Disrupted in Schizophrenia 1 forms pathological aggresomes that disrupt its function in intracellular transport

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Disrupted in Schizophrenia 1 (DISC1) is a key susceptibility gene implicated in major mental illnesses, such as schizophrenia, depression, bipolar disorder and autism, but the link between this protein and the pathology of these diseases remains unclear. Recently, DISC1 has been demonstrated to form insoluble protein aggregates in vitro and in human post-mortem brain tissue but the cellular dynamics of these DISC1 aggregates and their effects on neuronal function are unknown. Using a combination of biochemistry and live cell confocal and video microscopy, we characterize the properties of DISC1 aggregates and their effects on cellular function. We demonstrate that DISC1 protein aggregates are recruited to the aggresome and degraded there by the autophagic pathway. We show that there is a compromised exchange between DISC1 in aggresomes and the cytosolic DISC1 pool, and that the large DISC1 aggregates, which can also co-recruit endogenous soluble DISC1, exhibit altered trafficking. Moreover, we demonstrate that large DISC1 aggregates have a pathological effect in neurons by causing the disruption of intracellular transport of key organellar cargo, such as mitochondria. These data, therefore, show that DISC1 is recruited to aggresomes with negative effects on neuronal function, and suggests a novel DISC1-based mechanism for neuronal pathology.

INTRODUCTION

A balanced chromosomal translocation t(1;11)(q42.1;q14.3) in a Scottish family with a high incidence of schizophrenia and mood disorders revealed a gene, Disrupted in Schizophrenia 1 (DISC1), as a promising candidate susceptibility gene for major mental illnesses, such as schizophrenia, depression, bipolar disorder and autism (1). Many further studies have demonstrated association between major mental illness and the DISC1 locus (2). While several important roles for DISC1 in healthy neuronal function have emerged (1,3), the mechanisms by which DISC1 leads to neuronal dysfunction and disease pathology remain unclear.

DISC1 is important for correct neuronal development, influencing neuronal proliferation and migration in addition to intracellular signalling pathways, such as the Wnt/β-catenin signalling pathway and PDE4 (4–7). DISC1 has been shown to play a role in axonal transport of mitochondria, synaptic vesicles and the kinesin-1 motor-dependent translocation of key neurodevelopmental proteins, including NDEL1 and LIS1 to axon tips (8–10). DISC1 has also been demonstrated to form insoluble protein aggregates (11,12) potentially reminiscent of other diseases where insoluble protein aggregates are a hallmark of the disorder; e.g. tau and amyloid-β aggregates in Alzheimer’s disease, mutant huntingtin aggregates in Huntington’s Disease and α-synuclein aggregates in Parkinson’s disease (13). The accumulation of insoluble aggregates in nerve cells is associated with an overload of the proteolytic degradation pathway and recruitment of these aggregates to the aggresome-autophagy pathway for protein removal (14). Protein aggregates in Alzheimer’s and Huntington’s disease can also lead to abnormal neuronal function, including intracellular trafficking and axonal transport defects (15,16). In contrast, whether DISC1 aggregates in neurons are recruited to aggresomes and whether they can disrupt neuronal function remains unknown. Characterizing the nature and cellular dynamics of DISC1 aggregates and determining whether they can disrupt functions such as intracellular cargo transport

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nuclear inclusions

Here we show, using a combination of molecular, biochemical and imaging approaches, that an insoluble form of DISC1 can be found both in cell lines and neurons, and that these DISC1 aggregates are recruited to the aggresome and degraded by the autophagic pathway, suggesting excess protein aggregation and therefore a potentially pathological role for this insoluble form of DISC1. Once recruited to this immobile aggresomal structure, the ability of DISC1 to interchange with cytosolic pools of the DISC1 protein is significantly reduced and a loss of DISC1 from the soluble fraction is seen, suggesting a sequestration of DISC1 away from regions where it is functional. Moreover, we find that driving DISC1 aggregate formation in neurons leads to disrupted intracellular transport of mitochondria. Thus, DISC1 aggregation in neurons appears to have a pathogenic function in the cell, leading to disturbed intracellular transport that may impact on neuronal development, synaptic function and synaptic plasticity (17), supporting the idea that via these mechanisms DISC1 aggregation may affect neuronal function.

