Sexual Differentiation of Identified Motor Terminals in *Drosophila* Larvae

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**ABSTRACT:** In *Drosophila*, we have found that some of the motor terminals in wandering third-instar larvae are sexually differentiated. In three out of the four body-wall muscle fibers that we examined, we found female terminals that produced a larger synaptic response than their male counterparts. The single motor terminal that innervates muscle fiber 5 produces an EPSP that is 69% larger in females than in males. This is due to greater release of transmitter from female than male synaptic terminals because the amplitude of spontaneous miniature EPSPs was similar in male and female muscle fibers. This sexual difference exists throughout the third-instar: it is seen in both early (foraging) and late (wandering) third-instar larvae. The sexual differentiation appears to be neuron specific and not muscle specific because the same axon produces Ia terminals on muscle fibers 2 and 4, and both terminals produce larger EPSCs in females than males. Whereas, the Ib terminals innervating muscle fibers 2 and 4 are not sexually differentiated. The differences in transmitter release are not due to differences in the size of the motor terminals. For the terminal on muscle fiber 5 and the Ia terminal on muscle fiber 4, there were no differences in terminal length, the number of branches, or the number of synaptic boutons in males compared to females. These sexual differences in neuromuscular synaptic physiology may be related to male-female differences in locomotion.


**Keywords:** sex; synapse; *Drosophila*; neuromuscular; larvae

**INTRODUCTION**

The sexual differentiation of the nervous system is well documented. Male-female differences in the size of brain regions have been reported for a variety of vertebrate organisms including rats (Gorski et al., 1978), birds (Nottebohm and Arnold, 1976), and humans (Swaab and Fliers, 1985). In some cases these sexual differences have been demonstrated at the cellular level. For example, male-female differences in dendritic morphology have been reported for birds (DeVoogd and Nottebohm, 1981), hamsters (Greenough et al., 1977), and rats (Gould et al., 1990). In addition, there are sexual differences in the density of dendritic spines in the rat hippocampus (Gould et al., 1990; Shors et al., 2001). These morphological studies suggest that there are male-female differences in the physiology of CNS synapses. Although a sexual difference in the efficacy of CNS synapses has not been demonstrated, there is evidence for sexual differences in the production of LTP in the rat hippocampus (Maren et al., 1994).

Sex-related differences in synaptic physiology have been clearly demonstrated at the neuromuscular junction. In *Xenopus laevis*, males produce a distinctive courtship song that differs from female vocalizations. Sex differences in transmitter release at laryngeal motor terminals contribute to these different vocalizations (Tobias et al., 1995). Surprisingly, there are no comparable studies in invertebrates. Identified synapses in invertebrates provide a very tractable sys-
Sexual Differences in Motor Terminals

Drosophila is an ideal system to study the sexual differentiation of synapses because the genetic cascade responsible for sexual differentiation has been well studied, and genes involved in sexual differentiation of morphological features and behavior have been identified (for reviews see Hall, 1994; Christiansen et al., 2002). In adult insects, sexual differentiation of the nervous system has been clearly demonstrated at the cellular level. In flies, male-specific neurons in the visual system are involved in tracking females (Stewart et al., 1994; Gilbert and Strausfeld, 1995). In moths, there are sex-specific olfactory projection neurons that respond to sex pheromones (Christensen and Hildebrand, 1987) and sex-specific motoneurons that innervate muscles in the reproductive tract (Thorn and Truman, 1994). In cicadas, there is sexual differentiation of the motoneuron that innervates the male timbal organ (Wohlers and Bacon, 1980).

Sexual differentiation in Drosophila is predominantly cell autonomous, rather than mediated by hormones (Baker and Ridge, 1980). However, hormonal influences cannot be totally ruled out (De Loof and Huybrechts, 1998), and it has been proposed that the fat bodies could function as an endocrine organ and secrete hormones in a sex-specific manner (Fuji and Amrein, 2002). In vertebrates, sex steroid hormones play a major role in the sexual differentiation of the nervous system (Breedlove, 1992). However, there is recent evidence that sex-determining genes may directly control some aspects of brain differentiation in mammals (De Vries et al., 2002) and possibly birds (Arnold, 1997).

