Astrocytes integrate local sensory and brain-wide neuromodulatory signals

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Supplementary Information

Online Methods

Animals

All animal protocols were approved by the Ethical Committee of the University of Leuven (KU Leuven, Belgium). The *Fgfr3-iCreER^{T2}* transgenic line[1] was obtained from William Richardson (University College London). Genotyping was performed by PCR using primers iCre250 (5'-GAGGGACTACCTCCTGTACC-3') and iCre880 (5'-TGCCCAGAGTCATCCTTGGC-3'). Adult male animals were eight weeks of age or older at the time of experiments.

Surgical Procedures

Anesthesia: For all surgical procedures, mice were administered dexamethasone (8 mg/kg) four hours prior to surgery. Procedures were performed using isoflurane anesthesia (induction 2.5 %, 1 l/min O_2; maintenance 1-1.5%, 0.6 l/min O_2). Animals were placed on a heating pad set to 37°C and the depth of anesthesia was checked using the toe pinch reflex.

Insertion of a cranial window: The procedure for insertion of a removable cranial window into the skull of a mouse was described previously[2]. Briefly, skin was removed from the skull with a surgical scalpel, and the temporalis muscle was separated from the skull. A custom-made titanium head plate, centred on the posterior left hemisphere, was attached to the skull using cyanoacrylic glue. Exposed muscles and skull were covered with Vetbond (3M) and dental cement (Metabond, C&B). A 5 mm craniotomy was performed, centred on the left primary visual cortex (1.6 mm anterior from lambda, 3.1 mm lateral from midline). Optical windows consisting of one 8 mm and two 5 mm coverslips, glued together with optical glue (NOA71, Norland), were fixed to the skull with dental cement (Kerr Tab 2000, KemDent). The cement also contained black pigment to block ambient light from the multiphoton microscope. Two neoprene o-rings were attached to the head-

plate, to form a well to hold distilled water during imaging. After surgery, mice were moved to a home cage and allowed to recover. All mice received post-operative treatment for 60 hours to minimize pain and prevent infection. Buprenorphine (0.2 mg/kg) and cefazolin (15 mg/kg) were supplied via intramuscular injection at 12 hour intervals for 2.5 consecutive days. Trimethoprim (0.1 mg/ml) and sulfamethoxazole (0.5 mg/ml) were supplied in the drinking water for a maximum of 10 days post-surgery. Training started five days after window implantation.

Mapping of primary visual cortex: To determine the precise position of primary visual cortex in each mouse, prior to AAV injection, retinotopic mapping was performed, based on intrinsic imaging of flavoproteins across the entire region visible through the cranial window[3]. We used a blue LED (470 nm, Thorlabs) for excitation and collected the emitted green light (510/84 nm filter, Semrock) using a 4x widefield lens (NA = 0.055, Edmund Optics) and EMCCD camera (EM-C2, QImaging; 1,004 by 1,002 pixels with 4 by 4 binning), at a rate of 10 frames per second (fps). To elicit visual responses, stimuli consisting of square-wave moving gratings (spatial frequency = 0.08 cycles per degree, temporal frequency = 4 Hz, horizontal and vertical orientations moving in four cardinal directions) were presented at six retinotopic locations on a calibrated 22-inch LCD monitor (Samsung 2233RZ, 1,680 by 1,050 pixels resolution, 60 Hz refresh rate, average luminance 59 cd/m^2). The screen was positioned 18 cm in front of the right eye. The visual field covered from 0 to 120 degrees central to peripheral field and ± 40 degrees lower to upper field. Stimuli were presented in a 2-by-3 matrix from the upper-central to bottom-peripheral right visual hemifield. Each stimulus lasted 8 s and stimuli were interleaved by equally-timed 'blanks' (50% luminance), followed by a seventh 'blank' phase of 8 s duration. This cycle was repeated 10 times. Fractional changes in fluorescence were normalized to baseline and averaged across 4 s intervals to capture the slow time course of the flavoprotein signal. The location of V1 was identified by using the (negative) flavoprotein signals and their spatial relationship to higher visual areas.

