Experience-dependent development of synaptic size in *Drosophila melanogaster* larval neuromuscular junctions

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Abstract

Changes in the nervous system induced by sensory experience are called experience- or activity-dependent plasticity; this subject has been extensively studied in many organisms. Experience-dependent changes are also found during development and there is no doubt that both programmed and non-programmed activity-dependent cellular processes are a requisite for the proper development of neural function. This review will primarily focus on experience-dependent regulation of synaptic size during early development of the *Drosophila* larval neuromuscular junctions and will discuss possible underlying mechanisms. Regulation of the size of presynaptic sites to match postsynaptic size is quite important for precise transduction of neural information. In *Drosophila*, sensory inputs after hatching and neural activity are necessary for the proper development of synaptic size. This matching is similar to homeostatic regulation of synaptic size during development and synaptic plasticity found in many systems including the vertebrate central nervous system. Further understanding of development and plasticity in the nervous system can provide valuable clues to how neural circuits are constructed to have appropriate functions during development and how they adapt to alterations in the environment.

Development of neural circuits begins during embryogenesis and proceeds with a well-programed series of gene expression and steps regulated by internal factors without sensory inputs. For example, in the mammalian visual system, topographic maps are formed during an early innate period of development in an experience-independent manner (Espinosa and Stryker, 2012). In *Drosophila* embryo and larvae, motor circuits required for the peristaltic motor pattern can develop in the complete absence of sensory inputs (Suster and Bate, 2002). These studies suggest that neural cells can develop mature synapses without sensory inputs from the environment. However, it has been clearly shown that sensory inputs and their induced neural activity as well as activity-independent processes are important in the proper development of neural function and refinements in neural circuits. In the case of the locomotion circuit of *Drosophila* embryo and larvae, the integration of this circuitry into actual patterns of locomotion requires information from the sensory system (Suster and Bate, 2002). Moreover, sensory experience is quite important for the development of the sensory system, especially the visual system of both vertebrates and invertebrates (Hubel and Wiesel, 1963, 1970; Kral and Meinertzhagen, 1989; Barth et al., 1997; Crair et al., 1998; Pyza and Gorska-Andrzejak, 2008; Yuan et al., 2011; Espinosa and Stryker, 2012, Kikuchi et al., 2012). For example, cats reared in limited visual environments show specific morphological alterations in the primary visual cortex (Tieman and Hirsch, 1982; Hirsch, 1985). Even in adult insects, which were once considered to have a

hard-wired neural network, experience is important for appropriate neural development. After eclosion, deprivation of light impairs the development of visual pattern discrimination in the fly *Boettcherisca peregrina* without impairing phototaxis (Mimura, 1986). In *Drosophila melanogaster*, rearing conditions after eclosion have been shown to have great influence on structural and behavioral development of the visual system (Barth et al., 1997; Yuan et al., 2011; Kikuchi et al., 2012). However, the morphological structure of high-order visual system neurons are not affected by rearing conditions (Karmeier et al., 2001; Scott et al., 2003). Thus, sensory experience/inputs together with internal processes are crucial for the proper development of synapses and neural circuits (Goodman and Shatz, 1993; Espinosa and Stryker, 2012).

Because molecular and genetic techniques used to study *Drosophila* are well established compared to those in other insects, *Drosophila* serves as a valuable model system for studying synaptic development and plasticity. I will begin by describing experience-dependent development in *Drosophila* larval neuromuscular junctions (NMJs). *Drosophila* larval NMJs are a simple and useful model system to understand the molecular mechanisms of synapse formation, maturation, and plasticity. NMJs are accomplished in late embryogenesis and keep growing during larval stages. I will provide an overview of studies including ours that demonstrate that sensory experience after hatching is critical for the proper development of synapses and matching of presynaptic size with postsynaptic size (Nakayama et al., 2006). The matching phenomena are hypothesized to share molecular mechanisms with homeostatic plasticity found in *Drosophila* NMJs and mammalian systems. Potential molecular candidates, such as calcium/calmodulin-dependent protein kinase II (CaMKII), in matching mechanisms will be discussed.