RESULTS

DISC1 aggregates form aggresomes

Expression of DISC1 in multiple cell lines leads to the formation of large intracellular accumulations, or aggregates, of DISC1 (18–21), but the identity and subcellular localization of these aggregates remains unclear. To further investigate the relationship of DISC1 aggregates to other key intracellular compartments, we carried out confocal imaging of green-fluorescent protein (GFP)-tagged DISC1 (GFP-DISC1) coexpressed with markers for various intracellular organelles in COS7 cells (SV40-transformed simian cells). On expression of GFP-DISC1, we observed some cytosolic DISC1, numerous small puncta distributed throughout the cell and in ~30% of cells large DISC1 aggregates located perinuclearily (Supplementary Material, Fig. S1, % of COS7 cells containing perinuclear inclusions >5 μm in diameter = 27.94 ± 2.27%, percentage of neurons containing perinuclear inclusions >3 μm in diameter = 29.93 ± 2.01%). Colocalization between GFP-DISC1 and markers of key subcellular compartments, including the endocytic pathway, endoplasmic reticulum and Golgi was not found (Supplementary Material, Fig. S2). Due to the recent findings of the existence of insoluble multimers of DISC1 (11,12), we investigated whether DISC1 was recruited to a protein degradation structure, such as the aggresome, to which excess or misfolded proteins are known to localize and which can be visualized as large perinuclear inclusions or ribbon-like distributions in cells (14). We investigated colocalization between DISC1 and several markers for the aggresome including heat-shock protein 70 (HSP70) (22,23), ubiquitin (22,24), γ-tubulin (25) and the 26S proteasome (23). We found that with all markers used, there was a large degree of overlap (Fig. 1A). This provides a direct demonstration of the localization of DISC1 aggregates to aggresomes. To address whether DISC1 aggregates also form in neurons, we extended these investigations to GFP-DISC1-transfected primary hippocampal cultures. Similarly to COS7 cells, expression of GFP-DISC1 in transfected neurons led to the formation of GFP-DISC1 aggregates that colocalize with aggresomal markers (Fig. 1B).

To further test whether large DISC1-positive aggregates were in aggresomes, we treated cells with MG132, which interferes with the proteasomal degradation pathway. MG132 causes enhanced recruitment of a number of proteins linked to neurological disease to the aggresomal pathway for removal, leading to protein accumulation in large perinuclear structures or aggresomes (25–27). We found that treatment of GFP-DISC1-transfected cells with MG132 led to a substantial increase in GFP-DISC1 accumulation in dense perinuclear aggregates, while in contrast the distribution of GFP alone was unaffected (Fig. 2A and B, % of cells containing large perinuclear aggregates on treatment with MG132; GFP-DISC1 treated with ethanol vehicle was 19.09 ± 3.52%; compared with 90.50 ± 3.14% on MG132 treatment, P = 0.01; GFP treated with vehicle was 3.61 ± 1.24%; compared with 9.587 ± 0.63% on MG132 treatment, not significant). An additional hallmark of aggresomal structures is the disruption of intermediate filaments leading to their collapse to form cages surrounding the aggresomes (28). To test this, in untreated GFP-DISC1-transfected cells containing large GFP-DISC1 perinuclear aggregates, we investigated the organization and subcellular localization of vimentin, a marker for the intermediate filament network. In COS7 cells, a clear disruption of vimentin distribution was seen compared with cells expressing GFP control (Fig. 2C), further demonstrating the nature of these large GFP-DISC1 aggregates as aggresomes.

Aggresomes are predominantly degraded via the autophagic pathway (29,30). To determine whether GFP-DISC1-containing aggresomes were degraded via this same pathway, we investigated the colocalization between GFP-DISC1 and the autophagy marker, microtubule-associated protein light chain 3 (LC3) (31). We observed high levels of colocalization between LC3 and GFP-DISC1 in perinuclear regions supporting a role for the autophagy pathway in degrading GFP-DISC1 aggregates (Fig. 2D). In agreement with this, some GFP-DISC1 aggregates were also found to colocalize with lysosomes, which fuse with autophagosomes to degrade their contents (data not shown). GFP-DISC1 aggregate formation was also assayed on treatment of COS7 cells with two drugs that inhibit the autophagy pathway (32). Treatment with both 3-methyladenine (3-MA) and Bafilomycin-A1 caused a significant increase in the number of GFP-DISC1-containing aggresomes in COS7 cells [Fig. 2E, % of cells containing large perinuclear aggregates on treatment with phosphate-buffered saline (PBS) (control) = 34.57 ± 2.42% compared with 3-MA treatment = 54.53 ± 6.30%, P = 0.041, % of cells containing large perinuclear aggregates on treatment with dimethyl sulfoxide (DMSO) (DMSO control) = 33.01 ± 2.29% compared with Bafilomycin-A1 treatment = 49.40 ± 3.10%, P = 0.013]. This demonstrates that GFP-DISC1 aggresomes are degraded by the autophagy pathway commonly used for degradation of aggresomal structures.

