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Krüppel, a *Drosophila* segmentation gene, participates in the specification of neurons and glial cells

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Abstract

We report that the *Drosophila* segmentation gene *Krüppel* (*Kr*) is expressed in neural precursor cells, neurons and glia cells at different stages of neurogenesis and that *Kr* mutants develop aberrant peripheral (PNS) and central (CNS) nervous systems. Expression derived from a *Kr* minigene rescues the segmentation defects but these embryos continue to lack most of the neural *Kr* activity. Phenotypic analysis of the rescued embryos indicates that, in addition to overall effects on the PNS and CNS structure via its segmentation role, *Kr* expression in the nervous system is functionally required for establishing particular neural and glial fates.

Keywords: Drosophila neurogenesis; Transcription factor; Glia cell differentiation; Minigene rescue; Serotoninergic neurons

1. Introduction

Genetic and molecular analyses have identified an elaborate cascade of segmentation genes which generate the pre-pattern of the *Drosophila* body within the single-layered epithelium of the blastoderm (reviewed in Pankratz and Jäckle, 1993). Shortly thereafter, during germ band extension, the process of neurogenesis begins. Founder cells of the central nervous system (CNS), the neuroblasts, originate from a sheet of neuroectodermal cells. Two classes of genes that participate in this process have been identified in *Drosophila*. The 'proneural' genes provide neuroectodermal cells with the competence to become neural precursors and the activity of the 'neurogenic' genes restricts this competence to a single cell within a proneural cluster (reviewed by Campos-Ortega, 1993). After delamination the neuroblasts begin to divide asymmetrically to eventually give rise to about 200 neurons per hemisegment (Hartenstein and Campos-Ortega, 1984; Hartenstein et al., 1987). At the time of germ band retraction neurons differentiate to produce specific neuronal types such as motorneurons, interneurons and neurosecretory neurons and to initiate axonogenesis (reviewed by Goodman and Doe, 1993).

Candidates for genes involved in the establishment of neural fates include members of the group of segmentation genes. The early activity of pair-rule genes controls the initial patterns of proneural gene expression (Martin-Bermudo et al., 1991; Skeath et al., 1992). Subsequently, the activity of segment polarity genes, for example *wingless* (Chu-LaGraff and Doe, 1993) or *gooseberry* (*gsb*) (Skeath et al., 1995) is important for the correct specification of individual neuroblast fates. Members of the pair-rule and segment polarity class of segmentation genes are later expressed in specific subsets of neuronal precursor cells and neurons (Carroll and Scott, 1985; Hiromi et al., 1985; Macdonald et al., 1986; Baumgartner et al., 1987; Frasch et al., 1987; Duffy et al., 1991). This suggests that pair-rule and segment polarity genes may be re-employed to control specific cell fates during neural development. Indeed, such a role has been already demonstrated for *fushi tarazu* (*ftz*), *even-skipped* (*eve*), *runt* and *gsb* (Doe et al., 1988a,b; Duffy et al., 1991; Gutjahr et al., 1993). In addition to pair-rule and segment polarity genes, gap genes such as *hunchback* (*hb*), *knirps* (*kni*) and

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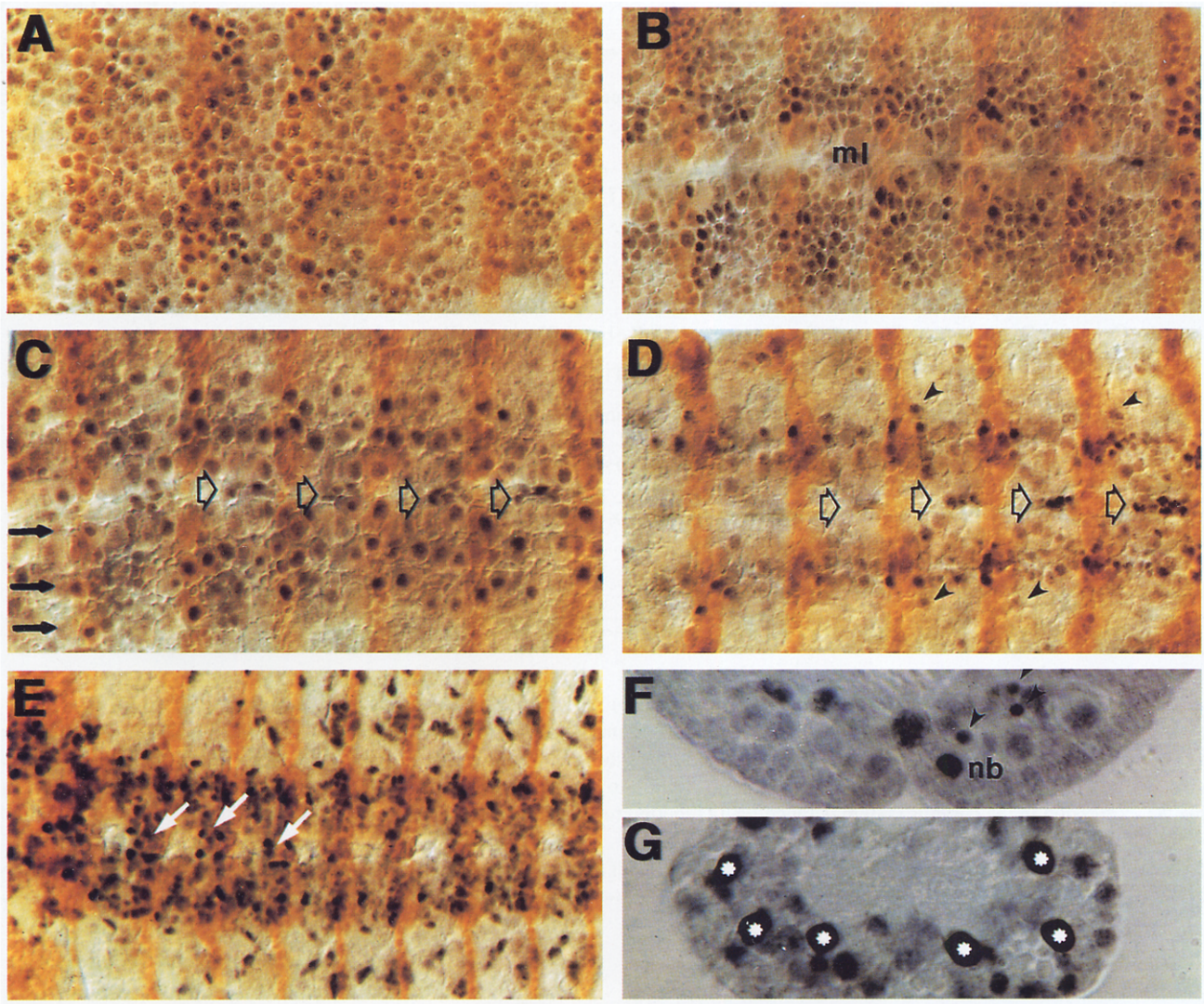


