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Advances in Thin Tissue Golgi-Cox Impregnation: Fast, Reliable Methods for Multi-Assay Analyses in Rodent and Non-human Primate Brain

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Abstract

In 1873 Camillo Golgi discovered a staining technique that allowed for the visualization of whole neurons within the brain, initially termed 'the black reaction' and is now known as Golgi impregnation. Despite the capricious nature of this method, Golgi impregnation remains a widely used method for whole neuron visualization and analysis of dendritic arborization and spine quantification. We describe a series of reliable, modified 'Golgi-Cox' impregnation methods that complement some existing methods and have several advantages over traditional whole brain 'Golgi' impregnation. First, these methods utilize 60–100μm thick brain sections, which allows for fast, reliable impregnation of neurons in rats (7–14 days) and non-human primates (NHP) (30 days) while avoiding the pitfalls of other 'rapid Golgi' techniques traditionally employed with thin sections. Second, these methods employ several common tissue fixatives, resulting in high quality neuron impregnation in brain sections from acrolein, glutaraldehyde, and paraformaldehyde perfused rats, and in glutaraldehyde perfused NHP brain tissue. Third, because thin sections are obtained on a vibratome prior to processing, alternate sections of brain tissue can be used for additional analyses such as immunohistochemistry or electron microscopy. This later advantage allows for comparison of, for example, dendrite morphology in sections adjacent to pertinent histochemical markers or ultrastructural components. Finally, we describe a method for simultaneous light microscopic visualization of both tyrosine hydroxylase immunohistochemistry and Golgi impregnation in the same tissue section. Thus, the methods described here allow for fast, high quality Golgi impregnation and conserve experimental subjects by allowing multiple analyses within an individual animal.

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Keywords

dendrite; spine; neuroanatomical method; Golgi-Cox impregnation; immunohistochemistry; electron microscopy

1. Introduction

In 1873, Camillo Golgi discovered the basic method of staining neurons in the nervous system that was initially termed 'the black reaction' (*la reazione nera*) and is now referred to as Golgi staining. After laborious attempts to stain elements of nervous tissue, Golgi uncovered a process involving nervous tissue hardening in potassium dichromate and impregnation with silver nitrate (Golgi, 1873). The original method required immersion of tissue in the potassium dichromate for several months and subsequent impregnation in silver nitrate for several additional days. One of the main strengths of the Golgi method is that it is capricious in that it stains only approximately 1–10% of neurons in any one select region (Shankaranarayana et al., 2004). This capricious staining allows a panoramic visualization of virtually all parts of an individual neuron including the soma, axon, dendrites, and dendritic spines.

Understanding brain connectivity, function and structural modifications associated with various pathological conditions has been of interest for more than a century. While neuroscientists have developed a vast array of methods to probe neuronal architecture (for review see Lanciego and Wouterlood 2011), Golgi impregnation remains a frequently used method for whole neuron visualization and elegant detailed analysis of dendritic arborization and dendritic spine phenotypes (e.g.: Robinson and Kolb, 1999; Diana et al., 2006; Marchetti et al., 2009; Srivastava et al., 2009; Hamilton et al., 2010; Li et al., 2012; Pinto et al., 2012; Krugers et al., 2012). In recent decades, there has been interest from neuroscientists to apply this classical approach of staining whole neurons in conjunction with newer approaches (Somogyi et al., 1981; Anderson and Felten, 1982; Buller and Rossi, 1993; Diane et al., 2006; Pilati et al., 2008; Spiga et al., 2011; Pinto et al., 2012) and, thus, numerous variations of Golgi impregnation have been developed. Each of the variant approaches has advantages and disadvantages compared to the classic Golgi approach. One specific disadvantage commonly associated with thin tissue impregnation has been the crystallization artifacts that are endemic in the 'rapid Golgi' method of staining (Gabbott and Somogyi, 1984; Friedland et al., 2006). These artifacts are thought to be due to a nonselective reaction between potassium dichromate and silver nitrate, resulting in the formation of bulk crystals on the surface of the specimen (Pasternak and Woolsey, 1975).

In the experiments described in this paper, we use a variation of the original Golgi recipe, termed Golgi-Cox (e.g.: Cox, 1891; Van der Loos, 1956; Glaser and Van der Loos, 1981), in which silver nitrate is replaced with mercury chloride to foster the impregnation of neurons. We describe a variation of the Golgi-Cox method, similar to that detailed by Landas and Phillips (1982), which reliably and effectively impregnates neurons in thin brain tissue sections (100μm) without significant crystallization artifact. We detail here the extensive characterization of this modified Golgi-Cox method that provides high quality, reliable Golgi impregnation of neurons in thin brain tissue sections from either rat or non-human primate (NHP). We have characterized the advantages and caveats of the Golgi-Cox method associated with various fixatives including paraformaldehyde (PF), acrolein, and glutaraldehyde. Fixative perfused sections are also compared with saline perfused sections, which were Golgi impregnated as thin tissue sections (100μm) or as blocks (~7 mm). Further, because thin sections were obtained on a vibratome prior to Golgi-Cox processing, we detail the reliability and potential caveats of using alternate sections of brain tissue for

additional analyses including immunohistochemistry (IHC) and electron microscopy (EM). Finally, we describe a method for performing and visualizing, with light microscopy, immunohistochemically stained and Golgi impregnated neurons in the same tissue section. This approach complements and extends the utility of dual Golgi/IHC reported previously by Buller and Rossi (1993), Spiga et al., (2011), and Pinto et al., (2012) using different experimental conditions.

Overall, the advantage of employing Golgi impregnation with thin tissue sections provides value by 1) eliminating the need for impregnating the entire brain, thus allowing for a more expeditious staining of neurons, and 2) conserving the number of animals required for a study by allowing for the use of the alternate brain sections, taken in series, for Golgi impregnation and additional analyses such as IHC or EM. The ability to obtain multiple measurements from each animal in a single experiment offers advantages including a determination of the strength of the association between the dependent measures, determining which of the dependent measures is most important, and an examination of the effects of the covariates. Throughout the manuscript we highlight several technical details associated with Golgi-Cox impregnation so that any investigator may tailor their various assays to work in tandem.

2. Materials and methods

2.1. Animals

Adult male Sprague Dawley rats (Harlan Laboratories, Indianapolis, IN), weighing 225–250g at the start of the experiment, were housed in groups of two. Food and water were available *ad libitum* in their home cages. Rats were maintained on a 12 hour light/dark cycle with lights on at 0700 hours. Rhesus monkeys (*Macaca mulatta*), weighing 6–20 kg at the start of the experiment, were housed separately in home cages and exposed to a 12 hour light/dark cycle. They were fed daily in amounts appropriate for the size and age of the animals and water was available *ad libitum*. All studies were carried out in accordance with the Declaration of Helsinki and with the Institute for Laboratory Animal Research of the National Academy of Science *Guide for the Care and Use of Laboratory Animals* (8^{th} edition, revised 2011) and were approved by the Michigan State University, University of Cincinnati, and University of California San Francisco Institutional Animal Care and Use Committees. All efforts were made to minimize the number of animals used and their pain or discomfort.

2.2. Preparation of the Golgi-Cox fixative solution

For all cases described herein, the Golgi-Cox fixative solution was prepared such that the final concentration of mercury chloride ($HgCl_2$, Fisher Scientific, Pittsburgh, PA), potassium chromate (K_2CrO_4, Sigma-Aldrich Corp., St. Louis, MO), potassium dichromate ($K_2Cr_2O_7$, Sigma-Aldrich Corp., St. Louis, MO) was 1%; each was dissolved in distilled water. For example, to prepare a 1L solution of Golgi-Cox fixative solution, 10g of mercury chloride was first dissolved into 200ml of distilled water at 80°C. The solution was allowed to cool to room temperature (RT) and was then slowly added to an equal volume of a 5% solution of potassium dichromate (e.g.: 10g of potassium dichromate was dissolved into 200ml of distilled water at RT). To this a 1.67% solution of potassium chromate (e.g.: 10g dissolved into 600ml distilled water at RT) was slowly added while stirring. The solution was wrapped in aluminum foil to protect it from light and allowed to sit for 12 hours at RT. Care was used when using aluminum foil as a light barrier with Golgi-Cox methods as aluminum is highly soluble in this fixative and shreds of dissolved foil that could fall into the sample containers will inhibit Golgi impregnation. The solution was decanted into

sample containers, with care taken to avoid decanting the reddish-brown precipitate at the bottom of the container.

2.3. Brain-block Golgi-Cox impregnation: saline perfused rat tissue

Adult male Sprague Dawley rats (Harlan Laboratories, 225–250g) were deeply anesthetized with sodium pentobarbital (200mg/kg) and transcardially perfused with 200ml of heparinized, RT 0.9% saline. Similar to the more traditional approach originally described by Golgi (Golgi, 1873) which employed whole brains, we bisected the brain in half coronally (Fig. 1) at the level of the hypothalamus using a steel brain mold (Stoelting Co., Wood Dale, IL) and a single-edged razor blade. The result was a brain "block" that measured ~7 mm (rostral to caudal length) (Fig. 1). For this study, the rostral portion of the block containing the striatum was placed into Golgi-Cox fixative. The container was wrapped again in aluminum foil to prevent exposure of the tissue and fixative to light. The fixative solution was exchanged with fresh solution ~24 hours after the tissue was first immersed in Golgi-Cox fixative. The samples were then allowed to impregnate in the dark for two weeks (14 days). At the end of this period, the Golgi-Cox fixative solution was replaced with a 1% solution of potassium dichromate (e.g.: 10g dissolved in 1L of distilled water) for 24 hours. The brains were sectioned on a vibrating microtome (Leica VT1000S, Leica Microsystems, Wetzlar, Germany) at a thickness of 100μm into a 1% solution of potassium dichromate. The sections were placed onto 4% gel-coated slides and gently blotted with filter paper. After drying in a humid chamber for 20 minutes at 37°C, the slides were placed in a solution of 28% ammonium hydroxide (NH_4OH, 28% NH_3 in distilled water, Sigma-Aldrich Corp.) for 30 minutes at RT, followed by a 5-minute rinse in tap water, a 15-minute rinse in 15% Kodak fixer (Kodak Polymax T Fixer, Electron Microscopy Sciences, Hatfield, PA) and an additional 5-minute rinse in tap water. Finally, the slides were dehydrated by exposing them to an ascending series of alcohol solutions (i.e., 3 minutes in 50% ethanol, 3 minutes in 70% ethanol, two 3-minute exposures to 95% ethanol, two 3-minute exposures to 100% ethanol), cleared in xylene (two 3-minute exposures to xylene) and coverslipped in DPX mountant (Fluka BioChemika, Buchs, Switzerland).

