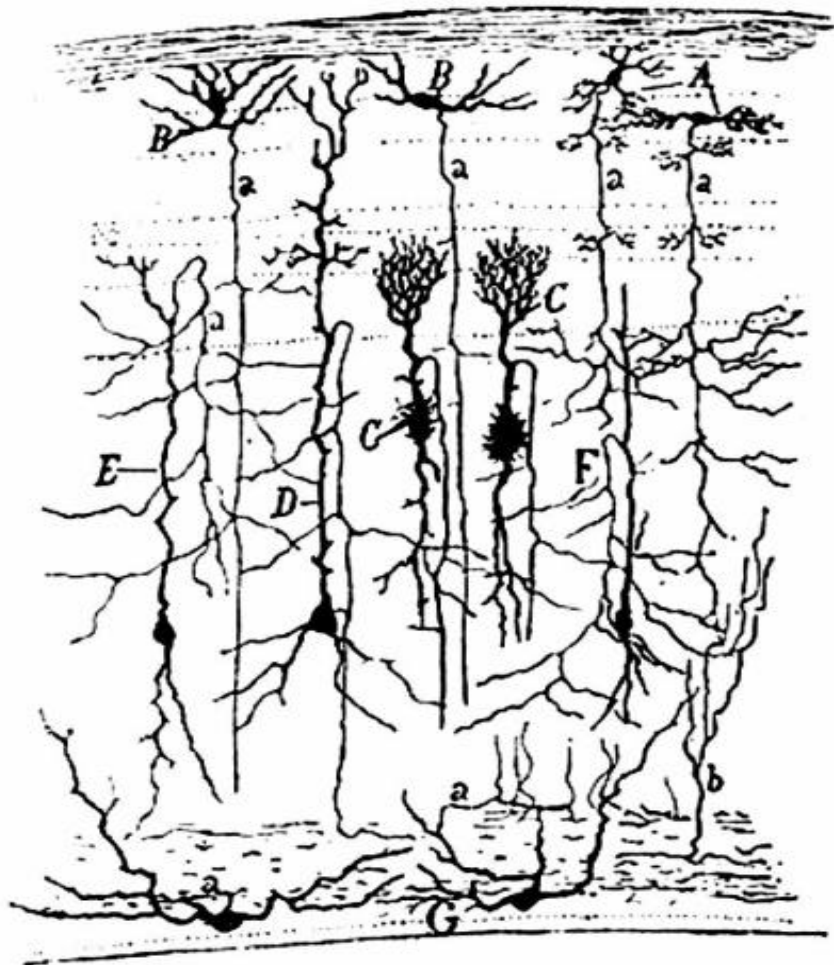


Conférences en Neurobiologie Ladislav Tauc
2004

The world of the synapse : molecular basis, pathologies and drug discovery



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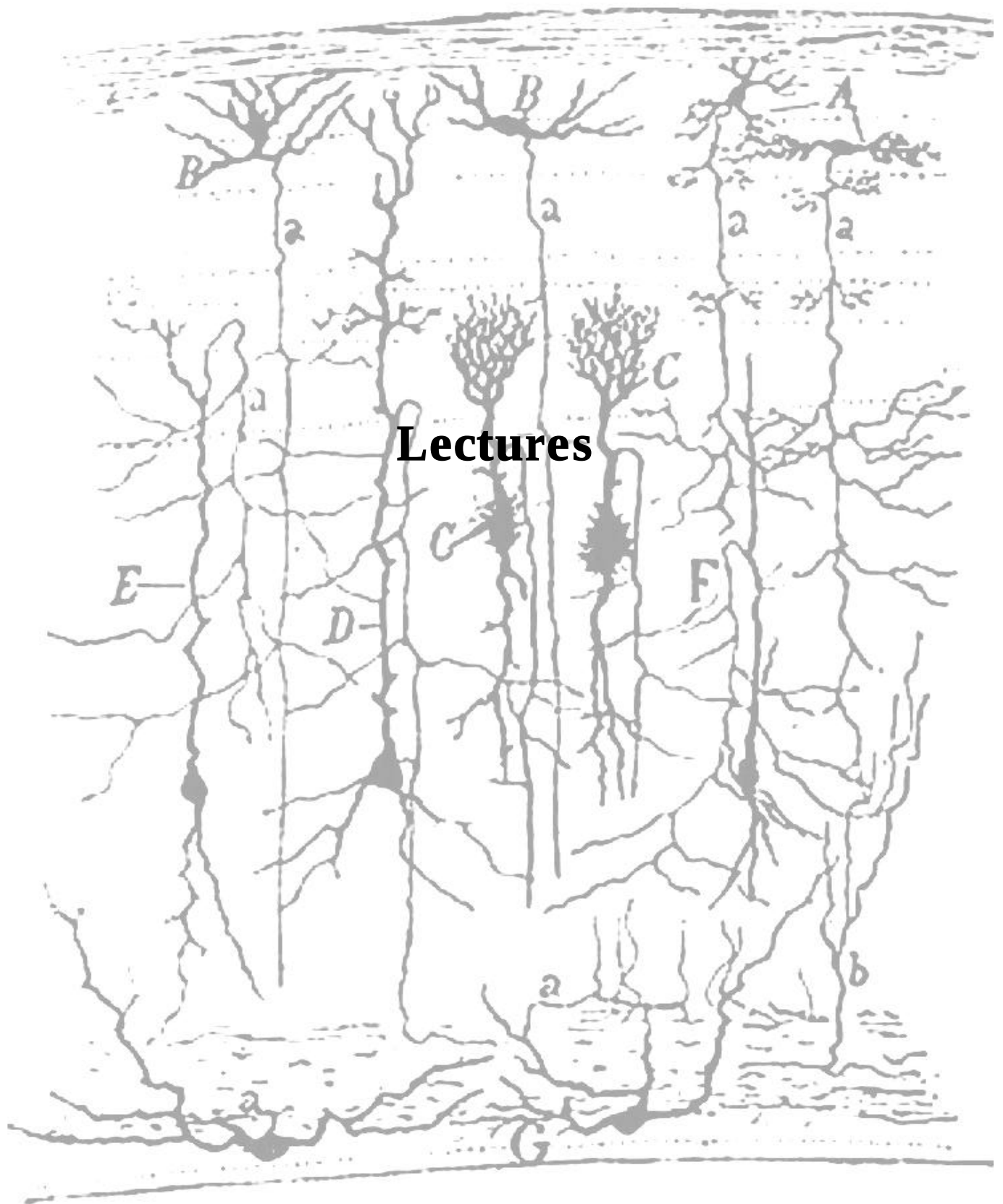
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Neuron-glia communication reveals synaptic information processing by astrocytes

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Accumulating evidence demonstrates that astrocytes play an active role in synaptic physiology by exchanging information with the synaptic elements. The synaptic control of the astrocyte intracellular Ca^{2+} is crucial in the reciprocal astrocyte-neuron communication. However, the properties and regulation of the synaptically-evoked astrocytic Ca^{2+} signal remain largely undefined.

Using electrophysiological and Ca^{2+} imaging techniques in rat hippocampal slices, we have investigated the properties and modulation of the astrocyte Ca^{2+} signal induced by activation of two different afferent pathways that use glutamate and acetylcholine as neurotransmitters, (i.e., Schaffer collaterals and alveus), and the electrophysiological consequences of the astrocyte Ca^{2+} signal on CA1 pyramidal neurons.

We have found that:

- 1) Astrocyte Ca^{2+} elevations were evoked by glutamate released from Schaffer collaterals, and by acetylcholine—but not glutamate—, released from alveus terminals, indicating that astrocytes discriminated the activity of different synapses belonging to different axon pathways.
- 2) The astrocyte Ca^{2+} signal was bidirectionally modulated by simultaneous activation of both pathways, being depressed at high stimulation frequencies and enhanced at low frequencies.
- 3) The Ca^{2+} signal modulation was required receptor activation, did not depend on neural network interaction, and as elicited by direct application of transmitters, suggesting that it was due to astrocytic intrinsic properties
- 4) This Ca^{2+} modulation occurred at discrete regions of the processes, supporting the idea that the astrocytic subcellular microdomains are the elementary units underlying the reciprocal communication between astrocytes and neurons.
- 5) While high frequency stimulation of either pathway induced Ca^{2+} elevations that initiated in a process and eventually propagated to the soma and other processes, upon simultaneous stimulation, the Ca^{2+} signal was confined to discrete regions, failing to propagate along the astrocyte.
- 6) The astrocyte Ca^{2+} elevations evoked NMDA receptor-mediated slow inward currents in pyramidal neurons.

We conclude:

- 1) Astrocytes show functional domains that discriminate between the activity of different synaptic pathways.
- 2) Astrocyte Ca^{2+} signal is modulated by interaction of different synaptic inputs, and this modulation is due to astrocyte intrinsic properties.
- 3) The simultaneous activity of both synaptic pathways controlled the propagation of the astrocyte intracellular Ca^{2+} signal.
- 4) Astrocytic Ca^{2+} can regulate the neuronal excitability in CA1 pyramidal neurons.

These results indicate that the astrocyte Ca^{2+} signal does not simply reflect synaptic activity, rather, the complex properties of the astrocyte response to neuronal activity indicate that astrocytes discriminate and integrate synaptic information.

We therefore propose that astrocytes can be considered as cellular elements involved in the information processing by the nervous system.

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Functional aspects of neuron-astrocyte reciprocal communication

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Synchronization of activity of anatomically distributed groups of neurons represents a fundamental event in the processing of information. While this phenomenon is believed to result from dynamic interactions within the neuronal circuitry, how exactly populations of neurons become synchronized remains largely to be clarified. We propose that astrocytes are directly involved in the generation of neuronal synchrony in the hippocampus. By using a combination of experimental approaches in hippocampal slice preparations, including patch-clamp recordings and confocal microscopy calcium imaging, we studied the effect on CA1 pyramidal neurons of glutamate released from astrocytes upon various stimuli that trigger Ca^{2+} elevations in these glial cells, including Schaffer collateral stimulation. We found that astrocytic glutamate evokes synchronous, slow inwards currents (SICs) and Ca^{2+} elevations in CA1 pyramidal neurons by acting preferentially, if not exclusively, on extrasynaptic NMDA receptors. In the virtual absence of extracellular Mg^{2+} , in paired recordings from two CA1 pyramidal neurons we also found that glutamate released from astrocytes evokes highly synchronous SICs. Due to desensitization, AMPA receptors were not activated by astrocytic glutamate unless cyclothiazide was present. In Ca^{2+} imaging experiments astrocytic glutamate was found to trigger in CA1 pyramidal neurons NMDA receptor-mediated Ca^{2+} elevations that occurred synchronously in domains composed of 2 to 12 CA1 neurons. In the presence of extracellular Mg^{2+} (1 mM) both synchronous SICs and synchronous Ca^{2+} elevations in neuronal domains were still observed, although with a reduced frequency. Our results reveal a functional link between astrocytic glutamate and extrasynaptic NMDA receptors that contributes to the overall dynamics of neuronal synchrony. Our observations also raise a series of questions on possible roles of this astrocyte-to-neuron signaling in pathological changes in the hippocampus such as excitotoxic neuronal damage or the generation of epileptiform activity.

Glutamatergic synapses, glia and D-serine: what is it all about?

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Although chiral molecules can be produced in both L- and D-stereoisomers, only L-amino acids have been considered to be present in higher organisms. Research over the past decade has challenged this dogma with the discovery that the mammalian central nervous system (CNS) contains high levels of D-serine. Increasing evidence is now supporting the idea that D-serine is an unusual and new glial-derived regulator for the key brain ionotropic NMDA receptors. First, D-serine is synthesized within discrete population of glial cells by the pyridoxal-5'-phosphate (B(6))-requiring serine racemase. Secondly, D-serine immunoreactivity is detected near NMDA receptors. Thirdly, D-serine can be released by glutamate through stimulation of glial AMPA receptors. Finally, treatment of brain slices or cell cultures with the D-serine degrading enzyme, D-amino acid oxidase, reduces drastically NMDA receptor dependent transmission. Despite recent progress in the understanding of D-serine functions in the CNS, the precise molecular mechanisms underlying D-serine release by glia are currently unknown. We have investigated the different hypothesis for putative mechanisms subserving the regulated D-serine release from cultured astroglial cells. Here, we are presenting evidence that astrocytes release D-serine through a Ca^{2+} - and SNARE protein-dependent mechanism and that vesicular D-serine is stored in synaptobrevins2/3-bearing vesicles. Our data highlights the importance of glial exocytotic pathway in influencing CNS levels of extracellular messengers and particularly D-serine. This pathway could be related to several pathologies including schizophrenia or ischemia.

The supraoptic nucleus as a model to study neuroglial interactions

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During intense physiological stimulation, such as dehydration or lactation, the supraoptic nucleus (SON) of the rat hypothalamus undergoes a striking and reversible morphological plasticity that is characterized by a reduced astrocytic coverage of its neurons. We are taking advantage of this anatomical neuroglial remodeling to investigate the contribution of astrocytes to the regulation of synaptic transmission in the central nervous system. Our recent studies indicate that the relative absence of astrocytic processes at the vicinity of glutamate release sites causes a local increase in glutamate concentration and results in an enhanced negative feedback control exerted by glutamate on its own release through the activation of presynaptic metabotropic glutamate receptors (mGluRs). Furthermore, measurement of diffusion parameters of the extracellular space (ECS) revealed that diffusion in the SON is facilitated under conditions of diminished glial coverage of neurons. In agreement with this result, mGluR-mediated heterosynaptic depression of GABAergic transmission is enhanced in the SON of lactating animals indicating that diffusion of glutamate at a distance from its release sites (spillover) is improved by the glial withdrawal. Taken together, our data indicate that astrocytes, by controlling glutamate concentration and diffusion in the ECS, regulate synaptic efficacy at glutamatergic terminals and intersynaptic crosstalk between glutamatergic and GABAergic synapses.

We also investigated whether the astrocytic environment of neurons influenced NMDA receptor (NMDAR)-mediated responses. Analysis of evoked excitatory postsynaptic currents (EPSCs) revealed that synaptic NMDA responses were impaired in the SON of lactating rats. Interestingly, D-serine, an endogenous agonist of the glycine site of NMDAR, is synthesized and released specifically by astrocytes. The deficiency in NMDAR activity in lactating rats, therefore, could result from lower concentrations of D-serine at glutamatergic synapses as a consequence of the reduced glial coverage of SON neurons. In agreement with this hypothesis, exogenous application of D-serine in lactating rats restored NMDAR EPSCs to values similar to those measured in virgin animals. Conversely, degrading D-serine with the specific enzyme D-amino acid oxidase in slices obtained from virgin animals resulted in impaired NMDAR-mediated EPSCs. Such a difference in NMDAR activity had some consequences on long-term synaptic plasticity. Whereas a classical pairing protocol induced long-term potentiation (LTP) in the SON of virgin rats, it caused long-term depression (LTD) in lactating animals. This LTD could be switched into LTP by supplying the slices with saturating concentrations of D-serine. Taken together, these data indicate that the level of occupancy of the glycine site of synaptic NMDAR, and therefore NMDAR activity, is highly dependent upon the astrocytic coverage of neurons.

Glia thus appears to be an important physiological contributor of synaptic transmission and plasticity both at pre- and post-synaptic levels. Our findings also indicate that the SON is a particularly appropriate experimental model to investigate neuroglial interactions in a physiological context.

Role of glia-derived cholesterol in CNS synaptogenesis

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Glial cells, namely astrocytes, regulate the function of chemical synaptic connections as they control the synaptic microenvironment, provide metabolic support and release modulatory signals. The development of synapses has long been regarded as a neuron-intrinsic process, but this view appears outdated given the increasing evidence for a glial influence on synaptogenesis (Slezak & Priege, 2003; Ullian et al., 2004). Studies on highly purified rat retinal ganglion cells (RGCs) revealed that glia-derived factors promote the formation and maturation of synapses (Priege & Barres, 1997; Nagler et al., 2001; Ullian et al., 2001), one of them being cholesterol (Mauch et al., 2001). These studies raised several questions including how cholesterol induces the synaptogenic effect and whether synaptogenesis in other CNS neurons also depends on glial signals. In my talk, I will summarize our efforts to address these issues. In the first part, I will show that 1) cholesterol together with other glial signals enhances synaptogenesis in rat RGCs directly at the pre- and postsynaptic level and indirectly by promoting specific stages of neuronal differentiation and 2) that evoked transmitter release is highly sensitive to the cholesterol level. In the second part, I will summarize our efforts to study the relevance of glia for synaptogenesis in other brain regions. To accomplish this, we established new methods to immunoisolate neurons from three different regions of the postnatal mouse brain (hippocampus, cerebellum and retina) and to culture them for at least one week under defined, glia-free conditions. Depending on the brain region, our isolation protocols yields between 30,000 to 500,000 neurons per animal with less than 0.5% of glial "contamination". Neuronal survival requires a combination of growth factors and an increase in cAMP levels as reported for rat RGCs (Meyer-Franke et al., 1995). Our electrophysiological recordings of spontaneous and evoked synaptic activity show that soluble, glia-derived factors including cholesterol enhance synaptic activity in all regions tested. Thus, our results corroborate the idea that CNS neurons need glia to develop synapses.

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Modulation of synaptic transmission and plasticity by glial cells

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In the central and peripheral nervous systems, glial cells were long considered passive elements, playing subservient roles in maintaining a proper environment to enable nerve cells to function adequately. However, in the past few years, the dogmatic view that glial cells only played subservient roles in the transfer of information in the nervous system has been shaken by a number of observations indicating that glial cells modulate neuronal activity and synaptic transmission. For instance, at the neuromuscular junction, glial cells have been shown to participate in the regulation of synaptic efficacy and plasticity. Activation of perisynaptic glial cells by synaptic activity is accompanied by feedback modulation of synaptic efficacy and short-term plasticity. The involvement of glial cells in such a role at CNS synapses has only recently begun to be explored.

Here we will test the involvement of glial cells in one form of short-term plasticity in the CNS, heterosynaptic depression (HSD). It occurs in parallel to long-term potentiation at Schaffer collateral (SC) synapses and is induced by NMDA receptors and caused by an adenosine-mediated inhibition of transmitter release involving interneurons. Interestingly, conditions causing HSD such as stimulation of SC or NMDA application also produce Ca^{2+} rises in hippocampal glial cells. Synaptic transmission in rat hippocampal slices was measured by field recordings simultaneously with Ca^{2+} imaging in glial cells.

Our data show that glial cells in rat CA1 hippocampal slices are activated by Ca^{2+} -mediated processes during heterosynaptic depression. Moreover, their activation involves local and network interactions between afferent fibers, inhibitory interneurons and glial cells, and is necessary for heterosynaptic depression. Our data show that glial cells are involved in HSD via Ca^{2+} - and GABA_B -dependent mechanisms, and suggest that heterosynaptic modulation of SC-pyramidal cell synapses is mediated by a complex network composed of GABAergic interneurons and glial cells. These findings underscore a novel physiological interactive role for interneurons and glial cells in synaptic plasticity at CNS synapses.

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Calcium signaling controlling neuronal network behaviour

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The generation of calcium signals following electrical activation is a fundamental property of neurons that controls many processes in the developing and the adult vertebrate nervous system. Activity-induced increases in the intracellular calcium concentration result from calcium entering neurons from the extracellular space through ligand and/or voltage-gated ion channels; these calcium transients can be amplified through calcium release from intracellular calcium stores. The N-Methyl-D-Aspartate (NMDA) receptor, a calcium permeable, glutamate-gated ion channel, plays a particularly important role in the mammalian central nervous system. NMDA receptors are present in the synapse but are also located extra-synaptically. Calcium entry through synaptic NMDA receptors activates mechanisms that affect synaptic connectivity and promote neuronal survival; these changes can be long-lasting and may depend on nuclear calcium signaling and gene expression mediated by the transcription regulators CREB and/or CBP. The pro-survival and plasticity programs triggered by synaptic NMDA receptors are antagonized by a signaling pathway stimulated by calcium entry through NMDA receptors that are localized outside synaptic contacts; extrasynaptic NMDA receptors couple to CREB shut-off and cell death pathways. The specification of the biological response by the localization of the receptor activated is a new concept in neuronal calcium signaling that can explain many of the opposing roles of NMDA receptors. The presentation will be focused on the role of nuclear calcium-regulated transcription in neuronal plasticity. A novel, very simple and experimentally accessible system will be described that allowed monitoring of the collective behaviour of small neuronal networks. The results obtained illustrate that both local, near-NMDA receptor signaling (activating the ERK1/2 MAP kinases) and the dialogue between the synapse and the nucleus are important to induce and uphold particular network activity patterns.

Neuronal activity, gene transcription, and synaptic development

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The ability to convert transient stimuli into long-term changes in brain function is central to an animal's capacity to adapt to and learn from its environment. Sensory experience and resultant neuronal activity are essential in shaping the connectivity of the mammalian central nervous system both at the level of individual synapses and neuronal networks. Insight into the mechanisms that underlie this persistent adaptability came from the observation that the stimulation of neurons with neurotransmitters can drive new gene expression. Activity-dependent transcription has now been shown to be crucial for many types of long-lasting plasticity, and work from a number of investigators has identified a class of genes that are regulated by neuronal activity and play key roles in neuronal survival, synaptogenesis, and synaptic plasticity.

Our laboratory has a long-standing interest in the mechanisms by which neuronal activity regulates new gene transcription. Two classes of genes are regulated by neuronal activity, immediate-early gene-encoded transcription factors that mediate cellular responses to extracellular stimuli and genes selectively expressed in the nervous system that directly regulate neuronal development and synaptic plasticity. Of the neural-selective activity-dependent genes, we have extensively studied the gene encoding brain-derived neurotrophic factor (BDNF). BDNF plays important roles in neuronal survival, development, and plasticity. Our studies, in combination with the work of other laboratories, have characterized signaling pathways that couple membrane depolarization to new *Bdnf* transcription. These intracellular signaling cascades converge to modulate the activity of promoter-bound transcriptional regulators, and we have identified several *trans*-acting factors that act through calcium-responsive elements present in the *Bdnf* promoter.

