Mice deficient for the chromosome 21 ortholog Itsn1 exhibit vesicle trafficking abnormalities

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Abstract

Enlarged early endosomes in the neurons of young Down syndrome (DS) and pre-Alzheimer’s disease (AD) brains suggest that a disturbance in endocytosis is one of the earliest hallmarks of AD pathogenesis in both conditions. We identified a chromosome 21 gene, *Intersectin-1* (*ITSN1*) that is up-regulated in DS brains and has a putative function in endocytosis and vesicle trafficking. To elucidate the function of *ITSN1* and assess its contribution to endocytic defects associated with DS and AD we generated *Itsn1* null mice. In knockout mice we found alterations in a number of parameters associated with endocytic and vesicle trafficking events. We found a reduced number of exocytosis events in chromaffin cells and a slowing of endocytosis in neurons. Endosome size was increased in neurons and NGF levels were reduced in the septal region of the brain. Our data is the first indication that *Itsn1* has a role in endocytosis in an *in vivo* mammalian model and that a disruption in *Itsn1* expression causes a disturbance in vesicle trafficking and endocytic function in the brain. These results imply a role for *ITSN1* in the early endocytic anomalies reported in DS brains which may have ramifications for the onset of AD.
**Introduction**

Down syndrome (DS) is caused by trisomy of all, or part, of human chromosome 21 and has an overall incidence of one in 700 live births (1). Many of the abnormalities that are characteristic of DS also occur in the general population, however in DS they occur earlier in life and at a higher incidence. All DS individuals are mentally impaired, most experience seizures and after 35 years of age, all develop Alzheimer's disease-like neuropathology, characterized by β–amyloid (Aβ) peptide-containing neuritic plaques and tau-containing neurofibrillary tangles (2).

Recently it was proposed that disturbances in endocytosis in the brains of individuals with DS and early Alzheimer's disease (AD) contribute to pathological processes. Enlarged early endosomes were reported as the earliest neuropathological alteration identified in sporadic AD, preceding Aβ peptide deposition and were not observed in normal ageing brains (3). Early endosomes were markedly enlarged in DS neurons apparent as early as 28 weeks of gestation (3) and in the partial trisomy 16 mouse, Ts65Dn, widely used as a mouse model for DS (4). These observations suggested an endocytic trafficking defect. Interestingly, early endosomes are the sites of internalization of proteins involved in the pathogenesis of AD, including amyloid protein precursor (APP) and apolipoprotein E (ApoE) and are a major site of Aβ peptide generation (5). Inhibiting endocytosis slowed Aβ generation (5) and forcing endosome enlargement in cells by the over-expression of Rab-5 increased Aβ levels significantly (6). In sporadic AD, endosome pathology heralds the initial abnormal rises in Aβ levels and many
neurons exhibit enlarged Rab-5-positive endosomes, most of which contain Aβ immunoreactivity (5).

Our efforts to identify genes on human chromosome 21 with the potential to cause the brain anomalies observed in DS led to the discovery of *ITSN1* (Intersectin1) (7, 8). *ITSN1*, located on chromosome 21q22.2 distal to *APP*, *SOD1*, and *IFNAR1/2* and proximal to *RCAN1*, *ERG1* and *ETS2*, is over-expressed in DS. The gene produces two major mRNA transcripts, a widely expressed mRNA of approximately 6 kb and a larger brain-enriched transcript of approximately 11 kb, encoding 140 kDa (ITSN1-S) and 200 kDa (ITSN1-L) proteins, respectively (7). The generation of the long isoform is the result of an alternative splicing event in which exon 29, which encodes the stop codon for the short isoform, is skipped. ITSN1 contains many motifs involved in protein-protein interactions including two Eps15 homology (EH) domains, five consecutive Src homology 3 (SH3) domains (SH3A-E) and a proline-rich domain. In addition, ITSN1-L contains a carboxy terminus which harbors Db1 homology (DH) and pleckstrin homology (PH) domains. DH domains serve as catalytic regions of guanine-nucleotide exchange factors, catalyzing the exchange of GTP for GDP (9) and PH domains always follow DH domains and mediate membrane phosphoinositide interactions (10).

The first *in vitro* studies on *ITSN1* suggested that it functioned in endocytosis by acting as a scaffolding protein for the assembly of endocytic components. For example, ITSN1 recruits dynamin1 and synaptojanin1, two endocytic proteins involved in the formation of clathrin-coated pits (11, 12) and ITSN1 over-expression blocks clathrin-mediated
endocytosis measured by transferrin uptake in COS cells (13) presumably through disruption of higher order protein complexes between ITSN1 and its binding partners. Studies in cell lines later demonstrated ITSN1 to be involved in signal transduction. ITSN1 regulates the Ras/MAPK pathway (14-17), activates actin assembly through interactions with Cdc42 and N-WASP (18), regulates epidermal growth factor receptor endocytosis (19) and influences neuron survival through a phosphoinositide 3'-kinase-C2β-AKT pathway (20). In addition, RNAi knockdown experiments in cell lines demonstrated that ITSN1 had a role in exocytosis by integrating the actions of Cdc42 on exocytosis and actin polymerization (21) and was involved in the mitochondrial apoptosis pathway via its regulation of MEK/Erk1/2 (22). Finally, at the Drosophila melanogaster neuromuscular junction, loss-of-function mutations of dap160, the fly ortholog of ITSN1, resulted in defective synapse bouton structure as well as the appearance of aberrant enlarged synaptic vesicles. A reduction in the synaptic expression of the endocytic proteins dynamin, endophilin, synaptojanin and AP180 and defects in synaptic vesicle recycling and synaptic transmission were also observed (23, 24) suggesting that Dap160 is directly involved in synaptic vesicle trafficking in Drosophila.

