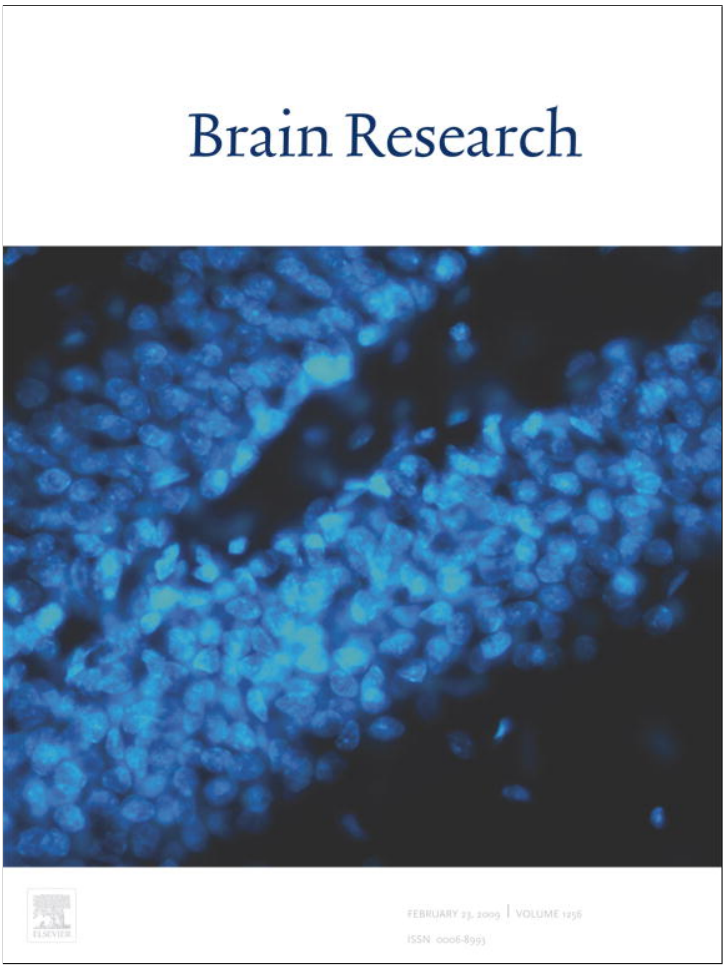

BRAIN RESEARCH 1256 (2009) 76–84

available at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/brainres

BRAIN RESEARCH

Research Report

Adiponectin acts in the nucleus of the solitary tract to decrease blood pressure by modulating the excitability of neuropeptide Y neurons

*Ted D. Hoyda, Pauline M. Smith, Alastair V. Ferguson**

Department of Physiology, Faculty of Arts and Science, Queen's University, Kingston, Ontario, Canada K7L 3N6

ARTICLE INFO

Article history:
Accepted 2 December 2008
Available online 11 December 2008

Keywords:
Adiponectin
Electrophysiology
Hypotension
ssRT-PCR
NPY
GABA

ABSTRACT

Adiponectin is an adipocyte derived hormone which acts in the CNS to control autonomic function, energy and cardiovascular homeostasis. Two 7-transmembrane domain receptors, AdipoR1 and AdipoR2, expressed in the hypothalamus and brainstem mediate the actions of adiponectin. The medulla's nucleus of the solitary tract (NTS) is the primary viscerosensory integration site and an important nucleus in the regulation of cardiovascular function. Here we show the localization of both AdipoR1 and AdipoR2 mRNA in the NTS. We have investigated the consequences of receptor activation in response to exogenous application of adiponectin on cardiovascular (blood pressure and heart rate monitoring *in vivo*), and single neuron (whole cell current-clamp recordings *in vitro*) function. Microinjection of adiponectin in the medial NTS (mNTS) at the level of the area postrema resulted in a decrease in BP (mean AUC = −2055 ± 648.1, n = 5, mean maximum effect: −11.7 ± 3.6 mm Hg) while similar commissural NTS (cNTS) microinjections were without effect. Patch clamp recordings from NTS neurons in a medullary slice preparation showed rapid (within 200 s of application) reversible (usually within 1000 s following washout) effects of adiponectin on the membrane potential of 62% of mNTS neurons tested (38/61). In 34% (n = 21) of mNTS neurons adiponectin induced a depolarization of membrane potential (6.8 ± 0.9 mV), while the remainder of mNTS cells influenced by adiponectin (n = 17) hyperpolarized in response to this adipokine (−5.4 ± 0.7 mV). *Post-hoc* single cell RT-PCR (ssRT-PCR) analysis of neurons showed that the majority of NPY mRNA positive mNTS neurons were depolarized by adiponectin (7/11), while 4 of these depolarized cells were also GAD67 positive. The results presented in this study suggest adiponectin acts in the NTS to control BP and suggest that such effects may occur as a direct result of the ability of this adipokine to modulate the excitability of discrete groups of neurons in the NTS. These studies identify the mNTS as a new CNS site which adiponectin may act to influence central autonomic processing.

1. Introduction

The adipocyte hormone adiponectin is positively correlated with insulin sensitization, energy homeostasis and endocrine control through actions at two receptors, AdipoR1 and AdipoR2, distributed throughout the CNS and peripheral tissue (Hoyda et al., 2007; Fry et al., 2006; Ahima and Lazar, 2008; Yamauchi et al., 2003; Kadowaki and Yamauchi, 2005).

* *Corresponding author.*
E-mail address: avf@queensu.ca (A.V. Ferguson).

0006-8993/$ – see front matter.
doi:10.1016/j.brainres.2008.12.012

Additionally much recent attention has focused on the cardiovascular effects of adiponectin treatment (Wang and Scherer, 2008). Hypoadiponectinemia has been positively correlated with high risk for hypertension (Iwashima et al., 2004), whereas reconstitution of adiponectin through adenoviral infection in the genetically obese KKAy mouse ameliorates elevated systolic BP (Ohashi et al., 2006). Evidence suggests that adiponectin may influence cardiovascular homeostasis through actions on endothelial cells to promote local nitric oxide (NO) production (Tan et al., 2004), through interaction with the renin–angiotensin system (Furuhashi et al., 2003), or potentially as a consequence of its modulatory effects on the sympathetic nervous system through actions at sites in the central nervous system (Fry et al., 2006; Tanida et al., 2007). Located in the medulla, the NTS is the principle integration site for cardiovascular and autonomic sensory afferents (Saper, 2002). Through its GABAergic interneurons, the NTS is a critical component of the baroreceptor reflex circuit that regulates sympathetic tone (Guyenet, 2006) and thus represents a potential site at which adiponectin could influence cardiovascular homeostasis. Therefore, using microinjection, current-clamp electrophysiology and molecular techniques we have investigated the actions of adiponectin in the NTS and provide evidence to suggest this site represents a potentially crucial autonomic site for actions of this adipokine.

