Directional *Trans*-Synaptic Labeling of Specific Neuronal Connections in Live Animals

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ABSTRACT Understanding animal behavior and development requires visualization and analysis of their synaptic connectivity, but existing methods are laborious or may not depend on *trans*-synaptic interactions. Here we describe a transgenic approach for *in vivo* labeling of specific connections in *Caenorhabditis elegans*, which we term iBLINC. The method is based on BLINC (*B*iotin *L*abeling of *IN*tercellular *C*ontacts) and involves *trans*-synaptic enzymatic transfer of biotin by the *Escherichia coli* biotin ligase BirA onto an acceptor peptide. A BirA fusion with the presynaptic cell adhesion molecule NRX-1/neurexin is expressed presynaptically, whereas a fusion between the acceptor peptide and the postsynaptic protein NLG-1/neuroligin is expressed postsynaptically. The biotinylated acceptor peptide::NLG-1/neuroligin fusion is detected by a monomeric streptavidin::fluorescent protein fusion transgenically secreted into the extracellular space. Physical contact between neurons is insufficient to create a fluorescent signal, suggesting that synapse formation is required. The labeling approach appears to capture the directionality of synaptic connections, and quantitative analyses of synapse patterns display excellent concordance with electron micrograph reconstructions. Experiments using photoconvertible fluorescent proteins suggest that the method can be utilized for studies of protein dynamics at the synapse. Applying this technique, we find connectivity patterns of defined connections to vary across a population of wild-type animals. In aging animals, specific segments of synaptic connections are more susceptible to decline than others, consistent with dedicated mechanisms of synaptic maintenance. Collectively, we have developed an enzyme-based, *trans*-synaptic labeling method that allows high-resolution analyses of synaptic connectivity as well as protein dynamics at specific synapses of live animals.

KEYWORDS biotin; circuit; connectome; labeling; synapse

UNDERSTANDING animal behavior requires the determination and analysis of their precise neural connectivity, *i.e.*, their connectome. Historically, this has been accomplished through reconstruction of serial sections of electron micrographs (White *et al.* 1986; Jarrell *et al.* 2012). Pioneered in the nematode *Caenorhabditis elegans* with its defined and invariant nervous system, partial circuits from *Drosophila* and the mouse retina have now also been reconstructed (Helmstaedter *et al.* 2013; Takemura *et al.* 2013). However, both the experimental effort and the static nature of the connectome at the time of analysis render investigations into the variability of synaptic connections between individuals, during development, or in different genotypes laborious at best. Live-imaging techniques to visualize synaptic connectivity have been developed such as GFP Reconstitution Across Synaptic Partners (GRASP) in *C. elegans* and, more recently, Synaptic Tagging with Recombination (STaR) in flies (Feinberg *et al.* 2008; Chen *et al.* 2014). GRASP takes advantage of the strong molecular interaction between split green fluorescence protein segments to create a fluorescent bridge across the synaptic cleft (Feinberg *et al.* 2008). However, the *trans*-synaptic interaction is strong and may not be reversible (Chen *et al.* 2014), thus precluding dynamic studies. STaR, on the other hand, relies on colocalization of pre- and postsynaptic labels without a direct *trans*-synaptic interaction (Chen *et al.* 2014).

To develop a methodology that involves a *trans*-synaptic enzymatic reaction, we adapted *B*iotin *L*abeling of *IN*tercellular *C*ontacts (BLINC), a technique established in cell culture (Liu

doi: 10.1534/genetics.115.177006
Manuscript received April 1, 2015; accepted for publication April 24, 2015; published Early Online April 27, 2015.
Supporting information is available online at www.genetics.org/lookup/suppl/doi:10.1534/genetics.115.177006/-/DC1.
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et al. 2013) and developed it for transgenic use in *C. elegans*. BLINC is based on enzymatic transfer of biotin onto a 15-amino-acid peptide (the acceptor peptide, or AP) by the *Escherichia coli* biotin ligase BirA (Figure 1A) (Liu *et al.* 2013). The *in vivo* BLINC method, which we term iBLINC, seems to capture the directionality of synaptic connections and permits the quantification of synaptic patterns in a population of animals. Based on the observed patterns we conclude that connectivity patterns between two given neurons may be variable between individual animals, whereas the overall strength of the connection remained relatively constant. Our experiments show excellent correlation with known electron microscopic studies both in an *unc*-104/kinesin mutant background and during aging. Thus, iBLINC is a useful tool for high-resolution studies of specific synaptic connections in live *C. elegans* and possibly other organisms.

Materials and Methods

Strains

Worms were cultured as previously described (Brenner 1974). For a complete strain list, see Supporting Information.

Molecular biology

The sequence of monomeric streptavidin (mStrep) (Lim *et al.* 2011) was codon-optimized for *C. elegans* and synthesized *de novo* by Genscript. Subsequently, mStrep was cloned *PmlI*/*Age*I into *Punc-122*::*HS4C3*::*tagRFP* (Attreed *et al.* 2012) to encode a fusion protein where streptavidin is N-terminally fused in frame to the SEL-1 signal sequence and C-terminally to tagRFP (Supporting Information, Figure S1A and Figure S2). The BirA::NRX-1/neurexin fusion was created in several steps. First, the *E. coli* biotin ligase (BirA) was PCR-amplified from *ceh-36prom2*::*NLS-myc-BirAo*::*unc-54-3′UTR* (kind gift of B. Tursun and O. Hobert) and fused in frame by PCR to part of the NLG-1/neuroligin complementary DNA (cDNA) amplified from a GRASP plasmid (*Psrh-128*::*nlg-1*::*spGFP11*, kind gift of M. vanHoven). Second, this PCR fusion was cloned in frame into *Psrh-128*::*nlg-1*::*spGFP11* to yield a BirA::NLG-1/neuroligin fusion. Third, the NLG-1/neuroligin sequence was replaced by the NRX-1/neurexin coding sequence, which was amplified from a N2 cDNA library with custom primers. Finally, the *Psrh-128* promoter was replaced by other promoters as necessary. This construct encodes a predicted protein with a heterologous PAT-3 β-integrin signal sequence, followed by BirA fused N-terminally to NRX-1/neurexin lacking its endogenous signal peptide (Figure S1A and Figure S2). Similarly, AP was PCR-amplified from pBALU-NtermAVI 2.0 (kind gift of B. Tursun and O. Hobert) and fused by PCR to a part of the NLG-1/neuroligin cDNA amplified from a GRASP plasmid (*Psrh-128*::*nlg-1*::*spGFP11*, kind gift of M. vanHoven). This PCR fusion was cloned in frame into *Psrh-128*::*nlg-1*::*spGFP11* to yield a AP::NLG-1/neuroligin fusion, and, finally, the *Psrh-128* promoter was replaced as required. This construct encodes a predicted protein with a heterologous PAT-3 β-integrin signal sequence, followed by two 15-amino-acid AP peptides fused N-terminally to NLG-1/neuroligin lacking its endogenous signal peptide (Figure S1A and Figure S2). All amplified sequences were verified by sequencing. For further details, see Supporting Information.

Fluorometric and microscopic analyses

For epifluorescence microscopy, young adult animals were anesthetized with 5 mM levamisole or 0.3 mM 2,3-butanedione monoxime (BDM) + 4 mM levamisole and analyzed on an AxioImager Z1 compound microscope (Zeiss); the signal is not visible under a dissecting microscope equipped for epifluorescence, at least not with the synaptic connections tested. In all images, the iBLINC signal was visualized using an exposure time of 1000 ms at ×630 magnification. The cytoplasmic markers were visualized with a 10- to 20-ms exposure time at ×630 magnification. Animals were optically sectioned using the Zeiss Apotome, and the sections were processed to produce maximum intensity projections in some cases. All images were processed subsequently with the Zeiss proprietary Axiovision software and assembled into figures using Adobe Photoshop and Adobe Illustrator.

Time-course analysis

To observe the signal at larval stage 1 (L1), an egg prep was performed and animals were allowed to develop without food over night. To image the signal during larval stages 2–4 (L2-L4), an egg prep was performed and the animals were left developing on a plate containing OP50 and staged by gonad morphology. To observe the signal during adulthood, L4 worms were picked and incubated at 20° for 1–15 days. Every 2 days the worms were transferred onto a fresh plate to avoid starvation and to eliminate the progeny. At relevant time points, animals were imaged as describe above.

Analyses of synapses pattern and statistical analyses

To compare the pattern of AFD–AIY synapses, the images of different genotypes were shuffled and blinded for analysis using ImageJ (Schneider *et al.* 2012). The staining patterns were isolated from background, and worms were placed in the same orientation. Individual punctae were circled and position, fluorescence intensity, and surface area of each puncta extracted. Statistical analysis and plots were generated using R (R Core Team 2013). The average size of punctae was analyzed using an ANOVA test with a Bonferroni correction for pairwise comparisons.

