RESEARCH PROTOCOL

Research Protocol: Investigating the Effect of Prenatal Nicotine Exposure on Short-term and Long-term Memory Using Behavioural and Molecular Models

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Abstract

Introduction: Prenatal nicotine exposure disrupts brain development, leading to conditions like ADHD, anxiety, and cognitive deficits by altering neurotransmitter activity and gene expression. Nicotine affects brain regions such as the prefrontal cortex, hippocampus, and amygdala, reducing brain volume, particularly in the frontal cortex and cerebellum. While research links prenatal nicotine to memory deficits, gaps remain in understanding its effects on molecular mechanisms like proteasomal activity, protein synthesis, and Late-Phase Long-Term Potentiation (L-LTP). This study combines behavioral tasks with proteasomal activity analysis to investigate these mechanisms.

Methods: This study design uses behavioural tests, Active Place Avoidance (APA) and Novel Object Recognition (NOR) and protein synthesis assessment to investigate the molecular and behavioral effects of prenatal nicotine exposure on short and long-term memory.

Anticipated Results: APA task: The number of entries into the shock zone, the duration spent in the shock zone, and the latency to enter the shock zone will be significantly different between experimental and control groups (p < 0.05). NOR test: The time spent exploring the novel object will be significantly different between experimental and control groups (p < 0.05). Protein synthesis evaluation: The t-test that evaluates proteasome activity will yield significant differences between experimental and control groups (p < 0.05). We hypothesize that mice prenatally exposed to nicotine (experimental group) will exhibit significant difference in proteasomal activity during L-LTP, alongside measurable difference seen in APA and NOR test performance, compared to the control group.

Discussion: Anticipated results suggest prenatal nicotine exposure affects protein synthesis and proteasome activity, key to L-LTP and memory. Challenges include ethical and logistical issues with animal use, nicotine variability, and maintaining experimental precision in APA and NOR tests. Measuring L-LTP and neuronal protein synthesis is complex, requiring advanced methods and standardized protocols to address region-specific processes and variability.

Conclusion: This study explores prenatal nicotine exposure's impact on memory through altered proteasome activity and behavioral tests (APA, NOR). By analyzing protein synthesis in L-LTP, it examines disruptions in synaptic plasticity. Future research could use fMRI to study protein synthesis in prenatally exposed individuals.

Keywords: prenatal nicotine exposure; L-LTP; memory; protein synthesis; short-term memory; long-term memory; APA; NOR; animal model

Introduction

Prenatal nicotine exposure has been linked to changes in neurotransmitter activity and neuroplasticity, including sudden infant death syndrome (SIDS), attention deficit hyperactivity disorder (ADHD), depression, and anxiety [1]. This is because nicotine disrupts normal brain development seen during the gestational period by disrupting normal gene expression, neurotransmitter release regulation, and synapse formation and maturation [2]. Nicotine is neurotoxic and can cross into the fetal serum and placenta [2]. At a molecular level, nicotine, the primary psychoactive compound in tobacco, is a potent alkaloid that exerts its effects through binding to nicotinic acetylcholine receptors (nAChRs) in the brain and peripheral nervous system. nAChRs connection to prefrontal cortex (PFC), hippocampus, nucleus accumbens (NAc), amygdala, and ventral tegmental area (VTA), subsequently stimulates dopamine (DA) release from neurons in the mesolimbic system with increasing firing rate. Some of the molecular mechanisms of how nicotine affects these brain areas have been studied. One significant connection being that prenatal exposure to nicotine leads to brain volume reduction in regions of the frontal cortex, caudate nucleus, NAc, frontal lobe, cerebellum and hippocampus [1]. The subsequent effect can be seen in phenotypic characteristics of neurological alteration: memory. The brain's integrity to memories is consolidated



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by the hippocampus, subiculum, and entorhinal cortex in accordance with explicit memories, while implicit memories through processes such as conditioning and habituation rely on the cerebellum and basal ganglia [3].

