

Transgenerational Inheritance of Odour-Specific Fear Memory

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Abstract. Transgenerational epigenetic inheritance suggests that environmental experiences can influence offspring biology through mechanisms that do not involve changes to DNA sequence. Previous studies have demonstrated that paternal fear conditioning to a specific odour can alter offspring sensitivity and behavioural responses to that same odour, indicating that parental experiences may be transmitted across generations. However, the neural mechanisms underlying these effects remain poorly understood. This study investigates the neural mechanisms underlying this phenomenon. Using ArcCreERT2; Ai14 mice, F0 males will be fear-conditioned to acetophenone to examine experience-dependent changes potentially associated with germline epigenetic modifications. F1 offspring will then undergo odour conditioning with three distinct odours, and their brains will be analysed using confocal microscopy to quantify activity-dependent engram formation. Comparisons between the offspring of conditioned and control fathers will assess whether paternal experience selectively biases the neural representation of acetophenone. The author hypothesises that F1 offspring of conditioned fathers will exhibit an increased acetophenone-associated engram compared to controls, suggesting that paternal experience may bias memory encoding in offspring.

Keywords: Transgenerational epigenetic inheritance, odour-specific fear conditioning, hippocampus

1. Introduction

Transgenerational epigenetic inheritance (TEI) refers to the transmission of non-DNA sequence-based epigenetic information across generations, whereby environmentally induced phenotypic traits can be passed to offspring over multiple generations [1]. Understanding the potential mechanisms of intergenerational memory transfer is of great significance for elucidating human cognitive adaptation, evolutionary processes, and the mechanisms underlying mental health disorders [1]. If fear responses formed by specific environmental cues can be inherited, this could challenge traditional cognitive models of learning and memory as processes confined to a single generation and may help re-examine intergenerational susceptibility to trauma-related disorders such as post-traumatic stress disorder (PTSD) [2].

Odour-specific fear conditioning is a classic behavioural paradigm in which animals learn to associate neutral odours with aversive stimuli, resulting in conditioned fear responses upon re-

exposure to the odour [3]. Earlier studies have suggested that this conditioned response not only forms stable memory representations at the individual level but may also be transmitted across generations through epigenetic mechanisms. However, a significant knowledge gap remains regarding the expression of transgenerational epigenetic fear memory at the level of neural circuits. Research has shown that the hippocampus plays a significant role in the encoding, retrieval, and reactivation of memory; however, it stays unclear whether hippocampal neuronal populations activated during initial fear conditioning are preserved in offspring or undergo reorganisation [4].

This unanswered question limits the understanding of how "inherited" trauma may manifest at the neural level. Therefore, this study proposes that the population of hippocampal neurons activated during parental odour fear conditioning may be partially reactivated when offspring recall the same odour-associated fear memory. However, the overall activity pattern may differ in intensity and spatial distribution, reflecting the plasticity of transgenerational epigenetic effects at the level of neural circuits.

To test this hypothesis, this study will use ArcCreERT2; Ai14 mice and their offspring, which are genetically modified lines used for activity-dependent neuronal tagging. Confocal microscopy will then be used to address two key questions: first, does paternal conditioning to odour A increase the number of odour A-specific engram neurons in F1 offspring, and second, how do hippocampal activity patterns differ between mice encoding an odour-specific fear memory and their offspring when recalling the same odour-associated memory?

2. Methods

The goal of this experiment is to find out if fear memories can be inherited and how they affect the distributions of activated neurons in the hippocampus when encoding and retrieving the fear memory, by looking at the neurons activated in the F0 mice and F1 mice. To achieve precise neuronal labelling, the author utilised a dual transgenic mouse model. The experimental F1 offspring were generated by crossing two parental lines: ArcCreERT2 Mice and Ai14 Reporter mice [5]. ArcCreERT1 mice express a tamoxifen-inducible Cre recombinase (CreERT2) under the control of the endogenous Arc promoter, achieved through homologous recombination. The Arc gene is an immediate-early gene robustly expressed in neurons upon activation. This design ensures that CreERT2 expression is restricted to neurons engaged during a specific experience. Ai14 Reporter Mice harbour a loxP-flanked STOP cassette preventing the expression of a red fluorescent protein called tdTomato, which is inserted into the ubiquitous Rosa26 locus [6].

Then, the experiment took the male parental generation, which is the ArcCreERT2 mice, and exposed them to Odour A (10% acetophenone) for 30 seconds and followed with a two-second, 50V foot shock. The reason why male mice were used is due to the elimination of hormonal variability from the female menstrual cycle that could affect behavioural results. The foot shock serves as the unconditioned stimulus, producing an aversive response, while the Odour becomes the conditioned stimulus [7]. All conditioning was performed in a standardised behavioural chamber equipped with a grid floor for foot shock delivery and a calibrated olfactometer for consistent odourant presentation. The ArcCreERT2 mice underwent three conditioning sessions (one session per day) over three consecutive days to consolidate the fear memory.

Afterwards, the author bred the fear-conditioned ArcCreERT2 mice with the Ai14 mice. The resulting double transgenic F1 offspring (ArcCreERT2; Ai14-tdTomato) provide a powerful activity-dependent tagging system. Upon administration of tamoxifen, CreERT2 is activated exclusively in neurons where the Arc promoter was recently active [8]. This catalyses the excision of the STOP cassette in the Ai14 reporter, leading to permanent tdTomato expression in those neurons. This

creates a stable, long-term fluorescent record of the neuronal ensemble activated during the tamoxifen-sensitive window.

