each tracer can be seen by calculating the ratio of contralateral to ipsilateral labelled cells (c/i ratio). There were significant differences between the c/i ratios calculated after injecting RITC, FG and TB into Rt ($F_{2,13}$=33.98, p<0.0001, one-way ANOVA). RITC led to a higher c/i ratio than either FG (mean ± SE: RITC 0.485±0.051 vs. FG 0.050±0.015, df=10, t=5.8, p=0.0002, unpaired 2-tailed t-test) or TB (RITC 0.485±0.051 vs. TB 0.027±0.012, df=10, t=6.1, p=0.0001, unpaired 2-tailed t-test). Thus, RITC labelled both the ipsilateral and contralateral tectal neurones, but FG and TB selectively labelled the ipsilateral tectal neurones only.

Consistent with previous reports (Bischof and Niemann, 1990; Ngo et al., 1994), the tectal efferents were found to project to the contralateral Rt via the ventral supraoptic decussation (SODv). Unexpectedly, SODv was labelled not only by RITC but also by FG and TB (Fig. 3.6). In fact, the axons in SODv were well labelled with FG and TB even though these tracers labelled only very few contralateral cell bodies in the optic tecta of the same animals.

3.3.2 Experiment 2: Effects of the interval between injection and sampling on FG and TB labelling of the tectofugal projections and the effect of DMSO

In some animals (R28, R42 and R44), every second section was used to count the labelled neurones in the optic tectum and, in others (135#, 160# and 157#), only every fourth section was mounted and used to count the labelled neurones. There were variations of volume of the injection site between the animals and between tracers used (Table 3-3). Therefore, only the c/i ratio, not the absolute counts of the labelled neurones in both the ipsilateral and contralateral optic tectum, was used to compare the labelling results between different treatments. As reported above, RITC, FG and TB all labelled the tectal neurones ipsilateral to the injection of these tracers into Rt, but

