or recurrently evoked electrical activity (15, 16), reducing the excitability of KCs is predicted to prolong reaction times.

We therefore targeted the inwardly rectifying potassium channel Kir2.1 under FoxP-GAL4 control to αβ core (αβ) and γ KCs while tuning expression levels with temperature-sensitive GAL80° (17). Flies expressing low Kir2.1 levels behaved like homozygous FoxP-GAL4;GAL80° mutants: Reaction times increased in a difficulty-dependent manner relative to parental controls (Fig. 3, D and E), but accuracy was maintained (Fig. 3C). Boosting the expression of Kir2.1 exacerbated this phenotype: Despite a further increase in reaction times (Fig. 3, G and H), FoxP–Kir2.1 flies now performed near chance level in difficult discriminations (Fig. 3F), echoing the accuracy defects of severe FoxP alleles (Fig. 2K).

To bolster and refine our identification of FoxP-GAL4–positive KCs as sites of FoxP action, we compared the consequences of introducing Kir2.1 with those of reducing FoxP expression, using a panel of GAL4 lines whose expression domains included all or parts of the FoxP-GAL4 pattern: OK107-GAL4 targets all KCs (18), NP60924-GAL4 and NP71755-GAL4 label αβ, neurons (14, 19), and NP131-GAL4 marks γ KCs (14, 20) (Fig. 4, A and B). Knockdown of FoxP in αβ, but not γ, KCs prolonged reaction times in difficult discriminations (Fig. 4, C and D, and fig. S4B), mirroring the differential impact of Kir2.1 on these neuronal populations (Fig. 4, E and F). Attempts to disrupt FoxP expression with the help of FoxP-GAL4 itself produced marginal effects (fig. S5A), probably due to inadequate FoxP-GAL4 levels. Consistent with this interpretation, significant decision phenotypes were seen with all three of the GAL4 drivers capable of expressing high levels of FoxP-GAL4 in αβ KCs (Fig. 4C and fig. S5, B and C).

The evolution of a decision toward commitment requires the progression of neural activity from a choice-neutral to a choice-specific state. Mutations in FoxP evidently slow this progression, at least in part by interfering with the function of αβ neurons. The same neurons have been implicated in value-based decisions, such as choices between odors associated with punishments or rewards (19). It remains untested whether value judgments also incur a difficulty-dependent cost of decision time. Nonetheless, the available evidence suggests that ~80 FoxP-GAL4–positive αβ KCs form part of a versatile decision circuit that processes sensory information in one context and remembered value in another.

As a transcription factor (21, 22), FoxP could act during development to specify synaptic connections and/or throughout life to regulate neuronal function. Vertebrate FoxP homologs have been linked to both types of processes (23–26) and have been attributed critical roles in cognitive development (24, 27, 28), vocal communication (21, 26, 29), and motor control (23, 25). A potential commonality between these processes and decision-making is their unfolding over time: Neurons representing trains of thought, strings of syllables, chains of motor commands, or accumulating evidence must all step through ordered activity sequences. It is therefore tempting to speculate that understanding the function of an ancestral FoxP gene (22) might reveal fundamentals of temporal processing (30).

REFERENCES AND NOTES

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NEUROSCIENCE
Rapid Hebbian axonal remodeling mediated by visual stimulation
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We examined how correlated firing controls axon remodeling, using in vivo time-lapse imaging and electrophysiological analysis of individual retinal ganglion cell (RGC) axons that were visually stimulated either synchronously or asynchronously relative to neighboring inputs in the Xenopus laevis optic tectum. RGCs stimulated out of synchrony rapidly lost the ability to drive tectal postsynaptic partners while their axons grew and added many new branches. In contrast, synchronously activated RGCs produced fewer new branches, but these were more stable. The effects of synchronous activation were prevented by the inhibition of neurotransmitter release and N-methyl-D-aspartate receptor (NMDAR) blockade, which is consistent with a role for synaptic NMDAR activation in the stabilization of axonal branches and suppression of further exploratory branch addition.

