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Supplemental Information

Cell Replacement Therapy Improves Pathological Hallmarks in a Mouse Model of Leukodystrophy Vanishing White Matter

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Supplemental Figures

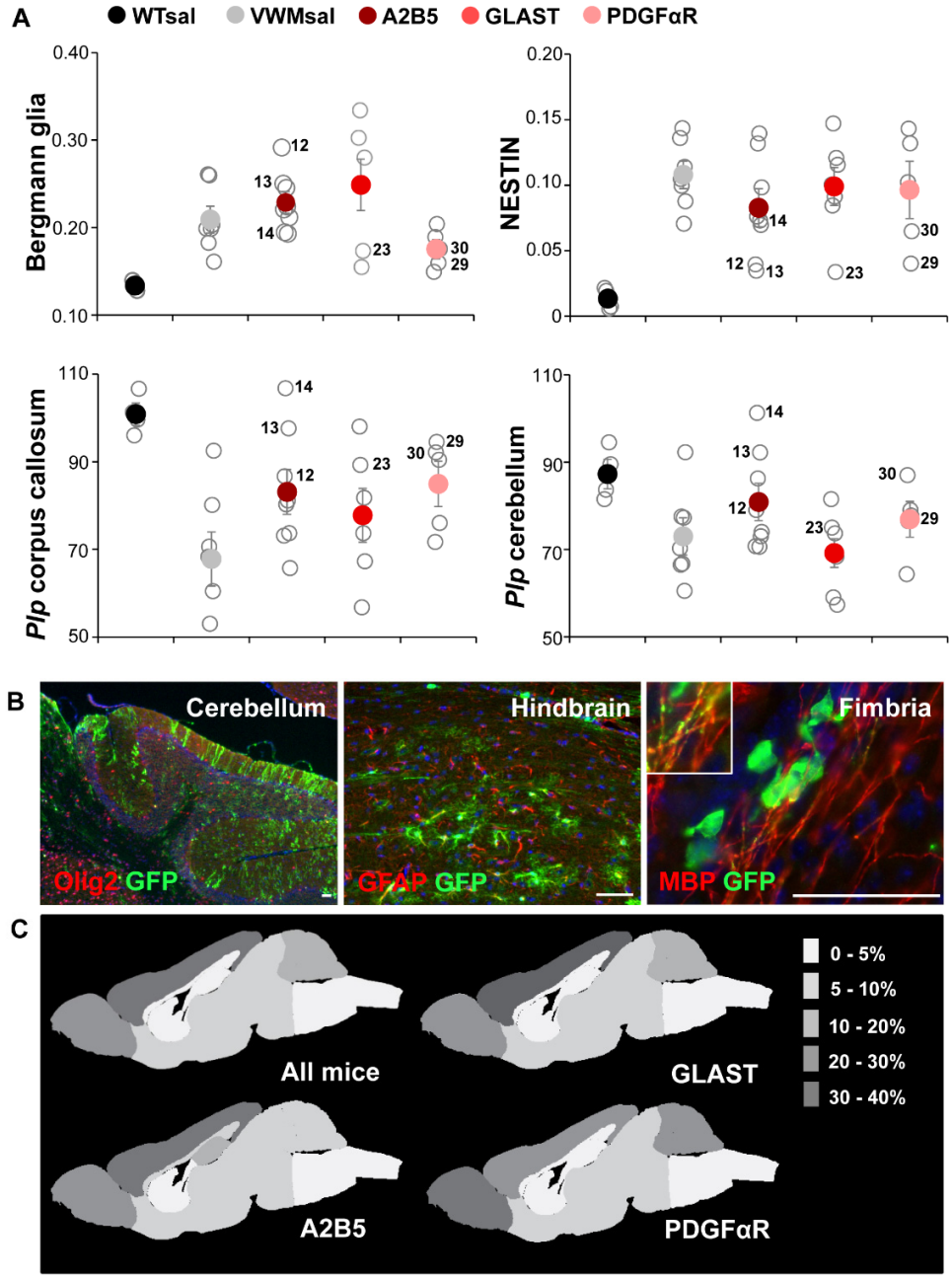

Figure S1. Pathology scores and cell dispersal after transplantation. Related to Figure 1 and 2. (A) shows the data of all mice on the three disease markers: Bergmann glia translocation (left top), the number of NESTIN$^+$ astrocytes in the corpus callosum (right top), and the number of *Plp*$^+$ oligodendrocytes in the corpus callosum (left bottom) and cerebellum (right bottom). Open data points indicate individual mice, with solid data points indicating group means ± SEM. The animals that showed improvement according to the discriminant analysis are indicated by numbers. WTsal = WT saline injected mice n=4, VWMsal = VWM saline injected mice n=7, A2B5 = VWM A2B5$^+$ cells injected mice n=8, GLAST = VWM GLAST$^+$ cells injected mice n=6, PDGFαR = VWM PDGFαR$^+$ cells injected mice n=5. (B) shows that injected (GFP$^+$) cells have migrated to different brain areas such as the cerebellum, hindbrain or fimbria. (C) shows the percentage of the total number of injected cells that have migrated to a specific