2. Results

2.1. *The NTS contains mRNA for both AdipoR1 and AdipoR2*

The NTS is a primary integration site for autonomic control and a site at which adiponectin could potentially act. We first examined if adiponectin receptors were expressed in NTS tissue through the use of RT-PCR. Acutely dissociated slices containing medial NTS neurons were cut out and used to make cDNA (Fig. 1B). Primers designed to detect AdipoR1 and AdipoR2 mRNA were used to probe mNTS cDNA and the resulting products of the PCR reaction were visualized on an agarose gel. cDNA of both AdipoR1 and AdipoR2 was detected indicating adiponectin receptor mRNA is localized in the mNTS (Fig. 1A). cDNA of GAPDH was amplified along with the adiponectin receptor primers as a positive control and an RT (–) reaction was performed to verify the reactions contained no genomic DNA.

2.2. *Adiponectin microinjected into the NTS decreases BP*

A total of 15 animals were used in this study. Microinjection of 0.5 μl of 10^{-7} M adiponectin into mNTS resulted in a clear decrease in BP (mean AUC=−2055.0±648.1 mm Hg*s, $n=5$, mean maximum effect: −11.7±3.6 mm Hg), without a significant change in HR (mean AUC=−40.9±19.3 beats, $n=5$). These depressor effects showed a rapid onset (occurring within 15 s of adiponectin administration) with a peak decrease in BP occurring approximately 60 s after microinjection and lasting several minutes (at least 5 min) before returning toward baseline (see Figs. 1B, C). These effects were shown to be the result of peptide administration as microinjection of aCSF (vehicle control) was without effect on BP (mean AUC=−7.3±183.9 mm Hg*s, $n=6$, $p<.01$) or HR (mean AUC=−5.3±15.9 beats, $n=6$). The effect of adiponectin microinjection into mNTS was also shown to be site specific as adiponectin administration into the immediately adjacent cNTS was without effect on BP (AUC=−380.9±96.0 mm Hg*s, $n=4$, $p<.05$) or HR (AUC=−13.6±17.8 beats, $n=4$) (Figs. 1C, D).

2.3. *Adiponectin influences the membrane potential of NTS neurons*

Current-clamp recordings were made from 61 mNTS neurons (identified anatomically based on their position in the slice) and the effects of bath administration of 10 nM adiponectin was examined. Following the development of a stable control baseline membrane potential for a minimum of 50 s, adiponectin was perfused on to these medullary slices at a rate of 1–2 ml/min. Adiponectin influenced the majority of cells (38/61 affected), inducing both clear depolarizing (mean 6.8±0.9 mV, $n=21$) or hyperpolarizing (mean −5.4±0.7 mV, $n=17$) effects on NTS neurons (Figs. 2A, B). Both depolarizing and hyperpolarizing effects were reversible upon removal of adiponectin from the bath and most cells recovered to control membrane potential within 1000 s. In some cells the depolarizing effects of adiponectin were accompanied by an initiation of action potentials or an increase in action potential frequency (in previously active cells) which returned to baseline upon washout. Similarly cells which hyperpolarized in the presence of adiponectin decreased or ceased firing APs, an effect that was also reversible. Intriguingly cells which depolarized reached peak effect faster than those that hyperpolarized in the presence of adiponectin (depolarization: normally within 200 s, hyperpolarization: normally 500 s) suggesting two independent signaling mechanisms with different time courses of activation. These observations suggest that there are at least two distinct adiponectin responsive cell populations within the mNTS, that adiponectin controls the excitability of a majority of these neurons and that these effects are potentially mediated through different intracellular signaling pathways.

2.4. *Adiponectin effects are diverse among electrophysiologically defined NTS neurons*

Considerable effort has been made to define the unique electrophysiological properties of cell groups in the NTS, studies which have led to the classification of three different subpopulations of neurons defined according to the presence (in response to a large hyperpolarizing current pulse) of a delayed return to baseline and first spike (DE cells), a post-inhibitory rebound (PIR cells), or neither of these characteristics (NON cells) (Vincent and Tell, 1997). Analysis of the effects of adiponectin on the membrane potential of DE (27% depolarize, 27% hyperpolarize, 46% unaffected, $n=26$), PIR (36% depolarize, 18% hyperpolarize, 46% unaffected, $n=11$) and NON (33% depolarize, 25% hyperpolarize, 42% unaffected, $n=24$) cells demonstrated heterogeneity in the response characteristics in each group (Fig. 3A). These data suggest that adiponectin actions on mNTS neurons are not correlated

