Adiponectin selectively inhibits oxytocin neurons of the paraventricular nucleus of the hypothalamus

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Adiponectin is an adipocyte derived hormone which acts in the brain to modulate energy homeostasis and autonomic function. The paraventricular nucleus of the hypothalamus (PVN) which plays a key role in controlling pituitary hormone secretion has been suggested to be a central target for adiponectin actions. A number of hormones produced by PVN neurons have been implicated in the regulation of energy homeostasis including oxytocin, corticotropin releasing hormone and thyrotropin releasing hormone. In the present study we investigated the role of adiponectin in controlling the excitability of magnocellular (MNC – oxytocin or vasopressin secreting) neurons within the PVN. Using RT-PCR techniques we have shown expression of both adiponectin receptors in the PVN. Patch clamp recordings from MNC neurons in hypothalamic slices have also identified mixed (27% hyperpolarization, 42% depolarization) effects of adiponectin in modulating the excitability of the majority of MNC neurons tested. These effects are maintained when cells are placed in synaptic isolation using tetrodotoxin. Additionally we combined electrophysiological recordings with single cell RT-PCR to examine the actions of adiponectin on MNC neurons which expressed oxytocin only, vasopressin only, or both oxytocin and vasopressin mRNA and assess the profile of receptor expression in these subgroups. Adiponectin was found to hyperpolarize 100% of oxytocin neurons tested ($n = 6$), while vasopressin cells, while all affected ($n = 6$), showed mixed responses. Further analysis indicates oxytocin neurons express both receptors (6/7) while vasopressin neurons express either both receptors (3/8) or one receptor (5/8). In contrast 6/6 oxytocin/vasopressin neurons were unaffected by adiponectin. Co-expressing oxytocin and vasopressin neurons express neither receptor (4/6). The results presented in this study suggest that adiponectin plays specific roles in controlling the excitability oxytocin secreting neurons, actions which correlate with the current literature showing increased oxytocin secretion in the obese population.

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Adipose tissue plays an active role in modulating energy homeostasis through secreting factors which act at both peripheral and central targets (Ahima, 2005). Adiponectin is an adipose tissue derived hormone with antidiabetic, insulin sensitizing and energy regulatory properties (Fruebis *et al.* 2001; Yamauchi *et al.* 2001; Berg *et al.* 2001). Intriguingly, in contrast to leptin, the cellular expression of adiponectin mRNA and serum concentrations are inversely correlated with adiposity (Hu *et al.* 1996; Arita *et al.* 1999; Yamauchi *et al.* 2001). Two receptors (AdipoR1 and AdipoR2) have recently been characterized and localized to tissues throughout the body including the central nervous system (Yamauchi *et al.* 2003). When administered centrally, adiponectin stimulates thermogenesis, promotes oxygen consumption and significantly decreases body weight (Qi *et al.* 2004). Furthermore, intracerebroventricular (I.C.V.) administration of adiponectin increases the numbers of c-fos positive neurons in the paraventricular nucleus of the hypothalamus (PVN) (Qi *et al.* 2004) suggesting that adiponectin may act in this nucleus to activate subpopulations of PVN neurons.

The PVN is a bilateral hypothalamic nucleus located on either side of the third ventricle and is recognized as a pivotal autonomic control centre which plays essential integrative roles in the control of fluid and electrolyte balance, cardiovascular regulation, the immune response, energy metabolism and stress responses (Ferguson & Washburn, 1998; Swanson & Sawchenko, 1980). Within

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this nucleus a subpopulation of neuroendocrine cells exist which control the release of vasopressin and oxytocin through axonal projections to the posterior pituitary. These magnocellular (MNC) neurons are located mainly in the lateral region of the nucleus and are responsible for modulating cardiovascular function, fluid balance and the initiation of the milk ejection reflex (Brody, 1988; Gimpl & Fahrenholz, 2001). We have previously reported that magnocellular neurons depolarize in response to leptin administration supporting the hypothesis that these neurons are sensitive to adiposity hormones (Powis *et al.* 1998) and therefore the circulating concentrations of neurohypophysial peptides may be regulated by adipokines. Intriguingly, oxytocin neurons in the PVN have been suggested to play a vital role in coordinating feeding cessation (Olson *et al.* 1991*a*,*b*; Verbalis *et al.* 1993; Blevins *et al.* 2003) by acting as targets for factors which induce anorexigenic behaviour such as CCK (Olson *et al.* 1992) and peptide histidine isoleucine (PHI) (Olszewski *et al.* 2003). The concentrations of oxytocin in brain and periphery have also been shown to be compromised during pathological states of energy homeostasis such as obesity (elevated) (Stock *et al.* 1989) and restrictive anorexia (reduced) (Demitrack *et al.* 1990).

The present study was therefore designed to examine the role of adiponectin in controlling the excitability of MNC neurons of the PVN.

Methods

Preparation of hypothalamic slices

All experiments performed in this study used 50–100 g male Sprague–Dawley rats (Charles River, PQ, Canada), maintained on a 12 h–12 h light–dark cycle and provided with food and water *ad libitum*. Animals were decapitated and brains removed from the skull and placed in ice-cold slicing solution containing (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, and bubbled with 95% O_2–5% CO_2 for 1–2 min. Brains were then trimmed and mounted on a stage, and 300 μm coronal sections of the hypothalamus were cut using a vibrating microtome (Leica, Nussloch, Germany) while bathed in ice-cold slicing solution. All procedures were approved by the Queen's University Animal Care Committee, and were in accordance with the guidelines of the Canadian Council for Animal Care.

PVN RNA extraction and reverse transcription polymerase chain reaction (RT-PCR)

RNA extraction and cDNA synthesis. The PVN was microdissected from 300 μm coronal hypothalamic slices. Total RNA was extracted with TRIzol as per the manufacture's instructions (Invitrogen, Burlington, Ontario, Canada), and was then subjected to DNase treatment by adding RNA to a reaction mixture containing 1 μl 10× buffer with $MgCl_2$, 7 μl diethylpyrocarbonate (DEPC) treated-H_2O and 1 μl deoxyribonuclease I (Sigma, St Louis, MO, USA). This solution was then incubated at 37°C for 30 min, 1 μl of 25 mM EDTA was added, and the solution was then incubated at 65°C for 10 min. Oligo-dT based cDNA was then synthesized using a RETROscript cDNA synthesis kit (Ambion, Austin, TX, USA) as per the manufacturer's instructions to make a final reaction volume of 20 μl.

