DSCR1/RCAN1 regulates vesicle exocytosis and fusion pore kinetics: implications for Down syndrome and Alzheimer’s disease

Damien J. Keating1,2, Daphne Dubach3, Mark P. Zanin1, Yong Yu3, Katherine Martin3, Yu-Feng Zhao2, Chen Chen2, Silvia Porta4,5, Maria L. Arbonés5, Laureane Mittaz3 and Melanie A. Pritchard3,*

1Molecular and Cellular Neuroscience Group, Department of Human Physiology and Centre for Neuroscience, Flinders University, Adelaide, Australia, 2Endocrine Cell Biology Group, Prince Henry’s Institute of Medical Research, Clayton, Victoria, Australia, 3Centre for Functional Genomics and Human Disease, Monash Institute of Medical Research, Monash University, Clayton, Victoria, Australia, 4CIBER Epidemiología y Salud Pública (CIBERESP), Barcelona, Spain and 5Genes and Disease Program, Center for Genomic Regulation (CRG), UPF, Barcelona, Spain

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Genes located on chromosome 21, over-expressed in Down syndrome (DS) and Alzheimer’s disease (AD) and which regulate vesicle trafficking, are strong candidates for involvement in AD neuropathology. Regulator of calcineurin activity 1 (RCAN1) is one such gene. We have generated mutant mice in which RCAN1 is either over-expressed (RCAN1ox) or ablated (Rcan1−/−) and examined whether exocytosis from chromaffin cells, a classic cellular model of neuronal exocytosis, is altered using carbon fibre amperometry. We find that Rcan1 regulates the number of vesicles undergoing exocytosis and the speed at which the vesicle fusion pore opens and closes. Cells from both Rcan1−/− and RCAN1ox mice display reduced levels of exocytosis. Changes in single-vesicle fusion kinetics are also evident resulting in the less catecholamine released per vesicle with increasing Rcan1 expression. Acute calcineurin inhibition did not replicate the effect of RCAN1 overexpression. These changes are not due to alterations in Ca2+ entry or the readily releasable vesicle pool size. Thus, we illustrate a novel regulator of vesicle exocytosis, Rcan1, which influences both exocytotic rate and vesicle fusion kinetics. If Rcan1 functions similarly in neurons then overexpression of this protein, as occurs in DS and AD brains, will reduce both the number of synaptic vesicles undergoing exocytosis and the amount of neurotransmitter released per fusion event. This has direct implications for the pathogenesis of these diseases as sufficient levels of neurotransmission are required for synaptic maintenance and the prevention of neurodegeneration and vesicle trafficking defects are the earliest hallmark of AD neuropathology.

INTRODUCTION

Down syndrome (DS) is caused by the trisomy of human chromosome 21. DS individuals are mentally impaired, many experience seizures, and most develop Alzheimer’s disease (AD)-like neuropathology by their mid-40s, characterized by β-amyloid (Aβ) peptide-containing plaques, tau-containing neurofibrillary tangles, basal forebrain cholinergic neuron degeneration and dementia (1). The mechanisms underlying the etiology of sporadic AD remain undefined although mechanisms involving dysfunctional processing of APP, abnormal tau protein handling, mitochondrial dysfunction and abnormal vesicle trafficking are currently being explored. Abnormal vesicle trafficking precedes neuronal Aβ deposition in DS and sporadic AD. Enlarged early endosomes, which are associated with abnormal vesicle trafficking (2), are
observed in DS neurons from as early as 28 of weeks gestation, at 2 months in the DS mouse model, Ts65Dn, and in sporadic AD, preceding Aβ peptide deposition (3,4). Proteins related to vesicle endocytosis and exocytosis have been linked to DS and AD pathology. SNAP-25 expression is decreased in brains of DS individuals and AD patients (5), synaptotagmin levels are increased in DS brains (6) and the neuronal dynamin kinase, Cdk5 (7), is implicated in neurodegeneration in AD brains (8,9).

