RNA-Seq Analysis Reveals that Human eSC- or iPSC-derived Motor Neurons Exposed to Microneurotrophins or Neurotrophin Proteins Have Similar But Not Identical Gene Family Expression Changes

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Abstract

Microneurotrophins (MNT) are small molecule derivatives of the naturally occurring brain steroid dehydroepiandrosterone (DHEA) that do not have significant interactions with sex steroid receptors but retain high-affinity binding to neurotrophin (NT) protein tyrosine kinase (Trk) receptors. Because MNT’s cross the blood-brain barrier and can mimic many of the pleiotropic actions of NT proteins on neurons, they offer therapeutic potential for neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS) that affects mainly motor neurons (MN).

We utilized human H9 eSC- (embryonic stem cell) and CTL subject iPSC- (induced pluripotential stem cell) derived motor neuron cell lines exposed to NT proteins or MNT molecules, followed by paired-end RNA sequencing. After alignment to the hg38 human genome, transcript expression relative to that occurring in the presence of DMSO vehicle was analyzed with Cufflinks2. Expression ratios (relative to DMSO vehicle) of the top 500 genes were calculated, and the resulting gene lists were analyzed in DAVID for Gene Ontology (GO) representation. All three MNT’s studied (BNN20, BNN23, BNN27) showed overlap of GO terms with NGF (nerve growth factor) and BDNF (brain-derived neurotrophic factor) in the H9 eSC-derived MN’s. In the iPSC-derived MN’s two (BNN20, BNN27) showed overlap of GO terms with NGF or BDNF. Each NT protein had GO terms that did not overlap with any MNT in the MN cell lines. In the H9 eSC-derived MN’s, BNN20 had 2 GO terms that did not overlap
with any NT protein. In the iPSC-derived MN's BNN23 did not show any overlap in its two GO terms with either NT protein.

Our findings, based on gene expression changes in human eSC-derived and iPSC-derived motor neurons, support the further development of MNT drugs for use in ALS. (278 words)

Keywords: Microneurotrophin, ALS, iPSC-derived motor neuron, RNA-sequencing, gene ontology

Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal degenerative disease in which motor neurons in the motor cortex and brainstem/spinal cord become dysfunctional and die for unclear reasons. Named after Lou Gherig, the famous

1 Abbreviations used:
ALS= amyotrophic lateral sclerosis
BDNF= brain-derived neurotrophic factor
CTL= control
DMSO= dimethylsulfoxide
eSC= embryonic stem cell
FACS= fluorescence-activated cell sorting
fALS= familial ALS
FPKM= fragments per kilobase of exon sequenced per million reads
GO= gene ontology
iPSC= induced pluripotential stem cell
MN= motor neurons
MNT= microneurotrophin
NGF= nerve growth factor
NSC= neural stem cell
NT=neurotrophin
PBMC= peripheral blood mononuclear cell
RNA-seq= RNA sequencing
sALS= sporadic ALS
UCSC= University of California Santa Cruz
A baseball player who died from this illness in 1941, ALS has no FDA-approved disease-slowing treatment beyond riluzole that extends life for only a few months.

~90% of ALS occurs sporadically (sALS) without any inheritance patterns, with ~10% of ALS being familial (fALS) and arising from mutations in at least 14 known genes[1]. Transgenic mice expressing fALS gene mutations develop clinical phenotypes resembling human disease, but therapies prolonging life of these Tg mice have not translated well into sALS patients.

Additional models of sALS are needed for therapy development. One approach is to use human eSC or iPSC cells differentiated into motor neurons, the vulnerable population in both fALS and sALS [2]. This approach has several limitations, among those being the relative “youth” of the motor neurons derived from iPSC cells compared to native motor neurons that have lived for decades in a natural environment, and lack of innervation of physiological targets (muscle cells) and surrounding cell types (astrocytes) normally abundant in CNS tissues.[2]

