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Mutations in *spalt* cause a severe but reversible neurodegenerative phenotype in the embryonic central nervous system of *Drosophila melanogaster*

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SUMMARY

The gene *spalt* is expressed in the embryonic central nervous system of *Drosophila melanogaster* but its function in this tissue is still unknown. To investigate this question, we used a combination of techniques to analyse *spalt* mutant embryos. Electron microscopy showed that in the absence of Spalt, the central nervous system cells are separated by enlarged extracellular spaces populated by membranous material at 60% of embryonic development. Surprisingly, the central nervous system from slightly older embryos (80% of development) exhibited almost wild-type morphology. An extensive survey by laser confocal microscopy revealed that the *spalt* mutant central nervous system has abnormal levels of particular cell adhesion and cytoskeletal proteins. Time-lapse analysis of neuronal differentiation in vitro, lineage analysis and transplantation experiments confirmed that the mutation causes cytoskeletal and adhesion defects. The data indicate that in the central nervous system, *spalt* operates within a regulatory pathway which influences the expression of the β-catenin Armadillo, its ligand N-Cadherin, Notch, and the cell adhesion molecules Neuroglian, Fasciclin 2 and Fasciclin 3. Effects on the expression of these genes are persistent but many morphological aspects of the phenotype are transient, leading to the concept of sequential redundancy for stable organisation of the central nervous system.

Key words: *Drosophila*, *spalt*, Neurodegeneration, Cell adhesion, Neuronal differentiation, Nervous system development

INTRODUCTION

In the fruitfly *Drosophila melanogaster*, the paralogue genes *spalt* (*sal*) and *spalt related* (*salr*) encode putative transcription factors with spaced sets of zinc-finger motifs. They belong to a gene family expressed from early development in nervous tissue in humans and several animal groups (Kühnlein et al., 1994; Barrio et al., 1996). The importance of *sal* homologues for neuronal development has been confirmed in the nematode *C. elegans*, where *sem-4* controls the migration of two serotonergic motoneurons (Basson and Horvitz, 1996). In *Drosophila*, *sal* is involved in the formation of sensory organs in the embryo and in the adult (Rusten et al., 2001; Elstob et al., 2001; de Celis et al., 1999). In *Drosophila*, loss-of-function mutations in *sal* cause several embryonic phenotypes, including a severe disruption of the respiratory system (Kühnlein and Schuh, 1996) and are embryonic lethal. In the embryonic central nervous system (CNS), Sal and Salr proteins are expressed in a substantial proportion of cells and localise to the nuclei of partially overlapping neuronal populations (Barrio et al., 1996), but it is not clear which function they have in this tissue.

To investigate this question, we have studied the CNS in mutant embryos, initially with the aid of transmission electron microscopy (TEM). Tissues fixed at 60% of embryonic development had a strong neurodegenerative phenotype but, surprisingly, those fixed at a slightly later stage (80% of development) looked almost normal. A simple explanation to this finding would be that the phenotype defined by electron microscopy reverted in the few hours between the two stages. To test such a provoking possibility, we studied the dynamics of the phenotype with a combination of techniques applied either in situ, in vitro or after transplantation of single mutant precursors into wild-type tissue. The results indicate that during embryonic CNS development *sal* is important for cell adhesion and the physical integrity of neuronal axons and membranes, probably during a short developmental period around 60% of embryonic development.

A related phenotype is that mutations in this gene affect the expression of particular adhesion and cytoskeletal proteins in

the CNS. Although many of the morphological effects are transient, specific branching projection defects of certain neurones persist. Thus, the absence of Sal compromises the capacity of some central neurones to differentiate correctly. We discuss the concept of genetic redundancy in the context of this result.

MATERIALS AND METHODS

Drosophila strains

Flies were raised on standard *Drosophila* medium and kept at 25°C. We used the deficiency *Df(2L)32FP-5*, which removes the genes *sal* and *salr* (Barrio et al., 1999), and the point mutation sal^{445}, which expresses a truncated Sal protein (Kühnlein et al., 1994).

Transmission electron microscopy

Embryos were manually dechorionated, mounted in halocarbon oil to avoid dessication, and their genotype (GFP-expression) and stage determined upon examination at 40× on a Zeiss Axiovert microscope at room temperature. At least six embryos of each genotype and stage were processed. Heterozygotes (GFP positive) and wild type (w^{1118}) were also compared. A minimum of 100 histological sections were prepared from each embryo according to standard procedures (2 μm thickness and stained with 0.1% boracic Toluidine Blue) and carefully analysed, covering representative regions of brain and nerve cord. Three embryos from each sample were also analysed with a transmission electron microscope (JEOL 100CX) operated at 60 kV. As GFP-negative mutant embryos develop at slower and somewhat erratic rates, individual fixation of carefully staged mutant embryos was used to obtain samples of similar developmental age. Upon reaching the desired stage, as defined by morphological and temporal criteria, the embryo was quickly pre-fixed by shaking in a cold mixture of heptane and aldehydes under 10 minutes, devitellinized by hand, post-fixed in dialdehyde solution for 1 hour, and for another hour in 1% osmium tetroxide. The dialdehyde fixative was freshly prepared in 0.1 M sodium cacodylate buffer pH 7.3, and contained 4% paraformaldehyde, 3% glutaraldehyde and 2% tannic acid (Afzelius, 1992). Ultrathin sections (silver), contrasted by serial incubation in lead citrate, uranyl acetate and lead citrate (Daddow, 1982), were mounted directly onto 300 mesh copper grids.

Immunocytochemistry, laser confocal microscopy and image analysis

Transheterozygous *Df(2L)32FP-5;*sal^{445} mutant embryos of two ages (stage early 16 and 17) were processed for whole-mount immunocytochemistry according to standard techniques (Patel, 1994) with a double-staining protocol in which one of the two primary antibodies always was mouse or rabbit anti-β-galactosidase for genotype assignment. The other antibody probes were specific for N2-Armadillo (Peifer and Wieschaus, 1990; Loureiro and Peifer, 1998), DN-Cadherin (Iwai et al., 1997), Faint Sausage (Lekven et al., 1998), Fasciclin 2 (Schuster et al., 1996), Fasciclin 3 (Patel et al., 1987), Neurexin IV (Baumgartner et al., 1996), Neuroglian (Bieber et al., 1989), Neurotactin (Speicher et al., 1998), Notch (Johansen et al., 1989), Tubulin (Sigma BioSciences) and Futsch/22C10 (Developmental Studies Hybridoma Bank). Stained embryos were mounted in PBS-buffered glycerine and studied with laser confocal microscopy on a Zeiss LSM 510. For general surveys either XY-sections or Z-series covering selected CNS areas were produced. For quantification of fluorescence intensity, single optical sections scanned with exactly the same parameters and under the same session were compared, always on exactly the same anteroposterior and dorsoventral location in the thoracic neuromeres. Care was taken to choose a CNS area relevant for each marker (for example, longitudinal tracts for Fasciclin 2, but ventral cell body cortex for Notch). Histograms of pixel intensity for each channel were prepared directly with the Histogram tool of LSM 510, or, after import into Photoshop, with the Histogram tool of the Adobe software. Each staining was made at least twice, each time with embryos collected from a different cross.

