Postsynaptic receptors regulate presynaptic transmitter stability through trans-synaptic bridges

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Abstract

Stable matching of neurotransmitters with their receptors is fundamental to synapse function and reliable communication in neural circuits. Presynaptic neurotransmitters regulate the stabilization of postsynaptic transmitter receptors. Whether postsynaptic receptors regulate stabilization of presynaptic transmitters has received less attention. Here we show that blockade of endogenous postsynaptic acetylcholine receptors at the neuromuscular junction destabilizes the cholinergic phenotype in motor neurons and stabilizes an earlier, developmentally transient glutamatergic phenotype. Further, expression of exogenous postsynaptic $GABA_A$ receptors in muscle cells stabilizes an earlier, developmentally transient GABAergic motor neuron phenotype. Both acetylcholine receptors and GABA receptors are linked to presynaptic neurons through trans-synaptic bridges. Knockdown of specific components of these trans-synaptic bridges prevents stabilization of the cholinergic or GABAergic phenotypes. Bidirectional communication can enforce a match between transmitter and receptor and ensure the fidelity of synaptic transmission. Our findings suggest a potential role of dysfunctional transmitter receptors in neurological disorders that involve the loss of the presynaptic transmitter.

Significance Statement

Sites of presynaptic neurotransmitter release are tightly correlated with the postsynaptic expression of cognate neurotransmitter receptors. At the same time, many neurons express more than one neurotransmitter and their synaptic partners express more than one population of transmitter receptors. It is essential for information transfer at synapses that transmitters and receptors are matched. Failure to achieve a transmitter-receptor match would cause failure of synaptic transmission. Using pharmacological, immunocytochemical, neurophysiological and molecular

methods, we show that postsynaptic neurotransmitter receptors are necessary and sufficient to achieve the stabilization of their cognate neurotransmitter in the presynaptic neuron. This retrograde signal from different receptors is mediated by physical bridges of proteins involving synapse adhesion molecules. These trans-synaptic bridges specify neurotransmitter identity.

Introduction

Postsynaptic cells differentiate morphologically in response to presynaptic signals. For example, filopodia on the dendrites of cultured mouse hippocampal pyramidal neurons respond to release of glutamate from developing axons, leading to physiological and morphological maturation (1). Dendrites of mouse cortical pyramidal neurons in acute brain slices respond to extracellularly uncaged glutamate or GABA by forming spines that express glutamate or GABA receptors (2, 3). Muscle cells respond to the release of agrin from motor neurons by clustering acetylcholine receptors (4). Cultured skeletal muscle cells respond to cultured glutamatergic neurons by forming functional glutamatergic synapses (5). Also, when neurotransmitters switch, the postsynaptic cells respond to the newly expressed transmitter by expressing a matching receptor (6–9). Conversely, presynaptic cells respond to retrograde signaling by endocannabinoids and neurotrophins, which regulate many functions in the nervous system (10, 11). During synapse development, retrograde signaling by postsynaptic neurotransmitter receptors regulates presynaptic neurotransmitter identity (12). It is unclear whether retrograde signaling regulates neurotransmitter stabilization after synapses have been established.

Glutamate and GABA are transiently expressed in *Xenopus* motor neurons at neural plate and early neural tube stages (13). Following formation of the neuromuscular junction, motor neurons lose

these neurotransmitters and the cholinergic phenotype appears (13–15). In loss-of-function and gain-of-function experiments, we took advantage of the presence of the canonical transmitter, acetylcholine, and the earlier transient expression of glutamate and GABA during development, to address the role of postsynaptic receptors in transmitter stabilization. We demonstrate that retrograde signaling by postsynaptic transmitter receptors is necessary and sufficient to stabilize expression of their cognate transmitter in presynaptic motor neurons and that this retrograde signaling is blocked by disruption of receptor-specific trans-synaptic bridges.

Results

Blocking endogenous acetylcholine receptors at the neuromuscular junction

We first tested whether blockade of acetylcholine receptors (AChR) at cholinergic neuromuscular junctions in *Xenopus* larvae affects the expression of acetylcholine in motor neurons. To achieve local unilateral inhibition of AChR, we implanted 120 μm diameter agarose beads containing AChR antagonists, pancuronium or curare, or saline into developing mesoderm at 19 hours post fertilization (hpf) for drug delivery by diffusion (6, 9, 13) (**Fig. 1*A***). We immunostained wholemounts of larvae for choline acetyltransferase (ChAT), the enzyme that synthesizes acetylcholine (ACh), and for synaptic vesicle protein 2 (SV2), a marker of nerve terminals, to determine the capacity for ACh synthesis in nerve terminals in the myocommatal junctions at the boundaries between chevrons of myocytes. We then compared the percentage labelled area of ChAT-stained nerve terminals adjacent to pancuronium beads to the percentage labelled area of ChAT-stained terminals adjacent to saline beads in sibling larvae (**Fig. *1B-E*, left**). We also compared the SV2-stained percentage labelled area of the same nerve terminals adjacent to pancuronium beads to SV2-stained percentage labelled area of the same nerve terminals adjacent

to saline beads (**Fig. 1*B-E*, right**). At 2 days post fertilization (dpf), the areas of ChAT and SV2 staining in larvae with beads containing pancuronium were not different from the staining in saline bead controls and larvae not implanted with beads (**Fig. 1*B*** and ***E*** and *SI Appendix* **Fig. S1*A***). Unaltered SV2 and ChAT expression at 2 dpf suggests that pancuronium beads have not affected assembly of the neuromuscular junctions. Neuromuscular junctions have been assembled by 1dpf, but not before, as evidenced by the presence of cholinergic mEPPs and EPPs in myocytes (14, 15) and confirmed in recordings from larvae at this age (*SI Appendix* **Fig. S2*A-D***). By 3 dpf, the percentage labelled area of ChAT-stained nerve terminals adjacent to pancuronium beads decreased to 19% of saline controls with no change in staining for SV2 (**Fig. 1*C*** and ***E*** and *SI Appendix* **Fig. S3**). Because loading of a transmitter into synaptic vesicles by transporter proteins is an essential component of a transmitter phenotype, we examined the expression of VACHT, the vesicular ACh transporter. Staining for VACHT was reduced to 40% of saline controls at 3 dpf, with no change in staining for SV2 (*SI Appendix* **Fig. S1*B***). By 4 dpf the staining for ChAT had fallen to 5% of saline controls in response to either pancuronium or curare (**Fig. 1*D*** and ***E*** and *SI Appendix* **Fig. S1*C*, *D***), while SV2 staining was 73% of saline controls (**Fig. 1*E*** and **Table S1**). Reduction of SV2 staining likely reflects withdrawal of nerve terminals (16, 17). We observed similar loss of ChAT staining when beads contained 5 μM pancuronium (**Fig. S3**), a concentration which blocks only postsynaptic myocyte AChR and spares presynaptic neuronal AChR (18). These results suggest that the loss of ChAT precedes the loss of SV2 and show that blockade of postsynaptic AChR impairs the stability of ChAT expression.

