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Auditory and Olfactory Integration in the Round Goby, Neogobius melanostomus

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Auditory and Olfactory Integration in the Round Goby, *Neogobius melanostomus*

by

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ABSTRACT

Auditory and olfactory signaling can be used in courtship and mating. In some fish

species, the signaler is the male and the female needs to receive the signals to respond. In

some species of fish, the female has an increased receptiveness for the male's call and/or

pheromones when reproductive and this receptiveness decreases after the breeding

season. In the round goby, *Neogobius melanostomus*, the male readies a nest and when

reproductive, produces a call and pheromones to attract females. The two main objectives

of this thesis were to determine the female's behavioural response to a reproductive

male's call and pheromones over the breeding season and to determine if integration of

these two sensory systems was occurring in the brain. These two objectives were

accomplished through behavioural trials and tract tracing experiments. This

multidisciplinary approach gives an overall better view of reproductive female behaviour

and brain integration in this species.

DEDICATION

I dedicate this thesis to my family for all their love and support over the years.

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CHAPTER I

INTRODUCTION

Fish communication

For survival it is essential that fish are able to communicate with conspecifics and perceive their surrounding habitat. Fish often have complex social interactions involving signals that originate from different modalities (Myrberg Jr, 1997). Fish can use many sensory modalities for communication including audition, olfaction, vision, gustation, electrosensation, and mechanosensation; which includes both lateral line and hearing. Some of these modalities, depending on the fishes' habitat type, are not used in long distance communication.

Visual signals tend to be more important for short distance communication because of the reduction in light transmission through water (Hawryshyn et al., 1988). The fish chemosensory system includes olfaction and gustation, and solitary chemosensory cells, the latter of which are used for short distance communication but remained largely unstudied (Hansen & Reutter, 2004). The mechanosensory system, which includes the lateral line, is used for short-distance communication with the fishes' environment because it is used to detect the flow of water at the surface of the fish (Montgomery et al., 2001). The gustatory system is also used in short distance communication with the fishes' surroundings for detection and affirmation of food sources (Hansen & Reutter, 2004). Electrosensation, used in short distance communication, can allow electroreceptive fish to navigate its environment, communicate with conspecifics, detect and localize heterospecifics and conspecifics (Keller, 2004). These sensory systems are important for many of the behaviours fish

exhibit; however I chose to focus on two sensory modalities for long distance communication - audition and olfaction.

Auditory signals are used as a long distance mode of communication due to the low attenuation of sound in water; the attenuation of sound is lower in freshwater than saltwater (Rogers & Cox, 1988). Fish can use auditory signals for predator avoidance, spawning, courtship, mate attraction and mate choice (Ladich, 2004). Acoustic signals can be involved in conspecific communication to reduce aggression and assessing the ability of a conspecific prior to a fight (Ladich, 2004). Auditory signals can be disrupted not only by objects in the environment but can be lost because of anthropogenic noises, which include sources like sonar systems or can be by-products of activity such as shipping (Popper & Schilt, 2008).

Olfactory signals are generally used as a long distance mode of communication and can aide a fish in locating food, find a mate, avoid a predator from a distance (Hansen & Reutter, 2004). Sometimes olfactory signals are used for short distance communication, if the signal can only be detected at high concentrations, which occurs in the guppy, *Poecilia reticulata* (Archard et al., 2008).

Auditory and Olfactory Systems

The auditory system of fishes consists of the inner ear connected to the auditory nerve. The inner ear of bony fishes and sharks consists of three semicircular canals and three otoliths; the saccule, lagena and utricle (Popper & Schilt, 2008; Figure 1.1, Audrey Rollo). Each otolith is associated with an epithelium containing sensory and nonsensory cells; there can be thousands or hundreds of thousands of sensory cells depending on the
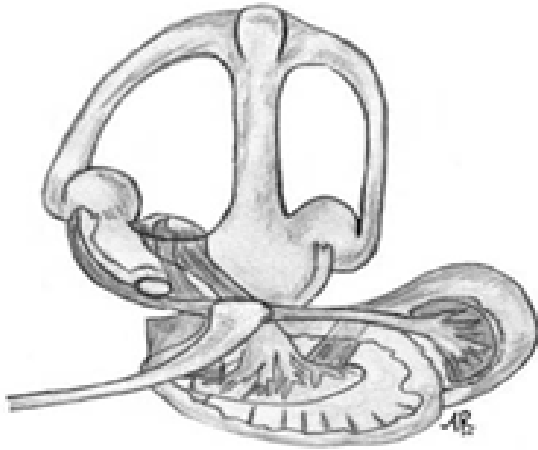
Figure 1.1: The inner ear of a round goby, *Neogobius melanostomus*.

species and size of the fish (Popper & Schilt, 2008). Each otolith is coupled to the epithelium by a thin membrane. The sensory cells have a ciliary bundle on their apical surface which is composed of many stereocilia and one kinocilium (Popper & Schilt, 2008). When sound pressure contacts the fish, it causes the movement of both the epithelium and otolith, however the otolith moves slower because it is denser than the epithelium which causes the cilia on top of the sensory cells to bend (Popper & Schilt, 2008). When the cilia bend, it causes ion channels to open thus causing an intracellular cascade of events to occur, which eventually lead to the release of neurotransmitter and stimulation of the auditory nerve (Popper & Schilt, 2008).

The olfactory system is comprised of a peripheral olfactory organ which includes the olfactory nerve, which is composed of the axons from the olfactory receptor neurons, and the central olfactory organ which includes the olfactory bulbs and higher brain areas (Hansen & Reutter, 2004). In the round goby, water flows into the anterior naris, over the olfactory sensory epithelium and out of the posterior naris (Belanger et al., 2003; Figure 1.2, Belanger et al., 2003). The anterior naris leads into an olfactory chamber that has two posterior accessory sacs. Water is helped to flow across the sensory epithelium by the ciliated olfactory chamber and by accessory sac compression, where odourant molecules can come into contact with one of three possible types of olfactory sensory neurons: ciliated, microvillar and crypt (Belanger et al., 2003). When an odourant molecule comes into contact with a G-protein-coupled olfactory receptor, it will bind to the receptor protein allowing gated channels to open and a signal cascade to occur inside the cell (Zielinski & Hara, 2007). The olfactory sensory neurons form synaptic contacts onto
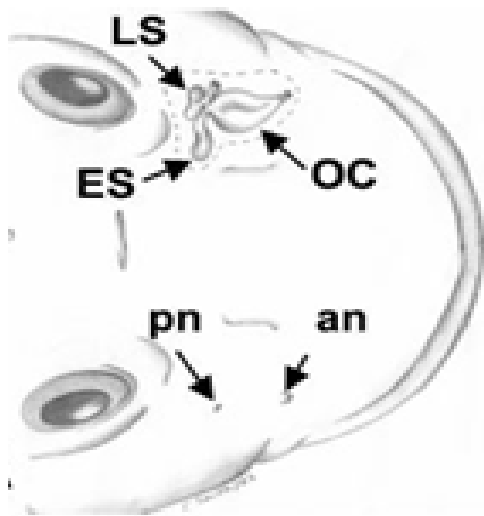
Figure 1.2: The nose of the round goby. an: anterior naris, pn: posterior naris, OC:

Olfactory chamber, LS: lachrymal accessory sac, ES: ethmoidal accessory sac (modified

from Belanger et al., 2003)

mitral cells in the olfactory bulb and these mitral cells project to telencephalic regions

(Finger, 1975).

Multisensory responses

Multisensory or multimodal communication is defined as communication

occurring with signals received through more than one sensory channel (Partan & Marler,

2005). There are some functions of multisensory communication that are unique because

of the use of more than one sensory channel. Detection and localization of stimuli can be

improved, the receiver`s attention can be attracted by the signaler with one sensory

channel while the second channel contains a message or the distance of the signaler can

be estimated by the difference in time in receiving the two signals (Partan & Marler,

2005). Multiple signals are often sent because one signal is effective within a certain

range and the other is more effective outside of the range of the other modality (Braun et

al., 2002).

Multimodal signals can be classified based on the signal components and the

responses they elicit. The signal components may be redundant or non-redundant (Partan,

2004; Partan & Marler, 2005). The benefit of sending redundant signals is that the

signaler has more assurance that the message will be received with fewer errors due to

biotic or abiotic noise (Partan, 2004; Partan & Marler, 2005). However, non-redundant

signals send more information without an increase in energy costs compared to redundant

signaling (Partan & Marler, 2005).

Redundant signaling can be further sub-divided into equivalence and

enhancement (Partan, 2004) or synergy (Braun et al., 2002) of the response. Equivalence

is not as common as enhancement; but is seen in the honeybee, *Apis mellifera*, where

vibratory and acoustic components of a signal to others detailing the location and

distance of a food source both contained the same information when tested separately

(Michelsen et al., 1992). Enhanced or synergistic responses have been argued to be the

rule in animal communication involving redundant signals (Partan, 2004) and occurs by

higher level mechanisms such as bi-modal neurons that may be the common target of two

sensory pathways (Braun et al., 2002). Enhanced responses can be further broken down

into three categories: the response to the multimodal signal can be just above the response

of either unimodal signal, it can be equal to the sum of the two unimodal responses or it

can be more than the sum of the two components or multiplicative (Partan, 2004).

If the signals however are non-redundant, there is a possibility of four different types of

responses. The response to the multimodal signal can include the responses that are seen

to each unimodal signal which is termed independence (Partan, 2004; Partan & Marler,

2005). However, the response to the multimodal signal may contain only one of the

responses of one of the unimodal signals which is termed dominance (Partan, 2004;

Partan & Marler, 2005). One signal may influence the effect of the other signal which is

termed modulation; which can also occur through learning (Partan, 2004). The final

possible type of response to non-redundant signals is termed emergence, which occurs

when the response to the multimodal signal is not the response that occurs to either

unimodal signal (Partan, 2004; Partan & Marler, 2005).

Influence of reproduction on responsiveness

Reproductive status has been shown in other fish species to affect their

behavioural responses (e.g. Sisneros & Bass, 2003; Clement et al., 2005; Lengkeek &

Didderen, 2006; Sisneros, 2009). Some behaviour and physiology will change in both

males and females in some species of fishes depending on whether they are reproductive

or not. For example, females of the gobiid species *Bathygobius soporator* release fluids

when they are reproductive or gravid, which cause the males of this species to exhibit

courtship behaviour (Tavolga, 1976), not exhibited outside of the breeding season.

A vocalizing species of fish called the plainfin midshipman, *Porichthys notatus*, has

females that cycle in auditory sensitivity through the breeding season (Sisneros & Bass,

2003). The female is able to encode more harmonic components of the male`s

advertisement call during the breeding season or when the female is reproductive than

when in a non-reproductive state (Sisneros & Bass, 2003). This species of fish

experiences seasonal variations in steroid hormones; the levels in males peak during the

early nesting season when they begin vocalizing, while females` levels peak when their

eggs were developing (Sisneros et al., 2003). Brain aromatase levels also fluctuate in

males and females at the same time their hormone levels fluctuate; aromatase converts

androgens to estrogens and one of its functions is that it activates adult sexual behaviour

(Forlano & Bass, 2005). Whereas in the cichlid, *Astatotilapia burtoni*, the reproductive

state of the female changes the preference the female has for males (Clement et al.,

2005). Females that are gravid prefer to associate with males that are also reproductive

but they also prefer more active males; which females show no preference for when not

reproductive (Clement et al., 2005). Female river blennies, *Salaria fluviatilis*, alter their

behaviour when they are reproductive (Lengkeek & Didderen, 2006). When female

blennies are reproductive, they display courtship behaviour to males that have been

displaying courtship behaviour, which is not observed at any other time (Lengkeek & Didderen, 2006).

The round goby, *Neogobius melanostomus*, has been found to have a shift in olfactory preference when reproductive (Gammon et al., 2005) and had an increased physiological olfactory response when reproductive (Belanger et al., 2007). Reproductive females show a preference for the conditioned water of reproductive males over untreated dechlorinated water, when non-reproductive females show no preference (Gammon et al., 2005). The round goby shows cyclical olfactory responses coincident with the reproductive season and changes in behaviour (increasing gill ventilation in reproductive fish) but other behavioural changes in this fish have not been investigated.

Round Goby, Neogobius melanostomus

The round goby is an invasive species in the Laurentian Great Lakes (Corkum et al., 2004). The goby came to the Great Lakes in the early 1990s by way of ballast water in ships coming from the Ponto Caspian region of Europe (Johnson et al., 2005). The goby is a bottom dwelling fish and has outcompeted many native species like the mottled sculpin, *Cottus bairdi*, and logperch, *Percina caprodes*, for habitat and reduced other populations of native species by feeding on their eggs (Corkum et al., 2004). The goby has a long reproductive season, during which it can spawn multiple times (MacInnis & Corkum, 2000). Females mature at year one, which is one year earlier than in their native habitat, and have a high fecundity when compared to native species in the Great Lakes (MacInnis & Corkum, 2000).

Male round gobies are nest guarders (Wickett & Corkum, 1998); they will prepare the nest and release odourants (Belanger et al., 2004) and calls (Rollo et al., 2007) to

attract females to the nest. The male fans the nest using pectoral fins (Meunier et al., 2009), and in doing so, water is pushed out of the nest which contains his urine and pheromones. In laboratory settings, reproductive females were found to be attracted to reproductive male conditioned water (Gammon et al., 2005) and female round gobies have been shown to be attracted to the male's call (Rollo et al., 2007; Rollo & Higgs, 2008). Gobies will increase their gill ventilation rate in response to steroids and conspecific odours (Murphy & Stacey, 2002; Belanger et al., 2006) and the physiological response to these odours changes with the reproductive status of the fish (Belanger et al., 2004; Belanger et al., 2007).

Thesis Objectives

The overall objective of my thesis was to determine how multimodal signals, specifically auditory and olfactory stimuli, are received and interpreted by female round gobies (*Neogobius melanostomus*). This overall framework of my thesis was accomplished by splitting the work into two separate, but related, objectives.

The first objective of my thesis, presented in chapter 2, was to determine the behavioural responses of female round gobies to auditory and olfactory stimuli from reproductive male fish over the breeding season.

The second objective of my thesis, presented in chapter 3, was to map the auditory and olfactory pathways of the round goby and test for the convergence of secondary olfactory and secondary auditory neurons in the torus semicircularis, a midbrain integrative structure in fish. By combining two approaches, I hoped to ascertain how multimodal signaling affects reproductive communication in the round goby.

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CHAPTER II

REPRODUCTIVE STATUS INFLUENCES MULTISENSORY INTEGRATION
RESPONSES IN FEMALE ROUND GOBIES, *NEOGOBIUS MELANOSTOMUS*

Abstract

Reproductive signaling often requires multimodal signals to be sent between
conspecifics. It has often been easier to study one sensory modality at a time but it is
more realistic to study multiple sensory modalities together to fully understand how
sensory signals are integrated together. Often when integration occurs, behavioural
responses are modified from the responses that occur with one sensory modality. I
presented female round gobies, *Neogobius melanostomus*, with a reproductive male call
and reproductive male conditioned water on their own and simultaneously, to examine
differences between unimodal and multimodal responses. I also tested females of varying
reproductive status over the breeding season to see the effect of reproductive status on
responsiveness. When round goby females, both reproductive and non-reproductive, were
presented with either unimodal stimulus in a swimming flume, females showed no
significant response compared to the control period in which no stimuli were
administered. However, when reproductive females were presented with multimodal
stimuli, they spent significantly more time in a zone surrounding where the stimuli were
placed in than they did when given either unimodal stimulus but the same pattern was not
seen for non-reproductive females. This study shows that female round gobies cycle in
their attraction and localization abilities to male stimuli through the breeding season and
reproductive females have a synergistic response to multimodal stimuli from reproductive
males.

Introduction

Many aspects of animal communication involve complex behaviours requiring that more than one type of signal is sent, which frequently occurs across multiple sensory modalities (Hebets & Papaj, 2005). While much of the previous research in animal behaviour has tended to focus on one sensory modality at a time (McLennan & Ryan, 1997; Andersson & Amundsen, 1997; Atema et al., 2002; Smith et al., 2002; Honkavaara et al., 2002; Schaefer et al., 2006), more recent work has begun to focus on how multiple sensory modalities are used in behavioural contexts (Braun et al., 2002; Hebets et al., 2006; Kulahci et al., 2008; Uetz et al., 2009; Barry et al., 2010; O'Loghlen & Rothstein, 2010). Sensory stimulation over multiple sensory modalities can either provide redundant information or each signal can provide different information (Uetz et al., 2009). When the signal provides redundant information it can produce either a response that is equivalent to receiving sensory information through one modality or it can produce a synergistic response (Braun et al., 2002; Uetz et al., 2009).

Synergistic responses to multimodal signals can enhance the response (Uetz et al., 2009). Enhanced responses to multimodal stimuli occur when the intensity of the response is greater than that of the response to any of the unitary stimuli composing the multimodal stimulus (Partan, 2004) and can be seen across varying taxa. In general, when animals are given visual and auditory stimuli, both are able to localize the source with more accuracy (Knudsen & Knudsen, 1985; Stein et al., 1989; Boyd & Fabrivius, 1965). Wolf spiders (Lycosidae) use visual and vibrational signals during courtship; the presence of the vibrational signal enhances the response of the female (Hebets et al., 2006; Uetz et al., 2009). Praying mantids (*Pseudomantis albofimbriata*) use chemical and

visual stimuli to locate mates and for mate assessment (Barry et al., 2010) while in the

brown-headed cowbird (*Molothrus ater*), male mating success is enhanced when females

can receive both auditory and visual stimuli (O'Loghlen & Rothstein, 2010). Eastern grey

squirrels (*Sciurus carolinensis*) show enhanced alarm responses to a conspecific robot

displaying auditory and visual cues when compared to being given each stimulus on its

own (Partan et al., 2009); an enhanced response is greater than the response to any of the

unimodal signals (Partan & Marler, 2005). When given both olfactory and visual signals,

bumble-bees (*Bombus impatiens*) do not make faster decisions but are able to decide with

more accuracy which flower contains sucrose instead of water, based on previous training

(Kulahci et al., 2008). Enhanced responses are seen in the majority of organisms when

given a multimodal stimulus as opposed to a unitary stimulus; but for some organisms

like the mantid (Barry et al., 2010), both stimuli are required for the behaviour to occur.

To date there have been few studies examining multimodal signal reception in fish.

Electroreceptive fish (*Gnathonemus petersii*) are able to locate prey faster by combining

electroreception and vision than when only one modality is available (Moller, 2002) and

olfaction decreased the time spent searching for prey (Von der emde & Bleckmann,

1998). Electroreceptive fish also use feedback from electric organ discharge and vision to

stay close to one another and stay in a group (Moller et al., 1982). In non-electroreceptive

fish, visual signals and signals to the mechanosensory lateral line are required for two

predatory fish, largemouth bass (*Micropterus salmoides*) and muskellunge (*Esox

masquinongy*) to determine their optimal strike distance when foraging (New & Kang,

2000). In salmon (*Oncorhynchus nerka*), males require both visual and vibrational stimuli

from the female to spawn (Satou et al., 1994). In the freshwater eel (*Anguilla astralis*),

the time and distance travelled is shortened when tracking an odour by using both

olfactory and mechanosensory stimuli (Montgomery et al., 2002). In a gobiid fish

(*Bathygobius soporator*), some males require only an olfactory stimulus to show

courtship behaviour but a visual stimulus aided them in orientation. However, other

males required the combination of an olfactory and visual stimulus to elicit full courtship

behaviour (Tavolga, 1956). The female goby was found to orient towards the male if

given both an auditory and visual stimulus (Tavolga 1956). Fish are thus able to use

multimodal signals to respond to conspecifics and show synergistic responses, as found

in other animals, but the breadth of these signals has not studied as frequently as in other

taxa.

In the current study, I examine female responses to a male sexual display using a

species of nest guarding fish, the round goby, *Neogobius melanostomus*. In the round

goby, reproductive males sit at their nests and try to attract females to them (Arbuckle et

al., 2005). Male round gobies emit calls from their nest when reproductive (Rollo et al.

2007). The call has been shown in laboratory settings to attract females towards an

underwater speaker (Rollo et al., 2007; Rollo & Higgs 2008) and in the field, the call has

been shown to bring round gobies into the area of the underwater speaker (Rollo et al.,

2007). Reproductive male round gobies release odorants that attract females (Belanger et

al., 2004) and Gammon et al. (2005) used reproductive male conditioned water, in a

laboratory setting, which was found to attract reproductive females. When a reproductive

male is in the nest, the male fans the nest using pectoral fins (Meunier et al., 2009), which

therefore pushes water containing odour out of the nest to attract females. Both of the

auditory and olfactory signals from the male have been shown to attract reproductive

females on their own (Gammon et al., 2005; Rollo et al. 2007; Rollo & Higgs, 2008) but, the interaction of these two signals has not been examined. I hypothesized female round gobies would have a synergistic response to multimodal signaling from reproductive males. In addition, I wanted to test whether the reproductive status of females affected their response to a multisensory stimulus because reproductive status has been shown in other fish species to affect their behavioural responses (*e.g.* Sisneros & Bass, 2003; Clement et al., 2005; Lengkeek & Didderen, 2006; Sisneros, 2009).

Methods

Sampling Methods

Round gobies were caught by angling and seining in the Detroit River in Windsor, ON in the summer of 2009. The fish were transported back to the University of Windsor's Animal Quarters where they were kept under a constant photoperiod (16L: 8D). Fish were fed fish flakes daily (Wardley Essentials; Hartz Mountain Corporation, Secaucus, NJ) and housed according to the University of Windsor's Animal Care Committee guidelines. All fish were held no more than one week prior to testing to avoid testing the fish after they changed reproductive states from what they were in the field or during that period of the breeding season.

Experimental Procedure

Behavioural experiments were conducted in a swimming flume (Figure 2.1, designed by Dr. K Tierney & constructed by the Technical Support Centre) consisting of a 1 m long central tube (internal dimensions of 10 cm x 10 cm) that

49.5 cm

94.5 cm
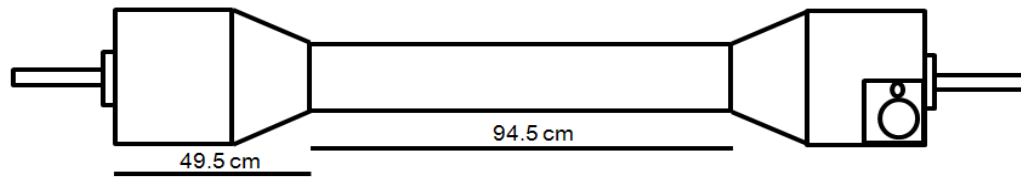
Figure 2.1: The swimming flume operating as a flow through system was used for all trials. The end box on the right is the upstream box; the smaller box inside represents the zone, the large circle represents the underwater speaker and the small circle represents the odour delivery hole.

transitioned over 25 cm on both ends to chambers (boxes) that were 25 cm x 25 cm x 25 cm. Each end box had a removable lid with valves allowing odorant to be administered; there was an additional hole added to the upstream box to allow an underwater speaker (details below) to be placed into the flume.

Fish were sexed before testing by examination of gonopodium shape (Marentette & Corkum, 2008). Females were termed reproductive if they had a swollen belly (Gammon et al., 2005) and a swollen, orange coloured papilla (pers. obs.); in two-spotted gobies (*Gobiusculus flavescens* Fabricius 1779) females develop orange colouration to their belly when reproductive (Svensson et al., 2005). Once a female's reproductive state was determined, the fish was placed into the downstream box of the flume and given 5 hours to acclimate. A pilot study determined a 5 h acclimation period was needed for the fish to settle into the experimental flume and respond to sensory cues. At the end of 5 hours, the fish was given 3 treatments each consisting of an additional 10 minutes of acclimation to background water followed by a 10 minute trial, to keep each treatment a total of 20 minutes (10 minutes of acclimation and 10 minutes of treatment for a total of 60 minutes). The treatments the fish received were odour, call, or odour & call. The order in which these were presented was randomly picked on the day of the trial. Acclimation and treatment periods were not started until the female returned to the downstream box. The sound stimulus used was a reproductive call from a nest guarding male recorded in the field (Rollo et al. 2007). This call has been shown to be attractive to female gobies in previous studies (Rollo et al. 2007, Rollo & Higgs 2008) and thus represents a natural cue to assess responsiveness. The call was played from a laptop computer connected to a stereo amplifier (Alesis RAA300, Alesis Inc., Santa Monica, CA, U.S.A.) which in turn

was connected to an underwater speaker (UW-30, Lubell Labs, Columbus, OH, U.S.A.) placed in the upstream box of the flume. The call was 7 s in duration and was played on a continuous loop over the 10 minute trial period. Before each day's trials, call output was set to a sound level of 150 dB re 1 μPa in the downstream box using a hydrophone (Interocean Inc., San Diego, CA, U.S.A.). Call structure did not change between the upstream and downstream boxes of the flume but intensity of the call was lower by 4 dB in the downstream box.

