Immunoablation of cells expressing the NG2 chondroitin sulphate proteoglycan

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ABSTRACT

Expression of the transmembrane NG2 chondroitin sulphate proteoglycan (CSPG) defines a distinct population of NG2-glia. NG2-glia serve as a regenerative pool of oligodendrocyte precursor cells (OPCs) in the adult CNS, which is important for demyelinating diseases such as Multiple Sclerosis, and are a major component of the glial scar that inhibits axon regeneration after CNS injury. In addition, NG2-glia form unique neuron-glial synapses with unresolved functions. However, to date it has proven difficult to study the importance of NG2-glia in any of these functions using conventional transgenic NG2 'knock-out' mice. To overcome this, we aimed to determine whether NG2-glia can be targeted using an immunotoxin approach. We demonstrate that incubation in primary anti-NG2 antibody in combination with secondary saporin-conjugated antibody selectively kills NG2-expressing cells *in vitro*. In addition, we provide evidence that the same protocol induces the loss of NG2-glia without affecting astrocyte or neuronal numbers in cerebellar brain slices from postnatal mice. This study shows that targeting the NG2 CSPG with immunotoxins is an effective and selective means for killing NG2-glia, which has important implications for studying the functions of these enigmatic cells both in the normal CNS, and in demyelination and degeneration.

Introduction

The NG2 chondroitin sulphate proteoglycan (CSPG) is expressed in both the developing and adult central nervous system (CNS), where it marks a specific population of glial cells (Butt et al., 2002). A primary function of NG2-glia is to act as oligodendrocyte progenitor cells (OPCs), generating oligodendrocytes during development and in the adult (Rivers et al., 2008; Etxeberria et al., 2010). Most significantly, NG2-glia have been shown to form functional synapses with neurons in hippocampal, cortical, and cerebellar slices (Bergles et al., 2000; Lin et al., 2005; De Biase et al., 2010; Etxeberria et al., 2010; Ge et al., 2006; Kukley et al., 2008; Vélez-Fort et al., 2010), and with nodes of Ranvier in white matter (Butt et al., 1999; Kukley et al., 2007, Ziskin et al., 2007; Hamilton et al., 20010). In addition, NG2-glia may also perform a structural function at synapses (Stegmuller et al., 2003). A key aspect of NG2-glia is that they are highly reactive cells and respond to most forms of CNS injury by a rapid increase in proliferation and glial scar formation (Levine et al., 2001; Tan et al., 2005). Notably, NG2 is considered one of the main molecules in the glial scar that contributes to the failure of axon regeneration in the adult CNS (Sandvig et al., 2004; Tan et al., 2005). Neurotransmission may serve as a regulatory mechanism for controlling the developmental progression of NG2-glia (Etxeberria et al., 2010), and a further possibility is that it regulates an injury response in NG2-glia (Hamilton et al., 2010). Hence, regulating NG2-glia has potential significance in normal brain development and function, and in demyelination and degeneration.

The functions of NG2-glia are in part directly related to the properties of the CSPG molecule (Stegmuller et al., 2003; Levine et al., 2001; Nishiyama et al,

1996b; Stallcup, 2002). However, a line of NG2-null mice has been produced, and these did not manifest gross phenotypic differences (Grako et al., 1999). Subsequent studies have indicated subtle changes in oligodendrocytes, with a reduction in the numbers and proliferation of OPCs in developing white matter and a delay in myelination in the NG2 null mouse (Kucharova and Stallcup, 2010). However, there are no apparent effects on hippocampal neurogenesis (Thallmair et al., 2006), or on axonal regeneration in the CNS and PNS (Hossain-Ibrahim et al., 2007). These genetic studies did not result in the loss of NG2-glia, and so we wished to develop a system that specifically ablates these cells. Immunotoxins have been used successfully for eliminating particular neural populations on the basis of their expression of specific antigens (Wiley, 1996). Here, we demonstrate that as a membrane spanning molecule, the NG2 CSPG is an amenable target for selective immunoablation of NG2-expressing glia *in vitro*, and that this secondary immunoablation protocol achieves selective destruction of NG2-glia *in situ* in cerebellar slice organotypic cultures.

Methods

Animals and Tissue

Experiments were performed on cell lines (C6, CTX-TNA2 and NG108-15) and on cerebellar slices from GFAP-EGFP transgenic mouse line (Nolte et al., 2001), in which the expression of enhanced green fluorescent protein (EGFP) was under the control of the human glial fibrillary acidic protein (GFAP) promoter [line *TgN(GFAPEGFP) GFEC-FKi*] (gift from Frank Kirchhoff, University of Goettingen). Mice aged postnatal day (P)12 were killed humanely in accordance with the Home Office Animals (Scientific) Act 1986 (UK), and brains were prepared fresh for cerebellar slice cultures or immersion fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for immunohistochemistry.

Cell Lines and Culture Conditions

C6, CTX-TNA2 and NG108-15 cell lines were obtained from European Collection of Cell Cultures as rat glioma line, transfected astrocytes and mouse neuroblastoma × rat glioma hybrid, respectively. The F98 cell line was obtained from ATCC-LGC Promochem as a rat glioma line. CTX-TNA2 and F98 cells were maintained in culture with D-MEM medium (Invitrogen) supplemented with 5% foetal bovine serum (FBS) (Invitrogen); C6 cells with F12 medium (Invitrogen) supplemented with 5% FBS and L-Glutamine (Invitrogen) to 2 mM final concentration; NG108-15 cells with D-MEM supplemented with 5% FBS, L-Glutamine to 2 mM final concentration, 0.6% HAT supplement (Sigma) and with a solution of Penicillin-Streptomycin (Penicillin G sodium 10000 U/ml, Streptomycin sulphate 1000 µg/ml) diluted 1:100. Cells were cultured in an incubator at 37°C with 5%CO_2 and 95% air; 75 cm^3 flasks were used for maintenance of the cell lines, and 24-well plates were used for experimental

purposes. For passaging steps, subconfluent cultures were digested with 0.25% trypsin/EDTA solution (Invitrogen) for 3-5 min, and then resuspended in culture media in a 1:10/1:20 ratio.

Cerebellar slice cultures

Cerebellar brain slice cultures were prepared as previously described, with some modifications (Leoni et al., 2009). Brains from P11-13 mice were rapidly removed and placed in chilled Gey's balanced salt solution (GBSS, Invitrogen), supplemented with 25 mM D-glucose. Cerebellar slices were cut at 300 µm using a McIlwain tissue chopper and slices were transferred into 6-well culture plates with 1 ml serum-free culture medium per well. Medium was composed of Neurobasal™ media (Invitrogen) supplemented with B27 supplement (Invitrogen), with 1 mM glutamine (Invitrogen) and with 25 mM D-glucose (Sigma), and a solution of Penicillin-Streptomycin (Penicillin G sodium 10000 U/ml, Streptomycin sulphate 1000 µg/ml, Invitrogen) diluted 1:500. Slices were cultured at 37°C in 5% CO_2 and 95% air for up to 4 days in vitro (DIV), and the culture medium was replaced every two days. Slices were regularly examined and discarded if contamination occurred. At the end of the experiments, slices were immersion fixed in 4% PFA for 30 min at room temperature (RT) for immunohistochemistry.

