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RAPID REPORT

Intracellular energy status regulates activity in hypocretin/orexin neurones: a link between energy and behavioural states

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Non-technical summary A growing body of evidence has shown that energy status has a significant impact on the behavioural states in animals and that the availability of nutrients (energy state) in the brain may modulate animal behaviours. In this study, we report that the intracellular energy stores determine activity in a selective group of nerve cells (hypocretin-containing neurones) in the brain. The unique energy state of hypocretin neurones correlates with behavioural states of animals, i.e. the energy level is low during sleep and high during wakefulness. These results suggest that hypocretin neurones may act as an 'energy gauge' in the brain, which integrates nutritional, energetic and behavioural signals critical for the survival of animals.

Abstract The hypocretin/orexin (Hcrt)-containing neurones within the lateral hypothalamus integrate nutritional, energetic and behavioural cues to generate the final output in order to exert their functions. It is still not clear how Hcrt neurones monitor changes in energy status in animals. In brain slices from transgenic mice expressing green fluorescent protein (GFP) exclusively in Hcrt neurones, we examined the roles of intracellular levels of ATP ($[ATP]_i$) in regulating activities in these cells with conventional and perforated whole-cell recording. By using 'ATP clamp' we demonstrated that membrane potential (V_m) correlated with the $[ATP]_i$ in Hcrt neurones. Perforated recording revealed a V_m of -46.1 ± 1.6 mV ($n = 18$), close to the level measured with an $[ATP]_i$ equal to 5–6 mM (–48.7 ± 1.4 mV, $n = 16$, 5 mM ATP), suggesting that a unique demand for energy is required to maintain normal functionality in Hcrt cells. A direct disruption of ATP production or reduction in ambient glucose levels resulted in an inhibition of activity in Hcrt neurones. The V_m was significantly depolarized in Hcrt neurones in sleep-deprived mice as compared with controls ($P < 0.01$, t test), which was eliminated by experimental manipulations causing the same level of $[ATP]_i$ and K_{ATP} channel opening in both groups, suggesting a decrease during sleep and an increase during sustained wakefulness in $[ATP]_i$ in Hcrt cells. In summary, these data demonstrate that a delicate control of activity by monitoring the availability of intracellular energy stores in Hcrt cells may serve as a novel mechanism regulating energy expenditure and behavioural state dependent upon the energy state in animals.

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Abbreviations ARC, arcuate nucleus; GFP, green fluorescent protein; Hcrt, hypocretin/orexin; LH, lateral hypothalamus; V_m, membrane potential;

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Introduction

The behavioural state of animals is determined by many internal and external environmental factors and the energy status of animals is highly correlated with their behavioural state (Shulman *et al.* 2003). On the one hand, the brain consumes more energy in the activated and even the so-called 'resting state' than in the anaesthetized state (Shulman *et al.* 1999); on the other hand, a low energy state leads to unconsciousness in animals (Shulman *et al.* 2009). It has been hypothesized that insufficient energy may lead to attenuation of wakefulness and promotion of sleep (Benington & Heller, 1995; Scharf *et al.* 2008). Therefore, sensing of the availability of energy in the brain and allocating energy expenditure between the brain and the rest of the animal body is not only necessary for the maintenance of the functioning of the nervous system, but also essential for survival. Evolutionarily, foraging for food (the energy source for animals) consists of a complex array of behaviours, which are governed by the brain through continuously monitoring the intake and expenditure of energy. It is not yet clear how the brain circuitry responsible for energy homeostasis is influenced by the availability of energy and how it adjusts the animal's behaviour to accommodate the energy state.

The neurones in the lateral hypothalamus (LH) that synthesize the neuropeptide hypocretin/orexin (Hcrt) play a key role in many brain functions critical to the survival of animals (de Lecea *et al.* 1998; Sakurai *et al.* 1998). It has been established that Hcrt participates in the determination of the behavioural state of animals, and that dysfunction of the Hcrt system leads to impairments to arousal and wake maintenance (Chemelli *et al.* 1999; Lin *et al.* 1999). It has also been reported that Hcrt neurones are modulated by molecules relevant to energy intake (such as leptin, insulin and glucose) in animals (Yamanaka *et al.* 2003; Burdakov *et al.* 2006; Guyon *et al.* 2009; Parsons & Hirasawa, 2010). However, the question remains whether the energy state of Hcrt neurones determines the activity of these cells and in turn shapes the functions governed by these neurones, particularly the behavioural state of animals. Adenosine-5′-triphosphate (ATP) is an important intracellular molecule utilized by cells to operate critical biochemical and biophysical processes, and the intracellular concentration of ATP ($[ATP]_i$) may be an indicator of the energy state of cells (Peters *et al.* 2004). The sensing of ATP levels via the ATP-sensitive potassium channels in terms of modulating the excitability of excitable cells is particularly critical to neurones and other cell types that sense the energy state of animals (Nichols, 2006).

In this study, we investigated the role of $[ATP]_i$ on the excitability of Hcrt neurones. Importantly, our results suggest that the energy state of Hcrt neurones as represented by the $[ATP]_i$ might shape the response of these neurones to external stimuli and thus the behavioural state of animals.

Methods

Animals

Male Hcrt-GFP mice (14 to 21 days old, on a C57BL6 genetic background) were group housed and maintained on a 12–12 h light–dark cycle with food and water available *ad libitum*. Sleep deprivation by gentle handling was performed as described previously (Rao *et al.* 2007). Briefly, mice in the SD group were sleep deprived (SD) for 4 hours (08.00 h to 12.00 h) by being gently touched with a small soft paintbrush on the back upon eye closure by an experimenter, while mice in the control group were allowed to undergo normal sleep. Mice were habituated to the experimenter and the paintbrush for 3 days before the SD experiment. All animal procedures were performed in strict accordance with NIH Care and Use of Laboratory Animals Guidelines and were approved by the Yale University Animal Care and Use Committee.

