LETTERS

Optogenetic dissection of a behavioural module in the vertebrate spinal cord

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Locomotion relies on neural networks called central pattern generators (CPGs) that generate periodic motor commands for rhythmic movements[1]. In vertebrates, the excitatory synaptic drive for inducing the spinal CPG can originate from either supraspinal glutamatergic inputs or from within the spinal cord[2,3]. Here we identify a spinal input to the CPG that drives spontaneous locomotion using a combination of intersectional gene expression and optogenetics[4] in zebrafish larvae. The photo-stimulation of one specific cell type was sufficient to induce a symmetrical tail beating sequence that mimics spontaneous slow forward swimming. This neuron is the Kolmer–Agduhr cell[5], which extends cilia into the central cerebrospinal-fluid-containing canal of the spinal cord and has an ipsilateral ascending axon that terminates in a series of consecutive segments[6]. Genetically silencing Kolmer–Agduhr cells reduced the frequency of spontaneous free swimming, indicating that activity of Kolmer–Agduhr cells provides necessary tone for spontaneous forward swimming. Kolmer–Agduhr cells have been known for over 75 years, but their function has been mysterious. Our results reveal that during early development in zebrafish these cells provide a positive drive to the spinal CPG for spontaneous locomotion.

We searched for novel spinal neurons that trigger the CPG in the zebrafish larva by using 'intersectional optogenetics', a combination of transgene expression in specific cell types[7] and genetic tools for manipulating neuronal activity with light[4]. The light-gated channel LiGluR[8,9] was selectively expressed in distinct subsets of spinal cord neurons by crossing transgenic animals carrying *UAS:LiGluR*[10] with a series of fish lines[11] that express GAL4, the transcription factor that activates the UAS promoter, in distinct cellular patterns. We looked for common behavioural outcomes induced by light stimulation in lines with partly overlapping expression patterns that could be attributed to the activity of a common cell type (Supplementary Fig. 1).

Five-day-old zebrafish larvae exhibit spontaneous forward slow swims[12]. These occur in brief bursts, with each burst consisting of a series of symmetrical, dampening left–right oscillations (Fig. 1a). We chose several *Gal4* transgenic lines to drive expression of LiGluR in different subsets of spinal neurons, and asked whether optical activation of these neurons elicits a forward swim-like behaviour. We first tested the *Gal4*^{s1020t} line, which labels a heterogeneous population of ventral spinal neurons. When crossed to *UAS:LiGluR* and labelled with the chemical photoswitch MAG1 (refs 8–10), 94% of the double-transgenic animals ($n = 37$) exhibited robust tail oscillations upon stimulation of the caudal spinal cord with a short light pulse (see Methods) (Fig. 1b and Supplementary Movie 2). The frequency and initial deflection angle of these oscillations closely resembled the spontaneous slow swim that we observed in unrestrained animals (Fig. 1c–f). The optical stimulation had no effect on non-transgenic larvae ($n = 12$) or on LiGluR-expressing larvae not incubated with MAG1 ($n = 12$).

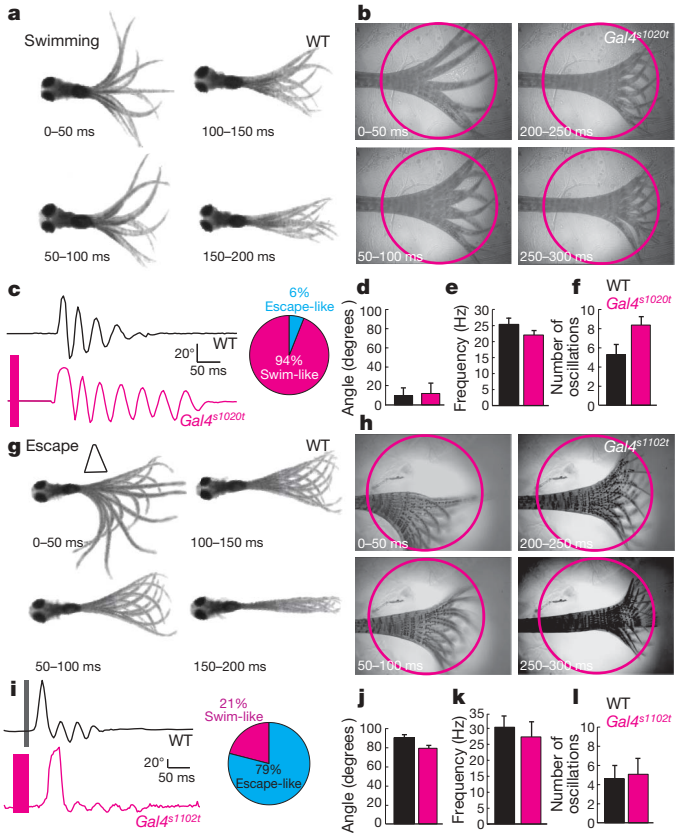

Figure 1 | Optical stimulation of specific spinal neurons leads to distinct locomotor behaviours. **a**, Spontaneous swim (superimposed frames). **b**, Optical stimulation (circle) of *Gal4*^{s1020t}*/UAS:LiGluR* evokes a 'spontaneous swim'-like behaviour. **c**, Comparison of deflection angle traces corresponding to **a** (top, black) and **b** (bottom, magenta, bar for stimulation) (inset: 94% of responses were a swim ($n = 18$)). No difference in angle ($P = 0.51$) (**d**) or frequency ($P = 0.09$) (**f**), but more oscillations ($P < 0.01$) (**e**) in light-induced swims. Values are given + s.e.m. ($n = 9$). **g**, Escape elicited by a water jet (triangle) consists of sharp C-turn away from stimulus followed by a forward swim. **h**, Light-induced escape induced by stimulation of Rohon-Beard cells in *Gal4*^{s1102t}*/UAS:LiGluR* larvae. **i**, Tail deflection traces corresponding to **g** (top) and **h** (bottom) (inset: 79% of responses were an escape ($n = 11$)). **j–l**, No difference in (**j**) deflection angle ($P = 0.13$), (**k**) frequency ($P = 0.42$) and (**l**) number of oscillations ($P = 0.41$). Values are given + s.e.m. ($n = 7$).

