

Population Density Regulates *Drosophila* Synaptic Morphology in a Fasciclin-II-Dependent Manner

Bryan A. Stewart, Jesse R. McLean

Department of Life Sciences and Zoology, University of Toronto at Scarborough,
Toronto, ON, Canada

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ABSTRACT: Genetic analysis of the *Drosophila* larval neuromuscular junction has identified some of the key molecules that regulate synaptic plasticity. Among these molecules, the expression level of Fasciclin II (FasII), a homophilic cell adhesion molecule, is critically important for determining the final form of the neuromuscular junction. Genetic reduction of FasII expression by 50% yields more elaborate nerve terminals, while a greater reduction in expression, to 10% of wild-type, yields a substantial reduction in the nerve terminal morphology. Importantly, regulation of FasII expression seems to be the final output for several genetic manipulations that transform NMJ morphology. In an effort to understand the importance of this regulatory pathway in the normal animal, we have undertaken studies to identify environmental cues that might be important for initiating FasII-dependent changes in synaptic plasticity. Here we report on the relationship between larval population density and synaptic morphology, synaptic

strength, and FasII levels. We raised *Drosophila* larvae under conditions of increasing population density and found an inverse exponential relationship between population density and the number of synaptic boutons, the number of branches, and the length of branches. We also observed population-dependent alteration in FasII levels, with lower densities having less FasII at the synapse. The correlation between density and morphological change was abrogated in larvae constitutively expressing FasII, and in wild-type larvae grown on soft culture medium. Together these data show that environmental cues can induce regulation of FasII. Interestingly, however, the quantal content of synaptic transmission was not different among the different population densities, suggesting that other factors contribute to maintaining synaptic strength at a defined level. © 2004 Wiley Periodicals, Inc. *J Neurobiol* 00: 000–000, 2004
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INTRODUCTION

Analysis of synaptic plasticity at the *Drosophila* neuromuscular junction (NMJ) has revealed a complex repertoire of alterations that can be observed with

specific genetic lesions. Some mutations or transgenics show very elaborate expansions or retractions of the larval NMJ while others show changes that are more modest depending upon the mutation under study (Budnik et al., 1990; Zhong et al., 1992; Jarecki and Keshishian, 1995; Stewart et al., 1996, 2002; Wan et al., 2000; Sweeney and Davis, 2002). Among the identified molecules thought to play an important role at this synapse, Fasciclin II (FasII) has emerged as a key molecule that can mediate growth- and activity-dependent changes in synapse morphology (Schuster et al., 1996a,b; Baines et al., 2002). Indeed, this molecule seems to be the final common output for several activity-dependent processes that can regulate synaptic plasticity.

Correspondence to: B. Stewart (stewart@utsc.utoronto.ca).
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While identifying the key molecules important for synaptic plasticity has been successful using the genetic approach, it is presently not clear to what extent the identified mechanisms are used in the normal animal to coordinate the growth and function of the synapse. Recent work has shown that manipulations of *Drosophila* larval activity by temperature, or crawling substrate, can increase glutamate receptor density and decrease FasII levels at the NMJ (Sigrist et al., 2003). There is an increase in synaptic strength and synaptic bouton number accompanying these changes in protein expression. This study begins to address the *in vivo* mechanisms that contribute to synaptic plasticity at this synapse, and we seek to identify additional environmental cues that might use similar molecular pathways and contribute to normal synaptic development.

One parameter that is well known to regulate normal *Drosophila* development is population density (Joshi and Mueller, 1988; Joshi, 1997). Indeed, it is easy to observe that extreme overcrowding of *Drosophila* cultures yields smaller larvae and adults. The relationship between population density and synaptic morphology at larval NMJs has been anecdotally noted by some authors, and indeed many studies attempt to control for population density by limiting the amount of egg-laying time available to gravid females. However, a quantitative examination of the relationship between the number of larvae in a vial and synaptic morphology has not been formally presented. As a first step towards identifying potential environmental cues that might regulate synaptic plasticity, here we carefully control the number of larvae in each culture and we report that even small changes in population density have substantial impacts on the larval NMJ morphology, and these changes occur in a FasII-dependent manner.

