SUMMARY OF THE MAJOR PROJECT ACHIEVEMENTS SINCE THE START OF THE PROJECT

Describe what you would consider to be the most outstanding or more particularly significant outcome of the work performed during the period covered by this report, in terms of scientific/technological results, research training methodologies, opening up of career opportunities to researchers, international networking of the concerned scientific community, etc.

NP01 -Knipper/Engel
During the first period of Cavnet funding, we have established new methodology for analysing and understanding the role of Cav1.2 channels in hearing and auditory pathology. Conditional Cav1.2 mice with deletion of Cav1.2 in Pax2 locus are generated and are in cooperation with P05 under investigation (MS in preparation). The role of VGCC channel subunit alpha2delta3 for hearing is under investigation using a new mouse model (Jackson Laboratories) deficient for a particular isoform of the auxiliary alpha2delta VGCC subunit, alpha2delta3, termed CACNA2D3-/-. Cooperative work with P05, P02 will be included. The elucidating of the role of the Ca2+ channel subunit alpha2delta3 in auditory processing promises novel insights in the function of Ca2+ channel complexes (MS in preparation).
A double labeling method for simultaneous detection and differential expression analysis of Cav1.3/Cav1.2 mRNA and protein was established and through labrotations tranfered to most of the Cavnet partners. Moreover in cooparation with P09 modulation of Cav1.3 through Rim2 was successfully investigated (MS in preparation).
Aiming to analyse the function of L-type Ca-channels for BDNF expression changes during environemental enrichment an in vitro system (CFP-YFP-BDNF) and in vivo system (conditional BDNF mice) is under investigation. Fellows were successfully trained in gene cloning, generation of riboprobes, in situ hybridization, Western, generation of fusion proteins, cell transfection, ELISA and recording of Ca2+ currents in immature and mature hair cells. Fellows also have are trained during a curriculum (4h/week during term).

NP02 -Bartsch
Aiming to generate conditional alleles of the CaV1.3alpha (Cacnad1) gene for molecular, pharmacological, physiological and behavioral analysis of the channel function in different tissues two different strategies are used. (1). The FLEX strategy generating, upon cre recombination, gene replacement of the CaV1.3alpha allele by the green fluorescent protein (GFP). This strategy allows direct visualization of CaV1.3alpha knockout in targeted tissue. (2). Conditional, loxP flanked (floxed) allele of CaV1.3alpha. This allele does not allow direct positive identification of cells with CaV1.3alpha knockout, but requires antibody staining to determine the extent of CaV1.3alpha ablation. All conditional alleles are available for the member of the CAVNET consortium and represent common tool for research in the consortium.
In collaboration with P012 HN, P05 EF, P01 EK, a Task5-cre transgenic mice is in the process to be generated aiming cell specific deletion of L-type Ca-channels within the auditory tract. The construct is ready and ES injection has started.
In collaboration with P09 a GFP - Cav1.3 reporter knockin mice is generated.
ESR, Siri Malmgren was trained on characterization of transgenic rats with genetically modified Cav1.3 expression, including the establishing of learning and memory test, Object Recognition Test, examination of tamoxifen-induced behavioral changes as well as analysis and presentation of behavioral data. In September 2008, Dr. Anita Hansson (Experience researcher) joined our group. In the first months, Dr. Hansson started with in-situ hybridizations to quantify CaV1.3alpha #expression in knockout and transgenic rats.

NP04 -Carbone
Since the beginning of the CavNET project we have been in close contact with the Innsbruck group (Prof. Joerg Striessnig) and worked on the role of Cav1.3 and Cav1.2 channels in WT and Cav1.3-/- mice in the pace-maker current of mouse chromaffin cells. The group also helped us with the qRT-PCR analysis of the Cav1.2 and Cav1.3 mRNA expressed in WT and Cav1.3 KO mice. A manuscript related to the contribution of Cav1.3 and Cav1.2 to the pace maker current responsible for the spontaneous firing of mouse chromaffin cells has been submitted for publication. Two other papers are now in preparation related to the same aspect. One dealing with the role of L-type channel

in setting the firing mode of cultured hippocampal neurons and one on the role of Cav1.2 and Cav1.3 on the exocytosis and endocytosis in mouse chromaffin cells.
We also started a strict collaboration with the Montpellier group (Dr. Matteo Mangoni) on the electrophysiological characterization of the contribution of Cav1.3 and Cav1.2 channels in setting the pace-maker current of mouse sino-atrial nodes (SNAs). The work is still in progress and concerns a direct interaction between Pietro Mersica (ER, Montpellier) and Andrea Marcantoni (postdoc, Torino).
In collaboration with P01 the degree of co-localization of Cav1.3 and BK channels in MCCs on mouse chromaffin cells is under investigation. In cooperation with P01 and P08 a transfection method for over expression of Cav1.2 and Cav1.3 in MCCs is developed. Related to the tight coupling between L- and BK channels there is now growing interest to start a project using BK KO mice available at Tubingen in Prof. Peter Ruth Lab.

