AST-1, a novel ETS-box transcription factor, controls axon guidance and pharynx development in C. elegans

Christina Schmid, Valentin Schwarz, Harald Hutter *

Max Planck Institute for Medical Research, Jahnstr. 29, 69120 Heidelberg, Germany

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Abstract

Neurons send out axons and dendrites over large distances into target areas where they eventually form synapses with selected target cells. Axonal navigation is controlled by a variety of extracellular signals and neurons express receptors only for that subset of signals they need to navigate to their own target area. How the expression of axon guidance receptors is regulated is not understood. In genetic screens for mutants with axon guidance defects, we identified an ETS-domain transcription factor, AST-1, specifically required for axon navigation in certain classes of interneurons. In addition, ast-1 has a role in the differentiation of the ventral cord pioneer neuron AVG. Outside the nervous system, ast-1 is essential for morphogenesis of the pharynx. Ast-1 is transiently expressed in several classes of neurons (including AVG) during neuronal differentiation with a peak expression during late stages of neuronal differentiation and axon outgrowth. Ast-1 genetically interacts with other transcription factors controlling neuronal differentiation like lin-11 and zag-1 as well as components of the netrin pathway suggesting that ast-1 might control the expression of components of the netrin signal transduction machinery.

Keywords: Development; Neuron; Axon guidance; Ventral cord; Transcription factor; ETS-box

Introduction

In the developing brain, neuronal processes (axons and dendrites) have to navigate over large distances into targets areas where they form synapses to establish neuronal circuits. Several extracellular signaling molecules are known to control directed axon outgrowth. Ephrins and their receptors form gradients in the visual system leading to precise connections between the axons leaving the retina and brain regions where visual information is processed further (Drescher, 1997; Kullander and Klein, 2002; Wilkinson, 2001). Certain members of the BMP/TGF-β family are involved in guiding axons in dorsolateral direction in C. elegans and also within the spinal cord of vertebrates (Charron and Tessier-Lavigne, 2005). Semaphorins are a family of secreted or cell surface bound molecules mediating predominantly repulsive responses preventing axons from erroneously entering certain territories (Van Vactor and Lorenz, 1999). Several signals guide axons at the ventral midline of the developing embryo. Secreted Slit proteins were found to be repulsive cues preventing axons from crossing the ventral midline (Brose and Tessier-Lavigne, 2000). UNC-6/Netrin is a secreted protein with structural similarity to laminin, a component of the basement membrane. It has an evolutionarily conserved role in guiding axons towards the ventral midline in vertebrates and invertebrates like Drosophila and C. elegans (Culotti and Merz, 1998). Receptors for netrin were first discovered in C. elegans. They mediate distinct responses to netrin (Chan et al., 1996; Leung-Hagesteijn et al., 1992). Neurons expressing the UNC-40 receptor interpret UNC-6/Netrin as an attractive cue and send their processes towards the ventral midline. By contrast, neurons expressing the UNC-5 receptor in addition interpret UNC-6/Netrin as a repulsive cue and send their processes away from the ventral midline (Hedgecock et al., 1993). Consequently precise control of axon guidance receptor expression is a crucial aspect...
for directed axon outgrowth. How this is achieved is essentially unknown.

Cascades of transcription factors are known to be involved in earlier steps of neuronal differentiation from the selection of neuronal precursors to the point of neuronal subtype specification. LIM-homeobox proteins play a prominent role in these later stages of neuronal differentiation. This has been demonstrated for motoneurons in the vertebrate spinal cord, where LIM-proteins act in a combinatorial fashion to specify neuronal subtype identities (Jacob et al., 2001; Shirasaki and Pfaff, 2002). *C. elegans* LIM-proteins are among the founding members of this family (*lin-11* and *mec-3*) and have also been implicated in neuronal differentiation (Hobert et al., 1998; Xue et al., 1992). Among other things, *lin-11* is essential for the proper differentiation of the ventral cord pioneer neuron *AVG* (Hutter, 2003). More recently, a zinc-finger and homeobox containing transcription factor, *zag-1*, was identified in genetic screens for mutants with ventral cord axon guidance defects (Clark and Chiu, 2003; Wacker et al., 2003).

Neurons and their processes can be visualized in vivo in *C. elegans* animals by expressing the green fluorescent protein (GFP) under the control of neuron-specific promoters. In genetic screens using such reporters to identify mutants with defects in the navigation of interneuron axons of the motorcircuit, we identified two mutants defining the gene *ast-1* (ast for axonal steering). *Ast-1* was found to encode an ETS-domain transcription factor expressed in neurons during their differentiation. Defects in *ast-1* mutants are limited to axon navigation in certain classes of neurons, indicating that *ast-1* specifically controls this aspect of differentiation. *Ast-1* genetically interacts with other transcription factors like *zag-1* or *lin-11*, indicating that it is part of a transcriptional network controlling neuronal differentiation. *Ast-1* also interacts with components of the UNC-6/netrin pathway, suggesting that *ast-1* controls the expression of components of this signaling pathway.

Materials and methods

**Nematode strains**

The following mutants were used for mapping: *dpy-10(e128), dpy-17(e164), dpy-13(e184), dpy-11(e224), dpy-13(e184), unc-104(e1265) rol-6(e187), mab-20(yk105(e128) unc-104(e1265) rol-6(e187), mab-20(yk105(e128)) and CB4856 (wild type from Hawaii see (Hodgkin and Doniach, 1997)).

