Transsynaptic labeling and transcriptional control of zebrafish neural circuits

Cagney Coomer[1], Daria Naumova[1,2], Mustafa Talay[3,#], Bence Zolyomi[3], Nathaniel J. Snell[3], Altar Sorkaç[3], Jean-Michale Chanchu[4], Ji Cheng[1,2], Ivana Roman[5], Jennifer Li[5], Drew Robson[5], Gilad Barnea[3] and Marnie E. Halpern[1,2,4*]

1. Department of Molecular and Systems Biology, Geisel School of Medicine at Dartmouth, Hanover NH
2. Department of Biology, Johns Hopkins University, Baltimore, MD
3. Robert J. and Nancy D. Carney Institute for Brain Science, Department of Neuroscience, Brown University, Providence RI
4. Department of Embryology, Carnegie Institution for Science, Baltimore MD
5. Max Planck Institute for Biological Cybernetics, Tübingen, Germany

Current address: Howard Hughes Medical Institute, Department of Molecular and Cellular Biology, Harvard University, Cambridge, MA

*Corresponding author
Dr. Marnie E. Halpern
Tel.: (603) 646-5251
E-mail: marnie.e.halpern@dartmouth.edu

Abstract

Deciphering the connectome, the ensemble of synaptic connections that underlie brain function, is a central goal of neuroscience research. Here, we report mapping of connections between presynaptic and postsynaptic partners in a living vertebrate nervous system, that of the zebrafish, through the successful adaptation of the *trans*-Tango genetic approach, first developed for anterograde transsynaptic tracing in *Drosophila*. Neural connections were visualized between synaptic partners in the larval retina and brain and followed over development. Results were corroborated by functional experiments in which optogenetic activation of retinal ganglion cells elicited responses in neurons of the optic tectum, as measured by *trans*-Tango-dependent expression of a genetically encoded calcium indicator. Transsynaptic signaling through *trans*-Tango reveals predicted as well as previously undescribed synaptic connections in the zebrafish brain, providing a valuable *in vivo* tool to monitor and interrogate neural circuits over time.

Introduction

Ever since the application of Golgi staining to the nervous system, neuroscientists have sought to understand the structural organization of the brain and its underlying neural connections ([1]). Labeling with dyes or fluorescent proteins, transsynaptic tracing through application of viruses and, more recently, reconstruction by serial electron microscopy (EM) have helped resolve aspects of the connectome in invertebrate and vertebrate models. Each approach has its benefits and limitations ([2]). For example, anterograde and retrograde viral tracers have been widely used to map neural connections in the mouse brain ([3, 4, 5]), but transduction of virus is variable in different cell types and inevitably causes death of infected neurons. The labor-intensive sample collection and computational complexity required for 3-D EM reconstruction, ([2, 6]), precludes surveying large brain regions or comparisons between samples.

Genetic methods for anterograde transsynaptic tracing permit precise labeling from identified presynaptic neuronal populations in multiple individuals and can be used to both identify synaptic partners and modify their activity. The genetically encoded synthetic transsynaptic labeling system, *trans*-Tango ([7]) has been successfully used to map a variety of neural circuits in *Drosophila* ([8-12]). The Tango system relies on a synthetic signaling pathway involving two fusion proteins that convert the activation of a receptor into reporter expression ([13]). To determine its effectiveness in a vertebrate central nervous system (CNS), we modified the components of *Drosophila trans*-Tango for anterograde transsynaptic labeling in zebrafish, a powerful vertebrate model for correlating whole brain imaging of neural activity with behavior.

Large numbers of zebrafish embryos can be readily injected at the 1-cell stage with plasmids bearing all *trans*-Tango components, and subsequent neuronal labeling assessed in the larval brain. Such transient assays enable a variety of constructs to be rapidly tested, compared, and optimized. The transparency of larval stages permits live confocal imaging of uniquely labeled pre- and postsynaptic neurons throughout the brain and connections can be followed over time. Notably, neuronal morphologies are distinguished at the single-cell level by sparse labeling resulting from mosaic expression of injected DNA constructs. Many transgenic tools are also already available that can be used in conjunction with the

73 *trans*-Tango reagents. As first described by Talay et al., ([7]), the *trans*-Tango approach relies on two
74 binary transcriptional regulatory systems, Gal4/UAS from *Saccharomyces cerevisiae* and QF/QUAS from
75 *Neurospora crassa* (Fig. 1a), that both function in zebrafish ([14, 15]). Adopting *trans*-Tango for zebrafish
76 capitalizes on existing Gal4 lines to drive expression of a ligand derived from human glucagon (hGCG) in
77 presynaptic neurons of interest. In this construct, hGCG is fused to the cytosolic and transmembrane
78 domains of a zebrafish synaptic protein, either *neurexin 1a* or *1b*, and the extracellular domain of the
79 *human intercellular cell adhesion molecule 1* (ICAM1). With these modifications, the ligand is tethered
80 to the presynaptic membrane and extends into the synaptic cleft. The corresponding G protein-coupled
81 glucagon receptor (hGCGR) is expressed on the surface of all neurons under control of a nearly pan-
82 neural promoter. The cytoplasmic tail of the receptor is fused to the QF transcription factor via a
83 sequence containing the cleavage site for the N1a protease from the tobacco etch virus (TEV) ([13]). Upon
84 binding of the ligand across the synapse, the activated G protein-coupled receptor recruits human
85 β-arrestin2 that is fused to the N1a protease of the tobacco etch virus (hArr-TEV). Recruitment of
86 hArr-TEV results in proteolytic cleavage of the TEV cleavage site (TEVcs) and release of the receptor-
87 bound QF transcription factor in postsynaptic neurons. QF can then translocate to the nucleus where it
88 activates transcription of any gene downstream of the multicopy upstream activating sequence (QUAS).
89 As we demonstrate, different reporters can thus be placed under UAS and QUAS control to label pre- and
90 postsynaptic neurons, respectively, with distinct fluorescent proteins. Additionally, target neurons can
91 express any gene under QUAS control such as genetically encoded calcium indicators (i.e., GCaMP).
92

93 **Results**

94 <u>Adaptation of *trans*-Tango components for zebrafish</u>

95 To adapt the *trans*-Tango system for use in zebrafish, the ligand, receptor, and arrestin-TEV constructs
96 were cloned into vectors containing the short flanking arms of the Tol2 transposon ([16]). The presence of
97 each component was assessed by the addition of a secondary marker on the same plasmid, consisting of a

98 tissue-specific promoter driving expression of a fluorescent protein (Fig. 1b). Six ligand constructs that
99 differed in the length of the ICAM1 extracellular domain and in zebrafish *neurexin1a* or *1b* domains were
100 placed under the control of multicopy UAS sequence (10xUAS) ([17]) in a Tol2 plasmid that also contained
101 the *crystalline, alpha A* promoter driving mCherry (*cryaa:mCherry*). For transcription of the hGCGR-QF
102 and hArr-TEV constructs, we used the promoter from the zebrafish *ELAV like neuron-specific RNA*
103 *binding protein 3* (*elavl3*) gene that directs widespread expression in post-mitotic neurons throughout the
104 larval zebrafish CNS ([18]). The receptor and arrestin-TEV constructs were cloned into Tol2 plasmids
105 containing the promoter from the *hatching gland enzyme1.1* (*he1.1*) gene ([19]) upstream of sequences
106 encoding cyan or yellow fluorescent protein, respectively. The *he1.1* promoter drives expression in
107 hatching gland cells at 24 hours post fertilization (hpf); however, labeling is not detected after 72 hpf.
108 This permits imaging of neural connections in the larval brain unobscured by bright fluorescence from a
109 secondary marker.
110
111 Gal4-dependent anterograde labeling by *trans*-Tango
112 To assess the effectiveness of the individual ligand, receptor-QF, and arrestin-TEV constructs, we injected
113 plasmids encoding each along with mRNA encoding Tol2 transposase into zebrafish embryos. The
114 embryos were derived from matings between *pt1fa:Gal4-VP16* driver ([20]) and *QUAS:mApple-CAAX*
115 reporter lines. The *pancreas-specific transcription factor 1a* (*ptf1a*) driver because it promotes
116 transcription broadly in the hindbrain ([21-23]), as well as in retinal amacrine and horizontal cells and a
117 subset of ganglion cells ([24, 25]), providing a substantial target area for testing the functionality of *trans*-
118 Tango. We determined which of six UAS:ligand constructs resulted in extensive labeling of putative
119 postsynaptic neurons. Three constructs contained domains from the *neurexin 1a* gene and three from
120 *neurexin 1b* (Supplementary Fig. 1) that either included the full-length extracellular domain from ICAM1,
121 a truncated version, or lacked these sequences altogether ([7]). Criteria for determining the optimal ligand
122 construct were mApple-CAAX reporter labeling that was robust, consistent between samples, and Gal4-

123 dependent. We co-expressed the receptor and arrestin-TEV constructs with each ligand configuration and
124 determined that 10x*UAS:sGCG-ICAM(1235)-nrxn1b* produced the most extensive mApple-CAAX
125 labeling of target neurons with minimal Gal4-independent labeling (Supplementary Fig. 1). We, therefore,
126 used this ligand construct for all subsequent experiments. In 89% of larvae that were screened positive
127 for *pt1fa:Gal4-VP16* and all three *trans*-Tango components (n=1,042), mApple-CAAX labeled neurons
128 were found in close proximity to GFP-labeled presynaptic neurons throughout the hindbrain (Fig. 2a,a').
129 In addition, we observed labeling of putative postsynaptic neurons in the optic tectum, presumably
130 because the *pt1fa* promoter is active in a subset of retinal ganglion cells ([25]) whose axonal projections
131 terminate in this midbrain region.

132 To address the specificity of *trans*-Tango, we compared the location of mApple labeled neurons
133 with those observed using two other Gal4 driver lines. Cis-regulatory sequences from the *tyrosine*
134 *hydroxylase* (*th*) gene in *th:Gal4-VP16;UAS:GFP* direct expression in dopaminergic neurons throughout
135 the larval CNS ([26]). Those from the *ISL LIM homeobox 2b* (*islet2b*) gene in *isl2b:Gal4* drive expression in
136 retinal ganglion cells ([27]), as well as in Rohan-Beard sensory neurons, neurons in cranial ganglia, dorsal
137 root ganglia, the anterior and posterior lateral line and populations in the brain ([28-30]). At the 1-cell stage,
138 embryos derived from matings between fish bearing these Gal4 driver lines and a UAS:GFP transgene
139 with fish from the *QUAS:mApple-CAAX* reporter line were injected with the *trans*-Tango ligand,
140 receptor, and arrestin constructs. At 6 days post fertilization (dpf), each driver line produced a distinct
141 pattern of mApple-CAAX labeled neurons and, in all cases, putative postsynaptic cells were located near
142 GFP-labeled neurons or their processes (Figs. 2a,a', 2 b,b' and 2c,c'). mApple-CAAX labeled cells were
143 not detected in larvae bearing all *trans*-Tango components except the ligand (Figs. 2d,e,f). These
144 observations indicate that *trans*-Tango functions effectively in the zebrafish nervous system with a high
145 signal-to-noise ratio and patterns of labeling determined by the presynaptic subpopulation that expresses
146 the ligand.