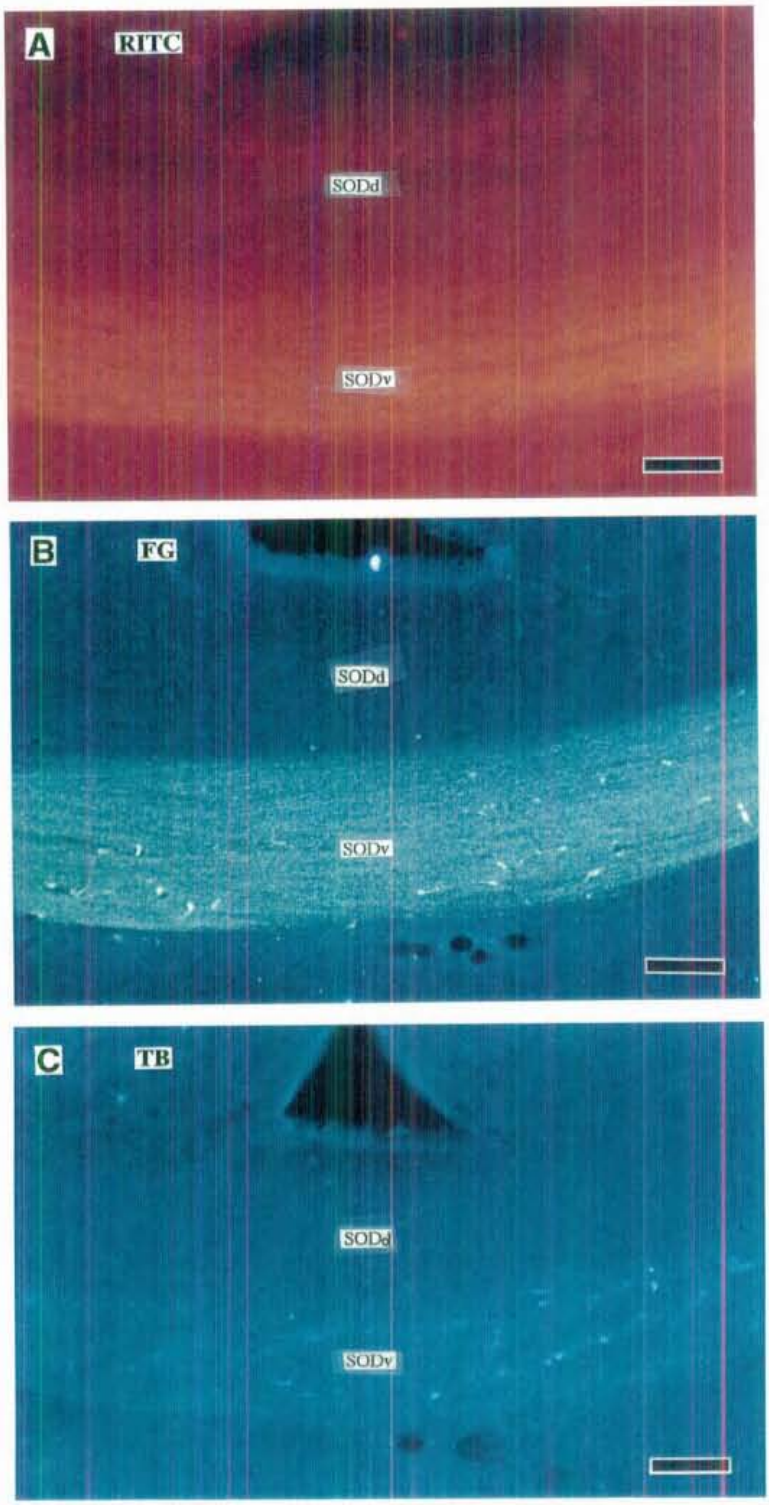

Figure 3.6 The ventral supraoptic decussation (SODv) was labelled following injection of RITC (A), FG (B) or TB (C) injection into Rt. Note particularly the number of axons labelled with FG, despite the fact that cell bodies in the optic tectum were not labelled in the same chick. The structure of the supraoptic decussation has been presented in Fig.3.4C. Scale bars: 100μm.

Table 3-3 Number of labelled neurones in the optic tectum of animals with different survival periods after injecting RITC, FG and TB with DMSO into Rt

No.	Survival period (Days)	RITC Injection side	RITC Injection volume (mm^3)	RITC Ipsi-lateral	RITC Contral-lateral	RITC c/i ratio	FG Injection side	FG Injection volume (mm^3)	FG Ipsi-lateral	FG Contral-	FG c/i ratio
R28*	2	Right	0.291	12183	8983	0.737	Left	0.608	16823	193	0.012
R42**	4	Left	0.522	17519	5877	0.336	Right	0.524	1973	9	0.048
				RITC					TB		
R44**	4	Right	0.24	12300	7904	0.643	Left	0.042	1984	61	0.007
				FG					TB		
135#	8	Left	0.134	3617	42	0.012	Right	0.057	1722	23	0.013
160#	8	Right	0.339	7109	35	0.005					
157#	10	Left	0.371	6096	929	0.031	Right	0.038	929	9	0.010

*: In R28, FG was prepared with water.
**: In R42 and R44, TB and FG were prepared with 1% DMSO. Every second section was used to count the labelled neurones.
#: In animals 135, 160 and 157, both TB and FG were prepared with 1% DMSO and only every fourth section was used to count the labelled neurones.

variation of the number of contralateral labelled neurones occured.

As shown in Table 3-3 , injection of FG or TB prepared with 1% DMSO (in animals R42, R44, 135#, 160# and 157#), labelled only a few contralateral tectal neurones (Fig. 3.7). The c/i ratio of all animals was less than 0.05 (compared with c/i ratios ranging from 0.336 to 0.737 following injection with RITC). Thus, use of DMSO did not improve the labelling of contralateral cell bodies in the tectum by FG and TB.

