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A carbohydrate epitope expressed uniquely on the cell surface of *Drosophila* neurons is altered in the mutant *nac* (neurally altered carbohydrate)

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Antibodies against horseradish peroxidase (anti-HRP) recognize neural specific cell surface antigens in *Drosophila* and other insects. The nature of these antigens was investigated in *Drosophila* and found to include a complex set of developmentally regulated proteins. Their common epitope appears to be a carbohydrate that shares features with the sugar moiety of pineapple stem bromelain, a plant glycoprotein whose carbohydrate structure has been determined. A mutation was identified that eliminates staining by the antibody in imaginal and adult neural tissue. Tissue specific glycoconjugates, although widespread in the animal kingdom, are little understood. This mutation provides a unique opportunity to address the consequences of altering a neural specific carbohydrate moiety in an otherwise intact and behaving animal. The mutation maps to 84F. A second mutation, contained on the third chromosome balancer, TM3, eliminates anti-HRP staining in embryos. These mutations appear to be separate genes.

Key words: anti-HRP/carbohydrate epitope/*Drosophila* nervous system/*nac* gene

Introduction

The molecular details of neurodevelopment remain largely unknown. It seems reasonable to expect, however, that cell–cell interactions mediated by cell surface determinants are involved in the specification of where a neuron arises, what pathways its growth cone and neurites will follow and with whom it will form functional contacts. One way of identifying candidate molecules for such functions is by the use of antibodies that recognize neuronal membrane proteins during the relevant growth periods. Antibodies against horseradish peroxidase (HRP) recognize specifically neural tissue in *Drosophila* as well as in other insects. Moreover, they define molecules on the cell surfaces of neuroblasts, sprouting neurons, and growth cones, as well as mature neurons (Jan and Jan, 1982). All growth cones express this antigen, and it is often expressed by 'guidepost' cells in the grasshopper that growth cones interact with and use to set up pioneer pathways (Bentley and Caudy, 1983). In the grasshopper limb bud, it is transiently expressed on segment boundaries where growth cones are observed to pause, and on non-neuronal cells in the periphery of limb pathways, toward which filopodia have been seen to divert (Caudy and Bentley, 1986a,b). In fact, Caudy and Bentley (1986b) observed that extrinsic cues for growth cone guidance on three different cell types are correlated with anti-HRP expression, and that differences in growth cone affinity for these cues correlate with the degree of expression of the anti-HRP epitope. They therefore suggested that the anti-HRP binding site may be a molecular cue for neuronal recognition, or that its expression is closely linked with the expression of such a cue. A similar transient expression on epithelial cells in the vicinity of growth cones has been observed in the wing imaginal disc of *Drosophila* (Murray *et al.*, 1984). It therefore seems possible that, together with other cues, this epitope is itself involved in developmentally significant cell–cell interactions.

In this paper we explore the nature of the anti-HRP epitope in the imaginal nervous system of *Drosophila*. Snow *et al.* (1987) have studied this epitope in the embryo and shown it is a carbohydrate contained on multiple proteins. We further demonstrate here the nature of this carbohydrate and show it is displayed on a developmentally regulated array of proteins. Moreover, we have identified a mutation that affects its expression in imaginal nervous tissue. This mutation allows us to probe the function of a neural-specific glycoconjugate in an intact developing and behaving organism.

Results

Antibodies against HRP recognize an epitope shared by many proteins

To identify proteins recognized by antibodies against HRP, we initially used immunoprecipitation of radiolabeled 3rd instar larval central nervous system (CNS). A large number of proteins were precipitated with approximate M_rs ranging from 45 to >300 kd (Figure 1b). Although we could preabsorb the antibody activity with HRP (Figure 1a), suggesting the precipitation was specific, it was possible the proteins were precipitated as a complex, only one of which was recognized by the antibody. In addition, despite the presence of protease inhibitors, it was possible that proteolysis increased the complexity of the pattern. Western blots proved to be unreliable, as the majority of proteins are large and transfer poorly to nitrocellulose. As a result, homogenates were prepared directly in boiling SDS to minimize proteolysis, and immediately run on SDS–PAGE gels. The SDS was removed from the gel and antibody incubations were done directly in the 'renatured' gel (Burridge, 1978). A comparison of embryo, larval CNS and adult CNS is shown in Figure 2B. The diversity of proteins recognized is comparable to that seen after immunoprecipitation. The protein sets are not identical at each stage, although some in each set are shared. The embryo contains the least protein both quantitatively and qualitatively. These proteins are not observed following the use of non-immune IgG (Figure 2A).

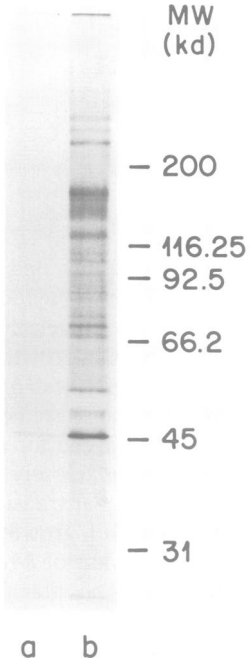

Fig. 1. [^{35}S]methionine-labeled proteins precipitated by anti-HRP. The ventral ganglion and brain of Oregon-R third instar larvae were radiolabeled with [^{35}S]methionine and precipitated with anti-HRP in the presence (**a**) or absence (**b**) of exogenous HRP. Immunoprecipitates were run on 5–15% SDS–PAGE gels. An autoradiogram of the dried gel is shown.