brain region, to give an overview of cell dispersal. The percentages are calculated for all mice (left top; n=40 mice) and separately for mice that received A2B5^{+} (left bottom; n=13 mice), GLAST^{+} (right top; n=10 mice) or PDGFαR^{+} (right bottom; n=17 mice) cell injections. Note that although white matter areas as the corpus callosum did not receive a high percentage of the total injected cells, the corpus callosum is also a much smaller area than for example the cortex which has not been taken into account in these calculations. Scalebars = 50 µm.

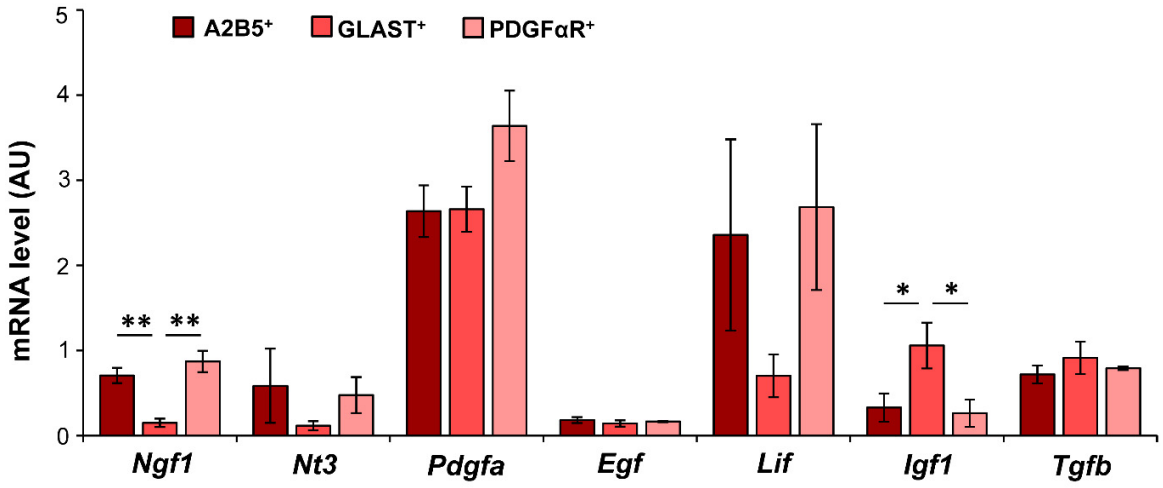

Figure S2. In vitro analysis of GPCs. Related to Figure 3. mRNA levels of neurotrophic factors in A2B5^{+}, GLAST^{+} and PDGFαR^{+}, based on qPCR analysis of 3 independent experiments. Only minor differences between GPC populations are observed, with a significant decrease of *Ngf1* and a significant increase of *Igf1* in the GLAST^{+} cells. Bars represent mean ± SEM. Shown are $2^{-\Delta CT}$ values in arbitrary units (AU). N=3 independent sortings for all. * = $p<.05$, ** = $p<.01$

Table S1. All pathology data. Related to Table 1.

