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SHORT COMMUNICATION

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# In vivo evidence for a regulatory role of the kinase activity of the *linotte/derailed* receptor tyrosine kinase, a *Drosophila* Ryk ortholog

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**Abstract** The RYK subfamily of receptor tyrosine kinases is characterised by unusual, but highly conserved, amino acid substitutions in the kinase domain. The *linotte/derailed* gene encodes a *Drosophila* RYK subfamily member involved in embryonic and adult central nervous system development. Previous studies have shown that the kinase activity of this receptor is not required in vivo for its embryonic function. In this study, we have investigated the role of the cytoplasmic domain and the kinase activity of the *linotte/derailed* receptor tyrosine kinase in adult brain development. Our results indicate that these domains are not essential for adult brain development but they are required for the proper regulation of the activity of this receptor. This sheds light on a regulatory role for the kinase activity of a RYK subfamily member.

**Keywords** Mushroom body · *Drosophila* · Receptor tyrosine kinase · *linotte/derailed* · Ryk ortholog

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## Introduction

The *linotte/derailed* (LIO/DRL) receptor tyrosine kinase (RTK) belongs to the RYK subfamily of RTKs, which possesses members in mammals, *Caenorhabditis elegans* and *Drosophila melanogaster* (Callahan et al. 1995; Dura et al. 1995; Halford et al. 1999; Hovens et al. 1992). Although each member has the invariant lysine that is essential for the phosphotransfer reaction (Carrera et al. 1993), all members of this subfamily display unusual, but highly conserved, amino acid substitutions in their kinase domains. These substitutions and biochemical evidence (Hovens et al. 1992; Katso et al. 1999) have led to the hypothesis that the RYK subfamily members are catalytically inactive.

The LIO/DRL RTK has been implicated in different aspects of *Drosophila* central nervous system (CNS) development. In the embryonic CNS, it is expressed by neurons sending contra-lateral projections via the anterior commissure. In *lio/drl* mutants, the axons of these neurons abnormally cross the midline via the posterior commissure (Callahan et al. 1995). Conversely, misexpression of the LIO/DRL RTK in neurons that normally project via the posterior commissure switches their axonal projections to the anterior commissure. Moreover, it has recently been shown that the LIO/DRL receptor interacts genetically and physically with the secreted Wnt5 protein and is an essential component of Wnt signalling in the embryonic CNS (Yoshikawa et al. 2003). These data suggest that the LIO/DRL protein acts autonomously in the neurons projecting via the anterior commissure as a receptor for the Wnt5 protein. The LIO/DRL RTK is also involved in adult brain development since *lio/drl* mutants display axonal guidance defects in the mushroom bodies (MB; Moreau-Fauvarque et al. 1998; Simon et al. 1998), the *Drosophila* learning and memory centres. These defects impair MB function and *lio/drl* mutants exhibit learning and memory deficits (Dura et al. 1995; Moreau-Fauvarque et al. 2002). During MB development, the LIO/DRL protein is not detected in the intrinsic neurons of the MB but in a transient structure at the brain midline (Simon et

al. 1998). These results suggest that, in contrast with its function in the embryonic CNS, the LIO/DLR RTK may act non-autonomously during MB development.

To test the requirement for the kinase activity of the LIO/DRL RTK in the embryonic CNS, Yoshikawa et al. have produced a LIO/DRL mutant protein, catalytically inactive, and shown that this mutant protein behaves like the wild-type protein (Yoshikawa et al. 2001), providing the first evidence that catalytic activity is not required in vivo for the function of a RYK subfamily member. In the present study, we have used the same kinase-dead mutant and a cytoplasmic-domain-deleted mutant to study the requirement for kinase activity during MB development. We found that these mutant proteins rescued the MB phenotype of *lio*/*drl* mutants and that, when expressed in wild-type background, induced gain-of-function phenotypes in the MBs more efficiently than the wild-type protein. These results lead us to conclude that, as in the embryonic CNS, the kinase activity of the LIO/DRL receptor is not essential during MB development but that it significantly contributes to the regulation of LIO/DRL RTK function.