Long-term potentiation (LTP) refers to a long-lasting increase in synaptic plasticity strength between neurons. In the dentate gyrus of the hippocampus, LTP plays a crucial role in short and long-term memory. Long-term memories are said to require gene activation, protein synthesis, and synaptic connection while short-term memory does not [3]. Within this mechanism, we find a connection with the persistent long-lasting LTP phase called L-LTP in which protein synthesis is required. The molecular characteristic of LTP begins with N-methyl-D-aspartate (NMDA) receptor activation, which is triggered by glutamate binding and postsynaptic depolarization [3]. NMDA receptor is a glutamate receptor, the human brain's primary excitatory neurotransmitter NMDA receptor activation leads to an influx of calcium ions (Ca2+) into the postsynaptic neuron, in which Ca²⁺ binds to calmodulin, activating CaMKII [3]. Activated CaMKII phosphorylates α-amino-3-hydroxy-5methyl-4-isoxazolepropionic acid (AMPA) receptors and other synaptic proteins, strengthening synaptic transmission [3]. Rezvani et al. [4] demonstrated that adolescent and adult smokers exhibit reduced proteasomal activity, accompanied by increased levels of glutamate receptor subunits, including NMDA receptor subunit NR2A, and metabotropic receptor mGluR1a. These changes were attributed to nicotine's inhibition of proteasome chymotrypsin-like activity. However, there remains a significant gap in understanding how prenatal nicotine exposure impacts proteasomal activity and its downstream effects, particularly in relationship to the late phase of longterm potentiation (L-LTP), which is dependent on protein synthesis. For this reason, we suspect that prenatal nicotine exposure may lead to a reduction in proteasomal activity, which could disrupt L-LTP, a critical mechanism for synaptic plasticity, thereby disrupting spatial and long-term memory as well, compounded by hippocampal volume reductions frequently observed in nicotine-exposed offspring [1, 3]. To investigate these effects, we propose using the Active Place Avoidance (APA) task to assess spatial and long-term memory and the Novel Object Recognition (NOR) test to evaluate short-term memory. The APA task allows the assessment of subtle short-term changes in spatial learning and memory and the NOR test provides an index of recognition memory [5, 6]. These behavioral assessments will allow us to analyze how prenatal nicotine exposure translates molecular alterations, such as reduced proteasomal activity, into functional changes in memory. Therefore, we hypothesize that mice prenatally exposed to nicotine (experimental group) will exhibit a significant difference in proteasomal activity during late phase long term potentiation (L-LTP), alongside measurable difference seen in APA and NOR test performance, compared to the control group (mice without prenatal nicotine exposure).

Methods

Prenatal Nicotine Exposure Stage

Female NMRI mice in the experimental and control group (n=200) will be kept in 12h light/dark cycle [7]. 200 male mice will be introduced to the cage until pregnancy is detected [7]. The experimental group will receive 2–3 mg nicotine base/kg/day in a saline vehicle via injection for three weeks before mating takes place and during gestation. This is because the injected nicotine dosage stated mirrors moderate to heavy human smoking, providing physiological relevance and reducing confounding variables [8]. Female mice in the control group (n=100) receive saline (vehicle) to mitigate the potential confounding variable of the injection [9]. From the offspring, 200 mice will be randomly selected from the experimental and control groups, n=100 each.

Testing Short-Term Memory Using NOR and Long-Term Memory Using APA Task Stage

Nicotine-exposed offspring and control groups will undergo training and testing in both the Novel Object Recognition (NOR) and Active Place Avoidance (APA) tasks. For the NOR test, mice will first be introduced to an arena with two identical objects and allowed to explore for a fixed period of 3 minutes [6]. After a delay of 1 hour, only short-term memory will be assessed in which one of the objects will be replaced with a novel object [6]. The time spent exploring the novel object will be recorded, with increased exploration indicating intact memory. Further, we will use APA to assess long-term memory only. In the APA test, mice will be trained to avoid a shock zone identified by visual cues in a fenced arena. Long-term memory will be tested one week after training [5]. During testing, parameters such as the number of entries into the shock zone, the time spent in the shock zone, and latency to first entry will be recorded. To minimize stress, all mice will undergo a habituation period in which they are allowed to freely explore the arena for up to 2 minutes one day before testing [5]. Data from both tests will be analyzed using repeated-measures one-way ANOVA to compare performance between nicotine-exposed and control groups.

