Regulation of central neuron synaptic targeting by the Drosophila POU protein, Acj6

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SUMMARY

Mutations in the Drosophila class IV POU domain gene, abnormal chemosensory jump 6 (acj6), have previously been shown to cause physiological deficits in odor sensitivity. However, loss of Acj6 function also has a severe detrimental effect upon coordinated larval and adult movement that cannot be explained by the simple loss in odorant detection. In addition to olfactory sensory neurons, Acj6 is expressed in a distinct subset of postmitotic interneurons in the central nervous system from late embryonic to adult stages. In the larval and adult brain, Acj6 is highly expressed in central brain, optic and antennal lobe neurons. Loss of Acj6 function in larval optic lobe neurons results in disorganized retinal axon targeting and synapse selection. Furthermore, the lamina neurons themselves exhibit disorganized synaptic arbors in the medulla of acj6 mutant pupal brains, suggesting that Acj6 may play a role in regulating synaptic connections or structure. To further test this hypothesis, we misexpressed two Acj6 isoforms in motor neurons where they are not normally found. The two Acj6 isoforms are produced from alternatively spliced acj6 transcripts, resulting in significant structural differences in the amino-terminal POU IV box. Acj6 misexpression caused marked alterations at the neuromuscular junction, with contrasting effects upon nerve terminal branching and synapse formation associated with specific Acj6 isoforms. Our results suggest that the class IV POU domain factor, Acj6, may play an important role in regulating synaptic target selection by central neurons and that the amino-terminal POU IV box is important for regulation of Acj6 activity.

Key words: POU domain, Transcription factor, CNS, Neuron-specific, Axon targeting, Synapse, POU IV box, Interneuron

INTRODUCTION

Although significant progress has been made in characterizing cell surface proteins and secreted molecules involved in the direction of terminal axon targeting and synaptic selection, the transcriptional regulation of these processes remains relatively poorly understood. Recent studies have demonstrated that members of the LIM homeodomain and ETS domain family are necessary for terminal neuron differentiation (reviewed in Bang and Goulding, 1996; Curtiss and Heiling, 1998; Goulding, 1998). The expression of variable combinations of these regulators appears to play an essential role in determining the identity (Sharma et al., 1998; Tanabe and Jessell, 1996; Thor et al., 1999; Tsuchida et al., 1994) and precise patterns of connectivity for motor neurons with their targets, such as corresponding sensory afferents (Lin et al., 1998).

The POU domain family of transcription factors also plays a critical role in the development of specific cell types. Many POU domain proteins have distinct expression patterns in the nervous system and have been shown by genetic studies in numerous organisms to regulate neuronal development (reviewed in Ryan and Rosenfeld, 1997; Sharp and Morgan, 1996; Wegner et al., 1993). Of particular interest are the neuron-specific class IV POU domain proteins including Unc-86 from C. elegans (Finney et al., 1988), zebrafish Brn-3.1 (Sampath and Stuart, 1996), and the mammalian Brn-3a/Brn-3.0, Brn-3b/Brn-3.2 and Brn-3c/Brn-3.1 proteins (Collum, 1992; He et al., 1989; Lillycrop et al., 1992; Xiang et al., 1993). All of these proteins have been shown to perform essential functions in the differentiation of specific neuronal cell types (Erkman et al., 1996; Finney and Ruvkun, 1990; Finney et al., 1988; Gan et al., 1996; McEvilly et al., 1996; Sze et al., 1997; Vahava et al., 1998; Xiang et al., 1997, 1996).

The abnormal chemosensory jump 6 (acj6) gene encodes the single class IV POU domain family member found in Drosophila. acj6 mutants were first isolated in a behavioral screen for mutations lacking a response to odorants (Ayer and Carlson, 1991). Further studies demonstrated that Acj6 is required to specify the odor sensitivities of a subset of olfactory receptor neurons (Clyne et al., 1999a). In addition to its function in olfactory sensory neurons, results from our analysis of Acj6 spatial and temporal expression patterns suggest that Acj6 may regulate events necessary for the establishment of synaptic connections. Genetic studies indicate that Acj6 is...
required in optic lobe neurons to form wild-type R cell and lamina synaptic structures. Ectopic expression studies suggest that this regulation may occur through a system of protein-protein interactions, since structural alterations in the amino-terminal POU IV box have profound effects upon Acj6 activity in vivo.

MATERIALS AND METHODS

Drosophila stocks

Flies were raised on standard cornmeal-yeast-agar medium. All stocks and balancer chromosomes not specifically mentioned in the text are described in Lindsley and Zimm (1992). All genetic crosses were performed at 25°C. Stages of embryonic development are according to Campos-Ortega and Hartenstein (1985).

