Fig.S1
Miller
et. al.

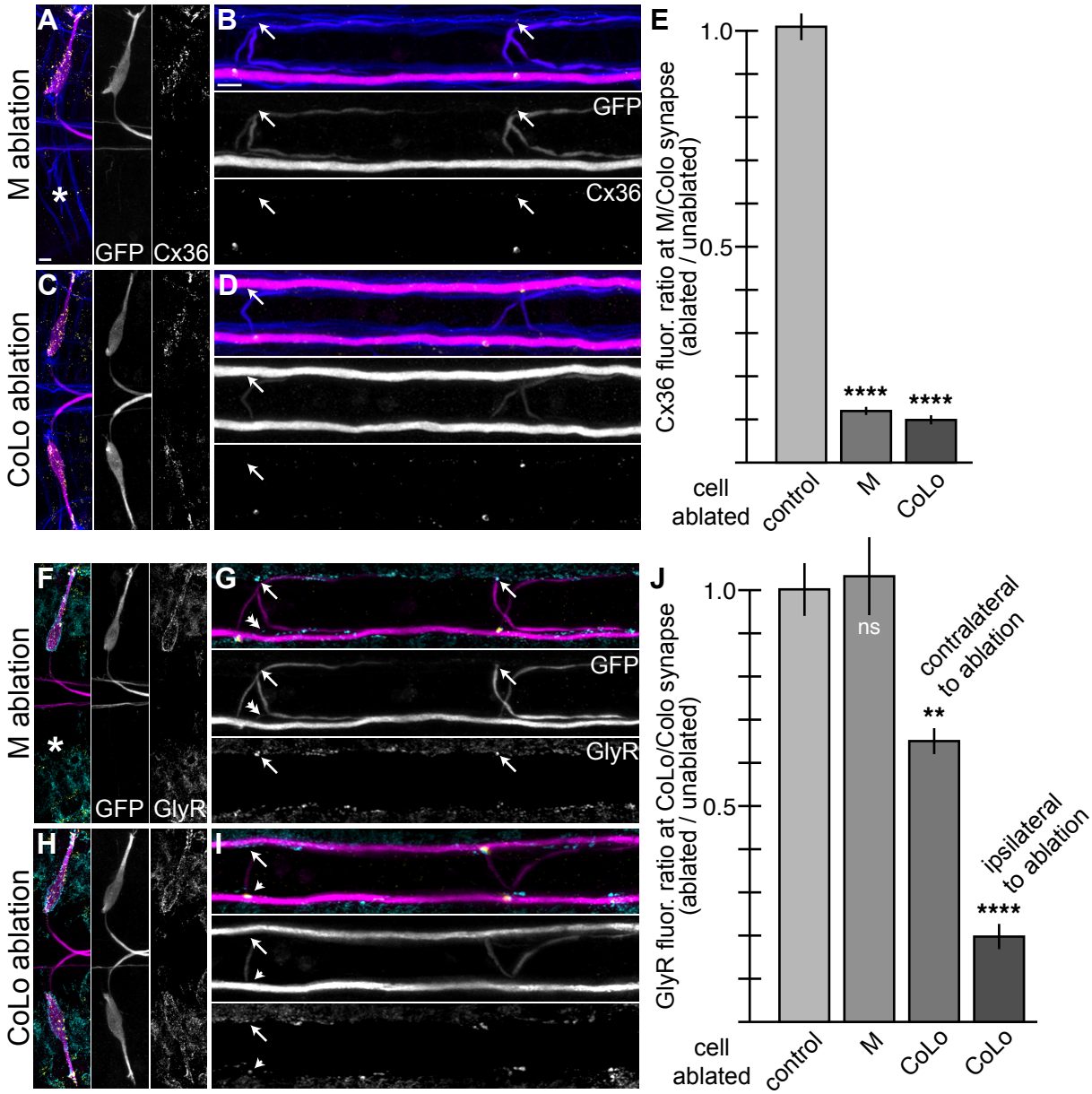


Fig.S2
Miller
et. al.

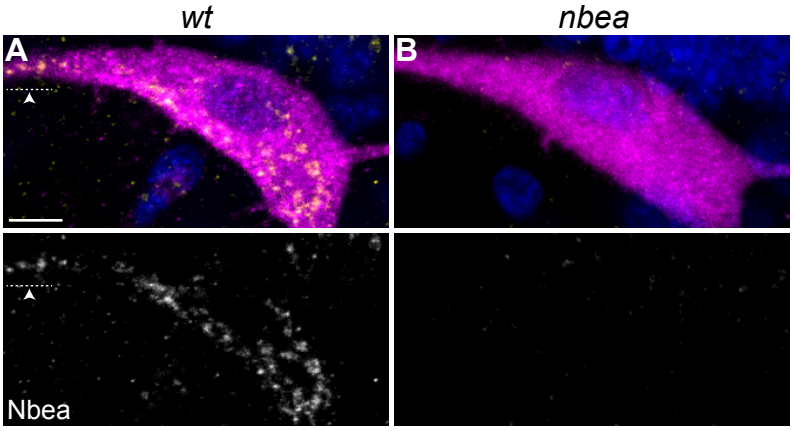


Fig.S3
Miller
et. al.