neuronal activity and molecular cues cooperate to form precise neuronal circuits (1, 2). Experimental blockade of action potential firing or synaptic transmission (3–5), particularly involving N-methyl-D-aspartate receptors (NMDARs) (6–9), degrades axonal projections in the developing nervous system. The precise pattern of neuronal firing is believed to be important for instructing connection refinement because disrupting the temporal correlation of firing between neighboring neurons, even while sparing overall activity levels, results in axons with diffuse terminal arbors (10, 11). Hebbian plasticity, an appealing model for activity-dependent refinement of circuits, posits that synapses may be strengthened or stabilized when the presynaptic cell participates in making its postsynaptic partner fire (12). Convergent inputs firing synchronously

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would cooperatively excite the postsynaptic neuron to fire. Thus, Hebbian plasticity in principle should aggregate active inputs, effectively leading to circuit refinement (6, 13, 14). The detailed mechanisms by which such remodeling actually occurs remain poorly understood.

The developing retinotectal system of the albino *Xenopus laevis* tadpole is amenable both to live imaging and in vivo electrophysiological characterization. RGC axons in *Xenopus* normally project to the contralateral optic tectum. Occasionally, a single mistargeted ipsilaterally projecting retinal ganglion cell (RGC) axon is observed (Fig. 1, A and B) (15). Using post mortem intracocular 1,1-dioctadecyl-3,3,3,3′-tetramethylindocarbocyanine perchlorate (DiI) injections to label all RGCs (fig. S1) in stage-46 to -48 tadpoles when the retinotectal projection is established but still refining (16), we detected no ipsilateral RGC axon in the majority of cases (61%). However, animals with one (21%), two (9%), or more (9%) ipsilaterally projecting neurons were occasionally observed (Fig. 1C). Ipsilaterally projecting axons are thus unlikely to represent a specific class of RGCs but rather reflect random pathfinding errors at the optic chiasm. We exploited the fact that the lone ipsilateral and surrounding contralateral RGCs could be independently visualized in order to test the role of correlated activity on synaptic maintenance and axonal refinement.

We first tested whether ipsilaterally projecting RGCs form functional synapses onto tectal neurons. To identify postsynaptic partners of a single ipsilateral RGC (Fig. 1D), we made transgenic tadpoles expressing photoactivatable green fluorescent protein (PA-GFP) (17, 18) under the neuronal β-tubulin promoter and then bulk-electroporated plasmid encoding tdTomato into one eye so as to label an ipsilaterally projecting axon (16). Scanning 780-nm light in a volume immediately surrounding the ipsilateral axon (Fig. 1E) resulted in photoactivated PA-GFP backfilling a few tectal cells from their dendrites (Fig. 1F). In vivo perfused patch recordings of compound synaptic currents (CSCs) from these cells, in response to alternating light flashes presented to each eye through optical fibers (Fig. 1D), revealed that all visually responsive neurons could be driven through the contralateral eye (Fig. 1G). Some cells also responded to stimulation of the ipsilateral eye (Fig. 1H). The ipsilateral CSC (5.31 ± 1.06 pC) was consistently smaller than the contralateral CSC (11.80 ± 2.33 pC).

We next investigated how correlated visual stimulation modifies synaptic strength. Ten min of baseline light-evoked CSCs were measured by alternating a 10-ms light flash to each eye every 30 s, holding the cells at −60 mV (16). Cells were then switched to current clamp mode so as to allow spiking, and one of two training paradigms was applied: “synchronous stimulation,” in which both eyes were stimulated together every 2 s, or “asynchronous stimulation,” in which the eyes were stimulated 1 s apart (Fig. 1I). Both eyes experienced light flashes every 2 s, with only the relative timing differing across paradigms. After 10 min of training stimulation, CSCs were again measured at −60 mV in order to test the relative efficacy of the two eyes at driving a response. This cycle of training and testing was repeated three times per cell. After the first 10 min of training, asynchronous stimulation resulted in a loss of...
synaptic strength for the ipsilateral eye, whereas synchronous stimulation maintained the relative contribution of the ipsilateral eye at or above baseline levels (Fig. 1J and Fig. S2). By the third round of visual training, five of the eight eyes with asynchronously stimulated inputs, but none of the synchronously stimulated cases (n = 10 cells) were weakened to <20% of baseline synaptic strength. These changes stably persisted for at least 13 min after training stimulation (Fig. 1J, open symbols).

