How may a widely distributed, secretory glycoprotein contribute to the consolidation of specific neuronal circuits during memory formation ?

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Introduction

Ependymin, a secretory glycoprotein, was first detected by its increased metabolism after vestibulomotor conditioning in fish (Shashoua, 1976) and named according to its early immunohistochemical localization in the subependymal zone of goldfish brain (Benowitz and Shashoua, 1977). Recent studies revealed that ependymin provides a good substrate for the outgrowth of retinal ganglion cell axons in vitro (J. Schmidt et al., 1991) and may qualify as a prominent member of a hitherto not much studied class of calcium and zinc binding cell adhesion molecules (Schmidt and Makiola, 1991). Cell biological and molecular properties of ependymin now suggest a functional explanation for the involvement of this glycoprotein in plastic adaptations of the central nervous system, which range from the activity dependent sharpening of receptive fields during regeneration of the retinotectal projection (J. Schmidt and Shashoua, 1988) to classical (Pironi and Schmidt, 1988; Shashoua and Hesse, 1989) and operant (Shashoua and Moore, 1978; Schmidt, 1987) conditioning.

Molecular characterization of ependymin polypeptide chains

The full length sequence of ependymin was deduced from cDNA cloning (Königstorfer et al., 1989). The precursor comprises 216 amino acids, exceeding the mature molecule by 21 lipophilic N-terminal residues with a recognition site for cleavage by signal peptidase, as is typical for a secretory protein. Point mutations of the gene result in expression of closely related forms of the mature protein in goldfish, and two N-glycosylation sites provide the basis for mono- and bi-N-glycosylated variants of 31 and 37 kDa (Schmidt et al., 1990).

Regulation of ependymin conformation by metal cations

Ependymin forms a hetero-dimer and the two homo-dimers from the 31 and 37 kDa subunits, respectively (Schmidt and Shashoua, 1981). It binds radioactive calcium and zinc with high affinity at independent binding sites (Schmidt and Makiola, 1991). Stoichiometric calculations suggest that two monomeric ependymin subunits chelate one zinc cation, whereas only one out of 43 ependymin molecules binds calcium. This percentage corresponds to the portion of ependymin that forms monomers under physiological conditions (Schmidt and Shashoua, 1981). These results support the assumption that monomeric ependymins are stabilized by calcium, whereas dimeric conformations are stabilized by zinc ions.

Furthermore, ependymin was shown to polymerize *in vitro* to insoluble fibres on addition of calcium chelating agents (Shashoua, 1988). It was co-purified with EDTA-sensitive metallo-

protease and -esterase activities from goldfish brain (Shashoua and Holmquist, 1986). Obviously, polymerization, on the one hand, and monomerization and proteolysis on the other, offer the means for a calcium dependent bimodal regulation. Calcium concentrations are known to change in neuronal and in extracellular brain compartments during neuronal activity (see below).

Carbohydrate contents and cell adhesion properties

Contents in carbohydrates is high as compared with the calculated molecular weight of 22 kDa for the naked ependymin polypeptide chain. When treated with N-glycosidase F, carbohydrates are removed from the ependymin molecule. Amongst other sugars they comprise 3-sulfated glucuronic acid typical of the L2/HNK-1 epitope observed at cell adhesion molecules of the N-CAM family (Shashoua et al., 1986; Schmidt et al., 1990).

In order to test for a possible function of ependymin as a cell adhesion molecule, we explanted goldfish retinae on glass that had been covered with ependymin. It provided a good substrate for the growth and adhesion of retinal ganglion cell axons (J. Schmidt et al., 1991). When we administered the protein in form of stripes of different concentrations, axons followed the concentration gradient. Furthermore, axons preferred a mixture of ependymin plus laminin over pure laminin. Anti-ependymin antisera inactivated the adhesion properties. Although ependymin may fit into the IgG superfamily (Kõnigstorfer et al., 1989), we detected only very limited homology to any other sequenced proteins.

