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N-Methyl-D-Aspartate Receptor Antibodies in Herpes Simplex Encephalitis

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Pruss et al.

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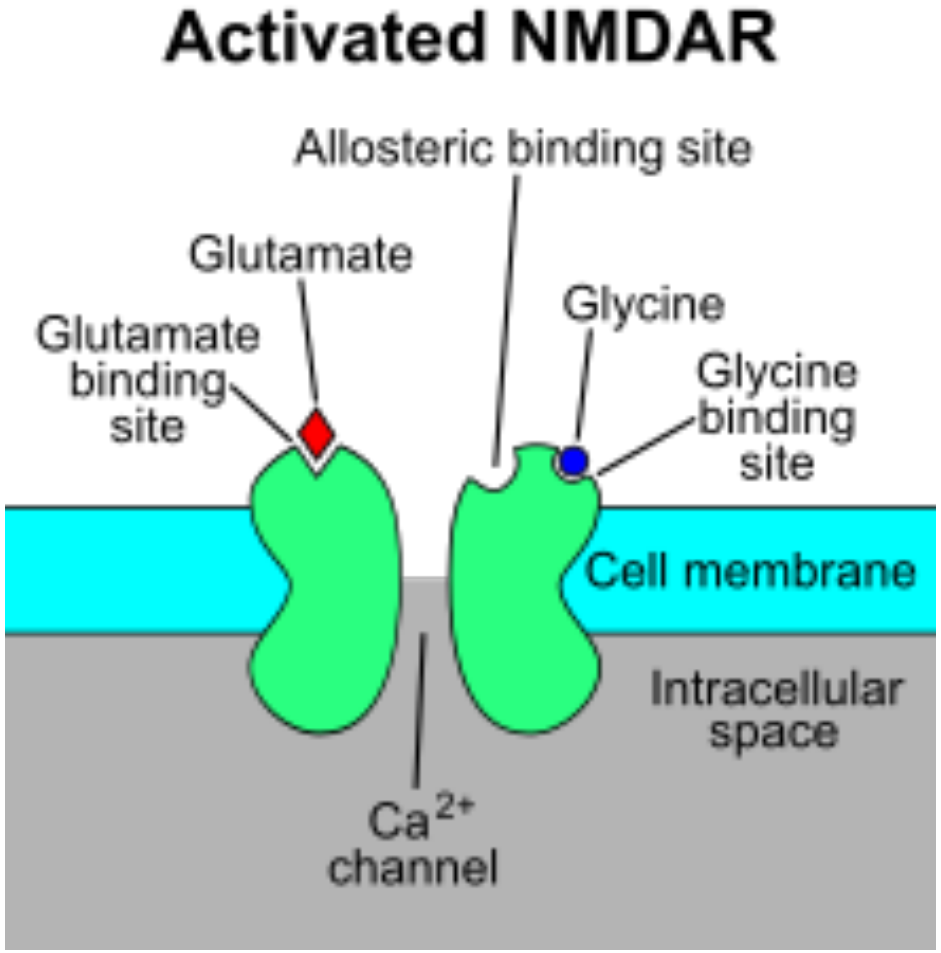
MJC Presentation

7/8/21

NMDAR

- Stands for “N-Methyl-D-Aspartate Receptor”, which a is a synaptic receptor believed to be present in >90% of synaptic junctions
- However, the receptor accepts glutamate (a key excitatory neurotransmitter) and glycine as its ligands
- Activating the NMDA receptor leads to an inhibitory effect

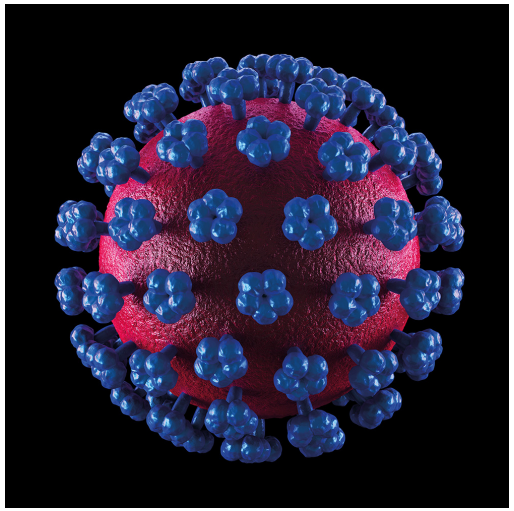


NMDAR Immunity

- Despite its necessity, antibodies that downregulate the receptor can be developed, leading to higher levels of brain stimulation and excitement
- The body can present subunits for the protein (such as NR1) as antigens and thus develop specific immunity to the protein
- This effect is particularly common in the case of HSV infection, as observed by Dr. Pruss et al's research team

