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Research Report

Callosal oligodendrocyte number in postpartum Sprague-Dawley rats

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ABSTRACT

Prolactin (PRL), an anterior pituitary hormone with neurogenic properties associated with pregnancy, has been implicated in oligodendrocyte proliferation during gestation, contributing to increased myelination in the maternal brain. However, PRL is elevated during lactation as well, suggesting that the postpartum (PP) period may contribute to additional gliogenesis in lactating females. In the present study, we assessed oligodendrocyte number in the corpus callosum (CC) of female Sprague-Dawley rats near the end of gestation, and at two weeks postpartum in both lactating and non-lactating dams, and in virgins. Though pregnant females did not differ significantly from any other group, lactating females had significantly more oligodendrocytes in the CC than virgins ($p = .01$), and in medial regions of the CC than non-lactating dams ($p < .02$). Oligodendrocyte number in the CC of pregnant and PP females correlated positively with the number of pups in their litter ($r^2 = .68$, $p < .005$). These results suggest that the gestational period contributes to oligodendrocyte proliferation or survival, likely mediated by an endocrine hormone whose concentration varies with the size of the litter. The PP period also contributes to increases in CC oligodendrocyte number, though it is unclear whether endocrine influences and/or pup-interaction underlie the differences in myelination between lactating and non-lactating groups. Further investigation is required in order to confirm whether the effects observed are mediated by members of the PRL-family, experience, and/or other gestational/PP endocrine hormones.

1. Introduction

In rats, the subventricular zone (SVZ) produces multipotential progenitor cells that migrate and differentiate into neurons and glia (Levison and Goldman, 1993). Glial precursors from the SVZ migrate radially during gliogenesis to the corpus callosum (CC) and cerebral cortex, where some differentiate into oligodendrocytes, the myelinating cells of the central nervous system (CNS) (Nait-Oumesmar et al., 1999). Following focal brain injury, post-mitotic oligodendrocytes are unable to provide remyelination (Keirstead and Blakemore, 1997). However local and SVZ progenitors continue to produce oligodendrocytes throughout adulthood (Gensert and Goldman, 1997; Levison et al., 1999), and these cells have been shown to contribute to repair and remyelination after a demyelinating injury (Polito and Reynolds, 2005). The profile of oligodendrocyte myelination varies

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between sexes in rodents, with females possessing fewer myelinated axons in the CC (Kim and Juraska, 1997), and having greater oligodendrocyte turnover in adulthood (Cerghet et al., 2006).

Research suggests that one mechanism subserving the increased oligodendrocyte turnover seen in females may be prolactin (PRL). PRL, produced primarily in the anterior pituitary (Riddle et al., 1933), has been shown to act as an astroglial mitogen (DeVito et al., 1992) and has general neuro- and gliotrophic properties. PRL is involved in glial responses following hypoxic ischemic injury in the rat, and infusion of exogenous PRL is sufficient to facilitate repair in damaged brain areas (Moderscheim et al., 2007). In addition, PRL-mediated neurogenesis during pregnancy leads to significant increases in the number of new cells in the SVZ, and another smaller increase in cell proliferation is seen just after parturition (Shingo et al., 2003).

More recently, research has begun to focus on the role of glial plasticity in the onset and maintenance of maternal behaviour. Primiparous rats display increased numbers of astrocytes in the medial preoptic area, a region critical for maternal behaviour, while in multiparous females astrocyte number is decreased in the medial amygdala (Featherstone et al., 2000). Lactating rats display higher concentrations of glial fibrillary acid protein (GFAP) and basic fibroblast growth factor (bFGF) than virgins or late-term pregnant females (Salmaso et al., 2005), and once this occurs, maintenance of changes in GFAP and bFGF expression is not dependent upon continued suckling stimulation (Salmaso and Woodside, 2006). Taken together, these results are suggestive of a role for both neural and glial plasticity in the expression of maternal behaviour.

