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Research Report

Actions of adiponectin on the excitability of subfornical organ neurons are altered by food deprivation

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ABSTRACT

Adiponectin (ADP) is a peptide produced by adipose tissue, which acts as an insulin sensitizing hormone. Recent studies have shown that adiponectin receptors (AdipoR1 and AdipoR2) are present in the CNS, and although adiponectin does appear in both circulation and the cerebrospinal fluid there is still some debate as to whether or not ADP crosses the blood brain barrier (BBB). Circumventricular organs (CVO) are CNS sites which lack normal BBB, and thus represent sites at which circulating adiponectin may act to directly influence the CNS. The subfornical organ (SFO) is a CVO that has been implicated in the regulation of energy balance as a consequence of the ability of SFO neurons to respond to a number of different circulating satiety signals including amylin, CCK, PYY and ghrelin. Our recent microarray analysis suggested the presence of adiponectin receptors in the SFO. We report here that the SFO shows a high density of mRNA for both adiponectin receptors (AdipoR1 and AdipoR2), and that ADP influences the excitability of dissociated SFO neurons. Separate subpopulations of SFO neurons were either depolarized (8.9±0.9 mV, 21 of 97 cells), or hyperpolarized (−8.0±0.5 mV, 34 of 97 cells), by bath application of 10 nM ADP, effects which were concentration dependent and reversible. Our microarray analysis also suggested that 48 h of food deprivation resulted in specific increases in AdipoR2 mRNA expression (no effect on AdipoR1 mRNA), observations which we confirm here using real-time PCR techniques. The effects of food deprivation also resulted in a change in the responsiveness of SFO neurons to adiponectin with 77% (8/11) of cells tested responding to adiponectin with depolarization, while no hyperpolarizations were observed. These observations support the concept that the SFO may be a key player in sensing circulating ADP and transmitting such information to critical CNS sites involved in the regulation of energy balance.

1. Introduction

Adipose tissue-derived hormones, known as adipokines, control immune, cardiovascular, metabolic, and endocrine functions. Adiponectin (ADP) is one such adipokine that is an insulin sensitizing hormone and is associated with obesity-related diseases such as diabetes type 2 and metabolic syndrome (Scherer et al., 1995; Kadowaki and Yamauchi, 2005). Overexpression of ADP in mice caused an increase in adipose tissue clearance, while ADP knockout mice developed obesity (Kubota et al., 2002; Maeda et al., 2002). Human studies have shown that inadequate ADP production increases

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susceptibility to obesity, insulin resistance, diabetes, and hypertension (Kondo et al., 2002; Ukkola and Santaniemi, 2002; Iwashima et al., 2004; Damcott et al., 2005; Hara et al., 2005; Kadowaki and Yamauchi, 2005). ADP expression in circulation is inversely proportional to adipose mass (Hu et al., 1996; Arita et al., 1999; Yamauchi et al., 2001), and CSF concentrations are 100-fold lower than those found in circulation (Kos et al., 2007). Centrally administered adiponectin has been shown to stimulate thermogenesis, promote oxygen consumption, decrease body weight and increase CSF concentrations of ADP (Qi et al., 2004).

Two 7-transmembrane receptors for ADP (AdipoR1 and AdipoR2), which are distinct from G-protein coupled receptors, have been localized throughout the body, including the CNS (Yamauchi et al., 2003). Expression of these receptors in critical hypothalamic metabolic control centers including the paraventricular nucleus (PVN) (Hoyda et al., 2007), arcuate nucleus (ARC) (Kubota et al., 2007), the medullary nucleus of the solitary tract (NTS) (Hoyda et al., 2009), and the area postrema (Fry et al., 2006) have all been reported.

The subfornical organ (SFO) is a midline sensory CVO that protrudes into the third ventricle that plays an important role in the regulation of fluid balance (Simpson and Routtenberg, 1975, 1978; Mangiapane and Simpson, 1980; Simpson et al., 1978). Recent work demonstrating SFO neurons to be sensitive to circulating metabolic signals including amylin (Riediger et al., 1999), ghrelin (Pulman et al., 2006) and leptin (Smith et al., 2009), have strongly suggested that this CVO may also play important roles in the regulation of energy balance. SFO neurons can transmit information regarding these circulating signals through their efferent projections to autonomic control centers in the hypothalamus (Miselis, 1981; Lind et al., 1982).

Our own recent microarray analysis of the SFO reported the presence of both AdipoR1 and AdipoR2, suggesting potential actions of adiponectin in this CVO (Hindmarch et al., 2008). The present study was therefore undertaken first to validate these microarray observations, and then to determine the effects of ADP on the excitability of dissociated SFO neurons. Finally we have examined the effects of 48 h of food deprivation on the responsiveness of SFO neurons to ADP.

2. Results

2.1. *Adiponectin receptors 1 and 2 are expressed in SFO tissue*

While recent microarray analysis of SFO tissue from our laboratory suggests the presence of AdipoR1 and AdipoR2 expression in the SFO (Hindmarch et al., 2008) such observations had yet to be validated. We therefore performed RTPCR on cDNA prepared from mRNA harvested from SFO tissue to confirm whether these receptors were in fact expressed in the SFO. The total cDNA underwent PCR amplification using

Table 1 – Primer sets used for RT-PCR.

