Neto-Mediated Intracellular Interactions Shape Postsynaptic Composition at the *Drosophila* Neuromuscular Junction

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**Abstract**

The molecular mechanisms controlling the subunit composition of glutamate receptors are crucial for the formation of neural circuits and for the long-term plasticity underlying learning and memory. Here we use the *Drosophila* neuromuscular junction (NMJ) to examine how specific receptor subtypes are recruited and stabilized at synaptic locations. In flies, clustering of ionotropic glutamate receptors (iGluRs) requires Neto (Neuropillin and Tolloid-like), a highly conserved auxiliary subunit that is essential for NMJ assembly and development. *Drosophila neto* encodes two isoforms, Neto-α and Neto-β, with common extracellular parts and distinct cytoplasmic domains. Mutations that specifically eliminate Neto-β or its intracellular domain were generated. When Neto-β is missing or is truncated, the larval NMJs show profound changes in the subtype composition of iGluRs due to reduced synaptic accumulation of the GluRIIA subunit. Furthermore, *neto*-β mutant NMJs fail to accumulate p21-activated kinase (PAK), a critical postsynaptic component implicated in the synaptic stabilization of GluRIIA. Muscle expression of either Neto-α or Neto-β rescued the synaptic transmission at *neto* null NMJs, indicating that Neto conserved domains mediate iGluR synaptic transmission. However, only Neto-β restored PAK synaptic accumulation at *neto* null NMJs. Thus, Neto engages in intracellular interactions that regulate the iGluR subtype composition by preferentially recruiting and/or stabilizing selective receptor subtypes.

**Author Summary**

Ionotropic receptors assembled from different subunits have strikingly different properties and uses. In mammalian brain, the molecular mechanisms controlling the subunit composition of glutamate receptors are critical for the formation of neural circuits and for the long-term plasticity underlying learning and memory. Here we investigate how subunit composition is regulated at the *Drosophila* neuromuscular junction (NMJ) to examine how specific receptor subtypes are recruited and stabilized at synaptic locations. In flies, clustering of ionotropic glutamate receptors (iGluRs) requires Neto (Neuropillin and Tolloid-like), a highly conserved auxiliary subunit that is essential for NMJ assembly and development. *Drosophila neto* encodes two isoforms, Neto-α and Neto-β, with common extracellular parts and distinct cytoplasmic domains. Mutations that specifically eliminate Neto-β or its intracellular domain were generated. When Neto-β is missing or is truncated, the larval NMJs show profound changes in the subtype composition of iGluRs due to reduced synaptic accumulation of the GluRIIA subunit. Furthermore, *neto*-β mutant NMJs fail to accumulate p21-activated kinase (PAK), a critical postsynaptic component implicated in the synaptic stabilization of GluRIIA. Muscle expression of either Neto-α or Neto-β rescued the synaptic transmission at *neto* null NMJs, indicating that Neto conserved domains mediate iGluR synaptic transmission. However, only Neto-β restored PAK synaptic accumulation at *neto* null NMJs. Thus, Neto engages in intracellular interactions that regulate the iGluR subtype composition by preferentially recruiting and/or stabilizing selective receptor subtypes.
and B. Mutations that eliminate or truncate the Neto-β isoform fail to accumulate the type-A receptors, as well as other postsynaptic proteins important for the synaptic stabilization of type-A receptors. This result indicates that Neto may use its cytoplasmic domains as signaling hubs and organizing platforms to sculpt postsynaptic composition. Neto proteins modulate the formation and function of glutamatergic synapses from worms to humans. Our findings expand the repertoire of Neto proteins and illustrate the richness in synapse modulation brought about by the growing family of auxiliary proteins.

### Introduction

Ionotropic glutamate receptors (iGluRs) play major roles in excitatory transmission in the vertebrate brain and at the insect neuromuscular junction (NMJ). The synapse properties are primarily shaped by the subunit composition of the receptors, which could be further modified by RNA editing and alternative splicing [1]. Changes in the subunit composition of postsynaptic iGluRs, in particular the AMPA-type receptors, have a tremendous impact on the development and plasticity of glutamatergic synapses [2,3]. Mechanisms controlling the recruitment of selective receptor subtypes exist and they integrate signals from multiple signaling pathways and regulate synaptic trafficking via posttranslational modifications within the iGluRs intracellular domains [4,5]. In addition, several auxiliary subunits, which primarily modulate the channel properties, have been implicated in the subcellular distribution of receptors [6]. Whether and how the auxiliary subunits modulate the subunit composition of iGluRs remains unclear.

The *Drosophila* NMJ is a glutamatergic synapse similar to mammalian central synapses. In flies, the iGluRs are heterotetrameric complexes composed of three shared subunits, GluRIIC, GluRIID and GluRIIE, and either GluRIIA (type-A receptors) or GluRIIB (type-B) [7–11]. The function of the fly NMJ also requires Neto (Neuropillin and Tolloid-like), an obligatory auxiliary subunit of the iGluR complexes [12]. In the absence of Neto, or any of the shared iGluR sub-units (or GluRIIA and GluRIIB together) the receptors fail to cluster at synaptic locations and the animals die as paralyzed embryos unable to develop into larval stages [12,13]. Genetic manipulation of Neto and iGluR levels indicated that Neto and the shared iGluR subunits are limiting for the synaptic localization of receptors and that GluRIIA and GluRIIB compete with each other for synaptic localization [9,14].

The type-A and type-B receptors differ in their single-channel properties, synaptic currents, regulation by second messenger, and sub-synaptic distribution [13]. The type-B channel desensitizes ten times faster than the type-A [8]. Also, the postsynaptic response to the fusion of single synaptic vesicles (the quantal size) is much reduced when only the type-B receptors are present; in fact, the dose of synaptic GluRIIA versus GluRIIB is a key determinant of quantal size. The interplay between the type-A and type-B synaptic receptors modulates synapse strength and plasticity.

The synaptic accumulation of type-A and type-B receptors at the fly NMJ is differentially regulated. Two hybrid and genetics screens identified Coracle, the *Drosophila* homologue of the mammalian cytoskeletal protein 4.1, as a direct binding partner for the GluRIIA cytoplasmic domain [15]. Disruptions of the actin cytoskeleton or mutations in *coracle* caused selective reduction in GluRIIA synaptic accumulation, suggesting that Coracle anchors the GluRIIA to the actin cytoskeleton. Mutations in the p21-activated kinase (PAK) also disrupt the localization and function of the GluRIIA. PAK co-localizes with the iGluR complexes at postsynaptic densities (PSDs) and, in conjunction with the guanine nucleotide exchange factor Pix and the adaptor protein Dreadlocks, promotes the synaptic accumulation of GluRIIA [16,17]. The synaptic
accumulation of GluRIIB appears reduced in mutants of the PDZ (PSD-95/Dlg/Zona occludens-1) domain-containing scaffolding protein Discs large (Dlg) [18]. However, there is no evidence for a direct interaction between GluRIIB and Dlg, and except for GluRIIC [9], the iGluR subunits of the Drosophila NMJ lack a PDZ binding domain, suggesting that Dlg influences the synaptic abundance of GluRIIB indirectly. Also, Dlg localizes perisynaptically, at subsynaptic reticulum (SSR) [19]. In contrast, mammalian PSD-95 is a major component of the PSD, and it directly binds and modulates the synaptic targeting of iGluRs [20] and some of their interacting partners, such as the 4.1N protein [21]. The PSD-95-mediated interactions can be further regulated by post-translational modifications such as palmitoylation and phosphorylation [5].