We next examined whether correlated activity also regulates the growth of developing RGC axons. After retinal electroporation of enhanced GFP (EGFP) plasmid, we selected animals with single EGFP-labeled ipsilaterally or contrateraterally projecting axons (16). Using a video display directly beneath rearing tanks, free-swimming tadpoles were continuously presented with full-field stroboscopic flashes (0.5 Hz) to synchronize both eyes, or large moving black dots to independently activate the two eyes (Fig. 2A). Dot-rearing leads to asynchronous stimulation of the ipsilateral RGC axon but synchronous activation of contralateral RGC axons relative to other contralateral eye inputs. RGC axons were imaged in vivo with two-photon laser scanning microscopy daily for 5 days (Fig. 2B). All three groups that were reared under synchronizing conditions exhibited comparable axon growth and branch elaboration (Fig. 2, C and D). In comparison, dot-reared ipsilateral RGC axons, which experienced asynchronous stimulation relative to neighboring inputs, grew faster, with larger, more diffuse arbors by 2 days of stimulation.

To investigate axonal growth and branching at higher temporal resolution, we collected two-photon time-lapse images of individual ipsilateral axon arbors in tadpoles every 10 min for 5.5 hours while simultaneously presenting stimuli to the eyes through a pair of optical fibers (Fig. 3, A to C) (16). One of two stimulation protocols was applied (Fig. 3B). In the first protocol [dark-asynchronous-synchronous (DAS)], baseline images in darkness were collected for 90 min, after which each eye was stimulated at 0.5 Hz with a 5-ms light flash. Asynchronous stimulation (1 s apart) was presented for the first 2 hours, followed by synchronous stimulation for the last 2 hours. The second protocol [dark-synchronous-asynchronous (DSA)] was identical, except that the 2-hour asynchronous stimulation followed the synchronous stimulation.

Under the DAS protocol, asynchronous visual stimulation rapidly produced a robust increase in axon branch dynamics compared with that in darkness (Fig. 3D and movie S1). Within 20 min of asynchronous stimulation, the rate of new branch additions significantly increased (Fig. 3, E and F). Branches were also eliminated more rapidly during asynchronous stimulation (Fig. 3, G and H), which is consistent with an overall increase in dynamic remodeling. Furthermore, asynchronous stimulation significantly augmented the elongation lengths of branch tips as compared with growth in darkness (Fig. 3, I and J).

Changing from asynchronous to synchronous stimulation, without altering stimulation intensity or frequency, significantly decreased the numbers of branches added (Fig. 3, E and F) and lost (Fig. 3, G and H), in addition to reducing branch elongation (Fig. 3, I and J, and movie S2). The decrease in newly added branch tips was more gradual, only reaching significance by 40 min after the onset of synchronized stimulation. Consistent with these observations, animals that were presented first with synchronous stimulation (DSA protocol) did not show a significant increase in branch additions (Fig. 3, E and F) or elongation (Fig. 3, I and J) until asynchronous stimulation was presented.

Although synchronous stimulation decreased the addition of new branches compared with asynchronous stimulation (Fig. 3F), new branches formed during synchronous stimulation were in fact more stable than those added during asynchronous stimulation. They had longer lifetimes, with a significantly larger fraction surviving longer than 30 min (Fig. 3, K and L). Thus, activation of a RGC rapidly results in highly dynamic exploratory branches when its firing is mismatched to that of surrounding inputs, but coactivation with its neighbors suppresses this probing and stabilizes contacts.

