CHAPTER 3

TO DETERMINE THE ROLE OF MeCP2 ON ASTROCYTE SECRETED FACTORS REGULATING MYELINATION

3.1 Introduction

Astrocytes are unarguably the most abundant cells in the CNS and play crucial roles in regulating and maintaining normal brain homeostasis (Volterra and Meldolesi, 2005). Astrocytes greatly influence the functions of other brain cells- neurons, oligodendrocytes and even neighbouring astrocytes. Many of the physiological functions of astrocytes are orchestrated by the secreted factors that they produce, collected as astrocyte condition medium (ACM). ACM consists of a wide range of bioactive molecules including trophic factors, cytokines, chemokines (Lafon-Cazal et al., 2003; Delcourt et al., 2005), mitogens and differentiation factors (Bhat and Pfeiffer 1986) that affect oligodendrocytes and neurons at all stages of development.

ACM acts as a mitogen for OPCs by secreting PDGF which times oligodendrocyte development (Raff et al., 1988, Luo et al., 1999) by regulating proliferation and migration (Frost et al. 2009). Additionally, in response to ATP from axons, astrocytes secrete LIF which increases the number of myelinated fibers (Ishibashi et al., 2006) resulting from an enhanced oligodendrocyte differentiation. ACM increases the survival of neurons after insults and play a major role in neuroprotection (Yamamuro et al., 2003; Zhu et al., 2006; Ji-wen et al., 2013) and regulate neurogenesis, neural function and communication as well (Barres 2008).

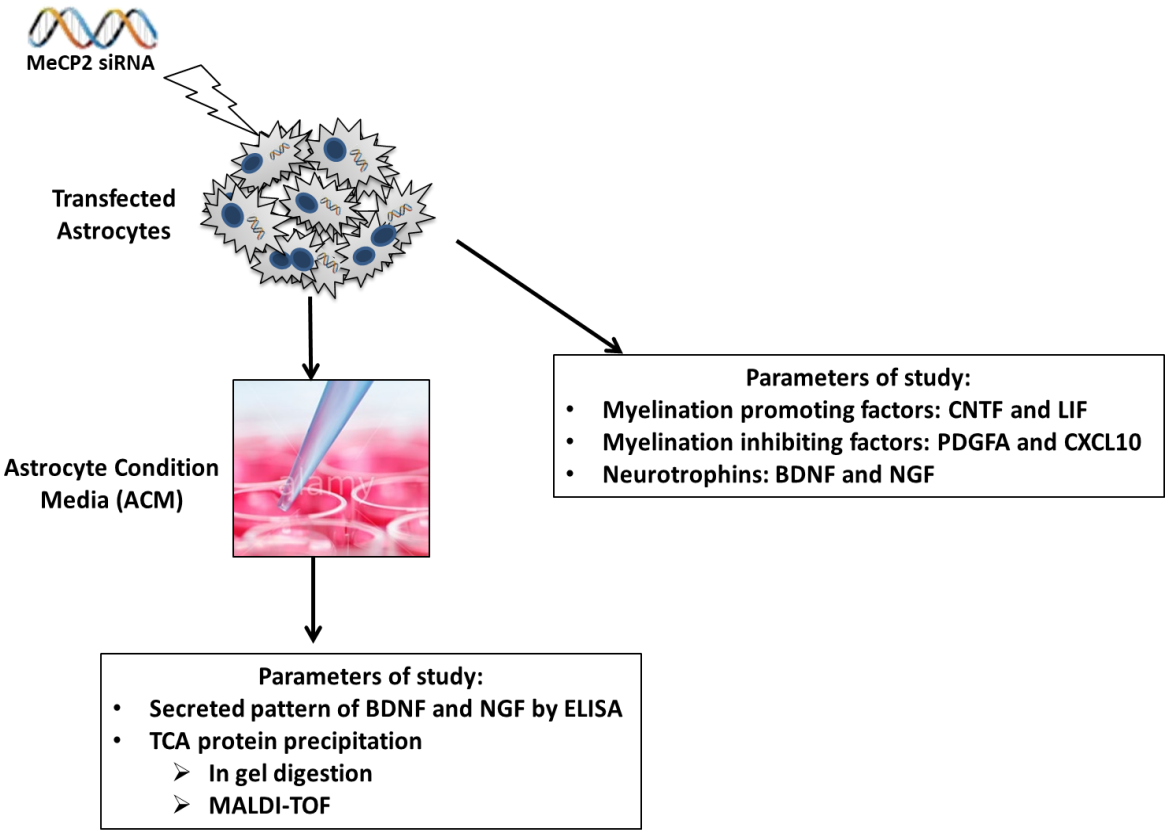
However for a long time the functional importance of astrocytes in the context of myelination was not studied mainly because majority of studies on myelination were done on purified oligodendrocytes or neurons or co-cultures of both. Astrocytes are major sources of neurotrophins- BDNF and NGF (Zafra et al., 1992; Lu et al., 1991; Kimoto et al. 2009) which in turn play very proactive roles in regulating CNS myelination (Xiao et al., 2010, Yin et al., 2012). ACM also consists of secreted factors that stimulate or inhibit myelination (Barnett and Linington, 2013) and are involved in myelin pathology of multiple sclerosis (Moore et al., 2011).

MeCP2 (Methyl-CpG binding protein 2) favourably binds to methylated CpG dinucleotide in DNA, mutations of which have been linked to Rett syndrome, an autism spectrum disorder (Amir et al., 1999). Originally, MeCP2 was known to be a gene repressor by binding to various transcriptional repressors, but recently studies have elaborated on functions of MeCP2 as an activator (Ben-Shachar et al., 2009;

Chahrour et al., 2008), thus earning it more apt title of a global transcriptional regulator. Moreover, recent studies have focussed on the presence and functions of MeCP2 in glial cells, loss of which also contributes to Rett syndrome (Ballas et al., 2009; Maezawa and Jin, 2010; Nguyen et al., 2013). One of the major and most studied target genes of MeCP2 is BDNF, which is studied to be involved in synaptic plasticity and myelination (Chen et al., 2003; Khorshid Ahmad et al., 2015; Xiao et al., 2010).