Here we show that Neto is key in regulating the subtype composition of iGluRs at the Drosophila NMJ. We characterized a novel Neto isoform, Neto-β, which appears to be the predominant isoform at the fly NMJ. The two Drosophila isoforms, Neto-α and Neto-β, share the highly conserved extracellular and transmembrane domains, the hallmark of the Neto family of proteins [6], but have distinct cytoplasmic domains, rich in protein interaction motifs. We generated neto-β isoform specific mutants and found that the synaptic accumulation of type-A receptors is selectively impaired at neto-β mutant NMJs. Furthermore, Neto-β-deficient synapses fail to recruit PAK, a critical PSD signaling molecule required for the synaptic stabilization of type-A receptors and for the recruitment of other postsynaptic components. Muscle overexpression of either Neto-α or Neto-β isoforms could rescue the lethality and NMJ function of neto null mutants, indicating that the shared domains of Neto function to mediate iGluRs clustering. However, only Neto-β could restore the recruitment of PAK at neto null synapses. Our data demonstrate that Neto-mediated cytoplasmic interactions control the subtype composition of iGluRs and shape postsynaptic composition.

Results

Neto-β, a novel Neto isoform at the Drosophila NMJ

Drosophila neto codes for two transcripts, cDNA references GH11189 and RE42119. They share the first 10 exons, encoding the extracellular and transmembrane part of Neto, but have alternative exons that generate different intracellular domains (Fig 1A). Exon 11 encodes the cytoplasmic domain of Neto-α, a 206-residue acidic domain (pI 3.88). Exons 12–14 encode the 351-residue Neto-β intracellular domain (pI 8.90). The two domains are predicted to contain multiple phosphorylation and protein interaction motifs (Neto-β shown in S1 Fig), but they show no homology with other Neto proteins. In fact, unlike the highly conserved extracellular and transmembrane domains of Neto proteins, the intracellular parts are highly divergent or even missing, such as in the C. elegans Neto (Fig 1A) [22–24].

Previous RNA-Seq analyses indicated that neto-α and neto-β transcripts are expressed throughout development, including larval stages. Using RT-PCR and qPCR analyses we also detected both transcripts in the third instar larval carcasses and estimated that the neto-α transcript was ~4 fold less abundant than neto-β. To examine if Neto-β is present at the NMJs, we generated Neto-β isoform specific antibodies (details in Materials and Methods and S2 Fig) and compared them with previously described Neto antibodies, raised against the extracellular CUB1 domain [12]. The Neto-β positive signals co-localized with the Neto-ex puncta at control and at neto-β-rescued NMJs, but not at neto null NMJs rescued with neto-α transgenes (Fig 1B). Importantly, muscle overexpression of either isoform could rescue the embryonic lethality and paralysis of neto null mutants. This suggests that the shared part of Neto, including the CUB1, CUB2, LDLα and the transmembrane pass, contains all the components essential for iGluRs clustering and NMJ development. Indeed, muscle expression of a Neto variant in which the intracellular domains were replaced by eGFP rescued the neto null paralyzed embryos to fertile
Fig 1. Neto-β is a novel Neto isoform at the *Drosophila* NMJ. (A) Diagram of the *Drosophila* neto locus. Two isoforms are generated by alternative splicing, Neto-α and Neto-β. They have different cytoplasmic domains, but share highly conserved domains, CUB (complement C1r/C1s, Uegf, BMP1), LDLα (LDL receptor class a) and transmembrane (TM), with Neto proteins from vertebrates and *C. elegans*. (B) Confocal images of third instar larvae NMJ4 from neto<sup>null</sup> animals rescued with neto-α (*neto36;G14>neto<sup>α</sup>*), neto-β (*neto36;G14>neto<sup>β</sup>*), and a neto transgene lacking any intracellular part, neto<sup>Δ intra</sup> (*neto36;G14>neto<sup>Δ intra</sup>)*. These Neto variants can rescue the viability and NMJ development in neto<sup>null</sup> animals. The Neto-ex signals mark all NMJs, but the Neto-β
antibodies specifically label control and neto-β rescued NMJs. (C-D) Confocal images of late embryos ventral muscle fields (indicated in white) labeled for (C) Neto-ex (red), Neto-β (green), HRP (blue) indicating the presence of Neto-β in the early stages of the larval development. Genotypes: control (w¹¹¹B); neto³⁶G14>neto⁺ (neto³⁶/Y; G14-Gal4/UAS-neto-A9); neto⁻⁻G14>neto⁰ (neto⁻⁻, UAS-neto-B6/Y; G14-Gal4/+); neto⁻⁻G14>netoΔ⁺⁶⁰⁰ (neto⁻⁻/Y; G14-Gal4/ UAS-netoΔ⁺⁶⁰⁰-H4). Scale bars: (B) 20 μm (C-D) 10 μm; 5 μm in details.

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adults (Fig 1B). The caveat of these rescue experiments is that Neto overexpression highly exceeds the endogenous levels and may obscure the contribution of individual isoforms. The function of the extracellular and transmembrane domains of Drosophila Neto will be examined elsewhere. Here we will focus on characterizing the new isoform, Neto-β, and its role during NMJ development.

We have previously demonstrated an essential role for Neto in the formation of iGluR clusters during synapse assembly and development. Likewise, Neto-β started to accumulate at the NMJ synapses at the onset of synaptogenesis (Fig 1C and 1D). In late embryo fillets, Neto-β antibodies labeled distinct synaptic puncta, which were also Neto-ex positive and co-localized with the iGluR synaptic signals (GluRIIA shown in Fig 1D). Neto-β signals remained at junctional locations throughout larval development and appeared to represent a significant fraction of the total Neto at the Drosophila NMJ (S2 Fig).

### Generation of neto-β isoform specific mutants

To examine the role of Neto-β in synapse assembly and development we generated isoform specific mutants by imprecise excision of a transposable element Mi(ET1)Neto[MB07125] [25]. Several lines including precise excisions and small deletions were isolated and characterized by PCR amplification and sequencing (Fig 2A and Materials and Methods). The neto²⁰³ allele is partly missing neto-β specific exons; neto²⁰⁴ lacks the entire neto-β specific coding sequence and is likely a neto-β genetic null. No neto-β transcript was detectable in neto²⁰⁴ animals, but in neto²⁰³ larvae we found a truncated transcript, predicted to encode a short Neto-β variant (S1 Fig). For clarity we will refer to neto²⁰³ as neto⁺⁺, neto²⁰⁴ as neto⁺⁻, neto⁻⁶ as neto⁻⁻, and neto¹⁰⁹ as neto⁻⁻⁻⁻ [12]. A diagram of the neto-β alleles and predicted Neto-β proteins is shown in Fig 2A and 2B.

The predicted Neto-β variants were detected by Western analysis in larval muscle extracts (Fig 2C): a full-length Neto-β was detected in control, and a band of 65 kD, corresponding to the truncated Neto-β variant, was detected in neto⁺⁺ larval muscle. Interestingly, no band corresponding to Neto-α was detected suggesting that the endogenous Neto-α levels are very low, even in the absence of Neto-β. The Neto-β variants (full length and short) appeared expressed at equivalent levels relative to Tubulin. However, the synaptic Neto levels were decreased by 34% at neto⁺⁺ NMJs, suggesting that the cytoplasmic domain influences Neto distribution (Fig 2C and S3 Fig). The Neto levels were decreased by 70% at neto⁻⁻⁻⁻ NMJs, consistent with the estimated 4-fold difference in the transcript levels. Further purification of Neto-β polyclonal sera against the two antigenic peptides (β1 and β2) produced two pools of antibodies (Fig 2B and S1 Fig). As expected, only Neto-β2 antibody labeled the neto⁺⁺ synapses, whereas no Neto-β1 signals were found at neto⁺⁺ or neto⁻⁻⁻⁻ NMJs (Fig 2D and 2E). In contrast, clear Neto-β1 synaptic signals were detected at neto⁻⁻⁻⁻ NMJs. The neto⁻⁻⁻⁻ allele lacks the exon containing the translation initiation codon [12]. This lesion should affect both Neto isoforms.