In addition to a role during nervous system development, several lines of evidence suggest that misregulation of activity-dependent transcription contributes to human disease. For example, in the course of our study of the transcriptional regulation of the *Bdnf* gene we found that the methyl-CpG binding protein 2 (MeCP2) binds selectively to *Bdnf* promoter III and functions to repress expression of the *Bdnf* gene. MeCP2 has been implicated in long-term silencing of gene expression, and inactivating mutations in the *MECP2* gene cause the majority of cases of Rett syndrome (RTT), a human X-linked disorder characterized by arrested neurological development and subsequent cognitive decline. In response to membrane depolarization and subsequent calcium influx into neurons, MeCP2 is rapidly phosphorylated and released from *Bdnf* promoter III, allowing for activity-dependent transcription of the *Bdnf* gene. While RTT has previously been hypothesized to result from a disruption in the long-range developmental silencing of gene expression, our findings raise the intriguing possibility that defects in activity-dependent gene transcription may contribute to RTT pathology.

NAADP, a new Ca^{2+} releasing messenger

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Hormones and neurotransmitters, in order to control cell functions, generate Ca^{2+} signals such as local and global Ca^{2+} elevations, and also Ca^{2+} oscillations. Accumulative evidences suggest that nuclear Ca^{2+} play an important part in the control of synaptic plasticity or gene expression. Recent works reported that in addition to IP_3 other Ca^{2+} releasing messengers such as cyclic ADP-ribose (cADPr) and nicotinic acid adenine dinucleotide phosphate (NAADP) are of functional importance. For example, in pancreatic acinar cells, a model of polarized cell, at low concentrations, acetylcholine evokes local repetitive Ca^{2+} spikes, whereas cholecystokinin, in addition to such local signals also, from time to time, induces global Ca^{2+} transients invading the nucleus. We have shown that the transformation of local Ca^{2+} spikes to global Ca^{2+} transients require the combinatorial effects of NAADP, cADPR and in some extend IP_3 .

It has recently been shown in the laboratory that NAADP enhances evoked quantal acetylcholine release at the presynaptic terminal of an identified synapse in the buccal ganglion of *Aplysia californica*. We have also investigated the role of NAADP in the generation of global Ca^{2+} transient in *Aplysia* neurone and in particular, its role in the control of nuclear Ca^{2+} homeostasis. Indeed, Ca^{2+} has been reported in these neurones to be important in the regulation of plasticity and in the regulation of transcription factor CREB. The main advantage of this model is that *Aplysia* neurones are giants with nuclei of big size perfectly adapted high resolution imaging experiment (around 40-60 μm diameter). To investigate the effect of NAADP, cADPR and IP_3 on nuclear homeostasis, we have established a preparation of isolated nuclei from *Aplysia* neurons. The changes of the Ca^{2+} concentration were assayed in the nuclear envelope as well as in the nucleoplasm using confocal microscopy. Addition of IP_3 , cADPR and NAADP induced a release of Ca^{2+} from the nuclear envelope and increased nucleoplasmic Ca^{2+} concentration. Our data suggest that the NAADP, cADPR and IP_3 receptors are localized in the inner nuclear membrane. We found that the nuclear Ca^{2+} pumps require ATP and are sensitive to thapsigargin. Depletion of the thapsigargin-sensitive stores significantly reduced the Ca^{2+} response evoked by IP_3 or NAADP. Interestingly, a NAADP dose-response experiment indicated that the NAADP receptor may have desensitizing property. Our data bring new insight in the regulation of nuclear Ca^{2+} homeostasis by several intracellular messengers suggesting a multiple control of gene expression.

TRP channel proteins and neuronal Ca^{2+} signaling

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TRP channel proteins constitute a large and diverse family of proteins that are expressed in many tissues and cell types and it is conserved through evolution. This family was designated TRP because of a spontaneously occurring *Drosophila* mutant lacking TRP that responded to a continuous light with a Transient Receptor Potential (and hence TRP). Besides responses to light, TRP channels mediate responses to nerve growth factor, pheromones, olfaction, mechanical, chemical, temperature, pH, osmolarity, vasorelaxation of blood vessels and metabolic stress. Furthermore, mutations in several members of TRP related channel proteins are responsible for several diseases, such as several tumors and neurodegenerative disorders. TRP related channel proteins are found in variety of organisms, tissues and cell-types, including non-excitabile epithelial and red blood cells, smooth muscle and neuronal cells. The large functional diversity of TRP channels is also reflected in their diverse permeability to ions, although, in general they are classified as non-selective cationic channels, which are permeable to Ca^{2+} . The molecular domains that are conserved in all members of the TRP family constitute parts of the transmembrane domains and in most members also the ankyrin-like repeats at the amino terminal of the protein and a "TRP domain" at the COOH-terminal, which is a highly conserved 25- amino acids stretch with still unknown function. All of the above features suggest that members of the TRP family are "special assignment" channels, which are recruited to diverse signaling pathways. The channels' roles and characteristics such as gating mechanism, regulation and permeability are determined by evolution according to the specific functional requirements. During the last years there is increasing interest in TRP channels because these channels are emerging as essential cellular switches that allow animals to respond to their environment. TRP channels thus appear to be responsible for fine tuning of wide variety of essential cellular processes including sensory signaling and synaptic transmission.

Regulated exocytosis: not only secretion but also a number of other processes of great physiological importance

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Regulated exocytosis is universally recognized as the process by which specialized organelles fuse with the plasma membrane in response to adequate intracellular signals. In many laboratories, however, regulated exocytosis is considered a synonymous of regulated secretion, and the exocytic organelles are identified, in all cases and only, with secretory granules and vesicles. However, many regulated exocytic processes are not secretory. In fact, they serve either to transfer to the cell surface specific membrane components; such as channels, receptors, transporters and pumps; or to rapidly enlarge the surface, often to compensate for events that either lead to a reduction or require an enlargement. I propose these two exocytic processes, that play key roles in cell physiology, to be called protein exposing exocytosis and expansive exocytosis, respectively.

In my presentation I will discuss two issues related to various forms of exocytosis.

1. The nature of a well known type of exocytic secretory organelles, the dense-core vesicles, present in neurons and neurosecretory cells. Previous studies had suggested these organelles to appear in any type of cell following the expression of one or more proteins of the core, such as chromogranin B. In our hands no dense core vesicle form in those conditions. The transfected secretory protein is addressed in part to some cytoplasmic puncta which however are positive for lysosomal enzymes and do not share the minimum requirements that we have established for the secretory organelle identification. I believe that expression of secretory organelles requires activation of a specific gene programme.

2. The enlargeosome, the new type of non-secretory exocytic organelle identified in our laboratory. The enlargeosome exocytosis appears to be of the expansive type. I will show evidence about its distinction of enlargeosomes from the other cytoplasmic organelles, its properties, its exocytosis-endocytosis cycle, its involvement in a variety of cell functions, all requiring a rapid enlargement of the cell surface.

Molecular motors, KIFs, key molecules for life: structure, dynamics and functions

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Cells transport and sort various proteins and lipids following synthesis as distinct kinds of membranous organelles and protein complexes to the correct destinations at appropriate velocities. The intracellular transports are fundamental for cell morphogenesis, functioning and survival. To elucidate this mechanism we have identified and been characterizing kinesin superfamily proteins, KIFs, using molecular cell biology, molecular genetics, biophysics, X-ray crystallography and cryoelectron microscopy. In this conference I will focus on KIF1A, KIF1B β , KIF17, KIF5s, KIF3 and KIF2A, and present our recent studies about the structure, dynamics and functions.

KIF1A, KIF1B β , KIF17, KIF5s and KIF2A are deeply involved in neuronal functions and network formation. KIF1A and KIF1B β transport synaptic vesicle precursors and play essential roles on neuronal function and survival. Molecular genetic study revealed that KIF1B β is a responsible gene of human hereditary neuropathy. KIF17 conveys NMDA type glutamate receptors, important for memory and learning, in dendrites through the interaction with scaffolding protein complex and NR2B subunit of NMDA receptors. Transgenic mice study showed that overexpression of KIF17 improved spatial and working memory. On the other hand, conventional kinesin (KIF5) transports AMPA type glutamate receptors in dendrites via GRIP1 and GluR2 interaction. The middle motor domain type KIF2A is fundamental for correct wiring of brain by suppressing elongation of axon collaterals through depolymerizing microtubules in growth cones. Recently using X ray crystallography of KIF2C we have elucidated how unique KIF2 depolymerizes microtubules at their ends. Our very recent study unraveled also that KIF5 transports mRNAs such as CaMKII mRNA and Arc mRNA in dendrites through the interaction of KIF5 tail and a large RNA-Transporting protein complex (~1000s). Thus, KIFs play a number of significant roles not only on various cell functionings but also on brain wiring and higher brain functions such as memory and learning.

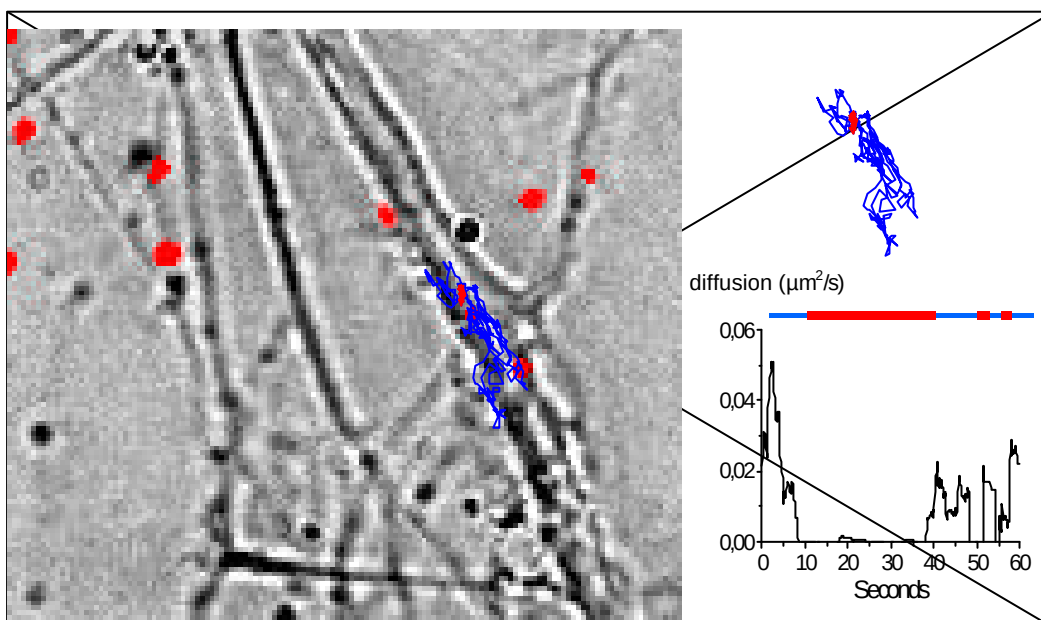
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Dynaynamic imaging of AMPA and NMDA receptors trafficking in and out synapses

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We will present the application of single molecule tracking to follow glutamate receptor dynamics in living neurons. AMPA subtype of glutamate receptor trafficking in and out of synapses is one of the core mechanisms for rapid changes in the number of functional receptors during synaptic plasticity. We directly imaged AMPA and NMDA type glutamate receptor movements inside, at the periphery and outside synapses of live cultured hippocampal neurons using single-molecule fluorescence microscopy and single particle tracking of quantum-dot coupled receptors. We could also record exchanges of receptors through lateral diffusion between these different membrane compartments. NMDA receptors are less mobile than AMPA receptors. Changes in neuronal network activity strongly modified AMPAR but not NMDAR lateral mobility, whereas protein kinase C activation modified both receptor mobilities. Differences in mobility were mainly detected for AMPARs present in the extrasynaptic membrane. In conditions of glutamate induced synaptic depression, AMPA receptor mobility increased inside synapses and a higher fraction of mobile receptors was found in a juxtasympatic annulus. Conversely, during the processes which lead to increased AMPARs numbers at synapses, receptors are initially mobile in synapses and are then stabilized. Altogether, our data show that rapid exchange of receptors between a synaptic and extra-synaptic localization occurs through regulation of receptor diffusion inside and outside synapses. Changes in this dynamic equilibrium could participate in plasticity processes.



Left : Composite image of an interferential contrast image of cultured hippocampal neurons (grey level) superimposed with image of AMPA receptor bound Quantum dots (red) and with the trajectory of an individual QDot recorded over 1 minute.

Right : Plot of the diffusion coefficient versus time for the displayed trajectory. Note that receptors alternate between periods of fast and slow diffusion.

Up-regulation by nicotine of its own receptors: identification of the molecular mechanisms

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Nicotine is the primary substance responsible for tobacco addiction, a major cause of death in western societies. Chronic exposure to nicotine is mediated by the interaction with neuronal nicotinic acetylcholine receptors (nAChRs), a class of pentameric ligand-gated ion channels engaged in cholinergic transmission in the brain. The effect of acute nicotine on nAChRs is extensively documented, and results in a transient activation through the opening of their cation selective channel (Corringer *et al.*, 2000). In contrast, few are know about the long-term effects of chronic nicotine. In particular, nicotine has been shown to produce up-regulation of its own receptors (defined as an increase in the number of nicotine high affinity binding sites), in the smoker brain, in rodents as well as in cell lines expressing various combinations of neuronal nAChRs (Buisson and Bertrand, 2002). This paradoxical effect of chronic nicotine might contribute to its long-term effects, including addiction, tolerance and/or sensitization.

To get insights into the molecular and cellular mechanism underlying this adaptative process, we investigated the fate of the $\alpha 4\beta 2$ nAChR expressed in HEK 293 cell from their synthesis in the endoplasmic reticulum (E.R.) to their surface export, using metabolic labeling and immunoprecipitation techniques. We showed that high-mannose glycosylated subunits mature and assemble into pentamers in the E.R., and that only pentameric receptors reach the cell surface following complex sugar processing in the Golgi apparatus. We found that nicotine, which efficiently penetrate into the cells, acts very early after the subunit synthesis to promote their maturation toward species that are recognized by a conformation-dependant antibody. Thus nicotine enhances a critical step in the intracellular maturation of these nAChRs, leading eventually to an increased number of high-affinity functional receptors.

To further progress in the molecular mechanism of up regulation, we studied the $\alpha 3\beta 2$ and $\alpha 3\beta 4$ nAChRs, that display very contrasted up-regulation properties (Wang *et al.*, 1998). By the systematic construction of chimeras between the $\beta 2$ and $\beta 4$ subunits, we identify 16 amino acids that govern the different up-regulation properties of $\alpha 3\beta 2$ and $\alpha 3\beta 4$ receptors (Sallette *et al.*, 2004). On an atomic three-dimensional model of the extracellular domain of nAChRs (Le Novère *et al.*, 2002), these amino acids form a compact microdomain, that paves the interface between the subunits, and also contact the nicotinic binding site. We propose the nicotine, by binding to immature oligomers within the E.R., elicits a conformational reorganization of the microdomain, strengthening the interaction between adjacent subunits and thus, facilitating maturation process toward high affinity receptors.

This novel mechanism of "maturational enhancer" for a drug of addiction like nicotine may contribute to the long term effect associated with tobacco consumption, by may also contribute to neural plasticity in physiological conditions. This work thus open new perspectives to develop specific "maturational enhancers", designed to modulate the nicotinic system response, or to rescue receptors that are misfolded, for instance due to congenital mutations such as those observed in myasthenic patients (Engel *et al.*, 2003).

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Virtual screening of compound and target libraries: Application to the identification of novel GPCR ligands

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G-Protein-Coupled receptors (GPCRs) represent the most important family of macromolecular targets amenable to therapeutical intervention. Until very recently, only very few targets have been investigated (>50) despite the huge number of druggable GPCRs (350-400) in the human genome. Herewith, we present a framework aimed at getting a reliable 3-D model for most human non-olfactory GPCRs that permits to address ligand design by taking into account the GPCR universe. The GPCR database currently contains 350 nonredundant entries and can be used for several purposes: (i) to prioritise binding studies of a given ligand to GPCRs supposed to be close enough to the cognate receptor and thus address the ligand selectivity as early as possible (ii) to locate poorly-studied or orphan targets in the GPCR universe and predict their druggability at the light of known data on already investigated receptors, (iii) to map transmembrane binding cavities from automatically-generated 3-D models, (iv) to search for novel ligands by screening focussed compound libraries on a defined target, (v) to search for the most likely target(s) of a known bioactive ligand by a reverse screening procedure. Each of the above-described directions will be described by test cases applied to widely divergent GPCRs.

Linking molecular motors to Alzheimer's disease

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Aberrant generation of amyloid- β peptides (Ab) and their aggregation into senile plaques are thought to play a critical role in the pathogenesis of Alzheimer's disease (AD). The knowledge that the microtubule-dependent motor protein, kinesin-I, mediates anterograde axonal transport of APP prompted us to investigate whether this transport may contribute to the formation of Ab. To this end, we analyzed a genetic cross between mice expressing APP harboring mutations linked to Swedish familial AD kindreds (Tg-swAPP), which exhibit characteristic axonal swellings, and mice deficient in the neuron-enriched kinesin light chain subunit 1 of kinesin-I (KLC1). We found that benign reductions in KLC1 were sufficient to increase the formation of axonal swellings and to enhance the amyloidogenic processing of APP. Our results reveal an interaction between kinesin-I and APP that leads to changes in Ab reminiscent of the ones caused by familial AD mutations found in presenilin proteins.

Pharmacological and molecular enhancement of learning in aging and AD

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The functional alterations in the brain during learning are beginning to be understood. As a new association is formed, neuron responsiveness or excitability to the newly significant stimulus input is enhanced. Neurons in aging brains have more difficulty increasing excitability during learning, a cause for age-related learning deficits. Calcium and calcium-activated potassium currents of neurons, that help control intrinsic neuronal excitability and are altered during learning and in aging, have been extensively studied and will be described. Behavioral studies in which drugs are administered to reverse the neuronal excitability changes during aging have demonstrated that age-associated behavioral changes can be reversed pharmacologically. These studies are being extended in mouse models of Alzheimer's Disease using molecular approaches to understand and treat the disease.

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Progressive degeneration of human mesencephalic neurons triggered by dopamine-dependent oxidative stress is dependent on the mixed lineage kinase pathway

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Models of Parkinson's disease (PD) based on selective neuronal death have been successfully applied to simulate the symptoms of PD, and subsequently to validate symptomatic therapies. Nevertheless, for investigation of putative neuroprotectants, it is essential that the conditions leading to neurodegeneration in experimental models replicate those occurring in the disease. We have developed a human-based *in vitro* model of PD based on the assumption that dys-regulated cytoplasmic dopamine levels trigger neurodegeneration in PD. Differentiated human mesencephalic neurons were exposed to methamphetamine (METH), in order to promote cytoplasmic dopamine accumulation. In the presence of elevated iron (Fe^{2+}) concentrations, also observed in PD, the increased cytosolic catecholamine levels lead to oxidative stress, activation of the c-jun N-terminal kinase (JNK) pathway, neurite disintegration, and eventually apoptosis. We examined the role of the mixed lineage kinases (MLK) in this complex degenerative cascade by using the potent inhibitor CEP1347. Selective block of MLKs not only inhibited Fe^{2+} /METH-induced JNK activation and apoptosis, but also early degenerative steps like neurite degeneration and lipid peroxidation. This broad neuroprotective action of CEP1347 was associated with increased transcription of the oxidative stress response element ATF4 and of the cystine-glutamate transporter. As a functional consequence, cellular cystine uptake and intracellular levels of the redox buffer glutathione (GSH) were augmented. Thus, this new human model of parkinsonian neurodegeneration has the potential to yield new insights into neuroprotective therapeutics. In the case of CEP1347, our findings suggest that the enhancement of cellular protective mechanisms may be essential for the disease-modifying properties of new PD drugs.

Synaptic plasticity and cell signaling in Alzheimer's disease

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The neuropathological correlates of Alzheimer's disease (AD) include amyloid- β (A β) plaques and neurofibrillary tangles. To study the interaction between A β and tau and their affect on synaptic function, we derived a triple transgenic model (3xTg-AD) harboring PS1_{M146V}, APP_{Swe}, and tau_{P301L} transgenes (Oddo *et al.*, 2003). The 3xTg-AD mice progressively develop amyloid plaques and neurofibrillary pathology in AD-related brain regions such as the hippocampus, cortex, and amygdala. Synaptic dysfunction, including LTP deficits, manifests in an age-related manner, but notably before plaque and tangle pathology. Deficits in short- and long-term synaptic plasticity correlate with the accumulation of intraneuronal A β . More recently, we further show that the onset of cognitive deficits also correlates with intraneuronal A β and, these deficits can be reversed by immunotherapeutic clearance of A β . Taken together, these studies suggest a novel pathogenic role for intraneuronal A β with regards to synaptic plasticity and cognitive deficits. In addition, our lab has is investigating the role that altered cell signaling plays in the pathogenesis of AD. In this regard, a role in nuclear signaling is one physiologic function mediated by a fragment of APP, called the APP intracellular domain (AICD). AICD rescues calcium signaling deficits in APP null cells (Leissring *et al.*, 2002) implicating calcium genes as a potential target for this fragment. Here we show that AICD governs the expression of the sarco-endoplasmic reticulum (ER) calcium ATPase, isoform 2b (SERCA2b), the ubiquitously expressed calcium pump that maintains ER calcium store levels, and further show that A β levels can be modulated by manipulating SERCA activity.

