Synscaff

Project no. **511995**

Project acronym
**SYNSCAFF**

Project title
**Synaptic scaffolding proteins orchestrating cortical synapse organisation during development**

Instrument
**SPECIFIC TARGETED RESEARCH PROJECT**

Thematic Priority **LSH-2003-2.1.3-5**
**[Cortical development]**

**Publishable final activity report**

Period covered from *1st January 2005* to: 31 December 2007

Start date of project: *1st January 2005* Duration: 36 months

Project coordinator name: Prof. Monica Di Luca

Project coordinator organisation name : Università degli Studi di Milano

**Table of contents**

## 1. Project execution

### Summary description of project objectives

The development of neural circuitry is a complex process, and the role of many of its components - notably the control of neuronal morphology, the formation of specific synaptic connections as well as the exquisite localisation of key synaptic proteins - is still very poorly understood.
This represents the starting scenario for the Synscaff project who aimed at reaching a more comprehensive understanding of cortical networking during development and in particular of genes and their products governing the complex molecular mechanisms driving synaptic structuring and organisation during development of cortical networks and circuitries. This project integrated the information about the role of different candidate genes/proteins in synaptic formation, remodelling and function and it capitalises on results obtained in *in vitro* systems and translated them to increase our knowledge on mental retardation, identifying both key steps responsible for defect of development and new genes/proteins and mechanisms responsible for a defect in development..

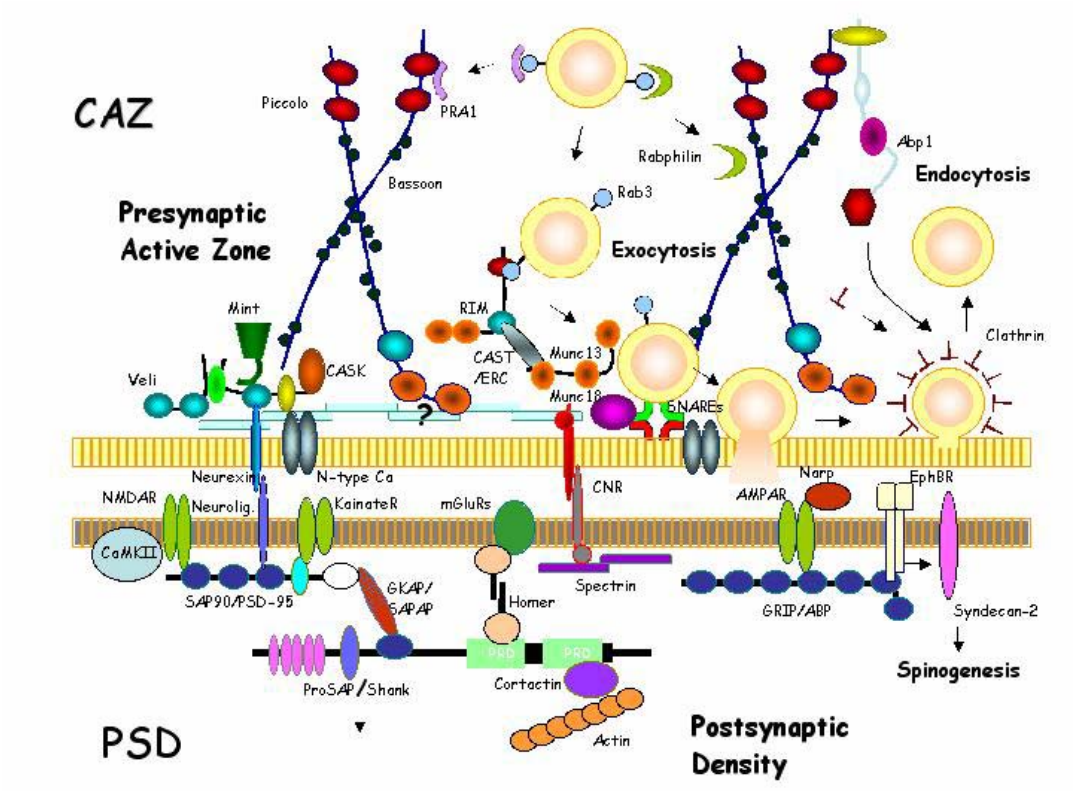
The Consortium was formed by European laboratories leaders in the field of neuronal circuitries and synaptic structuring, who contributed with a strong collaborative effort to the achievement of the main goal.
The groups involved in addition were highly complementary in term of skills and technologies. The intense interaction and osmosis among the Synscaff groups in the time frame of the project surely contributed not only to increase the current knowledge on cortical development and excitatory synapse formation and function but also to increase the technological level of each participating group.

The final goal to "define the molecular portrait of cortical synapse during development, defining the key localization of gene products within the synaptic structure" was accomplished. In fact, the Synscaff consortium had the capability to characterize gene products responsible for synapse formation, addressing the role of both presynaptic proteins and postsynaptic ones. Further, we identified and localized in defined synaptic domains new proteins involved in synaptic formation, analysed their role in synaptic function in vitro and by means of adequate animal models.
The impact of mutation of key genes ruling excitatory synaptic function in mental retardation was also addressed. Transgenic animals for scaffolding proteins as well as for key elements in mental retardation were characterized in great molecular, functional and behavioural details, reflecting the transferability of results obtained in *in vitro* systems. Animal model for mutated scaffolds, i.e. Bassoon ko mice, were clearly identified and characterised for molecular electrophysiological and behavioural aspects.

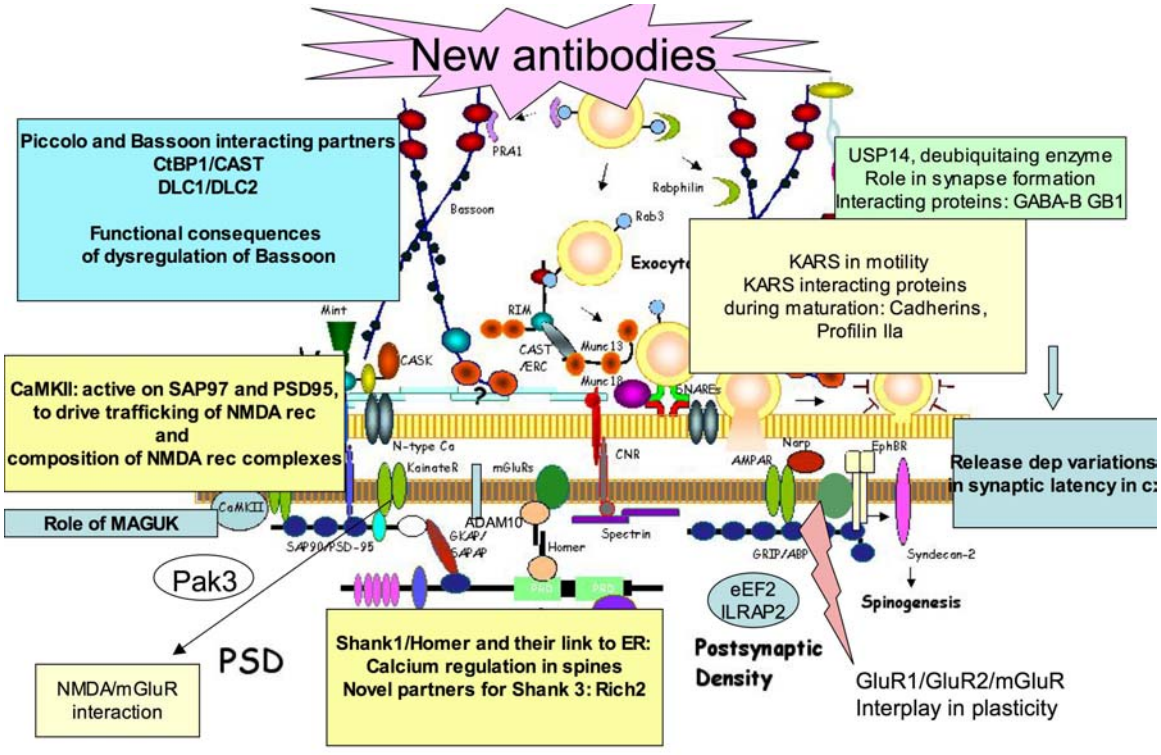
The key results of the consortium have been further exploited by Biotest, who designed and produced new antibodies for proteins analysed by the consortium, thus defining new tools that will be now available to the scientific community.

The scientific contribution of Synscaff to the knowledge on assembly and function of the excitatory synapse, can be appreciated and deducted by the following schemes; the first one represents the current knowledge on excitatory synaptic structure at the start of our project, whilst the second one reflects the actual knowledge implemented by the recent findings of our consortium.

The strong scientific value of Synscaff and its strong impact on the scientific community is more evidently assessed by the high number of publications arising from the consortium: 42 accepted papers in peer review journals, 11 manuscripts submitted and in preparation. These results represent the major outcome of Synscaff and testify the dissemination capability of the consortium.




*Scheme 1: Structural organization of the excitatory, 2004-2005*




*Scheme 2: Structural organization of excitatory synapse after Synscaff implementation.*

**Publications 2005-2008 related to Synscaff consortium**

**2005**

Bellone, C. & Lüscher, C. 2005. mGluRs induce a long-term depression in the ventral tegmental area that involves a switch of the subunit composition of AMPA receptors, Eur. J. Neurosci. 21:1280-88.

Boda B., Alberi S., Nikonenko I., Node-Langlois R., Jourdain P., Moosmayer M., Parisi-Jourdain L. and Muller D. 2004. The mental retardation protein PAK3 contributes to synapse formation and plasticity in hippocampus. J. Neurosci. 24:10816-25.

Debanne D 2004. Information processing in the axon. Nature Reviews Neuroscience 5: 304-316.

Roussignol G, Ango F, Romorini S, Tu JC, Sala C, Worley PF, Bockaert J, Fagni L 2005. Shank expression is sufficient to induce functional dendritic spine synapses in aspiny neurons. J Neurosci 25:3560-3570.

Sala C, Roussignol G, Meldolesi J, Fagni L 2005. Key role of the PSD scaffold proteins, Shank and Homer, in the functional architecture of $Ca^{2+}$ homeostasis at dendritic spines in hippocampal neurons. J Neurosci 25:4587-92

tom Dieck S, Altrock WD, Kessels MM, Qualmann B, Regus H, Brauner D, Fejtová A, Bracko O, Gundelfinger ED, Brandstätter JH 2005. Molecular Dissection of the Photoreceptor Ribbon Synapse: Physical Interaction of Bassoon and RIBEYE is Essential for the Assembly of the Ribbon Complex. J. Cell Biol, 168, 825-36.

**2006**

Bellone, C. & Lüscher, C. 2006. mGluRs induce a long-term depression in the ventral tegmental area that involves a switch of the subunit composition of AMPA receptors, Nat. Neurosci. 9:636-41.

Boda B, Nikonenko I, Alberi S. and Muller D. 2006. Central Nervous System Functions of PAK Protein Family: From Spine Morphogenesis to Mental Retardation. Mol Neurobiol. 34(1-2):67-80.

Bouzioukh F, Daoudal G, Falk J, Debanne D, Rougon G & Castellani V. 2006. Semaphorin 3A regulates synaptic function of differentiated hippocampal neurons. European Journal of Neuroscience 23: 2247-2254.

Carlier E, Sourdet V, Boudkkazi S, Déglise P, Ankri N, Fronzaroli-Molinieres L & Debanne D 2006. Metabotropic glutamate receptor subtype 1 regulates sodium currents in rat neocortical pyramidal neurons. Journal of Physiology (London) 577: 141-154.

Debanne D, Gastrein P & Campanac E 2006. Queer channels in hippocampal basket cells : h-current without sag. Journal of Physiology (London) 574: 2.

Debanne D, Thompson SM & Gähwiler BH 2006. A brief period of epileptiform activity strengthens excitatory synapses in the rat hippocampus in vitro. Epilepsia 47: 247-256.

Dresbach T, Fejtová A, Gundelfinger ED 2006. Assembly of presynaptic active zones. In *Molecular Mechanisms of Synaptogenesis* (Dityatev A & El-Husseini A eds). Springer Science+Business Media LLC, pp. 235-245

Fejtova A, Gundelfinger ED 2006. Molecular Organization and Assembly of the Presynaptic Active Zone of Neurotransmitter Release. In: Cell Communication in Nervous and Immune System (Gundelfinger ED, Seidenbecher CI & Schraven B, eds). Results Probl Cell Differ 43:49-68

Finardi A., Gardoni F., Bassanini S., Lasio G., Cossu M., Tassi L., Caccia C., Taroni F., LoRusso G., Di Luca M., Battaglia G. 2006. NMDA Receptor Composition Differs Among Anatomically Diverse Malformations of Cortical Development.
J. Neuropathol. Exp. Neurol., 65(9):883-893.

Gardoni F, Picconi B, Ghiglieri V, Polli F, Bagetta V, Bernardi G, Cattabeni F, Di Luca M, Calabresi P 2006. A critical interaction between NR2B and MAGUK in L-DOPA induced dyskinesia. J Neurosci 26:2914-2922

Gardoni F., Polli F., Cattabeni F. and Di Luca M. 2006. Calcium/Calmodulin-dependent protein kinase II phosphorylation modulates PSD-95 binding to NMDA receptor. Eur. J. Neurosci, 24(10):2694-704

Gerrow G, Romorini R, Nabi SM, Colicos MA, Sala C, El-Husseini A. 2006. A preformed complex of postsynaptic proteins is involved in excitatory synapse development. Neuron 49:547-62.

Gundelfinger ED, Boeckers TM, Baron M, Bowie JU 2006. A role for zinc in post-synaptic density asSAMbly and plasticity? Trends Biochem Sci 31, 366-373.

Ko J, Yoon C, Piccoli G, Chung HS, Kim K, Lee JR, Lee JW, Kim J, Sala C, Kim E 2006. Organization of the presynaptic active zone by ERC2/CAST1-dependent clustering of the tandem PDZ protein syntenin-1. J Neurosci 26:963-70.

Lüscher, C & Ungless, M. 2006.The mechanistic classification of addictive drugs, PLOS Med, 3:e437.

Nodé-Langlois R., Muller D. and Boda B. 2006. Sequential implication of the mental retardation proteins ARHGEF6 and PAK3 in spine morphogenesis. J. Cell Sci. 119: 4986-93.

