Establishing Immunohistochemistry Protocols in Early Neonatal Rat Brain for Retinoid X Receptor Gamma (Rxrγ), A Labile Biological Target

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ABSTRACT

Objective:
To establish immunohistochemistry protocols in early neonatal rat brain for retinoid X receptor gamma RXR γ, a labile biological target.

Methodology:
A series of experiments were carried out to optimize the immunohistochemistry (IHC) protocols for RXRγ in early neonatal rat brains. IHC protocol for RXRγ was modified from the protocol available for adult rat brain in literature. We have done RXRγ IHC for rat brains at the ages of 3, 10, 18 and 60 days. We have compared the immunostaining (IS) with the Nissle-stained sections of the same brains to show the staining pattern of RXRγ IHC.

Results:
Our experiments revealed characteristics of IS for RXRγ. It was shown that RXRγ protein was present in the cytoplasm as well as nuclei. The cellular processes also took the IS. Additionally our IHC results clearly showed that more cells are visible through Nissle staining as compared to RXRγ IHC.

Conclusion:
We could present expression of RXRγ in early neonatal and adult rat brains through IHC. We have documented the sensitivity of RXRγ to various commonly practiced methodological components for IHC. Our study has not only developed a successful IHC protocol for RXRγ in early neonatal rat brains but it may help future researchers to tackle sensitive proteins for IHC.

Keywords:
Immunohistochemistry staining, RXRγ, Immunohistochemistry protocols.

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INTRODUCTION

Vitamin A is very important for development of multiple organ systems in the body. Retinoid X receptor γ (RXRγ) is one of the nuclear receptors through which vitamin A helps in various biological processes. The role of vitamin A in metabolism and embryologic development is well documented.[1,2] Deficiency of vitamin A during development leads to gross morphological and ultrastructural abnormalities of multiple systems including central nervous system and cardiovascular system.[3] Lack of vitamin A in certain experimental models during development leads to embryonic death.[4] Hence, vitamin A receptors have been capturing the interest of researchers for many decades. Vitamin A acts through two groups of nuclear receptors, RARs and RXRs. Both RARs and RXRs have α, β, and γ gamma isoforms.[5,6] We decided to explore expression of RXRγ in early neonatal rat brains. However IHC following the standard protocols in our lab yielded poor results. RXRγ IHC following this protocol showed high background staining. RXRγ localization through IHC in adult rat brains was already documented by Moreno and Colleagues which will be referred to as Moreno protocol in this manuscript.[7] The IHC protocols established by Moreno and colleagues were not suitable for early postnatal rat brains. Moreno and colleagues sectioned non-post-fixed adult rat brains on vibratome, which would not give acceptable results for delicate neonatal rat brains. Hence we decided to modify the already documented IHC protocols for RXRγ in terms of fixation and sectioning.

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METHODOLOGY

Animals:

Long-Evens rats bred in the Centre for Biology of Memory at NTNU, Norway were used for this study. All animals were kept at room temperature ranging from 25 to 30^{0}C. The animals were given free access to food and water. The day the pups were seen in a cage was taken as P0 (post-natal day 0) and IHC experiments were conducted on postnatal day 3 rat brains. RXRγ IHC was done for rat brains at the ages of third (P3), tenth (P10), eighteenth (P18) and sixtieth (P60) postnatal day.

Establishment of immunohistochemistry methods:

We optimized IHC protocols for RXRγ after passing through a series of experiments. Our experiments for establishing the IHC protocols are summarized as following figure. The routine IHC protocol in the lab resulted in a lot of background staining Figure 1-A.

1. The IHC of Moreno protocol gave good staining but poor sectioning for young rat brains - Figure 1-B.

2. Multiple experiments of microtome sectioning with different modifications of cryoprotection resulted in better sectioning but poor tissue morphology - Figure 1-C.

3. Different modifications of post fixation were tried to render the tissue consistency to bear sectioning and IHC procedure. However post fixation of even half an hour of sections resulted in low signal strength - Figure 1-D and E.