Mouse#	Group[a]	Genotype[b]	Gender	Nestin[c]	BG[d]	*Plp* cc[e]	*Plp* cer[f]
1	Saline	WT	M	0.021	0.140	101	82
2	Saline	WT	M	0.019	0.132	96	84
3	Saline	WT	M	0.006	0.136	107	90
4	Saline	WT	M	0.007	0.128	100	95
5	Saline	VWM	M	0.136	0.199	80	77
6	Saline	VWM	M	0.105	0.261	60	61
7	Saline	VWM	M	0.099	0.260	50	67
8	Saline	VWM	M	0.143	0.198	93	92
9	Saline	VWM	F	0.070	0.203	71	67
10	Saline	VWM	F	0.114	0.161	68	70
11	Saline	VWM	F	0.088	0.182	53	78
12[g]	**A2B5**	**VWM**	**F**	**0.039**	**0.291**	**87**	**79**
13[g]	**A2B5**	**VWM**	**F**	**0.035**	**0.250**	**98**	**92**
14[g]	**A2B5**	**VWM**	**F**	**0.076**	**0.195**	**107**	**101**
15	A2B5	VWM	F	0.132	0.245	73	71
16	A2B5	VWM	M	0.139	0.193	74	73
17	A2B5	VWM	M	0.073	0.212	66	74
18	A2B5	VWM	F	0.070	0.221	81	71
19	A2B5	VWM	F	0.098	0.224	80	86
20	GLAST	VWM	M	0.084	0.302	82	70
21	GLAST	VWM	M	0.147	0.334	67	68
22	GLAST	VWM	F	0.091	0.155	98	82
23[g]	**GLAST**	**VWM**	**F**	**0.034**	**0.173**	**89**	**75**
24	GLAST	VWM	F	0.121	0.280	57	59
25	GLAST	VWM	F	0.115	0.248	74	74
26	PDGFαR	VWM	F	0.102	0.149	72	64
27	PDGFαR	VWM	M	0.143	0.189	76	78
28	PDGFαR	VWM	M	0.132	0.204	90	79
29[g]	**PDGFαR**	**VWM**	**M**	**0.040**	**0.160**	**95**	**77**
30[g]	**PDGFαR**	**VWM**	**F**	**0.065**	**0.175**	**92**	**87**

[a] Saline = saline injected mice; A2B5, GLAST and PDGFαR = mice injected with primary mouse cells sorted on marker expression of the named proteins.

[b] Genotype of mice: WT = wildtype, VWM = VWM mice,

[c] Ratio of Nestin$^+$ cells to total number of DAPI$^+$ cells in the corpus callosum

[d] BG = Bergmann glia. Ratio of translocated Bergmann glia to total number of Bergmann glia

[e] cc = corpus callosum. Average number of *Plp*$^+$ cells in a 100x200 micron square in the corpus callosum

[f] cer = cerebellum. Average number of *Plp*$^+$ cells in a 100x200 micron square in the cerebellum.

[g] Bold script indicates transplanted animals that were classified as WT in the discriminant analysis.

Table S2. Injection coordinates for cell transplantation in P0 pups.

Weight of pup	Injection site	X[a]	Y[b]	Z[c]
0.9 gr	Posterior corpus callosum	±1.4	0	-.5
	Anterior corpus callosum	±0.9	-2	-.4
	Cerebellum	±1.1	1.9	-1.5
1.0 gr	Posterior corpus callosum	±1.4	0	-.5
	Anterior corpus callosum	±1	-2.1	-.4
	Cerebellum	±1.1	2	-1.5
1.1 gr	Posterior corpus callosum	±1.4	0	-.5
	Anterior corpus callosum	±1	-2.2	-.4
	Cerebellum	±1.2	2	-1.5
1.2 gr	Posterior corpus callosum	±1.6	0	-.5
	Anterior corpus callosum	±1.1	-2.2	-.5
	Cerebellum	±1.2	2.1	-1.6
1.3 gr	Posterior corpus callosum	±1.6	0	-.6
	Anterior corpus callosum	±1.1	-2.3	-.5
	Cerebellum	±1.2	2.1	-1.7
1.4 gr	Posterior corpus callosum	±1.6	0	-.6
	Anterior corpus callosum	±1.2	-2.3	-.5
	Cerebellum	±1.2	2.2	-1.7
1.5 gr	Posterior corpus callosum	±1.6	0	-.7
	Anterior corpus callosum	±1.2	-2.4	-.5
	Cerebellum	±1.2	2.2	-1.8
1.6 gr	Posterior corpus callosum	±1.6	0	-.7
	Anterior corpus callosum	±1.3	-2.4	-.6
	Cerebellum	±1.3	2.3	-1.8
1.8 gr	Posterior corpus callosum	±1.6	0	-.7
	Anterior corpus callosum	±1.3	-2.4	-.6
	Cerebellum	±1.3	2.3	-1.8

[a,b,c] The coordinates are in mm distance to the lambda, which is clearly visible in P0 pups.