Aβ plaques and tau-containing neurofibrillary tangles are characteristic of AD but whether these are a cause of the disease or a consequence of some earlier malfunctioning of the brain remains unknown. Reports linking endocytic abnormalities with the earliest signs of AD make a compelling argument for investigating the potential contributions of endocytic proteins that are over-expressed in DS, such as ITSN1, in the disease process. As a prelude to understanding how ITSN1 over-expression may contribute to parts of the
DS phenotype, we decided to elucidate the in vivo function of ITSN1 by the generation of two strains of mice deficient for Itsn1. In one strain we disrupted both Itsn1 isoforms, in the second only the long isoform was disrupted. In the Itsn1 total null (Itsn1-TK), the gene is disrupted in all tissues i.e. both the short and long isoforms are lacking. In the long isoform-specific null (Itsn1-LK), the long isoform is missing, leaving the short isoform intact. We demonstrate that in both models Itsn1 is essential for correct vesicle trafficking in neuronal and non-neuronal cells and that in support of an endocytic trafficking defect, nerve growth factor (NGF) levels are abnormal.

**Results**

**Targeted disruption of the Intersectin-1 gene**

Targeting vectors for the generation of both total and long isoform-specific Itsn1 null mice are shown in Supporting Figure 1. Deletion of both long and short isoforms (total knockout) was achieved by removing exon 3 and deletion of the long isoform only was achieved by removing exon 32. Western blotting of protein extracts derived from brain and heart tissue of homozygous null mice revealed the expected absence of Itsn1 long and short isoforms in both lines (Supporting Fig. 2A). Heterozygous mice from both lines produced pups with a typical Mendelian distribution, demonstrating that Itsn1-deficiency did not cause embryonic lethality (Supporting Fig. 2B), however, a small proportion of homozygous null pups from both lines (13% of Itsn1-TK and 12% of Itsn1-LK) appeared to be unhealthy. They were smaller than their littermates and failed to thrive, often dying between 3 and 8 weeks of age. The remaining homozygous null mice appeared to develop and reproduce normally and had no gross abnormality of any major organ (data
not shown). With the exception of the endosome size measurements which were performed on brains derived from both healthy and unhealthy mice, all experiments were carried out on healthy mice and all defects were apparent in these mice.

**Expression of the Itsn1 protein isoforms in brain cells**

Protein expression studies by Western blot on neuronal or glial cultures from rat brains had demonstrated that the Itsn1 long isoform was neuronal-specific and that the short isoform although predominantly expressed in glial cells, was also in neurons (12, 25), although one study suggested that the presence of the short isoform in neurons may have been due to glial cell contamination of the cultures. In an attempt to clarify this issue we prepared cultures containing various proportions of neurons and glia for immunoblotting using an α-Itsn1 antibody that recognizes both isoforms. We confirmed that the long isoform was neuronal specific and determined that the short isoform was expressed specifically in glial cells, not in neurons (Fig. 1A).

Because of our strategy for generating *Itsn1* long isoform knockouts, where exon 32 was removed leaving the upstream exons intact, we examined the possibility that the short isoform would now be produced in neurons. Western blotting of neuronal cultures from *Itsn1*-LK mice showed no evidence of short isoform expression in neurons (Fig. 1B). Supporting Figure 2A also shows no increase in short isoform expression in *Itsn1*-LK brain tissue extracts (compare lanes 1 and 2).
Enlarged early endosomes in \textit{Itsn1} null brains

Since enlargement of endosome size has been taken as an indicator of disrupted endocytic function, we measured endosome volume in \textit{Itsn1} deficient mice. We performed immunohistochemistry using an $\alpha$-Eea1 antibody to determine the sizes of early endosomes in brain sections and found no differences between control or healthy \textit{Itsn1}-TK or \textit{Itsn1}-LK mice (data not shown). Intriguingly, when we performed the same experiment on brains derived from the subset of \textit{Itsn1} null mice that failed to thrive, significantly enlarged endosomes were evident in the brains of both \textit{Itsn1}-TK and \textit{Itsn1}-LK mice compared to controls (Fig. 2).