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Regulation of glutamate receptor function and synaptic plasticity

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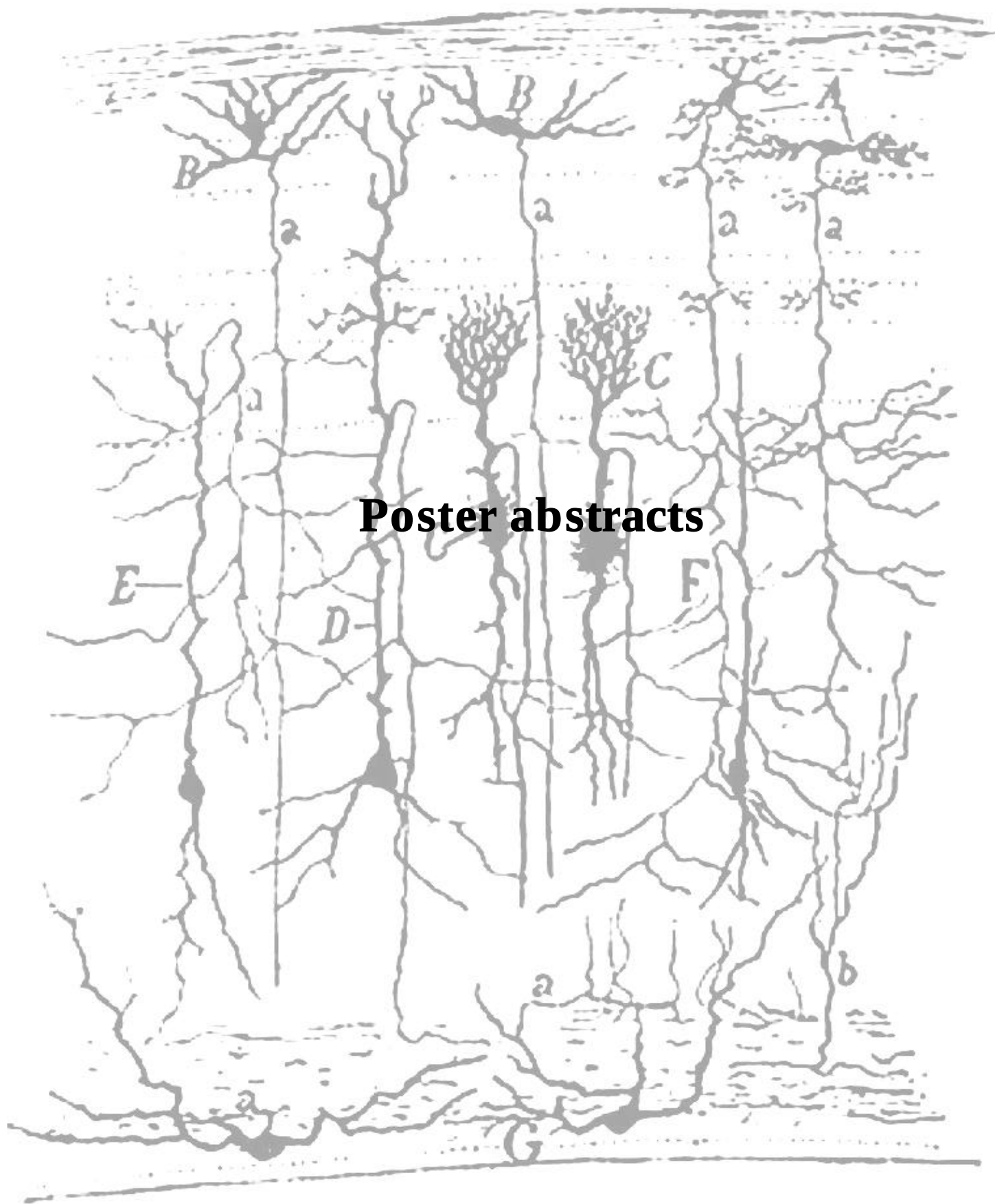
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Neurotransmitter receptors mediate signal transduction at the postsynaptic membrane of synaptic connections between neurons in both the central and peripheral nervous systems. We have been studying the molecular mechanisms in the regulation of neurotransmitter receptor function. Recently we have focused on glutamate receptors, the major excitatory receptors in the brain. Glutamate receptors can be divided into two major classes: AMPA and NMDA receptors. AMPA receptors mediate rapid excitatory synaptic transmission while NMDA receptors play important roles in neuronal plasticity and development. Studies in our laboratory have found that both AMPA and NMDA receptors are multiply phosphorylated by a variety of protein kinases. Phosphorylation regulates several functional properties of these receptors including conductance and membrane targeting. For example, phosphorylation of the GluR1 subunit of AMPA receptors by multiple kinases including PKA, PKC and CaM kinase II regulates its ion channel function. Recent studies have demonstrated that the phosphorylation of AMPA receptors is regulated during cellular models of learning and memory such as long-term potentiation (LTP) and long-term depression (LTD). Moreover, phosphorylation of GluR1 is required for the expression of these forms of plasticity and for the retention of spatial memory.

We have also been examining the mechanisms of the subcellular targeting and clustering of glutamate receptors at synapses. We have recently identified a variety of proteins that directly or indirectly interact with AMPA and NMDA receptors. We have found a novel family of proteins that we call GRIPs (Glutamate Receptor Interacting Proteins) that directly bind to the C-termini of the GluR2/3 subunits of AMPA receptors. GRIPs contain seven PDZ domains, protein-protein interaction motifs, which crosslink AMPA receptors to each other or link them to other proteins. In addition, we have found that the C-termini of GluR2 also interacts with the PDZ domain of PICK1, a protein kinase G-binding protein that is found at excitatory synapses. Finally, the GluR2 subunit also interacts with the NSF protein, a protein involved in the regulation of membrane fusion events. These AMPA receptor interacting proteins are critical in the proper subcellular trafficking and synaptic targeting of these receptors. We have recently shown that the binding of PICK1 is required for specific form of LTD in the cerebellum that is a cellular model for motor learning.

In addition to these studies on AMPA receptors, we have been characterizing a separate NMDA receptor associated protein complex that is important in synaptic targeting and downstream signaling of NMDA receptors. We have recently identified an excitatory synapse specific rasGAP, which we call synGAP that regulates synaptic Ras signaling and has profound effects on synaptic plasticity

In summary, we have examined the molecular mechanisms underlying the regulation of neurotransmitter receptor function. Our studies have suggested that regulation of receptor function may be a major mechanism for the regulation of synaptic plasticity in the nervous system and may be an important determinant of animal behavior.



Poster abstracts

**Expression of the transcriptional repressor gene myoneurin
at the neuromuscular junction, during mouse development and in the adult**

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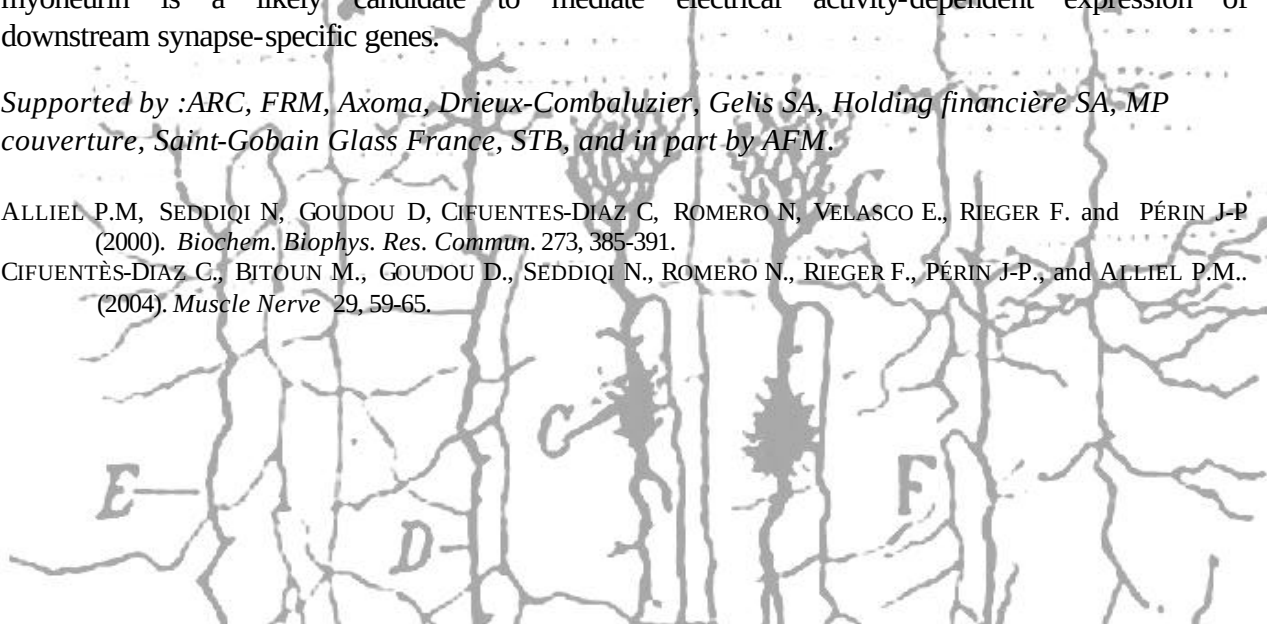
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The mixed agonist/antagonist activity of Selective Steroid hormone Receptors Modulators (SSRM), such as RU486, could be finely tuned by the relative amount of coactivators and corepressors within a given cell. To date, numerous coactivator proteins are characterized, but only few corepressor proteins have been identified. In the course of our investigation to identify novel repressors, we have cloned myoneurin, a novel protein with both a btb/poz domain and eight tandemly repeated kruppel-type zinc finger motifs. The btb/poz domain is found in various nuclear and cytoplasmic proteins mainly involved in ubiquitination and transcription repression. The human gene spans 16.5 kb, and is composed of seven coding exons and two non-coding exons. Three transcript variants have been identified in the 5' non-coding region and six alternatively spliced mRNAs affecting the coding region, could give rise to six isoforms. Rodent genes have similar intron/exon arrangement, but the 2nd non-coding exon is lacking. Promoter beta-galactosidase constructs were used to define the region (first intron) that conferred basal expression of the myoneurin gene. By using a GAL-4/luciferase reporter gene assay, we demonstrate that myoneurin represses gene transcription by an histone deacetylase-independent mechanism. The identification of N- and E-boxes elements within the 5'-upstream region, which are required for regulating the expression of genes coding for components of the neuromuscular junction (NMJ), prompted us to analyze the myoneurin expression at the motor end-plate and in mouse denervated muscle, by immunohistochemical and *in situ* hybridization studies. Myoneurin is a nuclear protein, whose expression is associated with subsynaptic nuclei at the neuromuscular junction, and is developmentally regulated, during the late embryonic period (E16, P0). Nerve crush or axotomy both lead to a burst of myoneurin transcription and to a dysregulated myoneurin expression in extrasynaptic myonuclei. Myoneurin was detected, by electron microscopy analyses, within the peripheral chromatin and in the euchromatin/heterochromatin borders, clearly associated with functional chromatin. Myoneurin exhibits both an innervation-dependent repression of transcription, and an enhanced expression following denervation, therefore the repressor myoneurin is a likely candidate to mediate electrical activity-dependent expression of downstream synapse-specific genes.

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Subunit c of F₀F₁-ATPase as a structural element of mitochondrial permeability transition pore

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The DCCD-binding proteolipid of F₀, the subunit c of F₀F₁-ATPase, was recently found to be in phosphorylated form. Dephosphorylation of subunit c was promoted by unselective Ca²⁺-induced mitochondrial permeability transition (PT) and prevented by cyclosporine A (CsA). Additionally, preincubation of the mitochondrial suspension with isolated dephosphorylated subunit c decreased the membrane potential, promoting “permeability transition pore” (PTP) opening¹. Incorporation of the subunit c into black-lipid membranes (BLM) increased the membrane conductivity by inducing single channel formation with conductances 15-360 pS. Their open to close state transition times varied in the ms-s range. In contrast the channels formed by the dephosphorylated form of subunit c had much longer open times (tens of minutes). Channels, produced by both phosphorylated and dephosphorylated forms of subunit c are cation-selective. Since subunit c was dephosphorylated during PTP opening, channel properties were investigated mostly on channels formed by dephosphorylated subunit c. These channels were found to be permeable to Na⁺ and to K⁺ ions. Mg²⁺ in range of 0.5-6 mM was observed to promote a linear increase of channel conductivity. Ca²⁺ in range of 10⁻⁶-10⁻⁴ M had no effect on transmembrane conductivity of open channels, that at the same time were permeable for calcium. It is known that PTP opening is accompanied by swelling of mitochondria. We showed that purified dephosphorylated subunit c added to mitochondrial suspension stimulated the swelling of mitochondria. Thus we demonstrated that Ca²⁺ and CsA-sensitive dephosphorylation of subunit c was correlated with the opening of PTP (decreasing of the membrane potential, swelling of mitochondria and ability to keep opened channels in BLM). It is thus suggested that subunit c of F₀F₁-ATPase might be a structural component of the unselective mitochondrial pore. It is worth to note some data that could serve as support of our findings. Subunit c homologues are known to be present in the plasma and intracellular membranes performing a variety of functions. One is a 16 kDa proteolipid, subunit c of the vacuolar V type H⁺-ATPase, involved in H⁺ transport, another one is the 16 kDa mediato-phore, component of synaptosomal membranes, participating in neurotransmitter transport and finally, ductin a 16 kDa poly-peptide of gap-junction-like structures that form megachannels during intercellular communications for small molecules. The channel-forming ability of these protein is dependent on their orientation in the membrane, on interactions with other proteins in multiprotein structures and can be regulated by Ca²⁺. Taking into consideration the data described above we may suggest that the c subunit of F₀F₁-ATPase in mitochondria also could be a multifunctional peptide.

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δ -cono peptide EVIA isolated from *Conus ermineus* is a new pharmacological tool for discriminating sodium channel subtypes

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Delta-conotoxin EVIA (δ -EVIA), isolated from the west-african gastropod *Conus ermineus* venom, contains 32 amino acid residues and a six cysteine/four loop framework similar to that of previously described conopeptides of the O-superfamily i.e., ω -, δ -, μ O-, and κ -conotoxins. However, it displays low sequence homology with the latter conotoxins.

Native and chemically synthesized δ -EVIA inhibit voltage-sensitive Na^+ channel inactivation with unique tissue specificity upon binding to receptor site-6 of neuronal Na^+ channels as detected in binding studies with rat brain synaptosomes. Using amphibian myelinated axons and spinal neurons, we demonstrate that δ -EVIA increases the duration of action potentials by inhibiting Na^+ channel inactivation, and enhances nerve terminal excitability and synaptic efficacy at the neuromuscular junction, but does not affect directly-elicited skeletal muscle action potentials.

The neuronal-selective property of δ -EVIA has been confirmed by showing that a fluorescent derivative of δ -EVIA labels motor nerve endings but not skeletal muscle fibers. Moreover, in a heterologous expression system, δ -EVIA inhibits inactivation of rat neuronal Na^+ channel subtypes (rNav1.2a, rNav1.3 and rNav1.6), but does not affect rat skeletal (rNav1.4) nor human cardiac muscle (hNav1.5) Na^+ channel subtypes. δ -EVIA, in the range of concentrations used, is the first conotoxin found to affect neuronal Na^+ channels without acting on Na^+ channels of skeletal and cardiac muscles.

Hence, it is a unique tool for discriminating voltage-sensitive Na^+ channel subtypes, and it may serve as a lead to design new drugs adapted to treat diseases characterized by defective nerve conduction.

Functional study of SG2Na by siRNA technique

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In neurons, scaffolding proteins play a crucial role in the establishment, maintenance and structure of dendritic spines. These proteins are also involved in many post-synaptic signaling pathways. In mammals, the three members of the striatin family (striatin, SG2NA, zinedin) constitute a new group of scaffolding proteins specifically expressed in dendritic spines. Information concerning the physiological role of the striatin family is beginning to emerge. First, we have shown that striatin expression is important for both dendritic growth in cultured rat embryonic motoneurons and for the control of motor function in adult rats. Second, striatin and SG2NA have been proposed to be regulatory subunit of proteins phosphatase 2A. At the molecular level, each protein of the striatin family shares identical protein-protein interactions domains : from the N to the C-termini, a caveolin-binding domain, a coiled-coil structure a Ca^{2+} /calmodulin-binding domain and a large WD-repeat-domain. To precise the implication of these proteins in signaling and trafficking in neurons, we used siRNA technique to inhibit SG2NA expression.

By semi-quantitative immunoblotting, we demonstrated that the selected siRNAs specifically and dramatically inhibited the exogenous expression of SG2NA. This effect was independent of the type of siRNA expressing vectors and was observed in different cell types (COS-7, N2a, B104 and HEK). To visualize siRNA expressing cells, we constructed bi-cystronic vectors expressing EGFP-Actin and siRNA. These vectors allowed us to observe a robust decrease in SG2NA expression in cultured hippocampal neurons. SG2NA inhibition was found to alter neuronal survival, presumably by apoptotic caspase dependent phenomenon. Thus, we concluded that SG2NA (and potentially other striatin family members) is probably involved in neuronal survival.

Antagonistic effect of calcium permeating pathways on embryonic *Xenopus laevis* neuronal differentiation

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Recent highlights in nervous system development demonstrate that frequencies of intracellular calcium transients code specifically for neuronal differentiation, axon extension and growth-cone pathfinding.

We found that the main effect of spontaneous calcium influx on cultured neuroectodermal cells was an inhibition of their differentiation into neurons. This inhibition was observed on pure neuroectoderm derived neurons independently of neurites outgrowth and it significantly decreased between the developmental stages 13 (neural plate) and 20 (neural tube). This effect was voltage-insensitive and could be reverse by micromolar concentration of nickel and lanthan. Patch-clamp experiments performed on immature differentiated neurons revealed a nickel-sensitive and voltage-insensitive calcium permeable cationic conductance that could mediate the inhibitory action of calcium.

On the other hand toxins that block specifically voltage-gated calcium channel (300 nM ω -conotoxin) and nicotinic receptors (100 nM α -bungarotoxin) significantly reduced neuronal differentiation while extracellularly applied ATP (1 mM) enhanced neuronal differentiation in a calcium dependent manner.

Taken together these results suggest that calcium influx through voltage-gated calcium channels, nicotinic receptors and purinergic receptor enhance neuronal differentiation whereas its influx through an unidentified nickel- and lanthan-sensitive channel has the opposite effect. Moreover these data suggest that the permeating pathway of the calcium ions is a spatial determinant of neuronal differentiation.

Control of nuclear calcium homeostasis of *Aplysia* neurons by multiple messengers : roles of NAADP, IP₃ and cADPR.

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Calcium release from intracellular stores in response to hormone or neurotransmitter stimulation is important in the control of synaptic plasticity or gene expression. Accumulative evidences suggest that nuclear calcium plays an important part in these processes. However, the mechanisms involved in the control of nuclear calcium homeostasis remain largely unexplored in neurons. In this study, we investigated the possible role of three intracellular messengers, known to trigger calcium release from the intracellular stores, inositol 1,4,5 trisphosphate (IP₃), cyclic ADP-ribose (cADPR) and the most recent messenger nicotinic acid adenine dinucleotide phosphate (NAADP) (Cancela JM *et al.*, 1999, Cancela JM *et al.*, 2002). The effects of these messengers were tested on isolated nuclei from *Aplysia* giant neurons.

The changes of the calcium concentration were assayed in the nuclear envelope as well as in the nucleoplasm using confocal microscopy. Our data showed that addition of IP₃ (10 μM), cADPR (10 μM) and NAADP (500 nM) induced a release of calcium from the nuclear envelope and increased nucleoplasmic calcium concentration. Our data suggest that the NAADP, cADPR and IP₃ receptors are localized preferentially in the inner nuclear membrane. Using a calcium sensitive fluorescent probe, located within the nuclear envelope, we found that the fluorescence intensity increased when ATP (5 mM) was added in the medium bathing the isolated nuclei, indicating the involvement of Ca²⁺ ATPases in the calcium uptake by the envelope. The nuclear calcium pumps were found sensitive to thapsigargin. Depletion of the thapsigargin-sensitive stores significantly reduced the calcium response evoked by IP₃ or NAADP. Interestingly, a NAADP dose-response experiment indicated that addition of nanomolar concentrations (10, 100 and 500 nM) of NAADP resulted in calcium release from every nuclei tested (n=9) whereas addition of micromolar concentrations of NAADP failed to evoke a response in 9 out of 12 nuclei. In those 3 responsive nuclei, the release of calcium was weak compared to the effect of nanomolar concentrations of NAADP. These results are reminiscent of the response obtained in intact cells of the mouse exocrine pancreas in which high concentration of NAADP (50 - 100 μM) does not generate any Ca²⁺ signal suggesting a complete desensitization of the receptor. Our work show that the bell-shape curve of NAADP effect also applies to neurons.

Our data show the first evidence that the nuclear envelope of a neuronal cell is a calcium store sensitive to several intracellular messengers suggesting a multiple control of gene expression.

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A critical role for astrocytes in the age-related alteration of long-term synaptic depression and depotentiation

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Synaptic plasticity depending on the activation of N-Methyl-D-Aspartate subtype of glutamate receptors (NMDAr) is altered in the aged rat and has been correlated to memory impairments. In the hippocampus, activation of NMDAr requires the binding at the strychnine insensitive glycine site of D-serine, an amino acid strictly synthesized in astrocytes. We previously reported that age-related deficits of synaptic plasticity were correlated to a decrease in D-serine efficacy since supplementing with the co-agonist prevented: 1) the depression of NMDAr-dependent postsynaptic potentials recorded in CA1 hippocampal slices of aged rats and 2) the deficits in long-term synaptic depression and depotentiation induced by low frequency stimulation of the Schaffer collaterals. In the present study, autoradiographic studies using ³H L689.560 as a radioligand and increased concentrations (3 nM to 300 μM) of the unlabeled competitor D-serine, exclude any age-related alteration in the affinity of the NMDAr for D-serine. Quantification of endogenous levels of the D-serine reveals a severe decrease of the amino acid in aged rats. Although semi-quantitative western blottings confirm an age-related gliosis, they show that the expression of the enzyme, serine racemase, which synthesizes D-serine, is significantly lowered in aged animals. On the contrary, the expression of the D-serine-degrading enzyme D-amino acid oxidase is not affected. Other putative mechanisms to account for the age-related decreased level of D-serine such as altered enzymatic activities or reduced level of the precursor L-serine are under investigation.

In conclusion, our results document for the first time that a reduction in synaptic deposition of the glial-derived factor D-serine is related to functional deficits occurring during normal brain aging underlying the physiological importance of maintaining the glial-neuronal cross-talk in the active brain..

