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Synaptic dynamism measured over minutes to months: age-dependent decline in an autonomic ganglion

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Naturally occurring rearrangements of synaptic terminals are common in the nervous systems of young mammals, but little is known about their incidence in adults. Using transgenic mice that express yellow fluorescent protein (YFP) in axons, we repeatedly imaged nerve terminals in the parasympathetic submandibular ganglion. We found that the pattern of synaptic branches underwent significant rearrangements over several weeks in young adult mice. In older mice, rearrangements were less common, and synaptic patterns on individual neurons were recognizable for many months to years. Axonal branches frequently retracted or extended on a time scale of minutes in young adult mice, but seldom in mature animals. These results provide direct evidence for a decrease in plasticity of interneuronal connections as animals make the transition from young adulthood to middle age. The long-term stability of synaptic patterns could provide a structural basis for the persistence of memory in the adult nervous system.

During postnatal development, synaptic connections are both established and eliminated by processes that depend in part on early experience[1–4]. A variety of physiological, anatomical and behavioral studies suggest that, to some degree, the adult nervous system retains a capacity to reorganize in the face of experience and pathology[5–12]. Nonetheless, the malleability in the nervous system decreases with age[1,2,13–17]. For example, visual deprivation causes amblyopia in children up to 7 or 8 years of age, and recovery from amblyopia after sustained treatment can be obtained in teenagers, but generally not in adults[17]. It remains unknown, however, what causes this loss of functional malleability. One possibility is that it results from an age-related decline in the structural plasticity of synapses. Here we tested the plausibility of this hypothesis by asking whether naturally occurring changes in nerve terminal structure take place in adults, and, if so, whether the incidence of these modifications changes with age.

Previous work using time-lapse imaging shows that at least one synaptic connection, the neuromuscular junction, is structurally quite stable in adult animals but highly dynamic in neonates. For example, an individual neuromuscular junction in an adult mouse is structurally recognizable following intervals of many months[18,19], whereas axons in young mice remodel extensively over several days[20]. The degree to which synapses on neurons are stable is more controversial. It has been shown that dendritic processes undergo substantial remodeling in sympathetic ganglia[21,22], and changes in presynaptic axonal terminals have been observed over weeks in submandibular ganglia of young adult mice[23]. More recently, transgenic mice expressing green fluorescent protein (GFP) and its spectrally distinct variants in the cytoplasm of axons and dendrites[24] have significantly improved the quality and ease of intravital time-lapse microscopy. Two *in vivo* studies of GFP-positive dendrites generated different views on the stability of postsynaptic sites on adult neurons[16,25]. Whatever the case for dendritic spine stability, however, imaging postsynaptic structures does not provide direct evidence of the stability (or lack thereof) of presynaptic terminals.

To investigate presynaptic axon terminal stability as a function of age, we imaged individual synapses in a parasympathetic ganglion over weeks, and, in some cases, years. By using laser scanning confocal microscopy to image the preganglionic innervation to the same ganglion cell terminals over time, we assessed the stability of terminal arbors made by preganglionic axons onto parasympathetic neurons. Our results suggest that synapses become progressively more stable as animals age.

RESULTS

We used the parasympathetic submandibular ganglion of the neck because its neurons are either completely isolated or distributed in small clusters on salivary ducts, making them easy to image and relocate[23,26]. Moreover, these neurons are almost completely adendritic, so nearly all of their inputs are on their somata[27,28]. We found that preganglionic axons innervating submandibular neurons are YFP-positive in several previously characterized lines of transgenic mice that express YFP under the control of neuron-specific regulatory elements from the *Thy1* gene[24]. Importantly, postganglionic neurons are largely YFP-negative in these mice, so axon terminals can be viewed without interference (**Fig. 1a,b**). Finally, to confirm that synaptic terminals were YFP-positive, we immunostained ganglia from transgenic mice with antibodies against the synaptic vesicle protein SV2. YFP labeled both synaptic boutons and inter-bouton regions of