We next examined the viability and behavior of neto-β mutants. Under optimal culturing conditions, 75% of neto⁻⁻⁻⁻ embryos and 90% of neto⁺⁺ developed into larval stages compared with control (n>200). In both cases, the third instar larvae were sluggish and exhibited a significant delay in rolling back when placed with dorsal side down. The neto⁺⁺ adult
Fig 2. Generation and characterization of neto-β isofrom specific alleles. (A) Schematics of the Minos transposomal elements and the small lesions corresponding to various neto alleles. MB07125 was mobilized to generate precise excision control and isoform specific neto-β alleles, neto\textsuperscript{short} (short cytoplasmic tail) and neto\textsuperscript{null}. The breakpoint coordinates are indicated. (B) Diagram of the predicted Neto-β proteins. The bars mark the antigens for Neto-ex, Neto-β1 and Neto-β2 antibodies. (C) Western blot analysis of lysates from S2 cells (left) and larval muscle (right). S2 cells were transfected with empty vector (lane 1), Neto-β (2), Neto-β\textsuperscript{short} (3), and Neto-α (4) expression constructs and the lysates were compared with muscle extracts from control (5).
flies have no apparent defects, but the neto\textsuperscript{null} homozygous have impaired climbing ability and are outcompeted by their heterozygous siblings. Further reduction to one copy of neto-\textalpha, such as in neto\textsuperscript{null}/neto\textnull trans allelic combinations, induced 100% lethality in more than 600 progenies examined. This lethality was rescued by a duplication covering the neto gene, Dp(1;3)DC270 [26], indicating that the molecular lesion is confined to the neto locus. In contrast, neto\textsuperscript{short}/neto\textnull were viable indicating that one copy of neto-\textalpha and neto-\textbeta\textshort could support adult viability.

neto-\textbeta mutants have impaired NMJ physiology and morphology

To test the functionality of NMJ synapses lacking an intact Neto-\textbeta isoform we recorded spontaneous miniature potentials (minis or mEJPs) and evoked excitatory junctional potentials (EJPs) from muscle 6 of third instar larvae. The mean frequency of mEJPs was decreased in both neto-\textbeta alleles compared to control (Fig 3A–3C and S1 Table). Mini amplitude or quantal size, the postsynaptic response to the fusion of a single vesicle, was also reduced in both neto-\textbeta alleles (to 62% of control in neto\textshort, and respectively 43% in neto\textnull). We found no significant change in the resting potential and input resistance in the neto-\textbeta mutant larvae.

The reduced mEJP frequency and amplitude at neto-\textbeta mutant NMJs were similar but less severe than at Neto- or iGluR-deprived NMJs [9,12]. But while Neto-deprived NMJs have severely reduced EJPs, the amplitude of EJPs was reduced by 12–14% compared to the control in neto\textshort, mutant larvae and was relatively normal at neto\textnull NMJs (Fig 3D–3F and S1 Table). This suggests that neto-\textbeta mutant NMJs must compensate for the decreased postsynaptic sensitivity by a compensatory increase in quantal content, the number of vesicles released in response to each action potential. We found that quantal content, estimated as ratio of average EJP amplitude to the mEJP amplitude, was increased 1.4 fold in neto\textshort larvae and 2.7 fold in neto\textnull compared with control (Fig 3F). In contrast, the neto\texthyper or neto\textRNAi larvae show no presynaptic compensatory response to reduced quantal size [12,27].

These studies show that the loss of Neto-\textbeta significantly alters the number, type and/or density of postsynaptic iGluRs and elicits a homeostatic compensatory increase of presynaptic release. These changes resemble the Glur\textIIIA loss-of-function phenotypes, and differ from Neto- or iGluR-deprived NMJs [7–12]. Thus, the defects observed in neto-\textbeta mutants do not seem to originate from a general decrease in Neto levels and suggest isoform specific Neto activities at the NMJ.

NMJ morphological analyses further emphasized these specific defects. We have previously shown that Neto-deprived NMJs have longer and fewer branches, with significantly reduced number of boutons [27]. In contrast, at similarly reduced Neto levels, the neto\textnull NMJs were reduced in length and had shorter branches (Fig 3G and 3H). Compared with controls, neto-\textbeta mutant NMJs had fewer but larger type Ib boutons (Fig 3I and 3J). In particular, the distal boutons had an estimated 2.4 fold volume increase (neto\textshort: \(18.9 \, \mu m^3 \pm 2.6, n = 29\), neto\textnull: \(20.8 \, \mu m^3 \pm 2.3, n = 45\), and control: \(7.8 \, \mu m^3 \pm 1.3, n = 29\)). Together these data indicate that perturbations of Neto-\textbeta have profound effects on the NMJ morphology and function that are different from a reduction in Neto levels.
Fig 3. Physiology and morphology defects at neto-β mutant NMJs. (A–F) Electrophysiological recordings of control (precise excision) and neto-β alleles of third instar animals. Representative traces of mEJPs and EJPs at 0.8 mM Ca^{2+} are shown in (A) and (D), respectively, and the results are summarized in S1 Table. The number of NMJs examined is indicated in each bar. The mEJPs amplitude (B) and frequency (C) were reduced for both neto-β alleles. The EJPs amplitude was reduced in neto^βshort compared to controls, but was normal at neto^βnull NMJs (E). Both alleles had increased quantal content, indicating a presynaptic compensatory response (F). The muscle resting potential and the input resistance were not affected. (G–H) Representative confocal images at Neto-Mediated Interactions Shape Postsynaptic Composition

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Loss of Neto-β affects PSD composition

At the Drosophila NMJ, Neto activities are essential for trafficking and clustering of iGluRs at synaptic sites. To assess the contribution of Neto-β to these functions we examined the iGluRs distribution at neto-β mutant NMJs. For all the quantitative analyses below control/neto-β mutant sets (5–10 larvae/genotype) were processed together and imaged under the same conditions to capture and compare the synaptic signals. At the fly NMJ synapses, the sites of neurotransmitter release are marked by presynaptic specializations called T-bars, where Bruchpilot (Brp), the fly homolog of the vertebrate active zone protein ELKS, accumulates [28]. Opposite to the T-bars, the iGluRs are concentrated and stabilized at synaptic sites by a myriad of PSD-associated proteins. We found that neto-β mutant boutons contained more synaptic contacts, as visualized by Brp/GluRIIC juxtaposing puncta, and their density was increased at proximal boutons but not at the distal ones (Fig 4A and 4B). This increase in synaptic density is consistent with the compensatory response observed at neto-β mutant NMJs (Fig 3F). The intensity of Brp signals was largely constant, but the GluRIIC levels were diminished to 78% from control at netoβshort NMJs (n = 10) to 39% at netoβnull NMJs (n = 10) (Fig 4A and 4C). The reduction of GluRIIC synaptic signals at neto-β mutant NMJs occurred without a change in the GluRIIC net protein indicating a defect in the synaptic localization of the receptors (Fig 4D). Importantly, the GluRIIC synaptic signals paralleled the levels of synaptic Neto observed at these NMJs (Fig 2D and 2E, S3A Fig), as Neto is limiting for receptors localization.