Immunoablation

Cell cultures were exposed to different concentrations of mouse primary anti-NG2 antibody (monoclonal from Upstate), alone or in combination with different concentrations of saporin-conjugated anti-mouse antibody (Mab-ZAP, Advanced Targeting Systems). Both Mab-ZAP and primary antibody were diluted in the

culture medium and the secondary conjugate was added first to avoid internalization of the primary antibody before complexing with the immunotoxin. Control groups included an untreated group (no primary antibody and no secondary immunotoxin), and single-treatment groups, where either one of the two antibodies was presented on its own at different concentrations. For each treatment, representative pictures of the cells were acquired using a digital camera adapted on a Nikon Eclipse TS100 microscope, while cell death was measured using the MTT assay as described below.

Cerebellar slices were treated with two different immunotoxin combinations: (A) NG2/Mab-ZAP, consisting of mouse monoclonal anti-NG2 primary antibody (from Upstate, 1:1000), with an anti-mouse secondary antibody linked with the toxin saporin (Mab-ZAP; from ATS, 5 µg/ml); or (B) NG2/Rab-ZAP, consisting of rabbit polyclonal anti-NG2 primary antibody (from Chemicon, 1:1000) and anti-rabbit secondary immunotoxin, Rab-ZAP (from ATS, 5 µg/ml); control slices (untreated group) were cultured in culture media. Slices were treated with the anti-NG2-saporin immuno-cocktail for between 2 to 4 days, and sections were analysed by immunohistochemistry (see below).

Western blot and RT-PCR

Cell cultures were examine for NG2 expression using western blot and RT-PCR. For western blot, proteins were extracted from the cell cultures with a lysis buffer composed of 50 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, 2 mM EGTA, 0.5 mM PMSF (phenylmethanesulphonylfluoride), 10 µg/ml leupeptin, 10 µg/ml antipain, 1 µg/ml chimostatin, 1 µg/ml pepstatinA, 5 mM $Na_2P_2O_7$, 1 mM Na_3VO_4 and 50 mM NaF. Lysates were centrifuged at 4000 rpm

and 4ºC and supernatants were used for the Stanford colorimetric determination of the total protein content of each sample. Bovine serum albumin was used to make a standard curve and BioRad reagent was used for the protein assay. The remaining supernatants were mixed with 5X Laemmli buffer (312 mM Tris, 0.1 g/ml SDS, 50% glycerol, 25% mercaptoethanol, 0.125 mg/ml bromophenol blue) to prepare the loading samples for the SDS-PAGE. 50 µg of total proteins were run on a 8% polyacrylamide gel and blotted onto nitrocellulose membranes by semi-dry electroblotting at 1.5 mA/cm^2. Membranes were then blocked for 1 h at RT in a blocking solution consisting of 4% skimmed milk powder dissolved in TRIS-buffered saline (TBS) (150 mM NaCl, 10 mM Tris, pH 7.4) added of 0.05% Tween-20 (TTBS). Membranes were next incubated overnight at 4ºC with a polyclonal anti-NG2 antibody (Chemicon), diluted 1:2000 in blocking solution. The membranes were subsequently washed three times for 5 min in TTBS and then incubated for 1 h at RT in peroxidase-conjugated goat anti-rabbit IgG (1:1000, Sigma). After three further washes for 5 min in TTBS, the membranes were finally processed with the ECL™ Western blot detection system (GE Healthcare) and the Hyperfilm-ECL (GE Healthcare) for the detection of the protein bands. In some cases, clarified homogenates were digested with chondroitinase ABC in order to cleave the GAG lateral chain from the native NG2 proteoglycan. 150 µl aliquots of homogenates were added of 5 mU of chondroitinase ABC (Seikagaku America Inc.) and digested for 30 min at 37°C. Digested samples were then added to 5X Laemmli buffer as described above, and stored at -20°C until use.

For RT-PCR, cell cultures were washed twice in PBS and incubated for 5 min with 1 ml RNABee (Biogenesis) per well. Each solution was transferred to Eppendorf

tubes and 100 µl chloroform was added to each tube, shaken by hand and left on ice for 5 min; samples were then centrifuged at 15000 rpm for 15 min at 4°C. The upper colourless phase containing the RNA was transferred in new Eppendorf tubes containing 600 µl isopropanol and centrifuged at 15000 rpm for 15 min at 4°C. The supernatant was discarded and 1 ml of 75% ethanol was added to wash the pellet. Samples were then centrifuged at 8000 rpm for 8 min at 4°C, ethanol was discarded, and pellets were vacuum dried for 5 min. The resulting RNA was resuspended in 35 µl RNA resuspension buffer (10 nM Tris pH 8.0, 10 mM NaCl, 1 mM EDTA) at 65°C in water bath. 5 µl aliquots of each sample were used to determine the RNA concentration and purity by determining the A_{260}/A_{280} ratio with a GeneQuant spectrophotometer. The remaining RNA samples were stored at -70°C until use for the reverse transcriptase reaction. 1 µg RNA of each sample was reverse transcribed into complementary DNA (cDNA) in a final reaction volume of 20 µl containing 4 µl $MgCl_2$ (25 mM), 2 µl 10X Buffer, 2 µl dNTPs (10 mM), 0.5 µl/ml oligo $d(T)_{15}$, 0.5 µl RNase Inhibitor and 0.25 µl of M-MLV Reverse Transcriptase, RNase H minus (all reagents were from Promega). The reaction was carried out at 42°C for 1 h, followed by 5 min at 95°C. Negative controls for the PCR reactions were carried out as described above, but omitting the Reverse Transcriptase and the RNase Inhibitor. cDNA samples were stored at -20°C until use. The PCR was carried out in a final reaction volume of 25 µl containing 2.5 µl of cDNA, 1.5 µl $MgCl_2$ (25 mM), 2.25 µl 10X Buffer, 1 µl of each forward and reverse primers (both 2.5 pmol/µl, synthesised by Sigma), 0.125 µl dNTPs (10 mM) and 0.625 U of Taq DNA polymerase. All reagents were from Promega, except for the primers that were synthesised by Sigma as follows: 5'-CCTCAGAGCCCTATCTCCACGTAGC-3' and 5'-CATCACCAAGTAGCCAGCGTTCG-3' (accession number NM139001). The PCR

reactions were performed in a thermal cycler (GeneAmp 9700, Applied Biosystem) with an initial denaturating step of 5 min at 94°C, and 35 cycles of annealing (55°C), extension (72°C) and denaturation (95°C), 30 sec per each step. Negative controls of each sample were also amplified in order to test that genomic DNA was absent from the cDNA samples. PCR products were run on a 1% agarose gel together with a 200 bp DNA ladder (Hyper-Ladder I from Bioline Ltd) and amplified bands were visualised by fluorescence with the SYBR Safe DNA gel stain 10.000X concentrated in DMS (Invitrogen), through ultraviolet illumination with UVI/PRO transilluminator system (UVIsoft UVIband Windows Application V10.01).