Sensory input-induced synaptic matching in *Drosophila* larval neuromuscular junctions

The *Drosophila* NMJ has unique identifiable pre- and postsynaptic cells that establish precisely specified connections (Keshishian et al., 1993). Initial synaptic connections are formed without any sensory inputs in embryos, and neither pathfinding nor target selection are affected by blocking neural activity (Broadie and Bate, 1993). Synaptic elimination, characteristic of vertebrate NMJs, was previously thought to be absent in the formation of *Drosophila* NMJs since synapses remain polyinnervated throughout larval life. Contrary to this idea, a study demonstrated that neural activity has an important role in synaptic elimination and refinement during embryonic and larval development similar to that of vertebrate NMJs (Jarecki and Keshishian, 1995). In this study, the authors observed extra ectopic synapses after suppressing neural activity during both late embryogenesis and first instar larval stages; these findings suggested that neural activity during this period of early larval development plays a role in the suppression of inappropriate ectopic connections and thereby maintaining precise connectivity (Jarecki and Keshishian, 1995). Retraction of synaptic connections during normal synaptic growth has also been reported (Eaton et al., 2002). Furthermore, several forms of experience- or activity-dependent plasticity have been observed in third instar larval NMJs. The double mutant *ether-a-go-go* (*eag*) *shaker* (*Sh*), which affects potassium channels and leads to enhanced nerve excitability, induces larval nerve endings with increased numbers of branches and varicosities (Ganetzky and Wu, 1983; Budnik et al., 1990). Mutations result in chronic effects on the NMJ;

however, acute experience-dependent synaptic plasticity can also be induced in NMJs. A study showed that larval locomotor activity was increased by shifting the rearing temperature from 18 or 25°C to 29°C; this shift resulted in the enlargement of nerve endings, potentiation of signal transmission, and an increase in the number of large subsynaptic translation aggregates (Sigrist et al., 2003). Population density of fly culture vials has been shown to regulate synaptic morphology as an inverse exponential relationship between population density and the number of synaptic boutons (Stewart and McLean, 2004). Sensory inputs and neural activity are also required for the establishment of locomotion circuits (Suster and Bate, 2002). Nevertheless, how sensory inputs and the induction of neural activity just after hatching regulate synaptic development of early-stage larval NMJs has not been well established. We found that sensory inputs after hatching were necessary for proper control of presynaptic size matching to postsynaptic size (Nakayama et al., 2006). By observation of unique synapses on muscle 6 (M6) and M7, which are innervated by the same neurons, we showed the significance of sensory inputs in the regulation of synaptic size. In fully developed larval NMJs, synaptic size on M6 is normally larger than that on M7, in accordance with the difference in muscle volume. This ensures the same extent of contraction of both muscles; we refer to this correspondence as “matching.” Matching was apparent in larvae 8 h after hatching but not in newly hatched larvae despite the difference in muscle volume (Fig. 1A and B). When sensory inputs after hatching were suppressed by the expression of tetanus toxin in all sensory neurons, synapses formed normally in newly hatched larvae. However, matching did not occur in larvae 8 h after hatching as the synaptic size on M6 was the same as that on M7 and synapses on both muscles were growing at the same extent as that in newly hatched larvae (Fig. 1C). These results suggest that sensory experience through sensory neurons is required for the proper control of presynaptic size matching to the postsynaptic cell, which in turn ensures postsynaptic excitability. Since changes in sensory experience cause alterations in larval locomotion, the activity pattern of presynaptic

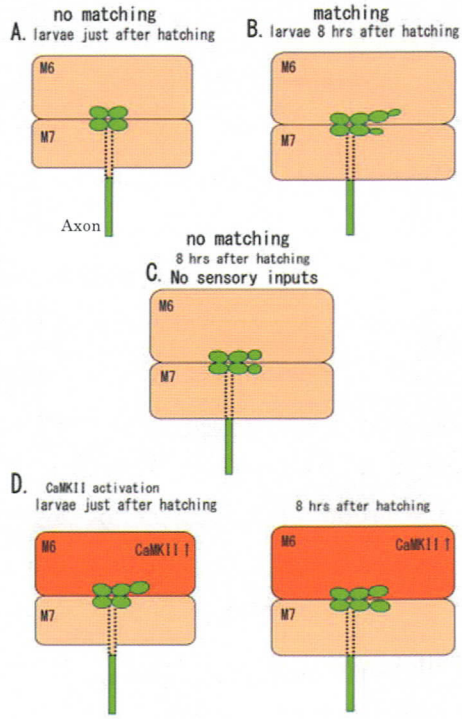

Figure 1. Schematic diagram of the development of *Drosophila* larval NMJs. A. Synapses on M6 and M7 of wild-type larvae just after hatching. The same neurons innervate these muscles. Size of synapses has not matched to the muscle volume. Small ellipses show nerve terminals. Squares are muscle cells. M6 is bigger than M7 at all larval stages. B. Synapses on M6 and M7 of wild-type larvae 8 h after hatching. Synaptic size matches the size of the postsynaptic cell. C. Synapses on M6 and M7 of larvae 8 h after hatching in which activity of sensory neurons is inhibited. Synapses grow, but the size of synapses does not match the differences in muscle size. D. Synapses on M6 and M7 of larvae when CaMKII was activated only in M6 (dark color). Left, larvae just after hatching, Right, larvae 8 h after hatching. Just after hatching, enhancement of synaptic maturation is induced only in M6 where CaMKII activity is increased. At 8 h after hatching, synaptic size on M6 is not enhanced, while that on M7 where CaMKII activity is not modulated is potentiated. CaMKII activity may determine the size of synapses.

motoneurons must be affected in a sensory experience-dependent manner. Thus, changes in sensory experience may lead to changes in motoneuronal activity, resulting in the regulation of synaptic size during development and synaptic plasticity at NMJs.

