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Age, but Not Sex, Modulates Foxp3 Expression in the Rat Brain across Development

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Abstract—The interconnectivity between brain development and the immune system has become an area of interest for many neuroscientists. However, to date, a limited number of known immune mediators of the peripheral nervous system (PNS) have been found to influence the development of the central nervous system (CNS). *FOXP3* is a well-established mediator of regulatory T-cells in the PNS. However, the expression pattern of *FOXP3* in the CNS and the PNS throughout development is unknown. To fill this void, we have characterized, in several brain regions, the developmental profile of *Foxp3* for both sexes using rats. We found different patterns of *Foxp3* in the CNS and PNS. In the CNS, we found *Foxp3* was ubiquitously expressed, with the levels of *Foxp3* varying by brain region. We also found both *Foxp3* mRNA and protein levels peak during embryonic development and then steadily decrease with a peak increase during adulthood. In adulthood, the protein but not mRNA increases to the equivalent levels found at the embryonic stage of life. In the PNS, Foxp3 protein levels were low embryonically and increased steadily over the life of the animal with maximal levels reached in adulthood. Patterns observed for both the PNS and CNS were similar in males and females across all developmental timepoints. Our novel findings have implications for understanding how the neural immune system impacts neurodevelopmental disorders such as autism and schizophrenia.

Key words: genes, brain development, sex differences.

INTRODUCTION

FOXP3 is a member of the Forkhead box gene family of transcription factors. There are four members of the *FOXP* subfamily, *FOXP1–4* and denoted with human forms are capitalized (FOXP3-protein), murine forms are in lowercase (Foxp3-protein) (Kaestner et al., 2000; Sin et al., 2015). Three of the *FOXP* genes, *FOXP1/2/4,* are abundantly expressed throughout the brain (Teramitsu et al., 2004; Bowers et al., 2013), whereas FOXP3 has been recognized as being expressed primarily in the immune system (Brunkow et al., 2001; Walecki et al., 2015). Numerous studies have focused on understanding the function of *FOXP1/2/4,* due to their well-established link to brain development, autism, and language impairment (Lai et al., 2001; Vernes et al., 2008; Girirajan et al., 2011; Bacon and Rappold, 2012; Bowers and Konopka, 2012; Rappold et al., 2017). In contrast, *FOXP3* has been regarded as an immune system regulator being expressed in the peripheral nervous system (PNS) with low expression in the brain (Takahashi et al., 2009; Mailer, 2018). It is also known that *FOXP3* is the only member of the FOXP subfamily that is located on a sex chromosome, specifically the X chromosome (Lu et al., 2017). Additionally, *FOXP3* impacts immune system functions by its key role in the development and function of regulatory T-cells (Treg) (Fontenot et al., 2003, 2005; Hori et al., 2003). In humans, *FOXP3* is present in two isoforms, one of which is encoded by a complete mRNA, and the other by a truncated variant that lacks exon 2 (Walker et al., 2003; Allan et al., 2005; Du et al., 2008). The expression levels of these two isoforms appears to be equivalent in Treg cells (Du et al., 2008). Previous findings show that the truncated variant of *FOXP3* lacks the DNA binding domain, which results in an autoimmune disorder in humans called IPEX, (immunodysregulation, polyendocrinopathy, enteropathy, X-linked disorder), and in mice this truncation results in scurfy (Deng et al., 2012). *FOXP3* has also been found to be expressed in fetal and adult human brains (Frattini et al., 2012), and in the adult hippocampus of mice (Yi et al., 2016), but no information is known about *Foxp3* in the rat brain.

We propose that investigating the role of *Foxp3* in the brain can provide insights into how mediators of the PNS immune are connected with and influence the development of the central nervous system (CNS). There already exists a strong connection for how

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Abbreviations: ASD, autism spectrum disorder; IPEX, immunodysregulation, polyendocrinopathy, enteropathy, X-linked disorder; PNS, peripheral nervous system; qRT-PCR, Quantitative real-time PCR; Treg, regulatory T-cells.

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dysregulations of the immune system contribute to brain development through the occurrence of disorders in the brain. For instance, irregular immune system functioning is often comorbid with neurodevelopmental disorders (e.g., schizophrenia and Rett syndrome) (Patterson, 2009; Zhao et al., 2017; Gottfried and Bambini-Junior, 2018; Benros and Mortensen, 2019), as well as neuropsychiatric disorders (e.g., depression and anxiety) (Khandaker et al., 2014; Rainville et al., 2018). Likewise, immune system disorders are over-represented in the population of individuals with autism or autism spectrum disorder (ASD) (Kohane et al., 2012; Bauman et al., 2013; Rossi et al., 2013; Brimberg et al., 2016).

In the present research, we considered several important characteristics such as age of animal, the specific brain region of expression, and sex. For instance, *FOXP1*, *FOXP2*, and *FOXP4* are known to be expressed together in neural tissue and play an important role in the initial stages of neurodevelopment (Bowers and Konopka, 2012; Bowers et al., 2014). Moreover, *Foxp1* and *Foxp2* mRNA and protein levels have been shown to vary across the lifespan (Dallaire et al., 2003; Campbell et al., 2009; Bowers et al., 2014). In addition, *FOXP1/2/4* are each known to be either expressed separately in a specific brain region or to be co-expressed in the same cell, which is also dependent on the specific region of the brain (Ferland et al., 2003; Takahashi et al., 2008; Campbell et al., 2009; Tam et al., 2011; Bowers et al., 2014; Fong et al., 2018). It has also been shown that mRNA and protein levels for Foxp1 and Foxp2 are expressed differently in males and females in some, but not all, regions of the brain (Bowers et al., 2013, 2014; Frohlich et al., 2017). These sex differences in the brain, for both Foxp1 and Foxp2, have been linked to sex differences in neonatal ultrasonic vocalizations (Bowers et al., 2013; Frohlich et al., 2017). Androgens have also been found to regulate both Foxp2 and Foxp1 protein levels differentially in males versus females (Bowers et al., 2014; Frohlich et al., 2017). These data strongly suggest age, brain region, and sex are relevant factors potentially mediating *Foxp1/2/4* expression in the brain. It is not known whether *Foxp3* shares these distinctive features in the brain like the other Foxp family members.

We aimed to measure the expression of *Foxp3* in the CNS and the PNS across development for both male and female rats. We quantified the mRNA and protein levels for Foxp3 assaying tissue from across the lifespan (i.e., from embryonic to adulthood), and from different brain regions from both males and females, as well as from the spleen. The results showed that in both the CNS and the PNS, expression of Foxp3 protein and mRNA varied across development. Furthermore, we found there were different patterns of expression of *Foxp3* in the CNS and the PNS. In the CNS, we found Foxp3 mRNA and protein levels in the brain are at peak levels during embryonic development and decreased across the lifespan until adulthood. At adulthood, there was an increase in Foxp3 to the equivalent levels found during embryonic development. Secondly, we found *Foxp3* did not have the same expression profile across the brain (i.e., Neocortex, striatum, hippocampus). Each brain region had a unique expression pattern across the lifespan. In tissue from the spleen, which we are using as a proxy for the PNS levels, we found that Foxp3 protein levels in the PNS were found to have low levels of protein during the embryonic stage and steadily increase over the lifespan into adulthood. Additionally, we found overall Foxp3 protein levels were higher in the spleen than in the brain. The low levels of Foxp3 in the brain may be a reason why Foxp3 has not been reported to be measured in the brain for a number of publications investigating the Foxp genes (Lu et al., 2002; Li et al., 2004; Mendoza et al., 2015; Sin et al., 2015; Mendoza and Scharff, 2017). For both the CNS and PNS, patterns of results were similar for males and females with no sex differences in *Foxp3*.