Fig. 1. Evolution of *Kr* expression in the CNS of wild type embryos. Anterior is left unless otherwise indicated. (A–E) Embryos double stained with anti-*Kr* (dark brown) and anti-En (orange-brown) antibodies. (F,G) Sections of the ventral region of embryos stained with anti-*Kr* antibody. In the neuroectoderm of stage 8 (A) and 9 (B) embryos, *Kr* expression undergoes dynamic changes; i.e. the midline (ml) in (B) has ceased to express *Kr* as compared with (A). Optical sections focusing on the neuroblast layer of a late stage 10 (C) and stage 11 (D) embryo reveal that *Kr* expression is at different levels in the individual neuroblasts. This is most clearly seen in (C) where the neuroblasts are organised in three rows (arrows). There is also transient expression in a subset of the midline precursors (hollow arrows in C,D), and *Kr* is also found in the precursor of the longitudinal glia cells located behind the *en* stripe (arrowheads, D; see also Fig. 7C). The pattern of *Kr* expression in the CNS of stage 15 embryos (E) is only detected in a subset of lineages. The medial most cell body glia cells (MM-CBG) is strongly labelled (arrows, see also Fig. 7A,B). They comprise four cells in the thoracic segments and two cells in every abdominal segments. They have been reported previously as VUM's support cells (Klämbt et al., 1991). (F,G) Cross-sections through embryonic CNS of embryos at stage 12 and 15, respectively, stained with anti-*Kr* antibody. Dorsal is up. (F) *Kr* expression in neuroblasts (nb) and ganglion mother cells (arrowheads). (G) Note the strongly labelled cells that correspond, according to size, shape and position, to the MM-CBG (white asterisk).

Krüppel (*Kr*), which act at the first level of the zygotic segmentation gene cascade (reviewed by Pankratz and Jäckle, 1993), are also expressed in the developing CNS. However the function of gap genes during neural development has not yet been revealed.

The gap gene *Kr* encodes a zinc finger transcription factor (Rosenberg et al., 1986). It is expressed in the central region of the syncitial blastoderm and in a cap at the posterior of the embryo during late cellular blastoderm (Knipple et al., 1985; Gaul et al., 1987a). Expression of *Kr* in these domains is necessary to establish the thoracic and anterior abdominal segments and the kidney-like excretory organ, termed Malpighian tubules, respectively (Wieschaus et al., 1984; Hoch et al., 1994). Here, we show that *Kr* is also expressed during neural development. Our results indicate that the absence of *Kr* activity affects the overall architecture of the nervous system via its segmentation function and that the correct specification of distinct neurons and of a subset of glial cells require *Kr* expression in the developing CNS.

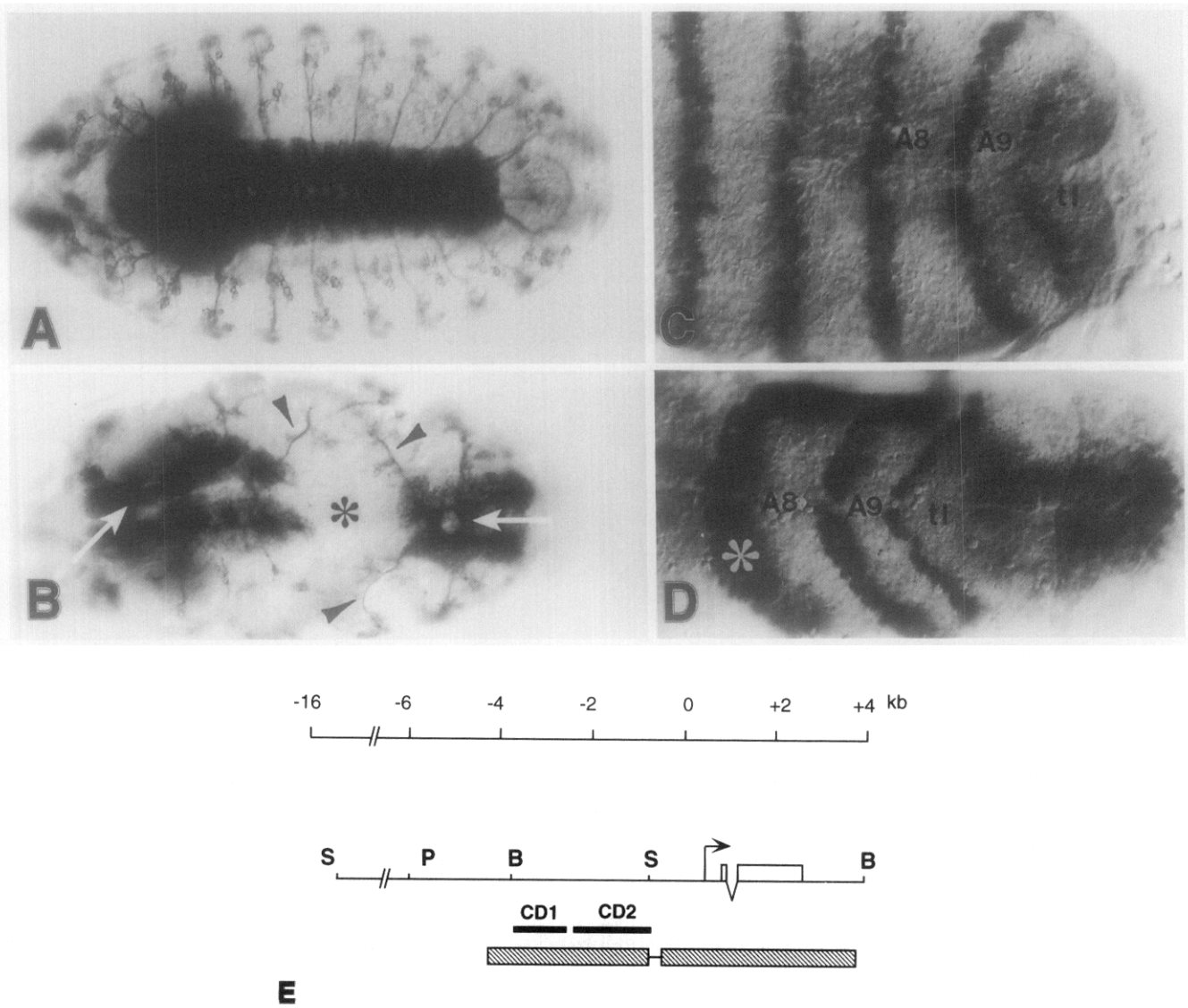


Fig. 2. Nervous system defects in *Kr* mutant embryos and construction of the Kr^{CD+} minigene. (A,B) Double staining with mAbs BP102 and BP104 in wild type (A) and Kr^{1} (B) embryos. The CNS in the mutant (B) is interrupted in the central region (asterisk) and the commissures in the remaining neuromers are fused (white arrows). The PNS is almost absent. The few remaining axons show abnormal patterns (arrowheads). (C,D) Stage 10 embryos. Posterior abdominal segments of wild type (C) and Kr^{1} (D) embryos stained with anti-Engrailed antibodies. Note that in Kr^{1} embryos the region corresponding to the non-segmented posterior terminal region (tl) is enlarged and that the posterior compartment of A7 is marked by an enlarged *en* stripe (asterisk); anterior pole is to the left. (E) Diagram representing the DNA containing the *Kr* enhancer elements and the *Kr* coding sequence used in the cloning of the Kr^{CD+} minigene. The bold line indicates the genomic DNA with the *Kr* coding sequences depicted as white boxes. The black boxes marked CD1 and CD2 represent the enhancer elements required for expression in the central domain of the blastoderm. Hatched boxes show the cloned region in the *Kr* minigene. Restriction sites: B, *Bam* HI; P, *PstI;* S, *Sal*I. The scale along the top of the diagram indicates the length of the *Kr* genomic region in kilobases; 0 is the start of transcription indicated by an arrow.

