

Investigating the Contribution of Agouti-Related Peptide (AGRP) on  
Ghrelin's Ability to Promote Social Interaction in Mice

by

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## **Abstract**

Ghrelin is a stomach-derived peptide that increases food intake by acting as an endogenous ligand for growth hormone secretagogue receptor (GHSR). GHSR is abundantly expressed in the brain with high expression found in agouti-related peptide (AGRP) and neuropeptide-Y (NPY) neurons within the hypothalamic arcuate (ARC) nucleus. Recent data suggests that, in addition to increasing food intake, ghrelin promotes social interaction whereas ghrelin receptor antagonists increase social vigilance. In this present thesis, we aimed to investigate the effects of ghrelin on promoting social interaction in mice and the role of AGRP in mediating these effects. Sixty-three male and female C57BL/6J mice were assigned to groups receiving intraperitoneal (IP) injections of saline or ghrelin (1 mg/kg of ghrelin in .1cc of isotonic saline) followed by injections of saline or the MC3/4 receptor agonist melanotan-II (100ug of MTII in .1cc of isotonic saline) which competes with AGRP, an endogenous antagonist to the MC3/4 receptors. Thirty minutes later, mice were tested on a social interaction test to investigate how these drug treatments affected social behaviors. Results showed that, regardless of sex, ghrelin injected peripherally promotes the motivation for social interaction as reflected by a significant decrease in the latency to approach a novel-conspecific. In contrast, MTII injected peripherally enhanced social vigilance as reflected by a significant increase in the frequency of corner observations. No interaction effects between the two drugs were found, suggesting that ghrelin promotes the motivation for social interaction, and these effects are independent from activation of AGRP neurons.

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## List of Abbreviations

**ACTH**, adrenocorticotrophic hormone;  **$\alpha$ -MSH**, alpha-melanocyte-stimulating hormone; **AGRP**, agouti-related peptide; **ANOVA**, analysis of variance; **ARC**, arcuate nucleus; **AP**, area postrema; **BAT**, brown adipose tissue; **BNST**, bed nucleus of stria terminalis; **CART**, cocaine amphetamine regulated transcript; **CeA**, central nucleus of the amygdala; **CNS**, central nervous system; **CPPT**, conditioned place preference test; **CVO**, circumventricular organs; **DMH**, dorsomedial nucleus; **GABA**, gamma-aminobutyric acid 2; **GPCR**, G-protein coupled receptors; **GH**, growth hormone; **GHSR**, growth hormone secretagogue receptor; **GHSR KO**, growth hormone secretagogue receptor knockout; **GOAT**, ghrelin O-acyltransferase; **HFD**, high fat diet; **IP**, intraperitoneal; **LH**, lateral hypothalamus; **LHA**, lateral hypothalamic area; **MeA**, medial nucleus of the amygdala; **ME**, median eminence; **MCH**, melanin-concentrating hormone; **MC1R**, melanocortin-1 receptor; **MC2R**, melanocortin-2 receptor; **MC3R**, melanocortin-3 receptor; **MC4R**, melanocortin-4 receptor; **MC5R**, melanocortin-5 receptor; **mRNA**, messenger ribonucleic acid; **NAc**, nucleus accumbens; **NPY**, neuropeptide Y; **NTS**, nucleus of the tractus solitaries; **PBN**, parabrachial nucleus; **PFA**, paraformaldehyde; **PNS**, peripheral nervous system; **POMC**, pro-opiomelanocortin; **PC-1**, protein convertase-1; **PC-2**, protein convertase-2; **PVN**, paraventricular nucleus; **VMH**, ventromedial nucleus of the hypothalamus; **VTA**, ventral tegmental area; **WAT**, white adipose tissue; **WHO**, world health organization; **WT**, wild-type

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## **Introduction**

### ***Characterizing Social Interaction***

Social interaction is an essential facet of the behavioural repertoire of animals and the reward associated with it can be strongly reinforcing (Trezza, Campolongo, & Vanderschuren, 2011; Vanderschuren, Achterberg, & Trezza, 2016). From an evolutionary perspective, this is critical because social behavior ensures survival and reproductive success across all species, including humans. Positive social interactions are crucial for the well-being, development, and reproductive success for both human and animals. They serve as a buffer against stressors (Smith & Wang, 2014) and are often incorporated into treatment programs against substance use disorders due to their protective effects (Roozen et al., 2004; Schéle, Pfabigan, Simrén, Sailer, & Dickson, 2020). In general, social interaction encompasses different types of behaviours such communication, allo-grooming, aggression, mating behavior, and parental behavior (Gammie, 2010). In laboratory rodents, social interaction can be operationally measured through various behavioural measures that evaluate social approach, social avoidance, and social vigilance.

Social approach can be characterized as the amount of time it takes to approach an unfamiliar animal in a novel environment (Williams et al., 2020). This measure allows laboratory animals to investigate the environment for potential competitors for resources such as food or mates as well as exchanging cues that provide information about the novel environment itself (Bluthé, Gheusi, & Dantzer, 1993; Hsu et al., 2018; Lindzey, Winston, & Manosevitz, 1961). In contrast, social avoidance can be described as the behavioural withdrawal displayed in a novel environmental and social context to avoid engaging in unfavorable and costly energetic contests (Neat, Taylor, & Huntingford, 1998; Wright, Hostinar, & Trainor, 2020). This behaviour has been observed across a wide array of species across the animal kingdom including rodents, birds,

primates as well as humans (R. J. Blanchard, McKittrick, & Blanchard, 2001; Carere, Welink, Drent, Koolhaas, & Groothuis, 2001; Huhman, 2006; Shively, Laber-Laird, & Anton, 1997), Subsequently, social vigilance is broadly defined as orienting towards an unfamiliar animal without approaching it and can acts as key inhibitor of behaviour (Duque-Wilckens et al., 2018). It is a behavioural strategy commonly used in adverse or changing social environments as means to enhance monitoring of social cues while simultaneously avoiding social contexts (Wright et al., 2020). This behaviour is displayed among rats when they are faced with a predator (Blanchard, Griebel, Pobbe, & Blanchard, 2011) as well as Syrian hamsters and female C57Bl6/J mice following social defeat (Gray, Norvelle, Larkin, & Huhman, 2015; Newman et al., 2019).

Social defeat acts as a potent stressor for species that live in groups such as rodents (Jianhua, Wei, Xiaomei, & Shao-Hui, 2017; M. B. Solomon, 2017), primates (Arce, Michopoulos, Shepard, Ha, & Wilson, 2010; Shively et al., 1997) and humans (Björkqvist, 2001; Valmaggia et al., 2015). Studies using behavioural paradigms with social defeat as a stressor demonstrate that social approach and vigilance are negatively correlated, as social defeat can significantly reduce social approach while simultaneously increase social vigilance (Duque-Wilckens et al., 2018; Limin Wang et al., 2018; Williams et al., 2018). Social avoidance and vigilance may serve as a protective response to avoid potentially dangerous confrontations against aggressive individuals; however, prolonged social avoidance and vigilance is associated with an increased risk in neuropsychiatric disorders such as mood, anxiety, and stress-related disorders (Wright et al., 2020).

Many rodent studies that measure different aspects of reward have found that rats will show a conditioned place preference for a variety of rewarding stimuli such as food, sex, drug use, and social interaction (Achterberg et al., 2016; Calcagnetti & Schechter, 1992; Normansell

& Panksepp, 1990; Panksepp, Siviyy, & Normansell, 1984). Specifically, rodents display a strong preference for social interaction activities and will actively work towards learning how to rapidly traverse a T-maze for the opportunity to interact with a conspecific from the same age.

Furthermore, it has been reported that drug-dependent rats display a preference for social interaction over drugs such as cocaine, when given the choice between the two (Venniro et al., 2018; Zernig, Kummer, & Prast, 2013). Rodents that are group-housed display a significant decrease in drug (i.e., cocaine) self-administration, reinstatement, and conditioned place preference (CPP) compared to rodents housed individually (Bardo, Neisewander, & Kelly, 2013; Solinas, Chauvet, Thiriet, El Rawas, & Jaber, 2008; Zlebnik & Carroll, 2015). In addition, the presence of a drug-naïve conspecific can result in a significant decrease in cocaine self-administration, highlighting the reinforcing properties of social interaction among rodents (Smith, 2012; Strickland & Smith, 2014). Also, studies have demonstrated that operant access to social reward can inhibit compulsive self-administration of drugs such as heroin and methamphetamine as well as prevent methamphetamine craving and relapse in drug-dependent rats (Venniro et al., 2018).

In contrast, negative social interactions and social isolation appear to promote drug self-administration and relapse in both humans and animal studies (Bardo et al., 2013; Nader & Banks, 2014; Venniro et al., 2018). In addition, social withdrawal is displayed as a core symptom across a wide range of neuropsychiatric disorders such as autism, early-onset schizophrenia, attention-deficit/hyperactivity disorder (ADHD), mood disorders, anxiety disorders as well as eating disorders such as anorexia nervosa (Alessandri, 1992; Jordan, 2003; Kaye, Bulik, Thornton, Barbarich, & Masters, 2004; Møller & Husby, 2000). Social deficits associated with eating disorders suggest an important role for social context in feeding behaviour. In fact, data

suggests that we eat differently when we are alone compared to eating with others, highlighting a role of social context as it pertains to feeding behaviour (Cavazza, Graziani, & Guidetti, 2011; Higgs & Thomas, 2016). To expand, we tend to eat more when around other people and our dietary choices tend to converge with individuals in our close social connections. In laboratory animals, chronic social defeat has a strong influence on feeding behaviour as it leads to increased caloric intake, weight gain and adiposity (Bartolomucci et al., 2005; Moles et al., 2006; Sanghez et al., 2013). This is because the neurobiology underlying social behaviour is strongly interconnected with autonomic, endocrine and other homeostatic processes that facilitate adaptive functions associated with feeding and reproduction to promote survival (Jennings, Rizzi, Stamatakis, Ung, & Stuber, 2013; Wright et al., 2020). This includes important adaptive functions such as the modulation of feeding behaviour displayed among social hierarchies as well as maladaptive consequences such as social withdrawal displayed among individuals with eating disorders (Behrens, Hunt, Woolrich, & Rushworth, 2008; Jennings et al., 2013; Kelley, Baldo, Pratt, & Will, 2005; Kennedy & Adolphs, 2012; Via et al., 2015).

That being said, the majority of research investigating the neuropeptides underlying social behaviours have primarily focused on oxytocin (OT), arginine vasopressin (AVP), and dopaminergic reward circuits. In non-human mammals, neuropeptides such as OT and AVP are critical mediators of complex social behaviours such as parental care, pair-bonding and attachment, as well as social aggression (Heinrichs, von Dawans, & Domes, 2009). Specifically, data shows that OT can facilitate social approach behaviour in animals by inhibiting their defensive behaviours and enabling them to overcome their natural social avoidance (Carter, 1998; Carter & Altemus, 1997; Donaldson & Young, 2008; Pedersen, 1997). On the other hand, AVP has been primarily implicated in facilitating male-typical social behaviours such as social

aggression, pair-bond formation, scent marking, courtship and mating behaviours (Carter, 1998; Carter & Altemus, 1997; Lim & Young, 2006; Young & Wang, 2004).

Interestingly, emerging evidence suggests that ghrelin, a peptide primarily involved in feeding, may have an important role in social behaviours. To expand, various studies have demonstrated that ghrelin promotes the motivation for social interaction by enhancing social approach towards a novel-conspecific, whereas ghrelin receptor antagonists or the deletion of GHSR increase social vigilance (Park et al., 2021; Schéle et al., 2020). Similarly, evidence shows that ghrelin signaling is required for animals to seek reward from various reinforcers, both natural, such as food (Egecioglu et al., 2010) and sex (Egecioglu, Prieto-Garcia, Studer, Westberg, & Jerlhag, 2016; Hyland et al., 2018) and drugs, such as alcohol (Jerlhag et al., 2009) and cocaine (Wellman, Davis, & Nation, 2005; Wellman, Hollas, & Elliott, 2008). In addition, it has been demonstrated that ghrelin increases the activity of dopamine neurons in the ventral tegmental area (VTA) as well as dopamine release into nucleus accumbens (NAcc), which has been implicated in mediating the effects of ghrelin on social behaviours (Abizaid et al., 2006; Park et al., 2021; K. P. Skibicka, Hansson, Alvarez-Crespo, Friberg, & Dickson, 2011). However, the exact mechanisms by which peripheral ghrelin targets the VTA directly to exert its effects on social behaviours remains to be elucidated.

In this present thesis, we aimed to investigate the effects of ghrelin on social behaviours and the role of the central melanocortin system in mediating these effects. This aim was formulated based on the evidence demonstrating a role for ghrelin signaling in promoting the motivation to seek reward from various reinforcers such as social interaction as well as a role for the central melanocortin system, particularly AGRP, in mediating these effects. To expand, evidence has implicated melanocortin receptors, particularly the antagonism of MC4R in directly

promoting social interaction and social approach for copulation as well as inhibiting key stress and anxiety-related processes (Chaki & Okuyama, 2005; Shimazaki & Chaki, 2005). Therefore, the role of the central melanocortin system in feeding and energy balance as well as its contribution to ghrelin's ability to influence social interaction, approach, and vigilance will be reviewed in this thesis. The overarching goal is to identify peptides and mechanisms that can promote social interaction and social approach. This can improve our overall understanding of social behaviors and can ultimately provide benefit to patients experiencing a wide range of neuropsychiatric disorders, especially disorders where social deficits are displayed as a core symptom.

