

Orexin neurons and inhibitory Agrp→orexin circuits guide spatial exploration in mice

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Key points

- Photoinhibition of endogenous activity of lateral hypothalamic orexin neurons causes place preference and reduces innate avoidance
- Endogenous activity of orexin neurons correlates with place preference
- Medialbasal hypothalamic Agrp neurons inhibit orexin neurons via GABA, and chemogenetic suppression of Agrp neurons increases avoidance in an orexin receptor-dependent manner.

Abstract Hypothalamic orexin/hypocretin neurons integrate multiple sensory cues and project brain-wide to orchestrate diverse innate behaviours. Their loss impairs many context-appropriate actions, but the motivational characteristics of orexin cell activity remain unclear. We and others previously approached this question by artificial orexin stimulation, which could induce either rewarding (positive valence) or aversive (negative valence) brain activity. It is unknown to what extent such approaches replicate natural/endogenous orexin signals, which rapidly fluctuate during wakefulness. Here we took an alternative approach, focusing on observing and silencing natural orexin cell signals associated with a fundamental innate behaviour, self-paced spatial exploration. We found that mice are more likely to stay in places paired with orexin cell optosilencing. The orexin cell optosilencing also reduced avoidance of places that mice find innately aversive. Correspondingly, calcium recordings revealed that orexin cell activity rapidly reduced upon exiting the innately aversive places. Furthermore, we provide optogenetic evidence for an inhibitory GABAergic Agrp→orexin hypothalamic neurocircuit, and find that Agrp cell suppression increases innate avoidance behaviour, consistent with orexin disinhibition. These results imply that exploration may be motivated and oriented by a need to reduce aversive orexin cell activity, and suggest a hypothalamic circuit for fine-tuning orexin signals to changing ethological priorities.

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Introduction

Across the animal kingdom, key aspects of behaviour and physiology are controlled by the orexin/hypocretin

system. This system comprises orexin neuropeptides and G-protein coupled receptors mostly (but not always) coupled to neuronal excitation (de Lecea *et al.* 1998; Sakurai *et al.* 1998; Burdakov, 2004; Belle *et al.* 2014).

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C. Garau was responsible for research governance.

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In the brain, orexin neurons are located exclusively in the lateral hypothalamus (LH), but project to multiple brain areas (Peyron *et al.* 1998). Loss of orexin signalling reduces sleep-wake stability, stress responses, and innate risk-avoidance, and increases body weight (Chemelli *et al.* 1999; Thannickal *et al.* 2000; Hara *et al.* 2001; Kayaba *et al.* 2003; Gonzalez *et al.* 2016b; Blomeley *et al.* 2018; Bassetti *et al.* 2019; Adamantidis *et al.* 2020). Orexin neurons are therefore thought to promote stress-escaping (and/or reward-seeking) and arousal-stabilising processes that overall deplete body energy (Boutrel *et al.* 2005; Mahler *et al.* 2014; Sakurai, 2014; Burdakov, 2019). Orexin neurons are innately (without training) activated by external and internal sensory signals across different modalities (Cai *et al.* 2001; Yamanaka *et al.* 2003; Lee *et al.* 2005; Mileykovskiy *et al.* 2005; Gonzalez, 2016a,b; Williams *et al.* 2007, 2008; Karnani *et al.* 2011, 2020; Blomeley *et al.* 2018; Giardino *et al.* 2018; Bracey & Burdakov, 2020). Many of these signals can be viewed as indicators of stress or deficiency (Boutrel *et al.* 2005; Hassani *et al.* 2016; Burdakov, 2019). Consistent with this, artificial activation of orexin neurons recapitulates the autonomic and behavioural stress responses (Boutrel *et al.* 2005; Suzuki *et al.* 2005; Johnson *et al.* 2012; Bonnnavion *et al.* 2015; Blomeley *et al.* 2018), and their inhibition prevents appropriate adaptations to diverse kinds of stress (Kayaba *et al.* 2003; Yamanaka *et al.* 2003; Boutrel *et al.* 2005; Kuwaki & Zhang, 2012; Karnani *et al.* 2020). This literature is consistent with a view of orexin cells as non-specialised stress-sensors driving multiple responses that counteract stress, by seeking missing rewards and/or by escaping dangers.

While the involvement of orexin neurons in fast and slow sensorimotor processing is clear, their motivational characteristics (aversive *vs.* rewarding) remain incompletely understood. In some contexts, orexin has been reported to increase midbrain dopamine signals, leading to suggestions that it signals reward (Korotkova *et al.* 2003; Harris & Aston-Jones, 2006; Borgland *et al.* 2008). However, some dopamine signals have since been found to signal aversion (Lammel *et al.* 2012), and so it is unclear which (aversive or rewarding) dopamine branches are controlled by orexin. Apart from innervating midbrain dopamine circuits, orexin also activates aversion-causing (negative valence) neural signals, such as accumbal D2 neurons, locus coeruleus noradrenaline neurons, and CRH neurons (Hagan *et al.* 1999; Horvath *et al.* 1999b; McCall *et al.* 2015; Blomeley *et al.* 2018). It is therefore difficult to infer, from orexin actions at individual projections, whether orexin signals associated with specific behavioural contexts have positive or negative valence. We and others previously approached this issue by activating orexin signals. However, such investigations do not replicate the intensity and timing of natural, behaviour-associated orexin signals.

Here we took an alternative approach of determining motivational characteristics, and upstream modulators, of natural orexin signals underlying a specific ethologically relevant behaviour. We chose innate spatial exploration behaviour, because it allows clear observation of behavioural choice (place avoidance *vs.* preference) without prior training, and avoids startle responses to experimental stimuli. Using observation and silencing of orexin cell activity, we provide evidence that natural orexin signals increase place avoidance. Furthermore, we show that hypothalamic AgRP neurons, which signal nutrient deficit and contain the inhibitory neurotransmitter GABA (Horvath *et al.* 1997; Cowley *et al.* 2001; Mandelblat-Cerf *et al.* 2015; Betley *et al.* 2015; Chen *et al.* 2015; Gropp *et al.* 2005; Luquet *et al.* 2005), suppress orexin neuron activity via a GABAergic circuit, and that AgRP neuron inhibition increases avoidance in an orexin receptor-dependent manner.

Methods

Ethical approval

The animal study protocols were legally and ethically approved by the United Kingdom Government (Home Office Project License PPL 70/7600), and conformed to principles and regulations outlined in Grundy (2015).

