Expression of IL-6 in a Rat Model of Infantile Spasms

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Acknowledgements: I would like to thank my mentor, teacher, and parents for their support.

Abstract

Background: West Syndrome, or infantile spasms, is a seizure disorder characterized by axial spasms in clusters, hypsarrhythmia, and psychomotor delay within the infant's first year of life. Infantile Spasms are believed to have originated in the brain stem with projections going to the cortex to cause hypsarrhythmia and to the spinal cord causing seizures. In this research, we were looking to see if IL-6 plays a role in the development of infantile spasms before seizures and looking at the expression of IL-6 before and after NMDA in beta vs. saline primed animals.

Methods: Immunohistochemistry, or IHC, is a staining technique used to amplify certain proteins to make them more visible under the microscope. Betamethasone is a drug administered to the rat mothers whilst pregnant at G15. Betamethasone is a way to mimic prenatal stress to see its affect on the mice after they are born and its role in seizure susceptibility to induced Infantile Spasms. Density analysis is one method used instead of counting because the staining did not have defined cellular patterns visible enough to accurately count. Using a computer program (ImageJ), we subtracted the foreground intensity from the background.

Results: In our research, it was found that the controls (saline-injected) had a foreground to background density number of roughly 16. Anything above that would imply there is greater background to foreground staining, therefore there are more positive proteins being accounted for. Where the staining is darker, or the number is greater than 16, it implies that there is a greater amount of IL-6 positive cells. It was concluded that there was a lower number and lighter staining in the beta animals than the saline animals. There was a 25% decrease in color density meaning there was a decrease in the IL-6 presence in the beta animals.
Conclusions: Prenatal stress (ie. beta) affects the development of the immune system during infancy. Changes in IL-6 expression may underlie mechanisms affecting seizure susceptibility in stressed animals however further experimentation is needed to test this. From these results it is clear that prenatal stress affects development and expression of inflammatory proteins, such as IL-6, and this knowledge will contribute to a better understanding of the pathology of infantile spasms leading to development of better, more effective treatments in the future. It can be said that priming alone (prenatal saline vs beta) does not affect baseline expression of IL-6 protein in the superior cerebellar peduncle (scp) however after induction of spasms on postnatal day 15 with NMDA there is a greater increase in IL-6 expression in beta primed animals compared to saline (beta NMDA vs saline NMDA).

Discussion: This data suggests that although beta priming does not immediately affect IL-6 expression or regulation, it does change the regulatory pathways associated with controlling its expression (ie. immune pathways) and therefore after an insult, such as NMDA-induced spasms, there is an overcompensation and increased expression of IL-6. IL-6 is proinflammatory therefore it may suggest a general increase in inflammatory response after NMDA in pups exposed to betamethasone. The increased IL-6 presence may also contribute to differences in seizure threshold in beta animals, which are lower. IL-6 can be proconvulsant therefore after initial spasms, increases in IL-6 may lead to increased vulnerability to future spasms. Prenatal stress (ie. beta) affects the development of the immune system during infancy. Changes in IL-6 expression may underlie mechanisms affecting seizure susceptibility in stressed animals however further experimentation is needed to test this. From these results it is clear that prenatal stress affects development and expression of inflammatory proteins, such as IL-6, and this knowledge will contribute to a better understanding of the pathology of infantile spasms leading to development of better, more effective treatments in the future.

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Review of Literature

West Syndrome, or infantile spasms, is a seizure disorder characterized by axial spasms in clusters, hypsarrhythmia, and psychomotor delay within the infant’s first year of life (1). Infantile Spasms is believed to have originated in the brain stem with projections going to the cortex to cause hypsarrhythmia and to the spinal cord causing seizures. These seizures typically consist of hundreds of 3 to 5 second convulsions summing up to about 8 to 20 minutes (1).