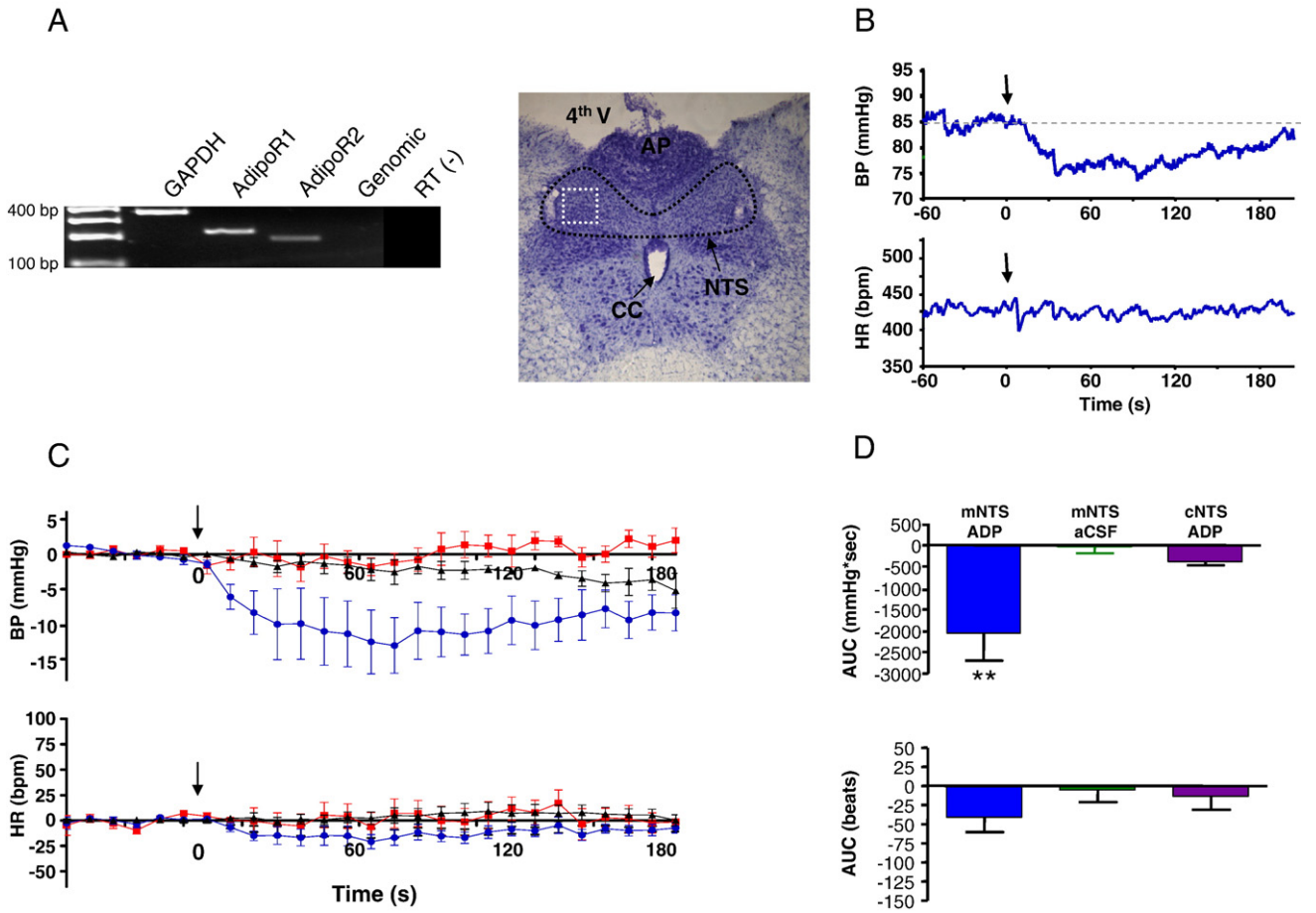

Fig. 1 – Microinjection of adiponectin into the mNTS decreases BP. (A) This agarose gel shows RT-PCR analysis of mNTS cDNA for AdipoR1 and AdipoR2. PCR products for GADPH are observed (positive control) while no products were observed for genomic cDNA (Genomic) or in the RT-lane, in which reverse transcriptase has been omitted from the cDNA synthesis reaction. The photomicrograph to the right is a cresyl violet stained coronal section of the medulla outlining NTS (black line) highlighting the portion of mNTS removed for RT-PCR analysis (white line) (AP = Area Postrema, CC = central canal, 4th V = fourth ventricle). (B) Raw BP (upper) and HR (lower) recordings showing the response to a bolus microinjection of adiponectin into mNTS in a single animal (time of injection is indicated by the arrow). The photomicrograph to the left shows the anatomical location of the microinjection site in mNTS (black circle) in this animal. (C) Normalized mean BP (upper) and HR (lower) traces showing the response to adiponectin microinjection into mNTS (●, $n=5$) or cNTS (▲, $n=4$). Microinjection of vehicle (aCSF) into mNTS (■, $n=6$) is also shown. (D) These summary bar graphs show mean area under the curve for BP (top) and HR (bottom) in response to adiponectin microinjection in mNTS (blue bar) or cNTS (purple bar) or to vehicle control (aCSF) microinjection into mNTS (green bar) (indicates $p<0.001$ between mNTS adiponectin vs. mNTS aCSF or mNTS adiponectin vs. cNTS adiponectin, determined by repeated ANOVA).**

to the electrophysiological properties unique to the three cell groups.

2.5. *NPY/GAD67 neurons in the NTS are depolarized by adiponectin*

A number of different neurotransmitters and neuromodulators are expressed within the NTS (Lawrence and Jarrott, 1996), each of which has a unique ability to shape the output characteristics of the nucleus. To assess whether adiponectin controls specific groups of chemically defined NTS neurons, following current-clamp recordings we used *post-hoc* ssRT-PCR to identify the presence of GAPDH, AdipoR1, AdipoR2, cocaine amphetamine related transcript (CART), melanocortin 4 receptor (MC4R), glutamate (VGLUT), pro-opiomelanocortin (POMC), neuropeptide Y (NPY) and GAD67 (a GABAergic cell marker) mRNA in recorded neurons. Cytoplasm was obtained from a total of 41 mNTS neurons of which 37 cells showed expression of the housekeeping gene GAPDH indicating successful collection and amplification of cytoplasmic mRNA. AdipoR1 mRNA was found to be expressed in 17 of these neurons (depolarization $n=8$, no response $n=7$, hyperpolarization $n=2$) while AdipoR2 was *co-expressed* with AdipoR1 in only one cell and *never* expressed independently. Of the 37 GAPDH positive cells a total of 11 of these identified neurons expressed NPY, and the majority (7/11) of these NPY

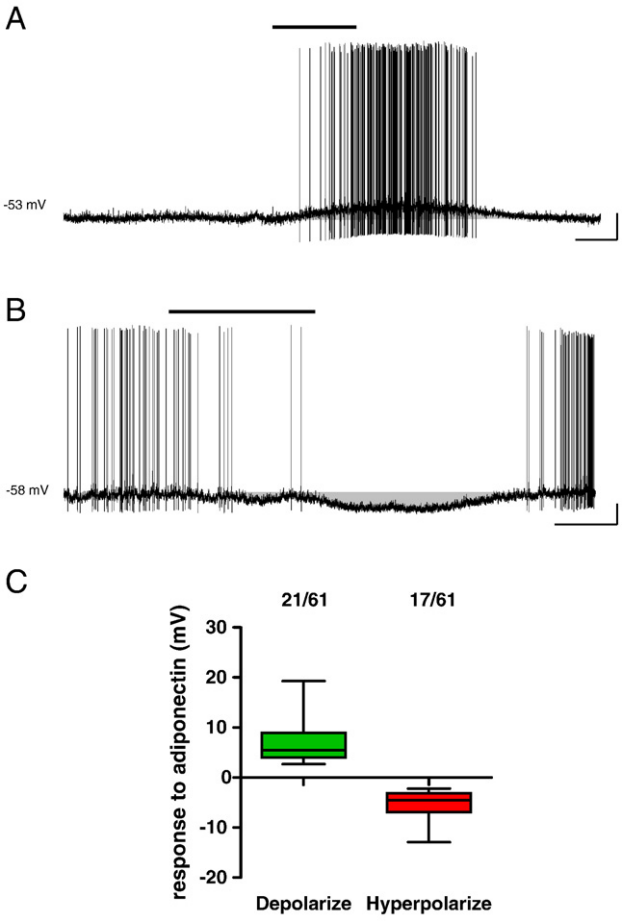

Fig. 2 – Adiponectin's effects on the membrane potential of medial NTS neurons. Current-clamp recordings showing two different medial NTS neurons (A) depolarizing and (B) hyperpolarizing to bath administration of 10 nM adiponectin (Black bar, scale bars: 10 mV and 50 s) and recovery of membrane potential to control following the removal of adiponectin from the bath. Adiponectin influenced the excitability of a majority of neurons tested (38/61). (C) A box-and-whisker plot indicating both depolarizing and hyperpolarizing responses seen in medial NTS neurons: mean depolarization: 6.8±0.8 mV, $n=21$, mean hyperpolarization: −5.4±0.7 mV, $n=17$ (mean±SEM).

cells depolarized in response to adiponectin (7.1±1.6 mV), while the remaining 4 NPY cells were unaffected (mean membrane potential change 0.0±1.3 mV) (Figs. 3B, C). Five of the depolarized NPY cells also expressed mRNA for AdipoR1 suggesting depolarization of NPY neurons is mediated through activation of this receptor. Four NPY neurons also expressed GAD67 and all of these cells depolarized in response to adiponectin (7.1±2.0 mV). In addition, 3/4 NPY/GAD67 neurons expressed AdipoR1. One cell expressing GAD67 but not NPY did not respond. While our experiments also identified small populations of CART ($n=2$), MC4R ($n=3$), VGLUT ($n=1$), POMC ($n=1$), and AdipoR1/AdipoR2 ($n=1$) neurons, these small subpopulations did not show any homogenous patterns of responsiveness to adiponectin.

