Synaptic Target Recognition at Drosophila Neuromuscular Junctions

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ABSTRACT Every synaptogenesis begins with “synaptic target recognition,” a cell-cell recognition event in which a neuron and its target stably adhere. Despite its importance in developing nervous systems, synaptic target recognition has been difficult to study in complex systems. The relatively simple and genetically accessible Drosophila NMJ model system provides a repertoire of target recognition cues. We describe how these molecules control the targeting of specific growth cones in either a positive (synaptogenic) or negative (anti-synaptogenic) manner. We also propose two alternate signaling paradigms to explain how these initial cell recognition events are coupled to the intracellular signaling pathways that begin the process of synapse maturation. Microsc. Res. Tech. 49:3–13, 2000. © 2000 Wiley-Liss, Inc.

INTRODUCTION

Synaptogenesis is a dynamic process, involving at least three different stages of cellular and molecular events. First, axons must navigate through correct pathways in order to reach a specific site of synaptogenesis, a process during which an axon may have up to thousands of cellular contacts (Tessier-Lavigne and Goodman, 1996). Second, when an axonal growth cone and its future synaptic target cell finally come in contact and recognize each other as compatible synaptic partners, the axon stops elongating and starts converting its tip into a presynaptic terminal (Chiba and Keshishian, 1996; Garrity and Zipursky, 1995). Third, presynaptic and postsynaptic cells undergo extensive local reorganization both at the cell surface and in the cytoplasm. At its completion, the mature synapse exhibits very precisely defined protein expression patterns on both presynaptic and postsynaptic sides (Prokop, 1999; Sanes, 1997; Sheng, 1996; Suedhof, 1995).

Of these three, the second stage, which we call “synaptic target recognition,” is the least described of all in molecular terms. Yet, such specific cell recognition is the very basis for the power of information processing achieved by neuronetworks. The lack of our knowledge in this area is due not to a lack of interest on the part of neuroscientists, but primarily to the fact that it is technically very challenging to study this specific form of cell recognition in well-defined and reproducible in vivo contexts. While the ultimate goal of neuroscientists is to understand the origin of functional neuronetworks in the vertebrate central nervous system (CNS), its numeric and molecular complexity is prohibitive. Many, therefore, find the neuromuscular junctions (NMJ) an excellent model system in which guiding principles behind establishing neuronetworks may be investigated with high cellular and molecular resolution.

In this review, we will outline the history of the research on synaptic target recognition, which has revolved around the central concept of chemoaffinity. In doing so, we will pay attention to the fast advances made through the use of one of the most manipulable NMJ systems known, the embryonic NMJ system of the fruitfly Drosophila melanogaster. The studies we describe have demonstrated as well as refined the chemoaffinity hypothesis. We will also discuss a major remaining issue in this field, i.e., how the specific target recognition molecules are linked to synaptogenesis machinery, and attempt to predict where future experiments may possibly take us.

CHEMOAFFINITY HYPOTHESIS

The idea of biomolecular matching, rather than electromagnetic or supernatural forces, as a basis of neuronal networking in the brain can be traced back a century to the days of the patriarchal neuroanatomist Santiago Ramon y Cajal (Ramon y Cajal, 1892) and the pioneer electrophysiologist John Langley (Langley, 1895). It is, however, Roger Sperry, who in the early 1960s first put forth explicitly the idea of “chemoaffinity” matching in terms compatible with a modern molecular biological framework of thinking. Experimenting with retino-tectal synaptic specificity, Sperry and his colleagues suggested that: The establishment and maintenance of synaptic associations [are] regulated by highly specific cytochemical affinities [italics added] that arise systematically among the different types of neurons involved via self-differentiation, induction through terminal contacts, and embryonic gradient effects (Sperry, 1963).

In essence, the chemoaffinity hypothesis proposes that neurons and their targets are each paired up by the appropriately matched expression of receptors and ligands. Many putative ligand/receptor complexes and homophilic cell adhesion molecules (CAMs) have since been identified in the developing brains of a variety of...
animal systems, and some have been proposed to affect binding between groups of growth cones and their targets (Chiba and Keshishian, 1996; Rutishauser et al., 1988).

