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NTPDase2 as a Surface Marker to Isolate Flow Cytometrically a Müller Glial Cell Enriched Population from Dissociated Neural Retinae

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Abstract

Müller glial cells are large neuroglial cells that extend throughout the entire retina, they function to maintain homeostasis and retinal integrity. In teleost fish, in response to retinal damage, Müller cells can re-enter the cell cycle, dedifferentiate and regenerate neuronal cells. Therefore, increasing our knowledge about these cells might open new avenues to regenerative medicine. Here, we present a reliable method to isolate Ectonucleoside triphosphate diphosphohydrolase 2 (NTPDase2) positive Müller glial cells by Fluorescence-Activated Cell Sorting (FACS). For this, cell suspensions were made from immature and mature mouse neural retinae by incubation in collagenase I and DNase I and repeated trituration. We confirmed by flow cytometry that glutamine synthetase positive Müller glial cells were also NTPDase2 positive. Thereafter, the surface marker NTPDase2 was used to sort by FACS a population enriched for Müller glial cells. NTPDase2 successfully marks an enriched population of glutamine synthetase positive cells. This is a reproducible method to sort Müller glial cells from mouse retina. The FACS isolated Müller glial cells can be used to study their properties in retinal degeneration, regeneration and cell function.

Article Highlights

- NTPDase2 successfully marks an enriched population of glutamine synthetase positive cells.
- NTPDase2 can be used as a surface marker to sort a population enriched for Müller glial cells from dissociated mouse neural retinae.

Keywords

Müller glial cells; NTPDase2; Retina; Fluorescence-Activated Cell Sorting (FACS)

Introduction

The vertebrate neural retina is organized into a laminar structure comprising six types of neurons and one type of glial cells. Müller glial cells are the only glial cells derived from retinal progenitors and are among the latest cells to be born during retinogenesis in all vertebrate retinae. They stretch from the inner limiting membrane to apical villi at the outer limiting membrane and their cell bodies are localized in the inner nuclear layer [1-3]. Müller glial cells provide metabolic, homeostatic, functional and structural support to neurons and are indispensable for the correct cellular organization of the retina. The latter became clear from studies showing that perturbation of adherens junctions between Müller glial cells and photoreceptor cells results in anomalous blood vessel formation, retinal degeneration and disease [4-6].

Transcriptomic analyses revealed great similarities between the genetic repertoire of Müller glial cells and multipotent late retinal progenitors [7-8]. Müller glial cells acquire specialized glial functions but still maintain a genetic signature of late progenitors [9]. Müller cells have the potential to de-differentiate [10-12], this together with the common gene expression profile seems to support the ability of these cells to become progenitor-like cells.

In several vertebrate classes, in response to acute retinal damage, Müller glial cells are able to de-differentiate into progenitor cells, re-enter the cell cycle and undergo proliferation [13-15]. In the zebra fish retina, Müller cells have properties of progenitor cells and generate new neurons after injury, suggesting that Müller cells might be endogenous stem/progenitor cells of retinae. Likewise, in chicken retinae, Müller cells are able to acquire progenitor-like phenotypes and produce new neurons after N-methyl-D-aspartate-induced damage [16]. In contrast to the above species, the mammalian retina is not able to self-repair. Müller glial cells are quiescent in the adult healthy mammalian retina although known to be reactive in disease or following injury [1,4,17,18].

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Establishing protocols to sort Müller glial cells will allow to isolate and study these cells in more detail. Studies with Müller cells are quite often restricted to the use of cultured cells [19]. However, taking Müller cells into cell-selective-culture results in changes in the Müller cells expression profile and morphology that do not mimic the one in the native tissue. Identification of proteins expressed at the surface of Müller glial cells will allow to sort/isolate these cells without the need to culture them and therefore maintain their original identity. Others have shown that in rodent retinae, the membrane bound Ectonucleoside triphosphate diphosphohydrolase 2 (NTPDase2) localizes throughout the Müller glial cell membrane [20,21]. NTPDase2 is expressed in the germinal zones of the developing and adult brain and by subpial astrocytes [22]. NTPDase2 is an Ectonucleoside triphosphate diphosphohydrolase 2 (NTPDase2) enzyme that catalyses the dephosphorylation of Adenosine 5′-triphosphate (ATP) to Adenosine 5′-diphosphate (ADP), generating a ligand for P2Y1, P2Y12 and P2Y13 receptors [23]. In retinal explants of newborn mice, treatment with exogenous ATP or ADP stimulated retinal progenitor cells to progress in the cell cycle [24]. Similarly, adenine nucleotides modulate the proliferation of rat retinal progenitors cells via activation of P2Y1 receptors regulating transition from G1 to S phase of the cell cycle [25]. Furthermore, extracellular nucleotides mediate glia-to-neuron signalling in the retina and are implicated in the volume regulation of Müller glial cells under osmotic stress conditions [26]. NTPDase2 is the major ATP-degrading ectonucleotidase of the retinal parenchyma and NTPDase2 expressed by Müller glial cells is involved in the regulation of purinergic calcium responses and cellular volume [20].