Electrophysiology

The preparation of hypothalamic slices containing Hcrt neurones was performed as described previously (Rao *et al.* 2007). Briefly, after mice were anaesthetized with isoflurane and decapitated, the brains were rapidly removed and immersed in cold (4°C) oxygenated high-sucrose solution containing (mM): sucrose 220, KCl 2.5, NaH_2PO_4 1.23, $NaHCO_3$ 26, $CaCl_2$1, $MgCl_2$ 6 and glucose 10, pH 7.3 with NaOH. After being trimmed to a small tissue block containing the hypothalamus, coronal slices (350 μm thick) were cut on a vibratome and maintained in a holding chamber with artificial cerebrospinal fluid (ACSF, bubbled with 5% CO_2 and 95% O_2) containing (in mM): NaCl 124, KCl 3, $CaCl_2$ 2, $MgCl_2$ 2, NaH_2PO_4 1.23, $NaHCO_3$ 26, glucose 2.5, pH 7.4 with NaOH. After a one-hour recovery period, slices were transferred to a recording chamber and were constantly perfused with ACSF (33°C) at a rate of 2 ml min^{-1}.

Both conventional and perforated whole-cell patch clamp recording were performed in Hcrt-GFP neurones under voltage- and current clamp as reported previously (Rao *et al.* 2007). The micropipettes were made of borosilicate glass (World Precision Instruments) with a Sutter micropipette puller (P-97) and back filled with a pipette solution containing (mM): potassium gluconate 135, $MgCl_2$ 2, Hepes 10, EGTA 1.1, Mg-ATP 1–6 (according to our experimental design), Na_2-phosphocreatin 10 and Na_2-GTP 0.3, pH 7.3 with KOH. In the perforated whole-cell recording, amphotericin B (Sigma, final concentration 3%) was added to a modified pipette solution with 0 ATP and

GTP. Both input resistance and series resistance were monitored throughout the experiments and the former was partially compensated. Only those recordings with stable series resistance and input resistance were accepted. The membrane and spontaneous action potential were recording in Hcrt neurones under current clamp, while whole-cell K_{ATP} current was recorded under voltage clamp. Tolbutamide, diazoxide and NaN_3 were from Sigma and were applied to the recording chamber via ACSF. All data were sampled at 3–10 kHz, filtered at 1–3 kHz and analysed with an Apple Macintosh computer using AxoGraph X (AxoGraph Scientific). Statistics and plotting were performed with KaleidaGraph (Synergy software) and Igor Pro (WaveMetrics).

Results

$[ATP]_i$ modulates the excitability via K_{ATP} channels in Hcrt neurones

ATP level is an essential parameter representing energy availability and serves as the link between the energy state and excitability in neurones. Although Hcrt neurones are critical in sensing glucose and lactate levels in the brain of animals (Yamanaka *et al.* 2003; Burdakov *et al.* 2006; Parsons & Hirasawa, 2010), it is not yet clear whether the $[ATP]_i$ has any effects on neuronal activity in these cells. In Hcrt neurones under current clamp recorded with a pipette solution containing 2 mM ATP (a typical concentration in patch pipette solution), a few minutes after the establishment of whole-cell recording configuration, the spontaneous action potentials (APs) gradually disappeared and membrane potential (V_m) hyperpolarized significantly from -58.3 ± 2.2 mV ($n = 20$) at the start of current clamp recording to -67.5 ± 1.5 mV ($n = 13$) and maintained this steady-state level throughout the experiment ($P < 0.05$, t test, Fig. 1*A*). Next, we performed conventional whole-cell recording in Hcrt neurones with a fixed ATP concentration ranging from 1 to 6 mM in the pipette solution ('ATP clamp') in the absence or presence of tetrodotoxin (TTX, 0.5 μM), which eliminates the influence of APs on the V_m. As the V_m measured in the presence and absence of TTX at each ATP concentration was not significantly different, the results obtained under these two conditions were combined. The V_m of Hcrt neurones underwent a similar hyperpolarization and reached a plateau 5 min later when $[ATP]_i$ was lower than 5 mM (Fig. 1*B*). The final stabilized V_m was as follows: -69.6 ± 1.9 mV ($n = 14$, 1 mM ATP), -65.1 ± 1.4 mV ($n = 29$, 2 mM ATP), -61.9 ± 2.7 mV ($n = 13$, 3 mM ATP), -59.1 ± 2.3 mV ($n = 14$, 4 mM ATP), -48.7 ± 1.4 mV ($n = 16$, 5 mM ATP) and -49.7 ± 2.2 mV ($n = 16$, 6 mM ATP). When Hcrt neurones were examined with perforated recording with amphotericin B to preserve intracellular content (including $[ATP]_i$), the V_m and APs remained at a stable level throughout the experiment and V_m (-46.1 ± 1.6 mV, $n = 18$) was close to that measured with 5–6 mM ATP in the pipette solution (Fig. 1*A*). Our further experiments verified that the K_{ATP} channels mediated the effects of $[ATP]_i$ on Hcrt neurones as seen in other types of cells (Nichols, 2006). A selective K_{ATP} channel blocker tolbutamide (100 μM) applied in the ACSF significantly reversed hyperpolarization in Hcrt neurones in conventional recording mode with 2 mM ATP in the pipette solution (control: -71.9 ± 1.7 mV, $n = 11$; tolbutamide: -53.7 ± 2.6 mV, $n = 11$; washout: -66.2 ± 2.6 mV, $n = 11$; $P < 0.01$, ANOVA, Fig. 1*C*). In contrast, V_m was unchanged in the presence of tolbutamide in perforated mode (control: -47.6 ± 2.0 mV, $n = 5$; tolbutamide: -48.0 ± 1.7 mV, $n = 5$; washout: -49.0 ± 1.9 mV, $n = 5$; $P > 0.05$, ANOVA, Fig. 1*C*). Furthermore, the K_{ATP} channel opener diazoxide (500 μM) applied in ACSF significantly hyperpolarized Hcrt neurones (control: -50.5 ± 2.1 mV, $n = 12$; diazoxide: to -69.1 ± 1.7 mV, $n = 12$; washout: -53.2 ± 1.6 mV, $n = 11$; $P < 0.01$, ANOVA, Fig. 1*E* and *F*). Together, these data indicate that the physiological concentration of ATP may be at 5–6 mM in Hcrt neurones and that the alteration in $[ATP]_i$ leads to changes in V_m via the opening of K_{ATP} channels in these cells.