The extrinsic molecules that affect axon pathfinding and synaptic targeting are usually classified into four classes: contact attraction, diffusible attraction, contact repulsion, and diffusible repulsion (Tessier-Lavigne and Goodman, 1996). These classifications, however, must be viewed as context specific, since certain molecules can trigger either positive or negative response from growth cones depending on the particular combination of receptors that the growth cones happen to express (Ming et al., 1997; Rutishauser et al., 1988). Furthermore, the functional differences between diffusible and cell-bound molecules often become blurry. While some secreted molecules are quickly trapped by the extracellular matrix surrounding the source, many presynaptic and postsynaptic cells extend filopodia through which cell-bound molecules can exert their influence for long distance. Therefore, the actual roles of each candidate molecule need to be determined separately in its normal in vivo context.

Perhaps the best-studied example of a vertebrate molecule thought to control many aspects of neuronal development is the neural cell adhesion molecule (NCAM). One of the most abundant adhesion/signaling molecules in the vertebrate nervous system, NCAM possesses both immunoglobulin and fibronectin repeats in its extracellular domain (Rutishauser et al., 1988). This transmembrane glycoprotein has been shown to mediate homophilic adhesive interactions, some of which are modulated by its polysialic acid (PSA) content (Muller et al., 1996; Rutishauser et al., 1988). The addition of PSA to NCAM not only attenuates homophilic NCAM adhesivity, but also interferes with other CAMs, such as L1, present in the same cells (Rutishauser et al., 1988). This post-translational modification provides a means by which cells can locally control their general adhesivity. Whether such a mechanism is used during synaptic binding remains unknown but synapses in rat hippocampal neuronal cultures require NCAM for the induction of long-term potentiation, a form of synaptic plasticity commonly associated with learning and memory (Luthi et al., 1994). Thus, NCAM is important in processes at least as varied as axon fasciculation and synaptic plasticity.

Many of the NCAM experiments have relied on in vitro cultures, in which the in vivo architecture is largely lost and it is impossible to correlate particular cells to their intact tissue counterparts. Recently, mouse knockouts have provided an opportunity to assess the in vivo role of NCAM at the NMJ. However, the phenotype observed was mild and described only in general terms, thus offering limited clues for a concrete role of NCAM in synaptic target recognition (Moscoso et al., 1998). These situations highlight the limitations that vertebrate systems often have when addressing questions of synaptic specificity. In general, expression patterns and biochemical binding properties of candidate matchmaking modules are determined before
Fig. 2. Molecules involved in axon targeting, synaptic target recognition or synaptogenesis in Drosophila embryos. These cell surface and secreted molecules can be divided into 3 basic classes: synaptogenic (A–D), anti-synaptogenic (E–H), and general adhesive signaling (I–L). Each panel shows the structure of the molecule and its expression pattern during the embryonic stage in which synaptic targeting occurs. The references given are the most recent descriptions of the molecules' individual effects on neural patterning.
their in vivo roles are demonstrated. Only a handful of the candidate molecules have received rigorous experimentation, particularly when it comes to observing their roles in intact neural tissues.

**DROSOPHILA NMJ AS A MODEL SYSTEM TO STUDY SYNAPTIC TARGET RECOGNITION**

Synaptic target recognition is typically manifested as a particular morphological change, accompanied by molecular, ultrastructural, and electrophysiological differentiation. In the embryonic *Drosophila* NMJ, these aspects have all been well correlated at the level of a single synapse (Fig. 1). The RP3 motor neuron has been well studied in the embryonic *Drosophila* NMJ system and many aspects of its axon pathfinding and synaptic maturation are known (Chiba and Rose, 1998; Halpern et al., 1991; Rose et al., 1997; Sink and Whiting, 1991). The transition of RP3 growth cone morphology from active and undifferentiated to stable and differentiated occurs during hours 14–20 of embryogenesis. As the growth cone head contacts the ventral sides of muscles 6 and 7, filopodia begin to retract and the growth cone aligns along the external edge of the cleft between 6 and 7 (Fig. 1B; see also Fig. 3B). This morphological change correlates well with many other observed measures of synapse maturation. For example, evoked endplate potentials at muscles 6 and 7 can be detected by hours 18–20 of embryogenesis (Broaddie and Bate, 1993). This time period corresponds to the accumulation of synaptic vesicle proteins such as the v-SNARE synaptotagmin at the swollen pre-synaptic “prevaricosities” of RP3 (Yoshihara et al., 1997). At the ultrastructural level, pre-synaptic T-shaped electron dense bodies of the type associated with synaptic vesicle cycling are observed, as well (Prokop et al., 1996). Similar sequences of the events seen in RP3 synaptic maturation are also present at other NMJs of the *Drosophila* embryonic system (Rose et al., 1997; Prokopet al., 1996; Yoshihara et al., 1997). Therefore, although underlying cytoplasmic rearrangements likely encompass a number of different molecular components, the initiation of synaptogenesis can be easily and reliably scored based upon morphological criteria. In particular, one can define the beginning of synaptogenesis by two discrete parameters, both of which are visible at the light microscopic level: retraction of most growth cone filopodia and the lining of a muscle edge (see Fig. 3B; see Rose et al., 1997, and Chiba, 1998, for in-depth discussion of synaptic target recognition).