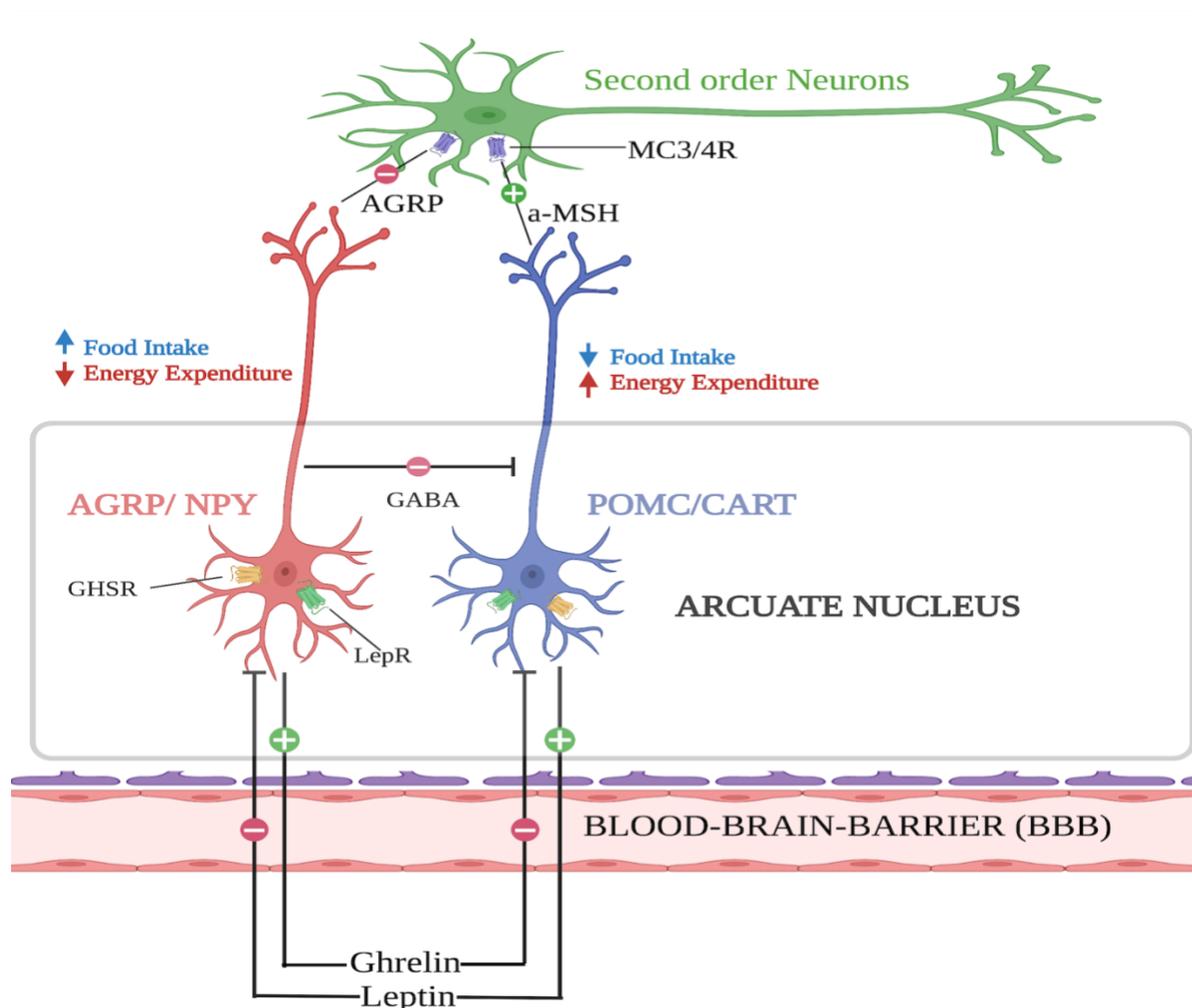
### ***An Overview of the Central Melanocortin System***

The central melanocortin system is a circuitry characterized by a number of hypothalamic peptides and hormones involved in the regulation of feeding behaviours and energy balance as well as other key physiological functions, including social behaviours. These include appetite-stimulating neuropeptides such as neuropeptide-Y (NPY) and the agouti-related peptide (AGRP), both of which are co-released from neurons located in the hypothalamic arcuate nucleus (ARC) (Cone, 2005; 1999). In contrast to NPY and AGRP, a different set of neurons within the ARC produce  $\alpha$ -melanocyte-stimulating hormone ( $\alpha$ -MSH), an appetite-suppressing neuropeptide that is cleaved from the pro-opiomelanocortin peptide (POMC). These neurons also produce appetite-suppressing neuropeptide, cocaine-amphetamine-regulated transcript (CART) (Bittencourt et al., 1992; De Lecea et al., 1998). These peptides are derived from POMC through posttranslational processing by prohormone convertase (PC-1 and PC-2) and are collectively known as melanocortins (Benjannet, Rondeau, Day, Chrétien, & Seidah, 1991; Chaki & Okuyama, 2005).

These two populations of neurons within the ARC, along with the downstream target regions that express melanocortin receptor 3 (MC3R) and melanocortin receptor 4 (MC4R) make up the central melanocortin system (Cone, 2005; De Barioglio, Lezcano, & Celis, 1991). This includes the paraventricular nucleus (PVN), amygdala (AMY), bed nucleus of stria terminalis, (BNST), lateral hypothalamus (LH), ventromedial hypothalamus (VMH), lateral parabrachial nucleus (LPB), ventral tegmental area (VTA) and nucleus tractus solitaries (NTS). Melanocortins are involved in a wide array of physiological functions such as learning and memory (De Wied & Jolles, 1982) thermoregulation (Murphy, Richards, & Lipton, 1983), feeding behaviours and energy balance (Poggioli, Vergoni, & Bertolini, 1986), stress response (Adan et al., 1999; De Barioglio et al., 1991; Von Frijtag, Croiset, Gispen, Adan, & Wiegant, 1998) as well as social behaviours (Shimazaki & Chaki, 2005). Additionally, this system is critical for the precise control of food intake and energy expenditure as it senses and integrates an array of hormones, nutrients, and afferent neural inputs. It is unique in its regulatory role as it consists of fibers that express agonists and antagonists for hypothalamic melanocortin receptors (MCRs) (Cone, 2005). Melanocortins exert their effects on five melanocortin receptors, MC1-5 receptors, which are seven-transmembrane G-protein coupled receptors. The MC1R, MC2R, and MC5R are mainly expressed throughout peripheral tissue, while MC3R and MC4R are predominantly expressed in the brain, although can still be found in the periphery (Mountjoy, 2010; Mountjoy, Mortrud, Low, Simerly, & Cone, 1994; Wikberg, 1999). Melanocortin receptors 3 and 4 are expressed across a wide range of brain nuclei that are targeted by agonist expressing POMC fibers and antagonist-expressing AGRP fibers for the central regulation of feeding behaviour and energy balance as well as other key physiological functions (Haskell-Luevano et al., 1999).

To expand, both POMC and AGRP neurons have parallel projections to various brain regions expressing MC3R and MC4R (Cone, 2005; Kistler-Heer, Lauber, & Lichtensteiger, 1998; Roselli-Rehfuss et al., 1993). They have opposite physiological effects primarily due to the contrasting effects of  $\alpha$ -MSH and AGRP on MC3/4Rs;  $\alpha$ -MSH acts as an endogenous MC3/4R agonist, while AGRP acts as an inverse MC3/4R agonist (Haskell-Luevano & Monck, 2001; Nijenhuis, Oosterom, & Adan, 2001; Tolle & Low, 2008). Substantial experimental evidence shows that the agonism of MCRs via the binding of  $\alpha$ -MSH reduces appetite and enhances energy expenditure (Hwa, Ghibaudi, Gao, & Parker, 2001; Wei Fan, Bruce A. Boston, Robert A. Kesterson, Victor J. Hruby, 1997). In contrast, AGRP binds to the same receptor site as an inverse agonist to enhance appetite and reduce energy expenditure in the absence of  $\alpha$ -MSH. In addition, AGRP acts as an MC3/4R antagonist in the presence of competing  $\alpha$ -MSH. This is consistent with evidence demonstrating that deletions or mutations within MC3R and MC4R genes causes obesity in rodents and humans (Butler et al., 2000; Farooqi & O'Rahilly, 2005; Huszar et al., 1997). Similarly, rodent studies show that chemogenetic or optogenetic activation of AGRP neurons leads to the induction of voracious feeding in sated mice (Aponte, Atasoy, & Sternson, 2011; Krashes, Shah, Koda, & Lowell, 2013). Conversely, acute, but not chronic inhibition of AGRP neurons can result in the cessation of feeding which leads to starvation and ultimately death (Gropp et al., 2005; Luquet, Perez, Hnasko, & Palmiter, 2005; Xu et al., 2005). This provides further support to their critical role in the control of feeding behaviour and the regulation of energy homeostasis, as well as established their necessity for survival. Given that AGRP and POMC neurons produce the sole ligands for MC3/4Rs, a functional opposition exists between them and their interaction is linked to being a critical control point for feeding behaviour (Cowley et al., 2001; Tong, Ye, Jones, Elmquist, & Lowell, 2009). AGRP neurons are

capable of negatively modulating the anorexigenic effects of POMC neurons by directly hyperpolarizing these neurons. This is mediated through an increase in AGRP-induced GABAergic synapses which decreases the production and release of  $\alpha$ -MSH (Roseberry, Liu, Jackson, Cai, & Friedman, 2004; M. A. Smith et al., 2007). In contrast, during positive energy balance states, leptin, an anorexigenic hormone, inhibits the transcription of NPY and AGRP genes in the hypothalamus (Mizuno & Mobbs, 1999; Schwartz et al., 1996; Stephens et al., 1995). This leads to a decrease in the GABAergic mediated tone induced by AGRP neurons on neighboring POMC neurons. This results in the disinhibition of POMC activity to ultimately promote reduced appetite and enhanced energy expenditure. These findings have led to an accepted model by which AGRP and POMC neurons act as critical sites that detect hormonal and nutrient changes in blood and adjust their activity according to nutrient-state. As a result, they regulate the activity of other hypothalamic nuclei that express MC3/4R which are implicated in a variety of physiological functions including social behaviours. These brain regions contain second order neurons which integrate and relay neuropeptides associated with energy balance to the thalamus and brainstem (Gebhart & Schmidt, 2013). For example, cell bodies within the LH release melanin-concentrating hormone (MCH), a potent orexigenic peptide as well as orexin to increase food intake and decrease energy expenditure (Farooqi & O'Rahilly, 2005). In contrast, cell bodies within the PVN release anorexigenic thyrotropin-releasing hormone (TRH) and thermogenic corticotrophin-releasing hormone (CRH) to decrease food intake and increase energy expenditure.



**Figure 1. Schematic Representation of the Central Melanocortin System.** Neurons in the ARC receive energy homeostatic signals from the periphery and as a result, release orexigenic AGRP or anorexigenic  $\alpha$ -MSH onto secondary order neurons in various brain regions in accordance with nutritional state. Ghrelin, a stomach-derived orexigenic hormone, binds to GHSR on AGRP/NPY neurons (red) to stimulate the release of orexigenic AGRP into second-order neurons containing MC3/4R (green). This leads to AGRP-induced GABAergic inhibition on POMC/CART neurons (blue) to facilitate an increase in food intake and decrease in energy expenditure. In addition, AGRP/NPY project to neurons in the LH to stimulate the release of orexigenic orexin and anti-thermogenic MCH to facilitate an increase in food intake and decrease in energy expenditure. In contrast, leptin, an anorexigenic hormone derived from white adipose tissue, binds to LepR on the POMC/CART neurons to stimulate the release of anorexigenic  $\alpha$ -MSH onto second-order neurons containing MC3/4R. As a results, PVN neurons release anorexigenic TRH and thermogenic CRH to facilitate a decrease in food intake and increase in energy expenditure.