Since the ATP metabolite ADP has also been shown to modulate K_{ATP} channels and the ratio of ATP/ADP triggers the energy-sensing mechanisms in neurones (Vesce *et al.* 2005; Nichols, 2006), we verified whether the ratio of ATP/ADP participated in the modulation of V_m in Hcrt neurones. To achieve this goal, we performed conventional whole-cell recording with both ATP and ADP in the pipette solution in Hcrt neurones. Specifically, we examined the effects of the ATP/ADP ratio found under physiological conditions on V_m in Hcrt neurones (Vesce *et al.* 2005). When the ATP/ADP ratio was adjusted to levels at 10:1, 5:1 and 2:1, the V_m was not significantly different from that without ADP in the pipette solution ($P > 0.05$, ANOVA) (Fig. 1*G*). V_m was -70.1 ± 2.1 mV ($n = 12$, 2 mM ATP only), -68.3 ± 1.7 mV ($n = 14$, ATP/ADP at 10:1), -72.3 ± 0.6 mV ($n = 11$, ATP/ADP at 5:1) and -67.6 ± 1.8 mV ($n = 12$, ATP/ADP at 2:1). These results suggest that the increase in ADP levels resulting from the use of ATP does not induce significant changes in V_m as long as the ATP levels are maintained stable through cellular metabolism in neurones and that the intracellular levels of ATP dominate the effects of adenosine nucleosides on V_m in Hcrt neurones. Lastly, to verify the specificity and validity of examining the effects of $[ATP]_i$ on V_m in Hcrt neurones, we used the same approach to examine the correlation of $[ATP]_i$ with V_m in neurones in the arcuate nucleus (ARC), since it was reported that $[ATP]_i$ was close to 0.9 mM in a previous report (Ainscow *et al.* 2002). As shown in

