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Spatial analysis of Fos-like immunoreactivity, during amino acid chemoreception in the brain of the zebrafish, Danio rerio

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Spatial Analysis of Fos-like Immunoreactivity, during Amino Acid Chemoreception in the Brain of the Zebrafish, *Danio rerio*

By

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Abstract

Spatial analysis of neuronal activation by assessing the localization of proteins upon an external stimulus has been well established. In particular, analysis of the expression of c-Fos protein has been widely used for investigating pathways of neural activity. In the current study, this approach was expanded to zebrafish by assessing Fos-like protein immunolocalization in the brain structures known to integrate olfactory sensory information, and to neural pathways that may link olfaction to locomotor control. The Fos-like protein was visualized in zebrafish brain tissue by western blot (sc-253) following amino acid exposure, and we found that it produced two bands at 64 and 77 kDa. These molecular weights were very similar to those seen in a previous study which probed for c-Fos protein in zebrafish tissue. We hypothesized that during exposure to amino acid odours, Fos-like immunoreactivity would be localized in brain regions that integrate olfactory sensory information, locomotor control centers, gustatory centers, and in neural pathways that link these regions. An increase in Fos-like immunoreactive cells was found in the olfactory bulb, and the lateral and posterior zones of the dorsal telencephalon. Both these telencephalic areas are target regions of the olfactory tracts. We did not find any significant changes in Fos-like immunoreactivity in the midbrain and hindbrain regions that we investigated. Some of these regions are responsible for locomotion and gustation, and therefore we did not find any integration of these sensory systems with the olfactory system using a Fos-like protein marker. This study is the first to apply Fos-like immunolocalization in zebrafish brain, and provides the potential for further applications of this tool for identifying neural activation during various sensory stimuli.

This thesis is dedicated to all the wonderful people in my life – Amarjit Bhogal, Sukhwinder Bhogal, Rupinder Bhogal, Dawinder Bhogal, and Jasdeep Rai – without whom I would not be who I am. I love you all.

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List of Abbreviations

AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid
AP-1	Activator protein-1
CC	Crista cerebellaris
cOSN	Ciliated olfactory sensory neurons
CP	Central posterior thalamic nucleus
Dc	Central zone of dorsal telencephalon
Dd	Dorsal zone of dorsal telencephalon
Dl	Lateral zone of dorsal telencephalon
Dm	Medial zone of dorsal telencephalon
DON	Descending octaval nucleus
Dp	Posterior zone of dorsal telencephalon
ERK	Extracellular signal-regulated kinase
FLi	Fos-like immunoreactive
FLI	Fos-like immunoreactivity
FLP	Fos-like protein
Hv	Ventral zone of periventricular hypothalamus
ICC	Immunocytochemistry
IMRF	Intermediate reticular formation
IRF	Inferior reticular formation
lMOT	Lateral medial olfactory tract
LOT	Lateral olfactory tract
LTP	Long term potentiation
MaON	Magnocellular octaval nucleus
MON	Medial octavolateralis nucleus
mOSN	Microvillous olfactory sensory neurons
MOT	Medial olfactory tract
NIII	Oculomotor nucleus
NIV	Trochlear nucleus
NMLF	Nucleus of medial longitudinal fascicle
NMDA	N-methyl-D-aspartate
OSN	Olfactory sensory neuron
PPv	Periventricular pretectal nucleus, ventral part
PTN	Posterior tuberal nucleus
PTZ	Pentylenetetrazole
SGN	Secondary gustatory nucleus
SRE	Serum response element
SRF	Serum response factor
SRF (Brain region)	Superior reticular formation
TCF	Ternary complex factor
TGN	Tertiary gustatory nucleus
TPp	Periventricular nucleus of posterior tuberculum
Vc	Central nucleus of ventral telencephalon
Vd	Dorsal nucleus of ventral telencephalon
Vv	Ventral nucleus of ventral telencephalon

1. Background

1.1. Introduction

All animal species depend on sensory stimuli for various everyday activities and survival. Sensory stimuli allow animals to perceive their environment and make vital decisions, such as moving towards prey, moving away from predators, locating food sources, finding mates, etc. For many of these activities, animals residing both on land and in water rely on olfactory cues in their environment. For fish, olfactory chemical cues include pheromones (Sorensen et al, 1988), bile acids (Zhang et al, 2001), alarm cue (McCormick & Manassa, 2008), and amino acids (Cagan & Zeiger, 1978). Amino acids are important chemical cues because they initiate feeding behaviours in teleost fish (Valentincic & Caprio, 1994), and processing of this olfactory input allows fish to locate the food source. In some species of fish, the inability to sense food odours and perceive this information would make it very difficult for fish to locate food, and survival would be compromised. In nature, zebrafish eat zooplankton and phytoplankton, in which the dominant molecules are amino acids, and other amino-acid-rich foods such as insects, insect larvae, worms, and crustaceans. The focus of this study is to observe active regions in the zebrafish brain during the delivery of amino acids (food odours) into the water.

1.2. Zebrafish

Zebrafish are teleosts (bony fish) that belong to the superorder Ostariophysi, which includes a broad range of fishes that have an inner ear and gas bladder, the class Actinopterygii which are the ray finned fishes, the order Cypriniformes which have a dorsal fin and pharyngeal teeth, and the family Cyprinidae which includes stomachless fish. There are many olfactory

studies which are reviewed in this thesis, that have used other species of fish related to zebrafish. In relationship to zebrafish, the rainbow trout, catfish, and pacific jack belong to the class Actinopterygii, and the more closely related crucian carp and goldfish belong to the family Cyprinidae. Zebrafish have a short generation time, transparent embryos that can be manipulated, sequenced genome, and are easily attainable, making them an excellent vertebrate model to study.

1.3. Zebrafish Behavioural Response to Amino Acids

Stimulation of the olfactory system with amino acids is observed in behavioural studies, in which zebrafish showed an attraction to amino acids. When L-alanine and L-valine were delivered, compared to a synthetic fragrance, there was an increase in number of turns per thirty seconds exhibiting appetitive behaviour (Braubach et al, 2009). In this study, the anosmic zebrafish did not display appetitive behaviours towards the amino acid odours, rendering the olfactory system as the primary sensory modality for amino acid odours. Tierney et al (2008) also examined the behavioural response of zebrafish following exposure to L-alanine (Fig. 1). The results revealed that zebrafish spent more time surrounding the delivery input area of the tank, confirming their affinity towards amino acids. In subsequent experiments, cauterization of the olfactory epithelium diminished this behavioural response, and zebrafish in amino acids spent as much time around the delivery input as negative controls (delivery of background water) (Fig. 1). This finding indicated that the observed attraction to L-alanine is primarily due to olfaction in zebrafish.

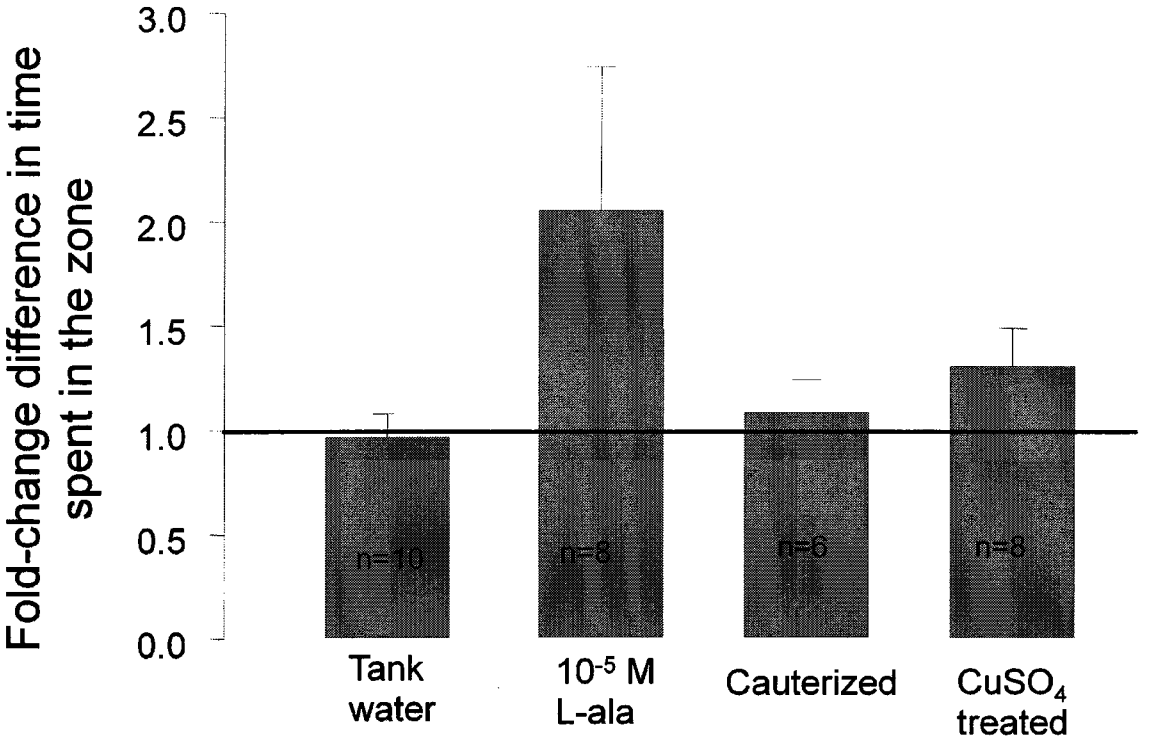


Figure 1 – Zebrafish attraction response to L-alanine. Zebrafish spent more time in the region surrounding the delivery input of L-alanine, compared to controls. After olfactory sensory deprivation by cauterization of olfactory epithelium, the olfactory response to L-alanine diminished and swimming activity was similar to controls. After exposure to copper, there was a decrease in time spent around the region. (Tierney et al, 2008).

1.4. Olfactory Epithelium

Zebrafish have externally located nares that lead into the peripheral olfactory organ, which is made up of lamellae (Hansen & Zeiske, 1998). The lamellae contain the non-sensory epithelium which is lined with ciliated non-sensory cells surrounding the medially located sensory epithelium. The sensory epithelium contains basal cells, support cells, and most importantly sensory receptors cells which are the primary olfactory sensory neurons (OSNs) (Byrd & Brunjes, 1995; Hansen & Zeiske, 1998; Hansen & Zielinski, 2006).

1.4.1. Olfactory Sensory Neurons

In the zebrafish, there are three types of olfactory sensory neurons: microvillous olfactory sensory neurons (mOSNs) lined with microvilli; ciliated olfactory sensory neurons (cOSNs) lined with cilia; and crypt cells lined with microvilli and cilia (Byrd and Brunjes, 1995; Hansen & Zeiske, 1998). All three OSN types have high levels of the neurotransmitter glutamate (Edwatds & Michel, 2002). Lipschitz and Michel (2002) delivered amino acid odours directly onto the olfactory epithelium of zebrafish for 10 seconds per minute and a total time of 10 minutes. They used agmatine, an ion channel permeant probe, to label OSNs stimulated by amino acids, and found that the majority of stimulated neurons were mOSNs. In rainbow trout, *Oncorhynchus mykiss*, recordings using the whole cell voltage clamp method showed that both cOSNs and mOSNs responded to single amino acids, amino acid mixtures, or both (Sato & Suzuki, 2001). Furthermore, mOSNs responded only to amino acid odours, whether the exposure was a single amino acid or an amino acids mixture, whereas cOSNs responded to amino acids, urine, and pheromones. Vielma et al (2008) recorded from crypt cells in the Pacific Jack, *Tracharus symmetricus*, using the patch-clamp method during and following 5 second intervals of amino acid exposure. They discovered that crypt cells were stimulated by amino acids, and found increased levels of intracellular calcium using calcium imaging (Vielma et al, 2008). Therefore, evidence from previous studies suggests that all three OSN types can be stimulated by amino acids, although the mOSNs are specialized for detecting only amino acid odours. Although the basis for having two other OSN types which also detect amino acid odours is unknown, it implies the importance of amino acids as an olfactory cue.

1.5. Olfactory Bulb

The olfactory bulb is a structure of the forebrain which is attached to the olfactory epithelium via olfactory nerves (Fig.2). It is responsible for receiving, sending, and processing olfactory information, and also receives centrifugal fibres from downstream regions of the brain (Ichikawa & Ueda, 1979). The olfactory bulb in zebrafish is made up of four distinct layers (Byrd & Brunjes, 1995) (Fig. 3). The outermost layer is the olfactory nerve layer containing axons of the incoming OSNs from the olfactory epithelium. The second layer is the glomerular layer, which contains glomeruli, the synaptic region between axons of the OSNs and dendrites of mitral cells. The mitral cell layer includes mitral cells and other cell types which will be discussed below, and the innermost layer is the internal/granule cell layer which contains granule cells.

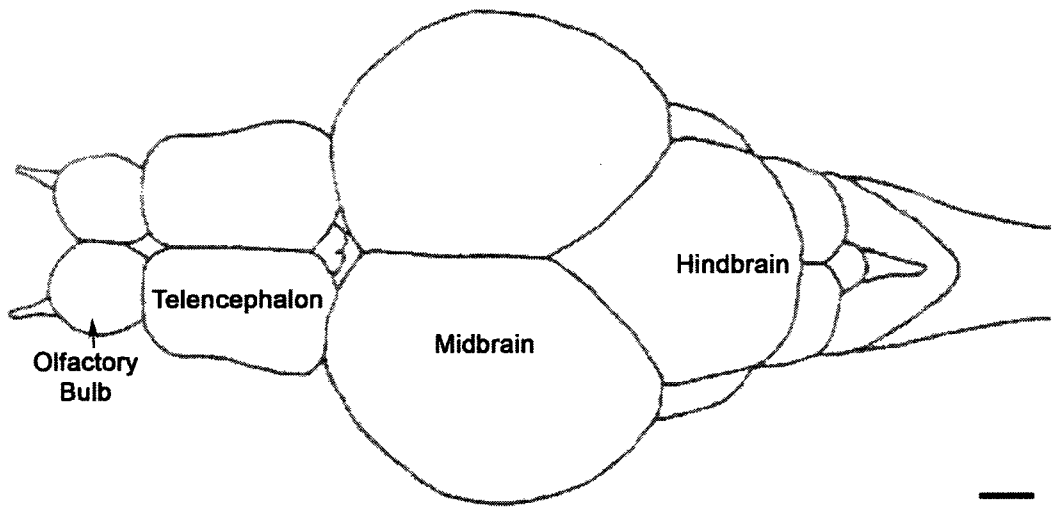


Figure 2 – A schematic horizontal view of the zebrafish brain, showing the location of the olfactory bulb, telencephalon, midbrain, and hindbrain. Image adapted from zebrafish neuroanatomy atlas (Wulliman & Reichert, 1996). Micrometer bar = 200 μm

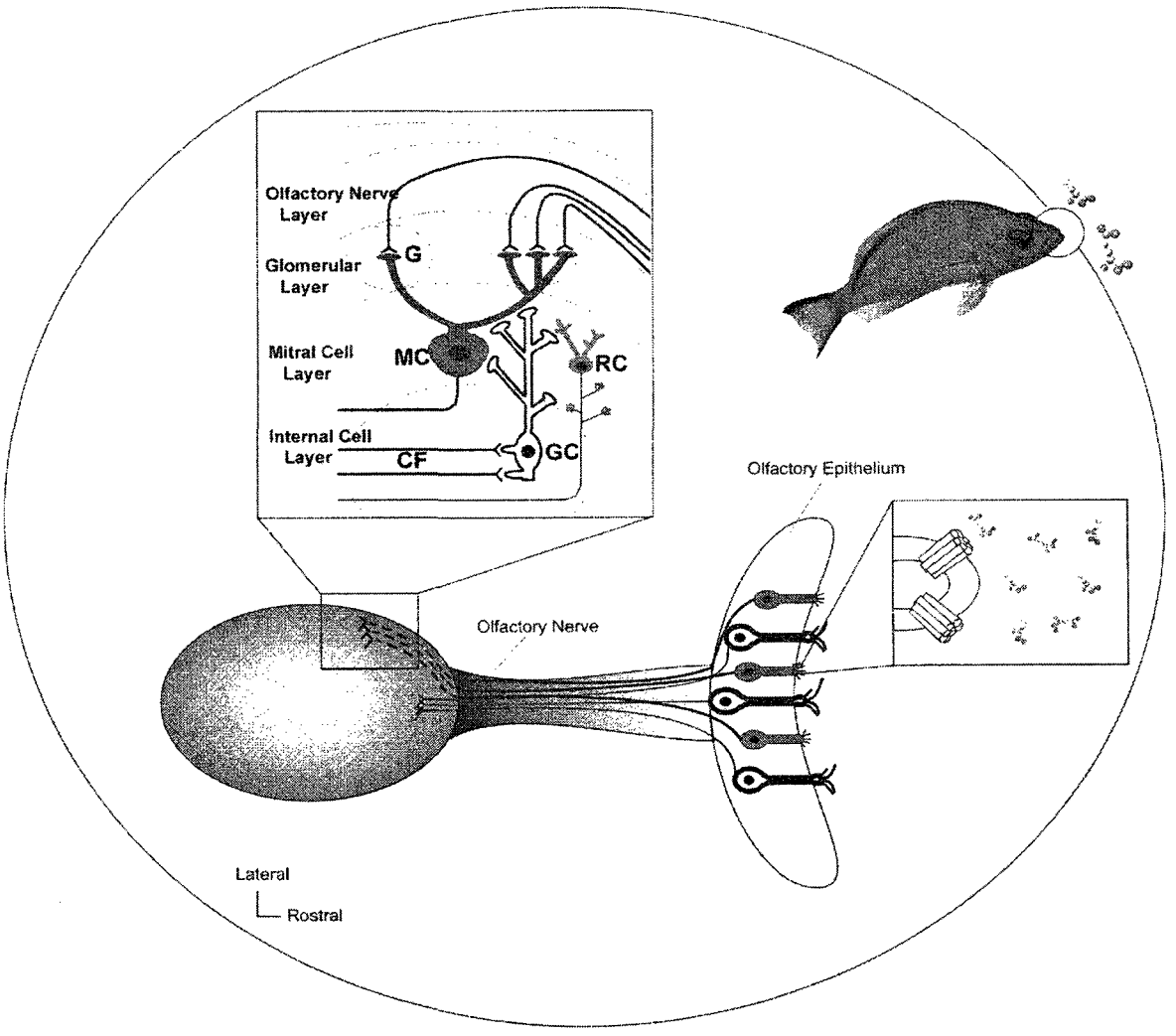


Figure 3 – A schematic view of the fish olfactory system. Odours, such as amino acids bind to olfactory sensory neurons in the olfactory epithelium. The olfactory sensory neurons project to the glomerular layer of the olfactory bulb, where the axon terminals synapse onto dendrites of mitral cells – the olfactory bulb output neurons. Mitral cells also form dendrodendritic synapses with granule cells in the internal cell layer, which receive input from centrifugal fibers projecting from more caudal brain regions. The mitral cell layer also contains ruffed cells, another type of olfactory bulb output neuron. Copyright for this image has been requested.

1.5.1. Cells of the Olfactory Bulb

In zebrafish, mitral cells are the main output neurons in the olfactory bulb and are distributed throughout the glomerular layer, mitral cell layer, and granule cell layer (Fuller et al, 2006). Mitral cells have AMPA (alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid), and NMDA (N-methyl-D-aspartate) receptors, which are ionotropic glutamate receptors, stimulated by the neurotransmitter glutamate (Edwards & Michel, 2002). Pre-synaptic neurons synthesize and release glutamate that binds to AMPA and NMDA receptors on post-synaptic neurons (reviewed by Shepherd, 1994). Although glutamate binds to both receptors, AMPA receptor activation leads to sodium influx which is necessary for depolarization of the neuron. Calcium channels that open following NMDA receptor activation have an Mg^{2+} block which is released after the cell is depolarized to allow calcium into the neuron, which acts like a second messenger to initiate the cascade of events for the transcription of genes.

High levels of glutamate were found in the olfactory nerve layer, the glomerular layer, and in all mitral cells (Edwards & Michel, 2002). Therefore, mitral cells are stimulated primarily by glutamate released by OSN axons, and release glutamate to stimulate granule cells. Tabor and Friedrich (2008) took electrophysiological recordings from stimulated mitral cells from the zebrafish olfactory bulb following delivery of amino acids. They also used antagonists to block both AMPA and NMDA receptors, which resulted in a diminished response by mitral cells, signifying the importance of both receptors during exposure to amino acid odours.

Although mitral cells are the most commonly known output neurons, a second population of output neurons in the olfactory bulb of zebrafish was identified by Fuller and Byrd (2005). These are the ruffed cells which are larger than mitral cells, present in smaller numbers, and their axons were also observed in the lateral and medial olfactory tracts. Although the presence of

ruffed cells has previously been discovered in a few fish species (Kosaka & Hama, 1980; Kosasa & Hama, 1981), the possibility of these being output neurons was unknown. However, ruffed cells do not receive input from OSNs or interact with mitral cells (Fuller & Byrd, 2005), but do form synapses with granule cells (Kosaska & Hama, 1981).

Granule cells are GABAergic, small interneurons (Mack-Bucher et al, 2007) that synapse with mitral cells and ruffed cells (Kosaka & Hama, 1981). Granule cells also have AMPA and NMDA receptors, and contain low levels of glutamate and high levels of GABA (Edwards & Michel, 2002). Mitral cells activate granule cells by releasing glutamate, and granule cells in turn release GABA and can inhibit them and other surrounding mitral cells that have weak responses or were activated by spontaneous firing. This results in the fine-tuning of the response that leaves the olfactory bulb (Yokoi et al, 1995). The olfactory bulb also contains juxtaglomerular cells, which are large interneurons that surround the glomeruli in the glomerular layer (Fuller et al, 2006). In mice, juxtaglomerular cells infiltrate the glomeruli and are suspected to be responsible for cross-talk between activated glomeruli (Kosaka & Kosaka, 2008). Juxtaglomerular cells also have AMPA and NMDA receptors, low levels of glutamate, and high levels of GABA, similar to granule cells (Edwards & Michel, 2002).

Therefore, the olfactory bulb contains different types of neuron specific for certain functional roles to efficiently process a response and further transmit it to target regions.

1.5.2. Olfactory Bulb Glomeruli

In zebrafish, mammals and other teleosts, the glomerular layer consists of glomeruli that are made up of OSN axon end clusters and mitral cell dendrites (Friedrich & Korsching, 1997). OSNs that express receptors for a particular odour are widespread in the olfactory epithelium but

converge onto a single glomerulus in the olfactory bulb (Fuss & Korsching, 2001). Mitral cells (output neurons of the olfactory bulb) relay sensory information through olfactory tracts (mitral cell axons), to higher brain centers that process odours (Hamdani & Doving, 2007). DiI injections into the ventro-lateral olfactory bulb labelled mOSNs in the olfactory epithelium in catfish, *Ictalurus punctatus* (Valentincic et al, 2005). Friedrich & Korsching (1997) found structures similar to the glomeruli, and designated them 'glomerular modules' due to their smaller size. Glomerular modules and larger sized glomeruli from ventro-lateral region of the olfactory bulb responded strongest to amino acids of L-configuration, with basic and neutral pH, and activity was documented using calcium imaging. Amino acids of D-configuration or an acidic pH produced very weak responses in the olfactory bulb. Fuss and Korsching (2001) also observed no glomerular stimulation following exposure to amino acids of D-configuration. These studies provided evidence towards understanding that glomeruli in the ventro-lateral olfactory bulb are most responsive to the L-configuration of amino acids, and those with neutral or basic side chain properties.

Yaksi et al (2009) exposed zebrafish to amino acids, used calcium imaging to observe activated neurons in the olfactory bulb, and labelled mitral cells and interneurons with markers to distinguish between which neurons are stimulated during the exposure. They found that mitral cells were predominantly stimulated in the ventro-lateral region of the olfactory bulb. Activated interneurons were distributed throughout the olfactory bulb and did not display chemotopic organization as seen by mitral cells, following exposure to amino acids.

