Synopsis of the Proposal

Food intake and energy balance are regulated by various brain regions in response to internal and external cues. Specific regions of the brain that lie outside of the blood brain barrier (BBB) are uniquely positioned to sense the circulating factors from the body that signal the state of energy balance, but remarkably, little attention has been paid to these specialized regions. In published work and exciting preliminary data we have shown that one of these regions, the subfornical organ (SFO), responds to, and integrates, circulating information regarding energy status, and is involved in the control of food intake. Intriguingly, the SFO plays similar roles in sensing and integrating information critical to the regulation of cardiovascular and immune function, systems that contribute critically to the comorbidities associated with obesity. In the current proposal we will focus on the SFO to delineate the precise roles of this structure in collecting and integrating information critical to the hypothalamic regulation of energy balance. We will describe physiologically induced changes in gene expression in SFO. We will investigate potential dysregulation of the SFO in obesity and diabetes, as well as the roles of specific ion channels and intracellular signaling pathways in integrating information from physiologically diverse circulating signals. These studies will elucidate not only the roles of SFO as a critical player in the regulation of energy balance, but will also describe how dysfunction in SFO contributes to dysregulation of cardiovascular and immune function associated with the comorbidities of obesity.

Feedback Control Pathways in the Regulation of Energy Balance

The health challenge posed by the obesity epidemic, which now reports over 60% of the Canadian population to be overweight/obese (Stats Canada 2008), cannot be underestimated, either in terms of human suffering, or the costs to the health care system. In fact, obesity was declared a formal disease state for the first time by the American Medical Association in July 2013. Overweight and obese individuals are vulnerable to the comorbidities of diabetes and hypertension, and collectively these three diseases contribute to the triad referred to as “metabolic syndrome”. Considerable research has focused on understanding glucose intolerance and insulin resistance, as well as the defects in vascular function, and triglyceride and cholesterol handling which underlie the comorbidities of metabolic syndrome. However, far less attention has been paid to understanding the homeostatic abnormalities underlying what is effectively a breakdown in integrated central autonomic control culminating in these collective comorbidities. Increased awareness of the obesity epidemic in the 1990’s, combined with the discovery of leptin, led to many studies directed toward understanding the central nervous system (CNS) circuitry through which energy intake and energy expenditure are controlled in normal and pathological states. Such studies have led to the development of models describing the networks through which the brain controls feeding and metabolism (6;49;93). An integral part of such models is the inclusion of feedback control circuitry, through which circulating signals (e.g. glucose, adiponectin, amylin, cholecystokinin (CCK), insulin, leptin, peptide YY (PYY), ghrelin) provide information regarding energy status to the CNS, despite the fact that most of these messengers do not diffuse freely across the BBB.

Many of these models suggest a central role in such blood to brain signaling for the arcuate nucleus (ARC) in the hypothalamus (The Arcuate Theory), and a secondary role for the nucleus tractus solitarius (NTS) in the medulla. Although it has been suggested that these two regions are able to perform such sensory functions as a consequence of a “leaky” BBB (16;59), anatomical studies clearly show that the vast majority of capillaries in the ARC and NTS are not fenestrated (36;82). In addition, systemic horseradish peroxidase only reaches the ARC as a result of retrograde transport to ARC neurons from the median eminence (11), confirming that this nucleus is protected by the BBB. **Thus, the critical question as to how essential circulating signals describing energy status influence CNS control centres protected by the BBB remains unanswered.** Peptide specific transporters have been suggested to mediate blood to brain signaling (8), but their roles are not well defined and for many important metabolic/satiety signals they do not exist (63;87). The focus of my laboratory’s efforts over the last five

years was to study regions of the brain that lie outside of the BBB, collectively termed circumventricular organs (CVOs), as we believed that these would be involved in sensing and responding to circulating signals. We tested the hypothesis that ***"The sensory circumventricular organs play essential roles sensing and integrating information from circulating signals which inform the CNS of the energy status of the organism."*** Our studies have confirmed the medullary area postrema (AP) can collect and integrate such circulating information and, have been the first to describe similar roles for the forebrain SFO. The studies outlined in this proposal will be the first to examine the detailed roles of the SFO in collecting and integrating information from circulating signals of metabolic, cardiovascular and immune status, and subsequently controlling integrated physiological state.

Circumventricular Organs and the Regulation of Energy Balance

The CVOs are specialised, structurally unique CNS nuclei that lack the normal BBB. The AP, SFO, and organum vasculosum of the lamina terminalis (OVLT) are the only CVOs containing neuronal cell bodies, as opposed to nerve terminals. These three regions are classified as the "sensory CVOs" in view of their roles as critical integrative centres where circulating peptides act to regulate the cardiovascular, immune and neuroendocrine systems (18;28;33;56;92). Evidence suggests that the AP and SFO also play roles in sensing circulating metabolic signals. Importantly, our transcriptomic analysis of the SFO (39) and AP (40) showed that both express receptors for many of the critical circulating energy balance signals, including adiponectin, amylin, apelin, α-melanocyte stimulating hormone (αMSH), CCK, endocannabinoids (CB_1), glucagon like peptide 1 (GLP-1), leptin, insulin and PYY. These findings support the conclusion that the CVOs play important roles in monitoring these signals, and transmitting this information to critical autonomic control centres in the hypothalamus (SFO) or medulla (AP).

These microarray studies also showed that the transcriptome of the SFO (but not the AP) is dynamic, as it is modified by physiological state. Food deprivation (48hr) causes twofold changes in 687 transcripts, and fluid deprivation twofold changes in 44 genes (39), observations which suggest roles for SFO in the regulation of energy homeostasis. This suggestion is supported by our demonstration that electrical activation of SFO neurons stimulates food intake in satiated animals (86). We have also shown that while lesion of either AP or SFO in isolation does not influence long term food intake or body weight, lesion of both CVOs reduces both parameters (10), suggesting complementary sensory functions of these CVOs. Such double lesions also result in reduced patterns of *fos* activation in paraventricular nucleus (PVN) – the hypothalamic location of adrenal (CRH) and thyroid (TRH) control neurons, and in NTS in response to systemic GLP-1 and PYY receptor activation, confirming vital roles for these structures in sensing circulating signals (9;10).

While the AP was initially known for its ability to sense circulating toxins and trigger nausea and emesis (41), this CVO also plays roles in the regulation of energy balance (17;45;70), and in central cardiovascular regulation (12;27;60). Receptor localisation, (78), electrophysiological (67), and lesion studies (73) collectively identified the AP as a CNS site at which circulating amylin acts to inhibit food intake. Other studies have shown effects of GLP-1 (72), CCK (23;88), adrenomedullin (79;95), orexin (94), adiponectin (34), PYY (62), and ghrelin (33;48) on AP neurons.

Subfornical Organ

The SFO is a forebrain midline structure located on the dorsal floor of the third ventricle, and is primarily known for its well established roles in cardiovascular and neuroendocrine regulation (29;55). Roles for the SFO in anorexia and emaciation (89) have also been suggested. Primary projections of SFO neurons to the PVN, supraoptic nucleus (SON), median preoptic nucleus, OVLT, and ARC (37;50;58), ideally position this CVO to regulate energy balance. SFO neurons sense circulating signals involved in fluid (osmolarity, sodium, calcium), cardiovascular (angiotensin II (ANG), endothelin, vasopressin (VP)), and immune (interleukin 1β) regulation (18). Specific excitatory projections have been found to VP and oxytocin (OT) neurons in the SON and PVN, as well as to parvocellular areas of the PVN that

in turn project either to the median eminence (CRH, TRH neurons), the medulla (OT, VP, TRH neurons), or the spinal cord (OT,VP neurons) (28). More recent single cell recordings have shown direct effects of metabolic signals such as calcitonin (77), amylin (66;67), and ghrelin (64) on SFO neurons. Our recent transcriptomic analysis of the SFO (39) was the first to identify receptors for additional signaling molecules, resulting in the description of actions of adiponectin (4), apelin (20), insulin (47), leptin (84), and CCK (2) on SFO neurons.

Our data showing that food deprivation changes the expression of hundreds of genes in the SFO, identified the adiponectin receptor (increased AdipoR2) as one particularly interesting target. Altered levels of AdipoR2 expression were confirmed using qRTPCR, and functional consequences confirmed with patch clamp recordings showing that adiponectin depolarized 77% (compared to only 28% in controls) of SFO neurons from food deprived animals (4), thus providing direct evidence of functional consequences associated with these transcriptome changes induced by physiological state. Similarly, in the longer term diet induced obesity (DIO) model, we have shown that cardiovascular responses to leptin microinjection in the SFO are modified in DIO, effects that are not observed in age matched diet resistant (DR) animals (85). Additional dynamic changes in the SFO genes associated with CNS regulation of energy balance following food deprivation include the calcitonin receptor, cocaine and amphetamine related transcript (CART), OT, pro-melanin-concentrating hormone (PMCH) and signal transducer and activator of transcription 3 (STAT3) (39). Finally, we have identified true glucose sensing neurons in the SFO (57), and have preliminary data showing that acute (<24hr) changes in glucose concentration modify the responsiveness of SFO neurons to CCK. These findings highlight the role of physiological state in modifying the sensory abilities of SFO in the regulation of energy balance.

Thus, the SFO has now been identified as a new region of the CNS which performs critical roles in continually monitoring circulating metabolic, cardiovascular and immune signaling molecules. Intriguingly, the SFO, through its efferent connections to hypothalamic autonomic control centres, may then coordinate the integrated regulation of metabolic, cardiovascular, immune and neuroendocrine outputs. The studies outlined in this proposal are directed toward **four Specific Aims** (Figure 1) designed to systematically describe the integrative pathways through which the SFO exerts essential influence on the CNS circuitry controlling energy balance and associated autonomic function.

Specific Aim 1: We will describe the integrative roles of SFO in the regulation of energy balance

Until recently, the only studies assessing the roles of the SFO or AP on food intake and body weight were conflicting reports showing that AP lesions either increase or decrease feeding (24;46). We recently reported that while neither AP nor SFO lesion alone had long-term effects on food intake or body weight, combined lesions of these two CVOs results in long term reductions in body weight and food intake (10). We also have intriguing preliminary data showing that the inhibitory effects of **leptin on food intake are reversed by combined AP/SFO lesion** (Figure 2). In addition such lesions reduce the short term hypophagic effects of PYY (9), but are without effects on exendin-4 (GLP-1 receptor agonist) induced inhibition of feeding (10). Single and double lesions also influence patterns of PYY and exendin-4 induced *c-fos* activation in in PVN, SON, ARC and NTS (9;10).

Ultimately, these integrative roles of the SFO are dependent on the combined sensory roles of the entire population of SFO neurons. Thus, describing the properties of these single neurons will also be critical to understanding the functional physiological roles of the SFO. The fact that significant proportions (>25%) of SFO neurons respond to each signal described above clearly suggests that each neuron has receptors (i.e. sensors) for a number of these molecules. Further, two studies have reported that single SFO neurons can sense two different signals (64;84).