We here showed that NTPDase2 is a suitable surface marker to isolate Müller glial cells from immature and mature mouse retina. Isolation of the NTPDase2 Müller glial cell enriched population will allow studies on the roles of the purinergic system in these cells.

Materials and Methods

Animal husbandry

Animal care and use of mice were in accordance with protocols approved by the Animal Care and Use Committee at the Netherlands Institute of Neuroscience. Adult (8-12 weeks old), postnatal day (P)5, P8 and P14 wild type C57BL/6J mice from Envigo (The Netherlands) were used.

Neural retina cell suspension

Murine neural retinae were isolated from enucleated eyes by dissection of the anterior part of the eye and attached vitreous, following a trans scleral incision and peeling the sclera and attached retinal pigment epithelium. A retinal cell suspension was prepared using 14 mouse retinae , by incubation in 1.2 ml of enzyme incubation solution (4 g/L $MgCl_2$, 2.55 g/L $CaCl_2$, 3.73 g/L KCl, 8.95 g/L NaCl, pH 6-7) containing 92.5U Collagenase I (Worthington, Lakewood NJ, USA) and 0.25 mg DNase I (Roche, Mannheim, Germany) for 1 hour at 37°C. The tissue was triturated every 10-12 minutes using a Gilson P1000 pipet and tips with decreasing bore size. Cell suspensions were washed in 20 ml GKN/BSA (8 g/L NaCl, 0.4 g/L KCl, 1.41 g/L $NaHPO_4$ anhydrous, 0.69 g/L $NaH_2PO_4.H_2O$, 2g/LD-glucose, pH 7.4, containing 0.2% BSA) and cells recovered in a pellet following centrifugation at 1,700^{x}g for 10 minutes, resuspended in 7 ml GKN/BSA and stained immediately or stored 16-18 hours at 4°C. For (Figure 4) single retina analysis of NTPDase2 expression was done at P5, 3 mouse retinae used and at P8, P14 and adult retinal stages 4 retinae were used.

Flow cytometric cell isolation from neural retina

For flow cytometric sorting the cell suspension was stained in subsequent steps, in GKN/BSA containing 1:45 anti-NTPDase2 (Enzo Life Sciences, Lausen, Switzerland) in 2 ml for 60 minutes, 1:100 biotinylated goat-anti-Rabbit (Jackson ImmunoResearch, West Grove PA, USA) in 2 ml for 30 minutes, 1:125 streptavidin-APC-Cy7 conjugate (BD Biosciences, San Jose CA, USA) in 1.5 ml for 30 minutes and finally by adding 25 µl 7-AAD solution (BD Biosciences) to 1900 µl cell suspension 10 minutes before analysis. Alternatively our custom made polyclonal antibody (Eurogentec) directed against NTPDase2 was used as an alternative to the Enzo antibody, at a dilution of 1:20. Flow cytometric sorting was performed using a FACSAria (BD Biosciences) or InFlux (BD Biosciences), collecting events that were FSC-low, SSC-low, 7-AAD-negative and NTPDase2-positive and data was analysed using the Diva software or FlowJo (Mac V9.5.3; Tree Star, Ashland OR, USA). The data regarding the sorting of NTPDase2 from immature and mature mouse retinae were the result of three independent experiments.

Statistical analysis

Values were expressed as mean ± Standard Error of the Mean (SEM). The graphic on Figure 4 was generated using Graph Pad Prism 5.