Primer Name	Position	Sequence	Product size (bp)
AdipoR1 (outside)	F	GTCCCCTGGCTCTATTACTCCT	509
	R	AGCACTTGGCTGTGATGT	
AdipoR1 (nested)	F	TCTTCCTCATGGCTGTGATGT	223
	R	GGCTCAGAGAAGGGAGTCATC	
AdipoR2 (outside)	F	GGAGCCATTCTCTGCCTTTC	464
	R	CCAGATGTCACATTTGCCA	
AdipoR2 (nested)	F	ACTGTAACCCACAACCTTGCTTC	191
	R	TCAGGAACCCTTCTGAGATGAC	
GAPDH (outside)	F	GATGGTGAAGGTCGGTGTG	469
	R	GGGCTAAGCAGTTGGTGGT	
GAPDH (nested)	F	TACCAGGCTGCCTTCTCT	360
	R	CTCGTGGTTCACACCCATC	
Synaptotagmin (outside)	F	GCTTTGAAGTTCCGTTCGAG	431
	R	AGGGCAGTGGTATGATGGAG	
Synaptotagmin (nested)	F	TGACAAGATTGGCAAGAACG	198
	R	TGCAGAAAGGCTTCGTTTT	
GFAP (outside)	F	ATAGAGGAGGGCTCTCTGGC	516
	R	ATACGAAGGCACTCCACACC	
GFAP (nested)	F	ACCGGTGGAGATAACTTGGA	276
	R	AGCTAGTGGTTGTGGGGATG	

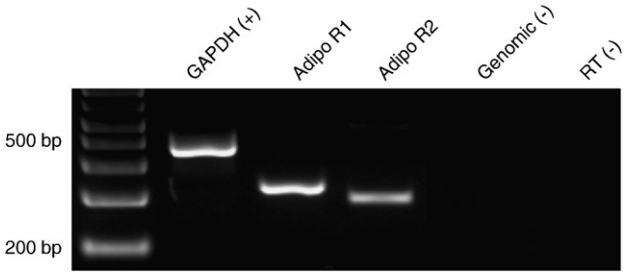

Fig. 1 – Adiponectin receptor expression in SFO tissue. This agarose gel shows RTPCR analysis from whole SFO tissue using primer sets designed to detect GAPDH, AdipoR1, AdipoR2 and genomic contamination. The clear expression of both AdipoR1 and AdipoR2 indicate mRNA expression in the SFO, while the lack of bands in either the genomic or (–) RT(–) lanes represents controls confirming the absence of genomic DNA or other contamination.

primer sets designed specifically to amplify AdipoR1 and AdipoR2 cDNA. GAPDH was used as a positive control, and RT (–) was used as a negative control, where reverse transcriptase was not used in the RTPCR reaction (Table 1). Genomic primers were used to detect possible genomic contamination. As illustrated in the gel shown in Fig. 1, the RTPCR reaction amplified both AdipoR1 and AdipoR2 indicating that both receptors are expressed in the SFO.

2.2. *Adiponectin influences the activity of SFO neurons*

Confirmation of the presence of AdipoR1 and AdipoR2 in SFO tissue, led us to next examine the effects of activation of these receptors with ADP on the excitability of SFO neurons. Whole-cell current-clamp recordings were obtained from 97 dissociated SFO neurons and their responsiveness was then characterized based on changes in membrane potential in response to bath administration of ADP. All cells included in our analysis showed action potentials >60 mV in amplitude, while the mean resting membrane potential of these SFO neurons was −59.0±0.9 mV. A minimum 100 s stable baseline period of recording was obtained from all neurons tested prior to bath application of 10 nM globular ADP, and membrane potential was then monitored for a minimum of 200 s after return to ACSF perfusion. In all neurons at least a partial recovery of membrane potential to baseline values was observed, although complete recovery could take more than 45 min and therefore was not observed in cells where recordings were not maintained for this extended period of time. We observed changes in mean membrane potential at intervals of 100 s after ADP application in order to determine the effects of ADP on membrane potential and the peak 100 s mean change in membrane potential from baseline values. Of the SFO neurons that were tested, we observed two distinct responsive groups that either depolarized (22% of cells (21/97); 8.9±0.9 mV) or hyperpolarized (35% of cells (34/97); −8.0±0.5 mV) in response to ADP application, as illustrated in Fig. 2 and summarized in Fig. 3. The remaining cells tested were unaffected by ADP (membrane potential change of less than 2 times SD obtained in 100 s baseline recording 43% of cells (0.3±0.4 mV) as illustrated in Fig. 3a. Peak mean effects on membrane potential in cells that depolarized in response to ADP were observed after 200 s (n=13), while cells that showed hyperpolarization showed a plateau at the 200 s time interval and in some cases a further hyperpolarization that peaked at the 900 s time interval followed by a return toward baseline (n=23) (Fig. 3b). In a small proportion of neurons (n=8), recordings were maintained for long enough periods of time that the effects of a second administration of ADP could be examined, and in all cases the second response to ADP was smaller than the initial effect observed (depolarizations — mean 4.2±1.9 mV, n=3; and hyperpolarizations — mean −2.1±2.2 mV, n=5).

During whole-cell current-clamp recordings, current pulses of −5, −10 and −15 pA were applied and the change in membrane potential was measured and used to determine the input resistance of responsive neurons before and after the application of ADP. Based on comparisons of the slope on the VI curve of baseline control to ADP treated neurons, both hyperpolarizing (n=13 cells — Control 1.87±0.21 GΩ, ADP 1.96±0.21 GΩ mean± SEM) and depolarizing (n=7 cells — Control 2.08±0.46 GΩ, ADP 2.16±0.54 GΩ) SFO neurons showed no significant change in input resistance (P>0.05; data not shown).

Adiponectin also influenced the spike frequency of SFO neurons in accordance with the effects of membrane potential as summarized in Fig. 3c. These effects were analyzed by counting action potentials occurring in sequential 10 s intervals before and after ADP application and calculating the mean change in action potential frequency for each response, which were found to be significantly different between all groups using the Kruskal–Wallis one-way ANOVA (P<0.001, n=26; Fig. 3c). These observations demonstrate clear reversible, but not readily repeatable (within the time frame of our recordings), effects of ADP on the excitability of SFO neurons, and in addition suggest the existence of separate differentially sensitive subpopulations of SFO neurons.

The effects of different concentrations of ADP on SFO neurons were examined using bath administration of 10 fM, 1 pM, 10 pM, 100 pM, 1 nM and 10 nM ADP, all of which are far lower than the circulating concentration of 100 nM (Arita et al., 1999). The decrease in ADP concentration is correlated to a decrease in the level of response, as measured by the mean change in membrane potential. Depolarizing cells showed a significant decrease in the mean change in membrane potential at 100 pM (3.6±0.7 mV) from 10 nM (8.9±0.9 mV; t-test, $^{**}P$<0.005) and hyperpolarizing neurons had a significant decrease in the mean change in membrane potential at 10 pM (−3.0±0.9 mV) compared to 10 nM (−8.0±0.5 mV; t-test, $^{**}P$<0.005); with no effects (hyperpolarizing or depolarizing) of ADP observed at a concentration of 10 fM ADP (Fig. 2d).