DNA manipulation and P-element transformations

Multiple acj6 cDNAs were isolated from embryonic poly(A)+ RNA as previously described (Clyne et al., 1999a). The coding regions (nucleotides 131-1577) of individual acj6 cDNAs were amplified using PCR with unique EcoRI restriction sites added in the primers to assist in subcloning. Each acj6 PCR fragment was sequenced to verify the absence of polymerase errors and then subcloned into the pUAST vector (Brand and Perrimon, 1993) to create individual UAS-acj6 transposons.

Germ-line transformants were generated as previously described (Spradling, 1986). Multiple insertion lines were isolated and established for each construct. Strains were confirmed to contain single copy inserts of the appropriate P-element vector by Southern blot analysis of genomic DNA.

Preparation of crude protein lysates and immunoblot analysis

To generate crude protein extracts exclusively from neural tissue, the brains from 15  

\[ \text{acj6}^{1/18} \] or  

\[ \text{acj6}^{6} \] adult flies were dissected in ice-cold 1× PBS, homogenized, and a portion of the lysate mixed with an equal volume of 2× sample loading buffer (125 mM Tris, 20% glycerol, 4% SDS, 10% β-mercaptoethanol, pH 6.8). Protein samples were separated on 12% SDS-polyacrylamide gels and electrotransferred to nitrocellulose using a Panther semi-dry electrobetter (Owl Scientific). Nitrocellulose membranes were blocked in BLOTTO (Pierce) + 0.5% Tween 20 for 2 hours followed by incubation with the mAb 9C52 anti-Acj6 (Clyne et al., 1999a) at a 1:500 dilution in 1× PBS + 0.5% Tween-20, overnight at 4°C. Primary antibodies were detected using a biotinylated goat anti-mouse secondary antibody (1:5000) (Vector Laboratories), Neutra-avidin (1:10,000) (Pierce) and chemiluminescent substrate (Pierce SuperSignal) followed by exposure to X-ray film.

Immunohistochemistry

Staged embryos, larval, pupal and adult brains were labeled using a modification of protocols previously described (Cortel and Johnson, 1996). The following primary antibodies were used: mAb 9C52 (anti-Acj6), mAb 1D4 (anti-FasII), mAb BP102, mAb 4D9 (anti-Engrailed/Inverted), mAb 3C10 (anti-Even-skipped), mAb 7G10 (anti-FasII), rabbit α-β-gal (Cappel), rat polyclonal RK2 serum (A. Tomlinson), mAb 2B10 (anti-Chaoptin), mAb dac2-3 (anti-Dachshund), mAb 78EA10 (anti-Elav) and mAb 4B1 (anti-Choline acetyltransferase; P. Salvaterra). Monoclonal antibodies BP102, 7G10, 2B10, 78E10 and dac2-3 were obtained from the NIH-supported Developmental Studies Hybridoma Bank maintained by the University of Iowa, Dept of Biological Sciences. Secondary antibodies included biotinylated goat anti-mouse (Vector), Oregon Green-conjugated goat anti-rabbit (Biosource), preabsorbed rhodamine-conjugated rabbit anti-rat (Chemicon) and FITC-conjugated goat anti-mouse (Biosource). Double-labeled images were captured using a BioRad 1024 laser-scanning confocal microscope maintained by the University of Iowa Central Electron Microscope Resource Facility.

RESULTS

acj6 mutants have larval and adult motor activity defects

The first acj6 mutant was isolated in a behavioral screen for mutations affecting the olfactory jump response (Ayer and Carlson, 1991). Through single-unit recordings, it was demonstrated that the odorant behavioral defects are due to a loss of Acj6 function in a subset of olfactory sensory neurons (Clyne et al., 1999a). However, initial examination of acj6 flies indicated that these mutants were also less active than wild type (Ayer and Carlson, 1991). Although a diverse group of mutants with odorant defects as their primary phenotype have been isolated and characterized (reviewed in Carlson, 1996; Smith, 1996), no apparent reduction in coordinated movement similar to the acj6 phenotype has been observed (J. Carlson, unpublished data), suggesting that the loss of olfactory information itself is unlikely to account for a decrease in motor activity.

