# Role of retinoic acid degradation in the maturation of inhibitory neurons in the mouse

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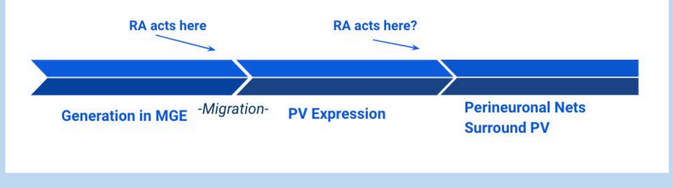
## INTRODUCTION

Within the last decade, psychiatric genetics have hit a turning point due to an increased amount of findings that can be placed in the neurobiological context. This has enhanced the understanding of disease pathogenesis, and has resulted in an increased capacity for developing therapeutics. This has led to more research being done on genetic signaling pathways[1]. One such pathway that may play a role in brain disorders is regulated by retinoic acid (RA), a derivative of Vitamin A. RA is a small, lipophilic molecule that plays an important role in cell-cell signaling during organ development in vertebrates[2]. In the brain, RA regulates gene expression and plays a role in the central nervous system[3]. Furthermore, RA signaling is involved in the patterning of the brain[4], memory, and synaptic plasticity[5]. It also regulates cortical synchrony in adults[4]. When impaired, it may play an important role in the causation of schizophrenia[6]. Due to the important role RA plays in the brain, the Nakagawa Lab has been studying the roles RA signaling plays in the development of inhibitory neurons (interneurons) in the prefrontal cortex (PFC). Interneurons are important regulators of excitation-inhibition balance and synchronous firing which forms the basis of our cognition and emotion. Previous work in this lab has found that the *Cyp26b1* gene, which encodes an enzyme responsible for the breakdown of RA, is developmentally regulated in the PFC. The expression of this gene begins at the neonatal stages of development and mice that lack the connection between the PFC and the thalamus are unable to express the *Cyp26b1* gene in the PFC. The lab has generated mutant mice in which the *Cyp26b1* gene is knocked out specifically in the frontal cortex, and has found that in these knockout mice, the number of a particular subtype of interneurons called parvalbumin (PV) interneurons is increased in the postnatal PFC. The lab has also found these neurons are a major target of RA signaling during early postnatal stages. These results indicate that in normal brains RA either enhances the survival or promotes the maturation of these PV interneurons. Previous work in the lab has found non-significant difference in survival, which leads us to test the other possibility, which is that maturation of PV interneurons are accelerated in *Cyp26b1* knockout mice. The mechanism of PV neuron maturation is not well understood. The objective of this research was to determine how the maturation of PV neurons differs between control and *Cyp26b1* mutant mice. I hypothesized that the maturation of PV neurons was accelerated in the *Cyp26b1* mutant mice, which would be indicated by the increase in the number of PV neurons surrounded by perineuronal nets.

## EXPERIMENTAL METHODS

1. Animals
   - Frontal cortex specific *Cyp26b1* mutant mice: [*Cyp26b1 flox*/+; *Syt6Cre*/+] x [*Cyp26b1 flox/flox*] (expression of *Syt6 (synaptotagmin6)* gene is layer 6- and frontal cortex- specific)
2. Tissue Processing
   - P21 pups were perfused with 0.1 M phosphate buffer and 4% paraformaldehyde
   - Brains were taken out of skull and washed in 0.1 M phosphate buffer
   - Brains were then post fixed in 4% paraformaldehyde for 2 hours
   - Brains were then sunk in 30% sucrose/0.1M phosphate buffer
   - 50 µm-thickness coronal sections were cut with a sliding microtome and mounted on glass slides
   - Immunostaining techniques were used to detect PV protein and perineuronal nets
     - Rabbit antibody used against PV and a biotin-conjugated wisteria floribunda lectin (WFL) against the perineuronal nets
     - Biotin-WFL (for nets) and anti-PV were detected by Cy2-conjugated streptavidin and Cy3-conjugated secondary antibody against rabbit IgG, respectively.
3. Cell Counts
   - Each section was imaged in Cy2 and Cy3 channels
   - Images in different channels were overlaid in Photoshop and upper, middle and lower layer bins were mapped on each section
   - PV cells were counted using ImageJ
   - PV and WFA positive cells were counted

