Evolution of neural precursor selection: functional divergence of proneural proteins

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Summary

How conserved pathways are differentially regulated to produce diverse outcomes is a fundamental question of developmental and evolutionary biology. The conserved process of neural precursor cell (NPC) selection by basic helix-loop-helix (bHLH) proneural transcription factors in the peripheral nervous system (PNS) by atonal related proteins (ARPs) presents an excellent model in which to address this issue. Proneural ARPs belong to two highly related groups: the ATONAL (ATO) group and the NEUROGENIN (NGN) group. We used a cross-species approach to demonstrate that the genetic and molecular mechanisms by which ATO proteins and NGN proteins select NPCs are different. Specifically, ATO group genes efficiently induce neurogenesis in *Drosophila* but very weakly in *Xenopus*, while the reverse is true for NGN group proteins. This divergence in proneural activity is encoded by three residues in the basic domain of ATO proteins. In NGN proteins, proneural capacity is encoded by the equivalent three residues in the basic domain and a novel motif in the second Helix (H2) domain. Differential interactions with different types of zinc (Zn)-finger proteins mediate the divergence of ATO and NGN activities: Senseless is required for ATO group activity, whereas MyT1 is required for NGN group function. These data suggest an evolutionary divergence in the mechanisms of NPC selection between protostomes and deuterostomes.

Key words: Neural precursor, *Drosophila*, *Xenopus*, bHLH, Proneural gene, Evolution

Introduction

The development of multicellular organisms involves a complex interplay of pathways regulating gene expression and mediating cell-cell interactions. Most of these pathways are highly conserved, even between very distantly related species. A central question of both developmental and evolutionary biology is how the reiterative use of highly conserved signaling cascades can generate such a large diversity of cells, tissues and organisms. Although it is conceptually clear that differential interactions among highly conserved proteins can result in dramatically different outcomes, little is known about the genetic and molecular basis of these interactions. A well-studied example of cell fate determination and the generation of cellular diversity is the selection of diverse types of early neural precursor cells (NPCs) from the neuroectoderm. The extensive body of knowledge of the genes and signaling pathways which specify different lineages within the peripheral nervous system (PNS) in many different model organisms makes the selection of NPCs in the PNS an excellent platform for addressing these questions.

The initiation event in neural lineage development is the selection of NPCs. The study of the PNS of various model systems, such as *Drosophila*, *Xenopus* and mouse, shows that expression of basic helix-loop-helix (bHLH) proteins in the neuroectoderm confers the ability to generate NPCs (Anderson, 1999; Brunet and Ghysen, 1999; Campuzano and Modolell, 1992; Chitnis, 1999; Guillemot, 1999; Jan and Jan, 1994; Okano et al., 1997). bHLH proteins are the key proteins in nervous system development and evolution. Their expression determines the position, timing and extent of neural stem cell selection as well as the identity of the neural cells in each lineage. These proteins, which are known as proneural proteins, promote NPC formation by forming heterodimers with a widely expressed bHLH protein, called Daughterless (DA) in *Drosophila* (Cabrera and Alonso, 1991), and E12/E47 in vertebrates (Murre et al., 1989). The proneural-DA heterodimer regulates transcription of target genes by binding, via some of the residues in the two basic domains, to a DNA motif called the E-box. The function of bHLH proteins is thought to reside mostly within the bHLH domain, a structural motif, encoded by a stretch of 50-60 highly conserved amino acid residues.

Expression of a proneural gene in a presumptive NPC regulates, and is regulated by, a cell-cell communication

process mediated by the highly conserved Notch signaling pathway (Artavanis-Tsakonas et al., 1999). Expression of Notch receptor ligands, such as Delta (DL), is under the transcriptional control of proneural genes (Fode et al., 1998; Kunisch et al., 1994). Ligand engagement in a signal receiving cell leads to the repression of proneural genes partly by activation of the *Enhancer of split E(spl)* complex genes (Bailey and Posakony, 1995; Jennings et al., 1994; Lecourtois and Schweisguth, 1995). Thus, the signaling cell elevates levels of proneural genes and adopts the NPC fate, while at the same time preventing the neural specification of its neighbours. The genes required for these steps are highly conserved both structurally and functionally across species.

What determines which cell becomes the NPC is not clearly established. In some cases, the future NPC autonomously expresses a higher level of proneural proteins (Culi and Modolell, 1998). A zinc (Zn)-finger transcription factor, Senseless (SENS), has been shown to be an essential element in the cascade of events that allows cells to differentiate as NPCs. SENS appears to interact synergistically with proneural proteins in a positive genetic feedback loop in *Drosophila* (Nolo et al., 2000). Similarly, the Zn-finger protein X-MyT1 appears to be involved in the selection of NPCs in *Xenopus*: it synergizes with NGN1 rendering cells apparently less sensitive to Notch inhibition (Bellefroid et al., 1996). However, SENS and X-MyT1 belong to different classes of Zn-finger protein, and what mediates their synergy with proneural proteins remains unclear.

Two families of proneural bHLH proteins have been found and are conserved across species: the Achaete-Scute proteins (AS) and the Atonal related proteins (ARPs) (Bertrand et al., 2002; Hassan and Bellen, 2000). The ARPs consist of several subgroups, two of which, Neurogenin (NGN) and Atonal (ATO) groups appear to act at the earliest steps of NPC selection (Fode et al., 1998; Goulding et al., 2000; Huang et al., 2000b; Jarman et al., 1993; Ma et al., 1996). In the ATO group, gene substitution and misexpression studies within and across species suggest that there is a very high degree of functional similarity, and sometimes, but clearly not always, functional identity (Ben-Arie et al., 2000; Goulding et al., 2000; Wang et al., 2002). Although this has not been directly tested by gene replacement, expression and mutant analyses suggest that it may be true for the NGN group as well (Begbie et al., 2002; Ma et al., 1999). Both flies and vertebrates have PNS expressed genes belonging to the NGN and ATO groups. These two groups of proteins show very high similarity in the bHLH domain. Interestingly, TAP, the fly NGN group protein is not expressed during NPC selection in the fly PNS and does not appear to have proneural activity (Bush et al., 1996; Gautier et al., 1997). Conversely, ATO proteins are generally not expressed during early NPC selection in vertebrate neural plate (Ben-Arie et al., 2000; Brown et al., 1998; Helms et al., 2001; Kanekar et al., 1997; Kim et al., 1997). Therefore, does this reversal in the use of ARP proteins in NPC selection represent (1) a divergence in the mechanisms by which these genes act to specify NPCs, or (2) a functionally inert change in expression patterns?

To answer this question, we initiated a comparative study of the proneural capacities of ATO and NGN group proteins using *Drosophila* and *Xenopus* as model organisms. First, we find that ATO group proteins, potent neural inducers in the fly, are extremely weak NPCs inducers in *Xenopus*. By contrast, NGN proteins, which are potent neural inducers in vertebrates, are extremely weak inducers in flies. Second, the functional specificities of ATO proteins and NGN proteins are differentially encoded within the bHLH domain. We identify the specific residues responsible for proneural activity in each protein. Third, this differential activity between ATO proteins and NGN proteins is not mediated by DA or Notch. Fourth, the specific residues encoding the proneural activity mediate the specificity of genetic interactions with the appropriate Zn-finger proteins. The correct combination of bHLH protein and Zn finger protein is highly specific, and necessary for NPC formation. In summary, we identify both extrinsic and intrinsic factors responsible for specificity of NPC selection and demonstrate a mechanistic divergence in bHLH protein function.