Specificity of distribution in the central nervous system

Radioimmunoassays (Schmidt and Shashoua, 1981) were applied to analyse tissue specificity, regional (Schmidt and Lapp, 1987a), and subcellular (Schmidt and Lapp, 1987b) distribution of ependymin in goldfish, in other cyprinides, amphibians and the mammalian brain (Schmidt et al., 1986). The protein is very specifically enriched in the CNS, with highest steady-state concentrations measured in microsomal and cytoplasmic fractions derived from the goldfish optic tectum and vagal lobes and in particular in extracellular fluid compartments both, within the brain ventricles and outside the leptomeninx. Our histological studies confirmed earlier reports on ependymin immunoreactivity in the subependymal zone (Benowitz and Shashoua, 1977) and in radial glial cells of goldfish brain (J. Schmidt and Shashoua, 1988). In addition, however, we observed staining for ependymin in the leptomeninx, at brain capillaries, in type XIV neurons of the periventricular cell layer and in type I neurons of the superficial grey and plexiform layer after perfusion fixation of the optic tectum (Schmidt, 1989; Schmidt et al., 1990). Furthermore, in (unperfused) cryostat sections we recognize pronounced staining all over the extracellular space, in particular in the brain ventricles suggesting a possible role of ependymin as a constituent of the extracellular matrix.

Immunogold labelling at the electron microscopic level now enabled us to localize ependymin to the reticular shaped cells above the basal membrane in the inner layer of the teleost endomeninx (nomenclature according to Momose et al., 1988). These cells are characterized by their elaborated rough endoplasmatic reticulum and Golgi apparatus, which were found to be packed with anti-ependymin immunogold particles (Rother et al., 1990; Schmidt et al., 1991).

Immunolabelled apical dendrites of the type I neurons were seen to traverse the optic layer, where unlabelled myelinated retinal ganglion cell axons invade the tectum, and they extend into the marginal layer of the tectum (Rother et al., 1990; Schmidt et al., 1991). On their dendritic spines

these type I neurons receive synaptic input from unmyelinated marginal fibres of the torus longitudinalis in characteristic horse-shoe shaped S1-synapses. Myelinated optic fibres terminate on the same apical dendrites (Meek, 1981).

In situ hybridization

Oligodeoxynucleotide probes against ependymin mRNA were used to search for the site(s) of synthesis. Despite the wide distribution of the ependymin antigen, synthesis was found to be restricted to the leptomeninx and meningeal invaginations into the brain (Rother et al., 1990, 1991; Schmidt et al., 1991). These results obtained by *in situ* hybridization were unexpected, but have independently been confirmed by Königstorfer et al. (1990). We conclude that secreted ependymin molecules have to be taken up by neurons, that do not synthesize them, and suggest a crucial role for meninx derived cell adhesion molecules in development and behavioural adaptations of the vertebrate central nervous system. Attempts to demonstrate a receptor for ependymins on neuronal membranes, however, have as yet been unsuccessful. Furthermore, it remains to be elucidated how ependymin molecules reach the brain ventricles and interstitial space after synthesis and secretion from the reticular shaped cells of the leptomeninx. Transport via radial glial cells or through the perivascular space may possibly provide distribution mechanisms.

Involvement in neuronal regeneration

Ependymin supports functional modifications during neural plasticity: In cyprinides, retinal ganglion cell axons decussate and innervate the optic tectum in a topically refined order. When the goldfish optic nerve is cut, axons regenerate and reinnervate their projection area in the contralateral tectum within approximately two weeks in a topically correct manner. However, the fish cannot yet see, since the tectal neurons respond to an enlarged area of the visual field. In the following weeks synchronous activity in neighbouring ganglion cell axons sharpens the receptive fields to their physiological value (J. Schmidt and Buzzard, 1990). Synthesis of ependymin is enhanced during this period, and anti-ependymin antibodies interfere with the sharpening of the retinotectal projection, when they are infused into the tectal ventricles during this critical period (J. Schmidt and Shashoua, 1988).

Involvement in memory formation

Changes in ependymin synthesis were first observed after training fish to swim with an attached float (Shashoua, 1976; Schmidt, 1987). In order to test a possible involvement of ependymin in an associative classical conditioning, we trained goldfish in a shuttle-box to cross a hurdle and to avoid mild electric shocks (unconditioned stimuli) that were preceded by a conditioning light signal. Fish learn this task within 20 one-minute cycles and remember it for many weeks (Piront and Schmidt, 1988).