Inhibition or overload of the proteasomal system (for example with MG132) drives the increased formation of aggresomes (33, see Fig. 2). We investigated whether, conversely, enhancement of the proteasomal system is able to
Figure 1. Identification of DISC1 localization to the aggresome. (A) DISC1 colocalizes with aggresomal markers in COS7 cells and (B) neurons. (Left) Colocalization of GFP-DISC1 with aggresomal inclusion bodies using antibodies against common aggresomal proteins. White arrows indicate regions of strong colocalization. Scale bar = 20 μm for neurons, 10 μm for COS7 cells. (Right) Line scans showing colocalization (intensity peaks at the same location) between GFP-DISC1 and markers of the aggresome.
reduce aggresome formation via cotransfection of GFP-DISC1 with the proteasomal activator subunit PA28γ. PA28γ expression has been shown to enhance proteasomal activity, reduce protein aggregation in cell lines (34) and increase cell survival in mutant huntingtin striatal cell lines (35). On coexpression of the proteasome activator PA28γ with GFP-DISC1 in COS7 cells, we found a significant reduction in the number of aggresome-containing cells (Fig. 2E, % of cells containing large perinuclear clusters >5 μm in diameter (n ≥ 100 cells per condition, three experiments). (C) Vimentin cages are seen collapsed around GFP-DISC1 aggregates but not GFP alone in COS7 cells. (D) GFP-DISC1 (green) colocalization with autophagy marker, LC3 (top panel—red), and proteasomal marker PA28γ (bottom panel—red). White arrows indicate regions of strong colocalization. (E) Treatment of COS7 cells with inhibitors of autophagy lead to increased GFP-DISC1 aggregation and proteasomal activators reduce GFP-DISC1 aggregation. Graph shows % of cells containing large perinuclear clusters >5 μm in diameter under different treatment conditions (n ≥ 100 cells per condition, three experiments). Scale bars = 10 μm.

may additionally act directly at the site of the aggresome to facilitate protein degradation.