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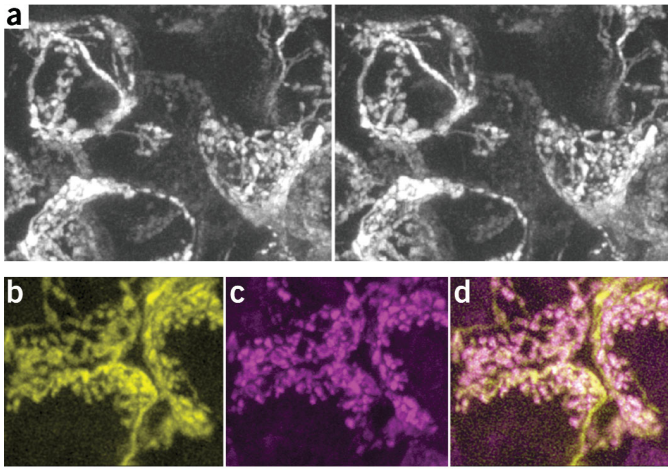

Figure 1 YFP labeling of preganglionic axons and synaptic boutons in the submandibular ganglion. (**a**) Stereo pair of a confocal reconstruction. The preganglionic axons and nerve terminals ramify around the spherical cell bodies of the ganglion cells. (**b**) YFP labeling of axonal terminals in three neurons. (**c**) Immunostaining of synaptic boutons with antibodies against synaptic vesicle protein SV2. (**d**) Colocalization (pink) of YFP (yellow) and antibody (purple) labeling of synaptic boutons. All YFP-positive varicosities are rich in synaptic vesicles, whereas pre-terminal and inter-bouton branches are YFP-rich but SV2-poor.

preganglionic axons (**Fig. 1b–d**). SV2 immunolabeling was indistinguishable in YFP-expressing and control mice at several ages (data not shown), suggesting that the expression of YFP throughout postnatal life does not alter the number or distribution of synapses.

To monitor the stability of neuronal connections, preganglionic arbors and their synaptic boutons were reconstructed from image stacks acquired *in vivo* with confocal microscopy (see Methods). We were able to relocate and image synaptic connections on the same ganglion cell multiple times over minutes to years. **Figure 2** shows four views of the preganglionic innervation to a ganglion cell that was first imaged at postnatal day 40 (P40), about the time of sexual maturity (5–6 weeks). Because this ganglion cell was well separated from other neurons, the preganglionic terminal axon branches observed were unambiguously the source of its innervation. Both addition (arrows in **Fig. 2b**,**c**) and loss (asterisk in **Fig. 2b**) of some terminal branches occurred between views. However, the arrangement of synapses remained distinctive and recognizable over 109 d; regions of the cell soma having synaptic boutons at the first view possessed boutons at subsequent views, and most areas that were not innervated at the first view remained unoccupied subsequently.

To quantify the extent to which synaptic patterns remained stable over various time intervals, we calculated the degree to which the terminal arbor imaged in a first view overlapped with that imaged in a later view. Because the innervation would appear stable if the cell surface were completely covered with boutons or completely uninnervated, we controlled for this false apparent stability by calculating the degree to which the labeled arbor overlapped with a randomized redistribution of the labeled arbor (see Methods). The index of stability is defined as the difference between two measurements: the change in pattern observed between the first view and a later view, and the change seen between the first view and a randomization of the pattern of innervation. The larger the index, the more stable the synaptic pattern remained over time. **Fig. 2e** shows the stability index of the arbor shown in **Fig. 2a–d**. Over each interval, the arbor appeared significantly more stable than could be accounted for by chance (different from randomized distribution, $P < 0.0001$, *t*-test). Similar results were obtained in four isolated ganglion cells over similar intervals.

