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Investigation of anti-IgLON5-induced neurodegenerative changes in human neurons

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Abstract

Background: Anti-IgLON5-mediated autoimmune encephalitis is a progressive neurological disorder, associated with antibodies against a neuronal cell adhesion molecule, IgLON5. In human postmortem brain tissue neurodegeneration and accumulation of phosphorylated-Tau (p-Tau) has been found. Whether IgLON5 antibodies induce this neurodegeneration or the neurodegenerative changes provoke an immune response remains to be elucidated.

Aim: To investigate the pathological effect of anti-IgLON5 antibodies on human neurons.

Methods: Human neural stem cells were differentiated for 14 days, and exposed from day 9-14 to either 1) purified serum IgG from a patient with confirmed anti-IgLON5 antibodies; 2) purified serum IgG from a healthy person or 3) standard culture conditions (negative control). After fixation, cells were immunostained for neuronal (β-tubulin III) and astroglial (Glial Fibrillary Acidic Protein) markers and counterstained with DAPI (nuclei). Other cultures were immunostained for p-Tau. Random images were obtained and analyzed using ImageJ software. Cell counting and a neurite outgrowth assay were performed using the Cell Counter, Analyze Particles and NeuronJ ImageJ applications.

Results: The proportion of cells with a degenerative appearance (blebbing) was significantly higher for neurons exposed to anti-IgLON5 IgG when compared to IgG treated or untreated neurons. There was also a significantly higher proportion of neurons with fragmented neurites in anti-IgLON5 IgG exposed cultures compared to controls (IgG treated or untreated). Furthermore, the relative content of cells with p-Tau accumulation was higher for cultures exposed to anti-IgLON5 IgG compared to the control groups. No differences in neurite morphology and neuronal or astroglial cell death were detected.

Conclusions: Pathological anti-IgLON5 antibodies induce neurodegenerative changes in human neurons along with increased accumulation of p-Tau. The findings support the hypothesis that these antibodies are causative for the neurodegenerative changes found in patients with anti-IgLON5-mediated autoimmune encephalitis.

Introduction

Anti-IgLON5 disease is a neurological disorder that was first described in 2014 (Sabater et al 2014). It is thought to be caused by an autoimmune reaction towards cells of the central nervous system, involving a component of neurodegeneration (Sabater et al 2014; Gelpi et al 2016). It was initially recognized as a sleep disorder with non-rapid eye movement (NREM) and REM parasomnia, breathing dysfunction, gait instability and bulbar symptoms, with serum and cerebrospinal fluid (CSF) antibodies against a neuronal adhesion molecule, named IgLON5 (Sabater et al 2014). The disease is now known to be more clinically heterogeneous in regards to presenting symptomatology and in its response to immunotherapy (Gaig et al 2017; Nissen and Blaabjerg 2019). The disease onset is insidious, progressing slowly over several years and has a fatal outcome if left untreated (Gaig et al 2017). Post-mortem analyses have revealed neuronal deposits of hyperphosphorylated Tau protein in the hypothalamus and the brainstem tegmentum (Gelpi et al 2016). This deposition, consisting of three repeat (3R) and four repeat (4R) isoforms, has a different distribution when compared to other neurodegenerative conditions and therefore the disorder has been described as a novel tauopathy (Sabater et al 2014; Braak and Braak 1991; Braak et al 1992). Men and women are equally affected, with a median age at diagnosis in late adulthood (62 years, ranging from 45 to 79 years). Despite the neurodegenerative features in post-mortem brain tissue, a strong association with Human Leukocyte Antigen (HLA) haplotypes, DRB1*10:01 – DQB1*05:01, indicates an immunological association (Gaig et al 2019). The clinical manifestation of anti-IgLON5 disease has been divided into four main phenotypes: 1) predominant sleep disorder, 2) a bulbar syndrome, 3) Progressive Supranuclear Palsy (PSP)-like syndrome and 4) cognitive impairment that may associate chorea (Gaig et al 2017). In addition, isolated ataxic gait and nervous system hyperexcitability syndrome has been included to the spectrum (Gaig and Compta 2019). The sleep disorder is described as characteristic, with altered non-REM (NREM) sleep initiation, vocalizations and finalistic movements (NREM parasomnia) (Sabater et al 2014; Gaig et al 2018). REM behavior disorder (RBD) and sleep-breathing difficulties such as obstructive sleep apnea (OSA) and stridor, can present early but also late in the course of disease (Gaig et al 2018). Brain imaging usually shows normal or nonspecific findings on cerebral magnetic resonance imaging (MRI) with patterns of brainstem/cerebellar atrophy or hyperintensities in regions most affected by phosphorylated Tau (p-Tau) deposition (hypothalamus, brainstem, hippocampus, cerebellum) (Nissen and Blaabjerg 2019; Gaig and Compta 2019). CSF analysis can show normal to mildly increased protein levels and slight pleocytosis in the range of 5 – 10 leukocytes/ µL (Nissen and Blaabjerg 2019). More commonly, low to moderate intrathecal protein elevation presents without pleocytosis or oligoclonal bands (OCB) (Blinder and Lewerenz 2019). Therefore, the diagnosis relies heavily on the clinical presentation and the presence of anti-IgLON5 antibodies in serum and CSF. The precise functions of the antibody targeted protein are unknown. The IgLON protein family belongs to the Immunoglobulin (Ig) protein superfamily and is characterized by the presence of three Ig-like domains, attaching to the membrane through a GPI-anchor (Karagogeos 2003; Sabater et al 2016). They are expressed in synapses and engage with each other forming homo- or heterodimers