Furthermore, evidence shows that effects of MC3/4R extend beyond feeding behaviours and energy balance. Specifically, MC4R has been implicated in a wide array of functions including social interaction and social approach associated with copulatory behaviour (Shimazaki & Chaki, 2005; Van der Ploeg et al., 2002), stress-related regulation of HPA axis (Lu, Barsh, Akil, & Watson, 2003; Von Frijtag et al., 1998), anxiety and depressive-related behaviours (Chaki et al., 2003), pain-related processes (Vrinten, Adan, Groen, & Gispen, 2001; Vrinten, Gispen, Groen, & Adan, 2000) and addictive behaviours (Alvaro, Taylor, & Duman, 2003; Cabeza de Vaca, Kim, & Carr, 2002; R. Hsu et al., 2005). To expand, evidence shows that administration of selective MC4R agonist in male mice enhances social approach towards receptive females as demonstrated through a decrease in the latency for mounting and intromission (Van der Ploeg et al., 2002). In addition, these effects were attenuated in mice lacking MC4R, highlighting its role in social behaviours. Moreover, it has been reported that melanocortins such as ACTH and  $\alpha$ -MSH induce anxiogenic effects through their agonism on MC4Rs. The anxiogenic effects of ACTH and  $\alpha$ -MSH have been demonstrated through various behavioural tests that measure anxiety-related behaviours such as the Vogel conflict (Corda, Orlandi, & Fratta, 1990), aggressive behaviour (Gonzalez, Vaziri, & Wilson, 1996), isolation-induced vocalization (Panksepp & Normansell, 1990), grooming behaviour (Adan et al., 1999) and social interaction (File & Clarke, 1980). To expand, the administration of  $\alpha$ -MSH into the medial preoptic area (MPOA) and VMH reduces the total time spent in the open arms of the elevated-plus maze test, highlighting its anxiogenic effects (Gonzalez et al., 1996). In addition, the administration of MC4R agonist (MTII) results in excessive grooming behaviours, whereas the administration of MC4R antagonist blocks the display of excessive grooming behaviour, a measure of anxiety-like behavior (Adan et al., 1999). Specifically, studies show that

administration of  $\alpha$ -MSH into the MPOA reduces exploratory behaviours and may enhance vigilance, whereas the administration of  $\alpha$ -MSH into the VMH increases aggressive behaviours (Gonzalez et al., 1996). Similarly, data shows that peripheral and intraventricular administration of ACTH reduces the motivation for social interaction as reflected through a significant reduction in the total duration of social interaction between male rat pairs (File & Clarke, 1980; File & Vellucci, 1978). Consistent with these results, data shows that administration of MC4R antagonist (MCL0129) attenuates anxiety-related behaviours displayed on the rat elevated-plus maze test (Gonzalez et al., 1996). Under non-stressful conditions, the administration of MC4R antagonist in mice produces anxiolytic effects as demonstrated by spending more time in the light area during the light/dark exploration test. Interestingly, the repeated administration of MC4R antagonist (MCL0129) for one week significantly increases the total duration of social interaction with a novel-conspecific (Shimazaki & Chaki, 2005). In contrast, the administration of MC4R agonist (MTII) significantly decreases the total duration of social interaction with a novel-conspecific, providing further evidence for the involvement of the central melanocortin system in social behaviours.

### ***Ghrelin and the Motivation for Social Interaction***

There is a long list of hormones that influence the activity of the central melanocortin system, and these include leptin, insulin, estrogen, corticosterone, and ghrelin, all of which have a large impact on the regulation of a variety of metabolic and physiological functions through their actions on the ARC. Of these, ghrelin is the only known signal produced by the gastrointestinal system that stimulates appetite, reduces metabolic rate and energy expenditure as well as promotes social behaviours (Park et al., 2021). Indeed, Kojima and Colleagues (1999)

identified ghrelin as an endogenous ligand for a previously identified orphan receptor that was called the growth hormone secretagogue receptor (GHSR). They did this by purifying ghrelin from rat stomach extracts and subsequently cloning it in rats and humans. While at first Kojima established the role of ghrelin in the secretion of growth hormone, his group and that of others established that ghrelin also stimulated food intake.

Ghrelin is unique in that its biological effects are dependent on a number of post-translational modifications. The ghrelin pre-propeptide generated after translation of the mature ghrelin mRNA is cleaved by protein convertase 1 (PC-1) to derive two peptides: unacyl-ghrelin and obestatin. Unacyl-ghrelin is then modified by ghrelin O-acyltransferase (GOAT), an enzyme that links the third amino-acid of the unacyl-ghrelin molecule to n-octanoic acid, converting the unacyl-ghrelin molecule into acyl-ghrelin, the active form of the peptide. The enzyme GOAT is unique as it does not appear to modify any protein targets beyond ghrelin, making it the only substrate for ghrelin in the human proteome (Darling et al., 2015). Without this modification, ghrelin does not bind to the GHSR. The ester bond in acyl-ghrelin is broken down by esterase enzyme activity, converting acyl-ghrelin into des-acyl ghrelin, a process that occurs within minutes of secretion into blood circulation. Because of this, acyl-ghrelin makes up about 10-30% of total circulating ghrelin (Yoshimoto et al., 2002). Experimental evidence shows that the loss of acyl-ghrelin in circulation due esterase enzyme activity can result in negative metabolic and neurological consequences (Stark et al., 2016). Specifically, studies investigating proteins in human serum associated with ghrelin esterase activity show that butyrylcholinesterase (BChE) acts as an important contributor to the deacylation of acyl-ghrelin in humans (Brimijoin, Chen, Pang, Geng, & Gao, 2016; P. Chen et al., 2015; Schopfer, Lockridge, & Brimijoin, 2015). Some work suggests that des-acyl ghrelin and obestatin are biologically active and have opposite

effects to ghrelin although these data remain controversial (Bang, Soule, Yandle, Richards, & Pemberton, 2007; Harada et al., 2008; J. V. Zhang et al., 2005). Acyl-ghrelin will be referred to as ghrelin for the remainder of this thesis.

Once activated, ghrelin binds to the GHSR, the only known active receptor for this hormone (Gnanapavan et al., 2002). The GHSR has a widespread distribution in the central nervous system and in many peripheral tissues. Within the brain, the GHSR is most abundant in the hypothalamus, and particularly in the ARC where ghrelin binds primarily to NPY and AGRP neurons to increase their activity. It is through the actions of ghrelin on these cell groups that ghrelin influences feeding and appetite through the central melanocortin system (Tschop 2000; Nakazato, 2001). Experimental evidence shows that mice lacking AGRP and NPY neurons (i.e. dual knockout mice) do not show increases in food intake in response to peripheral ghrelin treatment (H. Y. Chen et al., 2004; Luquet, Phillips, & Palmiter, 2007). In addition, the orexigenic effects of ghrelin were completely abolished in mice models with postembryonic ablations of NPY/AGRP neurons (Bewick et al., 2005). This is consistent with data from Nakazato et al., (2001) and Kamegai et al., (2001) which demonstrated that ghrelin increases the expression of AGRP and NPY transcripts. Also, evidence shows that antibodies and antagonists for NPY and AGRP neurons can attenuate ghrelin-induced generation of a positive energy balance state. Furthermore, other studies show that expression of Fos protein, which is used as a marker for neuronal activity, is significantly higher in NPY and AGRP neurons following IP and ICV administration of ghrelin (Nakazato et al., 2001; Lixin Wang, Saint-Pierre, & Taché, 2002). Under conditions of negative energy balance, circulating ghrelin levels are increased which enhances the expression and release of AGRP and NPY. This occurs as ghrelin stimulates the release of inhibitory GABAergic projections onto POMC neurons which ultimately results in

increased food intake and decreased energy expenditure (Cowley et al., 2003; Kamegai et al., 2001; Nakazato et al., 2001).

Under conditions of negative energy balance, hunger provides one of the strongest homeostatic motivations for behaviours in the animal kingdom which ultimately promotes foraging behaviours. Therefore, despite a wide range of stimuli and competing demands that innately influence their behaviours, animals always pursue food in times of caloric insufficiency to ensure survival (Burnett et al., 2016; Toates, 1980). In addition, animals must inhibit a range of competing innate motivational drives and behaviours such as fear, stress, thirst, and reproduction to forage for food highlighting the tremendous plasticity in feeding behaviours. Therefore, it is expected that functions of ghrelin extend beyond the observable physiological effects in relation to the stimulation of food intake and adiposity to regulate energy balance. Ghrelin can act on the central melanocortin system to influence motivational processes associated with need-based behaviours such as feeding, thirst, copulation, and social behaviors. Specifically, emerging evidence shows that ghrelin promotes the motivation for social interaction by enhancing social approach towards a novel-conspecific, whereas ghrelin receptor antagonists increase social vigilance (Park et al., 2021; Schéle et al., 2020).

In a recent study by Schele et al. (2020), they were able to demonstrate that ghrelin signaling can significantly increase the motivation for social interaction in the heavier partner of a male rat pair. To expand, they showed that ghrelin signaling enhances the motivation to seek social interaction in a manner that reflects the divergence in body weight between social pairs, suggesting that ghrelin could play a role in implementing social hierarchies in rats. In contrast, they showed that blocking ghrelin signaling through an antagonist can attenuate these effects and decrease the motivation for social interaction. This is consistent with evidence showing that

GHSR knockout mice or mice treated with a ghrelin antagonists have decreased motivation for social interaction by displaying longer latencies to approach receptive females compared to WT mice or mice treated with saline (Egecioglu et al., 2016; Prieto-Garcia, Egecioglu, Studer, Westberg, & Jerlhag, 2015). Similarly, GHSR knockout mice display a decreased place preference for rewarding stimuli such as palatable foods and social interaction following chronic social defeat, highlighting a role for ghrelin signaling in social reward (Chuang et al., 2011). Furthermore, data shows that ghrelin signaling is required for animals to seek reward from various reinforcers, both natural, such as food (Egecioglu et al., 2010) and sex (Egecioglu et al., 2016; Hyland et al., 2018) and drugs, such as alcohol (Jerlhag et al., 2009) and cocaine (Wellman et al., 2005, 2008), providing further support to the notion that ghrelin signaling is required for the motivation to seek reward from various reinforcers, including social interaction.

The mesolimbic dopaminergic system is a core element of the underlying neurobiology of motivated and reward-related behaviours, especially dopamine neurons within the VTA that project to the NAc and other limbic areas. The activation of this system is associated with the expectation or exposure to pleasurable stimuli such as palatable food, sexual experience, social interaction and drug use (Berridge, 1996; Blackburn, Pfaus, & Phillips, 1992; Richardson & Gratton, 1998; Wise, 2006). These reward-driven behaviours are initiated as a result of complex neurobiological mechanisms that lead to altered incentive motivational value of conditioned reward-predictors within the environment (Wise, 2002). Specifically, the activation of dopamine cells in the VTA plays a major role in facilitating the motivation for social interaction (McHenry et al., 2017). These cells receive an array of sensory and hormonal inputs from various regions within the hypothalamus and projects to brain regions associated with reward-related behaviours. This includes forebrain regions such as the nucleus accumbens (NAc), pre-frontal cortex,

amygdala and hippocampus (Gunaydin et al., 2014; Wise, 2004). Specifically, this system has been linked with the integration of social stimuli to promote the motivation for social interaction and produce social reinforcement (Dulac, O'Connell, & Wu, 2014; Fang, Yamaguchi, Song, Tritsch, & Lin, 2018; Hung et al., 2017).

Interestingly, data shows that ghrelin targets dopamine cells in the VTA to promote the motivation to seek rewards from various stimuli, such as palatable food and social interaction (Abizaid et al., 2006; Park et al., 2021; Karolina P. Skibicka et al., 2013). This is consistent with previous findings from Guan et al. (1997) demonstrating the presence of the GHSR within the VTA as well as evidence from Zigman et al. (2006) showing the expression of GHSR within several nuclei with direct or indirect connections to the mesolimbic reward system. To expand, fluctuating peripheral levels of endogenous ghrelin can activate GHSRs within the VTA which results in a rapid reorganization to the synaptic inputs of dopaminergic (DA) neurons to ultimately increase their firing rate (Abizaid et al., 2006). This leads to a GHSR-dependent increase in DA turnover within the NAc. This makes DA neurons more likely to be activated by other inputs which would result in increased sensitivity of the VTA to reward-seeking or reward-inducing stimuli. Both systemic and central administration of ghrelin into the VTA can increase operant behaviour for a food reward, whereas peripheral administration of GHSR-1A antagonist decreases operant responses for a food reward (Egecioglu et al., 2010; King, Isaacs, O'Farrell, & Abizaid, 2011; Perello et al., 2010; Karolina P. Skibicka, Hansson, Egecioglu, & Dickson, 2012). Similarly, intra-VTA administration of ghrelin enhances the motivation and intake of a food reward as well as body weight gain, whereas the administration of GHSR antagonist into the VTA attenuates these effects. Studies using a GHSR-1A knockout model or the peripheral administration of GHSR-1A antagonist demonstrate a decreased preference for a reward-paired

environment in a conditioned place preference test (CPP) as well as decreased sexual motivation in mice and rats, further highlighting the role of ghrelin signaling in motivated and reward-related behaviours (Chuang et al., 2011; Egecioglu et al., 2016; Hyland et al., 2018; Prieto-Garcia et al., 2015).

