AuPS/ASB Meeting - Adelaide 2010

Free communications: Neuronal development and pathology

Monday 29th November 2010 - Broughton Room - 17:15

Chair: Louise Tierney

Peroxiredoxin 4 is confined to the endoplasmic reticulum in human brain and associated with Lewy body formation in Parkinson's disease and dementia with Lewy bodies

L.J. Turnbull and J.H.T. Power, Department of Human Physiology, School of Medicine, Flinders University, Bedford Park, Bedford Park, SA 4042, Adelaide.

Parkinson disease (PD) is a progressive neurodegenerative disorder characterised by formation of Lewy bodies within the doperminergic neurons within the *substantia nigra* and depending on the staging of the disease, marked loss of these neurons. Similarly, dementia with Lewy bodies (DLB) involves the formation of Lewy bodies within the cortical neurons resulting in marked synaptic loss and eventually neuronal loss and dementia.

Lewy bodies contain a range of cellular proteins including a high proportion of α-synuclein, a small protein whose suspected role is in synaptic vesicle recycling. There is still debate as to whether Lewy bodies are protective or destructive to neurons. Yeast models expressing alpha synuclein suggest that α-synuclein blocks trafficking from the endoplasmic reticulum to the Golgi apparatus and results in endoplasmic reticulum stress.

Peroxiredoxin IV is the least characterized of the peroxiredoxin family of antioxidant enzymes and is different from other 2-Cys members in that it contains a hydrophobic leader which suggests that peroxiredoxin IV is a secreted protein. Recent pulse chase experiments have indicated that peroxiredoxin IV is in fact confined to the endoplasmic reticulum and possibly involved in protection against endoplasmic reticulum oxidative stress and as a molecular chaperone. This study was conducted to determine the cellular localization of this enzyme in the human brain and whether peroxiredoxin IV is present in the endoplasmic reticulum and associated with the formation of Lewy bodies in PD and DLB.

Human brain proteins from control, PD and DLB tissue were separated using PAGE, transferred to a Polyvinylidene difluoride membrane (PVDF) and then probed with a peroxiredoxin IV antibody to determine the specificity of the antibody and the molecular forms. Light immunohistochemistry using the same antibody was performed using paraffin embedded sections from the same tissue to determine the general distribution of peroxiredoxin IV. Confocal immunofluorecence using the peroxiredoxin IV antibody and specific cellular and organelle markers was used to determine the specific cellular and sub-cellular distribution. In addition, colocalization with α-synuclein was used to determine if peroxiredoxin IV and the endoplasmic reticulum was associated with Lewy body pathology in PD and DLB.

On Western blotting, peroxiredoxin IV stained as two prominent proteins at approximately 27kd and 65kD under reducing conditions. Peroxiredoxin IV is predicted to be 31kD with a 4kD leader sequence indicating the monomer in human brain is the cleaved mature form. This protein functions as a committed dimer and the 65kD form is probably the dimer although it is slightly larger than predicted. Peroxiredoxin IV was abundant in neurones, both light and confocal immunohistochemistry showed prominent granular staining in the neurones with low level staining in oligodendrocytes. Astrocytes did not appear to be labelled and low level staining microglia appeared to be from ingested material. Peroxiredoxin IV co-localised with the ER/Golgi marker SAR1 indicated that peroxiredoxin IV is confined to the ER in human brain supporting cellular pulse chase studies. SAR1 had a slightly larger area of distribution suggesting that peroxiredoxin IV does not translocate to the Golgi. However, peroxiredoxin IV did not co-localise with the lysosomal marker Lamp2 indicating it is not associated cellular degradation *via* lysosomes.

Co-localization of peroxiredoxin IV with the Lewy body marker α-synuclein showed that there is a close association between Lewy body formation and the ER. In pre-Lewy body neurones, α-synuclein and peroxiredoxin IV positive vesicular aggregations were observed to be coalescing between the ER and Golgi supporting recent findings in yeast models that α-synuclein blocks docking of ER vesicles with the Golgi apparatus. A variety of Lewy body forms were observed and all forms were closely associated with the ER as determined by the peroxiredoxin IV staining.

In conclusion these results show for the first time that peroxiredoxin IV is present in human neurones and that it is confined to the ER. These results also suggest that Lewy bodies are seeded from α-synuclein vesicular aggregations unable to dock with the Golgi apparatus giving new insights into the formation of this pathologic structure.