Genes located on chromosome 21 which are over-expressed in DS and AD and involved in the regulation of vesicle trafficking are strong candidates for involvement in the neuropathology of DS and AD. Down syndrome candidate region 1 (DSCR1 recently renamed RCAN1, Regulator of calcineurin 1) is one such gene. RCAN1 is highly expressed in brain, heart and skeletal muscle (10), in endocrine tissues including the adrenal gland (11) and is over-expressed in the brains of DS fetuses (12) and in the cerebral cortex and hippocampus of sporadic AD patients (11). Primary neuronal cultures transfected with a Rcan1–EGFP construct illustrate that Rcan1 co-localizes with synaptophysin, suggesting Rcan1 may be expressed on synaptic vesicles (13). This over-expression of Rcan1 causes aggresome-like inclusion bodies similar to those observed in DS and AD brains and reduces synaptophysin expression in neural processes (13), implying that Rcan1 upregulation may compromise exocytosis. Loss-of-function and over-expression mutants of nebul, the Drosophila ortholog of RCAN1, both display severe learning defects in several basic learning assays (14). Neurons from these mutant flies have increased numbers of mitochondria which are reduced in size, increased reactive oxygen species (ROS) levels and reduced ATP production (15). While this work in Drosophila illustrated severe learning and memory defects caused by the altered expression of nebul, no evidence is provided on the cellular processes underlying such changes.

There are indications that over-expression of RCAN1, as observed in DS and AD, may adversely affect exocytosis, endocytosis and vesicle trafficking. As we are interested in the brain/neuronal phenotypes associated with DS, we decided to generate transgenic mice overexpressing the brain predominant isoform of RCAN1, isoform 1.1 (16). In this study, we use mutant mice in which RCAN1.1 is transgenically over-expressed (RCAN1\textsuperscript{ox}) or ablated (Rcan1\textsuperscript{−/−}) and examine whether exocytosis from chromaffin cells, a classic cellular model of neuronal exocytosis, is regulated by Rcan1.

RESULTS

RCAN1 transgenic mice are viable, develop normally and show no overt abnormal phenotype

For the generation of transgenic mice, the isoform 1 splice variant was placed under control of the endogenous promoter to mirror as closely as possible the endogenous situation in DS and AD (Fig. 1A). Rcan1 null (Rcan1\textsuperscript{−/−}) and RCAN1 transgenic (RCAN1\textsuperscript{ox}) mice were viable, developed normally and showed no overt abnormal phenotypes.

Expression of the endogenous mouse Rcan1 gene and the RCAN1 transgene was assessed in adrenal gland tissue by RT–PCR (Fig. 1C). Using human-specific oligonucleotide primer pairs, the human transgene mRNA was found to be expressed in the transgenic mice, with no expression in the wild type as expected (Fig. 1B). Protein expression was assessed in adrenal gland using an antibody specific to RCAN1. This antibody detects both the endogenous mouse protein and the human transgene. Figure 1C shows a western blot performed on protein extracts derived from transgenic, knockout and wild-type (WT) adrenals. When we quantified these results, the transgenic mice express ~4 times more RCAN1 protein than their WT counterparts (P < 0.001 compared with WT littermates, n = 3) and no Rcan1 protein was produced in Rcan1 null mouse adrenals (P < 0.01 compared with WT littermates, n = 3). Similar levels of expression were seen in both our WT groups.

Rcan1 regulates the rate of exocytosis and fusion pore dynamics in chromaffin cells

Amperometry is a technique which monitors the release of oxidizable neurotransmitters at a carbon fibre surface. This oxidation produces a current spike representing the exocytosis of a single-secretory vesicle. As only oxidizable transmitters are able to be measured using this technique, its use is limited to cells and neurons which release oxidizable transmitters such as dopamine, noradrenaline, adrenaline and serotonin. In order to test whether altered Rcan1 expression had any effect on vesicle recycling or neurotransmission, we investigated exocytosis from adrenal chromaffin cells of Rcan1\textsuperscript{−/−} and RCAN1\textsuperscript{ox} mice using amperometry (17). Chromaffin cells have long been used as a model neuron system when studying exocytosis. WT control chromaffin cells exhibited strong levels of exocytosis when stimulated with an external solution containing 70 mM K\textsuperscript{+} for 1 min (Fig. 2A). Cells from Rcan1\textsuperscript{−/−} mice displayed reduced levels of exocytosis (Fig. 2B) under these conditions. When the number of exocytotic events during this stimulation period was quantified, it was clear that initial rates of exocytosis were similar between these groups only for the first 5–10 s, after which time the number of events occurring in Rcan1\textsuperscript{−/−} cells rapidly fell away (Fig. 2C, n = 21). In control cells, 60.8 ± 6.9 exocytotic events occurred during this minute compared with 30.5 ± 2.9 events in Rcan1\textsuperscript{−/−} cells (P < 0.0005, n = 21, Fig. 2D). To test whether an increase in RCAN1 expression would also affect exocytosis, we measured secretion from chromaffin cells obtained from RCAN1\textsuperscript{ox} mice. Surprisingly, over-expression of RCAN1 also resulted in a significant reduction in the number of vesicles undergoing exocytosis (Fig. 3), similar to that observed in Rcan1\textsuperscript{−/−} cells. Over the stimulation period, the number of events occurring in RCAN1\textsuperscript{ox} cells fell away over time (Fig. 3C, n = 23). In RCAN1\textsuperscript{ox} cells, the number of exocytotic events in 1 min was 37.5 ± 5.4 compared with 58.9 ± 6.5 in cells cultured from control animals (Fig. 3D, P < 0.001).