Despite the overall stability of synaptic patterns, the arbors never appeared identical between views. Rather, unequivocal changes in the position and number of boutons occurred on every cell imaged, leading to the prediction that alterations would eventually accumulate to the point that the entire pattern would be completely unrecognizable. To test this idea, we imaged neurons for up to 518 d, starting at 4 or 12 months of age. Of 32 cells imaged over an interval of >200 d, all but one (97%) could be relocated at the second view. The distribution of axonal branches and boutons on many neurons was still similar over these very long time intervals (**Fig. 3**). Thus, on some neurons at least, synaptic connections can be stably maintained for large portions of an animal's lifetime.

Next, we asked whether the stability of synaptic patterns varies as a function of age. Terminal branches were reconstructed from a total of 67 neurons, each viewed at least twice at intervals of 2 or 4 weeks. We found that the degree of stability between views increased significantly with age. In almost every neuron viewed at ~1.5 months

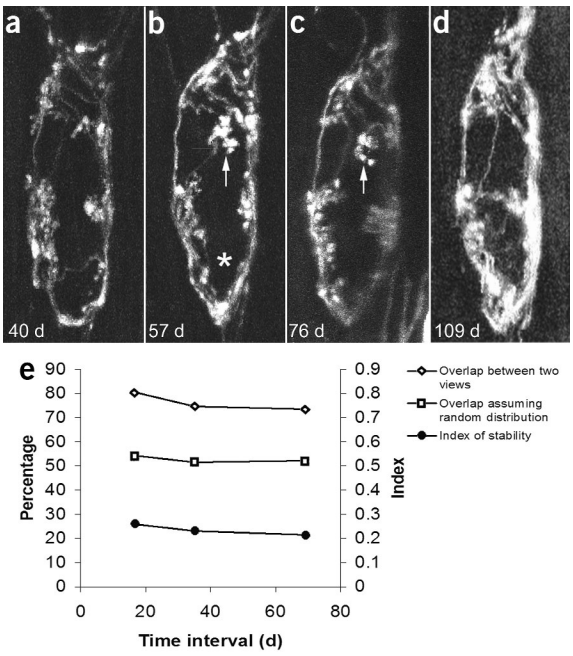

Figure 2 Overall stability of synaptic connections as well as some local remodeling over weeks to months. (**a**) Three-dimensional reconstruction of synaptic terminals on an individual submandibular ganglion neuron viewed initially at the age of 40 d. The same neuron was revisualized three times at the ages of 57 (**b**), 76 (**c**) and 109 (**d**) days. The general distribution of synaptic terminals was recognizably similar between views with addition (arrows) and elimination (*) of a few axonal branches. Small changes in cell shape reflect rotation and/or stretching of the submandibular duct. Such changes do not hinder assessment of either the overall stability or the localized changes in synaptic pattern. (**e**) Innervation patterns on this ganglion cell were compared to assess the extent of change. At all intervals examined, the degree of overlap was significantly higher than would occur if synapse location had randomized. Using an index of stability (Methods) we found relatively little change in the index in the same cell over 17, 36 or 69 d. Scale bar, 15 μm.