Materials and methods

Fly stocks and genetics

Transgenic fly lines of *uasngn1*, *uasngn2*, *uasMath3*, *uasngn^{bato}* and *uasngn^{H2ato}* on different chromosomes were generated by standard fly transformation procedures (Rubin and Spradling, 1983). All crosses involving mutant and transgenic stocks were performed at 25°C. The fly strains used in this study are: *Df(1)N^{8}/FM7,ftzlacZ[ry+]*, *Df(2L)da^{KX136}/Cyo*, *yw;;Sens^{E1},red,e/TM6,Tb*, *w;;uasm8[w+]*, *w;;uasmδ[w+]*, *w;;dppGal4[w+]/TM6*, *w;daGal4[w+]*, *w;;Gal4-7/TM6B*, *w;;A101-lacZ[W+]/TM6B* and *w;;FRT,ato^{1}/TM6B*.

Plasmid construction, microinjection and in situ hybridization

The *ato* and math1 cDNA and coding region of NGN1 were subcloned into pCS2+ vector. The pCS2+X-MyT1 plasmid was described earlier (Bellefroid et al., 1996). DNA coding for *ngn^{bato}*, *ngn^{H2ato}*, *ato^{bngn}* and *ato^{H2ngn}* were obtained by site-directed mutagenesis PCR. The *ngn^{bato}* and *ngn^{H2ato}* fragments were cloned into pUAST vector. The *ato^{bngn}* and *ato^{H2ngn}* fragments were cloned into pCS2+. The mRNAs were injected in a volume of 5 nl at a concentration of 20-200 pg/nl, into a single blastomere of *Xenopus laevis* embryos at the two-cell stage. Embryos were collected at stage 15 and 19. Whole-mount in situ hybridisation was performed as described (Harland, 1991), using a digoxigenin labelled antisense *N-tubulin* probe.

Immunohistochemistry

Third instar larval wing discs were dissected in PBS. Embryos were bleached for 3 minutes. Discs and embryos were fixed with 4% formaldehyde in PBT for 15 minutes. Blocking and antibody incubation were performed as described (Mardon et al., 1994). The antibodies used were: mouse anti-β-Gal (Promega, 1:2000), rabbit anti-ATO (1:1000), rabbit anti-NGN1 (1:250), rabbit anti-Math1 (1:100), rabbit anti-ASE (1:1000), guinea pig anti-SENS (1:1000) and monoclonal antibody 22C-10 (1:100). Secondary antibodies were always used 1 in 500. Samples were mounted in Vectashield mounting medium (vector) and detected using confocal microscopy (BioRad 1024). Adult fly wings and scutella were mounted in 70% ethanol and documented using Leica microscopes and software.

Evolutionary trace analysis

A multiple sequence alignment and a sequence identity tree were generated using the pairwise sequence comparisons algorithm PILEUP (Feng and Doolittle, 1987), from the GCG sequence analysis package (Devereux et al., 1984). The Evolutionary Trace was performed as described previously (Lichtarge et al., 1996b).

Results

ATO and NGN proteins share 47% identity in the bHLH domain including eight out of 12 amino acid residues in the DNA binding basic domain and are expressed in both the *Drosophila* and vertebrate PNS. However, differences in their usage for early NPC specification in the PNS between vertebrates and invertebrates have been noted (Hassan and Bellen, 2000). NGN proteins do not act early in NPC specification in invertebrates, whereas ATO proteins do not act early in NPC specification in vertebrates. Does this switch in the use of proneural proteins reflect a mechanistic difference or an inert change of expression pattern of otherwise functionally equivalent genes? To address this issue, we initiated a comparative analysis using *Drosophila* and *Xenopus* as model systems. To assay the proneural activity of mouse NGN1 and fly ATO in vertebrates, the mRNA of each was injected into a single blastomere of a two cell-stage *Xenopus* embryo. Neuronal induction was detected at stage 15 via whole-mount in situ hybridization for *N-tubulin*, an early marker of neuronal differentiation (Chitnis and Kintner, 1995). Compared with uninjected embryos (Fig. 1A), injection of *Ngn1* mRNA induces a large number of ectopic neuronal precursors on the injected side (Fig. 1B). By contrast, injection of *Ato* mRNA does not induce a significant increase in *N-tubulin* expression (Fig. 1C). Similarly, the injection of mRNA for *Math1*, a mouse ortholog of *ato* does not significantly increase *N-tubulin* expression at stage 15 (Fig. 1D) but very few scattered *N-tubulin* positive cells can be seen at stage 19 (see below, Fig. 4H). Similar observations have been made for *Xath1* which has been shown to be a much weaker inducer of neuronal precursors than NGN1 (Kim et al., 1997). These data suggest that the *Xenopus* ectoderm responds robustly to NGN group proteins, but very weakly to ATO group proteins, to induce neurogenesis.

One explanation for the weakness of ATO and MATH1 activity in *Xenopus* is that NGN proteins are more potent neural inducers than ATO proteins and that, in parallel, stronger induction is needed in the vertebrate neuroectoderm than in the *Drosophila* neuroectoderm. To test this possibility, we misexpressed ATO proteins and NGN proteins in *Drosophila* using the UAS/Gal4 system (Brand and Perrimon, 1993) and assayed neural induction by counting the number of sensory bristles produced. Consistent with the fact that *ato* and *Math1* completely rescue each the loss of function of each other (Wang et al., 2002), the two genes show very similar phenotypes and we use them interchangeably throughout the study. Expression of *ngn2* with four different wing disc Gal4 drivers (*C5-Gal4*, *71B-Gal4*, *32B-Gal4* and *dpp-Gal4*) showed no neural induction (data not shown). Sixteen out of 23 *ngn1* transgenic lines

