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guide RGC axons, we conducted a detailed spatiotemporal expression analysis using whole-mount in situ hybridization (*10*). We were unable to detect *robo2* mRNA in the retina at 28 hpf. At 31 hpf, weak expression was detectable in a ventronasal patch of cells adjacent to the ventral fissure (Fig. 4A). These are presumably the first-born RGCs, which appear in this location between 27 and 28 hpf (*20*) and project axons across the midline at 33 to 35 hpf (*21*). At 36 hpf (Fig. 4B), the expression had spread dorsally and temporally, reflecting the pattern of early RGC differentiation (*20*, *21*). By 41 hpf, *robo2* was expressed in all quadrants of the RGC layer (Fig. 4, D and E). Although the RGCs expressing *robo2* were initially located centrally (Fig. 4C), expression later became peripherally restricted, and we were unable to detect expression in the older central RGCs at 72 hpf (Fig. 4F). Thus, *robo2* is first expressed in RGCs shortly after their differentiation and turns off later, consistent with what might be expected for an axon guidance receptor. We have not yet successfully generated antibodies to study Robo2 protein regulation. Intriguingly, *robo2* was also expressed at certain points adjacent to the retinotectal projection (*10*) and in the inner nuclear layer (INL) (Fig. 4, B, E, and F).

Our genetic mapping and allele sequencing data show that the *ast* phenotype is caused by mutations in zebrafish *robo2*. Together with the phenotypic analysis, this shows that *ast*/*robo2* is essential for establishing the retinotectal projection. Because transplanted ast^{ti272z} RGC axons navigate incorrectly in a WT environment, and from its structural similarity to *Drosophila* Robo, we conclude that Ast/Robo2 acts as a guidance receptor in RGC axons. Conversely, because WT axons project normally in an ast^{ti272z} host, it is likely that *ast*/*robo2* function is not required in the environment. The only caveat is that ast^{ti272z} homozygotes still express some *robo2* mRNA, and thus could produce a secreted Robo2 fragment encoded by ast^{ti272z}. We cannot exclude the possibility that this truncated protein could mimic a normal non–cell autonomous function of Ast/Robo2 and thus guide the transplanted WT axons.

In *Drosophila*, Robo acts as a guidance receptor that recognizes the repulsive signal Slit, produced by midline glia, and prevents inappropriate crossing of the midline (*14*, *22*), whereas the combination of different Robos determines the medial-lateral position of the longitudinal fascicles (*23*, *24*). In zebrafish RGC axon guidance, *ast/robo2* functions not only to prevent inappropriate midline recrossing, but also to form the optic chiasm and prevent abnormal anterior and posterior projections and optic tract defasciculation. The *ast* phenotype is thus more reminiscent of the *C. elegans sax-3* axon guidance phenotype (*12*, *13*).

Coculture experiments have shown that mammalian Slit2 can repel RGC axons (*25*), inhibit RGC axon outgrowth (*26*, *27*), and cause tighter fasciculation of retinal axons (*27*). The complex pattern of *slit1*, *slit2*, and *slit3* expression along the optic pathway suggested that mammalian Slits might guide retinal axons at positions other than the midline (*25–27*). From its expression in RGCs, mammalian Robo2 is likely to be the receptor that mediates their response to Slits (*26*, *27*). Together with preliminary observations that two zebrafish Slits are expressed along the optic pathway (*28*), our Astray/Robo2 functional data suggest a conserved role for this ligand-receptor system in the vertebrate visual system.

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Fast Backprojections from the Motion to the Primary Visual Area Necessary for Visual Awareness

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Much is known about the pathways from photoreceptors to higher visual areas in the brain. However, how we become aware of what we see or of having seen at all is a problem that has eluded neuroscience. Recordings from macaque V1 during deactivation of MT+/V5 and psychophysical studies of perceptual integration suggest that feedback from secondary visual areas to V1 is necessary for visual awareness. We used transcranial magnetic stimulation to probe the timing and function of feedback from human area MT+/V5 to V1 and found its action to be early and critical for awareness of visual motion.

