Cannabinoid receptor 1 in goldfish *Carassius auratus*: protein expression and a role in appetite

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ABSTRACT

Cannabinoid receptor 1 (CB1), the first identified receptor subtype of the endogenous cannabinoid system (ECS), has been implicated in the control of appetite and the regulation of energy balance in vertebrates and invertebrates alike. This study investigated a potential role for CB1 in goldfish appetite regulation. A CB1 protein of ~ 53 kDa, corresponding in size to rat CB1 was found to be widely expressed in the brain and peripheral tissues of goldfish. The effects of peripheral (intraperiotoneal, IP) and central (intracerebroventricular, ICV) administration of various ECS agonists and antagonists were examined on food intake in goldfish. The agonists examined were the endogenous CB1 partial agonist anandamide and a synthetic analogue of this endocannabinoid methanandamide, while the antagonists examined were the CB1 selective antagonist/inverse agonist AM251, and the CB1 'silent antagonist' O-2050. IP but not ICV administration of anandamide increased food intake. Conversely, IP administration of methanandamide reduced food intake, while ICV administration had no effect. AM 251 did not alter food intake relative to saline treated controls, however at 24 h post-injection food intake levels were dose-dependently elevated among AM 251 treated fish. O-2050 was a potent anorexigenic agent, inhibiting food intake by IP and ICV administration, the former being effective up to 48 h post-injection. Food deprivation significantly elevated hypothalamic CB1 protein expression, while low doses of cortisol decreased hypothalamic CB1 expression. Taken together, these studies provide evidence for a functional ECS that appears to be involved in the control of food intake in goldfish and may be mediated, at least in part, by CB1.

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LIST OF ABBREVIATIONS

2-AG	2-arachidonoyl glycerol
AC	Adenylyl cyclase
ACC1	Acetyl-coenzyme-A carboxylase-1
Acrp30	Adiponectin
AgRP	Agouti-related peptide
ARC	Arcuate nucleus
cAMP	Cyclic adenosine monophophate
CART	Cocaine amphetamine related transcript
CB1	Cannabinoid receptor 1
CB2	Cannabinoid receptor 2
CCK	Cholecystokinin
CNS	Central nervous system
CRH	Cortisol releasing hormone
DMN	Dorsal medial nucleus
DTT	Dithiorthretiol
ECL	Enhanced chemiluminescence
ECS	Endogenous cannabinoid system/Endocannabinoid system
FAAH	Fatty acid hydrolase
FAS	fatty acid synthase
GABA	Gama aminobutyric acid
GAL	Galanin
GI	Gastrointestinal
GPCR	G-protein coupled receptor
HPI/A	Hypothalamic-pituitary-interrenal/adrenal
ICV	Intracerebroventricular injection
IP	Intraperitoneal injection
LH	Lateral hypothalamus
MCH	Melanin concentrating hormone
MS-222	Tricaine methanesulfonate
NADA	*N*-arachidonoyl-dopamine
NPY	Neuropeptide Y
OXA	Oxrein-A
OXB	Oxrein-B
PLD	Phospho lipase D
PVDF	Polyvinylidene fluoride
PVN	Paraventricular nucleus
SDS	Sodium dodecyl sulfate
SREBP	Sterol regulatory element binding protein
VMN	Ventromedial nucleus
α-MSH	Alpha-melanin stimulating hormone
Δ^9-THC	Delta 9-Tetrahydrocannabinol

1. INTRODUCTION

1.1 Appetite regulation in teleosts

The control of appetite is a highly regulated and complex multifactorial process. Appetite regulation involves the interplay of multiple hormones and neurotransmitters that signal within and between the brain, digestive track, and adipose tissue. In the vertebrate central nervous system (CNS), the hypothalamus is a well known "feeding centre" involved in the overall control of food intake (Jenson, 2000). Hypothalamic neuronal networks act to integrate appetite-stimulating (orexigenic) and appetite-reducing (anorexigenic) signals of central and peripheral origin to maintain energy homeostasis (Lin et al., 2000; Volkoff et al., 2005). Understanding of the complex circuitry involved in the regulation of appetite has greatly increased in mammals, however, understanding of appetite regulation and the control of food intake in teleosts remains limited. The following section will review appetite regulation in teleosts with a particular focus on orexigenic factors and briefly introduce key appetite regulating factors that have been shown to interact with the endocannabinoid system (ECS).

Much of our understanding of the neural control of food intake in teleosts was obtained from early brain lesioning and electrical stimulation experiments (for review see, Lin et al., 2000, Volkoff et al., 2005). From these studies, forebrain brain regions such as the olfactory bulbs, telencephalon, hypothalamus, and the optic tectum were implicated in the control of feeding behaviour and food intake in teleosts (Savage and Roberts, 1975; Peter, 1979; Lin et al., 2000). The olfactory bulbs and telencephalon are believed to be involved in the olfactory detection of food, while the hypothalamus represents the

primary brain region in the control of food intake in teleosts (Savage and Roberts, 1975; Peter, 1979). The lateral hypothalamus (LH) and inferior lobe of the hypothalamus are two hypothalamic areas that have been identified to have a role in the control of food intake in goldfish. Lesions of the LH resulted in aphagia in goldfish, while electrical stimulation of the LH evoked feeding behaviour and food intake, thus implicating this hypothalamic region as a feeding center (Roberts and Savage, 1978; Peter, 1979). Furthermore, electrical stimulation of the inferior lobe of the hypothalamus evoked robust feeding behaviour in goldfish, additionally implicating this hypothalamic region in the control of food intake (Savage and Roberts, 1978). More recently the focus has changed from the identification of discrete brain regions that are involved in appetite control to identifying specific appetite regulatory factors. Various peptides that have been demonstrated to have a role in the regulation of appetite in mammals have now been identified in fish (Lin et al., 2000; Volkoff et al., 2005). Food intake experiments examining the central (intracerebroventricular, ICV) and peripheral (intraperitoneal, IP) effect of exogenous peptides and or pharmacological agents (receptor agonists/antagonists) on feeding behaviour have elucidated the role of many appetite regulatory factors in fishes. Additionally, gene expression studies examining mRNA levels of appetite regulating peptides in relation to a single feeding event and under conditions of negative energy status (e.g. food deprivation) have provided further evidence in support for their role in the regulation of appetite and have contributed to our understanding of the neuroendocrine control of food intake in teleosts.

Multiple factors of central and peripheral origin partake in the complex interplay to maintain energy homeostasis. In mammals, several hypothalamic regions including the paraventricular nucleus (PVN), dorsalmedial nucleus (DVN), the ventromedial nucleus (VMN), arcuate nucleus (ARC) and the lateral hypothalamus (LH) have all been identified to have a role in the regulation of appetite and energy balance (Williams et al., 2001; Sainsbury et al., 2002). Currently, the anatomical and functional interaction between these various hypothalamic nuclei is a major area of research. The aforementioned hypothalamic nuclei are the site of synthesis of numerous appetite regulating neuropeptides. For example, the ARC has several neuronal populations that synthesize and release key appetite regulators, including two orexigenic peptides, neuropeptide Y (NPY) and agouti-related peptide (AgRP), and two anorexigenic peptides, α-melanocyte-stimulating hormone (α-MSH) and cocaine-amphetamine related transcript (CART) (Sainsbury et al., 2002). The ARC receives input from the gastrointestinal (GI) tract on short term meal-related conditions and also receives input from adipose tissue regarding overall, long term energy status (Jenson, 2000). Cholecystokinin (CCK) and ghrelin are two meal-related peptide hormones signaling energy availability of peripheral origin (Konturek et al., 2004). CCK is a prominent satiety hormone whose effect on food intake is mediated partially through the ARC and by the vagal afferent sensory neurons to the brainstem, an additional brain region that has been implicated in the regulation of energy homeostasis (Sainsbury et al., 2002; Burdyga et al., 2004). Ghrelin is a potent orexigenic peptide mainly produced by the stomach in mammals (Cani et al., 2004; Unniappan et al., 2004). CCK and ghrelin are considered to act as satiety and hunger

signals respectively as the levels of these two GI tract appetite regulators have been demonstrated to fluctuate in relation to food intake (Konturek et al., 2004; Unniappan et al., 2004). Finally, leptin, a secreted peptide factor of adipose tissue, plays a crucial negative feedback role on food intake and is believed to act as a long-term signal of energy status (Di Marzo et al., 2001; Sainsbury et al., 2002).

The majority of research examining the control of food intake in teleosts concerns appetite regulatory factors that act as a short-term signal (i.e meal-related conditions). Neuropeptide Y (NPY) (Lopez-Patino et al., 1999; Narnaware et al., 2000), orexin-A and orexin-B (OXA/OXB) (Volkoff et al., 1999), galanin (GAL) (Unniappan et al., 2004a), agouti-related peptide (AgRP) (Cerda-Reverter and Peter, 2003), and ghrelin (Unniappan et al., 2004b) are among the various peptides that have been shown or implicated to have an orexigenic role in fish. Furthermore, OXA, NPY and GAL have been demonstrated to functionally interact in the control of food intake in goldfish, as NPY and GAL actions on food intake are, in part, dependent on OXA actions, while OXA orexigenic effect on food intake is partly mediated by the NPY pathway (Volkoff and Peter, 2001). Additionally, central administration of recombinant murine leptin was demonstrated to attenuate the orexigenic actions of NPY and OXA and also found to decrease NPY mRNA expression in the goldfish brain (Volkoff et al., 2003), suggesting that leptin also has an anorexigenic role in fish. Aside from the elucidation of the physiological role of various peptide hormones in the control of feeding behaviour of fish and investigations examining the interactions between peptide pathways, little is known in regards to their mechanism of action.

1.2 Overview of the Endocannabinoid system

Historically the effects of exogenous cannabinoids on a myriad of physiological processes of the nervous, digestive, reproductive, cardiovascular and immune systems were commonly acknowledged as nonspecific, although these effects are now known to be mediated though the ECS. Research since the early 1990's has revealed the ECS to have a pivotal role in the modulation of endocrine function (Pagotto et al., 2006). Discovery of a CNS receptor capable of mediating the effects of the principal psychoactive component of *Cannabis sativa,* Δ^9-tetrahydrocannabinol (Δ^9-THC), indicated the existence of an endogenous cannabinoid system (Matsuda et al., 1990). To date, two cannabinoid receptor subtypes have been identified in vertebrates, denoted CB1 and CB2 (Matsuda et al., 1990; Munro et al., 1993). The CB1 receptor subtype is widely expressed in the CNS and periphery (e.g. eye, heart, intestine, and liver) and has been implicated in many physiological processes including the regulation of appetite and energy balance. CB2 is mainly expressed in cells of the immune system and is believed to modulate immune function (Matsuda et al., 1990; Galiegue et al., 1995; Munro et al., 1993).

Lipid ligands of the cannabinoid receptors, known as endocannabinoids were identified shortly after the discovery of the CB1 and CB2 receptor subtypes. A number of putative endocannabinoids are now recognized, including *N*-arachidonoylethanolamine (anandamide; Devane et al., 1992), 2-arachidonoyl glycerol (2-AG; Mechoulam et al., 1995), 2-arachidonoyl-glycerl-ether (noladin ether; Hanus et al., 2001), *O*-arachidonoyl-ethanolamine (virodhamine; Porter et al., 2002), *N*-arachidonoyl-dopamine (NADA;

Bisogno et al., 2000) and oleamide (Leggett et al., 2004). The regulation of ligand levels in tissues and in circulation is dictated by ECS enzymes; the biosynthetic and degradative pathways have been elucidated for anandamide and 2-AG (see De Petrocellis et al., 2004 for a review).

In summary, cannabinoid receptor subtypes (CB1 and CB2), the endocannabinoids and the enzymes responsible for their synthesis and degradation represent the primary components of this novel signaling system (Di Marzo et al., 1998). Cannabinoid receptor type 1 orthologs and endocannabinoids have been identified across animal phyla, however the vast majority of research investigating the physiological roles of the ECS concerns mammals.

1.3 Cannabinoid receptor 1 (CB1)

The existence of a specific cannabinoid receptor was first suspected with the observation that cannabinoid derivatives inhibited adenylyl cyclase (AC) activity in neuroblastoma cell cultures (Howlett et al., 1984). This finding was followed by autoradiographic studies revealing specific cannabinoid binding sites in the rat brain (Devane et al., 1988). The subsequent cloning of a G-protein coupled receptor (CB1) from a rat cDNA library provided further evidence indicating the existence of an endogenous cannabinoid system (Matsuda et al., 1990).

Cannabinoid receptors belong to the seven transmembrane domain superfamily of G-protein coupled receptors (GPCR) and are primarily coupled to G i/o class of G-proteins (Matsuda et al., 1990). The rat CB1 cDNA sequence encodes a 473 amino acid

protein with a predicted molecular weight of approximately 53 kDa (Matsuda et al., 1990). However, the N-terminal domain of the CB1 receptor has three potential glycosylation sites and this cannabinoid receptor subtype has been demonstrated to be an *N*-linked glycoprotein; thus the molecular weight of the CB1 receptor is known to vary from 53 kDa to approximately 72 kDa among vertebrates (Matsuda et al., 1990; Song and Howlett, 1995, Cottone et al., 2005a).

The CB1 receptor gene has been cloned and sequenced in numerous mammalian species (Murphy et al., 2001) and comparison of the cDNA sequences of model organisms such as the mouse or rat to the human CB1 cDNA sequence reveals greater than 97% identity at the amino acid level (Chakrabarti et al., 1995). High identity CB1 orthologs have also been identified in various non-mammalian vertebrate phyla, indicating that the CB1 receptor is highly conserved across vertebrate phyla. This observation suggests an important role for CB1 in animal physiology. In fish, CB1 has been cloned from two species of teleost, the puffer fish *Fugu rubripes* and the zebrafish *Danio rerio*, which respectively share 72% and 74% identity with human CB1 protein (Yamaguchi, 1996; Lam et al., 2006). The roughskin newt *Taricha granulosa* is the amphibian representative in which a CB1 ortholog has been cloned and displays 84% identity with human CB1 amino acid sequence (Soderstrom et al., 2001). Lastly, the zebra finch *Taeniopygia guttata* CB1 amino acid sequence is 92% identical to the human CB1 (Soderstrom and Jonhson, 2001).

The CB1 receptor is considered to be the most abundant G-protein coupled receptor of the mammalian brain. Receptor localization studies in the rat brain have

demonstrated there are particularly high CB1 receptor expression levels in the cerebral cortex, basal ganglia, cerebellum and hippocampus (Herkenham et al., 1990). The distribution of the CB1 receptor in the brain is consistent with many of the behavioural effects induced by exogenous cannabinoids, including alterations in mood, memory, cognition and motor coordination (Herkenham et al., 1990) and the long known hyperphagic effects of exogenous cannabinoids corresponds with the expression of CB1 receptor in areas of the CNS known to be involved in the regulation of appetite and reward centers of the brain (Herkenham et al., 1990; Tsou et al., 1998). Immunohistochemical investigation of the CB1 receptor distribution in the rat brain demonstrated that CB1 receptors are primarily expressed by GABAergic neurons (Tsou et al., 1998) and electron microscope studies have shown that the CB1 receptor is almost exclusively expressed on presynaptic cell terminals (Hajos et al., 2000). In line with the above mentioned findings, a neuromodulatory role has been proposed for the CB1 receptor, as activation of presynaptic expressed CB1 receptors results in the modulation of neurotransmitter release by inhibiting voltage-dependent calcium channels and activating potassium channels (Mackie et al., 1995; Twitchell et al., 1997).

CB1 receptor expression is also widely distributed in the periphery of mammals and has been detected in tissues involved in energy balance, namely the liver (Osei-Hyiaman et al., 2005), gastrointestinal tract (Casu et al., 1995), and adipocytes (Bensaid et al., 2003). Additionally, CB1 receptor mRNA and protein is also expressed in the eye (Porcella et al., 2000), heart (Galiegue et al., 1995), ovary (Galiegue et al., 1995), testis (Gerard et al., 1991), and peripheral nervous tissue (Burdyga et al., 2004), illustrating that

the ECS has a wide spectrum of activity in the periphery in addition to the central nervous system.

