Invertebrate Neuroscience, 1, 25-31 (1995)

# Immunocytochemistry of a novel GABA receptor subunit *Rdl* in *Drosophila melanogaster*

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ABSTRACT Following our recent cloning of a novel γ-aminobutyric acid (GABA) receptor subunit gene *Resistance to dieldrin* or *Rdl* from the cyclodiene resistance locus in *Drosophila melanogaster*, we were interested in defining its pattern of expression during development. Here we report the raising of an anti-*Rdl* polyclonal antibody that recognizes a single protein of the expected 65 kDa size in immunoblots of *Drosophila* head homogenates. *In situ* hybridization using *Rdl* cDNA probes and the anti-*Rdl* antibody shows that *Rdl* message and protein are highly expressed in the developing central nervous system (CNS) of 15–17 h embryos. Interestingly, despite the use of GABA in both the peripheral and CNS of insects, *Rdl* GABA receptor subunits appear to be confined to the CNS. Detailed immunocytochemistry of *Drosophila* brain sections showed particularly strong anti-*Rdl* antibody staining in the optic lobes, ellipsoid body, fan shaped body, ventrolateral protocerebrum and the glomeruli of the antennal lobes. Results are compared with the distribution of staining observed in the insect CNS with antibodies against GABA itself and synaptotagmin, a synaptic vesicle protein.

KEY WORDS: *Drosophila*; GABA receptors; insecticide resistance; cyclodiene insecticides; immunocytochemistry

## Introduction

γ-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in both vertebrates and invertebrates (Kuffler and Edwards, 1965; Usherwood and Grundfest, 1965; Otsuka *et al.*, 1966; Sattelle, 1990). In vertebrates the $GABA_A$ receptor is a complex hetero-multimer of different subunits that assemble in the postsynaptic membrane to form a chloride ion channel pore. A large number of $GABA_A$ receptor subunit types (α, β, γ, δ and ρ) and subtypes (e.g. α1–6) have now been identified and functional expression studies have shown that a number of different subunits need to be co-expressed in order to restore all the aspects of receptor pharmacology (Olsen and Tobin, 1990). These subunits show differential expression throughout the vertebrate nervous system (Olsen and Tobin, 1990), implying that their expression is coordinately controlled. While the number and distribution of vertebrate $GABA_A$ receptor subunits is well studied, the mechanisms underlying their spatial and temporal regulation are poorly understood. We are therefore interested in studying the pattern of expression of *Drosophila* GABA receptor genes as a prelude to a molecular genetic dissection of their regulation.

In contrast to the diversity of cloned subunits in vertebrates only two GABA receptor subunit genes have been cloned to date in *Drosophila*. One of these is a homolog of the vertebrate $GABA_A$ receptor β subunit (Sattelle *et al.*, 1992; Henderson *et al.*, 1993). The other GABA receptor subunit gene *Resistance to dieldrin* (*Rdl*) was cloned from a *Drosophila* mutant resistant to the GABA receptor antagonist picrotoxinin and to cyclodiene insecticides (ffrench-Constant *et al.*, 1991). Unlike vertebrate $GABA_A$ receptors the *Rdl* subunit forms highly functional GABA gated chloride ion channels as a homo-multimer in heterologous systems (ffrench-Constant *et al.*, 1993; Lee *et al.*, 1993; Chen *et al.*, 1994). Its sequence is divergent from that of any $GABA_A$ receptor subtype and shows somewhat higher amino acid identity to glycine receptors (ffrench-Constant and Rocheleau, 1993). This gene therefore appears to belong to a novel class of GABA receptor subunits.

We intend to use *Drosophila* as a model system to understand how GABA receptor genes are coordinately expressed in different tissues at different times through development. As the first step in this analysis we describe here the pattern of expression of the message and protein of the novel GABA receptor subunit *Rdl*. To our knowledge this is the first report of the distribution of a GABA receptor subunit in *Drosophila*.