Early electrophysiological abnormalities in lumbar spinal motoneurons in a transgenic mouse model of amyotrophic lateral sclerosis

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Amyotrophic lateral sclerosis (ALS) is an adult-onset and lethal disease characterized by a selective degeneration of motoneurons. However, some data suggest that ALS, as others neurodegenerative diseases, could be a consequence of an abnormal development of nervous network (Amendola *et al.*, 2004) and neurons properties. In order to test this hypothesis we used *in vitro* neonatal spinal cord preparations from SOD1^{G85R} mice, a transgenic model of ALS, and from wild type (WT) mice at the same age. This preparation allowed us to investigate, by intracellular recordings *in situ*, electrical properties of lumbar spinal neurons and motoneurons identified by their antidromic stimulation. There was no differences between the two strains in membrane resting potential, action potential and afterhyperpolarisation characteristics. But, we observed a larger input conductance (Gin) for SOD1^{G85R} motoneurons (Gin = 54.3 ± 6.9 nS, n = 31 SOD1^{G85R} motoneurons; Gin = 30.3 ± 2.8 nS, n = 14 WT motoneurons, * p<0.05 t-test) whereas the Gin from other spinal neurons were similar for the two strains (Gin = 37.4 ± 6.1 nS, n = 29 SOD1^{G85R} neurons; Gin 24.7 ± 3 nS, n = 25 WT neurons). The motoneurons of transgenic mice presented lower rheobase and slope of the current-frequency curves. Again the modifications observed between the two strains were specific to motoneurons and did not affect the other spinal neurons. We also noted that recurrent post-synaptic potentials appeared more frequently for SOD1^{G85R} motoneurons following antidromic stimulation of the ventral root (occurrence: n = 12/18 SOD1^{G85R} motoneurons *versus* n=4/17 WT motoneurons, Fisher exact test * p = 0.017). Furthermore, the dorsal root evoked excitatory post-synaptic potentials (EPSPs) recorded from mutant motoneurons exhibited a longer duration although no changes occurred in latency, amplitude and time to peak. These results show that SOD1^{G85R} motoneurons present very early altered electrophysiological properties and might provide a new key for the understanding of the pathogenesis of ALS.

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Catecholamine release from fetal rat chromaffin cells

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Catecholamines (CA) secreted by fetal adreno medullary chromaffin cells (AMCs) play an essential role during the adaptation to extrauterine life. Experiments were performed on primary culture of AMC from E15 and E19 rat fetuses. The techniques of Ca^{2+} current, amperometry and capacitance measurements were used.

At E15, application of digitonin (20 μM) and 10 μM free Ca^{2+} induce secretion of CA. At both stages, the exocytotic process requires the expression of HVA calcium channels (Bournaud et al. 2001), this current density is not significantly different and its exocytotic efficiency remains the same.

The quantal secretion of CA during this prenatal period, was compared by the cumulative integral of amperometric currents induced by depolarisation with 20 s exposure to 70 mM KCl, together with the characteristics of individual spikes. At E19, the value of cumulative integrals of amperometric currents at 1 min after KCl application was larger than at E15; such difference is the consequence of the increase in the number of molecules of CA per granule. The time delay to release 50% of the total amount of quanta mobilized by KCl was shorter at E19 than at E15. During the perinatal period, AMCs are not innervated and the CA release is supposed to be controlled by an oxygen sensing mechanism (Thompson et al., 1997). At E19, hypoxia induces a membrane depolarisation and CA release but at E15, hypoxia induces membrane hyperpolarisation. Anoxia differentially modulates multiple K^{+} currents (Thompson and Nurse, 1998). In fact, the dual effect over membrane potential is related to the changing contribution of different types of K^{+} channels. We compared the effect of glibenclamide (50 μM) over the total K^{+} current with or without external Ca^{2+} . At E19, IK_{Ca} is the main component (61 %) and IK_{ATP} is 14.25 %, of the total IK^{+} currents. At E15, the predominant K^{+} current is IK_{ATP} (48.7 %) and IK_{Ca} is 29.46% of the total. At E19, both Apamin (400 nM) and Iberiotoxin (100 nM) induce membrane depolarisation, but hypoxia failed to generate further membrane depolarisation in the presence of apamin.

These data show that at E15, the exocytotic process is functional but the quantal size is small compared to E19. The non-neurogenic CA secretion appears when the Apamin-sensitive K^{+} current is the main component of the total K^{+} current.

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Tonic release of glutamate in hippocampal slices

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Tonic glutamate release may regulate the baseline excitability of neurons. It has been suggested to be non-vesicular and by cystine/glutamate exchange. We assessed tonic glutamate release by measuring the inward NMDA receptor-mediated current produced when glutamate transporters were blocked by TBOA. (Control experiments assessed the effects of the manipulation used on the NMDA receptors used to sense glutamate). This release was unaffected by reducing vesicular release with cadmium, but was potentiated by blocking glial glutamine synthetase. It was unaffected by blocking swelling-activated ion channels, prostaglandin-mediated release from astrocytes, P_2X_7 receptors or gap junction hemichannels. Superfused cystine increased glutamate release, but block of cystine/glutamate exchange had no effect on release in the absence of superfused cystine. DIDS reduced the release, and this was not mimicked by propionate, implying this suppression did not simply result from the acidification of internal pH that DIDS produces.

Knockdown of amyloid precursor protein gene corrects altered intracellular Ca^{2+} signals in a cell line derived from the cerebral cortex of trisomy 16 mouse: an animal model of Down Syndrome

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Murine trisomy 16 (Ts16) is an animal model for Down syndrome (DS). We have generated immortal neuronal cell lines from normal and Ts16 mice. Our Ts16-derived lines overexpress DS-related genes (APP, amyloid precursor protein; SOD-1; Cu/Zn superoxide dismutase) and show altered cellular responses to glutamatergic stimuli. Here, we present evidence of APP overexpression in our CTb cell line, derived from mouse Ts16 cerebral cortex, compared to the CNh line, derived from a normal animal. RT-PCR studies showed that CTb cells express 38% more APP mRNA than CNh cells. Confocal microscopy and Western blot (WB) showed intracellular accumulation of APP products. To relate this excess APP to glutamatergic dysfunction, we transfected CTb cells with specific mRNA antisense sequences in order to knockdown APP expression. WB studies showed knockdown of APP expression by 39.4%, 48 h post transfection (n=4, p<.05). We then studied intracellular Ca^{2+} responses to 200 μM external glutamate in Fluo-3 loaded cells. The decay of Ca^{2+} signals showed significant reductions (p<.05) of the time constant at 24 h ($31,53 \pm 27,2$ s; n=8) and 48 hrs ($34,42 \pm 4,99$ s; n=7) post transfection, compared to non-transfected cells ($97,25 \pm 21,22$ s; n=7). The results suggest that APP overexpression in CTb cells could contribute to impaired glutamatergic function, and that gene knockdown in cell models is a relevant tool to study DS-related pathophysiology.

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A coiled-coil interaction mediates the formation of a ternary complex between dystrophin, dystrobrevin and kinesin heavy chain: possible implications in the mechanisms of transport

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Dystrobrevin, a dystrophin-related protein, is a cytoplasmic component of the dystrophin-associated protein complex (DPC) that, in muscle, localizes to the sarcolemma. Mutations of DPC-components are associated with various forms of muscular dystrophy. DPC-like complexes are expressed in non-muscle tissues including kidney, lung and brain. Recent studies have shown that DPC not only functions as a mechanical link between extracellular matrix and cytoskeleton but also as a highly dynamic scaffold for signaling proteins. Among several protein isoforms coded by two different dystrobrevin genes, beta-dystrobrevin is restricted to non-muscle tissues, and in brain it has been found highly enriched at the postsynaptic densities. To understand the function of DPC-like complexes in brain, we have focused on molecular associations of beta-dystrobrevin and identified, by yeast two-hybrid screening of a mouse brain cDNA library, the neuronal kinesin heavy chain Kif5A as a new interacting partner for beta-dystrobrevin. The kinetic rate constant of beta-dystrobrevin-Kif5A interaction *in vitro*, as evaluated by surface plasmon resonance, is in good agreement with other previously reported association kinetics of kinesin with other cargoes. By *in vitro* and *in vivo* association assays, we have found that this interaction is not restricted to beta-dystrobrevin and Kif5A, but reflects a more general mechanism involving alpha-dystrobrevin and Kif5B as well, thus suggesting a role for dystrobrevins as motor protein receptors.

Since coiled-coil regions of both kinesin and dystrobrevin are involved in the interaction, and dystrobrevin and dystrophin are known to interact through their coiled-coil motifs, we have investigated whether dystrophin can affect the dystrobrevin-kinesin interaction. *In vitro* experiments indicate that dystrophin does not interact directly with Kif5, but it is pulled-down by the dystrobrevin-kinesin complex, with whom it associates to form a ternary complex.

These data suggest that not only dystrobrevin is involved in the signaling function of the DPC, but may also play a role in the transport and targeting of its binding partners, such as dystrophin, to specific sites in the cell.

Modulation of a presumptive $\text{Na}_v1.9$ channel by the activation of NK_3 tachykinin receptors in enteric sensory neurons

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Voltage-gated Na^+ currents play a critical role in shaping electrogenesis in neurons. We have identified a tetrodotoxin-resistant Na^+ current in duodenum enteric sensory neurons of the Guinea Pig. The voltage dependent properties of this current are similar to those reported for $\text{NaN}/\text{Nav}1.9$ in dorsal root ganglion neurons. Because the activation of NK_3 tachykinin receptors (NK_3r) induces slow synaptic post synaptic potentials in enteric sensory neurons we examined whether senktide, a specific agonist of NK_3r could modulate the NaN like current in enteric sensory neurons.

Whole-cell patch clamp recordings from *in situ* neurons were performed. Senktide applied by short applications (15-20s) induced systematically a fast increase of the peak current. This effect is due to a shift of the activation threshold as well as of the whole current-voltage relationship. The activation curve before senktide was characterized by a mid point activation ($V_{1/2}$) of $-22.4 \pm 6 \text{ mV}$; this value shifted to $-34.7 \pm 5.1 \text{ mV}$ after senktide ($P < 0.05$, $n=12$). The sensitivity to voltage expressed by the slope factor k was unchanged (control $k = 4.96 \pm 1.6 \text{ mV}$; senktide $k = 4.36 \pm 1.4 \text{ mV}$). The conductance (g_{max}) of the neurones was not significantly affected by senktide (control g_{max} : $25 \pm 12.3 \text{ nS}$; g_{max} after senktide: $28 \pm 16.3 \text{ nS}$).

Preliminary results have shown that the inactivation curve was similarly shifted. Superimposing the activation curve to the steady state inactivation one gives a bell shape curve ranging from -40 to 0 mV (control conditions) indicating that a persistent Na^+ current should be produced in this window and should amount to 20% of the peak current. After NK_3r activation, this window current is preserved but shifted to more hyperpolarized values (from -60 mV to -20 mV).

Because it is well known that the activation of NK_3r triggers PKC pathways, we tested a Ca^{2+} -dependent PKCs activator (OAG; $10 \mu\text{M}$) and a non selective PKC activator (PDBu; $10 \mu\text{M}$). OAG shifted the mid point of activation from $-23.3 \pm 4.43 \text{ mV}$ to $-31.3 \pm 6.2 \text{ mV}$ ($n=3$) without affecting the slope factor and the maximal conductance. Similar results were obtained with PDBu (control $V_{1/2} = -19.13 \pm 3.3 \text{ mV}$; PDBu $V_{1/2} = -27.5 \pm 0.47 \text{ mV}$; $p < 0.05$). Preliminary results indicate that the effect of NK_3r activation is partly blocked by Ca^{2+} -dependent-PKC inhibitors (chelerythrine $10 \mu\text{M}$) as well as by a general PKC inhibitor (calphostine $10 \mu\text{M}$).

This is the first demonstration that the NaN current can be modulated by NK_3r activation. This modulation occurs mainly via PKC pathways. Under control conditions, the range of potentials that allows a persistent current is reached only transitory during action potentials. When modulated by the NK_3r activation, the NaN current can be persistent in a range of potentials that is experienced by enteric sensory neurons during slow excitatory post synaptic potentials that last around 2 min.

Conditional expression of two reporter genes in a transgenic mouse

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The nervous system is composed of some millions of nervous cells. From their functional role and the anatomical position, we can distinguish three kind of nervous cells, the sensory neurons, interneurons and motor neurons. These nervous cells are bound by specific synaptic connexions that are established during the embryonic life and can be modified later during activity-dependant modifications. These networks are responsible for learning and behaviour processes

To analyse the formation of synaptic connections and its dynamics after birth, it is important to image it in the living animal. We use in the laboratory, a genetic maker composed of C- terminal fragment of tetanus neurotoxin (TTC fragment) fused to the *gfp* gene (Green Fluorescent Protein). The hybrid protein is non toxic and retains the neuronal binding and retrograde trans-synaptic transport properties of the holotoxin. For these studies, I have made one transgenic reporter mouse (by microinjection of the linear transgene in the egg at stage one cell), which contains the hybrid gene *gfp-ttc* under the control of *Rosa26* ubiquitous promoter. The tracer transneuronal is coupled to an internal ribosome entry site (IRES) sequence to coexpress a *nls-LacZ* (nuclear localization marker LacZ) reporter gene as a nuclear marker of the cell where protein synthesis occurs. This “stationary” marker thus identifies the neuron actually expressing the transgene, and distinguishes it from the neurons receiving the fusion construct because those will be Bgal negative, but GFP positive. The activation of the *Rosa26* promoter depends of the activation of the recombinase Cre because we have introduced a loxP cassette in front of the *gfpttc* gene. By crossing this transgenic mice with an inducible mice *PGK-Cre* mice (Lallemand and al, 1998) which expressed the recombinase Cre everywhere, we observe the expression of our transgene only in certain region of the central nervous system of the mice. There is a very strong labelling punctate of the two reporter genes in the trigeminal ganglion during embryogenesis since embryonic day 12,5 and in adult life. However, this region contains only sensory neurons and does not permit us to follow an eventual transport of GFP-TTC protein because of the sense of the transport. We have also observed the expression of the two reporter genes in the retina. In the retina there are several types of neurons. Briefly, photoreceptor cells send nervous signal to bipolar cells which are connected to the ganglion cells. It appears that ganglion cells and bipolar cells expressed the two genes. Furthermore, in the bipolar cells, we remarked an accumulation of GFPTTC protein in the dendrites of the bipolar cells.

The mice can provide a tool to visualize some particular cells in the trigeminal ganglion and in the retina. In the retina, reporter genes are only expressed in bipolar cells and ganglion cells. We could eventually visualize a transport between the bipolar cells and the photoreceptors. Also this mice can be use to study the transport of the TTC in the neuron because we have an endogenous expression of the TTC in some neurons and we can follow *in vivo* its transport in a system like retina.

The NO pathway to increase utrophin in muscle: preclinical tests with an arginine derivative

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Strategy for palliative treatment of Duchenne (DMD) and Becker muscular dystrophies is to compensate for dystrophin absence by up-regulation of utrophin, another cytoskeletal protein of the muscular membrane. Treatment with L-arginine, the substrate for nitric oxide synthase or with molsidomine, a nitric oxide donor, increases utrophin levels in muscles of *mdx* mice (the animal model for DMD) and is associated with a global regression of the major symptoms of the disease. To propose a clinical trial for DMD and Becker patients we tested the ability of arginine butyrate, an arginine derivative already used to treat children affected by sickle cell anaemia, to produce the same beneficial effects as L-arginine. Treating *mdx* mice for 6 weeks with arginine butyrate (100 mg/kg/day, i.p. injection) resulted in decrease in serum creatine kinase levels by 55 %, attesting to the improvement of the whole musculature. Magnetic resonance imaging of lower limbs revealed a 40 % reduction of the necrotic zones, confirming the reduction of muscle pathology evidenced by histological staining. As expected, utrophin levels and transcript in the muscles were increased by 45% and β -dystroglycan, a member of the dystrophin-associated protein complex which disappears from the membrane in dystrophic muscle, was relocalised with utrophin. During exposure to hypercapnic conditions (6 and 8 % CO₂), the average respiratory tidal volume was significantly increased by 14 % in treated *mdx* mice. This improvement of diaphragm performance was supported by the reduction of the number of fibers stained by Evans blue, a vital dye, in treated mice. No weight variation was noticed between treated and non-treated mice when the treatment was applied to young animals during growth, or to adults for a longer period (one year), suggesting that the treatment was not toxic. Finally, utrophin levels in cultured human myotubes were increased (2 fold) after a 48 hour treatment with 0.5 mM arginine butyrate. Accordingly, and as arginine butyrate efficiently increases utrophin levels and reverses the dystrophic phenotype in mice, a move towards clinical trials appears justified.

GPR54 a new receptor involved in the neuroendocrine regulation of the gonadotropic axis and the onset of the puberty

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The control of GnRH secretion is determinant for reproductive maturation. It leads to normal sexual differentiation during foetal life, normal puberty and therefore normal fertility. GnRH neurons arise from olfactory placode and migrate to forebrain and hypothalamus along olfactory tractus. In mammals, transient activation of the gonadotropic axis occurs during late pregnancy and postnatal. An inhibitory phase of GnRH secretion occurs during childhood. A new increase of pulsatile GnRH release leading to secretion of glycoprotein hormones by the pituitary and sexual hormones by the gonads define the onset of the puberty. The molecular mechanisms involved in this sequence activation-inhibition-reactivation remain largely unknown.

For several years, we have investigated these mechanisms by a human genetic approach. Functional defect of the hypothalamo-pituitary unit of the gonadotropic axis leading to hypogonadism (hypogonadotropic hypogonadism or HH) has been described in several human genetic diseases. HH is defined by low circulating plasma levels of sex hormones associated with paradoxal normal or low plasma gonadotropin levels. Cases of isolated congenital hypogonadotropic hypogonadism are classified into 2 categories : those associated with anosmia (Kallmann syndrome) and those apparently isolated without anosmia. Mutations and deletions of the KAL-1 gene which encodes a protein involved in cell adhesion have been observed in many cases of the X-linked form of Kallmann syndrome. Very recently, loss of function mutations of fibroblast growth factor receptor 1 was shown to be linked to dominant form of Kallmann syndrome. Cases of hypogonadotropic hypogonadism without anosmia (IHH) were considered as idiopathic until the recent description of mutations of the GnRH receptor, LH or FSH genes. However, defects in these genes only account for only a small percentage of familial cases with mendelian transmission. These results clearly suggested that other unknown proteins play a major role in the physiology of the gonadotropic axis. Our group recently described GPR54 as one of these proteins by a genome mapping strategy performed in a very informative family. GPR54 is a G-protein coupled receptor express within hypothalamus and pituitary. It is the receptor of a RF-amide peptide derived from KiSS-1 protein known as a metastasis suppressor. Invalidation of GPR54 in the mouse, peripheral or central KiSS-1 injection and two other descriptions of GPR54 loss of function mutations in human have confirmed the key role of this receptor in the regulation of LH and FSH synthesis. These results open a new field in the physiology of the onset of the puberty and lead to suggest a new pharmacological modulation of this axis which may occur at the level of the hypothalamus or the pituitary.

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Two-component action of ATP on transmitter release from the motor nerve endings at the neuromuscular junction.

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Neuromuscular junction is composed by three active partners such as the motor nerve ending, the postsynaptic specialization of the muscle fiber and the perisynaptic glial Schwann cells (Auld and Robitaille, 2003). ATP coreleased from the motor nerve endings with ACh could potentially activate any of these three partners culminating in modulation of synaptic efficacy. We previously showed that, on skeletal muscle, ATP inhibited ACh release via presynaptic P2 receptors. The receptor identification (P2X or P2Y), and receptor transduction mechanism remained, however, unknown. In the present study using the voltage-clamp technique and imaging technique we analyzed the properties of presynaptic ATP receptors, its subsequent effector mechanisms and potential involvement of postsynaptic membrane and glia. ATP depressed multiquantal end-plate currents without changes in the amplitude of miniature currents indicating reduced quantal release. This depressant effect of ATP had a longer latency than similar inhibitory action of adenosine operating via P1 receptors suggesting a multistep or multicomponent activation. ATP γ S, agonist at P2X receptors, or Bz-ATP, agonist at P2X₇ receptors, were ineffective. The action of ATP was prevented by suramin and unchanged by PPADS or TNP-ATP, antagonists of P2X receptors, RB-2, a blocker of certain P2Y receptors, or MRS-2179, a specific blocker of P2Y₁ receptors. The depressant action of ATP was reproduced by UTP, metabotropic P2Y receptor agonist. Pertussis toxin, antagonist of Gi/o-proteins, and inhibitors of phosphatidylcholine specific PLC and PKC prevented the effect of ATP. The blockers of PLA₂ and COX significantly attenuated the depressant action of ATP. Similar attenuation of depression was obtained with several antioxidants. Imaging technique with dichlorofluorescein revealed an increased production of reactive oxygen species in Schwann cells overwhelming the fluorescence of motor nerve terminal and muscle fiber. However, upon activation by ATP muscle fiber became a major source of fluorescence suggesting that reactive oxygen species produced by muscle could retrogradely participate in presynaptic inhibitory effect. Thus, our data indicate, the existence at the neuromuscular junction of inhibitory PTX-sensitive P2Y receptors probably coupled to two distinct executive cascades one of which includes PLA₂ and COX with subsequent generation of reactive oxygen species, new messengers mediating the action of extracellular ATP. This divergent presynaptic P2 signalling could provide feedback inhibition of transmitter release and perhaps be involved in presynaptic plasticity.

Synaptic efficacy and remodeling at the neuromuscular junction of knockout mice deficient in acetylcholinesterase

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At the vertebrate skeletal neuromuscular junction two closely related enzymes can hydrolyze acetylcholine (ACh): acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). The classical function of AChE in regulating the duration of ACh action in the synaptic cleft is well established. However, the function of BChE remains controversial. Therefore, the present study was designed to evaluate neuromuscular transmission and the eventual role of BChE in knockout mice deficient in AChE activity. For this purpose we used AChE knockout mice (AChE^{-/-}) (Xie *et al.* 2000, Minic *et al.* 2003), transgenic mice with deletions of alternatively spliced exons of the *AChE* gene (deleted exons 5 + 6, and deleted exon 5/Neo) (Feng *et al.* 1999), collagen Q-deficient mice (ColQ^{-/-}) (Feng *et al.* 1999), and PRiMA (Perrier *et al.* 2002) knockout mice (PRiMA^{-/-}).

The kinetics of the current flowing through the endplate membrane in response to an ACh quantum was analyzed in isolated phrenic-hemidiaphragm neuromuscular preparations from the various transgenic mice, as well as nerve-evoked synaptic responses, muscle twitch and tetanic tensions. In addition, complementary information was obtained by confocal laser scanning microscopical examination of the endplate region of the various transgenic muscles in order to determine the characteristics of muscle nicotinic ACh receptor clustering.