These data suggest that adiponectin depolarizes populations of both NPY and GAD67/NPY cells in the NTS, a subpopulation which we speculate may be responsible for changes in BP caused by adiponectin microinjected into this nucleus.

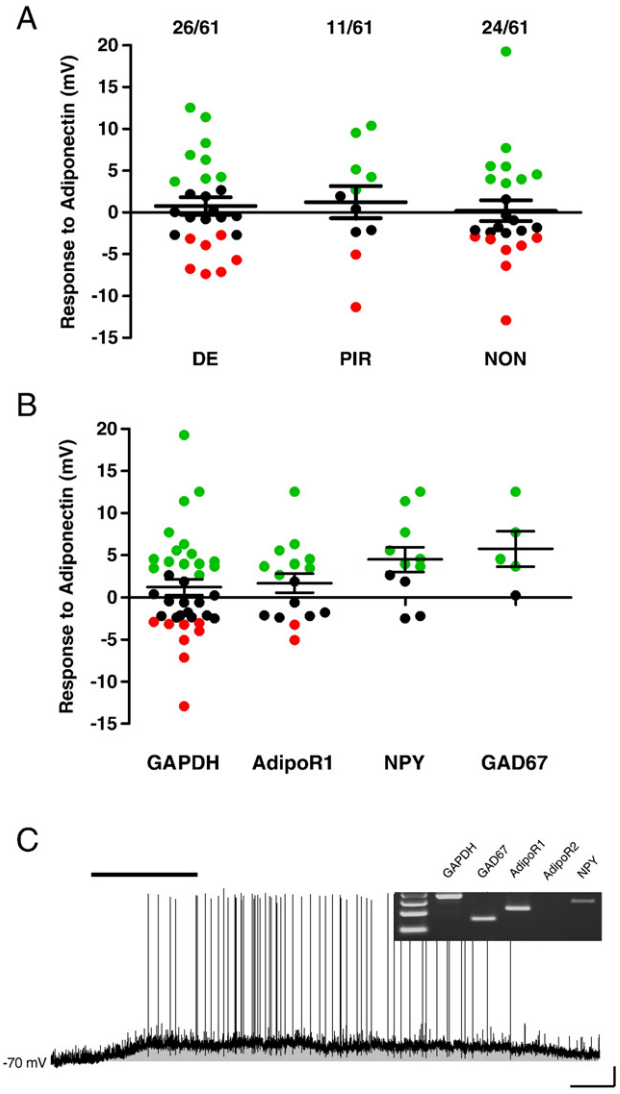

Fig. 3 – Adiponectin depolarizes neurons expressing NPY and GAD67. (A) Scatter plot separating recorded neurons according to their electrophysiological fingerprint; DE, PIR and NON. The plot indicates that each classification group contains neurons that depolarize, hyperpolarize or do not respond. (B) A scatter plot of ssRT-PCR results for the effect of 10 nM adiponectin on the membrane potential of cells that expressed GAPDH, only AdipoR1, NPY and GAD67. Green data points indicate a depolarizing response, red a hyperpolarizing response while black indicate no response to adiponectin. The four GABA cells which depolarized also expressed NPY and thus have been included twice in this graph. (B) Current-clamp recording of the membrane potential response to bath administration of 10 nM adiponectin (black bar) onto a cell whose chemical phenotype was assessed *post hoc* for GAD67, AdipoR1 and NPY by ssRT-PCR (gel inset). Scale bars: 10 mV, 50 s.

3. Discussion

Here we demonstrate mRNA for both adiponectin receptors to be localized in the NTS, and have provided evidence that adiponectin acts in the mNTS to decrease BP in the whole animal. Through current-clamp recordings and ssRT-PCR we have shown that adiponectin influences the membrane potential of mNTS neurons, depolarizing a subpopulation of mNTS neurons which express NPY and in some cases GAD67. A second subpopulation(s) of unidentified mNTS neurons which do not appear to express AdipoR2, VGLUT, MC4R, POMC or CART are hyperpolarized by adiponectin. These results highlight the mNTS as a CNS site at which adiponectin potentially acts to control autonomic function and energy homeostasis.

These results are the first to identify the presence of adiponectin receptors in the NTS. Our group and others have localized adiponectin receptors to central sites involved in autonomic control and feeding behavior including the paraventricular nucleus of the hypothalamus (PVN) (Hoyda et al., 2007), the arcuate nucleus (ARC) (Kubota et al., 2007) and the area postrema (AP) (Fry et al., 2006), a circumventricular organ adjacent to the NTS. Adiponectin has been shown to attenuate hypertension through interaction with the RAS-system (Furuhashi et al., 2003), at the endothelial level (Tan et al., 2004) and through actions in the CNS at the suprachiasmatic nucleus (Tanida et al., 2007) (for review see Wang and Scherer, 2008). Additionally our lab has reported *hypertensive* effects when microinjected into the area postrema (Fry et al., 2006). Intriguingly, while microinjections of adiponectin into the mNTS caused a decrease in BP, injections of the peptide into the commissural NTS exhibited no effect on BP. While only one dose was used for microinjection studies, the clear hypotensive effects following mNTS and not cNTS injections suggest a site specific action for adiponectin in controlling BP. These results suggest that adiponectin plays diverse roles in regulating BP depending on the predominant site of action. Opposite effects of adiponectin on BP seen in two adjacent brainstem nuclei may be due to availability and concentration *in vivo*. Unlike the NTS the AP is a circumventricular organ with fenestrated capillaries capable of readily sampling membrane impermeable substances from the blood (Price et al., 2008a). Blood brain barrier studies have suggested that adiponectin is unable to access the CNS (Spranger et al., 2006), although Qi et al. (2004) have suggested that adiponectin can access sites in the hypothalamus to confer its effects on energy homeostasis. Recent reports have indicated the presence of adiponectin in human cerebrospinal fluid albeit at 1000 fold lower concentrations (Kos et al., 2007; Kusminski et al., 2007) indicating that it is either derived in, or has access to, the CNS. Whether the difference in concentration seen by each nucleus is responsible for their opposite effects on BP is unknown at this time.