By mimicking neuronal survival-promoting actions of natural neurotrophin proteins, microneurotrophins (MNTs) offer the potential to help prevent neuronal death in ALS and other degenerative conditions [3]. MNT’s are small, blood-brain barrier permeable derivatives of the endogenous sex steroid dehydroepiandrosterone (DHEA) [4] that are free of interactions with sex steroid receptors but retain high-affinity binding to and agonist interactions with neurotrophin tyrosine kinase (Trk) receptors. MNT’s thus possess multiple
survival-promoting, pleiotropic actions of large neurotrophin proteins such as nerve growth factor and brain-derived neurotrophic factor. [5-7]

We used RNA-sequencing technologies [8] to assess gene expression changes in a human motor neuron cell line that we derived from either commercially available neural stem cells of human eSC origin, or peripheral blood mononuclear cells (PBMC) transformed by electroporation of a reprogramming plasmid into iPSC’s [9]. We compared 24 hours exposure to three MNT’s with exposure to human neurotrophin (NT) proteins and DMSO vehicle. We then used publicly available DAVID (Database for Annotation, Visualization and Integrated Discovery) [10, 11] to determine significant Gene Ontology (GO) groupings of the gene expression changes. In the human eSC-derived MN’s we found that incubation with all three MNT’s showed gene expression GO term overlap with both NGF and BDNF proteins. In the human CTL iPSC-derived MN’s we found that two out of the three MNT drugs possessed significant overlap with GO terms following incubation with NT proteins. Each of the two tested NT proteins showed GO term expression changes not produced by any of the three MNT drugs. Our findings support continued development of MNT drugs for ALS, while also providing a cautionary note about the need for improved sALS models for therapy screening.

**Materials and Methods.** Motor neurons were differentiated for 21 days from neural stem cells (NSC), as described [21]. H9 NSC’s were obtained from Gibco/ThermoFisher®. The CTL PBMC’s were reprogrammed to iPSC’s using
electroporation of a reprogramming plasmid (from Addgene®) as described [9]. Motor neuron preparations were incubated with human NGF or human BDNF (both from Sigma Chemical Co®) at 100 ng/ml. Motor neurons were incubated with MNT drugs (Figure 1) at 100 nM. All cell culturing was performed at 5% oxygen to reflect brain in vivo oxygen levels.

![Figure 1](image)

**Figure 1.** Structures of the microneurotrophin (MNT) drugs used.

Multiplex RNA sequencing on Illumina NextSeq 500® platforms was carried out by CofActor Genomics®, using RNA libraries we constructed using an Illumina TruSeq HT® kit according to manufacturer’s instructions and quantitated with a KAPA Illumina library quantification kit® (KAPA Biosystems). RNA alignment was performed using tophat2, bowtie2 and cufflinks2 running in Unix on a 12-core MacPro computer with 64GB of RAM and data saved on a 32TB HD array. The hg38 reference genome and transcriptome were downloaded from the UCSC site (http://hgdownload.cse.ucsc.edu/goldenPath/hg38/bigZips/)
Results

We used Cufflinks2 to acquire FPKM (Fragments Per Kilobase of exon per Million reads) values in which NSC’s made from H9 eSC/NSC’s or iPSC’s derived from PBMC’s of a 62 year-old control individual without any neurological illness were differentiated into motor neurons for 21 days, then exposed in duplicate for 24 hours to neurotrophin proteins (NGF, BDNF) at 100 ng/ml or MNT’s BNN20, BNN23, BNN27 (see Figure 1) at 100 nM, or to 0.001% DMSO vehicle. Total RNA was then extracted, analyzed for quality, bar-coded sequencing libraries were made and quantitated, followed by multiplex, paired-end read RNA sequencing. The reads were checked for quality using FastQC®, trimmed of bar codes using Trimmomatic® [12], then aligned against the hg38 version of the human genome using tophat2 [13]/ bowtie2 (http://bowtie-bio.sourceforge.net/bowtie2/index.shtml), then quantitated using Cufflinks2 (http://cole-trapnell-lab.github.io/cufflinks/install/) . The ratios of averaged FPKM values for each incubation condition divided by DMSO vehicle controls were ranked from largest to smallest. Genes from the top 500 ratios for each incubation condition were then submitted to DAVID and analyzed using gene annotation clustering.