147 We tested whether transsynaptic labeling could also be achieved with another nearly pan-neural
148 promoter, that of the *Xenopus tubulin beta 2B class IIb* (*Xla.tubb2b*) gene ([31]), which drives expression in
149 the zebrafish CNS from larval to adult stages. Injection of the receptor and arrestin-TEV constructs under
150 *Xla.tubb2b* control together with the ligand plasmid and Tol2 RNA into embryos from matings between
151 the *isl2b:*Gal4 and *QUAS:mApple*-CAAX lines resulted in robust mApple-CAAX labeling as early as 1
152 dpf, which increased as development proceeded (Fig. 3). Uniquely identified neurons were tracked over
153 time, including commissural primary ascending (CoPA) interneurons, V2a interneurons, and primary
154 motoneurons such as CaP that are all known post-synaptic targets of ligand-expressing Rohon-Beard cells
155 ([32, 33]). Labeling from the *Xla.tubb2b* driven constructs persisted, as mApple positive neurons present in
156 the optic tectum at 6 dpf could still be detected in sections prepared from 2-week-old larvae
157 (Supplemental Fig. 2a-b').
158
159 <u>Validation of anterograde labeling in the larval retina</u>
160 The retina is a highly conserved structure that is populated by distinct neuronal cell types distinguished by
161 their morphology, position and connectivity, making it an ideal substrate for testing the fidelity of *trans*-
162 Tango. We identified a total of 102 cells individually labeled by mApple-CAAX in retinal samples from
163 112 larvae that contained the *pt1fa:Gal4* driver and *QUAS:mApple-CAAX* reporter transgenic lines and all
164 *trans*-Tango plasmids. The *ptf1a* gene is expressed in a retinal progenitor population that gives rise to all
165 amacrine and horizontal cells ([24]). The postsynaptic partners of amacrine cells are retinal ganglion cells
166 and bipolar cells as well as other amacrine cells, whereas horizontal cells form reciprocal synapses with
167 photoreceptors [34]. Consistent with this, we detected mApple-CAAX labelled cells located in the ganglion
168 cell layer with axons extending to the brain within the optic nerve (Fig. 4a,a'; n=20), in amacrine cells
169 (Fig. 4b,b'; n=35) and bipolar cells (Fig. 4c,c'; n=25) in the inner nuclear layer, and photoreceptor cells in
170 the photoreceptor layer (Fig. 4 d,d'; n=57).
171 To corroborate the identities of *trans*-Tango labeled neurons, we performed
172 immunohistochemistry using cell-type specific antibodies and imaged double-labeled cells in cryosections

173 of 6 dpf larval retinas. mApple-CAAX labeled retinal ganglion cells co-labeled with HuC/D antibody
174 were detected in close proximity to GFP-labeled presynaptic amacrine cells (n=8; Fig. 4a''). Amacrine
175 cells situated in the inner nuclear layer with extensive dendritic processes in the inner plexiform layer also
176 co-labeled with the HuC/D antibody (n=9; Fig. 4b''), bipolar cells were distinguished by PKCa
177 immunoreactivity (n=6; Fig. 4c''), and photoreceptors by zpr-3 monoclonal antibody ([35]) (n=; Fig.4d'').
178 These results indicate that *trans*-Tango reliably identifies known synaptic partners in the retina.
179

180 <u>*Trans*-Tango in stable transgenic lines</u>

181 An advantage of plasmid injections is that diffuse labeling enables the identification of individual
182 neurons; however, for some applications, labeling an entire neuronal sub-population is desired. To
183 accomplish this, we established stable lines for each of the *trans*-Tango constructs. Plasmids were
184 injected into 1-cell stage *QUAS:mApple-CAAX* embryos. Those with labeled hatching gland cells at 24
185 hpf were raised to adulthood and their progeny screened to recover transgenic founders with germ-line
186 clones. Founders were mated with *QUAS:mApple-CAAX* fish and their heterozygous progeny used to
187 establish stable lines. Wholemount in situ hybridization (WISH) was performed at 6 dpf to verify
188 transgene expression with RNA probes designed to capture unique sequences only found in the *trans*-
189 Tango component (refer to Supplementary Fig. 3). For the ligand line *10xUAS-E1B:sGCG-ICAM(1235)-*
190 *nrxn1b*, the probe detected transcripts in the same pattern as endogenous *ptf1a* expression ([25]), but not in
191 the absence of the *pt1fa:Gal4-VP16* transgene (Supplementary Fig 3a). The arrestin-TEV and receptor
192 transgenes were broadly expressed throughout the CNS (Supplementary Figs. 3b,c) similar to endogenous
193 *elavl3* transcripts ([18]). Sibling embryos that lacked YFP labeling in the hatching gland at 1 dpf did not
194 show any WISH signal as expected (Supplementary Fig. 3b,c).

195 To confirm that the stable ligand line functioned effectively at transsynaptic signaling, we mated
196 the *pt1fa*-Gal4 driver line with F1 fish bearing *QUAS:mApple-CAAX* and *10xUAS-E1B:sGCG-*
197 *ICAM(1235)-nrxn1b* and injected the receptor and arrestin-TEV constructs into their 1-cell stage

198 embryonic progeny. At 6 dpf, over 80% of larvae (n=257/312) displayed mApple-CAAX labeled
199 postsynaptic neurons (Supplementary Fig. 3d-d''). We observed similar results with *elavl3:hArr-TEV*
200 larvae that, as embryos, had been injected with the ligand and receptor plasmids (n=132/164;
201 Supplementary Fig. 3e-e''). Only larvae from the receptor line, *elavl:hGCGR-TEVcs-QF*, failed to
202 produce transsynaptic labeling following injection of the ligand and arresti-TEV constructs. This is
203 unlikely due to transgene position effects as experiments were performed on three independently
204 established transgenic lines, whose larvae showed robust hGCGR-QF expression, but no mApple-CAAX
205 labeled neurons (n= 0/276; data not shown). Moreover, mApple-CAAX neurons were not observed using
206 a stable line for a receptor construct that was codon optimized for zebrafish (hGCGR*) and fused to QF
207 (n= 0/171; Supplementary Fig. 3f,f',f''). All transgenic lines did produce functional receptor because
208 robust mApple-CAAX labeling was observed at 6 dpf after a subthreshold concentration of the receptor
209 plasmid (Supplementary Fig. 3g,g') was injected into *elavl:hGCGR*-TEVcs-QF* embryos (n=21/25;
210 Supplementary Fig. 3h,h'). along with the ligand and arrestin-TEV constructs.
211 We further examined transsynaptic labeling using the ligand and arrestin-TEV transgenic lines,
212 either individually or together, with a different Gal4 driver, *isl2b:Gal4-VP16*, in which individual neurons
213 could be more easily resolved (Supplementary Fig. 4). Notably, on average, the number of mApple-
214 CAAX labeled neurons was significantly greater (>5-fold) after the receptor was introduced into embryos
215 doubly transgenic for both the ligand and arrestin-TEV (Supplementary Fig. 4d,d' and f) compared to the
216 number observed following injections of all *trans*-Tango plasmids (Supplementary Fig. 4a,a') or
217 injections into embryos with either the ligand (Supplementary Fig. 4b,b') or arrestin-TEV transgene alone
218 (Supplementary Fig. 4c,c'). Image registration of ten *isl2b:Gal4-VP16*; *QUAS:mApple-CAAX* 6 dpf larvae
219 bearing the ligand and arrestin-TEV transgenes and injected receptor plasmid revealed overlap in the
220 location of *trans-Tango* labeled postsynaptic neurons (Supplementary Fig. 4g).
221

222

223 *trans*-Tango validates retinotectal circuitry

224 Calcium imaging and sparse labeling experiments suggest that tectal neurons with distinct morphologies
225 are the targets of retinal ganglion cells ([36, 37]). To test this connectivity directly, we used the *isl2b:Gal4-*
226 *VP16* driver line in fish containing *UAS:GFP* and *QUAS:mApple-CAAX* to express *trans*-Tango ligand
227 selectively in retinal ganglion cells. We injected the ligand, receptor and arrestin-TEV constructs with
228 Tol2 mRNA in 1-cell stage embryos, sorted out those having all secondary markers at 1 and 2 dpf, and
229 then screened for *mApple-CAAX*-labeled tectal neurons at 6 dpf.

230 Seven neuronal subtypes previously proposed as postsynaptic to the optic tectum were uniquely
231 identifiable based on their position and shape (Fig. 5a-i'). These included non-stratified periventricular
232 neurons (nsPVIN, n=46; Fig. 5b,b'), superficial interneurons (SIN, n=11; Fig. 5c,c'), mono-stratified
233 periventricular neurons (msPVIN, n=67; Fig 5d,d'), bi-stratified periventricular interneurons (bsPVIN,
234 n=43; Fig. 5e-e'), periventricular projection neurons (PVPN, n=21; Fig. 5f,f' and h,h') and tri-stratified
235 periventricular interneurons (tsPVIN, n=72; Fig. 5g,g'). Using Fiji software, we produced renderings of
236 individually identified cells (Fig 5i) whose morphology closely corresponded to the previously described
237 tectal neurons ([37, 38]).

238 With *trans*-Tango we were also able to identify a postsynaptic neuron that exhibited a previously
239 undescribed morphology characterized by a long process in contact with the axon terminals of retinal
240 ganglion cells and a cell body located in the hindbrain (n=6; Fig. 5j-j''). These data indicate that *trans*-
241 Tango consistently labels predicted synaptic partners and is also a powerful tool for revealing unsuspected
242 postsynaptic targets.