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The swim-like response induced by light in *Gal4*^{s1020t}/*UAS:LiGluR* larvae differed from the well-described touch–escape response[13], in which larvae respond to touch on one side of the tail by an initial sharp bend of the tail ('C-bend') to the opposite side that propels the fish away from the touch (Fig. 1g and Supplementary Movie 3). A C-bend to either the left or right side was elicited by bilateral illumination of the tail in *UAS:LiGluR*/*Gal4*^{s1102t}-line[10] larvae expressing the LiGluR in Rohon-Beard touch sensing neurons of the tail. A C-bend was evoked in 79% of trials (seven larvae tested five times each) (Fig. 1h, i and Supplementary Movie 4), resembling the natural escape of free-swimming fish (see initial one-sided tail bend, the frequency and the number of the ensuing tail beats in Fig. 1i–l). The left/right symmetry of the beating oscillations and small deflection angle seen in the *Gal4*^{s1020t} line distinguish it from this Rohon-Beard-cell-induced asymmetric escape response of the *Gal4*^{s1102t} line (compare Fig. 1c, d with Fig. 1i, j).

Gal4^{s1020t} drives expression in several cell types in the ventral spinal cord (Fig. 2a). Inverse PCR cloning indicates that the transposon is integrated near the *Olig2* gene[11]. Indeed, the expression pattern of *Gal4*^{s1020t} is indistinguishable from that seen in an *Olig2:GFP* transgenic line[14] (Supplementary Fig. 2). Using the *BGUG* expression system to determine which cell types express GAL4 in the *Gal4*^{s1020t} line (Supplementary Methods), we found that 79% of the 250 cells imaged in 73 fish were motor neurons (26.4% primary motor neurons: Fig. 2b, top panel; 52.4% secondary motor neurons: Fig. 2b, bottom panel). The remaining cells (20.4%) were neurons with a central ascending axon and lacking dendrites (Fig. 2c, d, f). A few cells (two out of 250 cells (0.8%)) resembling oligodendrocytes were also green fluorescent protein (GFP)-positive (data not shown).

The neurons with a central axon appeared to represent a single cell type. They are located near the central canal and have an ascending axon that projects ipsilaterally, making terminals in a series of two to six consecutive segments (Fig. 2c, d, f). Instead of dendrites these cells have a brush of cilia emanating from the somata, which appear to contact the cerebrospinal fluid (CSF), as shown by the alignment of the cilia with the central canal (Fig. 2e). Antibody staining showed that these neurons are GABA (γ-aminobutyric acid)- and GAD65/67-positive (Fig. 2g, h and Supplementary Fig. 3) as well as somatostatin-positive (Supplementary Fig. 4). Combined, these features are consistent with these neurons being Kolmer–Agduhr cells[5,15].

To find out whether the Kolmer–Agduhr neurons are responsible for triggering the swim-like behaviour in the *Gal4*^{s1020t}/*UAS:LiGluR* fish, we screened more *Gal4* lines and found one line, *Gal4*^{s1003t}, in which expression in the spinal cord is restricted to Kolmer–Agduhr cells. These cells shared morphology, cell body position and marker expression with the sensory neuron labelled in *Gal4*^{s1020t} (Fig. 3a–d and Supplementary Fig. 5). As in *Gal4*^{s1020t}/*UAS:LiGluR*, the light-induced response in *Gal4*^{s1003t}/*UAS:LiGluR* consisted of an alternating symmetrical tail beat at the slow swim frequency (Fig. 3e–i and Supplementary Movie 5), confirming that Kolmer–Agduhr cells are indeed able to trigger the CPG. The properties of the light-induced swim in the *Gal4*^{s1003t}/*UAS:LiGluR* larvae were indistinguishable from those of *Gal4*^{s1020t}/*UAS:LiGluR* (Fig. 3), which suggests that at the intensities applied, motor neurons are not activated in *Gal4*^{s1020t}/*UAS:LiGluR*. Indeed, calcium imaging in tetrodotoxin (to block action potentials and confine activity to the optically stimulated cells) revealed that the light pulses used in the behavioural