1.5.3. Olfactory Tracts

The olfactory tracts extend from the olfactory bulb into the telencephalon and are made up of mitral cell axons (Fuller et al, 2006). Mitral cells from medial and rostral regions of the olfactory bulb project to the medial olfactory tract (MOT), and from the lateral and caudal regions of the olfactory bulb project to the lateral olfactory tract (LOT). The LOT generally projects into the dorsal telencephalon, and the MOT projects into the ventral telencephalon (reviewed by Wulliman et al, 1996). Similar to zebrafish, the olfactory system of crucian carp, *Carassius carassius*, involves medial (MOT) and lateral (LOT) olfactory tracts that process alarm cue and feeding odorants, respectively (Hamdani et al., 2001a; Hamdani & Doving, 2001b) (Fig.4). A previous study used a DiI tract tracing technique by injecting DiI into synaptic areas between OSNs and mitral cells in the lateral olfactory bulb to label OSNs and the olfactory tracts (Hamdani et al., 2001a). They found that applying DiI within the synaptic regions mostly labelled mOSNs in the olfactory epithelium, and axons that made up the LOT. Since previous studies had shown that mOSNs are stimulated by amino acids (Sato & Suzuki, 2001), Hamdani et al (2001a) indirectly concluded that the LOT must be responsible for processing amino acid odorants. In a subsequent study, Hamdani and Doving (2001b) showed that when the LOT was severed, crucian carp did not exhibit feeding behaviours such as biting, snapping, mouth opening, and vertical posture, after administration of food extract. These studies provided evidence towards the concept that the LOT is primarily responsible for feeding cues.

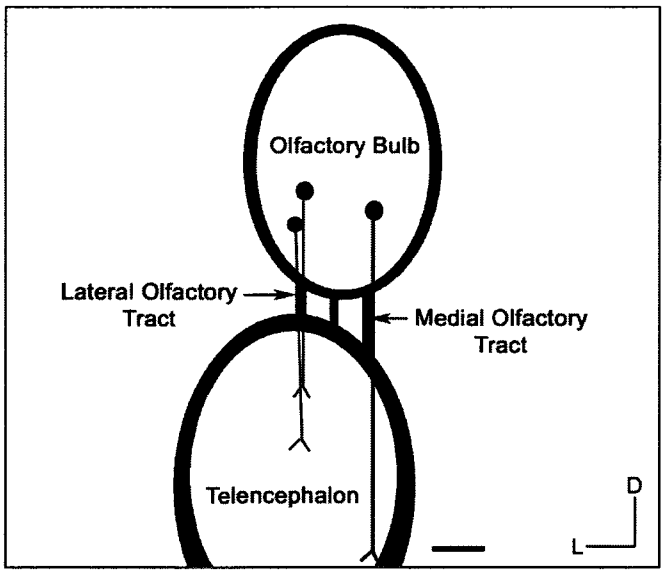


Figure 4 – A schematic view of olfactory tracts in zebrafish. Mitral cell axons from the olfactory bulb form the lateral and medial olfactory tracts, which project into and terminate in the telencephalon.

1.6. Telencephalon

The telencephalon, another structure of the forebrain, is attached caudal to the olfactory bulb of zebrafish (Fig. 2). The telencephalon is made up of the dorsal and ventral regions, also called the pallium and subpallium respectively (Rink & Wulliman, 2004). There are regions of the telencephalon which receive input from the olfactory bulb and midbrain, and other telencephalic regions which output to the midbrain (Rink & Wulliman, 2004).

1.6.1. Connections of the Telencephalon

The telencephalon sends and receives a vast amount of information to and from other regions of the brain. Using DiI labelling in the goldfish, *Carassius auratus*, brain, it was determined that the medial zone of dorsal telencephalon (Dm) receives input from areas of the preglomerular nuclei in the midbrain responsible for processing auditory and other

chemosensory information; the central posterior thalamic nucleus which processes auditory and visual information; the posterior thalamic nucleus which processes gustatory information; and a variety of information from the dorsal and ventral nuclei of the ventral telencephalon (Northcutt, 2006). The lateral zone of dorsal telencephalon (Dl) predominantly receives input from the olfactory bulb, areas of the preglomerular nuclei that process auditory and visual information, and the dorsal and ventral nuclei of the dorsal telencephalon (Northcutt, 2006).

In the zebrafish, the dorsal nucleus of ventral telencephalon (Vd) and ventral nucleus of ventral telencephalon (Vv) receive input from the olfactory bulb, and from other telencephalic regions such as the entopeduncular nucleus, Dm, posterior zone of dorsal telencephalon (Dp), supracommissural nucleus and, postcommissusal nucleus; and they output to the Dp, and supracommissural nucleus (Rink & Wullimann, 2004). The Vd and Vv also receive input from midbrain structures such as parvocellular preoptic nucleus, suprachiasmatic nucleus, thalamic nucleus, rostrolateral nucleus, periventricular nucleus of the posterior tuberculum, posterior tuberal nucleus, hypothalamic nuclei, and dorsal tegmental nucleus; and they output to the habenula, periventricular pretectum, paracommissural nucleus, posterior dorsal thalamus, preoptic region, posterior tuberculum, tuberal hypothalamus, and interpeduncular nucleus. Finally, the Vd and Vv regions also receive input from hindbrain structures such as the superior reticular nucleus, locus coeruleus, and superior raphe nucleus (Rink & Wullimann, 2004).

1.6.2. Response to amino acids

Yaksi et al (2009) used calcium imaging to investigate responses to amino acids in the olfactory bulb and telencephalon in zebrafish, and further examined the two telencephalic targets which responded the strongest: Vv and Dp. They established that there was neuronal activation

in the Vv and Dp regions of the telencephalon during odour perception of amino acids. Using neurophysiological methods, Nikonov et al (2005) recorded from different regions of the catfish forebrain following delivery of basic and/or neutral amino acids. Both basic and neutral amino acids stimulated the lateral regions of the dorsal telencephalon (Nikonov et al, 2005). From these previous studies, we know that in the catfish and zebrafish, the Dl, Dp, and Vv regions of the telencephalon are stimulated following exposure to amino acids.

1.7. Gustation

The gustatory system (taste) also functions as a processing system for sensory input involving food. Fish possess taste buds that are located in the oral cavity and distributed externally throughout the entire body surface (Finger & Silver, 1987). Taste buds are innervated by the facial, glossopharyngeal, and vagal nerves, which output to the primary gustatory nucleus, composed of the facial, glossopharyngeal, and vagal lobes, in the brainstem (Finger & Silver, 1987; Hara, 2007). From the primary gustatory nucleus, gustatory information is sent to the secondary gustatory nucleus (SGN), then to the tertiary gustatory nucleus (TGN), and finally to regions of the telencephalon such as Dd, Dm, and various ventral regions (Hara, 2007; Yoshimoto et al, 1998). An electrophysiology study in catfish by Lamb & Caprio (1992) found activation of neurons in the SGN after stimulating the oral cavity and the extraoral surface, with amino acids. Although there are projections from the SGN to TGN, the TGN has not yet been investigated for neuronal activation using amino acid stimuli.

Within the gustatory cells of taste buds are receptors which are stimulated by a few chemical types (Hara, 2007). The L-isomer of amino acids was found to be more stimulatory than the D-isomers in catfish, and their taste receptors showed affinity towards amino acids such

as L-serine, L-glutamine, D-alanine, and glycine (Caprio, 1975). Finger et al (1996) also found that taste bud cells in catfish have receptors which are specific for L-alanine and for L-arginine, and other non-specific receptors which bind to either amino acid (Finger et al, 1996). A study in the rainbow trout found that of the naturally occurring amino acids, taste receptors are stimulated by proline, alanine, and leucine (Yamashita et al, 2006). Therefore, taste receptors on different fish species have variable affinities towards a range of amino acids.

1.8. Midbrain

In the zebrafish, the midbrain is a large structure and is attached to the telencephalon. The main functions of the midbrain include processing visual cues, processing auditory input, locomotor control, and learning and memory. Although the forebrain structures of zebrafish have been investigated following exposure to amino acids using different techniques, the midbrain region has not yet been studied for responses to chemical feeding cues. A recent study by Derjean et al. (2010) showed that a midbrain region, the posterior tuberculum, relayed activation from the olfactory bulb to the reticulospinal neurons, when sea lampreys were exposed to odours (including amino acids), forming a direct olfactory-locomotion pathway. In zebrafish, areas of the ventral telencephalon receive input from the posterior tuberal nucleus (PTN) and the periventricular nucleus of posterior tuberculum (TPp) (Rink & Wulliman, 2004).Therefore, in this study we looked into the posterior tuberculum, and other midbrain regions, to understand if there are midbrain structures involved in the processing of amino acid odours in zebrafish.

1.9. Hindbrain

In the zebrafish brain, the hindbrain is located behind the midbrain, and attaches to the spinal cord. The hindbrain is primarily responsible for processing motor activity resulting from various types of stimuli. Bosch et al (2001) used c-Fos-like immunoreactivity to discover midbrain and hindbrain structures in the rainbow trout which were stimulated during the startle response. The startle response in teleost fish is initiated by the onset of any danger stimulus and followed by an evasion from the immediate danger (Liu & Westerfield, 1988; Bosch et al, 2001; Burgess et al, 2009). Although studies in zebrafish have investigated swimming and escape behaviours (Ritter et al, 2001), there aren't any that have explored the relationship between amino acids and locomotion. However, Gahtan et al (2005) explored the relationship between vision and locomotion in zebrafish. Zebrafish larvae with intact sight in a well-lit environment captured paramecia very effectively, but had difficulty in the dark or during vision impairment. They did laser ablations on groups of reticulospinal cells and found that this visual-motor behaviour diminished with ablation of a specific set of reticulospinal neurons in the nucleus of the medial longitudinal fascicle (nMLF) (Gahtan et al, 2005). Therefore changes in swimming and motor behaviours upon sensory stimuli can activate specific hindbrain regions in zebrafish.

1.10. The Neural Activity Marker – c-Fos

Active neurons in the brain can be monitored by quantifying expression levels of protein markers. In the brain, immediate early genes are expressed when neurons are stimulated by depolarization, and participate in events associated with synaptic plasticity (reviewed in Pinaud & Tremere, 2006). Some immediate early genes are translated to become transcription factors which are responsible for controlling the expression of other genes. The monitoring of these

transcription factors has been used to mark activity patterns in the brain, the development of neural pathways, and as well as signals that are associated with input processing. The most commonly used transcription factor for marking neural activity is c-Fos. Also included in the c-Fos family are FosB, Fra-1, and Fra-2, which combine with proteins in the Jun family such as c-Jun, JunB, and JunD. The resulting heterodimer is called the activator protein-1 (AP-1), and is a complex which consists of one Fos protein and one Jun protein. Depending on the proteins of the heterodimer, AP-1 can up-regulate or down-regulate transcription of genes, by binding to the AP-1 site on the promoter region of DNA.

The OSNs and mitral cells release glutamate which binds to NMDA receptors, causing an influx of Ca^{2+} (reviewed in Shepherd, 1994). Following the activation of NMDA receptors, it is expected that the signals generated by glutamate activate extracellular signal-regulated kinases (ERKs) located in the cytoplasm of the cell, which transport the signal to the nucleus and phosphorylate Elk-1 (Xia et al, 1996). Elk-1 then binds to the ternary complex factor (TCF) binding site in the serum response element (SRE), which uses the serum response factor (SRF) to initiate c-fos gene transcription (Fig. 5). This cascade of events is the most likely pathway to be responsible for transcription of c-fos when NMDA receptors are activated following sensory stimuli. The protein product, c-Fos, is translated between 60 – 120 minutes following activation of the cell, depending on the species.

This expression of c-fos in activated neurons is also important in long term potentiation (LTP) (Xia et al, 1996; Malenka & Nicoll, 1999; Ryoung-Hee et al, 2004). When neurons are undergoing LTP, stronger synapses are formed, and the resulting expression of genes ensures permanent changes in neurons (Malenka & Nicoll, 1999). The c-*fos* mRNA is translated to its protein product and then transported to the nucleus because it is a transcription factor responsible

for further transcribing a broad range of genes (reviewed in Pinaud & Tremere, 2006). Overall, the activation of NMDA receptors, calcium influx, and transcription of genes are important during LTP, which is a necessary factor in learning behaviours associated with sensory stimuli.

1.10.1. c-Fos as a Marker for Neuronal Activation in Mammalian Tissue

Localization of c-Fos protein has been used by numerous studies in mammals to mark neuronal activation in the brain following many types of stimuli (Kawamato et al, 2003; Sun et al, 2007; Mashoodh et al, 2008; Gonzalez-Cuello et al, 2004; Zimmer et al, 1997; Bennett & Semba, 1998). The molecular weight of c-Fos protein in mammalian tissue was found to be 62 kDa (Ezquer & Seltzer, 2003; Olesen & Auger, 2005). Sagar et al (1988) showed direct evidence linking c-Fos-immunoreactivity to neural activity by implanting electrodes in the hindlimb of rats to stimulate regions of the motor/sensory cortex. They observed an increase in c-Fos labelled nuclei in stimulated animals compared to controls, in the motor/sensory cortex and in regions of the brain that are known targets of the motor/sensory cortex. This study confirmed the validity of labelling c-Fos protein as a marker for activated neurons.

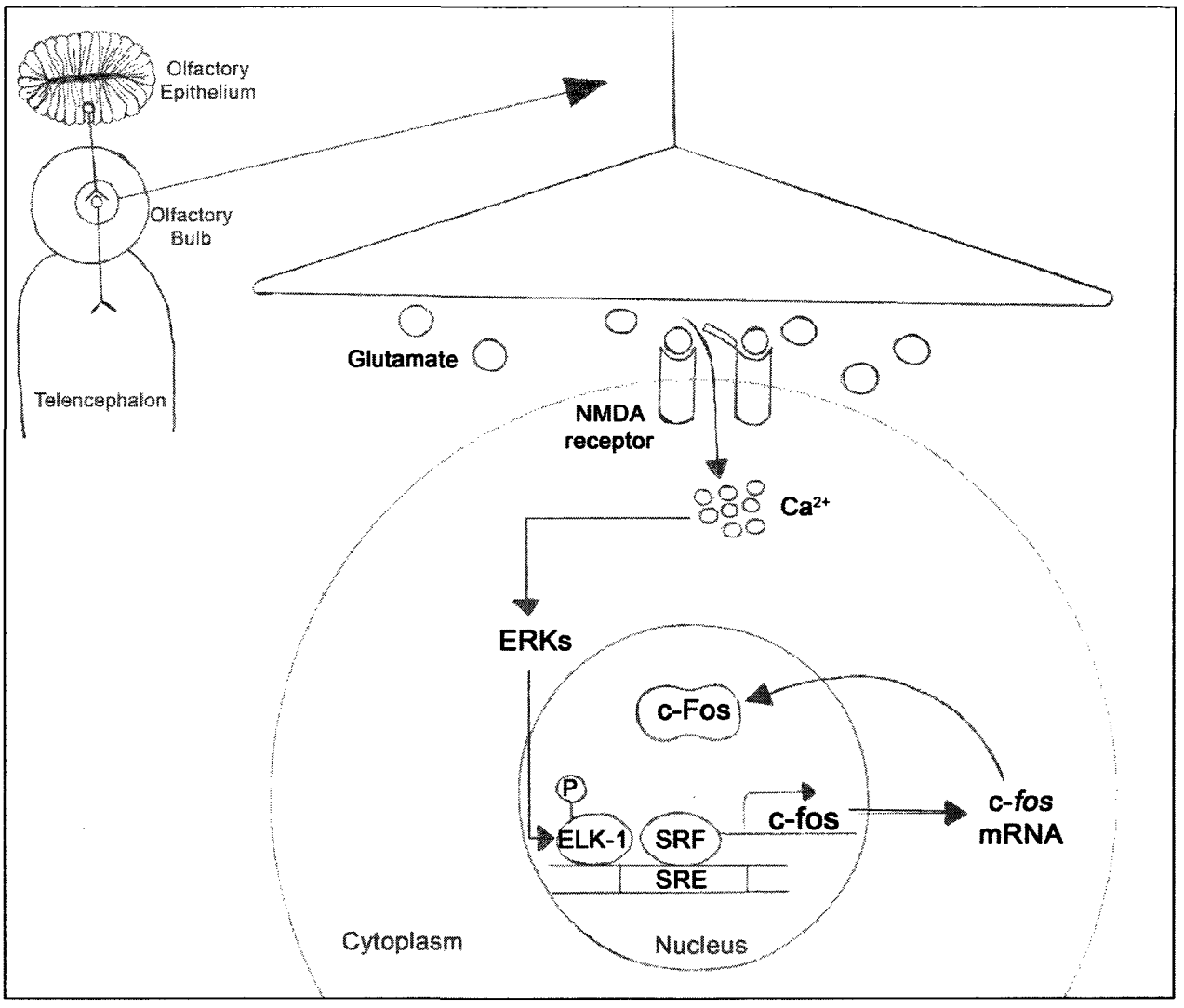


Figure 5 – A schematic model of c-fos stimulation in the olfactory system. The inset on the left shows location of the olfactory epithelium and mitral cells in the zebrafish olfactory bulb. Amino acids bind to olfactory sensory neurons, which form synapses with mitral cells in the olfactory bulb. The diagram on the right shows the sequence for c-fos activation, previously investigated in mammalian CNS. Pre-synaptic neurons release the neurotransmitter, glutamate, which binds onto NMDA receptors of the post-synaptic neuron, causing an influx of calcium ions into the cytoplasm. The intracellular calcium activates ERKs, which phosphorylate ELK-1 in the nucleus, and causes SRF to transcribe c-fos. In the cytoplasm, c-*fos* mRNA is translated into c-Fos protein and this protein is transported into the nucleus, where it acts on gene transcription.

1.10.2. c-Fos as a Marker for Neuronal Activation in Fish Tissue

Localization of c-Fos protein has also been studied in fish species (Bosch et al, 2001; Salierno et al, 2006; Salierno et al, 2007). In rainbow trout, brain regions stimulated by the startle response were investigated by localization of c-Fos-like protein (Bosch et al, 2001). Increase of c-Fos-like protein was observed in regions that have been linked to motor control and sensorimotor integration, such as the superior reticular formation, magnocellular octaval nucleus, Mauthner cell, and motor nuclei of the cranial nerve. Since the startle response is an escape mechanism, the increase in c-Fos-like immunoreactivity in regions of motor control validated the use of labelling c-Fos-like protein for stimulated neurons, in fish species.

Baraban et al (2005) used in situ hybridization to localize c-*fos* mRNA in zebrafish larvae, after exposure to the pesticide pentylenetetrazole (PTZ). The pesticide caused seizures in the fish, and analysis revealed that there was an increase of c-*fos* mRNA in the cerebellum, and midbrain regions such as the optic tectum (Baraban et al, 2005). This study was significant because it revealed that monitoring increases in c-*fos* expression may be applicable to experimental investigations using zebrafish.

Matsuoka et al (1998) used the RT-PCR technique to probe for c-fos related genes in rainbow trout brain following stimulation by kainic acid, which activates neurons and induces them to release the neurotransmitter glutamate. Following the stimulus, there was an increase in expression of two cDNA sequences that were similar to c-fos in mammals and other fish species. The two sequences, designated RT-fos1 and RT-fos2, were 88% identical to one another, and most likely translate two isotypes of c-Fos protein. Hirayama et al (2005) probed for c-Fos protein in zebrafish cells that displayed circadian oscillations using western blotting, and found that there were two bands, which is in accordance with the two c-fos related genes isolated by

Matsuoka et al (1998) in rainbow trout. The molecular weights of the two bands were 52 kDa and 68 kDa (Hirayama et al, 2005), although the molecular weights of bands on a western blot are approximate values, and the actual weights could be +/- 10 kDa of the estimated weights. Therefore, similar to the rainbow trout, zebrafish may have two isotypes of c-Fos protein with molecular weights similar to mammals and other fish.

When zebrafish are exposed to amino acids or food odours, sensory input to the olfactory bulb and processing of this information allows the fish to identify and locate a nearby source. Previously, electrophysiology and imaging studies have looked at stimulation in the olfactory bulb and telencephalon following delivery of amino acids. We propose to analyze the response to amino acids in these forebrain structures using a novel methodology in zebrafish. This technique of localizing Fos-like immunoreactivity (FLI) is advantageous because activation can be seen in all brain regions simultaneously, whereas other techniques have allowed investigators to examine single cells or single brain regions. Therefore we have been able to take this study a step further by investigating the response to amino acids in all brain structures including forebrain, midbrain, and hindbrain. This technique allows for a very complete study of spatial mapping, and also reveals active regions of higher brain centers which may be involved in olfactory processing, decision-making, memory, and movement.

1.11. Objectives

In this study, we investigated regions of the zebrafish brain which had FLI following exposure to amino acids. The first objective was to investigate the olfactory bulb in zebrafish because it is the main region responsible for processing various olfactory chemical stimuli

including amino acids. We hypothesized that after exposing zebrafish to an amino acids mixture, there will be an increase in FLI in the olfactory bulb.

The second objective was to investigate regions of the telencephalon in zebrafish, for changes in FLI following exposure to amino acids. Previous studies have found stimulation by amino acids in telencephalic regions, so we hypothesized that there will be an increase in FLI in regions of the telencephalon which receive olfactory input from the olfactory bulb and are stimulated by amino acids.

The third objective was to investigate changes in FLI in gustatory regions in the zebrafish brain. The gustatory regions are known, but whether the cells in these regions have NMDA receptors and are activated by glutamate is still unknown. Furthermore, the extent of the role that the gustatory system plays in zebrafish brain is also unknown. We hypothesized that if gustation plays an important part in amino acid processing, and cells possess NMDA receptors, there will be an increase in FLI in gustatory regions following exposure to amino acids.

The fourth objective was to investigate midbrain regions in zebrafish to determine if there was a change in FLI following exposure to amino acids. Two particular midbrain regions, PTN and TPp, were of great interest, because of the olfactory-locomotion pathway discovered in sea lamprey by Derjean et al (2010) following exposure to amino acids. However, stimulation of midbrain regions by amino acids has not yet been studied in teleost fish, rendering this investigation as the first that examined locomotor control regions using an amino acids stimulus. We hypothesized that if there are midbrain regions responsible for processing of amino acids, and neurons contain NMDA receptors which are activated by glutamate, there will be an increase in FLI in those regions.

The fifth objective was to investigate hindbrain regions in zebrafish which change in FLI following exposure to amino acids. Hindbrain regions predominantly control movement, and although this structure has been immensely investigated using danger stimuli, there are no studies conducted for an amino acid stimulus. However, Braubach et al (2009) observed changes in swimming behaviour (greater number of turns), following exposure to amino acids. In contrast, Tierney et al (2008) exposed zebrafish to amino acids, and found that the fish spent more time swimming around the delivery input. Therefore, we hypothesized that there will be an increase in FLI in hindbrain regions that integrate the olfactory response with locomotion.

This study provides a holistic investigation of the zebrafish brain following stimulation by amino acids. Furthermore, it is the first to employ the technique of localizing Fos-like protein (FLP) using an immunocytochemical approach in zebrafish, and allows for visualization of immunoreactive cells throughout the entire brain.

2. Materials and Methods

2.1. Experimental Animals

Adult zebrafish obtained from Profish center in Windsor, ON were used in all experiments, in compliance with the regulations of Canada Council of animal care. The zebrafish were held in the Biology department at the University of Windsor, for a minimum of three days to allow for acclimation.

2.2. Experimental Setup for Exposure to Amino Acids

In this experiment, zebrafish were exposed to background water in negative control tanks and amino acids in treatment tanks. Two-gallon tanks were used with 1-2 zebrafish in each experimental tank filled with 6 liters of water. The zebrafish were acclimated to dechlorinated water treated to remove metals which cause olfactory deprivation (McIntyre et al, 2008). The experimental fish were deprived of food for 72 hours, because the amino acid odour is a food stimulus. The amino acid odour stimulus contained eight amino acids: 0.1 mM L-alanine (Sigma-Aldrich, A-7627); 0.1 mM L-arginine (Sigma-Aldrich, A-5949); 0.1 mM glycine (Sigma-Aldrich, G-7126); 0.1 mM L-histidine (Sigma-Aldrich, H-8000); 0.1 mM L-phenylalanine (Sigma-Aldrich, P2126); 0.1 mM L-serine (Sigma-Aldrich, S4500); 0.1 mM DL-cysteine (Sigma-Aldrich, C-4022); and 0.1 mM L-lysine (Sigma-Aldrich, L-5626), dissolved in dechlorinated background water. These amino acids (at the same concentration) were used previously by Friedrich and Korsching (1998), Friedrich and Laurent (2004), and Mack-Bucher et al (2007), for olfactory studies. Only basic and neutral amino acids of L-configuration were used, because previous studies found minimal to no response with acidic amino acids (Friedrich & Korsching, 1997), and amino acids of D-configuration (Fuss & Korsching, 2001).