(Ranaivoson et al 2019). IgLON protein dimerization involves the first Ig-domain and they seem to take part in processes such as neuronal outgrowth and synaptogenesis (Ranaivoson et al 2019; Hashimoto et al 2009). Autoantibodies directed towards IgLON5 bind specifically to an epitope in the second Ig-domain (Sabater et al 2016). This binding is thought to be pathogenic and believed to be dependent on the IgG subclass. The IgG1 fraction has been shown to cause irreversible internalization of IgLON5 clusters while IgG4 fraction may affect protein-protein interaction (Sabater et al 2016). As such, the predominant subclass may be affecting the clinical features of disease. The pathological properties of anti-IgLON5 antibodies were recently described in cultured hippocampal rat neurons with increased neurodegenerative features such as neuronal blebbing and fragmentation. However, in these cells no Tau deposition was seen (Landa et al 2020). To further elucidate this intriguing link between neuroinflammation and neurodegeneration, we studied the effect of patient anti-IgLON5 IgG on neurons derived from human neural stem cells.

Materials and Methods

Ethical approval

The study was approved by the ethical committee at the Region of Southern Denmark (approval nr. S-20170134). Written informed consent was provided. All use of human stem cells was performed in accordance with the Danish national regulations, the ethical guidelines issued by the International Society for Stem Cell Research (ISSCR).

Isolation of anti-IgLON5 IgG fractions

Serum samples containing both anti-IgLON5 IgG1 and IgG4 were obtained from a patient with a positive cell-based assay (CBA) and clinically verified anti-IgLON5 disease. A commercial CBA kit using human embryonic kidney (HEK) 293 cells transfected with the IgLON5 complementary DNA and fixed with 1% formalin (Euroimmun, Lübeck, Germany) was used to verify presence of specific IgG anti-IgLON5 antibodies. Sera were analyzed in a 1:10 dilution and titrated to endpoint. Presence of other encephalitis-causing autoantibodies where excluded by CBA and indirect immunofluorescence on primate cerebellum. Control human IgG was provided from sera of healthy donors and tested using CBA to exclude presence of encephalitis-causing autoantibodies.
The IgG fraction of patient and control serum was purified using a Protein A gel (MabSelect™, Amersham Biosciences) and then concentrated and transferred into sterile Phosphate-Buffered Saline (PBS) by centrifugation at room temperature (3000G), using protein concentrator tubes (VIVASPIN 20, Sartorius). Both purified IgG fractions from patient and controls were re-tested on CBAs and the anti-IgLON5 IgG concentration was determined (titer 1:100). The antibody eluate was stored at -80°C until usage.

Human stem cell-derived neurons

Human neural stem cells (hNS1 – kindly provided by Dr. Alberto Martínez-Serrano) were propagated and proliferated in a Poly-L-Lysine (10 ug/mL, Sigma-Aldrich) coated T-25 flask (Nunc Easyflask). Proliferation was maintained using HNSC.100 media (composed of DMEM/F12, glucose, Hepes, AlbuMAX-I, N2 supplement, and NAAA (Gibco)), with addition of Pen/strep and growth factors EGF 1.0 µl/ml and bFGF 0.8 µl/ml (R&D systems). Media was changed every third day. Once an adequate confluence of 80- 90% was reached, the cells were trypsinized using trypsin/EDTA and a single cell suspension was plated in 24-well plates (24 Well Cell Culture Cluster, Costar) with Poly-L-Lysine coated 15mm coverslips (Hounisen, Denmark) with a seeding density of 50.000 cells/cm^2. Plates of monolayer cultures were incubated at 37°C: in a 5% CO2, 95% humidified air environment and differentiated for 14 days in HNSC.100 media without growth factors. A 50% media change was conducted every third day.

Exposure of human neurons to anti-IgLON5 IgG

Cells were divided into three treatment groups: One third received 2% purified anti-IgLON5 IgG, one third 2% control human IgG and one third regular media as non-exposed negative control. Cells were exposed to IgG from differentiation day 9 to 14. At day 14 cells to be used for immunocytochemistry were fixed in 4% paraformaldehyde (PFA) for 20 minutes and washed with D-PBS and kept at 4°C until processing.

IgLON5 live cell staining

At day 14 of differentiation, selected cultures were washed once in Dulbecco's Modified Eagle's Medium (DMEM/F12) followed by incubation with 2% primary antibody (patient anti-IgLON5 IgG) in HNSC.100 media for 30 minutes. After a second wash cells were incubated with secondary antibody (Alexa 488, goat-anti human IgG, 1:500) for 30 min. A final washing step was performed and cultures were fixed in 4% PFA for 10 minutes, washed in D-PBS and mounted with ProLong Diamond Antifade (Life Technologies, P36970).