2.3. *Food deprivation increases mRNA expression of AdipoR2 in the SFO*

Microarray experiments from our laboratory have recently suggested changes in adiponectin receptor expression in the SFO following food deprivation; specifically, AdipoR2, mRNA levels significantly increased (Student's t-test, P<0.0001), while AdipoR1 expression levels were unchanged during food deprivation (Fig. 4a). Using qPCR, we attempted to validate these observations using qPCR, and were able to show that expression of AdipoR2 increased 1.45-fold±13% after food deprivation compared to control, changes that were statistically different

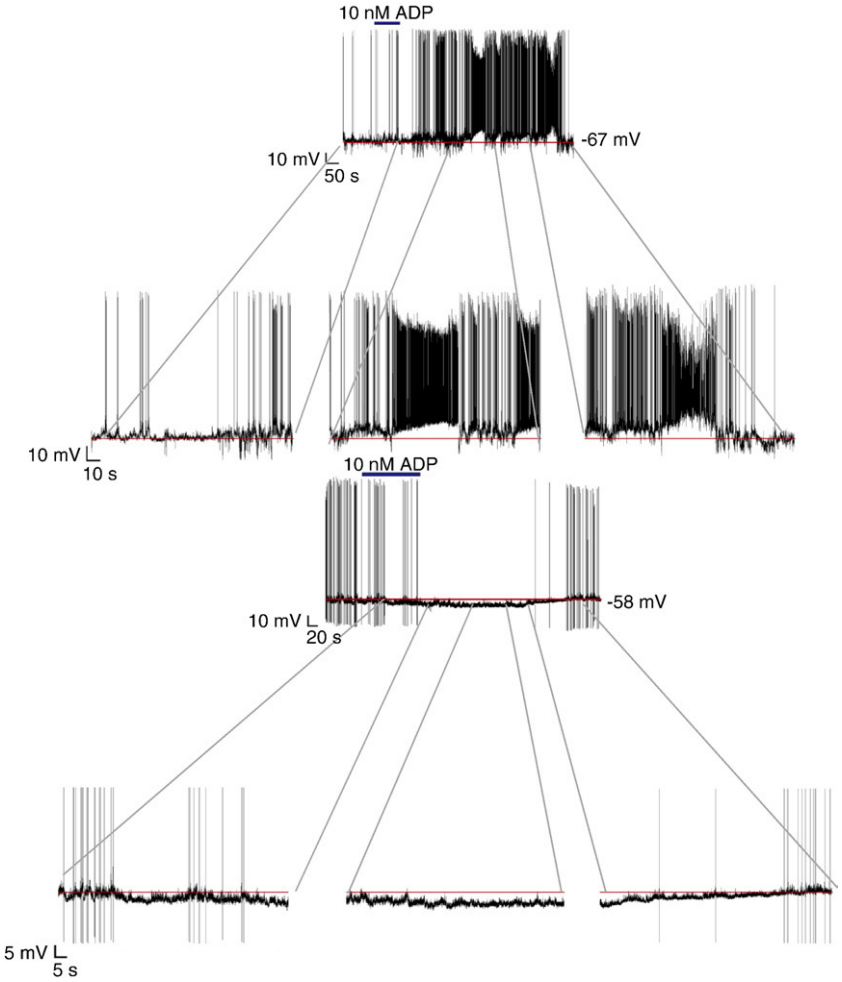

Fig. 2 – Adiponectin influences membrane potential of SFO neurons. These traces show current-clamp recordings from two separate SFO neurons, which illustrate the effects of bath administration of 10 nM ADP (time of application indicated by the horizontal bar above the traces). The top set of traces show depolarizing effects (mean change in membrane potential from baseline (indicated by red line) for this neuron was 10.4 mV) of ADP with an increase in spike frequency and a return to baseline membrane potential approximately 12 min after return to ACSF. The breakout traces show expanded timescale representations of the recording period as illustrated. In contrast the lower set of traces are from an SFO neuron which hyperpolarized (mean change of −5.3 mV) in response to similar bath administration of ADP, in this case with a decrease in action potential frequency, and again a return to baseline membrane potential approximately 5 min after ACSF was returned to the bath.

from the control (as determined by one sample Wilcoxon Signed Rank test, $n=5$, $P<0.05$). In contrast there was no statistically significant change in the expression of AdipoR1 following food deprivation (1.26-fold±8% as compared to control $n=5$, $P>0.05$). Together, the gene array data and qPCR validation show that AdipoR2 mRNA expression is increased by 48 h food deprivation, and raise the possibility that the profile of AdipoR1 and AdipoR2 expression in SFO neurons may be modified by this challenge.

We therefore next carried out experiments where we harvested of the cytoplasm of single SFO neurons and using single-cell RTPCR techniques determined whether individual cells express the mRNA for different combinations of adiponectin receptors following food deprivation. Using this technology, we classified 11 neurons from control animals and 8 neurons from animals that were food restricted for 48 h. Neurons were identified by confirming mRNA expression of neuronal marker synaptotagmin. All cytoplasmic contents were also GAPDH positive (+control), while none of them showed genomic DNA contamination. Of the 11 SFO neurons from control animals tested we found that 4 expressed AdipoR1 only, 0 expressed AdipoR2 only, 4 expressed both AdipoR1 and AdipoR2 and 3 expressed neither AdipoR1 nor AdipoR2 as summarized in Table 2. When compared to the expression profiles of neurons from rats that were food restricted for 48 h, we found that there was no obvious difference between the two conditions, where of the 8 neurons tested, 2 expressed AdipoR1 only, 1 expressed AdipoR2 only, 4 expressed both AdipoR1 and AdipoR2 and 1 expressed neither (Table 2).