Picconi B, Tortiglione A, Barone I, Centonze D, Gardoni F, Gubellini P, Bonsi P, Pisani A, Bernardi G, Di Luca M, Calabresi P 2006. NR2B subunit exerts a critical role in postischemic synaptic plasticity. Stroke 37:1895-1901

Schoch S, Gundelfinger ED 2006. Molecular organization of the presynaptic active zone. Cell Tiss Res 326, 379-91

**2007**

Angenstein F, Niessen HG, Goldschmidt J, Lison H, Altrock WD, Gundelfinger ED, Scheich H 2007. Manganese-enhanced MRI reveals structural and functional changes in the cortex of Bassoon mutant mice. Cereb Cortex 17, 28-36

Angenstein F, Hilfert L, Zuschratter W, Altrock WD, Niessen HG, Gundelfinger ED 2007. Morphological and metabolic changes in the cortex of mice lacking the functional presynaptic active zone protein Bassoon: A combined $^{1}$H-NMR spectroscopy and histochemical study. Cereb Cortex, [ePub ahead of print July 25, 2007]

Beri S, Tonna N, Menozzi G, Bonaglia MC, Sala C, Giorda R. 2007. DNA methylation regulates tissue-specific expression of Shank3. J Neurochem 101:1380-1391.

Campanac E & Debanne D 2007. Plasticity of neuronal excitability: Hebbian rules beyond the synapse. Archives Italiennes de Biologie 145, 277-287.

Centonze D, Rossi S, Napoli I, Merclado V, Lacoux C, Ferrari F, Ciotti M, De Chiara V, Prosperetti C, Maccarrone M, Fezza F, Calabresi P, Bernardi G, Bagni C 2007. The brain cytoplasmic RNA BC1 regulates dopamine D2 receptor-mediated transmission in the striatum. J Neurosci. 27:8885-92

Ferrari F, Mercaldo V, Piccoli G, Sala C, Cannata S, Achsel T, Bagni C. 2007. The fragile X mental retardation protein-RNP granules show an mGluR-dependent localization in the post-synaptic spines. Mol Cell Neurosci 34:343-354.

Gardoni F, Mauceri D, Marcello E, Sala C, Di Luca M, Jeromin A. 2007. SAP97 directs the localization of KV4.2 to spines in hippocampal neurons: Regulation by CaMKII. J Biol Chem.

Ibarretxe G, Perrais D, Jaskolski F, Vimeney A, Mulle C 2007. Fast regulation of axonal growth cone motility by electrical activity. J Neurosci 27:7684-7695.

Jose M, Nair DK, Altrock WD, Dresbach T, Gundelfinger ED, Zuschratter W 2007. Investigating interactions mediated by the presynaptic protein Bassoon in living cells by FRET-FLIM. Biophys J. [ePub ahead of print Oct 12, 2007]

Khelfaoui M, Denis C, van Galen E, de Bock F, Schmitt A, Houbron C, Morice E, Giros B, Ramakers G, Fagni L, Chelly J, Nosten-Bertrand M, Billuart P. 2007. Loss of X-linked mental retardation gene oligophrenin1 in mice impairs spatial memory and leads to ventricular enlargement and dendritic spine immaturity. J Neurosci. 27:9439-50

Kreis P., Thévenot E., Rousseau V. Boda B., Muller D. and Barnier J.-V. 2007. The p21-activated kinase 3 implicatge in mental retardation regulates spine morphogenesis through a Cdc42-dependent pathway. J. Biol. Chemistry 282: 21497-21506

Lüscher, C & Frerking, M. NMDAR- LTD, mGluR-LTD. In Squirre, L. [Ed.] The New Encyclopedia of Neuroscience, *in press*, 2007

Mameli, M.; Balland, B.; Luján, R. & Lüscher, C. 2007. Rapid synthesis and synaptic insertion of GluR2 for mGluR-LTD expression in the ventral tegmental area, Science 317:530-3,

Marcello E, Gardoni F, Mauceri D, Romorini S, Jeromin A, Epis R, Borroni B, Cattabeni F, Sala C, Padovani A, Di Luca M. 2007. Synapse-associated protein-97 mediates alpha-secretase ADAM10 trafficking and promotes its activity. J Neurosci 27:1682-1691.

Mauceri D., Gardoni F., Marcello E., Di Luca M. 2007. Dual role of CaMKII-dependent SAP97 phosphorylation in mediating trafficking and insertion of NMDA receptor subunit NR2A. J Neurochem 100(4):1032-46.

Piccoli G, Verpelli C, Tonna N, Romorini S, Alessio M, Nairn AC, Bachi A, Sala C. 2007. Proteomic analysis of activity-dependent synaptic plasticity in hippocampal neurons. J Proteome Res 6:3203-3215.

Saglietti L, Dequidt C, Kamieniarz K, Rousset MC, Valnegri P, Thoumine O, Beretta F, Fagni L, Choquet D, Sala C, Sheng M, Passafaro M. 2007. Extracellular interactions between GluR2 and N-cadherin in spine regulation. Neuron 54:461-477.

Siksou L, Rostaing P, Lechaire J-P, Boudier T, Ohtsuka T, Fejtová A, Kao H-T, Greengard P, Gundelfinger ED, Triller A, Marty S. 2007. Three-dimensional architecture of presynaptic terminal cytomatrix. J. Neurosci 27, 6868-6877

**2008 (in press)**

Boudkkazi S, Carlier E, Ankri N, Caillard O, Giraud P, Fronzaroli-Molinieres L & Debanne D Release-dependent variations in synaptic latency: a putative code for short- and long-term synaptic dynamics. Neuron (in press)

Centonze D, Rossi S, Zalfa F, De Chiara V, Prosperetti C, Febbraro F, Calabresi P, Bernardi G, Bagni C. Abnormal striatal GABA transmission in the mouse model for the fragile X syndrome. Biological Psychiatry (in press)

Coulon V, Audet M, Homburger V, Bockaert J, Fagni L, Bouvier M, Perroy j. Subcellular imaging of dynamic protein interactions by bioluminescence resonance energy transfer. Biophys J. (in press)

Cudmore RH, Goaillard JM & Debanne D Inhibition facilitates depression. Journal of Physiology (London) (in press)

Hung AY, Futai K, Sala C, Valtschanoff JG, Ryu J, Burgoon M, Kidd FL, Sung CC, Miyakawa T, Bear MF, Weinberg RJ, Sheng M. 2008. Smaller dendritic spines, weaker synaptic transmission but enhanced spatial learning in mice lacking Shank1. JNeurosci (in press).

Julie Perroy, Fabrice Raynaud, Vincent Homburger, Marie-Claude Rousset, Ludovic Teyller, Joel Bokaert and Laurent Fagni. Direct interaction enables cross-talk between iontropic and group-I metabotropic glutamate receptors. J. Biol. Chem. (in press)

**Submitted**

Blanchet C, Lanore F, Pinheiro P, Fetjova A, Gundelfinger E, Mulle C Impaired synaptic maturation in Basson-deficient mice. (in preparation)

Boda B., Jourdain L. and Muller D. Distinct, but compensatory roles of PAK1 and PAK3 in spine morphogenesis (submitted).

Campanac E & Debanne D. Spike-timing-dependent plasticity: a learning rule for dendritic integration in CA1 pyramidal neurons. Journal of Physiology (London) (in revision)

Campanac E, Daoudal G, Ankri N & Debanne D. Enhanced synaptic integration associated with LTP is mediated by the down-regulation of dendritic h-current. (submitted)

Di Filippo M, Tozzi A, Ghiglieri V, Picconi B, Costa C, Tantucci M, Belcastro V, Calabresi P. Synaptic striatal cholinergic dysfunction in a genetic model of Down syndrome. Brain (submitted)

Gardoni F., Malinverno M., Sala C., Cattabeni F. and Di Luca M. ADAM10 regulates dendritic spine morphology and glutamate receptor composition through N-cadherin cleavage. (Submitted)

Ghiglieri V, Picconi B, Sgobio C, Altrock W, Gundelfinger ED, De Sarro G, Bernardi G, Ammassari-Teule M, Di Luca M, Calabresi P. The presynaptic cytomatrix protein Bassoon regulates striatal cell-type specific synaptic plasticity. J Neurosci (submitted)

Gianfelice G, Pallotto M, Pavlowsky A, Bassani S, Valnegri P, Zanchi A, ChellyJ, Giustetto M, Billuart P, Passafaro M, Sala C. (2008) The role of the X-linked mental retardation gene IL1RAPL1 on synapse function. (manuscript in preparation)

Nikonenko I., Boda B., Steen S., Knott G., Welker E. and Muller D. PSD-95 promotes presynaptic terminal differentiation and synapse formation with nearby axons through nitric oxide signalling. (submitted).

Piccoli G, Verpelli C, Nairn AC, Sala C. (2008) A role for the eEF2 in regulating local protein synthesis and dendritic spine formation. (manuscript in preparation)

Romorini S, Pisani R, Falley K, Perroy J, Stucchi C, Fagni L, HJ Kreienkamp, Sala C. (2008) The role of the scaffold protein GKAP in the assembly of the postsynaptic density complex. (manuscript in preparation)

**Contractors involved**

*Arcachon, March 2007*

A brief overview of the tasks allocated to each contractor involved is included in this section. The details related to the work performed by each of them all over the project duration is shown in section "Work performed".

**UMIL, M. Di Luca**
The main goal of her lab was to study of the structural organisation of post-synaptic densities (PSD) at the excitatory synapse and the characterisation of the signal transduction processes associated to PSD. They described mechanisms regulating the correct assembly of the different components of the glutamatergic PSD, related to the activation of signalling elements, i.e. CaMKII. Further, her lab identified ADAM10, a metalloprotease involved in mechanisms of spine formation, as a new partner for MAGUK protein family members.

**CNRS, C. Mulle**
His main task was the full characterization of the role of KARs in sinaptogenesis and development. His lab addressed this issue both at pre-synaptic level and the post-synaptic one. He explored the clusters of KARs in the pre- and post-synaptic compartment and their functional role; further, he addressed the role and mechanisms of kainate receptors in the regulation of growth cone motility.

**CNRS, L. Fagni**
His main goal was to study the role of mGluRs and interacting proteins in cortical development. In particular he focused his efforts on the role of Shank/homer functional architecture in dendritic spines. He described new Shenk3 interacting proteins.

**INSERM, D. Debanne**
His main goal is the understanding the role of PSD-95 and other MAGuK in neuronal excitability. In particular he examined with electrophysiological recording techniques and molecular biology tools the consequences of MAGUK members over-expression on intrinsic neuronal excitability. In addition he revealed mechanisms regulating variation of synaptic latencies in the cortex.

**UNIGE, D. Muller**
His main goal was to understand the role of intracellular signalling processes in the regulation of spine morphogenesis. In particular he obtained data on a new proteins and signalling elements important regulators of spine morphogenesis.

**UNIGE, C. Luscher**
His laboratory focused in synaptic transmission in the ventral tegmental area (VTA) and forms of synaptic plasticity induced by addictive drugs. He described that cocaine triggers a redistribution of AMPA receptors leading to the synaptic insertion of receptors devoid of the subunit GluR2 as well as a form of LTD that reverses cocaine-evoked plasticity and depends on mGluRs. The mechanistic characterization involves scaffolding proteins of glutamate receptors in the expression of mGluR-LTD.

**LIN.NCMB, E. Gundelfinger**
The major task of his lab was the understanding of the molecular scaffold organizing the presynaptic terminal. In particular, questions concerning the role of Bassoon and Piccolo in the assembly of synapses during synaptogenesis and synaptic transmission were addressed.

**CNR-IN, C. Sala**
His task was focused on setting up a new protocol for proteomic analysis. Further he described new interacting proteins at the GluR N-terminal region and their functional role as well as new proteins driving spine formation.

**UNIPG, P. Calabresi**
His main goal was addressing the functional role of dysregulation of synaptic structure. In particular, he focussed on the behavioural and electrophysiological characterization of several mutants mice, describing differential synaptic changes of corticostriatal pathways in mutant mice including Bassoon, fmr1 and Ts65Dn mice.

**CASPHAR, J. Blahos**
His main goal was to characterize a deubiquinating protein USP14 reported to show a overlapped expression pattern with a GPCR (GABAb, subunit GB1), and to show a role for deubiquitination localized in the nerve terminal in regulating receptor activation.

**BIOTESTCZ, L. Jebavy**
Their main task was to produce sera and purified antibodies with the aim to manufacture sets of antibodies useful for studies of proteins associated with the receptors for neurotransmitters and for proteins involved in synapse organisation and regulation.

**CFc, C. Finocchiaro**
She represents the CF consulting company which supports the Consortium for the internal and external dissemination activities, for monitoring the quality assurance procedures (standard format of documents and procedures), collection of periodical reports to be submitted to the European Commission, knowledge management by monitoring the implementation of the Consortium Agreement.

**Work performed (by contractor)**

**CO1 UMIL**

**Introduction**

In the last decade, the identifications of scaffolding proteins as essential components of the postsynaptic density (PSD) structure, has revealed new insights into the molecular and assembly mechanisms of the synaptic apparatus. Among scaffolding elements, the MAGuK family comprises at least four closely related proteins (SAP97, SAP102, PSD-95 and PSD-93), each of which contains five protein-binding domains. These scaffolding proteins assemble a specialized protein complex around glutamate receptors that functions in signal transduction, cytoskeletal anchoring, and trafficking of the postsynaptic elements. Further, It is well known that the complex interplay among scaffolds and other synaptic proteins is a highly dynamic event governed by intracellular processes such as phosphorylation.

The main goal of our Unit was the characterization of MAGuK protein function in the PSD as well as the comprehension of the role of signal transduction elements, within the PSD, in the correct assembly of excitatory synaptic structure. In particular, we focus our attention on the phosphorylation processes of MAGUK members mediated by CaMKII, since this enzyme is the most active and it is expressed in the PSD.

**Activities carried out and results**

CO1 UMIL contributed to the following workpackages:

- **WP2**: Organisation and development assembly of postsynaptic scaffolds and receptors (IGLOOS, MGLURS, SHANK, MAGUK)
- **WP3**: Intracellular mechanisms regulating synaptic proteins assembly/disassembly
- **WP5**: Morphological, electrophysiological and biochemical analyses in engineered calls
- **WP6**: Characterisation of mouse mutants for scaffolding proteins

WP2 – Task 5: Understanding the role of PSD-95 and other MAGuK in neuronal excitability

The task consisted in examining, with the combination of electrophysiological recording techniques and molecular biology tools, the consequences of over-expressing PSD-95 and SAP-97 on intrinsic excitability in hippocampal pyramidal cells. This task was assigned to CR3 INSERM; our Unit collaborated to this task providing to CR3 on November 2005 the PSD95-GFP & SAP97-GFP constructs for transfections in COS-7 cell line and in hippocampal slices.