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## Materials and Methods

### *In situ* hybridization of *Rdl* cDNA

*In situ* hybridization of the *Rdl* cDNA to whole-mount embryos was performed according to standard protocols (Ashburner, 1989), with the following modifications to the fixing procedure: formaldehyde was used in place of paraformaldehyde and the fixative buffer was 10% formaldehyde, 1x phosphate buffered saline (PBS) and 50 mM ethylenediamine tetra-acetic acid (EDTA). Digoxygenin-labeled cDNA probes were synthesized by random priming using the Genius Kit (Boehringer Manheim). Labeled probes were resuspended in PBS + 0.1% Tween 20 (PBT) and an equal volume of 100% deionized formamide, and hybridized at 45 °C in hybridization fluid (50% formamide, 5x SSC (0.75 M NaCl and 0.075 M sodium citrate pH 7), 100 µg/ml salmon sperm DNA, 50 µg/ml heparin and 0.1% Tween 20). Staining was performed using alkaline phosphatase conjugated anti-digoxygenin antibodies diluted 1:2000 in PBT, 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrozolium according to the manufacturer's instructions. Reactions were stopped in PBT and 50 mM ethylene glycol-bis-tetra-acetic acid (EGTA). Embryos were cleared in a glycerol gradient and mounted in 80% glycerol.

### *Rdl* antisera and Western blotting

The production of the *Rdl* fusion protein (378 amino acids of maltose binding protein and 202 of *Rdl*) in the bacterial expression vector pMAL-c2 and the raising of the rabbit polyclonal anti-*Rdl* antibody have been described previously (Lee *et al.*, 1993). For immunoblotting the anti-*Rdl* antiserum was purified using a maltose binding protein covalently attached to Affi-gel 10 or Affi-gel 15. The purified antiserum was used on immunoblots of *Drosophila* head homogenates (five heads per lane). Controls lacking the *Rdl* specific signal were developed by pre-incubating the anti-*Rdl* antibody with varying amounts (50 µg or 100 µg) of *Rdl* fusion protein.

### Preparation and immunostaining of *Drosophila* brain sections

Adult OregonR flies were anesthetized with $CO_2$, their heads dissected and fixed overnight at 4 °C in HistoCHOICE (Amresco Inc.). Fixed heads were then embedded in 2% agar/1% formalin. Prior to sectioning, tissues were washed four times for 15 min. at 37 °C in ethanol, twice for 15 min. at 37 °C in xylene, twice for 30 min. in 1x paraffin and then for 15 min. in 2x paraffin at 58 °C. The agar blocks were then embedded in paraffin and sectioned at 5 µm in a cryomicrotome. Sections were mounted directly onto Superfrost Plus glass slides (Fisher Scientific).

For immunocytochemistry sections were dewaxed (three times for 2 min. in xylene, three times for 2 min. in 100% ethanol, once for 2 min. in 80% ethanol and once for 2 min. in 70% ethanol), rehydrated for 5 min. in 1x PBS, incubated in 3% $H_2O_2$ for 2 min. and finally washed for 2 min. in 2x PBS. Blocking was performed in a moist chamber for 30 min. in blocking buffer (50 mM Tris pH 6.8, 150 mM NaCl, 0.5% NP 40 with 5mg/ml bovine serum albumin (BSA)). The anti-*Rdl* antiserum was then diluted 1:100 in incubation buffer (50 mM Tris pH 6.8, 150 mM NaCl, 0.5% NP 40, 1mg/ml BSA and 5% normal goat serum) and applied to the sections. Incubation with the antibody was performed for one hour in a moist chamber at room temperature. Controls included pre-immune serum used under the same conditions and prior incubation of 500 µl of the diluted anti-*Rdl* antibody with 10 µg or 20 µg of the purified *Rdl* fusion protein.

After incubation with the primary antibody, slides were washed twice for 10 min. in 1x PBS and then incubated for 30 min. with the secondary antibody (biotinylated goat anti-rabbit IgG, Vector Lab. Inc.) diluted 1:200 in incubation buffer. Unbound antibody was removed by washing twice in 1x PBS for 10 min. and sections were then incubated for 30 min. with a horseradish peroxidase (HRP)–streptavidin conjugate (Vector Lab. Inc.) diluted 1:200 in PBS. Finally, slides were washed twice for 5 min. in 1x PBS, equilibrated in 0.1M Tris pH 6.8 and stained with 0.5 mg/ml DAB and 30% $H_2O_2$ (diluted at 1:1000 in 0.1M Tris pH 6.8). Development of staining was monitored under a dissecting microscope.

