

## **MESENCHYMAL STEM CELLS RESCUE PURKINJE CELLS AND IMPROVE MOTOR FUNCTIONS IN A MOUSE MODEL OF CEREBELLAR ATAXIA**

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## **ABSTRACT**

Mesenchymal stem cells have been proven to be potentially effective in the treatment of a large variety of diseases, including neurodegenerative disorders. Of these, cerebellar ataxia is a group of disorders characterized by the degeneration of the cerebellum, particularly the Purkinje cells, responsible for motor coordination and control of the motor functions. To analyze the possibility of using bone marrow-derived mesenchymal stem cells in treating ataxia, we transplanted these cells in the cerebellum of newborn Lurcher mutant mice, a very aggressive mouse model characterized by the selective early post-natal death of Purkinje cells in the cerebellum. Two months after the surgical procedure, the treated mice presented significant improvements in the motor behavior tests performed. Histological analysis of the cerebellum indicated that the donor cells had migrated throughout the cerebellum, as well as a significant increase in the number of Purkinje cells. Many grafted stem cells were located adjacent to the Purkinje cell layer, and expressed BDNF, NT-3 or GDNF, neurotrophic factors implicated in Purkinje cell survival. Also, a small percentage of the grafted stem cells had fused with Purkinje cells. Thus, we have shown that mesenchymal stem cells are capable of integrating into the central nervous system, migrate towards the areas where neurodegenerative processes are occurring, and rescue the degenerating cells through cell trophic effects. This is an adequate and feasible model that could be translated into a therapeutic approach for clinical assays in neurodegenerative diseases.

**KEY WORDS:** Mesenchymal stem cells, Lurcher mouse, cerebellar ataxia, neurotrophic effect, Purkinje cells, bone marrow, cell fusion

## **INTRODUCTION**

Bone marrow transplantation has been demonstrated to be a feasible therapeutic approach for the treatment of a variety of diseases, such as leukemia and lymphoma. Also, many studies have shown that stem cells in the bone marrow, particularly mesenchymal stem cells, are potentially a useful tool for the rescue and protection of target cells in degenerative disorders, including neurodegenerative diseases. In this type of diseases, the rescue has been proven to occur for several reasons. For example, several studies have demonstrated that mesenchymal stem cells are capable of releasing neurotrophic factors such as NGF, BDNF and NT-3, among others (Chen Q. et al 2005, Caplan A.I. et al 2006, Rodrigues-Hell R.C. et al 2009), which protect the degenerating cells and increase their chance for survival. Also, stem cells can fuse with the host's degenerating neurons (Alvarez-Dolado M. et al 2003, Weimann J.M. et al 2003a and 2003b, Johansson C.B. et al 2008). In general terms, this process occurs at a very low frequency, thus was initially considered to be therapeutically inefficient (Wagers A.J. et al 2002). However, a recent publication has shown that under certain circumstances, the fusion process may occur more frequently (Johansson C.B. et al 2008), reconsidering this process as a feasible therapeutic method. Thus, the objective of this study was to analyze the processes by which bone marrow-derived mesenchymal stem cells rescue neurons in a neurodegenerative mouse model of cerebellar ataxia.

To this end, we used the Lurcher mutant mouse. This mouse model, first described in 1960 by R.J.S. Philips, is a spontaneous semidominant mutation characterized by a wobbly ataxia due to the selective postnatal degeneration of Purkinje cells as well as the majority of their primary afferents, granule cells and

olivary neurons (Phillips R.J.S. et al 1960). The cause for this degeneration is due to a mutation of the  $\delta 2$  glutamate receptor (GluR $\delta 2$ ) (Zuo J. et al, 1997). Homozygous animals die around birth, whereas heterozygous animals are viable, and have been extensively used in studies of neurodegeneration and survival. Heterozygotes begin to present abnormalities in the Purkinje cells as soon as 3-4 days after birth. These cells, due to the mutation in the glutamate receptor, present a constitutive cationic current, which ultimately causes their death. The process by which this cell death occurs is not well understood, where autophagy, necrosis and apoptosis processes have been described. By 3 months of age, virtually all Purkinje cells, and the vast majority of granule cells and olivary neurons, have disappeared. For a review on the Lurcher mice, see Vogel et al, 2007.

In our approach, we transplanted either whole bone marrow or bone marrow-derived mesenchymal stem cells into the cerebellum of newborn Lurcher mice, before the neurodegeneration processes commence. Several authors have used this strain as a mouse model of neurodegeneration, usually transplanting neural progenitors or adult Purkinje cells (Tomey D.A. et al 1993, Dumesnil-Bousez N. et al 1993a and 1993b, Heckroth J.A. et al 1998, Cendelin J. et al 2009), however this is the first report to use bone marrow-derived stem cells in this model. The mice, once they reached a certain age, were submitted to behavior tests on the rotarod to analyze their possible motor function recovery compared to sham operated and non-grafted heterozygous Lurcher mice. After the tests, the mice were sacrificed and their brain analyzed for the detection of the grafted cells, as well as to study the processes induced by these cells in the cerebellum.

## **MATERIALS AND METHODS**

### **1. Animals**

All the experiments with animals have been performed in compliance with the Spanish and European Union laws on animal care in experimentation (Council Directive 86/609/EEC), and have been analyzed and approved by the Animal Experimentation Committee of our university. Green fluorescent protein (GFP) transgenic mice and Lurcher mutant mice were bred and maintained in our animal facilities. GFP mice ubiquitously express the green fluorescent protein under the control of the  $\beta$ -actin promoter (Okabe M. et al 1997).

### **2. Bone Marrow Isolation and Mesenchymal Stem Cell Culture**

Unless stated otherwise, all the materials and substances were purchased from Sigma-Aldrich. Femurs were dissected from 6- to 8-week old GFP mice, sacrificed by cervical dislocation. Bone marrow was extracted, and single-cell suspensions were obtained by mechanical dissociation. In the case of whole bone marrow (BM) transplantation, the cells were re-suspended in D-MEM and taken to the surgery room. In the case of mesenchymal stem cell (MSC) culture, the bone marrow suspension was washed and centrifuged, and the pellet was then re-suspended in D-MEM (Invitrogen) supplemented with 15% FBS (Biochrom AG, Berlin), 100 U/ml penicillin/ streptomycin, 1 mM sodium pyruvate, 1% non-essential amino acids, and 1 ng/ml FGF2. These cells were placed in culture flasks and the plastic-adherent population was isolated and allowed to proliferate for 3-4 weeks, changing the media every 2-3 days.

Approximately every 5-7 days, the cells were trypsinized with trypsin/EDTA 0.25% and replated in 75 cm<sup>2</sup> flasks. After 3-4 weeks, all the cells in culture expressed CD90 and CD44 and were negative for CD34 and CD45, confirming that the cell population was mainly composed of mesenchymal stem cells (data not shown).

### 3. Cell Transplantation

Mesenchymal stem cells were injected into the cerebellum of newborn Lurcher mice. To this end, the mice were cryoanesthetized before the surgical procedure. With a glass capillary attached to a Hamilton syringe (Hamilton, Reno, Nevada), either 500,000 BM or 250,000 MSC cells in 2 µl of D-MEM were unilaterally injected into the cerebellum, located 2.5 mm caudal to Lambda (beginning at the midline), 0.5 mm deep. The treated mice were compared to control Lurcher mutants which were not submitted to the transplantation process, and to sham controls, which were injected with 2 µl of D-MEM.

After the injection, the mice were reanimated and quickly placed with their mothers to avoid rejection.