Increased oligodendrocyte proliferation and myelination in the CC and spinal cord have been reported in pregnant mice, and these increases were sufficient to repair acute demyelinating damage to the spinal cord (Gregg et al., 2007). In virgin mice, systemic administration of PRL resulted in increased oligodendrocyte proliferation (Gregg et al., 2007), supporting the hypothesis that oligodendrocyte proliferation observed during gestation was mediated by PRL secretion at the onset of pregnancy.

During pregnancy, women with multiple sclerosis (MS), a demyelinating disease of the central and peripheral nervous system, experience a remission of their symptoms proportional to the decrease observed in active white-matter lesions (van Walderveen et al., 1994; Confavreaux et al., 1998; Voskuhl, 2003). PRL mediated oligodendrocyte proliferation has been implicated in this improvement: Gregg et al. (2007) demonstrated that precursor cells that had undergone mitotic division on gestational day 7 in female mice had migrated and differentiated into myelinating oligodendrocytes in the CC by gestation day 18. In addition, these authors found increased myelin density in the genu of the CC at two weeks PP (Gregg et al., 2007), however, there was no examination of the contribution of the PP period to myelination. Since PRL has been implicated in oligodendrocyte proliferation and is elevated during lactation (Freeman et al., 2000), a further examination of the PP period may elucidate whether PRL has similar gliogenic effects after parturition.

The purpose of the present study was to examine the effect of gestation and PP lactation on oligodendrocyte number in the CC of the rat. Oligodendrocytes were assessed near the end of the gestational period (GD18 group) and two weeks postpartum in both lactating (L-PPD14) and non-lactating dams (NL-PPD14). We hypothesized that females near the end of gestation would have higher numbers of oligodendrocytes in the CC than virgins. In addition, it was expected that lactating dams would display more CC myelination than non-lactating PP females.

2. Results

Virgins age-matched to GD18 and PPD14 groups did not differ on any measure and were therefore combined for the remainder of the analyses (called virgins hereafter). Three *a priori* hypotheses were tested using independent samples *t*-tests. It was hypothesized that gestational (GD18) females would display greater numbers of oligodendrocytes in the CC than virgins. It was also predicted that oligodendrocyte number in lactating postpartum females (L-PPD14) would differ from both virgins and non-lactating postpartum animals (NL-PPD14).

There was no significant difference between the mean number of oligodendrocytes in the CC of virgins and GD18 females, however, L-PPD14 animals had a significantly greater mean number of oligodendrocytes than the virgin group ($t=-3.381$, $df=8$, $p=.01$) (Fig. 1B). A trend toward greater oligodendrocyte number in L-PPD14 compared with NL-PPD14 animals did not achieve significance (Fig. 1B).

We suspected that the trend observed between L- and NL-PPD14 groups might represent genuine differences that failed to reach significance as a result of an uneven distribution of new oligodendrocytes. While little research has examined adult oligodendrocyte migratory patterns under normal physiological conditions, during early postnatal development oligodendrocyte precursors appear to migrate to the CC primarily from the dorsolateral SVZ along the lateral ventricular wall (Kakita and Goldman, 1999). This migratory route would lead OPCs to relatively medial regions of the CC in the areas we examined (between +2.28 mm and +1.32 mm anterior to Bregma). Kakita and Goldman (1999) have reported migratory speeds of 53–88 μm/h for these precursor cells, suggesting that over the two week period prior to sacrifice, precursors would be unlikely to migrate very far laterally before differentiating and beginning to express makers of mature oligodendrocytes. Thus, we suspected that group differences might be concentrated in medial regions of the CC. As expected, virgin and L-PPD14 oligodendrocyte counts differed significantly in medial regions (defined as ±1 mm from midline) of the CC ($t=-2.706$, $df=8$, $p<.05$), and L-PPD14 females had significantly higher numbers of oligodendrocytes in the medial CC than NL-PPD14 dams ($t=3.139$, $df=7$, $p<.02$) (Fig. 2). Finally, examination of CNPase-positive cells in the anterior commissure revealed that there were no significant differences in oligodendrocyte number between groups (Fig. 3).

