

Neuromuscular synapses can form *in vivo* by incorporation of initially aneural postsynaptic specializations

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Summary

Synapse formation requires the coordination of pre- and postsynaptic differentiation. An unresolved question is which steps in the process require interactions between pre- and postsynaptic cells, and which proceed cell-autonomously. One current model is that factors released from presynaptic axons organize postsynaptic differentiation directly beneath the nerve terminal. Here, we used neuromuscular junctions (NMJs) of the zebrafish primary motor system to test this model. Clusters of neurotransmitter (acetylcholine) receptors (AChRs) formed in the central region of the myotome, destined to be synapse-rich, before axons extended and even when axon extension was prevented. Time-lapse imaging revealed that pre-existing clusters on early-born slow (adaxial) muscle

fibers were incorporated into NMJs as axons advanced. Axons were, however, required for the subsequent remodeling and selective stabilization of synaptic clusters that precisely appose post- to presynaptic elements. Thus, motor axons are dispensable for the initial stages of postsynaptic differentiation but are required for later stages. Moreover, many AChR clusters on later-born fast muscle fibers formed at sites that had already been contacted by axons, suggesting heterogeneity in the signaling mechanisms leading to synapse formation by a single axon.

Key words: Acetylcholine receptor, Muscle, Neuromuscular junction, Zebrafish

Introduction

Pre- and postsynaptic elements coordinate the differentiation of one another as synaptogenesis proceeds, leading to the precise apposition of pre- and postsynaptic specializations. The current view of how these interactions elicit postsynaptic differentiation is derived in large part from studies of the skeletal neuromuscular junction (NMJ) *in vitro* and *in vivo* (reviewed by Sanes and Lichtman, 1999; Sanes and Lichtman, 2001). Myotubes cultured alone form high-density aggregates of acetylcholine receptors (AChRs), called hot spots, which are co-localized with numerous extracellular, intracellular and transmembrane proteins that are normally concentrated in the postsynaptic apparatus (Sytkowski et al., 1973; Cohen and Fischbach, 1973; Sanes et al., 1984). When neurons are added to these cultures, they do not selectively innervate hot spots, but rather organize new subneural AChR aggregates; subsequently, the aneural hot spots disperse (Anderson and Cohen, 1978; Frank and Fischbach, 1979; Ziskind-Conhaim et al., 1984). These results suggest that nerves bear molecules that pattern postsynaptic differentiation, and led to the identification of several candidate organizers, based on their effects on cultured myotubes (Sanes and Lichtman, 2001). One of them, agrin, clearly plays a crucial role *in vivo*. An alternatively spliced form of agrin (z-agrin) is selectively expressed by motoneurons, is concentrated at the NMJ *in vivo*

and potently promotes AChR clustering *in vitro* (McMahan, 1990; Ruegg et al., 1992; Ferns et al., 1992; Gesemann et al., 1995). Mutant mice lacking either all forms of agrin or only the z-isoform display lethal defects in postsynaptic differentiation (Gautam et al., 1996; Burgess et al., 1999). Conversely, forced expression of z-agrin in denervated muscle leads to the formation of aneural postsynaptic specializations (Cohen et al., 1997; Jones et al., 1997). These results suggested that z-agrin is a crucial nerve-derived organizer of postsynaptic differentiation.

Despite this impressive evidence, recent observations have raised questions about the ‘synaptic organizer hypothesis’ in general and the ‘agrin hypothesis’ (McMahan, 1990) in particular. In mice genetically engineered to lack either DNA topoisomerase or the HB9 transcription factor, the phrenic nerve fails to form and the diaphragm muscle develops aneurally. Nonetheless, AChR clusters form on myotubes in these muscles, and most reside in the central ‘end-plate’ band through which the nerve usually grows (Yang et al., 2000; Yang et al., 2001; Lin et al., 2001). Likewise, AChR clusters form initially in agrin mutant mice, although they subsequently disperse (Yang et al., 2001; Lin et al., 2001; Misgeld et al., 2005).

At least three explanations can be envisioned for these results. One is that the aneural AChR clusters *in vivo* are akin to ‘hot spots’ on cultured myotubes; growing axons use agrin to organize new postsynaptic sites and disperse ectopic clusters

in vivo as they do in vitro. Second, clustering observed in topoisomerase, HB9 or agrin mutants might be a compensatory response to failed innervation rather than part of a normal developmental progression. Both of these explanations are consistent with the organizer and agrin hypotheses. Less consistent is a third alternative, that the initially aneural AChR clusters might be recognized by axons and incorporated into synapses. In this case, the role of agrin could be to stabilize clusters or to organize subsequent steps in synaptic maturation.

Distinguishing among these alternatives requires analysis of the earliest steps in NMJ formation. Such analysis is difficult in mammals because live imaging of muscles is infeasible at the embryonic stages when NMJs form, and because muscles are innervated by numerous axons that form NMJs asynchronously. We therefore used zebrafish embryos because their transparency allows in vivo imaging and their myotomal muscles are initially innervated by individual, identified axons. We show that, contrary to the predictions of the organizer and agrin hypotheses, motor axons form some NMJs, particularly those on early-born, slow adaxial muscle fibers (Devoto et al., 1996; Blagden et al., 1997; Barresi et al., 2000; Stickney et al., 2000), by incorporating pre-existing postsynaptic structures, and that axonal outgrowth is guided by components associated with pre-existing clusters. Postsynaptic differentiation is nonetheless nerve dependent, in that axons are crucial for the maturation and maintenance of the postsynaptic apparatus and may be required for the formation of AChR clusters on later-born, fast muscle fibers.

Materials and methods

Animals

Wild-type zebrafish were obtained from Fish2U (Gibson, FL) and maintained as previously described (Akimenko et al., 1995). Embryos were raised at 28.5°C in 0.3×Danieau's solution with 100 units/ml penicillin and 100 µg/ml streptomycin. Staging was by the criteria of Kimmel (Kimmel, 1995). In some cases, 0.003% (w/v) 1-phenyl-2-thiourea (PTU) was added to the medium to block pigmentation in the trunk.

Transgenic lines

To label motor axons, we generated transgenic lines in which the expression of GFP or a membrane-associated (farnesylated) derivative of GFP (mGFP) was regulated by genomic elements from the zebrafish *hb9* gene. A PAC clone containing the genomic *hb9* locus (BUSMP706I09178Q2) was isolated by screening the gridded PAC-library 706 (RZPD, Berlin, Germany) with a radioactively labeled *hb9* cDNA probe (AY445044). Restriction analysis and Southern blotting revealed that the *hb9*-coding region, together with 3 kb of 5' sequence, were present on a 5 kb *EcoRI*-fragment. This fragment was subcloned, then the regulatory sequences were amplified by PCR and inserted upstream of cDNAs encoding GFP or mGFP (GAPGFP4) (Moriyoshi et al., 1996). The plasmid DNAs were digested with *EcoRI* and *Acc651*, purified, and injected into one-cell embryos (20 pg/embryo). Resulting transgene-positive adults were mated, and embryos were inspected by fluorescence microscopy, revealing germ-line transmission for seven *hb9*:GFP and two *hb9*:mGFP founders. Motoneurons were labeled in all lines. For each construct, the line with the strongest expression of GFP was selected for study. Further description of the regulatory elements and of expression in non-neural cells will be presented elsewhere (D.M., unpublished).

