Ultrastructure of Mauthner Cells in Fish Adapted to Long-Duration Vestibular Stimulation and the Effect of Ethanol

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ABSTRACT

Adaptation or resistance of fish Mauthner cells (M-cells) to long duration (2 h) vestibular stimulation (LDS) was produced by daily brief and gradually increasing vestibular stimulation (training). The LDS resistance was accompanied by an increase in the number of desmosome-like junctions in the afferent axosomatic synapses. F-actin, the main component of desmosome-like contacts, has been suggested to be responsible for the increased resistance of M-cells to LDS. The purpose of the present study was to investigate the capacity of M-cells to adapt to LDS under the influence of ethanol, which alters the content of F-actin in cells. The experiments were carried out in goldfish fry. Vestibular stimulation (training and LDS) was performed in special drums that were rotated in two planes. The training time was increased from 1 min on day 1 to 30 min on day 30. For ethanol exposure, fish were immersed daily in a 2% ethanol solution for 20 min. To assess the level of resistance to LDS, motor activity indicating the functional state of M-cells was evaluated before and after LDS. The results show that exposure to ethanol reduces the resistance to LDS in both untrained and trained fish. Electron microscopic data demonstrated some structural changes in the synaptic endings located on M-cell soma in ethanol-exposed fish. Wrapping of boutons by cytoplasmic outgrowths and myelin- like structures was observed. Morphometric analysis revealed that exposure to ethanol without training decreases the number of desmosome-like contacts, probably due to ethanol-induced depolymerization of cytoskeletal actin. Ethanol exposure also partly suppressed the increase in the number of desmosome-like contacts that occurs as a result of training. In ethanol-treated trained fish, however, a concomitant increase in the length of desmosome-like contacts was observed. As training alone leads to the formation of additional desmosome-like contacts of standard length, it is possible that although a sufficient amount of such structures cannot be formed in the M-cells of ethanol-exposed trained fish, the existing contacts can be elongated. Thus, possibly changes of the actin state are involved in the adaptation of M-cells to LDS.

KEY WORDS

Mauthner cells, adaptation, synapse, ethanol

INTRODUCTION

Previously we have shown that daily brief and gradually increasing natural stimulation (training) of the vestibular apparatus, a source of afferent input to Mauthner cells (M-cells) (Zottoli & Faber, 1979), results in adaptation (resistance) to sub-sequent long-duration vestibular stimulation (LDS), leading to drastic fatigue in untrained fish (Moshkov & Masyuk, 1981). Moreover, previous training was found to protect M-cells against

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kainic acid (Tiras *et al.,* 1990), a neurotoxic structural analogue of the excitatory neurotransmitter. glutamate, to which M-cells are sensitive (Diamond & Huxley, 1968). Certain biologically active substances, such as phalloidin, isonitrosin, and ethaminal, stabilize M-cells and prevent LDS-induced fatigue (Moshkov *et al.,* 1984; Potyomkin, 1989; Zherdev *et al.,* 1991). At the synaptic level, the adaptation of M-cells is accompanied by an increase in the desmosome-like junctions in the afferent axosomatic synapses contacting the M-cells (Moshkov & Masyuk, 1981). Similar changes of desmosome-like structures occur after treatment with phalloidin, which binds to actin filaments and prevents filament depolymerization (Moshkov *et al.,* 1980). Actin filaments have also been detected in desmosome-like structures within synaptic appositions (Tiras *et al.,* 1990). Actin, the main component of desmosome-like junctions and the cytoskeleton (Geiger *et al.,* 1985; Tiras *et al.,* 1992), is likely to be involved in such changes. Thus, on the basis of available evidence, it can be assumed that the enhanced resistance of M-cells to LDS after training and after administering certain biologically active substances proceeds by a common mechanism involving neuronal actin. Studying the adaptation of M-cells under the influence of agents that bind to actin and alter its polymerization can provide insight into this mechanism.

One agent affecting the content of F-actin in the cell is ethanol (Hassler & Moran, 1986; Mozhenok *et al.,* 1992). When compared with other substances having a similar effect, ethanol is less toxic and its effect is reversible. Owing to the ability of ethanol to penetrate the blood-brain barrier, intracerebral injections are not necessary. The latter circumstance is particularly important, considering the long duration of experiments on the adaptation of fish to LDS. The sensitivity of the cytoskeleton to ethanol and changes in neural function after administering ethanol have been demonstrated experimentally (Kulkarni *et al.,* 1990; Lynch *et al.,* 1983; Paula-Barbosa *et al.,* 1985; Smith & Davis, 1990). On the axolotl neural crest, both chronic and acute exposure to ethanol have been shown to destroy the actin cytoskeleton and cell contacts (Hassler & Moran, 1986). A decreased number of synaptic contacts has been described in the embryos of ethanol-exposed rats (Tanaca *et al.,* 1991). In mice, chronic ethanol exposure was found to affect the shape of hippocampal dendritic spines, which is determined by the actin cytoskeleton (Markharm *et al.,* 1987; Pavlik & Moshkov, 1992). Ethanol may be expected to affect also the functional properties and the ultrastructure of M-cells. The purpose of the present study was to determine the effect of ethanol on the capacity of M-cells to adapt to LDS and to describe the associated ultrastructural changes. Therefore, investigating the changes in desmosome-like contacts was essential for under-standing how actin is involved in processes underlying the adaptation.