WP3 - Task 1: Characterisation of the signal trasduction machinery in the PSD.

Specific aims of this task was the study of the structural organisation of post-synaptic densities (PSD) at the excitatory synapse and the characterisation of the signal transduction processes associated to PSD as well as identification of the possible mechanisms regulating the correct assembly of the different components of the glutamatergic PSD.

Previous studies performed in our laboratory showed that activation of CaMKII is capable of starting a mechanism of delivery of NMDA receptor subunits through SAP97 (Gardoni et al., JBC, 2003; Mauceri et al., JBC, 2004). Thus, in this project we evaluated the key role of CaMKII as novel mechanism for the regulation of synaptic delivery of members of MAGUK family and interacting proteins - i.e. glutamate receptors - during spine formation and organisation. These processes might be of great relevance in the functional modulation of ionotropic glutamate receptor complex and therefore of the neuronal response during development.

As first, we confirmed SAP97, as an *in vivo* substrate for CaMKII. In particular, we identified two different sites as *in vivo* phosphosites for CaMKII within SAP97 sequence; one at Ser29 and the second at Ser232 (Mauceri, Gardoni et al., JNC, 2007). We found that CaMKII-

mediated phosphorylation of SAP97-Ser39 is necessary and sufficient to drive SAP97 to the postsynaptic compartment in cultured hippocampal neurons. CaMKII-dependent phosphorylation of the other site -Ser232- disrupts SAP97 interaction with NR2A subunit thereby regulating synaptic targeting of this NMDA receptor subunit. Here we show by means of phospho-specific antibodies that SAP97-Ser39 phosphorylation represents the driving force to exit SAP97/NR2A complex from the endoplasmic reticulum (ER). Ser39 phosphorylation does not interfere with SAP97 capability to bind NR2A. On the other hand, SAP97-Ser232 phosphorylation occurs within the postsynaptic compartment and it is responsible for both the disruption of NR2A/SAP97 complex and in turn for NR2A insertion in the postsynaptic membrane. Thus, CaMKII-dependent phosphorylation of SAP97 in different time frames and space within the neurons governs both NR2A trafficking and insertion.
Further, we found that CaMKII can phosphorylate, within the PSD, another member of MAGUK protein family, PSD-95 (Gardoni et al., EJN, 2006). We identified PSD-95 phosphorylation as a molecular mechanism responsible for the dynamic regulation of both PSD-95 and CaMKII interaction with NR2A subunit. CaMKII-dependent phosphorylation of PSD-95 occurs both in vitro, in GST-PSD-95 fusion proteins phosphorylated by purified active CaMKII, and in vivo, in transfected COS-7 as well as in cultured hippocampal neurons. PSD-95 Ser73 phosphorylation causes NR2A dissociation from PSD-95, while it does not interfere with NR2B binding to PSD-95. These results identify CaMKII-dependent phosphorylation of the PDZ1 domain of PSD-95 as a mechanism regulating the signalling transduction pathway downstream NMDA receptor.

WP3 - Task 2: Synaptic expression of protein kinases during cortical development

To investigate the synaptic distribution of protein kinases as well as proteins constituent of the glutamatergic synapse during cortical development a biochemical approach was used. Western blot analysis was performed in cortical tissue dissected from rats at P0, P8 and P24. A well characterized subcellular fractionation method was used to prepare an highly purified postsynaptic fraction (TIF, Triton insoluble fraction). The protein composition of this preparation was carefully tested for the absence of presynaptic markers (i.e. synaptophysin). Western blotting (WB) analysis was then performed from homogenate and TIF prepared form P0, P8 and P24 cortical tissue. Three main groups of postsynaptic proteins were considered: i) NMDA receptor regulatory subunits NR2A and NR2B, ii) scaffolding proteins, i.e. members of the MAGUK protein family (SAP97, SAP102 and PSD-95) and iii) protein kinases (CaMKII, src and CREB). WB analysis for NMDA receptor subunit NR2A and NR2B shows, as expected the presence of NR2B subunit at earlier stage during cortical development when compared to the NR2A subunit. In fact, NR2B was detected at synaptic sites (TIF) already at P0. On the other hand, NR2A becomes detectable in synapses only starting from P8.

Analysis of subcellular localisation and activity of kinases during cortical development was then performed by means of WB in parallel samples for the total and active form of src, CaMKII and CREB from homogenate and TIF. These experiments show the presence of the active form of all kinases at synaptic sites during the early phases of development. In particular, psrc, p286-CaMKII and pCREB were detected at P0 in both homogenate and TIF fractions; conversely, the antibodies able to recognize the total form of kinases show a clear detectable band only starting form P8. These data suggest that kinases are mainly in the phosphorylated-active form at P0.

Finally, WB was performed for members of MAGUK protein family SAP97, SAP102 and PSD-95, shown to be substrates for kinases (Gardoni et al., JBC, 2003) . SAP102 and PSD-95 were found very early during cortical development (P0), while SAP97 appears detectable starting from P8 both in homogenate and TIF.

Phosphorylation mechanisms and specifically CaMKII mediated activity appears to be critical for various aspects of dendritic development and synapse formation. Recent data of our laboratory showed that CaMKII-dependent phosphorylation of SAP97 in Ser39 regulates the cellular localization of SAP97, providing a fine molecular mechanism responsible for the synaptic delivery of SAP97 itself as well as SAP97 interacting proteins, i.e. glutamate receptor subunits (Mauceri et al., JBC, 2004). To this, we used a phosphospecific antibody (p39-SAP97) to study CaMKII-dependent phosphorylation of SAP97 during cortical development. SAP97 phosphorylation by CaMKII is detected from P0 suggesting this intracellular events as

key early step for targeting of glutamate receptor subunits during cortical development.

WP5 - Task 2: Role of SAP97 and other MAGUK in modifying synaptic structure

The main aim of the present task was the evaluation of the role of the scaffolding protein SAP97, in modifying synaptic structure and organisation acting through and downstream glutamate receptor activation. As first, we demonstrated that SAP97, a protein involved in dynamic trafficking of proteins to the excitatory synapse, is responsible for driving ADAM10 (a disintegrin and metalloproteinase 10, the most accredited candidate for alpha-secretase) to the postsynaptic membrane, by a direct interaction through its SH3 domain. NMDA receptor activation mediates this event and positively modulates alpha-secretase activity. Furthermore, perturbing ADAM10/SAP97 association in vivo by cell-permeable peptides impairs ADAM10 localization in postsynaptic membranes, and consequently decreases the physiological amyloid precursor protein (APP) metabolism. Our findings indicate that glutamatergic synapse activation through NMDA receptor promotes the non-amyloidogenic APP cleavage, strengthening the correlation between APP metabolism and synaptic plasticity (Marcello, Gardoni et al., JN, 2007).
Further, recent studies (Reiss et al., 2005) demonstrated that neuronal cadherin (N-cadherin) is cleaved specifically by the ADAM10 in its ectodomain. ADAM10 is not only responsible for the constitutive, but also for the regulated, shedding of this adhesion molecule in neuronal cells directly regulating the overall levels of N-cadherin expression at the cell surface. Cell-cell adhesion molecule N-cadherin is involved in many important physiological events such as synapse formation during development and activity-dependent spine remodelling; N-Cadherin is also essential for the correct functioning of excitatory synapse, i.e. induction of long-term potentiation.
Based on these observations, here we have demonstratated that SAP97 mediated trafficking of ADAM10 is essential in modulating N-Cadherin metabolism in the postsynaptic compartment both in primary hippocampal neurons and in vivo in mice. Inhibition of ADAM10 trafficking/localization at synaptic sites and, consequently, its alpha-secretase activity, using a cell-permeable peptide able to disrupt its interaction with SAP97 leads to a decreased ADAM10 mediated N-Cadherin metabolism. This event is paralleled by a significant modification of spine morphology and molecular composition of AMPA receptors. In conclusion, these data show that ADAM10 plays an important for spine morphogenesis and can influence Glutamate Receptor composition in Post Synaptic Density, suggesting an implication in functional (plasticity) events of the glutamatergic synapse.

WP6 - Task 3: Role of scaffolding in activity dependent plasticity

In this task, Bassoon lacking mice (Bsn) are employed to address the role of pre- and post-synaptic scaffolding proteins in activity-dependent corticostriatal LTP and LTD in brain slices prepared from mice at different postnatal age. In particular, in the present task cohordinated by CR7 UNIPG we have performed molecular analysis of the composition of the postsynaptic compartment in Bassoon mice. To this aim, a triton-insoluble fraction (TIF) has been purified from blind samples of single striatum of wt and Bsn mutant animals using a previously validated biochemical fractionating method. We first measured protein levels of NMDA receptor subunits and other PSD-associated signaling proteins in both striatal homogenates and purified TIFs from control and Bsn mutant mice by Western blot analysis. Levels of the NMDA receptor subunit NR1, PSD-95 and SAP97 members of MAGUK protein family, alphaCaMKII, p286-(autophosphorylated)-alphaCaMKII and actin were not altered in striatal homogenate and TIF from Bsn mutant mice ($p > 0.05$), suggesting that the gross composition of the TIF was not affected in Bsn mutant animals. Interestingly, the synaptic levels of NR2A and NR2B showed a profound modification with a significant increase of NR2A ($p < 0.05$) and a concomitant decrease of NR2B ($p < 0.01$) in Bsn mutant striata. A significant decrease of the NR2B levels ($p = 0.01$) were found also in the total homogenate from Bsn mutant animals.

## CR2 CNRS (C. Mulle group)

### Introduction

Our participation in the projects of the SYNSCAFF consortium has mainly dealt with two lines of research. The first was related to the possible role that presynaptic ionotropic glutamate receptors of the kainate type could play during the maturation of synaptic connections, in particular through there possible interaction with presynaptic scaffolding proteins. Within this frame, we have attempted to map interacting proteins for presynaptic KARs and describe how these interactions develop with postnatal maturation of the brain. Our plan was also to understand better the role of presynaptic KARs in the motility of growth cones. The second line of research considered the development of synaptic connections with hippocampal granule cells and CA3 pyramidal cells as an interesting model to study processes and dysfucntion of maturation in mutant mice.

### Activities carried out and results

CR2 CNRS (C. Mulle group) was involved in WP1, WP2, WP5 and WP6.

**WP1 Organisation and developmental assembly of presynaptic scaffolds**

For this task, the objective was to characterize which identified KAR protein partners (Cadherin/Catenin, CASK, PSD-95, SAP 102, syntenin, GRIP, PICK1,…) specifically interacted with presynaptic KARs at critical stages of cortical synaptic maturation. This task was reoriented towards the GluR7 subunit, which was shown (after the start of the SYNSCAFF project) to be a purely presynaptic subunit in association with GluR6. We then decided to produce and use a GluR7 specific antibody to immunoprecipitate GluR6 from the GluR6/GluR7 complex, as well as proteins interacting with this putative presynaptic complex from the brain. In collaboration with the group of Jaroslav Blahos and Biotest, we have produced antibodies against an extracellular epitope of GluR7. After initial unsuccessful results, we have now characterized and purified antibodies that will likely be useful. Because of technical problems with the obtention of suitable antibodies, our task has been deviated, although we are confident that the newly produced antibodies will be instrumental in achieving our task (after the end of the contract). We have produced GST fusion constructs with the C-ter domain of GluR7a and GluR7b to use them for pull-down experiments. Three candidate interacting proteins have been identified: COP1, 14.3.3 and calmodulin. Ongoing work shows that interaction with COP1 regulates the membrane expression of GluR7b. A publication should be written during the year 2008.

- **WP2:** Organisation and development assembly of postsynaptic scaffolds and receptors (IGLOOS, MGLURS, SHANK, MAGUK)

We hypothesized that different combinations of scaffold proteins interact with KARs at different stages of cortical development. Relevant stages of synaptic maturation in the hippocampus and in the neocortex during the course of development have been compared. We have examined if KARs interact with already identified protein interactors (Cadherin/catenin, PSD-95, profilin) at these stages of development. We have used transgenic mice expressing myc-GluR6 already available n the laboratory (Coussen et al, 2002). Brains from myc-GluR6 transgenic mice at P0, P3, P15 and adults have been dissected, and myc-GluR6 containing receptors immunopurified. We have quantified the binding of partner proteins to GluR6, and we show that heteromerization between GluR6 and KA2 is stable during postnatal development. In addition, the association between the N-Cadherin complex through beta-catenin and GluR6 decreases throughout maturation. Finally, the quantities of PSD-95 and Profilin IIa associated with GluR6a are maximum at P7 and decrease with time.

- **WP5**: Morphological, electrophysiological and biochemical analyses in engineered calls

We have proposed to analyse the effects of focal kainate application on axonal growth cone motility, imaged with time-lapse videomicroscopy of actin-GFP fluorescence. The objective was to identify KAR subunit splice variants responsible for this effect.
We have first set up the experimental conditions (neuronal culture conditions, videomicroscopy, transfection) and the methods for analysis of axonal filopodila motillity. This method has allowed for a quantification of the effects of the application of kainate on the motility of axonal growth cone filiopodia. We have found that kainate transiently inhibits filopodia motility in neuronal cultures (6-9 DIV). We have further identified the subunit splice variants GluR6a and GluR6b as critical fro these effects. In parallel, we have futher examined the mechanisms of action of kainate receptors to inhibit axon growth cone motility. This effect is inhibited by TTX, and thus requires neuronal spike discharge. Using electrophysiological recordings, we found that kainate application increased spike discharge in conditions where growth cone motility was inhibited. Furthermore, applying a train of spikes at 10 Hz induced a stalling of the growth cones. We have imaged the level of intracellular calcium concentation in parallel with motility and with direct stimulation of the recorded neuron. We have found that calcium microdomains in the axonal growth cone are essential for the regulation of motility by spike activity. Thus we come to the important conclusion that neuronal activity is critical for the regulation of growth cone motility, and that increasing neuronal activity by kainate receptor activation can be an important physiological mechanism for this regulation. This work has been published in Journal of Neuroscience in august 2007.
GluR6a and GluR6b only differ in a peptide sequence in their C-terminal cytoplasmic domain, and do not show functional differences. We further showed that the targeting of GluR6 receptors to the axonal growth cone did not require the coassembly of the two proteins. We then transfected a mutant form of GluR6 that does not bind to profilin 2a, a protein potentially involved in the regulation of actin polymerization. No difference was observed, indicating that GluR6b/profilin2 interaction does not play a major role in this process. In addition, we have attempted to test the role of Pfn in this regulatory process by using kainate on Pfn knock-out mice. The results are negative: no change in the regulation of motility of growth cones was found in Pfn-/- mice.