Changes in single-vesicle fusion kinetics were observed in these recordings, indicating that Rcan1 regulates a late stage of exocytosis at the point of vesicle and plasma membrane fusion. While the average peak of each exocytotic spike was not different between control and Rcan1\textsuperscript{−/−} cells (Fig. 4A, n = 16), the amount of catecholamine released per fusion event was significantly increased in Rcan1\textsuperscript{−/−} cells.
This was caused by significantly slower rise ($P < 0.05$) and decay ($P < 0.05$) phases of current spikes in $Rcan1^{-/-}$ cells (Fig. 4C and D). As well as this, the half-width (width of a spike at half its maximal height) was significantly increased in $Rcan1^{-/-}$ cells ($P < 0.01$, Fig. 4E). A comparison of a representative spike from control and $Rcan1^{-/-}$ mice is shown in Figure 4F illustrating the difference in spike parameters when $Rcan1$ expression is ablated.

The release of catecholamine during an amperometric event is often preceded by a so-called ‘foot’ signal which occurs due to catecholamine release through the formation of an initial transient fusion pore (17,18). In $Rcan1^{-/-}$ cells, the percentage of events preceded by a foot signal was unchanged (Fig. 5A). The height of these foot signals was also unchanged in $Rcan1^{-/-}$ cells (Fig. 5B). Foot signals in $Rcan1^{-/-}$ cells lasted longer, however (Fig. 5C, $P < 0.05$), resulting in more neurotransmitter being released per foot signal (Fig. 5D, $P < 0.05$).

Increased expression of RCAN1 had the opposite effect to $Rcan1$ ablation on vesicle fusion kinetics. The average peak of each exocytotic spike was not different between control and $RCAN1^{ox}$ cells (Fig. 6A, $n = 16$), but the amount of catecholamine released per fusion event was significantly decreased in $RCAN1^{ox}$ cells ($P < 0.01$, Fig. 6B). This was caused by significantly faster rise ($P < 0.05$) and decay ($P < 0.05$) phases in amperometric spikes from $RCAN1^{ox}$ cells (Fig. 6C and D). As well as this, the half-width (width of a spike at half its maximal height) was significantly decreased in $Rcan1^{ox}$ cells ($P < 0.01$, Fig. 6E). A comparison of a representative spike from control and $Rcan1^{ox}$ mice is shown in Figure 6F illustrating the difference in spike parameters when RCAN1 is over-expressed.

In $RCAN1^{ox}$ cells, the percentage of events preceded by a foot signal was unchanged (Fig. 7A). The height of these foot signals was decreased in $Rcan1^{ox}$ cells ($P < 0.05$, Fig. 7B). In $RCAN1^{ox}$ cells, the foot duration was reduced (Fig. 7C, $P < 0.05$) resulting in less neurotransmitter being released per foot signal (Fig. 7D, $P < 0.01$). Similar results were found in a separate transgenic line we created, named M1 (Supplementary Materials, Fig. S1 and Table 1), ruling out any erroneous results which may have occurred due to insertional position effects of the transgene.
Figure 2. Loss of RCAN1 reduces chromaffin cell exocytosis. Typical responses of chromaffin cells to stimulation with a 70 mM K⁺ solution (indicated by dashed line under traces). Spikes indicate release of catecholamines from a single vesicle in (A) WT and (B) Rcan1⁻/⁻ cells. The frequency of these events, placed in cumulative bins of 5 s, decreases over the stimulatory period in (C) Rcan1⁻/⁻ cells and a significant difference over this period shown in (D) is found between controls and Rcan1⁻/⁻ cells (n = 21, P < 0.001). Light grey bars indicate WT and dark grey bars indicate Rcan1⁻/⁻ cells. Scale bar in (B) represents 10 s and 100 pA for (A) and (B).