[a] The X-coordinates represent the medial-lateral or left-right plane.

[b] The Y coordinates represent the rostral-caudal or front-back plane.

[c] The Z coordinates represent the ventral-dorsal or up-down plane.

Supplemental Experimental Procedures

Experimental animals
For this study mice with a homozygous mutation in the *Eif2b5* gene were used (*Eif2b5*$^{R191H/R191H}$; called *2b5*^{ho} or VWM mice, background strain C57Bl/6J (Dooves et al., 2016)). Littermates with heterozygous *Eif2b5* mutations were used as WT animals for the transplantation experiments as these mice do not show any disease phenotype. For cell sortings WT C57Bl/6J mice were used. Untreated *2b5*^{ho} mice show astrocytic abnormalities from P14 onwards, followed by myelin abnormalities from 1 month of age and ataxia from 4 months of age. Between 7 and 10 months of age untreated *2b5*^{ho} mice reach their humane endpoint, defined as the moment they become unable to take food or water or when they lost more than 15% of their body weight (Dooves et al., 2016). Pups were injected with cells or saline at postnatal day 0 (P0) as described below. Pups were weaned at P28 and housed in groups separated by sex.

Mice were sacrificed at 2 months, 5 months and 9 months of age by transcardial perfusion with 4% paraformaldehyde. Half of the brain was post-fixed for 24h, cryoprotected in 30% sucrose overnight and snap-frozen in Optimal Cutting Temperature mounting medium (Sakura Finetek Europe BV). The other half was embedded in paraffin. For every age group and experimental group 5-10 *2b5*^{ho} animals were collected: WT saline (9m n=11), VWM saline (9m n=22), VWM A2B5 (2m n=5, 5m n=5, 9m n=8), VWM GLAST (2m n=5, 5m n=5, 9m n=6), VWM PDGFαR (2m n=6, 5m n=5, 9m n=5). Due to differences in litter size and number of homozygous mutant animals per litter, the group sizes were slightly different for each condition. All mouse procedures were carried out according to the guidelines of the Dutch government and approved by the Animal Approval Committee of the VU University Amsterdam.

Motor tests
The motor skills of mice were tested at 7 months of age on WT saline (n=11), VWM saline (n=22), A2B5 injected (n=8), GLAST injected (n=6) and PDGFαR injected (n=5) mice. On the balance beam test, mice were trained to run over a narrow beam into an enclosed box. The number of slips and the time it took them to cross the beam were recorded. The grip strength meter was used to measure the strength of the front paws and the front and hind paws combined, by pulling mice over a grid that records the strength that mice use to hold on to the grid. The footprint test was used to analyse gait ataxia. The front paws of the mice were painted with red ink, and the hind paws with blue ink. Mice were then allowed to run over a paper. Papers were later scanned and gait parameters like stride length and gait width were recorded. See (Dooves et al., 2016) for more information.

Primary cell isolation
Forebrains from embryonic day 18 WT pups were dissociated with the GentleMACS dissociater (Miltenyi Biotec) as previously described (Dooves et al., 2016). After overnight recovery on an anti-adhesive plate, different cell populations were sorted with magnetic activated cell sorting (MACS) according to manufacturers' protocol (Miltenyi Biotec). Different glial progenitor populations were sorted for marker expression of A2B5, GLAST or PDGFαR. After the sorting cells were transduced by centrifuging for 1 hr at 37°C at 600 g in DMEM/F12 supplemented with polybrene (10 µg/ml) and GFP lentivirus. After transduction cells were washed with PBS twice and resuspended as $1x10^5$ cells per 400 nl in saline with DNAse (100 µg/ml) and immediately used for cell transplantation.