\textbf{Itsn1 regulates exocytosis, but not fusion pore kinetics, in chromaffin cells}

As studies in cell lines (13, 21) and in \textit{Drosophila} (23, 24) imply a role for \textit{Itsn1} in vesicle trafficking, we examined whether exocytosis is regulated by \textit{Itsn1}. We applied carbon fiber amperometry to measure catecholamine release from chromaffin cells, a classic cellular model of neuronal exocytosis. Chromaffin cells were cultured from phenotypically normal \textit{Itsn1} null mice. \textit{Itsn1} long and short isoform gene expression was confirmed in adrenal tissue (Fig. 3) and we found that, as in WT chromaffin cells (Fig. 4A), both \textit{Itsn1}-LK (Fig. 4B) and \textit{Itsn1}-TK (Fig. 4C) chromaffin cells displayed some exocytosis. However, exocytosis levels were lower at the earliest stages of stimulation and this continued throughout the stimulatory period in both \textit{Itsn1}-LK and \textit{Itsn1}-TK cells (Fig. 4D). In \textit{Itsn1}-LK cells the number of exocytic events detected was $23.8 \pm 2.5$ compared to $71.9 \pm 6.8$ in WT controls ($n=22$, $p<0.001$). Similarly, in \textit{Itsn1}-TK cells the number of vesicles undergoing exocytosis was $26.2 \pm 2.8$ compared to $66.9 \pm 6.6$ ($n=20$,
p<0.001) (Fig. 4E). Analysis of single spike kinetics demonstrated that neither isoform of
Itsn1 seemed to regulate fusion pore kinetics or the amount of vesicle content released per fusion event (Table I).

**Vesicle trafficking is disrupted in *Itsn1* null neurons**

Based on the chromaffin cell data showing that exocytosis was disrupted in a neuronal cell model we next investigated whether *Itsn1* deficiency caused deficits in synaptic vesicle recycling in neuronal cells. Synaptophysin (SpH), a pH-sensitive green fluorescent protein (GFP) fused to the luminal domain of vesicle-associated membrane protein (VAMP2), provides an optical tracer to track the net balance of exocytosis and endocytosis at nerve terminals (26, 27). Neurons in culture were stimulated by a field electrode placed in the bathing solution. A train of stimuli (40 stimuli at 20Hz) induced an increase in the fluorescence of synaptic regions of interest similar to that previously described using hippocampal neuron cultures (27). Average fluorescence changes corrected to baseline (ΔF/F) representing net vesicle exocytosis-endocytosis over an individual synaptic region of interest (bouton) are presented in Figure 5A. We measured various parameters from each individual bouton as indicated in Figure 5B and present these data in Table II. No significant difference was found in the rate of exocytosis (10-90% rise time), or the peak change in fluorescence following stimulation in this preparation. However, endocytosis was significantly slower in both *Itsn1*-TK and *Itsn1*-LK neurons compared to controls with longer T_{0.5} (Figure 5C) and decay time constant τ (Figure 5D). These data are further indicative of a defect in clathrin-mediated endocytosis, the primary method by which vesicles are recycled in central neurons (27).
**Altered NGF levels in *Itsn1* null mice**

Nerve growth factor (NGF) signaling is required for the survival and maintenance of certain neurons, including the basal forebrain cholinergic neurons (BFCN). NGF is produced in the hippocampus where upon binding to receptors on BFCN axons it is internalized by endocytosis and retrogradely transported to the cell bodies (28). Transport of NGF is defective in the Ts65Dn model of DS and as a result NGF levels were increased in the hippocampus and decreased in the septal region compared to normal controls (29). Since we had observed defective endocytosis in *Itsn1*-deficient neurons we reasoned that NGF levels may be similarly altered in *Itsn1* null mice. NGF levels were examined by ELISA in the hippocampus and septum of mice at 8-10 weeks of age and in mice at 12 months (Fig. 6). We found a significant increase in NGF levels in the hippocampi of younger *Itsn1*-TK mice compared to littermate controls (p=0.02) and observed a trend towards increased NGF levels in *Itsn1*-LK mice, however, in the hippocampi of older mice, there were no differences between either of the knockouts and controls. At both ages, *Itsn1* null mice displayed a trend toward reduced NGF levels in the septal region, where BFCN cell bodies are located, but the differences were only significant for *Itsn1*-TK mice (8-10 weeks, p=0.04; >12 months, p=0.03).

**Discussion**

In this study we generated two different *Itsn1*-deficient mouse models: one with no functional *Itsn1* protein and another that retained the ubiquitously expressed short isoform (*Itsn1*-S) but lacked the long, brain-predominant isoform (*Itsn1*-L). Both lines
were viable and once reaching adulthood reproduced and aged normally. We were surprised to observe that Itsn1-TK mice were not more severely affected than Itsn1-LK mice. However, in this study we concentrated on brain phenotypes, therefore, we cannot rule out the possibility that other organ systems may be more severely affected in Itsn1-TK mice or that, when presented with a challenge, Itsn1-TK mice may exhibit a more severe deficit. It is worth noting that others have removed components of the vesicle trafficking machinery in mice with no loss of viability although functional defects in vesicle trafficking were apparent. For example, amphiphysin1-deficient mice survive to adulthood and breed normally (30) as do mice deficient for synaptophysin (31).

We confirmed that the long Itsn1 isoform is neuron-specific and determined that the short Itsn1 isoform is expressed only in glial cells. We investigated brain/neuronal phenotypes in these mice with a particular emphasis on vesicle trafficking. Both models exhibited strikingly similar deficits. This indicates that at least for the parameters tested, functional Itsn1-L is required because the additional removal of Itsn1-S had no additive effect. This is consistent with an absolute requirement for the extra protein motifs encoded by Itsn1-L for neuron-specific functions. Both models exhibited vesicle trafficking defects in chromaffin cells and in neurons and mice of both genotypes showed alterations in brain NGF levels, suggestive of a failure of NGF trafficking via endocytosis and retrograde transport. Notably, our results are similar to those found in Ts65Dn mice where increased levels of NGF in the hippocampus were observed without a corresponding increase in NGF in the septal region. Indeed, similar to our findings in Itsn1 null mice, NGF was decreased in the septal region of Ts65Dn compared to 2N
controls implying that a disconnect exists between the production of NGF and its transportation (29). In both models neuronal endosomes were of normal size yet for reasons that we do not currently understand, enlarged endosomes were evident in brain sections derived from the subset of knockout mice that failed to thrive.