Crawling activity of acj6 mutant larvae was quantitated
each group calculated. The Ten groups of 40 flies were each tested three times and the average of $=30$ for each group.

homozygous acj6

(A) Quantification of the crawling activity of wild-type and unpaired significant differences from wild-type values, as determined by the null allele. Values are means ± s.e.m. Asterisks indicate statistically

scores for within a narrow tube in 1 minute. In contrast, corresponding type flies in each group were able to climb past a 15 cm mark

modified version of assays previously used to characterize walking/climbing activity of

8.5 and 7.3 cm respectively (Fig. 1A, asterisks). The combination, acj6 6

the null allele moved significantly shorter distances of

while larvae homozygous for the hypomorphic

Wild-type larvae crawled an average of 18.65 cm in 5 minutes

using a simple assay as described in Materials and Methods. Wild-type larvae crawled an average of 18.65 cm in 5 minutes while larvae homozygous for the hypomorphic acj6 1 allele and the null acj6 6 allele moved significantly shorter distances of 8.5 and 7.3 cm respectively (Fig. 1A, asterisks). The walking/climbing activity of acj6 adults was tested using a modified version of assays previously used to characterize mutations in sluggish-A and no-bridge (Hayward et al., 1993; Strauss et al., 1992; see Materials and Methods). 91% of wild-type flies in each group were able to climb past a 15 cm mark within a narrow tube in 1 minute. In contrast, corresponding scores for acj6 1 hypomorphic and acj6 6 null flies were 47% and 11%, respectively (Fig. 1B). The heteroallelic combination, acj6 1/ acj6 6, also exhibited a severe deficit in motor coordination (31%) (Fig. 1B), suggesting that the activity defect is due to a disruption of acj6. Results from these experiments led us to search for a molecular explanation for the quantifiable reduction in coordinated movement exhibited by acj6 mutant flies.

Acj6 is also expressed in the optic lobes in regions functioning as hierarchical processing sites for visual information (Figs 2C,E, 3A) (reviewed in Meinertzhagen and Hanson, 1993). We do not detect any Acj6 expression in photoreceptor cells (R cells) but instead Acj6 optic lobe expression is initiated in differentiated neurons that receive R cell synaptic input. Differentiated lamina and medulla neurons as well as neurons in the lobula express Acj6 through adult stages (Fig. 2E).

Acj6 function is not required for initial neuron differentiation events

Adult behavioral deficits caused by a loss of Acj6 activity could occur as a result of a continuing requirement for Acj6 in mature neurons or by cumulative developmental defects occurring at earlier stages. Acj6 protein is not expressed in neuroblasts or GMCs, suggesting that it does not play a significant role in neuronal cell lineage determination. To verify this observation, we examined the expression of several well-characterized neuronal markers, such as Engrailed and Even-skipped, in acj6 loss-of-function backgrounds. No detectable differences in the expression patterns of any markers

Regulation of synaptic targeting in Drosophila 2397 (Campbell et al., 1994), and a neuron-specific marker, Elav (Robinow and White, 1991), indicated that Acj6 expression is restricted to neurons (Fig. 2H and data not shown). Furthermore, Acj6 protein expression is limited to a subset of post-mitotic neurons and was never detected in embryonic neuroblasts (NBs) or ganglion mother cells (GMCs). Neuronal Acj6 expression is maintained at high levels in larval, pupal and adult stages (Fig. 2B,C) suggesting that Acj6 activity may also be needed in functioning adult central neurons. To determine the identity of Acj6-expressing neurons, motor neurons were marked by β-galactosidase expression in transgenic embryos containing a Latebloomer enhancer trap (C. C. Kopczynski and C. S. Goodman, personal communication) (Kopczynski et al., 1996). Latebloomer is a neural tetraspanin expressed in all embryonic motor neurons (Kopczynski et al., 1996). Colocalization of β-gal and Acj6 expression was not observed (data not shown), indicating that Acj6 expression is restricted to interneurons within the ventral nerve cord and brain lobes.

We were particularly interested in the pattern of Acj6 expression in regions of the adult brain lobes comprising the central brain neuropils (Fig. 2C,F) (reviewed in Hanesch et al., 1989; Meinertzhagen and Hanson, 1993). The central brain neuropils include the antennal lobes, the first central region for processing olfactory information (reviewed in Carlson, 1996), the mushroom bodies, higher order structures involved in complex behaviors (Davis, 1993; Heisenberg, 1989; Yang et al., 1995) and the central complex, a region necessary for the coordination and modulation of motor activities (Bouhouch et al., 1993; Ilus et al., 1994; Strauss and Heisenberg, 1993). A large subset of central brain interneurons express Acj6 including central complex neurons (Fig. 2F), which function to receive, process and convey information from one site within the nervous system to another (Bouhouch et al., 1993; Strauss and Heisenberg, 1993). A loss of Acj6 function could therefore affect the ability to carry commands necessary to direct motor activity and provides us with a reasonable hypothesis for the quantifiable reduction in coordinated movement exhibited by acj6 mutant flies.