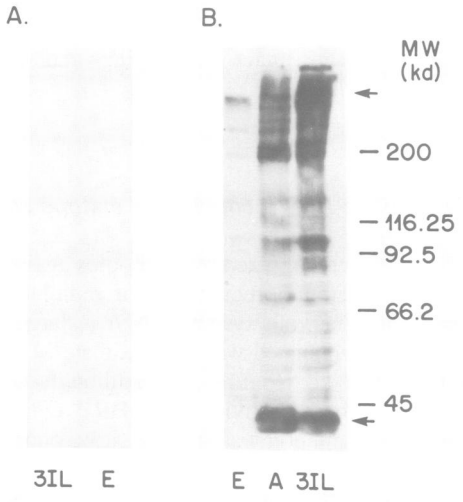

Fig. 2. Developmental profile of the antigens recognized by anti-HRP. Extracts from embryo (E), third instar larval CNS (3IL), and adult CNS (A) were analyzed on 6% SDS–PAGE gels. Gels were stained with either nonspecific rabbit IgG (**Panel A**) or rabbit anti-HRP (**Panel B**) followed by [^{125}I]Protein A. An autoradiogram of the dried gels is shown. The arrows indicate the most prominent developmentally regulated bands. **Lanes E** contain 50 μg of protein, **lanes A** and **3IL** contain 100 μg protein, each.

The epitope is carbohydrate

As the antibodies were generated against HRP, the *Drosophila* proteins must share some epitope with this molecule. HRP is a plant glycoprotein containing 6–8 asparagine-linked neutral carbohydrate side chains (Welinder and Smillie, 1972) which are recognized by concanavalin A (Con A; Clarke and Shannon, 1976). Since many proteins

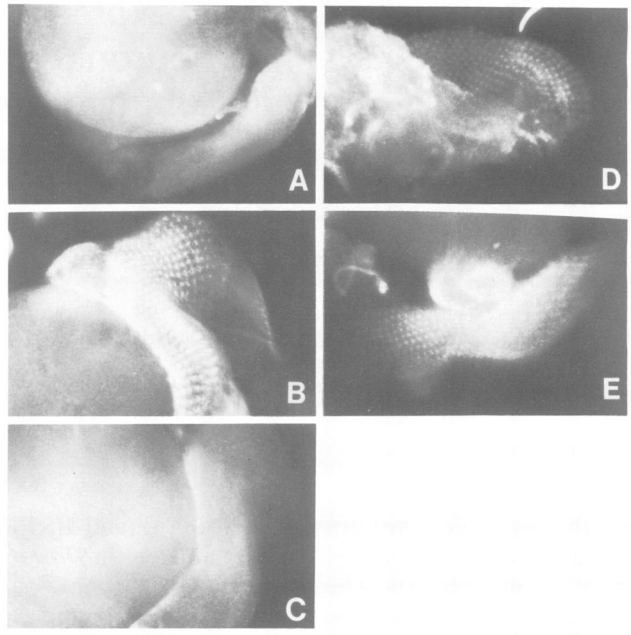

Fig. 3. Indirect immunofluorescence of third instar larval eye disc and brain lobes stained with anti-HRP alone (**B** and **E**) or after pre-absorption of the antibody with HRP (**A**), enriched HRP carbohydrate (**C**), or HRP pretreated with periodate (**D**). Anti-HRP was used at a dilution of 1:3000 in **A–C**, and 1:1000 in **D** and **E**, followed by FITC-conjugated goat anti-rabbit IgG.

share the anti-HRP epitope in *Drosophila*, it seemed possible that the relevant epitope might be carbohydrate. This appears to be the case. In immunoprecipitations, the epitope is resistant to heating at 95°C or boiling in 2% SDS/5% BME with subsequent renaturation in nonionic detergent (unpublished data). The ability of HRP to completely preabsorb anti-HRP staining of *Drosophila* neural tissue was used as an assay to assess the relevant features of the epitope. If the carbohydrate portion of HRP is enriched by exhaustive pronase digestion of HRP, selection of mannose-containing carbohydrate units on Con A, and elution of the bound fraction, the eluate retains the ability to preabsorb the antibody activity (compare Figure 3B and C) comparable to equivalent amounts of intact HRP (Figure 3A), suggesting the epitope is contained within this fraction. Consistent with this, when HRP is first treated with sodium metaperiodate (which oxidizes terminal sugar residues), its ability to preabsorb antibody activity is destroyed (compare Figure 3D and E).

The carbohydrate composition of HRP (Clarke and Shannon, 1976; Phelps *et al.*, 1971) is similar to that of pineapple stem bromelain, a plant glycoprotein protease whose carbohydrate structure has been determined (Yasuda *et al.*, 1970). Anti-HRP recognizes both HRP (Figure 4a) and bromelain (Figure 4b) on Western blots but not two other mannose-containing glycoproteins, fetuin and RNase B (Figure 4d and e). Mild periodate treatment of bromelain compromises its ability to be recognized by anti-HRP on Western blots (Figure 4c) suggesting that the epitope on bromelain recognized by the antibody is also carbohydrate. Moreover, bromelain, after inactivation of its proteolytic activity, is able to preabsorb anti-HRP staining of *Drosophila* neural tissue (data not shown). It therefore seems likely that the relevant epitope is contained within the structure of the

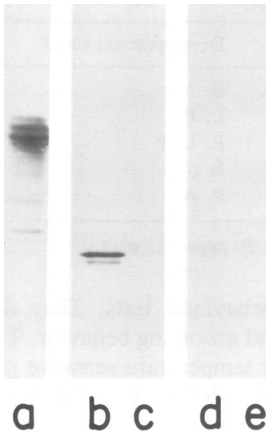

Fig. 4. Western blot of HRP (**a**), bromelain (**b,c**), fetuin (**d**) and RNase B (**e**) stained with anti-HRP. Bromelain in lane c was pretreated with periodate before blotting.