![Fig. 2. Rearing tadpoles with asynchronous binocular stimulation enlarges ipsilateral RGC axon arbors.](image)

**Fig. 2. Rearing tadpoles with asynchronous binocular stimulation enlarges ipsilateral RGC axon arbors.** (A) Animals were reared for 4 days with stroboscopic or moving-dot stimuli. (B) Ipsilaterally and contrateraterally projecting RGC axons from animals reared under each experimental condition, imaged once daily. (C) Total axonal arbor size and (D) branch-tip number were greatest in the asynchronous stimulation condition (ipsilateral axon, dot-reared) (*P < 0.05, two-way ANOVA mixed design, indicating a difference between ipsilateral dot-reared and all other groups by Tukey’s post-test, n = 8 axons for ipsilateral strobe, 8 axons for ipsilateral dots, 6 axons for contralateral strobe, and 12 axons for contralateral dots).
Axonal growth may be controlled by retrograde cues from postsynaptic cells (29). One option is that RGCs up-regulate dynamic growth in response to increased firing, but synaptic activity correlated with the postsynaptic cell leads to branch-suppressing or -stabilizing retrograde signals that inhibit this growth. Another possibility is that asynchronous input may be detected by the postsynaptic neuron, prompting it to deliver growth-promoting signals to the dissenting axon terminal. These two putative mechanisms predict opposite outcomes of blocking synaptic transmission between an axon and its partner, with increased branching under the first model and reduced branching under the second.

We tested the effects of blocking synaptic transmission by expressing tetanus neurotoxin light chain fused to EGFP (TeNT-Lc:EGFP) (2) in the ipsilaterally projecting RGC axon (Fig. 4A and movie S3). Because synaptic NMDARs have been proposed to act as correlation detectors because of their voltage-dependent response to glutamate (9) and have previously been implicated in activity-dependent retinotectal map refinement (7, 13), we also tested the effects of NMDAR blockade by treating animals with the blood-brain barrier permeant noncompetitive NMDAR antagonist MK-801 (Fig. 4B and movie S4). Because synchronized stimulation requires ~1 hour to achieve its full effect on branch addition (Fig. 3E) and loss (Fig. 3G) rates, we separately analyzed dynamics during the first and second hours of each stimulation period (Fig. 4, C and D).

Control ipsilateral axons significantly increase dynamic branch additions (Fig. 4C) and losses (Fig. 4D) during the first hour of asynchronous stimulation and return to baseline levels by the second hour of synchronous stimulation. In contrast, both TeNT-Lc:EGFP–expressing and MK-801–treated axons showed their largest increases in branch additions and losses during synchronous stimulation. To further ascertain the relative responses of axons to asynchronous versus synchronous stimulation on a cell-by-cell basis, we divided the mean rate of branch addition or loss during the last 90 min of asynchronous stimulation by that during the last 90 min of synchronous stimulation so as to generate addition and loss ratios for each cell (Fig. 4, E and F). Expression of TeNT-Lc:EGFP and blockade of NMDARs both reduced these ratios to ~1, indicating that these axons could not differentiate synchronous and asynchronous stimulation. We also examined the stability of branches in TeNT-Lc:EGFP–expressing (Fig. 4G) and MK-801–treated (Fig. 4H) ipsilateral RGC axons. Branch survival times did not significantly differ between stimulation conditions, which is in clear contrast to control axons, which form longer lasting branches under conditions of synchronous stimulation (Fig. 3, K and L). Taken together, these findings suggest that NMDAR-mediated synaptic transmission leads to increased branch stability and a reduction in branch dynamics during synchronous activation through the action of a branch-suppressing signal.