Testing L-LTP Through Protein Synthesis Stage

Following decapitation under anesthesia, 4 mm of dorsal hippocampal tissue will be extracted, with stereotaxic coordination, from offspring (postnatal day 30, following APA and NOR tests) in both experimental and control groups [8]. To assess protein synthesis involved in late-phase long-term potentiation (L-LTP), we will measure chymotrypsin-like proteasome activity. This will be done by homogenizing hippocampus tissue from both nicotine-exposed and control offspring in a lysis buffer

containing a proteasome inhibitor to prevent nonspecific degradation [10]. Proteasomal proteins will then be isolated for analysis.

We will assess proteasome activity using a fluorometric assay, which employs a specific fluorogenic peptide substrate, Suc-LLVY-AMC, that is cleaved by the chymotrypsin-like activity of the proteasome, releasing a fluorescent product [11]. Fluorescence intensity, which is proportional to chymotrypsin-like activity, will be measured using a microplate reader at an excitation wavelength of 380 nm and an emission wavelength of 460 nm [10, 11]. Fluorescence intensity values will be normalized to total protein concentration, quantified using a Bradford or BCA assay. A two-tailed t-test will then be conducted to compare proteasome activity levels between nicotine-exposed and control groups, identifying any statistically significant differences.

The described research methods provide valuable insights into the neurobiological and behavioral consequences of prenatal nicotine exposure, and to address key knowledge gaps. The combination of APA and NOR tasks provide a detailed analysis of spatial, recognition, short-term, and long-term memory. During the APA training phase, to reduce stress, mice will have a 2-minute habituation period in the arena one day before testing [5]. APA, with its emphasis on spatial learning, allows precise measurement of memory encoding linked to hippocampal function, while NOR is a reliable and non-invasive method to assess recognition memory, reducing inter-individual variability and stress-related interference. The inclusion of delays in NOR testing further evaluates short-term memory phases, enhancing the accuracy and translatability of the results. The gestation period of mice and the first two weeks of postnatal development are critical for neuronal cell growth, nAChR development, and synaptogenesis, particularly in the hippocampus and cerebellum. These stages align with human third-trimester brain development, ensuring that findings are translational to human health contexts. The nicotine dosage of 2-3 mg nicotine base/kg/day mirrors moderate to heavy human smoking, providing physiological relevance and reducing confounding variables [8]. Furthermore, measuring chymotrypsin-like proteasome activity in hippocampal tissue bridges the gap between molecular mechanisms and behavioral outcomes. This analysis sheds light on the role of proteasomal activity in late-phase LTP (L-LTP) and synaptic plasticity, providing insights into how prenatal nicotine exposure alters cellular processes fundamental to memory encoding and retention. This research method creates new knowledge by identifying pathways through which prenatal nicotine exposure impacts synaptic plasticity and memory at both molecular and behavioral levels. It provides a platform to explore targeted therapeutic interventions aimed at mitigating cognitive deficits resulting from developmental neurotoxicity of nicotine exposure at the prenatal level.

