Published in final edited form as:

J Neurogenet. 2009 ; 23(4): 395–404. doi:10.3109/01677060902878481.

Notch signaling is required for activity-dependent synaptic plasticity at the *Drosophila* neuromuscular junction:

Notch is required for NMJ synaptic plasticity

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Abstract

The cell-surface signaling protein Notch is required for numerous developmental processes, and typically specifies which of two adjacent cells will adopt a non-neuronal developmental fate. It has recently been implicated in long-term memory formation in mammals (Costa et al., 2003) and *Drosophila* (Ge et al., 2004; Presente et al., 2004). Here we investigate whether activity-dependent synaptic plasticity at the neuromuscular junctions (NMJs) of third instar *Drosophila* larvae depends on Notch signaling. The length and number of axonal branches and number of pre-synaptic sites (boutons) in NMJ vary with the level of synaptic activity (Budnik et al., 1990), so we increased activity at the NMJ by two complementary methods: increasing the chronic growth temperature of third instar larvae from 18 to 28°C (Sigrist et al., 2003; Zhong and Wu, 2004), and using the double mutant *ether-a-gogo, Shaker (eagSh)* (Budnik et al., 1990), both of which increase NMJ size and bouton count. Animals homozygous for the functionally-null temperature sensitive Notch alleles *N^{ts1}* and *N^{ts2}* (Shellenbarger and Mohler, 1975) displayed no activity-dependent increase in NMJ complexity when reared at the restrictive temperature. Dominant-negative Notch transgenic expression also blocked activity-dependent plasticity. Ectopic expression of wild type Notch and constitutively active truncated Notch transgenes also reduced activity-dependent plasticity, suggesting that there is a “happy medium” level of Notch activity in mediating NMJ outgrowth. Lastly, we show that endogenous Notch is primarily expressed in the presynaptic cell bodies where its expression level is positively correlated with motor neuron activity.

Keywords

Notch; synaptic plasticity; neuromuscular junction; *Drosophila melanogaster*; activity; axonal outgrowth

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Introduction

The *Drosophila* third instar body wall NMJ (Keshishian et al., 1996) is a popular model of synapses generally because of its accessibility for visualization, electrophysiology and the diversity of genetic tools available in the fruit fly. The size and complexity of the axonal arbors forming the NMJ vary with the synapse's level of firing activity; typically, mutations and conditions that increase activity lengthen and complexify the NMJ (Budnik et al., 1990) (Zhong et al., 1992; Wang et al., 1994; Sigrist et al., 2003), and vice-versa (Lnenicka et al., 2003; Xing et al., 2005). However, the relationship between synaptic activity and NMJ arbor complexity is complex, and cannot be entirely reduced to that simple rule; genetic perturbations that change the quality of synaptic transmission (Bogdanik et al., 2004) or interfere with axon/bouton outgrowth (Coyle et al., 2004) can exhibit negative correlations between synaptic activity and arbor complexity (Wan et al., 2000; Fischer and Overstreet, 2002).

Several pathways have been implicated in the causal relationship between synaptic activity and morphological changes. The cell adhesion molecule Fasciclin II is down-regulated in association with synaptic growth (Schuster et al., 1996) (Meinertzhagen et al., 1998), and appears to be a downstream proximal determinant of bouton size, itself regulated by the Ras-MAPK pathway (Koh et al., 2002). The wingless pathway has been recently implicated in rapid activity-induced morphological changes at the NMJ such as filopodial outgrowth (Ataman et al., 2008).

The protein Notch, traditionally considered an important protein in neuronal cell-fate determination, has become increasingly intriguing as a molecular component of synaptic plasticity. While canonical Notch signaling is required for, e.g. neural and glial differentiation in mammals (Lutolf et al., 2002), myelination (Givogri et al., 2002), and the proper development of *Drosophila* sensory bristle cells (Lyman and Yedvobnick, 1995), more recent work has indicated that non-canonical Notch signaling regulates the cytoskeleton and cellular morphology (Major and Irvine, 2005; Ferrari-Toninelli et al., 2008). Control of the cytoskeleton is essential for the neurite and synapse morphological modification thought to underlie long-term memory formation. Consistently, Notch activation has been found to inhibit neurite outgrowth in cultured mammalian neurons (Berezovska et al., 1999; Salama-Cohen et al., 2006), enhance LTP in hippocampal slices (Wang et al., 2004), and mediate long-term olfactory memory in *Drosophila* (Ge et al., 2004; Presente et al., 2004).

