Novel Vaccine for Neurological Disorders and Stroke

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Epidemiology of Stroke
Prevalence of Stroke in USA

- Stroke is the third leading killer in the United States, resulting in the following statistics annually (CDC 2001)
  - 750,000+ victims
  - 160,000 deaths (3rd leading cause of death)
  - 266,000 survivors with permanent disabilities
  - 30,000 new permanent admissions to nursing homes
  - Over 4 million living survivors of stroke
  - Every 45 seconds in the USA, someone has a stroke
  - Huge economic impact with costs of $40-$70 billion per year
Pathobiology of Stroke & Neurological Disorders
Inside the Network of human Brain

The communication network in brain has multi-trillion connections capable of performing 20 billion operations per second.

1. Neurons, which power the message
2. Neurotransmitters, which create the message
3. Receptors, which receive the message

However, Human brain biology is less understood.
Brain Receptors

- **Glutamate** is the principal neurotransmitter in the brain. Glutamate receptors are abundant in membranes of neurons. Glutamate is the most prominent neurotransmitter in the body, present in over 50% of nervous tissue. During normal conditions, glutamate concentration can be up to 1 mM. During abuse, higher glutamate concentrations cause the Neuron to kill itself by a process called apoptosis. This pathologic phenomenon can also occur after brain injury.

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- Glutamate receptors play a role in neurodegenerative diseases due to excitotoxicity and their prevalence throughout the central nervous system. The major glutamate receptor type is NMDA.
The NMDA receptor is an ionotropic receptor that allows for the transfer of electrical signals between neurons in the brain and in the spinal column. For electrical signals to pass, the NMDA receptor must be open. To remain open, an NMDA receptor must bind to glutamate and to glycine. An NMDA receptor that is bound to glycine and glutamate that open ion opening is blocked by a single magnesium ion (Mg$^{2+}$). An Mg$^{2+}$ ion is removed only when the electrical charge inside the cell rises to a specific value. The NMDA receptors have the special ability to let in large amounts of calcium ions (Ca$^{2+}$), an important mediator of glutamate’s toxic effects in humans.

As glutamate continually binds to non-NMDA receptors and allows the entry of positive ions, the cell’s voltage rises. Ultimately, the voltage reaches a certain value that causes the Mg$^{2+}$ ion to be removed from the opening of the NMDA receptor. Ca$^{2+}$ flows through the open NMDA receptor and causes various activities to occur that lead to cell death.
NMDA (N-methyl-D-aspartic acid) is an amino acid derivative acting as a specific Agonist at the NMDA receptor, and therefore mimics the action of the neurotransmitter Glutamate on that receptor.

In contrast to glutamate, NMDA binds to and regulates the above receptor only, but not other glutamate receptors. NMDA has three subunits of receptors NR1, NR2 and NR3.

Among these subunits, NR1 subunit is the most harmful factor in excitotoxicity. Antagonists of the receptors have held much promise for the treatment of conditions that involve excitotoxicity, including traumatic brain injury, Stroke, and neurodegenerative diseases such as Alzheimer's, Parkinson's, and Huntington's.
**In-Vitro** proof of our vaccine concept

NMDA (N-methyl-d-aspartate) receptors have 3 subunit families NR1, NR2 and NR3. We have chosen the NR1 subunit for blocking GLUTAMATE induced ionic toxins in the brain to prevent death of the nerve cells.

Receptors containing NR1 release excessive toxins into the nerve cells. Inactivation occurs when Glutamate (Glu) mediated NMDA NR1 is blocked by Anti-NMDA NR1 antibodies. A channel within the receptor complex can NOT be formed across the cell membrane. Excessive magnesium (Mg++) calcium (Ca++), sodium (Na+) and potassium ions (K+) do not enter the cell.

A. *In vitro* Optimized Glutamate mediated Ca++ toxicity blocked by antibodies to NMDA receptors. The cells were treated with mouse anti-NMDA-NR1 antibodies.