Immunolabelling

Cell cultures and cerebellar slices were rinsed once with PBS, and fixed with 4% buffered paraformahldehyde (pH 7.4) for 30 min at RT. Cells and tissues were washed in PBS, and a blocking stage was performed by incubation for 1h at RT in blocking buffer composed of 0.1% bovine serum albumin plus 10% normal goat serum (NGS) and, in the case of brain slices, addition of 1% Triton X-100 in phosphate buffered saline (PBST). Samples were then incubated overnight at 4°C with primary antibodies diluted in blocking buffer: mouse primary anti-NG2 antibody (monoclonal from Upstate, 1:500); rabbit anti-NG2 (either from Dr Stallcup, 1:500, or Chemicon, 1:200); chicken anti-GFAP (1:200; Chemicon); rabbit anti-calbindin D-28K (1:300; Chemicon). After three washes in PBST, samples were incubated for 1h at RT with the appropriate secondary antibodies conjugated with ^{488}Alexafluor or ^{567}Alexafluor (1:500, Molecular Probes), and in some cases Hoechst dye (Molecular Probes, 1:1000) was used for cell nuclei counterstaining. For double immunofluorescence labelling, primary antibodies of

different origin were diluted together in blocking buffer and co-dilutions of the appropriate secondary antibodies were used. After final washes in PBST, samples were mounted on poly-lysine coated glass slices with Vectashield mounting media (Vector Laboratories), and images were acquired using a LSM 5 Pascal Axioskop2 confocal microscope (Zeiss).

MTT cell death assay

Cell cultures were washed once with 1X HBM solution (20 mM HEPES, 140 mM NaCl, 5 mM KCl, 5 mM $NaHCO_3$, 1.4 mM NaH_2PO_4) containing 55 mM glucose and 0.4 mM $CaCl_2$. The cells were then incubated with 0.5 ml per well of 1.2 mM MTT ((3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide, Sigma) for 1 h at 37ºC, and the MTT solution was subsequently replaced with 300 µl of DMSO to solubilise the resulting formazan product. 200 µl aliquots of each sample were finally transferred into 96-well plates, and absorbance measured at 480 nm on a POLARstar Optima microplate reader (BMG LABTECH). Data were statistically analysed with the Origin Lab software. ANOVA was followed by *post hoc t*-test for comparing individual treatment groups.

Cell counts and statistical analysis

Two to four images from random fields of view (FOV; 460×460 µm at x20 magnification, or 230x230 µm at x40 magnification) from n≥3 cerebellar slices were acquired on a LSM 500 confocal microscope (Zeiss), using LSM Image software (Zeiss). Acquisition parameters of magnification, laser energy source and pinhole aperture were kept constant between different treatment groups. Numbers of immunolabelled cells were counted in each FOV and means (±SEM) calculated for each treatment group. Data were analysed with GraphPad Prism

v3.02 software (GraphPad Software Inc.), and one-way or two-way ANOVA analysis was performed for all the experimental groups, followed by a *post hoc* t-test or Tukey's test, as appropriate.

RESULTS

Efficacy and specificity of NG2-cell immunoablation *in vitro*

The immunotoxin system is based on a primary anti-NG2 antibody, and a secondary antibody that is directed against the primary antibody and to which the toxin saporin is chemically linked. The primary antibody binds specifically to the epitope on the cell surface, and provides a vehicle for the internalisation of the secondary immunotoxin, resulting in the intracellular release of the toxin saporin and cell death (Wiley, 1996; Kohls et al., 2000). To develop a specific immunoablation system for NG2-expressing cells, it was necessary to first identify cell lines which strongly expressed NG2, and others that did not express the proteoglycan. Four cell lines were selected on the basis that NG2 CSPG is expressed in glioma cells, but not neurons or astrocytes (Chekenya and Pilkington, 2002); C6 and F98 glioma cell lines, the CTX-TNA2 astrocyte cell line, and the NG108-15 neural cell line. The four cell lines were characterised for NG2 expression using immunocytochemistry, Western blot, and RT-PCR (Fig. 1). The results demonstrate that the C6 and F98 glioma cells expressed high levels of NG2, whereas no NG2 protein was detected either in the astrocytic CTX-TNA2 cell line or in the neuronal hybrid NG108-15 cells. Immunocytochemistry clearly demonstrate that the two glioma cell lines are immunopositive for NG2 (Fig. 1A, B), whereas CTX-TNA2 and NG108-15 cell lines are immunonegative (Fig. 1C, D). This was confirmed by western blot, where both the 300 kDa core protein and the 400 kDa native form of NG2 proteoglycan were detected in C6 and F98 glioma cells (Fig. 1E); enzymatic digestion of the homogenates with chondroitinase ABC (Ch ABC) resulted in the complete conversion of the high molecular weight form of NG2 into the 300 kDa one, due to the cleavage of the GAG lateral chain from the core protein, confirming the identity of the protein

bands detected. Finally, differential expression of NG2 was demonstrated at the mRNA level by RT-PCR (Fig. 1F), amplified products displaying the predicted size of 502 bp for NG2 mRNA in both C6 and F98 glioma cell lines, whereas no mRNA was detected in the astrocytic CTX-TNA2 cells and was barely detectable in the neuronal hybrid NG108-15 cell line.

On the basis of the expression analyses, the NG2-positive C6 glioma and NG2-negative CTX-TNA2 astrocyte cell lines were used to optimise the secondary immunotoxin protocol. The results show that compared to controls incubation with either the primary anti-NG2 antibody alone (1:5000 or 1:500 dilutions), or the saporin-conjugated anti-mouse secondary antibody (Mab-ZAP; 1.0, 0.5 or 1.0 µg/ml) had no effect on cell densities (Fig. 2A-F) or cell viability, as determined by the MTT assay (Fig. 2 G, H; $p>0.05$, one way ANOVA). Having shown that both primary and secondary antibodies on their own are not toxic for either C6 or CTX-TNA2 cell lines, we examined the combined effect of a range of concentrations of the mouse anti-NG2 and Mab-ZAP antibodies presented together (Fig. 3 and Table 1). Cells were examined after 72 h, and in NG2-expressing C6 glioma cells immunoablation occurred in a concentration-dependent manner (Fig. 3), whereas the viability of NG2-negative CTX-TNA2 cells was unaffected by any of the treatments (Table 1). Thus, only NG2-positive cells were ablated in the presence of both anti-NG2 antibody and the secondary saporin-conjugated secondary antibody, demonstrating the selectivity and specificity of the NG2-immunotoxin, with greatest cell death using 1:5000 dilution of primary antibody and 0.5-1.0 µg/ml of saporin-conjugated secondary antibody, consistent with a previous study targeting C6 glioma cells using the 192 IgG primary antibody against the $p75^{NTR}$ (Kohls & Lappi, 2000).

Interestingly, the 1:5000 dilution of primary anti-NG2 antibody was more effective than the higher concentration (1:500), most likely due to excess primary anti-NG2 competitively inhibiting the binding of conjugated anti-NG2-Saporin to NG2 epitopes and preventing its internalisation into the cells (Adam et al. 2006).