Drosophila NMJ utilizes two types of iGluRs, type-A and type-B, that differ in their subunits composition and properties [13]. The single channel current amplitude of both receptor types is identical, but the type-B channel desensitizes nearly ten times faster, thus the relative ratio of synaptic type-A/type-B receptors is a key determinant of quantal size [8]. Also, reduced levels of postsynaptic type-A receptors trigger a robust presynaptic compensatory response [7]. The physiological similarities between the GluRIIA and neto-β mutant NMJs suggest that Neto-β may primarily influence the synaptic accumulation of type-A receptors. We tested this possibility by examining the relative intensity of GluRIIA and GluRIIB signals at neto-β mutant NMJs. The netoβshort larvae had severely reduced GluRIIC synaptic levels (to 26% from control, n = 13), accompanied by no significant change in the GluRIIB levels (Fig 4C and 4E). The netoβnull NMJs showed an even stronger reduction in the GluRIIA synaptic levels (to 18.5% from control, n = 14) and also decreased GluRIIB levels (Fig 4C and 4E). In contrast, the netoβnull NMJs showed uniformly reduced levels for all iGluR subunits examined (S3 Fig). The drastic decrease of GluRIIA synaptic signals at neto-β mutant NMJs exceeded the reduction of GluRIIC and Neto synaptic levels observed at these NMJs, indicating a key role for the Neto-β isoform in regulating the GluRIIA synaptic abundance.

How does Neto-β regulate the selective accumulation of type-A receptors at synaptic sites? Neto-β may preferentially recruit the type-A receptors and/or influence their synaptic stabilization. If Neto isoforms promote the synaptic accumulation of selective iGluRs, and Neto-β influences the type-A receptors, the inference is that Neto-α should preferentially impact the distribution of type-B receptors. In support for this possibility, in RNAi knockdown experiments we found that Neto-α-deprived NMJs showed a dramatic loss of GluRIIB synaptic signals (by 68%) and a modest, variable increase of synaptic GluRIIA (by 18%) (Fig 4F and 4G). The efficiency of the RNAi-mediated knockdown was verified by Western blot analysis of muscle extracts from netoβnull larvae rescued with a V5-tagged neto-α transgene (Fig 4H and [12]).
iGluRs synaptic accumulation is perturbed at neto-β mutant NMJs. (A) Confocal images of NMJ4 boutons in larvae of indicated genotypes labeled for Brp (red), GluRIIC (green), and HRP (blue) (quantified in B-C). neto-β mutant NMJs have increased number of synaptic contacts. The intensity of the presynaptic active zone marker Brp appears to be normal, but the GluRIIC synaptic signals are reduced at neto-β mutant NMJs compared with control (precise excision). (D) Western blot comparison of GluRIIC protein levels in muscle lysates from neto-β third instar larvae. Tubulin was used as a loading control. (E) Confocal images of NMJ4 boutons in larvae of indicated genotypes labeled for GluRIIA (red), GluRIIB (green), and HRP (blue). The synaptic
Lack of Neto-α-specific reagents precluded us from expanding these experiments. Nonetheless, these results raise the possibility that Neto isoforms utilize their distinct cytoplasmic domains to modulate the synaptic abundance of specific receptor subtypes. In the case of Neto-β, the last 260 C-terminal cytoplasmic residues appear to be critical for the synaptic accumulation of type-A receptors. Neto-β recruits postsynaptic components and organizes postsynaptic structures

In *Drosophila*, several postsynaptic and perisynaptic proteins influencing the synaptic abundance of type-A and type-B receptors have been reported [15,16,18]. For example, PAK, a PSD component that co-localizes with the GluRIIA subunit, was shown to promote accumulation of type-A receptors at synaptic sites [16,17,29]. PAK also influences the size of the SSR, a stack of membrane folds that underlines the postsynaptic cell membrane of the type Ib boutons [16,17].

We found that PAK signals were severely reduced at neto-β mutant NMJs (Fig 5A). PAK synaptic levels dropped to 4% from control in neto<sup>short</sup> larvae (n = 11) and to 19% in neto<sup>null</sup> (n = 12). This drastic decrease exceeded the reduction in PAK signals observed at neto<sup>bypo</sup> NMJs, suggesting that the low level of Neto-β at neto<sup>bypo</sup> NMJs could partly mediate PAK stabilization at PSDs (Fig 2E and [12]). A similar, albeit less pronounced effect was observed for Dlg (Fig 5B–5D). The Dlg junctional signals were reduced to 48% from control in neto<sup>short</sup> third instar larvae (n = 14) and to 27% from control in neto<sup>null</sup> (n = 11). Importantly, the levels of PAK and Dlg net proteins were not changed in extracts from neto-β larval muscles (Fig 5E). Also, the accumulation of PAK and Dlg at junctional locations was completely restored in neto-β mutants by a duplication covering the neto locus (S4 Fig), indicating that the observed phenotypes are neto specific. In contrast, cysteine string protein (CSP), which labels clusters of synaptic vesicles in the vicinity of presynaptic membranes [30], and α-Spectrin, which labels the presynaptic axon and surrounds the bouton postsynaptically [31] appeared normal at neto-β mutant NMJs (S5 Fig).

Under electron microscopy, control synapses are marked by presynaptic specializations called T-bars and electron-dense membranes, corresponding to PSDs (Fig 6A–6C). At neto-β mutant boutons, serial sections revealed that the PSDs were significantly reduced in size but increased in number (Fig 6B–6D and Fig 4). The diminished PSD length (up to 2 fold in neto<sup>null</sup> mutants) matched the observed reduction of postsynaptic receptor fields (Fig 4). A thick stack of SSR membranes surrounds the control type Ib boutons. In contrast, the enlarged neto-β mutant boutons had diminished or even absent SSR structures, consistent with the reduced Dlg levels observed (Fig 6E–6H). In addition, neto-β mutants appeared to have misshaped T-bar structures, including double T-bar structures (Fig 6B and 6C, details). GluRIIA deprivation induces excessive recruitment of Brp at the active zones which sometimes results in active zone profiles with two or more T-bars [32]. Likewise, the excessive T-bar structures observed primarily in neto<sup>null</sup> mutant boutons may correlate to a homeostatic compensatory response triggered by the GluRIIA depletion (Figs 3F and 4B and 4E).
Neto-β, but not Neto-α, is required for PAK accumulation at PSDs

Since the SSR folds are reduced in neto-β mutants, it is possible that the reduced PAK and Dlg synaptic levels are indirectly caused by the lack of proper SSR structures. To test this possibility,
Fig 6. Ultrastructure defects at neto-β mutant boutons. (A–C) Electron micrographs of type Ib boutons in third instar larvae of indicated genotypes. The upper panels show entire boutons; the active zones (AZ, arrows), mitochondria (m), and subsynaptic reticulum (SSR, brackets) are indicated. The neto-β mutant boutons have numerous synaptic contacts, but their active zones often have abnormal T-bar structures (B′–B″, C′–C″), including closely spaced, distorted, fused, and floating T-bars. The PSDs length, quantified in serial section (D), is significantly reduced at neto-β mutant boutons. The boutons appear enlarged and the SSR area and thickness diminished in both neto-β mutants (quantified in E–H). Error bars indicate SEM. ***; p<0.001, **; p<0.005. Scale bars: 1 μm, 200 nm in details.

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we examined late embryos and first instar larvae, before the SSR develops [33]. If the SSR controls the synaptic accumulation of PAK, then PAK levels should be normal at neto-β mutant NMJs during the early stages of development. However, we found that PAK accumulated normally at the muscle attachment sites in control and neto-β mutant animals, but was severely diminished at neto-β mutant NMJs (Fig 7A). Thus, loss of synaptic PAK is not secondary to the loss of SSR and appears to be directly caused by impairments in Neto-β.