Adiponectin was also shown to have mixed effects on the membrane potential of NTS neurons, actions which we originally hypothesized might be associated with separate subpopulations of NTS neurons previously characterized by their electrophysiological fingerprint (Vincent and Tell, 1997). This was not the case as both depolarizing and hyperpolarizing effects of adiponectin were observed in DE, PIR and NON cells of the mNTS. We next examined if differential responses may correlate with genetic phenotype of recorded mNTS neurons using ssRT-PCR techniques. We were able to show that mNTS neurons which express NPY and AdipoR1 (and in some cases GAD67) represented a homogenous group the majority of which were depolarized by bath administration of adiponectin. Intriguingly NPY, while abundantly expressed in the NTS (Lawrence et al., 1998), is a well established *hypotensive* agent when injected into the brainstem nuclei (Tseng et al., 1989; Kubo and Kihara, 1990). The GABAergic interneuron also plays an important role in modulating BP regulation and baroreflex sensitivity in the NTS (McLean et al., 1996). Evidence suggests that activation of $GABA_A$ receptors mediate hypertensive responses following bilateral injections of GABA into the NTS (Catelli et al., 1987). We propose that this neuron which co-expresses NPY and GAD67 could potentially act as an autonomous state-dependent modulator of BP based on firing rate and release patterns of small-synaptic vesicles (containing GABA) and large dense-core vesicles (containing NPY; Paquet et al., 1996) from nerve terminals. Intriguingly the inefficacy of NPY in NTS seen in diabetes (Dunbar et al., 1992) has been suggested to play a role in the development of hypertension (Ergene et al., 1993; Lawrence and Jarrott, 1996). Indeed loss of adiponectin function is a key contributor in the pathogenesis of type 2 diabetes (Hara et al., 2002; Scherer, 2006). Whether adiponectin proves to be an important link between NPY neurons, the NTS and development of diabetic associated hypertension remains to be seen.

The NTS is the primary integration site for information regarding gustatory and gastrointestinal information in the CNS. It projects to major autonomic regulation sites in the brainstem and hypothalamus including the AP, ARC, PVN and lateral hypothalamus and participates in controlling energy homeostasis and feeding behavior (Williams et al., 2006). Adiponectin has recently been suggested to act in the ARC to increase food intake through AdipoR1 and AMPK activation suggesting a role for activation of the NPY neurons in the ARC (Kubota et al., 2007). If a reciprocal feeding pathway exists in the NTS regarding the activation of NPY neurons and the induction of feeding, our results would suggest that adiponectin may also act in the NTS to induce a control over feeding behavior. Further studies will be needed to assess this hypothesis.

Our ssRT-PCR analysis indicates that cells which hyperpolarize in response to adiponectin do not express AdipoR2, GAD67, NPY, VGLUT, CART, MCR4 or POMC. These data suggest that adiponectin may confer its effects on feeding and metabolism through inhibition of a chemically distinct group of neurons from the ones we have examined in this study. The lack of AdipoR2 mRNA in comparison to AdipoR1 mRNA expression in neurons is also intriguing. RT-PCR results from tissue experiments indicate that both receptors are present in the nucleus however ssRT-PCR studies suggest that with the exception of one cell, only AdipoR1 is present in neurons and thus mediates effects on neuronal excitability. Interestingly both depolarizing and hyperpolarizing effects of adiponectin are seen in neurons that express AdipoR1. Previous studies have shown that activation AMPK, the major

intracellular signaling molecule targeted by AdipoR1 (Kubota et al., 2007; Yamauchi et al., 2003), can lead to both excitation or inhibition of membrane potential in a cell type specific manner (Wen et al., 2008; Wyatt et al., 2007). The unique expression of ion channel complement in mNTS neurons and how they are affected by activated AMPK may be responsible for effects of adiponectin on the membrane potential of these cells. Alternately AdipoR2 may be expressed in supporting cells within the nucleus (endothelial and/or glial cells) and upon activation may contribute to signaling through paracrine actions on recorded neurons.

In conclusion we have shown that through the expression and activation of adiponectin receptors in the NTS, adiponectin decreases BP through controlling the excitability of neurons in the NTS potentially involving a NPY/GABA interneuron. Furthermore we have identified a novel excitatory effect of adiponectin on the orexigenic peptide NPY expressing neurons of the NTS. These results highlight the growing role of adiponectin in controlling autonomic systems through actions at sites in the CNS and provide important information into the physiology with which this peptide acts.

4. Experimental procedures

4.1. *Preparation of brainstem slices*

All procedures conformed to the ethical guidelines established by the Canadian Council on Animal Care and were approved by the Queen's University Animal Care Committee. Brains from decapitated male Sprague Dawley rats (p21–28) were quickly removed and immersed in a high sucrose based ice cold slicing solution (2–4 °C) 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, 75 sucrose bubbled with 95% O_2/5% CO_2 for 3–5 min. Brains were then blocked, mounted onto a vibratome (Leica Microsystems, Nussloch, Germany) and immersed in slicing solution bubbled with 95% O_2 while 300 μm slices containing NTS were cut and placed in 32 °C artificial cerebral spinal fluid (aCSF) containing (in mM): 126 NaCl, 2.5 KCl, 26 $NaHCO_3$, 2 $CaCl_2$, 2 $MgCl_2$, 1.25 NaH_2PO_4, 10 glucose saturated with 95% O_2/5% CO_2.

4.2. *Preparation of DNA free mRNA for adiponectin receptor mRNA detection*

All procedures involving molecular biology adhered to strict DNA–RNA contamination free protocol. Tissue sections containing exclusively mNTS tissue were microdissected out following slicing procedures (outlined above). NTS RNA was isolated from tissue and diluted a final volume of approximately 100 μl solution according to instructions in the ethanol free Ambion RNA isolation kit (Applied Biosystems, Toronto, ON, Canada). Isolated NTS RNA was then added to a reaction tube set up to run a DNase treatment with the following reagents provided by Fermentas (Burlington, ON, Canada): 10 μl 10× Reaction Buffer with $MgCl_2$ and 10 U deoxyribonuclease I. The DNase reaction proceeded at 37 °C for 30 min, then added to it 10 μM 25 mM EDTA and held at 65 °C for 10 min to inactivate the enzyme and placed on ice. 10 μl of DNA free RNA was used as a template for a reverse transcription reaction according to Superscript III (Invitrogen, Burlington, ON, Canada). A 50 μl reaction volume for the reverse transcription reaction included the following reagents: 50 ng random hexamer, 10 mM dNTP mix, 2 μl 10× RT buffer, 25 mM $MgCl_2$, 0.1 M DTT, 40 U RNaseOUT, 200 U Superscript III RT heated to 50 °C for 50 min then to 85 °C for 5 min. Two controls were run in addition to the reaction: one omitting the reverse transcriptase enzyme and one replacing NTS template with DNA/RNA free water. Following completion of the RT reaction, aliquots were stored at −80 °C or prepared for a polymerization chain reaction (PCR).