In the current study, myelination promoting (CNTF and LIF) and inhibitory factors (PDGF and CXCL10) were studied in MeCP2 deficient astrocytes. Since astrocytes are active sources of neurotrophins which, in turn, are involved in CNS myelination, transcript, protein and secretion pattern of BDNF and NGF were also analysed in ACM from MeCP2 deficient astrocytes. Additionally, the change in the secretome pattern of MeCP2 knockdown astrocytes was studied using MALDI-TOF.

3.2 Strategy of work



3.3 Results

3.3.1 Immunocytochemical characterization of astrocytic culture and MeCP2 siRNA knockdown

Mixed glial cultures were grown in vitro as described in materials and methods (section 2.1). As per the established lab protocol, after 10 days of seeding, oligodendrocytes and astrocytes were separated from the mixed glial cultures by a shake-off procedure. The astrocytic bed was enriched with >95% GFAP -positive astrocytes which were characterized by immunocytochemical methods (Figure 3.1 A).

MeCP2 expression was suppressed in astrocytes using specific siRNA at a final concentration of 10nM. The transfection was repeated after 24hrs to obtain optimum silencing. Transfected astrocytes showed 50-60% reduction in MeCP2 protein expression levels (Figure 3.1 B, C). These transfected astrocytes were employed for all further experiments.

3.3.2 Effect of MeCP2 on astrocyte secreted factors known to influence myelination

A recent review exhaustively enlisted astrocyte secreted factors which are known to be involved in myelination, either promoting or inhibiting myelination (Barnett and Linington, 2013). The transcript levels of myelination promoting factors- CNTF and LIF were analysed in MeCP2 knockdown astrocytes and significant down-regulation was observed in mRNA levels of both the genes compared to control (Figure 3.2 A). Also, transcript levels of myelination inhibiting factors- PDGFA and CXCL10 were evaluated and significant up-regulation was observed in MeCP2 knockdown astrocytes compared to control (Figure 3.2 B). These observations suggest indirect involvement of astrocytic MeCP2 in the regulation of myelination by altering the levels of myelination affecting molecules.

3.3.3 Effect of MeCP2 on neurotrophins expression in astrocytes

One of the most studied target genes of MeCP2 in neurons is BDNF (Bonni et al., 1999, Guy et al., 2011) which is essential for neuronal survival, differentiation (Ghosh et al., 1994), and synaptic plasticity (Poo 2001). In neurons, there exists an activity-dependent functional interaction between MeCP2 and BDNF (Chen et al., 2003). BDNF signalling is also altered in RTT disease pathogenesis and progression (Chang et al., 2006). Studies from our laboratory have shown that oligodendrocytes derived BDNF is also under MeCP2 regulation (Sharma et al., 2015). Present study focussed on the effect of astrocytic MeCP2 on neurotrophins (BDNF and NGF) protein as well as transcript levels. Condition media from MeCP2 deficient astrocytes were also collected post transfection and levels of secreted BDNF and NGF were measured by ELISA. BDNF transcript and protein levels were up-regulated significantly in MeCP2 knockdown astrocytes; however the same was not reflected in the secretion levels which were down-regulated but not significantly. While, NGF transcript and protein levels were also up-regulated, the secretion levels did not show any alterations (Figure 3.3 A- F).

3.3.4 Effect of MeCP2 on astrocyte secreted factors: MALDI-TOF analysis

Current study was carried out to identify the effect of MeCP2 knockdown on astrocytic secreted factors. ACM was collected post transfection and subjected to total protein precipitation. Further, 50μg of total protein was resolved by SDS-PAGE, bands of specific molecular weight were excised and subjected to in-gel trypsin digestion. Total protein as well as trypsin digested protein were analysed on MALDI-TOF Bruker UltrafleXtreme. Based on the mass spectra peaks, certain proteins/peptides were identified in astrocyte condition media. Astrocyte secretion of serine protease inhibitors and beta defensins has been described in earlier reports (Dowell et al., 2009, Hao et al., 2001), there were some other peptides which have not been identified in similar previous studies. Also, despite the relatively high sensitivity, no major growth factors or cytokines were detected and needs further investigation. The mass spectrum data suggests 2-3 fold decrease in the peptide peaks of MeCP2 knockdown ACM compared to control.

3.4 Discussion

Astrocytes are undeniably the most abundant cells in the CNS. They perform a wide range of functions right from early embryonic development to adult stage. In addition to their protective and house-keeping functions, they are also a principle source of ECM proteins, trophic factors, neurotrophins, growth factors which in turn perform a large number of functions in the CNS (Sofroniew and Vinters, 2010; Wang and Bordey, 2008).

For a long time, there existed an understanding that the presence and function of MeCP2 was limited to neurons and absent in glial cells (Shahbazian et al., 2002; Jung et al., 2003). However, studies in the last two decades have revealed expression of MeCP2 in astrocytes, oligodendrocytes and microglia (Ballas et al., 2009; Maezawa and Jin, 2010; Sharma et al., 2015) performing a number of essential functions in these cells. Current study also confirmed the gene and protein expression of MeCP2 in astrocytes.

An exhaustive review by Barnett and Linington, enlisted astrocyte secreted factors which have been studied to influence myelination either as promoting factors or as inhibiting ones (Barnett and Linington, 2013). Astrocytes are known to produce CNTF which promotes neuronal survival and neuroprotection (Yokota et al., 2005). Astrocyte secreted CNTF enhances OPC migration (Vernerey et al., 2013), stimulates oligodendrocyte maturation and differentiation *in vitro* and *in vivo* (Albrecht et al., 2007; Stankoff et al., 2002) thus leading to enhanced myelin formation via gp130-JAK pathway (Linker et al., 2002). On the other hand, CNTF knockout leads to reduced numbers of OPCs which is suggested to contribute to demyelination in EAE animals (Lu et al., 2009). LIF, astrocyte secreted neuropoeitic cytokine, is a promoter of oligodendrocyte survival (Kerr and Patterson, 2005), stimulates maturation and myelination in neuron-glial co-cultures (Stankoff et al., 2002) in response to electrical stimulation (Ishibashi et al., 2006). Reduced numbers of astrocytes and MBP levels have been observed in LIF knockout animals (Bugga et al., 1998) along with enhanced demyelination and oligodendrocytes loss (Marriott et al., 2008). Transcript levels of CNTF and LIF were found decreased in MeCP2 knockdown astrocytes compared to control. This suggests that MeCP2, via astrocytes, positively regulates

oligodendrocyte maturation and myelination possibly by binding to transcription factors which regulate secretion of CNTF and LIF by astrocytes.