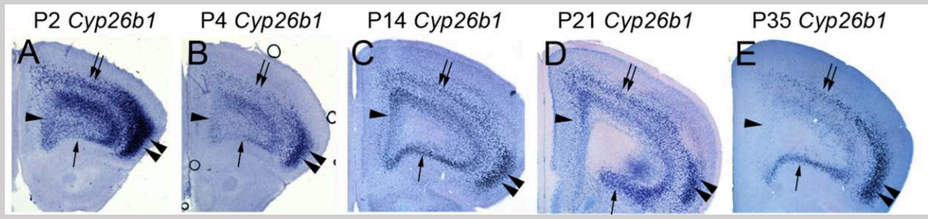
## Overview




PV development from generation in medial ganglionic eminence (MGE) to being surrounded by perineuronal nets, which indicates full maturity. The aim of this study was to determine which steps of PV neuron development are targets of RA. If RA acts sometime between generation in MGE and PV expression the number of PV cells would be affected. If RA was acting sometime between PV expression and nets surrounding PV, then the ratio of PV and WFA positive cells to PV positive cells would be affected. RA could act on more than one steps.

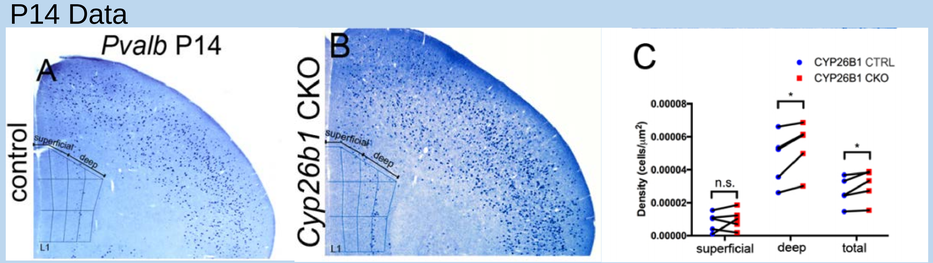
## RESULTS

### Cyp26b1 Expression




- Frontal section of control PFC at various stages postnatally. *Cyp26b1* expression starts in PFC, medially (arrowhead) and ventrally (single arrow) at P2 and continues until P14.
- At P21, medial PFC expression of *Cyp26b1* is reduced and at P35 is no longer detectable.
- *Cyp26b1* is also expressed in the lateral PFC, in both the motor area (double arrows) and the agranular insula (double arrowheads)[9].
- *Cyp26b1* expression strongest from P2 to P14 indicating that regulation of RA signaling by degradation occurs in this timeframe

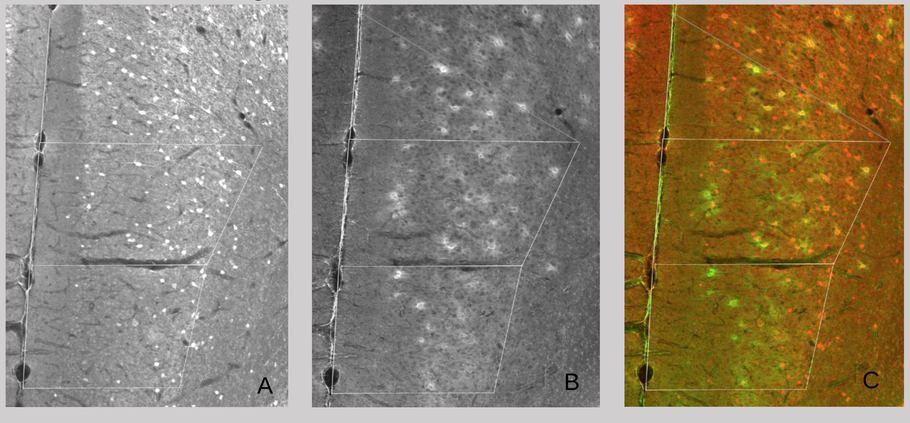
### P14 Data




In situ hybridization of frontal sections of P14 of control (A) and *Cyp26b1* conditional knockout (B) mice. Parvalbumin (*Pvalb*) expression is shown. The number of *Pvalb*-positive cells in each bin were added and compared between control and CKO mice. C is the result of statistical analysis. Each line connecting the red and blue dots represents a pair of brains from the same experiment[9].