NG2-cell immunoablation in cerebellar slice cultures

The results presented above demonstrated that a combination of primary antibody against NG2 and secondary immunotoxin antibody effectively and selectively killed cultures of NG2-expressing glia, with no indirect effects of either antibody on their own. In cerebellar slices, we used both the mouse anti-NG2 antibody and Mab-ZAP combination (Fig. 4), and the rabbit polyclonal anti-NG2 antibody with a secondary anti-rabbit immunotoxin Rab-ZAP (NG2/Rab-ZAP) (Fig. 5). Experiments were performed on cerebellar slices from P12-15 GFAP-eGFP mice in order to identify astrocytes (GFAP-EGFP+), in order to confirm the integrity of the slices and the selectivity of NG2 ablation. After 72h slices were fixed and immunolabelled for NG2 (n≥3 per group). In controls, NG2-glia and astroglia appeared normal as previously described (Leoni et al., 2009), with NG2-glia being uniformly distributed across the layers of the cerebellum intermingled with Bergmann glia with characteristic morphology, with somata in the Purkinje cell layer (PCL), and long primary processes extending through the molecular layer (ML) to the pia (Fig. 4A-C). Notably, there was no loss or disruption of NG2-glia following treatment with NG2-Mab-ZAP (Fig. 4D-I), whereas treatment with NG2-Rab-ZAP caused a marked loss of NG2-glia (Fig. 5). Immunolabelling for NG2 was performed following washout of the immunotoxin, and the results from NG2-Mab-Zap clearly demonstrate that NG2

immunostaining *per se* was not disrupted by incubation in the cocktail (Fig. 4); NG2-glia exhibited their characteristic morphology of small central somata and radially extended fine branching processes (Fig. 4G-I), as described previously (Leoni et al., 2009). The loss of NG2-glia was specific to treatment with the NG2-Rab-ZAP combination (Fig. 5), decreasing from 60.8 ± 8.35 cells per FOV in controls and 68.5 ± 7.64 cells per FOV in NG2-Mab-ZAP, to 22.0 ± 3.24 cells per FOV in NG2/RabZAP (Fig. 5D; $p<0.001$, one-way ANOVA and *post hoc* Tuckey's key test). In addition, NG2-Rab-Zap had no effect on immunostaining for NeuN and synaptophysin (Fig. 6A, B) or on the general distribution or cell density of $EGFP^+$ astrocytes (Fig. 6C, D); EGFP+ astrocytes were surrounded by degenerating NG2-glia and debris in immunotoxin-treated slices (Fig. 6E, F). The results demonstrate that NG2-Rab-Zap selectively and effectively ablates NG2-glia.

Following treatment with NG2-Rab-ZAP immunotoxin, the bulk of NG2 immunostaining appeared as diffuse and punctate, attributable to debris of dead or dying cells (Fig. 5C, 6E). In addition to this substantial loss of NG2-glia, compared to controls surviving NG2-glia appeared degenerative following NG2-RabZAP treatment (Fig. 7A, B); this was analysed further using morphological criteria to subdivide NG2-glia into, (i) normal process bearing cells (Fig. 7C), (ii) amoeboid reactive cells (Fig. 7D), or (iii) severely 'injured' cells characterised by fragmented immunostaining (Fig. 7E). The vast majority of NG2-glia in control slices (n=44 cells) were classified as normal (39%) or amoeboid reactive (61%) cells, and no visibly injured cells were identified (Fig. 7F). The situation was reversed following treatment with NG2/Rab-ZAP (Fig. 7G), with 81% of surviving NG2-glia appearing severely injured and 16% amoeboid reactive, with very few

cells appearing normal (3%). Note that compared to controls, only 30% of cells survived the immunotoxin treatment (Fig. 5D), and the vast majority of these were severely disrupted (Fig. 7G). Furthermore, we have previously reported that NG2-glia begin to express the astroglial EGFP reporter in cerebellar cultures (Leoni et al., 2009), and most of the NG2-glial cells surviving immunotoxin treatment appeared to express the EGFP reporter (Fig. 8), suggesting these cells may be less susceptible to immunoablation.

DISCUSSION

In the CNS, the NG2 CSPG is strongly expressed in NG2-glia, pericytes and certain gliomas (Butt et al., 2002; Nishiyama et al., 2007; Chekenya & Pilkington, 2002). One approach to study cellular function is to use genetic ablation, but NG2 knock-out mice did not result in the destruction of NG2-glia and there were only subtle effects on the CNS (Kucharova and Stallcup, 2010). Hence, we strove to develop an immunotoxin approach to target the destruction of NG2-glia *in situ,* using antibodies directed against the NG2 CSPG in combination with a secondary immunotoxin conjugated to saporin. The primary antibody targets the extracellular domain of the NG2 CSPG and serves as the vehicle by which the secondary immunotoxin antibody is internalised into the NG2-expressing cells. Our results show that this approach is highly effective for the selective ablation of NG2-expressing cells *in vitro* and *ex vivo* in cerebellar slices.

Immunoablation of NG2-glia was effective after 72h, consistent with *in vivo* studies examining immunoablation of neurons, which require long-term infusion of immunotoxins via cannulae (Kwok et al., 1999; Pizzo et al., 1999; Wiley et al., 1991). This reflects the time required for the anti-NG2 primary antibody to bind to the cell surface epitope, form a tertiary complex with the secondary immunotoxin, which must then be internalised for saporin to be released and initiate apoptosis (Wiley, 1996; Kohls et al., 2000). We found the mouse monoclonal anti-NG2-saporin was very effective at selectively ablating NG2-expressing C6 glioma cells *in vitro*, but was totally ineffective in brain slices. The reason for this is unknown, but presumably the NG2-Mab-Zap complex was not

411 effectively internalised in brain slices, demonstrating the importance of testing
412 multiple antibodies to identify the most efficacious combinations.
413
414 The anti-NG2-saporin immunotoxin effectively destroyed NG2-glia without
415 adversely affecting astrocyte or neuronal densities. Furthermore, the vast
416 majority of surviving NG2-glia appeared severely disrupted, possibly in the
417 process of dying. In addition, NG2-glia in cerebellar slice cultures begin to
418 express EGFP astroglial reporter after 48h (Leoni et al., 2009), and we found that
419 these cells preferentially survived immunoablation at 72h. These GFAP-
420 EGFP+/NG2+ cells appear to display an astrocyte-oligodendrocyte (AO) lineage
421 phenotype, which has been reported by Kirchhoff and colleagues (personal
422 communication) and is reminiscent of the O-2A cells described by Raff and
423 colleagues (Raff et al., 1983). NG2-glia have been reported to generate
424 astrocytes during development, but do not normally do so in the adult (Zhu et
425 al., 2008). It seems likely that AO cells generated in the cerebellar slice are in
426 the process of losing NG2 and gaining GFAP as they differentiate into astrocytes
427 (Leoni et al., 2009), and it is possible that down-regulation of the NG2 CSPG
428 protects them from the anti-NG2-saporin immunotoxin.
429
430 Our results show it is possible to target NG2-glia *in situ*, and this approach holds
431 promise for studying the effects of NG2-glial cell ablation *in vivo*. Saporin-
432 conjugated immunotoxins have been used to effectively target specific
433 populations of neurons *in vivo*, such as those directed against the $p75^{NTR}$ in
434 cholinergic neurons (Wiley et al., 1991, 1995), the dopamine transporter (DAT)
435 in dopaminergic neurons (Wiley et al., 2003), and a range of other neuronal
436 targets, including the norepinephrine synthesizing enzyme dopamine β

hydroxylase (DβH), NK1 receptor for the Substance P, the IL-2 receptor, vasopressin and others (Wiley, 1996). Our findings indicate it would be possible to use the same approach to examine the functions of NG2-glia *in vivo*.