The following integrated GFP reporter constructs were used for analysis of axonal defects and misexpression: *evhs111[F25B3.3::GFP], yEx173[opt-3::GFP, rol-6(sa1006)], rhls4[glr-1::GFP; dpy-20(+)], hds10[unc-129::CFP, glr-1::YFP, unc-47::DsRed, hsp16::rol-6], hds22[unc-129::CFP, unc-47::DsRed], hds26[odr-2::CFP, sra-6::DsRed] To score defects in the deletion allele *ast-1(hd92)*, we generated a strain with *ast-1(hd92) null* in trans to a (dominant) GFP marker, VHI100: *ast-1(hd92)*/msls12 [myo-2::GFP] II, rhls4[glr-1::GFP, dpy-20(+)]. III. The presence of deletion allele was confirmed by PCR. Animals homozygous for *ast-1(hd92)* were identified as animals without the *msls12 GFP* marker, which is strongly expressed in the pharynx.

All strains were cultured at 20°C using standard methods.

**Mutant isolation**

The *ast-1* alleles *rh300* and *hd1* were isolated after EMS mutagenesis of *rhls4[glr-1::GFP]* animals in a non-clonal screen for animals with axon outgrowth defects in *glr-1* expressing interneurons (Hutter et al., 2005). The deletion mutant *hd92* was isolated from an EMS mutagenized library using a poison primer approach (Edgley et al., 2002) targeting the sixth exon coding for the ETS domain.

**Mapping and cosmid rescue**

*Ast-1(rh300)* was mapped to a region left of *dpy-10(e128)* on chromosome II by classical genetic methods. For further mapping using single nucleotide polymorphisms, *ast-1(rh300) rol-6(187) recombinants were crossed into the CB4856 wild type strain. F2 animals showing axon guidance defects were scored for the presence of CB4856 SNP markers. The *ast-1* locus was determined to be between SNP marker 24 at position 13,374 on cosmid M03A1 and 32 at position 5769 on cosmid F56D3.

Transgenic animals injected with a cosmids pool (C15C4, R03H10, H37P12, B0473) at 1–2.3 ng/μl each or the individual cosmids at 1–10 ng/μl together with the co-injection marker pRF4 (rol-6(sa1006)) at 100 ng/μl were analysed for rescue of axonal defects. A digestion fragment of cosmid H37P12 (Aes/ Eco811) containing the entire coding region of *T08H4.3* plus 2.5 kb upstream was also tested for its rescuing ability.

**Sequencing**

Small PCR fragments 1 kb in size covering the entire coding region of *T08H4.3* were generated for sequencing to identify the mutations in *ast-1(rh300)* and *ast-1(hd1)*. cDNA clones yk533 and yk339 were excised according to the manufacturer’s instructions (Stratagene Kit #200253) and used for restriction analysis and sequencing. The start codon of *ast-1* is at position 37976 and the stop codon at position 31050 of cosmid T08H4. The deletion in *ast-1(hd92)* was defined by sequencing a 1.0 kb PCR fragment starting about 0.5 kb upstream of exon six (deleted region: position 32215 to 31492 of cosmid T08H4).

**Analysis of *ast-1* expression**

To generate reporter constructs, we used the Gateway™ cloning system according to the manufacturer’s instructions. A PCR fragment containing 2.5 kb upstream region of *ast-1* was cloned into an entry vector and recombined with a destination vector containing YFP to create *past-1::YFP* (gene-specific primer sequences: gccttgtccgcctacaaagctggcc/tccgcttccttttcctgtatctg). Similarly, the coding region of *ast-1* starting after exon 2 was fused to the N-terminus of YFP (gene-specific primer sequences: ggttaagttttagcgcgtatcgtatcgtcgtatcgt) to generate *Δast-1::YFP*. With a multistep cloning approach, we combined existing subclones of the 2.5 kb upstream region followed by the entire coding region with YFP to generate *AST-1::YFP*. Transgenic animals were generated by injecting 20 ng/μl reporter plasmid plus 50 ng/μl pRF4. The array containing the *AST-1::YFP* was integrated using UV-irradiation, followed by two backcrosses with N2.

**Analysis of neuronal defects**

Animals were grown at 20°C and different life stages analysed from a growing population. For image acquisition, animals were incubated with 10 mM T08H4.3 were generated for sequencing to identify the mutations in *ast-1(rh300)* and *ast-1(hd1)*. cDNA clones yk533 and yk339 were excised according to the manufacturer’s instructions (Stratagene Kit #200253) and used for restriction analysis and sequencing. The start codon of *ast-1* is at position 37976 and the stop codon at position 31050 of cosmid T08H4. The deletion in *ast-1(hd92)* was defined by sequencing a 1.0 kb PCR fragment starting about 0.5 kb upstream of exon six (deleted region: position 32215 to 31492 of cosmid T08H4).

**Sequencing**

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**Analysis of neuronal defects**

Animals were grown at 20°C and different life stages analysed from a growing population. For image acquisition, animals were incubated with 10 mM NaNO3 in M9 buffer for 1 h and mounted on agar pads. Stacks of confocal images with 0.3 to 0.4 μm vertical pitch were recorded with a Leica TCS SP2 microscope. Maximum intensity projections of all images from a given animal were generated using the ImageJ software package.

