RESEARCH PROTOCOL

Introduction of Gamma-Aminobutyric Acid into the Bloodstream to Negate NMDA Receptor Hypofunction Induced by Delta 9-THC

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Abstract

Glutamate is the most abundant neurotransmitter found in the brain, controlling fast signalling throughout all sections and being especially involved in memory recollection and learning. Long-Term Potentiation (LTP) is the strengthening of neural connections through receptor synthesis over consistent usage, first triggered by synapse activation by a small amount of glutamate. However, in heavy (prolonged instance of exposure) and habitual users of cannabis, the effects of LTP are exacerbated by N-methyl-D-Aspartic Acid (NMDA) Receptor Hypofunction (NRHypo) which in turn affects memory, learning, reasoning and other aspects of one's function. Emerging evidence has associated the inhibition of long-term potentiation by Delta 9-Tetrahydrocannabinol (D9-THC) activating presynaptic Cannabinoid Receptor Type 1 (CB₁) receptors to the inhibition of the ability to stop production of glutamate (GLU). An excess of glutamate will overstimulate the postsynaptic NMDA and α-Amino-3-Hydroxy-5-Methyl-4-Isoxazolepropionic Acid (AMPA) receptors in the neurons commonly in the hippocampus, basal ganglia, and prefrontal cortex, which allow excessive influx of calcium Ca^{2+} ions, causing neurotoxic conditions. Glutamate Decarboxylase 67 molecule has been shown bind in high concentrations with GLU and lower the harmful effects of D9-THC on the brain by converting GLU to Gamma-Aminobutyric Acid (GABA), an inhibitory neurotransmitter. GAD67 will be distributed to mice in this proposed experiment and the behaviour of the mice will be monitored. D9-THC affected, D9-THC and GAD67 affected, and normal mice will be subjected to behavioral interaction and maze tests which will show differences in their learning, spatial awareness and orientation, and reasoning abilities. Chemical analysis of cerebral fluid and brain slices will determine chemical concentrations of GAD67 and D9-THC in the brain. Using direct injections into the cerebrospinal fluid (CSF) and bloodstream in mouse models, our aim is to determine the selectivity of the blood brain barrier (BBB) to enzymes such as GAD67 via both channels as well as assess the interaction GAD67 has with cascading neurological effects caused by NRHypo and LTP.

Keywords: blood brain barrier; long-term depression; long-term potentiation; memory; neurotransmitters; neurotoxicity; NMDA receptor hypofunction; synapse

Introduction

Glutamate (GLU) utilizes multiple pathways to act as a fast-excitatory neurotransmitter within the synapse. Upon the arrival of the action potential at the presynaptic axon terminal, the stored GLU is released into the synapse, which activates receptors on the postsynaptic dendrite. Individual receptors have various effects, such as opening calcium channels to form graded potentials that continue through the dendrite and into the cell body and axon, forming the next action potential. In the glutamatergic pathway, GLU binds to AMPA and NMDA receptors (AMPAR and NMDAR) on postsynaptic neurons to induce an action potential by post-synaptic opening of calcium Ca^{2+} ion channels. The depolarization of the dendrite due to

Yan et al. | URNCST Journal (2020): Volume 4, Issue 3 DOI Link: <u>https://doi.org/10.26685/urncst.175</u> the influx of positive ions forms a graded potential, which travels to the cell body and summates with other graded potentials to form an action potential, conducting the signal forwards. If AMPARs and NMDARs are rapidly stimulated in minute amounts, long-term potentiation (LTP) is produced. LTP is the resultant cascading effect of AMPAR and NMDAR stimulation which leads to the proliferation of such receptors on postsynaptic dendrites [1]. As glutamate is not rapidly degraded or recycled by the cells, leftover concentrations in the synapse allow for lower amounts of future glutamate release to trigger NMDAR and AMPAR activation [1, 2].

