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Control of nerve cord formation by Engrailed and Gooseberry-Neuro: A multi-step, coordinated process

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ABSTRACT

One way to better understand the molecular mechanisms involved in the construction of a nervous system is to identify the downstream effectors of major regulatory proteins. We previously showed that Engrailed (EN) and Gooseberry-Neuro (GsbN) transcription factors act in partnership to drive the formation of posterior commissures in the central nervous system of *Drosophila*. In this report, we identified genes regulated by both EN and GsbN through chromatin immunoprecipitation ("ChIP on chip") and transcriptome experiments, combined to a genetic screen relied to the gene dose titration method. The genomic-scale approaches allowed us to define 175 potential targets of EN-GsbN regulation. We chose a subset of these genes to examine ventral nerve cord (VNC) defects and found that half of the mutated targets show clear VNC phenotypes when doubly heterozygous with *en* or *gsbn* mutations, or when homozygous. This strategy revealed new groups of genes never described for their implication in the construction of the nerve cord. Their identification suggests that, to construct the nerve cord, EN-GsbN may act at three levels, in: (i) sequential control of the attractive-repulsive signaling that ensures contralateral projection of the commissural axons, (ii) temporal control of the translation of some mRNAs, (iii) regulation of the capability of glial cells to act as commissural guideposts for developing axons. These results illustrate how an early, coordinated transcriptional control may orchestrate the various mechanisms involved in the formation of stereotyped neuronal networks. They also validate the overall strategy to identify genes that play crucial role in axonal pathfinding.

1. Introduction

Insect neuroblasts (NBs) are similar to mammalian neural stem cells in their ability to self-renew and to produce different types of neurons and glial cells (Qian et al., 2000; Skeath and Thor, 2003; Pearson and Doe, 2003; Egger et al., 2008; Knoblich, 2010; Brand and Livesey, 2011). In *Drosophila*, neurogenesis starts with the delamination from the neurectoderm of about 60 neuroblasts per segment. All NBs undergo multiple rounds of asymmetric divisions whereby they self-renew and produce intermediate progenitor cells, the ganglion mother cells. Each ganglion mother cell divides once to give rise to two post-mitotic cells that will differentiate as either neuron or glial cell.

The earliest neurons extend axons that have to navigate in an environment devoid of other axons. These "pioneer" axons (Bate, 1976) form a stereotyped network that will be used as a scaffold for later axonogenesis. One essential aspect of this early scaffold is its orthogonal organization, with axonal tracts extending either longitudinally (axon tracts) or transversally (commissures). Because the embryonic ventral nerve cord (VNC) comprises a chain of segmentally repeated motifs, the result of this orthogonal organization is a ladder-like structure with two longitudinal tracts extending all along the nerve cord, and two commissures, an anterior one (AC) and a posterior one (PC) within each segment. Axons that form the commissures have to cross the midline and project contralaterally. These axons initially respond to attractive signals emanating mostly from cells at the ventral midline (von Hilchen et al., 2010; Nawabi and Castellani, 2011). Once

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across, they respond to repulsive signals from the midline cells, which prevent them for re-entering the midline region (Evans and Bashaw, 2010). Attractive and repulsive systems must be appropriately controlled to ensure midline crossing, and prevent recrossing (Dickson and Zou, 2010).

Depending on their position, NBs express various combinations of transcription factors (Isshiki et al., 2001), which largely control the fate of their progeny (Doe, 1992; Bhat, 1999; Technau et al., 2006). Among the transcription factors present at early stages of neurogenesis, the homeodomain protein Engrailed (EN) is found in a subset of NBs, neurons and glial cells (Patel et al., 1989), and plays an important role in the organization of the embryonic nerve cord, as embryos that lack *en* do not form the segmentally repeated PC (Joly et al., 2007). A two-hybrid screen in yeast revealed a physical interaction between EN and another homeodomain transcription factor, Goosberry-neuro (GsbN) (Colomb et al., 2008). EN and GsbN are expressed in a subset of NBs (NBs 6.1; 6.2; 6.4; 7.1 and 7.3) out of the 30 NBs that delaminate from the neuroectoderm in each hemisegment (Colomb et al., 2008). Neuroblasts form through five waves (S1 to S5) that take place from embryonic stage 8 to stage 11 (Doe, 1992). Whereas EN is expressed when NBs delaminate (S1 wave), GsbN is only activated later in these EN-expressing NBs (NBs 6.1; 6.2; 6.4; 7.1 and 7.3) starting at stage 10 when the S3 wave takes place (Gutjahr et al., 1993). Note that NB7.3 is only formed during the S5 wave (Matsuzaki and Saigo, 1996). Although deficiencies removing either *en* or *gsbn* are fully recessive, doubly heterozygous embryos ($en^{-}/gsbn^{-}$) show defects in their PC (Colomb et al., 2008), suggesting that the two factors interact for commissural development (Botas et al., 1982). This EN-GsbN concerted action is required in neuroblasts, and is ineffective after neurons are formed (Colomb et al., 2008), suggesting a change in the competence of the cells (Pearson and Doe, 2004). Therefore an important issue is to understand how this transient EN-GsbN partnership takes over to achieve the construction of a normal VNC. To answer this question we asked what are the functions regulated by EN and GsbN that are required in VNC development.

