Video Article

An *In Vitro* Model for the Study of Cellular Pathophysiology in Globoid Cell Leukodystrophy

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Abstract

The precise function of multi-nucleated microglia, called globoid cells, that are uniquely abundant in the central nervous system of globoid cell leukodystrophy (GLD) is unclear. This gap in knowledge has been hindered by the lack of an appropriate *in vitro* model for study. Herein, we describe a primary murine glial culture system in which treatment with psychosine results in multinucleation of microglia resembling the characteristic globoid cells found in GLD. Using this novel system, we defined the conditions and modes of analysis for study of globoid cells. The potential use of this model system was validated in our previous study, which identified a potential role for matrix metalloproteinase (MMP)-3 in GLD. This novel *in vitro* system may be a useful model in which to study the formation and function, but also the potential therapeutic manipulation, of these unique cells.

Video Link

The video component of this article can be found at http://www.jove.com/video/51903/

Introduction

Globoid cell leukodystrophy (GLD), also known as Krabbe disease, is a fatal demyelinating disease resulting from loss of function mutations in the galatocerebrosidase (galc) gene[1]. The most prevalent form of GLD is the infantile variant which is typified by onset in early childhood and characterized by an aggressive clinical course of motor and cognitive decline leading to premature death often before five years of age[2,3]. Genetic testing is used to verify a diagnosis of GLD[4]. Neuropathology of GLD reveals widespread demyelination, neuronal atrophy, astrogliosis and presence of engorged multi-nucleated microglia called globoid cells[5-7]. The identification of globoid cells, often containing tubulous inclusions in their cytoplasm, has been a defining feature of GLD for the past 97 years, although the specific function of these conspicuous cells has remained elusive.

The involvement of non-myelinating glia (microglia and astrocytes) in the pathogenesis of GLD has long been considered a secondary response to the profound demyelination in this disease[8]. Interestingly, the first description of this disease, made by Knud Krabbe in 1916[5], reported formation of multinucleated phagocytes containing lipid debris that have been named 'globoid cells' and are a defining characteristic of this disease.

Globoid cells are the hallmark feature of GLD pathology, although their role in GLD has long been ignored. Interestingly, these cells are among the earliest characteristic changes in CNS tissue of GLD. This lack of knowledge may have been due to the assumption that the formation of multinucleated phagocytes, called giant cells in other diseases, are typically considered as a consequence of pathology rather than an initial pathogenic driving force[9]. Therefore, there have been few studies investigating the mechanism by which globoid cells are formed from phagocytes, particularly in the CNS of GLD. The procedure described in this report focuses on the importance of globoid cell formation in the CNS and our previous demonstration that psychosine-induced multinucleation of microglia *in vitro* and these cells exhibited higher levels of phagocytic activity. Consistent with these observations, globoid cells in twitcher brains frequently contain PAS-positive debris, suggesting high levels of phagocytic activity. Globoid cells are also found to be immunopositive for ferritin (a microglia marker)[10], KP-1/CD68 (a monocyte marker), and some are also positive for vimentin (an intermediate filament protein and marker of astrocytes and activated microglia)[11], HLA-DRa (an MHCII surface receptor), and TNF-α[7], and Iba-1 (a calcium binding protein used to identify microglia)[12]. Based on this collection of markers, globoid cells originate from microglia that develop a unique phenotype.

Despite their uniqueness, the specific function and contribution of GCs to GLD pathogenesis has been largely overlooked. Globoid cells have been thought to be a secondary consequence of chronic demyelination. However, past studies examining the temporal association of globoid cells to the white matter pathology of GLD have identified the presence of globoid cells in the late embryonic to early postnatal periods; times preceding oligodendrocyte apoptosis and overt demyelination[13]. Thus, the temporal sequence of development of the neuropathology in GLD suggests that globoid cells are formed in advance of demyelination in this disease[14]. This led to our hypothesis that the early formation of globoid

cells in GLD may represent a defining pathogenic event rather than a secondary, reactive response to oligodendrocyte damage[15]. Additionally, dysregulation of microglial activity in GLD has been considered a factor limiting the long-term efficacy of hematopoetic stem cell therapies for treating this disease[16]. Thus, investigating the cellular functions and regulation of microglia, and globoid cells, in response to psychosine is expected to provide new insights in the pathogenesis of GLD.