Production of lentivirus
A lentiviral GFP plasmid (LV-PGK-eGFP) was kindly gifted by Joost Verhaagen (Netherlands Institute for Neurosciences, Amsterdam, The Netherlands). Virus was produced by transfecting the plasmid and packaging plasmids (ViraPower™ Packaging Mix, Invitrogen) with FuGene in serum free opti-MEM medium in HEK293T cells overnight. The next day medium was replaced with serum free opti-MEM for virus collection. Medium was collected on two consecutive days. Virus-containing medium was concentrated by spinning down for 3 hr at 20.000 rpm. Afterwards, most of the supernatant was removed leaving about 200 µl of medium. The virus was resuspended by incubating on ice on a rocking platform for 1 hour, aliquoted and stored at -80°C. For every new virus batch the amount of virus needed to reach an ~70-80% transduction efficiency was tested on primary dissociated cells.

Cell injections
Pups were sedated before injections by incubation on ice for 7 min. Clay holders were used to keep pups in place under the stereotact. These clay holders were made by pressing (sedated) pups with different body weight in ~37°C warm low-melting-temperature agarose to make an impression of the pup. The agarose was cooled in the fridge until it was hard and filled with clay to make a clay pup. This clay pup was baked and pressed in clay to make a clay

holder. These clay holders were baked, marked with the pups weight and used for injections for pups of similar weight. Cells were stereotactically injected bilaterally in the anterior corpus callosum, posterior corpus callosum and the cerebellum. The injection coordinates used can be found in Table S2. It is important to note that the exact coordinates needed to target these specific areas will differ when the pup is placed underneath the stereotact at a different angle. For this reason, this coordinates are useful with the exact clay holders used in this study, and will need to be optimized on other setups. Per injection site $1x10^5$ cells were injected in a volume of 400 nl. Control animals got the same injections but with 400 nl saline. After injections pups were put on a heating mat for a few minutes until they were awake, and returned to their mother.

In vitro cell analysis

To analyse *in vitro* differentiation patterns cells were isolated from E18 mice in 3 independent experiments and sorted for marker expression of A2B5, GLAST or PDGFαR as described above. After sorting, cells were not transduced with GFP virus but immediately lysed in TRIzol® (Life Technologies) to extract RNA or plated at 100.000 cells/well on a PLO/laminin coated 8 well chamberslide in M41 medium (medium was refreshed after 3 days). Cells were fixed at 1 day and 1 week after sorting by incubation in 2% PFA for 20 min. Immunostaining on cell culture was performed as described below, but without the citrate buffer antigen retrieval step. Antibodies used targeted GFAP (1:1000, Sigma-Aldrich, G3893), OLIG2 (1:500, Millipore, AB9610), SOX9 (1:250, Cell Signalling, 82630), PDGFαR (1:250, eBioscience, 13-1401-82), MAP2 (1:20000, Abcam, AB5392) and CD11b (1:50, developed by Springer T.A. and obtained via the Developmental Studies Hybridoma Bank, M1/70.15.11.5.2). For cell counts 10 random pictures per well were taken. For each marker the number of positive cells as a percentage of the total number of $DAPI^+$ cells was calculated. Contamination of MAP2+ and $CD11b^+$ cells was lower than 5% after sorting.

