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Inventory of Supplemental Information

Our manuscript includes the following supplemental information:
Supplemental Table 1
Supplemental Figure 1
Supplemental Figure 2
Supplemental Methods

The reason for placement of this information into supplemental data is described for each table or figure below. In all cases we refer to the supplemental information (either figure or table) both in the manuscript text and in the figure legends of our manuscript. Finally, each supplemental figure has a legend that is present both in our manuscript text and with the supplemental figure itself.

Supplemental Table 1: This table includes all of the raw values for our electrophysiological analysis of synaptic transmission. These data support the display of information in each of our figures. As such, this is essential information for any reader who wishes to delve deeper into our electrophysiological analyses. We provide this information exactly as we have done in our past publications at Neuron (Frank et al., 2006; Frank et al., 2009).

Supplemental Figure 1: This figure presents quantification of synaptic bouton numbers at the NMJ for two mutations. The bouton numbers do not change. As there is no change, we decided to save space in our already long manuscript by placing this information in supplemental material.

Supplemental Figure 2: This figure presents quantification of EPSP amplitudes recorded in the presence of a Shal-specific toxin. There is no change in EPSP amplitude compared to control. As there is no change, we decided to save space in our already long manuscript by placing this information in supplemental material.

Supplemental Methods: We have provided additional, detailed methodology in this section for electrophysiology and PCR.

Bergquist et al., SUPPLEMENTAL MATERIALS

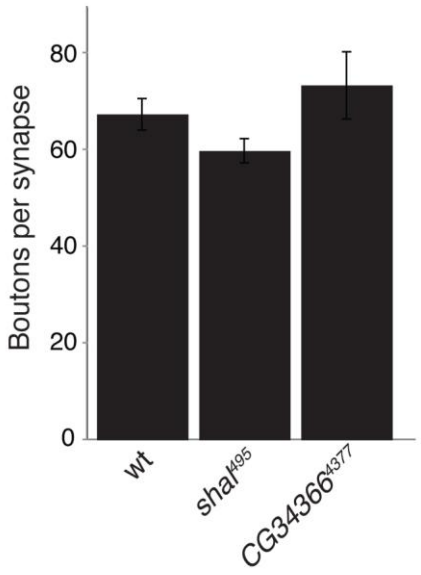

Figure S1. *shal*^{495} and *CG34366*^{4377} mutants have normal NMJ morphology. Synapse morphology is normal in *shal*^{495} and *CG34366*^{4377} compared to wt animals. Synapses were co-stained with anti-nc82 to visualize presynaptic active zones and anti-dlg to visualize postsynaptic membrane. The number of boutons per segment A3 muscle 6/7 NMJ were counted and averages are graphed (N= 6-8 animals, 12-14 NMJ). There is no significant difference between wt and mutant NMJ. There is no significant different in muscle surface area ($p>0.05$).

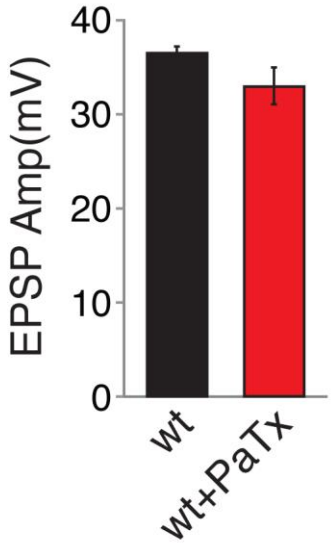

Figure S2. Wild-type NMJs are insensitive to the Shal specific toxin, Phrixotoxin. EPSP amplitude before (black) and after (red) incubation with 1μM Phrixotoxin (PaTx). Amplitudes are not significantly different following toxin application suggesting Shal protein does not localize to the NMJ.

Supplemental Methods

Neuronal recordings:

