Pupil fluctuations track rapid changes in adrenergic and cholinergic activity in cortex

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Rapid variations in cortical state during wakefulness have a strong influence on neural and behavioural responses and are tightly coupled to changes in pupil size across species. However, the physiological processes linking cortical state and pupil variations are largely unknown. Here we demonstrate that these rapid variations, during both quiet waking and locomotion, are highly correlated with fluctuations in the activity of corticopetal noradrenergic and cholinergic projections. Rapid dilations of the pupil are tightly associated with phasic activity in noradrenergic axons, whereas longer-lasting dilations of the pupil, such as during locomotion, are accompanied by sustained activity in cholinergic axons. Thus, the pupil can be used to sensitively track the activity in multiple neuromodulatory transmitter systems as they control the state of the waking brain.
Fifty years of pupillometry in humans and nonhuman primates support the view that in addition to changes in luminance and accommodation, spontaneous fluctuations in pupil diameter track changes in alertness, attention and mental effort1–3. In addition, rapid fluctuations in pupil diameter are highly correlated with alterations in electrophysiologically measured brain states, neural responsiveness and behavioural performance4–7. The precise pathways by which these alterations in brain state are coupled to pupil size are unknown. Pupil size has been widely assumed to be a reliable indicator of activity in the locus coeruleus (LC)8,9 and cortical state is powerfully controlled by the release of acetylcholine (ACh)10–12 and norepinephrine (NE)13–15. Here we studied the relationship of activity in cholinergic or noradrenergic axons in the neocortex in relation to spontaneous alterations in arousal (pupil diameter) and locomotion. We find that activity in noradrenergic projections in cortex tracks the phasic changes in pupil diameter better than the cholinergic terminals. On the other hand, cholinergic projections showed more tonic activation when the pupil was dilated for a longer time such as during locomotion.

Results

GCaMP6s imaging of cholinergic and noradrenergic projections.

We used two-photon microscopy to directly measure activity in ACh and NE projections to layer 1 (L1) of mouse cortex while tracking pupillary fluctuations (Fig. 1). To do so, we imaged GCaMP6s fluorescence in axonal projections in primary visual (V1) and auditory (A1) cortex, originating from choline acetyltransferase (ChAT)-expressing (cholinergic) neurons in the basal forebrain (n = 73 recordings from 25 imaging sites in six mice) or dopamine beta-hydroxylase (DBH)-expressing (noradrenergic) axons in V1 from neurons in the LC (n = 66 recordings from 18 sites in three mice; see Methods). Activity in NE and ACh projections in L1 of awakeful mice was highly coherent with pupil fluctuations, particularly at frequencies between 0.03 and 0.4 Hz for noradrenergic axons and <0.03 Hz for cholinergic axons (Fig. 1h and Supplementary Fig. 1C,D; example traces, Fig. 1d–h and Supplementary Figs 2–4). Consistent with this observation, electrical stimulation of the LC, but not adjacent brain areas, resulted in large, rapid dilation of the pupil after a 1.1 ± 0.1 s lag (Fig. 1C; N = 3 animals). Changes in pupil diameter, mediated by constriction and relaxation of smooth muscles, are well known to be lagged in response to neural activity in the sympathetic and parasympathetic pathways controlling these muscles16.

ACh and NE activity during fluctuations in pupil size.

During stillness both cholinergic and noradrenergic axonal activity was elevated while the pupil was dilating, and was reduced during constriction. NE activity levels were larger and shorter latency than ACh preceding the peak of a dilation (Fig. 2a–c and Supplementary Figs 2–4). Both ACh and NE activity showed a large, seconds-long peak in cross-correlation with pupil, but only NE activity showed a large peak in cross-correlation to the time derivative of the pupil (Fig. 2d,e). The time of the peak cross-correlation with pupil occurred earlier for NE than for ACh activity (P < 10−9), and both preceded pupil dilation (Fig. 2f, left; NE, lag = 0.98 ± 0.04 s, P = 10−16, n = 75; ACh, lag = 0.52 ± 0.13 s, P = 10−16, n = 140). Both neuromodulators were well correlated with pupil diameter during stillness (non-locomotion) (Fig. 2f, right). ACh activity was more correlated to pupil diameter than was NE, and both neuromodulators were more correlated to pupil diameter than were inactive auto-fluorescent blebs, which were not correlated with pupillary changes (see Methods). The reverse pattern was observed for the correlation to the time derivative of the pupil, which was large for NE and only slightly (but significantly) more correlated for ACh than for blebs (Fig. 2e, right, Supplementary Figs 5–8).