MATERIALS AND METHODS

Fly Stocks and Rearing Conditions

The *yw* strain of *Drosophila melanogaster* was used as the control strain throughout. We additionally used the UAS/Gal4 system to express FasII. B185 Gal4 (Davis et al., 1997) was used to drive expression of UAS-FasII motor neurons and muscle (Davis et al., 1997).

For our larval culture media, we used the Bloomington stock center recipe for *Drosophila* media (<http://flystocks.bio.indiana.edu/media-recipes.htm>). Each 10 L of food contained: 169 g of brewer's yeast, 98 g soy flour, 713 g yellow corn meal, 450 g of dehydrated light malt extract, 28 g agar, 0.75 L corn syrup, 47 mL propionic acid, and distilled water to 10 L. The amount of agar indicated here is less than that

called for in the Bloomington recipe, and it should be titrated for each brand to give appropriately hard food. As such, experiments that utilized "soft food" had one-half the amount of agar as our standard food.

Adult flies were allowed to lay eggs on grape-agar media overnight. Eggs were then collected, counted, and placed directly into 26 mm shell vials containing ≈ 10 mL of the food. The larvae then developed at room temperature until the wandering third instar stage.

Immunocytochemistry and Image Processing

Third instar larvae were dissected along the dorsal midline in zero calcium HL3 saline (Stewart et al., 1994). Upon removal of the digestive tract, fat bodies, and main trachea, the preparations were fixed in 3.7% formaldehyde in phosphate buffered saline (PBS) for 10 min. After fixation, the tissue was washed in PBS plus 0.1% Triton X-100 (PBT) for 10 min, blocked in 5% normal goat serum, and then incubated with a 1:1000 dilution of FITC-conjugated goat anti-HRP (ICN Biomedical), an antibody that cross-reacts with a glycoprotein on neural membranes. After 1–2 h incubation at room temperature, we then washed the tissues in PBT, prior to mounting in Vectashield (Vector Laboratories).

We imaged a z-stack of the NMJs on a Zeiss LSM 510 confocal microscope and projected the stack onto a single plane for morphometric analysis. We collected images of muscle 6/7 from abdominal segment 3 or 4. Projected images were exported to ImageJ software, which was used to measure all morphometric parameters. We quantified the muscle surface area of the same preparations on a Nikon microscope equipped with a Hamamatsu Orca ER CCD camera. The images from this camera were obtained, and the surface area measurements made, with SimplePCI (Compix, Inc.) software.

To quantitate the level of FasII expression at the NMJ, the larvae were dissected and processed as described above. Following blockade in NGS, the tissue was incubated in a 1:5 dilution of mAb 1D4 (anti-FasII; gift of Alex Kolodkin) overnight at 4°C. The tissue was then washed in PBT for 30 min and then incubated in 1:500 goat antimouse Alexafluor 546 (Molecular Probes, Inc.) for 2 h at room temperature. We then washed the tissues in PBT for 20–30 min prior to mounting the tissue in Vectashield (Vector Laboratories, Inc.). Larvae from each class were dissected and processed together in the same microcentrifuge tube to ensure all larvae were exposed to the same concentration of antibodies.

We measured FasII immunoreactivity from larvae raised in the 10 and 100 larvae per vial classes with the Zeiss LSM 510 confocal microscope. We first imaged type Ib boutons for muscle 4 from larvae of the 100 per vial class. Muscle 4 was used because its NMJ is relatively flat and can be imaged in a single 512×512 pixel frame. Confocal gain settings were optimized for these NMJs such that the brightest pixels were just below the saturating level. We maintained these settings for collecting images of muscle 4 from larvae of the 10 per vial class. To quantitate the level of

fluorescence, projected z-stacks were exported to ImageJ (<http://rsb.info.nih.gov/ij/>) and the entire NMJ was manually outlined. The mean pixel intensity from this was used as the measure of FasII levels. To account for small variation in fluorescence levels, we measured the background fluorescence on a nonsynaptic region of the muscle and subtracted this value from the mean synaptic value for each image. To create a false-color image, the grayscale confocal image was converted to an indexed color image using the spectrum lookup table in Adobe PhotoShop.