MATERIALS AND METHODS

Training, ethanol exposure, and LDS

The experiments were performed on 3-mo-old goldfish fry. Adaptation to LDS was carried out using a daily training protocol, as previously described in (Moshkov & Masyuk, 1981). For this purpose, fish were placed in a drum that was rotated in two planes (Moshkov *et al.,* 1991). The training time was gradually increased from 1 min on day 1 to 30 min on day 30. The fish were divided into 4 groups of 20 each: (1) untrained control fish, (2) untrained fish exposed to ethanol, (3) trained fish, and (4) fish exposed to ethanol immediately after each training session. In the experiments involving ethanol exposure, the fish were immersed daily in an ethanol solution [2% in a mixture of aquarium and distilled water (1:1)] until the fish became immobile and lost their main reflexes (Faber & Klee, 1976). Immobility ensued after 20 min. The fish were then returned to ordinary aquarium water. The sedative effect of ethanol usually persisted for 45 min after the fish were transferred from the ethanol solution to water. Four days after the termination of the 30-day training period and ethanol exposure, half of each group was subjected once to LDS (for 2 h) in the same drum

to assess the level of resistance to stimulation.

Swimming activity was evaluated as the number of sections of the narrow ring-shaped chamber that was crossed by the fish and as the number of turns performed in each consecutive 0.5 min epoch during a 10 min period. The measurements were carried out before and after LDS, using the method described in (Moshkov, *et al.*, 1980; Moshkov, *et al.*, 1991). These parameters allowed an indirect assessment of the functional state of the M-cells (Moshkov, *et al.*, 1982).

Electron microscopic study

Three fish from each group were placed on ice and sacrificed. Pieces of medulla oblongata with M-cells were isolated, fixed in a mixture containing: 2.04 ml glutaraldehyde (25%), 5.6 ml formaldehyde [4%) in cacodylate buffer (0.1 M)], 0.25 ml dimethyl sulfoxide, 0.44 ml cacodylate buffer (1M), and 1.57 ml distilled water; the pH of the fixative was 7.2 (Picard, 1976). After fixation for 12 h at room temperature, the brain pieces were rinsed for 10 min with cacodylate buffer (0.1 M), post-fixed for 2 h in a solution of osmium tetroxide (2% in the same buffer), dehydrated in alcohol, and then embedded in Epon 812. Sections (10 μm) were prepared from the blocks. The sections of M-cell soma containing the nucleus were transferred onto new blocks for ultrathin cutting. Ultrathin sections on grids were contrasted with uranyl acetate and lead citrate and then examined in a Tesla BS-500 electron microscope.

Morphometric study

For measurement, the synaptic endings (Fig. 1a) located along the perimeter of the somatic part of M-cells were photographed at equal magnification. The negatives were projected onto the screen of a "Mikrophot" device (Zeiss) at 9× magnification. Measurements were performed directly on the screen. The number of synaptic specialised contacts, (active zones, desmosome-like structures, and gap junctions) was estimated (Fig. 1b–e). We also measured the length of transversal axosomatic profiles (appositions) and of intersynaptic regions of the plasma membrane of M-cells, as well as the length of specialized contacts (Meyhew, 1979). From the data obtained, we calculated (a) the mean number of specialized contacts per 1 μm of plasma membrane of M-cells, (b) the portion of the plasma membrane occupied by synaptic appositions, (c) the percentage of synaptic endings containing specialized contacts, and (d) the proportion of planar, concave, and convex active zones. The significance of the differences in mean values was estimated using the Student's t-test.

RESULTS

Behavioural observations

We analyzed the effects of different types of vestibular stimulation and ethanol exposure on fish behaviour that was associated with the functional activity of M-cells (Table 1). We found that LDS, resulting in fatigue, reduced the swimming activity of untrained fish by approximately half; the speed of swimming and the number of turns decreased as well.