TUNEL staining

For the detection and quantification of apoptosis at a single cell level a fluorescein in situ cell death detection kit was used (Roche; standard labelling protocol for tissues). Staining was applied to flat preparations of embryos between stages 14 and 17. Z-series covering 5-6 abdominal neuromeres per embryo (n=14 for *sal* mutants or wild-type controls) were captured under a fluorescent microscope (Zeiss Axiophot) equipped with a digital camera (Sony MC-3255). Focal planes were combined using Photoshop (Adobe) and total numbers of TUNEL-positive cells were counted.

DiI labelling

To trace the cell lineages of individual neural precursors in *sal* mutant background, we applied the DiI labelling technique as described in detail elsewhere (Bossing and Technau, 1994; Bossing et al., 1996). Individual neuroectodermal precursor cells were labelled with DiI at stage 7 and allowed to further develop until stage 17. Clones generated by the labelled precursors were uncovered upon photoconversion of the dye in flat preparations and documented with a video camera (Sony MC-3255), Photoshop and Illustrator (Adobe).

Cell transplantations

Transplantations were performed as described previously (Prokop and Technau, 1993). Cells were removed from the neuroectoderm of horseradish peroxidase (HRP) labelled donors at stage 7 and individually transplanted into the neuroectoderm of wild-type hosts at the same stage. Hosts were allowed to develop until stage 17 when the clones derived from the transplanted precursors were uncovered and documented (see above) upon staining for HRP in flat preparations. Donor strain was kept over a GFP balancer, and the genotype of donor embryos was identified by GFP fluorescence.

Cell culture and time-lapse video analysis

Primary cultures of single precursor cells, time-lapse recordings and staining procedures of clones in vitro were performed as described elsewhere (Lüer and Technau, 1992). Cells were taken from the neuroectoderm of stage 7 donors and grown in culture for up to 4 days. As the donor strain was kept over a green balancer, the genotype of donor embryos was identified by GFP fluorescence. Clones were stained with anti-HRP (Dianova), anti-Repo (Halter et al., 1995) and anti-tubulin (Sigma) antibodies.

RESULTS

Sal is expressed by ~60 postmitotic neurones per hemisegment in the embryonic nerve cord of the CNS (Barrio et al., 1999) (data not shown). The Sal-positive neurones include motorneurones RP2 and aCC and interneurones dMP2,vMP2, pCC and serotonergic neurones (data not shown). Double staining with antibodies specific for Sal and the glia marker Repo (Halter et al., 1995) showed that *sal* is not expressed by this type of CNS glia, although it is expressed by glial cells of the peripheral nervous system (Rusten et al., 2001) (data not shown). Initial analysis revealed that mutations in the *sal* locus do not interfere with the development of the overall organisation of the CNS. Staining with the axonal markers Futsch/22C10 and BP102 revealed that the major axonal tracts are formed with only small, although variable and frequent

departures from the wild-type plan (data not shown). Staining with antibodies for Repo disclosed normal glial cell numbers although with a less regular distribution and more rounded morphology than in wild-type tissue (wild type: 61.6±0.23 glia per segment, *n*=13 segments; homozygous *Df(2L)32FP-5*: 59.2±0.35, *n*=14 segments; *Df(2L)32FP-5;sal*^{445}: 60.1±0.41, *n*=17 segments). The precise location of RP2 cell bodies was somewhat variable while the cell bodies of aCC, pCC and CQ neurones were in their correct locations (data not shown). During this light-microscopic analysis we also noticed that the mutant CNS appeared to be wider and less compact, and exhibited abnormal fragility during dissection. The combination of these initial data suggested that the mutation has a widespread effect, perhaps on the integrity of the tissue, and we decided to examine the ultrastructure of the mutant CNS.

sal mutant embryos develop a strong but reversible neurodegenerative phenotype

A strong and fully penetrant phenotype was detected in the CNS of *Df(2L)32FP-5;sal*^{445} mutant embryos by TEM at early stage 16 (~60% of embryonic development). Neuronal and glial cells in the brain and nerve cord were seen to be loosely attached or widely separated by a dramatic enlargement of the extracellular space (Fig. 1A). At this stage neuronal cell bodies are tightly packed in wild-type tissue (Fig. 1C). Membrane 'whorls', autophagosomes, and other membranous profiles typical of neurodegenerative processes were observed in the cytoplasm of neuronal and glial cells. The lacunar spaces between cell bodies and neuronal fibres in the neuropil contained large amounts of extracellular membranous material, mostly in the form of vacuoles of a wide size range, the largest approaching the size of whole cell bodies (Fig. 1A,E). These vacuoles either seemed empty or contained smaller vacuoles, but not organelles or cytoplasmic remnants characteristic of cellular debris resulting from cell death. We did not observe an increase in apoptotic profiles as compared with wild-type tissue either with TEM or TUNEL staining (see Materials and Methods, data not shown). Similar membranous formations result from mutations in *spongecake* (Min and Benzer, 1997). Axonal calibre was reduced in *sal* null mutants (compare insets in Fig. 1E,G) and filopodia emanating from growth cones were often clumped. The phenotype was observed in all *sal* mutant embryos, but not in heterozygous or wild-type embryos examined as controls. The phenotype was not observed in peripheral nerves.

Surprisingly, the CNS appeared to recover rapidly from the degenerative process, as embryos fixed a few hours later, by late stage 16 or stage 17 (between 80 and 90% of embryonic development), exhibited an almost normal organisation (see Fig. 1B for cell bodies, and 1F for neuropil; compare with wild-type morphology in Fig. 1D,H, respectively). At this stage, most of the extracellular membranous material had disappeared and the neuronal cell bodies in the mutant were almost as tightly packed as in the wild type.