The activity of both ACh and NE projections increased along with pupil diameter before the onset of walking (Fig. 3). After walking onset, NE activity began to decay whereas ACh activation, and a large pupil diameter, were sustained throughout the walking period in both cortical areas V1 and A1 (Fig. 3a,b, left and Fig. 3d; Supplementary Fig. 8c). The offset of locomotion recapitulated this pattern, with a slow decay of ACh activity that tracked the slow decay of the pupil, and a fast decay in the remaining NE activity (Fig. 3a,b, right). Monitoring of fluorescent blebs showed no walking-related modulation, indicating that the imaging plane was stable during walking (Fig. 3c). Consistent with this pattern, ACh (but not NE) activity was correlated to pupil during walking (Supplementary Fig. 8a). NE activity remained correlated to the pupil derivative during walking, suggesting that small and rapid changes in the pupil diameter during walking reflect NE activity, whereas, the pronounced long-lasting dilation around walking tracks ACh (Supplementary Fig. 8b,c).

Discussion

The waking state is associated with rapid variations that can strongly influence neural representations and behavioural responses. Remarkably, these rapid variations in state can be tightly tracked by changes in pupil diameter4–7. Through what neural mechanisms might brain state and pupil diameter be coordinated? The present study provides evidence for activity in cholinergic and noradrenergic projections underlying these rapid variations in cortical state, and provides a mechanistic foundation for the use of pupillometry in awake behaving animals. More generally, our results indicate that ACh and NE are not just regulators of slow changes in wakefulness and arousal, but are recruited on a timescale relevant to moment-to-moment fluctuations that track behavioural events, and that these rapid variations can be partially monitored through pupillometry.

For decades, brain state has been tracked by both changes in electrophysiological parameters19,20, and by changes in diameter of the pupil21–23. Human and animal studies demonstrate that non-luminance and non-accommodation changes in pupil diameter are related to a wide variety of mental and emotional factors, including arousal, attention, stress and cognitive load, and reveal a tight coupling between the state of the central and peripheral nervous systems19,21–40. At the network level, active behaviours such as locomotion and whisking are associated with a reduction in low-frequency rhythmic cortical activity5,41–46. Even in the absence of overt movement, increases in pupil diameter (dilation) are associated

CNIFER imaging of ACh and NE release in V1. To confirm the release of ACh and NE in relation to periods of locomotion, we performed two-photon imaging of HEK-293 cells overexpressing either muscarinic ACh (M1) or 21α NE receptors and a genetically encoded calcium indicator (CNIFERs17,18) injected into the supragranular layers of V1. These cells change their fluorescence in response to changes in the extracellular concentration of the neurotransmitter for which they express the receptor (for example, ACh and NE), although the response time of these fluorescent indicators is slow (seconds17,18) and therefore do not permit the examination of rapid changes in transmitter concentration. Bouts of walking were associated with marked increases in the activation of CNIFERs containing either M1-muscarinic or alpha1-noradrenergic receptors (n = 113 sites in 12 animals), confirming that both ACh and NE were released during the axonal activity around locomotion (Supplementary Fig. 9).
with increases in cortical activation and suppression of low-frequency rhythms. Likewise, low-frequency cortical activity is enhanced during pupillary constriction, especially below a critical level of pupil diameter. This striking relationship between changes in pupil diameter and cortical network activity, either at the local field potential level\textsuperscript{7} or membrane potential level\textsuperscript{5,6}, has been observed throughout broad regions of the cortex. Indeed, there is even a strong correlation between pupil diameter and the rate of sharp-wave ripples in the hippocampus\textsuperscript{6}, further emphasizing the generality of the relationship between pupil diameter and brain state.