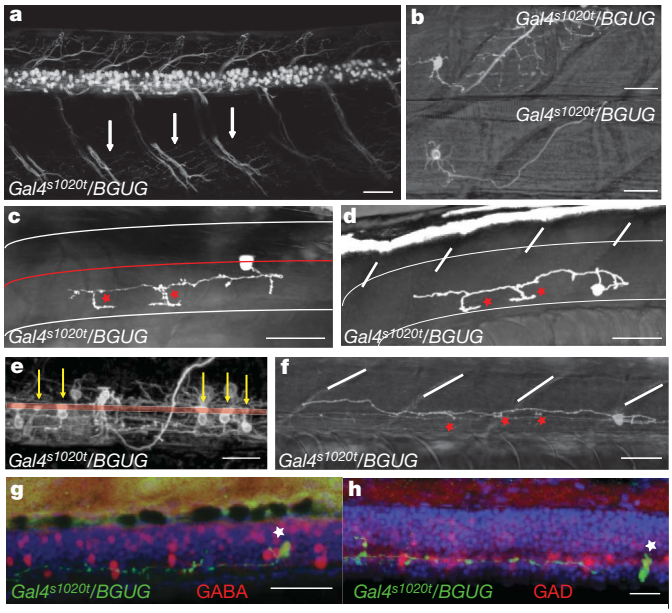

Figure 2 | The *Gal4*^{s1020t} line drives expression in motor neurons and Kolmer–Agduhr neurons. **a**, Expression in ventral cells including motor neurons projecting out of cord (arrows) (lateral view). **b**–**e**, Random labelling in *Gal4*^{s1020t}/*BGUG* identifies solely two cell types: **b**, motor neurons (primary motor neurons, top; secondary motor neurons, bottom) and **c**–**f**, Kolmer–Agduhr neurons (dorsal (**c**) and lateral (**d**–**f**) views of neuron with a central ipsilateral ascending axon; segment boundaries: white lines). Note contact feet (red stars in **c** and **d**) and 'toothbrush' morphology of cilia (yellow arrows in **e**) characteristic of Kolmer–Agduhr cells. In **c**, larva is tilted to show enlarged contacts on axon (midline, red line and segment, white lines). **e**, Dense *BGUG* pattern with multiple Kolmer–Agduhr cells shows the alignment of the brush of cilia (arrows) with central canal. **f**, The ascending axon runs near the ventral edge of the spinal cord before aiming dorsally. **g**, **h**, Kolmer–Agduhr cells at 5 days post-fertilization in *Gal4*^{s1020t}/*BGUG* (green) are GABAergic neurons (anti-GAD (**g**) and anti-GABA (**h**) immunostaining in red). Scale bars, 25 μm.

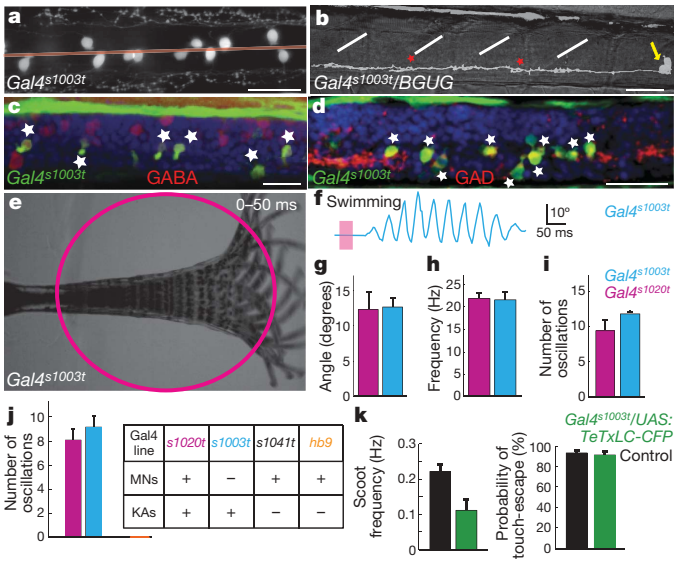

Figure 3 | Optical stimulation of Kolmer–Agduhr cells of *Gal4*^{s1003t} line induces a forward swim. **a**, Expression in spinal cord is confined to cells close to the midline (dorsal view). **b**, Lateral view in *BGUG* shows ventral neurons with central axons forming contact feet (red stars) and toothbrush morphology (arrow). White lines are segment boundaries. **c**, **d**, These cells (green) are GABAergic (anti-GAD and anti-GABA staining in red). **e**, **f**, Optical stimulation induces a swim-like response. **e**, Superimposed images. **f**, Deflection angle trace. **g**–**i**, No difference in deflection angle (**g**), frequency (**h**) and number of oscillations (**i**) between *Gal4*^{s1003t} (blue) and *Gal4*^{s1020t} (magenta) (respectively $P = 0.85$, $P = 0.98$, $P = 0.36$); values are given + s.e.m. ($n = 9$). **j**, Side-to-side comparison of number of oscillations evoked by a 100-ms pulse of light shows that only lines expressing in Kolmer–Agduhr cells show a swim-like response whereas line with motor neurons (MNs) and no Kolmer–Agduhr cells (KAs) (*Gal4*^{s1041t}, black ; *Hb9:Gal4*, orange) do not ($n = 6$ for each line). **k**, Reduction of the spontaneous swimming frequency in *Gal4*^{s1003t}/*UAS:TeTxLC-CFP* ($P < 0.0075$) but no change in the probability of touch-response ($P = 0.45$). Values are given + s.e.m. ($n = 10$ and 12, respectively).

experiments were strong enough to activate the narrow region surrounding the central canal where Kolmer–Agduhr cells are located, but not elsewhere in the ventral spinal cord where most motor neurons are situated (Supplementary Fig. 6).

To eliminate the possibility that motor neuron activation in *Gal4*^{s1020t}/*UAS:LiGluR* fish elicits swimming movements, we tested two additional *Gal4* lines with motor neuron expression but no expression in Kolmer–Agduhr cells: *Gal4*^{s1041t} and *Hb9:Gal4* (ref. 16) (Supplementary Fig. 7). Light pulses that reliably triggered swim-like behaviour in animals expressing LiGluR in the *Gal4*^{s1020t} and *Gal4*^{s1003t} lines produced no effect in either of these motor neuron lines (six LiGluR larvae tested for each line; Fig. 3j). However, increasing the intensity or duration of illumination by at least tenfold evoked contraction on the illuminated side of the tail, which was distinct from the forward swim-like behaviour (Supplementary Fig. 8). The requirement for stronger illumination to evoke the contraction is consistent with the larger size and lower input resistance of motor neurons[17]. Altogether, these observations show that the forward swim can be attributed specifically to the activation of the Kolmer–Agduhr cells.