Histochemistry

For histological analysis, dechorionated embryos were fixed at 4°C

for 4–6 hours with 4% paraformaldehyde, and rinsed in water. Fixed embryos were then stained with znp-1 (Zebrafish International Resources Center, University of Oregon, Eugene, OR), fluorophore-conjugated α -bungarotoxin (BTX; Molecular Probes) and anti-myosin (F59; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA), or fluorophore-conjugated phalloidin (Molecular Probes), followed by appropriate fluorophore-conjugated secondary antibodies. In some cases, embryos were stained live with BTX (see below) before fixation. Animals older than 24 hpf were permeabilized by immersion in acetone for 7 minutes at –20°C, after fixation and before immunostaining.

Some stained embryos were sectioned in a cryostat and counterstained with Neurotrace (Molecular Probes), but most were de-yolked and mounted on slides in glycerol. Initial morphometric analysis was performed on a Zeiss Axioplan2 fluorescent microscope. Confocal images were acquired using 20× (NA0.7), 40× (NA1.15) and 60× (NA1.2) long-working distance water objectives, or a 60× (NA1.45) oil objective. Stacks of optical sections were collected in the *z* dimension. The step size, based on the calculated optimum for each objective, was between 0.25 and 0.5 µm. Subsequently, each stack was collapsed into a single image (maximum intensity or *z*-projection), and additionally rendered to provide views from multiple angles. Analysis was performed offline using the Metamorph Universal Imaging software.

Time-lapse imaging

Embryos were staged, dechorionated, and incubated at room temperature for 45 minutes in L-15 medium containing 5% DMSO and 2.5 µM BTX (Molecular Probes). Embryos were then rinsed in L-15 and mounted on glass coverslips with 1% low-melting temperature agarose in embryo medium containing 0.003% PTU and 0.02% (w/v) tricaine (MS-222). A coverslip was inverted onto a central-well organ culture dish (Falcon #35-3037) containing medium/PTU/tricaine, and the dish was placed into a temperature-controlled stage (Cell Microtemp Systems; 28–30°C) and mounted on a confocal microscope. Stacks of optical sections were collected in the *z* dimension at intervals of 2–5 minutes for a period of 3–8 hours. Initial analysis used collapsed images, but for scoring axon-myotube contacts, we viewed expanded stacks plane by plane. Step size and processing were as described above.

Results

Each segmentally arranged myotomal muscle of the zebrafish trunk is initially innervated by just three primary motoneurons, termed CaP, MiP and RoP. During the first day of embryonic development, these neurons extend axons along stereotyped paths to innervate distinct portions of the myotome (Eisen and Myers, 1986; Myers et al., 1986; Westerfield et al., 1986; Beattie, 2000). Subsequently, additional (secondary) motoneurons extend axons along the paths pioneered by the primary axons (Fashena and Westerfield, 1999). To view NMJ formation in the simplest setting, we focused on the CaP axon, which exits the neural tube ventrally and extends adjacent to the notochord to innervate the ventral-most portion of the myotome (Fig. 1A–D). Moreover, we restricted our analysis to stages before secondary axons had entered the myotome [the first 24 hours post-fertilization (hpf) in rostral segments and 28 hpf in caudal segments]. We were therefore able to study the initial stages of NMJ development by a single, identified motor axon.

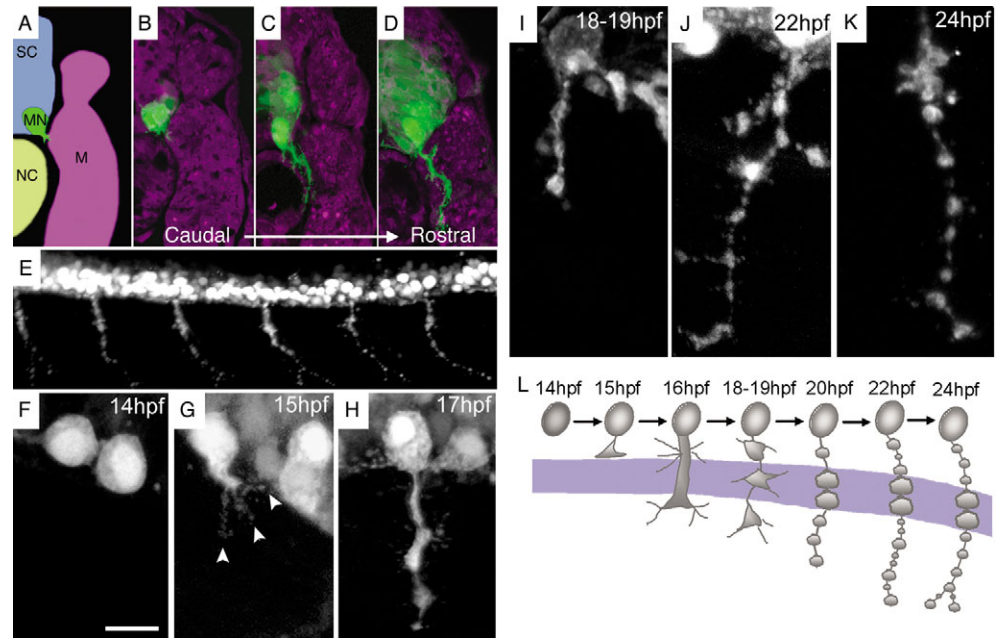
Labeling primary motoneurons with GFP

To image CaP, we sought to label it with Green Fluorescent

Fig. 1. Outgrowth of the CaP axon. (A-D) Cross-sections through a 20 hpf HB9-GFP fish counterstained with Neurotrace-435/455.

(A) Position of the spinal cord (SC), motoneurons (MN), notochord (NC) and myotome (M). (B-D) Caudal (B), central (C) and rostral (D) segments illustrating extension of the CaP motor axon between the notochord and the ventral myotome. Multiple stages are seen in one fish because of the rostrocaudal developmental gradient. (E) Lateral view of six segments from an HB9:GFP fish at 24 hpf, showing the labeling of segmentally arranged primary motor axons. (F-K) Time-course of outgrowth in rostral segments. Motoneurons were present at 14 hpf (F). They began to extend processes tipped by filopodia (arrowheads) by 15 hpf (G), and formed stocky axons by 17 hpf (H). Initially large varicosities at 18 hpf

(I) became interspersed with smaller varicosities by 22 hpf (J); varicosities become more uniform in size by 24 hpf (K). Axons were labeled by GFP in HB9:GFP fish (A-H,J) or by the *znp-1* antibody in wild-type fish (I,K). (L) Schematic of CaP axon outgrowth (not to scale). Lavender band indicates the position of cells that are, or will become, muscle pioneers. Scale bar in F: 10 μ m for F-K.