All experiments were performed on wandering third-instar larvae. Preparations were achieved by gluing both anterior and posterior extremities to sylgard (Silicone Elastomer 2-Part, World Precision Instruments, Inc) coated cover slips and then making a dorsal incision. The fat and guts were removed, care was taken to leave the ventral nerve cord (VNC) intact. The VNC was then glued to the body wall (Glue is Histoacryl, Braun Aesculap). Dissections were made in external saline (in mM, 135 NaCl, 5 KCl, 4 $MgCl_2 \cdot 6H_2O$, 2 $CaCl_2 \cdot 2\ H_2O$, 5 HEPES, 36 sucrose, pH 7.1). Protease (Protease, Type XIC: Bacterial from Streptomyces griseus, Sigma-Aldrich, St. Louis, MO) was focally administered to the ventral nerve cord using a glass pipette pulled to ~10 μm tip diameter. Debris was constantly cleared using the pipette with positive and negative pressure. With adequate exposure to the protease, the outer and inner membranes of the ganglion sheath are disrupted revealing the motor neurons. Motor neurons in the VNC are organized stereotypically with repeating segmental arrays of somata. We focused on the bilaterally symmetric dorsomedial clusters aligned parallel to the midline and containing motor neurons (MN) 1, 6/7, 14, and 30-Ib and MNISN-Is. These neurons form a cross like cluster and are easily isolated due to their dorsal location within the VNC (Choi et al., 2004). To further verify motor neuron identity, several cells were filled during recordings (alexa 488) and imaged post recording. Motor neuron axons project to the body-wall muscle through nerves that exit the VNC in each segment and positively identified the cells as motor neurons. Whole cell recordings were made in K^+ isolation saline (external saline with the addition of 1μM TTX). Thick-walled borosilicate glass electrodes (GC100TF-10; Harvard Apparatus, Edenbridge, UK) were pulled and fire polished to a resistance of 5-10 MΩ. Internal solution contained (in mM: 140 KCH_3SO_3, 2 $MgCl_2 \cdot 6H_2O$, 2 EGTA, 5 KCl, 20 HEPES, pH 7.4). Recordings were obtained with an axopatch 200B amplifier (Axon Instruments, Union City, CA, USA). Cells were held at -60mV. To isolate the I_A, two separate electrophysiological protocols were run. The first used a hyperpolarized conditioning pulse (-90mV), which removed inactivation from I_A, and was followed by depolarizing command pulses (-60 to +20mV), which activated both I_A and I_K. The second protocol was then run starting with a more depolarized (-40mV) conditioning pulse, which completely inactivated I_A, and was then followed by the same command pulses,

activating only I_K. Currents recorded under these two protocols were then electronically subtracted offline using Clampfit 9.0 (Axon Instruments) to give the isolated I_A.

Muscle recordings:

Recordings were made in HL3 saline containing 10mM Mg^{2+} and indicated calcium concentrations (see text). For PhTx treatment, semi-intact preparations, with the CNS, fat, and gut left intact were perfused with Philanthatoxin-433 (PhTx). PhTx (Sigma) was prepared as a stock solution (4mM in DMSO) and diluted in HL3 saline to 10μM. Following an incubation of 10 minutes, the preparations were rinsed and dissection was completed, including the removal of the CNS as previously described (Davis et al., 1998). The cut motor axon was stimulated as described previously (Davis et al., 1998). For 4-Amino Pyridine (AP) experiments, 4-AP (sigma) stock solution of 100mM was diluted in HL3 to a final concentration of 25μM. Preparations were either first incubated in PhTx for 10mins followed by 1min 4-AP incubation or just had the 1min 4-AP incubation. 4-AP was left on for the duration of the recording in both cases. Phrixotoxin-2 (PaTx2; Alomone Labs, Jerusalem, Israel) was kept frozen as 10X stock aliquots and thawed and diluted to 1μM final concentration immediately before use. PaTx was left on for the duration of the recording. Muscle input resistance (R_{in}) was monitored at the beginning and end of the recording. Recordings were excluded if R_{in} or V_m changed by more than 20%.

CNS Quantitative RT-PCR

Primer-probes specific for real-time PCR detection of Shal, Shaker, Ribosomal protein L32 (RpL32) were designed and developed by Applied Biosystems. The CNS was removed from 25 third-instar larvae per sample (3-6 samples/genotype). Total RNA was isolated from each sample using the standard Trizol protocol. A DNase digestion was then done to remove all potential DNA contamination (RQ1 RNase-free DNase Promega). RT was performed (Taqman reverse transcription reagents, Applied Bioscience) using random hexamers and 1μg total RNA. A no RT control was performed for each sample. Purified cDNA was used as a template in 30μl PCR reaction (TaqMan Universal PCR Master Mix, no AmpErase UNG, Applied Biosystems). This 30μl reaction was divided into three 10μl triplicates. In addition, one 10μl no RT reaction was used for each sample. The ABI Prism 7900 was used for all PCRs. Cycle threshold (C_T)