4. We kept the tissue in phosphate buffer while it was sectioned and incubation of the tissue with primary antibody was done one day after the perfusion. This resulted in decreased signal strength indicating time sensitivity of RXRγ expression - Figure 1-F.

5. Modifications in perfusion solution, perfusion procedure and vibratome sectioning resulted in establishment of successful IHC protocols for young rat brains.

PERFUSION:

P3 rats were anesthetized with isoflurane and perfused. Moreover the perfusion solution for the young animals consisted of 4% PFA plus 0.1% gluteraldehyde. This gave better tissue quality for the young brains.

FIXATION:

For the RXRγ antibody the optimal observed fixation conditions were to decapitate the animals directly after perfusion and then keep the heads at 4^{0}C by submerging them in ice for approximately 2 hours. The heads were then removed from the ice and the brains were carefully dissected. The brains were transferred to bottles containing phosphate buffer until they were sectioned with a vibratome on the same day.

SECTIONING:

After passing through different experiments vibratome sectioning was taken as method of choice for IHC of RXRγ antibody. The vibratome sectioning of freshly perfused early postnatal brains was improved by mechanically supporting the brain during sectioning. Gelatin/Albumin solution was made and just before embedding the specimen into it, glutaraldehyde was mixed to solidify the block as suggested by Levin.8 This block not only provides perfect support to the young rat postnatal brains but was also found to be useful for IHC of labile biological targets.[8]

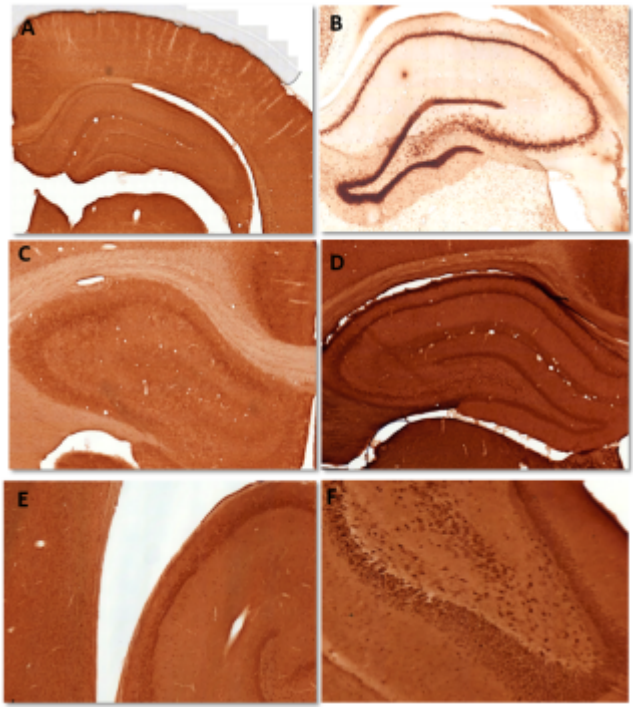

Figure 1: Results of some of the IHC experiments for establishing protocols for RXRγ immunoexpression, Magnified 2x. A: After routine protocol in the lab. B: Moreno protocol. C: Modification in cryoprotection experiment. D and E: Modifications in post-fixation. F: Incubation of the tissue with primary antibody after being kept in Phosphate buffer for 24 hours

IMMUNOHISTOCHEMISTRY:

IHC of the floating sections for DAB staining was conducted in the following steps:

- First the sections were washed with phosphate buffer\triton-X solution (0.1%) for 3 × 10 minutes each.
- The sections were incubated with 5% bovine serum albumin (BSA) solution for 1 hour to minimise the background signal.
- The sections were incubated in a solution containing anti-RXRγ antibody overnight at 4°C. The antibody was used at a concentration of 1:300 in PBS containing 0.1% triton-X and 2.5% BSA.
- On the next day the sections were washed with phosphate buffer\triton-X solution (0.1%) for 3 × 10 minutes each.
- The sections were incubated in the secondary antibody solution for one hour.
- They were washed with phosphate buffer \ triton-X solution (0.1%) for 3 × 10 minutes each.
- They were then incubated with ABC solution for 1 hour.
- Thereafter they were washed with phosphate buffer\triton solution (0.1%) for 3 × 10 minutes each.
- The sections were washed with Tris-HCl solution for 2 × 5 minutes each.
- The sections were treated with Diaminobenzidine (DAB) solution.
- The sections were washed with Tris-HCl solution for 2 × 5 minutes each. The tissue was kept in Tris-HCl solution until mounted on plain slides with gelatin solution. The mounted sections were left on the heating plate for one day at 40^0C to allow adequate dehydration before coverslipping.

MAGNIFICATION OF IMAGES CAPTURED THROUGH PANORAMIC VIEWER®:

All slides were digitalized using digital scanner from Carl Zeiss company (catalog no: ZEISS Axio Scan.Z1). Panoramic Viewer® is a software which allows studying and capturing the images from digital slides at variable magnifications. The magnifications of images presented in all histological figures of this manuscript have been set using the available knobs in the software GUI (graphic user interface). If an image was captured after magnifying the slides to 2X according to the software GUI, it was labeled as having magnification of Approximately 2X.

RESULTS

The IHC results for RXRγ for third (P3), tenth (P10), eighteenth (P18) and sixtieth (P60) postnatal day rat brains are shown in figure 2.

RXRγ IHC results in cerebral cortex and hippocampus of ten days old rat pups are compared with the Nissle-stained sections of the same animal in figure 3. Detailed histological study of the digital slides showed that the IHC staining for RXRγ was similar to Nissle staining in hippocampus.

In cerebral cortex of ten days old rats, the IHC staining was found in all parts of brain but it was not generalized. More neurons are seen in the deeper layers through IHC as compared to the superficial layers

Detailed examination of the digital slides on high power showed characteristics of the cellular staining through the IS - figure 4. It was shown that RXRγ protein was present in the cytoplasm as well as nuclei. The cellular processes were also taking the IS

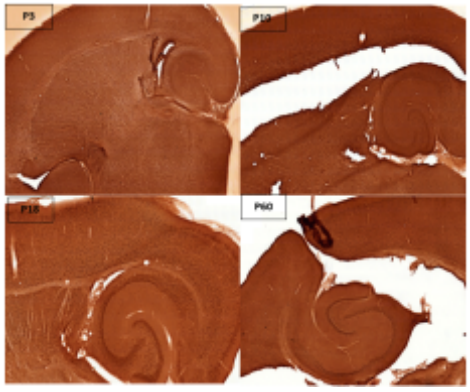

Figure 2: RXRγ IHC results for early neonatal and adult rat brains. The rat brains were DAB stained after incubation with RXRγ antibody P = post natal day

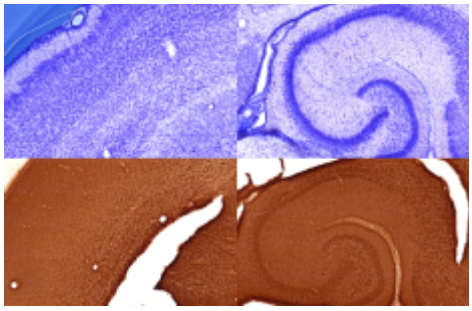

Figure 3: Comparison of RXRγ IS (bottom) with Nissle staining (top) in a P10 rat brain in the regions of cerebral cortex (right) and hippocampus (left). Apparently more cells are seen in Nissle stained sections as compared to the sections stained after RXRγ IHC

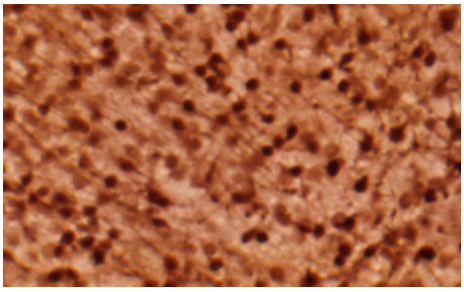

Figure 4: Characteristics of cellular staining through RXRγ IHC on high power (magnification=10 X)

DISCUSSION

It is necessary to establish IHC protocols for each protein of interest since the properties of antibody-antigen binding or antigen availability may vary. Biological researchers use different standardized IHC protocols in their labs. However many new proteins require optimization of IHC protocols. Optimized IHC protocols for interesting biological targets are commonly documented.[9] We have also presented a successful IHC protocol for RXRγ in early neonatal rat brains through this study.