### 4. Rotarod Assays

The rotarod test was performed on an 8500 Rota-rod (Leticia Scientific Instruments, Barcelona, Spain). The lane is 500 mm wide, and the rod has a diameter of 30 mm. First, once the mice reached three weeks of age and the symptomatic mice could be detected, they were trained daily for one week on the rotarod. Afterwards, they were analyzed on a weekly basis, taking note of

the time they spent walking on the rotating rod at a constant speed of 4 rpm. Also, their maximum walking speed was noted, by increasing the speed at a constant rate of 4 to 40 rpm in a 1-minute interval until the animal fell from the rotarod. The mice were placed on the rotarod 10 times for each test, and the average value was calculated. These tests were performed for a total of 5 weeks, until the mice reached the age of 8 weeks.

##### 5. Tissue Fixation and Immunohistochemistry

After the 5-week rotarod tests, the mice were anesthetized with isofluorane and fixed by intra-cardiac perfusion with 4% filtered paraformaldehyde (PFA) in phosphate buffer (pH 7.4). The brain was carefully excised and kept in 4% PFA overnight. After fixation, the samples were cryoprotected by increasing concentrations of sucrose (up to 30%) and finally embedded in Neg-50 Frozen Section Medium (Richard-Allan Scientific, Kalamazoo, Michigan), frozen, and stored at -20°C. Thirty micrometer sagittal sections were obtained using a Microm HM525 cryostat and mounted on slides.

The sections were first incubated at room temperature in 10% goat serum, 5% bovine albumin, 0.25% triton and PBS to permeate the tissue and block non-specific binding. Afterwards, the sections were incubated overnight at room temperature with the primary antibody, diluted in blocking solution (10% goat serum, 5% bovine albumin and PBS). The following primary antibodies were used: rabbit anti-calbindin (1:3000, Swiss Antibodies, Bellinzona, Switzerland), mouse anti-GFP (1:200, Molecular Probes, Eugene, OR), rabbit anti-GDNF (1:100, Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-BDNF (1:200, Santa Cruz Biotechnology, Santa Cruz, CA), sheep anti-

neurotrophin-4/5 (NT-4/5, 1:100, Chemicon/Millipore, Billerica, MA), sheep anti-nerve growth factor (NGF, 1:500, Chemicon/Millipore, Billerica, MA) and sheep anti-neurotrophin-3 (NT-3, 1:100, Chemicon/Millipore, Billerica, MA). The following day, the sections were incubated with the secondary antibody. For GFP staining, we used anti-mouse Alexa Green (1:500, Molecular Probes), and for the other antibodies biotinylated secondary antibodies were used (1:200, Vector Laboratories, Birmingham, California) followed by an incubation with streptavidin conjugated with Cy3 (1:500). DAPI (Molecular Probes) was used to stain nuclei. In some cases, the secondary antibody used was conjugated with peroxidase for DAB staining (Applichem, Germany), and the tissue counterstained with cresyl violet (Acros Organics, Belgium). Histological samples were observed under a fluorescence microscope (Leica DMR, Leica Microsystems) and micrographs taken with a confocal microscope (Leica DMR).

## 6. Cell Count

To count the number of Purkinje cells and grafted cells, immunohistochemistry was performed for calbindin and GFP, respectively. Then, the positive cells in the 10 center-most sections and the average number per section was calculated in the control and experimental cerebella. This was also performed to count the number of fused cells, as well as the grafted cells expressing neurotrophic factors.

## 7. Post-transplant Cerebellar Culture and Immunocytochemistry



After the MSC transplant and rotarod assay, 3 mice from 3 different experiments were sacrificed and brain extracted. The cerebellum was isolated and placed in a culture dish with D-MEM, minced into small pieces and mechanically disaggregated. The tissue was then centrifuged and the resulting pellet was re-suspended in standard MSC culture medium (described previously) and placed in several 35 mm treated culture dishes. After 5 days of culture, the cultures were fixed with 4% paraformaldehyde and stained for GFP and calbindin. The staining protocol used was similar to the tissue staining commented previously, only 0.025% Triton was used to permeabilize the cells. The samples were visualized under the fluorescence microscope.

## 8. Statistical Analysis

Statistical significance between control and experimental groups were calculated with SPSS v11.0 software, using ANOVA and the non-parametric Mann Whitney test.

## **RESULTS**

### 1. MSC-treated mice significantly improve in the behavior tests

A total of 38 lurcher mutant mice were used, distributed in 15 non-treated controls, 8 shams, 5 BM-treated and 10 MSC-treated mice. The experimental approach consisted in transplanting newborn Lurcher mice with either D-MEM (for shams), recently extracted bone marrow (BM-treated mice), or cultured mesenchymal stem cells (MSC-treated mice). Once the mice reached the age of 3 weeks, the symptomatic mice were isolated and trained in the rotarod at

both a constant speed of 4 rpm and at increasing speeds. This was performed during a total of 6 weeks, in which during the first week the mice were placed daily as training for the weekly tests performed afterwards. After the 5-week tests, the mice were sacrificed and brains extracted and processed for histological analysis (See Figure 1A for a chronogram of the experimental procedure).

During the first runs, the mice had many difficulties to walk on the rotarod, where in many cases they would just hold on to the rod until they fell, as previously seen by other authors (Hilber P. et al 2001). However, after the training period, the mice were capable of walking on the rod for several seconds in a similar fashion as a wildtype mouse would. The results of the rotarod tests are shown in Figure 1B. When the rotarod was placed at a constant speed of 4 rpm, the control mice were capable of walking, on average, for 12-20 seconds before falling (n = 10). Similar values were obtained with sham controls, which walked for 14-19 seconds (n = 8). These values were very stable throughout the weeks, with no evident improvement throughout time. As for the treated mice, the BM mice obtained values that were above the control and sham mice, though not significant (average 15-45 seconds, n = 5). On the other hand, MSC mice were capable of walking on average for 60-100 seconds, almost 5 times longer than the controls (n = 10). Both experimental groups seemed to obtain increasingly higher scores with each passing week, which was not observed in the controls. The average deviations of the charts, particularly in the MSC group, are very large, due to the variability of each mouse. For example, one of the MSC mice was capable of walking for over 400 seconds. Despite the variability, it was clear

that the MSC-treated mice obtained significantly better results than the control mice in this test ( $p < 0.05$ ,  $p < 0.001$  with Mann Whitney test).

As for the rotarod test at increasing speeds, on average, the control mice were capable of walking until reaching speeds of 9-12 rpm, while the sham averaged 9 rpm. As for the treated mice, the BM mice had scores that were similar to the controls (9-13 rpm) while the MSC reached slightly higher scores (12-14 rpm). Except for one of the weeks, the MSC-treated mice presented significantly higher values compared to the controls ( $p < 0.05$ ,  $p < 0.001$  with Mann Whitney).

Thus, MSC mice were capable of walking on the rotarod for much longer periods of time, indicating a significant improvement in the motor coordination of these mice.

## 2. After 1 month, mesenchymal stem cells have migrated throughout the cerebellum using the white matter

Several non-symptomatic treated mice were sacrificed at 1 month of age, to confirm that the injected cells survived the surgical procedure ( $n=5$ ). In these mice, the transfected cells could clearly be detected throughout the whole cerebellum (Figure 2). These cells were either spherical-shaped or elongated (Figure 2A-E). From the injection site, it was possible to detect GFP+ cells traveling around the exterior of the cerebellum, in between the lobes, until finally penetrating through the molecular layer into the white matter (Figure 2C-E). Once in the white matter, the cells migrated throughout the whole cerebellum. The vast majority of the cells were located in the white matter, although a few were found in the various layers of the cerebellar cortex. As

for the BMC-treated mice, the majority of the cells were detected outside the cerebellum (Figure 2F). These cells were spherical-shaped, and did not seem to be capable of penetrating into the cerebellar tissue as seen with MSC.