Anticipated Results

Rezvani et al. [4] demonstrated that adolescent and adult smokers exhibit reduced proteasomal activity, accompanied by increased levels of glutamate receptor subunits, including NMDA receptor subunit NR2A, and metabotropic receptor mGluR1a. These changes were attributed to nicotine's inhibition of proteasome chymotrypsin-like activity. Similarly, we anticipate a reduced fluorescence intensity in a chymotrypsin-like activity test, indicating decreased proteasome activity in prenatal nicotine-exposed hippocampal tissue. Additionally, a significant difference is expected in the normalized protein concentration, indicating decreased protein synthesis activity levels between the nicotine-exposed and control groups. with a t-test significance of p < 0.005. For this reason, we suspect that reduction in proteasome activity, important for the Late-phase Long Term Potentiation (L-LTP), disrupts the critical mechanisms of synaptic plasticity, thereby disrupting spatial and long-term memory as well, compounded by hippocampal volume reductions frequently observed in nicotine-exposed offspring [1, 3]. Subsequently, for this reason, we expect the number of entries into the shock zone, the duration spent in the shock zone, and the latency to enter the shock zone will be significantly different between experimental and control groups in the APA task (p < 0.05). While, for the NOR test, the time spent exploring the novel object will be significantly different between experimental and control groups (p < 0.05).

Discussion

The expected results indicate a relationship between prenatal nicotine exposure and proteasome chymotrypsinlike activity which has an established role in L-LTP. L-LTP plays a key role in learning. Therefore, disruption in L-LTP, caused by disrupted protein synthesis levels stemming from prenatal nicotine exposure affects short and long-term memory. Further investigation is required to establish a causational relationship.

The results of this study align with previous research and suggests that prenatal nicotine exposure can significantly impair proteasomal activity in the hippocampus. Rezvani et al. [4] demonstrated similar findings in adolescent and adult smokers, where reduced proteasomal activity was linked to elevated levels of glutamate receptor subunits, including NMDA subunit NR2A and metabotropic receptor mGluR1a. These alterations were attributed to nicotine's inhibition of proteasomal function. Our expected findings support this by predicting a reduction in chymotrypsin-like activity in hippocampal tissue from nicotine-exposed offspring, signaling decreased proteasome activity. Moreover, the anticipated decrease in normalized protein concentration suggests compromised protein synthesis, which is consistent with previous reports of disrupted synaptic plasticity and impaired memory functions in nicotine-exposed individuals. Specifically, this reduction in proteasome activity likely

hinders Late-phase Long-Term Potentiation (L-LTP), a critical mechanism for synaptic strengthening and long-term memory consolidation. This disruption, compounded by hippocampal volume reductions noted in nicotine-exposed offspring [1, 3], may explain the observed deficits in spatial memory and learning tasks. As hypothesized, we expect significant differences between the experimental and control groups in behavioral assays, such as the APA and NOR tests, further supporting the link between impaired proteasomal function and cognitive deficits. These findings underscore the long-lasting impact of prenatal nicotine exposure on hippocampal function and memory.

Prenatal Nicotine Exposure Stage Challenges

The present research method relies on the use of mice subjects pre and post-mortem. The cost and access to laboratory mice are potential challenges as are the ethical implications of confining animal subjects to a laboratory for their entire lifetime (offspring mice) and exposing gestating mice to nicotine. This proposed use of nicotine is also essential to our research and nicotine's effects are highly concentration dependent. This study will use concentrations around 2–3 mg nicotine base/kg/day during gestation [8], which closely mimic smokers' plasma levels. Slight variations could dramatically alter results [3].

APA Task and NOR Test Challenges

The experimental paradigm demands precise neuromotor coordination and cognitive flexibility, which means that any genetic or pharmacological intervention potentially compromising a mouse's locomotor capabilities could fundamentally undermine the task's validity. Specifically, interventions that impede a mouse's ability to move efficiently, escape shock zones, or navigate the rotating arena would render the experimental results uninterpretable. For instance, neurological treatments that cause motor neuron dysfunction, reduce muscle strength, or create balance impairments would prevent the mouse from demonstrating its genuine spatial learning capabilities. The task requires rapid, strategic movement and quick cognitive processing - any intervention disrupting these fundamental abilities would essentially invalidate the experimental outcomes. Researchers must, therefore, carefully screen and select interventions that preserve the mouse's core locomotor and cognitive functions [2].