A recent paper by Park et al. (2021) provided further support to the notion that GHSR signaling in the VTA is required to facilitate motivated behaviour, particularly social motivation in males. To expand, they demonstrated that mice lacking a functional GHSR or receiving a GHSR antagonist (JMV2959) display an increase in the latency to approach a conspecific and a reduced social interaction time compared to WT or vehicle-treated controls. This reflects an overall decrease in social motivation based on the parameters of social approach. Specifically, they were able to highlight a direct role of GHSR in the VTA as it pertains to social motivation. They showed that intra-VTA administration of GHSR antagonist (JMV2959) resulted in longer latencies to approach a novel-conspecific. Additionally, they showed that partial rescue of GHSR in the VTA can attenuate social approach deficits in GHSR KO mice. This is in line with evidence showing that rats and mice lacking the GHSR, or treated with GHSR antagonists peripherally or directly into the VTA display a significant decrease in sexual motivation (Hay et al., 2020; Hyland et al., 2018; Prieto-Garcia et al., 2015). Specifically, blocking GHSR signaling in the VTA seems to have an effect on the anticipation of sex and not the actual sexual performance, further highlighting the role of GHSR signaling in the motivational aspect associated with reward-related behaviours (Hyland et al., 2018)

One aspect that was not addressed in the study by Park et al. (2021) is that ghrelin has difficulties entering the brain and targeting the VTA directly due to poor BBB penetrance (Edwards & Abizaid, 2017). An alternative hypothesis would be that ghrelin targets the central

melanocortin system directly to exert its effects on social behaviours given that this system is, as described above, a primary target for ghrelin. This is possible because ghrelin can bypass the BBB through circumventricular organs which have a leaky BBB due to fenestrated capillaries (Edwards & Abizaid, 2017). This allows ghrelin to enter the brain and act on the ARC, which has been demonstrated to be an accessible brain region for peripheral ghrelin. This stimulates neurons within the central melanocortin system which can further relay the effects of ghrelin to brain regions that are protected by the BBB such as the VTA. Specifically, evidence shows that ghrelin acts on AGRP and NPY neurons in the ARC to influence various motivated behaviours including social behaviours. (Burnett et al., 2019). Interestingly, AGRP acts as an antagonist to MC4R which is directly implicated in promoting social interaction and social approach behaviours (Shimazaki & Chaki, 2005; Van der Ploeg et al., 2002). In addition, these neurons have direct connections to brain regions processing reward and motivation such as the VTA (Cansell, Denis, Joly-Amado, Castel, & Luquet, 2012). In fact, evidence shows that AGRP neurons have a direct role in the modulation of dopamine signaling (Dietrich et al., 2012). Studies using different animal models where AGRP neurons are either ablated from birth or selectively inactivated were able to demonstrate that AGRP neurons project directly to VTA dopamine neurons and influence their firing rate (Dietrich et al., 2012). To expand, the inactivation or ablation of AGRP neurons results in a reduced GABAergic inhibition on VTA dopamine neurons which translates to higher excitability within these neurons and that ultimately facilitates the induction of long-term potentiation. This highlights a critical role for AGRP neurons in directly influencing dopamine-dependent encoding for motivation and reward, as reduced AGRP activity results in enhanced dopamine-dependent encoding within the VTA (Dietrich et al., 2012). The behavioural outcomes in animal models where AGRP is inactivated

demonstrate an enhanced response to novelty and preference for an environment associated with drugs such as cocaine, further highlighting the role of AGRP neurons in the modulation of dopamine signaling (Dietrich et al., 2012; Palmiter, 2012). Furthermore, evidence shows that increased circulating ghrelin levels can bias motivational processes through AGRP neurons to promote feeding behaviours resulting in adaptive trade-offs between foraging and social behaviours (Burnett et al., 2019). For example, Burnett et al. (2019) were able to demonstrate that AGRP-induced hunger can significantly reduce resident male copulatory advances towards a female conspecific when food is present. Similarly, resident mice show significantly less aggression towards a subordinate male intruder in more intense hunger states when food is present (Burnett et al., 2019). However, effects of AGRP on the motivation for social interaction without the presence of a competing-stimuli remains to be determined. Given the evidence suggesting a role for ghrelin signaling in the motivation to seek reward from various reinforcers such as social interaction as well as evidence demonstrating that antagonism of MC4R can directly promote social interaction, we speculated that ghrelin promotes the motivation for social interaction in the absence of food and these effects are mediated in part, through AGRP neurons.

### ***Rationale for Present Thesis***

As stated previously, studies show that ghrelin signaling is required for animals to seek reward from various reinforcers, both natural, such as food (Egecioglu et al., 2010) and sex (Egecioglu et al., 2016; Hyland et al., 2018) and drugs, such as alcohol (Jerlhag et al., 2009) and cocaine (Wellman et al., 2005, 2008). Similarly, emerging evidence suggests that ghrelin receptor activation facilitates social interaction with a novel conspecific after both chronic defeat stress and in basal conditions (Park et al., 2021; Schéle et al., 2020). In addition, it has been

demonstrated that ghrelin increases the activity of dopamine neurons in the VTA as well as dopamine release into NAcc, which has been implicated in mediating the effects of ghrelin on social behaviours (Abizaid et al., 2006; Park et al., 2021; K. P. Skibicka et al., 2011). However, the exact mechanisms by which peripheral ghrelin targets the VTA directly to exert its effects on social behaviours remains to be elucidated. The ARC and particularly AGRP/NPY neurons in this area are rich in GHSR expression. Interestingly, AGRP has a wide-ranging influence on the central melanocortin system by acting as an antagonist at the MC3/4R, which is implicated in directly influencing social interaction, approach and vigilance (Shimazaki & Chaki, 2005; Van der Ploeg et al., 2002). Hence the goal of this present thesis was to investigate the contribution of AGRP to ghrelin's ability to promote social interaction with a novel-conspecific. Therefore, we hypothesized that ghrelin promotes the motivation for social interaction and these effects are mediated in part, through AGRP neurons. The following series of behavioural and immunohistochemical experiments were conducted to explore the role of ghrelin acting on AGRP neurons to promote the motivation for social interaction in mice.

### ***Specific Aim***

This issue was addressed in two experiments: In the first experiment, ghrelin administration was paired with either saline or the synthetic  $\alpha$ -MSH analog, melanotan-II (MTII) and the effects on behavior in the social interaction test were compared with that of mice given either saline alone or MTII alone. To limit the effects of stress on social behaviors, the social interaction test is conducted where a mouse is placed in a familiar environment and then a novel con-specific mouse is placed in the middle of the testing apparatus. Social motivation was operationally defined as the latency to approach and total duration spent interacting with the

novel-conspecific. Social vigilance was operationally defined as the frequency of corner observations and total time spent in the corner observing the novel-conspecific. In the second experiment, coronal brain sections were stained for c-Fos to investigate the neuronal activity following peripheral ghrelin and MTHI injections. This was done to validate the effectiveness of the injections by looking at activity in target brain regions within the central melanocortin system that drugs are expected to activate. The brain regions of interest are the arcuate (ARC) nucleus, paraventricular nucleus (PVN), bed nucleus of the stria, terminalis (BNST), central amygdala (CeA) and the medial amygdala (MeA). The ARC nucleus is investigated as it is a major target of ghrelin for its abundant expression of GHSR (Cone, 2005). The PVN is investigated for its high expression of GHSR and MCR3/4R which are a main target of ghrelin and AGRP projections from the ARC. The BNST, CeA, and MeA are investigated for being another target of AGRP/NPY projections as they express MC3/4R and for their role in anxiety, fear, and threat detection (Cone, 2005; Cowley et al., 2003; Wright et al., 2020). Based on such evidence, we would expect that ghrelin can directly target the ARC and PVN and indirectly target the BNST and amygdala through AGRP neurons.

## **Methods and Materials**

### ***Animals and Housing Conditions***

Male and female adult mice (C57/BL6, 18-22 grams) were obtained from Charles River farms (St. Constant, Quebec) at 3 months of age. All mice were individually housed in clear plexiglass cages with nesting material provided as enrichment at a temperature-controlled ( $22 \pm 1^\circ\text{C}$ ) and humidity-controlled ( $50 \pm 5\%$ ) environment. All mice were kept at a 12 hr light/12 hr dark cycle and were provided with 7 days to habituate to their environment before any

experimental procedures were conducted. All mice had *ad libitum* access to chow (2.9kcal/g, with 70% of calories derived from carbohydrates) and water throughout the habituation and experimental periods. All procedures were approved by Carleton University Animal Care Committee (protocol #102722) according to the guidelines of the Canadian Council of Animal Care (CCAC).

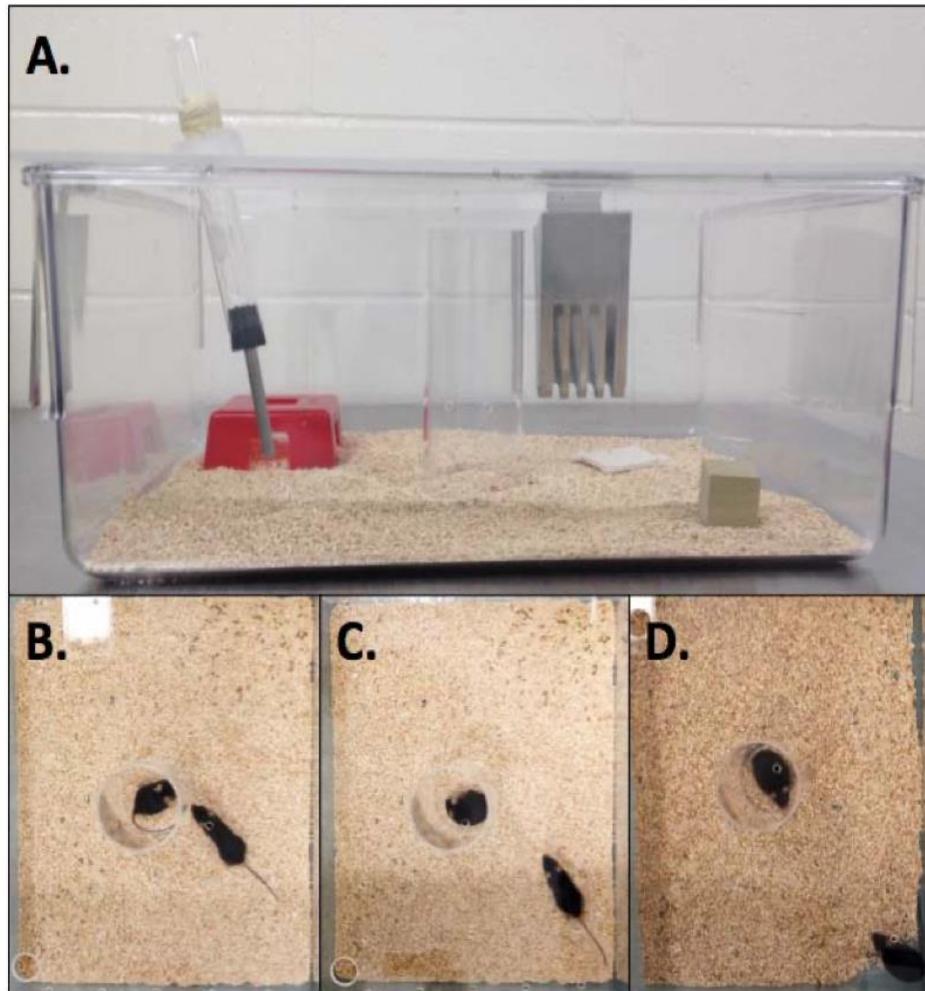
Mice were assigned to one of four groups based on the drug treatment they were to receive: (1) saline/saline (n=19), (2) ghrelin/saline (n=22), (3) MTII/saline (n=12), (4) MTII/ghrelin (n=10), with almost an equal distribution of males and females in each group. On the test day, subjects received two intraperitoneal (IP) injections according to their group, 30 minutes before the test. The doses of the drugs administered are the following: ghrelin (1mg/kg of ghrelin in .1cc of isotonic sterile saline), saline (0.1ml of 0.9% sodium chloride) or MTII (100ug of MTII in .1cc of isotonic saline). The rationale for choosing such doses is based on evidence demonstrating a strong response associated with ghrelin (1 mg/kg) and MTII (100ug) on the central melanocortin system (Dana I. Briggs, Enriori, Lemus, Cowley, & Andrews, 2010; Lu et al., 2003; Lv, Liang, Wang, & Li, 2018; Pierroz et al., 2002). Ghrelin and MTII were purchased from Tocris Bioscience and Vivitide, respectively and were dissolved in isotonic saline in the doses described. All injections were administered in the morning during the light cycle between 8:00 am-10:00 am.