B. Untreated cells showed Ca++ toxicity and **Apoptosis** or programmed cell death. Programmed cell death involves a series of biochemical events leading to a characteristic cell morphology and death.
Rationale behind Stroke Vaccine
The Vaccine concept

Since the interest in chemical form of NMDA approach has waned in the pharmaceutical industry. The chemotherapeutic rationale for most small molecule NMDA antagonists is poor due to ineffective efficacy to treat neurological diseases and adverse effects at clinically effective doses. Example: Namenda® – NMDA receptor antagonist for Alzheimer’s disease.

JN discovered a biological vaccine that blocks NMDA receptors by acting as an antagonist to prevent glutamate from harming neurons. Thus, we may find prevention and therapy for Stroke and other Neurological disorders in humans.

This is one of the most promising pathways for treating neurological disorders based on the pathobiology of the brain and central nervous system.

NMDA (N-methyl-d-aspartate) Receptor Blocker Antibodies
VACCINE COMPOSITION

NMDA-NR1 vaccine was prepared using NMDA NR-1 subunit receptor in the form of recombinant protein or DNA.

The vaccine is coated with polymer (PLGA) based microparticles to target multiple neurodegenerative diseases:

- Alzheimer’s disease
- Epilepsy
- Multiple Sclerosis
- Diabetes
- Stroke
- Huntington’s disease
- Parkinson’s disease
1) The gene encoding the NMDA-NR1 subunit was expressed in insect cells to produce recombinant protein (r-protein) and was encapsulated in poly (DL-lactide-co-glycolic acid) (PLGA) microparticles by solvent exchange and used for oral immunization.

2) DNA encoding NMDA-NR1 was encapsulated in PLGA microparticles and used as an oral DNA vaccine to test its utility for preventing stroke. NR1 protein and DNA were evaluated separately for their protective efficacy in the endothelin-1 (ET-1) model of middle cerebral artery occlusion (MCAO) in NR1-vaccinated and non-vaccinated pigs.
NMDA-NR1 Expression as a recombinant protein in Insect cells

**NMDA Protein Expression in insect cells**

**REPORT**

Fig. Western blot of sf9 expressed NMDA. Lysates from sf9 cells transfected with pIEx4-OS1 were resolved on SDS-PAGE and blotted with anti-NMDA McAb from ProSci.

Lane 1, Total extract of sf9 transfected with pIEx4-OS1;
Lane 2, Total extract of sf9 w/o transfection as control;
Lane 3, Supernatant of transfected sf9 cell lysed in low salt buffer;
Lane 4, Pellet of transfected sf9 cell in low salt lysis buffer, and treated with detergents.
Lane 5, final pellet from lane 4 dissolved in loading buffer.

Prepared by Ocimum Biosolutions Inc.
IN International Medical Corporation
Confidential
The *in vitro* release study data were fitted into various equations to explain the kinetics of drug release of NR1 r-protein from the microspheres. *In vitro* experiments were conducted with pig gastrointestinal fluids. Storage at 39.5 degrees C for 6 months did not affect release.
Scanning electron microscopy photograph of PLGA microsphere which contains 100 µg r-NR1 DNA/mg microparticles.
Oral Delivery system

- The antigen uptake by M cells does not result in the degradation of the antigen, but rather in the delivery of the intact antigen to the underlying antigen-presenting cells.

- R-Protein antigens or DNA coated with PLGA do not degrade as they transit through the stomach and small bowel at various pH levels of intestinal fluids and IgA antibody produced in mucosal surfaces.

- Immunogenicity and therapeutic activity of the stroke vaccine is maintained.
Pre-clinical Rat and Mice Model

Results

- A single dose of the vaccine was associated with strong anti-epileptic, anti-Stroke neuroprotective activity in rats for both a kainate-induced seizure model and a Middle Cerebral Artery occlusion stroke model at 5 months following vaccination to Rats and Mice.
Vaccine – NMDA- NR1

- NMDA NR1 subunit receptor DNA and r-Protein vaccines generated auto-antibodies that targeted a specific brain protein, the NR1 subunit.

- After per-oral administration of the vaccine, the NMDA-NR1 protein persisted for at least 5 months and was associated with a robust humoral response in the absence of a significant cell-mediated response.

