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RCAN1 regulates vesicle recycling and quantal release kinetics via effects on calcineurin activity.

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Running title: RCAN1 controls vesicle release

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Abstract

We have previously shown that Regulator of Calcineurin 1 (RCAN1) regulates multiple stages of vesicle exocytosis. However, the mechanisms by which RCAN1 affects secretory vesicle exocytosis and quantal release kinetics remain unknown. Here we use carbon fiber amperometry to detect exocytosis from chromaffin cells and identify these underlying mechanisms. We observe reduced exocytosis with repeated stimulations in chromaffin cells overexpressing RCAN1 ($RCAN1^{ox}$), but not in wild type (WT) cells, indicating a negative effect of RCAN1 on vesicle recycling and endocytosis. Acute exposure to calcineurin inhibitors, cyclosporine A and FK-506, replicates this effect in WT cells but has no additional effect in $RCAN1^{ox}$ cells. When we chronically expose WT cells to cyclosporine A and FK-506 we find that catecholamine release per vesicle and pre-spike foot (PSF) signal parameters are decreased, similar to that in $RCAN1^{ox}$ cells. Inhibiting calcineurin activity in $RCAN1^{ox}$ cells has no additional effect on the amount of catecholamine release per vesicle but further reduces PSF signal parameters. Electron microscopy studies indicate these changes are not due to altered vesicle number or distribution in $RCAN1^{ox}$ cells but reduced vesicle release may be cause by decreased vesicle and dense core size in $RCAN1^{ox}$ cells. Thus, our results indicate that RCAN1 may negatively affects vesicle recycling and quantal release kinetics via the inhibition of calcineurin activity.

Introduction

Regulator of Calcineurin 1 (RCAN1) is a chromosome 21 gene that is over expressed in Down syndrome (DS) and Alzheimer's disease (AD) brains (Ermak *et al.* 2001, Fuentes *et al.* 2000). Human DS and AD brains demonstrate neuronal changes including reduced noradrenaline signalling, basal forebrain cholinergic neuron degeneration, enlarged endosomes and dementia (Lai *et al.* 1999). Mouse models of both disorders demonstrate reduced synaptic transmission, LTP and altered learning and memory (Oddo *et al.* 2003, Siarey *et al.* 2005). Identifying proteins common to both DS and AD which have roles in cell signalling or neurotransmitter release may enhance our understanding of the mechanisms underlying the neuronal changes observed in these disorders.

We have previously identified RCAN1 as a novel regulator of vesicle release and quantal release kinetics (Keating *et al.* 2008). These experiments were undertaken in mouse adrenal chromaffin cells using carbon fibre amperometry, a method which measures the number of individual vesicles undergoing exocytosis and the amount released from each vesicle. Additionally, exocytotic events in these cells are often associated with a pre-spike "foot" (PSF) signal that represents release of catecholamine during the formation of a stable fusion pore. We previously illustrated that altering *RCAN1* expression reduces the number of exocytotic events occurring in chromaffin cells (Keating et al. 2008). We also observe that increased levels of RCAN1 reduced the amount released from individual vesicles and decreased PSF signal duration (Keating et al. 2008). These effects do not appear to be due to changes in vesicle loading or size, alterations in Ca^{2+} entry or the size of the readily releasable pool of vesicles.

The most well characterised role of RCAN1 is as an inhibitor of calcineurin activity (Fuentes et al. 2000, Kingsbury *et al.* 2000, Rothermel *et al.* 2000). There are several potential mechanisms by which RCAN1 might regulate exocytosis and quantal release kinetics via its effects on calcineurin activity. Reduced calcineurin activity caused by RCAN1 overexpression could alter the phosphorylation and activity of a number of calcineurin targets involved in exocytosis and vesicle recycling such as Munc18 (Craig *et al.* 2003), dynamin 1, amphiphysin 1/2 and synaptojanin (Cousin & Robinson 2001). Such an explanation focuses on the effect that relatively acute alterations in calcineurin activity could have on these processes. Chronic alterations in calcineurin activity may also have transcriptional effects via the well-characterised calcineurin/NFAT signalling node, which regulates presynaptic neurotransmitter release (Freeman *et al.* 2011). RCAN1 may also affect exocytosis and quantal release kinetics via direct mechanisms that are independent of its effect on calcineurin activity.

Here we identify the role of calcineurin activity associated with the regulation of exocytosis by RCAN1. Increased RCAN1 expression reduces vesicle endocytosis and recycling in a repeated stimulation protocol and this effect is caused by the inhibition of calcineurin in cell overexpressing RCAN1. Chronic (3 days *in vitro*) calcineurin inhibition in WT cells replicates the changes in quantal release kinetics observed in $RCAN1^{ox}$ cells and such calcineurin inhibition has no additional effect of the amount of catecholamine released per vesicle in $RCAN1^{ox}$ cells. This treatment in $RCAN1^{ox}$ cells does, however, have further effect on PSF signal kinetics illustrating an effect of RCAN1 on early quantal release stability that is independent of its effects on calcineurin activity. Electron microscopy confirms that RCAN1 overexpression does not affect vesicle distribution or morphology in chromaffin cells. Therefore, increased RCAN1 expression inhibits calcienrin activity to negatively effect the amount of vesicle content released as well as affects vesicle endocytosis and recycling.

Materials and Methods

Chromaffin cell culture

Adrenal glands were taken from 6-8 week-old male wild type (WT) and *RCAN1*^{ox} mice and chromaffin cells were prepared as previously (Keating et al. 2008) as approved by the Flinders University Animal Welfare Committee. Briefly, the adrenal medulla was dissected out in cold Locke's buffer (154 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 5.6 mM glucose, 5.0 mM HEPES, pH 7.4) and then incubated with collagenase type A (Roche, Germany) in Locke's buffer at a concentration of 3 mg/ml in a shaking bath at 37°C. The collagenase was diluted further in cold Locke's buffer, cells pelleted and resuspended in cell culture medium (DMEM medium supplemented with 100 units/ml penicillin and 100 mg/ml streptomycin (Invitrogen, Carlsbad, CA, USA), 2mM L-glutamine (Invitrogen, Carlsbad, CA, USA) and 10% FCS (JRH Biosciences, Lenexa, USA)) and filtered through nylon mesh. Cells were pelleted, resuspended in supplemented DMEM and plated on 35 mm culture dishes and incubated at 37°C with 5% CO_2. Cells were maintained in primary culture for 3 to 4 days prior to experiments.