Targeting reporters, actuators and photons

Adult male C57BL6 mice were used in this project. Mice were kept on a 12:12 h light-dark cycle (lights on at 07.00 h) in temperature ($21 \pm 2^\circ\text{C}$) and humidity ($50 \pm 5\%$) controlled rooms. Food and water were freely available. To silence or record genetically defined neural activity *in vivo*, we used either transgenic mouse lines (for AgRP neurons: AgRP-Cre mice = AgRP-IRES-Cre mice, Jax no. 012899; Tong *et al.* 2008) or custom viral vectors (orexin-GCaMP6s, characterised and described in Gonzalez *et al.* 2016b), and orexin-promoter-driven ArchT, validated in Fig. 1A–C and in Karnani *et al.* (2020); the proportion of orexin neurons tagged with ArchT is $\approx 60\%$; Karnani *et al.* 2020). To probe functional connectivity between AgRP and orexin neurons, we crossed the AgRP-Cre mice with the orexin-GFP mice (Burdakov *et al.* 2006). For optogenetic silencing of orexin cells, mice were bilaterally stereotactically LH-injected, under isoflurane general anaesthesia, with orexin-promoter-dependent ArchT, or, in control experiments, orexin-promoter-dependent GCaMP6s. Mice were then bilaterally implanted with intra-LH optical fibres at 1.35 mm caudal from bregma, 1.0 mm lateral from midline, and 5 mm ventral from brain surface. For pain management, on the day of surgery

mice were injected (s.c.) with Metacam (10 mg/kg) and Buprenorphine (0.1 mg/kg), and given a Metacam oral suspension the day after surgery. Starting from 3 weeks after surgery, mice were used for experiments, where green laser (532 nm, LaserGlow) was applied to LH via bilateral fibre implants at ≈ 20 mW light power at the fibre tip. For fibre photometry recordings from orexin cells, mice were unilaterally LH-injected (using the same surgical procedure as above) with the orexin-promoter-dependent GCaMP6s, and unilaterally implanted with intra-LH optical fibres at the same coordinates as above. For chemo-genetic silencing of Agrp cells, we targeted the inhibitory CNO receptor hM4Di to Agrp cells (Armbruster *et al.* 2007; Krashes *et al.* 2011), by injecting Cre-inducible

AAV (rAAV8/hSyn-DIO-hM4D(Gi)-mCherry; titre 5.30×10^{12} gc/ml, Penn Vector Core) injected into the arcuate hypothalamic nucleus of the Agrp-Cre mice. Two 100 nl injections of rAAV8/hSyn-DIO-hm4(Gi)-mCherry or a control Cre-dependent protein (AAV2/EF1a-DIO-ChR2(E123T/T159C)-mCherry, 7.3×10^{12} gc/ml, UNC Vector Core) were made at 1.4 mm caudal from bregma; ± 0.3 mm from midline; and 5.80 and 5.70 mm from skull surface. At the end of experiments, mice were given an overdose of injectable anaesthetic (ketamine/xylazone, i.p.) followed by secondary means of killing such as exsanguination, thoracotomy, and/or cervical dislocation. Targeting was confirmed using standard immuno-histochemical techniques as described in our previous

