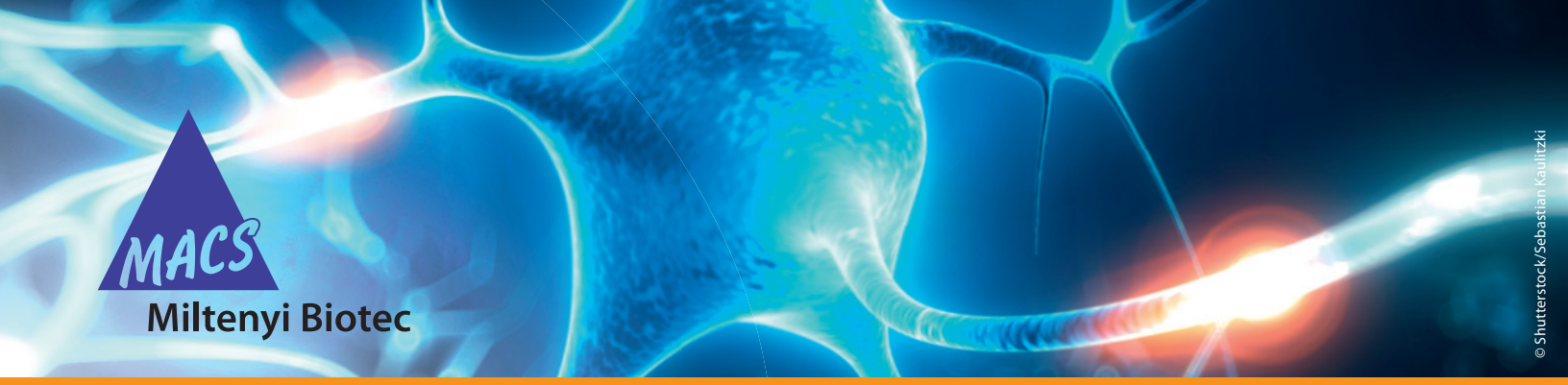

Isolation and cultivation of viable adult mouse cortical neurons

Introduction

During development, neurons extend axons throughout the nervous system, establishing connections with post-synaptic targets. The ability of these young neurons to robustly extend their axons dramatically diminishes in adulthood, where their intrinsic growth capacity is the major cause for the inability of central nervous system (CNS) neurons to regenerate their axons following injury[1–3]. As the nervous system begins to parcel out its peripheral and central components, the peripheral nervous system (PNS) retains its ability to regenerate axons spontaneously following injury in adulthood, while most CNS neurons do not. Activation of specific transcriptional and translational switches may account for this marked difference[4].

Due to the inability to culture adult cortical neurons, dorsal root ganglion (DRG) neurons have been used as a model system to study molecular mechanisms associated with axonal regeneration as these neurons extend axons through the peripheral sciatic nerve and into the CNS spinal cord. Following a sciatic nerve injury, regeneration is successful and neuromuscular junctions become re-innervated. Injury to the central branch of a DRG axon root, however, does not result in regeneration[5]. This difference in growth capacity has been associated with signaling pathways activated after peripheral nerve injury and relies on the coordinated expression of proteins in the axons and their somata[6–8]. Within the last few years, researchers have shown that adult CNS neurons do indeed retain their ability for growth. In fact, when the most refractory CNS tract is presented with the correct substrate within the lesion cavity, these corticospinal axons have the ability to regenerate[9]. Neural stem cells embedded into sites of CNS injury extend large numbers of axons over remarkable distances, where intrinsic neuron properties allow these axons to overcome the inhibitory milieu of the injured adult spinal cord[10]. These observations generate a set of conditions that allow us to utilize modern techniques in neuroscience to identify an unprecedented level of detail in the molecular mechanisms that allow for this regeneration.

Further evidence indicates that motor and sensory neurons differ in their molecular signatures as well as intrinsic cellular pathways that control regeneration[11]. A more robust screening method is therefore required to investigate molecular regulators of growth effectively. So far, most screening efforts are based on embryonic neurons; however, these differ from mature neurons in the molecular cues that navigate migration, cell division, and differentiation. In order to study axon growth and regeneration, mature neurons that do not undergo cell division, nor migrate, represent a more appropriate model system.

For the first time, we have successfully developed a culture protocol that enables efficient, high-throughput isolation and culture of cortical neurons harvested on postnatal days (P) 12 and 60. This approach will allow researchers to explore signaling networks associated with injured CNS neurons and may yield novel insights into the molecular switches that enable growth of an axon previously incapable of regeneration.