## Materials and methods

### Fly strains and culture

All strains were maintained on standard food at 25°C. $lio^{2}$ and $drl^{R343}$ are amorphic alleles of the *lio*/*drl* gene described in Dura et al. (1995) and Callahan et al. (1995), respectively. MBs are visualized with the pan-neural *elav* marker $GAL4^{c155}$ associated with *UAS-mCD8-GFP* which drives GFP expression in the membranes of neuronal processes of all post-mitotic neurons (Lee and Luo 1999).

### DNA constructs

Construction of the *UAS-drl* and UAS-*drlK371A* transgenes are described in Callahan et al. (1995) and Yoshikawa et al. (2001), respectively. The *UAS-drl*Δ*intra* construct was produced as a 0.9-kb *Pst*I-*Sal*I fragment from a complete *lio* cDNA (Dura et al. 1995) ligated to an oligonucleotide which provides a myc epitope (Munro and Pelham 1987). It encodes the first 295 amino acids of the LIO/DRL protein linked to the myc epitope by an isoleucine residue. This construct was then cloned in the pUAST vector (Brand and Perrimon 1993) and *P* element-mediated transformation was carried out as described by Spradling and Rubin (1982).

### Confocal microscopy

Adult heads were separated from the bodies and fixed in 3.7% formaldehyde in PBS for 15 min. The adult brains were dissected in PBS, fixed for 15 min in 3.7% formaldehyde in PBS and washed for 10 min in PBS containing 0.5% Triton X-100 (PBST). They were then mounted in Moviol or Vectashield (Vector Laboratories) and the samples were viewed with a DMRB Leica fluorescence microscope. Images of selected brains were taken with a Bio-Rad MRC 1024 laser scanning confocal microscope and processed using Adobe Photoshop.

### Real-time quantitative RT-PCR

This analysis was carried out on individuals having two copies of each transgene pan-neurally expressed by the $GAL4^{c155}$ driver. Total RNA was isolated from 30 *Drosophila* adult heads using RNAplus solution (Bioprobe Systems). Reverse transcription reactions contained 2 μg total RNA with 100 U SuperScript II (Life Technologies) and oligo $(dT)_{12\text{-}18}$ primers in 20 μl reaction volume. Real-time quantitative PCR experiments were performed in triplicate on a LightCycler apparatus (Roche Molecular Biochemicals) following the manufacturer's suggestions and using SYBR Green-based detection of PCR products. Serial cDNA dilutions were used to check amplification efficiency and melting curves were analysed after amplification to confirm single-product measurements. The primers used were lio1 (CTCAACTTGCACGAGGTGC TG) and lio2 (TTGCCACGTAAAGGTCACGTC) for amplification of *lio*/*drl* transcripts and tub3 (GTCAGACC TCGAAATCGTAGC) and tub4 (AATCTGGACACCAG CCTGACC) for amplification of *tubulin* transcripts.

## Results and discussion

### The LIO/DRL mutant proteins

We have compared the activity of the wild-type LIO/DRL protein with two mutant proteins: a kinase-dead mutant DRLK371A and an intracellular domain-deleted mutant DRLΔintra (Fig. 1). In the DRLK371A mutant, the lysine at position 371, which is invariant among tyrosine kinases and essential for catalytic activity (Carrera et al. 1993), has been mutated to an alanine (Yoshikawa et al. 2001). DRLK371A is thus predicted to be catalytically inactive but still display putative interaction sites with cytoplasmic proteins. The DRLΔintra mutant is a deletion of the intracellular domain. It encodes the extracellular and transmembrane domains but lacks most of the juxtamembrane domain and the kinase domain (Fig. 1). The DRLΔintra protein is thus catalytically inactive and unable to mediate interactions with putative cytoplasmic partners.

The DRL, DRLK371A and DRLΔintra proteins were expressed using the GAL4/UAS transactivation system (Brand and Perrimon 1993). Two or four copies of transgenes encoding each protein were combined in wild-type or *lio*/*drl* null mutant backgrounds and pan-neurally expressed using $GAL4^{c155}$, a *P[GAL4]* insertion in the *elav* gene. This driver was chosen because it allows the best rescue of the MB phenotype of *lio*/*drl* mutants (N. Grillenzoni, J.-M. Dura, unpublished results).