"Pearl chain" graphs were created by arbitrarily placing punctae into four differently sized bins (0–13; 14–18; 19–25; and >25 square pixels). Total synaptic length was defined by measuring the distance between punctae using a triangulation formula and summing the distances. Punctae were then aligned and binned depending on quartiles of synaptic length, the first quartile being the most distal part of the process (in relation to the cell body) and the last one being the proximal part of the synaptic length. The number of punctae in each quartile was compared between ages and genotypes by employing a two-way ANOVA test using GraphPad Prism (version 5.04 for Windows, GraphPad Software, www.graphpad.com). The

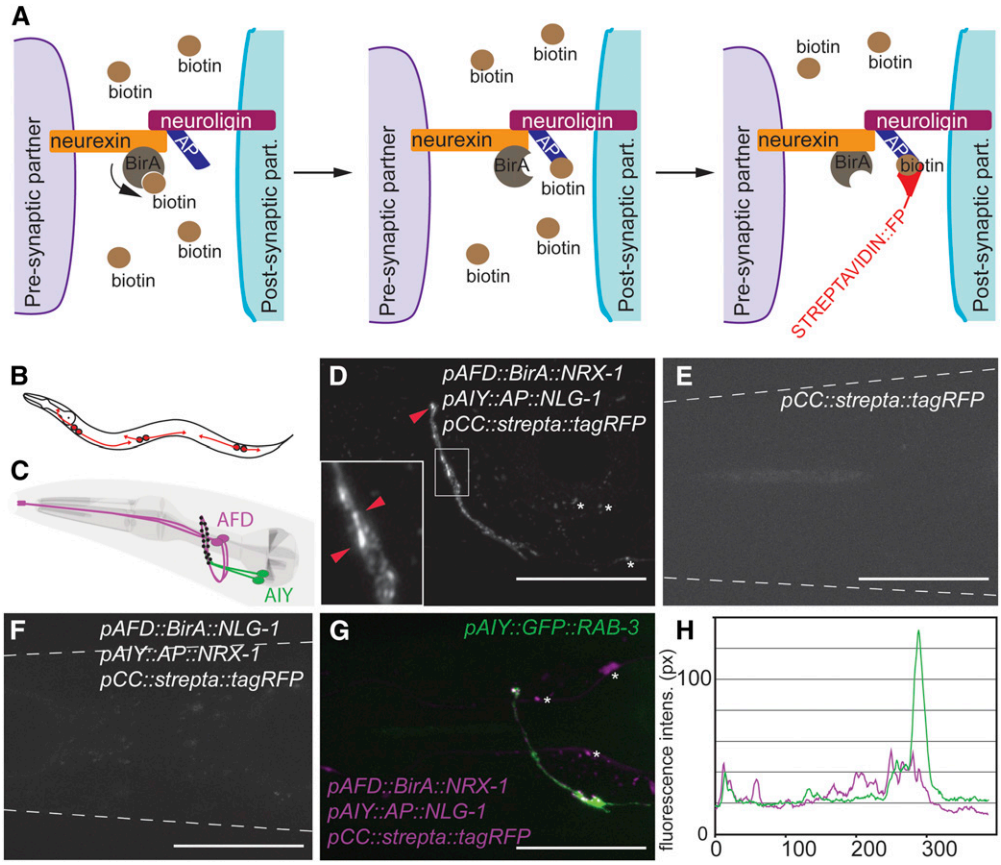

Figure 1 *Trans*-synaptic labeling of specific synapses in live animals. (A) Schematic of the iBLINC system. The biotin ligase (BirA) fused with neurexin biotinylates the acceptor peptide AP::neuroligin fusion, using biotin present in the extracellular space The ligated biotin is detected using a monomeric streptavidin fused to tagRFP and secreted from the coelomocytes (B). (B) Schematic of a worm showing the coelomocytes secreting a monomeric streptavidin–fluorescent protein fusion (detector). (C) Schematic of AFD and AIY neurons. Synapses are indicated as black dots. Adapted from the Virtual Worm Project (http://caltech.wormbase.org/virtualworm/). (D–F) Epifluorescent micrographs of the head region of transgenic animals expressing the presynaptic BirA fusion in AFD (using the *Pgcy-8* promoter), the postsynaptic AP fusion in AIY (using the *Pttx-3* promoter), and the streptavidin detector fusion from the coelomocytes (*Punc-122* promoter) (D), the streptavidin detector fusion alone (E), or the pre- and postsynaptic proteins in reversed order (F). Note that expressing the fusions from F correctly on the pre- and postsynaptic side, respectively, resulted in signal (Figure S3D). Some vesicular staining is usually seen in the postsynaptic cell body (not shown), which can be useful for cell identification. Arrowheads indicate labeling between AFD and AIY neurons in D and a dashed line the outline of the animals in E and F. In all micrograph panels, punctae marked by an asterisk are considered background staining as they are not seen consistently in different animals. Bars, 20 μm. Anterior is to the left. (G) Epifluorescent micrograph showing postsynaptic sites of AIY in magenta (iBLINC) and presynaptic sites in green [*wyIs45* (*Is[pAIY::GFP::RAB-3]*)]. (H) Colocalization of post- and presynaptic sites in AIY was measured by a line scan and show by and large mutually exclusive staining. One hundred percent of animals showed immediately juxtaposed pre- and postsynaptic sites in AIY neurons (n = 14).

analysis of 1-, 3-, 6-, or 9-day-old wild-type animals was performed using an integrated transgenic line (*dzIs68*: *Is[Pgcy-8::BirA::nrx-1*; *Pttx-3::AP::nlg-1*; *Punc-122::streptavidin::tagRFP*; *Pelt-2::GFP]*). The analysis of *unc-104(e1265)* mutant animals was performed using the parental extrachromosomal line *dzEx1240*.

Behavioral assays

Worms were grown as described at 20° and assayed at 25° (Tsalik and Hobert 2003). All plates and solutions were equilibrated at 25° before use, and all experiments were performed blindly. Worms of each genotype were picked the day before the assay at the L4 larval stage and placed on a plate with OP50 bacteria and incubated over night at 20°. On the day of the assay worms were picked and placed on a plate without food to "clean" worms from adhering food and then used as described below.

Radial locomotion: Ten worms were placed on the assay plate (lacking food), and their positions were marked on the plate at 5, 10, and 15 min. The distances were then measured and multiple *t*-tests were performed using the GraphPad software package.

Thrashing assays: Worms were picked individually and placed in a drop of M9 buffer. The number of thrashes in 1 min were counted and analyzed with a Mann–Whitney test using the GraphPad software package.

Reversal assays: Worms were placed on a fresh plate without food. After 1 min of recovery, the number of reversals in 3 min were counted. Every change of movement from forward to backward was considered as a reversal. Statistical analyses were performed using a Mann–Whitney test with the GraphPad software package.

Body bends: Worms were placed on a fresh plate without food, and the number of body bends per 20 sec were counted. Statistical analyses were performed using a Mann–Whitney test with the GraphPad software package.

Volumetric reconstruction of the C. elegans nervous system

Digitized serial-section transmission electron micrographs of an L4 animal (JSH series) and an ~3-day-old adult hermaphrodite (N2U) were aligned manually, segmented, and rendered

using TrakEM2 (White *et al.* 1986; Cardona *et al.* 2012). Synaptic connectivity between neurons was annotated by counting the number of consecutive serial sections containing a presynaptic density using Elegance (Xu *et al.* 2013).

Photo-conversion experiments

For photo-conversion experiments, young adult animals were anesthetized using 0.15 mM BDM + 4 mM levamisole and analyzed on an AxioImager Z1 compound microscope (Zeiss). The slide was sealed using Vaseline petroleum jelly. Images were taken first in the red channel (591–652 nm) at 400-ms exposure time and gain ×4 and then in the green channel (500–550 nm) at 400-ms exposure time and gain ×4 to avoid photo-conversion by the blue light. Then the worm was exposed for 30 sec to blue light using a DAPI filter (365 nm). A postphotoactivation image series was taken using the same exposure times. The worm was left on the slide for 1 hr at room temperature. Finally, the recovery images were taken using the same parameters.

Results

To develop BLINC for transgenic use in *C. elegans*, we modified GRASP constructs, which had previously been shown to be correctly trafficked to the cell surface (Feinberg *et al.* 2008). The resulting presynaptic BLINC construct encoded a protein in which the signal sequence of the presynaptic cell adhesion protein NRX-1/neurexin was replaced by the heterologous signal sequence of PAT-3 β-integrin followed by the *E. coli* biotin ligase BirA (Figure 1A, Figure S1, Figure S2). Similarly, the N terminus of the postsynaptic cell adhesion molecule NLG-1/neuroligin was modified such that the endogenous signal peptide was replaced by the PAT-3 β-integrin signal sequence followed by two copies of the 15-amino-acid AP (Figure 1A, Figure S1, Figure S2). NLG-1/neuroligin is a postsynaptic partner of neurexin (reviewed by Craig and Kang 2007). A NLG-1/neuroligin reporter shows expression in a subset of cells in the nervous system of *C. elegans*, including several motor neurons and interneurons such as the AIY interneurons (Hunter *et al.* 2010). In contrast, widespread expression in the nervous system is seen with a NRX-1/neurexin reporter (Haklai-Topper *et al.* 2011). We predicted that, in analogy to experiments in cell culture, transgenic pre- and postsynaptic expression of the two fusion proteins would result in enzymatic biotinylation of the AP on NLG-1/neuroligin across the synaptic cleft. To detect the biotinylated AP::NLG-1/neuroligin fusion *in vivo*, we designed a monomeric streptavidin::tagRFP "detector" that we transgenically secreted into the extracellular space of *C. elegans* from six scavenger cells termed coelomocytes (Figure 1, A and B; Figure S1A). We term this technique iBLINC, for *in vivo* BLINC.

Labeling of AFD–AIY synapses by iBLINC

To test the technique, we focused on part of the thermosensory circuit in the head of *C. elegans*, consisting of the bilateral pairs of AFD sensory neurons and AIY interneurons (Mori *et al.* 2007). The AFD neurons are localized laterally in the head of the animals whereas the AIY interneurons are localized in the ventral ganglion (Figure 1C). On both sides of the animal, the neurites of AIY and AFD run alongside each other in the ventral ganglion and nerve ring, the major neuropil of the nematode, where they form multiple *en passant* synapses (Figure 1C and Figure 2A) (White *et al.* 1986). We expressed the BirA::NRX-1/neurexin and the AP::NLG-1/neuroligin fusions presynaptically in AFD and postsynaptically in AIY neurons, respectively, using specific promoters. When the streptavidin detector was secreted from the coelomocytes at the same time, we observed punctate signals in the head (14/14 transgenic lines; n = 10–30 per line) (Figure 1D and Figure S1B). In addition, we usually saw vesicular staining in the cell bodies of the postsynaptic neurons (data not shown), suggesting that (i) the method is indeed "nontrapping" and fails to create a stable bond across the synaptic cleft and (ii) that the streptavidin–biotin–NLG-1/neuroligin complex is actively turned over. No fluorescent signal was observed in animals expressing the secreted streptavidin detector alone (4/4 transgenic lines; n = 20–30/line) (Figure 1E), indicating that no endogenous epitopes are recognized by the streptavidin detector. When the BirA::NRX-1/neurexin or AP::NLG-1/neuroligin fusions, respectively, were omitted, no signal was observed (Figure S3, A–C). These findings indicate that there are neither endogenous enzymes that can transfer biotin onto the AP::NLG-1/neuroligin fusion nor endogenous acceptor peptides for BirA present in the extracellular space of *C. elegans*. A similar signal was observed when the streptavidin was tagged with a superfolder GFP variant in tandem (2×sfGFP) or when it was secreted form the intestine instead of the coelomocytes (Figure S3, E–H). Thus, the observed signal is independent of the fluorophore or promoter used to drive the streptavidin detector.

