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Improvement of the research competitiveness in neuroscience
at the Ernst-Moritz-Arndt University of Greifswald

# Transport Processes in Neurodegenerative and Neuromuscular Diseases

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# Welcome to Greifswald!

*Heinrich Brinkmeier, Project Organisation*

*Silke Vogelgesang, Project Organisation*

*Christine Poehlke, Project Assistance*

Dear Colleague,

it is our pleasure to welcome you to Greifswald, a small but vivid university town at the beautiful shore of the Baltic Sea. The Ernst-Moritz-Arndt University is one of the oldest German universities and at the same time it developed to one of the modern ones during the last two decades. Biomedical research is one of the highlights in Greifswald, based on research in the Science Faculty, the University Medicine and the brand new University Hospital. We are delighted that you have joined us for the International Workshop on "Transport Processes in Neurodegenerative and Neuromuscular Diseases". In the next two days we are looking forward to exciting presentations and fruitful discussions on the pathophysiology of neurodegenerative and neuromuscular disorders. Topics will range from basic research to molecular therapies entering clinical studies. We thank the European Union for supporting our work with a grant committed to improve the research capacity in neuroscience.

Enjoy your time in Greifswald.

**Silke Vogelgesang, Heinrich Brinkmeier and Christine Poehlke**

## Corruptive protein seeding in the pathogenesis of neurodegenerative disorders

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The accumulation of specific misfolded proteins in the nervous system characterizes a variety of neurodegenerative disorders, including Alzheimer's disease and the prion diseases. Experimental evidence increasingly supports the hypothesis that the aggregation of susceptible proteins can be 'seeded' by corruptive protein templating, a process in which the normal protein is induced to misfold by interacting with abnormal proteinaceous seeds from affected brains. We have found that the self-assembly of Aβ, a key player in the pathogenesis of Alzheimer's disease, is seedable in the brains of β-amyloid precursor protein (APP)-transgenic rodents via the introduction of brain extracts rich in aggregated Aβ. Seeding occurs only if aggregated Aβ is present in the extract, and the characteristics of both agent and host govern the phenotype of induced protein deposition. In seeded mice, Aβ deposits occur mainly in the injected region, but Aβ-plaques and cerebral Aβ angiopathy also arise in non-contiguous brain regions, implicating neuronal and/or vascular transport mechanisms in the spread of the agent. We also have found that peripheral (intraperitoneal) administration of Aβ-rich brain extracts from APP-transgenic donor mice precipitates cerebral Aβ deposition in APP-transgenic recipient mice, though the means whereby the seeds reach the brain from the peritoneal cavity remain uncertain. Together, these studies suggest mechanistic similarities between Aβ-proteopathy and the prionoses, and indicate that corruptive protein templating is a possible molecular mechanism for the instigation and spread of Alzheimer-type pathology in the brain. The existence of aberrant proteinaceous assemblies in other neurodegenerative and systemic disorders argues that this mechanism is likely to be operational in a broad spectrum of diseases. Supported by the CART Foundation, NIH RR-00165, P01AG026423, P50AG025688, and University of Kentucky Faculty Support Grant 1012101660.

## Identification of pathways driving prion pathogenesis

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Prion diseases are a group of neurodegenerative protein-misfolding diseases involving the conversion of the cellular prion protein, PrPC, into the disease associated form PrPSc. We have recently established a model of prion infection in organotypic cerebellar slice cultures that allows us to study prion replication in a complex cellular model within a reasonably short timeframe (2-8 weeks) (Falsig et al. 2008a). We can now show that prion replication within tissue slices induces an innate immune response, astrogliosis, microgliosis, intra-neuronal vacuolization (spongiosis) and causes a dramatic loss of NeuN and Parvalbumin-positive neurons 6-8 weeks post-inoculation.
We have used this model to investigate the mechanism by which prions kill neurons. We find that neurodegeneration occurs in a caspase-independent manner and that the cell death pathway is calcium regulated. Prion replication induces increased production of reactive oxygen species and antioxidants are neuroprotective in the model. Using different biochemical approaches we have identified the NADPH oxidase complex as being a regulator of prion toxicity. We are still trying to elucidate the interplay between these two pathways.
Microglial inflammation has been implicated as a causal factor in neurodegenerative diseases. To mechanistically dissect the contribution of microglia to prion pathogenesis, we have established slice cultures from CD11b-HSVTK transgenic mice, allowing for the ablation of microglia in vivo and in vitro (Heppner et al. 2005). Depletion of microglia had no effect on the viability of non-microglia cells in brain slices. Furthermore, microglia depletion led to an increase in prion titers and the deposition of PrPSc in infected tissue, suggesting a protective function of microglia (Falsig et al. 2008b). We now find that depletion of microglia leads to dramatically enhanced prion neurotoxicity, suggesting that overall microglia is neuroprotective in prion-infected tissue. This observation has now been confirmed in vivo.