2.4. Thin tissue Golgi-Cox impregnation: saline perfused rat brain

Adult male Sprague Dawley rats (Harlan Laboratories, 225–250g) were deeply anesthetized with sodium pentobarbital (200mg/kg) and transcardially perfused with 200ml of heparinized, RT 0.9% saline, followed by 100ml of cold (4–10°C) saline. Brains were removed and sectioned immediately on a vibrating microtome at a thickness of 100μm into 0.1M phosphate buffer (PB). Two to four brain sections (100μm thick; collected at intervals per Fig. 1) were placed on the non-etched portion of a non-subbed glass slide. A second non-subbed glass slide was gently placed on top. This second slide was first wrapped with a single layer of standard vinyl electrical tape around each end, which acted as a 'spacer' to prevent compression of the tissue sections and allow for fluid flow; the two slides were then loosely banded together with a single piece of electrical tape. This two-slide construct was submerged in Golgi-Cox fixative and placed into a storage area that was completely devoid of light. After 24 hours, the Golgi-Cox fixative solution was exchanged for fresh Golgi-Cox fixative solution; the samples were then left undisturbed and allowed to impregnate for two weeks. At the end of this period, the Golgi-Cox fixative solution was replaced with a 1% solution of potassium dichromate for 24 hours after which the slices were transferred into a Petri dish containing 1% potassium dichromate by gently running a single-edged razor blade along the slide underneath the slice. The slices were then transferred onto 4% gel-coated slides and gently blotted with filter paper. After drying in a humid chamber for 20 minutes, the slides were placed into a solution of 28% ammonium hydroxide for 30 minutes, followed by a 5-minute rinse in tap water, a 15-minute rinse in 15% Kodak fixer and an additional rinse in tap water as described above. Finally, the slides were dehydrated by exposing them

to an ascending series of alcohol solutions, cleared in xylene, and coverslipped in DPX mountant as described above.

2.5. Thin tissue Golgi-Cox impregnation: paraformaldehyde perfused rat brain tissue with adjacent sections employed for tyrosine hydroxylase immunohistochemistry

Adult male Sprague Dawley rats (Harlan Laboratories, 225–250g) were deeply anesthetized with sodium pentobarbital (200mg/kg) and transcardially perfused with 200ml of heparinized 0.9% saline followed by 200ml of cold (4–10°C) paraformaldehyde (PF, Electron Microscopy Sciences, Hatfield, PA). Brains were removed and post-fixed in 4% PF at RT for 30 minutes and then transferred to 0.1M PB prior to immediate sectioning; although we have found that brains can be stored whole for approximately 1 week in 0.1M PB at 4°C prior to sectioning with no degradation of the quality of Golgi impregnation. However, tissue cannot be stored in standard cryoprotectant solution and frozen prior to Golgi impregnation. For the current study, sections through the rostral-caudal extent of the forebrain including the striatum were collected using a vibrating microtome. Beginning at the rostral pole of the forebrain, a series of 6 consecutive sections at 50μm thickness was collected for TH IHC and were stored in standard ethylene glycol based cryoprotectant solution (Watson et al., 1986). A 7th section, cut at 100μm, was used for Golgi-Cox impregnation and was either processed immediately for Golgi-Cox impregnation, or stored in 0.1M PB for up to one week. This sampling scheme was repeated until the caudal aspect of the forebrain was reached (Fig. 1). Each of the 100μm thick vibratome-cut sections was mounted onto a non-subbed glass slide for Golgi-Cox impregnation. Two 100μm thick brain sections were placed on the non-etched portion of any single non-subbed glass slide. A second non-subbed slide with the ends wrapped in a single layer of standard electrical tape was gently placed on top of the samples as described above. This double-slide construct was submerged in Golgi-Cox fixative. The fixative was exchanged ~24 hours after the tissue was immersed in Golgi-Cox fixative and the samples were allowed to impregnate in complete darkness for 14 days. At the end of this period, the brain slices were transferred into a Petri dish containing 1% potassium dichromate using a single-edged razor blade then transferred onto 4% gel-coated slides and gently blotted with filter paper. After drying in a humid chamber for 20 minutes, the slides were placed into a solution of 28% ammonium hydroxide (NH_4OH, 28% NH_3 in distilled water) for 30 minutes, followed by a 5-minute rinse in tap water, a 15-minute rinse in 15% Kodak fixer and an additional 5-minute rinse in tap water as described above. Finally, the slides were dehydrated by immersing them in an ascending series of alcohol solutions, cleared in xylene, and coverslipped in DPX mountant as detailed above.

The series of 50μm thick sections that were processed for standard TH IHC (e.g.; Fig. 5) were done according to previously published methods (Kordower et al., 1995; Steece-Collier et al., 1995; Maries et al., 2006). Briefly, sections were rinsed several times in 0.1M Tris-buffered saline (pH 7.4) then incubated in 0.3% H_2O_2 for 15 minutes to quench endogenous peroxidase activity. Sections were then blocked in 10% normal goat serum (NGS, Invitrogen, Inc., Grand Island, NY) containing 0.03% Triton X-100 (Tx) for 20 minutes at RT, then incubated for 24 hours at RT in a solution containing mouse anti-TH (1:4000, Millipore, Temecula, CA) and 1% NGS. Following extensive rinsing, sections were incubated in biotinylated goat anti-mouse IgG (1:400, Vector Labs, Burlingame, CA) and 10% NGS for 2 hours at RT and, again after rinsing, were incubated with avidin-biotin peroxidase complex (ABC, Vector Labs, Burlingame, CA) for 1 hour at RT. The sections were reacted with 0.05% 3, 3′-diaminobenzidine tetrahydrochloride (DAB, Sigma-Aldrich Corp.) containing 0.01% H_2O_2 for 3 minutes.

2.6. Thin tissue Golgi-Cox impregnation: paraformaldehyde/acrolein perfused rat brain tissue with adjacent sections employed for electron microscopy

To allow for optimal preservation of tissue ultrastructure for EM, adult male Sprague Dawley rats (Harlan Laboratories, 225–250g) were deeply anesthetized with sodium pentobarbital (200mg/kg) and transcardially perfused with RT 200ml heparinized 0.9% saline, followed by 50ml of cold (4–10°C) 2% PF containing 3.75% acrolein (Polysciences, Inc, Warrington, PA), followed by an additional 200ml of cold (4–10°C) 2% PF. Brains were removed and post-fixed for 60 minutes at RT in 2% PF then transferred to 0.1M PB prior to sectioning. The sampling scheme that we used for collecting alternate brain sections for Golgi impregnation and EM is illustrated in Figure 6 (inset diagram). For our purposes, the brain was blocked to allow for the vibratome sectioning of the entirety of the rat striatum. Sections designated for Golgi-Cox impregnation were cut, using a vibrating microtome at 60μm thickness (Fig. 6). All sections were collected into 0.1M PB. Sections were selected, according to systematic random sampling, and removed for Golgi impregnation or EM processing. Tissue sections from these brains selected for Golgi-Cox impregnation were processed according to the method reported above in section 2.5.

The sections from these brains that were designated for ultrastructural analyses were removed from the 0.1M PB within 48 hours of sectioning and placed into an EM grade cryoprotectant solution (referred to as Totterdell's solution). This cryoprotectant solution contains 0.2M sodium phosphate monobasic monohydrate ($NaH_2PO_4 \cdot 1H_2O$), 0.2M sodium phosphate dibasic dihydrate ($Na_2HPO_4 \cdot 2H_2O$), 30% ethylene glycol, and 30% glycerol in distilled water. For 1L of this solution, we dissolved 31.2g of the sodium phosphate monobasic and 35.6g of sodium phosphate dibasic in 400ml distilled water, then added 300ml of ethylene glycol and 300ml of glycerol. Samples were stored in this cyroprotectant at −20°C until processed for EM according to published methods (Morshedi et al., 2009; Rademacher et al., 2010) with minor modifications. Briefly, sections processed for EM were fixed in a solution of 1% osmium tetroxide and 1.5% potassium ferricyanide (Sigma-Aldrich Corp.) in 0.1M PB for 30 minutes at RT in the dark. The sections were dehydrated, stained with 1% uranyl acetate (Electron Microscopy Sciences) *en bloc*, and flat embedded in Durcupan epoxy resin (components A–D, Sigma-Aldrich Corp.) between two sheets of aclar fluorohalocarbon film. The epoxy resin was allowed to polymerize at 70°C for 72 hours. An area of known size (0.5 × 0.5 mm) was selected from the striatum. Ultrathin serial sections (silver, ~60–70 nm) were cut (~10 per grid; EM UC6; Leica Microsystems Inc., Bannockburn, IL), mounted on formvar-coated copper slot grids, and stained with uranyl acetate and lead citrate prior to viewing. The electron microscopic images were captured on a JEOL 100 CXII electron microscope equipped with a side mounted MegaView II digital camera coupled to analySIS® image analysis software (Olympus Soft Imaging Solutions GmbH, Berlin, Germany). The series of 60μm thick sections from these brains that were designated for TH IHC were processed as described in section 2.5.

2.7. Thin tissue Golgi-Cox impregnation: glutaraldehyde/paraformaldehyde perfused non-human primate brain tissue with adjacent sections employed for immunoelectron microscopy

Adult male and female NHPs (Rhesus macaque, *Macaca mulatta*, 6–20 kg) involved in a larger NIH-funded study were sedated via ketamine delivered intramuscularly and deeply anesthetized with sodium pentobarbital (>200mg/kg). They were transcardially perfused with cold (4–10°C) Ringer's solution (500ml–1L), followed by 2L of 0.1% glutaraldehyde (Electron Microscopy Sciences) in 4% PF. The brains were removed, cut into a series of 3-mm thick slabs through the entire rostral-caudal extent using a slotted metal brain mold. The 3-mm thick slabs were postfixed for 12 hours in 4% PF. The sampling scheme used for collecting tissue for Golgi impregnation, immunoelectron microscopy (immunoEM), and

TH IHC is illustrated in Figure 6 (inset diagram). A 3-mm thick brain slab, containing the striatal region of interest, was mounted onto a vibratome chuck and cut serially at either 60 or 100um, using a vibrating microtome as illustrated in Figure 6. Sections for Golgi-Cox impregnation were cut at 100μm thickness and collected into 0.1M PB. Sections for immunoEM and TH IHC were cut at 60μm thickness and collected into Totterdell's solution (described above), which allowed for storage at −20°C until processing for either IHC or immunoEM.