243

244 Activation of synaptically-coupled neurons

245 To verify that the *trans*-Tango labeling correlates with functional synaptic connections, we
246 optogenetically activated retinal ganglion cells in 6 dpf *isl2b:Gal4-VP16* larvae and assessed the response

247 in putative postsynaptic neurons in the optic tectum. Activity in post-synaptic cells was monitored with a
248 nuclear-tagged genetically encoded calcium indicator (H2B-GcaMP6s) ([39]) that we placed under QUAS
249 control. Receptor, arrestin-TEV and QUAS:H2B-GcaMP6s plasmids were injected into the 1-cell stage
250 progeny of *isl2b:Gal4-VP16; QUAS:mApple-CAAX* fish mated to the *10xUAS-E1B:sGCG-ICAM(1235)-*
251 *nrxn1b* ligand line and later screened for secondary markers for all *trans*-Tango components. We first
252 confirmed that GcaMP6s positive nuclei were detected in mApple-CAAX neurons in the 6 dpf larval
253 tectum (dashed boxes, Fig 6a-a''), indicating that both reporter genes were co-expressed through QF
254 transcriptional regulation. Next, we activated retinal ganglion cells using the same Gal4 line to drive
255 expression of the redshifted channel rhodopsin ReaChR under UAS control (*UAS:ReaChR-Tag-RFPT*)
256 ([40]) and recorded the change in fluorescence (ΔF/F) of GcaMP6s positive tectal neurons following retinal
257 exposure to a 561 nm laser. Individual neurons were imaged in the region of the tectum where axon
258 terminals from retinal ganglion cells form a layered neuropil and connect to dendrites and axons of
259 interneurons and projection neurons ([37, 41]). We observed a significant increase in calcium transients in
260 larvae containing *UAS:ReaChR-Tag-RFPT* compared to sibling larvae that lacked this transgene (p=
261 0.0048; Fig. 6b-d). Optogenetic activation of retinal ganglion cells, thus, leads to a response in tectal
262 neurons that is visualized through GcaMP6s, the product of *trans*-Tango transsynaptic signaling.
263 Another demonstration of *trans*-Tango mediated gene regulation is the targeted ablation of
264 postsynaptic neurons. We mated fish from the ptf1a:Gal4 driver line containing the *QUAS:mApple-CAAX*
265 reporter to a transgenic line that has an optimized version of the bacterial nitroreductase gene *nfsB* under
266 QUAS control *5xQUAS:GAP-tagYFP-P2A-NfsB_Vv F70A/F108Y* ([42]). At 5 dpf, larvae were imaged for
267 *mApple-CAAX* labeling in the hindbrain (Fig. 6e,e') and then incubated for 24 hours in 10 mM of
268 metronidazole, which when reduced is toxic to cells. Reimaging of the same hindbrain region revealed
269 that *mApple-CAAX* neurons were no longer detected (Fig. 6f-f'). The results indicate that *trans*-Tango
270 signaling can be used to identify, monitor, and disrupt neural circuits in the zebrafish brain.
271

272 **Discussion**

273 We established an anterograde transsynaptic labeling system, *trans*-Tango, for the zebrafish CNS. This is
274 the first genetically encoded system for transsynaptic labeling in a vertebrate nervous system. We used
275 *trans*-Tango to regulate the expression of transgenic reporters in the postsynaptic partners of genetically
276 identified presynaptic neurons. These reporters can be used to trace connectivity anatomically and to
277 identify postsynaptic partners of the starter neurons as well as monitor their activity or ablate them.

278 Functional investigations into neural pathways in zebrafish have been facilitated by optogenetic
279 modulators and genetically encoded calcium indicators that report neuronal responses throughout the
280 larval brain ([43-45]). However, genetic tools to validate synaptic connections directly have been lacking.
281 Retrograde tracing of afferents using recombinant rabies, vesicular stomatitis, or herpes simplex 1 virus
282 has been applied to the zebrafish CNS with some success ([46, 47]). However, drawbacks to viral approaches
283 include the potential for neuronal toxicity, the necessity to inject recombinant virus locally into brain
284 regions, and the maintenance of larval fish at higher than usual temperatures for effective expression.
285 Unlike the available tools for circuit interrogation in zebrafish, the *trans*-Tango genetic strategy is solely
286 based on the introduction of an optimized set of plasmids into 1-cell stage embryos. Hundreds of
287 embryos can be readily injected, providing a large sample size for testing constructs. Moreover,
288 concentration levels can be adjusted to recover broad or sparse labeling for assessing connectivity
289 between neuronal populations or single synaptic partners, respectively. Expression of the *trans*-Tango
290 ligand under UAS transcriptional control capitalizes on the large repertoire of neural-specific, transgenic
291 Gal4 driver lines available in zebrafish ([38, 48-52]), while plasmid injections avoid the transcriptional
292 silencing often associated with UAS-regulated transgenes in the zebrafish genome across generations ([53-
293 55]).

294 A major advantage of the *trans*-Tango approach is the ability to perform live imaging to monitor
295 neural connections in the same individual over time, as was demonstrated for postsynaptic targets of *isl2b*
296 neurons. With persistently expressing pan-neural promoters, *trans*-Tango can also be applied to examine

297 circuits in the juvenile and adult CNS. Ideally, performing analyses on adult brains will benefit from
298 providing *trans*-Tango reagents as stable transgenes, as in *Drosophila* ([7]). Although we were successful at
299 accomplishing this with transgenes for the ligand and arrestin-TEV components, we have not yet
300 achieved *trans*-Tango labeling using several independently isolated receptor transgenic lines. Similar
301 negative results were obtained when hGCGR or codon-optimized hGCGR* constructs were driven by
302 either the *elavl3* or *Xla.tubb2b* promoter. One explanation is that an insufficient number of receptors are
303 concentrated at postsynaptic sites, owing to their distribution throughout the extensive membranes of
304 neuronal processes. In support of this, individuals bearing four copies of receptor transgenes that had
305 been injected with ligand and arrestin-TEV plasmids did not exhibit labeling of the postsynaptic reporter
306 (data not shown). However, robust postsynaptic labeling was observed when a concentration of receptor
307 plasmid, that itself is too low to mediate *trans*-Tango signaling, was injected into animals that had only
308 one copy of the receptor transgene. These findings indicate that transgenic lines indeed produce
309 functional hGCGR. We further determined that injection of the hGCGR-QF construct into embryos
310 bearing both the ligand and arrestin transgenes, yielded significantly more labeled neurons compared to
311 injections into each transgenic line individually. Experimental approaches requiring widespread
312 transsynaptic signaling can therefore rely on receptors derived from plasmid injected into doubly
313 transgenic animals homozygous for the ligand and arrestin-TEV transgenes.

314 Distinct patterns of labeling were obtained using three different Gal4 driver lines. In all cases,
315 postsynaptic neurons demarcated by the QUAS-controlled membrane-tagged reporter were in close
316 proximity to neurons or their processes expressing the UAS-regulated reporter. Image registration also
317 revealed an overlap in the positions of labeled cells between individual larvae from a given Gal4 driver
318 line. To assess the fidelity of connections between neurons, we turned to the retina where there is
319 considerable information about the synaptic relationships between cell types ([34]). As expected, based on
320 their position and immunolabeling, horizontal cells expressing ligand yielded *trans*-Tango labeling of
321 bipolar cells, whereas ligand producing amacrine resulted in labeling of other amacrine cells or retinal
322 ganglion cells. Ganglion cells are the only retinal neurons that project to the brain via the optic nerve,

323 where they terminate in discrete arborization fields in the optic tectum ([56, 57]). Prior studies in several fish
324 species revealed the stereotypic morphology of defined classes of tectal neurons from Golgi labeling,
325 application of dyes, or calcium imaging of neural activity ([36, 37, 58]). Remarkably, by directing *trans*-Tango
326 in retinal ganglion cells, we identified the same classes of neuronal subtypes that were previously reported
327 in the tectum, supporting the validity of this genetic approach to confirm postsynaptic targets with high
328 specificity. We also observed connectivity with a previously undescribed cell type that extends a long
329 process from its cell body situated in the hindbrain. These findings further demonstrate the potential of
330 *trans*-Tango to reveal new synaptic partners.

331 An important feature of a genetic, anterograde transsynaptic signaling pathway is the genetic
332 access that it provides to postsynaptic neurons. With *trans*-Tango, expression is dependent on
333 translocation of the QF transcription factor from the cell membrane to the nucleus where it promotes
334 transcription of any gene under QUAS control, either from plasmids or genomic transgenes. Increasing
335 numbers of QUAS driven reporter and effector transgenic lines are available in zebrafish ([15, 59-62]) and all
336 can be used as outputs of *trans*-Tango signaling. In addition to the membrane-tagged fluorescent
337 reporter mApple-CAAX, we present functional evidence for activation of QUAS regulated genes
338 encoding a nuclear GcaMP and a bacterial nitroreductase. Together, such *trans*-Tango dependent tools
339 offer new ways to characterize synaptically coupled neurons and evaluate how their activity within neural
340 circuits underlies behavioral responses. Implementation in zebrafish of *retro*-Tango, a recently described
341 Tango-based strategy for retrograde transsynaptic signaling ([63]), will further increase the versatility and
342 power of genetic approaches to probe neural pathways both acutely and over time in a vertebrate brain.
343

344 **Methods**

345 Animals

346 Zebrafish were reared under a 14:10 light:dark cycle at 27°C in dechlorinated and filtered system water.
347 Transgenic lines for *trans*-Tango components were generated in the AB wild-type strain ([64]) including

348 two alleles of *Tg(10xUAS-E1B:sGCG-ICAM(1235)-nrxn1b; cryaa:mCherry)*[cd28, cd29] referred to as
349 (*10xUAS-E1B:sGCG-ICAM(1235)-nrxn1b)*, four alleles of *Tg(elavl3:hGCGR-TEVcs-QF; he1.1:CFP)*[c796,
350 c797, cd30, cd34] (*elavl3:hGCGR-TEVcs-QF*) and *Tg(elavl3:hArr-TEV)*[cd30] (*elavl3:hArr-TEV)*. The Gal4
351 driver lines *TgBAC(pt1fa:Gal4-VP16; UAS:GFP)*[jh16] (*pt1fa:Gal4-VP16*) ([65]), *Tg(th:Gal4-VP16;*
352 *UAS:GFP)*[m1233] (*th:Gal4-VP16)* ([66]) and *Tg(-17.6isl2b:Gal4-VP16;myl7:RFP;UAS:GFP)*[zc60] (*isl2b:Gal4-*
353 *VP16)* ([67]). And QUAS regulated lines *Tg(QUAS:mApple-CAAX; he1.1:mCherry)*[c636] (*QUAS:mApple-*
354 *CAAX*)[68] and *Tg(5xQUAS:GAP-tagYFP-P2A-NfsB_Vv F70A/F108Y,he:ECFP)*[jh562] ([42]) were also used in
355 this study. To inhibit melanin pigmentation, embryos and larvae were maintained in system water
356 containing 0.003% phenylthiourea (PTU; P7629, Sigma-Aldrich). All protocols and procedures were
357 approved by the Institutional Animal Care and Use Committee (IACUC) of Dartmouth College
358

359 Plasmids

360 Plasmids for the *trans*-Tango components were generated using the Hi-Fi DNA Assembly (NEB
361 #E5520S), BP Gateway Cloning (Thermo Fisher, Invitrogen #11789020) and LR Gateway Cloning
362 (Thermo Fisher, Invitrogen #11791020) kits. Relevant PCR primers indicated below are listed in Table 1.
363 All ligand constructs were generated in a similar manner. The plasmid pExpTol2-UAS-E1B-ReaChR-TS-
364 tagRFP-cryaa-mCherry was generated as an intermediate construct. The cryaa-mCherry sequence was
365 amplified from pTol2pA2-ubb:secHsa.ANNEXINV-mVenus, cryaa:RFP (Addgene #92388) using primers
366 3283 and 3284. The SV40 late polyA sequence was amplified from pKHR8 (Addgene #74625) using
367 primers 3285 and 3286. The pExpTol2-UAS-E1B-ReaChR-TS-tagRFP plasmid was digested with AscI
368 and MluI. Both PCR products and the digested vector were assembled using Hi-Fi DNA Assembly to
369 obtain pExpTol2-UAS-E1B-ReaChR-TS-tagRFP-cryaa-mCherry. For pExpTol2-UAS-E1B-sGCG-
370 Nrxn1a; cryaa-mCherry, the sGCG sequence was amplified from the *Drosophila trans*-Tango plasmid ([7])
371 using primers 3274 and 3279. To create the pExpTol2-UAS-E1B-sGCG-ICAM(1235)-Nrxn1a; cryaa-
372 mCherry vector, sGCG-ICAM(1235) sequences were amplified from the *Drosophila* plasmid ([7]) in two
373 separate reactions, using primer pairs 3274 and 2302 and 3275 and 2303. For pExpTol2-UAS-E1B-

374 sGCG-ICAM-Nrxn1a; cryaa-mCherry the sGCG-ICAM sequence was amplified using the primers 3274
375 and 3275. Nrxn1a sequence encoding the full-length protein without the signal peptide was amplified
376 using primers 3280 and 3264. Sequences encoding the transmembrane and the intracellular domains of
377 Nrxn1a swere amplified using primers 3276 and 3264 from pooled cDNA of 6 dpf zebrafish larvae. PCR
378 products were subsequently cloned pExpTol2-UAS-E1B-ReaChR-TS-tagRFP-cryaa-mCherry (Addgene
379 #43963) digested with SpeI and PacI using Hi-Fi DNA Assembly.