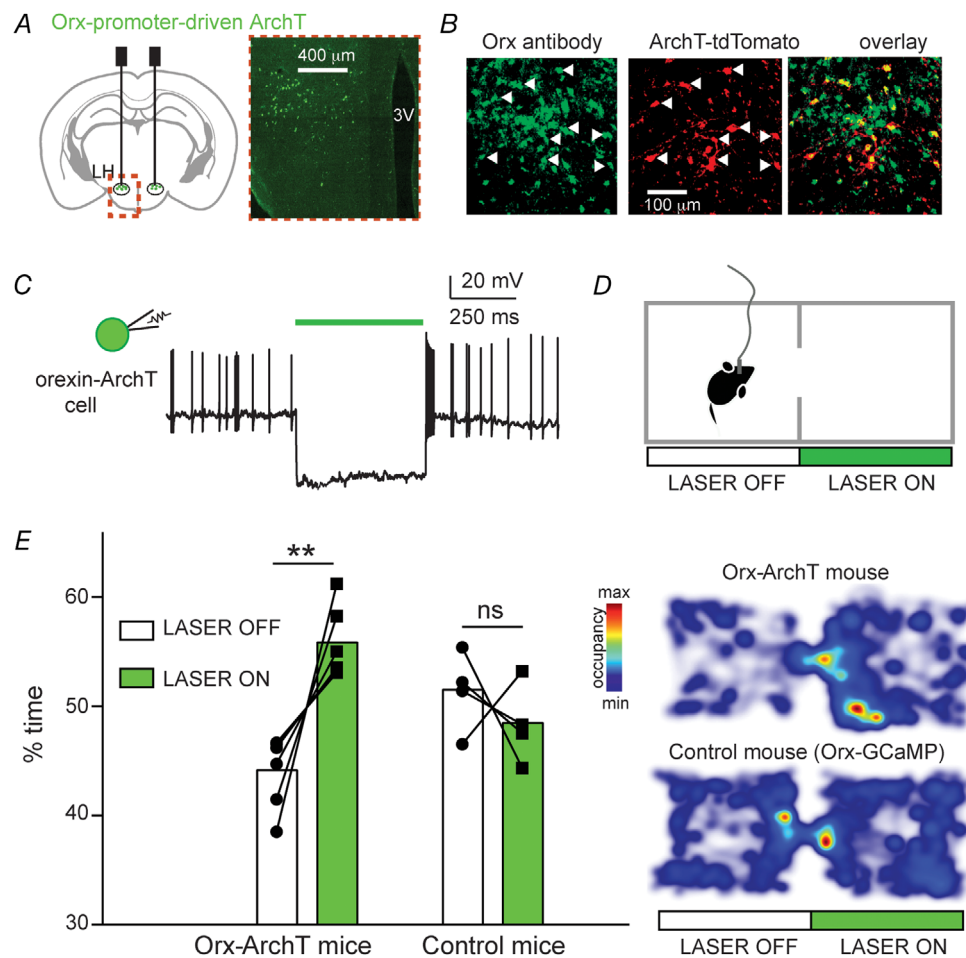


Figure 1. Effect of orexin cell silencing on real-time place preference

A, left: targeting ArchT to orexin cells. Right: expression of ArchT in LH, representative example of 10 brains. 3V, third ventricle. B, ArchT expression in orexin cells, with some ArchT cells arrowed to aid visual comparison. Specificity was $\approx 99\%$ (orexin immunoreactivity observed in 796/803 of ArchT-containing cells, $n = 5$ mice). C, brain slice patch-clamp recording (left) confirming silencing of orexin-ArchT cells (right) by green light (representative recording of $n = 5$ cells). D, schematic diagram of real-time place preference experiment. E, left: quantification of the effect of orexin cell silencing on chamber occupancy time (orexin-ArchT mice: $**P = 0.0077$, two-tailed paired t test, $t = 4.302$, $df = 5$, $n = 6$ mice; control orexin-GCaMP mice: ns, $P = 0.4721$, two-tailed paired t test, $t = 0.8205$, $df = 3$, $n = 4$ mice; the plots show individual datapoints and their means). Right: example heatmaps showing relative time spent in two chambers, illustrating increased occupancy of the chamber associated with orexin cell optosilencing in orexin-ArchT mice. [Colour figure can be viewed at wileyonlinelibrary.com]

work (Cains *et al.* 2017; Blomeley *et al.* 2018; Kosse & Burdakov, 2019).

Real-time place preference

Mice were placed in a rectangular 2-chambered (50 × 25 × 25 cm plexiglass) custom-made behavioural arena, and without any additional contextual cue, separated by a door, in two consecutive 20-min sessions with 30 s in between. In first session, laser stimulation was triggered by the mouse's entrance into the right chamber, and in second session, into the left chamber. We recorded behavioural data via a camera interfaced with Ethovision software (Noldus Information Technologies). Preference scores in each experiment were determined by computing the percentage of time spent in the 'light stimulation' side out of the total explored time during the tests. All measurements were quantified relative to the mouse body centre.

Open field test

The open field (OF) centre–border exploration test was conducted in an open arena made of opaque Plexiglas (50 × 50 cm floor area), which was dimly illuminated, and it was divided into a centre zone (25 × 25 cm) and an outer zone in the periphery or border. Rodents innately stay close to walls and avoid anxiogenic open areas (in this case, the centre). Each mouse was placed gently on the central zone, and its behaviours were recorded by a camera for 15 min and analysed by Ethovision XT software version 11 (Noldus). Tests were conducted between 10.00 and 15.00 h. Each individual mouse was tested once per week to maintain the effect of novelty, and within mouse groups, laser on and off trials were interleaved.

Light-dark box test

The light-dark (LD) test is based on the innate aversion of rodents to brightly illuminated areas and on the spontaneous exploratory behaviour of rodents in response to mild stressors, that is, novel environment and light. The test apparatus consisted of a cage (45 × 25 × 25 cm) divided into two sections of different size and light conditions separated by a door, a small dark-walled compartment (one third) and a large illuminated aversive compartment (two thirds). One chamber was brightly illuminated, whereas the other chamber was dark (dark-walled and covered by a lid). Mice were placed into the dark side then the door was opened. After each trial, all chambers were cleaned with ethanol and water to prevent a bias based on olfactory cues. Each individual mouse was tested once per week to maintain the effect of novelty, and within mouse groups, laser on and off trials were inter-

leaved. The lid covering the dark part of the arena was made compatible with studies of fibreoptic-tethered mice by the dimensions of the open inter-chamber door and the slackness of the fibreoptic.

Fibre photometry

For GCaMP6s excitation, we provided interleaved 405 nm and 470 nm excitation light pulses via LEDs (Kim *et al.* 2016; Kosse & Burdakov, 2019). Fluorescence emission produced by 405 nm excitation is calcium-insensitive thereby providing a real-time control for motion artefacts (Kim *et al.* 2016). Fluorescence signals were normalised to produce the plotted % $\Delta F/F$ values as follows: $\Delta F/F = 100 \times (F_t - F)/F$, where F_t is the raw signal and F is the mean of the first 10 s of trial. Behavioural testing started at least 3 weeks after surgeries, to allow for viral expression and recovery from surgery. Open field and light-dark test were performed as explained above. We wanted to study the dynamics of the natural orexin signals at the moment the animals move between the innately aversive and innately attractive parts of the arenas (centre and border in OF arena; light and dark zones in LD test). In LD test, the light part of the arena is considered an illuminated aversive zone, and the dark side is a safe/attractive zone for the animals. In both tests we studied the peri-events aligned to these transitions from the anxiogenic part of the arena to the safest zones (centre to border in OF; light to dark in LD). A separate cohort of LH-implanted control mice without GCaMP6s was used to validate the absence of potential movement artefacts, or artefacts relating to changes in ambient light (particularly important in the LD test).

Channelrhodopsin-assisted circuit mapping

Whole-cell patch-clamp recordings combined with optical excitation of ChR2-expressing cells were performed using standard techniques, as in our previous work (Schöne *et al.* 2014; Kosse *et al.* 2017; Kosse & Burdakov, 2019). Briefly, brain slices were prepared >4 weeks after stereotaxic virus injection, from 8- to 12-week-old male mice. Acute coronal (LH) slices of 250 μ m thickness were cut while immersed in ice cold ACSF solution using a Peltier-cooled Campden Vibroslice. Slices were then placed in a submerged-type chamber and incubated for 30 min in ACSF at 35°C, and then at room temperature (25°C) until recording. Fluorescent neurons were visualized with an upright Olympus BX51WI microscope with an oblique condenser and appropriate fluorescence filters. Excitation light for ChR2 was applied using a LAMBDA DG-4 fast beam switcher (Sutter) with a xenon lamp and ET470/40 nm bandpass filter. A 40 × 0.8 NA objective was used to apply 5 ms flashes of blue light (~10 mW/mm²)

onto ChR2-containing axons around the recorded neuron, while measuring postsynaptic currents or membrane potential, in order to map the connectivity between Arc and LH neurons. To probe a connection, pulses were typically delivered at 20 Hz, and each cell was tested in voltage-clamp mode at a range of holding potentials (as indicated) as well as in current-clamp.