Results and Discussion

In this study we established a means of isolating an enriched population of Müller glial cells from neural retina cell suspensions, using flow cytometry. For this, care should be taken to prepare the cell suspension using a proteolytic enzyme mix that is not detrimental to the cell surface markers. We tested several enzyme combinations, such as trypsin and Liberase Blendzyme cocktails (not shown) and found that a cocktail of collagenase I and DNase I worked optimally. This cocktail was used to dissociate pools of 14 neural retinae obtained from adult mice into single cell suspensions by intermittent trituration with pipet tips of decreasing bore size. This resulted in a suspension containing 75-100x10^6 cells. Markers, such as NTPDase2, that are equally expressed over the entire cell membrane are useful for cytometric sorting. NTPDase2 is present specifically on the cell membranes of both rat and mouse Müller glial cells with its expression being present throughout the cell including somata and endfeet as opposed to other ENTPDase family members which are not [20,21]. We first tested if glutamine synthetase positive Müller glial cells are also NTPDase2 positive. The cell suspension was stained intracellularly for glutamine synthetase and NTPDase2 (membrane), we observed that at least 58.7% of the glutamine positive cells are also NTPDase2 positive (Figure 1), confirming the immunohistochemistry data from others [21]. Using the reverse gating strategy we confirmed that 84.6% of the NTPDase2-positivepopulation (Figure1) is also glutamine synthetase-positive (Figure 2E), demonstrating that indeed the NTPDase2- positive cells are enriched for Müller glial cells.

Thereafter, we examined the use of a polyclonal antibody against NTPDase2 [20,21]. Using this antibody, in an indirect staining procedure, we isolated cells by flow cytometric sorting as a forward-scatter low, side-scatter low (small/non-granular), 7-AAD-negative (live), NTPDase2-positive population (Figure 2A-C). On average this sort yielded a live population of 85-95% and a NTPDase2-positive population that constituted 5% of the input population which is slightly lower than the reported size of the Müller glial cells population presumably because of conservative gating.

To assess if a similar strategy could also be applied to immature retinae, postnatal day 5 mouse retinae were dissociated as mentioned before and sorted. As in mature mouse retinae we yielded 5% of NTPDase2-positive cells (Figure 3). Interestingly, the percentage of NTPDase2-positive cells isolated at different stages of retinal development was very similar (Figure 4).

Conclusion

Our study provides a reproducible and robust method for isolation of Müller glial cells from immature and mature mouse retina laying a foundation on which cell based or cell transplantation approaches can be built.

We conclude that these techniques allow the isolation of Müller glial cells from both immature and adult retinae, with similar flow cytometric properties with respect to low forward- and side-scatter characteristics, intensity of NTPDase2 staining and percentage of cells isolated from the total population (ca. 5%). Isolation of the NTPDase2 Müller glial cell enriched population will allow studies on the roles of the purinergic system in these cells.

