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Respective roles of the DRL receptor and its ligand WNT5 in *Drosophila* mushroom body development

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In recent decades, *Drosophila* mushroom bodies (MBs) have become a powerful model for elucidating the molecular mechanisms underlying brain development and function. We have previously characterized the *derailed* (*drl*; also known as *linotte*) receptor tyrosine kinase as an essential component of adult MB development. Here we show, using MARCM clones, a non-cell-autonomous requirement for the DRL receptor in MB development. This result is in accordance with the pattern of DRL expression, which occurs throughout development close to, but not inside, MB cells. While DRL expression can be detected within both interhemispheric glial and commissural neuronal cells, rescue of the *drl* MB defects appears to involve the latter cellular type. The WNT5 protein has been shown to act as a repulsive ligand for the DRL receptor in the embryonic central nervous system. We show here that WNT5 is required intrinsically within MB neurons for proper MB axonal growth and probably interacts with the extrinsic DRL receptor in order to stop axonal growth. We therefore propose that the neuronal requirement for both proteins defines an interacting network acting during MB development.

KEY WORDS: Mushroom body, Developmental genetics, *Drosophila*, Receptor tyrosine kinase, *derailed* (*linotte*), *Wnt5* signaling, Brain development, Ryk ortholog

INTRODUCTION

The elaborate architecture of the adult brain is under the control of both genetic instructions and cellular interactions. How brain development is genetically controlled is still very poorly understood, even though the issue is central to neurobiology. The adult brain consists of discrete regions that can be specialized for unique functions, such as learning and memory. In the *Drosophila* brain, mushroom bodies (MBs) are substructures that are essential for olfactory learning and memory (Heisenberg et al., 1985; Connoly et al., 1996; de Belle and Heisenberg, 1994). It seems clear that the harmonious formation of a brain structure requires both intrinsic clues, i.e. molecular information provided by the cells belonging to the structure, and extrinsic clues provided by cells located outside the structure. The development and anatomy of *Drosophila* MBs have been rather well documented compared with other brain areas (Strausfeld et al., 2003), and a number of gene products have been described as being autonomously required to different extents for proper MB development (Lee et al., 2000; Reuter et al., 2003; Nicolai et al., 2003). While these gene studies included some cases of non-autonomy (Ng et al., 2002; Wang et al., 2002), these cases were interpreted as reflecting a strong community effect in axon guidance and branching within the MB neurons rather than genuine extrinsic molecular information.

The *derailed* (*drl*) gene, which is also known as *linotte* (*lio*), was first isolated based on its role in olfactory learning and memory (Dura et al., 1993). The DRL protein was subsequently shown to be a receptor tyrosine kinase (RTK) (Dura et al., 1995; Callahan et al., 1995) belonging to the RYK subfamily of RTKs (Halford et al., 1999; Hovens et al., 1992). *drl* mutants present structural brain defects in the MBs (Moreau-Fauvarque et al., 1998; Moreau-Fauvarque et al., 2002; Simon et al., 1998). However, one major unsolved question about the *drl* MB mutant phenotype is whether it is due to intrinsic (i.e. cell-autonomous) or extrinsic (i.e. non-cell-autonomous) factors. With respect to the former possibility, an enhancer trap line within the *drl* gene has been recovered that shows a weak adult MB expression pattern. This, and the weak anatomical rescue seen with a *GAL4* line that is expressed (although not exclusively) in the MBs, has led us to favor the cell-autonomous hypothesis in the past (Moreau-Fauvarque et al., 1998). Alternatively, the finding that DRL is expressed in interhemispheric glial cells supports the non-cell-autonomous hypothesis (Simon et al., 1998).

In a detailed analysis, we show here that DRL is not expressed within developing MB cells and that this is true as early as the embryonic stage and remains so throughout development. These results are supported by a clonal analysis of the *drl* loss-of-function (LOF) mutation, which shows that the gene is not required intrinsically within MB cells. Our results strongly support a neuronal requirement for *drl* close to, but not within, MB cells. Further, it has been shown that in the embryonic nervous system, DRL keeps axons out of the posterior commissure by acting as a receptor for WNT5, a member of the Wnt family of signaling molecules (Yoshikawa et al., 2003; Fradkin et al., 2004). Interestingly, the function and signaling mechanism of WNT proteins appear to be highly conserved (Fradkin et al., 2005). In particular, the mammalian Ryk-Wnt pair plays a role in neurite outgrowth and axon guidance (Lu et al., 2004; Keeble et al., 2006). We show here that *Wnt5* mutants have MB defects that can be rescued by expressing *Wnt5* cDNA specifically in MB cells. Strikingly, LOF *Wnt5* MB phenotypes resemble those induced by *drl* gain of function (Taillebourg et al., 2005) (this study), and we show that *Wnt5* and *drl* interact genetically. We propose that the gene pair *drl* and *Wnt5* work to build adult MBs, with *Wnt5* being required within MB neurons and *drl* being required within non-MB neurons.

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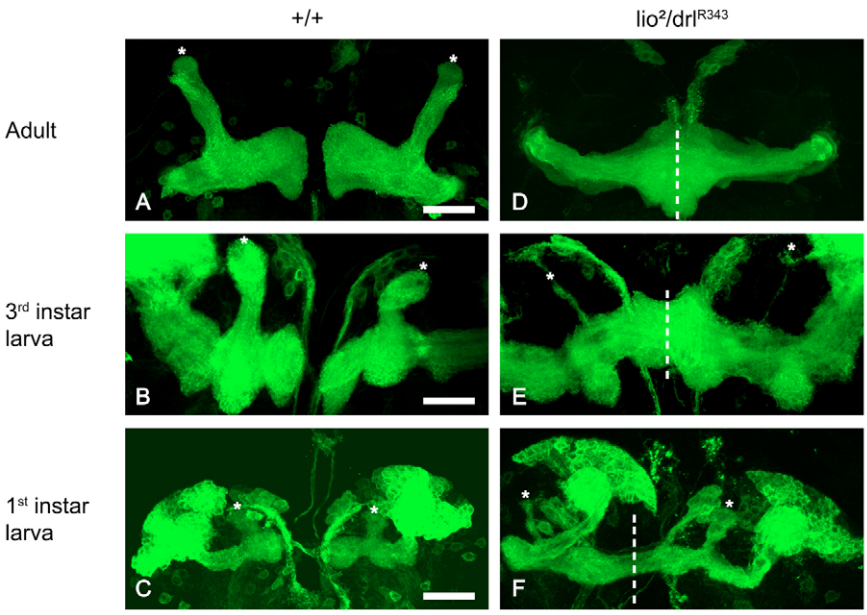

Fig. 1. MB defects in the *drl* LOF mutants are already present in newly hatched *Drosophila* first instar larvae and persist through metamorphosis. MBs are visualized in *GAL4-OK107 UAS-mCD8-GFP* individuals. (**A**,**B**,**C**) Wild-type brains, (**D**,**E**,**F**) *drl* LOF brains. Asterisks indicate the vertical lobes either present in the wild-type situation or reduced in the mutant situation. The midline is shown by a dashed line. The midline crossing is displayed at all stages in *drl* LOF mutant brains. In the displayed sample, the *drl* LOF mutant adult brain shows a disappearance of the αα′ vertical lobes (compare A with D). In late *drl* LOF mutant third instar larvae, the MB vertical larval γ lobes are absent in most of the analyzed brains (12 out of 15) (compare B with E). Much less reduction of the MB vertical larval γ lobes was observed in newly hatched first instar larvae (compare C with F). Scale bars: 50 μm in A and B; 20 μm in C.