If Neto-β recruits PAK at the cell membrane, then loss of synaptic PAK in neto-β mutants should be rescued by muscle expression of PAKmyr, a membrane-tethered PAK variant [34]. We found that muscle overexpression of PAKmyr in neto-β mutants induced accumulation of PAK signals at perisynaptic but not synaptic sites, and could not rescue the GluRIIA synaptic accumulation (netoβshort shown in Fig 7B). This indicates that Neto-β controls the recruitment of PAK at synaptic locations. Loss of PAK did not affect the synaptic recruitment of Neto-β: while PAK signals were lost at pak null NMJs (pak11/Df) or in mutants that fail to localize at cell peripheries (pak6/Df or pak6/11) [35], the synaptic Neto signals remained unaffected, irrespective of the pak genetic manipulations (Fig 7C). Also, the levels of GluRIIA were 4-fold reduced in neto-β mutants (Fig 4), but only 2-fold in pak mutants [16], suggesting that Neto-β has additional, PAK-independent functions in the synaptic recruitment/stabilization of GluRIIA. Together these data indicate that Neto-β is required for the synaptic accumulation of PAK. In flies and mammals, PAK membrane localization is controlled by Pix, a Rho-type guanine nucleotide exchange factor [16,36]. Mutations in dpix led to impairments in synaptic accumulation of PAK, GluRIIA, Dlg, and other postsynaptic components. Lack of Pix antibodies precluded us to test whether Neto-β recruits dpix at PSDs. Nonetheless, while the PAK synaptic signals were completely lost in dpix mutants, Neto signals accumulated at these mutant synapses albeit they appeared somewhat reduced, likely due to defects in the synapse organization (S6 Fig). Thus, dPix is required for PAK but not for Neto recruitment at synaptic sites.

We have previously demonstrated that the net levels of Neto are critical for synapse assembly and NMJ development [12,14]. In particular, Neto depletion has profound consequences for synapse assembly and function. The levels of synaptic Neto are reduced in neto-β mutants compared with controls (Fig 2 and S3 Fig) raising the possibility that the phenotypes observed in neto-β allelic series could be partly due to reduced Neto levels. To distinguish between isoform specific and suboptimal Neto phenotypes we manipulated the muscle expression of Neto-α and Neto-β isoforms and examined their effects on the development and function of NMJ. Similar to neto-α, we found that muscle expression of neto-β transgenes rescued the viability and NMJ development of neto-null mutants and induced gain-of-function phenotypes in a concentration-dependent manner (Fig 8A and [27]). Low to moderate levels of Neto-β induced formation of NMJs with relatively normal morphology and clearly defined synaptic Neto clusters, resembling the neto mutants rescued with low and moderate levels of Neto-α. Excess Neto-β appeared detrimental to the NMJ development and to the overall growth and viability of rescued animals (Fig 8A).

Intriguingly, Neto-β but not Neto-α enabled the stabilization of PAK at PSDs, over a wide range of concentrations tested (Fig 8A). Furthermore, increasing the levels of Neto-α in neto-β mutants could not restore the PAK synaptic accumulation, while introducing Neto-β effectively rescued this defect. This result cannot be explained by a difference between Neto-α and Neto-β cellular distribution, since both isoforms appear to concentrate at the NMJ. We confirmed that Neto-α and Neto-β synaptic levels correlate with the levels of Neto protein in the larval muscle, as detected by Western analysis (Fig 8B). Thus, both Neto isoforms can traffic and accumulate at synaptic locations where they mediate synapse assembly. However, only Neto-β isoform can enable PAK accumulation at PSDs.
Fig 7. PAK synaptic recruitment is impaired at neto-β mutant NMJs. (A) Representative confocal images of ventral muscle fields in late embryos (21 hours after egg laying) and early first instar larvae (2 hours after hatching) of indicated genotypes labeled for Brp (red), PAK (green), and HRP (blue). The synaptic PAK signals are weak at neto-β mutant NMJs, but normal at the muscle attachment sites. (B) Confocal images of NMJ4 in third instar larvae of indicated genotypes labeled for PAK (red), GluRIIA (green) and HRP (blue). Muscle expression of a constitutively membrane bound form of PAK (G14> pakmyr) does not rescue the GluRIIA synaptic abundance at netoβshort NMJs compared to control (G14+/+). PAK signals remain diffuse and localize.
Since PAK contributes to synaptic stabilization of GluRIIA, Neto-α-rescued NMJs are expected to exhibit a reduction in the synaptic GluRIIA signals and thus a reduction in quantal size. Indeed, we found that netonull mutants rescued with low/moderate levels of Neto-α had significantly reduced mini amplitudes and reduced GluRIIA/GluRIIB ratio (Fig 8C and 8D, S7 Fig). In contrast, netonull NMJs rescued with neto-β transgenes had relatively normal mini amplitudes, indicating normal GluRIIA/GluRIIB ratio at these synapses. The mini frequency was largely normal, except for the neto-β transgenes which showed increased mini frequency when reared at 18°C (Fig 8E). Interestingly, the evoked potentials were in the normal range in all Neto-α and Neto-β-rescued larvae tested, likely due to compensatory presynaptic response (Fig 8F–8H). These results indicate that Neto-α-rescued NMJs have deficits in the synaptic accumulation of type-A receptors. Such defects are partly obscured by excess Neto-α, likely because the conserved domains of Neto confer high iGluRs “clustering capacity” in these rescue experiments. But under normal condition, Neto is a low abundance protein and a limiting factor for iGluRs clustering in the muscle. Furthermore, Neto-β appears to be the predominant isoform at the Drosophila NMJ. Together our findings demonstrate that Neto-β, the major Neto isoform at the Drosophila NMJ, controls the subtype composition of iGluRs partly by regulating the recruitment of the PSD-associated kinase PAK.

Discussion

At the Drosophila NMJ, Neto enables iGluRs clustering at synaptic sites and promotes postsynaptic differentiation. Here we show that Neto-β, the major Neto isoform at the fly NMJ, plays a crucial role in controlling the distribution of specific iGluR subtypes at individual synapses. Similar to other glutamatergic synapses, the subunit composition determines the activity and plasticity of the fly NMJ. Our data are consistent with a model whereby Neto-β, via its conserved domains, fulfills a significant part of Neto-dependent iGluRs clustering activities during synapse assembly. At the same time, Neto-β engages in intracellular interactions that regulate iGluR subtypes distribution by preferentially recruiting and/or stabilizing type-A receptors. In this model, Neto-β could directly associate with the GluRIIA-containing complexes and/or regulate the synaptic abundance of type-A receptors indirectly, by recruiting PSD components such as PAK. Thus, Neto-β employs multiple strategies to control which flavor of iGluR will be at the synapses and to modulate PSD composition and postsynaptic organization.