Until recently, the lack of an appropriate model in which to study globoid cell formation had limited the understanding of the precise function and contribution of these cells to the pathology of GLD. In recent studies, it was determined that globoid-like cells can be formed in direct response to psychosine, a pathogenic lipid toxin that accumulates in GLD. We found that microglia, but not macrophages, are activated and transformed into globoid cells in primary glial cultures in response to psychosine[15]. This transformation into globoid cells was found to be mediated by the extracellular protease, matrix metalloproteinase (MMP)-3[15]. More recently, we have extended these findings and determined that psychosine-activated microglia and globoid cells developed in this *in vitro* model system are potently toxic to oligodendrocytes and oligodendrocyte progenitor cells. Hence, when considered in the context of GLD, the early accumulation of psychosine and formation of globoid cells prior to demyelination would support an emerging primary and possibly pathogenic role for microglia in this disease.

We propose that study of globoid cell formation will reveal new information about the pathogenesis of GLD that will contribute to our understanding of this disease. Moreover, this new cellular model of GLD may provide a new format from which novel therapeutic approaches to address pathological changes in this disease could be tested. Hence, in this report we provide a detailed protocol for the *in vitro* development of psychosine-induced globoid cells from primary cultures of non-myelinating glia.

Protocol

All procedures involving animals were performed in accordance with the Policy on Humane Care and Use of Laboratory Animals set forth by the Office of Laboratory Animal Welfare (NIH) and only with approval from the Institutional Animal Care and Use Committee (IACUC) of the University of Connecticut Health Center.

1. Preparation of Mixed Glial Cultures

1. Sterilize all instruments prior to use. Add 3 ml of ice-cold sterile Hank's balanced salt solution (HBSS) containing no cations (Mg^{2+} or Ca^{2+}) to three sterile 60 mm Petri dishes maintained on ice to keep cold.
2. Isolate the cortices from postnatal P0-P2 mouse pups, as previously described[17,18].
   NOTE: Subcortical structures, such as hippocampi, are not included in these cultures.
3. Carefully remove the meninges and then transfer the isolated cortices to fresh HBSS and dissociate the tissues enzymatically, using a neural tissue dissection kit according to the manufacturer's protocol.
4. Plate the cells into sterile T-175 flasks and incubate overnight in media [Dulbecco's modified eagle medium (DMEM) with 10% fetal bovine serum (FBS) containing 1x penicillin/streptomycin].
5. Replace all media the following day with fresh media. Continue to incubate cultures, with regular media changes, for approximately 3 weeks or until a cellular monolayer is established.
   NOTE: This monolayer will be comprised of astrocytes and microglia with an approximately 10:1 ratio[19].

2. Globoid Cell Induction in Mixed Glial Cultures

1. Prepare the ECM-coated coverslips.
   1. Soak circular coverslips with sterile 1 N hydrochloric acid (HCl) in a sterile Petri dish for 30 min. Wash coverslips with sterile water 3x and then soak in 95% EtOH for at least 20 min. and then let air-dry.
   2. Place droplets of diluted extracellular matrix (ECM) protein coating materials onto a sheet of Parafilm (*e.g.* 150-250 µl/droplet of laminin 10 µg/ml dissolved in sterile phosphate buffer solution [PBS]). Distribute the droplets so that coverslips will not touch when placed onto these droplets. Gently place each dried coverslip onto a droplet using forceps.
   3. Leave the coverslips on the droplets for approx. 4-6 hr inside the culture hood with the sash closed.
   4. Place the ECM-coated coverslips (ECM-coated side up) into each of the wells of a 24-well plate. Wash each well with sterile PBS 3x and keep at 4 °C until needed.