2. Results

2.1. *Kr expression in the wild type nervous system*

Kr is initially expressed at the blastoderm stage in two domains, one at the center of the embryo and one at the posterior cap which includes the anlagen of the Malpighian tubules (Gaul et al., 1987a). Subsequently, *Kr* begins to be expressed along the entire neuroectoderm during stage 8, when the first proneural clusters become resolved (Cabrera et al., 1987; Romani et al., 1987; Martin-Bermudo et al., 1991; Skeath and Carroll, 1992). This pattern of neuroectodermal expression of *Kr* persists until late stage 11. The spatio-temporal pattern of neuroectodermal *Kr* expression is heterogeneous, that is, certain areas accumulate higher levels of protein than others and the cells with highest expression vary with time (Fig. 1A,B). Likewise most neuroblasts express *Kr*, although the levels also vary, until at least stage 11 (Fig. 1C,D). *Kr* is also found in a large fraction of the ganglion mother

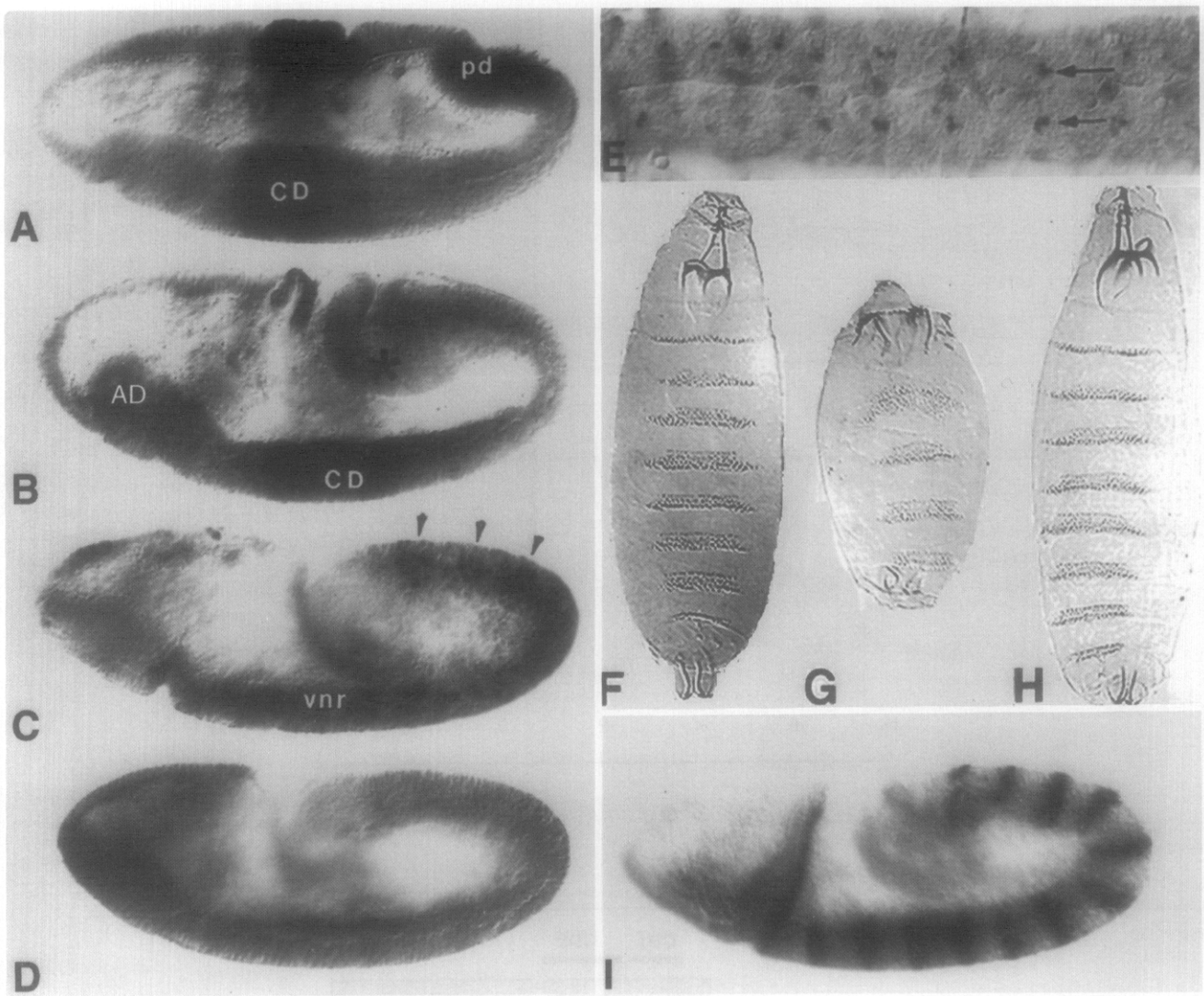


Fig. 3. Segmentation rescue of Kr^1 mutant embryos by the Kr^{CD+} transgene. Anterior pole is left unless otherwise indicated. Anti-*Kr* antibody staining of wild type (A,C) and Kr^1Kr^{CD+} (B,D,E,I) embryos. (A) Stage 7 embryo showing the central domain (CD) of *Kr* expression. The posterior domain (pd), which includes the anlagen of the Malpighian tubules, appears at this stage. (B) In a late stage 8 embryo *Kr* expression in the dorsal region of the CD begins to vanish. However the posterior domain, that at this time should be well established, is absent in Kr^1Kr^{CD+} embryos (asterisk). (C,D) After the central domain of expression has disappeared in an early stage 9 embryo, *Kr* protein accumulates broadly (C, arrowheads) in the ventral neurogenic region (vnr) while it is absent from stage 9 Kr^1Kr^{CD+} embryos (D). There is also some expression in the anterior region (AD). (E) Residual *Kr* expression in the nerve cord of a stage 15 Kr^1Kr^{CD+} embryo (arrows). (F,G,H) Cuticle preparations of wild type (F), Kr^1 (G) and Kr^1Kr^{CD+} (H) larvae showing that the segmentation pattern of *Kr* mutant embryos is fully restored in Kr^1Kr^{CD+} larvae. (I) The expression of *en* is detected in 14 stripes in stage 9 Kr^1Kr^{CD+} embryos as occurs in wild type (not shown) indicating that segmentation is restored.

cells (GMCs) and in their neuronal and glial progeny (Fig. 1E–G) where it persists until the end of embryogenesis.