Axial spasms consist of convulsions throughout the body, stiffening and relaxing. Many of these patients who make it to age five, have severe mental deficits and often times grow into another seizure disorder. The age of onset is typically between 3-7 months and sometimes later due to postnatal stress (2). These seizures can be differentiated from other seizures because high amplitude and a short wave length characterize them. Infantile Spasms can most effectively be diagnosed by hypsarrhythmia, or chaotic brain waves with abnormalities on the EEG (2). Though hypsarrhythmia can be effective in diagnosis, up to one third of patients suffering will not exhibit it. Psychomotor delay is another prevalent characteristic of infantile spasms (2). Psychomotor delay is a lesser word for mental retardation, as in the patients may experience mental impairments such as the slowing down of thoughts or reduction of physical movements (2). This separates Infantile Spasms from other seizure disorders because many times patients suffering from an epileptic disorder can live without having severe mental deficits. Many patients suffering from West Syndrome also begin to develop autistic features, making the disease that much more difficult to understand. Though some patients grow out of Infantile spasms, Autistic features typically continue to persist (2). Infantile Spasms differs from other epilepsies because of its age components and different mechanisms such as axial spasms, hypsarrhythmia, and psychomotor delay.

Currently, corticosteroids such as prednisone are administered to treat infantile spasms, but often result in adverse side effects.(12) Antiepileptic medications, such as topiramate, may erase some symptoms.(12) To treat infantile spasms in children ages one month to two years, Vigabatrin is administered.(12) Surgical removal of brain lesions that have been the cause of spasms serves as a more invasive treatment. The longer the spasms last before they are treated and controlled, the poorer the child may do developmentally. cite

Prognosis for children infantile spasms is dependent upon the underlying causes of the seizures.cite In most cases, intellectual prognosis is poor because many patients have neurological impairment prior to the onset of spasms. Epileptic spasms generally reduce by mid-childhood, but more than 50% of patients with infantile spasms will eventually develop other types of seizures. cite

In this research, we are trying to better understand the underlying mechanisms of Infantile Spasms. The ultimate goal of this research is to find a more effective and long-term treatment for children suffering from infantile spasms.
Inflammatory molecules, or mediators, are the main factors that play a role in inflammation. These inflammatory molecules are released when cells in the body are damaged and in need of repairing (3). Cytokines include many substances such as interferons, interleukins, and growth factors, that are secreted by certain cells of the immune system and have an effect on other cells stimulating cellular responses such as cell repair or clean up(4). There are two types of inflammatory molecules; anti-inflammatory and pro-inflammatory cytokines. Anti-inflammatory molecules help to suppress inflammation while pro-inflammatory molecules aid in stimulating inflammation (5).

IL-6 is the pro-inflammatory cytokine that is being studied in this research. It is secreted by the immune system under trauma, leading to further inflammation of the damaged tissue (6). Relating to Infantile Spasms, when the brain experiences trauma from seizures, the body releases inflammatory molecules, such as IL-6, to either increase or decrease inflammation when necessary (6). In this research, we are specifically looking at the expression of IL-6 in one part of the rat brain, the brainstem. The brainstem connects the cerebrum and the spinal cord consisting of the midbrain, medulla oblongata, and the pons. The main job of the brainstem is to allow the flow of neurons between the brain and the rest of the body (7).

Currently we are looking to see what role IL-6 plays in the development of Infantile spasms after betamethasone treatment and NMDA induced spasms. If IL-6 does play a role in the development of Infantile Spasms, we want to see if this affects seizure susceptibility and inflammatory protein expression aftermath.

**Methods**

**Immunohistochemistry**

Immunohistochemistry, or IHC, is a staining technique used to amplify certain proteins to make them more visible under the microscope. IHC makes it easier to visualize and find specific cellular components within cells. It works by targeting antigens by making antibodies bind to a specific antigen. Using a primary and secondary antibody it makes it possible to block out the background and make the foreground staining darker, making the specific cellular component more visible under the microscope. The role of the blocking buffer is to bind to the antigen, or protein, of interest making sure that the secondary antibody will bind to everything but the antigen of interest. The primary antibody is raised in the animal from a different species than what you are targeting. For example, to make a primary antibody that will detect a rat protein, is raised in a mouse. If it were raised in the same animal as you are targeting (rat) it would bind non-specifically to other proteins because of the homology/similarity other rat proteins have to one another. The secondary antibody, similarly, must be raised in a different animal as the primary antibody for the same reason (to remove non-specific binding). In this case, the secondary antibody was raised in horse that is specific for mouse IgG protein therefore it will detect the primary made in mouse as it has IgG associated. The secondary antibody then has a tag (Biotin) which reacts with the AB reagents you used in the next step to cause the enzyme reaction with 3’-3’-Diamino-benzidine (DAB) which leads to changing in color that shows a positively-stained cell which contains the protein of interest (IL-6). The research uses an enzyme-linked indirect method which means you use primary and secondary antibodies plus enzymes to amplify the signal (AB).