The odourant used was methanol-extracted GnRH treated reproductive male conditioned water (as used in Kereliuk et al., 2009). One reproductive male was placed in 1L of dechlorinated water for 16 hours after the fish was injected with gonadotropin releasing horomone (GnRH) one time, volume is GnRH injected was determined by [(fish weight (g) X 5)/1000], which makes males that may not be reproductive release steroids in their urine at the same rate a reproductive male does (unpublished data, Dr. Katare); water from 5 males was pooled after collection. The water was then collected, run through Sep-Pak cartridge (Waters Corporation, Milford, Mass, U.S.A.) and the cartridge was then rinsed with 5mL of distilled water followed by 5mL of methanol; 1mL of this methanol extract was then dried out through centrifuging in a CentriVap Concentrator (Labconco Corporation, Kansas City, Missouri, U.S.A.) and reconstituted with dechlorinated water to 200mL which made the conditioned water extract the concentration the male was releasing during the 16 hour collecting period. The amounts of the putative pheromones 11-oxo-etiocholanolone and its conjugates were estimated by an ELISA (enzyme linked immunosorbant assay) (Katare et al., 2010). The ELISA analysis of the methanol extracts revealed there was 24.75 ng of free 11-oxo-ETIO

immunoreactivity or 3.22 ng of free 11-oxo-ETIO and 20.85 ng of conjugated

immunoreactivity or 2.71 ng of conjugated 11-oxo-ETIO in 1mL of extract; 1mL of

extract was used per trial. The molarity of free 11-oxo-ETIO was 1.1×10^{-8} moles/L, the

molarity of the conjugated forms of 11-oxo-ETIO ranged from 7×10^{-9} moles/L for

sulphated 11-oxo-ETIO to 6×10^{-9} moles/L for glucuronated 11-oxo-ETIO. It is currently

thought that these steroids may act as pheromones in reproductive males (Jasra et al.,

2007; Kereliuk et al., 2009). The physiological threshold for detection, measured through

electro-olfactogram (EOG), of free and conjugated 11-oxo-ETIO is 10^{-9} moles/L

(Laframboise et al., 2008); thus the odourant I delivered had physiologically detectable

levels of free and conjugated 11-oxo-ETIO. The conditioned water extract was put into

the upstream box in the same area as the underwater speaker and was in front of the

inflow of water, to allow for the odour to be carried downstream (Figure 1). Odours were

delivered at 2 minute intervals to create a diffusion gradient down the swimming tunnel;

each delivery was 26 mL. When 26 mL was added to the swimming flume, it would be at

a concentration of 10^{-5} (as used in Gammon et al., 2005) by the time it mixed with the

96L in the flume and travelled to the downstream box. The swimming tunnel for all trials

was kept at a flow rate of 2.7 cm/s. Odour was delivered through a hole in the upstream

box by using a 30mL syringe with 10cm of Tygon tubing attached. Odour was delivered

in 2 minute pulses because dye mapping experiments with methylene blue, conducted as

a pilot study, showed that the upstream box and centre section of the swimming flume

would be almost completely cleared of odours after approximately 2 minutes at a flow

rate of 2.7cm/s.

Multimodal trials were run in a randomized order along with the single sensory stimulus trials for each fish. The 7 s call was played on a continuous loop starting as soon as the first odour delivery was made. The methanol extract, for multimodal trials, was prepared the same as for the single stimulus trials as described above. The odour was placed into the swimming flume in the same manner at 2 minute intervals. Time started for the trial as soon as the auditory and olfactory stimuli were started.

Control Fish Procedure

Control fish were tested in addition to the experimental fish, with the day of the week for control fish experimentation picked at random. Control fish were given a five hour acclimation to the flume and the experimental procedure was run the same as with experimental fish (60 minutes of trial time). The order in which the control stimuli were presented was randomized as done with experimental fish. The control condition for the sound trials was an underwater speaker not playing any sound to control for the visual stimulus of the speaker. The control for the odourant was dechlorinated water that was subjected to the same methanol extraction process as the GnRH treated reproductive male conditioned water. For multimodal controls, there was no call played from the speaker which remained in the same position in the upstream box and as in the odour control trials, and methanol extracted dechlorinated water was delivered into the upstream box at 2 minute intervals. The methanol extracted dechlorinated water was dried using the CentriVap Concentrator (Labconco Corporation, Kansas City, Missouri) and reconstituted the same way the GnRH treated reproductive male conditioned water was for experimental fish. A second set of syringes with attached Tygon tubing was used to

administer the control water to avoid contamination. Time for the control trial started just after the first odour delivery to be consistent with the other multimodal trials.

Behavioural and statistical analysis

All trials were recorded using a computer running surveillance software (EverSecure; Matco, St. Laurent, QC, Canada) connected to 4 digital CCD cameras (Matco, St. Laurent, QC, Canada) to allow for visualization of the entire flume. I used EthoVision Analysis software XL (Noldus Information Technology, Attleboro, MA, U.S.A.) to track the fish in the upstream box. The upstream box contained what I termed the 'stimulus zone' which was a virtual rectangle enclosing the underwater speaker and odourant delivery hole. From the videos I quantified if the goby responded to a trial, the amount of time fish spent in the upstream box excluding the stimulus zone and how much time they spent in the stimulus zone. A goby was counted to have responded to a trial if it swam from the downstream box to the upstream box after a stimulus was administered. Overall, 22 fish were given the auditory, olfactory and combined treatments, 5 additional fish were control fish. Of the 26 fish tested, 11 of these were in June, 9 were in July and 6 were in August. I used a Chi-square goodness of fit test to examine differences in the proportion of fish responding per month. For July fish, I did a repeated measures ANOVA (PASW Statistics 18, SPSS Inc., Chicago, Illinois, U.S.A.) for the relative amount of time spent in the upstream box and in the zone. Relative time spent in the upstream box was calculated with the following formula:

Relative time spent in upstream box (s) = [Time spent upstream during trial (s)] − [Time spent upstream during 10 minute acclimation (s)]

The relative time spent in the stimulus zone was calculated the same way as the relative time spent in the upstream box (see formula above). I used Mauchly's Test of Sphericity, for the repeated measures ANOVA, and because its value was significant I used the Greenhouse-Geisser correction (Zar 1999).

Because fish in the current study were needed for additional studies, reproductive status had to be estimated from live fish. To estimate reproductive status, the maximum width of the belly was measured (cm) and divided by fish total length (cm) to give a ratio of relative belly size for each fish. To correlate relative belly size with gonadosomatic indices (GSI), and hence reproductive status, in the summer of 2010, 10 reproductive females (RF) and 12 non-reproductive females (NRF) were caught to measure GSI and relative belly size to more accurately assess reproductive status of our experimental fish (See Appendix 2). A female is termed reproductive if the GSI is over 8% (Young et al., 2010). The belly size data from 2010 were first normalized with a log10 transformation and then analyzed with a one-way ANOVA and a Bonferroni post-hoc test was performed (Field 2009) in PASW Statistics 18 (SPSS Inc., Chicago, Illinois, U.S.A.).

Results

The behavioural responses of round gobies differed significantly by month in 2009 ($P<0.05$) (Figure 2.2). In June, 9 experimental fish were tested and 4 responded to one or more treatments; a fish was considered to have responded if it swam into the upstream box. In July, 7 fish were tested and 7 responded. In August, 6 fish were tested and 0 responded. One control fish showed a positive response in July to the control trial for the sound stimulus in which no sound stimulus is administered but this fish did not

Figure 2.2.
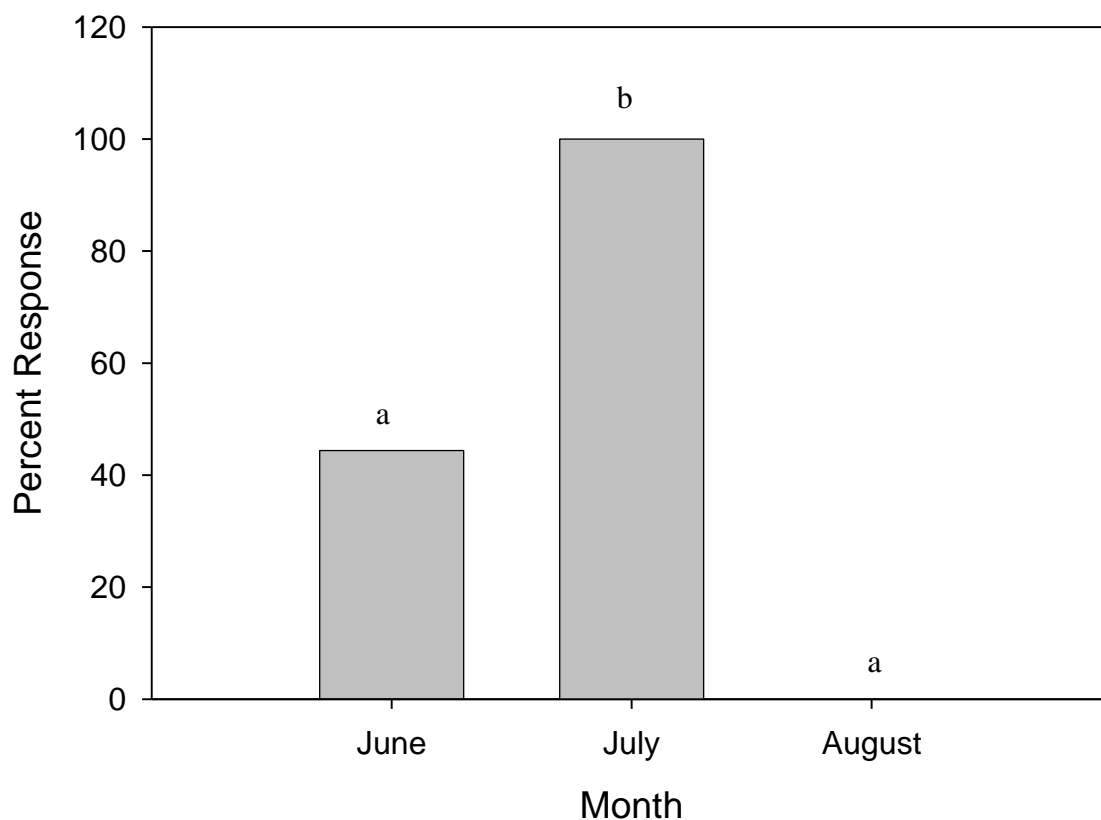
Figure 2.2. The percent of females that responded to behavioural trials. Percent of

responding female gobies to one or more treatments (call and methanol extracted GnRH

treated reproductive male conditioned water alone and simultaneously) was significantly

more in July (P>0.05) than in June or August. In June, 4 of 9 (44%) fish responded, 7 of

7 fish (100%) responded and 0 of 6 (0%) fish responded in August.

Figure 2.3.
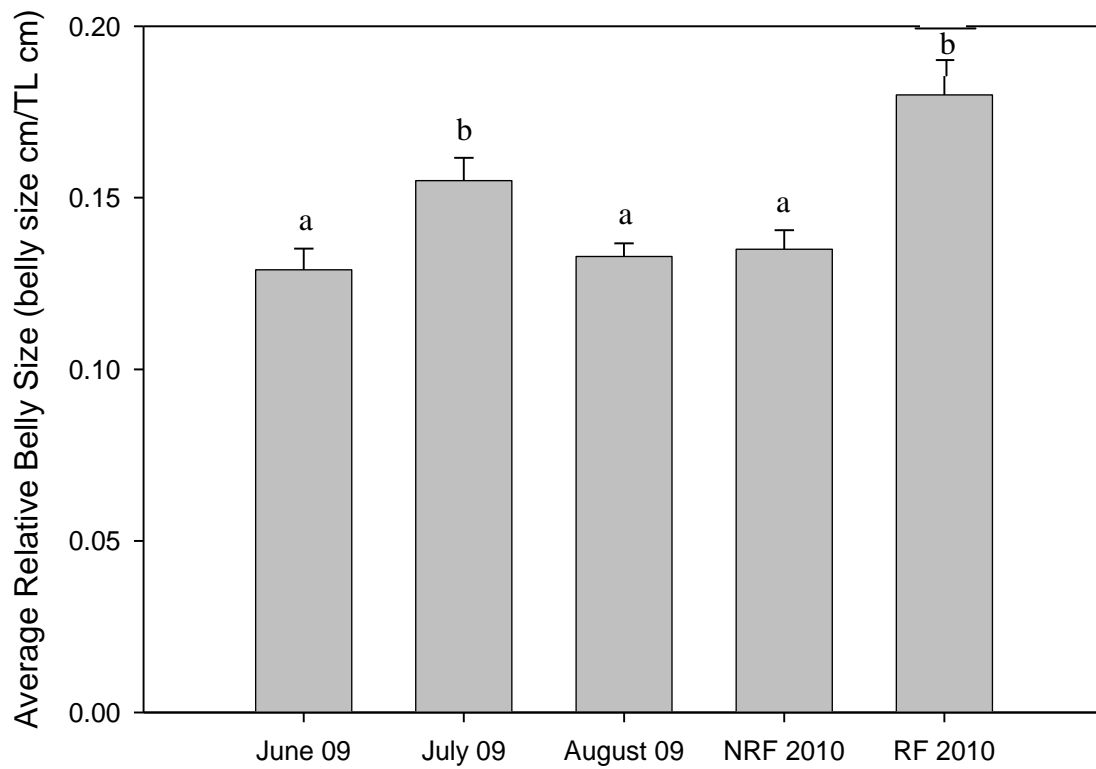
Figure 2.3. Average relative belly size of female fish from 2009 compared to fish in 2010 that had GSI measured. Females in July 09 and reproductive females (RF) 2010 had significantly larger relative belly sizes (P<0.001).

respond to methanol extracted dechlorinated water; no other control fish showed positive responses to sound, odour or multimodal control trials. Relative belly size was found to vary significantly across experimental fish from June, July and August of 2009 as well as RFs and NRFs from 2010 ($F_{4,48,0.05}$=10.55, P<0.001, Figure 2.3). Bonferroni post-hoc analyses showed that July 2009 fish and RFs from 2010 had significantly larger belly sizes than non-reproductive fish in June 2009, August 2009 or NRFs from 2010(P<0.001). Fish in July had a relative belly size of 0.15 while both June and August had a relative belly size of 0.13. July fish were judged to be reproductive based on comparison with known reproductive fish from 2010.

The analysis of the percent of fish responding in June did not differ significantly in response across treatment types (P>0.05). Of the 9 fish tested in June, 3 (33%) responded to the call on its own, 4 (44%) responded to the conditioned water extract on its own and 3 (33%) responded to the call and conditioned water extract when presented together. In July, fish tended to respond more when given the conditioned water and call together, although the response was not statistically significant (P>0.05, Figure 2.4). Of the 7 fish tested in July, 4 (57%) responded to the call on its own, 3 (43%) responded to the conditioned water on its own and 6 (86%) responded to the call and conditioned water when presented together. In August, 0 of the 7 (0%) fish tested responded to any of the three treatments.

My first hypothesis, which was that females would have a synergistic response to multimodal stimulus, was not supported by relative time spent upstream by fish in June. Fish in June, did not spend significantly more time upstream when given the call and

Figure 2.4.
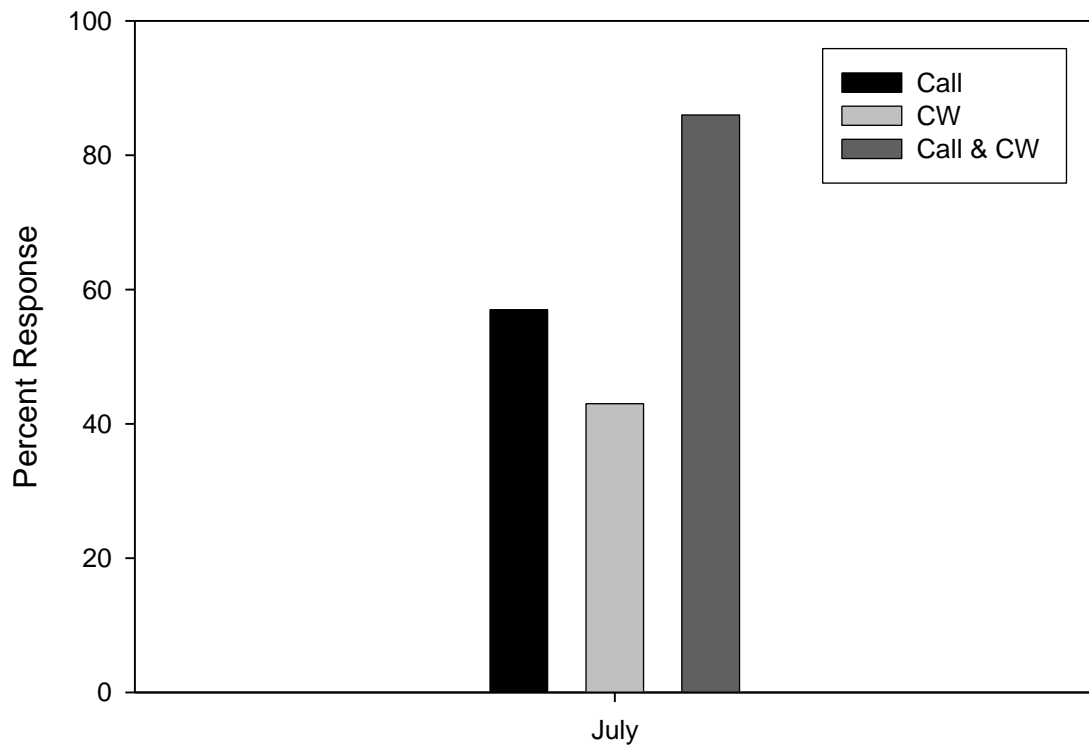
Figure 2.4. The percent of fish that responded to stimuli in July. Fish considered to

have responded, came into the upstream box after the trial start time. In July, 4 of 7

(57%) of female gobies responded to the call alone, 3 of 7 (43%) responded to

conditioned water (CW) alone and 6 of 7 (86%) responded when call and CW were

presented together.

Figure 2.5.
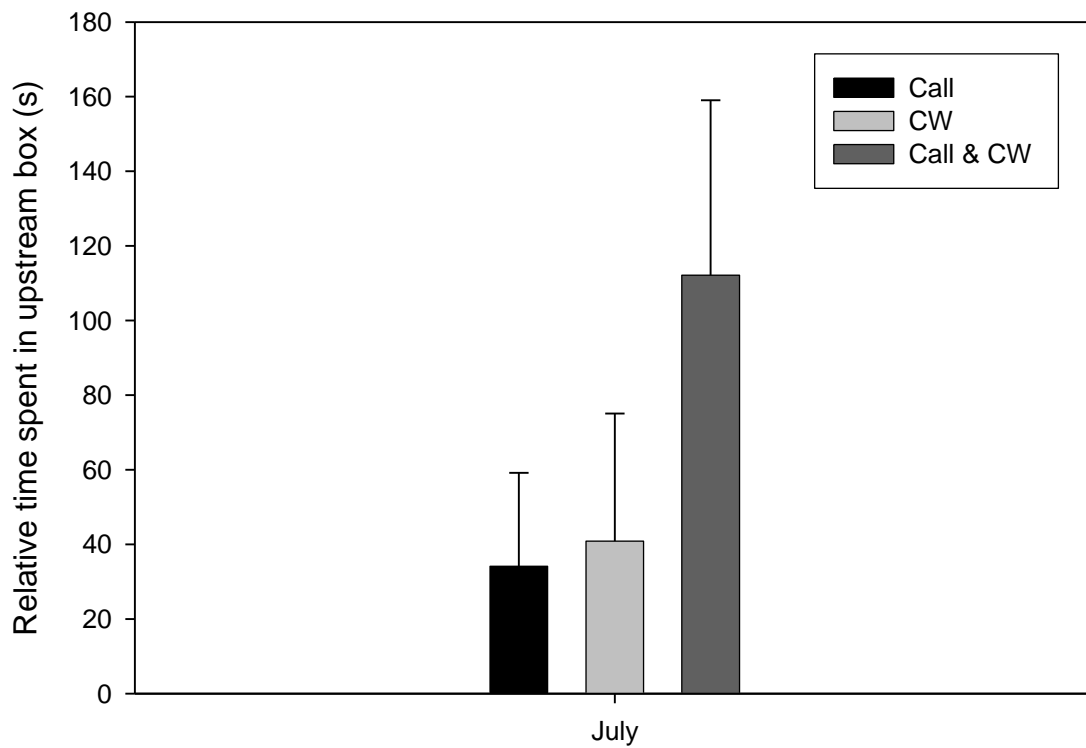
Figure 2.5. Relative amount of time spent in the upstream box (s) excluding the stimulus zone. Female gobies tended to spend more time in the upstream box (s) when presented the call and conditioned water (CW) together although not significant (P>0.05).

Figure 2.6.
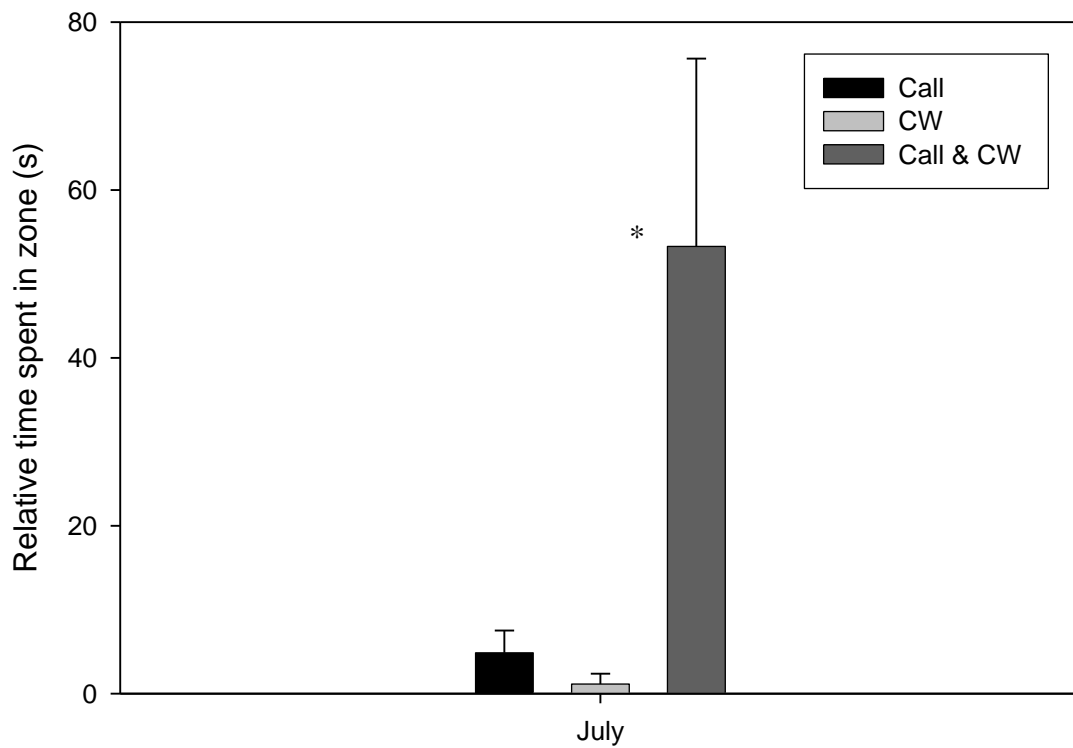
<u>Figure 2.6. Relative amount of time spent in the stimulus zone (s).</u> Female gobies

spent significantly more time in the stimulus zone (s) when presented with the call and

conditioned water (CW) together (P=0.036).

conditioned water together ($F_{2,8,0.05}=1.609$, P>0.05). Although fish in July did tend to spend more time in the upstream box when given the call and conditioned water together, they did not spend significantly more time upstream ($F=_{2,6,0.05}=1.333$, P>0.05, Figure 2.5). However, my first hypothesis was supported by the relative amount of time females spent in the stimulus zone ($F_{2,6,0.05}=9.485$, P=0.036) when given the call and conditioned water together in July (Figure 2.6). Fish in June did not spend significantly more time in the stimulus zone when given the call and conditioned water together ($F_{2,8,0.05}=1.661$, P>0.05).

My second hypothesis, that reproductive status affects behavioural responses, was supported by the significant difference in belly size between June, August, NRFs and July, RFs (P<0.001), this difference determined that females in July of 2009 were reproductive females. The reproductive females in July of 2009, spent significantly more time in the stimulus zone when given a multimodal stimulus in July (P=0.036).