In order to identify direct targets of EN-GsbN regulation, we used two complementary genomic approaches. First, we used chromatin immunoprecipitation (ChIP on chip) experiments to identify genes that are direct targets of EN and GsbN. Second we reasoned that such targets are likely to be deregulated in $en^{-}/gsbn^{-}$ double heterozygotes. Therefore, we compared the transcriptomes of $en^{-}/gsbn^{-}$ mutant embryos to that of wild type embryos. This dual approach allowed us to identify 175 genes that are putative direct targets of EN-GsbN regulation, with a clear preference for genes involved in neurogenesis, according to gene ontology.

One issue was to evaluate which genes among the 175 selected targets are effectively involved with *en* and *gsbn* in the construction of the VNC. To this end, we examined whether some of our targets would show defects in the architecture of the VNC when doubly heterozygous with a deficiency for either *en* or *gsbn*. We found that out of 23 candidates tested, 12 display defective PC in these conditions and present abnormal VNC when mutated. This suggests that these 12 genes are involved in the construction of the embryonic ventral nerve cord, indicating that at least half of our candidates are targets of EN-GsbN regulation involved in PC formation. The functions ascribed to these 12 genes reveal that EN-GsbN regulation may be potentially involved in at least three different pathways to achieve the construction of the VNC, including: temporal control of attractive-repulsive signaling, translational control within neurons, and glial behavior. For several of these genes, this is the first indication for a role in the construction of the embryonic VNC.

2. Materials and methods

2.1. Drosophila strains

Flies were maintained at 22 °C on standard fly food. Crosses were performed at 25 °C. The mutations used in this study were as follows: *Df(2 R)SFX31* (en^{X31}), *Df(2 R)IIX62* (gsb^{X62},BL2528), fra^{1} mutation, Df(3 R)ry^{75} (a deletion removing *paip2,* BL3808), $paip2^{GA716}$ (EP insertion in *paip2*, BL31788), Df(3 R)e^{BS2} (a deletion removing *glec,* BL3013), $glec^{EY22136}$ (EPgy2 insertion in *glec,* BL22541), $gish^{EY06451}$ (EPgy2 insertion in *gish,* BL19721), pum^{ET1} and pum^{13} (BL3260) (two EMS alleles), $lola^{e76}$ (BL28283), $lola^{5D2}$ (hypomorphic, LacW insertion in the promoter); $shot^{3}$ (BL5141), $sdc^{EY09287}$ (EPgy2 insertion in *sdc*, BL19695); Df(2 R)Exel6076 (a deletion removing *sdc*, BL7556); Df(3 R)Exel16169 (a deletion removing *sqd*, BL7648); P(lacW)sqd^{j4B4} (BL12134); $rapgap^{EY9287}$ (BL16930); Df(2 L)ED479 (a deletion removing *rapgap1*, BL9189); ctp^{GO207} (a P(lacW) insertion, BL11852); *Df(1)bi-D2* (a deletion removing *ctp*, BL3203); $lk6^{1}$ (hypomorphic loss of function, BL8706); Df(3 R)BSC469 (a deletion removing *lk6*, BL24973). Mutations were balanced with a chromosome marked with *Krüppel*-GFP (Casso et al., 2000), in order to select transheterozygous embryos. Several MiMIC RMCE lines: *pum* MiMIC EGFP (MI04825, BL59818); *gish* MiMIC EGFP (MI00340, BL63151); *lk6* MiMIC EGFP (MI02556, BL59795) have been used.

All strains were from the Bloomington Stock Center except en^{X31} provided by Thomas Kornberg, and $lola^{5D2}$, provided by Franck Girard. Oregon or w^{1118} were used as wild-type flies. INV-GFP Drosophila strain is a GFP protein trap line in the *invected* locus and reproduces the INV-EN pattern of expression (Laurent Perrin, personal communication).

2.2. Chromatin immunoprecipitation and microarray processing

ChIP experiments have been performed by the modENCODE consortium as previously described, using reagents that we provided (Negre et al., 2011). Briefly, whole embryos were collected and washed in PBS + 0.1%Triton 0–12 h after egg-laying, covering early embryogenesis to stage 15. Embryos were fixed in 1.8% formaldehyde for 15 min and crunched in a Douncer (pestle B). After quenching the fixation reaction with 225 mM Glycine, chromatin was sheared using a Bioruptor sonicator (Diagenode) for 15 min (30 s ON/ 30 s OFF cycles). Immunoprecipitations were carried out using custom-made anti-EN and anti-GsbN sera and protein-A Sepharose beads. The anti-EN antibody is a rabbit polyclonal raised against a truncated form of EN lacking the homeodomain, in order to avoid cross-reaction with other homeodomain-containing proteins. The anti-GsbN antiserum was also prepared in rabbits, using two specific peptides (NH2-CYSHPLPTQGQAKYWS-COOH and NH2-CRGSDRGSEDGRKDYT-CONH2) that are present in a region that does not share homology with the Gsb protein. The specificity of the antibody and the absence of cross-reactivity with Gsb was previously reported (Colomb et al., 2008). After IP, the DNA fraction was eluted and amplified by linker-mediated PCR before being used for hybridization onto microarrays. The microarrays used are the Affymetrix Drosophila Tiling v2.0 R arrays. All steps of hybridization and scanning of Affymetrix microarrays were performed according to manufacturer's instruction. The raw data were recorded as.CEL files. These files have been treated and analyzed using the MAT software (Johnson et al., 2006) or the TAS software from Affymetrix and visualized with the Integrative Genomics Viewer (IGV) application (Robinson et al., 2011; Thorvaldsdottir et al., 2013). The data for Engrailed in 0–12 h embryos can be retrieved from GEO with