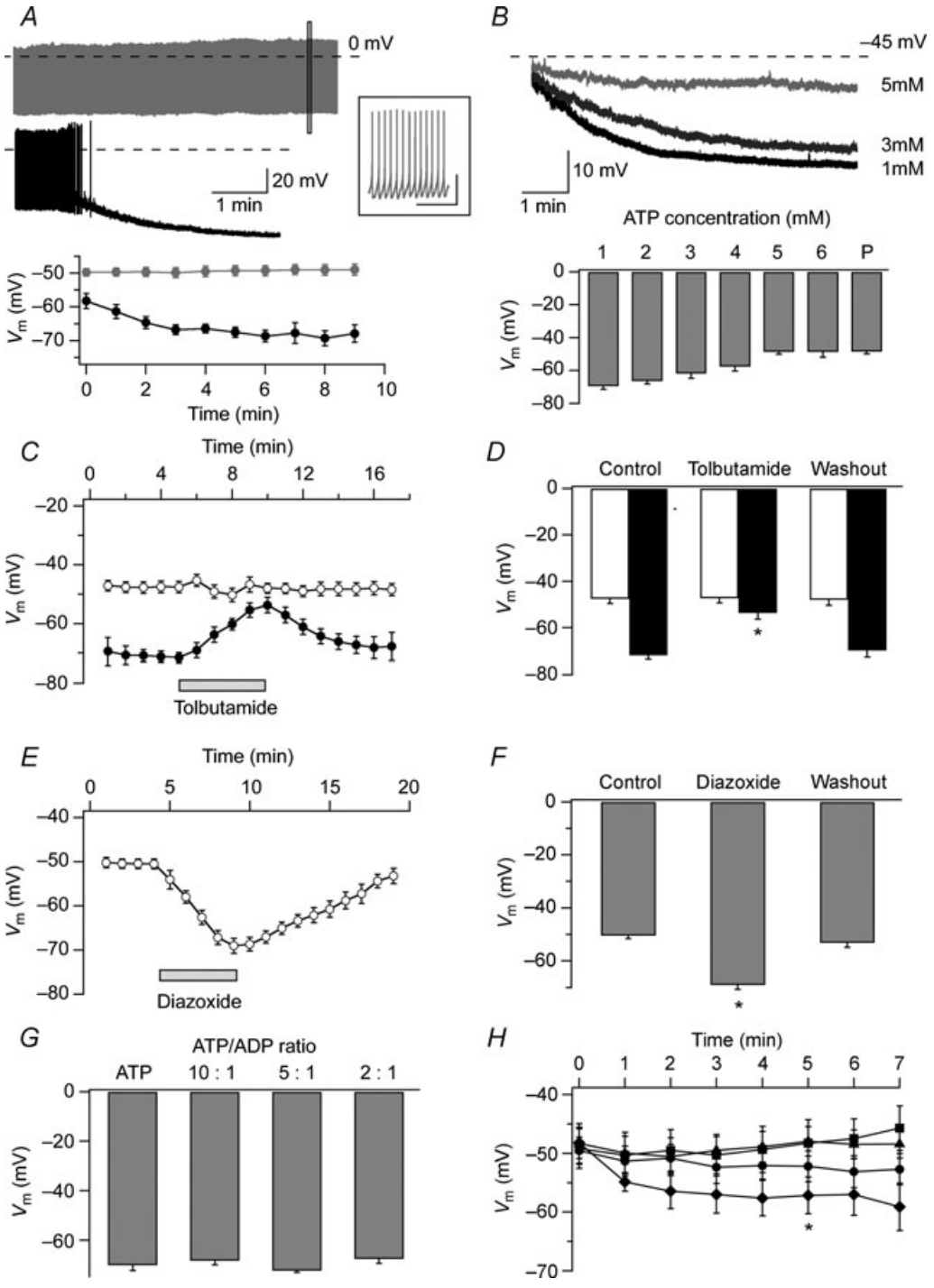

Figure 1. Intracellular ATP levels determine membrane potential (V_m) by modulating K_{ATP} channels in Hcrt neurones

A, 'run down' of V_m in Hcrt neurones after the dialysis of pipette solution containing 2 mM ATP into Hcrt neurones. Top and middle panels, raw traces of V_m and APs recorded in Hcrt neurones under current clamp in perforated (grey) and conventional (with 2 mM ATP in pipette solution, black) whole-cell modes. Bottom, time courses of V_m in Hcrt neurones examined under current clamp in perforated (grey, $n = 6$) and conventional (black, $n = 29$) whole-cell modes. Scale bar for the inset: 20 mV, 100 ms. *B*, the effects of $[ATP]_i$ on V_m in Hcrt neurones. Top, sample traces of V_m recorded with ATP levels at 1, 3 and 5 mM in the pipette solution. Bottom, pooled results from all cells tested at various ATP levels (1–6 mM) and undisturbed ATP levels (P, perforated recording). *C* and *D*, the time courses and pooled results show effects of the K_{ATP} channel blocker tolbutamide on V_m in Hcrt neurones at a low (2 mM, filled circles/bars) and an intact (open circles/bars) $[ATP]_i$. *E* and *F*, time course and pooled results show that diazoxide significantly

Fig. 1*H*, V_m maintained stable in ARC neurones under perforated (initial value: -49.6 ± 2.3 mV, $n = 7$; 6 min later: -53.1 ± 2.7 mV, $n = 7$; $P > 0.05$, *t* test; Fig. 1*H*, circles) and conventional whole-cell recording with 2 mM ATP (initial value: -49.2 ± 3.4 mV, $n = 6$; 6 min later: -50.3 ± 3.8 mV, $n = 6$; $P > 0.05$, *t* test; Fig. 1*H*, squares) and 0.5 mM ATP (initial value: -48.3 ± 3.4 mV, $n = 8$; 6 min later: -48.5 ± 2.5 mV, $n = 8$; $P > 0.05$, *t* test; Fig. 1*H*, triangles) in the pipette solution. The V_m was significantly hyperpolarized 5 min after the establishment of whole-cell configuration in ARC neurones when a pipette solution with 0 mM ATP was used (initial value: -48.2 ± 2.6 mV, $n = 7$; 5 min later: -57.2 ± 3.1 mV, $n = 7$; $P < 0.05$, *t* test; Fig. 1*H*, diamonds). These results suggest that the $[ATP]_i$ required to maintain a normal V_m was no more than 0.5 mM, which is close to the $[ATP]_i$ measured with the luciferase–luciferin approach (Ainscow *et al.* 2002).

To further verify the effects of $[ATP]_i$ on V_m via K_{ATP} channels, we examined the opening of K_{ATP} channels at different $[ATP]_i$ in Hcrt neurones. The whole-cell K_{ATP} current was measured when a voltage ramp from -120 mV to -40 mV was applied to Hcrt neurones under voltage clamp with a pipette solution containing 1 mM ATP (in our case, the Hcrt neurones could not tolerate a long period of recording with no ATP in the pipette solution). The K_{ATP} current responses in the same Hcrt neurones were recorded in control, tolbutamide- and diazoxide-containing ACSF sequentially (Fig. 2, bottom right panel). The net K_{ATP} current at 1 mM ATP and the total K_{ATP} current were obtained by subtracting current responses recorded in control and diazoxide-containing ACSF from that recorded in the presence of tolbutamide (non-K_{ATP} membrane currents) (Fig. 2*A*). The total whole-cell conductance of K_{ATP} currents (calculated as the slope of the tolbutamide-sensitive current–voltage curve between -70 mV and -95 mV in the presence of diazoxide) was 53.68 ± 38.18 pS pF^{-1} ($n = 7$) in Hcrt neurones. The K_{ATP} current responses at ATP levels of 3 and 5 mM were examined by the same approach (Fig. 2*B* and *C*). At ATP levels of 1, 3 and 5 mM in Hcrt neurones, the open probability of K_{ATP} channels (p), defined as $p = (I_{control} - I_{tolbutamide})/(I_{diazoxide} - I_{tolbutamide}) \times 100\%$, was $54.39 \pm 13.01\%$ ($n = 7$), $17.25 \pm 3.95\%$ ($n = 6$) and $4.52 \pm 3.26\%$ ($n = 6$), respectively (Fig. 2*E*). In perforated mode, the open probability of K_{ATP} channels measured with the approach described above was $0.3 \pm 0.36\%$ ($n = 4$), suggesting that the K_{ATP} channels are in the closed state in Hcrt neurones with intact intracellular ATP levels.

In summary, these results suggest that $[ATP]_i$ has a profound effect on the opening of K_{ATP} channels in Hcrt neurones, which may significantly dictate the V_m in these cells.