For the past several years, we have been using the *Drosophila* NMJ system to examine how molecular manipulations affect the initiation of synaptogenesis. In this system, the size and position of all 30 muscle cells and at least 32 identified motor neurons are precisely known (Chiba, 1998; Landgraf et al., 1997; Schmid et al., 1999). Therefore, specific cells can be consistently and reliably examined in a variety of genetic backgrounds. Furthermore, ease of genetic manipulation allows for the creation of various knockout and transgenic flies in a relatively short period of time. An increasingly popular transgenic approach is to design ectopic expression of the molecule of interest, as it is capable of discerning roles for molecules that would normally be missed in a knockout screen due to functional redundancy. Therefore, one can test both the necessity and sufficiency of many putative synaptic target recognition molecules in a context that stresses the 1:1 specificity between motor neurons and their target muscles (Keshishian et al., 1996).

Many *Drosophila* proteins involved in neural development possess a high degree of molecular homology with vertebrate proteins that are proposed to be involved in axon growth and synaptogenesis. For example, the well-known NCAM, Semaphorin, Integrin, Neulin, and Cadherin families all contain *Drosophila* homologues that are also speculated to have similar functions in neural development (Hoang and Chiba, 1998; Iwai et al., 1997; Kolodkin, 1996; Lin and Goodman, 1994; Winberg et al., 1998). The repertoire of cellular and molecular techniques available to *Drosophila* neurobiologists allows for the answer of many specific questions that are difficult to resolve in more complex systems.

**TESTING THE CHEMOCOGNITION HYPOTHESIS: SYNAPTIC MATCHMAKING**

The first and probably the best example among demonstrated *Drosophila* “synaptic target recognition” molecules is Fasciclin III (Fas3). A relatively small (80 kD) IgCAM (Fig. 2A), Fas3 has been shown to mediate homophilic adhesion when transfected into the *Drosophila* S2 cell line (Snow et al., 1988, 1989). In a developing embryo, Fas3 is expressed in a small subset of neuromuscular tissue. In contrast to other CAMs such as Fasciclin II (Fas2), Neuroglian, Integrin, and DN-Cadherin, which are ubiquitously expressed in neural or muscular tissue at this time (Fig. 2I–L) (Hall and Bieber, 1997; Hoang and Chiba, 1998; Iwai et al., 1997; Lin et al., 1994), Fas3 is present at high levels on the surface of both the RP3 motor neuron and its normal targets, muscles 6 and 7, but not other neurons or muscles (Fig. 2A) (Chiba et al., 1995; Kose et al., 1997; Snow et al., 1989). This has made Fas3 a good candidate for a matchmaking molecule specific to these particular synaptic partners.