Discussion

The goal of the current study was to investigate whether female round gobies would have a synergistic response to multimodal stimuli and to determine if responses change with reproductive status. Our results show that reproductive female round gobies have an enhanced response when receiving auditory and olfactory stimuli representative of a reproductive male round goby. Significantly more females responded to one or more treatment types (call, olfaction, call and olfaction) in July than in June or August. I correlated the female's responsiveness in July to their reproductive status. Females in July 2009 had larger belly sizes which were found to be statistically the same as female gobies that had their GSI taken in 2010 and were found to be reproductive. I found

female gobies had a change in responsiveness corresponding with their reproductive status, where reproductive females were more responsive to reproductive male signals. Females of vocalizing species can have an auditory system that cycles with the breeding season and are more sensitive to the male's call during the breeding season (Sisneros & Bass, 2003; Sisneros 2009; Lynch et al., 2004). Female mate preference can also change along with their reproductive state, which occurs in the cichlid, *Astatotilapia burtoni* (Clement et al., 2005). Heightened sensitivity during a reproductive state is not limited to the auditory system. Female tungara frogs, *Physalaomus pustulosus*, are more visually sensitive to the male's conspicuous vocal sac when reproductive (Cummings et al., 2008).

I found non-reproductive female round gobies to have differing responses to reproductive male signals compared to the responses seen with reproductive females, similar to the previous studies mentioned. The females in this study showed less responsiveness before entering their full reproductive state and no responsiveness after their breeding season. Female round gobies, in August 2009, did not respond to either the call or the conditioned water extract or the combined stimuli and were found to have belly sizes corresponding to non-reproductive females from 2010. Female round gobies have a quick turn over of eggs once their reproductive season is over (Kulikova, 1985) and the spawning season of round gobies only occurs until mid-summer (MacInnis & Corkum, 2000) and this time period corresponds to when I saw the strongest multimodal responses. Previous studies looking at unisensory modalities in round gobies found similar results corresponding with reproductive status. Reproductive females spend more time in the end of a tank reproductive male conditioned water is added to and they swam

faster and in a more linear path when responding to the male stimulus than during the control period but non-reproductive females showed no attraction response to reproductive male conditioned water (Gammon et al., 2005). Female round gobies will approach an underwater speaker closer if it is playing a conspecific call (Rollo et al., 2007) and will swim faster and in a more linear path when responding (Rollo & Higgs, 2008). Although some attraction was shown to unisensory stimuli in this study, reproductive females preferred the multimodal stimulus. Female round gobies, in this study, appear to show auditory cycling like that of the midshipman, where females have a lower threshold for a reproductive male's call when reproductive (Sisneros 2009). Female round gobies, in this study, may have also shown olfactory cycling with their reproductive status like some cyprinids including goldfish, *Carassius auratus*, and zebrafish, *Danio rerio* (Belanger et al., 2010) and male round gobies (Belanger et al., 2007). When changes in behaviour occur, there is usually a change physiologically as well. Some gonadotropin-releasing hormone (GnRH) receptors and sex steroid receptors have been found to fluctuate in expression, in the olfactory bulb, over the course of the reproductive season (Marsuka & Fernald, 2010) and sex steroid receptor levels have been found to fluctuate in the ear (Maruska & Fernald, 2010). Over the reproductive season, there are olfactory sensory neurons (crypt cells) that fluctuate in crucian carp (*Carassius carassius*), (Hamdani et al., 2007), and in the olfactory epithelium it is thought that crypt cells may respond to sex pheromones (Hamdani et al., 2007). Crypt cells have been found to vary in number over the breeding season of the crucian carp, with more crypt cells being present during the spawning season (Hamdani et al., 2007) leading to a heightened

sensitivity to pheromones, which is what reproductive female round gobies displayed in this study.

Reproductive females in July overall responded more to stimuli than non-reproductive females tested in June or August. The reproductive females spent significantly more time in the stimulus zone in the upstream box when given a multimodal stimulus (Figure 2.6), although they did not spend more time in the upstream box area which excluded the stimulus zone (Figure 2.5). Reproductive males of this species prepare the nests (Meunier et al., 2009) and mature males release attractants, which could be pheromones, to attract reproductive females (Arbuckle et al., 2005). It is advantageous for reproductive males that only reproductive females, as demonstrated with this study's results, show an enhanced ability to find the location of the nest when the male is producing a call and odour. Non-reproductive females, from the beginning of the breeding season in this study, appear to be attracted to the area but not to the male's specific location. The results of non-reproductive females from June, does not agree with the result of Gammon et al. (2005) which stated non-reproductive females did not respond more to conditioned water than dechlorinated water. It may be that Gammon et al. (2005) tested females late in the reproductive cycle, while I tested them throughout the reproductive season. I found that early in the breeding season when GSI values would indicate the fish to be non-reproductive, the females still show some response to GnRH treated reproductive male conditioned water over dechlorinated water, which elicited no response, but the females tested at the end of the breeding season showed no response to conditioned water or dechlorinated water. It appears fish at the beginning of the breeding season are starting to become responsive to reproductive male's signals but this response

is not fully developed until they are reproductive and ready to spawn. A possibility is that once females are done spawning, they lose their responsiveness to the male's signals.

Multimodal integration is shown through behaviour but occurs in the physiology of the animal. In fish, the midbrain integration centre is the torus semicircularis, it contains known acoustic and visual units (Schellart et al., 1987) and receives lateral line input (Schellart, 1983). The torus semicircularis is involved in ultraviolet vision in rainbow trout, *Oncorhynchus mykiss*, with juvenile fish having more sensitive or responsive units than adult fish (Coughlin & Hawryshyn, 1994). The torus semicircularis is not just an integration centre in fishes but also in amphibians. In the clawed toad, *Xenopus laevis*, the torus semicircularis has lateral-line, somatosensory, auditory inputs (Zittlau et al., 1985). The organization and connections of the torus semicircularis are similar to those of the inferior colliculus in mammals (Carr et al., 1981). Although no olfactory inputs have been found yet in the torus semcircularis or inferior colliculus, it remains a possible candidate for the integrative structure for auditory and olfactory inputs because it is a large integrative structure. Our behavioural results indicate that integration of auditory and olfactory stimuli are occurring in the round goby and causing an enhanced response to multimodal stimuli but the structure has not yet been located. Although the structure where multisensory integration is occurring has not been located, the behavioural responses have been examined. Reproductive female round gobies respond more to multimodal stimuli than unisensory stimuli. The three-spined stickleback, *Gasterosteus aculeatus*, uses visual and olfactory cues for mate choice (McLennan, 2003) and the guppy, *Poecilia reticulata*, may need visual cues if the olfactory cue is at a low concentration (Archard et al., 2008). Fishes' responses to

unimodal cues can vary depending on degradation in the environment and some may require a multimodal cue in order to elicit a response like the guppy (Archard et al., 2008). Fish, like other organisms, can use unimodal signals to respond to mates (Crow & Liley, 1979; Cole & Smith, 1992; Frade et al., 2002; Gammon et al., 2005; Rollo et al., 2007; Rollo & Higgs, 2008). However, in fish and other species, synergistic responses tend to be seen with multimodal signals (as seen in: Meredith & Stein, 1986; Acquistapace et al., 2002; Montgomery et al., 2002; Hebets, 2005; Archard et al., 2008). The increase in behaviour that is seen with multimodal signals in various organisms may be in part due to degradation of signal over the range in communication, making it more beneficial for the signaler to use two or more modes of communication. The range of detection for acoustic signals depends on the environment (Wiley & Richards, 1978) but acoustic signals are used in long distance communication in many organisms (Naguib & Wiley 2002), whereas olfactory cues may be more useful in short range communication because olfactory cues may not be detectable at low concentrations (Alberts, 1989; Archard et al., 2008). Although it is more energetically expensive to use two modalities for signaling as opposed to using a single sensory modality, it may be useful if one modality is better used for long range communication (acoustic cues) and one is better for short range communication (olfactory cues). In the current study, the female round goby was drawn to the area the male would have been with one sensory cue but straight to the location of the male with two sensory cues, so although more energetically expensive for the male, it decreases the time spent signaling if the female can find the location with more precision, thus making it advantageous to use multimodal signaling.

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CHAPTER III

AUDITORY AND OLFACTORY PATHWAYS IN THE ROUND GOBY,
NEOGOBIUS MELANOSTOMUS

Abstract

In fish, two sensory systems can be used for both long and short distance signaling – the auditory and olfactory systems. Fish use these systems for communicating with their environment and conspecifics. When fish receive signals in multiple sensory modalities, this information is sometimes integrated in one area of the brain. In the current study, I mapped the auditory and olfactory pathways with the post-mortem lipophilic tract tracer 1,1'–dioctadecyl-3,3,3'3'-tetramethylindocarbocyanine perchlorate (DiI), which travels both anterograde and retrograde, and the *in vivo* tract tracer dextran amines, which only travel anterograde when a high molecular weight is used. Tract tracing with DiI showed these sensory systems integrating in the torus semicircularis (TS), which is a midbrain integrative structure in fishes with known auditory, visual and lateral line projections. However, dextran application revealed anterograde projections from the auditory nerve to the TS but no anterograde projections from the labelled regions of the olfactory bulb to the TS. Together the results from the current study lead to the conclusion that olfactory and auditory integration occurs in the TS, as demonstrated with DiI, but does not happen with direct anterograde projections from the olfactory bulb, as demonstrated with dextrans.

Introduction

Communication is important for all organisms, including fish, in order to interact with their environment and conspecifics. While many sensory systems are available for this communication, audition and olfaction are of particular interest in the current study because both can be used for long-distance and short-distance signaling (Rogers & Cox, 1988; Hansen & Reutter, 2004). Fish can produce calls (McKibben & Bass, 1998) or odours like pheromones (Sorensen & Scott, 1994) for communication and conspecific fish need to receive and process the information. Fish without swim bladders, like the round goby, receive auditory signals as the displacement component of sound, unlike fish with swimbladders which receive auditory signals with the pressure component of sound (Popper & Schilt, 2008). When sounds reach the fish, otoliths inside the ear move thus causing the stereocilia and kinocilium of hair cells on the sensory epithelium under the otoliths to bend (Popper & Schilt, 2008). When the cilia bend, ion channels get opened causing a signal cascade within the cell body (Hudspeth, 1983; Popper & Schilt, 2008). This ionic change within the cell ultimately causes the release of neurotransmitter and an action potential to be sent down the auditory nerve and to the auditory brainstem (Popper & Schilt, 2008). When an odourant enters the naris of a fish, the molecule will bind with the corresponding receptor on the sensory epithelium within the peripheral olfactory organ; the peripheral olfactory organ can be shaped differently depending on the species of fish (Belanger et al., 2003; Lazzari et al., 2007; Zielinski & Hara, 2007). When the odourant binds to the correct receptor, it causes a signal cascade within the olfactory

sensory neuron which ultimately causes an action potential to be sent to the olfactory

bulb (Firestein, 2001).

The auditory and olfactory pathways have been mapped in numerous fish species

(*e.g.* Bass, 1981; Echteler, 1985; Bass et al., 1994; Hofmann & Meyer, 1995;

McCormick, 1997; Bass et al., 2000). When the neurotransmitter is released from the hair

cell onto afferent axons of the auditory nerve, it causes an action potential to propagate

along the axonal fibers located in the auditory nerve which projects to the hindbrain

structures of the auditory system. The auditory nerve enters the auditory brainstem and

some axons continue on to the descending octaval nuclei, some to the superior octaval

nuclei and others project to the torus semicircularis, a midbrain structure (Bass et al.,

1994; Bass et al., 2000). The axonal fibers that propagate the action potentials in

olfactory sensory neurons are located in the olfactory nerve, and terminate in the

olfactory nerve layer of the olfactory bulb (Hara, 1994), which is a forebrain structure.

Like with the auditory nerve fibers, not all the neurons end in the primary brain nuclei

(the olfactory bulb in the case of olfaction), some continue on to the telencephalon but

most synapse in the olfactory bulb, onto the dendrites of mitral cells located in the

olfactory glomeruli, and the axons of these mitral cells continue on to the telencephalon

(Hara, 1994). From these known pathways (the olfactory in the forebrain and the

auditory in the hindbrain and midbrain), it appears that at the level of the sensory input

from these two sensory systems is not integrated, at the level of the level of the secondary

sensory neurons. But with my data in chapter 2, I saw an increased behavioural response

in reproductive female round gobies, *Neogobius melanostomus*, when auditory and

olfactory cues from reproductive males were given simultaneously, leading to the hypothesis that integration should be occurring.

The torus semicircularis (TS) is a midbrain integrative center in fishes (Higgs et al., 2006) and it receives input from the auditory, lateral line and visual systems (Schellart et al., 1987). In nonelectrosensory teleosts the nucleus centralis (medial portion) of the TS processes auditory information, while the nucleus ventralis (ventral portion) of the TS processes lateral line input (Higgs et al., 2006). Visual units occur throughout the TS but the majority of visual neurons are located in the ventral portion (Schellart et al., 1987). The TS also contains bimodal neurons throughout that are sensitive to auditory and visual stimuli and are most likely concentrated in the middle of the torus, these neurons were located with physiology recordings (Schellart et al., 1987). When the TS was labelled with horseradish peroxidase dye, heavy labelling was seen in the auditory hindbrain (de Wolf et al., 1983), demonstrating there is communication occurring between the TS and auditory system, but such labelling has not been reported with the olfactory system. With the TS being a known integration centre for auditory, lateral line and visual stimuli, as well as containing bimodal neurons, it was hypothesized for the current study that it may also integrate olfactory stimuli, by receiving mitral cell axonal processes from the olfactory bulb.

Methods

Round gobies were caught by angling and seining in the Detroit River in Windsor Ontario and transported back to the University of Windsor's Animal Care Facility where they were kept according to animal care committee guidelines. All procedures conformed to the University of Windsor's animal care guidelines.

A preliminary study was done with the lipophilic tract tracer, 1,1'–dioctadecyl-3,3,3'3'-tetramethylindocarbocyanine perchlorate (DiI) (Cat. #D282, Molecular Probes, Invitrogen Canada Inc., Burlington, ON), a dye which travels anterograde and retrograde (Kobbert, 2000); retrograde is defined as movement from the axon terminal to the cell body (Altar et al., 1997) and anterograde is movement from the cell body to the axon terminals. This preliminary study was done to determine if there were any connections with the torus semicircularis before a refined technique was used to determine whether there was anterograde or retrograde transport occurring. A total of 12 gobies were euthanized with an overdose of clove oil, and the head was removed. An incision was made to the dorsal side of the head down to the skull and a section of the skull was removed to allow for quick fixation in 4% paraformaldehyde (PFA). The tissue was then kept in PFA for at least 4 days to ensure good fixation before application of DiI. Dye applications were done to the torus semicircularis (TS), n=4, the auditory nerve (VIII), n=5, and the olfactory bulb (OB), n=2 (Table 1; see Figure 1 for location of TS, VIII and OB). A crystal of DiI was placed onto the TS, VIII, or OB with an insect pin (0.2 mm tip diameter, Cat #26002-20, Fine Science Tools, Vancouver, B.C.) under a stereo microscope (Olympus SZX9, Olympus Canada Inc., Markham, ON). After dye placement, 5% agarose (Type IA, CAS #9012-36-6, Sigma Aldrich, Oakville, ON) was melted and poured over the exposed area. The tissue was then placed into 4% PFA and into an incubator (Gallenkamp model 1H-150, Artisan Scientific Corporation, Champaign, IL) set at 38-39°C. The incubation period was 4-6 weeks, to allow for the dye to diffuse; DiI travels at an estimated speed of 100-400 µm/day when incubated at 37 ^{o}C (Kobbert, 2000). I estimated that there is 140 µm between the VIII and TS and 250

μm between the olfactory bulb and TS from my histological atlas (see appendix 11).

After the incubation period, the brain was dissected out and embedded in 5% agarose; the

tissue was then sectioned horizontally at 125 μm using a vibratome (Leica VT 1000S,

Wetzlar, Germany). The tissue was viewed on a fluorescence microscope (Nikon Eclipse

E800, Nikon Canada Inc., Mississauga, ON) equipped with a camera (QI Cam Fast 1394,

QImaging, Surrey, BC) and Northern Eclipse software (Empix Imaging Inc.,

Mississauga, ON). The contrast and brightness were adjusted with Adobe Photoshop 7.0

(Adobe Systems, San Jose, CA). Plates were assembled and lettered with Adobe

Photoshop (Adobe Systems) and all images have a resolution of 300 dpi.

Table 1: **Location of DiI and dextran amine injection sites and structures labelled after injection**. Structures are listed rostral to caudal. The letter i before a structure indicated ipsilateral, while the letter c before a structure indicates contralateral labelling. C: cerebellum; DiI: 1,1'–dioctadecyl-3,3,3'3'-tetramethylindocarbocyanine perchlorate; DO: descending octaval nuclei; OB: olfactory bulb; PAG: periaqueductal gray; SO: superior octaval nuclei; Tel: telencephalon; TL: torus longitudinalis; TS: torus semicircularis; VIII: auditory nerve, VIII BS: auditory brain stem.

Tracer	Location of Injection	Structures Labelled
DiI	TS, n=4	iOB (n=2), iTel, cTel, iPAG, iC, iTS, cTS, iTL, cTL, iDO, iSO, cDO, cSO, iVIII BS, iVIII
DiI	VIII, n=5	iTel, cTel, iPAG, cPAG, iC, cC, iTS, cTS, iTL, cTL, iDO, iSO, cDO, cSO, iVIII BS, iVIII
DiI	OB, n=2	iOB, iON, iTel, cTel, iTS, cTS, iTL
Dextran Alexa Fluor® 488	VIII, n=4	iTS, cTS, iDO, iSO, cDO, cSO, iVIII BS, iVIII BS
Dextran Alexa Fluor® 488	Lateral OB, n=1	iOB, iTel
Dextran Alexa Fluor® 568	Medial OB, n=5	iOB, iTel, cTel

Figure 3.1: <u>Round goby brain</u>. Unlabelled horizontal section of a round goby brain, viewed on a steromicroscope; section is 150 μm thick, * represents PAG. DO: descending octaval nuclei; OB: olfactory bulb; ON: olfactory nerve; OT: optic tectum; PAG: periaqueductal gray; SO: superior octaval nuclei; Tel: telencephalon; TL: torus longitudinalis; TS: torus semicircuarlis; VIII: auditory nerve; VIII BS: auditory brain stem.

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A total of 10 dye applications were done with dextran amines (Molecular Probes, Invitrogen Canada Inc., Burlington, ON) with dextrans of high molecular weight (10kDa), which are predominately transported anterograde (Kobbert, 2000; Reiner & Honig, 2006) to determine the direction of the connections between the auditory nerve or olfactory bulb and the torus semicircularis. Dye applications were done to the auditory nerve (n=4) and the olfactory bulb (n=5) (Table 1; see Figure 1 for location of TS, VIII and OB); dextrans were placed on as crystals. I used dextran Alexa Fluor® 568 (Cat. #D22912, 10,000 MW, anionc, fixable, Molecular Probes, Invitrogen Canada Inc., Burlington, ON) and Alexa Fluor® 488 (Cat. #D22910, 10,000 MW, anionic, fixable, Molecular Probes, Invitrogen Canada Inc., Burlington, ON), for dye applications. Gobies were anesthetised with a 0.05% solution of 2-phenoxyethenol (Cat # 129H2503, Sigma Aldrich, Oakville, ON) and the same strength of solution was allowed to flow into the goby's mouth and over the gills at a flow rate of 0.05 L/min. For dye application to the auditory nerve, an incision was made on the dorsal surface of the fish's head, parallel to the groove in the operculum and in line with the top of the eye. For dye application to the olfactory bulb, an incision was made on the dorsal surface of the head caudal to the eyes. After the appropriate size opening was made, the dura was removed from the injection site and dextran crystals were placed into the area of interest using insect pins (0.2 mm tip diameter). The saccular auditory nerve was injected because it is the largest in diameter of the three nerves and thus it was easier to ensure a clean injection site. In the olfactory bulb, injections from the dorsal surface were done both medially and laterally to see if only medial or lateral mitral cell projections went into the torus semicircularis because the olfactory bulb is topographically organized (Hara & Zhang, 1997). Fish were

left for 5 hours after the dye was injected, to allow the dye to diffuse intracellularly after which the head was removed and placed into 4% PFA in 0.1M phosphate buffer for a minimum of 2 days for fixation. Additional fish were left for 10 hours of dye transport to ensure that dye travel time was not a limiting factor. The 10 hour treatments had no additional label as compared to the 5 hour incubation. After fixation the tissue was removed and cryoprotected by placing the tissue through graded sucrose solutions in phosphate buffered saline solution (PBS) (10% and 20% on day 1, 30% on day 2 and then left overnight) at 4°C and cryosectioned at $-16\,^{o}$C at a thickness of 25µm on a cryostat (Leica CM 3050S, Wetzlar, Germany). The tissue was placed on Fisherbrand Superfrost/Plus slides (Fisher Scientific Company, Ottawa, ON) and air dried overnight. Every other slide was counterstained with NeuroTrace® 640/660 deep-red fluorescent Nissl stain (Cat. #N21483, Molecular Probes, Invitrogen Canada Inc., Burlington, ON) to allow for visualization of neuronal cell bodies and to be able to later identify various structures in the brain. Sections labelled with dextran Alexa Fluor® 488 only, were photographed with a confocal microscope (Olympus Fluoview FV1000) equipped with Fluoview imaging software (FV10-asw version 1.7a). Specimens that were injected with dextran Alexa Fluor® 568 alone and those that were also injected with dextran Alexa Fluor® 488, were photographed with a fluorescence microscope (Zeiss Observer. Z1) equipped with AxioVision4 imaging software. The contrast and brightness were adjusted with Adobe Photoshop and Photoshop was also used to invert images. Plates were assembled and lettered with Photoshop and all images have a resolution of 300 dpi.

Results

DiI labelling of the torus semicircularis following labelling of auditory and olfactory structures

After DiI application to the auditory nerve, labelling was seen in the auditory brain stem, and the ipsilateral and contralateral descending and superior octaval nuclei (Figure 3.2A). Projections were also seen in the ipsilateral and contralateral TS (Figure 3.2B). After DiI application to the auditory nerve, projections were also seen to end in the periaqueductal gray (PAG), because they did not continue on to the optic tectum (Figure 3.2B), torus longitudinalis (TL) (Figure 3.2B), telencephalon (Figure 3.2C) and cerebellum (Figure 3.2D). When DiI was applied to the torus semicircularis, projections were seen in the ipsilateral and contralateral descending and superior octaval nuclei (Figure 3.3A), the auditory nerve (Figure 3.3A), the contralateral torus semicircularis (Figure 3.3B), the olfactory bulb (Figure 3.3C & 3.3D), telencepahlon (Figure 3.3C & 3.3E), torus longitudinalis (Figure 3.3B) and the cerebellum (Figure 3.3F). When DiI was applied to the olfactory bulb, projections were seen in the olfactory nerve (Figure 3.4A), the telencepahlon (Figure 3.4B), the contralateral torus semicircularis (Figure 3.4C), and the ipsilateral torus semicircuarlis and torus longitudinalis (Figure 3.4D). The ipsilateral and contralateral sides of the torus semicircularis were labelled after DiI labelling of one side of the torus semicircuarlis, the auditory nerve and the olfactory bulb.

Auditory and Olfactory pathways mapped with dextran amines

After dextran Alexa Fluor® 488 application to the auditory nerve, fibrous projections were seen in the auditory brain stem, ipsilateral and contralateral descending

Figure 3.2: <u>DiI application to the auditory nerve</u>. All images are inverted fluorescence images. All panels are from horizontal tissue sections. 100 μm micrometer bar in panel A is also for panel B, 50 μm micrometer bar in panel C is also for panel D. For all panels (except C which is the contralateral Tel), the right side of the image is the ipsilateral side and the left side of the image is the contralateral side.

(A) After DiI application to the auditory nerve (indicated by an arrow), projections can be seen to the ipsilateral auditory brain stem, descending octaval nuclei , superior octaval nuclei, due to large amounts of fluorescence, the individual areas cannot be identified . The contralateral descending octaval nuclei (1) and superior octaval nuclei (2) are circled.

(B) The ipsilateral and contralateral torus semicircularis (TS), the contralateral periaqueductal gray (PAG) which is indicated with a *, the ipsilateral and contralateral torus longitudinalis (TL) and cerebellum (Cb) also showed labelling after the auditory nerve was labelled.

(C) The telencephalon received projections after DiI was applied to the auditory nerve. Labelling in the contralateral telencephalon is shown.