## Results and Discussion

### Purification and analysis of the anti-*Rdl* polyclonal antibody

Following purification of the anti-*Rdl* polyclonal antibody by affinity chromatography using an immobilized maltose binding protein, a single protein of the expected size of 65 kDa was detected in immunoblots of adult *Drosophila* fly heads (Fig. 1(lanes 1–4)). This size is consistent with the predicted size of the 606 amino acid *Rdl* protein (ffrench-Constant *et al.*, 1991). This signal could be eliminated by previous incubation of the antibody with the *Rdl* fusion protein (Fig. 1(lanes 5–8)). These results confirm that the purified

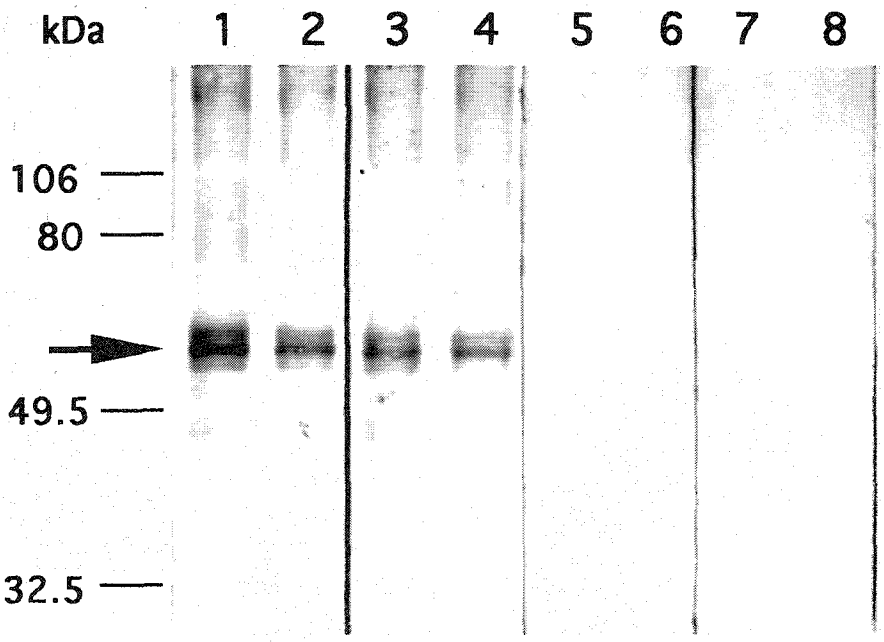


**Fig. 1. Immunoblots of *D. melanogaster* head homogenates probed with the anti-Rdl antibody previously purified over maltose binding protein attached to Affi-gel 10 (lanes 1–2) or Affi-gel 15 (lanes 3–4) columns.** *A signal corresponding to a protein of the predicted size of* Rdl *(65 kDa) is observed which can be blocked by pre-incubation of the anti-*Rdl *antibody with various concentrations (lanes 5–6, 50 μg and lanes 7–8, 100 μg) of the* Rdl *fusion protein.*

anti-*Rdl* antibody recognizes a protein of the correct size and that the *Rdl* fusion protein competes with this signal. This purified antiserum was therefore used for immunocytochemistry.