$[ATP]_i$ shapes the activity in Hcrt neurones

Next, we tested whether the interruption of ATP production by blocking oxidative phosphorylation or decreasing extracellular glucose has any effects on V_m in Hcrt neurones. In perforated mode, the application of NaN_3 (1 mM, an inhibitor of oxidative phosphorylation) through ACSF (Matsumoto *et al.* 2002) significantly hyperpolarized Hcrt neurones a few minutes later (control: -50.0 ± 1.4 mV, $n = 9$; NaN_3: -63.7 ± 1.2 mV, $n = 9$; washout: -48.9 ± 1.4 mV, $n = 9$; $P < 0.01$, ANOVA, Fig. 3*A*). Tolbutamide (100 μM) applied in the presence of NaN_3 significantly reversed the effect of NaN_3 on V_m in Hcrt neurones (control: -50.3 ± 1.6 mV, $n = 6$; NaN_3: -65.5 ± 0.8 mV, $n = 6$; NaN_3 plus tolbutamide: -44.5 ± 2.1 mV, $n = 6$; $P < 0.01$, ANOVA) (Fig. 3*B*). The same experiment was performed in Hcrt neurones under conventional whole-cell recording with an ATP level of 6 mM in the pipette solution. The application of NaN_3 did not induce any changes in V_m in this protocol (control: -47.4 ± 2.0 mV, $n = 5$; NaN_3: -47.7 ± 2.1 mV, $n = 5$; washout: -45.3 ± 1.7 mV, $n = 5$, $P > 0.05$, ANOVA, Fig. 3*E* and *F*). In a parallel set of experiments, we lowered the extracellular glucose concentration from 2.5 mM to 0.1 mM in the ACSF. The V_m of Hcrt neurones did not exhibit an acute change in response to the initial decrease in extracellular glucose concentration, but rather significantly hyperpolarized about 20 min later (control: -48.41 ± 1.66 mV, $n = 7$; low glucose: -53.46 ± 1.73 mV, $n = 7$, washout: -45.07 ± 1.81 mV, $P < 0.01$, ANOVA, Fig. 3*G*), which could be reversed by the application of tolbutamide (100 μM) (control: -51.64 ± 0.80 mV, $n = 7$; low glucose: -58.06 ± 1.93 mV, $n = 7$; low glucose + tolbutamide: -46.63 ± 1.44 mV, $n = 7$, $P < 0.01$, ANOVA, Fig. 3*H*). Together, these results suggest that the lowered $[ATP]_i$ resulting from the altered cellular metabolism induces the opening of K_{ATP} channels and hyperpolarization of V_m

induces a hyperpolarization of V_m in Hcrt neurones with undisturbed $[ATP]_i$. *G*, pooled results show that changes in intracellular ADP levels at the ATP/ADP ratios within the physiological range do not significantly alter V_m in Hcrt neurones. ATP (concentration was fixed at 2 mM) and ADP (at different concentrations according to the ATP/ADP ratios) were included in the pipette solution when conventional whole-cell recording was performed in Hcrt neurones. *H*, time courses of V_m in ARC neurones examined under current clamp in perforated (circles) and conventional whole-cell modes with 0 (diamonds), 0.5 (triangles) and 2 mM ATP (squares) in the pipette solution. $^*P < 0.05$, *t* test.

in recorded neurones. Replenishing the exogenous ATP could reverse this process.

$[ATP]_i$ is relevant to the activity state of animals

It has been shown that the cerebral consumption of glucose and oxygen falls during sleep in humans, and that glucose uptake is high during wake and low during sleep in animals (Boyle *et al.* 1994; Vyazovskiy *et al.* 2008). It is not clear, however, whether $[ATP]_i$ fluctuates in central neurones during the sleep–wake cycle. We examined whether $[ATP]_i$ altered in Hcrt neurones dependent upon the behavioural state of animals in control (normal sleep) and sleep-deprived (SD, from 08.00 h to 12.00 h) mice (Rao *et al.* 2007). As shown in Fig. 4*A*, in perforated mode the V_m was significantly depolarized in SD mice *versus* controls (control: -52.0 ± 0.5 mV, $n = 19$; SD: -47.6 ± 0.8 mV, $n = 17$; $P < 0.01$, t test). When conventional recording with 2 mM ATP in the pipette solution was performed in control and SD mice, the V_m was -60.3 ± 1.6 mV ($n = 8$, control) and -53.4 ± 2.9 mV ($n = 7$, SD) initially, declined gradually and reached almost the same level (control: -69.8 ± 1.8 mV, $n = 8$; SD: -69.3 ± 1.5 mV, $n = 7$; $P > 0.05$, t test, Fig. 4*B*) once V_m stabilized (after the completion of exchange between the pipette solution and intracellular content). This result suggests that the initial difference in V_m between control and SD mice may be due to the different levels of ATP in Hcrt neurones. To further verify the effects of intracellular ATP on V_m measured in control and SD mice, we examined the effects of diazoxide (a K_{ATP} channel opener) on Hcrt neurones in these mice (Fig. 4*C*). In perforated recording mode, the V_m was -52.4 ± 0.8 mV ($n = 11$, control) and -48.0 ± 1.3 mV ($n = 9$, SD) initially ($P < 0.05$, t test), then hyperpolarized to -66.0 ± 1.9 mV ($n = 11$, control) and -66.3 ± 2.8 mV ($n = 9$, SD) during the application of diazoxide ($P > 0.05$, t test) and recovered upon removal of diazoxide. Note that we also recorded V_m in Hcrt neurones in conventional recording mode with 5 mM ATP in the pipette solution in control and SD mice. In Hcrt neurones in control mice, V_m modestly depolarized from the initial value