was determined by automated threshold analysis using SDS2.3 software according to the manufacturer's instructions (Applied Biosystems, Foster City, CA). Comparative Shal and Shaker levels (between wt and mutant animals) were determined using the $\Delta\Delta C_T$ method (Applied Biosystems User Bulletin No. 2). To determine if the two amplification reactions have the same PCR efficiency, ΔC_T (C_T of experimental gene - C_T of reference gene) values are determined across the serial dilutions and plotted against the log of the cDNA dilution. A slope close to zero indicates equivalent amplification efficiency. This was done and both Shal and Shaker have approximately equal amplification efficiencies compared with the endogenous control, RpL32. Briefly, the $\Delta\Delta C_T$ method is as follows: ΔC_T values are determined as explained above. Next, experimental animal (mutants) ΔC_T values were subtracted from control animal (wt) ΔC_T values to give the $\Delta\Delta C_T$. Finally using the equation $2^{(-\Delta\Delta C_T)} \times 100$ the percent expression of each gene in experimental compared to control animals was calculated. Each experimental animal sample was compared to each wild type sample (Applied Biosystems User Bulletin No. 2). In four *shal*^{495} samples, Shal levels were below detection levels. To generate averages these samples were counted as 0.

Supplemental Table 1. Quantification of Synaptic Transmission

Condition	Genotype	PhTox	mEPSP	EPSP	QC	V_m	R_{in}	N
0.4mM Ca^{++}	wt	-	1.11 (0.05)	34.31 (1.19)	32.7 (1.79)	-69.4 (0.9)	10.5 (0.7)	31
	wt	+	0.53 (0.02)	31.35 (1.37)	62.42 (4.41)	-66.6 (1.4)	11.1 (1.0)	20
	shal^{495}/+	-	1.13 (0.08)	31.64 (1.69)	28.82 (1.47)	-68.9 (1.8)	5.7 (0.2)	13
	shal^{495}/+	+	0.52 (0.06)	24.72 (1.72)	51.11 (6.76)	-65.6 (1.9)	5.1 (0.1)	8
	shal^{495}	-	1.06 (0.05)	28.87 (1.05)	28.49 (1.59)	-72.4 (1.0)	10.4 (0.8)	27
	shal^{495}	+	0.6 (0.02)	12.21 (1.01)	21.18 (2.09)	-74.7 (1.3)	7.2 (0.9)	20
	shal^{495}/*DF*	-	1.11 (0.11)	29.96 (1.36)	29.00 (3.28)	-68.8 (1.6)	8.8 (1.0)	8
	shal^{495}/*DF*	+	0.51 (0.03)	17.81 (1.38)	37.18 (4.14)	-67.9 (1.7)	6.9 (0.9)	11
	shal^{495}/*shal*^{700}	-	0.82 (0.06)	22.25 (1.77)	28.58 (3.12)	-70.5 (1.8)	5.3 (0.3)	10
	shal^{495}/*shal*^{700}	+	0.33 (0.02)	9.82 (1.35)	30.49 (3.35)	-69.6 (1.5)	5 (0.3)	10
	shal^{700}	-	0.72 (0.05)	25.51 (1.47)	37.56 (3.83)	-73.2 (2.4)	5 (0.2)	9
	shal^{700}	+	0.41 (0.03)	9.30 (0.77)	24.39 (3.14)	-69.3 (0.8)	4.9 (0.1)	7
	shRNAi/+;shal^{495}	-	1.07 (0.06)	29.93 (2.4)	28.69 (2.42)	-77.9 (2.2)	5.8 (0.6)	13
	shRNAi/+;shal^{495}	+	0.61 (0.04)	11.28 (2.33)	18.79 (3.56)	-74.7 (1.7)	7.2 (1.1)	8
	C155/+;shRNAi/+;shal^{495}	-	1.31 (0.06)	33.77 (3.04)	26.02 (2.33)	-75.6 (2.3)	7.4 (0.4)	14
	C155/+;shRNAi/+;shal^{495}	+	0.64 (0.02)	34.77 (4.77)	54.87 (7.05)	-77.8 (2.7)	9.8 (0.8)	8
	Ok6/EKO-222	-	0.96 (0.08)	14.38 (2.05)	15.08 (1.9)	-78.1 (2.6)	7.3 (0.4)	10
	Ok6/EKO-222	+	0.39 (0.03)	7.56 (1.86)	20.14 (5.35)	-71.2 (1.5)	7.9 (0.5)	9
	CG34366^{4377}*(K_v3.2)*	-	0.76 (0.05)	29.18 (1.52)	39.51 (2.26)	-71.1 (1.6)	8.5 (0.7)	17
	K_v3.2	+	0.37 (0.02)	14.03 (1.77)	40.52 (5.42)	-70.1 (1.3)	6.6 (0.8)	14
	K_v3.2;shRNAi/+	-	1.11 (0.10)	21.68 (2.94)	20.69 (3.29)	-74.0 (2.0)	6.3 (0.6)	9
	K_v3.2;shRNAi/+	+	0.49 (0.03)	13.14 (2.03)	26.54 (3.39	-75.4 (1.3)	6.1 (0.5)	10
	C155/+;K_v3.2;shRNAi/+	-	0.90 (0.05)	32.37 (3.36)	36.00 (3.14)	-78.1 (1.1)	4.7 (0.2)	9
	C155/+;K_v3.2;shRNAi/+	+	0.49 (0.02)	17.15 (1.85	35.14 (3.83)	-66.1 (1.9)	5.7 (0.6)	11
4-AP (25µM)	wt	-	1.03 (0.06)	47.23 (2.16)	47.07 (2.47)	-69.6 (1.7)	7.1 (0.6)	12
0.4mM Ca^{++}	wt	+	0.54 (0.03)	39.62 (3.97)	76.32 (9.92)	-69.0 (2.5)	11 (0.6)	8
	shal^{495}	-	0.96 (0.05)	30.56 (2.04)	34.21 (2.80)	-72.4 (1.5)	5.0 (0.2)	22
	shal^{495}	+	0.55 (0.02)	28.49 (2.30)	52.81 (5.12)	-76.9 (1.7)	5.7 (0.3)	13