Thus, transplanted mesenchymal stem cells, but not whole bone marrow cells, are capable of integrating into the cerebellar tissue and migrate throughout the cerebellum.

### 3. Increased number of Purkinje cells in 2 month-old MSC-treated mice

After the 5 weeks of rotarod assay, control and treated mice were sacrificed, perfused and processed by immunohistochemical detection of the injected cells, as well as counter-stained with calbindin immunoreactivity to identify the Purkinje cells (n=10 in controls, n=8 in sham controls, n=5 in BMC-treated mice, and n=10 in MSCs-treated mice). Also, several control mice were sacrificed at 1 month of age (n=5). In the 1 month-old control mice, the wobbling ataxia of the mouse was very noticeable, and this coincided with a low number of Purkinje cells in the cerebellum found in these mice (10-12 Purkinje cells on average per serial section, see Figure 3A). At 2 months of age, this number further decreased to 0-4 Purkinje cells per section (Figure 3A, B, and E). Similar values were obtained in the sham controls and BM-treated mice. In the MSC-treated mice, however, at 2 months of age, approximately 12 Purkinje cells were detected per section, similar to the number found in the mice at 1 month of age (Figure 3A, C and F). As in the case of the 1 month-old control mice, the majority of the surviving Purkinje cells were located in lobule X. No significant differences were observed in the granule layer of both control and experimental groups (Figure 3D and G,

respectively). As for the BMC-treated mice, there was no significant increase in the number of surviving Purkinje cells compared to the controls. Also, as seen at 1 month (Figure 2F), there was practically no presence of bone marrow cells in the cerebellum. At this point, the injection sites of both MSCs and BMCs mice were practically undetectable except for a few cases.

As seen in Figure 3A, the number of Purkinje cells per serial section was calculated in the 10 sections. Also, the number of mesenchymal stem cells per section was calculated and compared to the number of Purkinje cells. As a result, we detected  $7.5 \pm 3.8$  GFP+ cells per surviving Purkinje cell. There was very little variability in the number of Purkinje cells detected ( $124.0 \pm 18.9$ ), while the number of grafted cells was very variable (418-1918). Thus, the number of engrafted cells detected in the cerebellum is not related to the number of surviving Purkinje cells.

Also, although differences in granule cells were not significant, we observed an increased immunoreactivity in parvalbumin and GFAP in the molecular layer of the treated mice (Figure 3H and J, respectively). Parvalbumin stains Purkinje cells as well as basket and stellate cells, which are also affected in this mutant mouse, whereas GFAP is a glial marker. Compared to controls (Figure 3I and K), treated mice presented a significant increase both cell types.

Our results indicate that MSCs, but not bone marrow cells, support the survival of the Purkinje cells detected at 1 month of age, considering the similarities both in number and distribution of 1 month-old controls and 2 month-old treated mice. Also, the grafted cells increased the survival of other

cell types in the cerebellum such as basket and stellate interneurons, as well as the GFAP immunoreactive glial cells.

4. Mesenchymal stem cells come in contact with dying purkinje cells, avoiding their death through neurotrophic factor release

Two months after the transplant, once the behavior assays have been completed, the MSC-treated mice were sacrificed and analyzed (n = 10). In this case, the majority of the transplanted cells were not located in the white matter, as seen after 1 month. At this moment, the GFP+ cells were generally located either throughout the cerebellar parenchyma forming multiple branched or spherical structures, or in the Purkinje cell layer (Fig. 3F, 4A). Clusters of grafted cells were always localized close or in physical contact with the surviving Purkinje cells (Fig. 4B), as well as surrounding blood vessels (Fig. 4C). In some mice, it was possible to detect a small fraction of GFP+ Purkinje cells with 2 nuclei (Fig. 4D-E), though the percentage of these cells was very low compared to the number of GFP+ cells (approximately 1 in 100 Purkinje cells were GFP+). This indicates a possible cell fusion of the transplanted cells with the native Purkinje cells. To further corroborate this observation, two months after the transplant, several MSCs-treated mice were sacrificed and their cerebella removed for culture (n = 3). The resulting cells were cultured in mesenchymal stem cell medium in order to obtain the surviving transplanted cells. Initially, it was possible to observe a few GFP+ cells floating among the tissue debris, which would eventually adhere to the culture dish (Fig. 5A). The cells were mainly spherical-shaped, and many

presented 2 nuclei (Fig. 5B-C). Also, some were calbindin positive. These cells did not proliferate in the 7 days the culture was maintained.

However, cell fusion can not be considered an important cause for the increased number of surviving Purkinje cells in the treated mice, as it accounts for only 1% of the Purkinje cells, thus other mechanisms must be in effect. Since surviving Purkinje cells were mainly localized close to clusters of grafted cells, we postulated that the grafted cells were either directly expressing or inducing the expression of neurotrophic factors. To this end, immunohistochemical analysis of several neurotrophic factors was performed (Fig. 6). Of the various neurotrophic factors analyzed, three were expressed in the majority of the grafted cells: BDNF, NT-3 and GDNF (Fig. 6A-C, respectively). These factors have been previously proven to be involved in the survival and proliferation of Purkinje cells (Mount H.T. et al 1994, Mount H. et al 1995, Lärkfors L. et al 1996, Tolbert D.L. et al 2003, Chen Q. et al 2005). The majority of the grafted cells expressed BDNF (56.0% +/- 35.2%), while a small fraction expressed NT-3 (7.9% +/- 1.6%) or GDNF (1.1% +/- 0.9%). Thus, the expression of these factors by the grafted cells may be one the main causes for the increase in survival of the Purkinje cells in the treated mice.

## **DISCUSSION**

We have demonstrated that bone marrow-derived mesenchymal stem cells avoid Purkinje cell death, as well as molecular layer interneurons and glial cells, in a neurodegenerative mouse model of cerebellar ataxia. Since the increase in the number of glial cells, basket and stellate neurons coincided with an increase

in surviving Purkinje cells, the survival of these cells seem to be the result of secondary trophic mechanisms. At least two processes seem to be responsible for this neuroprotection: neurotrophic factor release and to a much lesser degree cell fusion, without discarding the possibility of mechanical cell-cell trophic interactions. Whole bone marrow transplants, on the other hand, were not capable of improving the motor functions of the treated mice. Histological analysis showed that the cells were not capable of penetrating the cerebellar lobes, but rather remained on the outer regions of the cerebellum. Mesenchymal stem cells, however, were capable of penetrating into the cerebellum and come in contact with the Purkinje cells. In this manner, these cells could activate the necessary processes to induce neuroprotection of the Purkinje cells.

It is particularly interesting that mesenchymal stem cells but not whole bone marrow transplants induce a significant increase in Purkinje cells. In a previous report we observed a selective neurotrophic activity of whole bone marrow cells in motoneuron survival using an amyotrophic lateral sclerosis mouse model (Cabanés C. et al 2007). This indicates that the neurotrophic potential of a cell population for cell therapy in neurodegenerative diseases varies depending on the specific neuronal population to rescue. Thus, it is necessary to consider the possibility of selecting different cell populations (such as whole bone marrow or cultured mesenchymal stem cells) for an efficient neurotrophic effect on specific neuronal populations.