Protein (GFP). Unfortunately, primary motoneurons are not labeled in previously described transgenic zebrafish with strong neuron-specific expression of GFP (Higashijima et al., 2000; Park et al., 2000; Udvadia, 2001). We therefore generated new lines in which GFP was linked to regulatory elements of the zebrafish *hb9* gene (see Materials and methods). HB9 is a transcription factor that is expressed in motoneurons and required for early stages of their development (Jessell, 2000). Primary motor axons were intensely labeled in HB9:GFP fish (Fig. 1E). CaPs were labeled by 14 hpf, before their axons extended in the myotomes (Fig. 1F). Between 14 and 24 hpf, GFP labeled CaP axons at least as effectively as a well-characterized neuron-specific antibody, *znp-1* (Trevarrow, 1990) (Fig. 1). We conclude that the HB9:GFP line is suitable for following the earliest steps in NMJ formation.

Outgrowth of an identified motor axon

To monitor CaP outgrowth, sets of 30-50 confocal images through GFP- or *znp-1*-labeled fish were obtained at $\sim 0.4 \mu$ m intervals and used to digitally reconstruct blocks of 2-3 myotomes, which were then collapsed into a single projection.

By ~ 14 hpf (in rostral segments), the somata of primary motoneurons were prominent in the ventral portion of the neural tube (Fig. 1F), and the first adaxial myotubes had formed in the myotomes (see below). Over the next hour, CaP initiated axonogenesis by extending filopodia out of the neural tube (see below); in parallel, additional myotubes began to form adjacent to the pioneer cells. Following the filopodia, a broad foot-shaped axon extended, roughly perpendicular to the long axis of the myotubes and midway between their ends (Fig. 1G). Initially thick and uniform in diameter, the axon became variable in diameter by 17 hpf (Fig. 1H), and then broke into large varicosities separated by thin segments (19 hpf, Fig. 1I). Over the next few hours, small varicosities formed between the

larger ones (22 hpf, Fig. 1J). The smallest varicosities enlarged rapidly, so that varicosities were more (though not completely) uniform in size and shape by 24 hpf (Fig. 1K). This stereotyped sequence of steps is summarized in Fig. 1L.

AChR clustering precedes axon-myotube contact

To ascertain the relationship of AChR clusters to growing motor axons, we incubated live embryos with fluorophore-conjugated α -bungarotoxin (BTX), which binds specifically to AChRs, then visualized axons with anti-*znp-1* or GFP, as above. No clusters were detected at 14 hpf, before primary motoneurons extended axons (Fig. 2A). By 16 hpf, however, AChR clusters were present on myotubes at the medial edge of the myotome (Fig. 2B). As new myotubes formed, AChRs clustered on them, in advance of the extending axons (Fig. 2C). All AChR clusters on rostral segments were covered by axons by 20 hpf (Fig. 2D). Thus, AChRs cluster on myotubes before they are innervated.

Myotomes form in a rostral-to-caudal progression. Although the sequence of steps in NMJ formation was generally similar among segments, the degree to which AChR clustering preceded axon extension varied systematically along the rostrocaudal axis (Fig. 2E-I). In rostral segments, AChR clusters were initially present on only the few myotubes directly ahead of the advancing axon (Fig. 2B,C), and axons eventually extended beyond the clusters (Fig. 2D,E,I), consistent with the possibility that axons secrete (or their filopodia contain) an AChR-clustering agent. By contrast, a complete band of AChR clusters formed prior to axon outgrowth in caudal segments of the trunk (Fig. 2F). This difference appears to reflect a position-dependent delay between motoneuron and myotube development. In rostral segments, the first myotubes formed as the CaP axon exited the neural tube; AChRs clustered shortly after myotubes form, and

the clusters were rapidly innervated by the growing CaP axon. In more caudal segments, however, myotubes had formed, and AChRs clustered, across the full width of the myotome before the CaP axon exited the neural tube. This striking mismatch provides additional evidence that AChR clustering is initiated without axonal contact. Moreover, the presence of a full band of clusters in caudal segments does not support the possibility that postsynaptic differentiation is initiated by a mid-range cue emitted from growth cones as they extend.

Aneural AChR clusters are incorporated into NMJs

The observations presented so far suggested that axons incorporate initially aneural AChR clusters into NMJs. Before accepting this conclusion, however, we needed to consider two other possibilities. First, clusters that were aneural at the time of observation might have been transiently innervated at an earlier stage. Second, aneural clusters might have dispersed as axons extended, and been replaced by new, innervated clusters. To assess these alternatives, we imaged NMJ formation using HB9:GFP fish in which AChRs had been labeled with BTX. To ensure that fine filopodial processes did not go undetected, we generated an additional transgenic line that incorporated a membrane-associated (farnesylated) derivative of GFP (HB9:mGFP). Patterns of expression in HB9:mGFP fish were similar to those described above for HB9:GFP fish, but the membrane-targeted derivative provided superior labeling of

filopodia (Fig. 3). Stacks of optical sections were collected at intervals of 2-5 minutes for a period of 4-6 hours, beginning ~16-18 hpf. Segments 7-12 were filmed, because more rostral segments were occluded by yolk.

AChR clusters were present before any filopodia or other processes extended from GFP- or mGFP-labeled CaP axons. In the segment shown in Fig. 3A, for example, clusters were present from the initial view at 17 hpf, but no processes extended from CaP until nearly 19 hpf. (The time of initial CaP extension is later here than in Fig. 1 because it is a more caudal segment; see Fig. 2E.) At this point, a slender filopodium extended from the neuronal somata toward the myotome. Thereafter, numerous additional filopodia extended in multiple directions, many of which subsequently retracted. The filopodia were numerous (up to 20 per neuron at a single time), long (up to 100 μm from the axon shaft to the filopodial tip), and irregular in shape. They were highly dynamic, extending or retracting up to 65 μm in 2 minutes (the time between frames). AChR clusters were present for up to 2 hours prior to the extension of filopodia, and were observed in all 26 segments (from 16 fish) imaged starting at 16-17 hpf.

As filopodia and lamellopodia extended, some contacted previously aneural AChR clusters (Fig. 3A,B). A subset of these contacts remained stable for the remainder of the viewing period, during which time other filopodia extended, contacted additional AChR clusters, or remodeled. Because we collected