The amino acids mixture solution (100 ml) was then delivered at a point source using an air stone into the treatment tanks at an initial concentration of 1 mM, and was diluted by the tank water to a final concentration of 0.1 mM. The negative control treatment was 100 ml of background water removed from control tanks, and delivered back in during the experiment. Since c-Fos protein is generally translated between 1-2 hours in mammals (Pinaud & Tremere, 2006), we tested experimental times of 60 minutes, 90 minutes, and 120 minutes, to determine optimal c-Fos labelling. When comparing sections from fish that were exposed to these different time periods, optimal results were considered to have the darkest labelling in nuclei, which was achieved after 90 minutes. Therefore, in this study the experimental time for amino acids exposure was 90 minutes.

2.3. Tissue fixation

Following the background water or amino acids exposure for 90 minutes, zebrafish were removed into a beaker with MS-222 (anesthetic), immediately decapitated, and the heads were immersed in fresh 4% paraformaldehyde (PFA) in 0.1M phosphate buffer (PB) (pH 7.4) (Appendix G) for tissue fixation. Two hours later, the brains were removed from the cranial cavity and again immersed into fresh 4% PFA in 0.1M PB (pH 7.4) for another two hours. Brain tissue was cryoprotected through a sucrose gradient in 0.1M phosphate buffer saline (PBS) of 10% until the tissue sank (~ 30 minutes), 20% until the tissue sank (~ 2 hours), and 30% until the tissue sank (~ 2 hours). For cryosectioning, the brain tissue was immersed in Shannon M-1 Embedding Matrix (Thermo electron corporation, C0893). Cross sections were sliced at a thickness of 30 μm and horizontal sections were sliced at a thickness of 20 μm using a Cryostat (Leica CM 3050 S). Sections from negative control (background water) and treated (amino

acids) zebrafish brains were mounted on the same slide to ensure that both control and treated sections were exposed to the exact same conditions. The slides used were Adhesion microscope slides (Fisherbrand Superfrost Plus Slides, 12-550-15). The slides were left in room temperature for approximately 15 minutes until the sections dried.

2.4. Western Blot

The western blot was conducted to determine the molecular weight of protein recognized by anti-c-Fos (sc-253). The loading control in this experiment was an anti-neuronal cell surface marker (zn-12), which is abundant in zebrafish brain. Zebrafish were anesthetised with MS-222, decapitated, and the heads were homogenized using a mortar and pestle. For this assay, only fresh tissue was used, because problems with frozen tissue were previously observed. Lysis buffer (150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 1mM phenylmethanesulphonylfluoride [PMSF], and 50 mM Tris-HCl at pH 7.5, sodium dodecyl sulfate) was added to the homogenized tissue for cell lysis and degradation of proteases. The sample was sonicated for three seconds each three times, and centrifuged (Heraeus Instruments Biofuge 15, 3619) at 15,000 rpm for fifteen minutes at 4°C. The supernatant was separated from the pellet, and the Lowry method was used for protein quantification, so that the same amount of protein was loaded into in all wells.

For protein gel electrophoresis, 6% resolving gel and 5% stacking gel were used. 400 μg of protein was loaded into the wells with several duplicates, and the gel was run at 140 volts to allow proteins to migrate down the gel. The protein gel was transferred onto a nitrocellulose membrane for 1.5 hour at 140 volts. The membrane was stained with a ponceau red stain to visualize the protein bands. The membrane was then blocked using TBST (20 mM Tris-HCl pH

794, 200 mM NaCl, 0.2% Tween) with 5% non-fat skim milk, for one hour at room temperature on an orbital shaker with mild agitation. One membrane duplicate was incubated in a polyclonal primary antibody (rabbit anti-c-Fos, 1:500; Santa Cruz, sc-253) with 2% non-fat skim milk and TBST buffer, for 48 hours in 4°C on an orbital shaker. The membrane was washed three times for five minutes each in TBST buffer, and incubated in an HRP-conjugated secondary antibody (goat anti-rabbit, 1:20,000; Rockland, 611-1322) for one hour at room temperature with 2% non-fat skim milk in TBST buffer. The second membrane duplicate was incubated in a monoclonal primary antibody (mouse anti-neuronal cell surface marker, 1:2000; Developmental Hybridoma Bank, zn-12) with 2% non-fat skim milk in TBST buffer, for one hour in room temperature on an orbital shaker. After three washes for five minutes each in TBST buffer, the membrane was incubated in an HRP-conjugated secondary antibody (goat anti-mouse, 1:10,000; Rockland, 610-1319) with 2% non-fat skim milk in TBST buffer for one hour at room temperature on an orbital shaker. Both membranes were washed three times for five minutes each in TBST buffer at room temperature on an orbital shaker. The membranes were probed with a chemiluminescence HRP substrate reagent (Immobilon, WBKLS0500) to help visualize the protein bands in the AlphaImager 2200 Light Imaging system, and imaged using the software program ChemiImager v5.5. The molecular weight of the protein bands were estimated using AlphaImager software.

2.5. Immunocytochemistry

The immunocytochemistry (ICC) protocol was adapted from Minson et al (1996), and Burman et al (2004). Sections mounted on microscope slides were rehydrated using a 0.1M phosphate buffer saline solution with 0.1% Triton (TPBS), for 10 minutes in room temperature. The sections were immersed in a 1% hydrogen peroxide (ACP, 7722-84-1) in 0.1 M phosphate

buffer solution (PBS) (Appendix H) for 10 minutes at room temperature, to block endogenous peroxides. After rinsing sections in PBS, slides were immersed in a blocking solution with 10% normal donkey serum (Jackson ImmunoResearch, 017-000-121) and 0.2% bovine serum albumin (Vector Laboratories, SP-5050), for one hour at room temperature with agitation. Sections were then incubated in the polyclonal primary antibody (rabbit anti c-Fos, 1:4000; Santa Cruz, sc-253), for 48 hours at room temperature with agitation. This concentration was adapted from Laberge et al (2008), in which the same primary antibody was used. After rinsing with PBS, the sections were incubated in the secondary antibody (biotinylated donkey-anti-rabbit, IgG 1:500; Jackson Immuno Inc., 711-065-152) for 2 hours at room temperature with agitation. After rinsing with PBS, the slides were immersed in Extravidin horseradish peroxidise (1:400; Sigma, E2886) for 90 minutes at room temperature with agitation. Sections were rinsed with PBS, and for visualization of c-Fos protein, they were incubated in nickel-DAB (3,3 diaminobenzidine, Sigma-Aldrich, D5637) solution. The reaction was started using 0.1% glucose oxidase (Sigma, G0543-10KU), which generates hydrogen peroxide (Llewellyn-Smith et al, 2005). After optimal staining, the DAB-ni reaction was stopped with PBS, and the sections were dehydrated with an ethanol/water gradient (60%, 70%, 95%, 100%, 100%). The tissue was cleared with xylene, and slides were cover-slipped using Permount® (Fisher Scientific, SP15-100).

2.6. Controls

2.6.1. Pre-Adsorption control

A pre-adsorption control was used to confirm specificity of the primary antibody. The anti-c-Fos primary antibody (Santa Cruz Biotechnology, sc-253) was incubated with the blocking peptide (Santa Cruz Biotechnology, sc-253P), which is the antigen to which the

primary antibody is raised against, for 24 hours at 4 °C with mild agitation. The antigen-antibody solution was centrifuged at 100,000 g for 20 minutes, which resulted in a pellet and supernatant. The pellet contained the precipitated antigen-antibody complexes and the supernatant (pre-adsorbed serum) was used in place of the primary antibody during ICC. If there was no labelling in the tissue, the primary antibody would be specific to the antigens similar to those in the blocking peptide. Four slides with survey sections (section 1 is mounted on slide 1, section 2 is mounted on slide 2, section 3 is mounted on slide 3, section 4 is mounted on slide 4, section 5 is mounted back on slide 1, and so on) from one zebrafish brain were used. Two slides were used as positive controls (ICC with primary antibody), and two as pre-adsorption controls (ICC with pre-adsorbed serum).

2.6.2 Omission of antibodies

During the primary antibody incubation step of ICC protocol, one slide was incubated in a solution without the primary antibody. Since tissue sections did not have c-Fos-like labelling, we concluded that the secondary antibody was specific to the primary antibody in this tissue.

During the secondary antibody incubation step, one slide was incubated in a solution without the secondary antibody. Since tissue sections did not have c-Fos-like labelling, we concluded that the avidin bound specifically to the secondary antibody in this tissue.

2.6.3 Positive control

Baraban et al (2005) localized c-*fos* mRNA using in-situ hybridization in zebrafish after exposure to PTZ. Therefore, exposure to this pesticide induces c-*fos* expression in midbrain regions of zebrafish brain. In this experiment, zebrafish were exposed to 1 mM PTZ, and the

anti-c-Fos (sc-253) primary antibody was used to label FLP using ICC. Since the activation regions were already known, this positive control was used to validate the ICC protocol in zebrafish brain tissue.

2.7. Cell counts

The protein c-Fos is found in the nucleus, and therefore cells with dark labelling in the nucleus were counted from different regions of the brain, in both negative control (background water), and treated fish (amino acids), to determine whether there was an increase, decrease, or no change in FLI in certain areas. Regions of the olfactory bulb, telencephalon, midbrain, and hindbrain were identified by referring to the zebrafish neuroanatomy atlas (Wulliman & Reichert, 1996). Darkly labelled Fos-like-immunoreactive (FLi) cells were counted (Lau et al, 2003; Dielenberg et al, 2001; Laberge et al, 2008; Bosch et al, 2001) in sections from zebrafish exposed to background water (negative control), and amino acid mixture.

On each slide, analogous sections of olfactory bulb (according to bulb shape, size, and layers) were matched from negative control and treated fish, and cell counts were taken from one side only from all olfactory bulb sections (Chang et al, 2001; Bosch et al, 2001). For each fish, the number of FLi cells was counted in each section. The total number of FLi cells from all sections was calculated, and divided by the number of sections counted from, producing a value of average number of FLi cells per section in individual fish. These average values from each fish were used to calculate group means (Lau et al, 2003; Truitt et al, 2003; Kang et al, 2006; Larkin et al, 2010).

Cell counts were also taken from specific regions of the telencephalon, midbrain, and hindbrain in a similar method. For these structures, all sections were not counted from. Instead,

the sections as outlined in the Wulliman zebrafish anatomy atlas (Wulliman & Reichert, 1996) were used to identify corresponding sections, and cell counts from regions of interest were taken. In order to ensure accuracy of the regions that were counted from, it was important to follow the zebrafish neuroanatomy atlas (Wulliman & Reichert, 1996). The number of FLi cells was counted from each region in every section, and the total number of FLi cells was determined by adding the numbers from each section. The total number of FLi cells was then divided by the number of sections counted, to give an average value of FLi cells per section of each region. These average values from each region in each fish were used to calculate group means. This strategy was used for all the regions investigated from the telencephalon, midbrain, and hindbrain. Unpaired t-tests were done using the final group means obtained as described above, and the results from fish exposed to background water and amino acids were compared in the olfactory bulb, and regions of the telencephalon, midbrain, and hindbrain.

2.8. Data Analysis

Sections were analyzed using a brightfield microscope (Nikon Eclipse E800). Cell counts were taken from one side of olfactory bulb and telencephalon under 40X magnification. The zebrafish neuroanatomy atlas (Wulliman & Reichert, 1996) was used to look at different regions of the telencephalon, midbrain, and hindbrain (Table 1). Images were taken using a brightfield microscope (Zeiss Axioshop 2 mot plus) and camera (Imaging QICAM FAST 1394), with the Northern Eclipse software program, at different magnifications. Images were cropped and adjusted using Adobe Photoshop CS3.

3. Results

3.1. Western Blot

The western blotting method was used to validate the use of anti-c-Fos (sc-253) against protein in zebrafish brain tissue (Fig. 6B). Data from only one lane was shown because the purpose of conducting this assay is simply to label the proteins recognized by sc-253. For the loading control, the antibody zn-12 against neural surface markers was used (Fig. 6A), and the resulting molecular weight was approximately 123 kDa, similar to the molecular weight of 125 kDa found by Metcalfe et al (1990). The zn-12 antibody was used as a loading control because it recognizes various markers that are found on neurons in zebrafish brain; therefore if the protocol was accurate and the brain tissue was abundant in this preparation, there would be a prominent band close to 125 kDa after probing with the zn-12 antibody. Furthermore, since it is known that the antibody recognizes neural surface markers in zebrafish, if there were no protein bands on the blot, it would be suspected that there was an error in the protocol or protein degradation in the tissue, and the blot probing for FLP would be disregarded as well. In this assay, zebrafish heads rather than dissection were used to minimize the time between decapitation and adding preservative, which was done to avoid protein degradation. Using the anti-neuronal cell surface markers antibody ensured that there was an adequate amount of neural tissue, because the objective was to verify FLP in the brain.

The western blot probing for FLP using anti-c-Fos (sc-253) produced two bands (Fig. 6B). These two bands were previously observed in a zebrafish cell line from embryonic cells, which displayed regulated circadian oscillations by Hirayama et al (2005). The molecular weights of the two bands in this study were 64 kDa and 77 kDa. Since there is some ambiguity with getting the exact molecular weights of bands, the actual values can fall within the range of

64 +/- 10 kDA and 77 +/- 10 kDa. Therefore, the number of bands and molecular weights produced by the western in this study are similar to those previously observed by Hirayama et al (2005).

3.2. Pre-Adsorption Control

A pre-adsorption control was conducted to test if the sc-253 antibody against FLP was affected by the blocking peptide. The sc-253 antibody was raised against the blocking peptide which was synthesized using a 50 amino acid sequence (110 – 160) of the human c-fos gene. Using the NCBI protein blast program to compare protein sequences, this amino acid sequence was compared to the zebrafish genome for homologous sequences. The results showed that the human c-fos amino acids sequence 110-160 is similar in homology to c-Fos protein-like sequence in zebrafish by 88%, proto-oncogene c-Fos by 82%, c-fos by 82%, and fosB by 62%. The purpose of this experiment was to determine whether the antibodies saturated with the blocking peptide bound to other non-specific proteins in the zebrafish brain. There were FLi cells in zebrafish olfactory bulb tissue when the sc-253 antibody was used in the ICC protocol (Fig. 6C). When the pre-adsorbed serum (sc-253 with blocking peptide) was used instead of sc-253, there were no FLi cells found in the olfactory bulb tissue (Fig. 6D). Therefore this experiment verified that the anti-c-Fos (sc-253) antibody used in this study does not bind to other non-specific proteins in zebrafish brain.

3.3. Positive Control - Pentylenetetrazole Treatment

Baraban et al (2005) previously exposed zebrafish to the pesticide PTZ, and using *in situ* hybridization, discovered that there was an increase in c-*fos* mRNA in midbrain regions.

Zebrafish were exposed to PTZ, and this experiment was used as a positive control to confirm the localization of FLP using the anti-c-Fos (sc-253) primary antibody. After exposure to 1mM PTZ, there was an abundance of FLI in the nucleus of cells in the hypothalamus region of zebrafish brain (Fig. 7). Since there was an increase of FLP (product of *fos* mRNA) in zebrafish midbrain, combined with the results of the western blot and pre-adsorption control, using the sc-253 antibody with an immunocytochemical approach proved to be a good labelling technique in zebrafish brain tissue.

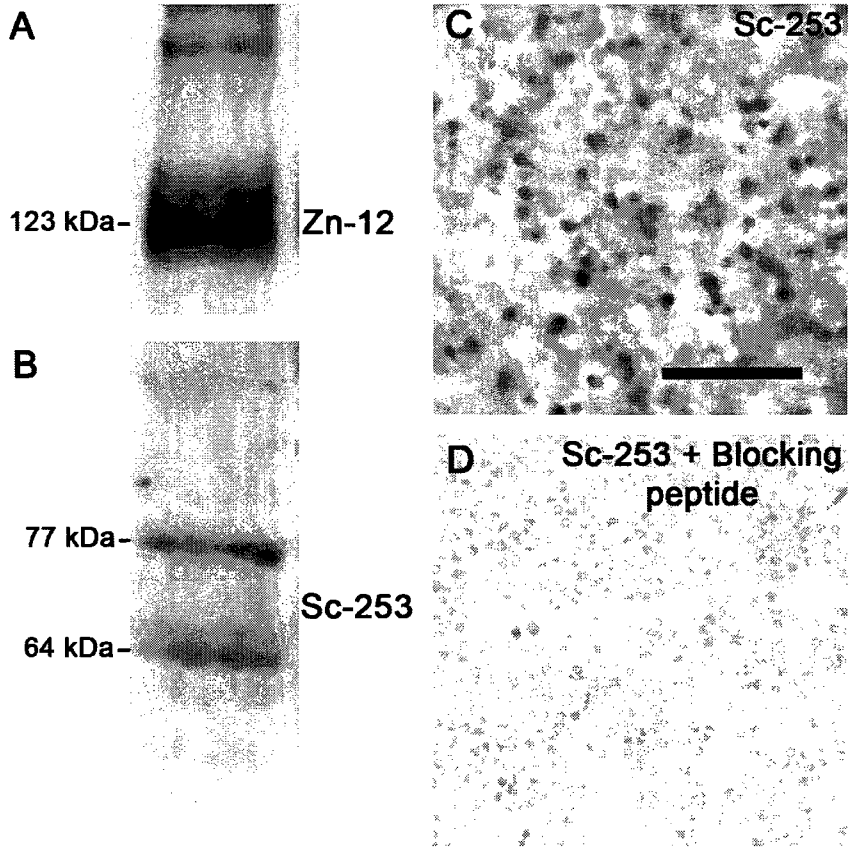


Figure 6 – Western immunoblot and ICC pre-adsorption control for Sc-253. A) The loading control – zebrafish anti-neuronal cell surface marker monoclonal antibody (Zn-12) has a molecular weight of approximately 123 kDa. B) The anti-c-Fos polyclonal antibody (sc-253) cross reacts to protein in zebrafish, and produces two bands at molecular weights of approximately 64 kDa and 77 kDa (courtesy of Dr. S. Ananvoranich). C) Dark nuclei show FLI in the olfactory bulb following ICC using anti-c-Fos primary antibody sc-253. D) No FLI was observed in the olfactory bulb following ICC using pre-adsorbed serum. Micrometer bar = 50 μm.

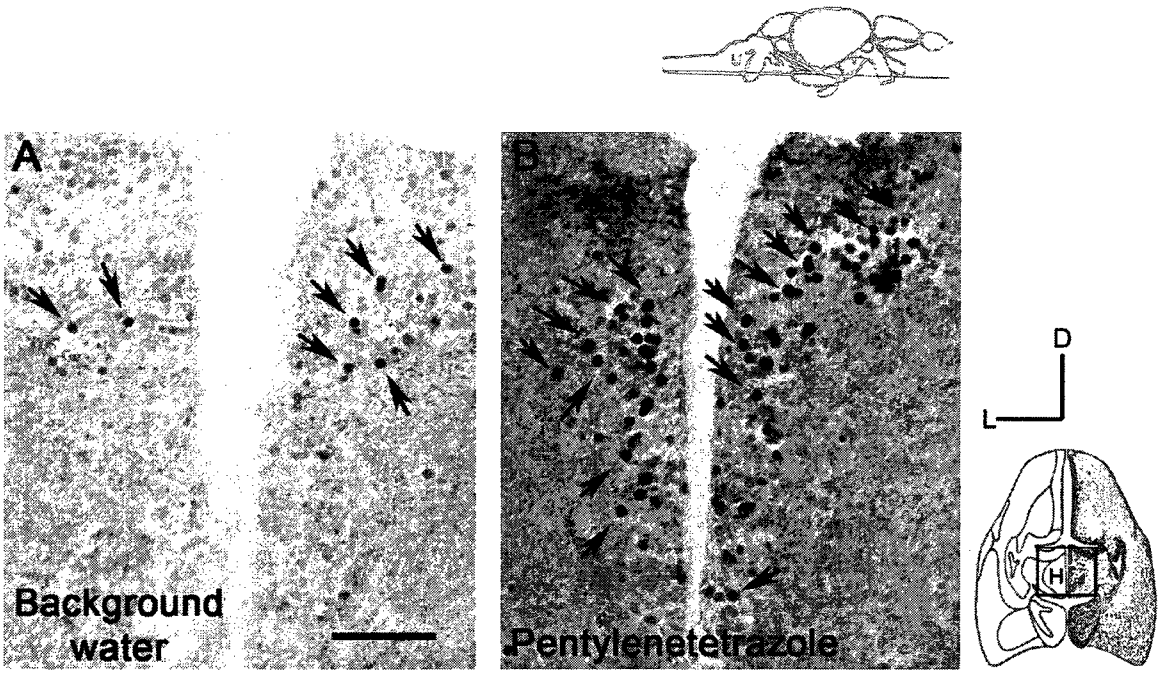


Figure 7 – Localization of FLi cells in the hypothalamus of zebrafish brain following exposure to the pesticide, pentylenetetrazole (PTZ). A) There were a few FLi cells in the hypothalamus in zebrafish which were exposed to background water (arrows). B) There was an abundance of FLi cells (arrows) in the hypothalamus of zebrafish brain following exposure to 1mM PTZ. Micrometer bar = 15 μm.

3.4. Changes in Fos-like Immunoreactivity following Exposure to Amino Acids

Zebrafish displayed attraction and appetitive behaviours towards amino acids (food odours) (Braubach et al, 2009; Tierney et al, 2008). Olfactory sensory deprivation caused by cautery of the olfactory epithelium diminished the attraction towards amino acids, validating that the attraction was due to olfaction (Tierney et al, 2008). This led to the investigation of brain regions that are involved in the processing of olfactory stimuli, during prolonged exposure to odours. Following stimulation by amino acids, there was a change in FLI in brain structures associated with olfactory activity, such as the olfactory bulb, and Dl and Dp regions of the telencephalon.

3.4.1. Olfactory Bulb

When background water was delivered to zebrafish, ICC revealed that FLI was normally present in olfactory bulb neurons (Figs. 8 A,B; 9A; 10A). When zebrafish were exposed to the amino acids mixture, there was an abundance of FLi cells in the olfactory bulb (Figs. 8 C,D; 9B; 10B), compared to fish in background water. FLi cells were counted from the entire olfactory bulb in both zebrafish in background water (n=12 fish), and in amino acids (n=12 fish), and there was a significant increase of FLi cells following exposure to amino acids (Fig. 11). The average number of FLi cells per section in the contralateral the olfactory bulb following delivery of background water was 5.04 +/- 1.22 cells. The average number of FLi cells per section in the contralateral olfactory bulb following delivery of amino acids was 18.44 +/- 5.61 cells. Unpaired t-tests showed that the increase observed in FLI was statistically significant.