Kolmer–Agduhr cells are GABAergic. To test the role of GABAergic transmission in the light-induced response of *Gal4*^{s1020t}/*UAS:LiGluR*, we injected the GABA-A antagonist bicuculline into the spinal cord. This treatment greatly reduced the number of oscillations evoked by light in *Gal4*^{s1020t}/*UAS:LiGluR* fish ($P < 10^{-6}$, $t(7) = 11.2950$; Supplementary Fig. 9), abolishing the light-induced response entirely in four out of eight larvae. These experiments indicate that the optical stimulation of Kolmer–Agduhr cells is sufficient to initiate a swim-like behaviour by a GABA-dependent process.

Having shown that activation of Kolmer–Agduhr cells is sufficient for inducing a swim-like behaviour, we next asked whether they are also necessary for spontaneous swimming by blocking synaptic transmission from Kolmer–Agduhr cells by a targeted expression of the tetanus toxin light chain (TeTxLC) fused to cyan fluorescent protein (CFP) (*UAS:TeTxLC-CFP* (ref. 18) crossed with *Gal4*^{s1020t} and *Gal4*^{s1003t}). Three- to five-day-old larvae expressing TeTxLC-CFP were easily identified by their CFP fluorescence (Methods). We compared the swimming behaviour of CFP-positive larvae with that of siblings that did not have CFP fluorescence (that is, did not express TeTxLC). *Gal4*^{s1020t}/*UAS:TeTxLC-CFP* larvae expressing the TeTxLC were paralysed at five days, as expected for expression of GAL4 in motor neurons. On the other hand, *Gal4*^{s1003t}/*UAS:TeTxLC-CFP* larvae, which lack motor neuron expression, were not paralysed, enabling behavioural assays. *Gal4*^{s1003t}/*UAS:TeTxLC-CFP* exhibited spontaneous burst-swimming, but the frequency of the swims was greatly reduced ($P < 0.0075$; $t(9) = -3.4278$) (Fig. 3k). These results indicate that Kolmer–Agduhr cells provide a positive drive to spontaneous swimming. It should be noted that only half of the Kolmer–Agduhr cells express the UAS transgene in the *Gal4*^{s1003t} line (Supplementary Fig. 3), which suggests that block of synaptic transmission in all of the Kolmer–Agduhr cells could have an even more profound effect on spontaneous swimming. Strikingly, we found that *Gal4*^{s1003t}/*UAS:TeTxLC-CFP* still respond to touch by a touch–escape ($P = 0.45$; $t(11) = 0.7863$) (Fig. 3k), indicating that Kolmer–Agduhr cells do not play a significant role in initiating touch–escape.

We further examined the Kolmer–Agduhr-induced swim and the Rohon-Beard-induced escape behaviours by performing local photoactivation. Because mechanical activation on one side of the larva elicits, as part of the escape response, a C-turn on the opposite side (Fig. 1g), we predicted that one-sided optical activation of Rohon-Beard cells would have the same effect. We tested this by confining the illumination to a small portion of the spinal cord with a digital light processing array (Fig. 4a). One-sided optical stimulation of Rohon-Beard cells in the *Gal4*^{s1102t} line triggered a reliable large-angle contralateral bend ($n = 9$ out of 9; Fig. 4c), resembling the C-bend induced by one-sided mechanical stimulation (Fig. 1i). In contrast, one-sided optical stimulation of *Gal4*^{s1020t} elicited a symmetrical forward swim-like behaviour (Fig. 4b), closely resembling the response to bilateral optical stimulation (Fig. 1c).

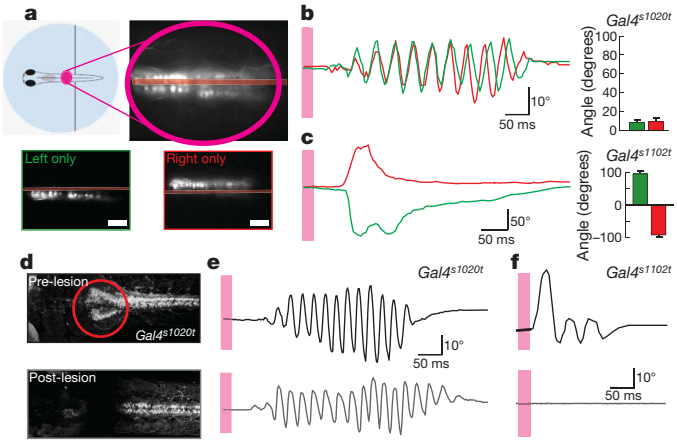

Figure 4 | Dissection of the light-evoked responses in *Gal4*^{s1020t} and *Gal4*^{s1102t} by unilateral stimulation and lesion studies. a–c, Patterned illumination for stimulation. **a**, Semi-restrained *Gal4*^{s1020t} larva aimed bilaterally (cartoon and fluorescence image of Kaede expression in three segments, top) and on left or right side (bottom panels; scale bar, 25 μm). **b**, **c**, Deflection angle traces and mean values induced by left (green) and right (red) stimulation. Values are given + s.e.m. **b**, Left and right activations induce similar symmetric oscillations of the tail in *Gal4*^{s1020t} line ($n = 5$). **c**, Left and right activations induce large and opposite-directed C-bends in *Gal4*^{s1102t} ($n = 9$). **d–f**, Effect induced by section of the cerebrospinal connections. **d**, Pattern in *Gal4*^{s1020t} pre- (top) and post- (below) lesion. **e**, **f**, No reduction of the light-induced swim behaviour in *Gal4*^{s1020t} (**e**, $n = 7$) but abolition of the light-induced escape behaviour in *Gal4*^{s1102t} (**f**, $n = 4$) (pre- and post-lesion, top and bottom).