Normally, embryonic *Xenopus* motor neurons express glutamate in their cell bodies at 1 dpf (13), which can potentiate ACh release at nerve terminals (19). The level of glutamate decreases by 2

dpf as ChAT begins to be detected (13) and by 3 dpf glutamate is no longer observed immunohistochemically in most neurons (13). Blockade of AChR with pancuronium led to increased expression of a vesicular glutamate transporter (VGLUT1) in motor neuron terminals at 4 dpf (**Fig. 1*F***), suggesting the stabilization of a glutamatergic phenotype. The expression of VGLUT1 in motor neuron terminals was accompanied by a corresponding increase in expression of glutamatergic AMPA and NMDA receptor subunits in myocytes adjacent to these terminals (**Fig. 1*G*** and ***H***), with staining patterns similar to those observed at earlier stages of development (6). Intracellular recordings from these myocytes at 4 dpf, when beads have lost most or all of their pancuronium, yielded two classes of miniature endplate potentials (mEPPs), characterized by blockade by different antagonists, rise and decay times, and frequencies (**Fig. 1*I-K*** and ***O-Q***). Mean frequency overall was 0.57±0.07 sec^{-1}. Rapid rise-rapid decay mEPPs with a mean frequency of 0.47±0.07 sec^{-1} were blocked by NBQX, indicating that they depended on AMPA receptors. The mEPPs remaining after the NBQX block had slower rise and decay times typical of AChR, a mean frequency of 0.07±0.00 sec^{-1} and were blocked by the additional application of pancuronium. mEPPs in control larvae implanted with a saline bead had a mean frequency of 1.03±0.07 sec^{-1} and were blocked by pancuronium (**Fig. 1*L-M*** and ***R***). Because quantal content is proportional to mEPP frequency (20, 21), the higher frequency of glutamatergic mEPPs than cholinergic mEPPs may indicate larger evoked glutamate release in response to ACh receptor blockade, which is a feature of homeostatic presynaptic scaling (22, 23). The local block of AChR did not result in immunostaining for GABA in motor neuron terminals (*SI Appendix* **Fig. S1*E***), in agreement with earlier reports that the number of neurons expressing glutamate, but not GABA or glycine, increases following a reduction in neuronal activity (6, 24). These results show that local block of ACh receptors leads to stabilization of expression and function of another excitatory

transmitter, glutamate, consistent with the inhibitory effect of ACh on glutamate signaling in CNS neurons (25).

Expressing exogenous GABA receptors in embryonic myocytes

Embryonic *Xenopus* motor neurons express GABA as well as glutamate in their cell bodies (13) and in their axons (**Fig. 2*A***). Ordinarily, the level of GABA decreases by 2 dpf as ChAT appears. We expressed $GABA_A$ receptors in a small number of embryonic myocytes (26) to determine whether these receptors would stabilize expression of GABA in the motor neurons that innervate them. We co-injected the transcripts for rat $GABA_A$ receptor α1-EGFP, β2, and γ2 subunits into the ventral blastomeres (V2) at the 8-cell stage to achieve assembly of $GABA_A$αβγ receptors. Injection of α1-EGFP transcripts alone served as control, since expression in this case is restricted to the cytoplasm and does not appear in the plasma membrane (27) (*SI Appendix* **Fig. S4*A*-*E***). The EGFP-tag (hence, GFP) on the α1 subunit labeled the transfected myocytes. $GABA_A$αβγ receptors were first reliably detected on the surface of myocytes at 27 hpf (*SI Appendix* **Fig. S5*A*-*C***) by fluorescence of BODIPY™ TMR-X-conjugated muscimol, a $GABA_A$ receptor agonist. As noted above, neuromuscular junctions have already formed.

At 1 dpf the presence of $GABA_A$αβγ receptors had not altered GABA expression at neuromuscular junctions, because the expression of GABA in motor neuron axons contacting $GABA_A$αβγ receptor-expressing myocytes was not different from that in motor neuron axons contacting either $GABA_A$α-expressing myocytes or myocytes in larvae that had not been injected with transcripts (**Fig. 2*A*-*D*** and ***E*-*H***). At 2 dpf, the normally decreasing expression of GABA in motor neuron axons was selectively stabilized when they contacted myocytes expressing

GABA$_A$αβγ receptors (**Fig. 2*I-L*** and ***M-P***). Strikingly, at 3 dpf, expression of GABA$_A$αβγ receptors in myocytes led to expression of GABA, VGAT and GAD65/67 in the nerve terminals that innervate them (**Fig. 3*A-M***). The expression of GABA in motor neuron axons in these embryos was increased specifically in axons contacting myocytes that express GABA$_A$αβγ receptors. Myocytes expressing GABA$_A$α alone did not elicit additional axonal GABA expression (**Fig. 3*N***), suggesting that GABA was stabilized by the presence of the cognate postsynaptic receptor. Examination of 20 GABA$_A$αβγ receptor-expressing 3dpf larvae showed no preferential distribution of GFP-expressing myocytes contacted by GABA-expressing axon terminals along the anterior-posterior axis of the trunk of the larvae (*SI Appendix* **Fig. S6**).

ChAT was coexpressed with GABA and VGAT, suggesting that the terminals are processes of motor neurons (*SI Appendix* **Fig. S4*F-L***). Further support that the nerve terminals expressing GABA and VGAT are from motor neurons came when we traced GABA-labeled axons in the myotome back to spinal cord cell bodies that consistently expressed motor neuron transcription factor Hb9 (*SI Appendix* **Fig. S7**). This conclusion was strengthened by the observation that GAD67 and ChAT, as well as GABA and Hb9, are coexpressed in neuronal cell bodies (*SI Appendix* **Fig. S4*M-P***). Moreover, neurons expressing ChAT and GABA in their cell bodies expressed the Lim3 and not the rAldh1a2 transcription factor. This result demonstrated that these were medial motor neurons produced during the primary wave of neurogenesis (28) and not the lateral motor neurons that are generated later (29) (*SI Appendix* **Fig. S8*A-D***). Expression of GABA$_A$αβγ receptors stabilized the GABAergic phenotype but did not stabilize expression of VGLUT1 or glycine in innervating axons (*SI Appendix* **Fig. S4*Q-T***).