RT-PCR reactions. One microlitre of PVN cDNA was added to a 50 μl reaction mixture containing 1× PCR buffer, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 1 U of Platinum® *Taq* DNA polymerase, 0.2 μM of each primer, along with H_2O to a final volume of 50 μl. Each reaction was denatured at 95°C for 2–4 min then cycled 35 times through a temperature protocol consisting of 94°C for 60 s, 55°C for 60 s, 72°C for 60 s, and a final extension at 72°C for 5 min. Two separate sets of primers were used to detect AdipoR1 and AdipoR2 mRNA along with a primer set for synaptotagmin (see Table 1) which acted as a positive control. PCR products were separated and visualized through gel electrophoresis on a 2% agarose gel containing ethidium bromide and sequenced to confirm identity (Robarts Institute, London, Ontario, Canada).

Localization of adiponectin receptors by *in situ* hybridization

Male Sprague–Dawley rats were anaesthetized with sodium pentobarbital and perfused transcardially with phosphate buffered saline (PBS) prepared with DEPC-treated water, followed by 10% neutral buffered formalin. Brains were removed, immersed in the same fixative overnight, followed by cryoprotection in 20% sucrose/PBS/DEPC at 4°C. Five series of 20 μm coronal sections were cut on a Reichert cryotome, mounted on Superfrost Plus glass slides (Fisher Scientific, Houston, TX, USA), air dried, and stored in desiccated boxes at −20°C. Before hybridization, sections were fixed in 4% formaldehyde in DEPC–PBS, pH 7.0, for 20 min at 4°C, dehydrated in increasing concentrations of ethanol, cleared in xylene for 15 min, rehydrated in decreasing concentrations of ethanol, placed in prewarmed sodium citrate buffer (95–100°C, pH 6.0), and then dehydrated in ethanol and air dried. The slides were hybridized with ^{35}S-cRNA probes against AdipoR1 and -R2, washed, dehydrated, air dried and exposed to film as previously described (Fry *et al.* 2006). After dipping in photographic emulsion, the slides were developed, counterstained with thionin, dehydrated in graded ethanols, cleared in xylene, and coverslipped with Permount (Fisher Scientific). Brain sections were examined with a Nikon E600 microscope,

Table 1. Primer sets used for RT-PCR analysis of receptor expression and single cell identification

Gene	Primer Name	Position	Sequence	Product size (bp)	Reference
Receptor Expression					
Adiponectin receptor 1	AdipoR1 (first set)	F	CTGGTCCCATCTTTCTAGG	210	This study
		R	TGATAGCAGGTTTCTTTTTAAAGC		
	AdipoR1 (second set)	F	TCTTCCTCATGGCTGTGATG	270	Kharroubi *et al.* (2003)
		R	AGCCACTTGGGAAGTTCCTCC		
Adiponectin receptor 2	AdipoR2 (first set)	F	TCTGGTTCAACATAGCACAAAC	367	This study
		R	AACCCCAACCTTCTATGATTC		
	AdipoR2 (second set)	F	GGAGCCATTCTCTGCCTTTC	467	Kharroubi *et al.* (2003)
		R	ACCAGATGTCACATTTGCCA		
Synaptotagmin	Synaptotagmin	F	AGGGGCTTTCCTATCTAAGGG	202	Richardson *et al.* (2000)
		R	GTTGGCAGTGTTGCAAGAGA		
Single Cell RT-PCR					
GAPDH	GAPDH	F (outside)	GATGGTGAAGGTCGGTGTG	469	This study
		R	GGGCTAAGCAGTTGGTGGT		
		F (nested)	TACCAGGCTGCCTTCTCT	360	
		R	CTCGTGGTTCACACCCATC		
GABA	GAD67	F (outside)	CACAAACTCAGCGGCATAGA	550	This study
		R	GAGATGACCATCCGGAAGAA		
		F (nested)	CACAAACTCAGCGGCATAGA	149	
		R	CTGGAAGAGGTAGCCTGCAC		
Tyrosine hydroxylase	TH	F (outside)	CACCTGGAGTATTTTGTGCG	1138	This study
		R	CCTGTGGGTGGTACCCTATG		
		F (nested)	TCGACCCAGTATATCCGCCA	376	
		R	TCGGACACAAAGTACACAGG		
Vasopressin	VP	F (outside)	CCTCACCTCTGCCTGCTACTT	237	This study
		R	AGCCAGCTGTACCAGCCTAA		
		F (nested)	ACCTCTGCCTGCTACTTCCA	216	
		R	AGCCAGCTGTACCAGCCTAA		
Oxytocin	OT	F (outside)	CTGCCCCAGTCTCGCTTG	281	This study
		R	CCTCCGCTTCCGCAAGGCTTCTGGC		
		F (nested)	CTGCCCCAGTCTCGCTTG	244	
		R	GCGAGGGCAGGTAGTTCTCC		
Adiponectin receptor 1	AdipoR1	F (outside)	GTCCCCTGGCTCTATTACTCCT	509	This study
		R	AGCACTTGGCTGTGATGT		
		F (nested)	TCTTCCTCATGGCTGTGATGT	223	
		R	GGCTCAGAGAAGGGAGTCATC		
Adiponectin receptor 2	AdipoR2	F (outside)	GGAGCCATTCTCTGCCTTTC	464	This study
		R	CCAGATGTCACATTTGCCA		
		F (nested)	ACTGTAACCCACAACCTTGCTTC	191	
		R	TCAGGAACCCTTCTGAGATGAC		

AdipoR1/R2 (second set) primer set adapted from Kharroubi *et al.* (Kharroubi *et al.* 2003), Synaptotagmin primer set adapted from Richardson *et al.* (Richardson *et al.* 2000). GAPDH = glyceraldehyde 3-phosphate dehydrogenase, GAD67 = glutamate decarboxylase 67.

and dark-field photomicrographs of the PVN were taken with a SPOT RT digital camera (Phase 3 Imaging Systems, Glen Mills, PA, USA).