MATERIALS AND METHODS

Drosophila stocks

The *drl* (*lio*) mutants were lio^2 (Dura et al., 1995; Moreau-Fauvarque et al., 2002), drl^{R343} and drl^{Red2} (Callahan et al., 1996; Bonkowsky and Thomas, 1999). The *Wnt5* null allele used was $Wnt5^{400}$ (Fradkin et al., 2004). The same mutant phenotypes were obtained with $Wnt5^{D7}$ (Yoshikawa et al., 2003). fz^{h51}, $fz2^{C1}$ and $fz3^{G10}$ (Srahna et al., 2006) were used. Enhancer-trap or regulatory region-GAL4 construct lines used were lio^1 (Dura et al., 1993; Simon et al., 1998), *GAL4-c739* (Yang et al., 1995), *GAL4-7B* (Ferveur et al., 1995; Moreau-Fauvarque et al., 1998; Nicolai et al., 2003), *GAL4-OK107* (Connolly et al., 1996; Adachi et al., 2003), *GAL4-C155* (Lee and Luo, 1999), *GAL4-247* (Zars et al., 2000) and *GAL4-442* (Hitier et al., 2000). For the rescue experiments, *UAS-drl* (Callahan et al., 1996) and *UAS-Wnt5* (Fradkin et al., 2004) were used. *UAS-nls-lacZ*, *UAS-tau*, and *UAS-mCD8-GFP* were used as reporter genes. Wild-type Canton-S was used as a control line. All the stocks were raised on standard corn medium at 25°C.

Antibodies and immunostaining

The following primary antibodies were used: 1D4 mouse anti-FASII (1/200) (DSHB), mouse anti-TAU (1/500) (Sigma), 8D12 mouse anti-REPO (1/20) (DSHB), rabbit anti-LIO (1/1500) (Simon et al., 1998), rabbit anti-β-galactosidase (1/5000) (Cappel) and goat CY3-coupled anti-HRP (1/200) (Jackson ImmunoResearch). Secondary antibodies were goat anti-mouse and goat anti-rabbit coupled with Alexa 488, Alexa 568, Alexa 647 (Molecular Probes) or HRP (Jackson ImmunoResearch) used at a 1/1000 dilution. HRP staining in embryos and first instar larval brains was revealed using the DAB substrate (Sigma). Antibody stainings and dissections at all stages were performed as previously published (Patel, 1994; Nicolai et al., 2003). Fluorescent samples were mounted in Fluoromount vectashield mounting medium. DAB-revealed samples were mounted in 90% glycerol.

MARCM mosaic analysis

For the *drl* clonal analysis (MARCM), the lio^2 mutation was recombined with the *FRT40A* chromosome and the clones generated by crossing this line with *GAL4-C155*, *hs-FLP*, *UAS-mCD8-GFP*; *tubP-GAL80 FRT40A* flies. A *tubP-GAL80* drl^{R343} *FRT40A* chromosome was also engineered. *Wnt5* clones were induced in *w hs-FPL tub-GAL80 FRT19A/w* $Wnt5^{400}$ *FRT19A; GAL4-c739 UAS-mCD8-GFP/+* individuals. Clones were induced in late-stage embryos/first instar larvae by applying a 1 hour heat shock at 37°C, as previously described (Lee et al., 1999; Lee and Luo, 1999). *fz* clones were induced in *y w hs-FLP*; *GAL4-c739 UAS-mCD8-GFP/+*; *tub-GAL80 FRT2A/*fz^{h51} $fz2^{C1}$ *FRT2A* individuals. In that case the heat shock was applied for an hour on 24- to 32-hour-old pupae.

Microscopy and image processing

Fluorescent samples were analyzed using a confocal microscope (LSCM 1024 BIORAD and a LEICA SP2). Image reconstruction was performed using NIH ImageJ and Photoshop software. DAB-revealed samples were observed under a LEICA microscope. Pictures were acquired on slide film, developed and subsequently digitized.

RESULTS

Developmental analysis of anatomical defects in MBs associated with *drl* loss of function

The development of MBs, which has been thoroughly described (Ito et al., 1998; Lee et al., 1999; Kurusu et al., 2002), is a continuous process that starts at the end of embryogenesis and continues until metamorphosis. Hence, it is possible that the anatomical defects in the MBs that have been previously described in *drl* (*lio*) LOF adult flies arise during early stages of development. In order to define at which developmental stage the defects are manifest, we analyzed MB morphology in *drl* LOF individuals at three developmental stages: adult, late third instar larva and newly hatched first instar larva. The *drl* mutant flies were obtained by crossing two independent null alleles, lio^2 and drl^{R343}. To label the MB axons at all stages, we used *GAL4-OK107 UAS-mCD8-GFP*. We first confirmed the adult MB defects that had been previously characterized using other techniques: overextension of the medial lobes and reduction or disappearance of the vertical lobes (compare A with D in Fig. 1). Second, we observed that similar defects are already present both at the end of larval life (compare B with E in Fig. 1) and immediately after larval hatching (compare C with F). Interestingly, at the earliest developmental stage analyzed, the reduction or disappearance of the vertical larval γ lobes was much less pronounced than at later stages. In conclusion, our results show that *drl* function is required at early stages of MB development, raising the possibility that the defects observed in adult MBs are a consequence of earlier-arising defects.

Clonal analysis reveals a non-cell-autonomous requirement for *drl* function during MB development

The MB defects displayed by *drl* LOF individuals could be due either to a need for the gene product in the Kenyon cells (KCs) themselves (cell-autonomous requirement) or to a requirement for