Viral vector administration and induction of GCaMP6m expression: For injection of an adeno-associated virus-based vector (AAV2/5) carrying a tamoxifen inducible version of GCaMP6m[4] driven by a CAG promoter (University of Pennsylvania Vector Core), mice were anesthetized (as

described above) and the coverslip was removed from the skull using an air-driven dental drill[2]. Vector was administered through bevelled glass capillaries (Drummond Science) with a tip diameter of 20 - 40 µm. Three to four injections were performed along the border of V1 to maximize the coverage of the area. Each injection used a total vector volume of 100 - 300 nl (titre approximately 8.6 x 10^{12} vector genomes/ml), at depths of 120 - 300 µm, using a microliter injection system (Nanoject II, Drummond Science). Injection speed was limited to 60 nl/min. To monitor injections, Alexa-563 was added to the solution. Following injections, capillaries were removed and the skull sealed with a fresh cranial window. Mice were allowed to recover as described.

Tamoxifen administration. After recovery, mice were administered tamoxifen (2 mg in a mixture 1 part ethanol - 9 parts sunflower oil; Sigma) by intraperitoneal injection (100 µl) for 5 consecutive days[5]. GCaMP6 expression was monitored under a widefield microscope and imaging started approximately one week after the last tamoxifen injection.

Behavioral Training and Treadmill Assay

During training and experiments, mice were kept on a water restriction schedule (1 ml free access per day), in order to use a water reward to facilitate spontaneous locomotion. Mice were first manually handled for 3 - 5 days and habituated to head-fixed treadmill locomotion. In case of signs of distress, habituation and the following training session were terminated. The treadmill equipment consisted of a 150 cm long, 5 cm wide belt of Velcro (Country Brook), mounted on two 10 cm diameter wheels, supported by a custom frame (Thorlabs)[6, 7]. Treadmill rotation was monitored with a resolution of 3.14 mm by a rotary encoder (Avago Tech), attached to the treadmill shaft. Once per lap, a photoelectric sensor (Omron) detected a reflective strip attached to the underside of the belt. This event triggered the opening of an electromagnetic pinch valve (MSscientific), leading to delivery of a drop of tap water, or 10% sucrose solution, through a spout that was accessible to the animal. Signals from the rotary encoder and photoelectric sensor were collected by a custom circuit board with a microcontroller (AT89LP52, Atmel), which linked

treadmill position to valve opening. All signals were acquired by a personal computer via a USB data acquisition board (MCC), sampled at 10 kHz and recorded with Presentation software (Neurobehavioral Systems). The duration of training increased gradually from a few minutes per day up to 1 hour per day, over a period of 2 - 3 weeks, to ensure that mice did not show signs of discomfort during spontaneous locomotion. Training was completed when animals reached the desired level of locomotor activity – typically 3 laps of the treadmill per minute. Animal training continued during the experimental phase, with at least two sessions per week, on days when experiments were not being performed.

Calcium Imaging

Visual stimulation: Presented stimuli were based on a 12 degree-wide bar in an alternating (6 Hz) black and white checkerboard pattern (with 0-100% contrast). Stimuli were moved across the screen in one of the four cardinal directions, at a speed of 6 degrees/s, such that a stimulus travelled across the display for approximately 20 s in the horizontal direction, or 16 s in the vertical direction. In experiments investigating the impact of altering the speed of bar movement on visually-evoked responses, the stimulus travelled at either 6, 12 or 24 degrees/s. These checkerboard stimuli were interspersed by regular 'blanks', consisting of a whole grey screen (10-30 s at 50% luminance). Stimuli and blanks presented in random order elicited identical results. One session typically consisted of 10 - 20 repetitions of a set of 4 visual stimuli with blank. Presentations of visual stimuli were synchronized to image acquisition in 1-photon (10 fps) or 2-photon (30 fps) modes, by using a trigger from the EMCCD camera (1-photon), or slow-axis galvanometer scan pulses (2-photon), controlled by Presentation software.