Carbon Fiber Amperometry

Catecholamine release from single chromaffin cells was measured using amperometry (Maritzen *et al.* 2008, Yu *et al.* 2008). A carbon fibre electrode (ProCFE, Dagan Corporation, USA) was placed on a chromaffin cell and +800 mV applied to the electrode under voltage clamp conditions. Current due to catecholamine oxidation was recorded using an EPC-9 amplifier and Pulse software (HEKA Electronic, Germany), sampled at 10 kHz and low-pass filtered at 1 kHz. For quantitative analysis, files were converted to Axon Binary Files (ABF Utility, version 2.1, Synaptosoft, USA) and secretory spikes analysed (Mini Analysis, version 6.0.1, Synaptosoft, USA) for a period of 60s from the start of stimulation. The standard bath solution (Krebs buffer) contained 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1mM $MgCl_2$, 5mM D-glucose, 10 mM HEPES, pH 7.4. High K^+-containing solution was the same as the control bath solution except that 70 mM K^+ replaced an equimolar amount of NaCl. All solutions were applied to cells using a gravity perfusion system, the outlet of which was placed within 500μm from the recorded cell. All experiments were carried out at room temperature (22–24°C). Acute and chronic calcineurin inhibition experiments were conducted using solutions containing FK-506 (5μM) and cyclosporine A (5μM) (Sigma Australia). Acute treatments involved incubating cells with inhibitors in Krebs buffer for 5 minutes at room temperature prior to stimulations, which were then carried out in the presence of the inhibitors. Chronic inhibition treatment involved incubating chromaffin cells, prepared the previous day, in cell culture medium containing the inhibitors for 3 days at 37°C with 5% CO_2. Stimulations were performed using Krebs buffers containing the inhibitors.

Amperometry data analysis

Data analysis was performed as previously described (Keating et al. 2008). Briefly, amperometric spikes were selected for analysis of event frequency if spike amplitude exceeded 10 pA and overlapping peaks were included. For kinetic analysis of spikes and PSF signals, only those events which exceeded 20 pA or were not overlapping with other spikes were included. Only pre-spike foot features longer than 1 ms and >2.5 times the RMS noise of the baseline in that recording were analyzed as foot signals.

Electron microscopy

Adrenal tissue was fixed and stained using a modified chromaffin reaction (Tranzer & Richards 1976). Adrenal glands were fixed for 6 hours at 4C in 0.1M phosphate buffer (PB) pH 7.2, containing 3% glutaraldehyde. The tissue was then transferred to

0.1M PB, pH 7.2 and kept overnight at 4C. The samples were washed 3 times in 0.1M PB before being post-fixed in 1% osmium tetroxide in 0.1M PB, pH 7.2 for 1hr at room temperature. After multiple washes the tissue was stained with 2% aqueous uranyl acetate for 30 minutes. Following a series of dehydration steps with ethanol solutions (50%-100%) and propylene oxide, the tissue was incubated for 1 hour in a 1:1 mixture of propylene oxide: Durcupan resin (Sigma; Sigma-Aldrich Pty Ltd, Castle Hill, Australia). The samples were then embedded in pure resin in capsules and polymerization was carried out for 48 hours at 60C. An ultramicrotome (RMC Mechanical Advance Ultramicrotome) was used to cut thick sections (1µm) which were stained with 1% toluidine blue in 1% borax for light microscopic examinations. Ultrathin sections (80-100nm) were cut using a diamond knife (Diatome) and sections were mounted on single-slot copper grids which were coated with 0.6% - 0.8% Butvar solution in chloroform. The sections were then stained with Reynolds' lead citrate solution (Reynolds 1963) and allowed to dry overnight. The ultra-thin sections were observed under transmission electron microscopy (JOEL1200-EX transmission electron microscope), and images were taken at 30,000 magnification. Ultrastructural images were captured with a Megaview3 camera using the ITEM interface program. Higher resolution montages of selected cells were compiled using the Multiple Image Alignment module in ITEM.

Chromaffin granule counting and statistics

Images were analyzed using ImageJ software (NIH). The number and spatial distribution of LDCVs were evaluated on micrographs covering the entire cell's cytoplasmic region. LDCVs were identified by their electron-dense core and circular membrane. To measure vesicle localisation, 200 nm-wide concentric zones starting from the cell membrane were defined within each cell and the number of dense core vesicles within each region counted and presented as a percentage of the total number of vesicles. Dense core and vesicle diameter were measured from 50 vesicles per cell. Representative regions within the cell were selected by overlaying a numbered grid on each electron micrograph. A random number generator was then used to identify the LDCV containing regions to be analysed. All electron micrographs taken at a magnification of 4000X and were analyzed using ImageJ image analysis software (NIH).

Statistical analysis

For amperometry whole spike analysis, as data is not parametrically distributed we obtain the median value in each recording and present the final data as the mean of these median values (Maritzen et al. 2008). This data is then analysed using a Student's unpaired t-test. For PSF analysis we often do not obtain sufficient data points to use the median value from each recording. Therefore for these parameters we obtain the mean of each individual value and gauge statistical differences between the means of different groups using the Mann–Whitney test. All data are presented as mean ± SEM. For electron microscopy analysis, data is represented as the average number of LDCVs per unit area ± SEM. Data for each genotype was compared using a Student's unpaired t-test. In all statistical tests significance was obtained at $p<0.05$.