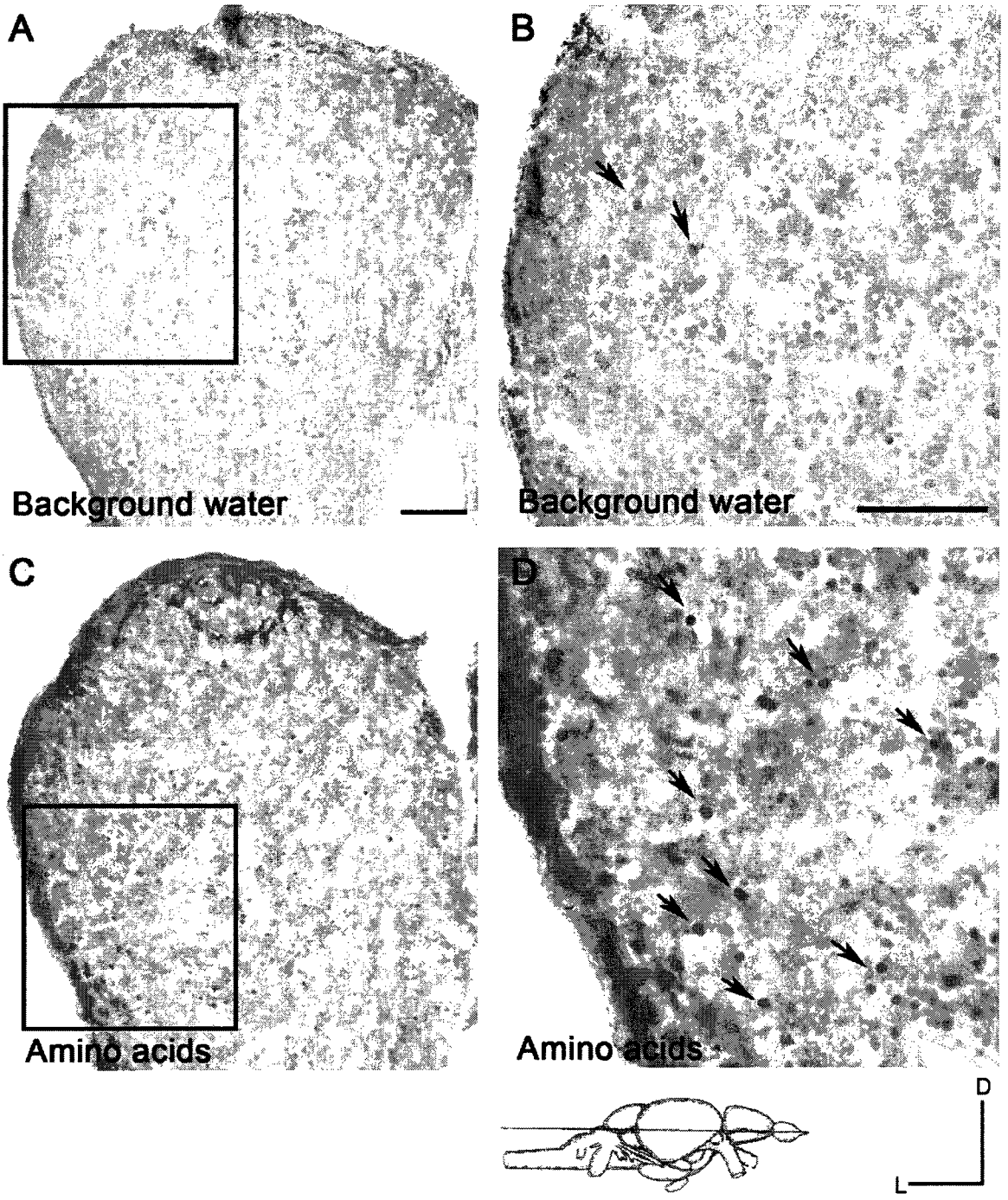


Figure 8 – Localization of FLi cells in the olfactory bulb after following treatment with 0.1 M amino acid mixture. A) Olfactory bulb following the delivery of background water. The area enclosed in the square is shown in higher power in B. B) Olfactory bulb section following delivery of background water. Arrows point to nuclei containing FLI. C) Olfactory bulb from zebrafish following delivery of the amino acids mixture. The area enclosed in the square is shown in high power in D. D) Olfactory bulb section following delivery of amino acids, showing an increase in cells containing FLI.

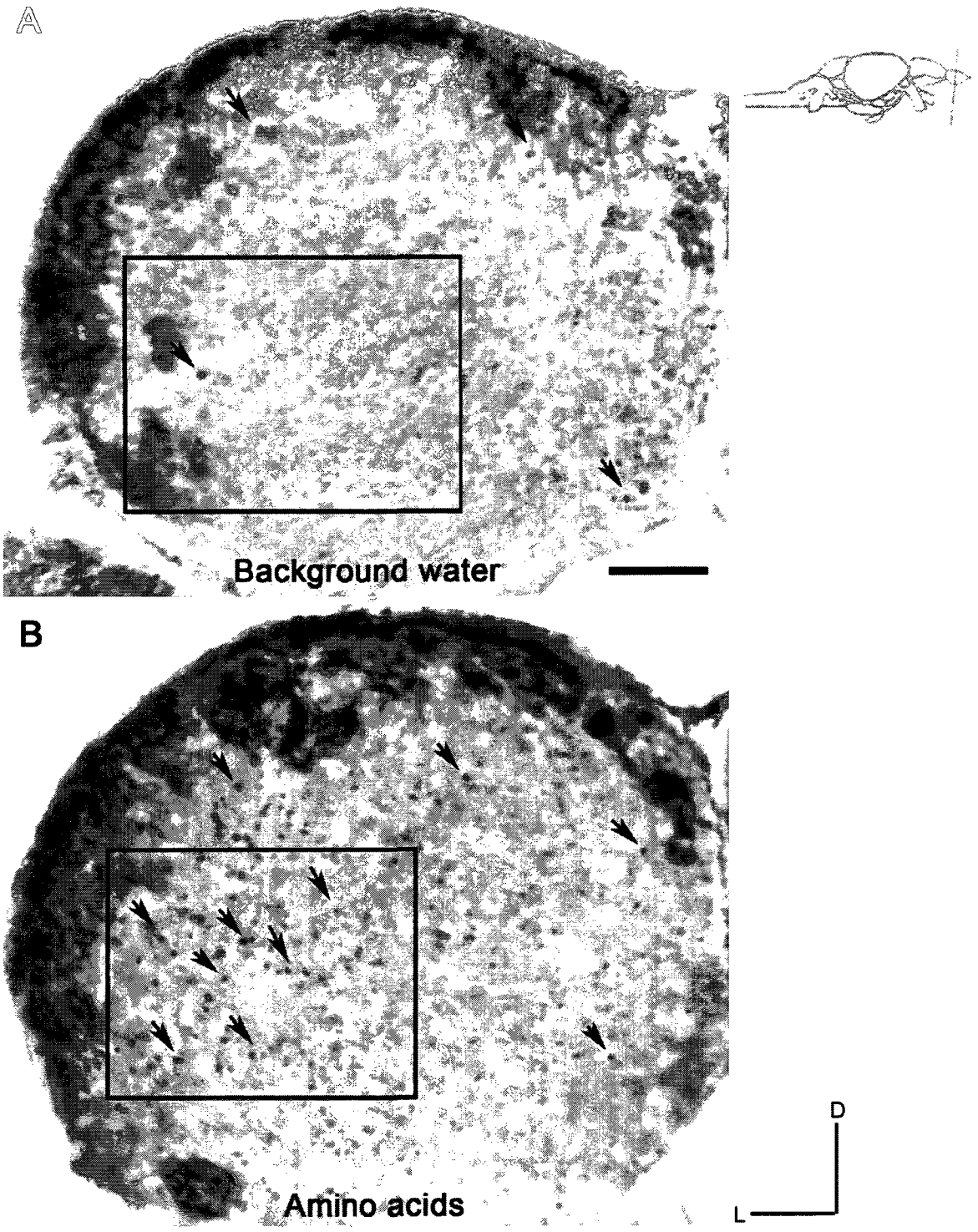


Figure 9 – Cross-sectional views of FLi cells in the olfactory bulb following exposure to background water and amino acids. A) Olfactory bulb section showing a few FLi cells (arrows) following delivery of background water. B) Olfactory bulb section showing an abundance of FLi cells following delivery of amino acids. Micrometer bar = 50μm.

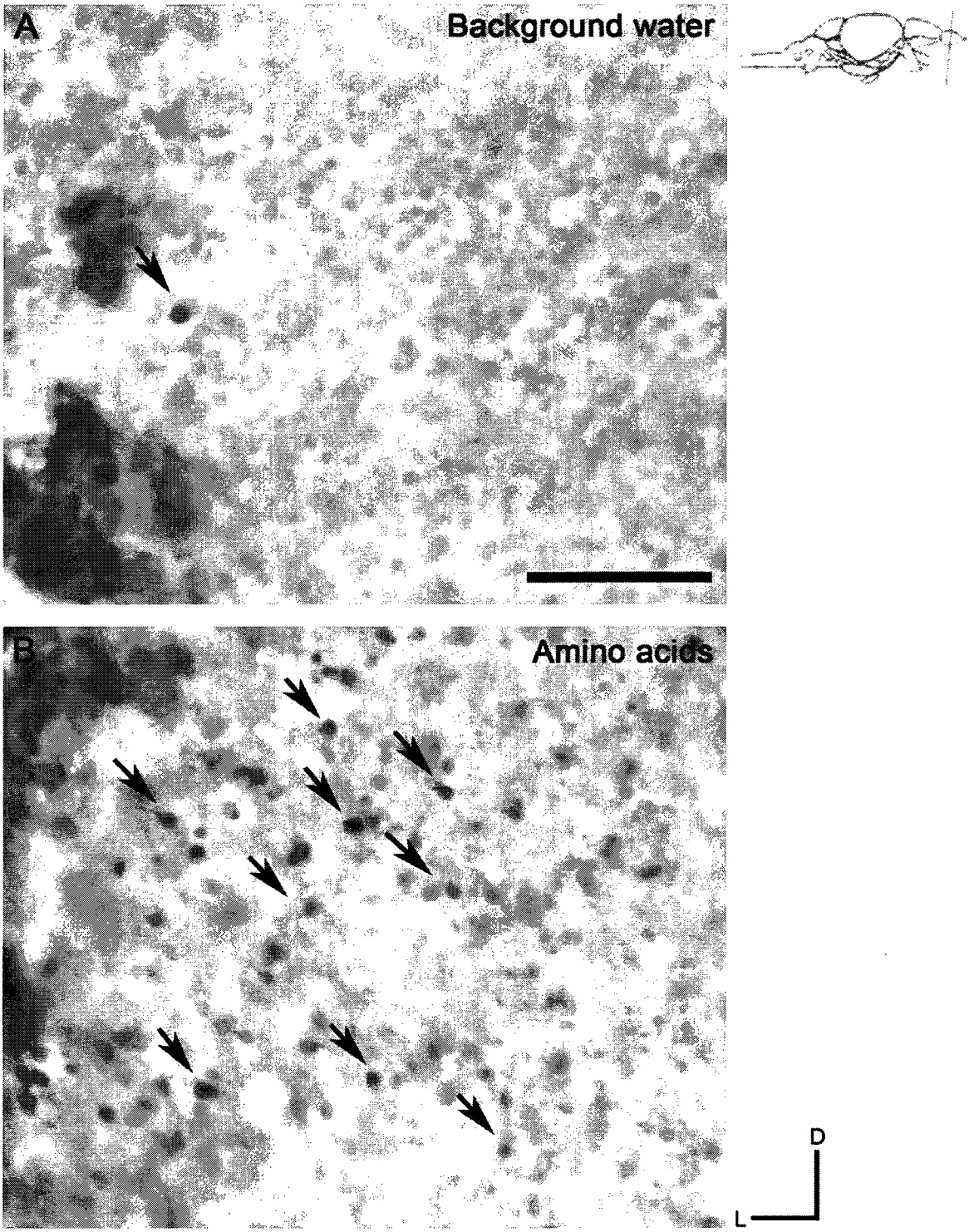


Figure 10 – High power views of FLI localization in the olfactory bulb following delivery of background water and amino acids. A) Very few cells were seen following treatment with background water. B) Abundant nuclei with FLI following amino acids treatment. Micrometer bar = 50μm.

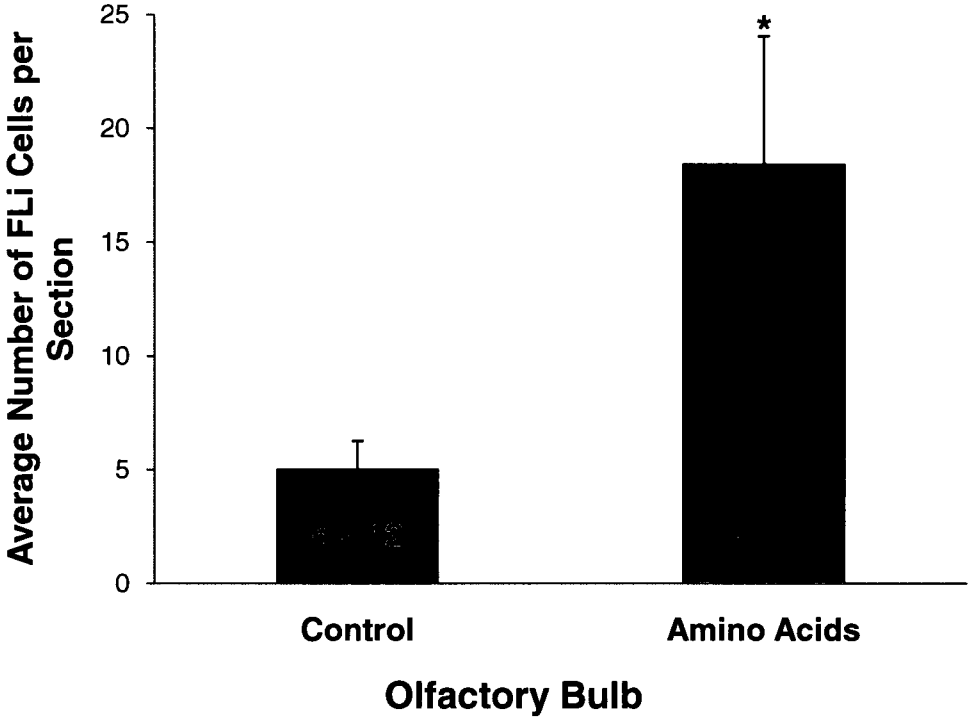


Figure 11 – Quantitative analysis of FLi cells in the olfactory bulb following exposure to amino acids. There was a significant increase in FLi cells following exposure to 0.1 mM amino acids. The average number of FLi cells per section of olfactory bulb was calculated from each fish, and group means from these values were taken and used in the graph (n=12). P-value = 0.029. Refer to Appendix A for cell counts.

Localization of FLi cells in the olfactory bulb was observed in lateral, medial, rostral, and caudal regions. Lateral regions of the olfactory bulb were compared to medial regions to determine whether there was a chemotopic map in the olfactory bulb as observed in previous studies. However, this pattern was not identified in all fish specimens in this study. In many fish, there was labelling of FLi cells in the glomerular layer, mitral cell layer, and granule cell layer, but based on FLI, different cell types could not be identified. However, most of the labelling was found in the mitral and granule cell layers of the olfactory bulb.

In some experiments when only background water was delivered into the tanks, some fish responded with high levels of FLI in cells of the olfactory bulb (Appendix A). Although not

known for certain, this may be a response to the presence of urine or pheromones in the tank from surrounding fish. Sato and Suzuki (2001) found that there are 35 amino acids found in urine of rainbow trout, and that urine stimulated cOSNs. Since urine and pheromone odours stimulate cOSNs, release of these in the water during the experiment could be responsible for responses seen in the olfactory bulbs of a few control fish. These fish were not included in the group means that were calculated for statistics and graphs because it is not certain whether the labelling was due to stimulation by other odours in the tank, or the low FLI in treated (amino acids exposure) fish was due to anosmia or genetic abnormalities. Therefore, it would not be accurate to use these fish to calculate statistics, when it is not known for certain whether the negative control tanks were in fact devoid of any stimulatory odours.

In some preparations, there was also dark labelling of OSN axons in the glomeruli of the olfactory bulb (Fig. 9A, B). This staining was also previously seen in rat olfactory bulb (Woo et al, 1996), and the labelling might be a preparation artefact. The possibilities of this type of labelling will be further discussed in the next section.

3.4.2. Telencephalon

The telencephalon was investigated because it receives input from mitral cells in the olfactory bulb, via the LOT and MOT. The telencephalic regions that were explored (Fig. 12) were the posterior zone of dorsal telencephalon (Dp) (Fig. 13), lateral zone of dorsal telencephalon (Dl) (Fig. 14), central zone of dorsal telencephalon (Dc) (Fig. 15A), dorsal zone of dorsal telencephalon (Dd) (Fig. 15B), medial zone of dorsal telencephalon (Dm) (Fig 15. C), ventral nuclecus of dorsal telencephalon (Vv) (Fig. 16A), dorsal nucleus of ventral telencephalon (Vd) (Fig. 16B), and central nucleus of ventral telencephalon (Vc) (Fig. 16C).

Following amino acids exposure, there was an abundance of FLi cells compared to controls in the Dp (Fig. 13), and Dl (Fig. 14). Unpaired t-tests showed that there were no significant changes in the average number of FLi cells per section from zebrafish exposed to background water (n=10 fish) and amino acids (n=10 fish), in the following regions of the telencephalon following stimulation by amino acids: Dc [control, 1 +/- 0.30 cells; amino acids, 1.65 +/- 0.52 cells], Dd [control, 0.5 +/- 0.19 cells; amino acids, 1.44 +/- 0.4 cells], Dm [control, 4.17 +/-1.21 cells; amino acids, 12.5 +/- 5.42 cells], Vc [control, 0.6 +/- 0.31 cells; amino acids, 0.3 +/- 0.3 cells], Vd [control, 4.2 +/- 1.32 cells; amino acids, 7.5 +/- 2.65 cells], and Vv [control, 0.8 +/- 0.3 cells; amino acids, 1.95 +/- 0.77 cells]. Cell counts and unpaired t-tests showed that there was a significant increase in the Dp [control, 0.46 +/- 0.14 cells; amino acids, 3.56 +/- 0.74 cells], and in the Dl [control, 7.2 +/- 1.38 cells; amino acids, 17.1 +/- 3.41 cells] (Fig. 17).

Therefore, there was a significant increase in FLi cells in the Dl and Dp, following amino acids exposure. There were no changes observed in FLI in the ventral telencephalon following amino acids exposure.

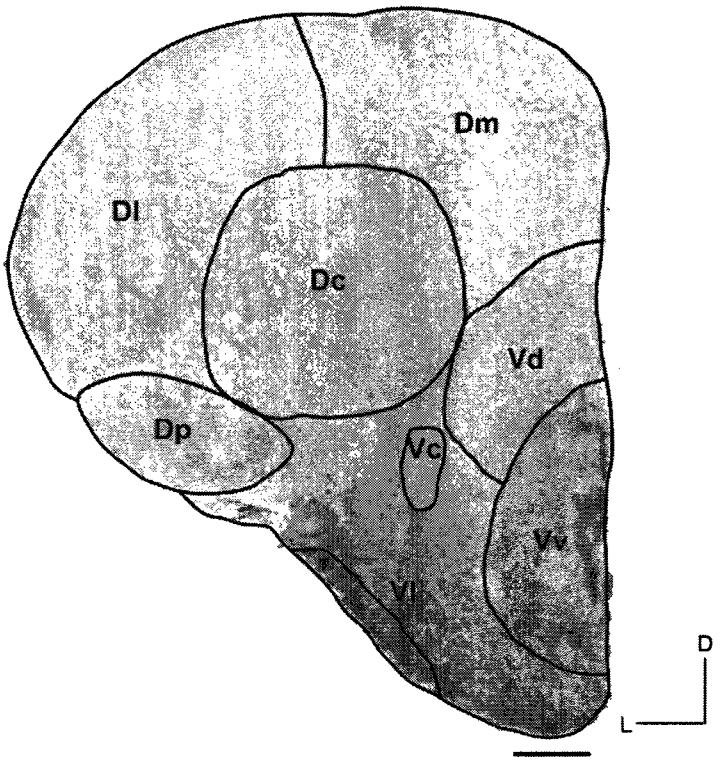


Figure 12 – Cross sectional view of the zebrafish telencephalon showing regions of the dorsal and ventral telencephalon. Micrometer bar = 100 μm.

Dc - Central zone of dorsal telencephalon
Dm - Medial zone of dorsal telencephalon
Dp - Posterior zone of dorsal telencephalon
Dl - Lateral zone of dorsal telencephalon
Vd - Dorsal nucleus of ventral telencephalon
Vv - Ventral nucleus of ventral telencephalon
Vc - Central nucleus of ventral telencephalon
Vl - Lateral nucleus of ventral telencephal

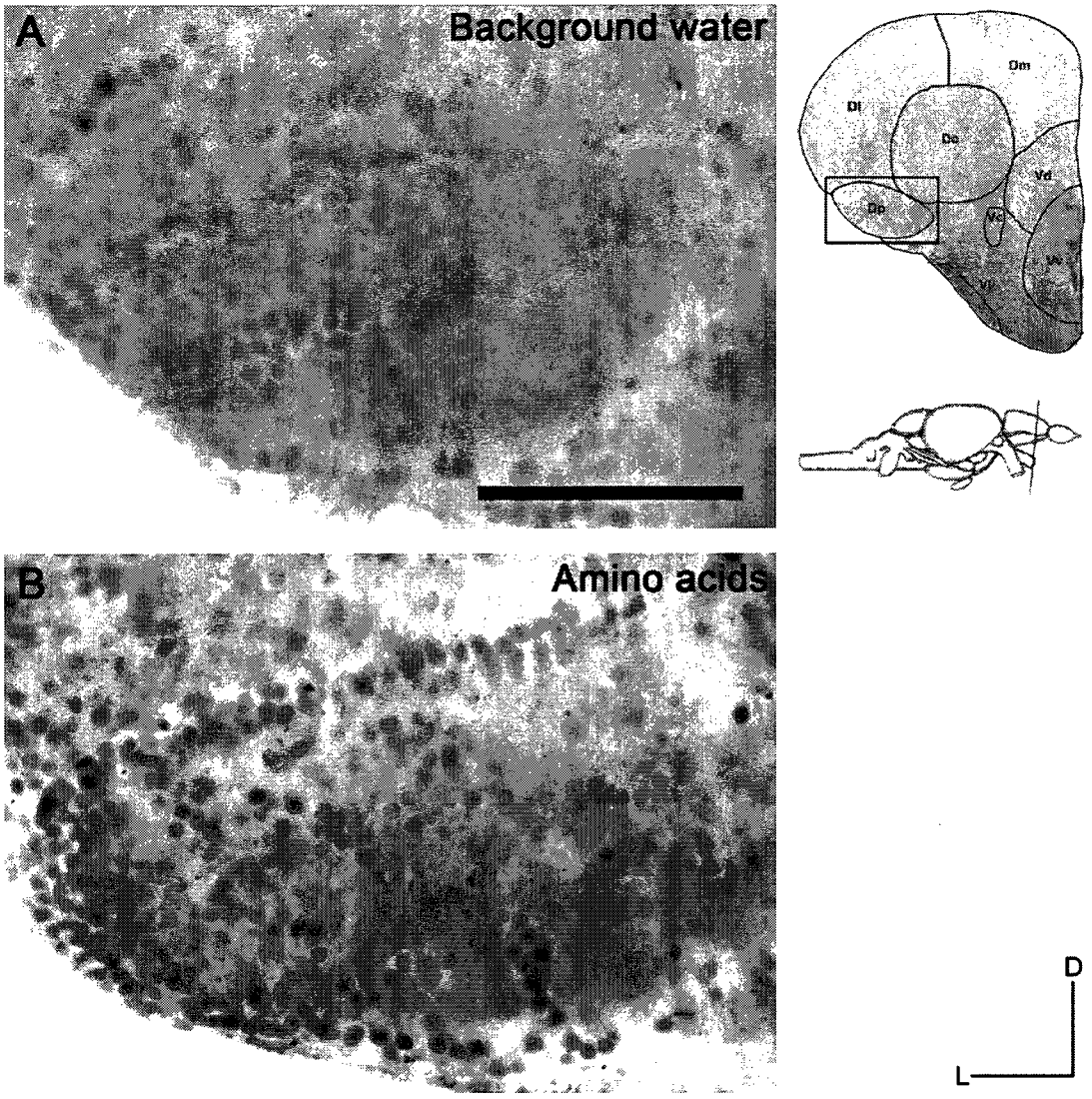


Figure 13 – Localization of FLi cells in the posterior zone of dorsal telencephalon (Dp), following exposure to amino acids. A) There are a few FLi cells in the Dp following exposure to background water. B) There is an abundance of FLi cells in the Dp following exposure to amino acids. Micrometer bar = 50μm.