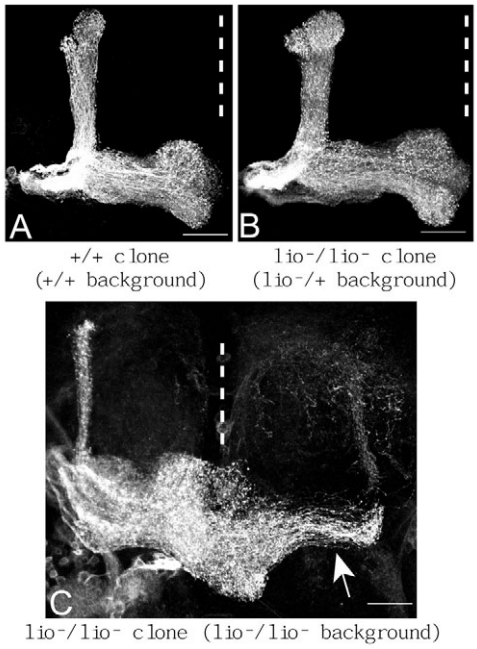

Fig. 2. MB mitotic clones induced in *Drosophila* early first instar larvae. In a heterozygous (*lio*^{2}/+) genetic background, *lio*^{2}/*lio*^{2} MB clones display wild-type morphology (compare **A** with **B**), meaning that the defects observed in the *lio*^{2}/*lio*^{2} genetic background are non-cell-autonomous. In a *lio*^{2}/*drl*^{R343} genetic background (**C**), labeling of a single MB neuroblast lineage reveals that the mutant axons, once having crossed the midline, follow the contralateral β lobe (arrow). The dashed line indicates the midline. The genotype is *GAL-C155*, *hs-FLP*, *UAS-mCD8-GFP; tubP-GAL80 FRT40A/FRT40A* in A, *GAL-C155*, *hs-FLP*, *UAS-mCD8-GFP; tubP-GAL80 FRT40A/lio*^{2} *FRT40A* in B and *GAL-C155*, *hs-FLP*, *UAS-mCD8-GFP; tubP-GAL80 drl*^{R343} *FRT40A/lio*^{2} *FRT40A* in C. Scale bars: 50 μm.

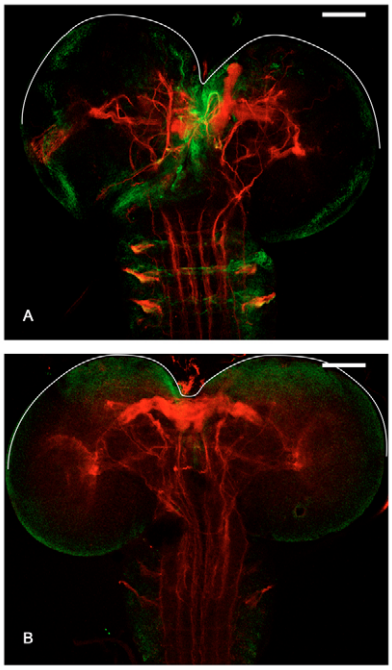

Fig. 3. Specificity of the anti-LIO antibody. Wild-type (**A**) and *lio*^{2}/*drl*^{R343} (protein null) mutant (**B**) *Drosophila* third instar larval brains were double stained with anti-LIO (green) and anti-FASII (red). Panels A and B are reconstructions of three confocal sections of 1 μm. No crossreaction with another protein is detected in null individuals. Some background staining may occur in the null individuals, but its localization and aspect are clearly unrelated to the true labeling seen in the wild type. The wild-type left hemisphere is somehow twisted, and its MB is not in the same focal plan, but it appears normally in subsequent confocal sections. Scale bars: 100 μm.

drl expression in surrounding cells (non-cell-autonomous requirement). In order to determine which of these two hypotheses is correct, we took advantage of the MARCM technique (Lee et al., 1999; Lee and Luo, 1999), which allows the generation of homozygous *drl* LOF KC clones in an otherwise heterozygous genetic background. Mitotic recombination was induced in late-stage embryos/early first instar larvae and the clones analyzed at the adult stage. We obtained 19 MB *lio*$^{-/-}$ neuroblast clones that include all three types of MB neurons. The *drl* LOF KC clones displayed an axonal morphology that was identical to that in wild-type clones (compare A with B in Fig. 2). These results demonstrate that the MB defects observed in the *drl* LOF individuals are not due to a cell-autonomous requirement for the *drl* gene product in the KCs.

Using the MARCM system, we also analyzed the morphology of MB clones induced in homozygous *drl* LOF individuals. With this method, we were able to observe KC axonal tracts deriving from only one side of the brain. These results showed that the *drl* LOF mutation leads to the complete crossing of the midline by KC axons (Fig. 2C).

DRL expression during embryonic brain development

We first confirmed the specificity of the anti-LIO antibody in the null combination *lio*^{2}/*drl*^{R343}. No crossreaction with another protein was detected in null individuals (compare Fig. 3A with 3B). As the *drl* LOF MB defects are already present in newly hatched larvae, we analyzed the expression pattern of the DRL protein in the developing embryonic brain. The protein was expressed from the onset of brain commissure formation in a subset of axonal projections linking the two hemispheres (data not shown). At later stages, two axonal tracts in the brain commissure expressed DRL (see Fig. 4A-F). Colocalization with an antibody raised against HRP, which is neuron-specific, confirmed the neuronal identity of the DRL-expressing tracts (see Fig. 4B,E).

In order to more clearly define the subsets of axonal tracts expressing the DRL protein, we performed double-labeling experiments using the anti-FASII antibody, the staining pattern of which has been previously well characterized in the embryonic brain (Noveen et al., 2000; Kurusu et al., 2002). No apparent colocalization was observed (see Fig. 4C,F). We next analyzed the embryonic pattern of DRL expression relative to the MB primordium. *GAL4-OK107* (Connolly et al., 1996) allows the visualization of the MB neuroblasts and siblings from early stages of development (Adachi et al., 2003). At late embryonic stage 16, the KC axons have already formed the pedunculus, while the dorsal and medial lobes are not yet formed (Fig. 4H). While the DRL protein was not present on the KC axons, their distal tips, most probably the growth cones, were in close contact with DRL-labeled axons (Fig. 4H). In conclusion, our overall results showed that the DRL protein is expressed by two commissural tracts during embryonic brain development, and that the axonal growth cones of embryonic MBs terminate close to these tracts in the

