The Neural Substrate of Sex Pheromone Signalling in Male Goldfish (*Carassius auratus*)

by

Wudu E. Lado

Thesis submitted to the
Faculty of Graduate & Postdoctoral Studies
University of Ottawa
in partial fulfilment of the requirements for the PhD degree in the
Ottawa-Carleton Institute of Biology

Thèse soumise à la
Faculté des études supérieures et postdoctorales
Université d'Ottawa
en vue de l'obtention du doctorat ès sciences
L'Institut de biologie Ottawa-Carleton

© Wudu E. Lado, Ottawa, Canada, 2012

AKNOWLEDGEMENTS

"It takes a village to raise a child" so goes the saying. This thesis is the fruit of the teachings and inspirations of others who made me what I am today. I will like to thank my late father, Alex Lado, and my mother, Mary Nyoka, for their dedication in shaping and getting me started on life's journey. Without their tireless efforts and support I would not have made it. A big credit also goes to my teachers (past and present, mentioned and unmentioned) beginning with my junior high History and Geography teacher, Mr. Guben, who challenged me to look beyond academia. To my former headmaster, Fr. Todd Riebe, thank you for inspiring me to reach for the stars. To Dr. Michael Persinger, a fellow warrior and traveler in life, thanks for helping me start my journey in research and academia. To Dr. Vance Trudeau and Dr. John Lewis, thank you very much for giving me the opportunity of a lifetime not only to hone my science skills but also to explore my abilities.

This journey will not be complete without mentioning professors and colleagues (past and present) who made this journey interesting. Thanks are due to the following:

Trudeau Lab: Dr. Dapeng Zhang, Dr. Jan Mennigen, Dr. Jason Popesku, Jake Zamora, Andrew Waye and Agnes Crnic., Dr. Susanna Wiens, Dr. Vicki Marlatt, Dr. Rosalie Awad, Dr. Christopher Martyinuk, Dr. Paula Durate-Guterman, Dr. Rob Zhao, Dr. Valerie Langlois, Dr. Helene Silva de Assis, Chantal Lanctot, Lei Xing, Dr. Natacha Hogan, Dr. Colin Cameron, Dr. Ahmed Al-Ansari, David Poisson Pare, Kate Crump and Andrey Massarsky.

Lewis Labmates: Dr. Ginette Hupe, Isabelle Shanks, Daniel Zysman, Zhahong Qin, Gerri Mileva, Colleen Young, Mayron Moorhead, Nick Tassone, Alan Tang, Mike Ben-Ezra, Phil Kaindl, Pierce McKennirey, Nichole and Sally Groothuis.

My other colleague in the Biology Department: Shahram Eisa-Besgyi, Teye Omlin, Yusuke Kumai., Dr. Houman Ghasriani (our honoury biology member) Saud Ayed, Ali Zidane and Chris Hart.

My PhD candidacy, thesis committee members and examiners: Dr. Tom Moon, Dr. Steve Perry, Dr. Kate Gilmour, Dr. Jayne Yack, Dr. David Lean, Dr. John Arnason, Dr. Frederic Laberge, Dr. Michael Jonz and Dr. Jeff Dawson.

Finally, Dr. Andrew Whyment and especially Dr. David Spanswick deserve a special mention for teaching me the mysteries of patch clamp electrophysiology. Without their patient mentoring I will not have know that there is an art in the science of patch clamp electrophysiology. Special thanks go to Dr. Laia Navarro-Martin and Aziz Al-Habsi for teaching me the intricacies of RT-PCR. Last but not least, a special thanks to the staff of the Biology Department, Gita and Doreen plus Bill Fletcher for all the technical advice and help with the care of the goldfish. Bill, you are a gem! This work was supported by funding from the University of Ottawa, NSERC and OGS.

ABSTRACT

The transmission of sex pheromone-mediated signals is essential for goldfish reproduction. However, the neural pathways underlying this reproductive signalling pathway in the goldfish brain is not well described. Lesioning experiments have shown previously that two brain areas, the preoptic area (POA) and the ventral telencephali pars ventralis (Vv) in particular, are important for reproduction. We used patch clamp electrophysiology to study the electrical activities of POA and Vv neurons. Based on the intrinsic properties of these neurons, we suggest there are five different functional classes of POA neurons and a single class of Vv neurons. In addition, by electrically stimulating the olfactory bulb (OB), we were able to show that this primary sensory structure makes monosynaptic glutamatergic connections with both POA and Vv neurons.

While electrophysiology measures signalling events occurring at short time scales on the order of milliseconds to minutes, we were also interested in studying sex pheromone signalling in the goldfish brain over a long time scale. Thus, we describe changes in gene expression in male goldfish exposed to waterborne sex pheromones ($17\alpha,20\beta$ dihydroxy-4-pregene-3-one and Prostaglandin-$F_{2\alpha}$) over 6 hours. We perform cDNA microarrays on Prostaglandin-$F_{2\alpha}$-treated fish to study the rapid modulation of transcription and define the signalling pathways affected. Our microarrays showed that 71 genes were differentially regulated (67 up and 4 down). Through gene ontology enrichment analysis, we found that these genes were involved in various biological processes such as RNA processing, neurotransmission, neuronal development, apoptosis, cellular metabolism and sexual reproduction. RT-PCRs were performed to validate our

microarrays and to facilitate direct comparisons of the effects of the two sex pheromones, 17α,20β dihydroxy-4-pregene-3-one and Prostaglandin-$F_{2\alpha}$.

By combining electrophysiology and gene expression analyses, we were able to study sex-pheromone signalling on two different time scales. One short, occurring on the order of milliseconds to minutes, that involves electrical activities in the brain through the glutamatergic amino-3-hydroxy-5-methylisoxazole-4-propionate and N-methyl-D-aspartate receptors; and the other long occurring several hours later that involves changes in the gene expression levels of calmodulin and ependymin among other genes underlying neuroplasticity. Reproductive neuroplasticity in the goldfish may therefore require the activation of glutamatergic receptors which then activate downstream signals like calmodulin and ependymin to transform the sex pheromones-mediate signal into gene expression.

RESUME

La transmission de signaux par les hormones sexuelles est essentielle à la reproduction chez les cyprins dorés. Cependant, les voies neuronales qui assurent la transmission de ces signaux dans le cerveau des cyprins dorés sont mal connues. Des expériences de sectionnement ont démontré que deux régions en particulier sont importantes pour la reproduction; la zone pré-optique (POA) et la zone telencephali pars ventralis (Vv). Nous avons étudié l'activité électrique des neurones POA et Vv à l'aide d'enregistrements patch clamp. Grâce aux propriétés intrinsèques de ces neurones, nous suggérons cinq différentes classes fonctionnelles chez les neurones POA et une seule chez les neurones Vv. De plus, en stimulant le bulbe olfactif (OB), nous avons démontré que cette structure sensorielle primaire forme des connections glutamatergiques monosynaptiques avec les neurones POA et Vv.

Bien que les enregistrements électrophysiologiques mesurent les signaux de courtes durées, de l'ordre de millisecondes aux minutes, nous avons aussi fait une étude des signaux de phéromones sexuelles dans le cerveau des cyprins dorés sur une longue durée. Ainsi, nous décrivons des changements dans l'expression de gènes chez le cyprin doré mâle exposé à des phéromones sexuelles d'origine hydrique ($17\alpha,20\beta$ dihydroxy-4-pregene-3-one et prostaglandine-$F_{2\alpha}$) pour plus de 6 heures. Nous avons choisi d'étudier la modulation de transcription et établir les voies affectées à l'aide de puces à ADNc générées à partir de poissons exposés à la Prostaglandine-$F_{2\alpha}$. Les résultats ont montré que 71 gènes sont régulés de manière différentielle (67 augmentés and 4 diminués). L'analyse d'enrichissement de gènes ontologiques a démontré que ces gènes étaient impliqués dans différents processus biologiques comme le traitement des ARN, la

neurotransmission, le développement neuronal, l'apoptose, le métabolisme cellulaire, et la reproduction sexuelle. Une RT-PCR a été faite non seulement pour valider la puce à ADN mais aussi afin de faciliter la comparaison entre les deux phéromones sexuels (17α,20β dihydroxy-4-pregene-3-one et prostaglandine-$F_{2\alpha}$)

En combinant ces deux approches, analyse électrophysiologique et expression de gène, nous avons été capable d'étudier les voies de signalisation reliées aux phéromones sexuelles à deux différentes échelles de temps. Une de courte durée et incluant des activités électriques dans le cerveau à travers les récepteurs glutamatergiques 2-amino-3-5-methyl-3-oxo-1,2- oxazol-4-yl-propanoic acid et N-methyl-D-aspartate, de l'ordre de millisecondes aux minutes, et l'autre incluant l'étude du niveau d'expression de gènes de calmoduline et ependymine durant plusieurs heures parmi d'autres gènes impliqués dans les processus caractéristiques de la neuroplasticité. La neuroplasticité reproductive chez le cyprin doré nécessiterais donc l'activation des récepteurs glutaminergiques, qui à leur tour activerais les signaux tels que la calmoduline et ependymine afin de transformer les signaux provenant des phéromones sexuelles en expression de gène.

ABBREVATIONS

17,20βP: 17α,20β-dihydroxy-4-pregene-3-one

AADC: aromatic amino acid decarboxylase

ACSF: artificial cerebrospinal fluid

ACSF: artificial cerebrospinal fluid

AD: Androstenedione

ADP: after-depolarization potential

AHP: after-hyperpolarization potential

ALDOC: aldolase C

AMPA: amino-3-hydroxy-5-methylisoxazole-4-propionate

AMPAR: AMPA receptor

ANOVA: analysis of variance

AOB: accessory olfactory bulb

AOS: accessory olfactory system

APA: action potential amplitude

APD: action potential duration

APV: DL-2-amino-5-phosphonopentanoic acid

$CaCl_2$: calcium chloride

CaM: calmodulin

cDNA: complementary deoxyribonucleic acid

cGnRH-II: chicken GnRH

CNQX: 6-cyano-7-nitroquinoxaline-2,3-dione

CO_2 : Carbon-dioxide

cORN: ciliated olfactory neuron

CV_{ISI}: coefficient of variation of the inter-spike interval

CV_L: coefficient of variation of the latency

CV_{RT}: coefficient of variation of the rise time

DA: dopamine

DAG: diacylglycerol

DAVID: database for annotation, visualization & Integrated discovery

EOG: electroolfactogram

EPD: ependymin

EPSP: excitatory postsynaptic potential

GABA: g-aminobutyric acid

GAD: glutamic acid decarboxylase

GtH: Gonadotropin hormone (see LH)

Glu: glutamate

GluR: glutamate receptor (AMPAR subunit)

GnRH: gonadotropin-releasing hormone

GO: gene ontology

GPCR: G-protein coupled receptor

GRIF: gonadotropin release inhibitory factor

I_H: h current

IV: current-voltage relationship

IP_3: inositol 1,4,5-triphosphate

KA: kainate

KAR: KA receptor

KCl: potassium chloride

KH_2PO_4: potassium bicarbonate

KW: Kruskal-Wallis

LH: luteinizing hormone

LOT: lateral olfactory tract

$MgCl_2$: magnesium chloride

MOB: main olfactory bulb

mORN: microvillous olfactory neuron

MOS: main olfactory system

MOT: medial olfactory tract

mRNA: messenger ribonucleic acid

MWW: Mann-Whitney-Wilcoxon

NaCl: sodium chloride

$NaHCO_3$: sodium bicarbonate

NMDA: N-methyl-D-aspartate

NMDAR: NMDA receptor

NPO: nucleus preopticus

NR1: NMDA receptor subunit 1

NR2A: NMDA receptor subunit 2A

NR2B: NMDA receptor subunit 2B

NR2C: NMDA receptor subunit 2C

NR2D: NMDA receptor subunit 2D

O_2: Oxygen

OB: olfactory bulb

OR: olfactory receptor

ORN: olfactory receptor neuron

PCA: principle component analysis

$PGF_{2\alpha}$: prostaglandin-$F_{2\alpha}$

PIT: pituitary gland

PLC: phospholipase C

POA: preoptic area

PSP: postsynaptic potential

PSP RT: PSP rise time

RD: rebound depolarization

RDS: rebound depolarization spikes

R_{input}: Input resistance

RMP: resting membrane potential

RNA: ribonucleic acid

rRNA: ribosomal RNA

RS: rebound spike frequency

RT-PCR: Real-time Polymerase Chain Reaction

SD: standard deviation

SEM: standard error of the mean

sGnRH: salmon GnRH

Tau: time constant

TF: threshold frequency

TI: threshold current

VNO: vomeronasal organ

VR: VNO receptor

Vs: ventral telencephali pars supracomissuralis

Vv: ventral telencephali pars ventralis

TABLE OF CONTENT

AKNOWLEDGEMENTS ... i

ABSTRACT ... iii

RESUME ... v

ABBREVATIONS ... vii

TABLE OF CONTENT ... x

TABLE OF FIGURES ... xiii

TABLE OF TABLES ... xv

CHAPTER 1 ... 1

GENERAL INTRODUCTION ... 1

1.1 Overview ... 1

1.2 Sequence of Events Leading to Goldfish Spawning ... 3

1.3 Chemical Communication in Vertebrates ... 4

1.4 Chemical Communication in Goldfish ... 5

1.5 Classes of Sex Pheromones That Have Effects in Male Goldfish ... 7

1.5.1 17α, 20β dihydroxy-4-pregene-3-one (17,20βP) ... 8

1.5.2 Prostaglandin-$F_{2\alpha}$ ($PGF_{2\alpha}$) ... 8

1.5.3 Androstenedione (AD) ... 8

1.6 Neurohormones Controlling Goldfish Reproduction ... 9

1.6.1 Gonadotropin-Releasing Hormone (GnRH) ... 9

1.6.2 γ-Aminobutyric acid (GABA) ... 10

1.6.3 Dopamine (DA) ... 11

1.6.4 Glutamate (Glu) ... 12

1.7 Hypothesis and Objectives ... 14

1.8 Model Organism ... 15

1.9 Thesis organization ... 17

CHAPTER 2 ... 22

GLUTAMATE MEDIATES EXCITATORY SYNAPTIC NEUROTRANSMISSION FROM THE OLFACTORY BULB TO THE VENTRAL TELENCEPHALON VIA THE MEDIAL OLFACTORY TRACT IN MALE GOLDFISH (*Carassius auratus*)............ 22
ABSTRACT... 22
INTRODUCTION 23
MATERIAL AND METHODS 25
Experimental Animals 25
Electrophysiological Recordings 26
Pharmacological agents 27
Data Analysis 28
RESULTS 30
1. Heterogeneity of Intrinsic Membrane Properties 30
2. Properties of Postsynaptic Potentials: inputs from the olfactory bulb................ 32
3. Biochemical properties of the postsynaptic potentials of vPOA and Vv neurons 32
4 Sectioning the lateral olfactory tract while recording from the POA 35
DISCUSSION 35
Heterogeneity of Neuronal Electrical Properties 36
Biochemical properties of the synaptic connections.. 38
Sectioning the Lateral Olfactory Tract 41
CONCLUSION... 41
CHAPTER 3 51
RAPID MODULATION OF GENE EXPRESSION PROFILES IN THE FOREBRAIN OF MALE GOLDFISH FOLLOWING EXPOSURE TO WATERBORNE SEX PHEROMONES 51
ABSTRACT... 51
INTRODUCTION 53
MATERIALS & METHODS 55
Animals 55
Chemicals and Experimental Procedure 55
Milt Volume... 56
RNA Extraction & cDNA synthesis.. 57

Microarray Hybridization 57
Microarray Data Analysis 58
Gene Ontology Analysis 59
Real-time Polymerase Chain Reaction (RT-PCR) 59
Statistical Analysis 61
RESULTS 62
Elevated Milt Volume 62
Expression profiling following exposure to PGF2α 62
Microarray validation using real-time RT-PCR 63
GnRH isoforms 64
DISCUSSION 66
$PGF_{2\alpha}$ regulates gene expression in the telencephalon 66
CHAPTER 4 84
GENERAL DISCUSSION 84
4.1 Major Contributions 84
4.2 Challenges and Future Directions 87
4.3 Conclusion 89
REFERENCES 91
APPENDIX A 109
EXPOSURES OF THE OLFACTORY EPITHELIUM OF MALE GOLDFISH BRAIN TO 17,20βP and PGF2α 109
APPENDIX B 111
EXPOSURES OF WHOLE MALE GOLDFISH BRAINS TO 17,20βP and PGF2α 111
APPENDIX C 112
SEROTONERGIC EFFECTS ON THE GOLDFISH BRAIN 112
APPENDIX D 113
THE INHIBITORY EFFECTS OF PPE ON FATHEAD MINNOW REPRODUCTION 113
APPENDIX E 114
ARTICLES PUBLISHED IN THE COURSE OF MY PhD 114

TABLE OF FIGURES

Figure 1.1: An illustration of the ventral-side up in vitro whole brain explant of the male goldfish we developed for patch-clamp electrophysiology. 18

Figure 1.2: A sagital diagram of the goldfish brain illustrating the detection and signalling of pheromones and odours from the OE to different parts of the telencephalon. 19

Figure 1.3: An illustrations of the interactions of the various hormones and pheromones coordinating goldfish reproduction between males and females. 20

Figure 1.4: Hypothesized role of glutamate in the OB-ventral telencephalon. LH release is facilitated by GABA and inhibited by DA. 21

Figure 2.1: Traces of some of the intrinsic membrane properties used in our statistical analyses. 44

Figure 2.2: Samples of a continuous recording showing superimposed EPSPs evoked in POA neurons 45

Figure 2.3: Intrinsic membrane properties of POA neurons. 46

Figure 2.4: High frequency stimulations of POA neurons with current delivered from the OB. 47

Figure 2.5: Evoked EPSPs in a POA neuron under different recording conditions. 48

Figure 2.6: Comparisons of OB synaptic inputs to POA (N = 7) and Vv (N = 6) neurons under different perfusions (ACSF, MFACSF and APV). 49

Figure 2.7: A ventral-side up diagram of the goldfish brain with the proposed neural circuitry of chemical communication showing synaptic connections. 50

Figure 3.1: Milt volume measured after exposures of goldfish to 6 hours of sex pheromones (17,20βP and $PGF_{2\alpha}$). 75

Figure 3.2: Gene Ontology (GO) analysis of differential expressed genes in the telencephalon of male goldfish exposed to $PGF_{2\alpha}$.. 76

Figure 3.3: Real-time RT-PCR validatation of cDNA microarray data. 77

Figure 3.4: Real-time RT-PCR of GnRHs mRNA in the telencephalon of male goldfish following pheromone exposure... 78

Figure 3.5: Gene expression of selected receptor subtypes in the telencephalon of male goldfish following pheromones exposure. 79

Figure 4.1: Schematic representation showing that sex pheromones (17,20βP and $PGF_{2\alpha}$) induce LH release, spermiation, steriodogenesis and sexual behaviour in male goldfish. 90

Figure A1: An illustrations of the possible effects of 17,20βP on POA neurons........ 110

TABLE OF TABLES

Table 2.1: The rotated component matrix of variables used in our principle component analysis.. 42

Table 2.2: Intrinsic membrane properties of POA and Vv neurons measured in ACSF. 43

Table 3.1: Real-time RT-PCR Primers used in the present study................................... 80

Table 3.2: Up-regulated and down-regulated genes identified by microarray analysis in male goldfish after exposures to waterborne $PGF_{2\alpha}$ (q value < 5%; fold change >1.5). .. 81

CHAPTER 1

GENERAL INTRODUCTION

1.1 Overview

Chemical communication plays a critical role in vertebrate reproduction by signalling social and reproductive status between conspecifics (Dulac and Torello, 2003). Animals have evolved broad classes of biologically active compounds for such purposes (Dulac and Torello, 2003). These so-called sex pheromones are effective because they are often cryptic, allowing signal transmission over relatively large distances while minimizing possible eavesdropping by potential predators (Stowe et al., 1995).

In nature, both the environment and behaviour of animals can determine and impose constraints on reproductive communication. For example, the solubility, volatility and polarity of sex pheromones determine their effectiveness in the environment, which allows for efficient and widespread distribution and transportation (Eisthen and Schwenk, 2008). In addition, for species that are solitary or living in high predation density, chemical communication is a cost-effective way to communicate safely. Therefore, given the importance of reproduction, animals have evolved sex pheromones in different milieu to advertise themselves covertly without compromising their reproductive fitness.

The neural circuitry mediating chemical communication in vertebrate reproduction has not been well-described. Most studies have provided indirect evidence based on brain lesioning, tract-tracing and electrical stimulation. We have, therefore, used an *in vitro* whole brain explant in goldfish (*Carassius auratus)*, which preserves the

neural circuitry from the olfactory bulb (OB) to the preoptic area (POA) parvocellular or nucleus preopticus (NPO) parvocellular for single-cell patch-clamp electrophysiology (Figure 1.1). An earlier version of the *in vitro* brain explant was first developed in 2000 by Vance Trudeau and David Spanswick to study the neural circuitry from the ventral telencephali pars ventralis (Vv) to the preoptic area (POA) (Trudeau et al., 2000b). At that time, little was known about the functional connections between Vv and POA, although some tract tracing studies were highly suggestive of the functional connections (Figure 1.2). For the present study, I optimized the preparation to allow effective OB stimulation to study the functional connections to the Vv and the POA. The rationale for choosing the POA is that, in addition to receiving inputs from the OB, many POA neurons are connected to the pituitary gland (PIT) (Anglade et al., 1993; Van Der Kraak, 2009). Furthermore, the POA is involved with the integration of reproductive behaviour and physiology (Hart et al., 1973; Peter, 1977; Koyama et al., 1984; Yamamoto et al., 1998; Kocsis et al., 2003; Dominguez et al., 2006; Dominguez, 2009) (Figure 1.2). We were also interested in the ventral telencephali pars ventralis (Vv), a homologue of the mammalian septal region (SR), because of its role in male reproduction in goldfish and rats (Butler and Hodos, 2005). In goldfish, the Vv is involved with behavioural responses in reproduction. Indeed, lesioning either the Vv or SR interferes with male reproduction in both goldfish (Kyle and Peter, 1982; Koyama et al., 1984) and rats (Gogate et al., 1995; Shimshek et al., 2006), respectively. This brain preparation allowed us to study the neural substrate of reproductive chemical communication in goldfish on the relatively short time scales of electrical signalling. In addition, we analyzed gene expression of male goldfish exposed to sex pheromones to study long-term changes. The goldfish is an

excellent model organism to study the neural basis of reproductive physiology and endocrinology because its sex pheromones and reproductive behaviours have been well studied and described (Peter and Gorbman, 1968; Peter et al., 1980; Peter and Paulencu, 1980; Sorensen et al., 1991b; Anglade et al., 1993; Dulka, 1993; Stacey and Sorensen, 2002; Hamdani and Doving, 2007).

1.2 Sequence of Events Leading to Goldfish Spawning

The sequence of events leading to spawning is mediated by a surge of luteinizing hormone (LH) in female goldfish which is induced by increasing temperature (e.g., from $12^{o}C$ to $20^{o}C$) and photoperiod (e.g. from 8 hours to 16 hours of daylight) in the spring (Dulka et al., 1987; Kobayashi et al., 2002). In females, the LH surge induces an increase in circulating 17α, 20β dihydroxy-4-pregene-3-one ($17,20\beta P$) and its metabolites, which promotes oocyte maturation leading to ovulation at late scotophase (Figure 1.2). When females release the preovulatory pheromone into the water through their gills (except for $17,20\beta P$-sulfate which is released through urine pulses), it primes males to increase their milt volume and promote courtship through stimulation of LH release (Stacey et al., 1994; Stacey et al., 2003; Stacey and Sorensen, 2009).

At ovulation, the release of prostaglandin Fs (PGFs) is triggered by the presence of oocytes in the female reproductive tract leading to spawning behaviour in the presence of a spawning substrate (Stacey et al., 1994; Kobayashi et al., 2002). When released by females, this postovulatory pheromone acts as a male releasing pheromone to induce males to engage in spawning behaviour (Figure 1.2). The stereotypical spawning

behaviour consists of females nudging or bumping into males which causes males to chase the now-sexually-receptive females (Kobayashi et al., 2002). After numerous such spawning behaviour, the females then lead one or several males into aquatic vegetation (which serves as a spawning substrate) where they both deposit their gametes. Both sexes will engage in this type of behaviour until all the oocytes are deposited and/or milt is depleted (Stacey et al., 1994).

1.3 Chemical Communication in Vertebrates

Sex pheromones comprise a subset of chemosensory cues that are detected through olfaction in vertebrates. These chemosensory cues are odours (olfactory cues from sources other than conspecifics) and pheromones (olfactory cues from conspecifics). Since vertebrate olfaction involves complex mixtures of odours and pheromones, this plethora of olfactory cues will naturally stimulate and activate different receptors, pathways, and brain regions to mediate diverse but cue-specific physiological responses and behaviours depending on the species.

The vertebrate olfactory system is divided into two parts functionally and neuroanatomically. These subdivisions are the MOS (main olfactory system) and the AOS (accessory olfactory system). Whereas these systems evolved to process different olfactory cues, functionally they are complementary. The MOS comprises the main olfactory bulb (MOB) and it detects a variety of volatile odours through odour receptors (ORs) on ciliated olfactory receptor neurons (cORNs) of the olfactory epithelium (OE) (Sato et al., 2005). On the other hand, the AOS is made up of the accessory olfactory bulb (AOB) and the vomeronasal organ (VNO), a specialized structure in tetrapods, that is

thought to detect pheromones and non-volatile compounds through the sensory epithelium that may contain vomeronasal receptors (VR) on microvillous ORN (mORN) (Dulka, 1993; Sato and Suzuki, 2001; Hansen et al., 2004). The VNO is thought to be an adaptation of tetrapods to the terrestrial environments but amphibians do develop VNOs before metamorphosis therefore suggesting that the VNOs did not evolve as an adaptation to terrestriality (Doving and Trotier, 1998). It is entirely possible that the VNO was co-opted during evolution in terrestrial tetrapods and gained new functionality to detect non-volatile compounds (Eisthen, 2000).

To some extent structure dictates function. Thus neuroanatomical differences across species are expected to occur within the olfactory systems that mediate odours versus pheromones although overlaps do sometimes occur depending on environmental constraints. For example, lesioning the VNO impairs reproduction in naïve guinea pigs but these impairments are lessened in guinea pigs that have had previous mating experiences suggesting that the MOS is also involved with reproduction (Doving and Trotier, 1998). Indeed domestic pigs do sense the sex pheromone, androstenone, through the MOS (Dorries et al., 1995). Snakes, for example, use the VNO for both prey tracking and food detection (Zippel et al., 1996). Teleosts do not have a VNO, and detection of food odours and sex pheromones occurs respectively in cORNs and mORNS which are located in the OE (Sato and Suzuki, 2001).

1.4 Chemical Communication in Goldfish

The current understanding of chemical communication in goldfish derives from numerous studies using electro-olfactogram (EOG) recordings (in both the OE and OB),

lesioning studies, tract tracings and electrical stimulation of the forebrain (Dulka and Stacey, 1991; Anglade et al., 1993). EOG recordings have played a role not only in pheromone identification but also in the measurement of the threshold of chemosensory detection. In terms of sensitivity, the detection thresholds of the sex pheromones in goldfish are in the picomolar to nanomolar range while other odorants are in the micromolar to millimolar range (Cao et al., 1998). In addition, severing either the medial olfactory tract (MOT) versus the lateral olfactory tract (LOT) has revealed that these fibres participate in specifically and exclusively transmitting sex pheromones and food cues, respectively (Stacey and Kyle, 1983; Dulka and Stacey, 1991). Furthermore, stimulating the MOT can elicit sperm release (Demski and Dulka, 1984a). Tract tracing studies have also described the connections from the OB to the pituitary gland (PIT) via both the Vv and POA (Bartheld et al., 1984; Anglade et al., 1993). Moreover, lesioning either the Vv or POA interferes with reproduction in male goldfish, impairing both behaviour and physiology (Peter et al., 1980; Kyle and Peter, 1982; Kyle et al., 1982; Koyama et al., 1984).

In goldfish, neural coding of chemosensory signals occurs in the rosette of the OE which is the primary detection site for chemical communication (Zippel et al., 1996; Hanson et al., 1998; Morita and Finger, 1998). The OE achieves this coding discrimination through cORNs and mORNs. Second order processing that participates in odour discrimination occurs in the glomeruli of the olfactory bulbs (OBs) where the terminal ends of axons from the OE converge to form a spatial “odotopic” map. In vertebrates, the odotopic map is made up of glomeruli that receive projections from

ORNs with the same receptors (Morita and Finger, 1998; Nikonov et al., 2005; Miyasaka et al., 2009).

Once coded and segregated, chemosensory signals are then relayed to different parts of the goldfish brain by mitral cells through the LOT and MOT for further processing (Figure 1.2). In goldfish, food odours are sensed in the OE by cORNs which then transmit the signals through LOT to higher brain feeding centres such as the posterior dorsal pallium, ventrolateral and / or ventroposterior hypothalamus (Cao et al., 1998; Canosa et al., 2004). Sex pheromones, on the other hand, are detected by mORNs (Dulka, 1993; Hansen et al., 2004; Hamdani and Doving, 2007; Hamdani el and Doving, 2007). This information then travels from the OE via MOT to Vv, POA and eventually to PIT to evoke LH release, courtship, sexual behaviour and ultimately spawning (Stacey and Kyle, 1983; Sorensen et al., 1991a; Dulka, 1993). Thus, it is believed that third order processing occurs in the POA and Vv in the goldfish (Figure 1.2).