It is a common approach to post-fix histological specimens after perfusion for IHC. The immunohistological specimens are post-fixed for a variable time to augment the initial preservation of the tissue histology done by the perfusion procedure. Though some recent studies suggest that formalin fixation does not affect IHC detection of many proteins.[10] Aldehyde fixatives like formaldehyde and glutaraldhyde are considered to show a "masking phenomenon" after post-fixation due to hiding of the antibody binding sites. [11] The solution to this problem is to find a balance between desired staining and the tissue histology.[12] We tried to find an appropriate amount of post-fixation not only for the purpose of fixation but also for the purpose of making the young soft rat brains relatively stiff to bear the cutting by a vibratome. We experienced problems in IHC with prolonged post-fixation of brains in PFA. The results of multiple RXRγ IHC experiments after varying degrees of post-fixation indicate that the RXRγ protein expression is sensitive to post-fixation with formaldehyde. Although methodological reports of previous studies about RXRγ IHC in adult rat brains indicate that no post-fixation was done for the specimens. [13] No clear reports about the fixation sensitivity of RXRγ was given.

Prolonged post-fixation was found to be incompatible with RXRγ IHC however there are a variety of antigen unmasking steps that can be taken if future researchers experience similar problems of post-fixation. For example, treating the post-fixed sections with ethanol solution prior to incubation with the primary antibody or microwave treatment of the post-fixed tissue has been a useful step for IHC.[14,15]

The IHC procedure has been carried out in rat or mouse brains after frozen sectioning [16] vibratome sectioning [17, 18] or paraffin embedded sectioning. It has been suggested that vibratome sections result in better tissue morphology as compared to frozen sections [19] or paraffin embedded sections.[20] Frozen sections gave lighter and displaced staining as compared to the vibratome sections in our experiments of RXRγ IHC. It has been suggested that freezing and thawing during frozen sectioning cause distorted neuronal morphology [19] . Possibly, rupture of cellular membranes in our histological specimen as a result of freezing and thawing was the cause of insufficient staining of RXRγ protein in frozen sections. Since RXRγ IHC results in vibratome sections incubated with the primary antibody on the next day after perfusion resulted in better staining than the frozen sections we conclude that RXRγ protein expression level is sensitive to freezing.

Additionally the incubation time of cryoprotection might be considered as a reason for lighter staining. Though Moreno protocol required incubation of the tissue with the primary antibody on same day when perfusion was done time sensitivity of RXRγ protein is neither reported by Moreno or other researchers.We experimented on the time elapsed between perfusion and incubation and showed that RXRγ protein expression level was found to be time sensitive.

Obtaining good quality vibratome sections requires the tissue to be relatively stiff otherwise the tissue tends to bend forward during cutting. Previously many techniques were used involving infiltration or embedding of the brain in gelatin or agar. The infiltrated specimens need to be hardened with PFA.[21-23] Any effort to make the tissue relatively stiff involving interaction of the brain with PFA for a longer duration after perfusion resulted in poor labeling of RXRγ protein. Therefore we did not try infiltration of the brain with gelatin or agar. However, the soft early postnatal rat brains were embedded in gelatin/albumin block which gave desirable sectioning.

Ultimately we were able to come up with a modified IHC protocol for RXRγ IHC. This study can help the future researchers to localize RXRγ in early neonatal rat brains. This study can help the future researchers to explore various aspects of CNS development. Additionally our documentation of IHC experiments can guide the biologists to deal with various problems encountered during IHC localization of labile biological targets.

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