Little is known about the regulation of CB1 receptor expression, although there is indications that receptor expression may be directly regulated by ligand availability (Hsieh et al. 1999; Childers, 2005). Chronic cannabinoid exposure *in vivo* is known to result in the development of tolerance to many of the physiological and behavioral effects elicited by Δ^9-THC and synthetic cannabinoids (Childers, 2005). This observation is believed to be partially attributed to receptor down-regulation by the loss of cannabinoid binding sites and/or the uncoupling of cannabinoid receptors to their G-protein intracellular effectors as demonstrated by cannabinoid-stimulated [^{35}S]GTPγS autographic studies (Breivogel et al., 1999). Hsieh et al. (1999) demonstrated that CB1 receptors rapidly undergo receptor internalization in response to effacious agonist binding *in vitro*. Short-term (i.e. 20 minutes) agonist treatment results in reversible internalization of the CB1 receptor, however, longer term (i.e. 90 minutes) agonist exposure was found to result in irreversible internalization and down-regulation of the CB1 receptor, thus suggesting that the activity of the ECS is highly regulated.

1.3.1 Cannabinoid receptor 1 in teleosts

Following the discovery of the CB1 receptor, many initial studies set out to detect and examine CB1 receptor distribution across animal phyla in order to understand the phylogeny of the ECS. Cannabinoid receptor 1 orthologs have been cloned and receptor distribution in the central nervous system and various peripheral tissues has been

investigated in several species of bony fish. Genes encoding two CB1-like receptors, denoted CB1A and CB1B have been cloned from the puffer fish *Fugu rubripes* (Yamaguchi et al., 1996) and one CB1-like receptor gene has been cloned from zebrafish *Danio rerio* (Lam et al., 2006). Sequence alignment analysis of the deduced amino acid sequences of the cloned and partial sequences of the CB1 receptor in fish with CB1 orthologues identified in other vertebrates reveals that this GPCR is highly conserved (Cottone et al., 2005; Lam et al., 2006). The zebrafish CB1 gene encodes a 474 amino acid protein that displays 74.8 % identity with the rat CB1 receptor at the amino acid level (Lam et al., 2006). The zebrafish CB1 receptor was found to be a functional homolog of the human CB1, since amino acid residues that have been demonstrated to be crucial for ligand recognition and receptor function were found to be conserved (Lam et al., 2006). Presently, only a partial nucleotide sequence for the goldfish *Carassius auratus* CB1 receptor is known (Cottone et al., 2005b). However, amino acid residues that were identified to be important in ligand binding and that have been demonstrated to impart functional activity were also found to be conserved in goldfish (Valenti et al., 2005).

The neuroanatomical distribution of the CB1 receptor was investigated in the African cichlid fish *Pelvicachromis pulcher* (Cottone et al., 2005) and revealed wide spread CB1-like immunoreactivity in various brain regions, including the hypothalamus (Cottone et al., 2005a). Recently, the ontogeny of CB1 mRNA distribution was investigated in the zebrafish brain (Lam et al., 2006). CB1 mRNA was detected early in the zebrafish embryo at 24 h post-fertilization in the preoptic area of the hypothalamus,

along with the first appearance of several neurotransmitter systems (Lam et al., 2006). In addition, abundant CB1-like immunoreactivity has also been detected in forebrain regions of the goldfish (Valenti et al., 2005). It is of note that CB1 receptor mRNA and immunoreactivity in the CNS of teleosts occurs in regions that are involved in the regulation of food intake, including the inferior hypothalamic lobes (Cottone et al., 2005a).

1.4 Endocannabinoids: Lipid messengers of the ECS

Endocannabinoids, the lipid ligands of the ECS, are derivatives of arachidonic acid. Several endocannabinoids have now been recognized, however *N*-arachidonylethanolamide (Devane et al., 1992) and 2-arachidonly glycerol, anandamide and 2-AG respectively (Mechoulam et al., 1995) have been characterized to the greatest extent. Anandamide was the first endogenous ligand of the CB1 receptor to be isolated and identified (Devane et al., 1992). This ethanolamine amide derivative of arachidonic acid was named anandamide after ananda, the Sanskrit word for "bliss", since it is capable of mimicking many of the *in vitro* and *in vivo* effects of the Δ^9-THC (Devane et al., 1992; Piomelli, 2003). The synthesis of anandamide is characterized by the hydrolysis of a membrane phospholipid precursor *N*-arachidonoyl phoshatidylethanolamine, which is catalyzed by phospholipase D (PLD) enzyme (Di Marzo et al, 1998; Okamoto et al., 2004). Unlike classical neurotransmitters, once anandamide is synthesized in the post-synaptic cell it acts as a retrograde signaling messenger to activate presynaptic expressed CB1 receptors (Di Marzo et al., 1998; Wilson and Nicol et al., 2001). Anandamide is

quickly deactivated by rapid re-uptake into neurons via carrier-mediated transport and hydrolyzed by fatty acid amide hydrolase (FAAH) (Di Marzo et al, 1998; Ligresti et al., 2004). An *in vivo* study examining the time course of anandamide metabolism in mice, showed that it is metabolized within minutes following intravenous injection (Willoughby et al., 1997), demonstrating that circulating levels of this endocannabinoid are tightly regulated, therefore many studies have used FAAH inhibitors in order to prolong the bioactivity of this lipid messenger.

Like CB1 receptors, endocannabinoids are present in the brain and periphery. Anandamide is a partial agonist of the CB1 receptor as it has been demonstrated to have lower efficacy in comparison to 2-AG and synthetic cannabinoid agonists in various *in vitro* assays of cannabinergic activity, including the inhibition of adenylyl cyclase and calcium conductance (Devane et al., 1992). Additionally, anandamide, like other cannabinoid receptor agonists has been shown to have a dose dependent variable effect on various classical behaviours of cannabinergic activity in rodents, collectively known as the mouse tetrad (e.g. motor activity, ring catalepsy, hypothermia, and analgesia tests). Low doses of anandamide were found to elicit an opposite effect to high doses (as low doses had a stimulatory effect, while high doses had an inhibitory effect, Sulcova et al., 1998).

1.5 ECS role in energy balance, appetite and food intake

1.5.1 Overview of the ECS role in energy balance

Behavioural pharmacology, CB1 knock out mice, and CB1 mRNA expression studies have implicated the ECS and the CB1 receptor subtype in the regulation of food intake and energy balance. Numerous reports that have investigated the central and peripheral effect of selective CB1 receptor agonists/antagonists and endocannabinoids on food intake have demonstrated that the ECS has an orexigenic role in the regulation of appetite in mammals (Kirkham and Williams, 2001). A myriad of selective agonists and antagonists of the CB1 receptor can stimulate and/or inhibit food intake in various mammalian models (Arnone et al., 1997; Williams et al., 1998; Colombo et al., 1998; Hao et al., 2000; Koch and Matthews, 2001). The orexigenic effect of cannabinoid derivatives is mediated by the CB1 receptor subtype, since the hyperphagic effects of Δ^9-THC and endocannabinoids are reversed by selective CB1 receptor antagonists and not by selective antagonists of the CB2 receptor subtype (Williams and Kirkham, 1999; Jamshidi and Taylor, 2001; Williams and Kirkham, 2002). Additionally, ICV administration of a selective CB2 receptor antagonist AM630 failed to block food deprivation-induced hyperphagia, whereas the CB1 antagonist AM281 significantly reduced food intake, suggesting that an endocannabinoid tone underlies the hyperphagia induced by food deprivation (Werner and Koch, 2003). The CB1 receptor has also been demonstrated to mediate the orexigenic effects of endocannabinoids, as stimulation of food intake induced by central and peripheral administration of anandamide and 2-AG is reversed by co-administration of the CB1 selective antagonist/inverse agonist SR 141716

(Williams and Kirkham, 1999; Jamshidi et al., 2001) and not by CB2 selective antagonist SR144528 (Williams and Kirkham, 2002). In summary, behavioural pharmacology studies have consistently expressed the involvement of ECS, specifically the CB1 receptor subtype in the control of food intake.

The development of CB1 deficient mice provided definitive evidence for the involvement of the ECS in the control of food intake and energy balance. Food intake levels of re-fed food-deprived CB1 receptor deficient mice were shown to mirror food intake levels of wildtype littermates treated with SR141716, a selective CB1 antagonist, whereas SR141716 had no effect on food intake of CB1 knockout mice (Di Marzo et al., 2001), implying that the CB1 receptor to mediates the orexigenic tone of the ECS. Furthermore, although CB1 knockout mice pups display hypophagia in the first few weeks of life (Cota et al., 2003; Trillou et al., 2004), energy intake of adult CB1 deficient mice was found to be comparable to adult wildtype mice, even though they display a lean phenotype and are resistant to diet-induced obesity. This observation signifies potential involvement of the ECS in the development of obesity. Moreover, the CB1 receptor was found to have a crucial role in the initiation of milk ingestion in new born mice, since mice pups treated with SR141716, a CB1 rec eptor antagonist failed to ingest milk, resulting in 100% mortality in the first week of life. However, mortality rates could be reversed by administration of exogenous CB1 receptor agonists (Fride et al., 2001), illustrating an important role for CB1 in the control of food intake during early life stages.

The ECS has also been shown to affect body weight through the expression of factors that regulate fat metabolism. The prototypical CB1 selective antagonist,

SR141716 was found to increase the expression of adiponectin (Acrp30) mRNA in cultured adipocyte cells and in adipose tissue of obese Zucker (fa/fa) rats, a genetic model of obesity (Bensaid et al., 2003). Acrp30 is a secreted adipocyte peptide hormone which has a role in fatty acid catabolism and has been demonstrated to reduce body weight in rodents (Bensaid et al., 2003). Furthermore, SR141716 had no effect on Acrp30 mRNA expression in CB1 deficient mice (Bensaid et al., 2003), suggesting that the CB1 receptor may play a role in the regulation of Acrp30 expression. These findings indicate that the ECS regulates Acrp30 expression in adipose tissue in a CB1 receptor-dependent manner and thus may represent a possible mechanism by which the ECS affects body weight. Additionally, CB1 receptor activation has been demonstrated to enhance lipogenesis in primary adipocyte cultures by increasing the activity of a lipoprotein lipase (Cota et al., 2003), and activation of hepatic CB1 receptor was found to stimulate fatty acid synthesis by increasing the gene expression of a lipogenic transcription factor, sterol regulatory element binding protein (SREBP-1c) and its target enzyme, namely acetyl-coenzyme-A carboxylase-1 (ACC1) and fatty acid synthase (FAS) (Osei-Hyiaman et al., 2005). In conclusion, the ECS appears to act on adipose tissue as well as the liver to modulate lipid metabolism and thus ultimately affect body weight.

The vast majority of functional studies examining the involvement of the ECS in the regulation of appetite and food intake concerns mammals. There are, however, several studies that suggest the ECS plays a role in appetite control and feeding in other animal groups, including invertebrates. For example, anandamide was shown to alter the

feeding response of *Hydra vulgaris*, a primitive metazoan and a representative of a group where the first nervous systems (nerve network) developed (De Petrocellis et al., 1999) The initial feeding response of *H. vulgaris* is characterized as a induced withering of the tentacles followed by mouth opening in response to glutathione release from captured or injured prey. When stimulated artificially by the addition of glutathione to prey-free water, De Petrocellis et al. (1999) sought to manipulate the duration of mouth opening by adding increasing doses of anandamide to the experimental water. Anandamide was shown to dose-dependently reduce the duration of the mouth opening response, an observation that was significantly attenuated by a selective CB1 receptor antagonist (De Petrocellis et al., 1999). Additionally, it is believed that a functional ECS is present in *H. vulgaris* as several components of the ECS have been identified and or suggested. Firstly, *H. vulgaris* has been shown to have selective binding sites for cannabinoids, anandamide has been isolated in addition to its biosynthetic precursor, and lastly *H. vulgaris* has been demonstrated to display FAAH-like activity (De Petrocellis et al., 1999).

Recently, the ECS has also been implicated to have a role in the control of feeding of the invertebrate chordate, the sea squirt *Ciona intestinalis*. Matias et al. (2005) investigated the potential role for the ECS in the feeding response of *C. intestinalis* by examining the effect of the CB1 agonist HU-210 on siphon reopening after closure induced by mechanical stimulation. This typical behavioural response of *C. intestinalis* was found to be inhibited by HU-210, which in turn was significantly attenuated by the combination of mammalian CB1 and CB2 cannabinoids receptor antagonists, AM251 and AM630 respectively (Matias et al., 2005). In vertebrates such as the goldfish and

zebra finch, hypothalamic endocannabinoid levels have been shown to be elevated in response to food deprivation (Soderstrom et al., 2004; Valenti et al., 2005). In addition, anandamide was recently demonstrated to have a variable effect on food intake in the goldfish *Carassius auratus*, with a low dose (1 pg/g i.p.) stimulating food intake, while a high dose (10 pg/g i.p.) reduced food intake (Valenti et al., 2005). In summary, a role for the ECS in the regulation of energy balance, appetite and food intake appears to be widespread across animal groups and its first appearance in cnidaria suggests that it is a phylogenetically ancient one.

1.5.2 CB1 receptor agonists

Anecdotal reports of the orexigenic effects of Δ^9-THC have been known for centuries. Early studies investigating the orexigenic properties Δ^9-THC in humans and non-rodent species presented contradictory results (Pagotto et al., 2006). Additionally, the effect of Δ^9-THC and synthetic cannabinoid receptor agonists on food intake in rats also produced inconsistent results from no effect to reducing food intake (Graceffo and Robinson, 1998; Giuliani et al., 2000). Williams et al. (1998) concluded that the inconsistency among early studies was potentially due to differences in experimental design and the use of relatively high doses of Δ^9-THC and went on to demonstrate that low doses of Δ^9-THC (0.5-2.0 mg/kg) resulted in hyperphagia in pre-fed rats. High doses of Δ^9-THC (>10 mg/kg) were found to generally decrease food intake in rodents and this observation was attributed to the sedative side effect of this plant cannabinoid. Additionally, Δ^9-THC effect on food intake was shown to be significantly attenuated by

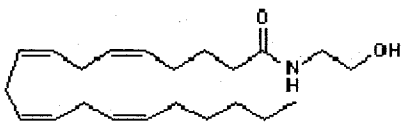
pre-treatment with the CB1 receptor antagonist SR141716, thus suggesting the CB1 receptor in mediating the hyperphagic effect of Δ^9-THC (Williams et al., 1998). Koch and Matthews (2001) investigated the effect of peripheral and central administration of Δ^9-THC on food intake in Lewis rats. Δ^9-THC (0.5-2.0 mg/kg) was found to have a short-term dose-dependent variable effect on palatable food intake when administered IP resulting in an "inverted U" dose-response curve. However, ICV administrations of Δ^9-THC had a stimulatory effect on food intake for the varying doses (2.5, 5, 10 and 25 μg) examined were also observed to effect food intake for a longer duration than those following IP administration (Koch and Matthews, 2001).

The endocannabinoids, anandamide and 2-AG have also been demonstrated to stimulate food intake in rodents (Williams and Kirkham, 1999; Kirkham et al., 2002). Central and peripheral administration of anandamide stimulates food intake in pre-satiated rats over a narrow dose range, as anandamide has also been demonstrated to have a dose-dependent biphasic effect on food intake (Williams and Kirkham, 1999; Jamshidi et al., 2001). The hyperphagia induced by anandamide was also shown to be reversed by the selective CB1 antagonist SR141716, suggesting that the orexigenic effect of this endocannabinoid is mediated by the CB1 receptor (Williams and Kirkham, 1999). 2-AG also potently and dose-dependently increases food intake in rats when injected into a limbic forebrain area; the nucleus accumbens shell and co-administration of CB1 receptor antagonist SR141716 attenuated this effect (Kirkham et al., 2002). Furthermore, given that anandamide levels are highly regulated, analogs that are more resistant to enzymatic hydrolysis have been developed. Methanandamide is a chiral analog of anandamide

which displays an increased affinity for the CB1 receptor and a higher degree of metabolic stability (Abadji et al., 1994). Methanandamide has been demonstrated to increase overall food consumption 3 h following IP administration in rats (Chambers et al., 2004). For a summary of CB1 agonist characteristics of anandamide and methanandamide see Table 1.