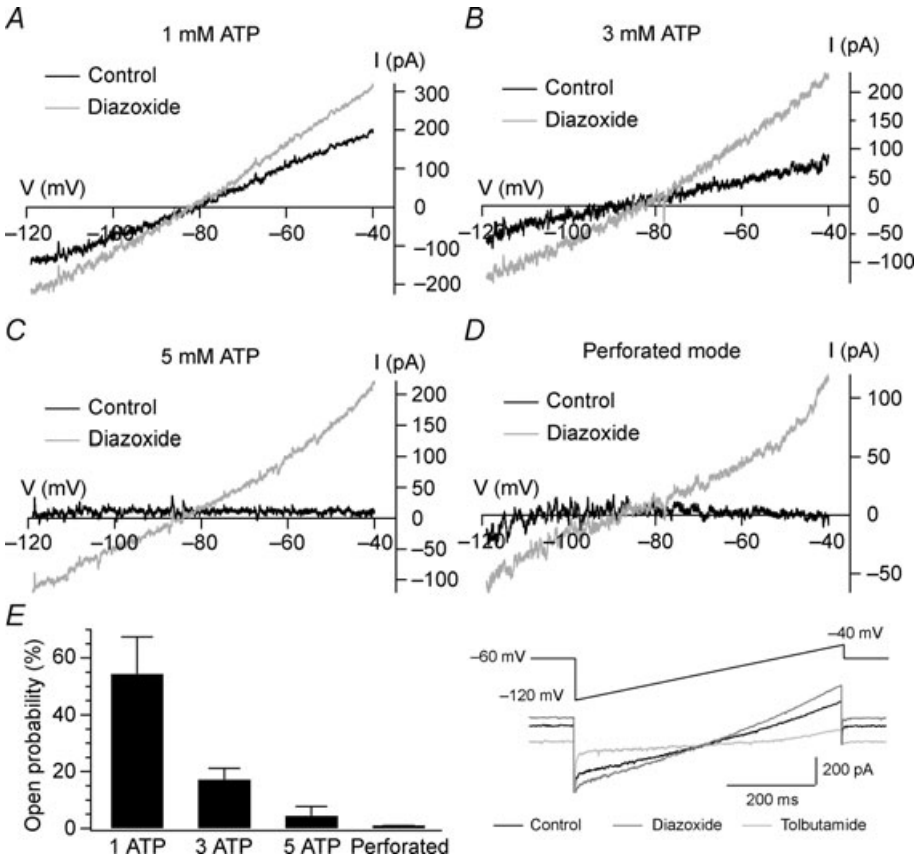

Figure 2. The state of K_{ATP} channels at different ATP levels in Hcrt neurones in LH slices
Lower bottom right panel, sample traces of whole-cell K_{ATP} current responses to a voltage ramp from −120 mV to −40 mV in the presence of control, diazoxide- and tolbutamide-containing ACSF, respectively. *A–D*, whole-cell K_{ATP} current *I–V* curves at various ATP levels in Hcrt neurones (black traces). The grey traces are *I–V* curves of total K_{ATP} currents measured under each condition. *E*, pooled results of open probability of K_{ATP} channels at different $[ATP]_i$ in Hcrt neurones.

of -55.1 ± 3.9 mV ($n = 7$) to -49.6 ± 2.7 mV ($n = 7$, $P > 0.05$, t test) 5 min after the establishment of whole-cell configuration, while it was unchanged in Hcrt neurones in SD mice (initial value: -49.8 ± 4.6 mV, $n = 7$; 5 min later: -49.2 ± 2.3 mV, $n = 7$). This result suggests that a concentration of 5 mM may be lower than (or close to) the level of ATP in Hcrt neurones in SD mice but higher than that in control mice. In a parallel experiment we measured V_m in ARC neurones from control and SD mice under perforated whole-cell recording. The