The punctate signal was juxtaposed with cytoplasmic fluorescent markers of both AIY and AFD neurons (Figure S4, A and B). It was adjacent to, but largely nonoverlapping with, a marker for presynaptic specializations in AIY interneurons consistent with the expectation that pre- and postsynaptic sites in AIY neurons are adjacent but nonoverlapping (Figure 1, G and H). This indicated that the signal is localized to, and probably labeled the connection between, AFD and AIY neurons. Finally, the observed fluorescent signal correlated with a volumetric reconstruction of the AIY and AFD neurons of an electron micrograph series of a L4 larval stage animal (Figure 2A and Figure S5).

To test whether the signal is dependent on the correct localization of pre- and postsynaptic proteins, we expressed a BirA::NLG-1/neuroligin fusion, *i.e.*, the postsynaptic protein, presynaptically in AFD, and the AP::NRX-1/neurexin presynaptic protein postsynaptically in AIY. We found no signal in these transgenic lines (0/19 lines; n = 3–15/line) (Figure 1F), suggesting that iBLINC can capture the directionality of synaptic connections. Finally, we compared iBLINC with GRASP using the same connection between the presynaptic AFD sensory neurons and AIY interneurons. We found iBLINC to result in similar labeling of synapses when compared to an analogous GRASP signal (Figure S1, B–D).

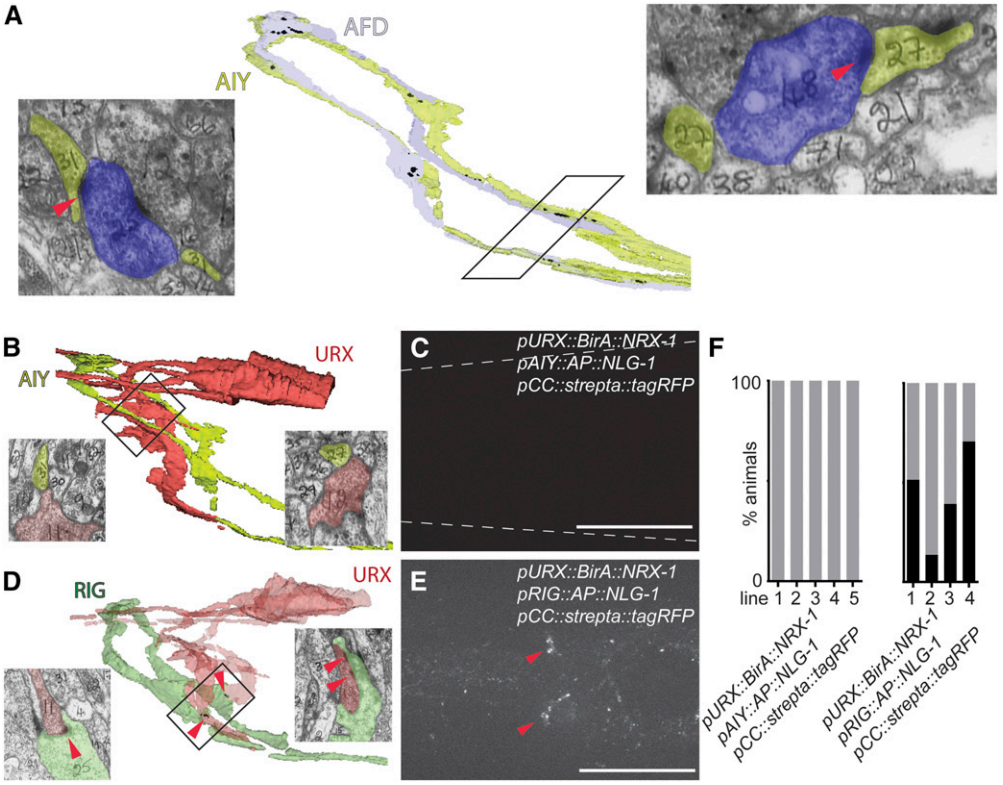

Figure 2 iBLINC is dependent on the formation of synapses. (A) Volumetric reconstruction of AFD sensory (shaded in light blue) and AIY interneurons (shaded in yellow) using the JSH electron micrograph series of an L4 larval stage animal. Electron micrograph section from the positions shows cellular contact with presynaptic specializations (red arrowheads). Synapses visible on the micrographs are reported as black punctae in the volumetric reconstruction. (B) Volumetric reconstruction of URX sensory (shaded in red) and AIY interneurons (shaded in yellow) using the JSH electron micrograph series of an L4 larval stage animal. Electron micrograph section from the boxed area shows cellular contact but no presynaptic specializations. Both URX and AIY were also seen to run along each other using cell-specific cytoplasmic fluorescent markers (Figure S4, D and E). (C) Epifluorescent micrographs of the head region of transgenic animals expressing the presynaptic BirA fusion in URX and the postsynaptic AP fusion in AIY. Dashed lines indicate the outline of the animal. (D) Volumetric reconstruction of URX sensory (shaded in red) and RIG interneurons (shaded in green) using the JSH electron micrograph series of an L4 larval stage animal; presynaptic densities observed between URX and RIG are represented in black (red arrowheads). Electron micrograph sections from the boxed area show cellular contact with presynaptic specializations in URX. (E) Epifluorescent micrographs of a ventral view of the head region of a transgenic animal expressing the presynaptic BirA fusion in URX (using the *Pgcy-32* promoter) and the postsynaptic AP fusion in RIG (using that *Pflp-18* promoter) together with the streptavidin detector. Specific signal is visible only between URX and RIG neurons (arrowheads) but not between URX and AIY (E) and likely corresponds to the connections boxed and shown in representative micrographs in D. Note that the *Pflp-18* promoter is also expressed in AVA command interneurons (Rogers *et al.* 2003), which may be weak postsynaptic partners of URX (White *et al.* 1986). (F) Quantification of animals that display fluorescent signal. Different transgenic lines are shown in each case (n = 19–51).

We next asked whether visualizing the synaptic connections between AFD and AIY compromises the functionality of the connection. Several behavioral paradigms of locomotion have previously been shown to require AIY function. For example, mutants in the homeobox transcription factor *ttx-3*, which is required for terminal differentiation of AIY interneurons (Altun-Gultekin *et al.* 2001), show temperature-dependent locomotion defects (Tsalik and Hobert 2003). We performed the same tests, including a thrashing assay, a reversal assay, a body bend assay, and a radial locomotion assay with *ttx-3* mutant animals serving as a positive control and N2 wild-type animals and nontransgenic siblings of the transgenic iBLINC line (*dzEx1240*) as negative controls. Transgenic animals expressing iBLINC did not show obvious differences in these assays compared to wild-type animals or their nontransgenic siblings (Figure S6). Collectively, these results suggest that iBLINC *trans*-synaptically labels connections from AFD sensory neurons to AIY interneurons without interfering with function.

iBLINC depends on the formation of a synapse

We next asked whether physical contact between two neurons was sufficient to allow formation of an iBLINC signal or whether formation of synapses was required. To this end, we sought to identify a neuron that is in cellular contact with AIY neurons, but fails to establish synaptic connections. Volumetric reconstruction of the original electron micrograph series from White *et al.* (1986) identified the neurites of the pair of URX sensory neurons to run along the neurites of AIY interneurons without forming synapses (Figure 2B). Using green and red fluorescent cytoplasmic markers of AIY and URX, respectively, we confirmed that the neurites of both pairs of neurons appeared immediately adjacent in 89% of the animals (n = 28) (Figure S4, D and E). Expression of the BirA::NRX-1/neurexin presynaptic fusion in URX with the postsynaptic AP::NLG-1/neuroligin in AIY failed to result in any signal (0/5 transgenic lines; n = 15–48) (Figure 2, B, C, and F). In contrast, expression of the BirA::NRX-1/neurexin presynaptic fusion in URX and the AP::NLG-1/neuroligin in RIG interneurons, *bona fide* postsynaptic partners of URX sensory neurons (Figure 2D) (White *et al.* 1986), resulted in specific labeling (4/4 transgenic lines; n = 22–51) (Figure 2, E and F). These results are consistent with the conclusion that the iBLINC signal requires the presence of synapses and that cellular contact alone is insufficient to bring the NRX-1/neurexin and NLG-1/neuroligin

components of the system into sufficient physical proximity for interaction.

iBLINC punctae correspond to synapses

To determine whether the punctae that we observe are synapses, we determined the number of punctae seen by iBLINC to compare them with the number of presynaptic densities seen in electron microscopic reconstructions. To that end, we circled and quantified the punctae in maximum-intensity projections of iBLINC patterns on either the right or the left side of transgenic iBLINC animals (Figure 3C and Figure S5) and arrayed them in order, thereby creating pearl chain graphs for each animal (Figure 3C). We found the number of iBLINC punctae to be in accordance with the number of presynaptic densities observed in electron microscopic reconstructions at two different ages, the L4 stage and older adult (Figure S5). We next determined and compared the number, average size, and total area of iBLINC punctae in wild-type animals and in a mutant of the KIF1A kinesin homolog UNC-104, previously shown by electron microscopy to contain fewer synapses with fewer synaptic vesicles (Hall and Hedgecock 1991). As in electron microscopic studies, we found a reduced overall number of punctae/synapses between AFD and AIY in *unc-104* mutant animals [17.5 ± 1.3 SEM (n = 17) per side] compared to wild-type animals of the same age [24.3 ± 1.1 SEM (n = 27)] (Figure 3D). Consistent with an unchanged average synaptic size, we observed a decreased total synaptic area, suggesting a weaker connection between both neurons (Figure 3, E and F). To obtain insight into the spatial distribution of punctae, we arbitrarily divided the length of the synaptic connections formed *en passant* into four equal bins/quartiles (Figure 3C). Surprisingly, we found the area proximal to the cell body to be possibly more affected than the distal portion of the synaptic zone, suggesting that UNC-104 may serve functions other than in anterograde transport (Figure 3G). Taken together, these findings suggest that the punctae in iBLINC correspond to one or a small number of synapses.

