Morphological Analysis of Early Cerebellar Development

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Biology

by
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Abstract

The Purkinje cell is the main motor output cell of the cerebellum and aberrant Purkinje cell morphologies can lead to ataxic disorders (Perkins et al., 2010). Early in Purkinje cell development the young cell sends out processes in all directions (Baptista et al., 1994; Morrison, 2004). The identity of these early processes is unknown. SMI31 and MAP2 antibodies can be used to label Purkinje cell axons and dendrites, respectively (Hayashi et al., 2010; Gianola et al. 2003). In this study, SMI31 and MAP2 primary antibodies were used to immunolabel P0, P7, P14, and P21 mouse brain sections in an attempt to identity these early processes. MAP2 staining was observed on the distal portions of Purkinje cell dendrites and in the internal granule cell layer on granule cell dendrites in all mouse brain section ages. SMI31 staining was observed on Purkinje cell axons in P21 mouse brain sections during a pilot study to determine appropriate primary antibody concentrations, but SMI31 staining failed in the ultimate experiment due to procedural errors. Future experiments should utilize the procedure established in this study to double stain P0, P7, P14, and P21 mouse brain sections with SMI31 and MAP2 primary antibodies to determine the identity of early Purkinje cell processes and further characterize cerebellar development.

Introduction

The cerebellum is a portion of the brain located within the lower, back region of the skull. It is responsible for coordinating balance and enabling fluid body movements. The Purkinje cell, a neuron or nervous tissue cell, is the basic, key, functional unit of the

cerebellum. Purkinje cell axons are long, fine, fibers that descend from the bottom of the cell and primarily send information to the deep cerebellar nuclei. Purkinje cell dendrites are highly branched, arborous fibers that primarily receive information from olivary nuclei climbing fibers, basket cells, and granule cells. During Purkinje cell development, the young cells first send out processes in all directions. The identity of these early processes is largely unknown. Will they express proteins similar to axons, dendrites, a mixture of the two, or something entirely different? The current study hopes to address this question through a cryostat section study of mouse, cerebellar, protein expression in Purkinje cells.

It is important to study Purkinje cell development because Purkinje cells that develop abnormally often have grossly aberrant dendritic morphologies. These aberrant morphologies affect signal transduction and often result in ataxias, or diseases categorized by a lack or degradation of smooth motor function (Perkins et al., 2010).

Mice are a good model in which to study cerebellar development and Purkinje cell morphology. Although mouse cerebella mature more quickly than human cerebella, the mechanisms and developmental stages of cerebellar maturation in mice are very similar to those in humans. In mice, at postnatal day zero, the Purkinje cell sends out the aforementioned early processes and withdraws them by postnatal day three (see Figure 1). At this point, the Purkinje cells are in an unorganized clump beneath the external granule cell layer (see Figure 2). At postnatal day three, one or two apical, bushy dendrites begin to form (see Figure 1). By postnatal day seven, the granule cells begin to migrate down through the Purkinje cell layer as the Purkinje cells migrate upwards and orient themselves in a straight line (see Figure 2). At this stage, climbing

fibers extend into the cerebellum from the olivary nuclei in the spinal cord and interact with specific sites along the proximal one third of the Purkinje cell dendrite (see Figure 2). Meanwhile, parallel fibers extend from the granule cells and interact with specific sites along the entire Purkinje cell dendrite (see Figure 2). By postnatal day twenty one, the Purkinje cells form a monolayer with their dendrites pointing towards the pia, the surface of the brain, and their axons diving down into the granule cell layer (see Figure 2). This fully mature Purkinje cell can be further characterized by its large, arborous dendrites into which climbing and parallel fibers have entwined and formed synapses (see Figure 2) (Baptista et al., 1994; Morrison, 2004).

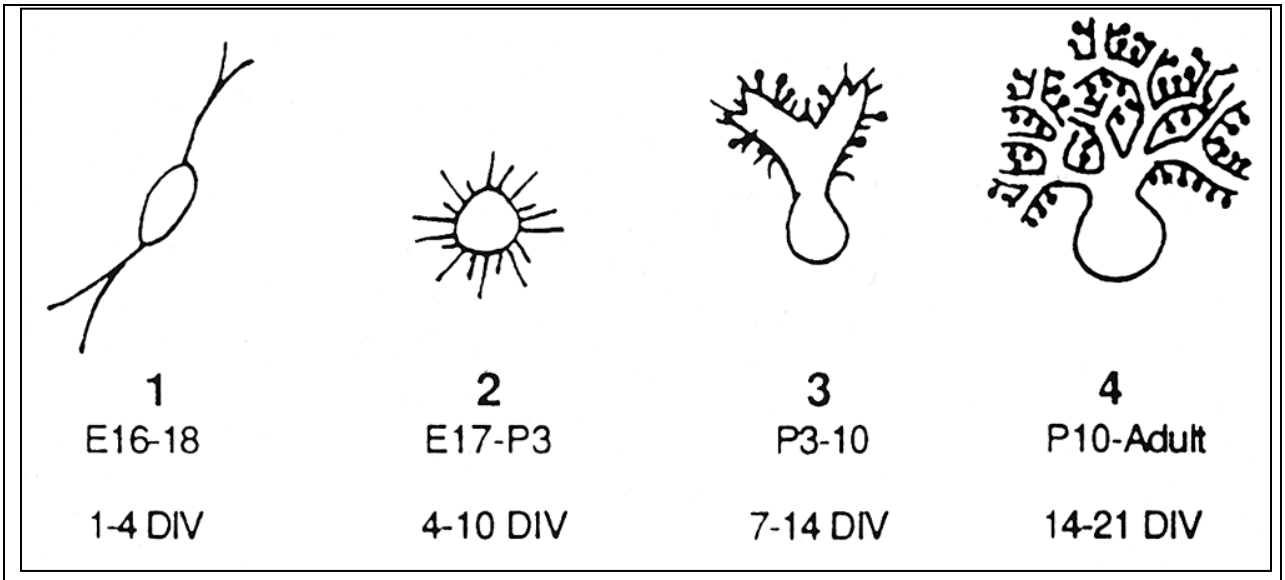


Figure 1: Purkinje cell growth and development. E16 stands for “Embryonic Day 16”, the sixteenth day of embryo life. P3 stands for “Postnatal Day 3”, the third day since the pup was born.

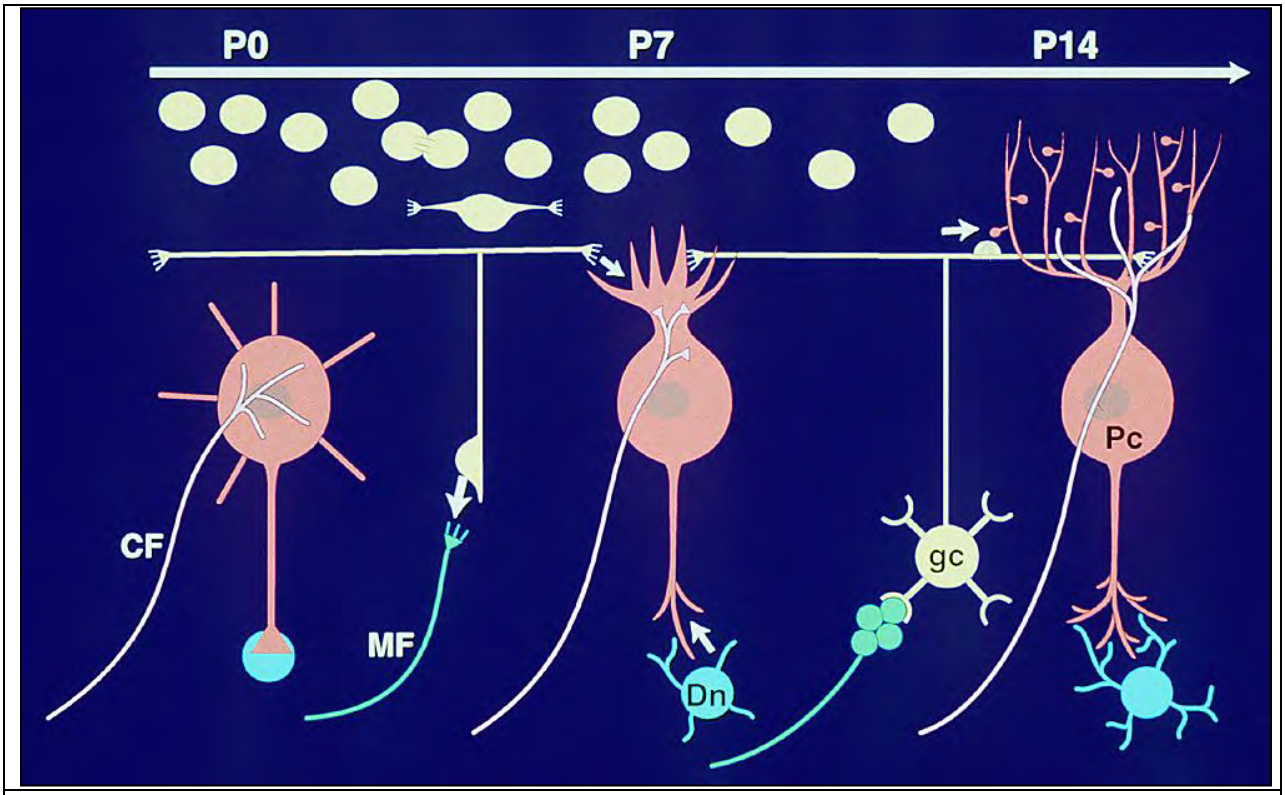


Figure 2: Purkinje cell development and interaction with other cerebellar cells. CF stands for climbing fibers from the olivary nuclei, MF stands for mossy fibers, Dn sands for deep cerebellar nuclei, gc stands for granule cell, and Pc stands for Purkinje cell.

Purkinje Cell Interactions with other Cerebellar Cells

To understand Purkinje cell maturation and morphology it is important to study interactions between Purkinje cells and other cerebellar cells. Although Baptista and his colleagues emphasized the importance of granule cells in Purkinje cell development, their results demonstrated that mixed cerebellar cultures actually had a much greater Purkinje cell survival rate than cultures containing only Purkinje cells and granule cells. After seven days, 15% of Purkinje cells in mixed cerebellar cultures survived whereas only 6% of Purkinje cells in Purkinje cell-granule cell cocultures survived (Baptista et al., 1994). These data suggest that healthy Purkinje cell development is dependent not just

upon interactions and signaling with the granule cells, but with other cells and processes of the cerebellum as well.

In contrast, Morrison and Mason later found that Purkinje cells co-cultured with granule cells actually survived better than Purkinje cells in mixed cultures. After 6 days in vitro, roughly half of the Purkinje cells in co-culture with granule cells survived whereas only about a third of Purkinje cells in mixed cultures survived. Furthermore, after 14 days in vitro, the Purkinje cells in granule cell co-cultures stabilized, but the Purkinje cells in mixed cerebellar culture lost about another third of the living cells that remained after 6 days in vitro. Morrison and Mason's results may have differed from those of Baptista and his colleagues because Purkinje survival and culture conditions were more closely examined. Alternatively, Purkinje cell survival may be intrinsically variable under these culture conditions (Baptista et al., 1994; Morrison, et al., 1998).

Morrison and Mason also found that the survival of Purkinje cells in granule cell cocultures was greater than that in whole cerebellar cultures at 14 days in vitro. Purkinje cells developed much bushier looking dendrites when co-cultured with granule cells and treated with Brain Derived Neurotrophic Factor, or BDNF, a signaling molecule that binds TrkB receptor proteins, a type of tyrosine kinase important in neural synapses. BDNF was included in this treatment because RNA which codes for the production of BDNF is found only in granule cells, but the resulting BDNF protein is detected primarily in Purkinje cells (Hofer et al., 1990). These dendrites likely appeared much bushier as a result of more dendritic spines, or tiny extensions from the dendrites. Furthermore, when cultured alone and treated with BDNF or NT4 (another neurotrophin that also binds the TrkB receptor), Purkinje cell survival after 14 days in vitro was about 50%

greater than untreated control cultures. However, when examining survival rate it was found that both co-cultures of granule cells and Purkinje cells, and mixed cerebellar cultures treated with BDNF and NT4, actually saw a significant decrease in Purkinje cell survival as compared to the untreated controls. Although other members of the scientific community suggested that serum commonly used in tissue culture would prevent the Purkinje cell die-off, Morrison and Mason determined that Purkinje cells in mixed cerebellar cultures on a medium containing serum were still not protected against the neurotrophin's induced toxicity (Morrison, et al., 1998).