Curve-fitting and statistical tests were done with Graph-Pad Prism 3.0 software. For comparison of two population means we used the Student's *t* test, with $p < 0.05$ chosen as the level of statistical significance. For comparison of the effects of multiple genotypes and densities we used two-way analysis of variance to test for significant variation; subsequently the Bonferroni post-test was used to test for statistical significance within genotypes at different densities, with $p < 0.05$ considered significant.

Electrophysiology

Measurements of spontaneous and evoked synaptic potentials were collected from muscle 6 of abdominal segments 3 and 4, and were performed exactly as previously described (Stewart et al., 1994) in HL3 physiological solution with 0.5 mM CaCl₂. The appropriate segmental nerve was stimulated with a suction electrode at a frequency of 0.2 Hz, at a voltage set to recruit both motor axons that innervate the muscle. Data were recorded from no more than two cells per larvae.

RESULTS

Relationship between Population Density and Synaptic Morphology

In this study, we collected and counted the number of eggs in a vial and then analyzed the NMJs for total branch length, the number of synaptic boutons, and the number of secondary branches on muscles 6 and 7 at the third instar larval stage. We found that each of these parameters followed an exponentially decaying relationship with the number of larvae, with each parameter changing rapidly between 10 and 50 larvae per vial and reaching a plateau by 100 larvae per vial (Fig. 1). For example, the measured length of all branches of the NMJ was $380 \pm 21.5 \mu\text{m}$ (mean \pm SEM throughout) from larvae raised in a vial with 10 larvae, compared to $262 \pm 15.5 \mu\text{m}$ from larvae raised with 50 larvae per vial, and $250 \pm 23.0 \mu\text{m}$ from larvae raised with 100 larvae per vial. The exponential relationship describing these data has a decay constant \pm SE equal to 20.1 ± 8.0 (# of larvae). Table 1(A)

summarizes the results of our curve fitting to these data and shows that each of the morphometric parameters that we analyzed changes rapidly within the narrow range of 10–50 larvae.

These observations were not due to differences in muscle size among the larvae raised at the different population densities. When we normalized branch length, bouton number, and the number of secondary branches to the muscle surface area there was no change in the relationship between these morphometric parameters and population density; all parameters continued to show an exponential relationship to population density [Table 1(B)]. Furthermore, there were no statistically significant differences in the muscle surface area recorded in larvae from the different conditions (data not shown). While it is known that severe overcrowding of *Drosophila* leads to smaller adults and larvae, the presence of up to 200 larvae per vial did not substantially alter the muscle size.

Population Density Regulates Synaptic Levels of FasII

In order to determine if population density invoked similar molecular and cellular mechanisms that have been previously described in mutant studies, we analyzed the amount of FasII present at muscle 4 NMJs of larvae from vials with 10 or 100 larvae per vial. We used quantitative immunofluorescence of mAb 1D4 (anti-FasII) to estimate the amount of FasII at the synapse and we found that larvae from 10 larvae per vial had significantly less ($p < 0.01$) perisynaptic FasII than their counterparts grown at 100 larvae per vial. Figure 2 shows characteristic NMJs from the two density conditions and a summary of the mean pixel intensity measured for all of the samples ($n = 9$ NMJs for each condition). We found that the pixel intensity was 35% greater from larvae raised at 100 larvae per vial.