Electrophysiology

Hypothalamic slices including the PVN were prepared as described above and were then incubated at 31.5°C for at minimum 60 min prior to recording in artificial cerebral spinal fluid (aCSF) containing (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. PVN neurons were visualized through a water immersion 40× objective mounted to an IR-DIC Nikon E600FN microscope (Tokyo, Japan). Borosilicate glass pipettes were pulled on a flaming micropipette puller (Sutter Instrument Co., Novato, CA, USA) to a resistance of 3–6 MΩ when filled with internal recording solution consisting of (mM): 140 potassium gluconate, 2 $MgCl_2$, 0.1 $CaCl_2$, 1.1 EGTA, 10 Hepes, 2

NaATP adjusted to a pH of 7.3 with (KOH). Electrophysiological recordings were made in the whole cell current-clamp configuration from type 1 MNC neurons, which were identified morphologically and according to distinct electrophysiological characteristics (Tasker & Dudek, 1991). Current clamp recordings were made with an EPC-7 amplifier (Heka Instruments, Germany) sampling at 10 kHz, filtered at 13 kHz, digitalized through a CED micro1401 (CED, Cambridge, UK) and displayed using Spike2 (CED) data acquisition software to be stored for off-line analysis. Adiponectin (10 nM) was perfused onto slices at a rate of 1–2 ml min^{-1}, only after stable membrane potential was obtained for more then 100 s. Recordings were performed at 32.5°C and a junction-potential correction of −17.2 mV was calculated using pCLAMP (Molecular Devices, Union City, CA, USA) and applied to the membrane potential of all recorded neurons.

Single cell RT-PCR

Following recording, negative pressure was applied to the pipette in order to collect the cytoplasm and care was taken as the pipette was withdrawn from the recorded cell to pull an outside-out patch in order to seal the cytoplasm contents within the pipette. Following withdrawal from the bath the pipette tip was broken off and the cytoplasmic contents expelled into a siliconized centrifuge tube and stored at −80°C. This withdrawn cytoplasm was then subjected to DNase treatment (TURBO DNA-*free* – Ambion, Austin, TX, USA) before undergoing cDNA synthesis using a random hexamer based Superscript™ III first strand synthesis kit (Invitrogen, Burlington, Ontario, Canada).

Due to the small amount of total RNA harvested from a single cell, RT-PCR was carried out in two separate steps. The first step was a multiplex reaction containing primers (outside) for all the genes of interest along with cDNA from the single cell. The second reaction was a nested PCR reaction using a single set of primers (nested) for each gene of interest. The multiplex reaction contained 10 μl 10× PCR buffer, 3 μl $MgCl_2$, 2 μl dNTPs, 0.5 μl Platinum® *Taq*, 1 μl cDNA, 9 μl H_2O, 2 μl of each outside primer set (2.5 mM) of interest (see Table 1) and ddH_2O up to a final volume of 100 μl. Each reaction was denatured at 94°C for 4 min then cycled 35 times through a temperature protocol consisting of 1 min at 94°C, 1.5 min at 55°C, 3 min at 72°C and finally for 5 min at 72°C and held at 4°C. The final product was diluted 1 : 1000 and used as a template for a second round PCR.

Second round PCR consists of a series of reactions for each of the genes of interest. Each reaction mixture contains 5 μl 10× PCR buffer, 1.5 μl $MgCl_2$, 1 μl dNTPs, 0.4 μl Platinum® *Taq*, 2 μl cDNA template, 1 μl of each nested primer (see Table 1) and sdH_2O up to a final volume of 50 μl. The reaction mixture was cycled through a temperature protocol as indicated above following which the products were run out on a 2% agarose gel containing ethidium bromide and sequenced to confirm their identity (Robarts Institute, London, Ontario, Canada).

Statistical analysis

Cells were classified as responders if within 1000 s after the application of adiponectin the membrane potential (mean measured over 100 s periods) changed by more than 2 standard deviations of the mean assessed in the 100 s prior to peptide application (baseline membrane potential). Cells were only included in the responder group if membrane potential showed a return towards baseline membrane potential following return to aCSF perfusion of the slice. Kruskal–Wallis one-way ANOVA tests were used to compare the changes in membrane potential in response to adiponectin between groups of cells. Wilcoxon signed rank tests were used to evaluate changes in spike frequency (paired) before and after adiponectin.

Peptides and chemicals

Adiponectin (human recombinant globular) was obtained from Phoenix Pharmaceuticals, Inc. (Belmont, CA, USA) and prepared daily from aliquots stored at −80°C. Adiponectin was made to a working solution of 10 nM in aCSF. Tetrodotoxin (TTX) was obtained from Alomone laboratories (Jerusalem, Israel) and prepared daily in aCSF to a working concentration of 1 μM. All other chemicals used were obtained from Sigma (Scarborough, Ontario, Canada).

Results

Adiponectin receptor 1 and 2 are expressed in the PVN

Based on evidence of receptor expression in the CNS (Yamauchi *et al.* 2003) and the induction of c-fos immunoreactivity in the PVN upon I.C.V. injections of adiponectin (Qi *et al.* 2004) we first examined if AdipoR1 and R2 were expressed in the PVN through the use of RT-PCR and *in situ* hybridization. RT-PCR reactions were performed on cDNA derived from mRNA isolated from acutely microdissected PVN tissue with two primer sets designed for different regions on the AdipoR1 and R2 cDNA (Fig. 1*A*). Synaptotagmin was used as a positive control along with an RT(−) in which the reverse transcriptase enzyme was omitted from the RT-PCR reaction. We amplified cDNA for both receptors indicating the presence of mRNA for AdipoR1 and R2 in PVN. Following this we performed *in situ* hybridization with probes specific for both receptor mRNA again confirming the presence of AdipoR1 and R2 in both magnocellular and parvocellular regions of the PVN as illustrated in Fig. 1*B*.