1.5 Classes of Sex Pheromones That Have Effects in Male Goldfish

There are two classes of sex pheromones in goldfish (Kyle and Peter, 1982; Kyle et al., 1982; Dulka, 1993; Stacey et al., 1994; Zheng and Stacey, 1996). These classes of sex pheromones differ not only in their chemical composition and mechanism of action but also in the timing of their release. The first are called preovulatory pheromones and are steroid derivatives that are released by females prior to ovulation to act as priming pheromones to prepare the male goldfish for reproduction (e.g., 17,20βP). The second class, called the postovulatory pheromones, are prostaglandins that are discharged by female after ovulation to stimulate male sexual behaviour; thus they are termed releasing

pheromones (e.g. $PGF_{2\alpha}$) because they are thought to "release" goldfish sexual behaviour which is under inhibition when the fish is not spawning (Figure 1.3).

1.5.1 17α, 20β dihydroxy-4-pregene-3-one (17,20βP)

The steroidal (priming) pheromones are comprised of 17,20βP and its glucuronidated and sulphated metabolites that are released in urine pulses into the water by female goldfish to stimulate LH from the anterior pituitary of the male (Figure 1.3). The 17,20βP primes male goldfish for reproduction through increased steriodogenesis and spermiation (Zheng and Stacey, 1997; Trudeau et al., 2000a). Behaviourally, 17,20βP and its metabolites promote male courtship and synchronizes the male's activities to female ovulation in anticipation of spawning (Dulka et al., 1987).

1.5.2 Prostaglandin-$F_{2\alpha}$ ($PGF_{2\alpha}$)

The second class of sex pheromones, known as the post-ovulatory or releasing pheromones, is comprised of $PGF_{2\alpha}$ and its metabolites including 15-keto-$PGF_{2\alpha}$ (Figure 1.3) (Zheng and Stacey, 1997). Prostaglandin induction and release in urine is triggered by the presence of oocytes in the female reproductive tract (Appelt and Sorensen, 2007). Prostaglandins stimulate and synchronize both male and female sexual behaviour (Cardwell et al., 1995).

1.5.3 Androstenedione (AD)

There are other factors that modulate goldfish reproduction. In addition to 17,20βP, $PGF_{2\alpha}$ and their metabolites, female goldfish release AD as a component of the preovulatory pheromones (Figure 1.3) (Stacey et al., 2003). Androstenedione suppresses sperm production leading to a decrease in milt volume. It is thought that females release AD so that males do not respond prematurely to 17,20βP (Sorensen et al., 2005). Males

also release AD during spawning as part of an intense inter-male rivalry for access to females and mating opportunities (Sorensen et al., 2005). AD is thought to increase male-to-male aggression which plays a role in establishing dominance thereby ensuring sperm competition and reproductive fitness (Kobayashi et al., 2002; Sorensen et al., 2005).

1.6 Neurohormones Controlling Goldfish Reproduction

Extensive studies in goldfish, as well as other teleosts, have now firmly established that the secretion of LH is stimulated by γ-aminobutyric acid (GABA) and inhibited by dopamine (DA) through both GnRH-dependent and GnRH-independent mechanisms (Figure 1.4) (Peter et al., 1986; Chang et al., 1990; Yu et al., 1991; Dulka et al., 1992; Yu and Peter, 1992; Trudeau et al., 1993c; Trudeau, 1997; Zheng and Stacey, 1997; Zohar et al., 2009). The exception so far is the Atlantic Croaker which uses serotonin (5HT) to stimulate LH release (Khan and Thomas, 1992; Trudeau, 1997; Ayala, 2009). In contrast to teleosts, glutamate and GABA are predominately stimulatory and inhibitory, respectively, in mammalian LH release in mammals (Trudeau et al., 2000b). While much is known about GABAergic and DAergic regulation of goldfish reproduction (Figure 1.4), relatively little is known about how and where in the goldfish brain glutamate exerts its effects.

1.6.1 Gonadotropin-Releasing Hormone (GnRH)

Gonadotropin-Releasing Hormone is an evolutionarily conserved decapeptide synthesized from the prohormone prepro-GnRH (Dong et al., 1997; Zohar et al., 2009).

In many vertebrates, GnRH is the main neuropeptide that stimulates LH and follicle stimulating hormone (FSH) release. Fourteen unique variants of this neuropeptide have been identified thus far with eight isoforms found exclusively in teleosts (Van Der Kraak, 2009). In teleosts, GnRH exists as two or three isoforms depending on the species (Anglade et al., 1993; Peter and Yu, 1997; Zohar et al., 2009). In goldfish, these isoforms are salmon GnRH (sGnRH) and chicken GnRH-II (cGnRH-II); cell bodies of both have been found in the OB, ventral telencephalon, POA and hypothalamus (Kah et al., 1986; Kim et al., 1995). Both GnRH isoforms exert stimulatory effect on LH release (Kim et al., 1995; Trudeau, 1997; Yu et al., 1998).

At the cellular level, GnRH neuropeptides bind to either of two GnRH receptors, GfA or GfB (Illing et al., 1999). Both GfA and GfB have greater sensitivity to cGnRH-II than sGnRH (Illing et al., 1999). These GnRH receptors comprise a family of seven transmembrane domain, G-protein coupled receptors (GPCRs) that stimulate phospholipase C (PLC) (Jobin et al., 1996). Phospholipase C then hydrolyzes phosphatidyl 4,5-biphosphate to produce diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3). Inositol 1,4,5-triphosphate mobilizes calcium (Ca^{2+}) which can activate protein kinase C (PKC) while DAG stimulates PKC to elicit LH release (Jobin et al., 1996). In addition, sGnRH can also stimulate LH release through activation of arachidonic acid and phospholipase A_2 (Chang et al., 1993).

1.6.2 γ-Aminobutyric acid (GABA)

γ-Aminobutyric acid is a predominately inhibitory neurotransmitter in vertebrates that is generated from glutamate by the rate limiting enzyme, glutamate decarboxylase (GAD), and the cofactor pyridoxal phosphate (Trudeau et al., 2000b; Martyniuk et al.,

2007). In goldfish, there are two GAD isoforms namely GAD65 and GAD67 (Martyniuk et al., 2005). GABA actions are mediated through both an ionotropic receptor ($GABA_A$) and a metabotropic receptor ($GABA_B$). The $GABA_A$ receptor is a chloride channel formed from five subunits (2α,2β,γ or ρ) while the $GABA_B$ receptor is a GPCR made up of a 7 transmembrane domain protein that regulates potassium channels (Trudeau et al., 1993b; Trudeau et al., 2000b).

Studies in goldfish have shown that intraperitoneal (i.p.) injections of muscimol, a $GABA_A$ receptor agonist, enhances LH release while bicuculline, a $GABA_A$ receptor antagonist, blocks this effect (Trudeau et al., 1993b). Whereas i.p. injections of baclofen (a $GABA_B$ receptor agonist) induces LH release, saclofen (a $GABA_B$ receptor antagonist) does not seem to block LH induction (Trudeau et al., 1993b). In addition, GABA also stimulates LH release through suppression of DAergic inhibition of GnRH in goldfish (Kah et al., 1992; Sloley et al., 1994).

1.6.3 Dopamine (DA)

The catecholamine neurotransmitter DA is synthesized from tyrosine through a two step process that involves the rate-limiting enzyme, tyrosine hydroxylase, and aromatic amino acid decarboxylase (AADC) (Popesku et al., 2008b; Zohar et al., 2009). In teleosts and other vertebrates, it exerts its action through its D_1 and/or D_2 classes of receptors that belong to the GPCR family and act through adenylyl cyclase.

In the context of goldfish reproduction, DA inhibits GnRH release in the POA through D_1-like receptors whereas in the PIT it exerts its inhibitory action on LH release through D_2-like receptors (Yu et al., 1991; Trudeau, 1997; Zohar et al., 2009). Indeed, 17,20βP stimulates LH release in male goldfish through its suppression of inhibitory

DAergic pathways in the POA (Kobayashi et al., 2002; Zohar et al., 2009). In addition, GABA inhibits DA neurons to disinhibit its suppression of GnRH leading to LH induction. The inhibition of DA by GABA may therefore be necessary to facilitate reproduction in male goldfish.

1.6.4 Glutamate (Glu)

Glutamate, the major excitatory neurotransmitter in vertebrate brains, is made primarily in neurons and astrocytes from glutamine by the rate limiting enzyme glutaminase, although some Glu is also produced from tricarboxylic acid cycle (Fonnum, 1984; Daikhin and Yudkoff, 2000). In vertebrates, it is involved in numerous physiological processes such as reproduction, memory, and respiration through its fundamental role in synaptic transmission (Spanswick et al., 1998; Hsu et al., 2000; Meldrum, 2000). Additionally, evidence is emerging that in vertebrates it is co-released with other neurotransmitters in monoamine neurons (Trudeau, 2004). Glutamate has two types of receptor classes, which are the ionotropic and metabotropic receptors. The ionotropic receptor class is made up of α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA), kainate and *N*-methyl-*D*-aspartate (NMDA) while the metabotropic receptor class is comprised of metabotropic glutamate receptors (mGluRs) that belong to the G-protein coupled receptor family (Meldrum, 2000).

α-Amino-3-hydroxy-5-methylisoxazole-4-propionate receptors (AMPAR) are formed of 4 heteromeric subunits (GluR1, GluR2, GluR3, and GluR4), and they are selectively permeable to Na^+ and K^+ ions. Kainate receptors (KARs) are also composed of four tetrameric subunits consisting of GluR5, GluR6, GluR7, KA1 and KA2. However, KARs are less abundant in the goldfish pallium than AMPARs (Henley and Oswald,

1988). Like AMPARs, KARs are permeable to Na^+ and K^+ ions but their activity is on a shorter time scale (Bleakman and Lodge, 1998). NMDARs are also made up of heterotetrameric subunits between 2NR1 and either 2NR2A 2NR2B or 2NR2C or 2NR2D and are permeable to Na^+, Ca^{2+} and K^+ ions (Harvey-Girard et al., 2007). NMDARs are dependent on non-NMDARs for activation because of a voltage-dependent Mg^{2+} block of NMDARs at resting membrane potentials (Spanswick and Logan, 1990; Spanswick et al., 1998). The mGluRs, on the other hand, belong to GPCRs family, and they mediate their activities through second messengers.

In vertebrates, Glu plays an important role to stimulate GnRH neurons, thus inducing LH release (Dhandapani and Brann, 2000; Trudeau et al., 2000b; Fraser et al., 2002; Kiss et al., 2003; Kocsis et al., 2003; Mahesh and Brann, 2005; Deviche et al., 2008). Since the reproductive axis is conserved in vertebrates, it is likely in goldfish as in other species that Glu in the POA stimulates GnRH, perhaps via both AMPARs and NMDARs (Peter et al., 1980; Flett et al., 1994; Trudeau et al., 2000b; Mahesh and Brann, 2005). Indeed *in vivo* intraperitoneal pre-treatment of goldfish with monosodium glutamate (MSG) potentiated the effects of GnRH-mediated LH release when the animals were injected later with a sGnRH analogue, thereby underscoring the importance of Glu in goldfish reproduction (Sloley et al., 1992a). However, the effects of MSG on LH release in the goldfish needs to be interpreted with caution as MSG has been known to cause necrosis in the POA when injected intraperitoneal (Peter et al., 1980). Furthermore, in seasonal breeders like the golden hamster (Colwell et al., 1991) Cassin's Sparrows (Deviche et al., 2008) and Siberian hamsters (Ebling et al., 1995), Glu modulates the responsiveness of the reproductive cycle to light by stimulating LH release

and subsequently activating the reproductive system . Since goldfish are seasonal breeders, photoperiod has also been shown to regulate their reproductive cycle (Razani et al., 1987; Blazquez et al., 1998). Glutamate may, therefore, regulate the responsiveness of goldfish to photoperiod in the same way as in the other species. Additionally, Glu, by serving as a precursor for GABA synthesis, also plays a role in LH release (see above).

1.7 Hypothesis and Objectives

Experimental studies in sexually mature male goldfish suggest that sex pheromones induce luteinizing hormone (LH) release through the suppression of dopamine (DA) inhibition of gonadotropin-releasing hormone (GnRH) in POA, which regulates male courtship and sexual behaviour (Stacey, 1983; Kobayashi et al., 1986a; Trudeau, 1997; Kobayashi et al., 2002). However, how the sex pheromone signals are transmitted from the OB to the ventral telencephalon and gene expression after exposures to sex pheromones have not been well studied. Therefore, this thesis focuses on the neural substrates of chemical communication in sexually mature male goldfish. My objectives and hypotheses are:

i. To characterize the neural circuitry from the olfactory bulb (OB) to the ventral telencephalon;

    Hypothesis: Glutamate is the main neurotransmitter that mediates olfactory cues from the OB to the POA and Vv.

ii. To determine which genes are differentially regulated when sexually mature male goldfish are exposed to sex pheromones.

Hypothesis: Sex-related genes will be differentially regulated in the telencephalon of male goldfish exposed to sex pheromones.

1.8 Model Organism

The goldfish is among a few vertebrates whose sex pheromones have been chemically identified and the mechanisms of action described (Kobayashi et al., 1986a; Peter et al., 1986; Stacey et al., 1989; Sorensen et al., 1991b; Dulka, 1993; Trudeau, 1997). In addition, the goldfish (like other teleosts) has direct innervation from the POA and hypothalamus to the pars distalis of the anterior pituitary. This is in contrast to the hypothalamo-pituitary anatomy in tetrapods where the connections between the hypothalamus and the hypophysis are neurohemal via the hypothalamo-hypophysis portal system (Anglade et al., 1999; Van Der Kraak, 2009). This feature, in the goldfish, has allowed the precise delineation of the hypothalamic inputs through retrograde tract-tracing (Anglade et al., 1999). Furthermore, goldfish are hypoxia-tolerant thereby making them excellent candidates for sex pheromone electrophysiology because their brains can endure extended sessions of recordings in potentially low oxygen situations (Van Waversveld et al., 1989; Trudeau et al., 2000b; Jibb and Richards, 2008; Roesner et al., 2008; Wilkie et al., 2008).

Since the teleosts diverged from the main vertebrate lineage more than 200 millions years ago (Dulka, 1993; Trudeau et al., 2000b), the neural circuitry that mediates

chemical communication in teleosts and tetrapods may have undergone modification to adapt to their different environments. Nevertheless, this circuitry retains many ancestral features such as the presence of an olfactory epithelium, olfactory bulbs plus main and accessory olfactory systems that all reflect on a common evolutionary origin (Dulka, 1993). It is expected that these similarities will allow the findings in goldfish to be extrapolated to other vertebrates (Dulka, 1993).

Goldfish are good candidates for sex pheromone electrophysiology because their olfactory system is simply organized, unlike tetrapods which have a separate accessory olfactory system (AOS) and main olfactory system (MOS) for the processing of pheromones and olfactory cues, respectively (Cao et al., 1998). In addition, it is easier to work with goldfish sex pheromones since they have been well characterized, are waterborne, soluble and easier to present to the animal than it is for the pheromones of tetrapod which are not yet well known and if known must be suspended in aerosols thereby making it difficult to maintain a consistent concentration in application (Dulac and Torello, 2003). Furthermore, the preservation of the underlying neural circuitry in the brain explant and the easy accessibility of POA to single cell patch clamp electrophysiology make goldfish attractive for studying the electrophysiological properties of pheromone communication.

1.9 Thesis organization

This thesis is made up of a series of chapters on the neurotransmission and gene expression of the neural substrates of sex pheromones. Each of the data chapters (Chapters 2 and 3) is written as a manuscript for publication. As such, there will be some level of repetition especially in the introductions. Chapter Two describes the characterization of the neural circuitry of chemical communication in male goldfish from the OB to POA and Vv. Chapter Three examines the role of sex pheromones exposure on gene expression. Finally, Chapter Four will provide a general discussion of the findings and recommendations for future research.

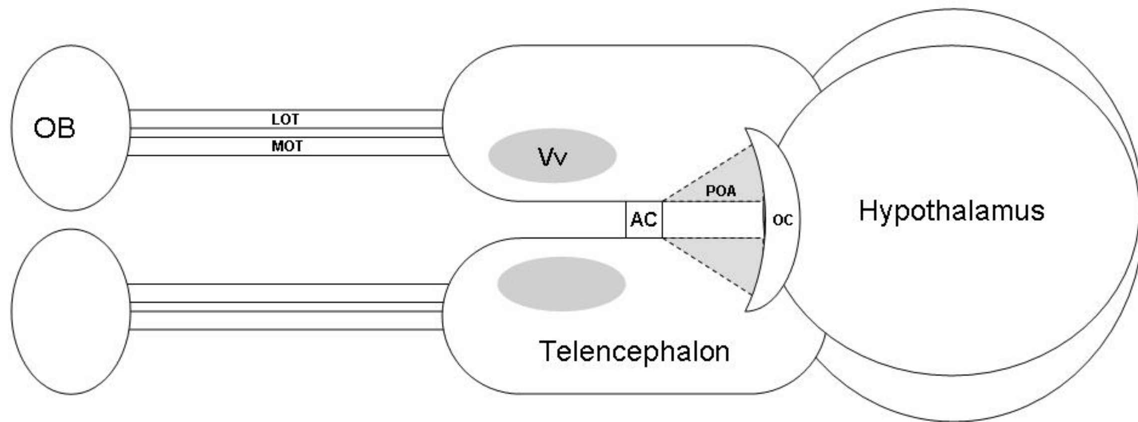

Figure 1.1: An illustration of the ventral-side up in vitro whole brain explant of the male goldfish we developed for patch-clamp electrophysiology. A stimulating electrode was inserted into one of the OBs and the stimulus measured from a single neuron in either Vv or POA. OB (olfactory bulb), MOT (medial olfactory tract), LOT (lateral olfactory tract), Vv (ventral telencephalon pars ventralis), POA (preoptic area), AC (anterior commissure) and OC (optic chiasm).

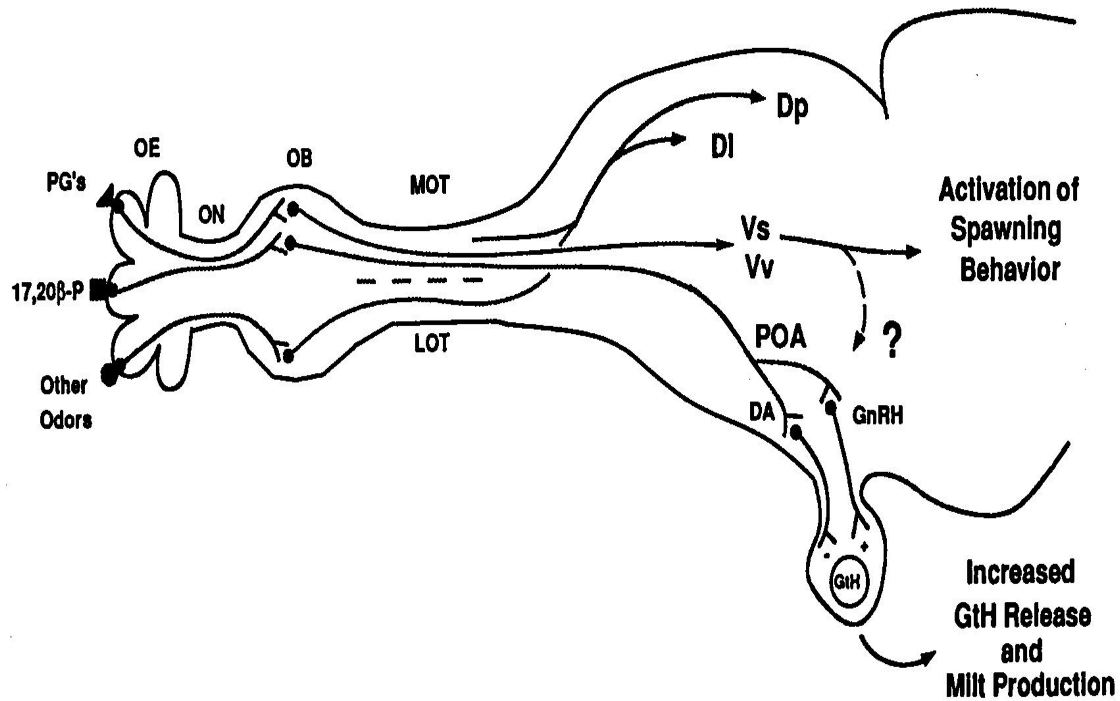

Figure 1.2: A sagital diagram of the goldfish brain illustrating the detection and signalling of pheromones and odours from the OE to different parts of the telencephalon. OE (olfactory epithelium), OB (olfactory bulb), MOT (medial olfactory tract), LOT (lateral olfactory tract), Vv (ventral telencephalon pars ventralis), POA (preoptic area), AC (anterior commissure) and 17,20βP (17α, 20β dihydroxy-4-pregene-3-one), PG (prostaglandin), GtH (gonadotropin hormone, LH (luteinizing hormone), as it is currently known). Taken from Dulka (1993) with permission from S. Karger AG©.

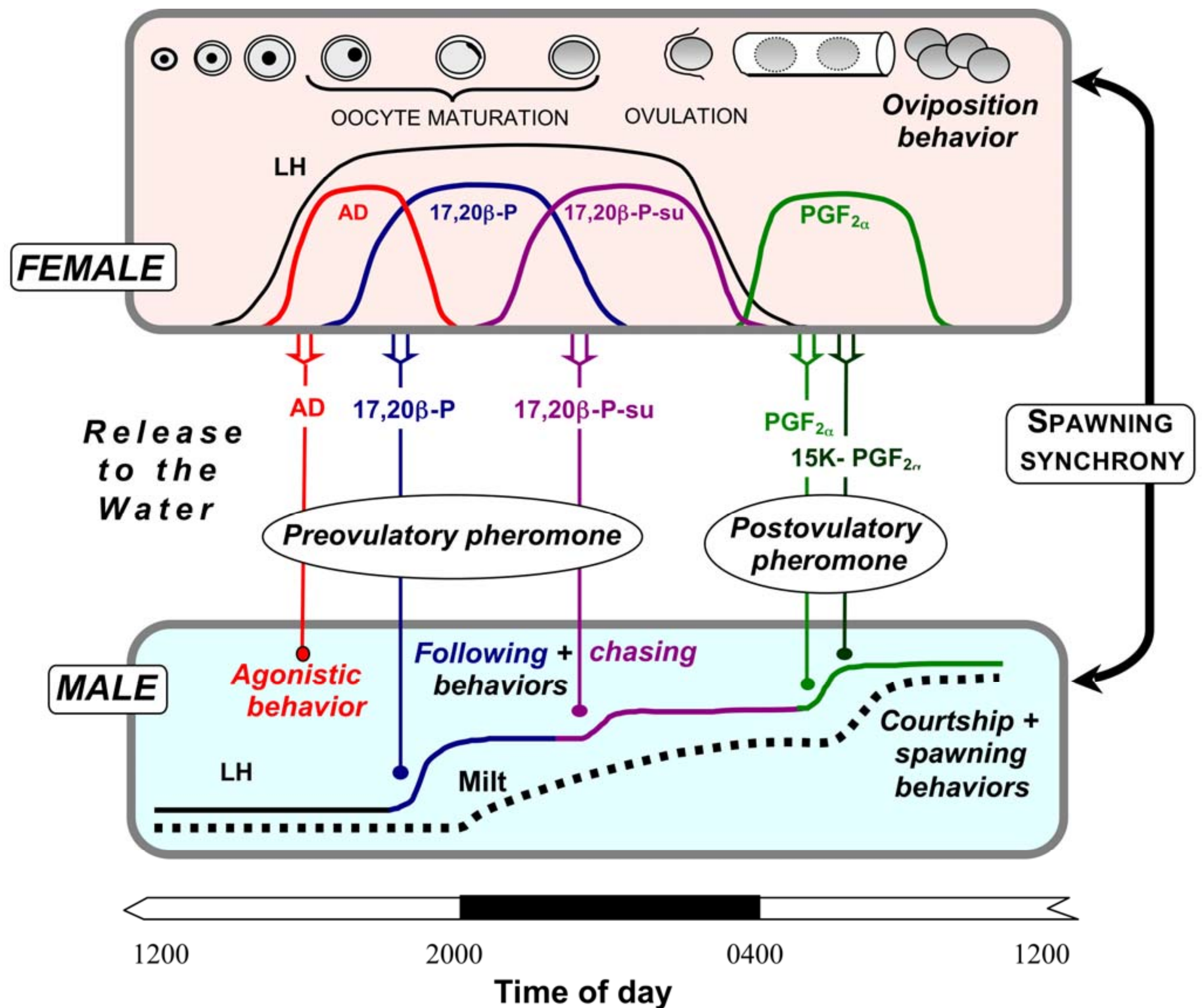

Figure 1.3: An illustrations of the interactions of the various hormones and pheromones coordinating goldfish reproduction between males and females. The process ultimately leads to spawning synchronization (see section 1.6 for detail). AD (androstenedione), 17,20βP (17α, 20β dihydroxy-4-pregene-3-one), 17,20βP-S (17α, 20β dihydroxy-4-pregene-3-one sulfate), $PGF_{2\alpha}$ (prostaglandin F 2 alpha), 15K-$PGF_{2\alpha}$ (15-keto-prostaglandin F 2 alpha) LH (luteinizing hormone). Taken from Stacey and Sorensen (2009). With permission from Elsevier ©.

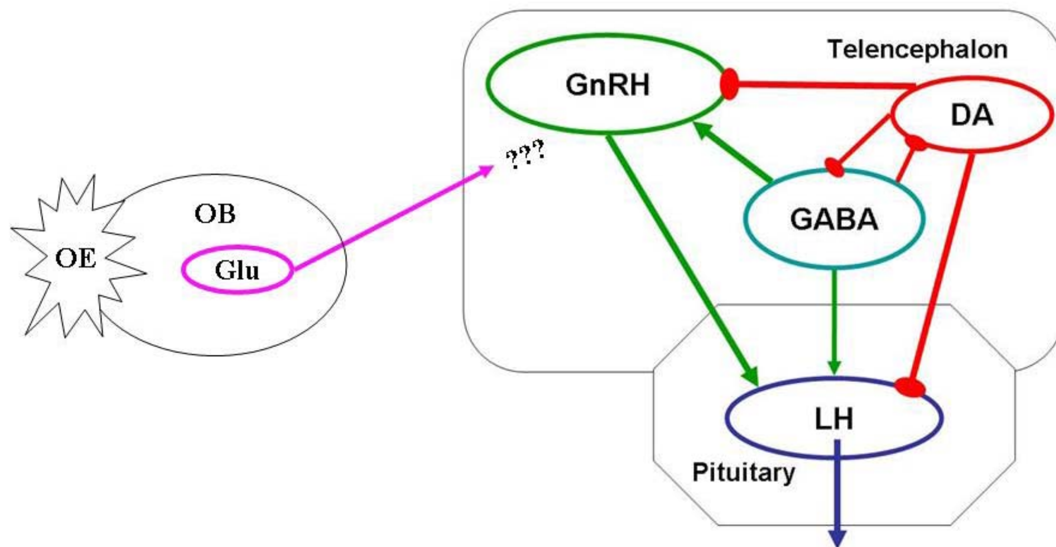

Figure 1.4: Hypothesized role of glutamate in the OB-ventral telencephalon. LH release is facilitated by GABA and inhibited by DA. The process of LH release can be dependent or independent of GnRH. GABA and DA reciprocally inhibit each other. Note that in the telencephalon DA acts through D1 receptors while in the pituitary it inhibits through D2 receptors. OB (olfactory bulb), OE (olfactory epithelium), Glu (glutamate), GnRH (gonadotropin releasing hormone), DA (dopamine), GABA (gamma aminobutyric acid); LH (luteinizing hormone).

CHAPTER 2

GLUTAMATE MEDIATES EXCITATORY SYNAPTIC NEUROTRANSMISSION FROM THE OLFACTORY BULB TO THE VENTRAL TELENCEPHALON VIA THE MEDIAL OLFACTORY TRACT IN MALE GOLDFISH (*Carassius auratus*)

ABSTRACT

Chemical communication via sex pheromones is critical for successful reproduction but the underlying neural mechanisms are not well-understood. The goldfish is a tractable model because sex pheromones have been well-characterized in this species. We used male goldfish forebrain explants *in vitro* and performed whole-cell current clamp recordings from single neurons in the ventral preoptic area (vPOA) and ventral telencephali pars ventralis (Vv) to characterize their membrane properties and synaptic inputs from the olfactory bulbs (OB). Electrical stimulation of the OB and application of receptor antagonists revealed monosynaptic glutamatergic projections from the OB to the vPOA via the medial olfactory tract. Principle component and cluster analyses based on intrinsic membrane properties of vPOA (N = 107) neurons revealed five (I-V) distinct cell groups. These cells displayed differences in their input resistance (R_{input}: I < II < IV < III < V), time constant (Tau: I = II < IV < III = V), threshold current (TI: I > II = IV = III = V) and connectivity to the OB [I (24%), II (40%), III (0%), IV (34%) and V (2%)]. The synaptic inputs from OB had a shorter latency in Vv neurons as compared to vPOA neurons. Our results suggest that there are at least 2 distinct glutamatergic pathways originating in the OB; one projects to the vPOA, the other to the Vv.