2.4. *Adiponectin depolarizes SFO neurons from food restricted animals*

In light of the data showing an increase in AdipoR2 in SFO tissue from food restricted animals, we hypothesized that an

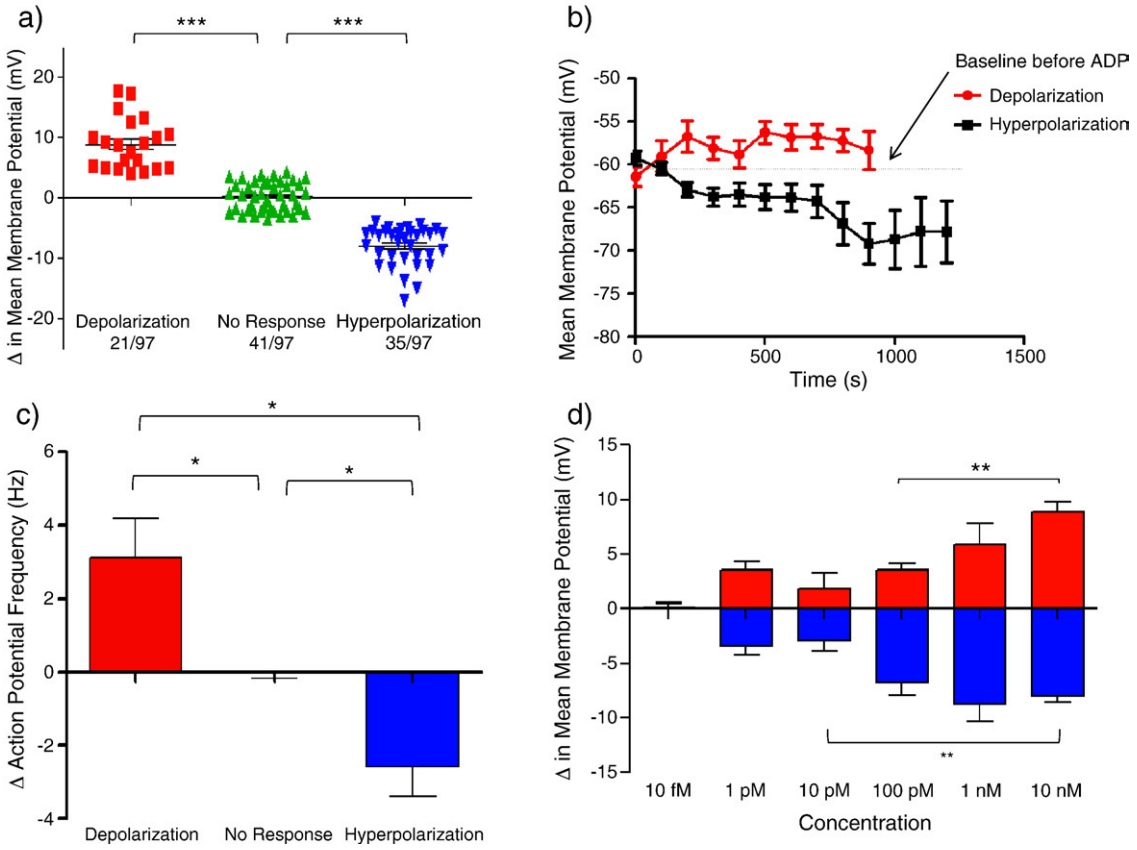

Fig. 3 – Summary data of adiponectin response. The graph in panel a) uses the peak change in membrane potential from each SFO neuron tested to generate a scatter plot graph summarizing the effects of 10 nM adiponectin in depolarizing (red; mean change 8.9±0.9 mV), hyperpolarizing (blue; mean change −8.0±0.5 mV) or having no effect (green; mean change 0.3±0.4 mV) on the membrane potential of SFO neurons. Mean change in membrane potential was determined to be significant between all groups by using Kruskal–Wallis test (*$P<0.001$). The data shown in b) illustrate the time course of mean changes in membrane potential during 100 s intervals after ADP application and show that SFO neurons that depolarized ($n=13$) reached their peak change in mean membrane potential earlier than SFO neurons that hyperpolarized ($n=23$). Time interval zero was 100 s before the application of ADP and mean change in membrane potential was normalized to baseline values before ADP application. Baseline before ADP application is shown as a grey line. C) Using 10 s intervals before and after application of ADP we determined the change in mean spike frequency. It was found that neurons from all three groups showed a significant difference in change of spiking frequency (Kruskal–Wallis one-way ANOVA, *$P<0.0001$). Whereas neurons that depolarized showed an increase in action potential frequency (change of 3.12 Hz), those that hyperpolarized showed a decrease in frequency (change of −2.59 Hz). Using decreasing concentrations of applied ADP, we found that ADP has concentration dependent effects on SFO neurons. The bar-graph in d) shows changes in membrane potential at different concentrations of ADP, where depolarizing neurons had a significant drop in response at 100 pM (**$P<0.005$) and hyperpolarizing neurons had a significant drop in response at 10 pM (**$P<0.005$) compared to the response of 10 nM.**

increase in AdipoR2 receptor induced a change in the responsiveness of SFO neurons, where either hyperpolarizations or depolarizations would be the dominant ADP-induced effect. We assessed this hypothesis by dissociating SFO neurons from food restricted animals, which were used within 36 h after dissociation after first demonstrating that cells from control animals recorded in this time period showed similar responses to adiponectin to those described above (28% depolarize — mean 9.6±1.9 mV, 26% hyperpolarize — mean −8.7±1.8 mV, 46% unaffected, $n=23$). Using whole-cell patch-clamp techniques, we found that the majority of SFO neurons obtained from food restricted animals depolarized in response to ADP ($n=10/13$; Fig. 5), while none of the neurons exhibited hyperpolarization effects, these results were shown to be significantly different from those observed in neurons from control animals (Chi-square test $p<0.001$).