Table 1 – Primers used in RT-PCR experiments

Primer Name		Outside	Nested
AdipoR1	Sense	gtcccctggctctattactcct	tcttcctcatggctgtgatgt*
	Antisense	agcacttgggaagttcctcc	gctcagagaagggagtcatc*
AdipoR2	Sense	ggagccattctctgcctttc	actgtaacccacaaccttgcttc*
	Antisense	ccagatgtcacatttgcca	tcaggaacccttctgagatgac*
GAPDH	Sense	gatggtgaaggtcggtgtg	taccaggctgccttctct
	Antisense	gggctaagcagttggtggt	ctcgtggttcacacccatc
GAD67	Sense	cacaaactcagcggcataga	cacaaactcagcggcataga
	Antisense	gagatgaccatccggaagaa	ctggaagaggtagcctgcac
VGLUT2	Sense	aggttggctaccacctcctt	cccgcaaagcatccaacca
	Antisense	tgagagtagccaacaaccagaagca	cctgcagaagtttgcaacaa
CART	Sense	tggacatctactctgccgtg	gctgtgttgcagattgaagc
	Antisense	aatatgggaaccgaaggagg	gtcacccccttcacaagcact
NPY	Sense	gcccagagcagagcaccc	cagagaccacagcccgcc
	Antisense	caagtttcatttcccatcacca	tcggacacaaagtacacagg
MC4R	Sense	tctgaaaagaccccgagtga	tcagccgagagtgagctttc
	Antisense	tcctccttgaagtggtccaa	tcctccttgaagtggtccaa
POMC	Sense	gattctgctacagtcgctca	ccctgttgctggccctcctg
	Antisense	tctcggcgacattggggtaca	ctccccccgccgtctcttcc

Sequences are always indicated in the 5′–3′ direction. Nested primer sets for AdipoR1 and AdipoR2 were used for tissue RT-PCR experiments (indicated by *).

4.3. PCR detection for adiponectin receptors in NTS

A multiplex PCR strategy (Qiagen, Mississauga, ON, Canada) was employed to detect PCR products with primers specific for adiponectin receptor 1 and 2, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) which acted as a positive control and genomic DNA, which acted as a negative control. PCR detection for adiponectin receptors in NTS was run in triplicate according to the following protocol. Multiplex reactions were performed in 50 μl volumes and contained 0.2 μM of each primer (Table 1), and reagents provided with the Qiagen kit. The reaction volume was denatured at 95 °C for 15 min and then cycled 30 times according to the following temperature protocol: 30 s at 94 °C, 90 s at 60 °C and 90 s at 72 °C followed by one final extension of 72 °C for 10 min. PCR products were run on a 2% agarose gel containing ethidium bromide and sequenced to confirm their identity (Robarts Institute, London, ON, Canada).

4.4. Microinjection

Urethane-anesthetized (1.4 g/kg) male Sprague Dawley rats (150–350 g) were fitted with a tracheal cannula (PE-205; Intramedic) to facilitate breathing and a femoral arterial catheter for the measurement of blood pressure (BP) and heart rate (HR). Animals were placed on a feedback-controlled heating blanket for the duration of the experiment to maintain body temperature at 37 °C. The animal was then placed in a stereotaxic frame with its head positioned vertically (nose down). A midline incision was made at the level of the obex to expose the dorsal surface of the medulla and a microinjection cannula (150 μm tip diameter; Rhodes Medical Instruments) was positioned into commissural nucleus tractus solitarius (cNTS) or mNTS. After a minimum 2 min stable baseline recording was obtained, 0.5 μl of either 10^{-7} M adiponectin (Phoenix Pharmaceuticals, Burlingame, CA, USA) or artificial cerebral spinal fluid (aCSF, vehicle control) was unilaterally microinjected into the region by a pressure driven 10 μl Hamilton micro-syringe over 10 s and the effects on BP and HR assessed. Only one injection was made per animal. The concentration of adiponectin for microinjection was chosen to reflect circulating plasma concentrations which, in the male Sprague Dawley rat, is approximately 3 μg/ml or 10^{-7} M (Yang et al., 2004).

At the conclusion of the experiment, animals were overdosed with anesthetic and perfused with 0.9% saline, followed by 10% formalin, through the left ventricle of the heart. The brain was removed and placed in formalin for at least 24 h. Using a vibratome, 50 μm coronal sections were cut through the region of NTS, mounted, and cresyl violet stained. The anatomical location of the microinjection site was verified at the light microscope level by an observer unaware of the experimental protocol or the data obtained.

4.5. Analysis of blood pressure and heart rate data

Animals were assigned to one of two anatomical groups (cNTS or mNTS) according to the location of the microinjection sites. Animals with injection sites that were not wholly confined within either of these regions were excluded from further analysis. Animals with confirmed microinjection sites in mNTS were further divided according to whether adiponectin or aCSF (vehicle control) was microinjected into the region. Normalized BP and HR data (mean baseline BP and HR data were calculated for 60 s before injection and subtracted from all data points before and after injection) were obtained for each animal 60 s before the time of microinjection (control period) until 180 s after microinjection. Area under the curve (AUC) (area between baseline and each BP and HR response) was calculated for each animal for the 180 s time period immediately after the injection, and the mean AUC for BP and HR responses for each group were then calculated. A one way analysis of variance (one way ANOVA) was used to determine whether BP and HR responses observed in response to adiponectin were different according to anatomical location of the microinjection site (cNTS vs mNTS adiponectin) or to substance administered (adiponectin vs aCSF).

4.6. Electrophysiology

Slices were placed in a recording chamber and continuously perfused with 30–32 °C oxygenated aCSF at a rate of approximately 2 ml/min. Neurons were visualized on an upright differential interface contrast microscopy system (Nikon, Tokyo, Japan). Whole cell current-clamp recordings were made using a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA, USA) sampled at 5 kHz, filtered at 2.4 kHz from a Micro 1401 interface equipped with Spike acquisition and analysis software (Cambridge Electronic Devices, Cambridge, UK) and then stored for offline analysis. Electrodes were filled with an intracellular solution consisting of (in mM): 125 potassium gluconate, 2 $MgCl_2$, 5.5 EGTA, 10 KCl, 2 NaATP and 10 Hepes (pH-7.2 with KOH). When filled with solution electrodes had resistances between 3–5 MΩ. Only those cells which had action potentials larger than 60 mV and a stable baseline membrane potential after 10 min were recorded from. Prior to experimentation a pulse protocol was used to classify neurons as either PIR, DE or NON (Vincent and Tell, 1997). After establishing 50 s of control baseline adiponectin was applied to the cell through bath perfusion. A cell was considered to be responsive to adiponectin if the membrane potential change measured in 50 s blocks for 500 s following the start of application changed more then 2 standard deviations of the baseline membrane potential. Neurons were excluded from analysis if they did not show a recovery towards baseline following removal of adiponectin from the bath.