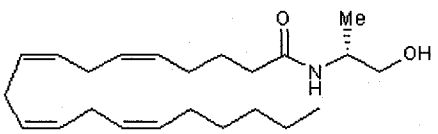
Table 1: Characteristics of the CB1 receptor agonists Anandamide and Methanandamide

Anandamide

Chemical structure	Agonist type	K_i (nM)
	Partial agonist	89

Chemical Name: N-(2-hydroxyethyl)-5Z,8Z,11Z,14Z-eicosatetraenamide

Methanandamide

Chemical structure	Agonist type	K_i (nM)
	Agonist	20

Chemical Name: (R)-N-(2-hydroxy-1-methylethyl)-5Z,8Z,11Z,14Z-eicosatetraenamide

1.5.3 Selective CB1 receptor antagonists

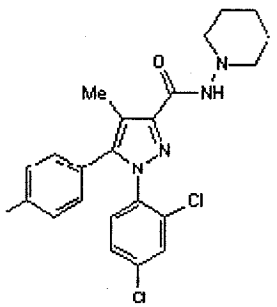
A myriad of cannabinoid receptor antagonists developed following the elucidation of Δ^9-THC structure have greatly advanced our understanding of the ECS. The effect of CB1 selective antagonists on food intake in mammals has been extensively examined for their potential use as an anti-obesity treatment. Those that have been comprehensively characterized in an appetite and food intake context include SR141716 and AM 251. AM 251 is structural analogue of the prototypical CB1 receptor antagonist, SR 141716, and is a potent CB1 selective antagonist (Gatley, et al., 1996; 1997). Moreover, AM 251 also acts as an inverse agonist as it has been demonstrated, *in vitro*, to produce opposite effects to those produced by cannabinoid agonists (Pertwee 2005). The effect of AM 251 on food intake has been investigated in rodents. AM251 has been demonstrated to have a prolonged dose-dependent anorectic effect in obese mice and rats (Hildebrant 2003; Chambers et al., 2004; 2005). The anorectic effect of AM 251 has been shown to supersede its half-life, which is approximately 6 h following intravenous injection and approximately 22 h when administered intraperitoneally in rats (Gatley, et al., 1996; McLaughlin et al., 2003). It should be noted that tolerance to the anorectic effect of AM 251 was shown to develop quickly in rats (Chamber et al., 2004), and this has been demonstrated to occur with SR141716 treatment as well (Colombo et al., 1998). However, the mechanism underlying tolerance and receptor desensitization remain unclear (Childers, 2006).

O-2050, a structural analogue of Δ^8-THC has been labeled as a putative 'silent antagonist' as it has been shown to lack inverse agonist properties *in vitro* (Gardner and

Mallet, 2006). O-2050 displays high affinity for CB1 receptors and was found to be equipotent to SR141716 in reducing food consumption in rats (Gardner and Mallet, 2006). Therefore, the anorexigenic effect of O-2050 was concluded to be due to antagonism of ECS activity, instead of inverse agonism at cannabinoid receptors (Gardner and Mallet, 2006). For a summary of CB1 antagonist characteristics of AM 251 and O-2050 see Table 2.

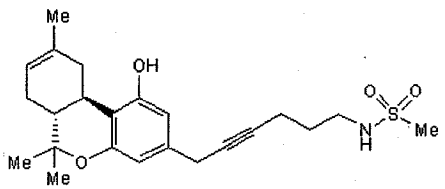
Table 2: Characteristics of the CB1 receptor antagonists AM 251 and O-2050

AM 251

Chemical structure	Antagonist type	K_i (nM)
	Antagonist/inverse agonist	7.49

Chemical Name: N-(Periperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dicholorophenyl)-4-methyl-1H-pyrazole-3-carboxamide

O-2050

Chemical structure	Antagonist type	K_i (nM)
	Silent antagonist	2.5

Chemical Name: (6aR,10aR)-3-(1-Methanesulfonylamino-4-hexyn-6-yl)-6a,7,10,10a-tetrahydro-6,6,9-trimethyl-6H-dibenzo[b,d]pyran

1.5.4 ECS central role in appetite regulation and energy balance

The activity of the ECS in the hypothalamus is believed to have a pivotal role in the homeostatic regulation of energy balance (Cota et al., 2003). Interestingly, although the density of CB1 receptors is relatively low in the mammalian hypothalamus in comparison to other brain regions, there is evidence indicating that the coupling efficiency of CB1 receptors to their G-protein intracellular effectors is the highest in the hypothalamus (Breivogel et al., 1997), signifying an important neuromodulatory role for the ECS in the hypothalamus. Moreover, *in situ* hybridization studies demonstrated that hypothalamic CB1 receptor transcripts are co-localized with several neuropeptides known to modulate appetite, including the anorexigenic peptides corticotrophin-releasing hormone (CRH), CART, MCH and the orexigenic peptide prepro-orexin (Cota et al., 2003). In CB1 deficient mice, CRH mRNA expression levels are elevated, implying that the expression of this potent anorexigenic neuropeptide is regulated by the ECS (Cota et al., 2003). Additionally, CB1 agonists were found to increase NPY release from hypothalamic explants, while CB1 receptor antagonist treatment inhibited the release of NPY (Gamber et al., 2005).

Furthermore, administration of a sub-anorectic dose of the cannabinoid receptor antagonist SR141716 was shown to facilitate the anorexigenic effects of α-MSH and block the orexigenic effects of intrahypothalamic ghrelin in rats (Verty et al., 2004; Tucci et al., 2004). These findings suggest a functional relationship between the ECS and several appetite regulating factors. In addition, hypothalamic endocannabinoids levels have been shown to be negatively regulated by leptin, an adipocyte secreted anoretic

factor that signals nutritional status to the hypothalamus (Di Marzo et al., 2001). Rodent models of obesity with defective leptin signaling have elevated hypothalamic levels of endocannabinoids, however, administration of exogenous leptin to *ob/ob* mice, which lack functional leptin, reduces both anandamide and 2-AG levels in the hypothalamus (Di Marzo et al., 2001). Furthermore, hypothalamic endocannabinoid levels have been demonstrated to fluctuate in relation to feeding and energy status (Kirkham et al., 2002; Hanus et al., 2003). Activity of the ECS in the hypothalamus is modulated by changes to nutritional status as hypothalamic levels of 2-AG were found to be elevated in food deprived rats and declined with feeding, while prolonged diet-restriction reduced hypothalamic 2-AG levels (Kirkham et al., 2002; Hanus et al., 2003). Additionally, both anandamide and 2-AG levels were also found to be significantly elevated in the limbic forebrain of food deprived rats, however, anandamide and 2-AG level remained unchanged in the cerebellum, a brain region not associated with the control of feeding (Kirkham et al., 2002). Interestingly, CB1 receptor density in the nucleus accumbens is reduced in rats with dietary obesity (Harrold et al., 2002), further supporting the notion that energy status affects the activity of the ECS in the central networks that govern appetite. Studies examining CB1 expression and hypothalamic endocannabinoid levels have suggested a central role for ECS in the regulation of appetite and energy balance.

1.5.5 ECS peripheral role in appetite regulation and energy balance

The ECS has also been suggested to play a role in the peripheral control of food intake. Similar to the hypothalamus, intestinal levels of endocannabinoids have been

demonstrated to fluctuate in relation to feeding (Gomez et al., 2002). Small intestinal levels of anandamide were found to increase sevenfold in food deprived rats, whereas refeeding normalized anandamide levels (Gomez et al., 2002). Furthermore, elevated levels of serum ghrelin in fasted rats were found to be significantly lowered by a selective CB1 receptor antagonist (Cani et al., 2004). In addition, CCK, a potent gastrointestinal tract satiety hormone was shown to be co-expressed with CB1 receptors in the vagal afferent neurons, which are involved in transmitting visceral sensory information to the CNS (Burdyga, et al., 2004). Using *in situ* hybridization and immunohistochemistry approaches, Burdyga et al. (2004) found that food deprivation increased CB1 receptor mRNA and protein expression in the rat nodose ganglion, although refeeding resulted in the rapid downregulation of the CB1 receptor. Furthermore, the expression of CB1 receptor in rat nodose ganglion appears to be modulated by CCK, as administration of the CCK-1 receptor antagonist lorglumide blocks the rapid loss of CB1 receptor expression that occurs with refeeding. Additionally, CCK administration in fasted rats results in the down-regulation of CB1 receptor expression, thus mimicking the effect of refeeding (Burdyga et al., 2004), signifying that peripheral CB1 expression is sensitive to CCK activity. In summary, the ECS also appears to play a peripheral role in the regulation of food intake as endocannabinoid levels and levels of CB1 receptor expression appear to be modulated by energy status and interact with several key peripheral signals of energy balance.

1.6 ECS and the stress response

Activation of the hypothalamic-pituitary-interrenal (HPI) axis in fish by physiological stressors results in elevated circulating levels of cortisol. The stress response in fish is characterized by a stressor induced release of corticotropin-releasing factor (CRF) from the hypothalamus, which elicits the secretion of adrenocorticotropin hormone (ACTH) from the pituitary. ACTH acts on the interrenal cells of the teleost head kidney to synthesize and secrete cortisol (Bernier and Peter, 2001). In turn, cortisol negatively regulates the release of CRF from the hypothalamus and the secretion of ACTH from the pituitary (Bernier and Peter, 2001).

The ECS has been demonstrated to have an inhibitory effect in the activation of the stress response in mammals, and this role may potentially be conserved in fish. The ECS was recently shown to play a role in the recovery from stress by mediating the negative feedback inhibition of cortisol on CRF release (Di et al., 2003). An electrophysiological study demonstrated that glucocorticoids rapidly decreased the glutamatergic excitatory synaptic input into CRF neurosecretory neurons of the PVN in a CB1 receptor-dependent manner (Di et al., 2003). This observation was followed by the finding that glucocorticoids can stimulate the biosynthesis and release of anandamide and 2-AG via a $G\alpha_s$-cAMP-protein kinase A-dependent mechanism (Malcher-Lopes et al., 2006), thus revealing that the CB1 mediated suppression of PVN excitation may potentially be attributed to glucocorticoids influencing endocannabinoid levels.

Several observations lend further support for an inhibitory role for the ECS in the stress response. CRF mRNA in the PVN is elevated in CB1 receptor deficient mice,

indicating that the ECS may exert tonic inhibition of the hypothalamic-pituitary-adrenal (HPA) axis in mammals (Cota et al., 2003). This observation was further verified by the finding that CB1 receptor SR141716 blockade dose-dependently elevated basal circulating levels of corticosterone in mice (Patel et al., 2004). Furthermore, the notion that the ECS exerts a tonic inhibitory tone on the HPA axis is supported by the observation that mice pretreated with a selective CB1 receptor antagonist prior to restraint-induced stress subjection results in a hyper elevation of serum corticosterone levels in comparison to vehicle treated mice (Patel et al., 2004). Low doses of a selective CB1 receptor agonist and an endocannabinoid transport inhibitor were found to decrease the restraint-induced corticosterone release (Patel et al., 2004). It has been speculated that the ECS may also have an inhibitory role in the stress response in teleosts, as IP administration of anandamide was demonstrated to lower plasma cortisol levels in goldfish (Valenti et al., 2005).

1.7 Hypothesis and objectives

Our initial working hypothesis was based on literature published until fall 2004. More specifically, we hypothesized based on the conserved involvement of the ECS in feeding behaviour, appetite and energy balance, the ECS would play a role in the regulation of food intake and appetite in teleost fishes. With the publication of Valenti et al. (2005), an orexigenic role for the ECS in the control of food intake was suggested for goldfish, as low doses of anandamide (1pg/g) stimulated food intake and food deprivation was shown to alter anandamide levels in the telencephalon. The objective of the present

study was to further investigate the orexigenic role of the ECS in the regulation of food intake in goldfish *Carassius auratus*. More specifically, the expression and tissue distribution of CB1 receptor in discrete regions of the goldfish brain and peripheral tissues was examined using Western blot analysis. Furthermore, hypothalamic CB1 protein expression levels were quantified under conditions of negative energy balance (i.e. food-deprivation) in order to determine if nutritional status can modulate CB1 receptor expression in a brain region involved in the control of food intake. Hypothalamic CB1 protein expression was also quantified in response to cortisol administration to further investigate the role of the ECS in response to HPI activation in teleosts. A functional role for the ECS in the control of food intake was examined by determining the effects of both peripheral (IP) and central (ICV) administration of the endogenous CB1 agonists anandamide, and its synthetic analogue, methanandamide. The CB1 selective antagonist/inverse agonist AM251 and 'silent antagonist' O-2050 effect on food intake were examined in order to establish CB1 receptor involvement. From a practical perspective, increasing our understanding of the physiological role of the ECS in the control of food intake in fish may have potential applications to the aquaculture industry. In addition, an increased understanding of the ECS role in the regulation appetite and food intake will advance our knowledge of the neuroendocrine control of energy homeostasis of teleosts.

2. MATERIALS AND METHODS

2.1 Experimental animals

Mixed sex goldfish (*Carassius auratus*) of the common or comet variety ranging from 10-40 g in body weight were purchased from a local supplier. Fish were held in 60 L tanks which received a constant flow of dechlorinated freshwater maintained at 18 ± 1 °C. Fish were held under a simulated photoperiod of 14L:10D. At least one week prior to all experiments, fish were entrained to a scheduled feeing regime (10:00 h). During this period, fish were fed *ad libitum* once daily with commercial koi and goldfish feed pellets (Martin Profishent, Elmira, ON). All experiments were carried out according to the guidelines established by the Canadian Council for Animal Care and according to the animal care and use policies of York University.

2.2 CB1 receptor protein expression in goldfish tissues

2.2.1 Protein extraction and western blotting

Goldfish were anaesthetized in 0.05% tricaine methanesulfonate (MS-222, Syndel Laboratories, Vancouver, B.C.) and killed by spinal transection. Brain tissue was removed, quickly frozen in liquid nitrogen and stored at -80 °C until use. Total protein was extracted from *C. auratus* tissues and Wister rat brain following a procedure described by Hungund et al. (2004). Briefly, goldfish tissues and rat CNS were homogenized in a threefold volume of ice-cold TME buffer (50 mM Tris-HCl, 3mM $MgCl_2$, 1 mM EDTA, 0.32 M sucrose, pH 7.4), containing freshly added protease inhibitor cocktail (1:200 dilution, Sigma-Aldrich). Homogenates were first centrifuged at

1000 x g (Eppendorf Centrifuge 5810 R) for 10 min at 4°C and supernatants were collected. Subsequently, supernatants were centrifuged at 22, 000 x g (Beckman J2-21 Centrifuge) for 20 minutes at 4°C; the resulting pellet was suspended in fresh TME buffer and recentrifuged at 22, 000 x g for 20 minutes at 4°C. The final pellet was resuspended in TME buffer and protein concentration of the samples was determined by Bradford assay (Bradford Reagent, Sigma-Aldrich) using bovine serum albumin (BSA, Sigma-Aldrich) as standards.

Following protein concentration determination at OD_{595nm} (Multiskan Spectrum, Thermo Fisher Scientific Inc), tissue homogenates were prepared with gel loading buffer (11.4 mM Tris-HCl, pH 6.8, 2 mM SDS, 30% glycerol, 3 mM DTT, 0.03% bromophenol blue) in a ratio of 1:5 and heated at 100 °C for 5 minutes. Appropriate amounts of protein were then resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using an Apollo Instrumentation gel apparatus (Interscience, Markham, ON). Following gel electrophoresis, gels and polyvinylidene fluoride (PVDF) Hypond-P membranes (Amersham Biosciences, Baie d'Urfé, QB) were incubated in transfer buffer (25 mM Tris base, 0.5 M gylcine, 20% methanol) for 20 minutes at room temperature (RT) with gentle agitation following PVDF membrane activation in 100% methanol. Proteins were then electrophoretically transferred to Hybond-P membranes for 2 hours at RT using a TE 70 semi-dry transfer unit (Amersham Biosciences, Baie d'Urfé, QB) at the maximum amperage of 0.8 mA/cm^2 calculated for total gel area. Following transfer, membranes were washed (3 x 5 minutes) with Tris-buffered saline (TBS-T: 10 mM Tris, 1.54 M

NaCl containing 0.5%-Tween-20, pH 7.2). Membranes were subsequently incubated in blocking buffer (5% non-fat dry milk in TBS-T) for 1 h at RT with gentle shaking.