Adiponectin influences the excitability of MNC neurons in the PVN

Current clamp recordings were obtained from a total of 55 morphologically (based on size) and electrophysiologically (based on an outward rectification caused by activation of the dominant I_A current) characterized MNC neurons (mean action potential amplitude of 96.7 ± 1.9 mV, mean resting membrane potential of -60.3 ± 0.9 mV and mean input resistance of 924 ± 56 MΩ). Following a minimum 100 s stable baseline recording period we examined the effects of bath application of 10 nM adiponectin for 200 s on both membrane potential and spike frequency. The membrane potential of MNC neurons responded to administration of adiponectin in one of three ways showing either depolarization (6.6 ± 0.5 mV (mean ± S.E.M.), $n = 23$), hyperpolarization (-6.6 ± 0.9 mV, $n = 15$) or no effect (0.2 ± 0.5 mV, $n = 17$) as illustrated in Fig. 2*A* and *B*. These observations suggest the existence of three distinct populations of MNC neurons with respect to adiponectin sensitivity. In addition the depolarizing effects of adiponectin on membrane potential suggest that 1 nM is the minimum effective concentration. Neurons examined in response to 1 nM adiponectin showed a mean change in membrane potential of 3.7 ± 0.9 mV ($n = 4$) while those subjected to 100 pM showed a change in membrane potential of 1.9 ± 1.0 mV ($n = 4$) (Fig. 2*C*). An EC_{50} value of 1.8 nM was calculated using:

$$Y = \text{Bottom} + (\text{Top} - \text{Bottom})/1 + 10\text{^}((\log EC_{50} - X)),$$

where $X = \log[\text{ADP}]$. Furthermore the duration of the effect and onset to peak response differed between the groups of adiponectin responsive neurons. The duration of the response in some cells lasted as long as 1500 s before seeing a return towards baseline in the membrane potential.

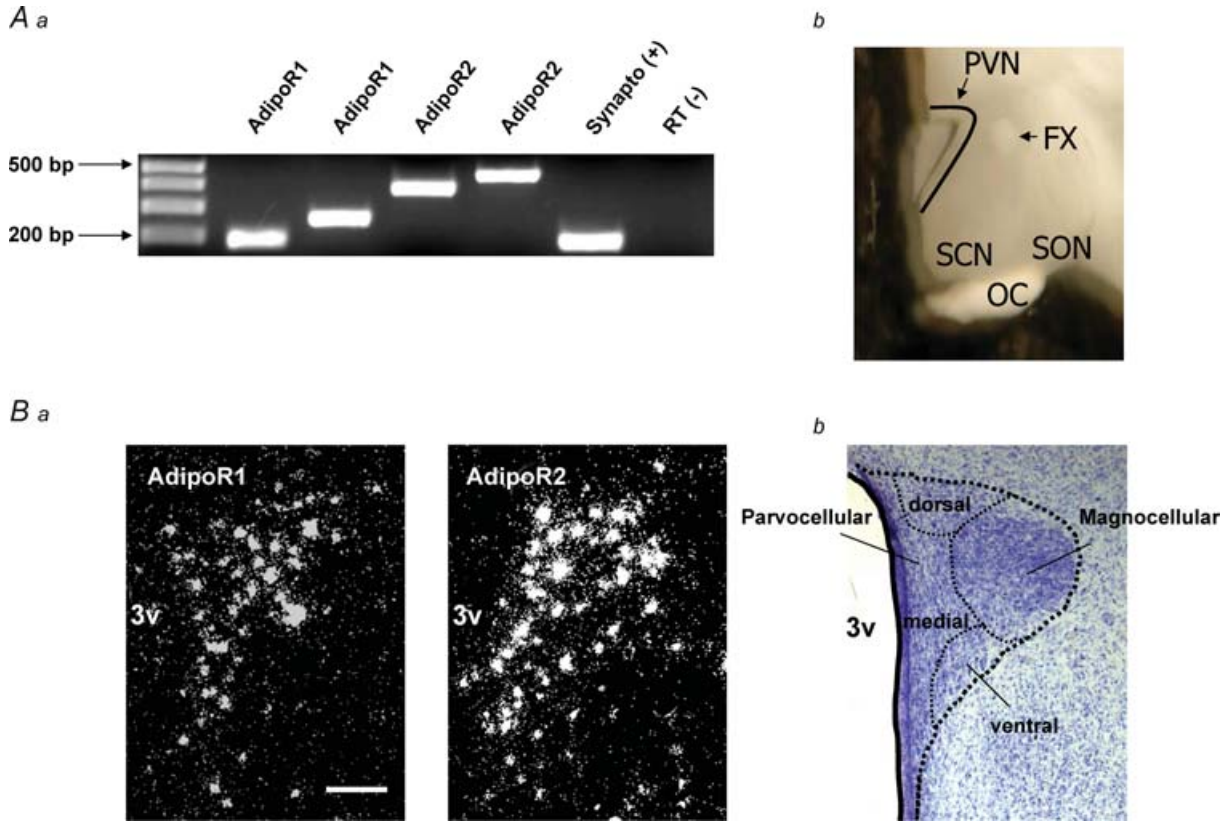

Figure 1. Adiponectin receptor mRNA is expressed in the PVN
Aa, agarose gel showing RT-PCR analysis of PVN cDNA using two primer sets specific for different locations of each adiponectin receptor mRNA and a primer set specific for synaptotagmin. PCR products for synaptotagmin are not observed in the RT(−) lane in which reverse transcriptase has been omitted from the cDNA synthesis reaction. *Ab*, a coronal section (300 μm) illustrating the tissue sample taken for mRNA extraction and the RT-PCR reaction. SON = supraoptic nucleus, SCN = suprachiasmatic nucleus, Fx = fornix, PVN = paraventricular nucleus. *Ba*, dark-field photomicrographs of *in situ* hybridization for AdipoR1 and AdipoR2, demonstrating mRNA expression in the paraventricular nucleus of the hypothalamus. 3v, third ventricle. Scale bar, 50 μm. *Bb*, a Nissl stained section of PVN with a schematic superimposed on it indicating relative anatomical positions of magnocellular and parvocellular neurons within the nucleus.

We also observed adiponectin induced changes in spike frequency with depolarization being associated with an increase in spike frequency (control: 0.18 ± 0.15 Hz, adiponectin: 1.69 ± 0.51 Hz). Conversely cells which hyperpolarized showed a decrease in firing rate (control: 0.62 ± 0.31 Hz, adiponectin: 0.04 ± 0.03 Hz) while spike frequency was not changed significantly in cells which showed no change in membrane potential in response to adiponectin (Fig. 2*D*).