I**mmunocytochemistry**

Immunocytochemistry was performed after fixation. Cells were permeabilized with 0.1% saponin wash buffer (WB) and blocked with a 5 % goat serum solution. Cells were then incubated with antibodies against neuronal or glial cytoskeletal associated proteins overnight at 4 °C: Anti-β-Tubulin III (β- Tub III) (Sigma, T8660, mouse monoclonal 1:2000) and anti-Glial Fibrillary Acidic Protein (GFAP)(DAKO, Z0334, rabbit polyclonal 1:4000); anti-p-Tau (Cell Signaling, E7D3E, Rabbit monoclonal 1:100). The following day, cells were washed in WB and incubated with secondary antibodies dependent on the primary antibody (anti-mouse Alexa 555 (Thermo Fisher Scientific, goat 1: 500) and anti-rabbit Alexa 488 (Thermo Fisher Scientific , goat 1: 500)) for 1 hr. at room temperature (RT) followed by washing steps. A counterstain was added as 10µM dihydrochloride hydrate 4´,6-Diamidino-2-phenylindole (DAPI) solution for 10 minutes at RT. Cells were washed and mounted with ProLong Diamond Antifade.

Image acquisition and analysis

Images were obtained using a fluorescent microscope (Olympus BX54), with a 60x objective for the live cell staining and a 10x objective for other immunocytochemical stainings. Five random images were collected from each 15 mm stained coverslip.

Image analysis was performed using ImageJ imaging software. For the IgLON5 live cell staining unspecific background staining was removed before the images were made binary. An automatic particle analysis gave the total number of IgLON5 clusters per image. For the total nuclear count, a Gaussian blur filter was applied with sigma (radius) of 6.00. Images were made binary and a watershed application segregated the individual corpuscular elements. Particle analysis gave the final nuclear count. During cell counting, a cell counter plugin tool was used to assist manual

counting of the number of β-Tub III and GFAP positive cells. Cells that co-stained with both β-Tub III and GFAP were counted as GFAP expressing cells to avoid overlap. Similarly, pyknotic and/or fragmented nuclei were counted to yield specific neuronal and glial cell death. Neuronal and glial content, along with neuronal and glial death fractions were normalized using total nuclear count (for neuronal and glial content) and total neuronal or glial count (for neuronal or glial death fractions). For analysis of neurodegeneration, neurons that contained multiple or localized swelling (blebbing), separate from a distally located growth cone, or fragmentation of the neuronal process, was counted. The percentage of neurons containing degenerative changes was calculated. In neurite outgrowth (NOG) analysis, β-Tub III pictures were uploaded in NeuronJ plugin application as 8-bit images. Color balance was optimized and standardized for all images. Tracings were added for assessment of the number and length of neuronal neurites, expressed as pixel length.
For p-Tau analysis, the number of neurons containing accumulations of p-Tau protein in processes was counted with the *Cell counter plugin* tool along with *particle analyzer* for a total nuclear count.

Statistical analysis

Statistical analysis was performed using GraphPad PRISM, version 6.0. IgLON5 cluster quantification data were analyzed using a two-tailed unpaired t-test. All other data were analyzed using a One-way, non-parametric ANOVA by application of Tukey's multiple comparisons test. Values in bar charts are presented as mean ± SEM. All experiments were run in triplicate.

Results

IgLON5 cluster internalization

Live cell staining for IgLON5 visualized IgLON5 clusters in the cultures (n=30, Figure 1, A-F). Quantification of IgLON5 clusters revealed a significant reduction in IgLON5 clusters in the anti-IgLON5 IgG treated cultures compared to cultures treated with control IgG (Figure 1, G, average of 54 and 104 clusters per image respectively, n=30; $P = 0.0021$).

Analysis of cell viability

We investigated the cell viability in cultures by counting different cell types, along with dead cells. The content of neuronal (Figure 2, A-C, G) and glial cells (Figure 2. D-F, I) was similar in all groups. There was no difference in neuronal or glial cell death (Figure 2, H, J).

Analysis of neurite outgrowth

We analyzed neurite outgrowth in cultures exposed to anti-IglON5 IgG, control IgG or unexposed controls. We found no differences between exposure groups in the length or number of primary or secondary neurites or neurite endpoints (data not shown).

Early neurodegenerative changes – neuronal blebbing and fragmentation

To investigate possible early neurodegenerative changes, we obtained random images (anti-IgLON5 IgG: n = 99; control IgG: n = 100; unexposed: control n = 94) in wells stained with β-Tub III and analyzed the number neurons with axonal blebbing and/or a fragmented appearance (Figure 3, A-B). The average percentage of cells with blebbing processes was significantly higher in neurons exposed to patient anti-IgLON5 IgG (14,27%), when compared to neurons exposed to control IgG (9,03 %: $P = 0.0096$) or unexposed controls (9,26 %: $P = 0.00346$) (Figure 3, A-B, D). In addition, there was more fragmentation of neuronal processes between groups (IgLON5 IgG: 8,15 %; Control IgG: 4,3 %; Neg. Control: 4,19 % $P = < 0,0002$) (Figure 3, B, E).

Accumulation of P-Tau protein

Since the analysis of unhealthy neuronal processes indicated differences in degenerative changes, more pronounced in the group receiving anti-IgLON5, we investigated the content of p-Tau protein in the neuronal processes of hNS1 cells in the three exposure groups. When looking at random culture areas (anti-IgLON5 IgG: n = 95; control IgG: n = 95; unexposed: control n = 90), we found that the mean percentage of cells with p-Tau accumulation was higher in the group exposed to anti-IgLON5 IgG compared to control IgG (8,63 % vs 5,57 %; $P = < 0.0001$) and unexposed controls (8,63 % vs 6,07 %; $P = < 0.0001$) (Figure 4, A-G).

Discussion

In this study, we describe the neuropathological effects induced by anti-IgLON5 antibodies providing the first demonstration of these effects on human stem cell-derived cells.