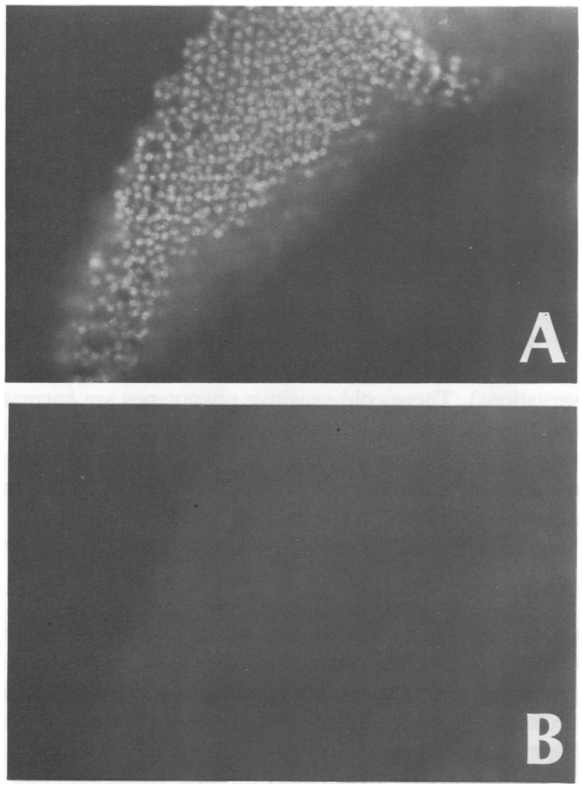

Fig. 5. Indirect immunofluorescence of a portion of a 4 h APF eye imaginal disc of *nac* double labeled with Mab 44C11 and fluorescein-conjugated goat anti-mouse IgM (**A**), or rabbit anti-HRP and rhodamine-conjugated goat anti-rabbit IgG (**B**).

bromelain carbohydrate unit, and that this structure is shared with HRP and *Drosophila* neural proteins.

A mutation eliminates anti-HRP staining in imaginal tissue

The anti-HRP epitope is thus an example of a class of tissue-specific glycoconjugates whose functions are, for the most part, unexplored. The significance of neural specific expression might best be tested in a mutant which lacks the epitope. In the course of an EMS screen for late pupal lethal

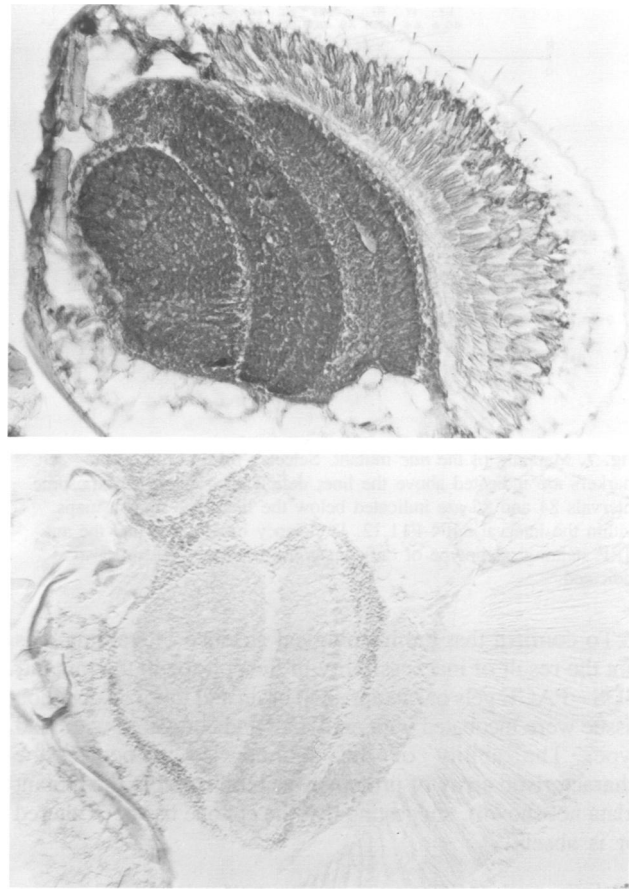

Fig. 6. Cryostat sections of adult brain reacted with anti-HRP. **Top**: *red cv-c sbd*^{2}e" (parent isogenic line on which mutant was induced). **Bottom**: Df(3R)*dsx*$^{D+R5}$*bx sr e/nac red cv-c sbd*^{2} *e"*. Cross section through the retina, lamina and optic lobe. Magnification, ~160×.

mutations on the 3rd chromosome, a mutant was identified that eliminated anti-HRP staining in the imaginal nervous system, and thus provided us with an opportunity to test the function of this neural-specific carbohydrate moiety. This mutation, *neurally altered carbohydrate* (*nac*), although it was isolated on a chromosome containing a recessive lethal mutation, was subsequently separated from it, and proved to be homozygous viable (see below).

Although the anti-HRP epitope is expressed on all neural tissue throughout the development of the fly, this mutation affects its expression exclusively in imaginal tissue. Whereas the embryonic and larval nervous system stain normally in the mutant, new neurons arising in the imaginal discs in the early hours of metamorphosis fail to label with the antibody (Figure 5B) although they readily label with other neuron-specific monoclonals (Figure 5A, Mab 44C11, Bier *et al.*, 1988). The ventral ganglion and brain lobes, which still contain many larval neurons during this period (0−6 h APF; Truman and Bate, 1988) contain antibody-positive cells, but this expression appears to be progressively lost as metamorphosis proceeds, and adult neural tissue (both central and peripheral) totally lacks the antigen (Figure 6). This progression mimics the second wave of neurogenesis, during which the embryonically derived larval nervous system is replaced or respecified for adult function (White and Kankel, 1978; Truman and Bate, 1988).