Effects of adiponectin on MNC neurons are maintained in TTX

We next examined if the effects of adiponectin on membrane potential were maintained when action potential induced release of neurotransmitter was inhibited by perfusing slices with 1 μM TTX. After the efficacy of TTX was established (i.e. the abolition of action potentials in response to depolarizing pulses), adiponectin was administered as above and the resulting

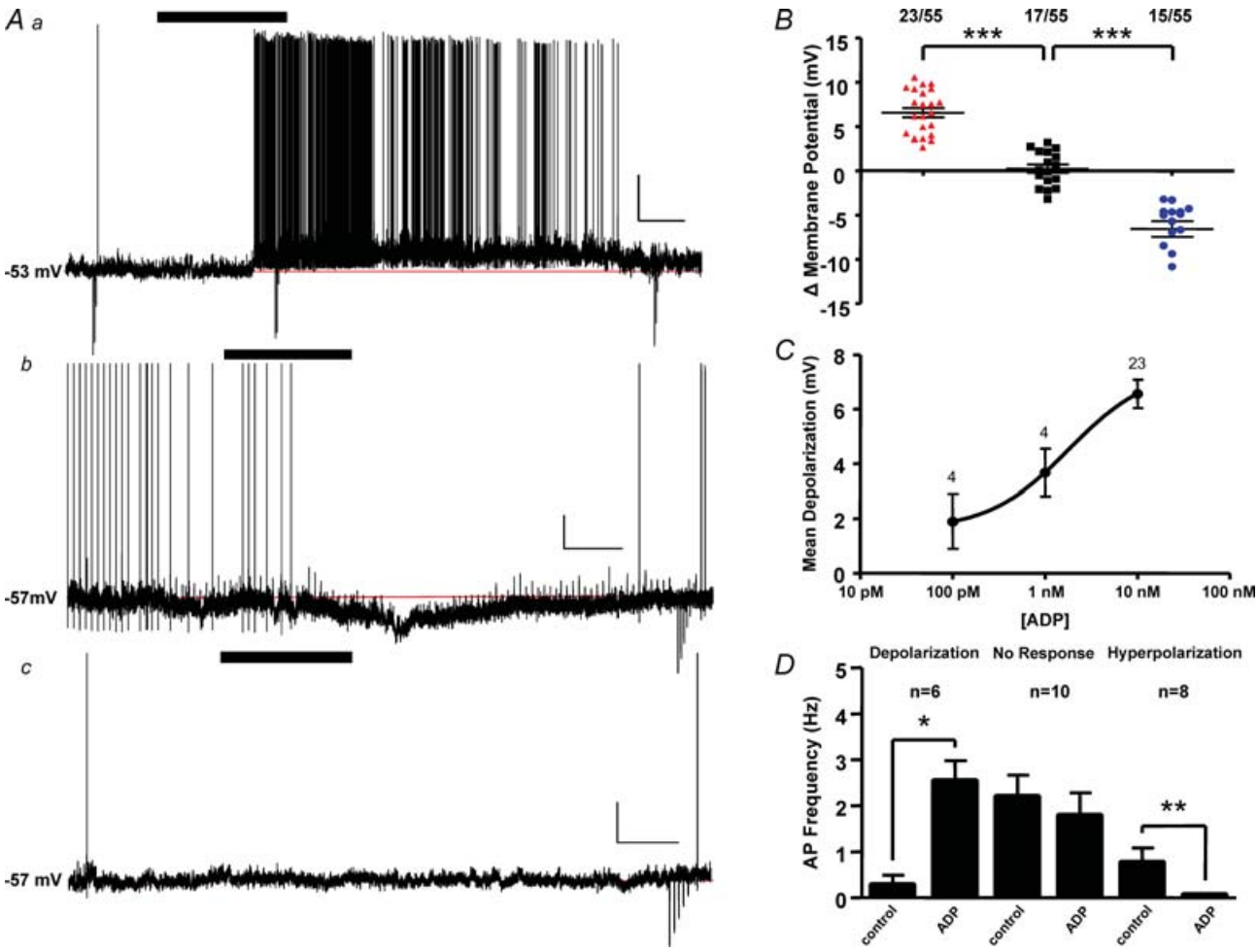

Figure 2. Adiponectin influences the membrane potential and spike frequency of MNC PVN neurons
A, representative current clamp recordings of MNC neurons showing responses to 10 nM adiponectin (black bars) depolarization (*a*) (scale: 20 mV, 100 s), hyperpolarization (*b*) (scale: 10 mV, 100 s), and no response (*c*) (scale bars: 20 mV, 100 s). Downward deflections in each trace show the voltage response to hyperpolarizing current pulses for the measurement of input resistance. *B–D*, graphs representing mean changes in membrane potential (*B*), concentration dependence of the depolarizing response (*C*) and spike frequency (*D*) for all cells examined in response to 10 nM adiponectin. Mean changes in membrane potential were significantly different between responsive and non-responsive groups (Kruskal–Wallis test, $^{***}P < 0.001$). Concentration–response curve was developed for neurons which depolarized to ADP, numbers above data points represent the cells tested with each dose. EC_{50} was calculated at 1.8 nM. Mean changes in spike frequency were assessed to be significantly different from control in each of the responding groups (Wilcoxon signed rank test, $^{*}P < 0.05$, $^{**}P < 0.005$) (ADP = adiponectin). Cells which depolarized but did not reach threshold to elicit action potentials and cells which were quiescent before hyperpolarization were not included in the analysis of spike frequency.

membrane potential changes were measured. Both depolarizing (5.50 ± 1.26 mV, $n = 3$) and hyperpolarizing (-4.65 ± 1.36 mV, $n = 3$) effects were observed in response to adiponectin indicating the site of action of adiponectin is most likely directly at the cell membrane of these magnocellular neurons (Fig. 3).

