Suppl. Figure 1: Examples of photomicrographs of brain slices from MCH-CFP mice taken using both fluorescence and light (transmission) microscopy

Along the ventral part of the third ventricle (3V), at the level of the lateral hypothalamus, fluorescent MCH fibers (arrow) reach the third ventricle (a, b), the basal membrane of the ciliated cell layer (c, d), or both (e, f). Few if any fluorescent fibers can be detected in the lateral ventricle (LV in g, h) or the dorsal part of the third ventricle (3Vd in i, j).
Suppl. Figure 2: MCH fibers are present in close apposition to the edge of the ventral part of the third ventricle.

Photomicrographs show the distribution of CFP-expressing MCH neurons along with immunoreactivity for vimentin and glu-tubulin in coronal sections from the tuberal region of the hypothalamus. a: low-magnification photomontage of CFP (green), glu-tubulin (red) and vimentin (white) immunofluorescence. b-e: High-magnification images showing CFP-containing fibers (green, white arrowheads) running close to the layer of ependymal cells (white) with glu-tubulin-immunoreactive cilia (red, white arrows), at the level of the ventromedial nucleus of the hypothalamus (VMH; b, c) and the dorsal part of the arcuate nucleus of the hypothalamus (ARH; d), but not the ME (e). Interestingly, the rare MCH-CFP fibers (green, arrowheads) running parallel to the ventricular wall appeared to selectively contact ciliated ependymal cells (arrows) (b, c, d). Note that glu-tubulin immunoreactivity was absent in vimentin-labeled tanycytes (empty arrows) of the ME, and that no MCH-CFP fibers appeared to contact their cell bodies (empty arrowhead) (e). However, MCH-CFP axon terminals were seen in the external zone of the median eminence, where tanycytic end-feet reside (asterisk) (e). LHA: lateral hypothalamic area; 3V, third ventricle. Scale bar = 100 μm, 10 μm in insets.
<table>
<thead>
<tr>
<th>Sex of the mice</th>
<th>MCH concentration (nmol. L(^{-1}))</th>
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<tbody>
<tr>
<td>Males (n=14)</td>
<td>1.49 ± 0.28</td>
</tr>
<tr>
<td>Females (n=5)</td>
<td>1.53 ± 0.51</td>
</tr>
<tr>
<td>Total (n=19)</td>
<td>1.50 ± 0.39</td>
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</table>

**Suppl. Table 1: MCH is detected at nanomolar concentrations in the CSF.**

For CSF sampling, mice (N=19) were anesthetized with sodium pentobarbital (70 mg.kg\(^{-1}\), i.p.) and CSF taken from the cisterna magna (Liu, L. & Duff, K. J. Vis. Exp. 21, 960 (2008)). The volume of CSF obtained from each mouse was 5-10 µl. MCH concentration was measured in 5 µl of CSF using an enzyme immunoassay kit detecting MCH (Phoenix Pharmaceuticals, Inc., Karlsruhe, Germanyin, 0-42 nmol.L\(^{-1}\) range). No significant difference was found between males and females (Mann Whitney test, U=32.5, \(P=0.85\)); data were therefore pooled. The results of our study are consistent with MCH levels in the CSF of ewes (Ungerfeld R. et al. Peptides 32, 2511-2513 (2011)).

**Suppl. Movie 1: Representative video recording of the CBF of ciliated ependymal cells in the third ventricle.**

The movie, recorded with a Leutron Picsight camera at a frame rate of 196 frames.s\(^{-1}\), is presented at a slowed speed of 20 frames.s\(^{-1}\).
Suppl. Figure 3: Effects of MCH, 5-HT and ATP on CBF on ciliated ependymal cells in the ventral third ventricle.

**a-** Normalized averaged (± s.e.m.) ependymal cell CBF responses to 5-HT and ATP as indicated by the bars.

**b-** Concentration-response curve (mean ± s.e.m.) of the effect of MCH on the CBF fitted to a Hill curve.

**c-** Lack of effect of MCH and the MCHR1 antagonist H6408 (250 µM) on the CBF in the MCHR1−/− mice.

(Number of slices in parenthesis, 1-3 slices per mouse)
Suppl. Figure 4: Electrophysiological recordings of ependymal cells from the ventral part of the third ventricle reveal that MCH activates Ca\textsuperscript{2+}-dependent K\textsuperscript{+} channels.

**a-** Photomicrograph showing the patch pipette sealed onto a ciliated ependymal cell. 3V: third ventricle.

**b-** Current obtained from a ciliated ependymal cell in response to potential ramps of -120 mV to 40 mV (0.056 mV.ms\textsuperscript{-1}) applied every 30 sec. under control conditions or in the presence of 1 µM MCH or 1 µM MCH + charybdotoxin (50 nM).

