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.

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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...
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>
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<th>Position</th>
<th>Sequence</th>
<th>Product size (bp)</th>
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<td>R</td>
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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, 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. 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.
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 the cytoplasm of single SFO neurons and using single-cell RT-PCR 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
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.,...
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 that SFO's response to ADP is modulated by different energy states.

We used patch-clamp electrophysiology to monitor changes in the membrane properties and action potential frequency of SFO neurons. 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 to food deprivation.

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

<table>
<thead>
<tr>
<th></th>
<th>R1 only</th>
<th>R2 only</th>
<th>Both R1/R2</th>
<th>Neither</th>
</tr>
</thead>
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<td>Control rats (n=11)</td>
<td>4/11</td>
<td>0/11</td>
<td>4/11</td>
<td>3/11</td>
</tr>
<tr>
<td>Food deprived rats (n=8)</td>
<td>2/8</td>
<td>1/8</td>
<td>4/8</td>
<td>1/8</td>
</tr>
</tbody>
</table>

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 RT-PCR. These neurons were screened for AdipoR1, R2, GAPDH (positive control) and synaptotagmin (neuronal marker, not shown).
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

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 36h following dissociation. Non responsive neurons are shown in green.
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 qRT-PCR 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 qRT-PCR, 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 system would explain the effects observed in SFO neurons. The 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 MgCl2, 7 µl diethylpyrocarbonate (DEPC) treated-H2O 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-H2O 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 The Nanogen (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 MgCl₂, 2 CaCl₂, 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 MgCl₂, 2 CaCl₂, 10 HEPES, 10 EGTA, 4 Na₃ATP 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 spontaneous or current evoked action potentials with a spike amplitude greater than 60 mV in current-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, Hanover MD) according to manufacturer’s directions, and treated with 1U of RNase-free DNase 1 (Fermentas, Hanover MD) against genomic DNA (a negative control; described in Price et al., 2008), synaptotagmin (neuronal marker; described in Hindmarch et al., 2008), genomic DNA (a negative control; described in Price et al., 2008), and adiponectin receptors (AdipoR1 and AdipoR2; described in Hindmarch et al., 2008) 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 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 30 s, 72 °C for 30 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 5x Q-Solution, 16 µl H₂O, 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 (qRT-PCR) 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 1U of RNase-free DNase 1 (Fermentas, Hanover MD) against manufacturer’s directions. This RNA was utilized as template for cDNA synthesis in a 30 µl reaction using Superscript II reverse transcriptase kit (Invitrogen, Burlington ON, Canada). The RT ran overnight at 37 °C, after which the single-cell 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 fibrillar 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 30 s, 72 °C for 30 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 5x Q-Solution, 16 µl H₂O, 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).

Adiponectin (human globular) was obtained from Phoenix Pharmaceuticals, Inc (Belmont CA, USA) in a lyophilized form. The lyophilized ADP was resuspended in DPC treated-H₂O 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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References