The results obtained indicate that focally recorded miniature endplate currents (MEPC) exhibit a prolonged decay time in all transgenic mice examined with the exception of PRiMA^{-/-} mutant muscles in which MEPCs were not affected.

Complementary morphological examination revealed that there is an important remodeling and different clustering of muscle nicotinic ACh receptors at junctions lacking AChE activity in which MEPCs were prolonged. These results will be discussed in terms of physiological adaptation or compensation for the absence of AChE activity, and in relation to the expression of AChE and BChE at the neuromuscular junction.

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Effect of Delta Sleep Inducing Peptide on NMDA and GABA evoked ion currents in cortical, cerebellar and hippocampal neurons.

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Delta Sleep Inducing Peptide (DSIP) is an endogenous substance that induces sleep in human and animal and blocks the NMDA elicited seizures in rats *in vivo*. Effect of DSIP on NMDA and GABA-evoked ion currents in cortical, cerebellar and hippocampal neurons was studied using whole-cell patch clamp technique. DSIP in range of concentrations 0.1 pM – 1 nM inhibited partially the ion currents elicited by NMDA in cortical neurons. DSIP reduced the NMDA evoked currents up to 40-60% of control value at all concentrations tested. DSIP affected the ionic currents within 1 min. The inhibitory effect of DSIP was fully reversible. DSIP in range of concentrations 1 pM – 1 nM partially inhibited the currents elicited by NMDA in hippocampal neurons excepted at 1 nM. The maximal inhibiting effect of DSIP, 44% of control value, was observed at 10 pM.

DSIP in range of concentrations 0.01 pM – 1 nM increased the ionic currents elicited by GABA in both hippocampal and cerebellar neurons. DSIP affected the ionic currents within 1 min. The inhibitory effect of DSIP was totally reversible. In hippocampal neurons, the maximal increase of the GABA-evoked currents, more than 200% relative to control value, was observed at DSIP concentrations of 0.1, 1 and 10 pM. The effect of DSIP was less prominent at concentrations less than 0.1 pM and higher than 10 pM. On the other hand in cerebellar Purkinje cells DSIP was most effective at 0.01 pM showing increase about 250% of control value. At 0.1 pM – 1 nM DSIP was less effective than at the lowest dose tested. Thus the inhibitory influence of DSIP on NMDA evoked ion currents suggests that the anticonvulsive effect of the compound might be due to blockade of responses to NMDA. Similarly, the potentiating influence of DSIP on GABA elicited ion currents suggests that the sleep inducing effect of the compound on animal might be due to an increase of responses to GABA and a decrease of responses to NMDA as well.

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Agrin mediates a rapid switch from electrical coupling to synaptic transmission during synaptogenesis

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In integrated biological systems, a finely tuned coordination between different mechanisms of cell-to-cell communication is required for an appropriate regulation of physiological function to take place. Although neuronal cells mainly use the synaptic transmission mode to exchange instructive signals, it is now well established that an electrical communication mediated by gap junctions is also a key component of intercellular communication in the nervous system. Compelling evidence argues that intricate interactions between electrical and chemical synapses play an essential role during synaptogenesis. As a common finding, the critical phase of synapse formation is accompanied by a switch from a predominant electrotonic coupling to a fully mature synaptic transmission. At present, the cellular mechanisms favoring the switch from electrical to chemical communication that occur during synaptogenesis still remain to be elucidated. We addressed this issue at the developing splanchnic nerve-chromaffin cell cholinergic synapse in newborn rat acute adrenal slices. At birth, the innervation of chromaffin cells by the cholinergic synaptic terminals arising from the splanchnic nerve is not fully competent but completely matures during the first postnatal week. Moreover, neonate chromaffin cells display a high level of electrical coupling (Martin *et al.*, 2003) compared to adult (Martin *et al.*, 2001). The neonate adrenal medulla therefore represents a suitable model to examine in parallel how these two major modes of cell-to-cell communication interact each other and are regulated during synaptogenesis. We reported here an entirely unanticipated role for the extracellular matrix protein agrin in the regulation of both electrical coupling and synaptic neurotransmission during postnatal development of the adrenal medulla. Immunofluorescent detection of agrin revealed an intense staining throughout the adult medulla outlining chromaffin cell clusters, whereas in neonates agrin was more weakly expressed. When bath-applied in neonate slices, agrin rapidly induced a decrease in gap junction-mediated electrical coupling between chromaffin cells (50% reduction in the gap junctional current amplitude recorded in cell pairs within 5-6 min). In parallel, agrin increased postsynaptic nicotinic currents. This switch from electrical coupling to chemical synaptic transmission was fully operational within one hour and depended on the activation of Src family-related tyrosine kinase-mediated signaling pathway.

Thus, in addition to its well-established function in the assembly of the neuromuscular junction, agrin may play a pivotal role in synaptogenesis and the maturation of stimulus-secretion mechanisms in favoring a rapid switch from electrical to chemical synapses. Because in the central nervous system agrin is expressed at early developmental stage in areas involved in synaptogenesis, we hypothesize that the unexpected role of agrin reported here might be extended to other developing neuron-neuron synapses.

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Mutations in *MUSK* cause congenital myasthenic syndrome

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We report the first case of a human neuromuscular transmission dysfunction due to mutations in the gene encoding the muscle-specific receptor tyrosine kinase (MuSK). Gene analysis identified two heteroallelic mutations, a frameshift mutation (c.220insC) and a missense mutation (V790M). The muscle biopsy showed dramatic pre- and postsynaptic structural abnormalities of the neuromuscular junction and severe decrease in acetylcholine receptor (AChR) ϵ -subunit and MuSK expression.

In vitro and *in vivo* expression experiments were performed using mutant MuSK reproducing the human mutations. The frameshift mutation led to the absence of MuSK expression. The missense mutation did not affect MuSK catalytic kinase activity but diminished expression and stability of MuSK leading to decreased agrin-dependent AChR aggregation, a critical step in the formation of the neuromuscular junction. In electroporated mouse muscle, overexpression of the missense mutation induced, within a week, a phenotype similar to the patient muscle biopsy: a severe decrease in synaptic AChR and an aberrant axonal outgrowth.

These results strongly suggest that the missense mutation, in the presence of a null mutation on the other allele, is responsible for the dramatic synaptic changes observed in the patient.

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Coupling between L-type calcium channels and phospholipase C in muscle cells

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Neonatal rat primary muscle cultures were subjected to trains of 400 pulses of 1 ms duration, delivered at 45 Hz. At this frequency, the myotubes developed action potentials in response to each pulse. During the stimulation train cells showed a fast calcium signal associated to E-C coupling and blocked by TTX and ryanodine. Several seconds post-stimulus, there is a second calcium signal, more prominent within the nucleus, which may last 5 to 10 minutes. This slow calcium signal was action potential dependent, the dihydropyridine receptors (DHPR) having a sensor role and calcium coming mainly from IP₃ sensitive stores (Biophysical J. 86: 3042, 2004).

Treatment of cells with a G protein inactivator (*pertussis toxin*, PTX) blocks both calcium signals and IP₃ transients. The viral transduction of a G $\beta\gamma$ scavenger (Ad β ARK) also inhibit the slow calcium response. These experiments suggest the participation of the G $\beta\gamma$ subunit of the G protein in the signaling of the DHPR to phospholipase C (PLC) activation in skeletal muscle.

Immunocytochemical studies using an anti PLC β 3 antibody show, under confocal microscope, a diffuse dotted pattern in the cytosol. 30 to 50 s post electrical stimulation, a distinct punctuate staining pattern in the nuclear envelope became evident. This staining reverses after 70 s. Translocation of PLC β 3 occurs both in the presence and in the absence of extracellular calcium but is inhibited by 10 μ M nifedipine.

These results suggest a novel mechanism of signal transduction coupled to the DHPR acting as a voltage sensor by which the G protein is involved in the PLC β 3 activation through translocation to the nuclear membrane. This mechanism may be part of the excitation-transcription coupling process in muscle cells.

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Identification of amino acids responsible for the effect of ivermectin at purinergic P2X₄ receptor

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The rat P2X₄ receptor is unusual among the purinergic P2X receptors in its potentiation by an antihelminthic drug ivermectin (IVM). Ivermectin increases the amplitude of ATP-induced currents and greatly prolongs the action of ATP at P2X₄ receptors in homomeric and heteromeric configuration, but does not affect ATP-induced currents in cells expressing other types of purinergic receptors. If applied alone in the absence of ATP, IVM induces a small current in P2X₄-expressing cells. Ivermectin binds to the P2X₄ receptor at an allosteric binding site, which has not yet been localized. The effect of IVM is time-dependent indicating that its accumulation in the membrane may be critical. We studied the effect of site-directed mutagenesis in the first transmembrane domain (TM1) and within the membrane-related part of ectodomain N-half of the rat P2X₄ purinergic receptor. The effect of ivermectin on ATP-induced currents was measured in HEK cells expressing recombinant and mutated rat P2X₄ receptors. In the presence of 3 μ M IVM, the amplitude of (1mM) ATP-induced P2X₄ current was potentiated by 450 $\pm$ 55% and the decay time constant of current induced by agonist removal increased from about 300 ms to 45 s. Mutation of crucial amino acid in ectodomain structure abolished the prolongation but caused IVM to retain potentiation effect. Mutations in TM1 caused partial loss of potentiation effect of IVM and ivermectin alone did not induce any current in these mutated receptors. These results suggest that IVM binding site responsible for ATP current modulation is localized both in TM1 and ectodomain N-half of the P2X₄ purinergic receptor.

An astrocytic control of excitatory transmission at granular cell synapses in hippocampus

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Astrocytes possess synaptic-like microvesicles containing glutamate and release the transmitter via a Ca^{2+} -dependent exocytotic process similar to that described in neurons (Bezzi et al., Nature Neurosci., 7(6):613-20, 2004). The question is to know what physiological roles this regulated glial glutamate release plays, notably during the synaptic function. Here, we report a modulatory control in the dentate gyrus in response to activation of P2Y1 purinoreceptors. By immunocytochemistry we show that P2Y1 is localized preferentially in astrocytic processes directly apposed to asymmetric synapses. In acute hippocampal slices, activation of P2Y1 receptor with the specific agonist 2MeSADP triggers a glutamate release that are both Ca^{2+} -dependent and sensitive to bafilomycin A1, a blocker of synaptic vesicle refilling. However, 2MeSADP is totally ineffective in releasing glutamate from hippocampal synaptosomes, suggesting that the release has a non-neuronal source. Functionally, P2Y1 activation with 2MeSADP reversibly increases mEPSC frequency in granule cells, indicating a pre-synaptic modulatory control. This control is NMDA-dependent because AP5 and ifenprodil, two NMDA receptor antagonists, prevented the facilitatory effect of 2MeSADP. To confirm the impact of glial glutamate on synaptic activity, we have studied a direct connection between astrocytes and neurons by a double patch approach (Kang et al., Nat Neurosci., 1: 683-92, 1998). Initial data show that direct electrical stimulation of astrocytes, leading to $[\text{Ca}^{2+}]_i$ elevation in these cells, triggers in 30% of the recorded granule cells an increased mEPSC frequency. This effect is blocked by ifenprodil suggesting an involvement of presynaptic NMDA receptors in this astrocytic control. Our data reveal the active control of astrocytes on granule cell synapses via the regulated release of glutamate.

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A role for phosphatases in altered hippocampal synaptic plasticity in the aged rat.

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Protein kinases and phosphatases activation following high or low levels of calcium entry regulate the direction of synaptic strength leading to LTP and LTD respectively. We studied synaptic plasticity following NMDA application on hippocampal slices from young (3-6 months) and aged (24 months) rats. In young rats, NMDA (20 μ M) induced opposite effects depending on the duration of the application. A short (1 min) or long (5 min) application induced a long term depression of synaptic activity while a 3 min application induced a potentiation. In aged rats, these responses were always depressant, whatever the duration used. Can we explain this difference in the 3 min application between young and aged rats by alteration of NMDA receptor and/or changes in kinase/phosphatase balance? First, the potentiation present in slices from young rats was not restored in aged rats by exogenous application of the co-agonist of NMDAR D-serine (which compensate NMDAR alteration in aged rats). In addition, NMDA applications never induced potentiation in aged rats. These results suggest that alterations involve mainly intracellular mechanisms. Depressions in neurons result from phosphatase activation. We compared the consequences of phosphatase inhibition in young and aged rats. Incubation of slices in okadaic acid (a PP1/PP2B antagonist), neither affect the depression induced by 5 min NMDA application in young rats nor the 3 min application in aged rats. The PP2B antagonist FK506 restored a potentiation in young rats (5 min NMDA application) and in aged rats (3 min NMDA application).

These results suggest that application of NMDA on slices from young rats induced a bi-directional plasticity through activation of kinase or PP2B. The respective activation depends on the time window of application. In hippocampal neurons from aged rats, we always observe a depression, suggesting that a preferential activation of PP2B occurs following induction of NMDA-dependent chemical LTD.

Characterization of new splice variants of PAK3 and their implication in mental retardation

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The three mammalian members of the p21-activated kinase (PAK) family are involved in the control of cytoskeletal dynamics and cell cycle progression. In brain PAK kinases are implicated in growth cone collapse and control of neurite outgrowth. More recently, PAKs have been involved in dendritic spine morphogenesis. Transgenic expression of a pan inhibitor of PAK leads to abnormal spine morphology and LTP defects. Moreover mutations in the human PAK3 gene are responsible for X-linked mental retardation. The main questions are: why is the loss of function of PAK3 not complemented by the two other PAK kinases? What is the specificity of PAK3 in neuronal signaling? What is the molecular mechanism by which PAK3 regulates the synaptic plasticity?

Here we report the characterization of three new mammalian PAK3 splice variants. We have identified two additional sequences of 63 and 45 bp in several cDNAs of mammalian PAK3 leading to four mRNAs. These inserts that correspond to alternatively spliced exons that we named Ec and Eb, are both located between exon 2 and exon 3. By RT-PCR we identified the four corresponding transcripts in mouse and human brains. We identified the four PAK3 proteins in mouse brain lysates with specific antibodies we have generated against the peptidic sequences encoded by either Ec or Eb.

These four mRNAs are expressed equally in the different brain areas. We further show that PAK3b isoform is present in equal amount to that of PAK3a, while PAK3cb is less abundant and the last isoform PAK3c is the least detected. We also show that PAK3 is expressed in neuronal but not glial cells whereas PAK1 and PAK2 are both expressed in these two cell types. These alternatively spliced exons encode 21 and 15 amino acids that are located inside the regulatory domain of PAK proteins, suggesting these PAK3 isoforms may have particular functions. Whereas the normal PAK3 kinase (PAK3a) has very weak kinase activity in resting cells and is strongly activated by recombinant active Cdc42 protein, the three new isoforms possess a high kinase activity in quiescent cells and were not stimulated by an active GTPase. Indeed, the presence of the inserts dramatically impedes the binding to activated Cdc42 or Rac GTPases. We are currently investigating their regulatory mechanism.

Three mutations have been identified in XLMR, located in the regulatory domain (R67C) and in the kinase domain. Because these mutations are present in the four PAK3 isoforms, the main question is whether the synaptic defect is due to the loss of a common function of the isoforms, or due to a defect of a specific function of one of these isoforms. To initiate this research, we have introduced the R67C mutation in the four isoforms and analyzed their kinase activity. We show a biochemical defect for the PAK3a-R67C protein which has a reduced activation by Cdc42-V12. The R67C mutation has no effect on the constitutive kinase activities of PAK3b, PAK3c and PAK3cb proteins. The main questions are: Is this defect sufficient to explain the plasticity impairment? Are the functions of the other isoforms altered by this mutation?

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Calcium modulated protein phosphorylation in rat brain mitochondria

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Protein kinase-dependent phosphorylation of membrane receptors, ion channels and transcription factors are key events of intracellular signalling, responsible for regulation of various enzymes and many cell functions. The biological role of protein kinase-dependent phosphorylation in mitochondrial systems such as respiratory chain, ion transport and ATP synthesis is not understood. It is not clear whether the 2d messenger, namely Ca^{2+} , can directly regulate the energy transforming system in the inner mitochondrial membrane. Besides, until now no data are available about any interrelationship between protein phosphorylation and Ca^{2+} -induced opening of the "permeability transition pore" (PTP). The aim of the present study was to investigate protein phosphorylation in brain mitochondria and to find out whether they belong to systems that could be regulated by phosphorylation/dephosphorylation, modulated calcium as a second messenger. Experiments were performed on isolated rat brain mitochondria (RBM).

Phosphorylation of 94, 66-67, 43, 28-30, 26, 17, 6.5 and 3.5 kDa proteins has been observed in mitochondria. Ca^{2+} regulatory effects on phosphorylation of these proteins were investigated in Ca^{2+} -EGTA buffers. Protein phosphorylation was found to be modulated by Ca^{2+} in the physiological concentration range (10^{-8} M - 10^{-6} M free Ca^{2+}). Phosphorylation of the 17 kDa and 21 kDa proteins was increased within the 10^{-7} - 10^{-6} M free Ca^{2+} range, whereas an opposite effect was observed for the 3.5 kDa. These effects were strengthened in the presence of calmidazolium, an antagonist of calmodulin. It was suggested that Ca^{2+} in physiological concentrations, as a second messenger, can control phosphorylation of the 3.5 kDa, 17 kDa, 21 kDa phosphoproteins in RBM.

Participation of protein kinase-dependent phosphorylation was investigated in the phenomenon of PTP. Dephosphorylation of the 3.5 kDa peptide (which was identified as subunit c of F_0F_1 -ATP synthase) and enhanced ^{32}P incorporation into the 17 kDa and 21 kDa proteins were found under conditions of mPTP opening. These effects were prevented or reduced in the presence of cyclosporin A (CsA), inhibitor of PTP. It was shown that the drop of m (registered with TPP^+ -selective electrode under the same conditions) was correlated with a dephosphorylation of the 3.5 kDa peptide and an increased phosphorylation of the 17 and 21 kDa proteins. CsA prevented mPTP opening and drop of m . Based on these data we suggested that phosphorylation/dephosphorylation of proteins in RBM was connected with mPTP opening. It should be reminded that CsA is considered to block PTP by interacting with mitochondrial cyclophilin. It is well known, however, that this complex of CsA and cyclophilin suppress also calcineurin. Calcineurin is calmodulin binding protein, representing a high percentage of total brain protein. Several CaM-binding proteins were earlier reported to be present in mitochondria. Using both polyclonal and monoclonal antibody against calcineurin we discovered that brain mitochondria contain calcineurin. Taking into consideration that mitochondria contain PKA and PKA anchoring proteins, which bind both PKA and calcineurin, it would be logical to suggest that regulation of mitochondrial protein phosphorylation and modulation of this phosphorylation by Ca^{2+} might be exert through PKA and calcineurin signalling system.

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Modulation of synaptic integration by nitric oxide in a neuronal network of rat visual cortex

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Neurons of the primary visual area continuously adjust their patterns of discharges in accordance with the environmental luminance changes. This adaptation level is modulated by information exchanges between primary visual area and associative area but also by numerous afferences (cholinergic fibers originating from the magnocellular nucleus, serotonergic fibers issued from the raphe, noradrenergic fibers issued from the locus coeruleus).

The aim of our work is to analyze the effects of various neuromodulators on a cortical neuronal network in order to determine the resulting change in the excitability of layer V pyramidal neurones which elaborate the output signals from the cortex. Using rat visual cortical slices, electrical stimulation in layer I evoked a composite synaptic response recorded in layer V pyramidal neurones patched in whole cell configuration. In this "physiological context", the recorded signal reflects the process of synaptic integration of excitatory inputs (glutamatergic) and inhibitory inputs (GABAergic) mainly received by the apical dendrite of the recorded pyramidal neurone. Briefly, the global evoked synaptic conductance is further decomposed in excitatory and inhibitory conductances assuming that reversal potentials are respectively equal to 0 mV for excitation and to -80 mV for inhibition. Given the experimentally derived excitatory and inhibitory conductance profiles, we can reconstitute the dynamics of the membrane potential.

Immunostaining of neuronal nitric oxide (NO) synthase reveals many positive neurons located between layer II and layer V with a larger density in layer II/III. To analyse the effects of the diffusible messenger NO on synaptic integration, we first applied on the cortical slice L-arginin (the substrate of NO-synthase) or PAPA-NONOate (an exogenous NO donor). Excitatory and inhibitory conductances were increased but the excitability of the pyramidal neuron did not change. However, L-NAME (a specific inhibitor of NO-synthase) application induced an increase of neuronal excitability showing that endogenous NO synthesis may decrease the excitability of the pyramidal neurone. The question was then to understand how the electrical activity of the network can trigger NO synthesis. We tried a protocol of stimulation of the cortical network classically used to induce LTP (long-term potentiation). This protocol consist in theta-burst stimulation (TBS) of layer I followed by the recording of responses to stimulations at low frequency (0.33 Hz) as in control conditions. An increase in excitatory conductance (by 38 % , G_{exc}) and in inhibitory conductance (by 55 % , G_{inh}) were observed and these effects were maintained from 15 to 60 min after the TBS. The analysis of these results shows that the excitatory inputs are attenuated under these experimental conditions compared to control conditions. Consequently, the pyramidal neuron excitability was decreased. The inhibition of NO-synthase prior TBS prevents any change of excitation and inhibition (only 4 % increase for G_{exc} and 1 % increase for G_{inh}).

It is concluded that in the neuronal network, NO can modulate excitatory and inhibitory synaptic transmission leading to a decrease in the excitability of the recorded pyramidal neurone in our experimental conditions. This action of NO could take part in the modulation of visual cortex output signals.

Chemokine fractalkine/CX₃CL1 reduces glutamate-mediated excitotoxic death of cultured hippocampal neurons

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Excitotoxicity is a form of cell death caused by excessive exposure to glutamate (Glu), contributing to neuronal degeneration in many acute and chronic CNS diseases including ischemia. In this work we investigated the role played by fractalkine/CX₃CL1, a chemokine abundantly expressed throughout the CNS, on the survival of hippocampal neurons exposed to the excitotoxic insult; and we show that CX₃CL1 reduces the Glu-mediated excitotoxicity through the activation of the ERK1/2 and PI3K/Akt pathways, being neuron survival abolished or reduced by the specific inhibitors PD98059 and LY294002, respectively. We also report that CX₃CL1 protects nerve cells from death not only when administered before Glu application, reducing the Glu-activated whole-cell current, but also when administered up to 8 h after excitotoxic stimulus. Evidence is also provided that CX₃CL1 is released from hippocampal cells upon stimulation with high doses of Glu, likely providing an endogenous protective mechanism against excitotoxic cell death.