The mean number of oligodendrocytes in the CC of females in GD18, NL- PPD14, and L-PPD14 groups correlated positively

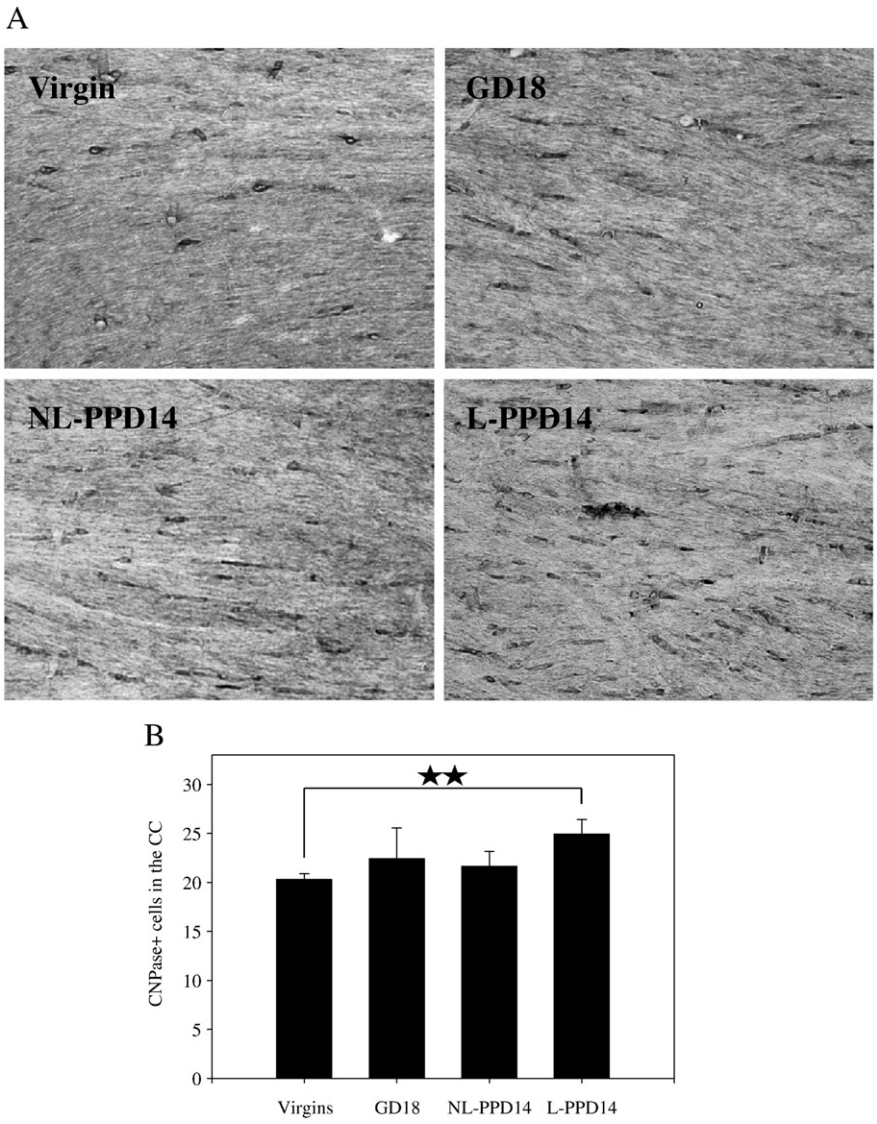

Fig. 1 – Lactating PP females differ from virgins in overall oligodendrocyte number in the corpus callosum (CC). (A) Representative CNPase stained micrographs showing oligodendrocytes in medial regions of the CC for virgin, GD18, NL-PPD14, and L-PPD14 rats. (B) Mean number of CNPase positive cells (+/− SEM) per field in the CC of virgin, GD18, NL-PPD14, and L-PPD14 rats. L-PPD14 females had significantly higher numbers of oligodendrocytes than virgins. ★★$p = .01$.

with the number of pups in the litter ($r = .822$, $df = 8$, $p < .005$) (Fig. 4). Partial correlations controlling for group confirmed that results were not due solely to group differences.