3. Discussion

Qi et al.(2004) have previously demonstrated that both AdipoR1 and AdipoR2 receptors are expressed in the brain, observations supported by our own work showing mRNA for the ADP receptors in the PVN (Hoyda et al., 2007), AP (Fry et al., 2006) and nucleus of the solitary tract (NTS) (Hoyda et al.,

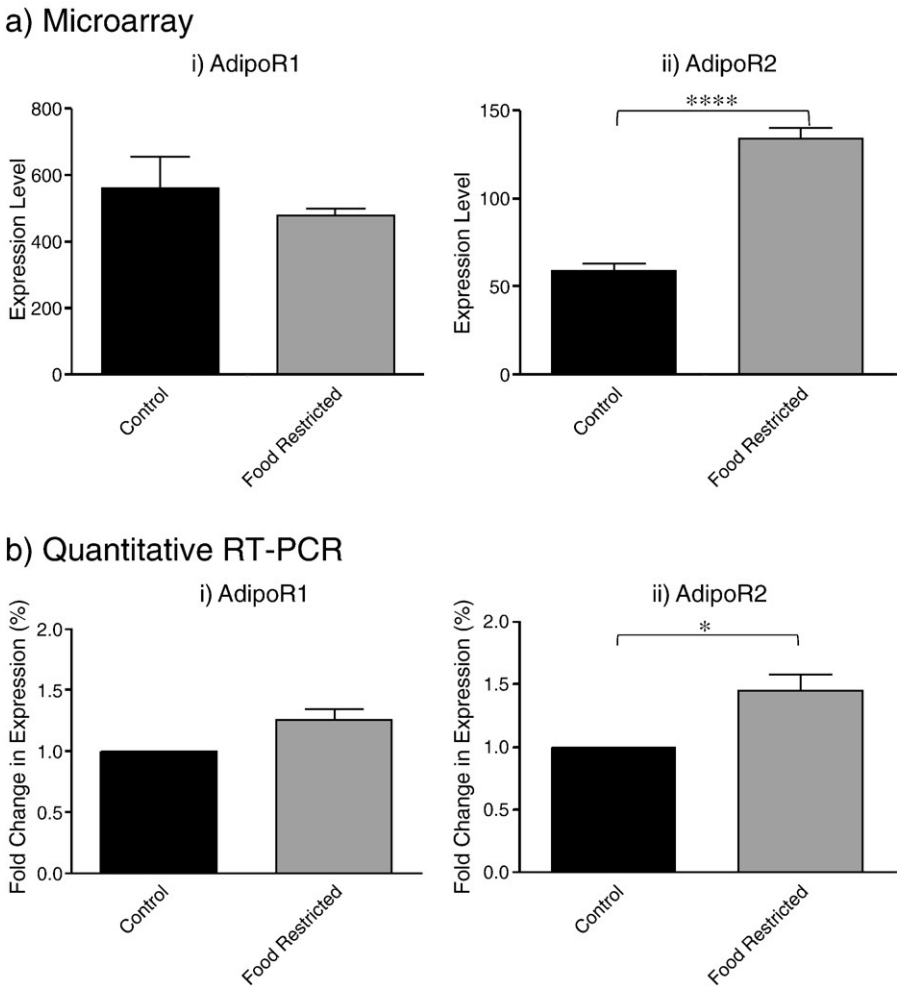

Fig. 4 – Analysis of AdipoR1 and AdipoR2 mRNA expression during control and food restricted states. Using *post-hoc* analysis of microarray data from Hindmarch et al. (2008), the bar graphs in parts a) and b) show mRNA expression of both adiponectin receptors in whole SFO obtained from either control (black) or food restricted (grey) animals. In these microarray experiments AdipoR2 exhibited a relative increase in mRNA expression after food deprivation (**$P<0.0001$; *$P<0.05$ d). Using qRTPCR and the $\Delta\Delta C_t$ method of quantification, AdipoR1 did not exhibit a significant change in expression after food deprivation (1.26-fold ± 8%; $n=5$), and AdipoR2 exhibited an increase in expression which was consistent with that observed in the microarray study (1.45-fold increase ± 13%; $n=5$).**

2009). In the present study we have identified the SFO as a potential target for adiponectin actions. We have shown that mRNAs for both adiponectin receptors are expressed in SFO tissue, and that activation of these receptors modulates the membrane properties of SFO neurons. Furthermore, our data indicate that the majority of SFO neurons are responsive to ADP and they express mRNA for at least one of the two adiponectin receptors. Our data also indicate that during food deprivation, there is a significant increase in the mRNA expression of AdipoR2 in SFO tissue, which is associated with a long-lasting change in the adiponectin response of SFO neurons when compared to controls. These data suggest that the SFO is a CNS site that is sensitive to ADP and that SFO's response to ADP is modulated by different energy states.

Table 2 – AdipoR1 and AdipoR2 mRNA expression in single SFO neurons.

	R1 only	R2 only	Both R1/R2	Neither
Control rats ($n=11$)	4/11	0/11	4/11	3/11
Food deprived rats ($n=8$)	2/8	1/8	4/8	1/8

Using cultured SFO neurons from both control rats ($n=11$) and 48 h food deprived rats ($n=8$), SFO cytoplasm was harvested and mRNA samples from each individual neuron underwent RTPCR. These neurons were screened for AdipoR1, R2, GAPDH (positive control) and synaptotagmin (neuronal marker, not shown).

We used patch-clamp electrophysiology to monitor changes in the membrane properties and action potential frequency of SFO neurons in response to ADP and found that the majority of cells tested were in fact responsive to ADP. These responses were categorized as being significant if they induced a change in membrane potential (means measured over 100 s time periods) greater than two standard deviations from the baseline level recorded in the 100 s time period prior

