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no-bridge and *linotte* act jointly at the interhemispheric junction to build up the adult central brain of *Drosophila melanogaster*

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Abstract

The *Drosophila* transmembrane protein Linotte (Lio) is expressed in a glial transcient interhemispheric fibrous ring (TIFR), which was hypothesised to serve as scaffold for adult brain formation during metamorphosis. We isolated TIFR specific enhancers from the *lio* locus and showed that only four interhemispheric cells give rise to this complex fibrous structure. We showed that *lio* controls the TIFR differentiation, and confirmed the major role played by this structure in central brain metamorphosis since its destruction by apoptosis led to a pronounced adult phenotype, which included defects of *lio* and *no-bridge* (*nob*) mutants. *lio* interhemispheric expression was specifically affected in a *nob*^{1} context, confirming that *nob* plays a key role in adult brain development via the TIFR.

Keywords: Brain development; Metamorphosis; Transcient Interhemispheric Fibrous Ring; *linotte*; *no-bridge*; *Drosophila melanogaster*

1. Introduction

While *Drosophila* has become a prominent model for studying central nervous system (CNS) development, most of the work has addressed the ventral ganglia, whereas brain development remains less understood. The differentiation of brain structures occurs mainly at two stages, embryogenesis and metamorphosis. At the embryonic stage anatomical studies revealed morphogenetic movements including cell migration, formation of proliferative clusters of neuroblasts, scaffolding of glia and pioneer neurones (for review, see Reichert and Boyan, 1997; Hartmann and Reichert, 1998; Hartenstein et al., 1998; Nassif et al., 1998). These events lead to the formation of a primitive brain. Genetic studies of embryonic brain development compared to ventral nerve chord (VNC) formation reveals similarities as well as remarkable differences (Therianos et al., 1995; Hartmann and Reichert, 1998) suggesting that specific genetic mechanisms underlie brain development. Interestingly, these mechanisms have been partially conserved through evolution. For example the *Drosophila* gene *otd* and its mammalian homologue *Otx* both control early neurogenesis of the anterior brain (Younossi-Hartenstein et al., 1997; Hirth and Reichert, 1999).

During larval life most neuroblast progeny accumulate without differentiating (Truman et al., 1993). The majority of neuroblasts stop dividing at early metamorphosis when the adult brain morphogenesis takes place. This step involves differentiation of immature neurones, neurodegeneration and axonal rearrangements (Technau and Heisenberg, 1982; Tissot and Stocker, 2000).

The *Drosophila* brain consist of seven segments or neuromeres (Schmidt-Ott and Technau, 1992). In the adult the largest and most complex neuromere is the protocerebrum which contains the mushroom bodies (MBs), the central complex (CX) and the optic lobes. The MBs (Fig. 1) are bilateral symmetrical structures comprising 2500 tightly associated neurones involved in olfactory learning and memory (see Heisenberg, 1998 for review). Each adult MBs consist of dorsal dendritic projections which form the calyx, a peduncle projecting rostrally and dividing to α and α' lobes dorsally and to β, β' and γ lobes medially. At the late embryonic stage primitive MBs are composed of γ lobes with dorsal and medial projections (Tettamanti et al., 1997). MBs undergo major reorganization during larval stages ($\alpha'\beta'$-lobe differentiation) and during metamorphosis (loss of dorsal γ-lobes projections, differentiation of $\alpha\beta$-lobes) (Armstrong et al., 1998; Lee et al., 1999). Several genes affecting MB formation have been characterized

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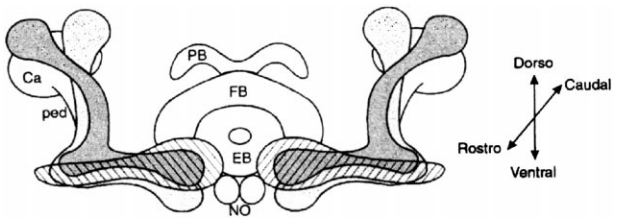

Fig. 1. Schematic representation of MB and CX neuropile (Boquet et al., 2000a). Dorsal is up; the most anterior is the first plane. Mushroom bodies: γ lobes, stripped; α′ and β′, dark gray; α and β, pale gray; ped, peduncle; Ca, calyx. Central complex: EB, ellipsoid body; FB, fan-shaped body; NO, noduli; PB, protocerebral bridge.

(Tejedor et al., 1995; Melzig et al., 1998; Awasaki et al., 2000; Lee et al., 2000).