2. Preparing Glial Cells for Plating onto Coverslips.
   1. Aspirate the media from the glial cultures and gently wash the glial monolayer 3x with 10 ml of sterile PBS. Next, add 8-10 ml trypsin-EDTA solution to the T-175, and incubate for 5-10 min at 37 °C.
   2. When most of the monolayer has been detached, add 20 ml of complete media to the flask to stop trypsinization. Collect the media containing the detached cells into a 50 ml conical tube and spin for 10 min at 300 x g at room temperature.
   3. Aspirate the supernatant without disturbing the pellet, and then re-suspend the cells in 2 ml of fresh complete media by gently pipetting the solution using a P1000 pipet.
   4. Determine the number of cells using a hemocytometer and then re-suspend cells in complete medium at the desired density (*e.g.* 10^5 cells/ml). Plate the cells onto the ECM-coated coverslips (*e.g.* 500 µl into each well of the 24-well plate). Add additional fresh complete media to each well for 3-5 days of incubation at 37 °C, or until cells grow to the desired visual confluence.

3. Treatment with Psychosine.
   1. Reconstitute the psychosine to a stock concentration (108.3 mM) in dimethyl sulfoxide (DMSO). Dilute this stock in 100% EtOH to make a working stock that can then be added into each well of 24-well plate to achieve a final concentration of 10 µM.
   2. At the time of adding the psychosine, gently swirl the culture plate to mix the psychosine into culture media. Supplement the psychosine by adding the equivalent of 10 µM every other day for one week.

4. After 7 days of psychosine treatment, collect the media and pipet 500 µl of cold 4% paraformaldehyde (PFA) into each well. Incubate at room temperature for 20 min to fix the cells. Then, wash each well 3x with PBS and then keep plates at 4 °C until processed for immunocytochemistry (see below).
   NOTE: cell culture media can be collected for enzymatic or ELISA assay at this point if desired.

3. Immunocytochemistry (ICC) to Visualize Globoid Cells

1. Replace PBS with blocking buffer [PBS + 0.3% Tween-20 + 5% normal goat serum (NGS)], and incubate the cells for 1 hr at room temperature.
2. Incubate the fixed cells in primary antisera against Iba-1 [(1:1,000) diluted in PBS + 0.3% Tween-20 + 2% NGS] for at least 1 hr at room temperature, then wash each well with PBS 3x 5 min/wash.
3. Add the appropriate secondary fluorescence-conjugated antibody to identify the primary antibody labeling and counter-stain with 4',6-diamidino-2-phenylindole (DAPI, 1:1,000) to identify nuclei. Incubate in secondary antibody solution for 1 hr at room temperature and wash with PBS 3x 5 min.
4. Mount each coverglass to microscope slides using a mounting medium. View slides on a fluorescent microscope for visual and quantitative analyses.

4. Analysis and Characterization of Globoid Cells in Primary Culture

1. Use the following three morphological criteria to identify a globoid cell in culture:
   1. Identify globoid cells using fluorescent microscopy by immunopositive labeling for the microglial marker, Iba-1+.
   2. Identify globoid cells as polynucleated (*i.e.* containing two or more DAPI positive nuclei).
   3. Identify globoid cells by a rounded morphology distinct from the highly branched appearance of resting microglial cells.
      NOTE: Cells may also be collected for cell surface marker analysis and quantification by flow cytometry from these cultures.

2. Measuring the phagocytic activation of microglia and globoid cells
   1. To assess the phagocytic activity of psychosine-treated microglia, add FITC-labeled latex beads to the wells in accordance with the manufacturer's instructions. Add fluorescent-conjugated latex beads 48 hr prior to fixation with PFA. Fix cells treated with beads (as mentioned in section 2.4), and process for immunocytochemistry (as described in section 3).
   2. Select fluorophore-conjugated secondary antibodies so that they do not overlap the fluorescent emission spectra of the fluorophore labeled beads.
   3. Analyze the phagocytotic profiles of Iba-1+ microglia by identifying those cells that co-localize with the fluorescently-labeled beads.
      NOTE: Psychosine will activate microglia. Phagocytosed beads will be identified within mononucleated and multinucleated Iba-1+ cells in this culture setting.