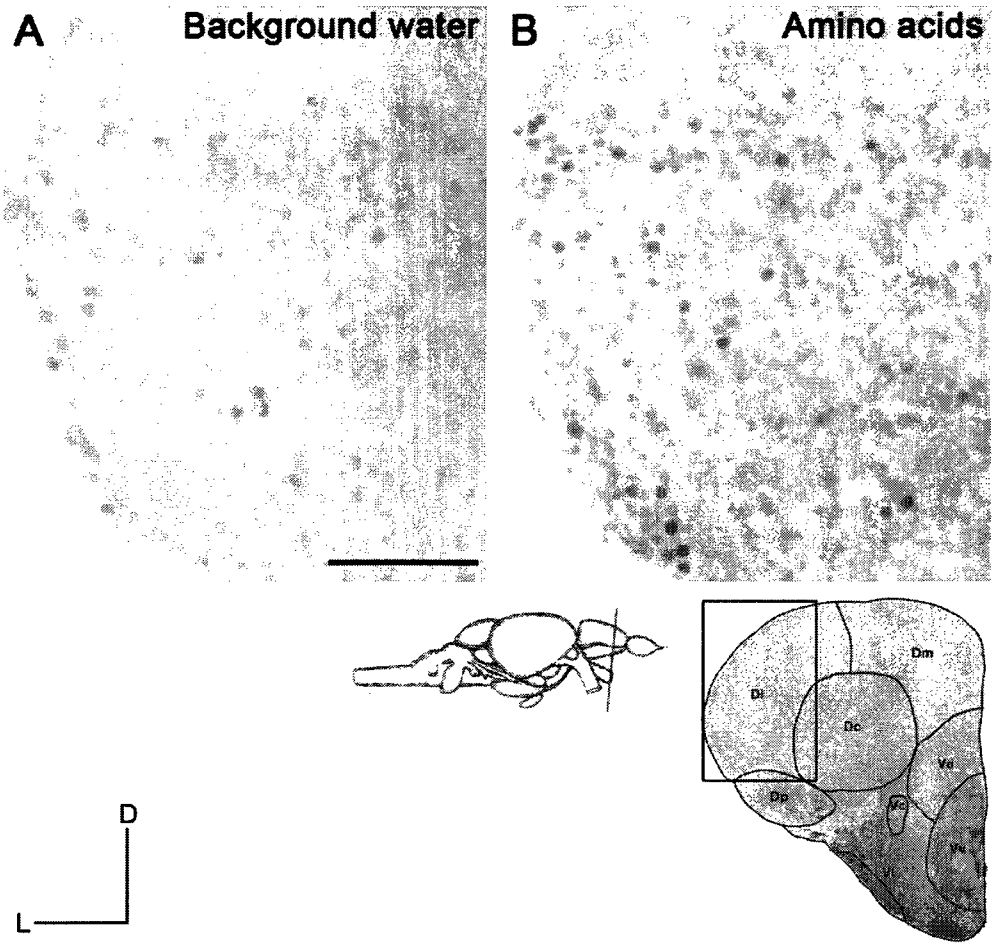


Figure 14 – FLi cells in the lateral zone of dorsal telencephalon (Dl) after exposure to amino acids. A) There are a few FLi cells in the Dl following exposure to background water. B) FLi cells following exposure to amino acids. Micrometer bar = 50 μm.

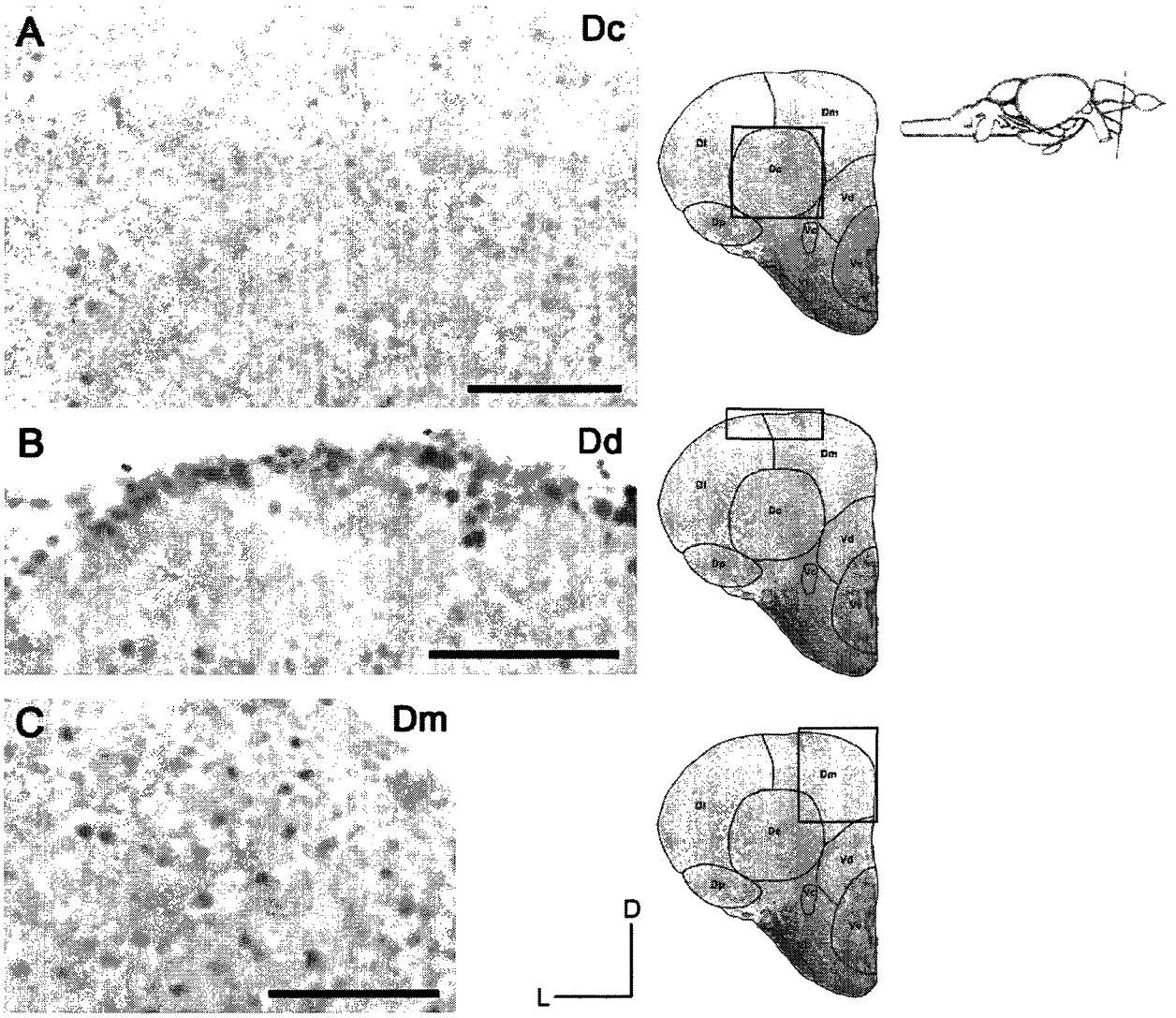


Figure 15 – FLi cells in the dorsal telencephalon following the delivery of amino acids. A) The central zone of dorsal telencephalon (Dc) showing faintly labelled FLi cells. B) Strongly labelled cells are concentrated at the periphery of the dorsal zone of dorsal telencephalon (Dd). C) The medial zone of dorsal telencephalon (Dm) contained FLi cells. Micrometer bar = 50 μm

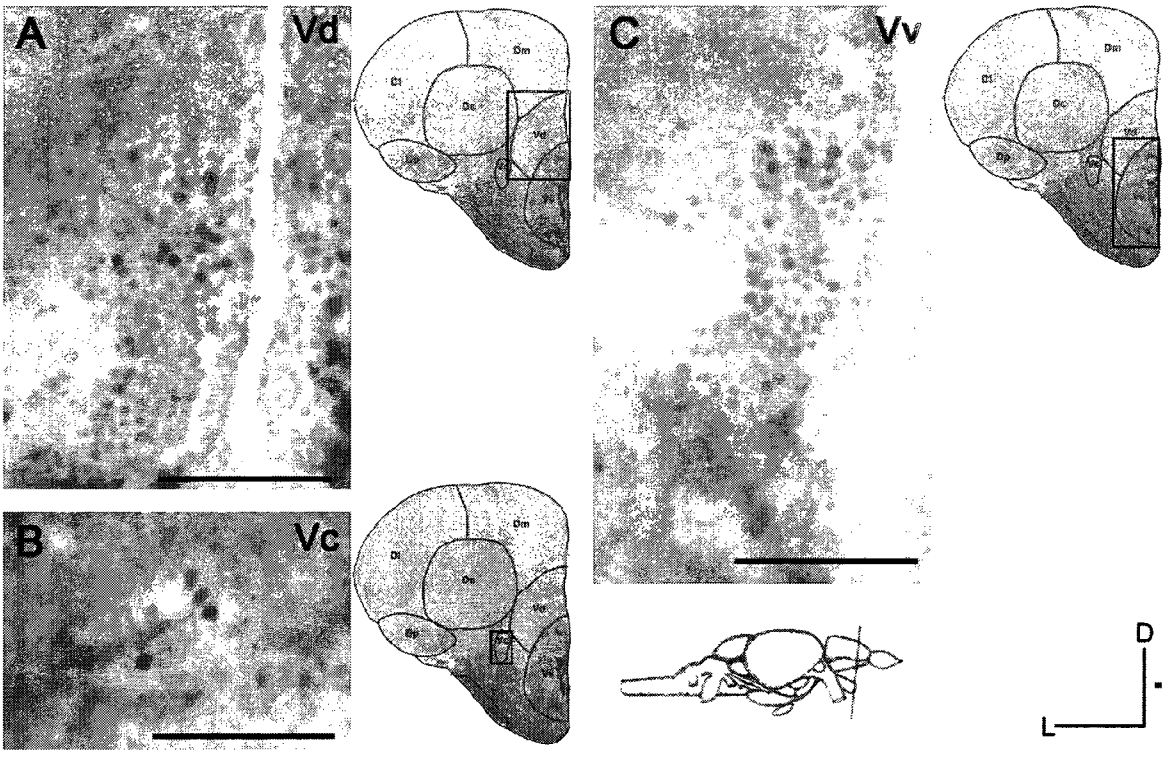


Figure 16 – Localization of FLI in the ventral telencephalon following exposure to amino acids. Micrometer bars = 50 μm A) The dorsal nucleus of ventral telencephalon (Vd) showing FLi cells. B) The central nucleus of ventral telencephalon (Vc) showing FLi cells. C) The ventral nucleus of ventral telencephalon (Vv) showing FLi cells.

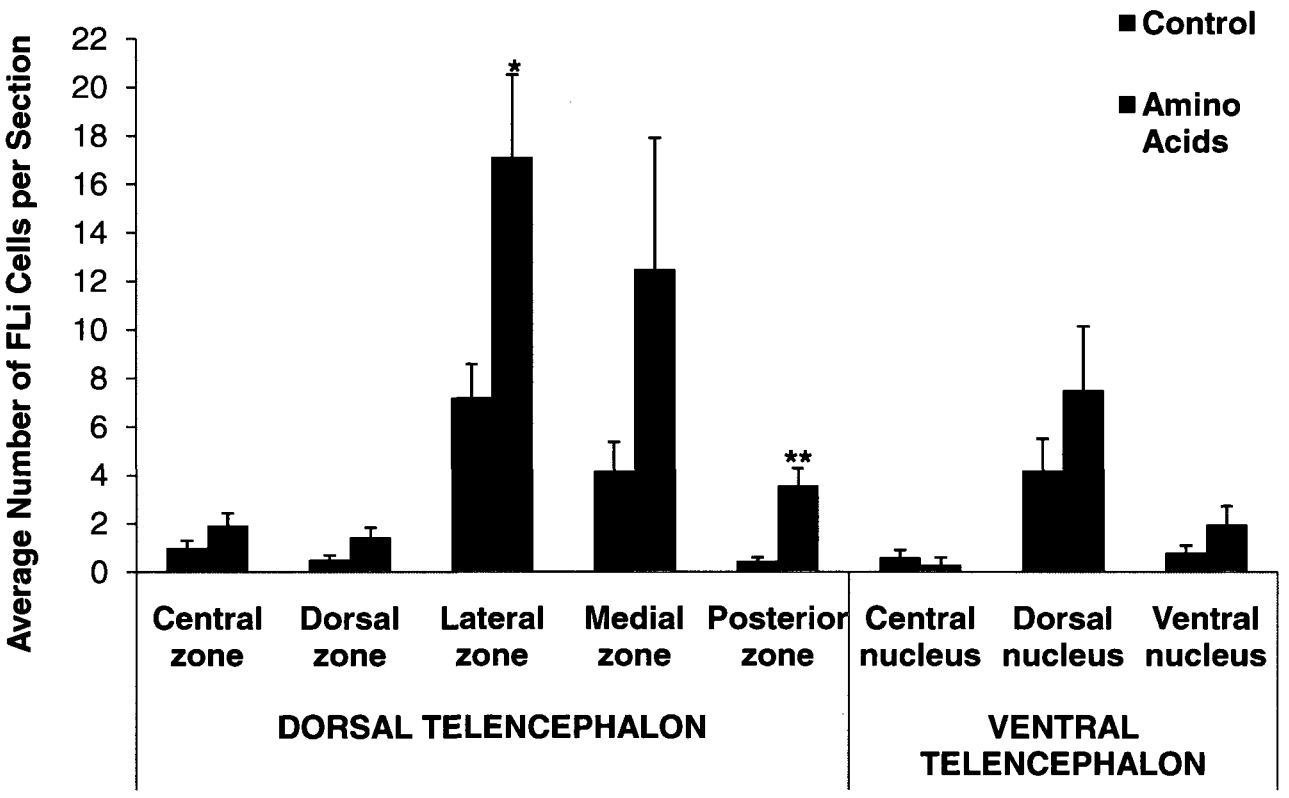


Figure 17 – Quantitative analysis of FLI in the telencephalon following exposure to amino acids. An average of FLi cells per section of telencephalic regions was calculated from each fish, and these values were used to calculate group means. (n=10 background water; n = 10 amino acids). Refer to Appendix B for cell counts.

A detailed view in the olfactory bulb and telencephalic areas (Dl, Dp) affected by the amino acids revealed consistent increase in FLi cells in the amino acid treated fish (Fig. 18). In this analysis, the FLI in the OB, Dl, and Dp from each individual zebrafish (Fish 1 – Fish 10) was compared to sections taken from the same regions in background treated fish (i.e. there were sections from 10 fish treated to background water). There was great variability of FLI following exposure to the same concentration and mixture of amino acids odour (but in all the amino acid

treated fish there was more FLi than the background water treated fish) in the olfactory bulb. The ratio values of FLi cells from treated (amino acids) to negative control (background water) fish were 1.07 (fish 8), 1.60 (fish 10), 1.83 (fish 2), 2.33 (fish 3), 2.44 (fish 7), 2.62 (fish 9), 2.70 fish 5), 4.73 (fish 6), 6.87 (fish 1), and 7.83 (fish 4). In the Dp, the ratio values of FLi cells from treated to negative control fish were 1.75 (fish 9), 2.00 (fish 10), 2.20 (fish 2), 2.50 (fish 4), 4.25 (fish 7), 4.67 (fish 5), 7.00 (fish 6), 8.00 (fish 3), 10.8 (fish 1), and 17.0 (fish 8, outlier not shown on graph). In the Dl, the ratio values of FLi cells from treated to negative control fish were 1.37 (fish 5), 2.03 (fish 2), 2.05 (fish 8), 2.58 (fish 1), 2.90 (fish 4), 3.00 (fish 3), 3.30 (fish 9), 3.38 (fish 7), 4.42 (fish 6), and 6.00 (fish 10).

There were some experiments in which the Dl and Dp regions of negative controls contained high levels of FLI (Appendix B). It is interesting to note that in these fish the FLI values were high in the olfactory bulb as well. This implies that there is a relationship between the olfactory bulb, Dl, and Dp, because even when the olfactory bulb was stimulated in negative controls for unknown reasons (perhaps by released urine), the Dl and Dp regions were stimulated as well (Table 2). The fish in which there was more FLI in negative controls compared to amino acids treated fish, were not included in the calculation of group means for graphs and statistics. Since there was an increase in FLI in the olfactory bulb, Dl, and Dp, following stimulation by amino acids, it is possible that in negative control fish which contained high levels of FLI, there was an odour stimulus present in the water (urine, pheromones). Therefore, including these fish in the group means is inaccurate because it is not certain that the negative controls were in fact only swimming in background water, when all the olfactory regions are stimulated.

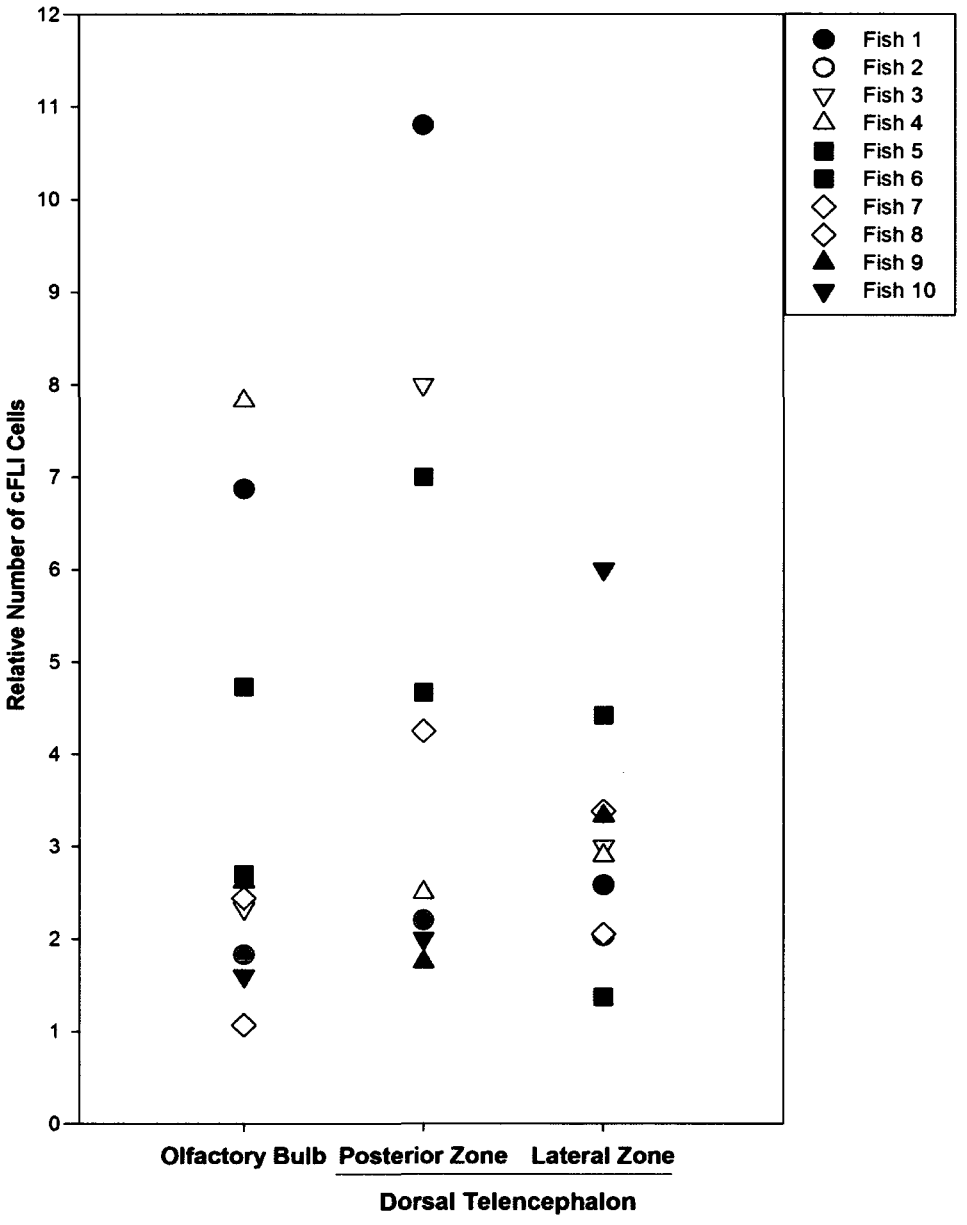


Figure 18 – Ratio of FLi cells in the olfactory bulb, and lateral (Dl) and posterior (Dp) zones of dorsal telencephalon, following exposure to amino acids, to FLi cells following exposure to background water. The average number of FLi cells per section from each fish exposed to amino acids, was divided by the average number of FLi cells per section from each fish exposed to background water. This resulted in a relative value showing how many times more FLi cells are present in treated (amino acids) fish compared to negative controls (background water) in the olfactory bulb, Dl, and Dp.

3.4.3. Gustation

The main gustatory regions in the brain were investigated to observe if there were changes in FLI following prolonged exposure to amino acids, in the absence of food, or other feeding cues. Since gustatory input is received by the secondary gustatory nucleus (SGN), and the tertiary gustatory nucleus (TGN), these two regions were assessed for FLI. There were no FLi cells found in the SGN (Fig. 19) and the TGN following delivery of background water (n=4 fish) or amino acids (n=4 fish) (Appendix F).

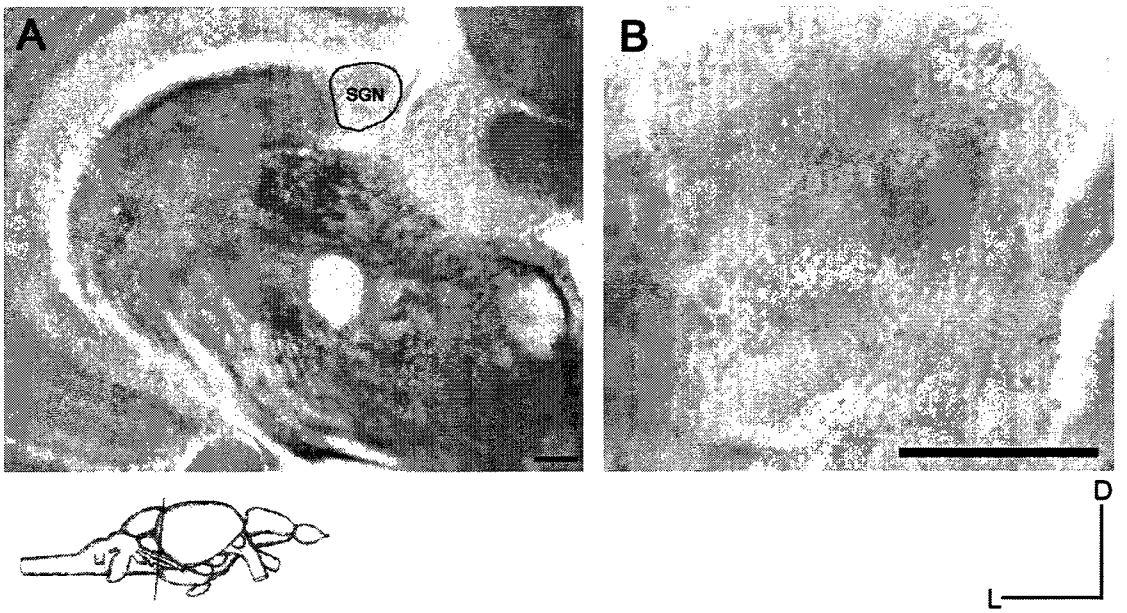


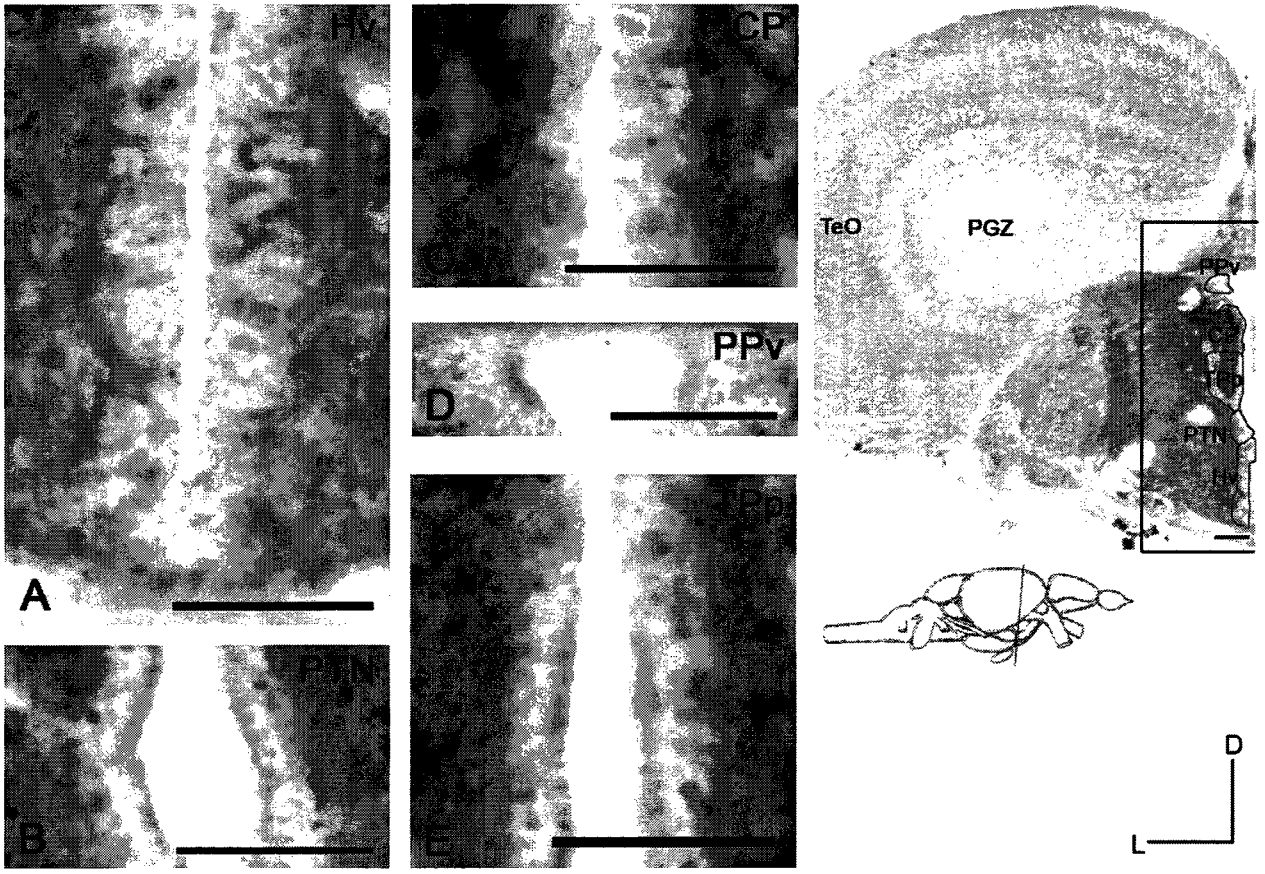
Figure 19 – FLI in the gustatory region, the secondary gustatory nucleus (SGN). A) One side of zebrafish midbrain with the SGN region labelled at low magnification. B) There were no FLi cells in this SGN section of the gustatory midbrain region. Micrometer bar = 50μm.