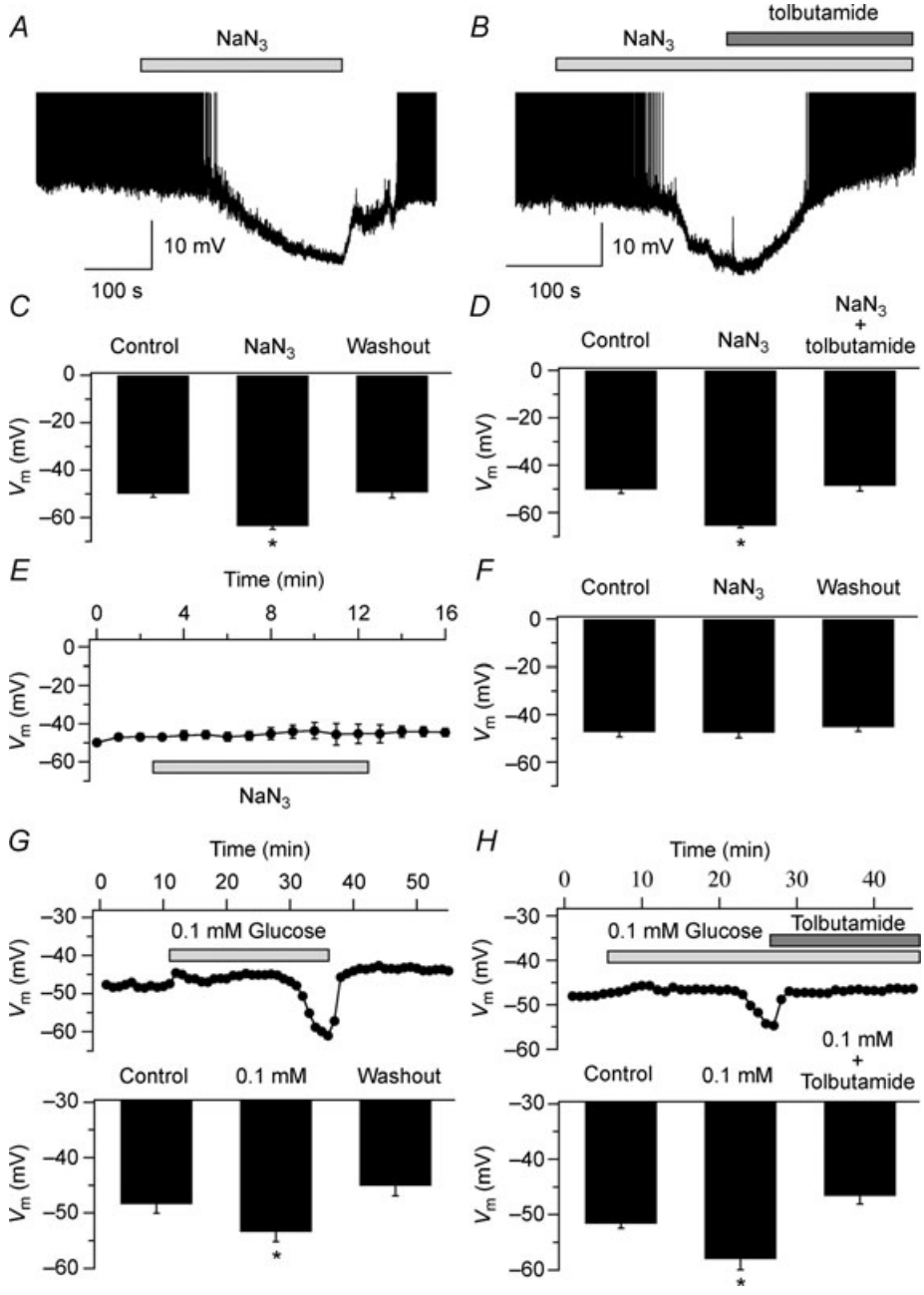

Figure 3. The production of ATP determines the excitability in Hcrt neurones
A and *C*, the inhibition of ATP production by NaN_3 induces hyperpolarization in Hcrt neurones under perforated whole-cell recording. A sample trace is shown in *A* while the pooled results are presented in *C*. $^*P < 0.01$, ANOVA. *B* and *D*, the effect of NaN_3 on V_m in Hcrt neurones is reversed by the application of tolbutamide. $^*P < 0.01$, ANOVA. *E* and *F*, the time course (*E*) and pooled results from all tested neurones (*F*) indicate that the effect of NaN_3 on V_m is eliminated in Hcrt neurones under conventional whole-cell recording with 6 mM ATP in the pipette solution. *G* and *H*, the time course (top) and pooled results from all tested neurones (bottom) indicate that the depletion of extracellular glucose significantly hyperpolarizes Hcrt neurones (*G*, $^*P < 0.01$, ANOVA), which is reversed by tolbutamide (*H*, $^*P < 0.01$, ANOVA).

V_m was not significantly different in ARC neurones in control and SD mice (control, -54.3 ± 2.7 mV, $n = 10$; SD, -55.6 ± 3.9 mV, $n = 6$, $P > 0.05$, t test). Together, these results suggest that V_m is significantly depolarized in Hcrt neurones in SD mice as compared to controls and that a higher level of ATP in Hcrt neurones in SD mice may contribute to this difference.

Discussion

In this study, by correlating $[ATP]_i$ with K_{ATP} current/membrane potential we demonstrate for the first time that Hcrt neurones in the LH rely on a high $[ATP]_i$ to maintain V_m and generate APs, and that the cellular metabolism may shape the activity in Hcrt neurones via changes in $[ATP]_i$ in these cells. Consistent with these results, our data that the $[ATP]_i$ was significantly higher in Hcrt neurones in mice with sustained wakefulness than in controls suggest a novel mechanism underlying the correlation between behavioural state and energy state in animals. We suspect that the difference in $[ATP]_i$ in Hcrt neurones between control and SD mice may be greater than what we report here, since the homogenized experimental condition in which brain slices were prepared and maintained, may lessen the difference in $[ATP]_i$ in Hcrt neurones between control and SD animals.

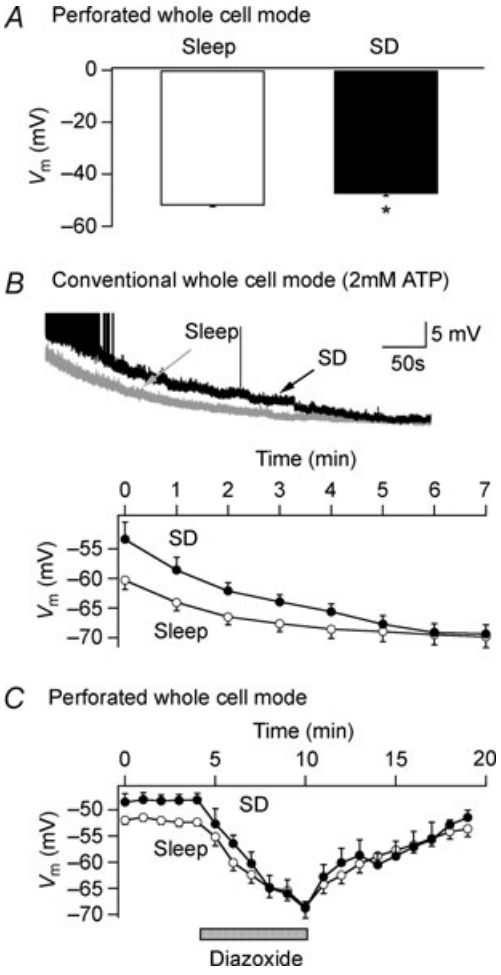

Figure 4. $[ATP]_i$ is elevated in Hcrt neurones in mice exposed to sleep deprivation (SD)
A, V_m of Hcrt neurones in brain slices recorded through perforated whole-cell recording in control and SD mice. $^*P < 0.01$, t test. *B*, the time course of changes in V_m in Hcrt neurones shows that the substitution of $[ATP]_i$ with the same low ATP level (2 mM) eliminates the difference in V_m in Hcrt neurones in control and SD mice. The top panel shows representative traces of V_m recorded in Hcrt neurones in control and SD mice. *C*, the opening of K_{ATP} channels to the same extent with diazoxide eliminates the difference in V_m in Hcrt neurones in control and SD mice.