5. Induction of Globoid Cells from Purified Microglial Cultures

NOTE: An alternate approach to decipher intercellular signaling between the astrocyte and microglia is another advantage of this *in* vitro globoid cell model. Purification of primary microglial cells for replating or co-culturing can be achieved by their lower adherence property in culture, as described in the following steps (see Section 5.2).

1. Collect the media from the confluent mixed glial cultures into 50 ml conical tube and maintain it at 37 °C in the incubator.
2. Microglial Isolation from Primary Mixed Glial Cultures.
   1. After removing the media from a confluent mixed glial culture T175 flask, add warm shaking media [consisting of complete media + 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) + 25 mM sodium bicarbonate (Sigma)]. Shake the flasks for 3-4 hr in an orbital shaker at 100 rpm (37 °C). Collect the media from these shaken flasks into 50 ml conical tubes.
      NOTE: This media will include the less adherent microglia from the flasks.
   2. Spin the media at 300 x g for 10 min at room temperature. Aspirate the supernatant and then re-suspend the cells in 2 ml of the astrocyte-conditioned media (collected in step 5.1).
   3. Establish the number of cells acquired using a hemocytometer and then plate the microglial cells onto substrate-coated coverslips (see section 2.1) for immunocytochemistry, or plate onto UltraLow adherence well plates for future collection of globoid cells for co-culture with other cell types, such as oligodendrocytes (as described in the following section).
      NOTE: Once plated the microglia can be treated with psychosine (10 µM) as described above (see section 2.3).

3. Co-culture with Primary Oligodendrocytes for Analysis of Cytotoxicity.
   NOTE: Collection of psychosine-treated microglia from the low adherence plates for subsequent co-culture onto primary cultures of oligodendrocytes, or oligodendrocyte progenitor cells, can be used to examine the potential cytotoxic function of these globoid-like cells *in vitro*.
   1. Develop cultures of primary oligodendrocytes using established methods[18,20]. To collect the psychosine-treated microglia, gently pipet to detach the loosely adherent cells from the bottom of the wells. Collect the cells, centrifuge at 300 x g for 10 min, and then carefully resuspend in oligodendrocyte differentiation media [composed of Neurobasal media, L-glutamine (1x), B-27 supplement (1x), and Triiodothyronine (T3, 10 ng/ml)].
   2. Count the number of cells using a hemocytometer and then seed the microglia onto the oligodendrocyte culture at 1 x 10^5 cells/ml, resulting in an approximate 1:2 microglia/oligodendrocyte ratio. Incubate as a co-culture for up to 3 days.
   3. Fix the cells for immunocytochemistry (see section 2.4). Collect cell culture media for ELISA or chemical analyses as needed.

Representative Results

This protocol, as written, is expected to take approximately 36 days to complete from start to finish (See **Figure 1**: Experimental Workflow Scheme). It has been our experience that the development of 'globoid-like' cells in this primary culture system is both reliable and reproducible: the formation of multinucleated cells in response to psychosine is consistently observed with 7 days of treatment.

Immunocytochemical staining of microglia using Iba-1 in conjunction with a nuclear counter-stain will enable identification of large, rounded multinucleated cells (**Figure 1b**). In some instances nuclear content in these globoid-like cells will appear with distinct nuclei. However, in many instances, the gross enlargement of the size of the nucleus reflects the multinucleated status of these cells (**Figure 1b**). The number of globoid-like cells can vary from visual field to visual field, but it is worth noting that the proportion of globoid cells formed *in vitro* represents <5-10% of the total microglial cell population. It is also important to point out that psychosine treatment also evokes a generalized activation of microglia (*i.e.* measurable increase in phagocytic activity) that occurs in response to psychosine treatment among mononucleated microglia and the multinucleated globoid cells (GCs) in these cultures. As shown in **Figure 1c**, phagocytically active profiles of Iba-1+ microglia can be readily identified when put in co-culture with oligodendrocyte progenitor cells (OPCs).