3.4.4. Midbrain

The midbrain was investigated because it may be responsible for relaying olfactory input to locomotor centers. The midbrain regions which contained darkly labelled cells were counted in zebrafish that were exposed to background water, and amino acids (Figs. 20-21). The following regions of the midbrain were explored using FLI: ventral zone of periventricular hypothalamus (Hv) (Fig. 20A); posterior tuberal nucleus (PTN) (Fig. 20B); central posterior thalamic nucleus (CP) (Fig. 20C); periventricular pretectal nucleus, ventral part (PPv) (Fig. 20D); periventricular nucleus of posterior tuberculum (TPp) (Fig. 20E); nucleus of medial longitudinal fascicle (NMLF); oculomotor nucleus (NIII); trochlear nucleus (NIV); and superior reticular formation (SRF) (Fig. 21). The PTN and TPp were investigated because these regions relayed olfactory input to locomotor regions in the hindbrain following amino acids exposure in sea lamprey (Derjean et al, 2010). Regions surrounding the PTN and TPp, such as Hv, CP, and PPv were also investigated because they contained cFLI cells, and may have been involved in an olfactory-locomotor circuitry. The NMLF, NIII, NIV and SRF were investigated because these regions contained FLI during the startle response behaviour in rainbow trout (Bosch et al, 2001), and therefore may be responsible for other locomotory behaviours as well.

The average number of FLi cells per section from fish exposed to background water (n=4 fish) and amino acids (n=4 fish) was calculated from the following regions: Hv [control, 0.13 +/- 0.13 cells; amino acids, 0.063 +/- 0.063 cells], TPp [control, 0.52 +/- 0.18 cells; amino acids, 0.3 +/- 0.24 cells], PPv [control, 0.08 +/- 0.08 cells; amino acids, 0.75 +/- 0.44 cells], CP [control, 1.9 +/- 1.2 cells; amino acids, 0.88 +/- 0.38 cells], PTN [control, 0.13 +/- 0.13 cells; amino acids, 0.23 +/- 0.16 cells], NMLF [control, 0.42 +/- 0.16 cells; amino acids, 0.83 +/- 0.4 cels], NIII [control, 0.25 +/- 0.14 cells; amino acids, 0.63 +/- 0.38 cells], NIV [control, 1 +/- 0.41

cells; amino acids, 0.5 +/- 0.5 cells], and SRF [control, 0.21 +/- 0.12 cells; amino acids, 0.14 +/- 0.1 cells]. There were no significant differences in FLI in any observed midbrain regions between amino acids and background water exposed specimens (Fig. 22).



CP = Central posterior thalamic nucleus
Hv = Ventral zone of periventricular hypothalamus
PGZ = Periventricular gray zone of optic tectum
PPv = Periventricular pretectal nucleus, ventral part
PTN = Posterior tuberal nucleus
TeO = Tectum opticum
TPp = Periventricular nucleus of posterior tuberculum

Figure 20 – Localization of FLI in midbrain regions. A) The ventral zone of periventricular hypothalamus (Hv) showing few FLi cells. B) The posterior tuberal nucleus (PTN) showing few FLi cells. C) The central posterior thalamic nucleus (CP) showing no FLi cells. D) The perviventricular pretectal nucleus, ventral part (PPv) showing no FLi cells. E) The periventricular nucleus of posterior tuberculum (TPp) showing one FLi cell. Micrometer bar = 50μm.

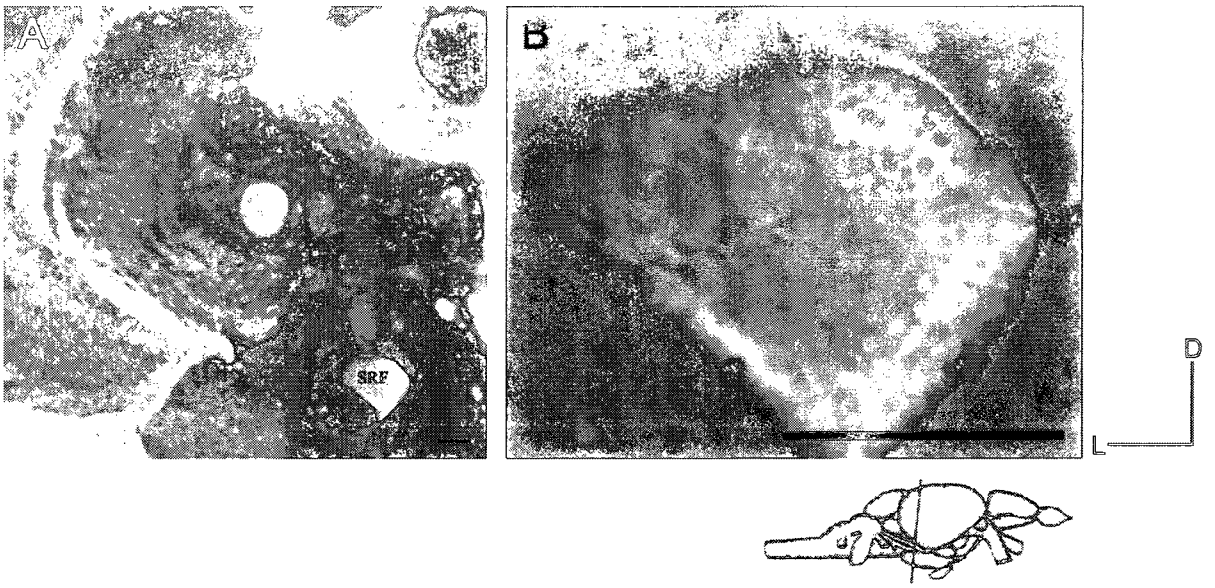
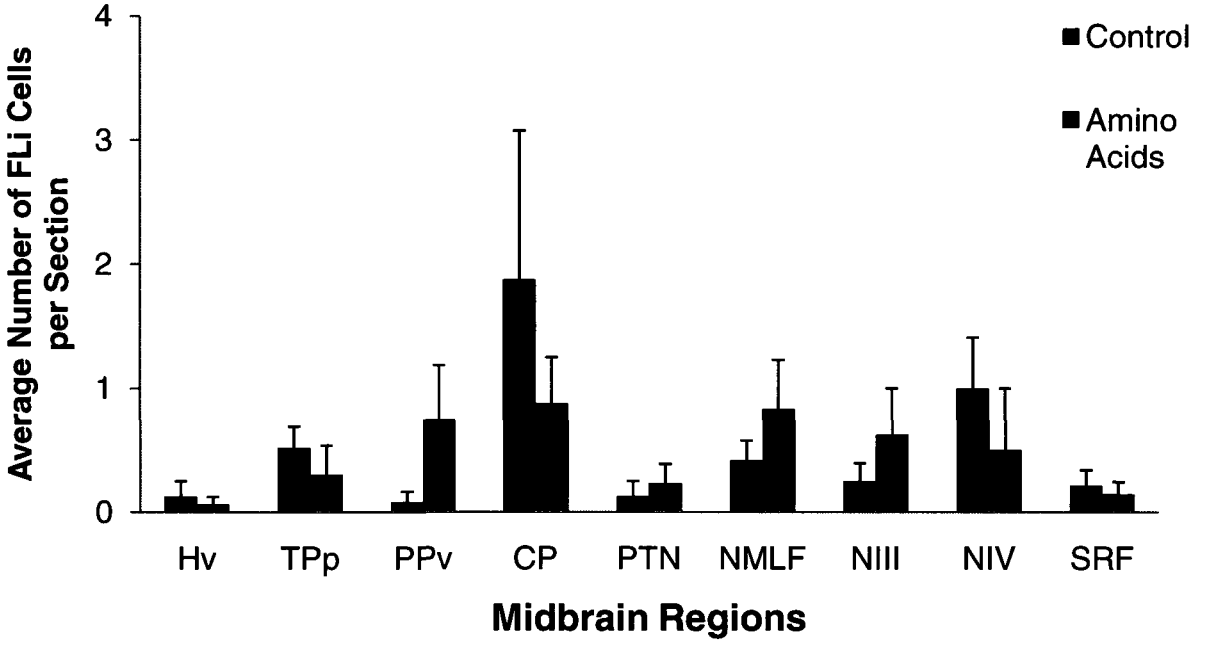


Figure 21 – Localization of FLi cells in superior reticular formation (SRF). A) One side of zebrafish midbrain with the SRF region shown at low magnification. B) There were no FLi cells in this SRF section in zebrafish midbrain. Micrometer bar = 50μm.



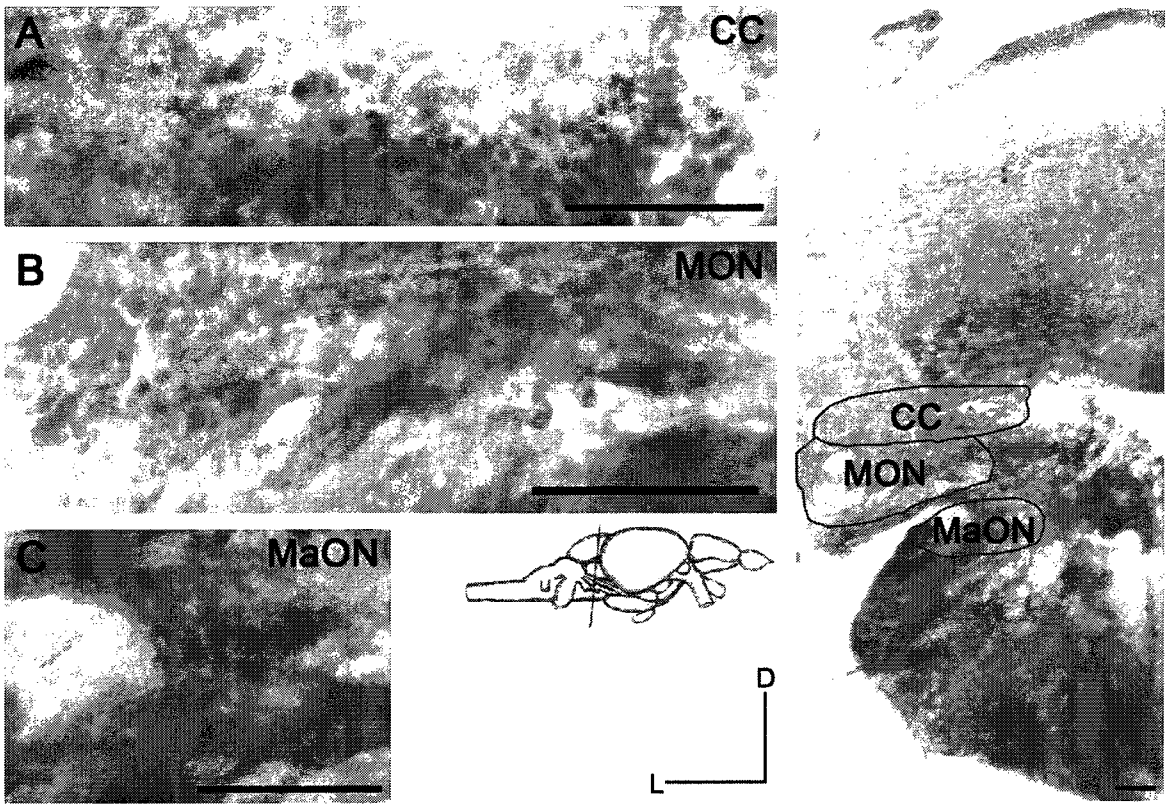
Hv	Ventral zone of periventricular hypothalamus
TPp	Periventricular nucleus of posterior tuberculum
PPv	Periventricular pretectal nucleus, ventral part
CP	Central posterior thalamic nucleus
PTN	Posterior tuberal nucleus
NMLF	Nucleus of medial longitudinal fascicle
NIII	Oculomotor nucleus
NIV	Trochlear nucleus
SRF	Superior reticular formation

Figure 22 – Quantitative analysis of FLi cells in midbrain regions following exposure to background water and the amino acids mixture. An average number of FLi cells per section of midbrain regions was calculated from each fish, and the group means were calculated for the graph (n=4 background water fish; n=4 amino acids fish). Refer to Appendix C for cell counts.

3.4.5. Hindbrain

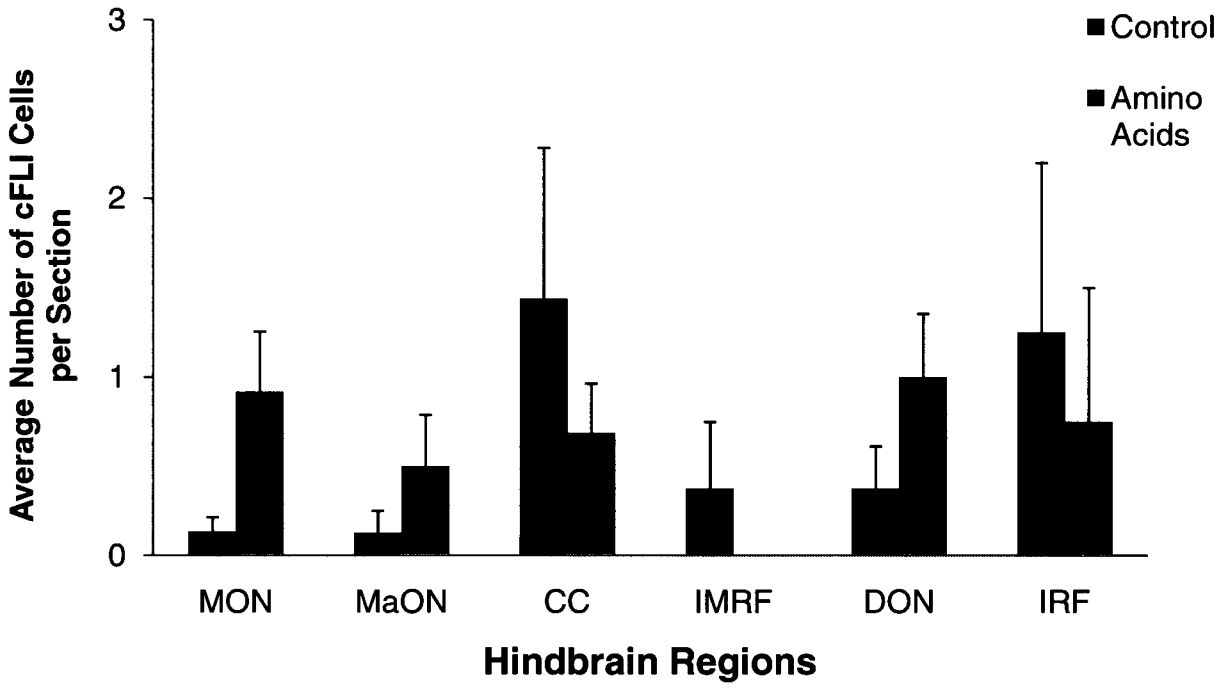
Hindbrain regions are important for controlling movement, and they were investigated to see if there was a change in FLI in cells following delivery of background water and mixture of amino acids (Fig. 23). The following hindbrain regions which had FLi cells were counted from: crista cerebellaris (CC) (Fig. 23A); medial octavolateralis nucleus (MON) (Fig. 23B); magnocellular octaval nucleus (MaON) (Fig. 23C); intermediate reticular formation (IMRF); descending octaval nucleus (DON); and inferior reticular formation (IRF). The MON, MaON, DON, and IRF were investigated because these regions increased in FLI following the startle response in rainbow trout (Bosch et al, 2001), and might be responsible for other swimming behaviours as well. The CC was investigated because it is located directly above the MON and DON, and contained FLI in most sections. The IRF was investigated because it is an extension of the SRF in the midbrain which was also examined in this study.

The average number of FLi cells per section from fish exposed to background water (n=4) and amino acids (n=4) was calculated from the following regions: MON [control, 0.13 +/- 0.08 cells; amino acids, 0.92 +/- 0.34 cells], MaON [control, 0.13 +/- 0.13 cells; amino acids, 0.5 +/- 0.29 cells], CC [control, 1.44 +/- 0.84 cells; amino acids, 0.69 +\- 0.28 cells], IMRF [control, 0.38 +/- 0.38 cells; amino acids, 0 +/- 0 cells], DON [control, 0.38 +/- 0.24 cells; amino acids, 1 +/- 0.35 cells], and IRF [control, 1.25 +/- 0.95 cells; amino acids, 0.75 +/- 0.75 cells]. There were no significant differences in FLI observed in any hindbrain regions between treated and untreated specimen (Fig. 24). There was very dark background staining present in the hindbrain regions, and this may be because the ICC protocol in this study was extensively modified for the olfactory bulb. Therefore, the protocol would have to be modified to achieve optimal labelling in the hindbrain.



CC = Crista cerebellaris
MON = Medial octavolateralis nucleus
MaON = Magnocellular octaval nucleus

Figure 23 – FLI in cross-sectional views of the hindbrain. A) The crista cerebellaris (CC) showing no FLi cells. B) The medial octavolateralis nucleus (MON) showing no FLi cells. C) The magnocellular octaval nucleus (MaON) showing no FLi cells. Micrometer bar = 50μm.



MON	Medial octavolateralis nucleus
MaON	Magnocellular octaval nucleus
CC	Crista cerebellaris
IMRF	Intermediate reticular formation
DON	Descending octaval nucleus
IRF	Inferior reticular formation

Figure 24 – Quantitative analysis of FLi cells in hindbrain regions following exposure to background water and amino acids mixture. There was no change observed in hindbrain regions from zebrafish brain following exposure to amino acids. An average number of FLi cells per section of each fish was calculated, and then group means were taken (n=4 background water fish; n=4 amino acids fish). Refer to Appendix D for cell counts.

3.4.6. Regions with Basal Levels of FLI

A principal midbrain region for visual integration, the periventricular gray zone of optic tectum (PGZ) (Gahtan et al, 2005), always contained FLi cells (Fig. 25). We consistently found that there was FLI in cells of the PGZ region in zebrafish that were exposed to background water, and those exposed to amino acids. This ICC technique of localizing FLP in zebrafish brain might prove to be worthy for future studies on vision.

The spinal cord is the brain structure responsible for executing various motor behaviours (Saint-Amant & Drapeau, 1998). There were always FLi cells labelled in the spinal cord of negative control (background water) and treated (amino acids) fish, with no significant change or relationship (Fig. 26). Therefore, using this technique could be beneficial in conducting studies on motor movements and the spinal cord.

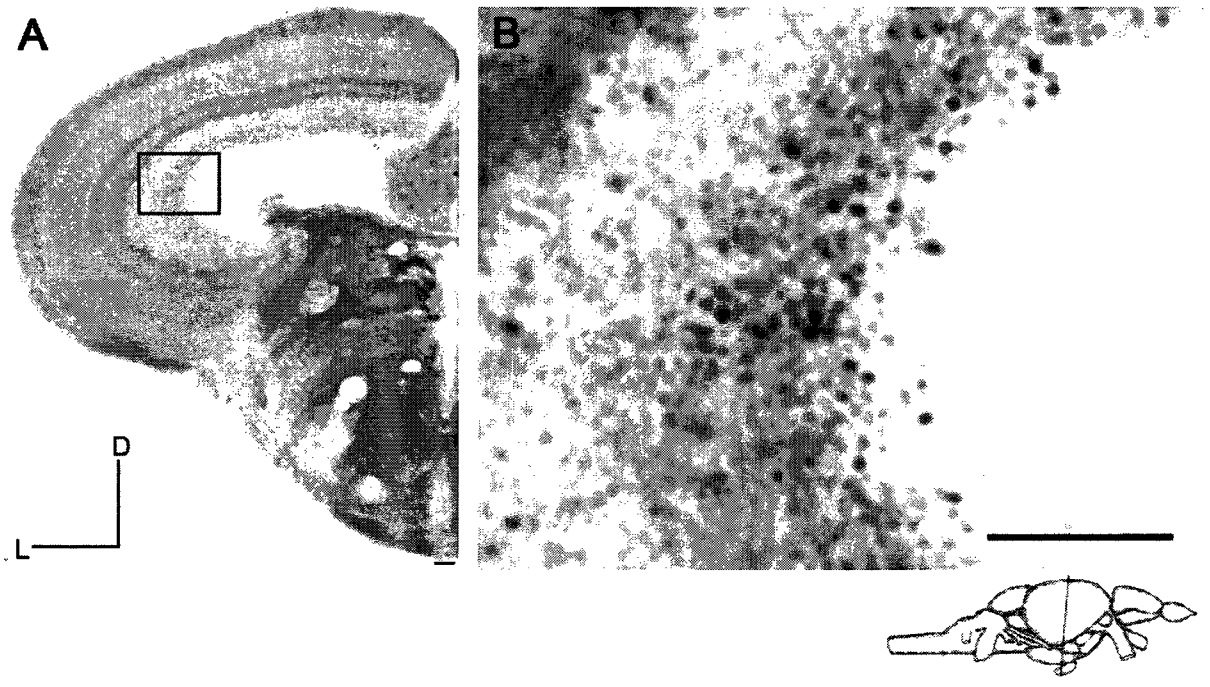


Figure 25 – Localization of FLi cells in the periventricular gray zone of the optic tectum (PGZ), in an amino acids treated specimen. A) Low magnification midbrain section which shows the location of the PGZ region. B) There were FLi cells found in the PGZ. Micrometer bar = 50μm.