185 The stabilization of GABA in axons innervating $GABA_A\alpha\beta\gamma$ receptor-expressing myocytes
186 persisted up to 7 dpf (*SI Appendix* **Fig. S8*E***). To further test whether $GABA_A\alpha\beta\gamma$ receptor-
187 expressing myocytes were specific in stabilizing the presynaptic GABAergic phenotype, we
188 removed mesoderm of 15 hpf uninjected embryos and transplanted into these hosts 15 hpf
189 mesoderm grafts from embryos expressing $GABA_A\alpha\beta\gamma$ receptors or $GABA_A\alpha$ alone. SV2-stained
190 axons that contacted $GABA_A\alpha\beta\gamma$ receptor myocytes in the grafts expressed ChAT and GABA;
191 axons that contacted $GABA_A\alpha$-expressing myocytes expressed only ChAT (**Fig. 4*A*-*F*** and **Table**
192 **S2**). Thus, stabilization of GABA in axons depended on the $GABA_A\alpha\beta\gamma$ receptor myocytes and
193 not on GABA expression within the spinal cord.
194
195 To assess the functional consequence of anatomical innervation by nerve terminals expressing both
196 ChAT and GABA, we recorded mEPPs from $GABA_A\alpha\beta\gamma$ receptor-expressing myocytes at 4 dpf.
197 We observed two classes of mEPPs, distinguished on the basis of their blockade by different
198 antagonists, their rise and decay times and their mean frequency (6), with an overall frequency of
199 0.79 ± 0.07 sec^{-1}. Those with faster times occurred at a frequency of 0.57 ± 0.03 sec^{-1} and were
200 blocked by pancuronium, showing that they depended on AChR. Those with slower times occurred
201 at a frequency of 0.24 ± 0.04 sec^{-1} and were blocked by bicuculline (**Fig. 5*A*-*C*** and ***G*-*I***), indicating
202 that they depended on $GABA_A\alpha\beta\gamma$ receptors. In contrast, only a single class of mEPPs at a
203 frequency of 0.98 ± 0.13 sec^{-1} was observed when recording from myocytes that expressed
204 $GABA_A\alpha$ alone. These mEPPs had rise and decay times similar to mEPPs recorded from
205 $GABA_A\alpha\beta\gamma$ receptor-expressing myocytes in the presence of bicuculline (**Fig. 5*D*-*F*** and ***J*)**. The
206 results of these recordings demonstrate that GABA can be functionally released from motor nerve
207 terminals and innervate myocytes that express $GABA_A\alpha\beta\gamma$ receptors.

208

209 **Signal transduction by trans-synaptic bridges**

210 We then considered the possible role of trans-synaptic bridges in regulating the stability of
211 presynaptic GABA and ACh (30–32). Presynaptic neurexins and postsynaptic neuroligins and
212 dystroglycans are synaptic adhesion molecules that are important for proper maturation and
213 function of synaptic contacts (33). They bridge the synaptic cleft and create a potential pathway
214 for retrograde signaling. However, in heterologous synapse formation assays with neuroligin 1 and
215 neurexin 1β, neuroligin 1 induced both a glutamatergic and a GABAergic phenotype at the same
216 time (34). To determine if trans-synaptic bridges could regulate cholinergic stability versus
217 GABAergic stability at the neuromuscular junction, we tested the role of postsynaptic receptor-
218 specific auxiliary subunits and associating proteins, which are components of endogenous trans-
219 synaptic bridges. The $GABA_A\alpha\beta\gamma$ receptor auxiliary subunit GARLH4 mediates interaction
220 between the γ subunit of the $GABA_A\alpha\beta\gamma$ receptor and neuroligin 2, which binds to neurexin (35).
221 A clue was provided by the finding that expression of α and β transcripts of the $GABA_A$ receptor
222 resulted in surface expression of receptors with channel properties similar to those of
223 $GABA_A\alpha\beta\gamma$ receptors (27). We found that none of 12 $GABA_A\alpha\beta$ receptor-expressing larvae
224 expressed GABA in motor neuron axons that contacted GFP-labeled myocytes, and in all these
225 cases GABA expression remained restricted to the spinal cord (*SI Appendix* **Fig. S9** and **Table**
226 **S2**). This result suggested that the presence of the $GABA_A$ receptor γ subunit of the $GABA_A\alpha\beta\gamma$
227 receptor might be required for a trans-synaptic bridge to stabilize the presynaptic GABAergic
228 phenotype.

229

To investigate directly whether trans-synaptic bridges stabilize presynaptic transmitters, we first used pan-neurexin and pan-neuroligin antibodies to confirm the presence of neurexin (36) and neuroligin at the *Xenopus* neuromuscular junction of larvae expressing $GABA_A$ receptor transcripts (*SI Appendix* **Fig. S10*A*** and ***B***). The presence of α-dystroglycan was previously established (37). We ascertained that GARLH4 is expressed in the myocommatal junctions of $GABA_A$αβγ receptor-expressing larvae (*SI Appendix* **Fig. S10*C-F***) and that Lrp4 is expressed in the myocommatal junctions of wild type larvae (*SI Appendix* **Fig. S10*G-J***). We then injected morpholinos into the V2 blastomeres at the 8-cell stage to knock down GARLH4 in myocytes and disrupt regulation by GARLH4-neuroligin-neurexin trans-synaptic bridges (**Fig. 6*A-C*** and ***F*** and *SI Appendix* **Fig. S10*C-F*** and *SI Appendix* **Fig. S11**). This experiment prevented the $GABA_A$αβγ receptor-mediated stabilization of GABA in motor neurons (**Fig. 6*D*** and ***E*** and ***G*** and ***H*** and *SI Appendix* **Fig. S12*A-C***) but did not alter ChAT expression (*SI Appendix* **Fig. S13A** and ***C*** and ***D***). These results indicated that GARLH4 is necessary for the expression of presynaptic GABA and implicated regulation by trans-synaptic bridges. Similarly, we injected morpholinos into the V2 blastomeres to knock down the postsynaptic AChR-complex-associating protein Lrp4 (38, 39) in myocytes and disrupt regulation by AChR-rapsyn-Lrp4-musk-dystroglycan-neurexin trans-synaptic bridges (40, 41) (**Fig. 6*I-K*** and SI Appendix **Fig. S10*G-J*** and *SI Appendix* **Fig. S11**). This experiment recapitulated the destabilization and loss of ChAT that we observed in the presence of pancuronium (**Fig. 6*L-Q***), without affecting the ability of $GABA_A$αβγ receptors to induce GABA in the motor neurons (*SI Appendix* **Fig. S13*B*** and ***D*** and ***E*** and **Table S3**). These results suggested that Lrp4 is required for expression of presynaptic ChAT and pointed to regulation by trans-synaptic bridges.