Adiponectin hyperpolarizes identified OT neurons

In view of the different responses of subsets of magnocellular neurons we next hypothesized that divergent effects of adiponectin may be due to different regulatory actions on oxytocin *versus* vasopressin neurons. We therefore undertook additional current clamp experiments in which we first defined adiponectin responsiveness of MNC neurons as described above (depolarization, hyperpolarization or no response) and then used a single cell RT-PCR technique to identify the chemical phenotype of each recorded MNC (see Methods). Single cell RT-PCR analysis suggests that there are three functional groups of MNC neurons in the PVN; neurons expressing vasopressin, oxytocin, or both vasopressin and oxytocin (Fig. 4*C*). Every neuron examined in this study which definitively expressed only oxytocin hyperpolarized in response to 10 nM adiponectin ($n = 6$). None of the neurons expressing both vasopressin and oxytocin responded to 10 nM adiponectin ($n = 6$). Although all identified vasopressin neurons were also responsive to adiponectin, these cells showed heterogeneous effects with equal proportions being either depolarized (3/6) or hyperpolarized (3/6) as shown in Fig. 4*C*.

MNC PVN neurons show diverse adiponectin receptor expression profiles

In light of the differential effects of adiponectin on the different chemical phenotypes of MNC neurons described above we hypothesized that each individual group expressed a different profile of adiponectin receptors. We assessed this hypothesis using the single cell RT-PCR technique in which aspirated cytoplasm from electrophysiologically identified MNC neurons was subjected to RT-PCR analysis as described above (see Methods) to examine the expression of adiponectin receptors in conjunction with peptidergic expression: vasopressin or oxytocin (Fig. 5). In neurons expressing exclusively oxytocin, both adiponectin receptors were detected in all but one cell (6/7) (Fig. 5*A*). The expression of adiponectin receptors in vasopressin neurons exhibited more complexity. Both adiponectin receptors were present in 3/8 neurons expressing vasopressin (Fig. 5*B*) whereas in 4/8 neurons, only AdipoR2 was present (Fig. 5*C*) and 1/8 neurons expressed only AdipoR1. A majority of MNC neurons (4/6) that coexpressed oxytocin and vasopressin did not express either receptor (Fig. 5*D*); however, 2/6 neurons expressing both vasopressin and oxytocin were found to express AdipoR2.

Discussion

We have shown that PVN tissue expresses both adiponectin receptor mRNAs and that activation of these receptors has direct consequences on the membrane properties of MNC neurons in the PVN. Our data indicate that

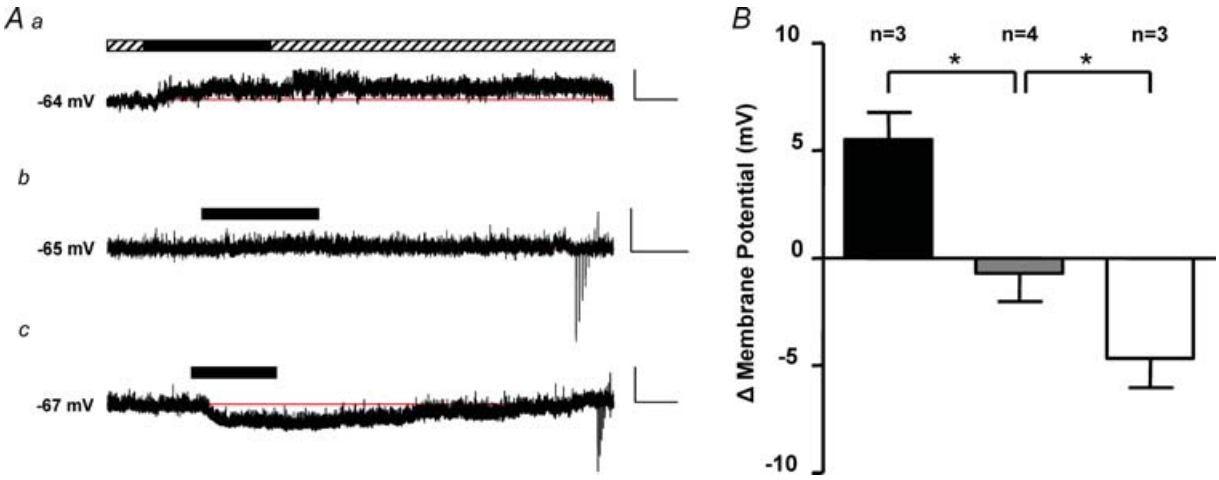

Figure 3. The effects of adiponectin are maintained when action potential induced release of neurotransmitter is inhibited
A, representative current clamp traces of MNC neurons showing the response to 10 nM adiponectin (black bars) in the presence of 1 μM TTX (diagonal lined bar). Scale bars: 10 mV, 100 s. *B*, bar graph showing mean membrane potential change of all cells treated with adiponectin (10 nM) in the presence of TTX (1 μM). Significant changes in mean membrane potential of the responding groups compared to the non-responding group was established through a Kruskal–Wallis test (*$P > 0.05$).

adiponectin has unique effects on distinct populations of MNC neurons as measured by single-cell RT-PCR, selectively hyperpolarizing oxytocin neurons, exhibiting no effect on those expressing oxytocin and vasopressin and either depolarizing or hyperpolarizing vasopressin neurons. Furthermore our results indicate that oxytocin and vasopressin neurons exhibit distinct adiponectin receptor expression profiles which may underlie the heterogeneous electrophysiological effects seen on these groups of neurons. These results indicate that this peptide plays important roles in regulating the release of neurohypophysial peptides into the circulation.

In order to examine the responsiveness of individual neurons, we used patch-clamp electrophysiology to monitor in real-time the membrane potential and action potential frequency of PVN neurons in a slice preparation of the hypothalamus. Previous reports from our lab and others have shown that MNC neurons maintained in this preparation respond to a variety of signalling factors and display diverse and repeatable responses to treatment (Hermes *et al.* 1999; Follwell & Ferguson, 2002; Latchford & Ferguson, 2003; Ferri *et al.* 2005). In addition, maintaining cells in a slice preparation allows us to examine a synaptic component, in that performing the experiments in the presence of TTX effectively isolates the recorded neuron from action potential induced release of neurotransmitter and allows us to measure sensitivity to treatment directly at the membrane.