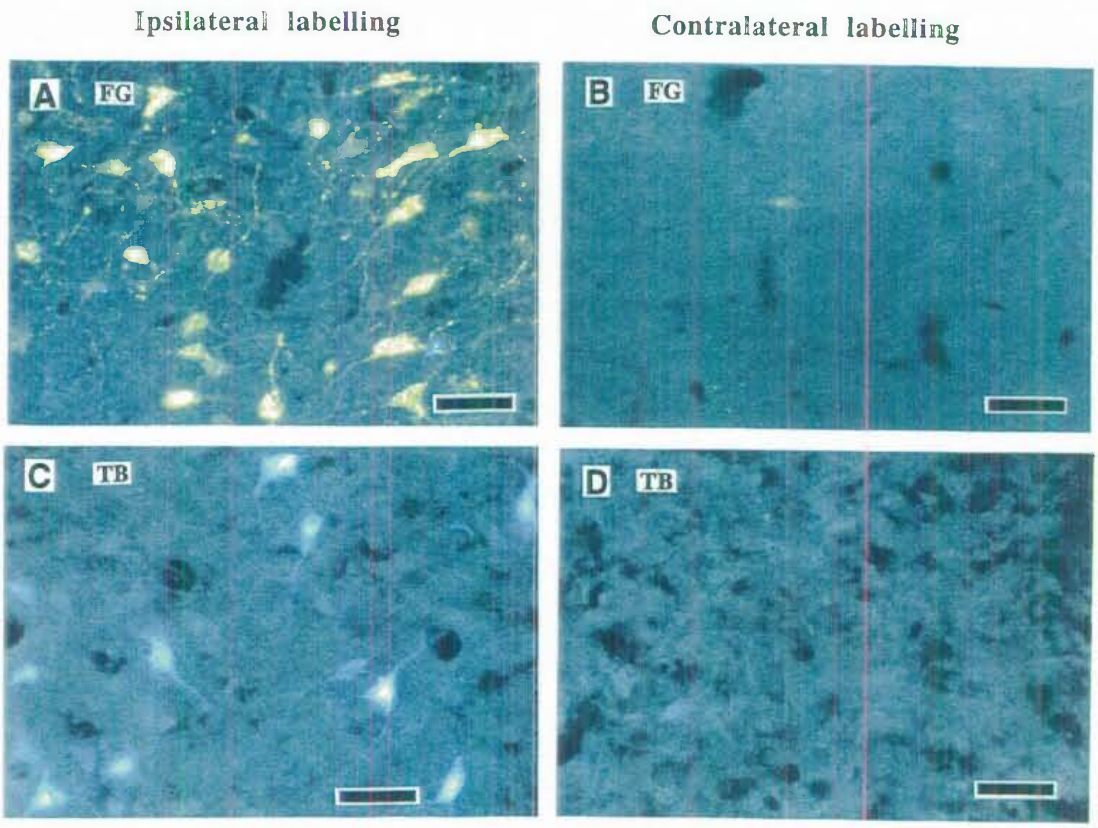

Figure 3.7 FG and TB labelling of the tectofugal projections of animals with 8 days post-injection survival. Both FG and TB were prepared with 1% DMSO.
Note the absence of cells labelled in the optic tectum contralateral to the injection site. Scale bars: 50 μm.

After injecting tracers into Rt, and allowing the animals to survive for periods between 2 to 10 days, there was no obvious difference in the number of cell bodies labelled. In chick R28, only 2 days of survival after injection of RITC into Rt, more than 8000 neurones were labelled in the contralateral tectum (c/i ratio was 0.737, Table 3-3). Thus, two days were sufficient for transport of the RITC from the Rt to the contralateral tectum and, therefore, to label the cell bodies of the contralateral tectal neurones. However, after injecting FG and TB into Rt, no matter whether the chicks were allowed to live 2 , 4, 8 or even 10 days, only very few neurones were labelled in the contralateral tectum, although the neurones in the ipsilateral tectum were labelled well (c/i ratio was consistently less than 0.05, Table 3-3 and Fig. 3.7). Thus, the survival period of up to 10 days was inadequate for labelling of cell bodies in the contralateral tectum with FG and TB (Table 3-3 and Fig. 3.7). Therefore, the survival period is not the factor that causes the difference between RITC and FG or TB labelling in the contralateral tectum.

The mean distance between the optic tectum and the contralateral Rt was found to be 10.17±0.06 mm (mean ± SE) and this was significantly shorter than that between GLd and the contralateral visual Wulst (12.32±0.14 mm; $p<0.01$, 2-tailed Mann-Whitney U-test). Therefore, the length of the axons is unlikely to be a factor explaining why FG and TB labelled the contralateral GLd neurones, but not the contralateral tectal neurones. Thus, the transporting distance also does not affect labelling effectiveness of the tracers.