This cell culture system is amenable to experimental manipulation as a means to assess the biology of globoid cells. For example, we have recently demonstrated a novel role for the extracellular protease, matrix metalloproteinase-3 (MMP-3/stromelysin-1) as an important mediator of psychosine-induced GC formation using this culture assay[15].

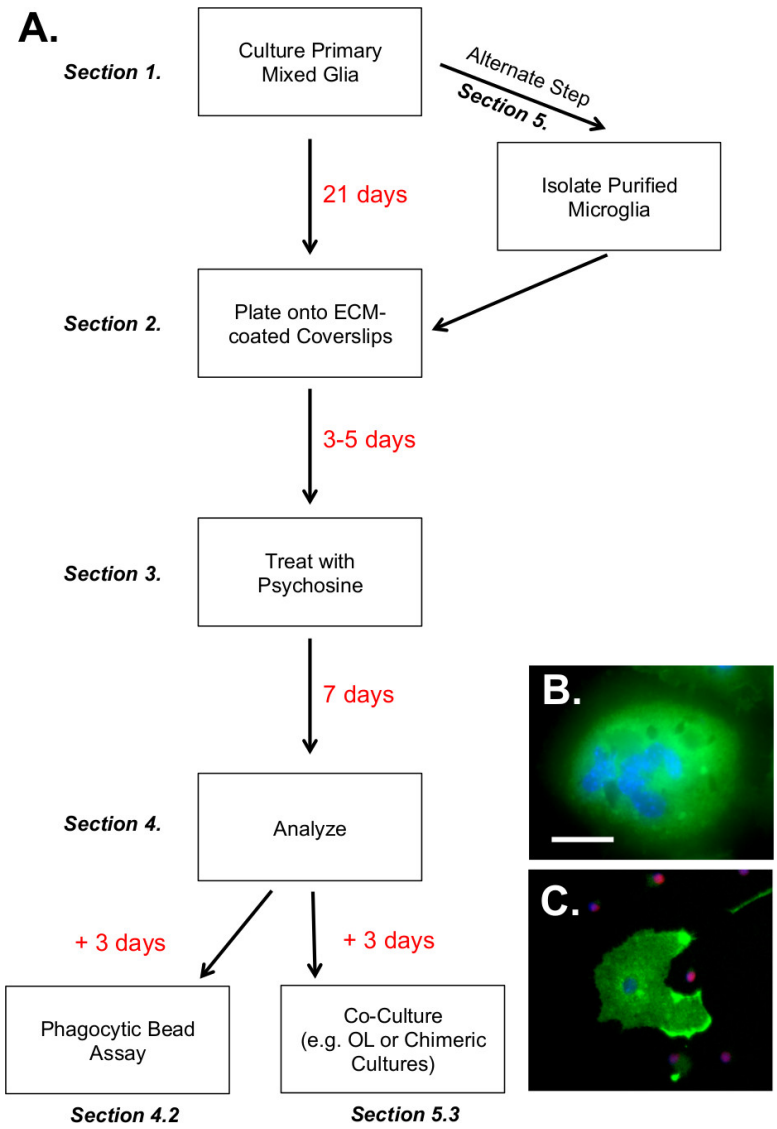

Figure 1: Experimental Workflow for Primary Culture *in vitro* Model of Globoid Cell Formation. **(A)** Flowchart depicting the primary steps and intervening times (in days) for development of globoid cells in culture. Each step is labeled with its associated text section from the protocol. This workflow provides both a logical progression from mixed (astrocyte and microglial cultures; Section 1) and an alternate starting culture of purified microglia (Section 5). **(B)** Representative immuncytochemical staining of a multinucleated microglial cell following 7 consecutive days of psychosine (10 μM) treatment as identified by the Iba1 (green) and DAPI (blue). **(C)** Example of presumptive phagocytic microglial cell (green) following psychosine treatment in co-culture with Olig2+ (red) OPC. Scale bar (in panel B) = 90 μm for 'B' and = 150 μm for 'C'. Please click here to view a larger version of Figure 1B, and here to view a larger version of Figure 1C.