Notch is a large transmembrane protein with two major domains: the Intracellular Domain (NICD), and the Extracellular Domain (NECD). The NECD contains a number of EGF-like repeats (Kumar and Moses, 2001) mediating binding of ligands such as Delta (Alton et al., 1989) and Serrate (Fleming et al., 1990). In canonical Notch signaling, the NICD is released following ligand binding and translocates to the nucleus (Schroeter et al., 1998) where it activates Suppressor-of-Hairless, an activator of the Enhancer-of-Split genes (Struhl and Adachi, 1998). Here we present evidence that Notch signaling is required for activity-dependent synaptic plasticity at the *Drosophila* NMJ. In particular, loss-of-function and

dominant-negative Notch mutations, as well as ectopic signaling, eliminate activity-dependent ramification of the NMJ arbors.

Materials and Methods

C-S (wild type), *dnc^{M14}*, *eag¹Sh¹³³*, *N^{ts2}*, and *N^{ts1};eag¹Sh¹³³* lines were maintained at room temperature. *hs-N cdc10rpts* and *N^{ts1}* flies were maintained at 18°C. To assay NMJ morphology, particularly bouton count and branch topology, eggs were collected by allowing 70 to 150 adults to lay eggs in vials containing corn food, dry yeast, and yeast paste for 2hrs. Vials were kept at 18°C for 28hrs (until the early 1st instar stage) – Notch deficiency is lethal during embryonic development (Welshons, 1971). After this period, the vials were transferred either to 29°C or kept in 18°C incubators.

After approximately three days, roaming third-instar larvae were removed and dissected in Low Ca⁺⁺ Saline (LCS). Body wall dissection was performed as in (Budnik et al., 1990). Samples were fixed in Bouin's fixative for 1.5 hrs and washed in PBT for 3–6 x 20min until the fixative's coloration was no longer visible. Overnight incubation in 1:1000 goat anti-HRP antibody was followed by PBT washes and 4h incubation in 1:100 HRP-conjugated rabbit anti-goat IgG secondary. Samples were then washed 3 x 20min in PBT, and once in 0.1M Tris buffer (pH 7.6) before being incubated in a 1:10 dilution in Tris Buffer of DAB-Peroxide solution (Sigma) until the NMJs could be seen easily under the dissecting microscope (approximately 10min). Samples were dehydrated in successive washes of ethanol of increasing concentration followed by xylene before mounting for microscopy.

The HRP stained NMJs were visualized using either stacked confocal microscope images or camera lucida. In both techniques, the 7, 6, 13, and 12 muscle fibers of the left side of the 4th abdominal segment were analyzed, unless that segment was damaged or malformed, in which case the right side was used. The images generated were then used to generate a branching/bouton count diagram by first identifying the nerve leading into each NMJ, and for each branch of the NMJ arbor, recording the total number of boutons along that branch. Branching diagram topology was standardized by minimizing the number of secondary and tertiary branches of the NMJ diagram. P-value significances between numbers of branches and boutons were calculated using the Student t-test.

Notch expression levels were measured using quantitative immunohistochemistry. NMJs of C-S, *eag¹Sh¹³³*, and *dnc^{M14}* lines were stained with 1:100 anti-NECD antibodies in a manner similar to the HRP staining. Secondary antibody was 1:1000 FITC-conjugated rabbit anti-mouse IgG. The motor ganglia of larval brains (subesophageal ganglia) were dissected and fixed for 30min in Bouin's fixative, washed 2 x 20min in PBT. Antibody treatments were the same as the body wall preparations, and incubated in the same tubes for comparability. Images of the muscle fibers and brains were acquired on a Zeiss AxioPhot compound microscope with a Kodak digital camera. The green channel was analyzed using the NIH ImageJ software. The Marquee Tool of that software was used to define an outline of either the subesophageal ganglia or muscle fibers. The outlines were designated without knowledge of which experimental group the sample belonged to, to eliminate bias, and the

average pixel luminosity within the outlined region was measured. These values were compared using t-test.

Evoked junctional currents between the motor neurons and muscle fibers were recorded in different transgenic fly lines using techniques previously reported (Stewart et al., 1994). As in the immunohistochemical analysis, roaming third instar larvae were chosen for physiological experiments. Dissection conditions and recording equipment were identical to previous studies (Guo and Zhong, 2006).