Hypothesis 1.1: The SFO and/or AP mediate metabolic responses to circulating signals

We propose a systematic analysis of the effects of ablation of these two CVOs on the regulation of feeding and metabolism to test the hypothesis that the SFO and/or AP mediate metabolic responses to circulating signals. *In vivo* studies will determine the effects of lesions of the SFO or AP, as well as

combined SFO/AP lesions, on body weight, feeding and drinking (using cages equipped for computer based monitoring) in response to a variety of circulating signals known to influence food intake and metabolism through actions in the CNS. Male Sprague Dawley rats (initially 150-180g) will be anaesthetised with sodium pentobarbital (65mg/kg, ip, as for all recovery surgeries), placed in a stereotaxic frame, and electrolytic lesions placed in either SFO, AP, or both CVOs by passing 1mA DC for 45s (parameters which produce localised lesions of these structures) through a monopolar tungsten electrode (tip exposure of 100μm) (31). Sham lesion animals (electrodes placed in SFO or AP but no current passed) will also be prepared. Animals will be housed individually and given appropriate post-operative care. Body weight, food and water intake will be measured daily 1 hour prior to lights off during all postoperative days. In order to ensure the effects on body weight and food intake induced by administered peptides are not a consequence of handling, restraint, and/or injections, rats will be treated with intraperitoneal (ip) injections of vehicle for 2 consecutive days, followed by 3 days of the test peptide. Peptide injection or vehicle volume will be calculated based on body weight obtained on the day of injection and administered (ip) 30 minutes prior to lights out, with the exception of ghrelin which will be given 4hrs into the light cycle. The following peptides will be tested in randomized order with each 3 day test period separated by at least 7 days recovery time: amylin (10μg/kg (51)), CCK (16μg/kg (2)), ghrelin (100μg/kg(35)) leptin (1mg/kg), and the αMSH analog MT-II (1mg/kg - (89), we have preliminary data showing cellular effects of αMSH in SFO – Figure 3). At the end of experiments, animals will be euthanized with urethane overdose, brains perfused, and 50μm coronal vibrato sections cut through hypothalamus and medulla. Sections will be mounted, stained with cresyl violet, and the locations of lesions identified by an investigator unaware of experimental outcomes. Animals will be grouped into SFO lesion, AP lesion, SFO/AP lesion, and Sham lesion groups, and data analysed to determine effects on food/fluid intake and body weight. Past experience and power analysis suggests 8 animals will be required in each group. Extensions of these studies will include similar lesion studies in DIO rats, and *c-fos* studies with specific peptides to identify roles for SFO in patterns of central activation (Coll. Dr. K. Sharkey, Calgary). Secondly, we will examine effects of selective lesion of SFO cell groups using toxins (CCK saporin – ATS, San Diego), or siRNAs for peptides, selected ion channels or critical components of cellular signaling systems (Coll. Dr. W Samson, St. Louis) on these circuits.

Anticipated Outcomes: We expect to confirm preliminary data showing that combined SFO/AP lesions **reverse** the inhibitory effects of leptin on food intake and body weight, but anticipate that SFO lesions alone may also have effects. We predict similar loss or reduction in the normal effects of amylin, CCK and MT-II (inhibition of feeding) and ghrelin (stimulation of feeding). These studies will determine roles for SFO or AP, or combined roles for these CVOs in mediating effects of these peptides.

Potential Pitfalls: All of these techniques are currently in use in my laboratory, and while the lesion approach may seem on the surface less sophisticated than modern targeted knockouts, we believe it represents the best technical approach for answering these specific questions. The most significant problem we envisage with these studies is obtaining enough double lesioned animals to meet our group size requirement, but extra animals will be prepared to allow for lesion misses.

Significance: These will be the first studies to provide a systematic analysis of the functional roles for these CVOs as **primary and essential** sites for the monitoring of critical circulating signals.

Hypothesis 1.2: Sensory abilities of SFO neurons projecting to ARC or PVN can be classified by expression of genes describing sensory function

We will describe both the sensory phenotype of SFO neurons and their connectivity by combining our now established single cell (sc) RTPCR technology (43) with retrograde labelling of single cells with fluorescent beads (5), allowing us to correlate the genomic sensory and signaling phenotype of single SFO neurons with their anatomical connectivity. Initially, we will prepare rats with microinjection of retrograde tracers into PVN and ARC in order to label SFO neurons as described previously (5). These output sites have been selected in view of established roles in the regulation of energy balance. Bilateral

microinjections (0.5µl) of fluorescent microspheres (Retrobeads, LumaFluor – selective uptake by terminals) will be made into ARC (red microspheres) and PVN (green - microspheres) of anaesthetized rats using stereotaxic techniques. Animals will be allowed to recover for 5 days (optimal time for retrograde transport) prior to dissociation of SFO neurons. The location of injections will be histologically verified in post mortem tissue (hypothalamic block retained during dissociation), and represents a critical component for these studies. Dissociated SFO cells will then be selected according to presence of fluorescent label (ARC *vs* PVN) and gentle suction applied through patch pipettes will be used to harvest the cytoplasm of single neurons (confirmed as neurons by the presence of voltage activated sodium currents). Cytoplasm will then be processed through two sequential rounds of PCR (multiplexed and then nested) with primer sets to detect mRNAs expressed in each neuron (34). We will use primer sets directed toward peptides (angiotensinogen (ATG), CCK, PYY, PMCH and OT), receptors (AT_{1a} (ANG), AdipoR1&2, calcitonin/RAMP2 (amylin receptor), CB_1, $CCK_{A\&B}$, GHSR (ghrelin), GLP-1, ObRb, IL1R (IL1β), $Y_{1\&2}$ (PYY)), ion channels from the transient receptor potential vanilloid (TRPV1, TRPV4) or inwardly rectifying potassium ($K_{ir}6.1$, $K_{ir}6.2$) families, and signaling proteins (EPAC1,2, PDE3B), shown to be expressed in SFO by our microarray data, which will form the focus of the studies described in Hypothesis 3 and 4. Neurons will also be processed for multiple housekeeping genes (GAPDH, HPRT), and for reactions in the absence of template or RT, providing robust controls. The identity of products will be confirmed by post hoc sequencing. Initially we will collect material from 120 SFO neurons, and data will correlate genomic sensory and signaling phenotype with connectivity.

Anticipated outcomes: We anticipate different profiles of neurons projecting to PVN *vs* ARC and anticipate that these data will identify complex single cell phenotypes (see preliminary data Figure 4) and highlight potential interactions between multiple metabolic and non-metabolic (e.g. AT_{1A}, IL1R), signals. It is also likely that TRPV *vs* K_{ir} expressing neurons may show different profiles of receptor expression, and/or different chemical phenotypes (e.g. OT vs CCK).

Potential pitfalls: scRTPCR technology is in routine use in my laboratory, and it is important to point out that 85% of neurons from which we harvest cytoplasm are GAPDH positive. Our two stage amplification process allows multiple primer sets to be run on mRNA from a single neuron (nb Qiagen kits in budget request). Anatomical labelling of neurons is also routine and does not interfere with PCR.

Significance: These will be the first studies to define the multisensory abilities of single SFO neurons, to correlate this with anatomical connectivity, and to begin to identify the ionic and signaling systems that transduce information in these cells. They represent a critical step forward in understanding the potential integrative abilities of neurons in the SFO.

Specific Aim 2: We will describe the role of physiological state in modulating the sensitivity of SFO neurons to circulating satiety signals through changes in gene expression.

We have used gene array technology to not only describe the transcriptome of the SFO, but also to identify changes in the expression levels of genes after physiological challenges. Thus, as described above, we have shown that food deprivation changes expression of many genes in the SFO (39), and have validated and described functional consequences of some of these changes (4). These findings show that there are functional consequences associated with regulation of the transcriptome by physiological state. However, this acute deprivation animal model, while simple, is neither well controlled nor ideally suited to understanding modifications to the transcriptome which may underlie the progressive physiological changes associated with development of the metabolic syndrome triad of obesity, hypertension and diabetes. We propose studies to investigate this changing transcriptome using a chronic physiological model of obesity and metabolic syndrome, and an acute cellular model of systemic glucose dysregulation relevant to diabetes.

We believe that the diet induced obesity (DIO) model is a more appropriate model to study chronic changes associated with metabolic syndrome (76). This model presents rats with a high fat diet and approximately 1/3rd develop the characteristics of metabolic syndrome, 1/3rd maintain the phenotype of chow fed (diet resistant (DR)), and these two groups are compared to age-matched normal chow fed animals (Chow). The final 1/3rd which have an intermediate body weight are excluded from analysis. Using this model, we have shown that the depressor effects of acute microinjection of leptin into the SFO are lost in DIO, but not DR or regular chow groups (85), and that AT_1 receptor blockade with losartan reduces food intake and stops all weight gain in both DIO and DR rats (Figure 5). These results suggest intriguing interactions between the high fat diet, leptin and AT_1 receptors, and speculate that such interactions occur at the SFO. Interestingly, preliminary data (Collaboration Dr. K. Sharkey) show that the high fat diet (DR and DIO) induces increased basal *c-fos* expression in SFO (Figure 6), and loss of inhibitory effects of CCK on food intake in these two groups of animals (Figure 7), further highlighting the dynamic functional phenotype of the SFO in the DIO model.

Many studies have clearly identified the critically important role of circulating glucose in not only providing the essential "fuel supplies" to maintain normal function in all tissues throughout the body, but also as a short term signal of energy status to the brain (52). Glucose sensing neurons have been identified in the ARC (90), ventromedial hypothalamus (22), lateral hypothalamus (13;14), and dorsal vagal complex (7), although their importance may have been overstated as these studies ignored the inability of glucose to diffuse across the BBB. In contrast to these areas **located behind the BBB**, the glucose sensing neurons in the SFO (57) and AP (1;68), can directly monitor circulating glucose concentrations and transmit this essential information to medullary (AP connections), and hypothalamic (SFO connections) metabolic control centres protected by the BBB within the CNS. Intriguingly, we now have preliminary data showing that acute (<24hr) changes in glucose concentration in the media in which dissociated SFO neurons are cultured modifies both CCK receptor expression and responsiveness of these neurons to CCK. We thus propose to use this model system to study the ability of acute changes in physiological state (hypo vs hyperglycemia, the latter presenting a pseudo diabetic state) to modify the functional sensory transcriptome of SFO neurons in a precisely controlled system.

Hypothesis 2.1: The high fat diet and subsequent diet induced obesity modifies the transcriptome and, thus, sensory functions of the SFO.