In summary, our study shows that immunoablation is a feasible option for specifically targeting NG2-glia *in situ*. In light of the potential importance of NG2-glia, it is perhaps surprising, or at the least disappointing, that NG2 knock-out mice have failed to reveal any critical effects in the CNS (Hossain-Ibrahim et al., 2007, Thallmair et al., 2006; Kucharova and Stallcup, 2010). However, a comparison can be made with astrocytes, where knock-out of GFAP, which characterises astrocytes, did not result in a loss of astrocytes or disrupt their functions (Pekny et al., 1995). The immunoablation approach provides a useful experimental tool for investigating the function of NG2-glia in both physiological and pathological conditions, such as demyelination and degeneration, and could also prove useful in experiments investigating the influence of oligodendroglial cells on cognitive functions, for example learning and memory.

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461 **Author Contributions**

462 GL performed the experiments, data analysis and preparation of the manuscript.
463 AB conceived and designed the study, and was responsible for organisation, data
464 analysis and interpretation, and preparing the paper. MR contributed to study
465 design and interpretation, and critical revision of the manuscript and approval of
466 the article. DF contributed to data interpretation discussions, critical revision of
467 the manuscript and approval of the article.

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Figure legends

Figure 1. NG2 expression in cell lines. C6, F98, NG108-15 and CTX-TNA2 cell lines were tested for NG2 expression using immunocytochemistry (A-D), western blot (E) and RT-PCR (F). (A-D) Cells were immunolabelled for NG2 (green) with a mouse monoclonal antibody from Upstate (1:500) and cell nuclei stained with Hoechst 33342 dye (blue). C6 (A) and F98 (B) rat glioma cells express the NG2 antigen on their cell surface, whereas the CTX-TNA2 (C) and NG108-15 (D) cell lines do not express NG2. Scale bar, 50 μm. (E) Western blotting revealed protein expression of the NG2 CSPG in the C6 and F98 cell lines, whereas the NG108-15 and CTX-TNA2 cell lines were NG2-negative. The arrows and the arrowheads indicate the 300 kDa core protein and the 400 kDa native form of NG2, respectively, in samples treated (+) with Chondroitinase ABC (Ch ABC), or prior to enzymatic digestion (-). Treatment with ChABC determined the complete conversion of the native form into the 300 kDa one, in both C6 and F98 cell lines. (F) RT-PCR confirmed C6 and F98 cell lines express NG2 mRNA, while NG108-15 and CTX-TNA2 cell lines were negative. A mouse brain extract was used in both Western blot and RT-PCR as a positive control, and molecular weight markers are shown on the left.

Figure 2. Lack of toxicity of either primary or secondary antibodies in vitro. (A-F) C6 glioma cells were exposed for 72 h to control culture media (A), or to either anti-NG2 primary antibody (α_1) alone (B-C) or saporin-conjugate anti-mouse secondary immunotoxin (ZAP) alone (D-F), at different concentrations. Neither of the two antibodies had any toxicity on the C6 cells, compared to the untreated control group. Scale bar, 100 μm. (G, H) C6 cells (G) and CTX-TNA2 (H) cells were exposed for 72 h to

either anti-NG2 primary antibody (α_1) or saporin-conjugated anti-mouse secondary immunotoxin (ZAP), at different concentrations, and cell viability measured using the MTT assay. Neither of the two antibodies had any significant toxicity on C6 or CTX-TNA2 cells, compared to the untreated control group. Data are expressed as mean±SEM.

Figure 3. Immunoablation of NG2-positive C6 cells. C6 cells were exposed for 72 h to a combination of Mab-ZAP and primary anti-NG2 antibody (α_1) at different concentrations. (A-H) Cell density was clearly reduced following treatment with NG2-Mab-ZAP; Scale bar, 100 μm. (I) Cell viability measured by the MTT assay was significantly decreased following treatment with the combined NG2-Mab-ZAP. For each treatment, n=4. One way ANOVA analysis ($p<0.05$) was performed over the three treatment groups. *$p<0.05$, **$p<0.01$, ***$p<0.001$, *post hoc* *t*-test vs intra-group controls (0 ZAP).

Figure 4. Mouse anti-NG2 immunotoxin (NG2/Mab-ZAP) does not affect NG2-glia in cerebellar slice cultures. Cerebellar slices from P13 GFAP-EGFP mice were cultured for 4DIV, and then fixed and immunolabelled for NG2. Confocal images illustrate the separate channels for NG2 immunolabelling (A, D, G, red), GFP expression (B, F, H, green), and the combined channels (C, F, I). (A-C) Control slices cultured in normal serum-free medium (CTR). (D-I) Slices exposed for 4 days to a combination of mouse anti-NG2 primary antibody and Mab-ZAP secondary antibody (NG2/Mab-ZAP). NG2 immunolabelling (A, D, G) and EGFP astroglial reporter protein (B, E, H) show no substantial toxicity of the

616 treatment to either population of cells. Arrowheads in (A) indicate NG2-
617 immunopositive vascular pericytes, whereas arrows in (F) indicate NG2-glial cells
618 expressing EGFP. Asterisks in (G) and (I) illustrate in detail the morphology of
619 NG2-glial cells that were unaffected by the NG2/Mab-ZAP treatment. These NG2-
620 glial cells were found closely associated with a presumptive Purkinje cell body,
621 indicated by the star in (G-I). GCL, granular cell layer; PCL, Purkinje cell layer;
622 ML, molecular layer.
623
624 **Figure 5. Effective immunoablation of NG2-glia in the cerebellum by the**
625 **NG2/Rab-ZAP immunotoxin.** Representative confocal micrographs of
626 cerebellum slices immunolabelled for NG2 after immersion culture for 2DIV, in
627 either control conditions (A), a combination of mouse anti-NG2 antibody and
628 Mab-ZAP (B), or rabbit anti-NG2 antibody and Rab-ZAP (C). Only NG2-Rab-Zab
629 caused ablation of NG2-glia; Scale bars, 20 μm. (D) Cell counts of NG2
630 immunopositive cells in cerebellum slices expressed as mean number of cells per
631 fields of view of 30 μm stack confocal images acquired at fixed acquisition
632 parameters of magnification, laser source energy, pinhole aperture and stack
633 interval (1 μm). *p<0.05, ***p<0.001, one-way ANOVA and *post hoc* Tukey's
634 test.
635
636 **Figure 6. Selective immunoablation of NG2-glia.** Confocal micrographs of
637 control and immunotoxin-treated (NG2/Rab-ZAP) cerebellum slices. (A, B)
638 Cerebellar slices obtained from P11 wt mice were cultured for 3DIV in culture
639 media (A) or Rab-ZAP (B) and then immunolabelled for NeuN (red) and
640 synaptophysin (green), revealing no gross differences between the groups. (C-
641 F) Treatment of P13 cerebellum slices for 3 DIV from GFAP-GFP mice

immunolabelled for NG2 (red), with normal medium (C) or NG2/Rab-ZAP immunotoxin (D-F). There were no gross differences in the number and distribution of astrocytes in the two treatment groups (C, D), and higher magnification shows viable astrocytes surrounded by NG2-immunopositive debris (E), and NG2-glia with fragmented labelling (F).

