The neuroprotective role of the GM1 oligosaccharide, II^{3}Neu5Ac-Gg$_4$, in neuroblastoma cells.

Elena Chiricozzi[1†]*, Margherita Maggioni[1†], Erika di Biase[1], Giulia Lunghi[1], Maria Fazzari[1], Nicoletta Loberto[1], Maffioli Elisa[2], Francesca Grassi Scalvini[2], Gabriella Tedeschi[2,3] and Sandro Sonnino[1]*.

[1]Department of Medical Biotechnology and Translational Medicine, University of Milano, Milano, Italy.

[2]Department of Veterinary Medicine, University of Milano, Milano, Italy.

[3]Fondazione Unimi, Milano, Italy.

[†] These authors equally contributed to this work

* Correspondence to:

Sandro Sonnino, Professor

Elena Chiricozzi, PhD

Via Fratelli Cervi 93, 20090 Segrate (MI), Italy

Phone: +39 0250330360

Fax: +39 0250330365

sandro.sonnino@unimi.it

elena.chiricozzi@unimi.it

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GM1 oligosaccharide neuroprotective role

ORCID ID

Elena Chiricozzi 0000-0001-7431-920

Sandro Sonnino 0000-0001-8180-5908

Abstract

Recently, we demonstrated that the GM1 oligosaccharide, $II^3Neu5Ac\text{-}Gg_4$ (OligoGM1), administered to cultured murine Neuro2a neuroblastoma cells interacts with the NGF receptor TrkA, leading to the activation of the ERK1/2 downstream pathway and to cell differentiation.

To understand how the activation of the TrkA pathway is able to trigger key biochemical signaling, we performed a proteomic analysis on Neuro2a cells treated with 50 µM OligoGM1 for 24 hours. Over 3000 proteins were identified. Among these, 324 proteins were exclusively expressed in OligoGM1-treated cells. Interestingly, several proteins expressed only in OligoGM1-treated cells are involved in biochemical mechanisms with a neuroprotective potential, reflecting the GM1 neuroprotective effect. In addition, we found that the exogenous administration of OligoGM1 reduced the cellular oxidative stress in Neuro2a cells and conferred a protection against MPTP neurotoxicity.

These results confirm and reinforce the idea that the molecular mechanisms underlying the GM1 neurotrophic and neuroprotective effects depends on its oligosaccharide chain, suggesting the activation of a positive signaling starting at plasma membrane level.

Keywords (6): GM1 ganglioside; GM1 oligosaccharide chain; TrkA neurotrophin receptor; Plasma membrane signaling; Neuroprotection; Shotgun label free proteomic.

Abbreviations

Ganglioside nomenclature is in accordance with IUPAC-IUBB recommendations [1].

GM1, $II^3Neu5Ac\text{-}Gg_4Cer$, β-Gal-(1-3)-β-GalNAc-(1-4)-[α-Neu5Ac-(2-3)]-β-Gal-(1-4)-β-Glc-Cer;

OligoGM1, GM1 oligosaccharide, $II^3Neu5Ac\text{-}Gg_4$;

Ctrl, control;

DMEM, Dulbecco's modified Eagles' medium;

ERK1/2, extracellular signal-regulated protein kinases 1 and 2;

FBS, fetal bovine serum;

HPTLC, high-performance silica gel thin-layer chromatography;

IPA, Ingenuity Pathway Analysis

MAPK, mitogen-activated protein kinase;

MPP^+, 1-methyl-4-phenylpyridinium;

MPTP, 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine hydrochloride;

MS, Mass Spectometry;

N2a, Neuro2a cells;

NGF, nerve growth factor;

PBS, phosphate-buffered saline;

p-ERK1/2, phosphorylated ERK1/2;

p-TrkA, phosphorylated TrkA;

PM, plasma membrane;

PVDF, polyvinylidene difluoride;

RA, retinoic acid;

ROS, reactive oxygen species;

RRID, research resource identifiers;

Trk, neurotrophin tyrosin kinase receptor;

Tyr490, tyrosine 490.

Introduction

The essential role of ganglioside GM1 ($II^3Neu5Ac\text{-}Gg_4Cer$) in neuronal differentiation, protection and restoration is a milestone as demonstrated by the disastrous consequences deriving from its genetic deletion and, on the other hand, by its therapeutic potential in relation to neurodegenerative diseases [2-6]. Despite these evidence, the molecular mechanisms explaining its physiological function and its pathological implication still remain to be elucidated. *In vitro* and *in vivo* studies have suggested that the GM1 oligosaccharide portion could have a key role. In a 1988 pioneering paper, Schengrund and Prouty observed that the GM1 oligosaccharide promoted the neuritogenesis process [7]. Afterwards it has been found an effective GM1 alternative in the semisynthetic derivatives LIGA4 and LIGA20 with a chemical modification in the GM1 ceramide structure, which renders the GM1 analogues membrane permeable maintaining the neurotrophic potency [8-10]. These two results suggest that the ceramide structure is not critical in determining the GM1 modulatory effects. Recently, we proved, in the neuroblastoma cell line Neuro2a (N2a), that within the entire molecule, the oligosaccharide chain, β-Gal-(1-3)-β-GalNAc-(1-4)-[α-Neu5Ac-(2-3)]-β-Gal-(1-4)-Glc-, $II^3Neu5Ac\text{-}Gg_4$ (OligoGM1), is actually the moiety responsible for GM1 neurodifferentiative properties by directly interacting with NGF specific receptor TrkA at the plasma membrane (PM), leading to the activation of ERK1/2 downstream pathway [11]. In this view, GM1 exerts its bioactive feature throughout the hydrophilic head, which protrudes in the extracellular environment and therefore acts at cell surface level across the interaction with PM proteins.

Starting from that, we questioned if over the differentiative properties, the oligosaccharide chain of GM1 could also contribute to explain the GM1 neuroprotective function. Since the GM1 protective efficacy has been reported for neurotoxins both *in vitro* and *in vivo* [9,12-17], here we investigate the OligoGM1 role in the protection from MPTP mediated cytotoxicity [18-20]. Our biochemical analysis highlighted that the GM1 oligosaccharide protects neuroblastoma cells from MPTP toxic effect as well as from mitochondrial oxidative stress starting with PM activation of TrkA-ERK1/2 signaling pathway.

Methods

Materials

Commercial chemicals were of the highest purity available, common solvents were distilled before use and water was doubly distilled in a glass apparatus.

Cell culture plates and Transfectagro™ reduced serum medium were from Corning (Corning, NY, USA). Mouse neuroblastoma N2a cells (RRID: CVCL_0470), phosphate-buffered saline (PBS), paraformaldehyde, Dako fluorescent mounting medium, bovine serum albumin, trypan blue, ethylenediaminetetraacetic acid (EDTA), retinoic acid (RA), 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine hydrochloride (MPTP), 3′,4′-dichlorobenzamil hydrochloride (DCB), sodium orthovanadate (Na_3VO_4), phenylmethanesulfonyl fluoride (PMSF), aprotinin, and protease inhibitor cocktail (IP) were from Sigma-Aldrich (St. Louis, MO, USA). TrkA inhibitor (CAS 388626-12-8) was from Merk Millipore (Billerica, MA, USA). Dulbecco's modified Eagle's (DMEM) high glucose medium, fetal bovine serum (FBS), L-glutamine, Penicillin and Streptomycin (10.000 U/mL) were from EuroClone (Paignton, UK). MitoSOX™ Red Mitochondrial Superoxide Indicator and Hoechst-33342 fluorescent stain were purchased from Thermo Fischer Scientific (Waltham, MA, USA). DC™ protein assay kit was from BioRad (Hercules, CA, USA). High-performance thin-layer chromatography (HPTLC) was from Merk Millipore (Frankfurten, Germany).

Preparation of OligoGM1

OligoGM1 was prepared by ozonolysis followed by alkaline degradation [11, 21] from GM1 ganglioside, which was purified from the total ganglioside mixture extracted from pig brains [22,23]. Briefly, GM1 was dissolved in methanol and slowly saturated with ozone at 23°C. The solvent was then evaporated under vacuum and the residue brought immediately to pH 10.5-11.0 by addition of triethylamine. After solvent evaporation, GM1 oligosaccharide was purified by flash chromatography using chloroform/methanol/2-propanol/water 60:35:5:5 v/v/v/v as eluent. GM1 oligosaccharide was dissolved in methanol and stored at 4°C.

NMR, Mass Spectrometry (MS) and HPTLC analyses [24] showed a purity over 99% for the prepared oligosaccharide (Fig. 1 of supplementary files).

Cell cultures

Murine neuroblastoma cells N2a were cultured and propagated as monolayer in DMEM high glucose medium supplemented with 10% heat inactivated FBS, 1% L-glutamine and 1% penicillin/streptomycin, at 37°C in a humidified

atmosphere of 95% air/5% CO_2. Cells were sub-cultured to a fresh culture when growth reached the 80-90% confluence (i.e. every 3-4 days).

Cell treatments

N2a cells were plated at 5 x $10^3/cm^2$ and incubated for 24 h to allow cells attachment and recovery in complete medium before treatments.

OligoGM1 or RA treatment

To induce neurodifferentiation, growth medium was removed and N2a cells were pre-incubated in pre-warmed (37°C) Transfectagro medium containing 2% FBS, 1% L-glutamine, 1% penicillin/streptomycin, for 30 min at 37°C. Sequentially, cells were incubated at 37°C with 50 μM OligoGM1 [11] or 20 μM RA [25]. Control cells were incubated under the same experimental conditions but omitting any addition of OligoGM1 or RA.

MPTP treatment

To induce neurotoxicity, following 24 h from OligoGM1 or RA treatment, cells were incubated with MPTP (250 μM) [18] (Supplementary Fig. 2). Control experiments were carried out under the same experimental conditions.

Inhibition of TrkA receptor

To block TrkA activity in N2a cells, TrkA inhibitor (120 nM) was added to the incubation medium 1 h before the addition of OligoGM1 [10,26].

Morphological analysis

Cultured cells, treated or not with OligoGM1 or RA, in the absence or in the presence of MPTP, were observed by phase contrast microscopy (200x magnification, Olympus BX50 microscope; Olympus, Tokyo, Japan). At least 10 fields from each well were photographed for each experiment.

Proteomic analysis

N2a cells were incubated in the absence (control) or in the presence of 50 μM OligoGM1 for 24 h. Then medium was removed and cells were rinsed twice with 1 mM Na_3VO_4, 1 mM PMSF, 2% (v/v) Aprotinin, 1% (v/v) IP in cold PBS. Cells were scraped in the same buffer and centrifuged 800 x g for 5 min at 4°C. Pellets were immediately frozen by liquid nitrogen and conserved at -80°C before proteomic analysis by a shotgun label free proteomic approach for the identification and quantification of expressed proteins. Proteins were subjected to reduction with 13 mM DTE (30 min at 55°C) and alkylation with 26 mM iodoacetamide (30 min at 23°C). Peptide digestion was conducted using sequence-grade trypsin (Roche) for 16 h at 37°C using a protein:trypsin ratio of 20:1 [27]. The proteolytic digested was desalted

using Zip-Tip C18 (Millipore) before MS analysis. LC-ESI-MS/MS analysis was performed on a DionexUltiMate 3000 HPLC System with a PicoFritProteoPrep C18 column (200 mm, internal diameter of 75 μm) (New Objective, USA). Gradient: 2% ACN in 0.1 % formic acid for 10 min, 2-4 % ACN in 0.1% formic acid for 6 min, 4-30% ACN in 0.1% formic acid for 147 min and 30-50 % ACN in 0.1% formic for 3 min at a flow rate of 0.3 μL/min. The eluate was electrosprayed into an LTQ OrbitrapVelos (Thermo Fisher Scientific, Bremen, Germany) through a Proxeon nanoelectrospray ion source (Thermo Fisher Scientific) as described in [28]. The LTQ-Orbitrap was operated in positive mode in data-dependent acquisition mode to automatically alternate between a full scan (m/z 350-2000) in the Orbitrap (at resolution 60000, AGC target 1000000) and subsequent CID MS/MS in the linear ion trap of the 20 most intense peaks from full scan (normalized collision energy of 35%, 10 ms activation). Isolation window: 3 Da, unassigned charge states: rejected, charge state 1: rejected, charge states 2+, 3+, 4+: not rejected; dynamic exclusion enabled (60 s, exclusion list size: 200). Data acquisition was controlled by Xcalibur 2.0 and Tune 2.4 software (Thermo Fisher Scientific).