(D) The ipsilateral and contralateral cerebellum showed labelling after DiI was applied to the auditory nerve, areas of label are indicated with arrows.
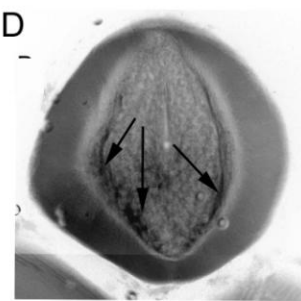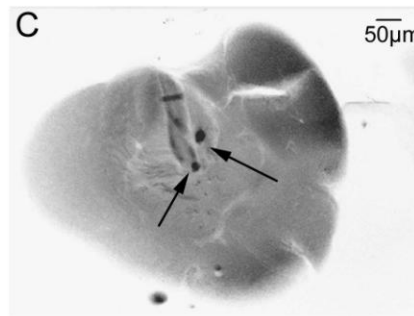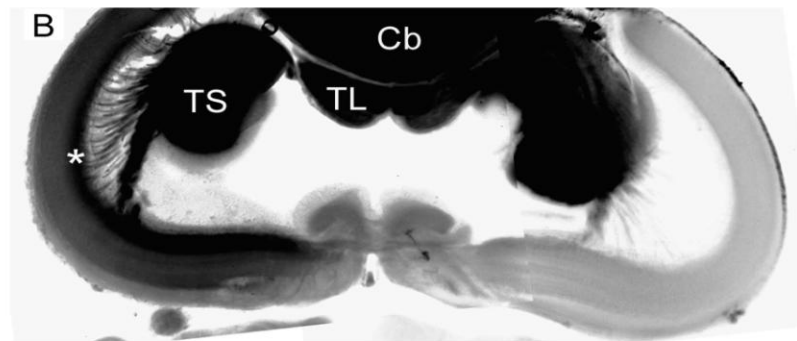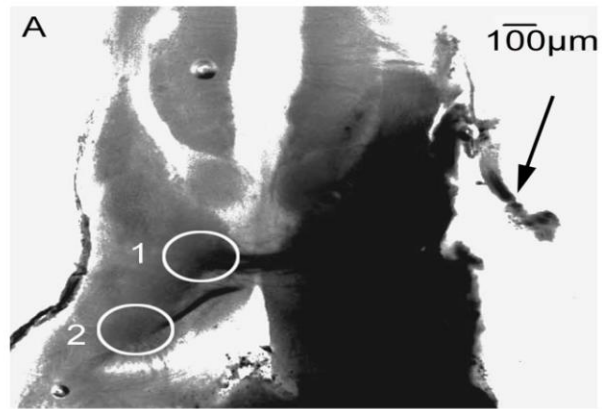
Figure 3.3: <u>DiI application to the torus semicircularis</u>. All images are inverted

fluorescence images. Panel A is from a cross section, all other panels are from

horizontally sectioned tissue. 100 µm micrometer bar in panel A is also for panel B, E

and F, 50 µm micrometer bar in C is also for panel D. For panels A and B, the left side of

the image is the ipsilateral side. For panels C &D only the ipsilateral side is shown. For

panels E & F, the right side of the image is the ipsilateral side.

(A) After DiI application to the TS, projections can be seen to the auditory nerve

(labelled with arrow), ipsilateral auditory brain stem (1), DO (2) and SO (3). The auditory

brain stem, DO and SO can also been seen on the contralateral side.

(B) The ipsilateral TS showed projections to the contralateral TS, ipsilateral torus

longitudinalis (TL) (indicated by circle) and ipsilateral PGZ (indicated by *).

(C) and **(D)** Projections were seen to the telencephalon (indicated with a circle) and the

olfactory bulb (OB) and olfactory nerve (ON). Differential labelling in (C) and (D) could

be due to differences in section thickness and/or depth through the structure.

(E) The ventral telencephalon (Tel) was labelled ipsilaterally and contralaterally.

(F) Labelled areas of the ipsilateral cerebellum are indicated with a circle, no labelling

was seen contralateral.
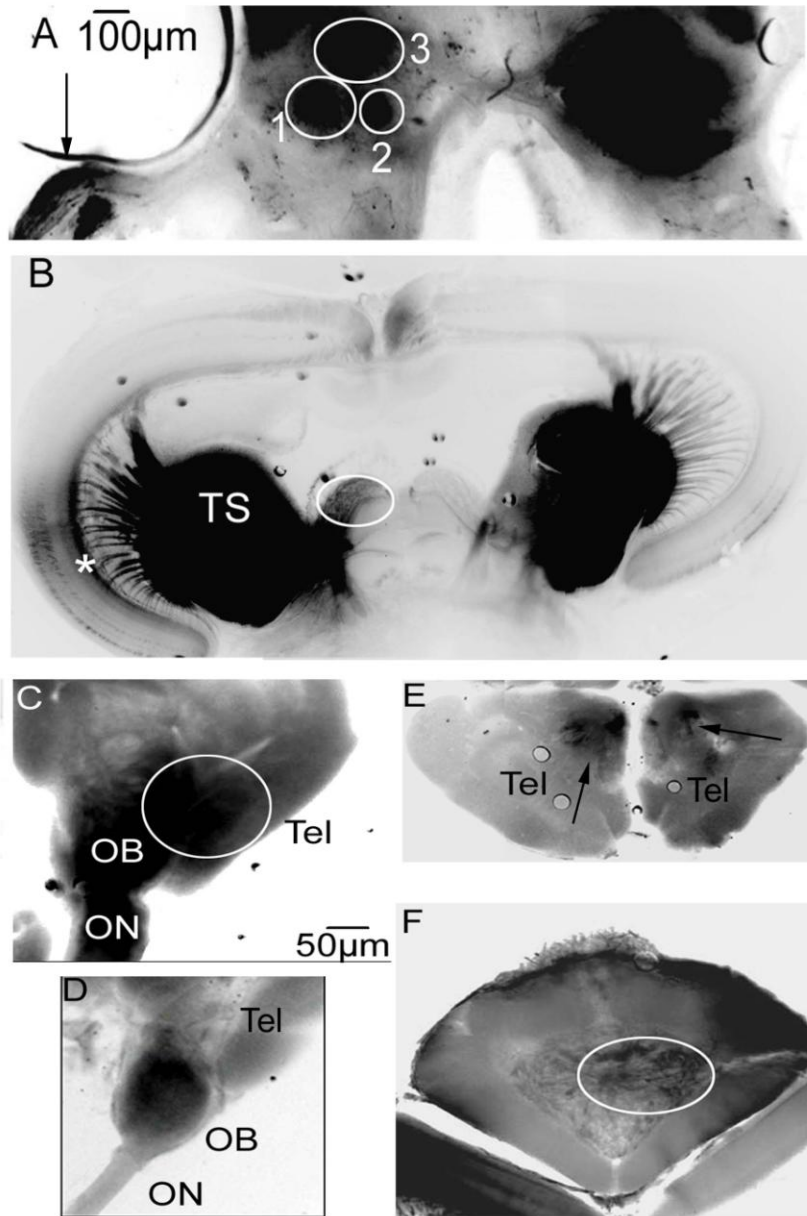
Figure 3.4: <u>DiI application to the olfactory bulb.</u> All images are inverted fluorescence images of horizontal sections. 100 μm micrometer bar in panel A is also for panel C and D. For panels A, B and D, only the ipsilateral side is shown. For panels B and C display only the contralaeral side. Rostral is at towards the bottom of all the panels.

(A) Label was seen in the olfactory nerve after DiI application to the OB..

(B) After DiI application to the OB, label was seen in the ventral area of the telencephalon.

(C) Label was seen in the contalateral TS and PAG (represented by a *).

(D) Label was seen in the ipsilateral TS, TL and PAG (represented by a *).
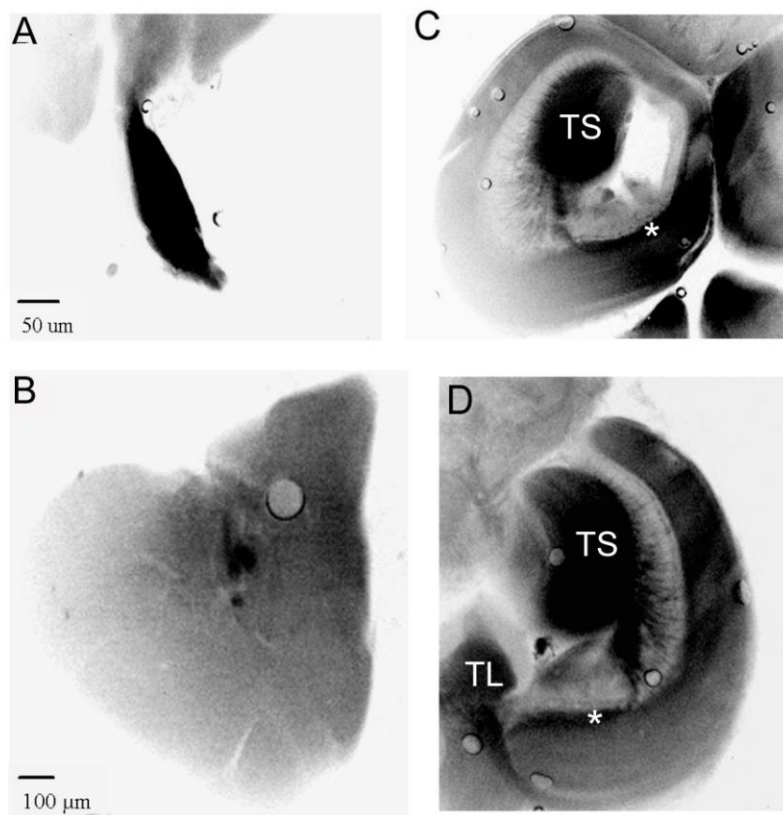
Figure 3.5: <u>Dextran Alexa Fluor® 488 application to the auditory nerve.</u> All images

are inverted fluorescence images, panel A is a cross section and panels B-D are from

horizontal sections. 20 μm micrometer bar in panel B is also for panels C and D. For

panels A and B, ipsilateral side is to the right of the panel; only ipsilateral TS shown in C

and only contralateral TS shown in D.

 (A) After dextran Alexa Fluor® 488 application to the auditory nerve (indicated by

arrow), fibrous projections were seen to the ipsilateral auditory brain stem (1), DO (2)

and SO (3). The SO (4) and DO (5) can also be seen on the contralateral side.

(B) Fibrous projections were seen running towards the ipsilateral TS.

(C) Fibrous projections were seen within the ipsilateral TS.

 (D) Fibrous projections were seen within the contralateral TS.
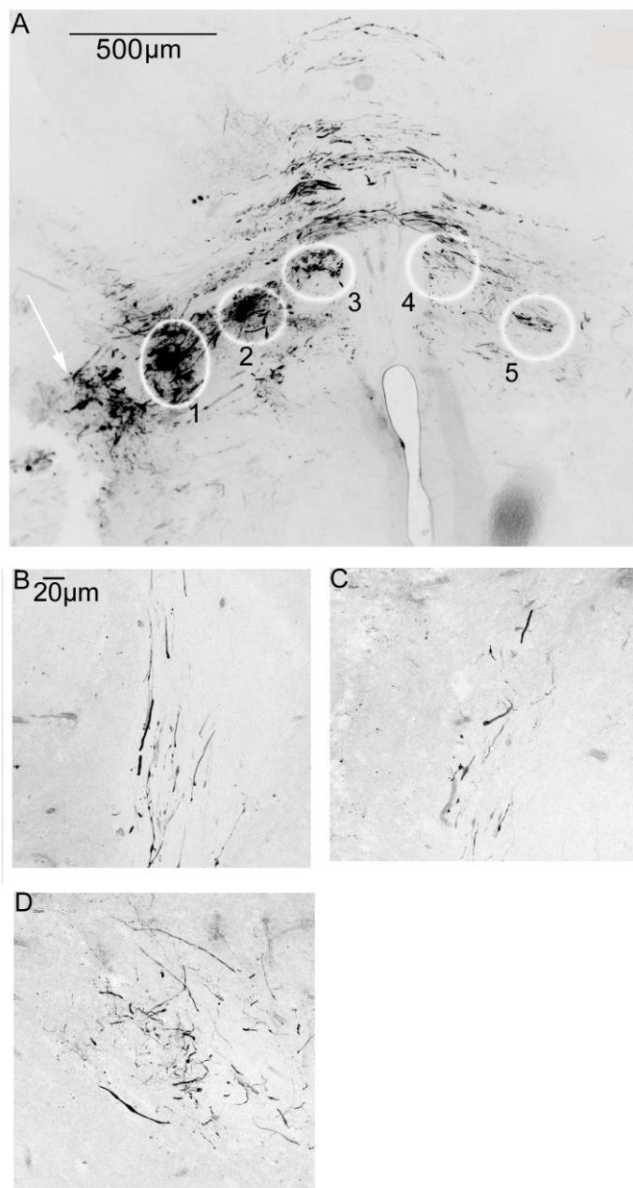
and superior octaval nuclei (Figure 3.5A). Projections were also seen traveling to the

ipsilateral TS (Figure 3.5B) and in the ipsilateral (Figure 3.5C) and contralateral TS

(Figure 3.5D). On the confocal, I manually focused through sections of the torus to see

nerve fibers terminating in this area, which were fibers that were present in the most

ventral part of the section and did not continue through the entire section (sections used

in Figures 3.5C &D; See Appendix 10) and terminating in cell bodies could also be

visualized in this area (See Appendix 11). A few fibers at the start of the section

terminated in that section and did not continue on to the next section, showing that fibers

were stopping in the TS and not just passing through. After dextran Alexa Fluor® 568

application to the medial region of the olfactory bulb, labelled mitral cells were observed

(Figure 3.6A) and after dextran Alexa Fluor® 488 to the lateral olfactory bulb, labelled

mitral cells were observed (Figure 3.6B). After applying dextran into the medial region of

the olfactory bulb, labelled fibrous projections were seen in the ipsilateral telencephalon

(Figure 3.6C) and after dextran application into the lateral region of the olfactory bulb,

labelled fibrous projections were seen in the ipsilateral telencephalon (Figure 3.6D).

Fibrous projections were also seen crossing to the contralateral telencephalon after

dextran was loaded into the medial olfactory bulb (Figure 3.6E). Labelled fibers can be

seen in both the ipsilateral and contralateral telencephalon after labelling of the medial

olfactory bulb (Figure 3.6F). When dextran was applied to the medial region of the

olfactory bulb, tracts were seen in the medial region of the telencephalon (Figure 3.6C).

When dextran was applied to the lateral region olfactory bulb, lateral tracts were seen in

the telencephalon (Figure 3.6D). Projections were confined to the telencephalon and no

projections were found in midbrain structures.

Figure 3.6. Dextran Alexa Fluor® 568 application to the medial olfactory bulb and dextran Alexa Fluor® 488 application to the lateral olfactory bulb. For all panels, rostral is towards the bottom of the panel and lateral is towards the left of the panel. For panels A-D only ipsilateral side is shown. For panels E and F, the ipsilateral side is to the left of the panel.

(A) After dextran Alexa® 568 application to the medial olfactory bulb (OB), mitral cells are labelled.

(B) After dextran Alexa® 488 application to the lateral olfactory bulb, mitral cells are labelled.

(C) Labelling of medial mitral cells shows fibrous projections through the telencephalon (Tel) to areas within the ventral telencephalon.

(D) After labelling of lateral mitral cells, fibrous projections were seen through the telencephalon to areas within the ventral telencephalon.

(E) Labelling of medial mitral cells shows fibrous projections to the contralateral telencephalon.

(F) Labelling of medial mitral cells shows fibrous projections within the ipsilateral and contralateral telencephalon.
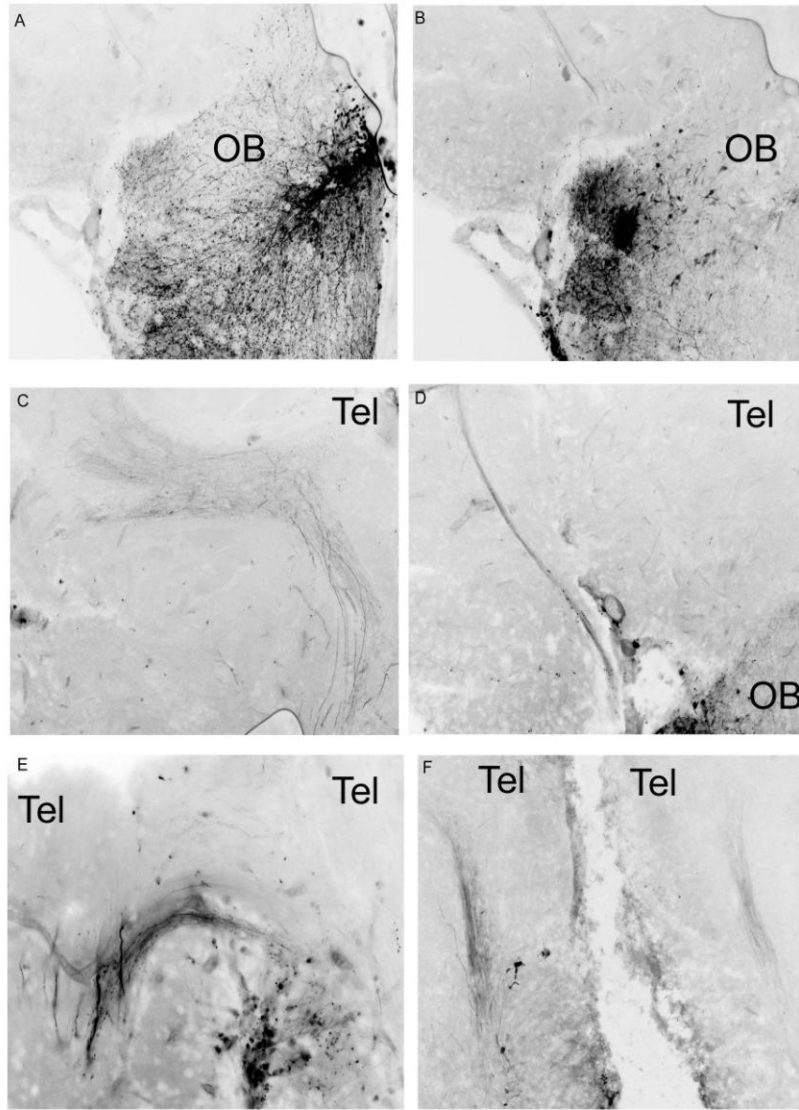
Discussion

My preliminary experiments with DiI, showed that the auditory and olfactory pathways both projected to the TS which meant the TS was possibly the centre where integration of these two pathways occurs. When DiI was injected into the auditory nerve, I saw labelled fibrous projections to auditory structures in the hindbrain, including the auditory brainstem, DO and SO. The DO and SO were seen labelled ipsilaterally and contralaterally. In a review of TS auditory circuitry by Bass et al. (2005), it was stated that the projection from the auditory nerve to the DO had not conclusively been demonstrated. However, in all of the DiI labelling of the auditory nerve, the contralateral DO was always seen to have label (as seen in Figure 3.1A). I also noted label to the contralateral SO and this part of the auditory circuitry was not in the review of Bass et al. (2005). After labelling in the auditory nerve, I saw label in the ipsilateral and contralateral TS also stated in the Bass et al. review. I also saw label in the PAG, torus longitudinalis (TL), telencephalon and cerebellum. The PAG was said to be part of the auditory circuitry in the review by Bass et al. and it is involved in behaviours such as locomotion and vocalizations (Kittelberger et al., 2006). The torus longitudinalis is used in visual circuitry and allows the eye to respond to varying amounts of light (Gibbs & Northmore, 1996) while the TS has known visual inputs (Schellart, 1985); so the connections between the TS and TL were expected. The cerebellum of teleosts has been noted to have functions similar to the cerebellum of mammals and be involved in motor and behavioural responses as well as spatial cognition (Rodriguez et al., 2005). Since the cerebellum is used in these functions it is conceivable that it would integrate with the TS which is involved with auditory, visual and lateral line circuitry which are all used for

spatial orientation to objects (Giske et al., 1998). Areas of the telencephalon have been indicated to be involved in reproductive behaviours such as nest building in fish (Demski & Knigge, 1971) and the auditory system and visual systems that are known to integrate in the TS, are both the auditory and visual systems are involved in reproductive behaviours (audition *e.g.* Brantley & Bass, 1994*;* vision *e.g.* Kodric-Brown & Johnson, 2002).

After DiI was injected into the TS, I saw label to the same auditory areas as were seen when the auditory nerve was labelled. Along with the auditory areas, I saw label in the contralateral TS, the ventral telencephalon and olfactory bulb. Areas in the ventral telencephalon in fish are involved in courtship and spawning behaviour (Kyle et al., 1982). Fish with lesions in the ventral telencephalon were not attracted to the olfactory stimuli of reproductive fish of the opposite sex (Kyle et al., 1982). The TS is showing connections with many areas involved in different aspects of reproductive behaviour and the olfactory system is a system with a large involvement in reproduction (van den Hurk & Resink, 1992; Hara, 1994; Kotrschal, 2000; Laberge & Hara, 2001) that has not been previously shown to integrate information in the TS.

The preliminary study with DiI was done to see what areas of the goby brain have either anterograde or retrograde connections with the TS. After my findings suggesting that there was integration occurring, I used dextrans to determine if these were anterograde connections from the auditory nerve and olfactory bulb. After dextran Alexa® 488 was injected into the auditory nerve, I saw fibrous projections to the auditory brain stem, ipsilateral and contralateral DO and SO, which confirmed my DiI results. I also saw fibrous projections to the ipsilateral TS and within the ipsilateral and

contralateral TS, also confirming my DiI results. However, labelling was limited to these areas suggesting the other areas that were seen to have projections after labelling the auditory nerve with DiI could be retrograde projections which would not be labelled with the high molecular weight dextran used. Labelling was then done to the olfactory bulb mainly with dextran Alexa® 568; double labelling was done to the olfactory bulb using dextran Alexa® 568 for either a medial or lateral injection and dextran Alexa® 488 was done on the other side of the bulb. However, double labelling of the olfactory bulb did not reveal any projections that were not seen with single labels to either the medial or lateral olfactory bulb. After labelling of the mitral cells in the medial olfactory bulb, fibrous projections were seen into the medial ventral areas of the telencephalon and after lateral labelling the fibrous projections seen were into the lateral ventral areas of the telencephalon. Medially labelled fibrous projections were seen crossing to the contralateral telencephalon. These results were expected because the olfactory bulb is known to be topographic in its projections (Hara & Zhang, 1997) and is known to project to the telencephalon (Laberge & Hara, 2001). However, I did not find any fibrous projections further than the telencephalon and therefore no direct projections to the TS. I could not confirm labelling of cell bodies in any areas other than the olfactory bulb because I was unable to image my far red Nissl stain, which only labels neuronal cell bodies. This does not conclude that there is no integration occurring in the TS with the olfactory system, just that there are no anterograde projections from the olfactory bulb to the TS. This does not rule out retrograde projections to the olfactory bulb, with cell bodies in the TS. It was not possible in this study to use dextrans to confirm the hypothesis of retrograde projections. For dextrans to travel the organism needs to remain

alive and thus injecting into the TS was not possible since it is completely covered by the optic tectum and making an incision or injection through the optic tectum would be fatal to the fish (when an area of brain was cut, the fish immediately bled out). Areas of the ventral telencephalon are involved in olfaction (Kyle et al., 1982) and the ventral telencephalon receives olfactory projections (Hara, 1994) and by injecting only into the olfactory bulb, I may have missed a projection from the ventral telencephalon to the TS. Further investigation is needed to support the preliminary DiI study which showed integration of the auditory and olfactory systems occurring in the torus semicircularis.

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CHAPTER IV

CONCLUSIONS AND RECOMMENDATIONS

Thesis Findings and Significance

In Chapter 2, I discussed my behavioural experiment with female round gobies, *Neogobius melanostomus*, which investigated the effect of simultaneous exposure of auditory and olfactory stimuli on response over the reproductive season. I used auditory and olfactory stimuli from reproductive male round gobies. The auditory stimulus used was a male call, provided by Dr. John Janssen (University of Wisconsin). The olfactory stimulus used was methanol extracted GnRH treated reproductive male conditioned water collected from reproductive male round gobies. I performed these experiments in a swimming flume and gave females time to acclimate to their new surroundings before beginning the trials. Trials that were administered were the auditory stimulus by itself, the olfactory stimulus alone and the auditory and olfactory stimuli together.

I found that females in the beginning of their breeding season, which occurred in June of 2009, while they are still considered non-reproductive [they were considered non-reproductive when their belly size was compared to the belly size of females determined to be non-reproductive by gonadal somatic index (GSI)] show some response to reproductive male stimuli by swimming to the other end of a swimming flume but do not show an increased response when receiving multimodal stimuli. But when females are reproductive, which occurred in the month of July in 2009, they have an overall increase in responsiveness to reproductive male stimuli and show a significantly enhanced response to multimodal male stimuli. However, once the female's reproductive season is over, seen in August 2009, the females no longer responded to reproductive male

auditory and olfactory stimuli. The round goby showed an increase in responsiveness to stimuli when reproductive and this also occurs in another vocalizing species of fish the plainfin midshipman, *Porichthys notatus* (Sisneros & Bass, 2003) that becomes more receptive to the male's call when reproductive. Female fish have been shown to have a lower threshold for the male's call when they are reproductive (Sisneros & Bass, 2005). Other species of fish show increased olfactory responsiveness when reproductive (Hamdani et al., 2007), as demonstrated with reproductive female fish in Chapter 2. Fish have also been shown to gain more crypt olfactory sensory neurons on the olfactory epithelium in their peripheral olfactory organ when reproductive (Hamdani et al., 2007) which could increase the receptiveness of a female for male pheromones.