NHP brain sections selected for Golgi-Cox impregnation were transferred from 0.1M PB to unsubbed (50×75mm) slides and 'sandwiched' as described above for rat brain sections with the exception that tissue remained in sealed vials of 0.1M PB for up to three months while we established the optimal method for Golgi-Cox impregnation. In addition, because of the larger size of the tissue sections/slides, a layer of Parafilm® was added to the electrical tape to increase the flow of fluid into the interior of the slide sandwich. The sandwiched sections were placed in Golgi-Cox fixative and allowed to impregnate in complete darkness. The Golgi-Cox fixative was replaced after the tissue had been incubating in the fixative for 2 weeks. After an additional 2 weeks (4 weeks total) of incubation in Golgi-Cox fixative, the fixative was replaced with 1% potassium dichromate, and the sandwiched tissue allowed to incubate in that solution for 12 hours at RT. The sections were transferred from the slides to a Petri dish containing a solution of 1% potassium dichromate. The sections were placed on a coverslip and submerged into a solution of 28% ammonium hydroxide (in distilled water) for 30 minutes at RT. This was followed by a 5-minute rinse in distilled water and a 5-minute incubation in 15% Kodak fixer. The sections were again rinsed for 5 minutes in distilled water, transferred to a fresh change of water, and then mounted on subbed slides. The slides were placed in a humid chamber at 37°C for 20 minutes, and then placed directly on a slide warmer to dry completely. It took ~10 minutes for the slides to dry completely. Care was taken to not to leave them unattended to dry longer than necessary. Next, the slides were dehydrated in 95% ethanol (twice for 30 seconds each), 100% ethanol (twice for 30 seconds each), cleared in xylene (twice for 2 minutes each) and coverslipped using DPX mountant. The series of 60μm thick sections that were processed for TH IHC were done as described in section 2.5.

Sections selected for immunoEM and depicted in this manuscript were subjected to one of three IHC reactions: 5HT (5-hydroxytrypamine, serotonin), TH and dopamine D1receptor (D1R), TH and dopamine D2receptor (D2R). Those selected for 5HT immunoEM were rinsed several times in 0.1M PB then incubated in a 1% solution of sodium borohydride for 30 minutes at RT. After rinsing, sections were then blocked in 10% NGS (Invitrogen, Inc.) containing 0.03% Tx for 1 hour at RT, followed by incubation for 36 hours at RT in a solution containing rabbit anti-5HT (1:100000, ImmunoStar, Hudson, WI). Following extensive rinsing, sections were incubated a 10% solution of NGS containing biotinylated goat anti-rabbit IgG (1:200, Vector Labs) for 2 hours at RT and, again after rinsing, were incubated with ABC for 1 hour at RT. The sections were reacted with 0.05% DAB containing 0.01% H_2O_2 for 3 minutes.

Sections selected for TH and D1R dual-label immunoEM were rinsed several times in 0.1M PB then incubated in a 1% solution of sodium borohydride for 30 minutes at RT. After rinsing, sections were blocked in 10% NGS (Invitrogen, Inc.) containing 0.03% Tx for 1 hour at RT, then incubated for 36 hours at RT in a solution containing mouse anti-TH (1:1000, Millipore). Following extensive rinsing, sections were incubated in a 10% solution of NGS containing biotinylated goat anti-mouse IgG (1:200, Vector Labs) for 2 hours at RT and, again after rinsing, were incubated with ABC (Vector Labs) for 1 hour at RT. The sections were reacted with 0.05% DAB containing 0.01% H_2O_2 for 3 minutes. After rinsing, sections were incubated for 36 hours at RT in a solution containing rat anti-D1R (1:500,

Millipore). Following extensive rinsing, sections were incubated in a 10% solution of NGS containing biotinylated goat anti-rat IgG (1:200, Vector Labs) for 2 hours at RT and, again after rinsing, were incubated with ABC for 1 hour at RT. The sections were reacted with Vector Slate Grey (SG) (Vector Labs) for 3 minutes. Sections selected for TH and D2R dual-label IHC (Fig. 7, N) were rinsed several times in 0.1M PB then incubated in a 1% solution of sodium borohydride for 30 minutes at RT. After rinsing, sections were blocked in 10% NGS (Invitrogen, Inc.) containing 0.03% Tx for 1 hour at RT, then incubated for 36 hours at RT in a solution containing mouse anti-TH (1:1000, Millipore). Following extensive rinsing, sections were incubated in a 10% solution of NGS containing biotinylated goat anti-mouse IgG (1:200, Vector Labs) for 2 hours at RT and, again after rinsing, were incubated with ABC for 1 hour at RT. The sections were reacted with 0.05% DAB containing 0.01% H_2O_2 for 3 minutes. After rinsing, sections were incubated for 36 hours at RT in a solution containing rabbit anti-D2R (1:500, Millipore). Following extensive rinsing, sections were incubated in a 10% solution of NGS containing biotinylated goat anti-rabbit IgG (1:200, Vector Labs) for 2 hours at RT and, again after rinsing, were incubated with ABC for 1 hour at RT. The sections were reacted with SG for 3 minutes. After the completion of the IHC reactions, the sections were fixed in a solution of 1% osmium tetroxide (Electron Microscopy Sciences) and 1.5% potassium ferricyanide (Sigma-Aldrich Corp) in 0.1M PB for 30 min at RT in the dark. The sections were dehydrated, stained with 1% uranyl acetate *en bloc*, and flat embedded in Durcupan epoxy resin (components A–D, Sigma-Aldrich Corp.) between two sheets of aclar fluorohalocarbon film. The epoxy resin was allowed to polymerize at 70°C for 72 hours. An area of known size (0.5 × 0.5 mm) was selected from the striatum. Ultrathin serial sections (silver, ~60–70 nm) were cut (~10 per grid), mounted on formvar-coated copper slot grids, and stained with uranyl acetate and lead citrate prior to viewing. The electron microscopic images were captured on a JEOL 100 CXII electron microscope equipped with a side mounted MegaView digital camera coupled to analySIS® image analysis software.

2.8. Dual label, thin tissue Golgi-Cox impregnation: paraformaldehyde perfused rat brain tissue with tyrosine hydroxylase immunohistochemistry employed in the same tissue section

Adult male Sprague Dawley rats (Harlan Laboratories, 225–250g) were deeply anesthetized with sodium pentobarbital (200mg/kg) and transcardially perfused with 100ml of cold (4–10°C) saline followed by 200ml of cold (4–10°C) 4% PF. Brains were post-fixed in 4% PF for 24 hours at RT. The rostral-caudal extent of the striatum was sectioned into 0.1M PB at 50um thickness using a vibrating microtome. The sections were first Golgi-Cox impregnated per the thin tissue method described above. After developing the slices in ammonium hydroxide and Kodak fixer, the slices were processed for TH IHC as described in section 2.5. Some tissue used for the development of this method was left in potassium dichromate for up to 11 days as sections were selected for IHC; a control sample was processed for Golgi every time tissue was selected for IHC during this period.

3. Results

In this study we demonstrate the dynamics of Golgi impregnation of neurons using the Golgi-Cox impregnation method (Cox, 1891), which utilizes mercury chloride in place of silver nitrate used in the original Golgi method (Golgi, 1873). Using the modifications presented here, the current study shows that this method allows for flexibility and reliability of staining in thin brain sections (50–100um) of rat and NHP under a variety of post-mortem tissue procurement approaches. As with all Golgi-type impregnation methods, there are strengths and weaknesses of the Golgi-Cox method. In this Results section, we detail the benefit and limitations of each approach.

3.1. Brain-block Golgi-Cox impregnation in saline perfused rat tissue gives good morphology with minimal overdevelopment

The use of the Golgi-Cox method to impregnate neurons in a block (~7 × 12 mm) of saline perfused rat brain resulted in complete staining of both whole neurons and individual dendritic spines in all brain regions examined (Fig. 2). While high quality impregnation was observed, we also noted that some areas showed minor overdevelopment in that there were clusters of impregnated neurons, which are seen best at low magnifications (Fig. 2D, G). Despite some level of clustering of impregnated neurons, we found that it was still quite easy to discern and trace individual dendritic trees at higher magnifications (e.g.: 60 and 100x) as can be seen in the tracing done with the Neurolucida and Neurolucida Explorer programs (Fig. 3).

3.2. Thin tissue Golgi-Cox impregnation in saline perfused rat brain gives suboptimal morphology

In contrast to high quality and extensive impregnation of individual neurons found in fresh brain tissue processed as a block of tissue, we found that processing thin tissue sections (100μm) from saline perfused rats with the Golgi-Cox methods provided suboptimal histological results. The perikarya, dendrites, and spines were noted to exhibit an appearance of incomplete impregnation (Fig. 4B, C, E, F) with an overall poor quality of neuronal anatomy. The stippled appearance of neurons in Figure 4 is typical of incomplete impregnation (Meller and Dennis, 1990).

3.3. Thin tissue Golgi-Cox impregnation in paraformaldehyde perfused rat brain tissue offers enhanced visualization of dendritic spine detail and allows for IHC staining of adjacent sections

The sampling scheme illustrated in Figure 1 was used to allow for adjacent sections to be processed for Golgi-Cox impregnation or TH IHC. Qualitatively, PF fixation yielded the appearance of greater numbers of impregnated neurons when compared to saline perfused tissue using either thin tissue or block impregnation. It can also be observed that this method produces areas that have a high density of impregnated neurons, particularly in the cortex and hippocampus, when compared to saline-perfused samples (thick black arrows in Fig. 5D, E; compare to Fig. 2D, E). Further, visualization of individual spine detail appears enhanced in PF perfused tissue compared to saline perfused tissue (Fig. 5C, F, I).

The brains employed for these methods of post-mortem analyses were obtained from unilaterally parkinsonian rats that were part of a larger study and had received a graft of embryonic ventral mesencephalon into the dopamine (DA)-depleted striatum. It can be noted that even in the Golgi-Cox processed sections, the region of the graft is apparent due to the presence of hemosiderin (black arrows, Fig. 5A, I), an iron storage complex found after the rupture of blood vessels that occurs during intracerebral surgery associated with the deposition of grafted cells. When TH IHC was performed in sections adjacent to those processed for Golgi, we observed specific staining of DA neurons in the striatum of rats that had received an intra-striatal graft of embryonic DA neurons (Fig. 5J-L). As can be seen in Figure 5 there is excellent morphology of the grafted TH positive neurons and evidence of extensive neurite outgrowth into the host parenchyma.