It has been previously shown in various reports that it is possible for cells of various origins to fuse with Purkinje neurons (Bae J.S. et al 2005, Bae J.S. et al 2007, Nygren J.M. et al 2008), although this is the first time to be reported in



Lurcher mice. In this model, where Purkinje cells begin to deteriorate at very early ages (postnatal day 3-4), we decided to transplant the cells at P0, when the cerebellum is still intact and Purkinje cells healthy. The transplanted cells were capable of integrating well into the tissue, and by the time the mouse reached the age of one month, the stem cells had already extended throughout the whole tissue. However, by this time the majority of the Purkinje cells had already disappeared. The few surviving neurons seem to be rescued from their inevitable death thanks to the mesenchymal stem cells, which can be detected throughout the whole tissue, although mainly localized in clusters near surviving Purkinje cells. In several cases, cell fusion could be detected by the presence of GFP+, calbindin+, double-nucleated Purkinje cells. This was corroborated by the extraction and culture of the cerebellum, which resulted in obtaining GFP+/calbindin+ cells with 2 nuclei. However, cell fusion accounts for a very small percentage of the surviving Purkinje cells, thus other mechanisms must be in effect to account for the increased number of neurons in the treated mice. Mesenchymal stem cells acting as trophic mediators have been widely studied (for a review see Caplan A.I. et al, 2006). We noticed that an important number of GFP+ cells did not fuse with Purkinje cells, but rather were located near the Purkinje cell layer. The majority of the grafted cells expressed BDNF, whereas a small percentage of cells expressed other factors such as NT-3 and GDNF. These three neurotrophic factors are implicated in Purkinje cell survival and differentiation (Mount H.T. et al 1994, Mount H. et al 1995, Lärkfors L. et al 1996, Tolbert D.L. et al 2003, Chen Q. et al 2005).

Despite the low number of Purkinje cells that are rescued, the treated mice respond more efficiently in the behavior tests, especially in the constant speed

tests. Thus, it is not necessary to recover a large amount of Purkinje neurons in order to detect a significant improvement. Also, many of the rescued Purkinje neurons were found in lobule X (or the vestibulocerebellum) of the cerebellum, which regulates balance and eye movements. This corroborates with the improved rotarod results in the treated mice. However, for the mice to improve more significantly in this test, it would be necessary to rescue a larger number of Purkinje cells.

In several reports, cell fusion was induced in response to an inflammation or injury process (Magrassi L. et al 2007, Johansson C.B. et al 2008). Here, besides the injection, there is no induced injury. Thus, no further damage, besides the injection of the cells, was necessary to obtain a positive result.

In conclusion, bone marrow-derived mesenchymal stem cells are capable of ameliorating the symptoms of a neurodegenerative disorder such as that found in Lurcher mice, by rescuing Purkinje cells from their inevitable death through at least cell fusion and neurotrophic factor release, and ultimately improving their motor functions, confirming their potential for a possible cellular therapy in neurodegenerative diseases.

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## FIGURE LEGENDS.

**FIGURE 1.** A) Diagram depicting the chronological scheme of the experimental procedure. B) Histograms comparing the results of control (black bars), sham controls (white bars), bone marrow (BM) treated mice (grey bars), and mesenchymal stem cell (MSC) treated mice (blue bars) in the rotarod tests. The top histogram presents the seconds the mice were capable of walking before falling when the rotarod was placed at a constant speed of 4 rpm, where the MSC-treated mice showed a significant improvement beginning on the second week ( $p < 0.05$ ,  $p < 0.001$  with Mann Whitney). The bottom histogram presents the maximum speed achieved by the mice when placed in the rotarod at increasing speeds (4-40 rpm in regular intervals). In this histogram, the asterisk marks the weeks in which the MSC-treated mice had significantly higher values than the controls ( $p < 0.05$ ,  $p < 0.001$  with Mann Whitney). In both histograms, the values are presented as mean  $\pm$  average deviation. Control mice  $n=10$ , Sham control mice  $n=8$ , BM treated mice  $n=5$ , MSC treated mice  $n=10$ .

**FIGURE 2.** Immunohistochemical analysis of the cerebellum in a 1 month-old non-symptomatic Lurcher mouse treated with either mesenchymal stem cells (A-E) or bone marrow (F). In all images, green is GFP and blue is nuclear DAPI. A) Sagittal section taken at 100X demonstrating the presence of spherical-shaped MSC in the white matter of the treated mice. B) Low-powered sagittal image (40X) of the cerebellum in which the injection site was detected as a cluster of GFP+ cells (white arrow). Also, additional elongated mesenchymal stem cells were detected between the cerebellar lobes (light blue arrow) and

penetrating through the molecular layer of one of the lobes and into the white matter (red arrow). C) Image of the white arrow in (B), taken at 400X, showing the injection site and the cells traveling between the cerebellar lobes. D and E) High powered images (630X) of the light blue and red arrows in (B), respectively, where it is possible to see the elongated GFP+ cells traveling between the cerebellar lobes and through the molecular layer of one of the lobes. F) Cerebellar sagittal section of a bone marrow-treated mouse taken at 40X. In this case, as opposed to the mesenchymal stem cells, the cells maintained a spherical shape and very few were detected inside the cerebellum. In all the images, PCL marks the Purkinje cell layer, ML the molecular layer, WM the white matter, and GL the granular layer. Scale bar measures 20  $\mu\text{m}$  in A, 50  $\mu\text{m}$  in B and F, 5  $\mu\text{m}$  in C, and 8  $\mu\text{m}$  in D and E.

**FIGURE 3.** Number and distribution of Purkinje cells in 1 and 2 month-old control and 2 month-old treated mice. A) Histogram presenting the number of Purkinje cells detected per sagittal section in 1 month-old controls (Non-TX Sympt 1 month, n=5), 2 month-old controls (Non-TX Sympt 2 months, n=10), 2 month-old sham controls (Sham-TX 2 months, n=8), 2 month-old BM treated mice (BM-TX 2 months, n=5) and 2 month-old MSC treated mice (MSC-TX 2 months, n=10). There was no significant difference among the 2 month-old controls, shams and BM-treated mice. However, the number of Purkinje cells in the MSC-treated group was significantly higher ( $p < 0.001$ ). Bars represent mean  $\pm$  average deviation. B, D, E) Immunohistochemical images of sagittal sections of the cerebellum in a 2 month-old symptomatic control mouse (100X magnification in (B), 400X in (D) and 40X in (E)). C, F, G) Immunohistochemical



images of sagittal sections of the cerebellum in a 2 month-old symptomatic MSC treated mouse (100X magnification in (C), 40X in (F) and 400X in (G)). In (B-C) and (E-F), Purkinje cells are stained with calbindin in red and DAPI is used to stain the nuclei, whereas in (D) and (G), calbindin staining is performed with DAB and the tissue counterstained with violet cresyl. In (C), (D) and (G), PCL identifies the Purkinje cell layer, ML the molecular layer, WM the white matter, and GL the granular layer. H, I) Parvalbumin staining in mesenchymal and sham-treated mice, respectively. J, K) GFAP staining in mesenchymal and sham-treated mice, respectively. Scale bar measures 20  $\mu\text{m}$  in B and C, 50  $\mu\text{m}$  in E and F, 5  $\mu\text{m}$  in D and G-K.