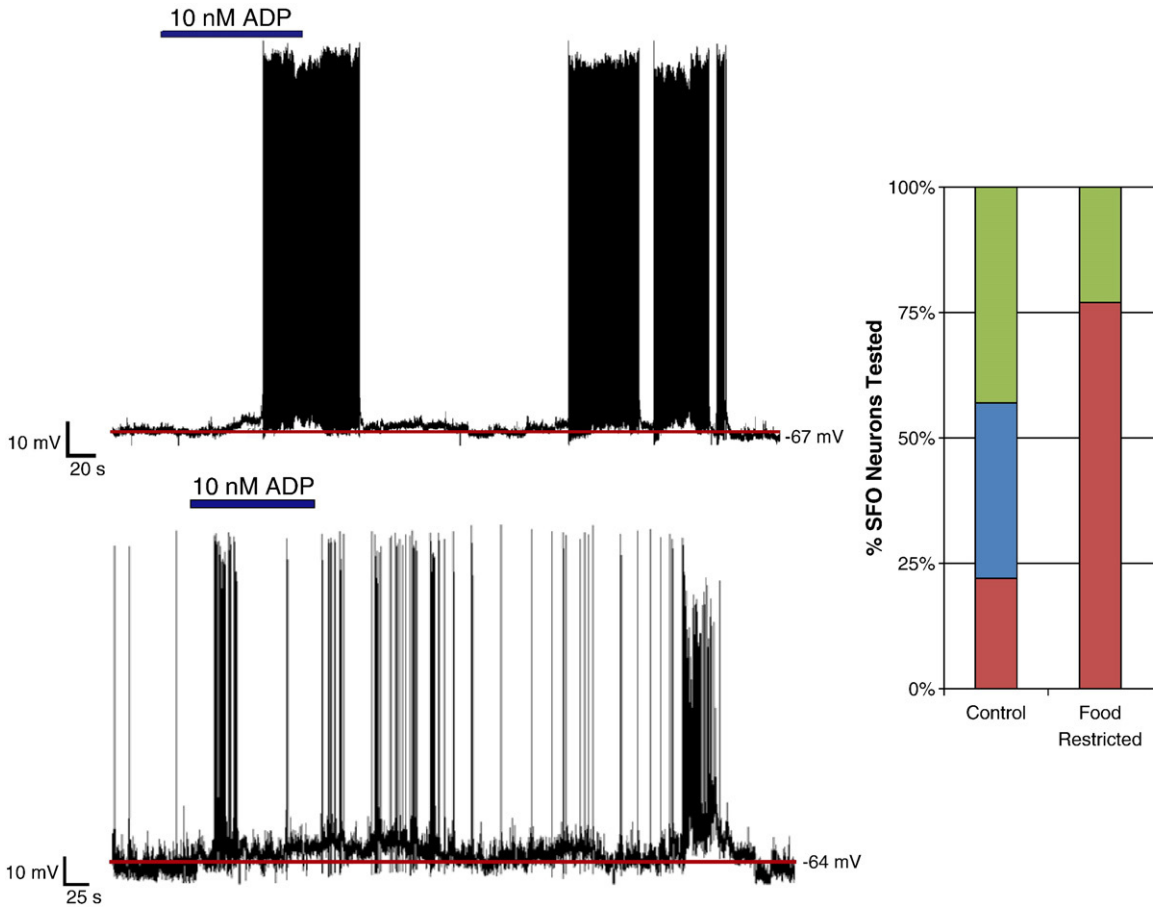

Fig. 5 – ADP depolarizes SFO neurons from food restricted animals. The traces above are two examples of SFO neurons from food restricted animals that depolarized in response to ADP treatment, while the summary data in the graph on the right shows the differences between the population responses of SFO neurons from control and food restricted animals. SFO neurons from control animals both hyperpolarized (26% of neurons; shown in blue) and depolarized (28% of neurons; shown in red) in response to ADP, while the majority of neurons from food restricted animals depolarized (77% of neurons) within 36 h following dissociation. Non responsive neurons are shown in green.

to ADP administration, and were split into subpopulations of SFO neurons that demonstrated hyperpolarizations or depolarizations. While this method for evaluation of responsiveness of individual neurons provides a definitive quantitative assessment, it should also be recognized that it may exclude small ADP-induced changes in membrane properties that are below the two standard deviation thresholds, a consideration which would result in our data underestimating the total proportion of SFO neurons that are responsive to ADP. Our observations that the input resistance of SFO neurons does not change in response to adiponectin induced changes in membrane potential suggest either that multiple channels are responsible for the change in membrane potential, or that effects may be on voltage activated channels that are not open during the hyperpolarizing pulses that we apply to measure input resistance. A third possibility is that modulation of an ion transporter may be responsible for the effects of adiponectin. Future voltage-clamp analysis will likely be necessary to identify the mechanisms through which ADP modulates the membrane potential of SFO neurons.

The concentration of adiponectin used to determine responsiveness of SFO neurons (10 nM) is close to circulating levels of this adipokine which have been reported to be approximately 100 nM (Arita et al., 1999), and in addition our studies have shown these effects on SFO neurons to be concentration dependent. We have also avoided potential problems of desensitization of SFO neurons to ADP in our studies by only using the first exposure of any SFO neuron to ADP for our primary analysis of the effects of this adipokine on membrane potential.

Our lab has previously shown that ADP modulates both membrane properties and action potential frequency in neurons from the PVN (Hoyda et al., 2007), AP (Fry et al., 2006) and the NTS (Hoyda et al., 2009). The latency, duration and responsiveness to ADP in SFO neurons is similar to that previously reported in these autonomic control centers in that both depolarizing and hyperpolarizing effects were observed, and that effects were normally relatively slow in onset and of long duration. Yamauchi et al. (2007) have shown that AdipoR1 and AdipoR2 activate second messenger systems for the AMPK

and PPAR-α pathways, respectively, and the activation of such systems would explain the effects observed in SFO neurons. These different effects of ADP on separate subpopulations of SFO neurons are perhaps not surprising in that previous work has already demonstrated separate populations of SFO neurons that respond to amylin and leptin (Smith et al., 2009), and amylin and ghrelin (Pulman et al., 2006), and again emphasize that different functional subgroups of SFO neurons exist.