Larvae in which Lrp4 was knocked down exhibited two classes of mEPPs at 4 dpf, differing in their blockade by different antagonists, their rise and decay times (*SI Appendix* **Fig. S14*A-C*** and ***G-I***) and their mean frequency, with an overall frequency of 0.63±0.15 sec^{-1}. Pancuronium-resistant NBQX-sensitive glutamatergic mEPPs had a frequency of 0.53±0.16 sec^{-1}. Pancuronium-sensitive, NBQX-resistant cholinergic mEPPs had a frequency of 0.05±0.01 sec^{-1}. These cholinergic mEPPs, for which there were also fewer AChR (42), were smaller in amplitude compared to cholinergic mEPPs in larvae expressing the control morpholino (1.7±0.1 mV vs 2.7±0.1 mV; n^{mEPP}=177, N^{larvae}=7; $p<0.0001$, two-tailed t-test). mEPPs in control larvae implanted with a saline bead occurred at a frequency of 1.20±0.09 sec^{-1} and were blocked by pancuronium (**SI Appendix Fig. S14*D-F*** and ***J*)**. The effect of knocking down Lrp4 may be phenocopied by pancuronium through binding to the α and γ subunits of the AChR (43) and altering the AChR-Lrp4 interaction, thereby disrupting signaling through cholinergic trans-synaptic bridges.

We next considered CASK (Ca^{2+}/calmodulin-activated Ser-Thr kinase) as a candidate that could receive signals from the presynaptic ends of the trans-synaptic bridges and stabilize transmitter expression in the motor neurons. CASK is a membrane-associated guanylate kinase (44) and transcription factor that binds to neurexin protein presynaptically (**Fig. 7*A***) and is present at both glutamatergic (45) and GABAergic (46) synapses. CASK pre-mRNA is subject to alternative splicing that yields proteins with preferences to interact with many targets (47). Autophosphorylated CASK translocates to the nucleus and induces transcription of genes essential for development (48, 49). Additionally, neurexin-1 competes as a CASK phosphorylation substrate, preventing CASK autophosphorylation (50). The splice variants, together with differential phosphorylation, identified CASK as a potentially significant player in stabilizing

presynaptic expression of different neurotransmitters. We found that CASK is expressed in the myocommatal junctions and spinal cord of normal *Xenopus* larvae (*SI Appendix* **Fig. S10*K-O***). Knocking down presynaptic CASK, by injecting morpholinos into the D1.2 blastomeres at the 16-cell stage to target the spinal cord (51) (**Fig. 7*B-D*** and ***H*** and *SI Appendix* **Fig. S10*N*** and ***O*** and *SI Appendix* **Fig. S11**), disrupted both $GABA_A\alpha\beta\gamma$ receptor-mediated GABA stabilization and AChR-mediated ChAT stabilization in motor neurons (**Fig. 7*E-G*** and ***I-N*** and **Table S3**). Knockdown of CASK, Lrp4 or GARLH4 did not alter laminin expression or the extent of labelling by synaptophysin (SYN) (*SI Appendix* **Fig. S15*A-C***), suggesting there was no change in the gross morphology of the myocommatal junction or postsynaptic myocytes.

Presynaptic localization of CASK in motor neuron nuclei and in myocommatal junctions, however, depended on integrity of the cholinergic or GABAergic trans-synaptic bridge **(Fig. 7*O*** and ***P*)**. In control larvae, in the presence of the cholinergic trans-synaptic bridge, CASK expression was observed in both the nuclei and the myocommatal junctions. Knocking down Lrp4, to disrupt the cholinergic trans-synaptic bridge, reduced CASK expression to 37% of controls in the synaptophysin- (SYN-) labeled myocommatal junctions but did not reduce CASK expression in the Hoechst-labeled nuclei. This result suggests that cytoplasmic expression of CASK in the motor neuron axon or cell body is required to stabilize cholinergic transmission. Expressing $GABA_A\alpha\beta\gamma$ receptors and simultaneously disrupting the cholinergic trans-synaptic bridge reduced CASK expression in the nuclei to 26% of controls but did not reduce CASK expression in the SYN-labeled myocommatal junctions. This finding suggests that reduction in nuclear expression of CASK is necessary to stabilize GABAergic transmission. As expected, disrupting the

GABAergic trans-synaptic bridge in the presence of the cholinergic trans-synaptic bridge recapitulated the CASK expression that was observed in control larvae.

Discussion

To address the role of postsynaptic receptors in transmitter stabilization, we took advantage of the presence of the canonical junctional transmitter, acetylcholine, and the early expression of glutamate and GABA as they are disappearing from motor neurons during development. Our results demonstrate that blockade of acetylcholine receptors at the neuromuscular junction destabilizes the cholinergic phenotype in motor neurons and stabilizes an earlier glutamatergic phenotype. Moreover, expression of $GABA_A\alpha\beta\gamma$ receptors stabilizes an earlier GABAergic motor neuron phenotype. Spontaneous mEPPs and EPPs are present prior to the reduction of ChAT expression by pancuronium and prior to enhancement of GABA expression by $GABA_A\alpha\beta\gamma$ receptors, indicating that these manipulations do not alter the initial formation of the synapse. Thus, postsynaptic neurotransmitter receptors regulate the stability of presynaptic neurotransmitters at newly formed neuromuscular junctions.

This regulation is achieved by non-canonical retrograde signaling by postsynaptic receptors (52, 53) that operates through trans-synaptic bridges (**Fig. 8**). The specificity of regulation relies on receptor-specific auxiliary subunits and associating proteins linked to neuroligin and dystroglycan at the postsynaptic end of the GABAergic and cholinergic bridges. When the cholinergic bridge was disrupted by knockdown of Lrp4, ChAT expression was reduced, and glutamate was stabilized. When the $GABA_A\alpha\beta\gamma$ receptor was expressed, GABA expression was stabilized unless GARLH4 was knocked down. For both GABA and ACh, the change in transmitter stability was

specific to the perturbation and no change was observed in the stability of the other transmitter. Our results are consistent with a model in which knockdown of Lrp4 or GARLH4, together with knockdown of CASK, knocks down the on-ramp and the off-ramp of the trans-synaptic bridges, preventing signal transmission across the synaptic cleft along the bridges formed by neurexins, neuroligins and dystroglycans (36).