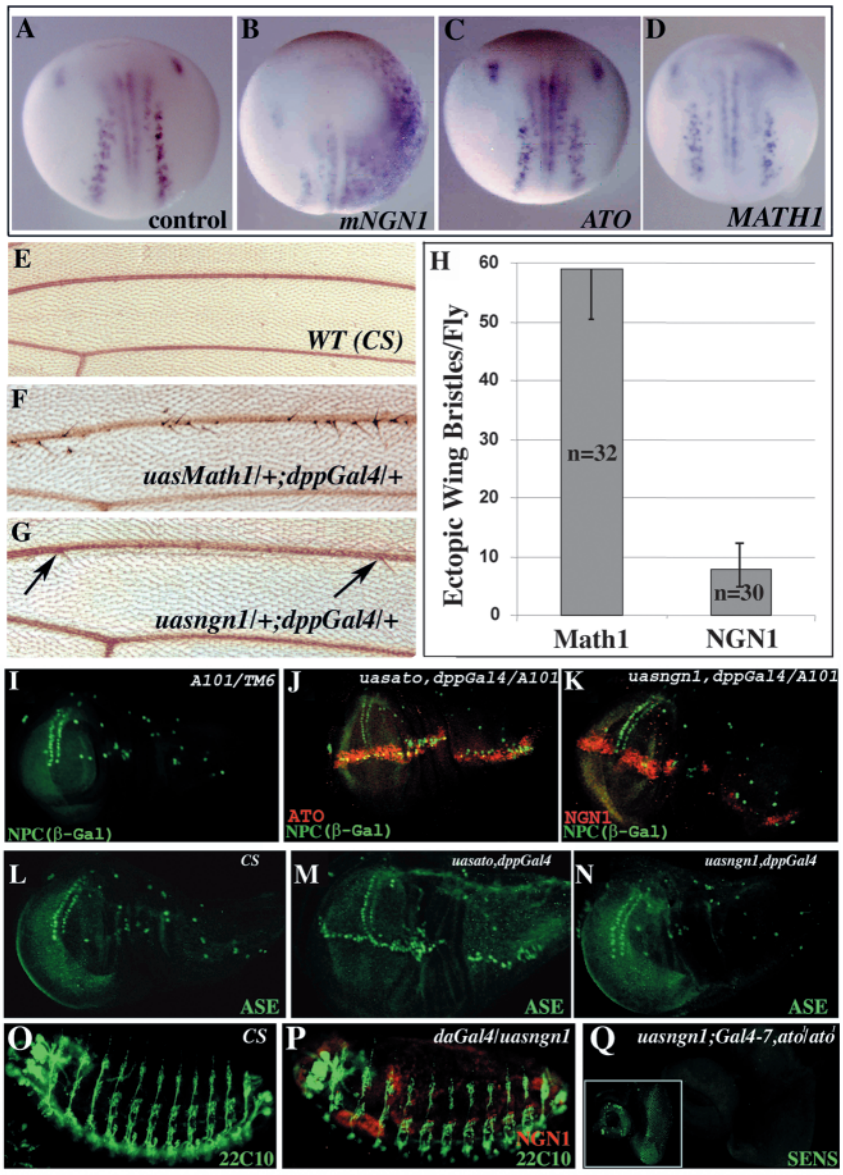

Fig. 1. The proneural activities of Atonal-related proteins. (A-D) Whole-mount in situ hybridisation with an N-tubulin probe to visualise neurogenesis in *Xenopus* embryos at stage 15. (A) Uninjected embryos. (B) 500 pg *Ngn1* mRNA. (C) 500 pg *Ato* mRNA. (D) 500 pg *Math1* mRNA. (E) Part of a wild-type fly wing showing no sensory bristles along the AP axis. (F) A *uasMath1/+; dppGal4/+* wing. (G) A *uasngn1/+; dppGal4/+* wing. (H) Quantitative analysis of the number of ectopic bristles per fly induced by expression of MATH1 or NGN1 using dppGal4 driver, '*n*' is the number of flies counted. (I-K) Third instar larval (L3) wing discs stained with anti-β-GAL (green) and proneural antibodies (red). (I) An *A101/TM6* wing disc. (J) A *uasato,dppGal4/A101* wing disc, anti-ATO (red) and anti-β-GAL (green). (K) A *uasngn1, dppGal4/A101* wing disc, anti-NGN1 (red), anti-β-GAL (green). (L-N) L3 wing discs stained with anti-ASE. (L) A wild-type fly (*CS*) wing disc. (M) A *uasato,dppGal4/TM6* wing disc. (N) A *uasngn1, dppGal4/TM6* wing disc. (O,P) Late stage embryos stained with 22C-10 (green) and anti-NGN1 (red). (O) A *CS* embryo. (P) A *daGal4/uasngn1* embryo. (Q) A *uasngn1;Gal4-7,ato^{1}/ato^{1}* L3 eye disc stained with anti-SENS. (Inset) A *uasngn1;Gal4-7,ato^{1}/TM6* L3 eye disc stained with anti-SENS revealing the R8 cells.

showed no neural induction. The other seven showed very weak induction (see below) with the strongest Gal4 driver, *dppGal4*. Therefore, the combination of *dppGal4* and the strongest *uas-ngn1* transgenic line were used in the rest of this study to determine the genetic and molecular basis of the difference in activity between ATO proteins and NGN proteins. The *dppGal4* driver in *Drosophila* is used to induce genes of interest along the anteroposterior (AP) axis of the wing disc. Wild-type flies have no external sensory bristles or chordotonal organs (CHOs) on the AP axis of the wing blade (Fig. 1E). By contrast, a large number of sensory bristles is found along the AP axis of the wing with 100% penetrance when either MATH1 (Fig. 1F) or ATO (data not shown) is expressed using dppGal4. In addition, both ATO and MATH1 induce CHOs (Jarman et al., 1993; Wang et al., 2002). Expression of the strongest NGN1 transgenic line results in very few bristles in only 70% of the flies examined (Fig. 1G) and no detectable CHOs. Quantitative analysis reveals that the number of sensory bristles induced by MATH1 is sixfold more than induced by NGN1 (P<0.001; Fig. 1H). Identical observations were made in the few surviving flies under the same conditions using strong UAS-ATO lines (data not shown). In the vertebrate PNS, NGN1 and NGN2 are sometimes co-expressed (Sommer et al., 1996), and activate the expression of NeuroD group proteins (Fode et al., 1998; Huang et al., 2000a; Sommer et al., 1996). Therefore, it is possible that the weak neural induction of mouse NGN1 is due to the lack of homologues of NeuroD proteins in flies. However, co-expression of NGN1 and NGN2 or NGN1 and MATH3, a NeuroD group protein (Tsuda et al., 1998), failed to enhance the proneural activity of NGN1 in *Drosophila* (data not shown).

One explanation for the very small number of bristles obtained after strong expression of NGN1 may be that the protein is able to induce NPCs, but most of these NPCs fail to differentiate properly and do not give rise to sensory organs. To test this possibility, we examined NPC formation directly upon expressing NGN1, ATO and MATH1 with *dppGal4* in *A101-lacZ* flies. *A101-lacZ* is an NPC specific enhancer trap (Huang et al., 1991). The normal pattern of NPCs is revealed by anti-β-GAL staining in third instar larval (L3) wing discs (Fig. 1I). Misexpression of ATO along the AP axis of the wing disc results in the induction of ectopic NPCs within the domain of ATO expression (Fig. 1J). By contrast, despite high levels of NGN1 expression, no detectable increase in NPCs is observed upon expression of NGN1 (Fig. 1K). Similarly, ATO, but not NGN1, induced *asense* expression, another marker of NPC specification (Fig. 1L-N).

Is the weak activity of NGN1 specific to ectopic expression in the wing disc? We find that wide expression of NGN1 in embryos using *da-Gal4* does not result in ectopic neurons (Fig. 1O,P). Finally, we attempted to rescue the loss of *ato* in the eye imaginal disc using *Gal4-7* and *uasngn1*. *Gal4-7* induces expression anterior to the morphogenetic furrow and has been used to restore photoreceptors to *ato* mutant eye discs using *scute* (Sun et al., 2000) and *Math1* (Wang et al., 2002). Expression of NGN1 in *ato* mutant discs did not result in any rescue (Fig. 1Q) nor did it induce ectopic R8 cells when expressed in control discs (Fig. 1Q, inset). For simplicity, we used the number of bristles as a quantitative assay for NPC formation for the remainder of the study.