Data analysis

MS spectra were searched against the mouse Uniprot sequence database (release 31.07.2017) by MaxQuant (version 1.3.0.5) [29]. The following parameters were used: initial maximum allowed mass deviation of 15 ppm for monoisotopic precursor ions and 0.5 Da for MS/MS peaks, trypsin enzyme specificity, a maximum of two missed cleavages, carbamidomethyl cysteine as fixed modification, N-terminal acetylation, methionine oxidation, asparagine/glutamine deamidation and serine/threonine/tyrosine phosphorylation as variable modifications. False protein identification rate (5%) was estimated by searching MS/MS spectra against the corresponding reversed-sequence (decoy) database. Minimum required peptide length was set to 6 amino acids and minimum number of unique peptide supporting protein identification was set to 1. Quantification in MaxQuant was performed using the built-in label-free quantification algorithms (LFQ) based on extracted ion intensity of precursor ions.

Three biological replicates, each one replicated twice, were carried out for treated and control cells. Only proteins present and quantified in at least 2 out of 3 biological repeats were considered as positively identified in a sample and used for statistical analyses performed by the Perseus software module (version 1.5.5.3, www.biochem.mpg.de/mann/tools/). A t-test (p value $\leq$ 0.01) was carried out to identify proteins differentially expressed among the two different conditions. Proteins were considered to be differentially expressed if they were present only in treated or control samples or showed significant t-test difference. Bioinformatic analyses were carried out by Panther (version 10.0) and DAVID software (release 6.7) to cluster enriched annotation groups of Molecular Function, Biological Processes, Pathways and Networks within the set of identified proteins. Protein-protein

interactions were analysed by Ingenuity Pathway Analysis (IPA) (QIAGEN Redwood City, www.qiagen.com/ingenuity) [30]. Functional grouping was based on p value ≤ 0.05 and at least two counts.

The MS proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD011682.

Evaluation of mitochondrial superoxide generation

The production of mitochondrial superoxide was performed with MitoSOX Red as previously reported [31, 32]. Briefly, N2a cells, cultured in six well-plate on glass coverslips, were incubated or not with OligoGM1 for 24 h. Then, cells were treated with MPTP for 6, 10 or 24 h and, sequentially, incubated with 5 μM MitoSOX (10 min, 37°C). Cells were washed three times with cold PBS and were fixed with 4% (v/v) paraformaldehyde in PBS (20 min, 23°C). Cells were stained with 0.0002% (v/v) Hoechst in PBS (5 min, 23°C) and mounted with Dako fluorescent mounting medium [33]. The fluorescence was detected using a NikonEclipse Ni upright microscope (400x magnification) and a Nikon DIGITAL SIGHT DS-U1 CCD camera. Images were analyzed and fluorescence intensity was quantified using ImageJ software (NIH, Bethesda, USA; http://rsbweb.nih.gov/ij/). At least 20 fields were examined for each sample.

Determination of cell viability

Cell viability was determined by Trypan blue exclusion assay after 24 h MPTP treatment. The number of living or death cells has been determined by counting cells after Trypan blue staining, as previously described [34,35]. At least 20 fields have been evaluated for each samples.

Protein determination

Protein concentration of samples was assessed using a DC™ protein assay kit according to manufacturer's instructions, using bovine serum albumin as standard.

Statistical analysis

Data are expressed as mean ± SEM and were analyzed for significance by Student's t-test or one-way ANOVA test. The analysis was performed with Prism software (GraphPad Software, Inc. La Jolla, CA, USA).

Other analytical methods

NMR spectra were recorded with a Bruker AVANCE-500 spectrometer at a sample temperature of 298 K. NMR spectra were recorded in CDCl3 or CD3OD and calibrate using the TMS signal as internal reference.

Ganglioside and oligosaccharide mass spectrometry analysis were performed in positive ESI-MS. MS spectra were recorded on a Thermo Quest Finnigan LCQTM DECA ion trap mass spectrometer, equipped with a Finnigan ESI interface; data were processed by Finnigan Xcalibur software system (Thermo Fisher Scientific, Waltham, MA, USA). All reactions were monitored by TLC on silica gel 60 plates.

Results

Proteomic profile of OligoGM1-treated cells

The information on TrkA-MAPK cascade activation by the OligoGM1 on N2a cells [11] suggests the triggering of other biochemical signaling processes besides that of cellular differentiation. To verify such hypothesis, we performed a proteomic analysis of cells treated with 50 μM OligoGM1 for 24 h.

Proteins were identified by a shotgun proteomic approach, using label free for the relative quantification of their expression levels. The proteomic analysis led to the identification of 3166 proteins in treated cells and 3132 in control cells with Pearson correlation of 0.94, suggesting that the two data sets are very similar in terms of protein composition (Fig. 1a). In accordance, the Venn diagram (Fig. 1b) shows that 2842 proteins are commonly expressed both in control and OligoGM1-treated cells, among which 70 proteins are differently expressed, 23 up and 47 down-regulated, in treated cells in comparison to controls (Table I), while 350 (Table II) and 324 (Table III) proteins are expressed only in control cells and in OligoGM1-treated cells, respectively.

Classification of the differentially expressed proteins based on bioinformatic analysis

In order to understand the effect of OligoGM1, we focused on the proteins expressed in OligoGM1-treated cells. The bioinformatic analyses by DAVID (p value ≤ 0.05, at least 2 counts) showed that in these cells there is a significant enrichment of Gene Ontology terms related to endocytic trafficking, ribosome biogenesis and regulation of transcription, regulation of cell cycle, mitochondrion, fatty acid metabolism and cell adhesion (Table IV). Panther analysis on the same data set suggested a significant enrichment of proteins related to organelle biogenesis and maintenance (R-MMU-1852241) (p value 0.006). In accordance, IPA analysis showed, among the top biofunctions in terms of p value, cellular assembly and organization, cell and organism survival, tissue morphology and nervous system development and function (Table V).

We then compared by IPA the proteins that are up-regulated or only expressed in treated cells (347 proteins) with the proteins that are down-regulated in treated cells or expressed only in the control (397) to highlight differences that may be triggered by the presence of OligoGM1 (Table VI). Interestingly, the proteome of OligoGM1-treated cells presents up-regulation or exclusive expression of proteins involved in biochemical mechanism of neuronal differentiation, protection and restoration, such as suppression of proinflammatory molecules and inhibition of oxidative stress. Sirtuins, for example, are described as a protein family whose activities cause activation of anti-apoptotic, anti-inflammatory, anti-stress responses, and the modulation of the aggregation of proteins involved in neurodegenerative disorders [36]. SUMOylation has emerged as a potential therapeutic target for neuroprotection in brain ischemia,

including both global and focal brain ischemia (ischemic stroke) [37]. EIF2 is a signaling pathway involved in cell proliferation and protein translation whose dysregulation is associated with a number of pathologies, including neurodegenerative diseases, metabolic disorders, and cancer [38]. In the central nervous system, the phosphatase and tensin homolog deleted on chromosome ten (PTEN) plays a fundamental role in development, synaptogenesis and synaptic plasticity and in neuronal death [39]. The renin-angiotensin pathway is involved in neurodevelopment and participates in cell growth inhibition, fetal tissue development, extracellular matrix modulation, neuronal regeneration, apoptosis and cellular differentiation [40]. Mostly, the proteomic analysis pointed out an increased expression of proteins typically involved in mitochondria bioenergetic and in oxidative stress protection in N2a treated with OligoGM1. Among them, we found the expression of *i)* mitochondrial ribosome (mitoribosome) proteins (MRPL53, MRPL1, MRPS34, MRPL4, MRPL21, MRPS18C, MRPL48), which synthesize essential components of the oxidative phosphorylation machinery [41,42]; *ii)* proteins involved in the mitochondrial bioenergetics (*i.e.* ACADM, MCAT, ATP5F1, NDUFC2, CBR4, TACO1, CYP27A1, L2HGDH, SURF1, ACSL5, ACOX1, SNCB, CKB, PPP3CA, ACSL5, NUT19, RAP1GDS1, NDUFC2, DHRS1); *iii)* frataxin (FXN) protein, whose loss of function activates an iron/sphingolipid/PDK1/Mef2 pathway leading to a neurodegeneration [43] and *iv)* CYP27A1 enzyme, a member of the cytochrome P450 superfamily, whose functions are related to iron ion binding, oxidoreductase activity and to vitamin D metabolism in the brain [44]. In addition, we found the expression of the mitochondrial serine protease HtrA2, which negatively correlates with mitochondrial dysfunction leading to neurodegenerative disease with Parkinson's feature [45].

OligoGM1 protection in MPTP-treated cells

MPTP is a lipophilic compound which easily crosses the blood brain barrier inducing *in vivo* selective degeneration of nigrostriatal neurons, thereby mimicking the Parkinson's disease. MPTP is converted by mitochondria MAO-B enzyme to its toxic metabolite MPP^+ [18-20]. Although, the mechanisms of cell death induced by MPP^+ are not fully clear, it is known that MPP^+ exerts its neurotoxic effect by the inhibition of mitochondrial respiration *via* complex I, leading to mitochondrial energy deprivation and eventual cell death. An additional mechanism of MPTP induced-toxicity involves oxidative stress resulting in generation of toxic mitochondrial reactive oxygen species (ROS) causing damage to critical biomolecules [18, 20]. Considering the reported GM1 efficacy *in vitro*, in mouse and non-humane primate MPTP model [4, 15-17], its ability to protect PC12 cells by the activation of MAPK against hydrogen peroxide toxicity [15] and what the proteomic analysis highlighted, we chose to verify whether the GM1 oligosaccharide has a neuroprotective potential in MPTP-treated cells.

OligoGM1 protects from MPTP induced cell death

N2a cells were exposed to 50 μM OligoGM1 which leads to activation of the TrkA-ERK1/2 pathway and differentiation in neuron-like cells (Supplementary Fig. 3). After 24 h from OligoGM1 treatment, N2a cells were exposed to MPTP (250 μM). MPTP-treated cells showed a morphological damage immediately after 24 h treatment, while the OligoGM1 pretreatment induced identifiable dose dependent signal of survival in the presence of MPTP (Fig. 2a). Trypan blue assay showed that the pretreatment with 50 μM OligoGM1 was effective in preventing the MPTP-mediated cell death (Fig. 2b), with a significant reduction in cell death over 50% with respect to MPTP treated cells.

To clarify if the OligoGM1 neuroprotective effect against MPTP was due only to the OligoGM1 induced neuro-like phenotype, N2a cells were incubated with RA, a well-known neurodifferentiative agent. The RA treatment induced cell differentiation but did not prevent from the MPTP toxic effect (Fig. 2), supporting OligoGM1 specificity in activating proper signal pathways.

OligoGM1 protective effect is abolished by TrkA inhibitor

To understand if the OligoGM1 protective effect is mediated by TrkA activation at the PM by the GM1 oligosaccharide interaction, we blocked TrkA activity using a specific inhibitor able to fit in the ATP pocket [11, 26]. The presence of inhibitor together with OligoGM1 abolished the OligoGM1 protective effect against MPTP (Fig. 2), suggesting that the OligoGM1 neuroprotective effect is associated to the TrkA-ERK1/2 pathway activation.

OligoGM1 protects from MPTP induced mitochondrial oxidative stress

N2a cells were pretreated with OligoGM1 for 24 h before MPTP. After 6, 10 and 24 h from MPTP administration, using the fluorescent probe MitoSOX Red, we carried out measurements of mitochondrial ROS levels via live-cell microscopy (Fig. 3; Supplementary Fig. 4). Interestingly we observed reduced MitoSOX Red signal in pretreated OligoGM1 cells compared to controls, for every time point analyzed (Fig. 3; Supplementary Fig. 4), suggesting that OligoGM1 limits MPTP-ROS production.

From the latter result, we can infer that the molecular mechanism underlying the OligoGM1 neuroprotective property relies on the modulation of mitochondrial ROS production conferring a protection against oxidative stress induced by MPTP.

Discussion

Gangliosides are sialic-acid containing glycosphingolipids, mostly abundant in the nervous tissues [46]. They have been considered master regulators of the nervous system development and homeostasis, due to their properties to organize membrane microdomains at the cell surface necessary to regulate numerous cell signaling pathways [2-6, 27-50]. Among the large family of gangliosides, which share the ceramide portion but differ for the structure of their oligosaccharide portion, the monosialo-ganglioside GM1 has been studied in detail, standing out as a modulator of several neuronal processes. Concerning that, most relevant is its interaction with neurotrophic factor receptors, to regulate neuronal differentiation, protection and restoration [2, 4, 30]. In cultured neurons, the GM1 enrichment in PM allows the dimerization and activation of neurotrophins receptors belonging to Trk family. This event in turn triggers a specific signaling cascade resulting in actin depolymerization, axon protrusion and elongation [51-55]. It has been shown that GM1 is able to induce a neuron-like morphology in N2a cells [55, 56] by the activation of nerve growth factor receptor (TrkA) enhancing the phosphorylation of Tyr490 [51, 57]. This event induces the MAPK phosphorylation cascade leading to neurodifferentiation [15, 58-62]. Moreover, it has been reported that the absence or the reduction in PM GM1 content negatively correlates with TrkA functions [14, 54, 60, 63-67] . Further, it has been hypothesized that the reduced level of PM GM1 in neurons can trigger the neurogenerative process by a failure of trophic signaling (GDNF/NGF signaling) [14, 68-70] .