1-photon imaging: GCaMP6 fluorescence in 1-photon imaging mode was monitored using the same conditions as for flavoprotein imaging.

2-photon imaging: 2-photon imaging was performed at a depth of 100 to 300 μm below the pial surface, using a custom-built 2-photon microscope (Neurolabware). 2-photon excitation light (920 nm) came from a MaiTai DeepSee laser with group-delay dispersion (Spectra Physics). Laser

scanning was controlled by galvo and resonant scanners (Cambridge 6215H and CRS 8K) through a 16× lens (NA = 0.8, Nikon). Fluorescence from GCaMP6 was collected using a band-pass filter (510/84 nm, Semrock) and a GaAsP photomultiplier tube (Hamamatsu). Images were collected at a rate of 30 fps (1,154 by 512 pixels, 620 by 380 μm field-of-view). The power of light entering the tissue was kept to the minimum needed to ensure reliable data collection and was usually 20 to 60 mW, depending on imaging depth. To block stray light from the visual display, an isolating shield, covered with blackout material (Thorlabs), was used to surround the neoprene o-rings, the objective and imaging chamber, as well as the photomultiplier tubes.

Behavior tracking: Locomotor behavior was tracked as described above.

Pupil tracking: Pupil size was measured using an infrared eye tracking camera, placed in front of the right eye. Infrared light was focused onto the eye with a far-red LED (735 nm, Thorlabs) and collimated lens (Thorlabs). Data was acquired at 30 fps with a CCD camera (AVT Prosilica GC660; Navitar Zoom 6000 lens) and StreamPix software (Norpix). Images were subsequently analyzed using custom software.

Analysis of Noradrenaline Levels

To deplete noradrenaline levels in the cortex, the neurotoxin DSP-4 (N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine hydrochloride; Sigma) was administered using a single intraperitoneal injection (75 mg/kg DSP-4 in saline). A final session of imaging experiments was performed 72 hours post-injection. Upon completion of imaging, mice were sacrificed, brains removed and cortical hemispheres collected. Tissue was snap-frozen in liquid N_2 and stored at -80°C until use. Five saline-injected animals were used as controls. High-performance liquid chromatography (HPLC) was used to confirm reduction of cortical noradrenaline levels. On the day of HPLC experiments, samples were rapidly thawed, followed by homogenization in 9 volumes (w/v) ice-cold 200 mM perchloric acid, containing 3 mM cysteine and 0.25 mM EDTA.Na_2 (as described[8]). Homogenization was performed in polypropylene tubes, using a hand-held ultrasonic processor (UP50H, Hielscher). After centrifugation (15,000 x g_{Av} for 10 min), the resulting supernatant was

transferred to HPLC vials and 20 µl was injected onto a reversed phase column (Alltima HP C18 AQ, inner diameter 4.6 mm, length 250 mm, particle size 5 µm), equilibrated in running buffer (20 mM acetic acid, 0.25 mM EDTA)[9]. After 1 min, bound analytes were eluted using a linear gradient of methanol (0 - 40% (v/v) in running buffer) with a flow rate of 1 ml/min. Signals were detected fluorimetrically (Waters 2475 multi-wavelength fluorescence detector), using an excitation wavelength of 279 nm and an emission wavelength of 320 nm. Chromatograms were analyzed with Breeze 3.20 software (Waters). Standardization was done against known amounts of external noradrenaline (Sigma). Noradrenaline was typically eluted at ~2.5 ml (against a void volume of 2 ml). In some experiments, 0.05% (v/v) trifluoroacetic acid was added to both solvents, improving the retention (~2.8 ml) of noradrenaline, allowing cleaner separation from a faster eluting contaminant.

Data Analysis

All imaging data were analyzed in MATLAB (The Mathworks). All figures were prepared in Adobe Illustrator CS6.