380 Constructs for the Nrxn1b plasmids were generated as described above. For pExpTol2-UAS:E1B-
381 sGCG, the sGCG sequence was amplified from the trans-Tango plasmid ([7]) using primers 3274 and 3281.
382 To create pExpTol2-UAS-E1B-sGCG-ICAM(1235)-Nrxn1b; cryaa-mCherry, the sGCG-ICAM(1235)
383 sequence was amplified from the trans-Tango plasmid ([7]) in two separate reactions using the primer pairs
384 3274 and 2302 and 3277 and 2303. For pExpTol2-UAS-E1B-sGCG-ICAM-Nrxn1b; cryaa-mCherry, the
385 sGCG-ICAM sequence was amplified from the *trans*-Tango plasmid ([7]) using primers 3274 and 3277.
386 Nrxn1b sequence encoding full-length protein without the signal peptide was amplified from zebrafish
387 cDNA using primers 3282 and 3265 and the transmembrane and the intracellular domains were amplified
388 using primers 3278 and 3264. PCR products were subsequently cloned into pExpTol2-UAS-E1B-
389 ReaChR-TS-tagRFP-cryaa-mCherry (Addgene #43963) digested with SpeI and PacI using Hi-Fi DNA
390 Assembly.

391 To produce hGCGR vectors, myl7_EGFP sequence was amplified from pDestTol2CG2-
392 U6:gRNA (Addgene #63156) using primers 3266 and 3267. The SV40 late polyA sequence was
393 amplified from pKHR8 (Addgene #74625) using primers 3268 and 3269. The Tol2 Gateway Destination
394 Vector ([43]) was digested with AscI and MluI. Both PCR products and the digested vector were assembled
395 using Hi-Fi DNA Assembly. The resulting plasmid was subsequently digested with SpeI and SacII. The
396 hGCGR-TEVcs-QF sequence was amplified from the *Drosophila trans*-Tango plasmid ([7]) using primers
397 3270 and 3271. The PCR product and the digested vector were assembled using Hi-Fi DNA Assembly.
398 The final plasmid was then subjected to LR Gateway cloning with pEntry-HuC ([43]) to obtain
399 elavl3:hGCGR-TEVcs-QF; myl7:EGFP. In later experiments, the secondary reporter labeling the heart

400 (myl7:EGFP) was replaced with he1.1:CFP that labels hatching gland cells. The he1.1:CFP sequence was
401 amplified from p3Ehe1a:ECFP (Addgene #113880) using primers 185 and 186.
402 The receptor construct was also placed under the control of the Xla.Tubb2b promoter
403 (Xla.Tubb2b:hGCGR-TEVcs-QF; he1.1:CFP). The hGCGR-TEVcs-QF sequence was amplified from
404 elavl3:hGCGR-TEVcs-QF;myl7:EGFP using primers 93 and 131 that contained flanking attB1 and attB2
405 sequences, respectively. A Gateway recombination reaction was performed using BP Clonase II with this
406 PCR product and donor vector pDONR221 (Invitrogen, #12536017) to obtain pME_hGCGR-TEVcs-QF.
407 Using Gateway LR Clonase II, p5E nbt ([59]), pME_hGCGR-TEVcs-QF, p3E_he1a:ECFP (Addgene
408 #113880) were introduced into the Tol2 Gateway Destination Vector ([69]) (#394) to obtain
409 Xla.Tubb2b:hGCGR-TEVcs-QF;he1.1:CFP.
410 The hGCGR sequence from the elavl3:hGCGR-TEVcs-QF;myl7:EGFP (this study) was codon
411 optimized for zebrafish (hGCGR*) following
412 https://www.nichd.nih.gov/research/atNICHD/Investigators/burgess/software ([70]) and synthesized by
413 Gene Universal (www.geneuniversal.com) into pUC57-BsaI. From this plasmid, the hGCGR* sequence
414 was amplified and attB1 and attB2 arms attached using primers 174 and 175. The PCR product was
415 placed in pME (pDONR221 #398, Addgene) using a Gateway recombination reaction with BP clonase II.
416 Subsequently, Gateway reactions were used to introduce either p5E_elavl3 or p5E_nbt with
417 pME_hGCGR*-TEVcs-QF and p3E_he1a:ECFP (Addgene #113880) into the Tol2 Gateway Destination
418 Vector ([69]) (#394) to produce elavl3:hGCGR*-TEVcs-QF;he1.1:CFP or Xla.Tubb2b:hGCGR*-TEVcs-
419 QF;he1.1:CFP.
420 For the arrestin construct elavl3:hArr-TEV;myl7:mCherry, the myl7:mCherry sequence was
421 amplified from pKHR8 (Addgene #74625) using primers 3266 and 3267. The SV40 late polyA sequence
422 was amplified from pKHR8 using primers 3268 and 3269. A Tol2 Gateway Destination Vector (Ahrens
423 et al., 2012) was digested with AscI and MluI. Both PCR products and the digested vector were
424 assembled using Hi-Fi DNA Assembly. The resulting plasmid was subsequently digested with SpeI and
425 SacII. The hArrestin2-TEV sequence was amplified from the trans-Tango plasmid ([7]) using primers 3272

426 and 3273. The PCR product and the digested vector were assembled using Hi-Fi DNA Assembly. The
427 final plasmid was then subjected to LR Gateway cloning with pEntry-HuC ([43]) to obtain elavl3:hArr-
428 TEV; myl7:mCherry.

429 For replacement of the myl7:mCherry secondary reporter with he1.1:YFP, the elavl3:hArr-TEV
430 sequence were amplified from the elavl3:hArr-TEV; myl7:mCherry plasmid using primers 183 and 184.
431 The he1.1:YFP sequence was amplified from p3E_he1a:eYFP (Addgene #113879) using primers 185 and
432 186. Both PCR products were assembled with the Tol2 Gateway plasmid backbone (pDestTol2pA2-
433 U6:gRNA; Addgene #63157) using Hi-Fi DNA Assembly to obtain elavl3:hArr-TEV;he1.1:YFP.

434 hArr-TEV was also placed under the control of Xla.Tubb2b by amplification of this sequence
435 from elavl3:hArr-TEV;myl7:mCherry using primers 203 and 51, with flanking attB1 and attB2
436 sequences, respectively. A Gateway recombination reaction was performed using BP Clonase II with the
437 PCR product and donor vector pDONR221 (Invitrogen, #12536017) to obtain pME_hArr-TEV. LR
438 Clonase II was used to recombine p5E_nbt, pME_hArr-TEV and p3E_he1a:eYFP (Addgene #113879)
439 into the Tol2 Gateway Destination Vector ([69]) (#394) to obtain Xla.Tubb2b:hArr-TEV;he1.1:YFP.

440 To generate the QUAS:H2B-GCaMP6s; he1.1:CFP plasmid, an H2B-GCaMP6s middle entry
441 clone was prepapred from Addgene plasmid #59530 and subsequently recombined with p5E_QUAS
442 (Addgene, #61374) and p3E_he1a:ECFP (Addgene, #113880) into a Tol2 Destination Vector ([69]) (#394).
443
444
445
446
447

448 Table 1: Primers for synthesis of plasmids. PCR amplified components of plasmids are indicated by the

449 text in red.

Component	Template	Primer #	Orientation	Sequence
sGCG-ICAM(1235)-Nrxn1a sGCG-ICAM(1235)-Nrxn1b sGCG-ICAM-Nrxn1a sGCG-ICAM-Nrxn1b sGCG-Nrxn1a sGCG-Nrxn1b	*trans*-Tango plasmid (Talay et a., 2017)	3274	Forward	ACGAATTCCTGCAGCCCGGG GGATCCGCGGCCGCCACCAT GAGTGCACTTCTGATCCTAG
sGCG-ICAM(1235)-Nrxn1a sGCG-ICAM(1235)-Nrxn1b	*trans*-Tango plasmid (Talay et a., 2017)	2302	Reverse	TCTGCTGGCTTGGGAAGCTGT AGATGGTCACGTC
sGCG-ICAM(1235)-Nrxn1a sGCG-ICAM-Nrxn1a	*trans*-Tango plasmid (Talay et a., 2017)	3275	Forward	GCCTACGACCATGCCCTCATA CCGGGGGGAGAG
sGCG-ICAM(1235)-Nrxn1a sGCG-ICAM(1235)-Nrxn1b	*trans*-Tango plasmid (Talay et a., 2017)	2303	Reverse	CAGCTTCCCAAGCCAGCAGA CCCCAATGTG
sGCG-Nrxn1a	*trans*-Tango plasmid (Talay et a., 2017)	3279	Reverse	ACCAGTGAACTCCATACTAGT ATTCCCGTTACCATTGCC
sGCG-ICAM(1235)- Nrxn1b sGCG-ICAM- Nrxn1b	*trans*-Tango plasmid (Talay et a., 2017)	3277	Forward	GCCCACCACCATCCCCTCATA CCGGGGGGAGAG
sGCG-Nrxn1b	*trans*-Tango plasmid (Talay et a., 2017)	3281	Forward	CCTGAGACTAGATCCACTAGT ATTCCCGTTACCATTGCC