If the recovery depends on the activity of another protein, with the capacity to compensate for the loss of Sal, simultaneous deletion of this protein should substantially diminish the capacity of the tissue to recover and perhaps make the phenotype irreversible. A potential candidate for this hypothetical redundant function could be the paralogous protein Salr. To test this hypothesis, we examined embryos lacking Sal and Salr due to a small deficiency. However, these homozygous *Df(2L)32FP-5; Df(2L)32FP-5* mutants exhibited the same phenotype caused by the lack of Sal alone and the

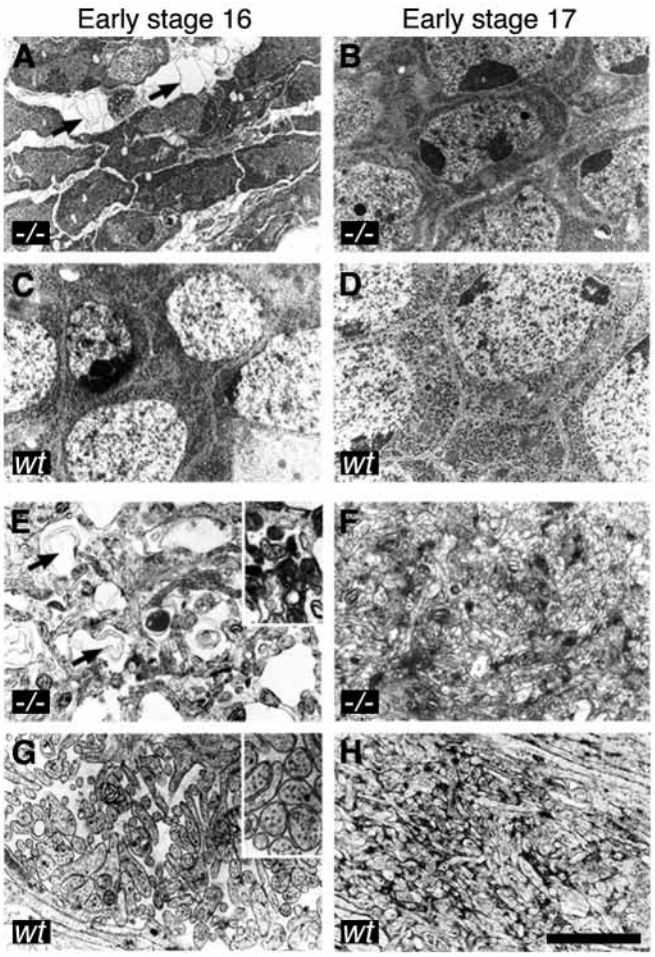

Fig. 1. Ultrastructural comparison of the central nervous system in transheterozygous *Df(2L)32FP-5;sal*^{445} mutant (A,B,E,F) and wild-type (C,D,G,H) embryos at early stage 16 (left panel) or 17 (right panel) of embryonic development. In the mutant CNS at early stage 16 (A), cell bodies are separated by large extracellular spaces occupied by vacuoles and other membranous material (arrows). At this stage neuronal cell bodies in wild-type tissue are tightly packed and the very thin extracellular space, free from vacuoles or other membranous material, is sharply outlined by the smooth cell membranes of CNS cells (C). The phenotype is not longer expressed a few hours later, when the extracellular space in the mutant CNS (B) has the same size as in wild-type tissue (D) and is mostly free from the membranous material found earlier. In the mutant neuropil at stage 16 (E), large vacuoles (arrows) separate 'clumps' of axons and filopodia, in contrast to the wild-type neuropil (G), where axons and filopodia are separated by a 'clean' extracellular space. The insets in E and G show representative axonal profiles at higher magnification. Notice the smaller diameter of axons in *sal* mutant CNS. By stage 17, neuronal processes in the neuropil are equally tightly packed in both mutant (F) and wild-type tissue (H) and most of the extracellular vacuoles previously found in the mutant have disappeared. All sections depicted here are from thoracic neuromeres of the nerve cord but the same morphology was observed across different regions of brain and nerve cord. Scale bar: 2 μm.

phenotype reversed within the same developmental interval (data not shown).

sal mutant embryos have abnormal levels of cell adhesion proteins

A possible interpretation of the phenotype defined above would be that components of cell adhesion are seriously compromised in the CNS of *sal* embryos during early stage 16. To test this hypothesis we used specific antibodies and laser confocal microscopy to survey the expression of molecules known to be important for cell adhesion in embryonic CNS at early stage 16. All the markers were detectably expressed in *Df(2L)32FP-5;sal*^{445} mutant embryos at both stages, and their spatial patterns of expression in the CNS were normal, showing that *sal* is not essential for any of these proteins to be expressed. However, the quantification of fluorescence intensity revealed that most markers were present in abnormally high or low levels. Representative examples are shown in Fig. 2A for seven of the analysed markers (see Materials and Methods for explanation of quantification of fluorescence levels using the laser confocal microscope's software). In transheterozygous *Df(2L)32FP-5;sal*^{445} mutants at early stage 16, when the strong TEM phenotype is manifest, we measured lower fluorescence levels for Armadillo, N-Cadherin, Neuroglian, Fasciclin 2 and Fasciclin 3; higher fluorescence levels for Notch; and levels similar to wild type for Neurotactin, Neurexin IV and Faint Sausage (Fig. 2B and data not shown). Comparison between wild-type, heterozygous and null *sal* mutant embryos revealed a stepwise decrease in the fluorescence levels for Armadillo and N-Cadherin (Fig. 2C), indicating that the effect of the mutation is dominant.

We next measured the fluorescence levels at the stage when the TEM phenotype is reverted (stage 17). The wild-type fluorescence for the three markers studied in this regard (Armadillo, Fasciclin 2, Neuroglian) changed between early stage 16 and stage 17, indicating that during this short developmental interval the levels of cell adhesion proteins are regulated (Fig. 3). Relative to these new wild-type levels, the three proteins that were not affected during the expression of the TEM phenotype (Neurotactin, Neurexin IV and Faint Sausage) remained normal in the mutant (not shown). The levels of Notch switched from abnormally high to slightly lower than normal. All other markers still exhibited lower-than-normal fluorescence levels, with the exception of N-Cadherin, which exhibited a partial recovery (data not shown).

Taken together, these data lead to the conclusions that the expression of *sal* is necessary to maintain correct dynamic levels of several adhesion molecules in the CNS and that *sal* exerts this function in a persistent and dominant fashion.