Constitutive Expression of FasII Prevents Population-Dependent Change in Synapse Morphology

If FasII contributes to density-dependent regulation of synaptic morphology, then blocking the animal's ability to regulate FasII, by constitutively expressing a FasII transgene, should remove the density-dependent effects. Accordingly we crossed B185-Gal4, a Gal4 enhancer trap expressed in both neurons and muscle, to UAS-FasII and collected eggs as described above. The B185-Gal4 line and the UAS-FasII line served as controls. We placed either 10 or 100 eggs in a vial and

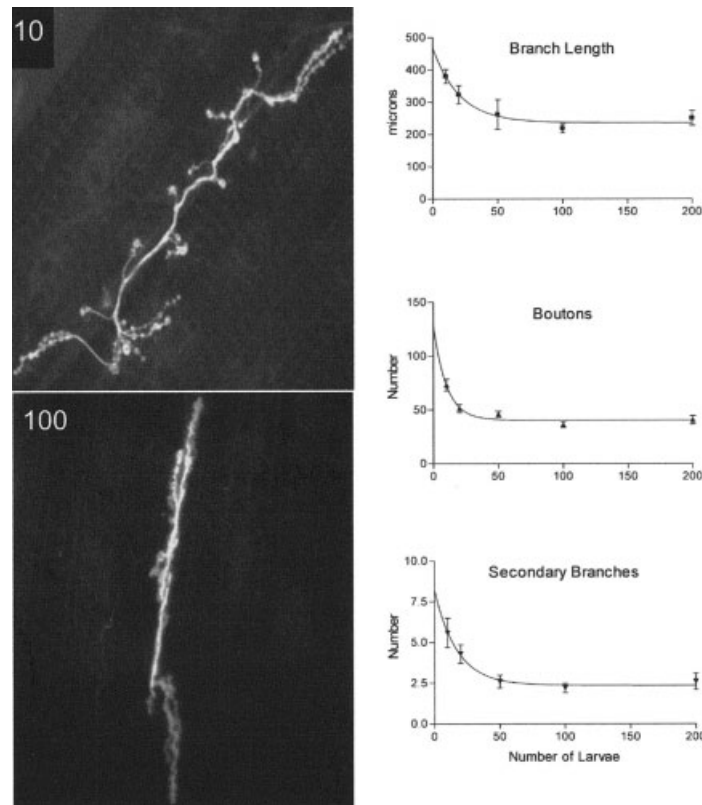


Figure 1 Synaptic morphology changes with population density. The left column shows neuromuscular junctions of third instar larvae grown in the 10 or 100 larvae per vial conditions. The right column shows the quantification of the total branch length, the number of synaptic boutons, and the number of secondary branches for larvae grown in each density. Each data point represents the mean value obtained from 14–19 individual NMJs. The lines represent the best fit of the data to the single phase exponential decay equation: $y = Ae^{-(1/k) \cdot x} + B$, where k represents the decay constant. Bars represent SEM.

Table 1(A) Exponential Curve Fit to Raw Data

Parameter	Total Length	Bouton Number	Secondary Branches
Decay constant (K) \pm std err (# of larvae)	20.6 ± 8.0	10.1 ± 4.3	17.5 ± 4.1
$\frac{1}{2}$ decay value (# of larvae)	14.3	6.9	12.2
Plateau (B)	235.7	40.3	2.3
R^2	0.99	0.99	0.99