While the involvement of GM1 in regulating neuronal processes is consolidated, the molecular mechanisms and the molecular portion responsible of these events are not completely understood. As all gangliosides, GM1 is composed by two portions: the oligosaccharide soluble portion protruding in the extracellular environment and the rigid lipid portion, the ceramide, inserted into the outer layer of the membrane and participating to the membrane lipid organization [71-75]. *In vitro* and *in vivo* past studies provided evidence that the oligosaccharide chain could have a central bioactive role. The successful exploit of GM1 analogs with modified ceramide structure led to the conclusion that the ceramide does not represent the critical portion responsible for GM1 modulatory properties. In fact the replacement of the stearoyl unit with the dichloroacetyl or with the acetyl group (respectively, LIGA20 and LIGA4 correspond to the codes for these GM1 analogs) maintained the same neuronal trophic, protective and restorative properties as GM1 [8, 9]. Even earlier, the oligosaccharide chain of GM1 was observed to induce the process of neuritogenesis in a neuroblastoma cell line [7]. Recently, the specific role of the GM1 oligosaccharide in the process of neurite elongation in murine neuroblastoma N2a cells has been explained [11]. The GM1 oligosaccharide, similarly to the entire GM1, but rather than the ceramide portion, directly binds to extracellular TrkA domain, and stabilizes the interaction of TrkA-NGF complex, allowing a rapid auto phosphorylation of the receptor's cytosolic portion and promoting the MAPK differentiation signaling [11].

In this paper, we investigated the cascade of processes following the interaction between the GM1 oligosaccharide (OligoGM1) and TrkA at cell surface. OligoGM1 was added to N2a cell culture medium at 50 μM concentration. As previously reported [11], OligoGM1 treatment induced TrkA-Erk1/2 pathway activation followed by N2a cells differentiation (Supplementary Fig. 3).

Comparing the protein patterns of OligoGM1 treated and untreated cells, we found restricted but significant differences of basal protein content, depending on the differential expression of specific groups of genes as a consequence of the downstream molecule cascade promoted by OligoGM1 induced TrkA activation (Table I; Supplementary Fig. 3). In addition, we found 324 proteins present exclusively in cells treated with the OligoGM1. Some of these are known to be involved in neuroprotection and survival processes (Table IV, V and VI). Thus we examined more in detail the protective potential of OligoGM1 against neurotoxicity induced by MPTP. MPTP-mediated toxicity is related to the inhibition of electron transport chain and to oxidative stress [18-20]. It has been reported, for example that MPTP caused H_2O_2 accumulation and inhibited catalase activity. Hydrogen peroxide can react with Fe^{2+} via Fenton reaction to generate $OH^{\bullet}$, a strong reactive oxidant.

For the first time, here, we showed that OligoGM1 pretreatment for 24 h before exposure of cells to MPTP reduces mitochondrial ROS production conferring a protection against oxidative stress and reduces the cell-death (Fig. 2 and Fig. 3).

To confirm that OligoGM1 induced TrkA activation represents the trigger event underling MPTP-protection, we specifically inhibited the TrkA activity: the administration of OligoGM1 together with TrkA inhibitor abolished the OligoGM1 protective effect against MPTP treatment (Fig. 2). On the other hand, to exclude that the OligoGM1 effect was simply due to the acquisition of a neuron-like phenotype, N2a cells were differentiated with retinoic acid and 24 h later were treated with MPTP. Retinoic acid was not able to confer any protection against MPTP toxicity. The latter result strengthened the specificity of OligoGM1 effect (Fig. 2).

The neuroprotective properties of GM1 ganglioside have been reported in many papers. Now, as for the GM1 differentiative properties [11] we confirm that the GM1 neuroprotection, at least in N2a cells, derives from a direct interaction between the GM1 oligosaccharide and the TrkA receptor, and the following signalling cascade capable to overexpress specific neuroprotective proteins.

Although much experimental work is needed to fully clarify the GM1 mechanism of action with respect to its potential, our findings sustain the idea that the oligosaccharide chain is the key molecular portion and the starting point for GM1 mediated protective function at the plasma membrane level.

We propose that GM1 oligosaccharide may stabilize the TrkA-NGF complex on the cell surface triggering the phosphorylation of Tyr490 promoting the MAPK differentiation signaling. This induces the activation of a complex

network of signaling processes that are involved in biochemical pathway of neuroprotection and neurorestoration (Fig. 4).

Conflict of interest

The authors declare that they have no conflict of interest.

Online supplemental material.

Fig. 1S shows the scheme of the reaction for OligoGM1 synthesis from GM1 and MS analysis.

Fig. 2S shows the time course of OligoGM1 and MPTP treatment of N2a in culture.

Fig. 3S shows the differentiation of N2a cells and TrkA-Erk1/2 activation induced by OligoGM1.

Fig. 4S shows the representative images of MitoSOX red fluorescence and nuclei staining 6 and 10 h later MPTP treatment in OligoGM1 treated N2a cells.

Supplemental Materials and Methods describe TrkA-Erk1/2 immunoblotting analysis.

Tables

Table I List of the proteins statistically differentially expressed in OligoGM1 vs CONTROL N2a cells

Student's T-test Difference OLIGO_CTR	Majority protein IDs	Protein names	Gene names
-3,97953	E9QMV2	Costars family protein ABRACL	3110003A 17Rik
-3,19309	Q9CQR2	40S ribosomal protein S21	Rps21
-3,07356	Q9CQZ1	Heat shock factor-binding protein 1	Hsbp1
-3,00908	Q3TIX9	U4/U6.U5 tri-snRNP-associated protein 2	Usp39
-2,89295	P60710	Actin, cytoplasmic 1	Actb
-2,47279	Q9CWZ3	RNA-binding protein 8A	Rbm8a
-2,46072	P55105	Bone morphogenetic protein 8B	Bmp8b
-2,12761	Q6ZWV3	60S ribosomal protein L10	Rpl10
-2,00293	A6H6S0	Hect domain and RLD 3	Herc3
-1,92439	Q8BJA2	Solute carrier family 41 member 1	Slc41a1
-1,91339	Q9ESW4	Acylglycerol kinase, mitochondrial	Agk
-1,75705	Q8VIB5	BarH-like 2 homeobox protein	Barhl2
-1,68246	P11157	Ribonucleoside-diphosphate reductase subunit M2	Rrm2
-1,61967	A0A0R4J0I2	Transmembrane protein 132D	Tmem132 d
-1,5279	E9Q2G7	Solute carrier family 2, facilitated glucose transporter member 3	Slc2a3
-1,51962	P54103	DnaJ homolog subfamily C member 2	Dnajc2
-1,49549	P58389	Serine/threonine-protein phosphatase 2A activator	Ppp2r4
-1,40454	E9PW66	Nucleosome assembly protein 1-like 1	Nap1l1
-1,37956	Q9Z1R2	Large proline-rich protein BAG6	Bag6
-1,37446	Q8VEH6	COBW domain-containing protein 1	Cbwd1
-1,37404	Q9CWF2	Tubulin beta-2B chain	Tubb2b
-1,31362	Q924T2	28S ribosomal protein S2, mitochondrial	Mrps2
-1,31199	Q9QZD8	Mitochondrial dicarboxylate carrier	Slc25a10
-1,23455	G3X8Y3	N-alpha-acetyltransferase 15, NatA auxiliary subunit	Naa15
-1,21106	A0A0R4J008	Histone deacetylase 2	Hdac2
-1,14768	E9Q6G4	ATP-binding cassette sub-family A member 7	Abca7
-1,13689	Q9Z2Q6	Septin-5	Sept5
-1,12486	Q4FE56	Ubiquitin carboxyl-terminal hydrolase	Usp9x
-1,09299	Q0VBD2	Protein MCM10 homolog	Mcm10
-1,0627	Q6P1J1	Crmp1 protein	Crmp1
-0,961719	A2AFI6	Transmembrane 9 superfamily member	Gm364
-0,908271	D3Z780	Translation initiation factor eIF-2B subunit delta	Eif2b4
-0,878172	E9QM77	Ataxin-2	Atxn2
-0,840163	Q9R0P4	Small acidic protein	Smap
-0,831308	Q9Z0V8	Mitochondrial import inner membrane translocase subunit Tim17-A	Timm17a

-0,814648	I1E4X0	Disks large-associated protein 4	Dlgap4
-0,801263	E9Q1P8	Interferon regulatory factor 2-binding protein 2	Irf2bp2
-0,750502	Q62465	Synaptic vesicle membrane protein VAT-1 homolog	Vat1
-0,7079	E9Q616	AHNAK nucleoprotein (desmoyokin)	Ahnak
-0,703338	Q9CQ22	Ragulator complex protein LAMTOR1	Lamtor1
-0,64407	P56480	ATP synthase subunit beta, mitochondrial	Atp5b
-0,641168	Q9DAP7	Histone chaperone ASF1B	Asf1b
-0,509192	Q9Z1N5	Spliceosome RNA helicase Ddx39b	Ddx39b
-0,448086	Q8BTW3	Exosome complex component MTR3	Exosc6
-0,387215	Q6PAM1	Alpha-taxilin	Txlna
-0,368086	Q9WTM5	RuvB-like 2	Ruvbl2
-0,316293	Q3UHJ0	AP2-associated protein kinase 1	Aak1
0,272641	Q99LC8	Translation initiation factor eIF-2B subunit alpha	Eif2b1
0,309552	Q8VE73	Cullin-7	Cul7
0,450782	Q9EQI8	39S ribosomal protein L46, mitochondrial	Mrpl46
0,484953	G5E8R4	Serine/threonine-protein phosphatase 6 regulatory subunit 3	Ppp6r3
0,550471	Q60790	Ras GTPase-activating protein 3	Rasa3
0,657146	E9Q0S6	Tensin 1	Tns1
0,700328	Q3U9G9	Lamin-B receptor	Lbr
0,71614	A0A087WPU8	Transcription factor Dp-2	Tfdp2
0,724323	P53994	Ras-related protein Rab-2A	Rab2a
0,868009	Q6PDI6	Protein FAM63B	Fam63b
0,886094	Q64378	Peptidyl-prolyl cis-trans isomerase FKBP5	Fkbp5
0,972225	P36552	Coproporphyrinogen-III oxidase, mitochondrial	Cpox
1,02418	P62827	GTP-binding nuclear protein Ran	Ran
1,11385	P61514	60S ribosomal protein L37a	Rpl37a
1,21209	A0A0U1RNX8	B-cell CLL/lymphoma 7 protein family member C	Bcl7c
1,25283	Q6GQT9	Nodal modulator 1	Nomo1
1,33364	P29595	NEDD8	Nedd8
1,35297	P62281	40S ribosomal protein S11	Rps11
1,53449	P35550	rRNA 2-O-methyltransferase fibrillarin	Fbl
1,60853	P62889	60S ribosomal protein L30	Rpl30
1,7774	Q6PIU9	Uncharacterized protein FLJ45252 homolog	N/A
1,84351	Q8CBB6	Histone H2B	Gm13646
2,89154	P02301	Histone H3.3C	H3f3c

Table II List of the proteins only expressed in CONTROL cells in the comparison OligoGM1 vs CONTROL N2a cells