**Genetic interactions**

To examine genetic interactions between *ast-1* and other genes, we looked at phenotypic aspects of the single and double mutants. Two genes are considered to act in the same pathway if the double mutant shows the same phenotype as the (stronger) single mutant. A chi square test was used to determine whether the double mutant had defects significantly different from the stronger single mutant. The following alleles were used for the genetic interaction studies: *ast-1 (rh300), unc-5(e53), unc-6(e490), unc-40(e271), unc-34(e566), sax-3(sy123), slt-1(oh15), nid-1(ur41), lin-11(n389), zag-1(rh315).*
Results

Ast-1 mutants show axon guidance defects in the ventral cord

Two ast-1 alleles – ast-1(rh300) and ast-1(hd1) – were isolated in genetic screens for mutants with axon guidance defects of the command interneurons of the motorcircuit which were visualized with a glr-1::GFP marker. These neurons have cell bodies in head ganglia and send their axons first into the nerve ring and from there further on into the ventral nerve cord (VNC, Fig. 1A). In ast-1 mutants, individual axons occasionally fail to reach the ventral cord and extend in variable lateral positions instead (Fig. 1B, Table 1). Within the VNC in wild type animals, these axons run in the right axon tract where they form a tightly fasciculated bundle. In 35% of mutant ast-1(rh300) animals, one or more axons cross the ventral midline and extend in the left axon tract (Fig. 1D, Table 1). Frequently, these axons eventually cross back into the right tract. Both ast-1 alleles show these defects with similar penetrance. Ventral cord axons labeled by the glr-1::GFP marker belong to the AVA, AVB, AVD, AVE, AVG, AVJ and PVC neurons. To more precisely define which of these neurons have defects we used two other markers labeling subsets of these neurons. An opt-3::GFP marker was used to specifically label the AVE neurons. No significant axon outgrowth defects were found with this marker, suggesting that the AVE axons are not affected in ast-1 mutants (Table 1). A nmr-1::DsRed marker was used to label AVA, AVD, AVE and PVC neurons in combination with a glr-1::YFP marker. In 77% of the animals, where glr-1::YFP labeled axons crossed the ventral midline, nmr-1::DsRed labeled axons were among them, suggesting that this subset of neurons is affected more frequently than the other interneurons.

Using a set of different GFP markers, we analyzed several classes of motoneurons as well as sensory neurons for axon outgrowth defects. Navigation defects were found only for the

PVPR and PVQL axons, which normally extend in the left ventral cord axon tract. In 26% of the ast-1(rh300) mutant animals, PVPR and PVQL axons were found to cross the ventral midline together. Neither the DD/VD motoneurons nor the DA/DB motoneurons showed any significant defects in axon navigation within the ventral cord or in navigation of commissures towards the dorsal cord. Neither amphid nor phasmid neurons stained with DiI nor inner labial neurons labeled with an odr-2::CFP marker had axon navigation defects (data not shown). Analysis with a pan-neuronal marker also failed to reveal any other defects outside the ventral cord (data not shown).

Partial differentiation defects of the ventral cord pioneer AVG in ast-1 mutants

The AVG neuron is the pioneer of the right ventral cord axon tract (Durbin, 1987). Using an odr-2::CFP marker to visualize this pioneer, we found AVG axon navigation defects in ast-1 mutants with a penetrance of 16% (Table 1, Fig. 2D). In 95% of these animals, glr-1::GFP expressing interneurons were found to cross the midline at the same position where the AVG axon crossed over (Fig. 2E). This emphasizes the importance of AVG as pioneer for interneurons in the ventral cord and suggests that almost half of the interneuron midline crossing defects (15% out of 35%) are secondary consequences of the pioneer navigation defect. In 22% of the ast-1 mutant animals, the AVG axon stopped prematurely (Table 1, Fig. 2G). This occurred at variable positions along the normal trajectory. Interneuron midline crossing defects in this fraction of animals (18%) were not enhanced, indicating that in the complete absence of the AVG axon other cues are used for navigation. Finally, in 8% of ast-1 mutant animals, the odr-2::CFP marker expression was drastically reduced or completely absent in AVG (Fig. 2J). In these animals, the glr-1::YFP marker was still expressed in AVG (Fig. 2K), suggesting that the AVG neuron is still present.

Ast-1 encodes an ETS-box transcription factor

To identify the ast-1 gene, we used standard genetic markers as well as single nucleotide polymorphisms between the N2 and CB4856 strains of C. elegans. Genetic mapping placed ast-1 on the right arm of chromosome II between SNP markers on cosmids M03A1 and F56D3. Rescue experiments with YACs, cosmids and genomic PCR fragments identified the predicted open reading frame T08H4.3 encoding an ETS-box transcription factor as the ast-1 gene (Fig. 3A). Sequencing of cDNAs confirmed the predicted exon–intron structure of the gene (Fig. 3B). To identify the nature of the mutations in the two ast-1 alleles, we sequenced the coding part of ast-1 in the mutants. In both cases, we found a point mutation within the ETS-domain. The ast-1(rh300) mutation corresponds to a ‘G’ to ‘A’ transition resulting in a glycine to arginine exchange at amino acid position 240 in the protein (Fig. 3B). In ast-1(hd1) a ‘C’ to ‘T’ transition leads to an alanine to valine exchange at amino acid position 251. Both amino acids are highly conserved among ETS-proteins and are close to (rh300) or within (hd1) the first
helix of the helix–turn–helix DNA-binding motif within the ETS-domain.