ProBDNF inhibits neurites outgrowth in neurons of mice by activating RhoA

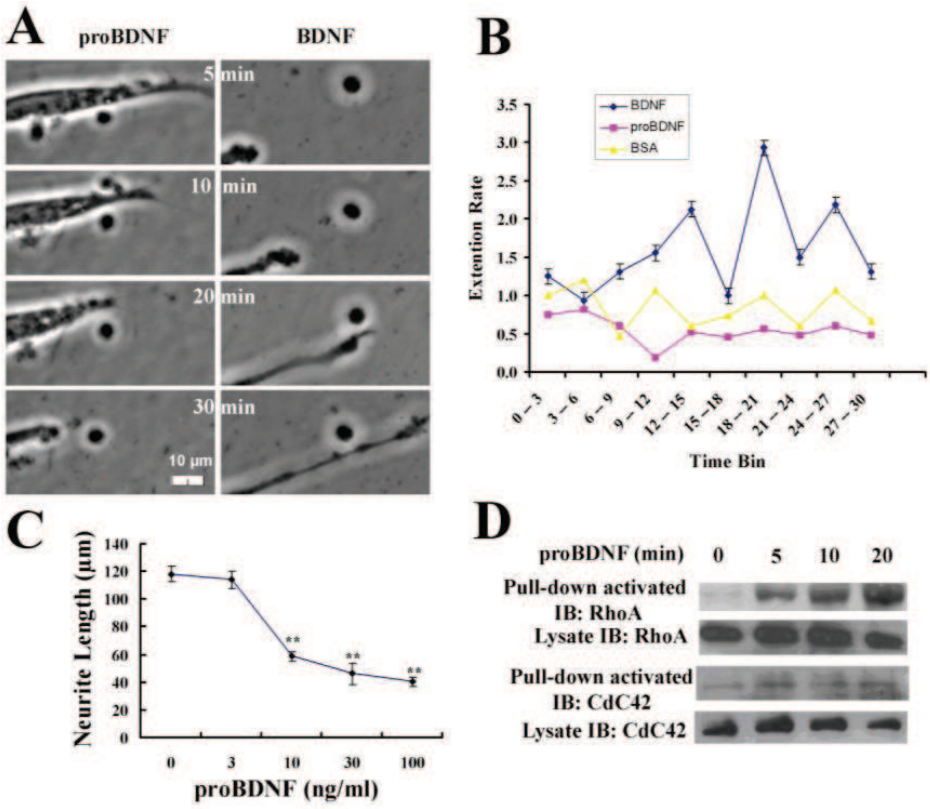
Y. Sun, Y. Lim, J.H. Zhong, M. Yang and X.F. Zhou, Department of Human Physiology and Centre for Neuroscience, Flinders University, GPO Box 2100, Adelaide, SA 5042, Australia.

Background: Brain-derived neurotrophic factor (BDNF) is a potent molecule regulating dendritic trees and synaptic plasticity, however, whether the BDNF precursor (proBDNF) plays any role in the neurite growth is unknown. This study investigated whether proBDNF has a physiological role in the neurite growth of neonatal cortex neuron *in vitro*. We focus on the following the aspects, the dose effect of proBDNF on neurite outgrowth, ELISA assay to test proBDNF and BDNF after stimulated neurons by 45 mM KCl for 30 min, distribution of RohA, cdc42, F-actin *via* immunohistochemistry after gradient or uniform of proBDNF added, and examined the down streaming signals of RhoA and cdc42.

Methods: The cortex from neonatal C57BL/6 (n=12) mice were rapidly removed and cultured. Live images were collected every 6 s for 30 min by using Nikon BioStation and analyzed *via* the advanced software. Primary neurons were incubated in proBDNF at various concentrations for 24 h and the lengths were measured. 8×10^6 cells were dissociated for 24 h, treated with 50 ng/ml proBDNF for 0, 5, 10, 20 min, the neuron cell lysates of 100 μg protein in 0.5 ml RIPA buffer were incubated in 10 μg Rho binding domain (RBD) agarose gel for RhoA and cdc42 activity assays. The pull-down and total proteins were evaluated by western blotting. Data are mean ± SEM from 3 independent experiments.

Results: Live imaging showed the clear collapse of growth cones in response to proBDNF (50 ng/ml) and the elongation to BDNF (50 ng/ml) at the indicated point (Figure A). proBDNF caused a 34% decrease in the extension rate after 6 min and maintained throughout a 30 min treatment. Meanwhile, neurons treated with BDNF spent 63% of the time extending (Figure B). proBDNF (10, 30, 100 ng /ml) resulted in a 50% decrease in length (58.6 μm *vs* 118.1 μm) compared with 0 ng /ml proBDNF group (Figure C) but proBDNF 3 ng /ml had no significant effects on the length. 5 min after proBDNF treatment, RhoA activity was increased by 5 fold 20 min after proBDNF incubation (Figure D). Interestingly, no difference in activated cdc42 level was seen during the proBDNF incubation.