2.2. *Lack of Kr activity causes multiple CNS and PNS defects*

Kr lack-of-function mutant embryos exhibit strong neural aberrations. They are not confined to the thoracic and anterior abdominal region affected by the absence of *Kr* segmentation function (Fig. 2A,B) (Nüsslein-Volhard and Wieschaus, 1980; Wieschaus et al., 1984; Preiss et al., 1985), but are detected also in the gnathal and three last abdominal segments. In the central region, CNS neuromers and PNS sensory organs are strongly affected or even absent. Anterior and posterior to the central domain strong disruptions and alterations of axonal pattern are observed, suggesting a specific requirement of *Kr* during neural development. However, the variability in the width of the posterior segments revealed by *en* staining (compare Fig. 2C,D) indicates that the early process of metamerisation is affected throughout the *Kr* mutant embryos and not only in the central domain. Therefore, to determine whether the neural defects are solely a conse-

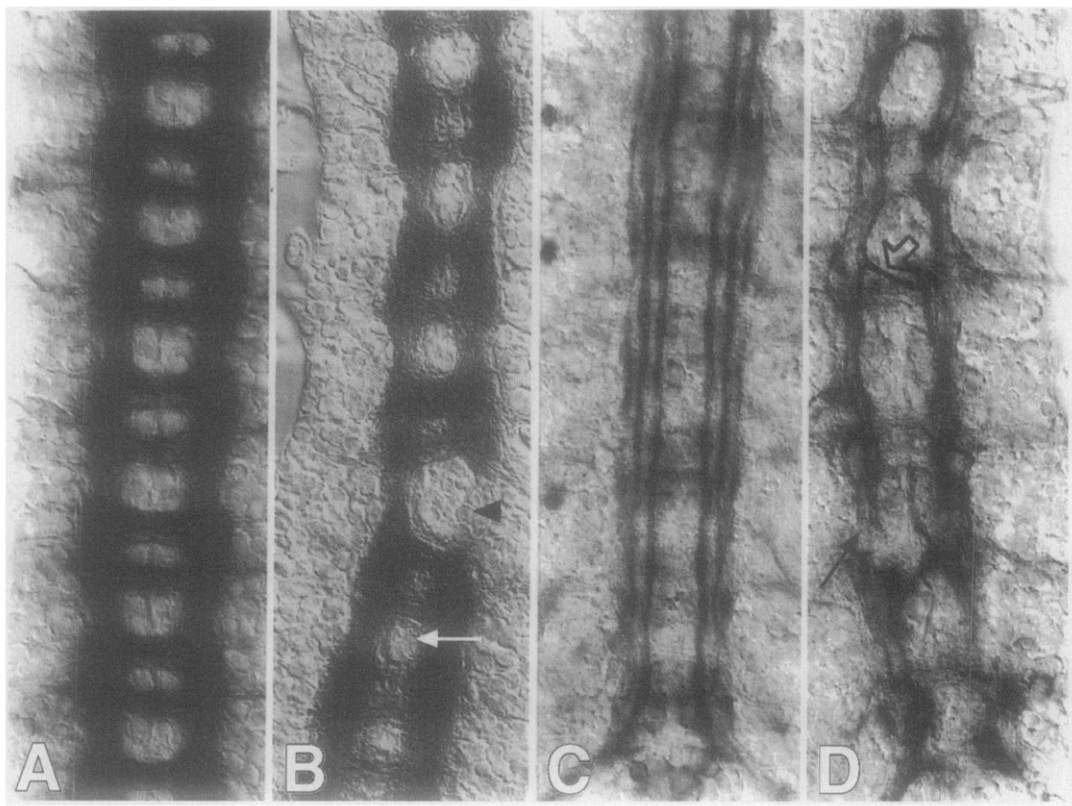


Fig. 4. Axonal pattern defects in Kr^1Kr^{CD+} embryos. Anterior is up. Flat preparations of wild type (A,C) and Kr^1Kr^{CD+} embryos (B,D) stained with mAbs BP102 (A,B) and 1D4 (anti-Fasciclin II) (C,D). (A,B), Nerve cords of stage 16 embryos. The space between the longitudinal tracts is narrowed in the Kr^1Kr^{CD+} embryos (white arrow in B) and the longitudinal connectives are thinner and disconnected at some points (black arrowhead). (C,D) Axons expressing Fas II appear very disrupted in Kr^1Kr^{CD+} embryos (black arrow); in some cases they cross the midline to fasciculate contralaterally (hollow arrow).

quence of the segmentation defects or whether they are also influenced by the lack of *Kr* expression in the developing nervous system, we used a *Kr* minigene to rescue the segmentation defect.

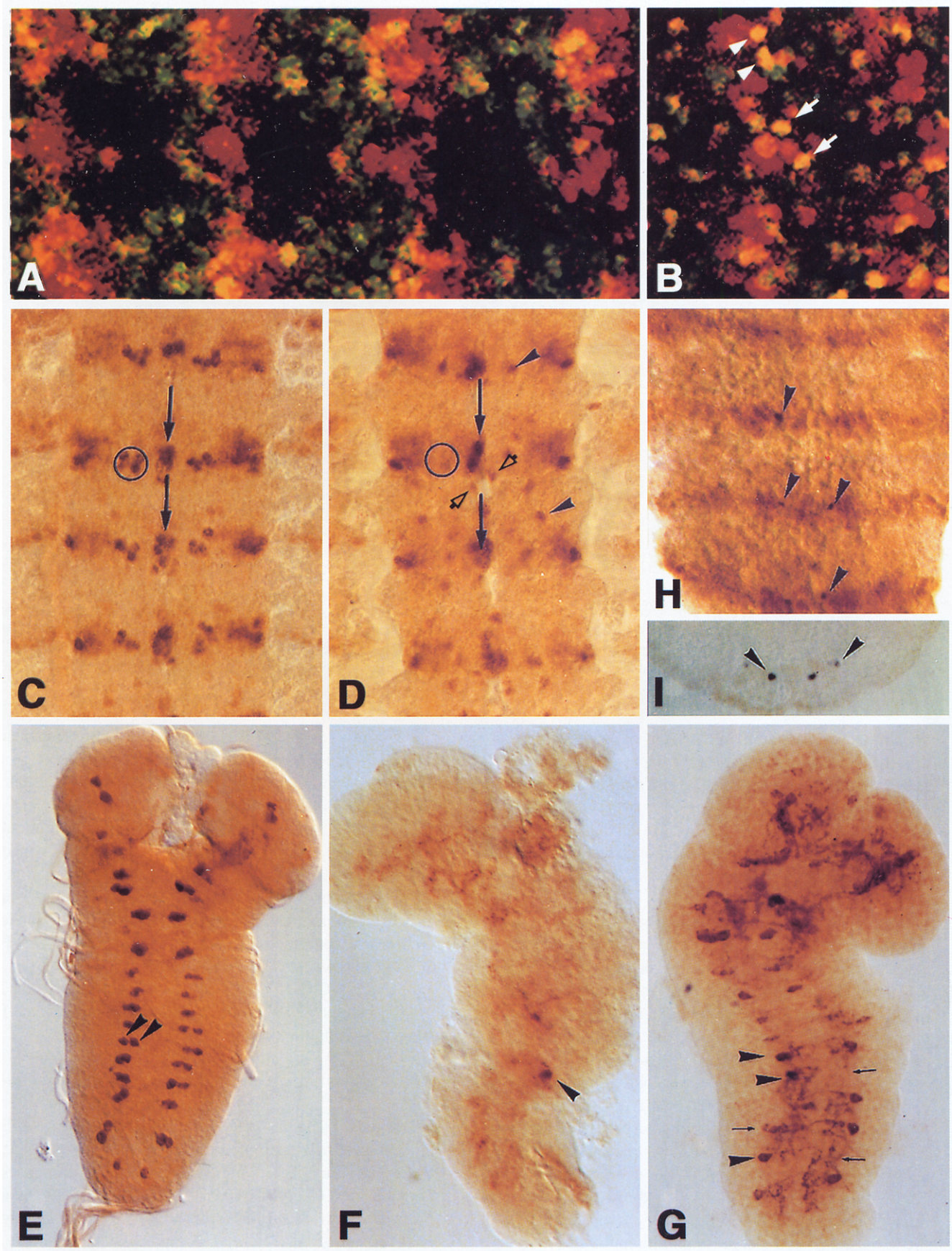
2.3. PNS defects and variable sized neuromers depend on Kr segmentation function