Majority protein IDs	Protein names	Gene names
A0A075B5Z7	T cell receptor alpha variable 5-1	Trav5-1
A0A087WPL5	ATP-dependent RNA helicase A	Dhx9
Q9CWU4	UPF0690 protein C1orf52 homolog	2410004B18Rik
Q8BXK8	Arf-GAP with GTPase, ANK repeat and PH domain-containing protein 1	Agap1
A0A087WRX8	Serine/arginine repetitive matrix protein 2	Srrm2
Q60862	Origin recognition complex subunit 2	Orc2
A0A087WSQ9	Zinc finger CCHC domain-containing protein 2	Zcchc2
G3UZM1	Probable JmjC domain-containing histone demethylation protein 2C	Jmjd1c
B7ZCJ1	Rho GTPase-activating protein 21	Arhgap21
A0A0A6YVS2	Transmembrane and coiled-coil domain-containing protein 1	Tmco1
Q64735	Complement component receptor 1-like protein	Cr1l
A0A0A6YVW3	Immunoglobulin heavy variable V1-23	Ighv1-23
A0A0A6YXG9	Uridine-cytidine kinase 2	Uck2
A0A0B4J1J5	Immunoglobulin heavy variable V9-3	Ighv9-3
E9PZ43	Microtubule-associated protein	Mtap4
A0A0G2JEG1	Serine/arginine-rich-splicing factor 11	Srsf11
O54946	DnaJ homolog subfamily B member 6	Dnajb6
A0A0G2JFP4	Ferric-chelate reductase 1	FRRS1
Q8BXV2	BRI3-binding protein	Bri3bp
A0A0G2LB90	Tubulin polyglutamylase TTLL7	Ttll7
A0A0J9YTZ5	Protein FAM193A	Fam193a
O35427	DNA-directed RNA polymerase III subunit RPC9	Crcp
F7BJB9	MORC family CW-type zinc finger protein 3	Morc3
Q3UEZ8	Sodium/bile acid cotransporter 4	Slc10a4
Q4VC33	Macrophage erythroblast attacher	Maea
P17183	Gamma-enolase	Eno2
Q80V62	Fanconi anemia group D2 protein homolog	Fancd2
Q9CQR6	Serine/threonine-protein phosphatase 6 catalytic subunit	Ppp6c
A0A0N4SVR5	RasGEF domain family, member 1A	Rasgef1a
A0A0R4J023	Methylglutaconyl-CoA hydratase, mitochondrial	Auh
A0A0R4J0D1	Store-operated calcium entry-associated regulatory factor	Tmem66
A0A0R4J0H8	Fibronectin type III domain-containing protein 3B	Fndc3b
A0A0R4J0J4	Atypical chemokine receptor 1	Ackr1
A0A0R4J0P1	Isobutyryl-CoA dehydrogenase, mitochondrial	Acad8
A0A0R4J0T5	CUGBP Elav-like family member 1	Celf1
A0A0R4J1C6	Butyrophilin-like protein 10	Btnl10
A0A0R4J1K1	CCR4-NOT transcription complex subunit 4	Cnot4
Q9QZR5	Homeodomain-interacting protein kinase 2	Hipk2
A0A0R4J220	Atypical chemokine receptor 1	Kifc3
A0A0R4J259	Heterogeneous nuclear ribonucleoprotein Q	Syncrip
Q8BX09	Retinoblastoma-binding protein 5	Rbbp5

A0A0U1RNX4	Unconventional prefoldin RPB5 interactor	Uri1
A0A140LJ04	Zinc finger ZZ-type and EF-hand domain-containing protein 1	Zzef1
A0A140LJ70	Protein arginine N-methyltransferase 1	Prmt1
O55222	Integrin-linked protein kinase	Ilk
Q6PD31	Trafficking kinesin-binding protein 1	Trak1
Q3TIR3	Synembryn-A	Ric8a
G5E8I8	Calcium homeostasis endoplasmic reticulum protein	Cherp
E9PZW8	Unconventional myosin-IXb	Myo9b
O54833	Casein kinase II subunit alpha	Csnk2a2
Q7TR45	Olfactory receptor	Olfr1131
Q9WU63	Heme-binding protein 2	Hebp2
Q8R4S0	Protein phosphatase 1 regulatory subunit 14C	Ppp1r14c
A0A1L1SUG9	Cadherin EGF LAG seven-pass G-type receptor 3	Celsr3
Q4VBF2	R3H domain-containing protein 4	R3hdm4
K3W4Q9	Golgi-associated PDZ and coiled-coil motif-containing protein	Gopc
Q8VHR5	Transcriptional repressor p66-beta	Gatad2b
A2A432	Cullin-4B	Cul4b
P02535	Keratin, type I cytoskeletal 10	Krt10
A2A654	Bromodomain PHD finger transcription factor	Bptf
A2AE27	AMP deaminase 2	Ampd2
Q9D711	Pirin	Pir
A2AIR7	Voltage-dependent N-type calcium channel subunit alpha-1B	Cacna1b
A2AMD0	Predicted gene 12666	Gm12666
A2AML7	Zinc finger protein 352	Zfp352
Q8JZX4	Splicing factor 45	Rbm17
A2APR8	Mitotic checkpoint serine/threonine-protein kinase BUB1	Bub1
Q80TY0	Formin-binding protein 1	Fnbp1
A2ASI5	Sodium channel protein type 3 subunit alpha	Scn3a
A2AUD5	Tumor protein D54	Tpd52l2
A2AVR2	HEAT repeat-containing protein 8	Heatr8
A2AWI7	Endophilin-B2	Sh3glb2
Q9D868	Peptidyl-prolyl cis-trans isomerase H	Ppih
A3KFM7	Chromodomain-helicase-DNA-binding protein 6	Chd6
A4FUP9	Glycosyltransferase 1 domain-containing protein 1	Glt1d1
A6H630	UPF0364 protein C6orf211 homolog	Armt1
Q9D3L3	Synaptosomal-associated protein	Snap23
Q9WTT4	V-type proton ATPase subunit G 2	Atp6v1g2
B1AQW2	Microtubule-associated protein	Mapt
Q9QWI6	SRC kinase signaling inhibitor 1	Srcin1
B1AUN2	Eukaryotic translation initiation factor 2B, subunit 3	Eif2b3
B1AVB2	Scm polycomb group protein-like 2	Scml2
B1AZM2	Predicted gene 15091	Gm15091
O35317	Pre-B-cell leukemia transcription factor 3	Pbx3
B2M1R6	Heterogeneous nuclear ribonucleoprotein K	Hnrnpk
B2RXS4	Plexin-B2	Plxnb2
B7ZC40	Glutaredoxin-2, mitochondrial	Glrx2
P47934	Carnitine O-acetyltransferase	Crat

B8JJZ4	Zinc finger protein 808	Zfp808
G3UZF7	Centrosomal protein C10orf90 homolog	D7Ertd443e
P62843	40S ribosomal protein S15	Rps15
E9Q425	Tubulin polyglutamylase TTLL5	Ttll5
P17515	C-X-C motif chemokine 10	Cxcl10
Q9Z1M4	Ribosomal protein S6 kinase beta-2	Rps6kb2
D3YXS5	Kinesin-like protein KLP6	Klp6
D3YXW1	Protein LLP homolog	Llph
Q99MN9	Propionyl-CoA carboxylase beta chain, mitochondrial	Pccb
Q9D6K7	Tetratricopeptide repeat protein 33	Ttc33
D3Z2J4	AKT-interacting protein	Aktip
Q9WTZ1	RING-box protein 2	Rnf7
Q9CQB5	CDGSH iron-sulfur domain-containing protein 2	Cisd2
D3Z4U8	DDB1- and CUL4-associated factor 11	Dcaf11
D3Z6B7	DNA damage-regulated autophagy modulator protein 2	Dram2
D3Z7P4	Glutaminase kidney isoform, mitochondrial	Gls
P97785	GDNF family receptor alpha-1	Gfra1
Q8CIG9	F-box/LRR-repeat protein 8	Fbxl8
D6RJI8	TBC1 domain family member 13	Tbc1d13
Q5SUQ9	CST complex subunit CTC1	Ctc1
E0CXT7	Cleavage and polyadenylation specificity factor subunit 4	Cpsf4
E9PU87	Serine/threonine-protein kinase SIK3	Sik3
P70170	ATP-binding cassette sub-family C member 9	Abcc9
E9PUR1	Opticin	Optc
P48774	Glutathione S-transferase Mu 5	Gstm5
E9PVN6	Synaptojanin-2-binding protein	Gm20498
E9PVZ8	Golgi autoantigen, golgin subfamily b, macrogolgin 1	Golgb1
E9PWQ7	Zonadhesin	Zan
Q8CCB4	Vacuolar protein sorting-associated protein 53 homolog	Vps53
E9PXU1	Integrator complex subunit 6-like	Ddx26b
E9PYD5	Transcription elongation factor A protein 1	Tcea1
Q6NZN0	RNA-binding protein 26	Rbm26
E9Q430		Gm2832
O08532	Voltage-dependent calcium channel subunit alpha-2/delta-1	Cacna2d1
Q8R0F3	Sulfatase-modifying factor 1	Sumf1
E9Q2E4	HECT domain E3 ubiquitin protein ligase 4	Gm15800
E9Q2N4	Vomeronasal type-1 receptor	Vmn1r184
E9Q394	A-kinase anchor protein 13	Akap13
E9Q4K7	Kinesin family member 13B	Kif13b
E9Q4R1	Protein FAM102B	Fam102b
E9Q545	Olfactory receptor	Olfr552
E9Q5L3	Short/branched chain specific acyl-CoA dehydrogenase, mitochondrial	Acadsb
G5E8P0	Gamma-tubulin complex component 6	Tubgcp6
E9Q7E2	AT-rich interactive domain-containing protein 2	Arid2
Q3UQ84	Threonine--tRNA ligase, mitochondrial	Tars2
E9Q7P5	Olfactory receptor	Olfr640
E9Q9J4	Inositol hexakisphosphate and diphosphoinositol-pentakisphosphate	Ppip5k2

	kinase 2	
Q6ZPL9	ATP-dependent RNA helicase DDX55	Ddx55
H3BJQ9	Homeobox protein cut-like 1	Cux1
F6QYZ5	Peptidase inhibitor 16	Pi16
F6S169	Tankyrase-2	Tnks2
O88384	Vesicle transport through interaction with t-SNAREs homolog 1B	Vti1b
Q810U5	Coiled-coil domain-containing protein 50	Ccdc50
Q6DFZ2	Nesprin-2	Syne2
F6XVH0	Predicted gene 12830	Gm12830
Q9CR21	Acyl carrier protein, mitochondrial	Ndufab1
F6ZGR6	RIKEN cDNA D430041D05 gene	D430041D05 Rik
J3QPW1	Phosphatidylinositol transfer protein alpha isoform	Pitpna
Q69ZR2	E3 ubiquitin-protein ligase HECTD1	Hectd1
Q99N84	28S ribosomal protein S18b, mitochondrial	Mrps18b
P19426	Negative elongation factor E	Rdbp
Q80VJ2	Steroid receptor RNA activator 1	Sra1
G3X9H5	Huntingtin	Htt
G3X9Z8	Innate immunity activator protein	5730559C18R ik
G5E852	Tyrosine-protein kinase	Jak2
H3BLL4	Heterogeneous nuclear ribonucleoprotein K	Hnrnpk
Q60766	Immunity-related GTPase family M protein 1	Irgm1
K4DI67	Condensin-2 complex subunit D3	Ncapd3
K7N678	Olfactory receptor	Olfr893
M9MMK5	Olfactory receptor	Olfr329-ps
O08675	Proteinase-activated receptor 3	F2rl2
O35350	Calpain-1 catalytic subunit	Capn1
O54732	Matrix metalloproteinase-15	Mmp15
O54774	AP-3 complex subunit delta-1	Ap3d1
O70456	14-3-3 protein sigma	Sfn
P01649	Ig kappa chain V-V regions	
P01897	H-2 class I histocompatibility antigen, L-D alpha chain	H2-L
P01942	Hemoglobin subunit alpha	Hba
P02772	Alpha-fetoprotein	Afp
P08122	Collagen alpha-2(IV) chain	Col4a2
P10922	Histone H1.0	H1f0
P13542	Myosin-8	Myh8
P15533	Tripartite motif-containing protein 30A	Trim30a
P20060	Beta-hexosaminidase subunit beta	Hexb
P22437	Prostaglandin G/H synthase 1	Ptgs1
P31938	Dual specificity mitogen-activated protein kinase kinase 1	Map2k1
P35585	AP-1 complex subunit mu-1	Ap1m1
P42227	Signal transducer and activator of transcription 3	Stat3
P43247	DNA mismatch repair protein Msh2	Msh2
P48432	Transcription factor SOX-2	Sox2
P49586	Choline-phosphate cytidylyltransferase A	Pcyt1a