Conclusion: Live imaging demonstrated that proBDNF repulsed growth cones and induced the neurite collapse of cortical neurons in neonatal mice. Statistical analysis showed the dose-dependent effect of proBDNF on neurite collapse. proBDNF rapidly activated RhoA without affecting the cdc42 level. Thus, proBDNF inhibits neurite growth *via* activating RhoA in neonatal cortical neurons of mice.



** $p < 0.001$, two-sample *t* test.

Postsynaptic $GABA_A$ receptor number and enhanced gaboxadol induced change in holding currents in Purkinje cells of the dystrophin-deficient mdx mouse

S.I. Head,[1] S.L. Kueh,[2] J. Dempster[3] and J.W. Morley,[1,2] [1]School of Medical Science, University of New South Wales, NSW 2052, Australia, [2]School of Medicine, University of Western Sydney, NSW 2560, Australia and [3]Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, Glasgow, Scotland, UK.

Duchenne muscular dystrophy (DMD) results from an absence of the protein dystrophin. It is characterized by severe wasting of skeletal muscle. In about a third of these patients, there is evidence of an accompanying cognitive and behavioral deficit. In the cerebellum dystrophin is normally localized at the postsynaptic membrane of GABAergic synapses on Purkinje cells. Here, we investigate the effect of an absence of dystrophin on the number of $GABA_A$ channels located at the synapse in cerebellar Purkinje cells of the dystrophin-deficient mdx mouse. Whole-cell patch-clamp recordings of spontaneous miniature inhibitory postsynaptic currents (mIPSCs) were performed in cerebellar slices from mdx and littermate control mice, which had been killed by an halothane according to UNSW ethics guidelines. Using non stationary noise analysis, we found a significant difference in the number of receptors at GABAergic synapses in mdx mice (38.38 ± 2.95; n=14) compared to littermate controls (53.03 ± 4.11; n=12) (p = 0.01). In response to the application of the GABA agonist gaboxadol we found a significant difference in the gaboxadol induced change in holding current in mdx mice (65.01 ± 5.89 pA; n=9) compared to littermate controls (37.36 ± 3.82 pA; n=8). The results show that in cerebellar Purkinje cells of dystrophin-deficient mdx mice there is a reduction in the number of receptors localised at GABAergic synapses, and an increase in extrasynaptic $GABA_A$ receptors, indicating that dystrophin plays an important role in ion channel localization and stabilization at the post-synaptic membrane. If similar changes occur in the CNS in boys with DMD, it may impact on the function of neural networks and contribute to motor, behavioral and cognitive impairment apparent in many boys with DMD.

Huntingtin associated protein 1 associates with amyloid precursor protein and regulates its trafficking and Aβ levels

X.F. Zhou, G.Z. Yang, J.J. Lu, Y. Lim, J.H. Zhong and M. Yang, Department of Human Physiology and Centre of Neuroscience, Flinders University, Adelaide, SA 5001, Australia.

Amyloid precursor protein (APP) is a type I transmembrane receptor-like molecule involved in the pathogenesis of Alzheimers disease. Following endocytosis, APP is delivered to endosomes, where β- and γ-secretases are localized and Aβ neurotoxic peptide is produced. Huntingtin associated protein 1(HAP1) is a brain-enriched protein and participates in intracellular trafficking in neurons. HAP1 interacts with kinesin light chain and dynactin p150Glued, regulates the anterograde and retrograde transport of a number of proteins including proBDNF and APP (McGuire *et al*,, 2006). However, how HAP1 regulates APP trafficking and the significance of this regulation remain unknown.