3.3.3. Experiment 3: Labelling of the tectofugal visual projections with red beads or green beads

Red beads and green beads were injected into Rt of 5 chicks. As shown in Table 3-4 and Fig. 3.8, there was no labelling difference between these tracers in the ipsilateral tectal neurones (mean number ± SE: red beads 4256±1530 *vs.* green beads 3546±1282; $p=0.77$, 2-tailed Mann-Whitney U-test). However, red beads labelled many more

contralateral tectal neurones than did green beads (mean ± SE: red beads 1461±427 *vs.* greed beads 194±85; p=0.04, 2-tailed Mann-Whitney U-test). Therefore, red beads had a higher c/i ratio than green beads (0.417±0.076 for red beds vs. 0.048±0.007 for green beads; p=0.02, 2-tailed Mann-Whitney U-test). Even if the survival period was extended to 8 days after the injection of the tracers, green beads still labelled only very few contralateral tectal neurones (Table 3-4 and Fig. 3.8C). Furthermore, even though in 3 animals (Table 3-4) red beads and green beads were injected into the same Rt and the injection sites of red beads and green beads were overlapping, only red beads labelled both the ipsilateral and contralateral tectal neurones. Green beads labelled only the ipsilateral tectal neurones well. It is most unlikely that the location of the injection sites influences labelling effectiveness.

Table 3-4 Number of labelled neurones in the optic tectum following injection of red beads and green beads into Rt

	Red Beads				Green Beads			
No.	Injection side	Ipsi-lateral	Contra-lateral	c/i ratio	Injection side	Ipsi-lateral	Contra-lateral	c/i ratio
T10*	Left	3507	2060	0.362	Left	1154	36	0.031
T29*	Right	5139	2060	0.401				
206*					Right	6861	412	0.061
133**	Right	7847	2181	0.274	Right	4226	242	0.057
173**	Left	530	334	0.630	Left	1941	85	0.043
	Mean ±SE	4256 ±1530	1461 ±427	0.417 ±0.076	Mean ±SE	3546 ±1282	194 ±85	0.048 ±0.007

Note: * indicates the chicks that were allowed to survive for 4 days.
** indicates the chicks that were allowed to survive for 8 days.
For all chicks, only every fourth section was used to count the number of the labelled neurones in the tectum. In the chicks T10, 133 and 173, red beads and green beads were injected into Rt on the same side of the brain.