3.4. Thin tissue Golgi-Cox impregnation in PF/acrolein perfused rat brain tissue provides for well preserved tissue ultrastructure compatible with EM, superior resolution of Golgi impregnated neurons, and dense clustering of impregnated neurons

Thin tissue sections were collected for both Golgi-Cox impregnation and EM from the same rat brain, according to the sampling scheme depicted in Fig. 6G. Golgi-Cox impregnation

following combined PF/acrolein fixation allowed for highly reliable and distinct Golgi impregnation of neurons throughout all brain regions examined. Similar to the PF fixed tissue, the resolution of individual dendrites and spines appears enhanced compared to that seen in saline perfused rat tissue, particularly in striatal medium spiny neurons (MSNs) (Fig. 6C). One potential caveat of this method using both PF and acrolein is the increased density and 'clustering' of impregnated neurons, which is particularly notable when the section is viewed at low magnification (Fig. 6A, D). Again, despite the high density of Golgi-Cox impregnated neuronal populations, it remains possible to discern individual neurites and their perikaryon of origin (Fig. 6C, F). As can also be seen in Fig. 6H, EM performed in adjacent sections taken from the same brain shows outstanding fine ultrastructural features of neurons (e.g.: mitochondria, synapses, synaptic vesicles, etc.).

3.5. Thin tissue Golgi-Cox impregnation in glutaraldehyde/paraformaldehyde perfused non-human primate brain tissue provides excellent neuronal detail for 3-D reconstruction despite a high level of Golgi-related debris; and allows exquisite ultrastructural immunoEM details in adjacent brain sections

Thin tissue sections from NHP brains were collected for Golgi-Cox impregnation, light microscopy TH IHC, and immunoEM according to the sampling scheme depicted in Figure 6G. As can be seen in Figure 7, we have found that the perfusion and post-mortem methods described here allow for excellent Golgi-Cox impregnation of neurons in the striatum as well as cortex. The Golgi impregnation is similar to what others have reported utilizing silver nitrate-based impregnation (Francois et al, 1984, Shankaranarayana et al, 1999). While it is possible to visual elegant examples of Golgi impregnated neurons, two Golgi-related caveats are worth mentioning.

First, there is a larger amount of debris in these NHP samples compared to what was observed in the rat tissue (compare Fig. 7 and 8 to Fig. 2 and 5). The debris can be seen as crystals in the parenchyma of the brain and as impregnation of the vasculature. At low magnification, the resolution of the neurons seems to be compromised by the debris (Fig. 8A, C), however, when visualized at a higher magnification, excellent dendrite and dendritic spine detail is easily seen (Fig. 7G, J). Interestingly, this varied from subject to subject despite all NHPs being processed identically (Fig. 8A, B versus C, D). As part of one of our studies reported here, we systematically increased the amount of time rat tissue spent in the secondary potassium dichromate step that follows the removal of tissue from the Golgi solution. An increase in the amount of time in potassium dichromate, which is also a component of the initial Golgi-Cox solution, does result in an increase in the crystal-deposit debris within the tissue (Fig. 10). This finding suggests that the increase in this type of debris in the NHP tissue, which had a 2x longer exposure time to potassium dichromate, may simply reflect an increase of potassium dichromate crystallization artifact.

Second, in the NHP tissue we found that in the same brain region (e.g.; cortex) and within the same brain section there can be differential impregnation of neurons (Fig. 7B, E). While we also noted this in rat brain tissue, it was much less common (data not shown). Further, there were rare examples where an entire set of sections taken from one subject that showed a lack of Golgi impregnation throughout the brain (data not shown). This later finding was observed in both rat and monkey subjects and may have to do with variability in sacrifice perfusion.

Despite the NHP tissue showing a higher level of debris following Golgi-Cox fixation, adjacent sections showed exquisite ultrastructural immunoEM (Fig. 7L–N) including a 5HT labeled axon and other processes (Fig. 7L), a TH labeled axonal process and a D1R labeled dendrite (Fig. 7M), and a TH labeled axonal process and a D2R labeled dendrite (Fig. 7N). Of note, for dual-label immunoEM studies, we see a similar pattern of staining (axonal/

presynaptic for TH; and primarily dendritic/postsynaptic for D2R) regardless of whether the immunohistochemical reactions both used ABC methods followed by TH visualized with DAB and D2R visualized with SG (Fig. 7N), or when TH was visualized via DAB and D2R was visualized via silver intensified gold labeling (data not shown). We also observed high quality TH IHC in the caudate nucleus and putamen in the intact hemisphere (Fig. 7K) in a third section, adjacent to both the Golgi-Cox stained section and the immunoEM section. There was an absence of TH immunoreactivity in the caudate nucleus and putamen of the hemisphere lesioned by the DA neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Fig. 7K).

3.6. Combination of Golgi-Cox impregnation with TH IHC

In our attempts to perform Golgi and IHC in the same brain tissue section, we found that it was necessary to perform TH IHC after the Golgi-Cox impregnation step similar to a recent study by Spiga et al. (2011). When the Golgi-Cox method described here was employed prior to TH IHC, we noted regionally specific, high-quality TH immunoreactivity as well as distinct Golgi-impregnated neurons (Fig. 9). The animal from which the tissue section shown in Figure 9 was collected had received a striatal injection of the neurotoxin 6-hydroxydopamine (6-OHDA) unilaterally. Thus, only one hemisphere reacted positively for TH IHC.

It is important to report here that performing IHC first interfered with Golgi impregnation when permeabilization of the tissue was done with the detergents TritonX-100 or Tween-20 typically used for IHC. However, without these detergents, antibody penetration was insufficient for adequate IHC staining. We also attempted using freeze-thaw techniques in place of detergents. Similar to the finding of Gabbot and Somogyi (1984) who attempted freeze thaw with the rapid Golgi method, we found that this approach that resulted in disruption of the structural integrity of cellular membranes was detrimental to Golgi-Cox impregnation methods. This was true even with pre-incubation of sections in increasing concentrations of dimethyl sulfoxide (DMSO). Thus, reversing the order of assay methods to first employ the Golgi-Cox thin tissue impregnation, followed then by our standard IHC assay allowed us to visualize the complete neuronal morphology of Golgi-impregnated neurons in the same tissue that had anti-TH antibody staining of nigrostriatal DA fibers. Of note, using the Golgi-Cox method described here, no de-impregnation of the Golgi stained brain sections was performed prior to IHC staining. It is pertinent to point out however, that the processing of previously Golgi impregnated brain sections for standard IHC staining (as detailed above) does cause a minor degree of apparent de-impregnation, particularly in cortical regions (Fig. 9A). While some regional de-impregnation appears to accompany secondary IHC processing, the majority of Golgi impregnated neurons maintain full, high quality impregnation (Fig. 9B–E).

4. Discussion

Golgi impregnation remains a superior method for obtaining elegant, panoramic, 3-D views of neurons in the nervous system. Several recent reports have focused on enhancing the utility of Golgi, for example, by combining bromodeoxyuridine (BrdU) IHC with Golgi-impregnation technique (Pinto et al., 2012) or performing simultaneous Golgi-Cox and confocal immunofluorescence of post-synaptic density-95kD protein (PSD-95) (Spiga et al., 2011). With the current studies, we contribute to this continuing interest in integrating Golgi methods with more innovative approaches by detailing the limitations and successes of the Golgi-Cox method in a variety of post-mortem conditions and done in tandem with other assays. One focus of ongoing studies in our laboratory is to determine how pathological alterations of neuron structure in the parkinsonian brain might impact therapeutic interventions. As we demonstrate in this manuscript, a single experimental subject can be

used to obtain high quality 3-D neuron morphology through Golgi-Cox impregnation, dual label immunoEM for assessment of synapse ultrastructure, and light microscopy level IHC to confirm reinnervation patterns. We also confirm that IHC and Golgi impregnation can be performed within the same tissue section for analysis with light microscopy without the need for de-impregnation of heavy metals (an approach often required for IHC in Golgi impregnated tissue; see Buller and Rossi 1993). Using the same brain to compare all experimental variables has the advantage of 1) decreasing inter-subject variability, 2) conserving the numbers of animals in a study, and 3) determining the strength of the association between the dependent measures.

To allow for a combined sampling scheme such as presented in this manuscript, thin section Golgi impregnation is needed. In our hands, the Golgi-Cox method on thin tissue sections gives optimal results when the brain has been perfused with standard fixatives like PF or glutaraldehyde. As we report here, the *thin section* Golgi-Cox method used with saline perfused brain sections provides suboptimal impregnation and poor cytoarchitectural detail. In contrast, when a saline perfused brain was Golgi impregnated as a block of tissue and subsequently sectioned, Golgi impregnation and cytoarchitectural details were outstanding. While the results in the saline perfused thin tissue sections were somewhat surprising, we believe that this was due in large part to the difficulty in manipulating unfixed/fresh tissue without compromising the integrity of the tissue. PF perfusion seemed to be the best fixative option for thin tissue Golgi technique. The use of acrolein plus PF perfusion also gave high quality Golgi impregnation but also gave rise to more ‘cluster staining’ of neurons. The reason that NHP brain sections, of the same thickness as rat brain sections required twice the amount of time for neuron impregnation is uncertain. It could be argued that the addition of the glutaraldehyde to the PF could be the cause since this was one factor that differed between rat and NHP. However, we have used the same fixative of glutaraldehyde plus PF in rats and found that the 14 days Golgi-Cox immersion method is sufficient for full impregnation (personal observation; data not shown). When these two fixatives were used in rats there was no increase in the crystal debris deposits that was seen in the NHPs fixed with this dual fixative approach. Thus, it seems reasonable to suggest that the increased length of time required for Golgi impregnation of NHP thin brain sections is responsible for the increased debris artifact. Again, despite the increased debris and inconsistent impregnation in the NHP tissue, regions that demonstrated good impregnation showed high quality impregnated neurons with extensive arborizations and excellent dendrite and spine detail.

