Abstract

Background

Primary neuronal cultures underpin diverse neuroscience experiments, including various protein analysis techniques, such as Western blotting, whereby protein extraction from cultured neurons is required. During immunoblotting experiments, we encountered problems due to a highly-abundant protein of 65-70 KDa present in the cell extracts, that interfered with total protein estimation, and immunodetection of target proteins of similar size. Previous research has suggested that serum proteins, specifically albumin, contained within commonly-used culture media, can bind to, or be adsorbed by, generic cell culture plasticware. This residual albumin may then be extracted along with cell proteins.

New Method

We made simple modifications to wash steps of traditional cell lysis/extraction protocols.

Results

We report that a substantial amount of albumin, accumulated from the standard culture media, is extracted from primary neuronal cultures along with the cellular contents. This contamination can be reduced, without changing the culture conditions, by modifying wash procedures.

Comparison with Existing Methods

Accumulated albumin from neuronal culture media, in amounts equivalent to cellular contents, can distort data from total protein assays and from the immunoreactive signal from nearby bands on Western blots. By altering wash protocols during protein extraction, these problems can be ameliorated.

Conclusions

We suggest that the standard extended culture periods for primary neuronal cultures, coupled with the requirement for successive medium changes, may leave them particularly susceptible to cumulative albumin contamination from the culture media used. Finally, we propose the implementation of simple alterations to wash steps in protein extraction protocols which can ameliorate this interference.

Keywords: BSA, band distortion, protein absorption, immunoblot problems, protein extraction, primary neurons

1. Introduction

Protein extraction from cultured cells is a key technique in a variety of biological sciences, including neuroscience, where primary neuronal cultures constitute a large component of basic research in the field. In particular, protein analysis, via a variety of techniques including Western blotting and a range of assays, is a corner stone of this research. In order to avoid protein degradation of sensitive proteins, many extraction protocols involve directly lysing cells from cell culture plasticware. These methods usually include an initial wash step with ice-cold phosphate-buffered saline (PBS), followed by the application of a cell lysis/extraction buffer, and brief centrifugation of the resulting cell extract to remove debris from the protein-rich supernatant. Most culture media are either supplemented by serum such as fetal bovine serum (FBS), or contain additional pro-survival factors which include serum components. For example, specialised Neurobasal medium supplemented with B27 is a widely used cell culture medium for the growth and maintenance of healthy primary neuronal cultures *in vitro* (Brewer et al., 1993). The B27 supplement includes bovine serum albumin (BSA) (Brewer & Cotman, 1989), an abundant protein of 65-69 KDa.

Interestingly, previous research, using common proliferative cell lines, has suggested that serum proteins contained in culture medium, in particular BSA, can bind or be absorbed by generically used cell culture plasticware (Hong et al., 2016, Miyara et al., 2016). Moreover, it appeared that the residual BSA from the culture medium could then be extracted along with cell proteins.

Our attention was drawn to this issue by problems in immunoblotting analysis of glutamic acid decarboxylase (GAD) expression in primary neuronal cultures. The GAD immunoreactive bands (at 65 and 67 KDa) were often distorted, as though by the presence of some highly-abundant interfering protein. Protein abundance (as assessed by housekeeping gene immunoreactivity) also seemed to vary between lanes, despite prior normalisation via a standard total protein assay. We considered the possibility that albumin from the culture medium might be accumulating on the culture well surface, and then contaminate the cell extract, distorting the GAD bands and the total protein measurement. This could be a particular issue with primary neuronal cultures, where, since optimal formation or maturation in terms of the peak of synaptic connectivity in cultured primary neurons at around 14 days *in vitro* (DIV) (Ichikawa et al., 1993), many research questions require the maintenance of cultures for at least this time period or longer. During this process, numerous culture medium changes are undertaken and the possibility for the aforementioned albumin binding may increase. Considering this, it may be that protein extracts and subsequent analysis from primary neurons cultured with B27 supplemented Neurobasal medium are particularly subject to the risk of contamination from BSA. This may impact data regarding total protein yields, resulting in incorrect protein concentration measurements and possibly interfering with experimental results. Given the popularity of Western blotting methods across many fields of molecular and cellular biology, this ~65 - 70kDa protein may inhibit the appropriate separation of other proteins of a similar weight. Moreover, equal loading of protein which is key to the accuracy of experimental data may also be affected by this.