For primary antibody treatment, membranes were incubated overnight at 4 °C gently agitating with a rabbit polyclonal antibody raised against a fusion protein containing the first 77 amino acid residues of the rat CB1 receptor (Affinity Bioreagents, Golden, CO) diluted 1:500 in blocking buffer. This antibody was selected since it has been previously used to detect CB1 receptor protein in the goldfish retina (Straiker et al., 1999). Following primary antibody incubation, membranes were subsequently washed (3 x 5 minutes) with TBS-T and incubated (1 h at RT) with a horseradish peroxidase conjugated goat anti-rabbit IgG (Biorad, Hercules, CA) diluted 1:8000 in blocking buffer. Following secondary antibody incubation, membranes were washed (3 x 5 minutes) with TBS-T and (2 x 5 minutes) with TBS (Tris buffered saline without 0.5% Tween-20). Immunoreactive bands were visualized by enhanced chemiluminescence (ECL Plus Western Blotting Detection Reagent, Amersham), followed by exposure to Bioflex autoradiography film (Interscience). Rat CNS was loaded as positive control and a prestained broad-range molecular weight marker (Precision Plus Protein Dual Colour Standards, Biorad) was loaded on each gel for molecular mass determination of the immunoreactive bands. Following visualization of CB1 receptor protein, membranes were stripped by washing membranes for 30 minutes at RT with stripping buffer (20mM $C_4H_6O_2$ $4H_20$, 30 mM KCl, 0.1 M gylcine, pH 2.2), washed (3 x 5 minutes) with TBS-T and incubated in blocking buffer (1 h at RT) and subsequently reprobed with mouse anti-α-tubulin monoclonal antibody (Developmental Studies Hybridoma Bank, University of

Iowa) diluted 1:5000 overnight at 4 °C with gentle agitation. Subsequently, membranes were then probed (1 h at RT) with horseradish peroxidase conjugated goat anti-mouse (1:5000 dilution, Biorad). CB1 and α-tubluin immunoreactive bands were imaged with Epichem^3 Darkroom (Diamed, Mississauga, ON).

2.2.2 CB1 protein distribution in the CNS and peripheral tissues of goldfish

Distribution of CB1 protein in discrete regions of the brain, olfactory bulbs, telencelphalon, hypothalamus, midbrain, hindbrain and the pituitary as well as peripheral tissues: eye, gill, heart, liver, spleen, intestine, kidney, testis, ovary and muscle was examined by Western blot analysis. Anesthetized fish were killed by spinal transection and tissues samples were collected, immediately frozen in liquid nitrogen and stored at -85°C until protein extraction. Total protein was extracted from samples as described previously.

2.3 Endocannabinoid administration and feeding studies

2.3.1 Drugs and vehicles

Various CB1 agonists and antagonists were examined in order to determine the role of the ECS on food intake. The CB1 agonists, anandamide [N-(2-hydroxyethyl)-5Z,8Z,11Z,14Z-eicosatetraenamide]; Sigma-Aldrich, Oakville, ON) and methanandamide ([(R)-N-(2-hydroxy-1-methylethyl)-5Z,8Z,11Z,14Z-eicosatetraenamide]; Sigma-Aldrich) were dissolved in vehicles consisting of 10% ethanol in 0.7% NaCl and 4% ethanol diluted in 0.7% NaCl respectively. These solutions were used for IP injections. For ICV

injections, the vehicle consisted of a 1.49% (v/v) ethanol saline solution for anandamide and a 0.75% (v/v) ethanol saline solution for methanandamide.

For IP injections, the CB1 antagonists, AM 251 ([N-(Periperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dicholorophenyl)-4-methyl-1H-pyrazole-3-carboxamide]; Torcris Bioscience) and O-2050 ([(6aR,10aR)-3-(1-Methanesulfonylamino-4-hexyn-6-yl)-6a,7,10,10a-tetrahydro-6,6,9-trimethyl-6H-dibenzo[b,d]pyran]; Torcis bioscience, Ellisville, MO) were dissolved in DMSO and then diluted with saline (0.7% NaCl) resulting in a final saline vehicle consisting of 3.3% DMSO (Sigma-Adrich). For ICV injections of the same antagonists, the vehicle consisted of a 1.36% DMSO saline solution for AM251 and a 2.5% DMSO saline solution for O-2050. Stock solutions for each test dose were made, aliquoted and stored at -20°C. For IP administration, aliquots were subsequently thawed and diluted in saline just prior to use. All units for test substance administration are expressed as ng or µg substance/g body weight (i.e. µg /g or ng/g).

2.3.2 Intraperitoneal (IP) injections

Fish were anesthetized in 0.05% tricaine methanesulfonate (MS-222, Syndel Laboratories, Vancouver, B.C.). Immediately after the loss of equilibrium, fish were weighed and a single injection of test solution was administered through the ventrolateral side of the body wall into the peritoneal cavity using a 100 µL Hamilton syringe (Hamilton Company, Reno, NV). The volume of each injection was standardized to 1 µL/ 6g body weight. Varying doses of test substance were administered intraperitonially

to determine the effect on food intake. Fish were then placed in anesthetic free water and given a 10 minutes to allow full recovery from anesthesia prior to conducting feeding experiments to determine food intake (outlined in section 2.3.4 below).

2.3.3 Intracerebroventricular (ICV) injections

Intracerebroventricular (ICV) injections were administered based on the stereotaxic atlas of the goldfish brain described by Peter and Gill (1975) and following the procedure standardized by Volkoff and Peter (1999). Briefly, following deep anesthesia in 0.05% tricaine methanesulfonate for 3 minutes, a three-sided flap was cut into the roof of the skull using a dentist drill (Microtechnique II, USA) equipped with a circular saw. With the skull flap folded to the side exposing the brain, fish were placed in a custom-fabricated stereotaxic apparatus and a 10-µl Hamilton microsyringe was used to inject test solution into the preoptic region of the brain third ventricle following the co-ordinates specified by the stereotaxic atlas of the goldfish brain (co-ordinates +1.0 M, D 1.2; Peter and Gill, 1975). The injection volume was standardized to 1 µL/ 15g body weight. All injection procedures were observed under a dissecting microscope (World Precision Instruments, Sarasota, FL, USA; Model# PZMIII BS) to ensure accuracy of procedures. In the rare event that injected fluid was seen to spill up and over the telencephalon, the fish in question was not used further. Following injection, the skull flap was replaced and secured with surgical thread (Seraflex USP 4/0, Naila, Germany). Fish were placed in fresh water to recover from anesthesia and given a 20 minute postoperative recovery period before feeding experiments were conducted.

2.3.4 Feeding experiments and food intake determination

Experiments to determine food intake began 10 (IP) or 20 (ICV) minutes following administration of a test solution. Each experiment was conducted with similar sized fish held in pairs, each receiving the same treatment (i.e. untreated, saline control, and test solution dose). Paired goldfish were given a pre-weighed excess of pellets and allowed to feed for 1 h undisturbed. Following the feeding period, all remaining pellets were collected and dried at 60 °C for 24 h to obtain dry food weight. Dry food weights were corrected for weight loss to water and food intake was calculated by dry food weight differences relative to the mass of the two fish and time spent feeding. Fish were tested in random order in terms of treatment and experiments commenced at the regular feeding time to which fish had been entrained (10:00h). Fish were monitored at various time points following administration of test solution (i.e. 1 h, 3 h, 6 h, 24 h). In order to verify that the administration procedure itself did not influence feeding, food intake was assessed for untreated fish subjected to only anesthesia and compared to saline-treated fish, which received saline vehicle void of CB1 agonists or antagonists. Fish that did not recover from anesthesia or those that exhibited abnormal post-operative behaviour (i.e. decreased locomotor activity, huddling at the bottom of the tank) were not used for further analysis.

2.4 Food deprivation and hypothalamic CB1 protein expression

2.4.1 Experimental animals

To examine the effects of food deprivation on hypothalamic CB1 expression, fish were food deprived for periods of 1, 2 and 4 weeks. Goldfish were held in 60 L opaque aquaria as previously described, with flow through dechlorinated tapwater at 18 °C. Six groups of fish (12 fish/group) were weighed and fed a scheduled regime of a 2% total body mass ration once daily for a minimum of two weeks prior to the start of the experiment. Following the acclimatization period, two groups of fish were randomly assigned to be fed (control) or food deprived for each respective week.

2.4.2 Tissue sampling

Hypothalamic tissues samples were collected as previously described, briefly anesthetized fish were killed by spinal transection and tissues samples were immediately frozen in liquid nitrogen and stored at -85°C until protein extraction. Total protein extracted from hypothalamic tissues was examined for alteration in CB1 protein expression using Western blot analysis, as described above and expressed relative to α-tubulin and then normalized as a percentage of the expression levels in the fed controls. CB1 protein expression was analyzed in protein samples extracted from individual hypothalamuses from fed or food deprived fish. Protein samples from each group for each respective week were run on the same gel in order to control for variation between gels. Protein band area density was measured using LabWorks image acquisition and analysis software (Ultra Violet Products Inc., Upland, CA). CB1 protein levels were

expressed as a ratio of CB1 band area densities to their respective α-tubulin and then normalized as a percentage of the expression levels of the corresponding control.

2.5 Cortisol administration and hypothalamic CB1 protein expression

2.5.1 Experimental animals

To investigate the effect of cortisol on hypothalamic CB1 protein expression, fish were administered cortisol using intraperioteoneal coconut oil (100% pure coconut oil, Westfair foods Ltd., Calgary, AB) implants. Coconut oil has been previously shown to be an effective vehicle to slowly release cortisol into circulation, and thus maintain elevated circulating levels over several days (Vijayan and Leatherland, 1989). Goldfish were held in 60 L opaque aquaria as previously described, with flow through dechlorinated tapwater at 18 ^{o}C. For the time course experiment, nine groups of fish (12 fish/group) were weighed and fed a scheduled regime of a 1.5 % total body mass ration once daily for a minimum of two weeks before the start of the experiment. Following the acclimatization period, three groups of fish were randomly assigned to be sham (cortisol free implant) or cortisol treated (75 μg/g or 400μg/g) for each respective time point (2, 4, or 8 days). For the dose response study, six groups of fish (12 fish/group) received intraperitoneal coconut oil implants containing cortisol at various doses (50, 100, 200, or 400 μg/g body) and untreated fish and sham-treated fish, which received only the coconut oil vehicle, were included as controls. For implantation, fish were anaesthetized and a small incision was made in the ventrolateral side of the body wall in order to receive an intraperiotoneal implant of coconut oil (5 μL coconut oil/g body weight) containing

dissolved cortisol (hydrocortisone 21-hemisuccinate, Sigma-Adrich) at various doses. Following injection, fish were briefly placed on ice to aid the solidification of the coconut oil and thus allow it to act as a cortisol releasing implant. Fish were then placed in anesthetic free water and given 5 minutes to allow for recovery from anesthesia. CB1 protein expression was analyzed in protein samples extracted from individual hypothalamuses from control, sham, and cortisol treated fish.

2.5.2 Tissue sampling

Hypothalamic tissues were sampled four days following cortisol administration with the exception of the time course experiment, which included a 2 and 8 day time point. Tissues samples were collected as previously described, briefly anesthetized fish were killed by spinal transection and tissues samples were immediately frozen in liquid nitrogen and stored at -85°C until protein extraction. Total protein extracted from hypothalamic tissues was examined for alteration in CB1 protein expression using Western blot analysis, as described above and expressed relative to α-tubulin and then normalized as a percentage of the expression levels in sham-treated fish. CB1 protein expression levels in sham-treated fish were normalized as a percentage of the expression levels in the untreated controls. Protein samples from each treatment were run on the same gel in order to control for variation between gels.

2.6 Statistical analysis

All data are expressed as mean values ± SEM. For multiple group comparisons a one-way analysis of variance (ANOVA) was used followed by a Student-Newman Keuls multiple comparison test. Unpaired Student's *t*-test was used for comparisons between two groups using GraphPad InStat software (GraphPad Inc., San Diego, CA). The significance level for all statistical tests was $P< 0.05$.

3. RESULTS

3.1 CB1 protein detection and distribution in the CNS and peripheral tissues of goldfish

Using an antibody directed against the N-terminal region of the rat CB1, a major immunoreactive band was found to resolve in an identical position to the rat CB1 protein receptor at approximately 53 kDa (Fig. 1A, 2). A standard curve of protein expression was constructed to determine what quantity of protein would be appropriate to examine for alterations in expression (Fig. 1B). CB1 was found in all regions of the CNS (Fig. 2A). In the pituitary, the protein band detected was marginally larger with a molecular weight of approximately 56 kDa. CB1 protein was differentially expressed in the various peripheral tissues examined. A band of the same molecular weight detected in the brain was found in all peripheral tissues investigated and was highly expressed in the heart and ovary and weakly expressed in the spleen and testis (Fig. 2B).

3.2 Anandamide administration and food intake

3.2.1 IP administration of anandamide and food intake

To determine the systemic effect of the endocannabinoid anandamide on food intake, fish were treated with varying doses of anandamide and food intake was examined over a 1 h period post-injection. There were no differences in food intake between control fish (i.e. untreated and saline treated fish) and fish treated with 25 or 250 ng/g anandamide (Fig. 3A). However, food intake was significantly elevated ($p \leq 0.05$) in fish treated with 500 ng/g anandamide in comparison to both the untreated and saline treated

controls and fish treated with the lower test doses of anandamide examined (i.e. 25 or 250 ng/g).

3.2.2 ICV administration of anandamide and food intake

The central effect of anandamide on food intake was determined over a 1 h period after ICV administration. Food intake was found to significantly differ between the untreated controls and saline treated goldfish (Fig. 3B). Food intake did not differ significantly between the saline treated fish and anandamide treated fish (2.5 and 5 ng/g).

3.3 Methanandamide administration and food intake

3.3.1 IP administration of Methanandamide and food intake

The systemic effect of methanandamide on food intake was examined immediately (0-1 h) following IP administration and then again at 3 (3-4 h), 6 (6-7h) and 24 (24-25 h) following a single injection of methanandamide. Food intake levels in fish treated with the varying doses of methanandamide (0.01, 0.1, 1 and 3 μg/g) were similar to the untreated and saline treated controls immediately following administration (Fig. 4A). However, food intake appeared to dose dependently decrease in response to increasing dose of methanandamide at 3 h, as food intake was significantly reduced in fish treated with 3 μg/g methanandamide in comparison to both the untreated and saline treated controls and in comparison to fish treated with the lowest test dose of methanandamide examined (i.e. 0.01 μg/g) (Fig. 4B). At 3 h post-injection, food intake

levels of fish treated 1 μg/g of methanandamide was significantly reduced in comparison to saline treated fish and 0.01 μg/g methanadmide treated fish (Fig. 4B). Fish treated with the higher test dose of methanandamide of 1 and 3 μg/g had significantly reduced food intake levels in comparison to fish treated with the lowest dose of methanandamide examined (0.01 μg/g) at 6 h following injection (Fig. 4C). However, food intake did not differ significantly between the controls and methanandamide treated fish at this time point. At 24 h post-injection a continued downward trend relative to increasing methanandamide dose was observed (Fig. 4D). However, food intake did not differ significantly between the untreated, saline treated fish and methanandamide fish treated at 24 h post-injection.

3.3.2 ICV administration of methanandamide and food intake

Food intake did not differ significantly between the controls (untreated or saline treated fish) and fish treated with the various test doses of methanandamide examined (i.e. 0.1, 1, 5, 10 ng/g) 1 h following ICV administration (Fig. 5).

3.4 AM 251 administration and food intake

3.4.1 IP administration of AM 251 and food intake

The effect of IP administration of the selective CB1 antagonist/inverse agonist AM 251 on food intake was examined at 1, 3, 6 and 24 h post-injection. Food intake levels were not significantly altered by the varying doses of AM 251 examined (i.e. 0.1, 1 and 5 μg/g) in comparison to the untreated and saline treated controls at all time points

food intake was monitored (Fig. 6A-D). However, food intake was significantly elevated 24 h post-injection in fish treated with the higher test dose AM 251 of 5 μg/g in comparison to fish treated with the lower test dose of AM 251 examined of 0.1 μg/g (Fig. 6D).

3.4.2 ICV administration of AM 251 and food intake

Food intake levels were not found to significantly differ between the controls (untreated and saline treated) and fish treated with the various test doses of AM 251 examined (i.e. 0.1, 1 and 5 ng/g) at each time-point that food intake was monitored following ICV administration (Fig. 7A-C).

3.5 O-2050 administration and food intake

3.5.1 IP administration of O-2050 and food intake

To determine the systemic effect of the selective CB1 antagonist O-2050 on food intake, fish were treated with varying doses of O-2050 and food intake was examined at 1, 3, 6, and once every 24 h up to 72 h after injection. There were no significant differences in food intake between the untreated controls and saline treated fish at all time points food intake was monitored (Fig. 8A-F). Food intake levels in fish treated with 0.1 μg/g O-2050 were similar to the saline treated controls throughout the course of the experiment (Fig. 8A-F). Fish treated with 5 μg/g O-2050 had reduced food intake levels following 3 h post-injection (Fig. 8B), however this observation was only significant in comparison to the uninjected fish. IP administration of 5 μg/g O-2050 significantly

reduced food intake in comparison to the saline treated controls at 24 h and 48 h post-injection (Fig. 8D, E). Food intake levels were not found to significantly differ between control fish (untreated and saline treated fish) and O-2050 treated fish at 72 h post injection (Fig. 8F).