We found no difference in total, specific neuronal or glial cell death in this acute exposure model, which may relate to the clinical observation that the disease course is protracted, progressing insidiously and does not lead to the typical acute prodromal symptomatic phases seen in other autoimmune encephalitides.

Neuronal cells with an unhealthy morphology were recognized in all cultures. We found that cultures exposed to anti-IgLON5 IgG displayed a significantly higher percentage of neurons with degenerative changes such as axonal blebbing and fragmentation. This is in line with recent observations in cultured rat hippocampal neurons and further provides evidence that exposure to anti-IgLON5 antibodies causes neurodegeneration (Landa et al 2020). These cells were moreover found to have smaller/pyknotic nuclei and were seen through a spectrum where they eventually released their neuronal processes. It is tempting to speculate that these cells were destined to die and if given more time would add to the total and specific neuronal cell death in our anti-IgLON5 exposed cell cultures. Axonal blebbing has been used to describe an unhealthy neuronal morphology in primary hippocampal and cortical rat cultures (Loehfelm et al 2020) and as marker for chronic degenerative changes caused by neuroinflammation in *in vivo* murine models (Chakravarty et al 2020). To our knowledge, this is the first demonstration of antibody mediated degenerative changes in cultures of human neurons.

A characteristic feature of neurodegeneration is the accumulation of p-Tau protein in aggregates (Saha 2019). Patients with anti-IgLON5 disease are found to have accumulation of p-Tau protein in post-mortem examinations (Gelpi et al 2016). Whether IgLON5 antibodies induce this neurodegenerative feature or these neurodegenerative changes provoke an immune response, leading to secondary antibody formation, is still debated. In our human cell model, the content of neuronal p-Tau was significantly higher when exposed to anti-IgLON5 IgG. This finding supports the hypothesis that neuroinflammation precedes neurodegeneration and that anti-IgLON5 antibodies contribute to the neurodegenerative changes found in patients with anti-IgLON5 disease.

A neurite outgrowth assay is commonly used to assess neuronal health and the neurotoxicity of substances at concentrations that may not affect cellular viability (Krug et al 2013). Further, it is used to investigate the growth of neurites in models of neurodegenerative diseases (Bogetofte et al 2019). As mentioned above, the IgLON protein family are intercellular adhesion molecules with a recognized role in synaptogenesis and neuronal outgrowth (Ranaivoson et al 2019; Hashimoto et al 2009; Pimenta et al 1995), but very little is known about the role of the latest discovered member, IgLON5. Despite an unhealthy morphology and increased p-Tau deposition, we did not find any differences in neurite number, length and branchpoints in cultures treated with anti-IgLON5 IgG compared to control and unexposed cells. Whether this is due to a very short antibody exposure period or because IgLON5 does not regulate neurite outgrowth remains unclear. If IgLON5 is indeed

not involved in neurite outgrowth, it would be sensible to investigate the effect of IgLON5 internalization on synaptogenesis and synapse conservation.

In this experiment, hNS1 cells were exposed for only a five-day period. Patients with anti-IgLON5 disease are exposed for much longer time periods. A recent case report indicates, that the typical brainstem tauopathy seen in this disease may be a late event (Erro et al 2020). Experiments with human neurons exposed over longer time periods are needed to further characterize p-Tau accumulation, due to anti-IgLON5 antibodies, in the *in vitro* setting.

Anti-IgLON5 disease is rare with an estimated incidence of 1/150 000 with only 2 cases documented in Denmark (Nissen and Blaabjerg 2019). One patient has died and we were limited to use serum samples from only one single patient. Our study is limited due to the use of serum samples from one patient with a combination of mainly IgG4 and less IgG1. However, it reflects the combined effect of antibodies in our patient and further studies of monoclonal antibody subtypes could help to dissect the cellular mechanisms leading to accumulation of p-Tau and morphological changes, seen in our neural cultures.

Conclusions

This study raises several important implications: 1) Patient anti-IgLON5 antibodies did not cause rapid general or specific cell death, which may help to explain the insidious onset and protracted course of disease; 2) These patient autoantibodies did not cause prompt changes in neurite outgrowth and did not indicate acute neurotoxicity, after 5 days of antibody exposure; 3) Cultures of human neurons exposed to patient anti-IgLON5 IgG lead to a significant increase in neuronal degenerative changes in the form of axonal blebbing and fragmentation and; 4) demonstrated the accumulation of p-Tau for the first time in human neurons. This supports the hypothesis that anti-IgLON5 antibodies lead to neurodegeneration and correlates with the neuropathological findings in patients postmortem.

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Conflicts of interest.

The authors have nothing to disclose.