Tables 1 and 2 summarize our DAVID findings after gene annotation clustering. We used a conservative strategy to report GO terms only and show only those GO terms with Benjamini (multiple comparison)-corrected p values <0.05.

For Table 1 (H9 eSC/NSC-derived MN’s): Overall, the most significant GO term overlaps occurred with BNN20 and BNN27, and both NGF and BDNF,
involving GO terms representing ribosome functions. The next most significant GO clustering involved BNN20 and BNN27, and NGF and BDNF, involving GO terms representing cytoskeleton. BNN23 showed significant GO term alignment involving GO terms representing nucleoplasm and synaptic function, but these GO terms were not significantly represented by either neurotrophin protein or other MNT drugs. BNN27 shared one GO term involving intracellular and protein transport with NGF, and one GO term involving microtubule cytoskeleton that did not appear with either NT protein. Finally, each NT protein showed three GO terms each that did not appear with any MNT drug.

For Table 2 (Ctl iPSC/NSC-derived MN's): Overall the most significant GO term overlaps occurred with all three MNT drugs and both NT proteins in areas of cytoskeleton, organelle lumen and nuclear lumen. BNN20 and BNN23 showed GO term overlap with NGF in RNA splicing, translation and ribonucleoprotein complex. BNN20 and BNN27 showed GO term overlap with both NT’s NGF and BDNF for intracellular organelle lumen, and all three MNT drugs showed GO term overlap with NGF in the area of growth cone formation and polarized growth. BNN20 by itself showed significant GO term clustering in areas of mitotic cell process and unfolded protein binding.

Figure 2 shows that at the gene expression level, H9 eSC/NSC-derived motor neurons and Ctl iPSC/NSC-derived motor neurons were very similar to each other. There was an excellent correlation (Pearson r=0.92) of the log2FPKM values for 8904 gene pairs from the two MN cell lines.
Discussion

To date no cell or animal model of ALS accurately predicts efficacy of neuroprotective treatments for the most commonly occurring, sporadic form of the illness (sALS). Because of the survival-promoting, pleiotropic actions of neurotrophins on neurons [14], and the demonstrated efficacy of neurotrophins or neurotrophin receptors in preventing motor neuron death following axotomy [15-17], we are pursuing the clinical development of microneurotrophins for ALS.

As part of this development effort, we examined the ability of three MNT drugs to activate gene programs in “young” motor neurons produced by differentiation of human neural stem cells produced from either human embryonic stem cells (hESC) or CTL human PBMC induced pluripotential stem cells (CTL iPSC). We carried out high-resolution, paired-end read RNA sequencing of libraries prepared from each MN cell type following incubation with either NT protein or MNT drug, and subjected the resulting lists of the 500 most activated genes to analysis in DAVID to determine GO groups. We used a conservative analysis of DAVID results and report only GO groups where the multiple comparison-corrected p values (by Benjamini method) were <0.05.

We found that in the CTL iPSC/NSC-derived MN’s, all three MNT drugs stimulated the same GO groups as did NGF and BDNF, whereas in the H9 human eSC/NSC-derived MN’s, two of the three MNT drugs activated similar GO groups as did NGF and BDNF. There were notable exceptions to these trends, and the “fit” is not perfect.
Because it is not clear which GO terms are most involved in MN dysfunction and death in ALS, it is not possible to select any one MNT drug based on the results shown in Tables 1 and 2. Rather, it seems reasonable to conclude that the MNT drugs tested are capable of stimulating the same GO families as do NT proteins in the MN cell lines.

There are many limitations to our study:

First, the RNA sequencing bioinformatics analysis has inherent biases [18], particularly with the Cufflinks quantitation of gene expression that in our experience overestimates gene expression relative to other approaches such as DEseq [19] or EdgeR [20].

Second, we examined only two MN cell lines, whereas a greater number of lines, particularly some from ALS subjects, would have increased the generalizability of our findings.

Third, we examined gene expression changes after 24 hours of NT or MNT exposure. We do not know if longer periods of incubation would have activated (or repressed) gene programs.