Results

RCAN1 overexpression does not alter vesicle localisation in chromaffin cells

We have previously demonstrated changes in exocytosis in RCAN1^{ox} chromaffin cells (Keating et al. 2008). We therefore used electron microscopy to analyse whether changes in exocytosis in RCAN1^{ox} cells may be due to alterations in vesicle number and localisation. The ultrastructural appearance of WT (Figure 1A) and RCAN1^{ox} (Figure 1B) chromaffin cells appears similar and we find that the average number of vesicles per unit area is not different between WT (n=7 cells) and RCAN1^{ox} cells (n=9 cells, Figure 1C). We also assessed the distribution of vesicles with regard to their distance from the plasma membrane in these cells. We find no differences in the average distance from the membrane between WT and RCAN1^{ox} cells (Figure 1D). These results indicate that altered exocytosis in RCAN1^{ox} chromaffin cells is not due to altered vesicle number or distribution. We additionally measured vesicle size in these cells and observe a significant decrease both in mean vesicle diameter (Figure 1E, $p<0.001$) and dense core diameter (Figure 1F, $p<0.001$). This data indicates that catecholamine loading into vesicles is negatively affected in RCAN1^{ox} chromaffin cells.

RCAN1 regulates vesicle recycling in chromaffin cells by inhibiting calcineurin.

Calcineurin controls endocytosis primarily through regulation of the activity of the endocytic proteins known as the dephosphins (Cousin & Robinson 2001). As endocytosis and exocytosis are directly interlinked, we wished to know whether endocytosis and vesicle recycling defects might underlie reduced levels of exocytosis in RCAN1^{ox} cells. To gauge this we implemented a repeated stimulations protocol, consisting of three one-minute stimulations with 5 minute intervals between them that reflects the release of vesicles that have been endocytosed in a dynamin-dependent manner after the initial stimulation (Elhamdani *et al.* 2001). This protocol induces consistent levels of exocytosis in WT cells (Figure 2A-D). However when we acutely inhibit calcineurin activity in WT cells using the calcineurin inhibitors cyclosporine A and FK-506, the result is a decline ($P<0.05$) in the number of vesicles undergoing exocytosis during subsequent stimulations (Figure 2E-H). This indicates that calcineurin controls vesicle recycling in WT chromaffin cells and that acute exposure to known inhibitors of calcineurin activity causes a rundown of secretion with this repeated stimulation protocol. We repeated these experiments in RCAN1^{ox} chromaffin cells. RCAN1^{ox} cells display a rundown in exocytosis in the absence of any calcineurin inhibitors using this protocol (Figure 3A-D), indicative of a recycling defect in RCAN1^{ox} chromaffin cells. Importantly, acute exposure to inhibitors of calcineurin in RCAN1^{ox} cells has no additional effect on vesicle recycling compared to RCAN1^{ox} cells in the absence of these inhibitors (Figure 3E-H). These results therefore support the possibility that calcineurin activity controls vesicle recycling in chromaffin cells and that RCAN1 overexpression in RCAN1^{ox} cells may inhibit vesicle recycling due to reduced activity of calcineurin.

Repeated acute inhibition of calcineurin does not alter quantal release kinetics.

Our previous studies identified a role for RCAN1 in the regulation of the amount of catecholamine released from individual vesicles and that this change was not replicated when we acutely inhibited calcineurin and stimulated cells a single time (Keating et al. 2008). However, we find in the present study that vesicle recycling is reduced following repeated stimulations in the presence of calcineurin inhibitors. We hypothesise that this is due to altered dephosphorylation of calcineurin target proteins involved in vesicle fission and fusion. As the phosphorylation status of some exocytosis proteins, such as Munc-18 (Craig et al. 2003), is affected by calcineurin activity, we therefore wished to identify if quantal release kinetics are also affected

during this repeated stimulations protocol in the presence of cyclosporine A and FK-506. We therefore analysed the spike kinetics from the previous experiments in which we acutely exposed cells to cyclosporine A and FK-506 for 10 minutes in WT cells and repeatedly stimulated cells in the continued presence of these drugs. We find that this has no effect on vesicle release kinetics such as amperometric spike area (Figure 4A), rise time (Figure 4B), half-width (Figure 4C) or decay time (Figure 4D). Therefore, short-term exposure to calcineurin inhibitors, even during repeated stimulations, does not affect quantal release kinetics and the amount of catecholamine released per vesicle.

RCAN1 overexpression may reduce the amount released per vesicle via the chronic inhibition of calcineurin activity.

As calcineurin activity is thought to be chronically, rather than acutely, inhibited in RCAN1^{ox} cells, we next investigated whether chronic exposure to calcineurin inhibitors could explain the effect of RCAN1 overexpression on vesicle release in RCAN1^{ox} chromaffin cells. We cultured chromaffin cells for 3 days in the presence or absence of cyclosporine A and FK-506 and compared the effect on amperometric spike kinetics in both WT and RCAN1^{ox} cells. Chronic exposure to calcineurin inhibitors reduced the total amount of catecholamine released per vesicle in WT cells (Figure 5A). Unsurprisingly therefore, the rise time (Figure 5B), half-width (Figure 5C) and decay time (Figure 5D) of WT spikes were also changed during chronic exposure to cyclosporine A and FK-506. When we undertook these experiments in RCAN1^{ox} cells we observe the reduced release from vesicles that we previously published (Keating et al. 2008). Importantly, when we chronically exposed RCAN1^{ox} cells to cyclosporine A and FK-506 we observe no additional decrease in these spike parameters. Thus it is possible that chronic inhibition of calcineurin activity reduces quantal release kinetics and that the reduced amount of catecholamine released from RCAN1^{ox} vesicles may be due to chronic inhibition of calcineurin caused by RCAN1 overexpression.

Calcineurin activity may also be important for regulating release during the earliest stages of exocytosis.

In amperometric recordings, a pre-spike foot (PSF) signal is often observed before a full secretory spike. This PSF represents the release of catecholamine through the developing fusion pore as it transitions to a stable, fully dilated pore. As we previously reported reductions in the PSF signal (Keating et al. 2008) when RCAN1 is overexpressed, we analysed changes in the PSF signal in WT and RCAN1^{ox} cells when cells are chronically exposed to cyclosporine A and FK-506. In WT cells we observe no change in PSF signal amplitude when cells are exposed to these drugs (Figure 6A) but do observe significant decreases in PSF signal duration ($P<0.001$, Figure 6B) and area ($P<0.001$, Figure 6C). In RCAN1^{ox} cells, chronic calcineurin inhibition caused a significant decrease in PSF signal duration ($P<0.001$) and area ($P<0.01$).