Ast-1 null mutants show pharynx differentiation defects in addition to axon navigation defects

The mutations in ast-1(rh300) and ast-1(hd1) are located within the DNA binding domain and could interfere with DNA-binding in a way that completely eliminates AST-1 function. Alternatively, some residual function could be left since both alleles are missense mutations leaving the AST-1 protein essentially intact. To test this, we isolated a deletion in ast-1, which is expected to completely eliminate all AST-1 function. The deletion in ast-1(hd92) is 724 base pairs in size and completely eliminates the second half of the ETS-domain. The deletion ends in an intron leading to a frameshift and subsequently to a stop codon effectively truncating the protein within the DNA-binding domain. We were not able to isolate viable animals homozygous for the deletion. We used a (dominant) GFP marker on chromosome II in trans to the deletion

Table 1
Neuronal defects in ast-1 mutants

<table>
<thead>
<tr>
<th>Defect</th>
<th>Marker used</th>
<th>Neurons</th>
<th>% animals with defects</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>WT</td>
<td>ast-1 (rh300)</td>
</tr>
<tr>
<td>Axon navigation defects</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lateral axon</td>
<td>gbr-1</td>
<td>All gbr-1::GFP expressing neurons</td>
<td>2 (n = 100)</td>
</tr>
<tr>
<td>Ventral cord midline crossing</td>
<td>gbr-1</td>
<td>AVE, AVB, AVD, AVE, AVJ, PVC</td>
<td>1 (n = 115)</td>
</tr>
<tr>
<td>Ventral cord midline crossing</td>
<td>opt-3</td>
<td>AVE</td>
<td>0 (n = 65)</td>
</tr>
<tr>
<td>Lateral axon</td>
<td>opt-3</td>
<td>AVE</td>
<td>11 (n = 65)</td>
</tr>
<tr>
<td>Ventral cord midline crossing</td>
<td>odr-2, sra-6</td>
<td>PVPR and PVQL</td>
<td>13 (n = 92)</td>
</tr>
<tr>
<td>Ventral cord midline crossing</td>
<td>unc-129</td>
<td>DA/DB motoneurons</td>
<td>0 (n = 151)</td>
</tr>
<tr>
<td>Ventral cord midline crossing</td>
<td>unc-47</td>
<td>DD/VD motoneurons</td>
<td>9 (n = 151)</td>
</tr>
<tr>
<td>AVG differentiation defects</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No expression</td>
<td>gbr-1</td>
<td>AVG</td>
<td>13 (n = 100)</td>
</tr>
<tr>
<td>Weak or no expression</td>
<td>odr-2</td>
<td>AVG</td>
<td>0 (n = 100)</td>
</tr>
<tr>
<td>Ventral cord midline crossing</td>
<td>odr-2</td>
<td>AVG</td>
<td>0 (n = 100)</td>
</tr>
<tr>
<td>Premature stop</td>
<td>odr-2</td>
<td>AVG</td>
<td>0 (n = 100)</td>
</tr>
</tbody>
</table>

Fig. 2. AVG defects in ast-1(rh300) II; hdsIs12[odr-2::CFP, glr-1::YFP, nmr-1::DsRed]. All animals are L1 stage and display a ventral aspect. Scale bars are 10 μm. Red: odr-2::CFP (panels A, D, G and J), green: glr-1::YFP (panels B, E, H and K). Panels C, F, I and L are overlays of the two individual channels. Images are maximum intensity projections of stacks of confocal images. (A–C) Wild type animal with normal expression level in AVG and no axon outgrowth defects. (D–L) ast-1(rh300) mutant animals. (A) The AVG cell body (arrowhead) is located at the anterior end of the ventral cord next to the two RIF neurons, which also express the odr-2 marker. The AVG axon extends all the way to the tail in the right axon tract. (B) glr-1::YFP expressing interneurons send their axons tightly bundled into the right axon tract. (D–F) AVG axon crosses erroneously in the left tract and back into the right tract (arrows in panel D). The majority of the interneuron axons follows the AVG axon (arrowhead in panel E), overlay (F). (G–I) Premature stop of the AVG axon along its normal trajectory (arrow in panel G). Interneuron axons continue normally but cross back and forth between right and left tract in the posterior part of the ventral cord (arrows in panel H). (J–L) Strongly reduced odr-2::CFP marker expression in AVG (arrowhead in panel J points to the AVG cell body, compare to panel A). glr-1::GFP is expressed at normal levels in AVG (arrowhead in panel K) and interneuron axons grow out normally in the ventral cord.
allele to identify animals carrying the deletion in homozygous form (they would not have the GFP marker) and found that all animals homozygous for \textit{ast-1(hd92)} failed to grow and arrested development as small larvae. Closer examination of the larvae revealed a pharyngeal defect leading to an inability to feed as the primary cause for the lethality. In \textit{ast-1(hd92)}, the pharynx fails to connect to the epidermis to form a functional mouth (Fig. 4D). As a consequence, animals are unable to feed and die shortly after hatching due to starvation. Time lapse recordings of \textit{ast-1} (hd92) animals showed that pharynx development is normal up to the 1 1/2-fold stage when the pharynx elongates and connects to the anterior hypodermis to form the mouth. This elongation fails in \textit{ast-1(hd92)} animals (Figs. 4E–H). Lineage analysis of ABara-derived pharyngeal lineages revealed no defects indicating that early inductions required to specify the ABara fate are normal (data not shown).