Fourth, we did not use a FACS-concentrated population of MN’s. It is likely that some of the cells studied are “young” neurons but not yet differentiated to “young” MN’s.

And fifth, and perhaps most important, we do not yet have any follow-up data on the actual efficacy of any MNT in ALS subjects. This awaits clinical studies of one or more MNT drugs in humans with ALS.
Conclusions

In spite of these limitations, we do conclude that our findings, although limited in interpretation, support going forward with MNT development in ALS. Our finding that MNT’s activate gene programs similar to NT proteins, in two human, derived MN cell lines, suggest that MNT drugs will mimic NT actions on spinal MN’s in ALS subjects. Clinical trials will be necessary to determine MNT efficacy, but our findings that 100 nM levels of MNT can activate gene programs in “young” MN’s is encouraging for achieving these drug levels in vivo.

We agree with the cautions advanced in a recent review of stem cell models of ALS [2]. We have found that increasing duration of MN differentiation from 21 days to 42 days substantially increases neurophysiological maturity (depolarization spikes following current injection) (O’Brien, et al, in preparation). We do not yet know to what extent neuromuscular synapse formation among iPSC-derived MN’s and muscle cells, presence of co-cultured astrocytes, or growth in a 3-D scaffold matrix will alter MN gene expression following NT or MNT exposure. These are conditions to be explored in future studies.

Author Contributions: JPB performed all RNA-seq bioinformatics and analyzed the data. LOB created the differentiation scheme for the MN’s, prepared the MN’s used, and carried out the incubations. DGB devised the scripts for bioinformatics algorithms used, assisted in the MN incubations, isolated the RNA’s and assayed quantities and qualities, prepared and quantitated the RNA sequencing libraries.
All authors contributed to writing of the paper and have seen/approved the final version.

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**Table and Figure Legends**

Table 1. DAVID [10, 11] GO terms’ enrichments and multiple comparison-corrected (Benjamini) significances for gene expression in H9 human embryonic stem cell (eSC)-derived neural stem cells (NSC) differentiated to motor neurons (MN) for 21 days and incubated for 24 hours with NGF or BDNF (100 ng/ml) or MNT analogues (100 nM). Shown are DAVID results from gene annotation clustering analysis of the top 500 genes in each group after normalization to gene expression in presence of DMSO vehicle (0.001%) controls.

Table 2. Same as Table 1, except MN’s were differentiated from CTL iPSC’s made from a CTL subject peripheral blood mononuclear cell preparation using electroporation of a reprogramming plasmid [9].

Figure 1. Structures of microneurotrophin drugs used

Figure 2. X-Y plot of log₂ of FPKM values (>5) for averaged gene expression in the CTL subject iPSC/NSC-derived MN’s vs. H9 eSC/NSC-derived MN’s.
References Cited


Figure 2. X-Y plot of log$_2$FPKM from CTL iPSC-derived MN vs. log$_2$FPKM from H9-derived MN. Both cell types were cultured and RNA sequenced in duplicate. Data are shown for mean FPKM's>5 for a total of 8904 gene pairs.
Table 1. Gene Ontology (GO) Terms from DAVID for Human iPSC-derived Motor Neurons Exposed to NGF or BDNF (100 ng/ml) or Microneurotrophins BNN20, BNN23, BNN27 (each at 100 nM) for 24 hrs and Analyzed by RNA Seq. X= present; En= range of GO enrichment (fold); Bj= range of Benjamini-corrected p values

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<th>Gene Ontology (GO) terms</th>
<th>nerve growth factor (NGF)</th>
<th>brain-derived neurotrophic factor (BDNF)</th>
<th>BNN20</th>
<th>BNN23</th>
<th>BNN27</th>
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Table 2. Gene Ontology (GO) Terms from DAVID for CTL Human iPSC/NSC-derived Motor Neurons Exposed to NGF or BDNF (100 ng/ml) or Microneurotrophins BNN20, BNN23, BNN27 (each at 100 nM) for 24 hrs and Analyzed by RNA Seq. X = present; En = range of GO enrichment (fold); Bj = range of Benjamini-corrected p values

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