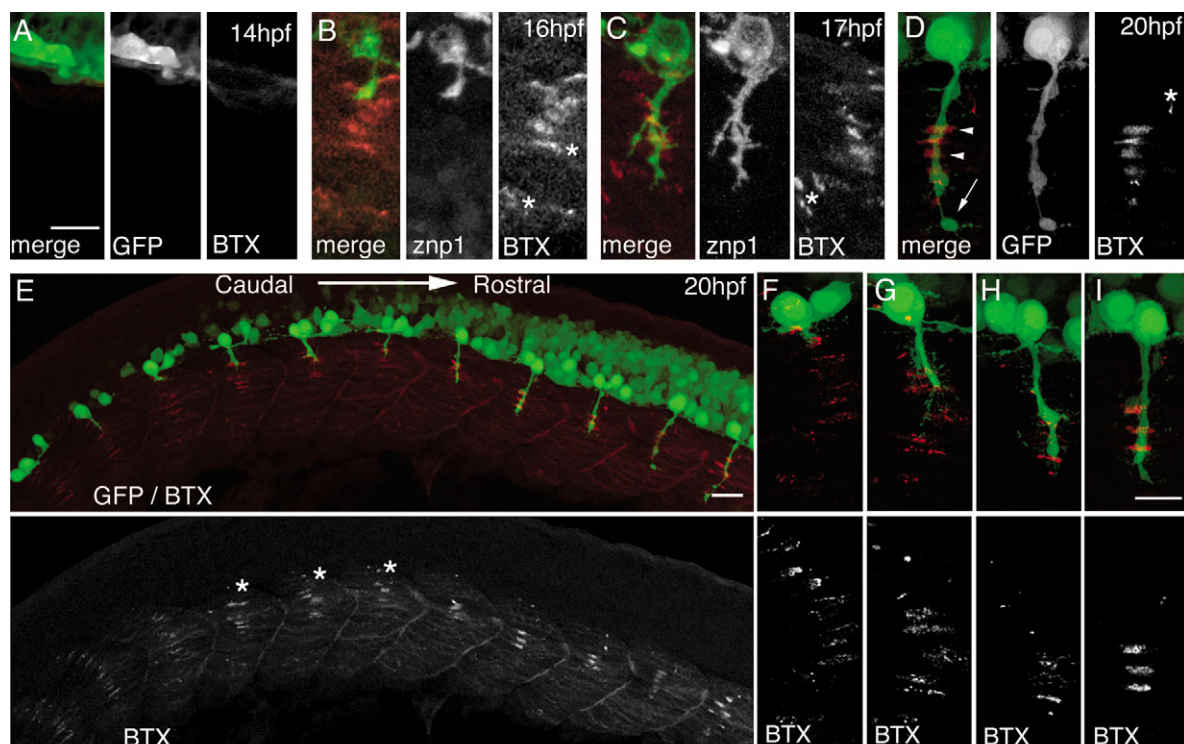
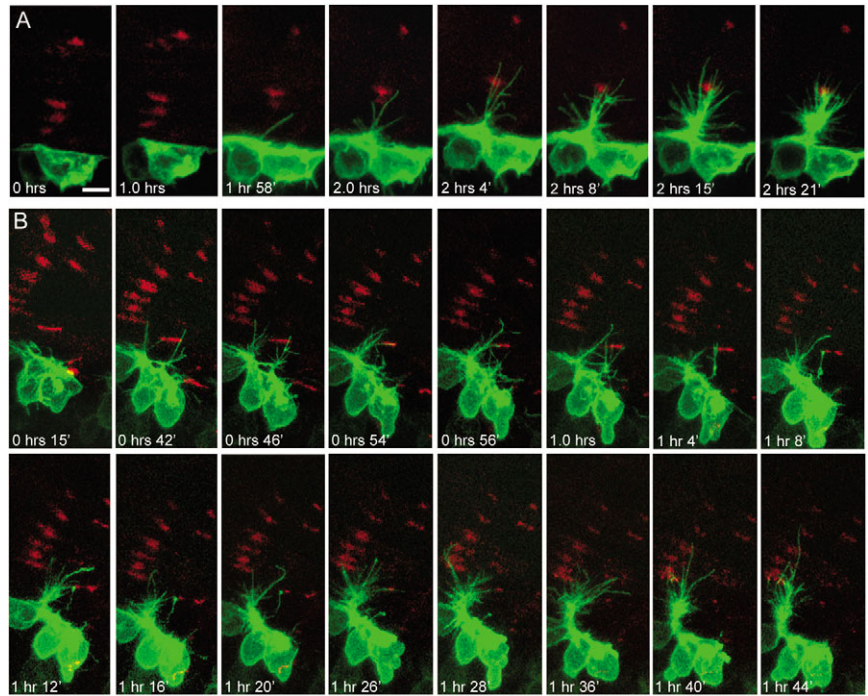


Fig. 2. AChR clustering precedes axon outgrowth. CaP axons (green) labeled with znp-1 (B,C) or GFP (A,D-I) as in Fig. 1; AChRs labeled with BTX (red). (A-D) Segments 1-3. Neither axons nor AChR clusters are present at 14 hpf (A), but clusters are present in front of the advancing axon by 16-17 hpf (B,C). By 20 hpf, (D) most AChR clusters are apposed by axonal varicosities, but some clusters are larger than varicosities (arrowheads) and some axonal processes extend beyond clusters (arrow). Asterisks in B-D indicate some of the AChR clusters that are not innervated; they are quantified in Fig. 6F. (E-I) Whole mount of a 20 hpf fish (E), and higher-power details of individual segments (F-I) showing the rostrocaudal developmental sequence (see also Fig. 1A). In caudal segments (F), AChR clusters have formed but no axon has yet extended. In intermediate segments (G,H), clusters on adaxial cells not contacted by CaP disperse (asterisks in E). In the most rostral segments (I), the CaP axon has extended beyond the ventral-most AChR clusters. Scale bars: in A, 10 μm for A-D; in E, 25 μm ; in I, 10 μm for F-I.

Fig. 3. CaP axons incorporate initially aneural AChR clusters into NMJs. (A,B) HB9:mGFP fish were counterstained with BTX (red) and imaged every 2 minutes for 2 hours starting at 17 hpf (0 hours). In both A and B, AChR clusters were present before filopodia approached them. The axon in B initially pioneered an incorrect path toward a group of clusters caudal to the end-plate band. Later, it retracted and then took a path down the central band. Dorsal is down and rostral to left in all panels. Scale bar: 10 μ m.



images at 2-5 minute intervals, and we never saw initially distinct AChR clusters completely disperse in <15 minutes, we can rule out the possibility that aneural clusters were replaced by distinct, nerve-induced clusters between views. Thus, many AChR clusters form aneurally and at least some NMJs are formed by the incorporation of these initially aneural clusters.

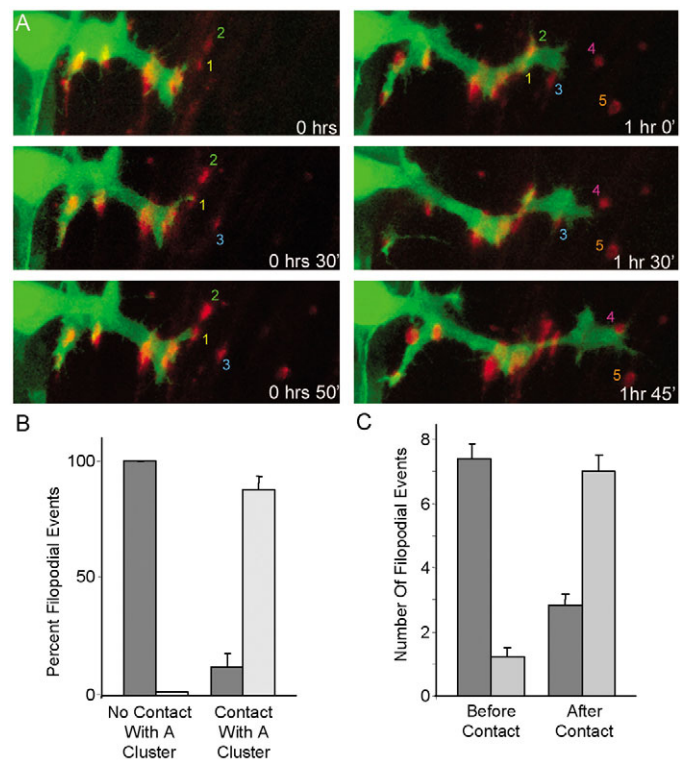
Motor axons recognize AChR clusters

If axonal behavior changed following contact with an AChR cluster, we could infer that axons 'recognize' components closely associated with the cluster. Analysis of time-lapse movies provided three lines of evidence that such changes occurred. First, axonal growth was frequently reoriented following contact with an AChR cluster. In the sequence shown in Fig. 4A, for example, the jagged path of the axon reflects association with a series of AChR clusters that were not arranged in a straight line. This pattern suggests that axons adhere to components associated with clusters.