Two hypotheses that were postulated about how brain activity mediates awareness have particular relevance to neurophysiology (*1–4*). There might be a class of neurons or neural pathways whose activity mediates awareness. Alternatively, awareness might be the result of specific forms of neuronal activity such as synchronous discharges or spike rate modulations. These hypotheses are not mutually exclusive, and a combination of both might be proposed (*4*). The role of striate cortex (V1) in visual awareness (*4–8*) is controversial; it may not have specialized "awareness-dedicated" neurons, but the spiking rate of V1 neurons appears to be modulated by perceptual context, correlated with

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awareness and dependent on backprojections from higher visual areas (*4*, *9*, *10–13*). The function and time course of these backprojections is beginning to be explored in nonhuman primates but has not been investigated directly in human subjects (*9–15*), although the organization and chronometry of feedforward-feedback loops provide a substrate that may give rise to visual awareness (*16*, *17*).

When transcranial magnetic stimulation (TMS) is applied to visual cortex, subjects may perceive phosphenes (flashes of light) (*18*, *19*) and, when applied to area MT+/V5, subjects can perceive moving phosphenes (*20–22*). To examine the temporal relation between events in V5 and V1, we applied TMS to both areas with variable delays of tens of milliseconds (*23–27*). TMS was applied such that stationary phosphenes evoked from V1 and moving phosphenes evoked from V5 would overlap in visual space (*28*, *29*) (Fig. 1). TMS was applied over V5 at 100% of V5 phosphene threshold and over V1 at 80% of the V1 phosphene threshold (*30*, *31*). After each pair of pulses, the subjects were asked to describe their perception of phosphenes and to rate them using a fixed, forced-choice scale (*32*).

If the V5 to V1 backprojection is critical for awareness, disruption of activity in V1 at the time of arrival of feedback should interfere with the perception of attributes encoded by the extrastriate area. The use of V1 stimulation below the threshold for production of a phosphene precludes a simple masking of a V5 phosphene by a V1 phosphene (*33*). Furthermore, the intensity of V1 stimulation used was below that required to disrupt psychophysical performance (*34*) or to induce scotomas (*35*). The use of a range of V5 to V1 TMS asynchronies controlled for nonspecific effects of TMS, such as the sound made by the discharge of the coil. In a control experiment, pairs of a subthreshold and a suprathreshold transcranial stimulus with variable interstimulus intervals were applied to V5 to assess the contribution of local V5 effects and fast V1 to V5 projections (*36*).

When TMS was applied to V1 before V5, there was no effect on the perceived movement of the phosphenes (Fig. 2). However, with a V5 to V1 asynchrony of +5 to +45 ms (that is, V5 TMS applied before V1 TMS), there was a marked decrease in the quantity and a change in the quality of the phosphenes elicited by V5 stimulation (Fig. 2). Five of the eight subjects reported an absence of phosphenes when TMS to V1 was applied up to 25 ms after V5. All subjects reported that in the majority of the trials the phosphene was stationary, rather than moving, when TMS was applied to V1 up to 45 ms after V5. Paired-pulse TMS to V5 had no effect on the perception of the moving phosphenes in any of the subjects, regardless of interstimulus interval (Fig. 2).

Our results correspond with findings from physiologic studies of monkey area MT+/V5 (*9–15*), suggesting that the V5 projection to V1 operates with a short time course (*37*). This finding contradicts the chronometrically naïve assumption that the "top-down" influences of feedback projections should occur late (*38*, *39*). The latencies of some MT+/V5 neurons and the conduction times of the V5 to V1 pathway in recordings from monkeys are sufficiently fast to account for the early effects seen here (*9–15*, *40–42*).

These results demonstrate the importance of V5 to V1 backprojections for perception and awareness of visual motion (*33*, *43–47*). TMS in blindsight patient G.Y. is unable to induce moving phosphenes from the hemisphere with the traumatic lesion of the left occipital area, including V1 (*3*, *8*, *22*, *48*). This supports the idea of the critical role of V1 in visual awareness. However, G.Y. can be aware of temporal change in his blind field (*3*, *8*, *48*). Functional magnetic resonance imaging (fMRI) studies reveal that MT+/V5 is always active when moving stimuli are presented to G.Y. in his blind field and that this activity correlates with G.Y.'s awareness for moving stimuli. An association between cortical activity as detected by fMRI and behavior (awareness in this case) does not establish a causal link between them. More important, awareness for moving stimuli in G.Y. may mean awareness of change (temporal change) rather than awareness of movement. Furthermore, G.Y.'s awareness of motion may well not be "visual awareness" but