Discussion

The protocol described herein provides a new model system in which to study the development and functional characterization of activated microglia and globoid cells. Prior work by Im *et al*. using a HEK293 cell line provided a template for the development of the present protocol for the study of globoid cell formation[21]. It is also important to point out that the globoid cells derived in the model differ from the native globoid cells identifiable in GLD. For instance, we have routinely observed quadranucleation of microglia in our murine cultures, while globoid cells in GLD have been frequently observed containing in excess of 10 nuclei. Secondly, the globoid cells formed in the *in vitro* system are also small in diameter as compared to those observed *in vivo*. There are likely to be several reasons for these phenotypic differences which may include the relative simplicity of the culture system used. For instance, there are no neurons or oligodendrocytes present during the induction phase of globoid cell formation using this primary glial culture procedure. Also, the timing of the seven day protocol yields a significant density of globoid cells; however, longer treatment times may further enhance the similarity of murine globoid cells formed by this protocol with the native features of globoid cells observed in GLD.

An important feature of this model is that it uses primary mixed glial cultures, which include both astrocytes and microglia[15,19]. This is an important advantage of this globoid cell model for its utility in assessing the contributions and interplay between astrocytes and microglia. Both of these non-myelinating cell types are activated in GLD, though their roles in this disease are, as yet, poorly characterized. Importantly, we had previously determined that astrocytes in GLD express higher levels of MMP-3 expression and that this presumably astrocyte-derived factor was

critical for the transformation of microglia in response to psychosine. Future applications of this *in vitro* model of globoid cell formation could employ chimeric cultures of astrocytes and microglia of differing genetic backgrounds, which could be employed to advance our understanding of the cell-specific contributions to the pathogenic effects of psychosine on microglia.

In summary, this protocol provides a new approach to study the pathogenesis of GLD. The role of the globoid cells has been enigmatic and hypotheses on their protective or deleterious functions in GLD have been proposed. The early identification of globoid cells in the natural history of GLD would further support our view at this time that activation of microglia in GLD are a primary pathogenic response and a likely mediator of myelin pathology in this disease. Adaptation of this *in vitro* model system is expected to further our understanding on the cellular etiology of GLD.

Disclosures

The authors declare no conflicts of interest.