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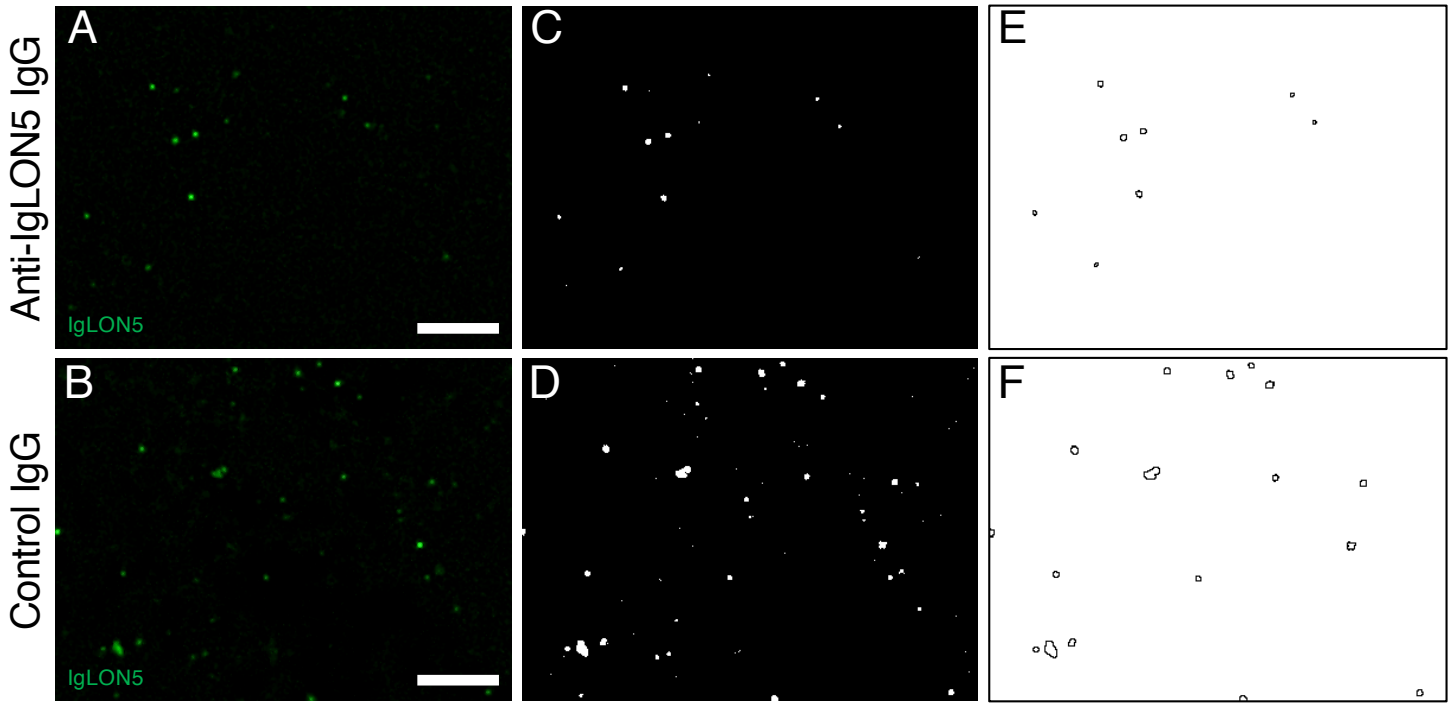