The CX (Fig. 1) connects the right and left hemispheres and consists of four substructures: the protocerebral bridge, the fan-shaped body, the ellipsoid body and the noduli. The CX is thought to be a higher centre controlling the fly's activity (Strauss and Heisenberg, 1993), and to establish information transfer between the brain hemispheres (Bouhouche et al., 1993). So far, no embryonic CX corresponding structure has been described (Nassif et al., 1998). In *Drosophila* the CX differentiates during metamorphosis out of the interhemispheric area (Hanesh et al., 1989; Renn et al., 1999; Boquet et al., 2000a), but few of the molecular events involved have been described (Boquet et al., 2000b).

Following an EMS mutagenesis a collection of CX mutants has been generated (Heisenberg and Böhl, 1979). For example, in *no-bridge* (*nob*) the protocerebral bridge is interrupted in the middle and occasionally a cleft is observed in the fan-shaped body and the ellipsoid body (Strauss et al., 1992). The corresponding genes are likely to be involved in CX differentiation during metamorphosis, but because these mutants were induced chemically their characterization has proceeded slowly. A *Pgal4* enhancer-trap mutagenesis aimed at genes expressed in the third instar larval brain led to the isolation of three new CX mutants (Boquet et al., 2000a). The corresponding genes are being characterized (Boquet et al., 2000b).

The *linotte* (*lio*) gene encodes a transmembrane tyrosine kinase homologous to the human RYK gene product (Dura et al., 1995; Callahan et al., 1995). It is expressed at the embryonic stage in neurones of the VNC and of the procephalic region (Callahan et al., 1995), and in the late third instar larvae in a glial transient interhemispheric fibrous ring (TIFR) which persists at the early pupal stages and disappears before adulthood (Simon et al., 1998). *lio* – also referred to as *derailed (drl)* – has been implicated in axon pathway selection in the embryonic VNC (Callahan et al., 1995; Bonkowsky et al., 1999), and adult brain development at metamorphosis (Simon et al., 1998; Moreau-Fauvarque et al., 1998). The brain of the lio^2 amorphic mutant exhibits an abnormal CX and a fusion of the β and β′ MB lobes (Fig. 5). The TIFR has been proposed to scaffold the organisation of the adult central brain preventing neighboring axons from crossing the midline abnormally and shaping correctly the CX (Simon et al., 1998).

To further address the question of how the TIFR controls brain hemisphere connectivity during metamorphosis we isolated genomic enhancers driving expression in four interhemispheric cells. Using this enhancer in combination with the Gal4 system it was shown that: (1) the four interhemispheric cells are sufficient to build a normal TIFR; (2) in the absence of the Lio product the four cells are normally present but the TIFR is strongly disorganized; (3) the destruction of the TIFR by apoptosis leads to a pronounced adult brain defect, confirming the major role played by this transient structure in central brain development. Interestingly, this phenotype resembles that of both *lio* and *nob*

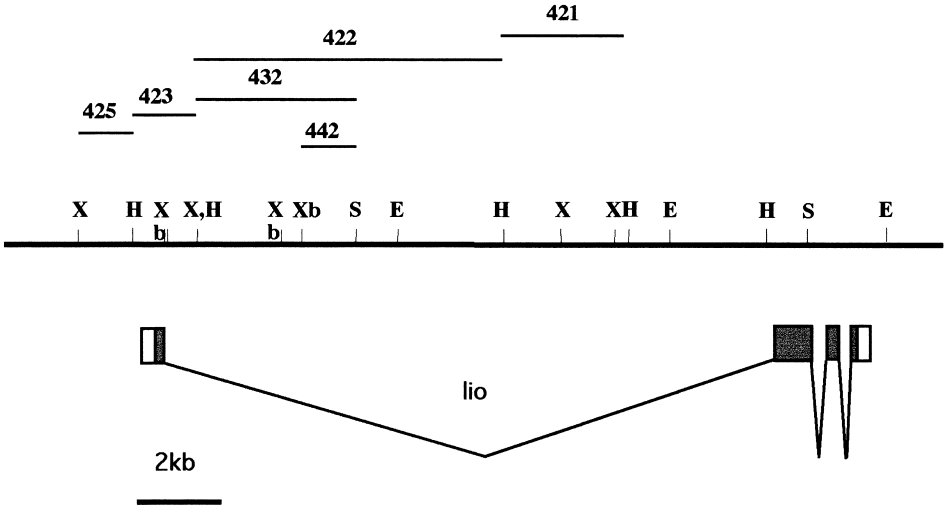

Fig. 2. Molecular dissection of *lio* enhancers. Genomic DNA fragments shown above the restriction map (X, *Xho*I; H, *Hin*dIII; S, *Sal*I; E, *Eco*RI; Xb, *Xba*I) were fused in front of a gene encoding either a nuclear β-galactosidase (PX425, PX423, PX432, PX421), or a cytoplasmic β-galactosidase (PZ442) or the Gal4 transcription factor (442-gal4). Gray areas represent the open reading frame in the transcript.

mutants. The link between *lio* and *nob* was further confirmed as *lio* interhemispheric expression specifically disappears in the *nob* mutant. This work is an important step toward a dissection of the genetic network driving the central brain development at the pupal stage, pointing out the importance of the TIFR in CX formation.