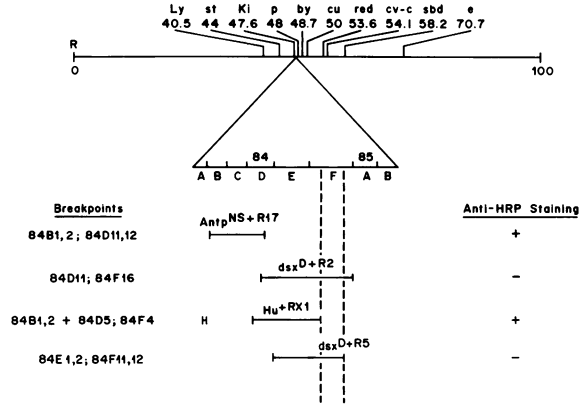

Fig. 7. Mapping of the *nac* mutant. Selected third chromosome markers are indicated above the line; deficiencies within chromosome intervals 84 and 85 are indicated below the line. The mutant maps within the interval 84F4-F11,12. Deficiency breakpoints and the anti-HRP staining phenotype of deficiency/*nac* heterozygotes are also indicated.

To confirm that the histological absence of staining was not the result of inaccessibility of the epitope in the mutant, SDS−PAGE gels of mutant adult brain and thoracic ganglion tissue were incubated with anti-HRP and compared with wild type. The ability of the antibody to recognize the characteristic array of proteins was eliminated in the mutant (data not shown), suggesting that the epitope has been altered or is absent.

Meiotic and deficiency mapping and relationship to an embryonic gene

The *nac* mutation was mapped by meiotic recombination to the interval between Ki and p^p on chromosome 3R. Using deficiencies across this region, we found it was uncovered by Df(3R)dsx^{D+R2} and Df(3R)dsx^{D+R5} but not by Df(3R) Hu^{+RX1} or Df(3R) $Antp^{NS+R17}$. This places it in the chromosomal interval 84F4-84F11,12 (Figure 7).

The third chromosome balancer, *TM3*, contains a mutation that abolishes anti-HRP staining in the embryo (Snow *et al.*, 1987; our unpublished observations). The *TM3* mutation complements *nac*, as *nac/TM3* larvae and pupae stain normally with anti-HRP. Moreover, Df(3R)dsx^{D+R5}, a deficiency which uncovers *nac*, fails to uncover the *TM3* mutation in Df(3R)dsx^{D+R5}/*TM3* embryos. This suggests that these two mutations are distinct genes. These observations are summarized in Table I.

Phenotype of the mutant nac

The alteration of a neural-specific glycoconjugate in *nac* allowed us to explore the role of such a tissue-specific carbohydrate. Histological examination of the developing sensory nervous system of 0−6 h pupae (Jan *et al.*, 1985) revealed no major defects in the formation of early pioneer pathways or in patterning of new imaginal neurons. Neurons were able to pioneer pathways, recognize other axons and fasciculate with them, defasciculate in the appropriate ganglia, and form normal projections (data not shown). Moreover, examination of both plastic sections and cryostat sections from mutant versus control pharate brains showed that the brain was intact and morphologically normal (cf. Figure 6). At 25°C, mutant flies [Df(3R)dsx^{D+R5}/*nac*] were both viable and fertile, and were consequently subjected to a battery of behavioral tests. They displayed normal jumping, flying and grooming behavior. They were neither bang sensitive nor temperature sensitive paralytic, and did not display abnormal behavior under ether (Ganetzky and Wu, 1986). They extended their proboscis in response to sucrose but not water on their tarsi (Duerr and Quinn, 1982), and responded to mechanosensory stimulation with appropriate brushing behavior (Vandervorst and Ghysen, 1980), suggesting that simple reflex circuits were formed correctly. Electrophysiological tests of muscle response to giant fiber stimulation were normal (Mark Tanouye, personal communication), as were responses at the larval neuromuscular junction (Leslie Timpe, personal communication). However, two defects were observed. After heat stress at 37°C for 5 min, subsequent banging produced a jittery phenotype not observed in controls. Individual animals seemed to have mild convulsions under this regime, from which they recovered over a period of minutes. In addition, although the *nac* epitope cannot be detected at any temperature, at 18°C *nac* flies born from heterozygous parents display morphological abnormalities (abnormal formation of the wing and assembly of the eye facets). Moveover, *nac* females are sterile at this temperature (W.Moats, Z.Smith and F.Katz, unpublished observations). These cold-sensitive phenotypes are currently being investigated. Under normal laboratory conditions, however, animals appeared to live normal (and productive) lives, despite the absence of correct glycosylation on multiple neuronal cell surface proteins.

Table I. Complementation of *nac* and the *TM3* embryonic mutation

Genotype	Developmental stage[a]	Anti-HRP staining
TM3/TM3	E	−
nac/TM3	L, P	+
Df(3R)dsx^{D+R5}/*TM3*	E, L, P	+
nac/Df(3R)dsx^{D+R5}	L	+
nac/Df(3R)dsx^{D+R5}	P, A	−

[a]E, embryo; L, larva; P, pupa; A, adult.

Discussion

We have shown that antibodies against HRP, previously demonstrated to recognize specifically the nervous system of *Drosophila* and other insects, recognize a complex array of proteins that share a common epitope. The larval and adult epitope appears to be carbohydrate in nature. It is resistant to denaturation and exhaustive pronase digestion, and is destroyed by mild periodate treatment. A similar conclusion has been reached from an analysis of the epitope in the embryo (Snow *et al.*, 1987). We demonstrate here that the epitope shares structural features with the carbohydrate moiety of pineapple stem bromelain, a plant glycoprotein whose carbohydrate structure has been determined to be α-D-Man (1→2)-α-D-Man-(1→2 or 6)-[α-L-Fuc-(1→6 or 2)]-α-D-Man-(β-D-Xyl)-β-D-GlcNAc-(1→3 or 4)-β-D-GlcNAc-(1→β-NH_2-N of Asn)-peptide (Yasuda *et al.*, 1970).