3. Discussion

The hypotheses that PP lactating rats would have greater numbers of oligodendrocytes in the CC than either virgins or non-lactating PP animals were supported. While virgins and lactating dams differed in total mean oligodendrocyte number, differences between lactating and non-lactating dams were concentrated in medial regions of the CC.

No significant differences in oligodendrocyte number were observed in the gestational group: CC oligodendrocyte counts of GD18 females were similar to those of virgins. Hence, our results differed from the findings of Gregg et al. (2007), which showed gestational increases in myelination in mice. However, here we did not assess oligodendrocyte proliferation or precursor (OPC) number, but absolute oligodendrocyte counts. It is possible that while significant increases in proliferation or OPC number are apparent by GD18 in mice, these differences are not yet sufficient to alter absolute counts. It should also be noted that the variance of our GD18 group was consistently larger than that of other groups. Since vaginal swabs were performed once a day to test for the presence of semen, GD18 females could have been inseminated as much as a day, and as little as some minutes prior to swabbing. That this temporal variation contributed to the variance we observed in the GD18 group's oligodendrocyte numbers must be considered. Furthermore, the length of gestation differs in mice and rats; performing perfusions later in gestation might have yielded different results.

The PP period appears to contribute to oligodendrocyte number, though it remains unclear whether this is due to differences in PP gliogenesis, oligodendrocyte distribution, or cell survival (OPC and/or oligodendrocyte). In addition, our results suggest a significant role for the gestational period: there was a robust correlation between the number of pups in dams' litters and the mean number of oligodendrocytes observed in the CC. Since the number of pups in the litter would have no PP effect in either gestational or non-lactating groups (that had no PP contact with pups), the correlation between CC oligodendrocyte and pup number is unlikely to be due to PP factors. As PRL has been established as a glial mitogen, a PRL-related hormone present during pregnancy may underlie the correlation we observed.

Maternal PRL secretion is high during the first half of pregnancy in rats, but drops at around gestation day 12 until the ante-partum period (Grattan and Kokay, 2008). During the second half of the gestational period, when maternal PRL secretion is low, PRL-like hormones (placental lactogens; PL) are produced by mural and polar trophoblast giant cells (Faria et al., 1990). PL are structurally similar to PRL and capable of binding PRL receptors (Grattan et al., 2008). Hence, PL could potentially account for the correlation observed between oligodendrocyte number and litter size, though would not be sufficient to explain NL- and L-PPD14 group differences.

It is unclear whether differences observed between NL- and L-PPD14 females were due to hormonal profiles or dams' experiences. After birth, pups' suckling triggers a transient PRL surge in the dam (Grattan and Kokay, 2008). Our non-lactating group was pup-deprived following parturition, thereby depriving these females of the tactile stimulation required to maintain elevated PRL levels PP. In lactating dams, this elevated PRL may have contributed to continued cellular proliferation PP. However, lactation necessarily involves mother-pup interaction, and pup-experience itself has been implicated in long-term alterations of the maternal brain, mediating both the onset, and continued expression of maternal behaviours (Orpen and Fleming, 1987). Pup-exposure, even briefly, has been shown to affect both cellular birth (Featherstone et al., 2000) and survival (Akbari et al., 2007) in a region-specific manner. Thus, either postpartum pup-experience or lactation, or both, may underlie our findings, contributing to additional gliogenesis or increased oligodendrocyte survival in the CC.