- **WP6**: Characterisation of mouse mutants for scaffolding proteins

We have characterized the electrophysiological properties of mossy fiber to CA3 synapses in Bassoon knock-out mice provided by partner 5 (planned month 18). After discussion with partner 5, we have changed the genetic background of mutant mice used in our study, because some of the results obtained so far might have been confounded with early epileptogenesis. We have compared the characteristics of mossy fiber to CA3 synaptic transmission and short term presynaptic plasticies in wild type and Bassoon knock-out mice in this new genetic background at key postnatal days of synaptic maturation: P7, P14 and P21. Our results indicate a less efficient synaptic transmission in knock-out mice at P14 compare to wild type. This is likely due to a lower number of functional release sites. Interestingly, at earlier time points, EPSCs of unusual large amplitude are observed, suggesting a dysregulation in the organization of the presynaptic active zone. A manuscript will be written within the next 3 months.

## CR2 CNRS (L. Fagni group)

### Introduction

The majority of excitatory synaptic transmission in the brain utilizes glutamate as a neurotransmitter. Glutamate activates both ionotropic (AMPA, kainate and NMDA) and metabotropic (mGlu1-8) receptors. Here we focused on the NMDA and mGlu1/5 receptors, which are both localized at post-synaptic sites.

Our studies concern WP2 and WP5. In WP2, a map of mGlu1/5 receptor interacting proteins was originally planned to be provided at midterm of the contract. However, after 6 months of investigation, no positive result was obtained. Therefore we redirected our task towards a closely related objective, that is the search for new post-synaptic glutamate receptor associated protein that could control dendritic spine morphogenesis. The core of the post-synaptic density is composed of multi-domain proteins that assemble glutamate receptors at the post-synaptic membrane, links these receptors to their intracellular signaling pathways and to cytoskeleton. The PDZ protein, Shank, is a master organizer of this multi-protein scaffold. It also induces synaptogenesis, shapes dendritic spines and controls synaptic arrangement of the receptors. Mutations of this protein are associated with mental retardation and autism. Several binding partners of Shank have already been identified by two-hybrid screening. However it is largely unknown which specific molecule could mediate the synaptic functions of Shank. Therefore we performed a proteomic study in order to identify new Shank interacting proteins involved in such processes. Since Shank is a relatively large protein, we focused on its PDZ domain, which has been shown to play an important role in spine maturation and synaptogenesis.

For WP5, the objective was to study the effect of blockade of mGluR1a/5 scaffolding interaction on localization and function of the receptors. The mGluR1a/5 receptors are localized at the periphery of the post-synaptic density via interaction with coiled-coil containing Homer protein dimers (Homer c-c) that bind to Shank. Disruption of this complex is achieved by inducing expression of the immediate early gene, Homer1a, a monomeric homolog of Homer c-c proteins that acts as a dominant negative regulator of Homer c-c-interactions. The Shank protein also binds to the GKAP-PSD95-NMDA receptor complex, thus linking together these receptors to mGluR1a/5. We studied the effect of Homer1a-induced disruption of the mGluR1a/5 complex on membrane targeting of the receptor and functional crosstalk between mGluR1a/5 and NMDA receptors.

### Activities carried out and results

CR2 CNRS (L. Fagni group) contributed to WP2 and WP5.

- WP2: Organisation and development assembly of postsynaptic scaffolds and receptors (IGLOOS, MGLURS, SHANK, MAGUK)

Pull-down 2D gel analyses, performed from brain extracts using GST-Shank PDZ region as a bait, revealed 6 spots that were identified by mass spectrometry. These proteins are : GIT1, ArfGAP6, Lasp, Calumenin, latrophilin and RICH2. Among these proteins, 3 of them (GIT1, ArfGAP6 and RICH2) are GTPases activating proteins (GAP), which are known to control dendritic growth and spine morphogenesis. Here we focused on RICH2. This protein displays a Rho-GAP domain, a BAR domain and a PDZ binding motif. We show that the Rho-GAP domain confers a GAP activity against all the members of the Rho family of GTPases (RhoA, Cdc42 and Rac1). The BAR and Rho-GAP domains are required for localization of RICH2 in early endosomes and the PDZ binding motif is required for colocalization and interaction of the protein with Shank, in dendritic spines. The Rho-GAP activity of RICH2 is inhibited in the presence of Shank3. RICH2 with Shank promote dendritic growth and enlargement of the spines. These results suggest a model where Shank would recruit RICH2 into spines and regulate recycling of membrane materials by controlling RICH2 Rho-GAP activity and promote spine maturation (Fig. 1).

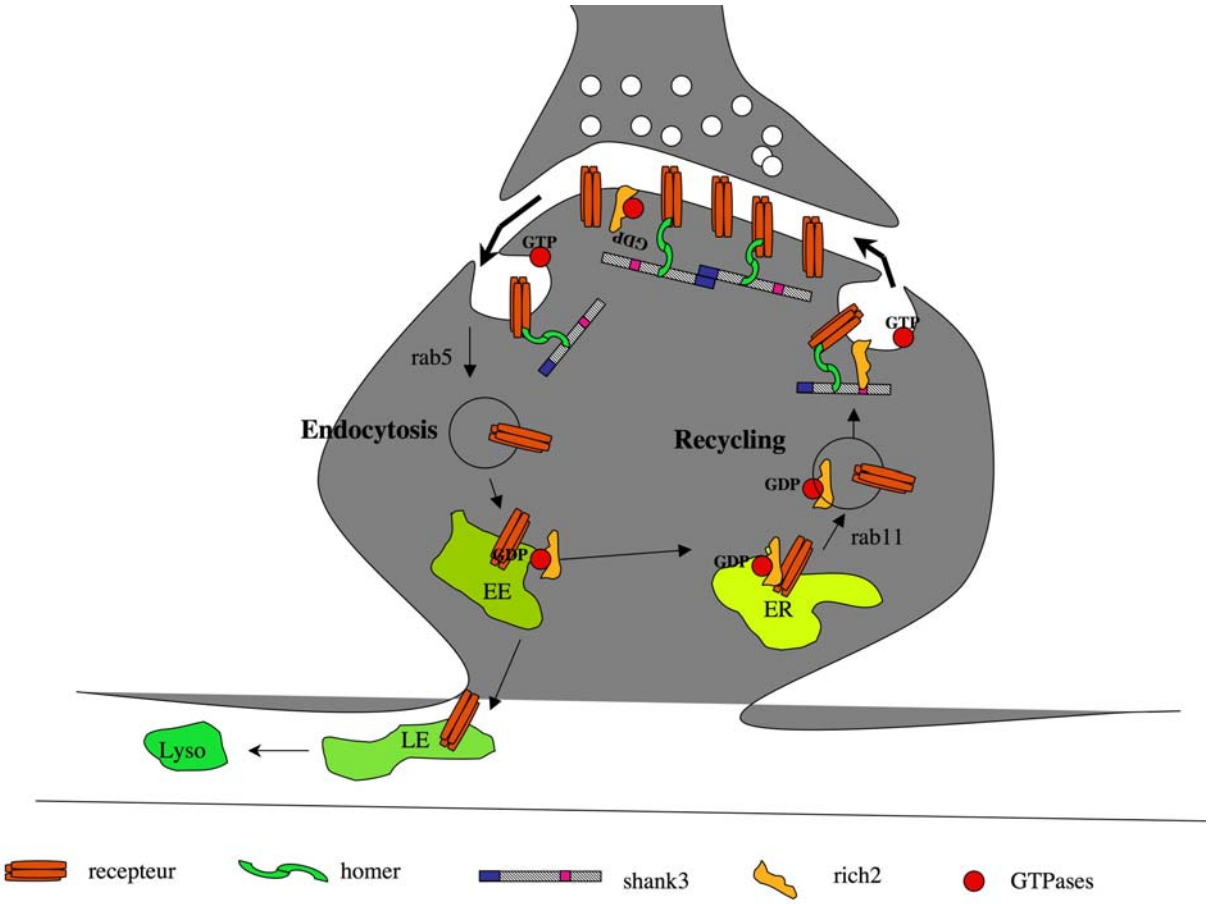



*Fig. 1: Involvement of the Shank-Rich2 complex in endocytosis/recycling process. During the recycling phase, formation the Rich2-Shank complex down-regulates the GTPase activity of Rho proteins allowing exocytose of receptors and channels to the post-synaptic membrane. Dissociation of Rich2 from Shank during endocytosis would allow internalization of synaptic constituents, which depends on Rho GTPase activity. These phenomena would contribute to neuritic development as well as spine formation and maturation.*

- **WP5**: Morphological, electrophysiological and biochemical analyses in engineered calls

In order to study structural determinants that control mGlu1a/5 receptor membrane targeting, we performed dynamic analyses of thrombin cleavable YFP-tagged mGlu1a receptors. We found a saturable surface insertion of mGluR1a, 120 min after the initial rest. Deletion of the C-terminus did not prevent the receptor cell surface insertion, but significantly slowed the rate of this process. This suggest a role of intracellular scaffolding proteins (Homer, tamalin, …), known to bind to the C-terminal PDZ domain of mGlu1a (and mGlu5) receptors, in membrane recycling of the receptor(s).
Parallel to this study we performed BRET measurements and found direct physical interaction between the mGluR5 and NMDA receptors, in transfected HEK cells. This interaction reciprocally decreased NMDA currents and mGluR5 intracellular calcium signaling. Interestingly, the effect of NMDA receptors on mGluR5 did not require activation of the ionotropic receptor and reciprocally. Deletion of the C-terminus of mGluR5 abolished both its interaction and reciprocal inhibition with NMDA receptor. This direct functional interaction may imply a higher degree of target-effector specificity, timing and subcellular signaling localization than could ever be predicted with complex signaling pathways.
In neurons, crosstalk between mGluR1/5 and NMDA receptors have long been studied. The majority of works have demonstrated enhancement of NMDA currents upon pre-treatment with group-I mGluR agonists, although an inhibitory effect mediated by mGluR1/5 has also been reported in organotypic hippocampal slices. It is worth noting that because of localization of NMDA-receptors within and mGluR1/5 at the edge of the PSD, synaptically released glutamate should either activate NMDA receptors solely, or both NMDA and mGluR1/5 receptors

concomitantly, rather than mGluR1/5 first and NMDA receptors subsequently. It was therefore of interest to investigate the functional consequence of co-activating the NMDA receptors and group-I mGluRs. In cultured cerebellar granule cells, we found no crosstalk under control condition, but disruption of the physical interaction between mGluR1a and Shank by induced Homer1a allowed a potent inhibition of NMDA currents by mGluR1 agonist. The effect was voltage-dependent and directly mediated by the mGluR1a-activated G-protein. We hypothesize that at rest, the distant location of mGluR1a from the NMDA receptor in the spine prevents binding of mGuR1 activated G protein to NMDA receptors. Disruption of the interaction between mGluR1a and Homer c-c allows lateral mobility and translocation of the receptor towards the PSD and NMDA receptors. This would facilitate membrane-delimited interaction of mGluR1-activated G protein with the NMDA receptors.
Despite numerous studies suggesting that proteins belong to functional networks, confirming the existence and monitoring the dynamics of these protein assemblies remain a challenge. Fluorescence- and Bioluminence-based Resonance Energy Transfer approaches (FRET and BRET respectively) become increasingly popular to monitor protein-protein interactions in living cells and thus could serve to dynamically follow spatio-temporal interactions. However only FRET is used in microscopic imaging. The BRET limitation relies on the low energy of bioluminescence reactions. This is unfortunate since BRET imaging circumvents many limitations of FRET (photo-bleaching, auto-fluorescence interference, photo-toxicity and undesirable photo-excitation of biological processes), because it does not depend on photo-excitation. We have developed BRET imaging technology that enabled us to reach spatio-temporal resolution of nuclear *versus* membrane dynamic interactions between V2-vasopressin receptor and *b*-arrestin in a minute time frame in COS cells and neurons, using covalently linked *renilla-luciferase* and YFP derivatives. This new technology will serve to study other receptor complex dynamics in neurons.

## CR3 INSERM

### Introduction

It is well accepted that all the glutamate receptors mediate a form of activity dependent plasticity. It has been shown that LTP and LTD in the CA1 region of the hippocampus are associated with long-lasting changes in EPSP-spike coupling, and these forms of plasticity require the activation of NMDA receptor, are synergic with LTD & LTP, input specific and are likely to results from long-lasting changes in intrinsic excitability. Ion channels involved in E-S plasticity are being identified. In addition, metabotropic glutamate receptors modulate induction of long-term synaptic plasticity. Debanne's lab showed that synaptic or pharmacological activation of mGluR5 induces long-term augmentation of excitability (>60 min) in developing layer V pyramidal neurons. This potentiation results from the long-lasting depression of the calcium-dependent $K^+$ current (SK). Thus, long-lasting facilitation of intrinsic excitability may contribute to the formation of active pathways in the cortex that could account for processes related to learning and memory but also participate to the maturation of cortical processing.