Chemicals

All drugs were from Sigma unless indicated otherwise. Artificial cerebrospinal fluid (ACSF), and the ice cold slicing solutions were gassed with 95% O₂ and 5% CO₂, and contained the following. ACSF (in mM): 125 NaCl, 2.5 KCl, 1 MgCl₂, 2 CaCl₂, 1.2 NaH₂PO₄, 21 NaHCO₃, 1 D-(+)-glucose. Slicing solution contained (in mM): 2.5 KCl, 1.3 NaH₂PO₄·H₂O, 26.0 NaHCO₃, 213.3 sucrose, 10.0 D-(+)-glucose, 2.0 MgCl₂, 2.0 CaCl₂. For whole-cell recordings, pipettes were filled with (in mM): 120 potassium gluconate, 10 KCl, 10 HEPES, 0.1 EGTA, 4 K₂ATP, 2 Na₂ATP, 0.3 Na₂GTP, 2 MgCl₂, pH 7.3 with KOH. For slice experiments, drugs were used at the following final concentrations: picrotoxin 10 µM, CGP 35348 10 µM; gabazine 3 µM (Tocris). Clozapine-n-oxide (CNO) was administered i.p. at 5 mg/kg in chemogenetic experiments. For orexin receptor activity manipulation *in vivo*, the orexin receptor antagonist SB-334867 (or control vehicle: 0.9% NaCl in 10% DMSO) was administered i.p. SB-334867 has a higher affinity for orexin type-1 receptor than for orexin type-2 receptor, but at higher concentrations, such as used in this study, it antagonizes orexin binding to both receptors, which makes it applicable for blocking both orexin receptors (Smart *et al.* 2001; Adamantidis *et al.* 2007; Karnani *et al.* 2011).

Statistical analyses, experimental design, and data presentation

Data were analysed using Matlab (MathWorks), Prism 6.0 (GraphPad), and Ethovision XT (Noldus) software. Control experiments estimating sample variance were used to pre-determine sample sizes according to institutional guidelines for reduction of animal use. Heatmaps depicting spatial exploration were produced using the 'over heatmaps' setting of Ethovision, in which a colour represents the same location frequency. Before parametric tests, data were tested for normality with a D'Agostino-Pearson omnibus test or Kolmogorov-Smirnov test for small sample sizes, and variances were tested for homogeneity with Bartlett's test. Based on results of these tests, appropriate data transformations were made where necessary to meet the assumptions of parametric tests. Where the assumptions of parametric tests could not be definitively assessed, the

hypotheses was also tested using suitable non-parametric alternatives. In brain slice recordings, cells were randomly chosen throughout the full anatomical extent of LH using an objective that blinded the experimenter to the exact intra-LH location of the orexin-GFP neurons due to its small FOV (×40 objective). In behavioural experiments, mouse groups were interleaved during trials in a Latin square design, to avoid order effects. Data collection and analysis were performed blind to the experimental conditions. Data extraction from videos was automated (EthoVision XT) to maximize objectivity and reproducibility.

Results

Role of exploration-associated orexin signals in place avoidance

Does the natural, exploration-associated activity in orexin neurons cause place avoidance? To answer this question, we performed closed-loop, place-dependent optogenetic silencing of real-time orexin cell activity in a real-time place preference assay. To achieve this, we specifically expressed orexin-promoter-driven red-shifted inhibitory proton pump ArchT in orexin cells (Fig. 1A and B), which enabled rapid and reversible hyperpolarization and electrical silencing of the orexin-ArchT cells by pulses of green light (Fig. 1C; full statistics are reported in the corresponding figure legends). In mice freely exploring an arena composed of two adjoining chambers, bilateral green-laser illumination of the LH specifically in one chamber caused orexin-ArchT mice (but not control mice) to spend more time in that chamber (Fig. 1E). This selective silencing of endogenous, exploration-associated orexin signals demonstrates that they normally cause mice to avoid places where they occur, as recently demonstrated for artificially stimulated orexin activity (Fig. 1g in Giardino *et al.* 2018).

During free exploration, mice innately avoid light or exposed places, preferring to stay near walls or in darkness (Walsh & Cummins, 1976; Crawley, 1985, 2006; Prut & Belzung, 2003). Does the natural, exploration-associated activity in orexin neurons affect these innate spatial avoidance behaviours? To investigate this question, we performed bilateral green-laser illumination of the LH of orexin-ArchT or control mice, while quantifying how many times they chose to enter light or exposed places in standard free-exploration arenas designed to quantify light-dark or centre-border preference (Fig. 2). In orexin-ArchT mice (but not in control mice), this optical manipulation increased the number of entries into light (Fig. 2A and B) or exposed central places (Fig. 2C and D). This demonstrates that endogenous, exploration-associated orexin cell signals normally stimulate the innate place avoidance behaviours,

as previously demonstrated for artificially stimulated orexin activity (Suzuki *et al.* 2005; Heydendael *et al.* 2014; Blomeley *et al.* 2018).

If the natural, exploration-associated orexin signals drive place avoidance as implied by the above data, then one would predict them to be higher in places that mice choose to avoid, and lower in places where the mice prefer to stay. To investigate this prediction, we selectively visualized the natural real-time dynamics of exploration-associated orexin cell activity, by fibre photometry calcium recordings of LH orexin-GCaMP6s population activity in freely exploring mice (see Methods). As predicted, we found that natural orexin cell activity rapidly reduced when mice voluntarily entered dark (Fig. 3A–C) or sheltered places (Fig. 3D–F). Overall, the above results reveal orexin-dependent place avoidance as an important variable governing the direction of self-paced spatial exploration.

Agrp→orexin inhibitory neural circuit and place avoidance

Apart from orexin neurons, other hypothalamic neurons have been implicated in place avoidance. For example, experimental activation of hypothalamic Agrp neurons reduces centre avoidance in the open field test (Dietrich *et al.* 2015). Does the contribution of orexin cells to place avoidance depend on intrinsic activity tone of these other

hypothalamic neurons? Agrp neurons are an inhibitory, GABAergic cell type (Atasoy *et al.* 2012). Anatomical evidence indicates that they densely innervate orexin neurons (Elias *et al.* 1998; Horvath *et al.* 1999a), suggesting that Agrp neurons inhibit orexin neurons. However, this inhibitory interaction was not investigated in the context of spatial exploration, nor confirmed functionally at the circuit level.

To confirm that functional inhibitory Agrp→orexin cell interactions exist at the circuit level, we optogenetically photo-stimulated LH Agrp cells, while recording membrane currents or action potential output from identified orexin cells in mouse brain slices (Fig. 4A). The Agrp cell photostimulation evoked rapid inhibitory membrane currents in orexin neurons, which were abolished by GABA-A receptor antagonism (Fig. 4B and C). The latency between photostimulation onset and current onset was 4.2 ± 2.6 ms (mean \pm SD, $n = 6$ cells). The stimulation of Agrp cell firing was sufficient to suppress orexin cell firing (Fig. 4D). Thus, Agrp cell inhibition would be expected to increase orexin cell firing via disinhibition, considering that orexin neurons are capable of generating intrinsic firing (Eggermann *et al.* 2003; Burdakov *et al.* 2004).