Q922U2	Keratin, type II cytoskeletal 5	Krt5
P51125	Calpastatin	Cast
P51807	Dynein light chain Tctex-type 1	Dynlt1
P51943	Cyclin-A2	Ccna2
P55200	Histone-lysine N-methyltransferase MLL	Mll
P57716	Nicastrin	Ncstn
P58058	NAD kinase	Nadk
P58871	182 kDa tankyrase-1-binding protein	Tnks1bp1
P59017	Bcl-2-like protein 13	Bcl2l13
P61967	AP-1 complex subunit sigma-1A	Ap1s1
P61971	Nuclear transport factor 2	Nutf2
P63147	Ubiquitin-conjugating enzyme E2 B	Ube2b
P70279	Surfeit locus protein 6	Surf6
P70333	Heterogeneous nuclear ribonucleoprotein H2	Hnrnph2
P70388	DNA repair protein RAD50	Rad50
P81117	Nucleobindin-2	Nucb2
Q6P4T3	Eyes absent homolog 3	Eya3
P97481	Endothelial PAS domain-containing protein 1	Epas1
Q0VBL3	RNA-binding protein 15	Rbm15
Q14AX6	Cyclin-dependent kinase 12	Cdk12
Q14CH7	Alanine--tRNA ligase, mitochondrial	Aars2
Q32KG4	Retrotransposon gag domain-containing protein 1	Rgag1
Q3TC93	HCLS1-binding protein 3	Hs1bp3
H9H9T1	Protein FAM107B	Fam107b
Q3TMP1	General transcription factor IIIC, polypeptide 3	Gtf3c3
Q3TTY0	Phospholipase B1, membrane-associated	Plb1
Q3TZR9	Cyclic AMP-dependent transcription factor ATF-7	Atf7
Q3U186	Probable arginine--tRNA ligase, mitochondrial	Rars2
Q3UHI4	Protein TMED8	Tmed8
Q6PDG5	SWI/SNF complex subunit SMARCC2	Smarcc2
Q3UM18	Large subunit GTPase 1 homolog	Lsg1
Q3UMR5	Calcium uniporter protein, mitochondrial	Mcu
Q3UMU9	Hepatoma-derived growth factor-related protein 2	Hdgfrp2
Q3USJ8	FCH and double SH3 domains protein 2	Fchsd2
Q64458	Cytochrome P450 2C29	Cyp2c29
Q3UWX6	RIKEN cDNA E330034G19 gene	E330034G19 Rik
Q561M1	Acp1 protein	Acp1
Q68FF6	ARF GTPase-activating protein GIT1	Git1
Q5ND29	Rab-interacting lysosomal protein	Rilp
Q8BFY7	Protein FAM64A	Fam64a
Q5RJG1	Nucleolar protein 10	Nol10
Q99N89	39S ribosomal protein L43, mitochondrial	Mrpl43
Q5SSW2	Proteasome activator complex subunit 4	Psme4
Q7TNE3	Sperm-associated antigen 7	Spag7
Q60596	DNA repair protein XRCC1	Xrcc1
Q60778	NF-kappa-B inhibitor beta	Nfkbib

Q60886	Olfactory receptor 147	Olfr147
Q61025	Intraflagellar transport protein 20 homolog	Ift20
Q61048	WW domain-binding protein 4	Wbp4
Q61136	Serine/threonine-protein kinase PRP4 homolog	Prpf4b
Q61838	Alpha-2-macroglobulin	A2m
Q63886	UDP-glucuronosyltransferase 1-1	Ugt1a1
Q641P0	Actin-related protein 3B	Actr3b
Q64676	2-hydroxyacylsphingosine 1-beta-galactosyltransferase	Ugt8
Q6A037	NEDD4-binding protein 1	N4bp1
Q6A152	Cytochrome P450 4X1	Cyp4x1
Q6DID3	Protein SCAF8	Scaf8
Q6NV83	U2 snRNP-associated SURP motif-containing protein	U2surp
Q6NXJ0	Protein WWC2	Wwc2
Q6NZR5	Superkiller viralicidic activity 2-like (S. cerevisiae)	Skiv2l
Q6P1C6	Leucine-rich repeats and immunoglobulin-like domains protein 3	Lrig3
Q6P5E6	ADP-ribosylation factor-binding protein GGA2	Gga2
Q6P9J9	Anoctamin-6	Ano6
Q6PGC1	ATP-dependent RNA helicase Dhx29	Dhx29
Q6V4S5	Protein sidekick-2	Sdk2
Q6ZQ73	Cullin-associated NEDD8-dissociated protein 2	Cand2
Q7TMM9	Tubulin beta-2A chain	Tubb2a
Q7TPM6	Fibronectin type III and SPRY domain-containing protein 1	Fsd1
Q7TQA6	Taste receptor type 2 member 38	Tas2r38
Q7TQT7	Olfactory receptor	Olfr1371
Q7TQZ0	Olfactory receptor	Olfr32
Q7TR71	Olfactory receptor	Olfr1062
Q7TRI9	Olfactory receptor	Olfr129
Q80X59	Transmembrane and coiled-coil domain-containing protein 5B	Tmco5b
Q80X71	Transmembrane protein 106B	Tmem106b
Q80X98	DEAH (Asp-Glu-Ala-His) box polypeptide 38	Dhx38
Q80ZX2	Zfp790 protein	Zfp790
Q8BFQ4	WD repeat-containing protein 82	Wdr82
Q8BGD6	Putative sodium-coupled neutral amino acid transporter 9	Slc38a9
Q8BGR9	Ubiquitin-like domain-containing CTD phosphatase 1	Ublcp1
Q9EQ28	DNA polymerase delta subunit 3	Pold3
Q8BH79	Anoctamin-10	Ano10
Q8BHF7	CDP-diacylglycerol--glycerol-3-phosphate 3-phosphatidyltransferase, mitochondrial	Pgs1
Q8BJ03	Cytochrome c oxidase assembly protein COX15 homolog	Cox15
Q8BP48	Methionine aminopeptidase 1	Metap1
Q9CTN4	Rho-related BTB domain-containing protein 3	Rhobtb3
Q8BVG4	Dipeptidyl peptidase 9	Dpp9
Q8BYL4	Tyrosine--tRNA ligase, mitochondrial	Yars2
Q8C052	Microtubule-associated protein 1S	Map1s
Q8C0L8	Conserved oligomeric Golgi complex subunit 5	Cog5
Q8C263	Spindle and kinetochore-associated protein 3	Ska3
Q8C3Y4	Kinetochore-associated protein 1	Kntc1

Q8C9W3	A disintegrin and metalloproteinase with thrombospondin motifs 2	Adamts2
Q8CD10	EF-hand domain-containing family member A1	Efha1
Q8CD92	Tetratricopeptide repeat protein 27	Ttc27
Q8CDA1	Phosphatidylinositide phosphatase SAC2	Inpp5f
Q8CE46	Pseudouridylate synthase 7 homolog-like protein	Pus7l
Q8CEC6	Peptidylprolyl isomerase domain and WD repeat-containing protein 1	Ppwd1
Q8CI03	FLYWCH-type zinc finger-containing protein 1	Flywch1
Q8CI61	BAG family molecular chaperone regulator 4	Bag4
Q8CIL4	Uncharacterized protein C1orf131 homolog	
Q8CJ27	Abnormal spindle-like microcephaly-associated protein homolog	Aspm
Q8CJG0	Protein argonaute-2	Eif2c2
Q8K202	DNA-directed RNA polymerase I subunit RPA49	Polr1e
Q8K248	4-hydroxyphenylpyruvate dioxygenase-like protein	Hpdl
Q8K4P0	pre-mRNA 3 end processing protein WDR33	Wdr33
Q8R000	Organic solute transporter subunit alpha	Osta
Q8R080	G2 and S phase-expressed protein 1	Gtse1
Q8R0A0	General transcription factor IIF subunit 2	Gtf2f2
Q8R293	Vomeronasal type-1 receptor	Vmn1r73
Q8R322	Nucleoporin GLE1	Gle1
Q8R3H7	Heparan sulfate 2-O-sulfotransferase 1	Hs2st1
Q8R3K3	Pentatricopeptide repeat-containing protein 2	Ptcd2
Q8R3N6	THO complex subunit 1	Thoc1
Q8R480	Nuclear pore complex protein Nup85	Nup85
Q8R4H4	Carboxypeptidase A5	Cpa5
Q8R5A6	TBC1 domain family member 22A	Tbc1d22a
Q8VCV2	Protein NDRG3	Ndrg3
Q8VDK1	Nitrilase homolog 1	Nit1
Q8VED5	Keratin, type II cytoskeletal 79	Krt79
Q8VGW6	Olfactory receptor	Olfr124
Q8VHZ7	U3 small nucleolar ribonucleoprotein protein IMP4	Imp4
Q8VI47	Canalicular multispecific organic anion transporter 1	Abcc2
Q8VIH1	Homeobox protein NOBOX	Nobox
Q91UZ5	Inositol monophosphatase 2	Impa2
Q91WG2	Rab GTPase-binding effector protein 2	Rabep2
Q91XL3	UDP-glucuronic acid decarboxylase 1	Uxs1
Q920G5	Olfactory receptor	Olfr713
Q99J10	Cytoplasmic tRNA 2-thiolation protein 1	Ctu1
Q99L85	Dermal papilla-derived protein 6 homolog	Derp6
Q99LB2	Dehydrogenase/reductase SDR family member 4	Dhrs4
Q99NI3	General transcription factor II-I repeat domain-containing protein 2	Gtf2ird2
Q99P31	Hsp70-binding protein 1	Hspbp1
Q99PL6	UBX domain-containing protein 6	Ubxn6
Q9CQA6	Coiled-coil-helix-coiled-coil-helix domain-containing protein 1	Chchd1
Q9CQT7	Desumoylating isopeptidase 1	Pppde2
Q9CWE0	Protein FAM54B	Fam54b
Q9CZH7	Matrix-remodeling-associated protein 7	Mxra7
Q9D0N7	Chromatin assembly factor 1 subunit B	Chaf1b

Q9D1M4	Eukaryotic translation elongation factor 1 epsilon-1	Eef1e1
Q9D2F1	PRAME family member 12	Pramef12
Q9D2H8	Fibronectin type III domain-containing protein 8	Fndc8
Q9D2R8	28S ribosomal protein S33, mitochondrial	Mrps33
Q9D6X6	Serine protease 23	Prss23
Q9D771	Transmembrane protein 206	Tmem206
Q9D7A8	Armadillo repeat-containing protein 1	Armc1
Q9D9S2	Transmembrane protein 225	Tmem225
Q9DC33	High mobility group protein 20A	Hmg20a
Q9DC70	NADH dehydrogenase [ubiquinone] iron-sulfur protein 7, mitochondrial	Ndufs7
Q9DCC4	Pyrroline-5-carboxylate reductase 3	Pycrl
Q9EP97	Sentrin-specific protease 3	Senp3
Q9EPQ8	Transcription factor 20	Tcf20
Q9EQG9	Collagen type IV alpha-3-binding protein	Col4a3bp
Q9ER38	Torsin-3A	Tor3a
Q9ER81	Torsin-1A-interacting protein 2, isoform IFRG15	Tor1aip2
Q9ESN1	Double C2-like domain-containing protein gamma	Doc2g
Q9JKS4	LIM domain-binding protein 3	Ldb3
Q9JL35	High mobility group nucleosome-binding domain-containing protein 5	Hmgn5
Q9QWV9	Cyclin-T1	Ccnt1
Q9QXV1	Chromobox protein homolog 8	Cbx8
Q9QXW0	F-box/LRR-repeat protein 6	Fbxl6
Q9QYC1	Pecanex-like protein 1	Pcnx
Q9QYX7	Protein piccolo	Pclo
Q9QZI8	Serine incorporator 1	Serinc1
Q9WTP7	GTP:AMP phosphotransferase, mitochondrial	Ak3
Q9WUK4	Replication factor C subunit 2	Rfc2
Q9WUN2	Serine/threonine-protein kinase TBK1	Tbk1
Q9WVL0	Maleylacetoacetate isomerase	Gstz1
Q9Z2U2	Zinc finger protein 292	Zfp292
S4R1D6	H-2 class I histocompatibility antigen, TLA(B) alpha chain	H2-T3
Z4YKJ7	Excitatory amino acid transporter 5	Slc1a7

Table III List of the proteins expressed only in OligoGM1 cells in the comparison OligoGM1 vs CONTROL N2a cells