Daily training resulted in adaptation to LDS

Daily training resulted in adaptation to LDS. This adaptation was manifest in trained fish because the swimming velocity and the ability to perform turns were not reduced after such stimulation (see Table 1). Indeed, the performance of these tasks even increased when compared with the values before LDS. This finding suggests that training significantly enhanced the resistance of fish and their M-cells to subsequent fatiguing stimulation.

Daily exposure to ethanol increased the swimming velocity of the untrained fish, but did not affect their ability to perform turns (as compared with untrained fish before stimulation). In this group, the ability to swim and to perform turns decreased after LDS (see Table 1). In fish

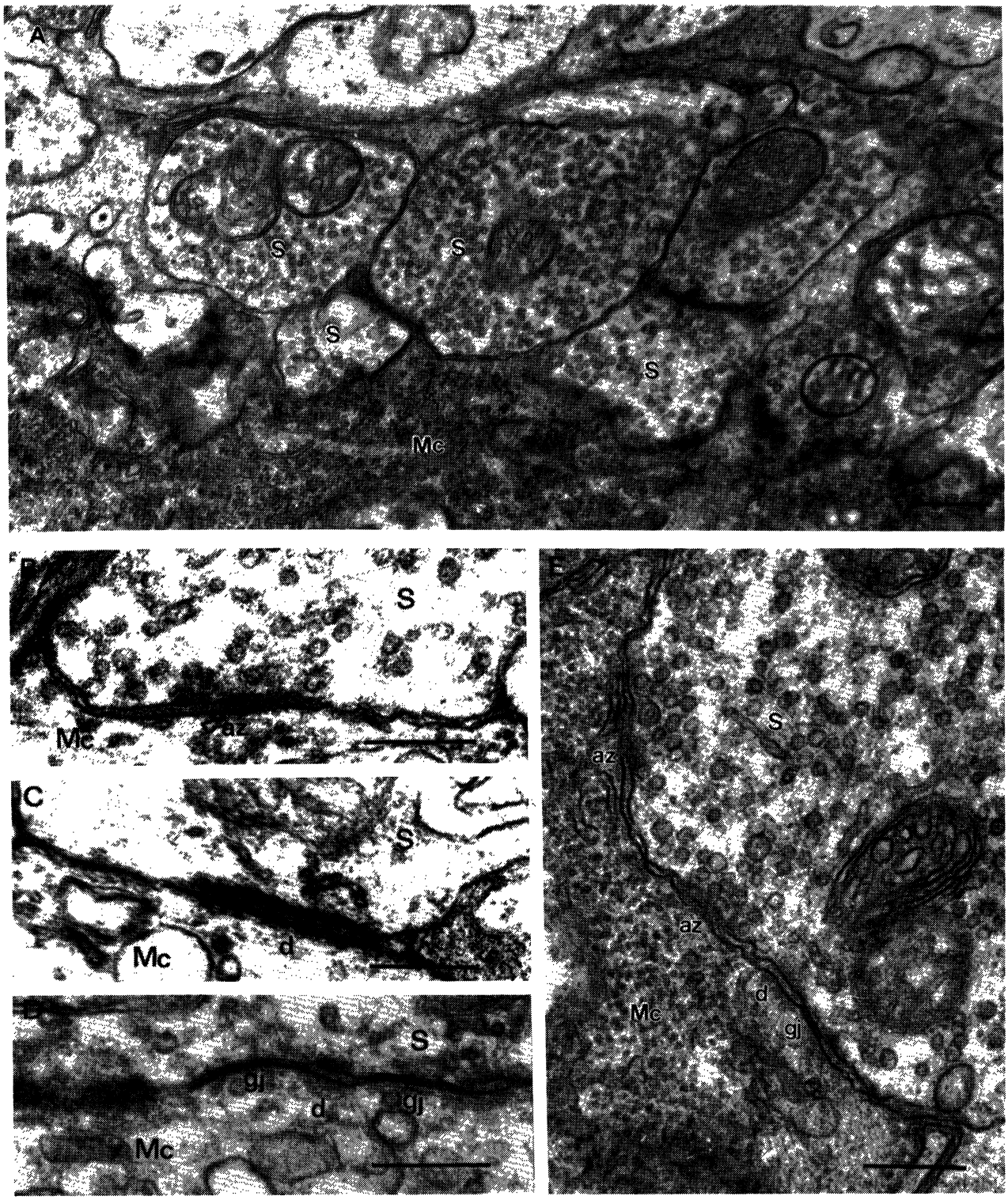

Fig. 1 Synaptic boutons (*S*) on M-cell soma (*Mc*). (a) Control fish; (b) (c) and (d): Specialized contacts -active zone (*az*), gap junction (*gj*), (*d*) desmosome-like structure in M-cells of fish from different groups; (e) Trained fish. No differences in the length and the number of active zones and of gap junctions were found in M-cells of control and ethanol exposed fish. The structure of synaptic endings in trained fish did not practically differ from control. Scale bar = 0.25 μ m