To probe the role of natural Agrp cell activity in spatial exploration, we targeted the inhibitory CNO receptor hM4Di to Agrp neurons (Fig. 5A). Subsequent inhibition of Agrp-hM4Di cells by CNO (Fig. 5B) reduced centre

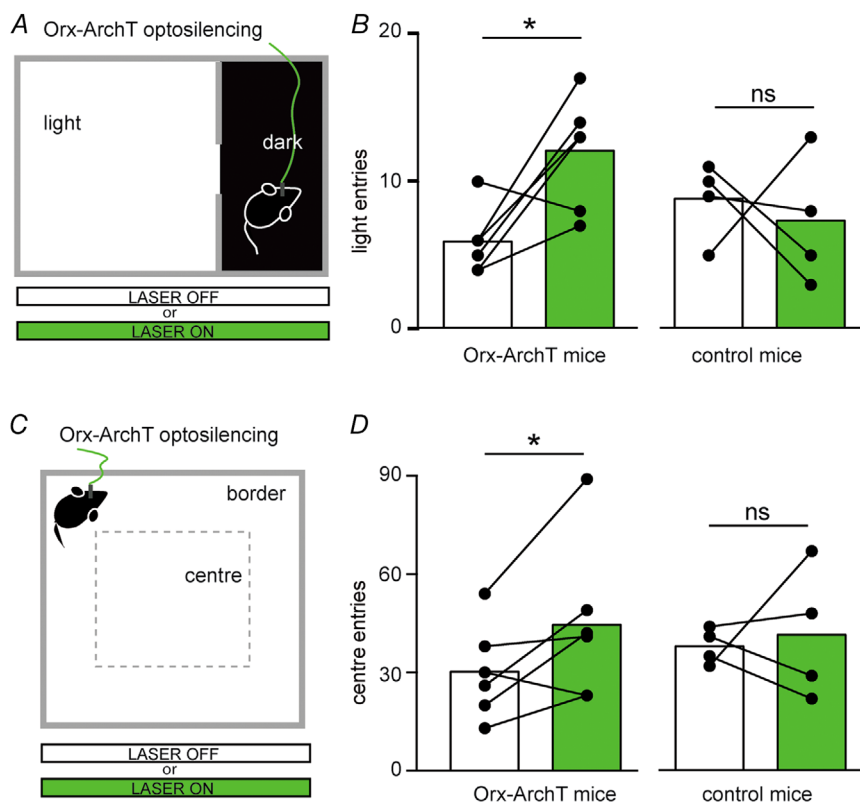


Figure 2. Effect of orexin cell silencing on self-paced entries into innately aversive places

A, schematic diagram of light-dark free exploration experiment. B, quantification of the effect of orexin cell optosilencing on the number of entries into the light part of the arena (orexin-ArchT mice: $*P = 0.0131$, one-tailed paired t test, $t = 3.125$, $df = 5$, $n = 6$ mice; control orexin-GCaMP mice: ns , $P = 0.3456$, one-tailed paired t test, $t = 0.4376$, $df = 3$, $n = 4$ mice; the plots show individual datapoints and their means). C, schematic diagram of the open field centre-border free exploration experiment. D, quantification of the effect of orexin cell optosilencing on the number of entries into the centre of the arena (orexin-ArchT mice: $*P = 0.0348$, one-tailed paired t test, $t = 2.302$, $df = 5$, $n = 6$ mice; control orexin-GCaMP mice: ns , $P = 0.3876$, one-tailed paired t test, $t = 0.3125$, $df = 3$, $n = 4$ mice; the plots show individual datapoints and their means). [Colour figure can be viewed at wileyonlinelibrary.com]

entries in the open field test (Fig. 5C; control data are in Fig. 5D). This effect of Agrp cell inhibition was not observed in the presence of the orexin receptor antagonist SB (Fig. 5C). This suggests that orexin receptor activation contributes to the suppression of centre entries evoked by Agrp cell inhibition.

Discussion

Our results provide evidence that the exploration-associated orexin signals are linked to spatial avoidance,

while spatial attraction correlates with a reduction in the natural orexin cell signals. Mice were attracted to places paired with optosilencing of natural orexin signals rapidly and without training, consistent with an acute role of ongoing orexin activity in guiding innate spatial exploration. The natural orexin signals contributed to place preferences in both familiar (Fig. 1) and novel (Fig. 2) arenas, confirming an innate coupling of orexin activity and spatial exploration. Our study also provides functional confirmation of earlier anatomical data (Elias *et al.* 1998; Horvath *et al.* 1999a) that arcuate Agrp