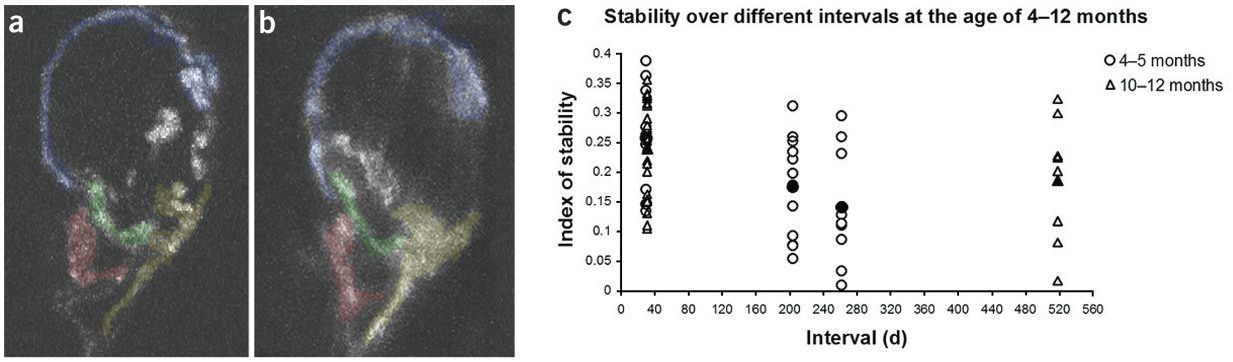

Figure 3 Imaging ganglion cells over months or years showed stability of synaptic patterns. (**a**,**b**) The terminal arbor of one neuron viewed at 1 year of age (**a**) and 518 d later (**b**). Regions of the axon terminal arbor are colorized to show the stability of certain branches. Branches that appeared or disappeared were also seen; these are not colored. (**c**) Scattergram of the index of stability for neurons such as the one shown in **a** and **b** at various intervals between views. Whether beginning at 4–5 months (○; mean, ●) or 10–12 months (△; mean, ▲) there is on average only a slight change in the arbors over substantial times, causing a slow decline in the stability index.

of age, some boutons appeared or disappeared over a 2–4 week period (**Fig. 4a**), whereas few changes were apparent in 1-year-old animals (**Fig. 4b**). Quantitation using the stability index described above confirmed that stability increased progressively within the first 4 months of postnatal life (**Fig. 4c,d**). A simpler analysis that compared positions of individual synaptic boutons regardless of the amount of innervation on cell somata showed that over 2-week (**Fig. 4e**) and 1-month intervals (**Fig. 4f**), synapses were also progressively less likely to move with advancing age.

Despite the age-related decrease in synaptic terminal remodeling, we noted some additions and losses of terminal branches at all ages studied. Given the long intervals between views, we could not determine whether these rearrangements actually took weeks to occur, or rather reflected remodeling that occurred on a faster time scale. To address this issue, we modified our procedure to monitor axonal inputs at intervals of 1–5 min over periods of 2 h. The gross structure of arbors changed little over this time, but some small branches underwent rapid extension and retraction within minutes (**Fig. 5a,b**). In some cases, rapidly extending or retracting processes reversed themselves, leading to no significant net alterations over the time intervals studied. If these rapid changes were the source of the long-term changes seen, then we would expect that the incidence of these changes should decrease with age. Indeed, significant rearrangements were found in ~70% of cells (9/13 cells)