The Kr^{CD+} transgene contains the *Kr* basal promoter and the cis-acting regulatory sequences required for *Kr* expression in the central region of the blastoderm embryo (Fig. 2E) (Hoch et al., 1990). It also contains some sequences for expression in the anterior domain (see an example in Fig. 3E) and larval photoreceptor organ (not shown) (Schmucker et al., 1992). One Kr^{CD+} transgene insertion, mapping to the second chromosome, was recombined to the lack-of-function mutation Kr^1 (Wieschaus et al., 1984), giving rise to the Kr^1Kr^{CD+} genotype. Homozygous Kr^1Kr^{CD+} blastoderm embryos show normal *Kr* expression in the central domain (Fig. 3A,B) but they lack the early *Kr* expression in the nervous system (Fig. 3C,D). These embryos also lack most of the late aspects of *Kr* expression although, due to not yet delimited cis-acting sequences within the proximal part of the *Kr* promoter (Jacob et al., 1991), they show transgene-dependent *Kr* expression in a few GMCs at late stage 11 (see below) and in a small number of scattered neurons at later stages (Fig. 3E).

The early pattern of *en* expression and the cuticular phenotype (Fig. 3F–I) as well as the position of sensory organs of the PNS (not shown) show that segmentation in Kr^1Kr^{CD+} embryos is normal. However, the overall pattern of commissures and connectives in the CNS of late embryos is abnormal (Fig. 4A,B). Staining with anti-Fas II antibody reveals apparent stalling and misrouting of axons (Fig. 4C,D). These findings suggest that neural *Kr* activity, not provided by the *Kr* transgene, is required for normal development of the CNS.

2.4. Kr mutants fail to establish particular neuronal fates

To trace back the late neural phenotype of Kr^1Kr^{CD+} embryos we first examined the overall neuroblast pattern using anti-Hunchback antibodies as a neuroblast marker (Jiménez and Campos-Ortega, 1990). Although *Kr* is expressed in most neuronal precursors, Kr^1Kr^{CD+} embryos have an apparently normal complement of neuroblasts (data not shown). Therefore, to assess possible defects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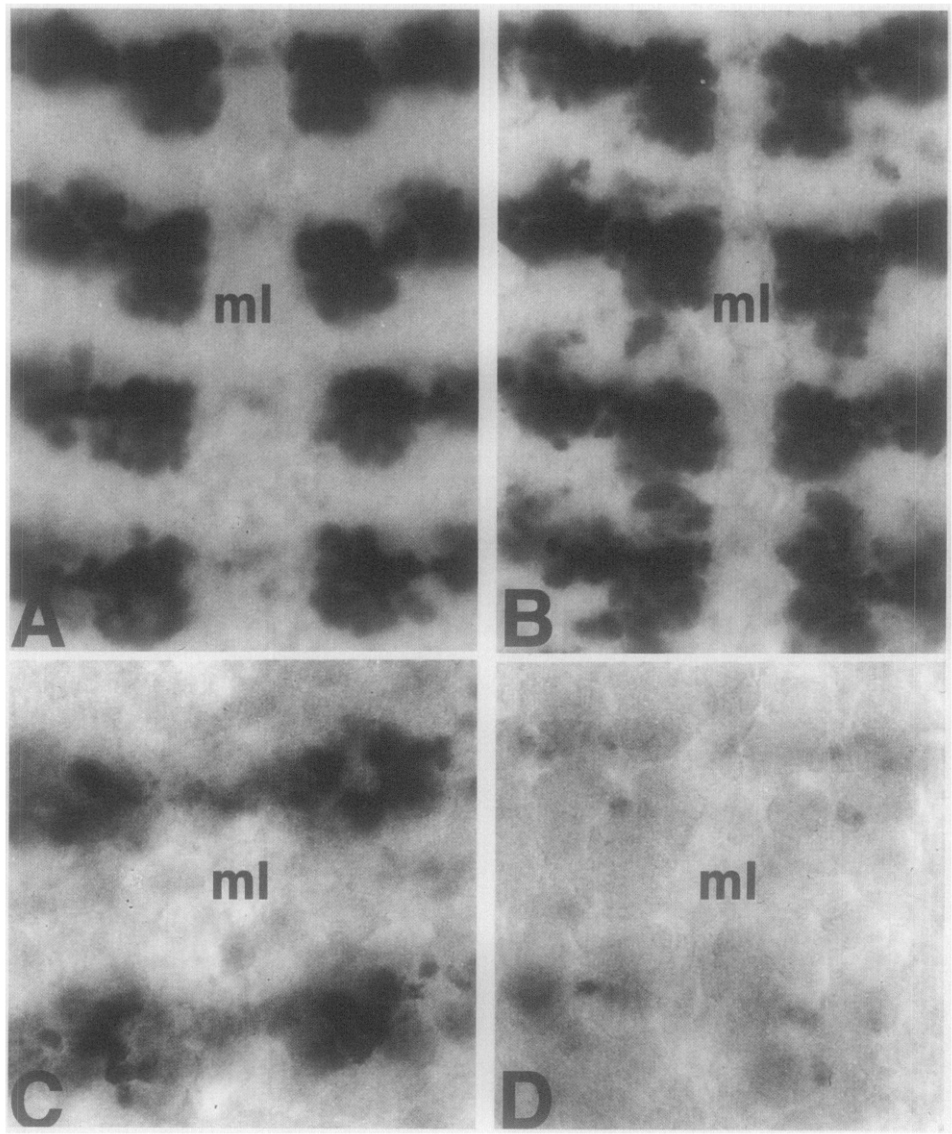