Age-dependent changes of synapse number and size in the AFD–AIY connection

The iBLINC signal between AFD and AIY interneurons was visible as early as the first larval stage and throughout life until at least 15-day-old adult worms (Figure 4, A–C). To determine the patterning of synapses between animals and over time, we created binned pearl chain graphs at different ages from L4 larval animals to 9-day-old adults. These analyses revealed no obvious regularity of punctae between animals at the L4 larval stage or as 1-, 3-, 6- or 9-day-old adults (Figure 4D). We next quantified the number and size of synapses at different ages. At the L4 larval stage we found an average of 18.4 ± 0.8 SEM (n = 16) punctae/synapses per side of the animal, which increased to 24.8 ± 1.6 SEM (n = 16) punctae in 1-day-old adults with no obvious bias for the left or right side of the animal [left: 24.5 ± 1.7 SEM (n = 13); right: 24.0 ± 1.3 (n = 14)] (Figure 4E). Consistent with a higher number and an unchanged average size of synapses, the total synaptic area increased during early adulthood (Figure 4, F–H). However, from 1-day-old animals onward, the number of synapses decreased significantly and stabilized at 17.4 ± 1.9 (n = 11), 18.1 ± 1.4 (n = 14), and 16.0 ± 1.6 (n = 7) synapses in 3-, 6-, and 9-day-old

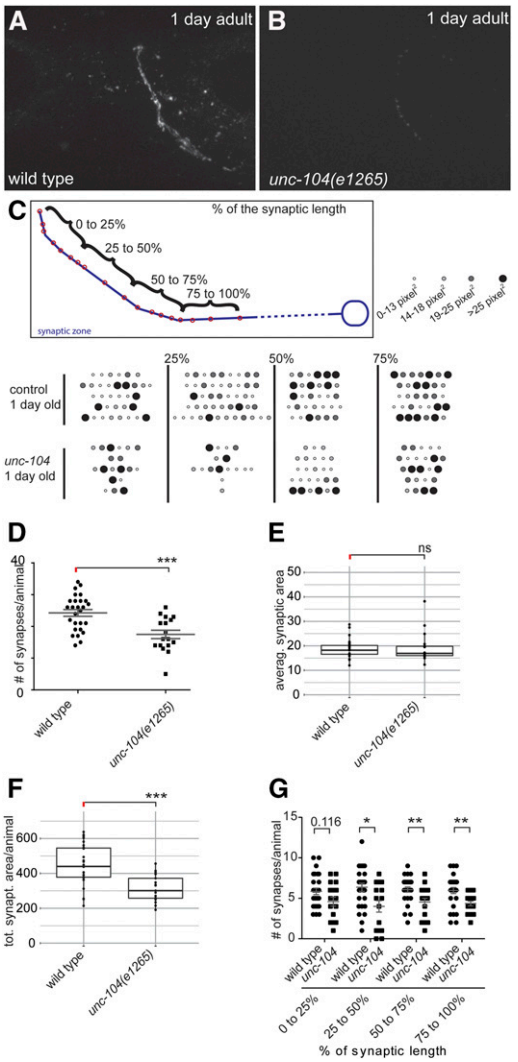

Figure 3 iBLINC labeling in live animals of different genotypes. (A and B) Epifluorescent micrographs of a 1-day-old wild type (A) or 1-day-old *unc-104 (e1265)* mutant animals (B). Anterior is to the left in all micrograph panels. (C) Pearl chain graphs illustrating the sequential pattern of synapses. Each row is an individual animal. Synapses were quantified and placed into four differently sized bins based on fluorescent area as indicated by different sizes and shades. Four different synaptic areas were defined as shown in the schematic. The total synaptic length was defined by the distance between the first and last puncta and then split into four equally sized areas. (D–G) Quantification of synapse patterns in the genotypes indicated. Shown are the number of synapses per animal (D) and box plots of the average synaptic area per animal (E), the total synaptic area per animal (F), and the number of synapses per animal in the different synaptic sections (as defined in panel C of this figure) (G). In E and F, each dot represents the mean of one animal (with the mean of means indicated by a line), and the box shows the 25th and 75th percentile, respectively. Statistical significance is indicated explicitly or as the follows: ns, not significant; *$P < 0.05$; **$P < 0.005$; ***$P < 0.0005$. Size units are square pixels.

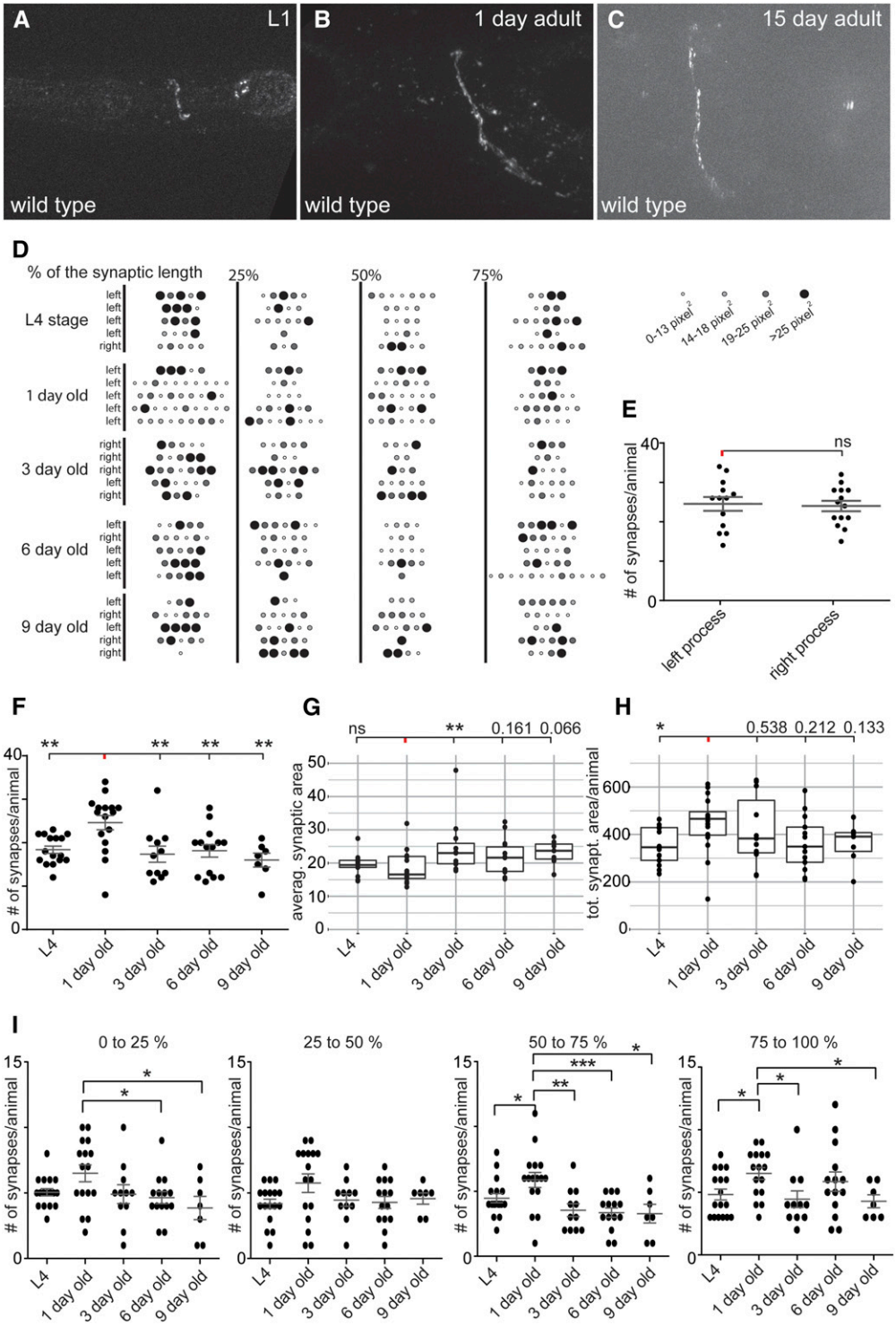

Figure 4 iBLINC labeling in live animals during aging. (A–C) Epifluorescent micrographs of animals at an early larval stage (L1) and 1- and 15-day-old adults. Anterior is to the left in all micrograph panels. (D) Pearl chain graphs illustrating the sequential patterns of synapses of worms at different stages: L4 (larval stage), 1-, 3-, 6-, and 9- days- old. Each row represents an individual animal. Synapses were quantified and placed into four different bins based on four different synaptic sections as defined in the schematic (Figure 3C). Total synaptic length was defined by the distance between the first and last puncta and then split into four equally sized sections. Fluorescent area of punctae is indicated by different sizes and shades. (E) Quantification of the number of synapses per 1-day-old adult animals on the left and right side, respectively. ns, not significant. (F–I) Quantification of synapse patterns at the developmental stages indicated. Shown are the number of synapses per animal (F) and box plots of the average synaptic area per animal (G), the total synaptic area per animal (H), and the number of synapses per animal in the different synaptic sections (as defined in panel Figure 3C)(I). at the indicated ages. In G and H, each dot represents the mean of one animal (with the mean of means indicated by a line), and the box shows the 25th and 75th percentile, respectively. Statistical significance is indicated explicitly or as the follows: ns, not significant; $*P < 0.05$; $**P < 0.005$; $***P < 0.0005$. Size units are square pixels.

adults, respectively (Figure 4F). This occurred primarily due to loss of smaller synapses (Figure S7). Consistent with this observation, the average synapse size significantly increased in 3-day-old adults and, with less statistical certainty, in 6- and 9-day-old adults (Figure 4G). On the other hand, the total synaptic area remained constant with a trend for decreasing total area in aging animals, in line with electron microscopic studies (Toth *et al.* 2012) (Figure 4H). These results suggest an expansion of connectivity during early adulthood, followed by a consolidation phase and eventual decline.