Our goal is to assess changes in the SFO transcriptome that accompany chronic obesity and to describe the functional consequences of such alterations. Our past transcriptomic analysis of the SFO in collaboration with Drs. Hindmarch and Murphy (Bristol University) utilised Affymetrix GeneChip microarrays for expression profiling of the SFO in normal, food deprived, and fluid deprived states (39). However, in order to maximise the efficiency of data acquisition and to ensure that all genes, coding as well as non-coding (including micro-RNAs) are included in the analysis, we will switch to Illumina deep-sequencing (RNAseq). Unlike microarrays, RNAseq is not limited to detecting transcripts that correspond to existing genomic sequences but can also quantitatively examine splicing diversity of genes, non-coding events, and potential single nucleotide polymorphisms (SNPs). We have obtained preliminary data using RNAseq in a small number of samples, illustrating the technical feasibility of this approach and suggesting intriguing changes in AT_1 receptor expression (Figure 8). We will profile global gene expression patterns in the SFO as well as critical forebrain structures receiving direct neural projections from this CVO involved in regulation of energy balance and cardiovascular regulation, namely the ARC and PVN. Upon arrival, male Sprague–Dawley rats (125–150 g) will be housed in pairs under a 12:12 h light/dark cycle in a temperature-controlled room and exposed to a high-fat diet (#D124521, composition 45% kcal fat, 35% kcal carbohydrate, and 20% kcal protein; Research Diets, New Brunswick, NJ, USA) with water available ad lib. Weight gain will be measured on a weekly basis and, after 10 weeks animals are divided into DIO or DR groups based on weight gain. Five rats will be required to provide enough tissue for each experimental group, and all 3 brain regions will be obtained

from each animal. The experiment will consist of: 3 experimental groups (Chow/DR/DIO) x 4 independent replicates x 3 tissues (SFO/ARC/PVN) = 36 indexed libraries. Thus, 20 animals will be required for each treatment group. Eight indexed libraries will be pooled and sequenced in a single lane of HiSeq2500 with 100bp paired end reads. Thus, 4 lanes of a flowcell will be required to generate between 30-40 million PE reads per sample. Total-RNA will be extracted from the different experimental groups and prepared according to a ribodeletion protocol allowing both coding and non-coding features to be resolved using RNA-sequencing (RNAseq; 100bp ~30million Paired-End reads). Read alignment, transcript identification, and expression/junction analysis will be performed using established pipelines created within the Galaxy environment (http://galaxyproject.org/) and using novel algorithms in development. We will assemble RNA libraries and identify the expression of the entire transcriptome from each sample, in order to build up a profile of gene expression in SFO of the DIO obese phenotype by comparison to Chow and DR groups. These experiments will also allow us to describe changes in the DIO group in both ARC and PVN, and also identify genes in all three regions changed by the high fat diet rather than obesity (changed in both DR and DIO groups compared to Chow).

Anticipated outcomes: These experiments will provide the first comprehensive description of the transcriptome of the SFO, PVN, and ARC using RNAseq techniques. They will provide information about changes in gene expression associated with high fat diet and diet induced obesity and allow us to distinguish between the two. Finally, they will produce detailed quantitative data on specific transcripts (receptors, ion channels, intracellular signaling molecules) which are the focus of Aims 3 and 4.

Potential pitfalls: We do not anticipate any major technical problems in completing these studies.

Significance: While these studies are **not specific hypothesis driven**, we believe this limitation is profoundly outweighed by the depth of transcriptomic detail obtained. The information we will obtain will identify new targets, and will highlight the complex reality of physiological control systems being dependent on a dynamic physiologically regulated transcriptome.

Hypothesis 2.2: Acute changes in glucose concentration modulate responsiveness of SFO neurons to circulating signals

We have recently discovered that SFO neurons are responsive to changes in extracellular glucose, identifying the SFO as a sensor of circulating glucose concentrations (57). Intriguingly, we have also obtained preliminary data showing that the responsiveness of SFO neurons to CCK is profoundly changed by the glucose concentration (1mM *vs* 5mM *vs* 10mM – Figure 9) the neurons are maintained in (culture and recording). In addition, CCK_1 receptor expression increases 4.1 fold in 5mM glucose and 4.9 fold in 10mM glucose compared to 1mM glucose, while CCK_2 expression is unaffected. Whole cell recordings will be obtained from dissociated SFO neurons (30) maintained in medium at glucose concentrations of 10mM (hyperglycemic), 5mM (normoglycemic), and 1mM (hypoglycemic) to mimic the broad physiological range in plasma, with osmolarity in the latter two adjusted to 290-295mOsm using mannitol. Changes in membrane potential will be used to classify neurons depolarized, hyperpolarized or unaffected by CCK while input resistance (slope of V/I curves run at 120s intervals), and spike frequency (% increase from control) will also be monitored to provide additional information about cellular mechanisms of action. Similar electrophysiological studies will be carried out to determine if basal glucose concentrations influence responsiveness of SFO neurons to other metabolic signals including insulin (10nM (47)), leptin (10nM (83)), and ghrelin (10nM (64)). We will also examine interactions of glucose with (traditionally) non-metabolic signals such as ANG (10nM (30)) and IL1β (100pM (21)), to probe for interactions between metabolic, cardiovascular and immune signals within SFO neurons. In all cases parallel experiments will assess changes in receptor expression in dissociated SFO neurons cultured in medium containing 1mM, 5mM, or 10mM glucose for 24 hours. While these experiments could be completed using total RNA extracted and prepared for real time qRTPCR (CCK receptor data above), we propose analysis of the whole transcriptome using RNAseq as described above (Hypothesis 2.1) in these 3 groups with 4 replicates (5 microdissected SFOs for each replicate) per group

to provide the same depth of analysis outlined above at a similar cost. We will also use this approach to examine the time course (6, 12, and 18hrs) of glucose induced changes.

Anticipated outcomes: We expect to confirm that at higher concentrations of glucose a much larger proportion of SFO neurons depolarize in response to CCK. These studies will identify interactions between metabolic, cardiovascular and immune signals which may identify SFO as an intriguing target contributing to the comorbidities of obesity, diabetes and hypertension. RNAseq will again provide critical parallel data with the added advantage on the identification of novel targets.

Potential pitfalls: No problems are anticipated as preliminary data and all techniques are in place.

Significance: These studies will describe the ability of one metabolic signal (glucose) to modify the transcriptome of the SFO thus changing the ability of neurons to monitor other signals. The concept of this **dynamic transcriptome** is, in my view, a profoundly important one for us to grasp if we are to ultimately understand the complex interactions underlying physiological control in these systems.

Specific Aim 3: We will describe integrative roles for non-selective cation (NSCC) and ATP sensitive potassium (K_{ATP}) channels in processing metabolic, cardiovascular and immune signals in SFO neurons

The membrane potential of SFO neurons is regulated by a large number of circulating signals which provide critical information to the CNS concerning current homeostatic state. While the ability of single neurons to sense multiple signals has been described (64;83), little is known about the cellular mechanisms through which these signals are integrated. One potential target for such integration are ion channels, whose modulation is at least partially responsible for the effects on membrane potential induced by circulating signaling molecules. Two channels known to play critical roles in regulating resting membrane potential in SFO neurons are NSCCs (primarily TRPVs) and K_{ATP} channels. In SFO neurons these channels are regulated by multiple signals, NSCCs being modulated by ANG, ghrelin, PYY, and apelin (20;44), and K_{ATP} channels by glucose, and insulin (47;57). We will first describe the effects of leptin, CCK, and αMSH on these channels, thus setting the stage for the critical subsequent analysis of interactions between different signaling molecules at specific ion channels.

Hypothesis 3.1: Non- selective cation channels represent a point for integration of metabolic, cardiovascular, and immune signals in SFO neurons

Whole-cell voltage- and current-clamp recordings will be obtained from dissociated SFO neurons (maintained in 5mM glucose) to examine the effects of leptin, CCK, and αMSH on NSCCs. We will use techniques which we have previously employed to describe effects of Ca^{2+} (91), ghrelin (64) and IL1β (21) on this conductance. We will use the voltage-clamp recording configuration to measure the currents evoked by slow voltage ramps (12 mV/s, -90mV to 0mV) applied first in control artificial cerebrospinal fluid (aCSF), followed by 5 minutes in 10nM leptin, CCK or αMSH (preliminary data for leptin – Figure 10), and finally by a return to aCSF. Ramps will be collected at 60s intervals and an average of at least 3 ramp induced currents will be used to establish mean responses. Control currents will be subtracted from currents recorded during peptide treatment, with the resultant difference current representing the peptide induced current. The recovery currents measured following a return to aCSF (must be similar to initial control) are also essential to this protocol to confirm that any observed effects are not simply the result of "run down" of currents. In neurons still displaying robust electrophysiological properties (input conductance <1nS, series resistance <20MΩ), further ramps will be run in the presence of ruthenium red (10μM (80)) to block the NSCC, allowing us to define the total magnitude of this conductance in these neurons (subtraction of final ramp from original ramp).

We will next examine interactions of different signals through NSCCs by determining the effects of acute (24hrs) pre-treatment of dissociated cells with pathophysiological concentrations of known modulators of NSCC including ANG (10nM - cardiovascular signal), leptin (10nM - increased in

metabolic syndrome), or IL1β (1nM -immune signal), and again assess effects of αMSH, CCK, on NSCC using voltage-clamp protocols and comparing population responses (min. 10 neurons) in each condition.
Anticipated outcomes: We predict confirmation of leptin effects on NSCCs, and anticipate that CCK and αMSH will also influence this conductance. Experiments looking at interactions are harder to predict but we anticipate convergence of these signals such that pre-treatment with ANG for example will occlude effects of CCK /leptin/αMSH on the NSCC.
Potential pitfalls: We do not anticipate technical problems assessing effects of single peptides on NSCC. Studies examining interactions may require adjustment of pre-treatment times and concentrations.
Significance: These studies will be the first to describe effects of leptin, CCK, and αMSH on NSCCs. In addition, they will describe how this conductance represents a point of convergence for multiple signaling molecules. They may explain how traditional cardiovascular, immune and metabolic signals have convergent actions on single neurons responsible for controlling integrated autonomic output, thus contributing to the comorbidities associated with obesity.

Hypothesis 3.2: K_{ATP} channels represent a point for integration of integration of metabolic, cardiovascular and immune signals in SFO neurons

We will undertake analysis of K_{ATP} channels by measuring whole cell currents (voltage clamp recordings) in response to slow voltage ramps (12 mV/sec from -100 to –20 mV) in SFO neurons before, during, and after bath administration of 10nM αMSH, CCK, insulin, leptin, adiponectin (concentration eliciting peak effects on membrane potential). Data will be analyzed by comparing the mean of three consecutive ramps in each condition, and by subtraction to determine the difference current which represents the peptide-induced current. The recovery currents measured following a return to aCSF will again be confirmed (as in 3.1 above). Finally, in each cell a set of ramps will be run in the presence of the K_{ATP} channel blocker glibenclamide (500nM) to assess the percentage of active K_{ATP} current blocked by each peptide. Using current clamp techniques, we will examine the ability of pre-treatment with glibenclamide to abolish effects of these signals on the membrane potential of SFO neurons. Furthermore, we will examine whether application of glibenclamide during αMSH, CCK, leptin, insulin, and adiponectin induced responses reverses the effect of these peptides.

We will examine interactions of different signals at K_{ATP} channels by, as for 3.1, determining the effects of acute (24hrs) pre-treatment of dissociated SFO neurons with physiological concentrations of adiponectin (10nM – (4)), or glucose (10mM – see 2.2), both of which modulate K_{ATP}, and again assess effects of αMSH, CCK, insulin and leptin on K_{ATP} current measured using voltage clamp protocols.
Anticipated outcomes: These studies will describe effects of αMSH, CCK, leptin, insulin, and adiponectin on K_{ATP} channels. We anticipate that we will show that glucose modifies the responses of SFO neurons to CCK, and may also identify permissive roles for adiponectin enhancing insulin actions.
Potential Pitfalls: Same as those outlined for Hypothesis 3.2.
Significance: These studies will be the first to describe effects of leptin, CCK, and adiponectin on K_{ATP} channels. In addition, they will identify the mechanisms through which this conductance represents a point of convergence for multiple signaling molecules.