EXPERIMENTAL PROCEDURES

Brain and spleen tissue from animals were dissected at several timepoints. We collected tissue before birth (embryonic, E) and after birth (postnatal, PN) at the following ages: E19, PN0, PN7, PN14, PN21, PN28, PN48 and PN65 for quantitative real-time PCR (qRT-PCR) and Western blot analysis. Five different litters were used to obtain embryonic tissue. Twelve different litters were used to obtained postnatal brain tissue. All timepoints had a comparable number of males and females. The pups from each litter were randomly assigned to a specific timepoint in order to distribute pups from any one litter across all timepoints. The total number of animals used for the qRT-PCR = (timepoint #1n = 20 and timepoint #2n = 42) and Western blot = (timepoint #1n = 34, timepoint #2n = 52, timepoint #3n = 51). The breakdown of male and females per age are noted in the figure legends with each data result. Brain dissections were conducted for: neocortex, hippocampus, and striatum. Dissections were performed using a rat coronal section brain block that had 1.0 mm interval divisions (Stoelting). The individual brain regions were obtained from serial sections using coronal slices going from anterior to posterior regions throughout the entire brain. For the qRT-PCR analysis, the analysis was divided into Timepoint 1: E19, PN0, PN7 and Timepoint 2: PN0, PN7, PN14, PN21, PN28, PN48, PN65. Due to size limitations of the Western blot membrane, the timepoints across the lifespan were separated as follows: Timepoint 1: E19, PN0, PN7; Timepoint 2: PN0, PN7, PN14, PN21; and Timepoint 3: PN21, PN28, PN48, PN65. This division of qRT-PCR analysis into two separate time points and the Western blot into three timepoints was due to the high number of individual samples being processed. It was impossible for use to put all our samples in one PCR plate, or in one Western blot membrane. We also choose to overlap one to two timepoints for qRT-PCR and Western blot in order to serve as an internal comparison. The internal comparisons served as a proxy for us to qualitatively determine any runtime changes between qRT-PCR plates or Western blot membrane processing. Spleen tissue was used as a

proxy for the peripheral immune tissue levels and was collected at the same timepoints and from the same animals we obtained the brain tissue.

Animals

Long Evans rats were mated and raised in our vivarium under a 12-h dark and 12-h reverse light cycle. Females were allowed to deliver naturally. The day of birth was designated as PN0. All animals were housed in polycarbonate cages (20 × 40 × 20 cm) in corncob bedding with water and food *ad libitum*. Virginia Tech Institutional Animal Care and Use Committee approved all animal procedures.

qRT-PCR

RNA was prepared from frozen rat brain tissue samples using Qiazol and purified on RNeasy columns (Qiagen) with DNase digestion. Using 1 μg of tissue, RNA was transcribed to cDNA using the Transcriptor first-strand cDNA Synthesis Kit (Roche Applied Science) using both anchored-dT and random primers. A quantity of 1.2–3.0 mg was used in a reaction to synthesize cDND. qRT-PCR was performed on a QuantStudio-6 Flex (Applied Biosystems) with Quant Studio Real-Time PCR software. qRT-PCR was performed with an annealing temperature of 60 °C, using Power SYBR Green master mix (Applied Biosystems). Specific primers for the gene *Foxp3* and the control normalizing genes *Rpl13a* and *Tata Binding Protein*, (*TBP*) were designed using Primer Express 3.0. Primers for *Foxp3* gene followed the sequence: forward, 5′-TTTATGCATCAGCTCTCCACTG TAG-3′, and reverse, 5′-GAGAAGACTCCAGTGGCAG CAGTA-3′. For sexing of embryonic tissue, we used primers targeting the *Sry* gene 5′-GCGCCCCATGAATG CAT-3′ and reverse, 5′-TGGGATTCTGTTGAGC CAACT-3′. The primers specific for *Sry* were obtained from a previously published manuscript (Turner et al., 2007) and used by us in a previous publication (Bowers et al., 2013). Primers for the first housekeeping gene *Rpl13a* gene followed the sequence: forward, 5′-AGG CAAAGATCCATTACCGG-3′, and reverse, 5′-GGCA CAAACAGTCTTTATTGGG-3′. Primers for the second housekeeping gene *TBP* gene followed the sequence: forward, 5′-ACCGAAGAAAGTGAGAGTCATGGA-3′, and reverse, 5′-CGTAAGGCATCATTGGACTGAAGA-3′. Data were analyzed using the standard curve method. This method is used as a standard or calibration curve for extrapolating relative expression level information for the gene of interest in unknown experimental samples. The relative quantification calibration curve results for the gene of interest is normalized to that of a control normalizing gene in the same samples, and then the normalized numbers are compared between samples to get a fold change in expression. We compared the two normalizing genes and found they were not statistically different from each other at any timepoint. We cross-compared the two normalizing genes to further confirm the expression pattern of *Foxp3*, but only report one, *Rpl13A,* in order to be succinct.

Western blot

Animals were euthanized either by rapid decapitation (< PN4) or using deep anesthesia (> PN5) to dissect and process brain tissue as previously reported (Bowers et al., 2014). Twenty-five micrograms of protein were electrophoresed in separate lanes using 4–20% Tris-Glycine polyacrylamide gels (Invitrogen Novex) and transferred to a low fluorescence PVDF membrane (Bio-Rad). Membranes were blocked in Odyssey blocking buffer (LI-COR) for 1-h and then incubated with the monoclonal anti-mouse Foxp3 antibody (1:1000; Santa Cruz) overnight at 4 °C. This Foxp3 primary antibody has been used in previous published work (Fu et al., 2019; Shu et al., 2019). After 1-h of incubation with the corresponding fluorescent secondary, antibody anti-mouse (IRDye 680 RD, LI-COR (1: 15,000; LI-COR), the immunoreactive bands were detected and digitalized using the Odyssey Fc Imaging System (LI-COR). Total protein stain was used for normalization of the Western blots. The Foxp3 protein was detected as a band with a relative molecular weight of ~49 kDa. For relative quantification purposes, the band for the protein of interest was divided by the total protein and is expressed as "Normalized Optical Intensity" in arbitrary units.

Statistical analysis

All data are expressed as mean ± SEM and effect size estimate calculations (η^2 and r^2) are reported. All datasets were first tested for normality using Levene's test for homogeneity of distribution (Levene, 1960). Homogeneity analysis found our distributions to be normally distributed. Thus, we analyzed our data using a parametric two-way ANOVA with age and sex as main factors, after we determined that litter was not a significant contributing factor by using litter as an additional covariate factor of analysis. All statistical analyses were followed by a *post hoc* pairwise comparison test. To control for familywise error for multiple pairwise comparisons, we used the Holm's sequential Bonferroni correction. Any pairwise comparisons not following this criterion were considered statistically non-significant. All statistical tests were computed in SPSS 26 and graphed using GraphPad Prism 8.2.1.

RESULTS

Foxp3 mRNA analysis by qRT-PCR

Whole brains were collected on E19, and for the postnatal timepoints we separated into the neocortex, striatum, hippocampus. Because the embryonic brain is not well differentiated at E19, we compared only these three brain regions.