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Fig. 3. Defining synaptic target recognition. Light microscopic overlays show differential interference contrast (DIC) images of the ventrolateral musculature and fluorescent RP3 axonal growth cones. A: Schematic shows RP3's normal outgrowth as it corresponds to Toll and Fas3 expression. B: In wild type embryos, RP3 consistently begins the process of synaptogenesis by retracting most of its filopodia and lining the edge between muscles 6 and 7. C: When Fas3, a synaptic protein, is removed, RP3 loses a slight degree of targeting accuracy, and sometime misinnervates muscle 13 as shown. D: Misexpression of Fas3 in the musculature causes frequent mistargeting of the RP3 growth cone onto neighboring muscles, including muscles 14 and 30 as shown. E: Such mistargeting depends on Fas3 being expressed in RP3, as muscle misexpression does not induce ectopic synaptogenesis in a Fas3 knockout background. F: When Toll an anti-synaptic molecule, is removed from the muscles, RP3 loses targeting accuracy and sometimes misinnervates the normally Toll positive muscles 15 and 16. G: Misexpression of Toll in the musculature causes a frequent failure of RP3 to initiate synaptogenesis, though it always reaches the correct target region (note that the focal plane of muscles 6 and 7 is shown for clarity). H: When both Fas3 (synaptogenic) and Toll (anti-synaptogenic) are concurrently misexpressed in the musculature, RP3 targeting is largely normal. Asterisks indicate the direction of CNS. Scale bar = 5 μm.
Fig. 3.
Our experiments have shown that entire removal of the Fas3 protein leads to a slightly impaired efficiency of RP3 targeting by 9% (Fig. 3C) (Kose et al., 1997). Since other NMJs in this system form normally, the overall effect of a Fas3 knockout is specific to the cells that normally express the protein. Initially, as scored by an immunocytochemical analysis of the neuropil, Fas3 knockouts looked identical to wild type (Chiba et al., 1995). Detecting this subtle defect in a single axon required specific intracellular labeling of the RP3 growth cone with fluorescent dye (Chiba and Rose, 1998). This single cell analysis approach provided the first hint that the protein contributes to RP3 synaptic target recognition.

A complementary, and more striking, demonstration of Fas3 function came from additional experiments. We utilized the same intracellular labeling technique in a transgenic mutant background. We tested whether misexpression of Fas3 is sufficient for the initiation of synaptogenesis between RP3 and the muscles that bear ectopic Fas3 (Chiba et al., 1995; Kose et al., 1997). Misexpression was driven by the pan-muscle promoter Mhc', which led to Fas3 expression in all muscles throughout the period of RP3 targeting. The RP3 growth cone was shown to be clearly capable of initiating synaptogenesis on ectopic muscle targets when they express Fas3 on their surfaces (Fig. 3D). Thus, Fas3 expression alone was sufficient to cause RP3 to initiate synaptogenesis at abnormal target sites.

A further series of experiments demonstrated that this ability of Fas3 to initiate RP3 synaptogenesis is dependent on Fas3 being expressed in a matched manner on both the growth cone and its synaptic targets (Kose et al., 1997). In a Fas3 knockout background, Fas3-deficient RP3 is no longer capable of responding to pan-muscle misexpression of Fas3, demonstrating that neural Fas3 is required to respond to muscle Fas3 (Fig. 3E). This result supports the idea that Fas3 acts as a matchmaking (synaptogenic) molecule, presumably promoting homophilic adhesion and subsequently initiating RP3 synaptogenesis at its normal site.

If Fas3-mediated homophilic synaptogenic effects were a generalizable phenomenon, we would expect that induced Fas3-expression in neuron-muscle pairs other than RP3 and 6/7 would show a similar synaptic effect. To test this, we created transgenic flies in which Fas3 was ectopically expressed in the aCC motor neuron and the dorsal musculature (Kose et al., 1997). The aCC motor neuron, which is normally Fas3-negative, was shown to respond to Fas3 misexpression by initiating ectopic synaptogenesis when, and only when, it itself expressed Fas3 (Kose et al., 1997). Thus, homophilic Fas3 matching is sufficient to induce synaptic target recognition, independent of other factors. These experiments with Fas3 provide a concrete demonstration of Sperry's matchmaking mechanism in vivo.

Several other molecules at the Drosophila NMJ provide further support that the presence of molecular matchmakers is important for many different synaptic pairs. One example is Connectin (CON). A leucine-rich repeat molecule, CON is normally expressed at high levels in a group of motor neurons that normally innervate the lateral musculature and the target muscles themselves (Fig. 2B) (Nose et al., 1992; Raghavan and White, 1997). Like Fas3, CON also mediates in vitro homophilic adhesion (Nose et al., 1992; Raghavan and White, 1997). Moreover, it is apparently capable of inducing ectopic synapse formation in vivo, being dependent on both neurons and muscles expressing CON, indicating the interaction is homophilic (Nose et al., 1997).