Environmental factors represent a critical challenge in the NOR Test. The protocol emphasizes the importance of precise experimental conditions, including light intensity (recommended at 15 lux), temperature, humidity, and circadian rhythms. Even minor deviations in these parameters can introduce significant experimental noise and potentially invalidate results. The sensitivity of these conditions is particularly pronounced with different mouse strains, especially when working with albino animals that have heightened light sensitivity. Additionally, exploration behavior measurement itself is complex and subjective. The

L-LTP Stage Challenges

There are also some measurement challenges associated with the L-LTP method we propose as distinguishing between early (E-LTP) and late (L-LTP) phases is important and extrapolating results between different experimental conditions can be problematic. As Lynch [3] concludes, while LTP provides valuable insights, definitively proving it as a biological substrate for learning remains a significant challenge.

Measuring Long-Term Potentiation's molecular changes presents profound scientific challenges due to the intricate, microscopic nature of neurological processes. Invasive techniques like electrophysiological recordings or genetic manipulations inherently risk altering the natural neurological environment, introducing experimental artifacts that could misrepresent genuine synaptic plasticity. Specialized microscopy and advanced imaging technologies offer glimpses into these processes, but each method carries inherent limitations in resolution, temporal tracking, and potential interference with delicate cellular interactions.

APA Task and NOR Test Limitations

The APA task, while innovative, presents several notable challenges that researchers must carefully address. One primary limitation is the task's complexity, which makes it less suitable for certain subject populations. Specifically, juvenile mice under 8 weeks old struggle with the paradigm, often attempting to jump out of the arena rather than engaging in strategic spatial learning. To mitigate this, researchers should strictly adhere to using adult mice (8+ weeks) and ensure consistent age matching across experimental groups [2].

One limitation of the NOR Test is the potential for inter-individual variability [6]. Mice can exhibit substantial differences in exploratory behavior due to factors like strain, sex, age, and individual temperament. To mitigate this challenge, researchers can implement the following strategic approaches:

- 1. Use a consistent mouse strain and age group to minimize biological variability
- Employ a robust selection criterion, such as the 20-second exploration time used by Leger et al. [6], which helps standardize data collection
- 3. Increase sample sizes and include comprehensive control groups, particularly when testing pharmacological treatments or transgenic animal models

4. Randomizing object placement and ensuring blinded data collection can further reduce potential experimental bias.

Another critical limitation is the test's sensitivity to environmental conditions, which can significantly impact experimental outcomes. The protocol emphasizes the importance of controlling factors like light intensity (recommended at 15 lux), temperature, humidity, and circadian rhythms [6]. To mitigate these environmental influences, researchers should create a highly controlled experimental environment with consistent parameters across all trials. This includes maintaining a stable room temperature ($22 \pm 1^{\circ}$ C), using a reversed light-dark cycle, and conducting experiments during the animal's active phase. Implementing standardized handling protocols - such as handling mice twice weekly for one minute in the week preceding experiments - can also reduce novelty-induced stress and create more consistent behavioral responses. Furthermore, using automated video tracking systems with nose-point detection can provide more objective and consistent data collection, reducing human observer variability.

By addressing these limitations through meticulous experimental design and rigorous standardization, researchers can enhance the reliability and reproducibility of the APA Task and NOR Test methods.

L-LTP Stage Limitations

Neuronal protein synthesis represents an extraordinarily complex and region-specific process. Different brain areas exhibit unique protein synthesis patterns, making standardized measurement protocols exceptionally difficult to develop. Neurons in the hippocampus, cortex, and other brain regions may respond differently to identical stimuli, creating significant variability in experimental outcomes. The molecular machinery driving protein synthesis involves mechanisms, including multiple regulatory genetic expression, epigenetic modifications, and environmental influences. These intricate interactions mean that even minor experimental variations can produce dramatically different results, challenging researchers' ability to create reproducible, generalizable findings about synaptic plasticity and memory formation.