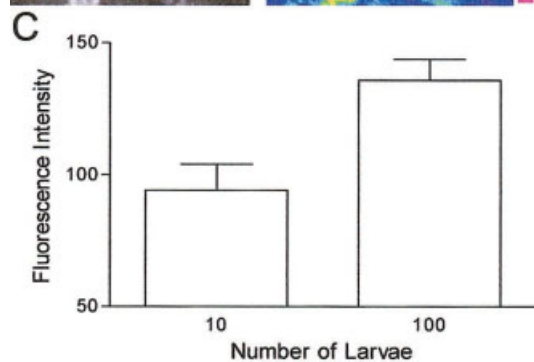
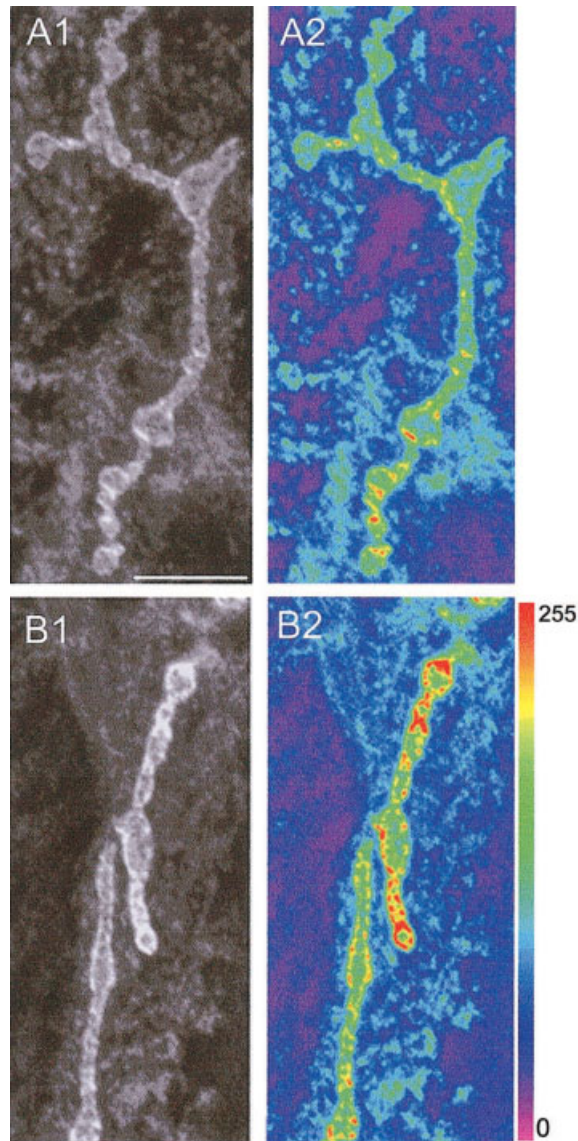
All data were fit to the single phase exponential decay equation: $y = Ae^{-(1/K) \cdot x} + B$.

Table 1(B) Exponential Curve Fit to Data Normalized for Muscle Area

Parameter	Total Length	Bouton Number	Secondary Branches
Decay constant (K) \pm std err (# of larvae)	8.0 ± 5.2	4.8 ± 3.9	11.6 ± 3.2
$\frac{1}{2}$ decay value (# of larvae)	5.5	3.3	8.0
Plateau (B)	9.2	1.5	0.1
R^2	0.91	0.91	0.98

All data were fit to the single phase exponential decay equation: $y = Ae^{-(1/K) \cdot x} + B$.

allowed the progeny to develop until the third instar, at which time they were dissected and processed for FITC anti-HRP immunocytochemistry, and morphometric analysis of muscle 6 and 7 NMJs was per-



formed. Two-way analysis of variance and post-tests revealed significant effects of population density in the control lines with respect to total branch length and total bouton number (Fig. 3). However, there were no population effects in the B185-UAS FasII larvae. For example, B185-UAS FasII larvae raised at a density of 10 per vial had total branch length of $243.3 \pm 26.5 \mu\text{m}$ and had 34.5 ± 3.9 boutons per NMJ while larvae raised at a density of 100 larvae per vial had total branch length of $227.8 \pm 20.2 \mu\text{m}$ and 29.9 ± 4.5 boutons per NMJ. The differences within B185-UAS FasII were not statistically significant. Therefore, these data demonstrate that constitutive expression of FasII eliminates the population-dependent effects on synapse morphology.