## Imaging structural and functional alterations in AD mouse models

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The neuropathological hallmarks of Alzheimer's disease (AD) are extracellular deposits of the amyloid beta (Aβ) peptide (amyloid plaques), intracellular accumulations of the tau protein (tangles) as well as neuronal death. The best correlate for the cognitive decline however is the loss of synapses, which is observed in AD patients and in animal models of the disease. Aβ is known to have detrimental effects on synapses, which are thought to be causally related to the cognitive deficits in AD. In order to provide an effective therapy, it is essential to understand the pathogenesis of the disease. Therefore, using mouse models of Alzheimer`s disease, we visualize amyloid plaque development and the morphological neuronal changes associated with it in vivo with 2-photon microscopy. Furthermore, we investigate the mechanisms underlying these neuritic changes and test the idea that they might have the potential to recover after treatments. One promising therapeutic approach to combat AD is to reduce or prevent Aβ production. By combining biochemical analyses with in vivo 2-photon microscopy we are able to monitor the impact of treatment on the deposition of new amyloid plaques as well as on dendritic spines.

## Blood-brain barrier P-glycoprotein: a new target for Alzheimer's disease?

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One hallmark of Alzheimer's disease (AD) is accumulation of neurotoxic amyloid-β (Aβ) in the brain. The mechanistic basis for this pathology is unknown, but reports indicate that elevated Aβ brain levels are in part due to a failure in clearing Aβ from the brain. Aβ brain clearance is a two-step process: Aβ? must first pass through the abluminal plasma membrane and then the luminal membrane of the brain capillary endothelium. Since Aβ is a peptide, both steps must be facilitated. At the abluminal membrane, LRP appears to be responsible for the first step of Aβ uptake. Our data suggest that the second step in clearing Aβ from the brain is mediated by P-glycoprotein (P-gp) suggesting P-gp plays an important role in AD pathology.
We show that P-gp transports Aβ from brain capillaries into the vascular space. We also demonstrate that P-gp is reduced in brain capillaries from Aβ-overproducing hAPP mice suggesting a link between high Aβ levels and reduced brain capillary P-gp in AD. Importantly, using hAPP mice we show that restoring P-gp expression and transport activity in brain capillaries significantly reduces Aβ brain levels within one week. Thus, restoring blood-brain barrier P-gp has the potential to increase Aβ clearance and reduce Aβ brain accumulation. This mechanism could potentially be used as a new therapeutic strategy in Alzheimer's disease.

## ABC-transporter at the blood-brain barrier: Impact for risk and therapy of neurological disorders

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The ATP binding cassette (ABC) membrane transporter P-glycoprotein (P-gp, ABCB1) is one of the best characterized human efflux transporters. At the blood–brain barrier ABCB1 acts as defense mechanism against damages of the brain by toxins and appears to be also most important regarding drug penetration into the CNS. Inhibition of P-glycoprotein or down-regulation after inflammatory stimuli led to a dramatic increment of typical substrates in the liquor. Accordingly, the anti-nociceptive effects of opioids increased after inhibition of P-gp in certain trials. Over-expression of P-gp however is hypothesized as one cause of drug resistance in the treatment of epilepsy, but pharmacogenetic association studies failed to explain interindividual differences in therapy response. Recent data however indicate an additional significance of ABCC2, being responsible for the transport of drugs such as carbamazepine rather than ABCB1. Interestingly some studies reported an impact of ABCB1 genetic variants also to therapy response in the treatment of depression with drugs such as venlafaxine, being an ABCB1 substrate, these data need to be confirmed. Moreover, there is some evidence that ABCB1 is involved in the clearance of cerebral amyloid beta peptide at the blood brain barrier (BBB), thereby possibly contributing to the risk of developing Alzheimer disease. Our own data indicate a role of ABCB1 genetic variants in carriers of ApoE ε4 negative genotypes, an established risk factor of AD. Especially the variants 2677G>TA and 3435C>T may influence the risk of increased Aβ-load and therefore the risk of AD. Moreover, the impact of these two variants is strengthened by the important risk factor age.
Overall, ABC efflux transporter plays an important role in drug distribution to the central nervous system but also for endogenous substances. Genotyping of genetic variants allows the explanation of certain interindividual differences in drug-response, it should be taken in mind however, that further factors lead to a broad inter- and interindividual variation of ABC transporter expression and function.

## Beta-Amyloid downregulates MDR1-P-glycoprotein (Abcb1) expression at the blood-brain barrier in mice

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The blood-brain barrier (BBB) protects the brain against endogenous and exogenous compounds and plays an important part in the maintenance of the microenvironment of the brain. In particular, the importance of brain-to-blood transport of brain-derived metabolites across the BBB has gained increasing attention as a potential mechanism in the pathogenesis of neurodegenerative disorders such as Alzheimer's disease, which is characterized by the aberrant polymerization and accumulation of specific misfolded proteins, particularly β-amyloid (Aβ). There is growing evidence that the ABC transport protein P-glycoprotein (P-gp), a major component of the BBB, mediates the efflux of Aβ from the brain.