PDGF is one of the most studied growth factor and its receptor (PDGFαR) is a phenotypic marker for OPCs (Hart et al., 1989). Astrocytes express PDGFαR and are a source of PDGF in the surrounding environment (Fruttiger et al., 1996). Noble and Murray demonstrated that astrocytes inhibited oligodendrocyte differentiation; this effect was regulated by astrocyte secreted PDGF (Noble and Murray, 1984; Durand and Raff, 2000). The classical function of PDGF has been in OPC migration as a mitogen and mitogen (Baron et al., 2000; Frost et al., 2009). An increased level of PDGF maintains the progenitor state of OPCs by inhibiting differentiation and promotes migration from the SVZ. Transcript levels of PDGF and CXCL10 were significantly up-regulated in MeCP2 knockdown astrocytes compared to control, suggesting that MeCP2 is involved in regulation of oligodendrocyte physiology via astrocytes.

Astrocytes are active sources of neurotrophins like BDNF, NGF and NT3. Astrocyte derived BDNF is associated with increased OPC proliferation and remyelination (Ramos-Cejudo et al., 2015) and help to restore myelin integrity in cuprizone induced demyelination model (Fulmer et al., 2014, Tsiperson et al., 2015). Moreover, astrocyte secreted BDNF was shown to have a direct effect on OPC maturation and white matter remyelination (Miyamoto et al., 2015). This non-cell autonomous function on oligodendrogenesis and remyelination is crucial for white matter homeostasis. Association of MeCP2 with BDNF has been extensively studied in neurons. MeCP2 is bound to the BDNF promoter and represses transcription whereas membrane depolarization leads to release of MeCP2 from the promoter site resulting in active gene transcription (Chen et al., 2003). Astrocytes are also principle producers of NGF (Goss et al., 1998) which is an important regulator for survival and differentiation of sensory and sympathetic neurons (Sofroniew et al., 2001) as well as regulation of myelination (Chan et al., 2004). NGF is found to be reduced in Rett syndrome post-mortem brain tissue (Lipani et al., 2000). In the present study, transcript and protein levels of both neurotrophins- BDNF and NGF were significantly up-regulated in MeCP2 knockdown astrocytes. These observations of astrocytes are in accordance to the negative regulation of BDNF by MeCP2 in neurons.

In neurons, the total effect of MeCP2 mutations on BDNF release is decided by region-specific changes in total BDNF content and the percentage available for release (Wang et al., 2006). Moreover, cell autonomous and autocrine role of BDNF has been explained in MeCP2 null glutamatergic neurons wherein their observations indicate a defect in BDNF synthesis leading to a decrease in BDNF availability for release (Sampathkumar et al., 2016). In the current study, secreted levels of BDNF were decreased in MeCP2 knockdown astrocytes, despite the increased levels of mRNA and protein content of BDNF, while no alteration was observed in NGF secretion patterns. This suggests regulation of astrocytic MeCP2 in the exocytosis of BDNF across the astrocytic cell membrane.

Secretomics, a subclass of proteomics, is a potent tool for understanding the cellular functions, finding unique biomarkers and therapeutic targets. The astrocyte secretome is a comprehensive set of secreted proteins and gives an understanding of astrocytic functions as well as their use as diagnostic or therapeutic agents for CNS disorders (Jha et al., 2013). Several studies have examined the astrocyte condition media and identified plenty of proteins; thus providing a catalogue of the secreted as well as cytosolic proteins (Lafon-Cazal et al., 2003; Thorsell et al., 2008; Dowell et al., 2009; Moore et al., 2009; Greco et al., 2010; Jha et al., 2018). In the present study, astrocyte secreted factors collected from the condition media were analysed using MALDI-TOF and the acquired mass spectrometry data were deferred to database search using Mascot software (Matrix Science, London, UK (Perkins et al. 1999)). The data did not suggest of any newly secreted proteins or any potent peptide modifications in response to MeCP2 deficiency in astrocytes. However, the intensity levels of peptides peaks in MeCP2 knockdown group were significantly down-regulated as compared to control.

3.5 Conclusion

Current study was directed towards understanding the involvement of astrocytic MeCP2 in regulation of factors involved in myelination. Astrocytic MeCP2 positively regulates the myelination promoting factors- CNTF and LIF, while has a negative regulation on myelination inhibitory factors- PDGF and CXCL10. MeCP2 in astrocytes also regulates BDNF and NGF expression along with influencing their secretome pattern. Peptide peaks of astrocyte secreted factors were also considerably reduced in response to deficient MeCP2 in astrocytes.

Figure 3.1 (A)