Results

Muscle fibers 7, 6, 13, and 12 (ordered from ventral (medial) to dorsal (lateral)), of the A4 abdominal segment of third instar larvae, were analyzed for the number of synaptic boutons, and the complexity of the branching of the synaptic arbor. Because the synaptic arbors of muscle fibers 7 and 6 originate from the same nerve and frequently cross between these muscle fibers multiple times, we considered the boutons on those muscles to be part of the same arbor. For all muscle fibers, the number of boutons and complexity of branching was determined in wild type, N^{ts1} , and N^{ts2} flies, at both 18°C and 29°C (Figure 1). N^{ts1} , and N^{ts2} animals show some small, but statistically significant reductions in bouton count at 18°C compared to wild type animals at 18°C. Specifically, in N^{ts1} flies, boutons count is reduced by 31% and 30% on muscle fibers 13 and 12 respectively, and in N^{ts2} animals bouton count is reduced by 15% on muscle fiber 12. All other bouton and branch counts between wild type and mutant animals are not statistically different at 18° and 29°C. The stereotyped structure (Figure 1A) and topology of the NMJs (Figure 2) in wild type animals is also conserved in the mutant lines.

Temperature-dependent synaptic plasticity was observed in wild type animals. NMJ arbors on muscle fibers 13 and 12 show enlargement and greater complexity in animals reared at 29°C compared to 18°C (Figure 1A), with 33 and 21% more boutons respectively (Figure 1B). While the arbors of muscle fibers 7 and 6 did not show significant increases in bouton count, their arbors were observed to have significantly more branches, as was the case on muscle fibers 12 and 13 as well. Specifically, the number of branches increased by 35, 47, and 58% on muscle fibers 7 and 6, 13, and 12 respectively. Type I boutons can be distinguished from type II and III boutons morphologically in anti-HRP stained NMJs. The NMJs on muscle fibers 7 and 6 is predominantly composed of type I boutons and rarely showed significant changes in number following temperature shifts, whereas the smaller type II and III boutons on muscle fibers 12 and 13 were variable. The number of large type I boutons on muscle fibers 13 and 12 did not change count in animals carrying the Notch alleles that reduced the baseline number of boutons (data not shown).

The increase in bouton count from 18°C to 29°C is absent in both the N^{ts1} and N^{ts2} cases (Figure 1), with no statistical difference between these experimental groups, except in the case of N^{ts1} muscle fiber 12 which shows a 10% decrease in boutons at the higher temperature. This effect was also present, though less consistently, in the degree of arbor branching. N^{ts1} animals show an average 35% increase in branches in the muscle fiber 13 arbor and N^{ts2} animals show a 42% increase, but in all other conditions the degree of

branching is unchanged from 18°C to 29°C. The number of branches, but not boutons were reduced in the *N* alleles at 18°C compared to wild type at 18°C, probably due to leakiness in the temperature sensitive alleles at this nominally permissive temperature.

The topologies of NMJ arbors in wild type animals at 18°C have the same distribution of complexity as the *N^{ts1}* arbors and *N^{ts2}* arbors at both 18°C and 29°C. Figure 2 shows randomly selected examples of branching diagrams of *N^{ts1}* and wild type muscle fiber 12 arbors at 18° and 29°C. A similar pattern was seen in the *N^{ts2}* allele, data not shown. That the mutant lines have the same topology as wild type at 18°C implies that the mutants do not impair the baseline level of growth or topological development of the arbors, even at the restrictive temperature.

The transgenic construct *hs-N^{cdc10rpts}* is a truncated form of *Notch* expressing ten copies of only the extracellular and transmembrane domains of the protein under the control of a heat-shock inducible promoter (Rebay et al., 1993). Endogenous Notch is also expressed in this line. *hs-N^{cdc10rpts}* acts as a *Notch* dominant negative, sequestering ligand away from the wild type copies of Notch, but inducing no signal due to its missing NICD. There was no significant difference in the number of boutons or branches in 18°C wild type animals and 18°C *hs-N^{cdc10rpts}* animals (Figure 3A). However, in animals reared at 29°C after first instar, there is a significant reduction in both the number of boutons and number of branches in *hs-N^{cdc10rpts}* compared to wild type, on muscle fibers 13 and 12. There was a statistically non-significant reduction in the number of branches or boutons on muscle fibers 7/6. While the transgene caused no statistically significant reduction in boutons or branches at 18°C, brief heat shocks of 1 hour at 29°C or 37°C per day were sufficient to induce statistically significant reductions in branch counts in the muscle fiber 12 NMJ (Figure 3B). No significant reductions were observed under these conditions in bouton number or branching on the other muscle fibers, indicating that neither leaky baseline expression of the dominant negative transgene nor genetic background, was responsible for the elimination of temperature-dependent synaptic plasticity.