Acj6 expression is restricted to post-mitotic neurons and maintained in all developmental stages

Acj6 protein expression is first observed at embryonic stage 13 in embryonic neurons that will form the larval olfactory organs (Fig. 2A) (Schmidt-Ott et al., 1994; Schneitz et al., 1993) and in a subset of cells in the ventral nerve cord (VNC) (Fig. 2A). Double-labeling experiments with a glial-specific marker, RK2

Fig. 1. Coordinated movement defects in acj6 larvae and adults. (A) Quantification of the crawling activity of wild-type and homozygous acj6 larvae. Distance traveled represents the mean length of larval tracings after a 5 minute assay, n=30 for each group. (B) Activity of adult acj6 mutant flies in the vertical walking assay. Ten groups of 40 flies were each tested three times and the average of each group calculated. The acj6 1 allele is hypomorphic and acj6 6 is a null allele. Values are means ± s.e.m. Asterisks indicate statistically significant differences from wild-type values, as determined by the unpaired t-test (larval: P<0.0001; adult: P<0.0001).
Acj6-expressing midline neurons were identified as SP interneurons using an antibody against the cell adhesion molecule, Connectin (mAb C1.427) (Meadows et al., 1994) (data not shown). SP neuronal axons project anteriorly to the next neuromere and are necessary for formation of embryonic longitudinal axon pathways (Jacobs and Goodman, 1989; Doe and Goodman, 1993). The SP interneurons still express Connectin as well as choline acetyltransferase (ChAT) in \textit{acj6} mutants, suggesting that these aspects of neuronal identity are maintained. In addition, the initial pathfinding steps of \textit{acj6} mutant SP axons are unaffected, although later stages of SP interneuron development could not be observed using this marker. A lack of observable defects in early stages of differentiation correlates with the post-mitotic initiation of Acj6 expression and suggests that Acj6 may regulate events following initial axon pathfinding such as terminal axon targeting or synaptic connectivity.

**Loss of Acj6 function in lamina neurons affects synaptic connections**

Although motor activity defects are probably due to Acj6 function in the central complex, as an initial step in determining whether Acj6 is required for events following lineage determination and initial axon guidance, we focused on the easily visible axonal projections and organized synapses found in the optic lobe. In the wild-type larval eye-brain complex, R cell axons project from the eye disc, through the optic stalk and into the optic ganglia (Fig. 3A,D). R1-R6 photoreceptors send their axons to the lamina ganglion layer of the brain where their growth cones form an array of postsynaptic ‘cartridge’ units. Each cartridge unit contains the set of R1-R6 axons, five lamina neurons (L1-5) and several glial cells (reviewed in Meinertzhagen and Hanson, 1993). As described above, Acj6 expression is observed in the synaptic partners of the R cells, the lamina and medulla neurons, but not in the R cells themselves.

To first analyze any effects that loss of Acj6 function might have on synapses as a post-synaptic target, we assessed the organization of R cell projections in \textit{acj6} mutants using the R cell-specific antibody mAb 24B10 (Zipursky et al., 1984). The array of expanded R1-R6 growth cones appears as a continuous line of immunoreactivity in wild-type larvae (Fig. 3D). In \textit{acj6} mutants, R cell axons project into the brain in a wild-type manner; however, these fibers do not uniformly form the optic lobe. In the heteroallelic combination, \textit{acj6} \textit{acj6}, we observe occasional gaps in the lamina plexus. In the \textit{acj6} \textit{acj6} mutants, the entire lamina plexus is of variable thickness generating irregular small breaks (Fig. 3E,F). Therefore, the loss of Acj6 function in the R cell synaptic partners affects the ability of these R cell growth cones to establish connections with the appropriate lamina neuron column.

Defects in R cell connectivity could be due to a loss of neurons or abnormal lamina neuron specification. In \textit{acj6} mutants, lamina precursor cell (LPC) proliferation and initial lamina neuron differentiation are wild type, as assessed using anti-Dachshund (Fig. 3B,C) and anti-Elav staining (data not shown). At the level of antibody labeling, the organization of lamina neurons into columns also appears largely normal (data not shown). Possible explanations for the connectivity defects may be the inability of the R cell growth cones to recognize
their synaptic partners or to adhere correctly and form a stable synaptic cartridge.

To investigate possible pre-synaptic changes in Acj6-expressing lamina monopolar neurons, we used mAb 1D4, which is directed against the cell adhesion molecule Fasciclin II (Lin and Goodman, 1994). The axons and synapses of a subset of lamina neurons, L1 and L3, express Fasciclin II during selected stages of pupal development as they project in a highly structured pattern into the distal medulla (Fig. 3G). In acj6 mutants, the L1 and L3 arborizations and terminals are disorganized and unevenly spaced (Fig. 3H). In addition, the structure of the synaptic terminals is diffuse and overlaps are observed. These results provide further evidence that Acj6 is not required for initial differentiation steps such as axon pathfinding, but instead may be necessary for target cell selection and the establishment of synaptic connections.