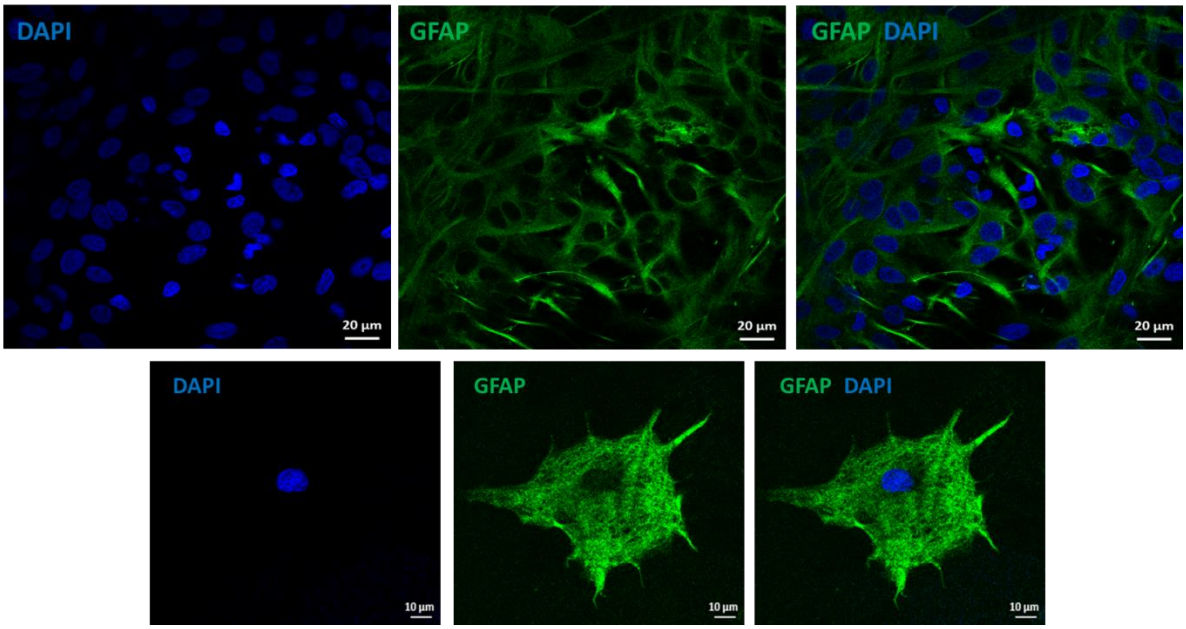


Figure 3.1 (B)

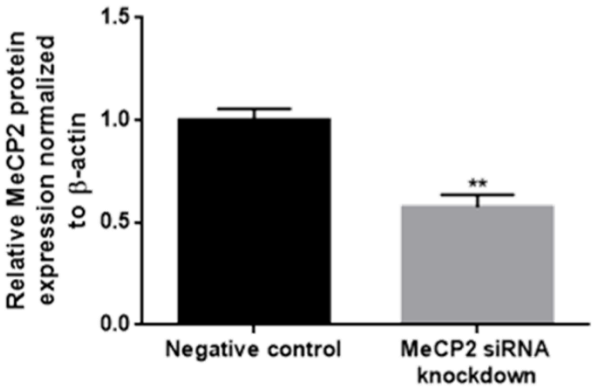


Figure 3.1 (C)

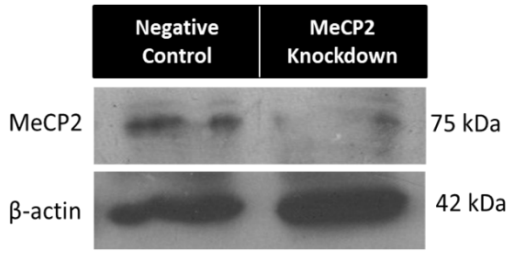


Figure 3.1: Immunocytochemical characterization of astrocytic culture and MeCP2 siRNA knockdown: (A) 63X Confocal microscopy of primary astrocytes positive for astrocyte cell-specific marker, GFAP (Green). Nucleus was stained with DAPI (blue). Scale Bar = 10-20µm. (B) Relative change in MeCP2 protein expression in MeCP2 knockdown astrocytes, normalized to β-actin, relative to control. (C) Representative western blot of MeCP2. Values represent mean ± SEM from 3–4 samples and evaluated by Student's t-test (*P<0.05;**P<0.01; ***P<0.001).

Figure 3.2 (A)

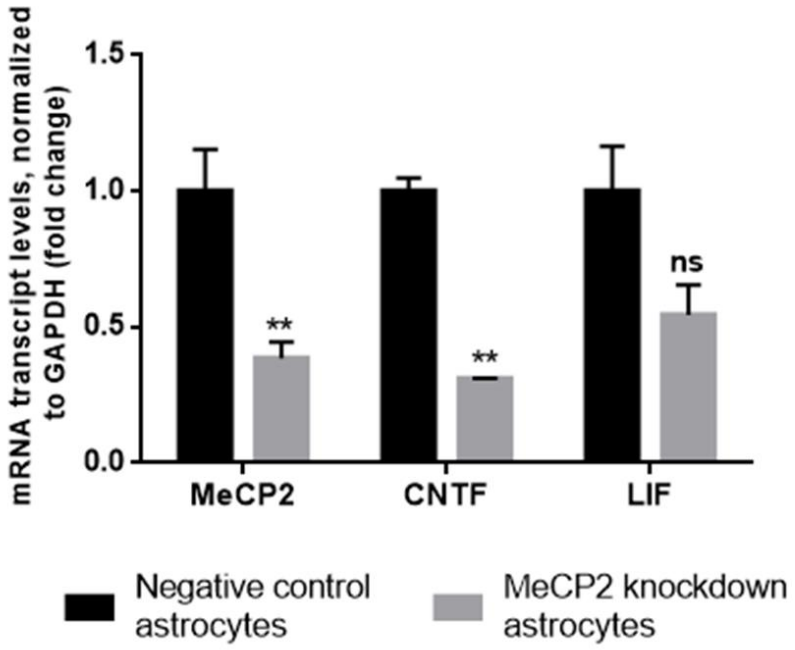


Figure 3.2 (B)

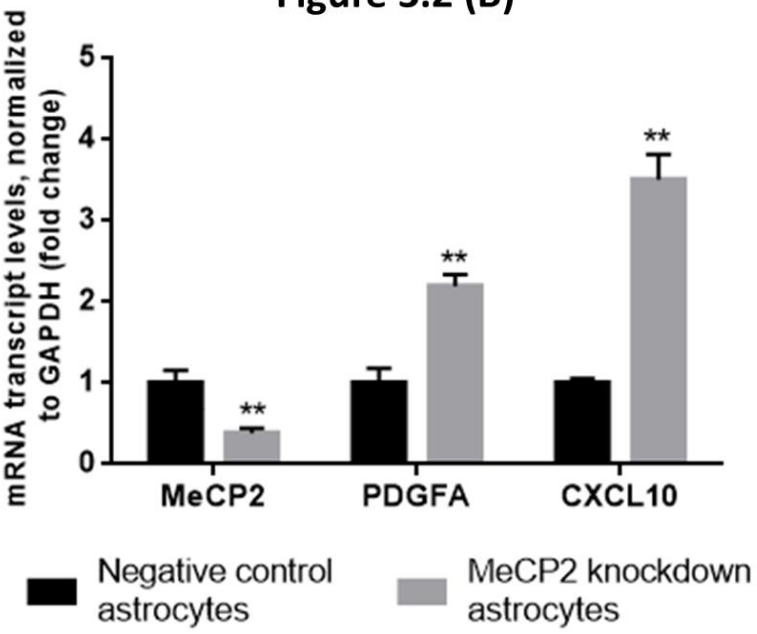


Figure 3.2: Effect on astrocytic MeCP2 on secreted factors promoting myelination: Astrocytes were transfected with Negative Control siRNA and MeCP2 siRNA after which (A) MeCP2, CNTF, LIF and (B) MeCP2, PDGFA, CXCL10 mRNA levels were detected by real time PCR, normalized to GAPDH and relative to control. Results are represented as mean ± SEM of 3-4 independent experiments and evaluated using Student's t-test (*P<0.05;**P<0.01; ***P<0.001).