3.5.2 ICV administration of O-2050 and food intake

The central effect of O-2050 on food intake was examined 1, 3, and 6 h after ICV administration. There were no significant differences in food intake between control fish and saline treated fish at all time points that food intake was monitored (Fig. 9A-C). Food intake levels did not significantly differ between controls (untreated and saline treated fish) and O-2050 treated fish (i.e. 0.1, 1, 5 ng/g) 1 h following ICV administration (Fig. 9A). At 3 h, following injection, food intake was significantly reduced in fish that received 1 ng/g O-2050 compared to the untreated controls (Fig. 9B). There were no significant differences in food intake between untreated, saline treated fish and fish treated with 0.1 ng/g or 5 ng/g 0-2050 at this time point. However, at 6 h after central administration of O-2050, food intake was significantly reduced by all test doses examined (i.e. 0.1, 1, and 5 ng/g O-2050) in comparison to both the untreated and saline controls (Fig. 9C).

3.6 Hypothalamic CB1 protein expression in response to food deprivation

To determine the effect of food deprivation on hypothalamic CB1 protein expression, fish were deprived of food for a period of 1, 2 or 4 weeks. Hypothalamic

CB1 protein expression was not significantly altered after 1 week of food deprivation (Fig. 10). In goldfish deprived of food for a 2 week period, CB1 protein expression in the hypothalamus appears elevated, although this observation was not found to be significant compared to the corresponding fed controls. However, hypothalamic CB1 protein expression was significantly elevated in fish food deprived for a 4 week period relative to the fed controls (Fig. 10).

3.7 Hypothalamic CB1 protein expression in response to cortisol administration

3.7.1 Time course study

The effect of elevated circulating levels of cortisol on hypothalamic CB1 protein expression was examined 2, 4, and 8 days following the administration of coconut oil implants containing cortisol. These implants have been demonstrated to elevate circulating cortisol levels to the range of 90-250 ng/mL (Chasiotis pers. comm.). Cortisol administration was not found to significantly alter hypothalamic CB1 expression, although some trends appear to be present (Fig 11, also see discussion).

3.7.2 Dose response study

The dose-dependent effect of cortisol on CB1 protein expression was examined at 4 days following the implantation of the cortisol containing coconut oil implants. These implants have been demonstrated to elevate circulating cortisol levels to the range of 50-400ng/mL (Chasiotis pers. comm.). Hypothalamic CB1 protein expression of sham treated fish did not significantly differ from untreated controls (data not shown).

Hypothalamic CB1 protein expression was significantly reduced in fish treated with 50 and 100 μg/g BW cortisol in comparison to the sham treated fish (Fig. 12). Hypothalamic CB1 protein expression in fish treated with 200 or 400 μg/g BW cortisol were not found to differ significantly from the sham treated fish.

4. DISCUSSION

4.1 Expression of CB1 protein in goldfish CNS and peripheral tissues

CB1 protein was found to be widely expressed in the goldfish brain. A protein band with a molecular weight of approximately 53 kDa was detected in all discrete regions of the goldfish brain by Western blot analysis (Fig. 2A). The wide distribution of CB1 protein expression within the goldfish brain is consistent with previous reports that have demonstrated ubiquitous CB1-like immunoreactivity in the CNS of the African cichlid fish *Pelvicachromis pulcher* and the zebrafish *Danio rerio* (Cottone et al., 2005a; Lam et al., 2006). The 53 kDa band was interpreted to be CB1 as it is consistent with the predicted molecular weight of the unglycosylated form of the rat CB1 (Matsuda et al., 1990) and with a recent report that also examined CB1 protein expression in the CNS of goldfish *Carassius auratus* (Valenti et al., 2005). Furthermore, goldfish CB1 corresponded in size with the band detected in the rat CNS positive control. A protein band with an apparent molecular weight of approximately 56 kDa was detected in the goldfish pituitary, which may represent a partially glycosylated form of the receptor, given that CB1 has three potential N-glycosylation sites (Mastuda et al., 1990) and has been demonstrated to be a N-linked glycoprotein in the rat brain (Song and Howlett, 1995). Furthermore, CB1 receptor size variations have been reported between fish and mammals, a protein band with an apparent molecular weight of approximately 61.5 kDa was detected in the CNS of the African cichlid fish *Pelvicachromis pulcher* while a 63 kDa band was detected in the rat brain using antiserum directed against the N-terminus

and C-terminus of the rat CB1 (Cottone et al., 2005a). The protein band detected in the above mentioned studies is believed to represent the glycosylated form of CB1. The widespread distribution of CB1 receptor protein in the CNS of the goldfish, as well as other fish species suggests numerous potential physiological roles for this receptor and its ligand in fishes. Of particular interest in our studies was its presence in the hypothalamus, a brain region with an important role in the regulation of appetite and feeding. Hypothalamic presence of CB1 observed in the present study is consistent with the detection of abundant CB1-like immunoreactivity in hypothalamic areas known to be involved in the control of food intake of teleosts, including the inferior hypothalamic lobes (Cottone et al., 2005a).

CB1 protein expression was also found to have widespread distribution in peripheral tissues of the goldfish. A band consistent in size with the goldfish CB1 detected within discrete brain regions was observed in all peripheral goldfish tissues examined by Western blot analysis (Fig. 2B). The widespread distribution of CB1 in the peripheral tissues of goldfish is in line with the peripheral expression of CB1 observed in other metazoan, including a urochordate (Matias et al., 2005), an amphibian (Meccariello et al., 2006) and in mammals. The pattern of expression of the CB1 receptor in goldfish peripheral tissues is consistent with mammalian CB1 mRNA and protein distribution, specifically in the eye (Porcella et al., 2000), heart (Galiegue et al., 1995), liver (Osei-Hyiaman et al., 2005), intestine (Casu et al., 2003), spleen (Galiegue et al., 1995), kidney (Deutsch et al., 1997), and gonads (Gerard et al., 1991; Galiegue et al., 1995). Moreover, CB1 protein expression in the eye, testis and ovary of the goldfish confirm previous

reports of CB1 immunoreactivity and CB1 mRNA expression in the goldfish retina and reproductive tissues respectively (Straiker et al., 1999; Cottone et al., 2005b). Lastly, CB1 protein expression in the goldfish spleen is in agreement with CB1A mRNA expression in the puffer fish *Fugu rubripes* spleen (Yamaguchi et al., 1996). In addition to its wide distribution in the goldfish CNS, CB1 receptor is also ubiquitously expressed in the goldfish periphery analogous to observations made for other vertebrates.

4.2 Effect of Anandamide administration on food intake

Peripheral administration of the 500 ng/g dose of anandamide significantly increased food intake in goldfish (Fig 3A), suggesting that this endocannabinoid acts as an orexigen in fish, similar to the demonstrated effect of anandamide in rodents (Williams and Kirkham, 1999; Hao et al., 2000; Williams and Kirkham, 2002). Recent studies have reported qualitatively similar alterations in food intake of goldfish after IP administration of anandamide (Valenti et al., 2005). However, the stimulatory effect of anandamide on goldfish feeding behaviour in the aforementioned report occurred at a dose of 1 pg/g. We were unable to repeat these results (data not shown). Instead, our data seems more consistent with the doses, and responses, of rodent models where anandamide stimulates food intake at doses varying from 1 μg/kg dose (Hao et al., 2000) up to 0.5-10 mg/kg (i.e. 500-10,000 ng/g, Williams and Kirkham, 1999). The discrepancy between our observations and previous findings for goldfish may be attributed to several factors concerning experimental design, including the use of unsatiated fish, as fish were deprived of food for 24 h prior to IP administration of

anandamide and subsequent food intake determination and potentially the use of short feeding period of only 1 h in the present study. Experimental design has been shown to affect the detection of the orexigenic dose for anandamide in rodents, as a greater dose range (0.5- 10 mg/kg) was required to stimulate food intake in satiated rats during a short feeding period (1-3 h), while a low dose of anandamide (1 μg/kg) was found to increase food intake in diet restricted mice over a 1 week period (Williams and Kirkham, 1999; Hao et al., 2000).

Central administration of anandamide (2.5 and 5 ng/g) was not found to significantly alter food intake in comparison to the saline treated fish (Fig. 3B). This contrasts with previous observations made by Jamshidi and Taylor (2001) who reported that administration of anandamide into the ventromedial hypothalamus (VMH) of satiated rats significantly increased food intake 3 h post-injection. Although, this finding would seem to suggest that anandamide does not have a central effect on food intake in goldfish, other factors may be at play. For example, the narrow dose range examined and various experimental differences may potentially explain the lack of effect of anandamide on food intake observed in the present study. Goldfish were not satiated prior to central administration of anandamide and were given only a 1 h feeding period, consequently this experimental design may lack the resolution required to see an effect on food intake. Another factor that should be taken into consideration is the biological stability of anandamide. Due to the use of low doses of anandamide, the chemical itself may have quickly degraded, since anandamide has been shown to rapidly metabolized in mice, a phenomenon presumably expedited by fatty acid amide hydrolyze (FAAH) (Willoughby

et al., 1997). Goldfish have recently been shown to exhibit FAAH-like activity (Valenti et al., 2005). All of the above mentioned factors may have contributed to the lack of effect observed in the present study. Conversely, food intake in saline treated fish exhibited significantly reduced food intake levels in comparison to the untreated controls (Fig. 3B), suggesting that the ICV procedure itself/or potentially the saline vehicle used may be influencing food intake.

4.3 Effect of Methanandamide administration on food intake

In the present study, the varying doses of methanandamide administered intraperitoneally did not alter food intake over a 1 h period post-injection (Fig. 4A), suggesting that methanandamide does not elicit an acute effect on food intake in goldfish. This observation is consistent with a previous study that reported methanandamide (1 mg/kg) to only significantly increase cumulative food intake 3 h following IP injection in rats and had no acute effect (Chambers et al., 2004). However, 3 h following administration of methanandamide, food intake levels were dose-dependently reduced with increasing dose of methanandamide (Fig. 4B). Goldfish treated with a high dose of methanandamide (3 μg/g) were found to have significantly reduced food intake levels in comparison to the untreated and saline treated controls and fish treated with the lowest dose of methanandamide examined (0.01 μg/g) (Fig. 4B). This result is contrary to our hypothesis that CB1 agonists would stimulate food intake in goldfish. However, ECS agonist (anandamide) was recently shown to have a variable effect on food intake in goldfish that was dependent on dose (Valenti et al., 2005). This finding would seem to

be applicable to methanandamide, given that it is an analogue of this endocannabinoid. Furthermore, the negative effect of methanandamide on food intake in goldfish observed in the present study, suggests that the doses investigated here, which are comparable to the mammalian literature, may be too high to elicit a stimulatory effect on food intake in goldfish. A potential explanation for this finding is that ECS agonists are known to alter motor behaviour. Romero and colleagues (1996) have shown methanandamide (2.5-10 mg/kg) to inhibit motor behaviour, decrease frequency of stereotypic behaviours and increase periods of inactivity in rats, thus potentially affecting food intake. In fact, cannabinoid agonists have been shown to reduce food intake in the zebra finch (*Taeniopygia guttata*) and in rats (Soderstrom and Johnson, 2001; Giuliani et al., 2000), potentially due to the administration of doses that may have affected motor control and/or be a result of experimental design (e.g. use of fasted or satiated animals). However, as food intake was the only endpoint examined in the present study it remains unclear if food intake in methanandamide treated fish was reduced due to appetite suppression or due to other behavioural effects of endocannabinoids. On the other hand, it should be noted that no obvious behavioural discontinuity was observed in the present study. Interestingly, food intake levels were significantly reduced in fish treated with the highest test doses of methanandamide examined (1 and 3 μg/g) in comparison to fish that received the lowest test dose (0.01 μg/g) at 3 h up to 24 h following IP administration (Fig. 4B-D), suggesting that a lower dose range may have a stimulatory effect on food intake. It should be noted that food intake measurements at 3 and 6 h post-injection was determined in the same fish and therefore, fish were partially satiated, consequently

mirroring mammalian studies. Despite a downward trend, food intake levels of methanandamide treated fish did not significantly differ from the untreated controls at 6 h post-injection. Nevertheless, food intake levels were marginally elevated in fish treated with 0.01 μg/g methanandamide and differed significantly from fish treated with 1 or 3 μg/g (Fig. 4C). This finding would seem to indicate that a lower dose range of methanandamide may potentially have a stimulatory effect on food intake. Given that methanandamide is a synthetic analogue of anandamide that displays greater metabolic stability against FAAH degradation and higher affinity for the CB1 receptor adds further support to the argument that the dose range investigated in the present study may have been too great to elicit a stimulatory effect on food intake in goldfish. At 24 h following IP administration, a general dose-dependent reduction in food intake in methanandamide treated fish was observed (Fig. 4D), however food intake levels methanandamide treated fish remained were not found to significantly differ in comparison to saline treated controls.

Central administration of varying doses of methanandamide did not significantly alter food intake levels in comparison to the saline treated controls (Fig. 5), suggesting that methanandamide does not elicit an acute centrally mediated effect on food intake. Again, various factors may have contributed to the lack of effect observed in the present study, including the use of unsatiated fish and a short feeding period. To the best of my knowledge, there are no previous reports examining the effect of central administration of methanandamide on food intake.

4.4 Effect of AM 251 administration on food intake

Systemic or central administration of varying doses of AM 251 (0.1-5 μg/g IP; 0.1-5 ng/g ICV) were generally not observed to significantly alter food intake in goldfish (Fig. 6 A-D, 7A-C). These findings indicate that the doses employed in the present study, although similar to those demonstrated to have a sustained anorexigenic effect in rodents (Chambers et al., 2004; Hildebrandt et al., 2003; Chambers et al., 2005) were not capable of reducing food intake in goldfish. At 24 h following IP administration, food intake was significantly reduced in fish injected with the low dose of AM 251 (0.1 μg/g) in comparison to the highest dose examined (5 μg/g) (Fig. 6D). Although, food intake levels in AM 251 treated fish did not significantly differ from the controls, the significant alteration in food intake among AM 251 treated fish suggests that the doses examined in the present study may have been too high and the lack of biological effect may be potentially attributed to receptor desensitization. Cannabinoid receptors has been shown to quickly desensitize both *in vivo* and *in vitro* (reviewed by Pertwee, 1997), since CB1 receptors are internalized in response to high concentration of agonist (Hsieh et al., 1999). However, AM 251 is an analogue of the CB1 selective antagonist/inverse agonist SR 141716, which has been shown to block receptor internalization *in vitro* (Hsieh et al., 1999). Although, it should be noted that tolerance to the anorectic effects of CB1 antagonism by SR141716 and AM 251 has been shown to rapidly develop within days of treatment in rodents (Colombo et al., 1998; Chambers et al., 2004), implying that receptor desensitization may also occur in response to CB1 receptor antagonist/inverse agonist

treatment. Furthermore, AM 251 displays greater affinity for the CB1 receptor in comparison to SR141716, and consequently more likely to cause receptor desensitization.

An alternative explanation for these findings is the possibility that AM 251 does not act as a competitive antagonist at the goldfish CB1 receptor, yet Valenti et al. (2005) reported AM 251 (1 μg/g) to have negative effect on food intake in goldfish, hence, the lack of effect of AM 251 on food intake in the present study remains unclear. Nevertheless, the significant reduction in food intake in fish treated with the 0.1 μg/g AM 251 and the dose-dependent increase in food intake among AM 251 treatment groups at 24 h following IP administration would seem to indicate that a lower dose range may have an inhibitory effect on food intake (Fig. 6D). Moreover, as AM 251 is an inverse agonist of the CB1 receptor it therefore has the potential to elicit effects that are contrary to physiological norm of the ECS; and given that ECS agonists have a dose-dependent variable effect on food intake this may explain dose-dependent stimulation of food intake among AM 251 treated fish.