Which features of this structure constitute the epitope is as yet undetermined. The core $ManGlcNAc_2$ linked to asparagine seems unlikely as it is a common core element in many glycoproteins, including RNase B and fetuin, which are not recognized by anti-HRP. Moreover, in contrast to the neurally restricted expression of this epitope,

$Man_3GlcNAc_2$ is a common feature of insect glycoproteins isolated from *Drosophila* and mosquito cell lines. Complex type N-linked oligosaccharides, on the other hand, are not observed (Hsieh and Robbins, 1984; Butters *et al.*, 1981). The fucose and/or xylose moieties found in the bromelain carbohydrate are therefore likely candidates to participate in the epitope. Neither, however, is individually sufficient, as hapten competition with these sugars is ineffective in blocking antibody recognition (unpublished data). Both xylose and fucose have been found associated with papilin, a proteoglycan-like glycoprotein isolated from *Drosophila* Kc cells (Campbell *et al.*, 1987), but their linkages are unknown.

Whereas all neurons throughout development express this epitope, the proteins that carry it change over development. It is therefore possible that the carbohydrate is specific for neural surfaces rather than particular proteins, and is added to most cell surface proteins in neurons as they are transported to the plasma membrane. In addition, as individual proteins that bear the epitope can be found both outside the nervous system and without the epitope (Snow *et al.*, 1987) it is likely that the carbohydrate is not necessary for cellular aspects of glycoprotein processing, such as intracellular transport or stability (cf. Olden *et al.*, 1982), but rather subserves some general role useful to neurons at their surfaces. The conservation of this epitope throughout the insect kingdom, moreover, suggests it has a necessary function. In this respect, the phenomenology of expression of this epitope is intriguing, as it is present on the cell surface of neurons at critical developmental periods. It is found on 5 h gastrulation stage cells that later become neurons (Salvaterra *et al.*, 1987) and is expressed on the growth cones of sprouting neurons. In the grasshopper limb bud, its expression is correlated with growth cone behavior (see Introduction) suggesting it may play a role in cell–cell interactions. Precedents for such a role for carbohydrates exist (cf. Sharon, 1975; Glabe *et al.*, 1982; Edelman, 1984; Florman and Wassarman, 1985; Yednock *et al.*, 1987; Kunemund *et al.*, 1988; Rutishauser *et al.*, 1988; Bayna *et al.*, 1988).

A mutation on the third chromosome, *nac*, has been identified that allows us to explore the function of this moiety during the development of the imaginal nervous system and in adult behavior. We find that the alteration of this epitope as expressed in this mutant has little effect on peripheral pathfinding or pattern formation under controlled laboratory conditions (at 25°C). Adult behavior is also little affected. A battery of behavioral tests suggest that normal neural circuits are established. However, under stress these flies exhibit aberrant behavior that uncovers a hidden dysfunction. Similarly, they display developmental abnormalities at 18°C, including defects in the assembly of the ommatidia of the eye. The origin of these stress responses is not known. It is of interest that lack of anti-HRP staining in TM3 homozygote embryos is correlated with small changes in neuronal architecture which leaves the overall pattern intact (Snow *et al.*, 1987). It is conceivable that this epitope is part of a system of redundant information necessary for correct neurogenesis, which can only be completely uncovered when multiple molecules are simultaneously disturbed. If absence of correct glycosylation leads to an increased mistake frequency during the assembly of neural networks, such mistakes might only be revealed when the animals are subjected to stress. On the other hand, in this mutant it is possible the functional aspect of the carbohydrate moiety is partially masked but not totally disabled, producing residual function that is further compromised under stress. In addition, we cannot rule out the formal possibility that two closely linked loss of function mutations map within the deficiency interval.

In that a single mutation is able to eliminate the expression of a carbohydrate epitope on multiple proteins, it appears likely that *nac* encodes a molecule involved in some aspect of carbohydrate metabolism or processing. We have been unable to directly demonstrate that loss of the epitope occurs without loss of the proteins that bear it. However, a monoclonal antibody, 3B2, has been shown to recognize exclusively a subset of the proteins recognized by anti-HRP (Snow *et al.*, 1987). *nac* imaginal neural tissue continues to stain with this antibody (unpublished data) consistent with the suggestion that a local perturbation of the carbohydrate moieties, rather than loss of the protein molecules themselves, occurs in the mutant. Similarly, TM3 homozygote embryos continue to stain with this reagent (Snow *et al.*, 1987). Given the mild effect of this mutation, it seems unlikely that some more global aspect of carbohydrate metabolism (for which the anti-HRP epitope serves as an indicator in neural cells) is affected. Loss of a carbohydrate epitope can be caused by physical absence of the relevant sugar moieties, by masking through the presence of secondary modifications such as phosphorylation or sulfation (Hubbard and Ivatt, 1981), or by additional sugar units attached to the epitope. Single monosaccharides are sufficient to mask an epitope (Feizi, 1985) and it has been suggested that transient expression of carbohydrate epitopes during development might be caused by the addition or deletion of monosaccharides (Feizi, 1985). We cannot distinguish between absence and masking at this time.

Finally, a second mutation, contained on the third chromosome balancer, TM3, eliminates anti-HRP recognition of embryonic neural tissue. We observe no interaction between this gene and the *nac* mutation, or a deficiency that uncovers *nac*, and conclude that these represent separate genes, either developmental counterparts or genes involved in different parts of the pathways concerned with the synthesis or modification of the glycoconjugate.

In summary, we have identified a new mutant in *Drosophila melanogaster* which results in the alteration (or loss) of a normally neuron-specific glycoconjugate. The analysis of this moiety has suggested that what is most likely a common plant carbohydrate structure is found unexpectedly in the nervous system of insects. The viability associated with the *nac* mutation should make it a useful marker for mosaic experiments in the adult nervous system of *Drosophila*. At the same time, an analysis of its behavior in combination with other mutations affecting the neuronal cell surface may contribute to an understanding of its function. Such studies are in progress.