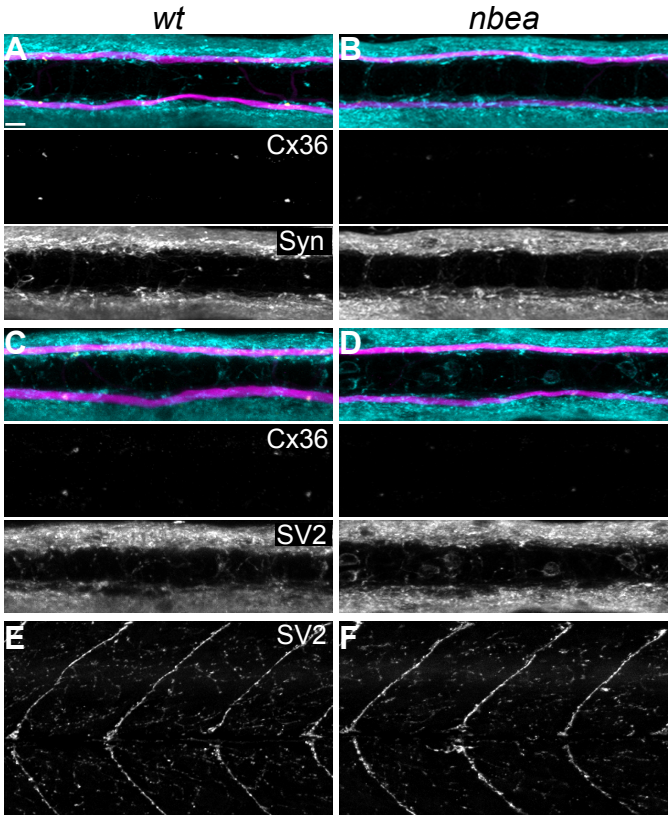
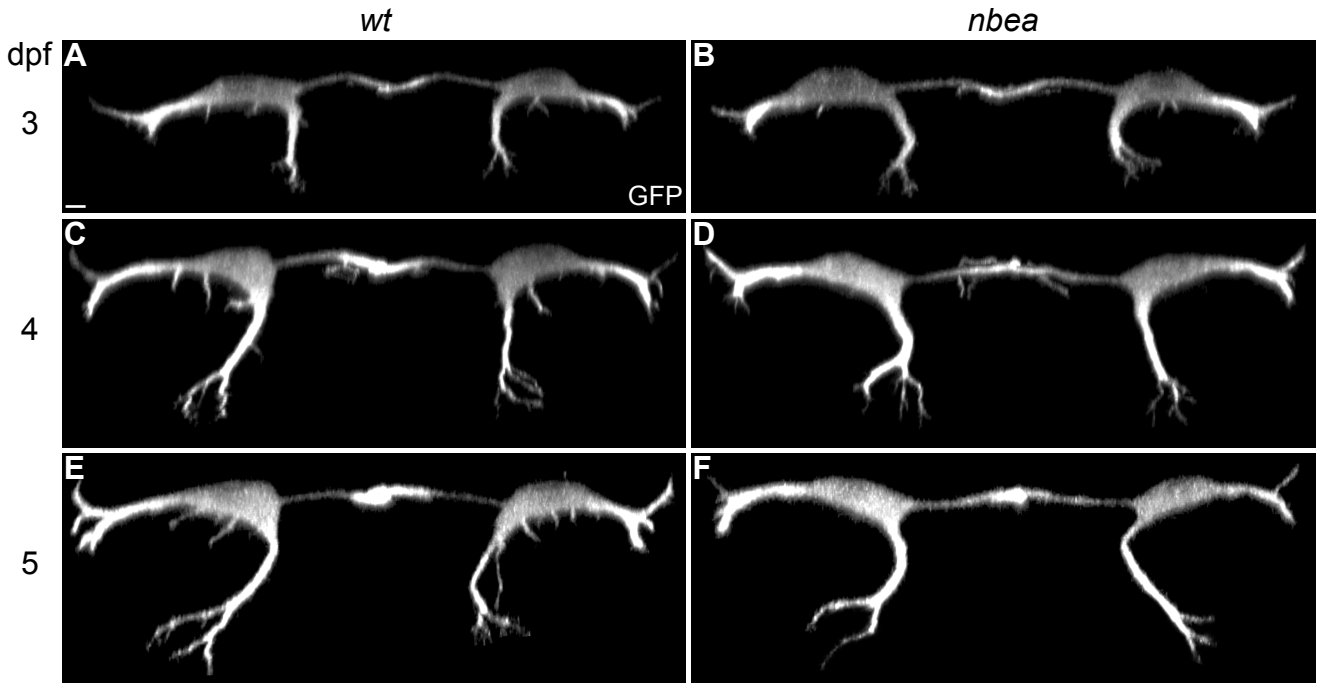


Fig.S4
Miller
et. al.



