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Free communications: Membrane transport

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A novel digestive complex and its role in Hartnup disorder: trafficking of the neutral amino acid transporter B^0AT1 by angiotensin converting enzyme 2 (ACE2)

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In the intestine, proteins are digested by proteases and brush-border peptidases into small peptides and amino acids, which are then absorbed by peptide and amino acid transporters. Neutral amino acids are absorbed by the amino acid transporter B^0AT1. B^0AT1 is also expressed in the kidney, where it mediates reabsorption of neutral amino acids from the primary urine. Mutations in B^0AT1 cause Hartnup disorder, a defect in neutral amino acid transport resulting in neutral aminoaciduria.

In the kidney, B^0AT1 requires the auxiliary protein collectrin for trafficking to the brush-border membrane. However, collectrin is not expressed in the intestine, suggesting that a different protein facilitates B^0AT1 trafficking in this tissue. Interestingly, the closest homologue of collectrin is a brush-border carboxypeptidase, angiotensin converting enzyme 2 (ACE2).

Coexpression of B^0AT1 and ACE2 in *Xenopus laevis* oocytes* caused a dramatic increase in the surface expression of B^0AT1. Addition of a peptide containing a carboxyterminal leucine residue to these oocytes resulted in leucine transport by B^0AT1, demonstrating that B^0AT1 and ACE2 form a complex that performs two consecutive steps in protein digestion and absorption. The Hartnup disorder associated mutations B^0AT1 (D173N) and B^0AT1 (R240Q) showed reduced interaction with both ACE2 and collectrin, thereby explaining how these mutations cause Hartnup disorder.

**Xenopus laevis* oocytes were harvested by surgery of anaesthesized frogs (MS-222, 1.5g/l). The procedure was approved by the Animal Experimentation Ethics Committee of the Australian National University.

Regulation of epithelial sodium channels by $G_{\alpha q}$

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Transepithelial Na^+ absorption mediated by epithelial Na^+ channels (ENaC) is important for Na^+ and fluid balance. The activity of ENaC is regulated by an array of physiological factors, many of which exert their effects on the channel *via* G-protein-coupled receptors. For example, intracellular ions exert their effect on ENaC *via* $G_{\alpha o}$ (Komwatana *et al.*, 1996) and $G_{\alpha i2}$ (Dinudom *et al.*, 1995), whereas the inhibitory effect of purinergic receptor activation on ENaC is mediated *via* an unidentified pertussis toxin-sensitive G-protein (Kunzelmann *et al.*, 2005). The G_q family, G_q, G_{11}, G_{14} and $G_{15/16}$, plays an important role in the regulation of the function of a variety of ion channels and transporters. For instance, G_q modulates L-type and N-type Ca^{2+} channels (Gamper *et al.*, 2004; Lu *et al.*, 2005), TRPC4 (Otsuguro *et al.*, 2008) and TASK-1 and TASK-3 K^+ channels (Chen *et al.*, 2006). So far, the mechanisms and extent to which ENaC is modulated by G_q family G-proteins remain largely unexplored.

To investigate the potential regulation pf ENC by G_q family members, we expressed constitutively active mutants of $G_{\alpha q}$, $G_{\alpha 11}$ and $G_{\alpha 14}$ in Fisher rat thyroid (FRT) cells cotransfected with ENaC and in mouse collecting duct (M1) cells. All α-subunits of G_q family proteins exerted a strong inhibitory effect on the activity of ENaC in both cell types. The effect of $G_{\alpha q}$ on ENaC, however, was not mediated *via* the traditional signaling molecules downstream of GPCR activation, such as PLC, PKC or MAP kinases. We also found that $G_{\alpha q}$ had no effect on the abundance of ENaC at the cell membrane and that its effect on ENaC was independent of Nedd4-2. We further found that the effect of $G_{\alpha q}$ on ENaC was inhibited by Grk2, although the kinase activity of Grk2 was not involved in its inhibition of the $G_{\alpha q}$ effect on ENaC. This effect of Grk2 was totally dependent on the presence of its Regulatory of G-protein Signalling (RGS) domain.

We conclude that the activity of ENaC is regulated by multiple G-protein signalling mechanisms that differentially influence the activity and the membrane expression of the channel. Grk2 acts as a negative regulator of G-protein-dependent regulation of ENaC in two different ways. The kinase activity of Grk2 renders G-protein-mediated regulation of ENaC, such as that of $G_{\alpha o}$, ineffective (Dinudom *et al.*, 2004). Additionally, similar to other RGS-like proteins, the structural domains of Grk2 impinge on the activity of free $G_{\alpha q}$, possibly by accelerating its hydrolysis of GTP, and so render them unable to propagate signals to modulate function of ENaC.