**FIGURE 4.** Immunohistochemical analysis of the cerebellum of 2-month old MSC treated mice. A) Low powered transverse image (40X) of the cerebellum, where green GFP+ MSC cells are detected throughout the section. Several cells (white arrows), particularly those detected in lobe X, co-express calbindin (red), a Purkinje cell marker. B) High powered image (400X) of a sagittal section of the cerebellum, in which GFP+ MSC were detected in contact with Purkinje cells. C) High powered image (400X) of a sagittal section of the cerebellum, in which GFP-positive cells were located surrounding or forming blood vessels. In this case, the MSC were also detected nearby or in contact with Purkinje cells. (D) High-powered immunohistochemical image (200X) of a GFP and calbindin-positive Purkinje cell. Blue is nuclear DAPI staining. (E) Close-up image of the Purkinje cell in (D), where only the DAPI staining is seen, detecting the presence of 2 nuclei in the cell, confirming cell fusion of a grafted mesenchymal stem cell with a native Purkinje cell. In this image, the green and white lines

delimit the two nuclei present in a single Purkinje cell. In (A-C), PCL identifies the Purkinje cell layer, ML the molecular layer, WM the white matter, and GL the granular layer. Scale bar measures 50  $\mu\text{m}$  in A, 5  $\mu\text{m}$  in B and C, and 10  $\mu\text{m}$  in D.

**FIGURE 5.** Cell culture of cerebellum extracted from MSC-treated mice. A) Phase contrast image of the cerebellar suspension after one day of culture, where several GFP-positive cells could be found floating among the debris (Image taken at 200X). These cells adhered to the plate after 2-3 days of culture. (B-C) Immunocytochemical analysis of the suspension culture after 5 days. In (B), several GFP-positive cells co-expressed calbindin (in red). C) DAPI staining of the image in (B), where it can be seen that several cells presented 2 nuclei (yellow circles). Scale bar measures 10  $\mu\text{m}$ .

**FIGURE 6.** Immunohistochemical images of sagittal sections of cerebella of 2 month-old MSC-treated mice staining for various neurotrophic factors. In all cases, green is GFP, red is the neurotrophic factor of interest, and blue is nuclear DAPI stain. Under each image, taken at 200X, is an orthogonal section of the image, proving that the green and red fluorescence correspond to the same cell. The neurotrophic factors expressed by the grafted cells were A) BDNF, B) NT-3 and C) GDNF. In the latter case, GDNF is a neurotrophic factor that is mainly detected in the Purkinje cells in the cerebellum, as seen in the image (white arrows). In the images, PCL marks the Purkinje cell layer, WM the white matter, and GL the granular layer. Scale bar measures 10  $\mu\text{m}$ .

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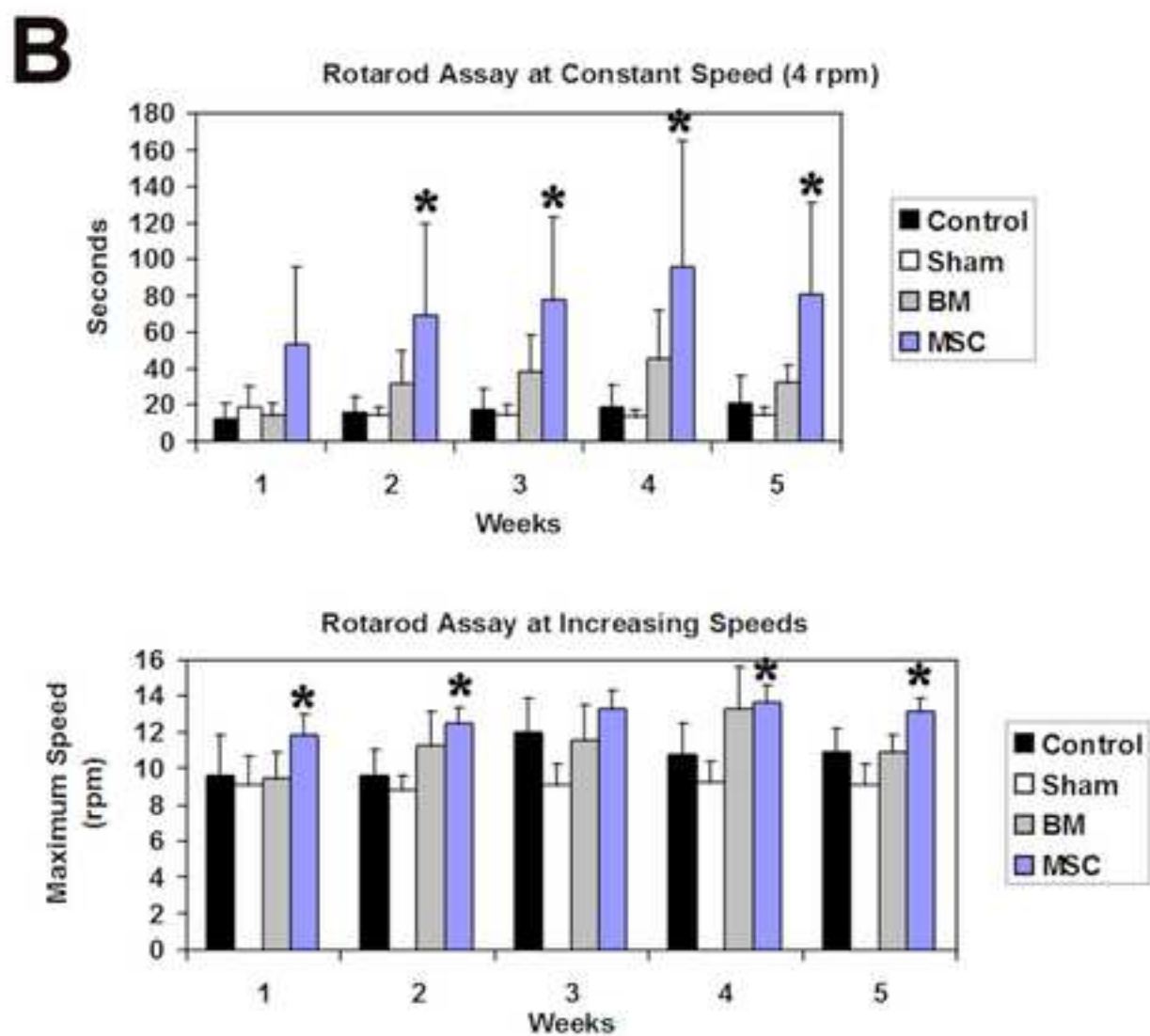
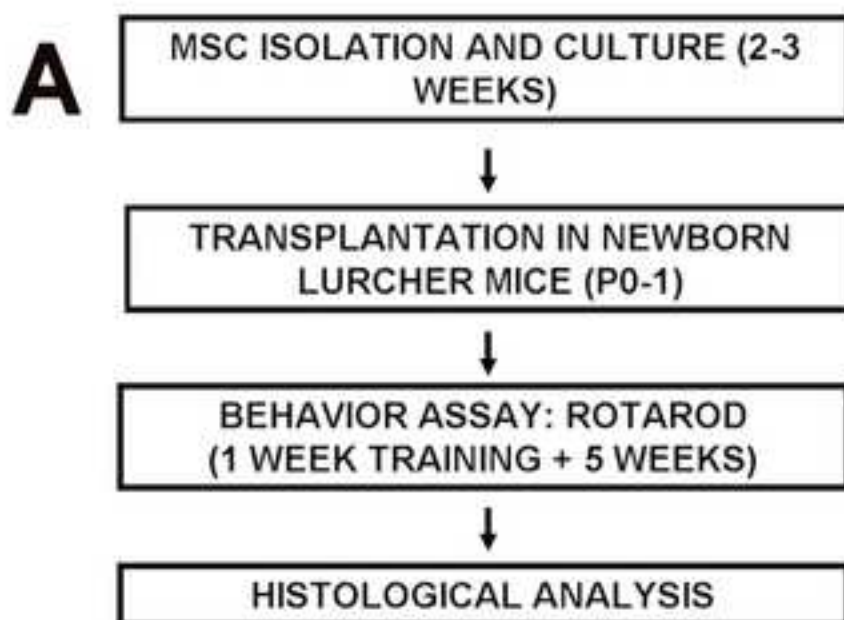