Supplemental Figure Legends

Figure S1 – Related to Fig. 1 and 3. Ablation of M circuit neurons reveals connectivity. Images are dorsal views of hindbrain and two spinal cord segments from *M/CoLo:GFP* larvae at 5 days post fertilization. Hindbrain and spinal cord images are maximum intensity projections of ~30 and ~10uM, respectively. Anterior is to the left. Scale bar = 10 uM. Larvae are stained for GFP (magenta) and Connexin36 (Cx36, yellow) in all panels, neurofilaments (RMO44, blue) in A-D, and Glycine receptor (GlyR, cyan) in F-I. Individual GFP, Cx36, and GlyR channels are shown in neighboring panels. Graphs represent data as mean +/- SEM. Control ablations were GFP+ neurons anterior to M in the hindbrain. Statistical significance compared to control is denoted as ** for P<0.01, **** for P<0.0001, and ns for not significant. Associated experimental statistics are found in Table S1. **A,B.** M ablation leads to a loss of associated electrical synapses in the hindbrain (A, ***** marks where the M should have formed) and the spinal cord (B, arrows marks where synapses should have formed). **C,D.** CoLo ablation has no effect in the hindbrain (C) but leads to a loss of the associated synapse in the spinal cord (D, arrow depicts where synapse should have formed – CoLo in upper left of image was ablated). **E.** Quantitation of the ratio of Cx36 fluorescence at M/CoLo synapses associated with ablated compared to unablated neurons within an animal. **F-I.** Each CoLo is both postsynaptic (ipsilateral to cell body) and presynaptic (contralateral to cell body) within the image. The arrows (postsynaptic) and arrowheads (presynaptic) refer to the CoLo located in the upper left of the image. Refer to Fig. 1A for schematic. **F,G.** M ablation leads to a loss of associated glycinergic synapses in the hindbrain (F, ***** marks where the M should have formed) but has no effect on GlyR staining in the spinal cord (G). Double arrowhead highlights a thin process extended from CoLo towards the site of the CoLo/CoLo glycinergic synapse – these processes are often not visible given the nearby bright M axon. **H,I.** CoLo ablation (CoLo in upper left of image was ablated) has no effect on the hindbrain (H) but causes a loss of associated ipsilateral, postsynaptic GlyR staining (I, arrow) and a slight diminishment of the contralateral, presynaptic GlyR (I, arrowhead). **J.** Quantitation of the ratio of GlyR fluorescence at CoLo/CoLo synapses associated with ablated compared to unablated neurons within an animal.

Figure S2 – Related to Fig. 2. Punctate cytoplasmic Neurobeachin staining is lost in *nbea* mutants. A,B. Cross section views of M from *M/CoLo:GFP* transgenic embryos at 5 days post fertilization.

Larvae are stained for GFP (magenta), Neurobeachin (Nbea, yellow), and DAPI (blue). Individual Nbea channels are shown in neighboring panels. **A.** Wildtype Nbea staining appears punctate throughout the cell body and extends out into dendrites (arrowhead). **B.** Nbea staining is lost in *nbea* mutants. Images are maximum intensity projections of ~20uM. Ventral is down, lateral is left. Arrowhead points to lateral dendrite. Scale bar = 10 uM.

Figure S3 – Related to Fig. 3. Synaptic vesicle markers are unperturbed in *nbea* mutants. A-D. Dorsal views of two spinal cord segments of *M/CoLo:GFP* transgenic line at 5 day post fertilization. **A-B.** Larvae are stained for GFP (magenta), Connexin36 (Cx36, yellow), and Synaptophysin (Syn, cyan). Individual Cx36 and Syn channels are shown in neighboring panels. **C-D.** Larvae are stained for GFP (magenta), Connexin36 (Cx36, yellow), and SV2 (cyan). Individual Cx36 and SV2 channels are shown in neighboring panels. E-F. Lateral views of SV2 staining in three segments at the level of the neuromuscular junctions.

Figure S4 – Related to Fig. 7. Neurobeachin is required to maintain dendritic complexity. Cross section views of individual larvae from *M/CoLo:GFP* transgenic embryos at noted days post fertilization (dpf). Individual GFP channels are shown. **A,C,E.** In wildtype (*wt*) dendritic branching in all compartments gets progressively more complex over time. **B,D,F.** In *nbea* mutants dendritic outgrowth and branching initiate normally but fail to maintain complexity at 5dpf. Images are maximum intensity projections of ~20uM from digitally rendered cross sections. For clarity, fluorescent signal outside of the GFP-labeled neurons was digitally removed. Scale bar = 10 uM. Ventral is down.

Movies S1 and S2 – Related to Fig. 5. *nbea* mutants have defects in touch-induced escape response. Movies were taken with a high-speed camera at 500-frame/second. Movie 1 is a wildtype sibling and Movie 2 is a mutant from a heterozygous incross ($nbea^{fh364/+}$ x $nbea^{fh364/+}$). Animals were genotyped after experiments.