Soft Culture Media Reduces Population-Dependent Change in Synapse Morphology

One parameter that clearly changes over time in these experiments is the condition of the culture medium. In our larval cultures it is clear that those with 50 or more larvae per vial churn up the food so that after a few days it is softer than in cultures with 10 larvae. In order to determine the effect of the differing medium we prepared vials with one-half the normal amount of agar. The goal of this experiment was to grow larvae at low density but with softer food to determine if the food consistency is a determining factor. When we compared larvae grown at 10 per vial ($n = 8$) with those grown at 50 per vial ($n = 7$) in the soft food we indeed observed that the differences in morphometric parameters were largely abolished (Fig. 4). Therefore, one factor that contributes to population-dependent morphological change is different food density, and the potential changes in neural activity that can effect different food substrates (Sigrist et al., 2003).

Figure 2 Fasciclin II levels at muscle 4 synapses. FasII level was measured as the amount of Mab1D4 (anti-FasII) immunoreactivity. (A1,B1) Example grayscale images acquired at identical microscope settings for larvae raised at 10 per vial (A1) and for larvae raised at 100 per vial (B1). (A2,B2) False color representations of the same grayscale images, where red indicates the highest pixel values. (C) Mean grayscale pixel intensities for nine individual NMJs were obtained for each condition and their average values calculated. Bars represent SEM.

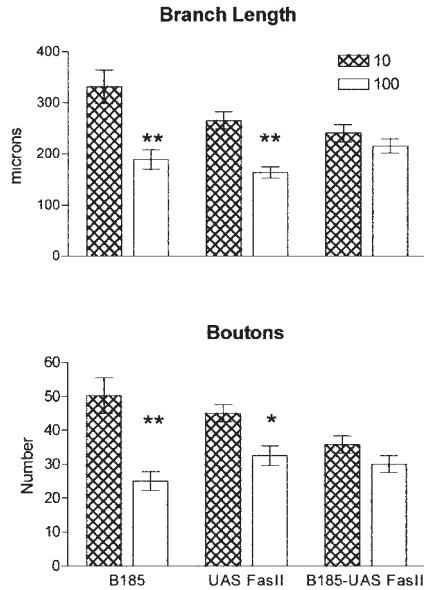


Figure 3 Constitutive expression of FasII. B185-Gal4 was used to drive expression of UAS-FasII in muscle and nerve while the number of eggs in each vial was controlled. Comparison of branch length and bouton number of muscle 6/7 NMJs revealed a significant population-dependent effect in the control lines (** $p < 0.01$; * $p < 0.05$) but not in B185Gal4-UAS FasII instar larvae. Sample size: $n = 11$ and 12 NMJs for the B185Gal4 line at 10 and 100 larvae per vial, respectively; $n = 10$ and 12 NMJs for the UAS FasII line at 10 and 100 larvae per vial, respectively; $n = 17$ and 20 NMJs for the B185Gal4 UAS FasII larvae at 10 and 100 larvae per vial, respectively.

No Change in Synaptic Strength in Larvae Grown at Different Densities

Given the significant change in synaptic morphology in the different population densities, we expected that this would lead to a corresponding change in synaptic strength. However, when we measured nerve stimulated excitatory junctional potentials (EJPs) from larvae raised at 10 and 100 larvae per vial we found that there was no significant difference (Fig. 5). The mean EJP size recorded from NMJs of larvae raised at 10 per vial was 23.7 ± 2.3 mV ($n = 17$), while the identical measure in larvae raised at 100 per vial was 21.0 ± 2.6 mV ($n = 19$). Furthermore, we measured the mEJP amplitudes from larvae grown under these conditions and found the mEJP amplitudes were also indistinguishable (1.1 ± 0.2 mV, 439 minis from $n = 6$ cells; 0.9 ± 0.1 mV, 377 minis from $n = 5$ cells). Lastly, there were no differences in the resting membrane potential of the muscle fibers; the mean values were -65.6 ± 1.2 mV and -63.8 ± 0.9 mV for larvae from the 10

per vial and 100 per vial conditions. Based upon these measurements we conclude that there is no change in synaptic strength in larvae raised under the present population densities.

