Role of cholesterol in neurotransmitter receptor trafficking and synaptic plasticity (**SynaptoChol)**

Learning and memory deficits are associated to multiple cognitive disorders, and they represent a devastating and costly problem in our society. In Europe, the prevalence of people at any given time with Alzheimer's disease and other dementias is over 7 millions, and over 4 million with schizophrenia. Aging is the most significant risk factor for the development of most neurodegenerative disorders. Alzheimer's disease incidence, for example, rises exponentially with time, roughly doubling every five years. One feature of brain aging is a decrease of cholesterol content in hippocampal neurons and several cholesterol metabolism markers are now associated with an increased risk factor for Alzheimer's disease. Therefore, we believe that advancing our understanding of the role of cholesterol in neuronal mechanisms for learning and memory is of the outmost importance.

Synaptic functions and in particular synaptic plasticity are widely thought to underlie learning and memory in both vertebrates and invertebrates. In this study, we investigated the role of cholesterol and its contribution to the modification of synaptic functions. To this end, we used a combination of pharmacological and molecular tools, together with electrophysiology, biochemistry and fluorescence imaging. The following specific aims have been pursued:

Aim 1. Analysis of cholesterol in neurons during synaptic plasticity:

It has been recently shown that glutamate-mediated excitotoxicity triggers acute cholesterol loss in hippocampal neurons and it was suggested that this may reflect the exaggeration of physiological excitatory transmission. Here we tested whether NMDA receptor activation during synaptic plasticity would also be able to regulate cholesterol levels.

We employed three complementary approaches: biochemical quantification of endogenous cholesterol, imaging of fluorescently labeled cholesterol derivatives, and monitoring intracellular distribution of a cholesterol binding reporter. These combined experiments strongly suggest that synaptic plasticity and more specifically Long Term Potentiation (LTP) triggers a rapid and sustained loss of cholesterol in neurons.

Aim 2. Development and validation of cholesterol perturbation tools:

Given these results, we then decided to test whether an acute cholesterol loss may in turn affect synaptic function. To alter cholesterol content we employed acute pharmacological approaches to remove cholesterol, rather than slower genetic interventions or inhibitors of enzymatic activities, to bypass compensatory effects and/or accumulation of intermediary products.

Endogenous cholesterol was reduced by short time applications (30 min) of MBCD (10 mM) or cholesterol oxidase enzyme (Chol Ox, 10 U/ml). These two manipulations offer complementary information to control for potential non-specific effects, since MBCD is only partially specific for cholesterol but does not generate additional metabolites, whereas Chol Ox is very specific for cholesterol but generates H_2O_2 and a ketone derivative of cholesterol as by-products. Both protocols produce a moderate but significant decrease of

cholesterol levels in hippocampal slices, as probed by a fluorescence quantitative enzymatic assay.

Aim 3. Study of cholesterol modification effects on basal synaptic properties

To evaluate the effect of cholesterol decrease on synaptic function, we measured CA3-to-CA1 responses in hippocampal slices using whole-cell electrophysiological recordings. We showed that MBCD or Chol Ox treatments do not affect passive membrane properties of the cell nor presynaptic properties of excitatory transmission. In contrast, cholesterol removal enhances postsynaptic function at excitatory CA1 synapses, specifically increasing AMPA receptor (AMPAR)-mediated synaptic transmission. Furthermore, we showed that GluA1-containing AMPARs are inserted at the synapse.

We confirmed these results on endogenous AMPARs by performing a double immunostaining of surface and total GluA1 receptors in dissociated hippocampal neurons. Taken together, these results indicate that cholesterol removal enhances excitatory synaptic transmission by inducing the delivery of new GluA1-containing AMPARs at synapses.

In order to ascertain the delivery route, we assessed the involvement of Rab11-dependent trafficking, which mediates the activity-dependent delivery of GluA1-containing-AMPAR from intracellular compartments into synapses during Long Term Potentiation (LTP). To evaluate the role of Rab11 in AMPAR synaptic delivery upon cholesterol removal we used a well-characterized dominant negative (DN) version of Rab11 (Rab11-S25N). Our data show that Rab11-DN blocks the potentiation of AMPAR currents produced by acute cholesterol depletion. Thus, we conclude that Rab11-dependent trafficking mediates the synaptic delivery of AMPARs triggered by cholesterol removal as observed during LTP.

It has been recently shown that a reduction of cholesterol levels in hepatic cells triggers the mobilization of Rab11 recycling endosomes, in a process mediated by Cdc42 activation. Therefore, we hypothesized that a similar mechanism may be operating in neurons. We first evaluated whether cholesterol removal alters Cdc42 activation in hippocampal slices. To this end, we measured the amount of active (GTP-bound) Cdc42 with a standard pull-down assay (GST-Pak3-CRIB) and showed that cholesterol reduction produces a significant activation of Cdc42 in hippocampal extracts. We then tested whether Cdc42 activation was required for AMPAR synaptic delivery triggered by cholesterol removal. Similar to the experiments for Rab11-DN, we expressed a well-characterized dominant negative form of Cdc42 (T17N) and demonstrated that Cdc42-DN blocks the enhancement of AMPAR responses induced by cholesterol removal. Therefore, we conclude that AMPAR synaptic potentiation induced by a reduction in cholesterol requires Cdc42 activation.

Altogether our data reveal the existence of a dynamic interplay between cholesterol levels in neurons and synaptic plasticity, and provide a mechanistic link between activity-dependent cholesterol changes, AMPAR trafficking and synaptic plasticity. Given the implication of cholesterol metabolism in brain aging and Alzheimer's disease, we believe that our work will help understanding these conditions.