With the legalization of marijuana, an interest for new innovations to use cannabinoids for medicinal properties



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has dramatically increased. Cannabidiol (CBD), a major constituent within marijuana, is opening new paths in biomedical research; adult neurogenesis [3, 4] and cancer therapeutics [5] are among the myriad of possible benefits of the cannabinoid [4]. CBD, however, is becoming increasingly absent in the composition of marijuana being sold, with delta 9-tetrahydrocannabinol (D9-THC) dominating the chemical composition of the plant [6, 7]. D9-THC contributes to many of the feelings of being "high", including but not limited to: euphoria, muscle relaxation, disorientation, increased sensitivity to stimuli, confusion, psychosis, cold sweats, and loss/impairment of memory and recollection of time. Additionally, it is a psychoactive cannabinoid and has been linked to negative long-term neurological effects such as neuron excitotoxicity and decreased sensitivity to ligands, requiring a higher concentration of ligands to achieve the same degree of stimulation [8–11].



Figure 1: NMDA (red) and AMPA (light blue) receptors are being activated by GLU (black) on a synapse. Presynaptic calcium (green) results from the action potential travelling to the terminal, while postsynaptic calcium triggers the graded potential by entering through voltage-dependent calcium channels (VDCC). LTP occurs when GLU is released in small amounts at high frequencies, when a concentration of leftover GLU combines with newly released GLU to stimulate larger graded potential amplitudes.

Cannabinoids, both endogenous and exogenous, bind to presynaptic receptors CB_1 and CB_2 on the axons of presynaptic neurons, the former being common specifically in the hippocampus, a structure of the brain associated with memory [12,1]. Cannabinoids binding to the CB_1 receptor inhibit the release of GLU, an excitatory neurotransmitter [11,13].

If, however, the presynaptic neuron is inhibited enough, a state known as NMDA Receptor Hypofunction (NRHypo) on the postsynaptic neuron is produced [14]. A positive feedback loop is created such that the presynaptic axon terminal increases the synthesis of GLU because of NMDA and AMPA inactivity on the postsynaptic dendrite [14]. However, GLU release remains inhibited by activated CB₁ receptors and accumulates in the presynaptic axon terminal. Later dissociation of the cannabinoid from the CB₁ receptor results in overstimulation of the postsynaptic neuron by the accumulated GLU constantly activating NMDA and AMPA receptors, which allows calcium Ca²⁺ to flood the

Yan et al. | URNCST Journal (2020): Volume 4, Issue 3 DOI Link: <u>https://doi.org/10.26685/urncst.175</u> dendrite. The resulting state is also commonly induced by phencyclidine, an anesthetic [14] which induces hallucinogenic effects, feelings of dissociation, and is found commonly laced in illicit or homemade marijuana [15]. While regulated marijuana does not contain phencyclidine, an effect of lower severity is still present due to the effects of D9-THC.

This overstimulation due to NRHypo would be regulated by LTP, as more NMDAR and AMPAR is synthesized in order to receive more GLU. However, with prolonged instances of D9-THC exposure, the effects of NRHypo are also prolonged and increase due to the upregulation of postsynaptic receptors which are signaling for release of GLU. Thus, NRHypo and LTP synergize to allow an increase of Ca^{2+} ions to enter the postsynaptic neuron, and in such excess, would result in a host of cascading events, the likes of which can result in neurodegeneration [2].

NRHypo has been reported to induce the creation of neurofibrillary tangles seen in Alzheimer's disease patients

[14] as well as neurotoxic effects seen in Schizophrenic patients [14, 16]. The same effects of chronic D9-THC usage can be seen in people with schizophrenia and neurodegenerative disorders [17]. The latter is also commonly exacerbated by chronic and/or heavy marijuana use [17, 18]. Hence, the consumption of marijuana, specifically D9-THC, and its inhibitory actions on the presynaptic release of GLU by activation of the CB₁ receptors, is a driving factor for NRHypo and, when coupled with the effects of LTP, pose a greater risk for heavy (prolonged instances) and or chronic marijuana users as they are more likely to develop neurodegenerative behavioral phenotypes, primarily, the decreased capacity to encode and recall memory.