When a behaviour occurs it can be the result of receiving stimuli from a conspecific, interpreting the stimuli and generating a response. In order for the response to happen, the signals need to be interpreted in the brain. The auditory and olfactory systems of fishes are both used in short and long distance communication (auditory: Rogers & Cox, 1988; Popper & Schilt, 2008; olfactory: Hansen & Reutter, 2004; Archard et al., 2008). When a reproductive male goby generates and releases his call and urine, reproductive female round gobies receive these stimuli and need to interpret them. When an auditory stimulus is received by a fish, it causes the otolith inside the inner ear to move slower than the body of the fish which bends the hair cells on the sensory epithelium (Popper & Schilt, 2008). Bending of the hair cells causes a signal cascade inside the cell bodies of the hair cells after ion channels are opened (Popper & Schilt, 2008). This signal cascade ultimately releases neurotransmitter which is received by the axon terminals of the axons composing the auditory nerve (Popper & Schilt, 2008). The

neurotransmitter causes an electrical impulse to be propagated down the axons of the auditory nerve to the ipsilateral auditory brain stem, descending and superior octaval nuclei (Bass et al., 2005). I also found fibrous projections to the contralateral descending and superior octaval nuclei which were not confirmed in the review by Bass et al. (2005). Some fibrous projections continued past hindbrain auditory areas to the midbrain. The midbrain structures that received projections, when the auditory nerve was injected with DiI, were the periaqueductal gray area, the cerebellum, the ipsilateral and contralateral torus semicircularis, and the ipsilateral and contralateral torus semicircularis. When the auditory nerve was injected with high molecular weight dextran amines, which predominantly travel anterograde (Kobbert, 2000), midbrain structures receiving fibrous projections were limited to the ipsilateral and contralateral torus semicircularis.

When the olfactory system receives a stimulus, like pheromones from a reproductive male round goby, the odorant comes into the nose of the fish and binds to an olfactory receptor on the sensory epithelium of the peripheral olfactory organ. After the odour molecule binds with the receptor, a signal cascade occurs within the cell, causing ion channels to open and ultimately neurotransmitter to be released from the olfactory sensory neuron (Firestein, 2001; Zielinski & Hara, 2007). The olfactory sensory neurons terminate in the glomerular layer of the olfactory bulb, where these sensory neurons synapse with mitral cells (Firestein, 2001). Mitral cells labelled with dextran in my experiment, projected to areas in the ventral telencepahlon. Areas of the ventral telencephalon have been implicated in olfaction and reproductive behaviour (Kyle et al., 1982). When the olfactory bulb was labelled with DiI, projections were seen in the torus semicircularis and vice versa. However, these same projections were not seen with the

dextran amines used, which travel only anterograde. Leading to the hypothesis that integration of olfactory and auditory stimuli are occurring in the round goby, demonstrated with DiI and my behavioural data from Chapter 2, but is not occurring because of direct anterograde projections from the olfactory bulb to the torus semicircuarlis. It is possible that retrograde projections between the torus semicircularis and the olfactory bulb are occurring or that the ventral telencephalon is the area for integration between the torus, a midbrain integrative auditory structure (Higgs et al., 2006), and the olfactory bulb because the telencephalon is an area involved in olfactory behaviour (Kyle et al., 1982). My behavioural data suggests that multimodal integration of auditory and olfactory cues is indeed occurring in reproductive female round gobies and this Chapter 2 data was supported by my preliminary study with DiI. However, further tract tracing work needs to be done to determine how the torus semicircularis is integrating auditory and olfactory cues or if there is another area of the brain that is infact the integrative center for these two sensory systems.

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APPENDICES

Appendix 1: Date and time behavioural trials were completed. * indicates control fish. Control fish are fish which received call, odour and multi control trials (no call, methanol extracted dechlorinated water, no call & methanol extracted dechlorinated water).

Fish Number	Date	Time Trials Began	Order of presentation
1	June 9/09	16:57	Call, multi, odour
2	June 10/09	15:30	Call, odour, multi
3	June 15/09	14:42	Multi, odour, call
4	June 16/09	14:23	Call, odour, multi
5	June 17/09	14:14	Odour, call, multi
6	June 18/09	14:08	Call, odour, multi
7*	June 19/09	15:30	Call, multi, odour
8	June 22/09	15:22	Odour, call, multi
9*	June 23/09	15:13	Multi, odour, call
10	June 24/09	15:05	Odour, multi, call
11	June 25/09	14:03	Multi, odour, call
12	July 14/09	12:49	Call, odour, multi
13*	July 15/09	15:07	Odour, multi, call
14	July 16/09	16:26	Odour, call, multi
15	July 17/09	15:19	Call, odour, multi
16	July 20/09	16:45	Multi, odour, call

17	July 21/09	15:10	Call, multi, odour
18	July 22/09	15:09	Odour, multi, call
19	July 23/09	14:46	Odour, multi, call
20*	July 24/09	12:45	Call, odour, multi
21	August 7/09	14:42	Call, multi, odour
22	August 10/09	14:48	Odour, multi, call
23	August 11/09	14:51	Call, odour, multi
24	August 12/09	15:06	Call, odour, multi
25*	August 14/09	15:40	Multi, odour, call
26	August 24/09	15:23	Call, odour, multi
27	August 27/09	15:01	Odour, call, multi

Appendix 2: Gonadal Somatic Index (GSI) values for 2010 fish and Belly Size Number

for fish from 2009 and 2010. Graph is for GSI vs. Relative Belly Size data for 2010.

Relative Belly Size 2009

June	July	August
0.14	0.14	0.12
0.15	0.14	0.13
0.13	0.15	0.13
0.09	0.14	0.15
0.12	0.17	0.13
0.10	0.14	0.14
0.14	0.15	0.13
0.15	0.19	
0.14	0.13	
0.12		
0.12		

Relative Belly Size and GSI 2010

GSI = Ovary weight (g)/ Total weight of fish (g)

Fish #	Date	GSI	Relative Belly Size
n/a	June 2	2.5%	0.13
1	June 3	10.3%	0.15
2	June 3	12.0%	0.17

3	June 3	17.7%	0.18
1	June 10	4.0%	0.15
2	June 10	11.0%	0.17
3	June 10	10.0%	0.16
1	June 16	3.0%	0.19
2	June 16	4.0%	0.12
3	June 16	6.0%	0.15
4	June 16	12.0%	0.24
5	June 16	7.0%	0.21
1	June 29	8.0%	0.16
2	June 29	3.0%	0.13
3	June 29	1.0%	0.12
4	June 29	10.0%	0.22
5	June 29	3.0%	0.12
1	July 5	10.0%	0.18
2	July 5	8.0%	0.15
3	July 5	10.0%	0.18
4	July 5	1.0%	0.13
5	July 5	1.0%	0.13
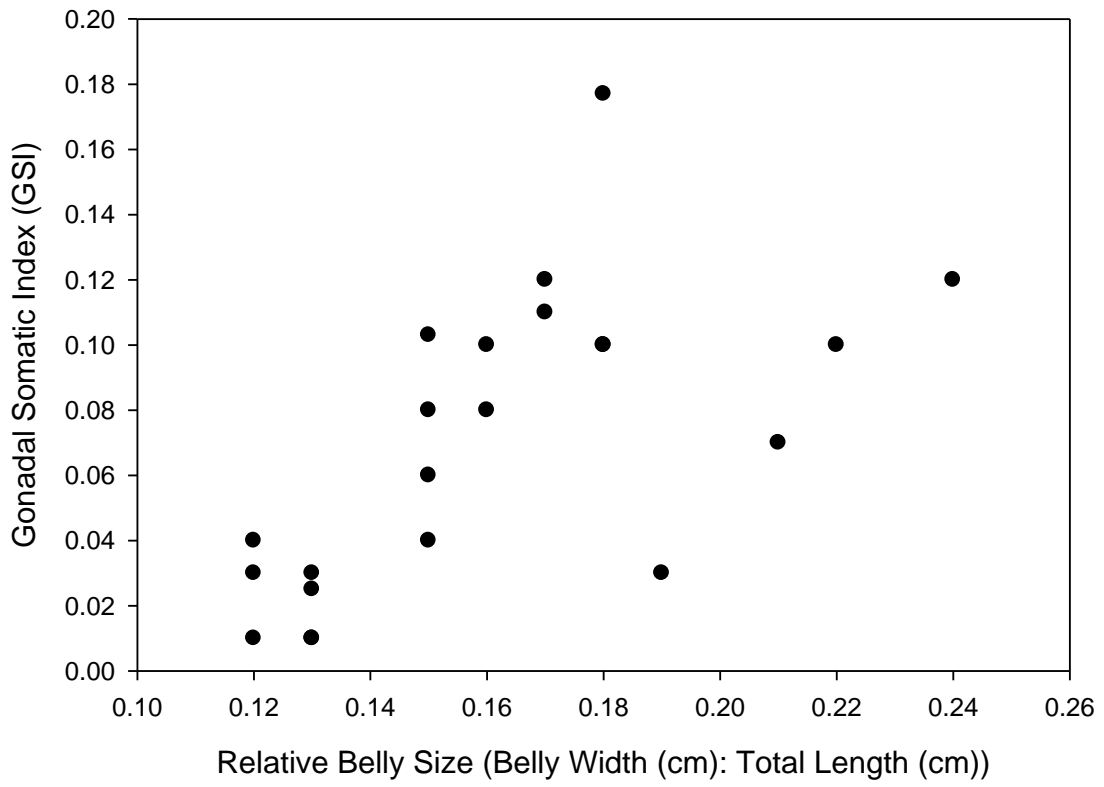
Appendix 3: Tissue dehydration in preparation for embedding in paraffin

Use 20 mL containers for 1 brain or 1 ovary

50% ethanol 1hour

95% ethanol 1hour

100% ethanol 1 hour

100% ethanol 1hour Can stop here overnight

Xylenes 30min x 2

1:1 xylenes:paraffin 1 hour (in oven) **Can stop here overnight**

Paraffin 1 hour x 3

Appendix 4: Cresyl violet staining procedure (used for brain after microtome sectioning)

70% ethanol 2min

95% ethanol 2min

100% ethanol 2min

Clearene/Histosol 2min

100% ethanol 2min

95% ethanol 2min

70% ethanol 2min

Distilled water wash x2

1% cresyl violet 15sec

Distilled water wash x2

Acetic acid solution (to desired level of staining)

Distilled water wash x2

70% ethanol 2min

95% ethanol 2min

100% ethanol 2min x2

100% ethanol 3min

Clearene/Histosol 2min x2

Clearene/Histosol 3min

Coverslip with Krystalon or Permount

Appendix 5: 4% Paraformaldehyde, PB and PBS

To make 1L of 4% PFA:

Heat 220mL of distilled water to $60^{\circ}C$

Add 20g of PFA powder, stir constantly

Stir for 10min under fumehood

Clear by adding drops of 1M NaOH

Add 500mL of 0.2M PB

405mL of Na_2HPO_4 (to make 0.2M, dissolve 28.4g Na_2HPO_4 into 1L of distilled water)

95mL of NaH_2PO_4 (to make 0.2M, dissolve 27.6g NaH_2PO_4 into 1L of distilled water)

Add 250mL of distilled water

To make PBS:

First make 0.2M PB

190 mL NaH_2PO_4

810 mL Na_2HPO_4

To make 0.1M PB add 1000mL of distilled water to 1000mL of 0.2PB

To make 0.1M PBS add to 1L of 0.1M PB

8.0g NaCl

0.2g KCl

Appendix 6: Biocytin protocol, solution not good for injecting small area precisely like auditory nerve, dye leaks into ventricles (Image is of telencephalon after Biocytin injection, labeling (seen as black areas) over entire telencephalon, demonstrates dye leakage).
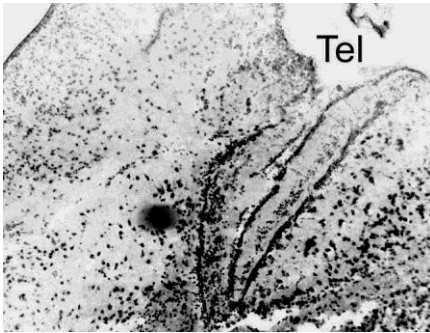
To make 4% biocytin solution:

8 µg biocytin powder

4 µL Triton X (need it to be 0.2%)

196 µL solution*

*When injecting into brain tissue, use teleost ringer's as the solution to dissolve biocytin powder

*If injecting into the nostril, use water as the solution to dissolve biocytin powder

Add fast blue to working biocytin solution to allow for visualization of the injection

To make biocytin crystal:

Add 4mg of biocytin powder to 100µL of distilled water, vortex solution in bullet tube

Add 1 drop of fast blue/green, place solution on glass slide and let evaporate in a dark place

Ex vivo process:

Incubate live/dye loaded tissue overnight in circulating, chilled ringers (12+ hours)

Fix tissue in 4% PFA for 24 hours

Put into 10% sucrose in until drops (10g sucrose in 100mL distilled water)

Put into 20% sucrose in until drops (same day as 10%) (20g sucrose in 100mL distilled water)

Put into 30% sucrose (next day) and leave overnight (30g sucrose in 100mL distilled water)

Cryosection

Avidin labelling:

Rehydrate tissue by placing slides in PBS for 10min

Place into streptavidin keepers for 24 hours @ 4°C or 4 hours at room temp

Rinse slides 3 x 10 min in PBS (in dark)

Coverslip with vectasheild (store slides in freezer in dark slide box)

Streptavidin keepr

12mL PBS

120µL Streptavidin Alexa 568 (Molecular Probes, Cat #S11226)

120 µL 10% Triton

120 µL of 5% Sodium Azide

Appendix 7: Ringer's solution

To make 500mL of Ringer's solution:

2.92g NaCl

0.09g KCl

0.11g $CaCl_2*H_2O$

0.01g $MgCl_2*6H_2O$

0.03g $NaH_2PO_4*H_2O$

pH needs to be 7.2

Appendix 8: NeuroTrace Protocol

Centrifuge solution before use for 5 min at 2000rpm to separate out DMSO. Keep

supernatant which contained NeuroTrace.

Rehydrate sections for 30min in 0.1M PBS, pH 7.2

PBS + 0.1% Triton X-100 for 10min (12mL of PBS in slide keeper, 12μL Triton)

PBS 5min x2

Dilute NeuroTrace in PBS. 1:100 (12mL PBS: 120μL NeuroTrace)

Apply approx. 200μL of diluted stain to slide for 20 min

Remove stain, wash for 10min in PBS + 0.1% Triton X-100

PBS 5min x2

PBS for 2 hours at room temp or overnight at 4°C

Coverslip with vectashield

Appendix 9: Hematoxylin/Eosin stain (used for goby ovaries after sectioning with microtome). Image shown is of a NRF goby ovary stained with Hematoxylin/Eosin stain.
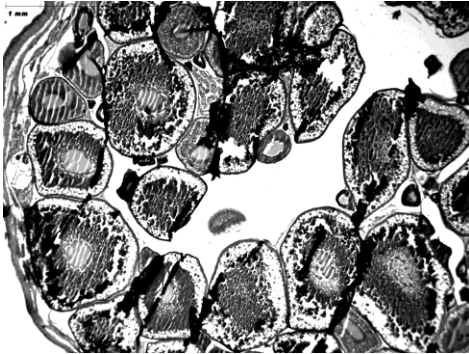
Xylenes 3min

100% ethanol 2min

95% ethanol 2min

70% ethanol 2min

Distilled water 5min

Harris' Hematoxylin 3min

Distilled water quick rinse

Tap water (running) 5min

Acid alcohol to desired level of staining (2mL glacial acetic acid + 98mL distilled water)

Tap water running 2min

Distilled water 1min

Eosin 1min (0.2% eosin in 95% ethanol = 2g/L of ethanol)

100% ethanol 2min

Xylenes 2 x 2min

Coverslip with Permount

Appendix 10: Z-series images of fibers ending in contralateral TS. All micrometer bars are 20μm. Images shown are from an Auditory nerve injection of Alexa Fluor® dextran 488, incubation time of 5 hours, tissue was cryosectioned horizontally in 25μm thick sections. White circles show fiber visible in panel 1 but not visible by panel 12 of 13; this demonstrates fiber ending in TS.