Neto-mediated intracellular interactions shape postsynaptic composition

Neto proteins have been initially characterized as auxiliary subunits that modulate the function of kainate (KA) and NMDA receptors [22,23]. In vertebrates, Neto1 and Neto2 directly interact with KAR subunits and increase channel function by modulating gating properties [23,37,38]. Since loss of KAR currents in mice lacking Neto1 and/or Neto2 exceed a reduction that could be attributed to alterations of channel gating, an additional role for Neto proteins in synaptic targeting of receptors has been proposed. The role for vertebrate Neto proteins in KAR membrane and/or synaptic targeting remains controversial and appears to be cell type-, receptor subunit-, and Neto isoform-dependent [23,39,40]. Furthermore, the C. elegans Neto has a very small intracellular domain (24 amino acids beyond the conserved domains) [24]. This implies that 1) Neto without an intracellular domain constitutes the minimal conserved functional moiety, and 2) the divergent intracellular domains of Neto proteins may fulfill tissue and/or
Fig 8. Neto-β, but not Neto-α, restores PAK recruitment and mEJP amplitude at neto<sup>Δnull</sup> NMJs. (A) Confocal images of larval NMJ4 labeled for Neto-ex (red), PAK (green) and HRP (blue). Neto-β, but not Neto-α, restores PAK synaptic accumulation over a large range of concentrations tested. (B) Western blot comparison of Neto levels in muscle extracts from control (first lane), and neto<sup>Δnull</sup> larvae rescued with neto-α transgenes (low, medium, and high expression) (magenta gradient), or neto-β transgenes (low, medium, high, and very high expression)(blue gradient). Arrows indicate unprocessed and processed Neto-α (magenta) and Neto-β (blue). (B’)—low exposure. Tubulin was used as a loading control. (C-H) Electrophysiological recordings of neto<sup>Δnull</sup> NMJs rescued by Neto-Mediated Interactions Shape Postsynaptic Composition
Neto-Mediated Interactions Shape Postsynaptic Composition

various levels of Neto-α or Neto-β. Data are reported relative to controls matched by rearing at 18°C (empty bars) or 25°C (hatched bars). Representative traces of mEJPs and EJPs are shown in (C) and (F), respectively. The mEJPs amplitude is largely normal (G), likely due to subtle, but significant increases in quantal content at Neto-α (D). The mEJPs frequency appears less dependent on Neto levels/isoforms, but is significantly increased in larvae reared at 18°C (E). The EJPs amplitudes traces of mEJPs and EJPs are shown in (C) and (F), respectively. The mEJPs amplitude is reduced at NMJs rescued by low and medium levels of Neto-α (E) and (I), and (J). doi:10.1371/journal.pgen.1005191.g008

In flies, Neto is an essential protein that plays active roles in synapse assembly and in the formation and maintenance of postsynaptic structures at the NMJ. The Drosophila Neto isoforms do not have PDZ binding motifs, but they use at least two different mechanisms to regulate the synaptic accumulation and subunit composition of iGluRs. First, Neto participates in extracellular interactions that enable formation of iGluR/Neto complexes; formation of stable aggregates is presumably prevented by the inhibitory prodomain of Neto [27]. Second, the two Neto isoforms appear to facilitate the selective recruitment and/or stabilization of specific iGluR subtypes. We speculate that Neto-β may selectively associate with and recruit type-A receptors, perhaps by engaging the C-terminal domain of GluRIIA, which is critical for the synaptic stabilization of these receptors [42,43]. Aside from a possible role in the selective recruitment of iGluR subtypes, Neto-β participates in intracellular interactions that facilitate the recruitment of PAK at PSDs; in turn, PAK signals through two independent, genetically separable pathways (a) to modulate the GluRIIA synaptic abundance and (b) to facilitate formation of SSR [17].

Whether Neto-β recruits PAK directly or via a larger protein complex remains to be determined. Neto-β contains an SH3 domain that may bind to the proline-rich SH3 binding domain of PAK. However, in tissue culture experiments, we failed to detect a direct interaction between PAK and Neto-β (full-length or intracellular domain). PAK synaptic accumulation is completely abolished at NMJ with mutations in dPix, although not all dpix defects are mediated through PAK [16]. Conversely, PAK together with Dreadlocks (Dock) controls the synaptic abundance of GluRIIA, while PAK and dPix regulate the Dlg distribution [17]. The reduction of GluRIIA and Dlg synaptic abundance observed at neto-β mutant NMJs suggests that Neto-β may interact with both dPix and Dock and enable both PAK activities. In addition, Neto-β may stabilize postsynaptic type-A receptors by enhancing their binding to Coracle, which anchors GluRIIA to the postsynaptic actin cytoskeleton [15].

Importantly, this study connects the complex regulatory networks that modulate the PSD composition to the Neto/iGluR clusters themselves. The Neto-β cytoplasmic domain is rich in putative protein interaction motifs (S1 Fig), and may function as a scaffold platform to mediate multiple protein interactions that act synergistically during synapse development and homeostasis. Loss of Neto-β-mediated intracellular interactions at neto-βshort NMJs reduced the GluRIIA synaptic abundance, but did not affect the GluRIIB synaptic signals (Fig 4E). It is unlikely that the remaining cytoplasmic part of Neto-β facilitates the GluRIIB synaptic accumulation at these NMJs at the expense of GluRIIA and PAK. Instead, we favor a model whereby the synaptic stabilization of GluRIIA requires a Neto-β-dependent intracellular network. Disruption of this network diminishes GluRIIA and increases GluRIIB synaptic abundance, pending the

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availability of limiting subunits, GluRIIC-E and Neto. Conversely, the presence of this network ensures that at least some type-A receptors are stabilized at synaptic sites, even at Neto-deprived synapses, such as in neto hypomorphic larvae [12]. Assembly of this network does not require GluRIIA since both Neto-β and PAK accumulated normally at GluRIIA mutant NMJs (Fig 8I). Furthermore, in the absence of Neto-β the synaptic abundance of GluRIIA can be partly restored by excess Neto-α or a delta-intracellular Neto variant, suggesting that excess iGluRs “clustering capacity” overrides the cellular signals that shape PSD composition [27]. What intracellular domain(s) of Neto bind to and how they are modified by post-translational modifications will be critical questions to understand how postsynaptic composition is regulated during development and homeostasis.

Expanding the repertoire of Neto functions at glutamatergic synapses

The discovery of Drosophila Neto isoforms with alternative cytoplasmic domains and isoform specific activities expands the repertoire of Neto-mediated functions at glutamatergic synapses. On one hand, all Neto proteins share the highly conserved CUB1, -2, LDLα and transmembrane domains that have been implicated in engaging and modulating the receptors, the central function of Neto proteins [22,23,44]. In flies this conserved part is both required and sufficient for iGluRs clustering and NMJ development. In C. elegans the entire Neto appears to be reduced to this minimal functional unit [24]. The only exception is a retina-specific CUB1-only Neto1 isoform with unknown function [45]. In contrast, the cytoplasmic domains are highly divergent among Neto proteins. This diversity might have evolved to facilitate intracellular, context specific function for Neto proteins, such as the need to couple the iGluR complexes to neuron or muscle specific scaffolds in various phyla. Alternatively, by engaging in different intracellular interactions, via distinct cytoplasmic domains, different Neto isoforms may undergo differential targeting and/or retention at the synapses and thus acquire isoform-specific distributions and functions within the same cell.

Phylogenetic analyses indicate that the intracellular domains of Neto are rapidly evolving in insects. Blocks of high conservations could be clearly found in the genome of short band insect Tribolium castaneum (Coleoptera) or in Apis mellifera (Hymenoptera). However, most insects outside Diptera appear to have only one Neto isoform, more related to Neto-β. In fact, the only Neto-α isoform outside Drosophila was found in Musca domestica (unplaced genomic scaffold NCBI Reference Sequence: XM_005187241.1). Other neto loci, from Hydra to vertebrates, appear to encode Neto proteins with unique and highly divergent intracellular domains. An extreme example is the C. elegans Neto/Sol-2, with a very short cytoplasmic tail, which requires additional auxiliary subunits, Sol-1 and Stargazin, to control the function of glutamate receptors [46,47]. Neto proteins appear to utilize their intracellular domains to connect to the signaling networks that regulate the distribution and subunit composition for iGluRs. Such cellular signals converge onto and are integrated by the intracellular domains of the receptors and/or by various auxiliary subunits associated with the iGluR complexes [1,6].