sGCG-ICAM- Nrxn1b*	UAS-E1B:hGCG-ICAM-Nrxn1b;cryaa:mCherry (this study)	87	Forward	GGGGACAAGTTTGTACAAAAAAGCAGGCTCACCATGAGTGCACTTCTGA
sGCG-ICAM- Nrxn1b*	UAS-E1B:hGCG-ICAM-Nrxn1b;cryaa:mCherry (this study)	88	reverse	GGGGACCACTTTGTACAAGAAAGCTGGGTAAAAAACCTCCCACACCTC
sGCG-ICAM(1235)-Nrxn1a sGCG-ICAM-Nrxn1a	6 dpf zebrafish cDNA	3276	Forward	TCCCCCCGGTATGAGGGCATGGTCGTAGGCATCGTTGCAG
sGCG-ICAM(1235)-Nrxn1a sGCG-ICAM-Nrxn1a sGCG-Nrxn1a	6 dpf zebrafish cDNA	3264	Reverse	TTAATTCGAGCTCCACCGCGGTGGCTTAATTAATCAGACGTAGTACTCCTTGTCCTTGTTCTTTTT
sGCG-ICAM(1235)-Nrxn1b sGCG-ICAM-Nrxn1b	cDNA	3278	Forward	TCCCCCCGGTATGAGGGGATGGTGGTGGGCATCGTGGCG
sGCG-ICAM(1235)- Nrxn1b sGCG-ICAM-Nrxn1b sGCG-Nrxn1b	cDNA	3265	Reverse	TTAATTCGAGCTCCACCGCGGTGGCTTAATTAATCACACGTAATACTCCTTGTTTTTGTTCTTCTTGC
sGCG-Nrxn1a	cDNA	3280	Forward	AACGGGAATACTAGTATGGAGTTCACTGGTGCAGAAGGCCAG
sGCG-Nrxn1b	cDNA	3282	Forward	AACGGGAATACTAGTGGATCTAGTCTCAGGTTTACAGGATCCGAC
hGCGR-TEVcs-QF	*trans*-Tango plasmid (Talay et a., 2017)	3270	Forward	CGTTCAGCTTTCTTGTACAAAGTGGTCACTAGTCACCATGCCCCCCTGCCAGCCACAGCG

hGCGR-TEVcs-QF	*trans*-Tango plasmid (Talay et a., 2017)	3271	Reverse	CGATGAATTAATTCGAGCTCCACCGCGGGACGTCCTATTGCTCATACGTGTTGATATCGC
elavl3:hGCGR-TEVcs-QF	elavl3:hGCGR-TEVcs-QF;myl7:EGFP (this study)	183	Forward	ACGCGTGATCTGCGAAGATAC
elavl3:hGCGR-TEVcs-QF	elavl3:hGCGR-TEVcs-QF;myl7:EGFP (this study)	184	Reverse	GGCGCGCCTGAATGATCAG
elavl3:hGCGR*-TEVcs-QF	elavl3:hGCGR-TEVcs-QF;myl7:EGFP (this study)	174	Forward	GGGGACAAGTTTGTACAAAAAAGCAGGCTGCCACCATGCCACCTTGTCA
elavl3:hGCGR*-TEVcs-QF	elavl3:hGCGR-TEVcs-QF; myl7:EGFP (this study)	175	Reverse	GGGGACCACTTTGTACAAGAAAGCTGGGTAAAAAACCTCCCACACCTCCCC
Xla.Tubb2b:hGCGR-TEVcs-QF	elavl3:hGCGR-TEVcs-QF; myl7:EGFP (this study) p5E_Xla.Tubb2b (gift from Paul Krieg)	93	Forward	GGGGACAAGTTTGTACAAAAAAGCAGGCTATGCCCCCCTGCCAGC
Xla.Tubb2b:hGCGR-TEVcs-QF	elavl3:hGCGR-TEVcs-QF; myl7:EGFP (this study) p5E_Xla.Tubb2b (gift from Paul Krieg)	131	Reverse	GGGGACCACTTTGTACAAGAAAGCTGGGTGCGCCTGAATGATCAGATT
hArrestin2-TEV	*trans*-Tango plasmid (Talay et a., 2017)	3272	Forward	CTCGTTCAGCTTTCTTGTACAAAGTGGTCACTAGTCACCATGGGGGAGAAACCCGGGAC
hArrestin2-TEV	*trans*-Tango plasmid (Talay et a., 2017)	3273	Reverse	CGATGAATTAATTCGAGCTCCACCGCGGTCACGAGTACACCAATTCATTCATGAGTTG

elavl3:hArr-TEV	elavl3:hArr-TEV;myl7:mCherry (this study)	183	Forward	ACGCGTGATCTGCGAAGATAC
elavl3:hArr-TEV	elavl3:hArr-TEV;myl7:mCherry (this study)	184	Reverse	GGCGCGCCTGAATGATCAG
elavl3_EGFP	pDestTol2CG2-U6:gRNA (Addgene #63156)	3266	Forward	TCTAGATCAGATCTAATCTGATCATTCAGGCGCGCCAAATCAGTTGTGTTAAATAAGAG
elavl3_EGFP	pDestTol2CG2-U6:gRNA (Addgene #63156)	3267	Reverse	ATCTTATCATGTCTGTTACTTGTACAGCTCGTC
SV40 late polyA	pKHR8	3268	Forward	GAGCTGTACAAGTAACAGACATGATAAGATACATTGATG
SV40 late polyA	pKHR8	3269	Reverse	GAGCACCCGTGGCCGTATCTTCGCAGATCACGCGTAAAAAACCTCCCACACCTC
cryaa:mCherry	pTol2pA2ubb:secHsa.ANNEXINV-mVenus, cryaa: RFP (Addgene #92388)	3283	Forward	CTAGATCAGATCTAATCTGATCATTCAGGCGCGCCATACAATACATTTCCTACAATAATG
cryaa:mCherry	pTol2pA2ubb:secHsa.ANNEXINV-mVenus, cryaa: RFP (Addgene #92388)	3284	Reverse	ATCTTATCATGTCTGTTACTTGTACAGCTCGTC
he1.1:CFP	p3E_he1a:ECFP	185	Forward	TCTGATCATTCAGGCGCGCCTCAACCACTCCAGGCATAG
he1.1:CFP	p3E_he1a:ECFP	186	Reverse	TATCTTCGCAGATCACGCGTCCATAGAGCCCACCGCATC

p5E_QUAS	Addgene, #61374			
pME_H2B-GCaMP6s	Addgene plasmid #59530		Forward	GGGGACAAGTTTGTACAAAA AAGCAGGCTATGCCAGAGCC AGCGAAG
pME_H2B-GCaMP6s	Addgene plasmid #59530		Reverse	GGGGACCACTTTGTACAAGA AAGCTGGGTAAAAAACCTCC CACACCTCC
p3E_he1a:ECFP	Addgene, #113880			

450

451 Microinjection

452 Progeny from matings between Gal4 driver lines and the *Tg(QUAS:mApple-CAAX; he1.1:mCherry)*^{c636}
453 reporter line were microinjected at the 1-cell stage with plasmids for all *trans*-Tango components
454 (10xUAS-E1B:sGCG-ICAM-(1235)Nrxn1b; cryaa:mCherry, *elavl3-hArr-TEV; he1.1:eYFP, elavl3-*
455 *hGCGR-TEVcs-QF; he1.1:CFP)* together with Tol2 transposase mRNA (25 ng/µl). Tol2 transposase
456 mRNA was produced by digesting pCS-zT2TP ([71]) with *NotI* and using the mMESSAGE mMACHINE
457 SP6 Transcription kit (AM1340, Thermo Fisher Scientific) followed by phenol/chloroform-isoamyl
458 extraction. Ligand and arrestin constructs (25 ng/µl) and the receptor construct (10 ng/ µl or 25 ng/µl or
459 125 ng/µl) were pooled in a 5 µl injection mixture with water and phenol red (.2%). At 24 hpf, injected
460 embryos were transferred to 0.003% PTU and system water and screened under an Olympus MVX10
461 Macro Zoom fluorescence microscope for labeling of hatching gland cells. Embryos positive for YFP,
462 CFP, and mCherry labeled hatching gland cells were reared to 4 dpf, then screened for mCherry labeling
463 of the lens and the presence of the UAS:GFP reporter. At 6 dpf, larvae were screened on the Olympus
464 microscope for mApple-CAAX labeling in the CNS. Those that were positive were individually mounted

465 in 1% low melting point agarose (50100, Lonza) and imaged on a Zeiss LSM 980 confocal microscope
466 using a 20x (NA=0.5) water immersion lens.
467
468 Whole mount in situ hybridization
469 Embryos from transgenic lines were presorted for labeling of appropriate secondary markers (refer to Fig.
470 1b) and, at 6 dpf, larvae were fixed in 4% paraformaldehyde (PFA; Sigma-Aldrich) in 1X phosphate-
471 buffered saline (PBS) at 4°C overnight and then dehydrated with methanol. To generate probes, primers
472 amplifying a 200-500 bp stretch of DNA sequence corresponding to each *trans*-Tango component were
473 designed using Primer3 software (https://primer3.org/). The location, size of DNA template, and primer
474 sequences for the ligand, receptor and Arrestin transgenes are provided in Table 2. The *trans*-Tango
475 plasmids were used as templates to generate PCR products, which were purified using a PCR cleanup kit
476 (QIAGEN QIAquick PCR purification kit #28104). Reverse primers included a promoter for in vitro
477 transcription using SP6 RNA polymerase (Roche RPOLSP6RO) with incorporation of UTP-digoxigenin
478 (Roche 11093274910). RNA was purified using G-50 columns (Cytiva 27533001) and diluted to 30
479 ng/ml. Larvae were rehydrated in PBS, permeabilized using Proteinase K (Roche 03115828001), post-
480 fixed in 4% PFA, and hybridized in 50% formamide containing 30 ng of the RNA probe at 70°C. Probes
481 were detected using alkaline phosphate-conjugated digoxigenin antibody (Sigma Aldrich 11093274910)
482 and visualized after incubation with substrate solution containing NBT (4-nitro blue tetrazolium, Roche
483 11383213001) and BCIP (5-bromo-4-chloro-3-indolyl-phosphate, Roche 11383221001). Larvae were
484 mounted in 100% glycerol under coverslips and bright-field images captured using a Leica DFC500
485 camera mounted on a Zeiss Axioskop
486

487

488 Table 2: Primers for synthesis of probes for RNA in situ hybridization

Construct	Location of DNA template	Primer sequences	Size
Ligand	GCG/linker/ICAM	CATTCACAGGGCACATTCAC	322 bp
Ligand	GCG/linker/ICAM	GTTTCAATTTAGGTGACACTATAGGGGTCTC TATGCCCAACAAC	322 bp
Receptor	QF	GTTCGAAACGCCAGAGAGTC	475 bp
Receptor	QF	GTTTCAATTTAGGTGACACTATAGGCCGAGA AAGTCAAGTGAGG	475 bp
Arrestin	hArr-TEV junction	TCCCTTCATCTGATGGCATA	312 bp
Arrestin	hArr-TEV junction	GTTTCAATTTAGGTGACACTATAGTCATGAG TTGAGTCGCTTCCT	312 bp
	Sp6	GTTTCAATTTAGGTGACACTATAG	