Panel 1 of 13
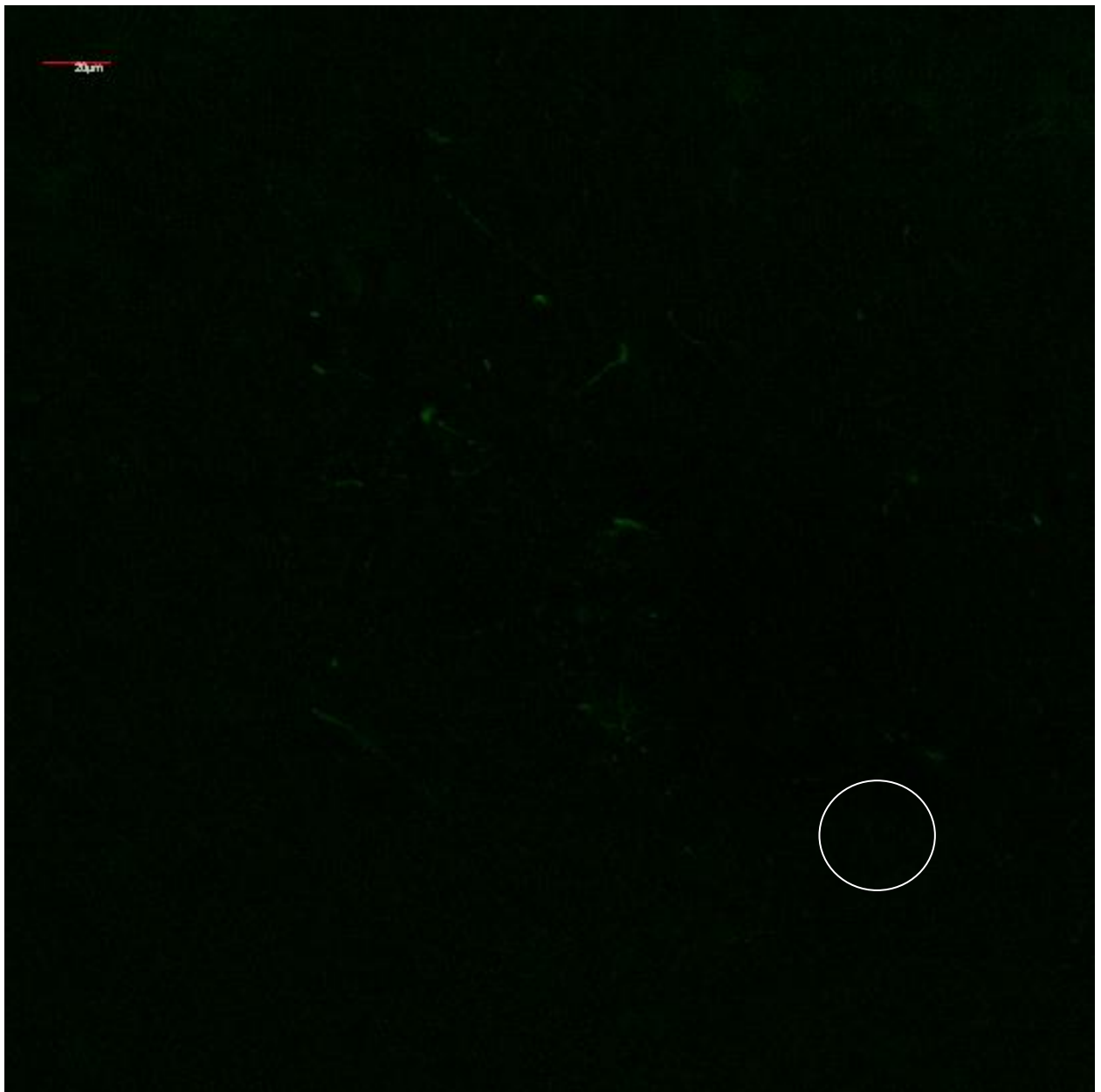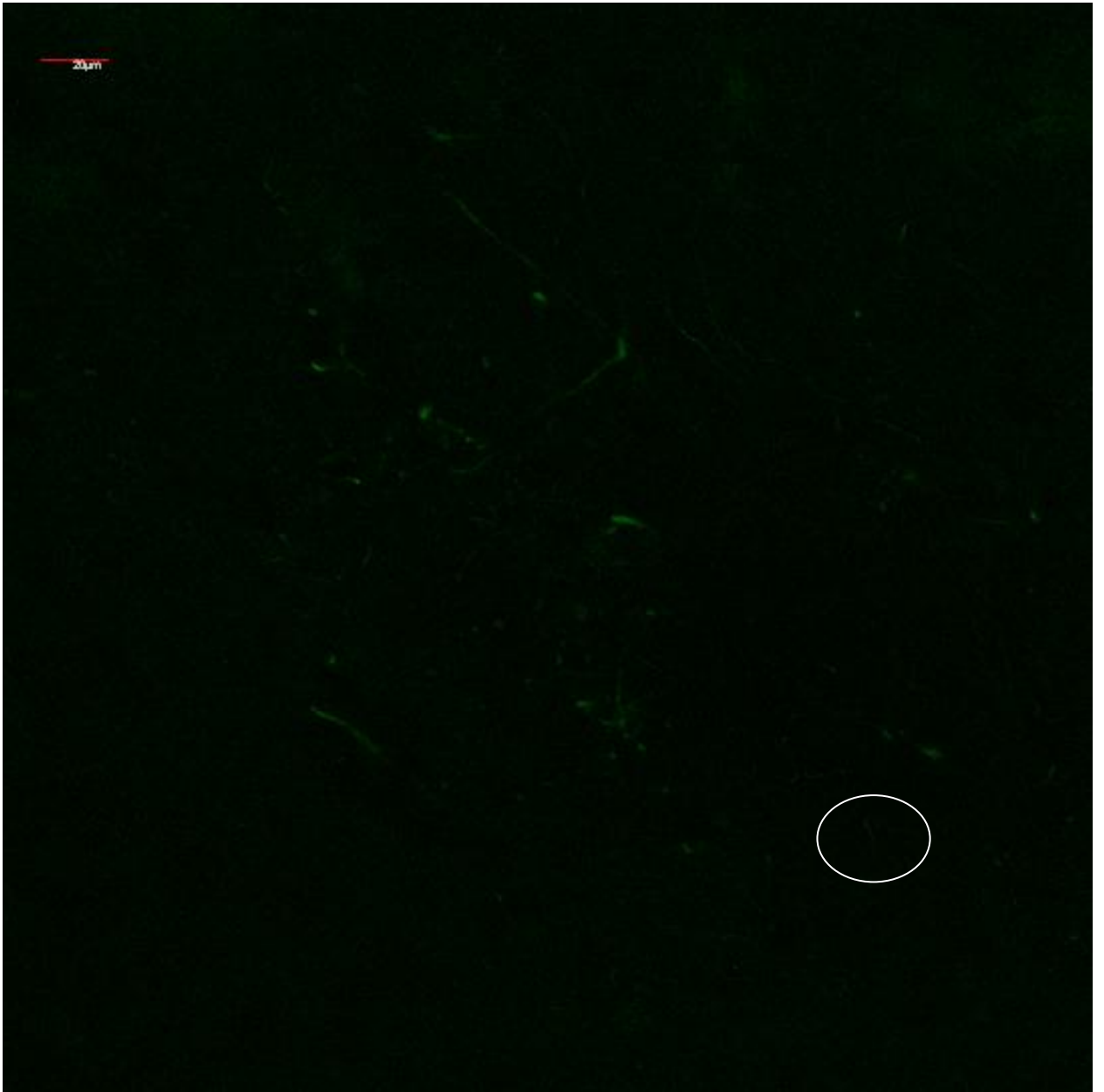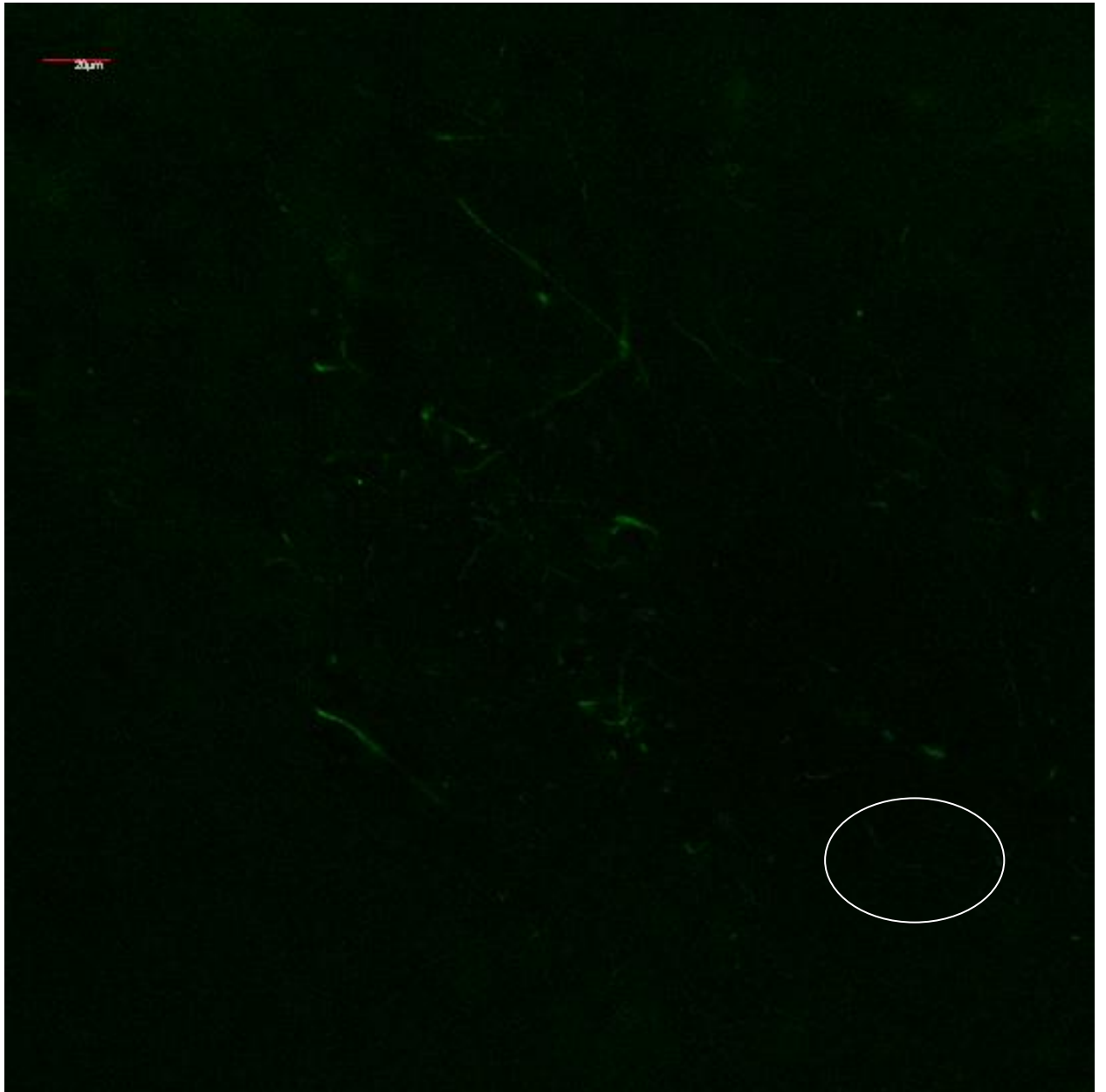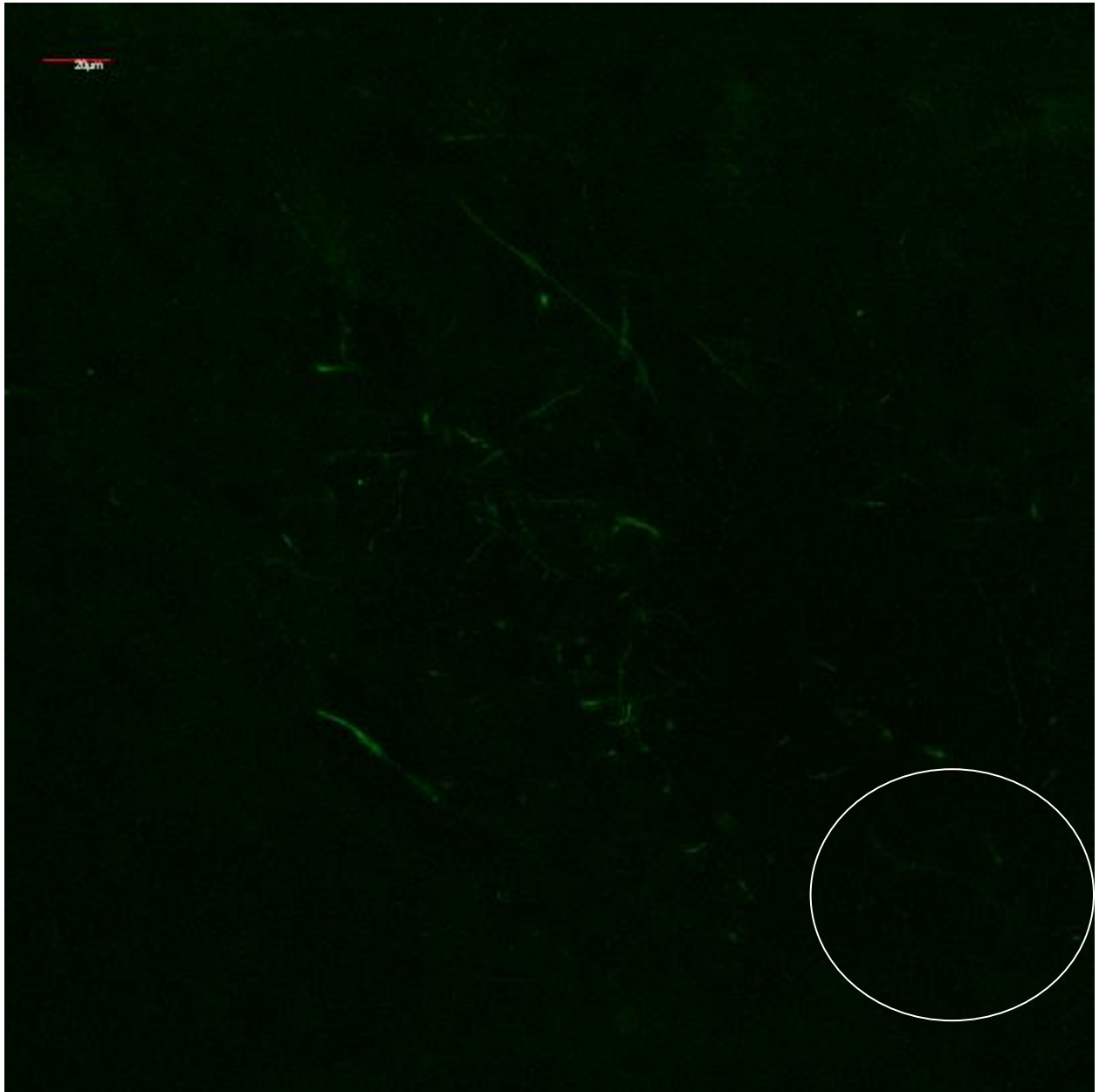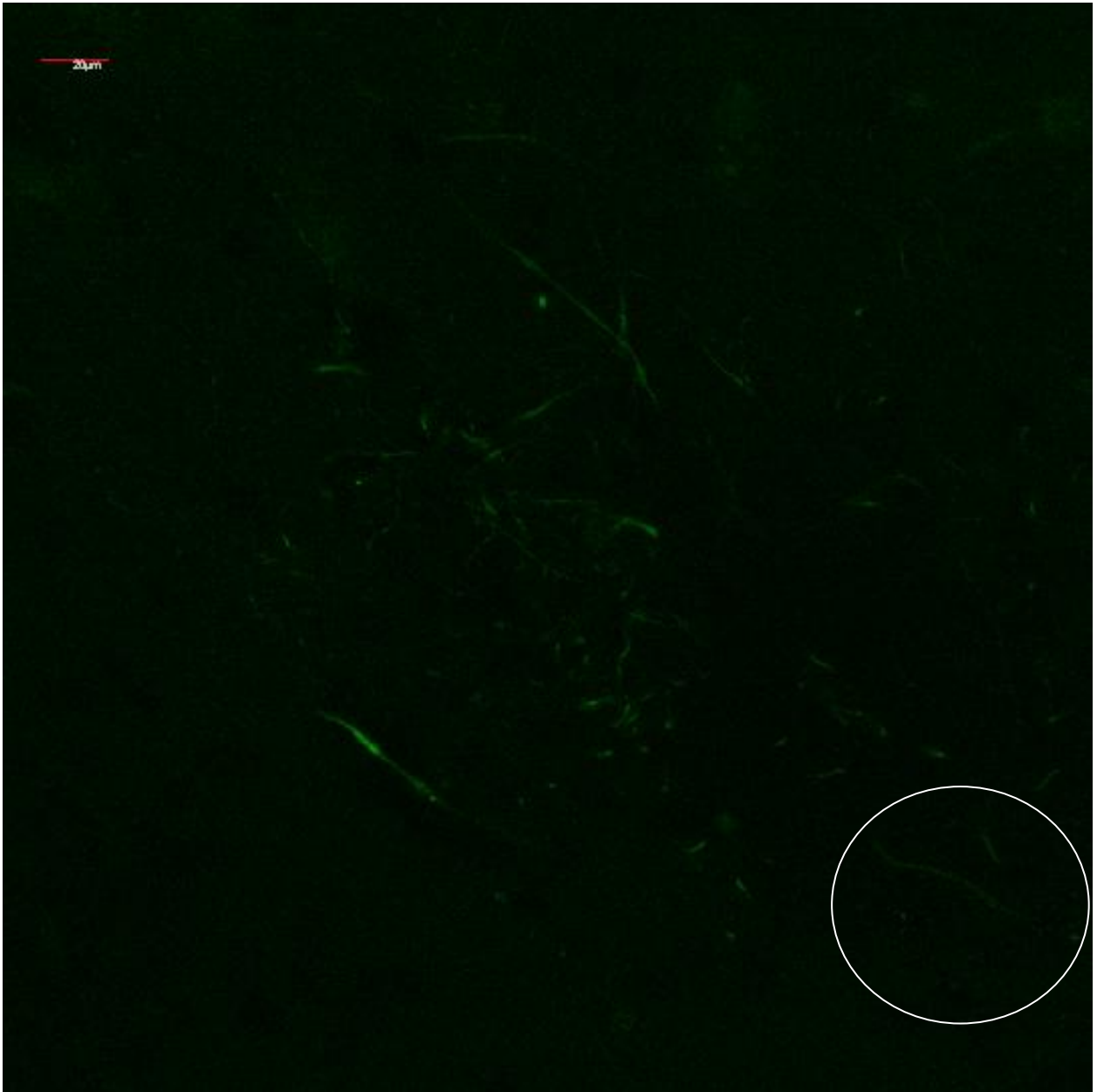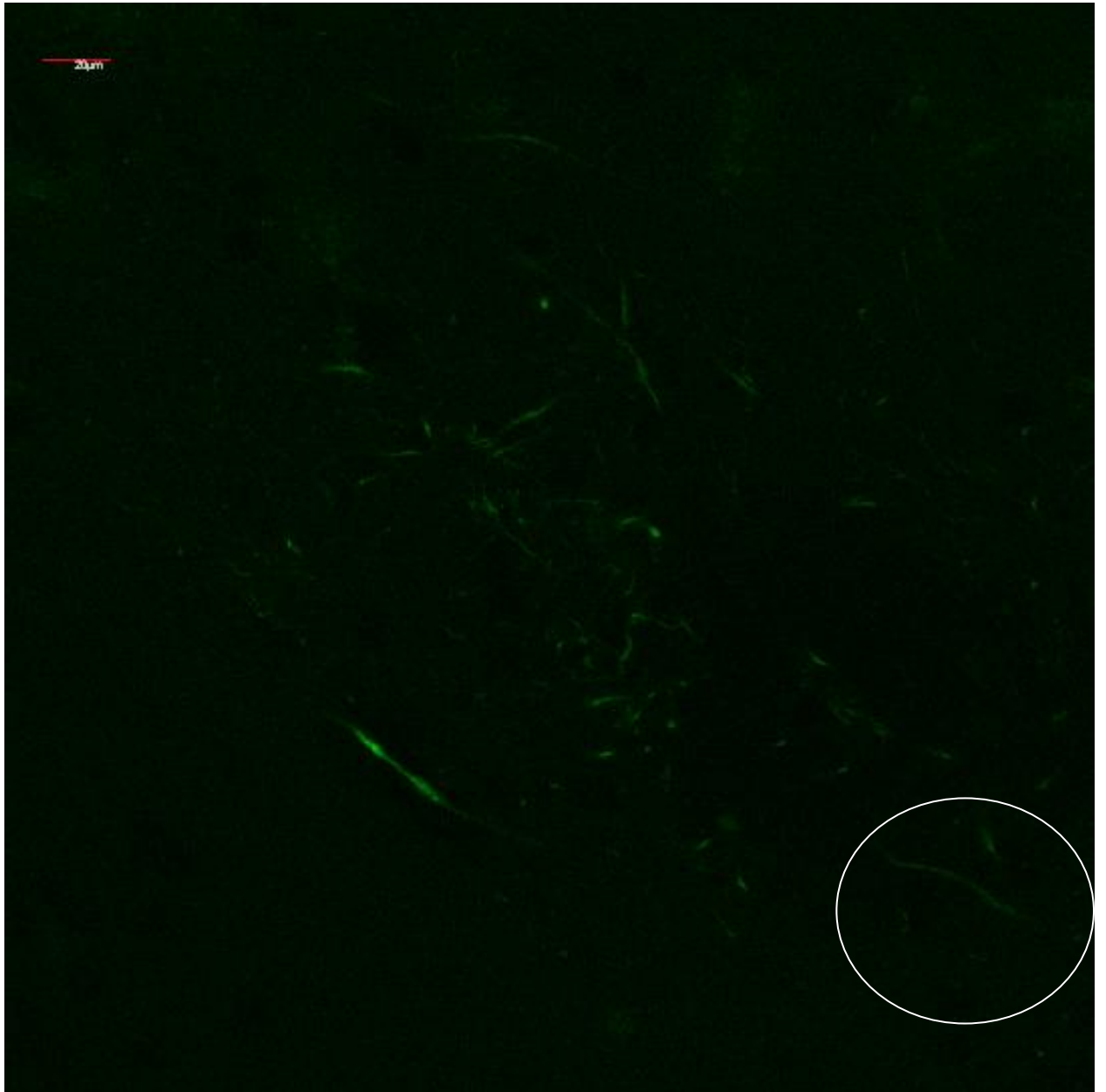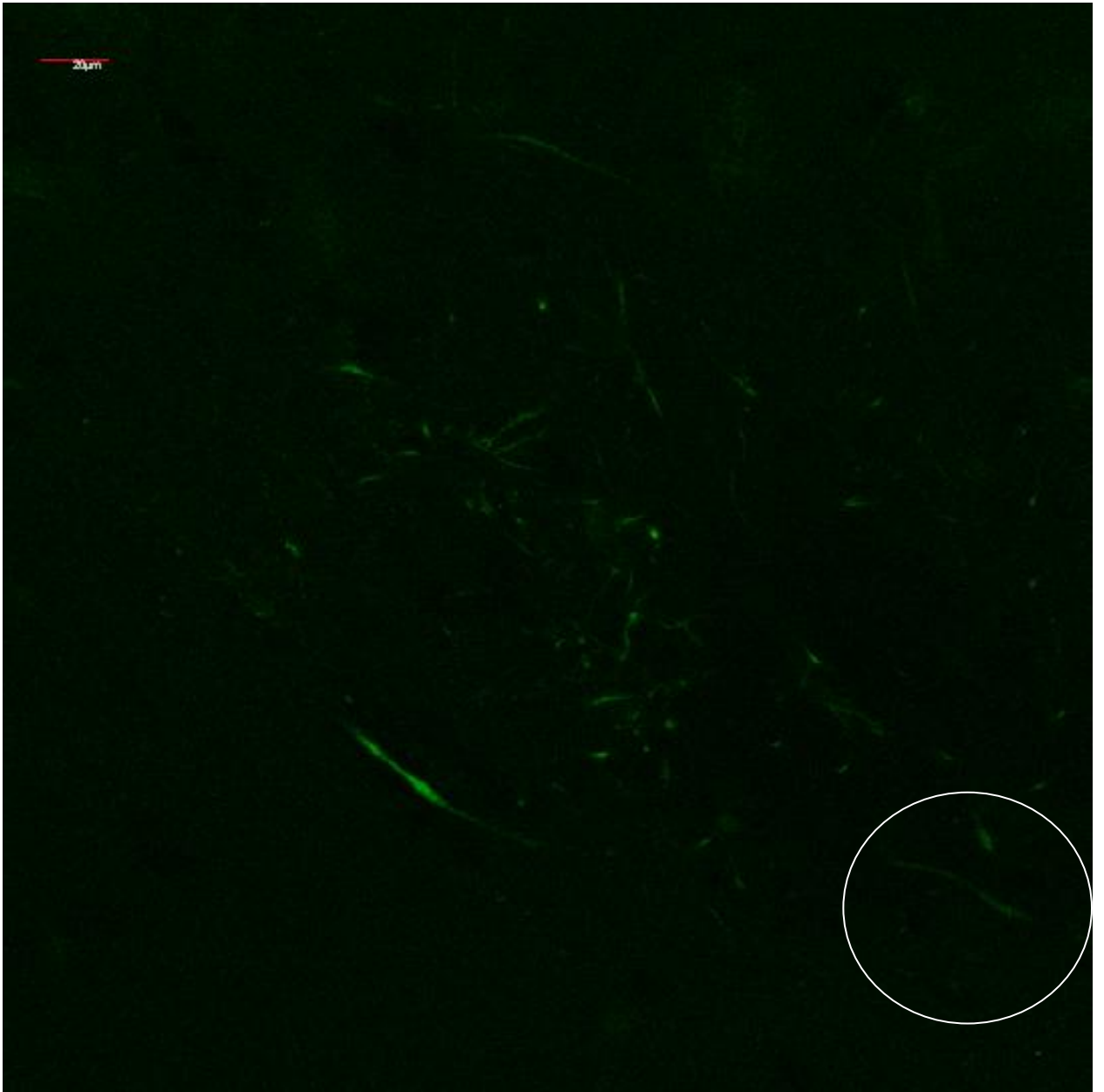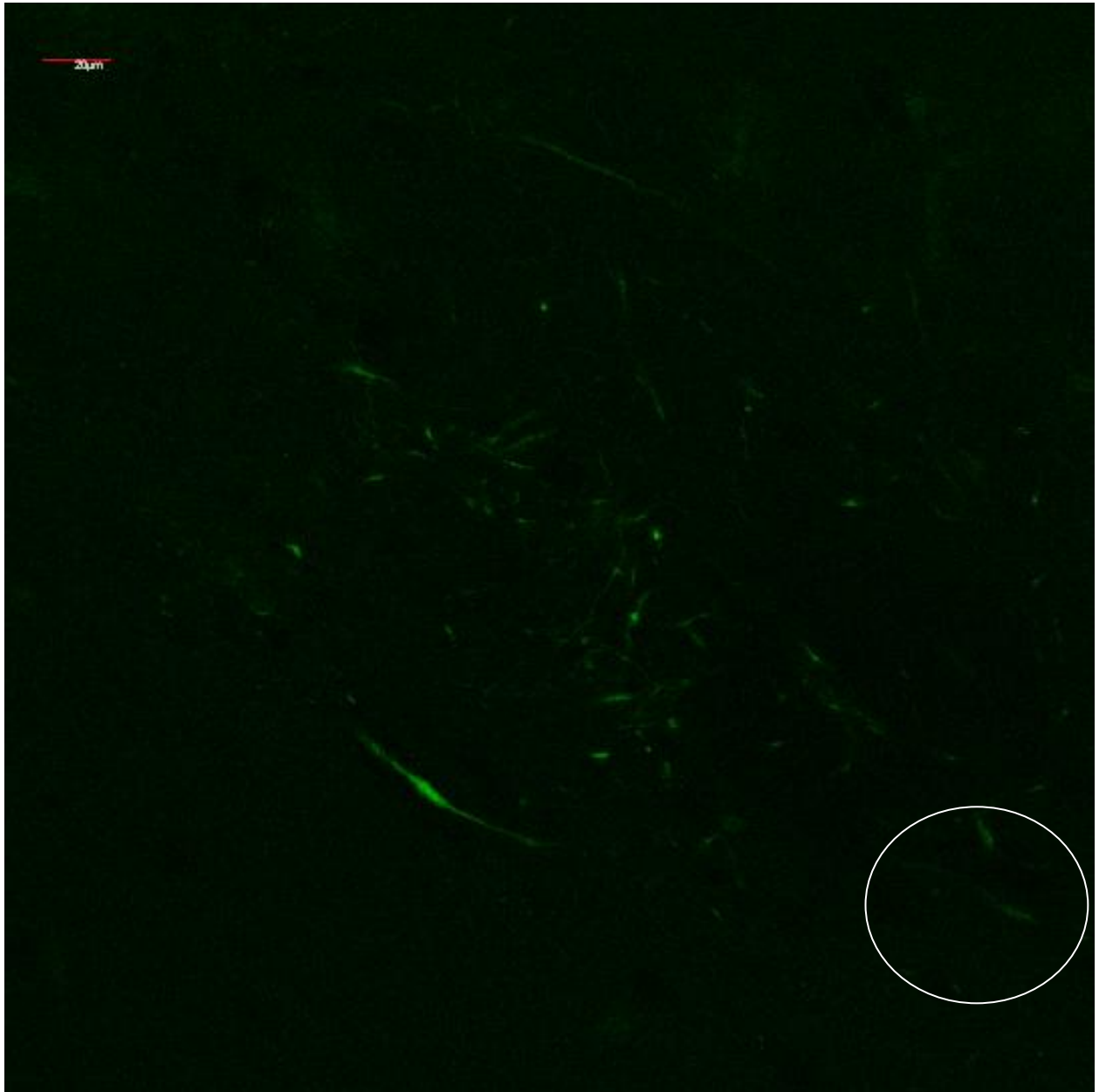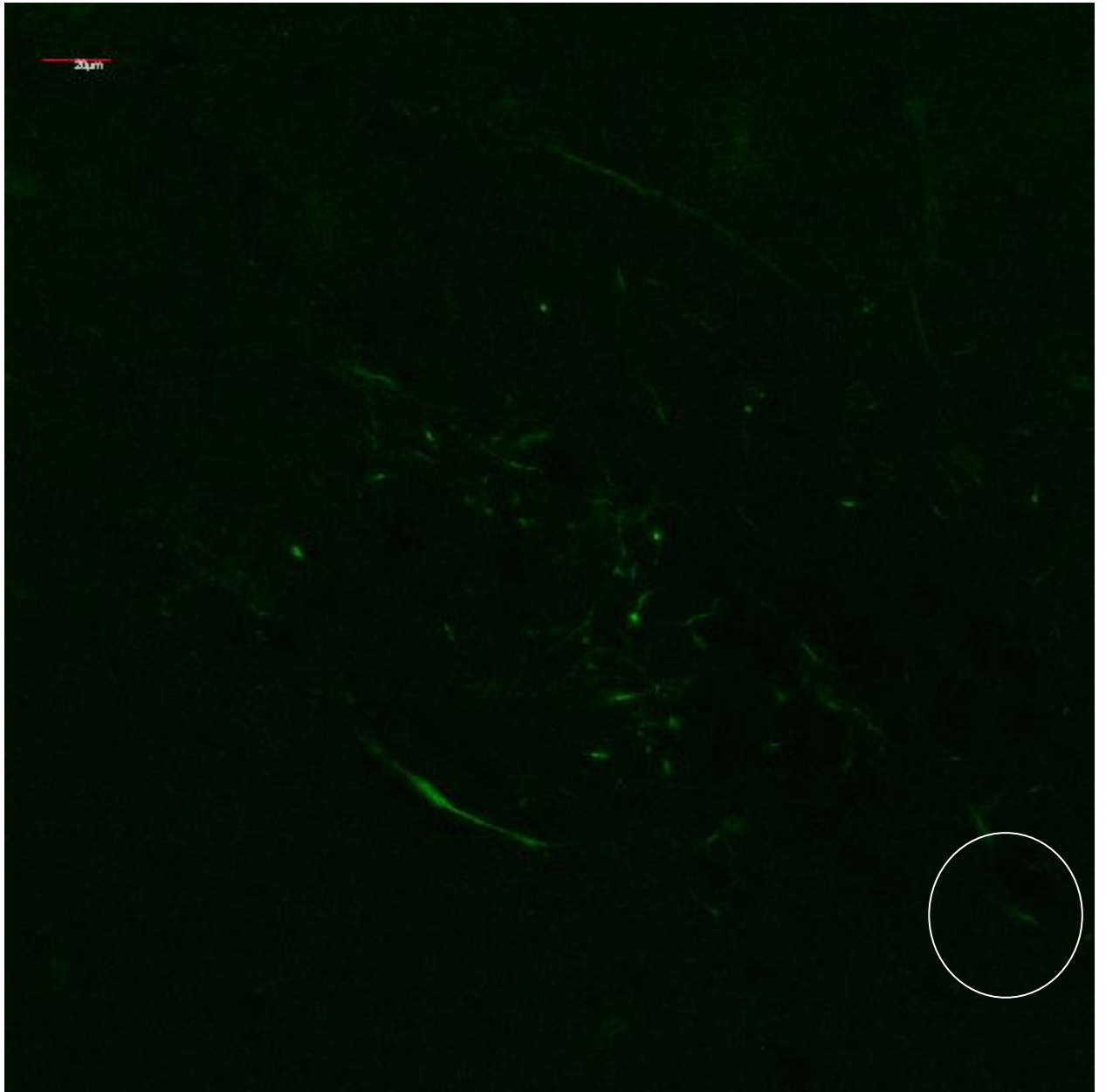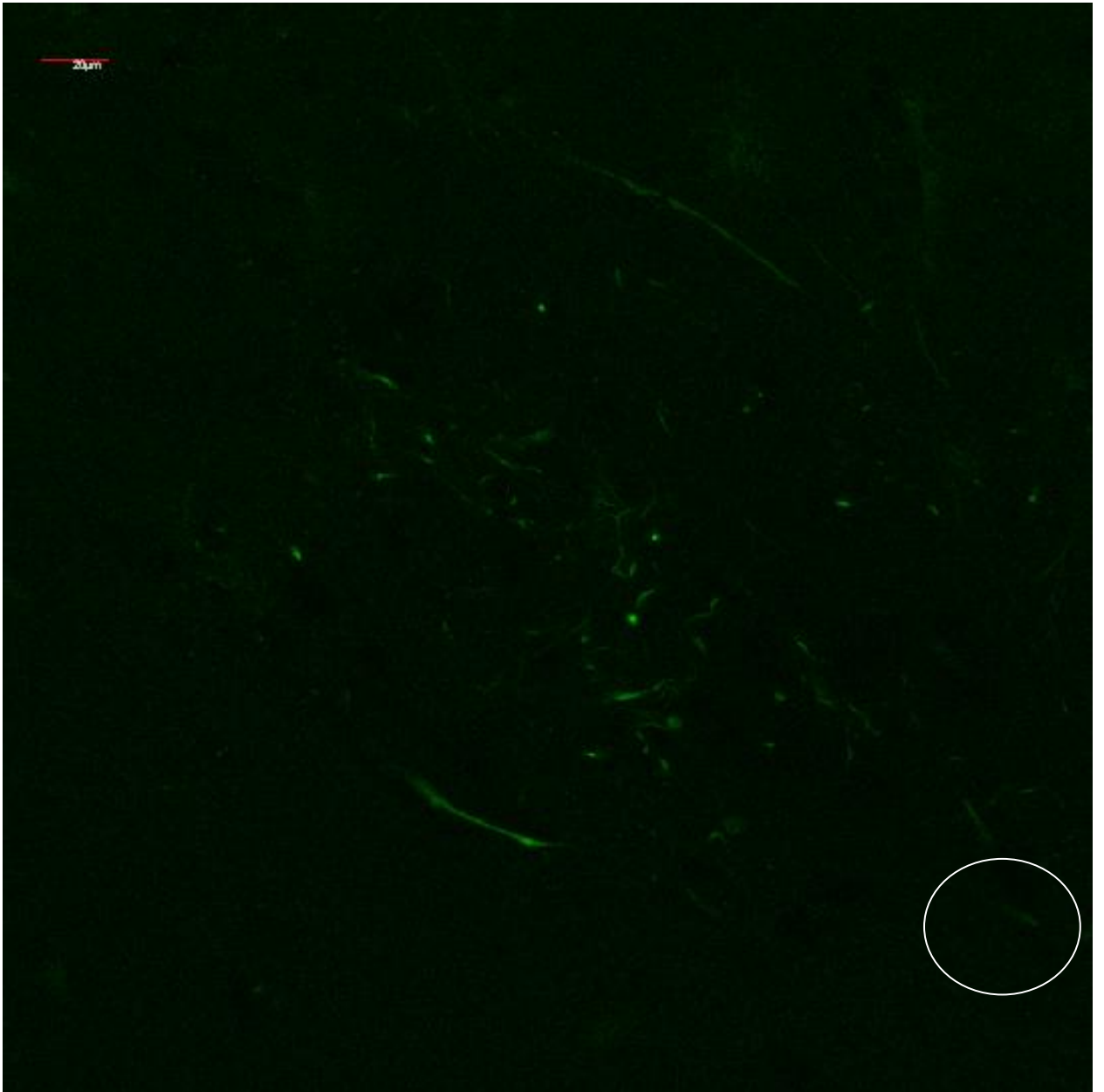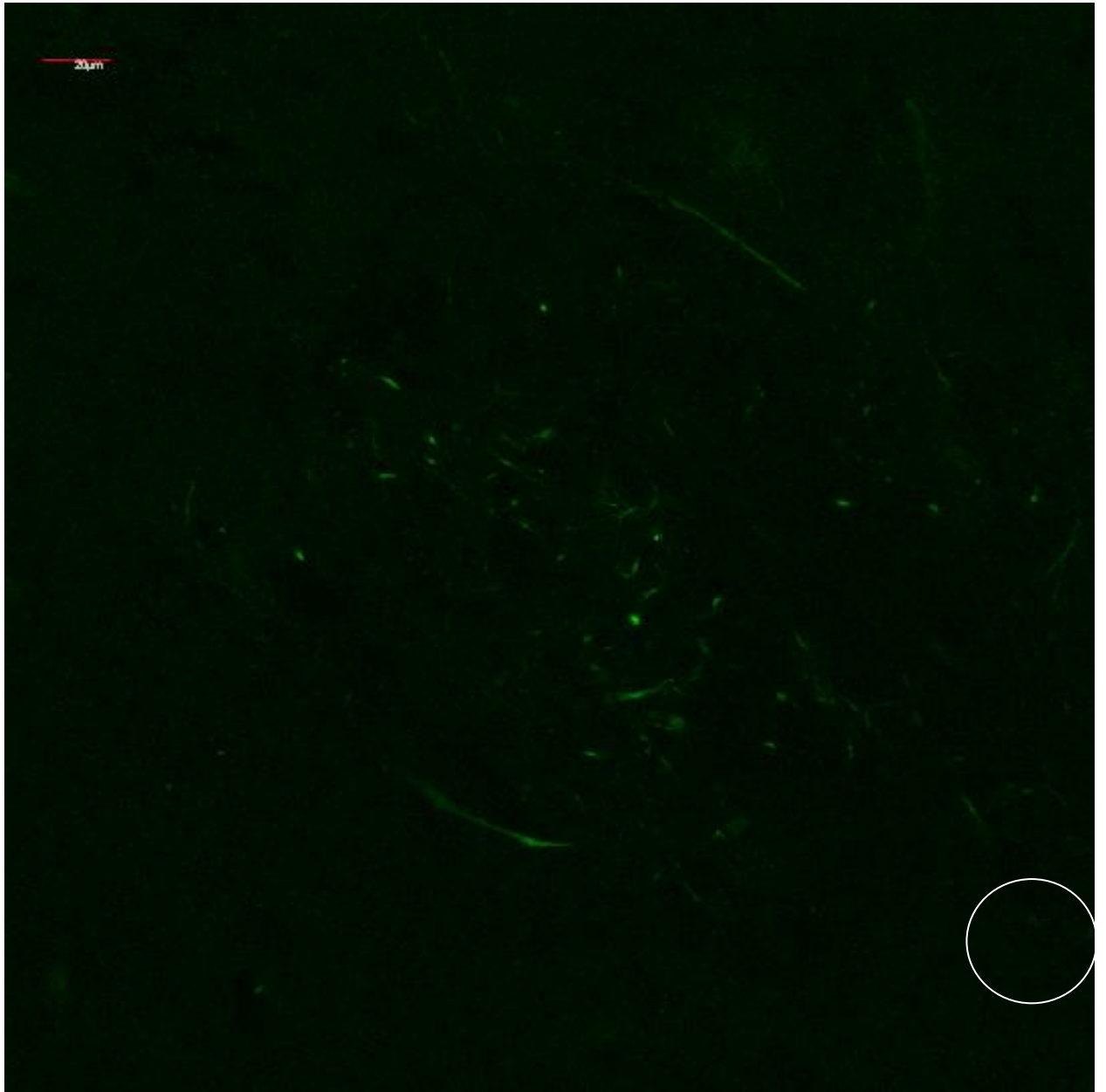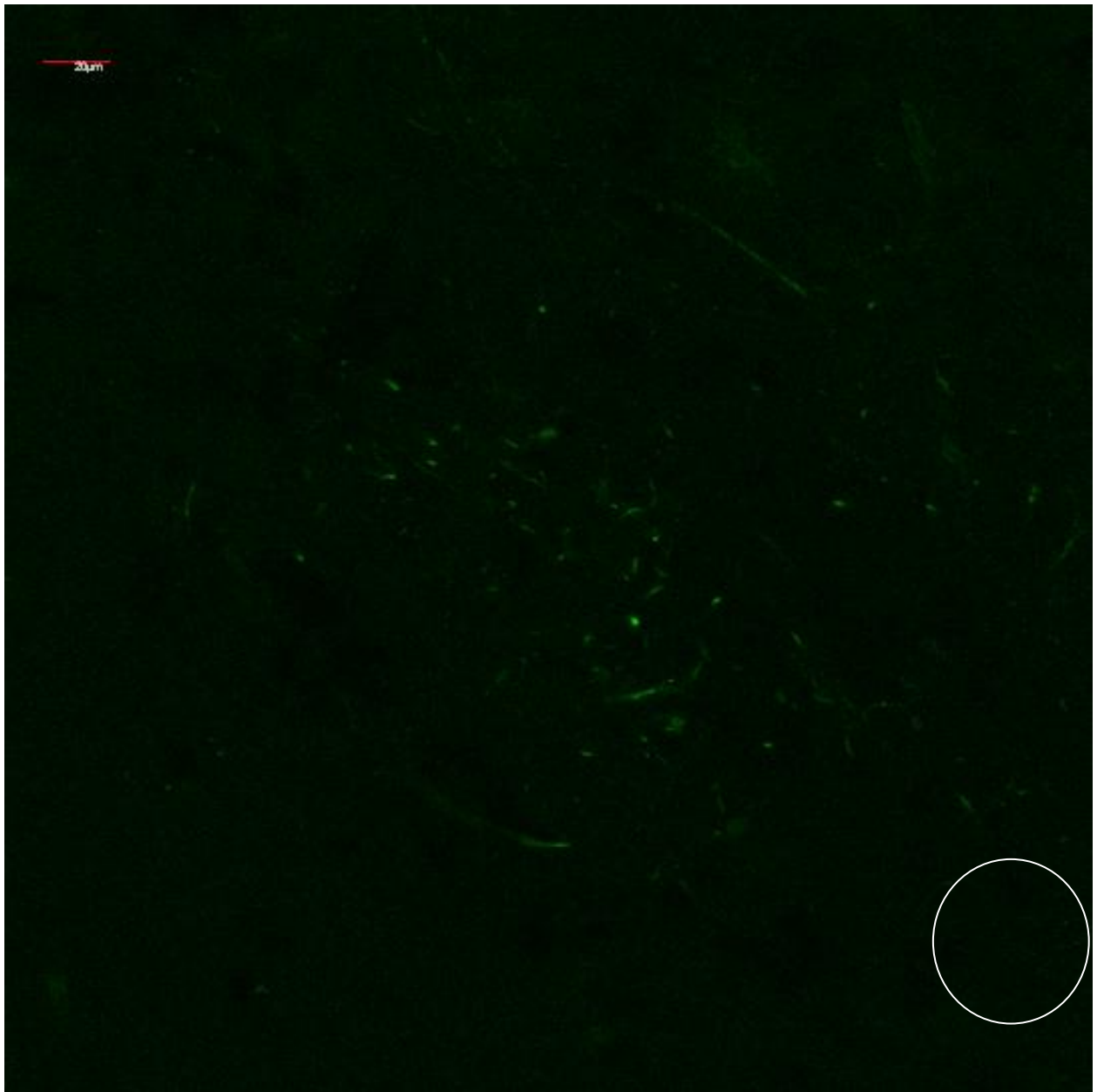
Appendix 11: Z-series images of fiber leading to cell body in ipsilateral TS. All micrometer bars are 20µm. Images shown are from an Auditory nerve injection of Alexa Fluor® dextran 488, incubation time of 5 hours, tissue was cryosectioned horizontally in 25µm thick sections. White circles show fiber visible in panel 7 and ending in a cell body in panels 12 and 13 of 13; this demonstrates fiber ending in TS.