Neuronal defects in \textit{ast-1(hd92)} mutants were analyzed with the \textit{glr-1} \(\bowtie\) GFP marker. Some cell bodies of \textit{glr-1} expressing neurons in the head region were found to be misplaced. This is probably a secondary consequence of the pharynx differentiation defect, since these cell bodies normally are clustered around that part of the pharynx which fails to differentiate.
properly. Midline crossing defects are similar to *ast-1(rh300)* mutants (Table 1, Fig. 4B), but lateral growing axons are found with a significantly higher penetrance in *ast-1(hd92)* compared to *ast-1(rh300)*. The frequency of failure of *glr-1:*GFP marker expression in AVG (Table 1) is similar in *ast-1(hd92)* and *ast-1(rh300)*, suggesting that the AVG differentiation defects are not stronger in the null mutant.

*Ast-1 is transiently expressed during neuronal differentiation*

To find out where *ast-1* is expressed, we generated transgenic animals using several different *ast-1* promoter and AST-1 protein YFP fusion constructs (Fig. 5). The *past-1:*YFP construct contains the entire upstream region required for rescue (2.5 kb) fused to YFP. Expression of this construct in the embryo was confined to a set of sensory neurons with 6-fold symmetry most likely the inner labial neurons (Figs. 5A–B). It was highest between comma and 3-fold stage, fading away afterwards and being absent in larval stages and adults. To test the possibility that control elements for *ast-1* expression reside in introns, a second construct was generated containing all larger introns and most of the coding region of *ast-1* (∆ *AST-1:*YFP construct, Figs. 4C–D). Expression of this construct also started at the end of gastrulation in a few neurons. The number of neurons expressing the ∆ *AST-1:*YFP construct increases until the 3-fold stage. Expression is maintained in the first larval stage, decreases during further development and is only weakly detectable in adults. Interestingly the YFP signal in the embryo is initially confined to the nucleus, but is redistributed later in larval stages to spots outside the nucleus. Finally, we generated a complete *AST-1:*YFP fusion construct containing the same 2.5 kb promoter region as in the *past-1:*YFP construct. This construct reflected the expression of both *past-1:*YFP and ∆ *AST-1:*YFP also starting at the end of gastrulation (Figs. 5E–F). At the 2-fold stage, about 60 cells in the head express *ast-1*, most of them neurons, but also a few cells in the pharynx (Figs. 5I–J). A single cell in the retrovesicular ganglion expresses *AST-1:*YFP throughout development from the 1/2-fold stage onward (Figs. 5G–H). Using an *odr-2:*CFP marker and checking for overlap in expression, this cell was identified as the AVG neuron. The ALN neurons in the tail weakly express *AST-1:*YFP in late embryo and early larval stages (Figs. 5O–P). Occasionally, a second pair of cells in the tail was seen based on cell body position most likely the rectV cells. Expression in the tail generally is weak and somewhat variable from animal to animal. In the L1 stage, we found *AST-1:*YFP to be coexpressed with *glr-1:*CFP in AVA, AVD (weak *ast-1*), AVE (weak *ast-1*) RMDD and SMDD as well as AVG. *AST-1:*YFP expression decreases during larval development and is restricted to few neurons, which continue to express throughout the entire life cycle. As with the ∆ *AST-1:*YFP construct, we initially see the YFP signal only in the nucleus consistent with *AST-1* being a transcription factor. Already beginning in the 3-fold stage, we see a redistribution of the YFP signal until most of the signal is outside the nucleus mainly in spots in the cell body but also in the neuronal processes (Figs. 5N, R).

*Ast-1 interacts with lin-11 and zag-1 transcription factor mutants*

In the genetic screens leading to the isolation of *ast-1* mutants, we also identified mutants in two other transcription factor genes, *zag-1* and *lin-11*, with similar axon outgrowth defects. *Zag-1* affects multiple aspects of neuronal differentiation in many classes of neurons (Wacker et al., 2003), whereas *lin-11* controls neuronal differentiation of the ventral cord pioneer AVG, which has indirect effects on the navigation of *glr-1:*GFP expressing interneurons (Hutter, 2003). These transcription factors could act in combination with *ast-1* or independently of *ast-1*. To distinguish between these possibilities, we evaluated
ventral cord midline crossing defects in ast-1; zag-1 double mutants as well as in ast-1; lin-11 double mutants. In both cases, double mutants had a phenotype not significantly different from the phenotype of the stronger single mutants (Fig. 6), suggesting that ast-1 and zag-1 and also ast-1 and lin-11 act in a common pathway. Ventral cord defects in zag-1 and lin-11 mutants are stronger than defects in ast-1 mutants, indicating that these mutants also affect pathways that are independent of ast-1. Since all these genes encode transcription factors, it is possible that the genetic interaction reflects a direct dependency at the transcriptional level. To test this, we analyzed expression of the AST-1::YFP fusion construct in lin-11 mutants and the expression of lin-11::GFP and zag-1::GFP reporters in ast-1 (rh300) mutants. Neither zag-1 expression nor lin-11 expression was substantially altered in ast-1 mutants, indicating that neither zag-1 nor lin-11 is directly regulated by ast-1. Ast-1 expression in AVG, however, is absent in lin-11(n389) mutants (Figs. 5S, T), suggesting that lin-11 acts upstream of ast-1. In zag-1(rh315) mutants, expression of AST-1::YFP was also absent in AVG (Fig. 5).