Another synaptic matchmaking molecule is Capricious (CAP). This leucine-rich repeat molecule is expressed in muscle 12 and the motor neurons that innervate it (Fig. 2C) (Shishido et al., 1998). In CAP knockout mutants, the motor neurons extend ectopic contacts onto the neighboring muscle 13. This was interpreted to indicate that these growth cones are normally restricted to muscle 12 through its expression of CAP (Shishido et al., 1998). When CAP was misexpressed via a pan-muscle promoter, more ectopic synapses of the type that normally innervate muscle 12 were seen. Like Fas3, then, CAP seems to be involved in the stabilizing of particular neuron-muscle contacts. Though CAP likely mediates its effect homophilically, it may do this primarily through signaling and not adhesion, since unlike Fas3 and CON, it is incapable of mediating in vitro cell adhesion (Shishido et al., 1998).

The Drosophila Netrin B (NETB) is a good candidate for a heterophilic matchmaking molecule. NETB has highly restricted muscle expression pattern, but is not expressed by motor neurons (Fig. 2D) (Mitchell et al., 1996). In knockout mutants lacking both of the known Netrins (A and B), partially penetrant defects in certain motor neurons are seen, suggesting that these molecules contribute to normal neural targeting. These effects include occasional failure of RP3 to innervate the normally NETB-positive muscles 6 and 7 (Mitchell et al., 1996). Pan-muscle misexpression of NETB leads to a high level of ectopic contacts originating from neighboring neurons, along with occasional stalls in various nerve branches (Winberg et al., 1998). Though no single cell analyses were performed and NETB-specific receptors have not been conclusively determined, these data suggest that NETB is involved in mediating heterophilic synaptogenic interactions between neurons and muscles.

The aforementioned matchmaking molecules (Fas3, CON, CAP, NETB) help explain how certain subsets of motor neuron-muscle matchmaking may be accomplished in the Drosophila NMJ system. They also provide some of the best examples to date for Sperry's chemoaффinity model.

EXPANDING THE CHEMOAFFINITY HYPOTHESIS: ANTI-SYNAPTOGENIC SIGNALING

In recent years, Sperry's chemoaффinity hypothesis has been expanded to include repulsive and anti-synaptogenic molecules. These are the proteins that either act to collapse growth cones or directly inhibit the formation of synapses. One large family of molecules thought to actively repel growth cones is the Ephrins (Flanagan and Vanderhagen, 1998). Acting through the conserved Eph receptor tyrosine kinases, Ephrins are proposed to be important in the formation of a stable neural topographic map by causing growth cones to collapse or avoid regions of high Ephrin expression. Another family of repulsive molecules is the Semaphorin family, which includes both cell bound and
secreted proteins, sharing similar extracellular Sema domains (Kolodkin et al., 1993). Through the activation of receptors such as the Neuropilins and Plexins, they function to repel neurons (Hung-Hsiang and Kolodkin, 1999; Kolodkin and Ginty, 1997). Both Ephrins and Semaphorins are now considered a critical element of the establishment of synaptic specificity in many different systems.

In Drosophila embryos, one widely used molecular repulsion mechanism seen during axon fasciculation is that mediated by the secreted glycoprotein Beat (BEAT). A small (43 kD) novel molecule expressed by motor neuron growth cones (Fig. 2E), BEAT appears to have a directly anti-adhesive effect on axon fascicles. The loss of BEAT protein leads to a highly penetrant lack of defasciculation in the major nerve branches of the neuromuscular system (Fambrough and Goodman, 1996). This phenotype is almost identical to that seen with misexpression of Fas2 (Fig. 2I), a close relative of NCAM, in motor neuron growth cones, suggesting that BEAT and Fas2 act antagonistically to regulate fascicle adhesivity. BEAT, therefore, is an example of a molecule that acts to directly inhibit adhesive contacts between neighboring growth cones (Fambrough and Goodman, 1996). Although similar anti-adhesive functions could in theory contribute to specific synaptic targeting, BEAT itself is an unlikely candidate due to its ubiquitous expression in motor neurons and lack of expression at potential target sites.

Two other examples of molecules that directly affect neural growth cones in a negative manner are D-Semaphorin I (Sema1) and D-Semaphorin II (Sema2). Sema1 is a transmembrane Semaphorin expressed on a large subset of motor neurons and CNS axons (Fig. 2F) (Yu et al., 1998). In mutant embryos lacking Sema1 protein, many nerve branches show improper development, suggesting motor neuron targeting errors. Misexpression of Sema1 in all muscles leads to a frequent stalling of nerve branches, a phenotype similar, at least superficially, to that seen in knockout embryos. These results were interpreted to mean that Sema1 provides a cell-bound inhibitory cue for growth cones (Yu et al., 1998).