Materials and methods

Brain dissection

On P12 and P60, whole brains were carefully removed from C57BL/6 mice and individually placed into a dissecting dish filled with D-PBS. Cortical hemispheres were isolated and transferred into a second dish filled with D-PBS. The dishes were kept on ice while harvesting all tissue. Each cortical hemisphere (0.14 g for P12 and 0.22 – 0.25 g for P60 mice) remained intact for further downstream processing.

Tissue dissociation

(A) gentleMACS™ Technology

Each cortical hemisphere was transferred to a gentleMACS™ C Tube (Miltenyi Biotec) containing the combined enzyme mixes 1 and 2 prepared from the Adult Brain Dissociation Kit, mouse and rat (Miltenyi Biotec). Mechanical enzymatic tissue dissociation, debris removal and red blood cell removal were performed using the kit, in combination with the automated gentleMACS Octo Dissociator with Heaters (Miltenyi Biotec), according to the manufacturer's intructions. The cell suspension was cleared using MACS® SmartStrainers (70 µM) (Miltenyi Biotec) and subsequently proceeded to neuron isolation.

(B) Conventional manual dissociation system

The Papain Dissociation System (Worthington, LK003150) was carried out according to the manufacturer's instructions. Cells that were collected after the dissociation procedure were further processed for isolation of neurons.

Neuron isolation

In order to purify neurons, non-neuronal cells were depleted using the Neuron Isolation Kit, mouse in combination with the QuadroMACS™ Separator and LS Columns (all Miltenyi Biotec), according to the manufacturer's instructions. This neuron isolation step was performed after both of the dissociation methods described above. Eluted neurons were plated directly onto Imaging Plates CG 1.5 (24 well) (Miltenyi Biotec).

Note: If electroporation is performed downstream, directly proceed to electroporation after brain dissociation.

Flow cytometry

Samples were stained with the following set of Miltenyi Biotec antibodies: Anti-O4-PE, human, mouse, rat (clone: REA576), CD45-VioBlue®, mouse (clone:REA737), Anti-ACSA-2-APC, mouse (clone IH3-18A3) and CD11b-VioBright™-FITC, mouse (clone: REA592) according to the manufacturer's instructions. 1 ug/mL Propidium Iodide Solution (Miltenyi Biotec) was added to each sample after antibody staining. The MACSQuant® Analyzer 10 (Miltenyi Biotec) was used for sample acquisition and the MACSQuantify™ Software (version 2.10.1647.17442) was used to analyze all samples.

Cell culture

Isolated P12 or P60 neurons were plated at 150,000 cells/well onto poly-D-lysine- and 10 µg/mL laminin-coated Imaging Plates CG 1.5 (24 well) and cultured in MACS® Neuro Medium supplemented with MACS NeuroBrew®-21 (all Miltenyi Biotec), 10% FBS, L-glutamine solution, and 0.02 µg/mL BDNF. Cells were maintained in culture for up to 14 days *in vitro*, replacing media every other day.

Immunofluorescent staining

Cells were fixed with 4% PFA and subsequently permeabilized and blocked in 5% horse serum + 0.25% Triton™ X-100 in 1× PBS for 1 hour at room temperature. Fixed cultures were stained overnight at 4°C with mouse Anti-βIII Tubulin Antibody G7121 (Promega®) at 1:2000 dilution, chicken Anti-MAP2 Antibody CPCA-MAP2 (EnCor Biotechnology) at 1:5000 dilution, and rabbit Cleaved Caspase-3 (Asp175) Antibody #9661 (Cell Signaling) at 1:1000 dilution. Secondary antibody labeling was performed with donkey anti-mouse IgG-Alexa 488 (Invitrogen), donkey anti-chicken IgG-Alexa 647 (Jackson Immuno), donkey anti-rabbit IgG-Alexa 594 (Invitrogen™), all at 1:1000 dilution. Neurite outgrowth was measured using the ImageXpress® Micro (Molecular Devices) as well as the SP8 confocal microscope (Leica™).

Results

Efficient isolation of functional adult neurons with gentleMACS Technology and MACS® MicroBeads

In order to study molecular mechanisms associated with growth in adult neurons, we aimed to develop a technique by which mouse late postnatal neurons could be isolated and cultured. Our results show that isolated P12 cortical neurons derived by gentleMACS Technology could be maintained in culture for up to two weeks, where they extended their neurites and differentiated into fully mature neurons with dendritic and axonal compartments (fig. 1). Positive staining of the neuronal markers neuron-specific class III β-tubulin (Tuj1) and microtubule-associated protein 2 (MAP2) confirms the presence of mature, viable neurons.