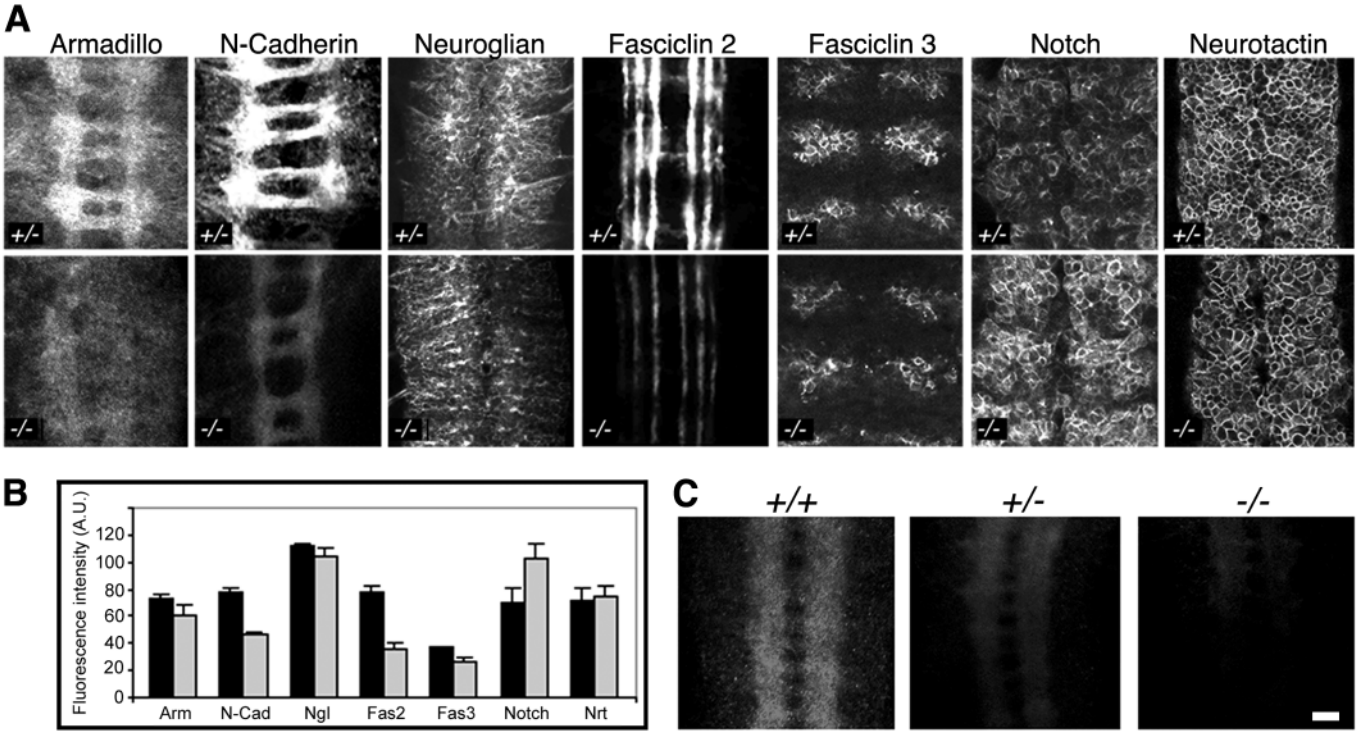

Fig. 2. Expression of cell adhesion markers in *sal* mutants. (A) Confocal optical sections show representative views of the expression of cell adhesion proteins in anterior nerve cord of embryos heterozygous (top row) or homozygous (bottom row) for the *sal* mutation at the stage when the strong adhesion phenotype was detected with TEM (early stage 16). (B) Fluorescence levels were consistently lower for Armadillo, N-Cadherin, Neuroglian, Fasciclin 2 and Fasciclin 3, but higher for Notch and normal for Neurotactin. Notice that Armadillo, N-Cadherin, Neurotactin and other markers are expressed by all CNS cells. The bars represent average levels for heterozygotes (black) and *sal* nulls (grey). Each bar shows the mean value for a minimum of five embryos processed, scanned and measured as detailed in Materials and Methods. Standard error is indicated. The most significant differences were found for Fasciclin 2 (P=0.001), Fasciclin 3 and N-Cadherin (P<0.005) and Armadillo (P<0.05). (C) When wild-type embryos were included in the comparison, the fluorescence levels for Armadillo, and N-Cadherin showed a gradual discrepancy from wild-type to null mutant embryos. The example shown here is Armadillo. Scale bar: 10 μm.

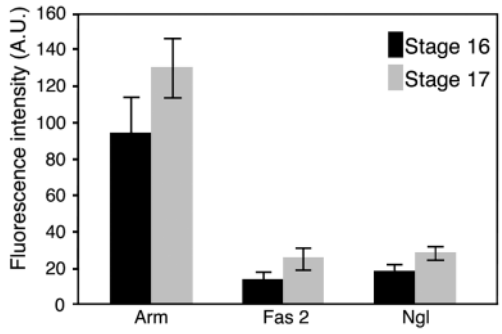

Fig. 3. Dynamic expression of adhesion proteins in the central nervous system. In wild-type embryos, the fluorescence levels measured by laser confocal microscopy for Armadillo, Fasciclin 2 and Neuroglian changed significantly within a short developmental interval. Each bar represents the mean value for a sample of at least six embryos and the s.e.m. is indicated. An increase was found for Armadillo (P=0.005), Fasciclin 2 (P=0.003) and Neuroglian (P=0.001); significance was calculated with the Student's t test. See Materials and Methods for the conditions used for immunostaining, confocal microscopy and quantification of fluorescence levels.

sal is necessary for normal neuronal growth and differentiation in vitro

To gain a more detailed understanding of the dynamics of the *sal* phenotype, we used cell cultures derived from single neuronal precursors isolated either from the neuroectoderm or the midline region of mutant embryos. Unlike the wild type, mutant cells were extremely fragile and sometimes disintegrated upon suction into the microcapillary. Moreover, the cells had a rounded morphology and showed obvious difficulties to establish and maintain a normal attachment to the substrate. Upon inspection of time-lapse recordings, we even found examples of cells that attached and lost contact with the substrate repeatedly. Wild-type neuroectodermal precursors, however, strongly adhered to the bottom of the culture chamber and adopted a more flattened morphology (see also Lüer and Technau, 1992). Proliferation of the *sal* mutant-derived cells did not appear to be affected. However, their progenies exhibited a clearly slower rate of branch growth and differentiation (Fig. 4A, compare with wild type in 4B). Even after several days of culture, most mutant clones still had a poor branching when compared with wild-type clones (Fig. 4C, compare with wild type in 4D). The fibres growing from *sal*-derived cells were often very thin (Fig. 4A), confirming the original TEM observations, and sometimes displayed abnormal fasciculation. Mutant-derived clones were often surrounded by debris, probably representing material shed from living cells (Fig. 4C).

Staining with antibodies specific for the glial nuclear protein Repo (Halter et al., 1995) showed that this glial marker is expressed in some cells derived from *sal*-mutant explants (Fig. 4E). An unusual morphology frequently found in *sal*-derived clones was an anastomosing network of distal branches intercalated with wider, flattened structures (see arrowheads in Fig. 4E). Time-lapse analysis of these anastomosing structures revealed that they were very dynamic, with new branches and connections being made and dismantled over intervals of some minutes (Fig. 5A-D).