**GFP-DISC1 is recruited to the insoluble fraction and can co-recruit endogenous DISC1 protein**

DISC1 has been found in the insoluble fraction of post-mortem brain tissue of patients who suffered from schizophrenia, depression and bipolar disorder (12). We wanted to investigate whether we could confirm the formation of insoluble DISC1 aggregates in primary dissociated neurons. Cortical neurons were used to provide cell numbers required for biochemical assays. A Sarkosyl-insoluble protein fractionation protocol was used based upon the methods used to identify insoluble protein aggregates in human disease brain both in schizophrenia and in other diseases commonly associated...
with aberrant protein aggregation, such as Huntington’s disease (12). Using this assay, we found low levels of aggregated DISC1 in the insoluble fraction of primary cortical neurons under basal conditions. As insoluble DISC1 was found largely in diseased brain and not in brain tissue from healthy individuals (12), we chose to investigate conditions that might mimic the disease state. Several studies have suggested the association of free radical generation with neuronal stress and schizophrenia (38). We therefore investigated whether free radical generation with hydrogen peroxide (H2O2), which has previously been used as a cell stressor (39,40), could lead to enhanced formation of DISC1 aggregates. We found that exposure of primary cortical neurons to H2O2 treatment led to a shift in distribution of DISC1 to the insoluble fraction (Fig. 3A and B). The presence of aggresome formation was seen in 30% of GFP-DISC1-transfected COS7 cells (Supplementary Material, Fig. S1). We wanted to investigate whether the aggregated GFP-DISC1 observed in aggresomes of COS7 cells could also correspond to the Sarkosyl-insoluble DISC1 identified in the brain tissue of patients with major mental illness (12), therefore linking these aggresomes to the previously demonstrated disease-linked insoluble form of DISC1. To do this, we carried out the same Sarkosyl-insoluble protein fractionation assays on GFP-DISC1-transfected COS7 cells. We found that GFP-DISC1-transfected cells express large amounts of insoluble GFP-DISC1 compared with the levels of GFP found in the insoluble fraction from control GFP-transfected cells (Fig. 3D and E). Levels of endogenous DISC1 in the insoluble fraction also increase in transfected with GFP-DISC1 compared with GFP alone assayed using DISC1 antibody. Lack of pronounced DISC1 detected at 130 kDa indicating a lack of reactivity of the antibody with the human GFP-DISC1 protein (n = 3). Expression of GFP-DISC1 in COS7 cells leads to accumulation in the insoluble fraction that corresponds with a loss of both exogenously expressed GFP-DISC1 (top panel) and endogenous DISC1 (bottom panel) from the soluble fraction. A loss of GFP from the soluble fraction is not seen (middle panel). Quantification of GFP protein levels of either GFP or GFP-DISC1 in the soluble fraction normalized to total input. (J) Quantification of endogenous DISC1 levels in the soluble fraction normalized to levels of endogenous DISC1 in the soluble fraction of the GFP control (n = 6).
Mutant huntingtin (PolyQ) aggregates are recruited to detergent-insoluble aggresomes (37) and have been demonstrated to sequester non-mutant soluble huntingtin into these aggresomal structures (41). To investigate whether aggregated GFP-DISC1 could similarly co-recruit endogenous, non-aggregated DISC1 to the aggresome, we transfected COS7 cells with either GFP-DISC1 or GFP alone and carried out high detergent separation assays to measure the amount of endogenous DISC1 in the Sarkosyl-insoluble fraction. Using an antibody to DISC1 and quantifying levels of full-length endogenous DISC1 in COS7 cells (molecular weight 97 kDa, no 72 kDa isoform is seen in these cells), we found that endogenous DISC1 levels present in the insoluble fraction were significantly higher when GFP-DISC1 was expressed compared with GFP alone. This demonstrated an enhanced recruitment of endogenous DISC1 to the insoluble fraction upon accumulation of aggregating DISC1 (Fig. 3F and G, normalized amount of endogenous DISC1 expression on transfection of GFP-DISC1 = 3.23 ± 1.00 compared with GFP alone, \( P = 0.05 \)). These findings show that aggregating DISC1 can recruit cytosolic DISC1 to the aggresomal compartment, suggesting a dominant disruption of DISC1 function throughout the cell.

In order to address whether the increase in DISC1 in the Sarkosyl-insoluble fraction produces a corresponding reduction in the soluble pool of DISC1, the levels of DISC1 in the soluble fraction were also quantified. When we quantified the levels of DISC1, both of exogenously expressed GFP-DISC1 and endogenous pools of DISC1, a significant reduction in the localization of DISC1 to the soluble fraction was found for both pools of DISC1 compared with controls (Fig. 3H and I, relative amount of GFP-DISC1 located in the soluble pool of GFP-DISC1 expressing COS7 cells = 0.85 ± 0.34 compared with GFP alone, \( P = 0.005 \) for the GFP antibody and Fig. 3H and J, relative amount of endogenous DISC1 located in the soluble pool of GFP-DISC1 expressing COS7 cells = 0.67 ± 0.11 compared with GFP alone, \( P = 0.005 \) for the DISC1 antibody). These findings suggest that aggregating DISC1 can lead to the co-recruitment of soluble DISC1 and to a loss of DISC1 from the soluble pool.

### Dynamics of DISC1 aggregates

To gain further insights into the properties of GFP-DISC1 in aggresomes and infer the behaviour of the co-recruited endogenous DISC1, we carried out live-cell imaging of these large perinuclear GFP-DISC1 aggregates and cytosolic GFP-DISC1 to investigate exchange properties of aggresomal GFP-DISC1 with the cytosolic pool of GFP-DISC1. We investigated this to determine whether DISC1 recruited to the aggresome could be released into the cytosolic pool of DISC1 where it would be required for function. To do this, we measured fluorescent recovery after photobleaching (FRAP) of GFP-DISC1 fluorescence to assay the recovery of fluorescence, representing a reintegration of cytosolic GFP-DISC1 into either large aggresomal GFP-DISC1 structures or cytosolic pools of DISC1. We found that there was a significant loss in FRAP of GFP-DISC1 in large perinuclear DISC1 aggregates compared with DISC1 in the cytosol, both in the rate of recovery and amount of recovery compared with pre-bleach levels (Fig. 4A–C, total recovery of aggresomal GFP-DISC1 as a % of pre-photobleach values = 43.12 ± 3.61 compared with 72.22 ± 4.64 for cytosolic GFP-DISC1, \( P = 1.15 \times 10^{-5} \)). This demonstrates a compromised interchange between the cytosolic pool of GFP-DISC1 and the aggresome recruited GFP-DISC1 compared with cytosolic DISC1, suggesting that sequestered DISC1 shows a reduced ability to exchange with cytosolic GFP-DISC1 pools where it is required to carry out its physiological roles.