Glial soluble factors regulate the activity and expression of the neuronal glutamate transporter eaac1: implication of cholesterol

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A coordinated regulation between neurons and astrocytes is essential for the elaboration of the glutamatergic synapse and the control of extracellular glutamate concentration. Here, we investigated the influence of astrocytes on high affinity Na⁺-dependant glutamate transport in primary neuronal cultures derived from rat embryonic cortices. The rate of glutamate uptake and the expression of the neuronal glutamate transporter EAAC1 were lower in neurons grown in the absence of astrocyte (pure neuronal culture) than in neurons grown with few astrocytes (partially purified neuronal culture). Treatment of the pure culture with Glial Conditioned Medium (GCM) induced an increase in glutamate uptake rate (increase in maximal velocity). Western blotting experiments show that at least part of the effect of GCM is attributable to an increase in the total amount of the neuronal glutamate transporter protein EAAC1.

Furthermore, we show that cholesterol (10µg/ml) increased also glutamate uptake activity when added directly to neurons but with no effect on glutamate transporter expression. The effects of GCM on glutamate transport activity were reduced when cholesterol was removed from GCM by mevastatin treatment of the glial culture (Low-Cholesterol or LC-GCM) and restored when cholesterol was added to LC-GCM. Furthermore, the effects of GCM and cholesterol were not additive, confirming that they share a common pathway. Altogether, these results confirm that cholesterol, present in GCM, regulates glutamate transport in neurons.

In conclusion, we have provided new evidences for neuronal glutamate transport regulation by astrocytes and identified cholesterol as one of the factors implicated in this regulation.

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Extrasynaptic NR2B and NR2D subunits of NMDA receptors are shaping «superslow» afterburst EPSC in hippocampus

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In conditions of facilitated synaptic release, CA3/CA1 synapses generate anomalously slow NMDA receptor-mediated EPSCs (EPSC_{NMDA}). Such a time course has been attributed to the cooperation of synapses through glutamate spillover. Imitating a natural pattern of activity we have applied short bursts (2-7 stimuli) of high frequency stimulation and observed a spike-to-spike slow-down of the EPSC_{NMDA} kinetics, which accompanied synaptic facilitation. It was found that the early component of the EPSC_{NMDA} and the burst-induced late component of the EPSC_{NMDA} have distinct pharmacological properties. The competitive NMDA antagonist R-(-)-3-(2-carboxypiperazine-4-yl)-propyl-1-phosphonic acid (*D*-CPP), which has higher affinity to NR2A than to NR2B subunits and lowest affinity at NR2D subunits, significantly slowed down the decay rate of the afterburst EPSC leaving the kinetics of the control current unaffected. In contrast, ifenprodil, a highly selective NR2B antagonist, and [β -cis-1-[phenanthren-2-yl-carbonyl]piperazine-2,3-dicarboxylic acid (PPDA), a competitive antagonist that is preferential for NR2D subunits, more strongly inhibited the late component of the afterburst EPSC_{NMDA}. The receptors formed by NR2B and (especially) NR2D subunits are known to have higher agonist sensitivity and much slower deactivation kinetics than NR2A-containing receptors. Furthermore, NR2B is preferentially and NR2D is exclusively located on extrasynaptic membranes. As the density of active synapses increases, the confluence of released glutamate makes EPSC decay much longer by activating more extrasynaptic NR2B- and NR2D-subunit containing receptors. Long-term potentiation (LTP) induced by successive rounds of burst stimulation is accompanied by a long-term increase in the contribution of extrasynaptic receptors in the afterburst EPSC_{NMDA}.

Difference in neurotransmitter release induced by calcium channel and acetylcholine receptor activation in adrenal chromaffin cells.

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Molecular mechanisms, which are involved in Ca^{2+} -induced neurotransmitter release have common features in excitable cells such as neurons and adrenal chromaffin cells. In our experiments we used the rat adrenal chromaffin cells as a model for study neurotransmitter release. The influence of different types of stimulation on the secretion of catecholamines from secretory vesicles has been investigated using an electrochemical technique in configuration “amperometry” on cultured adrenal chromaffin cells. We show that the mean frequency of quantal release during cell stimulation with ACh is definitely higher than during cell depolarization by KCl. A comparative analysis of single secretory events show that in case of stimulation by acetylcholine single secretory quanta have a definitely shorter duration, rise and decay times as compared with those during depolarization. Their amplitude in the first case was also definitely higher. However, the statistical analysis of the area of single secretory spikes did not reveal substantial difference during both types of stimulation. A mathematical modeling of the dynamics of the secretory processes led to the conclusion that the experimentally demonstrated deceleration of the secretory process during membrane depolarization by hyperpotassium solution as compared with stimulation by acetylcholine may occur due to a decrease in the diameter of the fusion pore formed during fusion of the secretory vesicle with the cell membrane.

β -Dystrobrevin links to the neuronal Protein Kinase A signaling pathway by binding the PKA-R1 α subunit

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The dystrophin-associated protein complex links the actin cytoskeleton to the extracellular matrix, providing mechanical stabilization to the sarcolemma during muscle contraction. However, the dystrophin complex is also present at membrane specialization in many non muscle cells, including synaptic sites in neurons. Recent studies in muscle and non-muscle tissues have shown that the dystrophin complex functions as a scaffold that localizes and stabilizes a variety of signaling molecules to the plasma membrane. It has been proposed that part of the signaling functions of the dystrophin complex is mediated by the dystrobrevins, but their role in this context is not fully understood. Dystrobrevins, a family of dystrophin-related and associated proteins, are encoded by two separate genes, α and β . Targeted disruption of α -dystrobrevin gene in mice has shown that α -dystrobrevin may serve a signaling function that is important for the maintenance of muscle integrity. β -Dystrobrevin is not expressed in adult muscle, and it is abundant in brain, where it has been found highly enriched at the postsynaptic densities of hippocampal and Purkinje neurons. Dystrobrevins have no enzymatic activity of their own, therefore their involvement in signaling pathways is dependent on their interaction with other proteins. Several dystrobrevin-associated proteins have been described but, with the exception of the syntrophins, whether they contribute to the signaling function of dystrobrevin and the dystrophin complex remains to be seen.

In order to improve our understanding of the role of β -dystrobrevin in brain, we searched for new interacting proteins using the yeast two-hybrid system. We have recently shown that dystrobrevin is a kinesin receptor that may be involved in the traffic and targeting of the dystrophin complex, or a part of it, to specific sites in the cell¹. Among the other positive clones characterized after the screen, we report here on one that encodes the type I α regulatory subunit (RI α) of cAMP-dependent protein kinase A (PKA). We have confirmed the direct interaction of β -dystrobrevin with RI α by pull-down experiments and co-localization in transfected cells. We have also localized the site for RI α binding to β -dystrobrevin by pull-down assays. Sequence comparison between β -dystrobrevin and known PKA anchoring proteins has identified this site as a conserved α -helical PKA anchoring protein motif.

Our results suggest a role for β -dystrobrevin as an AKAP (A Kinase Anchoring Protein), and imply a novel function of this multifunctional scaffold protein in connecting the dystrophin-associated protein complex to PKA signaling.

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Study of APP function in the development of cultured neurons

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The amyloid precursor protein (APP) has been the subject of a lot of studies to understand how the deregulation of its metabolism could be deleterious to neurons in Alzheimer disease. However, the physiological function of this transmembrane protein is still poorly understood. We investigated its possible role in cultured hippocampal neurons development using a peptide corresponding to the cytosolic part of APP coupled to the penetratin to enter the neurons. This peptide should compete with endogenous APP for the binding of its partners and inhibit APP function. This treatment had no effect on neurite outgrowth, neuron polarization or axonal targeting of L1. Nevertheless, peptide treatment is able to promote a patchy distribution in neurites of the cytosolic GTPase dynamin 3. Most of untreated cells present a homogenous punctate distribution of dynamin 3. In neurons, APP staining after fixation is also patchy but surprisingly the labeling of dynamin 3 and APP are apposed and not overlapping. The role of APP in dynamin 3 localization does not involve direct interaction as the two proteins do not co-immunoprecipitate. Dynamin 3 function is still unknown, however recent work involved this GTPase in dendritic spines morphogenesis and remodeling (Gray NW *et al.*, 2003). Thus, in regard of the proposed implication of APP in synaptogenesis, the APP/dynamin 3 interaction should be further investigated during this particular period of neuron development.

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NAADP Signaling in mice pancreatic acinar cells: characterization of the calcium stores involved

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Intracellular Ca^{2+} levels play a key role in several cellular processes, including secretion. Aside from the well established Ca^{2+} releasing messengers IP₃ and cADPr (Cancela et al., 2002), a new Ca^{2+} releasing messenger, NAADP has been recently discovered (Lee *et al.*, 1995). The NAADP transduction pathway is poorly understood. For example, its receptor hasn't been cloned yet and the identity of the Ca^{2+} store mobilized is still under investigation. Lysosome-like organelles have been proposed to be NAADP sensitive in sea urchin eggs (Churchill *et al.*, 2002) and pancreatic acinar cells (Yamasaki *et al.*, 2004). To test the hypothesis that lysosomes are a Ca^{2+} store recruited by NAADP, Ca^{2+} or agonists, we have simultaneously recorded Ca^{2+} signals and lysosomes distribution using confocal microscopy. Lysosomes have been visualized by the dye LysoTracker red and specifically disrupted by application of Gly-Phe- β -naphthylamide (GPN), a specific substrate of the lysosomal enzyme Cathepsin C, that provokes osmotic swelling of these organelles.

Our data show that:

- Lysosomes labelled using the LysoTracker red were disrupted by GPN. The lysis of lysosomes provoked a Ca^{2+} release that did not affect the endoplasmic reticulum Ca^{2+} stores (ER). Store characterization experiments were repeated in Ca^{2+} free media and showed similar results, indicating that the cytosolic Ca^{2+} increase evoked by GPN treatment originated from an intracellular store.

- We have observed that CCK or ACh stimulation induced lysosomes mobilisation. Interestingly, lysosomes were also mobilized when a Ca^{2+} elevation was induced by ER depletion using the SERCA pump inhibitor thapsigargin (Tg).

- We have used GPN to test the involvement of lysosomes as a Ca^{2+} store in cholecystokinin (CCK), Acetylcholine (ACh), and NAADP signalling. Two concentrations of GPN were tested. A low dose of GPN (50 μM) evokes a small release of Ca^{2+} due to lysosomes lysis and selectively disrupt the Ca^{2+} response to 5 μM CCK, whereas ACh at 25 nM evokes a normal Ca^{2+} response as previously reported (4). However, addition of a higher dose of GPN (200 μM) induced an even more important Ca^{2+} release, and prevented the Ca^{2+} response to 25 nM ACh, suggesting that lysosomes are required also for ACh signalling.

In addition, we have investigated the effect of lysosomes lysis on the NAADP response. Our data shows that 200 μM GPN abolishes NAADP Ca^{2+} response. In regard to our previous finding that NAADP is involved in CCK-evoked Ca^{2+} signals, these data suggest that ACh and CCK may recruit lysosomes through NAADP pathway, and ACh through ER Ca^{2+} mobilisation.

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Long action rhythmic activity and synaptic transmitter release in case of reduced intracellular calcium concentration.

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Long action rhythmic activity is one of the main forms of motor neuron function. This activity manifests itself by changes in the transmitter release, so-called synaptic plasticity. The aim of our work was to estimate the changes of nerve ending electrogenesis and transmitter release under long stimulation in the presence of reduced intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$). The experiments were performed on *cutaneous pectoris* muscle of frog *Rana ridibunda*. A modified Ringer solution ($[\text{Ca}^{2+}] = 0.5 \text{ mM}$, and $[\text{Mg}^{2+}] = 10 \text{ mM}$) was used to prevent muscle contractions. Postsynaptic currents and nerve ending (NE) responses were recorded using a single extracellular microelectrode. Nerve endings were stimulated at 10 Hz for 10 min and 50 Hz for 5 min. BAPTA-AM was used to reduce $[\text{Ca}^{2+}]_i$. Control 10 Hz-stimulations lead to a gradual quantal content release increase (up to 270%); the second phase of nerve ending response decreased by 24% and widened by 48%, and the third phase of NE response decreased by 40%. In case of 50 Hz stimulation, during the first minute quantal content increased (by 360%) followed by a complete disappearing of evoked transmitter release, as for NE response its second phase decreased by 70 % while the width increased by 290% the amplitude of the NE response's third phase decreased by 70%. After treatment with BAPTA-AM, the 10 Hz stimulation leads to an enhancement of evoked transmitter release to 290%, second phase of NE response decreased (by 28%) and widened (by 52%), and its third phase decreased by 51% at the end of stimulation. A stimulation frequency of 50 Hz leads to a diphasic change of transmitter release too, but the enhancement developed more quickly and reached a peak level of 860%. NE response's second and third phases decreased more quickly and their amplitudes decreased to the noise level by the end of the stimulation, making measurements impossible. These results allow us to conclude that long-action rhythmic activity leads to alteration of the kinetics of ion channels at presynaptic terminals, as evidenced by changing the shape of action potential, which in turn modifies calcium entry that considerably contributes to transmitter release.

Pregnenolone-sulfate enhances long term potentiation and memory through modulations of NMDA receptors, intracellular calcium and MAP kinases in rat hippocampus

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We have recently shown that Pregnenolone Sulfate (PREGS) enhances both hippocampal-associated spatial memory (Akwa *et al*, 2001) and in vitro long term potentiation (LTP) (Sliwinski *et al*, 2004) through NMDA receptors modulation. To further document the molecular mechanisms underlying these rapid effects of PREGS, the intracellular calcium mobilisation (Ca^{2+}_i) and the MAP Kinases pathways were investigated.

In primary cultures of embryonic rat hippocampal pyramidal neurons, we determined whether PREGS modifies NMDA receptors influx of $[\text{Ca}^{2+}]_i$ with brief applications of glutamate following a perfusion of PREGS (300 nM, 10 min) by using microspectrofluorometry of the Ca^{2+} -sensitive indicator Fura-2. Perfusion of 50 μM glutamate (1 min) induced a rapid initial increase of $[\text{Ca}^{2+}]_i$ followed by a plateau preceding a regular decrease to the baseline signal. This influx was completely inhibited when MK-801 (0.5 μM) was bath applied, suggesting that this influx was NMDA receptor-dependent. In absence of any preperfusion of glutamate, PREGS was not active on $[\text{Ca}^{2+}]_i$ influx while it modified the time-course of the $[\text{Ca}^{2+}]_i$ changes after NMDA receptor activation. This effect was essentially due to a decrease of the plateau-phase, suggesting that Ca^{2+}_i was more rapidly used in presence of PREGS. We then identified the time-course of the phosphorylation of MAP Kinases pathways (ERK, CREB) induced during LTP in control and PREGS perfused slices (300 nM, 10 min immediately followed by tetanus (3x100Hz/1s). Phosphorylations of ERK and CREB were estimated by western blotting using respectively Phospho p44/42 MAP Kinase and Phospho-CREB (Ser133) antibodies. Slices were prepared and used as for the electrophysiological recordings and were taken off at different times after the tetanus. All biochemical processes were stopped in N_2 liquid and CA_1 was dissected under ice and then homogenated in SDS. Slices without tetanus served as control amount of phosphorylated states. Surprisingly, a phosphorylation of ERK and CREB was observed 10 min after the end of the PREGS perfusion in slices without tetanus. Consequently the timing of their phosphorylations during LTP under PREGS were modified. Finally, we show that PREGS enhancement of spatial memory was dose-dependently attenuated by the coadministration of a selective inhibitor of MEK1/2 (U0126: 0, 0.037, 0.075, 0.15 nmoles, icv).

In conclusion, PREGS enhancement of hippocampal-associated memory was combined with a change of the Ca^{2+}_i function that induced timing changes of the MAP kinases pathways, thus being likely to allow a better consolidation of the memory.

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Selective prion protein binding to synaptic components of cholinergic synaptosomes is modulated by oxidative stress induced by copper and peroxynitrite unveiling a role for calcineurin B and thioredoxin.

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Choline acetyltransferase (ChAT) and choline transport are decreased after nitrosative stress. ChAT activity is altered in scrapie infected neurons, where oxidative stress develops. Cellular prion protein (PrP^C) may play a neuroprotective function in participating in the redox control of neuronal environment and regulation of copper metabolism, a role impaired when PrP^C is transformed into PrP^{Sc} in prion pathologies. The complex cross-talk between PrP^C and cholinergic neurones was analysed *in vitro* using peroxynitrite and copper treatments on nerve endings isolated from *Torpedo marmorata*, a model of the motoneuron presynaptic element. Specific interactions between solubilized synaptic components and recombinant ovine prion protein (PrPrec) could be demonstrated by Biacore technology. Peroxynitrite abolished this interaction in a concentration dependent way and induced significant alterations of neuronal targets. Interaction was restored by prior addition of peroxynitrite trapping agents. Cu²⁺ treatment of synaptosomes triggered a milder oxidative effect leading to a bell-shape increase of PrPrec binding to synaptosomal components, counteracted by the natural thiol agents glutathione and thioredoxin. Copper induced modifications of thiols in several neuronal proteins. A positive correlation was observed between PrPrec binding and immunoreactive changes for calcineurin B and its partners suggesting a synergy between calcineurin complex and PrP for copper regulation.

Sodium channels and compartmentalization at the axonal initial segment

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The axonal initial segment (AIS) is a specialized domain that maintains the polarized distribution of membrane proteins in neurons. Highly enriched in voltage-gated sodium channels (VGSCs), it contributes to electrogenesis. To obtain new insights into the mechanisms that determine the targeting and the clustering of VGSCs in this sub-domain, we analyzed, in cultured hippocampal neurons, the trafficking of a reporter protein containing the cytoplasmic loop connecting domains II and III of the Nav1.2 subunit. This region, called Nav1.2 II-III linker contains a 27-amino-acid motif (amino-acids 1102-1128) involved in sodium channels compartmentalization at the AIS (Garrido et al., Science 2003). Here, we show that this Nav1.2 II-III linker protein is preferentially inserted in the somatodendritic domain and is trapped at the axonal initial segment by interaction with ankyrinG, prior to its insertion in the axonal tips. The non-tethered population in dendrites, soma and the distal part of axons is subsequently eliminated by a mechanism of endocytosis controlled by a 19-amino-acid-motif (amino acid 1010-1030).

Our results suggest that at least two distinct and independent mechanisms operate in hippocampal neurons to achieve axonal initial segment accumulation of the Nav1.2 II-III linker protein : endocytosis and domain-selective tethering. However, whether Nav1.2 sodium channel follows a similar trafficking pathway remains to be explored.

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Study of an ADP-ribosyl cyclase from brain synaptosomes using the CD38 deficient mouse model

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Cyclic ADP-ribose (ADPRc) and ADP-ribose (ADPR), two metabolites of NAD⁺, are known to modulate intracellular calcium levels and signaling in various cell types, including neural cells. Cyclic ADP-ribose mobilizes ryanodine-sensitive intracellular calcium stores, whereas ADP-ribose acts on TRPM2 channel-mediated calcium entry. The enzymes responsible for producing cADPR and ADPR in the cytoplasm of mammalian cells remains unknown, however two mammalian enzymes that are capable of producing the metabolites extracellularly have been identified, CD38 and CD157.

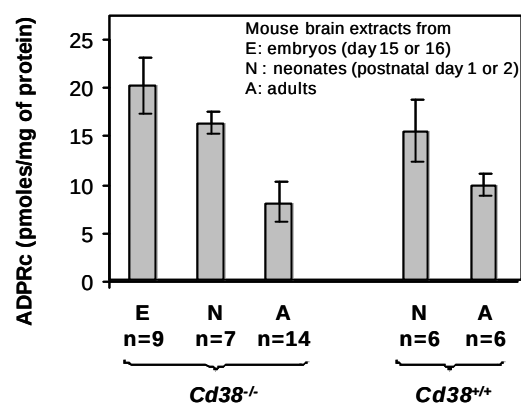
We previously have shown the presence of an intracellular membrane-bound ADP-ribosyl cyclase/NAD⁺-glycohydrolase distinct from CD38 and from CD157 in mouse brain (Ceni *et al*, *J. Biol. Chem.* (2003) 278, 40670-40). This novel enzyme, which is more active in the developing brain than in the adult tissue, was suggested to be regulated by a Gprotein-dependent mechanism.

In the present work, we have studied the ADPRc content in developing and adult brain tissue isolated from wild-type and *Cd38*^{-/-} mice. We confirm that this content is independent from CD38. We show an important reduction of the cADPR content (2.5 times) between embryonic (day 15-16) and adult tissues.

We have shown that intact synaptosomes prepared from *Cd38*^{-/-} brains (as in Phillips *et al. Neuron* (2001) 32, 63-77) have high cADPR levels and are enriched in ADP-ribosyl cyclase activity.

Permeabilisation of the synaptosomes with a detergent resulted in a drastic increase in cADPR and ADPR production, showing the intracellular localization of the protein. Moreover, the optimum pH of both cyclase and NADase activities are compatible with a cytosolic localization. The enzyme activities are higher in synaptosomes obtained from neonates compared to synaptosomes isolated from adults. We are currently characterizing the synaptosomes ADP-ribosyl cyclase by analysing its substrate affinity and sensitivity to free calcium.

Altogether, our results strongly suggest that the novel mammalian ADP-ribosyl cyclase could be involved in cADPR messenger production within pre- or post-synaptic structure. In addition, it might be regulated in response to some neurotransmitters Gprotein-coupled receptors activation.



Expression of Nav1.6 sodium channels by Schwann cells at neuromuscular junctions : role in the Motor Endplate Disease

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The neuromuscular junction is a “tripartite synapse” : the nerve terminal arborisation emits several branches which make synaptic contacts with muscle fibers at synaptic folds where acetylcholine receptors are concentrated. Terminal Schwann cells are non-myelinating cells, which wrap around the nerve terminal at the neuromuscular junction. These cells participate in the maintenance and the repair of neuromuscular synapses and modulate neurotransmission (Auld and Robitaille, 2003).