TABLE 1

The influence of training, ethanol and LDS on the swimming activity of fish

Experimental group	Speed of swimming (sections crossed)		Turns performed	
	number per 0.5 min			
	Before LDS	After LDS	Before LDS	After LDS
Without training	4.37±0.16	2.18±0.14§	2.91±0.15	1.47±0.12§
Training	4.07±0.15	4.81±0.24*	2.39±0.13†	2.48±0.17
Ethanol exposure	6.12 ±0.23‡	3.16±0.23§	2.88±0.17	1.19±0.13§
Ethanol exposure after training	4.56±0.19	3.30±0.20§	2.20±0.12‡	1.93±0.17

Data are presented as mean ±SEM, n=200 for each group.
†P< 0.05, ‡P< 0.01, Student's t-test; difference from the value in group without training before LDS;
*P< 0.05, §P< 0.01; difference from the value in group before LDS

that were exposed to ethanol after training, LDS decreased swimming velocity but did not affect the ability to perform turns (see Table 1).

Ultrastructure of synaptic endings

Control: The ultrastructure of synaptic endings on M-cells of control and adapted goldfish fry, before and after LDS, previously described in detail by Moshkov & Masyuk (1981), was found to be similar to that of adult fish (Nakajima, 1974).

Training: Synaptic endings in trained fish did not differ from those of control fish. The details of outlines of synaptic boutons, the filling of the endings with synaptic vesicles, the topography of their allocation within the bouton, the structure and the form of mitochondria, and the structure of specialized synaptic contacts and synaptoplasm were indistinguishable (Fig.1a,e).

Ethanol exposure

In ethanol-exposed fish (trained and untrained), certain structural changes of synaptic endings were observed. Firstly, in the trained ethanol-treated fish, cytoplasmic outgrowths into the synaptic boutons and perisynaptic spaces were present (Fig. 2a,b). The outgrowths into the perisynaptic spaces were narrow and long, contained up to six membranous layers, and enwrapped the boutons on one or two sides. Another morphological feature of the synaptic endings was the presence of groups of vesicles, that were separated from each other by two or more membranes (Fig. 2b).

In untrained ethanol-exposed fish, large myelin-like structures were observed in the cytoplasm of M-cells beneath synaptic endings and in perisynaptic spaces (Fig. 2c). Additionally, in boutons, horse-shoe-shaped, myelin-like