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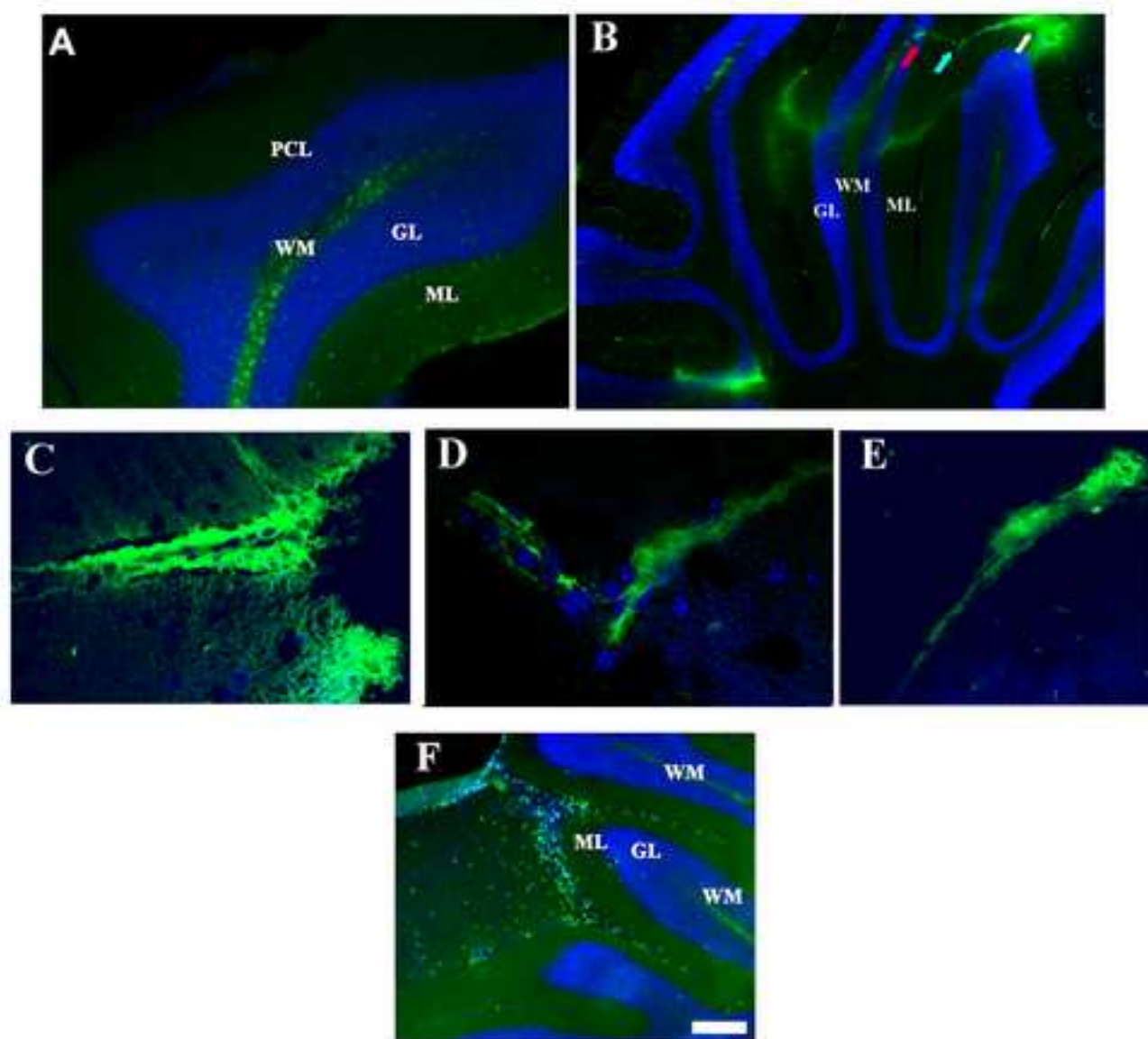