To confirm that the observed requirement for Notch signaling in temperature-dependent synaptic plasticity reflects its role in activity-dependent synaptic plasticity, we increased activity at the NMJ by a method other than increasing incubation temperature. We recombined *N^{ts1}* into the *eag,Sh* double mutant background to produce the *N^{ts1},eag,Sh* triple mutants (Figure 4). *eag* and *Sh* encode K⁺ channel subunits (Zhong and Wu, 1993) (Timpe et al., 1988), and display greater baseline synaptic activity than wild type animals, irrespective of temperature. They also have 48% more boutons than wild-type animals for muscle fibers 12 and 13 (Budnik et al., 1990; Zhong and Wu, 2004). *N^{ts1},eag,Sh* animals had significantly fewer boutons than *eag,Sh* animals on muscle fibers 13 and 12, but not 7 and 6, and branch number was also significantly less on muscle fiber 13. Branch number was reduced, but not significantly, on muscle fiber 12. Thus, Notch inactivation blocked the activity-dependent NMJ plasticity that would be otherwise induced by the *eag,Sh* mutations. We wanted to create animals with *N^{ts1},dnc* double mutations, which is expected to have the same effect as *N^{ts1},eag,Sh*. However, the *N* and *dnc* genes are separated 4,180 bases, making the generation of a recombinant impractical.

We tested whether ectopic activation of Notch signaling would affect the size or complexity of the NMJs after the temperature shift by analyzing their morphology in two additional transgenic lines: *hs-N* and *hs-N^{intra}* which express respectively full length and NICD-only Notch under the control of the inducible heat-shock promoter (Rebay et al., 1993). In both of these lines, rearing the animals at 29°C does not result in an increase in bouton count (Figure 5). In *hs-N^{intra}* animals, bouton counts are significantly lower on muscle fibers 7 and 6, and 12 at 29°C compared to 18°C, and in *hs-N* animals, muscle fiber 12 has significantly reduced bouton counts. In all cases, the transgenic animals have bouton counts at 29°C that are not statistically different from wild type at 18°C. Unlike the mutants and transgenes blocking Notch signaling, these constructs did not show significant reductions in the number of NMJ branches at the high temperature (data not shown).

Immunohistochemistry was used to assess locations and levels of Notch expression in the regions of cells interacting at the NMJ: the subesophageal ganglia, motor center of the larval brain containing the motor neuron cell bodies, and the muscle fibers. Antibody staining for the NECD was done in wild type animals as well as in the mutants *eag,Sh* and *dunce (dnc)* (Dudai et al., 1976), a cAMP phosphodiesterase (Byers et al., 1981) that also increases synaptic activity. Surprisingly, Notch was not observed at the NMJs nor on the muscle fibers, which were statistically indistinguishable from staining controls lacking primary antibody. Instead, Notch was detected in the motor ganglion of the larval brain (Figure 6), which contains cell bodies of the motor neurons as well as interneurons and motor neuropils. Varying NMJ activity levels with the mutants *dnc* and *eag,Sh* did not change the negligible levels of Notch expression in the muscle fibers, but there was a statistically significant increase in Notch expression levels between the ganglia of wild-type and *dnc* and *eag,Sh* flies, as determined by quantification of the fluorescence (Figure 6B).

Having observed that mutations in Notch can affect both the structure and the structural plasticity of the NMJ, we lastly asked whether perturbing Notch signaling affected the electrophysiological properties of the NMJ. We recorded activity in muscle fibers while stimulating the segmental nerve. The evoked junctional currents (EJCs) of wild-type and *hs-N*, *hs-N^{intra}* and *hs-N cdc10rpts* animals were indistinguishable (Figure 7), indicating that perturbing Notch signaling does not alter the electrophysiological activity of the NMJ.