### ***Social Interaction Test***

The social interaction test was adapted from a previous protocol (Tsuda & Ogawa, 2012). To mitigate any confounding anxiogenic effects that might be associated with a new environment, all mice were habituated to the social interaction chamber for 48 hours before the

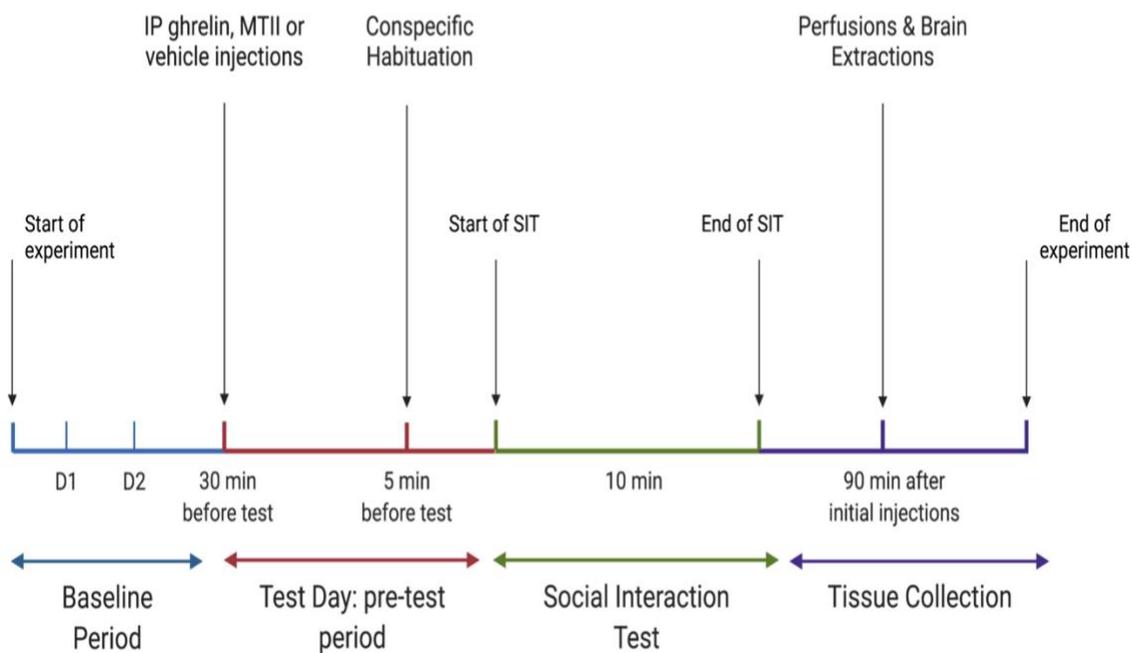
experimental procedure. The social interaction chamber contained a perforated cylinder (3.5"x7") custom made from Plastics Inc. Ottawa as well as red housing, nesting and a wooden block for enrichment. On the test day, all enrichment and food with the exception of the perforated cylinder were removed from the chamber and the mouse habituated to this condition for 30 minutes. Then, the mouse was removed from the social interaction chamber and placed in an empty plexiglass cage with nesting for a period of 5-minutes. During this 5-minute period, a novel-conspecific of the same sex was placed into perforated cylinder in the social interaction chamber. The experimental mouse was then placed in a corner of the social interaction chamber and social activity was video recorded for 10 minutes. Latency to approach the stranger mouse, frequency of sniffing, stretching, corner observation as well as the total time spent in corner and total duration of social interaction were quantified to assess social behaviours.

Behaviours such as the latency to approach and total duration of social interaction are indicative of social approach and motivation (Lim & Young, 2006). In contrast, behaviours such as the frequency of stretching, corner observation and the total time spent in corner are indicative of social vigilance. Latency to approach is operationally measured as the total time it takes to approach the novel-conspecific. Sniffing behavior is operationally measured when the mouse placed its nose in the perforated holes when the novel-conspecific was placed in the cylinder. Stretching behavior is operationally measured when the mouse stretches its trunk towards the novel-conspecific without getting close to the cylinder. Corner observation is operationally measured when the mouse stayed in the corners of the chamber while fixating its sight on the novel-conspecific.



**Figure 2. Social Interaction Test.** The social interaction test design was modified using the method by Tsuda and Ogawa (2012) (A) Social interaction cage with a perforated cylinder in the center and enrichment (B) Sniffing behavior is when the mouse placed its nose in the perforated holes when the novel-conspecific was placed in the cylinder (C) Stretching behavior is when the mouse stretches its trunk towards the novel-conspecific without getting close to the cylinder (D) Corner observation is when the mouse stayed in the corners of the chamber while fixating its sight on the novel-conspecific

## Experimental Timeline



**Figure 3. Experimental Timeline.** A schematic diagram showing the time course of the experimental design. Prior to the beginning of the experiment, all mice had ad libitum access to standard laboratory chow and tap water. Baseline period (blue): mouse habituated to the SIT chamber for 48 hours. Pretest period (red): the administration of drug treatments 30 mins prior to the test and conspecific habituation 5 minutes prior to the test. Social interaction test (green). Tissue collection period (purple).

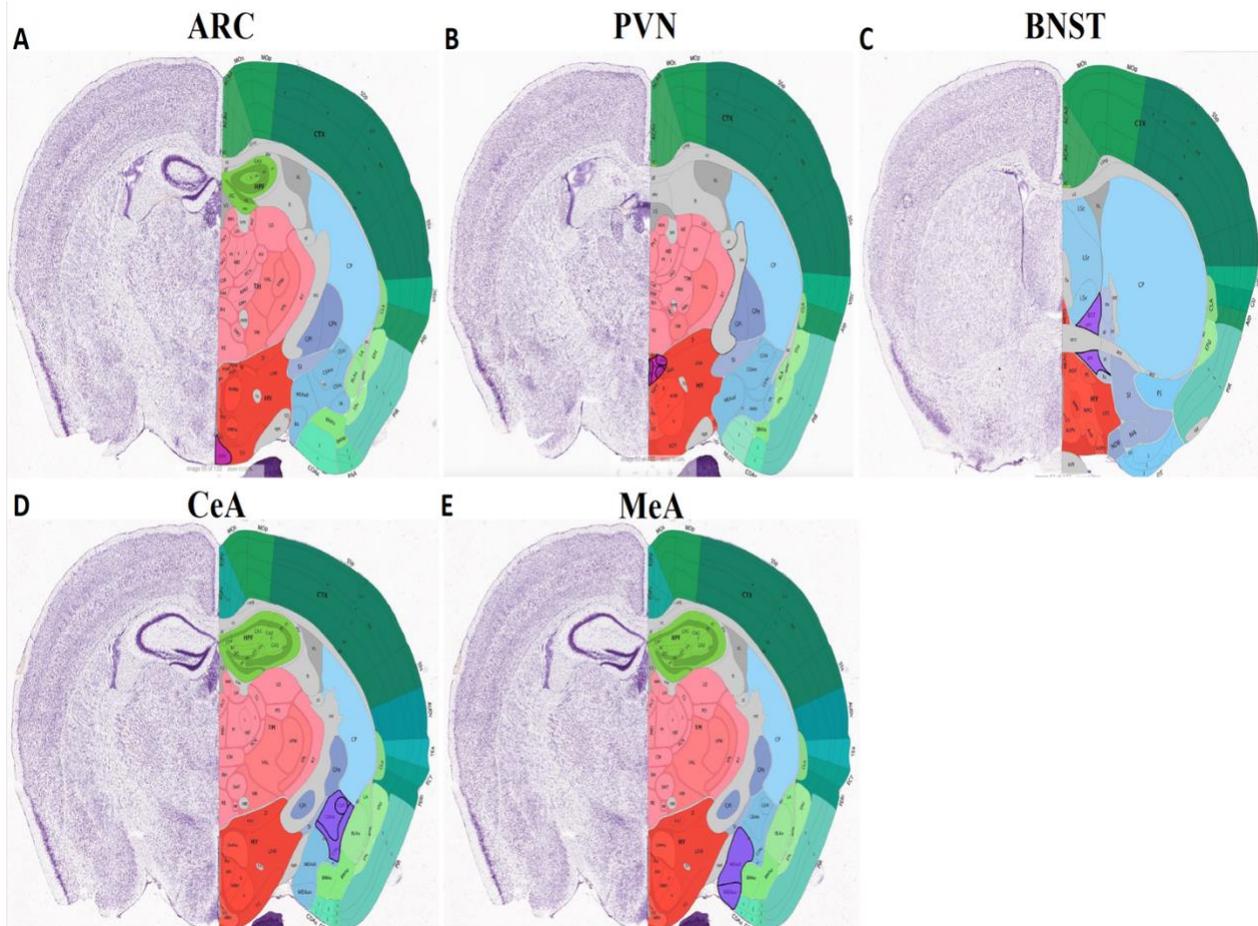
### ***Fos Immunohistochemistry***

Ninety minutes after the initial injections, mice were deeply anesthetized by isoflurane and transcardially perfused with 0.9% saline, followed by 4% paraformaldehyde (PFA) in 0.1M phosphate buffer. This time point was chosen based on evidence showing that fos protein can be detected in neurons by immunohistochemical techniques up to 90 mins after peripheral administration of ghrelin (Bullitt, 1990; Men  trety, Gannon, Levine, & Basbaum, 1989; Morgan, Cohen, Hempstead, & Tom, 1987; Toshinai et al., 2003). Brains were collected and post-fixed in 4% PFA at 4  C for at least 48 hours and transferred into a 30% sucrose solution containing sodium azide for dehydration and storage. Brains were frozen and coronally sliced at 30  m in a 1-in-4 series, on a cryostat (Fischer Scientific). Free floating brain tissues were washed in phosphate buffered (PB) solution 3-5 times and quenched with (30% H<sub>2</sub>O<sub>2</sub> in methanol and PB) for 15 minutes at room temperature. Sections were washed again for 3-5 times and placed in blocking solution (3  l Triton-X, 0.01g bovine serum albumen, and 50  l normal goat serum dissolved in 1ml PB) for 30 minutes. Immediately after the removal from blocking solution, tissues were incubated in an affinity-purified rabbit polyclonal antibody raised against cFOS diluted 1:20,000 with a solution of Triton X-PB with BSA for 48 hours. The tissues incubating in the primary antibody were then washed 5 times with PB, then incubated in a biotinylated anti-rabbit IgG made in donkey (Jackson ImmunoResearch Laboratories, Inc., Westgrove, PA, USA), diluted 1:250 with BSA in PB for 2 hours. Then, the tissues were washed for 3 times then treated with an ABC complex (2   l solution A and 2   l solution B dissolved in 1ml PB) for 1 hour, washed 3 times again and placed DAB solution (50  l of DAB stock and 35.29  l of Nickel Chloride stock solution dissolved in 900   l of PBS) for 3 minutes. Lastly, 25  l of solution B (10  l of 30% H<sub>2</sub>O<sub>2</sub> dissolved in 990  l of PBS per 1 mL) were added to the tissues in the DAB

solution for nickel enhancement. The enhancement reaction occurred for 4 minutes and then the reaction is stopped immediately by washing the tissues with PB 3 times. Lastly, sections were mounted on gel-coated glass slides, dehydrated with alcohol, soaked in Clearene (Surgipath, Leica Microsystems Inc., Concord, ON, Canada) and coverslipped with Permount mounting medium (Fisher Scientific, Toronto, ON, Canada). After that, sections were imaged using Olympus BX51 bright field microscope (Olympus Canada, Markham, ON, Canada) and associated digital capture device DVC-2000C (DVC Company, Austin, TX, USA) connected to a desktop computer with Windows XP operating system. Regions of interest were defined based on the Allen Mouse Brain Atlas and Allen Reference Atlas; Coronal Atlas (<https://mouse.brain-map.org>). For each region of interest, at least two sections per animal were counted bilaterally. The total number of stained cells per area were included and counted for each animal. The inclusion criteria for counting was defined as the mid-range from the lightest to the darkest point within the image. Quantification and cell counting was conducted manually using ImageJ software by two experimenters that were blind to group membership. The regions of interest were labelled by one experimenter to ensure both experimenters were counting the same regions.

### ***Statistical Analysis***

Data obtained from the social interaction test and cfos experiments were analyzed using a two-way analysis of variance (ANOVA) with drug treatments (ghrelin vs MTII) as independent subject factors. Main effects and significant interactions were investigated by performing simple main effects and post-hoc tests (i.e., Tukey's Multiple Comparison Test) where appropriate. All statistical analysis were performed using the GraphPad Prism software with the significance criterion set at  $p < 0.05$ . Data represented as mean  $\pm$  SEM. \*  $p < 0.05$ , \*\*  $p < 0.01$ .



**Figure 4. Regions of Interest (ROI).** Reference images of the brain regions of interest (purple) obtained from Allen Mouse Brain Atlas and Allen Reference Atlas; Coronal Atlas (<https://mouse.brain-map.org>) for Fos immunohistochemistry. (A) Coronal section highlighting the location of arcuate nucleus (purple) (B) Coronal section highlighting the location of paraventricular nucleus (purple) (C) Coronal section highlighting the location of bed nucleus of stria terminalis (purple) (D) Coronal section highlighting the location of central nucleus of the amygdala (purple) (E) Coronal section highlighting the location of medial nucleus of the amygdala (purple)

## Results

### *Experiment 1: Ghrelin Promotes the Motivation for Social Interaction Independent of AGRP*

Results showed no significant sex differences on most behavioural measures; this includes the latency to approach ( $F_{(1,59)} = 0.878$ ,  $P = 0.353$ ), frequency of sniffing ( $F_{(1,59)} = 3.173$ ,  $P = 0.080$ ), frequency of stretching ( $F_{(1,59)} = 0.470$ ,  $P = 0.496$ ), total time spent in the corner ( $F_{(1,59)} = 3.593$ ,  $P = 0.067$ ) and total duration of social interaction ( $F_{(1,59)} = 2.712$ ,  $P = 0.105$ ). There was only a significant sex difference on the frequency of corner observations ( $F_{(1,59)} = 4.640$ ,  $P = 0.036$ ) with a main effect of MTII ( $F_{(1,59)} = 5.737$ ,  $P = 0.002$ ). However, no significant interaction effects between sex and treatment were reported on the frequency of corner observations ( $F_{(1,59)} = 0.625$ ,  $P = 0.602$ ). Due to this, the subjects were combined according to group and analyzed using a two-way ANOVA to measure the effects of drug treatments (ghrelin and MTII) only.