Our TEM data showed that the enlarged extracellular space of the mutant CNS tissue appeared to be packed with vacuoles. Time-lapse recording of cultured cells was used to document directly the loss of membrane material that could be the origin of these vacuoles. We detected blistering along *sal* mutant-derived fibres, which could represent an early step in the formation of vacuoles. However, these structures were observed only rarely and were always reabsorbed (Fig. 5E-H). We were thus not able to directly document the loss of membrane or cell material from mutant cells in culture.

The neuronal cytoskeleton is modified in *sal* mutant embryos

The results obtained from time-lapse recordings suggested that the cells derived from *sal* mutant CNS have a deficient cytoskeleton. To investigate this possibility we stained for tubulin in vitro and found two major differences with wild-type neurones. In mutant neurones, tubulin did not reach into the growth cone (Fig. 6A) and patches of poor fluorescence were also detected along the axon (Fig. 6B), suggesting the existence of interruptions along the core of axonal

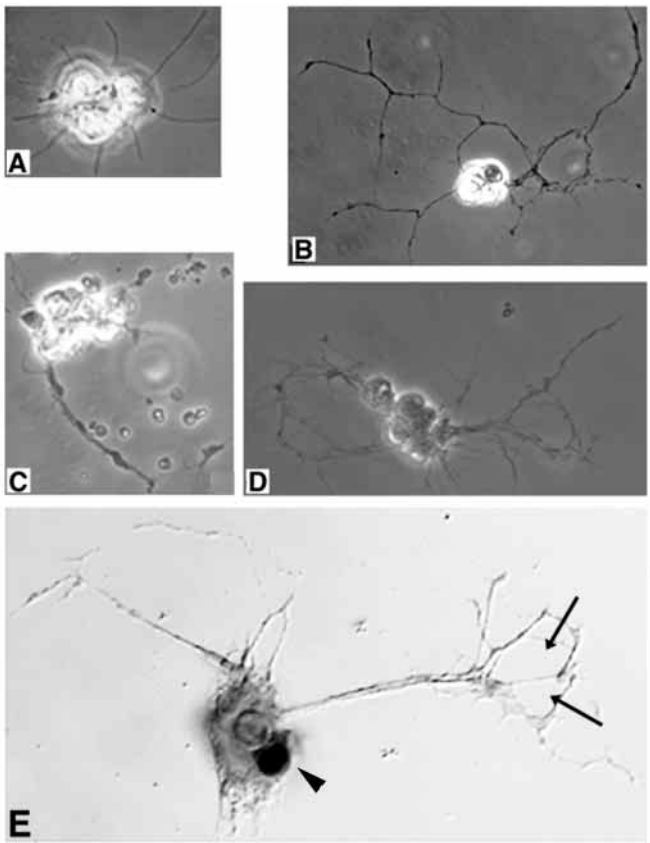

Fig. 4. In vitro analysis of neuronal clones derived from single CNS precursors taken from *sal* null (A,C,E) or wild type (B,D) embryos. After 18 hours of culture, mutant clones (A) had thinner and poorly branched fibres compared with wild-type clones (B). After 4 days in culture, the branches in mutant clones (C) were still less elaborated than in wild-type clones (D). Mutant clones were often surrounded by cell debris (C) and their branches formed 'anastomosing' structures (arrows in E) not observed in wild-type clones. Staining for the glial marker Repo (arrowhead in E) indicated that mutant clones comprised neuronal and glial cells.

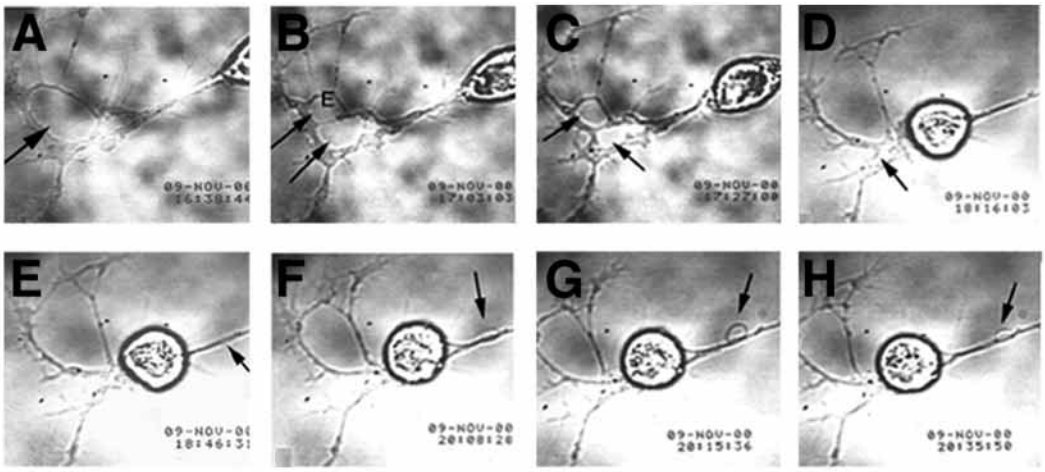

Fig. 5. Time-lapse video analysis of cell cultures derived from *sal* nulls single neuronal precursors. Abnormal networks were formed by mutant fibres in which individual anastomoses were formed and disassembled over short time periods (arrows in A-D). Another abnormal feature was the formation of vacuolar-like 'blisterings' along the axons that persisted for short periods before becoming reabsorbed (arrow in E-H).

microtubules. In wild-type neurones, anti-tubulin fluorescence extends homogeneously along the entire axons and reaches almost the distal border of the growth cone (Fig. 6C). Laser confocal microscopy of embryos stained with three cytoskeletal markers (F-actin, tubulin and the tubulin-associated protein Futsch) revealed additional differences. The three markers were correctly expressed across brain and nerve cord, with the typical accumulation along major axonal tracts (Fig. 6D), but the fluorescence levels were abnormally higher for F-actin, and lower for tubulin and its associated protein Futsch (Fig. 6E).

sal is necessary for correct neuronal differentiation in situ

The data gathered from our cell cultures showed on a single cell level that neurons derived from *sal* mutant neuroectodermal precursors differentiate poorly in vitro. Next, we tested to what extent the lack of Sal affects the differentiation of individual cell lineages in the developing CNS tissue. In *Drosophila* each neural precursor (neuroblast) produces a stereotyped combination of cells identifiable by cell body position and the pattern of axonal projections. Extensive data obtained from lineage analysis in wild type make it possible to identify each neuroblast lineage on the basis of its neuroanatomy (Bossing and Technau, 1994; Bossing et al., 1996; Schmidt et al., 1997). We decided to exploit this knowledge to investigate the capacity of *sal* mutant neuroblasts to produce normal cell clones in situ. Single neuroectodermal and midline precursors were labelled with DiI and cell lineages derived from these precursors were analysed at late stage 17.