In Drosophila, the synapses most amenable to physiological studies are those found on the body-wall musculature of larvae (Keshishian et al., 1996). Surprisingly, we have found that some of these identified neuromuscular synapses are sexually differentiated. In three of the four muscle fibers that we examined, females showed a larger synaptic response than males, apparently due to greater transmitter release from their motor terminals. We assume that the differences in transmitter release are due to ultrastructural or biochemical differences because male and female motor terminals are similar in length and synaptic bouton number. These synaptic differences may be related to the differences in male and female locomotion (Glossop and Shepard, 1998).

METHODS

These studies were performed on third-instar larvae of the wild-type strain Canton S, maintained at 25°C on JAZZ-Mix Drosophila media (Fisher Scientific). For most of the experiments, we used late (wandering) third-instar larvae collected from the sides of the culture bottles. In addition, we examined early (foraging) third-instar larvae that were collected less than 1 day after their second molt. To obtain early third-instar larvae, we collected larvae that hatched over a 4 h period and placed them in vials with media. Every 12 h a few of the largest larvae were removed from the vials and staged by examining their mouthparts (Bodenstein, 1950). Experiments were performed on larvae that were collected within 12 h of the first appearance of a third-instar larva in the vial. After performing physiology, larval mouthparts were examined to verify that they were third instars.

Neuromuscular Physiology

Larvae were dissected in HL3 saline (Stewart et al., 1994) to expose the body-wall muscles. After an incision through the dorsal body wall, the larvae were pinned out flat in a physiology chamber and the internal organs were removed. To evoke synaptic responses in muscle fibers in segments 3 or 4, the cut end of the segmental nerve was stimulated with a suction electrode (approximately 10 μm inside diameter). EPSPs or EPSCs were recorded using sharp microelectrodes (20–30 MΩ filled with 3 M KCl) connected to a preamplifier (Axoclamp 2A; Axon Instruments Inc., Foster City, CA). Data were acquired (sampling rate 5 KHz) and analyzed using a Digidata 1200A (Axon Instruments Inc.) interface and pCLAMP 9.2 (Axon Instruments Inc.) software. EPSPs were recorded from muscle fiber 5 and conventional two-microelectrode voltage-clamp techniques were used to record EPSCs from muscle fibers 2 and 4. For voltage clamping, a grounded shield was placed between the electrodes to reduce coupling capacity. The holding potential was set at −60 mV and the gain was adjusted to hold the membrane potential constant while recording EPSCs. In addition to recording EPSCs, we measured muscle fiber input resistance during 10 mV step hyperpolarizations.

To identify the EPSPs produced by the Ib and Is terminals on muscle fiber 6, we recorded simultaneously from muscle fibers 6 and 12 as previously described (Lnenicka and Keshishian, 2000). We used a similar procedure to identify the Ib and Is EPSCs recorded from muscle fibers 2 and 4. We simultaneously recorded EPSCs from fiber 4 and EPSCs from fiber 2, or EPSCs from fiber 2 and EPSCs from fiber 4. Because the same axon supplied the Is terminals to fibers 2 and 4 (Hoang and Chiba, 2001), recruitment of this axon produced a synchronous synaptic response in the two fibers. The Ib terminals innervating fibers 2 and 4 are supplied by separate axons and thus, the two terminals can be recruited separately. By carefully adjusting the stimulation...
voltage, we could determine whether the terminals were recruited synchronously or separately.

Motor Terminal Morphology

The methods for staining terminals and the morphological measurements have been previously described in detail (Lnenicka and Keshishian, 2000) and will only be briefly described here. We used a goat antibody to horseradish peroxidase (HRP) followed by antigoat IgG conjugated to HRP (Cappel; MP Biomedicals, Orangeburg, NY) to stain the motor terminals (Jan and Jan, 1982). For fibers 4 and 5, the motor terminals and muscle fibers were drawn using a drawing tube attached to an Olympus BHS microscope (Melville, NY). On fiber 4, the Is and Ib terminals were distinguished based upon relative bouton size. These drawings were digitized and the terminals and muscle fibers were measured using Scion Image beta 4 (Scion Corporation, Frederick, MD). In a few cases the Is and Ib terminals overlapped and individual terminals could not be easily followed; these terminals were not measured. Images shown in Figures 1 and 7 were obtained using a digital CCD camera (CoolSNAP HQ; Photometrics, Tucson, AZ) and Metamorph 6.1 software (Universal Imaging Corp., Downingtown, PA). All statistical comparisons of mean values were performed using Mann-Whitney U tests.