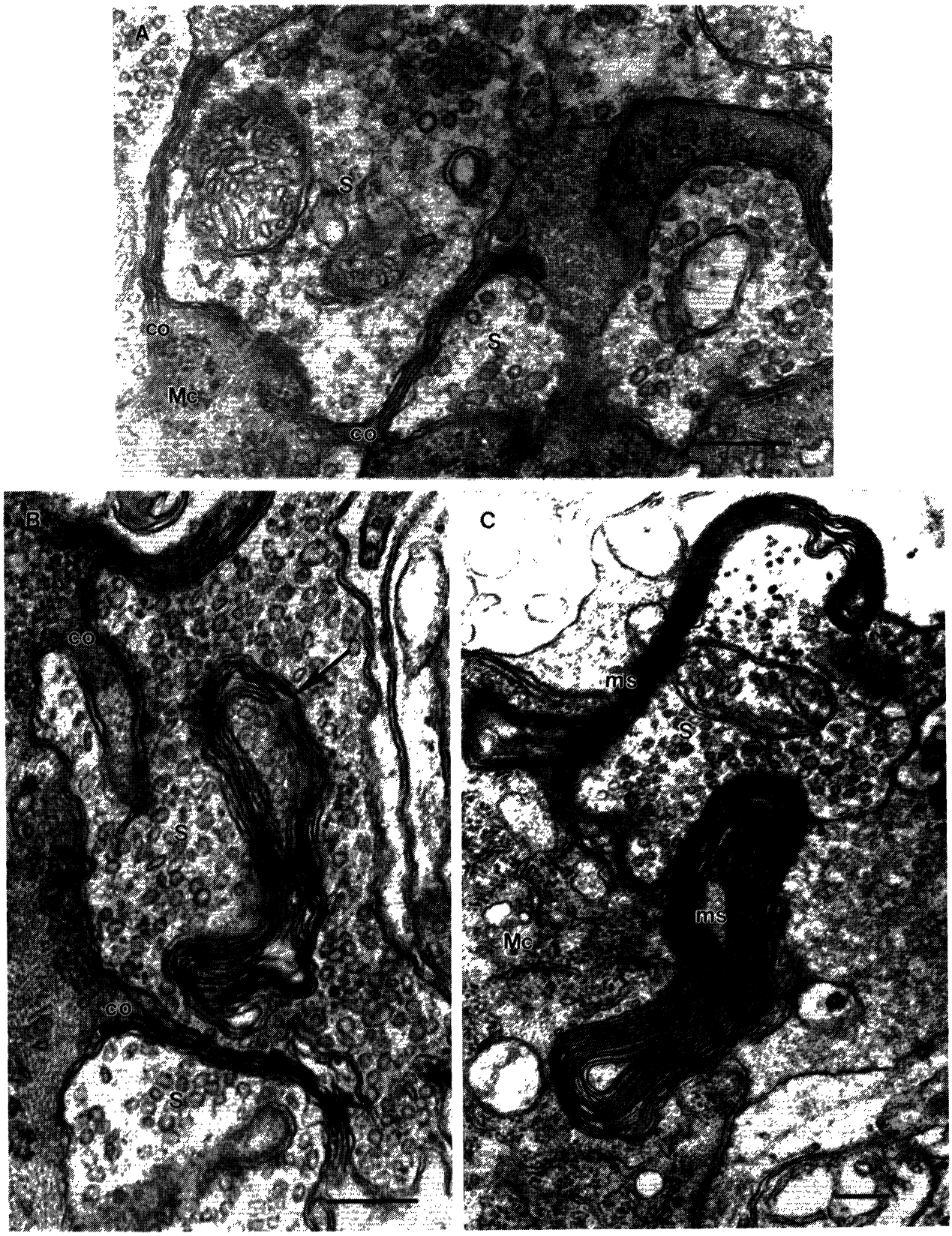

Fig. 2: Synaptic boutons on M-cell of ethanol exposed fish. (a) and (b) : Combined exposure to ethanol and training; (c) Exposure to ethanol without training. Note cytoplasmic outgrowths (*co*), myeline-like structures (*ms*), synaptic vesicles separated by membranes (arrow). Abbreviations as in Fig. 1. Scale bar = 0.25 μm.

structures with a small number of membranes, without any vesicles inside, were observed. Thus, the structural changes were most pronounced in the synapses of untrained fish exposed to ethanol, indicating that training gave some protection against the effect of ethanol.

LDS

LDS of untrained fish revealed the following essential morphological changes in M-cells and in synaptic endings:

- elongation of nuclei,
- non-uniform distribution of chromatin in the nucleus,
- increased smooth endoplasmic reticulum cisternae,
- prevalence of free ribosomes not aggregated in polysomes,
- appearance of myelin-like structures in the cytoplasm, just beneath the synaptic appositions and within synaptic boutons.

A decreased number of synaptic vesicles, their displacement towards active zones, and ruffled contours of synaptic membranes were also observed (Fig. 3a).

After LDS of trained fish, such ultrastructural changes were less expressed (Fig. 3b). A slight vacuolization of the synaptoplasm was observed. The vesicles in boutons were more abundant than at the afferent endings of M-cells of control fish. In trained fish, the profiles of the synaptic membranes were smoothed out. In the post-synaptic area, well-developed cisternae of the rough endoplasmic reticulum and many free ribosomes could be seen. The structures of the nuclei, the nuclear chromatin, and the matrix of trained fish did not differ significantly from and in some cases were even identical to those of the nuclei of M-cells from control fish.

LDS of ethanol-exposed untrained fish caused drastic morphological changes in synaptic boutons and in post-synaptic neurons. In addition to the ethanol-induced ultrastructural alterations described above, a strong depletion of synaptic boutons was noted. In addition, the remaining vesicles became pleomorphic and sometimes double-contoured. Some vesicles had a clearly visible dense core. Most vesicles were situated in close proximity to synaptic active zones. A strong vacuolization of the post-synaptic cytoplasm was also seen (Fig. 3c).

In trained fish that had been exposed to ethanol before LDS, the manifestations of abnormal changes were somewhat less pronounced (not shown).

Morphometric analysis

Morphometric data indicated that several parameters of M-cells (length of the active zones and of gap junctions, proportion of concave and convex active zones, number of active zones and of gap junctions per 1 μm of the M-cell plasmatic membrane, excluding the increased number of active zones after LDS of untrained fish) did not vary among the experimental groups and were not included in Table 2.

Training changed the quantitative characteristics of the axosomatic synapses on M-cells (when compared with those of control fish, see Table 2). The length of the synaptic apposition (2.25±0.11 μm, $P<0.01$) and the proportion of the M-cell plasma membrane occupied by synaptic appositions (94±1%, $P<0.01$) were increased. The proportion of synaptic endings containing desmosome-like structures (46±4%, $P<0.01$), as well as the number of these contacts per 1 μm of M-cell plasma membrane (0.32±0.02, $P<0.01$), were also increased. In such cases, the length of desmosome-like contacts remained unchanged.