DISC1 is actively transported to distal sites of action such as axon tips and growth cones (9,42,43), suggesting that it must be dynamically trafficked throughout the cell to carry out its correct function. We compared the movement of GFP-DISC1 in large aggresomal structures to the mobility and trafficking of distal smaller puncta of DISC1, a potentially functional form of DISC1. Notably, DISC1 puncta were found to be highly dynamic with ~20% of the puncta moving distances of >2 \( \mu \)m over the imaging period with an average velocity of 0.30 \( \mu \)m/s. This velocity is in the range of other cargo transported by fast axonal transport, suggesting that movement of DISC1 puncta occurs via microtubule-based transport mechanisms (8,44). In contrast, aggresomal DISC1 was immobile indicating a lack of recruitment to the microtubule network for transport throughout the neuron. Quantification of the mobility of GFP-DISC1-containing structures showed that aggresomal DISC1 was stationary compared with the more distal GFP-DISC1 puncta (Fig. 4D–G, % of clusters moving a distance >2 \( \mu \)m over 5 min, puncta = 21.19 ± 3.96%, aggresomes = 0.00 ± 0.00%, \( P = 0.0004 \). Velocity of puncta = 0.30 ± 0.01 \( \mu \)m/s). Thus, once GFP-DISC1 has been sequestered into large aggresomal structures in the cell, it can no longer be correctly trafficked to more distal regions such as growth cones and synapses where it may be necessary to maintain a pool of DISC1 required for correct neuronal function.

### DISC1 aggregate formation disrupts healthy neuronal function

Protein aggregation leads to disrupted cellular function as has been seen in Alzheimer’s, Huntington’s and Parkinson’s disease. Aggresomal DISC1 may sequester DISC1 away from the cytosolic pool and therefore reduce DISC1 availability at locations required for healthy function. To assay cellular function, we addressed effects on intracellular transport, which is regulated by DISC1 (8–10). We explored whether GFP-DISC1 aggregate formation would lead to compromised mitochondrial trafficking using live-cell microscopy to image mitochondrial movement in the axons of primary hippocampal neurons containing large GFP-DISC1 aggresomal structures. We compared this with mitochondrial movement in cells expressing GFP as a control. To visualize mitochondria, we co-transfected mitochondrial-targeted dsRed proteins, mtdsRed, together with either GFP-DISC1 or GFP. Mitochondria imaged over time in axons were visualized using kymographs (Fig. 5A). In GFP-transfected neurons, the percentage of mitochondria that were moving was ~25%, similar to previous observations (8) (Fig. 5C). We then imaged mitochondrial movement in GFP-DISC1 aggresome-containing neurons identified by large perinuclear inclusions of DISC1 >3 \( \mu \)m in...
diameter, and found a significantly decreased number of moving mitochondria compared with GFP transfection alone (Fig. 5A and C, % of mitochondria moving in GFP versus GFP-DISC1-transfected neurons: GFP = 24.41 ± 3.59%, for GFP-DISC1 = 13.00 ± 2.79%, \( P = 0.029 \)). In contrast, when we assayed the instantaneous velocity by tracking displacement of mobile mitochondria from their location in the previous frame (Fig. 5B and C), we found that the average velocity of mitochondrial movement was unaffected across all cell types demonstrating the lack of disruption of the microtubule network. These findings show that depletion of functional DISC1 caused by recruitment of DISC1 to aggresomes causes compromised DISC1 function and the loss of mitochondrial transport regulation in neurons. This demonstrates a key functional effect caused by the accumulation of insoluble DISC1 into aggresomes.