Recent microarray analysis has suggested that a 48 h food deprivation results in increases in the expression level of AdipoR2 mRNA in SFO tissue (Hindmarch et al., 2008), observations which we have confirmed in the present study using qRTPCR techniques. In addition our single-cell analysis of AdipoR expression in SFO neurons confirms subpopulations of cells which express mRNA for AdipoR1, AdipoR2, both receptors or neither under control and food deprived conditions. All of these cells were confirmed as neurons (as opposed to other cell types) using additional primer sets for GAPDH and synaptotagmin (neuron specific), and all were also negative for GFAP. Additional controls also indicated that there was no genomic contamination and that there was no DNA contamination. These observations are particularly important as they clearly show that response profile (depolarization or hyperpolarization), is not specifically linked to the expression of one or other AdipoR. Interestingly, the increase in AdipoR2 expression in SFO tissue did not result in a significant increase of AdipoR2 positive SFO neurons from animals following 48 h food deprivation. A possible explanation to these results is that unlike qRTPCR, single-cell RTPCR does not quantify mRNA expression so an increase in the absolute level of AdipoR2 mRNA in a single neuron cannot be determined using this method. It is also possible that changes in expression AR2 may be in non-neuronal cells during food deprivation which would result in a change in the mRNA expression profile in tissue but not in neurons. Finally, it may be that food deprivation results in modified receptor expression in different regions of the SFO, a possibility which we have not addressed in the present study.

In conclusion, we demonstrated that SFO neurons are sensitive to ADP and that the response to ADP changes during food restricted conditions, suggesting that SFO's response to ADP is modulated by energy status. The SFO is the second CVO that has been shown to be responsive to ADP and overall, this study suggests that the SFO is a CNS site that can detect circulating energy signals.

4. Experimental procedures

4.1. *Subfornical organ neuron preparations*

All animal protocols were approved by Queen's University Animal Care Committee. Male Sprague Dawley rats (125–175 g) maintained on a 12/12 light/dark cycle and allowed *ad libitum* access to food and water (except in food restricted state — see below) were used for all experiments. A food restricted group of animals was not allowed access to food 48 h prior to experimental procedures. SFO neurons were dissociated using protocols adapted from those described previously (Ferguson et al., 1997; Fry et al., 2006). Briefly, male Sprague Dawley rats were decapitated; the brain was removed immediately and was placed in oxygenated, ice cold slicing solution containing (in mM): 87 NaCl, 2.5 KCl, 25 $NaHCO_3$, 0.5 $CaCl_2$, 7 $MgCl_2$, 1.25 NaH_2PO_4, 25 glucose, and 75 sucrose. A block of tissue containing the hippocampal commissure was dissected from the whole brain and the SFO which can be readily distinguished from all adjacent tissue was isolated and removed. SFO was then placed in hibernate media (Brain Bits, Springfield, IL) with 2 mg/ml papain (Worthington, Lakewood, NJ) and incubated in a water bath at 30 °C for 30 min. SFO tissue was washed using hibernate media with 1×B27 supplement (Invitrogen, Burlington, ON, Canada), triturated, centrifuged at 200 rpm for 4 min, and resuspended in neurobasal-A/B27 (Invitrogen). Dissociated neurons were plated on glass-bottom dishes (MatTek Ashland, MA) and incubated at 37 °C in 5% CO_2. Electrophysiological experiments were normally performed within 1–5 days of dissociation, during which time period membrane properties (resting membrane potential, input resistance, and spike amplitude) remained stable, and contacts between neurons were not observed. In some experiments investigating the effect of food deprivation, acutely dissociated neurons were used within 6 h of preparation.

4.2. *Reverse-transcription PCR*

SFO tissue was acutely dissected out of rat brain as described above and total RNA was extracted using RNAqueous™ kit (Ambion) according to the manufacturer's directions. The total RNA from two SFOs was then DNase treated by adding a mixture of 1 μl 10× buffer with $MgCl_2$, 7 μl diethylpyrocarbonate (DEPC) treated-H_2O and 1 μl deoxyribonuclease to the total RNA and incubating the solution at 37 °C for 30 min. After incubation, 1 μl of 25 mM of EDTA was added to the solution and incubated at 65 °C for 10 min. Oligo-dT based cDNA was synthesized using Superscript™ III reverse transcriptase kit (Invitrogen, Carlsbad, California, USA) to make a final reaction volume of 20 μl.

The QIAGEN® Multiplex kit (QIAGEN, Mississauga, Ontario, Canada) was used for PCR reactions to amplify cDNA. Ten microlitres of the SFO cDNA was added to a PCR reaction containing: 50 μl 2× QIAGEN® Multiplex PCR Master Mix, 10× primer mix, 0.2 μM of each primer, 10 μl 5× Q-Solution, and DEPC treated-H_2O to a final volume of 100 μl. The reaction tube was first denatured at 95 °C for 15 min, and then cycled 30 times through a protocol of 94 °C for 30 s, 60 °C for 90 s, 72 °C for 90 s and finally 72 °C for 10 min. Primer sets previously described in Hoyda et al. (2007) were used to detect GAPDH (a positive control), Genomic DNA (a negative control; described in Price et al. (in press), synaptotagmin (neuronal marker; described in Dixon et al. (2000)) and adiponectin receptors (AdipoR1 and AdipoR2) mRNA (Table 1, outside primers). PCR products were run and visualized on electrophoresis gel containing 2% agarose and ethidium bromide. The surplus products from the PCR reaction were sequenced by Robarts Institute (London, ON, Canada) to confirm identity.

4.3. *Electrophysiology*

Whole-cell current-clamp recordings from SFO neurons were obtained using an Axopatch 200B patch-clamp amplifier (Molecular Devices, Palo Alto, CA). Recording and stimulation were controlled by Spike2 version 5 software (Cambridge

Electronics Design, Cambridge UK). Recordings were filtered at 5 kHz and acquired at 10 kHz. The external solution for all recordings was as follows: 140 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, and 10 glucose, pH 7.2 (adjusted with NaOH). Patch electrodes were made from borosilicate glass (World Precision Instruments, Sarasota FL) and had a resistance of 4 to 6 MΩ, and were filled with an internal solution of (in mM): 130 K-gluconate, 10 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, 10 EGTA, 4 Na_2ATP and 0.1 GTP (Sigma, ON, Canada).

Using a micromanipulator (Siskiyou Design Instruments, Grants Pass, OR and Sutter Instrument Company, Novato, CA) the electrode was lowered and brought into position so that the tip was just touching the cell membrane. With the application of gentle suction a seal was formed with a resistance of at least 1 GΩ, followed by a slight suction pulse which allowed whole-cell access by rupturing the cell membrane under patch. Neurons were identified based on the presence of voltage-gated Na^+ channels using simple depolarizing step protocols in voltage-clamp configuration, and the occurrence of spontaneous or current evoked action potentials with a spike amplitude greater than 60 mV in current-clamp configuration. During recordings, ADP was applied to the cell via bath perfusion.