Figure 3. Overexpression of RCAN1 reduces chromaffin cell exocytosis. Typical responses of chromaffin cells to stimulation with a 70 mM K⁺ solution (indicated by dashed line under traces). Spikes indicate release of catecholamines from a single vesicle in (A) WT and (B) RCAN1ox cells. The frequency of these events, placed in cumulative bins of 5 s, decreases over the stimulatory period in (C) RCAN1ox cells and a significant difference over this period shown in (D) is found between controls and RCAN1ox cells (n = 23, P < 0.01). Light grey bars indicate WT and dark grey bars indicate RCAN1ox cells. Scale bar in (B) represents 10 s and 100 pA for A and B.
Figure 4. Ablation of Rcan1 slows fusion pore kinetics. Ablation of Rcan1 expression does not alter spike amplitude (A, \(n = 16\)) but does increase quantal size (charge, B) and slows both the rise time (C) and decay time (D) and increases the half-width (E) of individual spikes. (F) A comparison of a spike from a control and Rcan1\(^{−/−}\) cell is also shown to illustrate the changes in spike shape which represent alterations in fusion pore kinetics. *\(P < 0.05\), **\(P < 0.01\). Light grey bars indicate WT and dark grey bars indicate Rcan1\(^{−/−}\) cells.

Figure 5. The initial fusion pore is longer lasting when Rcan1 is ablated. While the frequency of foot signals preceding an amperometric spike (A, \(n = 16\)) nor the amplitude of foot signals (B) are not altered, foot signals last longer in Rcan1\(^{−/−}\) cells (C) enabling more catecholamine to be released through this initial fusion pore (D). **\(P < 0.01\). Light grey bars indicate WT and dark grey bars indicate Rcan1\(^{−/−}\) cells.
Figure 6. Overexpression of RCAN1 accelerates fusion pore kinetics. Rcan1 overexpression does not alter spike amplitude (A, \( n=17 \)) but does decrease quantal size (charge, B) and accelerate both the rise time (C) and decay time (D) and decrease the half-width (E) of individual spikes. (F) A comparison of a spike from a control and RCAN1\(^{ox} \) cell is also shown to illustrate the thinning of spike shape which represent an acceleration of fusion pore kinetics. **\( P<0.01 \). Light grey bars indicate WT and dark grey bars indicate RCAN1\(^{ox} \) cells.

Figure 7. The initial fusion pore is more short-lived when RCAN1 is over-expressed. While the frequency of foot signals preceding an amperometric spike is not altered (A, \( n=17 \)) the amplitude of foot signals is decreased (B) and foot signal duration is shorter in RCAN1\(^{ox} \) cells (C) resulting in less catecholamine being released through the initial fusion pore (D). *\( P<0.05, **P<0.01 \). Light grey bars indicate WT and dark grey bars indicate RCAN1\(^{ox} \) cells.
Table 1. Effect of RCAN1 and acute calcineurin inhibition on single amperometric events

<table>
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<tr>
<th></th>
<th>No. of events</th>
<th>Amplitude (pA)</th>
<th>Charge (pC)</th>
<th>Rise time (ms)</th>
<th>Decay time (ms)</th>
<th>Half-width (%)</th>
<th>Foot frequency (Hz)</th>
<th>Foot area (pA)</th>
<th>Foot duration (ms)</th>
<th>Foot height (pA)</th>
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<td>WT</td>
<td>60.8 ± 6.9 (21)</td>
<td>46.0 ± 2.5</td>
<td>221.7 ± 22.1</td>
<td>0.56 ± 0.06</td>
<td>2.7 ± 0.3</td>
<td>2.9 ± 0.3</td>
<td>53.5 ± 5.8</td>
<td>30.4 ± 2.0</td>
<td>4.7 ± 0.2</td>
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<td>−/−</td>
<td>30.5 ± 2.9 (21)**</td>
<td>48.0 ± 4.1</td>
<td>338.2 ± 34.1**</td>
<td>0.83 ± 0.08*</td>
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<td>63.4 ± 4.7</td>
<td>42.4 ± 4.2</td>
<td>5.9 ± 0.4**</td>
<td>18.4 ± 1.2</td>
<td>16</td>
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<tr>
<td>WT</td>
<td>58.9 ± 6.5 (23)</td>
<td>52.9 ± 2.5</td>
<td>275.3 ± 25.6</td>
<td>0.50 ± 0.04</td>
<td>3.0 ± 0.4</td>
<td>3.0 ± 0.3</td>
<td>58.7 ± 4.2</td>
<td>35.2 ± 3.6</td>
<td>4.1 ± 0.2</td>
<td>21.2 ± 1.1</td>
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<td>M9</td>
<td>37.5 ± 5.4 (23)**</td>
<td>50.8 ± 2.5</td>
<td>171.6 ± 16.2**</td>
<td>0.38 ± 0.03**</td>
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<td>58.9 ± 4.3</td>
<td>21.3 ± 1.4**</td>
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<td>16.5 ± 0.9**</td>
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<tr>
<td>WT</td>
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<td>51.2 ± 4.2</td>
<td>274.8 ± 25.0</td>
<td>0.48 ± 0.03</td>
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<td>3.0 ± 0.3</td>
<td>61.5 ± 4.5</td>
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<td>M1</td>
<td>28.9 ± 4.7 (18)**</td>
<td>52.3 ± 3.9</td>
<td>167.5 ± 20.7**</td>
<td>0.39 ± 0.04*</td>
<td>1.8 ± 0.2**</td>
<td>2.0 ± 0.3*</td>
<td>61.2 ± 4.1</td>
<td>19.3 ± 1.1</td>
<td>4.0 ± 0.1*</td>
<td>17.2 ± 0.9*</td>
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<tr>
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<td>312.8 ± 33.3</td>
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<td>3.3 ± 0.4</td>
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Data shown as mean ± SEM of the cell median for each parameter. Differences between Reani+/− and Reani++ (two different lines named M9 and M1) and wild-type (WT) controls are indicated as "P < 0.05; **P < 0.01; ***P < 0.001. Cys indicates the calcineurin inhibitor cyclosporine A. Numbers in parentheses in the first column are number of cells used to calculate the number of exocytotic events (see Materials and Methods for further details).