Our results illustrating a similar level of reduction in exocytosis in both Itsn1-LK and Itsn1-TK chromaffin cells indicate that the long, but not the short Itsn1 isoform regulates the amount of secretory vesicle exocytosis in these cells. Given that both proteins exist in these cells (21) we can conclude from this result that the longer C-terminus which includes the three extra protein interaction domains contained in the long Itsn1 isoform is essential for regulating stimulation-induced secretion. This data is consistent with that of Malacombe and colleagues (21) who found that the Itsn1 long isoform regulates secretory granule exocytosis in chromaffin and PC12 cells. Our analysis of individual amperometric spikes indicates that Itsn1 does not regulate exocytosis at the point of fusion and that Itsn1 must therefore be regulating exocytosis at points either distal to or proximal to vesicle fusion. Our synaptophysin experiments in neuronal cultures showed no evidence of reduced exocytosis. In light of this result, we conclude that the reduced level of exocytosis in our chromaffin cell data is caused at least partially by reduced levels of endocytosis significantly reducing vesicle recycling and subsequently exocytosis. However, it should be noted that the situation in chromaffin cells may be different as both Itsn1 isoforms are expressed in this cell type.
In neurons we found that the rate of synaptic vesicle endocytosis was significantly reduced in both Itsn1-TK and Itsn1-LK mice. A longer $T_{0.5}$ (132% in Itsn1-TK and 190% in Itsn1-LK over controls) indicated that net exocytosis/endocytosis was slower than controls in both Itsn1-TK and Itsn1-LK mice. The rate of vesicle endocytosis was also slower than controls in Itsn1-TK and Itsn1-LK neurons indicated by an increase in the decay time constant $\tau$ (176% and 204% over controls, respectively). We can therefore conclude that deficiency of *Itsn1* in neurons impairs reuptake of synaptic vesicles due to disrupted endocytosis.

The presence of enlarged early endosomes hint at endocytic deficits in Ts65Dn mice (4) but apart from defective NGF transport which involves signaling endosomes, no studies have specifically examined endocytosis or exocytosis at a functional level in DS mouse models. Ts65Dn mice are segmentally trisomic for about 136 genes orthologous to those located on human chromosome 21 (32), including *Itsn1*, so it is difficult to assign a particular gene to a particular function. We have begun to do this by determining the biological function of one of these genes, *Itsn1*. We are cognizant of the fact that phenotypes associated with DS and DS mouse models arise from gene over-expression, yet we observe similar abnormalities with a gene knockout. This may not be surprising given the role of Itsn1 as a scaffolding protein. It is likely that any perturbation in *Itsn1* expression (either over or under expression) would disrupt protein complexes and result in the same gross phenotype. Indeed, loss of function mutations of *dap160* result in enlarged synaptic vesicles and disrupted endocytosis (23, 24). On the other hand, given our results in a null model, it is also possible that over-expression of *ITSN1* in DS has a
protective role by reducing some of the adverse phenotypes. For instance, elevated expression of \textit{ITSN1} may help to reduce the size of endosomes that are larger due to the effects of over-expression of other chromosome 21 genes, or if \textit{ITSN1} was not present at elevated levels, NGF transport may be more severely affected. It would be informative to reduce the dosage of \textit{Itsn1} in the Ts65Dn mouse model to 2N by crossing our \textit{Itsn1} nulls with Ts65Dn mice and determining whether phenotypes such as defective NGF transport or enlarged endosomes are reversed.

It will be interesting to determine the effects of \textit{Itsn1} over-expression in mice on vesicle trafficking in neurons. So far, only one study has reported on the application of a functional assay to assess endo- or exocytosis in mice over-expressing a chromosome 21 gene and this was in chromaffin cells, not in neurons (33). We would predict that subtle phenotypic changes would occur in single gene over-expressors. This would be consistent with the idea that the DS phenotype is due to disturbances in biological pathways resulting from the accumulation of subtle changes brought about by the over-expression of many single genes. Indeed, at least five genes on chromosome 21 have been implicated in vesicle trafficking, including \textit{RCAN1}, \textit{DYRK1A} and \textit{SYNJ1} (presumably via their interactions with the endocytic regulator, calcineurin), \textit{APP} and \textit{ITSN1} (4, 34-37) and at least two of these genes, \textit{RCAN1} and \textit{DYRK1A}, have been shown to act cooperatively (35).