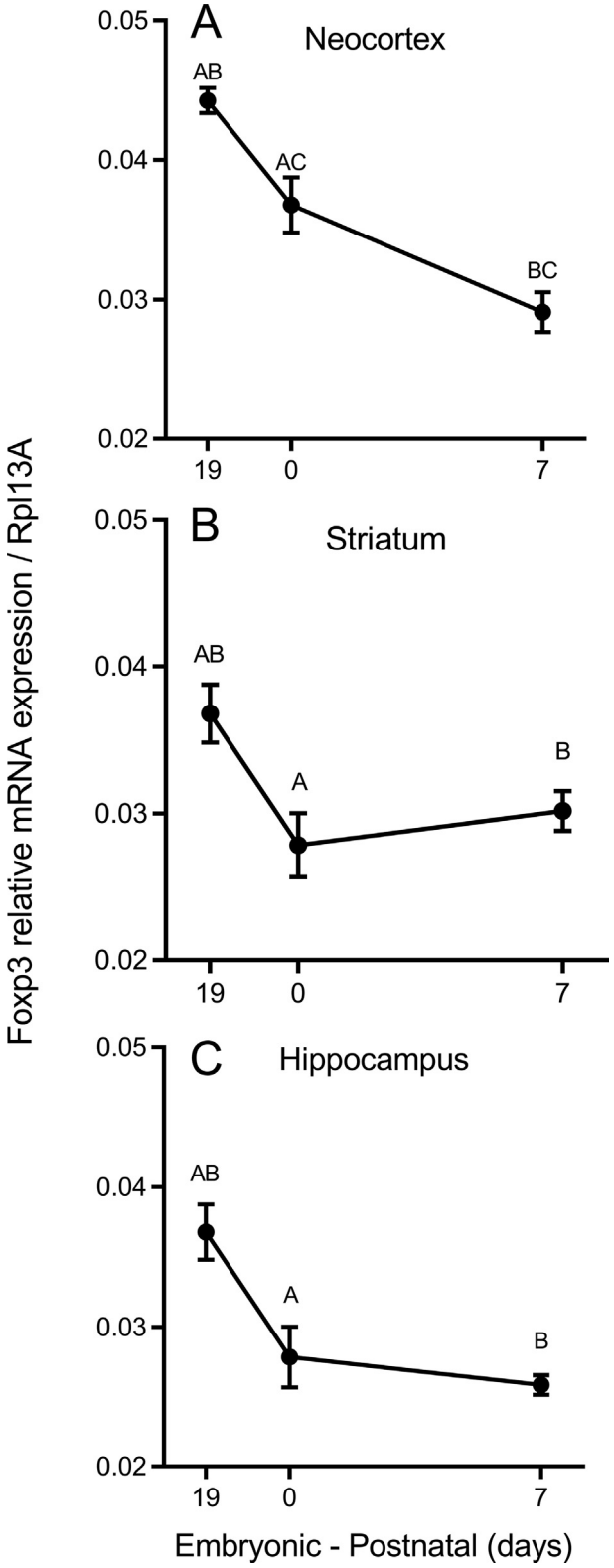
Timepoint 1: mRNA expression during embryonic and early postnatal points

Neocortex. We found a significant main effect for age, $F(2, 17) = 19.071$, $p < 0.000$, $\eta^2 = 0.692$. However, the main effect for sex was not statistically significant, $F < 1.0$. Pairwise comparisons found *Foxp3* mRNA

levels were higher at E19 when compared to PN0 and PN7, t (12) = 3.059, p = 0.010, r^2 = 0.66192 and t (12) = 8.912, p < 0.000, r^2 = 0.93206, respectively (Fig. 1A). Additionally, PN0 was found to have significantly higher levels of *Foxp3* mRNA than PN7, t (10) = 2.935, p = 0.012, r^2 = 0.68028.

Striatum. Levels of *Foxp3* mRNA showed a significant main effect for age, F (2, 17) = 6.229, p = 0.009, η^2 = 0.423, but not sex, F < 1.0. Pairwise comparisons found *Foxp3* mRNA levels were higher at E19 when compared to both PN0 and PN7, t (12) = 3.008, p = 0.011, r^2 = 0.65565 and t (12) = 2.554, p = 0.025, r^2 = 0.59343, respectively (Fig. 1B). PN0 and PN7 were not statistically different from each other, t (10) = 0.909, p = 0.385.

Hippocampus. Levels of *Foxp3* mRNA showed significant differences of age, F (2, 17) = 10.925, p = 0.001, η^2 = 0.562, but not sex, F < 1.0. Pairwise comparisons found *Foxp3* mRNA levels were higher at E19 when compared to both PN0 and PN7, t (12) = 3.010, p = 0.011, r^2 = 0.6559 and t (12) = 4.570, p = 0.001, r^2 = 0.79693, respectively (Fig. 1C). PN0 and PN7 were not statistically different from each other, t (10) = 0.872, p = 0.403.



Period 2: Postnatal timeline from P0 to P65

Neocortex. The mRNA levels of *Foxp3* were statistically significant for the main effect of age, F (6, 33) = 7.534, p < 0.000, η^2 = 0.578. However, there was no significant main effect for sex, F < 1.0. Pairwise comparisons found *Foxp3* mRNA was significantly higher at PN0 as compared to PN7 and PN14, t (10) = 5.160, p < 0.000, r^2 = 0.85262 and t (10) = 4.194, p = 0.002, r^2 = 0.79846, respectively (Fig. 2A). After correcting for multiple pairwise comparisons, there were no additional significant differences.

Striatum. There was no significant main effect for age, F (6,33) < 1.0, or for sex found, F < 1.0 (Fig. 2B).

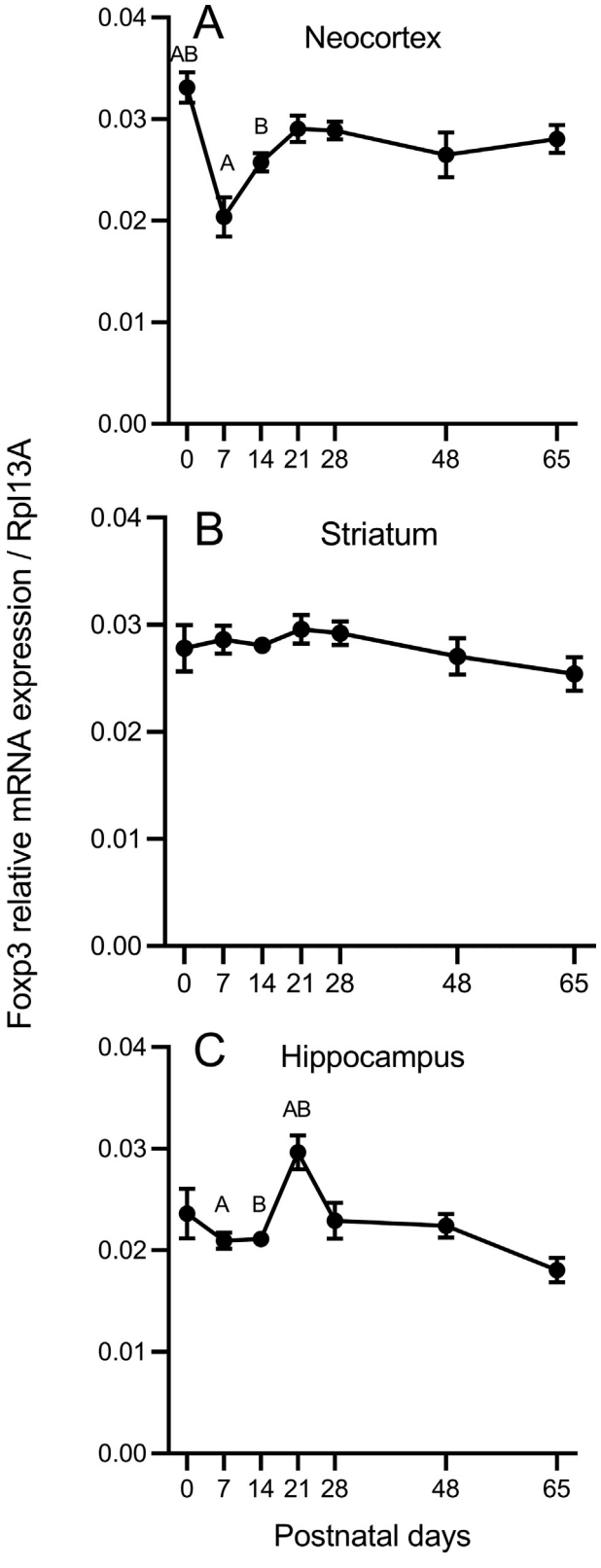
Hippocampus. There was a significant main effect for age, F (6, 33) = 5.661, p < 0.000, η^2 = 0.507, but no main effect for sex was found, F < 1.0. Pairwise comparisons found higher *Foxp3* expression at PN21 when compared to PN7 and PN14, t (10) = 4.641, p = 0.001, r^2 = 0.8264 and t (10) = 4.853, p = 0.001, r^2 = 0.83783 respectively (Fig. 2C).