2. Results and discussion

2.1. Identification of TIFR-expressing enhancers

To identify postembryonic *lio* enhancers we fused restriction fragments spanning the *lio* locus (Fig. 2) to a reporter encoding either a nuclear form of β-galactosidase (PX constructs), a cytoplasmic β-galactosidase (PZ construct) or the transcription factor Gal4. The PX423 construct leads to no obvious enhancer activity in the CNS (Fig. 3A). The PX421 construct shows enhancer activity restricted to the midline part of the VNC (Fig. 3B). The PX422 construct, corresponding to the 5′ part of the first intron, exhibits enhancer activity in the primordium of the optic lobes and in four cells at the interhemispheric junction (IJ) (Fig. 3C). The PX432 construct corresponding to the 5′ part of the 422 fragment, exhibits strong enhancer activity in the primordium of the optic lobes, the CNS, the lateral parts of the brain and in the four interhemispheric cells (Fig. 3D). The interhemispheric junction specific enhancer was further restricted to 1.3 kb as the PZ442 construct leads to a TIFR staining. Interestingly the 425 fragment which lies 5′ from the *lio* transcription start also showed specific expression in the four IJ cells (Fig. 3F). We thus isolated two independent TIFR specific enhancers.

The alternative use of nuclear (Fig. 4B) or cytoplasmic reporter (Fig. 4E) in combination with TIFR specific enhancers allowed to demonstrate that only four glial cells generate the complex fibrous structure of the TIFR.

The fragments we used are partially redundant with the fragments isolated for *lio* embryonic enhancer activity analysis (Bonkowsky and Thomas, 1999). The 425 and the 442 fragment are respectively comprised in the drlU and drlT fragments showing enhancer activity in midline crossing neurones at the embryonic stage. This observation suggests that the same enhancer elements might control expression at the midline of the embryonic VNC and of the larval brain, even though *lio* is expressed in neural cells in the embryo and in glial cells in the larval brain.

2.2. The Lio product is required for shaping the TIFR, not for cell survival

The expression of *lio* was known to be required to build up a correct TIFR (Simon et al., 1998). However, since the TIFR could be observed only in the hypomorphic lio^{drlP} mutant it was not clear if *lio* expression was required for the formation and survival of the four IJ cells or for the TIFR fasciculation per se. A staining experiment performed on lio^{2}; *PX422/+* third instar larvae revealed that the four interhemispheric nuclei are normally present in an amorphic *lio* background (Fig. 4C). At the pupal stage, using the 442-gal4 construct and the axon targeted *tau*-based reporter we showed that the TIFR is drastically disorganized in the amorphic context (Fig. 4F). *lio* is thus required for glial fibrous fasciculation and/or pathway selection in the interhemispheric junction, as it was observed for neurones at the embryonic midline (Callahan et al., 1995; Bonkowsky et al., 1999), but not for cell survival.