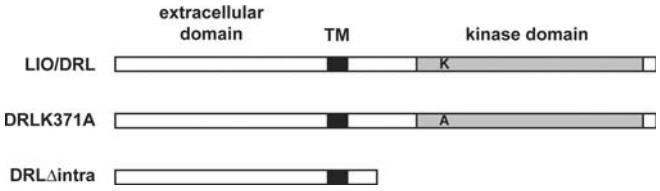


**Fig. 1** Schematic representation of the constructs used in this study. The wild-type LIO/DRL protein is 610 amino acids long with the typical hallmarks of a RYK subfamily member: a relatively short extracellular domain (239 amino acids) and a kinase domain with unusual, but highly conserved, amino acid substitutions. The invariant lysine at position 371 is essential for kinase activity and has been mutated to an alanine residue to produce the kinase-dead mutant DRLK371A (Yoshikawa et al. 2001). The DRLΔintra mutant is a deletion of the intracellular domain. It encodes the extracellular and transmembrane domains but lacks most of the juxtamembrane domain and the cytoplasmic kinase domain (TM transmembrane domain)

## The LIO/DRL mutant proteins induce defects in the mushroom bodies

Adult MBs are characterised by a set of orthogonal lobes (Fig. 2a): two dorsal lobes (α and α′) and three median lobes (β, β′ and γ). We have observed that pan-neural expression of the wild-type DRL protein induces the loss of some (Fig. 2b), or all (Fig. 2c), of these lobes. The severity of this gain-of-function phenotype is dosage-dependent since the percentage of MBs affected increases with the number of copies of the *UAS-drl* transgene and the absence of all the lobes is only observed with four copies of transgene (Table 2). Interestingly, we have observed that the DRLK371A and DRLΔintra mutant proteins produce the same kind of defects as the wild-type DRL protein (Table 2). This shows that the induction of gain-of-function phenotypes in the adult MBs requires neither the kinase activity nor the intracellular domain of the LIO/DRL RTK and suggests that the extracellular domain is sufficient to induce these phenotypes.

In order to compare the phenotypes induced by the pan-neural expression of DRL, DRLK371A and DRLΔintra proteins, the levels of expression of their mRNAs were determined by real-time quantitative RT-PCR (Table 1). We assumed that the levels of transgene expression would be proportional to those of the protein products. Statistical analysis revealed that the *drl* and *drlΔintra* constructs were not differently expressed ($P$ =0.391) whereas *drlK371A* was significantly less expressed than *drl* ($P$ =0.048) and *drlΔintra* ($P$ =0.007). This result must be taken into account when the phenotypes induced by the pan-neural expression of DRL, DRLK371A and DRLΔintra proteins are analysed (Table 2). Indeed, extrapolation from the results of mRNA quantification indicates that although DRLK371A is about three times less expressed, it induces more defects than DRL ($P$ <0.005 with four copies of transgene) whereas, for the same levels of expression, DRLΔintra induces many more defects than DRL ($P$ <0.001 with two and four copies of transgene). This implies that the mutant proteins are more potent in inducing gain-of-function phenotypes than the wild-type protein.

The DRLK371A protein induces significantly fewer defects than the DRLΔintra protein ($P$ <0.001 with two and four copies of transgene) but, since it is estimated to be about four times less expressed, it is not clear whether this result is solely due to quantitative effects or whether qualitative differences exist between the mutant proteins.

Our results show that the kinase activity and the intracellular domain of the LIO/DRL RTK are not essential for the induction of defects in the adult MBs but are required to negatively regulate the capacity of the extracellular domain to induce such phenotypes.