Figure 7. Effect of immunoablation on the morphology of NG2-glia. Cerebellar slices obtained from P12 mice were immersion cultured for 3DIV and immunolabelled for NG2. (A) NG2-glial cells in control slices had a characteristic stellate processes-bearing morphology. (B) NG2-glial cell in slice treated with the NG2/Rab-ZAP immunotoxin were severely disrupted and NG2 immunolabelling appeared in 'clumps'. (C-D) NG2-glia in slices treated with the NG2/Rab-ZAP immunotoxin were ranked in three morphological classes, corresponding to process-bearing (C), amoeboid reactive (D) and condensed and fragmented cells (E). (F, G) Pie-chart analysis of the proportions of NG2-glia with the different morphologies expressed as number of cells per FOV of 30 µm confocal stack.

Figure 8. GFAP-EGFP-expressing NG2-glia survive immunoablation. NG2-glial cells that expressed the astroglial reporter protein GFAP-EGFP displayed resistance to the immunotoxin treatment. (A) Confocal 30 µm *z*-stack (Ai) and single 1 µm z-zections (Aii, Aiii) illustrating viable NG2-glia expressing the GFAP-EGFP reporter, in the proximity of severely disrupted $NG2^{+}/EGFP^{-}$ cells. (B) Confocal z-stack of NG2-glial cell expressing the GFAP-EGFP reporter, showing the combined channels (Bi) and single channel analysis of NG2 immunolabelling (Bii) and EGFP expression (Biii). Three-dimensional reconstructions of the $NG2^{+}/EGFP^{+}$ cell in orthogonal projections (Biv) and following isoforming (Bv)

668 illustrate the cytoplasmic EGFP reporter and cell membrane NG2
669 immunolabelling.
670

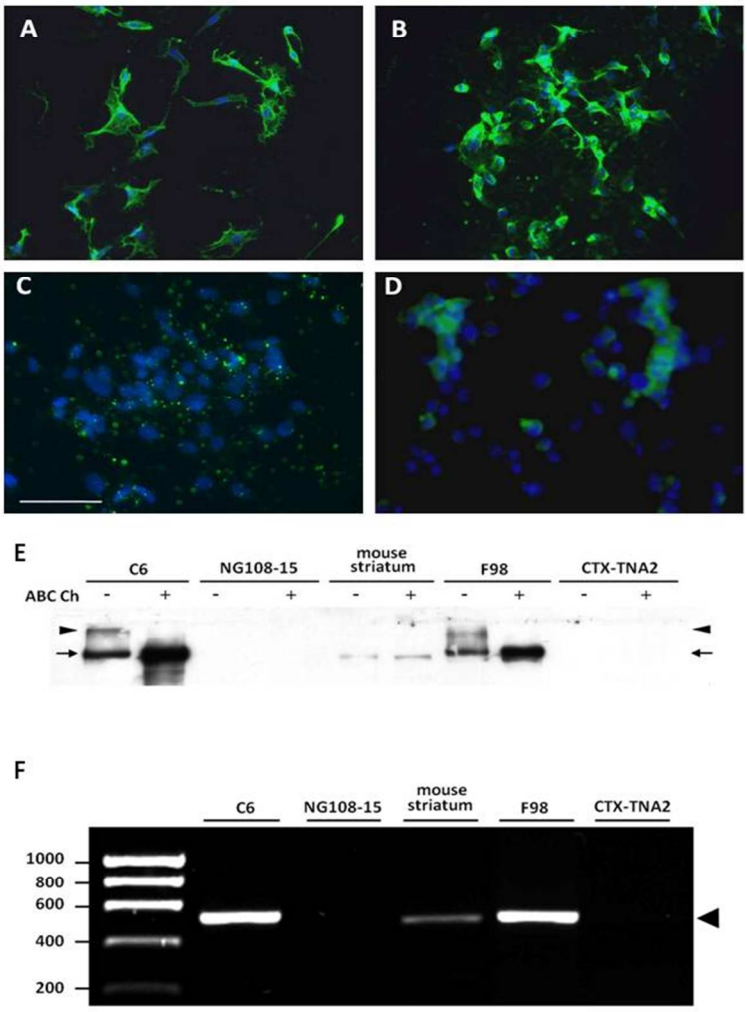

Figure 1
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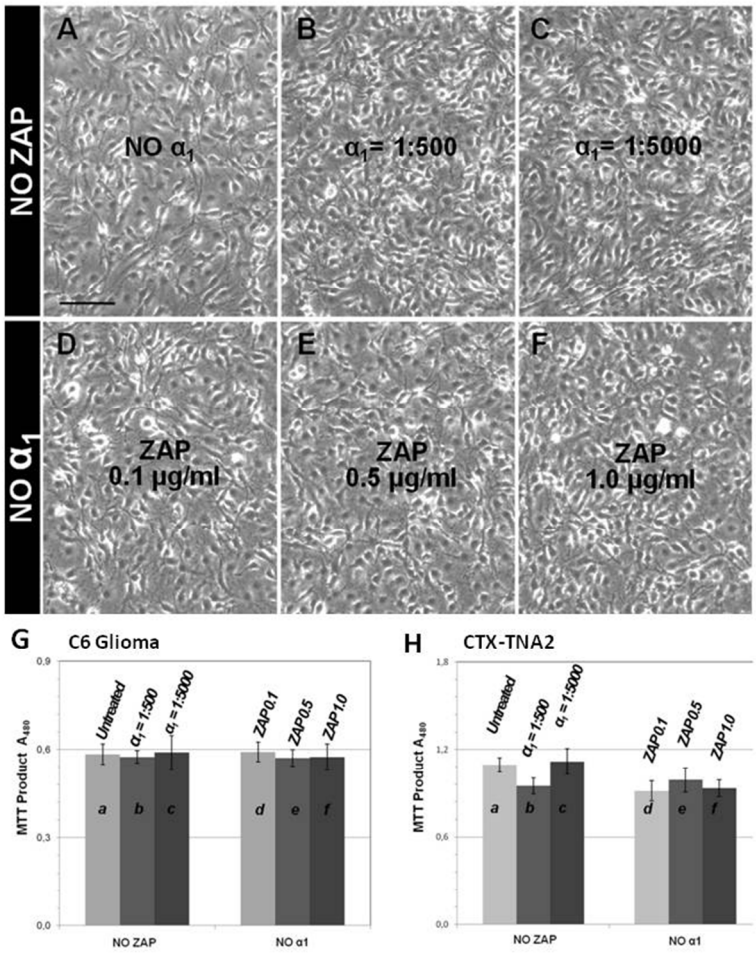