Condition	Genotype	PhTox	mEPSP	EPSP	QC	V_m	R_{in}	N
0.3mM Ca^{++}	wt	-	1.07 (0.03)	23.76 (1.57)	22.54 (1.66)	-68.2 (1.0)	7.0 (0.2)	21
	wt	+	0.54 (0.03)	18.25 (2.90)	35.72 (6.71)	-65.4 (1.0)	7.2 (0.4)	14
	shal^{495}	-	0.76 (0.05)	12.59 (1.65)	17.90 (3.22)	-71.8 (1.1)	5.0 (0.4)	8
	shal^{495}	+	0.55 (0.01)	5.73 (0.95)	10.45 (1.81)	-74.5 (0.6)	5.0 (0.3)	8
	sh^{14}	-	1.05 (0.07)	43.36 (3.26)	42.60 (4.02)	-77.8 (1.1)	5.3 (0.3)	10
	sh^{14}	+	0.55 (0.02)	48.23 (0.91)	88.43 (4.33)	-69.0 (1.8)	7.1 (0.2)	8
	sh^{14}*;;shal*^{495}	-	1.12 (0.05)	44.21 (2.5)	39.97 (2.72)	-74.7 (1.7)	6 (0.4)	10
	sh^{14}*;;shal*^{495}	+	0.69 (0.01)	54.83 (2.96)	79.44 (3.02)	-72.3 (1.6)	8.4 (0.7)	8
	sh^{14}/+	-	0.97 (0.04)	42.45 (2.02)	43.99 (1.33)	-72.9 (1.7)	5.1 (0.4)	8
	sh^{14}/+	+	0.51 (0.02)	35.29 (5.0)	69.06 (9.36)	-66.1 (6.9)	7.0 (0.4)	7
	sh^{14}/+*;;shal*^{495}	-	1.13 (0.07)	39.68 (2.70)	36.38 (3.16)	-72.6 (1.9)	7.6 (1.0)	12
	sh^{14}/+*;;shal*^{495}	+	0.62 (0.03)	41.0 (2.18)	66.7 (2.24)	-77.3 (1.4)	5.1 (0.4)	13
0.4mM Ca^{++}	*GluRIIA*	-	0.39 (0.01)	28.34 (1.21)	75.91 (5.06)	-71.3 (1.1)	9.9 (0.8)	24
	GluRIIA;shal^{495}	-	0.51 (0.03)	16.84 (1.19)	36.16 (3.95)	-73.7 (1.4)	5.8 (0.2)	19
	GluRIIA;shal^{495}*/Df*	-	0.38 (0.04)	10.30 (1.15)	30.37 (5.30)	-68.4 (1.4)	6.1 (0.4)	10

1. Genotypes to be compared, with and without PhTx, are presented sequentially and are shaded similarly.
2. Values are presented as mean (±standard error).
3. QC refers to quantal content (see Methods)
4. N refers to the number of neuromuscular recordings