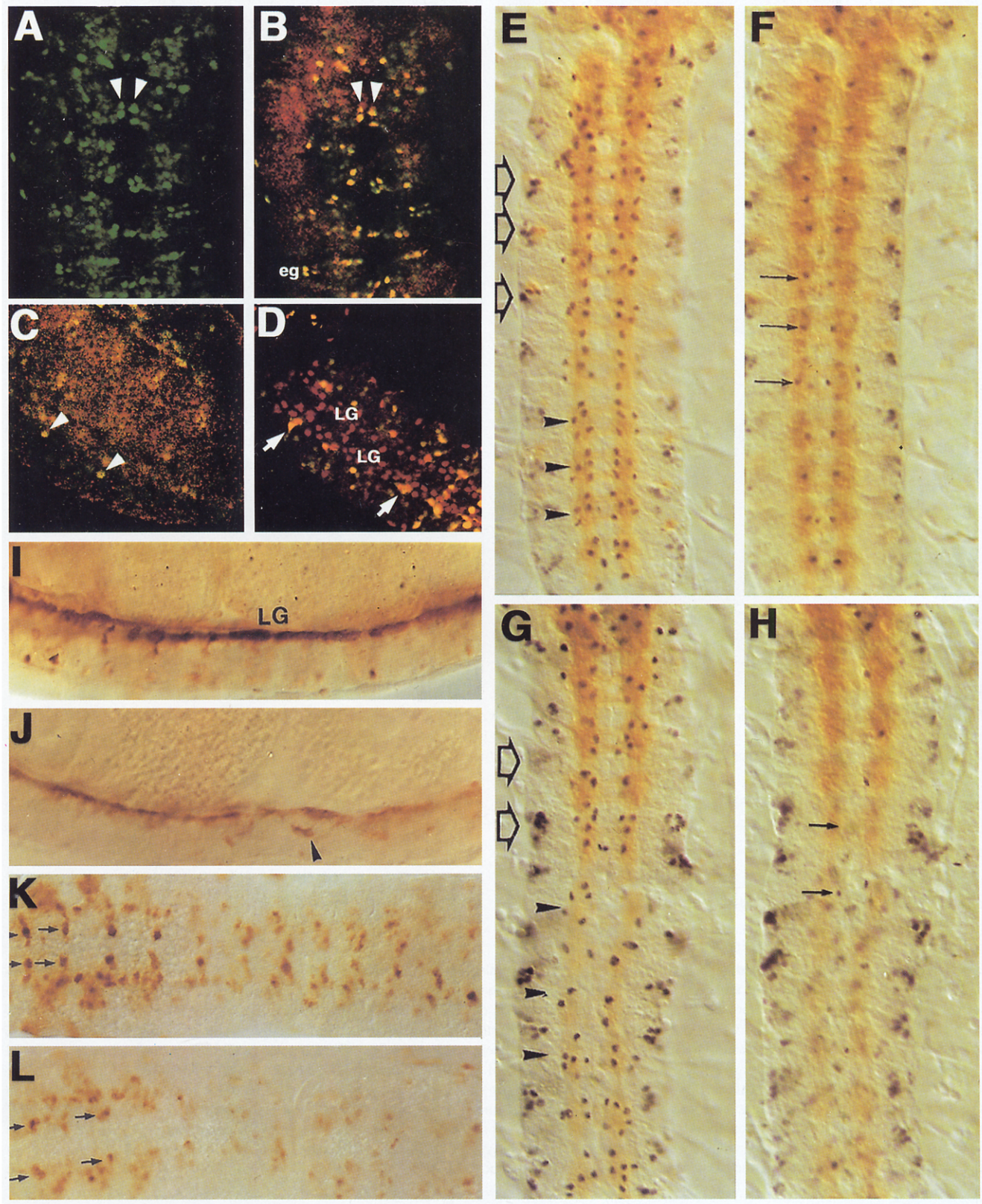
Fig. 6. Expression pattern of Gsb-n in Kr^1Kr^{CD+} embryos and heat-shock *Kr* embryos. Flat preparations of stage 13 wild type (A) and Kr^1Kr^{CD+} (B) embryos and late stage 11 wild type (C) and P(hs *Kr*) (D) embryos stained with anti-Gsb-n antibody. (A,B) show the three thoracic and two first abdominal segments; (C,D) the three thoracic segments. Abbreviation: ml, midline.

occurring in the neuroblast progeny, we examined the location and identity of different *Kr*-expressing neurons and glia cells.

Kr is coexpressed with *en* in a subset of neurons and glia which include the medial-lateral cluster of *en*-expressing neurons and the dorsal channel glia cells (Fig. 5A,B) (Patel et al., 1989b; Ito et al., 1995). In Kr^1Kr^{CD+} embryos the medial-lateral cluster is either absent or fails to express *en*, but the dorsal channel glia cells are not affected (Fig. 5C,D). The medial-lateral cluster of *en*-

Fig. 5. Alteration of *en* and serotonin expression patterns in Kr^1Kr^{CD+} embryos. Anterior is up unless indicated. (A,B) Anti-*Kr* (green) and anti-en (red) antibody stainings in wild type embryos at stage 13 (A) and 15 (B) as visualized by confocal microscopy. Cells coexpressing both proteins are seen in yellow. In (B) note the serotoninergic neurons (arrowheads) and the dorsal channel glia cells (arrows). Anterior is left. (C,D) Flat preparations of stage 15 wild type (C) and Kr^1Kr^{CD+} (D) embryos stained with anti-engrailed antibodies; the two last thoracic and the two first abdominal segments of the CNS are shown. The medial-lateral cluster which contains serotoninergic neurons (circle) is undetected in the mutant. Instead, some scattered *en*-positive cells can be observed (arrowheads). The cluster of the MNB and its progeny (arrows in the midline) show some mislocations of cells. The dorsal channel glia cells are present (hollow arrows). (E–G) Nerve cords of wild type (E), Kr^1 (F) and Kr^1Kr^{CD+} (G) stage 16 embryos stained with anti-serotonin antibodies. Note that at least one, and sometimes two, serotoninergic neurons per hemisegment (arrowheads) are missing in Kr^1Kr^{CD+} embryos (small arrows in G). Occasionally serotoninergic neurons were left in the Kr^1 mutant embryo (example shown by arrowhead in F). (H) Stage 11 Kr^1Kr^{CD+} embryo double stained with anti-*Kr* (dark brown) and anti-Engrailed (orange-brown) antibodies. Faint staining can be seen in a GMC in the *en* region (arrowheads). (I) Section of the abdominal region of a corresponding Kr^1Kr^{CD+} embryo; dorsal is up. Two GMCs per hemisegment are expressing *Kr* (arrowheads).

expressing neurons contains, amongst others, serotoninergic neurons. These form distinct clusters of three neurons per thoracic and two per abdominal hemisegment, the last one containing only one (Fig. 5E) (Budnik and White, 1988; Patel et al., 1989b). To see whether the development of these neurons is affected by the lack of *Kr* activity we looked at serotonin expression in Kr^1 embryos. Almost no neurons synthesising serotonin remain in these embryos (Fig. 5F). Consistent with a role for *Kr* in the development of these neurons, Kr^1Kr^{CD+} embryos



lack at least one serotoninergic neuron per hemisegment (Fig. 5G). The development of the remaining serotoninergic neurons could be accounted for by *Kr* activity derived from the transgene which is observed in some GMCs at late stage 11 (Fig. 5H,I).

We have used other markers which are expressed in subsets of GMCs and neurons. In Kr^1Kr^{CD+} embryos the number of *gooseberry-n* (*gsb-n*)-expressing cells is increased between 10 and 50% (Fig. 6A,B) with respect to the wild type (Gutjahr et al., 1993). To further investigate the relationship between *Kr* and *gsb-n* expression we induced ectopic *Kr* expression under the control of a heat-shock promoter. This led to a strong reduction of gsb-n (Fig. 6C,D) suggesting that *Kr* activity influences *gsb-n* expression.

We also studied *eve* and *ftz* expression in the CNS of Kr^1Kr^{CD+} embryos. In both cases we only observed slight derangement of the pattern of expressing cells which we interpret as secondary consequences of increased cell death occurring in these embryos (not shown).