The aforementioned results suggest that the excess BDNF added to the co-cultures and mixed cultures may have hyperexcited the granule cells so that they produced a glutamate excess, killing the Purkinje cells. To test this hypothesis, Morrison and Mason developed several methods to prevent the Purkinje cell's exposure to excess glutamate. In the first method, trkB-IgG protein was added to Purkinje-granule cell co-cultures treated with BDNF in order to bind excess BDNF. This increased Purkinje cell survival by 63% when compared to granule cell co-cultures treated only with BDNF, but still demonstrated a survival rate 11% lower than control cultures. Next, a BDNF-specific antibody that inhibits signaling by the BDNF receptor upon BDNF binding was added to Purkinje-granule cell co-cultures treated with BDNF. Once again, this increased Purkinje cell survival by 28% when compared to granule cell co-cultures treated only with BDNF, but still demonstrated a 46% lower survival rate than control cultures. Finally, CNQX, an AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) type glutamate receptor antagonist, was added to granule cell co-cultures treated with BDNF in hopes of preventing Purkinje cell exposure to excess glutamate. Although

AMPA-type glutamate receptors did appear to be the correct receptors to target, Purkinje cell survival rate was still 42% lower than control cultures, but 32% higher than Purkinje-granule cell co-cultures treated only with BDNF (Morrison, et al., 1998). These results demonstrate the complexities of the relationship between BDNF exposure and Purkinje cell development and survival. Some extra BDNF may boost survival, but too much BDNF appears to trigger production of excess glutamate that can become toxic to the Purkinje cells.

In a related study, the morphology of well-developed and less well-developed Purkinje cells was examined in cultures without treatment, with BDNF treatment, and with TrkB-IgG treatment. Both the well-developed and less well-developed Purkinje cells in the BDNF-treated culture appeared to have a greater number of stem dendrites and bushier dendrites than the Purkinje cells from the other cultures. To determine if this initial hypothesis was true, combined dendritic length, number of dendritic segments, number of branch termination tips, number of stem dendrites, and maximal branch order were all measured by analyzing camera lucidia reconstructions. After quantification using the aforementioned parameters it was found that the BDNF-treated cultures did have bushier dendrites due to increased spine density, but otherwise all three Purkinje cell cultures were morphologically similar (Shimada et al., 1998).

To determine what made the Purkinje cell cultures treated with BDNF appear bushier, the total spine density and percentage of filopodia-like spines, or spines which lack a bulbous head, was determined for cultures with no treatment, with BDNF treatment, with BDNF + TrkB-IgG treatment, and with only TrkB-IgG treatment. None of the treatments were significantly different across all three experiments in the percentage

of filopodia-like spines study. However the BDNF-treated cultures consistently had three to four more spines per micrometer, roughly 130% more than the other cultures. TrkB-IgG significantly increased the length of the spine necks in spines with heads. Because dendritic spines are thought to act like independent biochemical compartments, longer spine necks may decrease calcium and acetyl choline leak, thereby hampering signal transfer from the spine into the adjacent dendrite. This hypothesis is supported in the medical literature in that patients with intellectual disabilities, epilepsy, and neurodegenerative disorders typically have abnormally long spine necks (Shimada et al., 1998).

SMI31, MAP2, and Purkinje Cell Structure

In order to determine Purkinje cell morphology and development it is necessary to study Purkinje cell structure. Cellular structure is largely influenced by structural proteins such as microtubules and neurofilaments. Specifically, Purkinje cell axon and dendrite morphology is determined by unique structural proteins. Antibodies can be used to label and then image these structural proteins.

One study attempted to label structural proteins in the Purkinje cell axon by immunolabeling adult rat cerebella with two monoclonal antibodies, 04-7 and 02-135. The 04-7 antibody was a precursor to the SMI 31 antibody now commonly used to label Purkinje cell axons. This 04-7 antibody reacted with phosphorylated 200 kilodalton and reacted weakly with phosphorylated 150 kilodalton proteins. The 04-7 antibody appeared to label basket cell fibers in the area surrounding the Purkinje cell body and Purkinje cell fibers which descended into the granule cell layer. Alternatively 02-135

antibody reacted with only unphosphorylated 200 kilodalton proteins and appeared to label basket cell fibers around Purkinje cell bodies, Purkinje cell bodies, Purkinje cell dendrites, and Purkinje cell axons. This suggests that the 04-7 monoclonal antibody would be useful in selectively labeling only Purkinje cell axons (Langley et al., 1988).

Hayashi and colleagues later immunolabeled rat embryonic day 16 cerebellar cortex cultures to differentiate axons from dendrites. The cultures were fixed with 4% paraformaldehyde for 30 minutes each to ensure tissue adhesion and stained with a variety of primary antibodies including monoclonal SMI-31 antibody at a 1:1500 dilution. The monoclonal SMI-31antibody is known to label phosphorylated neurofilaments and the cerebellar cultures stained with the monoclonal SMI-31 antibody had presumptive Purkinje cell axons labeled. This seems to confirm Langley and colleagues' previous research which suggests that monoclonal antibodies against phosphorylated neurofilaments selectively label Purkinje cell axons (Hayashi et al., 2010).

Alternatively, microtubule-associated protein 2, or MAP2, has proven to be critical in formation and assembly of cellular cytoskeleton. It specifically has been found to interact with microtubules and actin to form cytoskeletal scaffolding within dendrites (Buddle et al., 2003). Further studies suggest its specific expression in Purkinje cell dendrites through selective immunolabeling of disassociated cultures of cerebellar cells with anti-MAP2 antibody (Gianola et al. 2003). However, it should also be noted that anti-MAP2 antibody can also bind and brightly stain granule cell dendrites (Huber, et al., 1984).

One as of yet poorly characterized aspect of Purkinje cell development is the identity of the aforementioned early processes that extend from the young Purkinje cell

before retreating back into the cell soma around postnatal day three. John Mastrobuono's independent study project aimed to identify these early neuronal processes extended by young Purkinje cells through antibody staining of whole cerebellar cultures. Anti-MAP2 antibody (1:3000, Molecular Probes cat. # AB5622) was used to label microtubule-associated protein 2 in dendrites and SMI-31 antibody (1:3000, Jackson Immuno Research cat. # 115-165-003) was used to label neurofilament H in axons. Mastrobuono found that some of the early processes stained with the Anti-MAP2 antibody (and therefore as dendrites), while some stained with the SMI-31 antibody (and therefore as axons). It was, however, impossible to determine if the processes stained for both dendrites and axons because the antibodies were applied to separate cultures (Mastrobuono, 2010).

The specific proteins expressed in Purkinje cells during development can be observed in whole brain sections through a special method of sectioning and staining the brain. A cryostat is an instrument that freezes the brain tissue and sections it into very thin (14 um) slices using a very sharp blade. The cerebellum can then be immunostained using antibody markers, which label specific proteins, and examined under a microscope. Karahlyn Troutman's independent study attempted to visualize cryostat sections of the mouse brain stained for several neuronal markers including calbindin, a cell marker for Purkinje cells. However, tissue section to slide adhesion issues resulted in massive tissue loss during the staining process and significant secondary antibody background staining rendered fluorescent images all but useless. Both problems resulted in tissue sections where gross neuronal structure was almost impossible to determine. Background in fluorescent images is typically due to non-

specific binding of antibodies throughout a tissue section or auto-fluorescence of tissue. In auto-fluorescence, electrons in unstained tissue become excited by the light, jump to a higher energy orbital, and then drop back to their ground state, releasing a photon of light (Jaafar et al., 2010; Troutman, 2010).

The current study will first address section adhesion issues that plagued Troutman. The use of “subbed” slides, slides coated with a chrome-alum gelatin complex (Kiernan, 1999), and the use of 4% paraformaldehyde as a post fixative (Hayashi et al., 2010) will both be explored to increase tissue adhesion. Next, monoclonal SMI 31 antibody and polyclonal MAP2 antibody will be used to label axons and dendrites, respectively, in post-natal-day 0, 4, 7, 14, and 21 mouse, whole-brain sections. Through this labeling, the expression of axonal and dendritic proteins during Purkinje cell development will be determined, which should help to better characterize the early processes which extend from the young Purkinje cell before retreating back into the cell soma around postnatal day three.

Materials and Methods

A Note on Methods in the Text

Because this document is intended as an aid for future researchers undertaking cryosection, immunohistochemistry studies, the level of detail included in the methods section at times exceeds what is necessary for a typical honors thesis.

Animals

The brains used in this study were dissected from a strain of C57Bl6/J mice obtained from Jackson Laboratories in Bar Harbor Maine and bred and maintained in conventional housing in a clean, but not completely sterile environment at Lycoming College. The pups used for this experiment were generated through setting up designated mating cages with two females and one male each and checking cages daily for pups. The gestation period for the mice is roughly 19 days, and the day of birth is referred to as postnatal day zero, or P0. The day after birth is referred to as postnatal day one, or P1, the second day after birth as postnatal day two, or P2, etc.

Boat and Chuck Preparation

Boats were prepared by heating a razor in a Bunsen burner and using it to slice 15 mL, conical, plastic tubes into 3-5 cm high cylinders open at either end. Next, a vertical slit was made down the side of each cylinder, leaving each boat in a “C” shape when observed on end, to ensure that it could later be removed from the frozen tissue section. Finally, the boat was frozen at -20 degrees Celsius onto a cryostat chuck using Tissue-Tek O.C.T. (Optimal Cutting Temperature) embedding medium (catalog number 62550-12).

Slide Subbing

The aforementioned subbed slides were prepared according to the methods outlined in Kiernan (1999). First, 0.02 g of Acros Organics chromium potassium sulfate (catalog number 222520050) was dissolved in 6.6 mL of distilled water. Then 0.2 g of

Electron Microscopy Sciences 175 Bloom Gelatin Powder (catalog number 16562) and 190 mL of distilled water was added to bring the solution to a final volume of 200 mL. This solution was then transferred to a glass staining tank and staining racks full of Fisher Scientific Superfrost/Plus Precleaned Microscope Slides (catalog number 12-550-15) were lowered into the solution. The slides were submerged for 30 s and constantly agitated throughout the process. The slides were then allowed to air dry in their racks in a dark cabinet overnight, and the leftover solution was transferred to a flask, covered with aluminum foil, and saved until needed for future use for up to three months. Once dry, the slides were transferred to slide containers at room temperature and ready for use.

Solution Preparation

The 1X Tok PBS used in immunohistochemistry staining was stored at room temperature as 10XTok PBS stock solution containing 1.4 M NaCl, 0.027 M KCl, 0.081 M Na_2HPO4, and 0.017 M $NaH_2PO_4•H_2O$ in distilled water. When needed, 100 mL of the 10XTok PBS stock solution was diluted in distilled water to a final volume of 1 L and brought to a pH of 7.3.

The 0.2 M Sorensen's Phosphate Buffer contained one part 0.2 M $NaH_2PO_4•H_2O$ and four parts 0.2M $NaHPO_4$ at pH 7.2-7.3 and was stored at room temperature.

The 8% paraformaldehyde was prepared in distilled water by heating to 60 degrees Celsius while stirring. 10 N NaOH was added drop-wise until all the solute had dissolved. The solution was then cooled to room temperature, separated into 50 mL conical tubes and frozen at -20 degrees Celsius until needed.

Dissection

At the time of dissection, P1 and P4 mice were removed from their mother and placed in a pup box lined with paper towels and Kimwipes to keep the pups warm and reduce stress. The pup's back was then held lightly between the thumb and forefinger of one hand and curved scissors were used for decapitation, separating the head from the body just beneath the base of the skull. Next, the severed head was positioned by grasping the nose gently with curved forceps. Straight scissors were then inserted into the spinal cord opening and a cut was made below each ear to the top of the eye on either side of the head. Next, a pair of straight forceps was inserted perpendicular to the head through the previously made cuts from eye to eye and, with a gentle roll of the wrist, used to remove the top of the skull. The brain lay nested in the top of this skull section. Next, the skull was elevated over O.C.T. so that, using gravity and forceps, it could be gradually guided from the skull and into the O.C.T., taking care not to introduce bubbles into the O.C.T. The brain was then coated in excess O.C.T and floated on additional O.C.T. added freshly to a previously prepared boat frozen to a -20 degree Celsius cryostat chuck. The brain was positioned so that it lay on its side, remaining space in the boat was filled with O.C.T., and the block was frozen at -20 degrees Celsius.

The P7, P14, P21, and P-1 month pups used in this experiment were first anesthetized with a mix containing 200 uL of Putney Ketamine (catalog number NDC 26637-411-01, ANADA number 200-073), 100 uL of Anased Injection Xylazine (NADA number 139-236), and 700 uL of 0.85% sterile saline, to give a final concentration of 20 mg/mL Ketamine and 2 mg/mL xylazine in saline. The resulting anesthetic was then

injected at an oblique angle into the crease between the mouse's thigh and body until the 26.5 gauge needle entered the peritoneal cavity. The quantity of anesthetic appropriate for the mouse's age was administered according to the American Veterinary Medical Association's Guidelines on Euthanasia (see Table 1) and the needle held steady for roughly 30 s to ensure that all anesthetic mix remained intraperitoneal (AVMA Guidelines on Euthanasia, 2007).

