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The rapid assembly of synaptic sites in photoreceptor terminals of the fly's optic lobe recovering from cold shock

(synaptic plasticity/synaptic ribbon/capitate projection/temperature reversal)

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ABSTRACT **When a housefly, *Musca domestica*, is subject to cold exposure (0°C for 24 hr), a number of obvious changes are seen in the first optic neuropil, or lamina, beneath the compound eye. In particular, the number of afferent photoreceptor synapses declines by about 30%. This loss is dramatically restored after warm recovery at 23°C for 24 hr. Synapses disappear at an average rate of 2–3/hr during cold exposure and reappear at a maximal rate of more than 20/hr during the first 2 hr of warm recovery. Thereafter their number temporarily overshoots control values, to increase at 6 hr of warm recovery to 60% above their cold-exposed minimum. The number subsequently returns more or less to normal. These changes demonstrate the lability of synaptic sites under these conditions, with individual sites forming and disappearing rapidly. The changes also interrupt the close correlation between synaptic number and the surface area of the receptor terminal, a correlation that normally conserves synaptic spacing density. The density is preserved during cold exposure but increases during warm recovery at a time when the addition of newly formed synapses exceeds the slower increase in receptor terminal size.**

Synaptic contacts between neurons are widely thought to be continually replaced in the adult nervous system (1), with the scrupulous preservation of particular synaptic partnerships by replacement synapses. The fission of large existing synaptic sites into smaller ones (2) provides one means to preserve synaptic specificity, but multiple-contact synapses, such as are found in the vertebrate retina (e.g., refs. 3 and 4) or many invertebrate nervous systems (5), cannot divide in this way. This is because the division of large synapses would entail not only the division of the single presynaptic site but also the integrated division of the several postsynaptic dendrites, so that at these synapses turnover must involve other means of forming new sites. In photoreceptors of the first neuropil, or lamina, of the fly's optic lobe, for example, the afferent synaptic contacts are tetrads (6, 7). Each tetrad synapse is generally of rather fixed size (8), and its population is regulated primarily through the number of such sites (9), which is remarkably constant in the terminals of an individual fly (7, 34).

The regulation of the photoreceptor synaptic population is dynamic, since even in an adult fly individual sites can both form and disappear. New tetrads have been shown to form, for example during light exposure, either after dark rearing (10) or as part of a circadian rhythm (11). Conversely, existing sites must be lost, because their number decreases, either with age (9) or with dark exposure (10). In no case, however, have stages in either the formation of new sites or the loss of existing ones been clearly identified, although there is evidence to indicate tentatively the accretion of protein monomers to new presynaptic ribbons (10). Furthermore, it is not directly established that a dynamic equilibrium between both losses and gains normally exists, to constitute a cycle of turnover. The existence of a circadian rhythm of synaptic number (11) does, however, imply that gains and losses must coexist within a 24-hr period and are thus probably concurrent. To quantify these further necessitates separation of the individual rates of loss or formation among synapses.

We report here that cold shock in a fly produces a reproducible decrease in the synaptic number that is entirely reversible. After the fly returns to a normal temperature, the rate of recovery of synaptic numbers from this loss provides a means to assess the rate of formation of new synaptic sites, using a thermal stress not routinely available in warm-blooded animals.

MATERIALS AND METHODS

Animals. Houseflies, *Musca domestica*, were reared in continuous culture at 23°C under a regulated light/dark cycle as described (12). One-day-old females were used exclusively. Flies were cooled in a glass vial placed in a cooling bath at 0°C. To test the effects of cooling on the lamina, flies were cooled for periods of 2, 12, 24, and 36 hr. Immediately after cold treatment they were dissected on ice, to prevent the heads from possible warm recovery. To examine the recovery from cold, flies were kept continuously for 24 hr at 0°C, after which they were returned to room temperature at 23°C and left to recover for 2, 6, 12, and 24 hr. They were then dissected at room temperature. Control flies of the same age as experimental flies, but that had undergone no treatment, were also examined.