2.5. *Kr is required for glial development*

We also analysed *Kr* expression in the glia by double staining with antibodies against *Kr* protein and the protein encoded by the *reversed polarity* (*repo*) gene, a specific marker for the glia (Campbell et al., 1994; Xiong et al., 1994; Halter et al., 1995). We found that Kr is expressed in several glia types like the cell body glia cells (CBG) including its medial component (MM-CBG), in the exit glia cells and in the intersegmental nerve root glia media cells (ISNG-M) (Fig. 7A–D) (Klämbt et al., 1991; Halter et al., 1995; Ito et al., 1995). However, it is not expressed in the differentiated longitudinal glia (LG) (Fig. 7C) but found in the corresponding precursor glioblast (Figs. 1D and 7C).

To determine whether the lack of *Kr* neural expression affects glia development, we first examined in Kr^1Kr^{CD+} embryos the expression of the *prospero* (*pros*) gene, a marker for a subset of the LG and the 'belt glia cells' (Doe et al., 1991). In Kr^1Kr^{CD+} embryos the *pros* expressing LG develops normally until stage 14. Then, it appears that fewer cells survive and they have an irregular distribution (Fig. 7D–G). The reduced number of LG cells was confirmed by the analysis of a *ftz-lacZ* reporter gene which is expressed in the longitudinal glioblast lineage (Jacobs et al., 1989) (data not shown). In addition, this marker reveals some defects in the migration of the LG (Fig. 7G,I,J).

To examine the behaviour of the CBG (Ito et al., 1995), which expresses Kr until late embryonic stages (see Figs. 1E,F and 7B), we used the PP3.0HZ lacZ reporter gene expression (Hoch et al., 1990) which is controlled by a *Kr* enhancer element. Low levels of reporter gene expression were found consistently in the mutant, suggesting that *Kr* regulates its own expression. We also observed a reduced number of lateral CBG which ultimately loses reporter gene expression (not shown). In addition, the medialmost component of the CBG fails to migrate properly (Fig. 7K,L). As the complement of neuroblasts looks normal in Kr^1Kr^{CD+} embryos and both components of the CBG are present at early stages, it is possible that *Kr* activity is required for the correct specification or differentiation of this glia. Its absence leads to abnormal behaviour (i.e. migration) and eventually cell death which is very increased in stage 14 Kr^1Kr^{CD+} embryos, as observed with acridine orange (not shown). To address this question it is necessary to know the lineage of this glia type and find the appropriate markers to demonstrate abnormal cell behaviour in the mutant.

3. Discussion

3.1. *Direct Kr requirement for CNS development*

We have rescued the segmentation defects of *Kr* mutant embryos using a transgene which has allowed us to analyse the role of the *Kr*-encoded transcription factor in the development of the nervous system. The widespread spatio-temporal expression pattern of *Kr* in the CNS suggests an extensive requirement for *Kr* activity throughout neural development. This is supported by the severe and

Fig. 7. Glial defects in Kr^1Kr^{CD+} embryos. (A–D) Double staining with anti-*Kr* (green) and anti-Repo antibodies (red) in wild type embryos at stage 12 (A,B), stage 11 (C) and stage 16 (D). (A) Green channel revealing *Kr* expression only. (B) Same specimen with red and green channels superimposed. Co-expression of *Kr* and *repo* in several cells including the MM-CBG (arrowheads) and the exit glia cells (eg) is seen. Anterior is up. (C) superimposition of the green and red channels to show coexpression in the longitudinal glioblast (arrowheads). (D) *Kr* and Repo co-expression in the ISNG-M (arrow). Note two rows of *repo*-expressing cells (LG) at both sides of the midline not expressing *Kr*. (E–H) *prospero* expression in wild type (E,F) and Kr^1Kr^{CD+} (G,H) embryos. Anterior is up. (E,F) Different focal planes of a flattened stage 16 embryo stained with anti-prospero antibodies (black) and mAb BP102 (brown); (E) LG (arrowheads) and 'belt glia cells' (hollow arrows) are shown; (F) arrows indicate the position of the ventral interface glia cells (V-IG). (G,H) Similar focal planes of stage 16 Kr^1Kr^{CD+} embryos as the one shown in (E,F) indicating that the belt glia cells form clusters in some segments and are missing in others (hollow arrows). The LG (arrowheads) and V-IG (arrows) fail to develop normally. (I,J) Expression of a *ftz* lacZ reporter gene in wild type and Kr^1Kr^{CD+} stage 15 embryos, respectively, stained with anti-β-galactosidase antibody. Note that the longitudinal glia cells (LG) have reached their final dorsal position in wild type (I) but occasionally not in Kr^1Kr^{CD+} embryos (J, arrowhead). (K,L) Expression of the PP3.0HZ in wild type (K) and Kr^1Kr^{CD+} stage 12 embryos (L). In the wild type, the MM-CBG has already migrated medially (small arrows in K) whereas in slightly older Kr^1Kr^{CD+} embryos it still remains in a more lateral position (arrowheads in L). Note that reporter gene expression is decreased in the mutant (compare K with L).

complex neural defects found in embryos after the rescue of the segmentation. We have focused our study on subsets of *Kr*-expressing cells in order to unravel the complex defects and to obtain some clues of how specifically *Kr* exerts its function.

One indication for the role of *Kr* in the developing nervous system emerged from the observation that lack of *Kr* neural expression increases the number of *gsb-n*-expressing cells, whereas the excess of *Kr* function represses *gsb-n* expression. These results are an indication that *Kr* influences cell fate perhaps through effects on *gsb* expression. Interestingly, at early stages *gsb* and *Kr* are likely to be coexpressed since we detect Kr in all neuroblasts. This apparent paradox may indicate that the effects of *Kr* on *gsb* only occur later, possibly mediated through interactions with other factors. Alternatively, only high levels of *Kr* may be able to repress *gsb*; it has been shown that *Kr* can function as a repressor or an activator of transcription in a concentration-dependent manner (Sauer and Jäckle, 1993) and its role in regulating *gsb* may require high concentration of the protein not present in the neuroblasts.

The medial-lateral cluster of *en*-expressing neurons, which includes the serotoninergic neurons, is strongly affected in Kr^1Kr^{CD+} embryos. It is thought that the fate of serotoninergic neurons is an early cell-lineage determination event both in grasshopper (Taghert and Goodman, 1984) and *Drosophila* (Huff et al., 1989), which takes place between stages 12 and 14 in the fly. This is precisely the period at which alterations of *en* expression in the medial-lateral cluster in Kr^1Kr^{CD+} embryos can be observed. Therefore, it is highly probable that the alteration observed in the serotoninergic lineage of *Kr* mutants occurs during the allocation of cell fates rather than at the final stage when the neurons differentiate and produce serotonin.

Kr expression is only observed in the LG precursors but not in their progeny. The first divisions and migration of their progeny are normal (S.R., unpublished observations) but the absence of Kr does not allow to complete this process. These defects can be best explained by a defective specification of the cell lineage in the corresponding glioblast. In contrast, *Kr* is expressed both in the CBG and in their corresponding precursors (e.g. NB 6-4; J. Urban, personal communication). Therefore, in this later lineage *Kr* might function at several levels, including the level of specification.