. The mouse was then placed in a box similar to the aforementioned pup box for comfort and safety until it was sufficiently anesthetized. Mice typically took between 10-15 minutes to enter a deep anesthetic sleep as determined by pinching the mouse's foot and looking for the lack of the leg jerk reflex.

Table 1: Anesthetic dose according to mouse age.

Age	P0	P4&P7	P14	P21&P-1 month
Anesthetic Dose	0.01-0.02mL	0.03-0.05mL	0.04-0.05mL	0.1mL

P7 mice were then dissected as in the above procedure for P1 and P4 pups. For P14, P21, and P-1 month pups the head was removed from the body at the base of the skull using Vantage V95-16 large dissection scissors. Next, the skin was cut down the center of the head with Roboz RS 6700 scissors and pinned down on a Sylgard plate to either side of the head to reveal the skull. The Roboz RS6700 scissors were then used to cut the skull down the middle starting at the foramen magnum and taking care to keep the blade up toward the inside of the skull, away from the brain (see Figure 3). One cut on either side of the skull was then made perpendicular to the first cut and sharp, fine Roboz Dumont #5 forceps were used to peel the skull away from the brain. The forceps were used again to expose and carefully tease out the flocculi on either side of the brain, a small spatula was used to carefully tilt the brain out of the remaining

skull, and the optic nerves were clipped to allow the brain to fall into a pool of O.C.T. The free, intact brain was then completely coated in O.C.T., placed on fresh O.C.T. in a pre-made boat attached to a cryostat chuck, covered in excess O.C.T., and frozen at -20 degrees Celsius.

Figure 3: Dissection of a P21 mouse brain

If the tissue blocks prepared during dissection were to be sectioned within a few days following the dissection, the plastic boat which surrounded the frozen tissue was removed with a straight razor and the block was sectioned. However, if the block was not to be sectioned within a week, the block was cut from the chuck using a razor. Then the plastic boat which surrounded the tissue block was removed, the block was placed

in an appropriately labeled plastic test tube, and it was stored at -80 degrees Celsius. The test tube was then moved from the -80 degree freezer into the cryostat chamber at -20 degrees Celsius two days prior to sectioning. The day before sectioning the tissue block was frozen to a chuck at -20 degrees Celsius using O.C.T., and the next day the block was sectioned as normal.

Defrosting of the LEICA CM1850 Cryochamber/Chuck Shelf and General Use

To turn on the Cryostat, the automatic main fuse switch was flipped. Then to set the chamber temperature to -20 degrees Celsius the [+] and [-] buttons next to the thermometer where used.

To manually defrost the cryochamber, the snowflake/water button was pressed at which point an audible chime sounded. Then the [+] and [-] buttons next to the thermometer were pressed to select the desired defrost temperature.

To manually defrost the chuck shelf the snowflake/water button was pressed and then the [***] button was pressed. The shelf gets quite hot during the defrost cycle so it is important not to touch it.

The maximum duration of a defrost cycle is 12 minutes and defrost will occur until the cryochamber reaches -5 degrees Celsius. To set an automatic defrost time, the [+] and [-] buttons below the snowflake/water button at the clock panel were pressed until the time read 12:00 AM.

Sectioning

To section the tissue blocks, the chuck was first placed in the specimen head and the screw was tightened to secure the chuck. The clearance angle was placed at -2 degrees, the tissue thickness was set at 30 to 50 um, and the hand wheel was unlocked and turned slightly to position it in front of the Feather Safety Razor Company LTD. Mechanical Division Acu Edge High Profile Microtome Blade (catalog number 05057594). The blade was then lowered until it just barely touched the tissue block and the hand wheel was turned to trim the face of the block until the brain's orientation could be determined. Then the tissue block was removed from the specimen head and a razor was used to cut a trapezoid shape around the exposed brain tissue as shown in Figure 4.

Figure 4: Sectioning with the Aid of a Red Sable Hair Brush

The tissue chuck was returned to the specimen head, positioned so that the brain was upright, and the screw was again tightened to secure the tissue chuck. The block was again trimmed at 30 microns or less until the block's surface was no longer slanted, but flat and complete sections were obtained. The block was then sectioned at 14

microns, being careful to take relatively parasagittal sections so as to obtain the most useful cerebellar tissue. To section the block a Loew Cornell Natural Hair 165-2 red sable hair brush was rested at a 30-45 degree angle, gently so that the bristles did not bend upon the lower right face of the tissue block where there was no tissue, just frozen O.C.T. Then the hand wheel was slowly turned as the brush was lowered in a gradual forward sweeping motion to guide the section flat on to the stage (see Figure 4). Lowering the brush and turning the wheel simultaneously is ideal, but nearly impossible, so it is better to lead with the wheel and follow with the brush. Once the very top of the section is all that is attached to the tissue block, the brush should be used to push down at the right angle where the section and tissue block meet as the hand wheel is turned so that the section detaches cleanly from the block. A room temperature chrome gelatin coated, or “subbed”, slide was then lowered onto the section which melted onto the comparatively warm slide. Three sections were added to each slide, all oriented in the same direction, using the same technique. Methanol and paper towels were used to clean the stage as needed throughout sectioning. The slides were stored at -20 degrees Celsius until the block was completely sectioned and then moved to storage in a slide container at -80 degrees Celsius. When finished sectioning the hand wheel was relocked, the tissue chuck was removed from the specimen head, and a razor was used to remove any remaining tissue block from the chuck. The remaining tissue block was discarded and the chuck returned to the -20 degrees Celsius cryostat chamber for future use.

Immunohistochemistry

Post-fixation:

In all staining experiments, slides containing brain sections were allowed to defrost in a fume hood and a Research Products International Corp. Super HT PAP Pen was used to isolate each group of sections using curvy lines. As the hydrophobic barrier was drying, 50 mL of frozen 8% paraformaldehyde was thawed in a hot bath at 60 degrees Celsius in a fume hood and mixed with 50 mL of 0.2 M Sorensen's phosphate buffer at room temperature. Once the barrier was dry, 350 uL of 4% paraformaldehyde post-fixation solution was pipetted on to the slides and the tip of the pipette was used to spread the solution evenly across the sections without addition of bubbles. At the conclusion of the desired time period the fixative was immediately pipetted off and replaced with 350 uL of 1XTok PBS for five minutes. The slides were then washed twice more for five minutes each in Coplin jars filled with 50 mL of 1XTok PBS.

Preblock:

Next, each slide received 200 uL of preblock solution for one hour in a room temperature, humidified container. The preblock solution always consisted of Sigma 10% normal goat serum (catalog number G-9023) and Sigma 0.2% TritonX100 detergent (catalog number T-9284) in 1XTok PBS.

Primary Antibody:

Then 200 uL of primary antibody solution was spread across each slide to completely cover the sections and the slides were stored overnight in a parafilm-sealed,

dark, humid, 4 degrees Celsius environment. The next day, the primary antibody was pipetted off and the slides were washed three times for 20 minutes each in Coplin jars filled with 1XTok PBS at room temperature.

Secondary Antibody:

Then 200 uL of secondary antibody solution was added to each slide and left on for 45 minutes in a humid, room temperature chamber. The secondary antibody was pipetted off and the slides were washed three times for 20 minutes each in Coplin jars filled with 1XTok PBS at room temperature. It is important to wash different antibodies separately to prevent unwanted antibody cross-binding.

Coverslipping

At the end of the washes, the excess tokPBS was blotted away from the slides using a Kimwipe and seven drops of Lerner Laboratories' Aquamount Aqueous Mountant (order number 13800) was placed onto the end of each slide, just inside the hydrophobic barrier. Next, one, and only one (check this by bending it or holding it up to a light to look for odd refractions) clean, #1 thickness, 24X60 mm cover slip was touched at a 45 degree angle to the Aquamount and drawn back to the edge of the frosting. Using needle thin tweezers, the cover slip was slowly lowered so that the Aquamount wicked cleanly around the sections the entire length of the slide. If needed, the slides were tilted slightly to facilitate the flow of Aquamount to cover all sections. Tweezers were used to gently push the cover slip back into place so that no edges extended beyond the slide. The slides were then left to dry overnight and the edges of

the slides were coated with clear nail polish to seal in the moisture. The slides were stored in the dark at 4 degrees Celsius until ready to be viewed so as to minimize fluorescence fading.

Imaging

The slides were imaged using a Nikon TE 2000 microscope and camera. Slides were placed face down on the stage so that light need only pass through the thin cover slip. The cerebellar tissue was identified and the sections were imaged, photographed, and processed using MetaView Software.

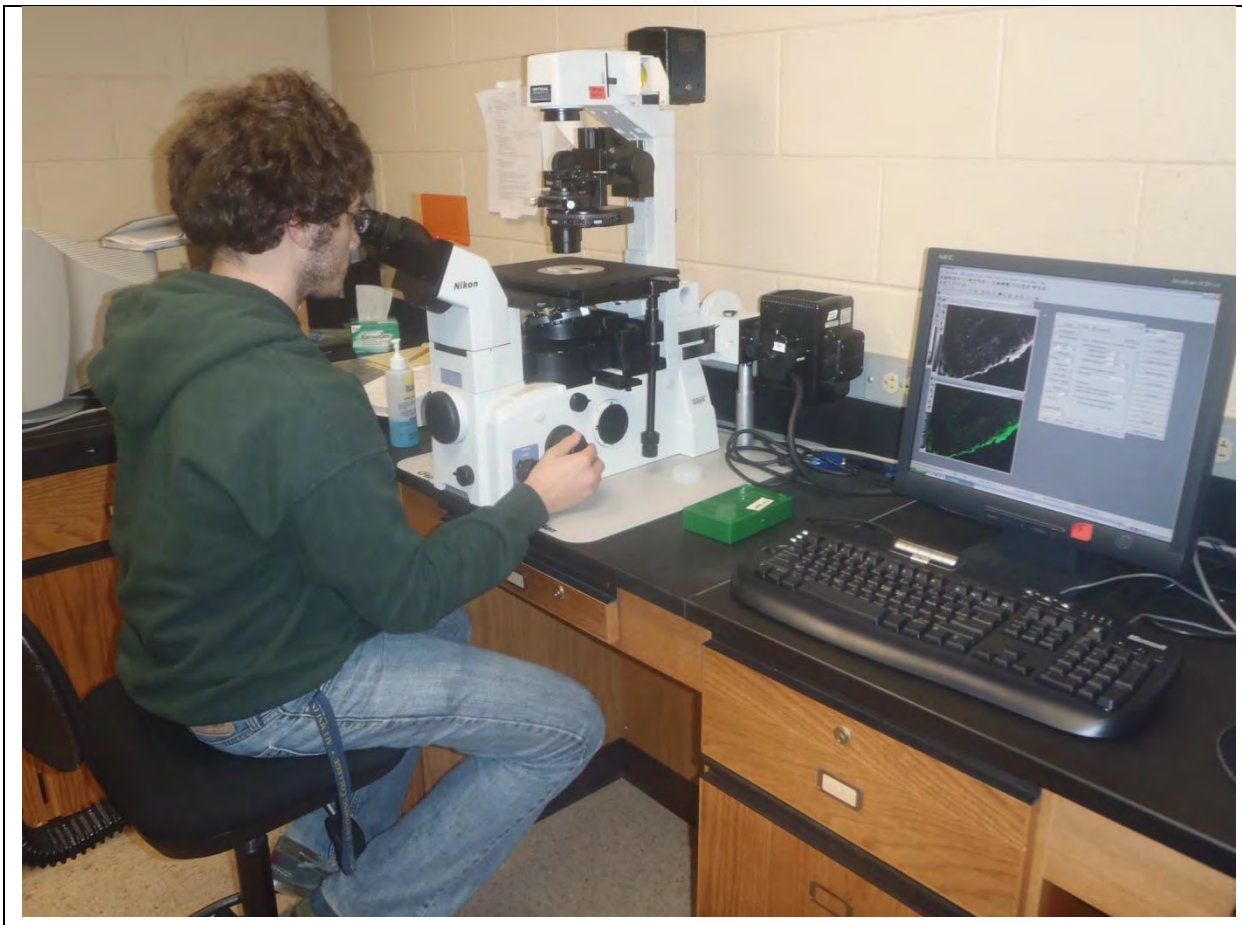


Figure 5: Imaging

Results

Pilot Study to Determine Ideal Fixation Time

Five slides with five sections each of P7 mouse brain were post-fixed with 4% paraformaldehyde for 20 minutes, 10 minutes, 5 minutes, 1 minute, and 0 minutes and stained with mock primary and secondary antibody solutions. To the naked eye, the slide post-fixed for 20 minutes seemed to have entirely intact sections, slides post-fixed for 10 and 5 minutes had some minor tissue loss, the slide post-fixed for 1 minute had almost complete tissue loss, and the slide that did not receive post-fixative had severe tissue loss (see Figure 6).