Matching is not only found in larval NMJs but also in sensory synapses of the adult fly's olfactory neurons (Kazama and Wilson, 2008). The amplitude of unitary synaptic currents of projection neurons matched to the size of their dendritic arbor. Although it is not clear if matching in the sensory system of adult flies requires sensory experience, matching is a common mechanism during development of the nervous system that is necessary for establishing synaptic structures and ensuring stable synaptic excitability. In addition, in terms of forming stable synaptic connections, the matching found in our studies seem to be one form of homeostatic regulation of synaptic function found in many neural systems; these systems may share similar molecular mechanisms as described in the next section. Homeostasis of synaptic excitability has been extensively studied in third instar larval NMJs. Larval NMJs grow in a homeostatic manner to maintain the ability to drive muscle contraction despite various changes, such as changes in synaptic efficacy or synaptic morphology (Purves et al., 1987; Schuster et al., 1996a, b; Davis and Goodman, 1998; Zito et al., 1999; Sanyal et al., 2002). For example, in *Drosophila* with mutations in glutamate receptor II A (GluRIIA), a major neurotransmitter receptor of *Drosophila* NMJs, synaptic strength to nerve stimulation is normal despite a reduction in synaptic response of the muscle to a single neurotransmitter vesicle (Peterson et al., 1997). This suggests an increase of presynaptic neurotransmitter release and the existence of retrograde signaling that regulates presynaptic functions. In mammalian synapses, homeostatic mechanisms that maintain neural activity at a certain level, named synaptic scaling, were also reported (Turrigiano, 1999; Turrigiano and Nelson, 2004). Although most studies were done *in vitro*, synaptic scaling was also observed in the developing visual system in an experience-dependent manner (Desai et al., 2002). An inverse relationship between synapse number and quantal amplitude has been observed in the developing rodent primary visual cortex, and dark rearing has been shown to prevent the developmental decrease in quantal amplitude (Desai et al., 2002). Homeostatic regulation of intrinsic excitability and synaptic transmission was also found in the developing *Xenopus* retinotectal circuit (Pratt and Aizenma, 2007). Taken together, these findings suggest that homeostatic matching is a fundamental mechanism for maintaining synaptic strength at a certain level despite changes during development in synaptic inputs and in neural activity as well as for establishing stable yet flexible neural circuits.

Molecular mechanisms underlying matching

The molecular mechanisms that induce matching have not yet been clarified; however, CaMKII is a candidate signaling molecule with a known role in homeostatic matching. In *Drosophila*, CaMKII has been reported as a key molecule for the activity-dependent regulation of synapses (Griffith et al., 1993; Wang et al., 1994; Jin et al., 1998; Koh et al., 1999; Kazama et al., 2003; Morimoto-Tanifuji et al., 2004). In larval NMJs, we have demonstrated that CaMKII is an important key molecule in the developmental stage-dependent regulation of synaptic size. Activation of CaMKII in muscle cells promotes coordinated