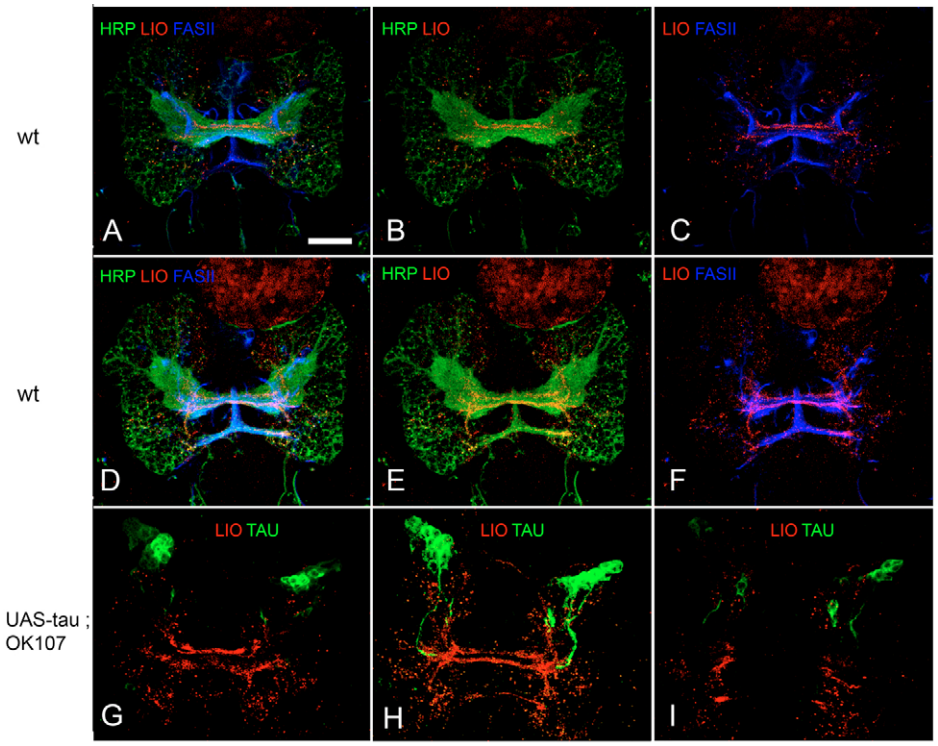

Fig. 4. DRL expression in the *Drosophila* stage 16 embryonic brain. (**A**,**D**) Confocal reconstructions of the same brain, A being dorsal to D. (**B**,**C**,**E**,**F**) Double-channel images derived from A and D. In the stage 16 wild-type embryonic brain, two commissural bundles express the DRL protein (red), one located posteriorly and slightly dorsally (A,B,C), the other being in the anterior part of the commissure (D,E,F). These bundles do not express the FASII marker (blue, C and F) and are HRP-positive (green, B and E), confirming their neuronal identity. (**G-I**) The LIO (red) pattern relative to the embryonic MBs (green *GAL4-OK107* line), going from a dorsal to a ventral view; no colocalization is observed. Scale bar: 10 μm.

neuropile before extending the dorsal and medial larval γ lobes. A schematic representation of MB primordia in the embryonic brain, such as Fig. 10A of Kurusu et al. (Kurusu et al., 2002), may help the reader understand the relationship between commissural tracts and MB axons.

No obvious morphological defects are detectable in the embryonic brain of *drl* LOF mutants

In view of the early expression of the DRL protein in the embryonic brain at a stage preceding KC axonal growth, we investigated whether the anatomical defects in the MBs linked to the *drl* LOF mutation could be due to an early alteration of the brain architecture. As no gross morphological defects were discernable using general axonal markers such as BP102 antibody (data not shown), we analyzed whether the lack of *drl* function leads to abnormalities in neurons that normally express the DRL protein. In order to perform this experiment, we used the drl^{Red2} allele of the *drl* gene, which is a null allele that has retained Tau-β-galactosidase activity from the enhancer trap (Bonkowsky et al., 1999). We first confirmed that adult drl^{Red2}/drl^{R343} flies display an MB phenotype identical to that of null *drl* flies (data not shown). Then, we analyzed the expression pattern of the reporter gene in the embryonic brain. At late embryonic stage 16, the Tau-β-galactosidase protein was detectable in neuronal cells that send their axons along two commissural tracts (see Fig. 5A). Colocalization experiments demonstrated that the two commissural tracts were those expressing the DRL protein (data not shown). Analysis of the morphology of the two commissural tracts in drl^{Red2}/drl^{R343} embryos revealed no differences compared to heterozygous embryos (compare A with B in Fig. 5). Finally, we showed that FASII-expressing axonal tracts, which do not express the DRL protein, were also unaffected by the *drl* LOF mutation (compare C with D and E with F in Fig. 5). In conclusion, our analysis failed to identify any obvious defects in the embryonic brain of *drl* LOF individuals.

DRL expression in the third instar larval brain

Previous studies characterized the expression pattern of DRL protein in the third instar larval brain, revealing the antibody pattern by enzymatic reaction (Simon et al., 1998). The obtained results, combined with enhancer trap data and transgenic reporter gene constructs (Hitier et al., 2000), showed an expression pattern in four cells located in the interhemispheric region. The shape of these cells, as well as their lack of ELAV immunoreactivity, led the authors to assume that they were glial cells. The authors also described, although in less detail, additional, uncharacterized cells that send cellular processes across the brain commissure. In order to gain a better understanding of the *drl* expression profile in the third instar larval brain, we performed a confocal analysis using different markers. We first positively confirmed that the four interhemispheric cells expressing β-galactosidase in the original enhancer-trap line lio^{1} are indeed glial cells, as they express the REPO transcriptional factor (see below).

Next, we analyzed the DRL expression profile relative to the MB axonal tracts, which were labeled by anti-FASII immunoreactivity. Our data were consistent with previously published results: the DRL protein is expressed in four cells located in the interhemispheric region (see Fig. 6F), which, based on their size and position, are most likely the same glial cells expressing the β-galactosidase reporter gene in the lio^{1} enhancer-trap line. The cellular processes of these cells enwrap the medial larval KC γ lobes (see Fig. 6B,C). We confirmed with the *GAL4-442* construct line, which was previously described as being expressed in the interhemispheric glial cells (Hitier et al., 2000), that glial cells processes enwrap medial larval MB lobes (not shown). Interestingly, we were able to detect DRL expression in many cells found all over the surface of the brain (see Fig. 6A-E). Most of them expressed DRL in cellular processes crossing the brain commissure. This pattern of expression is reminiscent of that described in the embryonic brain (see above), and we speculate that these cells are neurons. No colocalization was

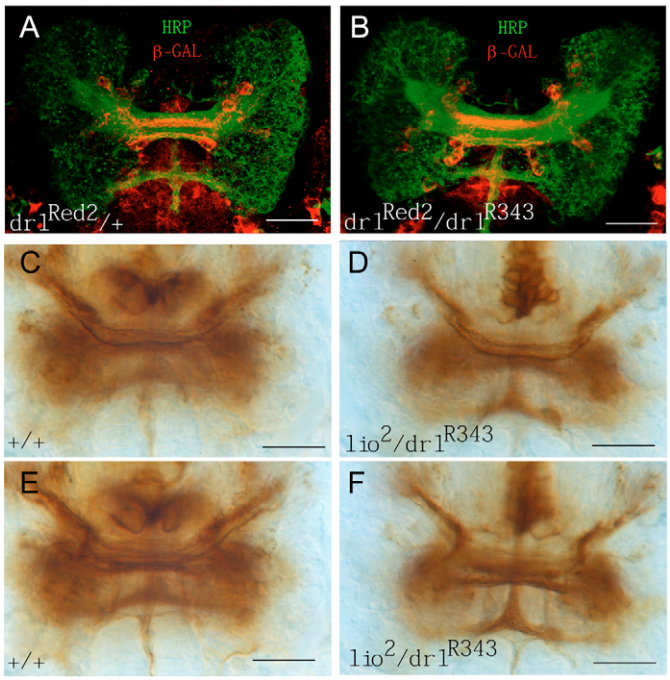

Fig. 5. *drl* LOF mutation induces no apparent defects in the embryonic *Drosophila* brain. Stage 16 brains of drl^{Red2}/+ (**A**) and drl^{Red2}/drl^{R343} (**B**) embryos labeled with anti-β-galactosidase (red) and anti-HRP (green) antibodies. No apparent differences were observed between the two genetic backgrounds. Wild-type brain (**C**,**E**, with C being dorsal to E) and lio^{2}/drl^{R343} brain (**D**,**F**, with D being dorsal to F) from stage 16 embryos labeled with an anti-FASII antibody. The FASII-positive fibers do not display any apparent morphological defects in the lio^{2}/drl^{R343} mutant compared to wild type (compare C with D and E with F). Scale bars: 10 μm.