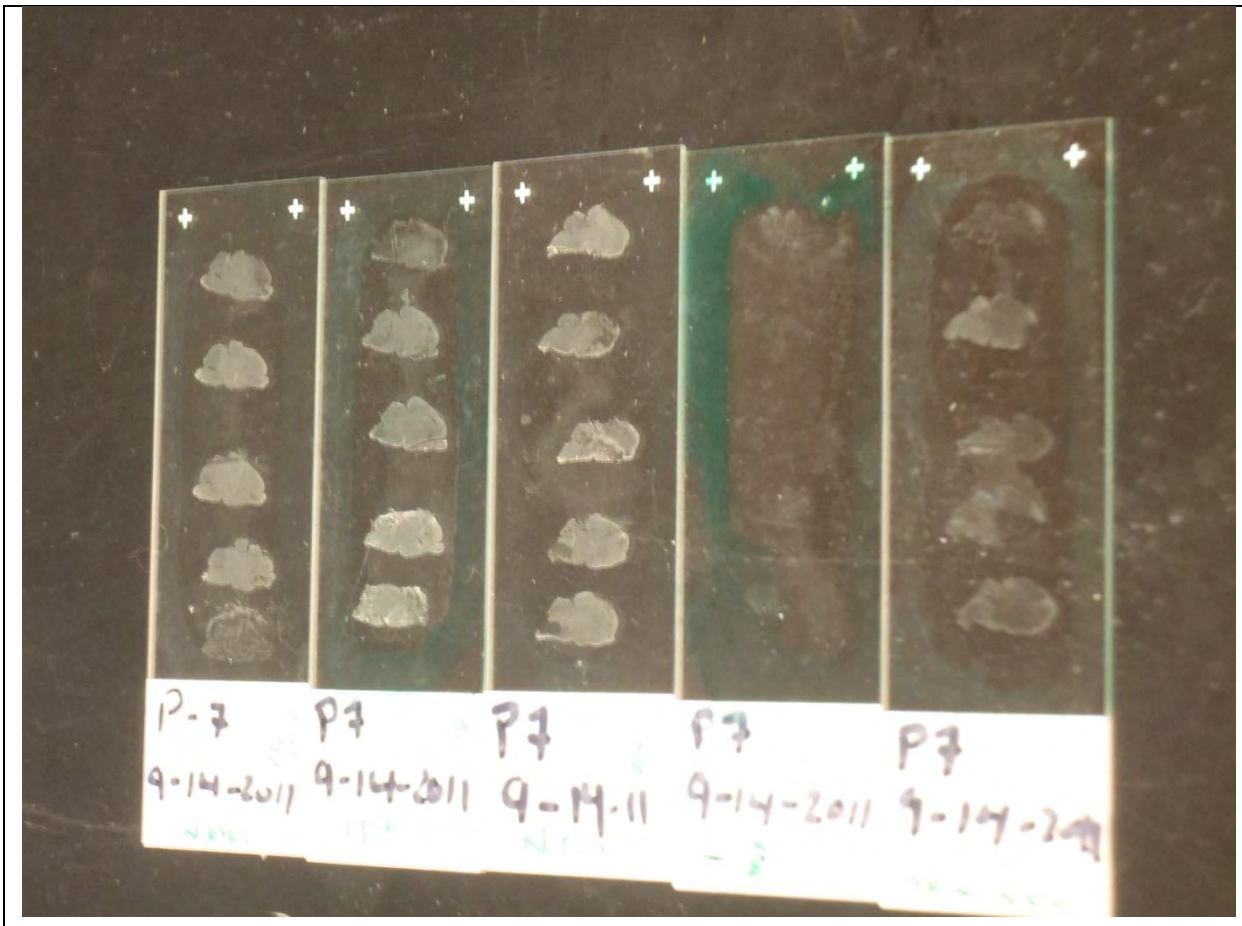


Figure 6: P7 section adherence with varying 4% paraformaldehyde post-fixation times. Fixation times are, from left to right: 20 minutes, 10 minutes, 5 minutes, 1 minute, and zero minutes. The slides received mock primary and secondary antibody solutions. All sections used were 14 um thick.

Pilot Study to Determine the Effect of Tissue Age on Fixation Success

Two slides with three sections each of P0 mouse brain, one slide with three sections of P4 mouse brain, and two slides with three sections each of P-1 month mouse brain were post-fixed with 4% paraformaldehyde for 30 minutes each and stained with mock primary and secondary antibody solutions. To the naked eye, all of the slides seemed to have entirely intact tissue sections (see Figure 7).

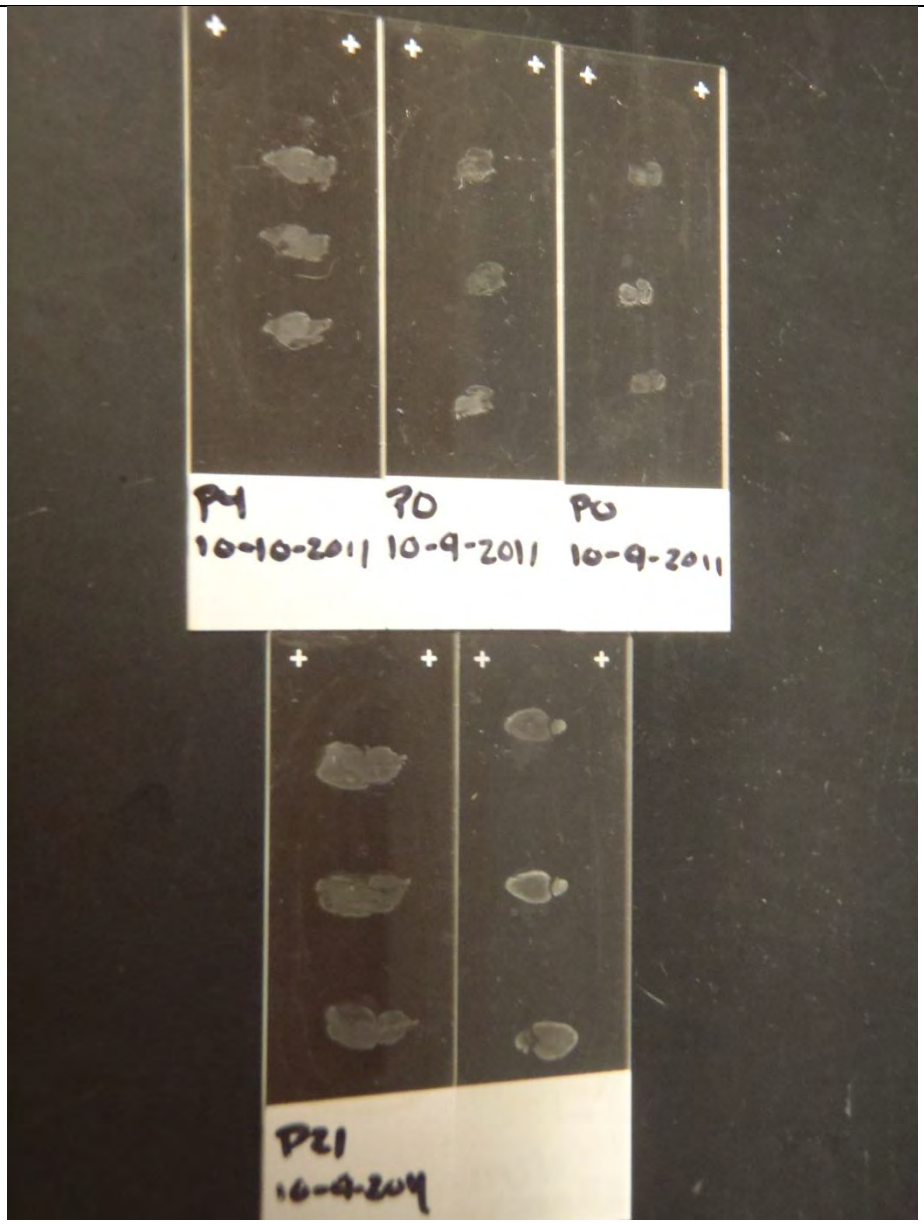


Figure 7: P0, P4, and P-1 month tissue section adherence when post-fixed with 4% paraformaldehyde for 30 minutes. Tissue section ages are, clockwise from top left: P4, P0, P0, P-1 month, and P-1 month. The slides received mock primary and secondary antibody solutions.

A Note on Image Autoscaling

One slide with three P21 mouse brain sections received 1:3000 calbindin primary antibody and 1:3000 Cy3 goat anti rabbit secondary antibody. This slide was imaged with and without image autoscaling with the same exposure time. When imaged without

image autoscaling, the section had dim, specific staining throughout the Purkinje cell layer and in the pia, but only very dim background staining in the internal granule cell layer (see Figure 8, image A). When imaged with image autoscaling, the same area had similar staining patterns except that everything was brighter (see Figure 8, image B). One slide with three P21 mouse brain sections received 1:3000 Cy3 goat anti rabbit secondary antibody. This slide was imaged with and without image autoscaling. When imaged without image autoscaling, the section had extremely dim background and looked nearly black (see Figure 8, image C). When imaged with image autoscaling, the same area looked to have non-specific, background staining throughout the tissue, but this was simply a result of the image autoscaling (see figure 8, image D).

Two slides with three P21 mouse brain sections each received 1:3000 Cy3 goat anti rabbit secondary antibody only and were imaged with autoscaling off. These images had barely perceptible, nonspecific, background staining (see Figure 9, Images A and B). Two slides with three P21 mouse brain sections each received either 1:1500 MAP2 primary antibody or 1:3000 calbindin primary antibody and 1:3000 Cy3 goat anti rabbit secondary antibody. When imaged with autoscaling off, the slide receiving MAP2 primary antibody had dim, specific staining in the region between the Purkinje cell body and the pial layer and in the internal granule cell layer directly below the Purkinje cell bodies, but only very dim background staining in the region where the Purkinje cell bodies are (see Figure 9, image C). When imaged with autoscaling off, the slide receiving calbindin primary antibody had dim, specific staining throughout the Purkinje cell layer and in the pia, but only very dim background staining in the internal granule cell layer (see Figure 9, image D). When similar regions on different sections of the

same slides receiving MAP2 and calbindin primary antibody were imaged, the same regions with dim specific staining appeared much brighter, but the very dimly labeled background areas also appeared brighter (see Figure 9, images E and F).

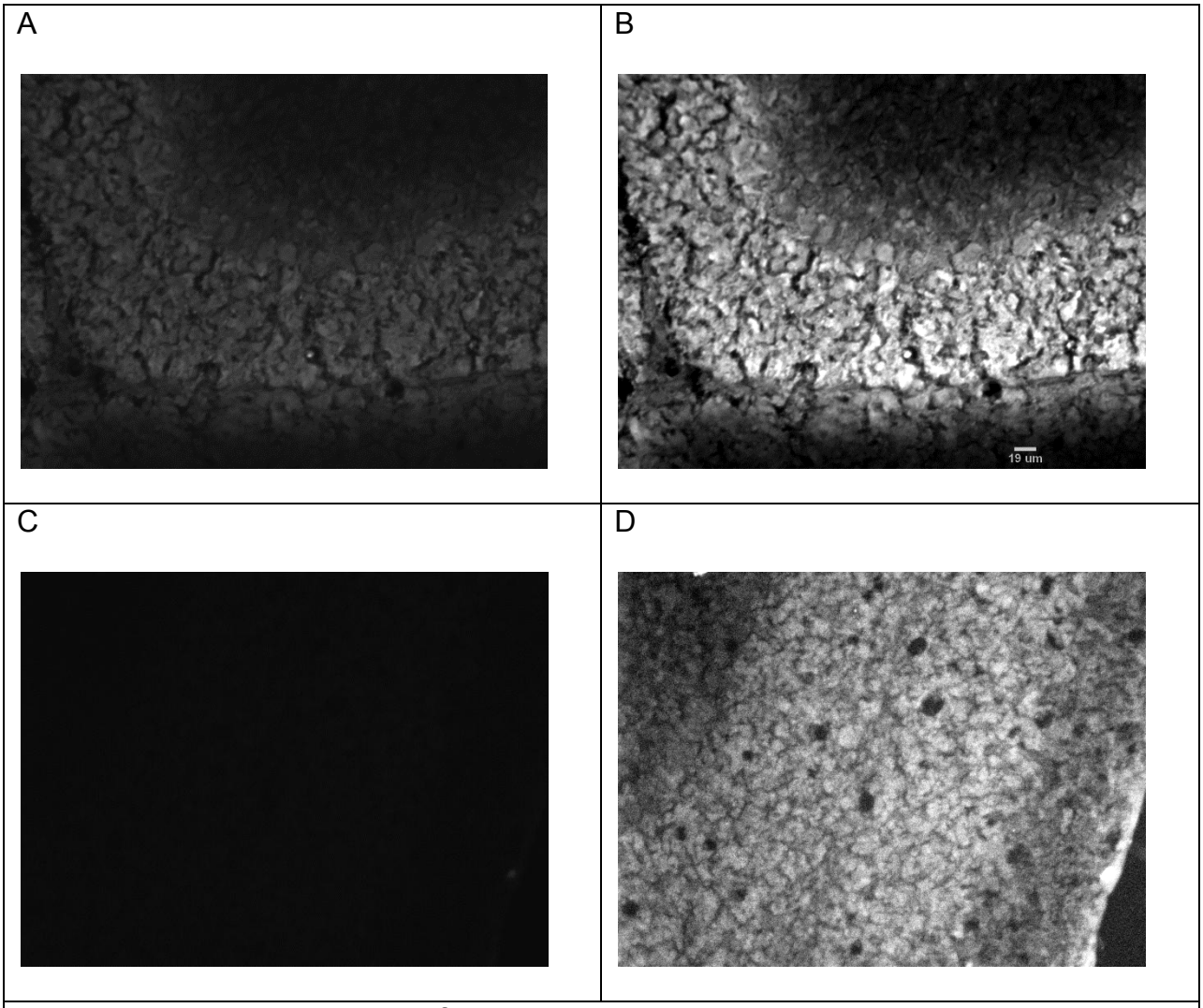


Figure 8: Fluorescent imaging of two mouse brain sections, with and without image Autoscaling. A, C – Autoscale off; B, D – Autoscale on. A, B – 1:3000 calbindin primary antibody. Cy3 goat anti rabbit secondary antibody dilution was 1:3000 for all sections. All sections were 20 um thick. Total magnification was 200X for all images.

