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Automated imaging system for fast quantitation of neurons, cell morphology and neurite morphometry *in vivo* and *in vitro*

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ABSTRACT

Quantitation of neurons using stereologic approaches reduces bias and systematic error, but is time-consuming and labor-intensive. Accurate methods for quantifying neurons *in vitro* are lacking; conventional methodologies are limited in reliability and application. The morphological properties of the soma and neurites are a key aspect of neuronal phenotype and function, but the assays commonly used in such evaluations are beset with several methodological drawbacks. Herein we describe automated techniques to quantify the number and morphology of neurons (or any cell type, *e.g.*, astrocytes) and their processes with high speed and accuracy. Neuronal quantification from brain tissue using a motorized stage system yielded results that were statistically comparable to those generated by stereology. The approach was then adapted for *in vitro* neuron and neurite outgrowth quantification. To determine the utility of our methods, rotenone was used as a neurotoxicant leading to morphological changes in neurons and cell death, astrocytic activation, and loss of neurites. Importantly, our technique counted about 8 times as many neurons in less than 5–10% of the time taken by manual stereological analysis.

Introduction

Neurons and glia are differentially affected by neurotoxins, neurodegenerative disease and multiple other insults, including trauma. Reliable and quantitative tools to measure neurodegeneration are needed, and the manual approaches currently used are insufficient. For neuronal analysis, it is not enough to just determine cell number; changes in cell morphology have been related to cell death and neurite quantification is also needed as neurodegeneration often begins in distal regions of the neuron.

Superficially, neuron counting would seem simple; however, the distribution of cells is not random and for this reason, stereological methods have been developed which do allow for accurate quantitation. The optical fractionator is generally accepted as the most efficient and accurate counting approach, combining the optical dissector with spatial sampling methods that are statistically optimized (West et al., 1991). This technique uses systematic random sampling (SRS) to generate unbiased data, but is extremely time-consuming.

In contrast to counting neurons within the exquisitely ordered structure(s) found in brain sections, neurons grown in culture are randomly organized and are not amenable to classic stereology. Accordingly, most investigators continue to utilize the traditional visual enumeration method, selecting representative fields of view and manually counting immunostained neurons (Caiazzo et al., 2011). It is possible to use flow cytometry to generate simple cell counts (Meyer et al., 1980) or tritium uptake to indirectly measure cell survival (Gao et al., 2011; Mytilineou and Cohen, 1984) but neither method allows the subtlety needed to define cell structure or health.

Chronic inflammation involving activated astroglia is a pathognomonic sign of many human diseases including neurodegenerative disorders. Astrocyte organization is regionally consistent and spatially distinct; however, morphology of individual cells may behave independently of region and can be considerably influenced by environmental factors (Bushong et al., 2003).

Specific morphologic changes such as cell elongation, cell shrinkage, condensation of chromatin, and changes in membrane morphology are consequence of cellular differentiation, cellular toxicity or pathology. In neurodegenerative disorders, cells undergoing apoptosis display typical morphological alterations (Mattson, 2000). Thus, alterations in cell structure are events of particular importance in

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the pathogenesis of neurodegenerative disorders and their quantitative assessment could be worthwhile for the development of effective new neuroprotective therapies.

Quantitative analysis of neurites is essential when studying factors influencing neuronal development (Brandt et al., 2007) and pathological changes related to neurodegeneration (Wu et al., 2010) or neuroprotection (He et al., 2009). The morphological properties of neurites comprise key aspects of neuronal phenotype and play essential roles in establishing neuronal network connectivity and information processing, and must therefore be measured. However, these methods tend to be manual and hence, time-consuming. Because neurons extend into space in all three dimensions, following a branching structure, a successful strategy for realistic tracing applications has to operate in 3D. In this regard, multiple different methods have been implemented with variable success (Zhang et al., 2007).

We have applied multiple dimension (XYZ) automated digital image collection methods to overcome the existing limitations for neuronal quantification and assessment of neurite morphometry. We have designed and engineered an efficient automated system using an upright microscope equipped with a linear encoded motorized stage capable of quickly scanning the entire surface of a specimen and assembling up to 400 images in 4 colors into a single high resolution montage for analysis. Initial goals were to optimize system reliability and sensitivity enough to detect physiological changes in neurons and provide results at least comparable to stereology. For this study, we used rotenone, a pesticide and complex I inhibitor that induces degeneration of dopamine (DA) neurons in the substantia nigra (SN) of rat (Betarbet et al., 2000) and in primary neuronal cultures of the ventral midbrain (Gao et al., 2011).