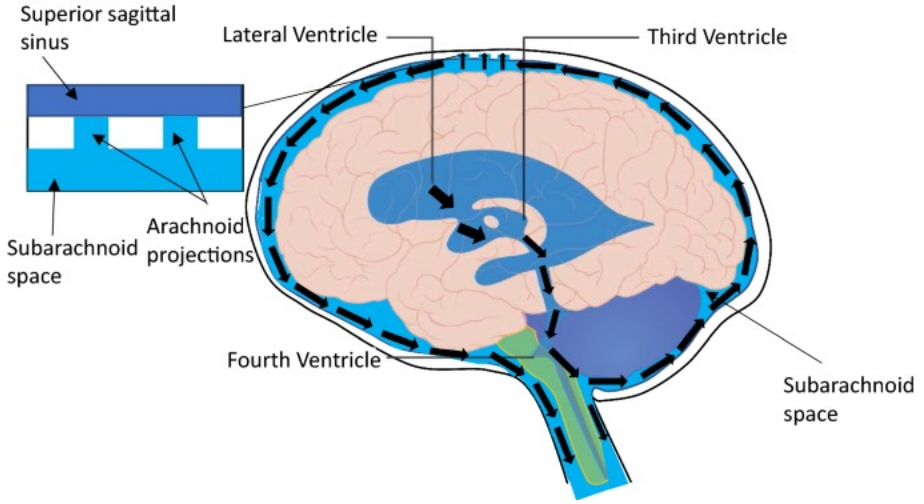
HSV – Herpes Simplex Virus

- Inflammatory response tends not to arise from the brain
- However, infection can occur when viral and bacterial antigens infiltrate the blood-brain barrier (especially prions)
- Microglia (phagocytes within the central nervous system) are typically responsible for eliminating antigens in the CNS, but their relative number is low
- Furthermore, HSV-1 (the variant used within the study) has been shown to be particularly infectious
  - ”[In 2016], an estimated 3752.0 million people had HSV type 1 infection at any site, equivalent to a global prevalence of 66.6% in 0-49 year olds” (James et al.)”



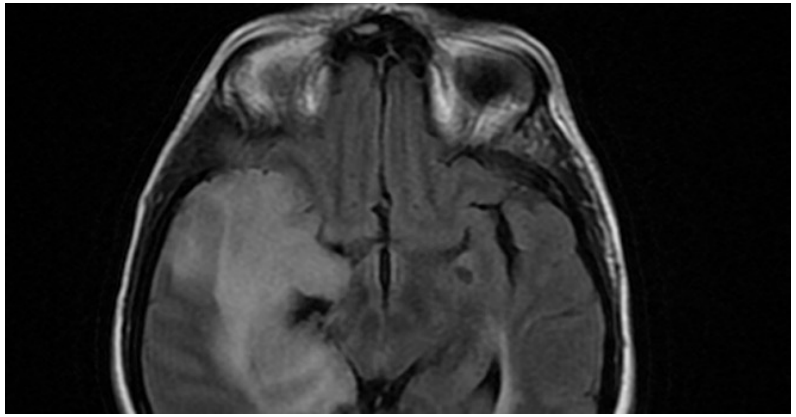
HSE – Herpes Simplex Encephalitis

- Despite the prevalence of HSV type 1, the encephalitis form is quite uncommon as it requires the centralization of the virus within the brain as opposed to more susceptible areas such as the eyes and nose (1 in 500,000 persons per year)
- The infection leads to an inflammatory response within the brain, leading to the accumulation of CSF (cerebral spinal fluid) and serum around the infected area
- In comparison with other infections that transmit themselves throughout the body, the brain blood supply is relatively isolated in the brain



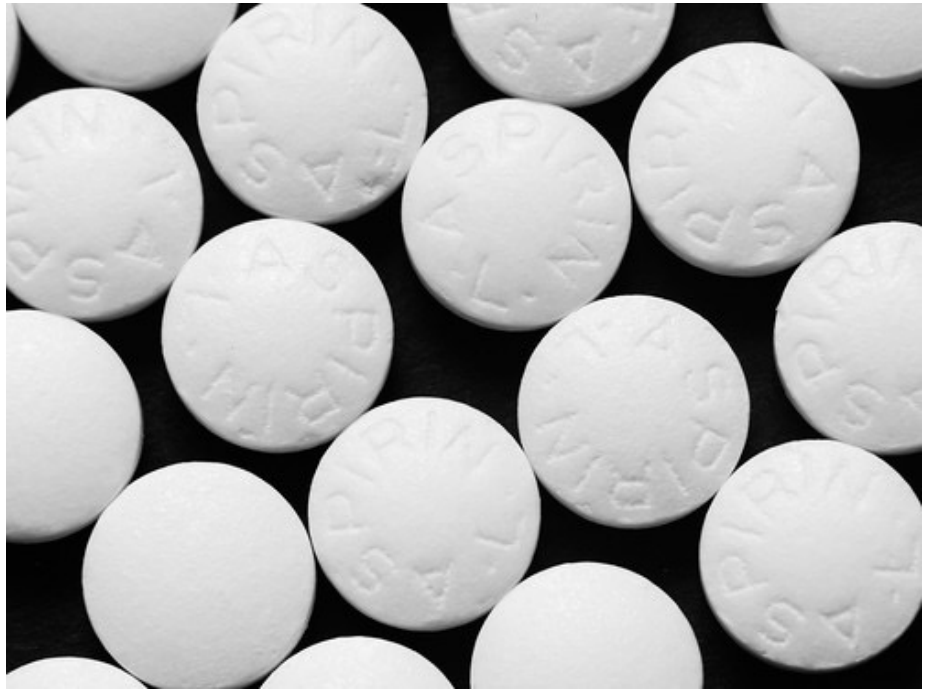
HSE - Herpes Simplex Encephalitis

- Due to the lack of immune support within the brain, the symptoms of HSE remain for extended periods of time
- What is encephalitis?
  - The inflammatory response induced by the viral infection
  - The inflammation leads to swelling, causing the brain to press against the meninges and skull
  - Patients with encephalitis usually complain of headache, mild-flu like symptoms, and neck stiffness



What is used to treat HSE?