489

490 Immunofluorescence

491

492 Larvae were raised to 6 dpf in 0.003% PTU in system water, anesthetized using tricaine (0.02%, Sigma
493 Aldrich E10521), and fixed in 4% paraformaldehyde overnight at 4°C. They were cryoprotected at 4°C
494 overnight, first in 10% sucrose and then in 30% sucrose. Specimens were individually mounted nosedown
495 in optimal cutting temperature (OCT) compound in plastic molds and frozen at -80°C. Sections (25 µm)
496 were prepared on a HM525 NX Cryostat (Epredia), layered onto Superfrost Plus slides (Fisher Scientific),
497 and dried overnight in the dark at room temperature. Subsequently, sections were post-fixed in 1%
498 paraformaldehyde, washed 3 times each in PBS and PBST, and incubated in blocking solution containing
499 PBST and 1% bovine serum albumin at room temperature for 1 hour. The sections were then incubated
500 overnight at 4°C in primary antibody diluted in blocking solution with 5% normal goat serum (Abcam,
501 ab7481). Antibodies were directed against GFP (Abcam, ab13970, 1:500), HuC/D (Thermo Fisher
502 Scientific, A-21271, 1:40), PKCa (Santa Cruz Biotechnologies, sc-17769, 1:100) and zpr-3 (ZIRC,

503 1:100). Following three washes in PBST, sections were incubated at room temperature for 1 hour with
504 either goat anti-mouse Alexa Fluor 647 (Thermo Fisher Scientific, a32728) or goat anti-chicken Alexa
505 Fluor 488 (Thermo Fisher Scientific, a11039) secondary antibody diluted 1:1000 in PBST: Slides were
506 washed in PBST, incubated 10 minutes with DAPI stain (1:1000 in PBS, Sigma Aldrich D9542-10MG),
507 and washed in PBS before incubation in 10% glycerol overnight at 4°C. Sections were covered in 30%
508 glycerol for at least one hour at room temperature before mounting in 40% glycerol.
509

510 Confocal imaging

511 Larvae were anesthetized with 0.02% ethyl 3-aminobenzoate methanesulfonate salt (Tricane, (E10521,
512 Ethyl 3-aminobenzoate methanesulfonate) and mounted in 1% low melting point agarose (50100, Lonza)
513 in a 60 mm x 15 mm petri dish. Once solidified, system water containing PTU (0.003%) and Tricaine
514 (0.02 %) was added to cover the agarose. Larvae were imaged using a Zeiss LSM 980 with Airyscan 2
515 under a 20X (NA 0.8) water immersion objective. Images were taken in Z-stacks and processed using Fiji
516 software. For tracing the morphology of individual neurons labeled by *trans*Tango transsynaptic
517 signaling, 3D renderings were also performed using Fiji ([72]). Image files were extracted using *File ->*
518 *Split channels -> image -> adjust -> threshold -> invert -> save as -> Tiff.*
519

520 Image registration

521 Confocal images of zebrafish larval brains were captured in two channels, one for the reference brain
522 (GFP) and one for *trans*-Tango labeling (mApple). The reference larva was select based on orientation,
523 straightness of head and lack of tilting and all other datasets (larvae) were registered to the reference
524 channel (ptf1a/islet2b) of the reference larva using ANTs (Advanced Normalization Tools). We
525 applied the code from Marquart et al. ([73]) with minor modifications to streamline the computation.
526 Computation was performed using the high-performance computer cluster managed by Research
527 Computing at Dartmouth College.
528

```
antsRegistration --dimensionality 3 --verbose 1 --float 1 -o
[${file//01.nrrd/01_},${file//01.nrrd/01_warped.nrrd}] --use-histogram-matching 0  --interpolation
WelchWindowedSinc --initial-moving-transform [${refbrain//01.nrrd/01.nrrd},${file//01.nrrd/01.nrrd},1]
-t rigid[0.1] --metric MI[${refbrain//01.nrrd/01.nrrd},${file//01.nrrd/01.nrrd},1,32,Regular,0.25] -c
[200x200x200x0,1e-8,10] --shrink-factors 12x8x4x2 -s 4x3x2x1vox -t Affine[0.1] --metric
MI[${refbrain//01.nrrd/01.nrrd},${file//01.nrrd/01.nrrd},1,32,Regular,0.25] --convergence
[200x200x200x0,1e-8,10] --shrink-factors 12x8x4x2 -s 4x3x2x1vox -t SyN[0.05,6,0.5]  --metric
MI[${refbrain//01.nrrd/01.nrrd},${file//01.nrrd/01.nrrd},1,32,Regular,0.25] --convergence
[200x200x200x200x10,1e-7,10] --shrink-factors 12x8x4x2x1 -s 4x3x2x1x0vox;

antsApplyTransforms --dimensionality 3 --float 1 -v 0 -n WelchWindowedSinc -i ${file//01.nrrd/02.nrrd}
-r ${refbrain//01.nrrd/02.nrrd} -o ${file//01.nrrd/02_warped.nrrd} -t ${file//01.nrrd/01_1Warp.nii.gz} -t
${file//01.nrrd/01_0GenericAffine.mat};
```

To compare *trans*-Tango labeling between different datasets, we devised an algorithm to assign a unique color to the neurons of individual larvae by dividing the spectra from violet to red (0-360 degrees) with the number of individuals (n) in the Hue, Saturation, Lightness (HSV) color system. The registered CNS labeling for each larva was then plotted in a single image stack.

<u>Optogenetics and calcium imaging</u>

Embryos from *Tg(isl2b:Gal4-VP16); Tg(UAS:ReaChR-Tag-RFPT)*^{jf50} intercrosses were injected with the QUAS:Hsa.H2B-GCaMP6s; he1.1:CFP plasmid along with the *trans*Tango ligand, receptor, arrestin plasmids at the 1-cell stage. Larvae containing all secondary markers were allowed to develop to 6 dpf, at which time they were paralyzed by a 1 min immersion in α-bungarotoxin (20 µl of 1 mg/ml solution in system water, B1601, ThermoFisher Scientific) and flushed with fresh system water. Individual larvae were mounted in a droplet of 1% low melting agarose (50100, Lonza) in the middle of a 60 mm x 15 mm Petri dish and immersed in system water after the agar solidified. For calcium imaging experiments, the xyt acquisition mode was used to capture images using a 20X (NA=0.5) water image objective on a Zeiss LSM 980. Images containing the optic tectum in the field of view were acquired with the 488 nm laser at 310 x 310-pixel resolution and a rate of 2.6 Hz while stimulating the left or right retina with 561 nm light.

Calcium transients were recorded in a series of 200 frames prior to retinal activation, 20 frames during activation with the 561 nm laser, and 150 frames following activation.

Image files were extracted in Fiji using *File -> Save as -> Image Sequence Files*. Frames were imported to MATLAB (Mathworks Inc.), and mean fluorescence intensities for individual regions of interest (ROI; areas adjacent to the left and right optic tectum) were calculated. For each larva, a highcontrast image was generated by calculating a maximum intensity projection for the series of images generated in Fiji. ROIs were drawn manually using the MATLAB *roipoly* function and the mean fluorescence intensity of pixels within each ROI was calculated. The change in fluorescence (ΔF/F) was calculated according to the following formula

$$F \leftarrow \frac{F_i - F_{min}}{F_{max} - F_{min}}$$

where F_i represents the mean fluorescence intensity in an ROI at each time point, and F_{max} and F_{min} are the maximum and minimum fluorescence values, respectively, for that ROI during the recording period. To calculate total activity for each larva before and after activation, the ΔF/F was averaged across the ROIs of each larva and total activity was obtained for a period by calculating the area under the curve using the MATLAB *trapz* function.

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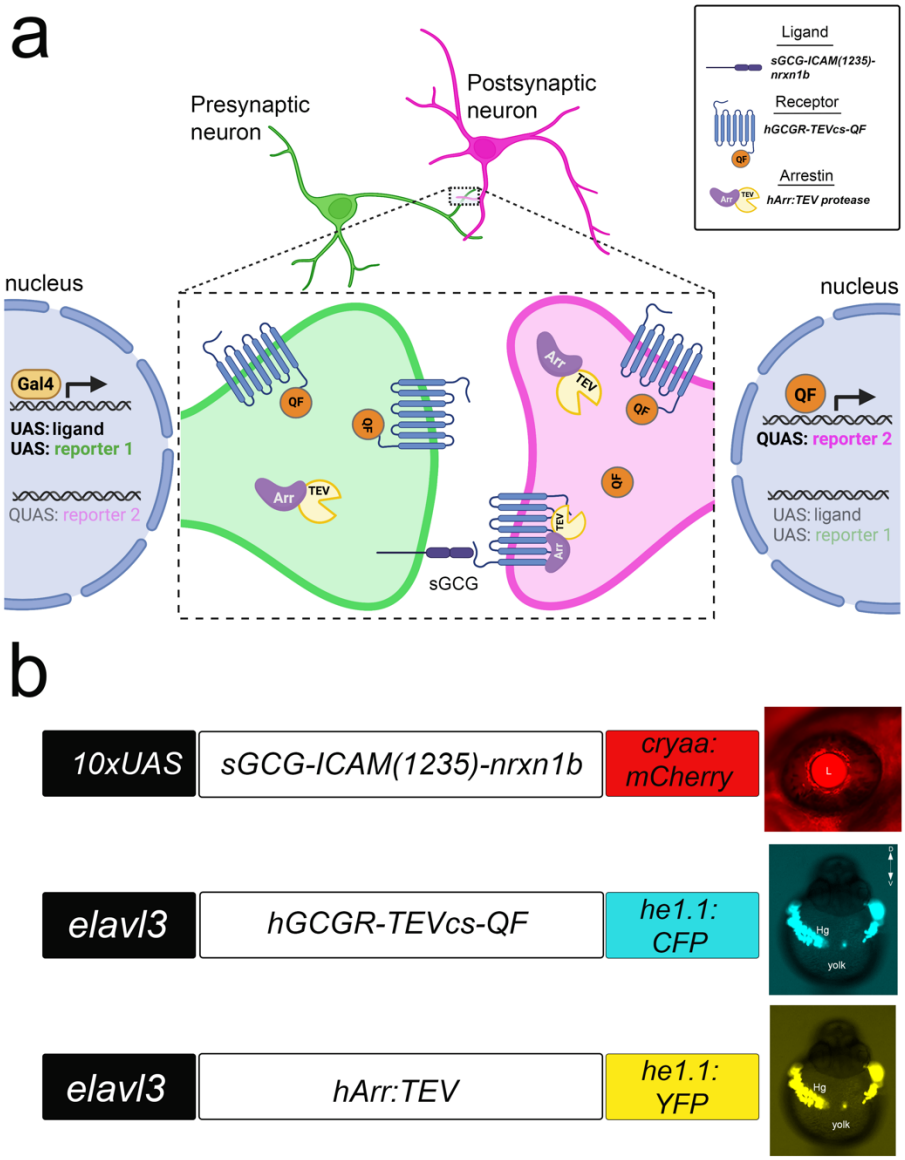
1 **Figure Legends**

2



3

4 Fig. 1: *trans*-Tango mediates transsynaptic signaling in zebrafish

5 (a) Gal4 driver lines direct ligand expression in presynaptic neurons of interest. Ligand derived from

6 human glucagon (sGCG) is tethered to the presynaptic membrane through the transmembrane domain of

7 zebrafish Nrxn1b and extended into the synaptic cleft through the ICAM(1235) linker sequence (Talay et

8 al., 2017). The receptor (hGCGR) is fused to the QF transcription factor through the protease cleavage

9 site (cs) of the N1a protease from the tobacco etch virus (TEV) and is expressed by neurons throughout

the CNS. Ligand binding to the receptor activates the pathway in postsynaptic neurons, resulting in recruitment of an Arrestin-TEV protease fusion protein and proteolytic cleavage of QF. QF can then translocate to the nucleus where it promotes transcription of genes downstream of the upstream activation sequence (QUAS) to which it binds. UAS and QUAS regulated reporters label pre- and postsynaptic neurons, respectively, with green and red fluorescent proteins. (b) Constructs containing modified ligand, receptor and arrestin-TEV components were cloned into Tol2 transposition vectors. To confirm the presence of each *trans*-Tango component in injected embryos, secondary markers consisting of tissue-specific promoters driving different FPs that label the lens of the eye (*cryaa*) or hatching gland cells (*he1.1*) were included in each Tol2 plasmid.