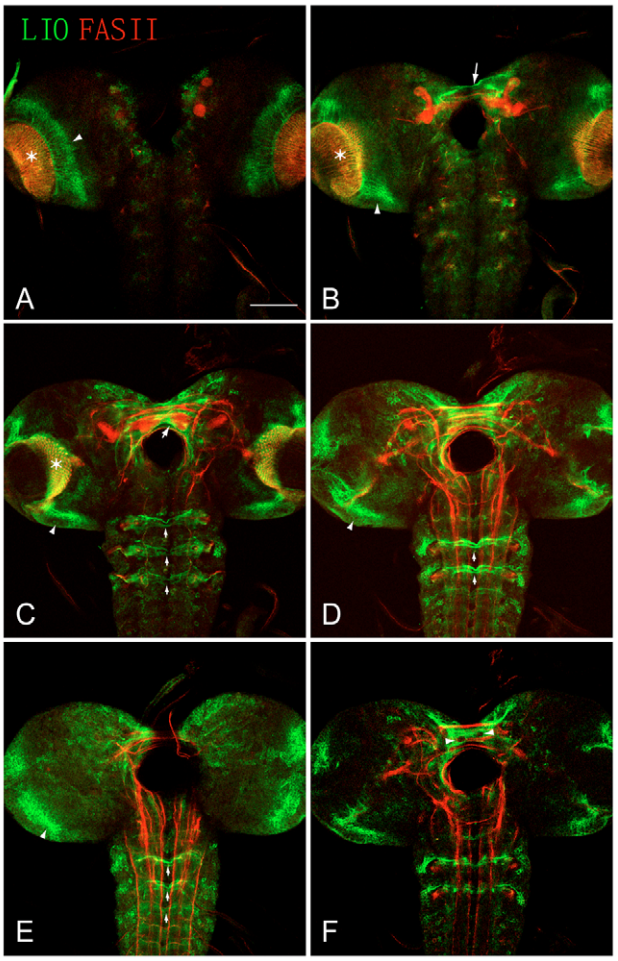

Fig. 6. DRL expression pattern in the *Drosophila* third instar larval brain. (**A-E**) Reconstructions of ten confocal sections spaced by 1 μm, with A being the most frontal section and E the most caudal. (**F**) A single confocal section at the level of the interhemispheric glial cells. The DRL protein (green) is expressed in the third instar larval brain in a complex pattern characterized by a lack of colocalization with MB axons well labeled by FASII (red in B). Different populations of cells, presumably neurons, send fibers crossing the brain commissure (arrow in B). About four cells (two are marked with arrowheads in F), which are glial cells (compare to A-C in Fig. 8) located in the posterior part of the brain, display cytoplasmic processes that wrap around the MB lobes (see arrow in C). DRL expression is also observed in the optic lobes (arrowhead in A-E) and in the incoming photoreceptor axons (asterisk in A-C). In the thoracico-abdominal part of the CNS, DRL protein is detected in commissural neurons (small arrows in C-E). Scale bar: 100 μm.

detectable between the DRL labeling and the FASII-positive MB axons (see Fig. 6A-E). We also detected intense DRL expression in the thoracic and abdominal ganglia (see Fig. 6A-E). This expression profile, which was stronger in the thoracic segments, is similar to that observed in the brain, although no glial cells expressed DRL in the midline region. Finally, strong DRL expression was observed in the optic lobe region. In conclusion, the overall results show that the DRL protein is expressed in more cells than previously characterized. The majority of them, very likely neurons, send their axons across the brain commissure. Interhemispheric glial cells also express the DRL protein, while KC axons appear to be DRL-negative.

Rescue of *drl* LOF MB defects using different GAL4 lines

As DRL expression is detected in brain commissural axons during both embryonic and larval stages, we investigated whether the expression of a transgenic *drl* construct solely in neuronal cells could rescue the *drl* LOF mutation. Expression of the *UAS-drl* construct (Callahan et al., 1996) by the pan neural *elav* driver *GAL4-C155* was able to almost completely rescue the adult MB defects in a *drl* LOF genetic background (Fig. 7). The rescuing effect was directly due to the *UAS-drl* construct, as the *GAL4-C155* line alone had no effect (Fig. 7). These experiments provide evidence that the *drl* gene is required in neuronal cells for correct MB axonal development, in apparent contradiction to previously published results linking the phenotype to DRL expression in interhemispheric glial cells (Simon et al., 1998). In order to verify that *GAL4-C155* expression is indeed exclusively neuronal, as is commonly believed, we performed double-labeling experiments in the third instar larval brain using a *UAS-nls-lacZ* reporter and the anti-REPO antibody. Surprisingly, although mainly expressed in REPO-negative cells, the β-galactosidase expression driven by the *GAL4-C155* line was also detected in REPO-positive cells located close to the brain commissure (see Fig. 8D-F). The size and position of these cells were similar to those in cells expressing the β-galactosidase reporter gene in lio^{1} individuals (compare panels A-C with D-F in Fig. 8).

In order to test whether expressing *drl* in glial interhemispheric cells can rescue the *drl* LOF MB phenotype, we used the *GAL4-442* line. Confocal analysis revealed that *GAL4-442* induces expression

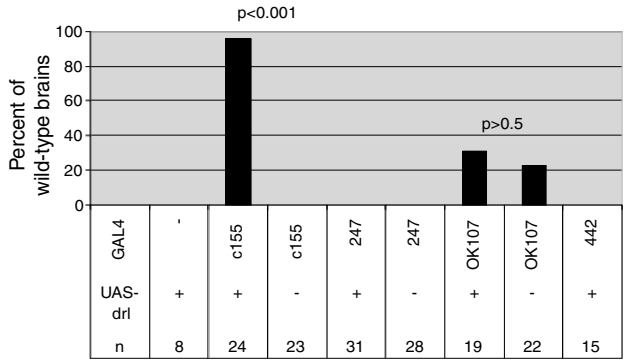

Fig. 7. Rescue of *drl* LOF MB defects using different *Drosophila* GAL4 lines. All the lines analyzed are in the lio^2/drl^{R343} genetic background. In the *x*-axis label, both the *GAL4* line used and the absence (–) or presence (+) of the *UAS-drl* construct are specified. *n*, the number of brains analyzed. On the *y*-axis, the percentage of wild-type brains is shown. MB morphology was assessed using the anti-FASII antibody. *P*-values were obtained after χ^2 test comparisons. Only the *GAL4-c155* was able to rescue; the other GAL4 lines (*247*, *OK107* and *442*) were unable to rescue the *drl* MB phenotypes. These results indicate that the *drl* MB defects are linked to the lack of expression of the *drl* RTK in neuronal cells extrinsic to the MBs.