Using HEK 293 cells transfected with HAP1-CFP and APP-YFP plasmids, we showed that these two proteins were highly colocalized. Immunohsitochemical data showed that these two proteins are present in a number of brain regions such as cortex, hippocampus and hypothalamus with a similar distribution patterns in the mouse and human brains. Confocal microscopy showed that they also co-exist in a number of subcellular structures. FRET analysis showed that the FRET efficiency between HAP-CFP and APP-YFP was over 20%, much higher than negative and positive controls, indicating these two molecules were close to each other *in vivo*. Immunoprecipitation experiments on over-expressed HEK293 cell lysates or on human brain homogenates showed that HAP1 and APP were present in the immunoprecipitated samples forming a complex. To see whether HAP1 regulates APP subcellular trafficking, we cultured cortical neurons from HAP1+/+ and HAP1-/- neonatal mice and analyzed the co-localization of APP with organelles marker proteins. The results showed that APP has a high co-localization ratio with giantin GM130 (cis-Golgi marker), Golgi97 (trans-Golgi complex marker), EEA1 (early endosome marker) and SEC22b (ER-Golgi intermediate compartment marker) in HAP1-/- cortical neurons, but has no significant difference with GRP78 (endoplasmic reticulum marker), CD71 (recycling endosome marker), Lamp1 (lysosome marker) and VPS35 (retromer marker) in neurons between HAP1+/+ and HAP1-/- mice. However, there was a lower co-localization ratio between APP and the autophagy marker beclin1 in HAP1-/- neurons, compared with wt neurons. These results suggest that APP is retained in cis-Golgi, trans-Golgi complex, early endosome and ER-Golgi intermediate compartment when HAP1 is deleted, and HAP1 may increase the APP trafficking to autophagy vesicles. Sucrose gradient fractionations on wt and HAP-/- brain homogenates showed that the APP distribution is altered. In the normal wt brain there was only one peak of APP, corresponding to membranous organelles such as Golgi and ER, whereas in the HAP1-/- mice, there were two peaks of APP distribution: one is near to the bottom of the gradient and the other was in the cytosol fractions. Interestingly, GM130, EEA1 and GRP78 had a similar distribution pattern to APP in HAP1-/- mice. APP internalization assay using antibody imaging and biotinylation techniques on HAP1-/- neurons showed that significant alteration in APP endocytosis in HAP1-/- neurons and abnormal re-insertion of APP into the cytoplasmic membrane. Live imaging analysis and FRAP assay on APP-YFP vesicles in HAP1-/- neurons showed that the trafficking speed was reduced and the number of motionless particles were increased. To see whether HAP1 regulates Aβ production, we cultured cortical neurons from Alzheimers disease mice and knocked down HAP1 protein with interference RNA. We found that the down-regulation of HAP1 increases Aβ levels.

Taken together, our data suggest that HAP1 associates with APP and regulates APP subcellular trafficking to the non-amyloidogenic pathway. Up-regulation of HAP1 may increase APP re-insertion into cytoplasmic membrane and reduce Aβ production.

McGuire JR, Rong J, Li SH, Li XJ. (2006) Interaction of Huntingtin-associated protein-1 with kinesin light chain: implications in intracellular trafficking in neurons. *Journal of Biological Chemistry* **281:** 3552-3559.

Huntingtin-associated protein 1 (HAP1) regulates exocytosis via multiple mechanisms

K. Mackenzie, X.F. Zhou and D.J. Keating , Discipline of Human Physiology, Centre of Neuroscience, Flinders University, Sturt Rd, Adelaide, 5042, Australia.

Subcellular localisation and protein interaction data indicate that HAP-1 may be important in vesicle trafficking and microtubule transport. However, no physiological evidence exists to verify this possibility. Our study reports a novel role of HAP-1 as a regulator of exocytosis by influencing the rate of exocytosis, fusion pore dynamics and the size of the readily releasable pool (RRP) which consists of vesicles released immediately upon stimulation. Chromaffin cells from the adrenal gland were used for our exocytosis assays. Cells were cultured from adrenal glands taken from dead 8 week old mice using collagenase (Type A, Roche). Carbon-fibre amperometry was used to investigate exocytosis in single chromaffin cells. We applied +800 mV to a carbon fibre electrode placed on the surface of a single chromaffin cell. We simultaneously measured current caused by the oxidation of released catecholamines and analysed the number of current spikes, representing single exocytotic events, occurring in this time. Chromaffin cells were cultured from HAP-$1^{-/-}$ (KO), HAP-$1^{+/-}$ (Het) and HAP-$1^{+/+}$ (WT) mice. Similar levels of exocytosis triggers by a 70mM K^+ solution wer found in WT (102.2 ± 10.2 exocytotic events, n= 29) and Het (90.8 ± 11.5, n=20) cells while exocytosis in KO cells was significantly reduced (60.4 ± 7.1, n=35) compared to WT (p<0.01) or Het (p<0.05) cells. The duration of the pre-spike "foot signal", an indicator of fusion pore opening, was found to be prolonged in KO cells (3.0 ± 0.1 ms) compared to WT (2.3 ± 0.1 ms, p<0.05) and Het (2.9 ± 0.1 ms, p<0.05) cells indicating that HAP-1 may function in stabilizing the formation of the fusion pore. The size of the RRP is also regulated by HAP-1 as the number of vesicles undergoing exocytosis following treatment with a hyperosmotic solution in KO cells (19 ± 5.3, n=7) is less than in WT (54.4 ± 8.9, n=7, p<0.01) or Het (46 ± 9.2, n=8, p<0.05) cells. Real-time PCR also indicates the downregulation of exocytosis-related genes in KO cells. Our findings implicate, for the first time, the involvement of HAP1 in the regulation of exocytosis at multiple levels including vesicle localisation, membrane fusion and gene transcription.