Specific Aim 4: We will describe integrative roles for cAMP in SFO neurons in the sensory processing of metabolic, cardiovascular and immune signals

Many circulating signals influence functions of SFO neurons by binding to G protein coupled receptors (GPCRs) and modulating intracellular signal transduction pathways (3). Many GPCRs act through either increasing (Gs – amylin, αMSH, GLP-1) or decreasing (Gi/Go – ANG, apelin, PYY) adenylate cyclase activity and thus regulating cAMP concentrations in individual cells (Figure 11). Other signals, including adiponectin, CCK, ghrelin, and leptin can indirectly regulate cAMP as a consequence of actions on cyclic nucleotide phosphodiesterases (PDEs), which catalyze the breakdown of cAMP (25),

or through systems utilizing nitric oxide or Ca^{2+} to indirectly regulate cAMP levels (Figure 11). Intracellular changes in cAMP levels modulate cell function through actions coordinated by either PKA or exchange protein activated by cAMP (EPAC)-dependent signaling pathways, or by coordinated actions of both effectors (54) (Figure 11). The majority of cAMP-mediated events in cells are now believed to be coordinated through local actions of cAMP within separate and largely non-overlapping pools (42) such that not all cAMP increases in cells, including neurons, are created equal. We propose specific studies to determine the role of cAMP as an intracellular molecule integrating information from a variety of circulating signaling molecules in SFO neurons. Importantly, since recent findings have shown that PKA and EPAC can both regulate the activities of the ion channels discussed in Aim 3 (15;53;97) we propose that these studies will provide mechanistic links between the studies in Aim 3 and this aim.

Hypothesis 4.1: Amylin, αMSH, and GLP-1 influence SFO neurons through cAMP-mediated mechanisms

Circulating amylin, αMSH and GLP-1 all inhibit food intake (38;51;61;65), activate SFO neurons (32) and signal through GPCRs linked to Gs (81). We will use Förster resonance energy transfer (FRET)-based reporters, built using the cAMP-binding domain of the cAMP-effector, EPAC1, to analyse real-time changes in cAMP levels in living cells. In collaboration with Dr. D. Maurice (Queen's University), using an adenovirus that encodes one such FRET-based cAMP reporter, we have developed a system for analysis of changes in cAMP in dissociated SFO neurons. Preliminary data (Figure 12) shows that amylin (10nM) increases cAMP levels in a subpopulation of SFO neurons to approximately 25% of that induced by a maximal concentration of the adenylate cyclase activator, forskolin (10μM) indicating that this peptide only controls a part of the total pool of cAMP in each neuron. We will describe the role of cAMP signaling in mediating the effects of amylin by first confirming these observations and then establishing the proportion of SFO neurons influenced by amylin, and the concentration-dependence (100nm-1pM) of this effect. Subsequently we will examine the roles of EPAC and/or PKA in activating SFO neurons in patch clamp experiments. In preliminary studies we have shown that the selective EPAC agonist [8-CPT-2Me-cAMP] promotes Ca^{2+} entry in individual SFO neurons, while the EPAC inhibitor CE3F4 decreases blood pressure in rats when applied *in vivo* to the SFO (Figure 13). Whole cell current clamp recording will be obtained from dissociated SFO neurons and effects of amylin on membrane potential established. Neurons showing depolarizing responses will then be tested a second time with amylin in the presence of EPAC (CE3F4, 1-10μM - (19)) or PKA (St-PKI 1-10μM) inhibitors to establish their respective roles in mediating the effects of amylin on membrane potential. Since localized cAMP events in cells are coordinated through the actions of the PDEs, experiments to delineate the roles of these enzymes in regulating cAMP-mediated effects in these cells will be studied using PDE inhibitors applied via our patch pipette. Should differential effects of PDE1, PDE3, PDE4 and PDE5 be observed, FRET experiments, similar to those described above, using FRET constructs engineered to be targeted to distinct subcellular domains, will be used to identify specific subcellular areas (soma vs dendrites) in which cAMP may be increased. Technically similar experiments will be carried out with GLP-1 and αMSH using both FRET, to directly assess changes in cAMP, and patch clamp techniques to determine the relative contributions of EPAC and PKA in the activation of SFO neurons.

Anticipated outcomes: We expect to identify amylin, GLP-1 and αMSH actions to increase cAMP in single SFO neurons. Our studies will describe complex interactions between these systems identifying subcellular pools of cAMP involved as well as the additive or occlusive nature of such integration.

Potential pitfalls: The primary technical issue with these studies is the potential complexity of the interactions we will uncover. The subcellular localisation of FRET reporters represent a significant technical asset as does the established expertise of the Maurice laboratory with these techniques in vascular smooth muscle and endothelial cells.

Significance: These studies will be the first to describe interactions between different circulating signals and their separate GPCRs, all of which are linked through Gs to cAMP in single SFO neurons.

Hypothesis 4.2: Leptin, adiponectin, CCK and ghrelin modulate cAMP mediated regulation of SFO neurons

Recent work has reported intriguing synergistic interactions between amylin and leptin (71) to cause weight loss in both rats and humans (clinical trials in progress for treatment of obesity using such combined therapy)(71). Amylin and leptin influence the excitability of SFO neurons (69;84), while leptin and adiponectin have been shown to act in the periphery and the CNS to modulate cAMP signaling as a consequence of the regulation of PDEs, especially PDE3B (26). Thus, actions of leptin, adiponectin, CCK and other non-Gs mediated signals may, at least in part, be related to the modulation of PDE3 (74;75;96). We will focus initially on determining if pre-treatment with leptin (10nM – 1hr) influences amylin induced increases in cAMP using the FRET-based approach detailed above, and if such effects result from actions on PDE3B (abolished by pre-treatment with PDE3B inhibitors) as has been shown in vascular endothelial cells. We will also determine if circulating signals such as ANG and CCK, which also modulate different PDEs, differentially modulate amylin actions. Experiments will next examine similar interactions of PDEs with αMSH and GLP-1 cAMP signaling described in 4.1. Confirmation of such interactions will lead to additional experiments to determine the relative role of different PDEs in the modulation of cAMP-mediated actions and to regulate separate or potentially integrated pools of EPAC and PKA which are involved in coordinating cellular events in SFO neurons.

Anticipated outcomes: We expect that separate Gs driven pools of cAMP will be regulated by differential effects on different but specific PDEs.

Potential pitfalls: The primary technical issue with these studies is again the potential complexity of the interactions we will uncover.

Significance: These studies will unravel the complex intracellular molecular interactions through which circulating signals control SFO neurons, and thus energy balance and cardiovascular regulation.

Timelines

The lesion and single cell classification (Aim 1) will be initiated on funding and anticipated to be completed in the first 2 yrs. of this grant. RNAseq analysis of the DIO model will be initiated in Yr. 1 and completed by the end of Yr. 3, while analysis of the effects of glucose on SFO neurons will begin in Yr. 3 and be completed by the end of Yr. 4 (Aim 2). Experiments outlined in Aim 3 and 4 will continue throughout the 5 Yr. term of this grant.

Significance

The studies described in this proposal will be the first to show that the SFO plays critical roles in sensing and integrating circulating information regarding energy status, and will likely provide the impetus for inclusion of these regions of the brain in models of the CNS circuitry controlling energy balance. Our work will also identify this forebrain CVO as a potential site for integration of metabolic and cardiovascular signals, which may contribute to the established comorbidities associated with obesity including diabetes, and cardiovascular disease. Our description of specific neurons, ion channels, and signal transduction pathways as important points of convergence for multiple signals will identify new targets for the treatment of these diseases. Thus, these studies will elucidate not only the roles of SFO as a critical player in the regulation of energy balance, but will also describe the potential for dysfunction in this structure to contribute to combined dysregulation of autonomic function associated with the comorbidities of obesity.

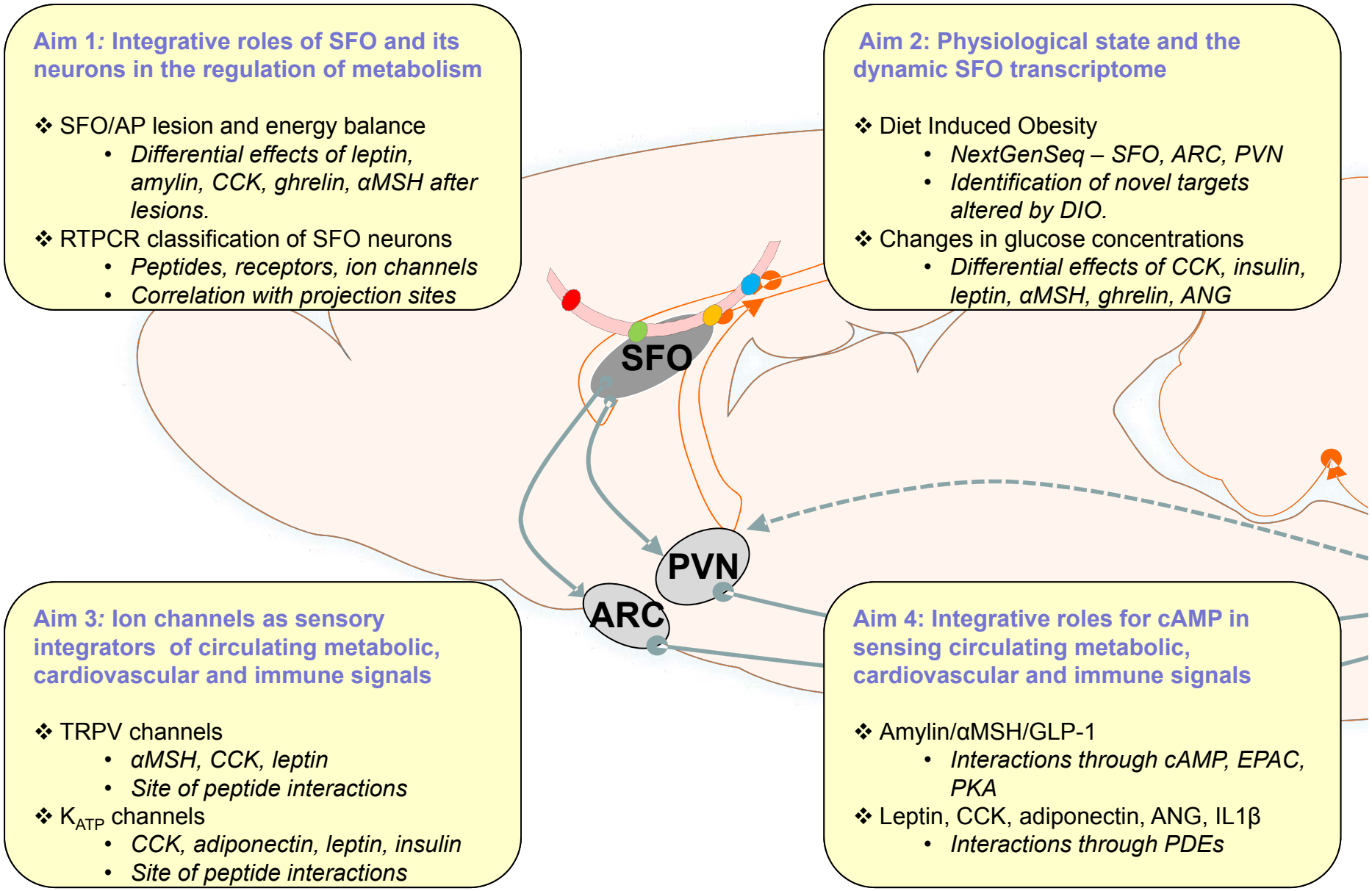


Figure 1: This schematic illustrates the anatomical location of the SFO, its ability to sense circulating signals delivered through fenestrated blood vessels, and its primary neural efferents. It also summarises the 4 Aims described in this proposal.