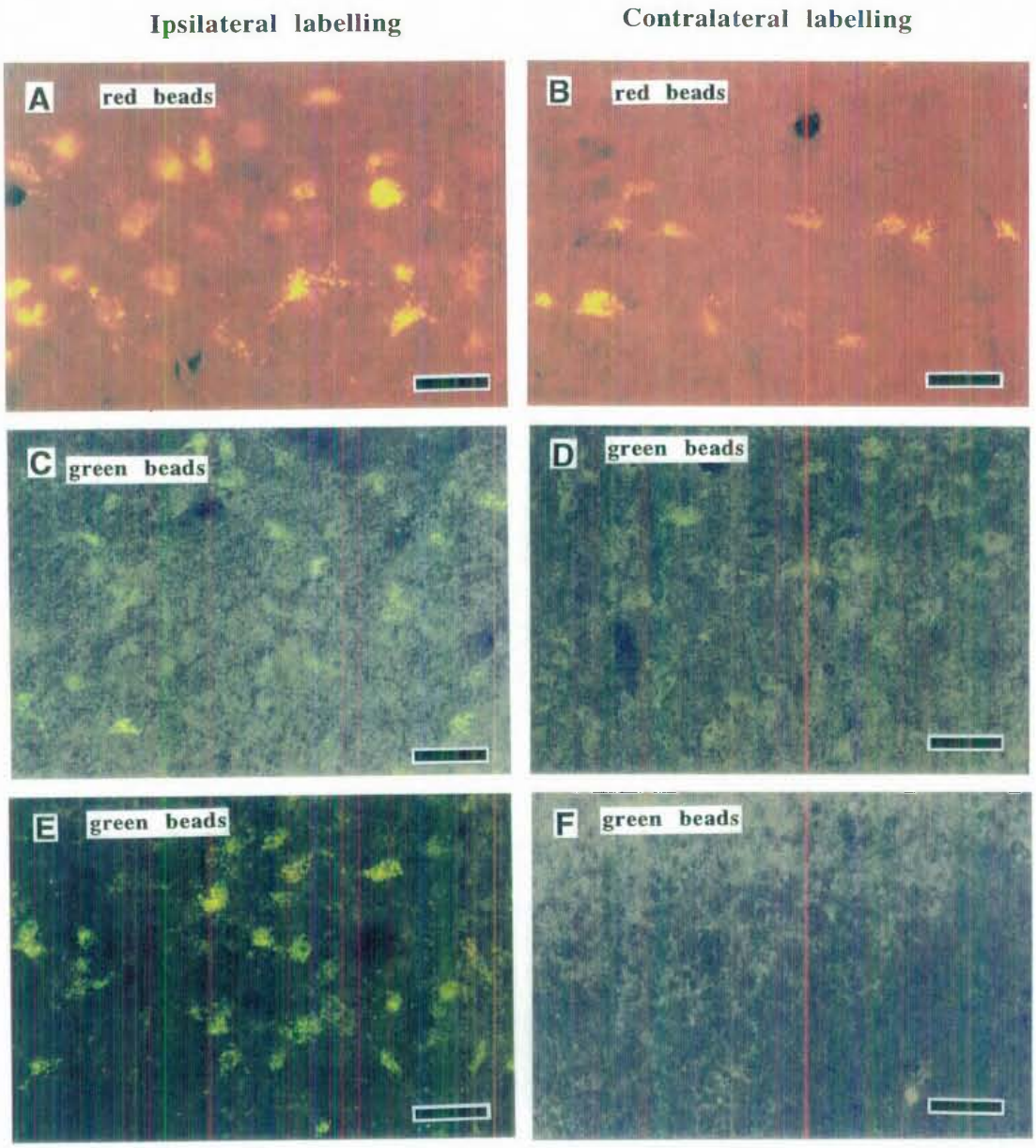

Figure 3.8 Labelling by red beads (A and B) and green beads (C-F) in the optic tectum after the tracers have been injected into Rt.
Red beads labelled both ipsilateral (A) and contralateral (B) tectal neurones. No matter whether the chick was allowed to survive for 4 days (C and D) or 8 days (E and F) after injection of the tracer into Rt, green beads labelled effectively only the ipsilateral tectal cells (C and E). They occasionally labelled a few contralateral tectal cells (D and F), but most of the contralateral cells were not labelled.

3.4 DISCUSSION

The results reveal that the three commonly used retrograde tracers, RITC, FG, TB, label the contralateral projecting neurones in the two main visual projections to the forebrain differentially, although they all are equally effective in labelling the ipsilateral projecting neurones. In the thalamofugal pathway, RITC, FG and TB label both the ipsilateral and contralateral GLd neurones following injection into the visual Wulst. However, in the tectofugal pathway, RITC labels both the contralateral and ipsilateral tectal neurones, but FG and TB label effectively only the ipsilaterally projecting neurones from the optic tectum to Rt. In addition, similar results have been obtained by using the other two tracers, red beads and green beads. Red beads label both ipsilateral and contralateral tectal neurones, but green beads label effectively only the ipsilateral tectal neurones. FG, TB and green beads labelled very few contralateral tectal neurones, although there are a large number of contralaterally projecting neurones in the optic tectum, as revealed by injection of the tracers RITC and red beads.

FG, TB and green beads are not simply unable to label all contralaterally projecting neurones, because they label the contralateral projections of the thalamofugal pathway. The possible reason for the specific inability of FG, TB and green beads to label the contralateral tectofugal projections was investigated.

TB is insoluble in water and diffuses to only a small extent from the site of injection. Therefore TB injection sites have a smaller volume than FG and RITC, which are soluble in water (for FG) or DMSO-water solution. However, although there was no significant difference in volume of the injection site between FG and RITC in both the Wulst and Rt, FG did not label the contralateral Rt neurones well. Thus, the extent of diffusion around the site of injection does not appear to be the reason for the differential labelling. The differences in the volume of the tracers injected are also not the reason for

the labelling differences between FG, TB and RITC, because the same injection volumes were used for all of these tracers. Although it can be argued that the smaller volume of the TB injection site itself (caused by less diffusion) compared to RITC may cause the labelling differences in which TB does not label the contralateral neurones, this is unlikely to be the explanation, since TB labels the contralateral GLd neurones. However, consistent with the smaller volume of the injection site, TB labelled fewer ipsilateral neurones in both the thalamofugal and tectofugal projections than FG or RITC. The fact that RITC has a larger volume of injection site than TB may allow RITC to be taken up by a larger number of terminals but this explains only the greater number of ipsilateral neurones labelled by RITC than by TB but not the difference in labelling of the contralateral neurones.