4.4. *Single-cell RTPCR*

In order to determine if 48 h food deprivation induced a change in expression of adiponectin receptors we carried out a series of experiments to analyze adiponectin mRNA from single SFO neurons using single-cell RTPCR. We harvested cytoplasm from single SFO neurons prepared from control animals and animals which were food restricted for 48 h. Cells for such analysis were dissociated as described above and then harvested between 6 and 24 h after dissociation. Cells were collected using patch pipettes filled for electrophysiology, which were brought close to the membrane of SFO neurons, gentle suction was applied to obtain a seal such that the cells could be removed from the bath and the tip was broken so that the contents could be emptied into a 0.5 ml DNAse/RNase-free Thermowell® tube. The contents were first treated with DNase (DNase I — Fermentas, Burlington ON, Canada), followed by reverse transcription for cDNA synthesis using Superscript™ II reverse transcriptase kit (Invitrogen, Burlington ON, Canada). The RT ran overnight at 37 °C, after which the cDNA was stored at −80 °C until analysis.

A two-step multiplex PCR reaction approach was employed. PCR primers for GAPDH (glyceraldehyde-3-phosphate dehydrogenase; a positive control described in Hoyda et al. (2007)), genomic DNA (a negative control; described in Price et al., 2008), synaptotagmin (neuronal marker; described in Dixon et al. (2000)), glial cell marker glial fibrillary acidic protein (GFAP; developed in this study), and adiponectin receptors (AdipoR1 and AdipoR2; described in Hoyda et al. (2007)) mRNA (Table 1) were all used in a multiplex reaction with the single-cell cDNA as template. The second reaction was a nested PCR reaction, which used a single nested set of primers for each gene of interest and 2 ul of the first round multiplex reaction as template. The multiplex reaction for single-cell RTPCR used the same QIAGEN® Multiplex kit (QIAGEN, Mississauga, Ontario, Canada) reagents as described above in the RTPCR reaction on SFO whole tissue. The multiplex reaction tube was first denatured at 95 °C for 15 min, and then cycled 20 times through a protocol of 94 °C for 30 s, 60 °C for 90 s, 72 °C for 90 s and finally 72 °C for 10 min, as previously described (Price et al., 2008). The nested reaction used the QIAGEN® Multiplex kit containing 2 μl of the multiplex products, 25 μl 2× QIAGEN® Multiplex PCR Master Mix, 5 μl 5× Q-Solution, 16 μl H_2O, and 2 μl of each nested primer set of interest, for a final volume of 50 μl. The nested products underwent 30 cycles of amplification using the same protocol as the multiplex reaction and the products were run and visualized on a 2% agarose gel containing ethidium bromide. Primers for both AdipoR1 and AdipoR2 targeted the transmembrane region of the receptor and the sequences were matched to established sequences in NCBI (BLAST — Bethesda, MD, USA).

4.5. *Quantitative RTPCR*

Quantitative RTPCR (qRTPCR) experiments were undertaken to validate the observations of Hindmarch et al., 2008 indicating that expression of AdipoR2 increased following a 48 h food deprivation. Briefly, SFOs were microdissected from brains of two food deprived or two control rats. The two SFO were pooled and total RNA was extracted using the RNAqueous Micro kit (Applied Biosystems, Austin TX) according the manufacturer's directions, and treated with 1 U of RNase-free DNase 1 (Fermentas, Hanover MD) according to manufacturer's directions. This RNA was utilized as template for cDNA synthesis in a 30 μl reaction using Superscript III kit as per manufacturer's directions. 2 μl of the cDNA was used as template in a 25 μl qPCR reaction containing 1× Quantifast SYBR Green Master Mix (QIAGEN) and 5 μl gene specific primers (QIAGEN). Reactions were performed with an Applied Biosystems 7500 real-time PCR instrument. The primer sets were specific for AdipoR1, AdipoR2 and GAPDH. Reactions including nonreverse transcribed total RNA and no template controls were used to verify absence of genomic DNA contamination and nonspecific amplification. Analysis of the dissociation curves was also carried out for each reaction to confirm specificity. Levels of RNA expression for the AdipoR1 and AdipoR2 were determined using the C_t analysis settings of 7500 Software v1.3 based on the $\Delta\Delta C_t$ method of relative quantification by standardizing against GAPDH expression. In total, SFOs from 10 food deprived rats (5 biological sample replicates) and 10 control rats (5 biological sample replicates) were used; each biological sample replicate was replicated 4 times (showing a coefficient of variation of less than 3%). Levels of AdipoR1 and AdipoR2 expression in food restricted condition are represented as fold change with respect to control. Significance was determined using the one sample Wilcoxon Signed Rank Test (Microcal OriginPro 8.1, Northampton, MA). Expression data are presented ± SD.

4.6. *Peptides*

Adiponectin (human globular) was obtained from Phoenix Pharmaceuticals, Inc (Belmont CA, USA) in a lyophilized form. The lyophilized ADP was resuspended in DPC treated-H_2O and separated into daily aliquots, which were stored at −80 C.

4.7. *Analysis of electrophysiological data*

Responsiveness of neurons was determined by comparing the mean resting membrane potential recorded in the 100 s immediately prior to ADP administration to consecutive 100 s periods following the application of the peptide. Effects on membrane potential were only attributed to an action of the initial change that occurred between 30 and 300 s after ADP perfusion of the tissue bath began. The peak membrane potential change during one of these periods was then used to determine the magnitude, and the number of periods with significant change to determine the duration of ADP effects on SFO neurons. A significant change in membrane potential was considered to have occurred if the peak change in membrane potential was greater than twice the standard deviation of the control baseline membrane potential. Kruskal–Wallis one-way ANOVA was used to compare changes in membrane potential and changes in action potential frequency between different groups. Student's *t*-test was used to compare changes in dose dependent responses. All analyses were carried out using Prism 5.0 (Graphpad Software, San Diego, CA) and all group data are presented as the mean ± SEM.

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