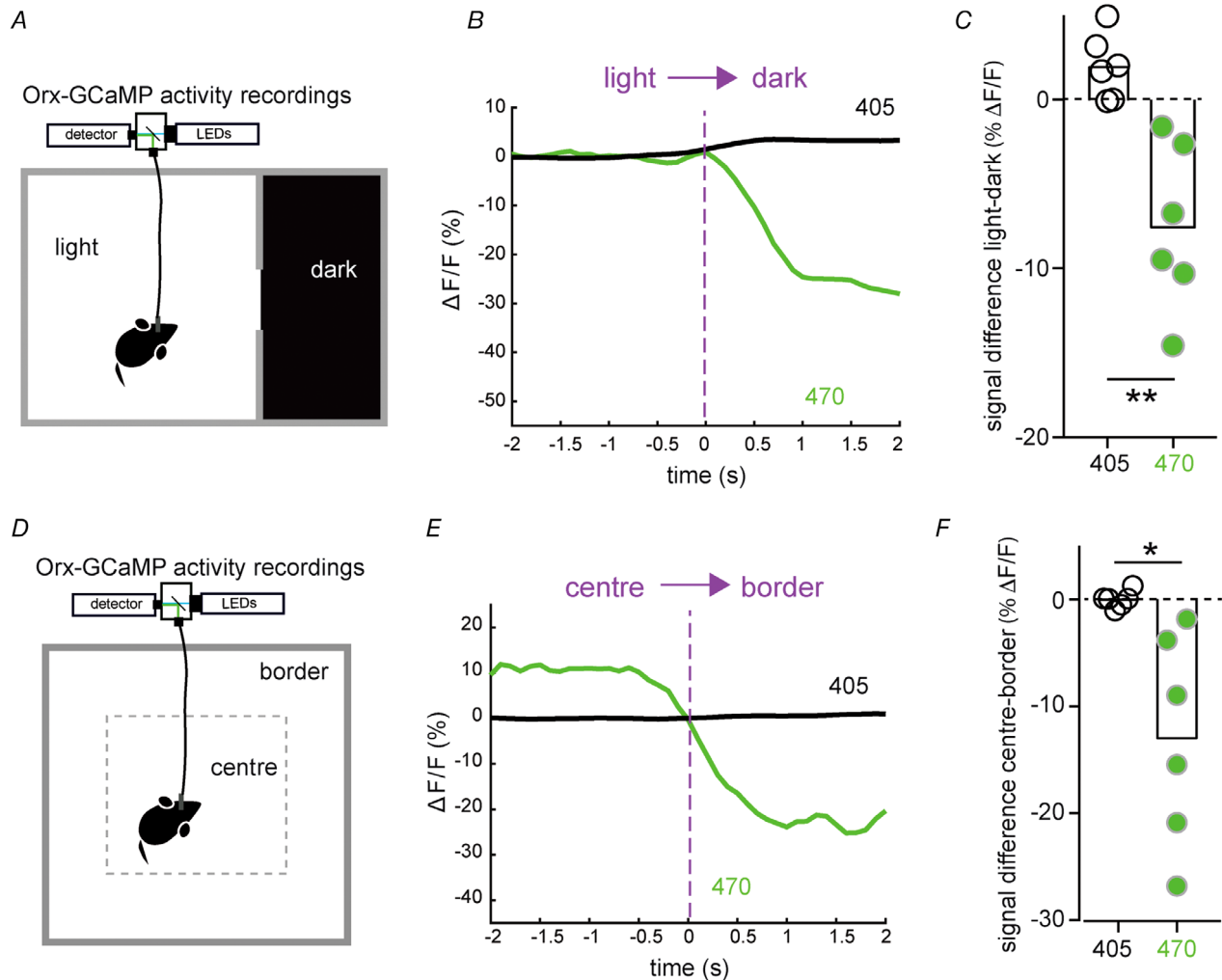


Figure 3. Natural orexin cell dynamics associated with exiting innately aversive places

A, schematic diagram of light-dark free exploration experiment. B, orexin-GCaMP6s signals associated with self-paced light→dark transitions (means of $n = 7$ mice, showing fluorescence emission at 470 nm excitation and 405 nm negative control excitation). C, quantification of data in B. Difference light – dark is dark signal minus light signal, where light and dark signals are average signals of 2 s before and 2 s after light→dark transition (i.e. signals shown in B). $**P = 0.0054$, two-tailed paired t test, $t = 4.697$, $df = 5$, $n = 6$ mice; the plot shows individual datapoints and their means. D, schematic diagram of the centre-border open field free exploration experiment. E, orexin-GCaMP6s signals associated with self-paced centre→border transitions (means of $n = 7$ mice, showing fluorescence emission at 470 nm excitation and 405 nm negative control excitation). F, quantification of data in E. 'Difference centre-border' is border signal minus centre signal, where centre and border signals are average signals of 2 s before and 2 s after centre→border transition (i.e. signals shown in E). $*P = 0.0239$, two-tailed paired t test, $t = 3.202$, $df = 5$, $n = 6$ mice; the plots show individual datapoints and their means. [Colour figure can be viewed at wileyonlinelibrary.com]