To determine whether Aβ influences the expression of key Aβ transporters, we studied the effects of 1-day subcutaneous Aβ1-40 and Aβ1-42 administration via Alzet mini-osmotic pumps on P-gp, BCRP, LRP1 and RAGE expression in the brain of 90-day old male FVB mice and found significantly reduced P-gp, LRP1 and RAGE mRNA expression in mice treated with Aβ1-42 compared to controls while BCRP expression was not affected. The expression of the four proteins was unchanged in mice treated with Aβ1-40 or reverse-sequence peptides. These findings indicate that, in addition to the age-related decrease of P-gp expression, Aβ1-42 itself downregulates the expression of P-gp and other Aβ-transporters, which could exacerbate the intracerebral accumulation of Aβ and thereby accelerate neurodegeneration in Alzheimer's disease and cerebral β-amyloid angiopathy.

## The impact of ryanodine receptor (RyR1) mutations on orthograde and retrograde triadic coupling

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In the process of excitation-contraction (EC) coupling, the depolarization of the transverse tubules leads to conformational signal transmission to the ryanodine receptor RyR1 (termed “orthograde coupling”) followed by massive Ca release into the myoplasmic space that eventually causes contraction. Two functionally distinct classes of RyR1 mutations associated with central core disease (CCD) have been identified that either lead to overactive RyR1 channels (“leaky channel mutations”) or to channels with diminished Ca permeability (“EC uncoupling mutations”). Knock-in mice for each group have become available providing new options to study the effects of these mutations on the organismic and the cellular level.

Using electrophysiological techniques combined with fluorometric Ca recording we investigated isolated fibers of mice with the Y524S (YS/+) and the I4895T mutation (IT/+), corresponding to human Y522S and I4898T. The former has been identified as a “leaky channel mutation” whereas there is still debate on the functional changes induced by the latter. Studying voltage step-induced Ca release in muscle fibers, we found that Y524S caused a displacement of the voltage dependence of activation to more negative potentials with no change in maximal amplitude, whereas I4895T caused a decrease in release without alteration of its voltage threshold. These findings are in line with the suggested opposite molecular changes, i.e. higher and lower Ca permeation, respectively, induced by the mutations. In addition to the orthograde transmission from the DHPR to the RyR1, feedback signaling (termed “retrograde coupling”) from the RyR1 to the DHPR has been reported. YS/+ fibers showed a shift in the voltage dependence of inactivation to negative potentials, leading to lower amplitudes of both Ca release and Ca current compared to WT fibers at potentials more positive than the normal resting potential. This finding indicates a novel feedback mechanism that signals from the mutant RyR1 to the DHPR enhancing DHPR inactivation. The voltage-dependent characteristics of activation and inactivation predict a range of voltages in which Ca release is permanently activated at low level. In agreement with this prediction, we found a steady increase in the basal Ca concentration in a window of voltages. In the YS/+ fibers this window is broader and shifted to more negative potentials. A rise in temperature causes a further substantial shift suggesting a role of window Ca release in the heat-induced malignant hyperthermia observed in these mice.

In summary, the results demonstrate clear differences in voltage-controlled Ca release in mice expressing the YS and IT-mutation reflecting different mechanisms in affecting RyR1 function. In addition, they suggest that both hyperpolarization and a change in the characteristics of DHPR inactivation are protective mechanism to counteract the effects of hyperactive RyR1 channels.

## Strictly controlled $Ca^{2+}$ influx through TRPC3 channels has a key role in insulin-mediated glucose uptake in striated muscle

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$Ca^{2+}$ influx into striated muscle cells through canonical transient receptor potential (TRPC) channels has recently been implicated in the pathology of muscular dystrophies. For instance, overexpression of TRPC3 channels causes muscle defects similar to those observed in Duchenne's muscular dystrophy. We, on the other hand, have investigated tentative functions of these channels under normal physiological conditions and our results indicate that TRPC3 channels are of key importance for insulin-mediated glucose uptake in striated muscle. The basis of these studies is our initial finding that insulin causes an increase in the $Ca^{2+}$ concentration immediately beneath the sarcolemma in skeletal muscle fibres, whereas the bulk cytoplasmic $Ca^{2+}$ remains virtually unaffected. Subsequent studies in or laboratory were aimed at revealing the functional importance and the channels involved in this sub-sarcolemmal $Ca^{2+}$ increase. We then showed that: (i) insulin induces an influx of $Ca^{2+}$ and maneuvers that increases or decreases this influx also increases or decreases glucose uptake; (ii) the insulin-mediated $Ca^{2+}$ influx is smaller in insulin resistant muscle; (iii) insulin causes a translocation of TRPC3 channels to the surface of control, but not of insulin resistant, cardiomyocytes; (iv) TRPC3 and glucose transporters (GLUT4) are co-localized in skeletal muscle fibres ; knock-down of TRPC3 decreased the insulin-mediated glucose uptake in skeletal muscle fibres. To conclude, strictly controlled $Ca^{2+}$ influx through TRPC3 channels appears have an integral role in insulin-mediated glucose uptake in striated muscle and decreased fluxes through the channels are associated with insulin resistance. On the other hand, uncontrolled $Ca^{2+}$ influx through the same channels may trigger deleterious effects in muscle dystrophies.