Discussion

We have previously shown that RCAN1 is a regulator of exocytosis and is involved in the regulation of both the amount of catecholamine released from vesicles and the number of vesicles undergoing fusion (Keating et al. 2008). In the present study we reveal further insights into the role of RCAN1 in the regulation of exocytosis. We firstly identify that overexpression of RCAN1 and the acute use of pharmacological agents which inhibit calcineurin activity both reduce vesicle recycling and re-use. Furthermore the combination of these two treatments has no additive effect in our vesicle recycling assay. These effects are not caused by changes in vesicle number or distribution as evidenced from our electron microscopy studies. Short term (minutes) use of drugs known to inhibit calcineurin activity does not affect the amount of catecholamine released per vesicle during our repeated stimulation protocol. However when we chronically (days) expose cells to these calcineurin inhibitors, as would occur if RCAN1 overexpression were inhibiting calcineurin in RCAN1^{ox} cells, we find the amount of catecholamine released from single vesicles to be drastically reduced to a simlar level to that seen in RCAN1^{ox} cells. Our finding that the chronic use of calcineurin inhibitors does not have any additional effect on vesicle release in RCAN1^{ox} cells suggests that RCAN1 overexpression in these cells reduces quantal vesicle release via the inhibition of calcineurin activity. Our EM analysis further illustrates that the underlying mechanism explaining reduced vesicle release in RCAN1^{ox} cells is a reduced catecholamine loading into vesicles. Intriguingly however, we still observe an effect of cyclosporine A and FK-506 in RCAN1^{ox} cells on the PSF signal.

The progressive decrease in exocytosis observed with our repeated stimulation protocol in the presence of cyclosporine A and FK506 suggests that vesicle endocytosis and recycling are impaired when calcineurin activity is blocked. This result is identical to the effect of dynamin inhibition in chromaffin cells using the same protocol (Elhamdani et al. 2001) and indicates the protocol provides a measurement of vesicle recycling and re-use in these cells. Overexpression of RCAN1 induces the same decrease in vesicle recycling. The lack of any further effect with pharmacological calcineurin inhibition indicates that RCAN1 overexpression may be inhibiting calcineurin activity to reduce recycling and re-use in subsequent stimulations. A likely mechanism underlying this is inhibition of the dephosphins, the group of structurally diverse proteins that are essential for vesicle endocytosis and which are all dephosphorylated by calcineurin. These proteins include dynamin, amphiphysin 1 and 2, synaptojanin, AP180, Epsin and Eps15 (Cousin & Robinson 2001).

We also tested whether spike kinetics are affected by RCAN1 overexpression or calcineurin inhibition during these repeated stimulations experiments. In these experiments we hypothesised that reduced calcineurin activity, which was potentially causing changes in endocytosis, may also affect quantal release kinetics. The phosphorylation state of most exocytosis proteins, including SNARE proteins, affects their function, and subsequently the kinetics of vesicle release. For example, Munc18-1 binds to syntaxin 1 with higher affinity when dephosphorylated by calcineurin (Craig et al. 2003). Therefore, if calcineurin activity is decreased in RCAN1^{ox} cells, Munc18-1 may bind to syntaxin1 with lower affinity, resulting in unstable fusion pore formation and accelerated fusion pore kinetics. Acceleration of fusion pore kinetics as a result of decreased Munc18 phosphorylation has been previously demonstrated (Barclay *et al.* 2003) using Munc18 phosphomimetic mutations. The lack of effect on vesicle release kinetics by acute exposure to cyclosporine A and FK-506 indicates that short term calcineurin inhibition, even during repeated stimulation, does not alter vesicle release.

Our experiments in which we chronically inhibit calcineurin activity in WT cells are the first example illustrating that this enzyme may control vesicle release kinetics. This effect may be due to changes in phosphorylation state of Munc18 or other exocytosis proteins as discussed or alternatively, longer term exposure to cyclosporine A and FK-506 may affect release due to changes in gene transcription that take longer than the total 10-30 minute duration of the acute inhibition experiments. Calcineurin is able to affect transcription through the NFAT transcription pathway (Clipstone & Crabtree 1992). Loss of NFAT function in pancreatic β-cells causes hypoinsulinemia due to downregulation of insulin gene transcription and insulin synthesis (Heit *et al.* 2006). NFAT also regulates activity-dependent plasticity in *Drosophila* (Freeman et al. 2011) and NFAT-dependent transcription controls both morphological and electrophysiological properties of neurons (Schwartz *et al.* 2009). As amperometry measures only the release of oxidisable catecholamines, we cannot be sure if altered fusion pore kinetics underlie the reduced catecholamine release from individual vesicles or whether such changes are due to factors affecting the amount of catecholamine packaged inside each vesicle. Our electron microscopy results demonstrating a decrease in dense core volume in RCAN1^{ox} cells clearly point towards a defect in catecholamine loading in these cells and would argue against an effect on fusion pore kinetics. Global gene expression analysis may provide useful information regarding potential transcriptional changes occurring in RCAN1^{ox} chromaffin cells and whether transcriptional changes affect various secretion-associated processes.

While we observe no effect of chronic exposure to cyclosporine A and FK506 on single vesicle catecholamine release in RCAN1^{ox} cells, effects on PSF signal duration and area do occur. The PSF signal represents catecholamine flux through the transient, unstable fusion pore as it transitions to an open, stable full fusion pore (Zhang & Jackson 2010, Zhou *et al.* 1996). Our results present at least two intriguing possibilities. Firstly, RCAN1 may have a direct effect on the earliest stages of fusion pore formation independent of its role as a calcineurin inhibitor. There are currently no identified protein binding partners of RCAN1 which might explain such an effect. RCAN1 contains two proline-rich domains (Fuentes *et al.* 1995) which typically bind to SH3 regions. SH3 regions are contained in a number of proteins associated with vesicle trafficking, exocytosis and endocytosis including ITSN1 and amphiphysin 1 and 2 (Keating *et al.* 2006) but whether such interactions occur or even regulate fusion pore development is an area of future investigation. Secondly, RCAN1 overexpression in these cells may not fully inhibit calcineurin and only when calcineurin is completely inhibited pharmacologically do we see the full effect of cyclosporin A and FK506 on the PSF signal. There may therefore be differences in calcineurin sensitivity associated with the mechanisms underlying the amount released per vesicle (spike area) compared to the regulation of the PSF signal.