Finally, we wanted to explore whether specific areas of the synaptic connection behaved differently. To this end, we quantified the number of synapses in each bin/quartile. We observed dynamic changes in some areas of the connection, whereas others remained relatively unaffected over time

(Figure 4I). These findings are consistent with the notion that dedicated maintenance mechanisms exist to ensure appropriate conservation of synaptic connections.

iBLINC allows dynamic studies

To explore whether iBLINC could lend itself to dynamic studies at the synapse, we fused the monomeric streptavidin detector to the photoconvertible fluorescent protein dendra2 (Chudakov *et al.* 2007). The dendra2 protein changes fluorescent properties upon exposure to blue light from green to red, allowing "chase" experiments in which "old" signal (converted/red) can be distinguished from newly formed signal (green). We find that the dendra2 iBLINC signal can be quantitatively converted into red fluorescence and that within <1 hr a new green iBLINC signal appears (Figure 5). Since the streptavidin–biotin interaction can be considered irreversible, this finding suggests that iBLINC could serve to investigate protein turnover at the postsynaptic site of specific synapses under different experimental conditions.

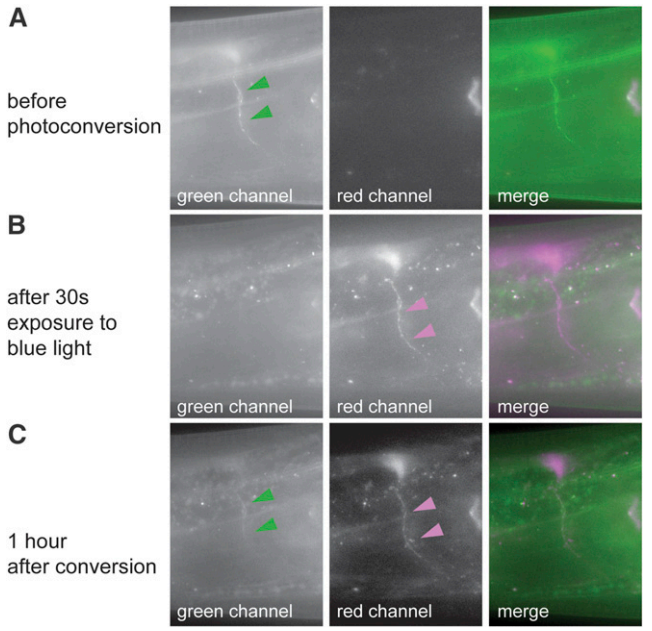

Figure 5 iBLINC may be used for dynamic studies. (A–C) Epifluorescent micrographs of the head region of transgenic animals expressing iBLINC with the detector tagged with a photoconvertible protein, Dendra2. Before photoconversion green fluorescence is observed in the head while no red is present (A). After a 30-sec exposure to blue light, green is no longer seen in the head while red appears (B). After only 1 hr of recovery, green is observed again. The green and red streptavidin colocalized, showing that new proteins are again at the membrane (C).

Discussion

We have developed a method for live imaging of specific synaptic connections in *C. elegans*, which we term iBLINC and which is based on BLINC (*B*iotin *L*abeling of *IN*tercellular *C*ontacts) (Liu *et al.* 2013). The method is rapid, simple, specific, and possibly brighter than existing methods, and the observed signals correlate well with electron microscopic studies of connectivity in *C. elegans*. Thus, iBLINC could serve as a useful additional tool to investigate synaptic connectivity and dynamics under different experimental conditions in nematodes and, possibly, other organisms.

Using iBLINC we were able to observe and quantify the variability of the synaptic connection between the AFD and AIY neurons, which are part of the thermosensory circuit of the small nematode *C. elegans*. The connectivity between these two neurons, at least in regard to size of the synapses, does not seem to follow any obvious pattern across a population of animals and varies from worm to worm, although the overall strength appears relatively constant. However, the number of synapses seems to be important and changes with age of the animals. For example, we observed an increased number of synapses between the last larval stage and 1-day-old adult worms. Our observations suggest a significant expansion of connectivity during early adulthood, followed by a consolidation phase and eventual decline. Similar observations of decline have also been made in the aging human brain (Bertoni-Freddari *et al.* 1990). It will be interesting to explore the impact of other factors on development of synaptic patterns, such as life history, environmental conditions, or neuronal activity. The observation that the functionality of the synaptic connection does not appear compromised in iBLINC animals could render such studies feasible in the future.

A concern with *trans*-synaptic approaches is that the synaptic connection that is being visualized is stabilized by the interactions of the transgenes used. Several observations suggest that this may not be the case for iBLINC. First, fluorescent vesicles can be observed in the postsynaptic neuronal cell body. This suggests turnover of the fluorescent streptavidin–biotin–AP-NLG-1/neuroligin complex and indicates that NRX-1/neurexin–NLG-1/neuroligin interaction may not be stabilizing. Second, the turnover at the postsynaptic side seems rapid, as new signal is observed within 60 min after photoconversion of the streptavidin-dendra2 detector. Finally, the signal does not obviously affect functionality of the tested connections, suggesting that, even if there were some stabilization, it may not be functionally relevant.

Nematodes could be uniquely suited for the use of iBLINC because of the convenience of coelomocytes as both source and sink of the streptavidin detector. However, even in the absence of these cells in vertebrates (*e.g.*, mice), it seems possible that a secreted version of the streptavidin detector could be used. Alternatively, transgenic mice that only express the pre- and postsynaptic fusions under the control of tissue-specific promoters could be envisioned, but without the streptavidin detector. Such animals could be subjected to staining with fluorescently labeled streptavidin in conjunction with methods such as CLARITY (Chung *et al.* 2013) to visualize global connectivity patterns under different conditions. Additionally, double-labeling experiments could be

imagined using alternative *trans*-synaptic techniques developed in cell culture, such as ID-prime where lipoic acid is enzymatically transferred across the synaptic cleft (Liu *et al.* 2013). In sum, we have developed a transgenic, enzyme-based method (iBLINC) for labeling specific synaptic connections in live animals. Using iBLINC, we have been able to analyze synaptic patterns and follow the dynamics of specific connections during development and aging across a population of animals.

Acknowledgments

We thank B. Tursun for the BirA and AVItag; M. vanHoven for GFP Reconstitution Across Synaptic Partners constructs; D. Colón-Ramos for *wyIs45*; J. White and J. Hodgkin for donating Medical Research Council/Laboratory of Molecular Biology archival electron micrographs, which are under the curation of D. Hall at the Albert Einstein College of Medicine; C. Brittin for assisting with electron micrograph analysis; J.-L. Bessereau and M. Pierron for help with analyzing colocalization; E. L. Snapp for the dendra2 plasmid and for assistance with photoconversion experiments; E. Klein for technical assistance; and B. Brugg, D. Hall, and members of the Bülow and Emmons labs for comments on the manuscript and helpful discussions. This work was supported in part by National Institutes of Health grants 5R01HD055380 and R01GM01313 (to H.E.B.), R01GM066897 (to S.W.E.), and 5T32GM007491 (to S.J.C.) and by a grant from The G. Harold and Leila Y. Mathers Charitable Foundation (to S.W.E.). H.E.B. is an Irma T. Hirschl/Monique Weill-Caulier Research Fellow.

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Communicating editor: O. Hobert

GENETICS

Supporting Information
www.genetics.org/lookup/suppl/doi:10.1534/genetics.115.177006/-/DC1

Directional *Trans*-Synaptic Labeling of Specific Neuronal Connections in Live Animals

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DOI: 10.1534/genetics.115.177006

Supplemental Material

to

Directional trans-synaptic labeling of specific neuronal connections in live animals

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Running title: directional trans-synaptic labeling

5 Figures

7 Supplemental Figures

File S1

Methods

Strains

Worms were cultured as previously described. The following strains and alleles were used: N2 Bristol wild-type, *oyIs17 [Pgcy-8::GFP]* (YU *et al.* 1997), *mgIs18 [Pttx-3::GFP]* (OH99) (ALTUN-GULTEKIN *et al.* 2001), *wyIs45 [Pttx-3::rab-3::GFP]* (TV392) (COLON-RAMOS *et al.* 2007), *dzEx1240 [Pgcy-8::BirA::nrx-1; Pttx-3::AP::nlg-1; Punc-122::streptavidin::tagRFP; elt-2::GFP]* (EB2217), *dzEx1321 [Pgcy-8::BirA::nrx-1; Pttx-3::AP::nlg-1; Pelt-2::streptavidin::tagRFP; rol-6(su1006)]* (EB2365), *dzEx1322 [Pelt-2::streptavidin::tagRFP; rol-6(su1006)]* (EB2366), *dzEx1323 [Pgcy-8::BirA::nrx-1; Punc-122::streptavidin::tagRFP; rol-6(su1006)]* (EB2367), *dzEx1324 [Pttx-3::AP::nlg-1; Punc-122::streptavidin::tagRFP; rol-6(su1006)]* (EB2368), *dzIs64 [Punc-122::streptavidin::tagRFP; rol-6(su1006)]* (EB2268), *dzEx1325 [Pgcy-8:nlg-1::spGFP1-10; Pttx-3::nlg-1::spGFP11; rol-6(su1006)]* (EB2369), d*zEx1326 [Pgcy-32::mcherry; Pttx-3::GFP; rol-6(su1006)]* (EB2370), *dzEx1330 [Pgcy-8::BirA::nrx-1; Pttx-3::AP::nlg-1; Punc-122::streptavidin::2xsfGFP; rol-6(su1006)]* (EB2374), *dzIs66 [Punc-122::streptavidin::2xsfGFP; Pelt-2::GFP]* (EB2268), *dzEx1327 [Pgcy-8::BirA::nrx-1; Pttx-3::AP::nlg-1; Punc-122::streptavidin::dendra2; rol-6(su1006)]* (EB2371), *dzEx1328 [Pgcy-32::BirA::nrx-1; Pttx-3::AP::nlg-1; Punc-122::streptavidin::tagRFP; rol-6(su1006)]* (EB2372), *dzEx1329 [Pgcy-32::BirA::nrx-1; Pflp-18::AP::nlg-1; Punc-122::streptavidin::tagRFP; rol-6(su1006)]* (EB2373), *dzEx1240 ; oyIs17* (EB2265), *dzEx1240; mgIs18* (EB2264), *dzEx1240; wyIs45* (EB2266), *dzEx1240; unc-104(e1265)* (EB2434).