Neto proteins modulate the gating behavior of KAR but also play crucial roles in the synaptic recruitment of glutamate receptors in vivo [12,40,48]. At the fly NMJ, Neto enables iGluRs synaptic clustering and initiates synapse assembly. In addition, the intracellular domain of Neto-β recruits PSD components and triggers a cascade of events that organize postsynaptic structures and shape the composition of postsynaptic fields. The cytoplasmic domains of Neto proteins emerge as versatile signaling hubs and organizing platforms that directly control the iGluRs subunit composition and augment the previously known Neto functions in modulation of glutamatergic synapses.
Materials and Methods

Fly stocks

To generate neto-β alleles, the Minos transposomal element Mi(ET1)Neto[MB07125] was mobilized with Minos transposase [25]. Several lines with precise excisions and imprecise excisions/small deletions were isolated and characterized by PCR amplification over the deficiencies and DNA sequencing. The genomic fragments removed in various neto alleles were as follows: neto\textsuperscript{203} X: 13,415,115–13,418,117, including part of the exon 13 and 14 of the predicted neto gene, and neto\textsuperscript{204} X: 13,414,477–13,417,931, containing exons 12, 13 and 14. The UAS-neto-β lines (B lines) were generated by insertion of the neto-β cDNA (from RE42119) in pUAST vector and germline transformation (BestGene, Inc.). Similarly, for the UAS-neto\textsuperscript{Δ}\textit{intra} (the H4 line) the Neto coding sequence M\textsuperscript{1}.R\textsuperscript{271} followed by a short linker (DVPALE) was placed in frame with the eGFP and cloned in pUAST. The neto-α\textsuperscript{RNAi} lines were generated by insertion of a neto-α specific PCR fragment in pUAST-R57 followed by germline transformation. The PCR primers utilized were: RNAi-F (5\textsuperscript{′}′-AAGGCCTACATGGCCGGACCGGCGAACAAATGGAGGAAGACG-3\textsuperscript{′}′) and RNAi-Rev (5\textsuperscript{′}′-AATCTAGAGGTACCTGATTTTGTGCAGGAACTTGAGG-3\textsuperscript{′}′).

Other fly stocks used in this study were as follows: neto\textsuperscript{36}, neto\textsuperscript{109}, and UAS-neto-α -V5 [12]; UAS-neto-α (A lines) [27]; neto(CUB1)\textsuperscript{R\textsuperscript{271}} [14]; Dp(1\textsuperscript{st}:3)DC270 [26]; pak\textsuperscript{6}, pak\textsuperscript{11}, and pak\textsuperscript{6\textsuperscript{yr}} [34]; dpx\textsuperscript{1} [16]; GluRII\textsuperscript{A\textsuperscript{3\textsuperscript{P\textsuperscript{16}}} and Df(2L)c\textsuperscript{P\textsuperscript{14}} [7] (from A. Di Antonio, Washington University). The G14-Gal4 was obtained from C. Goodman (University of California at Berkeley).

Antibody generation and protein analysis

The rabbit polyclonal anti-Neto-β antibodies were generated against two synthetic peptides: β1 (GRSHYGGLLVTSTAKQP) and β2 (LDDVSNRYYREAVPL) (21st Century Biochemicals) and separated by affinity purification. The rabbit polyclonal anti-GluRIIB and anti-GluRIIC were generated as previously described [8] against synthetic peptides ASSAKKKKTRRIEK, and respectively QGSGSNGTHGWAGRGEKEARV (Pacific Immunology Corp).

To analyze muscle proteins, wandering third instar larvae were dissected, and all tissues except for the body wall (muscle and cuticle) were removed. The body walls were mechanically disrupted and lysed in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, protease inhibitor cocktail (Roche) for 30 min on ice. The lysates were separated by SDS-PAGE on 4%–12% NuPAGE gels (Invitrogen) and transferred onto PVDF membranes (Millipore). Drosophila S2 cells were used for the production of recombinant proteins [12]. Full-length cDNA for neto-β from RE42119 was subcloned in pAcPA-based plasmids for expression in S2 cells under the actin promoter. The Neto-β truncation (neto\textsuperscript{203}-like) was generated by looping out the deleted fragment using the QuickChange site-directed mutagenesis kit. All constructs were verified by DNA sequencing.

Primary antibodies were used at the following dilutions: rat anti-Neto-ex [12], 1:1000; anti-Tubulin (Sigma-Aldrich),1:1000; rabbit anti-Neto-β, 1:1000; rabbit anti-GluRIIC, 1:1000; rabbit anti-PAK, 1:5000 (a gift from Nicholas Harden) [35]; mouse anti-Dlg (4F3), 1:1000; mouse anti-V5 (Invitrogen), 1:1000. Immune complexes were visualized using secondary antibodies coupled with IR-Dye 700 or IR-Dye 800 followed by scanning with the Odyssey infrared imaging system (LI-COR Biosciences).

Immunohistology

Wandering third instar larvae were dissected as described previously in ice-cooled Ca\textsuperscript{2+}-free HL-3 solution [49,50]. Embryos at 18h after egg laying (AEL) were dechorionated and
genotyped and, after an additional incubation of 2 h at room temperature, were dissected as described previously [12]. First instar larvae were similarly dissected within 2 h from hatching. The samples were fixed in 4% paraformaldehyde (Polysciences, Inc.) for 20 min or in Bouin’s fixative (Bio-Rad) for 3 min and washed in PBS containing 0.5% Triton X-100.

Primary antibodies from Developmental Studies Hybridoma Bank were used at the following dilutions: mouse anti-GluRIIA (8B4D2), 1:100; mouse anti-Dlg (4F3), 1:1000; mouse anti-Brp (Nc82), 1:200; mouse anti-CSP (6D6), 1:1000; mouse anti-α-Spectrin (3A9), 1:50; mouse anti-FasII (1D4), 1:10. Other primary antibodies were utilized as follow: rabbit anti-PAK, 1:2000; rat anti-Neto-ex, 1:1000 [12]; and Cy5-conjugated goat anti-HRP, 1:1000 (Jackson ImmunoResearch Laboratories, Inc.). Alexa Fluor 488-, Alexa Fluor 568-, and Alexa Fluor 647-conjugated secondary antibodies (Molecular Probes) were used at 1:200. All samples were mounted in ProLong Gold (Invitrogen).

Samples of different genotypes were processed simultaneously and imaged under identical confocal settings in the same imaging session with a laser scanning confocal microscope (Carl-Zeiss LSM780). All images were collected as 0.2m optical sections and the z-stacks were analyzed with Imaris software (Bitplane). To detect positive puncta we used the spot finding Imaris algorithm followed by manual inspection and correction. To quantify fluorescence intensities synaptic ROI areas surrounding anti-HRP immunoreactivities were selected and the signals measured individually at NMJs (muscle 4, segment A3) from ten or more different larvae for each genotype (number of samples is indicated in the graph bar). The signal intensities were calculated relative to HRP volume and subsequently normalized to control. Boutons were counted in preparations double labeled with anti-HRP and anti-Dlg. All quantifications were performed while blinded to genotype. The numbers of samples analyzed are indicated inside the bars. Statistical analyses were performed using the Student t-test with a two-tailed distribution and a two-sample unequal variance. Error bars in all graphs indicate standard deviation ±SEM. ***; p<0.001, **; p<0.005, *; p<0.05, ns; p>0.05.