The size of the RRP is altered by Reani

Following re-exposure of chromaffin cells to a hypertonic solution and cells exposed to these conditions (Supplementary Materials Fig. S3B) Rcan1+/- (control) (Supplementary Materials Fig. S3C). The number of vesicles released during the period of cell stimulation (Supplementary Materials, Fig. S2D). The number of vesicles released following a return to normal Ca2+ entry. Cells were loaded with the Ca2+ -specific indicator, Fluo-3, and stimulated with the same protocol used in the Rcan1+/- and Rcan1++ cells or were observed in control (Supplementary Materials, Fig. S2B). There was no difference in the peak level of Ca2+ entry between Rcan1+/− (n = 25) and control cells (n = 24) (Supplementary Materials, Fig. S2C) or RCAN1ox+/- cells (n = 26) and control cells (n = 25) (Supplementary Materials, Fig. S2D). The major regulator of exocytosis is stimulation-induced Ca2+ entry. We therefore carried out Ca2+ imaging experiments to verify whether the exocytosis phenotypes observed in Reani expression were due to alterations in normal Ca2+ entry. Cells were loaded with the Ca2+ specific indicator, Fluo-3, and stimulated with the same protocol used in the Rcan1+/− and Rcan1++ cells or RCAN1ox+/- cells (Supplementary Materials, Fig. S2C). The number of vesicles released following a gradual decline in intracellular Ca2+ concentration (Ca2+ entry) was also not different in the peak level of Ca2+ entry between Rcan1+/− (n = 25) and control cells (n = 24) (Supplementary Materials, Fig. S2D).

Inhibition of calcineurin activity does not regulate exocytosis

As Reani is an endogenous inhibitor of the phosphatase calcineurin, we first investigated whether changes in calcineurin activity might underlie the mechanism by which Reani regulates exocytosis. We first carried out a control experiment similar to previous amperometry experiments in which cells where stimulated for 10 min. We then allowed these cells to recover before exposing cells to the calcineurin inhibitors for 10 min. We then re-stimulated the same cell in the continued presence of the calcineurin inhibitors for 10 min. We then re-stimulated the same cell in the continued presence of the calcineurin inhibitors for 10 min. We then re-stimulated the same cell in the continued presence of the calcineurin inhibitors for 10 min. We then re-stimulated the same cell in the continued presence of the calcineurin inhibitors for 10 min. We then re-stimulated the same cell in the continued presence of the calcineurin inhibitors for 10 min. We then re-stimulated the same cell in the continued presence of the calcineurin inhibitors for 10 min. We then re-stimulated the same cell in the continued presence of the calcineurin inhibitors for 10 min.
the 30 s following a return to isotonic solution was 13.8 ± 1.7 in Rcan1−/− cells (n = 15) compared with 17.9 ± 4.2 in control cells (n = 9). In RCANIox cells, this number was 12.6 ± 3.0 (n = 9) compared with 16.2 ± 3.6 vesicles in control cells (n = 7) (Supplementary Materials, Fig. S3D). Thus, the effects of Rcan1 on exocytosis is not caused by changes in the localization of vesicles on the plasma membrane.