DISCUSSION

Here we show that DISC1 forms large perinuclear aggregates and conclusively identify these structures as aggresomes. We show that these DISC1-containing aggresomes colocalize with markers of the autophagic pathway and require this pathway for their degradation. Furthermore, we demonstrate that insoluble aggregates of DISC1 are formed in neurons. Large DISC1 aggregates can co-recruit soluble pools of endogenous DISC1 which once recruited demonstrate limited interchange with the surrounding cytosolic pools and a loss of the pool of soluble DISC1. Finally, we show that depletion of functional DISC1 via protein aggregation gives rise to compromised neuronal function, specifically the disruption of mitochondrial transport. This finding is similar to what we have previously observed upon depletion of DISC1 by
RNAi, that the loss of endogenous DISC1 from neurons causes a reduction in mitochondrial movement. Although DISC1 has been shown to genetically associate with major mental illness in multiple different populations and the role of DISC1 in neurons has begun to be illuminated (1,3,8,45), the disease mechanisms have remained elusive. Several studies have demonstrated an association with single nucleotide polymorphisms within the DISC1 gene and major mental illness, including schizophrenia, depression and bipolar disorder (46–48) and some mechanisms for disruption by these alterations have been suggested (8,49,50). Here we show that DISC1 in its most common variant is prone to forming large perinuclear aggregates that we identify as aggresomes. While it was recently proposed that DISC1 may be localized to aggresomes, no aggresomal markers or assays were used to confirm this (51,52). Here we show, both in cell lines and neurons, that DISC1 colocalized with four commonly used aggresomal markers, \(\gamma\)-tubulin, S26 proteasome, ubiquitin and HSP70 (22–25) in addition to being surrounded by vimentin cages. We also demonstrate enhanced aggresomal formation upon treatment with the proteasomal inhibitor MG132, whereas conversely, proteasomal activation reduces DISC1 aggresome formation. The aggresome is processed by fusion with the autophagosome (53); we find that aggresomal DISC1 colocalizes with the autophagy marker LC3 and that inhibitors of the autophagic pathway enhance DISC1 aggresome formation. These findings conclusively demonstrate that these GFP-DISC1 structures to be aggresomes. We then show that GFP-DISC1, in addition to endogenous DISC1, is found in the Sarkosyl-insoluble fraction, potentially linking this aggresomal DISC1 structure to the Sarkosyl-insoluble DISC1 aggregates found in human post-mortem brain tissue.

Aggresomes are structures formed to remove toxic build-up of misfolded or aberrant protein, suggesting DISC1 aggregation may contribute to neuronal pathology, as has previously been reported for other pathogenic proteins such as mutant huntingtin, tau, \(\alpha\)-synuclein or amyloid-\(\beta\) (13). These findings suggest that protein aggregation can be a feature not only of neurodegenerative disorders, but also of major mental illness. Supporting this, several studies have shown that the expression of multiple heat-shock proteins—chaperone proteins key for correct protein folding—are upregulated in post-mortem brain tissue of patients suffering from mood disorders (54–56). This may underlie a requirement for enhanced protein processing in response to build-up of aggregating proteins, such as DISC1. Interestingly, in our studies, we find that by enhancing proteasomal activity, we can reduce the build up of aggresomal DISC1 showing that increased protein processing can be neuroprotective. Furthermore, gene variations in HSP70 have been found to associate with schizophrenia and

Figure 5. GFP-DISC1 aggresome-containing neurons show a reduction in mitochondrial transport. (A, upper panel) GFP or GFP-DISC1-transfected hippocampal neurons showing either a diffuse distribution or aggresomal-like distribution, respectively. GFP-DISC1 neurons containing aggresomal structures were selected for imaging. (Lower panel) Kymographs showing movement of mitochondria through the axons over time. (B) Velocity of moving mitochondria was assayed, paths of moving mitochondria showing the displacement of mitochondria relative the previous frame. Paths of all moving mitochondria displayed in the kymograph are presented in the lower panel. (C) Graphs showing percentage of mobile mitochondria and velocity of the population of moving mitochondria. Scale bars = 10 \(\mu\)m (\(n = 7\)).
bipolar disorder in several studies (57–60), strengthening the link between protein misfolding and aggregation with mental illness.