of the GAL4 protein in more cells than previously published (data not shown). In addition to the interhemispheric glial cells, expression was detected in many cortical and perineural glial cells. No rescue of the *drl* LOF phenotype was observed when the *UAS-drl* transgene was expressed under the control of *GAL4-442* (see Fig. 7). These last results, combined with those obtained with *GAL4-C155*, suggested that the *drl* transgene product might need to be expressed by both neuronal and glial cells in order to rescue the *drl* LOF MB defects. In order to test this hypothesis, we characterized the expression pattern of the *GAL4-7B* line, which was previously shown to be able to rescue very well the *drl* LOF MB defects when combined with the *UAS-drl* transgene (Moreau-Fauvarque et al., 1998). Confocal analysis of the *GAL4-7B* line expression pattern together with REPO antibody staining revealed no positive REPO cells that also expressed the reporter gene under GAL4 control (see Fig. 8G-I).

These data suggest that neuronal *drl* expression is sufficient to allow appropriate MB development. Formally we cannot exclude that the interhemispheric glial cells indeed have a role in the normal situation, but what the *GAL4* rescue experiments tells us is that this glial cell expression seems neither necessary nor sufficient for appropriate MB development.

Finally, in order to functionally confirm the clonal analysis results presented above, showing that the *drl* LOF MB axonal defects are not cell-autonomous, we used two *GAL4* lines that are either specifically or mostly expressed in KCs to try to rescue the *drl* LOF MB defects. No rescuing effect was observed at all (see Fig. 7) when the *UAS-drl* transgene was expressed using the *GAL4-247* line (Zars et al., 2000). While a mild rescue effect was, however, induced by *GAL4-OK107* in combination with the *UAS-drl* transgene, a similar rescue was obtained using *GAL4-OK107* alone. The obtained values were not significantly different ($P>0.5$), suggesting that a dominant effect of the *OK107* insertion site might be responsible for the mild rescue (see Fig. 7). In conclusion, our functional rescue approach suggests that the *drl* LOF MB defects are linked to a lack of expression of the *drl* RTK in neuronal cells that are extrinsic to the MBs.

Role of WNT5 and its genetic interaction with the DRL receptor during MB development

As the WNT5 protein has been shown to act as a repulsive ligand for the DRL receptor in the embryonic central nervous system (CNS) (Yoshikawa et al., 2003), we wondered whether WNT5 might have a role in MB development. The *Wnt5* null MB phenotype was visualized directly using an anti-FASII antibody or using the *GAL4-c739* line combined with *UAS-mCD8-GFP*. We consistently observed a predominant mutant phenotype characterized by an absence of α and β lobes (about 75% of 30 to 50 MBs), indicating a complete arrest of axonal growth at the level of the peduncle (Fig. 9A). The other mutant MBs were variably distributed among three classes: no α lobes, no β lobes and 'wild-type-looking'. Strikingly, these *Wnt5* mutant phenotypes resemble those obtained as a consequence of *drl* pan-neuronal overexpression [see Fig. 2B,C and Fig. 3C of Taillebourg et al. (Taillebourg et al., 2005)] suggesting an antagonistic interaction between *Wnt5* and *drl*. To confirm that this *drl* gain-of-function (GOF) phenotype is still present when the expression is mainly restricted to the MB, we overexpressed two

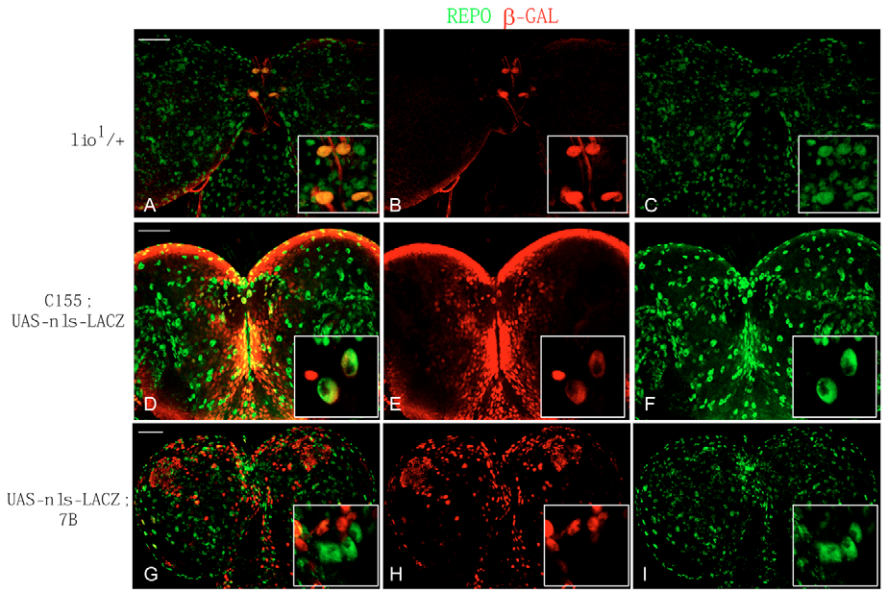

Fig. 8. Expression patterns of different enhancer-trap lines and REPO in the *Drosophila* third instar brain. Each row shows the merged and single channels of the same brain; the inset in each panel is a magnification of the interhemispheric region. (**A-C**) The lio^1 insertion drives the expression of nuclear β-galactosidase in four REPO-positive interhemispheric cells. (**D-F**) Colocalization of two REPO-positive cells with nuclear β-galactosidase driven by the *GAL4-C155* line is shown. (**G-I**) *GAL4-7B* does not drive expression of nuclear β-galactosidase in REPO-positive cells. Scale bar: 50 μm.

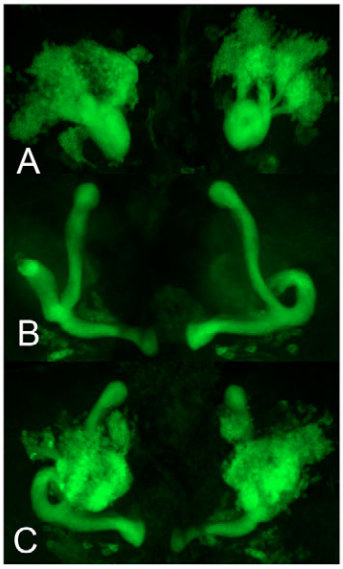

Fig. 9. MB defects of *Drosophila Wnt5* LOF mutants are rescued by *Wnt5*$^{+}$ expression in the MBs. (**A-C**) Composite confocal images of MB phenotypes visualized with the *GAL4-c739* line combined with *UAS-mCD8-GFP*. (A) In the *Wnt5* null mutation, there is a complete arrest of axonal growth at the level of the peduncle, although Kenyon cells appear to be unaffected. The genotype is *Wnt5*^{400}/Y; *GAL4-c739 UAS-mCD8-GFP*/+. B (excluding Kenyon cells) and C (including Kenyon cells) are images from the same brain and show a complete rescue of the MB mutant phenotype. The genotype is *Wnt5*^{400}/Y; *GAL4-c739 UAS-mCD8-GFP*/+; *UAS-Wnt5*$^{+}$/+.