Knockdown of MUSK (54) was not tested, as its absence would prevent assembly of the neuromuscular junction. Knockdown of dystroglycan would not distinguish between effects on cholinergic and GABAergic junctions (55–57). Knockdown of neurexin (58) and neuroligin (59) was not tested. Our data suggest that neurexin serves as the presynaptic end of both the cholinergic and GABAergic trans-synaptic bridges, receiving postsynaptic receptor-dependent signals and transmitting them through the neurexin-interacting protein CASK to achieve presynaptic cholinergic or GABAergic stabilization. The receptor-dependent distribution of CASK within the presynaptic neuron, likely coupled with its roles in scaffolding the synapse (60), organizing presynaptic voltage-gated calcium channels (61) and regulating neuronal gene transcription (48), appears to provide a mechanism by which different postsynaptic receptors stabilize cognate neurotransmitter expression. This signaling system of trans-synaptic bridges shares features with clustered protocadherin cell adhesion molecules (62), some of which change the nuclear versus cytoplasmic distribution of β-catenin to regulate the Wnt pathway (63).

The appearance of glutamate receptors following AChR blockade and the appearance of glutamatergic mEPPs following either AChR blockade or Lrp4 knockdown, as well as the expression of $GABA_A\alpha\beta\gamma$ receptors, are linked to a reduction in the frequency of cholinergic

mEPPs. Although not tested here, this would likely produce a reduction in the safety factor for transmission (64). The reduced frequencies could arise because of changes in the level of ChAT, competition for a limited pool of synaptic vesicles, changes in vesicle release, or changes in the level or extent of AChR (65). Interestingly, the loss of AChR precedes the loss of nerve terminals in a mouse model of myasthenia gravis (MG) (66). MG is often the result of an autoimmune attack on AChR function (67–69). Our findings suggest that reduced levels of presynaptic ACh, in addition to loss of AChR, may contribute to the muscle fatigue that is observed in MG.

The reduction we observed in presynaptic cholinergic markers upon blockade of postsynaptic ACh receptors is consistent with presynaptic homeostatic plasticity, in which a compensatory increase in neurotransmitter upon loss of receptor function is preceded by a compensatory change in receptor subunits (22, 23, 70–72). In *Xenopus* myocytes, where the AChR block cannot be rescued by alternative subunits (73, 74), reduction in presynaptic ChAT and VACHT was observed. Expression of glutamate receptors in myocytes (6, 75) led to stabilization of presynaptic glutamate (76, 77). Our results are consistent with the effectiveness of alternative, reserve receptor subunits in homeostatic synaptic plasticity at mammalian CNS synapses (78, 79). Our results are also consistent with the observation of a decrease in the *Drosophila* presynaptic active zone protein Bruchpilot, which is associated with presynaptic transmitter synthesis and release (80). Irrespective of differences between some of the molecular components at the *Drosophila* and *Xenopus* neuromuscular junctions, both junctions rely on neurexins and neuroligins and trans-synaptic bridges. The decrease in Bruchpilot occurs following the blockade of postsynaptic receptor subunits (81) that occurs early in homeostatic presynaptic scaling (70, 82). The decrease is reversed only when the receptor function is restored by expression and insertion of a different,

reserve receptor subunit (70, 71). These findings again link the stability of transmitter expression to the presence of an appropriate postsynaptic receptor.

What is the receptor-specific retrograde signal? Although we have identified one protein, CASK, with receptor-specific presynaptic distribution, the molecular mechanism by which this is achieved remains unknown. Our findings demonstrate the presence of neurexins at *Xenopus* neuromuscular junction. Further investigation will determine if different isoforms or splice variants of neurexins are recruited to GABAergic and cholinergic trans-synaptic bridges or if a receptor-specific conformational change in neurexin results in differential recruitment or phosphorylation of CASK. Other presynaptic signaling proteins that act independently or along with neurexin and CASK may also contribute to stabilization of the appropriate transmitter. In addition, the demonstration that trans-synaptic bridges are involved in transmitter stabilization does not preclude a role for postsynaptic diffusible factors, some of which have been shown to influence synapse formation and maintenance retrogradely (12, 83–86). It will be of interest to determine whether deficits in postsynaptic receptors or trans-synaptic bridges contribute to reduced transmitter levels at neuronal synapses in the mature nervous system and to pathological change in neurological disorders.

Neurotransmitter release is closely coupled with the expression of cognate neurotransmitter receptors postsynaptic to the release sites (70, 87). Mechanisms to accomplish this transmitter-receptor match are necessary to produce this precision, because neurons can have more than one neurotransmitter and their synaptic partners can express more than one population of transmitter receptors. If a neuron released one transmitter, and the postsynaptic cell expressed receptors for a different transmitter, synaptic transmission would fail. Spatially localized, bidirectional signaling,

as described here, can achieve the transmitter-receptor match essential for robust communication in neural circuits.

MATERIALS AND METHODS (526 words)

Animals

All animal procedures were performed in accordance with institutional guidelines and approved by the UCSD Institutional Animal Care and Use Committee. See *SI Appendix* for further details.

Local drug delivery

Spatial and temporal control of delivery of pharmacological agents was achieved using agarose beads (100-200 mesh, Bio-Rad) loaded with 2mM Ca^{2+} medium with or without drugs and implanted at 19 hours post fertilization (hpf) (Stage 18) (6, 9, 13). See *SI Appendix* for further details.

Whole mount immunohistochemistry

Whole *Xenopus* larvae were fixed at the indicated stages of development and processed for immunostaining with antibodies. See *SI Appendix* for further details.

Image acquisition

Confocal images of whole larvae were acquired with a Leica Stellaris 5 confocal microscope or on a Leica SP8 at the UC San Diego School of Medicine microscopy core, at a z-resolution of 0.5 μm. See *SI Appendix* for further details.

Image analysis

Images were analyzed in FIJI. Percent of labelled area was determined by measuring the fraction occupied by pixels of intensity at or above an empirically determined constant threshold. For

GABA receptor expression experiments, larvae were analyzed along the entire A-P axis. Representative images are maximum intensity projections of 5 consecutive slices. See *SI Appendix* for further details.

Electrophysiology

Recording techniques and analysis are described in *SI Appendix*.

Plasmids and morpholinos

Expression of $GABA_AR$ was achieved with $GABA_AR$ subunit plasmids for α1-EGFP, β2 and γ2 generated in the Czajkowski lab (27). The lissamine-tagged GARLH4, Lrp4 and CASK translation-blocking and splice-blocking morpholinos were supplied by GeneTools (Philomath, OR). See *SI Appendix* for further details.