4.7. Single cell RT-PCR

Following the completion of current-clamp recording, a *post-hoc* ssRT-PCR protocol was performed to examine the expression of molecular markers (see Table 1) as described previously (Price et al., 2008b). Briefly, gentle suction was applied to aspirate cytoplasm into the recording pipette. An outside-out patch was pulled sealing the cytoplasmic contents within the pipette. Cells which failed to form outside-out patches were eliminated from ssRT-PCR analysis but included in membrane potential analysis. The contents of the electrode were expelled into a 0.5 mL PCR tube. The following components were added and a DNase reaction was performed: (approximate concentrations) 10 U DNase I and 10 mM 10×

reaction buffer with $MgCl_2$ (Fermentas, Burlington, ON, Canada) and incubated at 37 °C for 30 min. 2.5 mM EDTA was added and incubated at 65 °C for 10 min to stop the DNase reaction. mRNA was reverse transcribed immediately after by adding Dithiothreitol (26 mM), dNTPs (3 mM), random hexamers (3 μM), $MgCl_2$ (4 mM), RNase inhibitor (20 U) and superscript II (100 U) (Invitrogen, Burlington, ON, Canada) and allowed to incubate overnight at 37 °C. An additional reaction mixture was made up without (RT) and used as a negative control for genomic contamination. Samples were subsequently stored at −80 °C until used in PCR detection.

A multiplex PCR protocol was used to amplify the cDNA produced in the RT reaction. This protocol consisted of two different amplification steps: outside and nested. First, 'outside' primer sets (0.2 μM) were added to the cDNA along with reagents provided in the Qiagen multiplex kit (Qiagen, Mississauga, ON, Canada) to a 100 μl volume. The reaction mixture underwent a temperature protocol: 95 °C for 15 min and then 20 cycles of 94 °C for 30 s, 60 °C for 90 s, and 72 °C for 90 s. The second step consisted of individual reactions for each gene of interest using 'nested' primer sets. Reactions were performed in 50 μl volumes consisting of Qiagen Multiplex reaction reagents, 0.2 μM of primers and 2 μl of solution from the multiplex reaction to act as template. The reaction mixture was subject to 35 cycles of the same cycling protocol. PCR products from the nested reaction were run on a 2% agarose gel containing ethidium bromide and sequenced to confirm their identity (Robarts Institute, London, ON, Canada). With each reaction a positive and negative control was performed in which primers were run against cDNA made from whole NTS (+control) and in a reaction which PCR grade water replaced the cDNA template (−control). Experiments which did not pass either control were eliminated from the study.

4.8. *Chemicals and peptides*

All chemicals used to make solutions were obtained from Sigma Pharmaceuticals (Sigma, Burlington, Ontario, Canada). Salts used to make intracellular solution used in ssRT-PCR analysis were from molecular biology grad stock and were kept separate from salts used for other solutions. Adiponectin (recombinant human globular) was obtained from Phoenix Pharmaceuticals (Belmont, CA, USA) reconstituted in distilled water and made up into working aliquots for experimentation.

Acknowledgments

This work was supported by grants from the Heart and Stroke Foundation (AVF) (PMS) and a Canadian Institutes of Health Research Doctoral Research Award (TDH).

REFERENCES

Ahima, R.S., Lazar, M.A., 2008. Adipokines and the peripheral and neural control of energy balance. Mol. Endocrinol. 22, 1023–1031.

Catelli, J.M., Giakas, W.J., Sved, A.F., 1987. GABAergic mechanisms in nucleus tractus solitarius alter blood pressure and vasopressin release. Brain Res. 403, 279–289.

Dunbar, J.C., Ergene, E., Anderson, G.F., Barraco, R.A., 1992. Decreased cardiorespiratory effects of neuropeptide Y in the nucleus tractus solitarius in diabetes. Am. J. Physiol. 262, R865–R871.

Ergene, E., Dunbar, J.C., Barraco, R.A., 1993. Visceroendocrine responses elicited by neuropeptide Y in the nucleus tractus solitarius. Brain Res. Bull. 32, 461–465.

Fry, M., Smith, P.M., Hoyda, T.D., Duncan, M., Ahima, R.S., Sharkey, K.A., Ferguson, A.V., 2006. Area postrema neurons are modulated by the adipocyte hormone adiponectin. J. Neurosci. 26, 9695–9702.

Furuhashi, M., Ura, N., Higashiura, K., Murakami, H., Tanaka, M., Moniwa, N., Yoshida, D., Shimamoto, K., 2003. Blockade of the renin–angiotensin system increases adiponectin concentrations in patients with essential hypertension. Hypertension 42, 76–81.

Guyenet, P.G., 2006. The sympathetic control of blood pressure. Nat. Rev. Neurosci. 7, 335–346.

Hara, K., Boutin, P., Mori, Y., Tobe, K., Dina, C., Yasuda, K., Yamauchi, T., Otabe, S., Okada, T., Eto, K., Kadowaki, H., Hagura, R., Akanuma, Y., Yazaki, Y., Nagai, R., Taniyama, M., Matsubara, K., Yoda, M., Nakano, Y., Tomita, M., Kimura, S., Ito, C., Froguel, P., Kadowaki, T., 2002. Genetic variation in the gene encoding adiponectin is associated with an increased risk of type 2 diabetes in the Japanese population. Diabetes 51, 536–540.

Hoyda, T.D., Fry, M., Ahima, R.S., Ferguson, A.V., 2007. Adiponectin selectively inhibits oxytocin neurons of the paraventricular nucleus of the hypothalamus. J. Physiol. 585, 805–816.

Iwashima, Y., Katsuya, T., Ishikawa, K., Ouchi, N., Ohishi, M., Sugimoto, K., Fu, Y., Motone, M., Yamamoto, K., Matsuo, A., Ohashi, K., Kihara, S., Funahashi, T., Rakugi, H., Matsuzawa, Y., Ogihara, T., 2004. Hypoadiponectinemia is an independent risk factor for hypertension. Hypertension 43, 1318–1323.

Kadowaki, T., Yamauchi, T., 2005. Adiponectin and adiponectin receptors. Endocr. Rev. 26, 439–451.

Kos, K., Harte, A.L., da Silva, N.F., Tonchev, A., Chaldakov, G., James, S., Snead, D.R., Hoggart, B., O'Hare, J.P., McTernan, P.G., Kumar, S., 2007. Adiponectin and resistin in human cerebrospinal fluid and expression of adiponectin receptors in the human hypothalamus. J. Clin. Endocrinol. Metab. 92, 1129–1136.

Kubo, T., Kihara, M., 1990. Modulation of the aortic baroreceptor reflex by neuropeptide Y, neurotensin and vasopressin microinjected into the nucleus tractus solitarii of the rat. Naunyn Schmiedebergs Arch. Pharmacol. 342, 182–188.