**Expression of alternatively-spliced Acj6 isoforms**

To further test our hypothesis that Acj6 may regulate the formation of synaptic connections, we wanted to misexpress Acj6 in motor neurons where it is not normally found in order to observe any distinct morphological effects upon the well-characterized neuromuscular junction (NMJ). However, multiple acj6 transcripts have been identified (Clyne et al., 1999a; current work) and it was not clear whether different Acj6 isoforms were capable of unique functions. The Acj6 protein contains two domains with extensive homology to the vertebrate class IV members, Brn-3a, Brn-3b and Brn-3c (Gerrero et al., 1993; Xiang et al., 1993). In addition to the DNA-binding POU domain, members of this group contain a class IV-specific 40-amino-acid POU IV box at the N-terminal end (Fig. 4A) (Gerrero et al., 1993; Xiang et al., 1995, 1993). Four of the five acj6 transcripts differ only in use of the four small exons encoding the N-terminal POU IV box (amino acids 88-119) (Clyne et al., 1999a) (Fig. 4B-D). Neither the POU IV box nor the POU domain are affected in the fifth alternatively spliced transcript, which utilizes an alternative consensus acceptor site to generate a short form of exon 5 (Fig. 4C).

It was therefore imperative to determine whether the predicted Acj6 isoforms are expressed in vivo so that the significance of any differences in functional capability could be evaluated. Alternatively spliced Acj6 transcripts should produce proteins with predicted molecular masses of 40.0, 41.2, 42.2 and 43.5 kDa. A cluster of bands corresponding to proteins of the predicted size was detected using the Acj6 antibody on western blots (Fig. 4E). The cluster of anti-Acj6 immunoreactive bands is absent in extracts from acj6 null flies (Fig. 4E) demonstrating that multiple Acj6 isoforms are expressed.

Based upon previous experiments with other members of the POU domain family (Gruber et al., 1997; Turner, 1996), we did not expect alterations in the Acj6 POU IV box to have significant effects upon DNA-binding activity. To verify that amino-terminal changes do not affect the ability of Acj6 isoforms to bind DNA, fusion proteins were generated and used in gel mobility-shift assays (GMSA). All of the Acj6 isoforms differing in the highly conserved POU IV box were capable of binding octamer (Muller et al., 1988; Sturm et al., 1988) and neuronal (Johnson and Hirsh, 1990; Turner, 1996) DNA recognition elements with affinities comparable to wild type (S. Certel, unpublished data). Although the POU IV box does not appear to be important for DNA-binding, in vitro studies indicate that this region is necessary for both the transforming activity of Brn-3a and activation of specific promoters (Budhram-Mahadeo et al., 1995; Smith et al., 1998; Theil et al., 1993). We used the Gal4/UAS system (Brand and Perrimon, 1993) to analyze the functional capabilities of two Acj6 isoforms,
Acj6(1,4) and Acj6(1,3,4), through misexpression studies (Fig. 4).

Acj6 misexpression affects axon outgrowth and synapse formation

Transgenic strains carrying a UAS-acj6(1,4) or UAS-acj6(1,3,4) transposon were mated with either scabrous-GAL4 (sca-GAL4) or elav-GAL4 flies to express the Acj6 isoforms at high levels in all neurons (Klaes et al., 1994; Luo et al., 1994). Acj6(1,4) and Acj6(1,3,4) differ only in the absence or presence of exon 3 encoding a portion of the conserved POU IV box (Fig. 4D). Multiple transgenic lines were tested to eliminate the possibility that differences in phenotypes might be due to the position of insertion. In addition, expression of Acj6 proteins at comparable levels from each of the UAS-acj6 transposons was confirmed by labeling with Acj6 antibody (data not shown).

In each abdominal hemisegment of the Drosophila embryo and larva, the axons of approximately 40 motor neurons exit the ventral nerve cord and specifically synapse with 30 identified muscle fibers (reviewed in Doe and Goodman, 1993; Keshishian et al., 1996). We have focused our analysis on the well described motor axons of the ISNb fascicle visualized using mAb 1D4 (Van Vactor et al., 1993). The ISNb fascicle contains motor axons from at least four motor neurons innervating the ventral muscles 6, 7, 12 and 13 (Landgraf et al., 1997; Sink and Whitington, 1991).