## TRPC3 channel blockers, but not TRPC6 channel blockers, inhibit background calcium influx and modulate calcium transients in mouse muscle fibers

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We have recently shown that the cation channels TRPC3 and TRPC6 are expressed in mouse skeletal muscle. The channel proteins seem to be, at least partly, present in the sarcolemma of mouse muscle fibers (Krüger et al. 2008, Neuromuscul Disord 18:501). To investigate the functional roles of the two TRP channels, we studied the effects of TRP channel activators and blockers on $Ca^{2+}$ influx, intracellular $Ca^{2+}$ levels and transient changes of $[Ca^{2+}]i$ induced by $K^+$ depolarization. Quench of Fura-2 fluorescence was recorded in the presence of 0.5 mM manganese (excitation at 360 nm). Changes of cytoplasmic calcium were measured using Fura-2 and alternate excitation at 340 and 380 nm. The application of 25 $\mu$M hyperforin, a TRPC6 activator, caused long-lasting increases of $[Ca^{2+}]i$ in single interosseus muscle fibers. Pre-incubation with ML-9, a specific blocker of TRPC6, significantly attenuated the effect of hyperforin (increase in 340/380 ratio after 260 s: 0.032 ± 0.018, n=9 with 100 $\mu$M ML-9 vs. 0.076 ± 0.051, n=17 control). Background $Ca^{2+}$ entry, as tested with the $Mn^{2+}$ quench technique, was not affected by ML-9. 2-APB inhibited background $Ca^{2+}$ influx by more then 50% (n=44, $p<0.01$). The half time of decay of KCl induced calcium transients was as well significantly influenced by 2-APB (control vs. 2-APB; 3.61 ± 0.18 s vs. 3.18 ± 0.15 s; n=27, 31; $p < 0.05$). The application of Pyr3, a specific inhibitor of TRPC3, resulted in a marked inhibition of Fura-2 quench rate in the presence of extracellular $Mn^{2+}$ (control vs. Pyr3; 6.4 ±0.6 vs. 2.5±0.4 %/min; n=46, 46; $p<0.01$). These data indicate that both channels, TRPC3 and TRPC6 are functional in the sarcolemma of isolated mouse muscle fibers. However, TRPC6 does not seem to have an activity at rest, while TRPC3 contributes substantially to the background calcium influx of muscle fibers.

## Lack of TRPC1 channels impairs skeletal muscle regeneration.

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We previously showed in vitro that calcium entry through TRPC1 ion channels regulates myoblasts migration and differentiation by activating calpain, a calcium-dependent protease which in its turn is able to cleave MARCKS protein (myristoylated alanine-rich C-kinase substrate), an actin binding protein involved in focal adhesion. To explore, in vivo, whether the absence of TRPC1 channel impairs skeletal muscle regeneration, we used cardiotoxin injections to induce muscle injury in adult $TRPC1^{+/+}$ and $TRPC1^{-/-}$ mice. Interestingly, we observed that $TRPC1^{-/-}$ muscles showed a smaller fibre size and a decreased muscle specific force after 10 days and 14 days of regeneration respectively. Moreover, we observed an increased proportion of centrally nucleated fibres at day 14 of regeneration in $TRPC1^{-/-}$. These observations pointed out a delay of muscle regeneration in $TRPC1^{-/-}$ mice in comparison with their controls. This was corroborated by the fact that, in comparison with their controls, $TRPC1^{-/-}$ muscles showed a decrease in myogenic transcription factors activity and, in particular, a decrease of MyoD , Myf5 and myogenin expression. As expected, the developmental Myosin Heavy Chain (MHCd), a well known downstream target of MyoD during muscle regeneration, was more expressed in $TRPC1^{+/+}$ than in $TRPC1^{-/-}$ muscles at day 3 of regeneration. Moreover, the Akt / m TOR/ P70S6k pathway which is involved in regeneration and in regulation of protein synthesis and muscles fibre size, was also down regulated in $TRPC1^{-/-}$ muscles. Indeed, we observed a decrease of phosphorylation of both Akt and P70S6k in $TRPC1^{-/-}$ muscles than in $TRPC1^{+/+}$ muscles. The possible involvement of PI3 Kinase, an upstream regulator of Akt pathway, is under study.
Altogether, our results emphasize the involvement of TRPC1 channels in skeletal muscles development and identify a calcium-dependent activation of the Akt /mTOR/P70S6k pathway during muscles regeneration which is decreased in TRPC1-/- mice.