Table S1 – Related to Fig. 1 and 3. Quantitation of neuronal ablation experiments. For all experiments, animals were mounted such that neurons on the right (R) side were ablated. At least three, but generally eight, synapses associated with the noted condition were sampled per animal

(n); e.g. when the R Mauthner (M) was ablated, eight CoLos on the left (L) side and eight on the R side were measured for the fluorescence intensity of Connexin36 (Cx36) or glycine receptor (GlyR) at synapses within each animal. For the M/CoLo electrical synapse experiments, M is labeled “pre”synaptic and CoLo “post”synaptic due to the physiological current flow from M to CoLo[S1]. Note that since the M axon crosses to the contralateral side measurements from the L CoLos are affected. When the R CoLo was ablated, there is no longer a definite location for where the synapse should have formed – therefore a location directly opposite that of the existing L CoLo synapse was measured at the level of the M axon. Note that the value obtained is similar to the “M axon” measurement in the “control location measures” section of the table. The M axon measure was sampled at a location halfway between two unaffected M/CoLo electrical synapses at the level of the M axon; this represents the background level of Cx36 in the M axon. For CoLo/CoLo glycinergic synapse experiments, because the CoLos receive synaptic input from their CoLo partners on the contralateral side of the spinal cord, each is both pre- and postsynaptic. Therefore, ablating a R CoLo causes the loss of the neuron that is “post”synaptic on the R (ipsilateral) side, and also a loss of the “pre”synaptic input onto the L (contralateral) side. See Fig. 1A for a circuit diagram. For the “midline” measurement, a location halfway between existing M/CoLo or CoLo/CoLo synapses was measured at the midline of the animal at the level of the M axon; this represents the non-axon background level of staining. Within each animal synapses associated with “unablated” neurons were measured to serve as the control. Synapses on the right side were measured in M ablations of “other” R hindbrain (hb) experiments, while synapses at least two segments away from ablated CoLos were used in all other experiments. Note the variance in the average Cx36 or GlyR staining between experiments – this is due to each experiment being a separately stained set of embryos, with variable antibody staining. However, the average ablated/unablated ratio, taken within each set of animals in an experiment normalizes each experiment allowing for comparison between groups, with the noted standard error of mean (SEM) for each experiment. Significance (sig.) is tested within each experiment against the “other avg. ablated/unablated” ratio using an unpaired, two-tail t-test with Welch’s correction. Blank entries were not determined. All animals were 5 dpf.

Table S2 – Related to Fig.1, 3, and 4. Quantitation of wildtype and *nbea* mutant electrical and chemical synapses. The fluorescence signals for Connexin36 (Cx36) and ZO-1 were measured at

the M/CoLo electrical synapses; glycine receptor (GlyR) and Gephyrin (Geph) were measured at CoLo/CoLo glycinergic synapses. At least eight synapses associated with the noted condition were sampled per animal, with the noted number of animals (n) being averaged with standard error of means (SEM). For the Neurobiotin experiments the fluorescence was measured for at least eight CoLo cell bodies, averaged, and divided by the fluorescence of the associated M cell body (CoLo/M ratio); this normalized for the amount of neurobiotin added to each M axon. Significance (sig.) is tested for each row against the wildtype (+/+ or +/+ with heterozygote combinations) within each genotype category using an unpaired, two-tail t-test with Welch's correction. Blank entries were not determined. All animals were 5 dpf.

Table S3 – Related to Fig.5. Quantitation of behavioral responses in wildtype and *nbea* mutants. Three rounds of testing were performed for each animal and the average number of trials with a response (# responses) or the number of animals on their sides (# on side) was recorded for the noted number of animals (n). From these three rounds, the average (avg.) was taken for each group and significance (sig.) was tested against the "wildtype" value using the Mann Whitney test. All animals were 6 dpf.

Table S4 – Related to Fig.6. Quantitation of electrical and chemical synapses in transplant experiments. For all experiments, at least four synapses associated with the noted genotype (geno.) were sampled per animal; e.g. when the Mauthner (M) was transplanted (Xplant), at least four CoLos associated with the GFP+ axon were measured, while at least four CoLos associated with the GFP- axon were measured as control within each animal (n). For the M/CoLo electrical synapse experiments, M is labeled "pre"synaptic and CoLo "post"synaptic due to the physiological current flow from M to CoLo[S1]. When synapses were associated with cases where both M and CoLo neurons were GFP+ those synapses were labeled as "both". For CoLo/CoLo glycinergic synapse experiments, because the CoLos receive synaptic input from their CoLo partners on the contralateral side of the spinal cord, each is both pre- and postsynaptic. Therefore, the CoLo "pre"synaptic measures are those associated with the synapses contralateral to the transplanted CoLo cell body, while the CoLo "post"synaptic measures are associated with the ipsilateral synapse. When two, paired CoLos within a segment were both GFP+ the associated measure was labeled as "both". See Fig. 1A for a circuit diagram. The Xplant / host ratio normalizes the

fluorescence measure within an animal to the host background. This ratio is computed for each animal and the average is reported in the table with the associated standard error of the mean (SEM). Significance (sig.) is tested for each ratio against the “+/+” control transplant within a “transplanted cell” category using an unpaired, two-tail t-test with Welch’s correction. All animals were 6 dpf.

Table S5 – Related to Fig.7. Quantitation of dendrites in transplants and at varying developmental stages. For all experiments, the Mauthner (M) dendrite was traced and quantified for a given genotype (geno.) with the noted number of animals (n). Each M dendritic compartment (ventral, lateral, somatic) was quantified separately and the noted measure was averaged and reported with the associated standard error of the mean (SEM). “Longest path” is the longest continuous main path from cell body to dendrite tip. “Total length” is the sum of the lengths of all the dendritic branches. “Branches” is the sum of the number of branches made off the main branch. “Branch depth” is the maximum depth of branching, with the main branch being primary, and all subsequent branches being labeled sequentially. Significance (sig.) is tested for each measure against the “+/+” donor “+/+” host in the transplants, or the wildtype “+/+ & +/fh364” at each developmental stage using an unpaired, two-tail t-test with Welch’s correction. dpf, days post fertilization. For the transplant experiments all animals were 6 dpf. n.a. not applicable.