A second, related line of evidence for the recognition of cluster-associated components by axons was that processes contacting an AChR cluster frequently became stably associated with it. To quantify the selectivity of this stabilization, we analyzed all processes that extended from one CaP axon in each of seven fish. Each process was scored for whether it contacted a cluster of AChRs, and whether it persisted to the end of the imaging period. All of the processes ($n=102$) that failed to contact an AChR cluster retracted, whereas most of the processes (63/71) that contacted an AChR cluster remained associated with it (Fig. 4B).

Fig. 4. CaP axonal behavior changes following contact with AChR clusters. (A) HB9:GFP-labeled CaP reorients its growth repeatedly following contact of its growth cone with clusters. Colored numbers 1-5 indicate initially aneural clusters that growth cones contact. (B) Most of the filopodia that contact an AChR cluster are retained (light bars) until the end of the imaging period, whereas most of those that do not make contact retract (dark bars). ($n=173$ filopodia from 13 cells in seven animals; $P<0.0001$ for both groups.) (C) Filopodial activity is lower in the quadrant surrounding the central band of AChRs (light bars) than in the sum of the other three quadrants (dark bars), prior to the initial contact of a filopodium with a cluster. After the first filopodium contacts a cluster, however, more filopodia extend in the quadrant containing the end-plate band than in the other three quadrants together ($n=1022$ filopodia from 10 cells in five fish; $P<0.0001$ both before and after contact).

Third, once a filopodium contacted an AChR cluster, additional filopodia selectively extended into the region surrounding the point of contact, resulting in a concentration of filopodia in the central band of AChR clusters. To quantify this directional bias, we compared the number of filopodia that extended toward the central band to the number that extended in other directions. Prior to the first contact between a CaP filopodium and an AChR cluster, 22% of the filopodia were



found in the quadrant centered on the end-plate band (90/420 from 10 cells in five animals, quadrants drawn to subdivide a circle centered on the motoneuronal somata), similar to the 25% that would have been expected if extension had occurred randomly in all directions. By contrast, following the initial filopodial contact with an AChR cluster, 71% (426/602) of the filopodia that extended subsequently were in the quadrant centered on the end-plate band (Fig. 4C). Interestingly, recent imaging studies have revealed a similar directed outgrowth of dendritic filopodia during central synaptogenesis in zebrafish (Niell et al., 2004).

The sequence shown in Fig. 3B illustrates several of the phenomena that appear to reflect the local guidance of axons. A filopodium initially contacted a cluster lying rostral to the central band, forming a contact that precisely matched the shape of the cluster. Following initial contact, filopodial activity became focused in its direction. Additional clusters were not encountered, however. Subsequently, the filopodium in contact with the AChR cluster retracted. Following this loss of axonal contact, multiple filopodia extended in all directions, a few of which contacted a proximal AChR cluster in the central band. Filopodia extending beyond this contacted cluster did encounter additional clusters, and the axon extended along the end-plate band. Thus, CaP processes became stably associated with AChR clusters, and the association preceded the reorientation of filopodial activity and axonal extension. Together, these observations suggest that axonal growth is influenced by components associated with AChR clusters.

Distinct AChR clustering patterns on slow and fast muscle fibers

Myotomal muscles contain two types of muscle fiber that differ

in developmental origin and molecular composition. The first-born fibers, called adaxial, form from myoblasts near the notochord. They contain slow myosin, and initially form a monolayer on the medial surface of the somite. Most of the adaxial fibers then migrate radially to the lateral edge of the myotome. The few adaxial fibers nearest the horizontal midline (2-6 per segment) do not migrate and remain near the notochord; they have been called 'muscle pioneers'. As migration occurs, myoblasts of a second population (lateral presomitic cells) fuse in the space between the notochord and the adaxial cells, giving rise to fast muscle fibers (Devoto et al., 1996; Blagden et al., 1997; Barresi et al., 2000; Stickney et al., 2000). These events, like the other aspects of neuromuscular development described above, occur in a rostrocaudal sequence (Fig. 5A'-D').

Examination of cross-sectioned myotomes from HB9:GFP fish revealed the relationship of the CaP axons to these two fiber types. Nearly all of the AChR clusters described above, which were imaged at ≤ 20 hpf, were found on adaxial fibers. Many of these fibers acquired AChR clusters before the CaP axon left the neural tube (Fig. 5A; see also Fig. 2B,F). The radial migration of adaxial fibers began as the CaP axons extended, so most of the fibers that CaP contacted were muscle pioneers (Fig. 5B,F; see also Fig. 2C,G). AChR clusters on these fibers were incorporated into NMJs (Fig. 3). AChR clusters on adaxial cells migrating laterally were not contacted by the CaP axon, which remained on the medial surface of the myotome; these clusters then dispersed (Fig. 2E).

After forming NMJs on muscle pioneers, the CaP axon extended past them. CaP remained on the medial surface of the myotome, and thereby contacted myoblasts of the later-born population, which were fusing to form fast muscle fibers (Fig.