Given the prevalence of such components in cell culture medium and the possible interference with data, it is important to know the extent of this binding, and adaptations that may be introduced to protein extraction protocols to ameliorate the problem. In this paper, we present findings which demonstrate that substantial amounts of albumin contained within culture medium can bind to generically used cell-culture plasticware, both with and without the presence of primary neurons. Furthermore, considering particular consequences this may have to primary neuronal cultures, we provide evidence that easily applied alterations to protocol wash steps may eliminate, or at least reduce, this issue.

2. Methods

2.1. Primary cortical neuronal cultures

Primary cortical neurons were prepared using tissue from C57BL/6 mouse embryos (E17), according to our standard procedures (Morris., 1995; McNair et al., 2010). In brief, whole brains were isolated and placed in ice-cold Hanks Balanced Salt Solution (HBSS), meninges were removed and cerebral cortices were isolated then transferred into clean ice-cold HBSS. Cortical tissue was chopped finely, washed twice in HBSS and then treated with 0.05% trypsin/EDTA (Gibco, 25300-054) at 37°C for 10 min. Following this, DMEM (10% HI horse serum, 1% Penicillin Streptomycin, 1% Glutamax) was added to inactivate the trypsin and tissues were centrifuged at 1500rpm for 5 min. Following trituration and resuspension, DMEM medium was added (9ml/embryo) and cells were seeded onto polystyrene based plastic 12-well plates (Corning, 3513) which had been pre-coated with 4µg/ml poly-D-lysine and 6µg/ml laminin. Neurobasal medium (Gibco; 21103049) supplemented with B27 (Gibco, 17504044) was added prior to cell seeding (1ml/well for 12-well plates). Cells were seeded at a 50% dilution. After 24 hours, 50% of the culture medium was replaced with Neurobasal/B27; this was repeated at 48 hours from time of seeding and then 50% changes with Neurobasal/B27 were made every 4 days for the duration of the cultures. An incubation length of 14 DIV was chosen in line with aforementioned research which suggests this period to be optimal for neuronal maturation *in vitro*; hence the majority of research protocols involving primary neuronal cultures will extract protein for analysis at this time-point or later.

All animal procedures were carried out in accordance with University of Glasgow ethics and animal research policies which subscribe to ARRIVE and UK Home Office guidelines.

2.2. Cell culture medium and mimetic incubation

In order to assess the ability of albumin to bind to cell culture plasticware within a 14-day period, we incubated 12-well plates just with culture medium (DMEM and Neurobasal+B27 medium) under the same protocol as used for primary neuronal cultures. All medium changes followed our primary neuronal culture protocol as above (ie. 50% changes with Neurobasal every 24 hours, 48 hour and then every 4 days). Incubation periods for all conditions were 14 DIV.

To confirm the role of albumin, the above experimental conditions were also employed using only BSA (Sigma, A9418)/PBS solution at a concentration equal to albumin contained within the previously used culture mediums. For serum, this was 3.5mg/ml BSA, in line with research which highlights an albumin concentration of 29.6g/L – 38.5g/L in serum from clinically healthy horses (Riond et al., 2009). To mimic the Neurobasal/B27, the BSA concentration was 2.5mg/ml in accordance with previously published B27 recipes successfully used for primary neuronal cultures (Brewer et al., 1993; Chen et al., 2008; Roth et al., 2010).

2.3. Protein extraction

Protein was extracted from 12-well plates at 14 DIV. Briefly, plates were placed on ice and culture medium or BSA/PBS solution was removed from wells. Ice-cold PBS (pH 7.5) was added to each well for ~15 seconds, the volume of which was manipulated dependent on experimental conditions (either 200µl, 500µl or 1000µl). The number of washes were also manipulated with wells being washed 1 or 3 times. The length of wash was also altered, with washes of ~15 seconds or 2 minutes. After removal of the wash buffer, 80µl of RIPA buffer (50mM Tris-HCL, 150mM NaCl, TritonX100 (1%), SDS (0.1%), Sodium deoxycholate (0.5%), 50ml dH_2O) + 1% Protease Inhibitor Cocktail (Sigma P-8340) was added. After a brief lysis period, wells were scraped for ~30 seconds, contents removed on ice within 10 minutes and centrifuged at 4°C, 13000rpm for 10 minutes. Supernatants were collected and protein concentration was determined using a Bradford protein assay (previously detailed, Bradford., 1976). All samples were stored at -80°.