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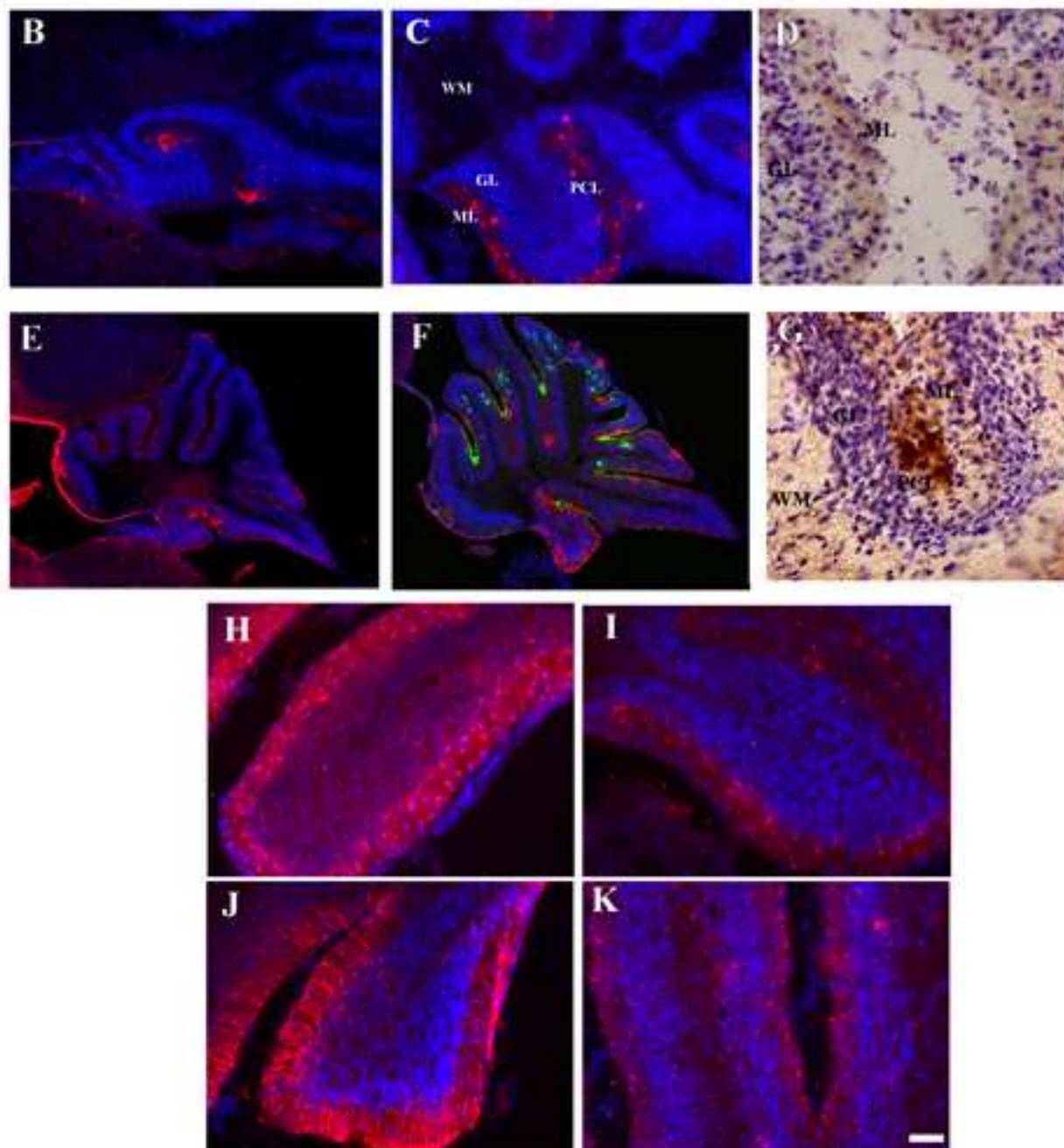
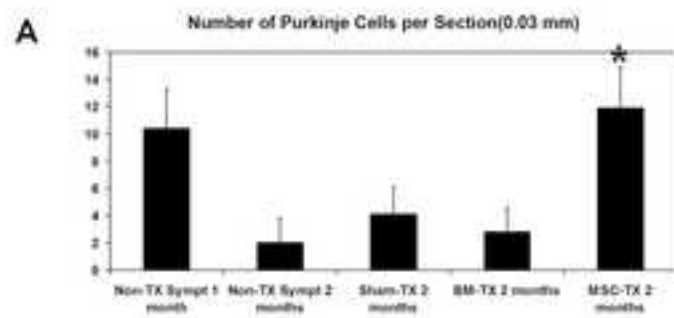
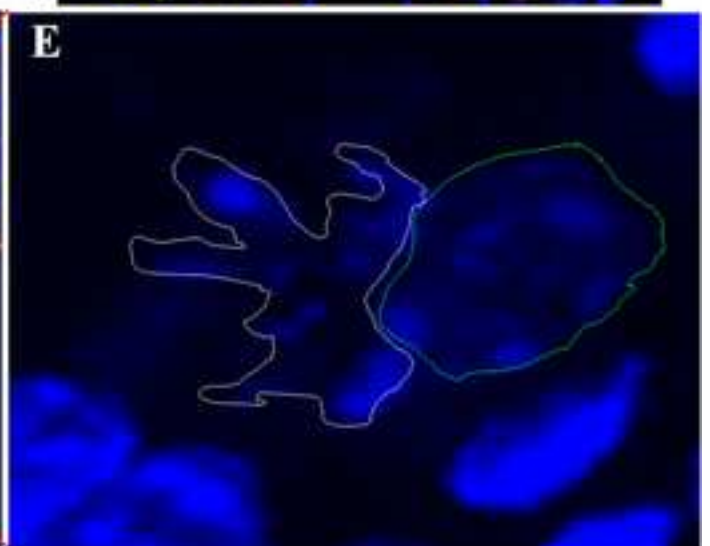
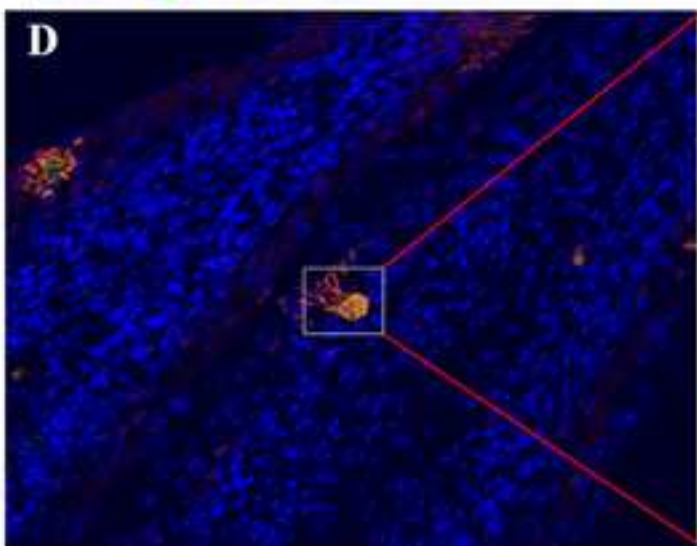
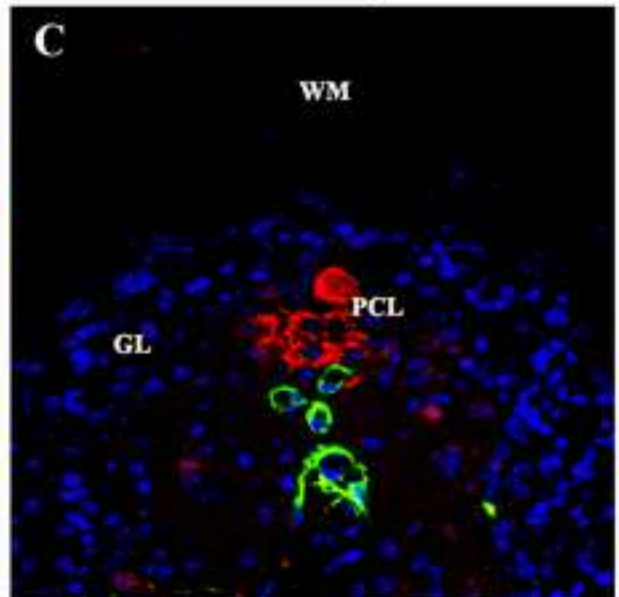
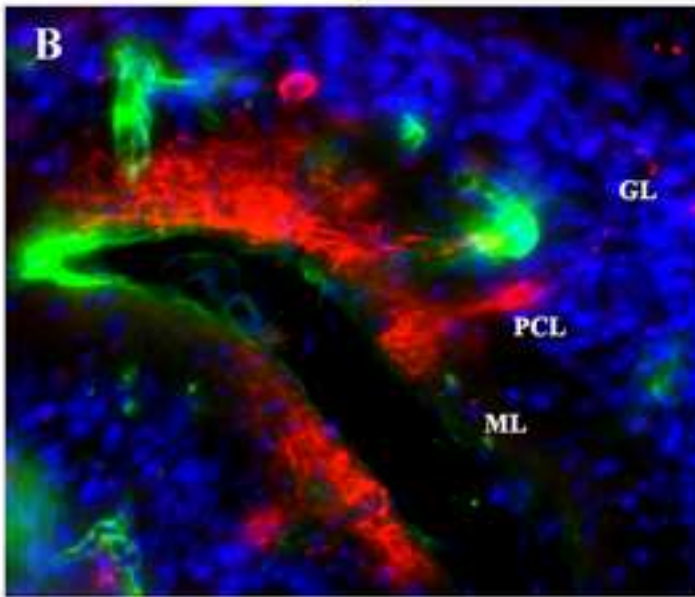
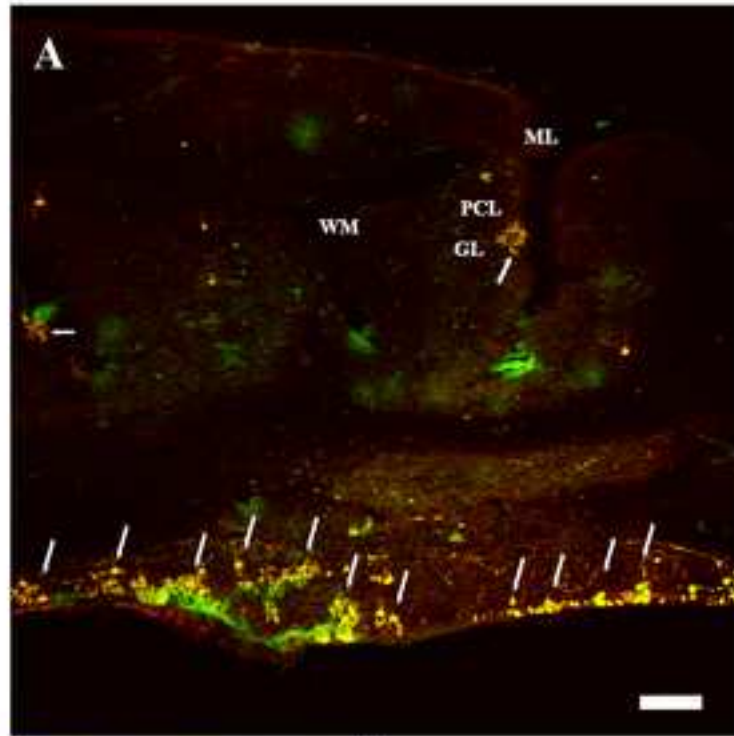