The clones obtained in *sal* null embryos can be classified into three categories according to their degree of neuroanatomical abnormality. Some clones differentiated into morphologies showing no obvious similarities to identified wild-type lineages (n=2; Fig. 7A), others exhibited abnormally projecting axons but were as a whole identifiable as particular wild-type lineages (n=7; Fig. 7B) and others, finally, were almost indistinguishable from their wild-type counterparts (n=7; Fig. 7C). Interestingly, some of the clones derived from labelled midline precursors also developed abnormalities, although *sal* expression has not been detected in these precursors (Fig. 7D and data not shown). At high magnification, spherical thickenings were found along the axons, which resemble the blistering observed in the time-lapse studies (Fig. 7E).

The function of *sal* during embryonic CNS development is probably cell autonomous

Although *sal* is expressed only in a subset of neurones, the embryonic *sal* mutant CNS exhibit a general disorganisation, indicating that the surrounding cells cannot overcome the lack of Sal. To test whether the phenotype is expressed cell autonomously, we used a transplantation assay. HRP-labelled neuroectodermal precursors (stage 7) were taken from *sal* mutant donors and singly transplanted into wild-type embryos, and their lineages were documented at stage 17. Again we could detect clones falling into the three categories listed above: non-identifiable lineages (n=8; Fig. 7F), partially abnormal morphologies (n=3; Fig. 7G) and apparently normal morphologies (n=17; Fig. 7H). Clones derived from midline precursors also showed abnormalities (Fig. 7I). These data together with the in vitro observations provide support for a cell autonomous function of Sal in the developing CNS. However, we can not exclude that non-autonomous aspects of Sal function also exist, e.g. considering that abnormalities occur in midline clones even though the expression of *sal* has not been detected in the midline.

DISCUSSION

We investigated the function of Sal during mid- to late embryonic development of the *Drosophila* CNS. We found that the ultrastructural organisation of this tissue is seriously distorted in null *sal* mutant embryos by early stage 16 (60% of development) when cell bodies and fibres are enormously separated by enlarged extracellular spaces containing membranous material. This situation is detected by electron microscopy across the brain and nerve cord, whereas the peripheral nervous system does not appear to be affected. This prominent CNS phenotype recovers after a few hours. Analysis of levels of cell adhesion and cytoskeletal proteins, of cell lineages in situ, and transplantation of single precursors into wild-type tissue, as well as time-lapse recordings of neurones growing in vitro, provided evidence that *sal* regulates directly or indirectly genes that are important for normal cell adhesion

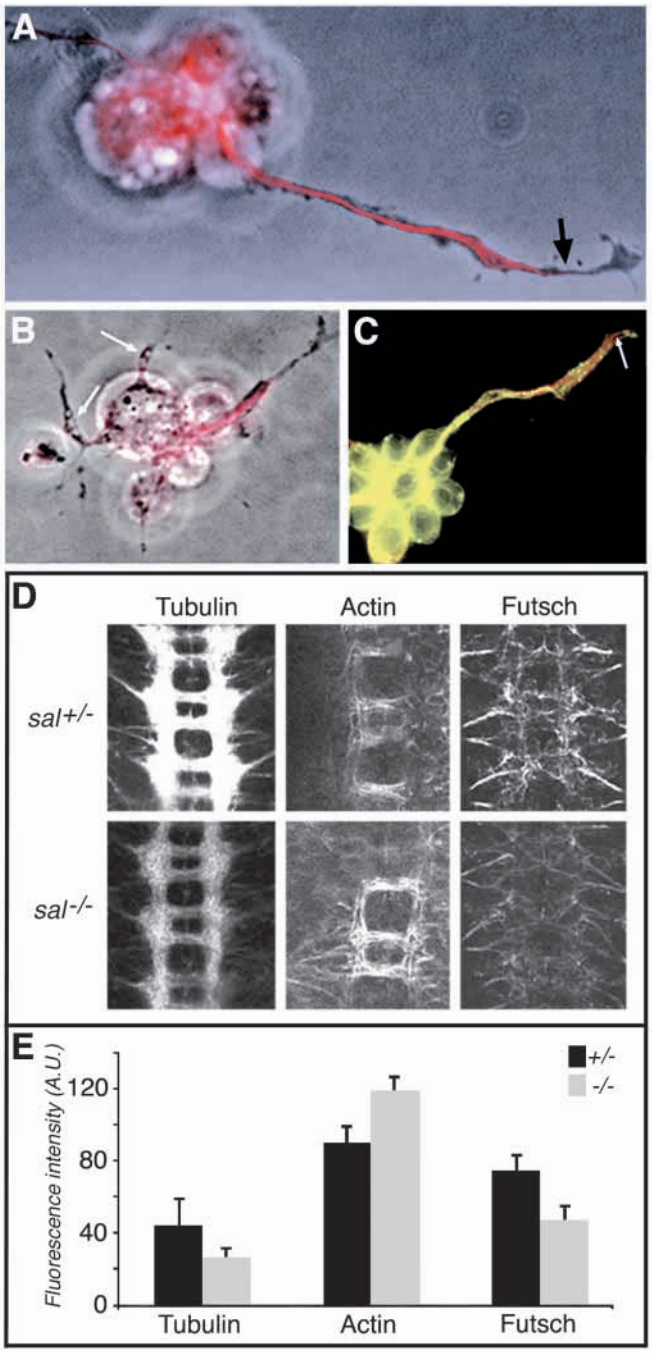

Fig. 6. Abnormalities in the cytoskeleton of *sal* mutant neurones in vitro (A-C) and in situ (D-E). Tubulin staining in vitro uncovered irregularities in the distribution of tubulin along the axon. The staining did not reach the growth cone (arrow in A) and had sometimes interruptions along the axon (arrows in B). Compare with the wild-type neurones in C [double stained with anti-tubulin (in red) and anti-HRP (in green)], where tubulin staining is uniformly intense along the axons and reaches almost the distal border of the growth cone (arrow). (D) At the stage of the strong TEM phenotype (early stage 16) laser confocal microscopy showed normal spatial distribution of F-actin, tubulin, and the microtubule associated protein Futsch/22C10 in *sal* mutant CNS. Notice the wild-type pattern in both heterozygotes and homozygotes, with accumulation of the marker along nerve roots and major axonal tracts. Careful analysis at higher magnification revealed frequent 'mild' malformations in the organisation of axonal tracts in *sal* null condition (not shown). (E) When the fluorescence levels were measured (see Materials and Methods) the homozygotes were found to express higher levels for Actin, and lower for Tubulin ($P<0.05$) and Futsch ($P=0.05$). Each bar represents the mean values for samples of at least six embryos each and the s.e.m. is indicated.