RESULTS

In the course of an earlier experiment, we observed male-female differences in the EPSPs recorded from muscle fiber 5 in control larvae. Here, we have examined the male-female differences in muscle fiber 5 in more detail and also examined muscle fibers 2, 4, and 6 (Fig. 1). Each larval hemisegment contains 30 identified muscle fibers. Most of the fibers receive at least two inputs, but muscle fiber 5 receives only a single input (Hoang and Chiba, 2001).

Sexual Differentiation of EPSPs Produced in Muscle Fiber 5

We measured the EPSPs produced by the motor terminal innervating muscle fiber 5 in wandering third-instar larvae. The EPSPs produced in muscle fiber 5 from segments 3 and 4 were larger in females than in males during both 0.1 and 10 Hz stimulation (Fig. 2). In males and females, 10 Hz stimulation produced synaptic facilitation. We averaged the amplitude of the first 10 EPSPs during 0.1 Hz stimulation and the first 20 EPSPs during 10 Hz stimulation for each fiber and compared the values for males and females. The EPSP amplitude was significantly greater for females compared to males at both 0.1 Hz (females: 5.9 ± 0.5 mV, n = 39; males: 3.5 ± 0.4 mV, n = 42; p < 0.001) and 10 Hz stimulation (females: 6.6 ± 0.6 mV, n = 35; males: 4.8 ± 0.4 mV, n = 34; p < 0.05).

In addition to the differences in EPSP amplitudes, the female synapses showed a low-frequency depression of EPSP amplitude, not seen at the male synapses. In females, the amplitude of the tenth EPSP during 0.1 Hz stimulation was significantly smaller than that of the first EPSP (p < 0.001). Low-frequency depression has been observed at a number of crustacean neuromuscular synapses (Bruner and Kennedy, 1970; Bryan and Atwood, 1981; Pahapill et al., 1987). Due to low-frequency depression, the male-female differences in EPSP amplitude are slightly greater if one compares the first EPSP (females: 6.6 ± 0.6 mV, n = 39; males: 3.4 ± 0.4 mV, n = 42; p < 0.001) rather than an average of the first 10.

The distribution of EPSP amplitudes seen during 0.1 Hz stimulation was much different for males and females (Fig. 3). In males about 60% of the fibers had EPSPs less than 3 mV, whereas in females only about 20% of the fibers had EPSPs less than 3 mV.

The sexual differentiation of neuromuscular synapses is not seen in all muscle fibers. When we compared the neuromuscular synapses on muscle fiber 6, we saw no evidence of sexual differentiation. Muscle fiber 6 is one of the ventral longitudinal muscles and is positioned on the medial side of muscle fiber 5 (Fig. 1). It receives Ib and Is terminals formed by axons 1 and 2, respectively (Atwood et al., 1993). Axon 1 only innervates muscle fibers 6 and 7, whereas axon 2 innervates a number of fibers includ-
The EPSP amplitudes produced by the Is and Ib terminals were very similar in males and females during 0.1 Hz (Is: males: 21.6 ± 2.4 mV and females: 21.7 ± 1.4 mV; Ib: males: 16.2 ± 2.5 mV and females: 16.1 ± 2.2 mV) and 10 Hz stimulation (Is: males: 11.6 ± 1.8 mV and females: 11.9 ± 2.0 mV; Ib: males: 13.5 ± 2.1 mV and females: 15.0 ± 1.0 mV). Thus, the terminals formed by axons 1 and 2 on muscle fiber 6 do not appear to be sexually differentiated.