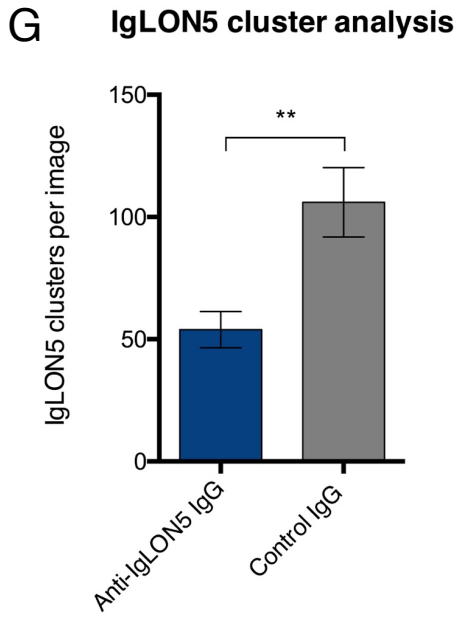
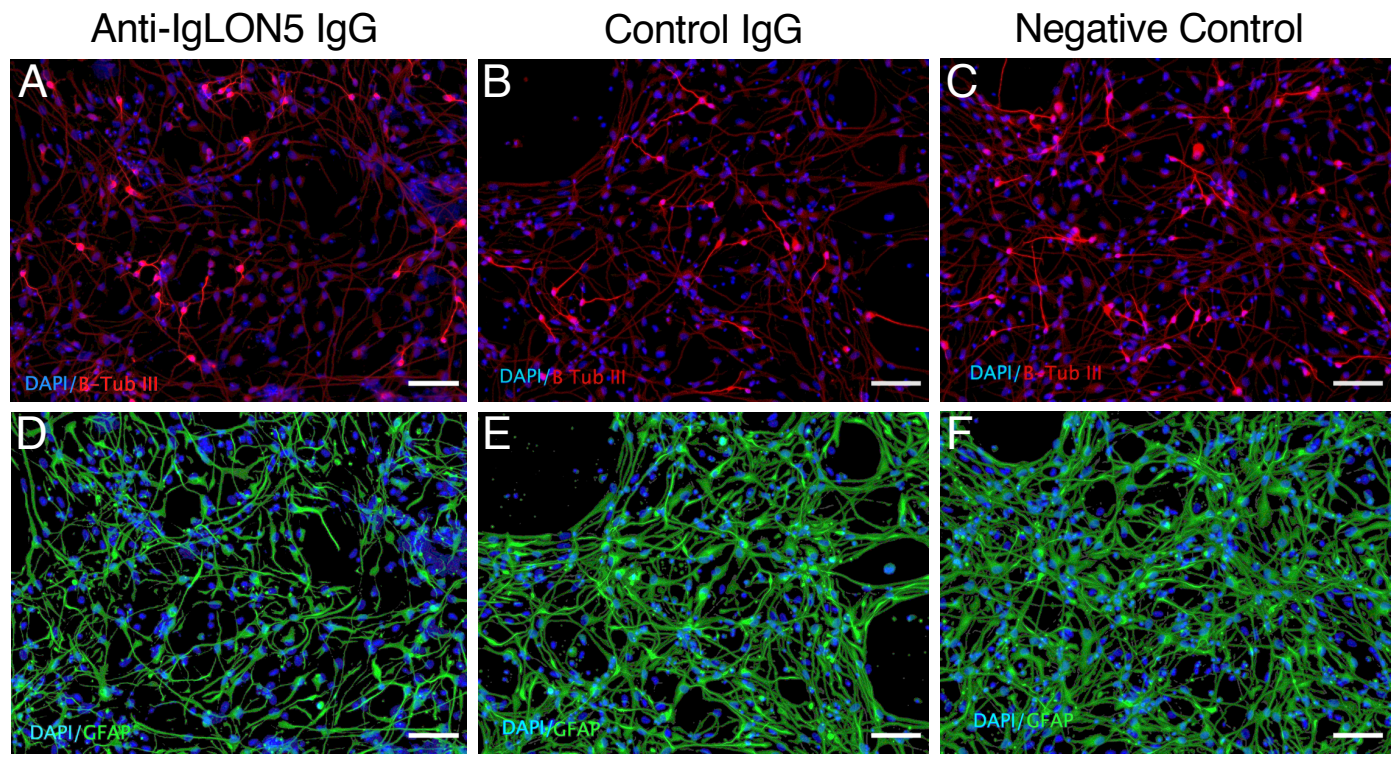
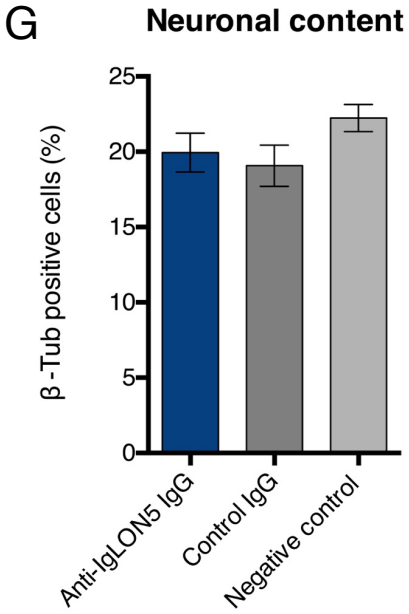
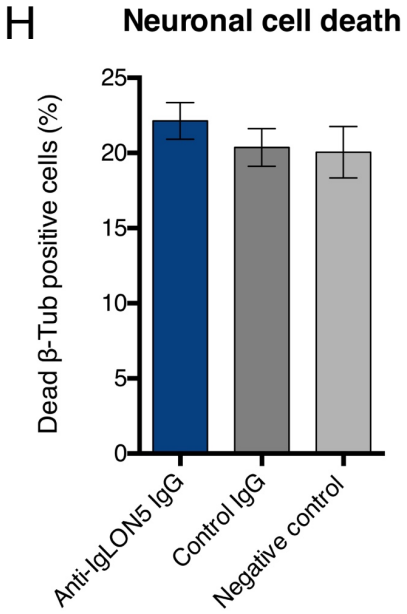
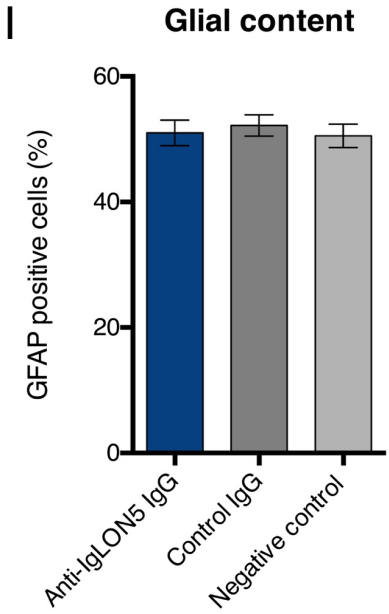


Figure 1. ***Patient antibodies decrease the amount of IgLON5 clusters.*** Indirect immunofluorescent staining of live hNS1 cells for IgLON5. Original images (A, B) binary versions (C, D) and an particle quantification analysis performed with the program ImageJ (E, F). The amount of IgLON5-positive clusters was significantly lower in the group treated with patient antibodies (mean: anti-IgLON5 IgG 54 clusters per image; Control IgG 106 clusters per image. n = 30) (G). Statistical analysis: two-tailed unpaired t-test (**: $p < 0.01$). Scale bars A-F: 5 µm