Materials and methods

Drosophila strains

Stocks were maintained at room temperature on standard cornmeal–molasses–agar medium. All mutations are as described in Lindsley and Grell (1968). The following deficiencies were used to map the mutant locus: Df (3R) Hu^{+RX1} (Hazelrigg and Kaufman, 1983); Df(3R)$Antp^{NS+R17}$ (Duncan and Kaufman, 1975); *Ki roe* Df(3R) dsx^{D+R2},

and Df(3R)dsx^{D+R5} *bx sr e* (Duncan and Kaufman, 1975; revised breakpoints as indicated in Figure 7: Bruce Baker, personal communication).

EMS mutant screen

Male flies isogenic for the third chromosome and marked with *red cv-c* sbd^2e" were fed ethyl methanesulfonate (EMS) according to the procedure of Lewis and Bacher (1968), and mass mated to virgin females that were *DTS-7 st* p^p/*TM1* (Holden and Suzuki, 1973) at room temperature. *DTS-7* is a dominant temperature-sensitive lethal mutation that dies at 29°C but is viable at room temperature. Male F1 progeny were selected and pair-mated to *DTS-7 st* p^p/*TM1* virgins and kept at room temperature for 4 days. On the fourth day the parents were discarded and the vials were put at 29°C. Surviving males were mated to sibling virgin females (*red cv-c* sbd^2 e"/*TM1*) at room temperature and the F3 were examined for late pupal lethals. Those vials containing red larvae and arrested late ebony pupae, but lacking red ebony adults, were selected and maintained as balanced stocks over *TM1*.

Meiotic and deficiency mapping

The mutation *nac* (on a chromosome containing *red cv-c* sbd^2 e^{11}) was mapped by meiotic recombination with the following chromosomes: *ru h st cu sr* e^s *ca* and *th st cp in ri Ch Ki* p^p. Recombinant flies (either homozygous or as heterozygotes with the original chromosome) were tested behaviorally and histologically (see below). As the original chromosome contained a pupal lethal mutation in addition to *nac*, some recombinants were maintained as balanced stocks. The mutant was further tested for complementation of the anti-HRP-staining negative phenotype with the deficiencies cited above (*Drosophila* strains).

Histology

Imaginal discs were prepared for histology as described (Jan *et al.*, 1985). Affinity-purified IgG fraction of rabbit anti-HRP (Cappel) was used at dilutions of 1:3000 unless otherwise indicated; rhodamine or FITC-conjugated affinity-purified goat anti-rabbit IgG (Cappel) was used at 1:250. Blocking was in 1% normal goat serum overnight at 4°C. Supernatant from Mab 44C11, which specifically recognizes neuronal nuclei (Bier *et al.*, 1988), was used at a dilution of 1:5 followed by FITC-conjugated goat anti-mouse IgM (Cappel) at 1:250. Slides were mounted in glycerol: phosphate buffer, pH 7.2 (4:1) for observation by fluorescence microscopy on a Zeiss ICM-405 microscope. Homozygous mutants and recombinants that retained the red marker mutation could be selected as larvae or pupae by their red malpighian tubules. Imaginal discs were then prepared for histology according to the standard protocol. Similarly, ebony homozygote pupae could be selected by the failure of their cuticle to tan by 1 h after puparium formation (APF). For those recombinants which had lost both red and ebony, stocks were created over a *TM6B Tb* e^s *ca* balancer (Craymer, 1984) and homozygotes were recognized as non-Tubby individuals. A similar use of the *TM6B* balancer for deficiency mapping in the absence of red and ebony was utilized.

Heads were removed from mutant and control flies and prepared for sectioning directly in OCT (Miles). Mutant and control heads were mounted in the same block so that all subsequent procedures would be identical. Sections of 12 μm were cut on a Reichart-Jung Frigocut 2800 and collected on microscope slides. Sections were fixed for 30 min in 4% paraformaldehyde/ 0.1 M phosphate buffer, pH 7.2, rinsed and incubated sequentially with anti-HRP and anti-rabbit Vecta staining solutions (Vector Labs) according to their procedure.

Embryos were collected on apple juice plates over 10−12 h, incubated until all embryos were 11−22 h old, and fixed according to the procedure of Bodmer and Jan (1987) with the following modifications: dechorionated embryos were fixed in heptane and 4% formaldehyde (Polysciences) in 0.1 M phosphate buffer pH 7.3 with 3% DMSO for 20−25 min on a shaker at 250 r.p.m. Embryos were removed from the fix−heptane interface and rinsed in 0.03% Triton X-100 in phosphate buffer. Excess liquid was removed and embryos were frozen in OCT for cryostat sectioning. To look at the complementation behavior of *nac* and the *TM3* balancer, egg lays from a Df(3R) dsx^{D+R5}/*TM3 Sb Ser e* stock were collected, sectioned and double labeled with rabbit anti-HRP and the mouse monoclonal 44C11. *TM3* homozygous embryos lack anti-HRP staining. The homozygous deficiency arrests before neuralization (unpublished observations). Therefore, if Df(3R) dsx^{D+R5} fails to complement *TM3*, all embryos in the egg lay that stain with 44C11 (that is, have neural tissue) should be anti-HRP negative. If, on the other hand, *TM3* complements the deficiency, only one third of the neuralized embryos (representing *TM3* homozygotes), should be negative with anti-HRP, which was observed.