Differential encoding of proneural activity in the bHLH domains of NGN proteins and ATO proteins

Three non DNA-binding basic domain residues determine the differential proneural activities of NGN proteins and ATO proteins

To explore whether the differential activities of NGN proteins and ATO proteins can be understood at the level of the proteins themselves, we turned to the comparative analysis of the amino acid sequence of the basic domain. Several studies have shown that important information is encoded by the basic domain, or specific residues therein (Chien et al., 1996; Davis and Weintraub, 1992; Talikka et al., 2002). In addition, the 12 amino acids in the basic domain are sufficient to phylogenetically delineate ATO proteins and NGN proteins, arguing that sequence differences within the basic domain are of functional significance (Hassan and Bellen, 2000). However, these studies did not investigate the genetic basis or address the evolutionary implications of the variation in basic domain sequence. ATO proteins and NGN proteins share eight residues out of 12 in the basic domain. One is variable, and the other three residues (4, 7 and 11) show almost absolute group specificity: they are highly conserved within each group but are essentially never the same between the two groups (Fig. 2A, green). To investigate whether this sequence specificity can explain the species-specific activities of ATO proteins and NGN proteins, we created a chimeric protein exchanging the three group-specific amino acids in the basic domain of NGN1 to those present in ATO, named NGN^{bATO} (Fig. 2B). Expression of NGN^{bATO} induces the appearance of bristles along the AP axis of the wing in all transgenic lines examined (Fig. 2C, inset). Strong UAS-NGN^{bATO} lines mimic strong UAS-ATO lines and result in significant lethality and more than 60 bristles per wing in the few surviving flies (data not shown). Moderate UAS-NGN^{bATO} lines behave like moderate UAS-ATO lines and induce an average of 33 bristles per fly along the AP axis (n=30) when compared with an average of seven for strongest UAS-NGN1 lines (n=45, Fig. 2C). Conversely, we generated a chimeric protein exchanging the three group-specific amino acids in the basic domain of ATO to NGN1, named ATO^{bNGN} (Fig. 2D). Whereas the injection of *Ato* mRNA in *Xenopus* embryos has no significant effect on the *N-tubulin* expression pattern (Fig. 2E), the injection of *Ato^{bNGN}* mRNA induces *N-tubulin* expression (Fig. 2F), indistinguishable from that caused by the injection of NGN1. Therefore, the NGN^{bATO} mutant recovers the NPC inducing activity of ATO in *Drosophila*, and the ATO^{bNGN} mutant recovers the NPC inducing activity of NGN1 in *Xenopus*.

It is worth to notice that only some of residues in the basic domain are directly contacting to DNA. The specific activities

Table 1. ET rank of functionally important residues in the bHLH domains of ATOs and NGNs

Rank	Alignment position	NGNs	ATOs
1	M13,L16,N17,A19,L23,R24,P28,L36, K38,E40,L42,A45,Y48,I49,L52	Invariant	Invariant
2	43	R	Q
3	20	L	F/Y
5	37	T	S
	44	F	M
7	39	I	Y/H/F

of ATO and NGN1 are unlikely to depend on differential DNA-binding activity as ATO proteins and NGN proteins have identical DNA contact amino acids (Hassan and Bellen, 2000) and can activate the NeuroD promoter via the same E-boxes in P19 cells (D. Castro and F. Guillemot, personal communication). Interestingly, biophysical and DNA-binding studies comparing MASH1 and MyoD have shown that they display similar binding preferences leading the authors to conclude that their different target specificities cannot be explained solely by differential DNA binding (Meierhan et al., 1995). Similar conclusions were made comparing *ato* and *sc* activities in neural subtype specification (Chien et al., 1996).

Five Helix2 residues are required for proneural activity of NGN proteins but not for ATO proteins

To investigate whether other functionally specific motifs exist in the bHLH domain of ARP proteins, we turned to the evolutionary trace (ET) analysis method. ET tracks residues whose mutations are associated with functional changes during evolution. This approach has been used to identify novel functional surfaces (Lichtarge et al., 1996a), and has recently been shown to be widely applicable to proteins (Madabushi et al., 2002). In practice, ET relies on the phylogenetic tree of a protein family and identifies residues of the alignment that are invariant within branches but variable between them. These positions are called 'class specific'. The smallest number of branches at which a position first becomes class specific defines its rank. The top ranked positions (1) do not vary. Very highly ranked positions (2-8) are such that they vary little and, whenever they do, there is also a major evolutionary divergence. By contrast, poorly ranked positions vary more often, and their variation does not seem to correlate with divergence. Thus, highly ranked positions tend to be functionally important, while poorly ranked ones tend not to be. When examining ARP bHLH domains, ET identified a number of positions that are jointly important in different bHLH domains, yet that undergo significant variation between them (Table 1). These residues varied in rank from 2 to 7, suggesting that they can undergo non-conservative mutations that are likely to correspond to functional divergence events. These positions tend to be most conserved between NeuroDs and NGN proteins and then undergo variations in ATO proteins, suggesting that they are important for an activity shared by NGN proteins and NeuroDs, but absent in ATO proteins. The data above show that the ability to induce NPCs in vertebrates is precisely such an activity. To investigate the role of these group-specific residues on functional specificity further, a chimeric protein, named NGN^{H2ATO} (exchanging amino acids 37, 39, 43, 44 and 46 in Helix2 of NGN1 to those present in ATO), was created and tested in *Drosophila* (Fig. 2G). Expression of the strongest NGN^{H2ATO} transgenic line induces a maximum of two bristles along the AP axis of the wing per fly in 50% of the flies. Quantitative analysis shows that, unlike ATO, NGN^{H2ATO} induces an average of 0.8 bristles along AP axis per fly (*n*=30, Fig. 2H). These data indicate that the group-specific motif in Helix2 of ATO does not encode proneural activity in