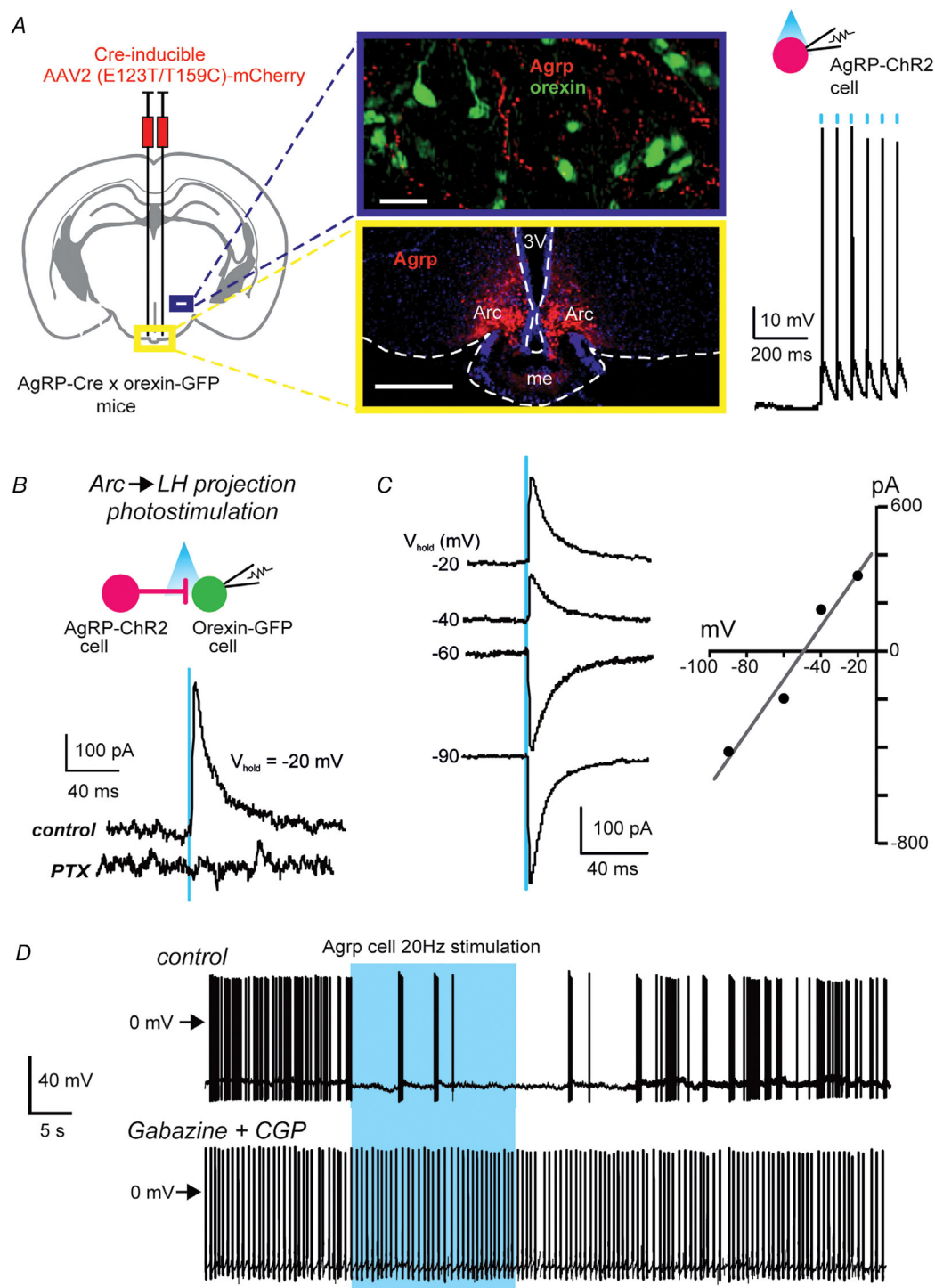


Figure 4. Functional circuit evidence for Agrp→orexin inhibition

A, targeting scheme (left), example expression (middle), and patch-clamp confirmation of Agrp-ChR2 cell optoexcitability (left, representative recording of $n = 5$ cells). B, recording schematic diagram (top), and voltage-clamp whole-cell recording demonstrating an Agrp-ChR2 cell stimulation-evoked outward current in orexin cells ($n = 17/24$ cells), that was blocked by GABA-A antagonist picrotoxin ($n = 17/17$ cells). C, current-voltage relationship of the current in B, demonstrating a reversal potential typical of GABA-A receptor chloride channels. Left, representative example; right, mean data of $n = 4$ cells. D, effect of Agrp cell stimulation on orexin cell firing rate under control and GABA blocker conditions. Control: change in firing rate = -1.87 ± 0.93 Hz, $P = 0.0044$; GABA blocker: change in firing rate = 0.058 ± 0.17 Hz (means \pm SD); $P = 0.4353$; P values from one-sample t tests, $t = 4.923$, $df = 5$ and $t = 0.8476$, $df = 5$, respectively, representative example of $n = 6/6$ cells. [Colour figure can be viewed at wileyonlinelibrary.com]

neurons provide inhibitory input to orexin cells. The short latency of the GABAergic Agrp→orexin synaptic inputs (Fig. 4) is consistent with the monosynaptic connectivity implied by the earlier anatomical work. On the other hand, a polysynaptic Agrp→orexin pathway (e.g. Agrp_{GABA}→GABA→orexin) is unlikely to account for our results because it would require Agrp neurons to excite an intermediate GABA neuron, and to the best of our knowledge there is no evidence that Agrp neurons elicit rapid postsynaptic excitation in the adult brain. In support of the idea that an Agrp→orexin inhibitory circuitry is operational during spatial exploration, suppression of natural Agrp activity increased spatial avoidance in an orexin receptor-dependent manner (Fig. 5). Overall, these results are consistent with models in which exploration is guided towards reducing orexin signals, and reveal a local circuit that may limit this orexin-dependent avoidance drive.

It should be noted that our conclusions only apply to neural populations that were naturally active during the behaviours studied here. It would be thus unwarranted

to state, based on the present data, that our conclusions are generally valid for the entire populations of orexin or Agrp neurons. This is because the silencing tools (ArchT, hM4Di, orexin receptor antagonist) manipulate only those neural signals that are naturally active at the time of the experiment (due to the fact that an absent neural signal cannot be silenced). This activity-dependence of silencing means that it probes neural populations that may be different from those activated when neural stimulators (e.g. ChR2, hM3Dq, exogenously applied orexin peptide) are used in an activity-independent manner. It should also not be assumed that the neural activity studied here during self-paced behaviour comes from the same orexin neurons that are activated by external stimuli or by reward-seeking (Harris *et al.* 2005; Gonzalez *et al.* 2016a; Hassani *et al.* 2016). Therefore, our findings do not necessarily contradict studies that propose different motivation properties for orexin cells – they may study different cells and/or the same cells at different activity states.

Nevertheless, our findings have implications for understanding motivational properties of orexin signals. In