2.4. Albumin extraction

In an attempt to deplete the existing albumin from primary neuronal culture extracts we utilised a differential precipitation-based extraction method (first outlined by Colantonio et al., 2005). In brief, samples were incubated in 0.1M NaCL for 1 hour, followed by a further 1 hour incubation in ice-cold ethanol. Samples were then centrifuged at 16000rcf for 45 minutes at 4°C, with the supernatant collected for further processing and the pellet (containing cell proteins) resuspended in 10mM Tris buffer. The pH of the supernatant was lowered to 5.7 and incubated for 1 hour, before centrifugation at 16000rcf. The resulting pellet was resuspended in 10mM Tris buffer and pooled with the previously collected pellet. The supernatant, containing the extracted albumin, was retained for further analysis. All incubations were performed at 4°C and all samples stored at -80°.

2.5. Protein electrophoresis and Western Blot

Immunoblotting was conducted as previously described (Guilding et al., 2007; McNair et al 2010). 25µl/lane of samples (~2.5µg/µl) from previously extracted protein were prepared with 4x sample buffer (NuPAGE, Novex, NP0007) and sample reducing agent (NuPAGE, Novex, NP0004). Protein was denatured at 80°C for 10min. Samples were then subjected to SDS-PAGE in 10 % Bis-Tris gel (NuPAGE, Novex, NP0302BOX) run at 200 volts for 1 hour and transferred to Invitrolon PVDF membrane (Novex, LC2005) (30 volts, 1 hour).
Following transfer, membranes were washed twice in dH_2O and blocked 0.5% Tween-tris-buffered-saline (TTBS) + 3% dried milk for 30 min at room temperature. Membranes were then incubated overnight at 4°C with anti-GAD65/67 (1:50,000, Sigma G5163, supplemented with 1% dried milk) while agitated. The following day, membranes were washed 3 times (10 min) in TTBS and incubated in HRP-conjugated anti-rabbit secondary antibody (1:10,000, supplemented with 1% dried milk) for 2 hours at room temperature. Membranes were washed again, once with TTBS and then twice in 10X Tris-Buffered Saline for 10 minutes. Blots were further probed using GAPDH-HRP antibody (1:20,000, GeneTexGTX627408, supplemented with 1% dried milk) and anti-Albumin (1:10000, GeneTex GTX102419, diluted in SignalBoost™, Merck 407207). Membrane-bound antibodies were detected on the blot with a Chemiluminescent HRP Substrate (Immobilon, Millipore, WBKLS0100), and digital images were captured by PXi4 (Syngene).

2.6. Coomassie staining

For experiments requiring Coomassie Brilliant Blue staining, samples were subjected to the same extraction and electrophoresis protocol as outlined above. The gel was then removed, washed twice with dH2O and placed in a 0.1% Coomassie Brilliant Blue (Fluka, 27815) solution (50% v/v methanol, 10% v/v acetic acid) for 30 minutes with agitation at room temperature. Following complete saturation of the gel, the Coomassie solution was replaced with a de-stain solution (50% v/v methanol, 10% v/v acetic acid). The gel was then agitated with the de-staining solution replaced as necessary until an adequate signal-to-noise ratio was achieved. Stained gels were then immediately imaged via a 12MP digital camera.

3. Results

3.1. BSA can bind to cell culture plasticware and be extracted with or without the presence of neurons

To investigate whether BSA contained within DMEM culture medium and B27 supplement can bind and be extracted from generically used culture medium, we first incubated plates with DMEM and Neurobasal/B27 medium under the same protocol as utilised for primary cortical neuronal cultures, but in the absence of primary neurons. Protein was then extracted as outline above and total protein quantified via Bradford assay.

Figure 1 (A) displays total protein yields from these plates under various wash conditions. We found that albumin could be extracted under all wash conditions, but was highest where PBS wash volumes were low (ie. 200ul x 1 for 15 seconds: 1.74 ± 0.02 mg/ml, compared to 1000ul x1 for 15 seconds: 1.09 ± 0.003 mg/ml). The same trends were observable under our BSA/PBS mimetic conditions (Fig.1B), again, with lowest wash volumes producing the highest amount of extracted protein (ie. 200ul x 1 for 15 seconds: 4.27 ± 0.11 mg/ml, compared to 1000ul x1 for 15 seconds: 2.28 ± 0.02 mg/ml). Increasing the number of PBS washes did produce an observable reduction in extracted protein amounts, but this was not as dramatic as reductions produced by wash volume increases.