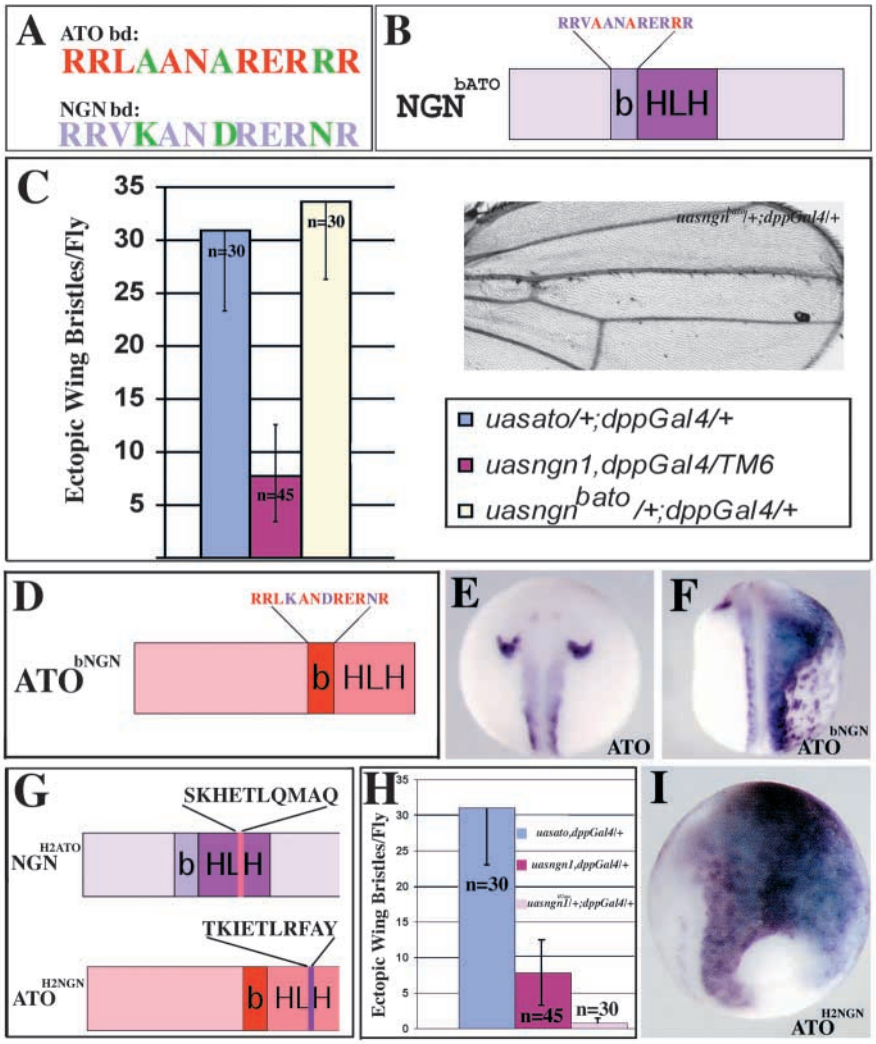

Fig. 2. Differential encoding of proneural activity in the bHLH domains of NGN proteins and ATO proteins. (A) The basic domains of ATO (red) and NGN1 (purple). Group-specific amino acids are in green. (B) Schematic representation of NGN^{bATO} with exchanged amino acids in red. (C) Quantitative analysis of proneural activity of misexpressed ATO, NGN1, NGN^{bATO} in a wild-type background. (Inset) A wing from a *uasngn^{bato}/+; dppGal4/+* fly. (D) Schematic representation of ATO^{bNGN} with the exchanged amino acids in purple. (E,F) N-tubulin stained *Xenopus* embryo at stage 19, injected with different mRNAs into one cell (right side) of two cell-stage embryos. (E) 1000 pg of *Ato* mRNA. (F) 1000 pg of *Ato^{bNGN}* mRNA. (G) Schematic representation of NGN^{H2ATO} and ATO^{H2NGN}. (H) Quantitative analysis of proneural activity of misexpressed ATO (blue), NGN1 (dark pink), NGN^{H2ATO} (light pink). (I) N-tubulin stained *Xenopus* embryo at stage 19, injected with 1000 pg of *Ato^{H2NGN}* mRNA into one cell of two-cell stage embryos.

Drosophila. Conversely, we generated a chimeric protein, named ATO^{H2NGN}, exchanging the same five amino acids in Helix2 of ATO to those found in NGN1 (Fig. 2G). Injection of ATO^{H2NGN} mRNA causes ectopic N-tubulin expression, indistinguishable from the injection of NGN1 (Fig. 2I). Therefore, ATO^{H2NGN} recovers the activity of NGN1 in *Xenopus*. Taken together, the mutational analysis results agree with the predictions of the ET analysis indicating that the identified residues in the Helix2 mediate the activity of NGN proteins but not of ATO proteins.

Mouse NGN1 can interact with daughterless and Notch in *Drosophila*

The data above support the hypothesis that ATO proteins and NGN proteins act via different genetic pathways to specify NPCs in different species. What might those pathways be? One simple explanation may be that NGN1 is not able to form heterodimers with fly Daughterless (DA), a required partner protein for NPC specification. In order to test this possibility, co-IP experiments were performed, in which ^{35}S-labeled ATO, MATH1 or NGN1 were co-precipitated with DA-Myc using anti-Myc antibodies (Fig. 3A, inset). In the presence of DA, mouse MATH1, fly ATO and mouse NGN1 are co-precipitated. Only background levels of NGN1 are detected in the absence of DA. These results suggest that mouse NGN1 can bind physically to fly DA in vitro. To test if DA and NGN1 can interact genetically in vivo, NGN1 was expressed in the absence of one copy of *da*. The number of sensory bristles produced by NGN1 along AP axis is greatly decreased in a heterozygous *da* background (Fig. 3A). Therefore, mouse NGN1 can physically and genetically interact with fly *da* in *Drosophila* in a dose-sensitive manner.

Next, we examined the possibility that mouse NGN1 does not respond to the *Drosophila* Notch signaling pathway (Fig. 3A). To test this, we examined neural induction by NGN1 in absence of one copy of *Notch* ($N^{+/-}$) or with the co-expression of *Notch* pathway genes. The proneural activity of NGN1 is enhanced in a $N^{+/-}$ background. Conversely, NGN1 activity is completely inhibited by co-expression of a constitutively active form, N^{intra} or members of the *E(Spl)* complex, *m8* and *mδ*. These data demonstrate that mouse NGN1 can be regulated by the Notch signaling pathway in *Drosophila*. It should be noted that overexpression of ATO in a *N* heterozygous background results in almost complete lethality and in extremely deformed wings owing, in part, to a very large number of bristles in the few surviving