DISCUSSION

Our results in this study clearly illustrate that Rcan1 regulates exocytosis at two separate stages: the number of vesicles fusing with the plasma membrane and undergoing exocytosis; and once this occurs, the speed at which the vesicle fuses with the plasma membrane. The manner by which Rcan1 regulates these two steps of exocytosis remains to be identified as no previous evidence exists to link Rcan1 to the regulation of vesicle exocytosis. Our results indicate, however, that Rcan1 does regulate exocytosis at stages of the process downstream of Ca2+ entry.

The reduction in the number of vesicles undergoing exocytosis in both the Rcan1−/− and RCANIox chromaffin cells indicates a careful balance in Rcan1 expression is required for optimal vesicle release to occur. A similar conclusion was drawn from work in over-expressing and loss-of-function nebula mutants, the Drosophila ortholog of Rcan1. Neurons of these two mutants displayed similar abnormalities, including mitochondrial anomalies such as increased numbers of smaller mitochondria, decreased levels of mitochondrial DNA, reduced ATP production and increased levels of ROS (15). Both these nebula mutants also displayed severe learning defects which are not caused by brain maldevelopment (14). If defects in CNS neurotransmission occur in Rcan1−/− and RCANIox mice similar to the changes we observed in chromaffin cells, this may explain learning difficulties in nebula mutants and potentially the cognitive defects occurring in individuals with DS. Care must be taken, however, when relating our results in RCANIox to what may occur in DS neurons due to the larger overexpression (four times normal) of Rcan1 in these mice compared with the 1.5–2 times overexpression of most chromosome 21 genes in DS. It should be noted that we did not investigate whether exocytosis is affected in chromaffin cells from heterozygous Rcan1+/− mice.

Enlarged early endosomes are observed in neurons of the Ts65Dn DS mouse model, DS patients and in sporadic AD patients (3,4). The enlargement of these early endosomes is associated with reduced rates of vesicle endocytosis (2). Such a reduction in the rate of endocytosis could explain how Rcan1 regulates exocytosis by reducing the amount of vesicle recycling occurring in our experiments. However, whether Rcan1 does regulate endocytosis and whether recycled vesicles even undergo exocytosis during our stimulation period in these current experiments requires further investigation. If vesicle endocytosis in chromaffin cells of these mice was reduced to such an extent as to diminish vesicle recycling, this could explain the reduced number of vesicles observed undergoing exocytosis. If this was also to occur in neurons of these mice then we may imagine that neuronal endosomes could also be enlarged. This type of neuronal profiling into pathological hallmarks of DS and AD in our Rcan1 mutant mice will provide further insight into the role of Rcan1 in these diseases. We do know that the number of

Figure 8. Calcineurin inhibition does not effect the rate of exocytosis or fusion pore kinetics. WT cells were stimulated before and after acute exposure to the calcineurin inhibitors FK-506 (5 μM) and cyclosporine A (5 μM). No differences were seen in (A) spike number, (B) amplitude, (C) charge, (D) rise time, (E) decay time (F) half-width, (G) foot duration or (H) foot area. Light grey bars indicate before and dark grey bars indicate after exposure to drugs.
vesicles located at the plasma membrane is not altered by changes in Rcan1 expression, leading to the conclusion that changes in the size of the readily releasable pool (RRP) do not contribute to the phenomenon we have observed. The fact that the RRP size is unaffected in Rcan1 mutant cells provides further strength to the possibility that slower vesicle endocytosis and recycling may at least partially underlie the reduced level of vesicle release we observe. Other possible explanations include a reduced size of the reserve vesicle pool or alterations in the number or localization of secretory vesicles.

While we observed changes in amperometric spike rise time, decay time, charge and half-width, spike amplitude remained unaffected. Spike amplitude is thought to be dependent on vesicle loading, as increasing the amount of catecholamine within chromaffin cell vesicles by pre-incubation with L-DOPA (21,22) or reducing levels by inhibiting the vesicular H+-ATPase pump (22) or the vesicular monoamine transporter (21), results in the spike amplitude increasing or decreasing, respectively. We therefore assume that the alterations in multiple amperometric spike parameters is not caused by changes in vesicle loading, but is more likely due to direct or indirect regulation of the fusion pore machinery by Rcan1.