INTRODUCTION

Chemical communication plays a vital role in vertebrate reproduction. Biologically-active sex pheromones have evolved across the animal kingdom to convey reproductive information to conspecifics (Dulac and Torello, 2003). However, in most cases, the neural circuitry associated with the processing of sex pheromones is poorly understood. Chemical communication is especially important in animals like goldfish because they rely on external fertilization and often live in turbid waters. These fish have evolved sex pheromones to synchronize spawning between the sexes and thus ensure reproductive success. Further, the goldfish is an attractive model to study the neural substrates of chemical communication because it is one of the only vertebrates whose sex pheromones have been fully characterized (Stacey et al., 1989; Sorensen et al., 1991b; Dulka, 1993).

Studies of male goldfish indicate that sex pheromones from females regulate male sexual behaviour and milt production by inducing the release of luteinizing hormone (LH) from the male pituitary gland through stimulation of gonadotropin-releasing hormone (GnRH) in the POA (Stacey, 1983; Kobayashi et al., 1986b; Trudeau, 1997; Kobayashi et al., 2002). The POA controls the release of LH (Peter et al., 1990; Chang et al., 2000; Trudeau et al., 2000a; Trudeau et al., 2000b) via a signalling pathway involving dopamine (DA), which tonically inhibits GnRH and LH release (Peter and Paulencu, 1980; Kah et al., 1987; Sloley et al., 1992b; Popesku et al., 2011). Coupled to the GABAergic inputs this area receives from the ventral telencephali pars ventralis (Vv) (Martinoli et al., 1990; Trudeau et al., 2000b), the vPOA may be the site where DA suppression of GnRH is removed to allow increased GnRH levels and elicit LH release.

Another reproductive area of interest in the goldfish brain is Vv. The goldfish Vv is homologous to the mammalian septal region, which in male rats plays an important role in reproduction (Illing et al., 1999; Butler and Hodos, 2005). The Vv is thought to serve as a relay between the olfactory bulb (OB) and the POA, and is therefore important for the transmission of pheromone-related signals in goldfish (Anglade et al., 1993; Dulka, 1993). Indeed, lesioning the Vv impairs reproductive behaviour in male goldfish (Kyle et al., 1982; Koyama et al., 1984), but it is not clear whether this impairment is due to destruction of Vv neurons or lesions to fibres of passage projecting to the POA.

To characterize the neural pathways underlying the OB, Vv and POA networks, we have developed a novel *in vitro* explant preparation of the goldfish forebrain (Trudeau et al., 2000b) . The adult goldfish brain is small and relatively unmyelinated making it attractive for patch clamp electrophysiology. In addition, the explant preserves the underlying neural circuitry yet allows for easy access to neurons on the ventral surface of the brain.

Here, we first describe the intrinsic membrane properties of neurons in the vPOA and Vv. Based on these properties, we suggest that vPOA neurons comprise five different subgroups. In contrast, our dataset is insufficient to support more than one uniform group of neurons in Vv. We then characterized the synaptic projections from the OB to the vPOA and Vv. In the goldfish and the closely related Crucian carp, the lateral olfactory tract (LOT) transmits food-related odours (Dulka, 1993; Hamdani et al., 2001b; Hamdani et al., 2001a) while the medial olfactory tract (MOT) conveys exclusively pheromonal and social signals (Demski and Dulka, 1984b; Sorensen et al., 1991b; Hamdani et al., 2000). Here, we demonstrate that there are functional monosynaptic glutamatergic

projections from the OB to the Vv and from OB to the POA through the MOT.

MATERIAL AND METHODS

Experimental Animals

This study was approved by the animal care committee of the University of Ottawa and carried out in accordance with the guidelines of Canadian Council on Animal Care. Common goldfish *(Carassius auratus)* weighing 15-40 g were purchased from a commercial supplier (Aleong's International Inc., Mississauga, ON, Canada). Fish were acclimated to 18°C, fed and maintained on a simulated photoperiod as previously reported (Trudeau et al., 1991). Only male goldfish were used throughout the study. During spawning season, sexually mature males were easily discernable by their distinctive tubercles and some readily expressed milt when their anogential area was gently pressed. After the spawning season and during recrudescence, when tubercles are not always evident, sex was confirmed post-mortem by visual inspection of the testes.

Fish were anaesthetized by immersion in 0.05 % tricaine methanesulphonate (TMS) prior to dissection of the brain explant. The method for this brain preparation is based on that from a previous study in our laboratory (Trudeau et al., 2000b). Briefly, after severing the spinal cord, the skull was carefully opened with surgical scissors to expose the brain. The brain was dissected out from the skull cavity; the optic nerves were cut, and then the whole brain was removed, with olfactory bulbs still attached. The explant was attached to a Petri-dish ventral side up at the level of the spinal cord and cerebellum with cyanoacrylate glue, and placed in a bath with ice-cold artificial cerebrospinal fluid (ACSF) of the following composition [mM]: 127 NaCl, 1.9 KCl, 1.2

KH_2PO_4, 2.4 $CaCl_2$, 1.3 $MgCl_2$, 26 $NaHCO_3$, 10 D-glucose; gassed with carbogen (95% 0_2, 5% CO_2); pH 7.4. When magnesium-free solution was used, $MgCl_2$ was omitted from the ACSF.

The meninges were removed with fine forceps to expose the ventral telencephalon and access the vPOA; then a transverse cut using a razor blade was made posterior to the hypothalamus to free the brain from the dish. The brain was then transferred carefully to a custom-built recording chamber perfused at room temperature with ACSF at a rate of 2 – 4 ml/min. The brain explant was mounted with the ventral side up and then held between two custom-made nylon grids where it was allowed to recover for 1 hour prior to recordings; all recordings were made in the 7 hours following. Neuroanatomical nomenclature in this study mostly follows that of Anglade *et al*., (Anglade et al., 1993).

Electrophysiological Recordings

Electrophysiological recordings were made based on previous methods for rat spinal cord and hypothalamus (Spanswick et al., 1998). Whole-cell patch clamp recordings using a Multiclamp 700B amplifier (Molecular Devices) in current clamp mode, were obtained from vPOA neurons (N = 107) and Vv (N = 6) in the *in vitro* forebrain explants at room temperature (~18 - 20°C) from 120 fish. Patch pipettes (5 – 8 MΩ) were fabricated from borosilicate filament glass (Sutter Instrument Co., Novata, CA, USA) using a horizontal pipette puller (P2000; Sutter Instrument Co., Novata, CA, USA) and filled with intracellular solution of the following composition [mM]: 140 K-gluconate, 10 KCl, 1 sucrose, 2 Na_2ATP, 1 EGTA-Na, 10 HEPES, 2 Na_2ATP and pH adjusted to 7.4 with KOH.

Using the anterior commissure and optic chiasm as landmarks, patch electrodes were guided to the vPOA under visual control of a dissecting microscope. Seal formation was monitored on an oscilloscope. Once a gigaohm seal (typically > 5GΩ) was achieved, whole-cell access was made by gentle suction. Series resistance was < 25 MΩ.

To measure synaptic connectivity, a bipolar stimulating electrode was inserted into one of the olfactory bulb (OBs). Postsynaptic potentials (PSPs) in the vPOA or Vv were elicited by electrical stimulation (5 - 30V, 0.2msec pulse duration) of the ipsilateral OB via a stimulus isolation unit (Digitimer Ltd, model DS2); stimuli comprised either single pulses or trains of four pulses (13.3 Hz) Data acquisition and experimental control was performed using pCLAMP 9.2 software (Molecular Devices). Data were low-pass filtered at 2 kHz and acquired at 10 kHz and later analyzed offline using CLAMPFIT 9.2 software (Molecular Devices).

Pharmacological agents

To characterize the pharmacological properties of the Vv and vPOA neurons and neural circuitry from the OB to the Vv and vPOA, the following drugs were used: 6-cyano-7-nitroquinoxaline-2, 3-dione (CNQX; Tocris), an α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate) receptor antagonist and D-2-amino-5-phosphonopentanoic acid (D-APV; Tocris), an N-Methyl-D-aspartic acid or N-Methyl-D-aspartate (NMDA) receptor antagonist. Drugs were made up as stock solutions, CNQX in DMSO (Sigma-Aldrich) and D-APV in distilled water, then, diluted in Mg^{2+} free ACSF. The final concentration of DMSO was always < 0.1%. Typically, Mg^{2+} free ACSF and the drugs were applied sequentially for 10 min each to the recording chamber before any attempts at recordings to allow sufficient equilibration time.

Data Analysis

Intrinsic membrane properties of vPOA and Vv neurons were characterized by patch clamp electrophysiology to determine whether they comprised distinct populations. After achieving whole cell access, the resting membrane potential (RMP) which is the baseline potential in the absence of any current stimulus was measured in current clamp mode (I = 0 nA). In addition, properties related to spontaneous action potential production was measured (Figure 2.1 A): action potential amplitude (APA) was measured from the shoulder of the threshold to the peak. Action potential duration (APD) was measured from the shoulder of the threshold. After-hyperpolarization potential (AHP) was determined from the threshold to the peak of the hyperpolarization. Note that only the fast component of the AHP was used in this study. After-depolarization potential (ADP) was measured from the hyperpolarization peak to the ADP peak. The coefficient of variation (CV_{spikes}= SD/mean) was calculated from the interval between successive spikes over a 90 second time window. Neurons were then stimulated with 1 sec hyperpolarizing and depolarizing current steps from a holding potential of -60 mV to measure a number of other intrinsic membrane properties such as (Figure 2.1 B-D): current threshold (IT) is the minimum current required to elicit an action potential. Spike threshold (ST) is the threshold at which a spike is elicited by IT. Presence or absence of a rebound depolarization (RD) after a hyperpolarizing current step. Presence or absence of rebound depolarization spikes (RDS). Rebound spike frequency (RS) which is similar to RDS but without the RD. Presence or absence of H current (I_H) which is associated with non-selective cation channels; Rectification which measures the current-voltage (IV) relationship through current steps to determine the movement of ions in and out of the

cell. If ions move linearly in one direction and non-linearly in the opposite direction when the driving force is reversed, then, the channel is rectifying (Siegelbaum and Koester, 2000). Conversely if the ions move linearly in both directions, then, it is non-rectifying (insets in Figure 2.1 C,D); the input resistance (R_{input}), which is the slope of the IV curve; membrane time constant (TC) is the time for the hyperpolarization response to reach two-thirds of its plateau value; soma membrane capacitance (C_{soma}) is the ability of the membrane soma to store charge at a given potential (Abbud and Smith, 1995).

A principle component analysis (PCA; SPSS Inc; 2006, v.15) was used to reduce the set of intrinsic membrane properties to a number of independent uncorrelated variables. For this analysis, all properties that were characterized by their presence or absence, i.e. RD, RDS, I_H and rectification were assigned binary values (0, 1). The PCA variables were then used to cluster (SPSS Inc; 2006, v.15) the cells into groups. An unsupervised cluster analysis was performed to classify neurons as has been demonstrated previously in other studies that have categorized neurons into groups (Ward, 1963; Krimer et al., 2005; Sosulina et al., 2006; Andjelic et al., 2009). This method consisted of grouping individual neurons based on the Euclidean distance between their respective PCA loadings. We used a bottom up approach in which neurons were grouped together based on their relatedness. With each repetition of group formations, progressively larger groups were formed.

Postsynaptic potentials (PSP) data were characterized by the peak amplitude (the height of the evoked PSP measured from baseline to peak), the latency (the time between the OB stimulus and beginning of PSP rise), (10% – 90%) rise time (measured from the shoulder of the rise to the peak) and (90%-10%) decay time (determined from the peak of

the PSP to the baseline) (Figure 2.1). To measure constant latency and rise time, the coefficient of variation ($CV_{latency}$ and $CV_{rise\ time}$) was derived by dividing the standard deviation (SD) by the mean of the traces (N = 4) measured in a typical recordings.

All data generated by the PCA were tested for normality and homogeneity of variance , and either an analysis of variance (ANOVA) or a Kruskal-Wallis (KW) analysis was used for between group comparisons where appropriate (SPSS Inc; 2006, v.15). Post-hoc analyses consisted of paired t-tests and Tukey's (SPSS Inc; 2006, v.15). Unless otherwise stated, data are reported as mean ± SEM.

RESULTS

1. Heterogeneity of Intrinsic Membrane Properties

To characterize the population of vPOA neurons, a number of intrinsic membrane properties were quantified (see Methods). We used a Principle Component Analysis (PCA) to determine the set of properties that could best distinguish neuronal subgroups. The PCA revealed six significant properties (loading factor) [TC (0.78), R_{input} (0.87), rectification (0.69), RS (0.91), ADP (0.79) and IT (0.81)] (Table 2.1). A subsequent cluster analysis of these variables revealed five distinct neuronal subgroups (denoted I, II, III, IV, and V) based on the variables distinguished by our PCA. In the following, we compare the membrane properties across the different subgroups (Table 2.2).

Since the data from our PCA failed normality ($P > 0.05$), it was transformed to its square root equivalent; and statistical analyses performed. Neuronal subgroups were found to differ in their R_{input} [$F(4, 106) = 325.93$, $P = 0.001$], TC [$F(4, 106) = 13.63$, $P = 0.001$] and TI [$F(4, 106) = 3.86$, $P = 0.006$] but not ADP [$F(4, 106) = 1.35$, $P = 0.25$]. Tukey's post-hoc

analyses showed that R_{input} was different in each neuronal cluster with V > III > IV > II > I (Figure 2.3A). Similarly, the TC of neurons in clusters III, IV and V were higher than those in clusters I, and II (Figure 2.3B). In addition, IT for neurons in cluster I was greater than for IV and V neurons (Figure 2.3C). Since the rectification and RS were categorical variables, we performed KW analyses on them. The rectification was not significant [$\chi^2(4)= 1.55$; $P = 0.818$] while RS were statistically significant [$\chi^2(4)= 10.28$; $P = 0.036$]. However, the post-hoc analyses for RS did not reveal any significance when we applied the Bonferroni correction ($P > 0.005$).

The intrinsic membrane properties of Vv neurons (N = 6) were also measured in ACSF and then compared to vPOA neurons under similar conditions. We used the subgroups of vPOA neurons derived from our PCA as a basis for comparison (Table 2.1). It was found that Vv neurons clustered with vPOA neurons in subgroups I (N = 3), II (N = 2) and IV (N = 1) suggesting at least three functional types of Vv neurons. However, due to the small sample size, these results cannot be considered conclusive.

From our analyses only R_{input}, TC and TI were significant ($P < 0.05$) while rectification and RS were not significant ($P > 0.05$). The TC is related to the R_{input} because it is directly proportional to the product of the R_{input} and C_{soma} [$TC = R_{input} \cdot C_{soma}$] (Molleman, 2003). Together, the R_{input}, TC and C_{soma} constitute the passive membrane properties of the cell. The calculated C_{soma} (after its transformation to the reciprocal of its square root to normalize the data) was statistical significant [$F(4, 106) =25.89$, $P = 0.001$]. Tukey's post hoc indicated that the C_{soma} was different with I > II > IV > V = III (Figure 2.3D). Since capacitance is also directly proportional to area [$C = A\varepsilon_r/d$; where A = area, ε_r is the dielectric constant of the membrane and d = the thickness of the membrane], it follows that the larger

the capacitance the larger the neuron. Putting all these in context, the capacitance will determine the threshold current. Large neurons will require large currents for elicitations of spikes. Therefore, neuronal spiking will depend on the passive membrane properties of a cell.

2. Properties of Postsynaptic Potentials: inputs from the olfactory bulb

Given these putative subgroups of vPOA neurons, we next set out to determine their connections to the olfactory bulb, OB. Of the 107 vPOA cells tested, 50 received synaptic inputs from the OB. The ratio of connected to unconnected neurons in each cluster was: I 59% (12/23); II 60% (20/38); III 0% (0/4): IV 53% (17/36); and V 19% (1/6). All Vv neurons were connected to the OB; this is consistent with the earlier observation that Vv neurons group with the vPOA subgroups I, II, and IV which were most likely to receive OB inputs.

The PSPs were reliably evoked with latencies of approximately 90ms (vPOA) and 60 ms (Vv). The coefficient of variation (CV) of the latency was low in both vPOA neurons (3.2%) and Vv neurons (3.6%), as was that for the rise-time of vPOA neurons (4.3%) and Vv neurons (6.7%) suggesting constant latency and rise time. Further, no failures were observed when stimulated with brief stimulus trains (Figure 2.4). Taken together, these data suggest there are monosynaptic connections from the OB to both the Vv and vPOA(Spanswick et al., 1998). Since the distance from the OB to the POA is about 9 mm, a latency of 100 msec suggests a conduction velocity similar to that of unmyelinated olfactory nerves in the tench (Carp family) (Dubois-Dauphin et al., 1980).

3. Biochemical properties of the postsynaptic potentials of vPOA and Vv neurons

To characterize the pharmacological properties of the PSP in both vPOA (N = 7)

and Vv (N = 6) neurons, goldfish brain explants were perfused sequentially with normal ACSF, Mg^{++}-free ACSF (MFACSF), 20μM D-APV and 10μM CNQX before washing off both drugs with normal saline. The latency, peak amplitude rise and decay time of the evoked PSPs were then measured and compared under the different recording conditions. The Mg^{2+}free ACSF potentiated the response to the evoked potential compared to normal saline. The antagonist, APV, partially blocked the evoked response which was subsequently blocked completely by CNQX indicating that the evoked potential was dual-component and mediated by glutamate acting on both NMDARs and AMPARs (Figure 2.5). The AMPARs may be required to depolarize the membrane sufficiently to relieve the Mg^{2+} blockage of the NMDARs. Indeed blocking AMPARs alone with CNQX (data not shown) failed to evoke any EPSPs implying the voltage-dependent block of the NMDARs is intact and requires preceding depolarisation via AMPARs to relieve this block.

The latency, rise times and decay times of the PSPs were found to be significantly different ($P < 0.05$) between the vPOA vs. Vv neurons while no significant difference ($P > 0.05$) was noted in the peak amplitude. For latency of the PSP, a main effect [$F(1,11)=14.38$, $P = 0.003$] was observed between type of neurons; no other effects were seen. Post hoc analysis indicated no within group differences ($P > 0.05$) were observed for either vPOA or Vv neurons (Figure 2.6A). In addition, group differences in the latencies between vPOA vs. Vv neurons were observed in ACSF [$t(11)=3.56$, $P = 0.005$], MFACSF [$t(11)=2.91$, $P = 0.014$] and APV [$t(11)= 3.55$, $P = 0.005$] (Figure 2.6A).

Rise time of the PSP was significantly different between the type of neurons [F(1,11)=5.73, P = 0.36]. There was also a significant type of neuron by perfusion medium interaction [F(1,11)=7.67, P= 0.018]. For the rise time of the PSP, post-hoc analyses did not find within group effects for either vPOA or Vv neurons. The only group differences in rise time between vPOA vs. Vv neurons was observed in ACSF [t(11)=2.95, P = 0.013] but not MFACSF [t(11)=1.28, P = 0.226] or APV [t(11) = 1.50, P = 0.162] (Figure 2.6B).

For the decay time of the PSP, there were main effects for type of neurons [F(1,11)=5.51, P = 0.038] and perfusion medium [F(1,11) = 50.91, P = 0.001] plus the interaction [F(1,11)=6.32, P = 0.029]. Post-hoc analyses for vPOA neurons revealed prolonged decay times in MFACSF vs. ACSF [t(6)=4.08, P = 0.006] and MFACSF vs. APV [t(6)=3.07, P = 0.022], but no significant differences was observed between ACSF vs. APV [t(6)=1.04, P=0.338] (Figure 2.6C). Additionally, for Vv neurons, the decay time PSP was prolonged in MFACSF vs. ACSF [t(5)=3.69, P = 0.014] and MFACSF vs. APV [t(5)=5.63, P = 0.002], but no statistical significance was found between ACSF vs. APV [t(5)=0.38, P = 0.717] (Figure 2.6C). Group differences were observed between vPOA vs. Vv neurons in ACSF [t(11)=2.51, P = 0.029] but not in MFACSF [t(11)= 0.69, P = 0.946] or APV [t(11)=1.84, P = 0.092] (Figure 2.6C).

Bathing the explant in MFACSF increased the decay times in both vPOA and Vv neurons, thereby prolonging the activation of NMDARs. These decay times in both ACSF and APV are considerably short. Therefore, the evoked PSPs had a biphasic response. APV partially blocked the slower and long lasting component of the evoked PSPs. leaving a faster and short lasting component that was subsequently and completely

blocked by CNQX suggestive of the involvement of both NMDARs and AMPARs in the synaptic transmission from the OB to the POA and Vv.

4 Sectioning the lateral olfactory tract while recording from the POA

To verify that the OB to vPOA projection is mediated exclusively through the medial olfactory tract, we sectioned the lateral olfactory tract (N = 3) while leaving the medial tract intact. We found that PSPs evoked in vPOA neurons by OB stimulation did not differ from those evoked under control conditions for latency, rise time, peak amplitude and decay times (KW analysis; $P > 0.05$). Furthermore, pharmacological manipulations influenced the EPSPs in similar ways for both cut and intact LOTs for latency, rise time, peak amplitude and decay times (KW analysis; $P > 0.05$). Sectioning both the medial and lateral olfactory tracts abolished the PSPs completely. This confirms that the pathway from OB to POA is via the medial olfactory tract.

DISCUSSION

Female sex pheromones regulate reproduction in male goldfish through the olfactory system (Partridge et al., 1976; Sorensen et al., 1991b; Dulka, 1993; Stacey et al., 2003; Chung-Davidson et al., 2008). Previous anatomical studies have shown direct connections from the OB to POA, and from the OB to Vv in the teleost brain (Forlano and Bass, 2011). With whole-cell patch clamp recordings, we show for the first time that both of these connections are functional glutamatergic monosynaptic connections. In addition, we show that both synapses involve the N-methyl-D-aspartate receptor (NMDAR) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR), which contributes significantly to the production of synaptic potentials in

both vPOA and Vv neurons in normal low frequency synaptic stimulation.

The POA is an important hypophysiotropic centre that regulates reproduction in vertebrates. Electrical stimulation of this area has been shown to elicit sperm release (Demski, 1983; Dulka and Demski, 1986; Dulka, 1993; Dominguez, 2009); sexual calling(Schmidt, 1968); nest-building and courtship (Demski and Knigge, 1971). Conversely, lesioning the POA impairs male reproduction thereby underscoring the importance of this neural system in goldfish reproduction (Hart et al., 1973; Kyle and Peter, 1982; Kyle et al., 1982; Koyama et al., 1984; Sorensen et al., 1991b; Dulka, 1993). In addition, the Vv, which is homologous to the mammalian septal nuclei, plays an important role in enabling reproductive behaviour. In males, lesioning either the Vv in goldfish or septal nuclei in rats interferes with responses to sex (Kyle and Peter, 1982; Gogate et al., 1995). The effects of the lesions on the goldfish Vv may either be due to damaged nuclei that process pheromonal cues and relay them to the POA or the severance of the fibres connecting the OB to the POA.

Heterogeneity of Neuronal Electrical Properties

We recorded from neurons in the vPOA while stimulating the OB. Since the identity of these neurons is not known, we characterized their intrinsic membrane properties. Our analysis revealed five subgroups of vPOA neurons, each with distinct intrinsic membrane properties and variable connections to the OB. Indeed, the POA has been shown to contain a plethora of cells immunoreactive to substance P (Sharma et al., 1989), GnRH (Peter et al., 1990; Parhar et al., 2001; Peter et al., 2003), γ-aminobutyric acid (GABA) (Martinoli et al., 1990), glutamate (Anglade et al., 1993), somatostatin (Canosa et al., 2004), CRF (Olivereau et al., 1984), secretoneurin (Canosa et al., 2011)

vasotocin(Parhar et al., 2001) and tyrosine hydroxylase (Hornby et al., 1987). Thus, this heterogeneity in electrophysiological profiles may reflect functionally diverse classes of vPOA neurons. Further work is required to determine if these electrophysiological “signatures” correspond to chemical phenotypes, projections and functions of these neurons. We also measured the intrinsic membrane properties of Vv neurons, in whole-cell current configuration to characterize them. Owing to their small sample size (N = 6), we compared them to vPOA neurons. We found that these Vv neurons clustered with group I, II and IV neurons in the vPOA.

Properties of Postsynaptic Potentials: inputs from olfactory bulb

Electrical stimulation of the OB evoked PSPs in both vPOA and Vv neurons in much the same way. These PSPs gave rise to action potentials at the peak of their responses in some cases. The evoked excitatory PSPs (EPSPs) had a consistent and constant latency and rise time with small coefficient of variations and no failures when stimulated repeatedly, indicative of monosynaptic inputs from the OB (Spanswick et al., 1998). These PSPs were not due to any artefacts of stimulations because some stimuli failed to evoke a response in some vPOA and Vv neurons when those neurons were not connected to the OB despite using the same stimuli that have been successful in eliciting responses from other neurons. This suggests that only neurons that are connected to the OB can be stimulated, but cells that are not connected cannot be stimulated by passive spreading current from the OB.

The conduction velocity of the inputs from the OB to the POA was similar to that found previously in other systems (Gasser, 1956; Potapov and Gusel'nikova, 1976a). The

relatively slow conduction is consistent with propagation through unmyelinated olfactory fibres (Potapov and Gusel'nikova, 1976b). The conduction velocity of the inputs from the OB to the vPOA was estimated to be 0.1 m/sec, which was similar to the pike (Gasser, 1956) and slower, by four times, than that reported by Kandel (Kandel, 1964) in the goldfish POA magnocellular neurons following stimulation of the pituitary gland. The slow conduction in our preparations may be either because the point of origin of our stimulation is cell bodies in the OB while in Kandel's preparation, the nerve terminals in the pituitary were stimulated but responses were recorded in the magnocellular cell bodies in the POA (Kandel, 1964). Nonetheless, the relative slowness of the conduction velocity in our preparation may be attributable to the slow conducting olfactory fibres that are either thinly myelinated or unmyelinated (Potapov and Gusel'nikova, 1976b).

Biochemical properties of the synaptic connections

Since anatomical connections between the OB, vPOA and the Vv have been established previously through tract-tracings (Levine and Dethier, 1985; Anglade et al., 1993), we were interested in defining the functional nature of these innervations. We hypothesized that the connections from the OB to the vPOA and OB to the Vv may be glutamatergic because glutamate is ubiquitous and widespread in the goldfish brain especially in areas that subserve reproduction (Peter et al., 1980).

To investigate the role of glutamate in chemical communication, we perfused the goldfish brain explant sequentially with ACSF, APV and CNQX in Mg^{2+}free ACSF (as shown in Figure 2.5) while stimulating the OB to measure the latency, amplitude, rise time, decay time and duration of the evoked potentials in either the vPOA or Vv. Mg^{2+}free ACSF enhanced the evoked EPSPs compared to normal ACSF. The antagonist

APV partially blocked the EPSPs leaving a fast acting and short lasting component that was subsequently completely blocked by CNQX irreversibly suggesting that the evoked PSP was excitatory, bimodal and mediated by glutamate acting on NMDARs and AMPARs, respectively. Receptors for AMPA may therefore be required to depolarize the cells (in their resting state) sufficiently to relieve the Mg^{2+} blockage of NMDARs so that glutamate can activate the receptor (Gotz et al., 1997; Spanswick et al., 1998).

The complete blockage of the evoked PSPs by the glutamatergic antagonists suggests that glutamate plays an important role in mediating chemical communication between the OB to the vPOA and OB to the Vv. Since both the POA and Vv are important for the regulation of reproductive behaviour, they may receive pheromonal cues from the OB to integrate milting and spawning in male goldfish (Kyle and Peter, 1982; Kyle et al., 1982). The use of glutamate signalling may therefore be a mechanism to induce sustained neuronal firing required to trigger sex hormones surge when sex pheromones are detected. To our knowledge, this is the first pharmacological characterization of second order neurons in the teleost olfactory system linked to reproduction.

We discovered that the connections from the OB to the vPOA and the OB to the Vv are monosynaptic and glutamatergic. This finding complements and extends our understanding of the neural circuitry involved with goldfish reproduction (Figure 2.7). The Vv neurons have not been fully characterized. However, previously Trudeau et al. (Trudeau et al., 2000b) demonstrated the existence of monosynaptic GABAergic projections from the Vv to the vPOA (Figure 2.7). In addition, other workers have revealed that this area contains neurons immunoreactive for GABA (Martinoli et al.,

1990). Indeed, GABA plays a central role in male goldfish reproduction by suppressing the DAergic inhibition of LH release [8,12]. There may well be projections from the Vv cells we recorded from to some areas of the POA especially areas posterior to the anterior commissure where DAergic cells have been found (Peter and Paulencu, 1980; Trudeau et al., 2000b). This suggests that there are interplays between diverse sets of neurotransmitters and neurohormones that regulate reproduction in male goldfish. The Vv may therefore modulate the glutamatergic inputs from the OB to the vPOA to regulate some aspects of reproductive behaviour or hormone release.

Role of Glutamate in Goldfish Reproduction

Previous studies have shown that intraperitoneal injections of male goldfish with monosodium glutamate (MSG) [58], NMDA [Trudeau 1993] or AMPA [12, 16] rapidly induces LH release. Furthermore, in rainbow trout it has been shown that the LH response to NMDA is blocked by APV or a GnRH receptor antagonist, indicating that glutamate modulates LH release through stimulation of GnRH (Flett et al., 1994), similar to the situation in mammalian models [61]. Moreover, Peter et al. (Peter et al., 1980) has shown that MSG injections in goldfish causes cellular degeneration in the POA, demonstrating excitotoxic actions of glutamate on POA neurons. Additionally, in rats it has been shown that glutamate injections in the POA or electrical stimulation of the POA decreases the latency between intromissions thereby increasing ejaculation frequency (Dominguez, 2009). Glutamate in the POA therefore plays an important role in vertebrate reproduction.