Foxp3 protein measurements by Western blot

We collected brain tissue from the neocortex, striatum, and hippocampus at the same time points as reported

Fig. 1. (A–C) Relative expression of *Foxp3* mRNA in the rat brain. Timepoint 1 (E19: ♀ = 4, ♂ = 4; PN0: ♀ = 3, ♂ = 3; PN7: ♀ = 3, ♂ = 3) late embryonic and early postnatal development in the neocortex **(A)**, striatum **(B)**, and hippocampus **(C)**. All data are expressed as mean (± SEM). Groups with the same alphabetic script are significantly different from each other. A group with more than two alphabetic scripts indicates that the individual group is significantly different from more than one group, e.g. ‘‘AB” listing over one group would denote that the group is different from any group with an ‘‘A” and is also significantly different from any group with a ‘‘B”.

for qRT-PCR (i.e., E19, PN0, PN7, PN14, PN21, PN28, PN48, PN65). To test the efficacy of the primary antibody, we compared the molecular weight from a tissue sample known to have high levels of Foxp3 protein, the spleen, to the three brain regions we quantified (Eufrasio de Figueiredo et al., 2019). The observed molecular weight in the spleen was identical to the protein levels from the brain, (Fig. 3A). We also noted that the molecular weight for Foxp3 did not change in the brain across the lifespan, (Fig. 3B).



Timepoint 1. Embryonic and early postnatal period

Neocortex: The analysis showed a significant main effect of age for Foxp3, F (2, 27) = 17.807, $p < 0.001$, $\eta^2 = 0.569$, but not sex, $F < 1.0$. In the neocortex, basal levels of Foxp3 protein were higher at E19 when compared to PN0, t (18) = 3.052, $p = 0.007$, $r^2 = 0.58396$ and PN7, t (18) = 6.804, $p < 0.001$, $r^2 = 0.84855$. Moreover, the protein levels were significantly higher when comparing PN0 to PN7, t (24) = 3.984, $p = 0.001$, $r^2 = 0.63093$, (Fig. 4A).

Striatum: Foxp3 protein levels were statistically significant for the main effect of age, F (2, 29) = 8.625, $p = 0.001$, $\eta^2 = 0.373$, but not sex, $F < 1.0$. Pairwise comparisons found Foxp3 protein levels where higher at E19 than at PN0, t (19) = 4.026, $p = 0.001$, $r^2 = 0.6785$. Likewise, Foxp3 protein was higher at E19 than at PN7, t (16) = 2.346, $p = 0.032$, $r^2 = 0.50591$ (Fig. 4B). However, after correcting for multiple pairwise comparisons, there were no statistically significant differences in protein levels between PN0 and PN7, t (23) = −2.086, $p = 0.048$.

Hippocampus: Foxp3 protein levels were statistically significant for the main effect of age, F (2, 28) = 13.897, $p < 0.000$, $\eta^2 = 0.498$, but not the main effect of sex, $F < 1.0$. Pairwise comparisons found significant higher levels of *Foxp3* protein levels at E19 when comparing this age to PN0 and PN7, t (16) = 2.864, $p = 0.011$, $r^2 = 0.58216$ and, t (18) = 6.730, $p < 0.000$, $r^2 = 0.84594$, respectively. Foxp3 protein levels were not statistically different when comparing PN0 to PN7, t (22) = 2.043, $p = 0.053$, (Fig. 4C).

Timepoint 2. First postnatal weeks of development

Four timepoints consisting of day of birth and weaning were assayed for this timepoint, (i.e., PN0, PN7, PN14, PN21). For the neocortex, there was a significant main effect of age for Foxp3 protein levels, F (3, 44) = 4.032, $p = 0.013$, $\eta^2 = 0.216$, but not for the main effect of sex, $F < 1.0$. Pairwise comparisons found significant higher levels of *Foxp3* protein levels at PN0 when

Fig. 2. (A–C) Relative expression of *Foxp3* mRNA from birth to adulthood. Timepoint 2 (PN0: ♀ = ♀ = 3, ♂ = 3; PN7: ♀ = 3, ♂ = 3; PN14: ♀ = 3, ♂ = 3; PN21: ♀ = 3, ♂ = 3; PN28: ♀ = 3, ♂ = 3; PN48: ♀ = 3, ♂ = 3; PN65: ♀ = 3, ♂ = 3), early postnatal development to adulthood: neocortex **(A)**, striatum **(B)**, and hippocampus **(C)**. All data are expressed as mean (±SEM). Groups with the same symbol are significantly different from each other. A group with more than two alphabetic scripts indicates that the individual group is significantly different from more than one group, e.g. ‘‘AB” listing over one group would denote that the group is different from any group with an ‘‘A” and is also significantly different from any group with a ‘‘B”.

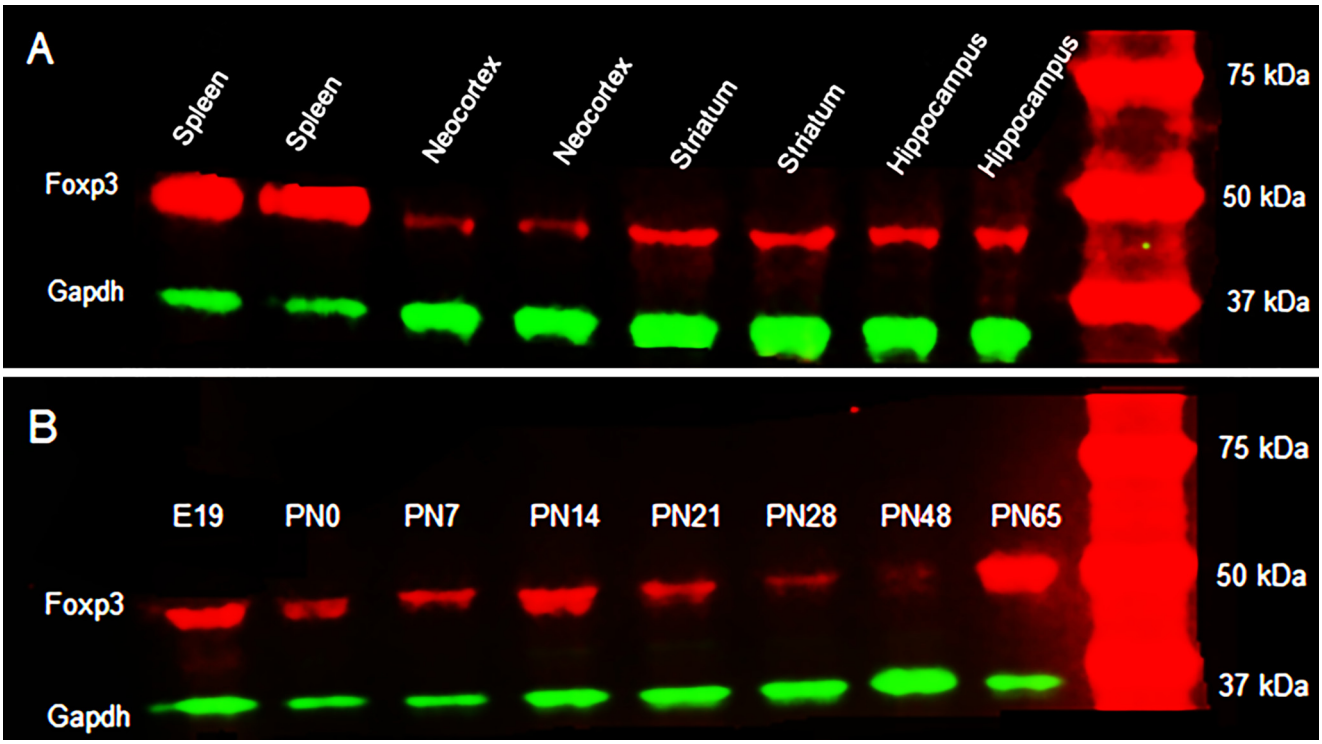


Fig. 3. (A, B) Foxp3 identification and protein assessment in the rat brain. **(A)** Detection of Foxp3 (~49 kDa) by western blot in the spleen, neocortex, striatum, and hippocampus. **(B)** A representation of Foxp3 developmental profile from embryonic stages to adulthood.

compared to PN7, t (24) = 5.862, $p < 0.000$, $r^2 = 0.76732$ and also when comparing PN0 to PN14, t (24) = 2.531, $p = 0.018$, $r^2 = 0.459$. No other pairwise comparisons were significant, (Fig. 5A).