While using adjacent sections for comparison of different variables is highly useful, there are some challenges to this approach. For example, there is differential contraction of the tissue treated for IHC and Golgi fixation, which presents some difficulty in identifying structures in the adjacent sections. However, it is possible to identify morphological landmarks that allow this challenge to be minimized. Our demonstration that this Golgi-Cox method is compatible with performing (TH) IHC in the same section, allowing for simultaneous examination of these two variables in the same tissue section, thus eliminates some of the aforementioned challenges. It is pertinent to reiterate that our description of dual IHC and Golgi impregnation complements and extends recent interest in this approach reported by Spiga et al., (2011) and Pinto et al., (2012), and discussed in the extensive review by Buller and Rossi (1993). Specifically, initial reports aimed at combining IHC with Golgi impregnation discuss the necessity of de-impregnating, or removing the mercuric deposits and subsequently bleaching the sections, and/or utilizing enzyme pre-treatments to restore antigenic reactivity and intensify immunohistochemical staining (e.g.: Somogyi 1990; Buller and Rossi 1993). While these pretreatments may still be a necessity for some antibody reactions, it is encouraging that our work, and that of others (e.g.: Somogyi 1990; Buller and Rossi, 1993; Spiga et al., 2011; Pinto et al., 2012) demonstrate that there is retention of antigenic reactivity in brain tissue after Golgi-Cox impregnation. Our protocol

of dual IHC and Golgi impregnation, and that of Spiga (2011) and Pinto (2012) do not require de-impregnation protocols prior to IHC, which allows visualization of immunostained neurons in combination with the salient features of Golgi stained neurons. The reports by Spiga et al. (2011) and Pinto et al. (2012) use fluorescent IHC and confocal microscopy thus necessitating visualizing Golgi impregnated neurons using a reflection mode at 488 nm wavelength, which creates Golgi-Cox impregnated neurons as shaded solid bodies and surface renderings (Spiga et al., 2011). This is in contrast to our approach where both Golgi and IHC stained neurons are visualized using light microscopy. Both light and confocal microscopy allow for 3-D reconstruction of neurons with optical sectioning through the z-plane using specific software such as the Neurolucida program described in this manuscript. Again, it is prudent to mention that not all antibodies may be compatible with Golgi impregnation methods. Further, from a purest standpoint, single processing of tissue with TH only (in the absence of Golgi) tends to provide a more unimpeded view of, in our case, the grafted cells and their extensive and delicate processes. Similarly, single processing of tissue for Golgi impregnation only provides for more consistent, high quality Golgi impregnation throughout the brain. Thus, each method holds specific advantages.

One other challenge that we have noted with the specific sampling scheme that we describe here, which employed brain section thicknesses ranging from 50 to 100um, is that quantifying the full extent of the dendritic arborizations in the z-plane is limited. As can be appreciated in Figure 3, 100um thick section provides a limited ability to trace the dendrites that extend into the plane above and below where the section is cut (i.e., the z-plane). Because the entire extent of the neuron can generally be traced across the x and y plane, 2-dimensional measurements are not compromised. Thus, if quantification of the entire 3-D arbor is desired, employing a section with a thickness that will allow such quantification should be anticipated. Accordingly, one advantage of whole brain impregnation compared to thin section impregnation is that entire dendritic arbors are available for serial reconstruction. However, if whole brain impregnation is employed, it is necessary to ensure that long-term exposure to the Golgi solution does not impact other endpoints if additional measures are desired in adjacent sections.

5. Conclusions

There are many variant approaches of the Golgi method that continue to be used by neuroscientists worldwide, each endowed with advantages and disadvantages (e.g.: Somogyi 1990; Buller and Rossi, 1993; Rosoklija et al., 2003; Melendez-Ferro et al., 2009; Spiga et al., 2011; Pinto et al., 2012). After attempting to use several approaches, we have found that the thin section Golgi-Cox method detailed here provides consistent, high quality, fast impregnation of neurons throughout the brain, providing the elegant and detailed features of these cells. This approach provides ease of use, requiring no constant agitation or daily pH checking, while still maintaining quality of impregnation and very little precipitate artifact under most conditions. As so eloquently stated by Somogyi (1990) “The Golgi method has been in service of neuroscience for over a hundred years… (it) continues to yield new information, because the organization of neuronal processes provides a solid framework on which to build all further knowledge for the understanding of neuronal networks and their operation”. Thus, understanding the limitations and advantages of various Golgi methods, their compatibilities and incompatibilities with other histological and histochemical methods will continue to assist in expanding our knowledge of structure-function in the nervous system. We have attempted to highlight several details in the development of combined Golgi-Cox/IHC/immunoEM approaches so to serve as a guide for the investigator who are interested in tailoring a Golgi-Cox or combined Golgi-Cox/IHC method for their own use.

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Abbreviations

3-D	3-dimensional
6-OHDA	6-hydroxydopamine
ABC	avidin biotin peroxidase complex
BrdU	bromodeoxyuridine
DA	dopamine
D1R	dopamine D1 receptor
D2R	dopamine D2 receptor
DAB	3,3′-diaminobenzidine tetrahydrochloride
DMSO	dimethyl sulfoxide
EM	electron microscopy
IHC	immunohistochemistry
immunoEM	immunoelectron microscopy
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MSN	medium spiny neuron
NGS	normal goat serum
NHP	non-human primate
PB	phosphate buffer
PF	paraformaldehyde
PSD-95	post-synaptic density-95kD protein
RT	room temperature
SG	Vector slate grey
TH	tyrosine hydroxylase
Tx	Triton X-100

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Revised Highlights

- We describe thin tissue ‘Golgi-Cox’ methods that allow fast impregnation without significant crystallization artifact.
- These methods are compatible with the tissue fixatives acrolein, glutaraldehyde, or paraformaldehyde.
- Fixative perfusion allows using alternate sections for analyses such as immunohistochemistry and electron microscopy.
- We also describe immunohistochemistry and Golgi impregnation in the same tissue section without de-impregnation protocols.
- We detail advantages and disadvantages to guide for the investigator interested in tailoring Golgi-Cox for their own use

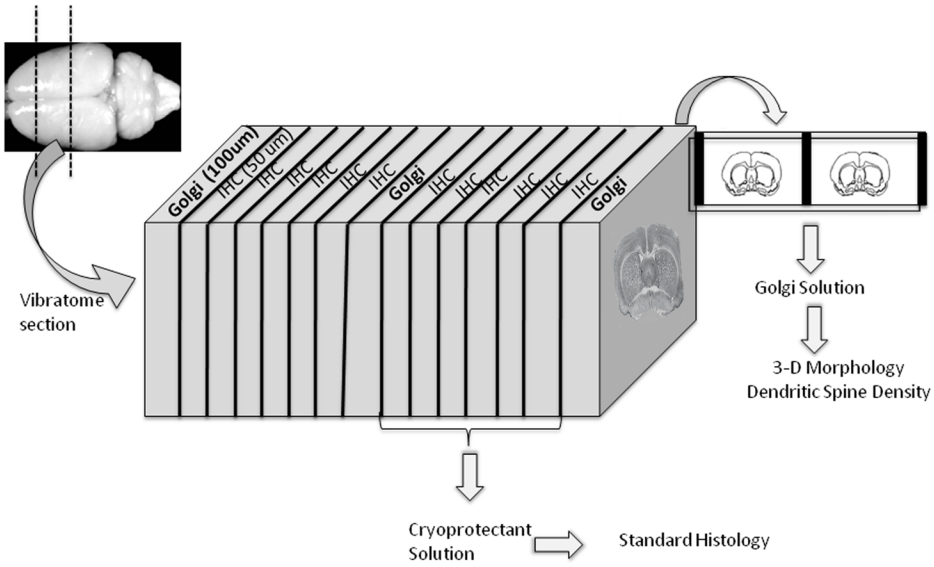

Figure 1. Demonstration of sampling method for employing a single brain for multiple postmortem analyses including IHC and Golgi impregnation

In this example, six serial slices though the striatum were collected for IHC in a 24-well plate in PB at a thickness of 50um, followed by a seventh at 100um. *Abbreviations*: IHC= immunohistochemistry; 3-D= 3-dimensional.

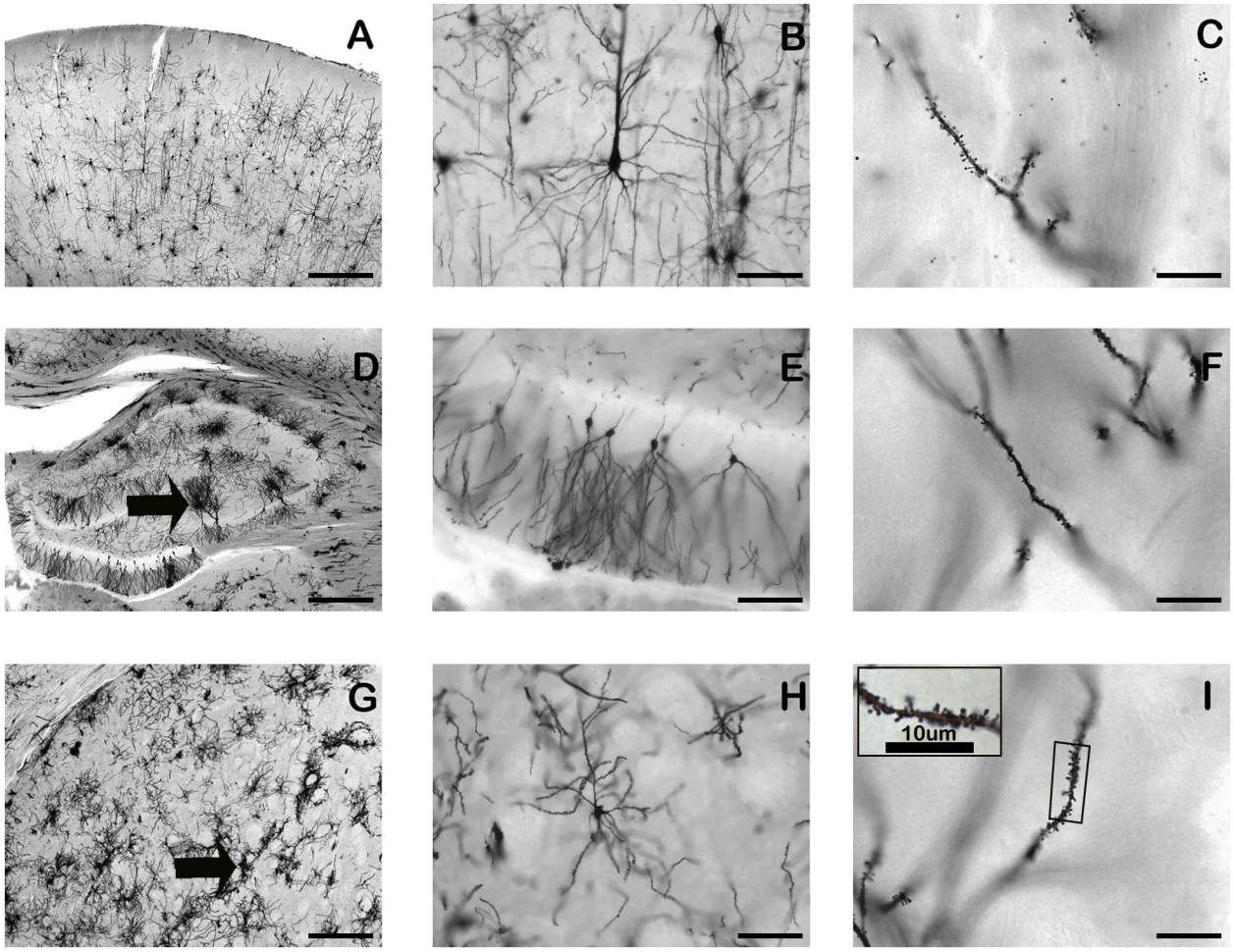

Figure 2. Micrographs of Golgi impregnated brain sections from a saline perfused, block impregnated rat brain