Microinjections

See *SI Appendix* for further details.

Protein extraction and ELISA

ELISA assays for GFP-tagged $GABA_AR$α subunit expression in the muscle and spinal cord were performed per the manufacturer's instructions (GFP ELISA Kit, Cell Biolabs). Dissection and sample preparation are described in *SI Appendix.*

Muscimol-BODIPY staining

Embryos or larvae at the appropriate stages of development (indicated in the Results) were skinned and incubated in 20 μM muscimol-BODIPY (reconstituted in 0.1X MMR). Samples were imaged immediately after mounting. See *SI Appendix* for further details.

Mesoderm grafting

To obtain myocyte-specific $GABA_AR$αβγ or $GABA_AR$α expression, presomitic mesoderm of normal embryos 15 hpf (St 13-14) was replaced with presomitic mesoderm explant dissected from

sibling embryos expressing either $GABA_AR\alpha\beta\gamma$ or $GABA_AR\alpha$ transcripts. The grafting technique was similar to that previously described (88). See *SI Appendix* for further details.

Western blotting

Protein for Western blotting of components of trans-synaptic bridges was extracted as described above except that larvae were anaesthetized on ice and RIPA lysis buffer was supplemented with phosphatase inhibitors (PhosSTOP, Roche). Protein detection by Western blotting was performed for different antibodies. Signal was detected using Clarity ECL Western substrate (BioRad) and imaged on a BioRad Chemidoc Touch Imaging System. See *SI Appendix* for further details.

Statistics

All statistical analysis was performed in GraphPad Prism. Values were expressed as mean ± SD. Statistical differences were analyzed using the unpaired, two-tailed Student's *t-test* or one-way ANOVA or Kolmogorov-Smirnov test. The statistical test used and the P values for each measurement are provided in the figure legends and Tables S1, S2 and S3. $P<0.05$ was considered statistically significant. See *SI Appendix* for further details.

Data availability statement

The authors declare that the data supporting the findings of this study are available within the paper and its supplementary information files.

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Author contributions

S.K.G and N.C.S conceived the study and designed the experiments with consultation and advice from H-q.L. and M.P. S.K.G. performed all the experiments. M.H. and H.T.C. performed electrophysiology experiments with S.K.G., Y.I. and L.S. contributed to the identification of motor neuron identity. J.B. and C.C. contributed $GABA_A$ receptor clones and advice regarding expression. J.B.L and L.N.B provided instruction in embryonic tissue transplantation. S.K.G. and N.C.S wrote the manuscript with contributions from all authors.

Competing interests statement

The authors declare no competing interests.

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Figure Legends

Fig 1. Local block of AChR in myocytes reduces ChAT expression in motor neuron axons and induces a glutamatergic phenotype. (***A***) Experimental design. A single agarose bead loaded with pancuronium, curare, or saline was implanted into the *Xenopus* mesoderm at 19hpf. (***B-D***) Wholemounts of bead-implanted larvae (lateral view) were stained at 2dpf, 3dpf and 4dpf for ChAT and SV2. Dotted lines outline the regions of myocommatal junctions (1/larva) analyzed for quantification of staining area. Dashed circles indicate positions of beads. (***E***) Area of expression (labelled area above threshold) quantified for ChAT and SV2. $n \geq 5$ larvae. (***F-H***) Expression and quantification of VGLUT1, GLUR1 and NR1 in the 4dpf myotome (lateral view) of control and

pancuronium-loaded agarose bead-implanted larvae. n≥8 larvae. A, anterior; D, dorsal. (***I-K***) Recordings from pancuronium-bead-implanted larvae reveal rapid rise and rapid decay AMPAR-mediated-PSP-like mEPPs (arrowheads) that are pancuronium-resistant and NBQX-sensitive, as well as pancuronium-sensitive and NBQX-resistant mEPPs with rise and decay times similar to those described for nicotinic receptor-mediated-EPPs. (***L-N***) Recordings from saline-bead-implanted larvae reveal only pancuronium-sensitive mEPPs. (***O-R***) Rise and decay time distributions for mEPPs in myocytes of pancuronium-bead implanted larvae and saline-bead implanted larvae. N, number of mEPPs. >155 mEPPs (≥3 larvae, 4dpf) for each group. Only mEPPs with decay times fit by single exponentials were included. Resting potentials were held near -60 mV. Arrowheads indicate median values. Kolmogorov-Smirnov test compared rise time and decay time in (*R*) with respective rise and decay time in (*P*) and (*Q*). *p<0.05, **p<0.01, ****p<0.0001, ns not significant, unpaired two-tailed t-test. See also *SI Appendix*, Fig. S1 - S3 and Table S1.

Fig 2. $GABA_AR\alpha\beta\gamma$ expression in myocytes does not affect GABA expression in motor neuron axons contacting these myocytes at 1dpf but increases GABA expression at 2dpf. (***A*** and ***B***) 1 dpf axonal GABA expression in the myotome of a normal larva and a $GABA_AR\alpha$ larva. Arrows identify myocommatal junctions. (***C***) 1 dpf GABA expression in the myocommatal junctions of a $GABA_AR\alpha\beta\gamma$ larva is not different from that in the $GABA_AR\alpha$ larva. (*A-C*) *Insets:* GABA, SV2 or GABA, SV2 and $GABA_AR$ expression in myocytes. (***D***) *Left,* magnified GABA+SV2+ process from the $GABA_AR\alpha$ larva contacts GFP+ myocytes (arrowhead and area indicated by yellow box in *B*). *Right,* magnified GABA+SV2+ processes from $GABA_AR\alpha\beta\gamma$ larva contact GFP+ myocytes