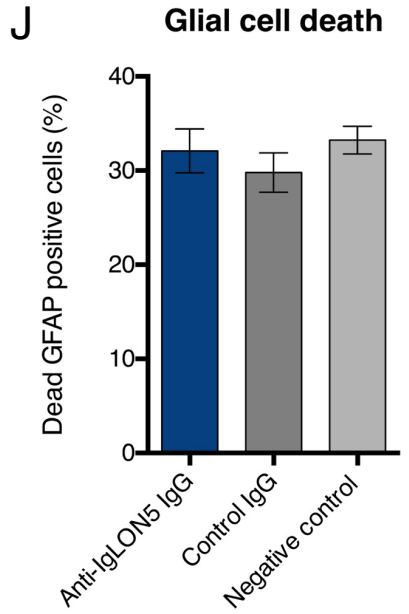


Figure 2. ***Patient and control serum did not cause differences in specific neuronal and glial cell content or specific death.***
Immunocytochemistry of hNS1 cells. Neuronal cell content visualized by β-Tub III (A- C) and glial cell content by GFAP (D-F). Neuronal content (mean: Anti-IgLON5 IgG 19.9%; Control IgG 19.1%; Negative control 22.2%; *n* = 30) and specific neuronal death (mean: Anti-IgLON5 IgG 22.1%; Control IgG 20.4%; Negative control 20.0%) was not different between groups (G, H). Neither, was there any difference in content of glia cells (mean: Anti-IgLON5 IgG 51.0%; Control IgG 52.2%; Negative control 50.5%; *n* = 30) and specific glial death (mean: Anti-IgLON5 IgG 32.1%; Control IgG 29.8%; Negative control 33.2%; *n* = 30)(I, J). Statistical analysis: One-way ANOVA followed by Tukey´s multiple comparisons test.
Scale bars A-E: 200 µm

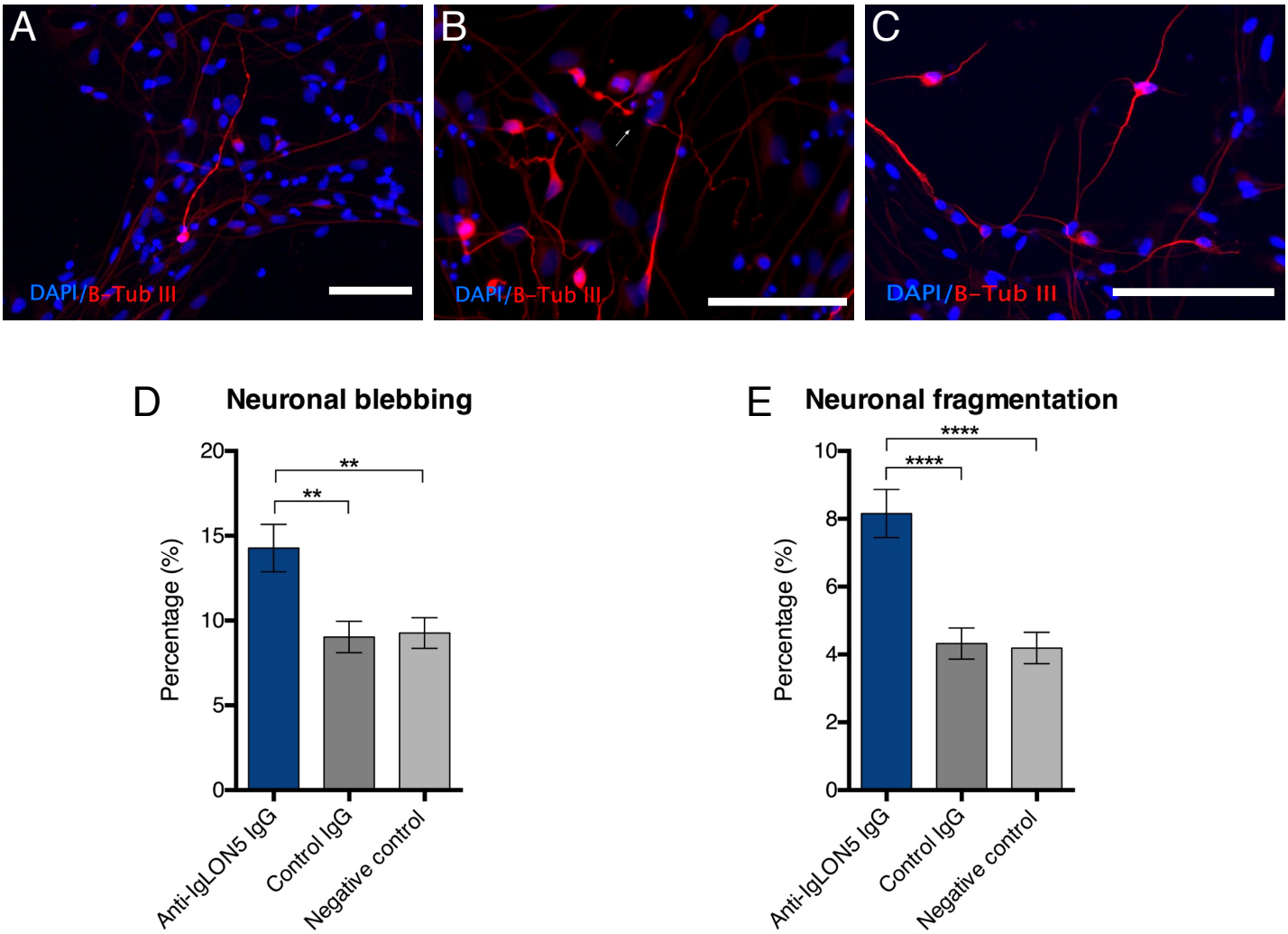
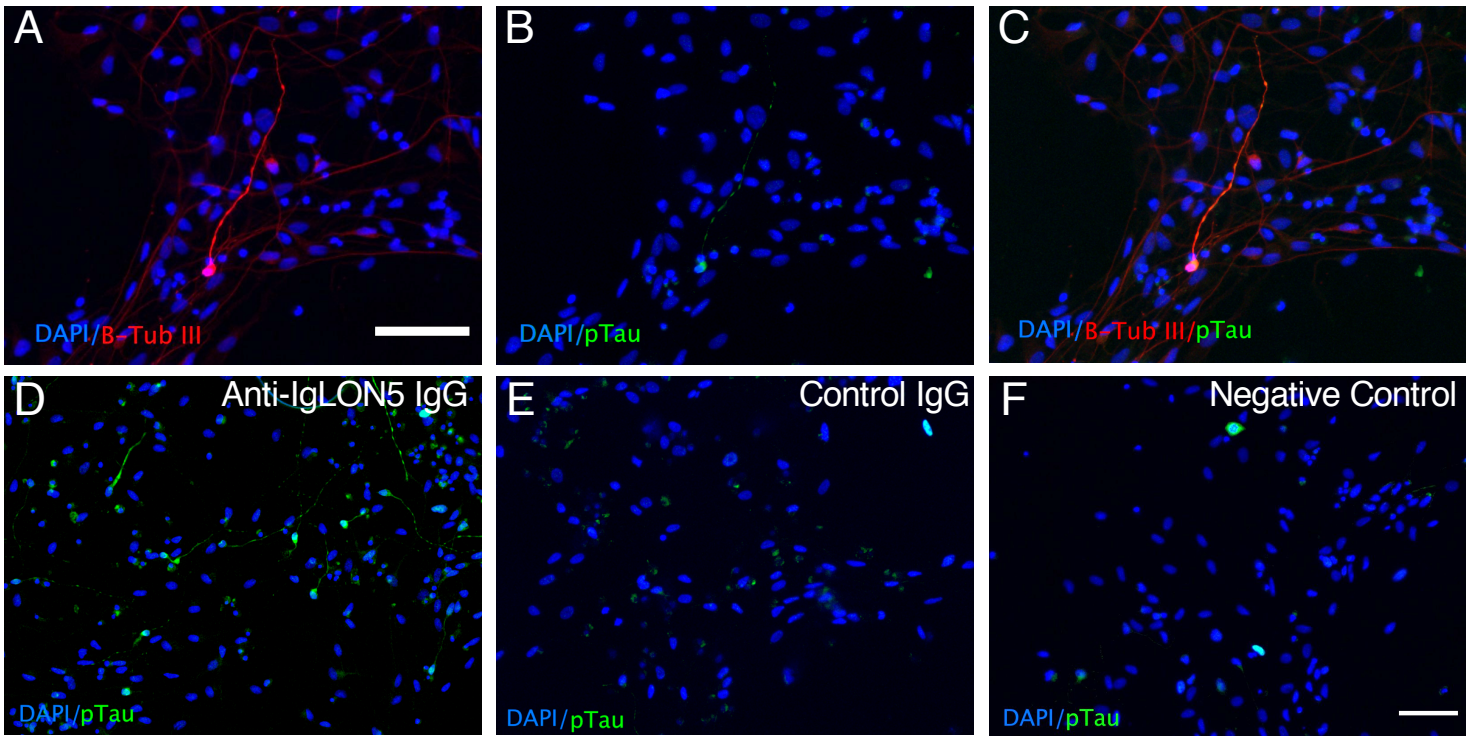