Sectioning the Lateral Olfactory Tract

We employed olfactory tract sectioning to determine if the glutamatergic projection to the vPOA was via MOT or the LOT. The EPSPs recorded in vPOA cells in explants with a transected LOT had the same amplitude and duration as those with the LOT intact. These EPSPs were modulated by APV and CNQX in much the same way as in intact preparations. Sectioning the MOT while leaving the LOT intact, eliminated OB-mediated PSPs in vPOA neurons. Our electrophysiological data therefore indicates that the OB to vPOA projection is via the MOT and not the LOT. This supports previous studies indicating unequivocally that sex pheromones signals in goldfish are mediated exclusively by the MOT (Sorensen et al., 1991b).

CONCLUSION

We show that the connections from the OB to the vPOA and from the OB to the Vv are monosynaptic and glutamatergic. Our results suggest there are two pathways to the vPOA from the OB – one directly to the vPOA and the other indirectly to the POA via the Vv. The direct connections from the OB to vPOA may facilitate spermiation and steriodogenesis (Peter et al., 1980; Peter and Paulencu, 1980; Kyle and Peter, 1982) while the indirect connections from the OB to the vPOA through GABA neurons in the Vv may play a role in modulating sexual behaviour and arousal (Kyle and Peter, 1982; Koyama et al., 1984; Levine and Dethier, 1985). While speculative at this point, the olfactory glutamatergic projections we identified may represent pathways that integrate pheromonal signals from females that stimulate reproductive hormone release, male sexual behaviour in the spawning period [63].

Table 2.1: The rotated component matrix of variables used in our principle component analysis. Loaded factors in bold show measurements that were subsequently used for our cluster analysis. See definitions in *Data Analysis* Section and Figure 2.1.

	Loaded factors					
Variables	**1**	**2**	**3**	**4**	**5**	**6**
TC (msec)	-.134	.081	.060	**.781**	-.105	-.162
RMP (mV)	.126	.829	.035	-.206	-.045	.146
R Input (MΩ)	-.110	**.865**	.044	.112	.028	.014
ADP (mV)	-.209	.151	**.791**	-.090	-.119	-.084
APA (mV)	.192	-.014	.535	-.515	-.072	.012
It (pA)	-.063	-.357	.033	-.269	**.805**	.034
ft (Hz)	.183	.321	.047	.126	.793	-.109
AHP (mV)	-.051	.085	-.788	-.172	-.239	-.059
CV	-.014	.220	-.106	-.007	.153	-.209
RD	.158	.116	-.129	.050	.061	-.187
APD (ms)	.476	-.373	.156	-.133	-.232	.018
Sag	.023	-.223	-.012	.614	.007	.414
Rectifying	-.049	.169	-.021	-.020	-.052	**.687**
RS	**.911**	-.074	-.082	-.135	.041	.070
RDS	.890	.140	-.036	-.018	.115	-.135

Extraction Method: Principal Component Analysis.
Rotation Method: Varimax with Kaiser Normalization.
Rotation converged in 6 iterations.

Table 2.2: Intrinsic membrane properties of POA and Vv neurons measured in ACSF. RMP (resting membrane potential); TC (membrane time constant); Rinput (input resistance); C_m (membrane capacitance); AHP (after hyperpolarization potential); APA (action potential amplitude); TI (action potential threshold current); TF (action potential threshold frequency); APD (action potential duration); CV (coefficient of variation); ADP (after depolarization potential). Note that I – V are clusters of POA neurons while Vv are neurons from the Vv. Not shown are variables for rectifying and RS. See definitions in *Data Analysis* Section and Figure 2.1.

CLUSTERS / ***VARIABLES***	***I*** *(N = 23)*	***II*** *(N = 38)*	***III*** *(N =4)*	***IV*** *(N = 36)*	***V*** *(N = 6)*	**Vv** (N = 6)
RMP (mV)	-56.5 ± 2.2	-58.2 ± 1.4	-57.1 ± 4.5	-56 ± 1.4	-54.3 ± 2.1	-62.0 ± 3.5
TC (msec)	47.6 ± 5.2	47.0 ± 2.7	92.9 ± 15.3	66.2 ± 3.9	110.3 ± 10.8	42.8 ± 9.8
Rinput (GΩ)	0.42 ± 0.04	1.0 ± 0.03	3.7 ± 0.15	2.0 ± 0.07	5.9 ± 0.04	1.3 ± 0.3
C_{soma} (pF)	144.3 ± 24	45.8 ± 2.8	24.9 ± 3.7	34.3 ± 3.4	19.2 ± 2.8	35.8 ± 10.3
AHP (mV)	12.2 ± 1.3	10.2 ± 0.8	13.7 ± 5.8	11.0 ± 1.0	15.4 ± 3.7	3.2 ± 0.4
APA (mV)	56.1 ± 2.7	52.6 ± 2.3	60.7 ± 5.7	53.1 ± 1.9	54.3 ± 4.5	57.3 ± 6.0
TI (pA)	13.0 ± 2.0	8.2 ± 1.2	3.9 ± 0.8	6.0 ± 0.7	3.3 ± 0.8	14.9 ± 4.0
TS (Hz)	3.3 ± 0.6	2.4 ± 0.3	2 ± 0.4	3.5 ± 0.4	5.3 ± 1.0	5.3 ± 1.5
APD (msec)	5.9 ± 0.5	7.0 ± 0.5	7.2 ± 1.0	8.2 ± 0.9	6.2 ± 0.9	11.3 ± 1.7
CV	1.1 ± 0.1	1.1 ± 0.1	1.0 ± 0.1	1.1 ± 0.1	2.3 ± 0.6	0.9 ± 0.3
ADP (mV)	1.4 ± 0.4	1.6 ± 0.4	-	0.6 ± 0.2	0.12 ± 0.0	0.9 ± 0.3

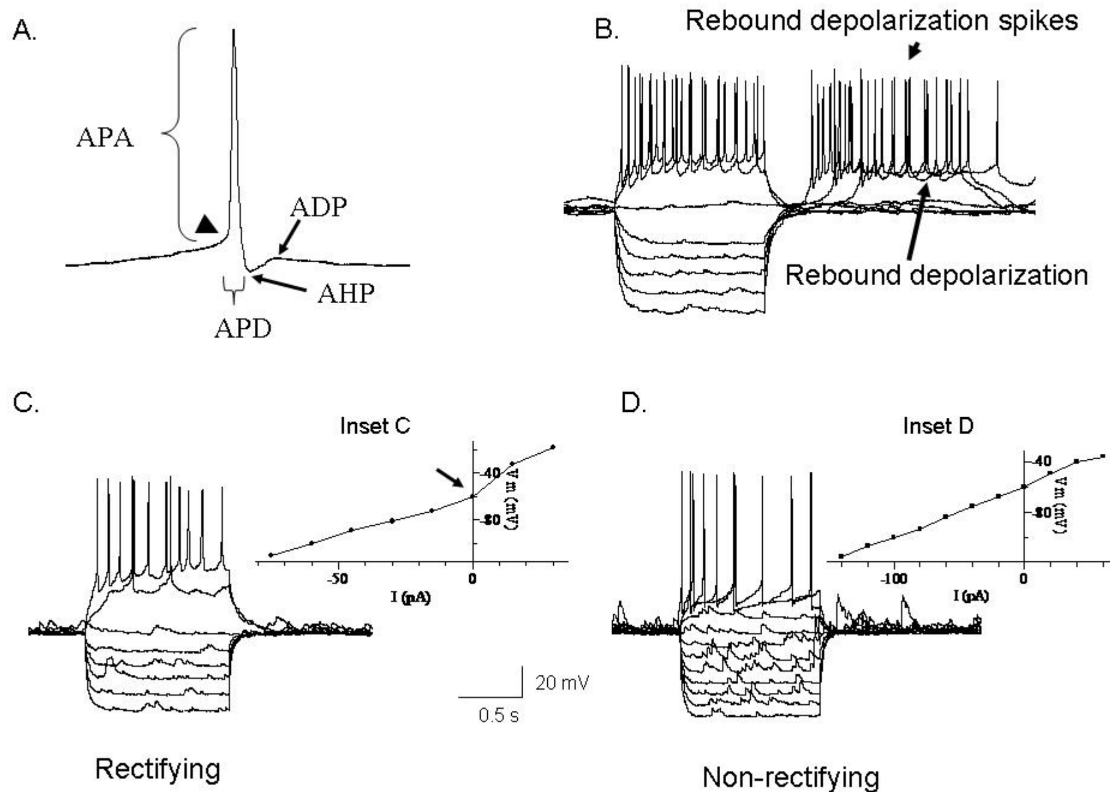

Figure 2.1: Traces of some of the intrinsic membrane properties used in our statistical analyses. Panel A: depicts the AHP (after hyperpolarization potential), APA (action potential amplitude), APD (action potential duration) and ADP (after depolarization potential). B shows the RD (rebound depolarization) and RDS (rebound depolarization spikes). C and D demonstrate the current to voltage relationship (IV) where C is rectifying as shown by the arrow at the inflection point (Inset C) and D is non-rectifying (Inset D). APA was measured from the shoulder at threshold shown by the arrowhead. APD was the width of the AP measured from the shoulder at threshold shown by the arrowhead. See definitions in *Statistical Analysis* Section.

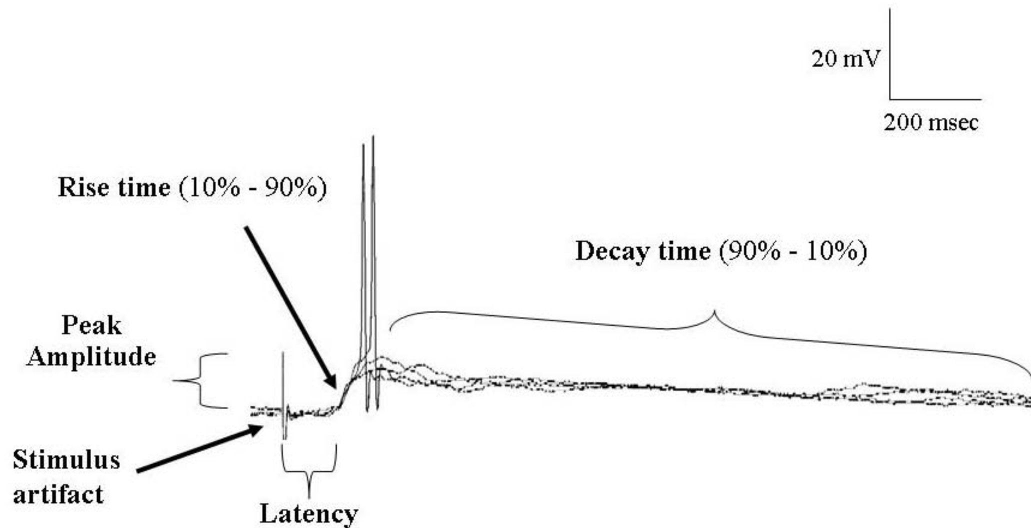

Figure 2.2: Samples of a continuous recording showing superimposed EPSPs evoked in POA neurons following stimulation of the olfactory bulb under normal (ACSF) conditions and highlighting the properties of EPSPs measured. Note that evoked EPSPs could give rise to action potential firing and showed constant latency and rise time, consistent with a monosynaptic origin.

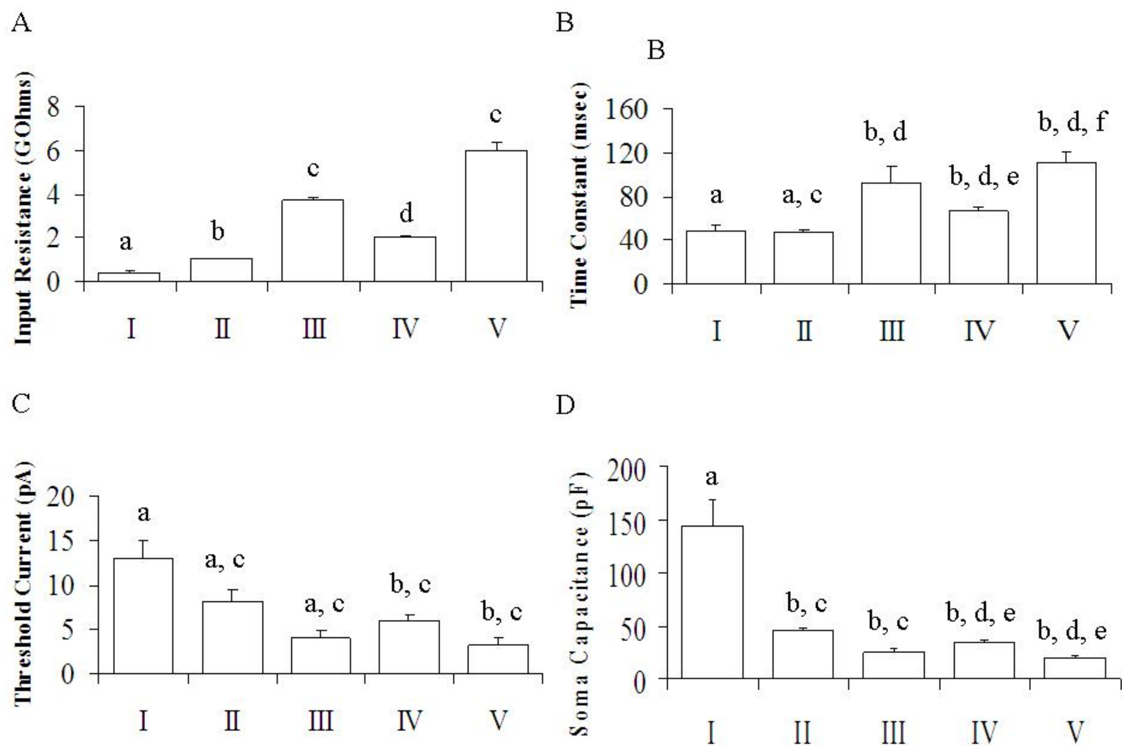

Figure 2.3: Intrinsic membrane properties of POA neurons. Neurons were made up of connected (N = 50) and unconnected (N = 57) neurons to the OB. Panel A: The input resistance showing group differences. Panel B: The time constant, indicating differences between groups. Panel C: The threshold current differences between groups. Results are presented as mean ± SEM for convenience. Letters represent groups that differed significantly ($P < 0.005$) from each other with Bonferroni corrections; same letters show no group differences. For detailed statistical significance see TABLE 1. I (N = 23); II(N = 38); III(N = 4); IV(N = 36) and V(N = 6) .

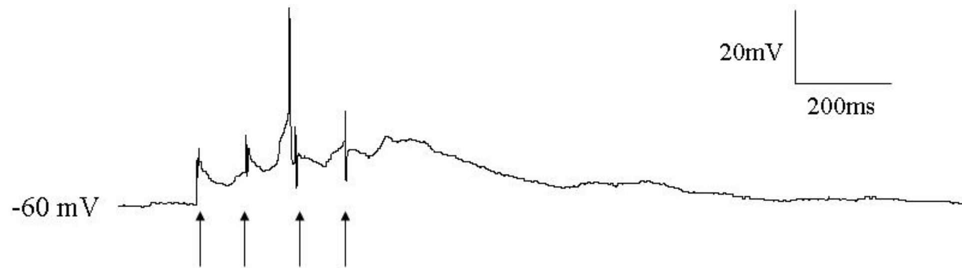

Figure 2.4: High frequency stimulations of POA neurons with current delivered from the OB. Holding potential was -60mV. Arrows showing stimulus artefacts of stimulus delivery, four times (30V, 0.2ms) with 100msec delay between stimulus deliveries.

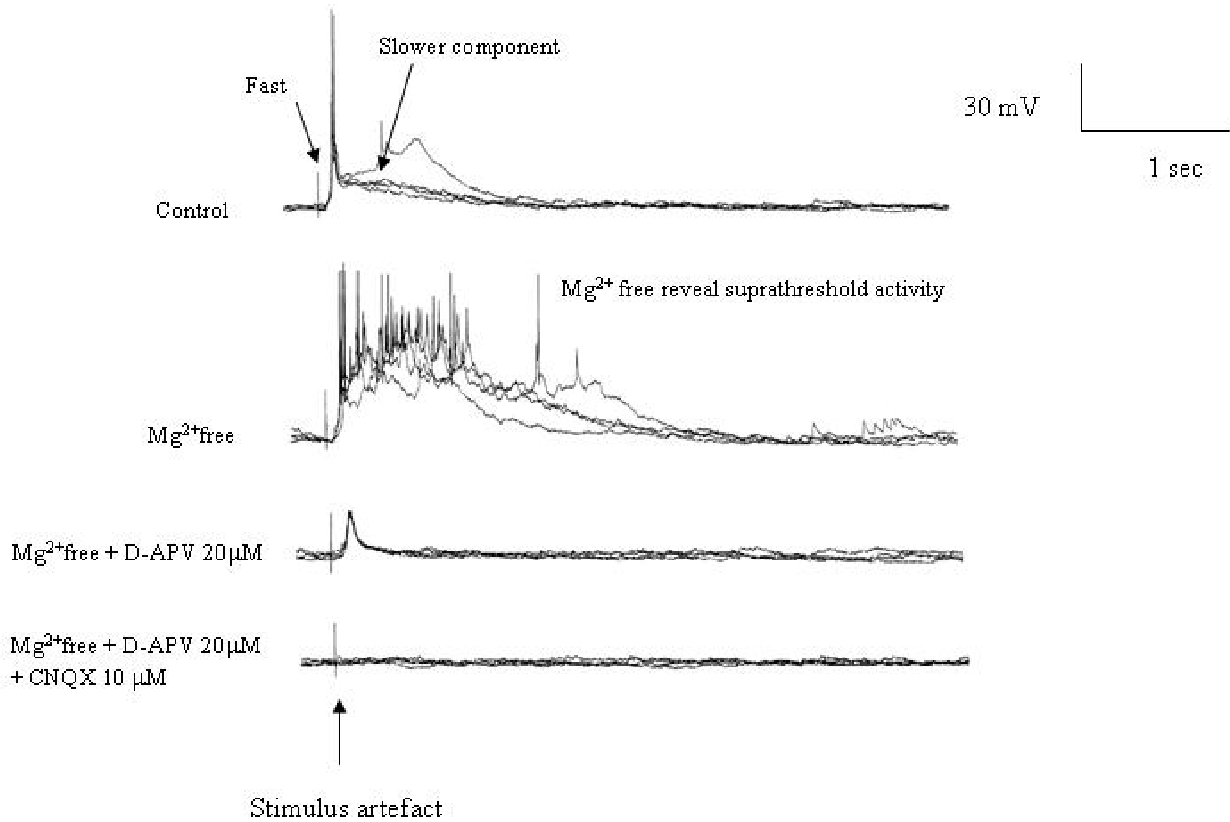

Figure 2.5: Evoked EPSPs in a POA neuron under different recording conditions. After achieving whole cell access, recordings were made in current-clamp mode. Typically, neurons were perfused sequentially with normal ACSF, Mg^{2+}free ACSF, 20uM AP-5 (in Mg^{2+}free ACSF) and 10uM CNQX in Mg^{2+}free ACSF) for 10min each before recordings. Not shown is the figure for Vv neurons which was similar to the figure above.

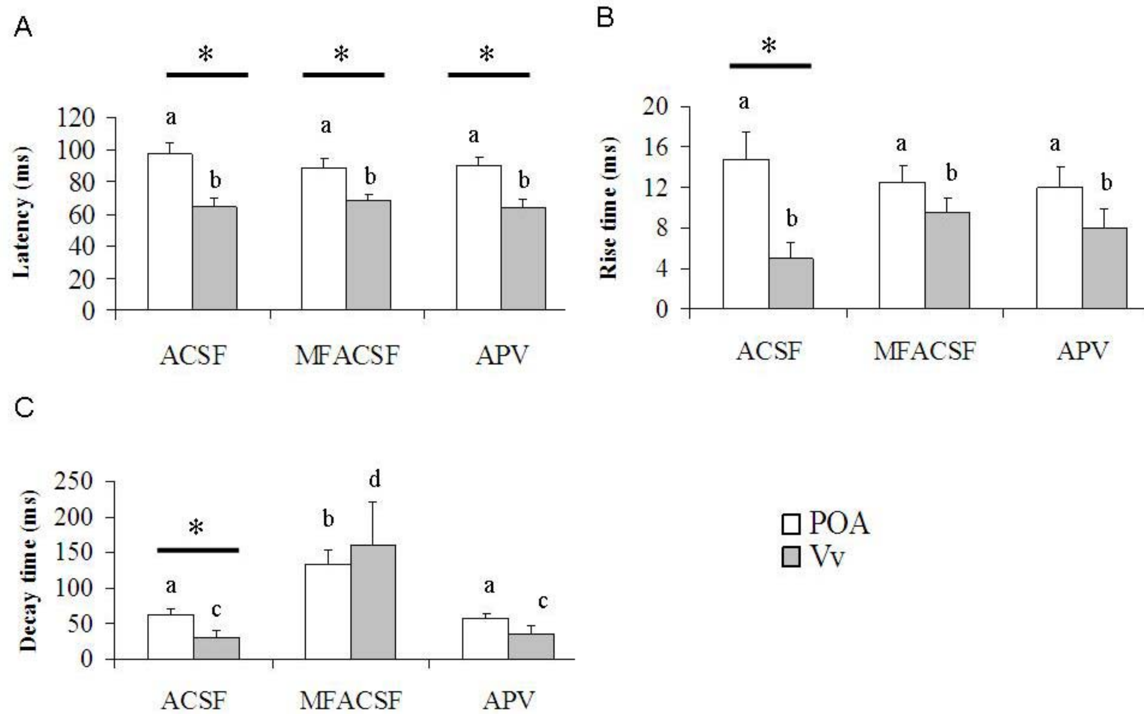

Figure 2.6: Comparisons of OB synaptic inputs to POA (N = 7) and Vv (N = 6) neurons under different perfusions (ACSF, MFACSF and APV). Note that values for CNQX and washout are not shown because CNQX irreversibly and completely blocked the EPSPs even after 10 min of washout. Most of the POA neurons clustered in group I (2/7), II (1/7) and IV (4/7). Panel A: The evoked EPSP took longer to reach POA neurons than Vv neurons in ACSF, MFACSF and APV. Panel B: The rise time for POA neurons was longer in ACSF than Vv neurons but was unaffected in MFACSF and APV. Panel C: The decay time of POA and Vv neurons was prolonged in MFACSF vs. ACSF and APV. Error bars denote SEM. Asterisks (*) and line bar (—) show statistical significance ($P < 0.05$) between groups while scripts denote differences within groups. Note that neurons from both groups were derived from clusters I, II and IV for both POA and Vv neurons.

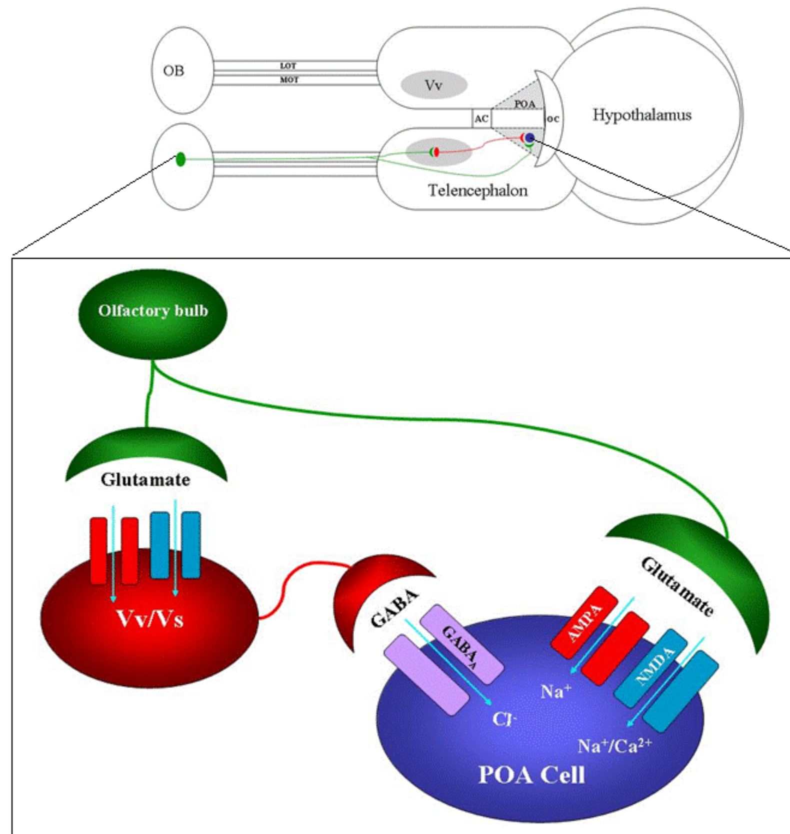

Figure 2.7: A ventral-side up diagram of the goldfish brain with the proposed neural circuitry of chemical communication showing synaptic connections. The connections from the OB to either the Vv or POA are glutamatergic while that from Vv to the POA is GABAergic (Trudeau et al, 2000). AC (anterior commissure), OB (olfactory bulb), OC (optic chiasm), LOT (lateral olfactory tract), MOT (medial olfactory tract), POA (preoptic area), Vv (ventral telencephali pars ventralis).

CHAPTER 3

RAPID MODULATION OF GENE EXPRESSION PROFILES IN THE FOREBRAIN OF MALE GOLDFISH FOLLOWING EXPOSURE TO WATERBORNE SEX PHEROMONES

ABSTRACT

Sex pheromones rapidly affect endocrine physiology and behaviour, but little is known about their effects on gene expression in the neuroendocrine tissues that mediate olfactory processing. In this study we exposed male goldfish for 6 h to waterborne 17,20βP (4.3 nM) and $PGF_{2\alpha}$ (3 nM), the main pre-ovulatory and post-ovulatory pheromones, respectively. Both treatments elevated milt volume (P = 0.001). Microarray analysis of male telencephalon following $PGF_{2\alpha}$ treatment identified 71 unique transcripts that were differentially expressed (q < 5%; 67 up, 4 down). Functional annotation of these regulated genes indicates that $PGF_{2\alpha}$ pheromone exposure affects diverse biological processes including nervous system functions, energy metabolism, cholesterol/lipoprotein transport, translational regulation, transcription and chromatin remodelling, protein processing, cytoskeletal organization, and signalling. By using real-time RT-PCR, we further validated three candidate genes, ependymin-II, calmodulin-A and aldolase C, which exhibited 3 - 5 fold increase in expression following $PGF_{2\alpha}$ exposure. Expression levels of some other genes that were hypothesized to be important for reproduction were also determined using real-time RT-PCR. Expression of sGnRH was increased by $PGF_{2\alpha,}$ but not 17,20βP, whereas cGnRH expression was increased by 17,20βP but not $PGF_{2\alpha}$. In contrast, both pheromones increase the expression of glutamate (GluR2a, NR2A) and γ-aminobutyric acid ($GABA_A$ γ2) receptor subunit mRNAs. This gene expression data link

milt release and rapid modulation of neuronal transcription as part of the response to female sex pheromones.

INTRODUCTION

Chemical communication is important for intra-species communication. Vertebrates have evolved cryptic pheromonal signalling to help navigate the complex environment of cues while avoiding eavesdropping. One such example of signalling is sex pheromones, which vertebrates have used to signal reproductive status and readiness. This strategy obviates the need for species to spend time and energy inspecting one another for reproductive cues, thereby allowing them to expand their range for feeding while expending their energy on growth, development and predator avoidance. Species-specificity of pheromone signalling is achieved through chemical form, size, polarity and blends depending on biosynthetic and environmental constraints (Brennan and Zufall, 2006; Symonds and Elgar, 2008).

Sex pheromones are important for goldfish reproduction because they are oviparous and live in murky waters where visibility is greatly reduced. Therefore, in order for male and female goldfish to coordinate their reproduction, they have established an elaborate and sophisticated chemical communication system to aid in advertising their reproductive status to conspecifics (Stacey, 2003). The sex pheromones in goldfish are sex hormones that have been co-opted through evolution and given new functionality. For example, 17α,20β dihydroxy-4-pregene-3-one (17,20βP) – the preovulatory priming pheromone – is an oocyte maturation-inducing steroid hormone in females, and when it is released in the water acts on males to induce milt production and courtship (Sorensen et al., 1998; Stacey, 2003). In addition, prostaglandin-$F_{2\alpha}$ ($PGF_{2\alpha}$) is a female hormone that stimulates female sexual behaviour but when released to the water by the female it becomes a postovulatory releaser pheromone that triggers milt production and male

sexual behaviour (Sorensen et al., 1988; Stacey et al., 2003). It is now well established that 17,20βP and $PGF_{2\alpha}$ are the primary sex pheromones controlling spawning in goldfish. Female goldfish, responding to environmental cues, release a priming pheromone (17,20βP and its metabolites) triggering an increase in luteinizing hormone (LH), milt production and courtship in males (Kobayashi et al., 1986a). A few hours later, the ovulating females emit the releasing pheromone ($PGF_{2\alpha}$ and its metabolites) which synchronizes the sexual behaviour of both sexes leading to spawning (Kobayashi et al., 2002).