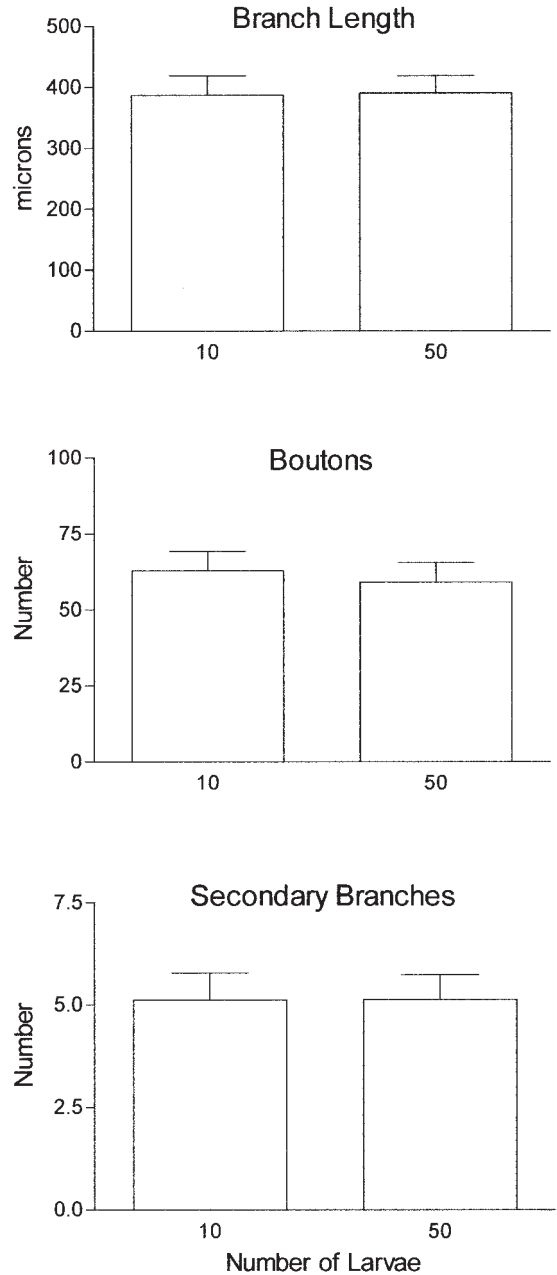


Figure 4 Larvae raised on soft culture medium show reduced density-dependent effects on morphology. Comparison of larvae raised in 10 or 50 larvae per vial, in culture medium that contained one-half the normal amount of agar showed no difference in total branch length, bouton number, or the number of secondary branches of muscle 6/7 NMJs. Sample size: $n = 8$ and 7 NMJs for larvae raised at 10 and 50 larvae per vial, respectively.

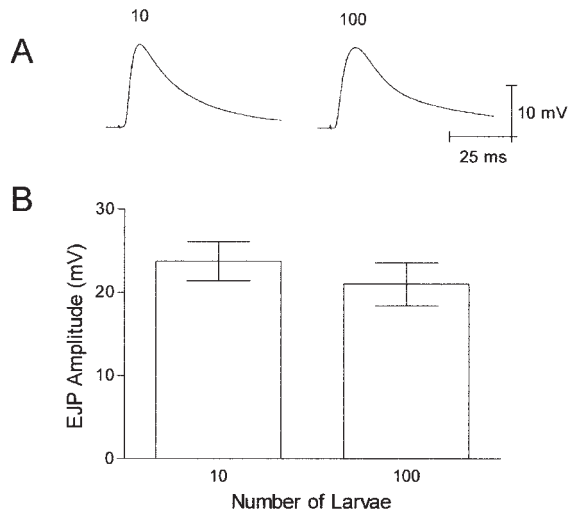


Figure 5 Synaptic strength at high and low population density. (A) Example excitatory junction potentials obtained from larvae raised at 10 or 100 larvae per vial. All EJPs were from muscle 6 at 0.2 Hz stimulation in HL3 solution with 0.5 mM Ca^{2+} . (B) The mean values obtained from $n = 17$ NMJs from the 10 per vial and $n = 15$ NMJs from the 100 per vial conditions. Bars are SEM.