To create transgenic worms, constructs were injected at 25ng/µl with *pRF4* (*rol-6(su1006)*) as a dominant injection marker or a gut fluorescent marker (*Pelt-2::*GFP). Several transgenic lines giving comparable results were obtained for each construct.

Cytoplasmic markers strains and iBLINC expressing strains were constructed using

standard procedures. Details for genotyping are available on request.

Molecular biology

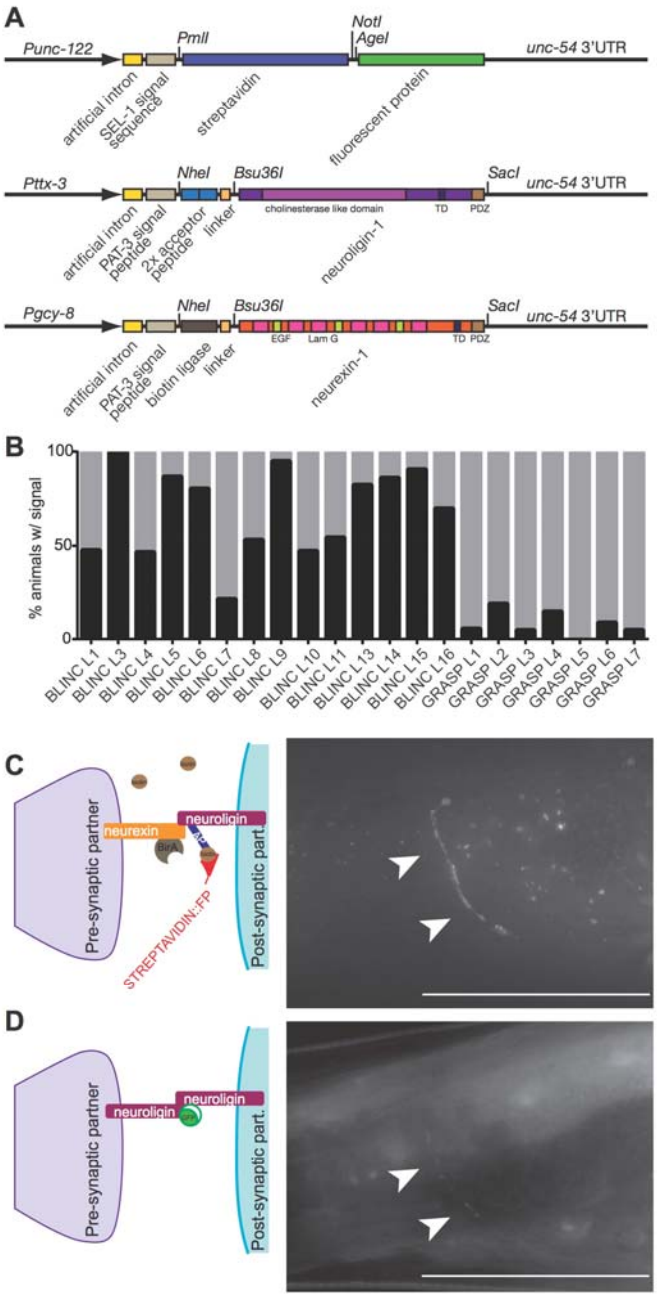
The acceptor peptide (AP) was PCR amplified from pBALU-NtermAVI 2.0 (kind gift of B. Tursun & O. Hobert) and fused by PCR to a part of the NLG-1/neuroligin cDNA amplified from a GRASP plasmid (*Psrh-128::nlg-1::spGFP11*, kind gift of M. vanHoven). This fragment was subcloned into *Psrh-128::nlg-1::spGFP11* using *Nhe*I and the *Age*I within the NLG-1/neuroligin cDNA so that the AP sequence was fused in frame between the PAT-3 signal peptide and NLG-1 sequence. The *Psrh-128* promoter was swapped to the *Pflp-18* promoter (ROGERS *et al.* 2003) using *Not*I/*BamH*I and to the *ttx-3* promoter with *Xma*I/*Apa*I.

The *E. coli* biotin ligase (BirA) was PCR amplified from *ceh-36prom2::NLS-myc-BirAo::unc-54-3'UTR* (kind gift of B. Tursun & O. Hobert) and fused in frame by PCR to NLG-1/neuroligin amplified from a GRASP plasmid (*Psrh-128::nlg-1::spGFP11*, kind gift of M. vanHoven) and subcloned using *Nhe*I and *Age*I into the same plasmid. Thus, the BirAo (o for optimized, hereafter named BirA) sequence is inserted in frame between the PAT-3 signal peptide and NLG-1 sequence. The *Psrh-128* promoter was switched to the *Pflp-18* promoter using *Not*I and *BamH*I sites and further to the *Pmec-7* promoter using *Sph*I/*Xma*I.

The *nrx-1* cDNA was amplified using custom primers from N2 cDNA and subcloned into *pPD96.41* using *Xho*I/*Xma*I sites; then subcloned into the plasmid *Pmec-7::BirA::nlg-1* to replace the NLG-1 sequence with the NRX-1 using *Bsu36*I and *Sma*I sites. Finally the *Pmec-7* promoter was switched to the *Pgcy-8* promoter using *Sph*I and *KpnI* enzymes. *Sph*I and *Xma*I sites were used to exchange the *Pgcy-32* promoter (YU *et al.* 1997) for the *Pgcy-8* promoter (YU *et al.* 1997). All PCR amplified sequences were verified, and primer and plasmid sequences are available on request.

The AFD-AIY GRASP specific plasmids were constructed by switching the *Pflp-18* promoter of *Pflp-18::nlg-1::spGFP1-10* for the *Pgcy-8* promoter, and the *Pshr-128* promoter in *Pshr-128::nlg-1::spGFP11* for the *Pttx-3* promoter using *Sph*I and *Xma*I sites.

SUPPLEMENTAL FIGURES



Supplementarry Figure S1

Figure S1. Schematic of iBLINC constructs.

A. The iBLINC constructs have a modular structure comprising a signal peptide, followed by various modules as indicated. Convenient restriction sites are indicated to allow easy replacement of promoters or fluorescent proteins. TD: transmembrane domain; Lam G: laminin G domain; EGF: Epidermal Growth Factor-like domain.

B. Quantification of fluorescent signal in several transgenic lines as indicated. The percentage of animals showing a fluorescent signal is shown by black bars (N=10-58/transgenic line). Variable penetrance of staining could be the result of variable expression from extrachromosomal transgenes.

C. Schematic of the *in vivo* BLINC system. Epifluorescent micrograph of the head region of a transgenic animal (d*zEx1240*) expressing iBLINC (using mstrep::tagRFP) in AFD/AIY.

D. Schematic of the *in vivo* GRASP system with an epifluorescent micrograph of the head region of a transgenic animal (d*zEx1325*) expressing GRASP (GFP) in AFD/AIY. Images in (c) and (d) were taken under exactly the same conditions (exposure time 1,000 ms; 630x) on the same microscope

Figure S2. Annotated sequences of key plasmids and fragments.

Punc-122::streptavidin::tagRFP (pCC::streptavidin::tagRFP)

unc-122 promoter

sel-1 Signal sequence

streptavidin

tagRFP

unc-54 3'UTR

ATGACCATGATTACGCCAAGCTTGGGCTGCAGgaagggattagtggaactggaaagacatacttgttaaatgctatttgt
ggactattcaaaagtgagtattacctagaaaaatatatgtattacggtgaaaaaatctcacctaaagtttgacatgaaaa
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Pgcy-8::BirA::nrx-1 (pAFD::BirA::NRX-1)

gcy-8 promoter

pat-3 Signal peptide

BirA coding sequence and BirA intron

Linker

Nrx-1 cDNA

unc-543'UTR

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Pttx-3::AP::nlg-1 (pAIY::AP::NLG-1)

ttx-3 promoter

pat-3 Signal peptide

acceptor peptide(AP)