Kubota, N., Yano, W., Kubota, T., Yamauchi, T., Itoh, S., Kumagai, H., Kozono, H., Takamoto, I., Okamoto, S., Shiuchi, T., Suzuki, R., Satoh, H., Tsuchida, A., Moroi, M., Sugi, K., Noda, T., Ebinuma, H., Ueta, Y., Kondo, T., Araki, E., Ezaki, O., Nagai, R., Tobe, K., Terauchi, Y., Ueki, K., Minokoshi, Y., Kadowaki, T., 2007. Adiponectin stimulates AMP-activated protein kinase in the hypothalamus and increases food intake. Cell Metab. 6, 55–68.

Kusminski, C.M., McTernan, P.G., Schraw, T., Kos, K., O'Hare, J.P., Ahima, R., Kumar, S., Scherer, P.E., 2007. Adiponectin complexes in human cerebrospinal fluid: distinct complex distribution from serum. Diabetologia 50, 634–642.

Lawrence, A.J., Jarrott, B., 1996. Neurochemical modulation of cardiovascular control in the nucleus tractus solitarius. Prog. Neurobiol. 48, 21–53.

Lawrence, A.J., Castillo-Melendez, M., McLean, K.J., Jarrott, B., 1998. The distribution of nitric oxide synthase-, adenosine deaminase- and neuropeptide Y-immunoreactivity through the entire rat nucleus tractus solitarius: effect of unilateral nodose ganglionectomy. J. Chem. Neuroanat. 15, 27–40.

McLean, H.A., Champagnat, J., avit-Saubie, M., 1996. A cholecystokinin-B receptor antagonist potentiates GABAergic and glycinergic inhibition in the nucleus of the solitary tract of the rat. Eur. J. Neurosci. 8, 1078–1084.

Ohashi, K., Kihara, S., Ouchi, N., Kumada, M., Fujita, K., Hiuge, A., Hibuse, T., Ryo, M., Nishizawa, H., Maeda, N., Maeda, K., Shibata, R., Walsh, K., Funahashi, T., Shimomura, I., 2006. Adiponectin replenishment ameliorates obesity-related hypertension. Hypertension 47, 1108–1116.

Paquet, L., Massie, B., Mains, R.E., 1996. Proneuropeptide Y processing in large dense-core vesicles: manipulation of prohormone convertase expression in sympathetic neurons using adenoviruses. J. Neurosci. 16, 964–973.

Price, C.J., Hoyda, T.D., Ferguson, A.V., 2008a. The area postrema: a brain monitor and integrator of systemic autonomic state. Neuroscientist 14, 182–194.

Price, C.J., Hoyda, T.D., Samson, W.K., Ferguson, A.V., 2008b. Nesfatin-1 influences the excitability of paraventricular nucleus neurones. J. Neuroendocrinol. 20, 245–250.

Qi, Y., Takahashi, N., Hileman, S.M., Patel, H.R., Berg, A.H., Pajvani, U.B., Scherer, P.E., Ahima, R.S., 2004. Adiponectin acts in the brain to decrease body weight. Nat. Med. 10, 524–529.

Saper, C.B., 2002. The central autonomic nervous system: conscious visceral perception and autonomic pattern generation. Annu. Rev. Neurosci. 25, 433–469.

Scherer, P.E., 2006. Adipose tissue: from lipid storage compartment to endocrine organ. Diabetes 55, 1537–1545.

Spranger, J., Verma, S., Gohring, I., Bobbert, T., Seifert, J., Sindler, A.L., Pfeiffer, A., Hileman, S.M., Tschop, M., Banks, W.A., 2006. Adiponectin does not cross the blood–brain barrier but modifies cytokine expression of brain endothelial cells. Diabetes 55, 141–147.

Tan, K.C., Xu, A., Chow, W.S., Lam, M.C., Ai, V.H., Tam, S.C., Lam, K.S., 2004. Hypoadiponectinemia is associated with impaired endothelium-dependent vasodilation. J. Clin. Endocrinol. Metab. 89, 765–769.

Tanida, M., Shen, J., Horii, Y., Matsuda, M., Kihara, S., Funahashi, T., Shimomura, I., Sawai, H., Fukuda, Y., Matsuzawa, Y., Nagai, K., 2007. Effects of adiponectin on the renal sympathetic nerve activity and blood pressure in rats. Exp. Biol. Med. (Maywood) 232, 390–397.

Tseng, C.J., Mosqueda-Garcia, R., Appalsamy, M., Robertson, D., 1989. Cardiovascular effects of neuropeptide Y in rat brainstem nuclei. Circ. Res. 64, 55–61.

Vincent, A., Tell, F., 1997. Postnatal changes in electrophysiological properties of rat nucleus tractus solitarii neurons. Eur. J. Neurosci. 9, 1612–1624.

Wang, Z.V., Scherer, P.E., 2008. Adiponectin, cardiovascular function, and hypertension. Hypertension 51, 8–14.

Wen, J.P., Lv, W.S., Yang, J., Nie, A.F., Cheng, X.B., Yang, Y., Ge, Y., Li, X.Y., Ning, G., 2008. Globular adiponectin inhibits GnRH secretion from GT1-7 hypothalamic GnRH neurons by induction of hyperpolarization of membrane potential. Biochem. Biophys. Res. Commun. 371, 756–761.

Williams, D.L., Baskin, D.G., Schwartz, M.W., 2006. Leptin regulation of the anorexic response to glucagon-like Peptide-1 receptor stimulation. Diabetes 55, 3387–3393.

Wyatt, C.N., Mustard, K.J., Pearson, S.A., Dallas, M.L., Atkinson, L., Kumar, P., Peers, C., Hardie, D.G., Evans, A.M., 2007. AMP-activated protein kinase mediates carotid body excitation by hypoxia. J. Biol. Chem. 282, 8092–8098.

Yamauchi, T., Kamon, J., Ito, Y., Tsuchida, A., Yokomizo, T., Kita, S., Sugiyama, T., Miyagishi, M., Hara, K., Tsunoda, M., Murakami, K., Ohteki, T., Uchida, S., Takekawa, S., Waki, H., Tsuno, N.H., Shibata, Y., Terauchi, Y., Froguel, P., Tobe, K., Koyasu, S., Taira, K., Kitamura, T., Shimizu, T., Nagai, R., Kadowaki, T., 2003. Cloning of adiponectin receptors that mediate antidiabetic metabolic effects. Nature 423, 762–769.

Yang, B., Brown, K.K., Chen, L., Carrick, K.M., Clifton, L.G., McNulty, J.A., Winegar, D.A., Strum, J.C., Stimpson, S.A., Pahel, G.L., 2004. Serum adiponectin as a biomarker for in vivo PPARgamma activation and PPARgamma agonist-induced efficacy on insulin sensitization/lipid lowering in rats. BMC Pharmacol. 4, 23.