Majority protein IDs	Protein names	Gene names
A0A075B607	T cell receptor alpha variable 14-3	Trav14d-2
A0A075B6D5	Immunoglobulin kappa chain variable 19-93	Igkv19-93
A0A087WQ44	Snf2-related CREBBP activator protein	Srcap
Q5DTW7	Uncharacterized protein C12orf35 homolog	Kiaa1551
A0A087WR36	Vomeronasal type-1 receptor	Vmn1r90
A0A1L1SQU7	FAT atypical cadherin 1	Fat1
A0A0A0MQA5	Tubulin alpha-4A chain	Tuba4a
A0A0A0MQD1	Tudor domain-containing protein 7	Tdrd7
P09925	Surfeit locus protein 1	Surf1
Q3TRR0	Microtubule-associated protein 9	Map9
A0A0A6YVV8	Muscleblind-like protein 1	Mbnl1
Q8VBV3	Exosome complex component RRP4	Exosc2
Q99PJ1	Protocadherin-15	Pcdh15
A0A0A6YX01	Protocadherin beta-6	Pcdhb6
A0A0A6YY47	Neural cell adhesion molecule 1	Ncam1
A0A0B4J1I7	Immunoglobulin kappa variable 4-68	Igkv4-68
A0A0G2JDF6	RIKEN cDNA I830077J02 gene	I830077J02Rik
A0A0G2JDW9	Immunoglobulin kappa variable 4-62	Igkv4-62
A0A0G2JE49	Paired immunoglobulin-like type 2 receptor alpha	Pilra
A0A0G2JEK2	Cysteine-rich protein 1	Crip1
A0A0G2JEY5	Immunoglobulin kappa variable 4-81	
Q920L5	Elongation of very long chain fatty acids protein 6	Elovl6
A0A0G2JFV8	Polypyrimidine tract-binding protein 2	Ptbp2
Q5DW34	Histone-lysine N-methyltransferase EHMT1	Ehmt1
A0A0J9YUP9	Transcription factor 4	Tcf4
Q91YP3	Putative deoxyribose-phosphate aldolase	Dera
A0A1N9MDH9	Probable G-protein coupled receptor 19	Gpr19
B2RPV6	Multimerin-1	Mmrn1
Q91WI7	Integrin-alpha FG-GAP repeat-containing protein 2	Itfg2
F6V2U0	Type I inositol 3,4-bisphosphate 4-phosphatase	Inpp4a
A0A0R4J0U3	Period circadian protein homolog 2	Per2
A0A0R4J187	X-ray repair cross-complementing protein 6	Xrcc6
A0A0R4J1N7	Ankyrin-1	Ank1
Q8R2Q6	Tectonic-3	Tctn3
A0A0U1RPA0	Pleckstrin homology domain-containing family A member 7	Plekha7
Q8BMB0	Protein EMSY	Emsy
A0A0U1RQ37	Ubiquitin-conjugating enzyme E2 S	Ube2s
Q9Z179	SHC SH2 domain-binding protein 1	Shcbp1
E9QPQ8	39S ribosomal protein L48, mitochondrial	Mrpl48
Q571B6	WASP homolog-associated protein with actin, membranes and microtubules	Whamm
Q9CQG6	Transmembrane protein 147	Tmem147

A0A140LIT2	7-dehydrocholesterol reductase	Dhcr7
A0A140LIW7	Ankyrin-repeat and fibronectin type III domain-containing 1	Ankfn1
Q8CG73	Protein fantom	Rpgrip1l
Q9D6T0	Nitric oxide synthase-interacting protein	Nosip
Q8JZK6	MCG22896, isoform CRA_b	Trim61
A0A1D5RLF0	F-box and WD-40 domain protein 27	Fbxw27
A0A1D5RLL4	Transformation/transcription domain-associated protein	Trrap
F8WI85	Leucine-rich repeat-containing protein 36	Lrrc36
A0A1L1SRQ8	Olfactory receptor	Olfr251
Q91XE8	Transmembrane protein 205	Tmem205
Q9DCU6	39S ribosomal protein L4, mitochondrial	Mrpl4
A0A1L1SSZ5	Olfactory receptor 1248	Olfr1248
Q9D083	Kinetochore protein Spc24	Spc24
A0A1W2P7F9	MCG50764, isoform CRA_a	Btg1
Q9CWV7	Zinc finger SWIM domain-containing protein 1	Zswim1
Q3TB48	Transmembrane protein 104	Tmem104
Q9D7S1	Transmembrane protein 54	Tmem54
A2A9L3	Serine/threonine-protein kinase PDIK1L	Pdik1l
E9Q0J2	Ral GTPase-activating protein subunit beta	Ralgapb
A2ACM0	Regulatory-associated protein of mTOR	Rptor
Q9ER67	Maged2 protein	Maged2
A2AI92	Predicted gene 9112	Gm9112
Q6P2L7	Protein CASC4	Casc4
A2AT37	UPF2 regulator of nonsense transcripts homolog (Yeast)	Upf2
Q80YN3	Breast carcinoma-amplified sequence 1 homolog	Bcas1
A2AX52	Collagen alpha-4(VI) chain	Col6a4
A4QPD3	Proto-oncogene c-Rel	Rel
A6PWV5	AT-rich interactive domain-containing protein 3C	Arid3c
A7TZG3	Selection and upkeep of intraepithelial T-cells protein 9	Skint9
B2RQS1	Striatin-3	Strn3
B2RXC1	Trafficking protein particle complex subunit 11	Trappc11
Q8BXQ2	GPI transamidase component PIG-T	Pigt
J3QQ27	Coiled-coil domain-containing 191	2610015P09Rik
B8QI34	Liprin-alpha-2	Ppfia2
D3YU71	3 beta-hydroxysteroid dehydrogenase type 7	Hsd3b7
D3YUK0	Predicted gene 3259	Gm3259
D3YUP1	Histone-arginine methyltransferase CARM1	Carm1
Q3TQR0	Post-GPI attachment to proteins factor 2	Pgap2
Q99LG0	Ubiquitin carboxyl-terminal hydrolase 16	Usp16
P30285	Cyclin-dependent kinase 4	Cdk4
Q9JIY5	Serine protease HTRA2, mitochondrial	Htra2
D3YYL7	40S ribosomal protein S29	Gm10126
D3YZP5	Ras-related protein Rab-3A	Rab3a
D3Z0X5	Pleckstrin homology-like domain family B member 1	Phldb1
Q9DCL8	Protein phosphatase inhibitor 2	Ppp1r2
D3Z3G0	Uncharacterized protein C12orf56 homolog	D930020B18

		Rik
D3Z3S5	Predicted gene 4744	Gm4744
Q9CXK9	RNA-binding protein 33	Rbm33
D3Z6C4	Carbonyl reductase family member 4	Cbr4
Q9D8N6	Protein lin-37 homolog	Lin37
Q9Z120	tRNA (guanine-N(7)-)-methyltransferase	Mettl1
D6RFB0	Adhesion G protein-coupled receptor L3	Lphn3
E9Q2M9	WD repeat and FYVE domain-containing 4	Wdfy4
E9PXJ8	Vomeronasal 2, receptor 90	Vmn2r90
Q8C2B3	Histone deacetylase 7	Hdac7
Q9CQJ6	Density-regulated protein	Denr
E9Q0J5	Kinesin-like protein KIF21A	Kif21a
E9Q0N0	Intersectin-1	Itsn1
E9Q163	X-ray repair cross-complementing protein 6	Xrcc6
E9Q1P2	Olfactory receptor	Olfr288
A7RDN6	Renalase	Rnls
Q9QVP9	Protein-tyrosine kinase 2-beta	Ptk2b
Q8BMQ2	General transcription factor 3C polypeptide 4	Gtf3c4
E9Q5A3	Histone-lysine N-methyltransferase EHMT1	Ehmt1
E9Q622	Protocadherin 11 X-linked	Pcdh11x
Q6ZQE4	Transmembrane protein 194A	Tmem194a
E9Q7Q3	Tropomyosin alpha-3 chain	Tpm3
E9Q876	ATP-binding cassette, sub-family A (ABC1), member 12	Abca12
E9Q912	RAP1, GTP-GDP dissociation stimulator 1	Rap1gds1
E9Q9M1	Cytosolic purine 5-nucleotidase	Nt5c2
E9QAN2	Poly(A) polymerase alpha	Papola
Q6RT24	Centromere-associated protein E	Cenpe
E9QKK4	Glucocorticoid-induced transcript 1 protein	Glcci1
Q70KF4	Cardiomyopathy-associated protein 5	Cmya5
Q8VEH3	ADP-ribosylation factor-like protein 8A	Arl8a
Q3UQN2	FCH domain only protein 2	Fcho2
		4933403O08
F6UK53	MCG62900	Rik
P10493	Nidogen-1	Nid1
F6VWU8	Zinc finger protein 946	Zfp946
Q8BND4	Protein DDX26B	Ddx26b
Q8K3A9	7SK snRNA methylphosphate capping enzyme	Mepce
F8VPP8	Zinc finger CCCH type-containing 7B	Zc3h7b
F8WIN2	AT-rich interactive domain-containing protein 3B	Arid3b
V9GX74	Zinc finger transcription factor Trps1	Trps1
Q9QUS9	Reg III delta	Reg3d
Q3UW64	Bifunctional UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosamine kinase	Gne
H3BJU3	Mitoguardin 2	Fam73b
Q4VGL6	Roquin	Rc3h1
Q549C9	Cellular tumor antigen p53	Trp53
Q9D572	UBX domain-containing protein 11	Ubxn11

J3QMK1	Shugoshin 2B	Sgo2b
J3QNW4	UPF0533 protein C5orf44 homolog	Trappc13
K3W4Q5	Protein FAM186A	FAM186A
O55057	Retinal rod rhodopsin-sensitive cGMP 3,5-cyclic phosphodiesterase subunit delta	Pde6d
O08643	Granzyme M	Gzmm
O35257	Prolactin-6A1	Prl6a1
O35615	Zinc finger protein ZFPM1	Zfpm1
O35654	DNA polymerase delta subunit 2	Pold2
O35943	Frataxin, mitochondrial	Fxn
O54786	DNA fragmentation factor subunit alpha	Dffa
O54988	STE20-like serine/threonine-protein kinase	Slk
O55142	60S ribosomal protein L35a	Rpl35a
O55183	Stanniocalcin-1	Stc1
O88495	Melatonin-related receptor	Gpr50
O88574	Histone deacetylase complex subunit SAP30	Sap30
O88630	Golgi SNAP receptor complex member 1	Gosr1
O89001	Carboxypeptidase D	Cpd
P01325	Insulin-1	Ins1
P01639	Ig kappa chain V-V region MOPC 41	Gm5571
P08508	Low affinity immunoglobulin gamma Fc region receptor III	Fcgr3
P11438	Lysosome-associated membrane glycoprotein 1	Lamp1
P11930	Nucleoside diphosphate-linked moiety X motif 19, mitochondrial	Nudt19
P12367	cAMP-dependent protein kinase type II-alpha regulatory subunit	Prkar2a
P16330	2,3-cyclic-nucleotide 3-phosphodiesterase	Cnp
P16382	Interleukin-4 receptor subunit alpha	Il4r
P19137	Laminin subunit alpha-1	Lama1
P22339	Growth arrest and DNA damage-inducible protein GADD45 beta	Gadd45b
P30549	Substance-K receptor	Tacr2
P34152	Focal adhesion kinase 1	Ptk2
P35546	Proto-oncogene tyrosine-protein kinase receptor Ret	Ret
P45952	Medium-chain specific acyl-CoA dehydrogenase, mitochondrial	Acadm
P46414	Cyclin-dependent kinase inhibitor 1B	Cdkn1b
P48455	Serine/threonine-protein phosphatase 2B catalytic subunit gamma isoform	Ppp3cc
P48542	G protein-activated inward rectifier potassium channel 2	Kcnj6
P54726	UV excision repair protein RAD23 homolog A	Rad23a
P61027	Ras-related protein Rab-10	Rab10
P61458	Pterin-4-alpha-carbinolamine dehydratase	Pcbd1
P61957	Small ubiquitin-related modifier 2	Sumo2
P62071	Ras-related protein R-Ras2	Rras2
P62911	60S ribosomal protein L32	Rpl32
P63166	Small ubiquitin-related modifier 1	Sumo1
P63328	Serine/threonine-protein phosphatase 2B catalytic subunit alpha isoform	Ppp3ca
P70213	Friend virus susceptibility protein 1	Fv1
P70298	Homeobox protein cut-like 2	Cux2