and the cytoskeleton Recently, it has been reported that *sal* regulates the expression of two adhesion proteins (Tartan and Capricious) in the imaginal wing disk (Milán et al., 2002)

At least five adhesion proteins seemed to be under-represented in *sal* null mutants and three cytoskeletal proteins were also quantitatively mis-expressed. At least some of the molecular phenotypes are also exhibited by *sal* heterozygotes to a lower level, indicating that *sal* acts in a dose-dependent fashion. Armadillo and N-Cadherin are expressed ubiquitously and the same values were obtained for each individual when different CNS areas were framed for the measurement of fluorescence levels, indicating that the differences between wild-type, heterozygous and *sal* null embryos do not represent changes in specific neuronal subpopulations but the state of the entire CNS. A likely explanation of these molecular phenotypes is that Sal, which acts as a transcription factor, regulates quantitatively transcription of genes that are directly or indirectly involved in cell adhesion and the cytoskeleton, thus offering a link to the observed cellular defects. It must be kept in mind, however, that some of the target genes can have functions other than cell adhesion. For example, Armadillo protein not only firmly attaches to the cell membrane but can also enter the nucleus to influence the transcription of genes involved in cell fate decisions (reviewed by Cavallo et al., 1997).

Adhesion-related abnormalities in attachment and branching were also exhibited by *sal* mutants at the cellular level. Both in tissue culture and in vivo, whether in situ or following transplantation into wild-type embryos, certain neuronal clones exhibited abnormal branching pattern. This suggests that these cellular phenotypes could be cell autonomously expressed. However, this does not seem to be the case for mutant CNS midline precursors, which showed abnormalities, although they normally do not express *sal* detectably. Defects were also seen in lineages derived from heterozygous precursors (data not shown), again indicating *sal* dominance. Importantly, both molecular and lineage defects were persistent. In these two respects, dominancy and persistency, these defects differ from the prominent ultrastructural phenotype, which is both recessive and transient. As the recessive ultrastructural defects undergo reversal when the molecular phenotypes that we have studied persist, we conclude that the proper organisation of the developing central nervous tissue is stabilised by genetic redundancy. The reversal of the ultrastructural defects suggest sequential redundancy. However, the fact that the ultrastructural defects are only seen in *sal* null mutants (contrasting with the co-dominance of molecular phenotypes) suggest coincident redundancy. The putative genes that are redundant with *sal* are as yet unknown.

It is generally assumed that many loss-of-function mutations for a given cell adhesion protein do not result in overt phenotypes throughout the CNS. Mutations in Armadillo, Fasciclin 2, Fasciclin 3, Neurotactin, Neurexin IV or