Striatum. The analysis did not show any significant differences in Foxp3 protein levels for the main effect of age or sex during this period from PN0-PN21, $Fs < 1.0$, (Fig. 5B).

Hippocampus. A significant main effect for age was found, F (3, 44) = 4.545, $p = 0.007$, $\eta^2 = 0.237$, but not for the main effect of sex, $F < 1.0$. Pairwise comparisons found no differences between PN0 and PN7, t (20) = 0.414, $p = 0.683$. However, significant increases in Foxp3 protein were found when comparing PN0 to PN14, t (20) = −2.519, $p = 0.020$, $r^2 = 0.49077$, and PN0 to PN21, t (20) = −2.451, $p = 0.024$, $r^2 = 0.48061$, (Fig. 5C).

Timepoint 3. Juvenile and adulthood stages

Four ages were assayed for this timepoint, (i.e., PN21, PN28, PN48, PN65). For the neocortex, there was a significant main effect of age, F (3, 43) = 10.595, $p < 0.000$, $\eta^2 = 0.419$, but no significant main effect or sex was found, $F < 1.0$. Pairwise comparisons found significant decreases in protein levels when comparing PN21 to PN28, t (25) = 3.828, $p = 0.001$, $r^2 = 0.6079$ and PN21 to PN48, t (25) = 4.103, $p < 0.000$, $r^2 = 0.63436$, but not between PN21 and PN65, t (22) = 0.012, $p = 0.991$. A significant increase in Foxp3 protein was found comparing PN28 to PN65, t (23) = −3.007, $p = 0.006$, $r^2 = 0.53122$, and PN48 to PN65, t (23) = −3.218, $p = 0.004$, $r^2 = 0.55719$ (Fig. 6A).

Striatum. A main effect for age was found, F (3, 41) = 10.078, $p < 0.000$, $\eta^2 = 0.424$, but no main effect for sex was detected during this period $F < 1.0$. Pairwise analyses found a significant decrease in Foxp3 protein levels at PN28 when compared with PN48, t (18) = 5.480, $p < 0.000$, $r^2 = 0.79072$, but no significant differences at any other timepoints were found (Fig. 6B).

Hippocampus. A significant main effect of age for was found, F (3, 44) = 10.595, $p < 0.000$, $\eta^2 = 0.419$, but not for the main effect of sex, $F < 1.0$. Pairwise comparisons found Foxp3 protein levels decreased when comparing PN21 to PN28 and PN48, t (25) = 3.316, $p = 0.003$, $r^2 = 0.5527$ and t (25) = 3.633, $p = 0.001$, $r^2 = 0.58782$, respectively. In contrast, Foxp3 protein levels were found to increase when comparing PN28 and PN48 to PN65, t (23) = −3.621, $p = 0.001$, $r^2 = 0.60257$, and t (23) = −4.021, $p = 0.001$, $r^2 = 0.64249$, respectively. Protein levels were not significantly different comparing PN21 to PN65, t (23) < 0.300, p > 0.05, (Fig. 6C).

Foxp3 across the lifespan in the PNS

We analyzed Foxp3 protein levels in the spleen across the lifespan as an estimate for the protein levels of Foxp3 circulating in the PNS. We found a significant main effect of age, but not sex, F (7, 23) = 12.564, $p < 0.000$, $\eta^2 = 0.793$ and ($F < 1.0$, $p > 0.05$) respectively. Foxp3 protein levels significantly increased at each timepoint when comparing E19 to PN0,

t (6) = −2.625, p = 0.03, r^2 = 0.73113, E19 to PN7, t (6) = −6.075, p = 0.001, r^2 = 0.92745, E19 to PN14, t (6) = −4.849, p = 0.003, r^2 = 0.89258, E19 to PN21, t (6) = −4.125, p = 0.006, r^2 = 0.85983, E19 to PN28, t (6) = 2.482, p = 0.049, r^2 = 0.71175, and E19 to PN65, t (6) = −5.550, p = 0.003, r^2 = 0.91486. When correcting for multiple pairwise comparisons, there were no statistically significant findings for E19 to PN48, t (6) = −0.0.136, p = 0.896, (Fig. 7).

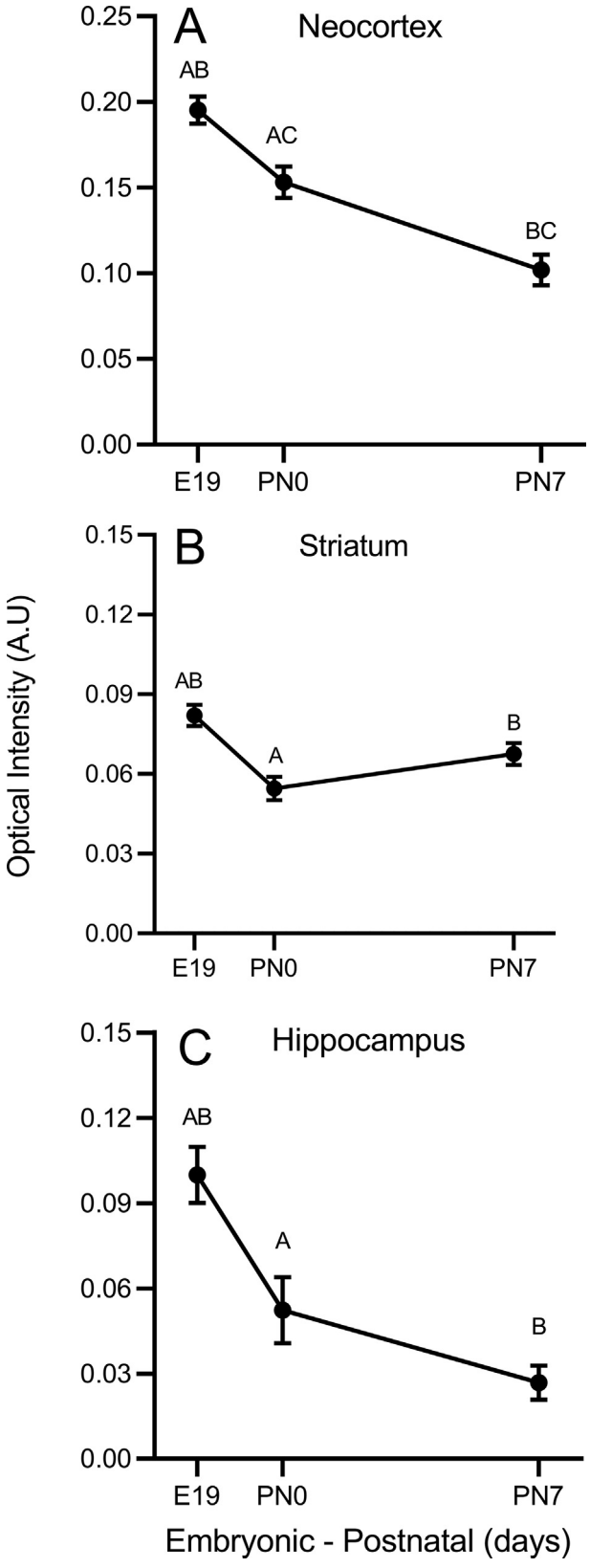


Fig. 4. (A–C) Foxp3 protein quantification around the perinatal period. (E19: ♀ = 4, ♂ = 4; PN0: ♀ = 7, ♂ = 6; PN7: ♀ = 7, ♂ = 6) in the neocortex **(A)**, striatum **(B)**, and hippocampus **(C)**. All data are expressed as mean (± SEM). Groups with the same symbol are significantly different from each other. A group with more than two alphabetic scripts indicates that the individual group is significantly different from more than one group, e.g. "AB" listing over one group would denote that the group is different from any group with an "A" and is also significantly different from any group with a "B".