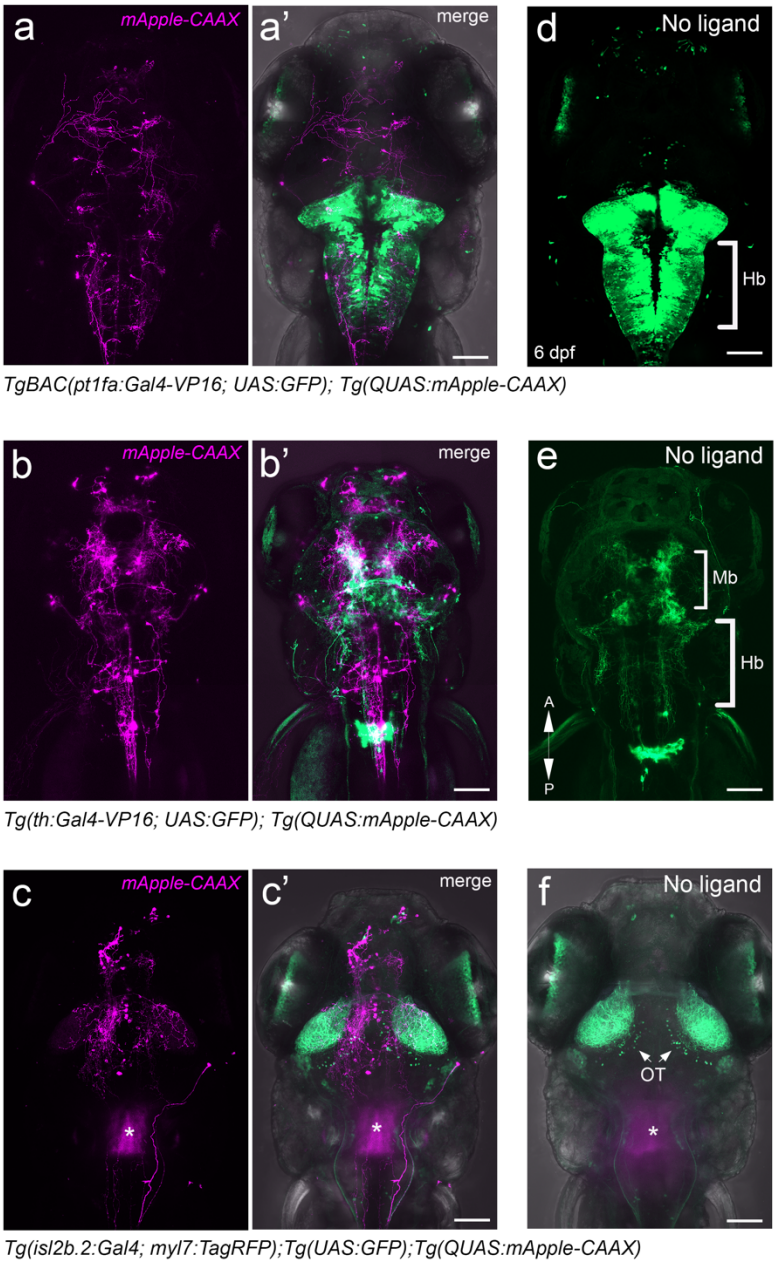


Fig. 2: Gal4-dependent *trans*-Tango labeling of synaptic partners

Progeny from Gal4 driver lines mated to *Tg(QUAS:mApple-CAAX;he1.1:mCherry)* were injected with *trans*-Tango constructs and Tol2 mRNA at the 1-cell stage. Larvae positive for all secondary markers were imaged by confocal microscopy at 6 dpf. (a) *TgBAC(ptf1a-Gal4-VP16;UAS:GFP)* produced *mApple-CAAX* labeling of hindbrain and midbrain neurons (n= 932/1042). (b) *Tg(th:Gal4-*

VP16;UAS:GFP) resulted in extensive labeling throughout the CNS (n= 548/656). (c) In *Tg(-17.6isl2b:Gal4-VP16;myl7:TagRFP;UAS:GFP)* larvae, mApple labeled neurons in the optic tectum (arrowhead; n= 223/297) were adjacent to GFP labeled axon terminals of presynaptic ganglion cells. Diffuse red fluorescent protein labeling (asterisk) is due to a secondary marker (*myl7:RFP*) expressed in the heart. (a', b', c') Merged images with GFP channel. (d, e, f) mApple labeling was not observed after embryos were injected with only the receptor and arrestin constructs (n=25 for each driver). Scale bars, 50 μm.

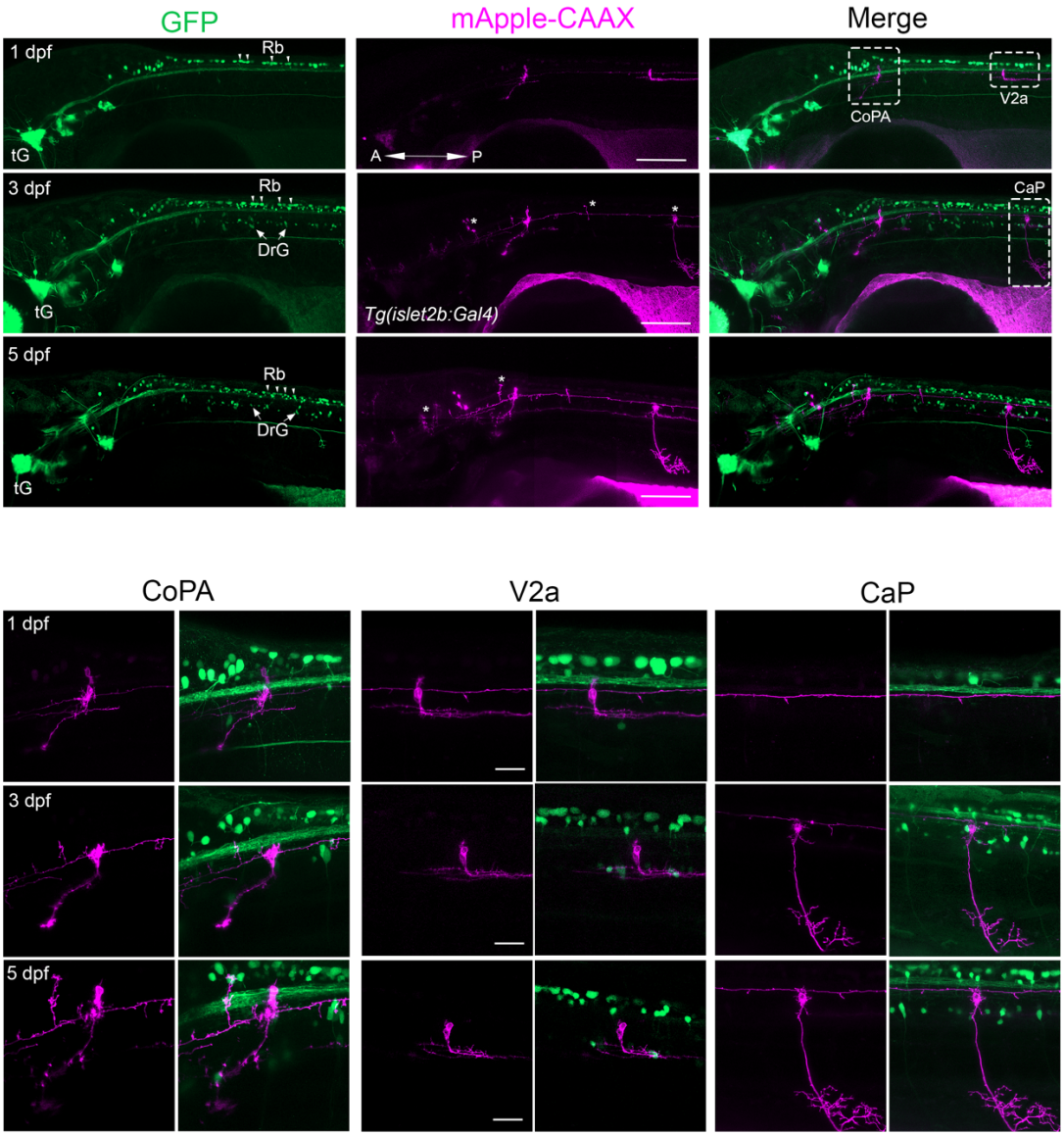


Fig. 3: Progression of *trans*-Tango labeling during neural development

Lateral views of GFP (pre-synaptic) and mApple-CAAX (post-synaptic) labeled neurons and axons in the ventral spinal cord of the same individual detected at (a) 1 (b) 3 and (c) 5 dpf. The larva contained *Tg(isl2b.2:Gal4; myl7:tagRFP); Tg(UAS:GFP)*; *Tg(QUAS:mApple-CAAX;he1.1:mCherry)* and secondary reporters for all *trans*-Tango components. Additional mApple-CAAX labeled cells were detected over time (arrowheads). Uniquely identified commissural primary ascending (CoPA) interneuron, V2a interneuron and caudal primary motor neuron (CaP) were imaged at each timepoint but, at 1 dpf, the CaP motoneuron was not yet labeled. For each time point, n=10 larvae were examined. Scale bars, 50 µm in upper panels and 100 µm in lower panels.