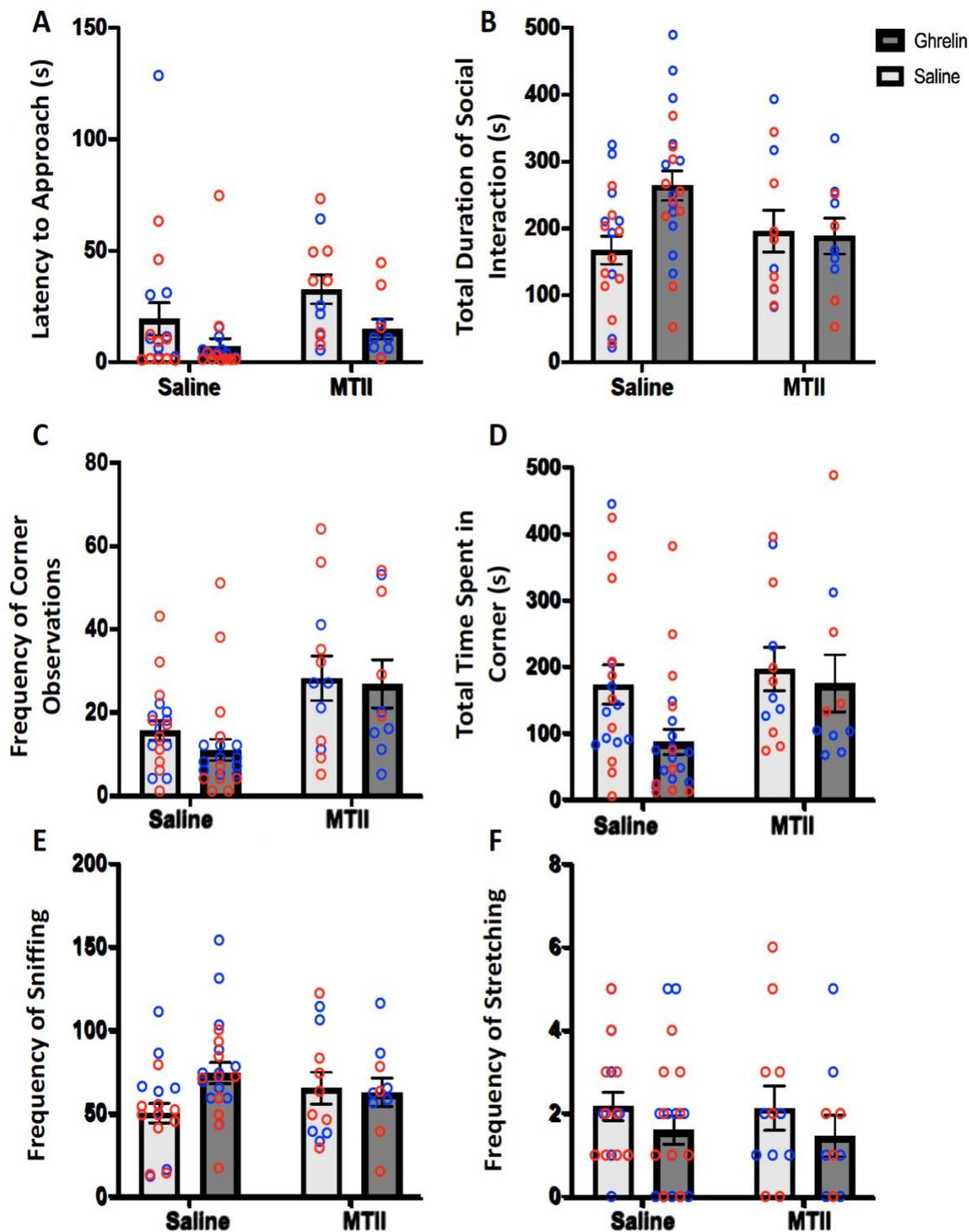
After grouping males and females together, results showed that ghrelin injected peripherally decreased the latency to approach a novel-conspecific (significant main effect of ghrelin,  $F_{(1,59)} = 6.211$ ,  $P = 0.0155$ ) (**Figures 5A**). No main effects of MTII ( $F_{(1,59)} = 3.002$ ,  $P = 0.0884$ ) were reported on the latency to approach. There were no significant interaction effects ( $F_{(1,59)} = 0.2341$ ,  $P = 0.6303$ ) between drug treatments (ghrelin and MTII) on the latency to approach and therefore pairwise comparisons were not conducted to investigate group differences.

In addition, there seemed to be an interaction effect ( $F_{(1,59)} = 3.974$ ,  $P = 0.0508$ ) between drug treatments (ghrelin and MTII) on the total duration of social interaction (**Figure 5B**), however the results are not significant. In addition, a low-moderate effect size ( $\eta_p^2 = 0.0674$ ) was associated with the interaction effect and therefore pairwise comparisons were not conducted to investigate group differences.

MTII injected peripherally increased the frequency of corner observation (significant main effect of MTII,  $F_{(1,59)} = 14.62$ ,  $P = 0.0003$ ) (**Figure 5C**). No main effects of ghrelin ( $F_{(1,59)} = 0.6355$ ,  $P = 0.4285$ ) were reported on frequency of corner observation. There were no significant interaction effects ( $F_{(1,59)} = 0.1965$ ,  $P = 0.6592$ ) between drug treatments (ghrelin and MTII) on frequency of corner observation and therefore pairwise comparisons were not conducted to investigate group differences.

No significant main effects of ghrelin ( $F_{(1,59)} = 3.158$ ,  $P = 0.0807$ ) and MTII ( $F_{(1,59)} = 3.390$ ,  $P = 0.0706$ ) were reported on the total time spent in the corner (**Figure 5D**). There were no significant interaction effects ( $F_{(1,59)} = 0.4876$ ,  $P = 0.4878$ ) between drug treatments (ghrelin and MTII) on the total time spent in the corner and therefore pairwise comparisons were not conducted to investigate group differences.

Lastly, there were no significant main effects of ghrelin ( $F_{(1,59)} = 2.717$ ,  $P = 0.1046$ ) and MTII ( $F_{(1,59)} = 0.005582$ ,  $P = 0.9407$ ) reported on the frequency of sniffing (**Figures 5E**). No significant interaction effects ( $F_{(1,59)} = 2.770$ ,  $P = 0.1014$ ) between drug treatments (ghrelin and MTII) were reported on the frequency of sniffing and therefore pairwise comparisons were not conducted to investigate group differences. Similarly, no significant main effects of ghrelin ( $F_{(1,59)} = 0.4876$ ,  $P = 0.4878$ ) and MTII ( $F_{(1,59)} = 1.061$ ,  $P = 0.3072$ ) were reported on the frequency of stretching (**Figures 5F**). There were no significant interaction effects ( $F_{(1,59)} = 0.4876$ ,  $P = 0.4878$ ) between drug treatments (ghrelin and MTII) on the frequency of stretching and therefore pairwise comparisons were not conducted to investigate group differences.

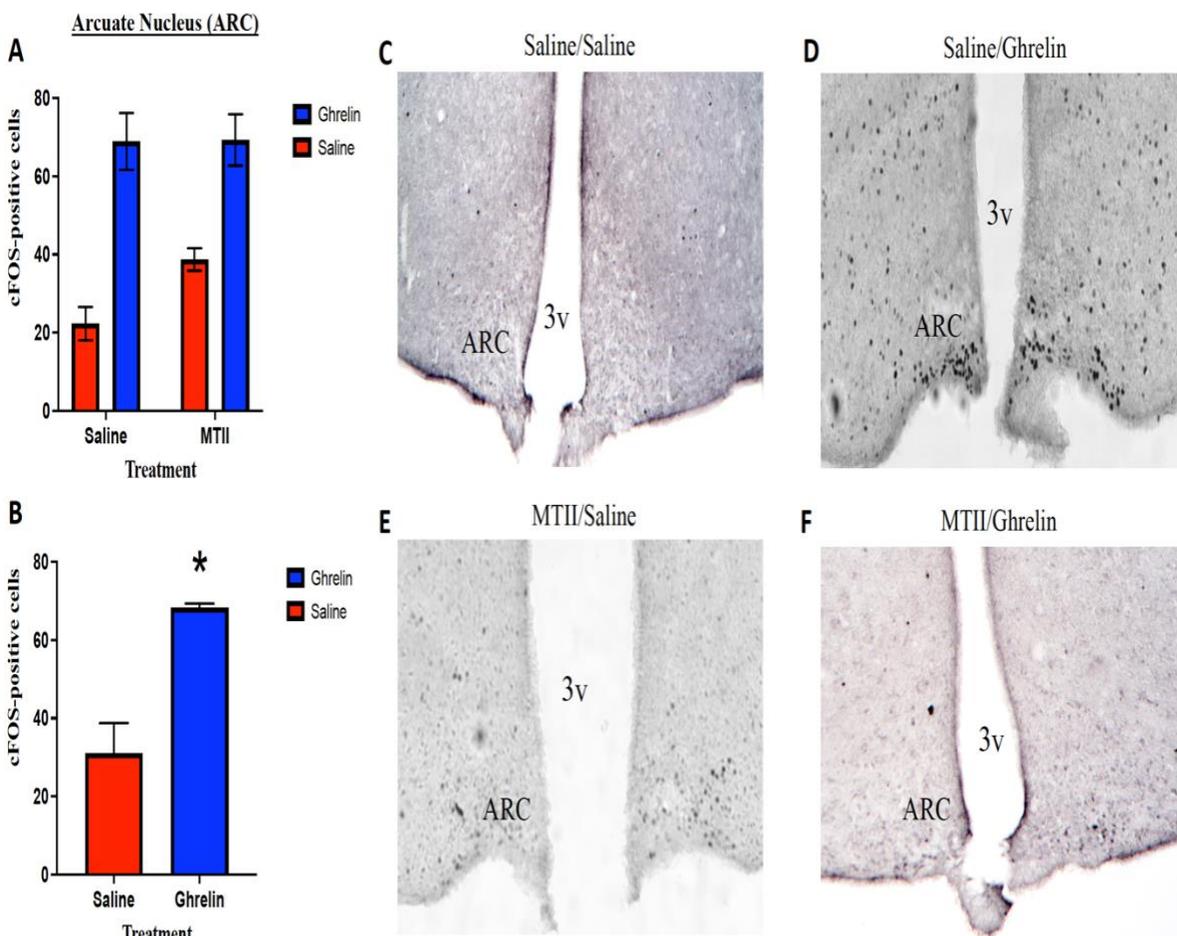


**Figure 5. Social Behaviour Measures.** (A) Latency to approach conspecific (sec) (B) Total duration of social interaction (sec) (C) Frequency of corner observations (D) Total duration of time spent in corner (sec) (E) Frequency of sniffing (F) Frequency of stretching. Data points in blue are representative of male subjects. Data points in red are representative of female subjects.