To test the involvement of supraspinal inputs in the Kolmer–Agduhr-elicited swim-like behaviour, we performed hindbrain lesions that ablated the connections between the brain and the spinal cord (Fig. 4d). Tactile stimuli sensed by Rohon-Beard cells are known to be transmitted to the hindbrain[13] where the command for escape is relayed back to the tail, and ablation of the hindbrain was shown earlier to suppress the fast contralateral C-bend that begins the escape[13]. Consistent with this, the C-bend component of the response to optical activation of Rohon-Beard cells in the *Gal4*^{s1102t}/*UAS:LiGluR* line was abolished by the hindbrain lesion ($n = 4$ out of 4; Fig. 4f). In contrast, the light-evoked swim-like behaviour in *Gal4*^{s1020t} remained intact after the lesion ($n = 7$; Fig. 4e), which demonstrates that intra-spinal activation of *Gal4*^{s1020t}-positive neurons is sufficient to drive the swim-like behaviour.

Previous work in vertebrates has implicated specific classes of spinal interneuron in regulating locomotion speed[19,20] or movement strength, and their activity was associated with specific states of the spinal networks recorded during fictive locomotion[16,21]. These studies were based on either loss of function[19] or on correlation of the activity of neurons with specific phases of ventral root activity during fictive locomotion[16,20]. We show that it is possible to employ a light-gated channel to show sufficiency of genetically targeted neurons in behaviour. Genetically encoded blockers of activity can then be used to also show the necessity of neuronal function in behaviour (Supplementary Fig. 1).

Previous studies in the lamprey showed that the GABAergic system is a strong modulator of fictive swimming[22], but there are many types of spinal GABAergic neuron and the neuronal basis for the observed modulation was not known. We demonstrate here that a single GABAergic cell, the Kolmer–Agduhr neuron, is a major modulator of locomotion in the awake behaving animal. Although Kolmer–Agduhr neurons were first described decades ago[5], their role in spinal circuits remained enigmatic. We show that Kolmer–Agduhr cells are necessary for the normal frequency of spontaneous swimming and are sufficient to drive the CPG in early development, when GABAergic transmission is excitatory[23].

The Kolmer–Agduhr neurons of zebrafish resemble the CSF-contacting cells of lamprey and other vertebrates, including mammals[24,25], in that they express GABA and the transcription factor olig2 (ref. 26) (Supplementary Fig. 2), are located next to the central canal and project a brush of stereocilia into the CSF, and have axons that run longitudinally[25]. It remains to be determined whether potential homologues of Kolmer–Agduhr cells may affect locomotor patterns in mammals.

Although we have determined that Kolmer–Agduhr cells provide a positive drive to the CPG in larval fish, in adult fish and in postnatal mammals, GABAergic transmission is inhibitory and blocking GABA receptor in the adult lamprey enhances swim frequency[22]. This suggests that Kolmer–Agduhr activity may suppress swimming in adult zebrafish. The 'liquor-contacting' cilia of the Kolmer–Agduhr neurons in the central canal[5,15,22] could allow them to sense mechanical deformation of the spine or chemical signals in the central canal, such as low pH, as proposed recently for mammalian CSF-contacting neurons[27]. The natural drive to Kolmer–Agduhr cells and their function later in life remain to be defined.

METHODS SUMMARY

To determine which cell types express in a GAL4 line and to quantify their abundance, we used the *BGUG* transgene (short for Brn3c:Gal4; *UAS: mGFP*), which labels a small, random subset of the GAL4-expressing cells owing to variegated expression of the gene encoding a membrane-targeted GFP[11,28]. The transgenic line *UAS:LiGluR*, as well as all Gal4 lines from the enhancer trap screen, were published previously[10,11]. To make the *Hb9:Gal4* transgenic construct, 3 kilobases of genomic sequence upstream of the *hb9* coding sequence[18,29] was amplified by PCR and inserted upstream of the *GAL4* coding sequence[30]. Synthesis of MAG-1 was performed as described[8–10]. Five-day-old larvae were bathed in 200 μM MAG-1, 4% dimethylsulphoxide (DMSO) for 45 min at 28.5 °C in the dark. The fast photoswitching light source was coupled to an upright Zeiss epifluorescence microscope. Patterned illumination was accomplished using a digital micromirror device. Motion of the tail was monitored at 250 frames per second by using a high-speed camera. Lesions were performed on anaesthetized larvae bathed in Evans solution with a fine tungsten needle. Embryos at appropriate stages were fixed in 4% PFA in PBS and processed for immunohistochemistry according to published protocols[28]. Injection of the calcium dye was performed 30–60 min after MAG-1 labelling. Fluorometric Ca^{2+} measurements were performed using a confocal Olympus laser-scanning microscope equipped with an ultraviolet SIM Scanner. Tracking of the tail position and calcium imaging analysis were performed using a custom-made script written in Matlab 2007 (Mathworks).