Comparing embryonic and adulthood timepoints for Foxp3 in brain and spleen tissue

In order to directly compare embryonic and adult tissue, from the periphery (i.e., spleen) and the brain, we quantified both the mRNA and protein level for Foxp3. For the mRNA, there was a significant interaction between tissue type (i.e., brain vs. spleen) and age (i.e., E19 vs. PN65), F (1, 8) = 45.046, p < 0.000, η^2 = 0.849. Pairwise comparisons for the E19 timepoints showed that there were significantly higher levels of *Foxp3* mRNA in the brain than in the spleen, t (4) = 9.311, p = 0.001, r^2 = 0.9777. In contrast, there was no significant difference in Foxp3 mRNA levels comparing E19 fetal brain to PN65 adult brain, t (4) = 1.195, p = 0.298. However, comparing E19 spleens to PN65 spleens, there was a significant increase in Foxp3 mRNA levels, t (4) = 6.848, p = 0.002, r^2 = 0.9599. Lastly, Foxp3 mRNA levels were significantly higher in the PN65 spleen as compared to the PN65 brain, t (4) = 6.171, p = 0.004, r^2 = 0.95129, (Fig. 8A).

For the protein, there was a significant interaction between tissue type (i.e., brain vs. spleen) and age (i.e., E19 vs. PN65), F (1, 13) = 23.667, p < 0.000, η^2 = 0.645. Pairwise comparisons for the E19 timepoints showed that there were significantly higher levels of Foxp3 protein in the spleen than in the brain, t (7) = 5.792, p = 0.001, r^2 = 0.90959. In contrast, there was no significant difference in Foxp3 protein levels comparing E19 brain and PN65 brain, t (8) = −1.706, p = 0.126. However, comparing E19 spleens to PN65 spleens, there was a significant increase in Foxp3 protein levels, t (6) = −4.906, p = 0.004, r^2 = 0.89468. Lastly, Foxp3 protein levels were significantly higher in the PN65 spleen as compared to the PN65 brain, t (7) = 6.340, p = 0.001, r^2 = 0.92287, (Fig. 8B).

DISCUSSION

The ability for the immune system to influence the peripheral and central nervous system is an area of special interest in understanding neurodevelopmental

disorders. This is because of the increasing information for how the immune system of the brain shapes neural development. A perfect example is the neurodevelopmental disorder, ASD. Individuals with ASD are reliably diagnosed by 24 months of age (Lord et al., 2006). This is a period of extensive neuronal development, including dendritic and synaptic spine growth and refinement (Rakic et al., 1986; Greenough et al., 1987; McGee et al., 2014). As dendrites are the main structures that receive information in neurons, the complexity of dendrites and synaptic spine numbers impact neuronal connectivity and communication (Vanleeuwen and Penzes, 2012; Raven et al., 2018). Development, refinement, and maintenance of the neuronal dendritic arbors are therefore crucial to normal brain function. The reported function of *FOXP 1/2/4* is to shape neural development (Bowers and Konopka, 2012). Thus, *FOXP 1/2/4* have been the focus of intense neural research, whereas the fourth member of the FOXP family, *FOXP3*, has not received this same attention. What is known about *FOXP3* suggests it is a regulator of the peripheral immune system (Haque et al., 2011; Ohkura et al., 2013). We are aware of one publication that has investigated *FOXP3* expression in the human brain (Frattini et al., 2012). What is known suggests that during human fetal development, *FOXP3* expression is found in the periventricular zone and cortical areas (Frattini et al., 2012). In the adult human brain, *FOXP3* expression has been detected throughout the brain (Frattini et al., 2012). In mice, *Foxp3* was found in the adult hippocampus (Yi et al., 2016).

A gap in our understanding of *FOXP3* is why has this transcription factor not been studied in the brain, as has been the other members of the FOXP family? We speculated this was due to the low expression levels of *FOXP3* in the brain, relative to the other FOXP members. Subsequently, we know nothing about expression profile of *FOXP3* across the lifespan in either the periphery or brain. Thus, to better understand the expression profile across the lifespan, we assayed several areas of the rat brain (i.e., neocortex, striatum, and hippocampus) and the spleen at various time points ranging from embryonic development to adulthood (i.e., E19, PN0, PN7, PN14, PN21, PN28, PN48 and PN65).

The qRT-PCR and Western blot analyses found an overall trend for *Foxp3* to have its highest expression in the brain for both mRNA and protein at E19 and the decrease throughout timepoint 1 (i.e., the first postnatal week) in all brain regions. This first week in the development of the rat is analogous to the third trimester of the developing human fetus (Patten et al., 2014; Zamudio-Bulcock et al., 2014). In rats, E1–E10 is the equivalent to the first trimester in humans with E10–E20 (just prior to birth in the rat) being equivalent to the

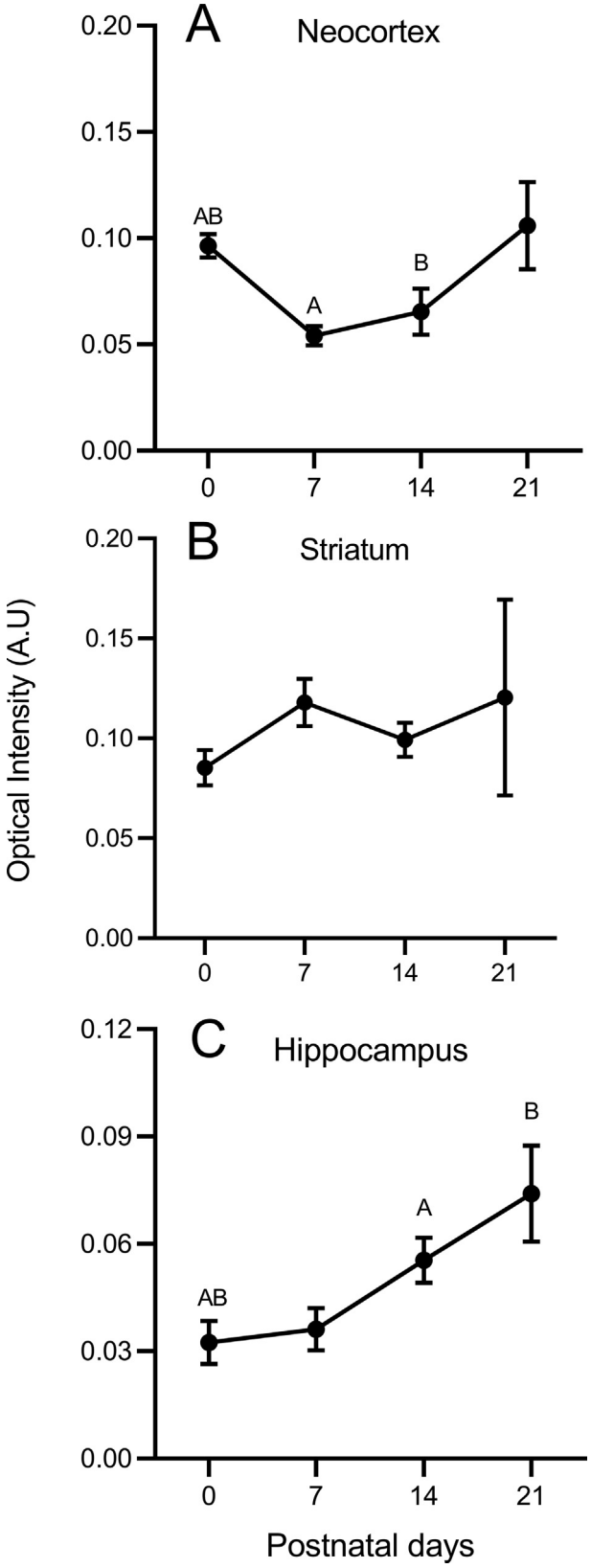
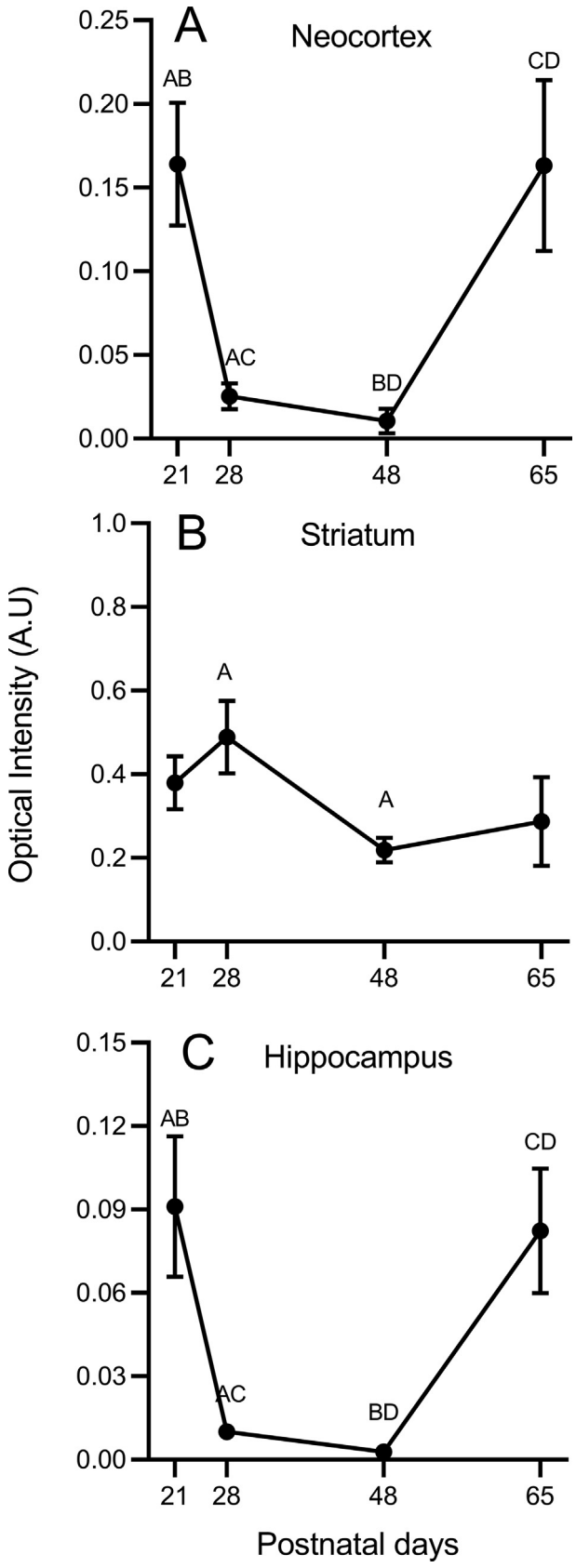