Although de Olmos and Heimer (1980) found that dissolving Granular blue, Nuclear yellow and Propidium iodide in 2% DMSO increased the number of the neurones labelled by these tracers, DMSO did not alter the labelling of the contralateral projecting neurones in the tectum by RITC and FG and TB. In the present study, irrespective of whether or not DMSO was used, TB and FG failed to label the contralateral tectal neurones. However, consistent with previous studies (Rogers and Sink, 1988; Adret and Rogers, 1989; Rogers and Bolden, 1991), TB and FG were prepared with water only before injection into the Wulst, and in this pathway both tracers labelled both the contralateral and ipsilateral GLd neurones.

The other two possible factors which may influence the retrograde labelling by fluorescent tracers (Kuypers and Huisman, 1984), the survival time and the transport distance, are also unlikely to account for the labelling differences between the tracers and between the pathways. Although the distance between GLd and the contralateral Wulst (12.32±0.14 mm) of the thalamofugal pathway is longer than that between the tectum and the contralateral Rt (10.16±0.06mm) of the tectofugal pathway, FG and TB label the

contralateral GLd neurones well, but not the contralateral tectal neurones. In addition, even when the survival time between injection and sacrifice was increased by up to 10 days, FG and TB still did not label the contralateral tectal neurones. This is in marked contrast to RITC which labels the contralateral tectal neurones after only two days (present study) or one day (in the adult pigeon; Güntürkün et al., 1993) post-injection.

Another factor which may influence the amount of retrograde labelling of contralateral neurones is the structure of the tracers. So far, it is known that only RITC and red beads label effectively the contralateral tectal neurones following injection of tracers into Rt (Bischof and Niemann, 1990; Güntürkün et al., 1993; present study), but FG, TB, green beads (present study), Fast blue (FB; Güntürkün et al., 1993) and HRP (Bischof and Niemann, 1990) do not label these same projections. In view of the fact that the main component of both red beads and RITC is rhodamine, it seems that only tracers with rhodamine are effective in labelling these contralaterally projecting neurones in the avian tectofugal pathway.

There are two possible explanations why FG, TB (and maybe also FB and HRP) do not label the contralateral projecting neurones in the avian tectal pathway, but RITC and red beads do. One explanation is differential uptake of the tracers by the nerve endings. Another explanation is differential transport of the tracers to the perikarya in the contralateral tectum. According to the first explanation, selective uptake of the tracers rhodamine and red beads but not other tracers may occur. Güntürkün et al. (1993) assumed that, in the tectofugal pathway, the contralaterally projecting neurones have a much smaller number of nerve terminals than the ipsilateral cells and thus RITC has higher effectiveness in labelling than FB. In other words, FB would fail to accumulate sufficiently to be detectable in the contralateral cell bodies. However, this assumption does not appear to explain our results, because the fibres of the ventral supraoptic decussation (SODv), via which the efferents of the tectum project to the contralateral Rt

(Bischof and Niemann, 1990; Ngo et al., 1994), were labelled densely by RITC, FG and TB. Thus, it would appear that the FG and TB (maybe also green beads) are taken up sufficiently by the nerve endings and are transported along at least part of the axon, but do not get transported into the cell body. Therefore, the second explanation, the differential transport of rhodamine versus other tracers, is the most likely explanation for the labelling differences of the contralaterally projecting neurones in the tectofugal pathway.