- Acyclovir, an antiviral, is commonly prescribed to treat HSV1
- Pharmacy names: Sitavig, Zovirax



A secondary immune response?

- It was observed that HSE patients who were immunocompetent had more severe symptoms that patients who were immunocompromised
- In essence, the younger individuals tended to be worse off than older patients with HSE
- This finding suggests the presence of a secondary immune response – an immune response targeted at something that is NOT the virus (an autoimmune response)
- This hypothesis is in line with improved outcomes “when combining acyclovir with corticosteroids”
  - Corticosteroids suppress an immune response

Immunity against NMDAR

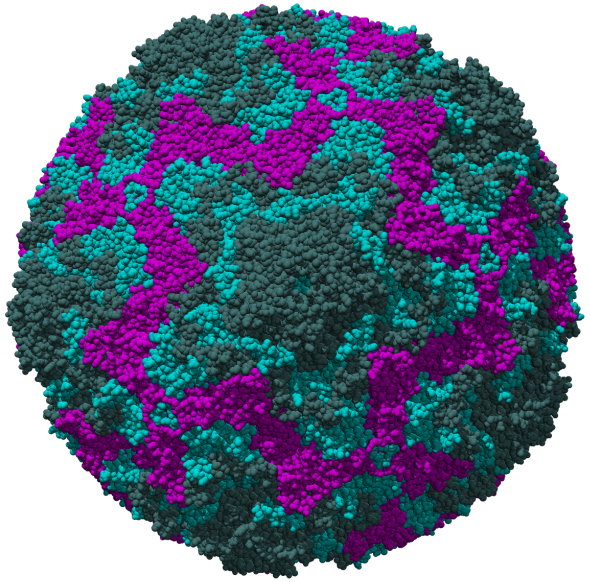
- When you add immunity against NMDAR, neurons are attacked because of two mechanisms as opposed to one:
  - 1. viral cytotoxicity
  - 2. detection of NMDA surface receptor (become the target of a specific immune response)
- HSE usually develops in the hippocampus, leading to cognitive (memory) and limbic (behavioral, emotional) deficits
- These symptoms are easily detected by family members, but they are difficult to identify on the part of the patients
  - “Interestingly, we detected significantly longer intervals between the first prodromal symptoms and hospital admission in patients with NMDAR antibodies.”

Experimental Methods

- “we performed a blinded retrospective study analyzing a large archived cohort of consecutive serum and cerebrospinal fluid (CSF) samples from patients with a definite diagnosis of HSE”
  - The identities of the patients were masked from the experimenters
  - The experimenters extracted only the CSF and serum of these patients, as these locations are where you can observe an immune response (test for antibody concentration, median white blood cell count, protein concentration)
  - All 44 patients studied were diagnosed with HSE as proved by PCR techniques
- Going forward: HSE ≠ anti-NMDAR encephalitis

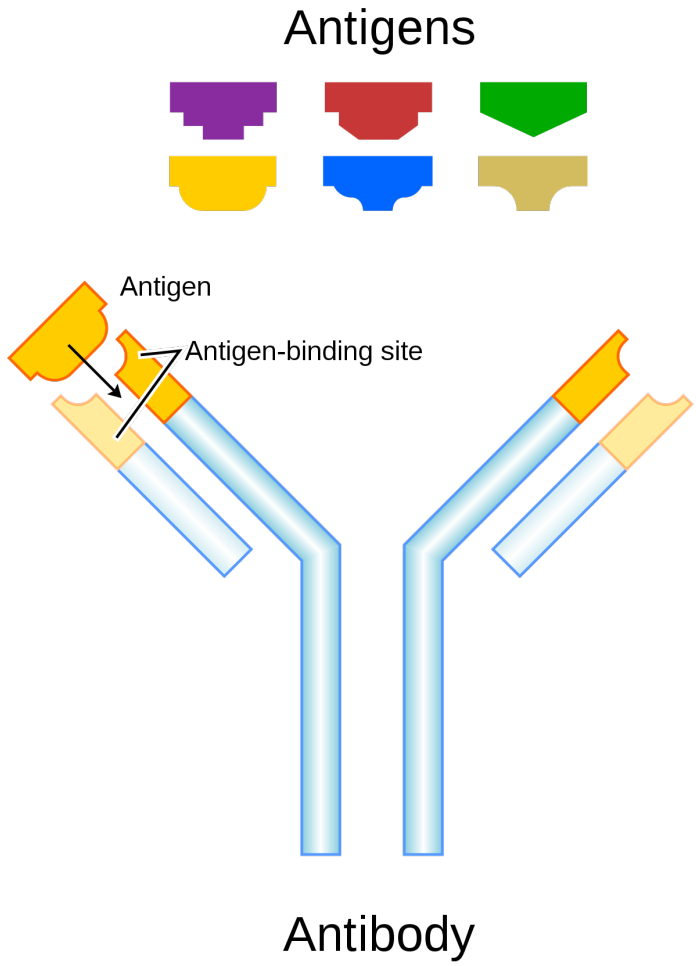
Patients

- All patients had presented three common characteristics:
  - Diagnosed with HSE in accordance with the German Society of Neurology standards
  - Had laboratory and imaging findings
  - Received IV acyclovir treatments for at least 2 weeks
- Control patients (“Entero”)
  - 10 patients with enterovirus encephalitis
  - 10 patients with varicella zoster virus
  - Both of these viruses are related to herpes simplex virus
- Informed consent was taken retrospectively