**c-** MCH-induced current obtained by subtraction of the control ramp from the 1 µM MCH ramp presented in b.

**d-** I/V curve of the mean (± S.E.M) MCH-induced current measured from ramps as in c for 5 different ciliated ependymal cells.

**e-** Time course of the small outward current induced by MCH application at a holding potential of -60 mV. This current was blocked by the application of charybdotoxin.

**f-** Time course of the current measured at 0 mV from ramps as in c (1 ramp every 30 sec). The kinetics of the current was similar to that in e.

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Suppl. Figure 5: Calcium imaging reveals that MCH induces Ca\(^{2+}\) elevations in ependymal cells from the ventral part of the third ventricle.

**a-** Representative image of brain slice fluorescence at 510 nm after Fura-2AM excitation at 340 and 380 nm, and the ratio of the two. The region of interest was chosen within the ciliated ependymal cell layer (black circle). A control area (white circle) was chosen in the third ventricle (3V) to determine background.

**b-** Plot against time of the ratio of the fluorescence obtained at 510 nm after Fura-2AM excitation at 340 and 380 nm, recorded at the level of ciliated ependymal cells bordering the lower part of the third ventricle (black circle in a) after subtraction of the background (white circle in a). The ratio was increased by MCH application (1 µM) to the medium (as indicated by the bar). H-6408 (250 nM) blocked this effect.

**c-** In another slice at the level of the ciliated ependymal cells bordering the lower part of the third ventricle, ATP (100 µM) and KCl (30 mM) applied to the bath (horizontal bars) induced an increase in the ratio of the fluorescence obtained at 510 nm after Fura-2AM excitation at 340 and 380 nm in areas corresponding to Fura-2AM-loaded ependymal cells.

**d-** Histogram showing the percentage variation of the fluorescence ratio obtained following various treatments. Changes in the ratio were calculated by subtracting the extrapolated baseline (obtained by fitting the local minimum values of the trace) from the peak values of the trace. The ΔF corresponded to a 9.0 ± 3.59% increase relative to the baseline ratio (N=11; df=10, t=2.519, P<0.05, unpaired t-test) for ATP and a 36.9 ± 7.1% increase (N=12; df=11, t=5.199, P<0.001, unpaired t-test; Fig 4c, d) for 30 mM KCl.

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Suppl. Figure 6: Acute intraperitoneal injections of DMSO alter CSF distribution and ventricular volume in the brain.

Brain-penetrating MCHR1 antagonists (such as DIVO1974) are poorly soluble in aqueous solution and need to be dissolved in solvents such as DMSO. Axial T$_2$-weighted brain images obtained from two control WT (MCHR1$^{+/+}$) mice before and 30 min after the i.p. injection of pure DMSO (100 µL/30 g body weight) or a solution of the MCHR1 antagonist DIVO1974 in DMSO show a hyperintense CSF. Note the enlargement of the lateral and third ventricles and the appearance of extraventricular CSF (dashed arrows) in both mice. Abbreviations: LV, lateral ventricles; 3V, third ventricle. Scale bar=1 mm.
Suppl. Figure 7: In vivo assessment of the ventricular system

a- Axial T2-weighted brain images from WT (MCHR1+/+), heterozygous (MCHR1+/–) and MCHR1 KO (MCHR1–/–) mice showing the whole ventricular system, and 3D surface-rendering of the ventricles. The reconstructed ventricular system represents a 3D surface mesh for each labeled ventricle; this explains the discontinuity between orange, red and green regions. Color coded-labels for the ventricular system: yellow, lateral ventricles; orange, 3V; red; Sylvian aqueduct; green; 4V. LV, lateral ventricles; Aq, Sylvian aqueduct; 3V, third ventricle; 4V, fourth ventricle. Ant, anterior; post, posterior. Scale bar=1mm.

b-c- Mean (± S.E.M.) body weight (b, df=2, H=0.4781, P>0.05) and brain volume (c, df=2, H=0.3231, P>0.05) of MCHR1+/+, MCHR1+/–, and MCHR1–/– mice before MRI.

d- Volumetric analysis of total ventricle size in MCHR1+/+, MCHR1+/–, and MCHR1–/– mice. Ventricular fractions are reported as means ± S.E.M. The total ventricular fraction (consisting of the lateral ventricles, the 3V, the Sylvian aqueduct and the 4V) tends to increase in MCHR1–/– (WT: 1.44±0.15; MCHR1+/+: 1.92±0.13; MCHR1–/–: 2.12±0.17, df=2, H=5.045, P=0.0803, Kruskal-Wallis test).

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Suppl. Figure 8: Schematic model of CBF modulation by MCH
MCH neurons, by sending fibers close to the ependymal cell layer bordering the ventral part of the third ventricle, could release MCH and thus increase the CBF through MCHR1 activation, resulting in an increase in intracellular Ca\(^{2+}\) concentrations.