## The LIO/DRL mutant proteins rescue the MB phenotype of *lio/drl* mutants

We have previously shown that mutations in the *lio/drl* gene produce axonal guidance defects in the adult MBs (Moreau-Fauvarque et al. 1998). Most of the *lio/drl* null mutants display a complete loss of the dorsal lobes and a

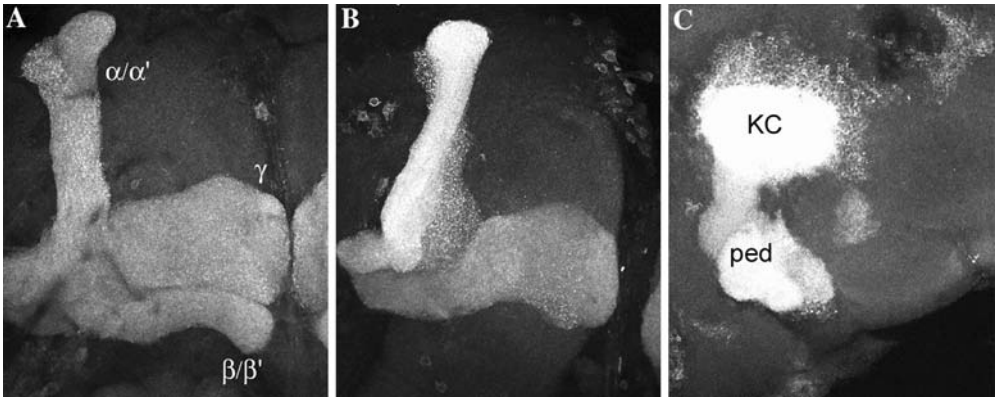


**Fig. 2** Composite confocal images of mushroom body (MB) phenotypes induced by the pan-neural expression of the wild-type and mutant LIO/DRL proteins. **a** Wild-type adult MB with the two dorsal lobes (α and α′) and the three median lobes (β, β′ and γ). **b** Adult MB with the median β and β′ lobes missing, archetypal of the "some lobes missing" category of Table 1. **c** Adult MB with all the lobes missing. In this case, the composite confocal image is more posterior showing the cellular bodies of the intrinsic MB neurons (Kenyon cells KC), and the pedunculus (ped) in which the axons have stopped before projecting into the lobes

**Table 1** Quantification of the expression levels of the LIO/DRL transgenic constructs used in this study. Transcript levels were determined by real-time quantitative RT-PCR from threshold cycle (*Ct*) values and normalised to *tubulin* transcripts (alphaTub84A). Ct and ΔCt calculations were based on three independent experiments on individuals having two copies of each transgene pan-neurally expressed by the $GAL4^{c155}$ driver. Statistical analysis (Student's *t* test) reveals that the *drl* and *drlΔintra* constructs were not differently expressed ($P$ =0.391) whereas *drlK371A* was significantly less expressed than *drl* ($P$ =0.048) and *drlΔintra* ($P$ =0.007). $\Delta Ct = Ct_{drl} - Ct_{tub}$

| | *drl* Ct | *tub* Ct | ΔCt |
|---|---|---|---|
| DRL[a] | 29.08±2.10 | 25.33±2.49 | 3.75±0.39 |
| DRLK371A[b] | 29.01±0.20 | 23.78±0.31 | 5.23±0.22 |
| DRLΔintra[c] | 27.19±0.53 | 23.78±0.67 | 3.41±0.19 |

[a] DRL: $GAL4^{c155}$, *UAS-mCD8GFP*/+; *UAS-drl(II)*/+; *UAS-drl(III)*/+
[b] DRLK371A: $GAL4^{c155}$, *UAS-mCD8GFP*/+; *UAS-drlK371A(II)*/+; *UAS-drlK371A(III)*/+
[c] DRLΔintra: $GAL4^{c155}$, *UAS-mCD8GFP*/+; +/+; *UAS-drlΔintra (IIIa)*, *UAS-drlΔintra(IIIb)*/+

fusion of the median lobes over the midline (Fig. 3a) whereas a few of them display a milder phenotype with dorsal lobes shorter or thinner than normal and a partial fusion of the median lobes (Fig. 3b). However, all the *lio/drl* null mutants have abnormal MBs (Table 3) indicating that the LIO/DRL RTK is required for MB development.