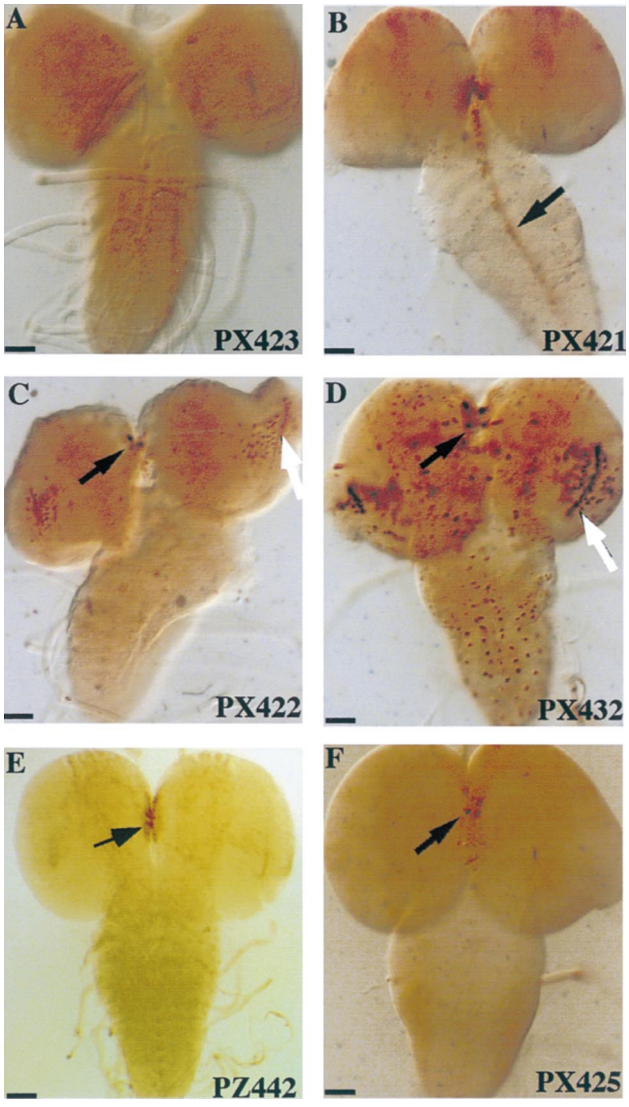

Fig. 3. Third instar larval CNS expression of *lio* enhancers as revealed with anti-β-galactosidase antibodies (PX corresponds to a nuclear β-galactosidase and PZ to a cytoplasmic one). Scale bars, 50 μm. (A) PX423 does not lead to any expression in the CNS. (B) PX421 is expressed at the midline in the VNC. (C) PX422 is expressed in four nuclei at the IJ (black arrow) and weakly in the optic lobe anlagen (white arrow). (D) PX432 lead to a strong expression in four nuclei at the IJ (black arrow), in the optic lobe anlagen (white arrow) and in many nuclei of the VNC. (E) PZ442 is expressed in a fibrous structure at the IJ. A weak signal is detectable in the optic lobe anlagen. (F) PX425 is expressed exclusively in four nuclei at the IJ.

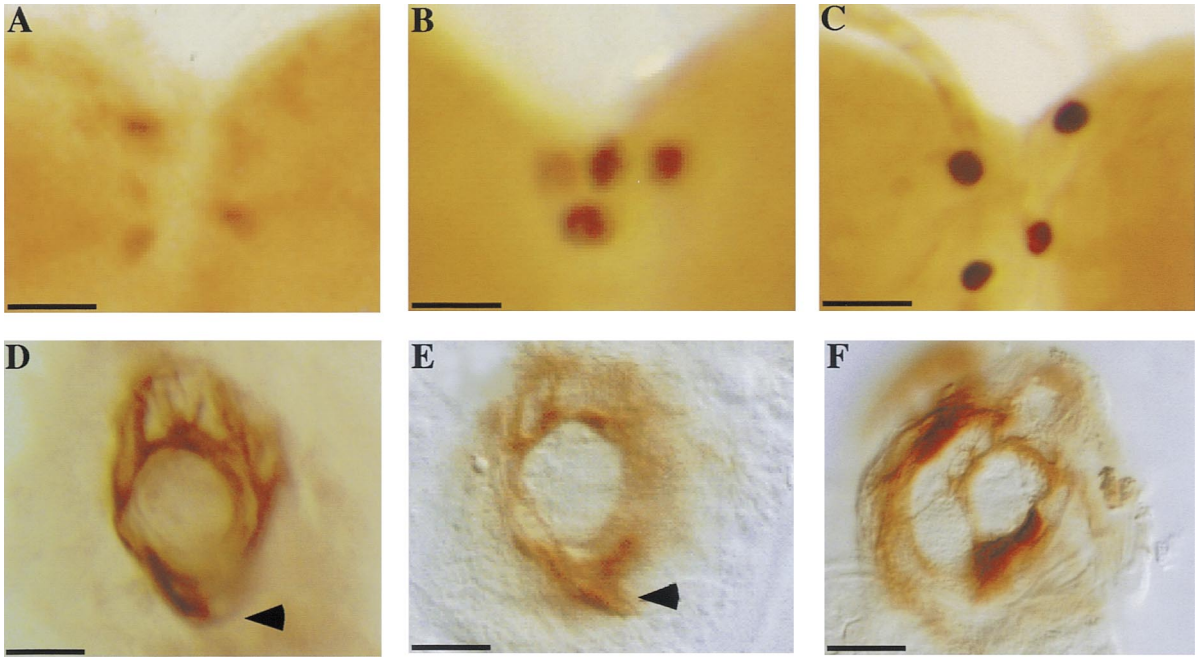

Fig. 4. The TIFR is present but disorganized in the *lio*^{2} amorphic mutant. (A–C) Expression in the central brains of third instar larvae (frontal view) and (D–F) at young pupal stages (sagittal view). Scale bar, 50 μm. (A) *lio*^{1}/+ staining. (B) *PX422/+* staining in wild-type context and (C) in *lio*^{2}*; PX422/+* individuals. (D) Normally shaped TIFR in *lio*^{drlP}/+ individual. Arrowhead in (D,E) indicates typical frontal hook of the TIFR (Simon et al., 1998). (E) *442-gal4/+; UAS-tau-lacZ/+* staining in wild-type context and (F) in *UAS-tau-lacZ/+; lio*^{2}*; 442-gal4/+* individuals.