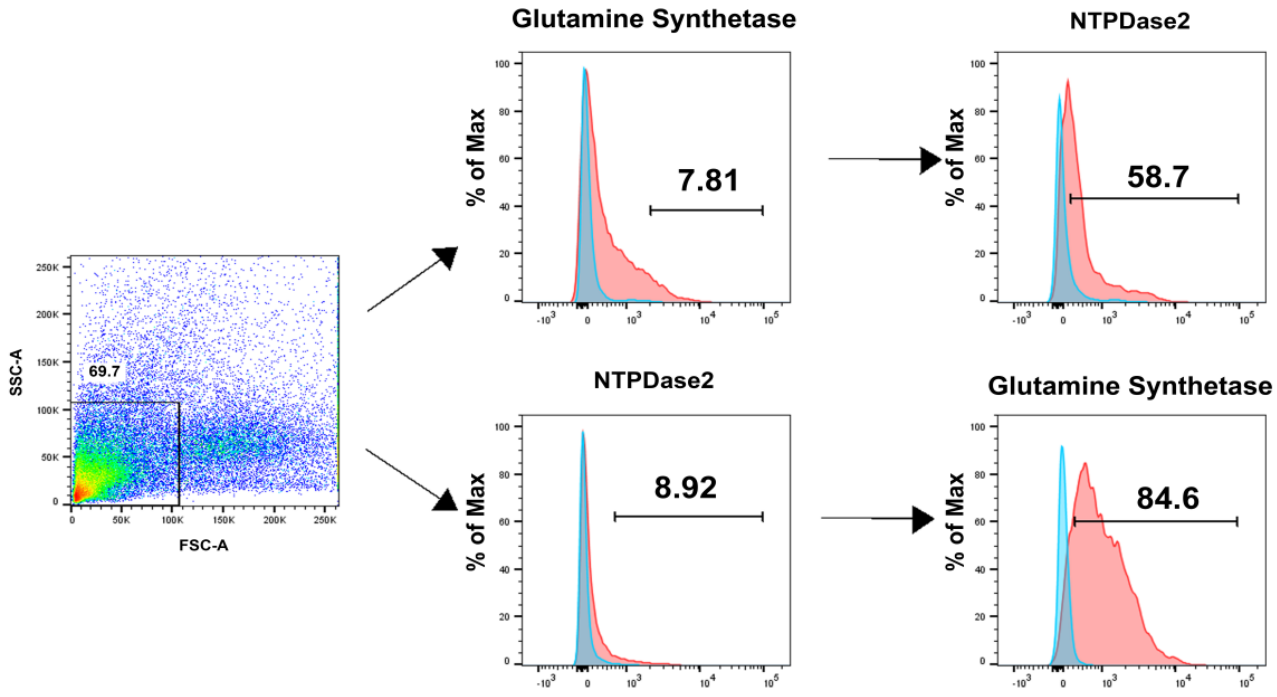

Figure 1: Glutamine synthetase positive cells express NTPDase2

Neural retina cell suspension from adult C57BL/6J mice stained with glutamine synthetase, a Müller glial cell marker and NTPDase2. Flow cytometry plots showed that 7.81% of the cells in suspension are glutamine synthetase positive Müller glial cells, 58.7% of these cells co-express NTPDase2. The neural retina cell suspension is composed by 8.92% of NTPDase2 positive cells from those 84.6% are glutamine synthetase. Blue curves represent negative isotype controls for red filled histograms (glutamine synthetase or NTPDase 2 stained).

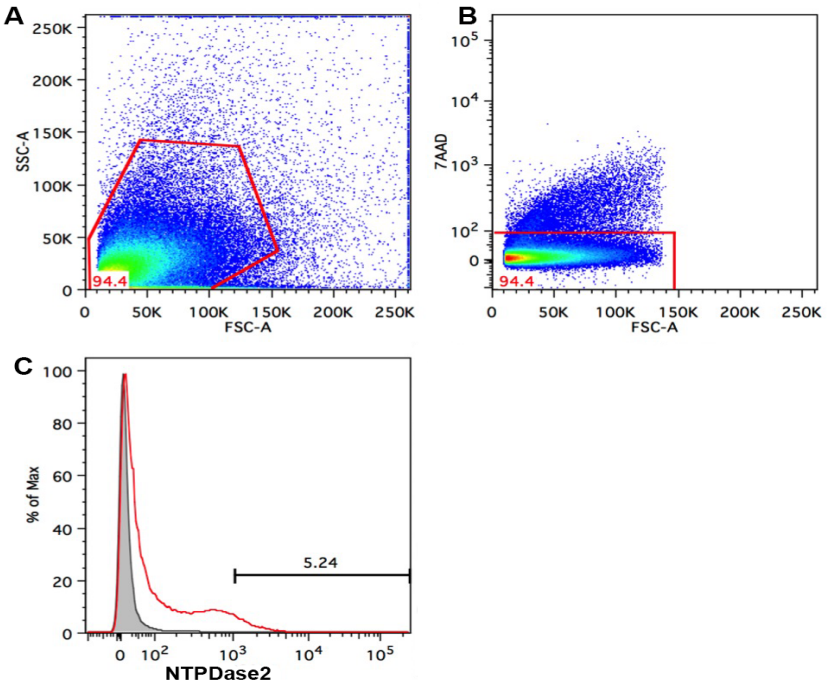

Figure 2: NTPDase2 positive Müller glial cells can be isolated from adult mouse retinas

NTPDase2 positive Müller glial cells can be isolated from neural retina cell suspension from adult C57BL/6J mice by flow cytometric sorting using their scatter characteristics (A), lack of 7-AAD staining (B) and expression of NTDase2 (C). Black line, isotype control. Red line, NTPDase2.