Sexual Differentiation of Neuromuscular Synapses on Muscle Fiber 5 Is Seen in Both “Early” and “Late” Third-Instar Larvae

Although there is considerable variability in larval growth rates, the third instar lasts approximately 48 h; they are foraging during the first half of the third instar and then leave the media and become wandering third instars (Bodenstein, 1950). The previous data were taken from these wandering third instars. To determine whether this sexual differentiation was seen throughout the third instar, we examined these early, foraging third instars less than 24 h after molting (see Methods). EPSPs were recorded from muscle fiber 5 in segments 3 and 4 during 0.1 Hz stimulation (Fig. 4). In early third-instar larvae, the average

Figure 2 EPSPs recorded from muscle fiber 5 in male and female wandering third-instar larvae. The axon innervating muscle fiber 5 was stimulated at 0.1 and 10 Hz, and EPSPs were recorded from muscle fiber 5. Top: Representative EPSPs recorded from muscle fiber 5 in segment 4 of male and female larvae. The traces are averages of the first 10 responses during 0.1 Hz stimulation and the first 20 responses during 10 Hz stimulation. Overall, the EPSPs produced during 0.1 and 10 Hz stimulation were larger in females than in males. Calibration: 5 mV; 10 ms. Bottom: The mean EPSP amplitudes recorded from muscle fiber 5 in segments 3 and 4. 0.1 Hz stimulation: n = 39 female fibers and 42 male fibers. 10 Hz stimulation: n = 35 female fibers and 34 male fibers.

Figure 3 Distribution of EPSP amplitudes from muscle fiber 5 recorded during 0.1 Hz stimulation in male and female larvae. The amplitudes of the first 10 EPSPs produced by 0.1 Hz stimulation were averaged to give a single value for each muscle fiber. The data were divided into 3 mV bins to give the histogram. The histogram shows that male larvae had a higher frequency of small EPSPs than female larvae. The data represent an approximately equal number of measurements from segments 3 and 4 for both male and female larvae.
amplitudes of the first 10 EPSPs recorded from females (5.1 ± 0.6 mV, \( n = 34 \)) were significantly larger than those recorded from males (3.5 ± 0.4 mV, \( n = 33 \); \( p < 0.05 \)). Similarly, the amplitude of the first EPSP was significantly greater for females (6.1 ± 0.5 mV, \( n = 34 \)) compared to males (4.1 ± 0.8 mV, \( n = 33 \); \( p < 0.05 \)). Although it appears that the male-female differences for early third instars were not quite as great as seen for late third instars, there were no significant differences when comparing EPSP amplitudes from foraging and wandering third instars for males or females (\( p > 0.10 \)).

**Female Motor Terminals Release More Transmitter than Male Motor Terminals**

For muscle fiber 5 in wandering third-instar larvae, the EPSP amplitude in females was 69% greater than that seen in males. To determine whether this difference is due to differences in transmitter release, we compared miniature EPSPs in males and females (Fig. 5). There was no significant difference between the amplitude of muscle fiber 5 miniature EPSPs in male (0.53 ± 0.04 mV, \( n = 7 \)) and female larvae (0.48 ± 0.06 mV, \( n = 8 \); \( p > 0.10 \)). Thus, the female motor terminals release more neurotransmitter than the male ones: during 0.1 Hz stimulation, the female motor terminals release approximately 12.5 quanta of transmitter and the male terminals release about 6.5 quanta.

**Sexual Differentiation of Neuromuscular Synapses Is Motor Axon Specific, Not Muscle Specific**

Next, we examined the motor terminals innervating muscle fiber 4 in male and female larvae. Muscle fibers...
fiber 4 is found on the lateral side of muscle fiber 5 and its anterior end shares an insertion with fiber 5 (Fig. 1). It is innervated by a type Is and Ib terminal. The motor axon supplying the Ib terminal only innervates fiber 4, but the axon producing the Is terminal innervates a number of muscle fibers including fiber 2 (Hoang and Chiba, 2001). To identify the motor terminals, we recorded simultaneously from muscle fibers 4 and 2 (see Methods). In these experiments, we recorded synaptic currents so that the individual Is and Ib currents could be determined by subtracting the current produced by a single terminal from the combined response (Fig. 6, top). When recording EPSPs, it was only possible to measure the EPSP that was evoked at the lower threshold because synaptic potentials are difficult to subtract accurately due to nonlinear summation (Lnenicka and Keshishian, 2000). The synaptic current was integrated to give the charge transfer and then the charge transfer for a single terminal was subtracted from the combined response. This approach allowed us to measure EPSCs produced by both Is and Ib terminals.