Discussion

Shifting wild type fly larvae from 18°C to 29°C induces temperature-dependent synaptic plasticity at the *Drosophila* neuromuscular junction. This plasticity is manifested, via synaptic arbor outgrowth, as an increase in the number of boutons and branches making up the NMJ (Sigrist et al., 2003; Zhong and Wu, 2004). This temperature-dependent effect is completely blocked, with respect to bouton numbers, by temperature-sensitive mutations in the *Notch* gene which are themselves induced by the ambient high temperature. Notch inactivation also blocks the increase in branching complexity of the NMJs brought about by high temperature, in most cases. Similarly, Notch inactivation by induction of Notch dominant negative proteins also blocks temperature-dependent plasticity. *Notch* mutation furthermore blocks activity-dependent synaptic plasticity induced by the double mutant *eag,Sh* which normally increases NMJ firing rate and NMJ morphological complexity.

Additionally, inducing ectopic Notch activity reduces temperature-dependent synaptic plasticity, implying that there is a bell-shaped response curve as a function of Notch activation levels, with the maximal level of activity/temperature-dependent synaptic plasticity resulting from intermediate levels of Notch signaling activity. Alternatively, these observations could be due to non-specific effects caused by the over-expression of dominant Notch alleles. However, this possibility is made less likely by the observation that manipulating the level of Notch signaling has no impairing effect on the electrophysiological properties of the NMJ. This suggests that Notch's effect on activity-dependent synaptic plasticity cannot be attributed to a Notch role in activity at the NMJ. These results are mutually consistent and imply that moderate levels of Notch signaling are required for activity-dependent synaptic plasticity.

In addition to an elimination of plasticity, there was a reduction in the number of boutons at 18°C from wild-type to the *N^{ts}* alleles. We speculate that at 18°C, some Notch was in the inactive conformation. This implies that in addition to having a synaptic plasticity phenotype, *Notch* may be involved in determining the baseline number of synaptic connections. That the reduction in number of boutons from wild-type to *N^{ts1}* is greater than the reduction from wild-type to *N^{ts2}* is consistent with the fact that *N^{ts2}* has generally weaker *Notch* phenotypes (Shellenbarger and Mohler, 1975).

NMJ boutons can be divided into three categories (Johansen et al., 1989). Type I boutons are large stimulatory, glutamatergic boutons characterized by deep and convoluted interweaving of the neuronal membrane with the muscle fiber membrane. Type II and III boutons are smaller and regulatory, and are most easily distinguished by the types of neurotransmitter that they release, octopamine (Monastirioti et al., 1995) and insulin-like peptide (Gorczyca et al., 1993) respectively. The 7 and 6 muscle fibers NMJ contains few or no small Type II and Type III boutons, instead being composed of Type I boutons. There was no significant difference in the number of boutons in wild-type muscle fibers 7/6, while there was in fibers 13 and 12, suggesting that most of the plasticity occurs in the Type II and Type III boutons. Consistently, perturbing Notch signaling had no effect on the muscle fiber 7 and 6 NMJs in most experiments.

We observed Notch expression in the motor ganglion of the larval brain, but not the NMJs or muscle fibers. The motor ganglion contains the motor neuron cell bodies as well as motor interneurons and neuropils, and here Notch expression correlated with the level of synaptic activity as induced by *dnc* and *eag,Sh* mutations. This is consistent with other experimental evidence that Notch expression levels are regulated by activity. Namely, Notch expression is attenuated in rats during memory consolidation following conditioning (Conboy et al., 2007). The effect of activity on Notch expression level appears to be uni-directional given our observation that the NMJ is electrophysiologically unaffected in Notch transgenic lines.

Our observation that ectopic Notch activation blocks synaptic plasticity the same degree as Notch inhibition, suggests a model in which negative feedback may limit the total flux of activity-dependent synaptic plasticity: while increasing activity will induce synaptic outgrowth, it will also induce greater Notch expression, which at sufficiently high levels blocks activity-dependent outgrowth. This could provide a simple mechanism to limit the

facility with which new long-term memories are formed, by limiting the amount of axonal outgrowth induced by synaptic activity. Thus, across synapses beyond the NMJ, Notch may have roles both in mediating plasticity-dependent memory formation as well as preserving the synaptic status quo when high levels of its expression block outgrowth, as has been observed here and in mammals (Berezovska et al., 1999). Our work constitutes further evidence that in addition to its traditional roles in cell fate determination, Notch now clearly has important roles in post-developmental processes, namely activity-dependent modification of neural morphology.

Acknowledgments

We acknowledge NIH research grant funding support to Y.Z. and the Undergraduate Research Program at Cold Spring Harbor Laboratory for support to B.L.d.B. We thank Jai Puthenveetil for experimental assistance and helping develop the project.