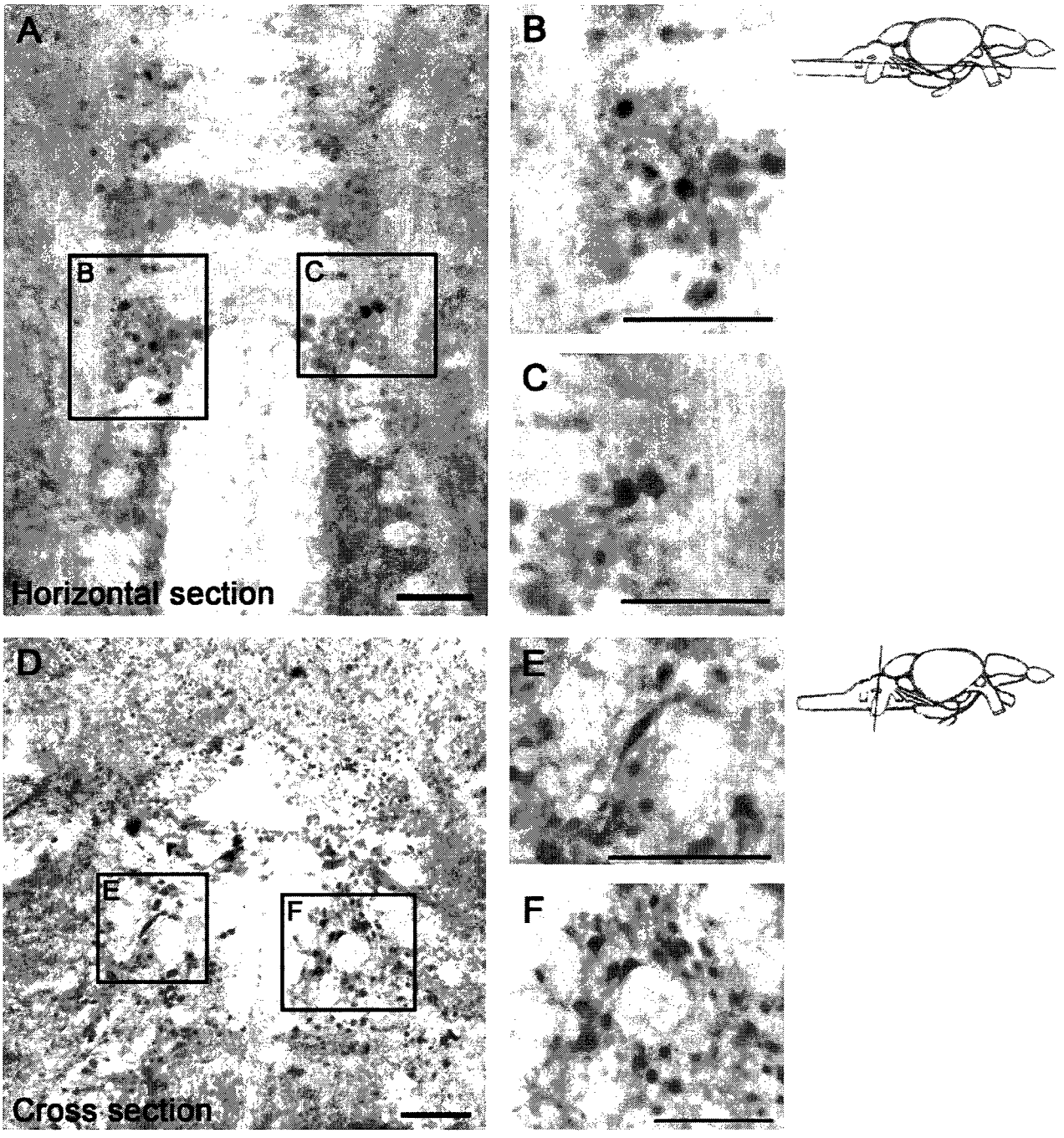


Figure 26 – Localization of FLi cells in the spinal cord. A) Horizontal section of the spinal cord showing FLi cells. B-C) FLi cells are magnified to show darkly labelled nuclei. D) Cross section of the spinal cord showing FLi cells. E-F) The FLi cells are magnified to show darkly labelled nuclei. Micrometer bar = 50 μm.

4. Discussion

This study is the first to localize FLP in zebrafish brain using ICC. This technique has been used extensively in mammalian studies, and with zebrafish becoming an important species for research, this method can help with many future studies using other olfactory cues, such as pheromones, urine, alarm substance, and bile acids. Furthermore, it has proven to be an effective method in observing active regions upon external stimuli, and it allows for the visualization of such regions throughout the entire brain. Previous studies have found that zebrafish with intact olfactory structures show attraction and appetitive behaviours towards amino acids (Braubach et al, 2009; Tierney et al, 2008). It was expected that there would be stimulation of neurons in brain regions following exposure to amino acids, and these could be labelled using localization of FLP.

In order to use the technique of localizing FLP, experiments were conducted to ensure that it would be an effective method for this study. The western blot was probed with sc-253 anti-c-Fos to label detected proteins in zebrafish head, and zn-12 anti-neuronal-marker to ensure the sample contained neural tissue and was still viable. The two bands produced after labelling with sc-253 were at molecular weights of 64 kDa and 77 kDa, comparable to the study in zebrafish by Hirayama et al (2005). The second band at 64 kDa was comparable to the molecular weight of c-Fos in mammalian studies, which is 62 kDa (Ezquer & Seltzer, 2003; Olesen & Auger, 2005), and there are many possibilities why there was labelling of a second band. The second band could correspond to FLP in a different post-translational modification stage (phosphorylated, glycosylated), which would increase the molecular weight of the protein. Generally, another cause for the second band on a western blot could be the dimerization of proteins. In this assay, sodium dodecyl sulfate (SDS) was used to break dimers, and the possibility of dimerization following the use of SDS is very low. Furthermore, the anti-fos (sc-253) was raised against a

specific portion from the human c-fos sequence (amino acids 110-160) which is similar to other genes (c-Fos protein like, proto-oncogene c-Fos, c-fos in zebrafish brain, CCAAT/enhancer-binding protein alpha) by more than 80%. The CCAAT/enhancer-binding protein alpha has a molecular weight of 43 kDa, and the second band on the western blot in this study was 77 kDa, so it is not likely that this protein is being probed in the western blot. Therefore, since the sequence against which sc-253 was raised against has high similarities (atleast >than 80%) to a c-Fos-like protein, proto-oncogene c-Fos, and c-fos, and all of these have molecular weights comparable to the proteins labelled in the western blot, sc-253 could be recognizing either of these proteins, and this may be the cause of the two labelled bands.

The pre-adsorption control showed that the FLi cells labelled with sc-253 possessed antigens like the blocking peptide (human c-fos). This was an important experiment because it confirmed that sc-253 does not bind to non-specific antigens in zebrafish tissue. In future studies, the c-fos sequence from zebrafish can be used to synthesize c-Fos protein, and can be used as a blocking peptide for the pre-adsorption control, and for western blotting to confirm that one or both of the bands is labelling c-Fos.

Zebrafish were exposed to PTZ to stimulate the regions observed by Baraban et al (2005), and to confirm the efficiency of using ICC to localize FLP. Baraban et al (2005) observed an increase in c-*fos* mRNA in midbrain regions following PTZ exposure, and in this study there was an increase in FLP in the hypothalamus, a midbrain region. This experiment provided support towards using an ICC technique to localize FLP.

Many experiments were conducted to test different concentrations and incubation times of the primary antibody, secondary antibody, and HRP-conjugated avidin. Several experiments were also conducted to determine the incubation time required for the DAB reaction to produce

labelling of optimal contrast (light background, darkly labelled nuclei) in the olfactory bulb of zebrafish.

The olfactory stimuli used in this study were amino acids because they are known to stimulate mitral cells in the olfactory bulb (Fuss & Korsching, 2001), and initiate feeding behaviours (Braubach et al, 2009) in zebrafish. Previous studies have shown that during amino acids delivery, all three types of OSNs can be stimulated, but the mOSNs are specific only to amino acids (Lipschitz & Michel, 2002; Sato & Suzuki, 2001; Vielma et al, 2008). Although studies have shown that mitral cells in the lateral region of olfactory bulb are stimulated by amino acids after receiving input from mOSNs (Hamdani et al, 2001), it is possible that cOSNs were also stimulated, and activated mitral cells in the medial region of olfactory bulb, because of the prolonged exposure (amino acids) time of 90 minutes.

Mitral cells have NMDA receptors and are stimulated by glutamate (Edwards, & Michel, 2002), which is proposed to be the beginning of a cascade of events resulting in the transcription of c-fos. Following exposure to amino acids, there was an increase of FLP distributed throughout the olfactory bulb. A chemotopic map suggesting that short exposure time (200 ms) to amino acids stimulated the lateral regions of the olfactory bulb, has been well established (Friedrich & Korsching, 1997; Friedrich & Korsching, 1998; Nikonov & Caprio, 2001). Yaksi et al (2009) also discovered that there was chemotopic organization of mitral cells in the olfactory bulb, but did not find this pattern in activated interneurons. In this study, there were no patterns observed in activated neurons in the olfactory bulb, and this may be because of labelled granule cells. In future studies, a double label against GABA would help distinguish between mitral cells and interneurons, and their organization in the olfactory bulb following amino acids exposure can be determined using FLI.

In zebrafish, after a few hundred milliseconds of amino acids delivery, the organization of odour processing in the ventro-lateral olfactory bulb began to change from a fixed glomerular pattern to a more broad distribution of mitral cells, and these changes were attributed to activated granule cells (Yaksi et al, 2007). Granule cells are inhibitory interneurons in the olfactory bulb, and they are responsible for fine-tuning of an olfactory response. When mitral cells are activated by OSNs, they release glutamate which activates granule cells. Granule cells can inhibit activity in the same pre-synaptic mitral cell and other mitral cells around them. They inhibit mitral cells with weak responses or spontaneous firing to produce a strong signal overall to send to telencephalic targets (Yokoi et al, 1995). Furthermore, the olfactory bulb also receives centrifugal fibers from other brain regions which synapse with granule cells (Luskin & Price, 1983), and could cause further inhibition in mitral cell activity. Therefore, after the initial chemotopic organization, the prolonged exposure time of 90 minutes allows for changes to start occurring in the olfactory bulb, caused by inhibitory interneurons and centrifugal fibers.

In this study, there was labelling of glomeruli structures observed in the olfactory bulb glomerular layer. Since hydrogen peroxide was used to initiate the staining reaction of DAB-Ni by metabolizing horseradish peroxidase (bound to avidin), there was a possibility of endogenous peroxidases in the glomerular region. Hydrogen peroxide was generated using the glucose oxidase method, and since activated neurons uptake glucose, this was another possible cause for the glomerular staining. In order to determine whether endogenous peroxidases and glucose uptake in axons, paired with the ICC technique, was labelling OSN axons, another experiment was conducted (data not shown) using immunofluorescence. In this method, the same primary and secondary antibodies were used as in ICC, but hydrogen peroxide and glucose oxidase were not required because the DAB-ni reaction is not used for immunofluorescence. However, there

was still glomerular staining using the immunofluorescence technique, and this experiment indicated that the staining must be due to an antigen on OSN axons that the primary antibody is binding to. Further studies need to be conducted to precisely understand why this labelling is occurring.

There was a significant increase in FLI in the Dl and Dp, following exposure to amino acids, compared to background water. The Dp region is homologous to the mammalian olfactory cortex, in which olfactory cues are processed in mammals (Wulliman & Mueller, 2004). Dp receives input from the olfactory bulb, Vd and Vv, and these two ventral regions also receive input from the olfactory bulb of zebrafish (Rink & Wulliman, 2004). Tract tracing experiments in goldfish have shown that Dl receives input predominantly from the olfactory bulb (Northcutt, 2006). Previous studies that used EOG in catfish (Nikonov et al, 2005), and calcium imaging in zebrafish (Yaksi et al, 2009) also observed activation in the Dl and Dp following delivery of amino acids. Therefore these studies supported the findings of the current investigation of olfactory processing in the Dl and Dp regions. Furthermore, the current study demonstrated the validity of localizing FLP in the olfactory structures of zebrafish brain.

When comparing the relative number of FLi cells from treated (amino acids) to negative control (background water) in the olfactory bulb, Dl and Dp, a great deal of variability is observed. Although there were consistently more FLI observed in the olfactory bulbs following exposure to amino acids compared to background water, there was no clear numerical relationship between the ratio values (treated/untreated) that encompassed all three regions in individual fish. Some variability was seen in negative controls; some were responsive under background water conditions in these regions, and the FLI values for amino acids treated fish were low. As previously discussed, the negative controls may have contained FLI in the

olfactory regions due to the presence of urine or pheromones in the water. The zebrafish exposed to amino acids which were not as responsive as expected could have deficiencies in their ability to smell, or simply were not hungry, as hormones are associated with satiation can affect olfactory sensory activity (Savigner et al, 2009). The variability in the Dl and Dp, can also be accounted for by the inhibitory effects and fine-tuning of the signal response by granule cells in the olfactory bulb.

In some cases, values for FLi cell counts in the olfactory bulb, as well as the Dl and the Dp were high in zebrafish exposed to background water. This demonstrated that when the olfactory bulb is stimulated by amino acids, or possibly even urine or pheromones (in the case of negative controls), we can expect to find stimulation in Dl and Dp regions.

There was an abundance of FLi cells in the Dm following amino acids treatment, but according to Northcutt (2006), the goldfish Dm receives input from many regions which process a variety of chemosensory information, such as the preglomerular nuclei, central posterior thalamic nucleus, posterior thalamic nucleus, and nuclei in the dorsal and ventral telencephalon. Therefore, the abundance of FLi cells in the Dm region may not entirely be due to olfactory input. Although there are known connections from the olfactory bulb to the ventral telencephalon (Rink & Wulliman, 2004), and the Vv region was stimulated using amino acids (Yaksi et al, 2009) in zebrafish, we did not find any significant results concerning FLI in any regions of the ventral telencephalon.

Gustatory regions which predominantly receive taste input, such as the secondary gustatory nucleus (SGN) and the tertiary gustatory nucleus (TGN) (Hara, 2007; Yoshimoto et al, 1998), were also investigated. There was no FLI found in either of these regions, although a previous electrophysiology study in catfish found activity in neurons of the SGN after

stimulating the oral cavity and extraoral surface with amino acids (Lamb & Caprio, 1992). Furthermore, a behavioural study in catfish found that even after olfactory deprivation, fish had no trouble in sensing feeding stimuli (Bardach et al, 1967). However, the extent of gustatory stimulation by amino acids can be variable across different species of fish. Some species rely predominantly on the gustatory sense to process amino acids, while others rely on the olfactory sense. In the behavioural study in zebrafish by Tierney et al (2008), following cauterization of the olfactory epithelium, the attraction to amino acids diminished. This suggested that the gustatory sense may not have played a big role during exposure of amino acids in zebrafish, or the concentration or mixture of amino acids used may not have been stimulatory. Furthermore, the presence of food might be important for the gustatory system to be stimulated. The lack of FLI in gustatory regions, specifically under these experimental conditions, suggested that the olfactory sense may be predominantly responsible for the processing of amino acids in zebrafish, or that the nuclei in these regions may not express c-Fos. However, since the gustatory response has not been well researched in zebrafish (Hara, 2007), further studies using electrophysiology or calcium imaging from gustatory regions would provide a more complete understanding of the involvement of the gustatory system in zebrafish.

In order to determine whether midbrain structures are responsible for processing input following an amino acids stimulus, we investigated regions in the midbrain which had cFLi cells. In this study, we did not find any patterns or significant changes in cFLI in any of the midbrain regions, including the posterior tuberal nucleus (PTN), and the periventricular nucleus of posterior tuberculum (TPp). In the sea lamprey, the posterior tuberculum was found to receive olfactory sensory input from the olfactory bulb after odour (including amino acids), and conveyed this information to locomotory hindbrain regions (Derjean et al, 2010). Therefore, the

lack of significant changes in FLI in the PTN and TPp suggested that the olfactory-locomotion pathway observed in the sea lamprey may not exist in zebrafish. Alternatively, this pathway may not be prone to expressing c-fos during activation.

When zebrafish were exposed to amino acids, there were no significant changes in FLI in any hindbrain regions that were investigated. Zebrafish swam towards the delivery input and spent more time in a small region surrounding the delivery source (Tierney et al, 2008). The swimming behaviour (activity, turns, etc.) was not investigated in the study, so it is not certain whether there was a change in motor activity. During the startle response in rainbow trout, the motor regions in the rainbow trout contained FLi cells (Bosch et al, 2001), confirming that those regions do contain glutamatergic neurons and are capable of inducing expression of c-fos. Since the posterior tuberculum (locomotor control region in sea lamprey) in the midbrain also did not significantly change in FLI in zebrafish, it is in accordance with the lack of change in FLI observed in hindbrain regions as well. In this study, a pathway between olfaction and locomotor regions was not observed. It is possible that amino acids were not a strong enough stimulant, and perhaps combining amino acids with food might have stimulated locomotor control regions in the zebrafish.

There was nuclear FLI in the periventricular gray zone of optic tectum (PGZ) in zebrafish that were exposed to background water and amino acids. The labelling intensity was generally always strong with darkly labelled nuclei spread throughout this region. The optic tectum primarily receives visual input from retinal ganglion cells (Smear et al, 2007). Therefore, the localization of FLP to mark activity using the ICC method may prove to be valuable for studies about the visual system in zebrafish.

There was dark labelling of FLi cells in the spinal cord of zebrafish following exposure to background water, and amino acids. In some zebrafish there were a few FLi cells labelled in the spinal cord, and in some zebrafish there was an abundance of FLi cells, regardless of the treatment. Localization of FLP could become a successful methodology for investigating activated neurons in the spinal cord in future investigations, using other stimuli which have a large effect in motor activity.

A drawback of this study was the dark non-nuclear background staining in the midbrain and hindbrain regions that were investigated. It is important to note that the protocol established in this study was adapted and modified to generate optimal labelling in the olfactory bulb. The concentrations of primary antibody, secondary antibody, and avidin-HRP may not have been the most favourable to get optimal labelling in regions other than the olfactory bulb. Furthermore, the amount of incubation time in DAB-ni reaction that produced good labelling in the olfactory bulb resulted in very dark background labelling in the midbrain and hindbrain. Therefore, to use this technique in future studies of the midbrain and hindbrain, modifications of the concentrations of materials and incubation time in DAB must be made.

5. Conclusion

In this study, FLP was used to mark neural activity in the brain using ICC, following exposure to an amino acids odour. Localization of FLP in zebrafish tissue is a novel methodology, and can now be used in future studies to further investigate the olfactory system using different odorant stimuli or in studies which explore vision and locomotion. However, when testing olfactory cues in an experiment such as this in which the experimental time is 90 minutes, there is a possibility of urine or pheromones interfering with the odour stimulus.

Furthermore to conduct studies in brain structures other than the olfactory bulb, modifications should be made to the concentrations or timing of incubation of primary and secondary antibodies, avidin-HRP and DAB, to ensure optimal contrast in background and nuclear labelling. Overall, this study was successful in using FLP to mark active regions of the olfactory system and provided a holistic approach to studying the processing of amino acids in the zebrafish brain.

Table 1 – Regions of the brain that were assessed for FLi cells

Brain Structure	Region	Abbreviations
Olfactory bulb	All	OB
Telencephalon	Central zone of D	Dc
	Dorsal zone of D	Dd
	Medial zone of D	Dm
	Posterior zone of D	Dp
	Lateral zone of D	Dl
	Central nucleus of V	Vc
	Dorsal nucleus of V	Vd
	Ventral nucleus of V	Vv
Midbrain	Ventral zone of periventricular hypothalamus	Hv
	Periventricular nucleus of posterior tuberculum	TPp
	Periventricular pretectal nucleus, ventral part	PPv
	Central posterior thalamic nucleus	CP
	Posterior tuberal nucleus	PTN
	Nucleus of medial longitudinal fascicle	NMLF
	Oculomotor nucleus	NIII
	Trochlear nucleus	NIV
	Superior reticular formation	SRF
Hindbrain	Medial octavolateralis nucleus	MON
	Magnocellular octaval nucleus	MaON
	Crista cerebellaris	CC
	Intermediate reticular formation	IMRF
	Descending octaval nucleus	DON
	Inferior reticular formation	IRF
Gustatory	Secondary gustatory nucleus	SGN
	Tertiary gustatory nucleus	TGN

Table 2 – Relationship of Fos-Like Immunoreactive Cells between Control (Background Water) and Treated (Amino Acids) Fish in Olfactory Bulb, and Lateral and Posterior Zones of Dorsal Telencephalon

Zebrafish Code	Olfactory Bulb (OB)	Dorsal Telencephalon	
		Lateral Zone of D (Dl)	Posterior Zone of D (Dp)
RB129	Control > Amino Acids	Control < Amino Acids	Control > Amino Acids
RB130	Control > Amino Acids	Control > Amino Acids	Control < Amino Acids
RB133	Control < Amino Acids	Control < Amino Acids	Control < Amino Acids
RB134	Control > Amino Acids	Control > Amino Acids	Control > Amino Acids
RB137	Control ~> Amino Acids	Control > Amino Acids	Control > Amino Acids
RB138	Control < Amino Acids	Control < Amino Acids	Control < Amino Acids
RB140	Control ~< Amino Acids	Control < Amino Acids	Control < Amino Acids
RB141	Control < Amino Acids	Control < Amino Acids	Control < Amino Acids
RB143	Control < Amino Acids	Control < Amino Acids	Control ~< Amino Acids
RB144	Control < Amino Acids	Control < Amino Acids	Control < Amino Acids
RB145	Control > Amino Acids	Control < Amino Acids	Control ~> Amino Acids
RB147	Control < Amino Acids	Control < Amino Acids	Control < Amino Acids
RB150	Control > Amino Acids	Control > Amino Acids	Control < Amino Acids
RB151	Control < Amino Acids	Control < Amino Acids	Control < Amino Acids
RB153	Missing Data	Control < Amino Acids	Control < Amino Acids
RB155	Control < Amino Acids	Control < Amino Acids	Control < Amino Acids
RB156	Control > Amino Acids	Control > Amino Acids	Control > Amino Acids
RB159	Control < Amino Acids	Control < Amino Acids	Control < Amino Acids

Same trend in OB, Dl, Dp

Same trend in OB, Dp

Same trend in OB, Dl

Same trend in Dl, Dp. No OB Data

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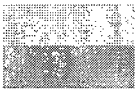
Appendix A – Counts of Fos-like Immunoreactive Cells from the Olfactory Bulb



These fish were used for statistics and graphs
This side was used for statistics and graphs

Olfactory Bulb					
		OB	# Fos-Like Labelled Nuclei per Section		
Fish #	Side	Total labelled nuclei/ # of sections	OB AVERAGE		
RB129 [Control]	L	48/2	24		
	R	15/1 section	15		
RB129 [Amino Acids]	L	9/2 sections	4.5		
	R	4/1 section	4		
RB133 [Control]	L	98/12	8.2		
	R	59/8	7.4		
RB133 [Amino Acids]	L	619/11	56.3		
	R	367/11	33.4		
RB144 [Control]	L	16/ 3 sections	5.3		
	R	11/3 sections	3.7		
RB144 [Amino Acids]	L	29/3 sections	9.7		
	R	36/3 sections	12		
RB147 [Control]	L	143/14	10.2		
	R	132/14	9.4		
RB147 [Amino Acids]	L	333/14	23.8		
	R	337/14	24.1		

RB151	L	14/6 sections	2.3		
[Control]	R	15/6 sections	2.5		
RB151	L	108/6	18		
[Amino Acids]	R	116/6	19.3		
RB155	L	20/10 sections	2		
[Control]	R	20/5 sections	4		
RB155	L	43/8 sections	5.4		
[Amino Acids]	R	47/7	6.7		
RB156	L	42/10	4.2		
[Control]	R	60/10	6		
RB156	L	22/11 sections	2		
[Amino Acids]	R	26/11 sections	2.4		
RB157	L	74/7	10.6		
[Control]	R	86/7	12.3		
RB157	L	No Bulbs	no bulbs		
[Amino Acids]	R	No Bulbs	no bulbs		
RB159	L	7/12 sections	0.6		
[Control]	R	18/12 sections	1.5		
RB159	L	No left bulb sections	no bulb sections		
[Amino Acids]	R	85/12	7.1		
RB130	L	186/10	18.6		
[Control]	R	112/7	16		
RB130	L	19/7 sections	2.7		
[Amino Acids]	R	62/11	5.6		
RB134	L	71/15	4.7		

[Control]	R	85/15	5.7		
RB134	L	8/13 sections	0.6		
[Amino Acids]	R	6/13 sections	0.5		
RB137	L	15/13	1.2		
[Control]	R	19/13	1.5		
RB137	L	14/12 sections	1.2		
[Amino Acids]	R	14/12 sections	1.2		
RB138	L	23/14	1.6		
[Control]	R	30/14	2.1		
RB138	L	43/11	3.9		
[Amino Acids]	R	42/14	3		
RB140	L	65/12	5.4		
[Control]	R	60/13	4.6		
RB140	L	76/13	5.8		
[Amino Acids]	R	60/11	5.5		
RB141	L	18/14	1.3		
[Control]	R	21/14	1.5		
RB141	L	47/14	3.4		
[Amino Acids]	R	19/7 sections	2.7		
RB143	L	0/12	0		
[Control]	R	3/12 sections	0.25		
RB143	L	4/12 sections	0.3		
[Amino Acids]	R	5/12 sections	0.4		
RB145	L	48/13	3.7		
[Control]	R	43/13	3.3		

RB145	L	28/14	2		
[Amino Acids]	R	32/13	2.5		
RB150	L	147/15	9.8		
[Control]	R	154/15	10.3		
RB150	L	101/12	8.4		
[Amino Acids]	R	30/12 sections	2.5		
RB153	L	No bulb sections	no bulb sections		
[Control]	R				
RB153	L	No bulb sections	no bulb sections		
[Amino Acids]	R				