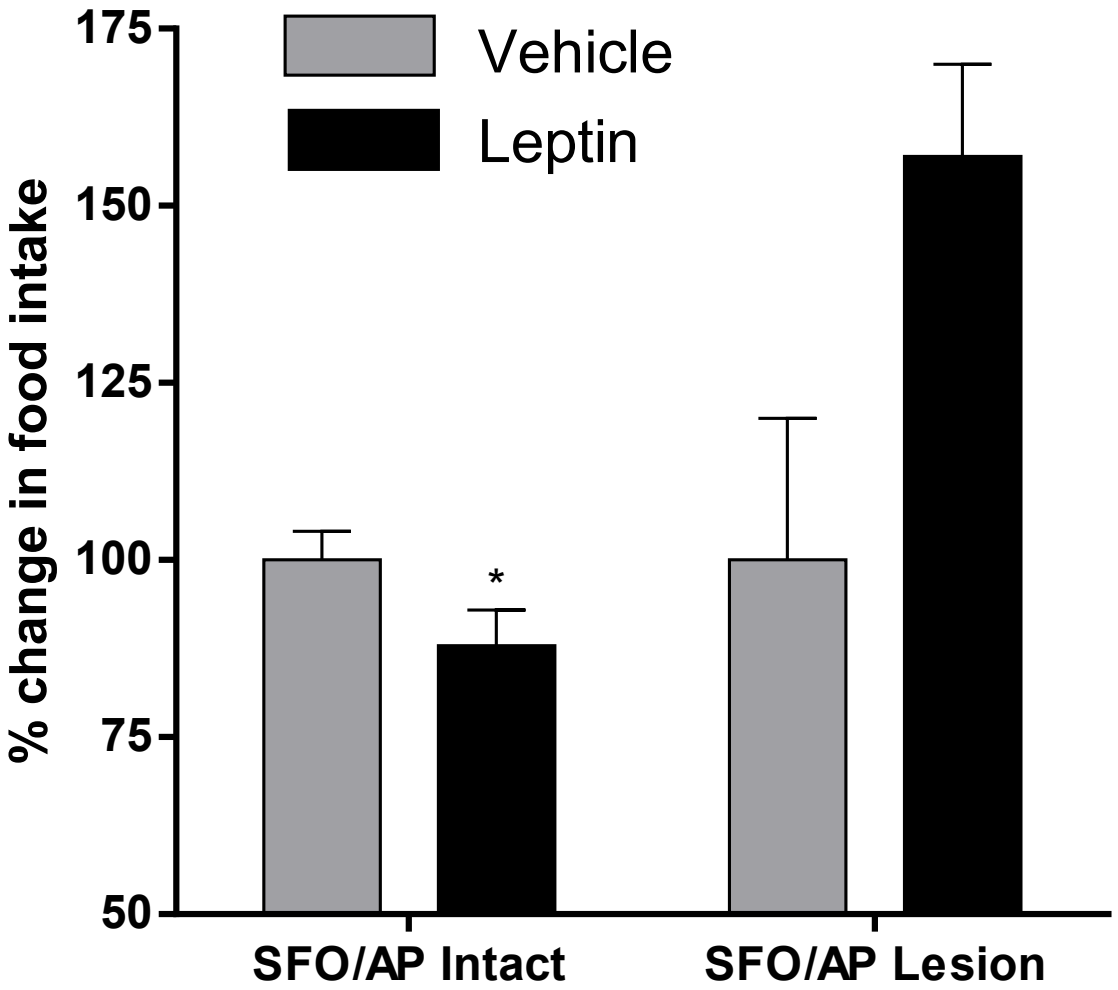


Figure 2: **SFO/AP ablation abolishes inhibitory effects of leptin on food intake:** This figure illustrates food intake in animals following IP injection of either vehicle or leptin in rats with SFO/AP intact (n=6) or these two CVOs lesioned (SFO/AP lesion n=4). Notice that the significant inhibitory effects of leptin on food intake are reversed, such that leptin actually enhances food intake in lesioned rats.

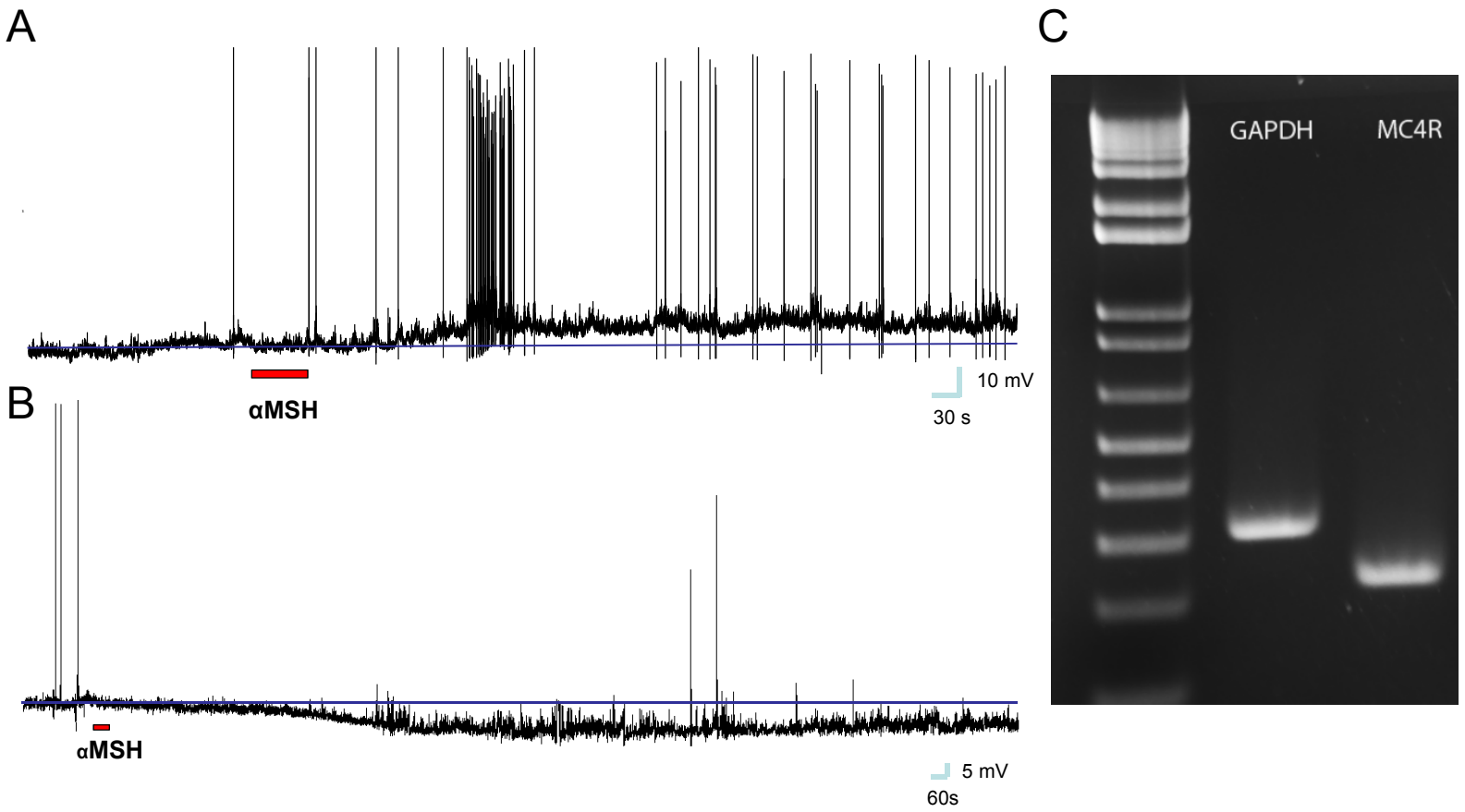


Figure 3: **The SFO is a target for circulating αMSH:** Current clamp recording from single SFO neurons are shown in A and B and illustrate the effects of bath administration of 10nM αMSH at the time of the red bar. Similar proportions of SFO neurons show either depolarization (A 4/11) or hyperpolarization (3/11) in response to αMSH. The PCR gel shown in C illustrates clear bands from whole SFO tissue confirming expression of the to αMSH receptor, MC4R, expression in the SFO.