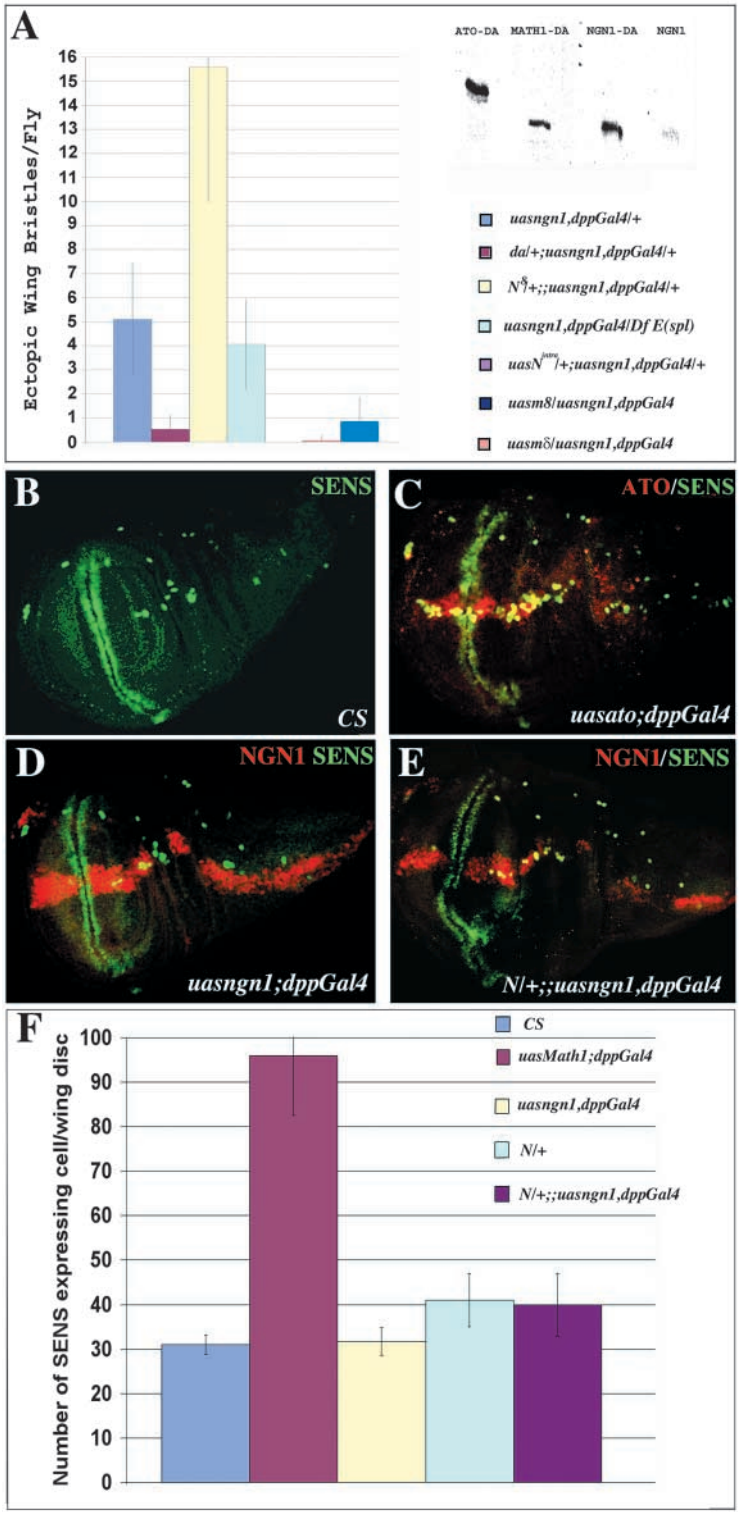

Fig. 3. Mouse NGN1 interacts with DA and Notch, but fails to induce SENS expression. (A) Quantitative analysis of the number of ectopic bristles per fly induced by expressing NGN1 in wild-type, $da^{+/-}$, $Notch^{+/-}$ or $E(spl)^{+/-}$ background, or co-expression with constitutively active Notch, or the members of *E(spl)* complex, *m8* or *mδ*. With the exception of the $E(spl)^{+/-}$ background, the effects of NGN1 expression in all backgrounds are significantly different from its effects in a wild-type background (P<0.001). (Inset) Autoradiograph of SDS-PAGE gels from co-immunoprecipitation using anti-Myc antibodies of ^{35}S-labeled ATO, MATH1 and NGN1 in the presence and absence of Myc tagged DA. (B) The expression pattern of SENS in *cs* L3 wing disc, stained with anti-SENS (green). (C) A *uasato; dppGal4/+* wing disc, stained with anti-ATO (red) and anti-SENS (green). (D) A *uasngn1,dppGal4/TM6* wing disc, stained with ant-NGN1 (red) and anti-SENS (green). (E) A N^{8}*/+; uasngn1,dppGal4/+* wing disc, stained with anti-NGN1 (red) and anti-SENS (green). (F) Quantitative analysis of the number of SENS positive cells in *CS* (wild type), *uasMath1/+;dppGal4/+*, *uasngn1,dppGal4/+* and N^{8}*/+* and N^{8}*/+;;uasngn1,dppGal4/+* L3 wing discs (n=5).

flies. As both ATO and NGN proteins can respond to levels of Notch signaling but only ATO proteins can efficiently specify NPCs, it is possible that ATO proteins and NGN proteins use different mechanisms to interact with the Notch signaling pathway. One possibility is that NGN proteins are more sensitive than ATO proteins to levels of transcriptional inhibitors of proneural activity encoded by the *E(spl)* genes because NGN1 activity, like that of ATO, can be repressed by ectopic expression of E(spl) proteins. However, in contrast to what is observed with *Notch*, removing a copy of the *E(spl)* complex does not alter NGN1 activity, suggesting that NGN1 is not more sensitive to levels of transcriptional inhibitors of proneural activity (Fig. 3A).

ATO proteins and NGN proteins interact genetically with different co-factors during NPC selection

NPC formation in *Drosophila* requires the Zn-finger protein Senseless (SENS). Fly proneural proteins first induce *sens* expression and then synergize with it in a positive feedback loop (Nolo et al., 2000). This appears to enhance the ability of proneural genes to downregulate Notch signaling in the presumptive NPC. In vertebrates, Senseless-like proteins appear not to act in NPC formation, although they are expressed in the PNS (Wallis et al., 2003). To test the possibility that SENS shows group specific interactions with bHLH proteins during NPC selection, we compared the abilities of ATO and NGN1 to induce SENS. SENS expression in wild-type L3 wing discs marks NPC formation (Fig. 3B). Ectopic SENS induction is detected along the AP axis of wing discs when ATO is misexpressed (Fig. 3C). However, SENS expression is not induced by NGN1 (Fig. 3D). These data suggest that unlike ATO, NGN1 does not efficiently induce SENS expression. We further tested whether lowering endogenous levels of Notch would allow NGN1 to induce SENS. Expression of NGN1 in Notch heterozygous animals, although significantly increasing the number of induced bristles (Fig. 3A), fails to induce SENS expression (Fig. 3E) when compared with $N^{+/-}$ controls, arguing that NPCs induced by NGN proteins are specified via a different mechanism not normally used in *Drosophila*. The data above are quantified in Fig. 3F. Although NGN1 does not induce SENS, it is possible that synergy might occur if the requirement for SENS induction is bypassed. We therefore compared the ability of NGN1 and MATH1 to synergize with SENS in vivo by co-expressing either NGN1 or MATH1 with SENS using a moderate scutellar Gal4 driver (*C5Gal4*). Neural induction was