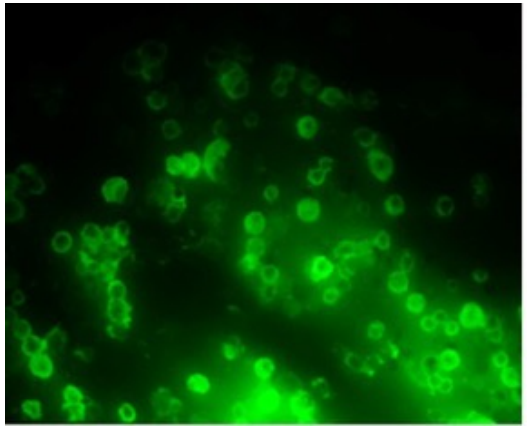
Testing for Antibodies

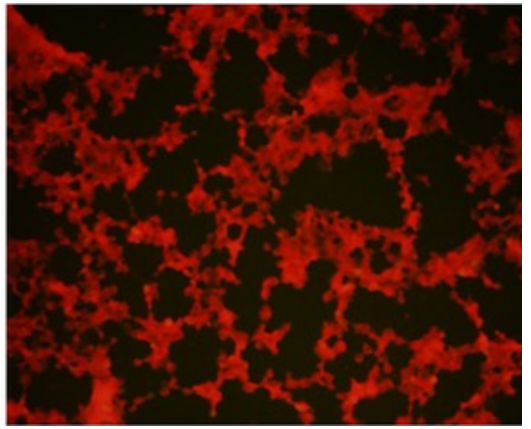
- Method: recombinant immunofluorescence
- Plasmids encoding for NMDAR were introduced to HEK293 cells (human embryonic kidney cells) and these cells were grown on slides
- Fragments from the slides were cut and put alongside rat hippocampus and cerebellum cells
- Finally, these samples were introduced to CSF and serum samples from the patients and incubated at room temperature



Testing for Antibodies

- After incubation, the samples were treated with fluorescein-conjugated goat-anti-human IgG, IgA, or IgM antibodies for 30 min
  - These antibodies, independent of the antibodies from the patient serum or CSF, would target the NMDARs
- The samples were then washed with PBS



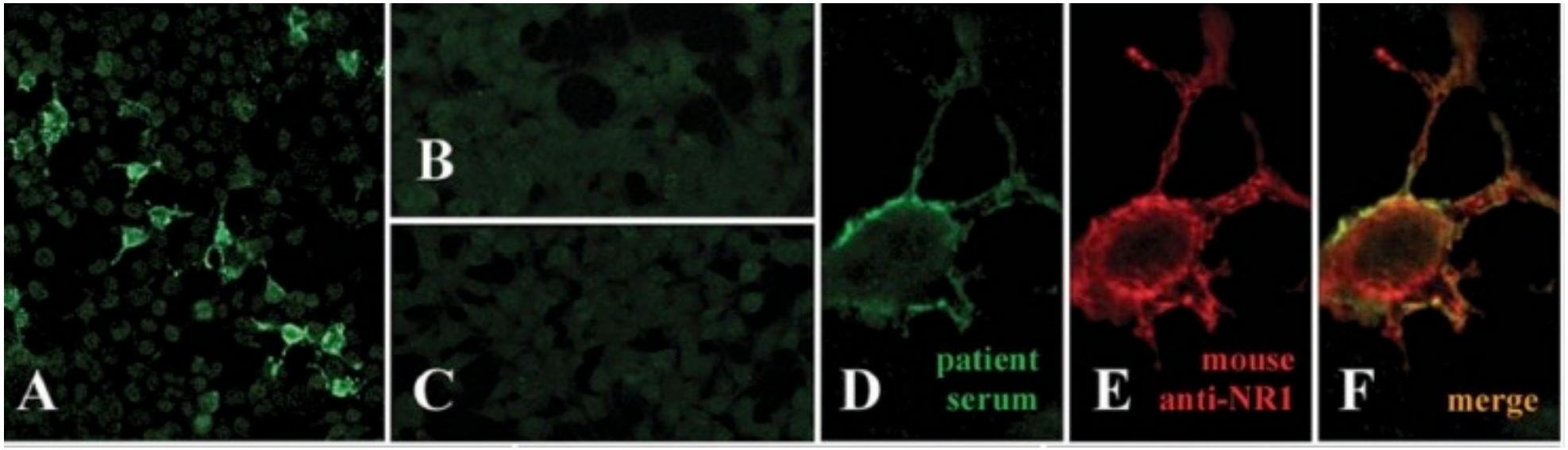


Testing for Antibodies

- The prevalence of antibodies could be determined based on the illumination of the antibody stains
- If the stains were sufficiently bright, the patient was considered immuno-positive toward anti-NMDAR antibodies
- When the antibody for NMDAR index was >4 for an Ig class prior to dilution, there was evidence of NMDAR-specific immunity for that Ig class (immuno-positive result)

$$antibody\ index = \frac{\left(\frac{CSF\ concentration_{antibodies}}{serum\ concentration_{antibodies}}\right)}{\left(\frac{CSF\ concentration_{rest\ of\ Ig\ for\ class}}{serum\ concentration_{rest\ of\ Ig\ for\ class}}\right)}$$

Testing for Antibodies – Results



- A: Immunity to NMDA receptors observed for IgM
- B + C: No immunity to NMDA receptors observed for IgA and IgG
- D - F: In IgM immunopositively samples, the stains from recombinant immunofluorescence from the patient antibodies and the goat antibodies are shown individually, as well as merged