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The epithelial sodium channel and blood pressure

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The epithelial sodium channel (ENaC) is involved in the long- and short-term regulation of blood pressure (BP) and has been implicated in the Mendelian BP diseases Liddle's syndrome and pseudohypoaldosteronism type 1. The β- and γ-ENaC subunit genes, *SCNN1B* and *SCNN1G* are located on a region of chromosome 16 linked to BP by a number of independent studies, including the Victorian Family Heart Study (VFHS) (Wong *et al.*, 1999; Harrap *et al.*, 2002). The VFHS consists of 767 Caucasian families (2880 healthy subjects) representative of the general Australian population. We investigated the association of *SCNN1B* and *SCNN1G* with SBP in the VFHS. Initially a total of 25 SNPs from each gene were genotyped using an extreme phenotyping approach, utilising unrelated subjects from the upper and lower deciles of the SBP distribution. This identified ten SNPs from with nominal evidence of association to SBP (*SCNN1B*: *rs1004749, rs239345, rs239346* & *rs3743966*. *SCNN1G*: *rs13331086, rs11074553, rs4299163, rs5740, rs4281710* & *rs4470152*). To independently test these findings, we genotyped these SNPs in 1971 relatives from 68 large Utah pedigrees selected for high risk of cardiovascular disease. Generalised estimating equations were used to test for association of the ENaC SNPs and BP while controlling for related observations in families. After adjusting for the covariates age, sex and body mass index, we detected significant association for the *SCNN1B* SNP *rs239345* with SBP and DBP ($p = 0.03$) at baseline and for the *SCNN1G* SNPs *rs13331086* and *rs11074553* with SBP and DBP at 25-year follow up ($p = 0.005$ and $p = 0.018$ respectively). Our findings suggest that both the β- and γ-ENaC genes are involved in BP determination in the general population.

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Huntingtin-Associated Protein 1 (HAP-1) is a novel regulator of exocytosis

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Huntington's Disease (HD) is a fatal neurodegenerative disorder, the genetic cause of which is a mutation in the gene encoding the protein Huntingtin (Htt). This mutation causes an expansion of an N-terminal CAG repeat sequence translating to a polyglutamine extension in the protein. Due to the ubiquitous expression of mutant Htt, the explanation for the selective neurodegeneration seen in HD may be the altered protein interactions of mutant Htt. Htt interacts with multiple proteins including Huntingtin-Associated Protein 1 (HAP-1) (Li *et al.*, 1995). HAP-1 may be an important factor in HD pathogenesis as mutant Htt has a greater binding affinity for HAP1 than Htt (Li *et al.*, 1995). Based on its subcellular localisation and protein interactions, HAP-1 is thought to play a role in vesicle trafficking and microtubule transport.

This study aims to determine whether HAP-1 has an influence on vesicle exocytosis. Carbon fiber amperometry was used to detect exocytosis from single chromaffin cells isolated using collagenase (Type A, Roche) from the adrenal glands of dead mice at P0. A carbon-fibre electrode at +800 mV was placed on the surface of a chromaffin cell which was stimulated with a high K^+ (70 mM) solution for 60 seconds. We 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. We found a similar level of exocytosis in WT (102.9 ± 13.4 exocytotic events, $n = 25$) and Het (91.3 ± 10.9, $n = 21$) cells whereas exocytosis in KO cells was reduced (60.1 ± 6.9, $n = 36$) significantly compared to either WT ($p < 0.01$) or Het ($p < 0.05$) cells. Analysis of individual amperometric spike shapes gives an insight into the characteristics of vesicle fusion pore kinetics. We observed no significant differences in spike shape between genotypes apart from the duration of the "foot signal". This signal precedes a full amperometric spike and is an indicator of fusion pore opening. We found foot duration to be prolonged in KO cells (2.09 ± 0.22 ms) compared to WT (1.55 ± 0.12 ms, $p < 0.05$) and Het (1.45 ± 0.08 ms, $p < 0.05$). The size of the readily releasable pool (RRP) is also regulated by HAP-1. We exposed cells to a hyperosmotic solution for 10 seconds and observed the number of exocytotic events subsequently occurring as a measure of the number of pre-fused vesicles, representing the RRP. The number of events that occurred in KO cells (19 ± 5.3, $n = 7$) was less than in WT cells (68.8 ± 8.3, $n = 4$, $p < 0.01$) or Het (46 ± 2.9, $n = 8$, $p < 0.05$) cells. These findings illustrate that HAP-1 has a previously unknown role in regulating exocytosis. Underlying this are alterations in the RRP size and in the rate of fusion pore formation. If HAP-1 is found to similarly affect neurotransmission in the brain then the potential may exist for an involvement of HAP-1 regulating synaptic activity and neuronal communication and possibly in HD pathogenesis.