We can only speculate at this stage as to the mechanisms by which Rcan1 regulates exocytosis. Rcan1 is an inhibitor of the protein phosphatase, calcineurin, which dephosphorylates a number of endocytotic proteins including dynamin 1, amphiphysins 1/2 and synaptotagmin (23) and the exocytotic protein, Munc-18 (24). Calcineurin is the main phosphatase involved in dephosphorylating the exocytotic protein Munc-18 in chromaffin cells (24). Munc18 and syntaxin bind with lower affinity when Munc-18 is in a phosphorylated state (25) resulting in a acceleration of fusion pore kinetics (26) similar to that reported here in Rcan1 mutant cells. With this in mind, it must be noted that calcineurin inhibition in chromaffin cells has previously been shown not to alter the rate of exocytosis (27,28) but to reduce the rate of endocytosis (27). We tested this ourselves using a combination of FK-506 and cyclosporine A to ensure complete calcineurin inhibition. Our results demonstrate that acute inhibition of calcineurin activity does not alter the amount or kinetics of exocytosis in chromaffin cells indicating that regulation of calcineurin activity by Rcan1 may not underlie Rcan1-dependent regulation of exocytosis. The changes in foot signal parameters including foot duration and foot area also signify a role for Rcan1 in regulating fusion pore stability and formation. The foot signals seen in these amperometric recordings occur due to the transient opening and closing of the fusion pore and the slow release of catecholamines during this time (17). The foot duration gives an indication of the time taken to form a stable fusion pore before complete dilation of the pore and release of vesicular contents, whereas the foot area relates to the amount of molecules able to pass through this transient fusion pore. Our results would indicate that an increase amount of Rcan1 reduces the time taken for the fusion pore to stabilize.

Regulation of exocytosis could also occur through protein interactions of Rcan1. Rcan1 contains a functional motif which binds calcineurin as well as two proline-rich SH3 binding domains (10), although the functions served by these domains are unknown. Apart from calcineurin (29), the only protein currently known to interact with Rcan1 is the protein kinase Raf-1 (30). While Raf-1 is known to link Ras to the MEK/ERK signalling pathway, there is no clear indication how this interaction might be linked directly to exocytosis. In primary neuronal cultures transfected with a Rcan1-EGFP construct, Rcan1 is detected as punctate staining in the cell body and neuronal processes and is co-localized with synaptophysin, suggesting Rcan1 may be expressed on vesicles (13). This localization may be of importance given the newly identified role of Rcan1 as a regulator of exocytosis. In this study, we identify a novel role of RCAN1 in the regulation of exocytosis and fusion pore dynamics. This is the first demonstration of any gene on human chromosome 21, which is over-expressed in DS having a role in regulating vesicle exocytosis. The fact that we see regulation occurring at two different stages, before and during vesicle fusion, indicates that RCAN1 may affect the exocytotic pathway via multiple mechanisms. The identity of these mechanisms remains to be discovered. If similar effects of Rcan1 are observed in neurons, these findings will have direct implications for the disease pathogenesis of both DS and AD in which Rcan1 is over-expressed and could potentially explain the early vesicle trafficking anomalies and subsequent cognitive decline observed in both these conditions.

MATERIALS AND METHODS

Generation of mutant mice

Dscr1/Rcan1 null mice were generated on a mixed 129SvJ × C57BL/6 genetic background as described (31). RCANI transgenic mice were generated using the human RCANI cDNA encoding the exon 1 splice variant. The minigene transgene was engineered to encompass 4 kb upstream of exon 1, parts of the flanking introns and exons 5–7 including the 3’-UTR. The final construct is represented in Figure 1A. The minigene DNA was prepared for microinjection by double digestion with HindIII and NotI and the gel purified insert used to microinject fertilized mouse embryos (mixed genetic background C57BL/6 × CBA). Thirty-four injected eggs gave rise to live offspring, five of which had the minigene DNA integrated in their genomes. Mouse genotyping was performed by PCR on ear-clip DNA by amplifying a region in the 3’-UTR.

RCAN1 expression in chromaffin cells

Adrenal glands from 8-week-old mice killed according to the guidelines approved by the Monash Medical Centre Animal Ethics Committee were collected, the RNA extracted and made into cDNA. RT–PCR was performed using either human-specific oligonucleotides hRCAN1-3utrF3 [GCA-CAGGACATTTGGGACTG] and RCAN1-3utrR4 [GTGGGGATGCTGAGTGAATG] or oligonucleotides that amplify both human and mouse genes. Exon 1 [AGCTT-CATCGACTGCGAGAT] and Exon 7 [GTGTACTCCGGCTCTCGTG] The reactions were performed with an annealing temperature of 62°C and an extension time of 45 s.
Western blot analysis

Adrenal protein extracts from knockout, transgenic and WT counterparts were assessed by western blot analysis for RCAN1 expression, using an anti-RCAN1 antibody that cross reacts with both human and mouse proteins (31). The mouse tissue extracts were prepared by homogenization in tissue-lysis buffer containing (10 mM Tris–HCL, 150 mM NaCl, 0.1% SDS, 1% Na deoxycholate, 1% TritonX100, pH 7.4), run through 12% SDS-polyacrylamide gels, and the proteins transferred onto Immobilon-P membrane (Millipore). The anti-RCAN1 antibody was used to probe the membrane at a dilution of 1/1000, and the secondary goat anti-rabbit antibody, conjugated to horse radish peroxidase (DAKO), was used at a dilution of 1/2000.