To clarify whether the protein extracted from these plates was albumin, we subjected samples to gel electrophoresis and Coomassie blue staining (Figure 2). As can be seen for both Neurobasal/27 (lanes 2-4) and BSA/PBS (lanes 5-7), clear and intense bands of a weight between 65 – 70kDa are present. Given the molecular weight of BSA (~66.5 - 69kDa) we consider this as a specific band. Moreover, this was the only reactive band present in samples extracted from Neurobasal/B27 incubated plates.

3.2. BSA is present in primary cortical neuronal culture extracts

We subjected extracts from primary cortical neuronal cultures to Coomassie gel staining, in order to address whether BSA from culture medium could bind to generically used cell culture plasticware and be extract with cell proteins in the presence of primary cultures. Figure 2A (lanes 8-10) displays the Coomassie staining for of these samples. A prominent and relatively large band can be seen at the weight of around 65-70kDa which is consistent with the molecular weight of BSA.

To assess potential removal of the albumin from cell extracts, we employed a chemical-based albumin extraction protocol on protein previously extracted from primary neuronal cultures. The resultant products (ie. pellet resuspension containing cell protein and albumin-rich supernatant) were subjected to gel electrophoresis and Coomassie staining (Fig 2. Lanes 11 and 12, respectively). We observed only one clear and strong reactive band from the supernatant, at the weight of ~ 65-70kDa, consistent with the size of bands seen in all other samples. Notably, this band was not present in the cell-pellet resuspension sample, indicating that this sample did contain albumin and that this had been effectively extracted. To further quantify these results, we performed a Bradford assay to assess the protein amount of the original sample and the subsequent products of the albumin extraction protocol (Fig.3). Our original extraction from cultured primary cortical neurons contained 1.76 ± 0.028 mg/ml total protein, while the pellet resuspension contained just 0.51 ± 0.001 mg/ml and the supernatant 0.71 ± 0.008 mg/ml. Approximately 0.54mg of protein appeared to have been lost through this protocol. We suggest this diminishment may be due to lengthy incubations steps involved in the albumin removal protocol, which may subject cell-proteins to the risk of degradation.

3.3. Implementing additional wash steps during extraction ameliorates BSA contamination in primary culture extracts and resolves interference with protein analysis

Results from our Bradford total protein analysis of plates incubated in the absence of primary neurons highlighted a reduction in extracted protein with increased wash volumes and number of washes. Considering this, we wished to investigate the effects of these wash steps on further protein analysis. We extracted primary neuronal cultures under a low volume (200µl) and high volume

(1000µl) wash protocol, and subsequently used immunoblotting with GAD65/67, albumin and GAPDH antibodies (Fig. 4).

The observable difference in the quality of these blots was relatively striking, particularly in the quality of separation of the 65 and 67kDa GAD bands. Under a low volume wash protocol (Fig. 4A), these bands were noticeably distorted and often bulging or compressed, this problem looks to be almost completely resolved by increasing wash volume (Fig. 4B). We suggest bands of this weight to be particularly at risk of interference from BSA contamination due to the overlap in their size, meaning that albumin present in the gel can distort the separation and clarity of these proteins. Figure 4C highlights the extent of albumin contamination under a low volume wash protocol. Moreover, it is clear that distortions and compressions of the 65 and 67kDa GAD bands are a direct effect of albumin contamination. Finally, BSA contamination impedes normalisation of protein loading amount as widely used protein assays (such a Bradford) do not separate between cell proteins and BSA. The improvement in equal protein loading is abundantly clear from GAPDH house-keeping protein bands after increasing wash volume (Fig.4).

4. Discussion

The present work investigated the hypothesis that BSA contained within components of culture medium (namely, serum and B27 supplement) can bind to generically used polystyrene-based cell culture plasticware. We found clear evidence which supports the above hypothesis, with BSA found to be present in samples extracted from 12-well culture plates with and without the presence of primary neuronal cultures. Moreover, our investigation highlighted the complications which can be created by BSA contamination of samples, in terms of the quality of analysis of a target protein and the ability to obtain a correct estimation of extracted cell protein.