Figure 3.3 (A)

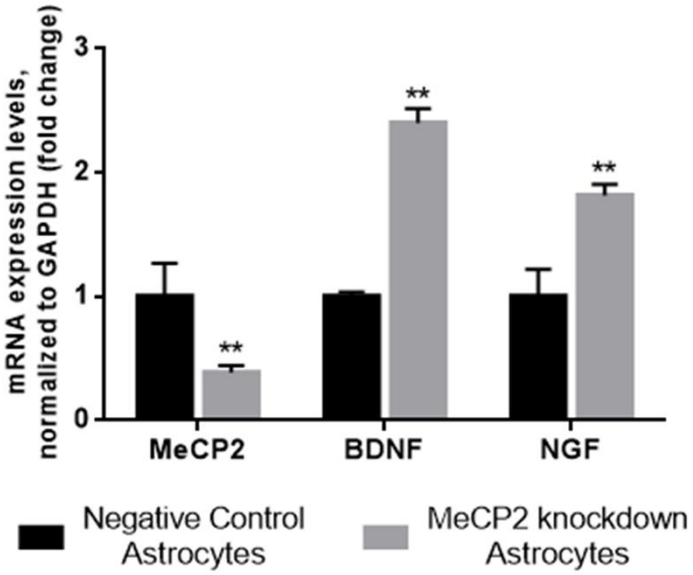


Figure 3.3 (B)

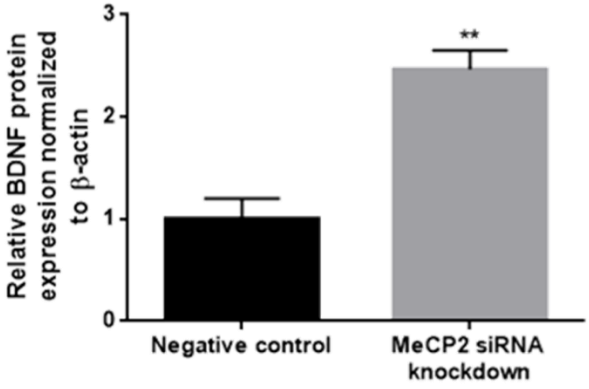


Figure 3.3 (C)

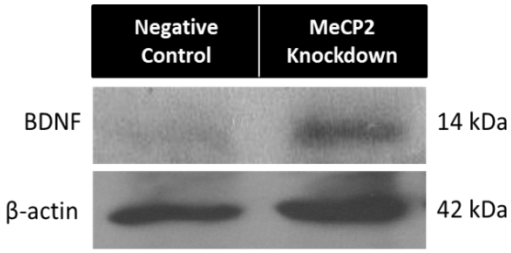


Figure 3.3 (D)

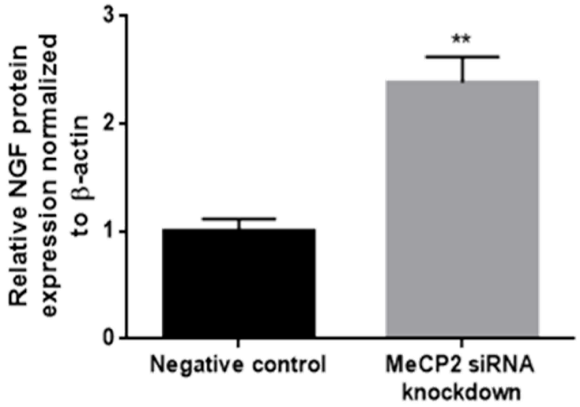
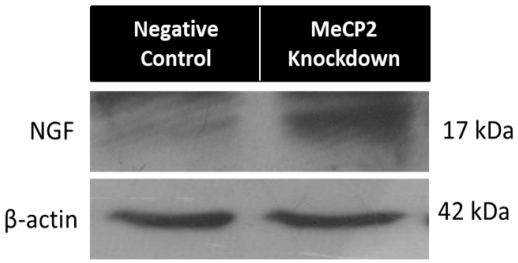


Figure 3.3 (E)



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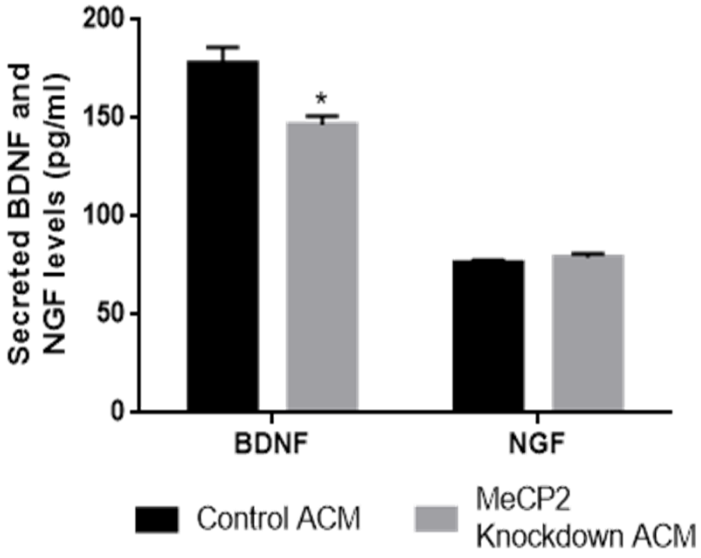

Figure 3.3: MeCP2 alters neurotrophins expression in astrocytes: Astrocytes were transfected with Negative Control siRNA and MeCP2 siRNA after which (A) Transcript levels of neurotrophins (BDNF and NGF) were detected by real time PCR, normalized to GAPDH and relative to control (B) Relative change in BDNF protein expression was analysed in MeCP2 knockdown astrocytes, normalized to β-actin, relative to control (C) Representative western blot of BDNF (D) Relative change in NGF protein expression was analysed in MeCP2 knockdown astrocytes, normalized to β-actin, relative to control (E) Representative western blot of NGF. (F) Astrocytes condition media (ACM) was collected and secreted neurotrophins (BDNF and NGF) levels were measured using ELISA following manufacturer's protocol. Results are represented as mean ± SEM of 3-4 independent experiments and evaluated using Student's t-test (*P<0.05; **P<0.01; ***P<0.001).