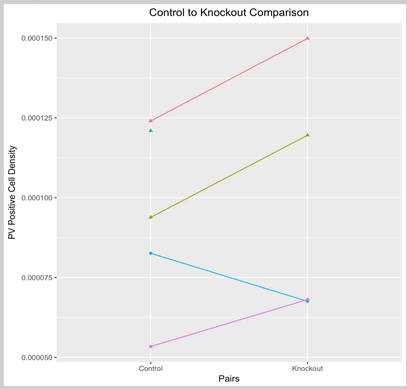
- Found increased expression of PV in medial PFC in *Cyp26b1* conditional knockout littermates
- Found no change in *Sst* and *Lhx6* in *Cyp26b1* conditional knockout littermates at same stage
- Results show that temporary expression of *Cyp26b1* in the medial PFC is needed to control the development of PV interneurons
- Indicates that overall populations of interneurons is not increasing; likely that PV maturation affected

### PV and WFA Staining

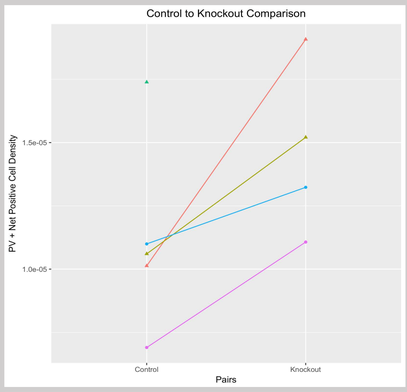



Representative staining of P21 CKO PV cells (A), perineuronal nets (B) and an overlay of PV cell and perineuronal net staining (C), where PV is red and nets are green.

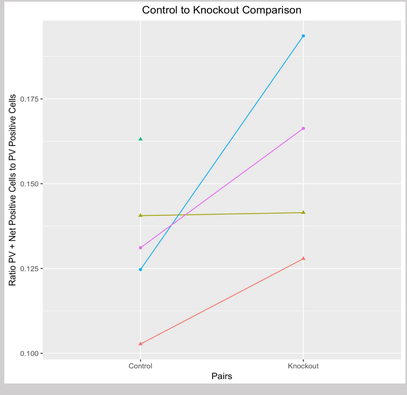
### P21 and P23 PV and WFA




- Comparison of the average density of PV positive cells per section in control and conditional knockout brains.
- Each line represents a pair. Triangles represent P21 pairs and circles represent P23 pairs.
- The decrease in the blue P23 pair is believed to be due to dark background staining that led to cells being lost when thresholding was done in ImageJ.
- Data was analyzed using a ratio paired t-test; p=0.1834.




- Comparison of average density of PV and net positive cells per section in control and conditional knockout brains.
- Each line represents a pair. Triangles represent P21 pairs and circles represent P23 pairs.
- The increase in density from control to knockout is consistent with the hypothesis that RA accelerates maturation of PV interneurons.
- Data was analyzed using a ratio paired t-test; p=0.0075.




- Comparison of the ratio of the average PV and net positive cell density to the average PV positive cell density per section in control and conditional knockout brains.
- Each line represents a pair. Triangles represent P21 pairs and circles represent P23 pairs.
- The steep increase in the blue P23 pair is due to the decrease in PV density from control to knockout while the density of PV and net positive cells in the knockout was as expected.
- Data was analyzed using a ratio paired t-test; p=0.0945

## SUMMARY/FUTURE WORK

- The general trend is that the number of PV interneurons surrounded by perineuronal nets increases from control to knockout brains which is consistent with the hypothesis that RA accelerates maturation of PV interneurons.
- The general trend is that the ratio of PV and net positive cells to PV cells increases from control to knockout which is again consistent with the accelerated maturation hypothesis.
- Future work will include working on the P23 counts to ensure that all PV cells are being included in the counts. Additionally, more pairs will be counted in order to determine statistical significance and draw conclusions.

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