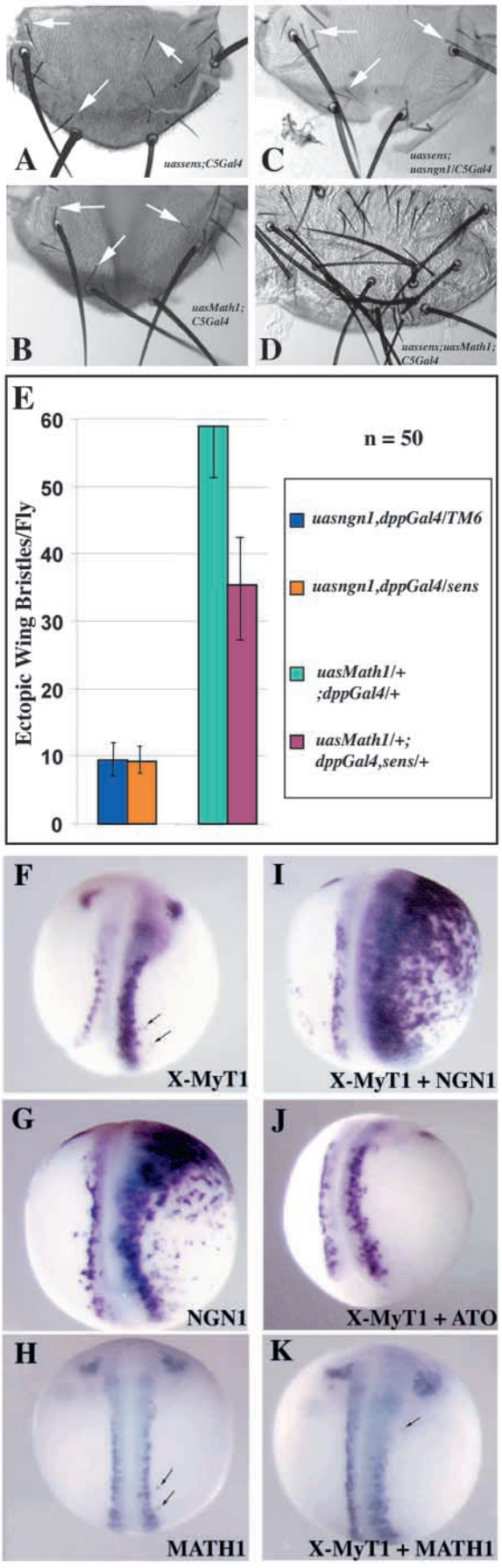

Fig. 4. NGN proteins and ATO proteins interact with different Zn-finger proteins. (A) A scutellum of a *uassens/+; C5Gal4/+* fly. Some ectopic microchaetes are indicated by arrows. (B) Ectopic microchaete on a *uasMath1/+; C5Gal4/+* fly scutellum. (C) Ectopic microchaete on a *uassens/+; C5Gal4/uasngn1* fly scutellum. (D) A scutellum of *uassens/+; uasMath1/+; C5Gal4/+* fly. (E) Quantitative analysis of the effect of SENS on NGN1 and MATH1.
(F-K) Detection of N-tubulin expression via whole-mount in situ hybridization in stage 19 *Xenopus* embryos, injected or co-injected with different mRNAs into a single blastomere at two-cell stage.
(F) 250 pg *X-MyT1* mRNA. (G) 250 pg *Ngn1* mRNA. (H) 1000 pg *Math1* mRNA. (I) 250 pg *X-MyT1* and 250 pg *Ngn1* mRNAs.
(J) 250 pg *X-MyT1* and 1000 pg *Ato* mRNAs. (K) 250 pg *X-MyT1* and 1000 pg *Math1* mRNAs.

examined by counting the ectopic bristles induced on the scutellum. Wild-type flies have four large bristles, or macrochaete, on their scutella. Expression of SENS (Fig. 4A) or MATH1 (Fig. 4B) alone with *C5-Gal4* induces a number of ectopic microchaete, or small bristles, on the scutellum. No ectopic sensory bristles were found when NGN1 was expressed alone (data not shown). Co-expression of NGN1 and SENS has the same effect on the scutellum as the misexpressing SENS alone (Fig. 4C). Co-expression of MATH1 and SENS, however, causes the appearance of a large number of both micro- and macrochaete (Fig. 4D). Finally, we misexpressed NGN1 or MATH1 in the absence of one copy of *sens* (Fig. 4E). No effect on NGN1 activity in a $sens^{+/-}$ background was observed. By contrast, the average number of sensory bristles produced by MATH1 along the AP axis was reduced by 42% (n=50, P<0.001) if a single copy of *sens* was removed suggesting dose-sensitive interactions. Thus, neither by loss nor gain of function criteria does NGN1 appear to interact with SENS, thus explaining its weak proneural activity and inability to efficiently antagonize Notch signaling in *Drosophila*. Therefore, SENS is a key extrinsic difference in how ATO proteins and NGN proteins regulate NPC selection.

In *Xenopus*, the C2HC-type Zn-finger protein X-MyT1 is expressed in primary neurons and can be induced by NGN proteins. In addition X-MyT1 has been suggested to play a role in NPC formation and to synergize with NGN proteins (Bellefroid et al., 1996). In order to test if X-MyT1, like SENS, shows specificity in its interaction with ARP proteins, we compared its ability to interact with NGN1 and ATO in *Xenopus*. *X-MyT1* mRNA was injected alone or co-injected with either *Ngn1* or *Ato* mRNA. As expected, the injection of *X-MyT1* increases the number of *N-tubulin*-expressing cells in the neural plate domains where neurons normally form (Fig. 4F), while the injection of *Ngn1* mRNA alone leads to induction of *N-tubulin* expression (Fig. 4G). Co-injection of *Ngn1* and *X-MyT1* mRNAs results in very strong *N-tubulin* induction, pointing to a synergistic interaction between the two proteins (Fig. 4I). By contrast, co-injection of *Ato* and *X-MyT1* mRNAs does not cause a detectable increase in *N-tubulin* expression compared with the injection of *X-MyT1* mRNA alone (Fig. 4J). Similarly, the few ectopic *N-tubulin*-expressing cells observed when *Math1* mRNA is injected (Fig. 4H) are not increased by co-injection of *Math1* and *X-MyT1* (Fig. 4K). Thus, X-MyT1 interacts specifically with NGN1 and not with ATO or MATH1. The data above demonstrate that the correct combination of ARP protein and Zn-finger protein is necessary for NPC induction.

NGN^{bATO} and ATO^{bNGN} have reversed interactions with Zn finger proteins

Does the coding sequence difference mediate the divergence in the genetic interactions of ARPs? To test this, we investigated whether the chimeric proteins recover the ability to interact with the respective Zn-finger proteins. Indeed, expression of NGN^{bATO} in *Drosophila* results in the induction of SENS (Fig. 5A-C), and the number of bristles induced by NGN^{bATO} in absence of one copy of *sens* ($sens^{+/-}$) is reduced by ~44% (n=30, P<0.001) (Fig. 5D). In addition, strong synergy was observed by co-expression of NGN^{bATO} and SENS using the *dppGal4* driver (data not shown). Therefore, NGN^{bATO} is able to induce and interact with SENS in *Drosophila*. In *Xenopus*, just like *Ngn1*, co-injection of Ato^{bNGN} and *X-MyT1* mRNAs (Fig. 5F) results in synergy and very strong ectopic *N-tubulin* expression when compared with the injection of *X-MyT1* (see