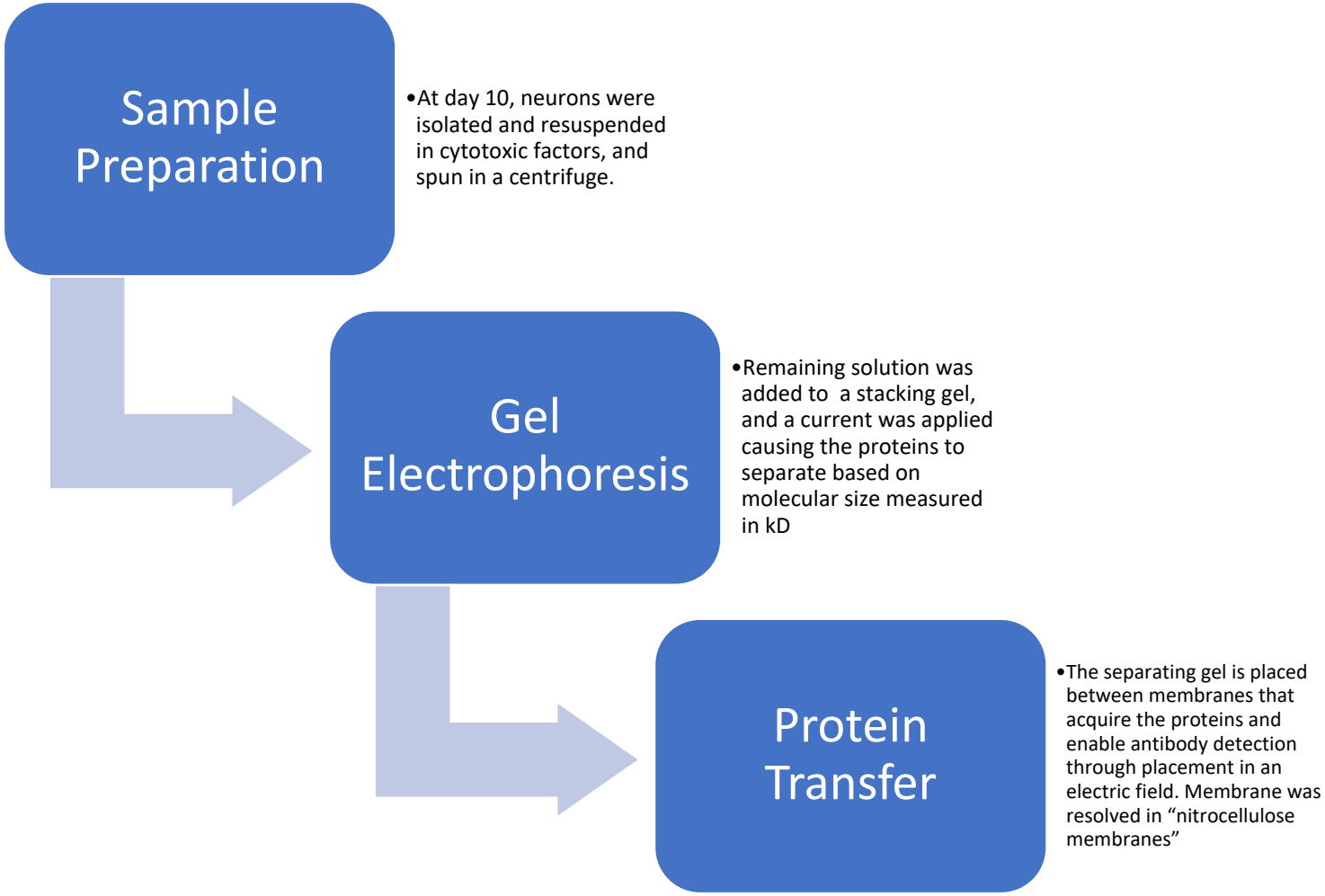
Obtaining mouse hippocampus cultures

Cultures of dissected mice hippocampal neurons were obtained as previously reported. [13] In brief, hippocampi at embryonic day 16 were dissociated in minimum essential medium supplemented with 10% fetal calf serum, 100IE insulin/l, 0.5mM glutamine, 100U/ml penicillin/streptomycin, 44mM glucose, and 10mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES). Following centrifugation, cells were resuspended (serum-free neurobasal medium supplemented with B27, 0.5mM glutamine, 100U/ml penicillin/streptomycin and 25μM glutamate), and 8×10^4 cells/well were plated in 24-well dishes on cover slips precoated with poly-L-lysine/collagen (all ingredients from Gibco/BRL, Eggenstein, Germany).