Supplemental Table 1 - Neuronal Ablations

	M/CoLo electrical synapse						
		avg. Cx36 fluor. (AU)					
fig.	ablated neuron	R CoLo	L CoLo	avg. ablated/ unablated ratio	SEM	sig.	n
1	control ablation						
	other R hb.	31.79	32.04	1.01	0.03		5
	R M "pre"	19.9	2.49	0.12	0.01	****	5
	R CoLo "post"	3.88	39.25	0.1	0.01	****	5
	control location measures						
		M axon	L CoLo	avg. axon/ unablated ratio			
		4.76	39.25	0.12	0.01		5
		midline	L CoLo	avg. midline/ unablated ratio			
		0.94	39.25	0.02	0.01		5

	CoLo/CoLo glycinergic synapse						
		avg. GlyR fluor. (AU)					
fig.	ablated neuron	R CoLo	L CoLo	avg. ablated/ unablated ratio	SEM	sig.	n
3	control ablation						
	other R hb.	52.04	52.82	1	0.06		5
	R M	73.65	74.49	1.03	0.09	n.s.	4
		avg. GlyR fluor. (AU)					
	ablated neuron	R CoLo "post"	L CoLo "pre"	adjacent unaffected CoLos	avg. "post"/ adjacent ratio	SEM	sig.
	R CoLo	9.63	31.84	48.38	0.2	0.03	****
	control location measures						
		CoLo axon		adjacent CoLos	avg. axon/ unablated ratio		
		10.53		48.38	0.22	0.02	
		midline		adjacent CoLos	avg. midline/ unablated ratio		
		5.85		48.38	0.11	0.05	

	avg. GlyR fluor. (AU)						
ablated neuron	R CoLo "post"	L CoLo "pre"	adjacent unaffected CoLos	avg. "post"/ adjacent ratio	SEM	sig.	avg. "pre"/ adjacent ratio	SEM	sig.	n
R CoLo	9.63	31.84	48.38	0.2	0.03	****	0.65	0.03	**	4

control location measures				
CoLo axon		adjacent CoLos	avg. axon/ unablated ratio	
10.53		48.38	0.22	0.02	4
midline		adjacent CoLos	avg. midline/ unablated ratio	
5.85		48.38	0.11	0.05	4

Unpaired t-test 2 tail with Welch's correction (doesn't assume equal variance in each distribution)

**** P < 0.0001

*** P < 0.001

** P < 0.01

n.s. P > 0.05

Supplemental Table 2 - Quantitation of synaptic markers

	M/CoLo electrical synapse							
figure	genotype	Cx36 (AU)	SEM	sig.	ZO-1 (AU)	SEM	sig.	n
1,4	+/+	28.06	0.89		27.2	1.55		5
	+/fh364	29.13	0.7	n.s.	27.51	2.26	n.s.	5
	fh364/fh364	8.97	0.13	****	15.66	1.42	**	5
2	+/+ & +/fh392	29.21	1.28					5
	fh392/fh392	10.32	0.33	***				3
	+/+ & +/fh380	32.37	0.67					5
	fh380/fh380	10.22	0.17	****				4
	+/+ & +/fh364 & +/fh392	29.33	0.82					5
	fh364/fh392	10.6	0.44	****				4
	+/+ & +/fh364 & +/fh380	29.86	0.53					5
	fh364/fh380	9.24	0.57	****				4
	+/+ & +/fh392 & +/fh380	30.84	1.16					5
	fh392/fh380	10.59	0.27	****				4

		Neurobiotin			
figure	genotype	CoLo/M ratio	SEM	sig.	n
1	+/+	3.84	0.35		4
	+/fh364	2.92	0.28	ns	10
	fh364/fh364	1.24	0.13	**	4

	CoLo/CoLo glycinergic synapse							
figure	genotype	GlyR (AU)	SEM	sig.	Geph. (AU)	SEM	sig.	n
na	+/+	39.54	1.61					5
	+/fh364	37.61	2.67	n.s.				5
	fh364/fh364	19.48	1.79	****				5
3,4	+/+ & +/fh364	29.25	1.04		44.64	1.38		5
	fh364/fh364	14.49	0.6	****	29.47	2.4	**	4

Unpaired t-test with Welch's correction (doesn't assume equal variance in each distribution)