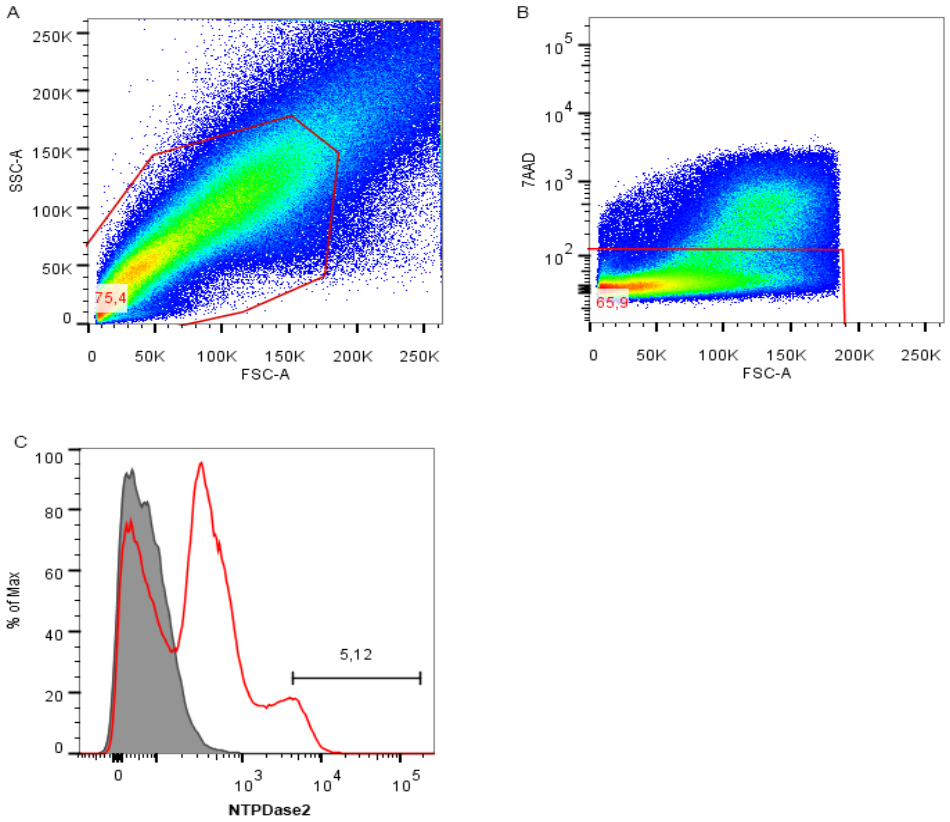

Figure 3: NTPDase2 positive Müller glial cells can be isolated from postnatal mouse retinas

NTPDase2 positive Müller glial cells can be isolated from neural retina cell suspension from postnatal day 5 mice by flow cytometric sorting using their scatter characteristics (A), lack of 7-AAD staining (B) and expression of NTDase2 (C).

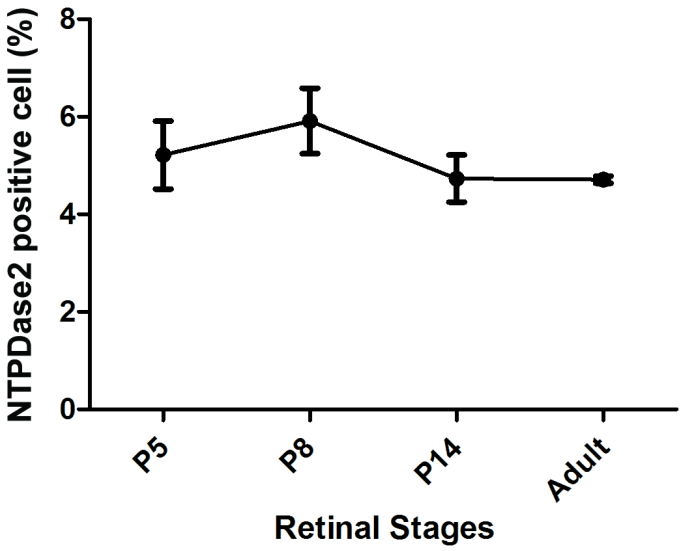

Figure 4: Percentage of NTPDase2 positive cells during postnatal retinal development

NTPDase2-positive cells can be isolated from neural retina cell suspension at different stages of retinal development (postnatal day 5, 8, 14 and adult retinas). The percentage of NTPDase2-positive cells at the different time points varies slightly between the different time points (P5= 5.2 ± 0.7, P8= 5.9 ± 0.7, P14= 4.7 ± 0.5, adult= 4.7 ± 0.1). Data are presented as mean ± SEM.

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