- Cells from the mouse hippocampus were first obtain from a lab
- Cells were then applied to a growth solution (“fetal calf serum”, ...)
- Cells were then spun in a centrifuge
- Cells were then resuspended (mixed in, usually after centrifugation), into another serum containing a mix of chemicals usually present within the cellular space of neurons

Western Blot of Mouse Hippocampus Cells

Goal: isolate antibodies from a protein cocktail



Western Blot of Mouse Hippocampus Cells

Goal: isolate antibodies from a protein cocktail

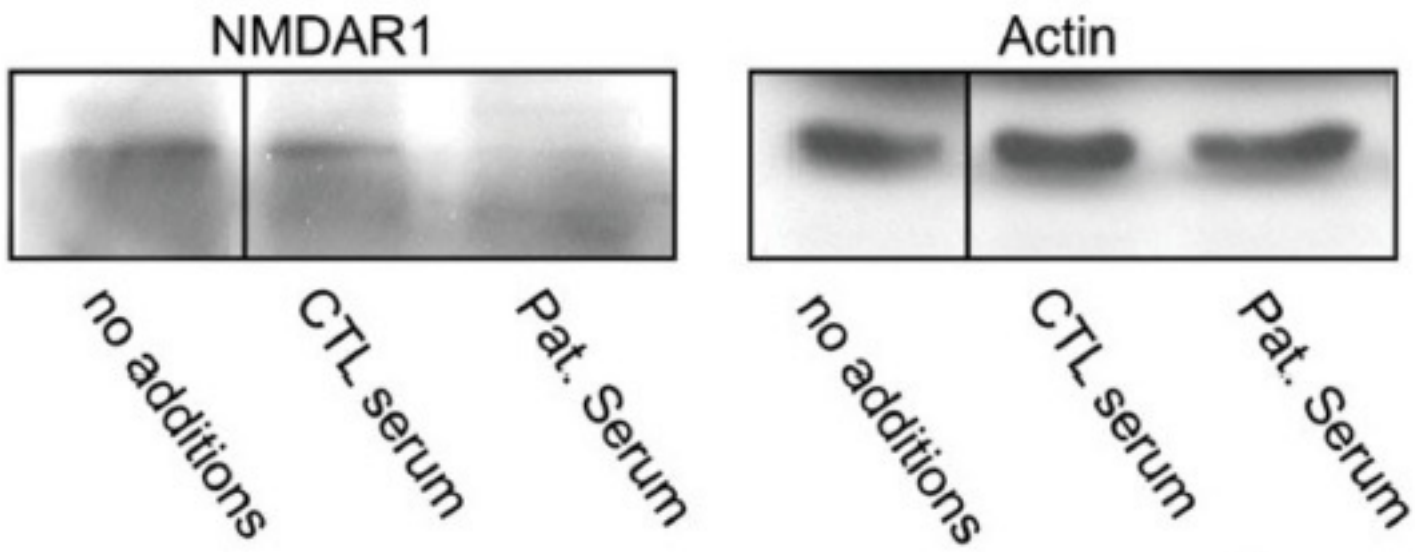
Blocking & Antibody Application

- Antibodies specific to the protein, protein subunit, or peptide of interest (in this case, the NR1 subunit) is introduced to the membrane in such a way that they do not bind non-specifically.

Observation & Indexing

- The antibodies are brought to illuminate. Based on the intensity of the light, the density of the protein can be assessed. The presence of luminescence also means that the protein of interest is present. The