Alzheimer’s disease research has focused on \textit{APP} yet it is unlikely that this is the only gene involved. After an analysis of the brain phenotypes in mice resulting from either
over-expression of the APP wild type protein (as in DS) or of a mutated version of APP that leads to Alzheimer-type pathology or by comparisons of the phenotypes in the brains of DS mouse models carrying three copies of App (Ts65Dn) or two copies (Ts1Cje), it was concluded that the APP gene is necessary, but not sufficient to cause endosome pathology and that other genes are required (4, 36). Based on the function that we have demonstrated for Itsn1 in this study, we propose that ITSN1 is one of these necessary additional genes. We should point out that in this study we have examined one form of endocytosis - synaptic vesicle trafficking, yet clathrin-mediated endocytosis also allows neurons to regulate the cell-surface availability of receptors and other plasma membrane proteins. Therefore, we may speculate that a lack of Itsn1 would also disrupt these processes. Indeed, ablation of Itsn1 may decrease internalization of membrane-bound APP and thereby decrease Aβ production since inhibiting endocytosis slowed Aβ generation (5). On the contrary, in DS over-expression of Itsn1 may contribute to an increased APP dosage effect by promoting Aβ synthesis through enhanced activity of the endocytic pathway.

Our results showing that Itsn1 is required for efficient endocytosis and exocytosis is the first in vivo demonstration of a functional role for Itsn1 in mammalian vesicle trafficking. In this paper, we have shown using functional tests in cells derived from a new mouse model that Itsn1 is involved in vesicle trafficking in chromaffin cells and crucially, that it is required for endocytosis in the mammalian brain. These are novel findings and of significant import to the field of DS, particularly since in vivo function has been ascribed to only a handful of chromosome 21-encoded genes and very little is known about the in
functions of genes with a potential involvement in neurophenotypes and known to be over-expressed in the DS brain. Our data also has implications for the onset of AD, particularly since the early appearance of abnormally large endosomes in DS and AD neurons is suggestive of vesicle uptake and trafficking defects, processes known to involve ITSN1. Finally, in vitro experiments have contributed towards an understanding of the function of ITSN1 in receptor-mediated endocytosis, actin polymerization, signal transduction and cell survival. These studies have concentrated on perturbing ITSN1 levels in transformed cell lines by forced over-expression or by conducting RNA interference experiments. Whilst both these approaches are valuable they possess inherent problems of specificity and physiological relevance. Our Itsn1 knockout mice will be useful for investigating the in vivo role of ITSN1 in these processes, particularly since disruptions in all these processes have been associated with the brain pathologies of DS and AD.

Materials and methods

Generation of Itsn1 knockout mice

Two different targeting vectors were constructed (SI Fig. 1). A lambda mouse genomic library from strain 129SV/J was screened for fragments containing either exons 3 or 32 using Itsn1-specific DNA probes. We had determined that flanking exon 3 or exon 32 with loxP sites would allow deletion of these exons causing a frame shift that would result in truncation of the protein via a stop codon in the adjacent downstream exon. A neomycin resistance cassette was placed between the loxP sites and a herpes simplex virus-1 thymidine kinase gene was placed at the 3′-ends of each targeting vector to allow
for selection in ES cells. Recombinant ES clones were transfected with Cre recombinase to remove the targeted exon along with the neomycin resistance cassette, then correctly targeted ES cell clones were microinjected into C57BL/6 blastocysts resulting in male chimeras that were subsequently bred with 129SV/J females to produce heterozygous *Itsn1* offspring on a pure 129SV/J genetic background which were interbred to produce *Itsn1* homozygous nulls. Animals were genotyped by PCR analysis using genomic DNA from ear biopsies (Supplementary Fig. 1). PCR oligonucleotide primers used were the following: for *Itsn1*-TK, TK5819f (5'-gTAgAgCTCTAAACTgCACCTTAG-3'), TK4942f (5'-CTggTATgTATCgATCACCATCCA, and TK6484r (5'-gTgACAACAgTCAACACAAGCTTtg-3') and for *Itsn1*-LK, LK2429f (5'-CTgACgTTggAAATACgATgAgA-3'), LK3743f (5'-gATAgACTCTAACCTgTAAgCCAg-3'), and LK2918r (5'-gATgTCgCCAATCATCTCCTCCAg-3'). All animal studies were conducted in accordance with the ethical guidelines approved by Monash University.

**RT-PCR**

Adrenal glands from 8 week old mice were collected, the RNA extracted and made into cDNA. RT-PCR was performed using oligonucleotides E3f: (5'-AAgCCgATAgCgggtATTAT-3') E3r: (5'-CTATTTCCAgTgCACCAgCA-3') for amplification of both isoforms and E32f: (5'-AgAAACCCCTgACggAgTCT-3') E32r: (5'-gAgAAACCATCCAgACCACAg-3') for amplification of the long isoform only.
Neuronal/glial cell cultures