**Distribution of *Rdl* message and protein in the developing embryo**

The embryonic *Drosophila* central nervous system (CNS) consists of two anterior ganglia, the sub- and supraoesophageal ganglia and the ventral nerve cord. *In situ* hybridization of the *Rdl* cDNA to whole mount embryos revealed that the message for this novel GABA receptor subunit is distributed globally in the developing CNS in 15–17 h embryos (Fig. 2B–D). In contrast message appeared to be absent from 12–13 h or earlier embryos (Fig. 2A). Immunocytochemistry with the anti-*Rdl* antibody showed that the receptor protein is similarly expressed in the CNS of 17 h embryos (Fig. 2E–F). Staining was predominantly of the longitudinal commissures of the ventral cord and ganglia of the brain. This is interesting as the commissures are thought to be composed mainly of axons and are not rich in synapses. This staining may therefore be accounted for by GABA receptors present in vesicles in the commissures as they are being transported to the synapses. Detailed examination of the anterior embryo revealed intense staining of the supraoesophageal ganglia and the connecting frontal and supraoesophageal commissures (Fig. 2F). The results from the analysis of *Rdl* GABA receptor protein are therefore consistent with those from the distribution of the message. Results from the expression of both the message and protein illustrate that *Rdl* subunits are highly expressed throughout the developing CNS and that expression may start at stage 13 when neurons are condensing in the CNS (Campos-Ortega and Hartenstein, 1985).

**Immunocytochemistry of *Rdl* in the brain**

Despite no reports on the distribution of GABA receptors in *Drosophila*, extensive descriptions of GABA-like immunoreactivity (GLIR) have been reported from a number of insects, including *Drosophila* (Buchner *et al.*, 1988; Restifo and White, 1990), *Manduca sexta* (Hoskins *et al.*, 1986; Homberg *et al.*, 1987), the honey bee (Schäfer and Bicker, 1986), the housefly (Meyer *et al.*, 1986) and the locust (Watson and Laurent, 1990). The anti-*Rdl* antibody was used to examine in detail the expression of *Rdl* in sections of *Drosophila* brain and we have compared these results on GABA receptor distribution with those obtained from GLIR in this and other insects. Frontal sections highlight intense staining in the suboesophageal ganglia, pedunculus and antennal lobes (Fig. 3A–B), while horizontal sections illustrate staining in the ventrolateral protocerebrum, the medulla, lobula and lobular plates, and the ellipsoid and fan shaped bodies (Fig. 3C–D). The horizontal section shown in Fig. 3(D) also shows intense staining in the spheroidal glomeruli of the antennal lobes. These correspond to the sites of numerous synaptic contacts between the receptor cells, local interneurons and the output relay neurons (Matsumoto and Hildebrand, 1981). The intense line of staining at the base of the ventrolateral protocerebrum (Fig. 3D) is extremely interesting and has not been previously described (L. Restifo and N. Strausfeld, personal communication). These results are consistent with distribution of anti-GABA antibody staining in *Drosophila*, except for our observation of *Rdl* receptor staining in the fan-shaped body which