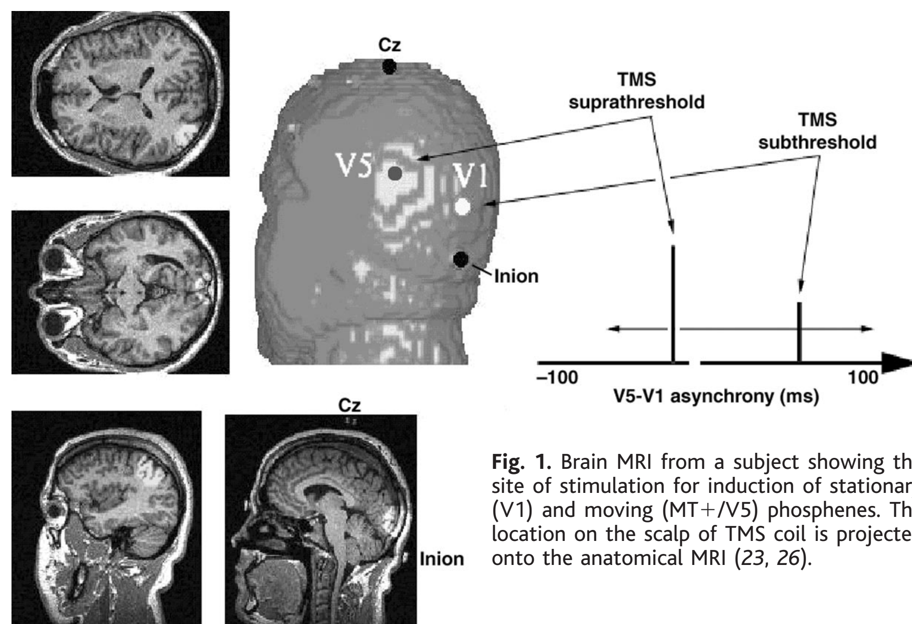

Fig. 1. Brain MRI from a subject showing the site of stimulation for induction of stationary (V1) and moving (MT+/V5) phosphenes. The location on the scalp of TMS coil is projected onto the anatomical MRI (*23*, *26*).

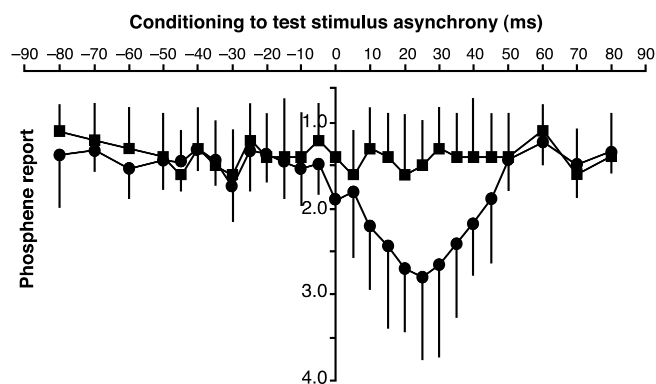

Fig. 2. Mean responses of all subjects (n = 8) to combined stimulation of V5 and V1 (*23*) are shown by the circles. Results of a control experiment of five subjects with paired stimulation to V5 are shown by the squares (*36*). In the V5 to V1 experiment, negative values indicate that V1 received TMS before V5, and positive values indicate that V1 was stimulated after V5. In the V5 to V5 stimulation experiment, the conditioning stimulus was a subthreshold stimulus to V5, and the test stimulus was suprathreshold (*36*). In both experiments, subjects made one of four judgements (*32*). The phosphene elicited by V5 TMS was (1.0) present and moving, (2.0) present but the subject was not confident to judge whether it was moving or moving differently, (3.0) present but stationary, (4.0) no phosphene was observed. TMS over V1 between 5 and 45 ms after TMS over V5 disrupted the perception of the phosphene. A conditioning stimulus to V5 did not affect the effect of the V5 test stimulus regardless of interval (squares). The individual data of all subjects in both experiments are available (*43*).

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rather a metamodal alerting response. Finally, the inability to perceive moving phosphenes evoked by TMS, despite being able to be aware of visually presented moving stimuli, may suggest a necessary distinction between awareness for internally generated percepts, such as imagery, and externally driven visual percepts normally associated with vision.