(arrowhead and area indicated by yellow box in *C*). (***E-H***) Magnified myocommatal junctions from dashed boxes in *B* and *C*. Quantification of 1 dpf expression of GABA and SV2. Dotted lines outline regions of myocommatal junctions analyzed. n=6 larvae. (***I*** and ***J***) 2 dpf axonal GABA expression in the myotome of a normal larva and a $GABA_AR\alpha$ larva have decreased compared to GABA expression at 1dpf (*A* and *B*). (***K***) 2dpf GABA expression in the myotome of a $GABA_AR\alpha\beta\gamma$ larva is greater than in the $GABA_AR\alpha$ larva. (***I-K***) *Insets*: GABA, SV2 or GABA, SV2 and $GABA_AR$ expression in myocytes. (***L***) *Left*, magnified GABA-SV2+ process from the $GABA_AR\alpha$ larva contacts a GFP+ myocyte (arrowhead and area indicated by yellow box in *J*). *Right,* magnified GABA+SV2+ process from the $GABA_AR\alpha\beta\gamma$ larva contacts a GFP+ myocyte (arrowhead and area indicated by yellow box in *K*). (***M-P***) Magnified myocommatal junctions from dashed boxes in (*J*) and (*K*). Quantification of 2dpf expression of GABA and SV2. n≥8 larvae. Five-fold increase in the 2dpf GABA-labeled area of motor neuron axons contacting $GABA_AR\alpha\beta\gamma$-expressing myocytes relative to $GABA_AR\alpha$-expressing myocytes (*N* and *K* versus *J*), with no difference in SV2-labeled area of motor neuron axons (*P*), indicates stabilization of GABA expression in axons contacting $GABA_AR\alpha\beta\gamma$-expressing myocytes. Dotted lines outline regions of myocommatal junctions analyzed. ****$p<0.0001$ using two-tailed *t-test*. A, anterior; D, dorsal. SC, spinal cord. M, myotome. See also *SI Appendix*, Fig. S4 and S5.

Fig 3. $GABA_AR\alpha\beta\gamma$ expression in myocytes leads to GABA expression in axons that contact them. (***A***) Injection of ventral blastomeres (V2) with $GABA_AR\alpha\beta\gamma$ mRNA at the 8-cell stage results in myocyte-specific $GABA_AR$ expression. (***B***) Expression of $GABA_AR\alpha\beta\gamma$ in sparse

749 myocytes. SV2 labels axons in the spinal cord and in the trunk myotome. (***C***) In an expansion of
750 the field of view in (*B*), staining for GABA reveals GABA+ axons in the spinal cord and coursing
751 ventrally and posteriorly over the trunk myotome. *Inset:* GFP+ myocyte contacted by the
752 GABA+SV2+ axon from the dotted box. (***D-F***) Higher magnification of contact in (*C*)
753 (arrowheads). (*B-F*) n=13 larvae. (***G-J***) A GABA+VGAT+SV2+ axon contacts a different
754 $GABA_AR\alpha\beta\gamma$ myocyte. n=6 larvae. (***K-M***) A GABA+GAD65/67+ axon contacts another
755 $GABA_AR\alpha\beta\gamma$ myocyte. n=10 larvae. (***N***) Injection of ventral blastomeres (V2) with $GABA_AR\alpha$
756 mRNA results in sparse expression of $GABA_AR\alpha$ in myocytes. SV2+ axons contact a $GABA_AR\alpha$-
757 expressing GFP+ myocyte (arrowheads). n=7 larvae. *Inset,* GABA+ axons are restricted to the
758 spinal cord. M, trunk myotome; SC, spinal cord; A, anterior; D, dorsal. All larvae 3dpf. Further
759 details in Table S2. See also *SI Appendix*, Fig. S4 – S8 and Table S2.
760
761 **Fig 4. Grafting $GABA_AR\alpha\beta\gamma$ receptor-expressing mesoderm into host embryos results in**
762 **$GABA_AR\alpha\beta\gamma$ receptor expression in myocytes and GABA expression in motor neurons**
763 **contacting these myocytes.** (***A***) Procedure for mesodermal transplantation. *Left to right:* donor
764 *Xenopus* embryo injected at the 8-cell stage with $GABA_AR\alpha$ or $GABA_AR\alpha\beta\gamma$ mRNA in the
765 ventral blastomeres (V2). The neural plate was lifted to access the presomitic mesoderm (green),
766 which was grafted from donor to wild-type host larva at 15 hpf (red arrow). GFP expression was
767 detected in the myotome of host larva at 3dpf. (***B***) *Left:* a GABA+ axon courses ventrally and
768 posteriorly over the trunk myotome (M) in $GABA_AR\alpha\beta\gamma$–expressing, mesoderm-grafted host.
769 *Right*: the GABA+SV2+ axon (arrowhead) contacts a $GABA_AR\alpha\beta\gamma$–expressing GFP+ myocyte.
770 (***C***) Another $GABA_AR\alpha\beta\gamma$ myocyte is contacted by a GABA+ChAT+ axon in the trunk myotome

(M). (***D***) Isolation of GABA+ChAT+ axons in (*C*). (***E***) Individual channels for GABA and ChAT in (*D*). n=5 larvae. (***F***) *Left:* No GABA is detected in the trunk myotome (M) in $GABA_AR\alpha$–expressing, mesoderm-grafted host. *Right*: A GABA-SV2+ axon (arrowhead) contacts a $GABA_AR\alpha$–expressing GFP+ myocyte. n=5 larvae. All larvae 3dpf. A, anterior; D, dorsal.

Fig 5. Neuromuscular junctions of myocytes expressing $GABA_AR\alpha\beta\gamma$ generate GABAergic and cholinergic mEPPs. (***A-C***) Recordings from $GABA_AR\alpha\beta\gamma$ myocytes reveal pancuronium-resistant and bicuculline-sensitive mEPPs (arrowheads; n=7 larvae), as well as pancuronium-sensitive and bicuculline-resistant mEPPs (n=7 larvae). (***D-F***) Recordings from $GABA_AR\alpha$ myocytes in the presence of saline, pancuronium and bicuculline reveal only pancuronium-sensitive and bicuculline-resistant mEPPs (n=5 larvae). (***G-I***) Recordings from $GABA_AR\alpha\beta\gamma$ myocytes reveal both pancuronium-sensitive, bicuculline-resistant mEPPs with kinetics of nicotinic receptor mEPPs and pancuronium-resistant, bicuculline-sensitive mEPPs with kinetics of $GABA_AR$-mediated mEPPs. (***J***) Recordings from $GABA_AR\alpha$ myocytes in presence of saline reveal rise and decay time distributions for mEPPs similar to (*I*). N, number of mEPPs. $N \geq 178$ mEPPs ($n \geq 5$ larvae) for each group. All recordings at 4dpf. Only mEPPs with decay times fit by single exponentials were included. Resting potentials were held near -60 mV. Arrowheads indicate median values. Kolmogorov-Smirnov test comparing rise time and decay time in (*J*) with respective rise and decay time in (*H*) and (*I*). ****$p<0.0001$, ns not significant. See also *SI Appendix*, Table S2.