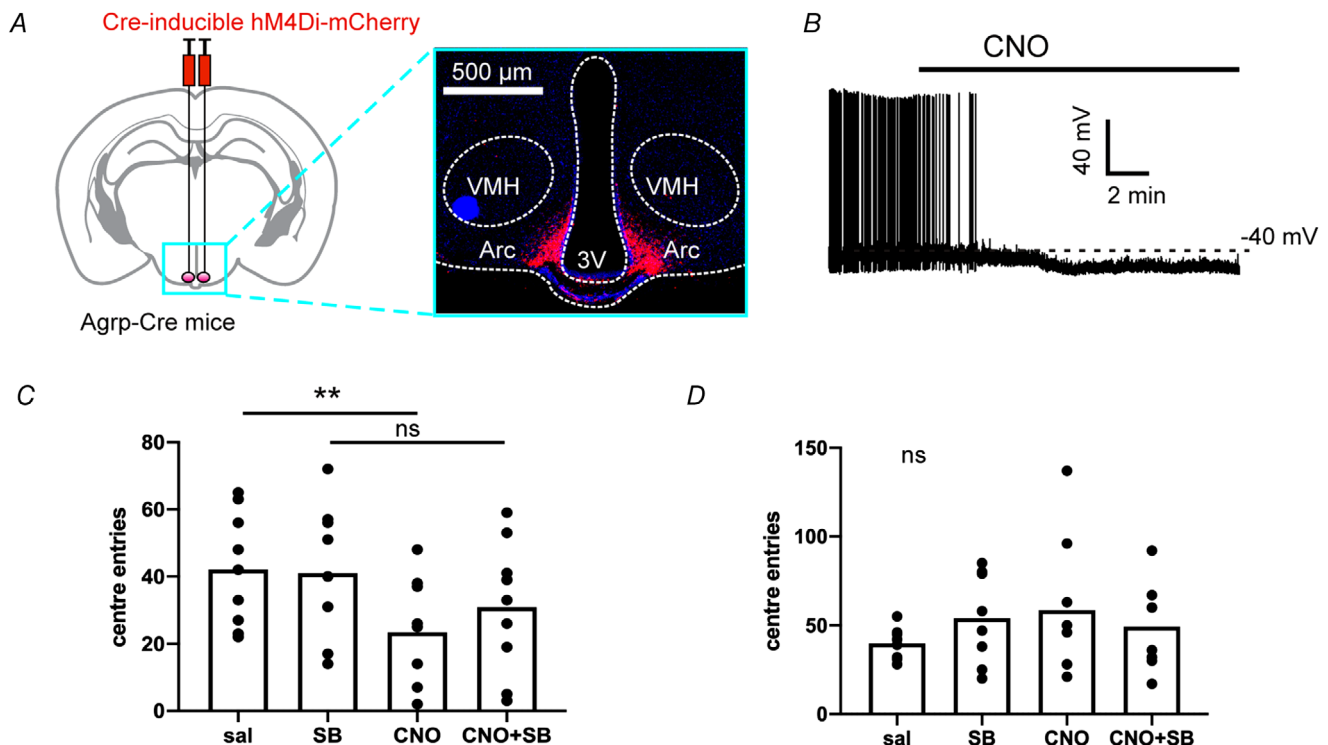


Figure 5. Effect of Agrp cell and/or orexin receptor inhibition on avoidance

A, left: targeting hM4Di-mCherry to Agrp cells. Right: expression of hM4Di-mCherry, representative example of 5 brains. Arc = hypothalamic arcuate nucleus, 3V = third ventricle, VMH = hypothalamic ventromedial nucleus. *B*, patch-clamp recording from an Agrp-hM4Di cell, confirming electrical silencing by CNO ($n = 5$ cells). *C*, number of centre entries (same centre-border experiment as in Fig. 2C) in Agrp-hM3Di mice ($n = 9$ mice; plot shows individual points and means; $**P = 0.0028$, $ns = P = 0.1628$ from Tukey's *post hoc* tests of RM one-way ANOVA, $F(3,24) = 7.173$, $P = 0.0013$), in the presence of CNO and the orexin receptor antagonist SB. *D*, number of centre entries (same centre-border experiment as in Fig. 2C) in Agrp-ChR2 control mice ($n = 8$ mice; plot shows individual points and means; $ns = P = 0.4674$ from RM one-way ANOVA, $F(3,21) = 0.8798$), in the presence of CNO and the orexin receptor antagonist SB. [Colour figure can be viewed at wileyonlinelibrary.com]

frameworks concerned with motivational characteristics of neural activity, a popular goal has been to assign a positive or negative valence to specific neural signals, i.e. to determine whether they drive attraction or avoidance (Tye, 2018). The real-time place preference test performed in Fig. 1 is a popular basis for such valence assignment (Kim *et al.* 2013; Namburi *et al.* 2016), since it clearly shows whether the animal prefers a place associated with particular neural activity. In our case, mice were attracted to the place where orexin cells were silenced, consistent with a view that mice avoid orexin signals (Fig. 1). The same conclusion was recently reached in optogenetic stimulation experiments, where mice were found to avoid places associated with orexin cells stimulation (Giardino *et al.* 2018). Earlier literature also concluded that orexin signals are anxiogenic or aversive, based on the tendency of rodents to 'hide' in dark or sheltered places when orexin receptors are stimulated (Suzuki *et al.* 2005; Burdakov, 2020), as also suggested by our silencing data (Fig. 2). The exploration-associated orexin signals studied here could therefore be viewed as aversive (negative valence). However, it cannot be ruled out that orexin signals may generate positive motivational states under different conditions. Indeed, it has been argued that orexin has the capacity to activate positive valence systems, based on findings of orexin-induced stimulation of dopamine systems (Korotkova *et al.* 2003; Borgland *et al.* 2008). However, it was subsequently found that some dopamine circuits can signal aversion (Lammel *et al.* 2012), and to the best of our knowledge it has not been shown whether orexin activates aversive or rewarding dopamine circuits. Increased orexin signals are observed during reward-seeking (Harris *et al.* 2005). Given that reward-seeking is often a response to reward deficit or stress, and that diverse stresses activate the orexin system, an alternative explanation could be that orexin signals represent reward deficit, i.e. an aversive state (Boutrel *et al.* 2005; Burdakov, 2019). From this perspective, reward cues (for drugs, food, etc.) can be viewed as creating an aversive state of 'reward hunger' that animals seek to avoid by identifying and consuming rewards (see Boutrel *et al.* 2005).

Our findings also have implications for understanding intrahypothalamic computations of context-appropriate behaviour. The reciprocal connectivity between orexin and arcuate NPY/AgRP neurons has been noted anatomically over 20 years ago (Hahn *et al.* 1998; Horvath *et al.* 1999a). Already in this early work, orexin was speculated to excite the NPY/AgRP neurons (subsequently confirmed electrophysiologically by van den Top *et al.* 2004), while AgRP/NPY cells were predicted to provide negative feedback control of orexin cells (Horvath *et al.* 1999a). Our results (Fig. 5) now provide functional circuit confirmation of the latter prediction. As expected from this inhibitory connectivity, chemogenetic inhibition of

AgRP cells activity increased innate avoidance behaviour (Fig. 5), consistent with disinhibition of orexin neurons. AgRP cell activity can acquire positive or negative valence characteristics after associative learning (Betley *et al.* 2015; Chen *et al.* 2016), and leads to inhibition of multiple neural targets, including negative valence CGRP neurons in the parabrachial nucleus (Campos *et al.* 2016, 2018). Therefore, our finding that AgRP neurons inhibit the negative-valence-like orexin signals do not necessarily contradict earlier conclusions on the valence of AgRP cell activity. We speculate that the inhibitory AgRP→orexin circuit may provide a negative feedback to orexin neurons during physiological states that activate AgRP cells (such as hunger), to reduce place avoidance when unrestricted exploration can favour survival.

Overall, our results elucidate motivational properties and an upstream control circuit of natural orexin signals during a specific ethologically relevant behaviour. These results are consistent with models in which spatial exploration is guided towards reducing exploration-associated orexin signals, and suggest a circuit logic for fine-tuning orexin signals to changing ethological priorities.

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Additional information

Data availability statement

All relevant data are available from the authors upon reasonable request.

Competing interests

None.

Author contributions

C.G. and C.B. performed the experiments and analysed data, D.B. and C.G. wrote the paper. All authors have approved the final version of the manuscript and agree to be accountable for all aspects of the work. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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Keywords

Agrp, behaviour, electrophysiology, exploration, hypothalamus, optogenetics, orexin

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Statistical Summary Document