Western Blot of Mouse Hippocampus Cells – Results

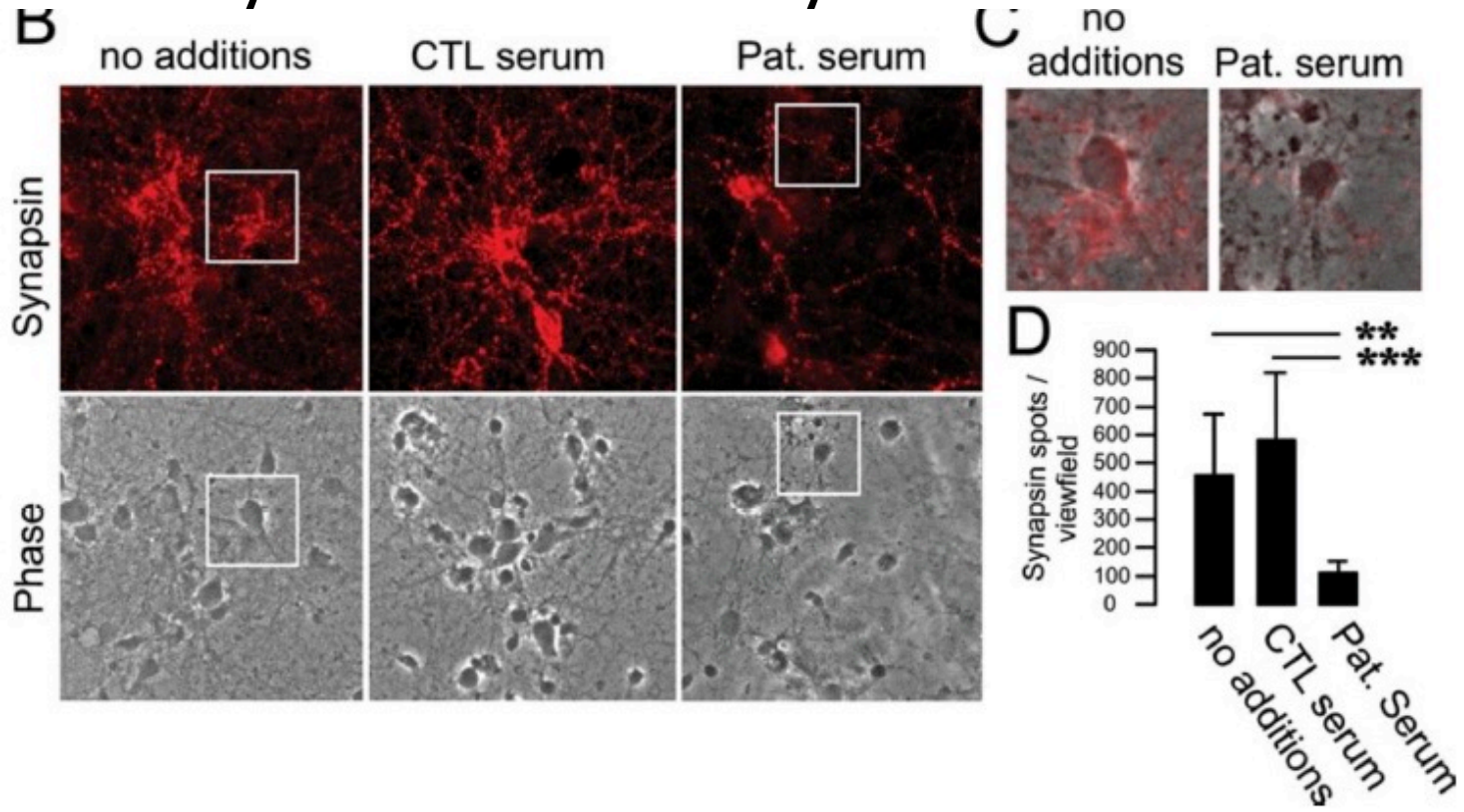


- Actin (control): no observed downregulation as no antibodies against actin were created as they had HSE
- NMDAR – NR1 subunit: In the patient serum, the presence of the NR1 subunit of NMDAR is lower than when no patient serum or CSF was added
- Thus, the patient antibodies lead to deterioration of NMDAR

Immunocytochemistry

- The researchers are also able to quantify the downregulation of NMDARs by staining the plates and comparing the presence of NMDAR for plates treated with patient serum/CSF and compare it to a control
- Anti-synapsin antibody was used for staining (does not downregulate NMDAR on its own – is just a staining technique)
- After three days of exposure to the patient serum, the fluorescence of each sample was observed
- However, without converting to grayscale, a complex image is derived and cannot be assessed quantitatively

Immunocytochemistry – Results



- On day 1, the presence of the red dots would be virtually the same
- However, after three days, the red dots have reduced in number, suggested a decline in the amount of NMDAR due to the presence of anti-NMDAR antibodies in the patient serum/CSF
- In grayscale, the brightness of the surrounding anti-synapsin antibodies is significantly lower in the patient serum, suggesting once again that NMDAR antibodies was downregulated

General Results

- In 13/44 patients, an immuno-positive result was observed
- Breakdown by Ig class:
  - IgA: 9
  - IgG: 5
  - IgM: 9
- Recombinant immunofluorescence confirmed the presence of NMDAR antibodies
- Furthermore, the Western Blot and immunocytochemistry procedures quantified the downregulation (decline) in the amount of NMDAR present in the mouse hippocampus cells after being exposed to the patient serum

General Results

- In 2 of the patients, they demonstrated immunity against both the NR1 and NR2 subunits of NMDAR
- Antibodies against cancer or other diseases were not detected, suggesting a strong correlation between HSE and the development of the anti-NMDAR antibodies
- Even when the researchers lowered the initial concentrations of the CSF and serum, 11/44 tests com out as immuno-positive (25%) and lead to the downregulation of NMDAR
- IgM wasfound to downregulate NMDAR at a similar level to IgA and IgG (novel insight)

General Results

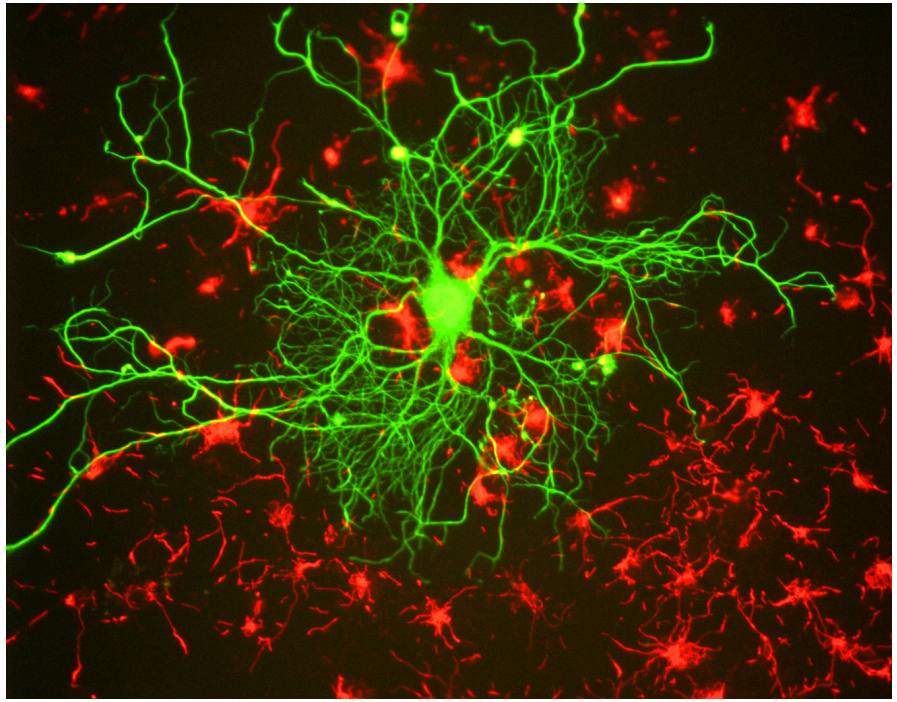
- In some patients, antibody titers for NMDAR were found in days 4-9 after diagnosis, suggesting that antibodies against NMDAR did not come about because of infection with HSV
  - Hypothesis: HSE symptoms were difficult to detect, and the antibodies began to develop after the onset of HSV instead of the point of diagnosis
- However, when treated with acyclovir, clinical symptoms reduced, and immunity toward NMDAR dropped
- After clinically presenting with HSE, patients largely experienced the same symptoms at the same intensities whether they were immuno-positive to NMDAR or not