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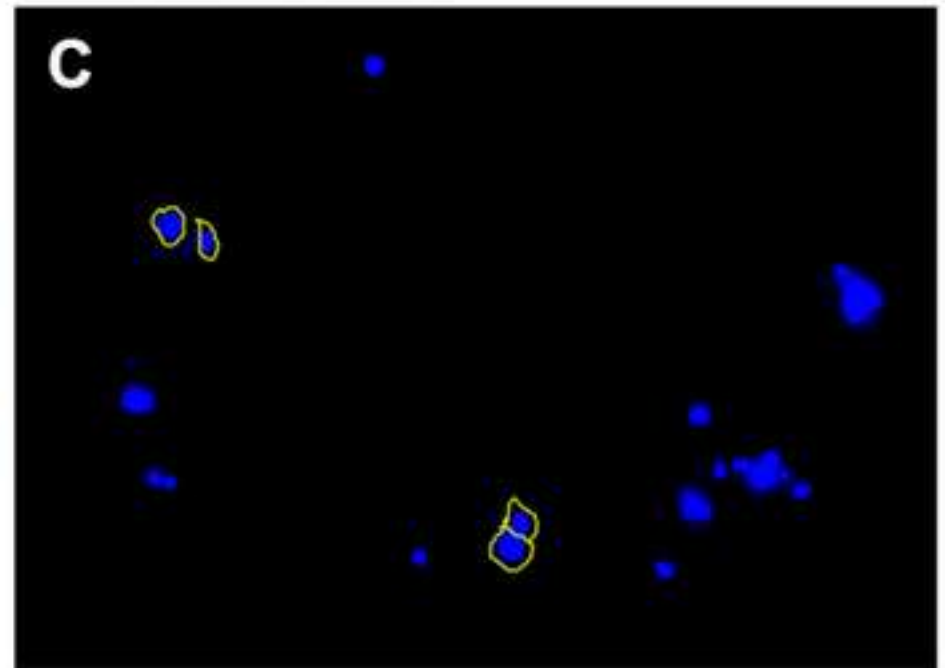
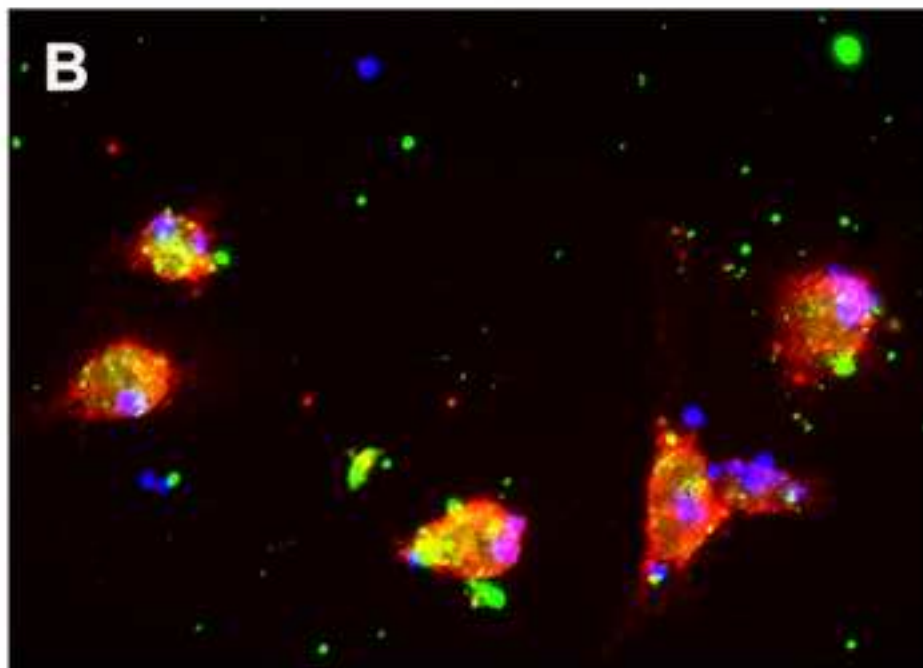
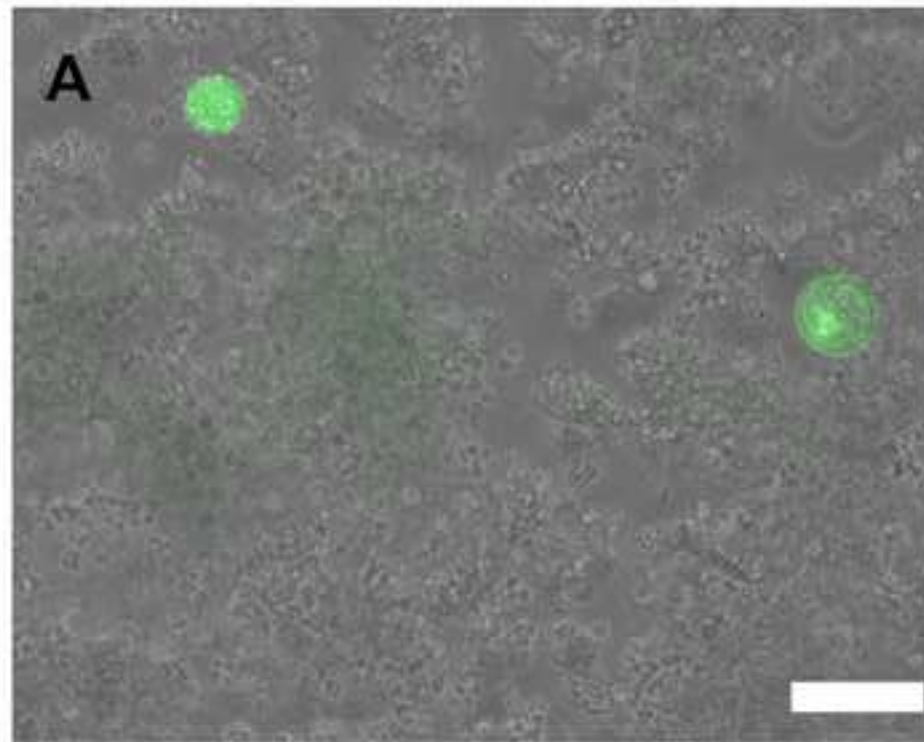


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