In ethanol-exposed untrained fish, both the length of axosomatic contacts and the proportion of the M-cell plasma membrane occupied by synaptic endings were increased (Table 2). It should be noted that in the synaptic endings of this group, the number of desmosome-like contacts per 1 μm of plasma membrane decreased. After LDS of ethanol-exposed, untrained fish, the length of the synaptic appositions and the length of the desmosome-like contacts increased, whereas the proportion of the plasma membrane occupied by synaptic endings

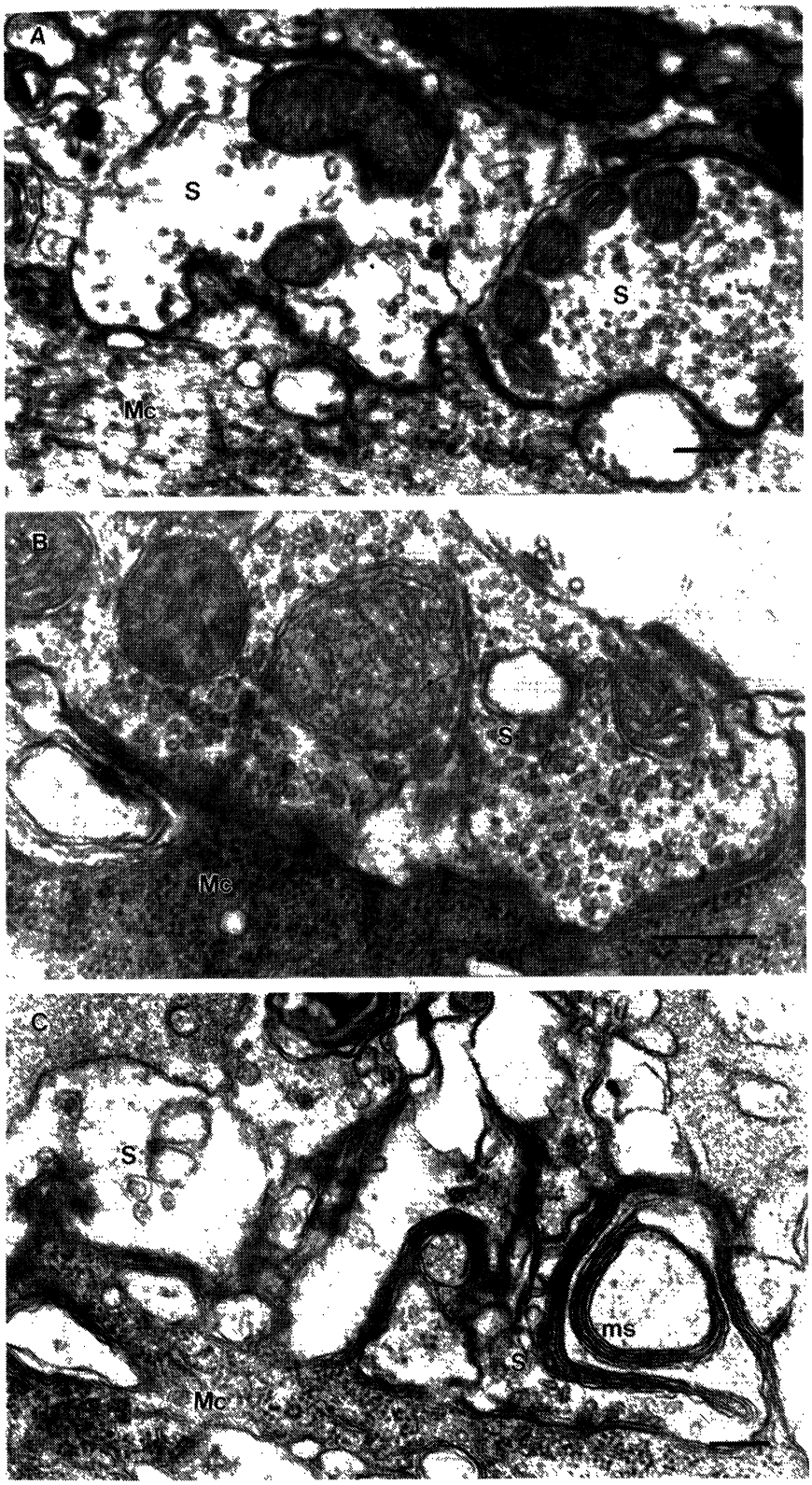

Fig. 3: Synaptic boutons on M-cell soma after LDS of (a) untrained fish, (b) trained fish, and (c) untrained fish exposed to ethanol. (a) Note a decreased number of synaptic vesicles, ruffled contours of synaptic membranes. (b) Training followed by LDS causes no changes in the number of synaptic vesicles. (c) The same structural features as in (a) and in Fig. 2b,c are observed: empty boutons, horseshoe-shaped myelin-like structures without synaptic vesicles inside them. Abbreviation as in Fig. 1 and 2. Scale bar for Fig. a and b= 0.25 μm, for c=0.2 μm.