Many compounds have been known to compete with D9-THC, including AM251 and Cytochrome P450, which are inverse agonists and antagonists respectively [19, 20]. We, however, propose alternative methods for tackling the synergistic effects of NRHypo and LTP by way of GLU regulation rather than D9-THC metabolism. Gamma-Aminobutyric acid (GABA) is a derivative of GLU through the enzyme Glutamic Acid Decarboxylase (GAD) and has few potential negative impacts on brain chemistry and neuron stability even in high concentrations. GABA is also an inhibitory neurotransmitter, which can help reduce overstimulation due to the excess GLU by the inhibition of graded potentials and the hyperpolarization of the postsynaptic dendrite. However, the efflux rate of GABA from the brain is at least 16 times higher than the influx rate with a concentration half-life of less than 20 minutes, making direct sustained introduction of GABA to the brain ineffective [21]. GAD65 and GAD67 are the two GAD isoforms found in mammals, where GAD65 is found exclusively in neuron endings while GAD67 can be found throughout the cell [1, 22-24]. D9-THC lowers active GAD67 concentrations, reducing the amount of GABA made from GLU and increasing risk of GLU-induced neurotoxicity [1, 11, 25]. While GAD65 is more beneficial to GABA synthesis, GAD67 synthesizes GABA for protection from neural injury while GAD65 mainly synthesizes GABA for neurotransmission, making GAD67 more relevant. In addition, GAD67 is consistently active due to tight adherence to its activation factor while GAD65 reacts only to GABA demand such as neurotransmission, making GAD65 less likely to be able to rapidly synthesize GABA from GLU [26].

While D9-THC can easily pass through the blood brain barrier (BBB) that separates the circulatory system from the brain, enzymes have difficulty passing through, leading to significant brain concentrations of D9-THC that cannot be metabolized by small brain concentrations of GAD67 [27, 28]. D9-THC is a non-polar molecule, allowing it to pass through cell plasma membranes without the need for special channels. However, the BBB is more permeable under high blood pressure and large concentration gradients of molecules, which could allow

Yan et al. | URNCST Journal (2020): Volume 4, Issue 3 DOI Link: https://doi.org/10.26685/urncst.175 easier passage of enzymes [28]. GAD67 is a relatively large enzyme, which has led to significant complications in attempting to cross the BBB. Using intrathecal injections into the subarachnoid space, the BBB is partially or completely bypassed as the protein can be directly introduced to the CSF surrounding the brain. However, injections directly into the CSF have shown a rapid efflux into the spine and absorption back into the blood [27], where the challenge of the BBB becomes pertinent once more.

Mouse models are commonly used due to several factors. The physiology of mice is well understood and similar to humans, including in the BBB. Mice can also be selectively bred to introduce or knock out specific genes and keeping an identical genotype. Using genetically identical mice with near identical physical condition will allow consistent results within each group.

The following proposed experiment aims to determine the permeability of the blood brain barrier to enzymes such as GAD67 and the possibility of permeation of GAD67 to the brain via intrathecal injections using mouse models, and to investigate and assess the effects GAD67 has with the neurological cascades caused by the prolonged exposure to D9-THC.

With the introduction of GAD67 into the bloodstream, some of the enzyme should permeate the BBB and catalyze more GABA from GLU both in the brain and in the bloodstream. Osmolytic differences should also trigger osmosis of GLU from the brain into the bloodstream, further reducing GLU concentrations and lowering risks of GLUinduced neurotoxicity by calcium Ca²⁺ flooding [28]. With the direct introduction of GAD67 into the CSF, we hypothesize the protein will bypass the BBB completely, providing a more efficient method of delivery directly into the brain, and permeate into synapses. Injections into the subarachnoid space have the potential to partially or completely bypass the BBB, which would provide effective and rapid delivery of GAD67. The high flow rate of CSF out of the brain would still be a determining factor in the efficacy of the injection.