Panel 7 of 13
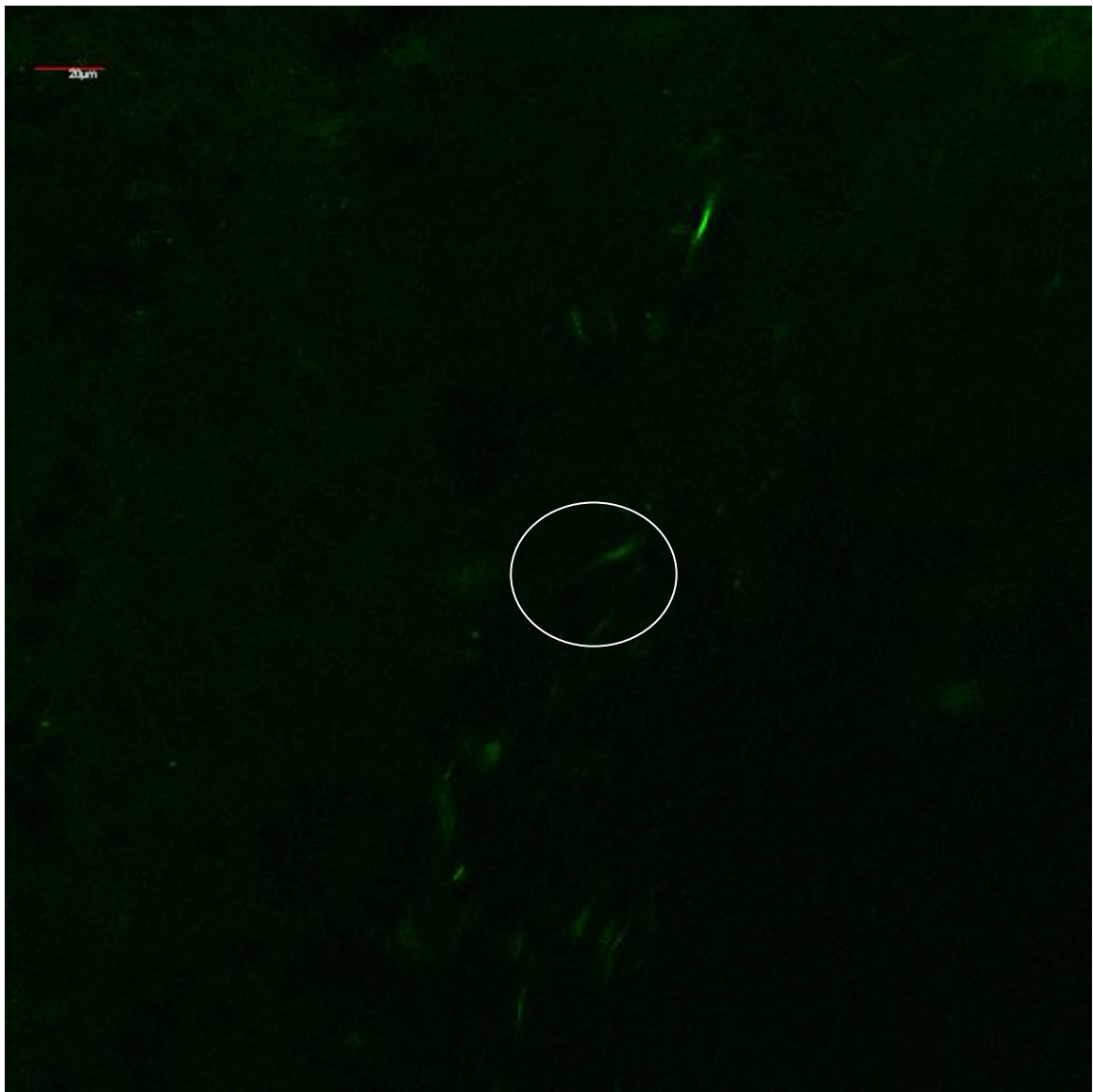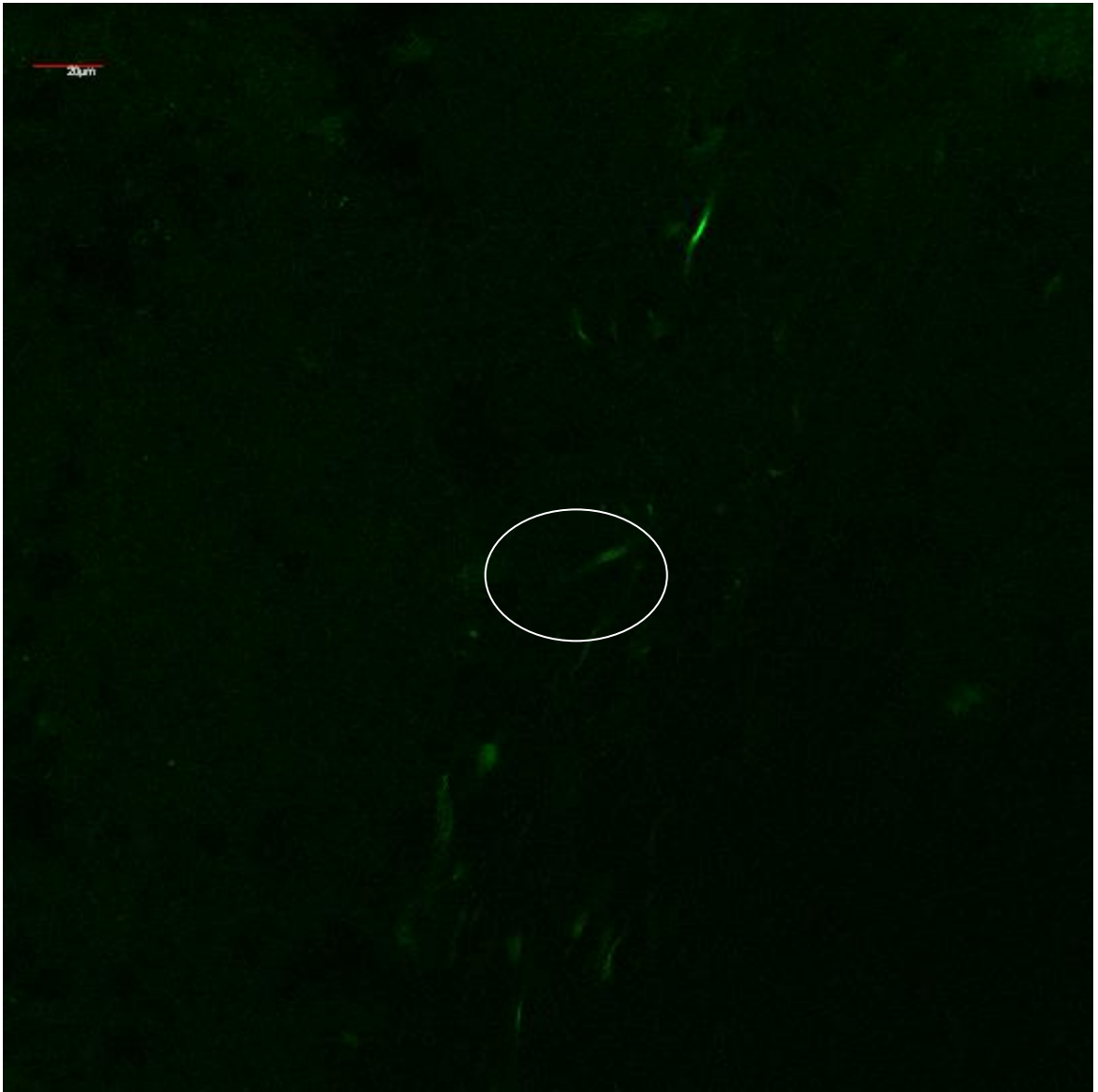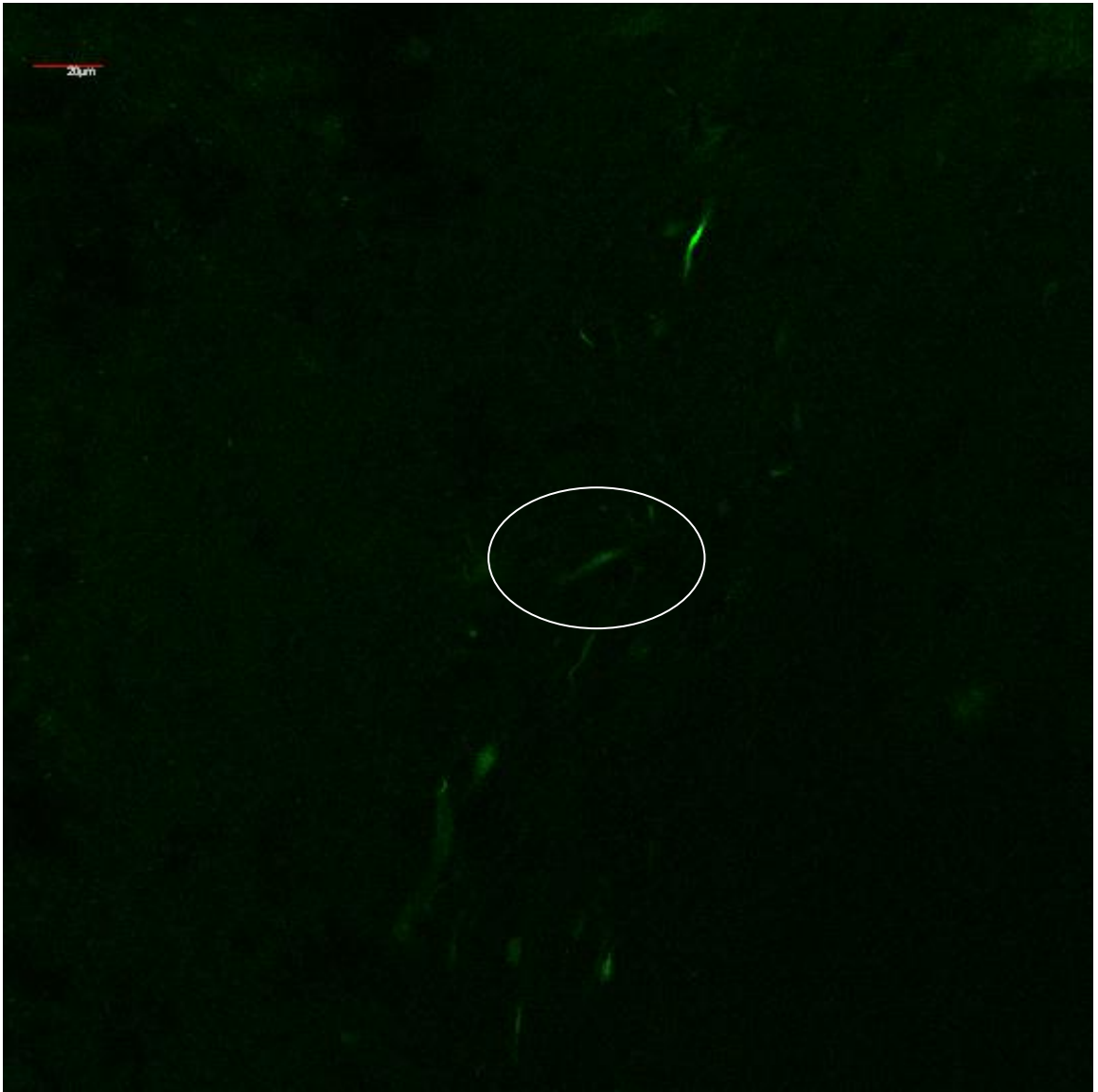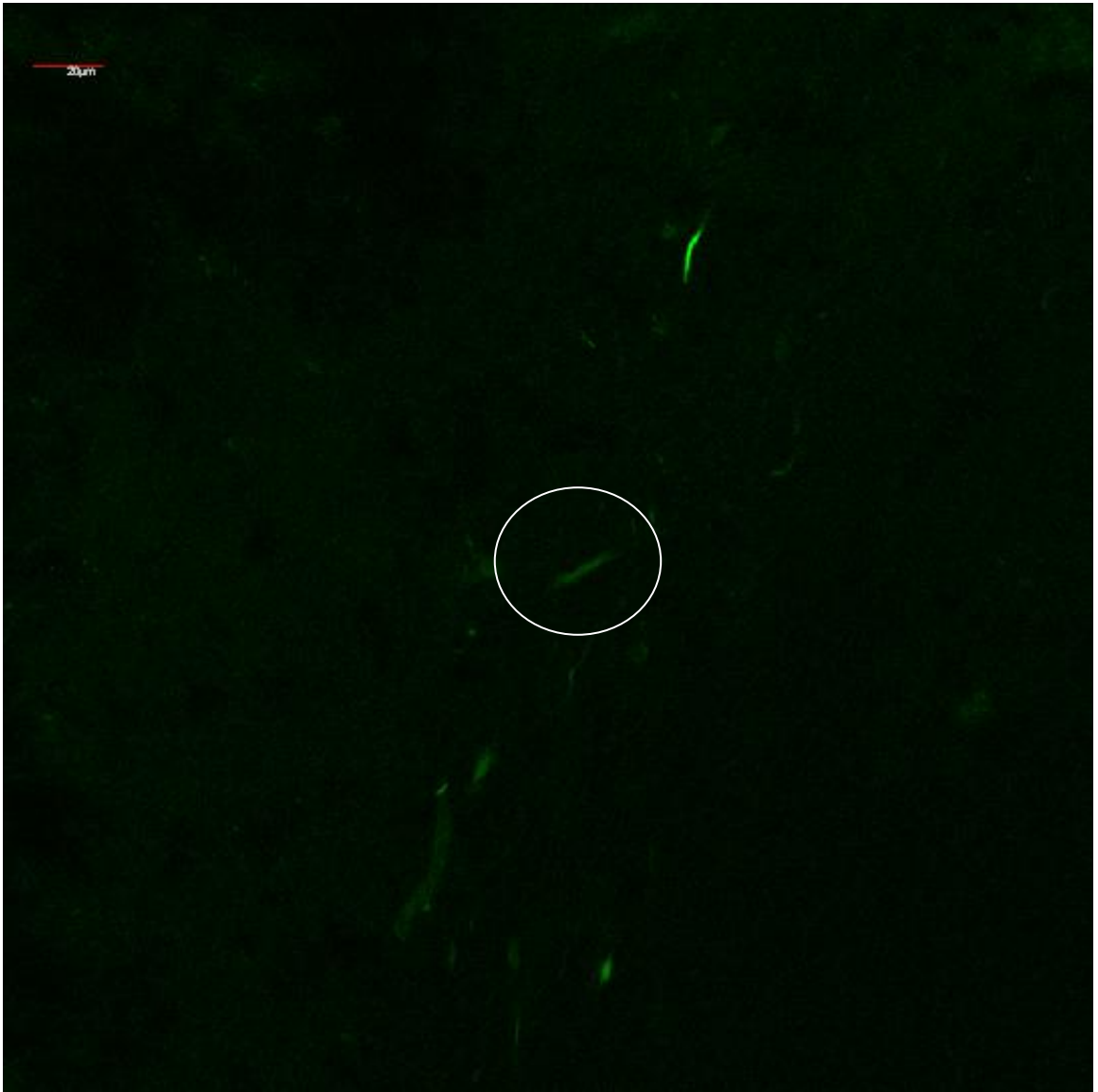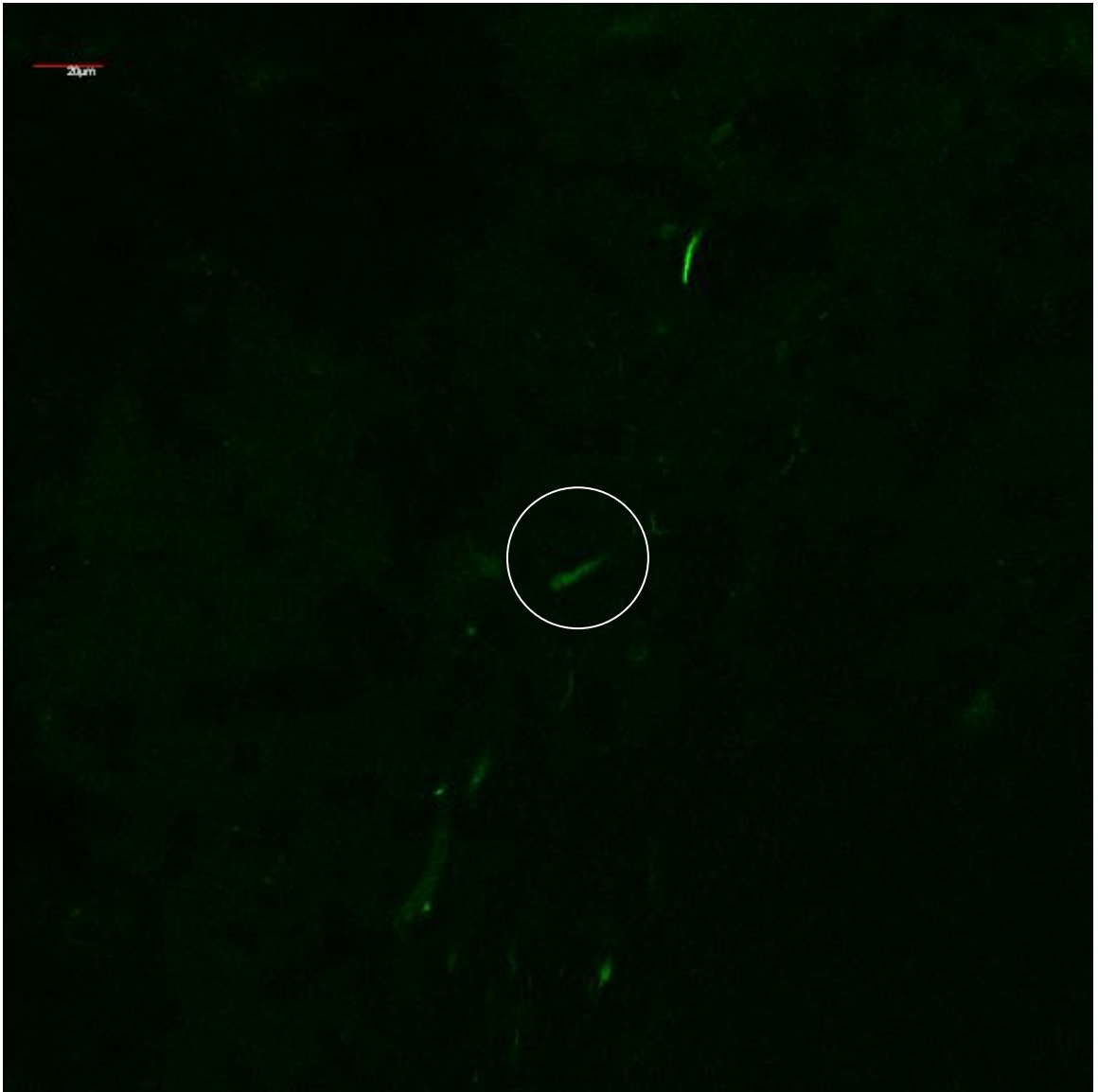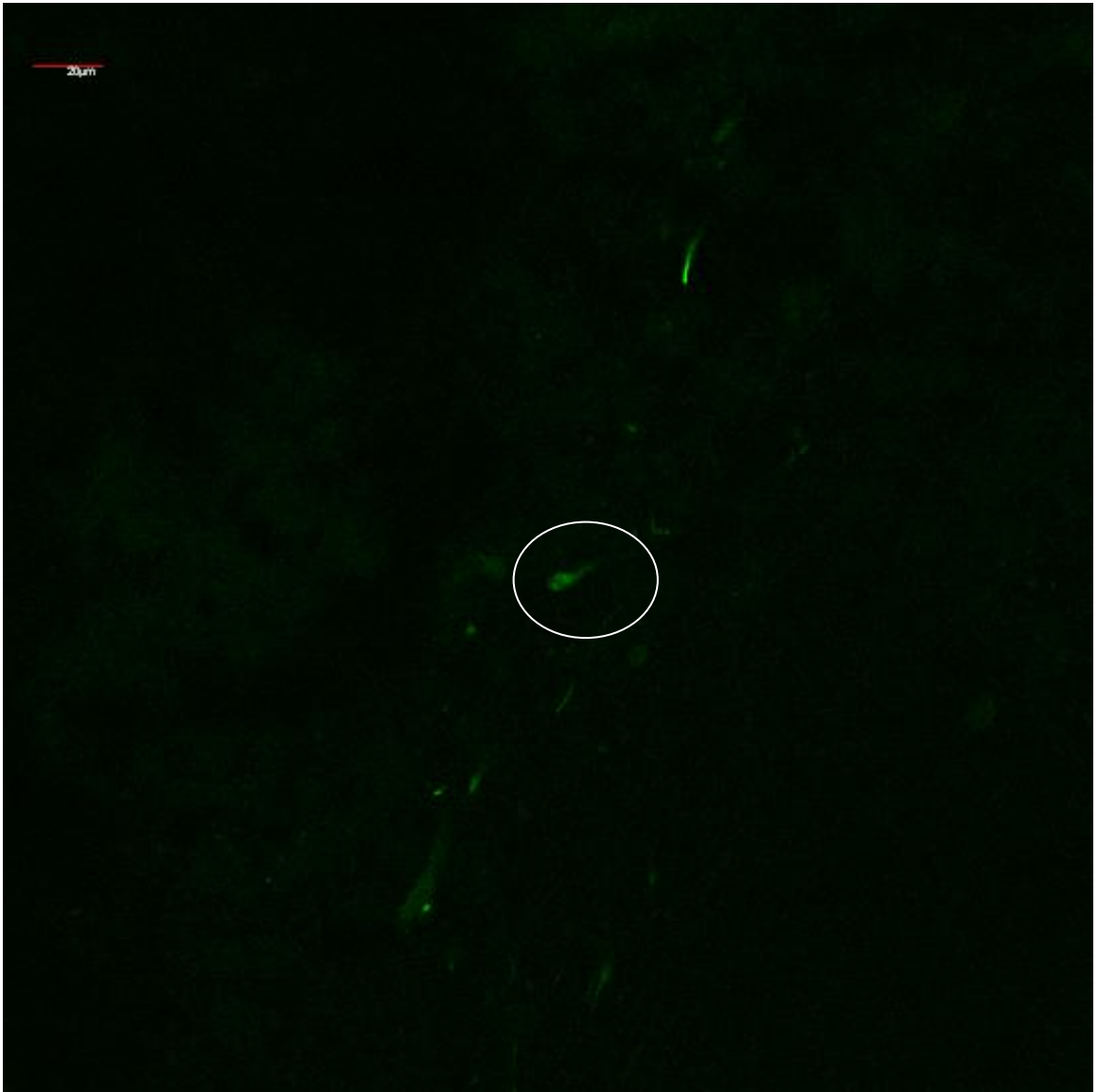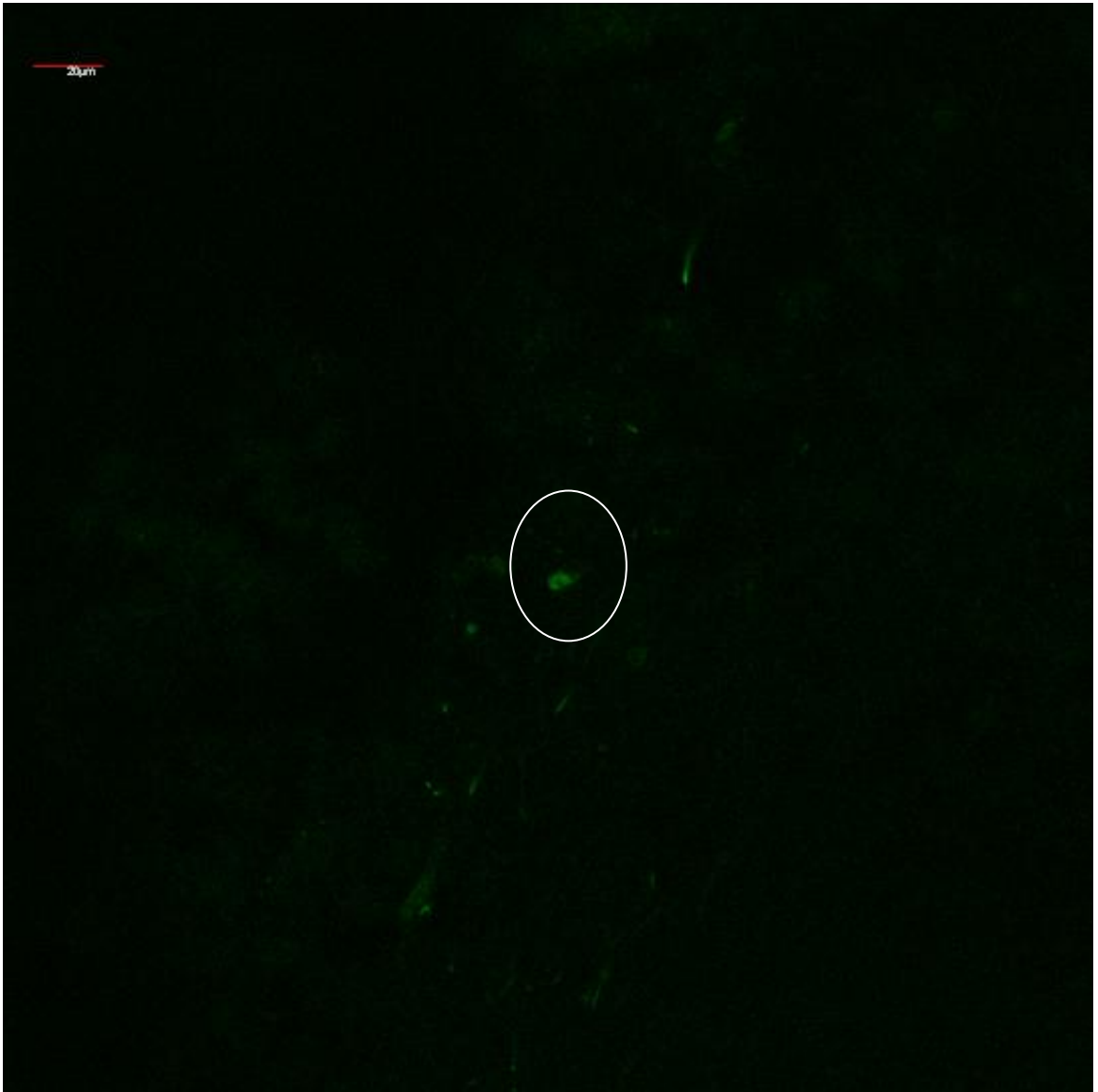
Appendix 12: Histological Atlas of round goby brain, *Neogobius melanostomus*. Brain was microtomed, cross sections are 10 μm thick, stained with cresyl violet.