For muscle fiber 4, we found that the Is terminal, but not the Ib terminal was sexually differentiated (Fig. 6, bottom). The charge transfer for the Is terminal was significantly larger in females (303.0 ± 22.9 pC, n = 55) than in males (209.0 ± 19.8 pC, n = 46; p < 0.01). For the Ib terminal, there was no significant difference in the charge transfer for female

**Figure 6** EPSCs recorded from muscle fibers 2 and 4 in male and female larvae during 0.1 Hz stimulation. Top: The procedure for identifying the type Is and Ib synaptic currents in muscle fiber 4. EPSCs were recorded from muscle fiber 4 (top trace) while simultaneously recording EPSPs from muscle fiber 2 (bottom trace). The motor axon supplying the Is terminal innervates both muscle fibers 2 and 4, whereas separate axons provide Ib terminals to muscle fibers 4 and 2. Therefore, when the Ib terminal on muscle fiber 4 was recruited there was no coincident EPSP seen in muscle fiber 2. However, recruiting the Is terminal on fiber 4 resulted in a coincident EPSP in fiber 2. To record and identify EPSCs in muscle fiber 2, we used the same procedure except EPSCs were recorded from fiber 2 and EPSPs were recorded from fiber 4. Bottom: The total charge transfer produced by the synaptic currents in muscle fiber 4 (top) and muscle fiber 2 (bottom). Again, note that the same axon produced the Is currents in fiber 2 and 4, however separate axons produced the Ib currents in the two fibers. The charge transfer was significantly greater for the Is terminals in both fibers 2 and 4; however, the charge transfer for the Ib terminals was not different in either fiber (p > 0.10). Numbers of fibers are given in parentheses. n values for Ib are the same as Is. * significantly different from male, p < 0.01.

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female larvae, the female muscle fibers were generally larger; however, the differences were significant for muscle fiber 4: (female: 28,284 ± 1374 μm², n = 21; male: 21,998 ± 964 μm², n = 20; p < 0.05), but not muscle fiber 5: (female: 10,181 ± 569 μm², n = 267; male: 9,491 ± 386 μm², n = 24; p > 0.10).

**DISCUSSION**

**Sexual Differentiation of Neuromuscular Synapses**

We have found that some neuromuscular synapses on *Drosophila* larvae body-wall muscles are sexually differentiated. In each case, the female neuromuscular synapse produced a larger response than the male counterpart. The single motor terminal innervating muscle fiber 5 produced a larger EPSP in female larvae than in male larvae due to greater transmitter release. For muscle fibers 2 and 4, the charge transfer produced by the Is terminals was greater for females than males. This is likely due to greater transmitter release from the female Is terminals based upon the findings for muscle fiber 5. We assume that this greater charge transfer for female Is terminals results in the production of larger EPSPs in these muscle fibers because the muscle fiber input resistance for fibers 2 and 4 was similar in males and females. Note that the muscle fiber 4 membrane resistance may be larger in females than males because the female muscle fibers appear to be larger.
The sex differences in neuromuscular synapses seem to be neuron specific and not all of the neuromuscular synapses appear to be sexually differentiated. For muscle fibers 2 and 4, the Is terminals, which originated from the same axon, were sexually differentiated, but the Ib terminals were not. In addition, we found no evidence for sexual differentiation of the motor terminals on muscle fiber 6.

For muscle fiber 5, the female neuromuscular synapses, but not male ones, showed low-frequency depression. Low-frequency depression has been reported for a number of neuromuscular synapses and is particularly prominent at synapses formed by phasic axons (Bruner and Kennedy, 1970; Bryan and Atwood, 1981; Pahapill et al., 1987). During stimulation at higher frequencies synaptic facilitation was seen in females as well as males. Thus, the female synapses show both low-frequency depression and synaptic facilitation; some other synapses that show low-frequency depression also produce facilitation when stimulated at higher frequencies (Bruner and Kennedy, 1970; Pahapill et al., 1987).

Previous studies have shown the sexual differentiation of neuromuscular synapses that mediate reproductive behaviors. For example, there are sex differences in neuromuscular junction size on mouse bulbocavernosus muscle (Balice-Gordon et al., 1990) and lizard ceratohyoid muscle (O’Bryant and Wade, 2002), and sex differences in transmitter release from motor terminals innervating *Xenopus* laryngeal muscles (Tobias et al., 1995). We found sexually differentiated neuromuscular synapses on muscle fibers that are not involved in a mating behavior. This may also be the case in other organisms; for example, in humans female skeletal muscle generally shows greater resistance to muscle fatigue (Hicks et al., 2001), which may involve sex differences in neuromuscular synapses (Hakkinen, 1993).