Radiolabeling of 3rd instar larval CNS and immunoprecipitation

The ventral ganglion and brain lobes of Oregon R third-instar larvae were dissected in minimal medium (MME; Siegel and Fristrom, 1978), treated wth 0.1 mg/ml collagenase (Worthington) for 10 min at room temperature, and washed in MME several times. [^{35}S]methionine (Amersham, 1055 Ci/mmol) was added to a concentration of 200 μCi/ml and the tissue was incubated at 25°C for 5 h, followed by washes with MME, then with buffer (IVT) containing 20 mM Tris−HCl, pH 7.4, 150 mM NaCl, 10 mM EDTA and an inhibitor mix (100 μM benzamidine, 10 μM leupeptin, 1 μM EGTA, 0.1 μM pepstatin A, 100 KIU/ml aprotinin, 10 mM sodium metabisulfite and 0.02 mg/ml soybean trypsin inhibitor). After sonication to disrupt the tissue, NP40 was added to a concentration of 1% and incubation proceeded for 30 min on ice, followed by clarification at 4°C for 15 min at 10 000 r.p.m. (Eppendorf microfuge). The supernatant was diluted with IVT to 0.1% NP40 and Staph A cells (Pansorbin, Calbiochem) with bound anti-HRP were added. After incubation at 4°C for 3 h or overnight, Staph A cells with bound protein were washed four times in IVT containing 0.5 M salt, protein was solubilized in Laemmli sample buffer (see below), and the cells were pelleted. Supernatants were analyzed by SDS−PAGE (see below).

Preparation of tissue homogenates and antibody incubations in SDS gels

Embryos (Oregon R) were collected at 25°C for 7 h and aged at 25°C for 11 h to give a population 11−18 h old. Embryos were dechorionated for 1.5 min in 50% bleach:50% water and frozen in liquid nitrogen. They were then ground to a powder on a mortar precooled on dry ice, and homogenized in a polytron homogenizer (Brinkman) in a buffer containing PBS, pH 7.3 with an inhibitor mix (see above). The homogenate was clarified by a low speed centrifugation. Aliquots of the supernatant were mixed with Laemmli sample buffer and run on SDS-PAGE.

Larval and adult brain and thoracic ganglia were dissected in Robbs (Martin and Schneider, 1978), homogenized in Laemmli sample buffer in a ground glass microhomogenizer, clarified by brief centrifugation at 10 000 *g*, and loaded on SDS−PAGE.

Gels to be incubated with antibodies were processed according to the procedure of Burridge (1978) using 3% acrylamide stacking and 6% acrylamide resolving gels (Laemmli, 1970). Rabbit anti-HRP at a concentration of 1:10 (0.4 mg/ml) was spread as a thin layer on top of the gel. Control gels were incubated with equivalent amounts of nonspecific rabbit IgG. Both were subsequently reacted with [^{125}I]Protein A (87.9 μCi/μg; NEN) at 10^6 c.p.m./ml.

Periodate treatment and enrichment of the HRP carbohydrate

Periodate oxidation of HRP and bromelain was according to the procedure of Yasuda *et al.* (1970). After termination of the reaction with ethylene glycol, the mixture was dialyzed against 0.1 M NaCl and then distilled water at 4°C. Control incubations were treated identically but sodium *meta*-periodate was omitted. Both treated HRP and its control were used to pre-absorb rabbit anti-HRP at a 4-fold molar excess of HRP to antibody. The pre-absorbed antibody was then used to stain third instar larval discs according to the standard protocol. Alternatively, aliquots of the treated and control proteins were run on SDS−PAGE, blotted to nitrocellulose and stained with anti-HRP (see below).

To enrich the HRP carbohydrate, HRP was digested with pronase (Sigma) according to the procedure of Spiro (1966). The pronase was then heat inactivated for 30 min at 85°C and the resultant material clarified by centrifugation. The supernatant was passed over a Con A−sepharose 4B (Pharmacia) column, previously calibrated to give >95% retention of HRP and subsequent release with α-methylmannoside, and washed extensively. The bound carbohydrate was released with 0.5 M α-methylmannoside (Katz *et al.*, 1977). The eluate, or a control eluate from a column without HRP, was used to pre-absorb anti-HRP, which was subsequently used to stain imaginal discs according to the normal procedure. The HRP eluate had no detectable ninhydrin positive material.

Electrophoresis and western blots

SDS−PAGE was performed as described by Laemmli (1970). Mol. wts were calibrated using the following standard proteins: lysozyme (14 400), soybean trypsin inhibitor (20 000), carbonic anhydrase (30 000), ovalbumin (43 000), bovine serum albumin (67 000), phosphorylase B (94 000), β-galactosidase (116 000) and myosin (200 000). Gels were stained with Coomassie blue (Fairbanks *et al.*, 1971) and destained (Weber and Osborn, 1969). Gels containing [^{35}S]methionine-labeled proteins were treated for 15 min with Enlightning (NEN) before drying. Autoradiography of the dried gels was performed using Kodak X-omat film with a double intensifying screen (Dupont Quanta III) at −80°C.

Western blots were prepared according to the procedure of Towbin *et al.* (1979) with the following modifications: rabbit anti-HRP was incubated with the blocked blot for 3 h at room temperature. The blot was washed three

times with TBS/0.05% Tween 20, then incubated with the Vectastain ABC reagents as recommended by the manufacturer (Vector Laboratories). Blots were developed using 4-chloronaphthol.

Protein was determined by the method of Bradford (1976; Bio-rad reagent).