Methods

Our experiment will use three test groups of mice, of 7-8 each, to reduce suffering while measuring reasonable variance over a period of seven weeks [29]. All mice would be subject to the same living conditions (ex. food and light exposure). Prior to experimentation, CSF samples will be drawn and isolated for each mouse. GAD67 can be extracted from each mouse's β -cells in the pancreas, which synthesizes GAD67. To reduce the probability of autoimmune rejection, CSF and GAD67 extractions will remain specific to each mouse. Multiple samples will be taken throughout the experiment for analysis of D9-THC, GAD67, and GLU concentrations and to minimize the possibility of rejection.

The control group of mice will be given CSF samples without additives as a placebo. The second and third groups of mice would be subject to high-concentration doses of

D9-THC every other day administered through the bloodstream via intravenous injections. The third group will be administered GAD67 alongside D9-THC into both the bloodstream and cerebrospinal fluid via the subarachnoid space. GAD67 will be administered via intrathecal injections into the subarachnoid space. A maze test, in which the mice must find the same exit from the same starting point, will run for two days without injections, to familiarize the mice with the maze. Thereafter, the mice will run the maze trial twice each day for six weeks, with their corresponding injections twice, once before and after the first run. The second run will immediately follow the second injection. This is done to mimic the effects of prolonged marijuana use.

After being fed, each subject group will be placed in a single large containment unit such that their interactions and behaviors can be observed within each group, measuring degrees of interaction by time spent performing grooming, sniffing, and other investigative behavior on each other [18]. After 2 days of large containment, one mouse from each group will be randomly selected and placed into a container together, following the procedure of a social novelty test. Various stimuli will be added throughout the interaction such as a strobe light or background noise at a constant volume and pitch to ascertain the effects of D9-THC on affected mice. The test will be repeated multiple times to interaction and familiarizing the mice [30]. Motivation can be recorded through time spent investigating the other mouse and the number of interactions between each other. At the end of the experiment, each mouse would have its cerebral fluid extracted to test levels of D9-THC and GAD67 using UV absorbance, and its brain sliced to determine intracellular D9-THC, GLU, GABA, and GAD67 concentrations in various areas, such as the hippocampus, prefrontal cortex, and cerebellum.

Results

hypothesize that there will be significant We differences between the behavior of each group. Both the control and GAD67 groups should have at least basic interaction with other mice, while the D9-THC group would show a significant decrease in motivation to interact with others. We hypothesize that mice affected by D9-THC will exhibit delayed reaction time and a diminished interest to a novel introduction, while also presenting significantly lower recollection of previously introduced mice. Erratic behavior aligning with symptoms of intoxication, such as sensitivity to stimuli and disorientation, may occur in both the D9-THC-only and D9-THC-GAD67 groups, where the frequency and severity as well as any potential patterns would be monitored and recorded. The various stimuli during the interactions would be at first a source of interest of the control mice but be quickly filtered out. D9-THC affected mice would suffer increased sensitivity and disorientation to stimuli, as well as consistently focus.

Additionally, we hypothesize that groups will all show differing behaviors when put in the maze with each group having variable rates of memory recall, curiosity, and reaction speed (ex. realizing dead ends) [25, 31]. Using the performance

Yan et al. | URNCST Journal (2020): Volume 4, Issue 3 DOI Link: <u>https://doi.org/10.26685/urncst.175</u> of the D9-THC-only group and the control groups as extremes, we can determine the effectiveness of the intrathecal injections in reaching the synapses. If the enzyme is able to reach the synapses and synthesize GABA from GLU, GAD67-injected mice would show increases in learning speed and retention rate in the maze relative to the D9-THC-only group, and potentially be able to match the control group times as the excess GLU is converted, increasing the rate of learning and memory retention [24, 32]. D9-THC-only mice will show significantly lower capacity for memory recall in less improvement for maze times. Chemical inspection should also show reduced levels of GLU in the brain for the GAD67 group compared to the D9-THC-only group, indicating that GAD67 was able to penetrate into the brain.