2.3. The TIFR integrity is required for the development of a normal adult brain

In order to characterize the TIFR function in adult brain development we analyzed the brain of *442-gal4/+*; *UAS-hid/+* flies, in which the TIFR was destroyed by *hid*-induced apoptosis (Zhou et al., 1997). Killing specifically the TIFR cells led to a late partial pupal lethality. Interestingly, the adult *442-gal4/+*; *UAS-hid/+* flies showed a strong *lio*-like phenotype, i.e. protocerebral bridge disorganization, fibers crossing the midline above the fan-shaped body, flat fan-shaped body and fused MB lobes (Fig. 5I–L). This result confirms that the lack of *lio* expression in the TIFR is responsible for most of the adult phenotype displayed by the *lio* mutant and that the TIFR is a key cellular structure for the midline choice in the central brain. However, unlike the *lio* amorphic mutant the α lobes are still present in *442-gal4/+*; *UAS-hid/+* individuals. In *lio* mutants the absent α lobe phenotype could thus be due to the lack of *lio* expression in the MBs itself (Moreau-Fauvarque et al., 1998).

The TIFR destruction lead to a new phenotype compared to the *lio* mutant: the protocerebral bridge was found to be severely disorganized or absent, and the ellipsoid body ventrally opened and flattened. The fact that the amorphic *lio* mutant does not exhibit such a drastic phenotype confirms the observation that *lio* is not required for the existence of the four interhemispheric cells. Interestingly, this additional phenotype partially matches that of the *nob* mutant phenotype in a *Canton-S* background (Fig. 5M–P). We hypothesised that the *nob* gene might play a role in the TIFR and interact with the *lio* gene.

2.4. linotte interacts with no-bridge

To assess the *lio-nob* interaction, *lio* expression in the *nob* mutant was followed in third instar larvae brains. In *nob* mutants *lio* expression is not detectable in the anterior fibers crossing the interhemispheric junction (Fig. 6A,B). In the optic lobes *lio* expression does not seem to be affected by the *nob* mutation and the Lio product is still detectable in the posterior fibers of the interhemispheric junction. In *nob/Y*; *lio*^{1}/+ larvae the β-galactosidase expression driven by the *lio*^{1} PlacZ insertion specifically disappears in the four interhemispheric nuclei corresponding to the TIFR (Fig. 6C,D). This effect is *nob*-specific as *lio* larval interhemispheric expression was normal in several other central brain mutants including *central body defect* (*cbd*), *central complex broad* (*ccb*), *central complex deranged* (*ccd*), *central brain deranged* (*ceb*), *central complex* (*cex*), *ellipsoïd body open* (*ebo*) and *mushroom body deranged* (*mbd*) (data not shown).

In *nob/Y; PX425/+* and *nob/Y; PX432/+* larvae the interhemispheric expression is specifically absent (Fig. 6E,F), as is the pupal TIFR expression (Fig. 6G,H). Altogether these results imply either that the *nob* gene controls *lio* expression in the TIFR or that the *nob* gene is required for the TIFR existence. The *gal1916* enhancer-trap line inserted in the *brain washing* gene was initially proposed to stain the TIFR (Boquet et al., 2000a). Recent confocal microscopy study indicated that *gal1916* line actually stains a neighbor-