Material and methods

Chemicals, reagents and other supplies

Chemicals and reagents were purchased as follows: Leibovitz L-15 medium, trypsin, neurobasal medium, B-27 supplement, fetal bovine serum, horse serum, L-glutamine, glutamax I, albumax I, Alexa Fluor 488, and 647 from Gibco (Invitrogen Life Technologies, Carlsbad, CA, USA). Minimum essential medium (MEM), sodium pyruvate, MEM non-essential amino acids, and penicillin–streptomycin were obtained from Mediatech Inc. (Cellgro, Manassas, VA, USA). Poly-D-lysine hydrobromide (PDL), sucrose, glucose, bisBenzimide H 33342 fluorochrometrihydrochloride, hydrogen peroxide (H_2O_2), dimethyl sulfoxide (DMSO), and 97.6% rotenone were acquired from Sigma Chemical Co. (St. Louis, MO, USA). Paraformaldehyde (PFA, 96%) was obtained from Acros Organics (New Jersey, NY, USA). Normal donkey serum and Cy3 secondary and biotin anti-mouse antibodies were ordered from Jackson ImmunoResearch labs, Inc. (West Grove, PA, USA). Phosphate buffered saline (PBS), Triton, glass coverslips, microscope cover glass, and microscope slides were obtained from Fisher Scientific (Pittsburgh, PA, USA). Vectastain avidin–biotin complex (ABC) kit, 3,3′-diaminobenzidine (DAB), and vectamount were acquired from Vector labs (Burlingame, CA, USA). Glial cell line derived neurotrophic factor (GDNF) was purchased from R&D Systems (Minneapolis, MN, USA). PFA (16%) was bought from Electron Microscopy Sciences (Hatfield, PA, USA). Miglyol 812N was obtained from Warner Graham (Baltimore, MD, USA). Magnesium chloride ($MgCl_2$) was ordered from Ambion (Austin, TX, USA). Aquamount mounting media were acquired from Lerner labs (Pittsburgh, PA, USA). We used antibodies to mouse anti-microtubule associated protein 2 (MAP2), sheep anti-tyrosine hydroxylase (TH), mouse anti-TH, rabbit anti-glial fibrillary acidic protein (GFAP) obtained from Millipore (Billerica, MA, USA).

Animals

Six-month-old male Lewis rats that weighed 400–450 g were purchased from Hilltop Lab Animals, Inc. (Scottdale, PA, USA) and used for the *in vivo* experiments. For the *in vitro* study, 2- to 3-month-old female timed-pregnant Sprague–Dawley rats, shipped to our animal facility on day 14 or 15 of pregnancy, were obtained from Charles River Laboratories International, Inc. (Wilmington, MA, USA). Conventional diets and water were available *ad libitum* and the animals were maintained under standard conditions (in a 22 ± 1 °C temperature-controlled room with 50–70% humidity) with a light–dark cycle of 12:12 h. The rats were randomly assigned to control and treatment groups. Housing and breeding of the animals and the experimental methods used in animal studies were approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh and were carried out in accordance with published NIH guidelines.

Experimental design for neurotoxic treatment

For the *in vivo* experiments, rats were injected intraperitoneally with a dose of 3.0 mg/kg/day of rotenone (Cannon et al., 2009; Tapias et al., 2010); the solution was administered at 1 mL/kg. The neurotoxin rotenone was initially prepared as a 50× stock dissolved in 100% DMSO then diluted in Miglyol 812N, a medium chain fatty acid. The control animals received an equivalent volume of the 2% DMSO + 98% Miglyol vehicle. The rats were randomized into 2 groups prior to rotenone administration. Each group was comprised of 5 animals.

For the *in vitro* experimental model, primary ventral midbrain cultures were prepared from embryonic day 17 (E17) rats; the embryos were obtained from 2 pregnant dams. Rotenone (50 nM) or vehicle was used to treat primary cell cultures for 5 days beginning on the fifth day *in vitro* (DIV 5). Rotenone was freshly prepared in DMSO and diluted to the final concentration in treatment medium. Ten days after seeding (DIV 10), the cultures were fixed and processed for subsequent analysis.

Histology and brain tissue processing

The experimental endpoint was established when a potentially debilitating phenotype for the animals was observed, *i.e.*, when clear signs of akynesia, rigidity, and postural instability were evident. Rats were euthanized by decapitation following CO_2 exposure at termination. The brains were carefully and quickly removed and fixed in 4% PFA in PBS for seven days and then cryoprotected in 30% sucrose in PBS for a minimum of 3 days until infiltration was complete. Next, brains were cut on a freezing sliding microtome into 35 μm transverse free-floating coronal sections, which were collected in 24 well-plates. Then, the sections were frozen in cryoprotectant (1 mL 0.1 M PO_4^{3-} buffer, 600 g sucrose, 600 mL ethylene glycol, pH = 7.2) and maintained at −20 °C until the subsequent DAB chromogen or immunofluorescent staining assays were performed.

Primary midbrain neuron cultures

Primary cells were prepared following a previously published protocol with some modifications (Gao et al., 2002). Ventral midbrain tissues were dissected from E17 Sprague–Dawley rat brains. After removal of the meninges, the pooled ventral midbrain tissues were dissociated by mild mechanical trituration and enzymatic digestion using trypsin. Cell viability and overall cell yield were evaluated using the trypan blue assay and a hemocytometer. Resuspended cells were seeded on circular coverslips pre-coated with PDL (0.1 mg/mL) in 24-well culture plates at a density of 5×10^5/well. Cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air in 0.5 mL/well of MEM containing 2% heat-inactivated fetal bovine serum, 2% heat-inactivated horse serum, 1 g/L glucose, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 μM non-essential amino acids, 50 U/mL penicillin, and 50 μg/mL streptomycin. Two days after the initial seeding, the culture medium was changed to 0.5 mL/well of fresh serum-free Neurobasal medium