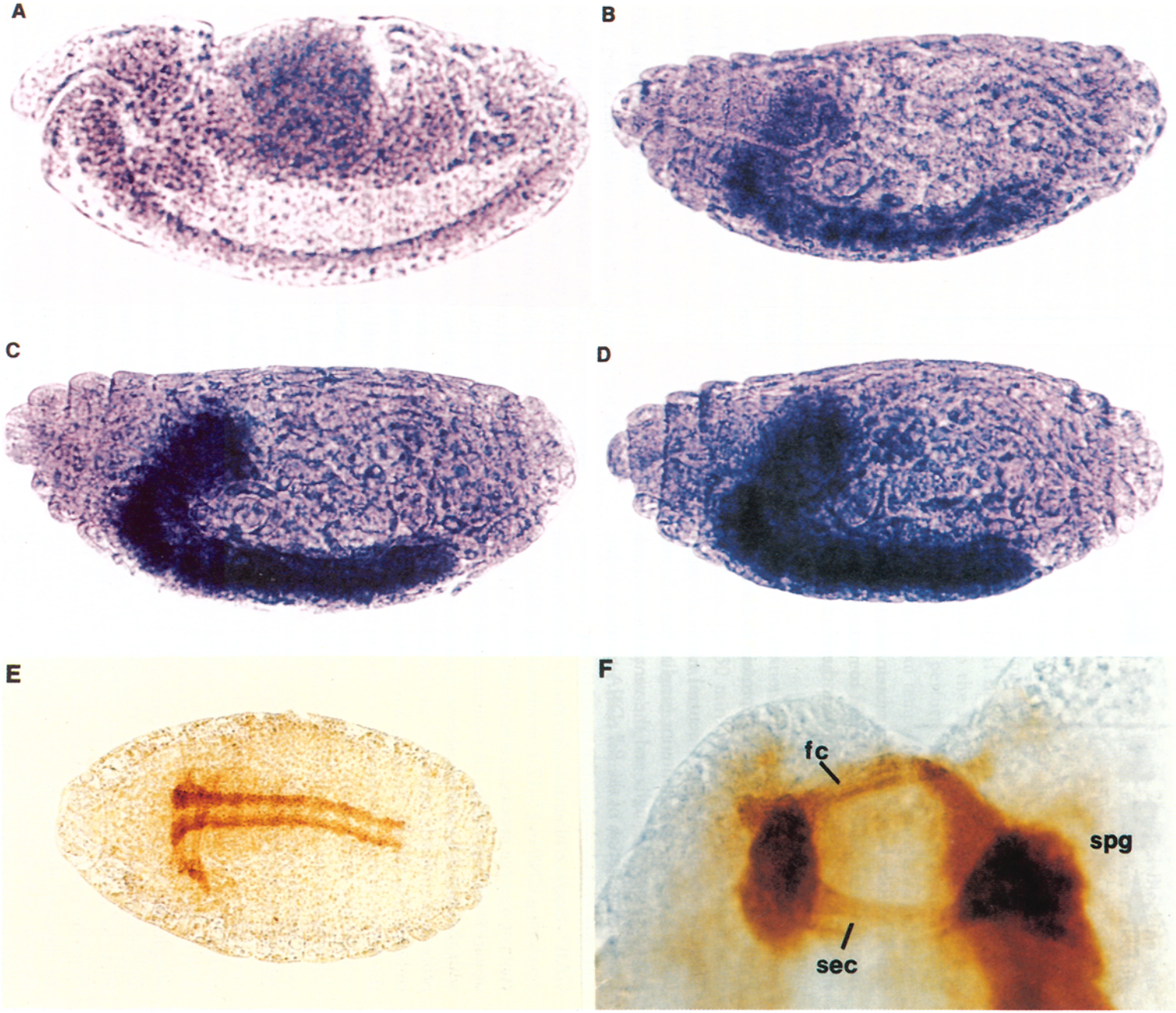


**Fig. 2. Pattern of expression of *Rdl* message (A–D) and protein (E–F) in the developing *Drosophila* embryo.** *A–D: Whole mount in situ hybridization of embryos using a digoxygenin labelled* Rdl *cDNA probe. A: Lateral view of 12–13 h embryo (stage 12) with no staining. B–D: Lateral views of 15, 16 and 17 h embryos (stages 14–16) showing increasing global staining of the developing central nervous system (CNS). E–F: Whole mount embryo staining of anti-*Rdl *antibody. E: Ventro-lateral view of 17 h embryo showing* Rdl *protein distribution in the CNS. Labeling is predominantly of the longitudinal commissures of the ventral cord and ganglia of the brain. F: Detailed view of the anterior of a 17 h embryo. Supra-oesophageal ganglia (spg) connected by the frontal commissure (fc) and the supra-oesophageal commissure (sec).*