NP05 -Friauf
Dr. Bohumila Tarabova (ER), recruited in 2008, has embarked on the characterization of voltage-gated calcium currents in neurons of the lateral superior olive (LSO) in the mammalian auditory brainstem. Aiming to elucidate why LSO neurons do not develop properly in mice lacking functional Cav1.3 channels, the nature and repertoire of the calcium channels and the quantification of the amount of current mediated through these channels in neonatal (P3), young (P12) and young adult animals (P30) is under investigation.
Trained to do whole-cell patch-clamp recordings in acute slices, BT has identified functional L-type channels in every LSO neuron of both wild-type and Cav1.3-/- mice. In wild-type animals, these L-type channels contribute by about 75% to the total amplitude of voltage-gated calcium currents. Likewise, we did not observe any age-dependent changes in the contribution of L-type channels. In Cav1.3-/- mice, the calcium currents were of significantly smaller amplitude than those of wild-type mice and are most likely mediated through Cav1.2 channels and/or channels other than L-type. In cooperation with P01 and P09 quantification of Cav1.3 channels in LSO is ongoing and a crucial role during development of synaptic circuitry and also in later life is under investigation.
The question if the developmental aberrations (morphological and physiological) that we observe in the LSO of Cav1.3-/- mice are the result of missing activity normally originating in the cochlea or the result of direct activity changes in the LSO will be adressed in conditional Cav1.3 animals with deletion of hair cell L-type (P01) or conditional with deletion of L-type in the auditory tract (P02 and P12).

NP06 -Lacinova
In collaboration with NP01 block of the CaV1.3 channel in inner hair cells by verapamil, gallopamil and diltiazem was characterized (MS is published in Eur J Pharmac).
The contribution of the Cav1.2 L-type calcium channels (LTCC) to hippocampal excitability was investigated in mice with a hippocampal knockout of the CACNA1C gene. Increased threshold for repetitive action potentials firing and lowered initial spiking frequency was observed.
Two models of neuronal excitability, PC12 cell line and primary culture of hippocampal neurons, were established. PC12 cells treated with NGF differentiated into nerve-like cells and cell surface and amplitude of calcium current increased significantly. PCR analysis done with NP01 proved the presence of mRNA for both CaV1.2 and CaV1.3 channels.
ER and ESR fellows learned the patch clamp technique, culturing of PC-12 cells and preparation of primary hippocampal cells. In collaboration with NP01 students learned the analysis of gene expression using PCR method and detection of mRNA and protein in sections. Anton Caro extended his proficiency in patch clamping in NP05.
Marianna Zana (an alumni) enhanced her potential for participation in international research. Anton Caro is working towards his PhD, for which he passed several mandatory examinations.
P06 in collaboration with P09 organized minisymposium "Role of L-type calcium channels in cellular excitability" during Joint Meeting of The Slovak Physiological Society, and The Physiological Society and FEPS, in September 2007 in Bratislava. In this minisymposium P01, P06 and P09 presented their results.

NP07- Mangoni / Nargeot
ER Pietro Mesirca has evidenced a critical role of Cav1.3 channels in pacemaking of atrioventricular node cells. Description of this unique role for Cav1.3 channels is now being implemented by ESR Angelo Torrente thanks to collaboration with partner P01. A second striking finding of Pietro

Mesirca's work is that Cav1.3 channels play an unexpected role in the muscarinic regulation of automaticity in primary pacemaker cells. Contrary to expectations, Cav1.3-/-/Kir3.4-/- pacemaker cells can respond to ACh only at very high concentrations. This finding raises the interesting hypothesis that Cav1.3 channels can influence intracellular basal cAMP concentration. ESR Angelo Torrente has identified a crosstalk pathway between Cav1.3 channels and intracellular Ca2+ release in primary pacemaker cells.
These important findings will fertilize research in our but also Cavnet partner groups during the next years. During these months of work, our fellows have been trained to perform patch-clamping, confocal imaging of intracellular Ca2+ dynamics, immunohistochemistry, MEA technology and PCR. In particular, collaboration with P04 is allowing ER Pietro Mesirca to open the possibility of studying cardiac pacemaker activity by a to-date unexplored technique (MEA technology) and to take advantage of the experience of P04 in action potential clamp experiments.
ESR Angelo Torrente had the possibility of learning fast Ca2+ imaging by confocal microscopy with Dr. Ana Gomez (Inserm U637, Montpellier), who is a leading expert of this technique.
On-going collaboration with P01 laboratory will allow Angelo Torrente to greatly benefit of the experience of this partner with immunohistochemistry and mRNA detection. He also started electrophysiological experiments in our lab to correlate Ca2+ signalling to electrical activity of the cell.