Linker

nlg-1 cDNA

unc-54 3'UTR

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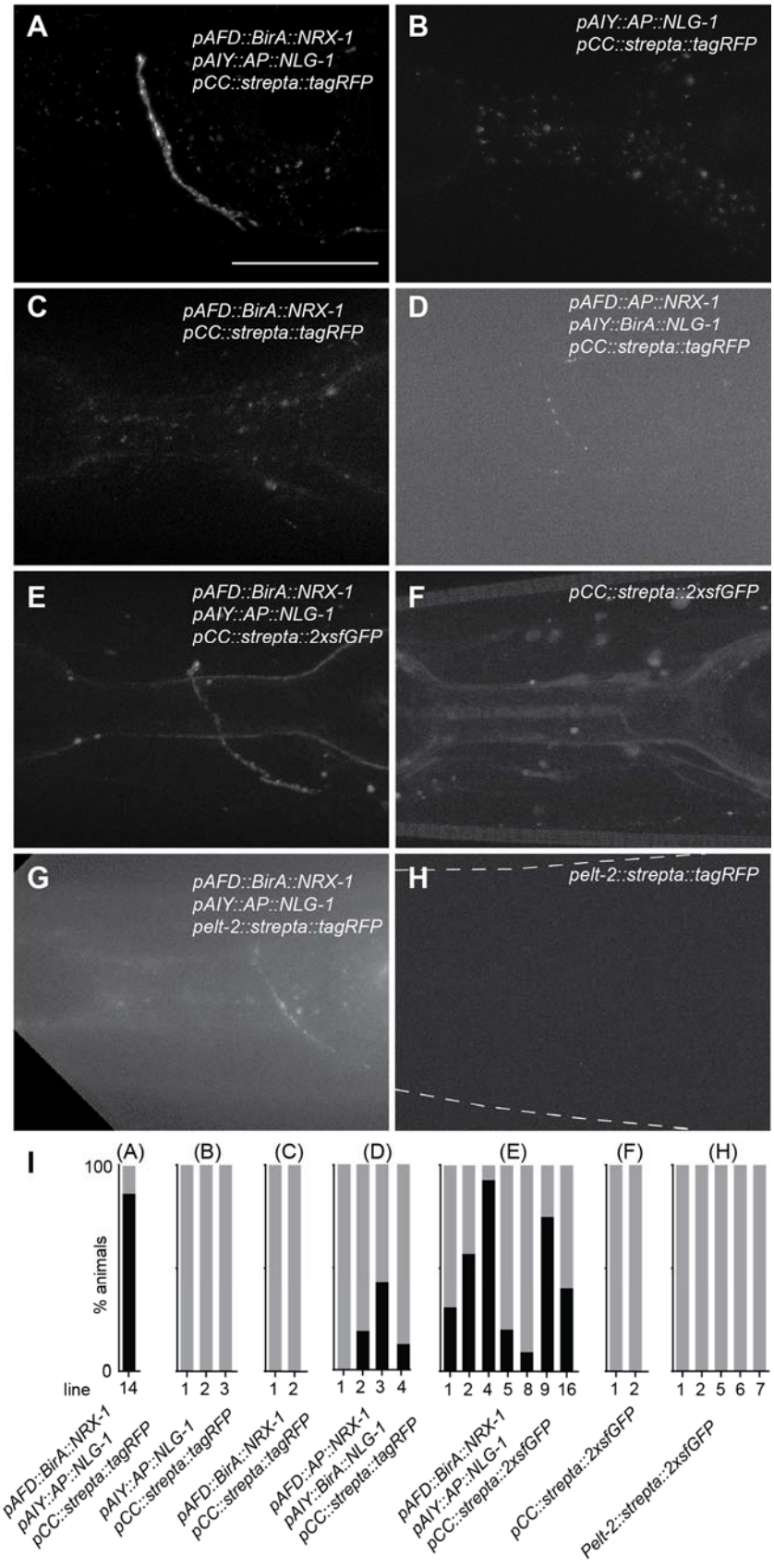


Supplemental Figure S3

Figure S3: Control experiments to demonstrate iBLINC specificity.

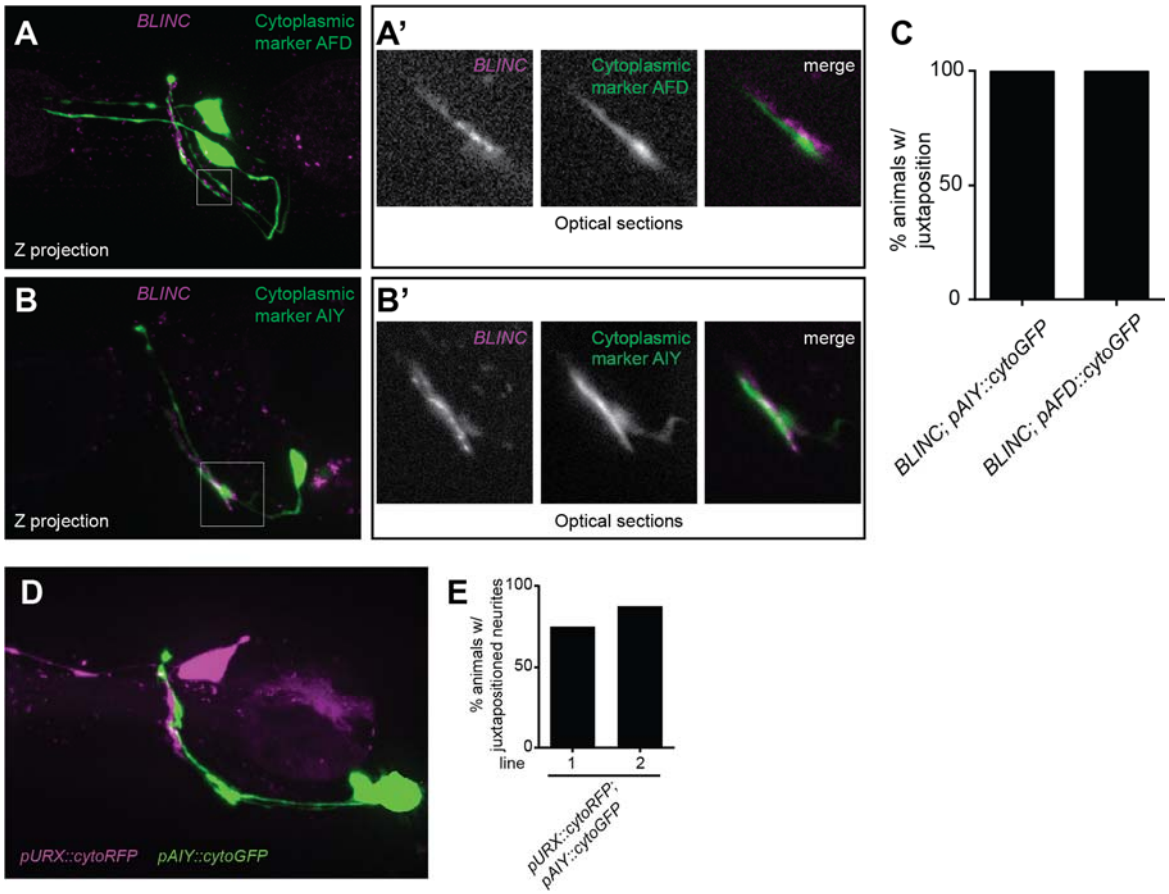
A. – C. Epifluorescent micrographs of the head region of transgenic animals expressing all three iBLINC constructs (A), the postsynaptic AP::NLG-1/neuroligin with the streptavidin 'detector' fusion (B), or the presynaptic BirA::NRX-1/neurexin with the streptavidin 'detector' fusion (C). Scale bar indicates 20 µm and anterior is to the left in all micrograph panels. The image in (A) is identical as in Figure 1D and shown for comparison only.

D. Epifluorescent micrograph of the head region of transgenic animals expressing the NRX-1 fusion with AP (instead of BirA) presynaptically and the NLG-1 fusion with BirA (instead of AP) also results in a signal albeit with reduced strength.

E. – F. Epifluorescent micrographs of the head region of transgenic animals expressing the iBLINC constructs using monomeric streptavidin fused to tandem superfolder GFP (2xsfGFP) as a detector instead of tagRFP (E, (*dzEx1330*)). The corresponding detector alone (F, *dzIs66*)) shows no signal.

G. – H. Epifluorescent micrographs of the head region of transgenic animals expressing the iBLINC constructs, but secreting the detector from the intestine using the *Pelt-2* promoter instead of the *Punc-122* coelomocytes specific promoter (G, (*dzEx1321*)). The corresponding detector alone (H, (*dzEx1322*)) shows no signal.

I. Quantification of transgenic lines as indicated. The percentage of animals showing signal is shown by black bars (N=8-54/transgenic line).



Supplementary Figure S4

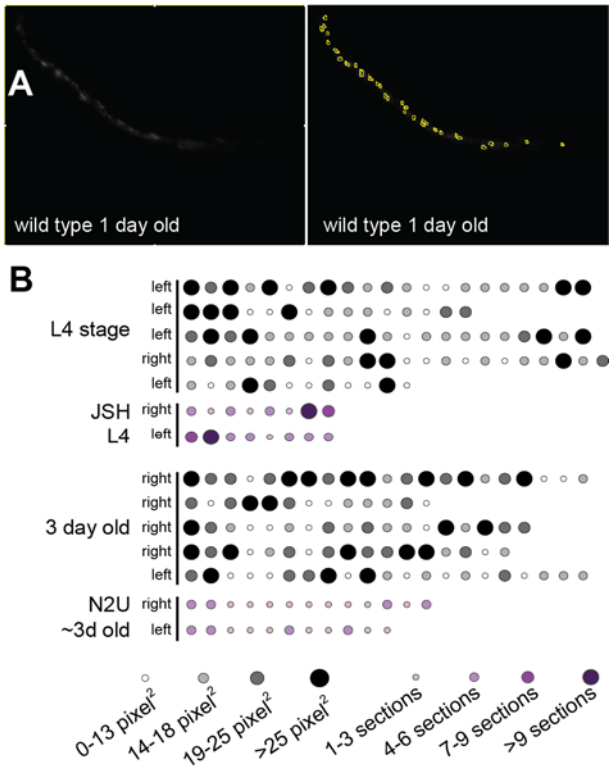
Figure S4: Neuroanatomy of AIY, AFD and URX neurons.

A. – B. Epifluorescent micrographs of the head region of transgenic animals expressing AFD/AIY iBLINC (*dzEx1240*) and a cytoplasmic marker for AFD (*oyIs17*) (a) or AIY (*mgIs18*)(b) showing juxtaposition of iBLINC signals with cytoplasmic markers. (B', C') Optical sections of the indicated area in (B, C), demonstrating juxtaposition of both signals.

C. Quantification of animals showing juxtaposition of iBLINC signals with the AIY or AFD cytoplasmic markers (N=24 and 30, respectively).

D. Epifluorescent images of animals expressing different fluorescent cytoplasmic markers in URX sensory neurons (magenta) and AIY interneurons (green). Anterior is to the left and dorsal to the top.