Rats were perfused with saline and the rostral half of the brain immersed in Golgi fixative and processed per the method described in the text. Micrographs demonstrate the exquisite staining of neurons in several different regions of the brain; **A–C**) cortex, **D–F**) hippocampus, **G–I**) striatum. As can be seen at lower magnifications, there is some minor overdevelopment of neuron impregnation restricted to small clusters in these brains (arrows in D, G). Despite some clustering of impregnated neurons, as can be observed at higher magnifications (micrographs B, E, H) it is still quite easy to discern and trace individual dendritic trees. Scale for A, D, G=500um; B, E, H=100um; C, F, I=20um.

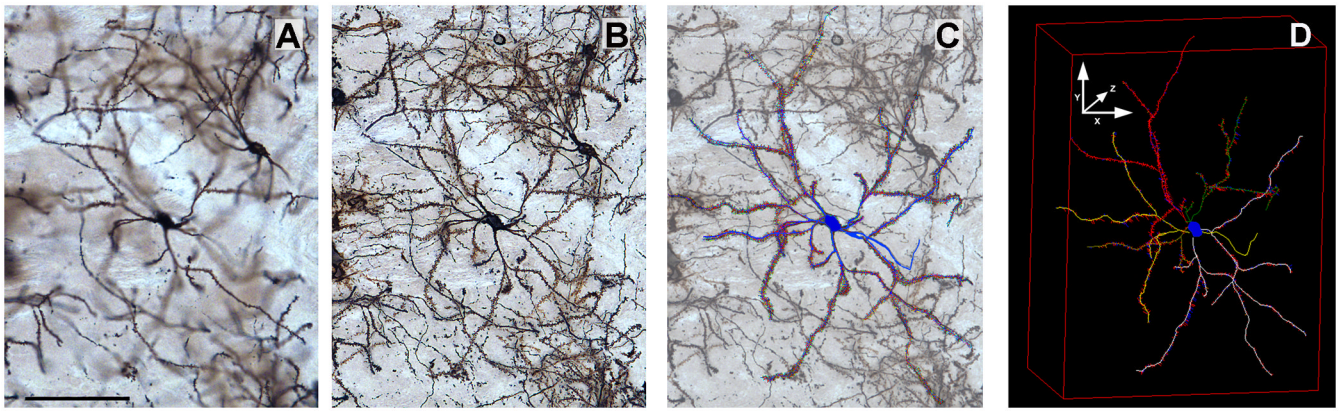

Figure 3. Computer assisted dendrite and spine tracing for neuron reconstruction of Golgi impregnated striatal MSNs in rat brain

These micrographs and photos demonstrate graphically the steps taken in neuron tracing and reconstruction via the Neurolucida® program (Microbrightfield Bioscience Inc, Williston, VT). **A**) An example medium spiny neuron identified for reconstruction and photographed on a single z-plane axis. This image demonstrates that Golgi impregnation penetrates the entire thickness of the tissue section showing some elements in the photographic focal plane and others above or below the focal plane and out of focus. **B)** Using the Neurolucida® program and optical sectioning through the z-plane, a series of images were taken at 1 um intervals and reconstruction into a single “deep focused” image. **C)** Shows the computer assisted neuronal tracing laid down over this Golgi impregnated neuron traced through the X-, Y- and Z-planes using the Neurolucida program from Microbrightfield Bioscience Inc. (Williston, VT); the Golgi image shown in ‘C’ was rendered 50% transparent to enhance viewing the tracing overlay for this photograph. **D**) A final 3-D image of the reconstructed neuron as viewed through the Neurolucida Explorer program, which can be rotated 360 degree for examination of 3-dimensional features of the traced neuron. Scale bar in A=100um.

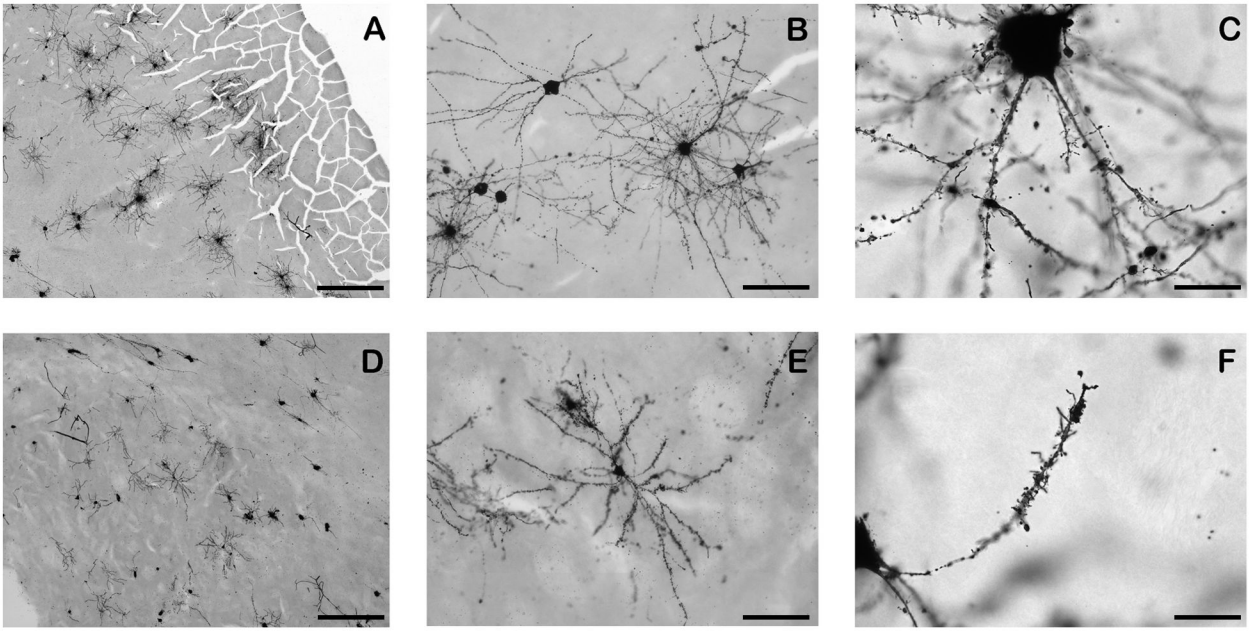

Figure 4. Micrographs of a Golgi impregnated brain section from a saline perfused rat following thin-tissue Golgi impregnation

Impregnation of thin tissue (100um) using our Golgi-Cox method produces suboptimal impregnation of neuronal elements, with abundant artifact related to the compromised integrity of brain tissue that occurs during processing; **AC**) cortex, **D–F**) striatum. Note the lack of prototypical layering of neurons generally present in Golgi impregnated cortical regions. The striatum shows exceptionally sparse impregnation of neurons. Scale for A and D=500um; B and E=100 um; C and F=20 um.

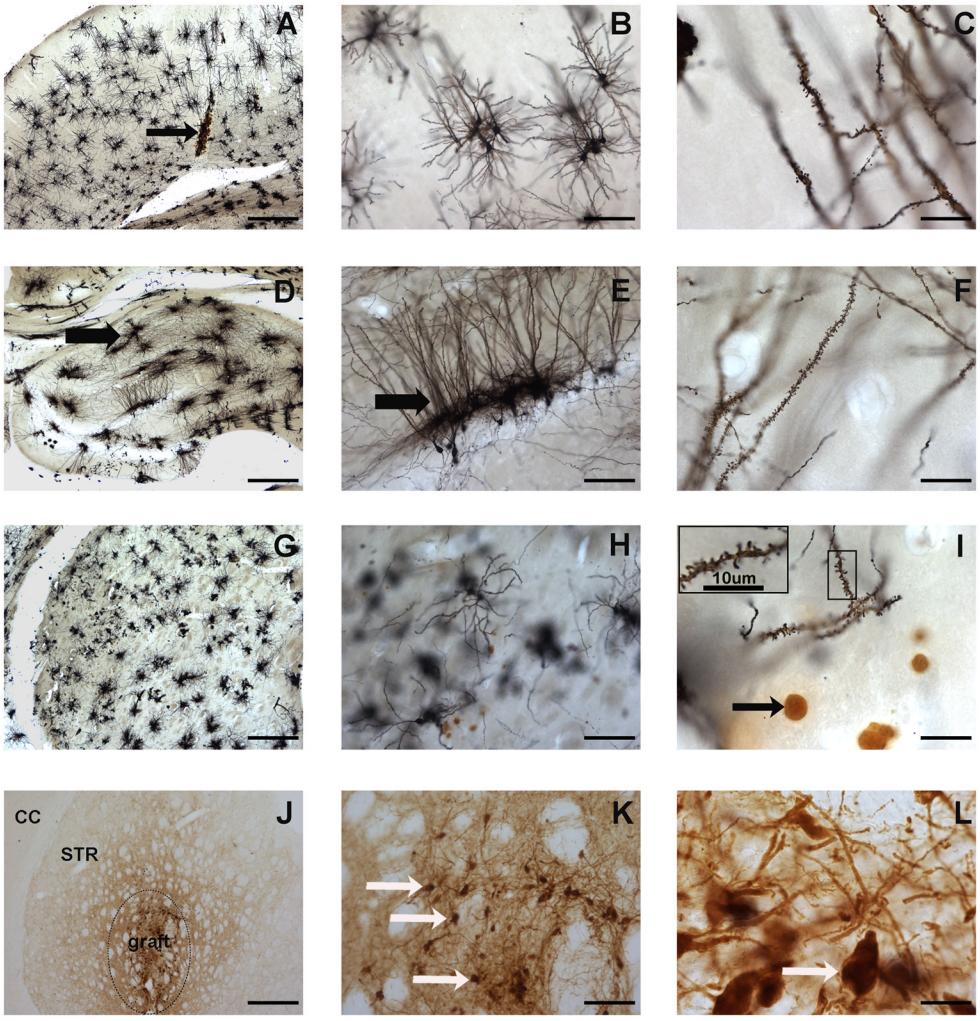

Figure 5. Micrographs of Golgi impregnated and TH immunostained brain sections from the same PF fixed rat brain