**** $P < 0.0001$

*** $P < 0.001$

** $P < 0.01$

n.s. $P > 0.05$

Supplemental Table 3 - Behavior

			Escape response			Balance				
figure	round	genotype	# responses (3 trials)	SEM	sig.	# on side	ratio	SEM	sig.	n
5	1	wildtype	2.14	0.22						21
		+/+ & +/fh364	1.91	0.19		2	0.06			35
		fh364/fh364	0.69	0.31		5	0.38			13
	2	wildtype	1.88	0.22						25
		+/+ & +/fh364	2.3	0.19		1	0.03			30
		fh364/fh364	0.67	0.28		5	0.42			12
	3	wildtype	2.23	0.28						13
		+/+ & +/fh364	2.47	0.15		1	0.03			34
		fh364/fh364	0.5	0.25		5	0.36			14
	avg.	wildtype	2.08	0.11						3
		+/+ & +/fh364	2.23	0.16	n.s.		0.04	0.01		3
		fh364/fh364	0.62	0.06	*		0.39	0.02	*	3

Mann Whitney

* $P < 0.05$

n.s. $P > 0.05$

Supplemental Table 4 - Transplants and synapses

	M/CoLo electrical synapse						
figure	Xplanted cell	geno. donor	geno. host	Xplant / host	SEM	sig.	n
6		+/+		1.04	0.02		17
	M "pre"	+/fh364	+/+	1.06	0.03	n.s.	12
		fh364/fh364		0.8	0.01	****	11
		+/+		1.04	0.03		35
	CoLo "post"	+/fh364	+/+	0.98	0.07	n.s.	15
		fh364/fh364		0.37	0.02	****	21
		+/+		1.03	0.04		4
	M/CoLo "both"	+/fh364	+/+	1.16	0.16	n.s.	2
		fh364/fh364		0.3	0.01	***	2
			+/+	1.04	0.02		7
	M "pre"	+/+	+/fh364	1.09	0.06	n.s.	16
			fh364/fh364	1.03	0.06	n.s.	12
			+/+	1.07	0.03		20
	CoLo "post"	+/+	+/fh364	1.1	0.06	n.s.	17
			fh364/fh364	3.47	0.25	****	17
			+/+	1.01	0.04		3
	M/CoLo "both"	+/+	+/fh364	1.04	0.04	n.s.	5
			fh364/fh364	3.4	0.25	**	3
	CoLo/CoLo glycinergic synapse						
	Xplanted cell	geno. donor	geno. host	Xplant / host	SEM	sig.	n
		+/+		0.98	0.02		13
	CoLo "pre"	+/fh364	+/+	1	0.04	n.s.	9
		fh364/fh364		1.09	0.06	n.s.	12
		+/+		0.99	0.02		13
	CoLo "post"	+/fh364	+/+	1.07	0.07	n.s.	8
		fh364/fh364		0.47	0.03	****	13
		+/+		1	0.03		8
	CoLo/CoLo "both"	+/fh364	+/+	1	0.06	n.s.	6
		fh364/fh364		0.44	0.03	****	6
			+/+	1.01	0.02		6
	CoLo "pre"	+/+	+/fh364	0.95	0.03	n.s.	6
			fh364/fh364	0.97	0.03	n.s.	10
			+/+	0.98	0.04		6
	CoLo "post"	+/+	+/fh364	0.99	0.05	n.s.	7
			fh364/fh364	2.16	0.14	****	10
			+/+	1.03	0.07		3
	CoLo/CoLo "both"	+/+	+/fh364	1.05	0.01	n.s.	4
			fh364/fh364	2.29	0.11	***	5

Unpaired t-test 2 tail with Welch's correction (doesn't assume equal variance in each distribution)