### Activities carried out and results

CR3 INSERM contributed to Workpackages 2 and 5

- **WP2 task 5**: Understanding the role of PSD-95 and other MAGUK in neuronal excitability

The initial idea here was to characterize the functional consequences of over-expressing scaffolding proteins such as PSD-95 or SAP-97 in cortical neurons. For this purpose, we have developed with success organotypic slice cultures of rat hippocampus (see Deliverable may 2006). This essential step obtained, we could focused on *transfection* of hippocampal neurons in order to over-express post-synaptic scaffolding proteins (PSD-95 and SAP-97).
In November 2005, we obtained from Monica Di Luca and Fabrizio Gardoni (CO1) these two constructions as shuttle plasmids (pGW1-PSD95-GFP and pGW1-SAP97-GFP). The first assays of pyramidal cell transfection using biolistic methods started in 2006. But the results were disappointing. The number of transfected neurons was insufficient to allow electrophysiological recordings in routine. More recently we have made some assays to infect slice cultures with *virus vectors* (Sindbis in collaboration with JC Poncer, Paris). In this case, the number of infected neuron is much higher and promising. However, the use of viruses presents security constraints when functional analysis is performed on infected neuronal tissue. We are now developing the electroporation technique on single cells (Haas, et al. 2001).
The slice cultures that were specifically developed for this task have been also used for the development of a project (**P1**) that examines the role of voltage gated ion channels in the temporal processing of neuronal information. We show that inactivating voltage-dependent potassium currents control the temporal precision of action potential firing (Cudmore et al. FENS abstract 2006; SFN 2007). The key conductance can be homeostatically down-regulated if cultures are grown in conditions in which network activity is reduced by pharmacological blockade of glutamate receptors (with kynurenate). When the current is down-regulated, synchronous network discharge are prominent and spike precision in individual neuron is enhanced. This work is the subject of a publication in preparation (Cudmore, Giraud, Campanac, Fronzaroli & Debanne).
In parallel, a second project (**P2**) involving the use of organotypic slice cultures of rat hippocampus has been developed. In the cortex, synaptic latencies display small variations (< ~1-2 ms) that are generally considered to be negligible. We show here that the synaptic latency at monosynaptically connected pairs of L5 and CA3 pyramidal neurons is determined by the presynaptic release probability: synaptic latency being inversely correlated with the amplitude of the synaptic response and sensitive to manipulations of the release probability (*Pr*). Changes in synaptic latency were also observed when presynaptic glutamate release was physiologically regulated during short- and long-term plasticity. Paired-pulse depression and

facilitation were respectively associated with increased and decreased synaptic latencies. Moreover, a presynaptic form of long-term depression (LTD) was found to prolong latency. Submillisecond increase in synaptic latency associated with a reduction in synaptic amplitude affected post-synaptic spiking indicating that amplitude-dependent latency variation is significant for the cortical network. We propose that the amplitude-related variations in latency represent a novel code for short- and long-term synaptic dynamics in cortical networks. This work is the object of a paper submitted for publication (Boudkkazi, Carlier, Ankri, Caillard, Giraud, Fronzaroli & Debanne, **Neuron** 2007).

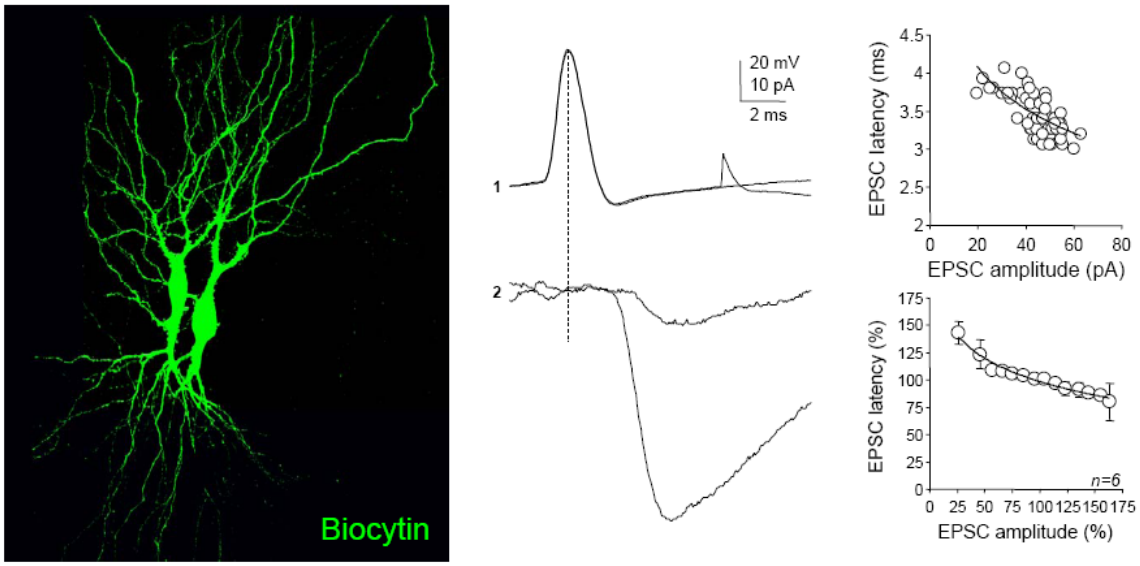



Figure 1

- **WP5 Task 4**: Developmental studies of plasticity of intrinsic excitability in neocortical neurons: the role of post-synaptic scaffolding proteins.

Developmental studies of plasticity of intrinsic excitability in neocortical neurons: the role of post-synaptic scaffolding proteins. This task is directly related to the WP2 task 5. The problems encountered in the achievement of the first task have, subsequently, reduced the progress for this task.

## CR4 UNIGE (D. Muller group)

### Introduction

Over the last few years, mutations of up to thirteen different genes have been identified to be responsible for x-linked forms of mental retardation. Despite this progress, very little is known about the physiological mechanisms involved. A most interesting hypothesis is that these mutations affect genes critically involved in mechanisms of synapse development, formation, plasticity or function. This could be particularly true for genes such as PAK3, oligophrenin, IL1RAPL1 and ARHGEF6, as their expression is modulated by neuronal activity or because they code for synaptic proteins. Recently, by overexpressing a mutated PAK3 protein in pyramidal cells of hippocampal organotypic slice cultures using gene gun transfection methodologies, it has been found that these neurons start to form morphologically abnormal spines similar to those reported in the cortex of mentally retarded infants. This model opens therefore the way to directly investigate the role of these genes in the development of synaptic networks and test the hypothesis that mental retardation induced by these mutations resulted from a defect in synapse formation or synapse function.

### Activities carried out and results

The activities carried out by CR4 UNIGE (Muller group) are referred to WP3 (task 3) and to WP5 (task 6).

Work carried out during the first year of the project allowed to identify the mental retardation gene PAK3 as an important regulator of spine formation and maturation (Boda et al., 2004). Specifically these experiments showed that suppression of PAK3 or expression of mutated variants of PAK3 resulted in a decrease in mushroom-type spines and an increased formation of elongated spines, characterized by small or even absent PSD. The morphological anomalies were correlated with defects in AMPA transmission and in induction of long-term potentiation. Further work then showed that a second mental retardation gene, ARHGEF6, which codes for an exchanged factor also regulating small Rho-GTPases, contributed in a very similar way to mechanisms of spine morphogenesis. Suppression of ARHGEF6 was found to produce a similar phenotype as suppression of PAK3, with also an increase in elongated, immature type spines. In addition we could demonstrate that the spine defects induced by suppression of PAK3 could be rescued by expression of a constitutively active form of PAK3 (Node-Langlois et al., 2006). Together these results indicated that the mechanisms underlying mental retardation associated with mutation of these two genes are likely to be comparable. We next undertook experiments in collaboration with the group of JV Barnier in Paris to investigate the type of Rho GTPase involved in the activation of PAK3. For this we took advantage of the existence of different human mutations affecting PAK3 gene and which affects different part of the PAK3 protein. We found that these different mutations somehow affected differentially the mechanisms of spine formation. Two mutations of the kinase domain resulted in very mild decrease in spine numbers associated with an increase in elongated, immature protrusions. In contrast a mutation of the Rho GTPase binding domain produced a marked alteration of the spine density only. Interestingly this mutation did not alter the kinase activity of PAK3, but markedly affected its ability to bind and respond to activated CDC42, but not Rac1. These results demonstrated therefore that CDC42 is likely to be the main GTPase responsible for PAK3 activation and that this pathway is important for regulating spine numbers (Kreis et al., 2007). More recently we started a comparison of the role of PAK3 and PAK1 in regulating spine morphogenesis. The two protins are expressed in hippocampal neurons with PAK1 being about 2-4 times more abundant than PAK3.

Using loss and gain of function experiments we found that PAK3 and PAK1 differentially affect spine morphology and in particular that PAK1 knockdown does not affect the number or shape of dendritic spines, while constitutively active PAK1 resulted in an increase in spines with a tiny head, a phenotype not observed with constitutively active PAK3. However we also found that constitutively active PAK1 can rescue the phenotype due to knockdown of PAK3, an important observation in the context of PAK3 mutation induced mental retardation. Thus it appears that

PAK1 and PAK3 differentially affect spine morphogenesis, but they seem to share similar substrates and could compensate for each other (Boda et al., submitted).
In a different study, we investigated the role of the scaffold protein PSD-95 in regulating the organization of the synapse and more specifically the formation of new synaptic contacts. For this, we used electron microscopy to reconstruct dendritic spines of PSD-95/EGFP transfected hippocampal pyramidal neurons. Over-expression of PSD-95 was associated with changes in spine shape, PSD organization and marked increases in spine volume and PSD area. More surprisingly, PSD-95 expression resulted in the formation of multi-innervated spines, with up to 7 different presynaptic terminals contacting a single spine. Deletion of $PDZ_2$ domain of PSD-95, which interacts with nitric oxide synthase (NOS), prevented formation of multiple contacts, whereas $PDZ_3$, deletion had no effect. Similarly, PSD-95 expression combined with siRNA mediated down-regulation or pharmacological blockade of NOS prevented axon differentiation into varicosities and multi-synapse formation, but not spine and PSD enlargement. Furthermore, NOS blockade under control conditions reduced synapse density. Together these results indicated that PSD-95 is not only a scaffold protein, but it also plays an important role, through interaction with NOS and NO signaling, in the regulation of synapse formation with nearby axons (Nikonenko et al., submitted).

**CR4 UNIGE (C. Luscher group)**

**Activities carried out and results**

Work has been carried out on the plasticity of excitatory afferents of dopamine neurons of the ventral tegmental area. This nucleus is the origin of the mesolimbic dopamine (DA) system, which is essential for reward-mediated learning and may be dysfunctional in mental retardation.

Since addictive drugs strongly stimulate this system, one can use them as a model system to study dopamine-mediated adaptations of synaptic transmission in this part of the brain.

In fact a single exposure to cocaine leads to an increase of AMPA/NMDA ratio in excitatory afferents onto DA neurons that lasts between 5 and 10 days. A first finding was that this synaptic strengthening does occur by the insertion of GluR2-lacking AMPA receptors, which therefore not only increases synaptic efficacy but also makes the synapse calcium permeable. We saw that this plasticity is expressed by a redistribution of postsynaptic receptors that is it requires a mobile pool of receptors: in a first step GluR2 containing receptors will be removed, in a second than GluR2-lacking inserted.

We next searches for a mechanism that could reverse this plasticity and found that the synaptic or chemical stimulation of mGluR1s was sufficient to abolish the inward rectification indicative of GluR2-lacking receptors. An in-depth analysis of the underlying molecular mechanism showed that this LTD was also expressed by an exchange of GluR2-lacking receptors for GluR-containing ones. Although occurring within minutes after the mGluR1 activation it requires protein synthesis. In a technical tour de force, we were then able to show that one of the proteins that needs to be synthesized is the glutamate receptor subunit GluR2 itself. Intrigued by the protein synthesis dependence and in the light of a large body of literature implicating FMRP in hippocampal mGluR-LTD, we looked whether in the FMR1 ko mice the plasticity in the VTA was also affected. However in contrast to the hippocampus we did not see an enhancement of the magnitude of the plasticity in the absence of the translational repressor FMRP.

Taken together, the support of SYNSCAFF did allow us to unravel the molecular mechanism of cocaine-induced plasticity and its reversal in the ventral tegmental area, which may a crucial role in several forms of learning and which may be altered in mental retardation.

## CR5 LIN.NCMB

### Introduction

Electron-dense cytosmatrices at both sides of the synaptic junction, i.e. the presynaptic cytomatrix at the active zone (CAZ) and the postsynaptic density (PSD), are hallmarks of excitatory synaptic contacts of the cortex. Both cytomatrices constitute specialized protein meshworks that are major substrates for developmental and functional plasticity of chemical brain synapses (Gundelfinger et al., 2006; Schoch & Gundelfinger, 2006). In the context of the SynScaff consortium, we have focussed on the role of two very large CAZ proteins called Bassoon and Piccolo in developmental assembly and molecular organization of cortical synapses (Dresbach et al., 2006; Fejtova et al., 2006; Schoch & Gundelfinger, 2006). Based on preliminary work, we have studied interaction partners of Bassoon and Piccolo (WP1, task 1.1), analyzed effects of mutations of these proteins on cortical development and function in vitro (WP5, task 1) and investigated consequences of CAZ dysfunctions in mice lacking functional Bassoon (WP6, task 1). In addition, we have contributed to studies of various aspects cortical synapse developmental dysfunctions in Bassoon mutant mice in collaborations with other partners of the consortium as well as other laboratories.

### Activities carried out and results

This partner has contributed to three workpackages of the project "**Synaptic scaffolding proteins orchestrating cortical synapse organisation during development**"

- WP1: Organisation and development assembly of presynaptic scaffold

  Task 1.1: Identify and structurally characterise interacting elements starting from known components of the CAZ.

- WP5: Morphological, electrophysiological and biochemical analyses in engineered cells

  Task 5.1: Analyses of the effects of mutation of Piccolo and Bassoon

- WP6: Characterisation of mouse mutants for scaffolding proteins

  Task 6.1: Dysregulation of the presynaptic scaffold in mouse mutants

WP1, task 1.1: Interaction partners of Bassoon and Piccolo

Work performed in this workpackage included the characterization of protein domains involved in the interaction between Bassoon and Piccolo and CtPB1 (deliverable D1-2) and mapping of the interaction between dynein-light chains (DLC) and Bassoon. Due to the exciting findings during the first period this task was extended at the expense of the other two tasks as approved during mid-term review.

*CtBP1:* CtBP1 is a homolog of Ribeye, a protein specifically occurring at ribbon synapses of retinal photoreceptors or inner ear hair cells. In photoreceptors the interaction between Ribeye and Bassoon is essential for proper anchoring of the CAZ (ribbon) to the presynaptic membrane (tom Dieck et al., 2005). CtBP1 has been discovered as a transcriptional co-factor. An isoform of the protein, known as BARS, is involved in vesicle fission from the trans-Golgi complex. Here, we have found that CtBP1 can physically interact with Bassoon and that CtBP1 is present at the majority excitatory and inhibitory brain synapses (tom Dieck et al., 2005). Like Bassoon, also Piccolo can bind CtBP1. We have performed a mutational analysis to map these binding sites at the molecular level (see D1-2). While Bassoon has a single interaction site, Piccolo harbours three of them, which are in close proximity in the centre of the molecule. In the absences of Piccolo and Bassoon CtBP1 is not targeted to developing synapses. Therefore we assume that CtBP1 is recruited to preassembled active zone transport packages via Piccolo and/or Bassoon.