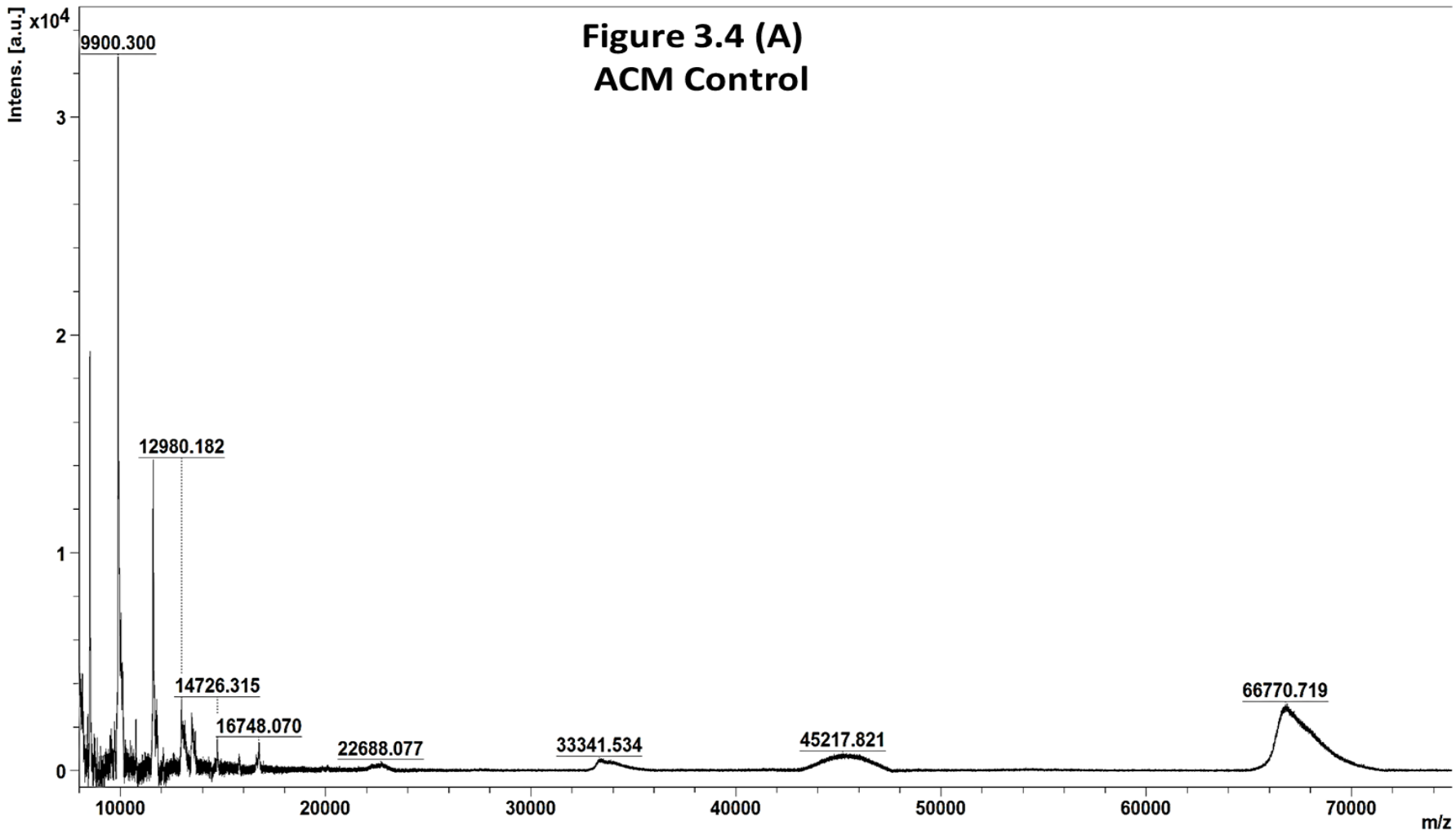
Figure 3.4 (A)
ACM Control
Intens. [a.u.]
x10^4
3
2
1
0
9900.300
12980.182
14726.315
16748.070
22688.077
33341.534
45217.821
66770.719
10000
20000
30000
40000
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Figure 3.4 (B)
MeCP2 knockdown ACM