Experimental studies on sexually mature male goldfish indicate that sex pheromones acting via the olfactory epithelium induce LH release from the pituitary gland through a suppression of dopaminergic inhibition of gonadotropin-releasing hormone (GnRH) in the preoptic area (POA) to regulate male courtship and sexual behaviour (Stacey, 1983; Kobayashi et al., 1986b; Trudeau, 1997; Kobayashi et al., 2002). However, how sex pheromones regulate genes important for reproduction is not well understood. Thus, we performed a cDNA microarray analysis to profile gene expression in the telencephalon of male goldfish exposed to waterborne $PGF_{2\alpha}$ for 6 hours. We chose to profile the effects of $PGF_{2\alpha}$ because it is the potent post-ovulatory pheromone responsible for induction of spawning behaviour and the final increase in milt production (Sorensen et al., 1988; Sorensen et al., 1989; Stacey et al., 2003). Importantly, $PGF_{2\alpha}$ exposure modulates neurogenesis in the male goldfish coinciding with behavioural changes during spawning (Kobayashi et al., 2002; Chung-Davidson et al., 2008). Additionally, we used targeted real-time reverse transcriptase polymerase chain reaction (RT-PCR) on a series of transcripts so that we could compare the effects of

the preovulatory pheromone, 17,20βP, to that of postovulatory pheromone, $PGF_{2\alpha}$ These transcriptomic data support the physiological data obtained to offer insights into the molecular mechanism underlying sex pheromones-mediated changes in the brain.

MATERIALS & METHODS

Animals

In the experiment to measure gene expression, goldfish (N = 36) were purchased from Aleong's International Inc., (Mississauga, ON, Canada) in April and sorted according to their sexual maturity based on tubercles on their pectoral fins and opercula. The sexually mature male goldfish were then acclimated to 18 ^{o}C in 70-liter round tanks, filled with dechlorinated tap water (12 per tank; three tanks total), for 3 weeks before commencing experimentation. The fish were fed daily on standard goldfish flaked food (Martin Mills, Elmira, ON, Canada) and maintained under natural photoperiod (L:D – 13: 11). For all dissections, fish were anaesthetized with 0.05% 3-aminobenzoic acid ethyl ester (MS222: Aquatic Eco-Systems, Apopka, FL). The study was conducted in early June and all experimental procedures were approved by the University of Ottawa Protocol Review Committee and followed the established Canadian Council on Animal Care guidelines on the ethical treatment of animals in research.

Chemicals and Experimental Procedure

Stock solutions of 17,20βP and $PGF_{2\alpha}$ (both from Sigma-Aldrich, Oakville, ON, Canada, purity ≥99%) were prepared by dissolving 1 mg of each of the respective chemical compounds in 1 ml of 95% ethanol (EtOH: Commercial Alcohols Inc.,

Brampton, ON, Canada, purity ≥99%) and stored at -20°C (Sorensen et al., 1989). The vehicle and pheromones were then applied to the fish tanks so that their final concentrations were 0.28 x 10^{-6}% (v/v) EtOH, 4.3 nM 17,20βP and 3 nM $PGF_{2\alpha}$. These concentrations are based on other studies showing robust effects at this levels (Sorensen et al., 1989; Chung-Davidson et al., 2008; Mennigen et al., 2010). Telencephalic tissues from the fish were rapidly removed, pooled (2 telencephalon per tube) and frozen on dry ice. Samples were then stored at -80°C until required for RNA isolation.

Milt Volume

Milting males were selected and randomly assigned to groups of 12 in flow-through 70-liter tanks. The fish were then exposed to waterborne 4.3 nM 17,20βP and 3.0 nM $PGF_{2\alpha}$ with the appropriate vehicle control for 6 hours. The exposures were timed to coincide with goldfish spawning in the early morning (Kobayashi et al., 2002). Fish were then anaesthetized, stripped of milt before being sacrificed.

Milt, extracted by gently squeezing the abdomen from the mid-ventral region posterior to the gonopore, was drawn by aspiration into a pre-weighted hematocrit tube which was weighed again to calculate the weight of the milt as described elsewhere (Kyle et al., 1985). Fish were stripped of milt twice. The first stripping was conducted before the start of the experiment to reduce a priori the intra-individual variability between fish by ensuring that all milt reserves are empty and they all start from the same basal level. The second stripping was to measure the replenishments of the strippable milt reserves following exposure to either sex pheromone.

RNA Extraction & cDNA synthesis

Homogenization of telencephalic tissues was performed using an MM301 Mixer Mill (Retsch, Newton, PA) at 20Hz for 2 min. Total RNA was, then, isolated from the telencephalon using Qiagen's RNeasy Plus Mini Kit (Mississauga, ON, Canada). Total RNA was extracted by RNeasy kit (Qaigen, Mississauga, ON, Canada). The quality of the RNA was assessed by an Agilent 2100 BioAnalyzer (Agilent, Palo Alto, CA), and the quantity determined on a NanoDrop spectrophotometer (Nano-Drop Technologies, Wilmington, DE). For real-time RT-PCR, the RNA (2ug), from control or treatment groups, was reverse transcribed (in a 20uL volume) into cDNA using Superscript II RNase H^- reverse transcriptase (SSII) with 200 ng random hexamer primers according to Invitrogen's protocol. A negative control, with RNase-free water instead of SSII enzyme (NRT), was also conducted for the cDNA synthesis at the same time.

Microarray Hybridization

We used version 1.1 of our carp-goldfish cDNA microarray which has been validated for use with neuroendocrine tissues (Martyniuk et al., 2006; Marlatt et al., 2008; Mennigen et al., 2008; Popesku et al., 2008a; Zhang et al., 2009a). The data from this experiment has been deposited in the Gene Expression Omnibus database (accession number GPL3735). Amplification and cDNA labelling with Cy3 and Cy5 for microarray hybridization followed the manufacturer's instructions (Genisphere, Hatfield, PA, USA).

A total of four microarray slides were hybridized to study the effects of $PGF_{2\alpha}$ on gene expression in the telencephalon. Control samples (N = 4) were pooled to form our reference or technical replicates while treatment samples (N = 4), which formed our

biological replicates, were ran separately(Allison et al., 2006). In addition, two dye swaps were performed for our biological samples for the treatments. This microarray design has also been extensively validated in our laboratory (Martyniuk et al., 2006; Marlatt et al., 2008; Mennigen et al., 2008; Popesku et al., 2008a; Zhang et al., 2009a). The arrays were scanned at full speed 10μ resolution with ScanArray 5000 XL system (Packard Biosciences/PerkinElmer, Woodbridge, ON, Canada) using both red and blue lasers. Images were obtained with ScanArray Express software using automatic calibration sensitivity varying photomultiplier (PMT) gain (PMT starting at 65% for Cy 5 and 70% for Cy3) with fixed laser power at 80% and the target intensity set for 90%. Resulting images were processed with QuantArray (Packard Biosciences/PerkinElmer, Woodbridge, ON, Canada), and raw signal intensity values were obtained for duplicate spots of genes.

Microarray Data Analysis

Microarray data analysis is described in detail in Martyniuk et al., (Martyniuk et al., 2006). Briefly, intensity-dependent Lowess normalization (Yang et al., 2002) was used to remove the signal bias of slides. The Significance Analysis of Microarray (SAM) method (Tusher et al., 2001) was used to assess the significance of the differential expression of the genes. The first criterion for significance was $q < 5\%$, which is based on the false detection rate (FDR). FDR is the percentage of significant genes identified by chance, while the q value is the minimum false discovery rate at which the genes are significant ($q < 5\%$). The q value is an adjusted P value, and it is designed for the FDR analysis using SAM. A fold-change >1.5 was a second criterion for the selection of genes for functional annotation.

Function was firstly assigned based on protein domain information that was retrieved from the Pfam database (Punta et al., 2012). For the protein domain family with functional involvement in multiple cellular processes, specific function of each subgroup of the family was inferred based on the reported literature on the human orthologs identified by the Basic Local Alignment Search Tool (BLAST) program in NCBI (Altschul et al., 1990).

Gene Ontology Analysis

Gene Ontology (GO) analysis for differentially expressed genes was carried out using the BLAST2GO program (http://www.blast2go.de/) to explore the potential biological processes affected by $PGF_{2\alpha}$ (Conesa et al., 2005). Functional gene clusters were defined by genes sharing a series of common GO terms in the category of biological process. Similarity threshold of 0.5 and other parameters with default setting were used. Significance was defined as enrichment thresholds larger than 0.5.

Real-time Polymerase Chain Reaction (RT-PCR)

We used the same mRNA samples (N = 4 per treatment) for cDNA microarray and real-time RT-PCR analyses. SYBR green real-time RT PCR reactions were carried out to measure the relative gene expression of ependymin II (EPDII), calmodulin-A (CaM-A), and aldolase C (ALDOC) to validate our arrays (Table 3.1). Ribosomal 18S subunit was chosen as our reference gene because previous work in our laboratory has shown that it is stable across a range of experimental conditions (Zhang et al., 2009a;

Mennigen et al., 2010). All primers were acquired from Invitrogen after designing them in either Primer3 (http://frodo.wi.mit.edu/primer3/) or Genscript (http://www.genscript.com/ssl-bin/app/primer) software. Primers (18-20 base pairs; bp) with optimal annealing temperatures between 58 and 60°C were designed to amplify sequences of 100 – 250 bp of genes of interest printed on the array. All primers tested have been validated before in our laboratory (Martyniuk et al., 2006; Zhang et al., 2009a) except for salmon GnRH (sGnRH) and chicken (cGnRH-II) primers which were from Chung-Davidson et al., (Chung-Davidson et al., 2008). These were chosen for RT-PCR because GnRH is the primary neuropeptide that stimulates reproduction in vertebrates (Yu and Peter, 1990). In addition, we were interested in comparing the expression levels of sGnRH and cGnRH-II in our experiments with the findings of Chung-Davidson et al., (Chung-Davidson et al., 2008).

Candidate genes from our arrays were amplified and detected using Mx4000 Quantitative PCR System (Stratagene, La Jolla, CA). Each real-time RT-PCR reaction contained the following: 2.5 - 3.5 mM $MgCl_2$, 1x PCR buffer, 0.25x SYBR green (Invitrogen), 0.1 uM ROX reference dye, 1x PCR buffer, 0.2 mM dNTPs, 1.25 unites of HotStar Taq (Invitrogen), 0.2 uM gene-specific primers (Table 3.1) and 50 ng template cDNA in a 25-µl reaction volume. In addition, each reaction was run with negative controls consisting of no template control (NTC) with RNase-free water instead of the template (cDNA) and a no reverse transcriptase (NRT) reaction. In all cases, no amplification was observed in NTC/NRT, indicating the reactions were free of contaminants.

The parameters for the thermal cycles were as follows: 1 cycle of Taq activation at 95°C for 15 min, followed by 40 cycles of 95°C for 15 s, 58 - 62°C for 5s (depending on primer set), 72°C for 30s, and a detection step at 80°C for 8s. The cDNAs from each sample were diluted (1:10 to 1:31,250), and the dilutions used to generate a relative standard curve for each primer set. Following the reactions, a dissociation curve was established, starting from 55°C (1°C /30s) to 95°C. Standard curves with slopes between -3.2 and -3.5 with $R^2 \geq 99\%$ and amplification efficiency between 90 and 110% were used. The amplification of PCR product was measured in real time as an increase in SYBR green fluorescence. The dissociation curves were all single peaks, indicating specificity. For all reactions, real-time RT-PCR samples were run in duplicates; and the data collected was analyzed with MxPRO, version 4.10 (Stratagene).

Statistical Analysis

Milt and RT-PCR data was analyzed using SPSS 15.0 and Excel software package. When data met the assumption of normality and homoscedasticity [Kruskal-Wallis Test: $P > 0.05$], an analysis of variance (ANOVA) and Tukey's post hoc test were used to test for statistical significance and identify differences between groups. Only data for ALDOC mRNA levels were skewed and violated kurtosis, and required log transformation to achieve normality prior to ANOVA. Results are reported as means and standard error of the mean (SEM).

RESULTS

Elevated Milt Volume

Milt volume was significantly increased [$F(2, 23) = 9.60$, $P = 0.001$] by both the pheromone treatments compared to vehicle but they did not differ significantly ($P > 0.05$) from each other (Figure 3.1). These data indicate that the pheromone exposures under our experimental conditions resulted in a robust physiological responses and confirms other reports about the effects of these pheromones (Sorensen et al., 1989; Kobayashi et al., 2002).

Expression profiling following exposure to PGF2α

Analysis of the microarray data identified 484 genes that were differentially expressed ($q < 5\%$) following pheromone exposure. Of these genes, 71 unique genes met our cut off threshold of ≥ 1.5 fold-change of which 67 were up-regulated while 4 were down-regulated (Table 3.2).

We performed a GO enrichment analysis to explore the potential biological processes related to the differentially expressed genes using BLAST2GO (Conesa et al., 2005). Primary biological process terms identified nine GO term classifications (Figure 3.2). These classifications are consistent with the general functional involvement of sex pheromones in various biological processes (Table 3.2).

Microarray validation using real-time RT-PCR

Using real-time RT-PCR, we further validated 3 genes identified by microarray as being up-regulated by $PGF_{2\alpha}$. Ependymin-II (EPDII) and calmodulin-A (CaM-A) are related to nervous system functions and we previously found that their expression in the hypothalamus was highest in the breeding season of female goldfish (Zhang et al., 2009b). Aldolase C is an important metabolic enzyme. Samples of telencephalon from both $PGF_{2\alpha}$-treated and 17,20βP-treated groups were chosen to measure expression level since we were interested in comparing gene profiles between the pre-ovulatory priming (17,20βP) and post-ovulatory releasing ($PGF_{2\alpha}$) pheromones of goldfish to determine any differential responses.

Differences [$F(2, 11) = 4.24$, $P = 0.05$] were observed in EPDII mRNA levels between treatment groups. Ependymin II mRNA was 3-fold higher in the telencephalon of $PGF_{2\alpha}$-exposed animals relative to the vehicle, confirming the microarray result. In contrast, no significant differences ($P > 0.05$) were detected between vehicle vs. 17,20βP-treated and 17,20βP-treated vs. $PGF_{2\alpha}$–exposed animals (Figure 3.2A).

Calmodulin mRNA expression levels differed significantly [$F(2,9)=6.60$, $P = 0.017$] between the experimental groups, and was three-fold higher in $PGF_{2\alpha}$-exposed males relative to the vehicle and 17,20βP-treated animal (Figure 3.2B). No differences ($P > 0.05$) were observed between the vehicle and 17,20βP-treated groups.

As shown in Figure 3.2C, there were significant differences [$F(2,11)=4.37$, $P = 0.047$] in ALDOC mRNA levels between vehicle, 17,20βP and $PGF_{2\alpha}$ –exposed animals. ALDOC mRNA was significantly up-regulated 5-fold in $PGF_{2\alpha}$ –treated fish in

comparison to the vehicle. In contrast 17,20βP-treatment did not affect ALDOC mRNA levels ($P > 0.05$) .

GnRH isoforms

We determined mRNA levels of sGnRH and cGnRH-II, the two isoforms of GnRH present in goldfish, because GnRH is the main regulator of reproduction in vertebrates (Kim et al., 1995). The sGnRH mRNA levels were significantly different [$F(2,10)= 5.69$, $P = 0.029$] between the treatment groups. $PGF_{2\alpha}$-exposed goldfish had a seven-fold higher level of sGnRH transcripts than 17,20βP-exposed fish and the vehicle (Figure 3.3A). No statistical difference ($P > 0.05$) was found for the vehicle vs. 17,20βP treatments.

The cGnRH-II mRNA levels were differential expressed [$F(2,11)=5.20$, $P = 0.032$] between treatment groups. The cGnRH-II mRNA levels in the telencephalon of 17,20βP-treated fish was elevated 2-fold times relative to $PGF_{2\alpha}$–exposed animals (Figure 3.3B). No differences ($p > 0.05$) were detected between cGnRH-II mRNA levels in the telencephalon of $PGF_{2\alpha}$–exposed vs. vehicle-exposed fish.

Amino acid neurotransmitter receptor subtype expression

We were also interested in assessing the possible effects of sex pheromones on selected neurotransmitter receptors that play a role in reproduction. Our electrophysiological studies clearly demonstrate that there are major glutamatergic projections from the olfactory bulb to the ventral POA and ventral telencephali pars ventralis (Vv; Lado et al., submitted) via the medial olfactory tract in male goldfish. Moreover, previous work indicated a GABAergic projection from the Vv to the ventral

POA (60). These receptors are α-amino-3-hydroxy-5-methylisoxazole-4-propionate receptor (AMPAR), *N*-methyl-*D*-aspartate receptor (NMDAR), and γ-aminobutyric acid $(GABA)_A$ receptor. The $GABA_A$ receptor is a pentamer composed of combinations of seven subunit classes (α_{1-6}, β_{1-4}, γ_{1-4}, δ, ε, θ and π) forming a chloride channel (Bormann, 2000; Martyniuk et al., 2005). On the other hand, AMPARs are formed of 4 heteromeric subunits (GluR1, GluR2, GluR3, and GluR4) that are permeable to Na^+ and K^+ (Bettler and Mulle, 1995; Gotz et al., 1997; Bleakman and Lodge, 1998). NMDARs are also made up of heterotetrameric subunits between NR1 and either NR2 (A, B, C, D) or NR3 (A, B), and they are permeable to Na^+, Ca^{2+} and K^+ ions (Harvey-Girard et al., 2007; Paoletti and Neyton, 2007; Chaffey and Chazot, 2008). We choose to look at the expression level of GluR2a, NR2A, NR2B and $GABA_A$ γ2subunits mRNAs and were not on the cDNA microarray.

The GluR2a mRNA was differentially expressed [F(2,11)= 4.63, $P = 0.041$] between experimental groups. There was a 3-fold increase in GluR2a mRNA in $PGF_{2\alpha}$–treated fish compared to the vehicle (Figure 3.4A). No differences were observed between vehicle vs. 17,20βP-treated or 17,20βP vs. $PGF_{2\alpha}$–exposed animals.

The NR2A mRNA was statistically significant [F(2,11)= 7.42, $P = 0.012$] between groups. It was up-regulated 3-fold times in $PGF_{2\alpha}$–exposed relative to the vehicle (Figure 3.4B). No differences were observed between vehicle vs. 17,20βP-treated and 17,20βP vs. $PGF_{2\alpha}$–exposed animals. The NR2B mRNA was significantly different [F(2,11)= 10.75, $P = 0.004$] in all treatments. The NR2B mRNA was down-regulated about 3-fold in both 17,20βP-treated and $PGF_{2\alpha}$–exposed fish compared to the vehicle

(Figure 3.4C). No statistical significant differences were observed between 17,20βP-treated and $PGF_{2\alpha}$–exposed goldfish.

The expression levels of $GABA_A$ γ2 subunit mRNA was significant [$F(2,11)= 6.73$, $P = 0.016$] between treatment groups. It was up-regulated 5-fold times in $PGF_{2\alpha}$–exposed animals relative to the vehicle (Figure 3.4D). No differences were observed between vehicle vs. 17,20βP-treated and 17,20βP vs. $PGF_{2\alpha}$–exposed animals.

DISCUSSION

Our analysis indicates that female sex pheromones in male goldfish induce sperm release and regulate gene expression in the telencephalon of male goldfish. After 6 hours of exposure to either 17,20βP or $PGF_{2\alpha}$, male goldfish exhibited an increase in milt volume, in agreement with several other studies (Sorensen et al., 1988; Stacey et al., 1989; Stacey et al., 1994; Mennigen et al., 2010). This confirmed that the male fish in our laboratory study had a robust physiological response to these pheromones.

$PGF_{2\alpha}$ regulates gene expression in the telencephalon

The $PGF_{2\alpha}$ pheromone not only synchronizes goldfish sexual behaviour to facilitate spawning, but it has previously been reported to enhance neurogenesis in male goldfish (Kobayashi et al., 2002; Chung-Davidson et al., 2008). We chose to perform our cDNA microarray analysis on the telencephalon because lesioning experiments of the preoptic area (POA), ventral telencephali pars ventralis (Vv) and ventral telencephali pars supracommissuralis (Vs) have all been shown to impair male spawning (Kyle and Peter, 1982; Koyama et al., 1984). We identified more than 450 transcripts that were

differentially regulated. We also employed a cut off of 1.5-fold change to increase the stringency criterion for further functional annotation. Extensive experience and validation of our cDNA microarrays indicates that this helps to identify the key genes that are regulated and can be confirmed readily by real-time RT-PCR analysis. This is evident when fold-change estimates between the microarray and the real-time RT-PCR are compared. For example, EPDII, CaM-A and ALDOC were up-regulated 2.2, 1.7 and 1.5 fold respectively, as determined by microarray. The corresponding fold-change inductions following $PGF_{2\alpha}$ exposures determined using real-time RT-PCR for EPDII, CaM-A and ALDOC were 3, 4 and 5-fold, respectively. All but 4 of the 71 regulated transcripts (94%) identified in the microarray experiment were up-regulated within 6 hr of $PGF_{2\alpha}$ exposure. The high transcriptional demand is reflected in the increased expression of numerous genes important for energy metabolism, translational regulation, transcription and chromatin remodelling, and protein processing (Table 3.2),

Our main interest is those genes specifically related to nervous system functioning since sex pheromones and milt release are also coupled with behavioural changes. First discovered in goldfish in 1985, ependymins (EPDs) are glycoproteins that function as neurotrophic factors (Shashoua, 1985, 1991; Adams et al., 1996; Suarez-Castillo and Garcia-Arraras, 2007). Since then, they have been found in teleostean cerebrospinal fluids and in several other species including humans (Shashoua, 1991). Recently, EPDs have been classified into four isoforms in fish (Suarez-Castillo and Garcia-Arraras, 2007). However, two of the isoforms are missing from tetrapods suggesting that the teleostean EPD underwent gene/genomic duplications giving rise to two new forms (Suarez-Castillo and Garcia-Arraras, 2007). Ependymin-II in our microarrays and

subsequent validation by real-time RT-PCR suggests that it plays an important role in $PGF_{2\alpha}$ -mediated neuroplasticity in relation to male goldfish reproduction. Moreover, EPD was also up-regulated 2-fold in the microarray experiment. Other studies have shown that EPD is involved with optic nerve regeneration and neuroplasticity in fishes (Shashoua, 1991; Suarez-Castillo and Garcia-Arraras, 2007). Our results therefore support and extend the observations of Chung-Davidson et al., (Chung-Davidson et al., 2008) who found that waterborne $PGF_{2\alpha}$ increased neurogenesis in male goldfish brain.

Calcium-modulated protein or calmodulin (CaM) is a Ca^{2+} sensor and signal transducer present in eukaryotic cells (Xia and Storm, 2005). We found that CaM-A was increased 4-fold in $PGF_{2\alpha}$-exposed animals relative to the other groups where the expression levels remained unchanged. Calmodulin is an effector of neuroplasticity through voltage-sensitive Ca^{2+}channels and NMDARs (Malenka and Bear, 2004; Xia and Storm, 2005). Activation of NMDARs leads to Ca^{2+} influx that stimulate a downstream cascade of second messengers which include CaM leading to the induction of neuroplasticity (Malenka and Bear, 2004; Xia and Storm, 2005). In is noteworthy that NMDARs mediate olfactory neurotransmission to the POA in goldfish (Lado et al., submitted). Taken together the up-regulation of the EPDs and CaM-A in response to $PGF_{2\alpha}$ suggests that these proteins are involved in sex pheromone-mediate neuroplasticity in the telencephalon. Neuroplasticity is the ability of the brain to change in response to various stimuli (Kinsley and Lambert, 2008). Since goldfish courtship leading to spawning synchrony occurs over several hours, it is conceivable that their brains undergo neuroplastic alternation to fully respond to the released sex pheromones.

Rapid increases in transcription and translation, and potential remodelling of the telencephalon required increased energy demands. This is reflected in the increased expression of metabolic enzymes. Gene ontology enrichment analysis revealed 9 gene clusters (Figure 3.2). Importantly, several genes such as netrin 4 (Serafini et al., 1994), ubiquitin C (Speese et al., 2003), and SNAP-25 (Verderio et al., 2004) are functionally involved in transmission of nerve impulses and neuronal development (Table 3.2). Some others such as spermatid perinuclear RNA and ubiquitin specific peptidase 9 classify as being involved in processed related to sexual reproduction. Spermatid perinuclear RNA, a microtubule-associated RNA-binding protein, is important for normal sperm flagellum function because sperms from mice lacking this protein show defects in sperm motility (Pires-daSilva et al., 2001). The role of spermatid perinuclear RNA in the brain remains unknown, but presumably it would be involved in similar RNA-binding functions. On the other hand, ubiquitin specific peptidase 9, a protein involved with vesicular trafficking and cell adhesion in the brain, is also involved in normal sperm development because its absence have been shown to result in azoospermia (Friocourt et al., 2005; Luddi et al., 2009). We performed real-time RT-PCR to validate mRNA increases in ALDOC identified by our microarray experiment. Aldolases are enzymes that belong to the fructose-bisphosphate family involved in the reversible aldol cleavage of fructose-1,6-bisphosphate and fructose-1-bisphosphate to dihydroxyacetone phosphate and either glyceraldehyde 3-phosphate or glyceraldehyde respectively (Hawkes and Herrup, 1995; Arakaki et al., 2004). There are three isoforms of aldolase: isoforms A is expressed in skeletal muscles, B in the liver and C in the brain (Penhoet et al., 1966; Kukita et al., 1988; Mukai et al., 1991; Hawkes and Herrup, 1995; Wang et al., 2007). The increase in

ALDOC mRNA that we observed supports the hypothesis that increased metabolism is required to mobilize energy reserves for brain remodelling and reproduction.

A second level of analysis employed targeted real-time RT-PCR and was conducted to compare the specificity of action of the two sex pheromones on genes already known to be important for reproduction, neurotransmission and neuroplasticity. We determined expression levels of sGnRH, cGnRH-II, $GABA_A\gamma2$, GluR2a, NR2A and NR2B. We found that cGnRH-II mRNA in the telencephalon was up-regulated about 2-fold in 17,20βP-exposed fish while it remained unchanged in $PGF_{2\alpha}$-exposed animals compared to the vehicle. The unchanging levels of cGnRH-II confirm the microarray results. Conversely, sGnRH mRNA expression was increased about sevenfold in $PGF_{2\alpha}$-exposed animals while it remained unchanged in 17,20βP-exposed fish. We were surprised why sGnRH did not appear in our list of genes regulated by $PGF_{2\alpha}$ as determined by the microarray. We do, however, note that the increase in sGnRH observed in the real-time data was rather variable and it is plausible that the rigorous statistics performed on the microarray dataset precluded sGnRH from appearing on the list owing to that variability. The effects of $PGF_{2\alpha}$ on LH and total immunoassayable GnRH in the male goldfish telencephalon is mediated by the medial olfactory tract (Yu and Peter, 1990). It was not possible to separately assess the effects of pheromones on cGnRH and cGnRH-II in that study because specific radioimmunoassays were not available at that time. Our results confirm those of Chung-Davidson et al. (Chung-Davidson et al., 2008) who found that $PGF_{2\alpha}$ increased sGnRH mRNA levels while cGnRH-II remained unchanged. The specific stimulation of cGnRH-II mRNA by the preovulatory pheromone 17,20βP may be important for stimulation of preparatory

increases in LH and milt production to ready the male for spawning several hours later (Zheng and Stacey, 1997). On the other hand, the effects of the postovulatory pheromone $PGF_{2\alpha}$ to stimulate courtship and spawning behaviours and to enhance LH release may be mediated at least partially by sGnRH. Treatments with a potent GnRH antagonist in male goldfish indicate that the final increases in milt at the time of spawning with females releasing $PGF_{2\alpha}$ is independent of changes in LH (Zheng and Stacey, 1997). This final important increase in sperm release may be dependent on other neuropeptides, for example, isotocin (Mennigen et al., 2010), or other neurotransmitters as suggested by changes in mRNAs encoding for synaptic proteins that we detected using microarray analysis.

Neuroanatomical studies indicate that numerous neuronal phenotypes send projections from the olfactory bulb to the telencephalon, hypothalamus and other brain regions. Our electrophysiological analyses indicate that there is a monosynaptic glutamatergic connection from the olfactory bulb to the ventral preoptic area of the male goldfish telencephalon (Lado et al., submitted). Additionally, given that specific AMPA and NMDA agonists injected into goldfish both stimulate LH release (Trudeau et al., 1991; Trudeau et al., 2000b), we wanted to examine whether sex pheromones modulate the expression of glutamate receptor subtypes. We found that the AMPAR GluR2a subunit and the NMDAR NR2A subunit mRNA in the telencephalon were up-regulated in response to $PGF_{2\alpha}$. This is consistent with our patch-clamp electrophysiological studies. We have shown that the AMPAR antagonist, CNQX, and the NMDAR antagonist, APV, respectively block the rapid and slow post-synaptic depolarisations of ventral POA neurons induced by electrical stimulation of the male goldfish olfactory bulb

(Lado et al., submitted). In contrast the NMDAR NR2B subunit mRNA was down-regulated in both 17,20βP– and $PGF_{2\alpha}$–exposed males. In the central nervous system, both AMPARs and NMDARs play different roles in neuronal depolarization and synaptic plasticity. The NMDARs are ligand-gated and membrane-bound receptors that have non-selective permeability to cations (Chaffey and Chazot, 2008). Activation of NMDARs is voltage-dependent because of the presence of Mg^{2+} ions, in their resting state, that block the NMDARs (Chaffey and Chazot, 2008). In order to remove this Mg^{2+} blocker, neurons have to be depolarized. AMPARs, therefore, play a role in the voltage-dependant activation of NMDARs in neuronal depolarization because of their low activation threshold (Gotz et al., 1997). The decrease in the expression level of NR2B subunit mRNA in the telencephalon may be due to activity-mediated receptor down-regulation following both pheromonal exposures. Electrophysiological evidence shows that NR2B in NMDARs desensitize slowly and take longer to recover than NR2A thereby prolonging receptor activity (Chaffey and Chazot, 2008). Additionally, studies of synaptic plasticity in rodent hippocampal and cortical neurons indicate that NR2A is important for long-term potentiation while NR2B for long-term depression (Massey et al., 2004; Chen et al., 2007). Therefore, NR2A and NR2B likely have distinct roles in mediating pheromonal actions.