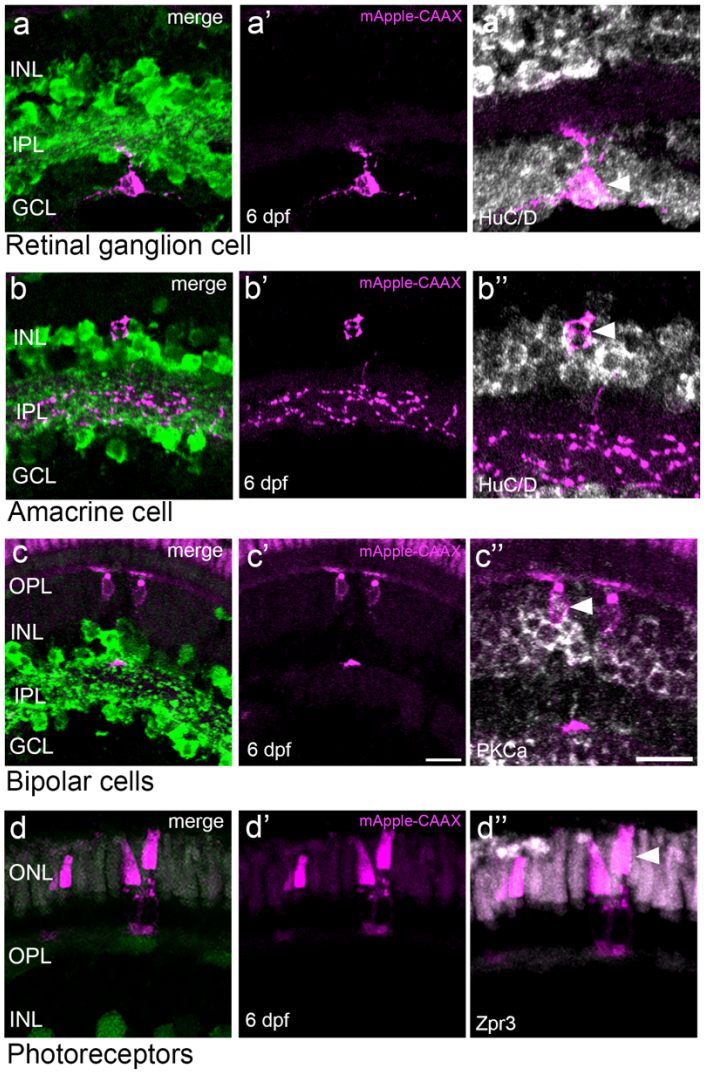


Fig. 4: Validation of *trans*-Tango labeling in the retina

Co-labeled neurons in cryosections (20 mm) of the retina from *Tg(ptf1a:Gal4-VP16)* and *Tg(QUAS:mApple-CAAX)* larvae that had been injected with *trans*-Tango components at the 1-cell stage. (a,a') mApple labeled cell in the ganglion cell layer (GCL) with dendrites in the inner plexiform layer (IPL) shows (a'') HuC/D immunoreactivity (arrowhead). (b,b') mApple labeled amacrine cell in the inner nuclear layer (INL) with extensive processes in the IPL is also (b'') HuC/D positive (arrowhead). (c,c'') mApple labeled bipolar cells in the INL with dendrites in the outer plexiform layer (OPL) variably express protein kinase C alpha (PKCa) (arrowhead). The cone photoreceptor outer segments are visible due to autofluorescence. (d,d'') Photoreceptors co-labeled with mApple and the zpr-3 monoclonal antibody (ref) are located dorsal to GFP labeled horizontal cells . All retinal sections are shown with photoreceptors oriented at the top. Scale bars, 10 µm.

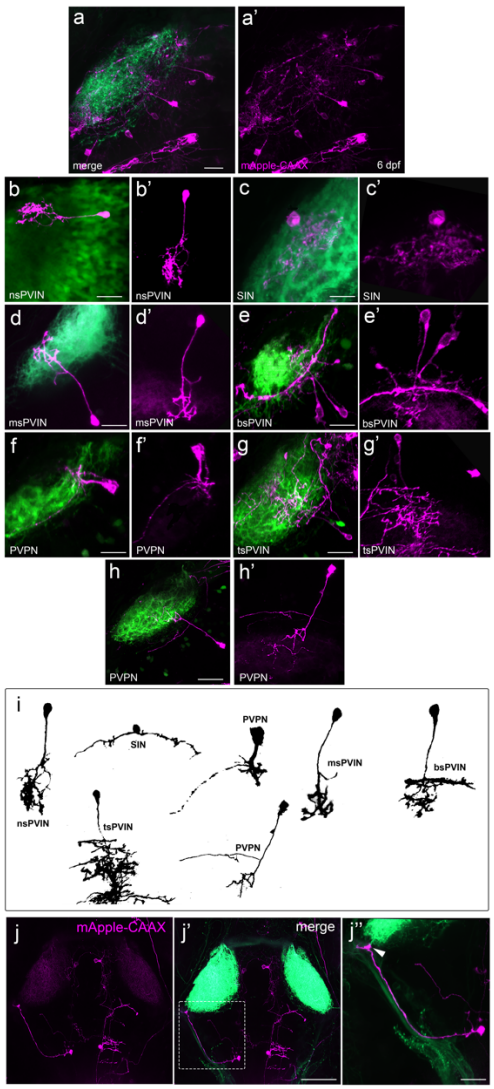


Fig. 5. *trans*-Tango confirms predicted postsynaptic partners of retinal ganglion cells (a,a') *Tg(isl2b.2:Gal4; myl7:tagRFP)* drives UAS:GFP in retinal ganglion cells that project to the optic tectum, where labeling from *Tg(QUAS:mApple-CAAX)* reveals their postsynaptic partners at 6 dpf. (b) Distinct morphology of non-stratified periventricular interneurons (nsPVN, n=46), (c) superficial interneurons (SIN, n=11), (d) mono-stratified periventricular interneurons (msPVIN, n=67), (e-e') bistratified periventricular interneurons (bsPVIN, n=43) (f) periventricular projection neurons (PVPN, n=21), (g) tri-stratified periventricular interneurons (tsPVIN, n=72) and (h) a distinct subtype of periventricular projection neurons (PVPN, n=26) labeled by *trans*-Tango. In (a'-h'), individual neurons

are reoriented with cell bodies anterior. Scale bars = 100 µm. (i) Drawings corresponding to each neuronal cell type. (j-j') Occasionally, hindbrain neurons with long processes extending to the tectum are also detected (n=6/297). Scale bar 50 µm in j' and 100 µm in j''.

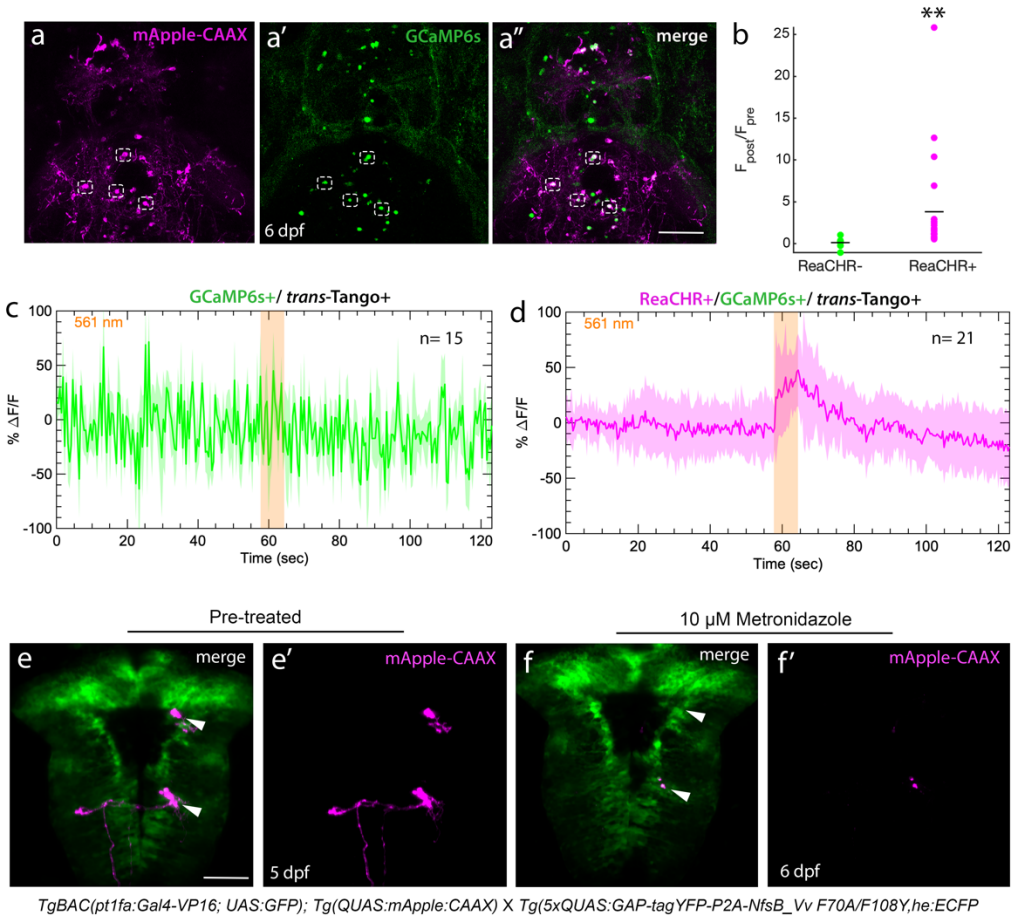


Figure 6. Functional validation of synaptic connectivity

trans-Tango signaling drives expression of any gene under QUAS control. (a,a'') In *Tg(isl2b.2:Gal4; myl7:tagRFP)*, *Tg(UAS:GFP)* and *Tg(QUAS:mApple-CAAX)* larvae that were injected with the QUAS:H2B-GCaMP6s plasmid, GCaMP6s labeled nuclei are only detected within mApple-CAAX labeled tectal neurons of *trans*-Tango positive larvae. (b-d) In larvae bearing all *trans*-Tango components, optogenetic activation of retinal ganglion cells expressing In larvae bearing all *trans*Tango components, optogenetic activation of retinal ganglion cells expressing ReaChR with 561 nm light activates GCaMP6s in tectal neurons. (b) Graphical representation showing 5-fold increase in calcium transients in

79 *Tg(UAS:ReaChR-Tag-RFPT)* larvae compared to siblings lacking this transgene (p = 0.0043). (c-d) Mean
80 and standard deviation of ΔF/F for GCaMP6s positive tectal neurons in (c) ReaChR negative controls
81 (n=15) compared to (d) ReaChR positive larvae (n=21) in response to 561 nm of light. (e,e') *trans*-Tango-
82 mediated ablation of postsynaptic neurons. In 5 dpf *TgBAC(ptf1a:Gal4-VP16)*^{jh16}; UAS:GFP) larvae
83 expressing the bacterial *NfsB* reducatase gene, mApple-CAAX neurons (arrowheads) were no longer
84 detected (f,f') following 1 day of incubation in 10 μM Metronidazole (n= 25). Scale bars, 50 μm.
85
86