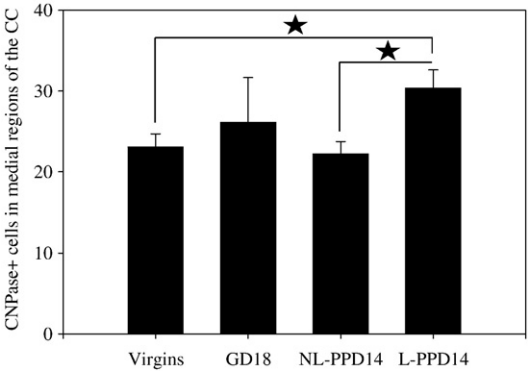

Fig. 2 – The number of oligodendrocytes in medial regions of the corpus callosum (CC) is increased in lactating PP females compared to both virgins and nonlactating PP animals. Mean number of CNPase positive cells (+/−SEM) per field in medial regions (+/−1 mm from midline) of the CC. L-PPD14 females had significantly higher numbers of oligodendrocytes in medial regions of the CC than virgins and NL-PPD14 groups. ★.05.

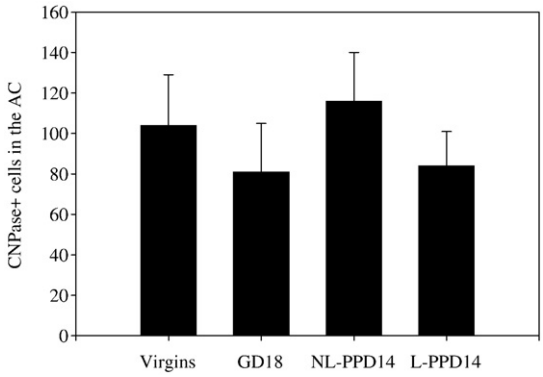

Fig. 3 – Groups did not differ in oligodendrocyte number or myelination in the anterior commissure (AC). Sum of CNPase positive cells (+/−SEM) of fields examined in the AC of virgin, GD18, NL-PPD14, and L-PPD14 rats.

Future research could examine the relative contributions, if any, of PL and pup-experience on PP CC myelination. Dissociating the role of PRL and pup-experience postpartum could be achieved via an examination of oligodendrocyte proliferation and survival in PP females allowed to interact with pups, but in which PRL-release is suppressed. Additionally, it is unclear whether multiparous females would display comparable increases in CC oligodendrocyte number following subsequent pregnancies, or whether the size of the effect would change with time and experience. If oligodendrocyte number increases in a site-specific manner, then would primiparous females display additional myelination in regions subserving the onset of maternal behaviour while in multiparous females, new myelination would be concentrated in areas subserving the maternal experience effect? Examination of PP myelination across pregnancies could elucidate the role CC oligodendrocytes play in the expression of maternal behaviour.

Primiparous female rats that nursed ten pups for two weeks displayed significantly higher numbers of oligodendrocytes in the CC than either virgins or PP females that did not interact with or nurse pups. Oligodendrocyte counts correlated positively with the number of pups in the dams' litter, suggesting that an endocrine hormone whose concentration varies in accordance with the number of pups in-utero contributes to oligodendrocyte proliferation and/or survival. The present study strengthens Gregg et al.'s (2007) hypothesis that oligodendrocyte proliferation associated with pregnancy contributes to re-myelination and the reduction of white-matter lesions observed in women with MS. Our results further suggest that, barring conditions that interfere with the process, breast-feeding may contribute to even greater white-matter repair. Alternately, offspring-interaction may

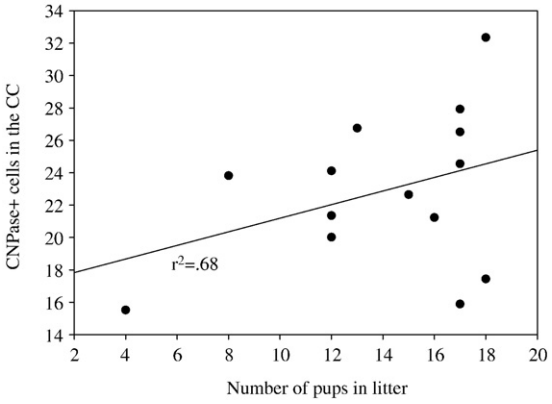

Fig. 4 – Scatter-plot depicting partial correlations controlling for group between mean number of CNPase positive cells in the corpus callosum (CC) of GD18, NL-PPD14, and L-PPD14 females, and the number of pups in females' litters ($r = .822$, $df = 8$, $p < .005$).

contribute to additional PP myelination, survival, or direct the fate of CC oligodendrocytes in a site-specific manner.