To mitigate the challenges of measuring neuronal protein synthesis across different brain regions, researchers can employ a multi-pronged approach that addresses the inherent complexity of the process. First, advanced computational modeling and machine learning techniques can be leveraged to develop more sophisticated analytical frameworks that account for regional variability. By creating adaptive algorithms that can recognize and normalize differences in protein synthesis patterns, researchers can develop more robust statistical models that accommodate the nuanced responses of neurons in different brain areas. Secondly, implementing comprehensive multi-omics approaches would provide a more holistic understanding of

protein synthesis mechanisms. This would involve integrating genomic, transcriptomic, proteomic, and epigenetic data to create a more comprehensive map of protein synthesis regulation. By utilizing techniques like single-cell RNA sequencing and advanced mass spectrometry, researchers can capture the intricate molecular interactions that drive region-specific protein synthesis with unprecedented detail. Furthermore, standardization efforts should focus on developing highly controlled experimental protocols that minimize environmental variables. This could include creating standardized cell culture conditions, precise environmental controls, and using isogenic animal models to reduce genetic variability [5]. Researchers should also employ multiple complementary techniques to crossvalidate findings, such as combining in vitro studies with in vivo imaging and molecular tracing methods.

Conclusions

This research study proposes the investigation of prenatal nicotine exposure's effect on long and short termmemory through altered proteasome activity and behavioural tests. We propose the assessment of protein synthesis involved in late-phase long-term potentiation (L-LTP) to understand how prenatal nicotine exposure impacts proteasomal activity and its downstream effects such as disruption of proteasome activity, L-LTP, and synaptic plasticity. We also propose behavioral tests (APA and NOR) to evaluate the effect prenatal exposure to nicotine has on the subjects' short-term, long-term, spatial, and emotional memory.

Using a molecular (protein synthesis) analysis of memory as well as a behavioural (APA and NOR) analysis allows us to draw connections between brain physiology and behaviour to establish prenatal nicotine exposure's effect on memory.

Hellyer et al. [12] demonstrated that fMRIs could be used to measure protein synthesis by using a graph theoretical approach and Dynamic connectivity approach. A future study could apply the methods proposed in the present study to investigate the effects of prenatal nicotine exposure among individuals who were already exposed to nicotine prenatally as exposing gestating mothers to nicotine for the purpose of studying their children are unethical as is extracting their hippocampal tissue postmortem. Therefore, utilizing fMRIs instead of hippocampal tissue to measure protein synthesis activity will provide a similar understanding of the molecular effect of prenatal nicotine exposure on memory. Using behavioral tests to assess memory will provide further insight into these effects as it will be a direct analysis of the human's memory. Further investigation into the molecular basis of memory in connection with other parts of the brain such as nucleus accumbens (NAc), amygdala, and ventral tegmental area (VTA), cerebellum, and basal ganglia that are necessary for the consolidation of memory and longterm potentiation.

List of Abbreviations

ADHD: attention deficit hyperactivity disorder AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid ANOVA: analysis of variance APA: active placement avoidance BMI: body mass index CaMKII: calcium/calmodulin-dependent protein kinase II DA: dopamine E-LTP: early phase long term potentiation L-LTP: late phase long term potentiation LTP: long-term potentiation mGluR1a: metabotropic glutamate receptor 1 alpha NAc: nucleus accumbens nAChRs: nicotinic acetylcholine receptors NMDA: N-methyl-D-aspartate NOR: novel object recognition NR2A: N-methyl-D-aspartate receptor subunit 2A PFC: prefrontal cortex RNA: ribonucleic acid SIDS: sudden infant death syndrome VTA: ventral tegmental area

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Ethics Approval and/or Participant Consent

This study does not require Research Ethics Board (REB) approval as our proposal is a research protocol.

Authors' Contributions

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

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

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