Pregnant female mice were humanely killed between 14 to 16 days post coitum and the fetuses dissected from the uterine horns in dissecting solution (1.24mM NaCl, 0.05mM KCl, 0.01mM NaH₂PO₄·H₂O, 0.25mM Hepes, 0.12mM MgSO₄, 0.26% D-glucose, 0.3% BSA). Fetuses were rapidly killed by decapitation and the cerebral cortices excised and pooled in 15 ml dissecting solution containing 0.025% Trypsin (Invitrogen, Carlsbad, CA USA) at 37°C. After 15 to 20 min, digestion was terminated by the addition of 15 ml dissecting solution containing 0.05% glycine max (Invitrogen, Carlsbad, CA USA) and 0.01% DNase (Sigma-Aldrich, St. Louis, MO USA). Tubes were inverted a few times, then the cells pelleted, resuspended in 2 ml dissecting solution containing 0.02% glycine max and 0.004% DNase and dissociated by passage through a glass Pasteur pipette (60 times). Finally, neuronal cells were collected after centrifugation and resuspended in 10 ml Neural Basal media containing 10% FCS, 2% B27 supplement (Invitrogen, Carlsbad, CA USA), 0.5mM glutamine (SAFC Biosciences, Lenexa, KA USA), 50µg/ml gentamicin (Invitrogen, Carlsbad, CA USA) and the cells counted. Five hours after plating the media was changed to the above minus FCS. Half the media was changed every two days during culture. All dishes and plates used for culture were treated with 0.005% Poly-L-Lysine (Sigma-Aldrich, St. Louis, MO, USA).

To obtain near pure neuronal cultures, 2.5µg/ml cytosine β-D-arabinofuranoside (Sigma-Aldrich, St. Louis, MO USA) was applied to cultures on day four post plating. Mixed neuronal/glial cell cultures were prepared as above except that no cytosine β-D-arabinofuranoside was added. Glial cell cultures were prepared as above but brains were
excised from 2 to 4 day old neonates with no addition of cytosine β-D-arabinofuranoside. Two antibodies were used to determine the relative proportions of neurons and glia in the cultures: anti-MAP2 (Chemicon, Temecula, CA USA) and anti-GFAP (Chemicon, Temecula, CA USA), specific to neurons and glia, respectively.

**Western blotting and antibodies**

Tissue or cells were homogenized in lysis buffer (50mM Heps, 100mM NaCl, 1mM EDTA, 10% Glycerol, 0.5% NP40, protease inhibitor cocktail tablets added just prior to use) and incubated at 4°C for 60 min with occasional shaking. These protein extracts were then combined with an appropriate volume of 2x sample buffer (10% sucrose, 0.125M Tris PH6.8, 4.5% SDS, 10% β-mercaptoethanol, 0.004% bromophenol blue) and boiled for 5 minutes. Following fractionation on SDS-polyacrylamide gels, proteins were transferred by electroblotting onto polyvinylidene fluoride (PVDF) membranes. Primary antibodies used were anti-Ese1 (BD Biosciences, San Jose, CA USA) a monoclonal antibody directed against the second SH3 domain encoded by exon 23 and anti-β tubulin (1: 2000) (Chemicon, Temecula, CA USA). Secondary antibodies were HRP-conjugated rabbit anti-mouse (1:2000) or goat anti-rabbit (1:1000) (Dako, Glostrup, Denmark). Membranes were incubated with SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL USA) and signal was visualized by exposure to Biomax film (Kodak Scientific, Rochester, NY USA).
**Immunohistochemistry and analysis of endosome size**

Mice were anesthetized by intraperitoneal injection of thiopental (Pentothal, 100 mg/kg body wt) and perfused through the apex of the heart with a fixative consisting of 4% paraformaldehyde and 0.2% picric acid. After fixation, brains were stored in 30% sucrose for 3 days and then embedded (OCT compound, Tissue Tek, Hatfield, PA USA) in standard biopsy moulds (Cryomold, Elkhart, IN USA) before sectioning at 10 µm. For labeling with early endosome antigen-1 (EEA-1), tissues were pre-incubated for 3 h at room temperature with 1% Triton X-100 in CAS Block (Zymed Laboratories Inc., San Francisco, CA USA). They were then incubated overnight at 4°C with an affinity-purified human polyclonal antibody against EEA-1 (1:5000 in incubation buffer) (a gift from Professor Marvin J. Fritzler, University of Calgary, Alberta, Canada). The sections were rinsed 4 times with PBS before incubation for 80 min at room temperature with the secondary antibody (goat anti-human IgG (H+L) Alexa Fluor 488, 1:1000). After being rinsed with PBS, the sections were mounted using Vectashield mounting medium (Vector Laboratories, Burlingame, CA USA), and then imaged by laser scanning confocal microscopy (Olympus FV1000) using a 100x, 1.4NA objective lens, 488nm excitation (argon ion laser) and detection through an emission window of 515-535nm. To measure endosome size, cellular detail was imaged slightly beyond nyquist rates and then subsequently deconvolved. To achieve this, the scanning area and zoom were set to achieve a final pixel resolution of 0.082µm in xy, and 20-30 optical sections were imaged at an interval of 0.120µm in z. Each optical series was deconvolved using a blind deconvolution algorithm (AutoDeblur) implemented as a plugin in the software program.
Analysis LS Professional (Soft Imaging System, GmbH, Münster, Germany). We determined that 20 iterations of the algorithm was an optimal deconvolution setting by testing deconvolution iterations of 10, 20, 30 and 50 and then assessing the impact of this on ease of segmentation and size measurements. With no deconvolution, endosome measurement was extremely difficult and variable as segmentation based on a single intensity level overestimated size of some endosomes, while underestimating others. Deconvolution provided significant improvement in the ability to segment and measure endosomes, but at 50 iterations, there was a marked decrease in size, indicating an over application of the algorithm. Therefore all measurements were performed after 20 iterations using the Analysis LS software. For each animal, three fields were imaged and deconvolved, and a sequence of 10 sections (i.e. a volume with 1.2µm depth) analyzed to ensure whole endosomes were assessed. The image series was projected by a MIP algorithm, with all endosomes remaining as discrete objects, and then detected, measured (area) and classed into 5 bins based on area (<0.17µm², 0.17–0.33µm², 0.34–0.48µm², 0.49–0.64µm², >0.64µm²). Means and standard errors of the means (SEM) of the area for early endosomes were calculated using Analysis LS Professional.