RNA was isolated by chloroform separation and precipitated with isopropanol. RNA concentrations and purity (260/280 and 260/230 values) were measured on a Nanodrop (Thermo Scientific) and cDNA was synthesized from 950 ng RNA with random hexamer primers. For qPCR cDNA samples were diluted 30 times and qPCR was performed with SensiFast Sybr Hi-ROX-kit (GC Biotech) on a Lightcycler480 (Roche) according to manufacturer's protocol. All reactions were performed in duplo. Primers were targeting housekeeping genes *Gapdh* (forward GTGCTGAGTATGTCGTGGAG; reverse TCGTGGTTCACACCCATCAC) and 18S (forward TTCTCGATTCCG TGGGTG; reverse GCGTAACTAGTTAGCATGCC) or target genes *Gfap* (forward AAGCCAAGCACGAAGCTA ACGA; reverse TTGAGGCTTTGGCCCTCC), *Olig2* (forward TGTGGATGCTTATTACAGACC; reverse ATCTAAGCTCTCGAATGATCC), *Glast* (forward TGTCATTGTGGGTACAATCCT; reverse ACAGCAATGAT GGTAGTAGTC), *Pdgfra* (forward CCGGGCTAAGGAAGAAGAC; reverse ATGCAGGAACTAGGGTTGAC), *Ngf1* (forward ACTTCCAGGCCCATGGTACAATCT, reverse TTGATGTCCGTGGCTGTGGTCTTA), *Nt3* (forward ACTACGGCAACAGAGACGCTACAA, reverse ATAGCGTTTCCTCCGTGGTGATGT), *Pdgfa* (forward CCAGCGACTCTTGGAGATAGAC, reverse GAATGGCTTCCTCAATACTTCTCT), *Egf* (forward CTG GACAGACAGTGGGAAGTC, reverse CGTCCGTCCAGAACAGTCTC), *Lif* (forward TGCCAATGGGACAGA GAAGACCAA, reverse TACTTGTTGCACAGACGGCAAAGC), *Igf1* (forward TGGCACTCTGCTTGCTCAC CTTTA, reverse TTGGTCCACACACGAACTGAAGAG), and *Tgfβ* (forward ACTGGAGTTGTACGGCAGTG, reverse GGCTGATCCCGTTGATTTCC). Primer sequences for *Ngf1*, *Nt3*, *Lif* and *Igf1* are obtained from Hawryluk et al. (2012). Data was analysed according to the $2^{-\Delta CT}$ method, using housekeeping CT values and target CT values per sample to calculate ΔCT values.

Immunohistochemistry and cell counts

Fluorescent immunostainings were performed on snap-frozen brain sections. Sagittal brain sections were cut on a Leica CM 1950 cryostat (Leica Microsystems) in 12 µm thickness and in series of 12 slides with 3-6 brain sections per slide. The number of brain sections was the same for all slides within each series of 12 slides, and within one series first one brain section is added on all slides, before proceeding with the second brain section on each slide. This way of cutting ensures that all slides within one series are from similar lateral distances.

For immunostaining slides were washed with PBS for 30 min and treated with citrate buffer at 90°C for 10 min to retrieve antigens. After cooling down, sections are incubated in blocking solution (PBS+ 5% normal goat serum + 0.1% bovine serum albumin + 0.3% Triton X-100) for 1 hour and incubated in primary antibody diluted in blocking solution overnight at 4°C. The next day, slides are washed for 30 min in PBS and incubated in secondary

antibody (Alexa Fluor 488- , Alexa Fluor 594-, or Alexa Fluor 647-tagged secondary antibodies; 1:1000, Fisher Scientific) in blocking solution for 2 hours at room temperature (RT). Pictures were taken with a Leica DM6000B microscope (Leica Microsystems). Brightness and contrast was optimized using Adobe Photoshop CS6. Antibodies used targeted GFP (1:500, Aves Labs, GFP-1020), GFAP (1:1000, DAKO, Z0334), OLIG2 (1:500, gift of J.H. Alberta, Harvard University, Boston, Massachusetts, USA), Nestin (1:500, BD Biosciences, 611658) and S100β (1:1000, ProteinTech, 15146-1-AP).

For Nestin cell counts, tissue sections were stained with Nestin and GFAP primary antibodies. All pathology measurements were performed on 9 months old mice of all groups: WT saline (n=4), VWM saline (n=7), A2B5 cells (n=8), GLAST cells (n=6) and PDGFαR cells (n=5) injected mice. Per animal at least 6 pictures of 100x magnification of the rostrum and splenium of the corpus callosum were taken. On each picture, the number of $Nestin^+/GFAP^+$ cells were counted, and expressed as a ratio to the total number of $DAPI^+$ nuclei.