Electron Microscopy. The eyes of control and experimental flies were fixed and processed for thin-section electron microscopy using previously described methods (12, 13) and sectioned at 65 nm; the sections were photographed on 35-mm film at magnifications of either 2200 or 3300. Synaptic counts were made from prints of these micrographs as described, and measurements of receptor terminal profiles were made by computer planimetry, also as described (12). In addition the frequencies of the profiles of capitate projections (14) and of mitochondria were counted.

Statistical Methods. Correlations were determined for various values during either cold exposure (the first 24 hr) or warm recovery (the second 24 hr). For both periods, the numbers of profiles of synapses, mitochondria, and capitate projections and the perimeters of the receptor terminals were each correlated using the Spearman's rank-correlation test (15). To compare any two consecutive values, either between control and experimental values or during the period of warm recovery or at the change from cold exposure to warm recovery, the *U* test of Wilcoxon, Mann, and Whitney (15) was chosen. Levels of significance are designated by R (Spearman's rank correlation) or U (*U* test). Each data point was

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derived from an examination of between 160 and 260 receptor terminals from at least two flies.

RESULTS

This study extends a body of quantitative data on the lamina of *Musca* (16, 17) in which cells are organized into modules, called cartridges, that are most propitious for anatomical analysis. Each cartridge is innervated by a group of receptor terminals that encircle a fixed group of uniquely identifiable lamina interneurons (Fig. 1). There are eight of these lamina cells in each cartridge, and these include two monopolar cells, L1 and L2, that are postsynaptic at all tetrad synapses (7). Not only does the cartridge comprise a fixed number of cells, but these are also ensheathed by epithelial glial cells (20, 21), delimiting each cartridge from others in the lamina's array (Fig. 1). The many cartridges, bundled in parallel like sheaves, are cut all together in a single transverse section to yield an array of receptor terminal profiles, from which an accurate average number of synaptic profiles per terminal can be readily counted.

Effects of Cold on the Lamina as a Whole. Initially, it was necessary to determine the period of cold exposure that produced the greatest effects on the lamina. Although all flies readily survived cold treatment for periods up to 36 hr, the effects at 36 hr were no different than at 24 hr, and since the longer period presented the flies with a larger stress, we chose 24 hr of cold exposure as the standard pretreatment for this study.

Cold caused all elements in the lamina to undergo extensive changes during the 24-hr period of exposure. Generally it caused the receptor terminals to shrink, so that cartridges in flies cold exposed for 24 hr were at least 30% smaller in diameter than cartridges in control flies. Cold had the greatest and most immediate effect on the epithelial glial cells, which showed in many cases, by contrast, extreme hypertrophy. Their mitochondria reacted most sensitively to cold, becoming enormously swollen with disintegrated cristae. These changes among the epithelial glial cells disturbed the normally regular organization of cartridges in the lamina of flies cold exposed for 12 or 24 hr. Remarkably, however, the changes were all reversible: all cells recovered very shortly after the flies were returned to 23°C.

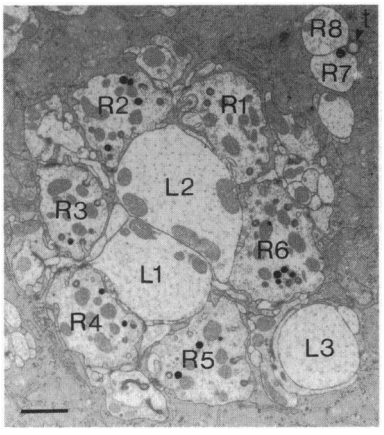

FIG. 1. Cross section of a lamina cartridge, from a fly at 23°C, revealing the circle of receptor terminals (R1–R6) surrounding the axial axonal profiles of L1 and L2. Two other photoreceptors, R7 and R8, penetrate the lamina where together with a tracheole (t) they form a characteristic triad (13). Profiles of all lamina cells are identifiable from their position within this cross section (18, 19), but only the position of a third monopolar cell profile (L3) is marked; additional profiles are neither labeled nor dealt with further in this study. (Scale bar = 1.0 μm.)