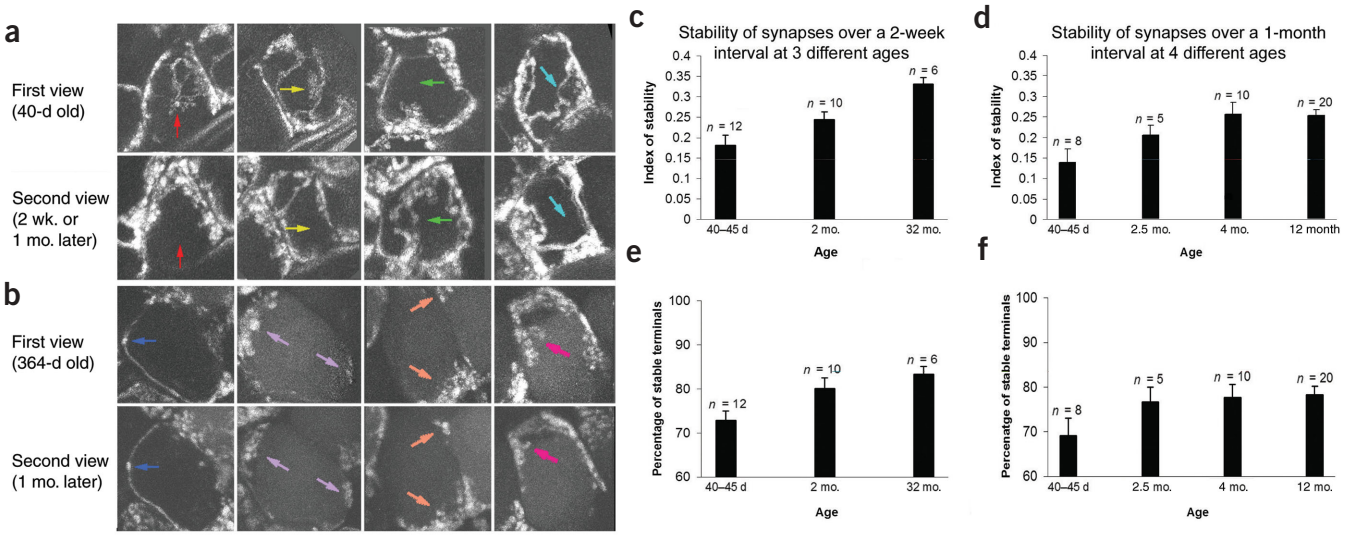

Figure 4 Distribution of synaptic connections is more stable in mature animals than in young adults. Neurons were imaged twice over 2 or 4 weeks in adult mice of various ages. (**a**) Upper two rows. In 40-d-old mice, ganglion cells were relocated over intervals of 2 weeks (first two columns) or 1 month (last two columns). Despite similarities in the shape of the neurons, the distribution of branches and boutons on the cell soma changed in each case. Arrows point to corresponding sites at the two time points for each pair of images where some significant change in the bouton distribution was evident. (**b**) In contrast, in 1-year-old animals, ganglion cells (which are somewhat larger) now have very similar bouton distributions over a one-month interval. Arrows point to features that are largely the same over one month. (**c**,**d**) Quantification of index of stability over intervals of 2 weeks (**c**) or a month (**d**) in mice of different ages shows that synaptic terminals showed significantly more stability as animals aged (over 2 weeks (**c**): $P < 0.0001$ between 40–45 d and 32 months of age; $P < 0.005$ between 2 and 32 months of age. Over 1 month (**d**): $P < 0.05$ between 40–45 d and 4–12 months of age). (**e**,**f**) Percentage of stable terminals over intervals of 2 weeks (**e**) or a month (**f**) in mice of different ages shows similar age-related synaptic stability as seen in **c** and **d** (over 2 weeks (**e**): $P < 0.04$ between 40–45 d and 2 months of age; $P < 0.002$ between 40–45 d and 32 months of age. Over 1 month (**f**): $P = 0.065$ between 40–45 d and 4–12 months of age).

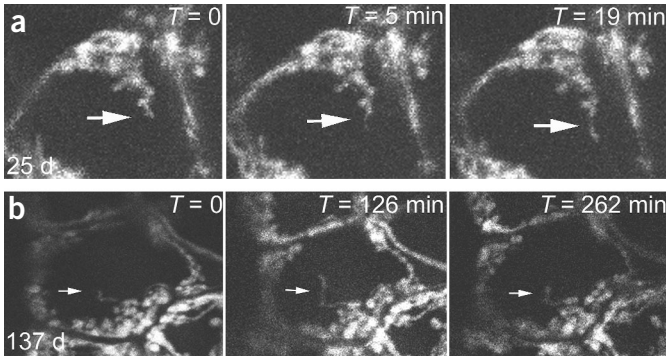

Figure 5 *In vivo* time-lapse recording of rapid change of synaptic terminal arbors in young mice. (**a**) Rapid arbor extension (arrow) was observed within minutes in this ganglion neuron at the age of 25 d. (**b**) Extension and retraction of an arbor was less common in older animals; this was the only case observed in ten cells monitored at >4 months of age.

imaged for 2 h at 25–29 d of age, but only in 1 of 10 cells imaged in >4-month-old mice. The age dependence of this rapid remodeling is consistent with the idea that it underlies the rearrangements seen over weeks to months.

DISCUSSION

We used transgenic and imaging tools to view synaptic patterns on identified neurons *in vivo* over periods of >1 year. By imaging presynaptic elements on identified postsynaptic cells, we observed that the degree of presynaptic rearrangement over intervals of weeks decreased as a function of age during the first 4 months of postnatal life. In addition, our studies show that most, if not all, individual neurons in the submandibular ganglion persist over much of the animal's lifetime (maximum interval observed, >2 years; mouse life-span, ~3 years). The persistence of neurons and the progressive stabilization of synapses led to retention of a substantial subset of synaptic connections over the animal's adult life.