Rats were perfused with 4% PF and adjacent sections processed for thin-tissue Golgi or standard TH IHC. **A–C**) cortex with Golgi impregnated neurons; **D–F**) hippocampus, with Golgi impregnated neurons; **G–I**) striatum, with Golgi impregnated neurons. While PF fixation appears to increase clusters of Golgi impregnated neurons (arrows in D and E), the resolution of individual dendrites and spines appears enhanced (e.g.; C, F, I). **J–L**) Micrographs of a striatal section stained with antiTH IHC and adjacent to the one stained for Golgi seen in G–I. As can be noted, there are abundant grafted embryonic dopamine neurons (arrows in K and L) extending dense neuritic processes into the surrounding striatal parenchyma. Arrows in **A** and **I** indicate hemosiderin related to graft surgery in this animal. Scale for A, D, G, J=500um; B, E, H, K=100um; C, F, I, L=20um. Abbreviations in 'J': CC= corpus callosum; STR= striatum.

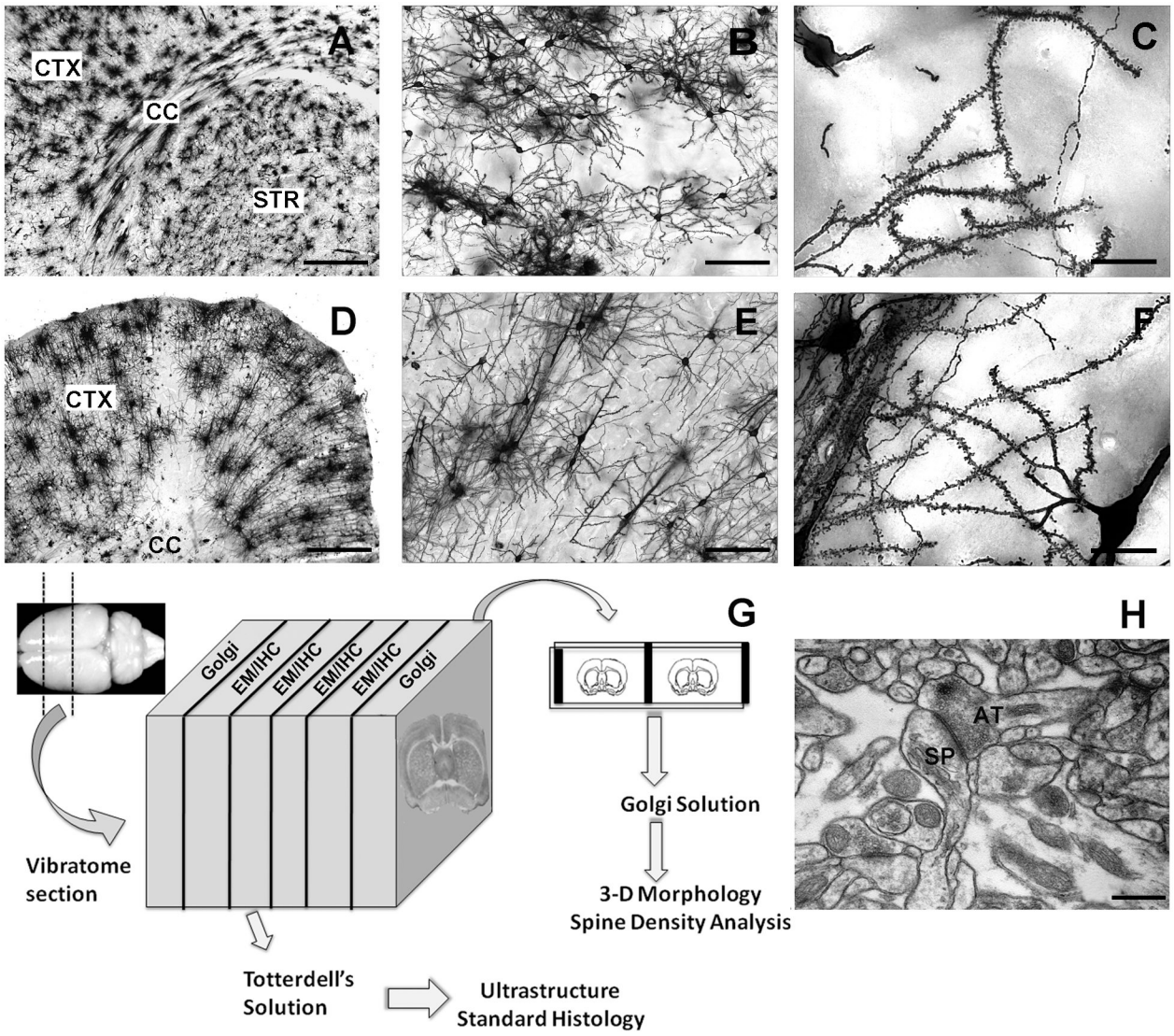

Figure 6. Schematic and micrographs demonstrating the utility of acrolein + PF fixation for multiple postmortem analyses

Rats were perfused with 3.75% acrolein in 2% PF to allow adjacent sections to be processed for thin-tissue Golgi impregnation and electron microscopic visualization central nervous system ultrastructure. **A–C**) striatum; **D–F**) cortex. This fixation notably increases the density and 'clustering' of Golgi stained neurons (evident in A and D). However, it also provides superior resolution of individual dendrites and spines in both cortex and striatum (C and F). **G**) Schematic demonstrating the sampling plan used for collecting tissue from a single brain for the Golgi and EM shown in these micrographs. **H**) Transmission electron micrograph of striatal ultrastructure from a brain section adjacent to the tissue section shown in A–F. Scale for A, D=500um; B, E=100um; C, F=20um; G= 500nm. *Abbreviations*: AT= axon terminal; CTX=cortex; CC= corpus callosum; EM=electron microscopy; IHC=immunohistochemistry; STR= striatum; SP= spine; 3-D=3-dimensional.

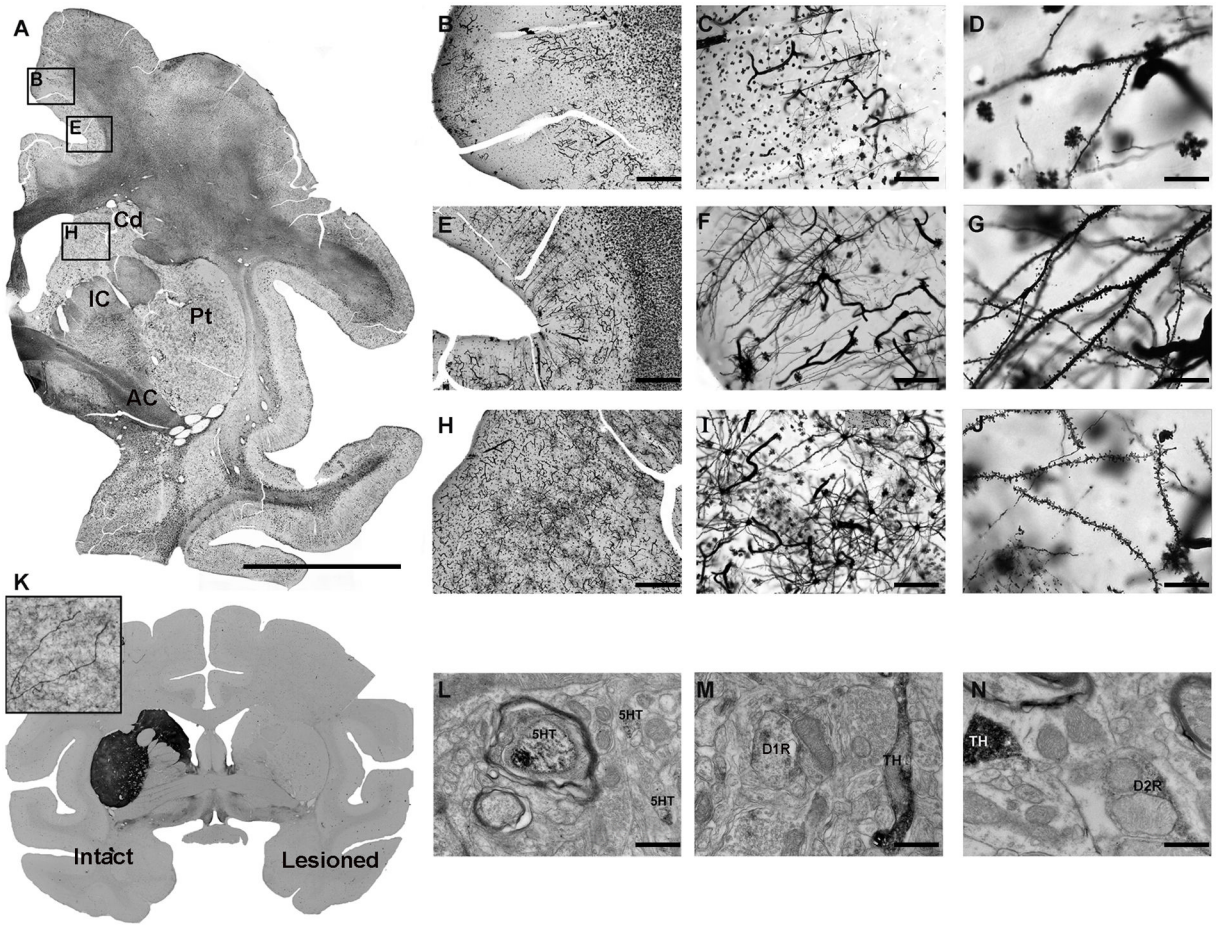

Figure 7. Micrographs demonstrating multiple post-mortem analyses of glutaraldehyde + PF perfused NHP brain tissue