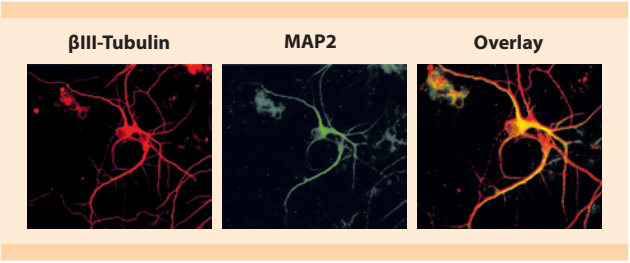

Figure 1: Neurons isolated from P12 mouse cortex, using gentleMACS Technology followed by the Neuron Isolation Kit, mouse were cultured on poly-D-Lysine-coated plates and maintained in cultured for 5 days. Cells established clear dendritic compartments (MAP2) while extending axons over long distances (βIII-Tubulin, Tuj1). The images depicted are representative for 3 independent experiments.

gentleMACS™ Technology yields higher numbers of viable and pure neurons than the conventional manual dissociation method

We further performed a side-by-side comparison of Miltenyi Biotec's gentleMACS™ Technology with the conventional manual dissociation system to determine which method (in combination with the Neuron Isolation Kit, mouse) would obtain a better yield of viable neurons, while minimizing contamination with non-neuronal cells. Using gentleMACS Technology, mouse cortices isolated on P12 yielded an average of 514,588 +/–108,739 neurons per animal with an average neuron viability of 87.35 % +/–1.27 % as assessed by flow cytometry. The same amount of cortical hemisphere wet weight yielded significantly less total neurons with the manual dissociation system as compared to gentleMACS Technology (fig. 2A) in age matched animals. Viability, assessed by PI staining and flow cytometry immediately after neuron isolation, showed no difference between the two dissociation methods (fig. 2B).

Flow cytometry analysis revealed that the gentleMACS Method resulted in pure P12 and P60 neuron cell populations that contained few contaminants from other cell types, such as astrocytes, oligodendrocytes and microglia, as well as very little debris (fig. 3). It is noteworthy to point out, however, that the amount of neurons isolated at P60 were 80 % fewer compared to the amount of neurons isolated at P12 (data not shown).

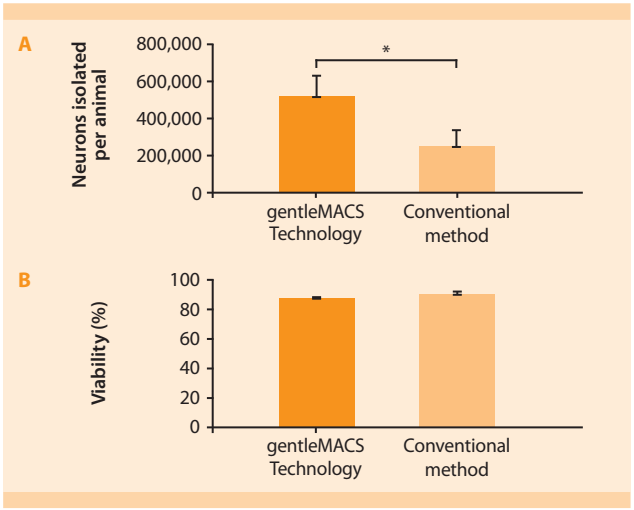

Figure 2: (A) gentleMACS Technology recovered approximately double the amount of total P12 neurons compared to the conventional manual dissociation system as determined by flow cytometry (p=0.035). **(B)** Viability was assessed by flow cytometry (PI staining) and did not vary between the two dissociation methods immediately after neuron isolation. Data represents n=3.