Pan-neural expression of two copies of the wild-type *UAS-drl* transgene rescues the phenotype of *lio/drl* null mutants with 68% of the MBs reverting to a wild-type phenotype (Fig. 3d, Table 3). We have observed that the DRLK371A and DRLΔintra mutant proteins can also completely rescue the MB phenotype of *lio/drl* mutants in 30% and 19% of the cases, respectively (Fig. 3e, f, Table 3). This shows that neither the kinase activity nor the intracellular domain is essential for LIO/DRL RTK function during MB development.

In the case of the wild-type DRL protein, 16% of the MBs remain mutant whereas 16% display a phenotype identical to the gain-of-function phenotype (Fig. 3c, Table 3). The mutant proteins are less efficient in rescuing the MB phenotype but induce either the same, or higher, levels of gain-of-function phenotypes than the wild-type protein (Table 3). This result is in accordance with our previous observations indicating that the intracellular domain and the kinase activity are required for negative regulation of the extracellular domain of the LIO/DRL RTK in its capacity to interfere with MB development.

In this report, we have compared the activity of the wild-type DRL protein with two mutant proteins: the kinase-dead mutant DRLK371A (Yoshikawa et al. 2001) and the intracellular domain-deleted mutant DRLΔintra (Fig. 1). We have shown that these proteins were able to rescue the MB phenotype of *lio/drl* mutants and that, when expressed in wild-type background, they induced gain-of-function phenotypes in the MBs more efficiently than the wild-type protein. This discrepancy (rescue of the mutant phenotype versus induction of a gain-of-function phenotype in wild-type for the same construct) is probably due to the fact that the MB phenotype of *lio/drl* mutants is highly sensitive to gene dosage (Moreau-Fauvarque et al. 1998). These results lead us to conclude that the intracellular domain of the LIO/DRL receptor is not essential for protein function during MB development and that at least the kinase activity contained therein is required for the negative regulation of the extracellular domain activity. Two alternative hypotheses may explain these results. In the first, the LIO/DRL protein would act as a ligand rather than a receptor. The kinase activity would negatively regulate the ability of the extracellular domain to interact with its "receptor". Alternatively, the LIO/DRL protein may act as a co-receptor negatively regulating the function of another receptor by the way of its kinase activity. The formation of this heterodimeric receptor would not depend upon the presence of the LIO/DRL intracellular domain. Study of the cellular autonomy of the MB phenotype of *lio/drl* mutants will discriminate between these hypotheses. However, in this paper, we only address the question of the role of the catalytic domain within the LIO/DRL protein. Moreover, it has recently been shown that, in the embryonic CNS, the LIO/DRL receptor interacts with the Wnt5 protein and is an essential component of Wnt

**Table 2** Quantification of the mushroom body (MB) defects induced by the DRL, DRLK371A and DRLΔintra proteins. Defects induced by the pan-neural expression of two or four copies of *UAS-drl*, *UAS-drlK371A* or *UAS-drlΔintra* transgenes were classified into three categories (Fig. 2) and quantified. Wild-type flies as well as control flies having only the UAS transgenes or the GAL4 driver do not display any MB phenotypes (data not shown). Statistical analysis (Chi-square test) reveals significant differences between DRL and DRLK371A with four transgene copies ($P$ <0.005), between DRL and DRLΔintra with two and four transgene copies ($P$ <0.001) and between DRLK371A and DRLΔintra with two and four transgene copies ($P$ <0.001)

| | Number of copies | Percentage of MBs | | | Number of MBs analysed |
|---|---|---|---|---|---|
| | | No lobes missing | Some lobes missing | All lobes missing | |
| DRL[a] | 2 | 77 | 23 | 0 | 64 |
| DRLK371A[b] | 2 | 72 | 28 | 0 | 61 |
| DRLΔintra[c] | 2 | 37 | 25 | 38 | 52 |
| DRL[d] | 4 | 17 | 78 | 5 | 101 |
| DRLK371A[e] | 4 | 0 | 84 | 16 | 43 |
| DRLΔintra[f] | 4 | 0 | 6 | 94 | 17 |