The *med* phenotype in mice is characterized by a severe impairment of neuromuscular transmission that results in death 20 days after birth (Duchen, 1970). Peripheral nerves remain capable of propagating action potentials, at least down to the intramuscular branches. This phenotype is due to a mutation in the *Scn8a* gene coding for the Nav1.6 isoform of voltage-gated sodium channel (Burgess et al., 1995).

In wild-type mouse Nav1.6 was immunodetected in nodes of Ranvier in CNS and PNS, in non-myelinated axons in PNS and retina, in the dendrites of cortical pyramidal cells and cerebellar Purkinje cells, and in radial glia in the cerebellum (Schaller and Caldwell, 2000 ; Caldwell et al., 2000). Nav1.6 is the major isoform in peripheral nodes of Ranvier in adults and replaces Nav1.2 during the first week of post-natal development.

In this study, fluorescent *in situ* hybridization, immunofluorescence and confocal microscopy were used to identify the neuronal sodium channel transcripts and protein isoforms expressed at the neuromuscular junction. In normal adult rats and mice, both Nav1.6 transcripts and protein are expressed in terminal Schwann cells. The Nav1.6 protein co-localizes with the Schwann cell marker S100 but is not detected in the SV2-positive nerve terminals. Pan sodium channel antibodies do not label nerve terminals in either wild-type or homozygous *med* mice. Strikingly, in mutant mice, the non-myelinated pre-terminal region of axons shows abundant sprouting at neuromuscular junctions and most of the α -bungarotoxin-labeled endplates are devoid of S100- or GFAP-positive terminal Schwann cells. In contrast, we found that a similar proportion of nodes of Ranvier express sodium channels in mutant and wild type animals and that Nav1.2 replaces Nav1.6 in *med* mice. Our data supports the hypothesis that the lack of expression of Nav1.6 in Schwann cells might play a role in the *med* phenotype.

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The adaptive mechanisms of muscarinic, α_1 -adrenergic and β -adrenergic receptors in CRH knockout mice

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The muscarinic receptors and β -adrenoceptors are the couple of receptors that can serve as an example of mutually interconnected receptor systems. In many cases, the heterologous regulation between these receptors appears. It can be comprehended as “fine tuning” of signal brought by neurotransmitters (1). Recently, we have demonstrated heterologous regulation of this pair of receptors in many cases in the heart tissue (2). Now, we wondered to know: 1) if this pair of receptors could adapt to targeted disruption of CRH (corticotropine releasing hormone) gene, 2) if there are some sex differences in receptor expression, 3) if there could be difference in the effect on α -adrenoceptors and β -adrenoceptors due to different affinity to noradrenaline (increased in CRH KO), and 4) what are the effects of immobilisation stress in CRH KO mice in comparison to their wild type (WT) counterparts.

We investigated the densities of muscarinic (MR) and of α_1 - (AAR), total number of β -adrenoceptors (BAR), β_1 - and β_2 -adrenoceptors in the lungs of CRH KO. Lung tissue can serve as a model of tissue in which the effects of sympathetic neurotransmitters (i.e. adrenaline or noradrenaline) are antagonised by the effects of parasympathetic neurotransmitters. Compounds [³H]quinuclidinyl benzilate, [³H]CGP 12177 and [³H]prazosin were utilised as specific markers of the muscarinic and adrenergic receptors, respectively. The respective subtypes were identified using specific antagonists (CGP 20712A, ICI 118.551).

The amount of receptor binding sites in CRH KO mice were decreased in comparison to their wild type counterparts. This effect was more prominent in males than in females and AAR were mainly affected. The stress brings about profound decrease in MR, BAR and AAR both in WT and KO females while lesser effect was seen in males. The changes in total BAR densities brought by stress were caused by β_2 -adrenoceptors, the β_1 -adrenoceptors were not changed or increased (in females, after 7th immobilization). On the other hand, the changes in the densities of MR and AAR brought by stress were more noticeable in WT animals.

We can therefore conclude that: 1) targeted disruption of CRH gene is able to change the densities of MR, AAR and BAR in the lung tissue; 2) stress reaction differs in CRH KO animals in comparison to their WT counterparts, 3) there are sex differences (both in receptor expression and reaction to stress). Our results showed that receptors adapt to the changed environmental condition through deep changes in the level of receptor binding sites.

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The enzymatic pathway of MMP2 is involved in synaptic remodeling induced by axotomy of rat superior cervical ganglion neurons

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We previously demonstrated an increase in the expression and activation of metalloproteinase2 (MMP2) in rat superior cervical ganglion (SCG) after ganglionic neuron axotomy. To clarify the enzymatic pathway leading to MMP2 activation, we investigated the expression pattern of both the membrane-type 1 MMP (MT1-MMP) and TIMP2, the main MMP2 activator and inhibitor, respectively. We used Western blot to analyze the expression levels of MT1-MMP and TIMP2 in SCG extracts at 15, 30, 60 min, and 1, 3 and 5 d post-axotomy. The level of MT1-MMP begins to increase at 30 min post-axotomy and remains higher than that of the control till 1 d after injury. On the other hand, TIMP2 level shows a first increase at 30 min, followed by a second one starting at 1 day after axotomy. The increase in MT1-MMP and TIMP2 levels suggests an activation cascade for MMP2 similar to that described in other experimental systems (Toth et al. J.Biol.Chem. 275:41415, 2000). RT-PCR analysis (15 min, 1, 6 h, and 1, 3 and 5 days post-axotomy) reveals that the level of MMP2 mRNA is not significantly affected by postganglionic nerve crush, while MT1-MMP and TIMP2 mRNAs gradually increase reaching a peak at 1 d. Recent data indicate β -dystroglycan (β -DG), a protein implicated in synaptic contact stabilization, as a possible MMPs substrate in the central nervous system. We investigated whether DG was modified in injured SCG. Western blot reveals an increase in the 30 kDa β -DG degradation product as early as 1 d after axotomy. In conclusion, our data indicate that both MT1-MMP and TIMP2, together with MMP2, are involved in intraganglionic synapses remodeling after SCG neuron axotomy and that DG may be one of MMP2 substrate.

Molecular determinants of allosteric modulators of the Calcium-Sensing Receptor

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The extracellular calcium sensing receptor (CaR) located at the cell surface of the parathyroid cell is stimulated by serum Ca^{2+} and controls parathyroid hormone release (Ruat and Petrel, *Med Sci*, 2004). In brain, the CaR on nerve terminals may regulate neurotransmitter release and its presence on oligodendrocytes suggests that it participates in the complex processes of myelination. The CaR belongs to G-protein-coupled-receptor (GPCR) class 3 characterized by an unusual long bilobed amino-terminal domain postulated to contain the ligand-binding site of these receptors. Low molecular weight synthetic molecules activating the CaR (calcimimetics) potentiate the effect of Ca^{2+} and are proposed to be of therapeutic benefit for the treatment of both primary and secondary hyperparathyroidism. Calcilytics inhibit the effect of Ca^{2+} on the CaR and may be of interest in the treatment of osteoporosis. We have constructed a model of the seven transmembrane domains of the human CaR based on the crystal structure of bovine rhodopsin (Palczewski K et al, *Science*, 2000). This three-dimensional model was used for docking (1S,2S,1'R)-N¹-(4-chlorobenzoyl)-N²-[1-(1-naphthyl)ethyl]-1,2-diaminocyclohexane (Calhex 231), a novel potent negative allosteric modulator (Petrel et al, *J Biol Chem*, 2003; Kessler et al, *ChemBioChem*, 2004) and NPS 2143, a reference calcilytic. This model allowed us to determine amino acid residues involved in the binding site of these negative allosteric modulators. To demonstrate the involvement of these residues, we have analyzed dose-inhibition response curves for the effect of these calcilytics on Ca^{2+} -induced [³H]inositol phosphate accumulation for selected CaR mutants transiently expressed in HEK293 cells. These mutants were further used for investigating the binding pocket of two chemically unrelated positive allosteric CaR modulators, NPS R-568 and (R)-2-[1-(1-naphthyl)ethylaminomethyl]-1H-indole (Calindol), a novel potent calcimimetic (Petrel et al, *J Biol Chem*, 2004; Kessler et al, *Bioorg Med Chem Lett*, 2004). Our data validate the involvement of W818^{6,48}, F821^{6,51}, E837^{7,39} and I841^{7,43} located in TM6 and TM7 in the binding pocket for binding of both calcimimetics and calcilytics, despite important differences observed between each family of compounds. Importantly, the observation that some mutations which have no effect on calcimimetics recognition but which affect the binding of calcilytics in TM3 and TM5, suggests that the binding pocket of positive and negative allosteric modulators is partially overlapping but not identical. Our CaR model should facilitate the development of novel drugs and the identification of the molecular determinants involved in the binding of allosteric modulators of class 3 GPCRs.

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Long term effects of rat prenatal ethanol exposure on Glu/GABA balance in central rhythmic neural network

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Fetal Alcohol Syndrom (FAS), due to ingestion of alcohol during pregnancy, is revealed by mostly cognitive problems which persist until adult age. Although the teratogenic effects of alcohol taken at early stage of development are known, long term effects of alcohol on synaptic transmission are still unclear. Some studies reported, however, that early chronic alcohol exposure induces long term alteration of neuronal discharge properties in some brain regions (e.g. ventral tegmental area). We studied the short and long term effects of early ethanol exposure on the excitatory/inhibitory balance (i.e. GLU/GABA) within the central rhythmic respiratory network of rats, and the consequences of such exposure on respiratory function using three different models: 1) an *in vitro* spontaneous rhythmic neonatal brainstem slice isolating the central pattern generator (CPG) for breathing, 2) an *in situ* preparation (with complete central respiratory network) for respiratory nerves recording at 3 weeks of age and 3) plethysmographic measurements of spontaneous breathing from intact neonatal rats. In addition, real time quantitative RT-PCR and [³H]MK801 binding were applied on neonatal slices to study NMDA-R1 and γ -GABA receptors subunits expression and NMDA receptors pharmacological properties. Rat dams were given 10% v/v ethanol solution as only source of fluid 4 weeks before gestation, then during gestation and lactation periods. Rat pups ("EtOH" group) were studied at either 5-7 days or 3 weeks old.

In spontaneous neonatal rhythmic slices of "EtOH" group, CPG rhythmic activity recorded from hypoglossal (XII) rootlet revealed an increased silent period duration of rhythmic activity but no changes in the active phase, thus decreasing rhythmic frequency. This specific disturbance was still present in spontaneously breathing intact neonatal animals, thus decreasing minute-ventilation and also on both XII and phrenic nerves recordings obtained at 3 weeks of age. Moreover, rhythmic activity from *in vitro* slices showed a decreased pharmacological sensitivity to NMDA applications (1.5 μ M-5 μ M) accompanied with an increased sensitivity to bicuculline (1-3 μ M). However, these changes were not due to neither a change in the number of NMDA receptors or [³H]MK801 binding affinity, nor to changes in mRNA levels for the NR1 subunit and the γ_2 subunit of GABA_A receptor.

Interestingly, we observed the same disturbance in all three types of preparation consisting in an increased silent period of rhythmic activity and a decreased rhythm frequency that was not compensated in intact or more mature animals. These results suggest a strong and long term central effects of alcohol on rhythmogenic properties of the network.

These preliminary data demonstrate that fetal ethanol exposure induces alterations on ventilatory function and on the balance between excitation and inhibition within the central spontaneous rhythmic neural network. It also strongly suggests that the gabaergic synaptic transmission is reinforced.

Three-dimensional word of synapse: 3D-reconstructions of hippocampal synapses using serial ultrathin sections for demonstration of multiple-synapses in both dendritic spines and presynaptic boutons

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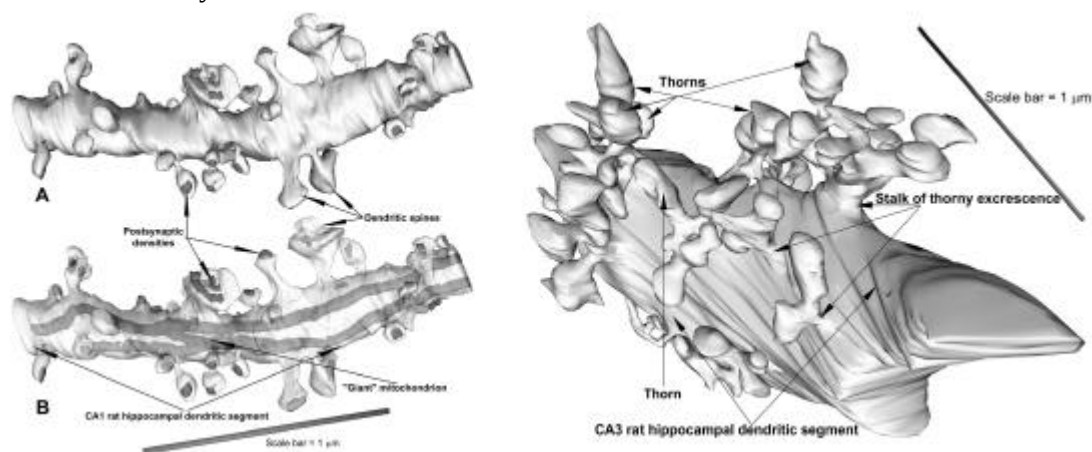
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Three-dimensional (3D) reconstructions of dendritic spines, presynaptic boutons, mitochondria and cisterns of smooth endoplasmic reticulum (sER) in dendrites and axons from CA1/CA3 and dentate gyrus hippocampal areas of both rats and ground squirrels were performed via serial ultrathin sections. Multiple-synapse boutons and multiple-synapse spines contacting with more than two axons were observed for all hippocampal areas. Quantitative changes in the shape and surface area of dendritic spines were obtained in vivo using various three experimental models: (1) hibernation of Yakutian ground squirrel; (2) long-term potentiation in anaesthetized rats; (3) audiogenic seizures in Krushinsky-Molodkina rats. Five categories of dendritic spines/synapses [(1) thin; (2) stubby; (3) branched; (4) mushroom; and (5) shaft synapses] were studied in CA1 hippocampal area and dentate gyrus. In contrast to dendritic spines from CA1 and dentate gyrus the apical dendrites of CA3 pyramidal neurons contain thorny excrescences. Mitochondria were not observed to penetrate into dendritic spines in either dentate gyrus or CA1, whereas CA3 thorny excrescences (which represent short dendritic branches) contained mitochondrial segments and numerous microtubules. Cisterns of sER originating from the outer membrane of the mitochondrial envelope penetrated into every spine examined. The changes in macular and perforated/segmented postsynaptic densities for all dendritic spines were studied. Our data demonstrate reversible retraction of dendritic spines and thorny excrescences during experimental manipulation in vivo, e.g. hibernation, long-term potentiation, stress, and spatial learning.

The results of the data presented suggest new paradigms of organization and functioning of synapses in central nerve system.



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D-serine and glutamatergic neurotransmission in rat vestibular system

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To conduct informations in the vestibular system either in the peripheral end organs or in the vestibular nuclei (VN), the main excitatory neurotransmitter is the glutamate which can act through ionotropic and metabotropic receptors. Our previous studies have shown first the timing of the expression of the glutamate receptors during postnatal development and second, their involvement in developmental plasticity.

Recently, it has been demonstrated that D-serine, an amino acid present in mammal central nervous system (CNS) is the endogenous ligand of glycine site of NMDA receptors. D-serine works in concert with glutamate to activate post-synaptic NMDA receptors. D-serine could regulate these receptors and could participate in synaptic plasticity related to LTP. D-serine is synthesized in astrocytic glial cells, by serine racemase and degraded by D-amino acid oxidase (DAAO). Therefore, in the vestibular system, D-serine could play a role in the maturation of the glutamatergic synapse in the VN as well as in the peripheral sensory end organ. By immunocytochemistry and confocal microscopy, we investigated if D-serine, serine racemase and DAAO are present in the vestibular system.

In the peripheral end organs, D-serine is present in the supporting cells localized on afferent nerve fibers which according to some data, are equivalent to glial cells in the CNS. But D-serine as well as serine racemase and DAAO are also present in transitional cells, which are epithelial cells highly involved in K⁺ recycling.

In the VN, we established spatiotemporal relationships among D-serine, serine racemase and DAAO during rat postnatal development. From birth to P21, high levels of D-serine were detected in glial cells. The drop of D-serine in adult corresponded to an increasing level of DAAO which has been shown to have a very high concentration in the brainstem. The levels of D-serine, serine racemase and DAAO and their immunocytochemical patterns from birth to adult may indicate that, in the mature VN, glycine might be an endogenous ligand of NMDA receptors as in the adult cerebellum. In the immature VN glutamatergic synapses, activation of NMDA receptors would occur through mechanisms involving the release of D-serine from astrocytes, suggesting a role for astrocytic D-serine in NMDA receptor-mediated synaptogenesis.

The carbamates that inhibit acetylcholinesterase by combined type exhibit a calcium antagonistic activity and low toxicity.

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Two series of carbamates, O-carbamoyl acylhydroxymoylchlorides and phenyl esters of N-substituted carbamine acid, have been examined as inhibitors of acetylcholinesterase as well as calcium antagonists. The carbamates from both series inhibited *in vitro* a partially purified acetylcholinesterase (AChE) from human red blood cells. Carbamates of the first series, the N-alkyl- and N,N-dialkylcarbamates of oximes inhibited AChE by progressive kinetics type while the N,N-methylphenylcarbamates of this series inhibited AChE by combined type: both with progressive and non-progressive kinetics. The latter exhibited calcium antagonistic activity *via* inhibition of the Ca^{2+} -induced contraction of the K^{+} -depolarised isolated rat ileum preparation as well as by blocking of the voltage-dependent L-type Ca^{2+} channels in rat cultured cerebellar granule neurons. They had lower acute toxicity on mice and weaker spasmogenic effect on intestine relative to their high anti-AChE activity; manifesting a paradoxical toxic effect at moderate doses. All of the phenyl esters of N-substituted carbamine acid inhibited AChE by both mechanisms exhibiting Ca^{2+} -antagonistic activity as well. The Ca^{2+} -antagonistic activity of the phenyl esters of N-substituted carbamine acid correlated highly with their efficiency in inhibiting AChE by non-progressive kinetics but not with efficacy of the enzyme inhibition with progressive kinetics. It is suggested that one of the carbamate-binding sites, of both AChE and the Ca^{2+} -channel, may have similar structure and that the physiological effects of the double-acting carbamates are based on balance of their efficacy in inhibiting AChE and potency as Ca^{2+} -channel antagonists.

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Regulation of synaptic properties by the microglial protein DAP12/KARAP

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Several proteins are expressed in both immune and nervous systems. However, their putative non-immune functions in the brain remain poorly understood. DAP12/KARAP is a transmembrane polypeptide associated with cell-surface receptors in hematopoietic cells (Tomasello *et al.*, 1998). Its mutation in human induces Nasu-Hakola disease, characterized by presenile dementia and demyelination (Paloneva *et al.*, 2000). Synaptic defects have been proposed to account for the early onset of some dementia. We thus hypothesized that DAP12/KARAP may impact synaptic function. In mice deficient for DAP12/KARAP function (named K^Δ75), LTP was enhanced (Fig.1A), and synaptic glutamate receptors were altered with AMPARs being more permeable to calcium. This last result was confirmed by biochemical analysis : in mutants, GluR2 expression was decreased only in the post-synaptic densities (PSD) but not in the whole membrane fraction, demonstrating specific impairment of synaptic receptor accumulation (Fig.1B). Alteration of BDNF/TrkB signaling in mutants was demonstrated by the dramatic decrease of synaptic TrkB (Fig.1B), with no change in other regulatory or scaffolding proteins. This decrease of synaptic TrkB was also observed by immunocytochemistry in cultured hippocampal neurons. Finally, DAP12/KARAP was detected only in microglia at perinatal stages, and never in neurons, astrocytes or oligodendrocytes.

DAP12/KARAP may thus alter microglial physiology, and subsequently synaptic function and plasticity through a novel microglia-neuron interaction. We are currently investigating by microarray analysis the factors involved in this link.

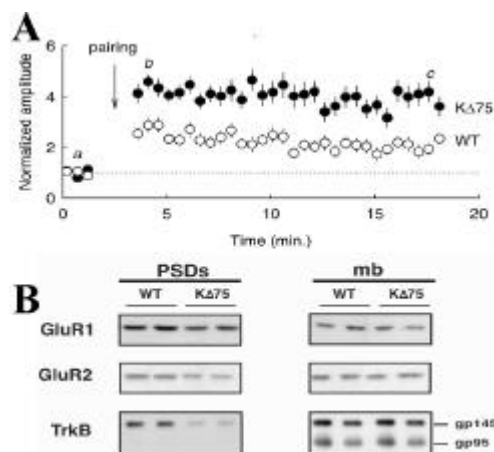


Figure 1 : Alteration of synaptic properties by KARAP/DAP12 mutation. A) Enhanced LTP in K^Δ75 mice. B) Diminution of GluR1, GluR2 and TrkB (gp 145) in Post-Synaptic Densities.

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Otoferlin, defective in DFNB9 human deafness, is a synaptic protein of sensory hair cells involved in exocytosis

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The molecular components underlying the high temporal precision and sustained neurotransmitter release of the auditory inner hair cell (IHC) ribbon synapse are still unknown. The elucidation of the molecular mechanisms governing these functional features is a major challenge, especially as the small number of IHCs in the cochlea precludes the use of standard biochemical and molecular genetics techniques. Thus, the identification of genes responsible for deafness, which may encode proteins involved in synaptic vesicle trafficking or neurotransmitter release seems a promising approach. Here, we provide evidence that otoferlin, a predicted transmembrane C2-domain protein defective in DFNB9 human deafness (Yasunaga et al., 1999), could be part of the IHC synaptic machinery.