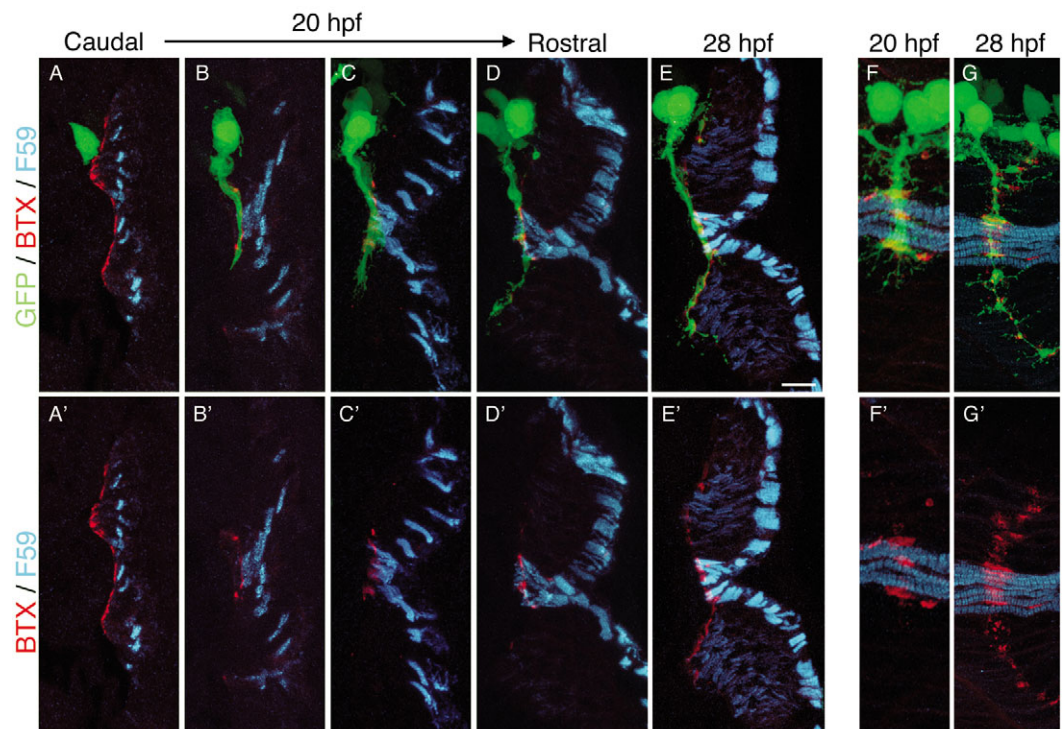


Fig. 5. Distinct modes of synapse formation on muscle pioneers and fast fibers. Cross-sections through HB9:GFP fish at 20 hpf (A-D,F) or 28 hpf (E,G) were stained with anti-myosin (F59) and BTX. (A-D) CaP extends to contact muscle pioneers as the other adaxial fibers migrate radially. (E) CaP then extends past the pioneers as fast, weakly F59-immunoreactive, fibers form. AChR clusters on migrating adaxial cells are lost and new clusters form on fast fibers as, or after, they are contacted by CaP. As in Figs 1 and 2, the rostrocaudal sequence of development allows the visualization of many stages in a single fish. (F,G) En face views at 20 hpf and 28 hpf to show that AChR clusters on muscle pioneers are larger and more elongated than those on fast fibers. A'-G' show F59 and BTX, but not axons, to highlight AChR cluster position and morphology. Scale bar in E: 10 μ m for all panels.

5E,G; see also Fig. 2D). New AChR clusters formed beneath the advancing axon, usually a few myotubes behind the tip of the growth cone (Fig. 5F,G; see also Fig. 2I). Clusters on the fast fibers were generally smaller than those on pioneers (Fig. 5G). Thus, AChR clusters formed on adaxial cells before CaP had contacted them but formed on fast fibers following axonal contact.

Once it reached the distal (ventral) edge of the myotome, CaP had contacted all fibers on the medial surface, and had initiated the formation of NMJs on them. Observations reported here are restricted to these NMJs. Subsequently, during the second day of embryogenesis, CaP, and the secondary motor axons that followed it, branched and turned to innervate fibers deeper in the myotome (Beattie, 2000).

Formation of precise synaptic appositions

Apposition of the CaP axon to AChR clusters was initially imprecise, in that some clusters were only partially covered by axonal processes, and some axonal segments extended beyond the borders of clusters (e.g. Fig. 2D-I, Fig. 6A,B). Apposition between axonal varicosities and AChR clusters became precise by 22-24 hpf in rostral segments. At this time, each axonal varicosity was centered over a myotube, each myotube bore a single AChR cluster, and each axonal varicosity was precisely aligned with the cluster (Fig. 6C-E).

Morphometric analysis of confocal images revealed five processes that contributed to the matching of axonal varicosities to AChR clusters. First, CaP axons became apposed to AChR clusters in the center of myotubes as they extended (Fig. 6F, dotted line). Second, aneural clusters lateral to the central endplate band (asterisks in Fig. 2B-D) were lost after the initial cluster had been contacted (Fig. 6F, solid line). Third, clusters on adaxial fibers other than the pioneers were lost as those fibers migrated laterally and became displaced from CaP (Figs 2E). Fourth, protrusions from the main axonal shaft that were not in contact with clusters were lost (Fig. 2C, Fig. 6A). Finally, clusters were often initially larger than even the largest varicosities that overlay them, but they decreased in size over a 3-5 hour period to match the size of the varicosities (Fig. 6G). The fact that matching occurred by shrinkage of the AChR cluster rather than by expansion of the varicosity suggests that the nerve terminal influences postsynaptic morphology.

Axons remodel and stabilize AChR clusters

Although AChR clusters form aneurally, axons might affect their fate. An opportunity to test this idea was provided by a fortuitous observation on HB9:mGFP transgenic fish. As expected, motor axons were unlabeled in approximately one-quarter of the offspring from matings between HB9:mGFP heterozygotes, and were labeled in approximately three-quarters; fluorescence was clearly brighter in approximately one-third of the mGFP-positive fish than it was in the remaining two-thirds, indicating that the brighter third were homozygous. Primary motor axons, including the CaP axons, failed to extend in ~70% of the segments of such putative homozygotes (called HB9:mGFP^{+/+}; Fig. 7A). Time-lapse imaging of HB9:mGFP^{+/+} fish from 14-22 hpf failed to reveal any axonal or filopodial extension in segments that were still axon-free at 22 hpf, whereas normal axon extension occurred in adjacent segments (data not shown). Thus, axon-free

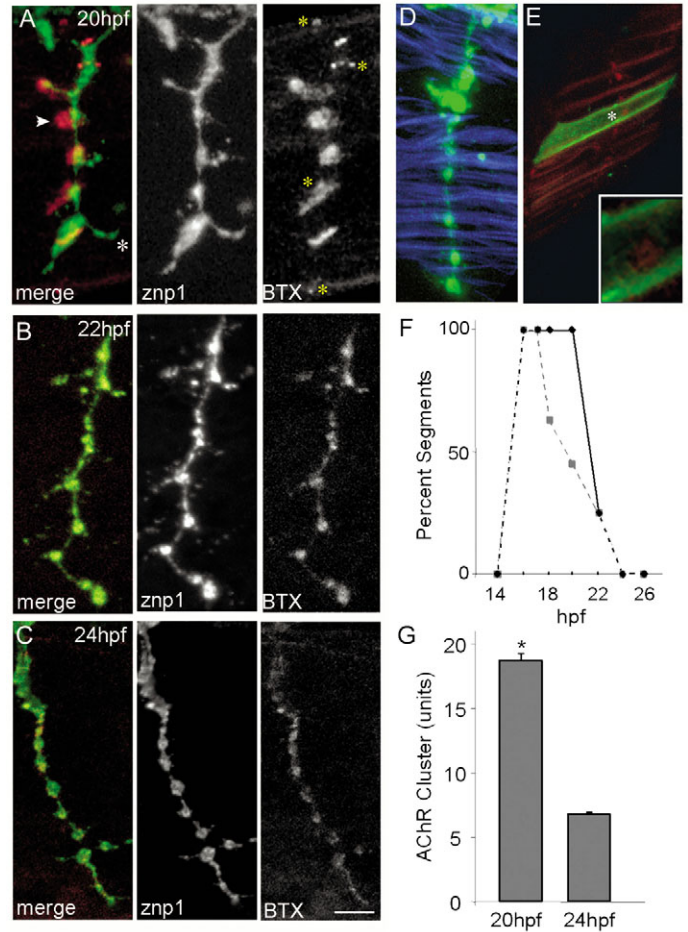


Fig. 6. Apposition of CaP axon varicosities to AChR clusters. (A-C) Axons and AChRs labeled with znp-1 (green) and BTX (red), respectively. By 20 hpf, axonal varicosities are apposed to AChR clusters, but some clusters are larger than varicosities (arrowhead) and some axonal processes extend beyond clusters (asterisk). Yellow asterisks indicate AChR clusters that are not innervated. Apposition becomes precise by 24 hpf (B,C). At this time, pre- and postsynaptic specializations are perfectly aligned (C), varicosities are centered on myotubes (D; phalloidin in blue) and each myotube bears a single AChR cluster (E; single myotube labeled with mYFP by mosaic expression of a transgene injected into a one-cell embryo). The inset in E is a higher-power view of the single cluster indicated by the asterisk. (F) Progressive loss of aneural clusters. Aneural clusters lying rostral or caudal to CaP disperse (solid line), whereas many of the aneural clusters in front of the axon (dashed line) are incorporated into NMJs ($n=10-64$, average=38, segments per stage). (G) Cluster size decreases between 20 and 24 hpf ($P<0.0001$ by t -test; $n=250$ clusters per stage). Scale bar in C: 5 μm for A-E.