Appendix B – Counts of Fos-like Immunoreactive Cells from Telencephalon Regions

This side was used for statistics and graphs

Telencephalon Regions					
	Number of Fos-like Immunoreactive Cells / Number of Sections				
	Dorsal Telencephalon				
	Central zone	Dorsal zone	Lateral zone	Medial zone	Posterior zone
RB133 [Control]	0/4 = 0	3/2 = 1.5	18/4 = 4.5	28/4 = 7	2/4 = 0.5
	5/5 = 1	2/3 = 0.67	26/5 = 5.2	13/5 = 2.6	5/5 = 1
RB133 [Amino Acids]	19/5 = 3.8	5/3 = 1.67	58/5 = 11.6	18/5 = 3.6	27/5 = 5.4
	6/5 = 1.2	5/3 = 1.67	66/5 = 13.2	21/5 = 4.2	20/5 = 4
RB144 [Control]	0/5 = 0	0/3 = 0	35/5 = 7	3/5 = 0.6	0/5 = 0
	0/5 = 0	0/3 = 0	44/5 = 8.8	1/5 = 0.2	0/5 = 0
RB144 [Amino Acids]	0/5 = 0	3/3 = 1	71/5 = 14.2	4/5 = 0.8	11/5 = 2.2
	3/5 = 0.6	0/3 = 0	74/5 = 14.8	5/5 = 1	9/5 = 1.8
RB147 [Control]	0/2 = 0	0/1 = 0	8/2 = 4	25/2 = 12.5	0/2 = 0
	0/2 = 0	0/1 = 0	7/2 = 3.5	42/2 = 21	0/2 = 0
RB147 [Amino Acids]	8/2 = 4	0/1 = 0	24/2 = 12	117/2 = 58.5	16/2 = 8
	8/2 = 4	3/1 = 3	34/2 = 17	143/2 = 71.5	20/2 = 10
RB151 [Control]	4/5 = 0.8	0/3 = 0	79/5 = 15.8	31/5 = 6.2	6/5 = 1.2
	13/5 = 2.6	1/3 = 0.33	64/5 = 12.8	28/5 = 5.6	7/5 = 1.4
RB151 [Amino Acids]	21/5 = 4.2	8/3 = 2.67	229/5 = 45.8	88/5 = 17.6	15/5 = 3
	21/5 = 4.2	1/3 = 0.33	196/5 = 39.2	65/5 = 13	17/5 = 3.4
RB155 [Control]	11/5 = 2.2	4/3 = 1.33	62/5 = 12.4	32/5 = 6.4	3/5 = 0.6
	13/5 = 2.6	6/3 = 2	44/5 = 8.8	43/5 = 8.6	0/5 = 0
RB155 [Amino Acids]	2/5 = 0.4	3/3 = 1	85/5 = 17	45/5 = 9	14/5 = 2.8
	1/5 = 0.2	4/3 = 1.33	96/5 = 19.2	40/5 = 8	8/5 = 1.6
RB159 [Control]	3/5 = 0.6	7/3 = 2.33	33/5 = 6.6	20/5 = 4	2/5 = 0.4
	2/5 = 0.4	5/3 = 1.67	39/5 = 7.8	4/4 = 0.8	1/5 = 0.2
RB159 [Amino Acids]	5/5 = 1	1/3 = 0.33	71/5 = 14.2	18/5 = 3.6	10/5 = 2
	0/5 = 0	0/3 = 0	53/5 = 10.6	13/5 = 2.6	7/5 = 1.4
RB138 [Control]	5/5 = 1	0/3 = 0	29/5 = 5.8	21/5 = 4.2	4/5 = 0.8
	2/5 = 0.4	1/3 = 0.33	16/5 = 3.2	16/5 = 3.2	9/5 = 1.8
RB138 [Amino Acids]	19/5 = 3.8	8/3 = 2.67	98/5 = 19.6	68/5 = 13.6	17/5 = 3.4

	16/5 = 3.2	16/3 = 5.33	91/5 = 18.2	67/5 = 13.4	8/5 = 1.6
RB140 [Control]	14/5 = 2.8	2/3 = 0.67	42/5 = 8.4	32/5 = 6.4	2/5 = 0.4
	17/5 = 3.4	1/3 = 0.33	52/5 = 10.4	44/5 = 8.8	0/5 = 0
RB140 [Amino Acids]	12/5 = 2.4	11/3 = 3.67	86/5 = 17.2	63/5 = 12.6	34/5 = 6.8
	17/5 = 3.4	3/3 = 1	101/5 = 20.2	35/5 = 7	19/5 = 3.8
RB141 [Control]	1/5 = 0.2	1/3 = 0.33	21/5 = 4.2	13/5 = 0.6	4/5 = 0.8
	8/5 = 1.6	2/3 = 0.67	27/5 = 5.4	9/5 = 1.8	2/5 = 0.4
RB141 [Amino Acids]	20/5 = 4	5/3 = 1.67	70/5 = 14	21/5 = 4.2	7/5 = 1.4
	11/5 = 2.2	5/3 = 1.67	77/5 = 15.4	25/5 = 5	19/5 = 3.8
RB143 [Control]	1/5 = 0.2	1/3 = 0.33	6/5 = 1.2	1/5 = 0.2	0/5 = 0
	1/5 = 0.2	0/3 = 0	1/5 = 0.2	1/5 = 0.2	0/5 = 0
RB143 [Amino Acids]	1/5 = 0.2	0/3 = 0	6/5 = 1.2	23/5 = 4.6	0/5 = 0
	5/5 = 1	0/3 = 0	30/5 = 6	5/5 = 1	1/5 = 0.2

Telencephalon Regions			
	Number of Fos-like Immunoreactive Cells / Number of Sections		
	Ventral Telencephalon		
	Central nucleus	Dorsal nucleus	Ventral nucleus
RB133 [Control]	0/1 = 0	26/2 = 12	10/2 = 5
	3/1 = 3	9/2 = 4.5	6/2 = 3
RB133 [Amino Acids]	0/1 = 0	3/2 = 1.5	1/2 = 0.5
	0/1 = 0	3/2 = 1.5	3/2 = 1.5
RB144 [Control]	0/1 = 0	4/2 = 2	1/2 = 0.5
	2/1 = 2	3/2 = 1.5	3/2 = 1.5
RB144 [Amino Acids]	0/1 = 0	8/2 = 4	0/2 = 0
	1/1 = 1	16/2 = 8	3/2 = 1.5
RB147 [Control]	0/1 = 0	28/2 = 14	0/2 = 0
	0/1 = 0	33/2 = 16.5	1/2 - 0.5
RB147 [Amino Acids]	0/1 = 0	55/2 = 27.5	15/2 = 7.5
	1/1 = 1	48/2 = 24	16/2 = 8
RB151 [Control]	0/1 = 0	12/2 = 6	3/2 = 1.5
	0/1 = 0	7/2 = 3.5	4/2 = 2
RB151 [Amino Acids]	0/1 = 0	11/2 = 6.5	4/2 = 2
	1/1 = 1	7/2 = 3.5	3/2 = 1.5
RB155 [Control]	0/1 = 0	13/2 = 6.5	0/2 = 0
	0/1 = 0	10/2 = 5	0/2 = 0
RB155 [Amino Acids]	0/1 = 0	4/2 = 2	0/2 = 0

	0/1 = 0	3/2 = 1.5	0/2 = 0
RB159 [Control]	0/1 = 0	0/2 = 0	0/2 = 0
	0/1 = 0	0/2 = 0	0/2 = 0
RB159 [Amino Acids]	0/1 = 0	0/2 = 0	0/2 = 0
	0/1 = 0	2/2 = 1	1/2 = 0.5
RB138 [Control]	1/1 = 1	10/2 = 5	2/2 = 1
	1/1 = 1	9/2 = 4.5	0/2 = 0
RB138 [Amino Acids]	0/1 = 0	23/2 = 11.5	10/2 = 5
	3/1 = 3	16/2 = 8	3/2 = 1.5
RB140 [Control]	1/1 = 1	3/2 = 1.5	2/2 = 1
	4/1 = 1	6/2 = 3	0/2 = 0
RB140 [Amino Acids]	3/1 = 3	27/2 = 13.5	3/2 = 1.5
	2/1 = 1	16/2 = 8	2/2 = 1
RB141 [Control]	2/1 = 2	2/2 = 1	0/2 = 0
	1/1 = 1	5/2 = 2.5	0/2 = 0
RB141 [Amino Acids]	2/1 = 2	7/2 = 3.5	2/2 = 1
	0/1 = 0	15/2 = 7.5	2/2 = 1
RB143 [Control]	0/1 = 0	0/2 = 0	0/2 = 0
	0/1 = 0	0/2 = 0	2/2 = 1
RB143 [Amino Acids]	0/1 = 0	0/2 = 0	0/2 = 0
	0/1 = 0	0/2 = 0	1/2 = 0.5

Appendix C – Counts of Fos-like Immunoreactive Cells from Midbrain Regions

Areas which barely had Fos-like labelled nuclei, so I did not add these regions into the graphs

Fish series (control and MIX) in which there were more Fos-like labelled nuclei in the MIX (treated) animals than control

This side was used for statistics and graphs

Midbrain Regions									
Fish Series	**Side**	**Hv**	**Hv MIX**	**ATN**	**ATN MIX**	**TPp**	**TPp MIX**	**Hav**	**Hav MIX**
RB129	L	1/4 sec	0/4	0/3	0/3	3/5 sec	1/5 sec	0/1	0/1
	R	2/4 sec	0/4	0/3	0/3	3/5 sec	0/5	0/1	0/1
RB134	L	0/4	0/4	0/3	0/3	0/5	4/5 sec	0/1	0/1
	R	0/4	0/4	0/3	0/3	0/5	7/5 sec	0/1	0/1
RB150	L	0/4	0/4	0/3	3/3 sec	1/5 sec	0/5 sec	0/1	0/1
	R	1/4 sec	0/4	0/3	3/3 sec	0/5	1/5 sec	0/1	0/1
RB151	L	2/4 sec	0/2	0/2	0/3	2/3 sec	0/5	0/1	0/1
	R	2/4 sec	2/2 sec	0/2	0/3	0/3 sec	0/5	0/1	0/1
RB153	L	0/4	0/4	0/3	0/3	4/5 sec	5/5 sec	0/1	0/1
	R	0/4	0/4	0/3	0/3	4/5 sec	1/5 sec	0/1	0/1
RB155	L	0/4	1/4 sec	0/3	0/3	3/5 sec	0/5	0/1	0/1
	R	0/4	0/4	0/3	0/3	2/5 sec	1/5 sec	0/1	0/1
RB156	L	0/4	0/4	0/3	0/3	7/5 sec	0/5	0/1	1/1 sec
	R	1/4 sec	1/4 sec	0/3	0/3	6/5 sec	0/5	0/1	0/1
RB157	L	0/4	0/4	0/3	0/3	0/5	1/5 sec	0/1	1/1 sec
	R	0/4	0/4	0/3	0/3	0/5	2/5 sec	1/1 sec	1/1 sec
RB159	L	0/4	0/4	0/3	0/3	0/5	1/5 sec	0/1	0/1
	R	0/4	0/4	0/3	0/3	0/5	0/5	0/1	0/1

Midbrain Regions									
Fish Series	Side	PTN	PTN MIX	NMLF	NMLF MIX	NIII	NIII MIX	NIV	NIV MIX
RB129	L	0/4	1/4 sec	3/3 sec	2/3 sec	0/2	0/2	0/1	0/1
	R	0/4	2/4 sec	2/3 sec	0/3	0/2	0/2	0/1	0/1
RB134	L	0/4	0/4	0/3	1/3 sec	0/2	0/2	0/1	0/1
	R	0/4	0/4	0/3	4/3 sec	0/2	0/2	0/1	0/1
RB150	L	0/4	12/4 sec	1/3 sec	3/2 sec	0/2	4/2 sec	0/1	0/1
	R	0/4	6/4 sec	3/3 sec	2/2 sec	0/2	2/2 sec	0/1	0/1
RB151	L	1/2 sec	0/4	0/3	0/3	1/2 sec	0/2	1/1 sec	0/1
	R	0/2	0/4	0/3	2/3 sec	0/2	1/2 sec	1/1 sec	1/1 sec
RB153	L	0/4	0/4	2/3 sec	4/3 sec	0/2	3/2 sec	2/1 sec	0/1
	R	0/4	0/4	2/3 sec	4/3 sec	0/2	1/2 sec	1/1 sec	0/1
RB155	L	0/4	0/4	1/3 sec	1/3 sec	1/2 sec	2/2 sec	1/1 sec	2/2 sec
	R	0/4	0/4	2/3 sec	2/3 sec	0/2	1/2 sec	1/1 sec	2/2 sec
RB156	L	0/4	1/4 sec	0/3	2/3 sec	0/2	2/2 sec	0/1	3/1 sec
	R	0/4	0/4	0/3	2/3 sec	0/2	1/2 sec	0/1	0/1
RB157	L	1/4 sec	0/4	1/3 sec	2/3 sec	0/2	0/2	0/1	0/1
	R	0/4	0/4	2/3 sec	2/3 sec	0/2	0/2	0/1	0/1
RB159	L	0/4	1/4 sec	2/3 sec	5/3 sec	0/2	0/2	0/1	0/1
	R	0/4	0/4	1/3 sec	1/3 sec	0/2	0/2	0/1	0/1

Midbrain Regions					
Fish Series	Side	SRF	SRF MIX	NVmd	NVmd MIX
RB129	L	0/7	0/7	0/3	0/3
	R	2/7 sec	1/7 sec	0/3	0/3
RB134	L	1/7 sec	6/7 sec	0/3	0/3
	R	3/7 sec	1/7 sec	1/3 sec	0/3
RB150	L	2/7 sec	0/7	0/3	0/3
	R	0/7	4/7 sec	0/3	2/3 sec
RB151	L	1/7 sec	0/7	4/3 sec	0/3
	R	2/7 sec	0/7	2/3 sec	0/3
RB153	L	4/7 sec	3/7 sec	0/3	0/3
	R	1/7 sec	0/7	0/3	0/3
RB155	L	1/7 sec	1/7 sec	0/3	0/3
	R	0/7	1/7 sec	0/3	0/3
RB156	L	0/7	0/7	0/3	0/3
	R	0/7	0/7	0/3	0/3
RB157	L	0/7	0/7	0/3	0/3
	R	0/7	0/7	0/3	0/3
RB159	L	0/7	0/7	0/3	0/3
	R	0/7	1/7 sec	0/3	0/3

Midbrain Regions						
Fish Series	Side	SRF	NVmv MIX	VIIs	VIIs MIX	
RB129	L	0/7	0/3	0/3	0/3	
	R	2/7 sec	0/3	0/3	0/3	
RB134	L	1/7 sec	0/3	0/3	0/3	
	R	3/7 sec	0/3	0/3	0/3	
RB150	L	2/7 sec	2/3 sec	0/3	0/3	
	R	0/7	3/3 sec	0/3	0/3	
RB151	L	1/7 sec	0/3	0/3	0/3	
	R	2/7 sec	0/3	0/3	0/3	
RB153	L	4/7 sec	0/3	0/3	0/3	
	R	1/7 sec	0/3	0/3	0/3	
RB155	L	1/7 sec	0/3	0/3	0/3	
	R	0/7	0/3	0/3	2/3 sec	
RB156	L	0/7	0/3	0/3	0/3	
	R	0/7	0/3	0/3	0/3	
RB157	L	0/7	0/3	0/3	0/3	
	R	0/7	0/3	0/3	0/3	
RB159	L	0/7	0/3	0/3	0/3	
	R	0/7	0/3	0/3	2/3 sec	

Appendix D – Counts of Fos-like Immunoreactive Cells in Hindbrain Regions

Areas which barely had Fos-like labelled nuclei, so I did not add these regions into the graphs

Fish series (control and MIX) in which there were more Fos-like labelled nuclei in the MIX (treated) animals than control

This side was used for statistics and graphs

Hindbrain Regions									
Fish Series	**Side**	**MON**	**MON MIX**	**MaON**	**MaON MIX**	**CC**	**CC MIX**	**T**	**T MIX**
RB129	L	1/5 sec	0/5	0/2	0/2	1/4 sec	7/4 sec	0/2	0/2
	R	1/5 sec	4/5 sec	0/2	0/2	1/4 sec	4/4 sec	0/2	0/2
RB134	L	0/5	1/3 sec	1/2 sec	5/2 sec	0/4	0/2	0/2	0/2
	R	0/5	0/3	0/2	9/2 sec	1/4 sec	0/2	0/2	0/2
RB150	L	6/5 sec	9/5 sec	0/2	4/2 sec	6/4 sec	2/4 sec	0/2	0/2
	R	0/5	7/5 sec	0/2	2/2 sec	4/4 sec	1/4 sec	0/2	0/2
RB151	L	1/3 sec	5/5 sec	0/2	0/2	0/2	5/4 sec	0/2	0/2
	R	0/3	0/5	1/2 sec	0/2	0/2	4/4 sec	0/2	0/2
RB153	L	0/5	9/5 sec	0/2	0/2	13/4 sec	4/4 sec	0/2	0/2
	R	0/5	8/5 sec	0/2	2/2 sec	8/4 sec	2/4 sec	0/2	0/2
RB155	L	1/5 sec	2/3 sec	0/2	0/1	9/4 sec	1/2 sec	0/2	0/1
	R	2/5 sec	1/3 sec	0/2	1/1 sec	2/4 sec	1/2 sec	0/2	0/1
RB156	L	0/5	0/5	0/2	0/2	2/4 sec	0/4 sec	0/2	0/2
	R	0/5	0/5	0/2	0/2	2/4 sec	0/4 sec	0/2	0/2
RB157	L	0/5	1/5 sec	0/2	0/2	0/4	2/4 sec	0/2	0/2
	R	1/5 sec	2/5 sec	0/2	0/2	0/4	1/4 sec	0/2	0/2
RB159	L	0/5	1/5 sec	0/2	0/2	0/4	0/4	0/2	0/2
	R	0/5	0/5	0/2	0/2	0/4	0/4	0/2	0/2

Hindbrain Regions									
Fish Series	Side	IMRF	IMRF MIX	SO	SO MIX	DON	DON MIX	IRF	IRF MIX
RB129	L	0/4	0/4	0/2	0/2	0/3	1/3 sec		
	R	0/4	0/4	0/2	0/2	0/3	2/3 sec		
RB134	L	0/4	0/2	0/2	0/2	1/3 sec	0/1		
	R	0/4	0/2	0/2	0/2	2/3 sec	0/1		
RB150	L	0/4	0/4	0/2	0/2	1/4 sec	6/4 sec	2/1 sec	6/1 sec
	R	0/4	0/4	0/2	1/2 sec	2/4 sec	3/4 sec	3/1 sec	7/1 sec
RB151	L	0/2	0/4	0/2	0/2	0/1	6/4 sec	no sec	0/1
	R	0/2	0/4	0/2	0/2	0/1	1/4 sec		0/1
RB153	L	0/4	0/4	0/2	0/2	4/4 sec	6/4 sec	0/1	3/1 sec
	R	0/4	0/4	0/2	0/2	5/4 sec	6/4 sec	0/1	2/1 sec
RB155	L	0/4	0/2	2/2 sec	0/1	4/4 sec	2/2 sec	4/1 sec	no section
	R	0/4	0/2	0/2	0/1	3/4 sec	3/2 sec	1/1 sec	
RB156	L	0/4	0/4	0/2	1/2 sec	0/4	0/4	3/1 sec	0/1
	R	0/4	0/4	0/2	1/2 sec	0/4	0/4	0/1	0/1
RB157	L	0/4	0/4	0/2	1/2 sec	2/4 sec	2/4 sec	1/1 sec	0/1
	R	0/4	0/4	0/2	0/2	0/4	2/4 sec	1/1 sec	0/1
RB159	L	6/4 sec	0/4	1/2 sec	0/2	5/4 sec	0/4	1/1 sec	0/1
	R	3/4 sec	0/4	2/2 sec	0/2	0/4	0/4	1/1 sec	0/1

Appendix E – Relationship between Olfactory Bulb, and Lateral and Posterior Zones of Dorsal Telencephalon

OB Relationship with Dl and Dp			
	# Fos-like Labelled Nuclei per Section		
Fish #	OB AVERAGE	Dl AVERAGE	Dp AVERAGE
RB129	24	3.2	0.6
[Control]	15	5.4	1.6
RB129	4.5	14.2	0.4
[Amino Acids]	4	18.4	1.6
RB133	8.2	4.5	0.5
[Control]	7.4	5.2	1
RB133	56.3	11.6	5.4
[Amino Acids]	33.4	13.2	4
RB144	5.3	7	0
[Control]	3.7	8.8	0
RB144	9.7	14.2	2.2
[Amino Acids]	12	14.8	1.8
RB147	10.2	4	0
[Control]	9.4	3.5	0
RB147	23.8	12	8
[Amino Acids]	24.1	17	10
RB151	2.3	15.8	1.2
[Control]	2.5	12.8	1.4
RB151	18	45.8	3
[Amino Acids]	19.3	39.2	3.4

RB155	2	12.4	0.6
[Control]	4	8.8	0
RB155	5.4	17	2.8
[Amino Acids]	6.7	19.2	1.6
RB156	4.2	7.6	1.8
[Control]	6	14.2	1.6
RB156	2	6.6	0.6
[Amino Acids]	2.4	5.6	0.4
RB157	10.6	11.2	1.4
[Control]	12.3	10.2	0.6
RB157	no bulbs	3.6	0.4
[Amino Acids]	no bulbs	6.6	0
RB159	0.6	6.6	0.4
[Control]	1.5	2.4	0.2
RB159	no bulb sections	14.2	2
[Amino Acids]	7.1	10.6	1.4
RB130	18.6	33	2.8
[Control]	16	21.8	4.6
RB130	2.7	23.8	4.4
[Amino Acids]	5.6	20.8	9.5
RB134	4.7	5.4	2.4
[Control]	5.7	12.4	1.6
RB134	0.6	4	0.4
[Amino Acids]	0.5	6.4	1.6

RB137	1.2	10.8	1.6
[Control]	1.5	13.8	0.6
RB137	1.2	9.2	0.4
[Amino Acids]	1.2	5.2	1.2
RB138	1.6	5.8	0.8
[Control]	2.1	3.2	1.8
RB138	3.9	19.6	3.4
[Amino Acids]	3	18.2	1.6
RB140	5.4	8.4	0.4
[Control]	4.6	10.4	0
RB140	5.8	17.2	6.8
[Amino Acids]	5.5	20.2	3.8
RB141	1.3	4.2	0.8
[Control]	1.5	5.4	0.4
RB141	3.4	14	1.4
[Amino Acids]	2.7	15.4	3.8
RB143	0	1.2	0
[Control]	0.25	0.2	0
RB143	0.3	1.2	0
[Amino Acids]	0.4	6	0.2
RB145	3.7	17.2	2.8
[Control]	3.3	23	1.4
RB145	2	21.4	2.2
[Amino Acids]	2.5	34	1.6
RB150	9.8	21	1.8

[Control]	10.3	15.8	2
RB150	8.4	15.4	4.4
[Amino Acids]	2.5	15.4	1.2
RB153	no bulb sections	8.8	0.8
[Control]		11.4	0
RB153	no bulb sections	17.4	3
[Amino Acids]		19.6	1.2

Appendix F – Counts of Fos-like Immunoreactive Cells in Gustatory Regions

Gustatory Regions					
Fish #	Side	TGN	TGNMix	SGN	SGN Mix
RB151	L	0	0	0	0
RB153	L	0	0	0	0
RB155	L	0	0	0	0
RB159	L	0	0	0	0

Appendix G – 4% Paraformaldehyde tissue fixative recipe

Makes 500 ml

Add 20 g of paraformaldehyde powder to 110 ml of distilled water.

Heat solution to approximately 55°C stirring for 10 minutes

Clear the solution by adding NaOH chips (approximately 2 chips) while stirring. Solution is clear when chips dissolve

Cool on ice if desired

Bring the volume of solution up to 250 ml with distilled water

Bring volume of solution up to 500 ml with 0.2M PB

Use HCl or NaOH to adjust pH to 7.4

Appendix H – 0.1M Phosphate buffer saline (PBS) recipe

Prepare Stock A:

- Dissolve 27.6 g of sodium phosphate monobasic into 1 liter distilled water

Prepare Stock B:

- Dissolve 28.4 g of sodium phosphate dibasic (anhydrous) into 1 liter distilled water

Add 190 ml of Stock A to 810 ml of Stock B. This makes 1 liter of 0.2M PB

Add 1 liter of distilled water to 0.2M PB. This makes 2 liters of 0.1M PB

Add the following to 1 liter of 0.1M PB

- 8 g NaCl
- 0.2 g KCl

Now there are 2 liters of 0.1M PBS. Use HCl or NaOH to adjust pH to 7.4

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