The severity of the global defects, like cell death (data not shown) and derangement of the cellular and axonal patterning, suggests that many more than the neurons and/or glia cells identified here are defective. This implies that *Kr* expression in the majority of neural cells is essential for the generation of the nervous system of *Drosophila*. As additional neural and lineage markers become available it will be possible to characterise the role of *Kr* in more neural cell lineages and to establish its common or specific function in establishing or maintaining cell fate decisions. It will also be interesting to ascertain whether the expression pattern and function of *Kr* in the nervous system is evolutionarily conserved in other insects as has been shown for its gap gene function in *Tribolium* (Sommer and Tautz, 1993) and *Manduca* (Kraft and Jäckle, 1994), and whether *Kr* has a role in the nervous system of vertebrates as seen with other segmentation genes (see Section 1).

3.2. Long-range effects of Kr segmentation function

In contrast to the pattern seen in the larval cuticle, which suggests that the absence of *Kr* only affects the thoracic and anterior abdominal region of the embryo, we find that mutations in *Kr* affect metamerisation throughout the trunk region, including the most posterior segments. This unexpected long-range effect of early *Kr* activity is documented by variably spaced stripes of *en* expression in *Kr* mutant embryos. The apparent inconsistency between the variably defined initial metameres and the resulting normal segmental pattern of the larva is similar to what has been observed in embryos which derive from females containing extra copies of *bicoid*. Such embryos are characterised by an enhanced prospective head region at the expense of abdominal segments at the beginning of gastrulation, but the resulting larval segmental pattern appears normal (Busturia and Lawrence, 1994). The control mechanism underlying this phenomenon is as yet unknown. We assume, however, that the posterior abdominal region of *Kr* mutant larvae becomes readjusted through the interdependent activities of segment polarity genes in the ectoderm as development progresses. The defects observed in the CNS and PNS in the posteriormost segments of Kr^1 embryos suggest that, in contrast to the cells remaining in the epidermis, the progenitor cells of the CNS and PNS might lose the ability to rearrange after their delamination and maintain instead the initial spatial orders derived from the segmentation genes (reviewed in Goodman and Doe, 1993). This proposal is consistent with the finding that segment polarity mutations affect the PNS pattern in parallel to the epidermal defects (Patel et al., 1989) and it is supported by our result that the rescue of the defective overall structures of the *Kr* mutant PNS and CNS is linked to *Kr* segmentation function.

So far *Kr* is the first gap gene whose function in nervous system development has been analysed, although the expression of others, e.g. *hunchback*, has been well documented and indeed is used to mark the developing neuroblasts. The gap genes act in a combinatorial manner to specify the domains of expression of pair-rule, segment polarity and homeotic genes. The Kr^1Kr^{CD+} embryos, in which the segmentation defects of *Kr* are rescued, provide a starting point for further analysis of gap gene function in nervous system development.

4. Experimental procedures

4.1. *Kr CD+ construct, germ line transformation and fly mutant strains*

The 3.5 kb upstream DNA fragment (*Kr* CD) containing the regulatory elements necessary for *Kr* expression in the central domain of blastoderm embryos (Hoch et al., 1990), and the 5.2 kb fragment comprising the *Kr* coding sequence and the basal *Kr* promoter (Rosenberg et al., 1986) were cloned into a Carnegie 20 vector DNA (Rubin and Spradling, 1983). The resulting minigene lacks a number of distinct elements required for *Kr* expression in the posterior blastoderm domain, the Malpighian tubules anlagen, the muscle precursor cells and the nervous system. It contains 500 bp of regulatory sequences necessary for expression in the anterior domain (Schmucker et al., 1992) The transgene construct was injected into ry^{506} embryos (see Rubin and Spradling, 1982; Hoch et al., 1990). Several independent transformed lines were established ('Kr^{CD+} lines'). A Kr^{CD+} line which had the Kr^{CD+} transgene inserted on the second chromosome was used to recombine the transgene with the Kr^{1} deficiency (Gloor, 1950) using standard genetic procedures. The resulting $Kr^{1}Kr^{CD+}$ stock was balanced over a second chromosome balancer containing a P-element *hunchback*-lacZ fusion vector (G. Struhl, unpublished). Thus, homozygous $Kr^{1}Kr^{CD+}$ embryos can be unambiguously identified by the absence of β-Gal expression in the *hunchback* domain. Additionally, they lack *Kr* expression at the posterior blastoderm cap and do not develop Malpighian tubules. To discard the possibility that the phenotype in this stock could be due to mutations in the recombinant chromosome other than *Kr* itself, embryos of the genotype *cn bw sp* Kr^{2}/*bw Kr* $^{CD+}Kr^{1}$ were analysed and similar defects were observed.

For the analysis of the glial phenotype the F263 P-element line (Jacobs et al., 1989) and the PP3.OHZ*KrlacZ* (Hoch et al., 1990) were crossed with $Kr^{CD+}Kr^{1}$/*CyO hb*-lacZ flies. Based on the *hb*-dependent *lacZ* expression pattern, homozygous $Kr^{1}Kr^{CD+}$ mutant embryos carrying the F263 and PP3OHZ*KrlacZ* bearing chromosomes could be identified unambiguously.

4.2. *Heat shock experiments*

P(ry+hs*Kr*)/CyO;ry (a gift from G. Struhl) and wild type embryos were incubated at 23°C until the desired stage and subjected to one 30 min heat shock at 38°C. After 30 min recovery they were processed for immunohistochemistry or left a further 10 min to develop at 25°C before processing.

4.3. *Immunohistochemistry*

Antibody staining for light field microscopy was done as described (Hoch et al., 1990) using the Vectastain ABC Elite-horseradish peroxidase system or according to the methods described by Patel (1994). When two antigens were to be detected simultaneously, the final precipitate of one of the reactions was darkened by the addition of NiCl.

The following primary antibodies were used: rabbit anti-β-galactosidase (Cappel), rabbit anti-Krüppel (Gaul et al., 1987a; P. Carrera unpublished), rabbit anti-Gsb-n (Gutjahr et al., 1993) and rabbit anti-Repo (Halter et al., 1995). Anti-Engrailed (Patel et al., 1989a), BP104 (Hortsch et al., 1990), BP102 (Bieber et al., unpublished data), 22C10 (Fujita et al., 1982) 1D4 (anti-Fasciclin II) (Helt and Goodman, unpublished), anti-Eve (Patel et al., 1994) and anti-Prospero (Oliver et al., 1993) were mouse monoclonal antibodies. The anti-Krüppel antibodies used for confocal microscopy were produced in rat (Wharton and Struhl, 1989). The following secondary antibodies and reagents were used: biotinylated anti-rabbit, anti-mouse and anti-rat (Vector and Amersham), anti-rabbit Texas Red conjugated (Vector) and avidin fluoresceine DCS (Vector). All the specimens were photographed under Nomarski optic unless otherwise indicated. Confocal microscopy was done on a TCS 40D inverted confocal microscope using a Fluotar objective lens of PH3 40×1.0 (oil) and the superimposition of images was done with an Adobe photoshop program.

4.4. *Other procedures*

Cuticle preparations were done according to Nüsslein-Volhard (1977). Embryonic nerve cords were dissected in ice-cold *Drosophila* Ringer (Budnik et al., 1986) and fixed in 4% paraformaldehyde/phosphate buffered saline (PBS) for 40 min at room temperature. After washing in PBS they were processed for immunohistochemistry. Plastic sections were done as described in Martin-Bermudo et al. (1995).

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