Figure 3. ***Patient antibodies caused neuronal degenerative changes.***
When evaluating degenerative changes in hNS1 cells after 5 days of antibody exposure, neuronal cell processes were stained with β-Tub III and inspected. There was a significant difference in neuronal blebbing (A, B, D) when comparing anti-IgLON5 IgG to control IgG (14.2% vs 9.0%) and negative control (14.4% vs 9.3%). In addition, there was a significant difference in fragmented processes (B arrow, D) between anti-IgLON5 IgG (8.2%), Control IgG (4.3%) and negative control (4.2%). Representative image of neurons with normal appearance (C). Statistical analysis: One-way ANOVA followed by Tukey´s multiple comparisons test.
(**: $p < 0.01$; ****: $p < 0.0001$) .
(anti- IgLON5 $n = 99$; Control IgG $n = 100$; Neg. Control $n = 94$)
Scale bars: A, 50 µm; B-C 100 µm



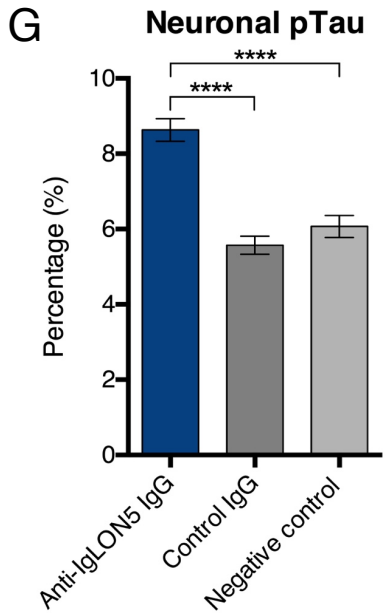


Figure 4. ***Exposure to patient antibodies causes increased accumulation of phosphorylated-Tau protein.*** Analysis of pTau in cultures. Images (A,B, C) depict a neuron with a blebbing process and accumulations of phosphorylated Tau. After 5 days exposure, the anti-IgLON5 IgG group showed a drastic increase in percentage of neurons with pTau (8.6%; $n = 95$) when compared to group exposed to control IgG (5.6%; $n = 95$) and negative control (6.1%; $n = 90$)(D,E,F,G). Statistical analysis: One-way ANOVA followed by Tukey´s multiple comparisons test.
(****: $p < 0.0001$)
Scale bars: 50 µm