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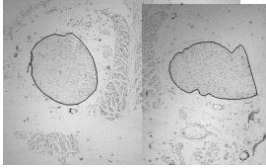
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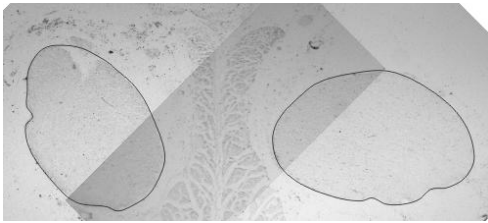
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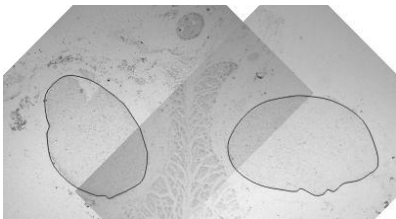
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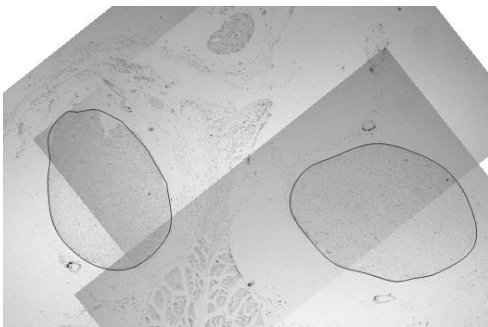
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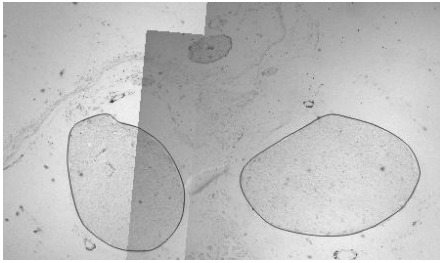
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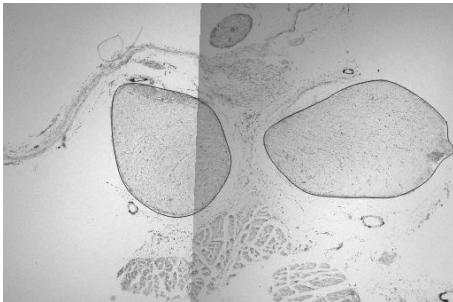
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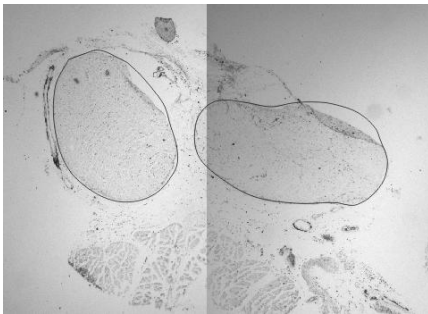
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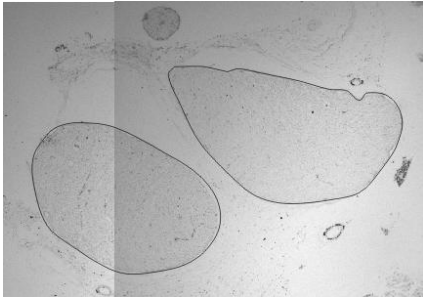
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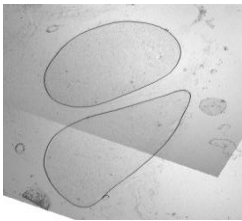
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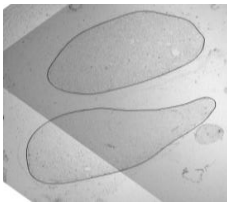
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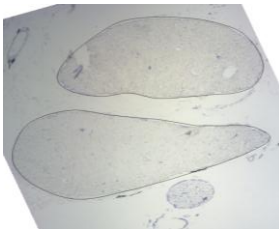
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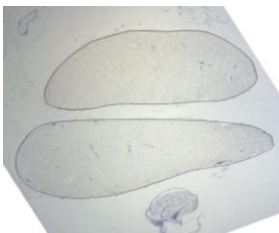
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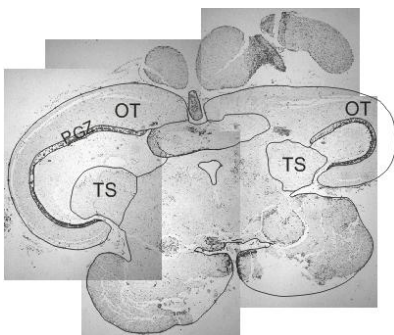

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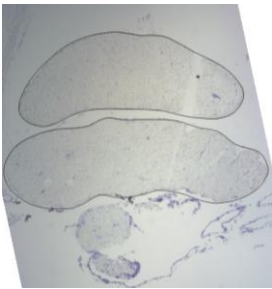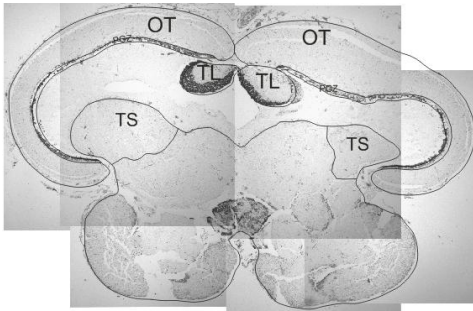

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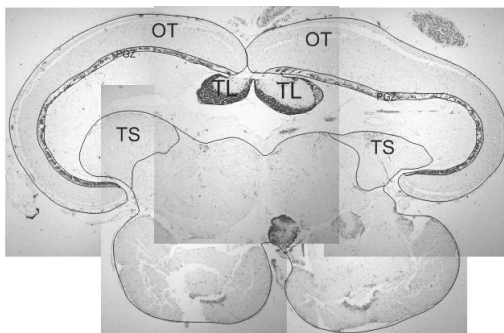

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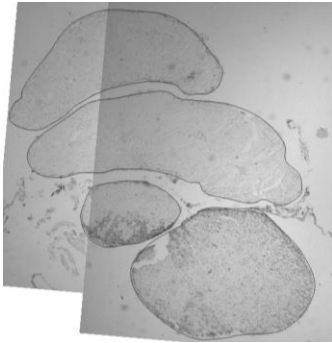
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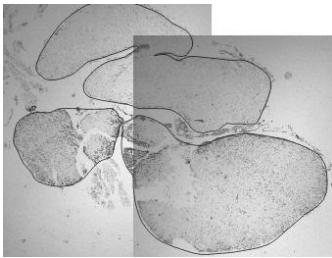
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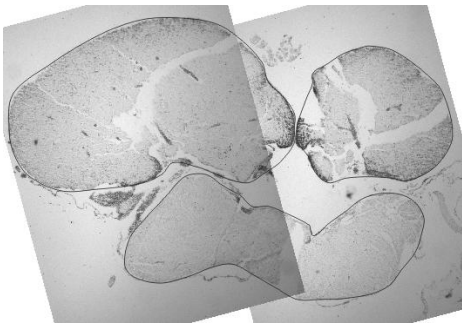
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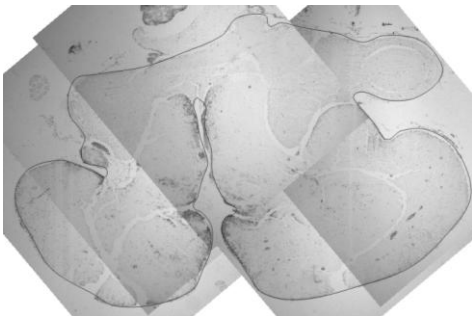
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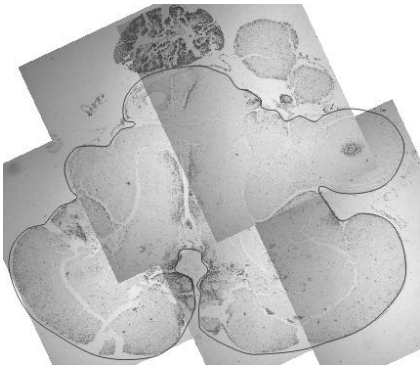
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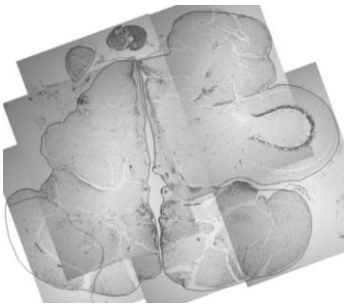
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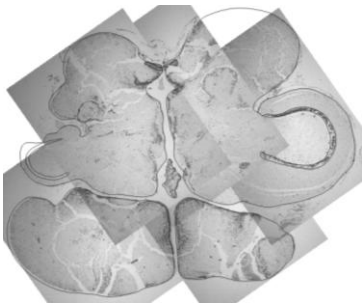
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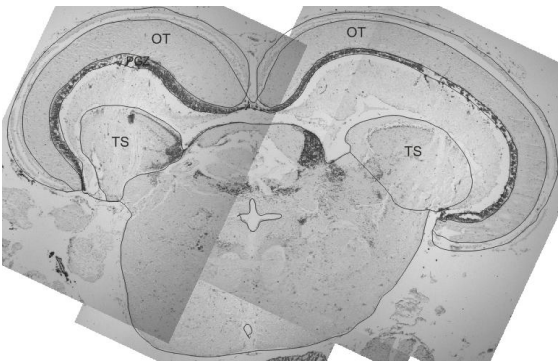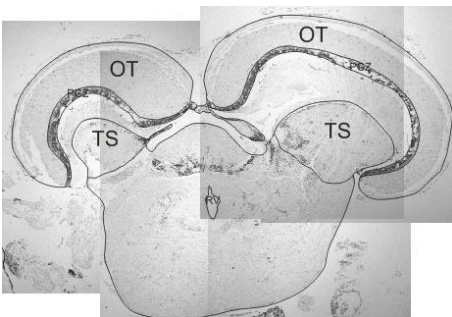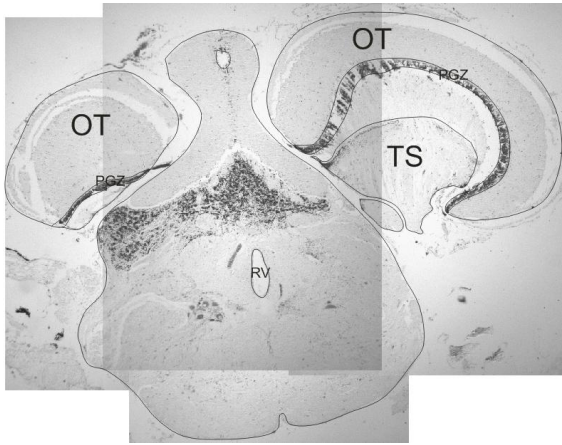
Slide 46

Slide 47

Slide 48

Slide 49

Slide 50

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