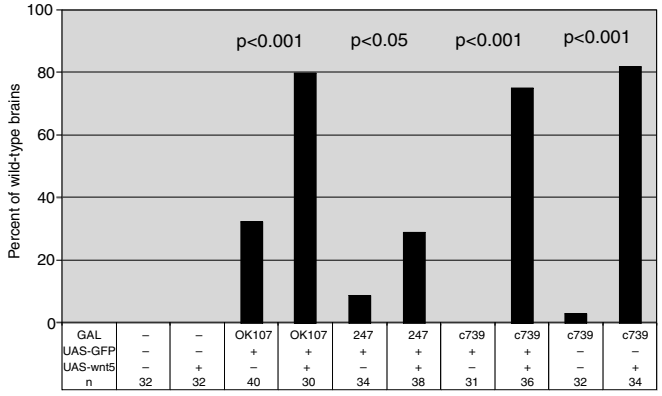

Fig. 10. Rescue of *Drosophila Wnt5* LOF MB defects using different MB GAL4 lines. All the lines analyzed are in the *Wnt5*^{400}/Y genetic background. In the *x*-axis label, both the *GAL4* line used and the absence (–) or presence (+) of the *UAS-GFP* and *UAS-Wnt5*$^{+}$ constructs are specified. *n*, the number of brains analyzed. The *y*-axis shows the percentage of wild-type brains. MB morphology was assessed using the anti-FASII antibody, or directly with GFP fluorescence when possible. *P*-values were obtained after χ^2 test comparisons. The three MB *GAL4* lines (*OK107*, *247*, *c739*) were able to rescue the *Wnt5* MB phenotype. These results indicate that the *Wnt5* MB defects are linked to the lack of expression of the WNT5 protein in intrinsic MB neurons.

doses of *UAS-drl* and two doses of *UAS-drlΔintra* with *GAL4-OK107*. We looked, in the two different genetic combinations with the anti-FASII labeling, to the MB phenotype: *UAS-drl*: 58 MB (all lobes missing: 0/58; some lobes missing: 2/58; no lobes missing or wild-type-looking: 56/58), *UAS-drlΔintra*: 62 MB (all lobes missing: 27/62; some lobes missing: 23/62; no lobes missing or wild-type-looking: 12/62). As was already seen (Taillebourg et al., 2005), the GOF effect is much more effective when the RTK is deleted of its intracellular domain. Therefore, clear MB axon pathfinding defects are obtained when *drlΔintra* is overexpressed mainly in the MB cells. This result is in good accordance with a titrating role of ectopic *drl* receptor domain on the WNT5 ligand and renders unlikely a negative regulation effect of ectopic *drl* on *Wnt5* transcription, as described in the embryonic CNS (Fradkin et al., 2004).

When a *Wnt5*$^{+}$ cDNA under a UAS promoter was principally expressed within the MBs using three different *GAL4* lines, a clear rescue of the *Wnt5* mutant phenotype was observed (Figs 9 and 10). This rescue strongly indicates that the WNT5 protein must be intrinsically expressed within MBs in order to insure axonal growth. Consistent with this hypothesis, WNT5 is highly and widely expressed in the central brain of early and late pupal brain (Srahna et al., 2006). However, WNT5 is a secreted protein, and as such, removal of *Wnt5* function in MB clones should not affect axonal outgrowth because these neurons should still be able to receive WNT5 signal from neighboring MB neurons. *Wnt5*^{400} homozygous MB neuroblast clones were obtained with the MARCM technique. Eighteen clones were produced and all look like wild type. An attractive hypothesis would be that axons grow under intrinsic *Wnt5* signaling during normal development and stop growing at the interhemispheric region because WNT5 becomes trapped by the extrinsic DRL receptor. One can propose that during the growing process, the secreted WNT5 protein is activating an MB intrinsic receptor, which may be of the Frizzled type (Logan and Nusse, 2004) in order to activate axonal growth. We were not able to uncover any involvement of *fz*, *fz2* or *fz3*. We looked, in different genetic combinations with the anti-FASII labeling, to the MB phenotype. *Wnt5*^{400}/+: 60 MB (all wild type), *fz*^{h51} *fz2*^{C1}/+: 70 MB (69 wild type, 1 without α lobe), *Wnt5*^{400}/+; *fz*^{h51} *fz2*^{C1}/+: 64 MB (62 wild type, 2 without α lobe), *Wnt5*^{400}/*fz3*^{G10}: 66 MB (all wild type). The *UAS-fz2GPI* bears a dominant-negative form of FZ2 and was associated with the *GAL4-OK107* line and assessed with anti-FASII: 36 MB (36 wild type). Finally, we induced 13 homozygous *fz*^{h51} *fz2*^{C1} MB clones (neuroblast or multiple single-cell), which all looked wild type, with the MARCM technique. These results indicate that another type of receptor for this unusual *Wnt5* signaling pathway might be at work. This hypothesis also predicts that *Wnt5* and *drl* should genetically interact during MB development. This is indeed the case, as *drl* overexpression mimicked *Wnt5* mutant MB phenotypes (see above), and reduced expression of *lio*$^{+}$/*drl*$^{+}$ associated with overexpression of *Wnt5*$^{+}$ within the MBs gradually led to a *drl*-like phenotype (Fig. 11). This last result indicates a strong antagonistic genetic interaction between the two proteins, where overproduction of WNT5 leads to a diminution of the activity of DRL, very likely by a titration mechanism.

DISCUSSION

We have shown that a *drl* (*lio*) receptor tyrosine kinase LOF mutation affects MB development as early as at the newly hatched first instar larval stage. It is at (or just before) this stage that the axons of the first MB intrinsic neurons to be born form the median and vertical lobes (Tettamanti et al., 1997). We can hypothesize that the MB defects displayed by *drl* LOF adult flies are at least partially due to aberrant early MB development. The DRL protein is not