DISCUSSION

Prior genetic analyses of *Drosophila* mutants have identified some of the key molecules important for functional development and synaptic plasticity of the larval neuromuscular synapse. In an effort to determine the extent to which these molecules normally contribute to synaptic development we have initiated studies on potential environmental cues. This study provides the first quantitative report on the relationship between larval population density and synaptic morphology, synaptic strength, and FasII expression at the synapse.

It is clear from our data that population density can regulate synaptic development in a FasII-dependent manner. This is demonstrated by the difference in FasII immunoreactivity at the NMJs of larvae raised at low and higher density, and from the abrogation of density-dependent effects on morphology when FasII is constitutively expressed by B185-Gal4. Therefore, our main conclusion is that synaptic development can be regulated by a global environmental parameter, such as population density, through the same molecular pathways that have been previously identified using a genetic approach.

From our data two surprises have emerged. The first is the very steep relationship between population density and the morphometric parameters that we examined. While our own experience, and the anecdotal

evidence of others, provided a basis to expect these results, we did not expect to find that small changes in the number of larvae would lead to such large changes in morphology. Like the study of Sigrist et al. (2003), which shows that prior locomotor activity can dramatically alter synaptic strength and morphology, here we show that population density can have profound impacts on synaptic form. On one hand, our result simplifies larval culturing conditions because there were near uniform measurements made from larvae grown at a density of 50 or more. On the other, the most robust changes, presumably an indicator of synaptic plasticity, occurred with very few larvae over a very narrow range of densities. These data suggest that in order to see the most robust response of the NMJ to experiments designed to manipulate synaptic plasticity, only a few larvae should be present in any vial.

The second surprising result is that we observed no effect of density upon synaptic strength, despite the marked changes in morphology. While previous genetic studies have invoked a homeostatic hypothesis to account for consistent neurotransmitter output in the face of altered NMJ morphology (Stewart et al., 1996), we anticipated that by manipulating more global parameters, such as density, we would see a congruence of synaptic morphometric and functional results, such as those reported recently (Sigrist et al., 2003). Our result might be explained if there is a homeostatic set point for synaptic strength that is regulated independently of synaptic morphology. This interpretation is consistent with previous observations made on hypomorphic alleles of FasII that reduce synaptic bouton number by 50% without any corresponding change in synaptic transmission (Stewart et al., 1996) and with studies in which FasII misexpression is used to bias synaptic growth onto one muscle, leading to homeostatic changes that maintain synaptic strength (Davis and Goodman, 1998).

Perhaps there are two variables that contribute to synaptic plasticity: neural activity and muscular power requirements. In the larval cultures it is clear that those with a higher density develop softer culture media than the low density cultures. We imagine that the amount of muscular force required to navigate the culture medium for the low density larvae is greater than for the high density larvae. Because these larval muscles likely use a graded mechanism to generate force, there should be more action potential activity in low density conditions. Such an increase in action potential frequency would not necessarily manifest itself as an increase in locomotor activity however, but it would be consistent with prior work that demonstrated genetic increases in neural activity reduce

FasII levels at the synapse, which is required for elaboration of the nerve terminal (Schuster et al., 1996b). Paradoxically, larvae raised at high density exhibit higher rates of locomotor activity than those at low density (Joshi and Mueller, 1988; Joshi, 1997), but here we've shown that higher density larvae have less elaborate NMJs. What remains unknown is if the outwardly visible larval movements accurately reflect action potential activity in individual motor neurons in the different population densities. A careful analysis of the relationship between neural activity of the motor neurons innervating the muscles studied here and population density is required to precisely identify the critical inputs that contribute to the functional development of the larval NMJ.

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