In the adult fly, there are well-documented differences in male and female morphology and behavior (Hall, 1994). Sexual differences in larvae are less obvious, but they also appear to exist. For example, female larvae are generally larger than male larvae; this is consistent with adult *Drosophila* where females weigh 40–50% more than males (Ashburner

![Figure 8](https://example.com/figure8.png)
et al., 2005). In addition, female larvae appear to be more motile than males (Glossup and Shepherd, 1998) and male and female larvae select different pupation sites (Bauer and Sokolowski, 1988). The sex differences in neuromuscular synapses occur on body-wall fibers that are presumably involved in locomotion, although the specific movements produced by these fibers are not known. These sex differences could produce differences in motility; for example, the larger EPSP in females could generate stronger contractions and more rapid movement.

This sex difference in physiology was not accompanied by differences in the size of the motor terminals: terminal length and the number of branches and boutons were similar in males and females. It appears that the differences in transmitter release are due to ultrastructural and/or biochemical differences. This is not surprising because it has been previously shown that the size of Drosophila motor terminals is not always a good predictor of transmitter release (Stewart et al., 1996; Angaut-Petit et al., 1998). In addition, it does not appear that the sex differences in transmitter release seen at Xenopus laryngeal motor terminals are accompanied by structural differences (Tobias et al., 1995).

Mechanism for the Sexual Differentiation of Motor Terminals

The sexual differentiation of the motor axons in the third instar could be an early event in the metamorphosis of the neuromuscular system. Many of the larval motoneurons survive metamorphosis and are remodeled into an adult form (Truman, 1990). It is possible that the sexual differences in the late third instar reflect some early event in metamorphosis that leads to sexual differentiation of the adult motoneurons. Although there is no evidence that the remodeling of larval neurons into their adult form begins this early, sexual differences in cell divisions by abdominal neuroblasts are seen in late third instar (Truman and Bate, 1988; Taylor and Truman, 1992). However, our observation that the neuromuscular synapses are sexually differentiated in the early third instar makes this scenario less likely.

Another possibility is that the sexual differences in transmitter release reflect differences in the use of the motor axons. This would be consistent with the observation that the sexual differentiation of the motor terminals was neuron specific. Because experimental increases in larval locomotor activity can produce an increase in transmitter release from motor terminals (Sigrist et al., 2003), the greater motility reported for female larvae (Glossup and Shepherd, 1998) might lead to stronger synapses. However, this male-female difference in motility would have to reflect differences in impulse activity in only a subset of the motoneurons because not all of the motor terminals are sexually differentiated. In addition, a prolonged increase in locomotor activity produced an increase in the number of synaptic boutons (Sigrist et al., 2003) and morphological differences were not seen in our study.

Finally, the sexual differentiation of the motor terminals could result from a direct genetic, or possibly hormonal, influence on selected motoneurons. Drosophila develop as females when the X chromosome:autosome ratio is 1 due to activation of the Sex lethal (Sxl) gene (for reviews see Hall, 1994; Schutt and Nothiger, 2000; Christiansen et al., 2002). Sxl is not activated when the ratio is .5 and flies develop as males. The Sxl protein activates transformer (tra), which along with transformer 2 (tra2), produces female-specific splicing of the doublesex (dsx) and fruitless (fru) genes. The absence of a Sxl protein in males results in male-specific splicing of the dsx and fru genes. These genes appear to encode transcription factors: dsx controls somatic sexual differentiation outside the CNS, whereas fru is involved in the sexual differentiation of the CNS. In addition, dissatisfaction (dsf) also appears to act downstream of tra to play a role in the differentiation of sex-specific motoneurons in adult Drosophila. Mutations in dsf result in females that lack innervation of the circumferential muscle of the uterus and males that have abnormal-looking motor terminals on abdominal muscles involved in copulation (Finley et al., 1997). These neuromuscular abnormalities contribute to behavioral deficits in male and female courtship and mating, as well as female sterility (Shupbach and Wieschaus, 1991; Finley et al., 1997).

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