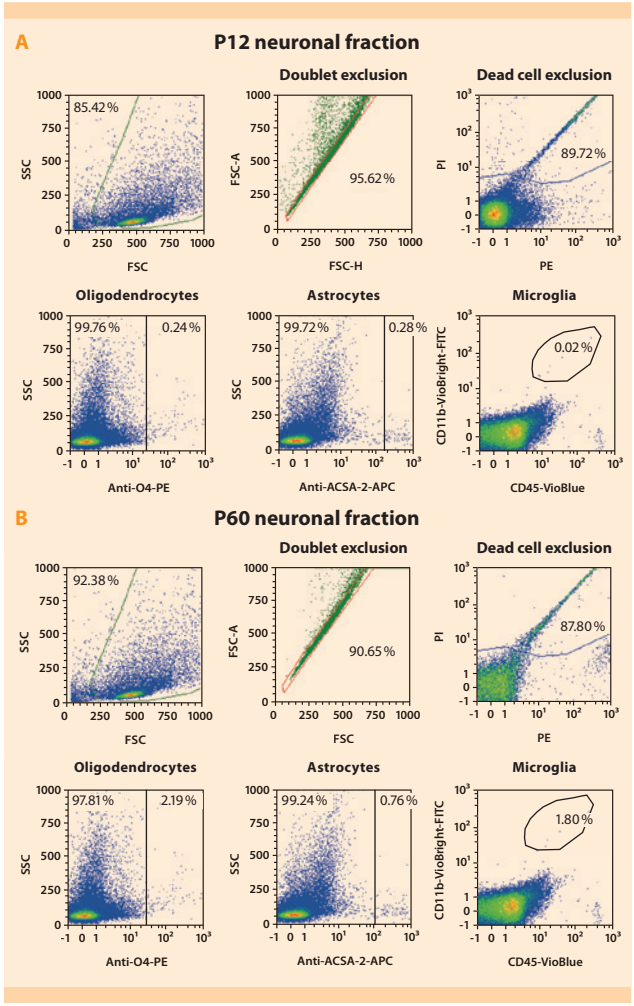

Figure 3: Flow cytometric analysis of **(A)** P12 and **(B)** P60 neurons derived with gentleMACS Technology shows minimal contamination with oligodendrocytes, astrocytes and microglia. The images depicted are representative for 3 independent experiments.

Viable culture of P12 and P60 isolated neurons with gentleMACS Technology

Immediately after neuron isolation, cell viability did not differ between the two dissociation methods (fig. 2B). Since programmed cell death requires 24–72 hours to complete, we sought out to compare neuron cell viability during prolonged cultivation *in vitro*. P12 and P60 neurons prepared by gentleMACS Technology survived in culture for 14 days (longer cultures were not tested), and extended both axons and dendrites (fig. 4). However, neurons derived from P12 brain tissue dissociated with the conventional manual dissociation system did not survive in culture and did not extend neurites (fig. 4). This loss in survival was accompanied by upregulation of the apoptotic marker cleaved-caspase 3, which was not induced in P12 and P60 neurons derived with gentleMACS Technology (fig. 4). This indicates that dissociation using this automated, mechanical technology allows isolation of healthy neurons that can be maintained in culture for extended periods of time.

In summary, gentleMACS™ Technology provides a reliable and reproducible procedure that will allow researchers to explore molecular mechanisms associated with adult CNS neurons and may yield novel insights into CNS neuron function not previously identifiable.

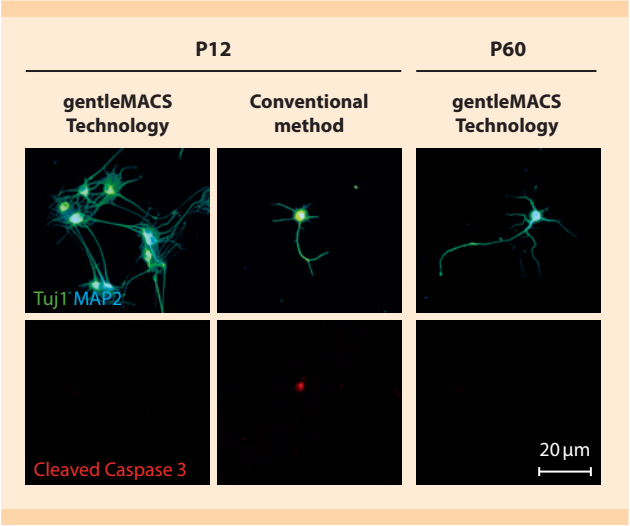

Figure 4: Neurons derived with gentleMACS Technology remain viable in culture for up to 14 days when isolated on postnatal days 12 and 60. Neurons isolated with the conventional manual dissociation method on P12 did not survive for longer periods of time and showed upregulation of the apoptotic marker cleaved caspase-3. The images depicted are representative for 3 independent experiments.

Conclusion

- With gentleMACS Technology and MACS® MicroBeads, high numbers of pure, viable neurons can be isolated from late postnatal and adult mouse brain.
- Neurons isolated from P12 and P60 mice maintain their functional integrity and survive in culture for at least 2 weeks, enabling *in vitro* molecular analyses that were not previously possible.
- Isolated neurons are free of contaminating non-neuronal cells like astrocytes, oligodendrocytes, and microglia.
- The gentleMACS Method provides a fast and easy protocol, which does not require much training (no animal perfusion), leading to reproducible and user-independent results.

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For identification purposes only, Dr. van Niekerk is a project scientist at the University of California, San Diego. Nothing herein should imply, indicate, or otherwise suggest that the University of California is connected or affiliated with, or endorses, favors, uses, or supports Miltenyi Biotec and/or any of its products or services.

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