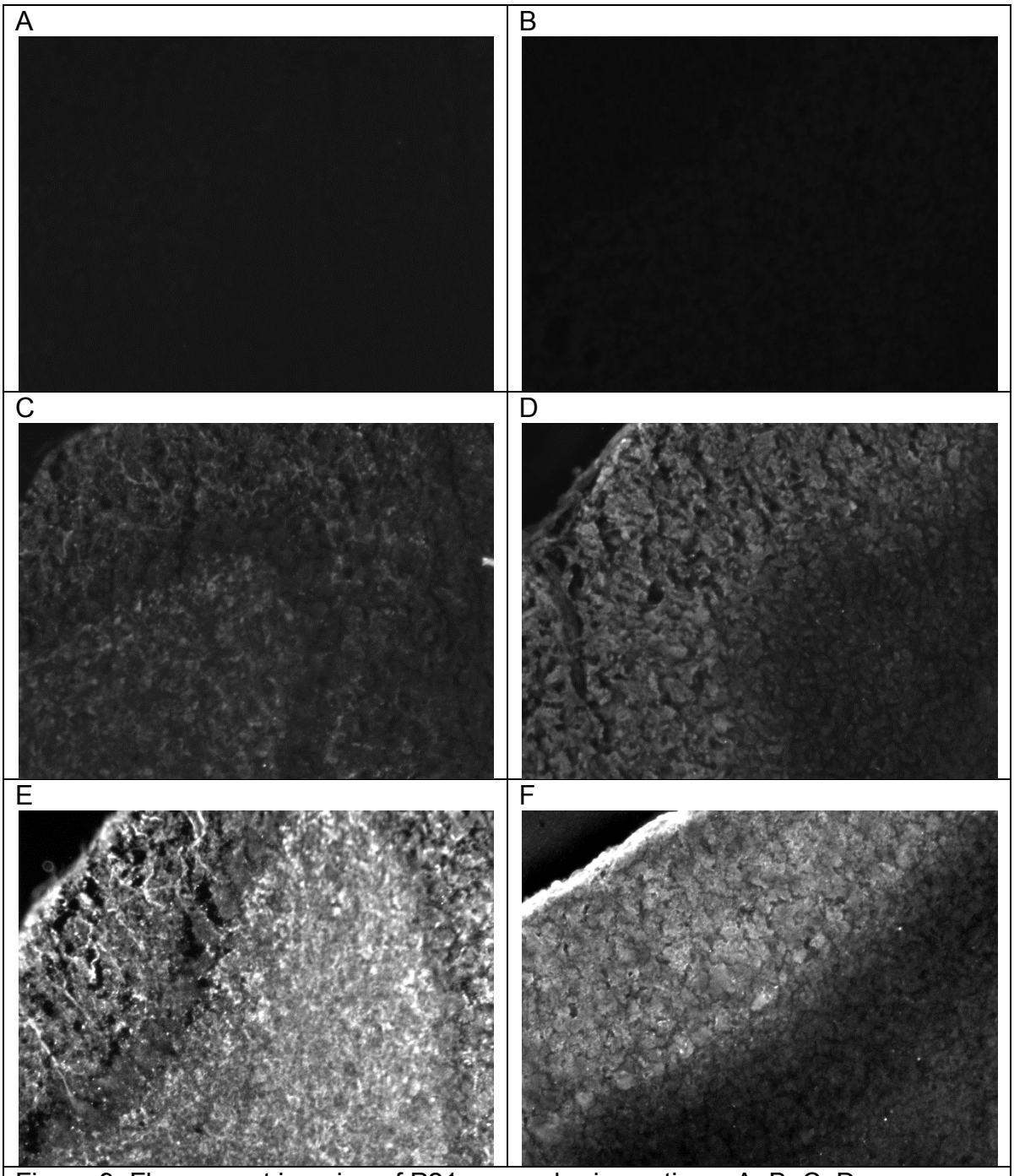
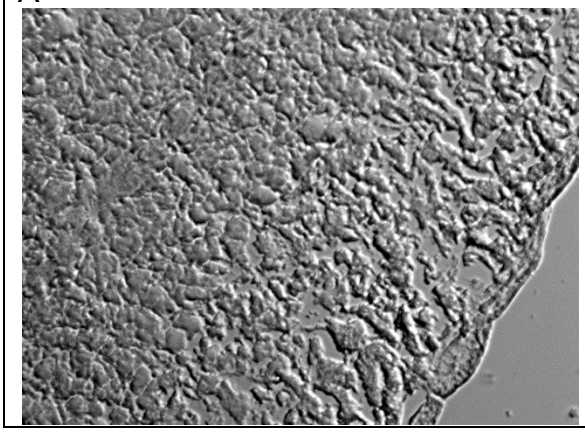
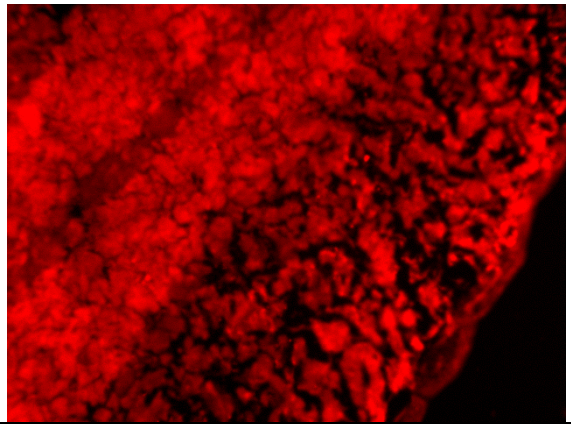
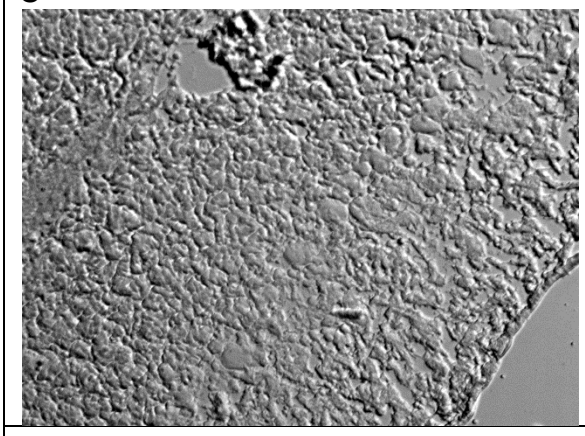
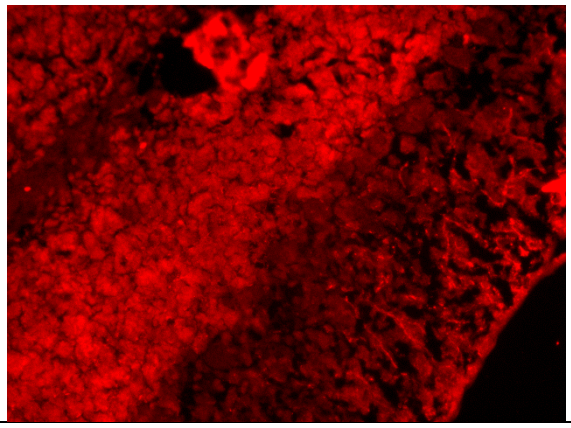
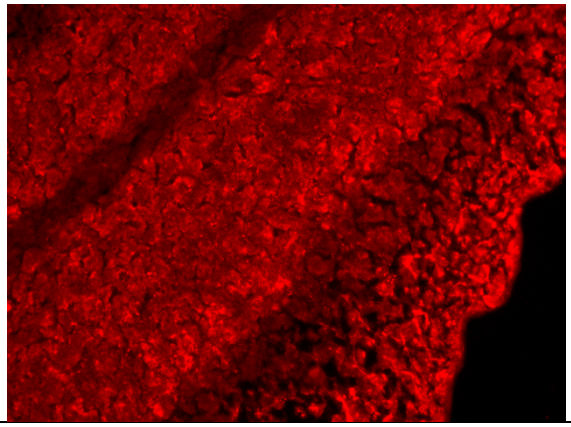
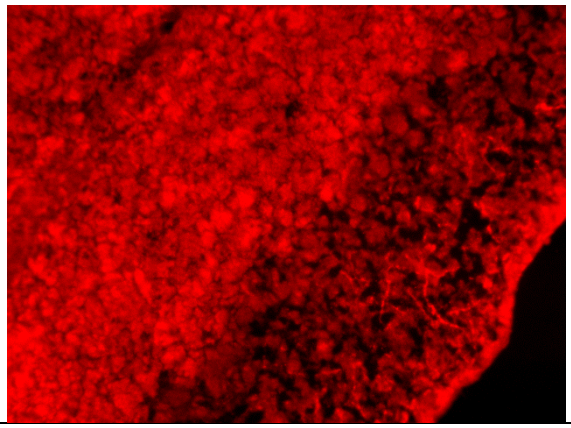


Figure 9: Fluorescent imaging of P21 mouse brain sections. A, B, C, D – Autoscale off; E, F – Autoscale on. C, E – 1:1500 MAP2 primary antibody; D, F – 1:3000 calbindin primary antibody. Cy3 goat anti rabbit secondary antibody dilution was 1:3000 for all sections. All sections were 20 um thick. Total magnification was 200X for all images.

Pilot Study to Determine Appropriate Primary Antibody Concentrations

Slides each with three P-1 month mouse brain sections received either 1:500, 1:1000, 1:2000, 1:5000, or no MAP2 primary antibody dilutions and 1:3000 Cy3 goat anti rabbit secondary antibody dilutions. Cerebella on each slide were then imaged at a 200X total magnification. The 1:500 MAP2 primary antibody stained section had broad, non-specific staining (see Figure 10, image B), but good section integrity (see Figure 10, image A). The 1:1000 and 1:2000 MAP2 primary antibody stained sections had weakly stained possible Purkinje cell dendrites and strongly stained fibers in the granule cell layer (see Figure 10, images D and E). The 1:1000 MAP2 primary antibody stained section had very slight tissue loss in the upper center of the visual field (see Figure 10, image C). The 1:5000 and no MAP2 primary antibody stained sections had weak, nonspecific staining (see Figure 10, images F and G).