In this study we also found that the $GABA_A\gamma2$ subunit mRNA was up-regulated five-fold in the telencephalon of $PGF_{2\alpha}$–exposed males but not in those exposed to 17,20βP. The $GABA_A\gamma$ subunit family is the extracellular ligand-binding domain of the $GABA_A$ receptor, a pentameric structure that forms a chloride channel (Schweizer et al., 2003). Previous studies from our lab indicate that GABA has a prominent stimulatory

effect on LH release by enhancing GnRH release and inhibiting dopamine turnover (reviewed in Popesku et al. (Popesku et al., 2008a)). The $GABA_A$ receptor agonist, muscimol, stimulates LH release *in vivo* in goldfish (Trudeau et al., 1993b). While it is not known if GABA-induced LH release leads to sperm release, it does provoke significant increases in serum testosterone levels in male goldfish (Sloley et al., 1992a). Moreover, electrophysiological studies indicate a monosynaptic GABAergic projection from the Vs/Vv region of the telencephalon to ventral POA neurons expressing functional $GABA_A$ receptors (Trudeau et al., 2000b). High levels of expression of glutamic acid decarboxylase (GAD) 65 and GAD67 mRNA in Vs and Vv (Martyniuk et al., 2007), and electrolytic lesions of this region impairs male spawning behaviour in goldfish (Kyle and Peter, 1982), The increase in $GABA_A\gamma2$ that we observed suggests that GABA is important for the effects of $PGF_{2\alpha}$ on male goldfish reproduction.

CONCLUSION

Using a combination of transcriptional profiling by cDNA microarray and targeted PCR we discovered that a total of 71 (67 up, 4 down) genes in the male telencephalon were affected by short term exposure to female sex pheromones. Many of these are known to be important for transcription, translation, protein processing and cellular signalling. Increased expression in metabolic enzymes and cytoskeletal elements emphasizes the rapid modulation of telencephalon function required for pheromone responses and subsequent reproductive activities.. The differential expression of the two GnRHs found in goldfish in response to different chemicals is indicative of the specificity of these pheromones. The GnRHs are the main regulators of LH release and reproductive

behaviours in vertebrates, so rapid transcriptional responses in association with milt release are not surprising. However, up-regulation of the ependymins, glutamate and GABA receptor subunits, along with netrin 4 (Serafini et al., 1994) and SNAP-25 (Verderio et al., 2004) are indicative of major changes in neuronal function. We hypothesize that these neuroplastic changes could help to enhance the male's ability for subsequent spawning with multiple females in a given breeding season.

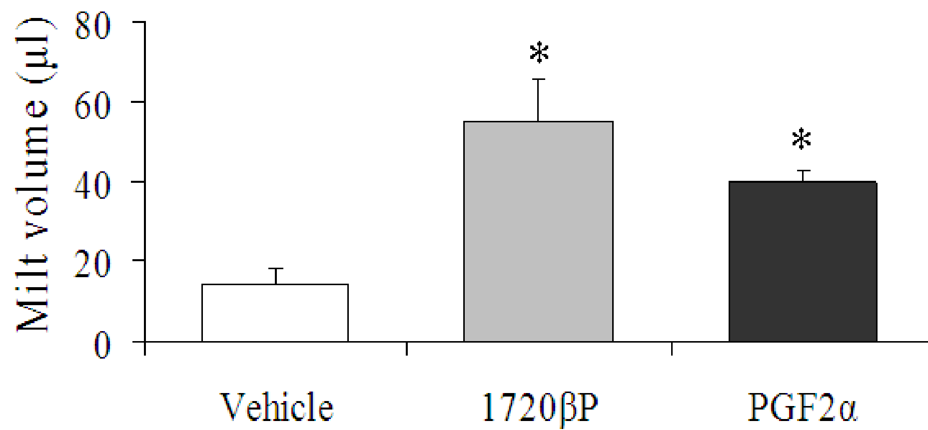

Figure 3.1: Milt volume measured after exposures of goldfish to 6 hours of sex pheromones (17,20βP and $PGF_{2\alpha}$). Error bars denote SEM. *Asterisks show significant differences at $P < 0.05$ from the vehicle.

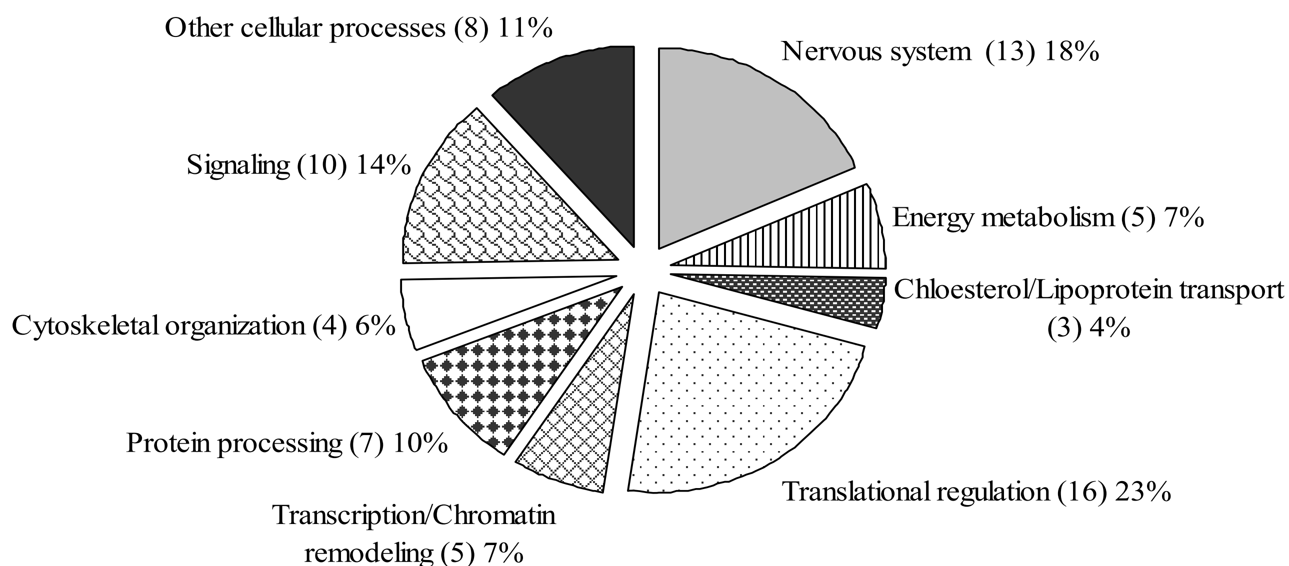

Figure 3.2: Gene Ontology (GO) analysis of differential expressed genes in the telencephalon of male goldfish exposed to $PGF_{2\alpha}$ as determined by BLAST2GO analysis of microarray results shown in Table 2. Number and % of genes falling into each GO category are indicated.

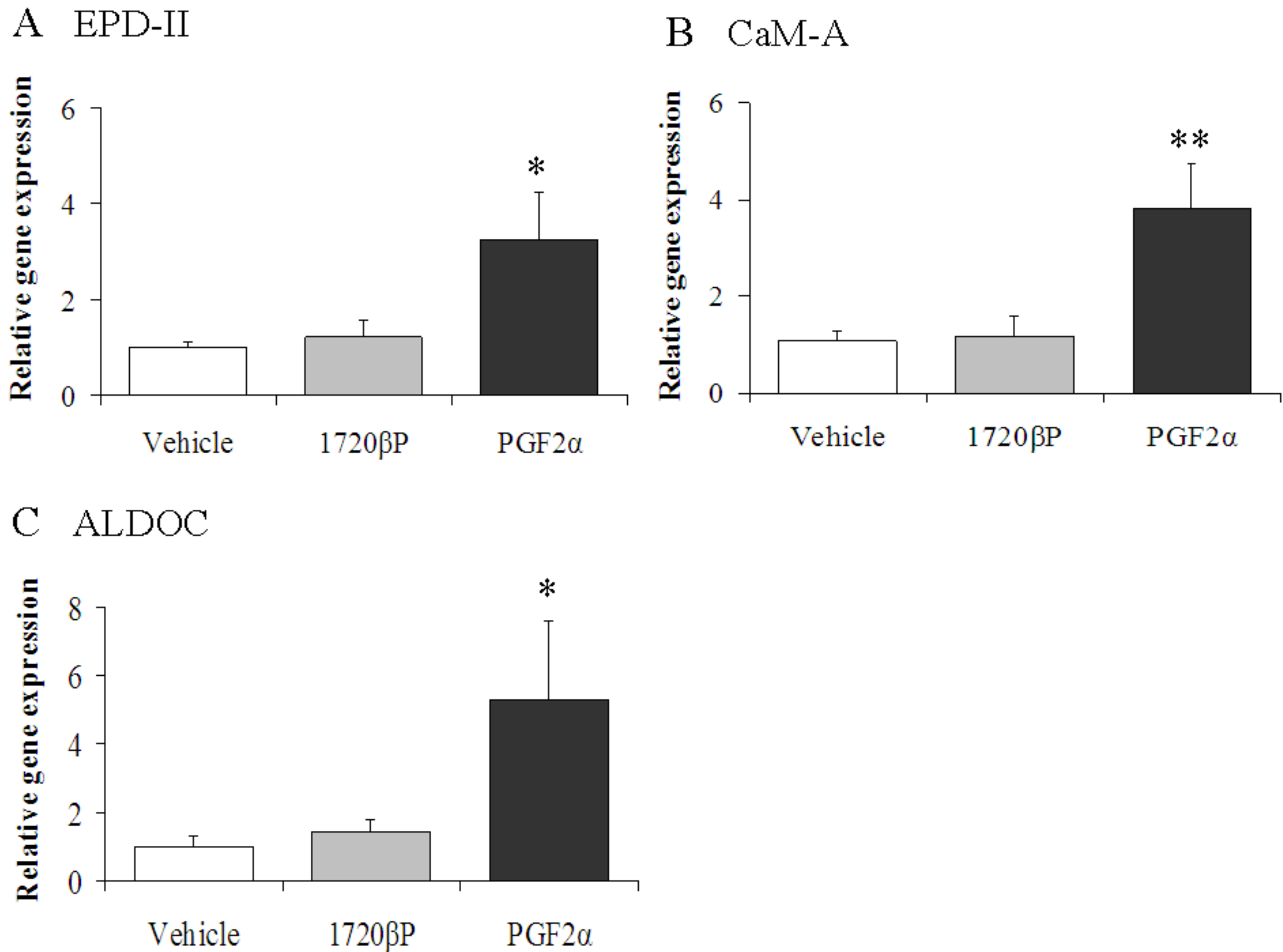

Figure 3.3: Real-time RT-PCR validation of cDNA microarray data. Panel A shows the 3-fold gene expression of EPD-II mRNA levels in $PGF_{2\alpha}$ relative to the vehicle. Panel B shows the 4-fold gene expression of CaM mRNA in $PGF_{2\alpha}$ relative to vehicle; CaM mRNA in fish exposed to 17,20βP was unaltered. Panel C shows the 5 fold gene expression of ALDOC mRNA in $PGF_{2\alpha}$ –treated fish relative to vehicle; ALDOC mRNA in 17,20βP-exposed fish was unchanged. (**)Asterisks denote statistical significance ($P < 0.05$) from vehicle and an experimental group. (*)Asterisk denotes significance ($P < 0.05$) from vehicle.

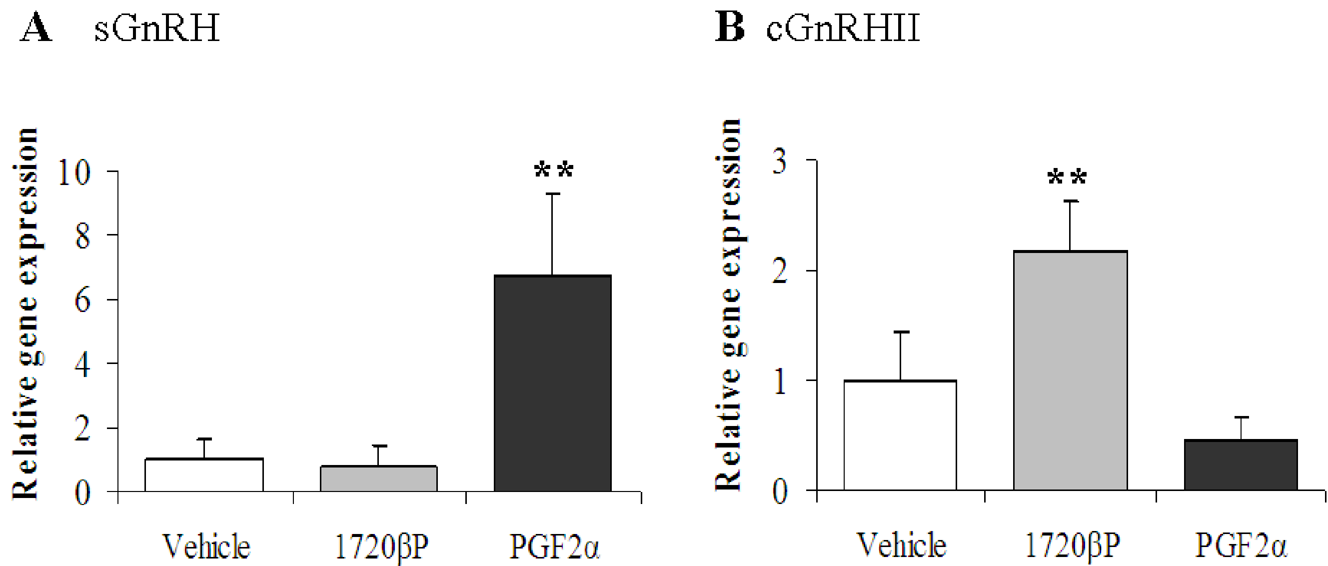

Figure 3.4: Real-time RT-PCR of GnRHs mRNA in the telencephalon of male goldfish following pheromone exposure. Panel A shows the 7 fold up-regulation of sGnRH in $PGF_{2\alpha}$–treated fish relative to vehicle; sGnRH was unchanged in 17,20βP -treated animals. Panel B shows the 2 fold up-regulation of cGnRH-II in 17,20βP -treated animals relative to vehicle. cGnRH-II mRNA levels in PGF-treated fish decreased but not significantly from the vehicle. $^{(**)}$Asterisks indicate statistical significance from vehicle and treatment groups ($P < 0.05$).

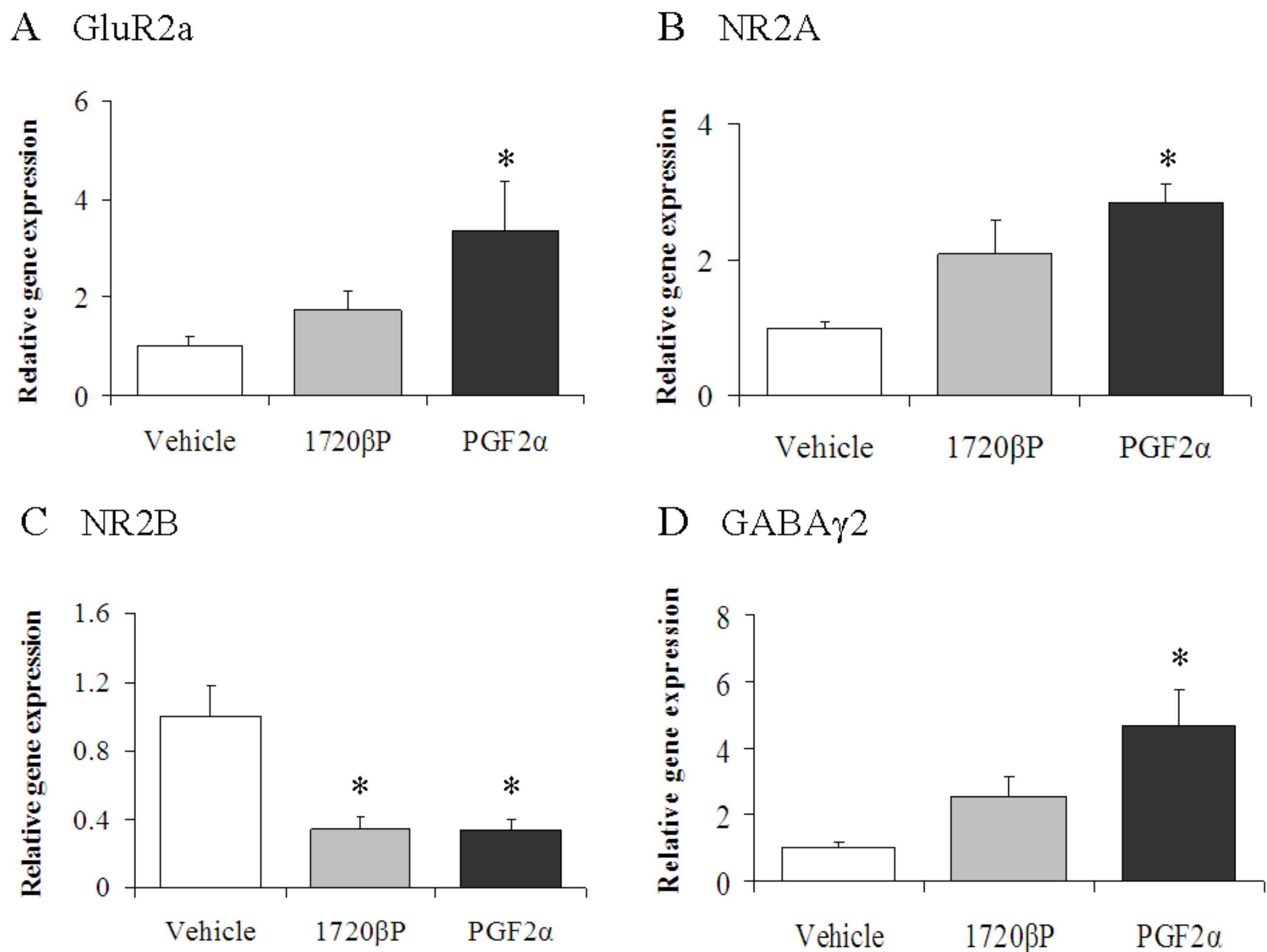

Figure 3.5: Gene expression of selected receptor subtypes in the telencephalon of male goldfish following pheromones exposure. Panel A shows the 3-fold increase of GluR2a subunit mRNA in $PGF_{2\alpha}$–exposed animals; the level of GluR2a subunit mRNA in 17,20βP -treated animals were not significant ($P > 0.05$). Panel B shows that NR2A subunit mRNA was increased 3-fold in $PGF_{2\alpha}$–treated subjects compared to the vehicle; levels of NR2A mRNA in 17,20βP-exposed fish did not differ ($P > 0.05$) from controls Panel C shows that NR2B subunit mRNA was down-regulated 0.3-fold in both 17,20βP- and $PGF_{2\alpha}$ -treated fish relative to the vehicle.. Panel D shows a 5-fold up-regulation of GABAγ2 subunit mRNA in $PGF_{2\alpha}$–exposed goldfish relative to vehicle; GABAγ mRNA remained unchanged in 17,20βP -treated animals. (*)Asterisks indicate statistical significance from vehicle.

Table 3.1: Real-time RT-PCR Primers used in the present study

Gene	*GenBank ID*	*Sequence 5'- 3'*	**Amplicon (bp)**
Ependymin II	J04986	F: TGAGCGGAACAATGAAAGTG R: TCAGACTCGTGAGTGGCATC	222
Calmodulin	AY656699	F: CATTTCCATCAGCGTCCA R: GCACCATCACGACCAAAGA	108
Aldolase C	U36777	F: GGCACGTCCATAAGAGAAGG R: GGAGGTCAGAGTGAGGAGGA	100
18S	AF047349	F: AAACGGCTACCACATCCAAG R: CACCAGATTTGCCCTCCA	166
sGnRH	U30301	F: TGGTGAAGTGGAGGCAACA R: TTCAGCGTCCACCTCACTCA	122
cGnRH-II	U30386	F: TGCAGGCTGTTTGTGGTGAT R: CCAGGATACCAGCCATGAGA	90
GABAγ2	AY640227	F: AGCTGGCACTCTGCATCAA R: CTGCGTCTCAACAGCAACA	173
GluR2a	AM886310	F:CACTGAGGAGTTTGAGGATGG R:TTAGCCGTGTAGGAGGAGATG	198
NR2A	EF645246	F: TGGATGTGGATAACATGGCT R: CCCAGAACAGAGACCAGTGA	131
NR2B	EF645245	F: TCATGGAGGAGTACGACTGG R: ACAGACATGTCCAGCAGCA	148

F, forward; R, reverse

Table 3.2: Up-regulated and down-regulated genes identified by microarray analysis in male goldfish after exposures to waterborne $PGF_{2\alpha}$ (q value < 5%; fold change >1.5).

Carp ID	*Gene ID*	*Gene Name*	*Function*	*Fold Change*
Nervous system				
24a13	83415122	Netrin 4	Cell-cell signalling/axon guidance	1.5
24p16	24233569	Pleiotrophic factor-alpha-2 precursor (PTF-alpha-2)	Cell-cell signalling/axon guidance	1.9
44k02	212952	**Ependymin II precursor**	Neuronal cell adhesion/component of CSF	2.2
23n11	292624213	Myelin basic protein	Neuron development/Structural component of myelin	2.2
23j16	18858295	Sodium/potassium-transporting ATPase alpha-1 chain precursor	Na+/K+ -ATPase-- electrical excitability/Neurotransmission	1.6
43k04	32331309	Rtn4 (reticulon 4-M)	Neuroendocrine pathway	1.7
19n13	548945	Synaptosomal-associated protein 25B (SNAP-25B)	Transport of synaptic vesicle	1.7
61i10	57526694	Synaptobrevin	Transport of synaptic vesicle	-1.6
27m22	1339869	Fast skeletal myosin light chain 3	Calcium signalling	1.9
27c21	33667057	**Calmodulin A (CaM A)**	Calcium signalling	1.7
28o06	91754190	Calpain small subunit 1	Calcium signalling	2.3
22p10	94733616	Parvalbumin 7	Calcium signalling	2.0
Energy metabolism				
42n15	35902900	**Aldolase C**	Glycolysis in brain	1.5
22e22	47085833	Glyceraldehyde-3-phosphate dehydrogenase	Glycolysis in brain	1.6
40p15	292625181	Cytochrome b-c1 complex subunit 9-like isoform 1	Mitochondrial electron transport	1.8
26m13	32482595	Creatine kinase M3-CK	Energy metabolism	3.3
28c12	157743330	Guanidinoacetate N-methyltransferase	Creatine biosynthesis	1.6
Chloesterol/Lipoprotein transport				
44i14	206598070	Apolipoprotein C-I	Chloesterol/Lipoprotein transport	1.7
53p22	71834428	Apolipoprotein L	Chloesterol/Lipoprotein transport	-1.5
27i16	372292222	Apolipoprotein Eb precursor (Apo-Eb)	Chloesterol/Lipoprotein transport	1.8
Translational regulation				
39b23	41152457	40S ribosomal protein S3a (V-fos transformation effector)	Translation/Ribosomal subunits	1.7
39h22	50540044	60S ribosomal protein L35a	Translation/Ribosomal subunits	1.6
22l18	47271398	60S ribosomal protein L7	Translation/Ribosomal subunits	1.6
13o21	209737924	40S ribosomal protein S4	Translation/Ribosomal subunits	1.6
39a13	41282078	60S ribosomal protein L23	Translation/Ribosomal subunits	1.6
21g19	27545217	60S ribosomal protein L35	Translation/Ribosomal subunits	1.7
14d20	41054305	60S ribosomal protein L5	Translation/Ribosomal subunits	1.6
40 e24	47086525	40S ribosomal protein S15a	Translation/Ribosomal subunits	1.9
39f01	91176292	60S ribosomal protein L32	Translation/Ribosomal subunits	1.5

40j13	157502233	60S ribosomal protein L37a	Translation/Ribosomal subunits	1.5
39n21	50344934	60S ribosomal protein L11	Translation/Ribosomal subunits	1.5
39k13	41152307	60S ribosomal protein L8	Translation/Ribosomal subunits	1.7
55o01	70887555	Eukaryotic translation initiation factor 1A, X-chromosomal	Translation/Ribosomal subunits	1.7
52j02	326671063	Spermatid perinuclear RNA-binding protein isoform 2	Translational regulator	1.6
23l22	189522121	Zinc finger protein 366	Translational regulator	2.0
35l04	72534835	Poly A binding protein, cytoplasmic 1 a [Danio rerio]	Post-transcriptional regulation	1.8
Transcription/Chromatin remodelling				
52g16	50539818	Negative elongation factor E (NELF-E)	Transcriptional regulation	1.7
52i02	28849910	Transcription factor AP-2 alpha	Transcription factor	1.7
25l04	46309493	Kruppel-like factor 12	Transcription factor	1.5
40f10	159155373	LOC799918 protein	Chromatin remodelling	2.1
22b22	58801528	SET protein (Phosphatase 2A inhibitor I2PP2A)	Chromatin assembly	1.7
Protein processing				
40g21	41152251	Ubiquitin-like protein FUBI	Ubiquitin pathway/Protein processing	2.0
30k16	209738556	Ubiquitin	Ubiquitin pathway/Protein processing	1.5
52p01	256419025	Ubiquitin--protein ligase EDD1	Ubiquitin pathway/Protein processing	1.7
52b06	116875773	Ubiquitin carboxyl-terminal hydrolase FAF-X	Ubiquitin pathway/Protein processing	1.9
13a11	255003733	Gamma-aminobutyric acid receptor associated protein	Ubiquitin pathway/Autophagy pathway	1.5
53h15	41056019	UPF0172 protein COX4	Ubiquitin pathway/Protein processing	1.8
25 e11	268837268	Influenza virus NS1A binding protein b	Ubiquitin pathway/Protein processing	1.7
Cytoskeletal organization				
26b14	18858249	Alpha-actin	Cytoskeletal organization	1.7
25d11	160773148	Tubulin alpha-6	Cytoskeletal organization	2.0
52p16	326680244	Electromotor neuron-associated protein 2	Cytoskeletal organization in neuron	1.5
27k16	41152171	Mid1-interacting protein 1-B	Cytoskeletal organization/induced by thyroid hormone	1.7
Signalling				
29k02	120538648	ICLP2 protein	Signalling	1.5
30e17	62955155	Tumour-associated calcium signal transducer 2 precursor	Signalling	1.5
23m24	41054729	Serine/threonine-protein kinase 10	Signalling	1.6
43f05	40538752	Casein kinase 2 alpha 1	Signalling	1.6
29p15	55742595	Serine/threonine protein phosphatase 2A, catalytic	Signalling	1.6

23k14	292617311	Cysteine-rich with EGF-like domains 1	Signalling	1.7
23m05	292619487	Neighbour of BRCA1 gene 1	Signalling	2.0
27p24	47085887	Myristoylated alanine-rich C kinase substrate 1	Signalling	1.6
52k02	29126917	Phosphatidylethanolamine-binding protein (PEBP)	Membrane Signalling	2.0
21p23	41054069	Target of rapamycin complex subunit lst8	G-protein signalling	1.5
Other cellular processes				
21n01	7804476	C-type lectin [Cyprinus carpio]	Antifreeze protein type II	-1.8
19l21	47085667	Gamma-glutamylcyclotransferase precursor	Antioxidant /drug detoxification	2.5
21k05	115496041	GPI transamidase component PIG-S	GPI anchor biosynthesis	1.6
53p16	47085973	ATP-binding cassette sub-family F member 1	Transport (ABC_ATPase)/Type I secretion	1.7
17p18	47086251	Nucleoporin Nup43 (p42)	Nuclear pore complex	-1.7
42j13	226823315	Hsp90b (heat shock protein 90-beta)	Chaperone/Protein folding	1.6
30a06	326670682	Pyridoxal kinase	Cofactor biosynthesis	1.5
53b14	77403694	ORF2-encoded protein, partial	Retroelement	1.6

*Genes shown in bold were used for our RT-PCR validations. *Note that Ependymin II precursor appears several times as different clones on the microarray.*

CHAPTER 4

GENERAL DISCUSSION

4.1 Major Contributions

The major goal of this research was to understand the nature of neurotransmission and gene expression mediating sex pheromone responses in the male goldfish brain. We, therefore, use patch-clamp electrophysiology and cDNA microarray analysis to address our two main hypotheses. Both techniques are different and measure different aspects of neuronal function, and therefore provide an integrated approach to the physiology of sex pheromone action in the vertebrate brain. Patch-clamp electrophysiology measures electrical activities as correlates of events occurring on the order of milliseconds to minutes allowing us to observe and characterize short-term phenomenon in single neurons. Microarrays, on the other hand, examine gene expression dynamics that happens over a longer timescale that is on the order of hours. These two approaches allow us to study both neuronal activities and the dynamics of gene expression as correlates of short-term and long-term neuroendocrine processes.