The technical challenges of monitoring presynaptic terminals over extended periods of time are greater than those associated with monitoring postsynaptic structures, as presynaptic terminals have fewer unambiguous structural features. This problem is somewhat ameliorated in the submandibular ganglion where the axon branches envelop the postsynaptic neuronal cell bodies.

A previous study in the submandibular ganglion in 2-month-old mice using mitochondria dye reported changes in the pattern of synaptic connections that occurred within weeks, leading to the impression that over months, innervation patterns should change completely[23]. We found, however, that as the mouse approaches middle age, there is a gradual increase in synaptic stability. This stabilization is likely to mark the end of a period of plasticity in young adults and may underlie age-dependent changes in functional malleability of the nervous system[1,14–17]. This age-dependence underscores the importance of paying attention to the maturity of the animal when analyzing stability of circuits[7,12,16,21–23,25].

Our results show that in young adults, axon branches undergo rapid growth and retraction over minutes, but the frequency declines with age. Many of these changes do not seem to lead to any long-lasting alteration in the size or number of synapses. Although the purpose of these rapid changes is not obvious, such dynamism may be the substrate for rapid synapse formation and/or elimination in young animals. If so, the age-dependence of these rapid movements may underlie the age-dependent decline of long-term rearrangements that we observed over weeks to years.

This work raises the question of what causes axonal arbors to stabilize with age. During an early postnatal period, synaptic circuitry is known to be refined such that some connections are strengthened by the addition of synapses, whereas other terminal axonal branches are eliminated. These modifications are a central feature of mammalian development, and it is possible that they extend further than traditional 'critical periods'[1–4,16]. In particular, in the submandibular ganglion there is a gradual decrease in the number of preganglionic axons that innervate each cell and a compensatory increase in the number of synapses that the remaining inputs establish[27]. This competitive process may continue at some low level for a considerable time in postnatal life and may only cease when competitive interactions between neurons or axons are largely settled. In addition, because axons grow in the presence of a variety of factors, it is possible that axonal plasticity decreases with age as humoral agents such as hormones and growth factors are downregulated[29,30]. Furthermore, as development proceeds, cytoskeletal elements of synapses become progressively stabilized[31,32], and adhesions between pre- and postsynaptic sites become stronger[33]. Both of these changes probably help maintain the structural stability of synapses in the adult. For example, at the neuromuscular junction, the postsynaptic specialization and the presynaptic ending undergo considerable morphological changes after synapses first form[34]. These changes include the deposition of a highly adhesive basal lamina between pre- and postsynaptic cells and the 'sinking' of the nerve into a synaptic gutter. Both probably help maintain the structural integrity of synapses in the face of the mechanical forces associated with muscle contraction. Whatever the root cause, the consequence is that synaptic terminals change little after middle age. Such stability may help assure that functionally appropriate connectivity will not undergo refinement without a good reason.

METHODS

Transgenic mice. Transgenic mice expressing YFP were described previously[24]. We used lines YFP-A, YFP-D and YFP-G interchangeably. All experiments were performed in accordance with animal protocols approved by the Institutional Animal Care and Use Committee of Washington University.

Preparing the mouse for *in vivo* imaging. Mice between 1 and 32 months of age were deeply anesthetized with a single intraperitoneal injection (5.0 ml per kg body weight) containing 0.17 mg ketamine (Ketaset, Fort Dodge Laboratories) and 1.7 mg xylazine (Anased, Lloyd Laboratories) per ml in 0.9% sodium chloride solution. During each imaging session, the mice were intubated with a thin polyethylene tube and mechanically ventilated with a small rodent respirator. Submandibular ganglion cells were exposed as described previously[23,26].