DIC	Fluorescence	Anti-MAP2 Dilution
A 	B 	1:500

C 	D 	1:1000
	E 	1:2000
	F 	1:5000

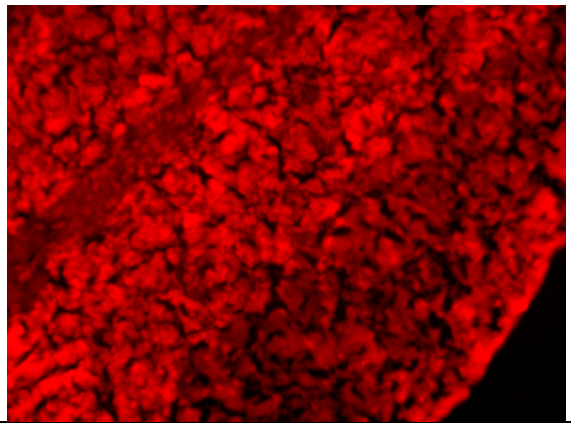
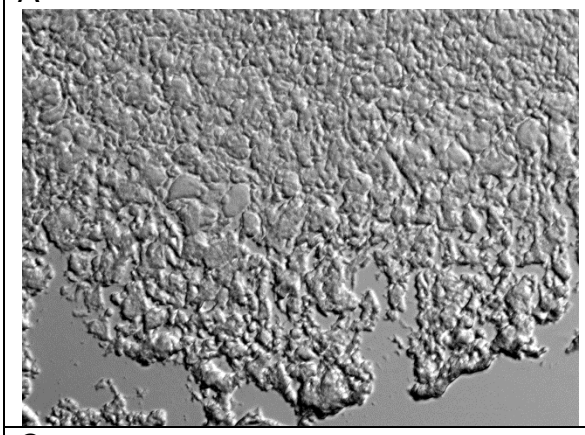
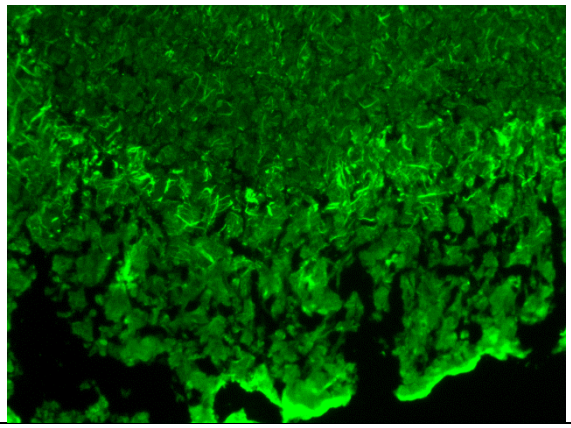
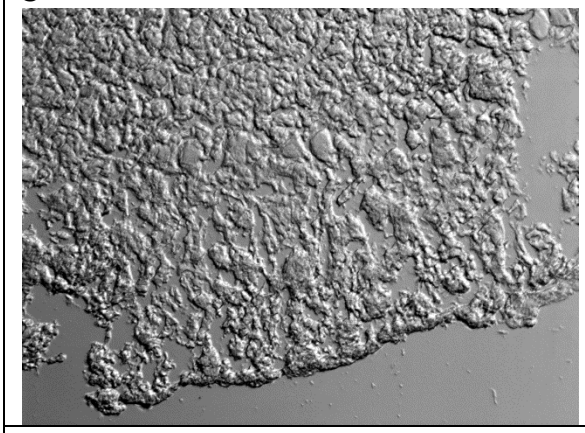
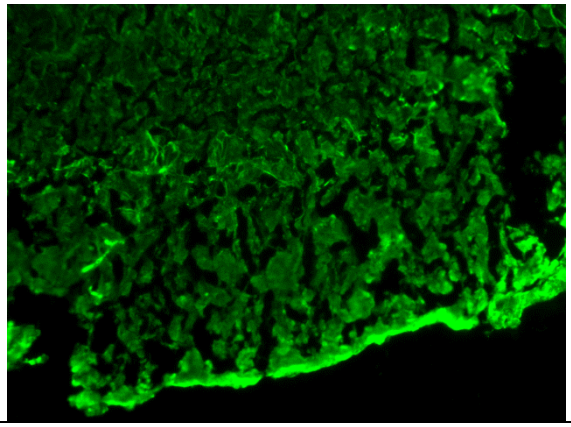
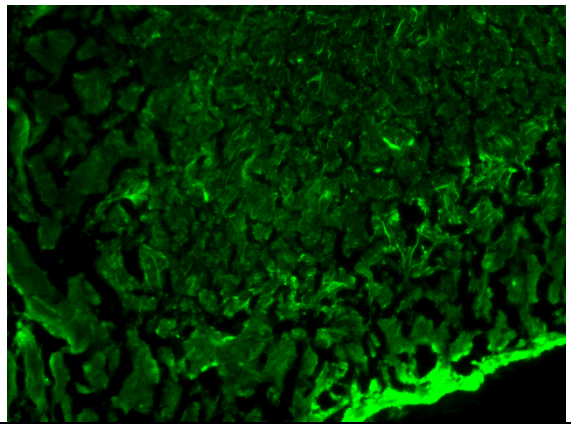
	G 	None

Figure 10: MAP2 primary antibody staining of P21 mouse brain sections. A, C – DIC images; B, D, E, F, G – Fluorescent images. MAP2 primary antibody dilutions: A, B – 1:500; C, D – 1:1000; E – 1: 2000; F – 1:5000; G – None. Cy3 goat anti rabbit secondary antibody dilution was 1:3000 for all sections. All sections were 20 um thick. Total magnification was 200X for all images. The Autoscale setting was on for all images.

Slides each with three P-1 month mouse brain sections received either 1:500, 1:1000, 1:2000, 1:5000, or no SMI31 primary antibody dilutions and 1:3000 Alexa488 goat anti mouse secondary antibody dilutions. Cerebella on each slide were then imaged at a 200X total magnification. The 1:500 SMI31 primary antibody stained section had broad staining throughout tissue, especially in possible Purkinje cell axons and possible blood vessels (see Figure 11, image B) and tissue loss and pia tearing in the lower region of the visual field (see Figure 11, image A). The 1:1000 and 1:2000 SMI31 primary antibody stained sections had weak, general staining, but strongly stained possible Purkinje cell axons and possible blood vessels (see Figure 11, images D and E). The 1:1000 SMI31 primary antibody stained section had minor tissue loss in the right and lower left of the field of vision and minor pia tearing in the lower left of the visual field (see Figure 11, image C). The 1:5000 SMI31 primary antibody stained section had weak, general staining, but strong staining in possible blood vessels (see Figure 11, image G) and very minor tissue loss in the upper right of the visual field (see

Figure 11, image F). The section without SMI31 primary antibody had very weak, nonspecific staining except for possible blood vessels which were strongly stained (see Figure 11, image I), but good section integrity (see Figure 11, image H).

DIC	Fluorescence	Anti-SMI31 Dilution
A 	B 	1:500
C 	D 	1:1000
	E 	1:2000

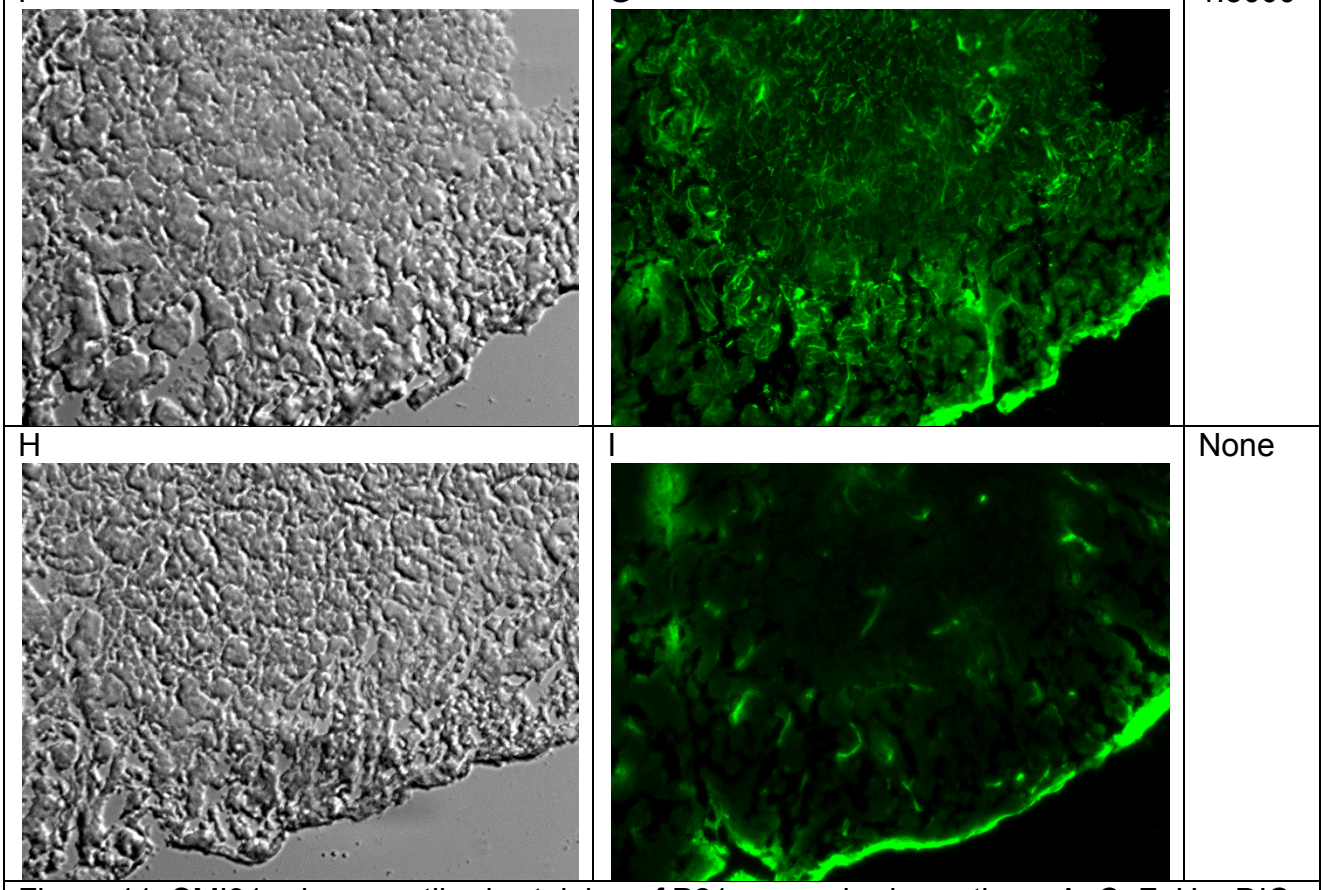


Figure 11: SMI31 primary antibody staining of P21 mouse brain sections. A, C, F, H – DIC images; B, D, E, G, I – Fluorescent images. SMI31 primary antibody dilutions: A, B – 1:500; C, D – 1:1000; E – 1: 2000; F, G – 1:5000; H, I – None. Alexa488 goat anti mouse secondary antibody dilution was 1:3000 for all sections. All sections were 20 um thick. Total magnification was 200X for all images. The Autoscale setting was on for all images.

Pilot Study to Determine Appropriate Secondary Antibody Concentrations

In the interest of time, not all slides used to determine the appropriate secondary antibody concentrations were photographed. Slides each with three P-1 month mouse brain sections were used for this pilot study. Two slides received 1:2000 polyclonal calbindin primary antibody dilutions and either 1:1000 or 1:3000 goat anti rabbit Alexa488 secondary antibody dilutions. At 200X total magnification and under fluorescent light, the slide which received 1:1000 goat anti rabbit Alexa488 stained brighter than the slide which received 1:3000 goat anti rabbit Alexa488. Both slides had

specific staining and very little background. Two slides received only 1:1000 and 1:3000 goat anti rabbit Alexa488 secondary antibody dilutions. Both slides had little to no staining and certainly no specific staining.

Two slides received either no primary antibody or 1:2000 polyclonal calbindin primary antibody dilutions and 1:3000 goat anti rabbit Cy3 secondary antibody. The slide which received no primary antibody was almost completely dark and the slide which received 1:2000 polyclonal calbindin antibody had bright, specific staining with very little background.

Two slides received either no primary antibody or 1:3000 monoclonal calbindin primary antibody dilutions and 1:3000 goat anti mouse Cy3 secondary antibody. The slide which received no primary antibody had very dim staining of only possible blood vessels and the slide which received 1:3000 monoclonal calbindin primary antibody had dim specific staining of possible blood vessels and possible Purkinje cells with very little background.

Two slides received either no primary antibody or 1:3000 monoclonal calbindin primary antibody dilutions and 1:3000 goat anti mouse Alexa488 secondary antibody. Both slides had very dim non specific staining and brighter possible blood vessel background staining.

One slide received a 1:1500 dilution of monoclonal SMI31 primary antibody and a 1:3000 dilution of goat anti mouse Cy3 secondary antibody. The slide had dim, but specific staining of possible Purkinje cell axons and no staining of possible blood vessels.

Two slides received a 1:1500 dilution of polyclonal MAP2 primary antibody and either 1:3000 or 1:6000 goat anti rabbit Cy3 secondary antibody dilutions. The slide that received a 1:3000 dilution of goat anti rabbit Cy3 secondary antibody had bright, specific staining of the granule cell layer and slightly less bright, specific staining of possible Purkinje cell dendrites. The slide which received a 1:6000 dilution of goat anti rabbit Cy3 secondary antibody was extremely dimly stained.