Sema2, a secreted Semaphorin structurally similar to chick Collapsin, is weakly expressed in all abdominal muscles (Fig. 2G) (Matthes et al., 1995). In embryos lacking Sema2 expression, several ectopic neural projections are seen, suggesting that neurons are more promiscuous in its absence (Winberg et al., 1998). The misexpression of Sema2 at high levels often leads to both the stalling of some growth cones just prior to their normal target sites, as well as the failure of certain nerve tracts to form altogether (Matthes et al., 1995; Winberg et al., 1998). Based on these observations, it was proposed that Sema2 acts to reduce axonal arborization and subsequent target recognition of growth cones.

The frequent stalling of entire nerve branches in embryos misexpressing Sema1 or Sema2 suggests that they may be involved in more than just synaptic target recognition. With the secreted Sema2 being able to diffuse some distance to bring about repulsive effects and Sema1 being expressed in a large number of growth cones, it is difficult to work out a specific wild type function for each protein. The apparent similarities seen between knockout and misexpression of either Sema1 further confuses the issue, though higher resolution analysis may eventually yield a better appreciation of each phenotype.

The aforementioned molecules provide evidence that synaptic targeting is more than just a simple process of matchmaking between partner cells. BEAT, Sema1, and Sema2, unlike the synaptogenic molecules, act to block the initiation of synaptogenesis of groups of neurons. However, the means by which their effects contribute to the accurate synaptic targeting of specific growth cones remains largely unknown.

Studies of a more discretely expressed muscle cell surface molecule, Toll, provides insights into how a specific molecule functions as an anti-synaptogenic molecule for specific subsets of motor neuron growth cones during their synaptic targeting. Toll is a transmembrane molecule of the leucine-rich repeat family with significant cytoplasmic homology to the signaling domain of the mammalian interleukin-1 receptor (Fig. 2H) (Halfon et al., 1995; Hashimoto et al., 1988; Heguy et al., 1992). During early pattern formation of the Drosophila embryo, maternally supplied Toll transduces the signal required for correct dorsal-ventral polarity (Hashimoto et al., 1988; Norris and Manley, 1992). Zygotically expressed Toll, on the other hand, is important in motor neuron and muscle development (Halfon et al., 1995; Halfon and Keshishian, 1998), as well as synaptic target recognition, where its effects are anti-synaptogenic (Rose et al., 1997). In the musculature, Toll is expressed on a specific subset of muscles by which the RP3 motor neuron normally passes on the way to its normal target. We showed that when Toll protein is removed from the musculature, RP3 reduces its normal targeting accuracy by 90% and instead initiates ectopic synaptogenesis on the normally Toll-positive muscles 15 and 16, which it would avoid in wild type embryos (Fig. 3F) (Rose et al., 1997).

In a subsequent study, we demonstrated that pan-muscle misexpression of the Toll protein leads to a failure of RP3 to initiate synaptogenesis with its normal targets or any other muscles (Fig. 3G). This effect is specific to synaptic target recognition, as RP3 path-finding remains normal. While correctly reaching its normal target region, the RP3 growth cone fails to retract its filopodia and line the edges of its normal target muscles, remaining in an undifferentiated state throughout the end of embryogenesis and even into larval development (Fig. 3G) (Rose et al., 1997). Thus, Toll provides an example of a transmembrane molecule expressed in a muscular pattern that correlates to the outgrowth of a particular motor neuron and specifically assures its correct synaptic target recognition of its growth cone through an anti-synaptogenic mechanism. These experiments illustrate the importance of anti-synaptogenic molecules in the creation of synaptic specificity.

FUNCTIONAL INTEGRATION OF SYNAPTGENIC AND ANTI-SYNAPTGENIC SIGNALING

The discovery that a variety of molecules could have strong effects on growth cone targeting led to the idea that overlapping sets of positive and negative cues could be functionally integrated at a given target site.
Recent experiments have suggested that the combined effects of synaptogenic (NETB) and anti-synaptogenic (Sema2) proteins could cancel each other out, recreating an overall NMJ phenotype similar to wild type (Winberg et al., 1998). In vitro experiments studying vertebrate primary neural cultures have shown that some cues can be interpreted as either attractive or repulsive based upon the internal cAMP or GMP state of a given growth cone (Ming et al., 1997; Song et al., 1997). These studies support the idea that a growth cone can integrate multiple inputs that converge upon a common second messenger pathway. Whether this neural signal summation occurs within a growth cone during its synaptic target recognition is an interesting issue.