Dynein light chains: DLC-1 and DLC-2 are small proteins originally described as part of the retrograde microtubule motor dynein. Meanwhile DLCs are known to fulfil multiple functions

including cargo adaptation to the actin-based motor protein myosin V and multiple adaptor and chaperonin functions. Bassoon, but not Piccolo, can bind DLC via three independent motifs. The binding motifs were identified and verified utilizing point mutations that disrupt the interaction. We expressed and purified fusion proteins containing the binding motifs, and their point mutated analogues and investigated their relative binding affinities to both DLC-1 and DLC-2 utilizing surface plasmon resonance technology. Further (still ongoing) experiments were performed in the context of WP5 to assess the functional relevance of Bassoon-DLC interactions.

WP5, task 5.1: Analyses of the effects of mutation of Piccolo and Bassoon

To further study the interaction of Bassoon and Piccolo with CtBP1 we performed expression studies wild-type and mutant primary neuronal cultures. We could confirm that Bassoon and CtBP1 do interact in living cells using FRET-FLIM (Foerster's Resonance Energy Transfer combined with Fluorescence Lifetime Imaging Microscopy) technology (Jose et al., 2007). Interaction occurs already at the trans-Golgi network suggesting that it is important for the assembly of presynaptic active zones during cortical synapse development. In the absence of functional Piccolo and Bassoon (double mutant cultures) CtBP1 is not targeted to synapses indicating that the interaction with at least one of these two proteins at the Golgi complex is essential for synaptic localization. The physiological function of CtBP1 at the active zone is currently unknown.

Studies on primary neurons also confirmed and cotransport of Bassoon and DLC1 in axons and their colocalisation in presynaptic boutons. Isolation and analysis of Piccolo-Bassoon transport vesicles confirmed the association of DLCs as well as of other motor components with these active zone precursors. Over-expression of dominant negative constructs carrying DLC-binding motifs of Bassoon and thus interfering with the binding of DLCs to endogenous Bassoon seem to affect Bassoon targeting to the synapse, whereas mutated constructs do not (Fejtova, unpubl. data) suggesting that the interaction of DLCs and Bassoon is essential for the developmental assembly of presynapses.

In collaboration with A. Triller and S. Marty we have investigated the ultra-structure of wild-type and mutant synaptic contacts using conventional and high-pressure cryo-electron microscopy (Siksou et al., 2007; unpubl. data). Deficiency of functional Bassoon and Piccolo seems to affect primarily synaptic maturation and plasticity as indicated by a reduced post-synaptic density in primary cultures of double mutant neurons.

In collaboration with partner CR9 new antibodies were generated against CtBP1 and DLC1. While the CtBP1 antibodies recognize their antigen specifically and can be used to discriminate CtBP/BARS isoforms, DLC antibodies turned out as not specific.

WP6, task 6.1: Dysregulation of the presynaptic scaffold in mouse mutants

To assess differences in mouse brains due to Bassoon and Piccolo deficiencies proteomes of mutants and wild-type mice were compared. We identified a number of changes in protein expression levels of various types proteins as reported in previous periodic activity reports. Furthermore, using magnetic resonance imaging as well as functional imaging and spectroscopy we found that Bassoon mutant mice display enlarged cerebral cortices and hippocampi and show alterations in their glutamate metabolism (Angenstein et al., 2007a; 2007b).

As the Bassoon mutant available is not a null and may display gain of function phenotypes we have generated a new Bsn-mutant mouse. Currently we have heterozygous animals of this new mutant.

Partner CR7 has analysed the epileptic phenotype of Bsn mutant mice in collaboration with CO1 and us. The analysis revealed a new form of short-term synaptic plasticity in the striatum. In addition CR7 discovered a significant decrease of BDNF-positive neurons in striatum of Bassoon mutant mice. Therefore we performed a detailed analysis of the expression of both BDNF and TrkB, the major BDNF receptor in neurons. Whereas the total level of TrkB is unchanged in homogenate of Bassoon mutant mice in comparison to wild type, it is increased 1,5fold in the insoluble fraction in Bassoon mutants. Moreover, we observed significant

decrease of BDNF in total homogenate (80% of wild type levels), which is in good agreement with initial observation of contractor CR7.

Partner CR2 performed an analysis of hippocampal mossy fibre synapses of Bsn mutant mice and discovered a developmental phenotype also at these specialized synapses. Currently ultra-structural analysis is underway to see whether physiological dysfunction can be correlated with structural deficits.

**CR6 CNR-IN**

**Introduction**
Although proteomics has identified some of the key elements of the postsynaptic specialisation, very little is known about their precise distribution and stoichiometry. Higher resolution analyses are used to investigate the subsynaptic localisation of Homer and Shank in distinct types of excitatory synapses in cortical circuits. Further, new approaches of proteomics are developed to identify unknown elements driving spine formation in cortical synapses.

**Activities carried out and results**

Our unit was involved in the following Work Packages:

- **WP2:** Organisation and development assembly of postsynaptic scaffolds and receptors
- **WP4:** Proteomic approach to study multi-proteins complexes modification induced by neuronal development and synaptic activity
- **WP7:** Characterisation of mouse models of mental retardation: morphological, structural, functional organisation of cortical synapses

WP2 - Task 3: Biochemical identification of factor(s) that bind to GluR2 N-terminal domain (NTD) and cooperate with GluR2 to induce dendritic spine formation

**Extracellular interactions between GluR2 and N-cadherin in spine regulation**
Via its extracellular N-terminal domain (NTD), the AMPA receptor subunit GluR2 promotes the formation and growth of dendritic spines in cultured hippocampal neurons. Here we show that the first N-terminal 92 amino acids of the extracellular domain are necessary and sufficient for GluR2's spine-promoting activity. Moreover, overexpression of this extracellular domain increases the frequency of miniature excitatory postsynaptic currents (mEPSCs). Biochemically, the NTD of GluR2 can interact directly with the cell adhesion molecule N-cadherin, in cis or in trans. N-cadherin-coated beads recruit GluR2 on the surface of hippocampal neurons, and N-cadherin immobilization decreases GluR2 lateral diffusion on the neuronal surface. RNAi knockdown of N-cadherin prevents the enhancing effect of GluR2 on spine morphogenesis and mEPSC frequency. Our data indicate that in hippocampal neurons N-cadherin and GluR2 form a synaptic complex that stimulates presynaptic development and function as well as promoting dendritic spine formation.
*Published in Neuron 54: 461-77 (2007)*

Further work related to this Work Package:

**Shank expression is sufficient to induce functional dendritic spine synapses in spiny neurons.**
Shank proteins assemble glutamate receptors with their intracellular signaling apparatus and cytoskeleton at the postsynaptic density. Whether Shank plays a role in spinogenesis and synaptogenesis remained unclear. Here, we report that knock-down of Shank3/prolinerich synapse-associated protein-2 by RNA interference reduces spine density in hippocampal neurons. Moreover, transgene expression of Shank 3 is sufficient to induce functional dendritic spines in aspiny cerebellar neurons. Transfected Shank protein recruits functional glutamate receptors, increases the number and size of synaptic contacts, and increases amplitude, frequency, and the AMPA component of miniature EPSCs, similar to what is observed during synapse developmental maturation. Mutation/deletion approaches indicate that these effects require interactions of Shank3 with the glutamate receptor complex. Consistent with this observation, chronic treatment with glutamate receptor antagonists alters maturation of the Shank3-induced spines. These results strongly suggest that Shank proteins and the associated glutamate receptors participate in a concerted manner to form spines and functional synapses.
*Published in J Neurosci 25:3560-3570.*

**Key role of the PSD scaffold proteins, Shank and Homer, in the functional architecture of $Ca^{2+}$ homeostasis at dendritic spines in hippocampal neurons.**
A key aspect of postsynaptic function, also important for plasticity, is the segregation within dendritic spines of Ca2+ rises attributable to release from intracellular stores. Previous studies have shown that overexpression in hippocampal neurons of two postsynaptic density (PSD) scaffold proteins, Shank1B and Homer1b, induces spine maturation, including translocation of the intracellular Ca2+ channel inositol trisphosphate receptor (IP3R). The structural and functional significance of these processes remained undefined. Here, we show that in its relocation, IP3R is accompanied by other endoplasmic reticulum (ER) proteins: the Ca2+ pump sarcoendoplasmic reticulum calcium ATPase, the lumenal Ca2+-binding protein calreticulin, the ER lumen-addressed green fluorescent protein, and, to a lesser extent, the membrane chaperone calbindin. The specificity of these translocations was demonstrated by their inhibition by both a Shank1 fragment and the dominant-negative Homer1a. Activation in Shank1B-transfected neurons of the metabotropic glutamatergic receptors 1/5 (mGluRs1/5), which induce IP3 generation with ensuing Ca2+ release from the stores, triggered considerable increases in Ca2+-dependent responses: activation of the big K+ channel, which was revealed by patch clamping, and extracellular signal-regulated protein kinase (ERK) phosphorylation. The interaction of Shank1B and Homer1b appears as the molecular mechanism linking mGluRs1/5, strategically located in the spines, to IP3R with the integration of entire ER cisternas in the PSD and with consequences on both local Ca2+ homeostasis and overall neuronal signaling.
*Published in J Neurosci 25:4587-92*.

**A preformed complex of postsynaptic proteins is involved in excitatory synapse development.**
Nonsynaptic clusters of postsynaptic proteins have been documented; however, their role remains elusive. We monitored the trafficking of several candidate proteins implicated in synaptogenesis, when nonsynaptic clusters of scaffold proteins are most abundant. We find a protein complex consisting of two populations that differ in their content, mobility, and involvement in synapse formation. One subpopulation is mobile and relies on actin transport for delivery to nascent and existing synapses. These mobile clusters contain the scaffolding proteins PSD-95, GKAP, and Shank. A proportion of mobile clusters that exhibits slow movement and travels short distances contains neuroligin-1. The second group consists of stationary nonsynaptic scaffold complexes that mainly contain neuroligin-1, can recruit synaptophysin-containing axonal transport vesicles, and are readily transformed to functional presynaptic contacts that recycle the vital dye FM 4-64. These results postulate a mechanism whereby preformed scaffold protein complexes serve as predetermined postsynaptic hotspots for establishment of new functional excitatory synapses.
*Published in Neuron 49:547-62.*

**DNA methylation regulates tissue specific expression of *Shank3***
Tissue-specific gene expression can be controlled by epigenetic modifications such as DNA methylation. *SHANK3*, together with its homologs *SHANK1* and *SHANK2*, has a central functional and structural role in excitatory synapses and is involved in the human chromosome 22q13 deletion syndrome. In this report we show by DNA methylation analysis in lymphocytes, brain cortex, cerebellum and heart that the three *SHANK* genes possess several methylated CpG boxes, but only *SHANK3* CpG islands are highly methylated in tissues where protein expression is low or absent and unmethylated where expression is present. An alternative chromatin conformation at the *SHANK3* locus is also apparent in some tissues. SHANK3 protein expression is significantly reduced in hippocampal neurons after treatment with methionine, while HeLa cells become able to express SHANK3 after treatment with 5-Aza-2'-deoxycytidine. Altogether, these data suggest the existence of a specific epigenetic control mechanism regulating *SHANK3*, but not *SHANK1* and *SHANK2*, expression.
*Published in J Neurochem 101:1380-91*

**The role of the scaffold protein GKAP in the assembly of the postsynaptic density complex**

The postsynaptic density (PSD) at the excitatory synapses consists of a network of proteins that anchor and link postsynaptic membrane proteins to cytoplasmic cytoskeletal elements and signaling pathways. Scaffold proteins such as PSD95, GKAP and Shank are core components of the PSD and form a major synaptic protein complex. Our laboratory has previusly demonstrated that PSD-95 and GKAP interaction is fundamental for the correct folding and synaptic localization of Shank (Romorini et al. 2004, JNeurosci. 24:9391-404) and that the formation of a post-synaptic complex constituted by PSD95, GKAP, Shank and NLG1 is able to recruit a pre-synaptic terminal leading the maturation of an excitatory synapse (Gerrow et al. 2006, Neuron. 49:547-62). We now further investigated the synaptic function of GKAP using an RNA interference approach in neuron cultures and whether the PSD-95/GKAP/Shank protein complex is assembled in the cell body or in the dendrites. As expected when GKAP expression is inhibited in mature neurons we observed a decrease in both post-synaptic scaffold proteins clusters and dendritic spines numbers and shrinkage of spine head size, suggesting the importance role of GKAP for maintaining the PSD structure. Interestingly we found that GKAP preferentially associate to the Shank protein synthesized in the dendrites, thus PSD protein complexes assembly might be differentially regulated between cell body and dendrite compartments (manuscript in preparation).

WP4 - Task 1: To set up a new protocol based on differential detergent fractionation (DDF), Task 2: To identify proteins in each fraction by means of mass spectrometry, Task 3: To identify the proteins that are regulated during development and synaptic activity in terms of synthesis, degradation, post-translation modifications or distribution in each fraction.

**Proteomic analysis of activity-dependent synaptic plasticity in hippocampal neurons.**

Following long-term treatment with bicuculline and tetrodotoxin (TTX) aimed at modifying synaptic activity in cultured neurons, we used a proteomic approach to identify the associated changes in protein expression. The neurons were left untreated, or treated with bicuculline or TTX, and fractionated by means of differential detergent extraction, after which the proteins in each fraction were separated by means of two-dimensional (2D) gel electrophoresis, and 57 proteins of interest were identified by mass spectrometry. The proteins that showed altered expression and/or post-translational modifications include proteins or enzymes involved in regulating cell and protein metabolism, the cytoskeleton, or mitochondrial activity. These results suggest that extensive alterations in neuronal protein expression take place as a result of increased or decreased synaptic activity.