P97489	Transcription factor GATA-5	Gata5
P97496	SWI/SNF complex subunit SMARCC1	Smarcc1
Q02614	SAP30-binding protein	Sap30bp
Q03402	Cysteine-rich secretory protein 3	Crisp3
Q04447	Creatine kinase B-type	Ckb
Q07563	Collagen alpha-1(XVII) chain	Col17a1
Q0V8T7	Contactin-associated protein like 5-3	Cntnap5c
Q0VAV1	Muc6 protein	Muc6
Q8BZH4	Pogo transposable element with ZNF domain	Pogz
Q3TCU5	Tapasin	Tapbp
Q3TDD9	Protein phosphatase 1 regulatory subunit 21	Ppp1r21
Q80W82	Mitogen-activated protein kinase 10	Mapk10
Q3TSN9	BTB/POZ domain-containing protein 3	Btbd3
Q3UDW8	Heparan-alpha-glucosaminide N-acetyltransferase	Hgsnat
Q3UQ17	MCG3834	Zbtb16
Q3UVL4	Protein fat-free homolog	Ffr
Q3UY93	Melanin-concentrating hormone receptor 1	Mchr1
Q3V3G9	Nardilysin, N-arginine dibasic convertase, NRD convertase 1	Nrd1
Q3V3Q4	Pyrin domain-containing protein 3	Pydc3
Q4ZGD9	Nuclear RNA export factor 3	Nxf3
Q569L8	Centromere protein J	Cenpj
Q91YI4	Beta-arrestin-2	Arrb2
A2AH25	Rho GTPase-activating protein 1	Arhgap1
Q5I043	Ubiquitin carboxyl-terminal hydrolase 28	Usp28
Q5SSE9	ATP-binding cassette sub-family A member 13	Abca13
E9Q284	Coilin	Coil
Q5SXG7	Vitelline membrane outer layer protein 1 homolog	Vmo1
Q60707	T-box transcription factor TBX2	Tbx2
Q61687	Transcriptional regulator ATRX	Atrx
Q61781	Keratin, type I cytoskeletal 14	Krt14
Q62048	Astrocytic phosphoprotein PEA-15	Pea15
Q62095	ATP-dependent RNA helicase DDX3Y	Ddx3y
Q62172	RalA-binding protein 1	Ralbp1
Q640M1	U3 small nucleolar RNA-associated protein 14 homolog A	Utp14a
Q64505	Cholesterol 7-alpha-monooxygenase	Cyp7a1
Q6DFV1	Condensin-2 complex subunit G2	Ncapg2
Q9WV80	Sorting nexin-1	Snx1
Q6P1G0	HEAT repeat-containing protein 6	Heatr6
Q6P539	Uncharacterized protein C17orf63 homolog	Fam222b
Q6P6J9	Thioredoxin domain-containing protein 15	Txndc15
Q6P8K3	Predicted gene 7978	BC061212
Q6P9R1	ATP-dependent RNA helicase DDX51	Ddx51
Q6PGF7	Exocyst complex component 8	Exoc8
Q6PR54	Telomere-associated protein RIF1	Rif1
Q6UJY2	Sodium/hydrogen exchanger 10	Slc9c1
Q6ZPY5	Zinc finger protein 507	Znf507
Q704Y3	Transient receptor potential cation channel subfamily V member 1	Trpv1

Q7M721	Taste receptor type 2 member 120	Tas2r120
Q7TS04	Olfactory receptor 301	Olfr301
Q80UW8	DNA-directed RNA polymerases I, II, and III subunit RPABC1	Polr2e
Q80WQ2	Protein VAC14 homolog	Vac14
Q8BHE0	Proline-rich protein 11	Prr11
Q8BHG9	CGG triplet repeat-binding protein 1	Cggbp1
Q8BLY2	Probable threonine--tRNA ligase 2, cytoplasmic	Tarsl2
Q8BNY6	Neuronal calcium sensor 1	Ncs1
Q8R2L5	28S ribosomal protein S18c, mitochondrial	Mrps18c
Q8BU03	Periodic tryptophan protein 2 homolog	Pwp2
Q8BUJ9	Low-density lipoprotein receptor-related protein 12	Lrp12
Q8BVT7	RIKEN cDNA 4921511C20 gene	4921511C20Rik
Q8BXZ1	Protein disulfide-isomerase TMX3	Tmx3
Q8BZR0	Probable G-protein coupled receptor 82	Gpr82
Q8C2E4	Pentatricopeptide repeat-containing protein 1	Ptcd1
Q8C5R8	Ribose-phosphate pyrophosphokinase	Prps1l1
Q8C754	Vacuolar protein sorting-associated protein 52 homolog	Vps52
Q8C845	EF-hand domain-containing protein D2	Efhd2
Q8CBF3	Ephrin type-B receptor 1	Ephb1
Q8CCX5	Keratin-like protein KRT222	Krt222
Q8CDK2	Cytosolic carboxypeptidase 2	Agbl2
Q8CF66	UPF0539 protein C7orf59 homolog	Lamtor4
Q8CH09	SURP and G-patch domain-containing protein 2	Sugp2
Q8CIV2	Membralin	ORF61
Q8JZM8	Mucin-4	Muc4
Q8JZR0	Long-chain-fatty-acid--CoA ligase 5	Acsl5
Q8K0Z7	Translational activator of cytochrome c oxidase 1	Taco1
Q8K394	Inactive phospholipase C-like protein 2	Plcl2
Q8K3H0	DCC-interacting protein 13-alpha	Appl1
Q8K3V4	Protein-arginine deiminase type-6	Padi6
Q8N7N5	DDB1- and CUL4-associated factor 8	Dcaf8
Q8R054	Sushi repeat-containing protein SRPX2	Srpx2
Q8R105	Vacuolar protein sorting-associated protein 37C	Vps37c
Q8R2E3	Vomeronasal type-1 receptor	Vmn1r36
Q8R2P1	Ectoderm-neural cortex protein 2	Klhl25
Q8R3F5	Malonyl-CoA-acyl carrier protein transacylase, mitochondrial	Mcat
Q8R420	ATP-binding cassette sub-family A member 3	Abca3
Q8R4Y8	Rotatin	Rttn
Q8VDV8	MIT domain-containing protein 1	Mitd1
Q8VFJ7	Olfactory receptor	Olfr1012
Q8VFV9	Olfactory receptor	Olfr1459
Q8VFZ3	Olfactory receptor	Olfr513
Q8VG32	Olfactory receptor	Olfr1408
Q8VGE3	Olfactory receptor	Olfr160
Q8VGL3	Olfactory receptor	Olfr535
F8WJ23	Hornerin	Hrnr

Q8VHP6	Cadherin-related family member 1	Cdhr1
Q8VHY0	Chondroitin sulfate proteoglycan 4	Cspg4
Q91XC9	Peroxisomal membrane protein PEX16	Pex16
Q91YP0	L-2-hydroxyglutarate dehydrogenase, mitochondrial	L2hgdh
Q91ZP4	MCG3105, isoform CRA_a	Slc5a4b
Q91ZZ3	Beta-synuclein	Sncb
Q921Y2	U3 small nucleolar ribonucleoprotein protein IMP3	Imp3
Q922B1	O-acetyl-ADP-ribose deacetylase MACROD1	Macrod1
Q99L04	Dehydrogenase/reductase SDR family member 1	Dhrs1
Q99LI5	Zinc finger protein 281	Zfp281
Q99LJ0	CTTNBP2 N-terminal-like protein	Cttnbp2nl
Q99MR1	PERQ amino acid-rich with GYF domain-containing protein 1	Gigyf1
Q99MZ3	Carbohydrate-responsive element-binding protein	Mlxipl
Q99N05	Membrane-spanning 4-domains subfamily A member 4D	Ms4a4d
Q99N96	39S ribosomal protein L1, mitochondrial	Mrpl1
Q9CPN9	RIKEN cDNA 2210010C04 gene	2210010C04Rik
Q9CQ54	NADH dehydrogenase [ubiquinone] 1 subunit C2	Ndufc2
Q9CQQ7	ATP synthase subunit b, mitochondrial	Atp5f1
E9PW43	Protein transport protein Sec61 subunit beta	Gm10320
Q9CR02	Translation machinery-associated protein 16	Tma16
Q9CXR1	Dehydrogenase/reductase SDR family member 7	Dhrs7
Q9CY97	RNA polymerase II subunit A C-terminal domain phosphatase SSU72	Ssu72
Q9CZN8	Glutamyl-tRNA(Gln) amidotransferase subunit A, mitochondrial	Qrsl1
Q9CZX5	PIN2/TERF1-interacting telomerase inhibitor 1	Pinx1
Q9D0M5	Dynein light chain 2, cytoplasmic	Dynll2
Q9D1C8	Vacuolar protein sorting-associated protein 28 homolog	Vps28
Q9D1H8	39S ribosomal protein L53, mitochondrial	Mrpl53
Q9D1N9	39S ribosomal protein L21, mitochondrial	Mrpl21
Q9D1Z3	Protein FAM173B	Fam173b
Q9D267	Epididymal-specific lipocalin-9	Lcn9
Q9D3Z8	RIKEN cDNA 4933425L06	4933425L06Rik
Q9D411	Testis-specific serine/threonine-protein kinase 4	Tssk4
Q9D9V3	Ethylmalonyl-CoA decarboxylase	Echdc1
Q9DAT2	MRG-binding protein	Mrgbp
Q9DBE0	Cysteine sulfinic acid decarboxylase	Csad
Q9DBG1	Sterol 26-hydroxylase, mitochondrial	Cyp27a1
Q9DBJ3	Brain-specific angiogenesis inhibitor 1-associated protein 2-like protein 1	Baiap2l1
Q9DCA5	Ribosome biogenesis protein BRX1 homolog	Brix1
Q9DCF9	Translocon-associated protein subunit gamma	Ssr3
Q9DD18	D-tyrosyl-tRNA(Tyr) deacylase 1	Dtd1
Q9EQ06	Estradiol 17-beta-dehydrogenase 11	Hsd17b11
Q9ERN0	Secretory carrier-associated membrane protein 2	Scamp2
Q9ES83	Blood vessel epicardial substance	Bves
Q9JIK9	28S ribosomal protein S34, mitochondrial	Mrps34
Q9JIX0	Enhancer of yellow 2 transcription factor homolog	Eny2

Q9JJ28	Protein flightless-1 homolog	Flii
Q9JJ78	Lymphokine-activated killer T-cell-originated protein kinase	Pbk
Q9JJ94	Sjoegren syndrome nuclear autoantigen 1 homolog	Ssna1
Q9JJT0	RNA 3-terminal phosphate cyclase-like protein	Rcl1
Q9R0H0	Peroxisomal acyl-coenzyme A oxidase 1	Acox1
Q9R0K2	Olfactory receptor	Olfr1264
Q9WTR1	Transient receptor potential cation channel subfamily V member 2	Trpv2
Q9WV54	Acid ceramidase	Asah1
Q9Z262	Claudin-6	Cldn6
Q9Z2X2	26S proteasome non-ATPase regulatory subunit 10	Psmd10
V9GXI9	Striatin-4	Strn4

Table IV Bioinformatic analysis by David of the proteins exclusively expressed in OligoGM1 cells in the comparison OligoGM1 vs CONTROL N2a cells

DAVID analysis p value ≤ 0.05 counts ≥ 2

	Category	Term	Count	p value	Genes
endosome/endocytic trafficking	Annotation Cluster 1	Enrichment Score: 1.9283871903046559			
	UP_KEYWORDS	Endosome	14	7,27E-03	RET, SCAMP2, VAC14, VPS52, SNX1, VPS37C, MITD1, APPL1, EPHB1, LAMP1, CDKN1B, ARL8A, VPS28, RAB10
	GOTERM_CC_DIRECT	GO:0010008~endosome membrane	7	8,49E-03	LAMP1, RET, VAC14, VPS52, SNX1, VPS28, APPL1
	GOTERM_CC_DIRECT	GO:0005768~endosome	15	2,66E-02	RAB3A, RET, SCAMP2, VAC14, VPS52, SNX1, VPS37C, MITD1, APPL1, EPHB1, LAMP1, CDKN1B, ARL8A, RAB10, VPS28
	Annotation Cluster 5	Enrichment Score: 1.2682691071296288			
	SMART	SM00282: LamG	4	1,77E-02	LAMA1, FAT1, CSPG4, CNTNAP5C
	INTERPRO	IPR001791: Laminin G domain	4	4,92E-02	LAMA1, FAT1, CSPG4, CNTNAP5C
	Annotation Cluster 8	Enrichment Score: 1.0091254512435084			
	UP_KEYWORDS	Coated pit	4	2,82E-02	ARRB2, LRP12, ITSN1, FCHO2
ribosome biogenesis and regulation of transcription	Annotation Cluster 2	Enrichment Score: 1.5686242450542756			
	GOTERM_CC_DIRECT	GO:0005840~ribosome	10	1,65E-03	MRPL53, MRPL1, RPL35A, MRPS34, MRPL4, MRPL21, MRPS18C, RPL32, TMA16, DENR
	UP_KEYWORDS	Ribosomal protein	9	6,36E-03	MRPL53, MRPL1, RPL35A, MRPS34, MRPL4, MRPL21, MRPS18C, RPL32, MRPL48
	UP_KEYWORDS	Ribonucleoprotein	10	2,42E-02	MRPL53, MRPL1, RPL35A, IMP3, MRPS34, MRPL4, MRPL21, MRPS18C, RPL32, MRPL48
	GOTERM_CC_DIRECT	GO:0030529~intracellular ribonucleoprotein complex	10	4,28E-02	MRPL53, MRPL1, RPL35A, IMP3, MRPS34, MRPL4, MRPL21, MRPS18C, RPL32, TDRD7
	Annotation Cluster 3	Enrichment Score: 1.561905533986465			
	UP_KEYWORDS	Ribosome biogenesis	5	8,66E-03	RCL1, IMP3, BRIX1, UTP14A, DDX51
	GOTERM_BP_DIRECT	GO:0006364~rRNA processing	6	3,71E-02	RCL1, RPL35A, IMP3, EXOSC2, UTP14A, DDX51
	GOTERM_BP_DIRECT	GO:0042254~ribosome biogenesis	5	4,09E-02	RCL1, IMP3, BRIX1, UTP14A, DDX51