Central administration of varying doses of AM 251 did not significantly alter food intake compared with the controls and no differences were found among AM 251 treatment groups at 3 or at 6 h following ICV administration (Fig. 7B, C). In comparison to systemic administration, the latter observation is not surprising given that the differences between groups were not apparent until 24 h following IP administration. To the best of my knowledge, mammalian studies that have investigated the effect of AM 251 on food intake have employed systemic administration (i.e. IP, oral), therefore, the central effect of AM 251 on food intake remains unexamined. There is little information

available regarding the central effects of selective CB1 antagonists on food intake, most likely due to this route of administration being therapeutically irrelevant. However, Gomez et al. (2002) demonstrated that central administration of SR 141716 (0.1-10 μg/g) had no effect food intake in food-deprived rats. In contrast, intracerebroventricular injection of CB1 antagonist/inverse agonist AM 281 (40 μg/g) reduced food intake up to 6 h post-injection (Werner and Koch et al., 2003), indicating that the doses of SR 141716 investigated by Gomez et al. (2002) may have been too low to antagonize CB1 receptors. The lack of a central effect of AM 251 on food intake in the present study may potentially be due to the doses examined, because the ICV procedure appeared to have a negative effect on food intake and lastly because the high variability among groups may have masked AM 251 effect. On the other hand, as AM 251 was ineffective in our hands, since neither peripheral nor central administration of AM 251 was found to significantly alter food intake from the controls, raises concerns regarding the integrity of this chemical.

4.5 Effect of O-2050 administration on food intake

In the present study, O-2050 was found to be the most potent anoreixgenic agent, significantly reducing food intake between 24 and 48 h following intraperitoneal administration of a 5 μg/g dose in comparison to the saline treated fish (Fig. 8D, E). These findings support our hypothesis that the ECS has a role in the control of food intake in goldfish and suggests that O-2050 may be a suitable antagonist of the ECS in goldfish. Furthermore, this observation is consistent with the demonstrated effect of O-

2050 significantly reducing food consumption in rats (Gardner and Mallet, 2006). However, Gardner and Mallet (2006), also reported that this 'silent antagonist' had a sedative side effect, inducing hypoactivity in rats treated with 0.3 and 3 mg/kg of O-2050, up to 4 h following intraperitoneal administration. Equally important, the authors reported that the time course of O-2050 effect on locomotor activity differed from O-2050 effect on food intake, suggesting that the reduction in food intake was not merely due to locomotor suppression (Gardner and Mallet, 2006). However, it should be noted that Gardner and Mallet (2006) only examined gross locomotor activity even though there are indications that impairments in gross locomotor activity do not necessarily affect motor control required for food handling and feeding in rats (MacLaughlin et al., 2005). These observations lend further support to the reduction in food intake induced by O-2050 may be distinct from its motor suppressive effects. Even though no obvious behavoiural discontinuity was observed in the present study, future investigations should include direct observation of feeding behaviour since it remains unclear if the reduction in food intake was due to O-2050 suppressing appetite or due to locomotor suppression.

Central administration of the varying doses of O-2050 did not significantly alter food intake levels in comparison to the saline treated fish until 6 h following administration (Fig. 9A, B). However, food intake levels in fish treated with 5 and 1 μg/g O-2050 were found to significantly differ from the untreated controls at 1 and 3 h following ICV administration respectively, which may potentially be attributable to the ICV administration procedure having a negative influence on food intake. Although, it should be noted that food intake levels in saline vehicle controls did not significantly

differ from the untreated fish for the various time points food intake was examined (Fig. 9A-C). Furthermore, at 6 h following ICV administration, food intake levels among O-2050 treated fish were significantly reduced in comparison to the untreated and saline treated controls (Fig. 9C). The lack of a dose-dependent effect of O-2050 on food intake would seem to suggest that the dose range examined was high, since the increasing doses of O-2050 (i.e. 1 and 5 ng/g) had no further effect on food intake. Furthermore, as food intake was reduced by lower doses via ICV (ng/g) versus IP administration (μg/g), implies that the anorexigenic effect of O-2050 is centrally mediated. Moreover, O-2050 is a putative CB1 'silent antagonist' devoid of inverse agonist properties (reviewed by Pertwee, 2005), this implies that O-2050 effect on food intake is due to antagonism of the orexigenic tone and not due to inverse agonist activity at CB1 receptors (Gardner and Mallet, 2006).

4.6 Food deprivation elevates hypothalamic CB1 protein expression

Hypothalamic CB1 protein expression was significantly elevated following four weeks of food deprivation (Fig. 10), suggesting that longer periods of negative energy balance can modulate CB1 protein expression levels in a brain region involved in the control of food intake. This finding further supports an orexigenic role for CB1 in goldfish, as up-regulation of CB1 protein expression in the hypothalamus would seem to suggest that negative energy status induced sensitization of the ECS. It has been proposed that the ECS is provoked by stimuli that challenges energy homeostasis (reviewed by Kirkham and Williams, 2001), thus our observation of starvation induced

CB1 up-regulation may be interpreted as the ECS acting to increase the 'drive' to eat, as CB1 receptors have been shown to have relatively high constitutive activity (Bouaboula et al., 1997). Furthermore, our finding is consistent with one previous report that demonstrated that fasting up-regulated CB1 receptors in a subset of the rat vagal afferent neurons, an observation which was reversed by re-feeding (Burdyga et al., 2004). Additionally, CB1 receptors have been shown to be down-regulated in various extrahypothalmaic regions of rats with dietary induced obesity (Harrold et al., 2002), which further implicates energy status in the modulation of CB1 receptor expression. Although, hypothalamic CB1 protein expression was elevated following two weeks of food deprivation, this observation was not found to differ significantly from the corresponding fed controls, which may potentially be attributed to the high variation in this experimental group.

4.7 Cortisol administration modulates hypothalamic CB1 protein expression

In the time course experiment, cortisol administration was not found to significantly alter hypothalamic CB1 expression (Fig. 11). However, a high degree of variation was observed among treatment groups. Our observations of the data collected thus far suggest that a trend of reduced expression at least, appeared to be present in the hypothalamus four and eight days following treatment. It would be interesting to repeat this experiment and examine an increased number of samples. If significant, it would suggest that cortisol has an inhibitory effect on the ECS when cortisol elevation is prolonged. Furthermore, in the dose-response experiment, hypothalamic CB1 protein

expression in fish implanted with low doses of cortisol (50 and 100 μg/g) was significantly reduced in comparison to sham treated fish (Fig. 12), indicating that low doses of cortisol can lower CB1 protein expression levels in the hypothalamus. The observation implies that the cortisol dose-response findings are in agreement with the time course study that demonstrated CB1 protein expression appeared to be reduced following four days of treatment.

The ECS has been implicated to mediate the inhibition of glucocorticoid release and the observation that CB1 antagonist SR141716 treatment significantly elevates serum corticosterone concentration in rats (Di et al., 2003, Patel et al., 2004), along, with the recent demonstration that anandamide reduced plasma cortisol levels of goldfish (Valenti et al., 2005) implicates the endocannabinoids to have an inhibitory role in the activation of the stress axis in mammals, as well as in fish. Consequently, an increase in CB1 protein expression in the hypothalamus in response to elevated serum cortisol levels would seem to suggest CB1 receptor sensitization, potentially to mediate recovery from perceived stress. However, a reduction in CB1 protein expression in the hypothalamus in fish implanted with cortisol for a longer period of time would seem to indicate that the activity of the ECS is reduced when stress is prolonged. The effect of elevated circulating cortisol levels on CB1 protein expression in the hypothalamus has not been directly examined, however, hypothalamic 2-arachidonlyglycerol levels have been shown to be differentially modulated in response to restraint-induced stress in rats; they are reduced by acute stressors, while prolonged stress elevates hypothalamic 2-arachidonlyglycerol levels (Patel et al., 2005). Taken together, these observations

suggest the possibility that hypothalamic CB1 receptor expression and endocannabinoid levels may be inversely modulated by stress.

5. CONCLUSION & FUTURE STUDIES

The present study examined CB1 protein expression in the CNS and peripheral tissues of the goldfish and investigated a potential role for CB1 in the regulation of food intake. CB1 was demonstrated to be widely expressed in the goldfish CNS and in the various peripheral tissues examined, indicating that the ECS has a wide spectrum of activity in goldfish. The stimulation and inhibition of food intake in fish treated with anandamide and O-2050 respectively, is consistent with the proposed orexigenic role for the ECS in the regulation of appetite, and may be mediated, at least in part, by CB1. Co-injections studies to determine if CB1 antagonists can block the endocannabinoid-induced stimulation of food intake are required to confirm CB1 involvement. The negative effect of methanandamide on food intake observed in the present study is inconsistent with the proposed orexigenic role for the ECS; however, due to the dose-dependent variable effects of CB1 agonists, an investigation of a lower dose range is warranted. The lack of effect of AM 251 on food intake in the present study remains unclear, prompting further examination of this CB1 antagonist. Furthermore, elevation of hypothalamic CB1 protein expression levels in response to negative energy status (i.e. food deprivation) suggests that nutritional status modulates the activity of the ECS in the hypothalamus and further supports an orexigenic role for CB1 in goldfish. Examination of hypothalamic CB1 protein expression in relation to a single feeding event (i.e. periprandial) may provide further evidence that implicates CB1 in the regulation of appetite. Finally, CB1 was also demonstrated to be regulated by cortisol administration, implying that CB1 protein expression in the hypothalamus may be differentially altered

by short-term and prolonged stress. However, future studies should confirm if cortisol directly modulates hypothalamic CB1 protein expression by examining if the effect is reversed by co-injection of the glucocorticoid antagonist RU 486.

This work has contributed to research examining a role for the ECS in the regulation of food intake and appetite in non-mammalian vertebrates. Cloning of the CB1 receptor in goldfish will allow us to investigate a role for CB1 in the control of food intake, appetite and energy balance at the transcriptional level. Furthermore, examination of endocannabinoid levels and FAAH activity in an energy balance context has not been fully explored in fish. The above mentioned future studies will provide additional insights into the physiological role of the endocannabinoid system in the regulation of appetite and food intake in goldfish and further our knowledge of the neuroendocrine control of energy homeostasis in teleosts.

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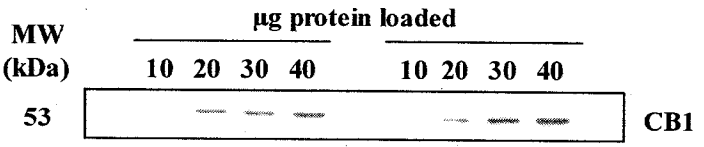
7. FIGURES

A



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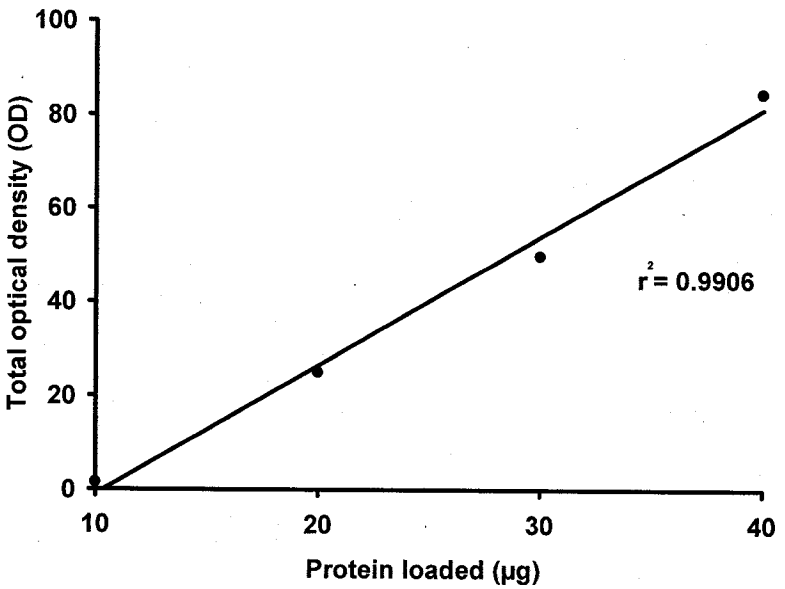

Figure 1. Protein standard curve of CB1 immunoreactivity in goldfish hypothalamus. A: representative immunoblots of protein gradients from the goldfish hypothalamus ranging from 10-40 µg of protein. B: standard curve of band densitometry plotted against protein loaded as determined by Bradford assay. The amount of protein used for quantifying hypothalamic CB1 protein expression was determined by constructing standard curves to establish band density (53 kDa immunoreactive band) as a function of total protein loaded. A total of 25 µg protein from the hypothalamus was used, since this amount falls in the mid range of the standard curve giving optimal resolution for experimentally induced changes in protein expression.

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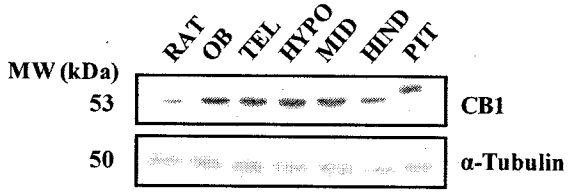

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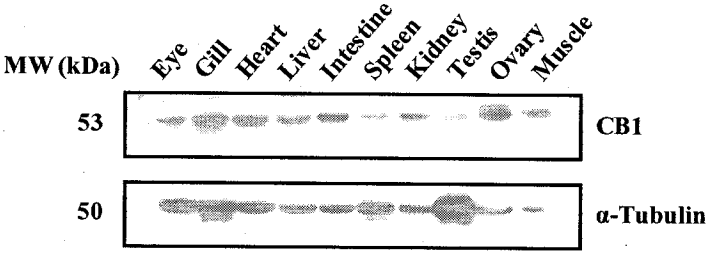

Figure 2. CB1 protein distribution in (A) discrete regions of the goldfish brain and (B) peripheral tissues as determined by Western blot analysis. A major band of approximately 53 kDa was detected in brain regions and peripheral tissues using a polyclonal antibody against the N-terminus of the rat CB1 receptor. Rat CNS was used as a positive control and α-tubulin was used as an internal loading control. Goldfish CB1 corresponded in size to rat CB1 in all discrete brain regions and tissues examined except the pituitary where an immunoreactive band of approximately 56 kDa was detected. Discrete brain regions are as follows: Olfactory bulbs (OB), telencephalon (TEL), hypothalamus (HYPO), midbrain (MID), hindbrain (HB), and pituitary (PIT).

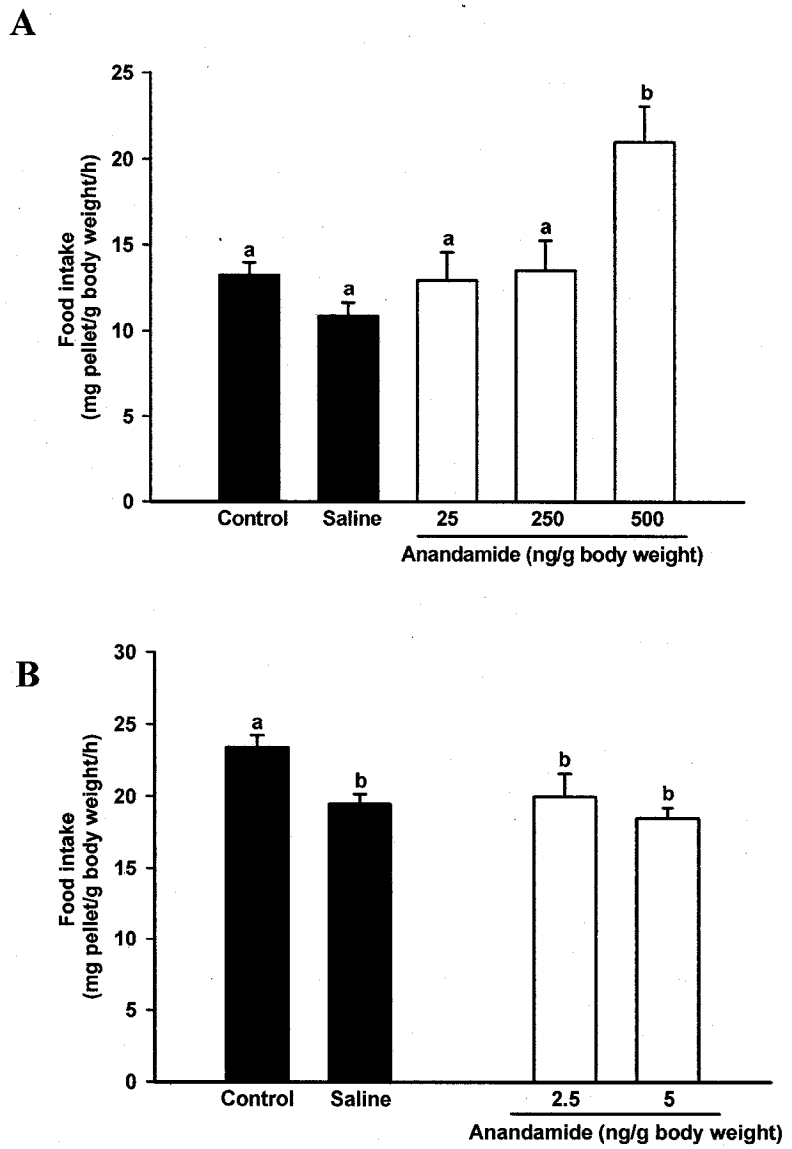

Figure 3. Food intake (mg pellet/g body weight/h) in goldfish after (A) intraperitoneal and (B) intracerebroventricular administration of anandamide. Food intake was examined over a 1 h period immediately following administration of anandamide. Values are expressed as the means ± SEM (n= 5-8). Means were compared using a one-way ANOVA; different letters denote statistical significance ($P< 0.05$). Control fish were uninjected, and saline fish were injected with saline vehicle.