According to previous research, the author suspects that a conditioned fear memory triggers heritable epigenetic modifications in sperm. This inherited information may then change the development of fear-related neural circuits in the offspring's hippocampus, ultimately leading to measurable differences in memory formation and the architecture of activated neuronal ensembles compared to the offspring of control fathers. In this case, the study predicts that A1 generation would be more sensitive to acetophenone.

To observe the difference in hippocampal activity in A1 mice when encoding and retrieving, the experiment grouped the A1 mice into three groups. Group 1, which involved 5 mice that received fear conditioning with 10% acetophenone paired with a 25V foot shock that lasted for two seconds. Group 2, which involved 5 mice that received fear conditioning with 1% 1-propanol and the same shock intensity (25V). Group 3 would be the control group, where 5 mice received no fear conditioning [9]. This protocol is particularly suited to sensitive strains or young mice, where lower foot shock intensity is used to avoid excessive stress. Tamoxifen was administered 5-30 minutes following each session to label the activated neuronal ensembles. When the mouse exhibits freezing behaviour, the study immediately prepares coronal sections of the hippocampus by slicing (thickness: 40-50 μm). Now the researchers have the mouse hippocampus brain sample for encoding.

To investigate the neural correlates of inherited memory retrieval, a separate cohort of mice was utilised. This cohort also consisted of three groups of the offspring (F1 generation) of parents conditioned to fear acetophenone, and each was given a different Odour followed by a foot shock. These F1 mice will be subjected to the identical three-day conditioning protocol as described before. Following the final training session, the mice were housed in a stable environment for five days, which is the consolidation period. This interval allowed the initial fear response to fade, ensuring that subsequent behaviour reflects a consolidated long-term memory. On day eight, these three groups of mice will all be re-exposed to 10% acetophenone for 30 seconds to recall the fear memory [9]. As the mice performed freezing activity, they were perfused transcranially with fixative to preserve brain tissue for further analysis.

3. Materials

Coronal brain sections containing the hippocampus were prepared using a cryostat. Neural activity from both the encoding and retrieval cohorts was visualised using confocal microscopy, a laser-scanning imaging technique. Specifically, neurons active during encoding were labelled with enhanced yellow fluorescent protein (EYFP) and excited using a 488 nm laser, while neurons active during retrieval were labelled with tdTomato and excited using a 561 nm laser.

Once the images had been obtained, image analysis was performed using the open-source software ImageJ. Following standard deconvolution procedures, the fluorescent channels were aligned to create composite images. ImageJ is a powerful tool for visualising activated neurons because it enables morphological analysis, allowing the density and distribution of activated neurons within hippocampal subregions to be assessed for each experimental group. It also facilitates the visualisation of neuronal population overlap. The colocalization of EYFP and tdTomato signals was quantified to determine the proportion of neurons that were active during both encoding and retrieval, as well as those uniquely active during only one of these states.

This approach, which combines confocal microscopy with activity-dependent neuronal tagging, was chosen because it provides excellent optical sectioning, reducing out-of-focus light and

allowing high-resolution three-dimensional reconstruction of neuronal ensembles. In addition, confocal microscopy can image multiple fluorophores simultaneously, making it well suited for directly comparing the two neuronal populations central to this study.

4. Predicted results

Based on established principles of engram biology and evidence for transgenerational epigenetic inheritance, some outcomes are predicted. First of all, all mice subjected to fear conditioning are expected to exhibit robust freezing behaviour and significant activation of hippocampal neurons upon exposure to acetophenone, which confirms the successful encoding and retrieval of the fear memory. Critically, a substantial overlap between the neuronal ensembles active during encoding and retrieval is expected to be observed, which validates the identification of the specific memory engram.

The central hypothesis will be tested by comparing engram characteristics across the F1 groups. The author predicts that the F1-Ace group will form a larger acetophenone-specific engram, evidenced by a greater number of activated neurons, compared to the F1-Pro and F1-Home groups [10]. The finding would provide direct neural evidence for experience-specific transgenerational inheritance, suggesting that paternal exposure may epigenetically influence offspring neural circuitry, increasing sensitivity to the same stimulus.

Conversely, the null hypothesis must also be considered: all three F1 groups may exhibit engrams of statistically similar size [11]. Such an outcome would indicate that paternal fear conditioning does not confer a stimulus-specific epigenetic predisposition for fear memory formation within the hippocampus.

5. Conclusion

Several limitations of this experimental design should be acknowledged. Even though the use of IVF helps control for the influence of paternal care, it cannot completely exclude potential prenatal effects arising from the surrogate mother. In addition, future studies could incorporate optogenetic inhibition of activity-tagged neurons to establish a causal link between these cells and memory expression.

Despite these limitations, the primary finding predicted by this study—that F1-Ace mice develop a larger acetophenone-specific hippocampal engram—would represent a significant advance in the understanding of transgenerational epigenetic inheritance. Such a result would provide direct evidence that paternal experiences can influence the neural architecture underlying memory formation in offspring.

Alternatively, if all F1 groups show engrams of similar size, this would challenge the hypothesis of stimulus-specific inheritance. Instead, it would suggest that paternal fear conditioning may induce a more generalised increase in threat sensitivity or vigilance, while the specific content of fear memories remains determined by the offspring's own experiences. Overall, this experiment aims to distinguish between these possibilities and determine how inherited epigenetic changes influence the neural circuits involved in memory formation.

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