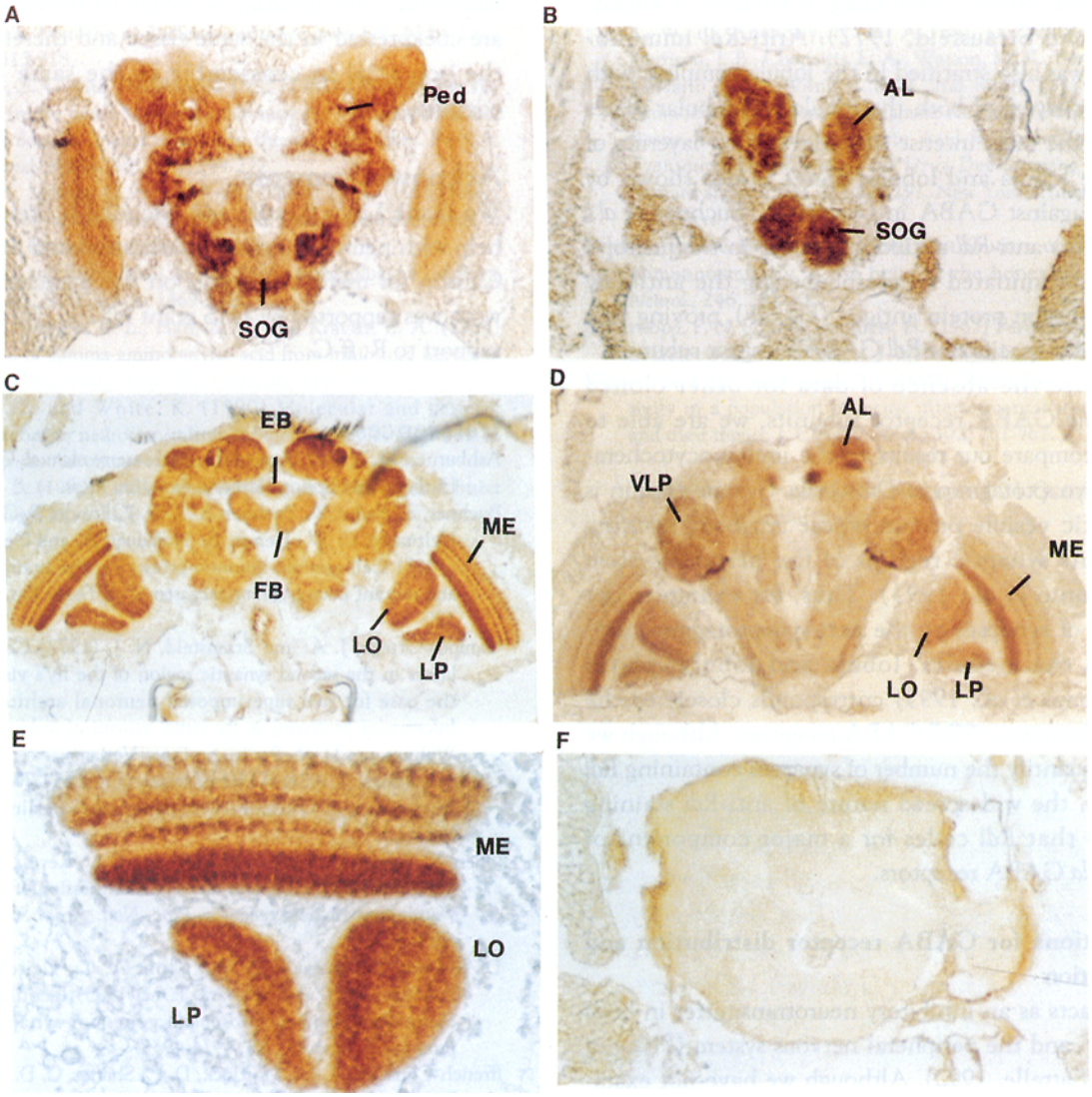


**Fig. 3. Sections showing the distribution of anti-Rdl antibody staining in the brain of *D. melanogaster*.** *A: Frontal sections, showing the sub-oesophageal ganglia (SOG) and pedunculus (Ped). B: Frontal section (anterior), showing the SOG and the antennal lobes (AL). C–D: Horizontal sections (C: dorsal and D: ventral) showing the lobular (LO), lobula plates (LP), medulla (ME), ellipsoid body (EB), fan shaped body (FB), ventrolateral protocerebrum (VLP) and the AL. Note the intense staining of the glomeruli of the AL and the base of the VLP in section D. E: Detail of the second order optic neuropil, showing the LO, LP and ME. Several distinct layers of immunoreactive staining can be seen in the ME. F: Control section preabsorbed with purified* Rdl *fusion protein (see text) showing the absence of specific staining. No staining was observed in the retina or the first order optic neuropil which are therefore cropped from the sections in order to increase detail.*

contrasts with the lack of anti-GABA immunoreactivity previously reported in this structure (Buchner *et al.*, 1988).