4. Experimental procedures

4.1. *Subjects*

Thirty-six female Sprague-Dawley rats aged 65–75 days at the beginning of the study were used for this experiment. Rats were housed in pairs in clear plexiglass cages (18×10×8 in.) at the Vivarium at the University of Toronto at Mississauga according to animal care guidelines. Animals were maintained on a 12 h light/dark schedule (lights on at 7 am) in a room maintained at 22 °C with 45%–55% humidity. Animals were provided with food and water *ad lib*.

Females were randomly assigned to five genetically-matched groups such that no two animals in any group shared a progenitor, and every group possessed one of five full- or half-siblings. The five groups consisted of: gestation day 18 group (GD18; $n=5$), PP day 14 females that were either lactating (L-PPD14; $n=4$), or non-lactating (NL-PPD14; $n=5$), and virgin age-matched control groups for GD18 and L- and PPD14 ($n=6$).

Females in GD18, and both PP conditions were placed with a proven stud male (two females and one male per cage) for one week and allowed to mate. Females in both virgin conditions were housed in pairs for the same duration as females in the mating condition. During mating, females were given daily vaginal swabs to test for the presence of semen. Females that failed to become pregnant following one week of being housed with a male were removed from the study. The presence of sperm marked GD0, at which time females were singly housed. Two days prior to expected females' parturition in the PP groups, virgin age-matched controls were moved to a pup-free room for the remainder of the study.

Twenty days after GD0, females in NL-PPD14 were monitored for parturition and the presence of pups at 15 min intervals over 24 h periods. Parturition marked PPD0. Pups

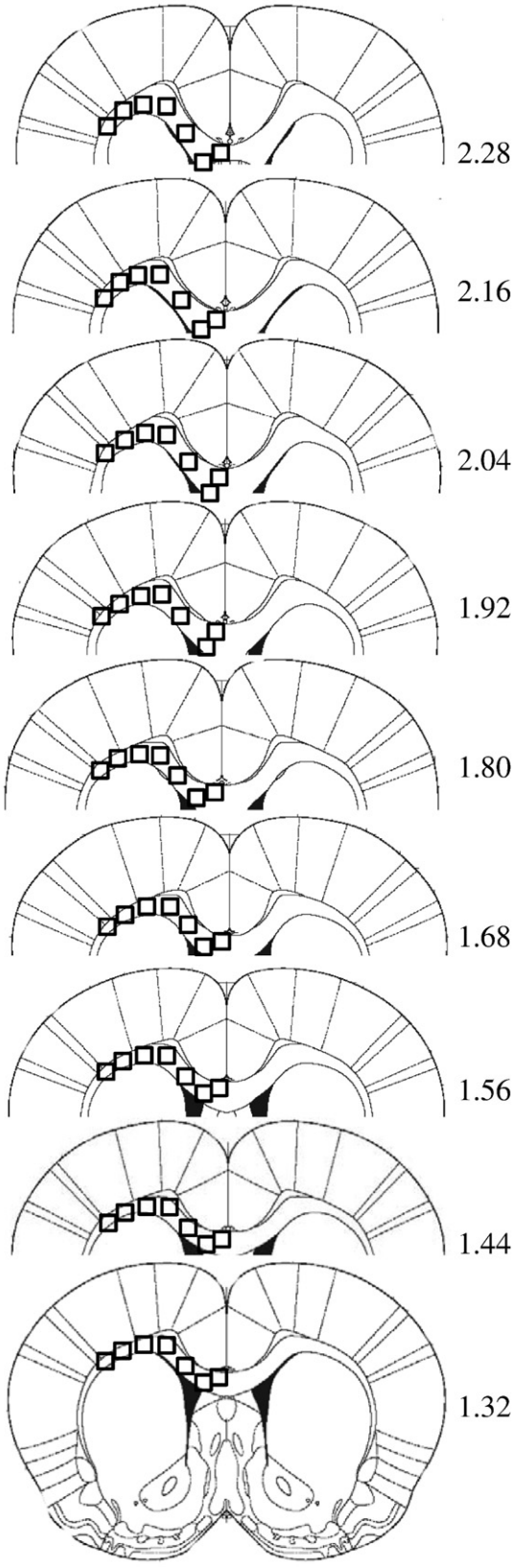

Fig. 5 – Brain schematics of areas imaged within the corpus callosum. Rectangles represent fields captured during imaging and analyzed.