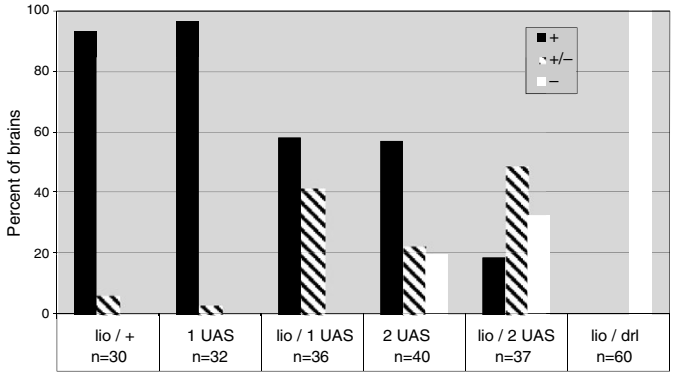

Fig. 11. Genetic interaction between *drl* and *Wnt5* during MB development in *Drosophila*. Different genotypes were assessed for the production of a *drl*-like phenotype. MB morphology was assessed using the anti-FASII antibody. These results indicate that reduced expression of lio^{+}/drl^{+} associated with overexpression of $Wnt5^{+}$ gradually leads to a *drl*-like phenotype. lio/+, lio^{2} *GAL4-c739*/+. 1 UAS, *GAL4-c739/+; UAS-*$Wnt5^{+}$/+. 2 UAS, *GAL4-c739/UAS-*$Wnt5^{+}$*; UAS-*$Wnt5^{+}$/+. lio/1 UAS, lio^{2} *GAL4-c739/+; UAS-*$Wnt5^{+}$/+. lio/2 UAS, lio^{2} *GAL4-c739/UAS-*$Wnt5^{+}$*; UAS-*$Wnt5^{+}$/+. lio/drl, lio^{2} *GAL4-c739/*drl^{R343}. *n*, the number of brains analyzed. +, wild-type; +/–, moderate midline crossing; –, complete midline crossing.

expressed within the MB intrinsic neurons at any developmental stage that we have analyzed. This result is strengthened by our clonal analysis experiments, which showed that the early removal of the wild-type *drl* gene in a subset of the three classes of MB intrinsic neurons does not alter their axonal morphology. The clonal analysis results demonstrate sensu stricto a non-cell-autonomous requirement for the *drl* gene in the MB intrinsic neurons; it does not, however, completely exclude the possibility that *drl* mutant clones develop properly due to the expression of the DRL protein in MB intrinsic neurons outside the clones. This would imply that the DRL expression level in the MBs is below the level required by the detection method used in our study. However, restoring the expression of the *drl* gene solely in a subpopulation of MB intrinsic neurons with the *GAL4-247* line, or even in most if not all MB intrinsic neurons with *GAL4-OK107* (see Fig. 7), was insufficient to rescue the MB defects induced by the *drl* LOF mutation. The partial rescue obtained previously with the *GAL4-c739* line (Moreau-Fauvarque et al., 1998) is likely to be due to some transient expression outside the MBs during development (Nicolai et al., 2003). The fact that the mutant phenotype cannot be rescued by two other GAL4 lines that are expressed either more specifically (*GAL-247*) or in more MB neurons (*GAL-OK107*) is ruling out a role of the MB expression of *GAL-c739* in the weak rescuing effect. Based on the overall results obtained, we favor the hypothesis that *drl* gene function is required extrinsically by MBs for their proper development. Finally, the MARCM technique allowed us to visualize the morphology of single-side median MB axons in *drl* LOF individuals. This analysis revealed that the mutant phenotype is not simply a fusion of the median contralateral lobes at the midline, but rather a real crossing of the axons, which then intermingle with their contralateral equivalents.

The function of the DRL protein is required extrinsically by the MBs for their proper development. Our data show that the protein is expressed from the onset of brain commissural formation in a subset of neurons crossing the midline. This pattern is remnant of DRL expression in the embryonic CNS, although at later stages the DRL-expressing brain commissural axons divide into two tracts. It is important to emphasize that the embryonic brain commissure is not identical to those of the ventral CNS, and that the molecular factors involved in their development, although often conserved, do not necessarily play the same role. Knowing that the DRL receptor is necessary cell-autonomously in the CNS to allow the correct midline crossing of a subset of anterior commissural axons, we analyzed whether similar defects could be observed in the embryonic brain. Such defects could be the primary cause of the MB observed phenotype. This is not the case, as no embryonic brain commissural tract abnormalities were detected using different axonal markers. It has been previously suggested that DRL expression in interhemispheric glial cells during late third instar larval and pupal stages is necessary for MB axonal development. Although we could detect DRL expression in interhemispheric glial cells of third instar brains, we were unable to rescue the MB phenotype by specific interhemispheric glial cell expression (Fig. 7). Moreover, no glial cells expressed the DRL protein at earlier developmental stages, even though MB defects were already present in *drl* LOF individuals. In addition, the observed DRL expression in commissural neurons and the positive rescue results using a pan-neuronal driver lead us to postulate that DRL is required in neuronal cells extrinsic to the MBs for the correct axonal development of the latter. In conclusion, our study suggests that in the *Drosophila* brain, DRL expression in a subpopulation of commissural neurons is necessary not for their own axonal development but rather for the guidance of MB intrinsic neurons that do not express the DRL protein.

This neuronal hypothesis is particularly attractive when we take into account the *Wnt5* results. We tested *Wnt5* mutants because WNT5 was described as being a ligand for the DRL receptor in the ventral CNS (Yoshikawa et al., 2003; Fradkin et al., 2004). We found clear MB phenotypes in *Wnt5* mutant brains. The *Wnt5* MB mutant phenotype is most consistent with WNT5 being required for neurite outgrowth. It is striking that these mutant phenotypes resemble those described for lio^{+}/drl^{+} overexpression (Taillebourg et al., 2005) (this study). We propose that this GOF phenotype is due to lio^{+}/drl^{+} expression within or close to MB cells, where the ectopic DRL protein can bind to the WNT5 protein and prevent its function. Therefore, we can propose a general model for the role of the *Wnt5-drl* pair in building normal MBs: WNT5 is expressed and required within MB cells in order to insure proper axonal growth. One can propose that during this process the secreted WNT5 activates an MB intrinsic receptor, which seems not to be of the *fz* type, in order to activate axonal growth. When WNT5 is absent, e.g. in a *Wnt5* mutant MB, then the axons fail to grow properly. In the normal situation, these MB intrinsic axons will stop growing at the midline when they reach extrinsic axons expressing DRL, because WNT5 is trapped by the DRL receptor. In *drl* mutant individuals, however, the MB axons will continue to grow, because WNT5 is not trapped by the DRL receptor. Although the biochemical relationship between the ligand and receptor is conserved from the embryonic ventral CNS to the adult brain, it should be stressed that MB development involves neurons that express WNT5 and not DRL, which is exactly opposite to the case in the embryo, where the mutant phenotype involves neurons that express the *drl* gene and not *Wnt5*. This is why *drl* and *Wnt5* mutants have the same phenotype in the embryonic ventral CNS but have opposite phenotypes in adult MBs.

The genetic control of brain development requires both intrinsic and extrinsic clues. The perfect crosstalk between both types of molecular information, coming from neurons of different types of brain substructures, ultimately ensures the development of a

harmonious and functional brain. It is central for neurobiology to decipher these interacting and developing neuronal networks at the cellular and molecular levels. Here, we describe a clear case in which *drl*, a receptor tyrosine kinase, is required within the brain for the normal development of MBs, although it is neither expressed nor required intrinsically within the MB neurons. Further, we propose that the WNT5 signaling molecule is the intrinsic MB axon target that needs to interact with the extrinsic DRL receptor in order to construct proper MBs within the brain.

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