Protein aggregation and aggresome formation can give rise to defects in neuronal function resulting from both the loss of functional protein and the pathogenic effects of large amounts of aggregated protein in the cell. This was investigated via assaying intracellular transport in neurons. Several forms of disease-related protein aggregation are known to disrupt intracellular trafficking and axonal transport, such as β-amyloid in Alzheimer’s disease and polyQ huntingtin in Huntington’s disease (15,44,61). Leliveld have shown that DISC1’s interactions with NDEL1 are disrupted by DISC1 aggregation (11,12). We find that formation of DISC1 aggregates not only leads to trapping of external pools of DISC1 and therefore their removal from other cellular locations, but also leads to mitochondrial transport defects which suggest that the DISC1-dependent transport of other cargo such as synaptic vesicles may also be disrupted (10). DISC1 interacts with a large number of microtubule-associated proteins, such as LIS1, NDEL1, MIPT3, 14-3-3, dynactin and MAP1 and disruption of DISC1 interactions with these proteins may contribute to the disruption of transport seen in our studies, suggesting that a more global disruption of transport may result from DISC1 protein aggregation (9,62,63). DISC1 localizes to the centrosome where it contributes to microtubule organization, and the recruitment of other centrosomal proteins to this structure to influence neurite outgrowth and neuronal migration (6,64–66), furthermore, DISC1 plays a role in neuronal proliferation via the Wnt/β-catenin signalling pathway (5,6) as well as at the synapse in regulation of spine maintenance and synaptic function (67,68). Our findings suggest that these processes might also be disrupted by DISC1 aggregation and loss of function and it will be interesting to further explore this possibility. It has recently been shown that aggregating DISC1 is cell penetrating (51,52). Whether these invasive structures are able to cause disruption of cellular function will also be an exciting avenue of study. Additionally, whether the aggregates formed by DISC1-Boymaw fusion constructs, which represent the fused gene product of the (1;11)(q42;q14.3) translocation potentially expressed in humans (69), can also disrupt neuronal function will also be important to investigate.

The findings presented here provide further evidence for the novel pathway of protein aggregation in DISC1 pathology, and demonstrate its effect on neuronal function. This opens further avenues for research investigating the role of DISC1 protein aggregation in pathology.

**MATERIALS AND METHODS**

**Constructs, antibodies and reagents**

Antibodies against β-lamin (IF 1:100) (goat), GFP (WB 1:100) (rabbit) and HSP70 (IF 1:200) (goat) were from Santa-cruz. 26S proteasome p27 subunit (IF 1:20) (mouse) was from ProGen and anti-DISC1-Midterm (WB 1:200) (rabbit) and Ubiquitin (1:500) (mouse) antibodies were from Invitrogen. cDNA construct encoding DISC1-FL in pEGFPc1 has been described previously (19). Mitochondrially targeted monomeric dsRed fluorescent protein (mtdsred2) has been previously described (8). Red fluorescent protein (RFP)-tagged cell markers were from Addgene: mRFP-Rab5—Addgene plasmid 14437 (70), DsRed-rab11—Addgene plasmid 12679 (71), pmRFP-LC3—Addgene plasmid 21075 (72), dsRed-Golgi (B14-galactosyltransferase, NM0014973, in pdsRed2-C1 vector) and dsRed-ER (calreticulin, NM004343.3, in pdsRed2 vector) were a kind gift from A.C. Dolphin (UCL) (73). Human myc-DDK-tagged PA28 cDNA (PSME3) cloned into the pCMV6-Entry vector was purchased from Origene. All other reagents were from SIGMA.

**Cell line and culture**

COS7 cells were maintained in Dulbecco’s modified Eagle medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum and penicillin/streptomycin and incubated at 37 °C, in a humidified 5% CO2 atmosphere. Transfections were carried out by electroporation (74) using the Amaxa Nucleofector (Amaza Biosystems). MG132 treatments were carried out at 10 μM (dissolved in ethanol). Control treatments were carried out in equivalent volumes of ethanol (vehicle). 3-MA treatments were carried out at 10 mM (dissolved in PBS) for 24 h. Control treatments were carried out in equivalent volumes of PBS (control). Bafilomycin-A1 treatments were carried out at 1 μM (dissolved in DMSO) for 24 h or with DMSO as a control (DMSO control). Hippocampal and cortical neuronal cultures were prepared from E18 Sprague–Dawley rats as previously described (8). Hippocampal neurons were transfected by calcium phosphate precipitation at 8 days in vitro (DIV) (8). Cortical cells were treated with H2O2 at 20 μM for 2 h at 10 DIV, or H2O2 at 20 μM for 2 h at 10 DIV followed by 24 h treatment with the autophagy inhibitor 3-MA at 10 μM.