**Chromaffin cell culture**

Catecholamine release from single chromaffin cells was measured using carbon fiber amperometry (38) and analyzed essentially as described in (39). Adrenal glands were taken from 6-8 week old male mice. The adrenal medulla was dissected out in cold Locke's buffer (154mM NaCl, 5.6mM KCl, 3.6mM NaHCO₃, 5.6mM glucose, 5.0mM 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 at 1000 rpm and resuspended in DMEM medium supplemented with 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA) and 10% FCS (JRH Biosciences, Lenexa, USA) and filtered through nylon mesh. Cells were pelleted at 1000 rpm, resuspended in supplemented DMEM and plated on 35 mm culture dishes and 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 carbon fiber amperometry (38). 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. Oxidative current 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 analyzed (Mini Analysis, version 6.0.1, Synaptosoft, USA) for a period of 60 s from the start of stimulation. The standard bath solution contained 140mM NaCl, 5mM KCl, 2mM CaCl₂, 1mM MgCl₂, 5mM D-glucose, 10mM HEPES pH 7.4. High K⁺-containing solution was the same as control bath solution except that 70mM 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 500 μm from the recorded cell. All experiments were carried out at room temperature (22-24°C).
Amperometric spikes were analyzed for event frequency if amplitude exceeded 10 pA. Cells with fewer than 10 events within the stimulation period were excluded from analysis. Non-overlapping spikes over 20 pA were included for kinetic analysis of spikes. The cell median of each spike parameter was taken from this analysis and compared between cell populations (39). Recordings of both wild-type and mutant cells used the same carbon fibers to eliminate potential effects of inter-fiber variability.

**Measurement of synaptic function**

Neuronal cultures (>95% pure) were transfected with 1-2 µg of superecliptic synaptophluorin (SpH) cDNA using Qiagen’s Effectine reagent (40, 41). After 24 hours, the media was changed and cultures were left for a further 5 days prior to experimentation to allow for proper synaptic expression of the marker. Prior to imaging, cultures were washed in a physiological saline comprised of: 125mM NaCl, 5mM KCl, 1mM MgCl, 10mM D-glucose, 2mM CaCl, 25mM HEPES. Transfected neurons were imaged on an inverted Olympus IX71, SpH excited with 490 ± 5 nm light and emission collected through a 510 nm long pass filter. Images were acquired at 10 Hz using an Andor 885 EMCCD camera and iXon software. A bipolar electrode connected to a Grass stimulator was used to deliver field stimuli comprised of 40 stimuli at 20 Hz for 2 seconds. Each image series was analyzed post-hoc using Image J software. Regions of interest were drawn around distinct synaptic puncta and plotted against time allowing comparisons of the rate of endocytosis.
NGF measurements

Hippocampal and septal regions of mouse brains were excised and NGF levels determined by ELISA using the NGF Emax immunoassay system kit from Promega (Madison, WI USA) according to the Manufacturer’s instructions.

Statistical analysis

All data is presented as ±SEM. For endosome size and NGF measurements, statistical comparisons were performed using one-way ANOVA. For measurement of synaptic function, statistical comparisons were performed using 2-tailed Student’s T tests. For amperometry, as the results are non-parametrically distributed, statistical differences between groups were evaluated using the Mann–Whitney test. P values < 0.05 were considered to be significant.

Acknowledgements

This work was supported by grants to MAP from the NH&MRC and The American Health and Assistance Foundation. This work could not have been started without generous support from The Jerome Lejeune Foundation, ANZ Charitable Services, APEX Foundation for Research into Intellectual Disability, The CASS Foundation, and The L.E.W. Carty Charitable Fund. DJK is supported by Bio Innovation, SA and the NH&MRC. DNB is supported by NH&MRC.
References


Figure legends

Figure 1. Cell-type expression of the \textit{Itsn1} protein isoforms. Western blots of protein extracts prepared from primary cultures containing different relative proportions of neurons and glia were probed with an antibody against Itsn1. (A) Wild type cultures show that the long isoform is neuronal-specific and that the short isoform is expressed in glia, not neurons. (B) Cultures from Itsn1-LK mice show that the short isoform is not expressed in Itsn1-LK neurons.

Figure 2. Enlarged early endosomes in \textit{Itsn1} null brains. The top panels are representative of endosomes in brain sections stained with an anti-Eea1 antibody. The genotypes are indicated. Endosomes are colored according to their size class (small to large: red, green, dark blue, yellow, light blue). Visual inspection reveals an obvious enlargement of endosomes in both Itsn1-LK, Itsn1-TK mice compared to their respective controls. These results for each size class (area) are shown graphically below. \( n=3 \). In all cases as indicated with an asterisk, \( p<0.05 \). Error bars are ±SEM. Scale bar = 10\( \mu \)M.