E. Quantification of the percentage of animals showing juxtaposition (defined as no visible gap between the two neurites in the nerve ring) of URX and AIY neurites in the nerve ring in two different extrachromosomal transgenic lines (N=22-33).

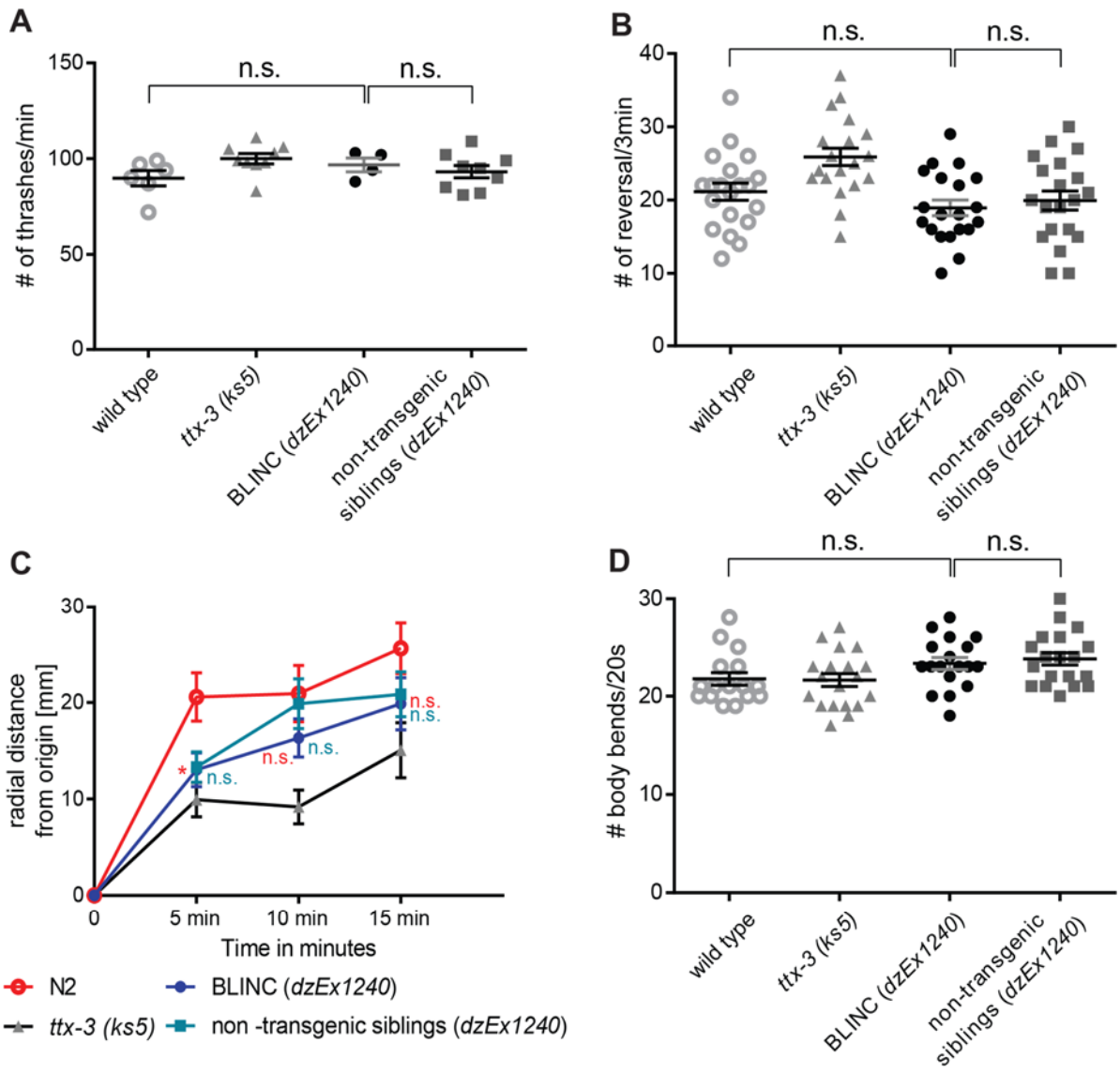


Supplementary Figure S5

Figure S5. Quantification of synapses between AFD sensory neurons and AIY interneurons.

A. Epifluorescent images of iBLINC expressing animals in one day old adult wild type animals. Synapses were circled and the area was quantified using ImageJ. Prior to analysis, images were randomized and analyzed with the investigator blind to genotype (or age).

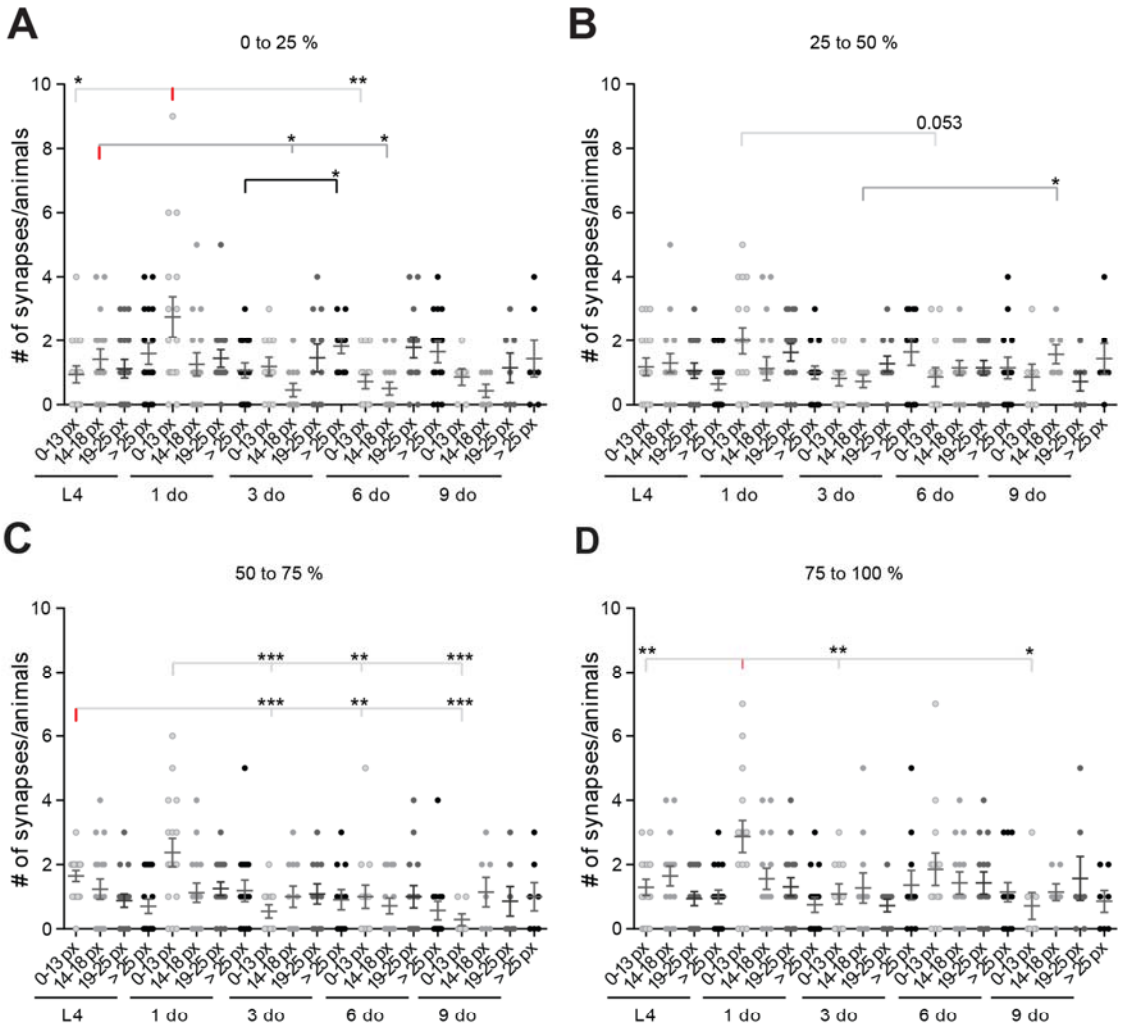
B. 'Pearl chain' graphs illustrating the sequential patterns of synapses by iBLINC at the L4 larval stage and in three day old adult animals compared to patterns obtained from electron microscopic series at the L4 larval stage (JSH) and three day old adults (N2U), respectively. Each row is an individual animal. Synapses/punctae were quantified and placed into four different sized bins based on fluorescent area (or number of sections seen in EM) as indicated by different sizes and shades. Size units are square pixels or number of sections. iBLINC animals used for the 'pearl chain' graphs are identical as in Figure 3C and shown for comparison only.



Supplementary Figure S6

Figure S6. iBLINC does not obviously compromise AIY functions during locomotory behaviors.

A. – D. Behavioral assays of locomotory behaviors were performed essentially as described (TSALIK AND HOBERT 2003), including thrashing rate (A), number of reversals (B), radial locomotion (C) and body bends/20s (D). The hypomorphic mutation in the homeobox transcription factor *ttx-3(ks5)* was used as a positive control and N2 wild type animals and non-transgenic siblings of the extrachromosomal iBLINC line (*dzEx1240*) as negative controls. Statistical significance compared to N2 and non transgenic siblings is indicated in red or blue, respectively: ns, not significant; *, *P<0.05.* Note that both iBLINC animals and their non-transgenic siblings display a slight reduction of radial locomotion (C), suggesting that this may not be due to the iBLINC transgene.



Supplementary Figure S7

Figure S7. Synaptic loss affects primarily smaller synapses

A. – D. Quantification of the number of synapses by size in each bin/quartile per animal (0 to 25 %, 25 to 50%, 50 to 75%, 75 to 100%) at the indicated ages: L4 larval stage, 1d-o adult, 3d-o adult, 6d-o adult, or 9d-o adult. Each dot represents the mean of one animal with the mean of means indicated by a line of each size and age as indicated. Statistical significance is indicated explicitly or as: *, $P<0.05$; **, $P<0.005$; ***, $P<0.0005$. Size units are square pixels.

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