Before beginning, pregnant mother rats were injected with either saline or betamethasone (0.4mg/kg x2) on gestational day 15. Rat pups were then sacrificed on postnatal day 15 (8). Rats were perfused transcardially with 410% buffered formalin containing 30% sucrose. Brains were removed from the
skull and post fixed in the same fixative overnight, and then embedded in a matrix to allow for cutting at -18°C. Staining was performed on free-floating coronal sections using our standard protocol for immunohistochemistry with the avidin–biotin peroxidase method (9). We used IL-6 antibody (1:1000 dilution, 72hr incubation) and anti-mouse secondary antibody. Sections were then mounted on gelatinized slides, dehydrated, and cover slipped with Permount. To minimize differences between the various sets of immunohistochemistry, all sections were treated exactly the same way at all steps. Negative control sections were prepared by incubating the sections without the primary antibody (8).

Prenatal Stress

In this research, we were seeing if IL-6 plays a role in the development of infantile spasms before seizures and looking at the expression of IL-6 before and after NMDA in beta vs saline primed animals. First to see if treatment affects expression in beta and saline and then if expression changes after NCMA-induced spasms (beta vs beta NMDA). Though these brain samples being studied have not yet experienced Infantile Spasms, they have been induced with betamethasone. Betamethasone is a drug administered to the rat mothers whilst pregnant at G15. Betamethasone is a way to mimic prenatal stress to see its affect on the mice after they are born and its role in seizure susceptibility to induced Infantile Spasms (10).

Density Analysis

Another technique used to analyze our data was density analysis. Density analysis is a method we used instead of counting because the staining did not have defined cellular patterns visible enough to accurately count. Using a computer program (ImageJ), we subtracted the foreground intensity from the background. After pictures are taken on the microscope they are sent to the computer to be analyzed. After getting at least five samples from the background you are given a number, or the density of the color, and then the same is done for the foreground, or the part of the cell that was being stained. The foreground should be darker than the background. The higher the number the greater the increase in the difference between the foreground and the background.

Data:
The two way ANOVA shows that NMDA is a factor that significantly affects IL-6 expression however the p value for Prenatal factor is >0.05 therefore is not significant however perhaps after adding more samples to the groups we may see this change. Currently, the NMDA significantly affects expression however prenatal priming does not. It is clear there is a trend that beta+NMDA has an increase in IL-6 after spasms which is not present in saline.

Results

In this research we were seeing if IL-6 plays a role in the development of infantile spasms after being administered betamethasone. Control animals were saline injected. Negative control sections were not given primary antibody. In our research, we found that the controls (saline-injected) had a foreground to background density number of roughly 16. Anything above that would imply there is greater background to foreground staining, therefore there are more positive proteins being accounted for. Where the staining is darker, or the number is greater than 16, it implies that there is a greater amount of IL-6 positive cells. We concluded that there was a lower number and lighter staining in the beta animals than the saline animals. There was a 25% decrease in color density which means there was a decrease in the IL-6 presence in the beta animals vs saline animals. We could say that priming alone (prenatal saline vs beta) does not affect baseline expression of IL-6 protein in the superior cerebellar peduncle (scp) however after induction of spasms on postnatal day 15 with NMDA we see a greater increase in IL-6 expression in beta primed animals compared to saline (beta NMDA vs saline NMDA). This data suggests that although beta priming does not immediately affect IL-6 expression or regulation, it does change the regulatory pathways associated with controlling its expression (ie. immune pathways) and therefore after an insult, such as NMDA-induced spasms, there is an overcompensation and increased expression of IL-6. IL-6 is proinflammatory therefore it may suggest a general increase in inflammatory response after NMDA in pups exposed to betamethasone. The increased IL-6 presence may also contribute to differences in seizure threshold in beta animals, which are lower. IL-6 can be proconvulsant therefore after initial spasms, increases in IL-6 may lead to increased vulnerability to future spasms.

Discussion

Prenatal stress (ie. beta) affects the development of the immune system during infancy. Changes in IL-6 expression may underlie mechanisms affecting seizure susceptibility in stressed animals however further experimentation is needed to test this. From these results it is clear that prenatal stress affects development and expression of inflammatory proteins, such as IL-6, and this knowledge will contribute to a better understanding of the pathology of infantile spasms leading to development of better, more effective treatments in the future.
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