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Figure 1. *Notch* mutant alleles block temperature-induced NMJ outgrowth

A) Representative *camera lucida* examples of NMJ arbors from *N^{ts1}* and *N^{ts2}* alleles, muscle fibers 7 and 6, 13 and 12, and low and high temperatures. B) Quantification of NMJ complexity is shown as the average number of boutons and branches per arbor under the above experimental conditions. Bars in the top half represent the mean number of boutons, and in the bottom half, the number of branches. Error bars are $\pm$ standard error of the mean. Horizontal lines at 100 boutons and 10 branches have been added for visual reference. P-values are given for the comparison between the adjacent 18°C and 29°C categories. For all figures, *** = p-value < 0.005, ** = 0.005 < p-value < 0.01, * = 0.01 < p-value < 0.05. The number of replicates per experiment is between 9 and 19 for all experiments in all figures unless otherwise noted. Bouton counts were not normalized for muscle fiber area, as the latter did not vary with statistical significance across experimental groups.

Figure 2. Representative Branching Diagrams

Nine muscle fiber 12 NMJ branching diagrams from each category were chosen at random to give a representative sample of the branching diversity and patterns for each experimental group. *N^{ts1}* arbors have the same topological distribution at 18°C and 29°C as the wild type arbors at 18°C. Wild type arbors at 29°C have many more branches and sub-branches.

Figure 3. *hs-N^{Δcdc10rpts}* dominant negative transgene blocks temperature-induced NMJ outgrowth

A) Expression of the temperature-inducible Notch dominant negative transgene *N^{Δcdc10rpts}* blocks temperature-dependent increase in bouton and branch counts on muscle fibers 13 and 12. P-value asterisks compare wild type to *N^{Δcdc10rpts}* NMJs in equivalent treatments. B) Mild heat shocks generally do not reduce NMJ complexity in *N^{Δcdc10rpts}* animals incubated primarily at 18°C, which do not appear statistically different from wild type when reared entirely at 18°C. One hour heat shocks at 29°C and 37°C per day are sufficient to reduce NMJ branching on muscle fiber 12 by ~27%. P-value asterisks compare each heat-shock condition to constant 18°C incubation.

Figure 4. Notch inactivation blocks NMJ plasticity induced by the activity double mutant *eagSh*
P-value asterisks compare *Notch*^{*ts1*},*eag*,*Sh* and *eag*,*Sh* for each muscle fiber at room temperature. *Notch*^{*ts1*},*eag*,*Sh* triple mutant lines grew weakly, and n=6 for each of these conditions. P-value asterisks compare high temperature to low temperature for each muscle fiber.

Figure 5. *hs-N* and *hs-N^{intra}* ectopic Notch expression transgenes reduce temperature-induced NMJ outgrowth

Bouton counts do not increase at high temperatures in lines ectopically expressing Notch (*hs-N*) or constitutively active Notch truncation *hs-N^{intra}*. All experimental lines were grown chronically at 29°C, inducing both temperature-dependent plasticity as well as the expression of the *hs*-dependent transgenes. P-value asterisks compare high temperature to low temperature for each genotype.

Figure 6. Notch expression levels vary with neural activity

A) Representative FITC confocal images of the subesophageal ganglion (motor ganglion) of the third instar larval brain, stained with antibodies against the NECD. Notch expression is higher in *dnc* and *eag,Sh* mutants which also increase neural activity. B) Average fluorescent intensity of subesophageal ganglion across 10 replicates of each genotype, quantified from the confocal images. P-value asterisks compare each genotype to wild type. C) Confocal images of Notch expression in the muscle fibers of the 4th abdominal segment. D) Quantified fluorescence in the muscle fibers. No significant variation was observed here in the mutants with higher activity. Vertical axes in B and D are in arbitrary units of fluorescent intensity with 256 as a sensor maximum.

Figure 7. NMJ electrophysiological responses are the same in Notch mutants and wild type
A) Typical EJCs recorded in muscle fiber 12 following stimulation of the incoming nerve. -hs animals were reared entirely at room temperature, +hs animals were incubated at 37°C for 1 hour per day, starting from the first instar. Heat shock caused a reduction in EJC magnitude that was independent of both Notch ectopic activation and inhibition. B) Quantification of EJC magnitudes. No statistically significant differences were seen between fly lines at fixed temperature conditions. $4 < n < 6$ for all experiments.