Discussions

Mice display highly social tendencies towards one another with emphasis on novel intruders and stimuli. The addition of stimuli throughout the interactions would attract attention from the control group but should be filtered out over time. Control mice, in resuming normal behavior, show no sensitivity to stimuli. D9-THC affected mice, including the GAD67-D9-THC group, would be constantly affected by constant stimuli due to the increased sensitivity induced by D9-THC. Memory of the stimuli would not be easily recalled and should affect the D9-THC mice nearly every time as an effect of D9-THC. Erratic behavior and decreased social functions would also serve as indicators on the effects of prolonged exposure to D9-THC, which have not yet been clearly analyzed [17].

As the mice become accustomed to the maze, the time for each trial should decrease in the control group through LTP and spatial recollection with reasonable deviation within the group. As D9-THC induces reduced recall of memory and disorientation with sense of time, affected mice would display little to no decrease in time, and may also deviate considerably between each mouse. Lower GLU levels in the GAD67-D9-THC group would suggest the permeability of GAD67 through the subarachnoid space and into the extracellular matrix surround synapses. GAD67 bonded with its activator rapidly converts extracellular GLU into GABA [26]. The effects of neurotoxicity induced by NRHypo and LTP will be at least partially reduced as the severity of NMDA overexcitation decreases and regulation to normal GLU levels will stimulate NMDAR and AMPAR normally. GABAergic pathways would also hyperpolarize the postsynaptic dendrite, reducing neurotoxicity and reducing erratic behavior caused by overstimulation of graded potentials sent to the cell body. With the flow of CSF, administered GAD67 would be pushed into the spine. CSF composition analysis could indicate the concentration of GLU to GABA, which would determine whether GAD67 had actually diffused to the synapse and converted GLU to GABA.

Conclusions

The main purpose of this study is to explore treatments and therapy options for chronic marijuana usage. Marijuana's recent legalization in Canada has been a

controversial process, attributed in part to the government's lack of preparedness for plant sales [33]. Statistics Canada claims 1 in 3 users of cannabis will develop a dependency at some point in their life [33]. Young users are significantly more susceptible to chemical changes in the brain due unfinished brain development, exacerbating pre-existing conditions and amplifying the effects of long-term usage [32]. In Canada, a large portion of adolescence have already and most likely will continue use cannabis [34]. Our proposed research will lead to therapeutic measures for Canadian youth, as well as a great number of people who have or will acquire dependency to cannabis. Moreover, any results from our research will open avenues for other research pertaining to schizophrenia and neurodegenerative diseases [16].

List of Abbreviations

AMPAR: α-Amino-3-Hydroxy-5-Methyl-4-Isoxazolepropionic Acid Receptor BBB: Blood Brain Barrier CBD: Cannabidiol CNS: Central Nervous System D9-THC: Δ^9 -Tetrahydrocannabinol GABA: Gamma-Aminobutyric Acid GAD: Glutamate Carboxylase GLU: Glutamate LTP: Long-Term Potentiation NMDAR: N-Methyl-D-Aspartate Receptor NRHypo: NMDA Receptor Hypofunction

Conflicts of Interest

The authors declare they have no conflict of interest.

Ethics Approval and/or Participant Consent

This proposal did not require ethics approval, as it was a protocol designed for a case competition.

Authors' Contributions

JA: made substantial contributions to the design of the study, drafted and revised the manuscript, assisted with the collection and analysis of data, and gave final approval of the version to be published.

MZ: made substantial contributions to the design of the study, drafted and revised the manuscript, and gave final approval of the version to be published.

MY: made substantial contributions to study design and planning, drafted and revised the manuscript, assisted with the collection and analysis of data, and gave final approval of the version to be published.

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