- This single dose vaccine was associated with strong Anti-epileptic and neuroprotective activity in rats and mice for both a kainate-induced seizure model and a middle cerebral artery occlusion Stroke model at 5 months following vaccination.

- Thus, a vaccination strategy targeting a brain protein is feasible and may have therapeutic potential for neurological disorders.
Experimental epilepsy

- We used the kainate model of temporal lobe epilepsy to determine potential anti-epileptic and neuroprotective efficacy of NMDA-NRI vaccination. Rats and mice received NMDA-NR1 or control vector, and a group of naive control rats received no vector.

- At 1 month following vaccination, animals were administered kainic acid (10 mg/kg body weight) intraperitoneally (i.p.). A blinded observer determined over 2 hours whether there were any signs of the characteristic progression through behavioral seizure stages, including immobility and staring, "wet-dog-shakes," unilateral and bilateral forelimb clonus, and facial clonus. (Clonus: a series of rapid repetitive contractions and relaxations in a muscle during movement, which is characteristic of epilepsy seizures).
Experimental epilepsy - Results

• Electroencephalographic (EEG) recordings showed the first signs of electrographic seizure activity within 10 min after drug administration in control animals, with all control animals developing facial and forearm clonus and proceeding to status epilepticus (SE).

• Seven vaccinated NMDA-NRI rats and mice showed neither EEG changes nor any behavioral changes after kainate treatment \( (p = 0.007, \text{ chi square analysis with expected } SE \text{ frequency of 78% reduced to 32% in the NMDA-NRI immunized group}) \).
Experimental Stroke

- We used the Endothelin-1 model of middle cerebral artery occlusion (MCAO) to determine the anti-stroke and ischemic neuro-protection efficacy of NMDA-NRI vaccination.

- Three groups of rats and mice, vector, control, and NMDA-NRI underwent MCAO 5 months after vaccination. At 3 days after endothelin-1 administration, rats were euthanized, the brains were removed, and the infarct volume was determined by a blinded investigator.

- **Vaccination effects on behavior.**
  1. To determine whether vaccination was associated with any changes in motor behavior and memory loss, rats and mice were tested by circular track and line crossing mobility paradigms. Vaccinated animals were hyperactive for a period of 3 days.
  2. We found no difference between vaccinated and un-vaccinated animals. Food flavor experiments did not demonstrate a difference between the groups.
### Exercise behavior for rats on treadmill

#### Control Rats

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#### Vaccinated Rats

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Behavior Experiments after Vaccination with NMDA-NR1
Rat Behavior after Experimental Stroke
Un-Vaccinated (Left and Right pictures) Vaccinated (Middle)
Experimental Stroke & Epilepsy - Histopathology of Rats

REDUCED ISCHEMIC DAMAGE IN CORTEX AND STRIATUM USING NMDA VACCINE.

Vaccine: Stroke: A: Undamaged brain cortex (Vaccinated), B: Damaged (unvaccinated)
Vaccine: Epilepsy: E: Undamaged brain cortex (Vaccinated) F: Damaged (unvaccinated)

C: This is interventricular hemorrhage induced by ET-1. IVH arose in the germinal matrix region in Rats. GMR is susceptible to "STROKE".

D: Hemiation compression and Duret hemorrhage, as seen here in the Epilepsy induced rat model
Rat model for post-traumatic Epilepsy and Stroke in un-vaccinated compared to vaccinated animals - EEG readings
ET-1 induced mice vaccinated with NMDA NR1 protein showing recovery from “STROKE”

Hypothetical mechanism of action of JN’s NMDA-NR1 Stroke Vaccine: The NMDA receptor is an ionotropic receptor that allows for the transfer of electrical signals between neurons in the brain and in the spinal column.

For electrical signals to pass, the NMDA receptor must be open. To remain open, an NMDA receptor must bind to glutamate and to glycine. An NMDA receptor that is bound to glycine and glutamate and has an open ion channel is called "activated."