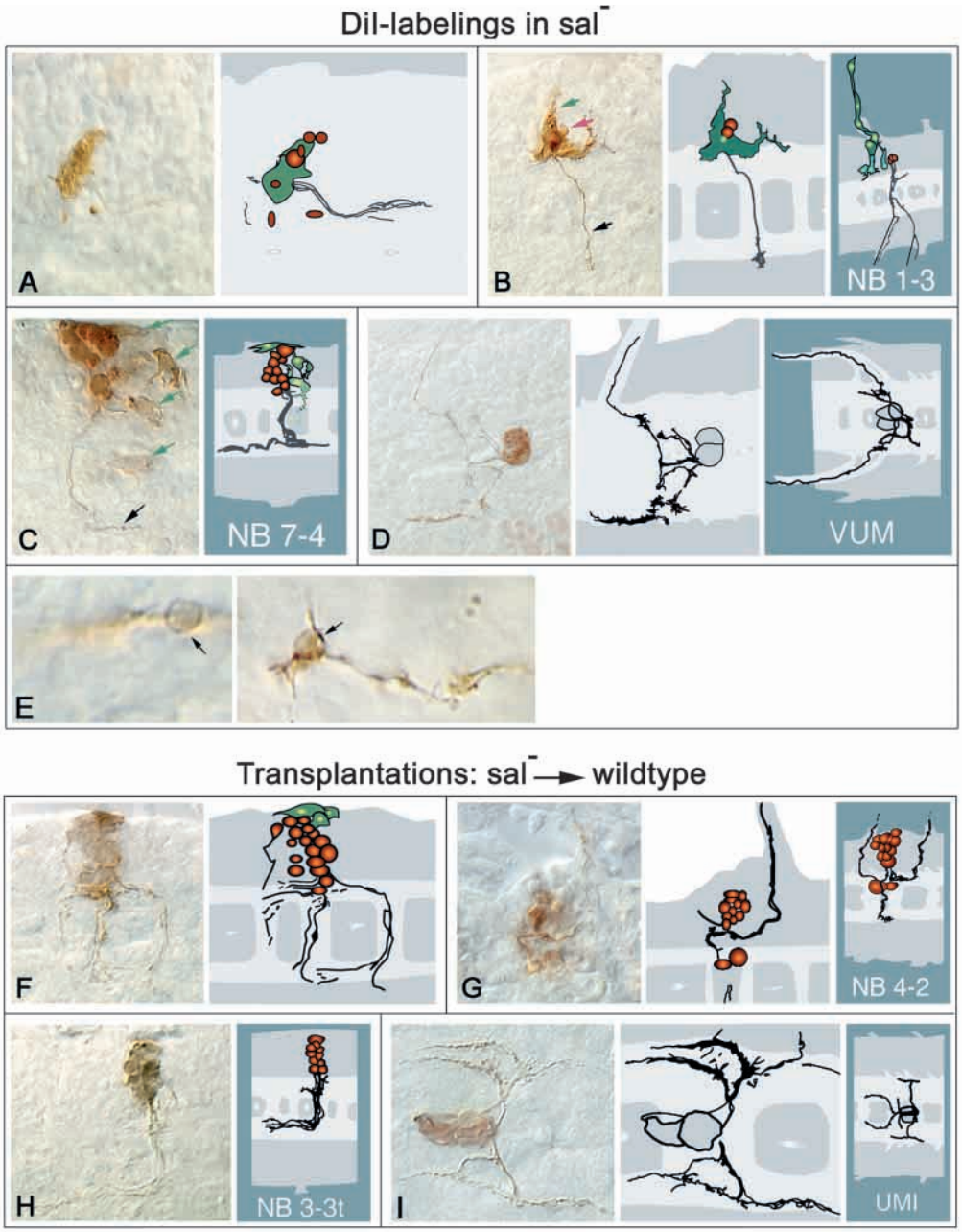

Fig. 7. *sal* mutant cell lineages (at stage 16) derived from single neuroectodermal precursors in mutant (A-E) or wild-type (F-I) background. Clones in A-E were obtained by labelling precursor cells (at stage 7) with DiI in situ, clones in F-I were obtained from HRP-labelled precursors upon transplantation (at stage 7) from mutant donors into wild-type hosts. All clones are located within abdominal neuromeres (A1-A4, shown as horizontal views, anterior towards the left). Drawings with light background (in A,B,D,F,G,I) show camera lucida tracings of the respective preparations. Drawings with dark background (in B-D,G-I) show identified wild-type lineages for comparison (see Bossing and Technau, 1994; Bossing et al., 1996; Schmidt et al., 1997). Glial cells are shown in green, neuronal cell bodies are in red and fibre projections are in black (see also arrows in B,C). In both series of experiments, there was a wide range of clonal phenotypes, including cases with no similarities to wild-type clones (A,F), cases with some components showing similarities to wild-type clones (B, NB1-3; G, NB4-2) and cases very similar to wild type clones (C, NB7-4; H, NB3-3). Along axons spherical thickenings were found (E, arrows). Although *sal* is not expressed in the CNS midline, some midline clones showed irregularities in their projection patterns (D, VUM clone; I, UMI clone).

Neuroglian have phenotypes restricted to de-fasciculation of particular axons, partial disorganisation of major tracts and nerve roots, or other 'localised' adhesion defects (Schuster et al., 1996; Patel et al., 1987; Peifer and Wieschaus, 1990; Speicher et al., 1998; Baumgartner et al., 1996) (reviewed by Goodman, 1996). The current interpretation is that normal adhesive properties in a complex tissue as the CNS depends on the combination of several molecules, some of which can possibly compensate for the absence of others. Examination of double mutants supports this hypothesis (Speicher et al., 1998), as stronger phenotypes are detected after simultaneous deletion of two cell adhesion proteins.

The rapid recovery of *sal* CNS during the course of stage 16 could be explained by the robustness inherent to a system in which adhesion is mediated by a combination of proteins and the possible compensatory effect mediated by upregulation of other members of the system. But we will also like to propose an alternative view. The ultrastructural recovery may as well reflect the normal dynamics of combinations of adhesion proteins expressed successively along embryonic development. From this point of view, the rapid recovery from the adhesion phenotype will reflect the normal transition between two particular combinations of adhesion proteins expressed at early or late stage 16. For this to be valid, the expression levels of several adhesion proteins must change along this interval during normal development. Interestingly, our data do support this possibility, as the fluorescence levels for Armadillo, Fasciclin 2 and Neuroglian did change between stages 16 and 17 in wild-type CNS. Whether *sal* is required for the regulation of a combination of cell adhesion and cytoskeletal proteins at

a particular developmental stage could be tested by deleting the expression of Sal exclusively in CNS tissue within short developmental intervals. This approach could now be possible using techniques based on combinations of the GAL4-UAS system and RNA interference (Piccin et al., 2001).

The fragility of the precursors during their manipulation with micro-capillaries and the difficulties they exhibited to settle down and maintain themselves adhered to the substrate clearly demonstrates, in combination with the previous data, that *sal* expression is necessary for the development of neural cells with normal adhesion capacity. Time-lapse recordings clearly showed that the axons of these cells often lose adhesion from the substrate and exhibited an unusual behaviour: They showed a 'vibrating' movement suggestive of increased tension, instead of the relaxed appearance of a well-attached wild-type axon. These two observations, and the previously commented fragility during micro-manipulation lead us to suspect an abnormal composition of the cytoskeleton. Staining for tubulin and actin confirmed this assumption. Cell adhesion and the cytoskeleton are functionally related, making difficult to clarify without further experiments which of these two cellular features is the original cause of the phenotype. Alternatively, *sal* could be necessary for the correct maintenance of both features. It is worth noting that loss of function in *sal* interferes with the migration of respiratory tubes (Kühnlein and Schuh, 1996), a phenotype that could also be explained by cytoskeletal defects.

An intriguing feature of the phenotype revealed by electron microscopy is the accumulation of 'vacuoles' in the enlarged extracellular space separating the loosely attached CNS cells. Vacuolation is a common feature in mammalian neuropathies and has also been recorded for mutations causing neurodegeneration in *Drosophila* (Coombe and Heisenberg, 1986; Min and Benzer, 1997; Wittmann et al., 2001). We were able to detect in vitro the formation of two types of structures that could represent the correlate of the membranous material detected with TEM. First, mutant clones were associated with more abundant extracellular debris than wild-type clones, which could represent loss of membrane material but probably also represent cell death if mutant cells are less tolerant to the culture conditions. Second, we detected blistering along the axons. However, these structures were seen only rarely and were reabsorbed, so we were not able to document directly in our time-lapse experiments any event of material loss through this mechanism. We also detected a possible morphological correlate to this axonal blistering in neurones labelled with DiI during our lineage studies. Axonal swellings are often correlated with defective axonal transport. In *Drosophila* they are caused by mutations in kinesin heavy chain, Dynein heavy chain (reviewed by Goldstein and Yang, 2000), and Lis1 (Liu et al., 2000). From this point of view, it is interesting to notice that neurones derived from *sal* mutant tissue exhibited both axonal blisterings and discontinuities in the tubulin staining along the axon.

Staining of nervous tissue with 22C10 and detailed clonal analysis also demonstrated the persistence of irregularities in neuronal projections at the end of embryonic development. Smaller departures from normal neuroanatomy of nerve tracts and cell body location where also detected in larval tissues (data not shown). These might be due to effects on axonal growth and path-finding during stage 16, which are not reversible once axons are formed, or to lack of maintenance of fasciculation patterns at later stages.

In conclusion, we show that embryonic *Drosophila* CNS possess a hitherto unknown capacity to recover from a strong adhesion phenotype, and that the zinc-finger nuclear protein Sal is necessary for the integrity of central nervous tissue, most probably acting in morphogenetic pathways that directly or indirectly control cell adhesion, the cytoskeleton and membrane integrity. Sal has a strictly nuclear localisation and is thought to function as a transcription factor (Kühnlein et al., 1994). An interesting question to be addressed will hence be whether the activity of genes, which mediates normal dynamics of neural cell adhesion and cytoskeleton, is under transcriptional control of Sal. Based on the phenotype reported here, the genes that codify Fasciclin 2, Fasciclin 3, Armadillo, N-Cadherin and Neuroglian should be regarded as probable candidates.

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