NP08 -Renström
During the first half of the CavNet, the Lund group has entered a new scientific community and found extensive new collaborations within the network. Intensely technical and scientific exchange exists with most partners (P01, P02, P04, P06, P09, P11). Successful method transfer for detection of Cav channels and their subunit is achieved in cooperation with P01, P09) for Cav trafficking studies. Collaborations with P12 have started to design studies on the role of ER in pancreatic beta-cell stimulus secretion coupling. Collaborations with researchers outside the CavNet (Anette Dolphin) have also been initiated.
This has promoted scientific advances in the areas of redox-regulation of Cav channels, Cav channel trafficking, and genetic studies of Cav-related genes in type 2-diabetes.
The participation in the CavNet has provided excellent technical training for the fellows, as well as enhanced in-lab training procedures. Soft skills and presentation abilities have also been boosted by the network. Careerwise the CavNet has been instrumental for both the PI and the ER and ESR fellows. For the PI (Erik Renström) it has facilitated extended networks science, as well as promoted his standing at home in Lund University, where he since the start of the project has become tenured and promoted to full professor. For the ER (Enming Zhang) CavNet has offered a possibility to enter the international scientific arena and establish himself as an independent researcher. For the ESR (Thomas Reinbothe) CavNet has meant faster scientific maturation and opportunities to develop his leadership skills. In summary, CavNet has and will mean a lot to these individuals in years to come.

NP09 -Striessnig
In collaboration with NP01 we could demonstrate that the two C-terminally spliced forms of Cav1.3 known so far exhibit different Ca2+- and voltage-dependent gating kinetics and are differentially expressed in different tissues, including cochlear inner hair cells (published PMID 18482979). Morevover, in cooperation with P01 the technique of differential expression of Cav1.3 C-terminal splice variants in the mouse brain with in situ hybridization was established and the presynaptic protein Rim2 was described to slow both Ca2+- and voltage-dependent inhibition of Cav1.3 (MS in preparation).
Together with NP-04 we could establish an important role of Cav1.2 and Cav1.3 for the pacemaker current underlying spontaneous action potentials in mouse chromaffin cells, an observation that was validated upon quantification of Cav1.3 and Cav1.2 in either mouse adrenal medulla and adrenal cortex (Cooperation with P04, P01; MS in NP-04 lab in preparation; see also Abstract, Neuroscience Meeting 2008).
The ER Jelka Cimmermann established non-radioactive in situ-Hybridisation (ISH; riboprobes) to identify adult Cav1.3 splice variants in normal mouse brain as well as in mouse models of anxiety and depression. Aiming to generate a GFP-reporter Cav1.3 mice for the use of all Cavnet, Jelka moreover was trained in the group of NP-02 in Mannheim for several months in state of the art recombineering (recombination-mediated genetic engineering) techniques. A genomic BAC clone containing the Cav1.3 coding exon/ intron 1-3 was obtained and a eGFP:Cav1.3 fusion gene with a NEO expression cassette was generated.

NP11 -Verkhratsky
We have engineered novel ER-targeted GFP constructs, (i) the ER-targeted fluorescent marker pIN-KDEL, and (ii) the photoactivatable probe LV-PA-pIN-KDEL into a 3rd generation replication-defective, self-inactivating lentiviral vector system capable of mediating gene transduction in diverse dividing and post-mitotic mammalian cells, including neurones. We demonstrated the ability of this vector to transduce astrocytes and neurones in culture and in cortical explants. Using these constract in combination with imaging approaches (photoprecovery after photobleching and photoactivation) we reveal the continuous nature of the ER lumen in neural cells, presenting the first direct evidence of an astrocytic ER lumenal continuum and providing more data to support the existence of a single ER lumen in neurones.
A second line of investigations was dedicated to studies of functional expression of ryanodine receptors/intracellular Ca2+ channels in neurones in triple transgenic model of Alzheimer disease. We found that the expression of RyRs/Ca2+ channels was significantly increased in dendritic compartments of hippocampal and cortical neurones at very early stages of AD, before investment of the brain parenchyma with #-amyloid plaques.

NP12 -Nothwang
In close collaboration with P02 and P01 a construct for generation of a mice for inducible ablation of LTCCs in the central auditory system under the promotor of TASK 5 is in the process to be generated. The ESR Mehmet Oktar Güloglu was trained to all basic and progressed molecular biological techniques required for the generation of the construct and succeeded to finish the construct in close cooperation and continuous exchange and advice with P02. As described in the last report in detail the construct is now finalized and sent to P02 (DB) for pronuclear injection. The ES injection has been performed.
The mice will be used in a first step in cooperation with P01 and P04 for deletion of floxed Ca-channels (Cav1.2, Cav1.3) in the auditory tract. Here in particular in the near future synaptic proteins that participate in Ca-regulated exocytosis and Depolarisation-Hypopolarizing Switch of GABA in the brainstem (KCC2) are of interest. Publications are expected to come out in the second period of the Cavnet with several cooperation partners.