Chemicals that “deactivate” the NMDA receptor are called antagonists (e.g., autoantibodies against JN’s NMDA-NR1 Vaccine). The NMDA receptor on nerve cells is unique in that it has various properties not found in other types of receptors.

A. First, the NMDA receptors have the special ability to let in large amounts of calcium ions (Ca2+), an important mediator of glutamate’s toxic effects in Human., e.g., autoantibodies against JN’s NMDA-NR1 Vaccine has the ability to stop Ca2+ influx into the brain (proved hypothesis by in vitro cell cultures)

B. A second important property of the NMDA receptor is that its opening is blocked by a single magnesium ion (Mg2+). An Mg2+ ion is removed only when the electrical charge inside the cell rises to a specific value., e.g., autoantibodies against JN’s NMDA-NR1 Vaccine may neutralize the excess Ca2+ and creates blockage or Mg2+ replacement (unproved hypothesis).
Evaluation of Preventive and Therapeutic Efficacy & Safety of NMDA-Receptor Blocker Stroke Vaccine in Swine Model
Swine Model - Pig's anatomy is similar in certain respects to human. Swine are increasingly used in research.

Humans and swine have similar anatomy, physiology, biochemistry and pathology. Pigs respond to drugs in the same way. We have similar lung size and functional capacity, analogous renal and pancreatic physiology;

Humans and pigs are monogastric (a simple single-chambered stomach) omnivores and the skin is of the same thickness and porosity.
Pigs allocation of treatment groups and Clinical Observations

**Allocation of animals per group:**
- Treatment 1: NMDA NR1 r-protein = 12 pigs;
- Treatment 2: NMDA NR1 DNA = 12 pigs;
- Treatment 3: PLGA = 12 pigs;
- Treatment 4: Vector = 12 pigs;
- Treatment 5: PBS = 12 pigs

**Clinical Observations**
Clinical signs and body temperatures of all pigs were collected daily for 8 days following vaccination with NMDA-NR1 r-protein or DNA PGLA microparticles. Clinical sign scores: labored breathing, abdominal breathing, anorexia, apathy and coughing. Blood was collected at 0, 4, 8, and 24 weeks. Scores of 0 (normal), 1 (slight), 2 (marked), or 3 (severe) were based upon the increase in respiratory rate, respiratory effort, presence of anorexia (eating disorder), and degree of lethargy.

**Detection of Antigen-Specific Antibodies**
The concentrations of NMDA-NR1-specific antibodies in serum (IgG), nasal secretions (IgA), and fecal samples (IgA) were determined by an enzyme-linked immunosorbent assay (ELISA) using NMDA NR1 r-protein.

**Cell Mediated Immune Response**
CMI responses were measured by the procedures described earlier by Furesz, S.E. et al. (1997) and Reddy. J. R. (2000).
Clinical Scores

All animals showed clinical scores less than 1.

[0 (normal), 1 (slight), 2 (marked), or 3 (severe)]
Cell Mediated Immune Response
There were no detectable cell mediated immune responses after vaccination with NMDA-NR1 r-protein or DNA. CMI response was also not detected in Rats in the previous study.
Figure 7. Neuroprotective efficacy of NMDA NR1 against Endothelin-induced MCAO reduction of cortical volume of brain damage (mm3) of Swine
Therapeutic efficacy for NMDA-NR1 against Stroke induced by ET-1 in unvaccinated (placebo) animals.

The temporal window of therapeutic efficacy for NMDA-NR1 with regard to its neuroprotective action was characterized in a separate group of animals. Although intravenous injection of NMDA-NR1 r-protein (200 µg/kg) at 1 or 30 min after endothelin-induced MCAO reduced the volume of cortical brain damage by 70% and 40% respectively, NMDA-NR1 was ineffective when administered after 60 or 80 min. NMDA-NR1 also reduced the volume of striatal damage in animals treated within 30 minutes.

The functional outcome paralleled the histopathology. Neurological deficit scores returned to normal within 80 hr in animals treated within 30 min.
A. The temporal window of neuroprotective efficacy for intravenous NMDA-NR1 protein (200 mcg/kg) administered after endothelin-induced MCAO is less than 30 min.