Fig. 5. Ast-1 expression. The three different YFP fusion constructs are indicated in the upper part of the figure. (A, B) 2-fold stage embryo showing expression of the past-1::YFP construct. Expression is limited to a set of sensory neurons. (C, D) 2-fold stage embryo showing expression of the ΔAST-1::YFP construct. Expression is seen in a larger number of neurons compared to the past-1::YFP construct in a pattern similar to the complete AST-1::YFP fusion construct (compare with panel J). Remaining panels: expression of the complete AST-1::YFP construct. (E, F) Embryo at the end of gastrulation/beginning of elongation. First time point, where AST-1::YFP expression is detectable in 6 cells. (G, H) 1 1/2-fold stage embryo. Cell number and expression levels increase. First time point, when expression is detectable in AVG (arrow). (I, J) 2-fold stage embryo, expression is now seen in about 60 cells in the head, 10 of which are within the pharynx primordium, the remaining cells are neurons surrounding the pharynx. (K, L) 3-fold stage embryo, first time point, where AST-1::YFP is no longer exclusively in the nucleus. (M, N) Head region of a L1 larvae, expression is still maintained in many neurons, but AST-1::YFP is found more and more outside the nucleus. (O, P) Tail region of an L1 animal, AST-1::YFP in ALN (arrowhead) and probably rectV (arrow). (Q, R) Adult animal, head region, expression decreases, but is maintained in some neurons and pharyngeal cells, predominantly outside the nucleus. (S, T) AST-1::YFP expression in lin-11(n389) mutants. Head region of an L1 animal. No expression in AVG (triangle). Scale bars are 10 μm, fluorescence images are maximum intensity projections of stacks of confocal images. Arrows in panels H, J, N and R point to the AVG neuron.
mutants, the AST-1::YFP fusion construct is not obviously changed, indicating that zag-1 does not directly control ast-1 expression.

Ast-1 interacts with nidogen and members of the netrin pathway

Ast-1 is likely to directly or indirectly control the expression of genes required for the navigation of interneuron axons; however, the actual targets are not known. To identify pathways of axon guidance cues affected in ast-1 mutants, we generated double mutants with members of the various known pathways like the netrin and slit pathways (Fig. 6). Double mutants between ast-1(rh300) and either unc-6/netrin or the unc-6 receptor unc-40 showed midline crossing defects of glr-1::GFP expressing interneurons with a penetrance not significantly different from the ast-1 single mutants, suggesting that these genes act in the same genetic pathway. Midline crossing defects in unc-6 and unc-40 single mutants are less penetrant than in ast-1 single mutants indicating that unc-6-independent signals are also affected in ast-1.

Slit is another extracellular cue important for axon navigation at the ventral midline (Hao et al., 2001). Sax-3 is the only known receptor for slt-1 (Hao et al., 2001). Defects in ast-1; sax-3 double mutants are significantly higher compared to ast-1 alone (Fig. 6), indicating that ast-1 and sax-3 act in different pathways. Slt-1 mutants have hardly any midline crossing defects and defects in slt-1; ast-1 double mutants are no more severe than ast-1 single mutants (Fig. 6).

Finally, we tested for potential interactions with nidogen (nid-1), a component of the basement membrane, which was show to be important for proper positioning of axon bundles in C. elegans (Kim and Wadsworth, 2000). We found that midline crossing defects in ast-1; nid-1 double mutants are not significantly different from defects in ast-1 alone, suggesting that ast-1 and nid-1 act in the same pathway (Fig. 6).

PVC neurons in the preanal ganglion send their axons towards the ventral cord and then further within the right ventral cord axon tract towards the brain. They are attracted to the ventral cord in part by UNC-6/Netrin, since PVC axons fail to enter the ventral cord and extend in lateral position in 48% of unc-6 mutant animals (Fig. 6). The unc-40 receptor is required here, but PVC defects are found in only 15% of unc-40 mutant animals, suggesting that another yet unidentified receptor is involved in this response. Ast-1; unc-40 double mutants have PVC defects with a penetrance significantly higher than unc-40 mutants alone and essentially identical to unc-6 mutants, suggesting that this unc-40-independent pathway is controlled by ast-1. Ast-1 does not seem to control expression of another signal, since defects in ast-1; unc-6 double mutants are not significantly different from defects in unc-6 mutants alone.