What are the neural pathways that couple brain state and pupil diameter together? A wide variety of neuromodulatory pathways have been implicated in the neural control of brain state (reviewed in refs \textsuperscript{47,48}). Two of these are the LC, which provides the source of noradrenergic innervation, and the basal forebrain, which is the source of cholinergic innervation to the cortex\textsuperscript{49}. These neurotransmitters can modulate the state of cortical activity through cell type-specific and subcellular mechanisms\textsuperscript{5,6,50–51}. Both cholinergic and noradrenergic neurons show graded and transient increases in firing in relation to increased attention to external stimuli, arousal and locomotion\textsuperscript{13,48,52,53}. Stimulation of the LC, but not immediately adjacent tissue, results in a large, rapid dilation of the pupil. Activity in NE axons precedes small, rapid pupil dilations during stillness. Activity in ACh axons also tracks rapid pupil dilations during stillness, but to a lesser extent. At the beginning of walking, strong NE activity occurs along with pupil dilation. ACh activity tracks the large, long-lasting dilation of the pupil that occurs around walking. NE activity is coherent with fluctuations in the pupil over a broad range of infra-slow frequencies (blue). ACh activity is also coherent with pupil, particularly at the lowest frequencies, such as occur around walking (orange). Error bands represent 68\% bootstrap confidence interval.

Figure 1 | The pupil tracks rapid fluctuations in ACh and NE cortical projections. (a, left) ChAT projections from BF (orange) and DBH projections from LC (blue). (right) A GCaMP6s-expressing axon traversing long distances in layer 1 of V1. (b) Simultaneous recording of (clockwise from upper left) treadmill velocity, pupil size, CNIFERs (see text) and axonal calcium activity. (c) Stimulation of the LC, but not immediately adjacent tissue, results in a large, rapid dilation of the pupil. Activity in NE axons precedes small, rapid pupil dilations during stillness. Activity in ACh axons also tracks rapid pupil dilations during stillness, but to a lesser extent. At the beginning of walking, strong NE activity occurs along with pupil dilation. ACh activity tracks the large, long-lasting dilation of the pupil that occurs around walking. NE activity is coherent with fluctuations in the pupil over a broad range of infra-slow frequencies (blue). ACh activity is also coherent with pupil, particularly at the lowest frequencies, such as occur around walking (orange). Error bands represent 68\% bootstrap confidence interval.
have observed a tight relationship between movement and basal forebrain cholinergic activity, which is also found when monitoring the activity of brainstem cholinergic nuclei. This raises the question—are the changes in cholinergic activity related simply to arousal or do they represent pre-motor planning, or both? Movement and arousal are intimately linked. Here we observed that periods of pupil dilation that were not associated with walking are often associated with increased activity in both cholinergic and noradrenergic fibres in the neocortex, although it is possible that smaller body movements (for example, postural adjustments, whisker movements and so on) may have occurred without locomotion.

Interestingly, although we observed increases in arousal and locomotion to be associated with increased activity in both cholinergic and noradrenergic pathways, we found that activity in cholinergic pathways more closely matched locomotion throughout the period of walking, while noradrenergic axon activity followed more closely the moment-to-moment fluctuations in pupil dilation, during both quiet rest and locomotion (Figs 2 and 3). These results suggest that the ascending cholinergic and noradrenergic pathways make unique, but overlapping, contributions to the control of cortical networks. Revealing the precise consequences, at the cellular and circuit level, of increased release of NE and ACh will reveal the role of these neuromodulatory pathways in the ascending control of brain state and whether this control is more closely associated with arousal or movement.

**Methods**

**Animals and surgery.** All procedures were carried out in accordance with the ethical guidelines of the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee (IACUC) of Baylor College of Medicine, Yale University and Columbia University. In this study, we used a total of 21 mice, including male and female. Twelve animals were injected with CNiFERS (see below), including one animal injected with z1a alone, six animals injected with M1 alone, and five animals injected with both z1a and M1. Several of these mice were Sst-Cre/Ai9 (n = 6) or PV-Cre/Ai9 (n = 2) crosses on a C57Bl/6 background. For imaging of ChAT and DBH axons, we used six ChAT-Cre (Jackson labs strain B6:129S6-Chattm1(cre)Walt/J) and three DBH-Cre mice (MMRRC Stock#016718-UCD B6.FVB(Cg)-Tg(Dbh-cre) K21J2Gsat/Mmucd). Expression of GCaMP6s in cholinergic or adrenergic neurons was achieved either via viral injection (four mice) of floxed AAV-GCaMP6s, U Penn Vector Core or AAVS-CAG-DIO-GCaMP6s, UNC Vector Core or reporter expression of GCaMP6s (five mice; B6:129S6-Gf(ROSA)26Sortm938f05778j/ reporter mouse, Jax strain number #024106).