**** $P < 0.0001$

*** $P < 0.001$

** $P < 0.01$

n.s. $P > 0.05$

Supplemental Table 5 - Dendrite quantitation

	Transplants		ventral dendrite										lateral dendrite										somatic dendrites										
			longest			total							longest			total							longest			total							
figure	geno. donor	geno. host	path	SEM	sig.	length	SEM	sig.	branches	SEM	sig.	depth	SEM	sig.	path	SEM	sig.	length	SEM	sig.	branches	SEM	sig.	depth	SEM	sig.	path	SEM	sig.	length	SEM	sig.	number	SEM	sig.	depth	SEM	sig.	n
7	+/+	+/+	90.58	2.75		205.2	12.1		17.92	1.75		4.54	0.27		65.21	2.18		98.97	5.09		5.62	0.55		3	0.16		14.5	1.28		39.76	3.81		4.31	0.33		n/a			13
	+/fh364	+/+	86.27	3.35	n.s.	186.5	12.41	n.s.	14.11	1.64	n.s.	4.22	0.22	n.s.	65.64	1.21	n.s.	97.16	4.66	n.s.	6	0.58	n.s.	3.11	0.2	n.s.	13.04	0.92	n.s.	34.5	4.91	n.s.	3.44	0.29	n.s.	n/a			9
	fh364/fh364	+/+	86.19	2.22	n.s.	134.4	11.82	***	8	1	***	3	0.27	***	63.15	2.68	n.s.	84.43	3.5	*	3.75	0.25	**	2.88	0.23	n.s.	5.81	1.14	****	8.23	2.82	****	1.25	0.31	****	n/a			8
	+/+	fh364/fh364	87.33	3.98	n.s.	205	20.29	n.s.	18.25	2.36	n.s.	4.75	0.41	n.s.	66.61	2.1	n.s.	107.6	6.08	n.s.	6.38	0.89	n.s.	3	0.27	n.s.	12.49	1.17	n.s.	33.83	4.08	n.s.	4.25	0.31	n.s.	n/a			8
	Developmental progression		ventral dendrite										lateral dendrite										somatic dendrites										
			longest			total							longest			total							longest			total							
	dpf	geno.	path	SEM	sig.	length	SEM	sig.	branches	SEM	sig.	depth	SEM	sig.	path	SEM	sig.	length	SEM	sig.	branches	SEM	sig.	depth	SEM	sig.	path	SEM	sig.	length	SEM	sig.	number	SEM	sig.	depth	SEM	sig.	n
	1	+/+ & +/fh364	4.2	2.7		8.4	2.9		1	0		1	0		29.45	1.36		33.63	2.46		1.5	0.29		1.75	0.25		5.4	n/a		9	n/a		0.75	0.75		n/a			4
		fh364/fh364	5	3.14	n.s.	10	3	n.s.	1	0	n.s.	1	0	n.s.	27.05	1.53	n.s.	32.8	3.99	n.s.	1.5	0.29	n.s.	1.25	0.25	n.s.	7	n/a	n/a	15	n/a	n/a	0.75	0.75	n.s.	n/a			4
	2	+/+ & +/fh364	34.78	2.32		42.55	3.13		3.1	0.41		2.3	0.15		31.6	2.44		39.41	3.04		3.4	0.4		2.6	0.22		7.51	0.6		18.18	2.36		2.9	0.23		n/a			10
		fh364/fh364	35.88	2.08	n.s.	44.3	3.68	n.s.	3.13	0.48	n.s.	2.13	0.13	n.s.	32.56	3.08	n.s.	45.21	3.87	n.s.	3.75	0.45	n.s.	2.63	0.26	n.s.	6.39	0.83	n.s.	13.51	1.76	n.s.	2.38	0.26	n.s.	n/a			8
	3	+/+ & +/fh364	53.84	1.85		66.72	3.96		3	0.42		2.2	0.13		49.93	1.83		61.71	2.73		2.9	0.31		2.3	0.15		8.16	0.71		15.03	1.78		2	0.3		n/a			10
		fh364/fh364	53.8	1.34	n.s.	65.39	3.01	n.s.	3	0.44	n.s.	2.43	0.3	n.s.	43.09	1.79	*	53.1	2.68	*	2.71	0.29	n.s.	2.14	0.14	n.s.	7.96	0.75	n.s.	14.29	0.84	n.s.	2.14	0.14	n.s.	n/a			7
	4	+/+ & +/fh364	81.01	1.94		144.3	12.57		10	1.38		4	0.21		60.58	2.46		99.91	10.41		4.4	0.48		2.5	0.17		15.33	1.27		30.96	2.71		2.9	0.31		n/a			10
		fh364/fh364	75.04	2.11	n.s.	123.9	7.32	n.s.	7.88	0.79	n.s.	3.88	0.23	n.s.	56.43	2.09	n.s.	87.36	5.03	n.s.	4.5	0.5	n.s.	2.63	0.18	n.s.	8.3	1.12	**	9.02	1.5	****	0.75	0.25	****	n/a			8
	5	+/+ & +/fh364	75.63	1.53		147.2	7.62		11.5	1.14		4.17	0.24		65.61	1.52		104.2	4.12		5.67	0.48		3	0.12		15.81	1.16		39.3	3.5		3.83	0.24		n/a			12
		fh364/fh364	73.98	4.23	n.s.	104.9	5.87	***	5.33	0.42	***	2.83	0.17	***	54.75	1.77	***	87.4	3.93	**	4	0.45	*	2.67	0.21	n.s.	6.1	n/a	n/a	6.1	n/a	n/a	0.17	0.17	****	n/a			6

**** P < 0.0001
*** P < 0.001
** P < 0.01
* P < 0.05
n.s. P > 0.05
n/a not applicable

Supplemental Experimental Procedures

Fish maintenance, mutants, and genotyping

All animals were raised in an Institutional Animal Care and Use Committee (IACUC)-approved facility at the Fred Hutchinson Cancer Research Center. Zebrafish (*Danio rerio*) were bred and maintained as previously described[S2]. Animal care is provided by Rachel Garcia and veterinary care is provided by Dr. Rajesh *K*. Uthamanthil, DVM. *nbea*^{fh364} was isolated from an early-pressure, gynogenetic diploid screen[S3] in a mixed *AB/Tu background; mutant carriers were maintained in the *M/CoLo*:GFP (*Et*(*Tol-056:GFP*)) background[S1], which itself is a mixed *AB/Tu background. Wildtype *nbea* sequence in exon21 of the transcript is:

CCGGAGGAGCAGAAGATCAC,

whereas the *nbea*^{fh364} mutation has a C to T transition (highlighted in bold below) introducing a nonsense codon (underlined) that truncates the protein approximately one third of the way through the protein:

CCGGAGGAG**T**AGAAGATCAC.

The frame shift deletions *nbea*^{fh392} and *nbea*^{fh380} were generated in an AB background by creating TALENs[S4] targeting the 1st or 21st exon of *nbea*, respectively, and stable lines were Sanger sequenced to verify deletions.