TABLE 2

Effect of training, ethanol exposure, and LDS on the afferent synaptic endings of goldfish M-cells

	Unexposed		Ethanol-exposed			
	Untrained		Untrained		Trained	
	Before LDS (control)	After LDS	Before LDS	After LDS	Before LDS	After LDS
1	89±2 (50)	90±1 (106)	96±1‡ (58)	91±1 (72)	92±2 (116)	94±1‡ (67)
2	1.79±0.09 (92)	1.95±0.06 (153)	2.31±0.13‡ (87)	2.66±0.14‡ (94)	1.96±0.01 (157)	2.14±0.09‡ (105)
3	0.19±0.02 (50)	0.21±0.01 (106)	0.12±0.02† (58)	0.16±0.02 (72)	0.24±0.02† (116)	0.26±0.02† (67)
4	0.18±0.01 (60)	0.20±0.01 (114)	0.19±0.01 (65)	0.21±0.01† (77)	0.23±0.01‡ (110)	0.21±0.01† (75)
5	28±3 (92)	30±2 (153)	22±3 (87)	30±4 (94)	36±4 (157)	42±3‡ (105)

Data are presented as mean ±SEM. Inside parenthesis is the number of measurements.
†P <0.05, ‡P <0.01, Difference from control group.
Row 1: Proportion of plasmatic membrane of M-cell occupied by synaptic apposition (%);
Row 2: Length of the profile of the synaptic apposition (μm);
Row 3: The number of profiles of desmosome-like contacts per 1 μm of M-cell membrane;
Row 4: The length of the profile of desmosome-like contacts (μm);
Row 5: The proportion of the synapses with desmosome-like contacts (%)

remained unchanged (Table 2).

The combination of ethanol and training caused no changes in either the length of apposition or in the proportion of the plasma membrane occupied by synaptic appositions, but did lead to an increase in the length of the desmosome-like contacts and their numbers per 1 μm of M-cells plasma membrane. In fish exposed to ethanol after training followed by LDS, all measured parameters were increased.

DISCUSSION

In this study, we investigated the effects of different types of vestibular stimulation and ethanol exposure on fish behaviour and on the ultrastructure of their M-cells. Our data indicate that the daily sedative effect of ethanol without training does not enhance the resistance of fish to LDS. Both groups of untrained fish, with and without exposure to ethanol, were fatigued. Their M-cells and afferent synapses had an abnormal morphology. Combined daily exposure of fish to ethanol and training somewhat enhanced the resistance to LDS, as compared with untrained fish and fish exposed to ethanol alone. Such fish, however, appeared to be less adapted than those trained without ethanol. Thus, we conclude that ethanol does not act as an adaptogen and moreover, that exposure to ethanol during training results in decreased adaptation to LDS. The present study clearly shows that a positive effect of training in ethanol-exposed fish exists: Training counteracts the daily harmful effect of ethanol, just as the hyperbaric exposure to a gas mixture antagonizes the depressant effect of ethanol in mice (Alcana *et al.*, 1985).

It is generally accepted that the effect of ethanol on neuronal structure and function is complex and incompletely understood at all levels of analysis, from molecular to behavioural (Babbini *et al.*, 1991; Dawson & Reid, 1997; Leonard, 1986; Samson & Harris, 1992; Wayner *et al.*, 1997). At present, two hypotheses have been advanced on the mechanism by which

ethanol affects nerve cells.

1. According to the first hypothesis, ethanol affects the plasma membrane of cells directly, disturbing the organization of the membrane lipid bilayer and altering its permeability and fluidity (Chin & Goldstein, 1977; Chin *et al.*, 1978; Leonard, 1986; Zeng *et al.*, 1993.
2. The second hypothesis suggests that ethanol interacts with membrane neurotransmitter receptor channels (Macdonald, 1995; Samson, 1992).