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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1. Grillner, S. Biological pattern generation: the cellular and computational logic of networks in motion. *Neuron* **52,** 751–766 (2006).
2. Fenaux, F. *et al.* Effects of an NMDA-receptor antagonist, MK-801, on central locomotor programming in the rabbit. *Exp. Brain Res.* **86,** 393–401 (1991).
3. Kiehn, O. *et al.* Excitatory components of the mammalian locomotor CPG. *Brain Res. Rev.* **57,** 56–63 (2008).
4. Luo, L., Callaway, E. M. & Svoboda, K. Genetic dissection of neural circuits. *Neuron* **57,** 634–660 (2008).
5. Agduhr, E. in *Cytology and Cellular Pathology of the Nervous System* (ed. Penfield, W.) Vol. 2 535–573 (Hoeber, 1932).
6. Higashijima, S., Mandel, G. & Fetcho, J. R. Distribution of prospective glutamatergic, glycinergic, and GABAergic neurons in embryonic and larval zebrafish *J. Comp. Neurol.* **480,** 1–19 (2004).
7. Douglas, J. R. *et al.* The effects of intrathecal administration of excitatory amino acid agonists and antagonists on the initiation of locomotion in the adult cat. *J. Neurosci.* **13,** 990–1000 (1993).
8. Volgraf, M. *et al.* Allosteric control of an ionotropic glutamate receptor with an optical switch. *Nature Chem. Biol.* **2,** 47–52 (2006).
9. Gorostiza, P. *et al.* Mechanisms of photoswitch conjugation and light activation of an ionotropic glutamate receptor. *Proc. Natl Acad. Sci. USA* **104,** 10865–10870 (2007).
10. Szobota, S. *et al.* Remote control of neuronal activity with a light-gated glutamate receptor. *Neuron* **54,** 535–545 (2007).
11. Scott, E. K. *et al.* Targeting neural circuitry in zebrafish using GAL4 enhancer trapping. *Nat. Methods* **4,** 323–326 (2007).
12. Budick, S. A. & O'Malley, D. M. Locomotor repertoire of the larval zebrafish: swimming, turning and prey capture. *J. Exp. Biol.* **203,** 2565–2579 (2000).
13. Liu, K. S. & Fetcho, J. R. Laser ablations reveal functional relationships of segmental hindbrain neurons in zebrafish. *Neuron* **23,** 325–335 (1999).
14. Shin, J., Park, H. C., Topczewska, J. M., Mawdsley, D. J. & Appel, B. Neural cell fate analysis in zebrafish using olig2 BAC transgenics. *Methods Cell Sci.* **25,** 7–14 (2003).
15. Dale, N. *et al.* The morphology and distribution of 'Kolmer–Agduhr cells', a class of cerebrospinal-fluid-contacting neurons revealed in the frog embryo spinal cord by GABA immunocytochemistry. *Proc. R. Soc. Lond. B* **232,** 193–203 (1987).
16. Liao, J. C. & Fetcho, J. R. Shared versus specialized glycinergic spinal interneurons in axial motor circuits of larval zebrafish. *J. Neurosci.* **28,** 12982–12992 (2008).
17. Drapeau, P. *et al.* In vivo recording from identifiable neurons of the locomotor network in the developing zebrafish. *J. Neurosci. Methods* **88,** 1–13 (1999).
18. Asakawa, K. *et al.* Genetic dissection of neural circuits by Tol2 transposon-mediated Gal4 gene and enhancer trapping in zebrafish. *Proc. Natl Acad. Sci. USA* **105,** 1255–1260 (2008).
19. Gosgnach, S. *et al.* V1 spinal neurons regulate the speed of vertebrate locomotor outputs. *Nature* **440,** 215–219 (2006).
20. McLean, D. L. *et al.* Continuous shifts in the active set of spinal interneurons during changes in locomotor speed. *Nature Neurosci.* **11,** 1419–1429 (2008).
21. Ritter, D. A., Bhatt, D. H. & Fetcho, J. R. *In vivo* imaging of zebrafish reveals differences in the spinal networks for escape and swimming movements. *J. Neurosci.* **21,** 8956–8965 (2001).
22. Alford, S., Sigvardt, K. A. & Williams, T. L. GABAergic control of rhythmic activity in the presence of strychnine in the lamprey spinal cord. *Brain Res.* **506,** 303–306 (1990).
23. Brustein, E. & Drapeau, P. Serotoninergic modulation of chloride homeostasis during maturation of the locomotor network in zebrafish. *J. Neurosci.* **25,** 10607–10616 (2005).
24. Vigh, B. & Vigh-Teichmann, I. Actual problems of the cerebrospinal fluid-contacting neurons. *Microsc. Res. Tech.* **41,** 57–83 (1998).
25. Stoeckel, M. E. *et al.* Cerebrospinal fluid-contacting neurons in the rat spinal cord, a gamma-aminobutyric acidergic system expressing the P2X2 subunit of purinergic receptors, PSA-NCAM, and GAP-43 immunoreactivities: light and electron microscopic study. *J. Comp. Neurol.* **457,** 159–174 (2003).
26. Furusho, M. *et al.* Involvement of the Olig2 transcription factor in cholinergic neuron development of the basal forebrain. *Dev. Biol.* **293,** 348–357 (2006).
27. Huang, A. L. *et al.* The cells and logic for mammalian sour taste detection. *Nature* **442,** 934–938 (2006).
28. Xiao, T. & Baier, H. Lamina-specific axonal projections in the zebrafish tectum require the type IV collagen Dragnet. *Nature Neurosci.* **10,** 1529–1537 (2007).
29. Flanagan-Steet, H., Fox, M. A., Meyer, D. & Sanes, J. Neuromuscular synapses can form in vivo by incorporation of initially aneural postsynaptic specializations. *Development* **132,** 4471–4481 (2005).
30. Koster, R. W. & Fraser, S. E. Tracing transgene expression in living zebrafish embryos. *Dev. Biol.* **233,** 329–346 (2001).

Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions C.W., F.D.B, H.B. and E.Y.I. made critical primary contributions to this study. C.W. built the photostimulation setup, performed behavioural experiments, lesions, pharmacology, calcium imaging, imaging of the immunolabelled larvae, anatomical analysis based on *BGUG* imaging and wrote the Matlab scripts for analysing behaviour and imaging. F.D.B. generated the transgenic lines *UAS:LiGluR*[10] and *Hb9:Gal4*, as well as performing the immunochemistry experiments. E.W. participated in the anatomical analysis of *BGUG*. E.K.S. and H.B. generated the enhancer trap *Gal4* screen, which made the 'intersectional optogenetic' approach possible[11]. E.Y.I. and D.T. developed chemical optogenetics with LiGluR[8]. C.W. and E.Y.I. wrote the manuscript with feedback from H.B. and F.D.B. H.B. and E.Y.I. supervised C.W. and F.D.B. and contributed to the planning of all aspects of this project.

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METHODS

Generation of transgenic lines. Zebrafish were maintained at 28 °C in the Tubingen Line genetic background. To make the *Hb9:Gal4* transgenic construct, 3 kilobases of genomic sequence upstream of the *hb9* coding sequence[18,29] was amplified by PCR and inserted upstream of the *GAL4* coding sequence[30]. This expression cassette was inserted between the Tol2 recognition sequences in the pT2KXIGΔin vector[31]. Wild-type TL embryos were injected at the one-cell stage with a solution of 25 ng μl^{-1} *Hb9:GAL4* DNA, 50 ng μl^{-1} transposase mRNA (prepared using the Ambion mMESSAGE mMACHINE T7 kit) and 0.04% Phenol Red. F1 embryos were pooled and screened for the transgene by crossing them with *UAS:Kaede* carriers. F0 founder animals were then mated to wild-type TL fish to create stable lines.

The following transgenic lines were used (designations according to official zebrafish nomenclature; previously published synonyms or abbreviated name are in parentheses): Tg(*UAS:iGluR6*(L439C))s1995 (*UAS:LiGluR*); *Tg(UAS-E1b:Kaede)s1999t/+ (UAS:Kaede); Et(-1.5hsp70l:Gal4-VP16)s1003t (Gal4^{s1003t}); Et(-0.6hsp70l:Gal4-VP16)s1020t (Gal4^{s1020t}); Et(fos:Gal4-VP16)s1041t (Gal4^{s1041t}); Et(E1b:GAL4-VP16)s1102t (Gal4^{s1102t}); Tg(pou4f3:GAL4,UAS:gap43-GFP)s314t (BGUG); Tg(5xUAS:TeTxLC-CFP)zf85 (UAS:TeTxLC-CFP)*. Because the GAL4 lines carry the *UAS:Kaede* construct, embryos positive for Kaede produced in a cross with *UAS:LiGluR* carriers were selected for behaviour experiments (50% were also *UAS:LiGluR* expressers). Dark embryos were used for calcium-imaging experiments. Genotyping of larvae for the LiGluR transgene was performed after behavioural experiments using the following primers: forward primer GGCTTGA GGATGGGAAATATGG and reverse primer GGGTTGCAAGGGTGTGGGTT ATACC.

To determine which cell types expressed GAL4 in the Gal4s1020t line and to quantify their abundance, we used the *BGUG* transgene (an abbreviation of Brn3c:Gal4; *UAS: mGFP*), which labels a small, random subset of the GAL4-expressing cells owing to variegated expression of the gene encoding a membrane-targeted GFP[11,28].

MAG-1 labelling and mounting of zebrafish larvae. Synthesis of MAG-1 was performed as previously described[8–10]. MAG-1 was first diluted to 5 mM in DMSO and pre-activated by ultraviolet light (365 nm) for 1 min. The medium E3 was then added to reach the final concentration of 200 μM MAG-1, 4% DMSO. Five days after fertilization, larvae were bathed in the labelling solution for 45 min at 28.5 °C in the dark. The larvae were then washed three times with fresh E3 medium. After recovery period of 1 h, all spontaneously swimming larvae were embedded in 2% agar and free-tailed. The agar was removed up to the fins for the behaviour experiments where illumination of the tail was global under a ×5 air objective (numerical aperture = 0.25), whereas the agar was removed only up to the anus for experiments where single (group of) cells were illuminated under the ×40 water immersion objective (numerical aperture = 0.8).

Photostimulation. The light source used for fast photoswitching was a DG-4 (Sutter Instruments) coupled to an upright Zeiss AxioImager epifluorescence microscope. Patterned illumination was accomplished using a digital micromirror device (Mosaic System, Photonic Instruments) coupled to the epifluorescence path of the microscope. For global illumination experiments, we illuminated the caudal portion of the tail bilaterally using a ×5 air objective (the light power was 0.24 mW mm^{-2} at 390 nm and 0.71 mW mm^{-2} at 500 nm; as a comparison the low power illumination previously used[10] was 0.04 mW mm^{-2} at 365 nm for 15 min), whereas local illumination of single segments or groups of cells was achieved using a ×40 water immersion objective (light power 24.6 mW mm^{-2} at 390nm and 37.7 mW mm^{-2} at 500 nm).