TALEN target binding sites and mutations:

Exon1 TALEN: forward = ATCAAGATGAAATTCGCAG, reverse = TGCTAACCTCCCCGACCTG,

Wildtype sequence: CAGTGCTCATCGGTTTGATC,

nbea^{fh392} sequence: CAGTGCTTTGATC.

There is a loss of 7 base pairs, causing a frame shift that is predicted to introduce a nonsense mutation (underlined) directly after the deletion at amino acid 95.

Exon21 TALEN: forward = ACATTAACCCAAAGAACCC, reverse = ATGTTGTAGACCATTTCGG,

Wildtype sequence: CCGGAGGAGCAGAAGATCAC,

nbea^{fh380} sequence: CCGGAGG*T*TCAC.

There is a loss of 9 base pairs, and an introduction of one base pair (italicized), resulting in a net change of 8 base pairs causing a frame shift. This change is predicted to introduce 43 different amino acids before encountering a new nonsense codon and truncating the protein.

Mutations were monitored through each generation by genotype and phenotype. Primers for detecting *nbea*^{fh392} were:

forward = CGGGGTGATAAACCCCTCGGTTC, reverse = CCAGATTTAAAACGGTCTCCACGATG, and *nbea*^{fh364} *and nbea*^{fh380} were:
forward = GGTGTGGCAGGACTGGATGTTCT, reverse = GGCGTGGTAGAGCAGGATACGGA.

Immunohistochemistry

Anesthetized embryos from 1-6 dpf were fixed in either 2% trichloroacetic (TCA) acid for 3 hours or 4% paraformaldehyde (PFA) for 1 hour. Fixed tissue was then washed in PBS + 0.5% TritonX100, followed by standard blocking and antibody incubations. Tissue was cleared step-wise in a 25%, 50%, 75% glycerol series and was dissected and mounted for imaging. Primary antibodies used were: chicken anti-GFP (abcam, ab13970, 1:250), rabbit anti-human-Cx36 (Invitrogen, 36-4600, 1:200), mouse anti-ZO-1 (Life Technologies, 33-9100, 1:200), mouse anti-Glycine-receptor-alpha-1 (Synaptic Systems, 146011, 1:100), mouse anti-Gephyrin (Synaptic Systems, 147111, 1:50), rabbit anti-Gephyrin (Synaptic Systems, 147002, 1:100), mouse anti-RMO44 (Life Technologies, 13-0500, 1:100), rabbit anti-Neurobeachin (Synaptic Systems, 194003, 1:50). All secondary antibodies were raised in goat (Life Technologies, conjugated with Alexa-405, -488, -555, -594, or -633 fluorophores, 1:250). DAPI was added for 2 hours after secondary antibodies were removed (Sigma, D9542, 1:500). Nb was detected using fluorescently-tagged streptavidin (Life Technologies, conjugated with Alexa-594 or 633 fluorophores, 1:500).

Laser ablation

Anesthetized embryos were immobilized on their right sides and imaged on an Ultraview spinning disk confocal microscope. M or CoLo, or other “control” GFP labeled neurons in the hindbrain, were identified using the 488-laser line of the confocal and then ablated using a Micro-Point nitrogen laser. Successful ablation was determined by the immediate loss of all GFP fluorescence within the cell body and stereotypical necrotic appearance of the nucleus and by later absence of the neuron when imaged for experiments. Laser ablation of M occurred at 1 dpf before axonal outgrowth, while CoLo was ablated at 2.5 dpf, the earliest timepoint at which GFP highlighted the neurons. Embryos were unmounted from the agar and allowed to grow until subsequent immunostaining at 5 dpf.

Cell transplantation

Cell transplantation was done using standard techniques[S5]. For “mutant into wildtype” experiments, *nbea*^{fh364} heterozygous animals carrying the *M/CoLo:GFP* transgene were incrossed while hosts were non-transgenic AB. For “wildtype into mutant”, *M/CoLo:GFP* animals were incrossed while hosts were an incross of *nbea*^{fh364} heterozygous animals that were not transgenic. Approximately 20 cells were deposited 3-12 cell diameters away from the margin at the sphere/dome stage (4-4.5 hpf) with a single embryo donating to 3-5 hosts. Embryos were allowed to grow until 6 dpf at which point they were processed for immunostaining. Donor (mutant to wildtype) or host (wildtype to mutant) embryos were genotyped.

Behavioral analysis

Behavioral experiments were performed on 6 dpf larvae and filmed using a high-speed camera (M3, IDT) capturing 500 frames per second. Movies were processed and analyzed using MotionStudio (IDT). On the day of testing, larvae were separated into individual 3.5 cM petri dishes and each animal was tested separately. The apparatus for testing startle was adapted from Satou *et al*.[S1]. Each trial started with three seconds of silence followed by a startling tone and a response period. Behavior was analyzed using the captured movies with a positive response being recorded if the animal produced a short- or long-latency turn triggered to either of the tones. Each animal was tested three times in a three-hour window, with the order of animals being randomized between trials. Three separate crosses between three unique pairs of parents were examined. The balance of the fish was assessed within the same escape trials – animals lying on their sides were noted. After behavioral analysis animals were genotyped.

Supplemental References

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S3. Walker, C., Walsh, G. S., and Moens, C. (2009). Making Gynogenetic Diploid Zebrafish by Early Pressure. JoVE.

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