Bergmann glia translocation was assessed with S100β staining. All pathology measurements were performed on 9 months old mice of all groups: WT saline (n=4), VWM saline (n=7), A2B5 cells (n=8), GLAST cells (n=6) and PDGFαR cells (n=5) injected mice. For each animal, at least 6 pictures of the cerebellum at 100x magnification were taken, all including the Purkinje cell layer and the molecular layer. With ImageJ software (NIH) the number of $S100\beta^+$ cell bodies in the Purkinje cell layer and the molecular layer were quantified as described previously (Dooves et al., 2017). The number of $S100\beta^+$ cell bodies in the molecular layer were considered translocated, and were expressed as the ratio of translocated cell bodies to the total number of $S100\beta^+$ cell bodies in the Purkinje cell layer and the molecular layer.

To analyse cell survival and cell fate one slide from each series of 12 slides was stained for GFP expression. The total number of engrafted cells was estimated in one hemisphere per animal based on counts of GFP+ cells in different brain sections. On each slide the number of GFP^+ cells per brain area was quantified. To estimate the total amount of GFP^+ cells present the total number of counted cells was multiplied by 12. This gives an estimation of the number of cells present in half of the brain. One brain half receives 3 injections of 100.000 cells, so 300.000 cells of which about 70-80% is GFP^+, so about 225.000 GFP^+ cells per brain half are injected. This amount is used to calculate the percentage of engrafted cells (# of GFP^+ cells divided by the # of injected cells). Cell survival was analysed in 2, 5 and 9 months old animals that received injections with A2B5 (2m n=4, 5m n=4, 9m n=5), GLAST (2m n=3, 5m n=5, 9m n=3) or PDGFαR (2m n=6, 5m n=5, 9m n=5) sorted cells.

To analyse cell fate pictures of GFP^+ cells were taken in the cortex (which contains transplanted cells in practically all animals). On each picture the total amount of GFP^+ cells was counted, as well as the number of $OLIG2^+/GFP^+$ and $GFAP^+/GFP^+$ cells. The cell fate is expressed as the ratio of $OLIG2^+/GFP^+$ or $GFAP^+/GFP^+$ cells over the total amount of GFP^+ cells. At least 50 cells were counted per mice. Cell fate was analysed in 2, 5 and 9 months old animals that received injections with A2B5 (2m n=4, 5m n=3, 9m n=4), GLAST (2m n=3, 5m n=4, 9m n=3) or PDGFαR (2m n=5, 5m n=5, 9m n=5) sorted cells.

In situ hybridization and cell counts

Tissue sections were probed against *Plp* mRNA as described previously (Dooves et al., 2016). The dig-labelled *Plp* probe was incubated overnight in hybridization buffer on tissue sections that were pre-treated with proteinase K. Anti-digoxygenin-AP (1:2000, Roche) was used to target the *Plp* probe and the staining was developed overnight with BM Purple (Roche). Nuclei were counterstained with 0.5% methylgreen at 60°C for 5 minutes. All pathology measurements were performed on 9 months old mice of all groups: WT saline (n=4), VWM saline (n=7), A2B5 cells (n=8), GLAST cells (n=6) and PDGFαR cells (n=5) injected mice. Pictures at a 100x magnification were taken of the white matter of the cerebellum and the corpus callosum. The total number of *Plp*-expressing cells was counted in a 100 x 200 µm rectangle. Per animal at least 6 fields per area were counted.

Statistical analysis

All data was analysed using SPSS software (IBM SPSS Statistics 20.0). A Shapiro-Wilk test was performed to test normal distribution of the data. Besides the count of cells *in vitro* after sorting, all data was normally distributed. Counts of cells *in vitro* were analysed with a Mann-Whitney U test. QPCR analysis of cells *in vitro,* cell fate *in vivo* and cell survival *in vivo* were analysed with an ANOVA and post-hoc Tukey test. A MANOVA was used to analyse the different treatment groups on all disease markers. Next a discriminant analysis was used to cluster saline WT and *2b5*^{ho} animals based on the different cell counts. The Box's M test was not significant, suggestion equality of the variance-covariance matrix, showing that the data met the assumptions of the discriminant analysis (normally distributed and equal variance/covariance matrixes). All disease markers had a significant mean difference between WTsal and VWMsal and were included as predictor variables, leading to a highly significant discriminant function that explained 95.1% of the variability ($p<.001$) and could classify all saline-injected animals correctly as WT or

VWM. The formula found in the discriminant analysis was then applied to all transplanted animals to cluster them either with the WT or the *2b5*^{ho} group.