The Effects of Cold on the Receptor Terminals. Generally receptor terminals shrank in the cold, and their mitochondrial profiles became fewer in number and long and spiny in shape.

After 2 hr of cold exposure, the main change in the receptor terminals was an abundance of invaginations between neighboring receptor terminals, which caused the appearance of membrane whorls (Fig. 2*a*) containing a large area of extra membrane. After 12 hr of cold exposure, the plasmalemma had equilibrated and receptor invaginations were rare, but in their place monopolar cells invaginated into the receptor terminals (Fig. 2*b*). The usually round or elliptical shape of many of the mitochondrial profiles had already clearly changed at this stage to a long, spiny shape (Fig. 2*b*). After 24 hr of cold exposure, the receptor terminals attained their smallest girth, and the cytoplasm, possibly in consequence, looked very dense, with the few mitochondrial profiles, mostly long and thin in shape (Fig. 2*c*). Monopolar cell invaginations into the receptor terminals were smaller and less frequent than after 12 hr of cold exposure. The most obvious feature in the receptor terminals after 24 hr was a significant increase in the number of pigment granules, which normally number no more than one or two per single cross section, but which were now densely packed at the core of the terminal (Fig. 2*c*). Neither the depth distribution of these within the photoreceptor nor whether those in the lamina represent a redistribution from the retina is known.

Recovery from Cold Exposure. Despite their appearance, the receptor terminals did not degenerate. All the previous changes reversed themselves, recovering significantly even 2 hr after the flies were returned to 23°C. After 2 hr of recovery, the receptor terminals looked somewhat emptier than normal, because the density of synaptic vesicles and microtubules was low (Fig. 2*d*). Invaginations were lacking. After 6 hr of recovery at 23°C (Fig. 2*e*) and increasingly after 24 hr of recovery (Fig. 2*f*), the receptor terminals and the numbers of their organelle profiles became progressively indistinguishable from control terminals (Fig. 1).

Changes in the Number of Afferent Photoreceptor Synapses. During cold exposure the number of tetrad synaptic profiles (Fig. 2*c Inset*) decreased significantly (R: $P < 0.001$) by about 30% over 24 hr (Fig. 3). Since the initial synaptic population numbers about 200 sites (7), this corresponds with an overall loss rate of 2–3/hr. Soon after recovery at 23°C, however, this loss was more than offset by an increase in the number of synaptic sites, leading to an overshoot in synaptic number after 6 hr of recovery (Fig. 3). After 2 hr of recovery from cold, synaptic sites already numbered about 30% higher than the lowest level after 24 hr of cold exposure, a significant (U: $P < 0.001$) increase that corresponds with an initial rate of reformation of ribbons of more than 20/hr. After 6 hr of warm recovery, the number of tetrad synapses overshot control values by about 10% (U: $P < 0.02$), a 60% increase over their number 6 hr earlier, which is significant (U: $P < 0.001$). After this overshoot at 6 hr, the number of tetrad synapses decreased again during warm recovery and returned to normal control values at 24 hr (Fig. 3). This decrease in the number of tetrad profiles was also significant (R: $P < 0.01$).

Changes in the Density of Tetrad Synapses. One of the most obvious effects of cold on the receptor terminals was the shrinkage in their girth. The mean perimeter of the terminals' profiles showed a steady decrease over the period of cold exposure, attaining a highly significant (R: $P < 0.001$) reduction of about 30% over the 24-hr period of cold exposure (Fig. 3). After the flies had returned to 23°C for 2 hr, there was already a highly significant (U: $P < 0.001$) gain in the receptor terminals' perimeter of about 15%, and this increase continued over the 24-hr recovery period (Fig. 3), even though the terminals did not fully recover their control size within this time.