Figure 3. \textit{Itsn1} long and short isoforms are expressed in mouse adrenal glands. Upper panel shows RT-PCR using the primers indicated; lower panel shows a Western blot using an \( \alpha \)-Itsn1 antibody.

Figure 4. \textit{Itsn1} regulates chromaffin cell exocytosis. Raw amperometric current traces are shown from (A) WT, (B) Itsn1-TK and (C) Itsn1-LK cells. (D) Progressive increase in number of amperometric spikes over the stimulatory period shows a similar response
in Itsn1-TK and Itsn1-LK cells and the final number of spikes at the end of stimulation is illustrated in (E). *** indicates p<0.001, n=22 (Itsn1-LK and WT controls) and n=20 (Itsn1-TK and WT controls). Dashed lines in A, B and C indicate period of stimulation. Error bars are ±SEM.

**Figure 5. Synaptic vesicle endocytosis is reduced in Itsn1-LK and Itsn1-TK neurons.**
(A) Averaged SpH fluorescence changes for control, Itsn1-TK and Itsn1-LK synapses. (B) Parameters measured from each individual bouton are indicated. (C) Half time (T0.5) of control, Itsn1-TK and Itsn1-LK experiments. (D) Decay time constant (τ) of control, Itsn1-TK and Itsn1-LK experiments. * P<0.01 compared to control. Error bars are ±SEM.

**Figure 6. NGF levels are altered in Itsn1 null brains.** NGF levels were quantified in either the hippocampus or septal regions of the brain in young adult and in aged mice. Genotypes are as indicated. n=3. NGF was significantly elevated in the hippocampus of younger Itsn1-TK mice (p=0.02) and significantly reduced in the septal regions of young and old Itsn1-TK mice (young, p=0.04; aged, p=0.03). Error bars are ±SEM.
Table 1: Effect of *Itsn1* on single amperometric events. Data shown as mean ± SEM of the cell median for each parameter. Differences between *Itsn1*-LK or *Itsn1*-TK and wild type (WT) controls are indicated as ***, p<0.001. Numbers in parentheses in the first column are number of cells used to calculate the number of exocytotic events (see Methods for further details).

<table>
<thead>
<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 (ms)</th>
<th>no. of cells</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>WT</strong></td>
<td>71.9±6.8</td>
<td>59.2 ± 4.9</td>
<td>265.1 ± 29.3</td>
<td>0.59 ± 0.09</td>
<td>2.8 ± 0.3</td>
<td>3.1 ± 0.4</td>
<td>16</td>
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<tr>
<td><strong>(22)</strong></td>
<td></td>
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<tr>
<td></td>
<td>66.9±6.6</td>
<td>51.8 ± 3.9</td>
<td>209.3 ± 29.3</td>
<td>0.47 ± 0.07</td>
<td>2.5 ± 0.5</td>
<td>2.5 ± 0.5</td>
<td>13</td>
</tr>
<tr>
<td><strong>(20)</strong></td>
<td></td>
<td></td>
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<tr>
<td><strong>Itsn1-LK</strong></td>
<td>23.8±2.5</td>
<td>55.6 ± 5.4</td>
<td>254.2 ± 37.6</td>
<td>0.52 ± 0.08</td>
<td>3.0 ± 0.7</td>
<td>3.2 ± 0.8</td>
<td>16</td>
</tr>
<tr>
<td><strong>(22)</strong>*</td>
<td></td>
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</tr>
<tr>
<td><strong>Itsn1-TK</strong></td>
<td>26.2±2.8</td>
<td>45.2 ± 3.9</td>
<td>192.3 ± 21</td>
<td>0.5 ± 0.08</td>
<td>2.7 ± 0.5</td>
<td>2.8 ± 0.4</td>
<td>13</td>
</tr>
<tr>
<td><strong>(20)</strong>*</td>
<td></td>
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</table>
Table 2. Measures of synaptic function in control, Itsn1-LK and Itsn1-TK neurons.

Data presented as the mean ± SEM. for (n synaptic boutons). * P <0.05 compared to controls.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Itsn1-TK</th>
<th>Itsn1-LK</th>
</tr>
</thead>
<tbody>
<tr>
<td>10-90% rise time (s)</td>
<td>0.17 ± 0.09</td>
<td>0.15 ± 0.09</td>
<td>0.17 ± 0.10</td>
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<tr>
<td>Peak ΔF/F</td>
<td>0.142 ± 0.019</td>
<td>0.126 ± 0.023</td>
<td>0.158 ± 0.018</td>
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<tr>
<td>T_{0.5} (s)</td>
<td>7.54 ± 0.09</td>
<td>9.95 ± 1.21*</td>
<td>14.29 ± 2.33*</td>
</tr>
<tr>
<td>τ (s)</td>
<td>9.13 ± 0.943</td>
<td>16.11 ± 2.66*</td>
<td>18.6 ± 3.21*</td>
</tr>
<tr>
<td>n</td>
<td>111 boutons</td>
<td>82 boutons</td>
<td>89 boutons</td>
</tr>
</tbody>
</table>
Figure 1

A

B
Figure 2
Figure 4
Figure 5
Figure 6
Abbreviations

ITSN1, Intersectin-1
DS, Down syndrome
AD, Alzheimer’s disease
APP, amyloid protein precursor
ApoE, apolipoprotein E