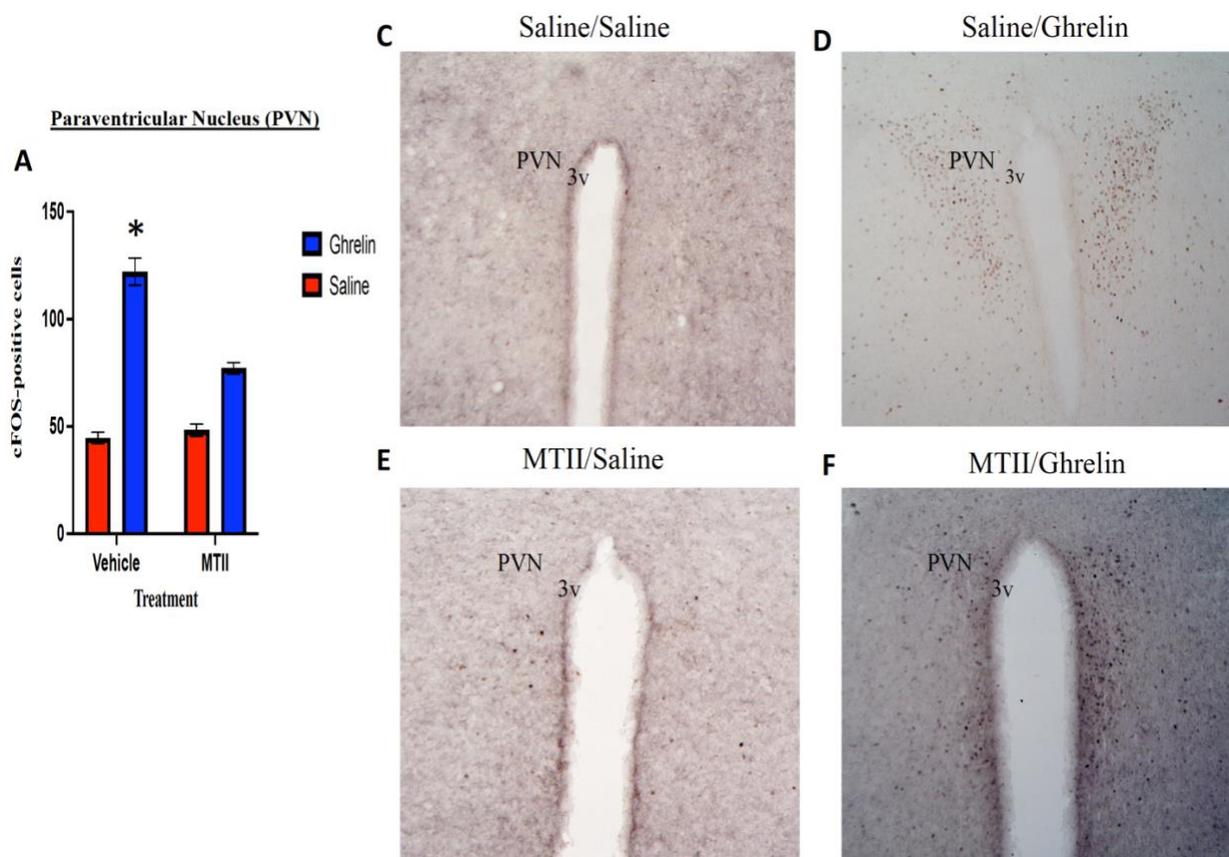
### ***Experiment 2: c-FOS Expression in Brain Regions of Interest***

As shown in figure 6, the expression of cfos in the ARC was higher in the group receiving IP ghrelin (significant main effect of ghrelin,  $N=16$ ,  $F_{(1,46)}= 35.12$ ,  $P<0.0001$ ) injections. No significant main effects of MTII ( $N=12$ ,  $F_{(1,46)}= 1.964$ ,  $P=0.1678$ ) were reported on cfos expression in the ARC. There were no significant interaction effects ( $F_{(1, 46)}= 1.635$ ,  $p=0.2067$ ) between drug treatments (ghrelin and MTII) on cfos expression in the ARC suggesting that effects of ghrelin on cfos expression in the ARC are not influenced by the presence of MTII. The changes in cfos expression within the ARC between different treatment groups are expected and therefore provide a validation that our peripheral injections of drug treatments have been successful.

As shown in figure 7, the expression of cfos in the PVN was increased after receiving IP ghrelin (significant main effect of ghrelin,  $N=16$ ,  $F_{(1,49)}= 91.36$ ,  $P<0.0001$ ) injections. In contrast, the expression of cfos in the PVN decreased after receiving IP MTII (significant main effect of MTII,  $N=13$ ,  $F_{(1,49)}= 11.38$ ,  $P=0.0015$ ) injections. There were significant interaction effects ( $F_{(1, 49)}= 16.37$ ,  $p=0.0002$ ) reported between drug treatments (ghrelin and MTII). This suggests that ghrelin has different effects on cfos expression in the PVN depending on whether MTII is present or not. If MTII is present, the increased cfos expression induced by ghrelin administration seems to be attenuated. These changes in cfos expression within the PVN between different treatment groups are expected and therefore provide another validation that our peripheral injections of drug treatments have been successful.



**Figure 6. c-Fos expression in the arcuate nucleus following ghrelin and MTII injections.** (A) Quantification of cfos-positive cells in the ARC following ghrelin and MTII injections (B) Main effect of ghrelin ( $F_{(1,52)} = 37.93$ ,  $P < 0.0001$ ) on cfos-positive cells in the ARC (C) Cfos expression in the ARC following saline/saline injections at 5x magnification (D) Cfos expression in the ARC following ghrelin/saline injections at 5x magnification (E) Cfos expression in the ARC following MTII/saline injections at 5x magnification (F) Cfos expression in the ARC following MTII/ghrelin injections at 5x magnification. Data represented as mean  $\pm$  SEM. \*  $p < 0.05$ , \*\*  $p < 0.01$ .

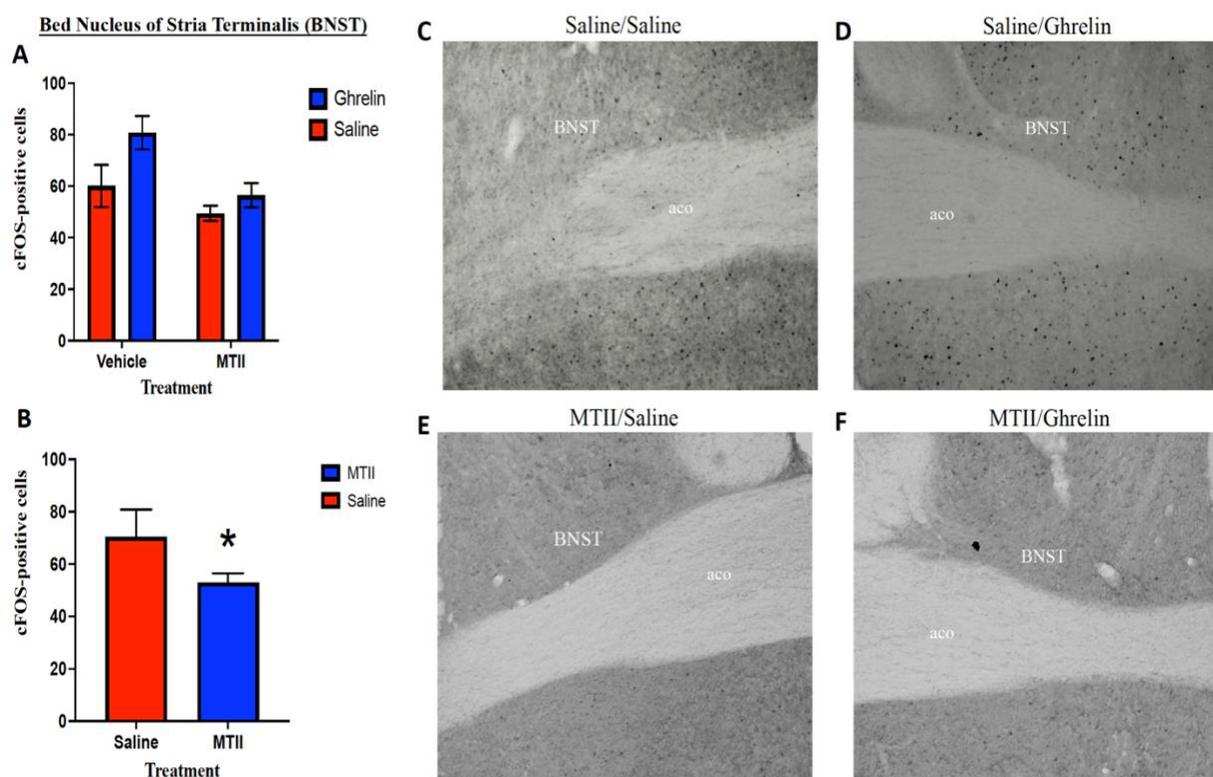


**Figure 7. c-Fos expression in the paraventricular nucleus following ghrelin and MTII injections.** (A) Quantification of cfos-positive cells in the PVN following ghrelin and MTII injections (B) Cfos expression in the PVN following saline/saline injections at 5x magnification (C) Cfos expression in the PVN following ghrelin/saline injections at 5x magnification (D) Cfos expression in the PVN following MTII/saline injections at 5x magnification (E) Cfos expression in the PVN following MTII/ghrelin injections at 5x magnification. Data represented as mean +/- SEM. \*  $p < 0.05$ , \*\*  $p < 0.01$ .

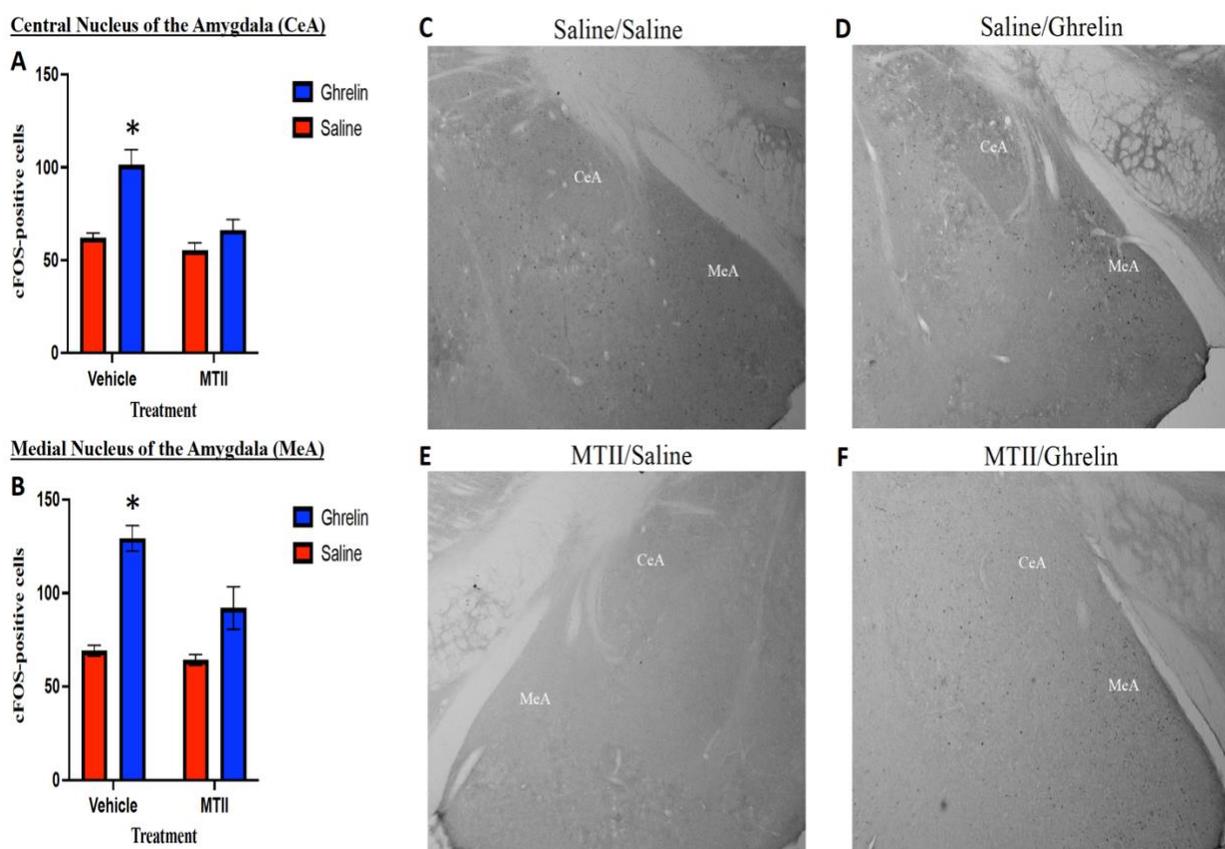
As shown in figure 8, the expression of cfos in BNST was significantly lower in the group receiving IP MTII (significant main effect of MTII,  $N=10$ ,  $F_{(1, 46)} = 5.789$ ,  $p=0.0202$ ) injections. No significant main effects of ghrelin ( $N=17$ ,  $F_{(1, 46)} = 3.642$ ,  $P=0.0626$ ) were reported on cfos expression in the BNST. There were no significant interaction effects ( $F_{(1, 46)} = 0.8882$ ,  $p=0.3509$ ) between drug treatments (ghrelin and MTII) on cfos expression in the BNST. This suggests that effects of MTII on cfos expression in the BNST are not affected by the presence of ghrelin.

As shown in figure 9A, the expression of cfos in the central nucleus of the amygdala (CeA) was increased following IP ghrelin (significant main effect of ghrelin,  $N=17$ ,  $F_{(1,46)}=16.51$ ,  $p=0.0002$ ) and decreased following IP MTII (significant main effect of MTII,  $N=11$ ,  $F_{(1,46)}=11.50$ ,  $p=0.0014$ ) injections. Significant interaction effects ( $F_{(1,46)}=5.264$ ,  $P=0.0264$ ) between drug treatments (ghrelin and MTII) were reported. This suggests that effects of ghrelin on increasing cfos expression in the CeA are attenuated in the presence of MTII.

As shown in figure 9B, the expression of cfos in the medial nucleus of the amygdala (MeA) was increased following IP ghrelin (significant main effects of ghrelin,  $N=17$ ,  $F_{(1,46)}=45.09$ ,  $p<0.0001$ ) and decreased following IP MTII ( $N=11$ ,  $F_{(1,46)}=11.65$ ,  $p=0.0014$ ) injections. Significant interaction effects ( $F_{(1,46)}=6.812$ ,  $p=0.0122$ ) between drug treatments (ghrelin and MTII) were reported. This suggests that effects of ghrelin on increasing cfos expression in the MeA are attenuated in the presence of MTII.