Considerable effort has been directed toward the identification of an electrophysiological fingerprint unique to either vasopressin or oxytocin expressing neurons (Armstrong, 1995; Armstrong & Stern, 1998*a*,*b*; Hirasawa *et al.* 2003) although the use of such fingerprints is recognized to leave doubt regarding the chemical phenotype of recorded neurons (Armstrong, 1995). In our studies we have identified cell type using single-cell RT-PCR analysis after whole cell recording (Lambolez *et al.* 1992; O'Dowd & Smith, 1996; Qiu *et al.* 2005) to identify the specific RNAs (OT/VP) expressed in recorded neurons. The single-cell RT-PCR system is remarkably

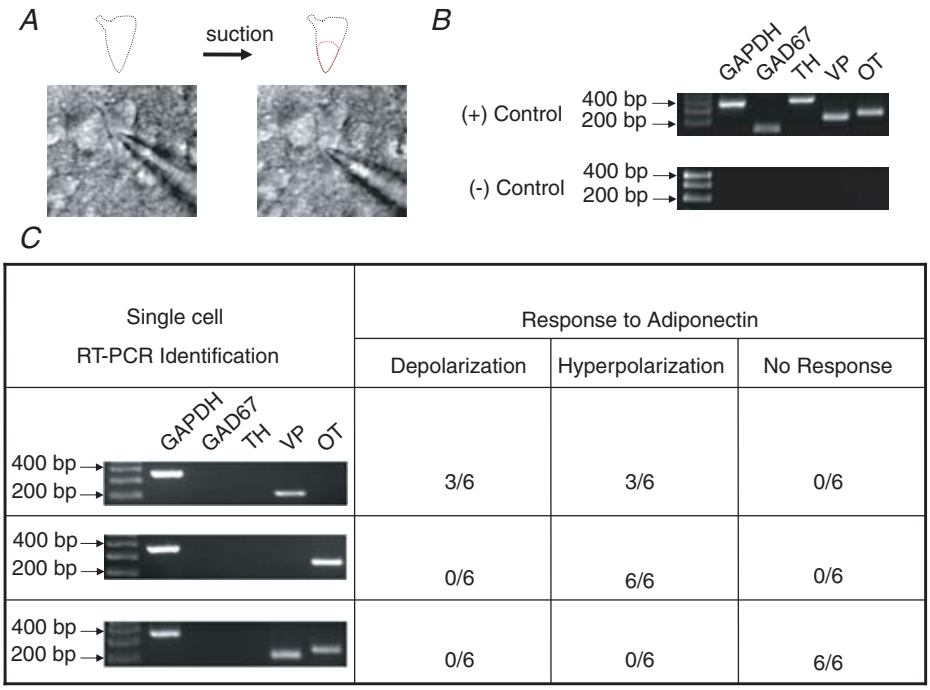

Figure 4. Adiponectin exhibits different effects on identified MNC neurons in the PVN
A, example of a MNC neuron in a slice preparation before (left) and after (right) cytoplasm has been aspirated into the recording pipette. Dashed lines above indicate the outline of the cell before (black) and after cytoplasm removal (red). Cytoplasm is then removed from the bath and subjected to RT-PCR analysis. *B*, agarose gels of control protocols run with each cytoplasm processed in this study. Top: agarose gel illustrating PCR products of each gene of interest derived from whole PVN cDNA which serves as a (+) control. Bottom: agarose gel showing an RT-PCR analysis in which PVN cDNA has been omitted from the reaction. RT-PCR analysis that did not show a full complement of PCR products in the (+) control and a clean (−) control were omitted from the study. *C*, correlation of the molecular phenotype of MNC neurons to the response seen after treatment with 10 nM adiponectin. Agarose gels on the left illustrate the single cell RT-PCR products of three distinct functional groups of MNC neurons. Each cell included in the study showed the presence of the housekeeping gene GAPDH in addition to the expression profile of the cell. (GAPDH = glyceraldehyde 3-phosphate dehydrogenase, GAD67 = glutamate decarboxylase 67, TH = tyrosine hydroxylase, VP = vasopressin, OT = oxytocin.)

sensitive, being able to detect individual mRNA transcripts from less then 1/100th the total mRNA contents of a single neuron (based on our whole PVN mRNA extraction and serial dilution sensitivity tests). However an important consideration when utilizing single-cell RT-PCR technology is to avoid the incorrect interpretation of results due to false-positives or false-negatives. To eliminate the possibility that the detected signal was due to genomic DNA contamination, cytoplasmic RNA was DNased (see Methods). A sample of bath solution was also taken and an RT(−) in which the reverse transcriptase enzyme was omitted from the reaction was included with each cell tested. A separate reaction which included no PCR template served as an additional negative control. In addition to negative controls, cytoplasm was tested for the presence of the housekeeping gene GAPDH and primer efficacy confirmed by a reaction with cDNA template derived from whole PVN tissue. Every cell included in this study showed a positive reaction for GAPDH and all target genes from whole tissue PVN. The success of each of these controls indicates to us that our single cell RT-PCR analysis is definitive and with it we have identified three functional groups of PVN MNC neurons based on peptidergic expression.

Our lab recently showed mRNA for both adiponectin receptors expressed in the area postrema; a sensory circumventricular organ important in autonomic control (Cottrell & Ferguson, 2004) and showed AP neurons to be responsive to adiponectin (Fry *et al.* 2006). The results presented in this study, however, are the first to show receptor expression in an adult nucleus which exists behind the blood–brain barrier (BBB). These observations raise interesting questions as to the source of peptide especially in view of permeability studies indicating that adiponectin likely does not passively cross the BBB (Spranger *et al.* 2006; Pan *et al.* 2006), presumably due to its large multimeric circulating size (Waki *et al.* 2003). However, I.V. injections of globular adiponectin have been reported to increase cerebral spinal fluid (CSF) concentrations (Qi *et al.* 2004) and adiponectin complexes have recently been detected in human cerebrospinal fluid (Kusminski *et al.* 2007) suggesting that circulating peptide could gain access to the CNS through the ventricular space. A recent report suggests CNS endothelial cells express adiponectin receptors activation of which decreases the release of interleukin-6 behind the BBB (Spranger *et al.* 2006). This mechanism could explain the effects seen on MNC neurons as vasculature is maintained in our slice, though it seems unlikely that decreases in cytokine release could be responsible for the diverse electrophysiological effects. A final possibility suggested by a recent report that adiponectin is also produced in the chicken diencephalon (Maddineni *et al.* 2005) at least raises the possibility that the CNS may also produce this adipokine. Specific neuronal localization, relevant interconnectivity with PVN MNC neurons and whether central adiponectin exhibits the same inverse adiposity correlation remains to be explored but may provide insight into the source and role of adiponectin in the CNS.