**Biochemical assays**

The Sarkosyl-insoluble fraction was isolated as described (11). In brief, 24 h after transfection, COS7 or cortical cells were lysed and treated with DNA lysis buffer, incubated at 37 °C for 30 min, then overnight at 4 °C (50 mM (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES), pH 7.5, 300 mM NaCl, 250 mM sucrose, 10 mM MgCl2, 1% NP-40, 0.2% Sarkosyl, 10 μg/ml antipain, pepstatin and leupeptin, 1 mM phenylmethanesulfonylfluoride (PMSF), and DNase 1 40 units/ml). Samples were centrifuged at 1800g for 30 min and resuspended in high salt buffer (50 mM HEPES, pH 7.5, 1.5 mM NaCl, 250 mM sucrose, 1% NP-40, 0.2% sarkosyl, antipain, pepstatin and leupeptin at 10 μg/ml, 1 mM PMSF). Subsequently, samples were centrifuged for 30 min at 1800g, and the resulting pellet was resuspended in 300 μl of cold 50 mM HEPES and 0.2% Sarkosyl. Finally, samples were subjected to ultracentrifugation at 100 000g for 45 min (TLA-100.3 rotor in an Optima Beckman Coulter Ultra Centrifuge), and the resulting pellet was solubilized in 1% SDS. All steps were performed at 4 °C unless otherwise specified. Samples were then solubilized in sample buffer and resolved on sodium dodecyl sulfate polyacrylamide gel electrophoresis gels and western blot analysis carried out as described (74).
Fixed and live imaging

For fixed imaging, cover slips were fixed with 4% paraformaldehyde/sucrose solution in PBS for 10 min before being permeabilized and blocked in block solution (PBS containing 10% horse serum, 0.5% Bovine serum albumin, 0.2% Triton X-100) for 10 min. Subsequent antibody dilutions were performed in block solution and washed in PBS. AF488- and AF568-conjugated anti-mouse and anti-rabbit secondary antibodies were from Invitrogen and used at 1:500.

For live neuronal imaging of GFP-DISC1 aggregates and mitochondria, cells were imaged under perfusion with extracellular solution pH 7.4 (125 mM NaCl, 5 mM KCl, 10 mM HEPES, 10 mM glucose, 2 mM CaCl₂ and 1 mM MgCl₂). Medium was warmed to 37°C and flowed at a rate of 5 ml/min throughout the duration of each experiment. MtdsRed-labelled mitochondria and GFP-DISC1 movement were analysed in the axons or cell body, respectively, and kymographs were created as described previously (8,74).

Briefly, using ImageJ software, curved processes were straightened using the ‘straighten’ macro and kymographs created by the ‘multiple kymograph’ macro. Resultant kymographs show the process along the x-axis and time across the y-axis. Mobility was assessed by counting the percentage of objects moving >2 μm during an imaging period. Velocity was measured using the ‘track points’ macro. The smallest change of position measurable was one pixel, i.e. ~0.3 μm. Periods of no movement within a track for 1 s or greater were discounted from the analysis.

Imaging was carried out using a Zeiss LSM 510 Confocal microscope and images digitally captured using LSM software. Excitation was via an argon laser at λ = 488 nm and a HeNe laser at λ = 545 nm.

Fluorescent recovery after photobleaching (FRAP)

COS7 cells were imaged in supplemented DMEM with 10 mM HEPES. Live cells were imaged in a 37°C heat-controlled chamber. FRAP was carried out as described (75). A single z-section at 2× zoom was acquired prior to FRAP protocol before and at time intervals after the bleach. A region of interest (ROI) was selected and photobleaching was carried out at 80% laser power for 10 iterations using the 488 nm line. Images were acquired for the whole frame every 250 μs for 100 s. Using ImageJ software, a ROI corresponding to the bleach area was selected for the analysis of signal intensity. Intensity values were normalized using the following equation; \( I_t = \frac{(A_t)}{(B)} \), where \( I \) is the intensity at time \( t \), \( A \) is the intensity at time \( t \) and \( B \) is the intensity immediately prior to photobleach. Significance between time points is calculated as difference between individual time points.

Statistical analysis

All data were obtained using cells from at least three different preparations. Individual differences were assessed using individual student’s t-tests. Data are shown as mean ± standard error of the mean (SEM).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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