These experiments demonstrate how correlated neural activity helps orchestrate the morphological remodeling of developing axons into precisely organized maps. Sensory stimulation promotes exploratory branching and outgrowth of RGC axons within their target structure. Axons that may have extended into inappropriate territory, where their firing patterns do not match those of nearby inputs, would fail to maintain...
stable functional and structural contacts and continue actively elaborating in search of appropriate partners. In contrast, axons that form synaptic contacts onto partners that receive other highly active inputs will engage cooperative mechanisms to stabilize those contacts and the branches on which they reside. Our experiments confirm that glutamate release and activation of NMDARs are critical steps in initiating a branch-stabilizing signal, although its molecular identity remains unknown. Because a single axon firing out of synchrony with numerous synchronized inputs does not appear to benefit from the stabilization signals that these active axons presumably receive, the stabilizing signal must be very precisely spatially or temporally restricted, which rules out long-lived, highly diffusible molecules as plausible candidates. The idea that this signal likely originates in the postsynaptic cell is supported by earlier experiments that demonstrated that calcium/calmodulin-dependent protein kinase II activity in tectal neurons can retrogradely modulate RGC axon growth (20).

However, our data do not exclude a potential contribution by the surrounding glial cells (27) or the possibility that putative presynaptic NMDARs (22, 23) on RGCs may be involved.

Our observation that visual stimulation drives a rapid increase in branching and growth is consistent with earlier studies in the retinotectal projections of zebrafish and mouse, in which suppression of RGC firing through expression of inward-rectifying potassium channels inhibited the dense elaboration of branches (4, 24). Similarly, the enlarged arbors reported in zebrafish RGCs expressing TeNT-Lc match our findings that this treatment prevents the down-regulation of branching during correlated activity (5). These authors argued for a model based on activity-dependent competition to explain their data; however, our results suggest that correlation detection may offer an important alternative explanation for these findings. Activity-dependent competition is useful to explain pathological conditions such as amblyopia but likely plays a minor role in normal map development. A correlated stimulation approach similar to ours was recently reported that used transgenic mice expressing channelrhodopsin-2 in RGCs for stimulation during early postnatal development. The consequences of synchronous and asynchronous stimulation in that system were highly consistent with our findings, with synchronous stimulation leading to ectopic ipsilateral eye projections presumably stabilized in contralateral eye territory (II).

Here, we present live observations of axonal structural plasticity directed by patterned visual stimuli in vivo that support the Hebbian prediction that coactive inputs are stabilized (25). Our data also demonstrate the up-regulation of exploratory growth over days in the absence of correlated firing (26) and growth suppression when inputs are inactive (27). The speed with which physiological visual stimuli can drive such changes—reducing the strength of synaptic currents evoked through the ipsilateral eye after just 10 min of asynchronous visual stimulation and significantly increasing the rate of new branch addition in under 20 min—was unanticipated.

**Fig. 4.** Synaptic neurotransmission and NMDARs mediate arbor stabilization during synchronous stimulation. (A and B) Cells (A) expressing TeNT-Lc:EGFP or (B) bathed in MK-801, with example reconstructions under different stimulation conditions, as in Fig. 3. (C and D) Transmitter release and NMDAR activation are both necessary to reduce branch dynamics during synchronous stimulation. (C) Mean branch additions for each cell during asynchronous versus synchronous stimulation. (D) Corresponding data for branches lost (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, two-way ANOVA mixed design with Tukey’s post-test). (E) Ratios of numbers of branches added for each cell during asynchronous versus synchronous stimulation. (F) Ratios for branches lost (*P < 0.05, **P < 0.01, Kruskal-Wallis test with Dunn’s post-test). (G and H) Survival plots for branches formed during asynchronous and synchronous stimulation in (G) TeNT-Lc:EGFP–expressing or (H) MK-801–treated EGFP-expressing cells. Unlike controls (Fig. 3, K and L), synchronous stimulation did not significantly enhance branch stability in these cells (log-rank test; n = 242 asynchronous and 300 synchronous branches from six TeNT-Lc:EGFP–expressing axons, n = 206 asynchronous and 191 synchronous branches from six MK-801–treated axons). Error bars indicate [(C) and (D)] SEM and [(G) and (H)] 95% CI.