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RCAN1 (Regulator of Calcineurin 1) is a novel regulator of secretory vesicle exocytosis and fusion pore kinetics

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Regulator of calcineurin 1 (*RCAN1*) is a gene located on chromosome 21 that is over expressed in the brains of Down syndrome and Alzheimer's disease patients, diseases in which synaptic activity is negatively affected. RCAN1 interacts with calcineurin to inhibit its activity (Fuentes *et al.*, 2000). Calcineurin is a protein phosphatase that regulates transcriptional activity via the NFAT pathway and also dephosphorylates a number of proteins, including several involved in exocytosis and endocytosis. We have used mice that transgenically overexpress *RCAN1* (*RCAN1^{ox}*) and mice in which *Rcan1* expression is ablated (*Rcan1$^{-/-}$*) to investigate the role of RCAN1 in exocytosis (Keating *et al.*, 2008). We studied exocytosis using carbon fibre amperometry on chromaffin cells, a neuroendocrine model of neuronal secretion. Chromaffin cells were obtained from the adrenal glands of dead, 6-8-week-old male mice. Catecholamine release from individual chromaffin cells was measured using carbon fibre amperometry, which involved placing a carbon-fibre electrode at +800 mV onto a chromaffin cell and recording a current trace for a period of 60 sec from the start of stimulation. Cells were depolarised using a bath solution containing 70 mM K^+, which consistently triggered exocytosis. Single exocytotic events were observed as spikes on the current trace and analysis of these spikes yielded information on the number of exocytotic events, speed of the events and the amount of catecholamines released per event. An increase in *RCAN1* expression resulted in fewer exocytotic events (*RCAN1^{ox}* 37.5 ± 5.4, control 58.9 ± 6.5, $p < 0.001$, $n = 23$) as did ablation of *Rcan1* (*Rcan1$^{-/-}$* 30.5 ± 2.9, control 60.8 ± 6.9, $p < 0.001$, $n = 21$). These data indicate that a careful balance exists between *RCAN1* expression and optimum levels of exocytosis. We also found that RCAN1 regulates fusion pore formation between the vesicle and plasma membranes. The speed of vesicle fusion was proportional to expression levels of RCAN1, evident as a decrease in catecholamine release with increasing speed of exocytosis (*Rcan1$^{-/-}$* 338.2 ± 34.1 pC, control 221.7 ± 22.1 pC, $p < 0.01$, $n = 16$, *RCAN1^{ox}* 171.6 ± 16.2 pC, control, 275.3 ± 25.6 pC, $p < 0.01$, $n = 16$). These effects were downstream of calcium entry, as determined by fluorescent imaging of cells loaded with the Ca^{2+} indicator dye Fluo-3 AM (5 μM). The ready releasable pool, representing the number of pre-fused vesicles, was also unaffected by RCAN1 expression, as determined by exposure to a hypertonic bath solution containing 500 mM sucrose for 10 sec and measuring the subsequent number of exocytotic events. To determine if these effects of RCAN1 were due to regulation of calcineurin activity, cells were chronically treated with the calcineurin inhibitors cyclosporin A and FK506 (1 μM each). Chronic inhibition of calcineurin in control cells resulted in more rapid fusion pore kinetics, evident as a 53.2% decrease in catecholamine release per vesicle ($p < 0.05$, $n = 10$), making secretion similar to that seen in *RCAN1^{ox}* cells. In contrast, chronic calcineurin inhibition had no effect on the speed of fusion pore formation in *RCAN1^{ox}* cells. Chronic calcineurin inhibition also had no effect on the number of exocytotic events in either *RCAN1^{ox}* or control cells. These data demonstrate a novel role for *RCAN1* as a regulator of exocytosis. We observed regulation at two stages; the number of vesicles undergoing exocytosis, regulated independently of calcineurin, and the speed of each exocytotic event, regulated by RCAN1-dependent control of calcineurin activity.

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