We have recently tested the idea that Toll and Fas3 are functional opposites that integrate to achieve specific synaptic target recognition of a neural growth cone. To do this, we created embryos that concurrently misexpressed both cues at similar levels in the muscle. The RP3 growth cone, when faced with simultaneous Fas3 and Toll expression, largely mimics the wild type phenotype, initiating synaptogenesis at the 6/7 muscle cleft (Fig. 3H) (Rose and Chiba, 1999). This contrasts the situations in which either cue was misexpressed alone, wherein RP3 was either seen to initiate ectopic synaptogenesis (Fas3 misexpression; Fig. 3D) or stall just prior to the 6/7 cleft (Toll misexpression; Fig. 3G) (Rose and Chiba, 1999).

Further supporting the notion of signal integration were dosage experiments. When the relative levels of each cue were manipulated, RP3 showed a phenotype severity that correlated with the amount of Toll/Fas3 disparity present (Rose and Chiba, 1999). RP3 was capable of responding not only to the absolute presence or absence of either cue, but to their relative levels, thus making a decision based upon the integration of synaptogenic and anti-synaptogenic signals present on target surfaces. These observations support the idea that anti-synaptogenic signaling is intimately integrated into the synaptic maturation pathway.

COUPLING SYNAPTIC TARGET RECOGNITION MOLECULES TO SYNAPTOGENESIS MACHINERY

Though many different cell surface and cell-associated molecules have been implicated in synaptic target recognition, a fundamental question remains: how is synaptic target recognition linked to the pre- and postsynaptic machinery necessary for the maturation of a functional synapse? Synaptic target recognition, achieved through integrated synaptogenic events, is only a required first step for the initiation of synaptogenesis. At some point, the specific cell recognition mediated by molecules such as Fas3, CON, and CAP, and inhibited by molecules such as Toll and Sema2, must be transduced to a pathway that leads to the induction of synaptic specialization and maturation, which involves cytoskeletal rearrangement, membrane re-allocation, and molecular complexing (Hoang and Chiba, 1999). How is this first step coupled to the activation of synaptic protein recruitment, and what are the molecular links between synaptic target recognition and synapse maturation?

A first-step towards answering these questions is to find what is common amongst all synaptic targeting regardless of the specific target recognition molecules involved. Through a collaboration with Emiko Suzuki at the University of Tokyo, we have begun to explore what “synaptogenic” means to a neuron and its partner cell at the ultrastructural level. Electron micrographs show that the RP3 growth cone and muscles 6 and 7, its synaptic partner cells, normally form close cellular contact sites (Suzuki et al., 2000). These contact sites are areas of tight membrane-membrane apposition with partner membranes being typically less than 10 nm apart from each other. Such membrane apposition, as well as the accumulation of subcellular electron-dense areas, appears at the same time that the characteristic morphological changes of synaptic target recognition, such as filopodial retraction and prevaricosity formation, are visible at the light microscopic level. The molecular components of such ultrastructural contact sites are not yet determined.

The mature synapse is known to contain a large complement of pre- and post-synaptic proteins specific to neural function and is modulated by a variety of factors, both chemical and electrical (Budnik, 1996; Craig, 1998; Prokop, 1999; Sheng, 1996; Suedhof, 1995). Accumulation of various cell adhesion molecules, such as NCAM, N-Cadherin, and Integrin, occurs consistently (Hynes, 1992; Martin et al., 1996; Redies and Takeichi, 1996; Rutishauser et al., 1988). Clustering of ion channels and neurotransmitter receptors required for proper synaptic function are also well described. Proteins containing PDZ domains, including the vertebrate PSD-95 and Drosophila Discs Large (Dlg), have been identified as adapter molecules necessary for the clustering of certain ion channels (Budnik et al., 1996; Cho et al., 1992). Other molecules shown to affect receptor clustering include gephyrin and rapsyn (Sheng, 1996). Many of these cell adhesion and adapter molecules are widely shared among a variety of synapses. What remains unknown, however, are the molecules responsible for coupling synaptic target recognition molecules, which are unique to synaptic pairs, to such maturation pathways, which are shared among many synaptic pairs.