Fig. 5. (A–C) Foxp3 protein assessment in the postnatally developing rat brain. Developmental profile of Foxp3 during the first three postnatal weeks (PN0: ♀ = 7, ♂ = 6; PN7: ♀ = 7, ♂ = 6; PN14: ♀ = 7, ♂ = 6; PN21: ♀ = 7, ♂ = 6) in the neocortex **(A)**, striatum **(B)**, and hippocampus. All data are expressed as mean (±SEM). Groups with the same symbol are significantly different from each other. A group with more than two alphabetic scripts indicates that the individual group is significantly different from more than one group, e.g. "AB" listing over one group would denote that the group is different from any group with an "A" and is also significantly different from any group with a "B".



second trimester and PN1- PN10 in the rat being equivalent to the third trimester (West, 1987; Patten et al., 2014). Our results for high expression of *Foxp3* during embryonic development support the findings reported for human fetal expression of *FOXP3* (Frattini et al., 2012). This suggests *Foxp3* may play an important role as a contributor to the development of the brain, especially since the first postnatal week is a critical period for forming neural connections (Bock et al., 2008).

During timepoint 2 (i.e., PN14–21), there is a different pattern of *Foxp3* expression, which is brain region specific. For the striatum, but not the neocortex or hippocampus, both mRNA and protein remain unchanged during this period. Furthermore, these levels were decreased compared to timepoint 1. However, the mRNA and protein levels for the hippocampus increased from timepoint 1, whereas in the neocortex they decreased during this timepoint. This divergent pattern is occurring during a critical transition phase in the animal (Bock et al., 2008). This timepoint (i.e., weaning) is one of the most demanding transitions for the animal behaviorally and cognitively because the animal is less reliant on mother's milk and moving towards solid food (Bateson and Feaver, 1990; Cook, 1999; Bock et al., 2008). Weaning in rats is not caused by a deficit in the dam's ability to produce sufficient milk relative to the nutritional requirements of the developing litter, but is rather induced by changes of the behavioral interactions between the weanlings and the dam (Alberts, 2005; Bock et al., 2008). Subsequently, weanling pups become nutritionally and behaviorally independent from their mother and show marked behavioral transformations (Cramer et al., 1990; Thiels et al., 1990). Therefore, it is not shocking the developmental impact of weaning has such a large influence on the maturation of the brain and behavior (Smith, 1991; Bateson and Kacelnik, 1997; Nakamura et al., 2003; Bock et al., 2008). Thus, we speculate that maintaining a relatively brain region independent expression of Foxp3 may allow the neuroimmune system to remain plastic and responsive for the refinement of the nervous system.

For timepoint 3 (i.e., PN28–PN65), the mRNA for all three brain regions remained relatively stable in their expression. However, the protein levels for Foxp3 in all three brain regions assessed showed a decreasing trend between PN28 and PN48. In contrast, from PN48 to PN65 there was a significant increase in Foxp3

Fig. 6. (A–C) Foxp3 protein measurement in the juvenile and adult rat brain. Developmental profile of Foxp3 after the first three postnatal weeks (PN21: ♀ = 7, ♂ = 6; PN28: ♀ = 7, ♂ = 7; PN48: ♀ = 7, ♂ = 6; PN65: ♀ = 6, ♂ = 5) in the neocortex **(A)**, striatum **(B)**, and hippocampus **(C)**. All data are expressed as mean (±SEM). Groups with the same symbol are significantly different from each other. A group with more than two alphabetic scripts indicates that the individual group is significantly different from more than one group, e.g. ''AB" listing over one group would denote that the group is different from any group with an ''A" and is also significantly different from any group with a ''B".

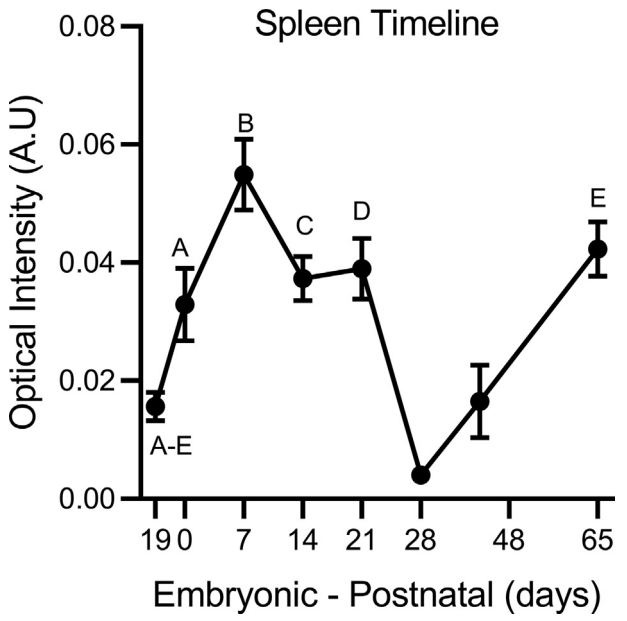


Fig. 7. Foxp3 in the spleen increases over the lifespan from embryonic development into adulthood. Protein values of Foxp3 in the rat spleen across the lifespan (E19: ♀ = 2, ♂ = 2, PN0: ♀ = 2, ♂ = 2; PN7: ♀ = 2, ♂ = 2; PN14: ♀ = 2, ♂ = 2; PN21: ♀ = 2, ♂ = 2; PN28: ♀ = 2, ♂ = 2; PN48: ♀ = 2, ♂ = 2; PN65: ♀ = 2, ♂ = 2). All data are expressed as mean (±SEM). Groups with the same symbol are significantly different from each other. A group with more than two alphabetic scripts indicates that the individual group is significantly different from more than one group, e.g. "AB" listing over one group would denote that the group is different from any group with an "A" and is also significantly different from any group with a "B".

protein for each brain region, except for the striatum. The distinctive pattern between the mRNA and protein is occurring at another critical transition phase in the life of the animal. At this stage, the animal is transitioning from adolescence (i.e., PN28–PN48) into young, adulthood (Evans, 1986; Sengupta, 2013). Pubertal changes (i.e., ~PN48) have been shown to impact myelination in several areas of the neocortex and contribute to the development of cognitive behaviors in humans and rats (Juraska and Willing, 2017; Darling and Daniel, 2019). Thus, we speculate that Foxp3 may be working to help refine plasticity in the brain during the maturation from adolescence to adulthood. Overall, we propose that the unique expression of Foxp3 across development to be suggestive of this gene's role in contributing to the maturation of the brain across the lifespan. Future research is required to test this idea.