Fig. 3E is a higher magnification view of the visual system. This complex consists of a series of optic neuropiles rich in synapses; the lamina, which contains most of the terminals of the photoreceptor cells; the medulla, which corresponds to the next stage in visual processing; and the lobula and lobular plates, which relay the signal to the other portions of the supra- and suboesophageal ganglia and thoracic neuropil. No expression of *Rdl* protein was observed in the peripheral retina or lamina (not shown), although anti-GABA immunoreactivity has been reported from the lamina of *Drosophila* (Buchner *et al.*, 1988; Restifo and White, 1990) and *Manduca* (Homberg *et al.*, 1987). The medulla shows seven distinct layers of anti-*Rdl* immunostaining. Differing layers of anti-GABA immunoreactivity have previously been identified in the medulla of insects; five in *Drosophila* (Buchner *et al.*, 1988), seven in the moth *Manduca sexta* (Homberg *et al.*, 1987) and nine in the honey bee (Schäfer and Bicker, 1986). However, the layers of anti-*Rdl* staining in the *Drosophila* medulla reported here correspond

more closely to the strata dense in synapses (Campos-Ortega and Strausfeld, 1972). Anti-*Rdl* immunostaining was also stratified in the lobula complex, with the inner layers of both the lobula and lobular plates showing the most intense staining. Similar layering of both the lobula and lobular plates is also shown by antisera against GABA in *Drosophila* (Buchner *et al.*, 1988). This anti-*Rdl* antibody staining in the neuropil could be eliminated by preincubating the antibody with the fusion protein antigen (Fig. 3F), proving that the signal is specific for *Rdl* GABA receptor subunits.

Despite the absence of data for other cloned *Drosophila* GABA receptor subunits, we are able to further compare our results to the immunocytochemistry of synaptotagmin in *Drosophila*. Synaptotagmin is a synaptic vesicle protein whose antibody staining highlights synaptic regions of the nervous system (DiAntonio *et al.*, 1993). Thus, for example, the staining of synapses by the anti-synaptotagmin antibody in the medulla, lobula and lobular plates (DiAntonio *et al.*, 1993) corresponds closely to the observed pattern of *Rdl* GABA receptors. Although we cannot quantify the number of synapses containing *Rdl* subunits, the widespread nature of anti-*Rdl* staining suggests that *Rdl* codes for a major component of *Drosophila* GABA receptors.

### Implications for GABA receptor distribution and composition

GABA acts as an inhibitory neurotransmitter in both the CNS and the peripheral nervous system (PNS) of insects (Sattelle, 1990). Although we have not examined the adult PNS for anti-*Rdl* staining, the apparent absence of *Rdl* expression in the embryonic PNS is extremely interesting and suggests that *Rdl* receptor subunits, at least in the embryo, function solely in the CNS. It can therefore be inferred that any GABA receptors present in at least the embryonic PNS must contain other subunits, perhaps homologs of vertebrate $GABA_A$ receptors, such as the *Drosophila* β subunit (Sattelle *et al.*, 1992; Henderson *et al.*, 1993).

To date the pattern of expression of the second GABA receptor gene cloned from *Drosophila*, the $GABA_A$ β subunit homolog, has not been reported. We are therefore currently unable to speculate whether *Rdl* coassembles with this or other subunits in the CNS. However, the precedent for GABA receptor composition formed from the study of vertebrate $GABA_A$ receptors is of complex hetero-oligomers resulting from the coassembly of a number of different subunits coded for by different genes (Olsen and Tobin, 1990). We will therefore compare the pattern of expression of other cloned GABA receptor genes, when they become available, with that described here for *Rdl*, in order to observe if they are coexpressed in the same tissue and therefore have the potential to coassemble in the same receptor complex.

## Acknowledgements

We thank Linda Restifo and Nicholas J. Strausfeld for help with neuroanatomical localization and Stanley D. Carlson for useful comments on the manuscript. This work was supported by PHS grant NS29623 and Hatch support to R. ff-C.

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Accepted 21 October 1994