As specific receptors for ethanol in neurons have not been detected, the possible targets for ethanol are suggested to be the receptors for γ-aminobutyric acid (GABA) and/or N-methyl-D-aspartic acid (NMDA) and α-amino-3-hydroxy-5-methyl-4-isox-azole propionic acid (AMPA) (Ahern *et al.*, 1994; Dildy-Mayfield *et al.*, 1995; Gonzales, 1990). Recently, a mechanism of ethanol's action has been studied on several glutamate and GABA receptors subunits that have been cloned and sequenced (Dildy-Mayfield & Harris, 1995; Macdonald, 1995). These studies demonstrated the role of calcium ions and protein kinase C in interactions between ethanol and neurotransmitter receptors. In rat pyramidal hippocampal cells, the ability of ethanol to increase intracellular Ca^{2+} concentration (Ahern *et al.*, 1994) and the mediation of Ca^{2+} mobilization by protein kinase C (Mironov & Hermann, 1996) were also shown.

The second hypothesis seems rather attractive to neuropharmacologists because it suggests the effect of ethanol on both excitatory and inhibitory synaptic transmission. Ethanol suppresses excitation and prolongs or facilitates inhibition, which probably underlies the anticonvulsant, depressive, sedative-hypnotic, and some other effects of ethanol (Fry *et al.*, 1991; Kulkarni *et al.*, 1990). The morphological data in the present study are consistent with both hypotheses. Indeed, the alterations revealed in afferent synapses of M-cells in ethanol-exposed fish, such as the presence of horseshoe-shaped membranous structures in presynaptic boutons, groups of vesicles surrounded by membranes, and the wrapping of boutons by cytoplasmic outgrowths, suggest a disturbance of membrane rigidity, which is probably due to the damage to the network caused by actin filaments adjacent to the cell membrane (Hassler & Moran, 1986). The direct effect of actin on synaptic membrane receptors is well known (Rosenmund & Westbrook, 1993), which agrees with the morphometric data presented here: the length of axosomatic contacts and the proportion of the M-cell plasma membrane occupied by synapses were increased. The observed morphological changes in synaptic endings on M-cells may be also interpreted as resulting from the squeezing of one synapse onto another from a direct effect of ethanol on the lipid component of the membrane and changes in its osmotic properties.

Quantitative analysis of synapse ultrastructure demonstrated significant differences between control fish and those exposed to ethanol alone and in combination with training. The synapses of fish that were exposed to ethanol daily and of those exposed to training revealed some similarity in the changes of the length of axosomatic contacts and the proportion of the M-cell plasma membrane occupied by synaptic endings. In the case of training, however, this process was accompanied by the formation of new desmosome-like contacts. In ethanol-exposed fish, the synapses were not stabilized by desmosome-like contacts, and a loss of some desmosome-like contacts was induced by ethanol treatment. Thus in this group of fish, disconnection of some synapses occurs during LDS, resulting in drastic morphological changes of M-cells and afferent synaptic endings.

The results of this study also suggest some mechanisms by which the M-cells of fish adapt to LDS. The results show that the adaptation of M-cells is related to an increased in the number of desmosome-like contacts of standard length. Ethanol suppressed this process partially. In the case of ethanol exposure combined with training, however, desmosomal individual length increased. How these two processes are interrelated in the cell remains unclear. That elongation of desmosome-like contacts is a compensatory response to a reduction in their numbers is likely because in the case of combined training and ethanol exposure, the number of

contacts is less than that seen in the case of training alone. A sufficient amount of new desmosome-like contacts probably cannot be formed in the M-cells of ethanol-exposed fish because of changes in the plasma membrane and in the cytoskeleton; but nevertheless, the existing contacts can be elongated.

The content of F-actin in the cells of ethanol-exposed animals is known to vary, depending on the ethanol concentration. At relatively high ethanol concentrations, the F-actin content decreases, whereas a low concentration has the opposite effect (Mozhenok *et al.,* 1992). Considering that the elongation of existing contacts and the formation of new desmosome-like contacts are related to the polymerization of actin, whereas the disassembly or disappearance of the contacts depend upon its depolymerization, it can be assumed that at the concentrations used in the present experiments, ethanol partially depolymerizes the actin in M-cells. As a result of training, which results in the adaptation of fish, actin is partly polymerized again, thereby restoring, to some extent, the ability of the M-cells to withstand LDS.

To summarize, the results of this study support our hypothesis that the changes of the actin state are involved in the adaptation of M-cells to LDS, and that the processes underlying adaptation are very resistant because ethanol fails to suppress them completely.

ACKNOWLEDGMENTS

This research was made possible in part by Grant JCD-100 from the International Science Foundation and the Russian Government and by Grant N98-04-48021 from the Russian Foundation for Basic Research. The authors wish to thank Professor O.S. Vinosgradova for helpful comments on the manuscript and L.I. Mikheeva, S.V. Sidorova, and M.A. Safronova for technical help.

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