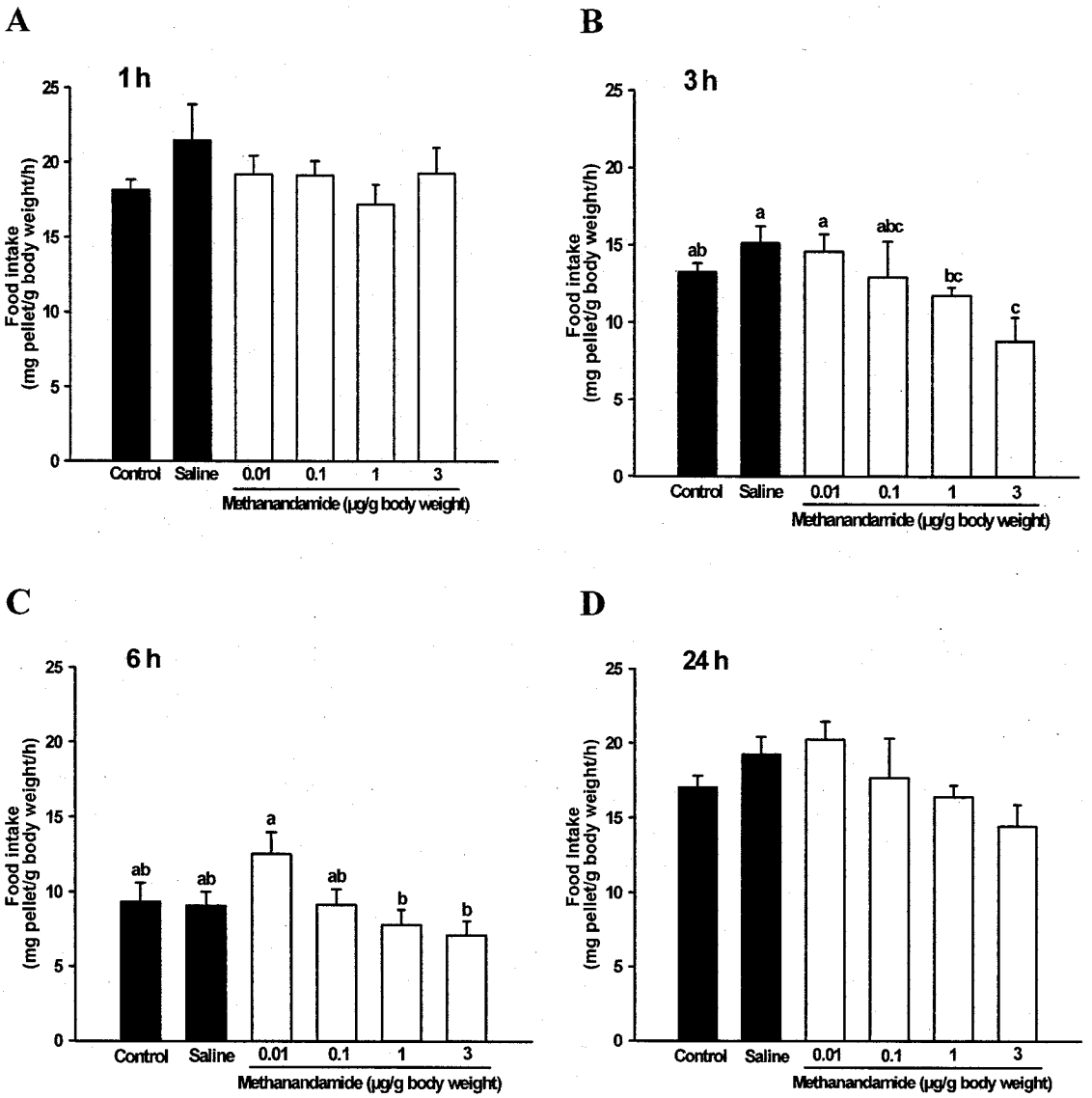

Figure 4. Food intake (mg pellet/g body weight/h) in goldfish (A) 1 h, (B) 3 h, (C) 6 h and (D) 24 h post-injection following intraperitoneal administration of varying doses of methanandamide. At the first time point that food intake was determined (i.e. 1 h) fish had been unfed for 24 h. Food intake at 3 and 6 h was determined in the same fish (i.e. previously fed). Values are expressed as the means ± SEM (n= 5-6). Means were compared using a one-way ANOVA; different letters denote significance ($P< 0.05$). Control fish were uninjected, and saline fish were injected with saline vehicle.

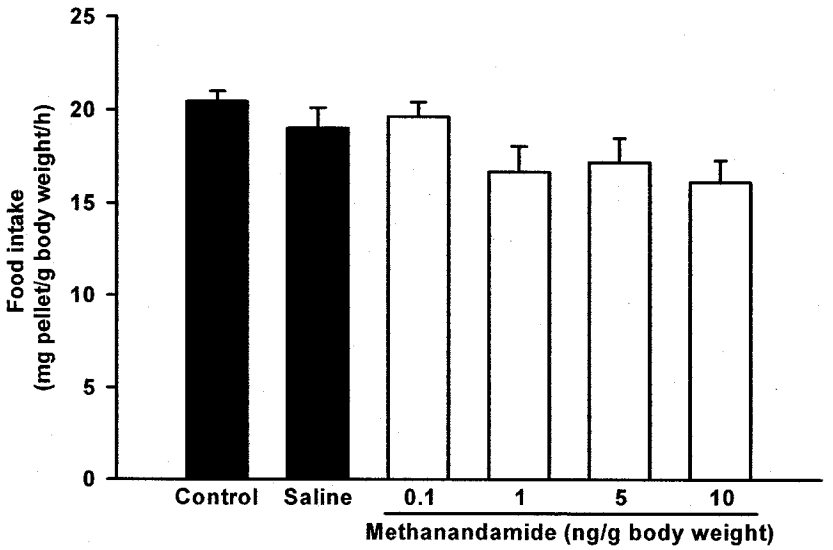

Figure 5. Food intake (mg pellet/g body weight/h) in goldfish following intracerebroventricular administration of varying doses of methanandamide. Food intake was examined over a 1 h period immediately following administration of methanandamide. Values are expressed as the means ± SEM (n= 6-8). Means were compared using a one-way ANOVA. Control fish were uninjected, and saline fish were treated with saline vehicle.

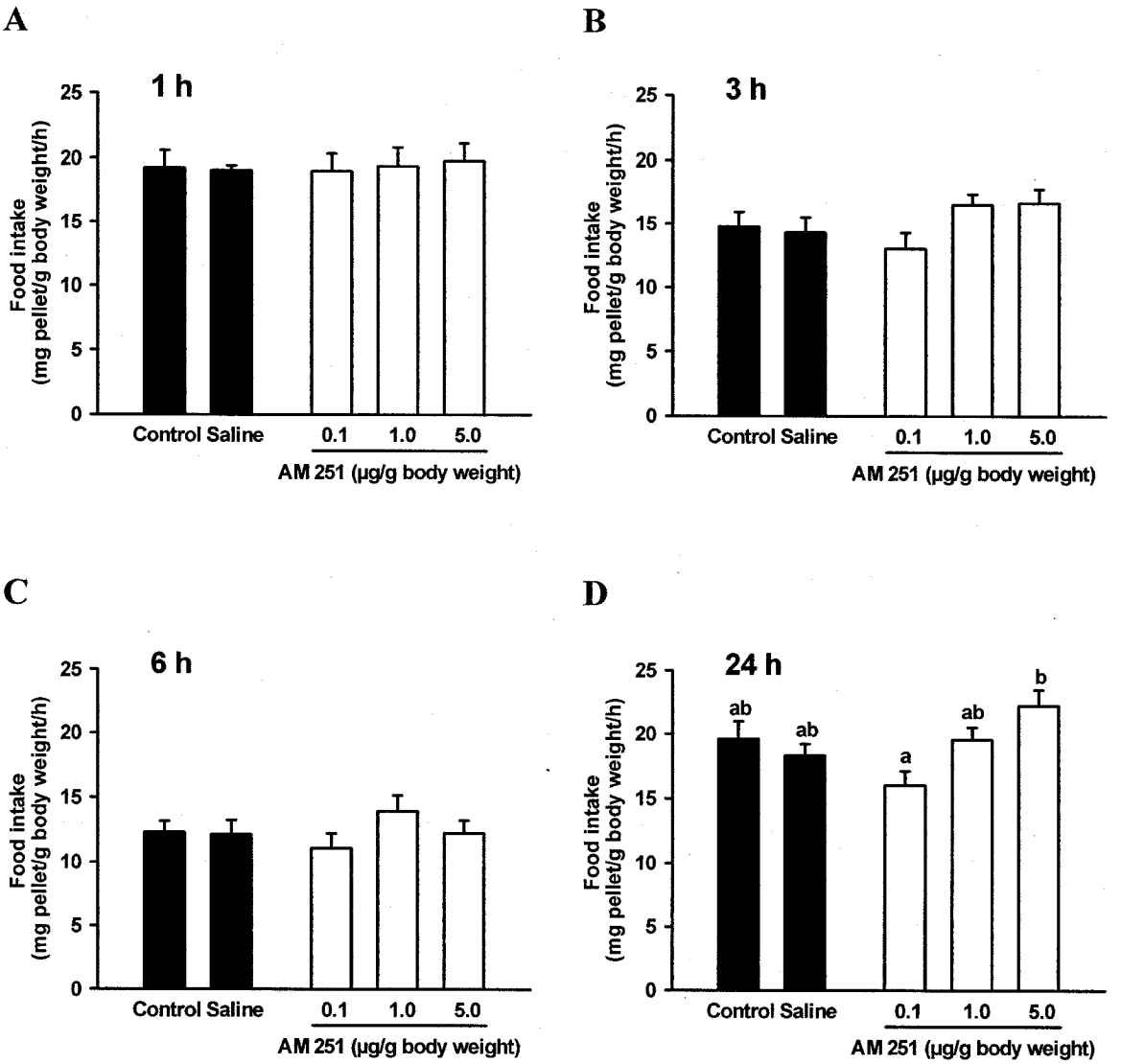

Figure 6. Food intake (mg pellet/g body weight/h) in goldfish (A) 1 h, (B) 3 h, (C) 6 h and (D) 24 h following intraperitoneal administration of varying doses of AM 251. At the first time point that food intake was determined (i.e. 1 h) fish had been unfed for 24 h. Food intake at 3 and 6 h was determined in the same fish (i.e. previously fed). Values are expressed as the means ± SEM (n= 8-11). Means were compared using a one-way ANOVA; different letters denote significance ($P< 0.05$). Control fish were uninjected, and saline fish were injected with saline vehicle.

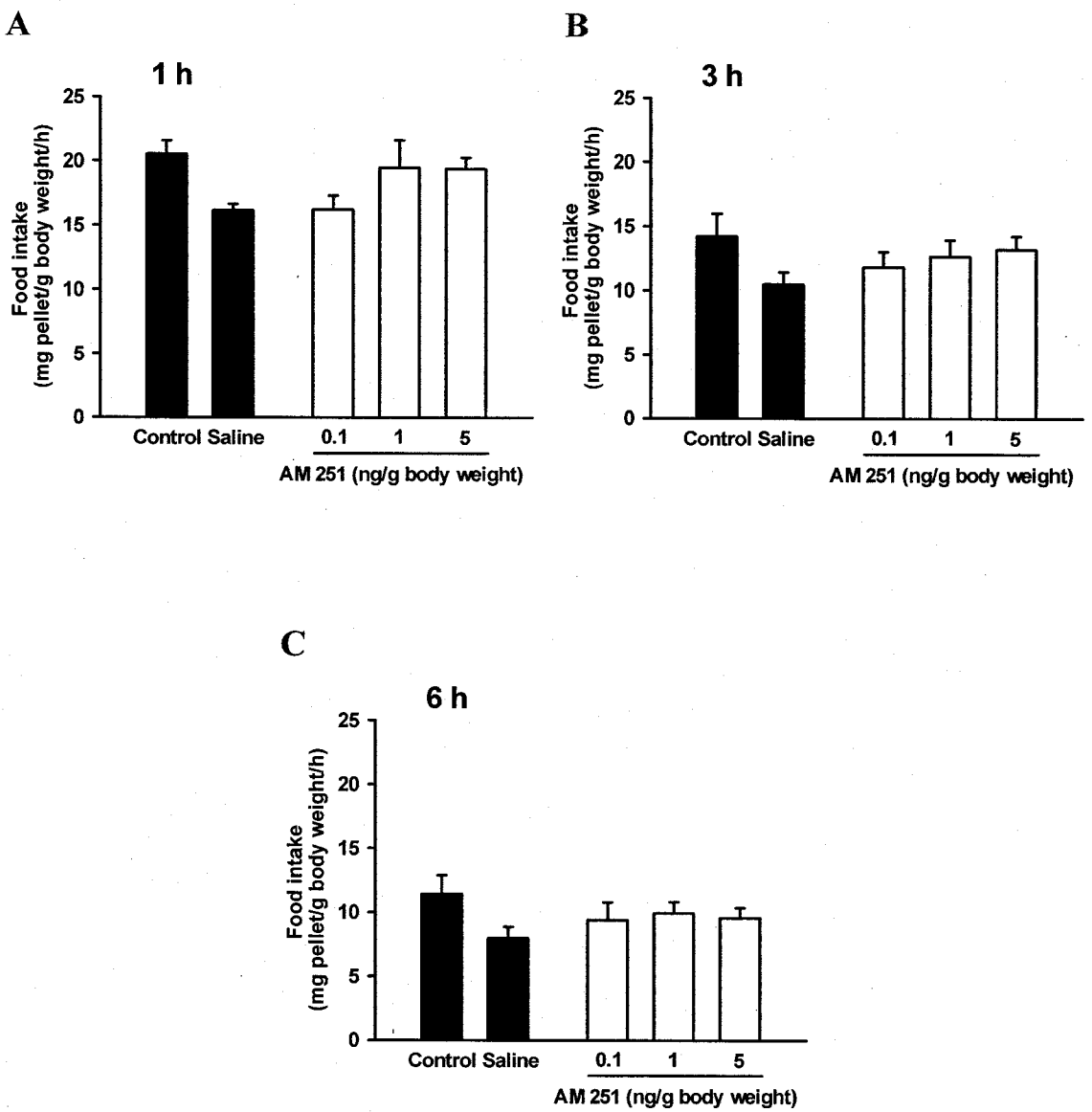

Figure 7. Food intake (mg pellet/g body weight/h) in goldfish (A) 1 h, (B) 3 h, (C) 6 h and (D) 24 h following intracerebroventricular administration of varying doses of AM251. At the first time point that food intake was determined (i.e. 1 h) fish had been unfed for 24 h. Food intake at 3 and 6 h was determined in the same fish (i.e. previously fed). Values are expressed as the means ± SEM (n= 6-7). Means were compared using a one-way ANOVA. Control fish were uninjected, and saline fish were injected with saline vehicle.