Discussion

AST-1 is an ETS-domain transcription factor controlling directed axon outgrowth

Ast-1 mutants were identified in genetic screens for mutants with axon guidance defects in glr-1::GFP expressing interneurons. The ast-1 gene was found to encode an ETS-box transcription factor, one of 10 ETS genes encoded in the C. elegans genome (Hart et al., 2000). Other members of this
family in *C. elegans* include *lin-1*, which is known to be involved in development of the vulva, excretory system, male tail and posterior ectoderm (Beitel et al., 1995; Ferguson and Horvitz, 1985; Han and Sternberg, 1990). The closest relative of *ast-1* in *C. elegans* (C42D8.4) is expressed in a subset of sensory neurons pointing to a function in neuronal development (Hart et al., 2000). Based on sequence similarity of the ETS-domain, *ast-1* and C24D8.4 are members of the fli subfamily of ETS proteins. The mammalian members of the subfamily, fli and erg, have a Pointed domain in addition to the ETS domain, which is missing in the *C. elegans* members of this subfamily. Fli-1 in mouse is implicated in endothelial and megacaryocyte development (Spyropoulos et al., 2000). Erg is predominantly expressed in mesodermal tissue during development. The fli subfamily is evolutionary old and it is speculated that the acquisition of the Pointed domain by mammalian members of the family is essential for their role in vertebrate-specific tissue differentiation (Hart et al., 2000). Two members of the ETS-family in vertebrates, Er81 and Pea3, are expressed in the developing nervous system, most notably in distinct subsets of motoneurons as well as the corresponding proprioceptive neurons (Arber et al., 2000; Lin et al., 1998; Livet et al., 2002). Analysis of mouse mutants suggests that Er81 is involved in the formation of specific connections between proprioceptive afferents and motoneurons (Arber et al., 2000). In Pea3 mutant mice, certain motoneuron axons fail to branch normally within their target muscle indicating that PEA3 controls the terminal arborization of specific motoneuron pools (Livet et al., 2002). In addition, in Pea3 mutants, branchial motoneurons fail to contact their normal muscle targets (Ladle and Frank, 2002). Both of these ETS transcription factors appear to control defined aspects of neuronal differentiation, in particular aspects of axon navigation and circuitry formation. The most obvious defects in the nervous system in *ast-1* mutants are axonal navigation defects in certain classes of interneurons. Other aspects of interneuron differentiation like expression of cell-type-specific markers are unaffected in *ast-1* mutants. Despite the obvious differences in the details of the phenotype, ETS domain transcription factors seem to have an evolutionary conserved function in controlling aspects of neuronal circuit formation: axon navigation in nematodes and terminal branching and target connection in mammals.

**AST-1 controls the differentiation of the ventral cord pioneer neuron AVG**

*Ast-1* controls various aspects of AVG differentiation. We found axon navigation errors, premature stop of axon outgrowth and failure to express certain markers. All these phenotypes were incompletely penetrant, indicating that other factors act together with *ast-1* to control individual aspects of AVG differentiation. *Ast-1* is expressed in AVG and this expression is abolished in *lin-11* mutants, suggesting that *lin-11* directly or indirectly controls *ast-1* expression in AVG. This idea also explains the results of the double mutants analysis which indicates that these two genes act in a common pathway. *Lin-11* is a LIM-homeobox transcription factor essential for proper differentiation of the AVG neuron (Hutter, 2003). Since AVG acts as a pioneer for later outgrowing axons in the ventral cord, AVG differentiation defects have secondary consequences for the navigation of inter- and motoneurons in the ventral cord. In *ast-1* mutants, almost half of the interneuron midline crossing defects appear to be direct consequences of AVG navigation errors. However, *ast-1* also has a role in the interneurons themselves, since we observe lateral outgrowing axons, a type of navigation error which cannot be explained as secondary consequence of an AVG differentiation defect. The *AST-1∷YFP* reporter construct is coexpressed with *glr-1∷CFP* in some neurons supporting this idea. We found most of the neuronal markers normally expressed in AVG. This is in contrast to the situation in *lin-11* mutants where none of the known cell-type markers is still expressed in AVG. No secondary ventral cord motoneuron axon navigation errors are apparent in *ast-1* mutants, suggesting that guidance cues provided by AVG for inter- and motoneurons are distinct and differentially affected in *ast-1*. Ventral cord defects scored with *glr-1∷GFP* in the putative null mutant *hd92* are not stronger than in the point mutants *rh300* and *hd1*. Those two alleles apparently represent already complete loss of function alleles for aspects of ventral cord axon navigation. The deletion allele has a higher penetrance of lateral outgrowing axons suggesting that navigation of interneuron axons towards the nerve ring is more severely affected in *hd92* compared to *rh300* and *hd1*. In contrast to these point mutations, the deletion allele also has defects outside the nervous system in the pharynx suggesting that *rh300* and *hd1* have residual function in certain cells. Maybe they are still able to bind to certain promoters despite mutations in conserved regions of the putative DNA binding domain. Alternatively, pharyngeal cells could be less sensitive to reduced levels of activity of *AST-1* in *rh300* and *hd1*. The deletion allele *hd92* removes a significant portion of the AST-1 protein; however, it does not completely eliminate it. The remainder of the protein could still have some function and we cannot exclude the possibility that it might interfere with some other protein’s function.