The *Gal4* transgenic lines carry the *UAS:Kaede* constructs. Therefore, in all behavioural experiments reported here with *Gal4* lines, we selected the larvae expressing Kaede. Half of the larvae tested were positive for *UAS:LiGluR*, meaning that the tests were always run blindly and genotyping was performed afterwards. The light-induced responses of *Gal4^{s1020t}/UAS:LiGluR*, *Gal4^{s1102t}/UAS:LiGluR* and *Gal4^{s1003t}/UAS:LiGluR* labelled with MAG-1 were abolished by bath application of tricaine (0.02%; $n = 8$, 8 and 7, respectively).

We used patterned illumination to ask whether optical stimulation of CSF-contacting neurons in three consecutive segments on either the left or the right side of the animal would also evoke asymmetrical tail motions (experiments were done in the *Gal4^{s1020t}* line because it reliably targets all Kolmer–Agduhr neurons, whereas *Gal4s^{1003t}* drives expression in only half of them).

Monitoring of behavioural responses. Five-day-old larvae exhibit spontaneous forward swims, also referred to as slow swims[12]. These occur in brief bursts, with each burst consisting of a series of symmetrical, dampening left–right oscillations, which can be analysed quantitatively in 'head-centred' movies, where the image of the fish in each frame is repositioned to register the head in one spot so that the movements of the tail can be visualized (Fig. 1a and Supplementary Movie 1). Swimming movements can also be observed in semi-restrained larvae with their heads fixed in agar and tails free to move (Methods), thus allowing targeted optical stimulation of the central nervous system to be combined with behavioural analysis and providing an easy comparison with the head-centred free swim. We chose several *Gal4* transgenic lines that allowed us to drive expression of LiGluR in different subsets of spinal neurons, and we asked whether optical activation of these neurons elicits a forward swim-like behaviour.

Motion of the tail was monitored at 250 frames per second using a high-speed camera (Fastec Inline, Itronx Imaging Technologies). The camera was coupled to the side port of the AxioImager microscope in global illumination experiments using the ×5 objective, or coupled to a ×4 objective under the stage when local photoswitching was performed using a higher magnification (×40) objective. The tracking of the tail position was performed using a custom-made script written in Matlab 2007 (Mathworks). Lesions were performed on larvae anaesthetized by a cold exposure and bathing in Evans solution using a fine tungsten needle at the boundary of the hindbrain and spinal cord. Animals were tested after a period of 60 min recovery. The quantification of spontaneous swimming in *Gal4^{s1003t}/UAS:TeTxLC-CFP* and dark siblings was performed by isolating six 5-day-old larvae at a time and counting swimming events in a 10-min recording. Thirty larvae were observed for each group. All experiments were conducted at room temperature.

Immunochemistry. Embryos at appropriate stages were fixed in 4% PFA in PBS and processed for immunohistochemistry according to published protocols[28]. The following primary antibodies and concentrations were used for whole-mount immunohistochemistry: antibody to GFP (rabbit anti-GFP; Molecular Probes A11122, or chick anti-GFP; GeneTex GTX13970) 1:500; antibody to GABA (rabbit anti-GABA; Sigma A2052) 1:2,000; antibody to GAD65/67 (rabbit anti GAD65+GAD67; Abcam ab11070-50) 1:500; antibody to Somatostatin (ImmunoStar 20067) 1:200. GABA immunostaining required fixation in 0.1% glutaraldehyde/4%PFA in PBS. Secondary antibodies conjugated to Alexa-488 or Alexa-555 (Invitrogen) were selected accordingly and used at 1:500 dilutions. Nuclei were counterstained with DAPI (Invitrogen).

Dye preparation and loading. Injection of the calcium dye was performed 30–60 min after MAG-1 labelling. Oregon Green Bapta 1-AM was freshly diluted in DMSO with 20% pluronic acid (Invitrogen) to yield to a 10 mM stock solution and further diluted in Evans solution with 0.01% Fast-Green (Sigma-Aldrich) to a final concentration of 1 mM. A fine borosilicate pipette of 1 mm outer diameter and 0.5 mm internal diameter (FHC; 5 MΩ resistance when filled with Evans solution) was used to inject the dye in the spinal cord using a Picospritzer (General Valve; 50 ms, 20 pounds per square inch, one to three puffs). Calcium recordings were performed between 1 and 5 h after the dye loading.

Calcium imaging. Fluorometric Ca^{2+} measurements were performed at room temperature using a confocal Olympus Fluoview laser scanning microscope equipped with a ×40 (numerical aperture = 0.8) water immersion objective. The 488-nm argon laser (20 mW) was used at 1–3% power for exciting the dye, and a 405-nm laser (50 mW) used at 1–30% power for photoswitching. Frames were acquired at 4–10 Hz. Local photoactivation of cells was performed by one scan at the same acquisition speed repeated every 10 s unless otherwise stated. Values of $\Delta F/F_s$ were computed using a custom-made script in Matlab 2007.

Pharmacology. Tricaine (0.02%) was dissolved in E3 and added to the bath. Bicuculline dissolved in fish Ringer's solution was injected at 40 μM, and TTX at 10 μM.

31. Masahira, N. *et al.* Olig2-positive progenitors in the embryonic spinal cord give rise not only to motoneurons and oligodendrocytes, but also to a subset of astrocytes and ependymal cells. *Dev. Biol.* **293**, 358–369 (2006).