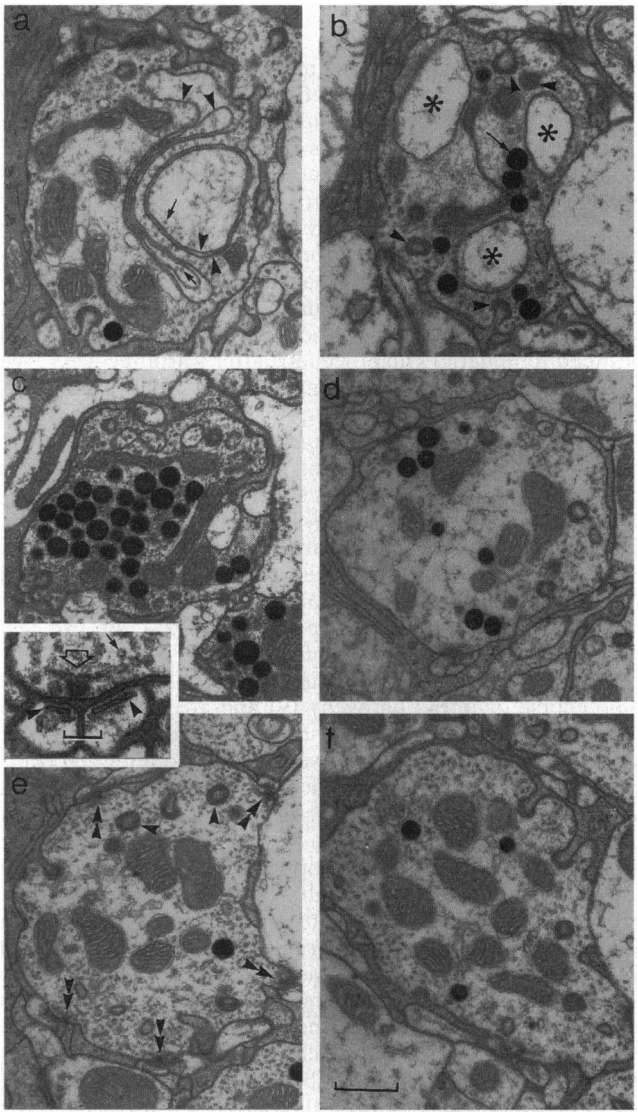

FIG. 2. Receptor terminals from flies at different times after cold exposure and warm recovery. (*a*) After 2 hr at 0°C, invaginations appear in the receptor terminal from its neighboring terminals. Two invaginating profiles visible are both double, as shown by the two double membranes (arrowheads). The receptor identity of the invaginating element is shown by occasional synaptic vesicles (arrows) similar to those within the terminal's own cytoplasm. (*b*) After 12 hr at 0°C, the terminal is smaller with more pigment granules (arrow) and fewer capitate projections (arrowheads). Three invaginations (asterisks) in the receptor terminal come from monopolar cells; their cytoplasm matches that of the parent axon (L) in confirmation of this origin. (*c*) After 24 hr at 0°C, the receptor terminal is most shrunken and the pigment granules pack its core. (*d*) After 2 hr of warm recovery at 23°C, the receptor terminal enlarges again, invaginations from monopolar cells disappear, and the number of pigment granules decreases. (*e*) After 6 hr of warm recovery at 23°C, the receptor terminal is almost completely expanded, and the numbers of capitate projections (arrowheads) and pigment granules return to normal, as do the apparent numbers of synaptic vesicles. Double arrowheads indicate tetrad sites. (*f*) After 24 hr at 23°C, the structure of the receptor terminal is again normal and indistinguishable from a control terminal. (For *a–f*, scale bar = 0.5 μm.) (*c Inset*) Enlarged view of tetrad. Synaptic vesicles (arrow) distributed throughout the receptor terminal cytoplasm are concentrated at presynaptic ribbons (open arrow), opposite which cisternae (arrowheads) confirm the spines of L1 and L2 as postsynaptic sites. (Scale bar = 0.1 μm.)