Two slides received a 1:1500 dilution of polyclonal MAP2 primary antibody and either 1:3000 or 1:6000 goat anti rabbit Alexa488 secondary antibody dilutions. The slide which received a 1:3000 dilution of goat anti rabbit Alexa488 secondary antibody had very dim, unspecific staining. The slide which received a 1:6000 dilution of goat anti rabbit Alexa488 secondary antibody dimly, specifically stained possible Purkinje cell dendrites.

Table 2: Pilot Study to Determine Appropriate Secondary Antibody Concentrations

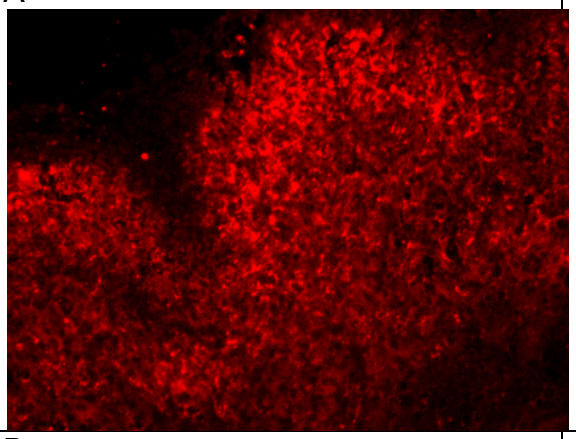
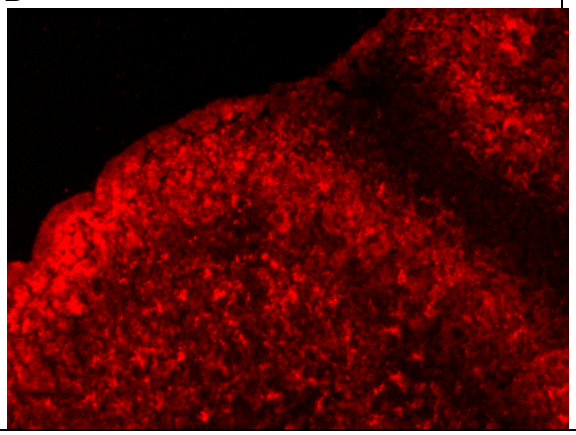
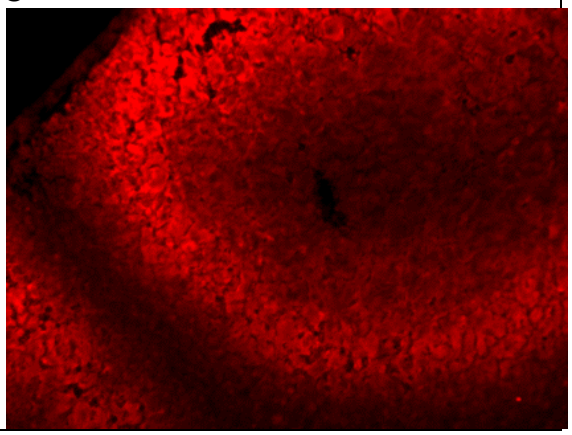
Primary Antibody	Secondary Antibody	Image
1:2000 polyclonal calbindin	1:1000 Alexa488 goat anti rabbit	Bright, specific signal
1:2000 polyclonal calbindin	1:3000 Alexa488 goat anti rabbit	Specific signal
No primary antibody	1:1000 Alexa488 goat anti rabbit	Relatively dim; no specific staining
No primary antibody	1:3000 Alexa488 goat anti rabbit	Relatively dim; no specific staining
1:2000 polyclonal calbindin	1:3000 Cy3 goat anti rabbit	Bright, specific signal, but some background staining
No primary antibody	1:3000 Cy3 goat anti rabbit	Almost no background staining; nearly black
1:3000 monoclonal calbindin	1:3000 Cy3 goat anti mouse	Dim, specific signal; strong blood vessel background staining
No primary antibody	1:3000 Cy3 goat anti mouse	Strong blood vessel background staining
1:3000 monoclonal calbindin	1:3000 Alexa488 goat anti mouse	Dim, specific signal; strong blood vessel background staining

No primary antibody	1:3000 Alexa488 goat anti mouse	Strong blood vessel background staining
1:1500 SMI31	1:3000 Cy3 goat anti mouse	Dim, specific signal and no blood vessel background staining
1:1500 MAP2	1:3000 Cy3 goat anti rabbit	Strong signal in internal granule cell layer, weak signal in Purkinje cell dendrites
1:1500 MAP2	1:6000 Cy3 goat anti rabbit	Too dim to use
1:1500 MAP2	1:3000 Alexa488 goat anti rabbit	Non-specific, very dim signal
1:1500 MAP2	1:1000 Alexa488 goat anti rabbit	Dim, specific signal

Morphological Analysis of Early Cerebellar Development

Two P0, P7, P14, and P21 slides with three mouse brain sections each, received either 1:1500 MAP2 or 1:3000 Calbindin primary antibody dilutions and 1:3000 Cy3 goat anti rabbit secondary antibody dilutions. Cerebella on each slide were then imaged at a 200X total magnification both with autoscaling on. The P0 sections which received 1:3000 Calbindin primary antibody and 1:3000 Cy3 goat anti rabbit secondary antibody lost too much tissue to merit photographing, but most of the primary antibody seemed to bind the pia (data not shown). The P7, P14, and P21 slides which received 1:3000 Calbindin primary antibody and 1:3000 Cy3 goat anti rabbit secondary antibody had dim, specific staining throughout the Purkinje cell layer and in the pia with low background (see Figure 12, images C, E, and G). The P0 section which received 1:1500 MAP2 primary antibody and 1:3000 Cy3 goat anti rabbit secondary antibody had no staining in the external granule cell layer; dim, specific staining in the area above the Purkinje cell bodies where prospective Purkinje cell axons and migrating granule cells should be; little staining in the area where the Purkinje cell bodies were; and dim,

specific staining in the internal granule cell layer (see Figure 12, image A; Figure 13, images A and B). The P7, P14, and P21 slides which received 1:1500 MAP2 primary antibody and 1:3000 Cy3 goat anti rabbit secondary antibody had dim, specific staining in the area above the Purkinje cell bodies where prospective Purkinje cell axons should be; little staining in the area where the Purkinje cell bodies were; and dim, specific staining in the internal granule cell layer (see Figure 12, images B, D, F).

Section Age	MAP2 Primary Antibody	Calbindin Primary Antibody
P0	A 	
P7	B 	C 

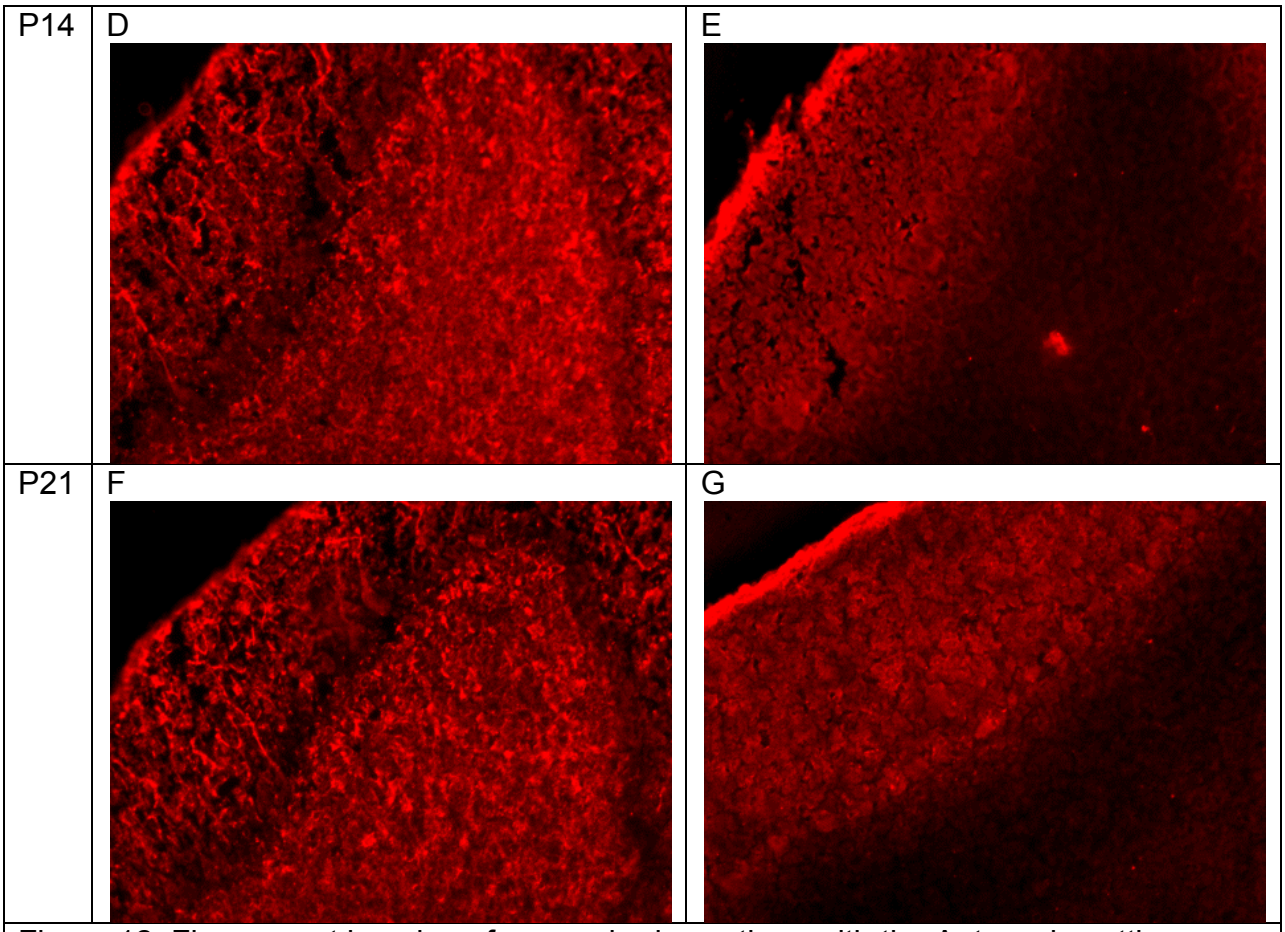


Figure 12: Fluorescent imaging of mouse brain sections with the Autoscale setting on. Section Age: A – P0; B, C – P7; D, E – P14; F, G – P21. A, B, D, F – 1:1500 MAP2 primary antibody; C, E, G – 1:3000 calbindin primary antibody. Cy3 goat anti rabbit secondary antibody dilution was 1:3000 for all sections. Section thickness: A, B, C, D, E – 14 um; F, G – 20 um. Total magnification was 200X for all images.

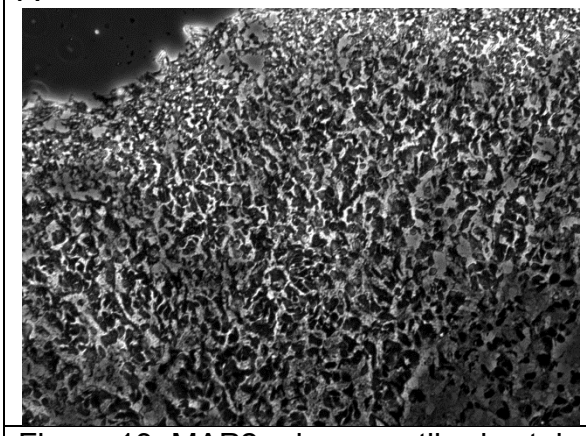
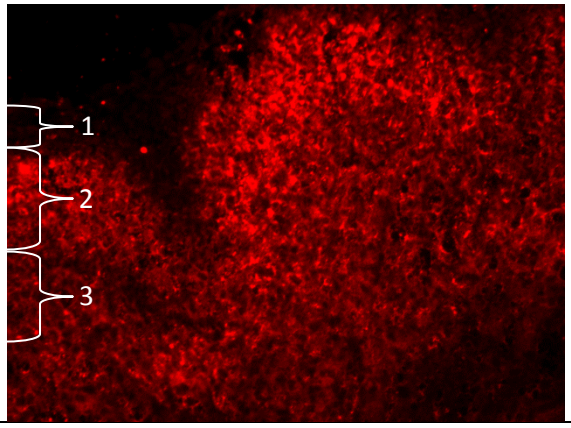
Phase	Fluorescence
A 	B 

Figure 13: MAP2 primary antibody stained P0 mouse brain sections. A – Phase Image; B – Fluorescent Image. A – Autoscale off; B- Autoscale on. MAP2 primary antibody dilution was 1:1500 for all sections. Cy3 goat anti rabbit secondary antibody dilution was 1:3000 for all sections. All sections were 14 um thick. Total magnification was 200X for all images. Bracket 1 – External granule cell layer; Bracket 2 – Purkinje cell layer; Bracket 3 – Internal granule cell layer.