Fig 6. Knockdown of postsynaptic components of trans-synaptic bridges prevents receptor-driven presynaptic neurotransmitter stabilization. (***A***) GARLH4 links $GABA_AR\alpha\beta\gamma$ to neuroligin in the postsynaptic membrane. (***B***) Simultaneous injection of ventral blastomeres (V2) with $GABA_AR\alpha\beta\gamma$ mRNA along with control morpholino ($control^{MO}$) or $GARLH4^{MO}$ (6 nl of 1 mM MO), to achieve widespread expression of $GABA_AR$ and MOs in myocytes. (***C***) Presence of GARLH4 and morpholino knockdown validation by Western blot. (***D***) GABA+ axon is observed in myotome of a $control^{MO}$ $GABA_AR\alpha\beta\gamma$ larva (dashed box, GABA channel only, 7/7). *Inset:* Area in the dashed box. GABA+SV2+ axon contacts a $control^{MO}$+GFP+ myocyte. (***E***) No GABA+ axon is observed in myotome of a $GARLH4^{MO}$ $GABA_AR\alpha\beta\gamma$ larva (dashed box, GABA channel only, 0/12), *Inset:* Area in the dashed box. SV2+ axon contacts a $GARLH4^{MO}$+GFP+ myocyte. n/N, larvae with GABA+ axon/total larvae examined. (***F***) Staggered injection of ventral blastomeres (V2) with $GABA_AR\alpha\beta\gamma$ mRNA followed 1 minute later by low dose of $control^{MO}$ or $GARLH4^{MO}$ (3 nl of 1 mM MO), to achieve sparse expression of MOs in myocytes. (***G***) GABA+ axon is observed in a $control^{MO}$ $GABA_AR\alpha\beta\gamma$ larva (dashed box, GABA channel only, 5/5). *Inset:* Area in the dashed box. GABA+ axon contacts a $control^{MO}$+GFP+ myocyte (arrowheads). (***H***) GABA+ axon observed in myotome of a $GARLH4^{MO}$/normal larva (dashed box, GABA channel only, 12/12). *Inset:* Area in the dashed box. GABA+ axon contacts a $GABA_AR\alpha\beta\gamma$ myocyte lacking $GARLH4^{MO}$ (arrowhead, 12/12). GABA+ processes were not observed contacting $GARLH4^{MO}$ $GABA_AR\alpha\beta\gamma$ myocytes (e.g. asterisk, 0/12). (***I***) Lrp4 links to AChR through rapsyn and to MUSK and dystroglycan in the postsynaptic membrane. (***J***) Injection of ventral blastomeres (V2) with a high dose of $Lrp4^{MO}$ or $control^{MO}$ (6 nl of 1 mM MO) to achieve widespread expression of MOs

in myocytes. (***K***) Morpholino knockdown validation by Western blot. (***L*** and ***M***) Control^{MO} and Lrp4^{MO} expression in the myotome. (***N*** and ***O***) Myocommatal junctions of larvae with control or Lrp4^{MO} stained for ChAT and SV2. (***P*** and ***Q***) Labelled area above threshold quantified for ChAT and SV2. n=4, **p=0.0092, two-tailed *t-test*. Red X in A and G, proteins knocked-down in B-H and J-Q. M, myotome; SC, spinal cord; NT, neurotransmitter; A, anterior; D, dorsal. All larvae 3dpf. See also *SI Appendix*, Fig S9 – S16 and Table S3.

Fig 7. Knockdown of a presynaptic component of trans-synaptic bridges prevents receptor-driven presynaptic neurotransmitter stabilization. (***A***) CASK links to neurexin in the presynaptic membrane. (***B***) Injection of a high dose of CASK^{MO} or control^{MO} (6 nl of 1 mM MO) in dorsal blastomeres 1.2 (D1.2) at the 16-cell stage. All MOs were lissamine-tagged. (***C***) Presence of CASK and morpholino knockdown validation by Western blot. (***D***) Injection of GABARαβγ mRNA in ventral blastomeres (V2) to achieve expression in myocytes, and a high dose of CASK^{MO} or control^{MO} (6 nl of 1 mM MO) in dorsal blastomeres 1.2 (D1.2) to achieve widespread transfection of spinal cord cells. (***E***) GABA+ axon in myotome of control^{MO}GABARαβγ larva (arrowhead, 6/6). (***F***) No GABA+ axons in the myotome of CASK^{MO}GABA$_A$Rαβγ larvae (0/12). (***G***) When the control^{MO} was injected into the dorsal blastomeres 1.2 (D1.2), most lissamine+ somata in spinal cords were ChAT+ (red) (77/83), indicating that the MO transfects motor neurons. When stained for GABA, only a small number of spinal cord lissamine+ somata were GABA+ (cyan) (3/82); the rest were either lissamine+GABA- or lissamine+ChAT- (magenta) (79/82). Total lissamine cells counted per larva ≥71. n=3. (***H***) Injection of control^{MO} or CASK^{MO} (6 nl of 1 mM MO) in dorsal blastomeres 1.2

(D1.2). (***I***) Larva expressing the control^{MO} in the SC. (***J***) Larva expressing the CASK^{MO} in the SC. (***K*** and ***L***) Myocommatal junctions of larvae with control^{MO} or CASK^{MO} stained for ChAT and SV2. (***M*** and ***N*****)** Labelled area above threshold quantified for ChAT and SV2**.** $n \geq 5$, ****$p<0.0001$, two-tailed *t-test*. (***O*** and ***P***) Myocommatal junctions of larvae with control^{MO}, Lrp4^{MO}, $GABA_AR\alpha\beta\gamma$-Lrp4^{MO} or $GABA_AR\alpha\beta\gamma$-GARLH4^{MO} stained for CASK in synaptophysin (SYN)+ motor neuron terminals along the myocommatal junction and in Hoechst+ nuclei in the spinal cord. (***O***) Area of expression of CASK and SYN. n=4, *$p<0.01$, one-way ANOVA ($F_{3,12}=6.316$, $p=0.0081$). (***P***) Area of expression of CASK and Hoechst. n=5, ***$p<0.0001$, one-way ANOVA ($F_{3,12}=16.5$, $p<0.0001$). n/N, larvae with GABA+ axon/total larvae observed. Red X in A, protein knocked down. M, myotome; SC, spinal cord; A, anterior; D, dorsal. All larvae 3dpf. See also *SI Appendix*, Fig. S10 and S11 and S13 and S15 and S16 and Table S3.

Fig 8. Model of receptor-driven neurotransmitter stabilization mediated by trans-synaptic bridges. Summary of interactions between neurotransmitter receptors and transsynaptic bridge proteins that signal retrogradely to achieve presynaptic transmitter stabilization.