Electrophysiology

The standard larval body wall muscle preparation first developed by Jan and Jan (1976) [51] was used for electrophysiological recordings [52]. Wandering third instar larvae were dissected in physiological saline HL-3 saline [49], washed, and immersed in HL-3 containing 0.8 mM Ca2+ or 0.3 mM Ca2+ using a custom microscope stage system [53]. The nerve roots were cut near the exiting site of the ventral nerve cord so that the motor nerve could be picked up by a suction electrode. Intracellular recordings were made from muscle 6. Data were used when the input resistance of the muscle was >5 MΩ and the resting membrane potential was between -60 mV and -80 mV. The input resistance of the recording microelectrode (backfilled with 3 M KCl) ranged from 20 to 25 MΩ. Muscle synaptic potentials were recorded using an Axon Clamp 2B amplifier (Axon Instruments) and pClamp 10 software. Following motor nerve stimulation with a suction electrode (100 μsec, 5 V), evoked EJPs were recorded. Four to six EJPs evoked by low frequency of stimulation (0.1 Hz) were averaged. For mini recordings, TTX (1 μM) was added to prevent evoked release [49]. To calculate mEJP mean amplitudes, 50–200 events from each muscle were measured and averaged using the Mini Analysis program (Synaptosoft). Minis with a slow rise and falling time arising from neighboring electrically coupled muscle cells were excluded from analysis [54,55]. Quantal content was calculated by dividing the mean EJP by the mean mEJP after correction of EJP amplitude for nonlinear summation according to previously described methods [56,57]. Corrected EJP amplitude = E[Ln E/(E—recorded EJP)], where E is the difference between reversal potential and resting potential. The reversal potential used in this correction was 0 mV [57,58]. Data are presented as
Electron microscopy

Wandering third instar larvae were dissected in physiological saline HL-3 saline and fixed for 30 min on dissection plate in fixation buffer (0.1 M Sodium Cacodylate buffer, pH7.2; 2 mM MgCl₂; 1% glutaraldehyde; 4% paraformaldehyde). The samples were trimmed to include only the abdominal segments A2 and A3, transferred in a 1.5mL Eppendorf tube, fixed overnight at 4°C, then washed extensively with 0.1 M Sodium Cacodylate buffer with 132 mM Sucrose, pH 7.2. The samples were further processed and analyzed according to published protocols [59] at the Microscopy and Imaging Facility, NICHD.

Supporting Information

S1 Fig. Sequence alignment for Neto-β cytoplasmic domain. ClustalW alignment of Neto-β cytoplasmic domains in several Drosophila species (Drosophila melanogaster, Drosophila ananassae and Drosophila pseudoobscura) reveals blocks of highly conserved sequences, including SH2 and SH3 binding domains, poly-H and poly-Q motifs, and putative phosphorylation sites (putative CaMKII phosphorylation site is indicated in red). The Neto-β truncated form (netoβshort allele) is predicted to retain the first 88 conserved intracellular residues followed by 8 additional, unrelated residues (shown in orange). The synthetic peptides used to generate the Neto-β isoform specific antibodies are marked by blue boxes.

(TIF)

S2 Fig. Characterization of Neto-β isoform specific antibodies. (A) Western blot of lysates from S2 cells transfected with control (mock), Neto-α or Neto-β expression constructs. The Neto-ex rat polyclonal antibodies label both Neto isoforms, while the Neto-β rabbit polyclonal antibodies can only recognize the Neto-β recombinant protein. Arrowheads point to unprocessed and processed Neto-α (white) and Neto-β (black). The apparent molecular weights are higher than predicted likely due to post-translational modifications: ~100/85 kD observed for unprocessed/processed Neto-α variants (75/62 kD calculated) and ~115/100 kD for Neto-β (92/77 kD calculated). (B) Representative confocal images of NMJ4 boutons in third instar larvae labeled for Neto-β (green), HRP (blue) and Neto-ex (red) (left panels) or GluRIIA (red) (right panels). Neto-β positive puncta co-localize with Neto-ex and GluRIIA signals at synaptic sites. Scale bars: 20 μm, 2 μm in details.

(TIF)

S3 Fig. The iGluRs synaptic signals at various neto mutant NMJs. (A) Representative confocal images of NMJ4 boutons in third instar larvae of indicated genotypes labeled for Neto-ex (red), GluRIIC (green) and HRP (blue). neto-β mutant NMJs have progressively reduced levels of Neto-ex positive synaptic signals (quantified relative to HRP in B). The levels of synaptic Neto closely match the GluRIIC synaptic signals. (C-D) Representative confocal images of NMJ4 boutons in third instar larvae of control (precise excision for neto-β allelic series), w1118 and netohypo labeled for HRP (blue), and Brp (red), GluRIIC (green) (C) or GluRIIA (red), GluRIIB (green) (D). The iGluRs signals are barely detectable at netohypo NMJs when imaged side-by-side with the precise excision and with w1118, the closest control for the neto hypomorphs. (E) Table summarizing the quantifications from the experiments presented above and in Fig 4. Error bars indicate SEM. ***; p<0.001. Scale bars: 20 μm.

(TIF)
S4 Fig. Recruitment of postsynaptic components at neto-β mutant NMJs is rescued by a duplication covering the neto gene. Representative confocal images of NMJ4 boutons (segment A3) in third instar larvae of indicated genotypes labeled for PAK (red), Dlg (green) and Neto-ex (blue). The synaptic accumulation of PAK and Dlg is restored at neto-β mutant NMJs by a duplication covering the neto locus. Genotypes: control (precise excision), netoβshort, netoβshort;Dp(netoβshort/Y;Dp(1;3)DC270/+), netoβnull, and netoβnull;Dp(netoβnull/Y;Dp(1;3)DC270/+).

Scale bars: 20μm, 2μm in details.

(TIF)

S5 Fig. CSP and -Spectrin have normal distribution at neto-β mutant NMJs. (A–B) Confocal images of NMJ4 boutons (segment A3) in third instar larvae of indicated genotypes labeled for HRP (blue), and Cystein string protein (CSP) (green)(A) or -Spectrin (green) (B). CSP and -Spectrin localize normally at neto-β mutant NMJs. Scale bars: 20μm, 2μm in details.

(TIF)

S6 Fig. The synaptic localization of Neto does not require dPix. Confocal images of NMJ4 boutons (segment A3) in third instar larvae labeled for Neto-ex (red), PAK (green), and HRP (blue). The Neto-positive synaptic signals but not PAK signals are present at dpix mutant NMJs. The Neto-ex staining is less uniform than in control (w1118) presumably because of generally altered NMJ morphology in dpix mutant larvae. Scale bars: 2μm.

(TIF)

S7 Fig. Neto-alone cannot ensure a normal GluRIIA/GluRIIB ratio at the PSD. Confocal images of NMJ4 boutons (segment A3) in third instar larvae labeled for GluRIIA (red), GluR-IIB (green), and HRP (blue) in the control and the netoαlow;G14>netoαlow (netoα/Y;G14-Gal4/UAS-neto-A9, reared at 25°C). These animals show a significant reduction of the GluRIIA synaptic levels (to 40% of control) and a more variable increase in GluRIIB signals. Error bars indicate SEM. **; p<0.005, ns; p>0.05. Scale bars: 20μm.

(TIF)

S1 Table. Summary of physiology defects at neto-β mutant NMJs.

(TIF)

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Author Contributions

Conceived and designed the experiments: CIR QW MS. Performed the experiments: CIR OI QW MS. Analyzed the data: CIR OI QW MS. Contributed reagents/materials/analysis tools: CIR OI QW MS. Wrote the paper: CIR MS.

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