The synaptic spacing density, a function of the number of synaptic sites (Fig. 3) and the surface area of the receptor terminals [derived from the average perimeter of their cross

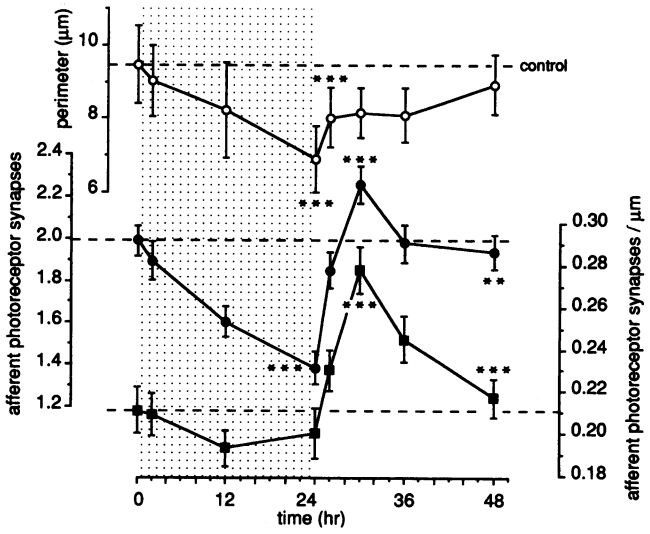

FIG. 3. Average number of profiles of tetrad synapses and the average plasmalemma perimeter per receptor terminal cross section, and their quotient, in flies subject to a period of cold exposure at 0°C (stippled), followed by recovery at 23°C. Changes in synaptic frequency (●) are significant when the following comparisons are made. Compared with control values, there are significantly fewer synaptic profiles after 24 hr of cold; compared with 24 hr of cold, there are significantly more after 6 hr of warm recovery; and, finally, compared with 6 hr of warm recovery, there are significantly fewer profiles after 24 hr of warm recovery. The perimeter of the receptor terminal profiles (○), a measure of membrane surface area, decreases significantly after 24 hr of cold treatment, compared with control values, and increases significantly during the first 2 hr of warm recovery. The average frequency of synaptic profiles per unit membrane perimeter (■), a measure of synaptic spacing density, remains constant throughout cold exposure, but compared with control values it increases significantly after 6 hr of warm recovery, with respect to which there is then a significant decrease after 24 hr of warm recovery. Levels of significance are $P < 0.001$ (***) and $P < 0.01$ (**).

section (Fig. 3)], is normally closely regulated (22). The number of tetrad synaptic sites per unit membrane perimeter showed no significant change during the 24-hr period of cold exposure (R: $P > 0.3$); the reductions in the number of synaptic sites matched reductions in the size of the terminals at different periods of cold exposure (2, 12, and 24 hr: Fig. 3). Covariance of synaptic number and membrane perimeter thus preserved the synaptic spacing closely during the slow changes during cold exposure. During the first 6 hr of recovery at 23°C, however, compared with the density for normal control terminals, there occurred a striking 35% increase in synaptic spacing density (Fig. 3), which was highly significant (U: $P < 0.001$). Thus during these first 6 hr of warm recovery, there were many more new synaptic sites gained than was commensurate for the increase in terminal size, indicating that the normally tight correlation of synaptic number and the surface area of receptor terminal membrane was lost. The loss was temporary, however, because after the increase in synaptic spacing density at 6 hr, spacing densities decreased again significantly by 24 hr of warm recovery (R: $P < 0.001$). By then they had nearly returned to their normal control value, showing no significant difference (U: $P > 0.5$).

Other Changes in the Receptor Terminals. To assess further the changes in synaptic organization after cold exposure and warm recovery, we also examined other features of the receptor terminals. We decided not to assess the changes in synaptic vesicle populations, which also appeared to change in a similar manner to the synaptic profiles, chiefly because we were not confident that our methods capture the entire population of undischarged vesicles in all terminals, given the 2-fold range of values seen in control receptor terminals (23) and because of