A–J) *Golgi Impregnated neurons****; K)*** *TH immunohistochemistry;* ***L–N)*** *Immunoelectron micrographs.* **A**) Micrograph of a coronal hemisection at the level of the crossing of the anterior commissure and containing the caudate/putamen and various cortical regions impregnated with the Golgi-Cox method. **B–G**) cortex, Golgi impregnated neurons; **H–J)** caudate nucleus, Golgi impregnated neurons. As can be noted in B–D versus E–G, the perfusion method employed in this animal results in differential degrees of Golgi impregnation of cortical neurons, with the region shown in E–G showing superior impregnation compared to the region shown in B–D. This is in contrast to a more uniform impregnation of neurons with Golgi fixative in the caudate and putamen. **K)** Micrograph of a brain section taken adjacent to the one used for Golgi impregnation and stained with anti-TH IHC. This monkey was rendered unilaterally parkinsonian by intracarotid artery infusion of the dopamine neurotoxin MPTP thus depleting all TH-positive immunohistochemical staining in the lesioned caudate and putamen. The TH-positive reaction product is readily seen in the intact caudate and putamen. The inset in K shows a high magnification of the TH-positive fibers in the intact hemisphere imaged at high magnification. **L–N**) Transmission electron micrographs performed on adjacent brain sections from this same subject showing immunolabeling for anti-5HT, anti-D1R and D2R and anti-TH antibodies. Scale bar in A=1cm; B, E and H=500um; C, F, I=100um; D, G, J=20um; L–N=500nm. *Abbreviations*: AC= anterior commissure; Cd= caudate; D1R= D1 receptor; D2R= D2 receptor; IC= internal capsule; Pt= putamen; TH=tyrosine hydroxylase; 5HT- serotonin

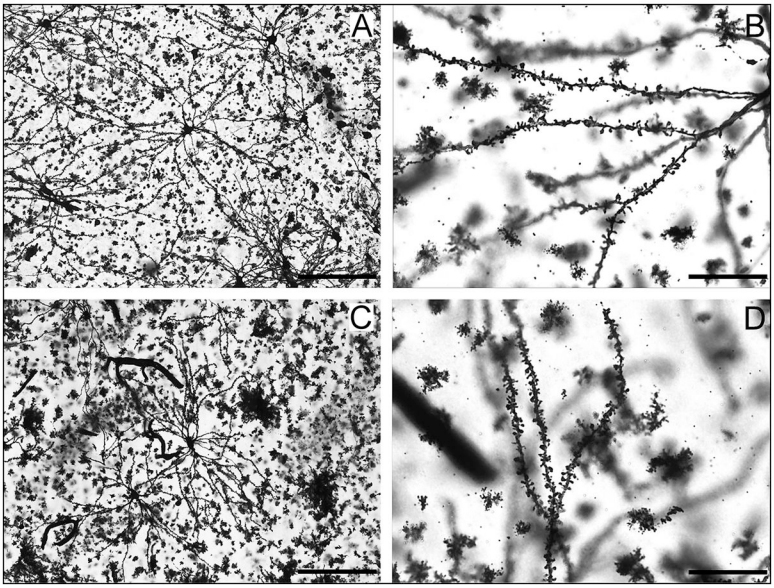

Figure 8. Micrographs demonstrating variability in the degree of Golgi neuron impregnation between subjects perfused with identical glutaraldehyde and PF fixation methods

These images were taken from the precommissural putamen of two different NHP brains fixed with identical perfusion methods. It is obvious that there is an apparent quantitative but not qualitative range of impregnation of striatal MSNs in these two subjects. **A**) This micrograph shows a representation of a more dense population of Golgi impregnated of striatal neurons, notable throughout this micrograph, in this first subject. **C**) This micrograph shows a representation of a relatively more sparse population impregnated striatal neurons in this second subject and more clustered debris. **B, D**) In both subjects the quality of impregnation appears equally high, with dendritic spines clearly visible in both animals. Scale bar in A and C=100um; B and D=20um.

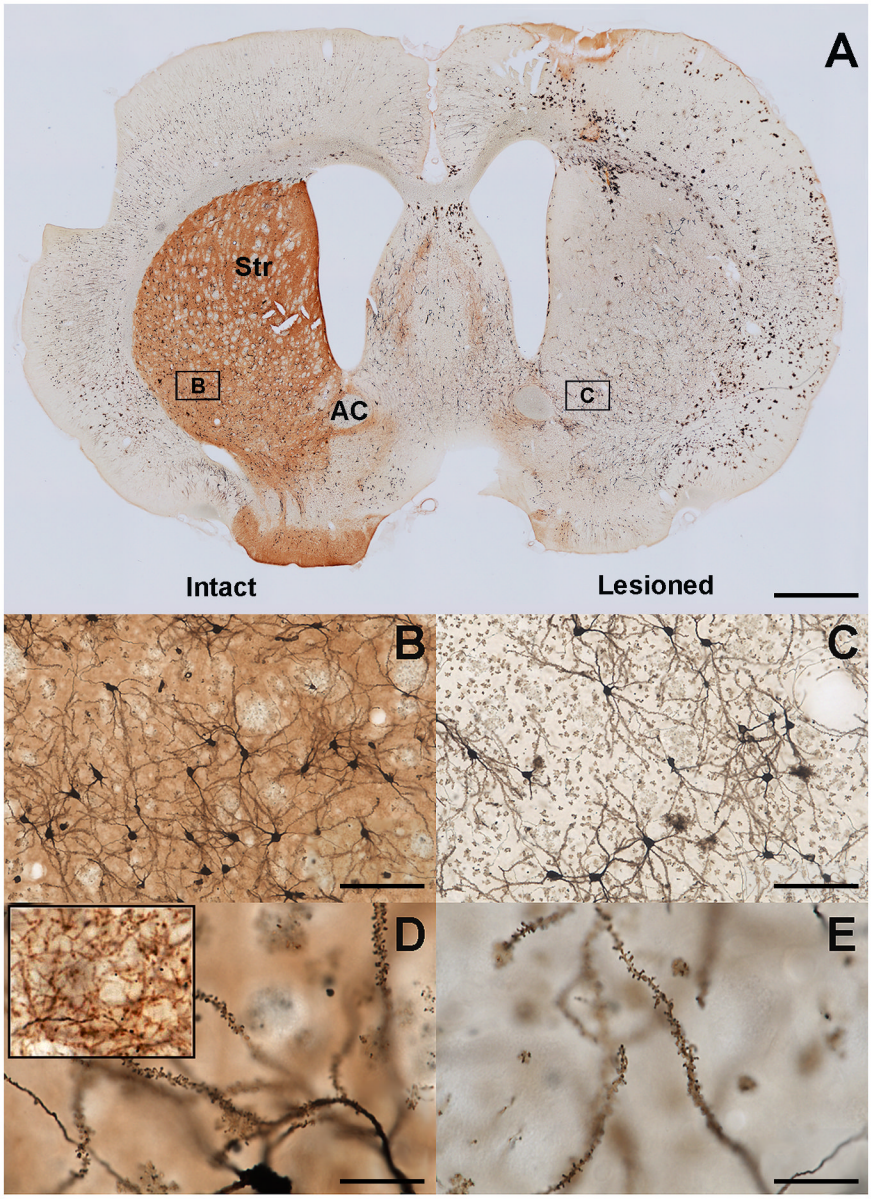

Figure 9. Dual label TH IHC and Golgi impregnation in the same tissue section from a PF perfused rat brain

The rat from which this sample was taken received a unilateral striatal injection of the dopamine neurotoxin 6-OHDA, which depleted TH-positive fibers in the lesioned striatum. As can be seen, on left side of micrograph **A**, and in **B** and **D**, there is abundant TH-positive staining on the intact side of the animal. In contrast, on the right side of micrograph **A**, and in **C** and **E**, there is an absence of TH immunoreactivity reflective of a near complete DA-depleting lesion on this side. **A:** While there are abundant high-quality Golgi impregnated neurons in both hemispheres, it can be noted that processing previously Golgi impregnated brain sections for standard TH-IHC staining can cause a minor degree of de-impregnation, particularly in cortical regions. **B–E:** While some regional de-impregnation appears to accompany secondary IHC processing, the majority of Golgi impregnated neurons maintain full, high quality impregnation. The micrograph in **A** was obtained by ‘stitching’ together multiple 4X images (using NIS Elements AR software, version 4.11). The inset photo in **D**

is a higher magnification view of the TH-positive fibers taken at a different focal plane from the Golgi-impregnated neuron in the intact striatal hemisphere. Interestingly, there is more debris on the lesioned side of the brain in both cortex and striatum. Scale bar in A=2000um; B–C=100um; D–E=20um. *Abbreviations:* AC= anterior commissure; OT= olfactory tubercle; Str= striatum.

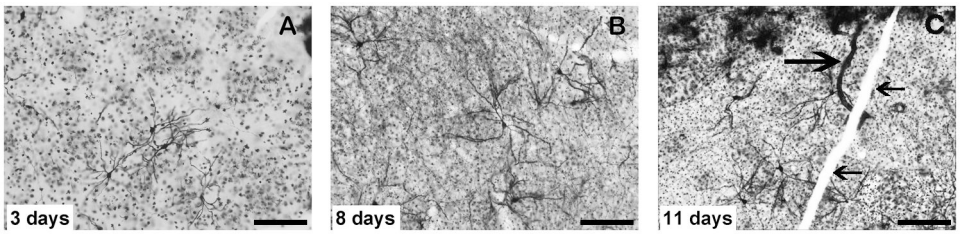

Figure 10. Time course of increasing potassium dichromate-related debris in Golgi impregnated rat tissue

A–C) Micrographs of three different rat brain sections that were differentially exposed to the secondary potassium dichromate step for either 3, 8 and 11 days; this differential exposure to potassium dichromate occurred after the usual 14 days exposure to Golgi-Cox solution. There is a marked increase in the amount of crystal-like debris staining with increased time in potassium dichromate. It can also be appreciated in **C** that the increased exposure time to potassium dichromate results in additional non-crystalline debris (i.e.: dark blotchy debris at top of image), cracking of brain tissue (two smaller arrows), and impregnation of blood vessels (large arrow). Scale bar for all micrographs=100um.