were removed immediately following birth in 15 minute intervals. The size of the litter of each dam was recorded. Litters of L-PPD14 females were randomly culled to 10 animals on PPD0 and the pups of NL-PPD14 females sacrificed. After pup-removal, NL-PPD14 females were transferred to clean cages and moved to a pup-deprived room until PPD14.

4.2. *Immunohistochemistry*

On either the eighteenth day of gestation (for GD18 and age-matched virgins) or two-weeks PP (for L-PPD14, NL-PPD14, and aged-matched virgins), females were injected with a lethal dose of Xylazine Hydrochloride (25 mg/kg) and Ketamine Hydrochloride (20 mg/kg) and perfused intracardially with .9% saline (300 ml) and 4% paraformaldehyde (300 ml; pH 7.4) kept on ice. The number of foetuses present in the uterine horns of GD18 females was determined. Following perfusion, female's brains were removed and stored over-night at 4 °C in 4% paraformaldehyde. Twenty-four hours later, brains were transferred to a 30% glucose solution until sliced into 30 μm sections using a cryostat (Leica VT1000 S). Slices were suspended in cryoprotectant solution and stored at −20 °C.

Every fourth 30 μm section was stained to mark the presence of oligodendrocytes. Brain sections were quenched in 20% methanol and 3% hydrogen peroxide in .9% Trizma Buffered Saline (TBS) solution for 20 min, then washed in TBS 3 times and preblocked in 3% normal goat serum (NGS; Vector Laboratories, Inc.; Burlington, Ontario, Canada) in TBS containing .3% Triton-X 100 (Sigma; Oakville, Ontario, Canada). Sections were washed in TBS and incubated at 4 °C over night in 1:3000 mouse monoclonal anti-CNPase (2′,3′-Cyclic Nucleotide 3′-Phosphodiesterase; GeneTex, Inc., San Antonio, CA) with 3% NGS in .3% Triton TBS. The sections were then incubated for an hour in biotinylated anti-mouse IgG (H+L) made in goat secondary antibody (1:200; Vector Laboratories, Inc.; Burlington, Ontario, Canada) in .3% Triton TBS with 3% NGS. Sections were washed in TBS and incubated for 30 min in avidin–biotin complex (ABC; 1:50) in .3% TBS. Slices were rinsed and the peroxidase complex was visualized using DAB peroxidise substrate kit (Vector Laboratories, Inc.; Burlington, Ontario, Canada) containing nickel chloride applied to darken the stain.

4.3. *Imaging*

CNPase-positive cells were counted in the CC beginning at +2.28 mm anterior to Bregma and at every 120 μm for 9 consecutive sections (Paxinos and Watson, 1998), and CNPase positive cells were counted in the anterior commissure (AC) beginning at +2.28 mm anterior to Bregma and at every 120 μm for 3 consecutive sections, using a light microscope (OLYMPUS BX41, Japan) at 20× magnification. Images were analyzed using Image-Pro Plus (Media Cybernetics, Inc., Silver Spring, MD) software. Brain regions in the CC and AC were identified using three landmarks per area, and pictures taken from identical sites in all brains based on these landmarks. For each animal, 7 fields in the right hemisphere CC were captured per section (see Fig. 5), and three fields encompassing the entire AC were captured per section. The background intensity was set to the same level for all pictures and the number of stained cells was counted with Image-Pro Plus (Media Cybernetics, Inc., Silver Spring, MD).

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