Our results are consistent with two previous reports, using non-neuronal cell lines, that albumin is absorbed from culture medium onto plastic cultureware surfaces, and then extracted along with cellular content (Hong et al., 2016; Miyara et al., 2016). However, to our knowledge, we are the first to identify that this effect is a particular issue in primary neuronal cultures. These findings are also in line with data showing high amounts of BSA in B27-supplemented culture medium which can interfere with subsequent target protein analysis (Trajkovic et al., 2018). This phenomenon may be a particular issue for primary neuronal culture, where cells are maintained for extended periods to allow development of a relatively mature neuronal phenotype, necessitating numerous replenishments of fresh medium. The amounts of albumin absorbed are rather substantial. From the albumin extracted from surface exposed either to neurobasal medium/B27, or BSA solution, we estimate that after washing with low volumes (200µl) 9.40% (Neurobasal Medium/B27) and 23.08% (BSA solution) of the BSA applied is retained. We note that BSA adsorption amounts appear to be substantially higher in plates incubated with the BSA/PBS solution compared to Neurobasal Medium/B27. Although we cannot be certain about which specific components of B27 may reduce BSA adsorption, it has previously been shown that BSA molecules adsorb in a more compact and stable conformation when in phosphate buffer (Larsericsdotter et al., 2005; Jeyachandran et al., 2009). Given this, we suggest that the increase in adsorption in the BSA/PBS incubated plates may be a consequence of the phosphate buffer itself, with more compact and stable molecules being adsorbed. This may lead to a densely packed adsorbed monolayer, which could increase the number of BSA molecules that can be adsorbed to the available surface area of the well. Another trend that has been observed is that less stable proteins generally lose more structure upon adsorption than more stable proteins, and the more extensive these structural alterations are, the less surface coverage of the protein layer (Norde et al.,1992). Hence, BSA molecules in phosphate buffer (having been noted as particularly stable) could potentially be more stable than those contained in Neurobasal/B27; this may be reflected in a more surface coverage and, hence, more adsorption and more recovered protein. The amounts of albumin are also in the same range as the total amounts of protein extracted from the neurones. The albumin therefore represents a large proportion of the protein detected by the Bradford assay used for protein normalisation, and hence leads to distortion of attempts to load equal amounts of cell extract on each lane. However, where extraction protocols include higher wash volumes (1000µl) we estimate the BSA retained is considerably lower at 5.98% and 12.32% for Neurobasal/B27medium

and BSA solution, respectively. Additionally, we propose that wash volume increases do not appear to impact the cellular content extracted from primary neurons, as evidenced by the Coomassie stain of lysed neuronal cultures (Fig.2, lanes 8-10). We observed a substantial reduction to albumin extracted in conditions with a higher wash volume (1 x 1000µl wash), but this did not appear to impact two additional prominent bands at ~37kDa and ~45kDa. Given the weight of the two bands, we suggest their identity to be GAPDH (35.8kDa) and Actin (42kDa) which are known to be abundant in primary neuronal cultures. Hence, it appears cell proteins are not adversely affected by shifting to a higher wash volume during extraction protocols.

Considering the importance of accurate protein quantification and analysis, we explored methods of ameliorating this issue. While albumin extraction protocols and commercially available albumin removal products are available, these are costly or time-consuming. Furthermore, our findings suggests a risk of cell protein loss through albumin extraction processes, which could prove particularly detrimental to studies which focus on a target protein of naturally low abundance. Hence, we trialled various easily implemented wash steps at the first stages of our extraction protocol, and found these to be sufficient in reducing BSA contamination in a timely manner. We suggest increasing PBS wash volumes to be most pragmatic with regards to time and avoids any consequences to protein integrity from excessive washing.

We believe this work is particularly pertinent to those working with primary neuronal cultures due to the longevity of incubations and numerous medium changes required. Consequently, cell culture plates are exposed to BSA-containing Neurobasal/B27 medium for an extensive period of time, possibly increasing the likelihood and extent of albumin binding. The issues that BSA binding and extraction with cell protein can cause to experimental outcomes can be substantial, and may interfere with protein analysis while remaining somewhat undetectable to researchers. A potential problem can be identified via a simple Coomassie stain of the gel. In view of the ease of implementation of steps which diminish this issue, we propose that future primary culture extraction protocols should consider refinement to ensure prevention of this potential issue.

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Figure Legends:

Fig. 1. Bar charts of total protein extracted (mg/ml) from polystyrene-based plastic cell culture plates as measured by Bradford assay. N= 3 for each condition. (A) Total protein yield from samples extracted from plates incubated in the absence of cells, under the same media conditions utilised for primary neuronal culture (i.e. B27 supplemented Neurobasal medium with the initial addition of DMEM 10% serum medium at 0DIV). (B) Total protein yield from samples extracted from plates incubated in the absence of cells with a BSA/PBS solution mimicking the total albumin content of a typical incubation with culture medium. All plate were incubated for 14DIV, and wash steps were varied in terms of volume of PBS (µl), number of washes, and length of incubation. Data are presented as mean ± SEM.