ATP is an essential commodity for biochemical and biophysical processes to occur in all cells in animals and humans. The determination of $[ATP]_i$ in intact neurones is critical to our understanding of energy metabolism in cells and eventually in whole animals. In this study we used an 'ATP clamp' approach to determine $[ATP]_i$ in neurones in brain slices. Compared to the luciferase–luciferin method (Ainscow *et al.* 2002), our approach can be used in neurones *in vitro* (acutely dissociated or cultured neurones and those in brain slices) and *in vivo*, as long as conventional and perforated whole-cell recording can be performed. In addition, our approach does not involve the expression of exogenous proteins in neurones, which adds an additional hurdle to overcome if using the luciferase-luciferin protocol. The resting cytosolic ATP concentration was estimated to be about 0.9 mM in cultured arcuate neurones and 0.6 mM in cultured cerebellar neurones (Ainscow *et al.* 2002). Consistent with these results, we showed that V_m and AP maintained intact with 0.5 mM ATP in the pipette solution in neurones in the ARC in hypothalamic slices in this study. The AP frequency and V_m remain intact for a long period of time (>10 min) under conventional whole-cell recording with 2 mM or less intracellular ATP in many central neurones, including substantia nigra, midbrain dopamine and cerebellar neurones (Jiang *et al.* 1994; Roper & Ashcroft, 1995; Stanford & Lacey, 1995). However, a concentration at this level seems insufficient to maintain normal V_m and APs in Hcrt neurones, in which an $[ATP]_i$ as high as 5–6 mM is required. It is noteworthy that basal forebrain cholinergic neurones may also require a high $[ATP]_i$ to maintain their normal function (Allen & Brown, 2004).

Previous results suggest that Hcrt neurones are inhibited by an acute elevation in the extracellular concentration of glucose, suggesting that Hcrt neurones act as an energy sensor to prevent excessive energy intake by animals (Yamanaka *et al.* 2003; Burdakov *et al.* 2006; Guyon *et al.* 2009). Our results that insufficient $[ATP]_i$ resulting from deficiency in ATP production or energy sources (low ACSF glucose supply) leads to inactivation of Hcrt neurones demonstrate heterogeneous mechanisms of

energy sensing in these neurones, consistent with a recent report (Parsons & Hirasawa, 2010). This mode of energy sensing has additional physiological significance. On the one hand, the high $[ATP]_i$ in Hcrt neurones may reflect an ample supply of energy in the brain and probably in the entire body. The closure of K_{ATP} channels depolarizes the V_m of Hcrt neurones enough to facilitate AP generation triggered by various cues, leading to sufficient arousal and more importantly the mobilization of energy expenditure to support animal behaviours. This is consistent with a report on the glucose utilization in skeletal muscle through the activation of the sympathetic nervous system by Hcrt (Shiuchi *et al.* 2009). On the other hand, the requirement of a high $[ATP]_i$ in Hcrt neurones to maintain a depolarized V_m and AP firing may lead to a rapid reduction in energy expenditure in whole animals in addition to a neuroprotective role in neurones under hypoglycaemia or anoxia (Ballanyi, 2004). When the energy source is scarce in the brain leading to a lowered $[ATP]_i$ inside neurones, the Hcrt cells would be less active due to the opening of K_{ATP} channels resulting from the fall in $[ATP]_i$ before the same change occurs in other types of neurones, in which lower ATP levels are required to maintain their excitability (Jiang *et al.* 1994; Roper & Ashcroft, 1995; Stanford & Lacey, 1995). Thus, the decline in the excitability of Hcrt neurones would, in turn, tune down the activity in its target systems, leading to reduced arousal and energy expenditure in animals to conserve energy. Therefore, the Hcrt neurones may provide an early alert to the shortage of energy in the brain.

In the light of changes in $[ATP]_i$ in Hcrt neurones between spontaneous sleep and prolonged wakefulness, our results are consistent with reports of elevated activity in ATP production machinery in the cortex during prolonged wakefulness and the fluctuation in ATP levels in several brain areas across the sleep–wake cycle in animals (Dworak *et al.* 2010; Nikonova *et al.* 2010). However, due to the technical limitations the measurements were performed in brain tissues but not neurones in both studies (Dworak *et al.* 2010; Nikonova *et al.* 2010). Therefore, our results provide a piece of direct evidence of changes in ATP levels in neurones according to the behavioural state of animals. Moreover, the correlation between the activity and $[ATP]_i$ in Hcrt neurones may serve as one mechanism underlying the energy hypothesis of sleep, in which the depletion of ATP may limit arousal or wake and promote sleep in animals (Benington & Heller, 1995; Scharf *et al.* 2008). In light of most recent results that the cerebral consumption of glucose and oxygen and glucose uptake fluctuates during the sleep–wake cycle in humans and animals (Boyle *et al.* 1994; Vyazovskiy *et al.* 2008), it is intriguing to examine whether a lowered energy state in neurones during sleep is a ubiquitous adaptation in all neurons. Also, our observations reported in this study may be applicable to other brain areas.

In conclusion, we demonstrate that the unique requirement of a high energy state in Hcrt neurones provides a novel mechanism for sensing the availability of energy in the brain and shape the behavioural state dependent on the energy status in animals.

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Author contributions

Z.-W.L., G.G. and X.-B.G. designed the experiments. Z.-W.L., G.G. and S.S. performed experiments and analysed results. Z.-W.L., G.G. and X.-B.G. wrote the manuscript. All authors approved the final version.

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