Figure 2
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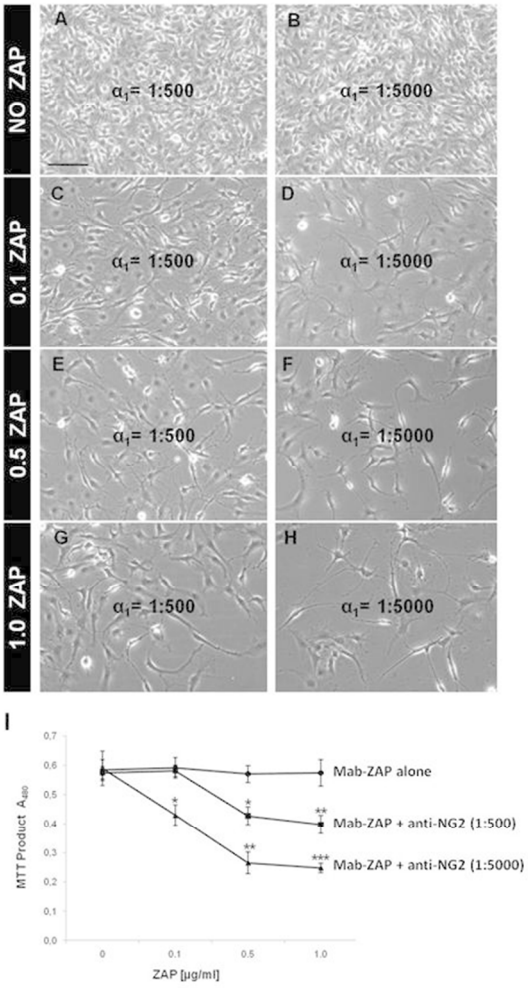
Figure 3



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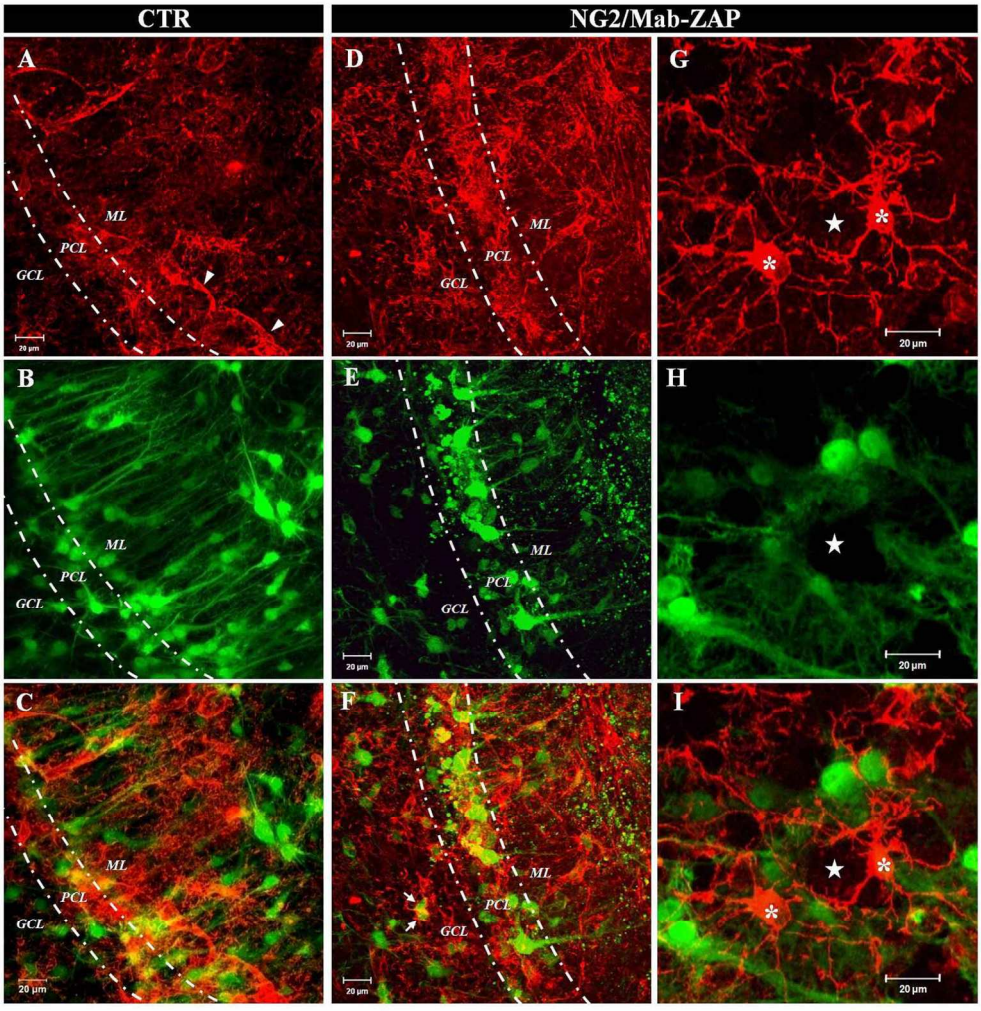

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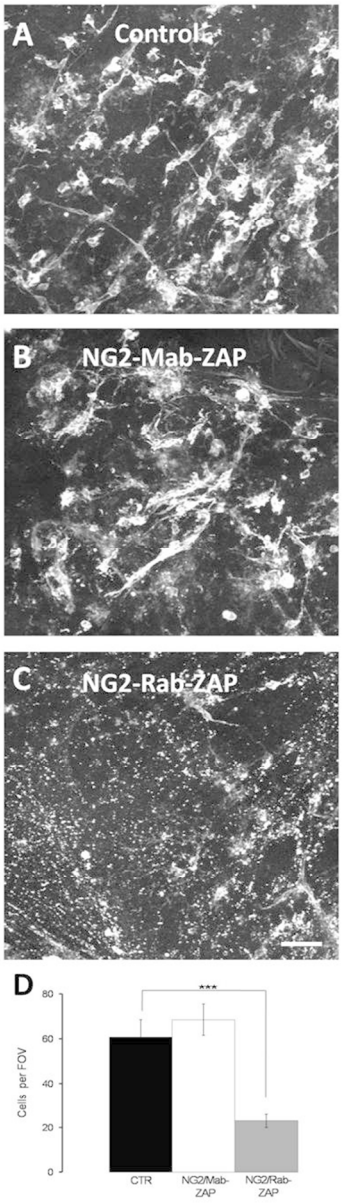

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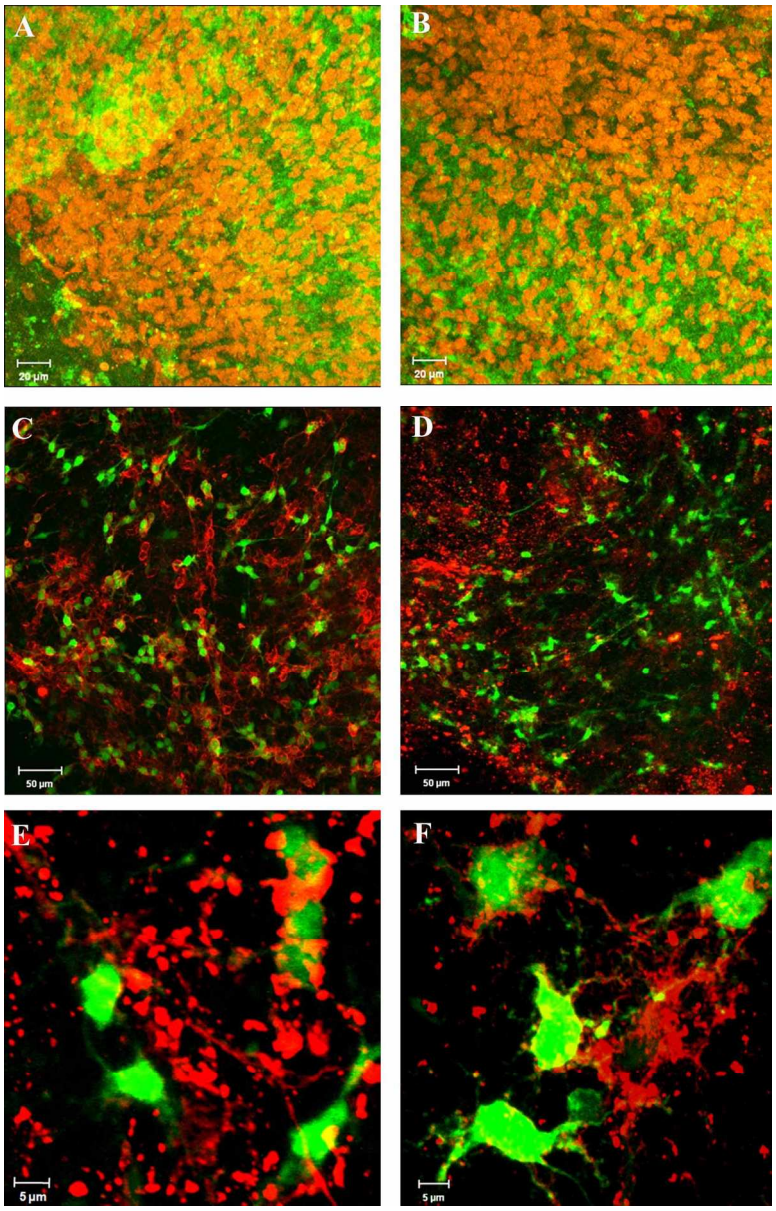