Our results highlight the importance of the fast feedback projections from V5 to V1 in visual awareness of motion and document the chronometry of the phenomenon. Whether these findings and this TMS-based approach to study perceptual awareness can be extrapolated to externally driven percepts or points out a fundamental distinction between types of perceptual awareness remains to be explored.

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23. TMS was performed using two magnetic stimulators discharged through an 8-shaped coil (wing diameter 43 mm). The current induced in the brain was around 10 to 15 mA/cm^2, the peak magnetic field strength approximately 3.5 Tesla, and the peak electric field 660 V/m. TMS was triggered by a CED 1401 controlled with Spike software. TMS stimuli were delivered with a V5 to V1 stimulation asynchrony (pseudorandomized and interspersed with single-pulse trials) of between –100 ms (that is, V1 before V5) and +100 ms (V5 before V1).
    We identified the scalp location from which single-pulse TMS induced moving phosphenes by stimulating at 100% of stimulator output over a grid of scalp positions centered around a site 4 cm lateral and 2 cm rostral from the inion (Fig. 1) (*20–22*). We identified the lowest intensity that induced a reliable, reproducible phosphene in at least five out of five trials. This moving phosphene threshold was used for stimulation of V5 during the rest of the experiment. Mean threshold was 71% of the stimulator output (range 57 to 94%; SD, 14.3%).
    We then stimulated over the occipital pole to evoke reliable stationary phosphenes in the same spatial location as the V5 moving phosphene (Fig. 1) (*29*). The V1 stationary phosphene threshold was then identified as for V5. An intensity 20% below V1 threshold was used for the V1 to V5 paired TMS experiment. Eighteen subjects were excluded at various stages for failure to perceive reliable V1 or V5 phosphenes.
    The scalp positions for induction of the V5 moving phosphene and the V1 stationary phosphene were marked on a tightly fitting Lycra swimming cap. Both coils were then held in position by using two articulated arms and clamps. An anatomical brain MRI was obtained to identify the brain area targeted by the TMS (Fig. 1). The coils used in our experiment affect functionally precise regions of cortex (*24–27*). When a TMS pulse is delivered to a cortical region, many neurons are simultaneously activated. Activation most likely occurs within 250 μs of the pulse (rise time of 200 μs). These neurons will fire a rapid series of impulses for a few milliseconds, followed by a period of (GABAergic) inhibition that will vary in duration depending on TMS intensity (*27*). At the point of maximal neuronal activation, the stimulated region will have its greatest disruption with respect to the task it is engaged in. However, as neurons recover from the GABAergic inhibition, lasting behavioral effects of TMS will depend on the level of signal required for the task being tested.
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28. Subjects (n = 26) were studied after giving written informed consent to the study, approved by the Institutional Review Board. As explained in (*23*), 18 were excluded, and the experiment was conducted with the remaining eight, five men and three women with a mean age of 31.6 years (range 22 to 39). All subjects were naïve to the aim of the study, but some (four out of eight) had participated in previous TMS studies of phosphene induction.
29. Stability of phosphenes is common, and subjects frequently perceive the same phosphenes in sessions separated by days or weeks. Spatial localization is similarly robust.
30. Subjects were blindfolded and allowed 30 min for dark adaptation. Light deprivation lowers phosphene threshold to TMS (*31*). Before the V1 to V5 experiment, thresholds for the V5 and V1 phosphenes were rechecked to rule out a drifting baseline secondary to the blindfolding.
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33. Backward masking may not be precluded entirely because the MT+/V5 stimulus (when applied first) may prime V1 activity; thus, a stimulus that is subthreshold for induction of phosphenes when applied alone, might become suprathreshold when preceded by a V5 stimulus. However, no subjects reported seeing the V1 phosphene during paired stimulation.
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44. Our results do not to distinguish backprojections from V5 to V1 from those via thalamic relay stations. However, the timing of the phenomenon described matches the physiologic findings (*9–15*) regarding connections between V5 and V1.
45. With occipital TMS it is difficult not to stimulate V1, but we cannot be certain that only V1 was stimulated. However, spread of TMS effects to V2 or V3 cannot account for our results. For example, there is no loss of awareness in patients or monkeys with damage to V2 or V3 (*46*, *47*).
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