Fig. 2. Digital image of gel stained with Coomassie Brilliant Blue. Lanes containing samples from (left to right): (1) size markers, (2-4) Neurobasal/B27-only incubation Wells are processed with: 200µl x 1 (A), 1000µl x 1 (B), 200 µl x 3 (C) PBS washes, (5-7) BSA/PBS , (8-10) primary cortical neuronal culture protein extracts, (11) cell-pellet resuspension from albumin removal protocol containing cell protein and (12) albumin-containing supernatant after removal protocol.

Fig. 3. Total protein (mg/ml) from original primary neuronal culture extract and the two remaining components following albumin removal procedures as measured by Bradford assay. N=2 for each condition. Data are presented as mean ± SEM.

Fig 4. Comparison of Western blot analysis of GAD65/67 and GAPDH from independent samples of primary cortical neuronal culture protein extracts subjected to a low volume (A) or high volume (B) wash protocol prior to protein extraction. (C) Membrane probed for Albumin and GAD65/67 under a low volume wash protocol. All samples extracted at 14DIV.

Fig 1:

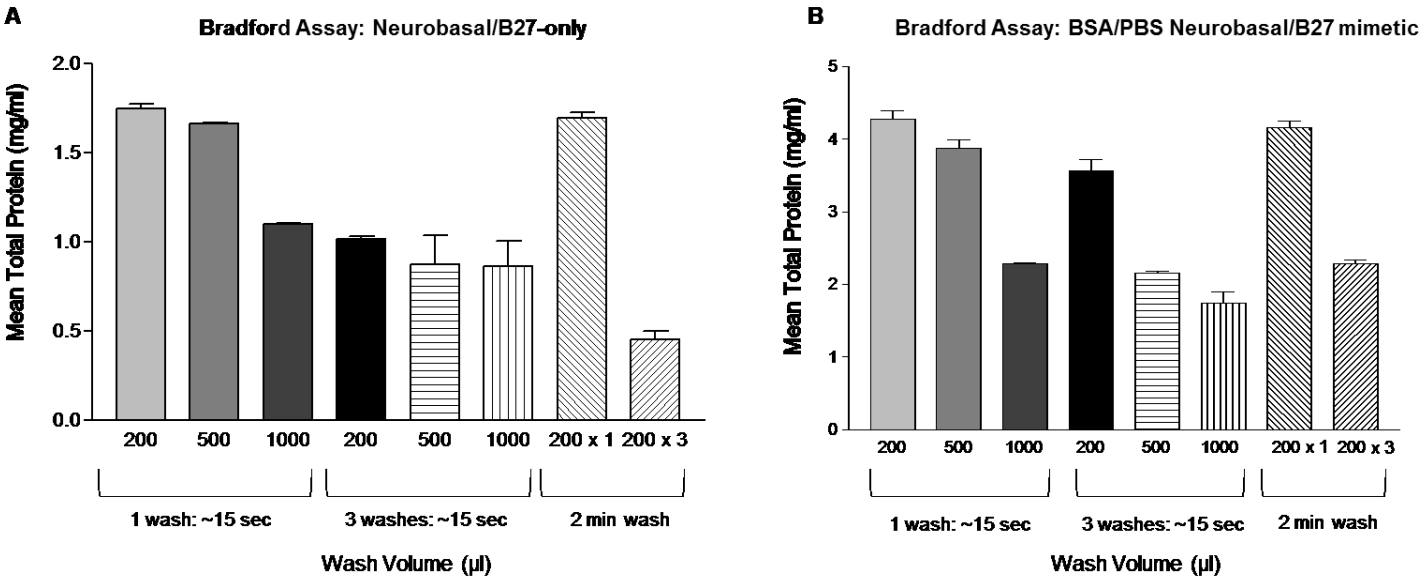

A
Bradford Assay: Neurobasal/B27-only
Mean Total Protein (mg/ml)
2.0
1.5
1.0
0.5
0.0
200
500
1000
200
500
1000
200 x 1
200 x 3
1 wash: ~15 sec
3 washes: ~15 sec
2 min wash
Wash Volume (µl)
B
Bradford Assay: BSA/PBS Neurobasal/B27 mimetic
Mean Total Protein (mg/ml)
5
4
3
2
1
0
200
500
1000
200
500
1000
200 x 1
200 x 3
1 wash: ~15 sec
3 washes: ~15 sec
2 min wash
Wash Volume (µl)

Fig 2:

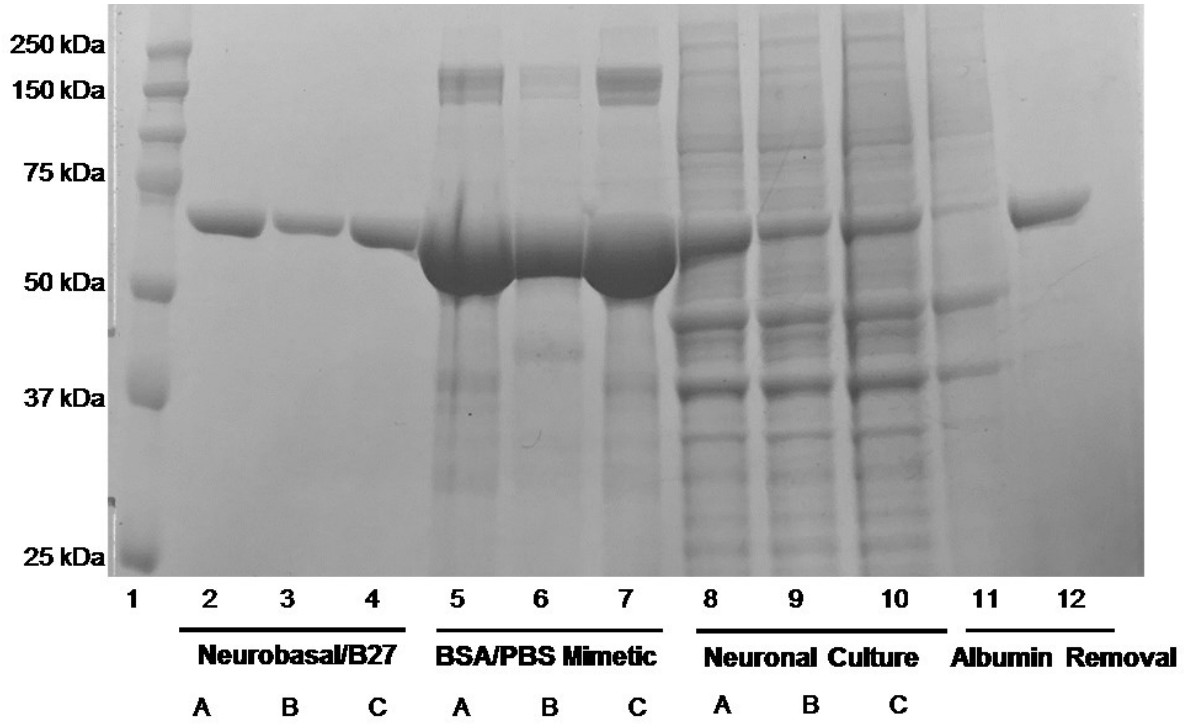

250 kDa
150 kDa
75 kDa
50 kDa
37 kDa
25 kDa
1
2
3
4
5
6
7
8
9
10
11
12
Neurobasal/B27
BSA/PBS Mimetic
Neuronal Culture
Albumin Removal
A
B
C
A
B
C
A
B
C

Fig 3:

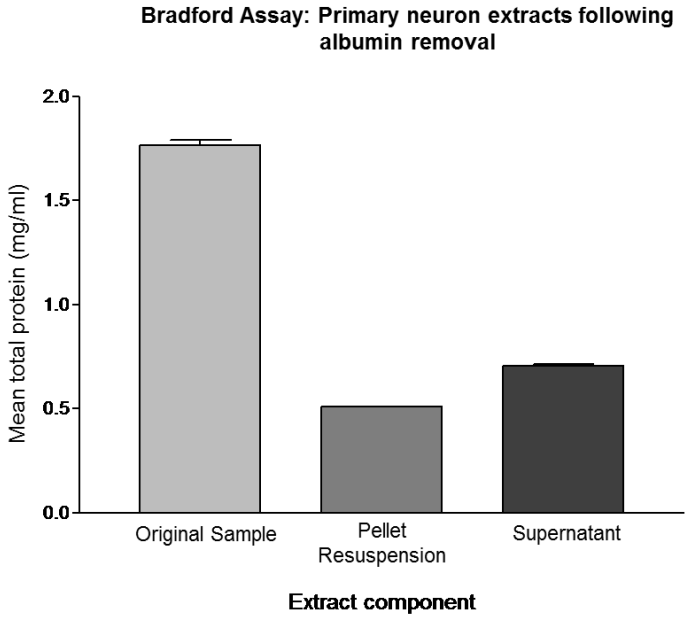
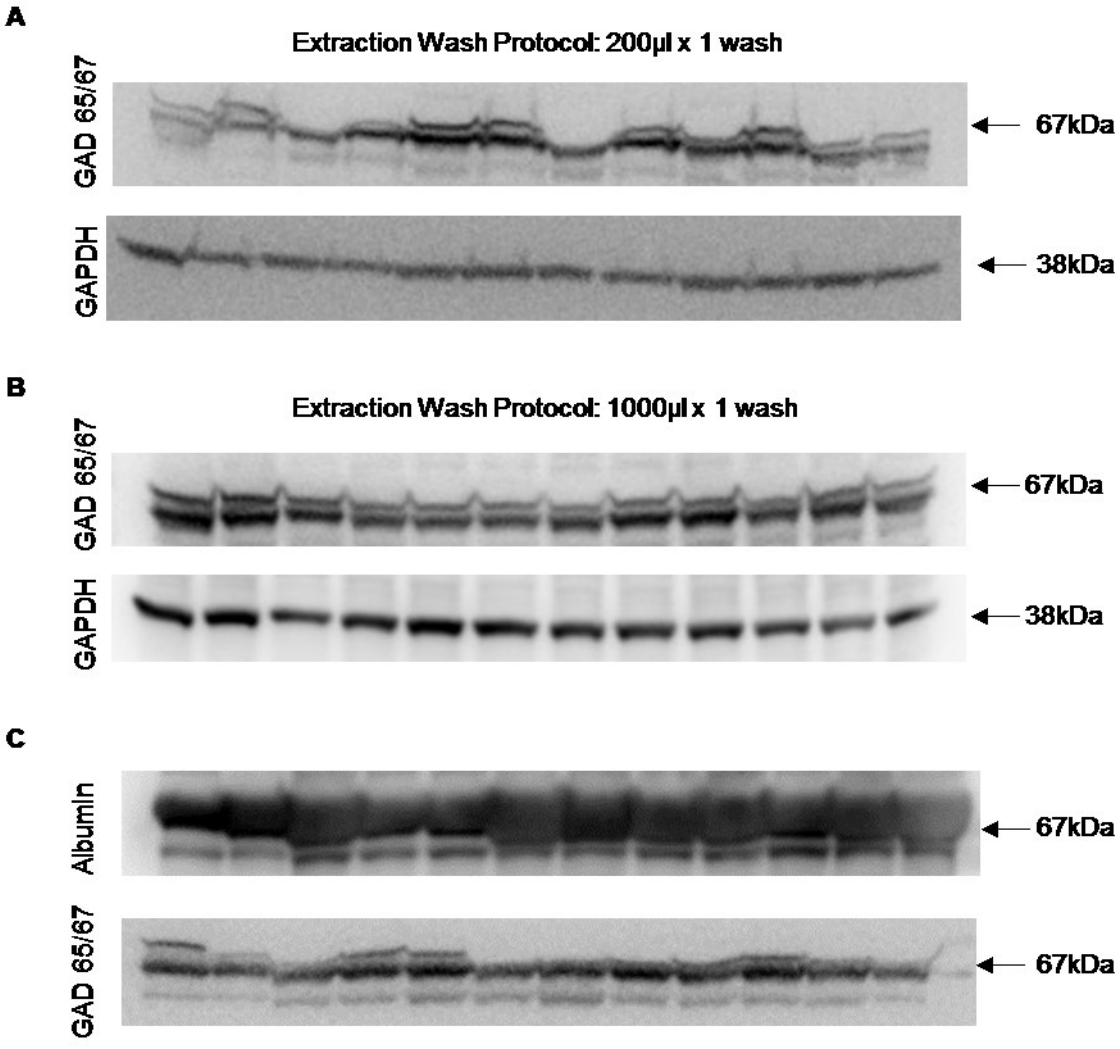

Bradford Assay: Primary neuron extracts following albumin removal
Mean total protein (mg/ml)
2.0
1.5
1.0
0.5
0.0
Original Sample
Pellet Resuspension
Supernatant
Extract component

Fig 4:


A
Extraction Wash Protocol: 200µl x 1 wash
GAD 65/67
67kDa
GAPDH
38kDa
B
Extraction Wash Protocol: 1000µl x 1 wash
GAD 65/67
67kDa
GAPDH
38kDa
C
Albumin
67kDa
GAD 65/67
67kDa