Two P0, P7, P14, and P21 slides with three mouse brain sections each, received either 1:1500 or no SMI31 primary antibody dilutions and 1:3000 Alexa488 goat anti mouse secondary antibody dilutions. Cerebella on each slide were then imaged at a 200X total magnification both with autoscaling on and off. These sections were not photographed because only very dim possible blood vessel background and no specific SMI31 Primary antibody labeling was observed, in contrast to the specific SMI31 staining seen previously (see Figure 11, images D and E).

Two P0, P7, P14, and P21 slides with three mouse brain sections each, received either 1:1500 SMI31 and 1:1500 MAP2 primary antibody with 1:3000 Alexa488 goat anti mouse and 1:3000 Cy3 goat anti rabbit secondary antibody dilutions or 1:3000 Alexa488 goat anti mouse and 1:3000 Cy3 goat anti rabbit secondary antibody dilutions only. Cerebella on each slide were then imaged at a 200X total magnification both with

autoscaling on and off. These sections were not photographed because only very dim possible blood vessel background and no specific SMI31 Primary antibody labeling was observed, although the MAP2 staining was visible.

Discussion

Pilot Study to Determine Ideal Fixation Time

Results from the pilot study to determine the appropriate length of time for tissue fixation clearly supports a direct relationship between fixation time and tissue integrity. The slides fixed for 20 and 10 minutes appeared to be almost entirely intact whereas the slides fixed for 5, 1, and 0 minutes had increasing degrees of tissue loss. Success with twenty minutes of fixation suggested that further fixation time may further ensure tissue integrity, so in the pilot study to determine the effect of tissue age on fixation success, sections were fixed for 30 minutes. Paraformaldehyde fixes tissue by cross-linking proteins, thereby increasing tissue stability. However, over-fixation can cross-link sections so severely that antibodies are unable to bind; therefore, no further attempts were made to increase fixation time beyond 30 minutes (Ramos-Vara, 2005).

Pilot Study to Determine the Effect of Tissue Age on Fixation Success

The pilot study to determine the effect of tissue age on fixation success demonstrates that 30 minutes of fixation is sufficient to ensure tissue integrity in P0, P4, and P-1 month mouse brain sections. Therefore, 30 minutes of fixation should be sufficient for all tissue age sections used in this experiment: P0, P4, P7, P14, P21, and P-1 month.

A Note on Image Autoscaling

Autoscaling is a MetaView Software function which alters an image which uses only a narrow range of pixel shade quantities so that the image uses the entire breadth of the Software's possible pixel shade quantities instead. In this way, the bright aspects of an image will appear brighter and the dark aspects darker, thereby increasing contrast between light and dark areas in an image. Although sections which receive secondary antibody only appeared to have dim, unspecific background in some figure (see Figures 10, image G and Figure 11, image I) this is really just a result of the fact that autoscaling was kept on to provide the best contrast for imaging antibody staining. Sections stained with Cy3 secondary antibody only and imaged with autoscaling off have very dim background (see Figure 8, image C and Figure 9, images A and B). This demonstrates the true background caused by the Cy3 secondary antibody. Alternatively, sections stained with MAP2 or calbindin primary antibody and Cy3 secondary antibody and imaged with autoscaling off have dim, but specific staining of probable Purkinje cell dendrites or dim, but specific staining of the entire Purkinje cell respectively (see Figure 8, image A and Figure 9, images C and D). These primary antibody stained slides also have extremely low background when imaged with autoscaling off (see Figure 8, image A and Figure 9, images C and D). This demonstrates the low Cy3 secondary antibody background and dim, but specific primary antibody staining. When autoscaling was turned on, there appeared to be dim, but significant background in these primary antibody treated slides, but this was just a result of the autoscaling (see Figure 8, image B and Figure 9, images E and F).

Pilot Study to Determine Appropriate Primary Antibody Concentrations

The 1:500 MAP2 primary antibody stained sections were broadly stained with no specific staining, likely due to a too concentrated dilution of MAP2. Conversely, the 1:5000MAP2 primary antibody stained sections were extremely dim as a result of too dilute a concentration of MAP2. Both the 1:1000 and 1:2000 MAP2 primary antibody stained sections had dim, but specific staining of probable Purkinje cell dendrites. Therefore, a 1:1500 dilution of MAP2, the midpoint between dilutions which provided the best results in this pilot study, is an appropriate choice for a MAP2 primary antibody dilution. It should be noted that the bright MAP2 staining in the granule cell layer was initially surprising, but is supported by prior literature which notes that MAP2 stains not only weakly stains Purkinje cell dendrites but also brightly stains granule cells (Huber, et al., 1984).

Results from the SMI31 staining were similar to the results of the MAP2 staining. 1:500 SMI31 primary antibody staining was general and non-specific because the antibody dilution was too concentrated and 1:5000 SMI31primary antibody staining was weak and non-specific because the antibody was too dilute. Once again, the 1:1000 and 1:2000 dilutions of SMI31 primary antibody best stained the P-1 month sections. The staining was specific to the Purkinje cell axons, with little background. Therefore, a SMI31 primary antibody dilution of 1:1500 was chosen for future staining.

It is hypothesized that the strong, specific staining of possible blood vessels throughout all SMI31 primary antibody concentrations, including the slide which received no primary antibody, is due to binding of the goat anti mouse Alexa488 secondary antibody. This is likely a result of the goat anti mouse Alexa488 secondary

antibody's reactivity to mouse tissue. This antibody to mouse not only binds to the monoclonal SMI31, but also to certain mouse tissue, in this case blood vessels. Although this makes sense because blood vessels, even in the brain, are known to express many different kinds of antibodies, it is still only a hypothesis and further research is needed to confirm this presumption.

Pilot Study to Determine Appropriate Secondary Antibody Concentrations

The 1:1000 goat anti rabbit Alexa 488 staining of 1:2000 polyclonal calbindin stained mouse brain sections was brighter than staining by a 1:3000 dilution of the goat anti rabbit Alexa488 and both secondary antibody concentrations had dim, non-specific staining of mouse brain tissue sections without primary antibodies. Therefore, the 1:1000 goat anti rabbit Alexa488 secondary antibody concentration is ideal for staining Purkinje cells green.

The 1:3000 goat anti rabbit Cy3 staining of 1:2000 polyclonal calbindin stained mouse brain sections was bright, but had some background. The 1:3000 goat anti rabbit Cy3 staining of mouse brain sections without a primary antibody was excellent with extremely dim staining. Therefore, if given the choice, sections treated with 1:2000 polyclonal calbindin should be stained with 1:3000 goat anti rabbit Cy3 secondary antibody rather than 1:1000 goat anti rabbit Alexa488 secondary antibody.

All sections stained with monoclonal calbindin primary antibody and either 1:3000 goat anti mouse Cy3 or Alexa488 had dim staining and strong possible blood vessel background staining. Therefore, secondary staining of polyclonal calbindin with 1:3000 goat anti rabbit Cy3 should be used whenever possible because the signal is more crisp

than that of monoclonal calbindin primary antibody by any of the goat anti mouse secondary antibodies.

Goat anti mouse Cy3 staining of monoclonal SMI31 stained possible Purkinje cell axons dimly, but specifically and did not stain blood vessels. To best image Purkinje cell axons, the goat anti mouse Cy3 secondary antibody should be used at the more concentrated dilution of 1:1000 to brighten the staining of possible axons.

1:3000 goat anti rabbit Cy3 secondary antibody staining of mouse brain sections with 1:1500 MAP2 primary antibody staining was strong in the granule cell layer, but only weakly stained probable dendrites. The 1:6000 goat anti rabbit Cy3 secondary antibody staining of mouse brain sections with 1:1500 MAP2 primary antibody staining was much too dim to efficiently use. Therefore, if it is necessary to stain Purkinje cell dendrites red, the 1:3000 goat anti rabbit Cy3 secondary antibody should be used.

1:3000 goat anti rabbit Alexa488 secondary antibody staining of mouse brain sections with 1:1500 MAP2 primary antibody staining was not specific and very dim. Alternatively, 1:1000 goat anti rabbit Cy3 secondary antibody staining of mouse brain sections with 1:1500 MAP2 primary antibody staining was still dim, but specifically stained probably Purkinje cell dendrites. Therefore, if Purkinje cell dendrites should be stained green, 1:1000 goat anti rabbit Cy3 secondary antibody staining should be used.

Morphological Analysis of Early Cerebellar Development

Staining in P7, P14, and P21 mouse brain sections treated with 1:3000 Calbindin primary antibody and 1:3000 Cy3 goat anti rabbit secondary antibody was indicative of expected cerebellar development. The Purkinje cells in the P7 mouse brain section are

slightly more clumped and not as neatly lined up as those in the P14 mouse brain section or P21 mouse brain section, which clearly portrays an ordered line of larger Purkinje cell bodies with staining throughout their dendritic layer. The internal granule cell layer is largely dark in all three ages, another expected result. Relatively bright staining of the pia is unexpected, but not without precedent: the pia contains numerous fibroblast cells known to absorb many different antibodies.

Staining in P0 mouse brain section treated with MAP2 primary antibody and Cy3 secondary antibody demonstrated some interesting staining patterns. It appears that MAP2 is expressed in the area above the Purkinje cell bodies where prospective early processes extended by the Purkinje cell could be (see Figure 13, image B, bracket 2). However, no MAP2 staining appears in the cell just under the pial surface, the external granule cell layer (see Figure 13, image B, bracket 1). This is expected the granule cells in the external granule cell layer are still dividing and have not yet begun producing axons. Cells in the internal granule cell layer, beneath the Purkinje cell bodies, do exhibit MAP2 staining, likely from granule cell dendrites, but not calbindin staining (see Figure 13, image B, bracket 3). Future experiments could double stain for MAP2 and calbindin to label early Purkinje cell processes and determine if they are expressing MAP2, if the granule cells migrating through the Purkinje cell layer are expressing MAP2, or if both are expressing MAP2.

Staining in P7, P14, and P21 mouse brain sections treated with MAP2 primary antibody further support expected Purkinje cell development. The area between the Purkinje cell bodies and the pial surface, where dendrites are expected, seems to express MAP2, as does the area below the Purkinje cell bodies where granule cell

dendrites should express MAP2. It is also worth noting that prospective Purkinje cell dendrites appear shorter in the P7 mouse brain sections relative to those in the P14 and P21 mouse brain sections (see Figure 12, images B, D, and F). This is also expected as Purkinje cell dendrite growth continues throughout the P7 to P21 time range (see Figure 2).

Staining in P0, P7, P14, and P21 mouse brain sections treated with SMI31 primary antibody was unsuccessful; the only visible staining was blood vessel background likely from the Alexa488 goat anti mouse secondary antibody. These poor results may be a result of procedural error (i.e. the SMI31 primary antibody was never added to the experimental primary antibody solutions from the stock tube, the SMI31 primary antibody was not mixed thoroughly enough, the slides did not receive SMI31primary antibody solutions, etc.). Alternatively, the SMI31 primary antibody stock solution may have somehow denatured, rendering it ineffective.

Tissue adhesion declined with tissue age in this experiment, rendering a number of the P0 sections completely useless and causing difficulty in several of the P7 sections. It is imperative that future experiments address this ongoing issue so that early Purkinje cell morphology can be properly and consistently studied. One way to potentially enhance tissue integrity is to continue to increase section thickness. Over the course of this honors project and the independent study which preceded it, section thickness has been increased twice, from 10um, to 14um, and finally to 20um. So far, antibodies have had no difficulty penetrating the thicker tissue and the increased thickness has even aided in Purkinje cell axon, dendrite, and body visualization. It seems reasonable to increase tissue thickness to 25um or even 30um to aid in tissue

integrity and Purkinje cell visualization. Perfusion of mouse pups prior to dissection is another effective and commonly used method of fixation to increase tissue integrity and adhesion. Although perfusion is typically expensive, cheaper methods are available and should also be explored to prevent future tissue loss.

Major Conclusions from this Study

Although this study did not answer the ultimate research question, "What is the identity of the early fibers extended by Purkinje cells between postnatal day 0 and 3?" it did provide the results and outline the protocol necessary to answer this question in future research. These results and protocol include:

- Tissue section thickness should be no less than 20um and section thickness can be increased.
- Slides should be post-fixed for thirty minutes with 4% paraformaldehyde in Sorenson's phosphate buffer.
- Calbindin primary antibody should be used at a 1:3000 dilution. Calbindin primary antibody binds entire Purkinje cells – dendrites, cell bodies, and axons (see Figure12, images C, E, and G).
- SMI31 primary antibody should be used at 1:1500 dilution. SMI31 primary antibody binds Purkinje cell axons and possible basket cell fibers (see Figure 11, image D and E) (Langley et al., 1988).
- MAP2 primary antibody should be used at 1:1500 dilution. MAP2 primary antibody binds the distal portions of Purkinje cell dendrites and granule cell dendrites (see Figure 12, images A, B, D, F and Figure 13, image B).

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