[a] DRL 2 copies: $GAL4^{c155}$, *UAS-mCD8GFP*/+; *UAS-drl(II)*/+; *UAS-drl(III)*/+
[b] DRLK371A 2 copies: $GAL4^{c155}$, *UAS-mCD8GFP*/+; *UAS-drlK371A(II)*/+; *UAS-drlK371A(III)*/+
[c] DRLΔintra 2 copies: $GAL4^{c155}$, *UAS-mCD8GFP*/+; +/+; *UAS-drlΔintra(IIIa)*, *UAS-drlΔintra(IIIb)*/+
[d] DRL 4 copies: $GAL4^{c155}$, *UAS-mCD8GFP*/+; *UAS-drl(II)*/*UAS-drl (II)*; *UAS-drl(III)*/*UAS-drl(III)*
[e] DRLK371A 4 copies: $GAL4^{c155}$, *UAS-mCD8GFP*/+; *UAS-drlK371A(II)*/*UAS-drlK371A(II)*; *UAS-drlK371A(III)*/*UAS-drlK371A(III)*
[f] DRLΔintra 4 copies: $GAL4^{c155}$, *UAS-mCD8GFP*/+; +/+; *UAS-drlΔintra(IIIa)*, *UAS-drlΔintra(IIIb)*/*UAS-drlΔintra(IIIa)*, *UAS-drlΔintra(IIIb)*