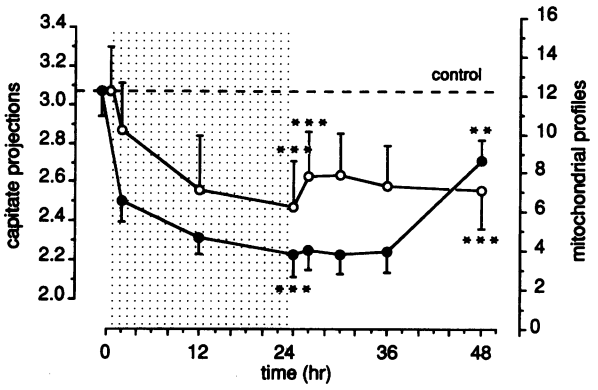

FIG. 4. The average frequencies of the profiles of capitate projections and of mitochondria in the same receptor terminals that were analyzed in Fig. 3. The numbers of capitate projection profiles (●) decrease significantly compared with control frequencies during 24 hr of cold exposure (stippled) and increase significantly on warm recovery during the next 24 hr. The mean frequencies of mitochondrial profiles (○) decrease significantly during cold exposure, in parallel with capitate projections, and increase significantly in the first 6 hr of warm recovery. Levels of significance are as given in Fig. 3.

local variation in the density of vesicles within different areas of the profile of any terminal.

Closely codistributed with the population of tetrad synapses in the terminals are characteristic glial invaginations into the terminals, which form conspicuous organelles of enigmatic function called capitate projections (14, 24) that are unique to Diptera, within which they are widely distributed (25).

The number of profiles of capitate projections (Fig. 4), like both the membrane perimeter (Fig. 3) and the number of its tetrad synapses (Fig. 3), dropped sharply during the 24-hr period of cold exposure. The decrease was about 30% and highly significant (R: $P < 0.001$). After returning the flies to 23°C, the number remained depressed over the first 12 hr of recovery, then significantly (R: $P < 0.01$) increased by about 20% over the next 12-hr recovery period, although it still failed to return to normal control values.

A similar response to cold exposure and warm recovery was seen in the mitochondrial profiles in the receptor terminals (Fig. 4). Their number showed a highly significant (R: $P < 0.001$) decrease of about 50% during the period of cold exposure but then showed a highly significant (U: $P < 0.001$) increase of about 30% in the first 6 hr of recovery at 23°C, decreasing again thereafter (R: $P < 0.001$).

DISCUSSION

Among the responses of the receptor terminals to cold shock, there seem to be two types of change, which we may distinguish on the basis of their time course.

The first type of change is manifest by the number and density of tetrad synapses (Fig. 3), which both exhibit a large overshoot on warm recovery, as if some buildup of synaptic precursors had occurred during their decrease in the cold. This overshoot is reminiscent of the synaptic responses in hippocampal mossy fibers of torpid ground squirrels, in which warm-induced arousal reactively increases the mean size of postsynaptic sites (26). At the tetrads, changes in synapse numbers are paralleled by changes in the number of receptor terminal synaptic vesicles, but for technical reasons we were unable to quantify these.

Synaptic changes seen in the fly occur faster than changes found in the brains of warmed lizards (27, 28), but the average peak rate for the formation of new synaptic sites after warm recovery compares with the rate of synaptic recovery from torpor in the ground squirrel (26), as well as with rapid rates of synaptogenesis under other conditions in the hippocampus (e.g., ref. 29). In the fly, it exceeds 20 sites in each terminal per hour—1 every 3 min. This rate matches the average peak rate of disassembly in tetrads, seen after photoablation of receptor terminals, when 85% of sites can be lost from a terminal in the first 8 hr (12). In both cases it is the appearance or loss of the presynaptic ribbon that designates whether a tetrad has disappeared from view or come into existence. The postsynaptic dendrites that persist at many former synaptic sites after photoablation are revealed by membranous postsynaptic cisternae (ref. 12; see intact synapse in Fig. 2*c Inset*). These identify the dendrites as belonging exclusively to L1 and L2 but are not visible as prospective postsynaptic ensembles at sites destined to undergo synaptogenesis after warm recovery. We have not studied the rearrangement of postsynaptic elements further, which would have required the analysis of serial sections, but our evidence is consistent with regrowth of L1 and L2's dendritic spines during synaptogenesis. Precedents elsewhere lead to no consensus for an overall action of either cold exposure or warm recovery on dendritic morphogenesis. Thus, in cold-exposed hibernating mammals, dendrites are either more branched and their spines are more numerous (supraoptic nucleus; ref. 30), or are larger yet possibly less numerous (cerebellar Purkinje cells; ref. 27), or are less numerous (hippocampal pyramidal cells; ref. 31).