General Results

- The presence of NMDAR antibodies did not lead to poorer symptoms?
  - The downregulation of NMDAR did not lead to worse cognition or pain
  - Undermines the usefulness of NMDAR in the nervous system
- However, being antibody-positive toward NMDAR did lead to a delay for clinical presentation
- However, the “clinical course of patients was not different”
- Downregulation of NMDAR was only observed in patients with IgG antibodies

Novel Insights

- IgG led to the worst effects in terms of the downregulation of NMDAR despite only playing an autoimmune role in 4/44 patients
- However, IgM also caused loss of NMDAR from the cell-membrane and a lower expression of synapsin (a general marker for synaptic receptors), similar to the previously-observed effects of IgG against NMDAR in other studies
- Thus, immunoglobulin of all classes play a role in NMDA-related encephalitis

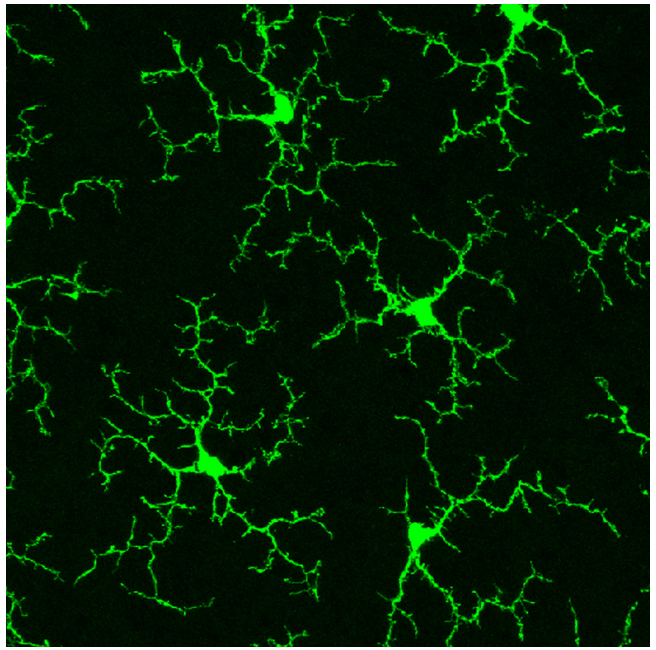


Conclusions

- At a rate of at least 25%, NDMAR antibodies were present in patients presenting with HSE and led to the downregulation of NMDAR
- Thus, physicians must way the potential effects of HSE on a macroscale (pressure from the skull) as well as a microlevel (lower expression of synaptic proteins)
- So, infection with HSV leading to encephalitis leads to autoimmunity to NMDAR
  - How?
  - Perhaps those 25% of HSE patients are presenting with anti-NMDAR encephalitis
  - Unlikely, given the low prevalence of both disease
  - There must be a causal factor

Conclusions

- Hypothesis: After cell lysis due to viral destruction, cellular membranes are presented to immune cells in the brain as antigens, leading to an autoimmune response
- Another hypothesis: The CNS inflammation in HSE patients caused an immune response and led to the identification of multiple antigens for a specific immune response, leading to the crossover between HSE and anti-NMDAR encephalitis
- Discussion Question: Which hypothesis serves as a more reliable reason for the autoimmune response to NMDAR?



Further Conclusions

- Despite the lack of more complicated clinical symptoms until the end of HSE treatment, NMDAR immunity does not bode well long-term
  - Has been linked to choreoathetosis in patients (jerky moments due to lack of inhibition in neurons) 1 month into treatment
  - However, this finding may be a side effect of acyclovir
  - Following treatment, seizures arise
- No long-term follow-up was conducted, preventing the study of continued NMDAR immunity

Clinical Implications

- Patients with HSE should be tested for NMDAR immunity
- Long-term follow-ups with patients immuno-positive for NMDAR after coming out of HSE should be conducted
- Additional screening on patients with IgA and IgM antibodies should be conducted since these antibodies were more frequent than IgG antibodies
- Patients with HSE and anti-NMDAR encephalitis may benefit from immunotherapy (ex: acyclovir)

Discussion Questions

- Should anti-NMDAR immunity treatments be a concern for researchers given the prevalence of other life-changing disorders such as diabetes and cancer?
- Would you expect HSV-1 immunity due to a vaccine to lead to lower levels of anti-NMDAR immunity?
- Technological tools allowed the researchers to observe the staining of the neurons at a cellular level and convert the synapsin-stained slides into grayscale images. Should we place a greater emphasis on technology instead of fundamental research?
- The antibody index of 4 is arbitrary (unless established from previous research). Should we rely on more quantitative means for research instead of trying to visualize the data qualitatively (ex: by using staining techniques)?

Citation

- Pruss, H., MD, Finke, C., MD, Holtje, M., MD, Hofmann, J., MD, & Klinbeil, C., MD. (2012). N-Methyl-D-Aspartate Receptor Antibodies in Herpes Simplex Encephalitis. *Ann Neurol, 72*(6), 902-911. doi:10.1002/ana.23689