Imaging the submandibular synapses. Preganglionic axons and synaptic boutons in submandibular ganglion were imaged with a confocal microscope (Bio-Rad MRC-1024 on an Olympus microscope). A 10× air objective was first used to locate ganglion cells on the submandibular ducts with standard epifluorescence microscopy. To avoid phototoxicity, the 100-W Mercury arc illumination was attenuated with neutral density filters to less than 5%. Once labeled ganglion cell clusters were found, a long-working distance (60×, 0.9 numerical aperture) water-immersion objective was used to locate superficial terminal arbors and obtain images. To obtain diffraction-limited resolution, the confocal laser scanning was zoomed 2.0 times. To collect as much light as possible, we opened the detector pinhole wider than its optimum diameter, sacrificing some optical sectioning. The laser intensity (1–3%) was kept low to minimize bleaching and photo damage. The respirator was turned off during image acquisition and immediately turned on afterward. It was necessary to acquire a stack of image planes (10–20 frames per cell at 0.8–1.0 μm steps) to have the full three-dimensional data set of synaptic connections around the cell soma.

Each cell was imaged at least twice to minimize distortion due to occasional animal movement. After imaging, the wound was sutured and the animal was returned to the cage.

Image processing. Movements induced by the mouse heartbeat sometimes caused displacements of individual images within stacks. MetaMorph (Universal Image Corp.) software was used to align adjacent images when such shifts occurred. The alignment of adjacent images was aided by the fact that labeled structures could be seen in three adjacent image planes because of the large pinhole size used (the interval between the adjacent planes is 0.8–1.0 μm). Because YFP-labeled axon terminals were observed in several focal planes of one stack, it is unlikely that any labeled synapses were missed in the stack. In addition, we imaged the same synaptic connections at least twice and usually three times each at a slightly different starting depth to make sure that each stack contained all the labeled synapses.

Quantification of stability. To quantify the similarity in the distribution of synaptic boutons on ganglion cell somata imaged over time, two images of the same cell were first aligned based on their shapes with MetaMorph software. The innervated (I) and uninnervated (U) areas of the two images were determined with IPLab software (Scanalytics, Inc.). We determined the areas that were innervated at each view that overlapped ($I_{overlap}$) and the areas that were not innervated that overlapped ($U_{overlap}$). The percentage of the surface area that overlapped (and hence did not change) between the two views was $(I_{overlap} + U_{overlap})/(I + U)$. To rule out the possibility that the similarity index was high between views because (i) there were so many synapses that even if they moved the same regions would be innervated or (ii) there were so few that most of the surface remained uninnervated at each view, we corrected the results relative to a randomization of the location of synapses seen in the first view. We generated a randomized distribution by redistributing YFP-positive pixels of the labeled arbor at random over the entire surface of the identified neuron. Then, assuming that the second view is derived from this randomized transformation of the first view, the density of boutons over the entire cell surface would be $I/(I + U)$; and the percentage of overlap (of innervated and uninnervated regions) under the randomized condition would be $(I^2 + U^2)/(I + U)^2$. The stability index is defined as the difference between the percentage of overlap observed and that under randomized condition $((I_{overlap} + U_{overlap})/(I + U) - (I^2 + U^2)/(I + U)^2)$. The correction eliminated a few spurious cases where synaptic contacts seemed largely unchanged for artifactual reasons.

In addition to the stability index described above, we also measured the areas that were innervated at each view that overlapped ($I_{overlap}$) and divided this by the innervated area in the first view (I). This ratio ($I_{overlap}$ /I) gives rise to the percentage of stable axonal terminals over time (**Fig. 4e,f**).

Immunostaining of synaptic boutons with antibody against SV2. The preparation was incubated first with a blocking solution (4% BSA, 0.4% TX-100 in PBS) for 2 h and then with primary antibodies against SV2 (Developmental Studies Hybridoma Bank) for 4 h. Rhodamine-conjugated secondary antibodies (Jackson ImmnoResearch Laboratories) were used for subsequent imaging of synaptic boutons.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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