In contrast, we found the spleen, which is rich in Foxp3, showed a different pattern to the one observed in the brain. Foxp3 protein in the spleen showed a steady increase from E19 to PN7, whereas in the brain Foxp3 protein levels were decreasing. Moreover, from PN21 to PN48, Foxp3 protein levels were decreasing in the spleen. This pattern is analogous to the observed trend for the protein levels in the brain. From PN48 to PN65, Foxp3 protein levels steadily increased in the spleen. This is an analogous trend observed in the data from brain tissue. Overall, the early developmental pattern of Foxp3 (i.e., E19–PN14) in the spleen (i.e., periphery) is opposite to what was found in the brain. Therefore, we hypothesize Foxp3 might be playing a dual role in mediating the immune system with one role

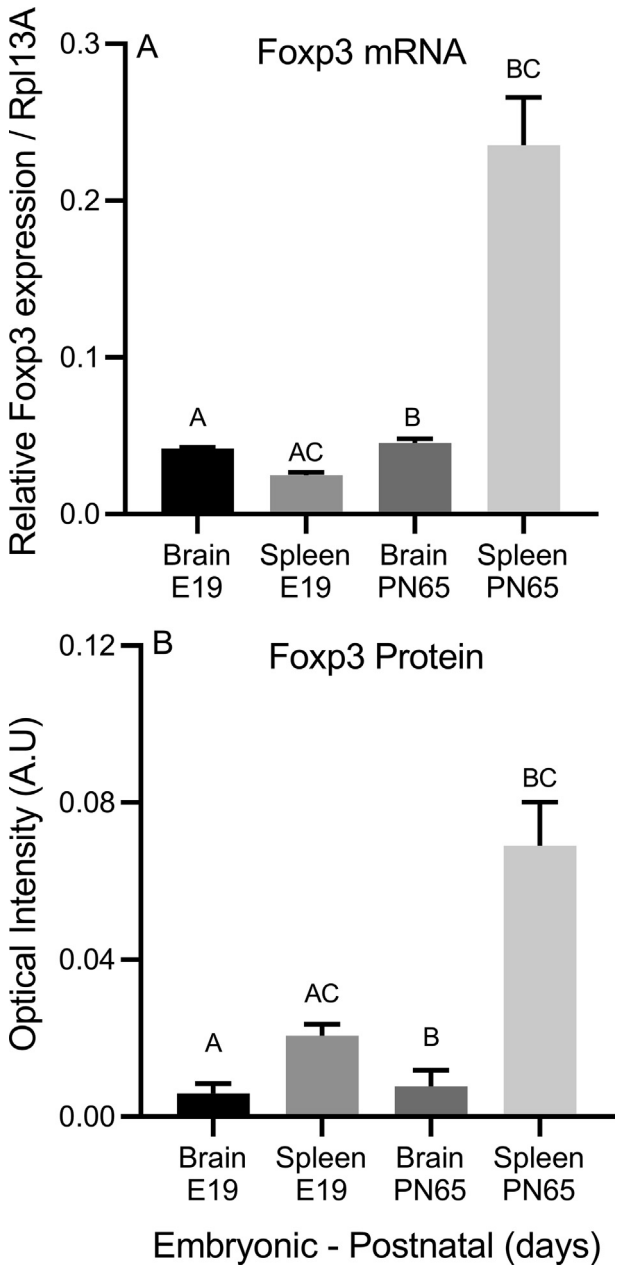


Fig. 8. (A, B) Foxp3 in the spleen increases over the lifespan from embryonic development into adulthood. **(A)** *Foxp3* mRNA in the brain and spleen at embryonic and PN65. For mRNA: (E19-spleen, $n = 3$, E19-brain, $n = 3$, PN65-spleen, $n = 3$, PN65-brain, $n = 3$). **(B)** Foxp3 protein levels of in the brain and spleen at embryonic and PN65. For protein: (E19-spleen, $n = 4$, E19-brain, $n = 5$, PN65-spleen, $n = 4$, PN65-brain, $n = 5$). All data are expressed as mean (±SEM). Groups with the same symbol are significantly different from each other. A group with more than two alphabetic scripts indicates that the individual group is significantly different from more than one group, e.g. "AB" listing over one group would denote that the group is different from any group with an "A" and is also significantly different from any group with a "B".

in the periphery and one in the brain during early development. More research is required to test this hypothesized function.

With respect to sex differences, the same pattern was found for males and females at all timepoints across development. This pattern for Foxp3 diverges from what has been reported for Foxp2 and Foxp1. Both Foxp1 and Foxp2 have been shown to have a sex difference in protein levels during early brain development in both rodents as well as the avians (Teramitsu et al., 2004; Bowers et al., 2013, 2014; Frohlich et al., 2017). The lack of sex differences in Foxp3 suggests that this gene functions similarly in both sexes. We hypothesize that this could be reflective of the fact that Foxp3 is located on the X chromosome, whereas no other member of the Foxp family is located on a sex chromosome (Lu et al., 2017). Moreover, our results are representative of the basal state of the organism. Thus, we would argue that atypical functioning of FOXP3 will negatively impact males more than females, as noted in IPEX (Deng et al., 2012). More research investigating the influence atypical functioning of Foxp3 has on male and females would be beneficial in identifying the etiology for the sex differences in neurodevelopmental disorders.

Our data are the first report of expression for Foxp3 across brain development. In comparison to the other Foxp family, Foxp3 expression is unique. Overall, Foxp1/2/4 have similar expression patterns in the brain that are brain region specific. The noted differences are observed in both humans and rodents where Foxp2 has been shown to have high expression during early embryonic brain development with decreasing expression across the lifespan (Ferland et al., 2003; Campbell et al., 2009; Li et al., 2018). In contrast, Foxp1 in both rodents and humans has been shown have a relatively stable expression across development (Ferland et al., 2003; Li et al., 2018), whereas Foxp4 expression is expressed similarly to Foxp2, but with marked reductions later in life (Takahashi et al., 2008; Li et al., 2018).

As a Foxp subfamily member, it implies *Foxp3* has the potential to dimerize with the other Foxp members and form heterodimers in the brain as noted to occur between the other Foxp family members (Li et al., 2004; Mendoza et al., 2015; Mendoza and Scharff, 2017). A likely candidate is *Foxp1* because this Foxp family member gene is known to play a role in immune cell function by its repression of immune regulatory genes in the brain (Tang et al., 2012). In the PNS, *FOXP1* has also been found to associate with *FOXP3* and it is thought that both transcription factors promote each other's binding with downregulation of *FOXP1* resulting in a marked decrease in *FOXP3* binding (Konopacki et al., 2019). Future experiments currently underway are testing the potential interactions between Foxp3 and the other Foxp family members in the brain. Interestingly, a recent publication has shown Foxp3 to be expressed by microglia (Chung et al., 2010; Yi et al., 2016). Microglia have been shown to be a contributor to the modification of not only brain development but also behavior (Lenz et al., 2013). No publication to our knowledge has investigated *Foxp3's* impact on microglia and how they may work together to sculpt the neural circuits responsible for developing the dendritic morphology and spine growth of neurons, as well as behavior. This could be an additional unique feature of Foxp3 in the brain given its cell type expression in the brain may not be on neurons, as observed with Foxp 1/2/4 (Tam et al., 2011; Bowers et al., 2014). Future investigations testing the cell type expression of *Foxp3* in the brain are clearly warranted.

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