Changes in tetrad frequencies superficially resemble the time course of tetrad ontogeny (9) and are presumed to lie behind the qualitatively similar time course of changes in the spacing density of tetrads. These also showed an initial overshoot on warm recovery, demonstrating quite clearly that, although the number of synaptic sites and membrane area are normally closely correlated, so as to conserve synaptic spacing densities under a wide variety of conditions (9, 22, 32), these two variables are perfectly capable of short-term independent variation. After the appropriate temporary perturbation, in this case cold exposure, the normally close relationship is no longer rigidly bound but becomes uncoupled.

The change in synaptic spacing density (Fig. 3) also reflects changes in receptor terminal membrane surface area, which exemplify the second type of change. Changes of this type, which typically show only a small increase on the first phase of warm recovery and a slow rise thereafter, are also seen in the number of profiles of capitate projections and of mitochondria.

Clear changes in the number of capitate projections occur in response to the appropriate perturbation, since the constant (150–170 nm) diameter of their heads provides assurance that the number of profiles per section for this type of organelle accurately reflects the true number of such invaginations. As a consequence, we can now report that these structures are not permanent, as previously claimed (24), but are lost with cold exposure. We have not sought intermediate forms, which would reveal the pathway for their loss. Their number covaries with the number of tetrads, not only during cold stress (when both decrease by about 30% and increase again during warm recovery) but also after light exposure in dark-reared flies (10). By contrast, neither population is affected after the axons of L1 and L2 are lesioned, whereas the number of mitochondrial profiles decreases with time after the lesion (33). Changes in the number of mitochondrial profiles, seen here after cold stress, are also seen at the lizard synapse (27). They may indicate changes in the true numbers of mitochondria, but further detailed interpretation is hindered by possible additional changes in the size or shape of these organelles, neither of which could be reliably estimated from our single sections. The increase seen during the first 6 hr of warm recovery, which goes hand in hand with the production of new synapses, indicates that the metabolic state of the receptor terminal alters rapidly, despite the degenerated appearance of the terminals at the end of cold exposure.

The occurrence of glial hypertrophy confirms other findings that the epithelial glial cells are particularly sensitive to the functional state of the neural elements of the lamina (B. Bausenwein, K. Plötze, R. Schulz, and E. Buchner, personal communication). For example, hypertrophy is seen after two experimental procedures: photoablation of receptor cells, causing the degeneration of their lamina terminals (12), and lesions causing degeneration of the axons of L1 and L2 (33). Neither of these causes direct glial injury, any more than does cold shock. The time course of all these changes presumably represents the time course of the cells' temperature adaptation response, although we have not systematically investigated the responses of the receptor terminals to temperature reversals of different magnitudes and durations. It does seem clear that the optic lobe's temperature changes more rapidly than the cellular responses to temperature change. The rates of cooling of the honeybee brain, for example (35), and of changes in the fly electroretinogram in response to cooling or warming (36) are both more rapid than synaptic changes seen here.

The degree to which long-term cold exposure alters the electrophysiological response of the lamina is not known. Temperature sensitivity of the fly's electroretinogram is known from an early study (36) that parallels our own and documents the transformation and restoration of the light-evoked "on" and "off" transients known to derive from L1 and L2 (37) but only after periods of relatively rapid freezing and thawing. Short periods of cooling abolish electrical activity of single units in the brain of the bee (38), but the threshold for synaptic excitation in an insect ganglion changes with temperature in a way that depends on the speed of temperature adjustment (39). Chilling in young flies also has a long-term effect on the development of visual pattern discrimination (40). A reexamination of the pattern of electroretinogram changes after long periods of cold exposure, of the sort used here, would now be timely.

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