**Figure 8. c-Fos expression in the bed nucleus of stria terminalis following ghrelin and MTII injections.** (A) Quantification of cfos-positive cells in the BNST following ghrelin and MTII injections (B) Main effect of MTII ( $F_{(1, 46)} = 5.789$ ,  $p = 0.0202$ ) on cfos-positive cells in the BNST (C) Cfos expression in the BNST following saline/saline injections at 5x magnification (D) Cfos expression in the BNST following ghrelin/saline injections at 5x magnification (E) Cfos expression in the BNST following MTII/saline injections at 5x magnification (F) Cfos expression in the BNST following MTII/ghrelin injections at 5x magnification. Data represented as mean  $\pm$  SEM. \*  $p < 0.05$ , \*\*  $p < 0.01$ .



**Figure 9. c-Fos expression in the amygdala following ghrelin and MTII injections.** (A) Quantification of cfos-positive cells in the central nucleus of the amygdala following ghrelin and MTII injections (B) Quantification of cfos-positive cells in the medial nucleus of the amygdala following ghrelin and MTII injections (C) Cfos expression in the CeA and MeA following saline/saline injections at 5x magnification (D) Cfos expression in the CeA and MeA following ghrelin/saline injections at 5x magnification (E) Cfos expression in the CeA and MeA following MTII/saline injections at 5x magnification (F) Cfos expression in the CeA and MeA following MTII/ghrelin injections at 5x magnification. Data represented as mean  $\pm$  SEM. \*  $p < 0.05$ , \*\*  $p < 0.01$ .

## Discussion

This present thesis aimed to investigate the contribution of AGRP to ghrelin's ability to promote social interaction in mice. This aim was formulated based on several studies highlighting a role for ghrelin signaling in reward-related and social behaviours. For example, ghrelin and AGRP neurons have direct projections to DA cells in the VTA and can directly impact their firing rate to influence reward-related behaviours (Abizaid et al., 2006; Dietrich et al., 2012; Menzies, Skibicka, Leng, & Dickson, 2013). Furthermore, data shows that ghrelin signaling is required for animals to seek reward from various reinforcers, both natural, such as food (Egecioglu et al., 2010) and sex (Egecioglu et al., 2016; Hyland et al., 2018) and drugs, such as alcohol (Jerlhag et al., 2009) and cocaine (Wellman et al., 2005, 2008). In addition, ghrelin signaling can significantly increase the motivation for social interaction as reflected by an increase in the latency to approach a novel-conspecific and total duration of social interaction (Park et al., 2021; Schéle et al., 2020). In contrast, GHSR antagonist treatment attenuates these effects and decreases the motivation for social interaction. Similarly, GHSR knockout mice or mice treated with a ghrelin antagonists display decreased motivation for social interaction by having longer latencies to approach receptive females compared to WT mice or mice treated with saline (Egecioglu et al., 2016; Prieto-Garcia et al., 2015). Moreover, AGRP neurons are rich in GHSR expression and have a wide-ranging influence on the central melanocortin system by acting as an antagonist at the MC3/4R, which is implicated in directly influencing social behaviours (Shimazaki & Chaki, 2005).

Data from our social interaction experiment showed that regardless of sex, ghrelin injected peripherally promotes the motivation for social interaction. This was concluded based on data from social behaviour measures showing a significant main effect of ghrelin on

decreasing the latency to approach a novel-conspecific, which is reflective of social approach and motivation. In contrast, MTII injected peripherally results in an increase in social vigilance. This was concluded based on data from social behaviour measures showing a significant main effect of MTII on increasing the frequency of corner observations, which is reflective of social vigilance. Our results are consistent with emerging evidence demonstrating that ghrelin signaling can increase the motivation for social interaction as reflected by a decreased latency to approach a novel-conspecific, whereas blocking ghrelin signaling can decrease the motivation for social interaction and promote social vigilance (Park et al., 2021; Schéle et al., 2020). Furthermore, our results are in line with numerous studies demonstrating an anxiogenic response associated with the agonism of MC4/R which may enhance vigilance (File & Clarke, 1980; Gonzalez et al., 1996; Shimazaki & Chaki, 2005). To expand, we were able to show that administration of MC3/4R agonist MTII increases the frequency of corner observation which is reflective of social vigilance. This is consistent with data showing that administration of MC4R agonist (MTII) can significantly decrease the total duration of social interaction and enhance vigilance, whereas the repeated administration of MC4R antagonist (MCL0129) for one week significantly increases the total duration of social interaction (Shimazaki & Chaki, 2005).

Results from our cfos experiments shed light on the brain regions targeted by ghrelin during the social interactions test. To expand, the expression of cfos in the ARC was significantly higher in mice receiving IP ghrelin injections compared to mice receiving IP MTII or saline. The changes in cfos expression in the ARC following IP ghrelin injections are expected considering it is rich in GHSR receptors. As previously stated, GHSR is most abundant in the hypothalamus, and particularly in the ARC where ghrelin binds primarily to NPY and AGRP neurons to induce its effects (Tschop 2000; Nakazato, 2001). It is through the actions of ghrelin

on these cell groups that ghrelin influences feeding, and behaviours associated with foraging for food. For example, mice lacking AGRP and NPY neurons (i.e. dual knockout mice) are insensitive to the orexigenic effects of ghrelin (H. Y. Chen et al., 2004; Luquet et al., 2007). In addition, the orexigenic effects of ghrelin are completely abolished in mice models with postembryonic ablations of NPY/AGRP neurons (Bewick et al., 2005). Moreover, several studies show that expression of *cfos* is significantly higher in NPY and AGRP neurons within the ARC following ICV and IP administration of ghrelin (Nakazato et al., 2001; Lixin Wang, Saint-Pierre, & Taché, 2002). This is in line with data from our *cfos* experiment showing that IP ghrelin injections result in a significant increase in *cfos* expression in the ARC.

In addition, our *cfos* results showed that mice receiving IP ghrelin injections display a significant increase in the expression of *cfos* in the PVN compared to mice receiving saline, MTII and MTII/ghrelin. In contrast, mice receiving IP MTII injections displayed a significant decrease in the expression of *cfos* in the PVN compared to mice receiving saline, ghrelin and MTII/ghrelin. These results are consistent with various studies demonstrating a role for ghrelin in influencing neuronal populations in the PVN (Morton, Cummings, Baskin, Barsh, & Schwartz, 2006; Shrestha, Wickwire, & Giraud, 2004; A. Solomon, De Fanti, & Martínez, 2006). To expand, the ARC plays a critical role in the integration of metabolic inputs between the periphery and the brain. It contains orexigenic AGRP/NPY neurons, and anorexigenic POMC neurons that project to the PVN in accordance with nutritional state (Cyr et al., 2013; Schwartz, Woods, Porte, Seeley, & Baskin, 2000). Ghrelin excites AGRP/NPY neurons and inhibits POMC neurons in the ARC to increase food intake during negative energy balance states. These effects are strongly mediated through AGRP/NPY projections to neuronal populations in the PVN (Morton et al., 2006; Shrestha, Wickwire, & Giraud, 2006; X. Zhang & Van Den Pol, 2016).

Several studies have shown that IP and ICV administration of ghrelin can cause a significant increase in cfos expression in PVN neurons in various animal models including rats, mice, and hamsters (Lawrence, Snape, Baudoin, & Luckman, 2002; Pirnik et al., 2011; Rüter et al., 2003). In addition, the administration of ICV GHSR antagonist can attenuate the orexigenic effects of ghrelin on the PVN, but not the ARC, highlighting an independent role for PVN as it pertains to central GHSR effects (Thomas, Ryu, & Bartness, 2016). Interestingly, studies show that administration of MTII can also attenuate the orexigenic effects of ghrelin on the PVN (Shrestha et al., 2004). This is consistent with our cfos results as we demonstrated significant main and interaction effects between ghrelin and MTII. To expand, we showed that effects of ghrelin on increasing cfos expression in the PVN are attenuated by the presence of MTII. Conclusively, the cfos data from the ARC and PVN demonstrate that both drug treatments were activating the expected brain regions target within the central melanocortin system and therefore serve as a validation that drug injections were successful.

Furthermore, results showed that mice receiving IP MTII injections display a significant decrease in the expression of cfos in the BNST compared to mice receiving saline, ghrelin and MTII/ghrelin. These results are supported by evidence showing that AGRP neurons project to various long-distant targets throughout the brain such as the PVN, PBN, BNST, CeA and MeA (Atasoy, Nicholas Betley, Su, & Sternson, 2012; Betley, Cao, Ritola, & Sternson, 2013; Dietrich, Zimmer, Bober, & Horvath, 2015; Padilla et al., 2016; Qi Wu, Clark, & Palmiter, 2012). Given the well-established role of the BNST in the regulation of fear states, stress and anxiety-related behaviour (Ahrens et al., 2018; Davis, Walker, Miles, & Grillon, 2010; Giardino et al., 2018), it is likely that AGRP projections to the BNST are involved in mediating AGRP-induced anxiolytic effects. In fact, emerging evidence shows that AGRP projections to the BNST are

critical in suppressing anxiety-like behaviour and territorial aggression (Burnett et al., 2016; Padilla et al., 2016). In addition, the optogenetic activation of AGRP axon terminals in the posterior BNST is sufficient in driving the preference for an immediate food reward compared to a delayed reward, suggesting an AGRP-induced anxiolytic effect. Similarly, our results highlight a role for AGRP projections into the BNST during social interaction. To expand, we showed that blocking the ability of AGRP from binding to its downstream MC3/4R through the administration of IP MTII significantly decreases AGRP activity as reflected by a significant decrease in the expression of *cfos* in the BNST.

Lastly, results showed that mice receiving IP ghrelin injections display a significant increase in *cfos* expression within amygdalar nuclei such as the CeA and MeA compared to groups receiving saline, MTII, and MTII/ghrelin. In contrast, mice receiving IP MTII injections displayed a significant decrease in the expression of *cfos* in these brain regions compared to mice receiving saline, ghrelin, and MTII/ghrelin. This is in line with evidence highlighting a role for ghrelin projections to the amygdala to affect food intake, memory, learning and motivated behaviours (Carlini et al., 2004; Diano et al., 2006; Tóth, László, & Lénárd, 2010). Similarly, studies show that GHSR is present among several amygdala nuclei such as the lateral and medial nucleus of the amygdala (Alvarez-Crespo et al., 2012). To expand, the release of GH protein from neurons in the amygdala is enhanced by the stimulation of GHSR (Meyer, Burgos-Robles, Liu, Correia, & Goosens, 2014). In addition, AGRP administered into the CeA results in a prolonged orexigenic effect, while MTII administered into the CeA can attenuate these effects (Boghossian, Park, & York, 2010). Similarly, evidence shows that AGRP projections to the MeA are involved in the suppression of territorial aggression and reduction of fear to promote foraging for food, highlighting a role for AGRP projections to the amygdala (Padilla et al., 2016).

Consistent results have been displayed in our cfos experiment as the administration of IP ghrelin resulted in a significant increase in cfos expression in the CeA and MeA, whereas the administration of IP MTII attenuated the effects of ghrelin on cfos expression in both brain regions.

## **Conclusion**

Based on the results from our experiments, our hypothesis predictions prove to be partially supported. In this present thesis, we were able to demonstrate a role for ghrelin administered peripherally in promoting the motivation for social interaction as reflected by a decreased latency to approach a novel-conspecific. In addition, we were able to demonstrate that effects of ghrelin maybe be independent of AGRP as no significant interaction effects were reported between treatments on any of the behavioural variables. Furthermore, our cfos data demonstrated that both drug treatments (ghrelin and MTII) were activating the expected target brain regions in the central melanocortin system and therefore provide a validation that the injections were successful. Our results are in line with various studies highlighting a role for ghrelin signaling in promoting the motivation for social interaction, whereas blocking of ghrelin signaling attenuates these effects. In addition, our results are consistent with data showing an anxiogenic response associated with the agonism of MC4R which enhances social vigilance. To expand, we were able to demonstrate that administration of MTII does in fact enhance social vigilance as reflected by an increased frequency of corner observations. One limitation to this conclusion is that effects of MTII on social vigilance during the social interaction test may likely be due to the agonism of MC3/4R, rather than the actual blocking of AGRP from binding to these receptors. Therefore, it is not possible to attribute the effects of MTII on social vigilance to

AGRP entirely. More research is required to investigate the direct contribution of AGRP on social behaviours. In addition, although no sex differences were reported on all behavioural measures except for the frequency of corner observations, it is important to note that we did not track the estrous cycle of females. To expand, evidence suggests that female social interaction with a novel-conspecific from the same sex is significantly different depending on the stage of the estrous cycle (Chari, Griswold, Andrews, & Fagiolini, 2020; Trainor et al., 2011). Therefore, it is not possible to make any conclusions regarding sex differences on the social interaction test without tracking the estrous cycle of females. Future studies are required to validate the sex differences on the social interaction test to provide better insights on the role of ghrelin in social behaviours.

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