	KEGG_PATHWAY	mmu03008: Ribosome biogenesis in eukaryotes	5	4,31E-02	RCL1, IMP3, NXF3, UTP14A, PWP2
	Annotation Cluster 24	Enrichment Score: 0.586830761089792			
	GOTERM_MF_DIRECT	GO:0001085~RNA polymerase II transcription factor binding	4	4,51E-02	TRP53, GATA5, TRPS1, ZFPM1
	Annotation Cluster 28	Enrichment Score: 0.547197473552621			
	GOTERM_BP_DIRECT	GO:0000122~negative regulation of transcription from RNA polymerase II promoter	19	2,07E-02	TRP53, EHMT1, TBX2, TRPV1, MLXIPL, ZBTB16, MAPK10, SAP30, REL, CGGBP1, PSMD10, TRPS1, PER2, ZFP281, CUX2, ZFPM1, TCF4, MEPCE, …
cell cycle	Annotation Cluster 9	Enrichment Score: 0.9624582129177877			
	GOTERM_BP_DIRECT	GO:0051301~cell division	11	4,77E-02	SPC24, POGZ, NCAPG2, ARL8A, CENPE, MITD1, MAP9, USP16, CDK4, CENPJ, UBE2S
	Annotation Cluster 11	Enrichment Score: 0.8865229236222075			
	GOTERM_BP_DIRECT	GO:0051726~regulation of cell cycle	6	2,39E-02	TRP53, PRR11, PER2, USP16, CDK4, GADD45B
mitochondrion, fatty acid metab.	Annotation Cluster 4	Enrichment Score: 1.2776717793217818			
	UP_KEYWORDS	Mitochondrion	23	2,91E-02	TRP53, MRPL53, MRPL1, MRPS34, MRPL4, ACADM, MCAT, ATP5F1, NDUFC2, CBR4, MAPK10, TACO1, QRSL1, MRPL21, MRPS18C, HTRA2
	Annotation Cluster 6	Enrichment Score: 1.0521935305885302			
	KEGG_PATHWAY	mmu01212: Fatty acid metabolism	5	8,61E-03	ACOX1, ACADM, MCAT, ELOVL6, ACSL5
	UP_KEYWORDS	Fatty acid metabolism	6	2,63E-02	ACOX1, ACADM, MCAT, CBR4, ELOVL6, ACSL5
	GOTERM_BP_DIRECT	GO:0006631~fatty acid metabolic process	7	2,67E-02	ACOX1, ACADM, MCAT, PER2, CBR4, ELOVL6, ACSL5
	KEGG_PATHWAY	mmu03320: PPAR signaling pathway	5	3,84E-02	ACOX1, ACADM, CYP27A1, CYP7A1, ACSL5
	Annotation Cluster 12	Enrichment Score: 0.8062496294708845			
	INTERPRO	IPR026082:ABC transporter A, ABCA	3	2,11E-02	ABCA3, ABCA13, ABCA12

cell adhesion	Annotation Cluster 7	Enrichment Score: 1.0256913451821696			
	INTERPRO	IPR002126: Cadherin	6	3,29E-02	RET, PCDHB6, PCDH11X, FAT1, CDHR1, PCDH15
	INTERPRO	IPR015919: Cadherin-like	6	3,48E-02	RET, PCDHB6, PCDH11X, FAT1, CDHR1, PCDH15
	Annotation Cluster 21	Enrichment Score: 0.6276318581765036			
	GOTERM_BP_DIRECT	GO:0030155~regulation of cell adhesion	4	2,30E-02	LAMA1, RET, PTK2, PTK2B

various	Annotation Cluster 18	Enrichment Score: 0.6872907708951744			
	UP_KEYWORDS	Nucleotide-binding	36	1,25E-02	RAB3A, GNE, TRPV1, XRCC6, ABCA3, EPHB1, CKB, QRSL1, PRKAR2A, PTK2, SLK, PTK2B, NT5C2, DDX3Y, 4933425L06RIK, KIF21A, …
	UP_SEQ_FEATURE	active site:Proton acceptor	19	1,51E-02	HSD17B11, ACOX1, RET, ACADM, HSD3B7, NRD1, CNP, CBR4, PBK, MAPK10, CDK4, EPHB1, DHRS7, DHRS1, PDIK1L, PTK2, SLK, PTK2B, TSSK4
	UP_KEYWORDS	ATP-binding	27	4,62E-02	TRPV1, GNE, XRCC6, ABCA3, EPHB1, CKB, QRSL1, PTK2, SLK, PTK2B, DDX3Y, KIF21A, TARSL2, ABCA13, ABCA12, ACSL5, RET, ATRX, …
	GOTERM_MF_DIRECT	GO:0000166~nucleotide binding	38	4,64E-02	RAB3A, RBM33, GNE, TRPV1, XRCC6, ABCA3, EPHB1, CKB, QRSL1, PRKAR2A, PTK2, SLK, PTK2B, NT5C2, DDX3Y, 4933425L06RIK, PTBP2, …
	Annotation Cluster 25	Enrichment Score: 0.5793190221422585			
	UP_SEQ_FEATURE	binding site:S-adenosyl-L-methionine; via carbonyl oxygen	3	4,30E-02	METTL1, CARM1, MEPCE

Table V Bioinformatic analysis by IPA of the proteins exclusively expressed in OligoGM1 cells in the comparison OligoGM1 vs CONTROL N2a cells

Molecular and Cellular Functions	p value	Molecules
Cell Death and Survival	1.24E-02-2.37E-06	86
Cell Morphology	1.16E-02-1.18E-05	54
Cellular Assembly and Organization	1.28E-02-1.18E-05	76
DNA Replication, Recombination, and Repair	1.18E-02-2.09E-05	28
Carbohydrate Metabolism	1.15E-02-3.99E-05	12

Physiological System Development and Function	p value	Molecules
Organismal Survival	3.56E-03-1.29E-05	75
Nervous System Development and Function	1.28E-02-4.21E-05	54
Tissue Morphology	1.24E-02-1.62E-04	33
Connective Tissue Development and Function	1.15E-02-1.89E-04	25
Embryonic Development	1.25E-02-3.14E-04	60

Table VI Bioinformatic analysis by IPA of the proteins differentially expressed in the comparison OligoGM1 vs CONTROL N2a cells

TOP BIOFUNCTION

Molecular and Cellular Functions		
	p value	Molecules
Cell Death and Survival	1.24E-02-2.37E-06	86
Cell Morphology	1.16E-02-1.18E-05	54
Cellular Assembly and Organization	1.28E-02-1.18E-05	76
DNA Replication, Recombination, and Repair	1.18E-02-2.09E-05	28
Carbohydrate Metabolism	1.15E-02-3.99E-05	12

Physiological System Development and Function		
	p value	Molecules
Organismal Survival	3.56E-03-1.29E-05	75
Nervous System Development and Function	1.28E-02-4.21E-05	54
Tissue Morphology	1.24E-02-1.62E-04	33
Connective Tissue Development and Function	1.15E-02-1.89E-04	25
Embryonic Development	1.25E-02-3.14E-04	60

Figure and Table Legends

Fig. 1 Proteomic characterization of OligoGM1 treated cells and control cells. **a)** Scatter plot of the proteome of N2a cells treated (OligoGM1) and untreated (CTRL) with OligoGM1. Pearson correlation coefficient R=0.94. **b)** Venn diagram showing the proteins identified in at least 2/3 replicates. Proteins common to both data sets were considered for differential expression.

Fig. 2 OligoGM1 neuroprotective effect versus MPTP treatment. **a)** Morphological analysis of N2a cells. 1, control; 2, OligoGM1; 3, MPTP; 4, OligoGM1 + MPTP; 5, TrkA-Inh + OligoGM1 + MPTP; 6, RA; 7, RA + MPTP. Details on the sequential treatments are reported in Methods section. Following 24 h treatment with MPTP cells were evaluated at 200x magnification with contrast phase microscopy. Images are representative of five independent experiments (*n*=5). **b)** Viability of cells under different treatments. The number of living (□) and dead (■) cells was determined by Trypan blue exclusion assay. Values represent the percentage mean of living (trypan blue negative) and dead (trypan blue positive) cells for three different experiments (*$p < 0.01$; 1way ANOVA, followed by Bonferroni's post-hoc, *n*=3).

Fig. 3 OligoGM1 effect on ROS production induced by MPTP. **a)** Representative fluorescence images showing mitochondrial superoxide (MitoSOX red fluorescence), nuclei (Hoechst blue fluorescence) and overlay of the two signals (merge) with 400x magnification. Images are representative of three independent experiments (*n*=3). **b**) MitoSOX Red signal was quantified using Image J software in percentage pixel intensity normalized on cell number. Data are the mean of three independent experiments and are expressed as fold increase over CTRL of mean ± SEM from three different experiments (*$p < 0.01$; 1way ANOVA, followed by Bonferroni's post, *n*=3).

Fig. 4 Diagram of the proposed mechanism for GM1 mediated neuroprotection in N2a cells. On the cell plasma membrane, ganglioside GM1 induces the TrkA activation by autophosphorylation. GM1 enhances TrkA activity stabilizing the TrkA-NGF complex by a direct interaction with its oligosaccharide chain. GM1 promotes the phosphorylation of Tyr490 triggering the differentiation, mitochondrial neuroprotective and calcium signaling. This image is updated from [11]. GM1 representation is according to [76]. ERK, extracellular signal regulated protein kinases 1 and 2; Grb2, growth factor receptor-bound protein 2; Gab1, Grb2-associated binder-1; HrtA2, serine protease; PINK1, PTEN (phosphatase and tension homologue)-induced putative kinase 1; RAS, GTP-binding protein; RAF, serine/threonine kinase; SHC, transforming protein 1; SOS, son of sevenless.

Table I – List of the proteins statistically differentially expressed in OligoGM1 vs Control N2a cells. Three biological replicates, each one replicated twice, were carried out for treated and control cells. Only proteins present and quantified in at least 2 out of 3 biological repeats were considered as positively identified in a sample and used for statistical analyses. A t-test (p-value ≤ 0.01) was carried out to identify proteins differentially expressed among the two different conditions.

Table II – List of the proteins only expressed in Control cells in the comparison OligoGM1 vs Control N2a cells. Statistical analyses were performed by the Perseus software module (version 1.5.5.3, www.biochem.mpg.de/mann/tools/).

Table III – List of the proteins expressed only in OligoGM1 cells in the comparison OligoGM1 vs Control N2a cells. Statistical analyses were performed by the Perseus software module (version 1.5.5.3, www.biochem.mpg.de/mann/tools/).

Table IV – Bioinformatic analysis by David of the proteins exclusively expressed in OligoGM1 cells in the comparison OligoGM1 vs Control N2a cells. Bioinformatic analyses were carried out by DAVID software (release 6.7) to cluster enriched annotation groups of Molecular Function, Biological Processes, Pathways and Networks within the set of identified proteins. Functional grouping was based on p value ≤ 0.05 and at least two counts.

Table V – Bioinformatic analysis by IPA of the proteins exclusively expressed in OligoGM1 cells in the comparison OligoGM1 vs Control N2a cells. Protein were analyzed by IPA (QIAGEN Redwood City, www.qiagen.com/ingenuity). Functional grouping was based on p value ≤ 0.05 and at least two counts.

Table VI – Bioinformatic analysis by IPA of the proteins differentially expressed in the comparison OligoGM1 vs Control N2a cells. Proteins were considered to be differentially expressed if they were present only in treated or control samples or showed significant t-test difference (p value ≤ 0.01). The proteins were analyzed by IPA (QIAGEN Redwood City, www.qiagen.com/ingenuity). Functional grouping was based on p value ≤ 0.05 and at least two counts.

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