Viral injections were performed stereotactically through a burr hole under isoflurane anaesthesia. Injections were targeted to the basal forebrain (ChAT-Cre mice, AAV1-Syn-FLEX-GCaMP6s), UNC Vector Core or AAVS-CAG-DIO-GCaMP6s) were performed similarly at coordinates 0.5 mm just above and below those used in a previous study to target the LC. Injections were performed 4–6 weeks before imaging to allow time for viral expression.

Cranial window surgeries over primary visual cortex were performed as described previously. Briefly, a 3 mm cranial window was opened under isoflurane anaesthesia and sealed with a 3 mm glass coverslip (Warner Instruments) and surgical glue (3M). In several experiments, the dura was removed before applying the coverslip to increase optical access to the cortex. In two mice used for imaging of ChAT axons, the craniotomy was made over primary auditory cortex instead of V1, and in two other mice, dual craniotomies were made over both primary visual and auditory cortical areas, so results were combined from both brain areas.

**LC stimulation.** Sprague-Dawley rats (n = 3) were anaesthetised with isoflurane. A small craniotomy was performed on the left hemisphere over the LC (stereotaxic coordinates: 3.1–3.7 mm caudal to Lambda, 1.2–1.4 mm lateral to the midline and 5.2–6.2 mm deep from brain surface). A single tungsten microelectrode (impedance: 1–2 MΩ) was slowly advanced into the LC using a hydraulic
Figure 3 | NE and ACh activity display different time courses around walking. (a-c) Mean calcium trace for NE axons (a), ACh axons (b) or blebs (c), aligned to the start (left) and end (right) of running. Pupil diameter (grey) and treadmill velocity (dark green) are plotted on the same time base. (d) A large increase in activity during the first second of walking is apparent in NE and ACh axons, but not blebs. During the last second of walking, NE activity has reduced significantly to near baseline, whereas ACh activity remains high. Error bands and bars are a 68% bootstrap confidence interval.

CNiFERs. In several experiments, HEK-293 cells expressing a calcium indicator and either cholinergic or noradrenergic receptors (CNiFERs) were injected in the cortex. CNiFER cells were plated from frozen stocks (gift of D. Kleinfeld) on 6 cm plates in 3 ml of standard media (high glucose DMEM with pyruvate, supplemented with 10% fetal bovine serum, 1 × non-essential amino acids, and 100 U ml⁻¹ penicillin/streptomycin; Life Technologies product numbers 11995065, 10437077, 11140050, 15140122). Cells were passaged or used for experiments when they were at or approaching confluence.

To prepare the cells for injection, media was removed and the plate was washed twice with PBS. Cells were dissociated without trypsinization by pipetting and resuspended in 1 ml ACSF (125 mM NaCl, 5 mM KCl, 10 mM Glucose, 10 mM HEPES, 2 mM CaCl₂, 2 mM MgSO₄). Aggregates were removed by passing the cells through a pipette-tip cell strainer (40 μm) into a 1.5 ml Eppendorf tube. The cells were then pelleted by lightly spinning the tubes for 5 minutes at 100 g. The supernatant was removed, leaving the cells resuspended in a thick slurry (50–100 μl). 0.5 μl of 1 mM Alexa 568 was added to the cells to facilitate visualization during injection under two-photon microscopy, and they were back-filled into a glass pipette with the tip broken to a diameter of ~30 μm.