The results of our analysis of Acj6(1,4) and Acj6(1,3,4) misexpression in motor neurons are summarized in Table 1. The ISNb fascicles of sca-GAL4/UAS-acj6(1,3,4) embryos correctly leave the nerve cord, defasciculate from the ISN and project to target muscle clefts in nearly all late stage 16/late stage 17 hemisegments examined (52/60) (Fig. 5A, Table 1). However, in many of the hemisegments with normal initial axon pathfinding (40/52), we observed a striking increase in the number of nerve terminal branches and processes arising from each motor axon. In addition, some of the ectopic terminal branches were able to extend and form connections onto inappropriate muscle fibers (Fig. 5B). To quantitate the observed increase in ectopic boutons, we allowed transgenic embryos to develop to crawling third instar larvae. Analysis of the larger NMJs in these larvae indicate that a subset of the ectopic terminal branches generated in the embryo were maintained, increasing the number of boutons by 27% at muscles 6 and 7 and 22% at muscles 12 and 13 (Fig. 6C). The ectopic boutons also

| Table 1. Frequencies of ISNb axon phenotypes in wild-type embryos and embryos ectopically expressing individual Acj6 isoforms |
|--------------------------|-----------------|-------------------|-------------------|
| Genotypes               | % complete      | % with bypass     | % showing ectopic |
|                         |                 | phenotype         | terminal branches |
| Wild-type               | 92 (55/60)      | 0 (0/60)          | 0 (0/60)          |
| sca-GAL4; UAS-acj6(1,3,4) | 86 (52/60)      | 3 (2/60)          | 77 (40/52)        |
| sca-GAL4; UAS-acj6(1,4) | 20 (14/69)      | 65 (45/69)        | 0 (0/69)          |

Actual number of embryos examined are given in parentheses.
expressed the synaptic markers synaptotagmin and cysteine string protein, consistent with functional synapses (data not shown).

In sharp contrast, misexpression of the Acj6(1,4) isoform caused a failure of motor axons of the ISNb fascicle to defasciculate from the ISN fascicle, resulting in defective innervation of the ventrolateral muscle field in approximately 65% (45/69) of embryonic hemisegments examined (Fig. 5C, Table 1). In approximately 15% of the hemisegments (10/69), the ISNb motor axons stopped at the correct location of their target muscles but did not branch out to innervate the ventral muscle groups. The remaining hemisegments (20%) show a wild-type pattern of muscle innervation. These studies suggest that distinct Acj6 isoforms can influence specific aspects of

![Fig. 5. Disruption of axon targeting and synaptogenesis by Acj6 misexpression in embryonic motor neurons. The schematic representation of each genotype includes the location of the motor neuron cell body in the CNS as well as the innervated muscles. The photograph of each embryonic fillet is restricted to the muscle region and is shown with anterior up and the midline of the VNC to the left. (A) Wild-type ISNb motor neuron innervation of muscles 6, 7, 12 and 13 visualized with mAb 1D4. Axon processes extend within the clefts between muscles in a stereotypical manner. (B-1, B-2) Misexpression of Acj6(1,3,4) in motor neurons using sca-GAL4 results in increased nerve terminal branching, as shown in these preparations from two independent embryos. Axons of the ISNb projection enter the ventrolateral muscles and innervate target muscles correctly (arrowhead); however, additional axon processes and branches are observed (long arrows). In addition, some of the ectopic nerve terminal processes extend to form connections onto inappropriate muscle fibers (small arrow). (C) Misexpression of Acj6(1,4) in motor neurons of late stage 16/early 17 sca-GAL4/UAS-acj6(1,4) filleted embryos. Axons of the ISNb projection leave the ventral nerve cord correctly but fail to defasciculate (arrow) from the ISN pathway. The axons do not enter the correct ventrolateral field leaving muscles 6, 7, 12 and 13 out of the plane of focus in this image.

![Fig. 6. Increased bouton number in larval nerve terminal branches generated by Acj6(1,3,4) misexpression. Synaptic boutons at the larval NMJ are visualized by immunostaining with mAb 1D4. (A) Wild-type innervation at muscles 12 and 13. (B) NMJ in transgenic sca-GAL4/UAS-acj6(1,3,4) larvae showing ectopic nerve terminal branching (arrows) with increased number of synaptic boutons. (C) Quantification of bouton number at muscles 6, 7, 12 and 13. Additional arborizations caused by Acj6(1,3,4) misexpression resulted in an increase in the number of boutons generated at each synapse. At sca-Gal4/UAS-acj6(1,3,4) synapses there is a significant increase (27%) at muscles 6 and 7 and muscles 12 and 13 (22%). Statistical significance was determined using the unpaired t-test. (muscles 6 and 7: P < 0.0007; muscles 12 and 13: P < 0.0001).]
nerve terminal branching and synapse formation. In addition, this activity appears to be mediated by differential activities of the N-terminal POU IV box.