It must also be noted that the calcineurin inhibitors used in this study have effects other than calcineurin inhibition. In order to competitively bind to and inhibit calcineurin activity cyclosprine A forms complexes with cyclophilin D while FK506 complexes with FK506 binding protein (FKBP) (Liu *et al.* 1991). Cyclophilin D can also bind the mitochondrial permeability transition pore to effect mitochondrial function (Elrod *et al.* 2010) while FK506 reduces the peptidyl-prolyl isomerase activity of FKBP (Brecht *et al.* 2003). Thus, while calcineurin inhibition is the primary therapeutic effect of these immunosuppressants, it is possible that the changes in secretion we observe with these drugs could be independent of changes in calcineurin activity. However, given the lack of additive effect on vesicle recycling and quantal vesicle release when we overexpress an endogenous calcineurin inhibitor (RCAN1) and add known calcineurin inhibitors, our primary conclusion is that these

treatments affect secretion through the same mechanism by reduced calcineurin activity.

In summary, our results demonstrate that RCAN1 regulates cell communication at several stages. Endocytosis and vesicle re-use as well as vesicle release is controlled by RCAN1 as well as by pharmacological calcineurin inhibitors, likely through the same mechanism of action of reduced calcineurin activity. What the sites of calcineurin/RCAN1 are in these instances remains to be identified. The reduction in vesicle size and catecholamine loading observed in our EM experiments seem to explain the decreased catecholamine release per vesicle in $RCAN1^{ox}$ cells. Catecholamine release through the developing fusion pore is additionally affected by RCAN1. This effect of RCAN1 may be either be independent of the inhibitory action of RCAN1 on calcineurin or be explained by a differential sensitivity to calcineurin activity between early and full fusion release. Our data therefore reveals a role of RCAN1 in regulating exocytosis at several stages. Recently published data illustrates that changes in hippocampal structure and function occur in these $RCAN1^{ox}$ mice brains (Martin *et al.* 2012). However these changes may not be associated with alterations in calcineurin activity and involve instead changes directly associated with RCAN1 function. Therefore, while increased expression of RCAN1 may play a significant role in the cognitive and neuronal pathologies associated with Alzheimer's disease and Down syndrome, elucidating which of these are associated with the role of RCAN1 as an inhibitor of calcineurin needs to be clearly defined and may well vary for individual types of cells or neurons. This point is further highlighted by recent evidence demonstrating that the effects of RCAN1 on calcineurin are dependent on a number of factors including the degree of RCAN1 expression and the presence of other RCAN1-bidning partners that effect the relationship between RCAN1 and calcineurin (Liu *et al.* 2009).

Acknowledgements
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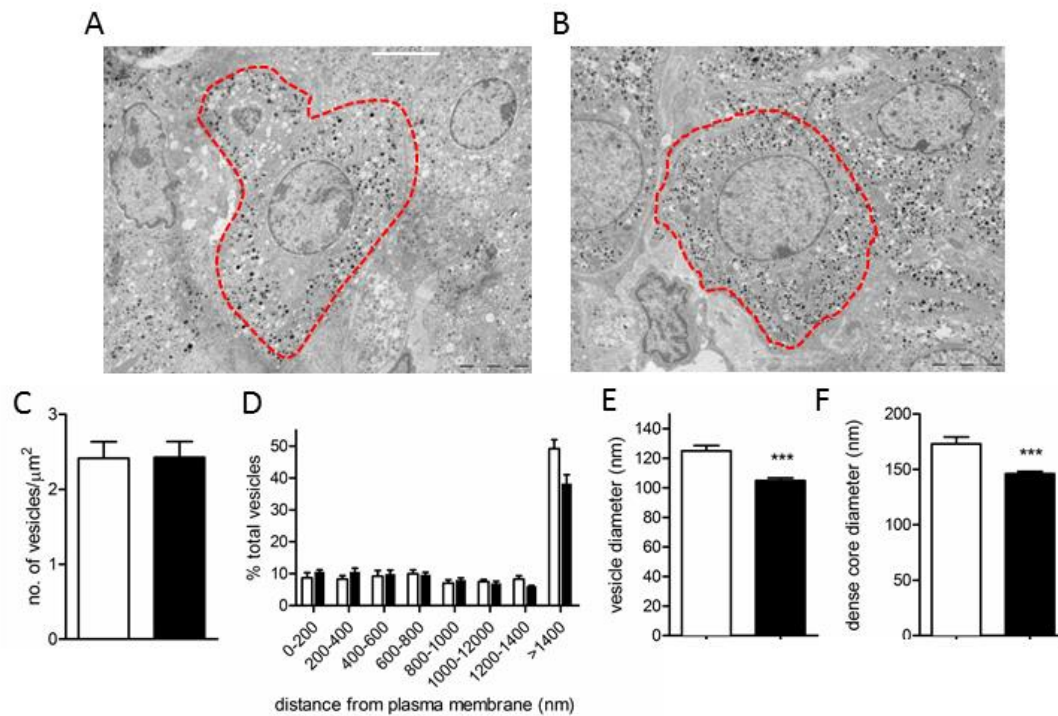

Figure 1

Figure 1: RCAN1 overexpression does not alter vesicle number or localisation. A representative electron micrograph of an adrenal medulla section from (A) WT and (B) RCAN1^{ox} mice clearly shows the large dense core vesicles throughout each chromaffin cell. The red line indicates the plasma membrane of a single chromaffin cell. (C) The average number of large dense core vesicles per unit area is not different nor is (D) the localisation of vesicle from the plasma membrane. (E) Vesicle diameter and (F) dense core diameter are significantly smaller in RCAN1^{ox} chromaffin cells indicating reduced catecholamine storage in RCAN1^{ox} vesicles. N=3 animals per genotype and n=7 and 9 different cells from WT and RCAN1^{ox} mice, respectively, ***$p<0.001$.