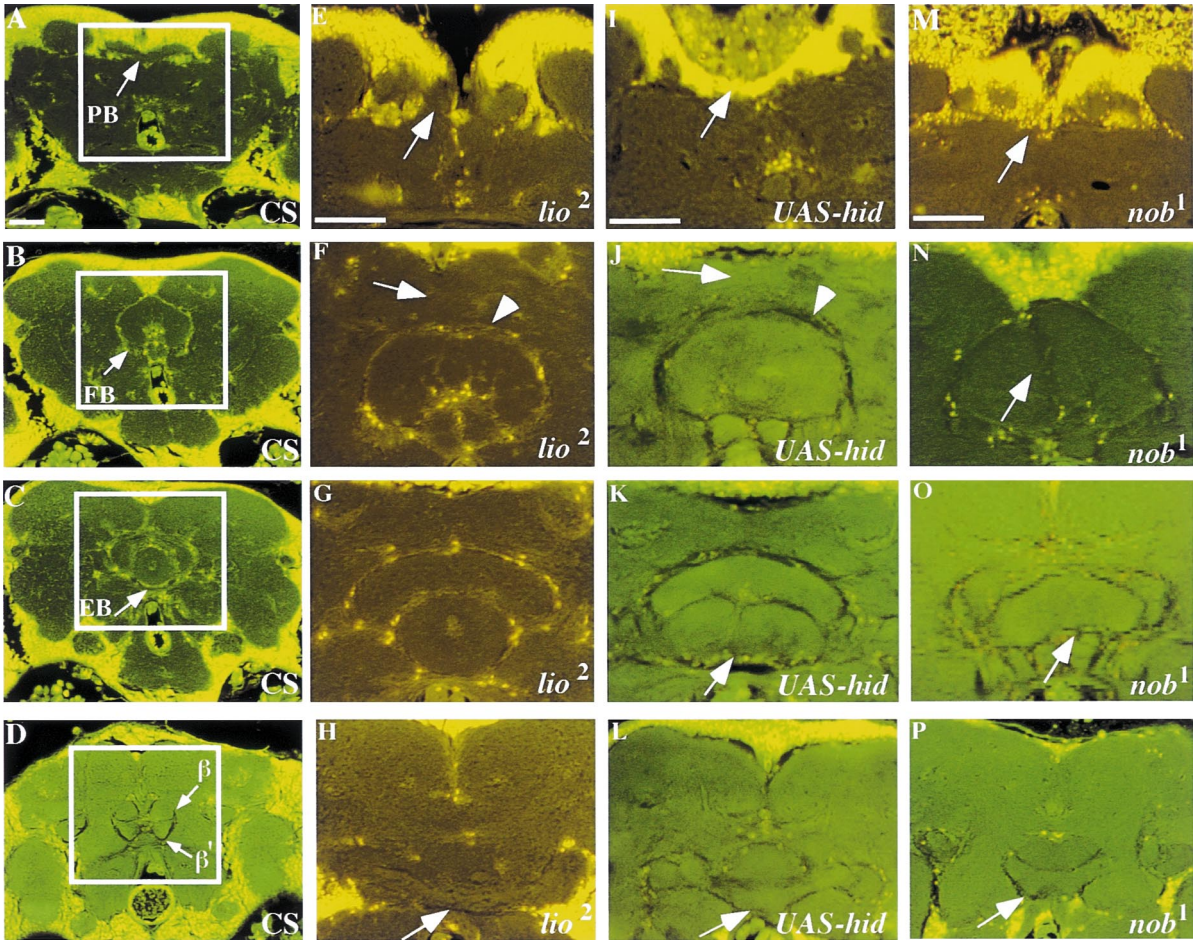

Fig. 5. TIFR destruction leads to a *lio*-like and a *nob*-like phenotype. Frontal paraffin sections of the adult brain. (A,E,I,M) sections at the protocerebral-bridge (PB) level. (B,F,J,N) sections at the fan-shaped body (FB) level. (C,G,K,O) sections at the ellipsoid body (EB) level. (D,H,L,P) sections at the mushroom-body lobes level. Scale bar, 50 μm. (A) *Canton-S*: the PB exhibits a continuous organisation. (B) *Canton-S*: the FB shows a fig-like shape and cell bodies reach the upper top of the structure. (C) *Canton-S*: the EB appears full and round. (D) *Canton-S*: the β/β′ lobes are separated by the median bundle fibers. (E) lio^2: the PB is fragmented (arrow). (F) lio^2: fibers abnormally cross the midline just above the FB (arrow) which exhibits a flat shape (arrow-head). (G) lio^2: the EB appears normal. (H) lio^2: the β/β′ lobes are fused across the midline (arrow). (I) *442-gal4/+; UAS-hid/+*: only the lateral extremities of the PB are present (arrow). (J) *442-gal4/+; UAS-hid/+*: fibers abnormally cross the midline just above the FB (arrow) which exhibits a flat shape (arrow-head). (K) *442-gal4/+; UAS-hid/+*: the EB is ventrally largely open (arrow). (L) *442-gal4/+; UAS-hid/+*: the β/β′ lobes are fused across the midline (arrow). (M) nob^1: the PB appears highly disorganized (arrow). (N) nob^1: the FB presents a medial cleft (arrow). (O) nob^1: the EB is ventrally largely open (arrow). (P) nob^1: the ββ′ lobes are fused across the midline (arrow).

ing structure (R.H., T.P., unpublished data). Thus, the *lio* expression remains the only marker available for the TIFR, so it was impossible to determine whether *nob* is required for *lio* expression in the TIFR or for the TIFR existence. However, the fact that *nob* flies do not exhibit the complete phenotype of *442-gal4/+*; *UAS-hid/+* flies, in which inter-hemispheric cells are missing argues for transcriptional regulation of the *lio* gene by *nob*. Such transcriptional regulation could nevertheless be indirect and cloning of the *nob* gene should help address this issue.