**DISCUSSION**

Mutations in the *Drosophila* class IV POU domain gene, acj6, have previously been shown to cause physiological deficits in odor sensitivity (Ayer and Carlson, 1991, 1992; Clyne et al., 1999a). Results from our current work demonstrate that Acj6 is also required for coordinated movement and is highly expressed in distinct neuronal regions in the *Drosophila* brain including the central brain or cellular cortex (Han et al., 1996; Strausfeld, 1976). The central brain region includes neurons constituting the central complex, mushroom bodies, antennal lobes, antennal mechanosensory centers, and other structures of the proto and deutocerebrum (Meinertzhagen and Hanson, 1993; Strausfeld, 1976).

Genetic mutants affecting central brain structure have been shown to influence walking activity, walking speed and leg coordination (Strauss et al., 1992; Strauss and Heisenberg, 1993). Analysis of the central complex structural mutant no-bridge indicates that initiation and maintenance of walking may be controlled specifically by the central complex (Strauss et al., 1992). Our results support the hypothesis that the reduction in motor activity associated with acj6 mutations may be attributed to a defect in the processing or relaying of information necessary to direct sustained movement. In support of this proposed role in synaptic communication, loss-of-function studies indicate that Acj6 is required for neuronal connectivity and synapse formation.

**Acj6 is necessary for the establishment of synaptic connections in a subset of central neurons**

Appropriate synapse selection, whereby each presynaptic axon is matched to its specific postsynaptic target neuron, must take place in order to produce the precise connections required for mature function. Several recent experiments have demonstrated the important role transcriptional regulation plays in the differentiation program of neurons. Lin et al. (1998) provide evidence that the establishment of appropriate synaptic connections between interconnected populations of mammalian sensory and motor neurons may depend on the coordinated expression of individual ETS domain proteins. In addition, analysis of the *C. elegans* LIM homeobox proteins, Lin-11 and Ttx-3, indicates that these gene products specify the functions of a distinct set of interneurons within a neural network, perhaps through the regulation of synaptic signaling (Hobart et al., 1998, 1997).

Although behavioral activity defects are likely due to a disruption of Acj6 function in central brain interneurons, we turned to the highly organized optic lobe neuropils to determine if Acj6 is required for the establishment and structure of synaptic connections. Acj6 is expressed in differentiated lamina and medulla neurons that receive information from R cells and in turn relay this information to the central brain. Our studies indicate that Acj6 is necessary for the establishment of synaptic connections between R cell afferents and their target neurons in the optic lobe. Although R cell axons provide signals to pattern the optic lobe, the target region itself produces guidance cues to the ingrowing axons. While specific target recognition signals remain unknown, the intimate association of R1-R6 growth cones with epithelia and marginal glia has led to the speculation that these cells provide at least some of the required signals (Perez and Steller, 1996). In addition, recent work indicates that the maintenance of connections between the R cells and their synaptic partners requires the signaling of lamina-derived nitric oxide (Gibbs and Truman, 1998).

Loss of Acj6 in optic lobe neurons results in a disorganized lamina neuropil, characterized by thinning and occasional gaps. Although the R cell axons themselves do not require Acj6, they fail to arborize properly upon entering the acj6 mutant target area. At later stages of development, the growth cones of acj6 mutant lamina neurons enter an acj6 mutant medulla target area. Mutant lamina neuron growth cones are able to navigate to their correct target regions, but display disorganized terminal arborizations and diffuse synaptic structures. The optic lobe phenotypes suggest that Acj6 function is required for the proper selection and/or maintenance of synaptic connections in the visual system.

Our results suggest that Acj6 regulated transcription may provide signaling molecules involved in axon target recognition such as cell surface receptors or secreted molecules. Although specific Acj6 transcriptional targets have not yet been identified, recently published results indicate that loss of receptor protein tyrosine phosphatase Ptp69D function in photoreceptor axons also causes a disruption of synaptic targeting at the lamina plexus (Garrity et al., 1999). This result raises the as-yet-untested possibility that Acj6 might regulate the expression of a retrograde target-derived signal required for modification of tyrosine phosphorylation pathways in the innervating photoreceptor axons. Alternatively, acj6-associated lamina ganglion defects could be caused by disruption of a more general process of cell adhesion required for synaptic targeting. Recent results from both vertebrates and invertebrates suggest that target selection and the formation of lamina-specific synaptic connections may involve a combinatorial signaling process requiring various cell adhesion molecules such as members of the cadherin family, NCAM or Connectin (Holt and Harris, 1998; Sanes and Yamagata, 1999).