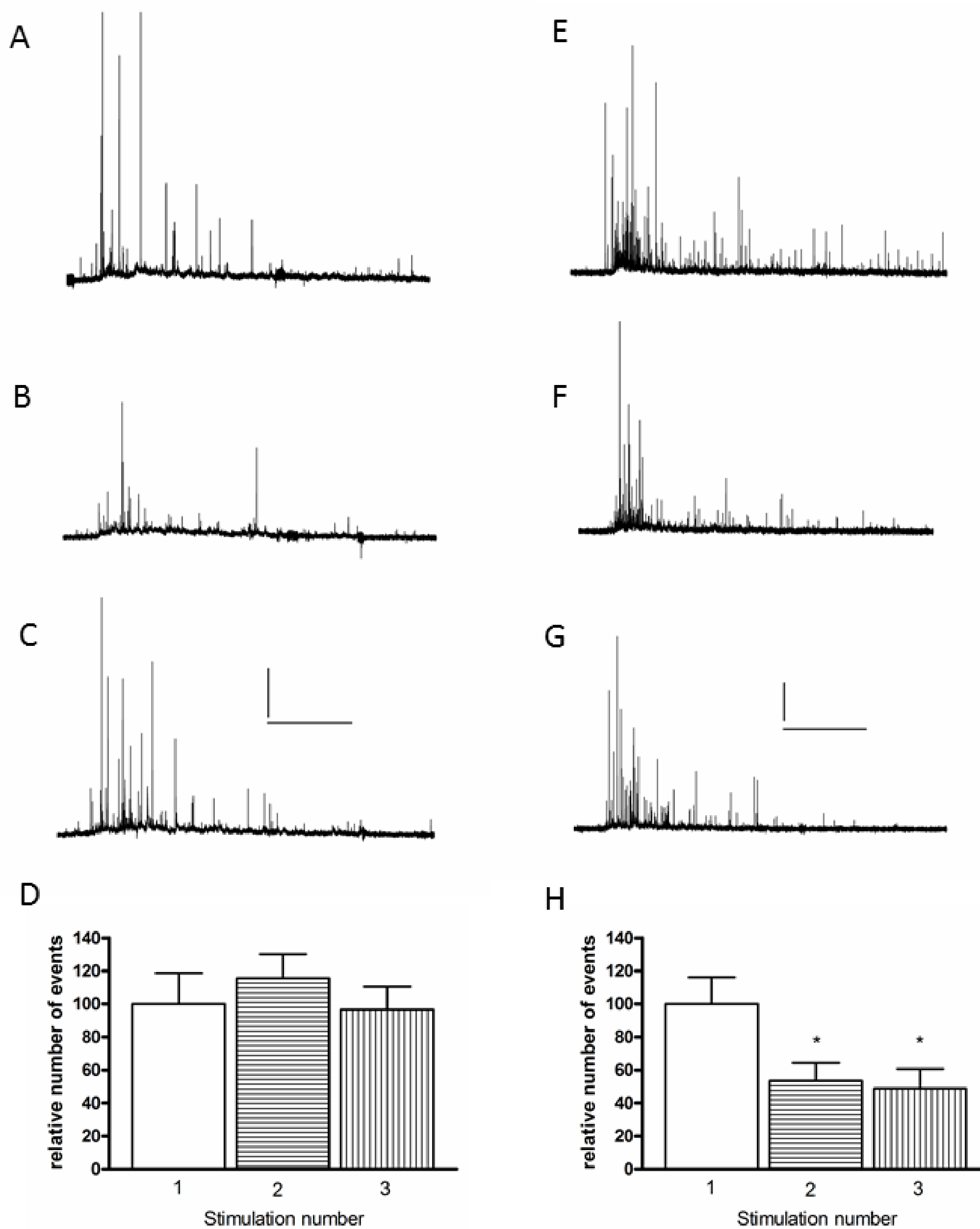

Figure 2

Figure 2: Calcineurin inhibition reduces exocytosis during repeated stimulations.
Cells were stimulated 3 successive times for 1 minute with 5 minutes rest between stimulations (Elhamdani et al. 2001). In WT cells we observe similar levels of exocytosis between the first (A), second (B) and third (C) stimulations. (D) The average number of exocytotic events does not decrease with this protocol (n=6 cells). Repeating these experiments in the acute presence of the calcineurin inhibitors, cyclosporine A and FK-506 results in exocytotic events (E) that reduce in frequency with a second (F) and third (G) stimulation. (H) This reduction was significant upon the second and third stimulations (H, n=16 cells, *$p<0.05$).