We provide the first evidence of second order glutamatergic monosynaptic connections from the olfactory bulb (OB) to either the preoptic area (POA) or ventral telencephali pars ventralis (Vv) of the ventral telencephalon of male goldfish. These data specifically support the first hypothesis proposed that the olfactory cues from the OB to the ventral telencephalon are mediated by glutamate (see page 14). These circuits may be involved with neurotransmission of sex pheromone signals detected at the olfactory epithelium. In addition, we show that exposures of sexually mature male goldfish to sex

pheromones not only elicit sperm release, but also differentially regulate gene expression in the telencephalon. These genes may play a role in male goldfish to facilitate spawning. Our second hypothesis that sex pheromones will differentially regulate sex-related gene expression was partially supported (see page 15), as the genes expressed were those that were involved with nervous system function, energy metabolism, cholesterol/lipoprotein transport, translational regulation, transcription/chromatin remodelling, protein processing, cytoskeletal organization, signalling and other cellular processes. Taken together, these results show that reproduction in goldfish is a complex process that involves glutamate and other biological molecules that are involved with diverse biological processes. This leads to the testable hypothesis that reproductive remodelling or neuroplasticity is required for a successful spawning (Figure 4.1).

Neuroplasticity is a process by which the brain adapts to signals to increase their efficacy, efficiency and fidelity (Spanswick et al., 1998). It involves either the remodelling of the brain through neurogenesis (Chung-Davidson et al., 2008; Kinsley and Lambert, 2008) or insertions or deletions of NMDARs (Prybylowski et al., 2002; Liu et al., 2004) and / or AMPARs (Malenka and Bear, 2004; Bartlett and Wang, 2010) at synapses or the strengthening of synaptic connections between neurons through dendritic growth and arborisation (Kinsley and Lambert, 2008). Glutamate, GluR2a, NR2A, NR2B, CaM and EPDII have all been shown in previous studies to be involved with neuroplasticity of the nervous system (Shashoua, 1985; Spanswick et al., 1998; Barria and Malinow, 2005; Xia and Storm, 2005). While in the short term glutamate may be required to trigger a sex hormone surge in response to sex pheromones, it is possible over the long-term that remodelling or neuroplasticity is needed for the brain to continually

respond to the sex pheromones because reproduction in goldfish takes place over hours and several days thereafter. We have yet to discover the mechanism or nature of neuroplasticity involved with the pathways identified ; it can either be through neurogenesis (Chung-Davidson et al., 2008) or structural remodelling (Kinsley and Lambert, 2008) (Chapter 2). However, we speculate that the short-term electrical activities of neurons get transformed to long term processes through neuroplasticity. Indeed, neuroplasticity requires the presence of both the AMPARs and NMDARs to initiate the process (Spanswick et al., 1998; Liu et al., 2004) that ultimately gets transformed to synaptic plasticity through downstream effectors like CaM (Xia and Storm, 2005) and EPDII (Shashoua, 1991; Pradel et al., 1999) that were shown in our microarrays to be up-regulated when goldfish were exposed to the sex pheromones 17,20βP and $PGF_{2\alpha}$. Our data supports a role for reproduction in neuroplasticity as has been shown in other studies (Kinsley and Lambert, 2008). However, the mechanism for reproductive neuroplasticity in the goldfish is not clear. Previously neurogenesis was shown to be one such mechanism because it occurs in the male goldfish brain after exposures to $PGF_{2\alpha}$ but not 17,20βP suggesting specificity in the actions of these pheromones (Chung-Davidson et al., 2008). In male goldfish, 17,20βP mediates arousal while $PGF_{2\alpha}$ stimulates sexual behaviour (Sorensen et al., 1989; Kobayashi et al., 2002). $PGF_{2\alpha}$ –mediate plasticity may, therefore, be a process to increase the reproductive fitness of male goldfish. Indeed other studies have found that reproduction promotes neurogenesis leading to the remodelling of the brain (Lau et al., 2011). However, we are interested in reproductive-mediate synaptic plasticity that has been shown to occur in seasonal reproducers (Tramontin et al., 2000; Kinsley and Lambert, 2008). It will

therefore be important to characterize the electrophysiology of this $PGF_{2\alpha}$-mediated neuroplasticity in goldfish to study how it shapes synaptogenesis and the efficacy of neural networks. Such studies would involve long-term potentiation and long-term depression studies as well as pair pulse and other short term neuroplasticity studies in the goldfish brain after the exposures of these fish to the sex pheromones. This will not only help to confirm our speculations but also to draw comparisons and parallels with mammalian models to help us better understand neuroplasticity in vertebrates and / or its role in reproduction.

4.2 Challenges and Future Directions

To functionally characterize the cells in the POA and Vv, we performed blind patch-clamp electrophysiology. Blind electrophysiological recording from the whole goldfish brain is fraught with difficulties and challenges because of the inherent instability of whole brain tissue (see Appendix A and B) and the fact that the immunohistochemistry and morphology of recorded neurons are unknown. In addition, it is challenging obtaining a fluorescent image of filled cells when only one neuron per session is gotten from either the POA or the Vv. Identification of neuronal immunohistochemistry and morphology is important for full characterization with the electrophysiological "signatures" obtained. Although we obtained five diverse classes of neuron from both the POA and Vv based on our cluster analysis, we have yet to determine the relevance of these electrophysiological "signatures" to cellular morphology or immunohistochemistry. Perhaps in future, single-cell PCR studies can be used to identify the identities of these neurons. However, single-cell PCR requires visualization

of the harvesting of the cytoplasmic contents of neurons which is difficult to achieve in blind patch-clamp electrophysiology. This may well necessity the horizontal dissection of the explant while ensuring that the connections from the OBs are preserved. Our experiments with sex pheromones (Appendix A and B) failed to yield results apart from a single recording owing to the difficulties of trying to record from the new explant preparation that had the entire olfactory system intact. It was technically challenging to apply pheromones to the olfactory epithelium, and at the same time maintain stability to be able to record using patch-clamp electrophysiology. However, the nature of the explant may offset these challenges and makes it very attractive to the study of synaptic connections. One of the challenges of working with the pheromonal explant, which involved turning the brain upside down in the cranium while ensuring that the olfactory bulbs were still connected to the olfactory epithelium, was that the skull below made the whole preparation unstable. Now that we have established that there is indeed a link between the OB and ventral telencephalon, it will be worthwhile to study neuroplasticity because the evidence for it is overwhelming (Chapter 2).

From many different studies, we know that sex pheromones elicit neurogenesis, behavioural responses, steriodogenesis, spermiation and LH release (Figure 4). To this body of literature, we now add the monosynaptic glutamatergic connections from the OB to the POA and Vv and also the differential expression of genes after male goldfish were exposed to sex pheromones (Figure 4). From studies in goldfish (Trudeau et al., 1993a; Trudeau et al., 2000b) and testosterone-primed immature trout (Flett et al., 1994), we know that NMDA and AMPA agonists elicit LH release through stimulation of GnRH. However, how glutamate is involved with sex pheromone mediated reproduction is still

not clear. As such to investigate the role of glutamate in goldfish sex pheromone-mediated reproduction, it will be worthwhile to pursue a study that looks at the effects of blocking NMDAR and AMPAR while exposing the fish to sex pheromones. Such an experiment will allow us to study not only gene expression but endocrine and physiological responses as well.

Blocking the sex pheromones responses with NMDA and AMPA antagonist as we show in Chapter 2 for the electrical stimulation will have easily validated our hypothesis about the role of glutamate in sex pheromone-mediate reproduction in goldfish.

4.3 Conclusion

Goldfish reproduction is a complex process that no only requires coordination between males and females but it also involves the interplay and integration of diverse molecules and processes to accomplish successful spawning. The present study, therefore, extends our understanding of the neural circuitry involved with reproduction. From our patch clamp electrophysiology, we have been able to show that POA and Vv neurons receive monosynaptic glutamatergic projections from the OB (Chapter 2). In addition, with our gene expression studies we have identified genes that are involved with neuroplasticity (Chapter 3). These two approaches (electrophysiology and gene expression) has enabled us to learn about both the short-term and long term effects of cellular processes in the goldfish brain associated with reproduction.

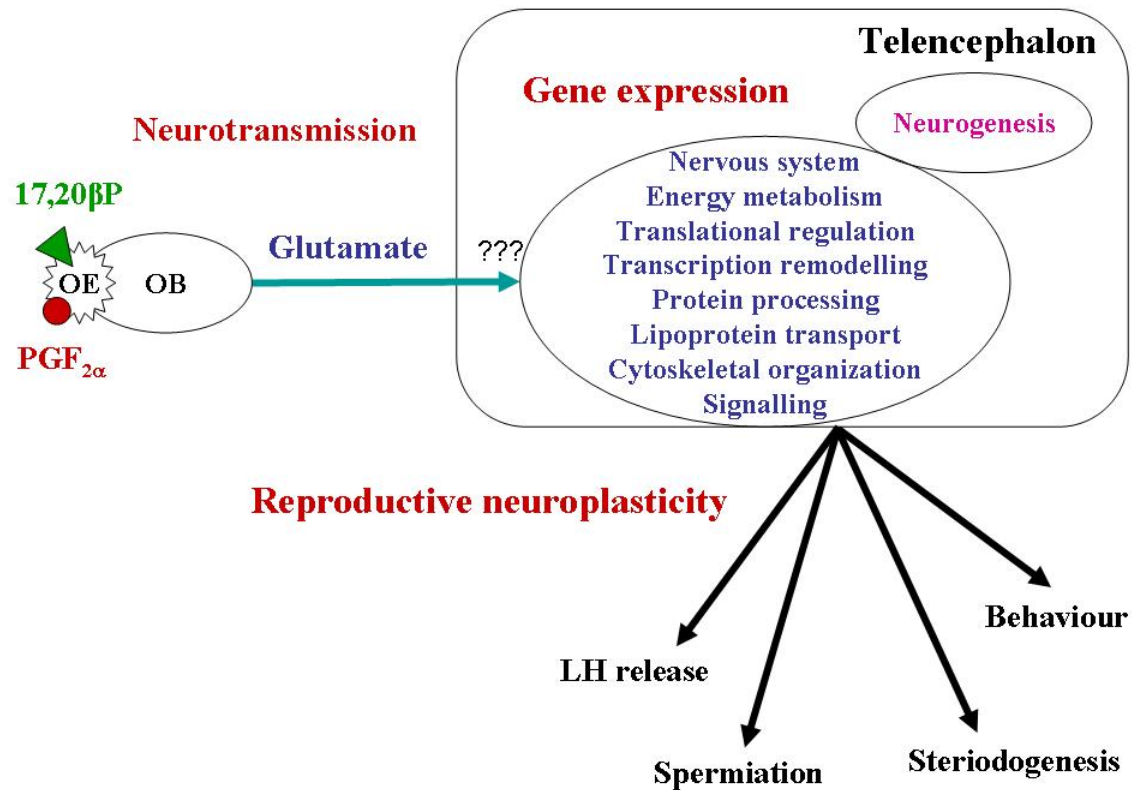

Figure 4.1: Schematic representation showing that sex pheromones (17,20βP and PGF2α) induce LH release, spermiation, steriodogenesis and sexual behaviour in male goldfish. We show that the connections from the olfactory bulb to the ventral telencephalon is monosynaptic and glutamatergic. These processes occur in the brain through reproductive neuroplasticity that involves differential gene expression (shown in blue) and neurogenesis as found by Chung-Davidson et al., (2008). Glutamate is thought to induce the initial sex hormone surge when sex pheromones are detected. However, how the sex pheromones are transduced and transmitted to the telencephalon still remains to be discovered.

REFERENCES

Abbud R, Smith MS (1995) Do GnRH neurons express the gene for the NMDA receptor? Brain Res 690:117-120.

Adams DS, Kiyokawa M, Getman ME, Shashoua VE (1996) Genes encoding giant danio and golden shiner ependymin. Neurochemical Research 21:377-384.

Allison DB, Cui X, Page GP, Sabripour M (2006) Microarray data analysis: from disarray to consolidation and consensus. Nature Reviews Genetics 7:55-65.

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. Journal of Molecular Biology 215:403-410.

Andjelic S, Gallopin T, Cauli B, Hill EL, Roux L, Badr S, Hu E, TamÃ¡s G, Lambolez B (2009) Glutamatergic nonpyramidal neurons from neocortical layer VI and their comparison with pyramidal and spiny stellate neurons. Journal of Neurophysiology 101:641-654.

Anglade I, Zandbergen T, Kah O (1993) Origins of the pituitary innervation in the goldfish. Cell and Tissue Research 273:345-355.

Anglade I, Mazurais D, Douard V, Le Jossic-Corcos C, Mananos EL, Michel D, Kah O (1999) Distribution of glutamic acid decarboxylase mRNA in the forebrain of the rainbow trout as studied by in situ hybridization. J Comp Neurol 410:277-289.

Appelt CW, Sorensen PW (2007) Female goldfish signal spawning readiness by altering when and where they release a urinary pheromone. Animal Behaviour 74:1329-1338.

Arakaki TL, Pezza JA, Cronin MA, Hopkins CE, Zimmer DB, Tolan DR, Allen KN (2004) Structure of human brain fructose 1, 6-(bis) phosphate aldolase: Linking isozyme structure with function. Protein Science 13:3077-3084.

Ayala ME (2009) Brain serotonin, psychoactive drugs, and effects on reproduction. Central Nervous System Agents in Medicinal Chemistry 9:258 - 276.

Barria A, Malinow R (2005) NMDA receptor subunit composition controls synaptic plasticity by regulating binding to CaMKII. Neuron 48:289-301.

Bartheld CS, Meyer DL, Fiebig E, Ebbesson SOE (1984) Central connections of the olfactory bulb in the goldfish, *Carassius auratus*. *Cell and tissue research* 238:475-487.

Bartlett T, Wang YT (2010) Preview: ionotropic glutamate receptor trafficking: AMPA receptors talk back. International Journal of Physiology, Pathophysiology and Pharmacology 2:45.

Bettler B, Mulle C (1995) Review: neurotransmitter receptors. II. AMPA and kainate receptors. Neuropharmacology 34:123.

Blazquez M, Bosma PT, Fraser EJ, Van Look KJ, Trudeau VL (1998) Fish as models for the neuroendocrine regulation of reproduction and growth. Comp Biochem Physiol C Pharmacol Toxicol Endocrinol 119:345-364.

Bleakman D, Lodge D (1998) Neuropharmacology of AMPA and kainate receptors. *Neuropharmacology*.

Bormann J (2000) The 'ABC' of GABA receptors. Trends in Pharmacological Sciences 21:16-19.

Brennan PA, Zufall F (2006) Pheromonal communication in vertebrates. Nature 444:308-315.

Butler AB, Hodos W (2005) Comparative Vertebrate Neuroanatomy: Evolution and Adaptation, Second Edition.628 - 629.

Canosa LF, Cerda-Reverter JM, Peter RE (2004) Brain mapping of three somatostatin encoding genes in the goldfish. J Comp Neurol 474:43-57.

Canosa LF, Lopez GC, Scharrig E, Lesaux-Farmer K, Somoza GM, Kah O, Trudeau VL (2011) Forebrain mapping of secretoneurinâ€ like immunoreactivity and its colocalization with isotocin in the preoptic nucleus and pituitary gland of goldfish. The Journal of Comparative Neurology 519:3748-3765.

Cao Y, Oh BC, Stryer L (1998) Cloning and localization of two multigene receptor families in goldfish olfactory epithelium. Proc Natl Acad Sci U S A 95:11987-11992.

Cardwell JR, Stacey NE, Lang SLC, Tan ESP, McAdam DSO (1995) Androgen increases olfactory receptor response to a vertebrate sex pheromone. Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology 176:55-61.

Chaffey H, Chazot PL (2008) NMDA receptor subtypes: Structure, function and therapeutics. Current Anaesthesia & Critical Care 19:183-201.

Chang JP, Jobin RM, Wong AOL (1993) Intracellular mechanisms mediating gonadotropin and growth hormone release in the goldfish, *Carassius auratus*. *Fish Physiology & Biochemistry* 11:25-33.

Chang JP, Yu KL, Wong AO, Peter RE (1990) Differential actions of dopamine receptor subtypes on gonadotropin and growth hormone release *in vitro* in goldfish. Neuroendocrinology 51:664-674.

Chang JP, Johnson JD, Van Goor F, Wong CJ, Yunker WK, Uretsky AD, Taylor D, Jobin RM, Wong AO, Goldberg JI (2000) Signal transduction mechanisms mediating secretion in goldfish gonadotropes and somatotropes. Biochem Cell Biol 78:139-153.

Chen Q, He S, Hu XL, Yu J, Zhou Y, Zheng J, Zhang S, Zhang C, Duan WH, Xiong ZQ (2007) Differential roles of NR2A-and NR2B-containing NMDA receptors in activity-dependent brain-derived neurotrophic factor gene regulation and limbic epileptogenesis. The Journal of Neuroscience 27:542-552.

Chung-Davidson YW, Rees CB, Bryan MB, Li W (2008) Neurogenic and neuroendocrine effects of goldfish pheromones. J Neurosci 28:14492-14499.

Colwell CS, Max M, Hudson D, Menaker M (1991) Excitatory amino acid receptors may mediate the effects of light on the reproductive system of the golden hamster. Biol Reprod 44:604-608.

Conesa A, Gotz S, Garcia-Gomez JM, Terol J, Talon M, Robles M (2005) Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics 21:3674-3676.

Daikhin Y, Yudkoff M (2000) Compartmentation of brain glutamate metabolism in neurons and glia. The Journal of nutrition 130:1026S.

Demski LS (1983) Behavioural effects of electrical stimulation of the brain. Ann Arbor: University of Michigan Press.

Demski LS, Knigge KM (1971) The telencephalon and hypothalamus of the bluegill (Lepomis macrochirus): evoked feeding, aggressive and reproductive behavior with representative frontal sections. The Journal of comparative neurology 143:1-16.

Demski LS, Dulka JG (1984a) Functional-anatomical studies on sperm release evoked by electrical stimulation of the olfactory tract in goldfish. Brain research 291:241-247.

Demski LS, Dulka JG (1984b) Functional-anatomical studies on sperm release evoked by electrical stimulation of the olfactory tract in goldfish. Brain Res 291:241-247.

Deviche P, Sabo J, Sharp PJ (2008) Glutamatergic stimulation of luteinising hormone secretion in relatively refractory male songbirds. J Neuroendocrinol 20:1191-1202.

Dhandapani KM, Brann DW (2000) The role of glutamate and nitric oxide in the reproductive neuroendocrine system. Biochemistry and Cell Biology 78:165.

Dominguez JM (2009) A role for preoptic glutamate in the regulation of male reproductive behavior. The Neuroscientist 15:11.

Dominguez JM, Gil M, Hull EM (2006) Preoptic glutamate facilitates male sexual behavior. J Neurosci 26:1699-1703.

Dong KW, Yu KL, Chen ZG, Chen YD, Roberts JL (1997) Characterization of multiple promoters directing tissue-specific expression of the human gonadotropin-releasing hormone gene. Endocrinology 138:2754-2762.

Dorries KM, Adkins-Regan E, Halpern BP (1995) Olfactory sensitivity to the pheromone, androstenone, is sexually dimorphic in the pig. *Physiology & Behavior* 57:255-259.

Doving KB, Trotier D (1998) Structure and function of the vomeronasal organ. *Journal of Experimental Biology* 201:2913.

Dubois-Dauphin M, DÃ¸ving KB, Holley A (1980) Topographical relation between the olfactory bulb and the olfactory tract in tench (Tinca tinca L). Chemical Senses 5:159.

Dulac C, Torello AT (2003) Molecular detection of pheromone signals in mammals: from genes to behaviour. Nat Rev Neurosci 4:551-562.

Dulka JG (1993) Sex pheromone systems in goldfish: comparisons to vomeronasal systems in tetrapods. Brain Behav Evol 42:265-280.

Dulka JG, Demski LS (1986) Sperm duct contractions mediate centrally evoked sperm release in goldfish. J Exp Zool 237:271-279.

Dulka JG, Stacey NE (1991) Effects of olfactory tract lesions on gonadotropin and milt responses to the female sex pheromone, 17-alpha, 20-beta, dihydroxy-4-pregnen-3-one, in male goldfish. *Journal of Experimental Zoology* 257:223-229.

Dulka JG, Sloley BD, Stacey NE, Peter RE (1992) A reduction in pituitary dopamine turnover is associated with sex pheromone-induced gonadotropin secretion in male goldfish. Gen Comp Endocrinol 86:496-505.

Dulka JG, Stacey NE, Sorensen PW, Kraak GJ, Marchant TA (1987) A Sex Pheromone System in Goldfish: Is the Nervus Terminalis Involved? a. Annals of the New York Academy of Sciences 519:411-420.

Ebling FJ, Alexander IH, Urbanski HF, Hastings MH (1995) Effects of N-methyl-D-aspartate (NMDA) on seasonal cycles of reproduction, body weight and pelage colour in the male Siberian hamster. J Neuroendocrinol 7:555-566.

Eisthen HL (2000) Presence of the vomeronasal system in aquatic salamanders. Philosophical Transactions of the Royal Society B: Biological Sciences 355:1209.

Eisthen HL, Schwenk K (2008) The chemical stimulus and its detection. Sensory evolution on the threshold: adaptations in secondarily aquatic vertebrates University of California Press, Berkeley, pg:35-41.

Flett PA, Van Der Kraak G, Leatherland JF (1994) Effects of excitatory amino acids on in vivo and in vitro gonadotropin and growth hormone secretion in testosterone-primed immature rainbow trout, Oncorhynchus mykiss. Journal of Experimental Zoology 268:390-399.

Fonnum F (1984) Glutamate: a neurotransmitter in mammalian brain. Journal of Neurochemistry 42:1-11.

Forlano PM, Bass AH (2011) Neural and hormonal mechanisms of reproductive-related arousal in fishes. Hormones and behavior 59:616–629.

Fraser EJ, Bosma PT, Trudeau VL, Docherty K (2002) The effect of water temperature on the GABAergic and reproductive systems in female and male goldfish (*Carassius auratus*). Gen Comp Endocrinol 125:163-175.

Friocourt G, Kappeler C, Saillour Y, Fauchereau F, Rodriguez MS, Bahi N, Vinet MC, Chafey P, Poirier K, Taya S (2005) Doublecortin interacts with the ubiquitin protease DFFRX, which associates with microtubules in neuronal processes. Molecular and Cellular Neuroscience 28:153-164.

Gasser HS (1956) Olfactory nerve fibers. J Gen Physiol 39:473-496.

Gogate MG, Brid SV, Wingkar KC, Kantak NM (1995) Septal regulation of male sexual behavior in rats. Physiology & Behavior 57:1205-1207.

Gotz T, Kraushaar U, Geiger J, Lubke J, Berger T, Jonas P (1997) Functional properties of AMPA and NMDA receptors expressed in identified types of basal ganglia neurons. The Journal of Neuroscience 17:204-215.

Hamdani EH, Doving KB (2007) The functional organization of the fish olfactory system. Prog Neurobiol 82:80-86.

Hamdani EH, Kasumyan A, Doving KB (2001a) Is feeding behaviour in crucian carp mediated by the lateral olfactory tract? Chem Senses 26:1133-1138.

Hamdani EH, Alexander G, Doving KB (2001b) Projection of sensory neurons with microvilli to the lateral olfactory tract indicates their participation in feeding behaviour in crucian carp. Chem Senses 26:1139-1144.

Hamdani EH, Stabell OB, Alexander G, Doving KB (2000) Alarm reaction in the crucian carp is mediated by the medial bundle of the medial olfactory tract. Chem Senses 25:103-109.

Hamdani el H, Doving KB (2007) The functional organization of the fish olfactory system. Prog Neurobiol 82:80-86.

Hansen A, Anderson KT, Finger TE (2004) Differential distribution of olfactory receptor neurons in goldfish: structural and molecular correlates. *Journal of Comparative Neurology* 477:347-359.

Hanson LR, Sorensen PW, Cohen Y (1998) Sex pheromones and amino acids evoke distinctly different spatial patterns of electrical activity in the goldfish olfactory bulb. Ann N Y Acad Sci 855:521-524.

Hart BL, Haugen CM, Peterson DM (1973) Effects of medial preoptic-anterior hypothalamic lesions on mating behavior of male cats. Brain Research 54:177-191.

Harvey-Girard E, Dunn RJ, Maler L (2007) Regulated expression of N-methyl-D-aspartate receptors and associated proteins in teleost electrosensory system and telencephalon. *The Journal of Comparative Neurology* 505:644-668.

Hawkes R, Herrup K (1995) Aldolase C/zebrin II and the regionalization of the cerebellum. Journal of Molecular Neuroscience 6:147-158.

Henley JM, Oswald RE (1988) Characterization and regional distribution of glutamatergic and cholinergic ligand binding sites in goldfish brain. *Journal of Neuroscience* 8:2101-2107.

Hornby PJ, Piekut DT, Demski LS (1987) Localization of immunoreactive tyrosine hydroxylase in the goldfish brain. J Comp Neurol 261:1-14.

Hsu C, Hsieh YL, Yang RC, Hsu HK (2000) Blockage of N-methyl-D-aspartate receptors decreases testosterone levels and enhances postnatal neuronal apoptosis in the preoptic area of male rats. Neuroendocrinology 71:301-307.

Illing N, Troskie BE, Nahorniak CS, Hapgood JP, Peter RE, Millar RP (1999) Two gonadotropin-releasing hormone receptor subtypes with distinct ligand selectivity and differential distribution in brain and pituitary in the goldfish (Carassius auratus). Proceedings of the National Academy of Sciences 96:2526.

Jibb LA, Richards JG (2008) AMP-activated protein kinase activity during metabolic rate depression in the hypoxic goldfish, Carassius auratus. Journal of Experimental Biology 211:3111.

Jobin RM, Neumann CM, Chang JP (1996) Roles of calcium and calmodulin in the mediation of acute and sustained GnRH-stimulated gonadotropin secretion from dispersed goldfish pituitary cells. General and comparative endocrinology 101:91-106.

Kah O, Dulka JG, Dubourg P, Thibault J, Peter RE (1987) Neuroanatomical substrate for the inhibition of gonadotrophin secretion in goldfish: existence of a dopaminergic preoptico-hypophyseal pathway. Neuroendocrinology 45:451-458.

Kah O, Trudeau VL, Sloley BD, Chang JP, Dubourg P, Yu KL, Peter RE (1992) Influence of GABA on gonadotrophin release in the goldfish. Neuroendocrinology 55:396-404.

Kah O, Breton B, Dulka JG, Nunez-Rodriguez J, Peter RE, Corrigan A, Rivier JE, Vale WW (1986) A reinvestigation of the Gn-RH (gonadotrophin-releasing hormone) systems in the goldfish brain using antibodies to salmon Gn-RH. Cell Tissue Res 244:327-337.

Kandel ER (1964) Electrical Properties Of Hypothalamic Neuroendocrine Cells. J Gen Physiol 47:691-717.

Khan IA, Thomas P (1992) Stimulatory effects of serotonin on maturational gonadotropin release in the Atlantic croaker, *Micropogonias undulatus*. General and Comparative Endocrinology 88:388-396.

Kim MH, Oka Y, Amano M, Kobayashi M, Okuzawa K, Hasegawa Y, Kawashima S, Suzuki Y, Aida K (1995) Immunocytochemical localization of sGnRH and cGnRH II in the brain of goldfish, Carassius auratus. The Journal of comparative neurology 356:72-82.

Kinsley CH, Lambert KG (2008) Reproduction-Induced Neuroplasticity: Natural Behavioural and Neuronal Alterations Associated with the Production and Care of Offspring. Journal of Neuroendocrinology 20:515-525.

Kiss J, Kocsis K, Csaki A, Halasz B (2003) Evidence for vesicular glutamate transporter synapses onto gonadotropin-releasing hormone and other neurons in the rat medial preoptic area. Eur J Neurosci 18:3267-3278.

Kobayashi M, Aida K, Hanyu I (1986a) Gonadotropin surge during spawning in male goldfish. Gen Comp Endocrinol 62:70-79.

Kobayashi M, Aida K, Hanyu I (1986b) Pheromone from ovulatory female goldfish induces gonadotropin surge in males. Gen Comp Endocrinol 63:451-455.

Kobayashi M, Sorensen PW, Stacey NE (2002) Hormonal and pheromonal control of spawning behavior in the goldfish. Fish physiology and Biochemistry 26:71-84.

Kocsis K, Kiss J, Csaki A, Halasz B (2003) Location of putative glutamatergic neurons projecting to the medial preoptic area of the rat hypothalamus. Brain Res Bull 61:459-468.

Koyama Y, Satou M, Oka Y, Ueda K (1984) Involvement of the telencephalic hemispheres and the preoptic area in sexual behavior of the male goldfish, Carassius auratus: a brain-lesion study. Behav Neural Biol 40:70-86.

Krimer LS, Zaitsev AV, Czanner G, Kröner S, González-Burgos G, Povysheva NV, Iyengar S, Barrionuevo G, Lewis DA (2005) Cluster analysis–based physiological classification and morphological properties of inhibitory neurons in layers 2–3 of monkey dorsolateral prefrontal cortex. Journal of neurophysiology 94:3009.

Kukita A, Mukai T, Miyata T, Hori K (1988) The structure of brain-specific rat aldolase C mRNA and the evolution of aldolase isozyme genes. European Journal of Biochemistry 171:471-478.

Kyle AL, Peter RE (1982) Effects of forebrain lesions on spawning behaviour in the male goldfish. Physiol Behav 28:1103-1109.