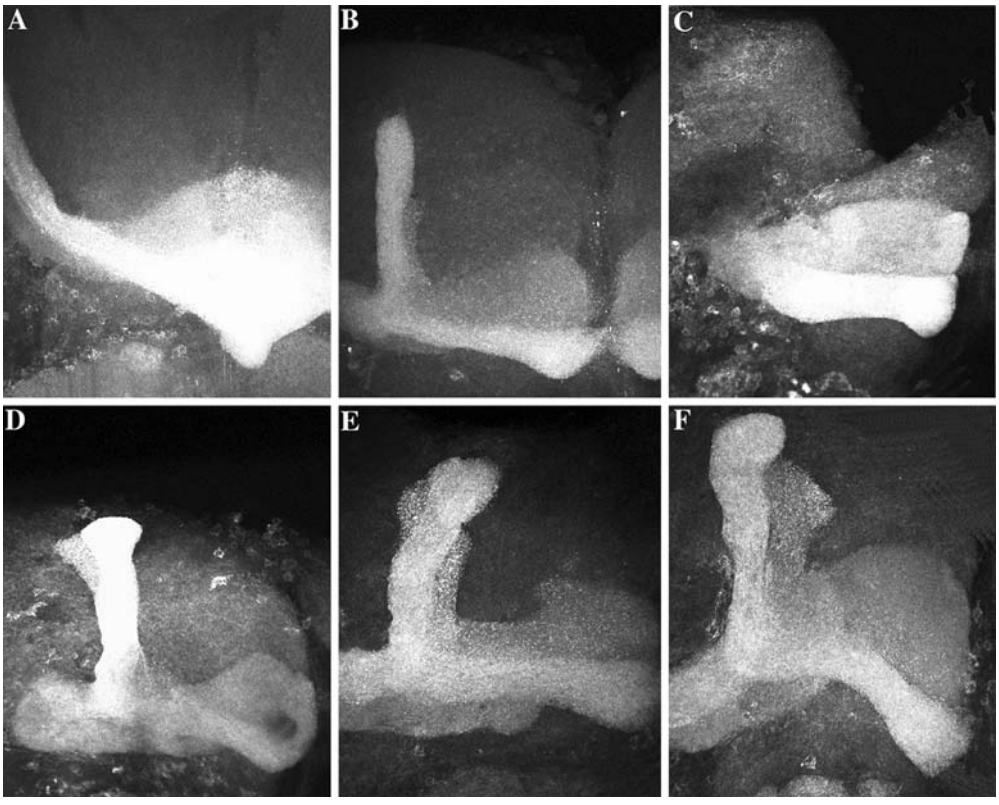


**Fig. 3** Composite confocal images of mushroom body (MB) phenotypes induced by the pan-neural expression of the wild-type and mutant LIO/DRL proteins in a *lio*/*drl* mutant background. **a** Null MB phenotype of *lio*/*drl* mutants with a complete loss of the dorsal lobes and a fusion of the median lobes over the midline. **b***lio*/*drl* mutants can display a mild MB phenotype with dorsal lobes shorter or thinner than normal and a partial fusion of the median lobes. Note that most of the "mild" phenotypes are closer to "null" phenotype than the one shown here. **c** In some cases, expression of the wild-type and mutant proteins does not rescue the mutant phenotype but leads to an MB phenotype identical to a gain-of-function phenotype (i.e. absence of some of the lobes: here, the α and α′ dorsal lobes are missing). **d–f** Complete rescue of the MB phenotype of *lio*/*drl* mutants by DRL, DRLK371A and DRLΔintra proteins, respectively

signalling (Yoshikawa et al. 2003). It will be of interest to determine whether Wnt signalling is also involved in MB development.

Yoshikawa et al. (2001) have previously shown that, in a dominant gain-of-function assay in the embryonic CNS and in a phenotypic rescue assay in the mesoderm, the DRLK371A mutant protein behaves exactly like the wild-type protein. Although our results confirm that the DRLK371A protein acts qualitatively like the wild-type LIO/DRL protein, we have also shown that this mutant protein is more active than the wild-type protein in inducing the gain-of-function phenotypes indicating a regulatory role for the kinase activity of the LIO/DRL RTK. Moreover, the same authors have recently shown that, in contrast with our results, a DRL protein lacking its intracellular domain (almost identical to the DRLΔintra mutant used in the present study) failed to induce gain-of-function phenotypes in the embryonic CNS (Yoshikawa et al. 2003). These results indicate that the LIO/DRL receptor functions differently in the embryonic CNS and during MB development raising the possibility that this diversity of mechanisms might extend to other members of the RYK subfamily of RTK.

**Table 3** Quantification of the rescue of *lio*/*drl* mushroom body (MB) phenotype by DRL, DRLK371A and DRLΔintra proteins. Effects of the pan-neural expression of two copies of *UAS-drl*, *UAS-drlK371A* or *UAS-drlΔintra* transgenes in a *lio*/*drl* mutant background were classified into four categories (Fig. 3) and quantified. Statistical analysis (Chi-square test) reveals significant effects of the three transgenes on the phenotypic distribution of *lio*/*drl* mutants ($P$ <0,001 for each transgene versus *lio*/*drl*) and significant differences between DRL and DRLK371A ($P$ <0.001) and between DRL and DRLΔintra ($P$ <0.001)

| | Percentage of MBs | | | | Number of MBs analysed |
|---|---|---|---|---|---|
| | Wild-type phenotype | Mild phenotype | Null phenotype | GOF phenotype | |
| *lio*/*drl*[a] | 0 | 18 | 82 | 0 | 51 |
| *lio*/*drl* + DRL[b] | 68 | 12 | 4 | 16 | 104 |
| *lio*/*drl* + DRLK371A[c] | 30 | 20 | 27 | 23 | 83 |
| *lio*/*drl* + DRLΔintra[d] | 19 | 3 | 3 | 75 | 64 |

[a] *lio*/*drl*: *GAL4*$^{c155}$, *UAS-mCD8DFP*/+; *lio*$^{2}$/*drl*$^{R343}$
[b] *lio*/*drl*+ DRL: *GAL4*$^{c155}$, *UAS–mCD8GFP*/+; *lio*$^{2}$/*drl*$^{R343}$; *UAS–drl(III)*/*UAS–drl(III)*
[c] *lio*/*drl* + DRLK371A: *GAL4*$^{c155}$, *UAS–mCD8GFP*/+; *lio*$^{2}$/*drl*$^{R343}$; *UAS-drlK371A(III)*/*UAS-drlK371A(III)*
[d] *lio*/*drl* + DRLΔintra: *GAL4*$^{c155}$, *UAS–mCD8GFP*/+; *lio*$^{2}$/*drl*$^{R343}$; *UAS-drlΔintra(IIIa)*, *UAS-drlΔintra(IIIb)*/+

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