We conducted immunohistochemistry analyses of otoferlin localization in the mice cochlea and investigated its biochemical properties with respect to the SNARE [soluble N-ethylmaleimide-sensitive factor attachment protein (SNAP) receptors] proteins, including syntaxin1 and SNAP25. Finally we explored the effect of otoferlin on vesicle exocytosis in transfected PC12 and chromaffin cells. Immunohistochemistry analyses showed that the distribution of otoferlin in developing and adult mouse hair cells parallels their afferent synaptogenesis. Immunoelectron microscopy detected otoferlin near the ribbons and at the presynaptic plasma membrane of the adult IHCs. Otoferlin colocalizes with presynaptic markers and directly interacts with syntaxin1 and SNAP25, two proteins of the SNARE complex, in a Ca²⁺-dependent manner. Otoferlin is able to form oligomers *in vitro*. Finally, we showed by amperometry that the presence of otoferlin in chromaffin cells increases the size of unitary exocytotic events. This effect was not observed with I515T (Mirghomizadeh et al., 2002) or P1825A (Migliosi et al., 2002) missense mutations that underlie DFNB9 deafness.

Our results suggest that otoferlin is a Ca²⁺-sensor that modulates IHC neurotransmitter release.

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The role of agrin during the maturation of human skeletal muscle fibres

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During synaptogenesis, motor axon terminals secrete trophic factors that bind to specific receptors located on the plasma membrane of the target cells. Signalling cascades activated via these receptors control cell maturation. Among these factors, agrin, a heparan sulfate proteoglycan released by nerve terminals, has been shown to promote clustering of nicotinic acetylcholine receptors and sodium channels at the neuromuscular junction (in McMahan, 1990; Caldwell, 2000).

Our work was aimed to detect the effect of agrin on the functional maturation of the mechanisms controlling calcium homeostasis and membrane electrical properties in developing human skeletal muscle cells. Patch-clamp and videoimaging experiments were performed on human myotubes aneurally cultured in the absence and presence of purified recombinant neural agrin. Voltage-clamp recordings in the whole-cell configuration revealed a decrease in membrane resistance and a more negative resting potential in agrin-treated myotubes.

It is known that apamin-sensitive small conductance Ca^{2+} -activated K^+ (SK) channels mediate the afterhyperpolarization and are responsible for changes in cellular excitability (Barrett et al., 1981). Moreover, in rat skeletal muscles a down-regulation of SK current has been observed during development, suggested to be dependent on muscle innervation (Schmid-Antomarchi et al., 1985). In our experiments, the density of apamin-sensitive SK currents, revealed in human myotubes as tail currents elicited after depolarizing pulses, decreased by 33% in agrin-treated cells.

The maturation of excitation-contraction (E-C) coupling mechanism was assessed by loading the myotubes with the fluorescent calcium dye Fura-2. A mature E-C coupling mechanism was detected in about 30% of human myotubes as shown by depolarization-induced intracellular calcium transients in the absence of extracellular calcium. The presence of agrin in the medium increased the percentage of responsive cells up to 70%.

Our results suggest that agrin *per se*, in the absence of nerve terminals, affects the *in vitro* myogenesis of skeletal muscle fibers, promoting the maturation of the mechanisms controlling electrical membrane properties and calcium homeostasis.

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Stimulation of post-lesional reinnervation processes in the hippocampus : the impact of a treatment increasing polyamine concentration

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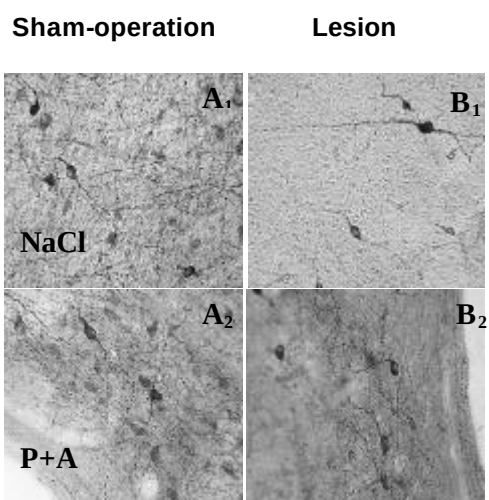
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In the Central Nervous System of the adult mammals, the post-traumatic regeneration is limited or non-existent. After lesion, the molecular signs of regenerative processes are nevertheless present, but the regeneration runs into several obstacles as the formation of a scar barrier or the inhibitory influence of the myelin proteins. Using a septo-hippocampal pathways section model, our experimental work consisted in neutralizing the inhibitory signalling mechanisms of the neuritic growth with i) the polyamine putrescine, ii) the inhibitor of the degradation of the polyamines, aminoguanidine or iii) a combined treatment with putrescine and aminoguanidine. These effects have been histochemically and immunocytochemically evaluated at the septal and hippocampal levels and more precisely, on both cholinergic soma and projections.



Images of rat septal cholinergic neurons, 6 weeks after a section of the fimbria-fornix (B) or a sham-operation (A). Note that ChAT expression by soma and fibers, clearly reduced after the lesion, can be restored after combined putrescine/ aminoguanidine treatment (2).

Scale bar : 50 μ m.

Our results, although preliminary, show that the pharmacological treatment having induced the most promising results was the one combining putrescine and aminoguanidine. Indeed, the combination of these two compounds induced, six weeks after lesion, an increase of i) the acetylcholine-transferase expression in the septal cell bodies, ii) the cholinergic fiber density in both septal and hippocampal regions, and iii) the number of phosphorylated neurofilament-immunoreactive axons in the hippocampus. It is possible that these modifications could be explained by an effective regeneration and/or an increased sprouting of the ventral cholinergic pathways, left intact by the lesion. This promising treatment, aiming at increasing the polyamine intraneuronal concentration, could thus favor the hippocampal reinnervation processes. Other studies are in course to verify if this treatment is, *in fine*, also able to promote a behavioural recovery of function.

Expression and role of Reelin during hippocampal synaptic maturation

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Extracellular matrix proteins are known to be involved in both synaptic embryonic development and postnatal maturation. The protein reelin, an extracellular protein secreted by Cajal Retzius cells, contributes to the regulation of migration and positioning of cortical and hippocampal neurons during embryonic development of laminated structures. Despite the fact that the population of Cajal Retzius cells disappears soon after birth, reelin is still present in the brain of adult rodents. Moreover, reelin has been shown to contribute to the early development of the hippocampus by playing a direct role in the modulation of hippocampal plasticity and stimulating dendrite outgrowth of hippocampal neurons. However, the precise role of reelin in the postnatal brain, particularly during hippocampal synaptic maturation, has not been elucidated yet. Therefore, we studied the developmental expression of reelin in mice either *in vitro* in cultured hippocampal cells or *in vivo* in the freshly extracted hippocampus. Using immunocytochemical approaches, we found that maturation of hippocampal neurons is characterized by an increase of reelin immunoreactivity from 6 days *in vitro* (DIV) to 9 DIV where it reaches a plateau, followed by a reduction from 12 to 21 DIV. Furthermore, the totality of reelin immunoreactive cells were identified as GAD67 (GABA-synthesizing enzyme glutamic acid decarboxylase)-labeled cells at any developmental stage. This indicates that during early postnatal development, reelin expression is restricted to a subpopulation of GABAergic neurons. In parallel, we determined the amount of reelin expressed in the hippocampus at postnatal days P0, P7, P14 and P21 by quantification of the three forms of the protein reelin (400, 320 and 180 kDa). We show that the expression of reelin which is stable during the first week of postnatal development, decreases from 7 to 21 days after birth. Thus, our results reveal a similar profile of reelin expression during postnatal maturation either *in vivo* or *in vitro*, characterized by an increase followed by a significative decrease in the expression of this extracellular matrix protein only few days after birth. Furthermore, by using electrophysiological and immunocytochemical approaches, we report that reelin controls the change in the subunit composition of synaptic N-methyl-D-aspartate subtype glutamate receptors (NMDAR) that usually occurs during postnatal maturation. Indeed, changes in the functional profile of NR1/NR2B receptors are timely correlated to the increase in reelin expression, and blocking the function of reelin by the CR-50 antibody prevents these changes.

Differential properties of GABAergic synaptic connections in rat hippocampal cell cultures

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According to the effect of prolonged tetanic stimulation (30 Hz, 4 s) we have divided GABAergic synaptic connections in hippocampal cell cultures into two groups: connections facilitated (~45%) and connections depressed (~55%) by the tetanic stimulation. In order to reveal possible reasons for the differential effect of the tetanisations we have compared several properties of the connections belonging to each group. We found that on average, evoked IPSCs in the connections facilitated by the tetanisations have smaller amplitude and larger coefficient of variation (CV) of IPSC amplitude compared to connections depressed by the tetanisation. We also estimated quantal parameters for both groups of the connections assuming that transmitter release is reasonably described by a binomial distribution. We have found that the background release probability (P) is substantially lower in the connections facilitated by the tetanisation ($P \sim 0.5$) than in the connections depressed by the tetanisation ($P \sim 0.9$) and suggest that this difference underlies the differential effect of the tetanisation.

We have also found that tetanisation induces opposite effects on connections made by distinct presynaptic neurons with the same postsynaptic cell (convergent connections) in a fraction of postsynaptic neurons studied (3 out of 9). These results support the idea that properties of the presynaptic neuron are of primary importance for the observed differential effect of the tetanisation, but do not exclude a role of the postsynaptic neuron in this effect.

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Generation of a mouse model for Schwartz-Jampel syndrome

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Schwartz-Jampel syndrome (SJS) is a rare human disease transmitted with an autosomal recessive mode of inheritance. Patients suffer from functional handicap with myotonia and chondrodystrophy, but their lifespan is not affected (Nicole and Fontaine, 2004). This syndrome is genetically homogeneous and results from hypomorph mutations in the perlecan gene. We observed that SJS homozygous truncating mutations were gathered in the C-terminal domain of perlecan. Perlecan is a heparan sulfate proteoglycan present in basal lamina (BL), where it is necessary for its structural maintenance. In skeletal muscle, perlecan has been involved in acetylcholinesterase clustering at the neuromuscular junction (NMJ) (Arikawa-Hirasawa *et al.*, 2002). To understand the physiopathological mechanism leading to muscle hyperexcitability in SJS, we have developed a mouse model for this human disease.

For this, we used a line of embryonic stem cells generated by a secretory gene trap approach (Skarnes *et al.*, 1995). In this line, the insertion of the reporter LacZ gene occurred in the 3' end of the perlecan gene at position similar to one SJS truncating mutation, leading to deletion of the C-terminal end of perlecan. Heterozygous mice were normal, whereas the homozygous were lethal *in utero*. We used this line to study perlecan expression during muscle development by X-Gal staining *in vivo*. We observed a down regulation of perlecan expression, as it was strongly expressed by myoblasts, whereas it was no more expressed by muscle fibers. Interestingly, a sustained expression at the adult NMJ by other cell type than the muscle fiber was observed.

The embryonic lethality of homozygous mice might result from the fusion between truncated perlecan and β -Gal. We therefore deleted the LacZ reporter gene (flanked by two FRT sequences) by crossing the heterozygous with transgenic mice expressing the FLP recombinase. We obtained homozygous deleted mice that were viable up to 6 months and suffered from chondrodystrophy and locomotor defects, strongly suggesting that they reproduce the SJS phenotype. If EMG analyses failed to reveal myotonia, electrophysiological recordings of synaptic transmission revealed slight modifications including higher frequency of giant miniature end plate potentials. Preliminary morphological data revealed major modifications of NMJ from 6 month-old mutants, suggesting a role of perlecan in NMJ formation and/or maintenance. Works are in progress to further characterize this mouse model and understand the role of perlecan in NMJ.

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René Couteaux (1907 - 1999): the first histologist who introduced the physiological term *synapse* in histology with successful staining of "subneural apparatus" and the first electron microscopist who observed an exocytotic opening of synaptic vesicles at the presynaptic "active zone"

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During a historical research, we realized that R. Couteaux (1909 - 1999) was the first histologist who stained postsynaptic structure and justified the use of physiological term "synapse" in histology. By means of Janus Green dye, he revealed membranous "subneural apparatus", a concave and lamellar sarcolemma (called also "synaptic gutter"), above which the motor nerve terminals (neurofibrils) were situated in a contiguous way (Couteaux, 1944 and 1947). With a modified histochemical thiocholine method, he localized acetylcholinesterase activity to this subneural apparatus (Couteaux & Taxi, 1952). Both his histological and histochemical stainings were superposable and presented a postsynaptic membranous structure (today we know that it was the basal lamina) of the muscle cells which was distinct from the nerve terminal. Thus, he not only supported Cajal's neuron theory but also gave morphological basis to the synapse. These optical microscopic works were done several years before the first electron microscopic observations of the synapses by american neurocytologists. (1954 - 1956). When the electron microscopy became available in Europe, Couteaux studied details of ultrastructure of the synapse and demonstrated the presynaptic "active zone" which was revealed as a restricted thickened zone of the presynaptic membrane and flanked with synaptic vesicles from both sides. Some of the synaptic vesicles were observed to be opened to the synaptic cleft by exocytosis at the level of the active zone (Couteaux & Pécot-Dechavassine, 1970). Furthermore, the synaptic vesicles are arranged in two rows in the close vicinity of linearly prolonged active zone which is situated just above the trench-like junctional folds (optical microscopic lamellar sarcolemma corresponded to these junctional folds) of the muscle membrane (Couteaux & Pécot-Dechavassine, 1974). Under the first high voltage electron microscope, he observed the neurofibrils, which was considered as frameworks of neurons in the classical histology and debated by neuronists and reticularists on their contiguity or continuity with neighbouring neurons. Couteaux confirmed intraneuronal network of the neurofibrils and absence of their anastomosis in the junctional regions (Carasso, Couteaux and Favard, 1975).

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Inhibition of presynaptic potentiation in neurons persistently infected with Borna disease virus caused by an interference with PKC signaling

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Borna disease virus (BDV) provides a unique system to study the mechanisms whereby a neurotropic virus can interfere with neuronal function without direct destruction of brain cells. BDV infects a wide range of warm-blooded animal species and causes a variety of symptoms, including well described behavioral abnormalities and learning deficits. As BDV persists mostly in limbic structures, we have developed an in vitro model of persistent infection, using primary rat hippocampal neurons. In a first step to study the functional consequences of viral persistence in neurons, we have analyzed the presynaptic activity by studying synaptic vesicle recycling. Our results show that viral persistence has no impact on synaptic vesicle recycling under resting conditions, or after continuous depolarization stimuli. However, infected neurons exhibited a severely impaired response to stimuli inducing long-term potentiation of presynaptic activity. This potentiation relies primarily on the activation of protein kinases, which phosphorylate presynaptic proteins involved in synaptic vesicle recycling. Using phosphosynapsin-1 antibodies we could demonstrate that the impairment of presynaptic potentiation observed in infected neurons was not due to a perturbation of PKA or CaMKI/CaMKII signaling. In contrast, we observed a perturbation of PKC signaling illustrated by a reduced phosphorylation of MARCKS following potentiating stimulus. In order to describe more precisely the viral interference with PKC signaling, we directly activated either PKA (as a control) or PKC using chemical stimulation. We found that potentiation of synaptic vesicle recycling was equivalent in infected and control neurons after PKA stimulation, whereas infected neurons showed a major impairment of potentiation following PKC stimulation. In BDV infected neurons, direct chemical stimulation of PKC also lead to a reduced level of phosphorylation of two major PKC substrates, namely MARCKS and Munc18. Taken together, our results suggest that BDV inhibition of presynaptic potentiation is due a specific viral interference with PKC signaling. These results raise the hypothesis that behavioral abnormalities found in BDV infected animals may be linked to impaired synaptic function, illustrating an intriguing aspect of the consequences of a persistent non cytolytic viral infection on neuronal function.

Effects of L-arginine on neuromuscular transmission

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L-Arginine is a widespread amino acid involved in many physiological processes. The main importance of L-arginine is attributed to its role as a precursor for the synthesis of nitric oxide (NO[•]). Besides, recently data about specific targets of action of L-arginine independent from NO[•]-synthesis emerge. In experiments on neuromuscular preparations of frog *cutaneous pectoris* muscle using extracellular microelectrodes the role of the nitric oxide precursor L-arginine on synaptic functions have been investigated. L-Arginine at a concentration of 0.1 mM decreased the quantum content of end plate currents (EPCs) and increased the 3rd phase of the motor nerve ending action potential which corresponds to the development of the voltage-dependent K⁺-currents. These effects reflect the elevated NO[•] synthesis in neuromuscular junction and correspond to the effects of exogenous NO[•] donors. Concentrations 0.5 and 1mM of L-arginine induced opposite effects on transmitter release without changing the nerve ending response.

It has been suggested that high concentrations of L-arginine were involved in a mechanism of NOS inhibition. NG-nitro-L-arginine methylester (L-NAME) – a NOS inhibitor – increased the transmitter release and decreased the voltage-dependent potassium currents of motor ending response. L-NAME abolished the effects of L-arginine (0.1 mM) and partially preserved the effects of L-arginine at 0.5 and 0.1 mM. These data suggests that L-arginine in high concentrations is involved in the mechanism of NO-synthase inhibition and has its own effects in motor nerve endings. The other precursor of NO synthesis, L-citrulline, at 0.1, 0.2 and 0.5 mM didn't influence the quantum content of EPCs and the amplitude of voltage-dependent potassium currents of nerve ending response. Thus we suggest that the enhancement of transmitter release under the influence of high concentrations of L-arginine is not linked with an increase of L-citrulline synthesis. Preliminary data indicated a participation of the adenylate cyclase system and changes of intracellular Ca²⁺-concentration in the effects of high concentrations of L-arginine.

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Congenital myasthenic syndrome caused by decreased receptor channel openings due to a novel mutation in the nAChR ϵ -subunit

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Congenital myasthenic syndromes (CMS) are a group of rare genetic disorders that affect neuromuscular transmission. Most of the CMS are caused by mutations of the nicotinic acetylcholine receptor (nAChR) in skeletal muscle. These mutations lead to low expression and/or kinetic anomalies of the nAChR such as slow channel CMS (SCCMS) and fast channel CMS (FCCMS).

Here we report an early-onset and recessively inherited highly disabling case of CMS. Symptoms were as follows: oculobulbar and limb involvement requiring wheel chair at 8 years of age. Cholinesterase inhibitors were efficient allowing walking. Electromyography revealed a decrement after repetitive stimulation. One of his brother was healthy while the other was also affected by the disease. Deltoid muscle histological examination showed highly abnormal neuromuscular junctions with denervation, reinnervation and sprouting features. Electron microscopy showed a drastic reduction of the postsynaptic folds. Molecular genetic analysis identified a homozygous mutation within the nAChR ϵ -subunit where the amino acid proline has been substituted by an arginine in position 282 (ϵ P282R).

In order to assess the functional consequences of this mutation, we carried out site directed mutagenesis of the ϵ -subunit. Co-transfection of the α , β , δ wild type nAChR subunits with either the wild type or mutant ϵ P282R subunits show the following: 1) upon transfection in Human Embryonic Kidney fibroblasts (HEK 293 cells), the mutation causes a 2-fold decrease in the total amount of ¹²⁵I-bungarotoxin binding sites, associated with a 10-fold reduction of the ACh apparent binding affinity. Moreover, whole cell electrophysiological recordings indicate that the mutation results in a 5-fold reduction of maximal ACh-evoked currents, a 10-fold decrease in ACh EC₅₀, and a strong acceleration of desensitization. Mutational analysis at this position further show that the positively charged arginine side chain causes the phenotype. 2) upon transfection in Chinese Hamster Oocyte (CHO) cells, used to avoid the interference of endogen currents existing in HEK cells, single-channel recordings show that ϵ P282R produces a shortened channel open time, a decreased open probability and decreased channel conductance.

Altogether, these data show that the ϵ P282R mutation causes a loss of function phenotype, due to a destabilization of the active conformation of the receptor, a decreased expression, and a decreased channel conductance. This study presents for the first time a myasthenic mutation within the loop linking the M2 and M3 transmembrane segments. On a three dimensional model of the muscle type nAChR, the mutation is located at the interface between the extracellular and transmembrane domains of the protein, suggesting that this position plays a key role in the long range allosteric coupling between the agonist binding site and the ion channel. This novel (ϵ P282R) mutation may therefore lead to a FCCMS, the functional endpoint being reduced responsiveness of the postsynaptic membrane to acetylcholine.

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Day-night variability in the postsynaptic sensitivity to GABA in the suprachiasmatic nuclei of rat brain slices

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The hypothalamic suprachiasmatic nucleus (SCN) operates as a central biological clock in Mammals. Neurons of SCN exhibit a circadian rhythm in the electrical activity driven by clock genes. In both day-active and night-active species, the frequency of action potentials firing in SCN neurons is higher during the day than during the night. The synaptic transmission mediated by gamma-amino butyric acid (GABA) is operative in SCN neurons, but it is unknown whether or not it undergoes circadian variations. Using whole-cell patch clamp technique, we have recorded spontaneous inhibitory postsynaptic currents (sIPSCs) in identified SCN neurons in hypothalamic slices during the day and night. For the day experiments, rats were killed 3-6 h after the lights came on and measurements were done in slices between circadian times 2 and 10. For the night experiments, rats were killed immediately before the light was turned off and measurements were done between circadian times 13 and 18. The frequency of sIPSCs, which reflects spontaneous GABA release by endogenous activity of neuronal network, was significantly higher when measured during the day ($15.3 \pm 5.9 \text{ s}^{-1}$, mean $\pm$ SEM) than during the night ($6.3 \pm 2.6 \text{ s}^{-1}$). In presence of $1 \mu\text{M}$ TTX, which blocked Na^+ -dependent action potentials, and $50 \mu\text{M}$ APV and $20 \mu\text{M}$ CNQX, which blocked glutamatergic transmission, the frequency of sIPSCs declined in both groups to comparable levels and reflected spontaneous GABA release by silenced neurons (miniature IPSCs). In contrast, the mean amplitude of sIPSCs was significantly higher during the night ($57.7 \pm 3.0 \text{ pA}$) than during the day ($45.0 \pm 3.3 \text{ pA}$) and was not affected by treatment with TTX, APV and CNQX. Furthermore, the application of $30 \mu\text{M}$ GABA to SCN neurons in slices evoked large currents, which amplitudes were also significantly higher during the night ($1228 \pm 171 \text{ pA}$) than during the day ($796 \pm 73 \text{ pA}$). These results indicate that there is a large circadian rhythmicity in the expression of postsynaptic GABA_A receptors, whereas the spontaneous GABA release from the presynaptic nerve terminals of silenced neurons does not undergo circadian variations.

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