*Published in J Proteome Res 6:3203-15*

**A role for the eEF2 in regulating local protein synthesis and dendritic spine formation.**

Network firing influences deeply neuron activity. Global inputs are traduced at the level of single post synaptic neuron in spine modification. Post synaptic plasticity needs local protein synthesis to adapt its molecular machinery to on-going situations.

We found that network activity modulates dendritic protein synthesis acting on eEF2K via glutamate receptors. Perturbations on eEF2K pathway result in a severe alteration of spine morphology and impair plastic response to pre-synaptic inputs. eEF2 phosphorylation by eE2FK impairs global elongation rate of newly born mRNA, but is also associated with a local increase in the rates of translation of certain mRNA species, as CaMK II, Arc and, as here suggested, BDNF.

We hypothesize global network state drives single neuron plasticity through an influence on local protein synthesis; it modulates at the level of mRNA elongation the expression of a specific pattern of protein playing a pivotal role in spine plasticity (manuscript in preparation).

WP7 - Task 1: Analysis on fmr 1 ko mice

Because most the experiments suggested in Task 1 of this WP7 have been published by other laboratories we have decided to study the functions of other proteins which mutation causes mental retardation in humans.

**Report on the role of IL1RAPL mutation in a non syndromic form of X-linked mental retardation disease**

Mutations of genes localized on X chromosome are responsible for 5% of the mental retardation patients. The related proteins, are involved in some cellular pathways important for neuronal network and/or synaptic plasticity. We focusing our attention on IL1RAPL1. IL1RAPL belongs to the IL-1/Toll receptor family. In addiction to the extracellular domain, consisting in 3 Ig-like domains, IL1RAPL contains an intracellular TIR domain, characteristic of the IL-1/Toll receptor family and 150aa C-term extension which is not present in any other mammalian IL1Rs or TLR families. We searched for proteins interacting with IL1RAPL by screening a human fetal brain library using Yeast Two-hybrid System and IL1RAPL C-term domain as a bait. Our results indicated different interacting proteins: PSD95 and SAP97, members of the MAGUK family of proteins. To confirm whether IL1RAPL1 is able to interact with PSD95, we perform a GST-Pull Down Assay and a Coclustering Assay on transfected COS-7 cells. We found a colocalization between IL1RAPL1 and PSD95 or SAP97 but not with Homer, another PSD protein. In particular, we observe a coclustering between IL1RAPL and PDZ1-2 domains of PSD95 and this interaction were mediated to the last 8aa of IL1RAPL.We also confirmed this observation in YTH Test, using PSD95's domains as prey. Moreover we overexpressed an HA-tag form of the protein in rat hippocampal neuron. We found IL1RAPL1 localized to dendrites and in the PSD. To better understand the effect of IL1RAPL1 on synapses formation, we overexpressed the wild type or different mutated proteins (also presents in the MRX patients) and we found that overexpression of IL1RAPL induced formation of new spines and this effect were mediated by it's C-term domain, while the mutated proteins were not localized to the PSD and were able to change spines morphology. Moreover, when overexpressing IL1RAPL in rat hippocampal neuron, we observe an enhanced immunostaining of presynaptic proteins. We hypotize a presynaptic effects, and we attempted to confirm these observation using cocultures assay. These data suggest that IL1RAPL1 by interacting with PSD proteins might play an important role in organizing the correct formation of postsynaptic complex and dendritic spines.

**Report on the characterization of the role of Shank3 on synapses function**

We have used siRNA technology to make *SHANK3* knock out (KO) mice. Specific siRNAs for *SHANK3* have been already identified and KO animals will be generated by infecting blastocyst cells with lentiviruses expressing the *SHANK3* siRNA in collaboration with the San Raffaele-Telethon Facility for Conditional Mutagenesis. The *SHANK3* KO mice will give us the possibility to produce an animal model of the 22q13 deletion syndrome that causes severe mental retardation. We have studied the role of *SHANK3* in regulating the functional development and maturation of synapses in neuronal cultures *in vitro* using the same *SHANK3* siRNAs. Primary neurons cultures have been infected by lentiviruses expressing the *SHANK3* siRNAs or control vector. Biochemical, morphological and functional studies have been performed on these cultures. Preliminary data suggest a role for Shank3 in regulating glutamatergic synapses organization.

**CR7 UNIPG**

**Introduction**

Behavioural and electrophysiological characterization of mouse mutants for scaffolding proteins was the main goal of this unit. Further dysfunctions in synapse formation, synapse turnover, synapse morphology or function responsible for human forms of mental retardation (bottom-up) were addressed in animal models.
In addition, with a opposite approach, mouse models of mental retardation are used with the aim to identify the synaptic defects in terms of morphology, function, organisation of scaffold proteins induced by expression of genes bearing mutations responsible for human forms of mental retardation (top-down). Fragile-X fmr1 mice, and trisomic mice that correspond to Down Syndrome are used.

**Activities carried out and results**

- **Workpackage 5 -** Task 2: Role of SAP97 and other MAGUK in modifying synaptic structure.

In collaboration with Di Luca Unit we have elucidated the role of MAGUK proteins in the trafficking within the post synaptic density (PSD) in physiological and pathological conditions (unilateral denervation of the DAergic projection by 6-hydroxydopamine, 6-OHDA, and chronic L-DOPA treatment). Abnormal function of NMDA receptor has been suggested to be correlated with the pathogenesis of Parkinson's disease (PD) as well as with the development of L-DOPA-induced dyskinesia. First, we have shown that NR2 subunits of the NMDA receptor display specific alterations of their subcellular distribution in striata from 6-OHDA-lesioned rats, L-DOPA-treated dyskinetic (DYS) and L-DOPA-treated non-dyskinetic rats (NON-DYS) (Picconi et al., 2004; Gardoni et al., 2006), accompanied by profound modifications of NMDA receptor NR2B subunit association with PSD-95, SAP97 and SAP102. The intrastriatal treatment of NON-DYS animals with a synthetic peptide (TAT2B) able to affect NR2B binding to MAGUK proteins - as well as synaptic localization of this subunit in NON-DYS rats - was sufficient to induce a shift of treated rats towards a dyskinetic motor behaviour (Gardoni et al., 2006). On the other hand, same duration treatment with TAT2A, a selective NR2A-MAGUK uncoupling peptide, in DYS rats was not able to reverse abnormal motor behaviour. The effects of TAT2A and TAT2B on corticostriatal synaptic plasticity in DYS and NON-DYS animals have been also investigated. As previously described (Picconi et al., 2003) corticostriatal spiny neuorns (MS) of L-DOPA-treated animals exhibit a normal LTP, but while NON-DYS similarly to controls, are able to show the depotentiation of LTP, induced by a low frequency stimulation protocol (LFS,), this depotentiation is completely lost in DYS rats (Picconi et al., 2003). Application of TAT2B was able to block the depotentiation in corticostriatal slices obtained from control and NON-DYS rats, inducing a DYS-like neuronal pattern and suggesting a possible mechanism for the behavioural/molecular shift previously observed following acute TAT2B treatment of these animals. Although in vivo treatment was not able to reverse abnormal motor behaviour, incubation with TAT2A was able to restore depotentiation in corticostriatal slices obtained from DYS rats. In control animals, application of either TAT2A/2B to corticostriatal slices, was able to block the induction of corticostriatal LTP in slices obtained from control rats. When applied after the tetanus, TAT2A/2B were not able to affect the maintenance of LTP. On the other hand, once induced the LTP, the depotentiation was not changed by the application of TAT2A, while it was blocked after the application of the peptide that alter the interaction between NR2B and the MAGUK-proteins complex. These results indicate that NR subunits-MAGUK interactions have a critical role in the bidirectional synaptic plasticity forms, necessary for motor control. Moreover our data indicate that, once established, mechanisms underlying dyskinetic phenomena lead to a more stable synaptic reorganization in the basal ganglia network.

- **Workpackage 6 -** Task 3: Role of scaffolding proteins in activity-dependent synaptic plasticity

Bassoon is a presynaptic protein essential for synapse development and for the correct assembly and release of neurotransmitter vesicles in glutamatergic synapses. Bassoon KO

(Bsn) homozygous mutant mice develop pronounced spontaneous seizures although at the ultrastructural level no obvious synaptic differences were observed compared to wild-type littermates (WT). In order to study the functional role of presynaptic protein Bsn on movement control, we characterized membrane properties and synaptic responses of spiny neurons (MS) and fast-spiking interneurons (FS) in Bsn KO mice. No significant changes of the intrinsic membrane properties (membrane potential, input resistances, firing discharge) of this neuronal subtype were found between Bsn KO and WT mice for both neuronal subtypes. Intracellular recordings from MS showed that LTD was equally expressed in Bsn KO and WT animals while amplitude of LTP observed in spiny neurons from KO mice was lower compared to WT animals. This defect was associated to rearrangement of striatal connectivity of MS neurons and profound alteration in NMDA receptor subunit composition. The altered pattern of MS connectivity indicates that an enhancement of striatal network functionality might try to compensate for the altered activity of seizures cells in these mutants. In order to further investigate on the adaptive mechanism of the striatal microcircuit, synaptic plasticity was also investigated in FS interneurons. Interestingly, in Bsn KO mice FS showed short term increased synaptic strength that was NMDA- and BDNF-dependent, while neurons recorded from WT showed either no change or a small transient synaptic depression. Moreover, comparison of striatal FS (as PV positive-cells) quantification between the two genotypes showed a higher number of FS interneurons in Bsn KO mice than observed in WT. Taken together these results suggest that a compensatory enhancement of FS interneurons function in the dorso-lateral striatum of these mutant animals may compensate for the changes in synaptic plasticity and morphology of MS and may account for the higher performance in behavioral tests. To find a behavioural correlate of the adaptive mechanisms observed at anatomical, molecular and cellular levels the corticostriatal-dependent learning capabilities of Bsn KO, animals were tested. Interestingly since the first session of active avoidance learning, Bsn KO mice become gradually able to escape the shock moving to the safe side of the box while the WT littermates slowly learned how to respond over the course of the five sessions. Although in open field testing BSN mutants resulted more active that controls, the increase in conditioned responses of Bsn KO mice was not due to enhancement of motor activity as shown by the number of crossings that was similar in both groups. In order to complete previous observations on hippocampal functions we moved to characterize the hippocampal synaptic plasticity, at CA3-CA1 synapse, in the genetically modified Bsn KO mice by in vitro electrophysiological study. Interestingly, extracellular recordings from hippocampal slices showed that the amplitude of LTP recorded in Bsn KO mice was lower compared to WT animals. Because of several indexes of ipovision previously evidenced in a battery of hippocampal-dependent test (Morris water maze, context fear conditioning, reactivity to spatial changes in open field) we decided to test olfactory learning and memory by the social transmission of food preference test (STFP), a paradigm in which animals learn that a particular odorous food is safe to eat (and therefore preferable) following exposure to the odour on a conspecific's breath. Several studies have shown that damage to hippocampus produces impairments in retention performance on this nonspatial task. In this way, we avoid the difficult to discriminate between visual and cognitive alteration. In the present study we used a variant of the ones described by Wrenn et al., 2002. Our results showed that Bsn KO had a low percentage of olfactory recognition, respect higher value in WT, confirming an impairment of hippocampal function in Bsn KO mice.

- **Workpackage 7** - Task 1: Analysis on fmr KO mice

Abnormal plasticity of excitatory transmission represents a prominent synaptic alteration described in mice knockout for the *fmr*1 gene encoding the fragile X mental retardation protein (FMRP) (*fmr*1 KO), a reliable model of fragile X syndrome (FXS) characterized by learning deficits, hyperactivity and dendritic spine abnormalities. In this model, the possible parallel alteration of inhibitory GABAergic transmission has also been postulated by neurochemical and molecular studies, but it has never been addressed physiologically. Whole-cell patch clamp recordings from striatal MS showed that spontaneous synaptic events could be blocked in both genotypes by bicuculline and that most event had amplitudes ranging between 5 and 40 pA and had kinetic properties indistinguishable in the two genotypes while the frequency of sIPSCs was increased in *fmr*1-KO mice, a result compatible with the idea that striatal GABAergic nerve terminals are dysinhibited in this model of FXS. This difference did not result from genotypic differences in release probability as supported by paired-pulse experiments. mIPSCs, recorded

following blockade of voltage-dependent sodium channels with TTX had a mean frequency significantly lower in both genotypes, while mean amplitude was unchanged. Also in this case the frequency, but not the amplitude, of mIPSCs was higher than normal in *fmr*1 KOs, supporting the idea that FMRP modulates transmitter release through a presynaptic action and independently of action potential generation. Also in mice lacking *BC1* gene (BC1 RNA is a small non-coding RNA which interacts with FMRP to regulate its functions) the frequency of sIPSCs and of mIPSCs was higher than in controls, while their amplitudes were unchanged, indicating that BC1 RNA ablation mimics the effects of genetic inactivation of FMRP on striatal GABA transmission. The use of double mutants for *fmr*1 and *BC1* confirmed that the effects of *BC1* ablation occluded those of *fmr*1 disruption since *fmr*1 and *BC1* disruption interferes with a common signaling cascade to increase striatal GABA transmission and that this effect was not due to saturating mechanisms. While detailed analysis failed to show any modifications of glutamate-mediate neurotransmission in all the mutants analyzed, dopamine DA D2 receptor-mediated transmission in the striatum was found profoundly altered in BC1 KO mice. Although in neurons from WT mice, application of the DA D2 receptor agonist quinpirole significantly reduced eIPSCs, in neurons from BC1 KO mice, the effects of quinpirole were remarkably potentiated indicating increased sensitivity of GABA synapses to D2 receptor stimulation. Further analysis demonstrated that the effect of quinpirole clearly reinforce a presynaptic dysregulation, DA D2 R-mediated, indicating an increased activity of the GABAergic neurons due to an iperfunctioning of the DA D2 receptors in the BC1 KO mice and supporting the concept that disruption of BC1 gene increases DA D2 receptor-mediated synaptic responses in the striatum.