## $Ca^{2+}$ influx and sarcoplasmic reticulum $Ca^{2+}$ leak in normal and dystrophic skeletal muscle

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In skeletal muscle, contraction is induced by a massive release of $Ca^{2+}$ from the sarcoplasmic reticulum (SR) in response to depolarization of the cell. Under resting conditions, early experiments have shown that external $Ca^{2+}$ enters muscle cell while $Ca^{2+}$ stored in the SR leaks into the cytosol. The pathways involved in the resting $Ca^{2+}$ entry and in the SR $Ca^{2+}$ leak are still under debate but several lines of evidence suggest that degenerative muscle pathologies such as Duchenne Muscular Dystrophy (DMD) are associated with an up-regulation of these $Ca^{2+}$ fluxes. Using the technique of $Mn^{2+}$ quenching of Fura-2 fluorescence in enzymatically isolated mouse skeletal muscle fibres under voltage control, we have shown that resting divalent cations influx depends on external cations concentrations and membrane potential, but was too small to be resolved as a macroscopic current. Yet, a number of studies have postulated that Transient Receptor Potential Canonical 1 (TRPC1) channels could be involved in the resting sarcolemmal $Ca^{2+}$ influx. However, our immunostaining experiments and in vivo gene transfer of a TRPC1-YFP construction revealed a striated expression pattern compatible with a longitudinal SR localization of the channel. Poisoning with the SR $Ca^{2+}$ pump blocker cyclopiazonic acid (CPA) provoked intracellular $Ca^{2+}$ increase whose rate was significantly higher in the TRPC1-YFP positive zones than in the TRPC1-YFP negative zones suggesting that TRPC1 operates as a $Ca^{2+}$ leak channel in the longitudinal SR. Recently we set up the measurement of $Ca^{2+}$ within the SR using a low affinity $Ca^{2+}$ dye (Fluo-5N) loaded into the SR of muscle fibres dialyzed with a high internal concentration of EGTA (50 mM). Depolarizing pulses of 1s duration evoked transient decreases of Fluo-5N fluorescence which recovered in less than 2 min and CPA inhibited the recovery phase, indicating that Fluo-5N fluorescence changes report $Ca^{2+}$ changes within the SR. At a holding potential of -80 mV, CPA induced decreases in Fluo-5N fluorescence whose rates were very significantly higher in muscle fibres from the mdx mouse, the murine model of DMD. These results indicate that in the absence of dystophin, SR $Ca^{2+}$ leak is up-regulated. Further experiments are needed to determine the nature of the pathway involved in the elevated SR $Ca^{2+}$ leak in dystrophin-deficient muscle fibres and the functional consequences of this leak.

## Surface and deeper networks structural alterations in mdx cardiomyocytes: a "plant staking" role for dystrophin?

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Duchenne muscular dystrophy (DMD) is a sex-linked recessive progressive disease that leads to the degeneration of skeletal and cardiac muscles. This fatal disease results from the loss of the protein dystrophin, which in turn causes striated muscle membrane instability and, in the heart, calcium dysregulation leading to cardiac arrhythmias.
Nevertheless, it remains unclear whether calcium dysregulation in dystrophic hearts are a result of wounded membranes caused by dystrophin deficiency or/and if dysregulated calcium transporters contribute to these defects. Here we describe membrane structural damages and disorganisations, from the surface to the depth, of cardiomyocytes from the 10-12 month-old mdx mouse (model of DMD).
Experiments have been performed with an innovative imaging technique, the Scanning Ion Conductance Microscopy (SICM), to characterize the loss of integrity of cardiomyocytes surface in dystrophin deficiency.
Furthermore, with the control of the SICM nanopipette, we were able to apply "smart microperfusions" of a depolarizing solution, stimulating single T-Tubule elements. This experimental approach revealed modification of Excitation-Induced Calcium Release involved in Excitation-Contraction (EC) coupling in mdx cardiomyocytes.
Finally, 2D Fourier transform analysis of labeled internal networks suggested local reorganizations of EC coupling proteins as observed in other cases of Heart Failure. Taking into account these data, we propose that, in the heart, dystrophin would play a “plant staking” role that is to be involved in maintaining the plasma membrane integrity not only at the surface but also in preserving T-Tubules structure in the depth of cardiomyocytes.