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Identifying the specific signals that mediate correlation-dependent structural plasticity will be greatly facilitated by exploiting the experimental protocol presented here.

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16. Materials and methods are available as supplementary materials on Science Online.
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MICROBIAL GENOMICS
Stop codon reassignments in the wild
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The canonical genetic code is assumed to be deeply conserved across all domains of life with very few exceptions. By scanning 5.6 trillion base pairs of metagenomic data for stop codon reassignment events, we detected recoding in a substantial fraction of the >1700 environmental samples examined. We observed extensive opal and amber stop codon reassignments in bacteriophages and of opal in bacteria. Our data indicate that bacteriophages can infect hosts with a different genetic code and demonstrate phage-host antagonism based on code differences. The abundance and diversity of genetic codes present in environmental organisms should be considered in the design of engineered organisms with altered genetic codes in order to preclude the exchange of genetic information with naturally occurring species.

Since the discovery of the genetic code and protein translation mechanisms (1), a limited number of variations of the standard assignment between unique base triplets (codons) and their encoded amino acids and translational stop signals have been found in bacteria and phages (2–7). Given the apparent ubiquity of the canonical genetic code, the design of genomically recoded organisms with noncanonical codes has been suggested as a means to prevent horizontal gene transfer between laboratory and environmental organisms (8–10). It is also predicted that genomically recoded organisms are immune to infection by viruses, under the assumption that phages and their hosts must share a common genetic code (6). This paradigm is supported by the observation of increased resistance of genomically recoded bacteria to phages with a canonical code (9). Despite these assumptions and accompanying lines of evidence, it remains unclear whether differential and noncanonical codon usage represents an absolute barrier to phage infection and genetic exchange between organisms.

Our knowledge of the diversity of genetic codes and their use by viruses and their hosts is primarily derived from the analysis of cultivated organisms. This is due to our limited access to genome sequences from uncultivated organisms, which are estimated to account for 99% in prokaryotes (11). Advances in single-cell sequencing and metagenome assembly technologies have enabled the reconstruction of genomes of uncultivated bacterial and archaeal lineages (12–14) and the discovery of a previously unknown reassignment of TGA opal stop codons to glycine (4, 5,14). These initial findings suggest that large-scale systematic studies of uncultivated microorganisms and viruses may reveal the extent and modes of divergence from the canonical genetic code operating in nature.

To explore alternative genetic codes, we carried out a systematic analysis of stop codon reassignments from the canonical TAG amber, TGA opal, and TAA ochre codons in assembled metagenomes and metatranscriptomes from environmental and host-associated samples, single-cell genomes of uncultivated bacteria and archaea, and a collection of viral sequences (Fig. 1A) (15). All sequence data were obtained from the Integrated Microbial Genomes (IMG) database (16). This global collection of sequences comprised 1776 samples from 146 studies, including 750 samples obtained from 17 human body sites (fig. S1) (17). In total, 5.6 terabases of sequence data, including 450 Gb of contiguous sequences (contigs) greater than 1 kb, were analyzed. All samples were classified into human-associated, other host–associated, soil, marine, or freshwater environments according to their metadata (15, 18).

We used a statistic of increased coding potential under alternate genetic codes as calculated by ab initio gene finder Prodigal (19), which was selected for its low rate of false-positive predictions (15). Contigs showing significantly higher coding potential when annotated with modified translation tables were forwarded to filtering and quality control to confirm stop codon reassignment through multiple sequence alignments to known homologs from the National Center for Biotechnology Information protein database (Fig. 1A) (15).

By applying this approach to 450 Gb of contigs larger than 1 kb in size, we identified 31,415 contigs with evidence of stop coding reassignment, adding up to a total of 198 Mb of recoded DNA (Fig. 1A). No recoding was observed in the metatranscriptome data. Varying ratios of reassigned stop codons to glycine (4, 5, 14). These initial findings suggest that large-scale systematic studies of uncultivated microorganisms and viruses may reveal the extent and modes of divergence from the canonical genetic code operating in nature.