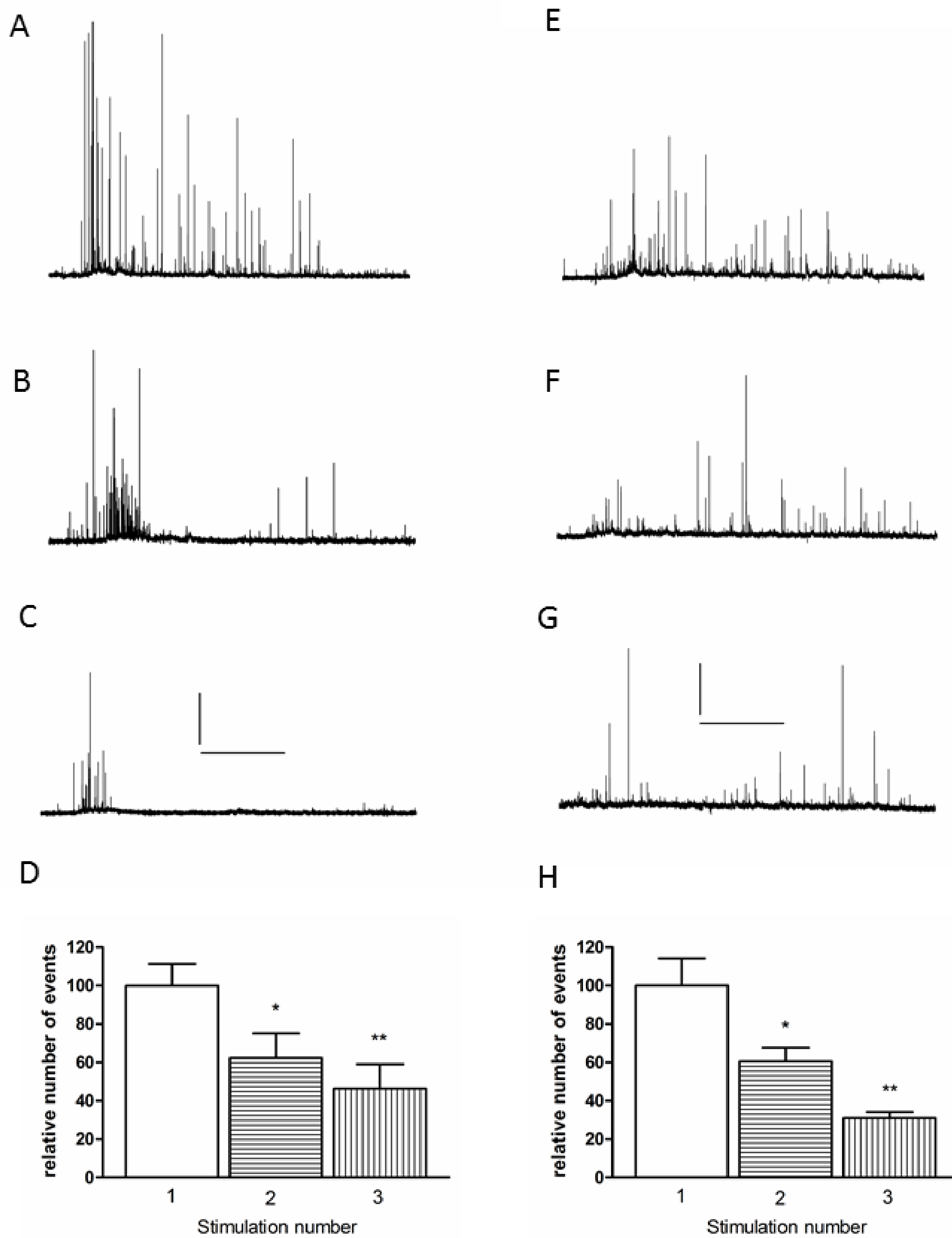

Figure 3: Exocytosis rundown occurs in RCAN1^{ox} cells with no additional effect of acute calcineurin inhibition. Cells were stimulated 3 successive times for 1 minute with 5 minutes rest between stimulations (Elhamdani et al. 2001). In RCAN1^{ox} cells we observe a gradual decline in exocytosis between the first (A), second (B) and third (C) stimulations. (D) The average number of exocytotic events significantly decreases with this protocol (n=7 cells, *$p<0.05$, **$p<0.01$). Repeating these experiments in the acute presence of the calcineurin inhibitors, cyclosporine A and FK-506 also results in exocytotic events (E) and these reduce in frequency with a second (F) and third (G) stimulations. (H) This reduction was significant upon the second and third stimulations (H, n=4 cells, *$p<0.05$, **$p<0.01$).

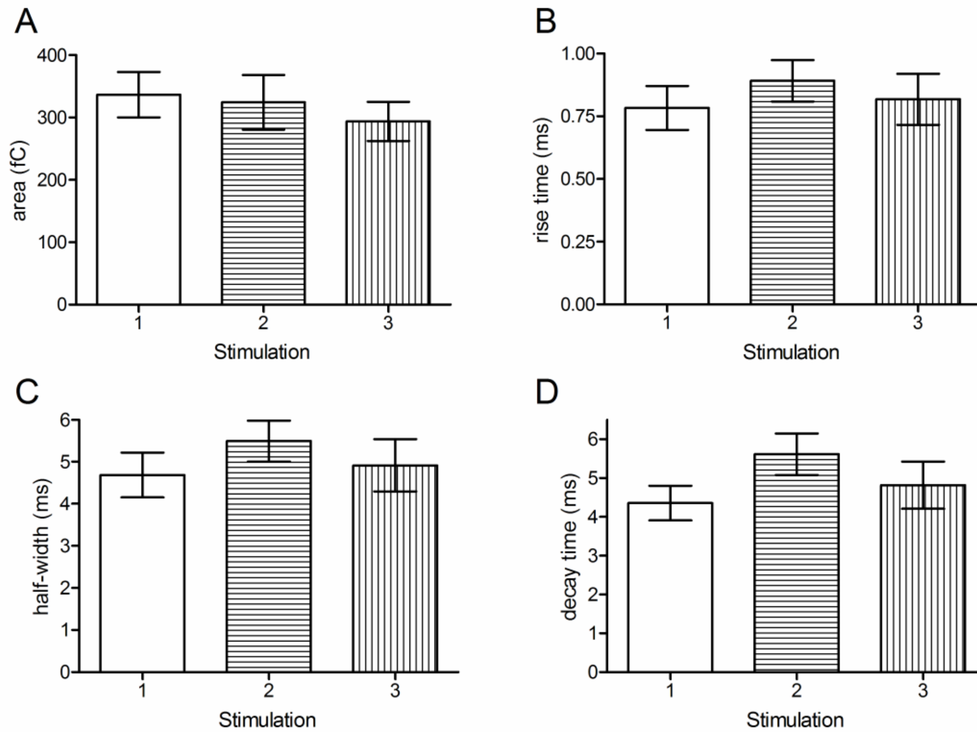

Figure 4: Repeated stimulation of WT chromaffin cells during acute calcineurin inhibition does not affect vesicle release kinetics. While acute calcineurin inhibition during repeated stimulations causes reduced exocytosis rates, it does not affect amperometric spike (A) area, (B) rise time, (C) half-width or (D) decay time (n=16 cells) when comparing these parameters between the first (white bar), second (horizontal stripes) and third (vertical stripes) stimulation.