- **Workpackage 7** - Task 2: Plasticity in Trisomy 21

Trisomy 21 or Down syndrome (DS) is the most frequent genetic cause of mental retardation, affecting 1/800 live born human beings. The Ts($17^{16}$)65Dn mouse has a segmental trisomy for a region of mouse chromosome 16 (MMU16) that exhibits perfect conserved linkage with distal HSA21 and develop a range of phenotypes that are analogous to those seen in DS. Ts65Dn mice show developmental delay during the postnatal period as well as abnormal behaviours in both young and adult animals and age-related degeneration of septo-hippocampal cholinergic neurons and impairment in hippocampal long term potentiation. It has been shown that intrastriatal cholinergic system does not show gross morphological alterations in these mutant mice. Because it is possible that functional alterations of cholinergic transmission are present in trisomic animals even if the interneurons are spared, we performed current clamp intracellular recordings from different striatal neuronal subtypes (spiny neurons MS, FS interneurons and Ach cholinergic interneurons) showing that the electrophysiological properties of the striatal neurons recorded were similar in Ts65Dn mice and their respective WT. To characterize the pre-synaptic modulation of glutamate release we performed extra- and intracellular recordings using different drugs with pre-synaptic sites of action. Interestingly, a dose-dependent reduction of the field potential/EPSP amplitude by the non-selective muscarinic receptor agonist, muscarine, was found in both groups. However, the ACh-esterase inhibitor neostigmine was able to induce a lower decrease of field potential/EPSP amplitude in the Ts65Dn compared to WT, and this effect was not due to genotypic differences in release probability, as shown by similar neostigmine-induced increase of paired-pulse ratio (PPR) in both groups. Although striatal MS recorded from Ts65Dn and WT mice did not show significant differences in both form of synaptic plasticity (LTP and LTD), HFS protocol failed to induce LTP in the Ach interneurons recorded from Ts65Dn mice but not from WT mice. This effect was also associated to morphological changes on Ach interneurons, in mutant mice. The FS interneurons did not show any form of synaptic plasticity in both groups of mice. Taken together these data suggest the presence of functional alterations in the presynaptic cholinergic transmission that might be functionally linked to the absence of a normal synaptic plasticity in the Ach interneurons of the Ts65Dn animals.

## CR8 CASPHAR

### Introduction

Presynaptic G-protein coupled receptors are modulated by various mechanisms. These regulatory mechanisms involve molecular machinery resulting from interactions with associated proteins. At a presynaptic level a deubiquinating protein USP14 has been reported to show an overlapping expression pattern with a GPCR (GABAb, subunit GB1), suggesting a role for deubiquitination in the nerve terminal in regulating receptor activation.
USP14 has overlapping expression pattern with GABAb receptor and both proteins co-localise at the synaptic terminals. Interestingly enough the natural mutant mice that are deprived from expression of the functional USP14 have strong presynaptic phenotype resulting from malfunctioning of the neurotransmitter release or its control. Thus, the role of USP14 in the process of synapse development and regulation is explored.

### Activities carried out and results

CR8 CASPHAR was involved in workpackage 1 (task 1.3), WP3 (task 3.4) and WP6 (task 6.2).

The aim was to investigate the role of a deubiquitinating enzyme USP14 in synaptogenesis. The mice lacking this enzyme have strong phenotype that was located to be predominantly of pre-synaptic origin. Moreover, we detected USP14 as an interacting protein with GABAb receptor GB1 subunit intracellular portion.
For the studies of USP14 role in synapse formation we developed several. First we designed antigens and produced fusion proteins with GST. These were administered in Biotest into rabbits and animals with positive screen on presence of antibodies recognizing the USP14 on immunoblots were bled and the sera were further characterized. Next we prepared set of constructs for biochemical studies. We also started yeast two hybrid screen against the USP14 itself. From this screen one candidate aroused. This molecule was identified, as Filamin C. Further biochemical verification of this protein-protein interaction however did not confirm this association, however. In search for other possible targets of pre-synaptic machinery involved in synaptogenesis we performed also yeast two-hybrid screen against the C-terminus of Cannabinoid CB1 receptor C-terminus. We identified several interesting candidates, most notably the Dynein light chain. Interestingly enough, our partner also detected this molecule as possible interactor with large pre-synaptic structural protein bassoon. The search for other possible target can be viewed as deviation from the original proposal in terms of procedures but meet the goals and fit the project with same integrity.
In agreement with the proposed experiments, animals with natural mutation of USP14 were obtained and the screen for wild type/heterozygots/ homozygots was set up. This PCR screen is based on detection of a cassette that in the mutant mice is introduced into the gene. Liability of the screen is crucial for our future plan that is distribution of the mice between the partners for collaborative effort. We started to do constructs of USP14in the lentiviral vectors with the aim to deliver in vivo various constructs into neurons. The lentiviral-based deliver vectors belong to method of choice lately for their high efficiency and low cellular toxicity. The use of these vectors is planned to be in delivery of mutant cDNAs for the USP14 in vivo in experiments aiming at re-introduction of the enzyme into deficient neurons.
Primary fibroblasts were derived from the USP14 deficient mice and are now available in our laboratory. These cells are to be used in functional assays where the interference with internal pool of naturally occurring enzyme interferes with the experiments.

The Laboratory was transferred to a new institute at the end of the year 2006 and beginning of 2007, as Dr. Blahos got a position in an Institute of Molecular Genetics, Academy of Science of Czech Republic. We are in much better equipped environment with access to wide spectrum of novel services (monoclonal antibodies semi-automated production, transgene unit, cryobank etc.). This move unfortunately meant unavoidable time lost. The scientific progress in the whole scope of this project will be balanced in this regard. The new animal facility was finished for the new institute and we prepare to move the USP14 deficient mice strain in there any time

after its approval. Within the new facility we have access to Cell'R Olympus fluorescent microscope system with controlled temperature and CO2 levels. This equipment will allow us to perform long term experiments with tagged proteins expressed in cells. We therefore prepared constructs of tagged GABAb subunits tagged with fluorophores in lentiviral expression system that is now used for transfection of primary neuronal cultures. The experiments are designed with the aim to compare distribution and mobility in cebellar neurons derived from wild type and the USP14 deficient mice.
The distribution of the GABAb receptor in the brain sections of the USP14 deficient mice is studied in the mean time. For this we prepared set of polyclonal antibodies recognizing the GABAb subunits and their splice variants. Also monoclonal antibodies that recognize GB1 and GB2 subunits are now under preparation for this purpose. In the control experiments (section from the wild type animals) the USP14 recognizing antibodies will be used to estimate the overlap with the GABAb receptor distribution. Within the new facility, we set up fluorescent Ca imaging. This is to be used for measurement of the GABAb responses. We will use the chimerical Gqi protein as an adaptor between the GABAb receptor that is Gi/o coupled and the Phospholipase C pathway. This approach we used successfully in our previous studies.

Besides the above mentioned antibodies, we were also involved in the antibody production that takes part mainly in Biotest for other partners. We tested and purified several antibodies both polyclonal and monoclonals for several partners from the Synscaff consortium. The antibodies are listed separately.

## CR9 BIOTEST

### Introduction

According to the general descriptions of WP8, we produced immunised sera. The immunizations of selected animals have already been started in the first year of the project. That is a positive outcome of intensive collaboration with our partner CASPHAR. The antigens have been designed in collaboration with our partners as well as species of immunised animals (rabbit or guinea pigs) were chosen according to the requirements of our partners.
Using the expertise of the other partners basic techniques, which would enable testing the sera for its antigenity and specificity, these were introduced in Biotest s.r.o. The sera were produced, based on the requirement of the consortium members, and sufficient amount of the product was made available to the consortium.

### Activities carried out and results

Biotest was involved in activities under workpackage WP 8, tasks 3 and 4.

During the first reporting period, we started to select rabbits and immunize them with peptides corresponding to portions of target proteins. These proteins are either synaptically localized receptors or their scaffolds.
Namely we injected rabbits with fusion proteins or synthetic peptides directed against portions of metabotropic glutamate receptors (mGluR1 splice variants, mGluR 5 etc.).
GABAb receptor GB1 and GB2 recognizing antibodies were made successfully. Antibodies recognizing C-terminal portion of CB1 cannabinoid receptor that is localized presynaptically were developed. Antibodies that recognize USP14 on western blots from transfected HEK 293 cells were also developed with partner 8. Scaffolding proteins such as Shank were designed with C. Sala were synthesized and the rabbits were immunized. We started to prepare new approaches such as genetic immunization in collaboration with partner 2. This approach was taken for long lasting problems with development antibodies against Kainate receptor subunits. We were involved in the antibody production for other partners. Immunization of both, rabbits and guinea pigs, was routinely processed in order to obtain antibodies from the two species. They tested and purified several antibodies.

### Immunization protocol

The antigens are delivered to the immunised rabbits subcutaneously on the animal's back in a scheme that uses two recurrent injections in a span of three months. After two months, the sera are tested again using small amounts of blood samples and the fate of each animal in the experiment is decided. Should the results look promising, more antigens (half of the first injection amount) are distributed subcutaneously. At the third month, the testing of animals is done again and the animals giving positive results are bleeding by cardiac puncture and later the immune reaction is boosted again. Such animals are kept for later bleed, usually for one in a month's time. This scheme is kept until the titer of the antibody is satisfactory.
The second option is to send obtained serum to our partners and to follow their instructions.

### Preparation, testing and delivery of the antibodies

The obtained blood is left to coagulate naturally and the sera are prepared later by centrifugation. These sera are divided into two portions. A small amount is preserved chemically and stored at 4° C for immediate use for testing and the rest is frozen in small aliquots for later usage. Testing of the antibodies is done on immunoblots against protein samples of mammalian cells transfected with the cDNA coding for the original protein from which the antigen was derived and also against samples of brain homogenate of several species and against another organ (liver). Protein samples are made in collaboration with CASPHAR.

**CR10 CFc**

**Introduction**
CR10 CFc, based on its experience, was involved in the SYNSCAFF project to give a concrete and complete support to:

- organisation of research project meetings and workshops;
- support to transfer of knowledge within and outside the consortium;
- dissemination process targeted to health authorities and politicians and public aimed to enlarge the awareness at European level on the project's results;
- knowledge management and quality assurance, in order to ensure the proper information availability for all partners and the correct activities' progression. Knowledge management in particular the creation of a documentation aimed to describe research project flow, activities' correlations, milestones & deliverables;
- design and maintenance of project websites;
- organisation of training activities.

**Activities carried out and results**

CR10 CFc contributed to WP9 and WP10.

As for WP9, we contributed during the first reporting period to the creation of the dissemination plan and the maintenance of the project website. During the second reporting period, a Final Plan of using and disseminating knowledge has been prepared (D9.2).
A report on raising public participation and awareness, demonstrating the extent to which actors beyond the research community have been involved to help spread awareness and to explore the wider societal implications of the proposed work has been also produced in the reporting period (D9.3).

As for WP10, we contributed to several tasks

- **Task 10.2:** Logistics

We contributed to the organisation of the meetings held all over the project duration. A section was always foreseen for separate discussion of administrative and financial aspects.

- **Task 10.3:** Legal and administrative issues

During the project, we participated to the short visits to update the European Commission on the project developments. We also supported the Coordinator in the collection of necessary documents for the two contract amendments requested during the first reporting period.

- **Task 10.4:** Reporting

We supported the Coordinator in the monitoring of project activities and in the collection of documents necessary for reporting.
An internal check of activities and expenses has been carried out every six months (month 6, 12, 24, 30). Also in these cases, CFc supported the Coordinator in the collection of material.
Collaboration with the administrative contact person of each contractor organisation has been also established in this context.

- **Task 10. 6:** Harmonisation of procedures and documentation

CR10 CFc created templates, forms and modules to be used by each participant for reporting activity (both scientific and administrative/financial), e.g. common format for all project deliverables, interim cost statements forms, time-sheets, audit certificates guidelines, etc.

This documents facilitated the reporting tasks of the partners and ensured an homogenous methodology of reporting. Each partner transmitted them to the administrative people in charge of the project.
All the templates are available on the partners' area of the SYNSCAFF project web-site.

- **Task 10.7:** Internal monitoring and risk analysis

During the start-up meeting in Milan, the CR 10 CFc's presentation introduced the participants to EU project management aspects, including main requirements and provisions (timeliness, progress and efficient deployment of resources and other important management issues). Risks and the EC review procedure was described and the role of the workpackage leader emphasised. The main issues of the consortium responsibilities (quality of work, participation to meetings, deliverables and report, justification of costs) were described. The management structure and roles were clarified and management tasks illustrated in details. Full details about the envisaged meetings and reports (both activity and financial reports) and their time schedule as required by the EC were given. Similarly deliverables and milestones were highlighted, with particular reference to the importance of complying with the envisaged schedule. The internal monitoring procedure was described (including the role of CR 10 CFc). The role and characteristics of the official national auditor for each partner in FP6 was clarified, including the supporting documents needed for carrying out statutory audit and to be kept at each contractor's offices. Publication procedures to be followed according the EC rules were described (including mention of the EC support through the SYNSCAFF contract). The project financial aspects were fully clarified (max EC contribution, modalities of pre-financing, procedure to keep the financial documents). A definition of eligible and non eligible costs according to the EC rules were described, together with cost models adopted by the partners). Main eligible costs items (personnel, travel, subcontracting, other specific costs) and related supporting documents were analysed.

Ethical procedures to be followed according to the contract were also described.

During the following periodic meetings, specific questions were made by the partners, in order to spread to all the partners information about the EC financial reporting, on topics of specific interest to the partners. In most cases, the replies from the EC officers to specific queries posed by the partners were communicated. During the same meetings basic information about the financial management of the project were given by CFc during the plenary session.

A tutorial on most common mistakes during the first exercise interim report was made, so to acquaint the partners with the EC forms. Again, supporting documents for each categories of costs were listed in order to facilitate the auditing process.

During Prague review meeting (May 2006) the results from the interim report at month 12 were analysed as far as the financial and man months situation is concerned. The presentation focused mainly on the financial and periodic activity report.

At any meeting corrective actions have been suggested to each partner whenever necessary and support was given as far as reporting procedure and eligibility of costs is concerned.

During all the period, CR 10 CFc provided an helpdesk for continuous assistance on any matter related to the implementation of the contract (by e-mail, phone and also organising meetings whenever necessary), liaising between the partners, the Project Coordinator and the European Commission.

CR10 CFc has been charged of internal financial audit all over the project duration. Interim financial reports at month 6, 12, 24 and 30 have been collected in order to monitor the status of the project expenditure.

- **Task 10. 9:** Financial external audits and certification

CFc supported the Coordinator in the monitoring of the appointment of qualified national auditors to evaluate expenses, justification of costs and to prepare audit certificates to be submitted to EU together with the annual financial report.

**2. Dissemination and use**

Not applicable