**The Acj6 misexpression phenotype resembles defects associated with alterations in cell adhesion**

Results from our misexpression studies indicate that ectopic Acj6 expression does not alter motor neuron specification or initial axon pathfinding choices. Instead, the motor neuron phenotypes generated from Acj6 misexpression strikingly affect the processes of terminal neuronal branching and connectivity. Similar to described acj6 phenotypes, defects in R cell connections and motor axon outgrowth are observed with mutations in the receptor tyrosine phosphatase (RPTP) encoding genes *Ptp69D, Ptp99D* and *Dlar* (Desai et al., 1996; Garrity et al., 1999; Krueger et al., 1996). The *sca-GAL4/UAS-acj6(1,4)* induced failure of ISNb defasciculation resembles the embryonic motor neuron ‘bypass’ phenotype that is thought to be due to excessive cell adhesion at essential choice points (Desai et al., 1997). Previous studies indicate that the regulated expression of cell adhesion molecules may direct or modify synapse morphology and specificity (reviewed in Goodman and Shatz, 1993; Martin and Kandel, 1996). Decreased activity
of the cell adhesion molecules FasI and FasII enhances nerve terminal arborization, as quantified by the numbers of nerve terminal branches and varicosities (Schuster et al., 1996a,b; Zhong and Shanley, 1995). Although expression of these proteins is initiated prior to Acj6, the phenotypes associated with Acj6 misexpression make the overall process of cell adhesion a potential target of Acj6 regulation.

Since Acj6 is found only in a subset of interneurons, it could not play a general role in connectivity for all neurons. However, Acj6 may regulate specific cell adhesion molecules necessary for specifying synapse selection among central neurons. Recent work describing the role of ETS domain proteins in the matching of sensory-motor neuron synaptic connections suggests that the individual ETS domain proteins may regulate members of the cadherin family of homophilic CAMs (Lin et al., 1998).

Additional possibilities are also consistent with the observed phenotypes. Acj6 could direct synapse selection by regulating the expression of genes that mediate synaptic activity. It has recently been demonstrated that Acj6 function is necessary for the expression of specific olfactory receptors (Clyne et al., 1999b). In vertebrates, several lines of evidence indicate that odorant receptors may first play an instructive role in guiding the axons of each olfactory neuron to their appropriate glomerular targets (Mombaerts et al., 1996; Wang et al., 1998). Subsequently, they function to receive and transduce chemosensory information to the antennal lobe or olfactory bulb through the generation of action potentials. In addition, transient transvection assays indicate that the vertebrate proteins Brn-3a and Brn-3b can both activate and repress the expression of synaptic proteins such as SNAP-25, synapsin I and the neuronal nicotinic acetylcholine receptor α2 subunit (Lakin et al., 1995; Milton et al., 1996; Morris et al., 1996).

Disruption of an Acj6-dependent transcriptional program could therefore cause defects in activity-dependent processes, resulting in the formation of ectopic neurite branching.

Class IV POU domain proteins

The acj6 locus yields multiple alternatively spliced transcripts, resulting in the production of distinct protein isoforms. Alternative splicing has been observed for a number of POU domain family members (reviewed in Latchman, 1996; Verrijzer and van der Vliet, 1993) and, in several cases, differential splicing generates proteins with distinct functional properties (Koncz and Moore, 1992; Voss et al., 1993). We have identified four Acj6 isoforms differing in amino terminal residues within the highly conserved POU IV box (Fig. 5B) and have tested two isoforms for in vivo function by ectopic expression in transgenic flies. Results from our work demonstrate a striking functional difference between the Acj6(1,4) and Acj6(1,3,4) isoforms. Misexpression of Acj6(1,3,4) promotes terminal branching and synapse formation while Acj6(1,4) inhibits this process, suggesting that Acj6 target genes may be affected either negatively or positively depending on the isoform expressed (Table 1).

How might the POU IV box influence the activity of distinct Acj6 isoforms? In vitro studies suggest that the amino-terminal region of Brn-3a is important for generating transcriptional activity from specific promoters and may be necessary for cell-specific protein-protein interactions (Budhram-Mahadeo et al., 1995; Theil et al., 1993). Therefore, the activity of individual Acj6 isoforms in a distinct cell or at a certain differentiation stage may depend on interactions with other proteins through the POU IV box. Alterations in the POU IV box may either positively or negatively influence these interactions through enhancement or disruption of protein binding or as a result of differential modifications by signal transduction pathways. It will be of great interest to distinguish between these possibilities.

Results from our studies underscore the importance of class IV POU domain transcription factors in terminal neuron differentiation and provide the first in vivo data indicating that the POU IV box structure is important. In the misexpression studies, at least two unique Acj6 isoforms appear capable of distinct functions. The high degree of conservation between Acj6 and the vertebrate Brn-3 proteins in this region indicate that the identification of specific co-activators may provide insight into the generation of distinct neuronal types in higher organisms as well.

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REFERENCES


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