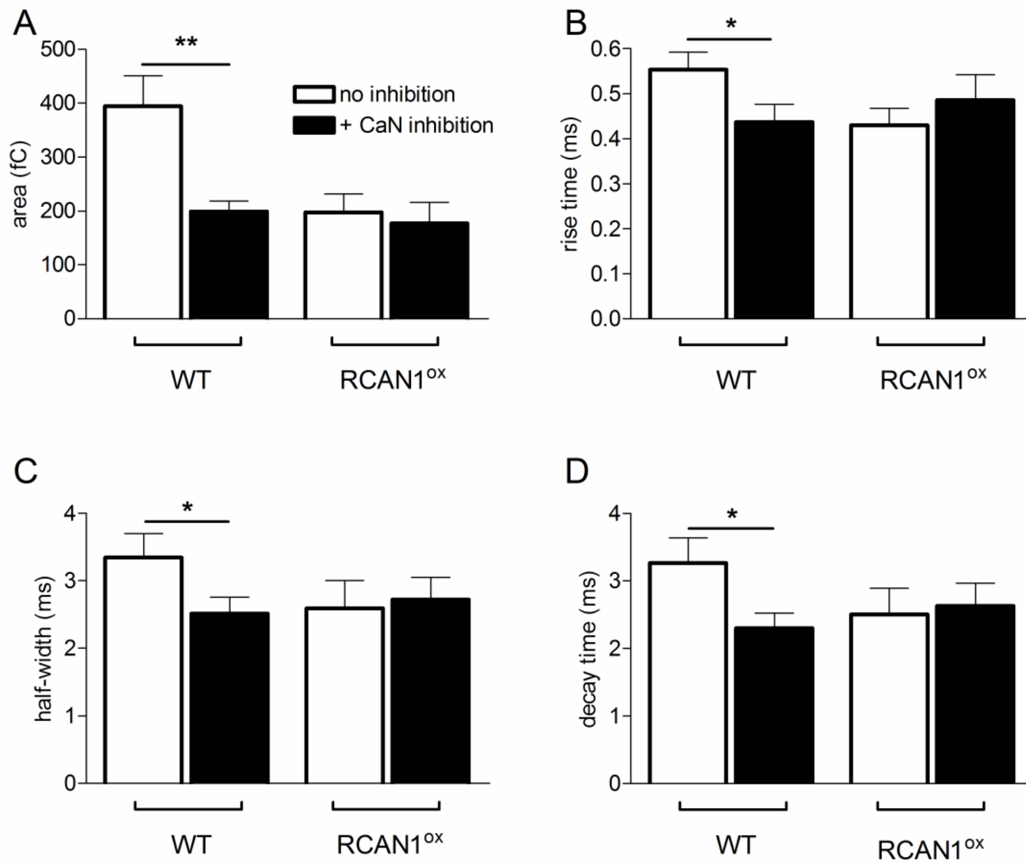

Figure 5: Chronic calcineurin inhibition underlies the altered quantal release kinetics observed in RCAN1^{ox} chromaffin cells. Cells were treated for 4-6 days in culture with calcineurin inhibitors, cyclosporine A and FK-506 (5 μM, indicated by + CaN inhibition). In WT cells this resulted in significant reductions in (A) spike area, (B) rise time, (C) half-width and (D) decay time. However this caused no change in these parameters in RCAN1^{ox} cells. *P<0.05, **P<0.01 In WT n=11 control conditions and 17 under calcineurin inhibition. In RCAN1^{ox} n=11 in control and 10 under calcineurin inhibition.

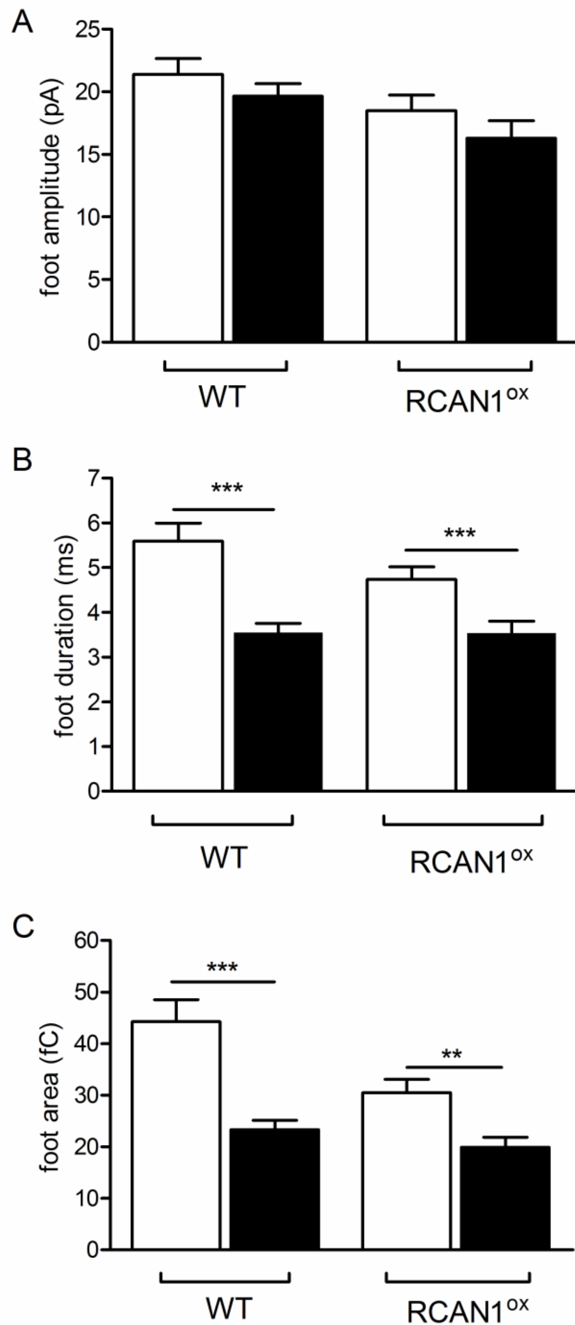

Figure 6: Chronic calcineurin inhibition reduces aspects of the PSF signal in both WT and RCAN1^{ox} cells. From the same experiments as those shown in Figure 4, we observe that chronic calcineurin inhibition has (A) no effect on PSF amplitude in WT or RCAN1^{ox} cells but decreases both (B) PSF signal duration and (C) PSF signal area in WT and RCAN1^{ox} cells. **P<0.01, ***P<0.001 In WT n=207 spikes in control conditions and 285 under calcineurin inhibition. In RCAN1^{ox} n=287 spikes in control and 166 under calcineurin inhibition.

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