Kyle AL, Stacey NE, Peter RE (1982) Ventral telencephalic lesions: effects on bisexual behavior, activity, and olfaction in the male goldfish. Behav Neural Biol 36:229-241.

Kyle AL, Stacey NE, Peter RE, Billard R (1985) Elevations in gonadotrophin concentrations and milt volumes as a result of spawning behavior in the goldfish. Gen Comp Endocrinol 57:10-22.

Lau BWM, Yau SY, So KF (2011) Reproduction: a new venue for studying function of adult neurogenesis? Cell Transplantation 20:21-35.

Levine RL, Dethier S (1985) The connections between the olfactory bulb and the brain in the goldfish. J Comp Neurol 237:427-444.

Liu L, Wong TP, Pozza MF, Lingenhoehl K, Wang Y, Sheng M, Auberson YP, Wang YT (2004) Role of NMDA receptor subtypes in governing the direction of hippocampal synaptic plasticity. Science's STKE 304:1021.

Luddi A, Margollicci M, Gambera L, Serafini F, Cioni M, De Leo V, Balestri P, Piomboni P (2009) Spermatogenesis in a man with complete deletion of USP9Y. New England Journal of Medicine 360:881-885.

Mahesh VB, Brann DW (2005) Regulatory role of excitatory amino acids in reproduction. Endocrine 28:271-280.

Malenka RC, Bear MF (2004) LTP and LTD: an embarrassment of riches. Neuron 44:5-21.

Marlatt VL, Martyniuk CJ, Zhang D, Xiong H, Watt J, Xia X, Moon T, Trudeau VL (2008) Auto-regulation of estrogen receptor subtypes and gene expression profiling of 17β-estradiol action in the neuroendocrine axis of male goldfish. Molecular and Cellular Endocrinology 283:38-48.

Martinoli MG, Dubourg P, Geffard M, Calas A, Kah O (1990) Distribution of GABA-immunoreactive neurons in the forebrain of the goldfish, *Carassius auratus*. Cell and Tissue Research 260:77-84.

Martyniuk CJ, Crawford AB, Hogan NS, Trudeau VL (2005) GABAergic modulation of the expression of genes involved in GABA synaptic transmission and stress in the

hypothalamus and telencephalon of the female goldfish (Carassius auratus). J Neuroendocrinol 17:269-275.

Martyniuk CJ, Awad R, Hurley R, Finger TE, Trudeau VL (2007) Glutamic acid decarboxylase 65, 67, and GABA-transaminase mRNA expression and total enzyme activity in the goldfish (Carassius auratus) brain. *Brain Research* 1147:154-166.

Martyniuk CJ, Xiong H, Crump K, Chiu S, Sardana R, Nadler A, Gerrie ER, Xia X, Trudeau VL (2006) Gene expression profiling in the neuroendocrine brain of male goldfish (*Carassius auratus*) exposed to 17α-ethinylestradiol. Physiological Genomics 27:328-336.

Massey PV, Johnson BE, Moult PR, Auberson YP, Brown MW, Molnar E, Collingridge GL, Bashir ZI (2004) Differential roles of NR2A and NR2B-containing NMDA receptors in cortical long-term potentiation and long-term depression. The Journal of Neuroscience 24:7821-7828.

Meldrum BS (2000) Glutamate as a neurotransmitter in the brain: review of physiology and pathology. The Journal of nutrition 130:1007S.

Mennigen JA, Lado WE, Zamora JM, Duarte-Guterman P, Langlois VS, Metcalfe CD, Chang JP, Moon TW, Trudeau VL (2010) Waterborne fluoxetine disrupts the reproductive axis in sexually mature male goldfish; Carassius auratus. Aquatic Toxicology 100:354 - 364.

Mennigen JA, Martyniuk CJ, Crump K, Xiong H, Zhao E, Popesku J, Anisman H, Cossins AR, Xia X, Trudeau VL (2008) Effects of fluoxetine on the reproductive axis of female goldfish (*Carassius auratus*). Physiological Genomics 35:273.

Miyasaka N, Morimoto K, Tsubokawa T, Higashijima S, Okamoto H, Yoshihara Y (2009) From the olfactory bulb to higher brain centers: genetic visualization of secondary olfactory pathways in zebrafish. The Journal of Neuroscience 29:4756-4767.

Molleman A (2003) Patch clamping: An Introductory Guide To Patch Clamp Electrophysiology: Wiley Online Library.

Morita Y, Finger TE (1998) Differential projections of ciliated and microvillous olfactory receptor cells in the catfish, Ictalurus punctatus. The Journal of comparative neurology 398:539-550.

Mukai T, Yatsuki H, Masuko S, Arai Y, Joh K, Hori K (1991) The structure of the brain-specific rat aldolase C gene and its regional expression. Biochemical and Biophysical Research Communications 174:1035-1042.

Nikonov AA, Finger TE, Caprio J (2005) Beyond the olfactory bulb: an odotopic map in the forebrain. Proceedings of the National Academy of Sciences of the United States of America 102:18688.

Olivereau M, Ollevier F, Vandesande F, Verdonck W (1984) Immunocytochemical identification of CRF-like and SRIF-like peptides in the brain and the pituitary of cyprinid fish. Cell and tissue research 237:379-382.

Paoletti P, Neyton J (2007) NMDA receptor subunits: function and pharmacology. Current Opinion in Pharmacology 7:39-47.

Parhar IS, Tosaki H, Sakuma Y, Kobayashi M (2001) Sex differences in the brain of goldfish: gonadotropin-releasing hormone and vasotocinergic neurons. Neuroscience 104:1099-1110.

Partridge BL, Liley NR, Stacey NE (1976) The role of pheromones in the sexual behaviour of the goldfish. Animal Behaviour 24:291-299.

Penhoet E, Rajkumar T, Rutter WJ (1966) Multiple forms of fructose diphosphate aldolase in mammalian tissues. Proceedings of the National Academy of Sciences of the United States of America 56:1275.

Peter RE (1977) The preoptic nucleus in fishes: A comparative discussion of function-activity relationships. American Zoologist 17:775-785.

Peter RE, Gorbman A (1968) Some afferent pathways to the preoptic nucleus of the goldfish. Neuroendocrinology 3:229-237.

Peter RE, Paulencu CR (1980) Involvement of the preoptic region in gonadotropin release-inhibition in goldfish, *Carassius auratus*. Neuroendocrinology 31:133-141.

Peter RE, Yu KL (1997) Neuroendocrine regulation of ovulation in fishes: basic and applied aspects. Reviews in Fish Biology and Fisheries 7:173-197.

Peter RE, Yu KL, Marchant TA, Rosenblum PM (1990) Direct neural regulation of the teleost adenohypophysis. J Exp Zool S4:84–89.

Peter RE, Kah O, Paulencu CR, Cook H, Kyle AL (1980) Brain lesions and short-term endocrine effects of monosodium L-glutamate in goldfish, *Carassius auratus*. Cell Tissue Res 212:429-442.

Peter RE, Prasada Rao PD, Baby SM, Illing N, Millar RP (2003) Differential brain distribution of gonadotropin-releasing hormone receptors in the goldfish. Gen Comp Endocrinol 132:399-408.

Peter RE, Chang JP, Nahorniak CS, Omeljaniuk RJ, Sokolowska M, Shih SH, Billard R (1986) Interactions of catecholamines and GnRH in regulation of gonadotropin secretion in teleost fish. Recent Prog Horm Res 42:513-548.

Pires-daSilva A, Nayernia K, Engel W, Torres M, Stoykova A, Chowdhury K, Gruss P (2001) Mice deficient for spermatid perinuclear RNA-binding protein show neurologic, spermatogenic, and sperm morphological abnormalities. Developmental Biology 233:319-328.

Popesku JT, Mennigen JA, Chang JP, Trudeau VL (2011) Dopamine D1 Receptor Blockage Potentiates AMPA-Stimulated Luteinising Hormone Release in the Goldfish. Journal of neuroendocrinology 23:302-309.

Popesku JT, Martyniuk CJ, Mennigen J, Xiong H, Zhang D, Xia X, Cossins AR, Trudeau VL (2008a) The goldfish *Carassius auratus* as a model for neuroendocrine signaling. Molecular and cellular endocrinology 293:43-56.

Popesku JT, Martyniuk CJ, Mennigen J, Xiong H, Zhang D, Xia X, Cossins AR, Trudeau VL (2008b) The goldfish (Carassius auratus) as a model for neuroendocrine signaling. Molecular and cellular endocrinology 293:43-56.

Potapov AA, Gusel'nikova KG (1976a) Electrotonic interaction between secondary neurons of the carp olfactory bulb. Neurophysiology 8:375-380.

Potapov AA, Gusel'nikova AA (1976b) [Electrotonic interaction of secondary neurons of the carp olfactory bulb]. Neirofiziologiia 8:490-496.

Pradel G, Schachner M, Schmidt R (1999) Inhibition of memory consolidation by antibodies against cell adhesion molecules after active avoidance conditioning in zebrafish. Journal of Neurobiology 39:197-206.

Prybylowski K, Fu Z, Losi G, Hawkins LM, Luo JH, Chang K, Wenthold RJ, Vicini S (2002) Relationship between availability of NMDA receptor subunits and their expression at the synapse. The Journal of Neuroscience 22:8902-8910.

Punta M, Coggill PC, Eberhardt RY, Mistry J, Tate J, Boursnell C, Pang N, Forslund K, Ceric G, Clements J (2012) The Pfam protein families database. Nucleic Acids Research 40:D290-D301.

Razani H, Hanyu I, Aida K (1987) Critical daylength and temperature level for photoperiodism in gonadal maturation of goldfish. Exp Biol 47:89-94.

Roesner A, Mitz SA, Hankeln T, Burmester T (2008) Globins and hypoxia adaptation in the goldfish, Carassius auratus. FEBS Journal 275:3633-3643.

Sato K, Suzuki N (2001) Whole-cell response characteristics of ciliated and microvillous olfactory receptor neurons to amino acids, pheromone candidates and urine in rainbow trout. Chemical senses 26:1145.

Sato Y, Miyasaka N, Yoshihara Y (2005) Mutually exclusive glomerular innervation by two distinct types of olfactory sensory neurons revealed in transgenic zebrafish. The Journal of neuroscience 25:4889.

Schmidt RS (1968) Preoptic activation of frog mating behavior. Behaviour:239-257.

Schweizer C, Balsiger S, Bluethmann H, Mansuy IM, Fritschy JM, Mohler H, Luscher B (2003) The γ2 subunit of GABAA receptors is required for maintenance of receptors at mature synapses. Molecular and Cellular Neuroscience 24:442-450.

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

Sharma SC, Berthoud VM, Breckwoldt R (1989) Distribution of substance P-like immunoreactivity in the goldfish brain. J Comp Neurol 279:104-116.

Shashoua VE (1985) The role of brain extracellular proteins in neuroplasticity and learning. Cellular and molecular neurobiology 5:183-207.

Shashoua VE (1991) Ependymin, a Brain Extracellular Glycoprotein, and CNS Plasticity. Annals of the New York Academy of Sciences 627:94-114.

Shimshek DR, Bus T, Grinevich V, Single FN, Mack V, Sprengel R, Spergel DJ, Seeburg PH (2006) Impaired reproductive behavior by lack of GluR-B containing AMPA receptors but not of NMDA receptors in hypothalamic and septal neurons. Molecular Endocrinology 20:219-231.

Siegelbaum SA, Koester J (2000) Ion channels. In: Kandel ER, Schwartz JH, Jessel MT, editors Principles of neural science New York: McGraw Hill:p 105 - 124.

Sloley BD, Trudeau VL, D'Antoni M, R.E. P (1994) Persistent elevation of tissue GABA and serum gonadotropin concentrations by GABA transaminase inhibition in goldfish (*Carassius auratus*). Endocrine 2:385–391.

Sloley BD, Kah O, Trudeau VL, Dulka JG, Peter RE (1992a) Amino acid neurotransmitters and dopamine in brain and pituitary of the goldfish: involvement in the regulation of gonadotropin secretion. J Neurochem 58:2254-2262.

Sloley BD, Kah O, Trudeau VL, Dulka JG, Peter RE (1992b) Amino acid neurotransmitters and dopamine in brain and pituitary of the goldfish: involvement in the regulation of gonadotropin secretion. Journal of neurochemistry 58:2254-2262.

Sorensen PW, Stacey NE, Chamberlain KJ (1989) Differing behavioral and endocrinological effects of two female sex pheromones on male goldfish. Horm Behav 23:317-332.

Sorensen PW, Hara TJ, Stacey NE (1991a) Sex pheromones selectively stimulate the medial olfactory tracts of male goldfish. Brain research 558:343-347.

Sorensen PW, Hara TJ, Stacey NE (1991b) Sex pheromones selectively stimulate the medial olfactory tracts of male goldfish. Brain Res 558:343-347.

Sorensen PW, Christensen TA, Stacey NE (1998) Discrimination of pheromonal cues in fish: emerging parallels with insects. Curr Opin Neurobiol 8:458-467.

Sorensen PW, Pinillos M, Scott AP (2005) Sexually mature male goldfish release large quantities of androstenedione into the water where it functions as a pheromone. Gen Comp Endocrinol 140:164-175.

Sorensen PW, Hara TJ, Stacey NE, Goetz FW (1988) F prostaglandins function as potent olfactory stimulants that comprise the postovulatory female sex pheromone in goldfish. Biology of Reproduction 39:1039-1050.

Sosulina L, Meis S, Seifert G, SteinhÃ¤user C, Pape HC (2006) Classification of projection neurons and interneurons in the rat lateral amygdala based upon cluster analysis. Molecular and Cellular Neuroscience 33:57-67.

Spanswick D, Logan SD (1990) Sympathetic preganglionic neurones in neonatal rat spinal cord in vitro: electrophysiological characteristics and the effects of selective excitatory amino acid receptor agonists. Brain Res 525:181-188.

Spanswick D, Renaud LP, Logan SD (1998) Bilaterally evoked monosynaptic EPSPs, NMDA receptors and potentiation in rat sympathetic preganglionic neurones in vitro. J Physiol 509 (Pt 1):195-209.

Speese SD, Trotta N, Rodesch CK, Aravamudan B, Broadie K (2003) The ubiquitin proteasome system acutely regulates presynaptic protein turnover and synaptic efficacy. Current Biology 13:899-910.

Stacey N (1983) Hormones and pheromones in fish sexual behavior. BioScience 33:552-556.

Stacey N (2003) Hormones, pheromones and reproductive behavior. Fish Physiology and Biochemistry 28:229-235.

Stacey N, Sorensen PW (2002) Hormonal pheromones in fish. Hormones, Brain and Behavior 2:375-434.

Stacey N, Chojnacki A, Narayanan A, Cole T, Murphy C (2003) Hormonally derived sex pheromones in fish: exogenous cues and signals from gonad to brain. Can J Physiol Pharmacol 81:329-341.

Stacey NE, Kyle AL (1983) Effects of olfactory tract lesions on sexual and feeding behavior in the goldfish. Physiology & Behavior 30:621-628.

Stacey NE, Sorensen PW (2009) Hormonal pheromones in fish, DW Pfaff, AP Arnold, AS Etgen, S. Fahrbach, R. Rubin, Editors. Hormones, Brain and Behavior:638-661.

Stacey NE, Sorensen PW, Van der Kraak GJ, Dulka JG (1989) Direct evidence that 17α,20β-dihydroxy-4-pregnen-3-one functions as a goldfish primer pheromone: preovulatory release is closely associated with male endocrine responses. Gen Comp Endocrinol 75:62-70.

Stacey NE, Cardwell JR, Liley NR, Scott AP, Sorensen PW (1994) Hormones as sex pheromones in fish. Perspectives in Comparative Endocrinology:438-448.

Stowe MK, Turlings TC, Loughrin JH, Lewis WJ, Tumlinson JH (1995) The chemistry of eavesdropping, alarm, and deceit. Proceedings of the National Academy of Sciences 92:23.

Suarez-Castillo EC, Garcia-Arraras JE (2007) Molecular evolution of the ependymin protein family: a necessary update. BMC Evolutionary Biology 7:23.

Symonds MRE, Elgar MA (2008) The evolution of pheromone diversity. Trends in ecology & evolution 23:220-228.

Tramontin AD, Hartman VN, Brenowitz EA (2000) Breeding conditions induce rapid and sequential growth in adult avian song control circuits: a model of seasonal plasticity in the brain. The Journal of Neuroscience 20:854-861.

Trudeau LE (2004) Glutamate co-transmission as an emerging concept in monoamine neuron function. J Psychiatry Neurosci 29:296-310.

Trudeau VL (1997) Neuroendocrine regulation of gonadotrophin II release and gonadal growth in the goldfish, *Carassius auratus*. Rev Reprod 2:55-68.

Trudeau VL, Peter RE, Sloley BD (1991) Testosterone and estradiol potentiate the serum gonadotropin response to gonadotropin-releasing hormone in goldfish. Biol Reprod 44:951-960.

Trudeau VL, Sloley BD, Peter RE (1993a) Testosterone enhances GABA and taurine but not N-methyl-D,L-aspartate stimulation of gonadotropin secretion in the goldfish: possible sex steroid feedback mechanisms. J Neuroendocrinol 5:129-136.

Trudeau VL, Sloley BD, Peter RE (1993b) GABA stimulation of gonadotropin-II release in goldfish: involvement of $GABA_A$ receptors, dopamine, and sex steroids. Am J Physiol 265:R348-355.

Trudeau VL, Sloley BD, Wong AO, Peter RE (1993c) Interactions of gonadal steroids with brain dopamine and gonadotropin-releasing hormone in the control of gonadotropin-II secretion in the goldfish. Gen Comp Endocrinol 89:39-50.

Trudeau VL, Kah O, Chang JP, Sloley BD, Dubourg P, Fraser EJ, Peter RE (2000a) The inhibitory effects of γ-aminobutyric acid (GABA) on growth hormone secretion in the goldfish are modulated by sex steroids. J Exp Biol 203:1477-1485.

Trudeau VL, Spanswick D, Fraser EJ, Lariviere K, Crump D, Chiu S, MacMillan M, Schulz RW (2000b) The role of amino acid neurotransmitters in the regulation of pituitary gonadotropin release in fish. Biochem Cell Biol 78:241-259.

Tusher VG, Tibshirani R, Chu G (2001) Significance analysis of microarrays applied to the ionizing radiation response. Proceedings of the National Academy of Sciences 98:5116.

Van Der Kraak G (2009) The GnRH system and the neuroendocrine regulation of reproduction. Fish Physiology 28:115-149.

Van Waversveld J, Addink ADF, van den Thillart G (1989) Simultaneous direct and indirect calorimetry on normoxic and anoxic goldfish. Journal of Experimental Biology 142:325.

Verderio C, Pozzi D, Pravettoni E, Inverardi F, Schenk U, Coco S, Proux-Gillardeaux V, Galli T, Rossetto O, Frassoni C (2004) SNAP-25 modulation of calcium dynamics underlies differences in GABAergic and glutamatergic responsiveness to depolarization. Neuron 41:599-610.

Wang CF, Yuan CZ, Wang SH, Zhang H, Hu XX, Zhang L, Wu C, Li N (2007) Differential gene expression of aldolase C (ALDOC) and hypoxic adaptation in chickens. Animal Genetics 38:203-210.

Ward JH (1963) Hierarchical grouping to optimize an objective function. J Am Stat Assoc 58:236-244.

Wilkie MP, Pamenter ME, Alkabie S, Carapic D, Shin DSH, Buck LT (2008) Evidence of anoxia-induced channel arrest in the brain of the goldfish (Carassius auratus). Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 148:355-362.

Xia Z, Storm DR (2005) The role of calmodulin as a signal integrator for synaptic plasticity. Nature Reviews Neuroscience 6:267-276.

Yamamoto N, Parhar IS, Sawai N, Oka Y, Ito H (1998) Preoptic gonadotropin-releasing hormone (GnRH) neurons innervate the pituitary in teleosts. Neurosci Res 31:31-38.

Yang YH, Dudoit S, Luu P, Lin DM, Peng V, Ngai J, Speed TP (2002) Normalization for cDNA microarray data: a robust composite method addressing single and multiple slide systematic variation. Nucleic acids research 30:e15-e15.

Yu KL, Peter RE (1990) Alterations in gonadotropin-releasing hormone immunoactivities in discrete brain areas of male goldfish during spawning behavior. Brain Res 512:89-94.

Yu KL, Peter RE (1992) Adrenergic and dopaminergic regulation of gonadotropin-releasing hormone release from goldfish preoptic-anterior hypothalamus and pituitary in vitro. Gen Comp Endocrinol 85:138-146.

Yu KL, Rosenblum PM, Peter RE (1991) *In vitro* release of gonadotropin-releasing hormone from the brain preoptic-anterior hypothalamic region and pituitary of female goldfish. Gen Comp Endocrinol 81:256-267.

Yu KL, He ML, Chik CC, Lin XW, Chang JP, Peter RE (1998) mRNA expression of gonadotropin-releasing hormones (GnRHs) and GnRH receptor in goldfish. Gen Comp Endocrinol 112:303-311.

Zhang D, Popesku JT, Martyniuk CJ, Xiong H, Duarte-Guterman P, Yao L, Xia X, Trudeau VL (2009a) Profiling neuroendocrine gene expression changes following fadrozole-induced estrogen decline in the female goldfish. Physiological genomics 38:351-361.

Zhang D, Xiong H, Mennigen JA, Popesku JT, Marlatt VL, Martyniuk CJ, Crump K, Cossins AR, Xia X, Trudeau VL (2009b) Defining global neuroendocrine gene expression patterns associated with reproductive seasonality in fish. PloS one 4:e5816.

Zheng W, Stacey NE (1996) Two mechanisms for increasing milt volume in male goldfish, Carassius auratus. Journal of Experimental Zoology 276:287-295.

Zheng W, Stacey NE (1997) A steroidal pheromone and spawning stimuli act via different neuroendocrine mechanisms to increase gonadotropin and milt volume in male goldfish Carassius auratus. Gen Comp Endocrinol 105:228-238.

Zippel HP, Sorensen PW, Hansen A (1996) High correlation between microvillous olfactory receptor cell abundance and sensitivity to pheromones in olfactory nerve-sectioned goldfish. Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology 180:39-52.

Zohar Y, Munoz-Cueto JA, Elizur A, Kah O (2009) Neuroendocrinology of reproduction in teleost fish. General and comparative endocrinology 165:438-455.

APPENDIX A

EXPOSURES OF THE OLFACTORY EPITHELIUM OF MALE GOLDFISH BRAIN TO 17,20βP and PGF2α

In order to validate our electrophysiological experiments in Chapters 2 and 3, we decided to perform experiments in which we exposed the goldfish olfactory epithelium (OE) to the sex pheromones, 17,20βP and $PGF_{2\alpha}$, dissolved in dechlorinated tap water while recording (see method & materials in Chapter 2) from the preoptic area (POA). The preparation was fraught with difficulties as we had to maintain two perfusions systems between the OE and the rest of the brain. The perfusion to the OE was tap water through which the pheromones were delivered while the rest of the brain was perfused with ACSF. One of the challenges was maintaining a barrier between the two perfusion systems - tap water and ACSF – because there was constant leakage between the two. We used Vaseline and later agar to try to form a leak-proof seal but our efforts were thwarted because the Vaseline and agar kept occluding the tips of our electrodes and also because the seals were not leak-proof. However, we managed to record from a single neuron over several months of recordings (Figure A1). It will be worthwhile to record from this set up but there is a need for a major redesign of the system as we could not completely remove the brain from the cranium but had to flip it upside down and record from inside the brain while the bottom of the skull form our base. This was one of the issues that caused instability with the recordings as it was difficult to hold down the whole brain.

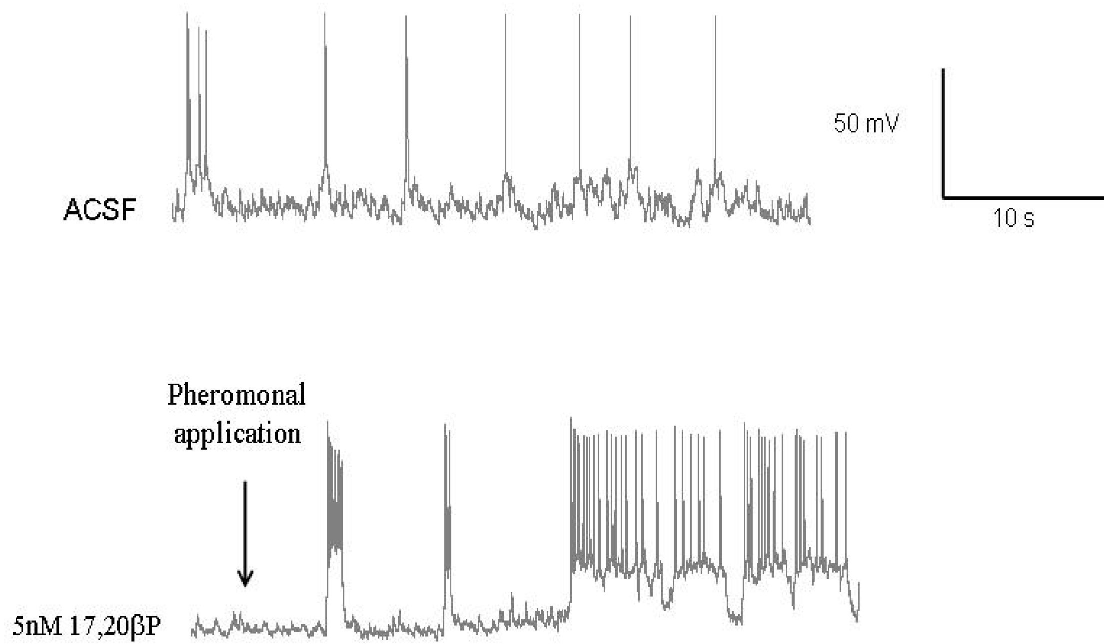

Figure A1: An illustrations of the possible effects of 17,20βP on POA neurons. Note that only the olfactory epithelium was exposed to the sex pheromones, which was dissolved in tap water, while the rest of the brain was in ACSF.

APPENDIX B

EXPOSURES OF WHOLE MALE GOLDFISH BRAINS TO 17,20βP and PGF2α

In order to rule out any non-specific effects from the sex pheromones, 7,20βP and $PGF_{2\alpha}$, we perfused the whole goldfish brain with 7,20βP and $PGF_{2\alpha}$ and recorded from the POA (Method and material in Chapter 2 and 3). Our rational for this experiment was to observe if there were any direct effects of the sex pheromones on the goldfish brain. However, we did not observe any changes suggesting that these pheromones act specifically through the olfactory epithelium to exert their effects (Appendix A).

APPENDIX C

SEROTONERGIC EFFECTS ON THE GOLDFISH BRAIN

Serotonin (5HT) has been known to exert GnRH release in male goldfish (Somoza et al., (1988). We next decided to study its effects in the goldfish brain. As such Agnes Crnic, our honours student, and I perfused the goldfish brain with 5HT while recording from the POA. However, we did not observed any changes in either the intrinsic membrane properties or the synaptic potentials. In addition, we did not obtain any results with fluoxetine (Flx), a selective serotonin reuptake inhibitor.

The reasons why we may not have observed any changes in the intrinsic membrane properties or synaptic potentials after perfusions with either 5HT or Flx may be that we were recording in current-clamp which did not allowed us to observe subtle changes in the intrinsic membrane properties of the POA neurons. In addition, perhaps the concentrations used (10μM, 20μM and 50μM) in the present study were not enough to elicit the expected responses. As such, it may be worthwhile to conduct these experiments under voltage-clamp conditions with increased dosages.

APPENDIX D

THE INHIBITORY EFFECTS OF PPE ON FATHEAD MINNOW REPRODUCTION

Andrew Waye, a PhD candidate in the Trudeau Lab, and I have collaborated with PAPICRAN to measure the effects of pulp and paper mill effluents (PPE) on reproduction in fathead minnows (FHM). Preliminary results indicate that the PPE, used in our experiment, have inhibitory effects on spermiation and ovulation in FHM since milting and egg released was prevented in the PPE but when the fish were transferred to tanks without the PPE they spawned normally. Since these experiments were conducted in sexually mature male and female FHM that were milting and ovulating, our results seem to indicate that the PPE affects the behavioural component of their reproduction.

APPENDIX E

ARTICLES PUBLISHED IN THE COURSE OF MY PhD

Lado WE, Persinger MA. 2012. Spatial Memory Deficits and Their Correlations with Clusters of Shrunken Neuronal Soma in the Cortices and Limbic System Following a "Mild'' Mechanical Impact to the Dorsal Skull in Female Rats. J *Behav & Brain Sci. 2: 333 - 342.*

Mennigen JA, **Lado WE**, Zamora JM, Duarte-Guterman P, Langlois VS, Metcalfe CD, Chang JP, Moon TW, Trudeau VL. 2010. Waterborne fluoxetine disrupts the reproductive axis in sexually mature male goldfish, *Carassius auratus. Aquatic Toxicololgy. 100(4):354-64.*

Cheung KW, **LadoWE**, Martin LJ, St-Pierre LS, Persinger MA. 2010. Cerebral Neurons in *Rattus norvegicus* Following a Mild Impact to the Skull: Equivalence of Modulation by Post-Impact Pregnancy or Exposure to Physiologically-Patterned Magnetic Fields. *Journal of Biological Sciences. 10(2): 84 - 92.*

Lado WE, Persinger MA. 2008. Increased conditioned immobility and weight loss in rats following mechanical impacts to the skull that do not produce loss of consciousness. *Central European Journal of Biology. 3(4): 422 – 430.*