## Syntrophin/dystrophin scaffold regulates TRPC/STIM1-dependent cation entry in developing skeletal muscle.

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Calcium mishandling in Duchenne Muscular Dystrophy (DMD) has suggested that dystrophin, a membrane-associated cytoskeleton protein, may regulate calcium signalling cascades such as calcium entries. Calcium overload in human DMD myotubes is dependent on their activity suggesting the involvement of channels being activated during contraction and/or calcium release. The TRPC protein has been shown to be part of non-voltage-gated heteromeric cation channels involved in $Ca^{2+}$ and $Na^{+}$ pathway. TRPC1 channels were shown to carry non-selective cation currents at the sarcolemma of skeletal fibres or divalent cation entries in cultured myotubes. We demonstrated that TRPC1 and TRPC4 were present in a costameric macromolecular complex constituted of dystrophin and α1-syntrophin, and that the PDZ domain of α1-syntrophin could interact with TRPC1 and TRPC4. Other groups have also shown that TRPC1 could interact with scaffolding protein like Homer-1 and caveolin3. Dystrophin-deficient skeletal muscle cells display higher store-dependent divalent cation entries, which could be restored at normal level by recombinant mini-dystrophin or α1-syntrophin expression. Moreover, deletion of PDZ- domain and siRNA silencing of α1-syntrophin led to the conclusion that α1-syntrophin is necessary for a PDZ-mediated regulation of TRPC-dependent cation entries. These cation entries are activated after a protocol of calcium store-depletion and also dependent on the expression of STIM1 as shown by siRNA silencing, which suppressed all store-operated cation entries in both dystrophin-deficient and mini-dystrophin expressing myotubes. This suggests that dystrophin/syntrophin complex regulates TRPC store-operated cation entries triggered by STIM1. We found that inhibition of PLC or PKC resulted in significant decrease of abnormal store-operated cation influx in dystrophin-deficient myotubes. Similarly, increased cation influx in α1-syntrophin-deficient myotubes was also dependent on PLC/PKC activity. All these results suggest an involvement of PKC/PLC pathway in impaired store-operated cation entry in myotubes deficient in dystrophin or in Alpha-syntrophin. We propose that the syntrophin/dystrophin scaffold normally repress the potentiation of TRPC store-operated cation entry by PLC/PKC pathway.

## Calcium homeostasis in extraocular muscles: implications for Duchenne Muscular Dystrophy

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Extraocular muscles (EOMs) are a unique group of skeletal muscles with unusual physiological properties such as being able to undergo rapid twitch contractions over extended periods and escape damage in the presence of excess intracellular calcium ($Ca^{2+}$) in Duchenne's muscular dystrophy (DMD). Enhanced $Ca^{2+}$ buffering has been proposed as a contributory mechanism to explain these properties; however, the mechanisms are not well understood. We investigated mechanisms modulating $Ca^{2+}$ levels in EOM and tibialis anterior (TA) limb muscles. Using Fura-2 based ratiometric $Ca^{2+}$ imaging we found that EOM myotubes reduced elevated Ca2+ ~2-fold faster than TA myotubes, demonstrating more efficient $Ca^{2+}$ buffering. Quantitative PCR (qPCR) and western blotting revealed higher expression of key components of the $Ca^{2+}$ regulation system in EOM, such as sarcoplasmic $Ca^{2+}$-ATPase 2 (Serca2) and calsequestrin 2 (Casq2). Interestingly EOM expressed monomeric rather than multimeric forms of phospholamban (Pln), which was phosphorylated at threonine 17 (Thr17) but not at the serine 16 (Ser16) residue. EOM Pln remained monomeric and unphosphorylated at Ser16 despite protein kinase A (PKA) treatment, suggesting differential signalling and modulation cascades involving Pln-mediated $Ca^{2+}$ regulation in EOM. In addition, high activity of store-operated $Ca^{2+}$ entry (SOCE) may play a critical part to replenish $Ca^{2+}$ for rapid and continuous cycles of contractions in EOM. The expression patterns of group 1 transient receptor potential (TRP) channels and the proteins Orai1 and STIM1 showed, that out of the group 1 TRP channels, TRPC1, TRPC6 and TRPV4 channel proteins in addition to STIM1 showed higher expression in EOM compared with TA. High TRPC1, TRPV4 and STIM1 levels could play a significant role in the high fatigue resistance, muscle differentiation and SOCE in EOM. Further, expression changes of $Ca^{2+}$ handling proteins and channels in dystrophic muscle from the mdx mouse model of DMD were investigated. Three channels were differentially expressed in mdx EOM compared with normal EOM: TRPM4 and TRPM7 were decreased in mdx EOM and TRPV4 was increased in mdx EOM. However, the changes in mdx EOM were of small magnitude. Increased expression of $Ca^{2+}$ handling proteins, $Ca^{2+}$ channels and channel regulators, differential post-translational modification of Pln, and superior $Ca^{2+}$ buffering is consistent with the improved ability of EOM to handle elevated intracellular $Ca^{2+}$ levels. These characteristics provide mechanistic insight for the potential role of superior $Ca^{2+}$ buffering in the unusual physiology of EOM and their sparing in DMD.