Fish were injected intracerebroventricularly with ^{3}H-valine as soon as they had reached a criterion of 80% correct responses. Cytoplasmic fractions separated 4 hours later on electrophoretic gels exhibited a slight, but statistically significant increase in ependymin synthesis. Eight hours after the acquisition, secretion of 37 and 31 kDa ependymins into the extracellular brain fluid was enhanced by 22% (Schmidt, 1989).

Changes were confirmed by RIA and ELISA measurements following several different classical and operant learning paradigms (Schmidt, 1987; Shashoua and Hesse, 1989). The peak concentration in cytoplasmic ependymins was detected 5 hours after acquisition. In the extracellular brain fluid, however, first a slight decrease in ependymin steady-state concentrations was measured, that was followed by an overshooting secretion (Schmidt, 1987; Shashoua and Hesse, 1989). Various groups of active, passive, stressed and yoked controls did not exhibit these changes.

Amnesic effect of anti-ependymin antisera

Inactivation of secreted ependymins by injection of antisera half an hour after acquisition of the shock avoidance behaviour inhibited memory consolidation. Fish did not recall the avoidance response on day 4, however, they learnt the task again after clearance of the antibodies from brain. The same result was obtained, when the antiserum was injected up to 24 hours after learning. At later time points, memory consolidation had been completed, and the antisera were without effect (Pironi and Schmidt, 1988). Furthermore, antisera did not interfere with acquisition or recall and were without influence on overtrained animals or on the performance of the behaviour as such. Analogous results have been obtained for the operant float-training paradigm (Shashoua and Moore, 1978; Schmidt, 1987).

Rapid induction of ependymin mRNA after active avoidance conditioning

The increase in the *in situ* hybridization signal after learning (studied by computer assisted quantitative image analysis) was even more dramatic than the changes observed in protein concentrations. After active avoidance conditioning ependymin mRNA was induced within 10 min and exhibited a characteristic biphasic shape with relative maxima at 20 min and 2 hours after acquisition (corresponding to 245% of untrained controls; Rother et al., 1990, 1991; Schmidt et al., 1991). Similarly rapid induction has been reported of gene regulatory proteins of the immediate early gene group, such as c-fos and the zinc binding protein zif/268, but further investigations will have to elucidate, whether zinc binding ependymin shares further properties with these gene regulatory proteins.

Learning and "stress" reactions

For immediate early gene messages a distinction between learning- and stress-induced changes proved difficult. This differentiation was easy for ependymin mRNA: Although a first peak of ependymin mRNA expression at 20 min was also observed in goldfish "stressed" by application of the same number of conditioned and unconditioned stimuli in a randomized, unpaired fashion, elevation of mRNA levels in yoked controls was less pronounced and only transient: One hour after the stress event, hybridization signals decreased below those of passive control animals and returned to the baseline level within 8 hours (Rother et al., 1991; Schmidt et al., 1991). Presumably, ependymin protein pools are neither depleted by incorporation into activated neurons nor by polymerization (see below) during the stress procedure. Under such conditions, the transiently enhanced mRNA production induces an extracellular surplus of ependymin, which may serve as a negative feedback signal to ependymin producing cells in the endomeninx.

Certainly, aversive (and possibly even appetitive) learning comprises physiological stress reactions, and stress hormones may be considered to regulate memory consolidation. The first peak in ependymin mRNA expression is not specific of the associative learning. Whilst an early

constituent of the arousal reaction may induce immediate ependymin mRNA expression and prime the CNS for plastic adaptations, prolonged elevation appears to depend on a decrease in the concentration of stress hormones during acquisition of the avoidance behaviour. By this mechanism the amount of "stress" involved in a new task, and the recovery thereof, may adequately evaluate the biological importance of the preceding learning situation and provide advantage for animals that successfully cope with the situation by acquisition of a novel behaviour.