Paraffin section analysis of *nob*; lio^1 and *nob*; lio^{drlP} brains did not reveal any synergetic effects as the double mutant phenotypes corresponded to the sum of both single mutant phenotypes (data not shown). This result might be due to the fact that *lio* and *nob* mutants already share several defects. The *nob;* lio^2 double mutant was found to be lethal. Since lio^2 is an amorphic allele, this result supports the idea that *nob* is also involved in a pathway parallel to that of *lio*, and that both pathways interact. This work constitutes a first step toward a dissection of the genetic network controlling CX metamorphosis. The identification of new genes expressed at the IJ (Boquet et al., 2000a,b) should strengthen this emerging field.

3. Experimental procedures

3.1. Drosophila stocks

Drosophila were maintained on a 12:12 h dark/light cycle on standard cornmeal-yeast agar medium at 25°C and 60% relative humidity. The wild-type strain was *Canton-Special*

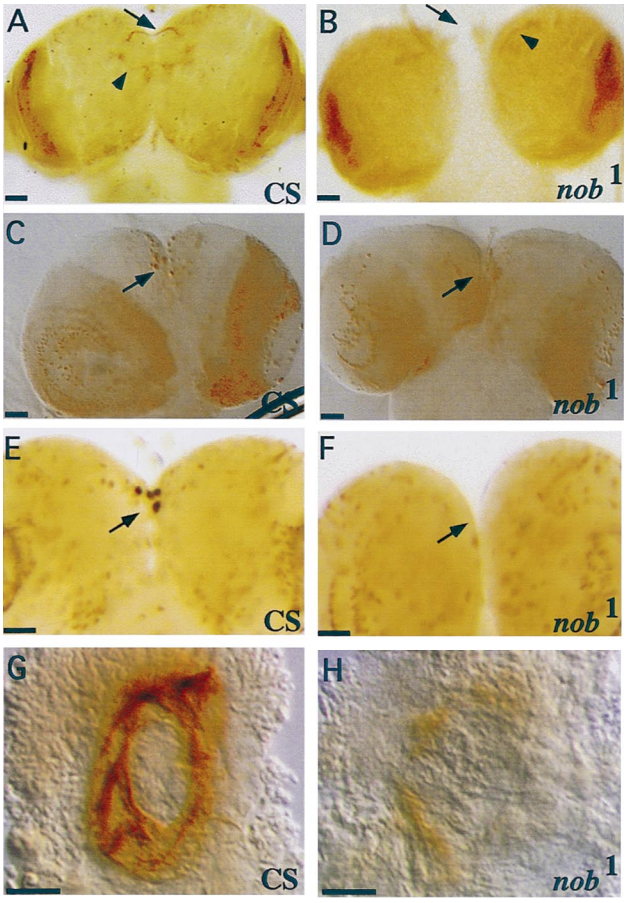

Fig. 6. *lio* expression in *nob*^{1} context. (A–F) Third instar larvae central brain. (G,H) Twenty-four hour pupal central brains (sagittal view). (A,B) Lio polyclonal antibody staining. (C–H) Anti-β-galactosidase antibody staining. Scale bars, 50 μm. (A) *Canton-S* individual showing strong expression in the optic lobe anlagen and in anterior fibrous element (arrow) and posterior fibrous element (arrow head) at the interhemispheric junction. (B) In *nob*^{1} individuals the anterior fibrous staining in the interhemispheric junction is missing (arrow) whereas staining in the posterior fibers at the interhemispheric junction (arrow head) and in the optic lobe anlagen is still detectable. (C) *lio*^{1} *PlacZ*/+: nuclei at the interhemispheric junction and cells in the optic lobe anlagen are stained. (D) *nob*^{1}; *lio*^{1}*PlacZ*/+: the interhemispheric junction staining is absent (arrow) whereas the staining is still present in the surrounding small nuclei (out of focus in the image) and in the primordium of the optic lobes. (E) *nob*^{1}/+; *PX432*/+ individual exhibits strong staining in four IJ nuclei. (F) In *nob*^{1}/Y; *PX432*/+ individuals, staining disappears specifically in the four IJ nuclei. (G) *lio*^{drlP}*-tau-lacZ*/+ TIFR staining. (H) *nob*^{1}; *lio*^{drlP}*-tau-lacZ*/+: the staining is almost completely missing.