## Calcium channel blockade as an intervention strategy in Duchenne Muscular Dystrophy

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Duchenne Muscular Dystrophy (DMD) is believed to be caused by membrane instability, but it is becoming clear that the disease is also associated with a disturbance of calcium homeostasis. Specific calcium channels have been proposed to directly initiate pathological events of DMD. By allowing calcium ions to excessively enter the muscle cells these channels have been suggested to trigger cell damage and degeneration events. We therefore wanted to investigate if a chronic blockade of calcium channels could prevent or alleviate the dystrophic symptoms. For this purpose we used the aminoglycoside antibiotic streptomycin, which unspecifically block calcium channels. The intervention study was performed in the mdx mouse model of DMD from developmental start. Treatment of the mice was started in utero of pregnant mdx females and continued after birth until the age of 6 weeks, 10 weeks and 6 months. Our results showed that the in utero treatment delayed the dystrophic symptoms of limb muscle pathology in 6 week old mdx mice but could not prevent degeneration and regeneration from occurring later in the disease course (from 10 weeks and onwards). Even though degeneration still occurred the overall outcome of calcium channel blockade showed a positive effect on limb muscle pathology. Here we observed reduced fibrosis and increased sarcolemmal stability combined with regeneration in 6 month old mdx mice. However, diaphragm and heart muscle pathology was not improved and we observed a worsening of the heart pathology, therefore we consider calcium channel blockade an unlikely treatment for DMD.

## Effects of green tea polyphenols, prednisolone and tamoxifen on mdx mouse muscle: Improvement of motor performance and calcium handling

Ruegg UT, Gallo C, Patte-Reutenauer J, Ismail HM, Gayi E, Johansson K and Dorchies OM

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Duchenne muscular dystrophy (DMD) is a fatal muscle disorder caused by the absence of dystrophin and characterized by progressive muscle wasting. Oxidative stress and excessive calcium influx are thought to contribute to the pathogenesis. We have previously shown that dietary interventions with powerful antioxidants such as green tea polyphenols (GTP) and EGCG (the major GTP component) improved muscle structure and function of the $mdx^{5Cv}$ mouse, a model for DMD. As clinical trials are being conducted with EGCG on DMD patients, we report additional therapeutic effects of GTP and EGCG on the dystrophic mouse. As in our previous study, 3-week old mice were given for 5 to 8 weeks a chow enriched with GTP, EGCG or pentoxifylline (PTX), a nonselective phosphodiesterase inhibitor and TNFα release inhibitor used as a positive control. Prednisolone (PDN) was also tested alone or in combination with EGCG. GTP, EGCG, and PTX ameliorated spontaneous locomotor activity and performance in a wheel running assay, and decreased plasma creatine kinase levels. The manganese quench technique was used to measure the calcium influx into fibers isolated from FDB muscles and loaded with the calcium probe Fura-2. Treatment of the mice with GTP, EGCG, or PTX reduced by up to two-thirds the excessive calcium influx in dystrophic fibers in resting conditions. Acute exposure to these agents had no effect. Similar findings were obtained from diaphragm strips in $^{45}Ca^{2+}$ influx experiments. Overall, PDN was less potent than EGCG and tended to obscure the beneficial effects afforded by EGCG when given in combination. Our findings suggest that GTP, EGCG, and PTX act through genome-dependent mechanisms to decrease the expression and/or the activity of over-activated calcium channels in dystrophic cells. The role of the calcium-insensitive phospholipase $A_2$ in triggering lysophospholipid production and stimulating store-operated calcium channels will be discussed. The inhibition of this enzyme likely contributes to the overall improvement of motor function on the awaken animal.

## Molecular Therapies in Muscular Dystrophies entering clinical studies

Lochmüller H, Guglieri M, Straub V, Bushby K

Institute of Genetic Medicine, Newcastle University

Muscular dystrophies are individually rare genetic disorders that cause much chronic disability, affecting young children and adults. In the past 20 years, more than 30 genetic types of muscular dystrophy have been defined. During this time, precise diagnosis, genetic counselling, and medical management have improved. These advances in medical practice have occurred while definitive therapies based on an improved knowledge of disease pathogenesis are awaited. A wide range of therapeutic options have been tested in animal models, and some are being tested in clinical trials. Various therapeutic targets are being investigated, from personalised medicines targeting specific mutations and drugs targeting cellular pathways to gene-based and cell-based therapies. We will specifically focus on completed and ongoing clinical trials in Duchenne Muscular Dystrophy.