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References

Bayna,E.M., Shaper,J.H. and Shur,B.D. (1988) *Cell*, **53**, 145–157.
Bentley,D. and Caudy,M. (1983) *Cold Spring Harbor Symp. Quant. Biol.*, **48**, 573–585.
Bier,E., Ackerman,L., Barbel,S., Jan,L.Y. and Jan,Y.N. (1988) *Science*, **240**, 913–916.
Bodmer,R. and Jan,Y.N. (1987) *Roux's Arch. Dev. Biol.*, **196**, 69–77.
Bradford,M.M. (1976) *Anal. Biochem.*, **72**, 248–254.
Burridge,K. (1978) *Methods Enzymol.*, **50**, 54–64.
Butters,T.D., Hughes,R.C. and Vischer,P. (1981) *Biochim. Biophys. Acta*, **640**, 672–686.
Campbell,A.G., Fessler,L.I., Salo,T. and Fessler,J.H. (1987) *J. Biol. Chem.*, **262**, 17605–17612.
Clarke,J. and Shannon,L.M. (1976) *Biochem. Biophys. Acta*, **427**, 428–442.
Craymer,L. (1984) *Dros. Inf. Serv.*, **60**, 234.
Caudy,M. and Bentley,D. (1986a) *J. Neurosci.*, **6**, 364–379.
Caudy,M. and Bentley,D. (1986b) *J. Neurosci.*, **6**, 1781–1795.
Duerr,J.S. and Quinn,W.G. (1982) *Proc. Natl. Acad. Sci. USA.*, **79**, 3646–3650.
Duncan,I.W. and Kaufman,T.C. (1975) *Genetics*, **80**, 733–752.
Edelman,G. (1984) *Trends Neurosci.*, **7**, 78–84.
Fairbanks,G., Steck,T.L. and Wallach,D.F.H. (1971) *Biochemistry*, **10**, 2606–2617.
Feizi,T. (1985) *Nature*, **314**, 53–57.
Florman,H.M. and Wassarman,P.M. (1985) *Cell*, **41**, 313–324.
Ganetzky,B. and Wu,C.F. (1986) *Annu. Rev. Genet.*, **20**, 13–44.
Glabe,C.G., Grabel,L.B., Vacquier,Y.D. and Rosen,S.D. (1982) *J. Cell. Biol.*, **94**. 123–128.
Hazelrigg,T. and Kaufman,T.C. (1983) *Genetics*, **105**, 581–600.
Holden,J.J. and Suzuki,D.T. (1973) *Genetics*, **73**, 445–458.
Hsieh,P. and Robbins,P.W. (1984) *J. Biol. Chem.*, **259**, 2375–2382.
Hubbard,S.C. and Ivatt,R.J. (1981) *Annu. Rev. Biochem.*, **50**, 555–583.
Jan,L.Y. and Jan,Y.N. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 2700–2704.
Jan,Y.N., Ghysen,A., Christoph,I., Barbel,S. and Jan,L.Y. (1985) *J. Neurosci.*, **5**, 2453–2464.
Katz,F.N., Rothman,J.E., Lingappa,V.R., Blobel,G. and Lodish,H.F. (1977) *Proc. Natl. Acad. Sci. USA.*, **74**, 3278–3282.
Kunemund,V., Jungalwala,F.B., Fischer,G., Chou,D.K.H., Keilhauer,G. and Schachner,M. (1988) *J. Cell Biol.*, **106**, 213–223.
Laemmli,U.K. (1970) *Nature*, **277**, 680–685.
Lewis,E.B. and Bacher,F. (1968) *Dros. Inf. Serv.*, **43**, 193.
Lindsley,D.L. and Grell,E.H. (1968) *Genetic Variations of Drosophila melanogaster*. Carnegie Institution of Washington, Publication No. 627.
Martin,P. and Schneider,I. (1978) In Ashburner,M. and Wright,T.R.F. (eds), *Genetics and Biology of Drosophila*. Academic Press, New York. pp. 219–264.
Murray,M.A., Schubiger,M. and Palka,J. (1984) *Dev. Biol.*, **104**, 259–273.
Olden,K., Bernard,B.A., White,S.L. and Parent,J.B. (1982) *J. Cell. Biochem.*, **18**, 313–335.
Phelps,C., Forlani,L. and Antonini,E. (1971) *Biochem. J.*, **124**, 605–614.
Rutishauser,U., Acheson,A., Hall,A.K., Mann,D.M. and Sunshine,J. (1988) *Science*, **240**, 53–57.
Salvaterra,P.M., Bournias-Vardiabasis,N., Nair,T., Hou,G. and Lieu,C. (1987) *J. Neurosci.*, **7**, 10–22.
Sharon,N. (1975) *Complex Carbohydrates, Their Chemistry, Biosynthesis and Functions*. Addison-Wesley Publishing Company, Inc., Reading, MA.
Siegel,J.G. and Fristrom,J.W. (1978) In Ashburner,M. and Wright,T.R.F. (eds), *The Genetics and Biology of Drosophila*. Academic Press, New York, pp. 317–394.
Snow,P.M., Patel,N.H., Harrelson,A.L. and Goodman,C.S. (1987) *J. Neurosci.*, **7**, 4137–4144.
Spiro,R.G. (1966) *Methods Enzymol.*, **8**, 26–52.
Towbin,H., Staehelin,T. and Gordon,J. (1979) *Proc. Natl. Acad. Sci. USA.*, **76**, 4350–4354.
Truman,J.W. and Bate,M. (1988) *Dev. Biol.*, **125**, 145–157.
Vandervorst,P. and Ghysen,A. (1980) *Nature*, **286**, 65–67.
Weber,K. and Osborn,M. (1969) *J. Biol. Chem.*, **244**, 4406–4412.
Welinder,K.G. and Smillie,L.B. (1972) *Can. J. Biochem.*, **50**, 63–90.
White,K. and Kankel,D.R. (1978) *Dev. Biol.*, **65**, 296–321.
Yasuda,Y., Takahashi,N. and Murachi,T. (1970) *Biochemistry*, **9**, 25–32.
Yednock,T.A., Stoolman,L.M. and Rosen,S.D. (1987) *J. Cell Biol.*, **104**, 713–723.

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