(*Canton-S*).The following mutant lines were used: hypomorphic *lio*^{1} (Dura et al., 1993), amorphic *lio*^{2} (Dura et al., 1995), hypomorphic *drl*^{P} referred here to as *lio*^{drlP}(Callahan et al., 1995) – the *lio*^{drlP} and *lio*^{1} alleles both correspond to a P insertion located at the same base (Taillebourg and Dura, 1999), and their anatomical phenotype are equivalent (Moreau-Fauvarque et al., 1998) – *nob* ^{KS49} (also referred to as *nob*^{1}) (Strauss et al., 1992), central complex mutants *cbd*^{1}, *ccb*^{2}, *ccd*^{1}, *ceb*^{1}, *cex*^{1}, *ebo*^{2} (Strauss and Heisenberg, 1993), *mbd*^{1} (Heisenber et al., 1985), *UAS-hid* (Zhou et al., 1997). The *442-gal4/+; UAS-hid/+* flies were obtained by crossing *w*^{1118}*; 442gal4/442gal4* males to *w*^{1118}*; UAS-hid/UAS-hid* females at 18°C. *nob*^{1}*/Y; PX432/+* males were obtained by crossing *nob*^{1}/*nob*^{1} females to *PX432/PX432* males. The *lio*^{2}/*lio*^{2}; *PX422/+* individuals were obtained by crossing *lio*^{2}/*lio*^{2}; *PX422/PX422* females to *lio*^{2}/*lio*^{2} males. *UAS-tau-lacZ/+; lio*^{2}*; 442gal4/+* individuals were obtained by crossing *UAS-tau-lacZ /UAS-tau-lacZ*; *lio*^{2}/*lio*^{2} males to *lio*^{2}/*lio*^{2}*; 442-gal4/442-gal4* females.

All the mutant lines used in this work were outcrossed beforehand with flies carrying a *Canton-S* background as genetic background is known to interfere drastically with anatomical brain phenotypes (de Belle and Heisenberg, 1996; Boquet et al., 2000a). Initially described in a *Berlin* background, the *nob* mutant was shown to exhibit a protocerebral-bridge disorganization and rarely a dorsal cleft in the fan-shaped body (Strauss et al., 1992). In a *Canton-S* background, as we show here (Fig. 5), the *nob* mutant exhibits a more drastic phenotype: the protocerebral bridge is disorganized or absent as in *Berlin* context but the fan-shaped body is often cleaved, the ellipsoid body is systematically ventrally largely open and the β and β′ lobes of the MBs are fused.

3.2. DNA fragment cloning

The pBS421, pBS422, pBS423, pBS425, pBS432 constructs were provided by the laboratory of J.M. Dura. Fragments were subsequently cloned in the PX27 vector (Segalat et al., 1994) driving β-galactosidase expression in the nucleus. Fragment 442 was cloned in PWHZ10 vector (Hiromi et al., 1986) driving cytoplasmic β-galactosidase expression. The 442-gal4 vector was constructed using the Casper3 vector (Thummel and Pirotta, 1991) as follows: The Hsp70 was introduced in the Casper3 vector by PCR amplification on the pHZ50 vector (Hiromi and gehring, 1987) containing the Hsp70 fragment. A 1.3 kb *Xba*I/*Bam*HI fragment from the pZ442 was cloned in Casper3 Hsp70 vector. The *gal4* gene was isolated from the GATN plasmid (Brand and Perrimon, 1993) and cloned in the Casper3 modified vector.

Several independent lines showing very similar expression patterns were observed with each fragment. Variable expression patterns were observed in the case of PX423, which did not overlap with normal *lio* expression. This situation probably reflects the fact that this fragment carries the *lio* promoter region (Dura et al., 1995) but no specific enhancer, and is therefore very sensitive to surrounding enhancers.

3.3. Immunohistochemistry

Staining with antibodies was performed as described in Simon et al. (1998). The anti-β-galactosidase monoclonal antibody was bought from Boehringer Mannheim and Chemicon International and used, respectively, at 1/2000 and 1/500 dilution. The anti-Lio polyclonal antibody (Simon et al., 1998) was used at 1/5000 dilution.

3.4. Brain paraffin section

Flies were placed in mass histology 'collars', fixed for 4 h in Carnoy's solution, dehydrated for 2 h, immersed overnight in methyl benzoate, and embedded in different baths of paraffin at 70°C for 4 h (Heisenberg and Böhl, 1979). Heads were cut in 7 μm serial frontal sections and inspected under a Leica fluorescence microscope.

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