Intriguingly, the data presented in this study suggest that adiponectin differentially modulates neuronal excitability of distinct chemical phenotypes of MNC neurons in the PVN. Our data indicate that adiponectin specifically hyperpolarizes oxytocin neurons thus inhibiting action potential generation and ultimately oxytocin release. Although many central signals involved in the regulation of energy balance including leptin (Hakansson *et al.* 1998; Ur *et al.* 2002), cocaine–amphetamine-related transcript (Vrang *et al.* 2000), CCK (Olson *et al.* 1992; Ueta *et al.* 1993) and PHI (Olszewski *et al.* 2003) play critical roles in controlling oxytocin secretion, the precise role of this neurohypophysial peptide in the regulation of energy balance has yet to be established. Modified serum concentrations of oxytocin have been reported in pathological states of energy imbalance. Serum oxytocin is approximately fourfold higher in clinically obese than control individuals (Stock *et al.* 1989), and is significantly elevated in a rodent model of diet induced obesity (Northway *et al.* 1989). Conversely, decreased central (no data on circulating levels) concentrations of oxytocin have been reported in the CSF of underweight restrictive anorexic patients (Demitrack *et al.* 1990) an abnormality which tends to normalize following

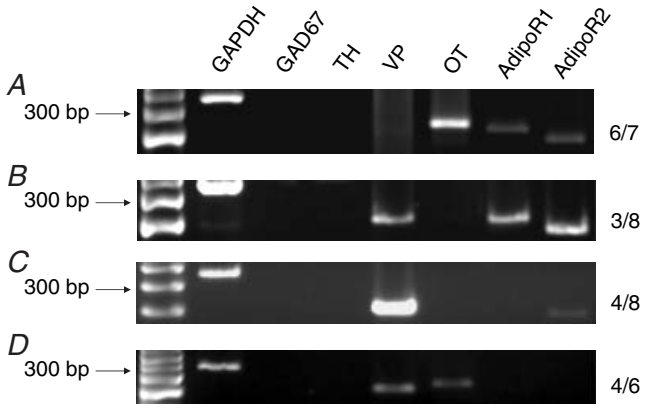

Figure 5. Subgroups of MNC neurons in the PVN express different profiles of adiponectin receptors
Left, agarose gels indicating PCR products from an RT-PCR reaction of the cytoplasmic contents of four different MNC neurons. Each gel is an example of the predominant expression pattern in each group. *A*, an oxytocin neuron expressing both adiponectin receptor 1 and 2. *B–C*, a vasopressin neuron expressing both receptors (*B*) or AdipoR2 (*C*). *D*, a coexpressing MNC neuron expressing neither receptor. Right, ratio of neurons in each group which expressed the profile of receptors indicated in each respective agarose gel.
(GAPDH = glyceraldehyde 3-phosphate dehydrogenase, GAD67 = glutamate decarboxylase 67, TH = tyrosine hydroxylase, VP = vasopressin, OT = oxytocin, AdipoR1 = adiponectin receptor 1, AdipoR2 = adiponectin receptor 2.)

restoration of normal weight (Kaye, 1996). There is, however, no direct information to link these changes in oxytocin to the predicted pathological changes in adiponectin function (decrease and increase, respectively) associated with such conditions.

The different responses of neurons expressing exclusively vasopressin suggest the possibility that a subgroup of MNC vasopressin neurons exist as a separate functional group. These may be caudally projecting neurons innervating autonomic centres in the brainstem but with similar electrophysiological characteristics to those of the neurohypophysial system (dominant I_A currents). The function of this group of neurons and what effect they have on the autonomic system in response to adiponectin remains to be determined.

The different expression profiles (as assessed by ssRT-PCR) of adiponectin receptors in subgroups of MNC neurons suggest that hyperpolarizing responses are associated with the expression of both AdipoR1 and R2 (oxytocin and some vasopressin neurons), while depolarizing effects may be associated with expression of AdipoR2 alone (proportion of vasopressin neurons only). As expected, a majority of the coexpressing vasopressin and oxytocin neurons showed no receptor expression correlating with the lack of response seen to adiponectin. These conclusions are slightly different from those from our previous study of area postrema neurons (Fry *et al.* 2006), in which such correlative data suggested that all cells showing responses to adiponectin expressed both AdipoR1 and R2, and suggest that in addition to receptor expression the broader phenotype of individual neurons (channel expression, etc.) may all play a role in determining responsiveness of individual neurons to adiponectin. The suggestion that hyperpolarization occurs through the activation of two receptors raises speculation about the mechanism by which this may proceed. A recent study (Kubota *et al.* 2007) examining the effects of adiponectin in the arcuate nucleus of the hypothalamus suggests that activation of AdipoR1 stimulates the phosphorylation of AMP-activated protein kinase (AMPK) thus increasing the production of ATP. It is well established that altering ATP concentrations within the cell has functional consequences on membrane potential and neuronal firing rate of the neuron through the alteration of K_{ATP} channel conductances (Spanswick *et al.* 2000). Microarray studies show the presence of K_{ATP} channel transcripts in the PVN (Hindmarch *et al.* 2006), but further studies will be required to examine their response to adiponectin. Additionally further studies will be needed to delineate the signalling pathways of both AdipoR1 and R2 and examine to what extent those contribute to the membrane potential and excitability of magnocellular neurons.

Finally, our recordings from chemically phenotyped vasopressin and oxytocin neurons confirm previous reports of their existence within the magnocellular PVN/SON (Mezey & Kiss, 1991; Xi *et al.* 1999). The consistently observed lack of response of these neurons to adiponectin suggests they may represent a separate functional group of magnocellular neurons whose properties have yet to be fully elucidated.

In conclusion we have shown that adiponectin modulates the membrane properties of MNC neurons in the PVN through the activation of AdipoR1 and/or AdipoR2. Furthermore through single-cell RT-PCR analysis, we have shown that adiponectin specifically regulates the oxytocinergic system by hyperpolarizing the membrane of neurons that express this peptide and both AdipoR1 and R2. Both these and previous results of adiponectin actions in the CNS highlight the growing complexity with which this peptide exerts its central effects and may provide insight into the pathology of neuronal systems that regulate energy homeostasis.

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