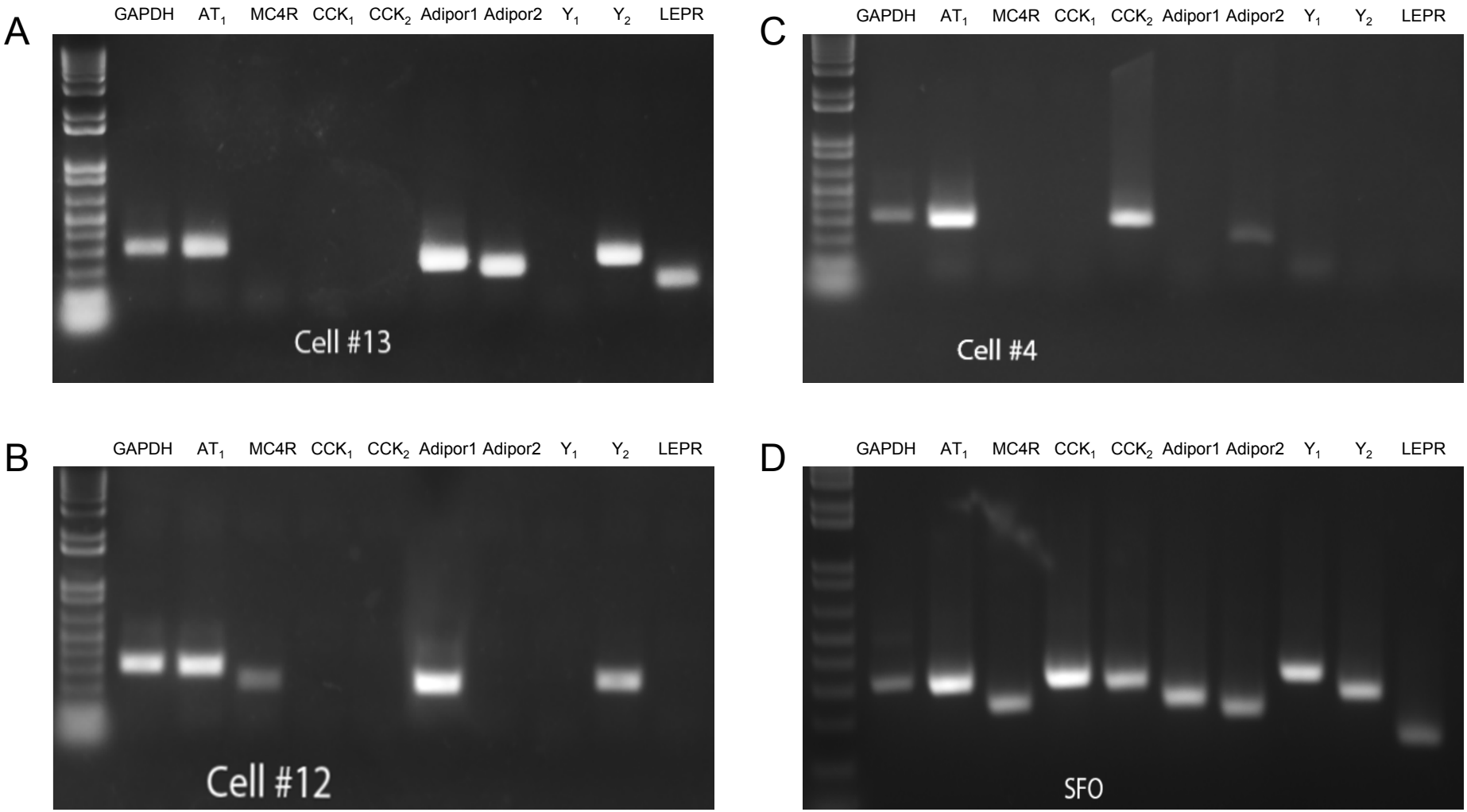
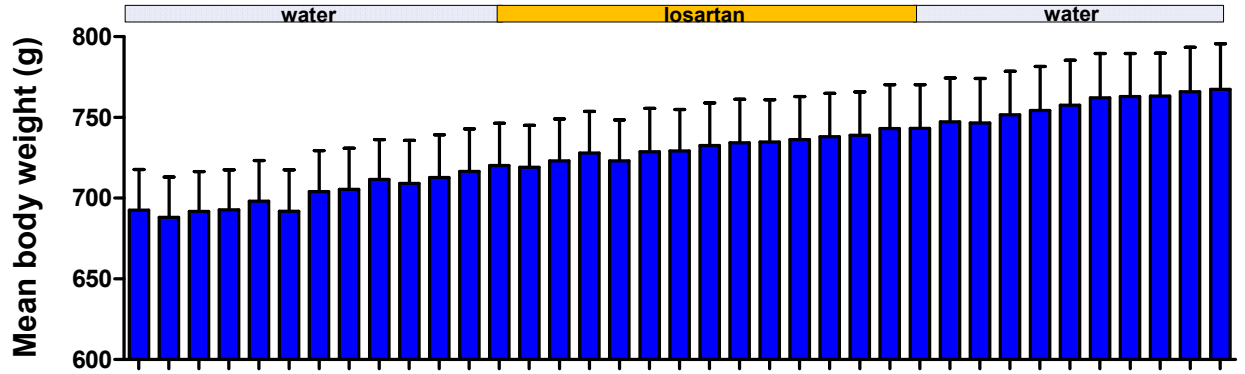


Figure 4: **SFO neurons show different phenotypes defined by single cell RTPCR:** Agarose gels of single cell RTPCR products in individual SFO neurons (A,B and C) and from whole SFO tissue (D). Lanes in all gels including the housekeeping gene GAPDH, the angiotensin type 1A receptor (AT_1), the αMSH receptor (MCR4R), the cholecystokinin receptors (CCK_1, CCK_2), adiponectin receptors (Adipor1, Adipor2), the PYY receptors (Y_1, Y_2), and the leptin receptor (LEPR). Note all genes are expressed in whole SFO tissue, in contrast to different combinations in each individual neurons.

Chow



DIO

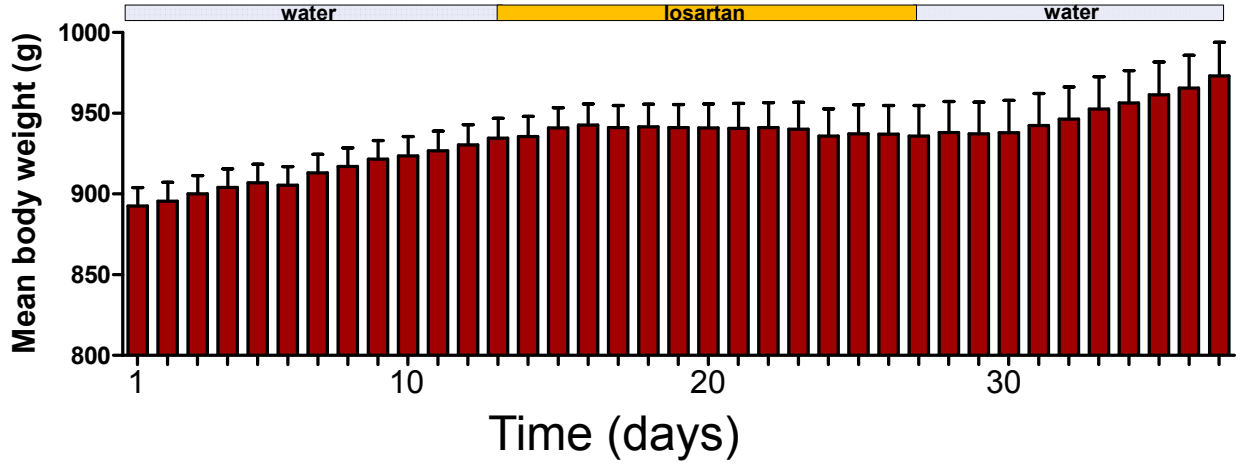


Figure 5: **AT_1 receptor blockade prevents weight gain in rats on a high fat diet:** These panels shown mean body weight in age matched control rats fed standard rat chow (Chow – upper panel) and DIO rats fed a high fat chow, before during and after oral administration of losartan (orange bar) for a period of 14 days.

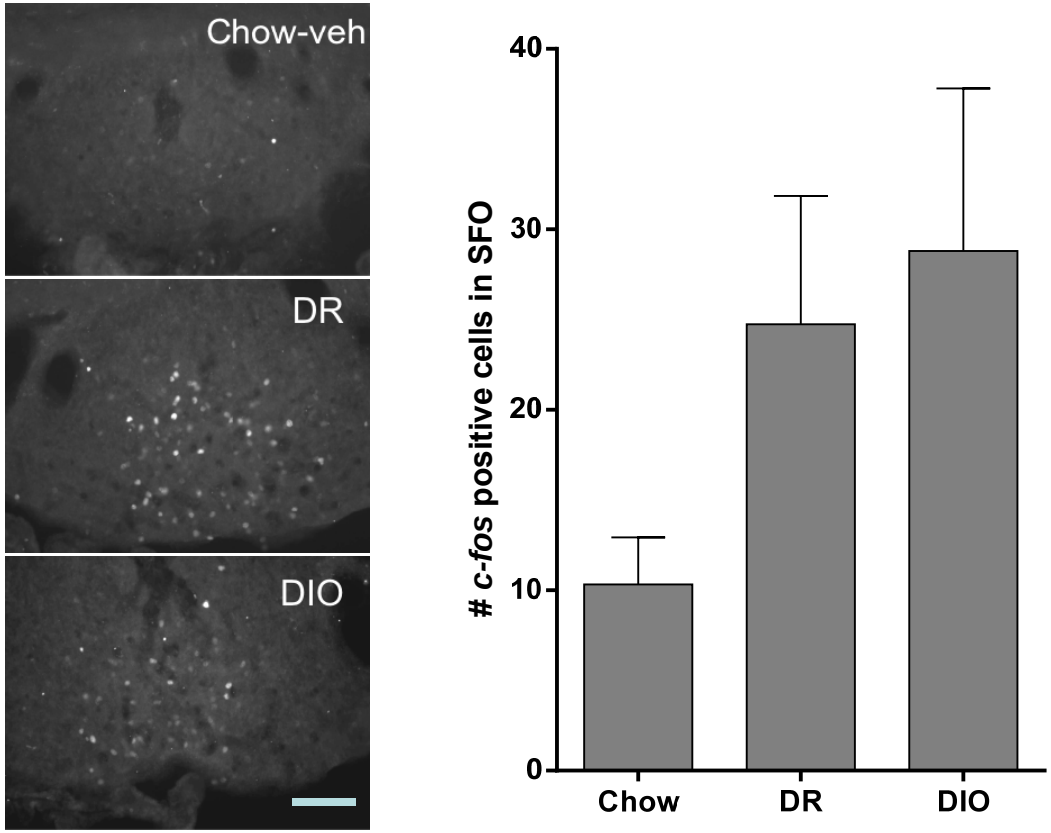


Figure 6: **High Fat Diet Increase Basal Activity in SFO Neurons:** The photomicrographs on the left show pictures of coronal sections through the SFO with *c-fos* labelled neurons (white dots – scale bar 0.1mm) in Chow, Diet Resistant (DR) and obese (DIO) rats, illustrating the increased labelling in the latter two groups. Data from 4 rats in each group are summarised in the bar graph on the right.

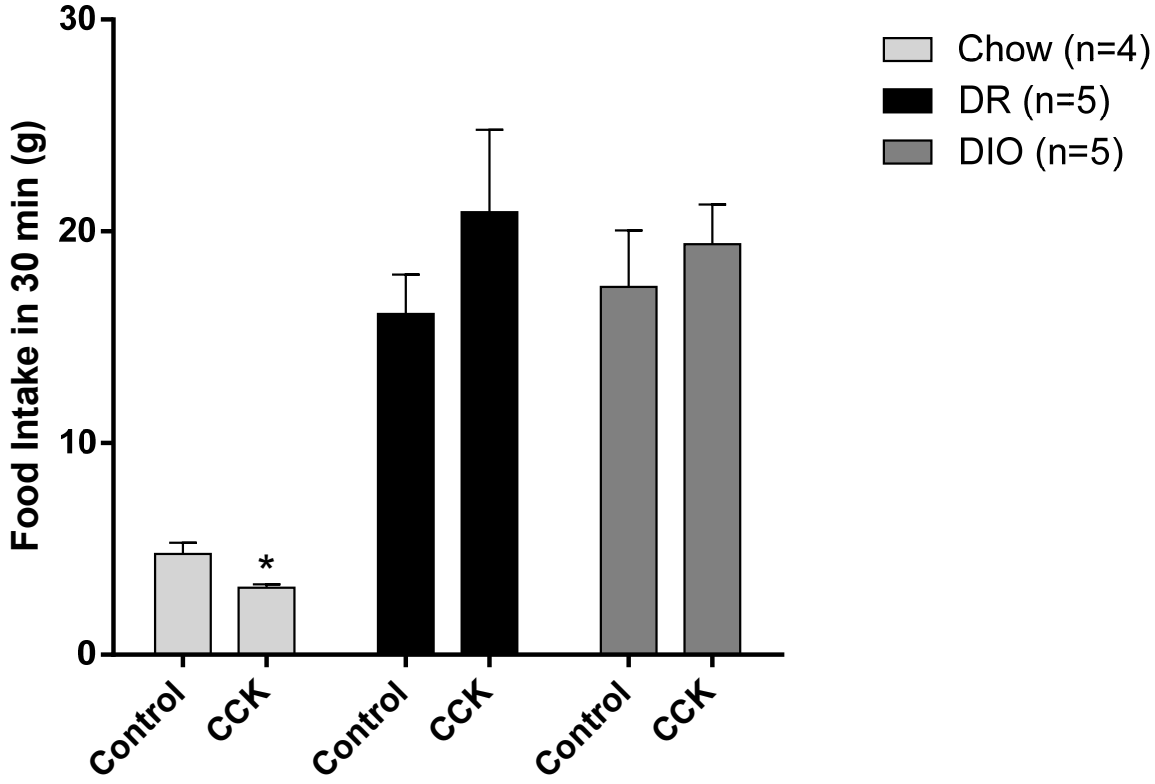


Figure 7: **DIO rats no longer respond to IP CCK:** This bar graph summarizes data examining the effects of ip CCK on food intake. Chow fed rats show the typical inhibition (*) of 30min food intake. In contrast, CCK is without effect on food intake in both DIO and DR rats suggesting that the high fat diet modifies CNS sensing of CCK.