Working-hypothesis: Towards a mechanism for the involvement of secreted cell adhesion molecules in neuronal plasticity

Our behavioural biological results may be interpreted independently from the regeneration studies and the cell-adhesion properties of ependymin. However, ependymin is involved in a late phase of memory formation, with a time course comparable to that of ultrastructural reorganizations. There is a further parallelism: The synchronous activity of neighbouring fibres, that promotes sharpening of the receptive fields, bears similarity to the contiguity in time, the crucial behavioural characteristic of classical conditioning, that was transponed by Hebb (1949) into a hypothetic physiological mechanism of converging synaptic activity: Synapses, which transmit the conditioned excitation, are strengthened only, if the postsynaptic neurons become activated via the unconditioned stimulus (for details, see Schmidt et al., 1991).

Our working-hypothesis is, that all synapses, which are involved in acquisition, become primed. But only if the new behaviour proves advantageous, cell adhesion molecules, such as ependymins, are secreted in concentrations high enough to consolidate long-term changes at the activated synapses. Whatever the exact regulation of ependymin synthesis may be, *in situ* hybridization revealed a clear difference between yoked animals and animals that were given the chance to reduce their "stress" reaction by adopting the active avoidance response. Although it is likely that the cell adhesion properties of ependymin molecules account for their functional involvement in plastic adaptations of the CNS via local structural alterations in neuronal circuits, increments in the overall rate of transcription and translation alone cannot provide a mechanism for specific changes at individual synapses.

How could extracellular ependymins find those synapses that are to be modified? Morris et al. (1986) described potentiation of tectal neurons by tetanic stimulation of the marginal fibres from the torus longitudinalis. Jointly with the establishment of posttetanic potentiation, calcium concentrations in the extracellular fluid decreased. Both, the potentiation and the attenuation in extracellular calcium, were more pronounced, if the cells had first been stimulated by a conditioning stimulus via the optic nerve. Although not explicitly identified, the potentiated tectal neurons may well correspond to the ependymin positive type I neurons described above, which are known to receive synaptic inputs from the retina and the torus longitudinalis and, therefore, favourably lend themselves towards an integration of vestibular and visual modalities during learning events. Probably ependymin polymerizes also *in vivo* at such activated synapses and the conformational change may alter its cell adhesion properties.

In conclusion, I suggest a *mechanism for memory consolidation* that involves humoral hormone factors to evaluate the preceding acquisition phase with regard to its importance for the biological fitness of the organism and to determine, when and how much of the cell adhesion molecule is synthesized and secreted. Local concentrations of metal cations in the neuronal micro-environment with its specific synaptic connections, on the other hand, reflect the semantic

aspect of the preceding neuronal activity and may, therefore, direct ependymin to - and polymerize it at - those synapses, that have to be consolidated in order to improve their efficacy for future activity. All organisms that consolidate exactly those neuronal circuits which had been activated before a decrease in the animals' arousal and stress reaction was achieved, will certainly gain selective advantage in evolution. Behavioural plasticity may thus become comprehensible as a local replay of tissue differentiation, a micro-event in cell recognition.

Ependymin-like immunoreactivity in higher vertebrates

Ependymin-like immunoreactivity was found in other vertebrates, including mammals, amphibians, birds, and many fishes, where the protein becomes first expressed at a time, when the optic fibres invade the tectum. In the rat the highest concentration of ependymin was measured in the embryonic hippocampus, in particular in pyramidal neurons cultured from embryos at 15 days of gestation and supported by media conditioned with neurotrophic factors from primary cultures enriched in glial cells (Schmidt et al., 1986). Probably, the adult fish brain retains some molecular features endowing it with a plastic and regenerative potential that is lost in the mammalian brain after the embryonic stage.

Increased synthesis and secretion of ependymin has also been described after a T-maze learning in mice (compare Shashoua, 1988/89). Fazeli et al. (1988) reported secretion of ependymin from the rat hippocampus during long-term potentiation and Shashoua detected polymerized ependymin at synapses of the Schaffer-collaterals (Shashoua, 1988/89).

It is anticipated that future collaboration with the colleagues who participated in the St. Goar Workshop on "Glycoproteins in Memory Formation" will provide the opportunity to search for an ependymin gene in higher vertebrates, to test the effect of our antisera on the maintenance of LTP, and to evaluate the functional significance of posttranslational modifications of ependymin molecules, such as fucosylation and sialylation, for cell adhesion and neuronal plasticity.

Acknowledgements

I am grateful to Susanne Gathmann for excellent technical assistance and to the Deutsche Forschungsgemeinschaft for financial support.

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