# International Workshop on Transport Processes in Neurodegenerative and Neuromuscular Diseases

## Sept. 12 – 14th, 2011

## Program

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| **Mo. Sept. 12.** | **Welcome and key note lecture** |
| 17:00-17:15 | Welcome<br>A Greinacher, Vice Dean for Research<br>A Popa-Wagner, Coordinator of EU Project<br>S Vogelgesang, Organizer |
| 17:15-18:15 | Key note lecture, Chair: S Vogelgesang<br>Corruptive protein seeding in the pathogenesis of neurodegenerative disorders<br>LC Walker, H LeVine III, Y Eisele and M Jucker |
| 19:30 | *Dinner at "Olive"* |

| Tu. Sept. 13. | **Session 1: Mechanisms of neurodegenerative disorders, Chair: S. Vogelgesang** |
|---|---|
| 8:30 – 8:45 | Welcome<br>M Zygmunt, Medical Director of University Medicine<br>C Kessler, Speaker of the Neuroscience Group in Greifswald<br>H Brinkmeier, Organizer |
| 8:45 – 9:20 | Identification of pathways driving prion pathogenesis<br>JF Pedersen, U Hermann, K Arroyo, A Varol, M Nuvolone, P Liberski, C Zhu, T Sonati and A Aguzzi |
| 9:20 – 9:55 | Imaging structural and functional alterations in AD mouse models<br>M Meyer-Lühmann |
| 9:55 – 10:25 | *Coffee break* |
| 10:25 – 11:00 | Blood-brain barrier P-glycoprotein:<br>a new target for Alzheimer's disease?<br>AMS Hartz, DS Miller and B Bauer |
| 11:00 – 11:35 | ABC-transporter at the blood-brain barrier:<br>Impact for risk and therapy of neurological disorders<br>I Cascorbi, S Haenisch, A Werk, U Stephani,<br>R Warzok, and S Vogelgesang |
| 11:35 – 12:00 | Beta-amyloid downregulates MDR1-P-glycoprotein (Abcb1) expression at the blood-brain barrier in mice<br>A Brenn, M Grube, A Fischer and S Vogelgesang |
| 12:20 – 14:00 | Lunch break |
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| **Tu. Sept. 13** | **Session 2: $Ca^{2+}$ regulation in skeletal muscle and functions of TRP channels, Chair: H Brinkmeier** |
| 14:00 – 14:55 | The impact of ryanodine receptor (RyR1) mutations on orthograde and retrograde triadic coupling<br>W Melzer |
| 14:55 – 15:30 | Strictly controlled $Ca^{2+}$ influx through TRPC3 channels has a key role in insulin-mediated glucose uptake in striated muscle<br>H Westerblad and J Lanner |
| 15:30 – 16:15 | *coffee break* |
| 16:15 – 16:30 | TRPC3 channel blockers inhibit background calcium influx and modulate calcium transients in mouse muscle fibers<br>YX Zhang, B Pritschow and H Brinkmeier |
| 16:30 – 17:05 | Ca2+ influx and sarcoplasmic reticulum $Ca^{2+}$ leak in normal and dystrophic skeletal muscle<br>B Allard |
| 17:05 – 17:40 | Surface and deeper networks structural alterations in mdx cardiomyocytes: a "plant staking" role for dystrophin?"<br>C Lorin, M Gueffier, P Bois, J-F Faivre, S Sebille and C Cognard |
| 19:30 | *Dinner at "Alter Fritz"* |
| | ... |

**We. Sept 14**

**Session 3: $Ca^{2+}$ regulation in dystrophin-deficient muscle and prospects for a therapy of DMD**
**Chair: C Cognard**

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| 8:30 – 9:05 | Syntrophin/dystrophin scaffold regulates TRPC/STIM1-dependent cation entry in developing skeletal muscle.<br>J Sabourin, R Harisseh, N Déliot, C Cognard and B Constantin |
| 9:05 – 9:40 | Calcium homeostasis in extraocular muscles: implications for Duchenne Muscular Dystrophy<br>U Zeiger, CH Mitchell, H Brinkmeier, TS Khurana |
| 9:40 – 10:15 | Calcium channel blockade as an intervention strategy in Duchenne Muscular Dystrophy<br>LH Jørgensen, A Blain, E Greally, SH Laval, AM Blamire, BJ Davison, H Brinkmeier, GA MacGowan, HD Schrøder, K Bushby, V Straub and H Lochmüller |
| *10:15 – 10:45* | *Coffee break* |
| 10:45 – 11:20 | Effects of green tea polyphenols, prednisolone and tamoxifen on mdx mouse muscle: Improvement of motor performance and calcium handling<br>UT Rüegg, C Gallo, J Patte-Reutenauer, HM Ismail, E Gayi, K Johansson and OM Dorchies |
| 11:20 – 11:35 | The role of the serum- and glucocorticoid-inducible kinase SGK1 for muscle function and fibrosis development in mdx muscle<br>M Steinberger, J Kasch, S Vogelgesang, D Kuhl, M Föller, F Lang and H Brinkmeier |
| 11:35 – 12:10 | Molecular Therapies in Muscular Dystrophies entering clinical studies<br>H Lochmüller, M Guglieri, V Straub and K Bushby |
| 12:10 – 12:20 | Concluding remarks, S. Vogelgesang and H. Brinkmeier |

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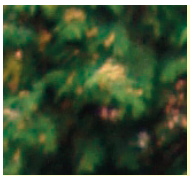

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# Improvement of the research competitiveness in neuroscience at the Ernst-Moritz-Arndt University of Greifswald

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