Images of the eye were recorded at 1,280 × 1,024 at 10 Hz (DCC1545M camera, Thorlabs, with TML-HP 1 × Telecentric lens, Edmund Optics). In some experiments, the eye was illuminated with a 720 nm light-emitting diode (ThorLabs), but in most cases, the infrared light transmitted from the pupil during two-photon imaging was sufficient. A moderate level of ambient illumination was maintained either by a grey screen presented on a 7” liquid crystal display monitor (Lilliput 665GL-70NP/HO/Y monitor; 60 Hz scan rate positioned 10 cm away from the eye, covering ~88° (azimuth) by 72° (elevation) of the contralateral visual field, or via an ultraviolet light-emitting diode (380 nm ± 20 nm, full-width at half-maximum). Post-hoc pupil segmentation was performed semi-automatically with custom MATLAB software as described previously.

Imaging. Two-photon imaging was performed with a fast resonant scanning system (ThorLabs) mounted on a Sutter objective manipulator. The imaging frame rate was 30–60 Hz. Excitation was via a Ti:sapphire laser (Chameleon Vision,
Coherent) tuned to either 800 nm (CNIfeRs) or 920 nm (GCaMP6s) with either a 26 × (0.8 NA, Nikon) or 25 × (1.1 NA, Nikon) objective. Power out of the objective was controlled by calibrated rotations of a half-wave attenuator and depended on the magnification of the scan but was typically 20–40 mW. We used ScanImage (Vidrio) to control the imaging system, and custom Labview software to acquire treadmill activity and pupil movies synchronized with the imaging scans.

Preprocessing of calcium imaging data. Imaging data was motion corrected and rasterized (Etienne et al., 2015) from video recordings with the ScanImage (Vidrio) software. Images were processed with custom MATLAB scripts. Fluorescence was quantified using the ImageJ (NIH) plugin FIJI (Johannes Schindelin et al., 2012), and traces were binned by the Hilbert phase of the filtered (0.1 to 1 Hz) calcium traces. For registration to a standard dilation/constriction cycle, the filtered fluorescence (datajoint-matlab) was used. We excluded from analysis and periods of dilation and constriction that were identified in the monkey somatosensory cortex revealed by pupilometrics. Neuron. 25, 173–181 (1996).

Activity around running onset and offset. Locomotion epochs were identified as described above, and normalized traces around the onset or offset of running for each axon or CNIfeR ROI were averaged as described above for dilation and constriction. For axon traces, locomotion periods were only used if they were preceded (onset) or followed (offset) by at least 10 s of quiet wakefulness. For CNIfeR PSTHs, locomotion periods were included only if they were preceded (followed) by at least 30 s of quiet wakefulness (Fig. 3 and Supplementary Figs 8 and 9).

Data availability. The data that support the findings of this study are available from the corresponding authors upon request.

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**Acknowledgements**

This work was supported by NIH grants DP1OD008301 and DP1EY023176 (A.S.T.) and 5R01NS26143 and the Kavli Institute for Neuroscience at Yale (D.A.M.), F32 DC012449 (M.J.M.) and P30EY002520. This work was also supported by the Intelligence Advanced Research Projects Activity (IARPA) via Department of Interior/Department of Justice, Office of Science and Technology (DoI/IBC) contract number D16PC00003. The US Government is authorized to reproduce and distribute reprints for Governmental purposes notwithstanding any copyright annotation thereon. The views and conclusions contained herein are those of the authors and should not be interpreted as necessarily representing the official policies or endorsements, either expressed or implied, of IARPA, DoI/IBC or the US Government. We thank David Kleinfeld (UCSD) and Paul Slesinger (Mount Sinai) for providing a2, M1, and control CNI95 and discussions that provided insights into their use and function, Russell Ray (BCM) for the DBH-Cre mouse line, and Dimitri Yatsenko and Saumil Patel for software used in this study.

**Author contributions**

J.R., A.S.T., M.J.M. and D.A.M. designed the study; J.R., M.J.M., Y.L. W. and C.R. performed the experiments; J.R. and M.J.M. analysed the data; and J.R., A.S.T., M.J.M. and D.A.M. wrote the manuscript.

**Additional information**

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

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How to cite this article: Reimer, J. et al. Pupil fluctuations track rapid changes in adrenergic and cholinergic activity in cortex. *Nat. Commun.* **7**, 13289 doi: 10.1038/ncomms13289 (2016).

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