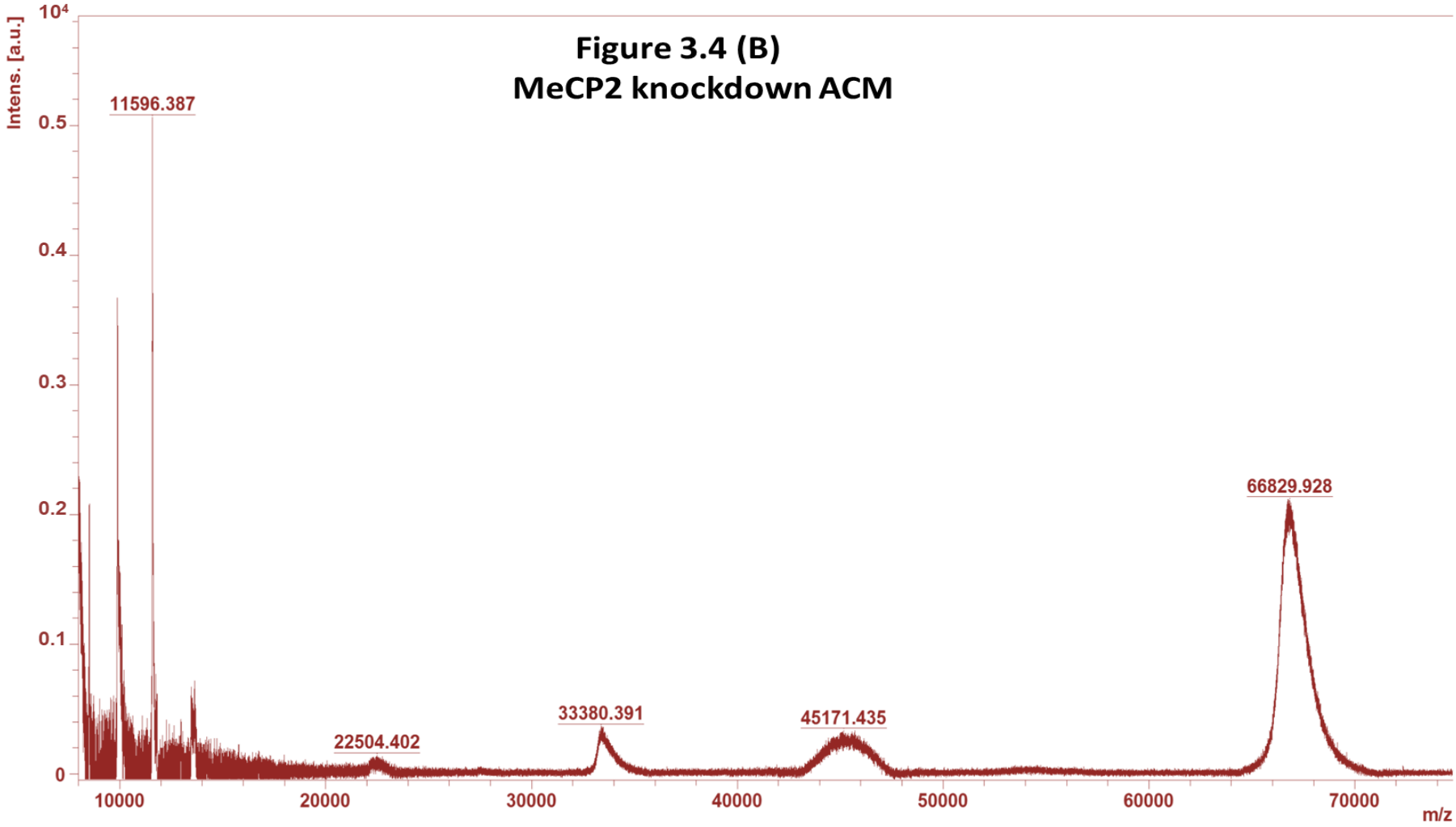

Figure 3.4 (C)
ACM Control

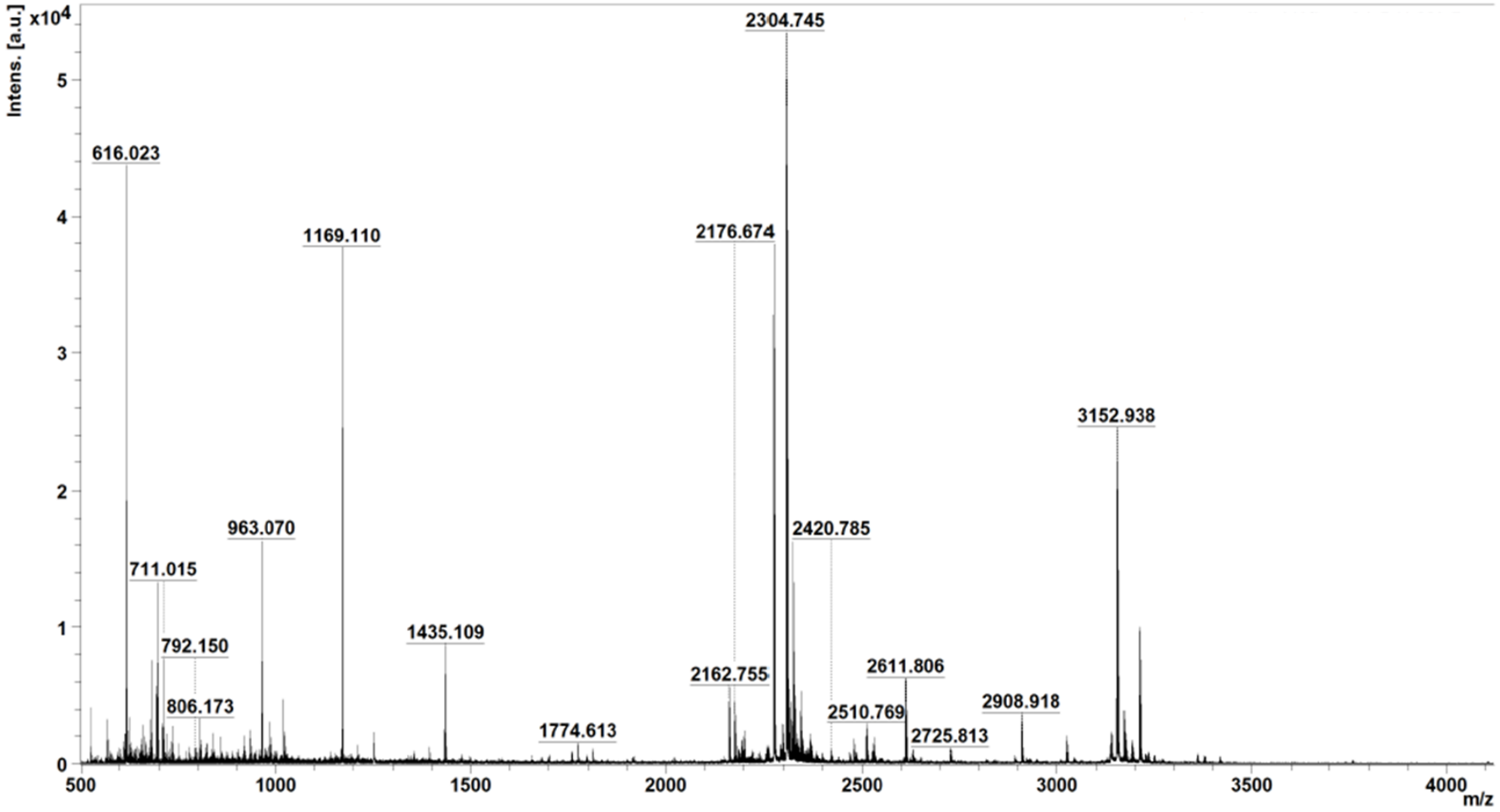

Figure 3.4 (D)
MeCP2 knockdown ACM

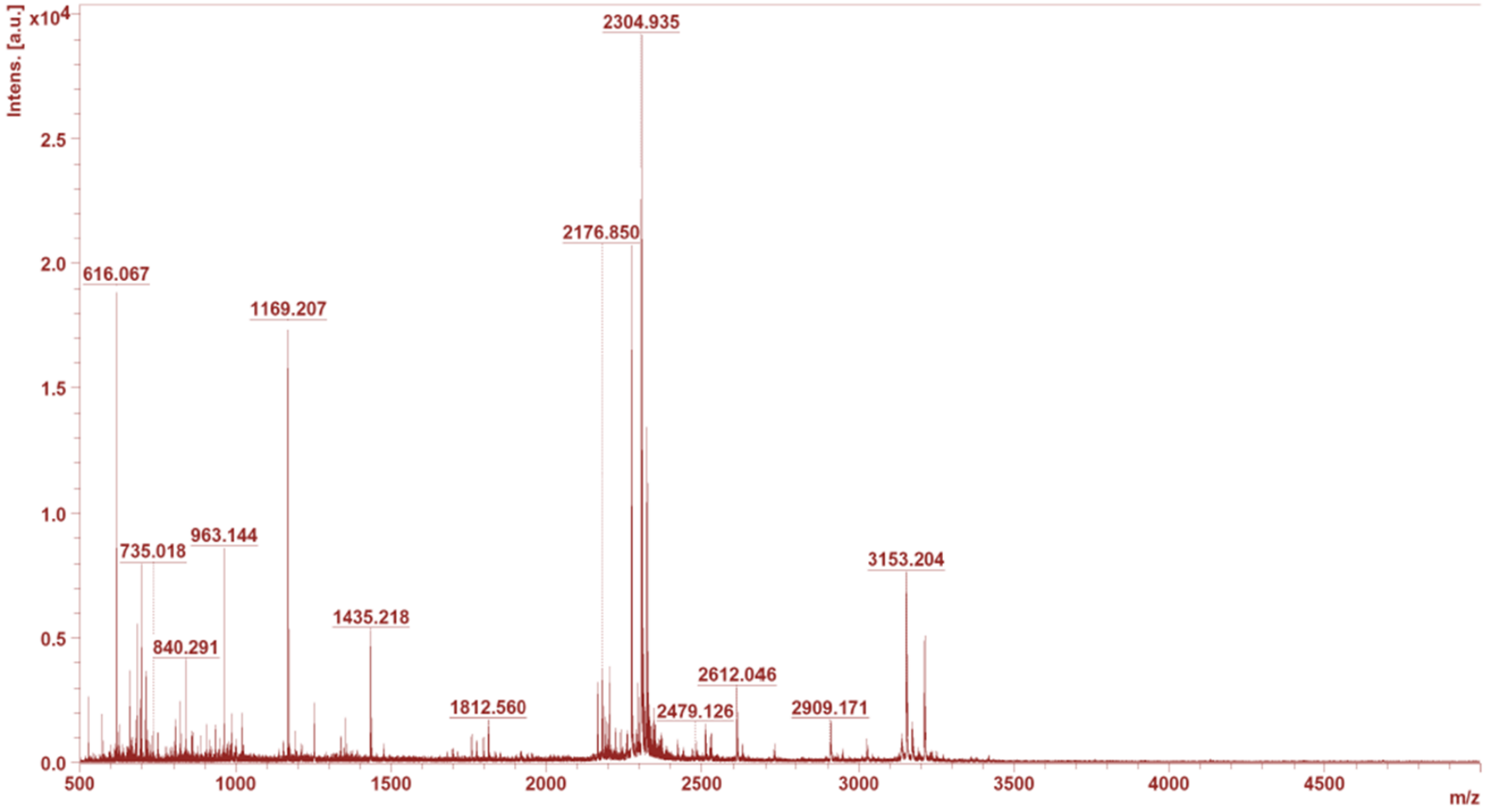

Figure 3.4: MALDI-TOF Analysis:

MALDI-TOF mass spectrum of ACM total protein (20-50 kDa) of (A) Control ACM and (B) MeCP2 knockdown ACM.

MALDI-TOF mass spectrum of ACM in-gel digested ~20 kDa band of (C) Control ACM and (D) MeCP2 knockdown ACM.

Table 3: Astrocyte secreted proteins identified by MALDI-TOF and MASCOT analysis

Sr. No.	Peak (m/z)	Proposed Protein/Peptide	Intensity (Control) a.u	Intensity (Treated) a.u	Function
1	616	Cytochrome c oxidase subunit 8A	43666	18849	COX8A is a subunit of cytochrome c oxidase and function for the efficacy of complex IV. Disorders can lead to a wide range of clinical manifestations like leukodystrophy, and severe epilepsy.
2	963	Beta-defensin	16327	8625	Beta-defensins are expressed by astrocytes as well as microglia and play a role in host defence against bacterial CNS pathogenesis.
3	1169	Serine protease inhibitor	37612	17326	Serine proteases play a key role in the fundamental biology of the CNS, and are also involved in the pathophysiology of neurodegenerative diseases.
4	1435	Vesicle-associated membrane protein 3	8701	5331	Belongs to the synaptobrevin family. VAMP3 is ubiquitously expressed and participates in regulated and constitutive exocytosis as a constituent of secretory granules and vesicles.
5	2176	Neuropeptide S	3152	2657	Cortical astrocytes synthesize both native and foreign neuropeptides and can secrete them in a stimulation-dependent manner.
6	2304	Beta-defensin 43	43730	22572	Expressed by astrocytes as well as microglia and play a role in host defence against bacterial CNS pathogenesis.
7	3152	Somatoliberin	10435	3262	Alias for GHRH which stimulates the secretion of growth hormone