**AST-1 is part of transcriptional networks controlling neuronal differentiation and pharyngeal development**

*Ast-1* expression is first detected after neurons become postmitotic and peaks during the final differentiation of neurons. Expression is seen in a large number of head neurons including sensory and interneurons. No expression is seen in ventral cord motoneurons indicating that *ast-1* is required only in distinct subsets of neurons. The specific function of *ast-1* in axon navigation is distinct from the role of other transcription factors known to affect neuronal differentiation. *Unc-42* encodes a transcription factor required for correct differentiation of *glr-1* expressing interneurons. *Unc-42* is essential for the correct expression of glutamate receptor subunits in these neurons (Baran et al., 1999). In addition, a subset of the *nmr-1* expressing neurons shows navigation errors like lateral growing axons in *unc-42* mutants (Brockie et al., 2001). Mutants in the zinc-finger/homeobox containing transcription factor *zag-1* have been identified in similar axon guidance screens, but show multiple neuronal differentiation defects indicating that *zag-1*
has a more general function in neuronal differentiation (Clark and Chiu, 2003; Wacker et al., 2003). Double mutant analysis between zag-1 and ast-1 suggests that the two genes act in a common pathway to control the expression of genes required for correct navigation of interneurons of the motor circuit. Since expression of zag-1 is not obviously reduced in ast-1 mutants and vice versa, it seems most likely that the two transcription factors act at the same level possibly binding together to promoters. Vertebrate ETS proteins are known to act in combination with zinc-finger transcription factors. Fli-1 activates expression at certain promoters together with the zinc-finger protein GATA-1 (Eisbacher et al., 2003). The vertebrate homolog of zag-1 (ZEB) blocks the activity of ETS proteins by binding to neighboring promoter elements (Postigo et al., 1997). These observations provide ideas how zag-1 and ast-1 could act together to control gene expression. Detailed analysis of this interaction requires the identification of common targets, which are not known yet.

We used genetic interaction studies to identify pathways where ast-1 targets are acting. For interneuron axon navigation in the ventral cord, we found that ast-1 and nidogen (nid-1) act in the same pathway. As a component of the basement membrane, nidogen could be responsible for presenting axon guidance cues (Kim and Wadsworth, 2000). Ast-1 acting in responding neurons could be involved in the control of the appropriate receptor. For the navigation of the PVC axon towards the ventral midline, ast-1 is in a common pathway with UNC-6/netrin. It seems to act in an unc-40-independent branch maybe controlling expression of a still unidentified receptor mediating part of the response to netrin. We were not able to unambiguously identify PVC as one of the neurons expressing AST-1 with the various YFP reporter constructs we generated. This might be due to low signal levels in PVC or reflect other limitations of the YFP reporter used in the expression studies. Given the nature of the ast-1 gene product (transcription factor) and the genetic interactions with the unc-40 receptor in PVC axon guidance, a cell autonomous action of ast-1 in the PVC neuron would be the simplest scenario, but remains to be proven. The lack of early cell-type-specific markers for PVC makes it difficult to address this idea at the moment directly by targeted rescue experiments.

Ast-1 is also expressed in some pharyngeal cells which is consistent with a role in pharyngeal differentiation. Pharyngeal defects in ast-1 mutants apparently are late differentiation defects. No early lineage defects are obvious in ast-1 mutants. This distinguishes ast-1 from other transcription factors like T-box factors txb-37 and txb-38 which are specifically required for differentiation of the AB-derived anterior part of the pharynx (Good et al., 2004). Pharynx defects in ast-1 are also distinct from defects in pha-4, a forkhead transcription factor acting early in all pharyngeal cells to specify pharyngeal identity (Homer et al., 1998). The posterior part of the pharynx differentiates normally in ast-1 mutants with a clearly visible grinder. Cells in the anterior part also adopt a pharyngeal fate and differentiate. The most obvious pharyngeal defect appears to be a morphogenetic problem, a failure of the pharyngeal primordium to elongate and attach to the hypodermis. Arcade cells and epithelial cells of the pharynx are required for this elongation (Portereiko and Mango, 2001). However, ast-1 does not appear to be expressed in those cells (and also not in anterior hypodermal cells eventually forming the mouth region) arguing against a primary defect in arcade and/or epithelial cells. The primary cause of the pharyngeal defect remains unclear at the moment.

YFP tagged AST-1 protein initially is localized to the nucleus of ast-1 expressing cells. However, starting already in the embryo, increasing amounts of AST-1::YFP are found in spots outside the nucleus. Engrailed homeoproteins expressed in COS cells were also found in non-nuclear compartments which were characterized as caveolae (Joliot et al., 1997). The EMX1 transcription factor in mouse is present not just in the nucleus but also in axons of olfactory sensory neurons along their entire length, including their terminals in glomeruli of the olfactory bulb (Briata et al., 1996). These and several other observations have led to the idea that transcription factors can be transferred to neighboring cells affecting their future development (Prochiantz and Joliot, 2003). A similar situation could be envisioned for AST-1, explaining aspects of the phenotype which do not seem to correlate with the expression. Alternatively, AST-1 could always act cell-autonomously. The GFP reporter might not completely reflect the AST-1 expression. Ast-1 expression in some cells is maintained throughout the life cycle suggesting that ast-1 not only controls developmental processes, but is also involved in maintaining a differentiated state.

Expression of ast-1 is rather dynamic yet with the exception of a few pharyngeal cells confined to the developing nervous system. Developmental defects are predominantly axon navigation defects pointing to a very specific role of ast-1 in neuronal differentiation. The genetic interactions with UNC-6/netrin and its receptors already point to certain pathways where ast-1 targets obviously play an important role. The identification of target genes controlled by ast-1 might directly identify genes responsible for directed outgrowth of axons like receptors for extracellular signals or components of their signal transduction pathways.

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