B. Data are the mean volume of ischemic brain damage in cortex for groups of 8-12 animals from control groups and NMDA-NR1 r-protein treated pigs.

C. Statistical comparisons were performed by using ANOVA with post hoc Scheffé's analysis (*p < 0.05 comparison of drug and vehicle groups; p < 0.05 for comparison of vehicle groups with the 1 min control groups).
R-Protein Quantities in Blood after Treatment
R-Protein Quantities in Cortical Tissue after Treatment

![Graph showing R-Protein Quantities in Cortical Tissue after Treatment](image)
The Electroencephalogram (EEG) in 10 seconds after treating with ET-1

- Three examples of short segments of EEG recordings are presented. In this example, the EEG trend was calculated for 30-minutes after ET-1 treatment epochs, overlapping 30-180 minutes.
- **A.** NMDA NR1 oral vaccinated animals: The left EEG shows minor seizures with an abrupt ending of electroencephalographic seizure activity within 10 seconds after induction with ET-1.
- **B.** Placebo vaccinated animals treated with NMDA NR1: The center EEG is from a pig with a minor neurological deficit (mild right-side hemiparesis, NIHSS=3) upon treated with NMDA NR1, 30 minutes after MCAO.
- **C.** Placebo animals: The right EEG is from a pig with a right cerebral infarct (NIHSS=13) showing polymorphic delta activity and development of subtle symptomatic focal seizures
ET-1 HEMORRHAGE IN PIGS

Hemorrhage involving the basal ganglia area tend to be non-traumatic and caused by ET-1. A mass with midline shift with secondary edema.
The large hemorrhage in brain arose in the basal ganglia region. This is one cause of human "stroke". This the ET-1 induced "STROKE" in pigs.
Potential use of NMDA NR-1 in Diabetic retinopathy

Considering these neural alterations manifest in non-proliferative diabetic retinopathy, we are now exploring the therapeutic potential of a novel neuroprotective factor of NMDA NR1 vaccine to protect retinal structure and function.

We are interested in the potential use of NMDA to treat an increasing number of neurological disorders following its successful use in the treatment of hypoxic-ischaemic brain damage, retinal ischaemia, light-induced retinal degeneration, diabetic neuropathy and stroke in humans.

ET-1 induced retinal effect can cause serious visual impairment of the affected eye. This may be due to irreversible damage to the retina, caused by hypoxia as well as ischemia of retinal cells, such as the photoreceptor or Müller cells. To analyze the damage to photoreceptor cells, we began studies to investigate the level of cyclic guanosine monophosphate (cGMP) in the surrounding fluids. cGMP was chosen as a marker since it is an important molecule in the metabolic cascade of photoreceptor signal transduction. The production of cGMP is catalyzed by guanylyl cyclases (GCs), which are found in photoreceptor cells and inner retinal neurons.
A. Vaccination resulted in marked reduction of neurological deficit scores and neuronal damage after Endothelin-1–induced MCAO. Posture (rota rod performance), sensory hemi-neglect, and neurological deficit scores returned to pre-ischemic levels in vaccinated animals within 80 minutes and pigs remained symptom free until the end of the study (24 weeks).

B. A significant reduction of cortical damage was observed in response to vaccination with NMDA-NR1 r-protein or with NMDA-NR1 DNA. In addition to cortical protection, NMDA-NR1 r-protein or DNA induced full-scale protection from neuronal stroke.

C. The area of brain damage at eight predetermined brains was assessed using light microscopy by an observer who was unaware of the treatment groups. The volume of brain damage was calculated by integration of the cross-sectional area of damage at each stereotaxic level and the distances between the various levels (Park et al., 1989; Sharkey and Butcher, 1994).
Blood Brain Barrier

Autoantibodies to NMDA NR1 would have minimal penetration into the brain under normal (basal) conditions, due to the blood brain barrier, and would avoid toxicity seen with small molecule antagonists.