It has been shown that both the ipsilateral and contralateral tecto-Rt projections are organized topographically (in the pigeon, Benowitz and Karten, 1976; in the chick see Chapter 5, p.158). When the tracers were injected into the same subdivisions of left and right Rt, double-labelled neurones were found in SGC of the tectum (see Chapter 5, p.153), and the number of the double-labelled neurones was up to 74% of the number of the contralateral labelled neurones. These double-labelled neurones are bilaterally projecting neurones with a main axon projecting to ipsilateral Rt and an axon collateral projecting to the contralateral Rt (Fig. 2-3, p.61; Fig. 5.12, p.167); the fibers projecting to the ipsilateral Rt are larger than the projecting to the contralateral Rt (Ngo et al., 1994). Thus, the contralateral tecto-Rt projections are mainly divergent axon collaterals, whereas the ipsilateral tecto-Rt projections are main axons without collaterals. There are also a few tectal neurones projecting only to the contralateral Rt. One advantage of fluorescent retrograde tracing techniques is the ability to reveal divergent axon collaterals by using combinations of two or more tracers (Kuypers and Huisman, 1984). It is generally accepted that all retrograde tracers are transported equally, via axon branches, to the perikarya. However, our present results show that, although all tracers used are taken up and transported retrogradely in the main axons of the bilaterally projecting neurones, this is not so in the collateral axon branches at least on the cell body side of the branch. We assume (Fig. 3.9) that the most crucial location for retrograde transport of tracers (and maybe other substances also) is the narrow point of the axon branch at which the axon