Selection of regions of interest (ROIs): 1-photon time stacks (from the initial imaging session) were down-sampled in space by a factor of 10 and mean and standard deviation (s.d.) projected. 200 by 200 µm wide ROIs were defined based on the local maxima detected in projections obtained from V1. These identified ROIs were then used in subsequent experiments with the same animal.

2-photon images were corrected for lateral movement using TurboReg[10]. ROIs corresponding to individual astrocytes were outlined manually, based on a maximum intensity projection of the acquired time stack, which allowed clear cell morphology to be determined. The same ROIs were used in subsequent experiments with the same animal and the same field of view.

Calcium transients: Changes in intracellular Ca^{2+} levels were calculated by averaging pixel intensities over each ROI. Data is expressed as fractional changes above baseline fluorescence (dF/F0). Baseline fluorescence (F0) was computed by linear regression and was set as the lowest 10 % of signal intensity measured in the unprocessed fluorescence images obtained during a time course experiment. Measures of peak dF/F0 (for the calculation of cumulative amplitude distributions) were extracted from 25 s windows following the onset of visual stimulation or the onset of locomotion. An increase in intracellular Ca^{2+} was recorded when the peak dF/F0 was greater than 2.5 s.d. above background.

Down-sampling and trial averages: For illustrations, imaging time stacks were 10 x down-sampled in time and averaged across trials (i.e. visual stimulation and locomotion onset windows). For illustration purposes, traces showing Ca^{2+} activity and locomotion were filtered using a 5^{th} order zero-phase Butterworth low pass filter.

Locomotion: Readout from the treadmill encoder was obtained at time points relevant to the 1- or 2-photon imaging stacks. For analysis purposes, we considered locomotion to be activity lasting 1 s or more, with a speed equal to (or greater than) 5 cm/s. Bouts separated by periods of inactivity (stationarity) of less than 0.5 s were merged. Transitions from stationarity to locomotion were detected after periods of inactivity lasting 8 s or longer[11] and are defined as the onset of locomotion.

Pupil diameter: Pupil tracking time stacks were filtered using a Gaussian 2D function and subject to a contrast threshold. Resulting images were then binarized to produce black and white images. The pupil was detected by fitting an ellipsoid to the centermost object and the pupil diameter was then estimated from the diameter of the ellipsoid. Eye blink artefacts were removed from further analyses. Pupil diameters, during visual stimulation or locomotion, were expressed as changes relative to the average pupil diameter in the 5 s period immediately preceding stimulus presentation or locomotion onset. Changes in pupil diameter were extracted from images acquired 10 s post-stimulus onset or 10 s post-locomotion onset.

Response maps: Pixel-wise response maps were calculated from dF/F0 trial averages from 1- or 2-photon time stacks, aligned to the onset of visual stimulation or onset of locomotion. We

analyzed a response window containing the first 25 s of the recorded images. We then determined, for each pixel, the preferred time of response by expressing dF/F0 as vectors in a polar plane, in which magnitudes correspond to the dF/F0 of the calcium transient and angles the duration of the response window - and then calculating the angle of the vector sum. Obtained values were translated into HSV (hue, saturation, value) maps, in which hue corresponded to the time point of the preferred response and value to the overall activity calculated as mean x s.d. of GCaMP6 fluorescence. Saturation was set to one.

Correlation analyses: Pearson's correlation coefficients and p-values were calculated for the average locomotion speed during visual stimulation (0 - 25 s windows) and the peak dF/F0 measured (0 - 25 s windows), or the maximum change in pupil diameter after stimulus onset (0 - 10 s windows) and the peak dF/F0 (0 - 25 s windows).

Statistical analyses: Two-sample Kolmogorov-Smirnov tests were used to test whether the distributions of peak amplitudes and response half-widths were independent, as well as to calculate whether the results were statistically significant. HPLC data was tested using a two sample Student's t-test.

Reagent and Data Access

All reagents, analysis code and raw data are available upon request from the corresponding authors.

Supplementary References.

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Supplementary Figure Legends.

Supplementary Figure 1. Responses properties of astrocytes.