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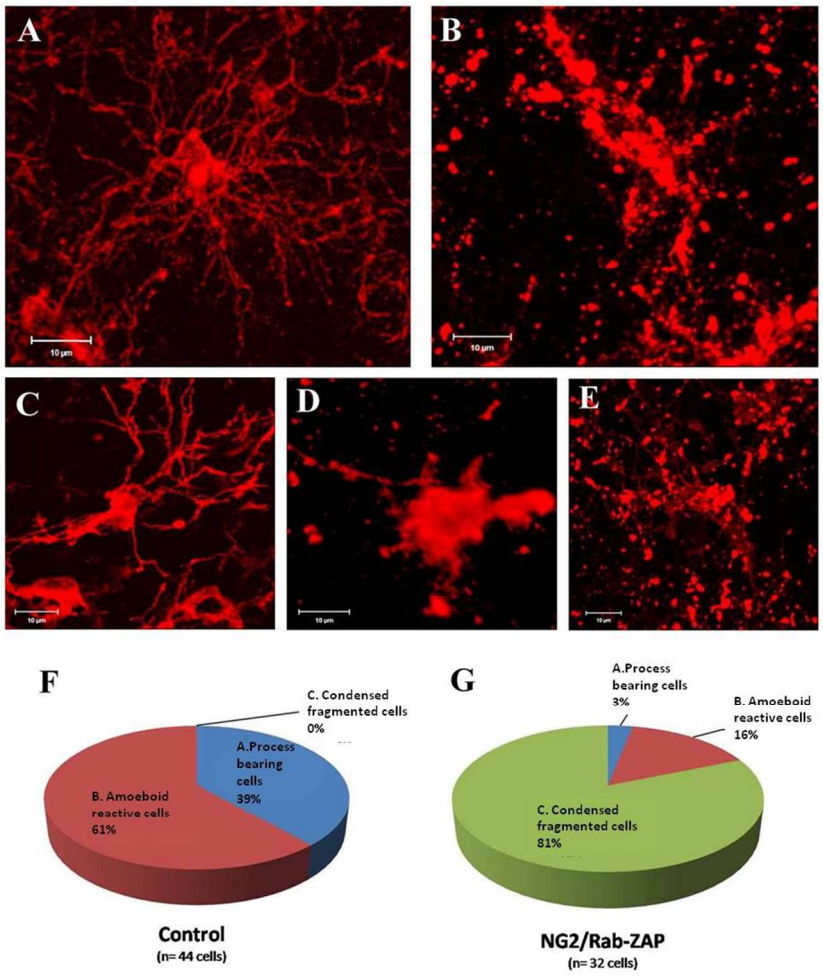

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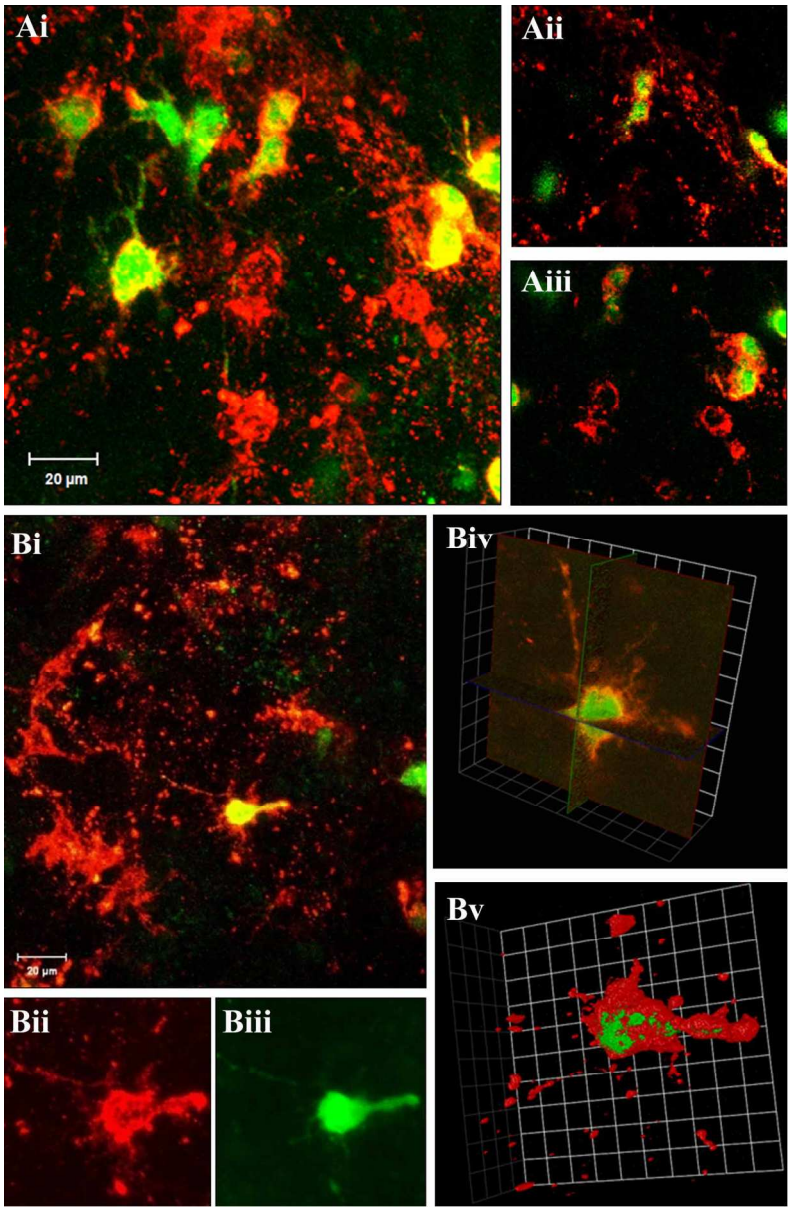

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