myotomal segments were aneural (not innervated) rather than being transiently innervated (denervated). The failure of extension reflected a toxic effect of high levels of mGFP rather than a lost function of a gene interrupted by transgene insertion, because attenuating mGFP expression by injecting a morpholino oligonucleotide complementary to sequences in the transgene restored axon formation in HB9:mGFP^{+/+} fish (data not shown). Thus, overexpression of mGFP in HB9:mGFP^{+/+} fish led to cell-autonomous blockade of axon outgrowth.

AChR clusters appeared on schedule in HB9:GFP^{+/+} fish, both in innervated segments and in segments that lacked CaP axons, as judged by comparison with wild-type fish. Clusters in segments that were not innervated were initially indistinguishable in number and size from those in adjacent innervated segments (Fig. 7B,F,G). By ~22 hpf, complete bands of AChR clusters extended along the dorsoventral axis of all segments, consistent with the idea that clusters form

without axonal contact. At later stages, however, AChR clusters in uninnervated segments differed from those in innervated segments in three ways.

First, between 19 and 22 hpf, the number of clusters increased >2-fold in segments that were not innervated but changed little in innervated segments (Fig. 7F). The lack of a net change in innervated segments reflects a balance between the formation of NMJs on newly formed myotubes, and the loss of aneural clusters from innervated myotubes ('side clusters', Fig. 6F) and from adaxial myotubes migrating laterally. Staining with anti-myosin or the actin-binding toxin phalloidin revealed no difference in myotube shape or number between innervated segments and segments that were not innervated (data not shown). Accordingly, the presence of supernumerary clusters in segments that were not innervated suggests that the dispersal of aneural clusters in innervated myotubes requires the presence of an axon.

Second, between 22 hpf and 24 hpf, the number of clusters increased in innervated segments, reflecting the formation of additional myotubes, but decreased by >80% in segments that were not innervated (Fig. 7C,D,F). By 26 hpf, most of the segments that lacked axons were completely devoid of AChR clusters (data not shown). Thus, although AChR clusters form aneurally, their maintenance requires innervation.

Third, between 22 hpf and 24 hpf, AChR cluster size decreased less in segments that were not innervated than in innervated segments or in control fish (Figs 7G). This result supports the hypothesis proposed above, that the generation of a perfect apposition between pre- and postsynaptic specializations involves axon-dependent remodeling of the AChR cluster to match the outline of the axonal varicosity.

Taken together, these results suggest that innervation is essential for the remodeling and maintenance of AChR clusters. Observations at a later stage provide evidence against an alternative interpretation, that a lack of innervation leads to defects in the ability of the myotube to participate in synaptogenesis. Although primary axon outgrowth was defective in HB9:mGFP^{+/+} fish, secondary axons grew into most segments, including those that were initially not innervated. The secondary axons formed NMJs in which terminal varicosities were apposed to AChR clusters (Fig. 7E);

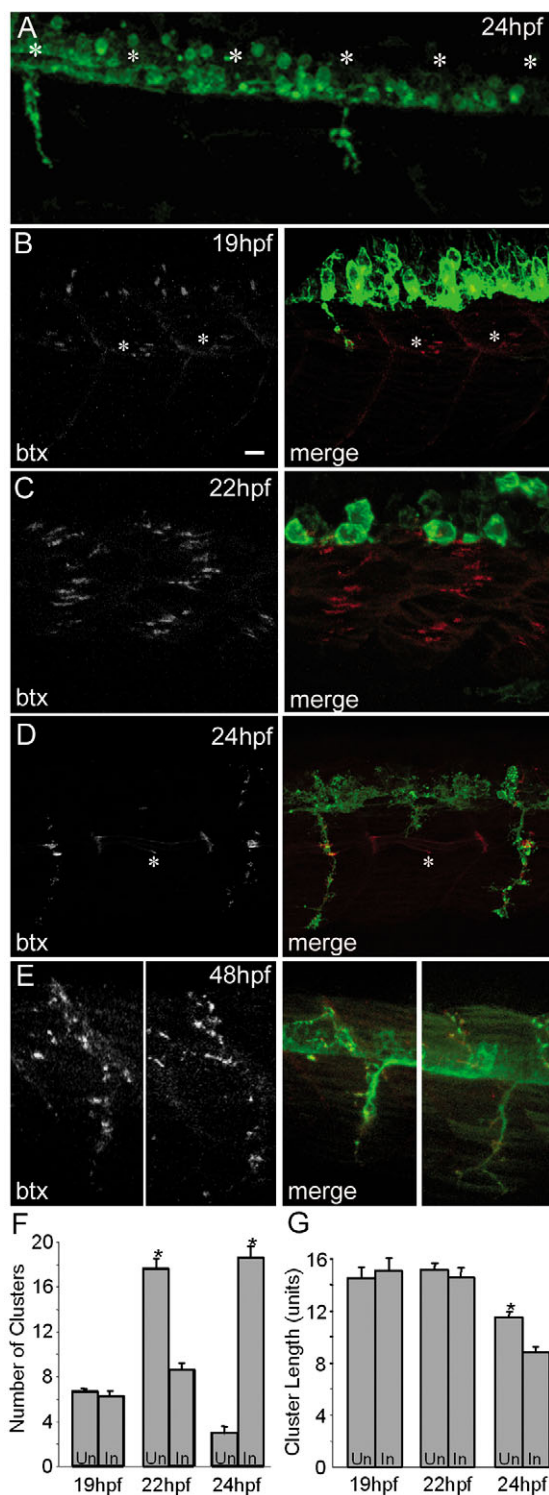


Fig. 7. AChR clusters form but do not mature or persist in the absence of axons. (A) An HB9:mGFP^{+/+} fish at 24 hpf. Out of six segments shown (asterisks), axons extend in only two. (B-E) HB9:mGFP^{+/+} fish at 19 (B), 22 (C), 24 (D) and 48 (E) hpf, counterstained with BTX (red). Bands of AChR clusters formed in segments that were not innervated, but did not persist. AChRs did cluster following outgrowth of secondary axons (E). Asterisks in B indicate aneural clusters; asterisk in D indicates muscle pioneers without clusters. (F) Analysis of cluster number in segments that were not innervated (Un) and in innervated segments (In) of HB9:mGFP^{+/+} fish. Segments that were not innervated contained the same number of clusters as innervated segments at 19 hpf, but contained nearly twice as many by 22 hpf. In the absence of innervation, most clusters disperse by 24 hpf ($n=10-35$ segments per bar, average=16; $P=0.82$ at 19 hpf, $P<0.0001$ at 22 hpf, $P<0.0001$ at 24 hpf). (G) Clusters present in innervated segments and in segments that were not innervated were similar in length at 19 and 22 hpf, but differed at 24 hpf ($n=30-102$ clusters per bar, average=60; $P=0.31$ at 19 hpf, $P=0.92$ at 22 hpf, and $P<0.05$ at 24 hpf). Scale bar in B: 10 μ m for A-E.

in some cases, these fish survived for at least 2 additional days. This result indicates that segments that were not innervated remained competent to form and maintain AChR clusters and NMJs.