We suggest two general paradigms to describe the mechanism by which synaptogenic or anti-synaptogenic molecules could be linked to the recruitment of general-purpose adhesion and adapter molecules to the membrane of a given motor neuron: (1) direct coupling between synaptogenic molecules and intracellular synaptic signaling pathways (Fig. 4A) or (2) indirect coupling of these events (Fig. 4B).

Direct coupling refers to the situation in which the molecule(s) primarily responsible for matchmaking directly transduce the initial signal for the initiation of synaptogenesis (Fig. 4A). In this scenario, the cytoplasmic domains of matchmaking molecules would be specialized for the intracellular signaling responsible for the recruitment of synaptic proteins, while their extracellular domains would be capable of mediating strong cell-cell adhesion between specific partner cells. In this paradigm, the removal of cytoplasmic signaling capability would lead to an inability to initiate synaptogenesis. Likely candidates for molecules involved in such direct coupling would be those sharing similar cyto-
plasmic domains but divergent extracellular domains, as such a combination would allow disparate ligand/receptor preferences to converge upon similar signaling pathways. Cytoplasmic domains identified as being necessary for synaptogenic pathway activation could then be further studied through both biochemical and genetic experiments in order to uncover the intracellular pathways common to most synaptogenesis.

Indirect coupling refers to the situation in which the molecule(s) primarily responsible for adhesive matchmaking are distinct from those that mediate the signal to carry out synaptogenesis (Fig. 4B). In this paradigm, the primary event required as the synaptogenic first step is cell-cell adhesion between specific partner cells. This could bring smaller or more weakly adhesive, general-purpose synaptic signaling molecules into close proximity, thus allowing them to begin the synaptogenic cascade. Removal or alteration of the intracellular domains of known synaptic target recognition molecules would lead to no difference in their synaptogenic effects.

Some supportive inference for indirect coupling comes from studies of the glycophasphatidylinositol (GPI)-anchored CON, which lacks any transmembrane or cytoplasmic domains (Nose et al., 1992). GPI-linked molecules as a class are in some cases thought to affect cell signaling indirectly by recruiting sets of signaling-capable proteins to discrete microdomains (Horejsi et al., 1998). Expression of CON by Drosophila S2 cells leads to homophilic aggregation (Nose et al., 1992). Since this protein is unable to directly initiate intracellular signaling on its own, any effects it has on synaptogenic target recognition would necessarily be mediated indirectly, likely as a side effect of its selective adhesion properties. Well-known CAMs such as Fas2, Neuroglian, Integrin, and DN-Cadherin are all expressed ubiquitously in motor neurons and/or muscles during the onset of synaptogenesis, making them potential indirect synaptic coupling molecules (Fig. 2I–L) (Hall and Bieber, 1997; Hoang and Chiba, 1998; Iwai et al., 1997; Lin and Goodman, 1994). Consistent with this idea, overexpression of Fas2 in specific cells has been shown to induce ectopic axon targeting (Lin et al., 1994). In addition to these molecules, Late bloomer, a novel tetraspanin expressed by all Drosophila motor neurons, is also thought to be a good candidate for a general-purpose synaptogenic signaling molecule (Kopczynski et al., 1996).

To date, evidence supporting either direct or indirect coupling is still incomplete. With a recently acquired molecular vocabulary, we can begin to study these questions and design in vivo experiments with more specific molecular contexts than was possible previously.

**CONCLUSIONS**

Synaptic target recognition is a specific form of cell recognition that precedes other synaptogenesis events. Historically, we have seen an evolution of the chemooactivity theory, from simple positive matchmaking to a combination of synaptogenic and anti-synaptogenic molecules, and now to the integration of multiple recognition signaling pathways within each growth cone. Offering high cellular resolution and genetic accessibility, the Drosophila NMJ system has been useful in isolating many of the molecular components in the synaptogenic pathway, as well as testing their in vivo roles. We anticipate that the continued use of this powerful model system will allow further investigations into the mechanisms by which neuron-target matchmaking and intracellular synaptogenesis pathways are linked.
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