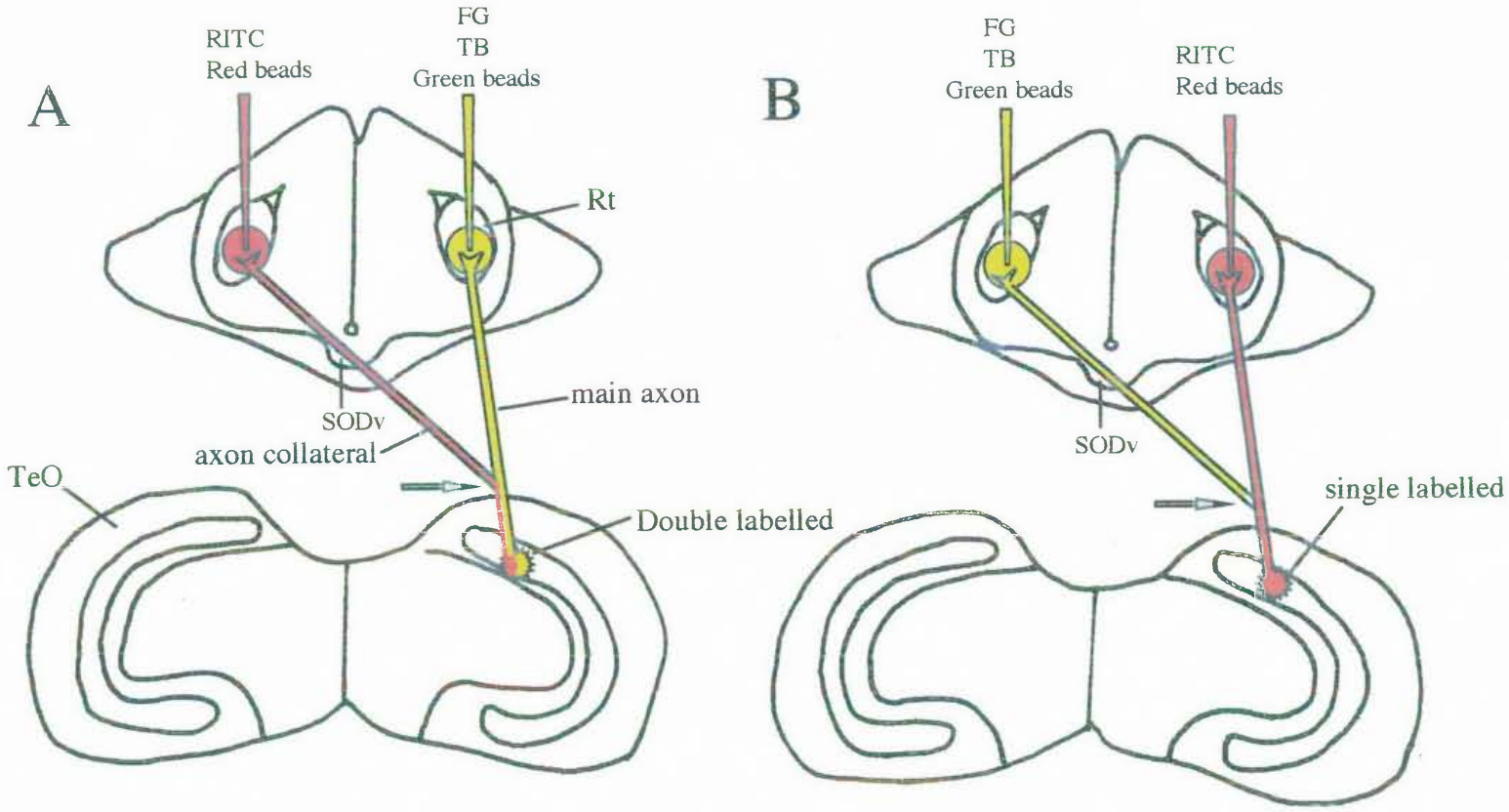

Figure 3.9 A proposed explanation of retrograde transport of various fluorescent tracers in the bilateral projecting neurones of the tectal-Rt projections in the chick.

A, RITC or red beads was taken up by the nerve endings of the axon collateral and FG or TB or green beads was taken up by the main axon. RITC and red beads could be transported retrogradely through the collateral and pass the arborization point that the axon collateral branching from the main axon, finally they reach the cell body of neurone. All of FG, TB and green beads could be transported retrogradely through the main axon to cell body.

B, FG or TB or green beads was taken up by the nerve ending of axon collateral and RITC or red beads was taken up by the main axon. FG, TB and green beads could be transported in the collateral but could not pass the arborization point. Therefore, FG, TB and green beads could not reach the cell body of the neurones. RITC and red beads can be transported through the main axon to the cell body.

Arrows indicate the narrow arborization point where the collateral diverges from the main axon.

collateral branches from the main axon. It appears that RITC and red beads are able to pass this arborization point, but FG, TB and green beads cannot. Thus, RITC and red beads are accumulated in the contralateral cell bodies, but FG, TB and green beads do not accumulate sufficiently to label the cell bodies.

There are, however a few tectal neurones that are labelled by injections of FG or TB into the contralateral Rt (Fig. 3.5). Possibly these FG- and TB-labelled neurones are contralaterally projecting neurones which do not have axon collaterals (Fig. 2.3, p.61; Fig. 5-12, p.167).

Miceli and Repérant (1982) have revealed that, in the pigeon, neurones of GLd provide either ipsilateral, contralateral or bilateral projections to the Wulst, the latter via collateral axon branching, since following injections of two fluorescent tracers (FB, Evans blue or Nuclear Yellow) into either the left or right Wulst double-labelled neurones have been found in sub-regions of GLd: the pars ventralis sub-division of the n. dorsolateralis anterior thalami, pars lateralis (DLLd, 9-13% double labelled neurones of the total neuronal population) and n. superficialis parvocellularis (SPC, 18-46% double labelled neurones of the total neuronal population). However, in the chick, extremely few double-labelled neurones (<0.01%) have been found in GLd following injection of RITC, FG or TB into the visual Wulst (Chapter 4, p.107). Thus there are very few bilaterally projecting neurones in GLd of the chick. Therefore, both the ipsilateral and contralateral GLd-Wulst projections consist of main axons. There are very few divergent collateral axons. This may be the reason why, in the thalamofugal pathway, FG and TB are able to label the contralateral projecting neurones as effectively as RITC, because all of these tracers have no difficulty in being transported retrogradely to the cell body via the unbranched axons.

In conclusion, the present study reveals that different retrograde tracers are

differentially effective in labelling divergent axon collaterals. Even the commonly used and efficient tracers FG and TB are not transported retrogradely in axon collaterals to label cell bodies. However, RITC and red beads can pass from the collateral to the main axon and so they label the cell bodies. Although, so far, we have not extended our investigation to all neural pathways with collaterals, the result suggests that caution must be exercised when choosing retrograde tracers to investigate the tracts by means of double or multiple labelling via collateral axon branchings.

Based on the results of this Chapter, in the following chapters, although RITC, FG and TB will all be used to investigate the organization of the thalamofugal projections, only the data of RITC and FG labelling will be used for quantitative analysis of the asymmetry (Chapter 4). In Chapter 5, for the organization of the tectofugal pathway, RITC, FG, TB, red beads and green beads will all be used to assess the ipsilateral tecto-Rt projection, but only RITC and red beads will be used to study the contralateral tecto-Rt projections. In Chapter 6, only RITC will be used to study the possible asymmetry of the tectofugal pathway.