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References

1. Rafi, M. A., Luzi, P., Chen, Y. Q., & Wenger, D. A. A large deletion together with a point mutation in the GALC gene is a common mutant allele in patients with infantile Krabbe disease. *Hum Mol Genet.* **4**, 1285-1289 (1995).
2. Duffner, P. K. *et al.* The long-term outcomes of presymptomatic infants transplanted for Krabbe disease: report of the workshop held on July 11 and 12, 2008, Holiday Valley, New York. *Genet Med.* **11**, 450-454, doi:10.1097/GIM.0b013e3181a16e04 (2009).
3. Duffner, P. K., Jalal, K., & Carter, R. L. The Hunter's Hope Krabbe family database. *Pediatr Neurol.* **40**, 13-18, doi:S0887-8994(08)00395-0 [pii] 10.1016/j.pediatrneurol.2008.08.011 (2009).
4. Duffner, P. K. *et al.* Newborn screening for Krabbe disease: the New York State model. *Pediatr Neurol.* **40**, 245-252; discussion 253-245, doi:S0887-8994(08)00618-8 [pii] 10.1016/j.pediatrneurol.2008.11.010 (2009).
5. Krabbe, K. A new familial, infantile form of brain sclerosis. *Brain.* **39**, 74-114 (1916).
6. Percy, A. K., Odrezin, G. T., Knowles, P. D., Rouah, E., & Armstrong, D. D. Globoid cell leukodystrophy: comparison of neuropathology with magnetic resonance imaging. *Acta Neuropathol.* **88**, 26-32 (1994).
7. Itoh, M. *et al.* Immunohistological study of globoid cell leukodystrophy. *Brain Dev.* **24**, 284-290, doi:S0387760402000578 [pii] (2002).
8. Reddy, A. S., Patel, J. R., Vogler, C., Klein, R. S., & Sands, M. S. Central nervous system pathology progresses independently of KC and CXCR2 in globoid-cell leukodystrophy. *PLoS One.* **8**, e64647, doi:10.1371/journal.pone.0064647 PONE-D-13-04655 [pii] (2013).
9. McNally, A. K., & Anderson, J. M. Macrophage fusion and multinucleated giant cells of inflammation. *Adv Exp Med Biol.* **713**, 97-111, doi:10.1007/978-94-007-0763-4_7 (2011).
10. Kaneko, Y., Kitamoto, T., Tateishi, J., & Yamaguchi, K. Ferritin immunohistochemistry as a marker for microglia. *Acta Neuropathol.* **79**, 129-136 (1989).
11. Graeber, M. B., Streit, W. J., & Kreutzberg, G. W. The microglial cytoskeleton: vimentin is localized within activated cells in situ. *J Neurocytol.* **17**, 573-580 (1988).
12. Kondo, Y., Adams, J. M., Vanier, M. T., & Duncan, I. D. Macrophages counteract demyelination in a mouse model of globoid cell leukodystrophy. *J Neurosci.* **31**, 3610-3624, doi:10.1523/JNEUROSCI.6344-10.201131/10/3610 [pii] (2011).
13. Pollanen, M. S., & Brody, B. A. Fetal globoid cell leukodystrophy. *Arch Pathol Lab Med.* **114**, 213-216 (1990).
14. Martin, J. J. *et al.* Fetal Krabbe leukodystrophy. A morphologic study of two cases. *Acta Neuropathol.* **53**, 87-91 (1981).
15. Ijichi, K. *et al.* MMP-3 mediates psychosine-induced globoid cell formation: Implications for leukodystrophy pathology. *Glia*, doi:10.1002/glia.22471 (2013).
16. Pellegatta, S. *et al.* The therapeutic potential of neural stem/progenitor cells in murine globoid cell leukodystrophy is conditioned by macrophage/microglia activation. *Neurobiol Dis.* **21**, 314-323, doi:S0969-9961(05)00210-X [pii]10.1016/j.nbd.2005.07.016 (2006).
17. Crocker, S. J., Milner, R., Pham-Mitchell, N., & Campbell, I. L. Cell and agonist-specific regulation of genes for matrix metalloproteinases and their tissue inhibitors by primary glial cells. *Journal of Neurochemistry.* **98**, 812-823, doi:JNC3927 [pii] 10.1111/j.1471-4159.2006.03927.x (2006).
18. Schildge, S., Bohrer, C., Beck, K., & Schachtrup, C. Isolation and culture of mouse cortical astrocytes. *J Vis Exp.* doi:10.3791/50079 (2013).
19. Crocker, S. J., Frausto, R. F., Whitton, J. L., & Milner, R. A novel method to establish microglia-free astrocyte cultures: comparison of matrix metalloproteinase expression profiles in pure cultures of astrocytes and microglia. *Glia.* **56**, 1187-1198, doi:10.1002/glia.20689 (2008).
20. Moore, C. S. *et al.* Astrocytic Tissue Inhibitor of Metalloproteinase-1 (TIMP-1) Promotes Oligodendrocyte Differentiation and Enhances CNS Myelination. *J Neurosci.* **31**, 6247-6254, doi:31/16/6247 [pii] 10.1523/JNEUROSCI.5474-10.2011 (2011).
21. Im, D. S., Heise, C. E., Nguyen, T., O'Dowd, B. F., & Lynch, K. R. Identification of a molecular target of psychosine and its role in globoid cell formation. *J Cell Biol.* **153**, 429-434 (2001).