(a) Pseudo-colour, pixel-wise maps of trial averages (N) for GCaMP6 fluorescence obtained from nine animals with 1-photon imaging in response to locomotion onset (top) or visual stimuli (bottom) moving along the 4 cardinal directions (shown on left).

(b) Trial averaged images recorded using 2-photon microscopy, at various times following stimulus onset. Maps are the average of 10 trials per stimulus direction in one animal. Images were obtained from Supplementary Movie 3 for stimuli moving in opposing directions.

(c) Varying the speed of the visual stimulus affects the amplitude of Ca^{2+} transients elicited in astrocytes. Cumulative distribution plots showing the peak response elicited by visual stimuli moving across the display at indicated speeds (black lines, solid, 6 degree/s: dashed, 12 degree/s: dotted, 24 degree/s) or elicited by locomotion onset (green line). Data were obtained with 2-photon imaging. Red line: s.d. threshold.

(d) Cumulative distribution plots illustrating that response probability is correlated to the speed of the drifting visual stimulus.

(e) Ca^{2+} transients induced by locomotion and visual stimulation display similar kinetics. Cumulative distribution plots of data comparing locomotion-induced responses to those elicited by a stimulus moving across the screen at 6 degrees/s. Data was recorded in 1-photon mode (left). Cumulative distribution plots showing the half-widths of responses in individual astrocytes elicited by visual stimuli moving across the display at indicated speeds (black curves, solid, 6 degrees/s:

dashed, 12 degrees/s: dotted, 24 degrees/s) or locomotion onset (green curve). Data was obtained in 2-photon mode (right). N = number of events recorded.

Supplementary Figure 2. Sensory-induced Ca^{2+} responses are influenced by behavioural state.

Single trial recordings of Ca^{2+} transients elicited by visual stimuli moving in the 4 cardinal directions (top traces) during spontaneous locomotion (bottom traces). Orange curves indicate 'stationary' trials. The shift in visual responses during presentation of stimuli (grey box) reflects the location of the imaged area (blue ROI, left) relative to the retinotopic organization of V1.

Supplementary Figure 3. Effects of DSP-4 administration on visually evoked Ca^{2+} transients.

(a) Example traces from indicated ROIs (blue boxes) from two animals before and after systemic DSP-4 administration. Ca^{2+} transients (top traces) are shown for the period when mice are exposed to visual stimuli (grey boxes) during spontaneous locomotion (bottom traces). Traces are shown for stimuli moving in each of the four cardinal directions (left).

(b) Pseudo-colour, pixel-wise maps of trial averages for changes in GCaMP6 fluorescence. Results from four animals imaged in 1-photon mode. Retinotopic responses were retained after DSP-4 treatment.

Supplementary Movies

Supplementary Movie 1. Ca^{2+} responses at locomotion onset (bottom trace) recorded with 1-photon widefield imaging of the whole cranial window in an awake, behaving mouse. Horizontal scale bar = 1 s, vertical scale bar = 20 cm/s.

Supplementary Movie 2. Trial-averaged responses to visual stimuli moving in the 4 cardinal directions, as indicated in the inset, recorded with 1-photon widefield imaging of the whole cranial

window in an awake, behaving mouse (N = 10 trials per animal). In reality, the actual stimuli used were not solid bars but were flickering checkerboards.

Supplementary Movie 3. Trial-averaged responses to visual stimuli moving from 0-120 degrees (top) and from 120-0 degrees (bottom) recorded with 2-photon microscopy from V1 in an awake, behaving mouse (N = 10 trials). Insets show the movement of each visual stimulus. In reality, the actual stimuli used were flickering checkerboards.

Supplementary Movie 4. Trial-averaged responses to presentation of visual stimuli moving in the 4 cardinal directions, as indicated in the inset, recorded with 1-photon imaging in 4 animals before (top row) and 72h after (bottom row) intraperitoneal administration of DSP-4 (N = 10 trials per animal). In reality, the actual stimuli used were not solid bars but were flickering checkerboards.