Chromaffin cell culture

Adrenal glands were taken from 6–8-week old male mice. The adrenal medulla was dissected out in cold Locke’s buffer (154 mM NaCl, 5.6 mM KCl, 3.6 mM NaHCO3, 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 DMEM medium supplemented with 5% FCS (JRH Biosciences, Lenexa, USA) and then incubated at 37°C with 5% CO2. Cells were maintained in primary culture for 3–4 days prior to experiments.

Amperometry

Catecholamine release from single chromaffin cells was measured using amperometry (17). 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 60 s from the start of stimulation. The standard bath solution contained 140 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 5 mM D-glucose, 10 mM HEPES, pH 7.4. High K+-containing solution was the same as control bath solution except that 70 mM K+ replaced an equimolar amount of NaCl. For evaluation of the number of vesicles contained in the RRP, cells were exposed for 10 s to a control bath solution to which 500 mM sucrose was added (19,20). All solutions were applied to cells using a gravity perfusion system, the outlet of which was placed within 500 μm from the recorded cell. All experiments were carried out at room temperature (22–24°C). FK-506 and cyclosporine A were acquired from Sigma (Australia).

Ca2+ imaging

For single cell Ca2+ imaging, cells were loaded with the Ca2+ indicator Fluo-3 AM (5 μM) in serum-free DMEM at 37°C for 45 min. Before recording, cells were rinsed in bath solution for at least 10 min to allow for full de-esterification of the dye. Cells were perfused at 2 ml/min. Confocal microscopy was applied using an argon ion laser (Olympus, Australia) scanning at a peak of 488 nm and emitted light was detected at wavelengths >515 nm. Images were captured and analyzed using in-built software (Fluoview-300, Olympus). Laser intensity was reduced to 5% of maximum by use of neutral density filters and the scan rates kept at one scan per 5 s to avoid photobleaching. Changes in intracellular Ca2+ levels were taken as the ratio of the maximum mean pixel value of the whole cell during stimulation compared with that in the control period, named ΔF/Fo, in order to rule out the influence of cell batch or Fluo-3 loading efficiencies (Keating, 2001 #167). This ratio is calculated according to the equation: ΔF/Fo= (F-Fmin)/Fmin, where Fmin, mean fluorescence intensity during control period and F, maximum fluorescence intensity during stimulation.

Data analysis

Amperometric spikes were selected for analysis of event frequency if spike amplitude exceeded 10 pA and overlapping peaks were included. Cells with fewer than 10 or >130 events within the 60 s stimulation period were excluded from analysis. For kinetic analysis of spikes and spike feet, 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.

Difficulties arise when analyzing amperometric spike data because of the large number of events per cell combined with a large cell-to-cell variability in spike parameters. Due to this, errors can occur when all the spikes are pooled for statistical testing (21). We analyzed all the spikes recorded in a cell that met our threshold criteria to estimate one statistic per cell (the cell median of each spike parameter) and compared this statistic between cell populations (21). For foot analysis, data was pooled from all recorded cells as many recordings contained a low number of foot signals so that an adequate median value could not be obtained for each cell. Foot onset was defined when the signal exceeded the peak-to-peak noise of a 5 ms time segment, while the end of the foot was defined by the inflection point between the foot and the main event. This threshold value was determined by mathematically taking the second derivative of the trace. Recordings of both WT and mutant cells used the same carbon fibres to eliminate potential effects of inter-fibre variability. When analyzing the number of vesicles contained in the RRP in contact with the plasma membrane, all spikes with an amplitude >2 pA were included. The same carbon fibre electrode was not utilized for WT and mutant cell recordings as event number only was analyzed, not the vesicle release kinetics. For the analysis of this data we included all events detected for 30 s after cells were exposed to the high sucrose solution. As the number of release events per cell...
and the amperometric spike parameters are non-parametrically distributed, differences between means of different groups were evaluated for statistical significance using the Mann–Whitney test. All data presented are shown as mean ± SEM.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

Conflict of Interest statement. None declared.

FUNDING


REFERENCES