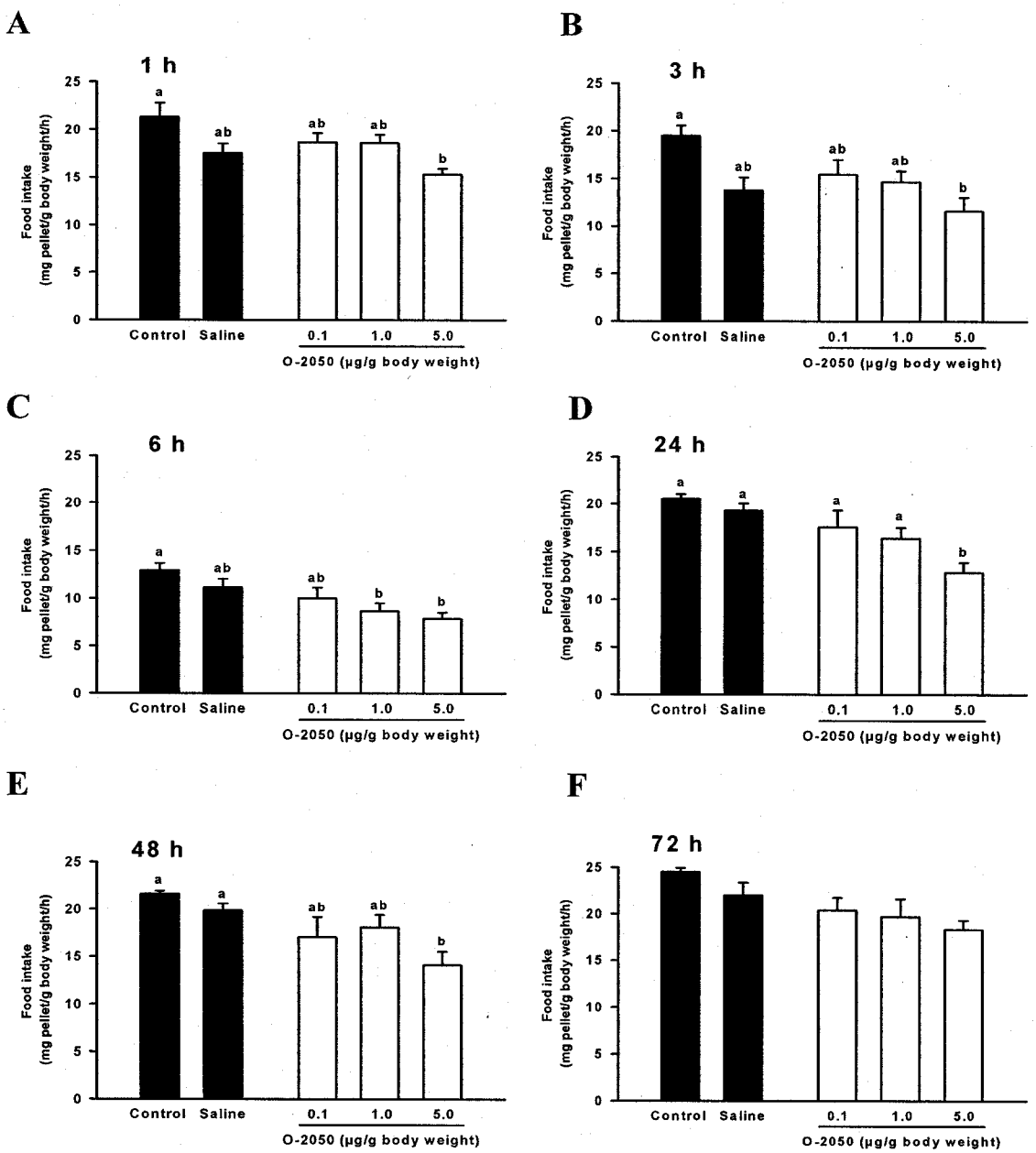

Figure 8. Effect of varying doses of O-2050 on food intake (mg food/g body weight /h) in goldfish (A) 1 h, (B) 3 h, (C) 6 h, (D) 24 h, (E) 48 h, (F) 72 h following intracerebroventricular administration. At the first time point that food intake was determined (i.e. 1 h) fish had been unfed for 24 h. Food intake at 3 and 6 h was determined in the same fish (i.e. previously fed). Food intake was then monitored once every 24 h up to 72 h after the injection. Values are expressed as the means ± SEM (n= 5-8). Means were compared using a one-way ANOVA; different letters denote significance ($P< 0.05$).

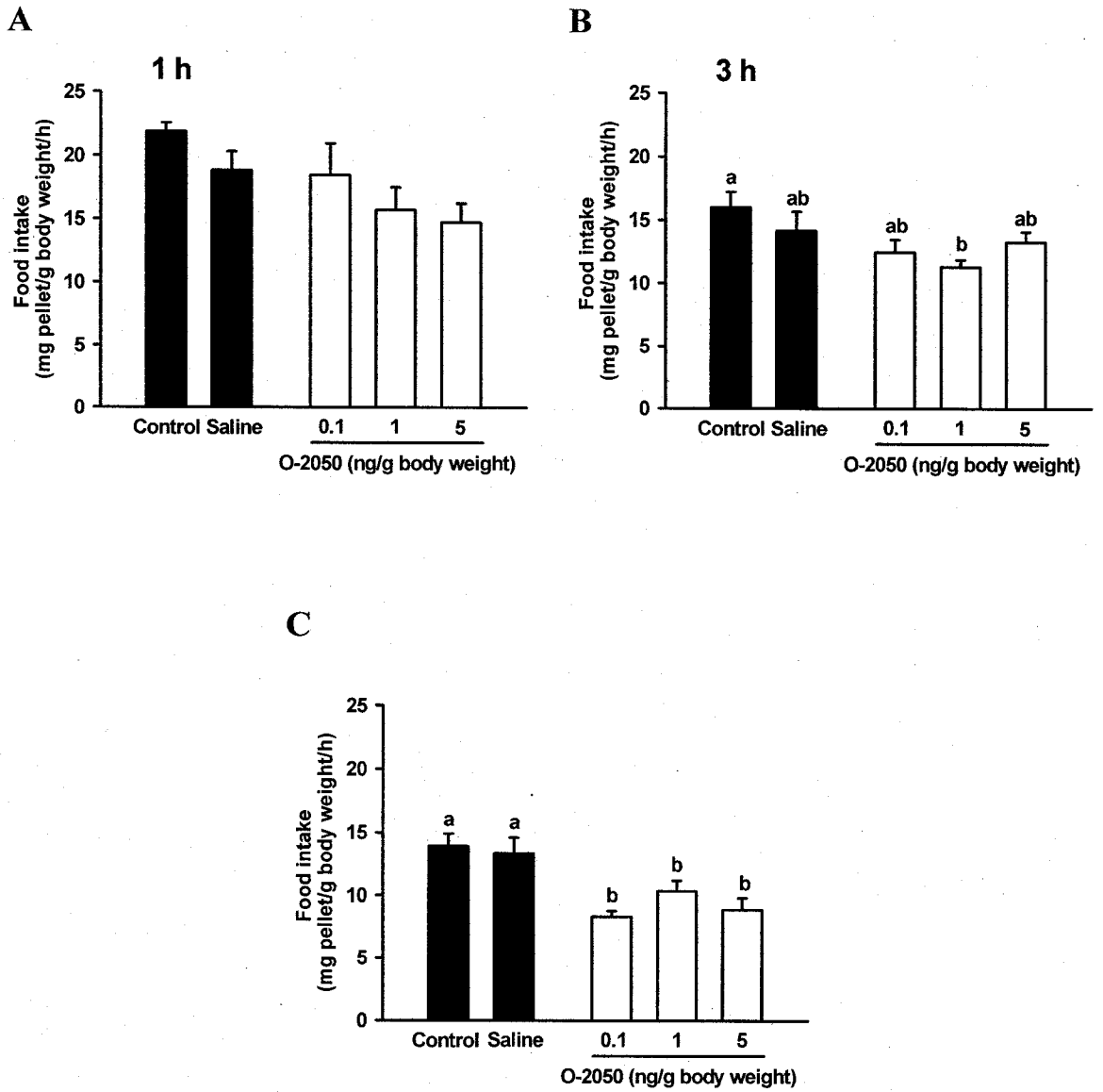

Figure 9. Effect of varying doses of O-2050 on food intake (mg pellet/g body weight/h) in goldfish (A) 1 h, (B) 3 h and (C) 6 h following intracerebroventricular administration. At the first time point that food intake was determined (i.e. 1 h) fish had been unfed for 24 h. Food intake at 3 and 6 h was determined in the same fish (i.e. previously fed). Values are expressed as the means ± SEM (n= 6-8). Means were compared using a one-way ANOVA; different letters denote significance ($P< 0.05$). Control fish were uninjected, and saline fish were injected with saline vehicle.

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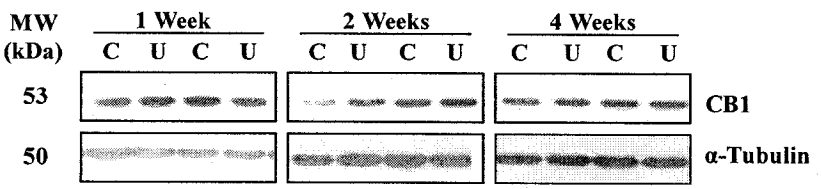

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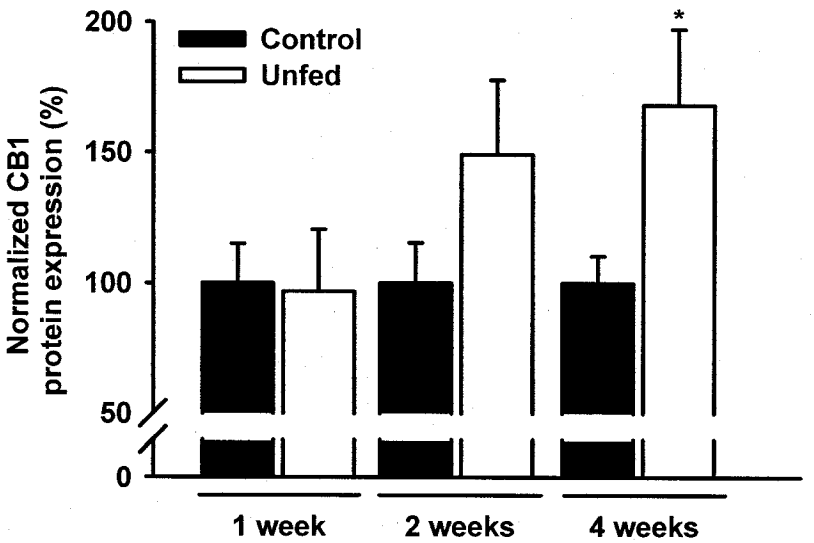

Figure 10. Hypothalamic CB1 protein expression in response to food deprivation. Fish were food deprived for periods of 1, 2 or 4 weeks. Control fish were fed once daily on a regular scheduled regimen. A: representative immunoblot of hypothalamic CB1 protein expression in control (C) and unfed (U) fish. B: quantitative densitometric analysis of CB1 immunoreactive bands. Values are expressed as the means ± SEM (n=6-8). Hypothalamic CB1 protein levels were significantly elevated in unfed fish following 4 weeks of food deprivation. Means were compared using an unpaired *t*-test; significant differences between control (fed) and corresponding unfed fish is denoted by an asterisk ($P<0.05$).

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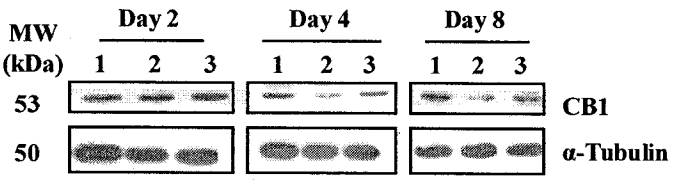

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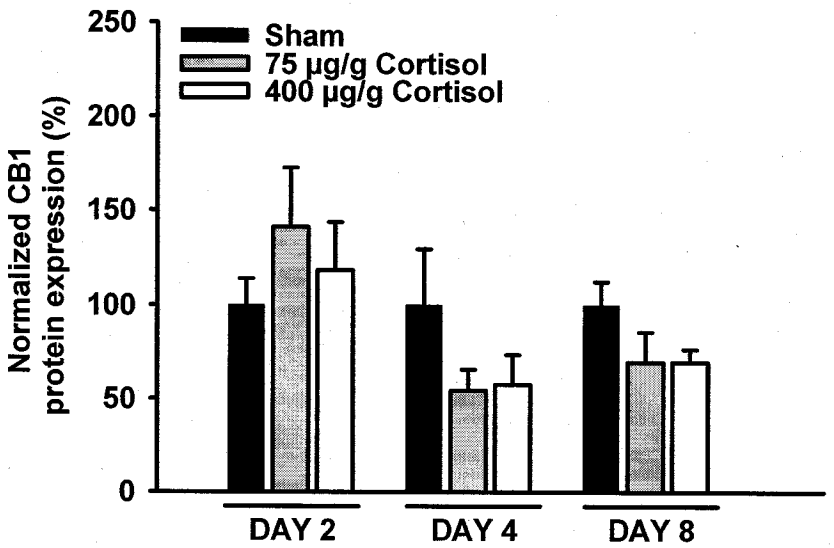

Figure 11. Hypothalamic CB1 protein expression in response to cortisol administration. Fish were either given an intraperitoneal coconut oil implant with dissolved cortisol (75 or 400 µg/g body weight) or vehicle (coconut oil alone) and sampled at 2, 4, and 8 days following implantation. A: representative immunoblot of hypothalamic CB1 protein expression in sham (1) and fish treated with 75 µg/g (2) or 400 µg/g (3) cortisol for each sampled time point. B: quantitative analysis of CB1 immunoreactive band by densitometry. Values are expressed as the means ± SEM (n=5-8).

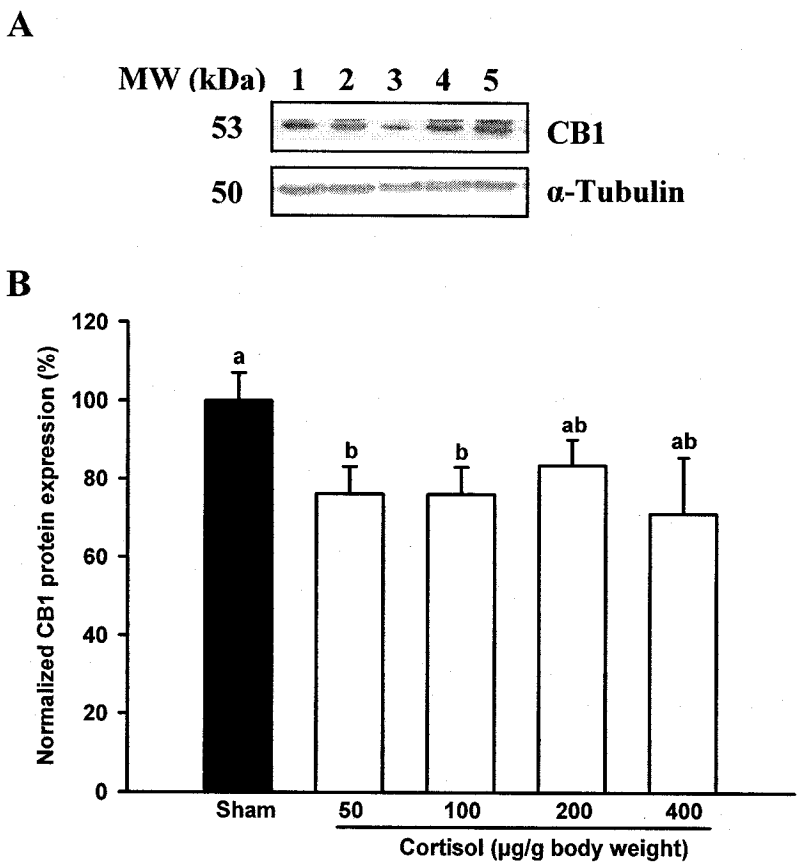

Figure 12. Effects of varying doses of cortisol on hypothalamic CB1 protein expression. Fish received a coconut oil implant containing varying doses of cortisol and were sampled 4 days following implantation. A: representative immunoblot of hypothalamic CB1 expression in sham (1) and fish treated with 50 μg/g BW (2), 100 μg/g BW (3), 200 μg/g BW (4) or 400 μg/g BW (5) cortisol. B: quantitative analysis of CB1 immunoreactive band by densitometry, values are expressed as the means ± SEM (n=6-8). Cortisol treatment of 50 or 100 μg/g BW significantly reduced hypothalamic CB1 protein expression levels. Means were compared using a one-way ANOVA; different letters denote significance ($P<0.05$).