Following cerebral insult, the autoantibodies would efficiently pass into the brain, block the receptor and reduce NMDA receptor mediated injury.
NMDA-NR1 r-protein or DNA vaccinated animals did not show the Stroke like symptoms and clinical abnormalities after ET-1 induced Stroke. We found no anatomical or histopathological abnormalities in the vaccinated animals.

The results of Swine and Rat model experiments showed similar response against ET-1 induced Stroke
NMDA mediated learning and memory - Food-flavor memory experiment

The NMDA class of glutamate receptors has a critical role in the induction of long-term memory.

Working memory is stored by the maintained firing of a memory-specific subset of neurons in networks of the prefrontal cortex.

We performed NMDA-NR1 receptor based vaccination. Treatment for Stroke did not damage the Hippocampus. We tested the animals’ ability to retain and maintain memory by FOOD-FLAVOR-EXPERIEMNTS in Pigs.

Pigs were trained to find food before induction of stroke.

Pigs’ favorite food was buried in a pit hole covered with hay and treatment groups of pigs were left in their pens to find the food.

Training

Buried food
## Treatment Effects on Memory Retention

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A method for producing therapeutic vaccine which consist of NMDA-NR1 subunit expressed in insect cells to produce recombinant protein and was encapsulated in poly(D-L-lactide-co-glycolic-acid) (PGLA) microparticles by solvent exchange and used for oral immunization. Thus the experimental model for stroke has been developed for the study of powerful N-methyl-d-aspartic acid (NMDA) NR1 sub units, their protective and therapeutic potential for treatment of the neurological disorder of stroke in animals and its practicability for therapy in humans.
**Preventive and Therapeutic Vaccine for Stroke and Neurological Disorders**

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<td>Abstract:</td>
<td>A method for producing therapeutic vaccine which consist of NMDA-NR1 subunit expressed in insect cells to produce recombinant protein and was encapsulated in poly[(D-L-lactide-co-glycolic-acid)] (PLGA) microparticles by solvent exchange and used for oral immunization. Thus the experimental model for stroke has been developed for the study of powerful N-methyl-D-aspartic acid (NMDA) NR1 sub units, their protective and therapeutic potential for treatment of the neurological disorder of stroke in animals and its practicability for therapy in humans.</td>
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<td>Eurasian Patent Organization (EPO)</td>
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<td>Publication Language:</td>
<td>English (EN)</td>
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<tr>
<td>Filing Language:</td>
<td>English (EN)</td>
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Conclusions

WIDE RANGE BENEFITS OF THE VACCINE

- Our stroke vaccine generates auto-antibodies whose access to the brain and neuroprotective regulation may hold promise as a prophylactic measure to protect the brain. Moreover, the ability of systemic immunization to generate auto-antibodies which bind to, and thereby alter the function of native brain proteins, opens new possibilities for modulating the nervous system and treating Neurological disorders.

- The present pre-clinical study showed stroke treatment and prevention, and could be a promising treatment or cure for other neurological disorders such as Stroke, Epilepsy, Diabetes, Alzheimer, Parkinson, and Huntington diseases.

- Oral immunization with recombinant NMDA-NR1 produced a humoral response which persisted over many months—and may possibly persist for years, resulting in life-time protection against some Neurological disorders.

- One possible limitation is that the chronic elevation of these auto-antibodies may eventually have undesirable effects on brain function.

- These experiments and additional safety studies must be investigated before any translation of this technology to the clinic.

- Markers to find side effects for human studies are necessary.
JN’s NMDA-NR1 biological vaccine may treat and prevent……….
References


5. Anti-NR1 N-terminal-domain vaccination unmasks the crucial action of tPA on NMDA-receptor-mediated toxicity and spatial memory.


References


3. Prevention of in Vivo Excitotoxicity by a Family of Trialkylglycines, a Novel Class of Neuroprotectants.


References

One human study in Russia with 270 patients showed the presence of NMDA receptor autoantibodies in the serum after a stroke.

Neurological recovery occurred in individuals with significant antibody titers but poor prognosis or death was associated with the absence of NMDA receptor autoantibodies.

This data further supports the clinical relevance of the vaccine.

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