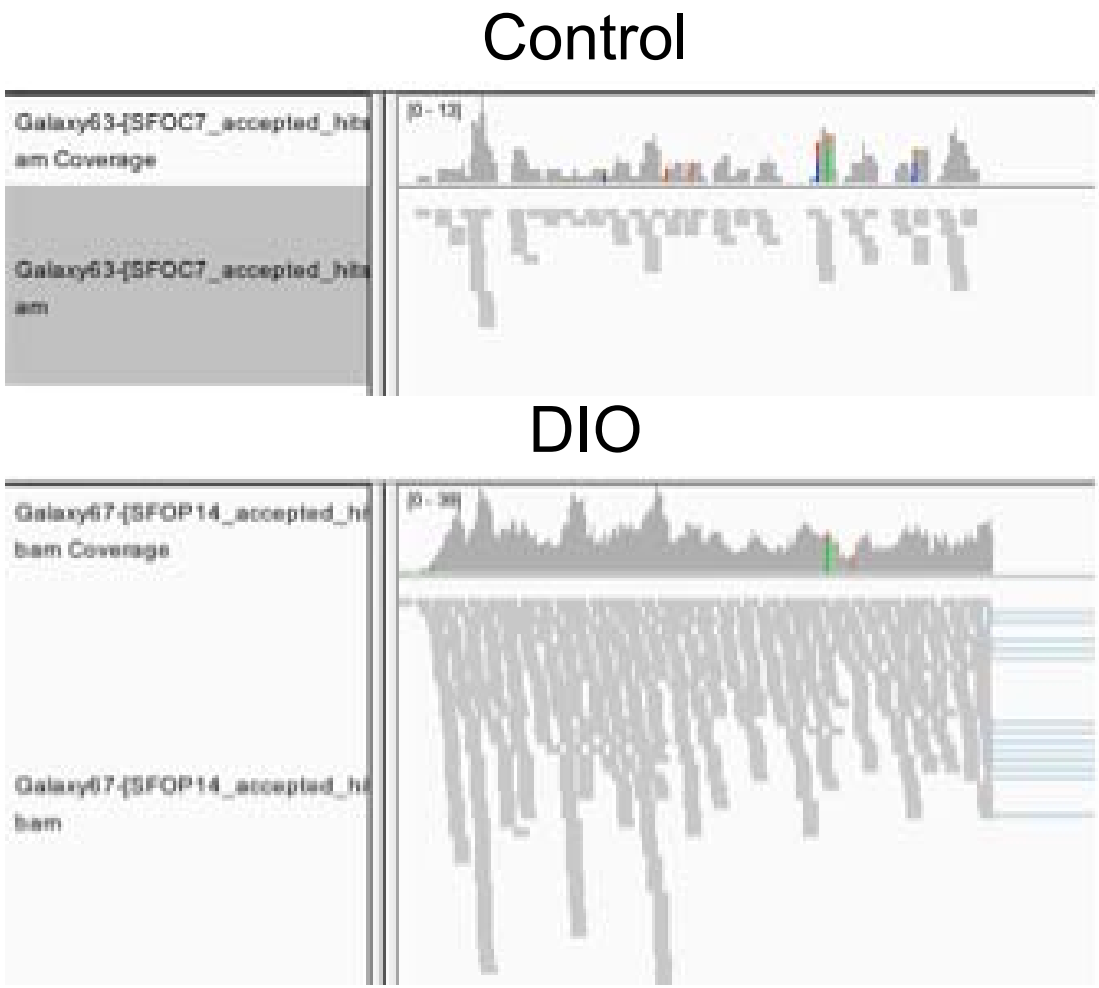


Figure 8: **AT_1 receptor expression is increased in SFO of DIO rats:** Preliminary RNAseq data from SFO of control and DIO rats summarizing 51bp reads in exon 3 of the AT_1 receptor gene. Each sample (control or SFO) consists of material from 5 microdissected SFOs from that particular animal group. In each example data above the line (bam coverage) represents the frequency of bases reported in that region of the genome. Each gray bar below the line (BAM) represents a 51bp read from that specific region of the genome, thus in simplistic terms the more grey bar the greater the expression level, and increased AT_1 expression in DIO rats.

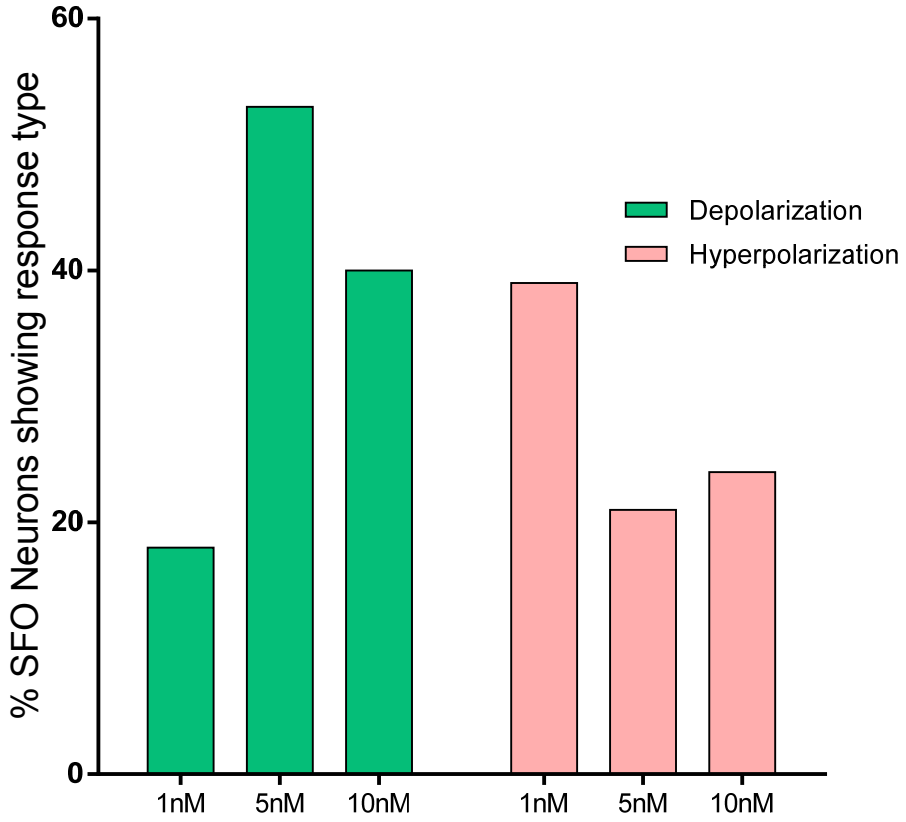


Figure 9: **Glucose concentration modifies the response of SFO neurons to CCK:** The graph above summarise the % of SFO neurons either depolarizing (green) or hyperpolarizing (pink) in response to bath administration of 10nM CCK. Notice that much larger proportions depolarize at higher glucose concentrations.

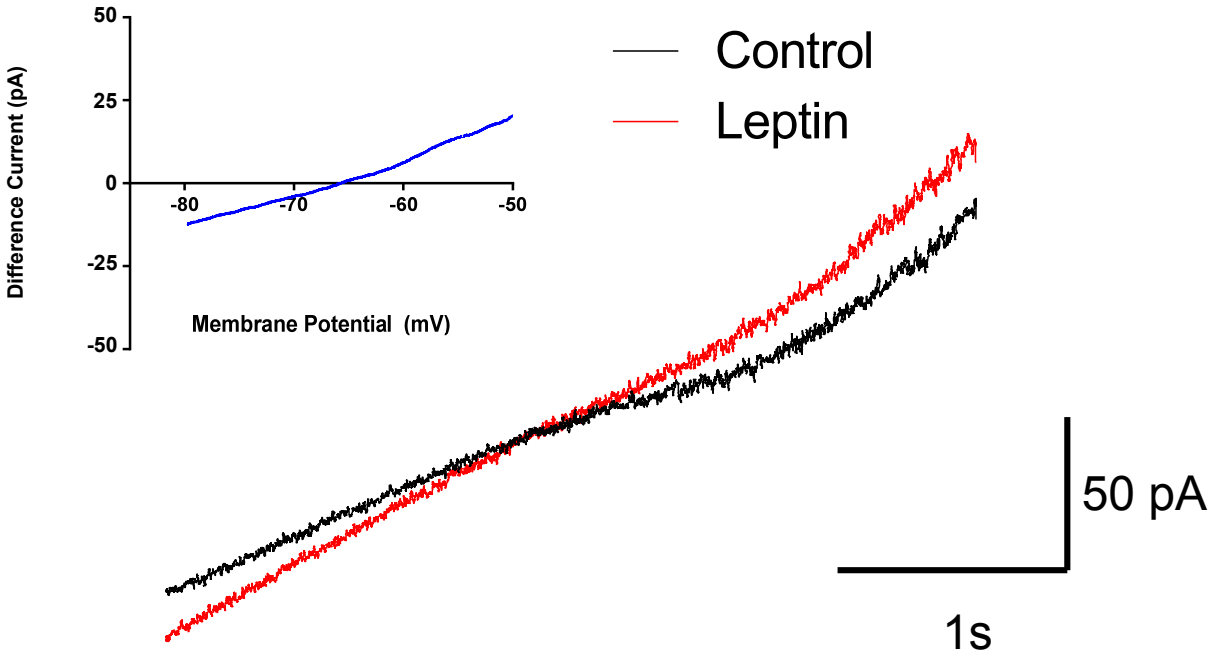


Figure 10: **Leptin activates a voltage independent current in SFO neurons:** This voltage clamp recording from an SFO neuron shows the currents measured in response to a slow voltage ramp (-80 → -20 mV, 10mV/s) during control (black) and 10nM leptin (red) perfusion. Subtraction of the former from the latter represents a measure of the leptin induced (or difference) current (blue) which is plotted in the inset, and shows the characteristic voltage independence and reversal potential of a non-selective cation current (NSCC).