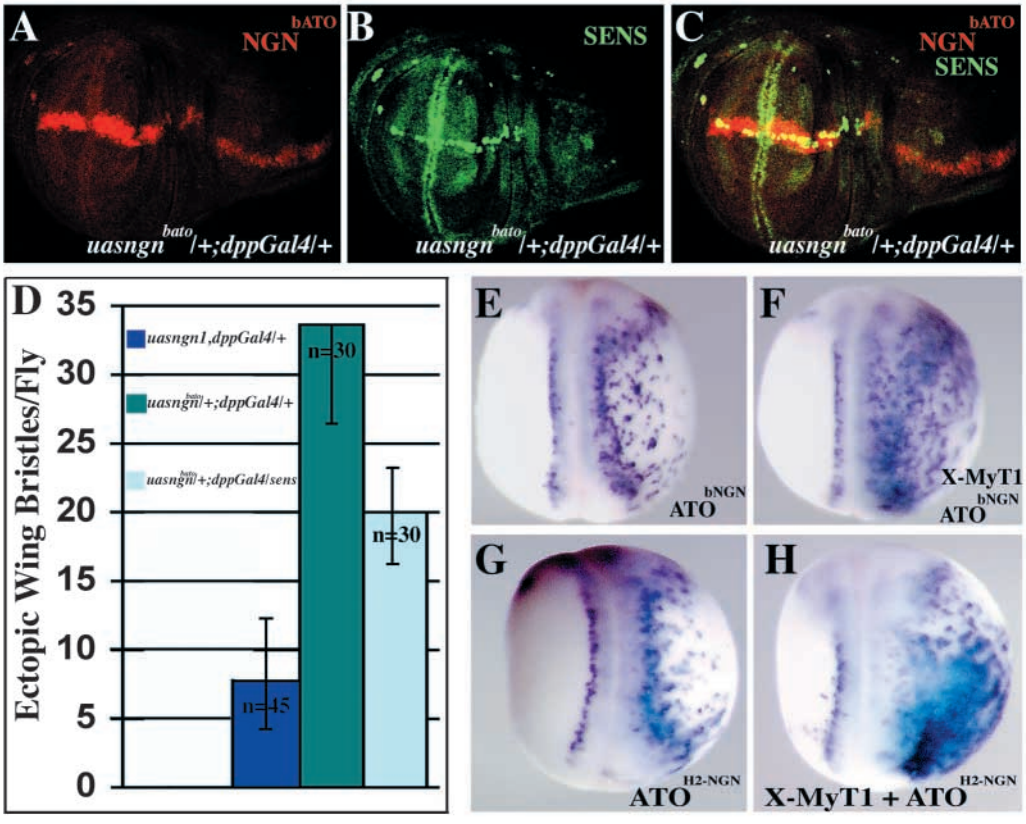

Fig. 5. NGN^{bATO} and ATO^{bNGN} have reversed interactions with Zn-finger proteins. (A-C) $uasngn^{bato}$/+; *dppGal4*/+ L3 wing disc stained with (A) anti-NGN1 (red) and (B) anti-SENS (green). (C) A merged image of A and B shows that misexpression of NGN^{bATO} induces SENS. (D) Quantitative analysis of the SENS effect on NGN^{bATO}. (E-H) Injected *Xenopus* embryos at stage 19, stained with N-tubulin. (E) 100 pg Ato^{bNGN}. (F) 100 pg Ato^{bNGN} and 250 pg *X-MyT1*. (G) 100 pg $Ato^{H2\text{-}NGN}$. (H) 100 pg of $Ato^{H2\text{-}NGN}$ and 250 pg *X-MyT1* mRNA.

Fig. 4F) or *Ato*^{bNGN} (Fig. 5E) alone. Similarly, co-injection of ATO^{H2NGN} and X-MyT1 mRNAs results in synergy and very strong induction of N-tubulin expression (Fig. 5G,H) suggesting that ATO^{H2NGN} and ATO^{bNGN} use the same mechanism of action as NGN1.

Discussion

The reiterative use of conserved signaling and regulatory pathways in various contexts is a defining biological principle. Paradoxically, it is precisely this reiteration and conservation that underlies the generation of developmental and evolutionary diversity. This raises the fundamental question of how gene-context interactions are regulated to produce diversity. In the vertebrate and invertebrate PNS, a difference in the use of bHLH proteins during NPC selection allows us to address this issue using an in vivo comparative analysis approach. In this study, we demonstrate that the invertebrate and vertebrate ectoderms respond differentially to ATO and NGN proteins to specify NPCs. The proneural activity is differentially encoded in the bHLH domains of ATO and NGN proteins. We further map the residues responsible for this specificity. Moreover, this divergence in the encoding of proneural activity is paralleled by a divergence in how bHLH proteins interact with Notch signalling.

ATO proteins and NGN proteins act by divergent mechanisms to regulate neural lineage development

At the developmental level, the data presented here can be explained by two possibilities (Fig. 6A,B). The first is that *Drosophila* and vertebrates use different bHLH proteins with divergent mechanisms for selecting similar cell types: the earliest born neural progenitors (Fig. 6A). Alternatively, NGN proteins may be involved in selecting neuronal (versus glial) rather than earliest born neural progenitors in vertebrates (Fig. 6B) (Nieto et al., 2001; Sun et al., 2001; Tomita et al., 2000). This is certainly the case in the mammalian inner ear (Bermingham et al., 1999; Ma et al., 2000) and it should be determined whether it is a more generally applicable rule, at least in the PNS. Given that there is no direct evidence in the literature to support a role for NGN proteins in selecting multipotent progenitors, we propose that the situation in Fig. 6B is likely to be more representative of the events in vivo. The latter scenario raises the question of whether *Drosophila*-like proneural proteins are needed in the vertebrate neural plate. If they are not, then the strict definition of a proneural gene as derived from work in *Drosophila* may need to be re-examined (Ledent and Vervoort, 2001). However, these two models for NGN function are not mutually exclusive. It is possible that in different lineages, NGN proteins select first neural, and then neuronal, precursors. This would be compatible with data from both flies and vertebrates showing that Notch signaling, in addition to having anti-neural effects, has also anti-neuronal and pro-glial effects during neural lineage development (Morrison et al., 2000; Udolph et al., 2001; Umesono et al., 2002; Van De Bor and Giangrande, 2001). Analysis of the fly NGN protein, TAP, may shed some light on this issue. At any rate, a comparative approach should provide a powerful tool for the systematic analysis of the pathways which program neural stem cells.

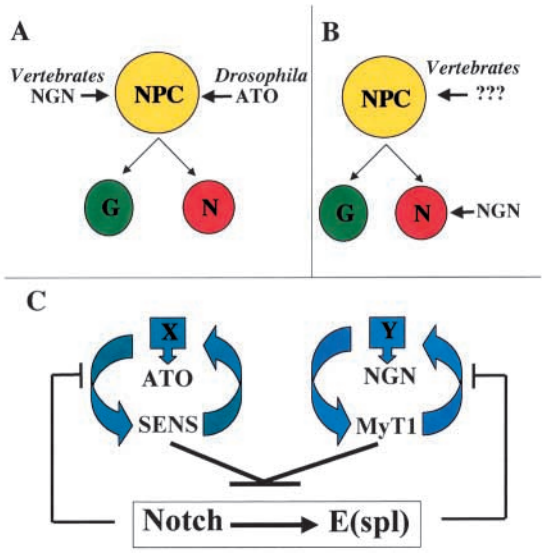

Fig. 6. The putative function of ATO proteins and NGN proteins during neuronal lineage development in *Drosophila* and vertebrates. (A) Both ATO proteins and NGN proteins may be specifying early neural progenitors (NPCs) in flies and vertebrates, respectively, using divergent mechanisms. N and G are neuronal and glial precursors, respectively. (B) In vertebrates, NGN proteins may specify neuronal, rather than neural, precursor cells. In this model, it is not known which genes specify neural precursor cells. (C) ATO proteins and NGN proteins use different genetic pathways to regulate Notch signalling and neural and/or neuronal precursors. X and Y refer to different unknown factors that mediate the synergy between ATOs and SENS on the one hand, and NGNs and MyT1 on the other.

Regardless of the precise developmental step at which ATO proteins and NGN proteins act, it is clear that the genetic and molecular mechanisms by which they act are different, suggesting that the functions of ATO proteins and NGN proteins are regulated by different factors (Fig. 6C). Furthermore, it is clear that the group-specific amino acids underlie these molecular differences. At this point it is difficult to interpret the precise role of the group specific residues in molecular terms. Nonetheless, three possibilities seem reasonable. The first is that currently unknown proteins bind to these residues. The second is that these residues are sites of differential post-translational modifications which in turn influence the choice of target gene specificity. Finally, it is possible that while these residues do not bind to DNA themselves, they influence the three dimensional structure or the conformational changes which DNA binding residues assume upon contacting DNA. In this scenario, these residues do ultimately influence the choice of the binding site without themselves contacting it. Our data illustrate the power of a comparative approach in identifying not only conserved, but also divergent, developmental mechanisms, and suggest a platform for screening for the genes mediating the divergence. It is noteworthy that NGN1, on the one hand, and XATH1 and MATH1, on the other, seem to have retained a type of proneural activity which is largely no longer needed in flies and vertebrates, respectively.

Finally, genes common to protostomes and deuterostomes including *atos*, *ngn* genes, *Notch* signaling genes, *sens* and *X-MyT1* most probably derive from the last common bilaterian ancestor (Erwin and Davidson, 2002). This implies that such an ancestor already possessed all the tools to specify a large diversity of neural cell types and lineages, suggesting a structurally, and consequently behaviorally, complex animal.

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