Discussion

Early steps in the formation of the vertebrate NMJ have been studied extensively using co-cultures and fixed tissue (e.g. Anderson and Cohen, 1978; Frank and Fischbach, 1979; Gautam et al., 1996; Burgess et al., 1999; Cohen et al., 1997; Jones et al., 1997; Yang et al., 2000; Yang et al., 2001; Lin et al., 2001; Misgeld et al., 2002) (reviewed by Grinnell, 1995; Sanes and Lichtman, 1999; Sanes and Lichtman, 2001). Time-lapse imaging has also enriched our view of NMJ development, but has been applied largely to later, postnatal aspects of synaptic rearrangement (Walsh and Lichtman, 2003). Here, we have exploited the simplicity and accessibility of the primary motor system in zebrafish to image embryonic NMJs as they form. Our results lead us to draw three main conclusions.

First, AChR clusters form on myotubes that have not yet been contacted by axons, and at least some NMJs form by the incorporation of these pre-existing AChR clusters. This view differs from the classical one, that growing axons organize AChR clusters at sites of contact with myotubes, and disperse aneural clusters that they do not contact (Anderson and Cohen, 1978; Frank and Fischbach, 1979; Ziskind-Conhaim et al., 1984; McMahan, 1990; Sanes and Lichtman, 1999). It is consistent with more recent studies showing that AChR clusters form in aneural mouse muscles (Yang et al., 2000; Yang et al., 2001; Lin et al., 2001) but, as explained in the Introduction, those studies were also consistent with the conventional view.

Second, although motor axons are not required for the formation of AChR clusters, they affect postsynaptic development in multiple ways. (1) Clusters remodel after they are contacted by axons, leading to the precise apposition of pre- and postsynaptic specializations (Fig. 6A-C). (2) Aneural clusters are lost soon after NMJs form (Fig. 6F). (3) All AChR clusters are eventually lost if axons fail to extend (Fig. 7). These observations support the idea that axons both disperse aneural and maintain innervated clusters, and thus suggest that nerves provide both stabilization factors that act locally and dispersal signals that act at a distance. A crucial local stabilization factor is likely to be z-agrin; indeed, defects in agrin mutant mice (Burgess et al., 1999; Yang et al., 2001; Lin et al., 2001) can be explained by a loss of this stabilizing influence (Misgeld et al., 2005), even though they were initially interpreted as supporting the idea that agrin acts as a clustering agent *in vivo*, as it does *in vitro* (McMahan, 1990; Ruegg et al., 1992; Ferns et al., 1992; Gesemann et al., 1995). Recent studies of mutant mice suggest that the neurotransmitter, acetylcholine, is a crucial dispersal signal *in vivo* (Misgeld et al., 2005; Lin et al., 2005), although there is also evidence that locally presented factors, such as agrin, can promote AChR cluster dispersal at a distance *in vitro* (Madhavan et al., 2003).

Third, a single motor axon can form synapses in two distinct ways: by the incorporation of initially aneural AChR clusters or by the organization of clusters following contact with a myotube. For CaP, it appears that these modes correspond to distinct fiber types, slow/pioneer and fast, respectively (Fig. 5).

The correspondence might reflect an intrinsic, qualitative difference in synaptogenic ability between the two fiber types, but it is also possible that it results only from the different times at which the two populations are born. Adaxial/pioneer fibers form considerably before they are contacted by CaP, whereas fast fibers fuse as CaP contacts them. If AChR clustering can occur aneurally a few hours following myotube formation, the developmental sequence would lead to the formation of CaP-encountering clusters on muscle pioneers but not on fast myotubes. Thus, consistent with results in mammals (Misgeld et al., 2005), axon-derived factors such as agrin might promote AChR clustering on some myotubes but serve to prevent dispersal on others.

Although the primary motor system of zebrafish is unique in some respects (Beattie, 2000), several observations suggest that our conclusions may be applicable to the more commonly studied NMJs of birds and mammals. (1) Some aneural AChR clusters are present in rodent and chick muscles, in the vicinity of axons (Lin et al., 2001; Smith and Slater, 1983), and, in one case, in advance of axonal contact (Dahm and Landmesser, 1991). (2) AChR clusters extend beyond nerve terminals at early stages in the formation of the mouse NMJ (Matthews-Bellinger and Salpeter, 1983). (3) AChR clusters shrink transiently perinatally in rodent muscle before growth resumes postnatally (Bevan and Steinbach, 1977).

Our work is based on the pioneering studies of Eisen, Westerfield and colleagues (Eisen and Meyers, 1986; Meyers et al., 1986; Westerfield et al., 1986; Fashena and Westerfield, 1999; Westerfield et al., 1990; Sepich et al., 1998; Liu and Westerfield, 1992). In general, our results are consistent with theirs, but they differ in one respect: they did not observe aneural AChR clusters prior to axon outgrowth or in segments rendered aneural by the ablation of primary motoneurons (Liu and Westerfield, 1992). We believe this difference is due to the improved sensitivity of fluorescence detection methods and the improved resolution of confocal imaging. Aneural AChR clusters are smaller and dimmer than those at mature NMJs, and thus might have been missed previously. Moreover, clusters on aneural segments are transient (Fig. 7), and many of the observations in those previous studies were made after they would have disappeared.

In summary, we have provided evidence that some embryonic NMJs are formed by the incorporation of initially aneural postsynaptic specializations. This scenario differs from the widely accepted one in which axons emit signals that organize the differentiation of postsynaptic sites beneath nerve terminals, and components organized by the axon then promote presynaptic differentiation. It is important to note, however, that the two scenarios are not mutually exclusive. Indeed, both appear to operate within a single myotome. It is possible that some differences among muscles in patterns of synaptogenesis and responsiveness to agrin (Pun et al., 2002) result from the predominant use of one mechanism or the other. Moreover, the roles we document for axons in remodeling postsynaptic sites once they are formed are common to both scenarios. Finally, most models for synaptogenesis in the central nervous system are based on the idea that postsynaptic differentiation occurs subsequent and consequent to axonal contact (Li and Sheng, 2003). However, neurons, like myotubes, can organize postsynaptic specializations in the absence of input (Rao et al., 1998). It will be interesting to learn whether some central

synapses form by incorporation of initially aneural postsynaptic sites, using mechanisms analogous to those described here for the NMJ.

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