maturation of pre- and postsynaptic sites of larvae just after hatching (Kazama et al., 2003, Fig. 1D left), while the effects are different in larvae 8 h after hatching (Morimoto-Tanifuji et al., 2004, Fig. 1D right). Interestingly, at 8 h after hatching, the expression of activated CaMKII only in M6 increases the growth of synaptic size in M7, which does not express activated CaMKII, thereby resulting in the loss of matching (Fig. 1D right). This suggests that matching is disturbed by modulating the balance of CaMKII activity between M6 and M7 when two muscles are innervated by the same neuron. In later larval stages, CaMKII plays a role in the regulation of postsynaptic GluR amount (Morimoto et al., 2010). Expression of an inhibitory peptide of CaMKII in muscle cells enhances the density of GluRIIA at synapses of third instar larval NMJs. On the other hand, postsynaptic expression of a constitutively active form of CaMKII reduces synaptic GluRIIA. Since GluRIIA is a major Ca^{2+}-permeable subunit of GluR, it is possible that Ca^{2+} influx through GluRIIA during synaptic response is coupled to CaMKII activity. Taken together, these findings suggest that the amount of GluRIIA is regulated by CaMKII such that the Ca^{2+} influx through GluRIIA can be maintained at a constant level and thus the level of CaMKII activity could also be maintained at a constant level. Accordingly, CaMKII could be a key factor for homeostatic matching of synapses in *Drosophila* NMJs. Moreover, in mammalian neural cells, accumulating evidence suggests that a family of CaMKs, CaMKII and CaMKIV, plays a role in homeostatic synaptic plasticity (Wang et al., 2012). In hippocampal neurons, an inhibitor of the calcium/calmodulin-dependent family of kinases (KN93) prevents the homeostatic modification of synaptic strength that is produced by activity blockade, thereby raising the possibility that calcium participates in this form of plasticity through the activation of calcium-dependent kinase (Thiagarajan et al., 2002). In cultured cortical neurons, postsynaptic expression of activated CaMKII was shown to increase the strength of transmission between pairs of pyramidal neurons, while it reduced overall excitatory synaptic density and increased the proportion of unconnected pairs, suggesting postsynaptic activation of CaMKII induces structural remodeling of presynaptic inputs that favors the retention of active presynaptic partners (Pratt et al., 2003). Further, CaMKII and CaMKIV have been reported to regulate the amount of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) (Thiagarajan et al., 2002; Ibata et al., 2008; Goold et al., 2010; Groth et al., 2011). AMPARs mediate fast synaptic transmission in the mammalian central nervous system and have an important function in neural development and synaptic plasticity (Dingledine et al., 1999). The subunit composition of AMPAR changes its Ca^{2+} permeability; therefore, regulation of the components as well as concentration of AMPARs is important for synaptic function and efficacy of neurotransmission (Hollmann et al., 1991; Verdoorn et al., 1991; Burnashev et al., 1992; Geiger et al., 1995; Liu and Zukin, 2007). Activation of CaMKIV reduces AMPAR currents and the surface amount of AMPARs (Ibata et al., 2008; Goold et al., 2010). In the case of CaMKII, α and β CaMKII, are inversely regulated by neural activity and have opposite effects on homeostatic regulation (Thiagarajan et al., 2002). It is not clear if changes in Ca^{2+} influx during neurotransmission are entirely due to CaMKII-mediated changes in AMPARs since there are also other Ca^{2+} permeable GluRs, such as *N*-methyl-D-aspartate receptors (NMDARs). In mammalian cells, there are many subtypes of functional proteins, whereas in *Drosophila* there are generally fewer subtypes. Thus, *Drosophila* NMJs may provide a

simple system to examine the role of these functional proteins in homeostatic regulation of synapses. It would be fascinating to demonstrate that CaMKII levels are also a key factor of homeostatic matching in the sensory system or the central nervous system of adult flies. Further, CaMKs are good candidate signaling molecules for sensory experience- or activity-dependent regulation of synaptic development *in vivo*, since changes in sensory experiences alter neural activity, leading to changes in Ca^{2+} influx and CaMK activity. CaMKII transforms a transient Ca^{2+} elevation signal induced by neural activity to prolonged cellular signaling through autophosphorylation (Lisman et al., 2002). Investigation of the downstream signal(s) of CaMKII will therefore help identify the factor(s) involved in synaptic size matching.

Several other molecular mechanisms of homeostatic regulation have been identified by using larval NMJs. Bone morphogenetic protein (BMP) is a major signaling molecule that may also function in homeostatic regulation of synaptic growth (Haghighi et al., 2003; McCabe et al., 2003). However, our results suggest that BMP signaling is unlikely to underlie the matching phenomenon since matching is intact in a mutant of BMP signaling (Nakayama et al., 2006). A recent report suggests that presynaptic Ca^{2+} influx through a *Drosophila* homolog of the CaV2 calcium channel, Cacophony, is important for homeostatic increase of neurotransmitter release in the *Drosophila* larval NMJ (Muller and Davis, 2012). Interestingly, electrophysiology-based genetic screening found that Rab3-GAP controls synaptic homeostasis in late stages of vesicle release (Muller et al., 2011). It is possible that these signaling molecules are important for synaptic size matching in addition to their role in regulation of synaptic release and function.

Conclusions

Development of the nervous system proceeds according to a sequence of genetically mediated and internal processes. However, synaptic modification is induced by sensory experience and the resulting changes in neural activity are indispensable for proper construction of neural networks. Experience-dependent regulation of presynaptic and postsynaptic sizes is prominent not only in larval NMJs but also in other synapses of the central nervous system. The *Drosophila* model opens a window to dissect the molecular mechanisms of experience-dependent and experience-independent development and plasticity of synaptic function. Recent progress in identifying molecules related to these phenomena will provide us with useful insights into how development of the neural system is genetically encoded and how it is regulated by sensory experience and neural activity.

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