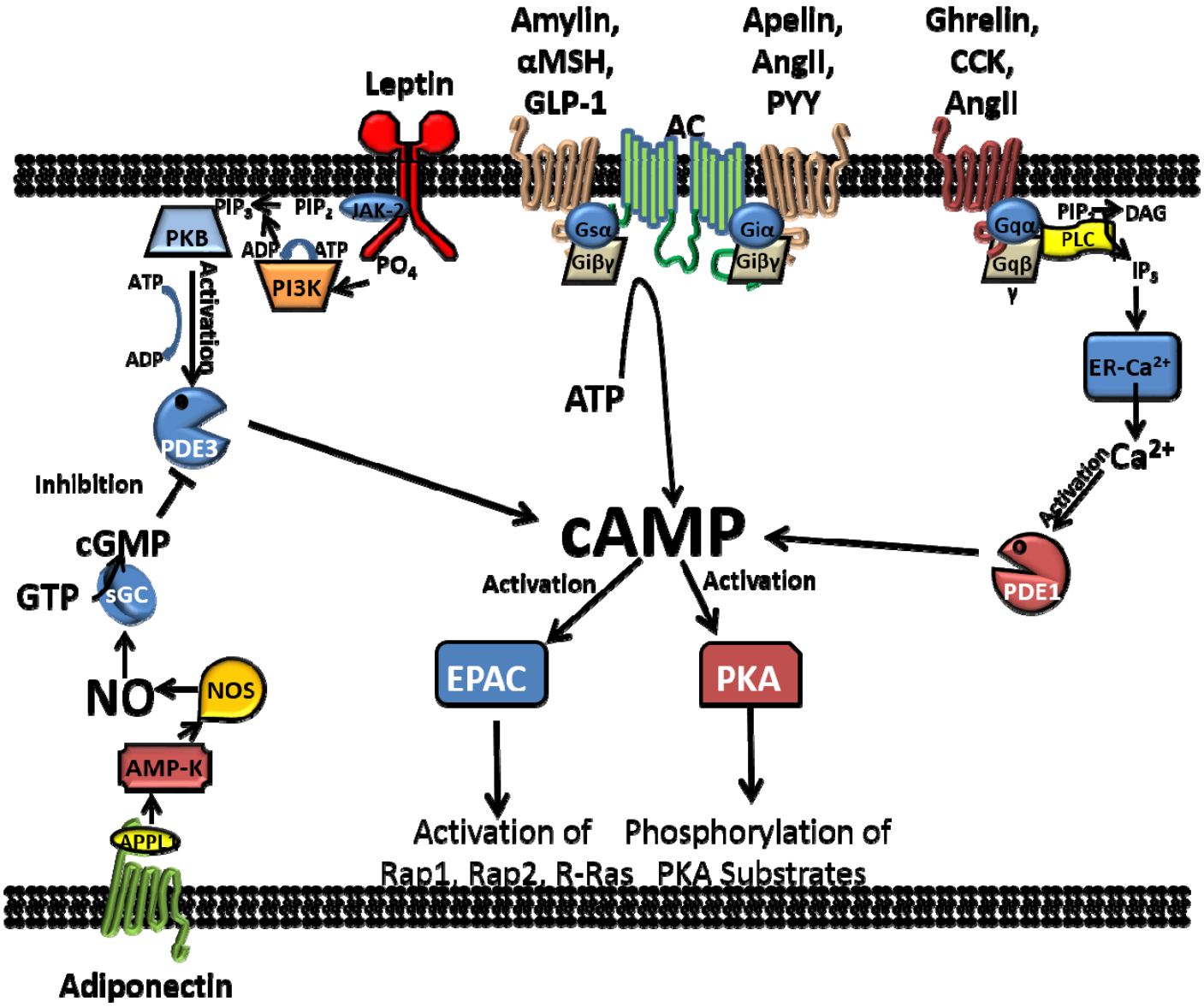


Figure 11: **cAMP signaling in SFO neurons:** This schematic illustrates the potential interactions between different signaling pathways in single SFO neurons which represent the focus of studies proposed in Aim 4.

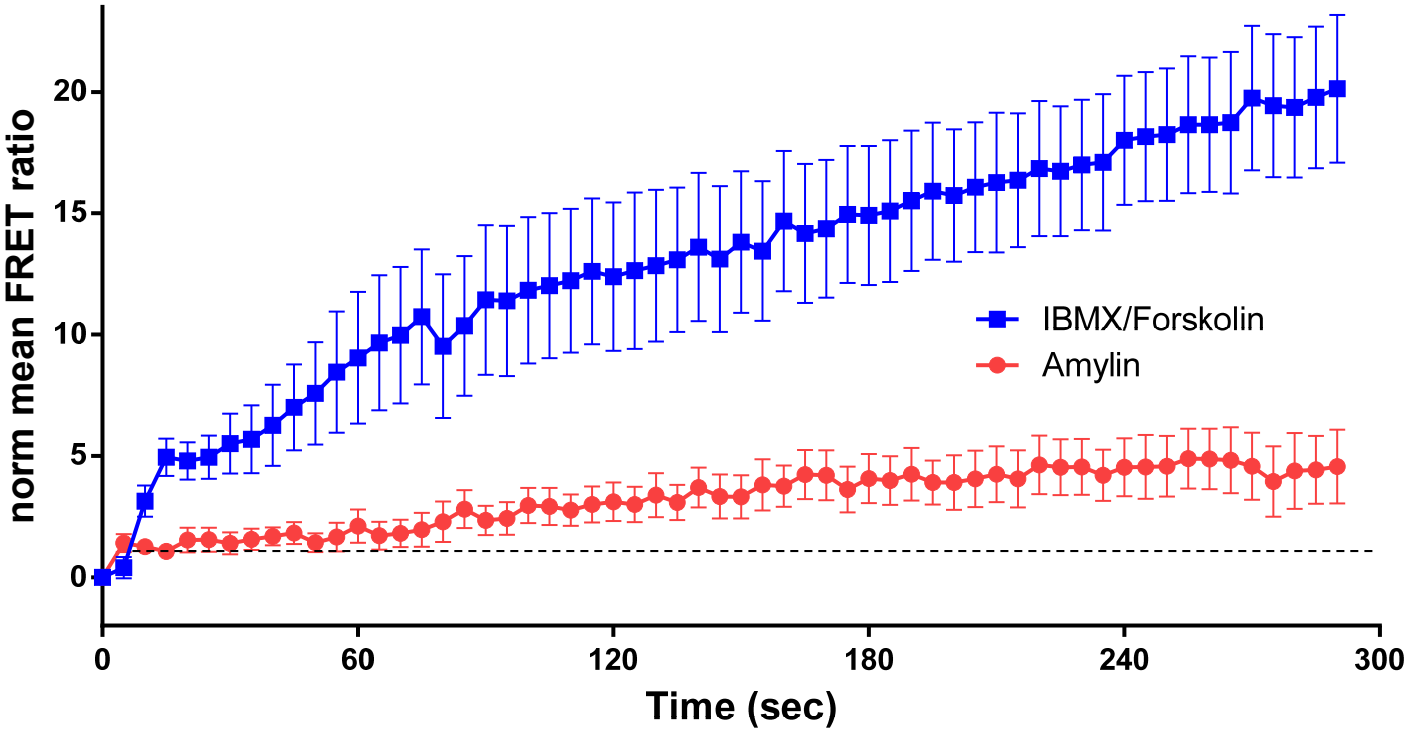


Figure 12: **Amylin elicits submaximal increases in cAMP in SFO neurons:** These graphs show FRET measurement of cAMP reporter in a subpopulation of single SFO neurons which respond to bath administration of 10nM amylin (4/9) and their subsequent responses to the cAMP activator IBMX/forskolin, with responses in both cases normalised to levels at the time of administration t=0.

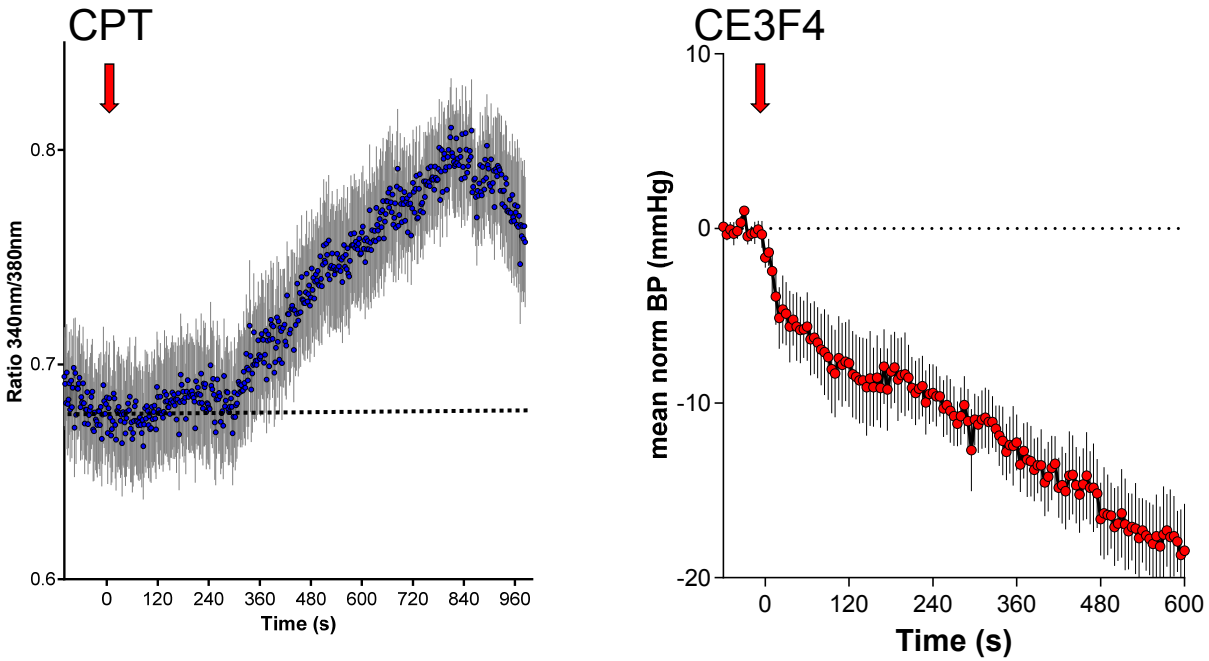


Figure 13: **EPAC influences SFO neurons and cardiovascular function:** The graph on the left shows that bath administration of the EPAC activator 8-CPT (100 μM red arrow) for 120s increases intracellular [Ca^{2+}] measured using ratiometric imaging (n=19). The graph on the right shows that microinjection of the EPAC inhibitor CE3F4 (0.5 μl 10 μM - red arrow) into the SFO decreases mean blood pressure in anaesthetised rats (n-3).

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